
Characterisation of PRC1 dynamics and chromatin binding in live cells

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A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

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Development of complex multicellular organisms depends on precise spatiotemporal regulation of transcription of genes in the genome. Although this regulation is known to depend on sequence-specific transcription factors, chromatin modifying proteins have also emerged as key regulators of transcription in development. Polycomb proteins represent a class of well studied transcriptional repressors which assemble into two main complexes with histone-modifying activity, Polycomb repressive complexes 1 (PRC1) and PRC2. PRC1 exists in mammalian cells as multiple complexes of distinct subunit composition, formed around a catalytic core of an E3 ubiquitin ligase and one of six PCGF proteins, which monoubiquitylate histone H2A. In addition to dictating the level of catalytic activity of the complex, inclusion of a different PCGF protein results in incorporation of different chromatin-binding modules in each complex. Although previous work has established that enrichment of PRC1 at the promoters of target genes is required to achieve repression, how the mechanisms by which PRC1 subunits interact with chromatin contribute to targeting PRC1 to specific sites in the genome remains unclear.

In order to advance our understanding of how Polycomb proteins interact with the genome, I have employed live cell imaging and single particle tracking to characterise the dynamics and subnuclear localisation of PRC1. I discover that PRC1 subunits have broadly similar and dynamic behaviour, although a small fraction of molecules exhibit stable binding. Quantification of the numbers of PRC1 subunits reveals they are of relatively low nuclear concentration, despite their role in depositing H2AK119ub1 across the genome. Using systems in which I can remove PRC1 catalytic activity or induce degradation of a core PRC2 subunit, I demonstrate that PRC1 catalytic activity and PRC2 contribute modestly to PRC1 interactions with chromatin. Despite this modest change, I find that both are required for enriching Polycomb proteins at nuclear foci. I investigate the contribution of DNA binding of PCGF1-PRC1 subunit to the dynamics of both PRC1 and PCGF1, and find that it is not required for PCGF1 chromatin binding. I show, furthermore, that PRC1 stable binding and localisation within Polycomb bodies depends on specific PCGF proteins. Together, these observations provide a quantitative characterisation of PRC1 chromatin binding and localisation, and provide insights into the contribution of major chromatin-binding mechanisms to Polycomb dynamics.

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DECLARATION OF AUTHORSHIP

I declare that all work presented in this thesis is my own, unless otherwise acknowledged.

This thesis has not been submitted, either partially or in full, for another degree, diploma, certificate or other qualification at this University or at any other institution.

Miles Kocur Huseyin

9th October 2019

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LIST OF COMMON ABBREVIATIONS

ac	Acetylation
AEBP2	AE binding protein 2
ANTC	Antennapedia complex
AUTS2	Autism susceptibility candidate 2
bp	base pair
BAF	BRG1- or HBRM-associated factors
BAP1	BRCA1-associated protein 1
BCOR	BCL-6 co-repressor
BCORL1	BCL-6 co-repressor-like protein 1
BXC	Bithorax complex
CAF1	Chromatin assembly factor 1
Cas9	CRISPR-associated protein 9
CBP	CREB binding protein
CBX	Chromobox protein
CGI	CpG island
ChIP	Chromatin immunoprecipitation
ChIP-seq	ChIP followed by massively parallel sequencing
CK2	Casein kinase 2
CpG	Cytosine-phosphate-Guanine
CPM	Conditional point mutation
cPRC1	Canonical PRC1
Cre-ERT2	Cre recombinase fused to the oestrogen receptor
CRISPR	Clustered regularly interspaced short palindromic repeats
CUT&RUN	Cleavage under targets and release using nuclease
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
dRAF	dRING associated factors
dRING	<i>Drosophila</i> Really Interesting New Gene protein, also called SCE for Sex combs extra
dRYBP	<i>Drosophila</i> RING1 and YY1-binding protein
dSFMBT	<i>Drosophila</i> Scm-like with four MBT domain-containing protein 1
EED	Embryonic ectoderm development
Elo	Elongin
EPOP	Elongin BC and Polycomb repressive complex 2 associated protein
ESC	Extra sex combs
ESC	Extra sex combs like
E(Z)	Enhancer of Zeste
EZH	Enhancer of Zeste
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FCS	Fluorescence correlation spectroscopy
FRAP	Fluorescence recovery after photobleaching
FT	Flowthrough
gDNA	Genomic DNA
H2AK118	Histone H2 lysine 118
H2AK119	Histone H2 lysine 119
H3K9	Histone H3 lysine 9
H3K27	Histone H3 lysine 27
H3K36	Histone H3 lysine 36
HDAC	Histone deacetylase

LIST OF COMMON ABBREVIATIONS

HDR	Homology directed repair
HILO	Highly inclined and laminated optical sheet
hnRNP	Heterogeneous nuclear ribonucleoprotein K
HOTAIR	HOX transcript antisense RNA
Hox	Homeotic
HP1	Heterochromatin protein 1
HT	HaloTag
IP	Immunoprecipitation
JARID2	Jumonji/ARID domain-containing protein 2
JF₅₄₉	Janelia fluor 549
JmjC	Jumonji C
KDM	Lysine demethylase
L3MBTL2	Lethal (3) malignant brain tumour-like protein 2
lncRNA	Long non-coding RNA
MAX	MYC-associated factor X
MBD	Methyl binding domain
me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
MEF	Mouse embryonic fibroblast
mESC	Mouse embryonic stem cell
MGA	MAX gene-associated protein
MSD	Mean squared displacement
NLS	Nuclear localisation sequence
NURF	Nucleosome remodelling factor
OHT	4-hydroxytamoxifen
PA	Photoactivatable
PALI	PRC2-associated LCOR isoform
PC	Polycomb
PcG	Polycomb group
PCGF	Polycomb group ring finger
PCL	Polycomb like protein
PCR	Polymerase chain reaction
PH	Polyhomeotic
PHD	Plant homeodomain
PHC	Polyhomeotic-like protein
PHO	Pleiohomeotic
PhoRC	Pho-repressive complex
PIC	Protease inhibitor cocktail
PRC	Polycomb repressive complex
PRE	Polycomb response element
PSC	Posterior sex combs
qPCR	Quantitative real-time PCR
RbAp	Retinoblastoma-associated protein
RBBP	Retinoblastoma-binding protein
RING1	RING finger protein 1
RNAPII	RNA polymerase II
RYBP	RING1 and YY1-binding protein
SAM	Sterile alpha motif
SCC	Sister chromatid cohesion protein

LIST OF COMMON ABBREVIATIONS

SCE	Sex combs extra, also called dRING
SCM	Sex comb on midleg protein
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SET	Su(var)3-9-enhancer of zeste-trithorax
sgRNA	Single guide RNA
SKP1	S-phase kinase-associated protein 1
SPT	Single particle tracking
SU(Z)2	Suppressor of Zeste 2
SUZ12	Suppressor of Zeste 12
SWI/SNF	SWItch/Sucrose non-fermentable
TIRF	Total internal reflection fluorescence
TMR	Tetramethylrhodamine
USF1	Upstream stimulatory factor 1
vbSPT	Variational Bayes SPT
vPRC1	Variant PRC1
Xist	X-inactive specific transcript
YAF2	YY1 associated factor 2
YY1	Yin Yang 1 transcriptional factor
ZF-CXXC	Zinc finger-CXXC

INTRODUCTION

1.1 Regulating expression of the genome

The capacity to control how genes in the genome are expressed is fundamental to life. The requirements for genes and their products change depending on the circumstances in which a cell finds itself, and the cell can adapt to its environment by changing its gene expression patterns. In multicellular organisms, regulation of gene expression is also key to cellular differentiation and the control of cell fate during development (for review, see Lemon and Tjian 2000). By controlling what complement of genes is used, differential gene expression can produce the vast array of cell types which make up a complex multicellular organism. DNA binding factors which exert control over transcription, often by binding to specific base pair sequences, or sequence motifs, are central to the regulation of gene expression.

1.1.1 Sequence-specific transcription factors

Early work in prokaryotes established that transcription factors bind to specific DNA sequences at gene promoters and promote transcription by enabling the binding of the core transcriptional machinery or other activators of transcription (Ptashne and Gann 1997). Although the core principle of transcription factor binding to sequence motifs remains in eukaryotes, increasing organismal complexity appears to correlate with increased regulatory complexity, and, from single celled eukaryotes to vertebrates, further layers of regulation and increasing numbers of transcription factors have evolved (Figure 1.1 and Tupler et al. 2001). Additional features involved in regulation include the expanded and widespread presence of non-coding sequences in and around coding regions, and the existence of regulatory sequences at extended linear distances along the DNA from their cognate core promoter (Levine et al. 2014). Despite these increases in complexity, higher eukaryote regulatory proteins still rely heavily upon sequence-specific DNA binding to achieve site specific occupancy required for transcriptional regulation. Many sequence-specific transcription factors are known to be essential for the array of gene regulatory networks involved in development and physiological function (Spitz and Furlong 2012; Davidson 2010). The sequence of the DNA itself therefore remains a key feature of the eukaryotic system for

regulating transcription.

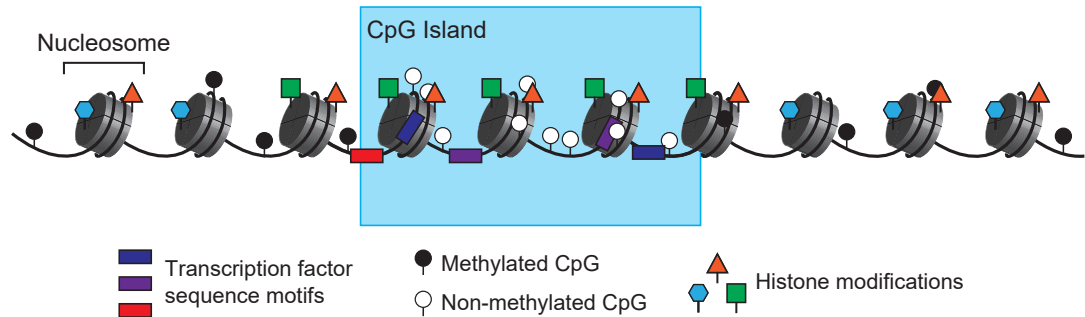


Figure 1.1: Features of vertebrate chromatin

The eukaryotic genome is packaged into chromatin, of which the basic repeating unit is the nucleosome, consisting of 147 bp of DNA wrapped around an octamer of histone proteins. Key features of chromatin in vertebrates which are linked to the regulation of transcription include sequence motifs recognised by transcription factors, DNA CpG dinucleotide methylation, and post-translational modification of histone proteins. Post-translational modifications of histones typically occur on the unstructured N-terminal tails, but these are not shown for simplicity.

1.1.2 Chromatin

Although DNA sequence alone plays a major role regulating transcription, the eukaryotic genome does not exist as naked DNA, but is packaged into chromatin, providing a further layer of complexity which is now understood to constitute a major part of the regulation of gene expression in eukaryotes (Narlikar et al. 2002; Orphanides and Reinberg 2002). Chromatin is comprised of the DNA genome, together with histone proteins and other non-histone protein components. The nucleosome, the fundamental repeating unit of chromatin, consists of 147 bp of DNA wrapped around an octamer of histones H2A, H2B, H3 and H4 (Figure 1.1 and Luger et al. 1997). Linker DNA of variable length occurs between nucleosomes and can be bound by histone H1 (Happel and Doenecke 2009). Together, these features of chromatin form the basis on which the genome is organised within the eukaryotic nucleus. In addition to organising the genome, chromatin also plays central roles in almost all other processes that take place on DNA, including transcription, repair, replication, and recombination (Li et al. 2007). Early work has demonstrated that chromatin can function to limit access of proteins to DNA and is therefore inhibitory to transcription (Knezetic and Luse 1986; Lorch et al. 1987). However, chemical modifications of both DNA and histone proteins have been associated with increased or decreased transcriptional activity, as well as providing binding sites for many factors which regulate transcription (Kouzarides 2007). Therefore, although the precise mechanisms which underpin the effects of chromatin on gene expression remain poorly understood, it is clear that chromatin is intimately involved in the

regulation of eukaryotic transcription.

1.1.3 DNA methylation

Vertebrate DNA undergoes extensive methylation, a feature of chromatin which extends the role of DNA in regulating gene expression beyond simple sequence motifs. Most occurrences of CpG dinucleotides are modified on the cytosine with a methyl group at the C5 position (Lister et al. 2009), deposited by a family of DNA methyltransferases which are responsible for both the *de novo* establishment of DNA methylation in development and its maintenance through replication (Jeltsch 2006; Bestor and Ingram 1983; Pradhan et al. 1999; Okano et al. 1999). Because maintenance of CpG methylation by the methyltransferase DNMT1 occurs coincident with replication, it serves to faithfully reproduce the pattern of DNA methylation on the newly synthesised DNA (Chuang et al. 1997; Vertino et al. 2002; Bostick et al. 2007; Hashimoto et al. 2008).

Methylated cytosine can spontaneously undergo deamination to produce thymine (Coulondre et al. 1978; Duncan and Miller 1980). Repair of the resulting T:G mismatch may lead to TpG conversion which may then be inherited, if it occurs in germline cells, and, as a result, CpG dinucleotides occur at approximately one fifth of the expected frequency (Josse et al. 1961; Swartz et al. 1962). Although the vertebrate genome at large is depleted of CpG dinucleotides, regions of CpG density approaching the expected frequency and with elevated GC content, typically around 1-2 kb in length, occur throughout the genome, with the majority lacking DNA methylation (Bird et al. 1985; Gardiner-Garden and Frommer 1987). These regions, known as CpG islands (CGIs), are present at approximately 50 - 70% of vertebrate promoters (Tykocinski and Max 1984; Bird et al. 1985; Saxonov et al. 2006), and typically lack DNA methylation regardless of cell type and gene expression (Figure 1.1 and Weber et al. 2007; Meissner et al. 2008). Importantly, methylation of promoters is associated with gene repression, and therefore maintenance of the non-methylated state of CGI promoters would appear to be required for normal transcription of the associated gene (Bird and Wolffe 1999; Bird 2002; Jones 2012). Methylated DNA can also serve directly as a binding site for certain proteins and is specifically recognised by a number of proteins involved in transcriptional repression (Meehan et al. 1992; Lewis et al. 1992; Nan et al. 1997; Cross et al. 1997; Hendrich and Bird 1998; Prokhortchouk et al. 2001) providing a functional link between DNA methylation and repression.

Because CGIs largely remain unmethylated despite changes in cell type and the level of transcription of their associated gene, it is clear that they cannot define the transcriptional state of the gene alone. Instead, these regions are proposed to act as a platform to allow other factors to bind to influence transcription (Blackledge and Klose 2011; Deaton and Bird 2011). Consistent with this, CGIs have been found to be hyperacetylated and depleted of linker histone (Tazi and Bird 1990), and more accessible and less nucleosome-occupied, as measured by micrococcal nuclease (MNase)-seq and deoxyribonuclease (DNase)-seq, and other nuclease-based assays (Stalder et al. 1980; Bird et al. 1985; Antequera et al. 1989; Sabo et al. 2004; Choi 2010; Fenouil et al. 2012). Increased accessibility of CGIs relative to promoters with lower CpG occurrence may therefore facilitate binding of the transcriptional machinery, transcriptional regulators and other chromatin-interacting proteins, thereby enhancing the capacity of the cell to regulate CGI promoters (Blackledge and Klose 2011; Deaton and Bird 2011).

Proteins have also been identified which specifically recognise non-methylated CpG dinucleotides via a cysteine-rich zinc finger (ZF-CXXC) domain (Voo et al. 2000; Lee et al. 2001; Long et al. 2013b). Proteins with ZF-CXXC domains tend to possess chromatin-modifying activities or associate with chromatin-modifying complexes, which may give them direct functions in regulating transcription (Lee and Skalnik 2005; Lee et al. 2007; Birke 2002; Bach et al. 2008; Blackledge et al. 2010; Farcas et al. 2012; Koyama-Nasu et al. 2007; Yamagishi et al. 2008). ZF-CXXC domains therefore target these chromatin-modifying and transcriptional regulatory activities to CGIs, allowing these sites to function as gene expression regulatory platforms. Other proteins, including CTCF, have been found to be unable to bind to their preferred sequence motif in the presence of DNA methylation, thereby also making them readers of the DNA methylation state (Watt and Molloy 1988; Iguchi-Ariga and Schaffner 1989; Comb and Goodman 1990; Bell and Felsenfeld 2000). Stable chemical modification of the DNA, therefore, constitutes another layer of regulation present in vertebrate chromatin.

1.1.4 Post-translational modification of histones

Core histone proteins possess an unstructured N-terminal tail which is exposed to solution, away from the globular core of the nucleosome (Luger et al. 1997; Harp et al. 2000). Amino acids within histone tails are known to undergo a wide variety of post-translational modifications mediated by histone-modifying enzymes, including: acetylation, methylation (which can occur as mono-, di-

or trimethylation), ubiquitylation, sumoylation, phosphorylation, ribosylation, biotinylation and glycosylation (Figure 1.1 and Shiio and Eisenman 2003; Camporeale et al. 2004; Kouzarides 2007; Zhang et al. 2011). Genomic approaches have been used to map the location of these modifications in the genome, and have revealed that specific modifications tend to co-localise at certain sites (ENCODE 2012). Some post-translational modifications, including methylation of histone H3 on lysine 4 (H3K4), H3K36 and H3K79 (Barski et al. 2007; Guenther et al. 2007; Krogan et al. 2003; Bannister et al. 2005; Steger et al. 2008), monoubiquitylation of H2BK120 (Zhu et al. 2005; Pavri et al. 2006), and lysine acetylation on histone H3 and H4 (Hebbes et al. 1988; Pokholok et al. 2005; Karmodiya et al. 2012) are associated with active transcription. Conversely, others, including methylation of H3K9 and H3K27 (Nakayama et al. 2001; Lehnertz et al. 2003; Cao et al. 2002), and ubiquitylation of H2AK119 (Wang et al. 2004a) are associated with transcriptional repression.

Although the genomic positions at which histone modifications occur have been mapped, whether or how they influence transcription remains poorly understood. Histone acetylation has been linked to a disruption of the contacts between the histone proteins and associated DNA, making nucleosomes easier to displace (Norton et al. 1989; Garcia-Ramirez et al. 1995; Anderson et al. 2001; Shogren-Knaak 2006), and therefore has been posited to make regions more accessible to DNA binding factors and transcription machinery. For other modifications, whether they contribute directly to regulating transcription remains unclear. Despite this, many proteins with established roles in regulating transcription are known to bind to specific histone modifications. Proteins which bind histone modifications often also have histone-modifying activity themselves, or are capable of interacting with a histone modifier, allowing modifications to be combinatorially placed, or for them to be propagated (Allis and Jenuwein 2016). Furthermore, many of these proteins or their interacting partners have characterised roles in regulating chromatin accessibility, organisation and transcription. Specific histone modifications may then act as a site to which they can bind in order to affect transcription. The effects of misregulation of histone modifications in cancer and other diseases, and their importance in development further underscore the central role these modifications play in the regulation of transcription (Dawson and Kouzarides 2012; Ronan et al. 2013; Lewis et al. 2013; Pavlova and Thompson 2016; Lu et al. 2016).

1.2 Polycomb repressive complexes

One of the most studied histone-modifying systems in higher eukaryotes is the Polycomb repressive system. The first Polycomb group (PcG) gene was identified in *Drosophila melanogaster* over 70 years ago (Lewis 1947), and was subsequently determined to function as a repressor of developmentally important *Homeotic (Hox)* gene expression (Lewis 1978). This first gene mutant, *Polycomb (Pc)*, was observed to lead to a phenotype matching the expected phenotype of ectopic expression of genes in the Bithorax complex (BXC) *Hox* cluster, with an overall transformation of more anterior body segments to resemble more posterior segments (Lewis 1978). This apparent failure to repress BXC genes thereby implicated wild type *Pc*, which is not itself part of the cluster, as a negative regulator of its expression. Another notable feature of *Pc* mutant flies is a gain of partial ectopic sex combs on the second and third pair of legs in heterozygous *Pc* male flies, while wild type flies only have sex combs on the first pair of legs. This particular feature of the *Pc* mutant phenotype arises from a failure to repress a gene, *Sex combs reduced (Scr)* of the other *Hox* cluster (Antennapedia complex, ANTC), leading to ectopic expression of *Sex combs reduced* in the second and third thoracic segments, and a gain of sex combs on the associated legs (Pattatucci and Kaufman 1991; Kennison 2004). The additional sex combs of this phenotype resulted in it being named Polycomb.

After identification of this first PcG gene, further mutants giving similar homeotic transformation phenotypes were discovered, expanding the list of annotated PcG genes (Jürgens 1985; Duncan 1982; Kennison 1995; Kennison 2004). By examining further homeotic transformation phenotypes, another family of transcriptional regulators, the Trithorax Group (TrxG), was discovered (Kennison 1995). Unlike PcG mutants, TrxG mutants were found to result in ectopic repression of *Hox* genes, and associated with the transformation of body segments to resemble more anterior ones, suggesting that they instead functioned to activate expression of *Hox* genes, in opposition to PcG proteins (Grimaud et al. 2006a).

With the discovery of a family of PcG genes in *Drosophila*, orthologues were identified across the five major animal lineages, as well as in plants and fungi (Schuettengruber et al. 2017). While the conservation of specific subunits varies across species, the major function of the Polycomb system as a regulator of transcription in development remains (Pien and Grossniklaus 2007; Sharif et al. 2013). Biochemical analysis of the proteins produced from identified PcG genes,

in *Drosophila* (Shao et al. 1999; Ng et al. 2000), and vertebrates (Alkema et al. 1997; Sewalt et al. 1998; Lohuizen et al. 1998) has revealed that PcG proteins form two main biochemically distinct complexes: Polycomb repressive complex 1 (PRC1) and PRC2.

1.2.1 PRC1 complexes

PRC1 has emerged as a particularly heterogeneous complex, especially in mammals, where many of the known *Drosophila* subunits have multiple homologues which are differentially expressed during development. Nevertheless, PRC1 complexes contain a common heterodimeric core, consisting in mammals of a RING1 protein (RING1A or RING1B, homologues of *Drosophila* dRING/SCE) and one of six Polycomb group ring finger proteins (PCGF, homologues of *Drosophila* PSC and SU(Z)2) (Figure 1.2 and Cao et al. 2005; Elderkin et al. 2007). RING1A/B have E3 ubiquitin ligase activity, catalysing the transfer of a single ubiquitin moiety from a cognate E2 ligase onto histone H2A lysine 119 (H2AK119ub1, or H2AK118ub1 in *Drosophila*), and this activity requires dimerisation with a PCGF protein (Cao et al. 2005; Elderkin et al. 2007). Furthermore, individual PCGF proteins appear to specify which additional PRC1 subunits are incorporated into the complex (Junco et al. 2013). Because of their highly variable subunit composition, PRC1 complexes are subdivided into canonical and variant complexes, whose composition I will discuss separately.

1.2.2 Canonical PRC1

Canonical PRC1 complexes (cPRC1) are defined by their incorporation of one of five CBX proteins in mammals (CBX2/4/6/7/8), homologues of the *Drosophila* PC protein (Figure 1.2). Polycomb CBX proteins contain a chromobox domain with affinity for H3K27me3 and are thought to play the major role in cPRC1 binding to chromatin (Bernstein et al. 2006; Zhen et al. 2016). Studies both *in vitro* and in live cells have suggested that this affinity varies between the different CBX proteins (Bernstein et al. 2006; Zhen et al. 2016), but it remains unclear as to how and to what extent these differences in affinity affect the function of the individual proteins, as their binding sites overlap when coexpressed (Pemberton et al. 2014).

Canonical complexes also specifically incorporate only two of the mammalian PCGF proteins, PCGF2/4, which are the closest homologues of *Drosophila* PSC and SU(Z)2, as well as one of three PHC proteins (PHC1/2/3), homologues of *Drosophila* Polyhomeotic (PH) (Figure 1.2). PHC

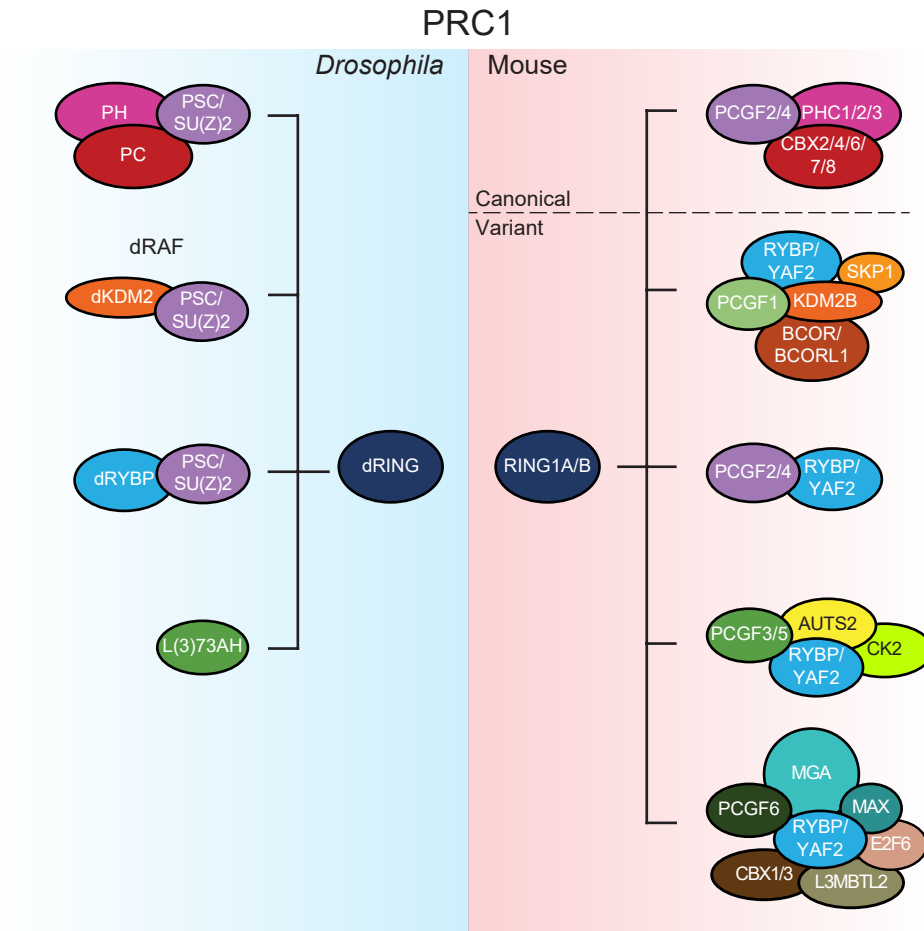


Figure 1.2: Diversity of mouse and *Drosophila* PRC1 complexes

Subunits of the characterised *Drosophila* (left) and mouse (right) PRC1 complexes are shown. Biochemically similar complexes between *Drosophila* and mouse are shown in line with each other. Mouse PRC1 complexes are divided into canonical and variant categories according to incorporation of CBX proteins or RYBP/YAF2. The names of mutually exclusive but related subunits are separated by a slash within the same object.

proteins are able to polymerise via their sterile alpha motif (SAM) domain (Kim et al. 2002), and this polymerisation has been found to be important in some cell types for the formation of microscopically visible nuclear foci into which Polycomb proteins are incorporated, known as Polycomb bodies (Isono et al. 2013). SAM domain polymerisation is required for the repressive activity of the *Drosophila* homologue PH (Robinson et al. 2012), while mutations of the PHC2 SAM domain cause a loss of repression at some Polycomb targets in mouse embryonic fibroblasts (MEFs) and induce posterior transformations in mice (Isono et al. 2013). CBX proteins, excluding CBX7, have also been recently found to have the capacity to form phase condensates both *in vitro* and in live cells, and are also implicated in the formation of these foci (Tatavosian et al. 2018b; Plys et al. 2019). PHC SAM domain polymerisation functions to bring Polycomb domains together (Isono et al. 2013), while CBX phase separation is linked to chromatin compaction by cPRC1 complexes (Eskeland et al. 2010; Grau et al. 2011; Lau et al. 2017; Tatavosian et al. 2018b; Plys et al. 2019), suggesting that cPRC1 complexes are involved in mediating chromatin structure. In mouse embryonic stem cells (mESCs), this structural function appears to contribute little to maintaining Polycomb-mediated repression as removal of cPRC1 complexes results in minimal changes in gene expression (Fursova et al. 2019), although it may play a role in differentiation (Noordermeer et al. 2011; Narendra et al. 2015; Narendra et al. 2016; Kundu et al. 2017).

1.2.3 Variant PRC1

Mammalian variant PRC1 complexes (vPRC1, also non-canonical PRC1) contain RING1 and YY1-binding protein (RYBP) or its paralogue YAF2 (homologues of *Drosophila* dRYBP), along with any one of the six PCGF proteins (Figure 1.2 and Gao et al. 2012; Kloet et al. 2016; Hauri et al. 2016). Incorporation of RYBP into PRC1 complexes has been found to enhance PRC1 E3 ligase activity *in vitro* and is required to achieve normal levels of H2AK119ub1 at Polycomb target genes *in vivo* (Gao et al. 2012; Rose et al. 2016). RYBP and YAF2 are also able to bind to H2AK119ub1 (Arrigoni et al. 2006), although the significance of this binding for PRC1 activity in general is unclear, as removal of RYBP/YAF2 does not appear to affect PRC1 occupancy (Rose et al. 2016). H2AK119ub1 binding by RYBP does appear to be involved in PRC1 binding to the X chromosome during X chromosome inactivation, as mutation of residues involved in this interaction results in a loss of enrichment of PRC1 subunits (Almeida et al. 2017). Interestingly, characterisation of the *Drosophila* homologue dRYBP has indicated that, while it forms equivalent

complexes to the mammalian proteins, it may serve a role in mediating robustness of gene expression which has not yet been observed in mammals (González et al. 2008; Fereres et al. 2014). This role in robustness was proposed because dRYBP appears to counteract repression by the Polycomb system and support Trithorax system-mediated activation of transcription in addition to interacting with Polycomb complexes as in mammals, and enhances the phenotypes of mutations in both systems.

Each PCGF protein appears to further specify which other proteins are incorporated into vPRC1 complexes (Junco et al. 2013), as well as affecting the *in vitro* E3 ubiquitin ligase activity of the PRC1 complex it forms (Taherbhoy et al. 2015; Rose et al. 2016). Furthermore, PCGF proteins also appear to contribute to genomic H2AK119ub1 and Polycomb-mediated gene repression to different extents in living cells (Fursova et al. 2019) and occupy distinct regions of the genome (Gao et al. 2012; Scelfo et al. 2019). As a result of these differences, vPRC1 complexes are categorised into four groups based on biochemical similarity: PCGF1-PRC1, PCGF2/4-PRC1, PCGF3/5-PRC1 and PCGF6-PRC1 (Figure 1.2).

Of these four types of mammalian vPRC1 complex, PCGF1-PRC1 is one of two for which an equivalent complex has been purified in *Drosophila* (dRAF) (Lagarou et al. 2008). PCGF1-PRC1 contains KDM2B, a lysine-specific demethylase with activity for H3K36me2 (Tsukada et al. 2006; He et al. 2008), while dRAF contains the *Drosophila* homologue, dKDM2 (Figure 1.2). In addition to its demethylase activity, KDM2B also contains an F-box domain and a ZF-CXXC domain (Farcas et al. 2012; Blackledge et al. 2014; Inagaki et al. 2014; Wong et al. 2016). Through its F-box domain, KDM2B can interact with SKP1-CUL1 E3 ubiquitin ligase complexes which target substrates for degradation, although only SKP1 appears to co-purify with other PCGF1-PRC1 subunits, and the contribution this interaction makes to PRC1 function is not known (Gearhart et al. 2006; Sanchez et al. 2007). Via its ZF-CXXC domain, KDM2B binds specifically to non-methylated CpG dinucleotides and is enriched at CGIs in the genome (see section 1.1.3, Farcas et al. 2012; Wu et al. 2013; He et al. 2013; Koyama-Nasu et al. 2007; Yamagishi et al. 2008). The function of the dRAF complex is less clear as there is no DNA methylation in *Drosophila*. dKDM2 does, however, contribute to removal of H3K36me2 and increases PRC1 catalytic activity (Lagarou et al. 2008). PCGF1-PRC1 complexes also incorporate BCOR or BCORL1, which generate a binding interface which is required for the interaction of KDM2B with PCGF1 (Wong et al. 2016; Rose et al. 2016).

PCGF2/4, which predominately have been characterised as forming cPRC1 complexes, have also been found to form vPRC1 complexes consisting of RING1A/B, PCGF2/4 and RYBP/YAF2 (Figure 1.2 and Gao et al. 2012; Tavares et al. 2012; Hauri et al. 2016). Interestingly, unlike all other vPRC1 complexes, PCGF2/4-vPRC1 complexes do not copurify with any additional subunits, at least in cell lines where these complexes have been characterised. As such, their biological relevance remains unclear.

PCGF3/5-PRC1 complexes incorporate a number of additional subunits, although the contribution of these additional factors to PRC1 function remains largely unstudied (Figure 1.2). AUTS2-containing PCGF3/5-PRC1 complexes appear to have an activating effect on transcription, in contrast to the otherwise repressive nature of the Polycomb system (Gao et al. 2014). Furthermore, AUTS2 incorporation leads to interaction with P300, a transcriptional co-activator, and Casein Kinase 2 (CK2), which was found to inhibit the E3 ubiquitin ligase activity of PRC1 (Gao et al. 2014). Similarly, PCGF3 has been shown to interact with the sequence-specific transcription factor USF1, which has been previously linked with active chromatin (Pognonec and Roeder 1991), and to be present at the promoters of some actively transcribed genes (Scelfo et al. 2019), suggesting this might function as a mechanism by which to target this activatory PRC1 complex to specific sites. In spite of this activatory role, PCGF3/5 have also been found to contribute to deposition of H2AK119ub1 genome-wide regardless of context (Fursova et al. 2019), presumably representing a complex lacking AUTS2 and CK2. Furthermore, PCGF3/5-PRC1 complexes can also interact with the protein hnRNPK, which itself binds to Xist RNA, serving to recruit PRC1 to the X chromosome during X inactivation, and mediating Polycomb-dependent silencing on the X chromosome (Almeida et al. 2017; Pintacuda et al. 2017). In *Drosophila*, the *l(3)73ah* gene was initially described as having significant homology to *Psc* and *Suz(2)* (Irminger-Finger and Nöthiger 1995), and was subsequently found to encode a homologue of PCGF3 with similar functions in depositing genome-wide H2AK118ub1 (Lee et al. 2015).

Similarly to PCGF3/5-PRC1 complexes, PCGF6-PRC1 complexes incorporate a large number of subunits, not all of which have been extensively characterised in the context of the Polycomb system (Figure 1.2). PCGF6 was first identified as a subunit of a complex containing the sequence-specific transcription factor E2F6 (Ogawa et al. 2002). It was later co-purified with L3MBTL2, a protein with a preference for binding mono- and dimethylated lysine residues and linked to gene repression (Bonasio et al. 2010; Trojer et al. 2011; Qin et al. 2012), and CBX1/3 (HP1 β/γ), which

bind to H3K9 methylation and also repress transcription (Kloet et al. 2016; Hauri et al. 2016). PCGF6 also interacts with the MGA/MAX heterodimer (Gao et al. 2012; Kloet et al. 2016; Hauri et al. 2016), which has been demonstrated to contribute to its binding to and repression of germ cell- and meiosis-specific genes in mESCs (Endoh et al. 2017). E2F6 and MGA/MAX appear to be the major factors contributing to chromatin occupancy of PCGF6, as measured by ChIP in mESCs, and there PCGF6 appears to be able to function with these factors independently of RING1 proteins (Endoh et al. 2017; Scelfo et al. 2019).

1.2.4 PRC2 core subunits

PRC2 complexes are formed around a well conserved set of core subunits, which, unlike PRC1, have undergone comparatively little expansion in mammals (reviewed in Mierlo et al. 2019b). PRC2 functions as a lysine methyltransferase, catalysing deposition of H3K27 mono-, di- and trimethylation, this activity being provided by the SET domain of the mutually exclusive enhancer of zeste (EZH) 1/2 subunits (E(Z) in *Drosophila*) (Cao et al. 2002). These proteins have relatively little catalytic activity alone *in vitro*, but form an active complex when bound to the remaining core subunits: embryonic ectoderm development (EED, and extra sex combs (ESC) or extra sex combs like (ESCL) in *Drosophila*), suppressor of zeste (SUZ12, and SU(Z)12 in *Drosophila*), and both RBBP4 and 7 (CAF1 in *Drosophila*) (Figure 1.3).

EZH1/2 are broadly similar in catalytic function, both catalysing H3K27me_{1/2/3} deposition, although they are differentially expressed during development (Shen et al. 2008; Ezhkova et al. 2011). However, EZH1- and EZH2-containing complexes appear to serve different functions, having different levels of catalytic activity (Margueron et al. 2008; Shen et al. 2008) and occupying different genomic loci in some cell types (Mousavi et al. 2012), suggesting that control of which subunit is expressed may allow for a further level of regulation. EED in mammals is expressed as four different isoforms, although in this case, the difference in their functions, if any, is not apparent (Kuzmichev et al. 2004; Martin et al. 2006). EED plays a key role in PRC2 function, both mediating the structure of the complex (Jiao and Liu 2015), as well as by enhancing the catalytic activity of the EZH protein through binding to H3K27me₃, causing a conformational change in the stimulation-response-motif in EZH2 (Hansen et al. 2008; Margueron et al. 2009; Jiao and Liu 2015; Justin et al. 2016). Through this binding, EED is therefore thought to mediate spreading of H3K27me₃ domains, an action which is supported by observations that H3K27me₃

binding can potentiate deposition of methylation on neighbouring nucleosomes (Yuan et al. 2012; Jiao and Liu 2015; Poepsel et al. 2018). Live-cell measurements of PRC2 dynamics have revealed, however, that H3K27me3 binding by EED only makes a minor contribution to PRC2 chromatin binding, resulting in a slight stabilisation of binding (Youmans et al. 2018).

SUZ12 also appears to play an essential role in coordinating the structure of EZH1/2 to enable their methyltransferase activity (Pasini et al. 2004), as well as mediating interactions between both core and auxiliary PRC2 subunits (Jiao and Liu 2015; Justin et al. 2016). SUZ12, along with other core PRC2 subunits, has also been found to be able to mediate interactions between PRC2 and RNA with relatively little specificity (Davidovich et al. 2013; Davidovich et al. 2015; Wang et al. 2017b). This binding has been shown to both inhibit catalytic activity of the complex (Cifuentes-Rojas et al. 2014; Herzog et al. 2014; Kaneko et al. 2014; Zhang et al. 2019) and to remove or compete for its binding to chromatin (Herzog et al. 2014; Beltran et al. 2016) and has been recently linked to the presence of G-tract DNA, which may produce RNA which forms G4 structures (Wang et al. 2017b; Beltran et al. 2019).

RBBP4/7 (or RbAp46/48) are histone-binding proteins which are incorporated into several histone modifying complexes, including a number of histone deacetylase complexes (Zhang et al. 1999; Nicolas et al. 2000; Korenjak et al. 2004), the histone acetylase complexes HAT1 and CBP (Parthun et al. 1996; Verreault et al. 1998; Zhang et al. 2000), the nucleosome remodelling complex NURF (Martinez-Balbas et al. 1998; Barak 2003), and the chromatin assembly factor-1 complex (Verreault et al. 1996; Kaufman et al. 1997), in addition to PRC2 (Kuzmichev et al. 2004). In PRC2, inclusion of these subunits serves to increase methyltransferase of the complex (Cao and Zhang 2004), although, given that RBBP4/7 are thought to only be able to bind to histone H3-H4 tetramers when they are not incorporated into nucleosomes, the mechanism for this is unclear (Verreault et al. 1996; Murzina et al. 2008). However, it has been found that mutation of the *Drosophila* homologue *Caf1* enhances the previously described *Pc* mutation when both mutations are present, indicating that these proteins do contribute to the developmental function of PRC2 (Anderson et al. 2011).

1.2.5 PRC2 auxiliary subunits

Besides its core subunits, PRC2 can also incorporate a small number of auxiliary subunits, present at substoichiometric levels. The mutually exclusive nature of the two sets of auxiliary

subunits that may be incorporated has led to the two forms of PRC2 being named PRC2.1 and PRC2.2. PRC2.1 complexes contain one of three PCL proteins (PCL1-3, also called PHF1, MTF2 and PHF19, homologues of *Drosophila* PCL), and one of EPOP or PALI1/2 (C17ORF96 and C10ORF12/LCOR or LCORL1, respectively), while PRC2.2 complexes contain JARID2 and AEBP2 (homologues of *Drosophila* JARID2 and JING, respectively) (Figure 1.3 and Li et al. 2011; Smits et al. 2012; Alekseyenko et al. 2014; Liefke et al. 2016; Hauri et al. 2016; Grijzenhout et al. 2016; Mierlo et al. 2019a).

The PCL proteins have been characterised as having a binding preference for unmethylated CpG dinucleotides, and function to allow PRC2.1 complexes to bind to CGIs (Casanova et al. 2011; Li et al. 2017; Perino et al. 2018). Characterisation of the DNA binding by the winged-helix domain of PCL2 indicates that it preferentially binds regions with reduced helical twist, and this has been suggested as a mechanism to allow the complex be enriched specifically at a subset of CGIs (Perino et al. 2018). DNA binding by PCL1 has also been proposed to increase the residence time of PRC2 on chromatin (Choi et al. 2017), and enhances PRC2 catalytic activity, potentially through this stabilisation (Sarma et al. 2008). PCL proteins also contain PHD domains, with PCL1 having been found to bind to H3K36me3 (Musselman et al. 2012). H3K36me3 is known to inhibit PRC2 methyltransferase activity (Yuan et al. 2011), and this binding may therefore represent a role of PCL proteins independent of PRC2.

EPOP does not appear to be required for PRC2 catalytic activity, but mediates interactions between PRC2.1 and EloB/C (Beringer et al. 2016). EloB/C interact with EloA, a positive regulator of RNA polymerase II (RNAPII) elongation (Aso et al. 1995; Liefke et al. 2016), and this interaction is proposed to allow for maintenance of low level transcription at otherwise Polycomb-repressed genes to maintain plasticity in gene expression in pluripotent stem cells. Consistent with this, removal of EPOP/EloB/C results in increased PRC2 occupancy at target genes (Beringer et al. 2016). Furthermore, PRC2 can monomethylate EloA, which is responsible for repression of a subset of PRC2 target genes, representing one of a limited number of examples of known mechanisms by which the Polycomb system represses transcription (Ardehali et al. 2017). The function of PALI1/2 is less clear, although PALI1 appears to moderately enhance PRC2 methyltransferase activity (Conway et al. 2018).

In PRC2.2 complexes, AEBP2 and JARID2 function together to increase PRC2 catalytic activity

(Son et al. 2013). This may, in part, be a result of JARID2 methylation on K116 being bound by EED, causing allosteric activation (Sanulli et al. 2015; Justin et al. 2016). Similarly, JARID2 can mimic an unfolded H3 histone tail in an interaction with RBBP7, which may act to stabilise the complex, thereby enhancing its overall activity, as well as stimulating PRC2 nucleosome binding (Kasinath et al. 2018; Lee et al. 2018). In addition to contributing to PRC2 catalytic activity, JARID2 also is able to bind to H2AK119ub1 deposited by PRC1 and thereby enrich PRC2 at sites already enriched with PRC1 binding and H2AK119ub1 deposition (Cooper et al. 2014; Kalb et al. 2014; Cooper et al. 2016; Grijzenhout et al. 2016).

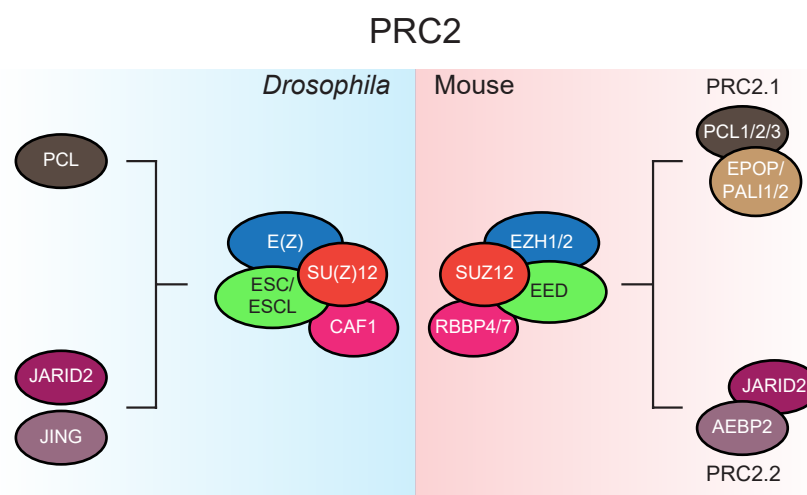


Figure 1.3: PRC2 complexes in *Drosophila* and mouse

Subunits of the characterised *Drosophila* (left) and mouse (right) PRC2 complexes. Biochemically similar complexes between *Drosophila* and mouse are shown in line with each other. Mouse PRC2 complexes are named PRC2.1/2 according to subunit incorporation. The names of mutually exclusive but related subunits are separated by a slash within the same object, with the exception of EPOP and PALI1/2, which are not related but are mutually exclusive.

1.2.6 Achieving Polycomb-mediated repression

Although the repressive functionality of the Polycomb system has been known almost since PcG genes were first discovered, the precise mechanisms by which targeted repression of specific genes is achieved by the system has remained elusive. Repression of transcription at specific genomic loci must necessarily entail at least two basic processes: some mechanism for repressing transcription, and a way to localise this repressive mechanism to target sites. Below, I will discuss our current understanding of these two processes for the Polycomb system.

1.2.7 Polycomb system targeting

In order to ensure that the Polycomb repressive system functions at the correct genes at the correct time, mechanisms to target specific genomic sites are required. There are two major ways in which the Polycomb system is targeted, through binding of the complexes to sites where the repressive activity is needed, and by restricting the repressive activity at sites where it is not needed.

In *Drosophila*, enrichment of the Polycomb system at target sites was first identified as requiring DNA elements termed Polycomb response elements (PREs) (Müller and Bienz 1991; Fauvarque and Dura 1993; Simon et al. 1993; Chan et al. 1994; Chiang et al. 1995; Gindhart and Kaufman 1995; Hagstrom et al. 1997; Mihaly et al. 1997; Busturia et al. 1997; Shimell et al. 2000; Maurange and Paro 2002). These regulatory elements contain motifs for several DNA binding proteins, including GAGA factor (GAF), Pipsqueak (PSQ), Zeste (Z), SPPS (SP1/KLF), Combgap (CG), Dorsal switch protein (DSP1), Grainyhead (GH), and Pleiohomeotic (PHO) and its homologue PHO-like (PHOL) (Huang et al. 2002; Ringrose et al. 2003; Déjardin et al. 2005; Blastyak et al. 2006; Brown and Kassis 2010; Ray et al. 2016). Notably, no component of *Drosophila* PRC1/2 has been identified with specific DNA binding. However, screens for mutations which induce Polycomb phenotypes also identified an additional complex, PhoRC, composed of zinc-finger protein PHO, one of the proteins with motifs occurring in PREs, and dSFMBT (Gehring 1970; Girton and Jeon 1994; Brown et al. 1998; Klymenko et al. 2006). This complex has no enzymatic activity inherent to it, but genome-wide characterisation of its genomic binding sites revealed that it colocalises with PREs, PcG proteins and H3K27me3 throughout the genome (Oktaba et al. 2008; Schuettengruber et al. 2009). Although a direct interaction between PRC1 and PhoRC was proposed (Mohd-Sarip et al. 2002), this link was subsequently disregarded because removal of PRC2 was observed to result in a loss of PRC1 chromatin binding (Wang et al. 2004b). Instead, a hierarchical model of Polycomb recruitment was proposed, with PRC2 binding first and placing H3K27me3, which is then bound by PRC1 through PC.

Initial efforts to understand Polycomb targeting in mammals focussed on attempting to identify the mammalian equivalent of the *Drosophila* PRE (summarised in Bauer et al. 2015). Despite the ability of YY1, the mammalian homologue of *Drosophila* PHO, to rescue the *Pho* mutant phenotype and Polycomb repression (Atchison et al. 2003), YY1 binding was found to overlap poorly with mammalian Polycomb proteins (Mendenhall et al. 2010), suggesting that it is not

a major determinant of Polycomb binding in mammals. Interactions of PRC1 with REST and RUNX1 (Dietrich et al. 2012; Yu et al. 2012), and of PRC2 with SNAIL1 (Figure 1.4 A and Herranz et al. 2008), all of which have well characterised DNA binding, only served to explain a small portion of Polycomb chromatin binding. As a result of a lack of an overarching principle for Polycomb targeting in mammals, the hierarchical recruitment model of PRC2 followed by PRC1, first proposed in *Drosophila*, initially remained the paradigm for Polycomb targeting in mammals. This was on the basis of studies in mammalian cells which also observed PRC2-dependent PRC1 chromatin binding (Figure 1.4 C and Boyer et al. 2006; Agger et al. 2007; Lee et al. 2007; Mujtaba et al. 2008).

Attempts to identify mammalian PREs, however, led to an observation that Polycomb chromatin occupancy correlated strongly with the presence of CGIs, a feature not present in *Drosophila*, more than specific sequence motifs (Boyer et al. 2006; Tanay et al. 2007; Mendenhall et al. 2010; Lynch et al. 2011). Subsequently, subunits of both PRC1 and PRC2 which preferentially bind to non-methylated CGIs were identified, revealing a new mechanism for Polycomb targeting (Figure 1.4 B). KDM2B was identified as a subunit of PCGF1-PRC1 which specifically binds non-methylated CpG dinucleotides and is sufficient to initiate PRC1 binding and repression (Farcas et al. 2012; Blackledge et al. 2014). This demonstrated a mechanism by which PRC1 could be targeted to chromatin independently of PRC2 and H3K27me₃, in line with a number of prior observations which had demonstrated PRC2-independent chromatin binding of PRC1 (Schoeftner et al. 2006; Pasini et al. 2007; Leeb et al. 2010; Tavares et al. 2012). Additionally, PCGF3- and PCGF6-PRC1 have both been found to contain sequence specific transcription factors (see section 1.2.3), giving vPRC1 complexes a number of mechanisms of interacting with chromatin independently of PRC2 (Figure 1.4 A).

PCL proteins and JARID2 were also identified as DNA-binding subunits of PRC2 with affinity for CGIs (Casanova et al. 2011; Li et al. 2017; Perino et al. 2018; Kim et al. 2003; Li et al. 2010). Although JARID2 was found to be important for PRC2 chromatin binding (Pasini et al. 2010; Landeira et al. 2010), it has subsequently been shown that the ubiquitin-interacting motif (UIM) of the protein allows it to bind H2AK119ub1 and is required to mediate PRC2 chromatin binding (Figure 1.4 C and Kalb et al. 2014; Cooper et al. 2016), suggesting that JARID2 DNA binding does not play a major role in PRC2 targeting. The finding that JARID2 is also able to mediate PRC1-dependent chromatin binding of PRC2, however, reveals that the mechanisms by

which the mammalian Polycomb system is targeted are not as straightforward as was assumed from *Drosophila*. Following these discoveries, further work in *Drosophila* has revealed that PRC1-dependent PRC2 binding is also present (Kahn et al. 2016) and that PRC1 can in fact directly interact with PhoRC through the SAM domains of SCM and dSFMBT (Frey et al. 2016), providing a mechanism for similar PRC1-initiated mechanisms for establishment of Polycomb binding. These discoveries thus complemented prior observations that PRC1 could be found at genomic sites lacking detectable PRC2 (Schwartz et al. 2006; Schaaf et al. 2013; Loubiere et al. 2016). The finding that PRC1 is capable of reciprocally stabilising PhoRC chromatin occupancy adds a further layer of complexity to the previously simple understanding of the *Drosophila* system for targeting Polycomb (Kahn et al. 2014; Schuettengruber et al. 2014).

PCL proteins allow PRC2 to bind to chromatin independently of PRC1 through DNA binding (Figure 1.4 B). Removal of PCL proteins results in reduced PRC2 occupancy (Casanova et al. 2011; Hunkapiller et al. 2012; Li et al. 2017; Perino et al. 2018). This PRC1-independent chromatin binding capability may therefore explain remaining PRC2 chromatin occupancy in the absence of PRC1 (Blackledge et al. 2014; Rose et al. 2016; Fursova et al. 2019; Blackledge et al. 2019).

DNA-based mechanisms for both PRC1- and PRC2-initiated Polycomb chromatin binding have therefore been described in mammals, as well as mechanisms by which each complex supports the other's binding through histone modifications. Binding of H2AK119ub1 by vPRC1 subunit RYBP (see section 1.2.3) and H3K27me3 by core PRC2 subunit EED (see section 1.2.4) also provide mechanisms for each complex to reinforce its own binding through deposition of histone modifications (Figure 1.4 C). Mutation of residues in RYBP involved in binding H2AK119ub1 lead to a loss of enrichment of vPRC1 on the inactive X chromosome during X inactivation (Almeida et al. 2017), although the role played by this binding on autosomes has not been studied. H3K27me3 binding by EED is important for allosteric activation of EZH1/2 methyltransferase activity (see section 1.2.4), but a study measuring PRC2 chromatin binding dynamics *in vivo* observed only a minor reduction in chromatin binding stability (Youmans et al. 2018). The small reduction in binding stability, however, occurred with a near-complete loss of PRC2 enrichment at an artificial CGI element. Therefore, the affinity of both Polycomb complexes for their own histone modification may play an important role in enriching complex binding at target sites.

In addition to DNA and histone modifications, RNA can also function to target Polycomb proteins. In X chromosome inactivation, PCGF3/5-PRC1 complexes are enriched on the X chromosome being silenced through interactions with hnRNPk, which, in turn, binds to the Xist long non-coding RNA (lncRNA) (Almeida et al. 2017; Pintacuda et al. 2017). Another lncRNA, HOTAIR, has also been associated with SUZ12 binding at the HoxD gene cluster (Rinn et al. 2007), and other specific non-coding RNAs might function similarly to target Polycomb binding (Pandey et al. 2008), although less specific RNA binding appears to antagonise Polycomb repression (discussed below).

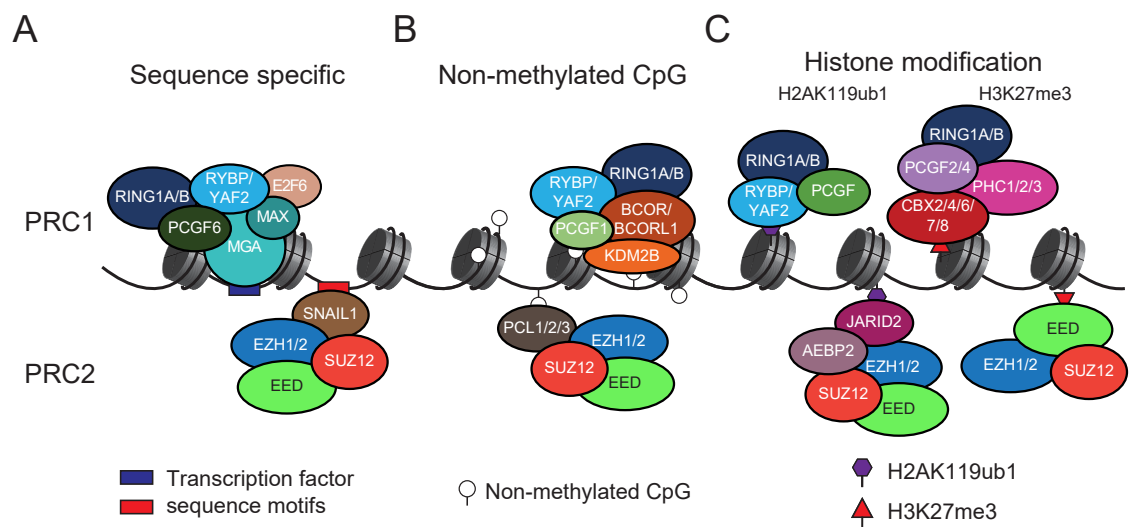


Figure 1.4: Mammalian Polycomb complexes can interact with chromatin by diverse means

A schematic of the major known mechanisms of PRC1 (above) and PRC2 (below) chromatin binding, divided according to the chromatin feature bound: (A) transcription factor binding to sequence motifs; (B) binding to non-methylated CpG dinucleotides; (C) binding to histone modifications, particularly those deposited by Polycomb complexes. Some complex subunits have been omitted for clarity.

1.2.8 Constraining Polycomb activity

Although a number of mechanisms for targeting Polycomb complexes to specific genomic sites are known, it is clear that Polycomb complexes also bind to regions of the genome outside these sites. PRC1 chromatin occupancy has been observed at low levels at all CGIs (Farcas et al. 2012), and this has been proposed as a possible method by which PRC1 might sample the genome for sites at which to initiate Polycomb-mediated repression (Klose et al. 2013). Furthermore, H2AK119ub1, deposited by PRC1, is present on approximately 10% of mammalian H2A histones (Albright et al. 1979; Matsui et al. 1979; Fursova et al. 2019), as is H3K27me3 on H3 histones, while H3K27me1/2 together account for a further 75% (Jung et al. 2010; Ferrari et al. 2014; Lee et al. 2015). This demonstrated that these complexes can engage in catalytically productive

interactions with the genome away from high-occupancy target sites, as these sites account for a relatively small proportion of the genome. It is not clear what the function of these genome-wide histone modifications is, although there is some suggestion that they may function to repress pervasive transcription (Lee et al. 2015). Although it is clear that both PRC1 and PRC2 must engage broadly with the genome, it is not known what mechanisms are driving this engagement. Recent work has shown that, in cells where all PRC2 auxiliary subunits with targeting functions have been removed, there is a loss of H3K27me3 at target sites, but little effect on the bulk histone modification occurring across the genome (Højfeldt et al. 2019; Healy et al. 2019), suggesting that this apparently promiscuous activity may be the result of a core function of these complexes.

Although there is no evidence for mechanisms that limit PRC1 catalytic activity at sites where repression would be inappropriate, there is evidence for displacement of the complex from chromatin by a TrxG system component, the SWI/SNF (or BAF) complex (Kadoch et al. 2016; Stanton et al. 2016), as well as for removal of H2AK119ub1 by histone deubiquitylases, most notably BAP1 (Scheuermann et al. 2010; Belle and Nijnik 2014). PRC2, conversely, is directly inhibited by histone modifications associated with active chromatin, namely H3K4 and H3K36 methylation, both of which are deposited by TrxG complexes (Schmitges et al. 2011; Yuan et al. 2011). Additionally, the TrxG MLL3/4 complexes, which are responsible for depositing H3K4me1 at enhancers (Hu et al. 2013), also incorporate the histone demethylase UTX, which removes H3K27me3 (Issaeva et al. 2006; Agger et al. 2007). Absence of H3K27 methylation allows CBP/P300, a histone acetyltransferase, to deposit H3K27ac (Schiltz et al. 1999; Weinert et al. 2018). This acts as a block on deposition of H3K27 methylation, as the two modifications are mutually exclusive.

There is also evidence that non-specific RNA binding by a number of PRC2 subunits can lead to sequestering of PRC2 from chromatin and inhibition of its methyltransferase activity in regions where RNA with appropriate features is being transcribed (Kanhere et al. 2010; Cifuentes-Rojas et al. 2014; Herzog et al. 2014; Kaneko et al. 2014; Beltran et al. 2016; Beltran et al. 2019). This indicates that general features of transcription can work to oppose PRC2 occupancy and methyltransferase activity, and, together with mechanisms for evicting PRC1, will aid in targeting Polycomb complexes by keeping them away from inappropriate genomic loci.

Polycomb complexes therefore have several mechanisms by which they can interact with

chromatin, through binding both DNA and nucleosomes, as well as mechanisms which work to counteract occupancy or activity of the complexes. Although the individual contributions of different Polycomb complexes to repression are being determined, a unifying model of how these interactions contribute together to enable the system to function correctly has yet to be described.

1.2.9 Polycomb-mediated repression

Although much has been learned regarding features of the Polycomb system, surprisingly little is understood about how repression of transcription is actually achieved. A number of functions of Polycomb proteins have been observed to contribute to some degree to gene repression, but there is currently no unified understanding of how Polycomb proteins work together to repress transcription.

One attractive mechanism by which the Polycomb system might repress transcription would be through the establishment of a chromatin environment at the promoters of genes to be repressed which is inhibitory to transcription. Given that both PRC1 and PRC2 have histone modifying activity, an obvious mechanism for repression would be deposition of H2AK119ub1 and H3K27me3. Although some earlier studies suggested that H2AK119ub1 might directly be related to transcriptional repression through means including preventing H3K4 methylation, restraining RNAPII at promoters, and inhibiting RNAPII elongation (Stock et al. 2007; Nakagawa et al. 2008; Zhou et al. 2008), later studies using point mutants (I53A in RING1B and I48A in SCE) which affect PRC1 ubiquitin E3 ligase activity suggested that H2AK118/9ub1 was not essential for development (Eskeland et al. 2010; Illingworth et al. 2015; Kundu et al. 2017; Pengelly et al. 2015). Crucially, however, these later studies employed only a single point mutation, for which it is now clear that it is not sufficient to fully remove PRC1 catalytic activity, requiring instead the mutation of two amino acids to achieve a full loss of catalytic activity in vertebrates (Buchwald et al. 2006; Tsuboi et al. 2018; Blackledge et al. 2019). In the presence of both mutations, gene regulation is clearly disrupted, as is mouse development (Tsuboi et al. 2018, Blackledge et al. 2019, and Klose lab, unpublished), indicating that PRC1 catalytic activity and likely H2AK119ub1 are involved, directly or indirectly, in Polycomb-mediated repression.

PRC1 also appears to compete with the SWI/SNF complexes for chromatin occupancy. While SWI/SNF can evict PRC1 (see section 1.2.8), PRC1 also appears to be capable of generating a chromatin state which prevents SWI/SNF binding, preventing chromatin remodelling necessary

for active transcription (Shao et al. 1999; Francis et al. 2001). Polycomb-repressed promoters also have been found to have a higher nucleosome density which is dependent on PRC1, suggesting the complex is playing roles in modifying the chromatin environment of promoters to influence transcription (King et al. 2018).

PRC2 H3K27 methyltransferase activity is also an attractive mechanism for repression. H3K27 methylation and acetylation are antagonistic modifications, with each only possible in the absence of the other. H3K27ac is associated with active transcription, and the presence of H3K27 methylation may therefore interfere with processes involved in transcription. Furthermore, mutation of H3K27 in *Drosophila* results in losses of gene repression and homeotic transformation similar to mutation of PRC2 itself, while deletion of ESC, the *Drosophila* methyltransferase, results in decreased RNAPII pausing at Polycomb target genes (Pengelly et al. 2013; Chopra et al. 2011). Together, this suggests that the modification is also involved in mediating repression, although this could indirectly result from downstream functions of the modification, such as mediating PRC1 and PRC2 chromatin binding. H3K27me2, which is present on 70% of histone H3, as well as H2AK118ub1, located away from Polycomb target sites, also may play a role in repressing pervasive transcription across the genome in *Drosophila* (Ferrari et al. 2014; Lee et al. 2015).

Polycomb complexes have also been found to be able to compact chromatin. This activity has been described for PRC2 as a whole (Margueron et al. 2008; Terranova et al. 2008), and may relate to the preference of PRC2 to interact with adjacent nucleosomes in close proximity to the currently bound nucleosome (Yuan et al. 2012; Jiao and Liu 2015; Poepsel et al. 2018; Leicher et al. 2019). Chromatin compaction by PRC1 has been well studied and appears to be mediated by cPRC1 subunits. While this appears to be largely mediated by PSC in *Drosophila*, in mammals, this function appears to be present instead in the PcG CBX proteins (excluding CBX7) (Francis et al. 2004; Eskeland et al. 2010; Grau et al. 2011; Lau et al. 2017). The same domain involved in compaction has recently been found to mediate phase separation of these proteins and is important for the formation of subnuclear clusters of Polycomb proteins known as Polycomb bodies (Tatavosian et al. 2018b; Plys et al. 2019). Polycomb bodies also appear to be dependent on SAM domain polymerisation of PHC proteins, and are known to incorporate strong Polycomb target sites, suggesting that they may serve some function in repression (Isono et al. 2013; Grimaud et al. 2006b). Phase separation of regions of high Polycomb occupancy may

serve as a mechanism for excluding factors associated with active transcription and maintaining repression. Interestingly, these subnuclear clusters are observed in a range of mammalian cells as well as in *Drosophila*, although it is not yet known whether they represent phase condensates in all cell types (Messmer et al. 1992; Buchenau et al. 1998; Saurin et al. 1998; Terranova et al. 2008). Removal of PCGF2/4 in mESCs, surprisingly, has a very limited effect on transcription (Fursova et al. 2019), suggesting that the structural roles mediated by cPRC1 complexes may not be of direct relevance to transcriptional repression in all cell types.

Polycomb chromatin domains have also been observed to interact in three dimensions over long linear distances in both mammals and flies (Lanzuolo et al. 2007; Tiwari et al. 2008; Comet et al. 2011; Gonzalez et al. 2014; Schoenfelder et al. 2015). The function of these interactions remains unclear, as does whether these interactions appear as Polycomb bodies in cells. Depletion of cohesin, which antagonises formation of these interactions, results in stronger Polycomb repression suggesting that these interactions may be involved in maintaining repression.

Other mechanisms by which Polycomb complexes might repress transcription have also been identified. This includes the interaction between PRC2.1 and EPOP, through which PRC2 appears to be able to repress transcription by monomethylation of EloA (see section 1.2.5), as well as binding and inhibition of CBP histone acetyltransferase activity in *Drosophila* (Tie et al. 2016).

Although a wide variety of possible mechanisms by which the Polycomb system might achieve repression have been identified, current knowledge of the system still resembles a collection of disparate observations, rather than a cohesive model for repression. As such, it remains unclear how Polycomb proteins function together to produce transcription repression.

1.3 Quantitative measurement of chromatin binding

Although we have discovered an extensive array of mechanisms by which Polycomb complexes can interact with chromatin, we still do not fully understand how these mechanisms interact to give a functioning system by which Polycomb repression is targeted to the correct sites. Understanding how Polycomb proteins locate and interact with target sites will provide a major advance in our knowledge of how the Polycomb system functions. To be able to further develop our understanding of Polycomb-chromatin interactions, it will be necessary to develop a model which can integrate our existing knowledge of individual mechanisms. Doing so will require quantitative descriptions

of Polycomb protein interactions with chromatin which we do not yet have. Below, I will introduce the genomic approaches that are now standard in the field, and their limitations with regards to obtaining such measurements, as well as techniques which are now beginning to be applied to quantitatively measure these dynamic processes.

1.3.1 Genomic approaches

The vast majority of experimental data that we have describing Polycomb protein interactions with chromatin comes from chromatin immunoprecipitation (ChIP) techniques or similar approaches, followed by sequencing of associated DNA (or use of DNA microarrays, quantitative PCR or other similar approaches) in order to determine where in the genome the protein is binding, and to what extent it is enriched in certain regions compared to others. Although data from these techniques are indispensable for our understanding of where these proteins localise, they also have major limitations in the quantitative information they can provide. One major problem with these techniques is that they typically represent an ensemble measurement of the chromatin interactions from many cells (on the order of thousands to millions of cells), although it is becoming increasingly possible to perform genome-wide sequencing experiments with very low input (Fossli et al. 2019). As a result, the data will represent a probability map of sites of interaction, which is especially hard to interpret when information, like the amount of bound protein in each cell, is unknown. Combined knowledge of the number of genomic sites typically enriched for protein and the total number of bound proteins per cell can then provide information not accessible by genomic approaches alone, like the fraction of time that a given site might be expected to be occupied.

Other limitations of this technique arise directly from the methodology. ChIP of chromatin-associated proteins requires cells to be cross-linked using a fixative agent like formaldehyde. Efficient fixation is dependent on penetration of the fixative agent, and it has been determined that, for example, formaldehyde fixation is unable to effectively capture events lasting less than 5 s, as well as causing other artefacts which result in, for example, substantial changes in protein localisation (Schmiedeberg et al. 2009; Teves et al. 2016). Our current understanding from these experiments may, therefore, be heavily biased towards stable binding events and against short-lived interactions. Indeed, despite evidence from the widespread nature of H2AK119ub1 and H3K27 methylation that PRC1 and PRC2 can interact with the entire genome, these notionally less specific

but nonetheless catalytically productive interactions are not captured (Albright et al. 1979; Matsui et al. 1979; Fursova et al. 2019; Ferrari et al. 2014; Lee et al. 2015). New techniques, for example CUT&RUN (Skene and Henikoff 2017), do not rely on fixation, but still generate a probability map of occupancy over the time of the experiment. All of these approaches depend on antibody recognition of an epitope on the protein of interest in order to pull down or otherwise allow DNA associated with the protein of interest to be purified. They are therefore susceptible to further bias if epitope accessibility is not uniform across different genomic contexts. The sequencing component of these experiments also adds a further complication to quantitative interpretation of the data, as different regions of DNA will not be sequenced equally well, complicating comparison of signal over different regions of the genome within a sample. Furthermore, repetitive regions and other sequences which are difficult to map correctly are typically excluded from analysis entirely. Finally, by their nature, these experiments capture a static picture of chromatin interactions. It is not possible to derive information about binding times, frequency of binding, or the time it takes for a protein to locate a binding site, measurements which are needed for a complete understanding of how proteins interact with chromatin. Instead, these features are convolved into the resulting dataset of chromatin occupancy, and must be measured using an alternative approach.

1.3.2 Measuring interactions with chromatin by microscopy

Advances in techniques for fluorescently labelling proteins and super resolution microscopy have begun to make it possible to make such measurements of protein dynamics directly (reviewed in Liu and Tjian 2018). Earlier studies of protein dynamics in live cells (McNally 2000; Stenoiien et al. 2000) have made use of fluorescent proteins (Tsien 1998) and fluorescence recovery after photobleaching approaches (FRAP, Axelrod et al. 1976), which measures the recovery of fluorescence in a bleached region of the cell, the rate of which depends on the diffusion and binding kinetics of the molecule being observed. This method is particularly effective for studying proteins with long-lived binding, and has been used to reveal differences in dynamics between different nuclear proteins, for example revealing that core histone proteins have highly stable binding times (Kimura and Cook 2001), while other chromatin and DNA-binding factors often have much shorter binding times, on the order of seconds (McNally et al. 1999; McNally 2000; Cheutin et al. 2003; Voss et al. 2011). FRAP has been applied previously to study the Polycomb system in both flies and mammals, revealing largely dynamic proteins with variable proportions

of highly stably bound protein (Ficz et al. 2005; Ren et al. 2008; Fonseca et al. 2012; Steffen et al. 2013; Vandenburg et al. 2014; Almeida et al. 2017; Youmans et al. 2018). FRAP, however, is limited in the molecular detail it can provide, as it depends on measurements of population averages of a large, likely heterogeneous population of molecules. As a result, FRAP does not measure the kinetics and diffusing behaviours of individual molecules directly, instead measuring recovery as a consequence of these kinetics. Extracting information about kinetics from FRAP data is possible, but requires the use of models which necessarily make assumptions about the behaviour of the molecules (McNally 2008; Mueller et al. 2008; Mueller et al. 2010; Mazza et al. 2012). Estimates of kinetics extracted from these data are strongly influenced by choice of model, and this choice will depend on the kinetics of the proteins being investigated (Mazza et al. 2012; Steffen et al. 2012). Although it is possible, though not trivial, to determine which models fit FRAP data well, validation of the model used is only possible with additional knowledge of the kinetics of the protein in question.

Fluorescence correlation spectroscopy (FCS) is another fluorescence microscopy technique by which molecular concentrations and diffusion dynamics may be measured, relying on measurement of changes in fluorescence intensity within a diffraction limited spot (Magde et al. 1972). The rate of fluctuation of fluorescence intensity is dependent on the concentration and diffusion of the molecules being observed. However, similarly to FRAP, these measurements are indirect measurements of dynamics and are subject to population averaging. Model fitting using prior knowledge of the protein's dynamics is again required to correctly interpret FCS data (Mazza et al. 2012).

Use of approaches by which the dynamics of proteins may be observed at the level of a single molecule, therefore, will allow for more direct measurement of the kinetics of diffusion and binding of a given protein, while avoiding obfuscating the existence of subpopulations of molecules with different behaviour as a result of population averaging. While single particle tracking (SPT) has been in use since it was first employed to study Brownian motion (Brown 1828), the advent of fluorescence microscopy has since enabled it to be applied to live cells. In a system where the number of fluorescent molecules can be controlled so that individual molecules can be observed, each molecule may then be accurately localised using the centroid of its fluorescence (Heisenberg 1949; Bobroff 1986; Betzig and Chichester 1993). Tracking of successive localisations across temporally separated images allows the dynamics of the track

protein to be measured.

The earliest SPT experiments on live cells used antibodies to track cell surface proteins (Ghosh and Webb 1994), and later SPT within cells was achieved using genetically encoded fluorescent tags (Fernández-Suárez and Ting 2008). The development of genome editing techniques now makes it routine to endogenously tag proteins of interest (Doudna and Charpentier 2014), enabling quantitative measurements of dynamics in a system which has been minimally perturbed (for example Teves et al. 2016, Hansen et al. 2017, Rhodes et al. 2017). Although initial experiments within cells were limited in their capacity to image due to the requirement for low numbers of visible molecules (Elf et al. 2007; Tokunaga et al. 2008), this limitation has subsequently been relieved with the use of photoactivatable fluorophores (Patterson and Lippincott-Schwartz 2002; Betzig et al. 2006; Hess et al. 2007; Manley et al. 2008). The use of fluorescent proteins, which are heavily limited in SPT by their low photon emission and photostability, has also been largely superseded by the use of protein tags (e.g. SNAP-tag and HaloTag (Gautier et al. 2008; Los et al. 2008)) alongside brighter and more photostable synthetic dyes which are cell permeable (Grimm et al. 2015).

Development of SPT techniques has now enabled measurements of the dynamic behaviours of a number of nuclear proteins and, though measurement of interactions at defined genomic loci using current techniques remains difficult, provides an approach for characterising the nature of the dynamics of nuclear proteins not possible using genomic approaches. These approaches have already begun to be applied to the Polycomb system, specifically investigating the contribution of H3K27me3 to PRC2 and CBX protein dynamics (Youmans et al. 2018; Zhen et al. 2016; Tatavosian et al. 2018a).

1.4 Aims of this thesis

Although we now know of many mechanisms by which Polycomb proteins can interact with chromatin, it remains unclear how these mechanisms contribute, independently and together, to chromatin binding which can be observed by genomic approaches, and to the targeted occupancy which is required for appropriate regulation of transcription. A major gap remains in our existing knowledge of the Polycomb system in that the dynamics of Polycomb proteins remain largely uncharacterised. Despite knowing the genomic loci at which they appear to be enriched in

a population of cells, we lack measurements of how frequently and how stably they bind to chromatin. Quantitative measurement of Polycomb complex dynamics therefore represents a key step in advancing our understanding of the function of the Polycomb system. PRC1 remains particularly poorly studied in spite of its key roles in transcriptional regulation and essential nature, with very little work performed on vPRC1 complexes. Characterising the dynamics of Polycomb proteins in live cells, with a focus on PRC1, and measuring how they change when components of the Polycomb system are perturbed are therefore the central aims of this thesis.

In order to characterise PRC1 dynamics, I employ genome engineering to generate cell lines compatible with SPT and other live cell microscopy approaches in mESCs. Through this approach, I characterise the dynamics of core PRC1 subunit RING1B (Chapter 3). Because PRC1 complexes are heterogeneous in their mechanisms for interacting with chromatin, it is possible that subunits specific to certain complexes may present different dynamics to PRC1 as a whole. Therefore, I further characterise the dynamics of additional PRC1 subunits by the same means, to provide a view of the differences in chromatin binding and diffusion of different PRC1 complexes (Chapter 4). In order to understand how individual mechanisms of associating with chromatin might contribute to PRC1 dynamics, I employ systems in which I can perturb features of the Polycomb system which have previously been characterised to disrupt chromatin binding of Polycomb proteins. To determine how Polycomb mediated histone modifications contribute to Polycomb chromatin binding, I characterise the changes in Polycomb protein dynamics following loss of H2AK119ub1 and H3K27me3 respectively (Chapter 5). Finally, I focus on the contribution to PRC1 dynamics made by DNA-binding vPRC1 complexes, and investigate how these functions of PCGF1- and PCGF6-PRC1 complexes contribute to PRC1 dynamics overall (Chapter 6).

MATERIALS AND METHODS

2.1 Cell lines and culture

2.1.1 Cell culture conditions

All mouse embryonic stem cell lines used in this thesis were grown at 37 °C and 5% CO₂ on gelatinised plates. Dulbecco's modified eagle medium (Gibco) was supplemented with 15% foetal bovine serum (FBS, biosera), 10 ng/ml leukemia-inhibitory factor, penicillin/streptomycin, beta-mercaptoethanol, L-glutamine and non-essential amino-acids. Cells were passaged using trypsin-EDTA (0.25%, Gibco) with 2% chicken serum.

Cells requiring feeders were plated onto gelatinised plates with mitomycin-inactivated SNLP (STO mouse fibroblasts expressing neomycin, puromycin resistance and Lif genes) as feeders.

2.1.2 Tamoxifen and dTAG-13 treatments

Tamoxifen treatments of conditional mESC lines were performed using 800 nM tamoxifen for 72 h to induce Cre-mediated recombination.

Cell lines expressing FKBP12^{F36V} fusion proteins were treated with 100 nM dTAG-13 for 96 h to induce and maintain protein degradation and depletion.

2.1.3 Embryonic stem cell lines

All mESC lines used in this study are listed in Table 2.1.

2.1.4 CRISPR guides and targeting constructs

Cell lines containing tags or loxP sites were engineered using CRISPR-mediated genome editing. Oligonucleotides encoding sgRNAs were cloned into the pSpCas9(BB)-2A-Puro plasmid as previously described (Ran et al. 2013). All guide sequences used are listed in Table 2.2.

Targeting constructs for homology directed repair (HDR) were prepared either by individual cloning of homology arms from genomic DNA extracted from mESCs and tag sequences from

MATERIALS AND METHODS

Name	Origin
RING1B-HaloTag	This study
iXist-Bgl stem-loop ₁₈ -ChrX	Derived from Nesterova et al. 2019 by Lisa Rodermund, with 18 Bgl stem-loops inserted
iXist-Bgl stem-loop ₁₈ -ChrX RING1B-HaloTag Bgl-SNAP	This study
HaloTag-CBX7	This study
CBX7-HaloTag	This study
HaloTag-RYBP	This study
HaloTag-PCGF1	This study
PCGF2-HaloTag	This study
HaloTag-PCGF3	This study
HaloTag-PCGF6	This study
PRC1 ^{CPM}	Blackledge et al. 2019
PRC1 ^{CPM} RING1B-HaloTag	This study
PRC1 ^{CPM} HaloTag-SUZ12	This study
PRC1 ^{CPM} RING1B-HaloTag FKBP12 ^{F36V} -SUZ12	This study
PCGF2-HaloTag FKBP12 ^{F36V} -SUZ12	This study
HaloTag-PCGF6 FKBP12 ^{F36V} -SUZ12	This study
<i>Kdm2b-CXXC^{fl/fl}; ROSA26^{Cre-ERT2}</i>	This study
<i>Kdm2b-CXXC^{fl/fl}; ROSA26^{Cre-ERT2}</i> RING1B-HaloTag	This study
<i>Kdm2b-CXXC^{fl/fl}; ROSA26^{Cre-ERT2}</i> HaloTag-PCGF1	This study
<i>Pcgf1/2/3/5^{fl/fl}; Pcgf4^{-/-}; ROSA26^{Cre-ERT2}</i>	Fursova et al. 2019
<i>Pcgf1/2/3/5^{fl/fl}; Pcgf4^{-/-}; ROSA26^{Cre-ERT2}</i> RING1B-HaloTag	This study

Table 2.1: mESC lines used in this study

appropriate template sequence, or were synthesised by GeneArt (ThermoFisher). Homology arms were designed to approximately match the length of the DNA sequence to be inserted. Where homology arms were cloned from template sequences, resulting PCR products were designed to contain homology overlaps compatible with Gibson assembly, and were assembled thus. Primers used to clone homology arms and tag sequences are listed in Table 2.3.

Tags were designed for either the N- or C-terminus based on previous tagging approaches, or to maximise the number of splicing isoforms that would be tagged. A *Kdm2b-CXXC^{fl/fl}* cell line was generated using an equivalent strategy to a previous study (Blackledge et al. 2014).

2.1.5 ESC transfection and screening

mESCs were transfected at approximately 60-70% confluency. Unless a different parental cell line was required, E14 mESCs were used. For CRISPR, 3 µg of guide plasmid and 10 µg

Gene	Guide sequence
<i>Ring1b</i> 3'	GCTCATTTGTGCTCCTTGGT
<i>Cbx7</i> 5'	ACAGCTCCATGCGGGGCGGC
<i>Cbx7</i> 3'	CTATTCACAGCTTCTCGTTG
<i>Rybp</i> 5'	GCCCATGGTCATGGACGGGC
<i>Pcgl1</i> 5'	GCCTCATCGCGATCGCAATC
<i>Pcgl2</i> 3'	CCCTTTCCTCAAGGGGGGCA
<i>Pcgl3</i> 5'	ATCTTCCTGGTCAACATCTT
<i>Pcgl6</i> 5'	TCTGATCCCCGCCATGGACG
<i>Suz12</i> 5'	GTGCTTCTGAGGCGCCATCG
<i>Kdm2b</i> 5' loxP insertion site	CAGCCGCCCTAGCTACAGTT
<i>Kdm2b</i> 3' loxP insertion site	CAGGAGTCGACAGCACGGCC

Table 2.2: Cas9 guide sequences used in this study

of targeting construct were transfected using Lipofectamine 3000, according to manufacturer protocol (Invitrogen). For stable integration to generate cell lines expressing either HaloTag-3xNLS or H2B-HaloTag, 10 µg of plasmid encoding appropriate the cDNA sequence was transfected in the same way. The following morning, transfected cells were passaged to new plates at a range of densities. After 5 h, selection was applied with 1 µg/ml puromycin for CRISPR or 400 µg/ml G418 for stable integration. Selection was maintained for 48 h for CRISPR and continuously to achieve stable integration.

Individual clones were isolated either by direct picking of colonies, or by FACS sorting of cells (see below) followed by colony picking where HaloTag fusions were generated. Isolated colonies were transferred to 96-well plates and positive clones identified by PCR screens of genomic DNA. PCR screens were performed using primers flanking the inserted sequence, and homozygous clones identified by an increase in product length corresponding to the desired insertion, with concomitant loss of the wild type product. Screening primers are listed in Table 2.4. Clones were validated by sequencing of PCR products, and western blot and fluorescence microscopy where appropriate.

2.1.6 FACS

Puromycin-selected cells targeted with HaloTag constructs were selected for presence of functional HaloTag protein by FACS. Cells were labelled with 500 nM Halo-TMR dye (Promega) and sorted in Fluorobrite DMEM, supplemented as described above. Cells with significantly

Purpose	Sequence
HaloTag-Linker (N-terminal) Fwd	GAAATCGGTACTGGCTTTCCATTCG
HaloTag-Linker (N-terminal) Rev	TCCGCCTCCGCCACTGCCGCCACCACCGCTTCCACCA CCTCCGCCTCCGCTACCGGAAATCTCCAGAGTAGACA G
HaloTag-Linker (C-terminal) Fwd	AGCGGAGGCGGAGGTGGTGGGAAGCGGTGGTGGCGGCA GTGGCGGAGGCGGAGAAATCGGTACTGGCTTTCCATT CG
HaloTag-Linker (C-terminal) Rev	ACCGGAAATCTCCAGAGTAGACAG
FKBP12 ^{F36V} -Linker (N-terminal) Fwd	ATGGGAGTGCAGGTGGAAACC
FKBP12 ^{F36V} -Linker (N-terminal) Rev	TCCGCCTCCGCCACTGCCGCCACCACCGCTTCCACCA CCTCCGCCTCCGCTACCTTCCAGTTTGTAGAAGCTCCA CATCGAAG
RING1B-HT 5' HA Fwd	GAAGGAAGGCCGTCAAGGCCGCATGGTTTTTTTGGC TCCATGATTAGGAAC
RING1B-HT 5' HA Rev	CGCTTCCACCACCTCCGCCTCCGCTTTTGTGCTCCT TGGTGGGT
RING1B-HT Tag Fwd	TTATGCACCCACCAAGGAGCACAAAAGCGGAGGCGG AGG
RING1B-HT Tag Rev	CTTAGAATTGACTTTTAAAAGCTCAACCGGAAATCTC CAGAGTAGACAG
RING1B-HT 3' HA Fwd	GCTGTCTACTCTGGAGATTTCCGGTTGAGCTTTTAAA AGTCAATTCTAAGACTGAAC
RING1B-HT 3' HA Rev	GAAAGGAAGGCCCATGAGGCCCAGAGAAATGAGGG GAAGCTTTGAACA
HT-CBX7 5' HA Fwd	GAAGGAAGGCCGTCAAGGCCGCATGCTGGTTTGGGT TGATGGGTG
HT-CBX7 5' HA Rev	CGAATGGAAAGCCAGTACCGATTTCATGCGGGGCG GC
HT-CBX7 Tag Fwd	GCCCACTCGCCCGCCGCCCGCATGGAAATCGGTAC TGGCTTTCCATTCG
HT-CBX7 Tag Rev	CCTGCTCGCCTATGGCTGACAGCTCTCCGCCTCCGC CACT
HT-CBX7 3' HA Fwd	TGGTGGCGGCAGTGCGGAGGCGGAGAGCTGTCAG CCATAGGCGA
HT-CBX7 3' HA Rev	GAAAGGAAGGCCCATGAGGCCCAGACATGCCTGCA CTTAAGCCAAAT
CBX7-HT 5' HA Fwd	GAAGGAAGGCCGTCAAGGCCGCATGCCACGTTTGAG ACCCAGAGA
CBX7-HT 5' HA Rev	CGCTTCCACCACCTCCGCCTCCGCTCAGCTTCTCGTT GCGGTC
CBX7-HT Tag Fwd	CTTCCGAGACCGCAACGAGAAGCTGAGCGGAGGCG GAGG

Table 2.3: Primers used to clone HDR targeting constructs

Purpose	Sequence
CBX7-HT Tag Rev	TTTAAAGAGGGGAAGCCGCTATTCAACCGGAAATCT CCAGAGTAGACAG
CBX7-HT 3' HA Fwd	GCTGTCTACTCTGGAGATTTCGGTTGAATAGCGGC TTCCCCTC
CBX7-HT 3' HA Rev	GAAAGGAAGGCCCATGAGGCCAGACTGACTGCCCA CATAACCACT
HT-RYBP 5' HA Fwd	GAAGGAAGGCCGTCAAGGCCGCATGGTCCTCTTATT GGTTGCCCCG
HT-RYBP 5' HA Rev	CGAATGGAAAGCCAGTACCGATTTCATGGACGGGC TGGCCCC
HT-RYBP Tag Fwd	GGCGCCGGGGGCCAGCCCGTCCATGGAAATCGGTAC TGGCTTTCCATTCTG
HT-RYBP Tag Rev	TCGGGCTCTTCTTGTCGCCCATGGTTCCGCCTCCGCC ACT
HT-RYBP 3' HA Fwd	TGGTGGCGGCAGTGGCGGAGGCGGAACCATGGGCG ACAAGAAGAG
HT-RYBP 3' HA Rev	CCCATGAGGCCAGAGCCCCACCTGGTTAAATCTC
HT-PCGF1 5' HA Fwd	GAAGGAAGGCCGTCAAGGCCGCATGCCTCTCGTGC ATCCATTTGT
HT-PCGF1 5' HA Rev	CGAATGGAAAGCCAGTACCGATTTCATCGCGATCG CAATCTGGC
HT-PCGF1 Tag Fwd	GGGGGGCCAGATTGCGATCGCGATGGAAATCGGTAC TGGCTTTCCATTCTG
HT-PCGF1 Tag Rev	CTGACTGGAGCTGGTTCCGAAGCCTTCCGCCTCCGC CACT
HT-PCGF1 3' HA Fwd	TGGTGGCGGCAGTGGCGGAGGCGGAAGGCTTCGGA ACCAGCTCC
HT-PCGF1 3' HA Rev	GAAAGGAAGGCCCATGAGGCCAGACTAAGGAGGG GTGAAGGGC
PCGF2-HT 5' HA Fwd	CGTCAAGGCCGCATGCCACCTTTCAATTCACTTACCT CTATCCCC
PCGF2-HT 5' HA Rev	CCTCCGCTTGGAGTGGGGGAAGTAGGATGG
PCGF2-HT Tag Fwd	CACTCCAAGCGGAGGCGGAGG
PCGF2-HT Tag Rev	CCTTTCCTCAGGAAATCTCCAGAGTAGACAGCCAG
PCGF2-HT 3' HA Fwd	GAGATTTCTGAGGAAAGGGACCCTCTCCTTC
PCGF2-HT 3' HA Rev	CCCATGAGGCCAGAGACCCACTAGTCAGCTAGGGC
HT-PCGF3 5' HA Fwd	CGTCAAGGCCGCATGGGAGCAAAGCAATATCTAGGT GGTG
HT-PCGF3 5' HA Rev	ACCGATTTCCATCTTTGGCTTCTGCAAGAAAAATAAA TACATGG
HT-PCGF3 Tag Fwd	CCAAAGATGGAAATCGGTACTGGCTTTCCATTCTG
HT-PCGF3 Tag Rev	CTGGTCAATCCGCCTCCGCCAC
HT-PCGF3 3' HA Fwd	AGGCGGATTGACCAGGAAGATTAACTCTGGGATATA AATG

Table 2.3: Primers used to clone HDR targeting constructs

Purpose	Sequence
HT-PCGF3 3' HA Rev	CCCATGAGGCCCCAGAGGACTGGCGAAAGCAAACTGT
HT-PCGF6 5' HA Fwd	CGTCAAGGCCGCGCATGGCATGTTTTGCTTGCATATATGTATGTTTCCAC
HT-PCGF6 5' HA Rev	CCGATTTCCATGGCGGGGATCAGAAAT
HT-PCGF6 Tag Fwd	CGCCATGGAAATCGGTACTGGCTTTCCATTTCG
HT-PCGF6 Tag Rev	GCCTCGTCTCCGCCTCCGCCACTG
HT-PCGF6 3' HA Fwd	AGGCGGAGACGAGGCTGAGACGGACG
HT-PCGF6 3' HA Rev	CCCATGAGGCCCCAGAGACATATAGGAACTAACAGTGAAAGGCACCTGT
HT-SUZ12 5' HA Fwd	GAAGGAAGGCCGTCAGGCCGCGCATGAAGCCACAACCAATTCCAC
HT-SUZ12 5' HA Rev	CGAATGGAAAGCCAGTACCGATTTCCATCGCGGTTCC
HT-SUZ12 Tag Fwd	GGGGGAGGAGGCAGGAACCGCGATGGAAATCGGTACTGGCTTTCCATTTCG
HT-SUZ12 Tag Rev	CCCCACCGCCGTGCTTCTGAGGCGCTCCGCCTCCGC
HT-SUZ12 3' HA Fwd	TGGTGGCGGCAGTGGCGGAGGCGGAGCGCCTCAGAGCACGG
HT-SUZ12 3' HA Rev	GAAAGGAAGGCCCATGAGGCCCAGAAGGTTTCAAA
FKBP12 ^{F36V} -SUZ12 5' HA Fwd	TGCTGCGTGTGAAGGAAGGCCGTCAGGCCGCGCATGAAGCCACAAC
FKBP12 ^{F36V} -SUZ12 5' HA Rev	CCAATTCCAC
FKBP12 ^{F36V} -SUZ12 Tag Fwd	ACTCCCATCGCGGTTCTCTGC
FKBP12 ^{F36V} -SUZ12 Tag Fwd	AACCGCGATGGGAGTGCAGGTGGAAAC

Table 2.3: Primers used to clone HDR targeting constructs

Insertion	Primer
RING1B-HaloTag	F: CTCACTGTTACTGAGCACCA R: TGAACATATCCCAGGTCTGTCC
HaloTag-CBX7	F: AACGTTGCGCAGGTTCAAAA R: CACTTTCTGCTCTGCCAAC
CBX7-HaloTag	F: CGCTCCCCTCAAGTGAAGTT R: GCCCCAAGAAGCACAGTTTT
HaloTag-RYBP	F: GACCGCGCTTCTCCTCTG R: CTCTTCTTGTCGCCCATGGT
HaloTag-PCGF1	F: CAATCCGGAGAATGTGAGGC R: GGTTACACAGCTAACGGAC
PCGF2-HaloTag	F: AGGAGGGAAAAGATGGCTGC R: CCCCAGCTACCCATCCTACT
HaloTag-PCGF3	F: GCAGCCCAGATCAGTCATCA R: AGTCACTGTGGTTGCGTCAA
HaloTag-PCGF6	F: CTTGTTTTCCGTGGCGTCC R: AGGTAGCCTAGGTGAACCACA
HaloTag-SUZ12 and FKBP12 ^{F36V} -SUZ12	F: AGCGGTTGGTGTAGCAGG R: CTTCTTCACCGGCAACACC
<i>Kdm2b</i> 5' loxP	F: TGTAGGAAGCAACCTGGCTG R: TGAGATGCCTGTGATGTCCG
<i>Kdm2b</i> 3' loxP	F: GGAGGGCAAAGTCTAAGCGT R: GGTTTAGGTGCCTTGTTGC

Table 2.4: PCR primers used to screen clones for homozygous or heterozygous tagging

higher 561 nm fluorescence than the similarly labelled parental cell line were FACS-selected. These cells were plated at low density to allow individual clones to be picked.

2.2 Protein methods

2.2.1 Acid extraction of histones

Unless otherwise stated, all centrifugation steps involving protein samples were performed at 4 °C. Histone extracts were prepared by washing pelleted cells in RSB (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂ and 20 mM NEM), followed by centrifugation at 240xg for 5 min and resuspension in RSB buffer supplemented with 0.5% NP-40. Following incubation on ice for 10 min, cells were centrifuged at 500xg for 5 minutes. The nuclear pellet was resuspended in 5 mM MgCl₂, an equal volume of 0.8 M HCl added, and incubated on ice for 20 min to extract histones. The supernatant was taken after centrifugation for 20 min at 18,000xg, and histones precipitated by addition of TCA up to 25% by volume and incubation on ice for 30 min. Histones were pelleted by centrifugation at 18,000xg for 15 min, and the pellet washed in cold acetone twice. The histone pellet was resuspended with vortexing in 1x SDS loading buffer (2% SDS, 100 mM Tris pH 6.8, 100 mM DTT, 10% glycerol and 0.1% bromophenol blue) and boiling at 95 °C for 5 min.

For histone extractions where H2AK119ub1 was required, all buffers prior to histone pelleting were supplemented with 20 mM NEM.

2.2.2 Preparation of nuclear extracts

Cell pellets were resuspended in 10 volumes of buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and cOmplete protease inhibitor (PIC, Roche)) and incubated for 10 min on ice. After centrifugation at 1500xg for 5 min, the cell pellet was resuspended in 3 volumes of buffer A supplemented with 0.1% NP-40. Following inversion, nuclei were pelleted again at 1500xg for 5 min. Pelleted nuclei were resuspended in 1 volume of buffer C (250 mM NaCl, 5 mM Hepes pH 7.9, 26% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and 1x PIC). The volume of the nuclear suspension was measured and NaCl concentration increased to 400 mM by dropwise addition. Nuclei were incubated at 4 °C for 1 h to extract nuclear proteins, which were recovered as the supernatant after centrifugation at 18,000xg for 20 min. Protein concentration was determined by Bradford assay (BioRad).

2.2.3 Immunoprecipitation

Immunoprecipitations were performed using 500 µg of nuclear extract. Extracts were diluted to 550 µL with BC150 (150 mM KCl, 10% glycerol, 50 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1x PIC) and incubated with antibody against the protein of interest overnight at 4 °C. Protein A beads (Repligen) were used to precipitated antibody-bound protein at 4 °C for 1 h. Beads were pelleted at 1000xg, and washed 3 times with BC300 (300 mM KCl, 10% glycerol, 50 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT). For subsequent western blotting, beads were resuspended in 2x SDS loading buffer and boiled at 95 °C for 5 min, and the supernatant taken as the immunoprecipitate.

2.2.4 Western blotting

Where not already done, protein extracts were mixed with SDS loading buffer and boiled at 95 °C for 5 min. Proteins were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels (0.1% SDS, 0.1% ammonium persulphate (Sigma), 0.1% TEMED (Sigma), 400 mM Tris HCl pH 8.8) were cast using the Mini-Protean Tetra Cell system (BioRad). Depending on the size of the protein of interest, resolving gel between 6% and 15% acrylamide/bis-acrylamide (BioRad) was used. Stacking gel contained 5% acrylamide/bis-acrylamide and 125 mM Tris HCl pH 6.8. Gels were run at 200 V in 1x SDS-PAGE running buffer (25mM Tris, 192mM glycine and 0.1% SDS).

Proteins were transferred to nitrocellulose membrane by semi-dry transfer using the Trans-Blot Turbo Transfer System (BioRad, 1.5 A for 7 min). Membranes were blocked in 5% milk in 1x PBS with 0.1% Tween 20 (PBST, Fisher) for 30 min at room temperate. Primary antibody incubations were carried out overnight at 4 °C in the same buffer. Membranes were washed for 3x 5 min with PBST, and then incubated with secondary antibody for 1 h in 5% milk in PBST. After 3x 5 min PBST washes and one PBS rinse, membranes were imaged using an Odyssey Fc system (LI-COR).

2.2.5 Antibodies

Table 2.5 lists the antibodies used in this study.

Antigen	Source of antibody	Dilution for western blotting
CBX7	Millipore 07-981	1:1000
FLAG	Sigma F1804	Used in IP only
H2AK119ub1	Cell signalling 8240	1:2000
H3	Cell signalling 3638	1:1000
H3K27me3	Cell signalling 9733	1:2000
HDAC1	Abcam ab109411	1:3000
KDM2B	Klose lab	1:1000
MAX	Santa Cruz SC197	1:1000
PCGF1	Klose lab	1:1000
PCGF2	Santa Cruz sc-10744	1:1000
PCGF3/5	Abcam ab201510	1:1000
PCGF6	LS Bio LS-C482495	1:1000
RING1B	Cell signalling 5694	1:2000
RING1B	Klose lab	Used in IP only
RYBP	Millipore AB3637	1:1000
SUZ12	Cell Signalling 3737	1:1000
TBP	Abcam Ab818	1:3000
Anti-mouse secondary (IRDye800)	LI-COR 32210	1:15000
Anti-rabbit secondary (IRDye680)	LI-COR 68071	1:15000

Table 2.5: Antibodies used in this study

2.2.6 Protein quantification

To quantify numbers of proteins in live cells, cells were first trypsinised and pelleted. A Countess II cell counter was used to count trypsinised cells. 5×10^6 cells were labelled with 500 nM HaloTMR for 15 min at 37 °C (previously described at near-quantitative labelling in live cells by Yoon et al. 2016). Cells were pelleted and counted again, and 3×10^6 labelled cells saved as a pellet.

HaloTag protein standard (Promega) at known concentration was labelled at 4 °C for 15 min with 5 μ M HaloTMR, a 10-fold molar excess relative to the protein standard. Pelleted cells were lysed by boiling in 1x SDS at 95 °C for 10 min and volumes equivalent to 1×10^5 and 2.5×10^5 cells were resolved by SDS-PAGE alongside known quantities of standard. Gels were imaged using a Typhoon FLA 7000 (Fujifilm) using the 532 nm laser. Band intensities were quantified using Image Studio software (LI-COR). Nuclear protein concentrations were calculated using the mean nuclear volume from segmented nuclei of RING1B-HT mESCs, as described below (Sections 2.4.2 and 2.5.7).

2.3 Chromatin immunoprecipitation

2.3.1 Preparation of chromatin

ChIP was performed as previously described (Blackledge et al. 2010; Schmidt et al. 2009), with minor modifications. 5×10^7 mESCs were fixed in 2 mM disuccinimidyl glutarate (Thermo Scientific) for 45 min, and then with added 1% formaldehyde for a further 12.5 min (methanol-free, Life Technologies). Fixation was quenched using glycine added to 125 μ M for 5 min. Cells were then pelleted at 1000xg for 5 min and washed with PBS.

The cell pellet was resuspended in LB1 buffer (50 mM HEPES-KOH pH 7.9, 140 mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton X 100 and 1x PIC) and rotated at 4 °C for 10 min. The nuclei were pelleted at 1000xg for 5 min, and then resuspended in LB2 buffer (10 mM Tris HCl pH 8.0, 200 mM NaCl, 1mM EDTA, 0.5 mM EGTA and 1x PIC) and incubated at 4 °C for 5 min. After a further centrifugation at 1000xg for 5 min, the pellet was resuspended in LB3 buffer (10 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine and 1x PIC).

Chromatin was sonicated using a BioRuptor Pico sonicator (Diagenode) at 4 °C. Sonication was performed using 25-30 cycles of 30 s on/ 30 s off at full power. The sonicated material was pelleted at 20,000xg for 20min, and the supernatant taken as chromatin. A ten-fold dilution of chromatin in 0.1 M NaOH was quantified by NanoDrop.

2.3.2 Chromatin immunoprecipitation

150 μ g chromatin was used per IP. Chromatin was diluted ten-fold in ChIP dilution buffer (1% Triton-X100, 1 mM EDTA, 20 mM Tris HCl pH 8, 150 mM NaCl and protease inhibitors) and then pre-cleared for 1 - 2 h with protein A magnetic Dynabeads (Invitrogen). Dynabeads were blocked prior to use with 500 μ g ml⁻¹ BSA and 50 μ g ml⁻¹ yeast tRNA. An input sample was taken from the precleared chromatin, and the remainder was immunoprecipitated overnight at 4 °C with 3 μ g of antibody. Antibody-bound chromatin was precipitated using blocked protein A Dynabeads. Washes were carried out for 4 min each at 4 °C, using low salt buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris HCl pH 8.1, 150mM NaCl), high salt buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20mM Tris HCl pH 8.1, 500mM NaCl), LiCl buffer (0.25 M LiCl, 1%

NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris HCl pH 8.1) and 2 washes with TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). DNA was eluted by vortexing for 30 min in elution buffer (1% SDS and 100 mM NaHCO₃), and cross-links reversed at 65 °C overnight with 200 mM NaCl and RNase treatment (1 µg, Roche). Samples were then incubated with Proteinase K (20 µg) for 1 h at 45 °C. DNA was purified using a ChIP DNA Clean & Concentrator kit (Zymo). Purified DNA was analysed using ChIP-qPCR.

2.4 Microscopy

2.4.1 Single Particle Tracking

Cells for single particle tracking were plated on gelatinised and glycine coated 35 mm petri dishes, 14 mm Microwell 1.5 coverglass dishes (MatTek, #P35G-1.5-14-C) at least 5 hours before imaging. Cells were labelled using 100 nM PA-Halo-JF₅₄₉ (Grimm et al. 2017) for 15 min at 37 °C, followed by 3 washes, changing medium to Fluorobrite DMEM (Thermo Fisher Scientific) supplemented as described for general ESC culture above. Cells were incubated for a further 30 min in supplemented Fluorobrite DMEM at 37 °C and washed once more before imaging.

Single molecule tracking experiments were carried out using a custom TIRF/HILO microscope as described in Wegel et al. 2016. The angle of the excitation beam is controlled by translation of the position of the focus in the objective (Olympus 100x NA1.4), and was positioned to maximise the signal-to-noise ratio. Sample temperature was maintained at 37 °C using an objective collar heater and heated stage, while pH of sample medium was maintained by adding HEPES, pH 7.4 to a concentration of 30 mM. For rapid tracking of diffusing and bound molecules, movies were acquired with continuous 561 nm and 405 nm excitation at 22 mW and 5 mW intensity at the fibre output, respectively, with a 15 ms exposure time. Movies were acquired for 4000 frames, and contained multiple nuclei. To measure binding times of single molecules, movies were acquired either with a frame rate of 2 frames/s and exposure time of 0.5 s, or 0.03 frames/s and exposure time of 1 s. Movies were acquired for 600 or 30 frames, respectively. Acquisition was started after activating sufficient numbers of fluorophores using the 405 nm laser and carried out with 561 nm excitation at 0.1 mW. Experiments were performed in at least three biological replicates.

For experiments also imaging Xist, cells were labelled instead for 30 min with both 100 nM PA-HaloJF₅₄₉ and 5 µM SNAP-Cell 505-Star (New England Biosciences) and incubated for 45

min following washes. Cells were imaged after 6 h induction of Xist expression with doxycycline. Tracking movies were acquired as normal, but prior to each acquisition, a short movie with 488 nm excitation was used to visualise inactive X domains.

2.4.2 Confocal microscopy and FRAP

To image Polycomb bodies and perform FRAP experiments, cells were plated on gelatinised 35 mm petri dish, 14 mm Microwell 1.5 coverglass dishes (MatTek, #P35G-1.5-14-C) at least 5 hours before imaging. Prior to imaging, cells were labelled with 500 nm Halo-JF₅₄₉ for 15 min at 37 °C, followed by 3 washes, changing medium to Fluorobrite DMEM (Thermo Fisher Scientific) supplemented as described for general ESC culture above. Cells were incubated for a further 30 min in supplemented Fluorobrite DMEM, and with added 10 µg ml⁻¹ Hoechst 33258 (Thermo Fisher Scientific) for Polycomb body imaging, at 37 °C and washed once more before imaging.

Confocal microscopy was performed on an IX81 Olympus microscope connected to a Spinning Disk Confocal system (UltraView VoX PerkinElmer) using an EMCCD camera (ImagEM, Hamamatsu Photonics) in a 37 °C heated, humidified, CO₂-controlled chamber. Z-stacks were acquired using either an Olympus PlanApo 60x/1.4 N.A. (FRAP) or PlanApo 100x/1.4 N.A. (Polycomb bodies) oil-immersion objective heated to 37 °C, using Volocity software (PerkinElmer).

Z-stacks for Polycomb body quantification were acquired by imaging Halo-JF₅₄₉ with a 568 nm laser at 1.25 s exposure at 15% laser power, while Hoechst was imaged with a 405 nm laser at 250 ms exposure at 20% laser power. Z-stacks were acquired at 150 nm intervals. Experiments were performed in at least two biological replicates.

Spot FRAP was performed by acquiring 10 prebleach frames at 1 s intervals, and then bleaching a circle 2.5 µm (normal FRAP) or 1.4 µm (Polycomb body FRAP) in diameter using the 568 nm laser at 100% power. Post-bleaching images were acquired at 0.5 s intervals for 10 min. Images were acquired using the 568 nm laser at 400 ms exposure at 15% laser power. Experiments were performed in at least two biological replicates.

2.5 Analysis of microscopy data

2.5.1 Tracking and localisation of SPT data

Analysis of SPT data was performed in MATLAB (MathWorks) using previously described software (Uphoff et al. 2014). Fluorescent molecules were detected in each frame based on an intensity threshold and localisations of molecules determined to 25 nm precision by fitting an elliptical Gaussian point spread function. Localisations in consecutive frames within a radius of 7.7 μm were linked to form tracks. Localisations were permitted to be lost for single frames within tracks as a result of fluorophore blinking or loss of focus through a memory parameter.

For experiments involving tracking within inactive X domains, molecules were tracked separately for each cell in two regions, one encompassing the inactive X and one outside of the region.

2.5.2 Calculation of apparent diffusion coefficients

Tracks with at least 5 localisations were used to determine apparent diffusion coefficients (D^*) based on the mean-squared displacement (MSD) of the total track. D^* was calculated by

$$D^* = \frac{\text{MSD}}{(4dt)} - \frac{\sigma^2}{dt}$$

where dt is the time between frames, with correction for localisation uncertainty σ .

2.5.3 Spot-On analysis of tracks

Analysis of tracks to determine the fraction of molecules in bound and diffusing states was performed using the online Spot-On tool (Hansen et al. 2018). Jump length distributions were prepared using a bin width of 0.01 μm , using 10 timepoints and 4 jumps, with a maximum jump length of 5.05 μm . Jump length distributions were fitted with a three state kinetic model with a localisation error of 40 nm, using Z correction with $dZ = 0.7$ and cumulative density function fitting with three iterations. Tracks from individual movies, each containing multiple cells, were fitted separately. All statistical analyses were performed on individual movie fits.

X inactivation experiments were analysed instead using pooled tracks, due to the lower number of tracks per region of interest, and averaged over each of three biological replicates.

2.5.4 vbSPT analysis of tracks

vbSPT (Persson et al. 2013) was used to determine how frequently molecules transition between states of diffusion, how long molecules reside in these states, and what fraction of molecules exist in each state. vbSPT state fitting was limited to a maximum of 3, 4 and 6 states. Fitting was performed with the following parameters: 2 step minimum trajectory length, 26 rounds of fitting, 100 bootstrap resamplings. Network maps from vbSPT outputs were plotted in R using the igraph package (Csardi and Nepusz 2006). Network node positioning was determined by clustering for maximal modularity score (Brandes et al. 2008) using the cluster_optimal function.

2.5.5 Single molecule binding time analysis

Tracking movies for measuring single molecule binding times were localised and tracked similar to the method described above, using instead a tracking radius of 1.92 μm or 6.72 μm for 0.5 s and 30 s experiments, respectively, and with a memory parameter allowing for 2 frames to account for missing localisations due to fluorophore blinking. Lengths of tracks of stationary molecules were used to determine apparent dwell times of chromatin bound molecules. Binding time constants were determined by fitting the observed distribution of apparent dwell times with a double exponential function. To correct for biases that result in underestimation of true binding times (Uphoff et al. 2013; Hansen et al. 2017), apparent dwell times for H2B-HaloTag, which is stably bound for hours, was used to correct for photobleaching/blinking or movement of the molecule out of focus, as this would account for loss of H2B tracks, rather than unbinding. This apparent dwell time, tBleach, was measured with each experiment to account for changes in imaging conditions. Binding times were calculated from fitted dwell time constants as follows:

$$t_{\text{Bound}} = \frac{t_{\text{Dwell}} \times t_{\text{Bleach}}}{t_{\text{Bleach}} - t_{\text{Dwell}}}$$

2.5.6 FRAP analysis

To measure fluorescence recovery, intensity measurements were made using ImageJ. Fluorescence intensity in the bleached region and a corresponding unbleached region within the same cell was measured. From each, the intensity of a background region outside of cells was subtracted. The relative intensity of bleached and unbleached regions was calculated by

dividing background-corrected unbleached intensity by background-corrected bleached intensity. Prebleach measurements were used to normalise all values so that the mean of the relative prebleach intensity was 1. The mean, normalised relative intensity of all repeats was plotted with error bars indicating standard error of the mean.

2.5.7 Polycomb body analysis

To segment Polycomb bodies in individual live cells for analysis, nuclei were first manually segmented based on Hoechst fluorescence using TANGO in ImageJ (Ollion et al. 2013). 561 nm channels of z-stacks were deconvolved using Olympus cellSens software (constrained iterative deconvolution, 5 cycles). Deconvolved 561 nm z-stacks were masked using outputs from TANGO, and individual Polycomb bodies identified using a custom script. Briefly, segmented nuclei were background subtracted using a 4 px rolling ball and a mask of Polycomb bodies generated using Otsu thresholding. 3D Objects Counter in ImageJ was used to quantify the properties of the masked Polycomb bodies, and its outputs (object volume, number, mean and total intensity) were processed and analysed using a custom R script to compile all data and calculate any values derived from the 3D Objects Counter measurements (fluorescence enrichment in foci, fractions of total nuclear volume and fluorescence in foci). The mean background intensity of each image stack, measured from a region containing no cells, was subtracted from all measured fluorescence intensities from the same stack. Apparent foci with volumes larger than $1.5\mu\text{m}^3$ were excluded from measurements of features of individual foci, as these likely resulted from segmentation failing to separate multiple nearby foci. Quartiles of foci by volume or mean fluorescence intensity were assigned based on the distribution of foci in untreated cells, and quartile boundaries were used to classify foci in treated cells.

MEASURING THE DYNAMICS OF PRC1

3.1 Introduction

PRC1 is a histone modifying complex involved in the regulation of developmental gene expression (Schuettengruber et al. 2017; Vidal 2019). Genomics approaches have shown that PRC1 binds to target genes, where it deposits H2AK119ub and represses transcription (Wang et al. 2004a). PRC1 complexes in mammals can bind to chromatin through proteins which recognise DNA features or sequences (Farcas et al. 2012; Gao et al. 2012; Hauri et al. 2016; Kloet et al. 2016; Ogawa et al. 2002; Endoh et al. 2017) or recognise histone modifications (Cao et al. 2002; Morey et al. 2012; Morey et al. 2013; Arrigoni et al. 2006). Furthermore, PRC1 also has the capability to self-interact (Isono et al. 2013; Plys et al. 2019), or interact with other chromatin-associated proteins (Pintacuda et al. 2017). The capacity to bind to chromatin is thought to be essential for Polycomb system function, but the array of potential modalities by which Polycomb proteins interact chromatin has made understanding their individual contributions difficult. Furthermore, the majority of our understanding of PRC1 chromatin binding comes from ChIP approaches which tell us nothing about the dynamics of the binding events they capture (Section 1.3.1). Approaches relying on crosslinking are further limited by under-representation of short-lived binding events due to the fixation process (Schmiedeberg et al. 2009; Teves et al. 2016). Therefore, there remains a fundamental lack in understanding of how PRC1 engages with chromatin and how PRC1 dynamics relate to its function.

Recent studies have begun to investigate dynamics of PRC1 proteins in *Drosophila* and mammals (Ficz et al. 2005; Fonseca et al. 2012; Steffen et al. 2013; Ren et al. 2008; Vandenbunder et al. 2014; Zhen et al. 2016; Tatavosian et al. 2018a). However, these studies have focussed on subunits of cPRC1 or their homologues, which have recently been shown to play a secondary role in Polycomb-mediated transcriptional repression in mESCs, in which the majority of the mammalian studies were performed (Fursova et al. 2019). Conversely, the dynamics of vPRC1 complexes, which play the major role in repression in these cells, remain unstudied. Furthermore, these studies have relied on exogenous expression approaches that do not recapitulate endogenous expression

levels, and in general, it was not confirmed that the tagged proteins used were functional and bound to chromatin as normal.

Here I sought to overcome the limitations of these previous approaches and characterise endogenous PRC1 dynamics using live-cell imaging approaches. I reasoned that this would provide the basis on which to dissect the mechanisms by which PRC1 interfaces with chromatin and how these interactions relate to Polycomb-mediated gene repression.

3.2 Results

3.2.1 Generation of an endogenous RING1B-HaloTag cell line

In order to begin characterising the dynamics of mammalian PRC1, I required a cell system in which I could fluorescently label PRC1 so that it could be imaged in live cells. The mammalian Polycomb system has been thoroughly characterised in mouse embryonic stem cells (mESCs), and therefore there is already extensive knowledge about PRC1 and the role it plays in chromatin modification and transcriptional regulation. Therefore, I decided to use mESCs as a model system and to tag RING1B, the core subunit of PRC1. Although RING1A, a paralogue of RING1B, can also form PRC1 complexes, RING1B accounts for the majority of PRC1 complexes in this cell type (Napoles et al. 2004), making it a good proxy for PRC1 dynamics as a whole.

To generate a system in which I could carry out live cell imaging, I chose to generate a fusion of RING1B to the HaloTag (RING1B-HT). HaloTag is not fluorescent alone, but instead can form a covalent bond to a ligand, which can be conjugated to a range of functional moieties (Los et al. 2008), including a range of bright, photostable and cell permeable dyes which are ideal for *in vivo* labelling and live-cell imaging (Grimm et al. 2015; Lavis et al. 2016).

Therefore, I employed CRISPR/Cas9-mediated genome editing (Garneau et al. 2010; Gasiunas et al. 2012; Ran et al. 2013) and homology directed repair to target a sequence encoding the HaloTag, preceded by a short glycine/serine linker, to the end of the final coding exon of *Ring1b*, producing a C-terminal tag (Figure 3.1 A). I identified homozygous targeted clones using a DNA-based screen and confirmed RING1B was homozygously tagged and expressed at endogenous levels by western blot (Figure 3.1 B). Following identification of homozygous clones, I labelled live cells with Halo-JF₅₄₉ to verify that the HaloTag was functional and the protein localised

correctly to the nucleus by fluorescence microscopy (Figure 3.1 C).

To confirm that RING1B binding to Polycomb genes was not disrupted by the HaloTag, I performed ChIP-qPCR of RING1B in the parental and tagged cell lines (Figure 3.1 D). RING1B-HT was enriched at levels similar to wild type RING1B at Polycomb-repressed gene promoters (*Bmp7*, *HoxA7*, *Fzd1*) and the CpG island of active gene *Ncoa2* relative to gene body regions and a region lacking genes. The tagged cell line showed a small reduction in RING1B occupancy, but the normal binding profile was retained. Importantly, western blotting of histone extracts indicated that RING1B catalytic activity was unaffected, as H2AK119ub1 levels were unchanged, and RING1B immunoprecipitation indicated that RING1B-HT interacted normally with PRC1 subunits (Figure 3.1 E and F). There was, however, a reduction in RING1B-HT signal in western blot analysis of both nuclear extract and IP compared to wild type. As the levels of interacting proteins in the RING1B immunoprecipitation remained constant despite the decrease in signal for RING1B, this suggests that expression of the tagged protein is not reduced, but instead the transfer efficiency of the larger tagged protein is reduced. I therefore have generated and validated a cell line expressing an endogenous, functional RING1B-HT fusion protein with which to characterise the dynamics of RING1B using live-cell microscopy approaches.

3.2.2 Single particle tracking and FRAP reveal a large diffusing fraction of PRC1

Having generated a RING1B-HT mESC line I then wanted to use it to directly measure the dynamics of RING1B using a single molecule tracking (SPT) approach. SPT represents one of the most direct techniques for measuring dynamics at a single molecule level in live cells that is currently available (Section 1.3.2). To achieve this, I combined the bright, photostable and photoactivatable Halo-PA-JF₅₄₉ dye and a custom TIRF/HILO microscope. HILO illumination (Tokunaga et al. 2008) uses a highly inclined beam of light to produce fluorescence from only a thin section of the sample being imaged. This dramatically reduces background from out-of-focus fluorescence, which is particularly problematic in thick mammalian cells like mESCs (>10µm), while allowing greater depth of imaging than TIRF, which only allows for surface imaging. The use of a photoactivatable dye enabled me to activate small numbers of fluorophores to allow for accurate tracking of molecules, as well as facilitating bleaching of autofluorescence which can also interfere with tracking. Using this approach, I was able to achieve a sufficient signal-to-noise ratio to follow the movement of single RING1B molecules in living cells (Figure

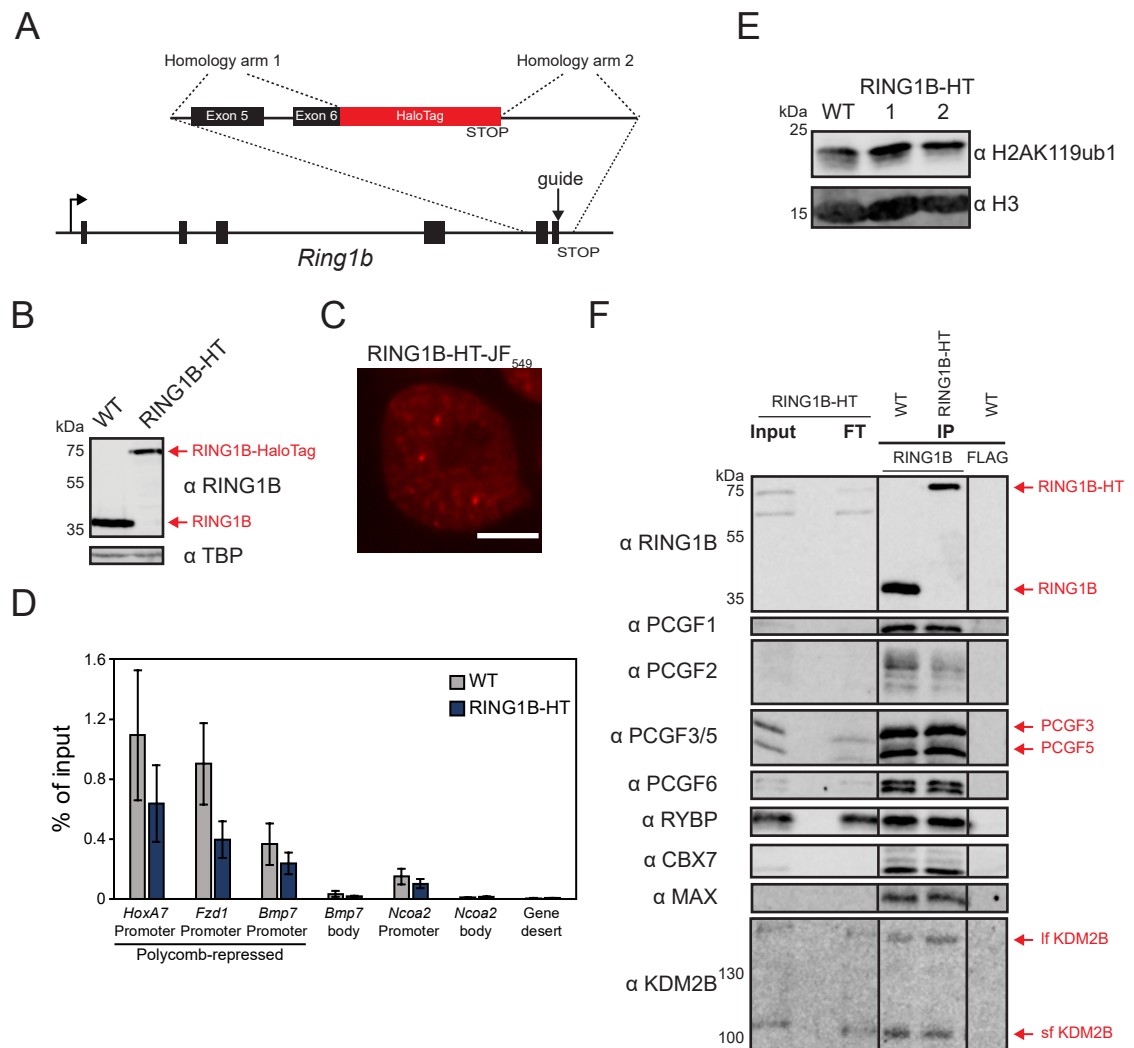


Figure 3.1: C-terminal tagging of RING1B with HaloTag

(A) Schematic depicting targeting of insertion of a sequence encoding a glycine/serine linker and HaloTag at the 3' end of *Ring1b* using the CRISPR/Cas9 system. Arrow at beginning of gene indicates transcription start site. Guide indicates the complementary site to the sgRNA used to target Cas9. Tag insertion was achieved using a template plasmid with homology arms flanking the tag as indicated.

(B) Western blot showing homozygous tagging of RING1B (37.6 kDa) with the HaloTag (34.5 kDa) to give a 72.1 kDa fusion protein (RING1B-HT). TBP is a loading control. WT indicates untargeted mESCs.

(C) A single Z-slice of a nucleus of a RING1B-HT cell labelled with JF₅₄₉. Scale bar = 5 μ m.

(D) RING1B ChIP-qPCR analysis in untargeted (WT) and RING1B-HT mESCs showing percent of input recovered to confirm normal chromatin binding by RING1B-HT. qPCR primers for promoters of Polycomb-repressed genes (*Bmp7*, *HoxA7* and *Fzd1* promoters), an active CGI promoter with lower RING1B enrichment (*Ncoa2* promoter), and gene bodies (*Bmp7* and *Ncoa2* bodies) and a gene-depleted region (gene desert) with no RING1B enrichment were used. Error bars indicate standard error of the mean of three biological replicates.

(E) Western blot showing levels of H2AK119ub1 in untargeted (WT) and two biological replicates (1 and 2) of RING1B-HaloTag mESCs. Histone H3 is included as a loading control.

(F) Western blot of a RING1B immunoprecipitation (IP) from nuclear extracts of untargeted (WT) and RING1B-HaloTag mESCs showing that PRC1 complexes form normally in RING1B-HT cells. 1% of input and flowthrough (FT) are loaded. A FLAG IP was included as a negative control.

3.2). Tracking individual molecules imaged at 65 frames per second (65 Hz) over the time they are visible (before photobleaching or diffusing out of focus) allowed me to measure the dynamic properties of RING1B and therefore PRC1.

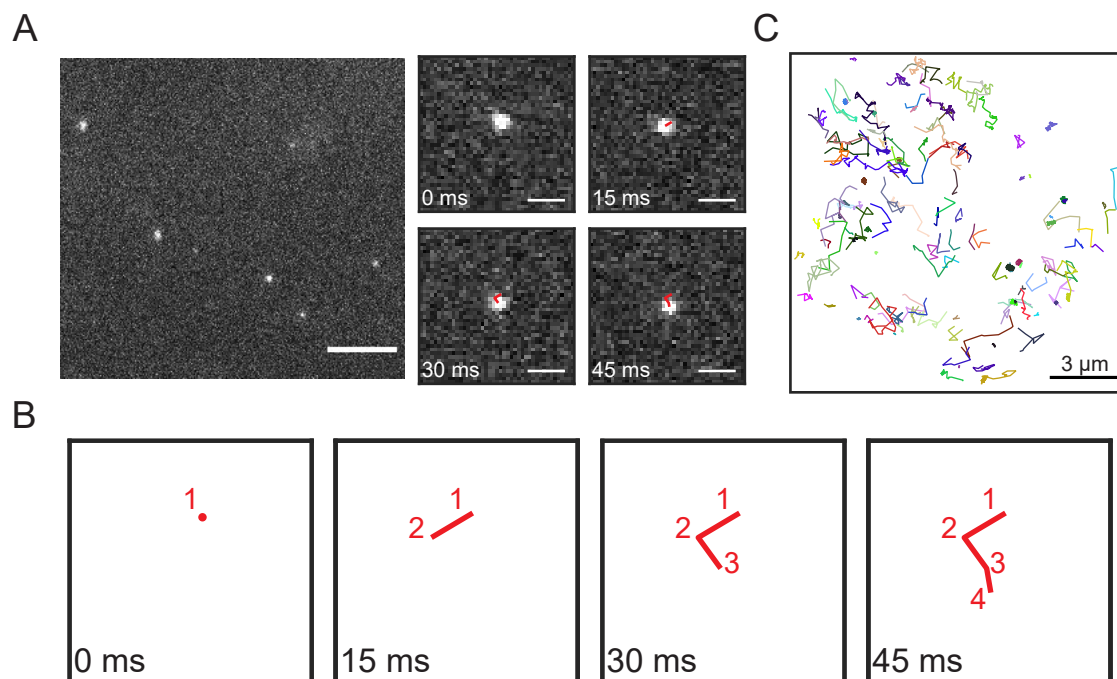


Figure 3.2: Single particle tracking of RING1B in mESCs

(A) Left: example frame from a 65 Hz tracking video of RING1B-HT-PA-JF₅₄₉ acquired using a custom TIRF/HILO microscope showing individual molecules of RING1B-HT. Scale bar = 5 μm . Right: zoomed frames of a single molecule. The tracked steps of the molecule between frames are superimposed in red. Scale bar = 1 μm .

(B) Zoom of the track in (A), with each localisation numbered, to clearly illustrate tracking of a molecule between frames.

(C) Example tracks for individual molecules from a single RING1B-HT cell showing a range of diffusive behaviours.

To begin characterising RING1B, and therefore overall PRC1, dynamics, I determined the apparent diffusion coefficient (D^*) for each RING1B molecule based on the total mean squared displacement (MSD) of the combined steps (the distance between localisations on adjacent frames) for its track. I then compared the range of observed D^* values for RING1B to those measured from cells stably expressing a histone-HaloTag fusion (H2B-HT) and HaloTag coupled to nuclear localisation signals (HT-3xNLS) as control proteins which are stably chromatin bound or have no specific binding, respectively (Figure 3.3 A - C). As expected, the majority of HT-3xNLS tracks had high D^* values ($\approx 2 \mu\text{m}^2 \text{s}^{-1}$), and were therefore rapidly diffusing, with a relatively small fraction showing slower diffusion ($\approx 0.4 \mu\text{m}^2 \text{s}^{-1}$) or immobile behaviour ($\approx 0.01 \mu\text{m}^2 \text{s}^{-1}$). The small slow and immobile fractions likely result from nonspecific interactions of the 3xNLS sequence, which has a positive charge, with negatively charged DNA. Conversely, a high proportion of immobile tracks were observed for H2B-HT, with a small number of fast- and

slow-diffusing tracks. Most H2B-HT therefore appeared chromatin bound and I hypothesise that diffusing tracks correspond to unincorporated H2B-HT that is expressed outside of S-phase (Ewen 2000) as a result of being under the control of a constitutive promoter. In contrast, RING1B showed a distribution with large proportions of fast and slow diffusing molecules, and a small proportion of immobile molecules which correspond to chromatin binding.

Although whole-track D^* measurements allow for an approximate characterisation of protein dynamics, they are not well suited to analysing complex diffusing behaviour in mammalian cells (Hansen et al. 2018). This is because combining behaviour over an entire track into a single D^* value averages out any changes in diffusing state (for example binding or unbinding) which occur while a molecule is visible. Bias toward diffusing molecules also occurs in mammalian cells due to the large volume of the nucleus relative to the small volume that is in focus. Molecules can therefore diffuse out of focus and back into focus, resulting in a single molecule being counted more than once, termed ‘defocalisation bias’ (Hansen et al. 2018). This can lead to an over-representation of diffusing molecules.

Therefore, to more accurately characterise the diffusion of RING1B, I took advantage of the Spot-On tool (Hansen et al. 2018), which was recently developed to account for bias in analysis of SPT data for mammalian cells. Spot-On uses individual step MSD calculations, rather than whole track MSD measurements, and therefore avoids averaging steps of different speeds within the same track. This approach could have the downside of overestimating the contribution of immobile tracks, as they do not diffuse out of focus and therefore are typically longer than diffusing tracks and would contribute more steps. However, Spot-On mitigates this by using only a fixed number of steps from the beginning of each track, as well as accounting for defocalisation bias.

Using Spot-On, the population of steps for all molecules is modelled as existing in two or three states: bound and diffusing, or bound and slow or fast diffusing. For RING1B, H2B and HT-3xNLS, two-state models fit the data poorly compared to three-state models based on Akaike and Bayesian information criteria, which assess model quality, taking into account potential overfitting. Therefore, I have used three-state models to analyse tracks. Analysis in Spot-On revealed that at any given time, 20% of RING1B molecules are chromatin bound, compared to 59% for H2B-HT and 11% for HT-3xNLS, while the majority of molecules are in either fast or slow diffusing states (Figure 3.3 D). The presence of a bound fraction of approximately 10% for

HT-3xNLS in Spot-On analysis has been reported previously (Hansen et al. 2018). This likely represents a combination of nonspecific interactions with DNA by the positively charged 3xNLS, as described above, and the consequence of analysing diffusion using single steps between frames, which will result in short displacements, which occur by chance even in random diffusion, being counted as representing a bound fraction. Some proportion of this fraction may therefore represent a ‘background’ bound fraction, which is observed even in a complete absence real binding.

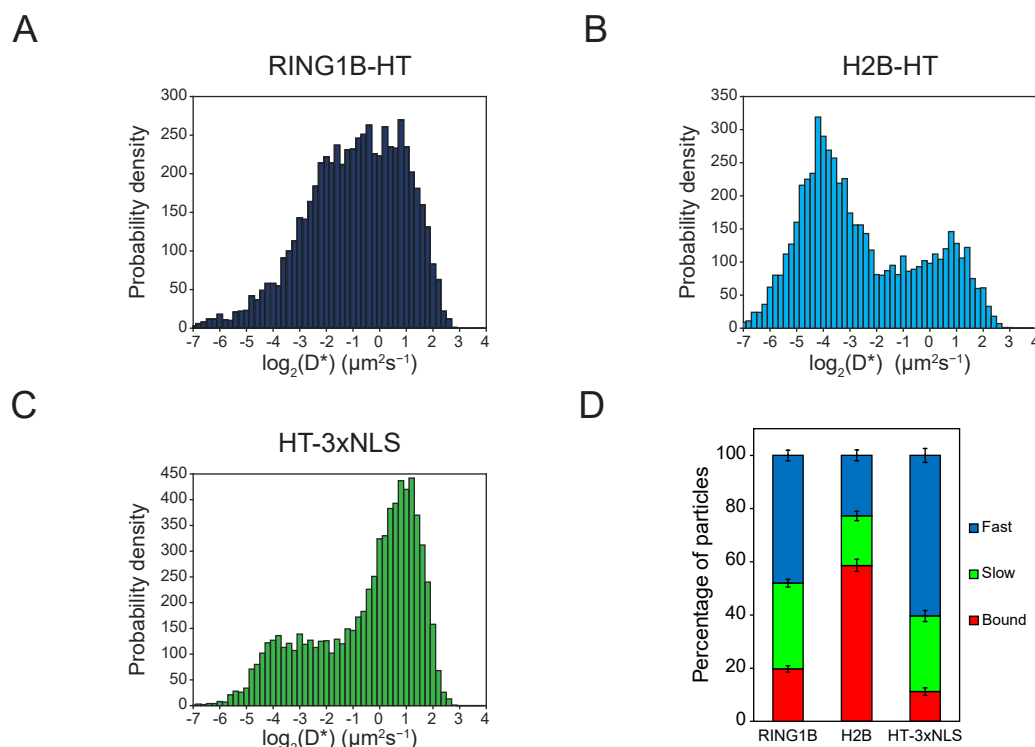


Figure 3.3: Single particle tracking reveals most RING1B is diffusing

(A) - (C) Example histograms of apparent diffusion coefficients (D^* , $\mu\text{m}^2\text{s}^{-1}$) calculated from tracking experiments for individual particles of RING1B-HT, H2B-HT and HT-3xNLS. Tracks analysed are from $n > 30$ cells for each protein. (B) Bar chart of percentages of particles in bound (red), slow diffusing (green), or fast diffusing (blue) states determined from tracking data using Spot-On. Error bars indicate standard error of the mean for model fitting for $n > 50$ cells.

In order to qualitatively confirm my observations from single particle tracking, I employed fluorescence recovery after photobleaching (FRAP) as an orthogonal approach to measure RING1B dynamics. By bleaching a region of the nucleus with high laser intensity, it is possible to infer information about protein diffusion by measuring recovery of fluorescence within the bleached spot (Figure 3.4 A). Although it is possible to derive diffusion coefficients and binding times from FRAP data, the method measures these parameters indirectly by affecting the rate of fluorescence recovery. Determining diffusion coefficients and binding times from FRAP data therefore requires fitting the observed fluorescence recovery with kinetic models, which require assumptions about the protein’s diffusion and binding behaviour (Mazza et al. 2012). Therefore,

I have not applied kinetic models to RING1B FRAP recovery here because RING1B is known to have several possible modes of interaction with chromatin and little is known about how these contribute to its dynamics. I reasoned, therefore, that the assumptions required to model the recovery, and derive diffusion coefficients and binding times would be inappropriate.

Nevertheless, qualitative interpretation of FRAP recovery curves is still possible. Examination of the photobleaching recovery curve for RING1B broadly confirmed the observations from single particle tracking (Figure 3.4 B). More than half of the bleached fluorescence intensity recovered within 20 s, indicative of a large diffusing fraction compared to H2B, which does not recover during this time. The remainder of RING1B fluorescence recovers slowly, likely as a result of slow diffusion and stable binding. Unexpectedly, even after 300 s, beyond which it becomes difficult to reliably track bleached spots due to recovery and cell movement, a small proportion of fluorescence has not fully recovered. This implies the existence of a small, stably bound fraction of RING1B.

Together, SPT and FRAP therefore reveal that RING1B molecules mostly exist in a diffusing state, with a relatively small proportion of molecules that are bound. FRAP further indicated the possibility that a subset of bound RING1B molecules may exist in a stably bound state lasting longer than tens of seconds.

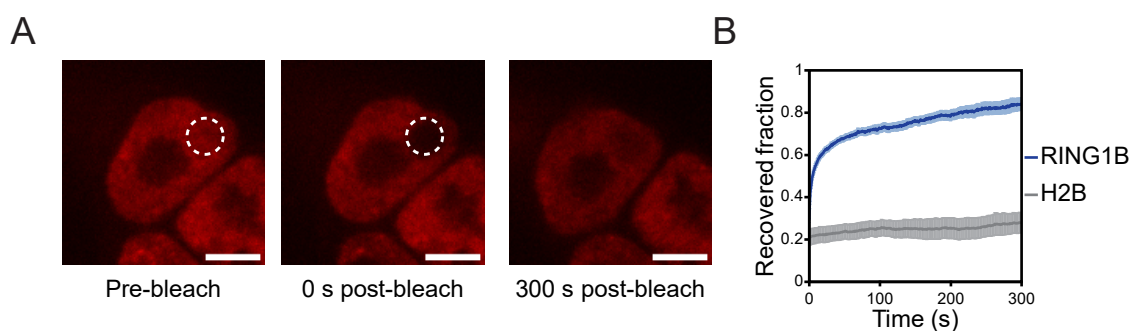


Figure 3.4: RING1B dynamics in FRAP are consistent with SPT measurements

(A) Example frames from a spot FRAP video of RING1B-HT-JF₅₄₉ showing photobleaching and recovery. The photobleached spot is marked by a dashed circle. Scale bar = 5 μ m.

(B) FRAP recovery curve for RING1B-HT and H2B-HT, a control for a stably bound protein. Recovered fraction is relative to initial fluorescence intensity, corrected using a reference unbleached region. Error bars denote standard error of the mean for $n = 38$ cells for RING1B and $n = 5$ cells for H2B-HT.

3.2.3 A small fraction of RING1B with stable binding can be directly observed with long exposure tracking

Although only a small fraction of RING1B molecules appeared to be bound to chromatin, I observed a fraction of RING1B which did not recover after 300s in FRAP. Therefore, I sought to determine whether this represented stable binding at the single molecule scale. To achieve this, I used much longer (0.5 s) exposure times in a single particle tracking experiment. This causes diffusing molecules to blur out, while immobile molecules remain visible as sharp foci (Figure 3.5 A), and enables the use of much lower laser power, reducing photobleaching of molecules and allowing them to be tracked over much longer times. Using this approach it is possible to measure how long each molecule is visible based on the length of its track (hereafter referred to as survival time), and thereby determine how long the protein resides on chromatin (dwell time). In addition to unbinding from chromatin, tracks of immobile molecules can end due to cell movement or chromatin diffusion taking the molecule out of focus, or by photobleaching. To account for this, I used H2B-HT as a control for a protein with a dwell time known to far exceed the photobleaching time of JF₅₄₉ (Kimura and Cook 2001). Loss of stably bound tracks for H2B will therefore result only from photobleaching, chromatin diffusion and cell movement, and not unbinding. The survival time of H2B can therefore be used to correct observed survival times for proteins which bind less stably in order to estimate their dwell time.

By imaging single molecules of RING1B with 0.5 s exposures and low laser power I was able to obtain tracks over 100 s in length, whereas tracks of at most a few seconds were captured for bound molecules in my previous 65 Hz tracking experiments (Figure 3.5 A). The observed decay curves for both RING1B and H2B were described well by a biexponential decay. This enabled derivation of long and short survival times for each, and the fraction of binding events each constituted (Figure 3.5 B). While long survival times will likely represent stable binding, it is less clear what behaviour shorter survival times (≈ 1 s) represent. These may arise from a less stable mode of binding, which results in shorter residence, or from transient blinking of the fluorophore, which occurs on a shorter timescale than photobleaching and would therefore give shorter residence times. The measured blinking properties for PA-JF₅₄₉ have previously been found to be shorter on average than a single exposure in these experiments (220 ms) and infrequent (1.4 events per molecule) (Lavis et al. 2016). Additionally, tracking of single particles in these videos was also

carried out with a memory function to account for missing localisations during individual tracks due to blinking. Together with the short and infrequent blinking of PA-JF₅₄₉, this suggests that the fraction of molecules showing shorter residence times are more likely to represent short binding events.

It was not possible to accurately determine the long dwell time for RING1B as the measured long survival time was close to that of H2B (≈ 50 s). Correction of survival time based on H2B depends on the measured survival time for the protein of interest being a small fraction of that of H2B. Nonetheless, this indicated that 38% of bound RING1B molecules exhibited stable binding of well over 50 s.

In order to try to better estimate RING1B dwell time, I acquired tracking videos with 1 s exposures at an interval of 30 s. This reduces the time for which molecules are illuminated with the laser, and therefore enabled tracks of hundreds of seconds in length to be observed (Figure 3.5 C). This was achieved at the expense of reduced density of tracked molecules, which was required to minimise the probability of tracks overlapping as a result of increased cell movement and chromatin diffusion between frames. Despite the increase in the length of tracks that were observed, the long survival time measured for RING1B remained too close to that of H2B to accurately estimate a dwell time (Figure 3.5 D). This confirmed, however, the existence of a fraction of stably bound RING1B observed in FRAP experiments (Figure 3.4 B), and revealed that RING1B molecules can remain stably bound for over 100 s.

3.2.4 RING1B is enriched at nuclear foci in mouse embryonic stem cells

Polycomb proteins have been observed previously to form nuclear foci, termed Polycomb bodies, in a range of mammalian cell types, including mESCs (Saurin et al. 1998; Suzuki et al. 2002; Ren et al. 2008; Isono et al. 2013; Vandenbunder et al. 2014). These sites represent an accumulation of Polycomb proteins and have been shown to incorporate Polycomb-repressed genes (Isono et al. 2013), which suggests they may be enriched for chromatin-bound Polycomb proteins. I also observed these foci in my RING1B-HT mESC line, and wanted to measure their number, size and enrichment of RING1B (Figure 3.1 C). To this end, I developed a workflow for segmenting and measuring Polycomb bodies in mESCs. I acquired Z-stacks of nuclei labelled with Halo-JF₅₄₉ and Hoechst 33342 using confocal microscopy and manually segmented nuclei in 3D based on Hoechst fluorescence. For each nucleus, I generated a 3D mask of Polycomb bodies from

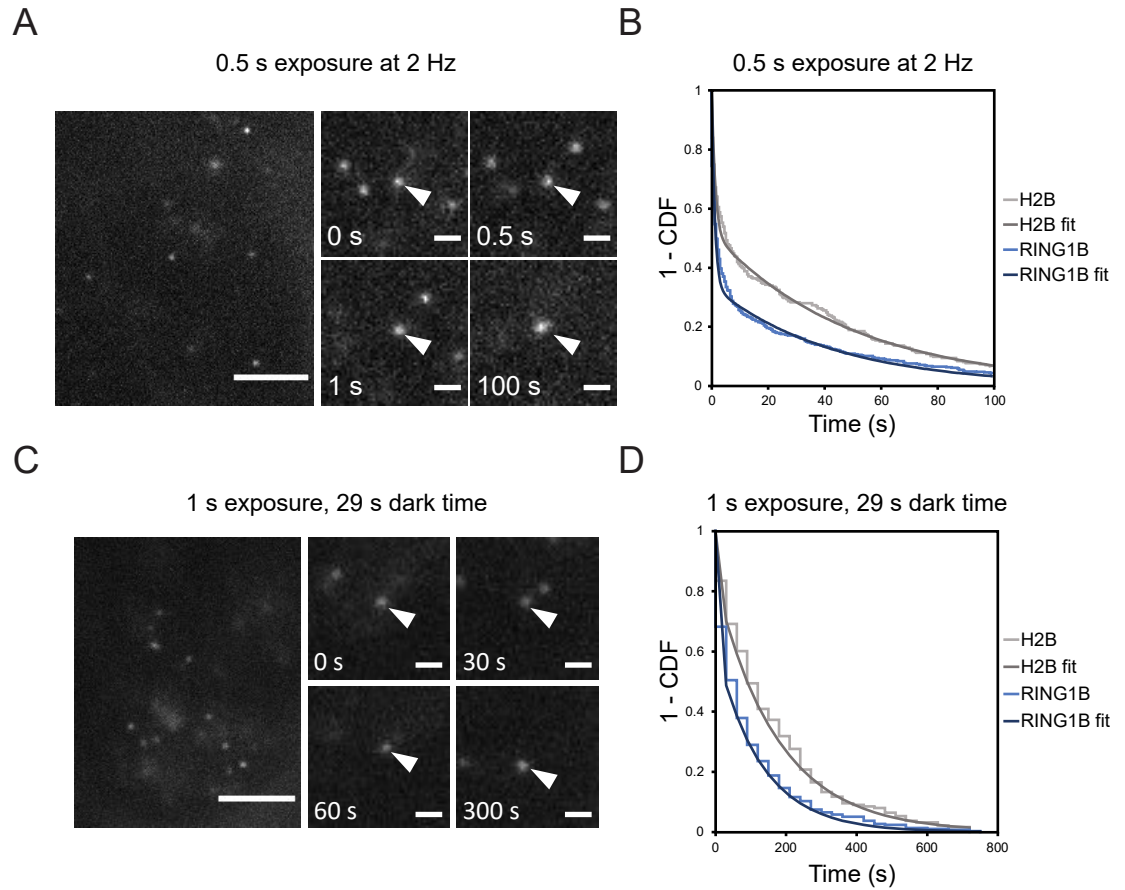


Figure 3.5: Long binding times can be observed directly for RING1B

(A) Left: example frame from a 0.5 s, 2 Hz tracking video showing individual bound RING1B-HT-PA-JF₅₄₉ and motion blur of diffusing molecules. Scale bar = 5 μ m. Right: zoomed frames showing a single molecule over the total time it was tracked. Scale bar = 1 μ m.

(B) Dwell time distributions (1 - cumulative distribution function, CDF) and fitted biexponential decay curves for immobile RING1B molecules for videos acquired as in (A). H2B is included as a control for highly stable binding. $n > 10$ cells.

(C) See (A), example frames from a tracking video with 1 s exposures at 30 s intervals.

(D) See (B), dwell time distributions and fitted curves for immobile RING1B for videos acquired as in (C).

deconvolved Z-stacks of JF₅₄₉ signal (Figure 3.6 A).

Using this approach, I was able to measure the number of Polycomb bodies in each nucleus, as well as their volume and concentration of RING1B relative to the rest of the nucleus. I discovered that Polycomb bodies numbered on average approximately 100 per nucleus and are small, having a mean volume of $0.25 \mu\text{m}^3$. Polycomb bodies were enriched in RING1B relative to the rest of the nucleus by a factor of 1.3-fold. They accounted for less than 10% of the nuclear volume, but roughly 15% of total RING1B fluorescence (Figure 3.6 B). Interestingly, the number of Polycomb bodies per nucleus showed a positive correlation with total nuclear volume, which itself has been shown to correlate well with cell cycle stage (Figure 3.6 C, Steen and Lindmo 1978). This may suggest that these foci are associated with particular genomic loci, which will increase in number as the cells progress through S-phase. Additionally, there was only a weak positive correlation between Polycomb body volume and brightness, suggesting that Polycomb bodies represent sites with differing degrees of compactness (Figure 3.6 D). I was therefore able to define RING1B nuclear localisation and observe that it forms a number of foci with variable enrichment and volume.

3.2.5 Sites of nuclear enrichment have a larger fraction of bound PRC1

Polycomb bodies have been proposed to represent genomic sites of high Polycomb enrichment, such as clusters of genes bound and repressed by the Polycomb system (Isono et al. 2013). Therefore, I wanted to investigate whether they were enriched for bound RING1B. Because of their small size and movement within the nucleus, localising single molecules within Polycomb bodies in tracking experiments was not feasible. Consequently, to test whether Polycomb bodies are enriched for bound RING1B, I performed FRAP experiments using a small ($1.4 \mu\text{m}$ diameter) bleaching spot size to measure fluorescence recovery of individual Polycomb bodies and compared this to equivalently sized regions without Polycomb bodies (Figure 3.7 A). Recovery for non-Polycomb body regions was faster than in my previous experiment (Figure 3.4 B) due to the smaller bleached spot size (Figure 3.7 B). However, spots containing Polycomb bodies recovered a smaller fraction within 20 s than non-Polycomb regions, and did not show any measurable recovery beyond 50 s, indicating the presence of a highly stable fraction of bound protein (Figure 3.7 B). This indicates that Polycomb bodies likely correspond to sites where RING1B is more likely to engage in stable chromatin binding.

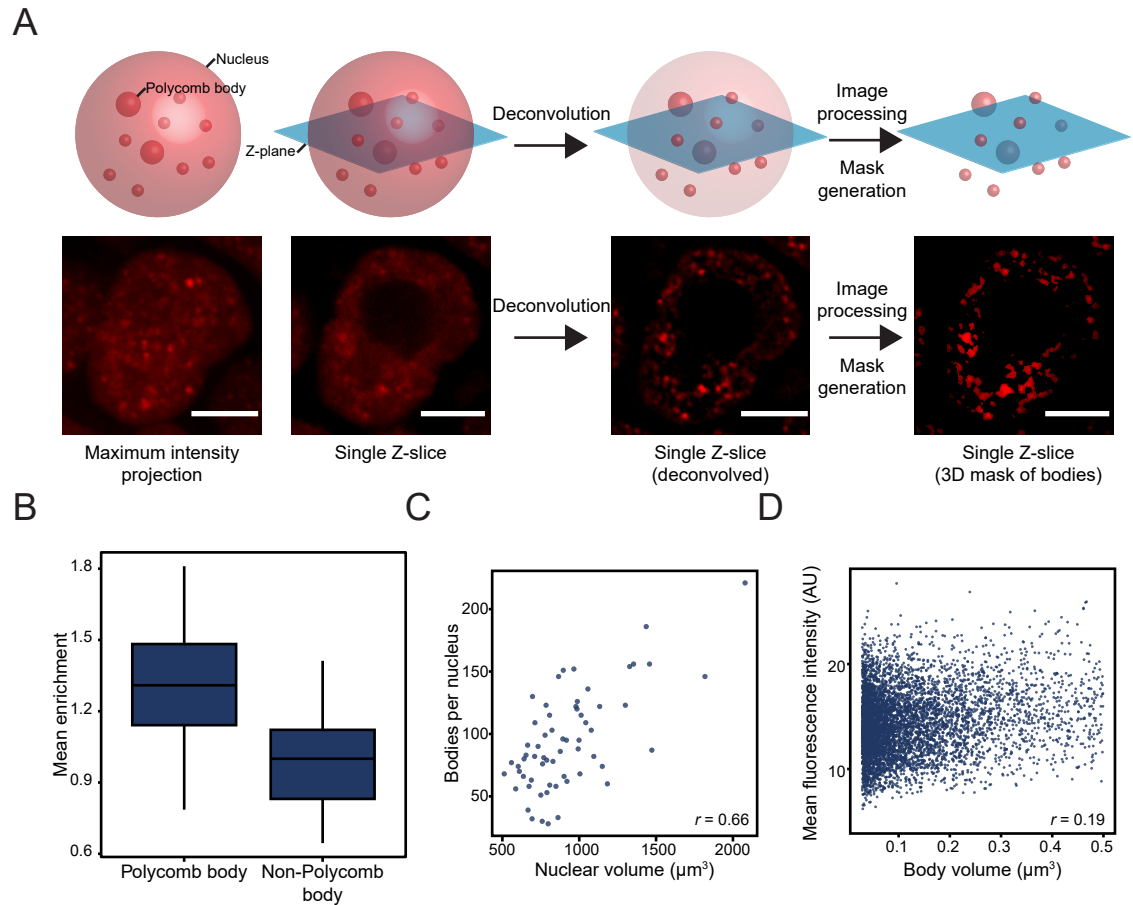


Figure 3.6: Polycomb bodies are small and numerous in mESCs

(A) The workflow used for Polycomb body analysis, illustrated using diagrams and example images. From left to right: maximum intensity projection of Z-stack of the nucleus showing all Polycomb bodies; single Z-slice through the centre of the same nucleus; single Z-slice following deconvolution, which decreases the signal outside of Polycomb bodies; single Z-slice through a 3D mask of Polycomb bodies created from the deconvolved Z-stack. Scale bar = 5 μm .

(B) Boxplot illustrating the relative mean fluorescence signal per unit volume of all RING1B Polycomb bodies in each cell compared to the remaining nuclear volume, normalised to the median non-Polycomb fluorescence. $n = 63$ cells.

(C) Scatter plot of number of Polycomb bodies per nucleus against nuclear volume. $r =$ Pearson correlation coefficient. $n = 63$ cells.

(D) Scatter plot of Polycomb body mean fluorescence intensity against volume. $r =$ Pearson correlation coefficient. $n = 63$ cells.

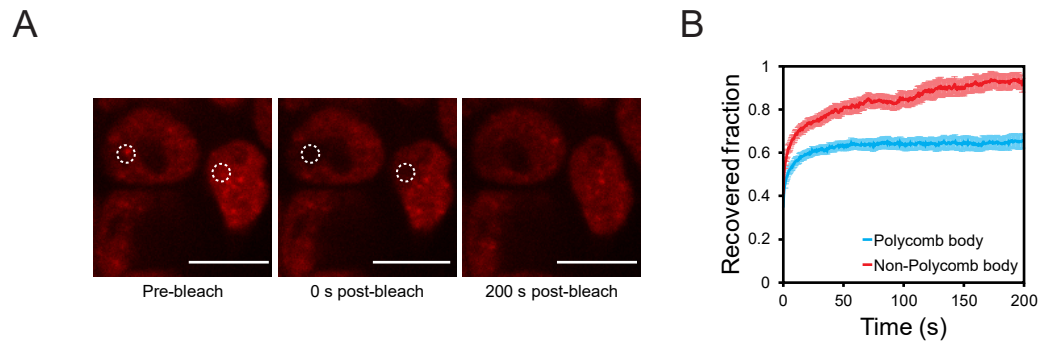


Figure 3.7: FRAP indicates Polycomb bodies are enriched for bound RING1B

(A) Example frames from a spot FRAP video of RING1B-HT-JF₅₄₉ showing photobleaching and recovery of a Polycomb body and a non-Polycomb body region. Photobleached spots are marked by a dashed circle. Scale bar = 5 μm.

(B) FRAP recovery curves for RING1B-HT in regions containing or not containing a Polycomb body. Recovered fraction is relative to initial fluorescence intensity, corrected using a reference unbleached region. Error bars denote standard error of the mean for $n > 20$ cells.

Given that it was not possible to track single RING1B molecules within Polycomb bodies, I instead aimed to track RING1B within a large domain of RING1B enrichment, the inactive X chromosome. Although the chromatin environment within the inactive X chromosome is not identical to that of an autosomal Polycomb target gene, it is similar in that it is also heavily enriched with Polycomb binding to chromatin. The inactive X chromosome therefore provides an opportunity to measure RING1B dynamics by SPT within an enriched region of the nucleus. To image the inactive X chromosome and RING1B together, I exploited a modified version of a female mESC line previously described by Nesterova et al. 2019, in which one X chromosome harbours an endogenous doxycycline inducible *Xist* gene, which produces a lncRNA containing multiple copies of the *bgl* stem-loop sequence. I targeted the HaloTag sequence into *Ring1b* as described above (Section 3.2.1). To image *Xist*, I stably integrated Bgl-SNAP, also under the control of a doxycycline inducible promoter. The SNAP tag functions similarly to the HaloTag for *in vivo* labelling of proteins (Keppler et al. 2002). Following addition of doxycycline, the cells initiate X chromosome inactivation and Bgl-SNAP binds to the Bgl stem-loops on the *Xist* lncRNA.

By simultaneous labelling of cells with SNAP-Cell 505-Star and PA-JF₅₄₉, it was therefore possible to visualise *Xist* domains, which represent X chromosomes undergoing inactivation, and track RING1B molecules (Figure 3.8 A). Crucially, *Xist* domains are large enough to allow for tracking single molecules within them. Analysis of tracks within and outside of *Xist* domains revealed that there was a significant enrichment of bound RING1B molecules observed inside the

regions marked by Xist, from 23% to 31% (Figure 3.8 B). Together, FRAP of Polycomb bodies and tracking within Xist domains demonstrate that regions enriched with RING1B have higher levels of bound protein.

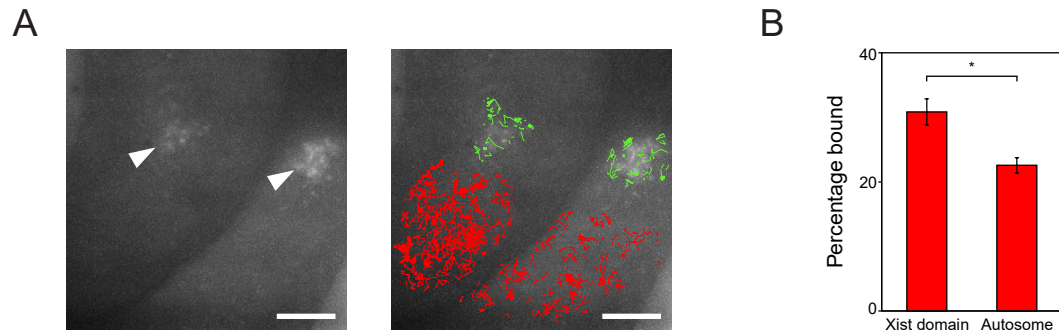


Figure 3.8: Bound and slow diffusing fractions of RING1B are larger within Xist domains

(A) Left: nuclei of cells expressing doxycycline-inducible Bgl-SNAP and Xist-Bgl stem loop after 8 hours of induction and labelled with SNAP-Cell 505-Star. Xist clouds resulting from induced X chromosome inactivation are indicated by white arrowheads. Right: tracks from 65 Hz tracking videos of RING1B-HT-PA-JF₅₄₉ are plotted in green (Xist domain) or red (autosome). Scale bar = 5 μ m.

(B) Bar chart of percentages of molecules in the bound state determined from SPT data using Spot-On. Error bars indicate standard error of the mean for n = 3 biological replicates of >20 cells each. * indicates p < 0.05 as calculated by Student's t test.

3.3 Summary and discussion

Here I set out to characterise the dynamics of PRC1 using single particle tracking in mESCs. This relied on the generation of a cell line expressing a fusion of endogenous RING1B with the HaloTag at wild type levels in order to accurately characterise protein dynamics. This was essential since changes in expression could lead to formation of unnatural subcomplexes, alter the normal incorporation of complex subunits, or produce excess free protein. Additionally, overexpression has been found to alter the measured dynamics of proteins even in the absence of complex formation (Hansen et al. 2017).

Using SPT, I discovered that RING1B has a small bound fraction (20%), and predominately exists in slow (32%) or fast (48%) diffusing states (Figure 3.2). This is similar to reported bound fractions for PRC2 subunits (Youmans et al. 2018; Tatavosian et al. 2018a) and CBX proteins, subunits of cPRC1 (Zhen et al. 2016) as well as p53 and CYCT1 (Loffreda et al. 2017; Lu et al. 2018). However, this is significantly lower than measured bound fractions for SOX2, CTCF and cohesin subunits SCC1 and SCC2 (Liu et al. 2014; Hansen et al. 2017; Rhodes et al. 2017). HP1 γ and CHD4, which are involved in mediating heterochromatin formation and nucleosome remodelling, and both associate with proteins with histone-modifying activity, have both been characterised as

having much higher bound fractions than RING1B, suggesting that different chromatin-interacting proteins can possess different types of dynamic behaviours (Zhang et al. 2016; Basu et al. 2018; Wiese et al. 2019). In its frequency of chromatin binding, RING1B therefore appears more similar to most transcription factors and other Polycomb proteins, although differences in methodology, particularly with less recent studies which do not use newer methods like Spot-On for analysis, can lead to large differences in estimates of fractions of bound molecules.

Three-state model fitting of diffusion also indicates a sizeable slowly diffusing state for RING1B. It is unclear from these experiments alone what molecular behaviour this slowly diffusing state accounts for. It is possible that it describes confined diffusion within particularly tightly packed regions of the nucleus, such as compacted heterochromatin, or within other such compartments. Slow diffusion could also result from the protein rapidly binding and unbinding from chromatin, below the time resolution of the imaging used here, thus appearing to diffuse more slowly.

The major fraction of RING1B molecules exist in a diffusing state, with only a small bound fraction. This is interesting given that PRC1 in some instances has been proposed to carry out its function on chromatin through mediating chromatin structure and compaction, which might be expected to require a large bound fraction, more similar to that of cohesin (Lanzuolo et al. 2007; Tiwari et al. 2008; Comet et al. 2011; Gonzalez et al. 2014; Schoenfelder et al. 2015; Francis et al. 2004; Eskeland et al. 2010; Grau et al. 2011; Lau et al. 2017). However, in mESCs, 10-15% of histone H2A is modified with H2AK119ub1, and the modification exists broadly across the genome (Fursova et al. 2019; Albright et al. 1979; Matsui et al. 1979). In addition to its high prevalence, evidence suggests this modification is rapidly turned over (Seale 1981) and therefore maintaining hundreds of thousands of modified nucleosomes genome-wide might require dynamic chromatin engagement. In this case, a large diffusing fraction capable of briefly binding to deposit H2AK119ub1 would be beneficial. A subset of PRC1 dynamics may therefore be accounted for by this type of ‘hit and run’ activity.

Although PRC1 ubiquitylates H2A genome-wide, both PRC1 and H2AK119ub1 are enriched at the promoters of Polycomb target genes (Ku et al. 2008; Farcas et al. 2012). This would suggest that Polycomb-repressed sites would likely be enriched for bound RING1B. On the inactive X chromosome, which is also known to be enriched for Polycomb occupancy and histone modifications (Plath et al. 2003; Silva et al. 2003; Napoles et al. 2004), I observed a significant

increase in the bound fraction of RING1B molecules (Figures 3.8). This suggests that sites which are subject to Polycomb enrichment and repression are sites at which RING1B is more likely to bind, consistent with the results from ChIP experiments.

I observed a small fraction of RING1B which exhibits stable binding longer than 100 s (Figure 3.5). Notably, this is considerably longer previous measurements for the PRC2 subunits EZH2 and EED, and cPRC1 subunit CBX7 (Zhen et al. 2016; Tatavosian et al. 2018a), along with the majority of DNA-binding transcription factors for which binding times have been characterised, including p53, FoxA1, NF- κ B and GR (Loffreda et al. 2017; Swinstead et al. 2016; Callegari et al. 2019; Paakinaho et al. 2017). This is also higher than measured for the more structural CTCF and SCC2 (Hansen et al. 2017; Rhodes et al. 2017). HP1 γ , another structural chromatin protein, has been described recently to have a similarly long binding time (Wiese et al. 2019). RING1B stable binding, therefore, is unlike previously observed behaviour for most mammalian nuclear proteins. Like measurement of fractions of bound molecules, direct comparison with measurements of residence time for Polycomb proteins between studies is also complicated by differences in methodology. Measurements for EZH2, EED and CBX7 (Zhen et al. 2016; Tatavosian et al. 2018a) use 30 ms exposures spaced by 170 ms dark times. Tracking at this rate will require a much higher laser power than used here, thereby bleaching molecules faster and resulting in shorter survival times. Comparisons are further complicated by use of tagged proteins which were not endogenously expressed and functionally validated.

Despite the differences in single particle measurements of residence time, similarities can be seen, comparing non- or slowly recovering fractions in FRAP between EZH2 (Youmans et al. 2018) and RING1B, with each showing 20% or more of fluorescence which does not recover within hundreds of seconds (Figure 3.4). These stably bound proteins may function to ensure that nucleosomes in occupied regions are fully modified by the catalytic activity of each complex and remain repressed. Alternatively, cPRC1 is proposed to mediate higher order chromatin contacts between Polycomb chromatin domains (Eskeland et al. 2010; Isono et al. 2013; Schoenfelder et al. 2015; Kundu et al. 2017), and the existence of a stably bound fraction of RING1B molecules not observed for transcription factors may therefore be related to this structural role. This would be analogous to other, structural proteins like cohesin subunits and CTCF, for which longer residence times have been observed (Rhodes et al. 2017; Hansen et al. 2017).

The structural role of cPRC1 has also been linked to the formation of Polycomb bodies, which contain Polycomb target genes with large Polycomb chromatin domains in mESCs (Isono et al. 2013), and which I also observed in RING1B-HT cells (Figures 3.6). These foci were enriched for stably bound RING1B compared to the rest of the nucleus (Figures 3.7). This has been observed previously for CBX proteins and PCGF4, both cPRC1 subunits, in U2OS cells, where Polycomb bodies are larger and less numerous (Vandenbunder et al. 2014; Hernández-Muñoz et al. 2005). The cPRC1 complexes which are responsible for forming these features are not involved in Polycomb-mediated gene repression in mESCs (Fursova et al. 2019), although the structures they form change in development and may be important for regulating transcription in differentiation (Noordermeer et al. 2011; Narendra et al. 2015; Narendra et al. 2016; Kundu et al. 2017). Therefore, stable binding may be a feature of the structural role of Polycomb, rather than directly related to the regulation of transcription, at least in mESCs.

In conclusion, live-cell dynamics measurements of endogenously tagged RING1B reveal that the protein exists in a predominately diffusing state, with a small fraction of stably bound molecules. Bound molecules are more abundant at sites of RING1B enrichment and bind more stably at these sites. These observed dynamics will result from a combination of the varied mechanisms of chromatin binding incorporated in PRC1 complexes. In chapter 4, I will examine the dynamics of subunits unique to specific PRC1 complexes to better understand how each contributes to overall PRC1 dynamics.

SUBUNITS OF INDIVIDUAL PRC1 COMPLEXES HAVE SIMILAR YET DISTINCT DYNAMICS

4.1 Introduction

The catalytic core of PRC1 complexes is comprised of a heterodimer of one of RING1A or RING1B and a PCGF protein. There are six different PCGF proteins in mammals, and these proteins correspond to four functionally distinct classes of PRC1 complex because the incorporated PCGF protein appears to specify which additional PRC1 subunits can interact with the complex (Section 1.2.2 and Gao et al. 2012; Junco et al. 2013; Kloet et al. 2016; Hauri et al. 2016). Each PRC1 complex has an individual role in regulating gene expression (Endoh et al. 2017; Fursova et al. 2019; Scelfo et al. 2019), and incorporates different mechanisms by which they can interact with chromatin.

PCGF2 and PCGF4 are thought to form biochemically similar PRC1 complexes. The major complexes formed by these PCGF proteins are also categorised as canonical PRC1 (cPRC1, Section 1.2.2). cPRC1 complexes further incorporate one of five CBX proteins, of which the most highly expressed protein in mESCs is CBX7. CBX proteins have a chromobox domain with affinity for H3K27me₃, which is deposited by the other major Polycomb complex, PRC2 (Zhen et al. 2016). cPRC1 complexes also incorporate one of three PHC proteins, which contain SAM domains, which can to polymerise (Kim et al. 2002). This polymerisation activity has been found to be required for formation of Polycomb bodies in mouse embryonic fibroblasts and mESCs (Isono et al. 2013).

Variant PRC1 complexes contain RYBP or YAF2 in place of a CBX protein and incorporate any of the six PCGF proteins (Section 1.2.3). PCGF1-PRC1 complexes contain KDM2B, a protein with a ZF-CXXC domain through which it binds to non-methylated CpG islands (CGIs) (Farcas et al. 2012; Wu et al. 2013; He et al. 2013). PCGF3 and PCGF5 have high sequence similarity and form nearly identical PRC1 complexes (Gao et al. 2012; Kloet et al. 2016; Hauri et al. 2016). The major function of the PCGF3/5-PRC1 complexes appears to be depositing H2AK119ub1

indiscriminately across the genome without preference for particular chromatin environments (Fursova et al. 2019). PCGF3 complexes may also bind to a small number of specific sites through the transcription factor USF1 (Scelfo et al. 2019). PCGF6-PRC1 complexes incorporate transcription factors MGA/MAX and E2F6, by which the complex binds to the promoters of germ cell-related genes and represses them (Gao et al. 2012; Kloet et al. 2016; Hauri et al. 2016; Endoh et al. 2017). Interactions of PCGF6-PRC1 with HP1 β and HP1 γ , which bind H3K9me3, have also been reported (Kloet et al. 2016; Hauri et al. 2016), although their relevance to PRC1 function is unclear. PCGF2 and PCGF4 have also been described as forming vPRC1 complexes with RYBP that lack CBX and PHC proteins, although no other interacting partners have been identified (Gao et al. 2012; Tavares et al. 2012; Hauri et al. 2016), and the function of these complexes and how they might engage with chromatin is unknown.

Individual PRC1 complexes therefore have diverse mechanisms for interacting with chromatin and differing roles in gene expression. Having characterised total PRC1 dynamics through RING1B (Section 3.2.2), I therefore aimed to characterise the dynamics of representative subunits of each class of PRC1 complex to determine whether having distinct modes of binding gives these complexes different binding dynamics and to better understand how each class of PRC1 complex contributes to the dynamics of PRC1 as a whole.

4.2 Results

4.2.1 Generation of endogenous HaloTag cell lines for individual PRC1 complexes

I sought to generate mESC lines expressing endogenous HaloTag fusions of subunits unique to individual PRC1 complexes in order to characterise their dynamics and understand how they shape the overall dynamics of PRC1. Because the six PCGF proteins define subunit composition for the four major classes of PRC1 complex, I generated HaloTag-fusion cell lines for one PCGF protein from each complex: HT-PCGF1, PCGF2-HT, HT-PCGF3 and HT-PCGF6. For PCGF2/4 and PCGF3/5, where both PCGF proteins form biochemically and functionally similar complexes, I chose to tag PCGF2 and PCGF3. PCGF4 is not expressed in mESCs, and expression of PCGF5 is lower than that of PCGF3. Although PCGF2 largely forms cPRC1 complexes with CBX proteins, it has been found to interact with RYBP, a protein exclusive to vPRC1 complexes which do not contain CBX proteins (Gao et al. 2012). Therefore, to compare dynamics between cPRC1 and

vPRC1 complexes as a whole, I also tagged CBX7, the major CBX protein in mESCs and RYBP, which is more abundant than YAF2 in mESCs.

Proteins were N-terminally tagged unless N-terminal tagging would not tag all annotated isoforms of the protein in question, in order to tag all copies of the protein within a cell to avoid bias in relative incorporation of tagged or untagged proteins into complexes. A C-terminal tag was used for PCGF2 as the gene includes several optional non-coding exons prior to the first coding exon, and I wanted to avoid disrupting splicing by inserting the tag sequence. The *Cbx7* gene contains three transcriptional start sites which produce mRNAs with different initial exons and shared final exons, and can further undergo alternative splicing to alter exon inclusion. I therefore opted for a C-terminal HaloTag which would allow me to label all expressed protein, and therefore avoid labelling only a single isoform. Because prior studies (Zhen et al. 2016; Tatavosian et al. 2018a) used an exogenously expressed N-terminal HaloTag fusion of one CBX7 isoform, I also generated a cell line expressing an endogenous fusion of the same isoform.

I used CRISPR/Cas9-mediated genome editing and homology-directed repair to target the HaloTag to the intended genomic locus, as described for RING1B (Section 3.2.1). As before, I verified that each protein was homozygously tagged and expressed at levels similar to wild type protein by western blot (Figure 4.1 A - F). I confirmed that the HaloTag was functional and the fusion protein localised correctly to the nucleus by fluorescence microscopy in cells labelled with Halo-JF₅₄₉ (Figure 4.1 G). Additionally, I performed ChIP-qPCR for CBX7 in wild type mESCs and both HT-CBX7 and CBX7-HT cell lines to validate that both the N- and C-terminally tagged proteins bound to chromatin normally (Figure 4.1 H). Surprisingly, I found that chromatin binding by CBX7 in the HT-CBX7 cell line, which expresses the previously used N-terminal HaloTag fusion, appeared to be substantially disrupted at Polycomb-repressed gene promoters (*Bmp7*, *HoxA7*, *Fzd1*) relative to both wild type and CBX7-HT, which showed normal binding. I therefore resolved to use only the CBX7-HT cell line for characterisation of CBX7 dynamics.

To confirm that tagged proteins were incorporated into PRC1 normally, I carried out immunoprecipitation of RING1B in wild type and tagged cell lines (Figure 4.2 A - F). All tagged proteins were pulled down similarly to wild type, as were known interactors of PCGF1, PCGF2 and PCGF6 complexes whose incorporation depends on the tagged protein. This indicated that addition of the HaloTag did not disrupt normal complex formation.

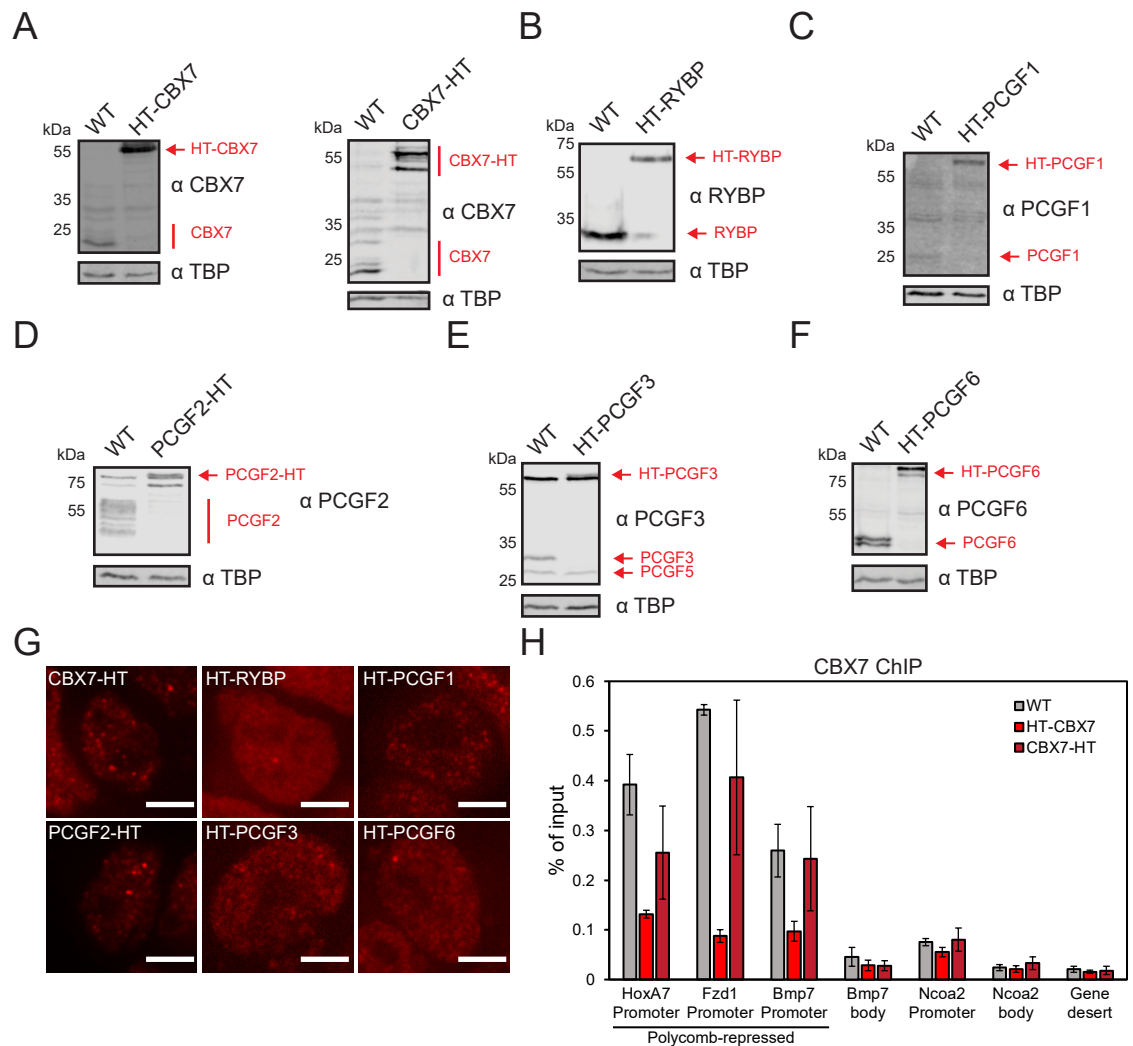


Figure 4.1: Generation of HaloTag cell lines for PRC1 subunits

(A) Western blots showing homozygous tagging of CBX7 (MW between 15.8 kDa and 28.6 kDa depending on splicing isoform) with the HaloTag (34.5 kDa) at N- and C-termini, resulting in a shift in molecular weight relative to the protein in wild type cells. TBP is a loading control. WT indicates untargeted mESCs.

(B) As (A), for RYBP N-terminus (24.8 kDa untagged).

(C) As (A), for PCGF1 N-terminus (29.2 kDa untagged).

(D) As (A), for PCGF2 C-terminus (37.7 kDa untagged). The tagged protein band is visible on top of a non-specific band present in both targeted and untagged cells.

(E) As (A), for PCGF3 N-terminus (28.0 kDa untagged). The tagged protein band is visible on top of a non-specific band present in both targeted and untagged cells.

(F) As (A), for PCGF6 N-terminus (37.6 kDa and 40.6 kDa untagged).

(G) Single Z-slice of nuclei of tagged cells labelled with Halo-JF₅₄₉ to confirm HaloTag function and subnuclear localisation. Scale bar = 5 μ m.

(H) CBX7 ChIP-qPCR analysis in untargeted (WT), HT-CBX7 and CBX7-HT mESCs to confirm normal chromatin binding by HaloTag fusion proteins. qPCR primers for promoters of Polycomb-repressed genes (*Bmp7*, *HoxA7* and *Fzd1* promoters), an active CGI promoter with lower PRC1 occupancy (*Ncoa2* promoter), and gene bodies (*Bmp7* and *Ncoa2* bodies) and a gene-depleted region (gene desert) with no PRC1 occupancy were used to measure percent of input recovered. Error bars indicate the standard error of the mean of three biological replicates.

To further characterise the tagged proteins, I performed FRAP on each cell line. All tagged proteins except RYBP showed fluorescence recovery similar to that of RING1B, with rapid initial recovery, followed by slow recovery of a fraction between 0.2 and 0.4, depending on the protein (Figure 4.2 G). RYBP behaved differently, with nearly complete recovery after 100 s. I used ChIP-qPCR of RYBP in untagged and HT-RYBP cells to confirm that the faster recovery in FRAP observed for RYBP was not due to the HaloTag compromising its chromatin binding (Figure 4.2 H). ChIP-qPCR indicated that RYBP enrichment at Polycomb targets was the same for tagged and untagged protein, suggesting that the faster recovery seen in FRAP compared to other PRC1 subunits is an intrinsic property of RYBP. Because RYBP is also not highly depleted from the flowthrough of RING1B IPs (Figures 3.1 F and 4.2 B), this may indicate that RYBP does not associate stably with the complex, and has a large fraction which is freely diffusing independently of PRC1.

I was therefore able to generate cell lines expressing endogenous HaloTag fusions of PRC1 subunits at wild type levels of expression. These cell lines therefore provided the basis for a systematic characterisation of the dynamics of PRC1 complexes.

4.2.2 Canonical PRC1 has dynamics resembling those of RING1B

To begin characterising PRC1 complex dynamics, I focussed first on cPRC1 subunits PCGF2 and CBX7. I applied the same SPT approach to characterise their dynamics as for RING1B (Section 3.2.2). I measured the bound fraction for each protein using 65 Hz SPT and analysis with Spot-On. This revealed that both PCGF2 and CBX7 have a similar bound fraction (18%) to RING1B (20%) (Figure 4.3 A). I then measured the survival times for CBX7 and PCGF2 using longer exposure times (Section 3.2.3). I found that higher fractions of bound CBX7 and PCGF2 molecules show stable binding than RING1B, at 0.5 and 0.44, compared to 0.38, and all three proteins gave survival times too close to the H2B photobleaching control to accurately estimate, indicating stable binding in excess of 50 s (Figure 4.3 B and C).

I had observed that many tracks obtained in my 65 Hz SPT experiments appeared to display more than one kind of diffusing behaviour, changing between fast or slow diffusion, or binding and unbinding in a single track (Figure 4.4 A and B). Because Spot-On considers steps independently of their context within a track, information about these changes in diffusive state cannot be extracted by this analysis method. With sufficient numbers of tracks containing transitions

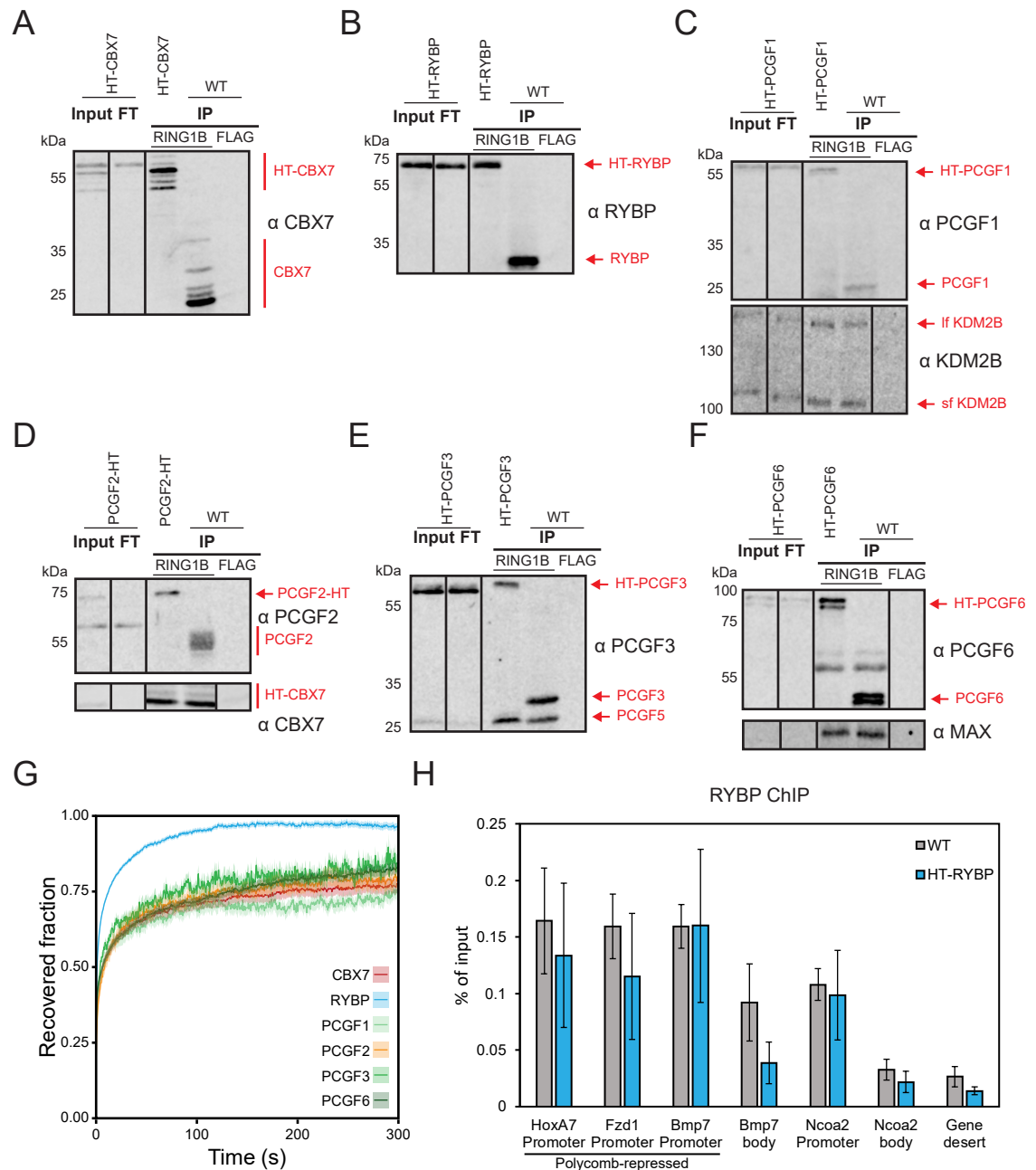


Figure 4.2: Characterisation of HaloTag cell lines

(A) - (F) Western blots of a RING1B immunoprecipitation (IP) from nuclear extracts of untargeted (WT) and HaloTag fusion cell lines showing that PRC1 complexes incorporate HaloTag fusion proteins and their interaction partners at normal levels. 1% of input and flowthrough (FT) are loaded. A FLAG IP was included as a negative control. If and sf KDM2B indicate long and short splicing isoforms of PCGF1-PRC1 subunit KDM2B.

(G) FRAP recovery curves for PRC1 subunits. Recovered fraction is relative to initial fluorescence intensity, corrected using a reference unbleached region. Shaded ribbons denote standard error of the mean for n > 25 cells for all proteins. (H) RYBP ChIP-qPCR analysis in untargeted (WT) and HT-RYBP mESCs to confirm normal chromatin binding by HT-RYBP. qPCR primers for promoters of Polycomb-repressed genes (*Bmp7*, *HoxA7* and *Fzd1* promoters), an active CGI promoter (*Ncoa2* promoter), and gene bodies (*Bmp7* and *Ncoa2* bodies) and a gene-depleted region (gene desert) with no PRC1 occupancy were used to measure percent of input recovered. Error bars indicate the standard error of the mean of three biological replicates.

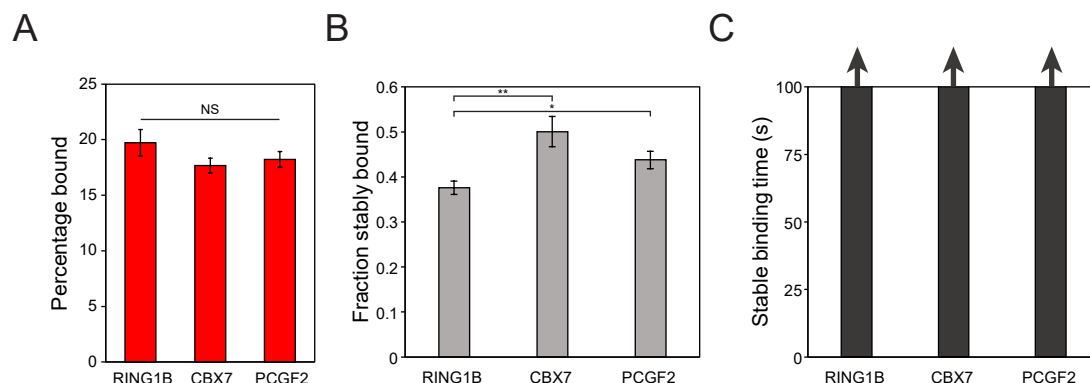


Figure 4.3: Canonical PRC1 binding is similar to RING1B

(A) Bar chart showing percentages of molecules in the bound state for RING1B, CBX7 and PCGF2 as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >50 cells. NS indicates $p > 0.05$ as calculated by Student's t test.

(B) Bar chart showing the fraction of RING1B, CBX7 and PCGF2 molecules exhibiting stable binding in 0.5 s tracking experiments determined by biexponential decay fitting. Error bars indicate standard error of the mean for >20 cells. * indicates $p < 0.05$ and ** indicates $p < 0.005$ as calculated by Student's t test.

(C) Bar chart showing stable binding times for RING1B, CBX7 and PCGF2 after photobleaching correction of stable binding times in 0.5 s tracking experiments determined by biexponential decay fitting. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Survival times were measured from >20 cells.

between diffusing states, it is possible to determine how long molecules spend in individual states.

In order to examine whether useful information on state transitions could be derived from my SPT data, I took advantage of the vbSPT (variational Bayes SPT) analysis tool (Persson et al. 2013). This tool is designed to determine the number of diffusive states and identify transitions between states from experimental SPT data. It then extracts information about the states it models, including the dwell time, diffusion coefficient and transition probability to any other state.

Provided with my data, vbSPT had a tendency to fit a large number (>10) states. Although these states may represent a variety of diffusive states within different nuclear environments, models of this size are too complex to meaningfully assess in the absence of further molecular information about diffusion of PRC1 in the nucleus. I therefore limited vbSPT to modelling a maximum of six states, which I have visualised as a transition probability network (Figure 4.4 C). The network shows the six modelled states, along with weighted arrows indicating the probability of a molecule transitioning from one state to another. In these networks, the state with the lowest diffusion coefficient represents bound molecules, while the state with the highest diffusion coefficient represents molecules which are freely diffusing. Intermediate states may result from confined diffusion, which decreases the distance molecules can travel, or from short binding or diffusing events that occur below the time resolution of the imaging approach in otherwise diffusing or bound movements, respectively.

In vbSPT analysis, RING1B, PCGF2 and CBX7 all formed transition probability networks with similar structures which largely progress linearly from lowest diffusion coefficient (stably bound) to highest (freely diffusing), with a small number of low probability transitions between states which are non-adjacent in the network. The similar overall structures of these transition probability networks suggests these proteins have largely similar diffusing behaviours. This is consistent with the observations from Spot-On analysis and residence time measurements which indicate these proteins have similar dynamics.

Fluorescence microscopy of PCGF2 and CBX7 appeared to indicate that cPRC1 subunits were more enriched at Polycomb bodies than vPRC1 subunits and RING1B (Figures 3.6 A and 4.1 G). By segmenting Polycomb bodies in PCGF2-HT cells as I had previously done for RING1B (Section 3.2.4), I was able to extract quantitative measurements of their properties. I identified a mean of 150 Polycomb bodies per nucleus, higher than the 100 present in RING1B-HT cells. This was likely due to more accurate segmentation of PCGF2 Polycomb bodies cells due to higher enrichment of PCGF2 in Polycomb bodies compared to RING1B (Figure 4.5 A). PCGF2 Polycomb body fluorescence was 2.2-fold higher than that of the rest of the nucleus, compared to only 1.6-fold higher for RING1B. FRAP of PCGF2 Polycomb bodies revealed a smaller diffusing fraction and enrichment for stably bound PCGF2 inside Polycomb bodies compared to the rest of the nucleus, similar to that of RING1B (Figure 4.5 B). These measurements reveal that PCGF2, and likely, therefore, cPRC1, is more enriched at Polycomb bodies than PRC1 overall, as well as having a similar increased bound fraction within these foci.

Together, these results indicate that the dynamics of cPRC1 subunits are largely similar to PRC1 as a whole, although cPRC1 subunits exhibit slightly higher fractions of stably bound molecules and stronger enrichment at Polycomb bodies.

4.2.3 Variant PRC1 complexes have unique dynamic behaviours

Comparison of cPRC1 and total PRC1 (RING1B) indicated that cPRC1 subunits have larger fractions of stably bound molecules. Because RING1B dynamics will represent an average of cPRC1 and vPRC1 dynamics, I hypothesised that vPRC1 subunits may have smaller fractions of stably bound molecules. To examine this possibility, I characterised the dynamics of vPRC1 complexes. Measurement of vPRC1 subunit bound fractions in 65 Hz SPT experiments revealed broad similarities in bound fractions between subunits, but with significant differences. RYBP

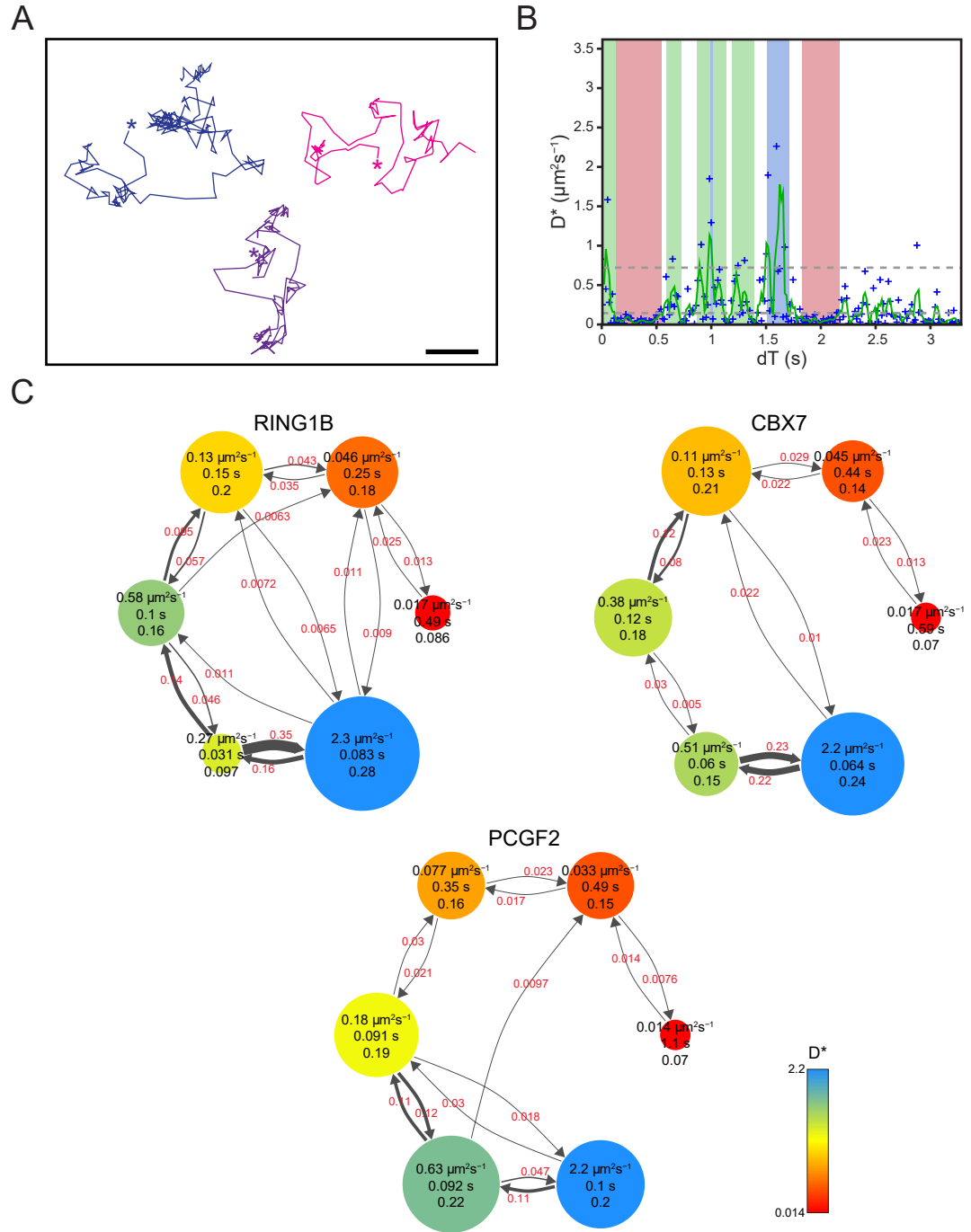


Figure 4.4: Canonical PRC1 dynamic behaviours are largely similar to RING1B

(A) Examples of long (215 localisations, 3.3 s (blue), 95 localisations, 1.4 s (purple), 131 localisations, 2.0 s (pink)) tracks of RING1B-HT molecules containing both diffusing and bound behaviours. Asterisk indicates the first localisation. Scale bar = 500 nm.

(B) A plot of the apparent D^* values for each step of the blue track shown in (A). Blue crosses represent individual steps, while the green line represents the moving mean of three steps. Grey dashed lines indicate approximate thresholds for the boundaries between immobile and slow diffusion (lower line, ($D^* = 0.05 \mu\text{m}^2 \text{s}^{-1}$)) and slow and fast diffusion (upper line, ($D^* = 0.6 \mu\text{m}^2 \text{s}^{-1}$)), based on estimated D^* values for each population. Shaded bars indicate regions of the track which likely correspond to fast (blue) or slow (green) diffusion, or to chromatin binding (red).

(C) Transition probability networks showing vbSPT outputs for RING1B, CBX7 and PCGF2. Diffusive states are represented as nodes. Node colour is scaled to apparent diffusion coefficient (D^* , $\mu\text{m}^2 \text{s}^{-1}$), and node size is scaled to the fraction of molecules in the state. Each state is labelled with its corresponding apparent diffusion coefficient, dwell time, and fraction of molecules in the state. Arrows between nodes indicate state transitions, and red numbers and arrow weight indicate the transition probability. Transitions with probability < 0.005 are not shown.

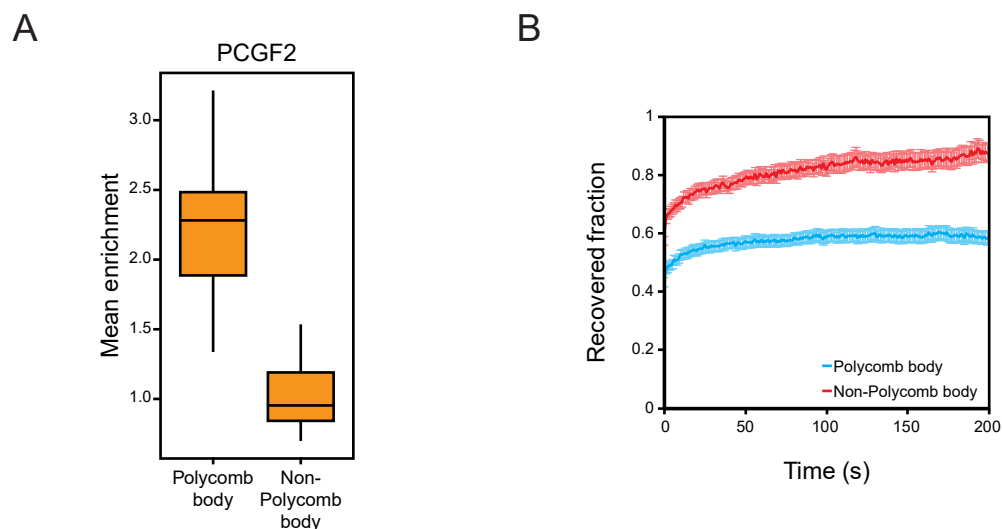


Figure 4.5: PCGF2 is strongly enriched in Polycomb bodies

(A) Boxplot illustrating the relative mean fluorescence signal per unit volume of all PCGF2 Polycomb bodies in each cell compared to the remaining nuclear volume, normalised to the median non-Polycomb fluorescence. $n = 36$ cells. (B) FRAP recovery curves for PCGF2 in regions containing or not containing a Polycomb body. Recovered fraction is relative to initial fluorescence intensity, corrected using a reference unbleached region. Error bars denote standard error of the mean for $n > 30$ cells.

(13%), PCGF3 (13%) and PCGF6 (17%) all have smaller bound fractions compared to RING1B (20%), while PCGF1 (26%) has a larger bound fraction (Figure 4.6 A). In 0.5 s tracking experiments, RYBP, PCGF1, PCGF3 and PCGF6 all had stably bound fractions similar to that of RING1B (Figure 4.6 B). However, while PCGF1, like RING1B, once again had a stable binding time that was too long to measure, RYBP, PCGF3 and PCGF6 all exhibited much shorter stable binding times, between 30 and 50 s (Figure 4.6 C). Together, this reveals that, unlike cPRC1 subunits, vPRC1 subunits exhibit distinct dynamics to each other and PRC1 overall.

vbSPT analysis of tracks for vPRC1 subunits further demonstrated a range of diffusive behaviours (Figure 4.7). RYBP and PCGF6 produced transition probability networks with similar structures to that of RING1B, although, as expected, RYBP has a higher proportion of freely diffusing molecules. PCGF1 and PCGF3, however, have networks which diverge substantially from the structure of that of RING1B. PCGF1 has a large fraction of molecules in chromatin bound states, while PCGF3 has a relatively small fraction of chromatin bound molecules. Both PCGF1 and PCGF3 showed high transition probabilities between fast- and slow-diffusing states. Because the dwell times for these states with slower diffusion are short, they may represent frequent, short-lived binding events below the temporal resolution of the imaging which appear to slow the molecules. vPRC1 subunits therefore also display varied diffusive behaviours in vbSPT analysis with a range of higher and lower bound fractions between PCGF1 and PCGF3.

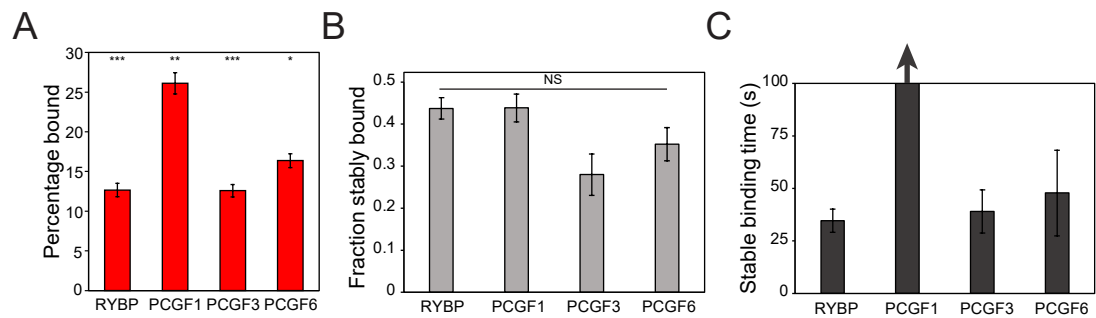


Figure 4.6: Variant PRC1 subunits have unique dynamics

(A) Bar chart showing percentages of molecules in the bound state for RYBP, PCGF1, PCGF3 and PCGF6 as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >50 cells. * indicates $p < 0.05$, ** indicates $p < 0.005$ and *** indicates $p < 0.0001$ relative to RING1B as calculated by Student's t test.

(B) Bar chart showing the fraction of RYBP, PCGF1, PCGF3 and PCGF6 molecules exhibiting stable binding in 0.5 s tracking experiments determined by biexponential decay fitting. Error bars indicate standard error of the mean for >20 cells. NS indicates $p > 0.05$ relative to RING1B as calculated by Student's t test.

(C) Bar chart showing stable binding times for RYBP, PCGF1, PCGF3 and PCGF6 after photobleaching correction of stable binding times in 0.5 s tracking experiments determined by biexponential decay fitting. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Error bars indicate standard error of the mean for >20 cells.

Overall, vPRC1 complexes demonstrate a range of dynamics. RYBP, which may not be stably associated with the complex (Section 4.2.1), and PCGF3 both have smaller bound fractions and shorter stable binding relative to RING1B. Conversely, PCGF1 has a larger bound fraction and similar stable binding. PCGF6 is largely similar to RING1B with the exception of shorter stable binding. RING1B and total PRC1 dynamics, therefore, represent a combination of similar yet distinct diffusive behaviours.

4.2.4 PCGF2 is the most abundant PCGF protein

Having determined that PRC1 dynamics represent a combination of different diffusive behaviours, I wanted to measure the relative numbers of each PRC1 subunit in mESCs to understand whether the abundance of each class of PRC1 complex would explain the how the dynamics of individual complexes contribute to the behaviour of total PRC1. There exist two previous measurements of relative PRC1 subunit levels in PRC1 complexes which both report PCGF6 as the most abundant PCGF protein in complex with RING1B (Kloet et al. 2016; Scelfo et al. 2019). These measurements differ, however, on the abundance of the other PCGF proteins. Both measurements depend on an immunoprecipitation-mass spectrometry approach to quantify protein abundance, and therefore can be affected by the efficiency of protein extraction and behaviour of each protein in the mass spectrometer. Given that I observed differences in chromatin binding between PCGF proteins, these differences may result in poorer extraction of more stably bound PRC1 complexes,

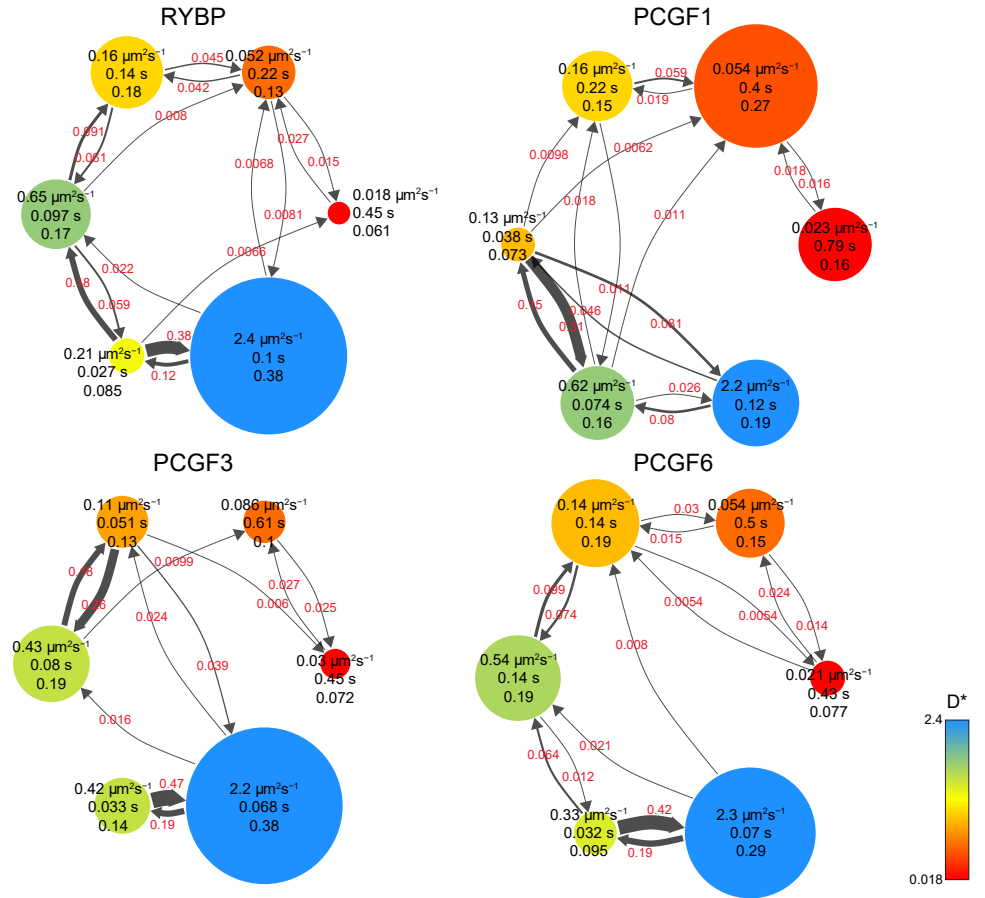


Figure 4.7: vPRC1 subunits have unique dynamics

Transition probability networks showing vbSPT outputs for RING1B, CBX7 and PCGF2. Diffusive states are represented as nodes. Node colour is scaled to apparent diffusion coefficient (D^* , $\mu\text{m}^2\text{s}^{-1}$), and node size is scaled to the fraction of molecules in the state. Each node is labelled with its corresponding apparent diffusion coefficient, dwell time, and fraction of molecules in the state. Arrows between nodes indicate state transitions, and red numbers and arrow weight indicate the transition probability. Transitions with probability < 0.005 are not shown.

thus giving a distorted view of complex abundance. I therefore leveraged my endogenous HaloTag fusion cell lines to measure relative total protein levels by two previously reported fluorescence methods (Cattoglio et al. 2019).

First, I measured nuclear fluorescence intensity in each HaloTag cell line under conditions of near-quantitative labelling with Halo-JF₅₄₉ (Yoon et al. 2016) and compared this to the fluorescence intensity I measured in the RING1B-HT cell line (Figure 4.8). I then prepared whole-cell lysates from defined numbers of Halo-JF₅₄₉-labelled cells and compared fluorescence intensity by in-gel fluorescence between tagged proteins for the lysate of each cell line. In order to derive estimates of the number of fluorescent molecules in each cell, I compared fluorescence intensity from cell lysates to known quantities of a HaloTag standard protein (Figure 4.8). Both approaches gave highly similar relative numbers of molecules, with low variability between cells or replicates, respectively. Furthermore, calculation of an average fraction of bound molecules or average stably bound fraction, weighted using the relative abundance for each PCGF proteins to represent total PRC1, gives values which resemble those of RING1B. Collectively, these observations suggest that these measurements of abundances are reliable.

These approaches reveal there are approximately 60,000 RING1B molecules per nucleus, and use of the nuclear volume from nuclear segmentation for Polycomb body quantification described above indicates that this accounts for a concentration of 114 nM. There exists also a pool of PCGF proteins stoichiometric with RING1B, with PCGF2 (25,000, 46 nM) and PCGF6 (15,000, 25 nM) being the most abundant. PCGF1 (5000, 9 nM) and PCGF3 (2500, 5 nM), by contrast, account for a small proportion of PCGF proteins in mESCs. RYBP (30,000, 53 nM) is present in broadly similar numbers to the vPRC1-specific PCGF proteins, while CBX7 (19,000, 34 nM) is present at lower numbers than PCGF2, which may be accounted for by the expression of other CBX proteins or the existence of variant PCGF2 complexes. The high abundance of PCGF2, and therefore PCGF2-PRC1 complexes, explains the similarity of its dynamics to total PRC1.

4.2.5 Polycomb-mediated gene repression and H2AK119ub1 are maintained by a small number of catalytically active PRC1 complexes

Applying my characterisation of the dynamics of PRC1 complexes, combined with my quantitative measurements of subunit levels and existing knowledge of features of the Polycomb system, it is possible to derive an overview of PRC1 function. Genes affected by perturbation of PRC1 in

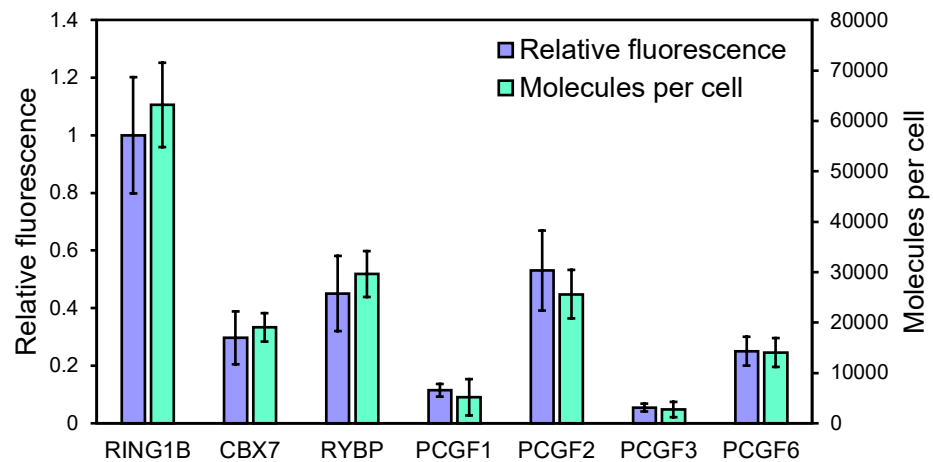


Figure 4.8: Fluorescence quantification reveals the abundance of PRC1 subunits

Bar chart showing relative nuclear fluorescence, measured by fluorescence microscopy, and molecules per cell, measured by in-gel fluorescence, for PRC1 subunits. Error bars indicate standard deviation for $n > 35$ cells (Relative fluorescence) or $n = 3$ biological replicates (Molecules per cell).

mESCs number on the order of a few thousand, depending on the type of perturbation (Fursova et al. 2019; Blackledge et al. 2019). Quantification of PRC1 subunits reveals that there are an average of approximately 60,000 RING1B molecules, and therefore a similar maximum number of PRC1 complexes (Figure 4.8). In mESCs, vPRC1 complexes appear to play the major role in Polycomb-mediated repression (Fursova et al. 2019), but only account for approximately half of potential PRC1 complexes based on the abundance of vPRC1-specific PCGF proteins (Figure 4.8). Furthermore, of these complexes, only around 20% are bound to chromatin at any given time (Figure 3.2 C), giving approximately 6000 chromatin-bound vPRC1 complexes, and, on average, a single complex bound at each repressed gene (Figure 4.9 A).

To mitigate this low abundance of PRC1 complexes, Polycomb bodies, which are enriched for PRC1 complexes, may represent condensed regions containing repressed genes. These foci account for 15% of RING1B (Figure 3.6), or roughly 9000 molecules. Although Polycomb bodies appear to be enriched for bound PRC1 (Figures 3.6 and 4.5), the number of bound molecules at these sites is still low relative to the total number of PRC1-repressed genes. Furthermore, Polycomb bodies likely do not contain all Polycomb target sites due to their low numbers in cells (approximately 100 - 150 foci), and therefore this concentration mechanism would not be sufficient to enable high PRC1 occupancy at all PRC1 target sites.

Because PRC1 likely cannot repress transcription by simply maintaining occupancy at target sites due to the low number of available complexes, an attractive hypothesis is that it may do so by

deposition of its major catalytic product, H2AK119ub1. It has recently been demonstrated that PRC1 ubiquitin E3 ligase activity is required for Polycomb-mediated transcriptional repression (Blackledge et al. 2019; Tamburri et al. 2019). Approximately 10% of histone H2A is modified with H2AK119ub1 in mESCs (Fursova et al. 2019), and, based on previous measurements of nucleosome spacing (Teif et al. 2012), this suggests there are a total of roughly 4×10^6 modified histones across a diploid mouse genome (equivalent to 9 μ M). Although H2AK119ub1 is present across the genome at low levels as well as enriched at Polycomb-repressed genes, only a small fraction of the total number of modified histones would be required for more H2AK119ub1 to be present at the promoters of repressed genes than PRC1 complexes. The decay half life of H2AK119ub1 has previously been measured at 90 minutes (Seale 1981). This is likely more stable than the vast majority of PRC1 binding events, and could therefore explain the maintenance of transcriptional repression with a low abundance of PRC1 complexes.

vPRC1 complexes are responsible for almost all H2AK119ub1 in mESCs (Fursova et al. 2019), and therefore 3×10^4 complexes are required to maintain a genome-wide pool of modified histones two orders of magnitude larger (Figure 4.9 B). Based on the previously measured decay half life for H2AK119ub1 of 90 minutes, this would suggest that each vPRC1 complex would be required to place approximately 1 modification per minute to maintain a constant level of H2AK119ub1. Quantitative measurements of PRC1 chromatin binding and abundance therefore reveal that a small number of PRC1 complexes maintain gene repression and modification of nucleosomes at Polycomb-repressed genes, suggesting that deposition of H2AK119ub1 may be a mechanism by which PRC1 maintains gene repression.

4.3 Summary and discussion

Here I have systematically characterised the dynamics of PRC1 subunits to describe the behaviour of PRC1 complexes in live cells. I generated cell lines expressing HaloTag fusions of PRC1 subunits unique to individual classes of PRC1 complex. I verified that these proteins were expressed and incorporated into PRC1 normally, enabling me to use them to carry out characterisations of the dynamics of their respective complexes.

SPT of PCGF2 and CBX7, which are both subunits of cPRC1, revealed that these proteins have similar dynamics to RING1B and therefore total PRC1. All three proteins have similar bound

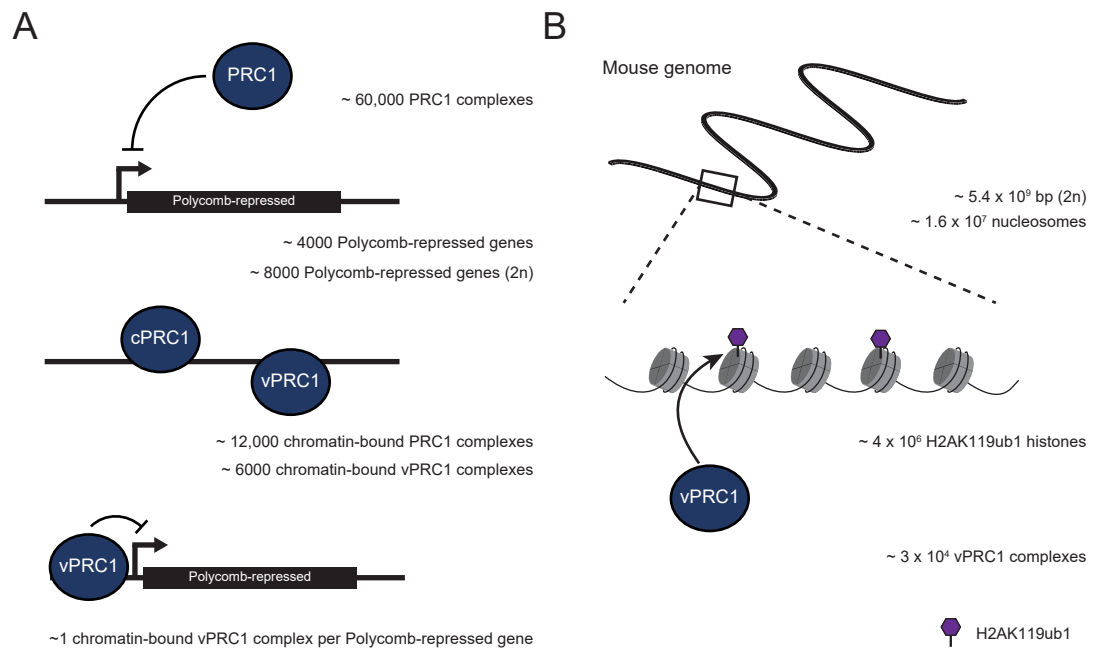


Figure 4.9: Applying quantitative measurements to PRC1

(A) A diagram showing quantitative features of PRC1-mediated gene repression.

(B) A diagram showing features of H2AK119ub1 (purple lollipops) deposition by PRC1.

fractions and long stable binding times (Figure 4.3). CBX7 and PCGF2 have larger stably bound fractions than RING1B, and this is likely due to RING1B being representative of both cPRC1 complexes with higher stably bound fractions, and vPRC1 complexes with lower stably bound fractions. Both CBX7 and PCGF2 are enriched at Polycomb bodies, and enrichment of PCGF2 in Polycomb bodies was higher than that of RING1B (Figure 4.5). PHC proteins, which are unique to cPRC1 complexes, play a central role in the formation of Polycomb bodies (Isono et al. 2013), and therefore enrichment of cPRC1 complexes in Polycomb bodies is expected. The highly stable cPRC1 binding I have observed may be required to maintain these structural entities. The high abundance of PCGF2 compared to other PCGF proteins in mESCs might therefore be a requirement of this structural role, despite its lack of a major role in repressing transcription.

In contrast to cPRC1, vPRC1 subunits displayed a variety of dynamic behaviours (Figure 4.6). RYBP, which is present in all vPRC1 complexes, appeared to be an outlier as it recovered rapidly in FRAP relative to all other PRC1 subunits I investigated. This suggested that RYBP may not be a stable component of PRC1 complexes, instead having a large fraction of freely diffusing molecules which bind to PRC1 complexes infrequently. This is consistent with the large diffusing fraction of RYBP observed in SPT experiments. Despite its smaller bound fraction, RYBP has otherwise similar diffusing and binding behaviour to the major vPRC1 PCGF protein, PCGF6.

These similarities likely result from RYBP which is incorporated into PCGF6-PRC1 complexes.

I found that PCGF6 is the most abundant vPRC1 PCGF protein, and the second most abundant overall after PCGF2. Prior measurements of PRC1 complex abundance have found instead that PCGF6-PRC1 is the most abundant (Kloet et al. 2016; Scelfo et al. 2019). This may be due to cPRC1 complexes having higher fractions of stably chromatin-bound molecules, resulting in less efficient extraction of these complexes from chromatin for immunoprecipitation. Given the high abundance of PCGF6, PRC1 dynamics appear to be strongly influenced by PCGF6-PRC1 complexes. The major difference between PCGF6 and RING1B is that PCGF6 shows much less stable binding than RING1B, with a mean bound time of roughly 50 s. PCGF6-PRC1 chromatin binding has been found to depend on its interaction with transcription factors (Endoh et al. 2017). However, this stable binding time is considerably longer than other transcription factors, which have reported binding times of typically less than or around 10 s (Loffreda et al. 2017; Swinstead et al. 2016; Callegari et al. 2019; Paakinaho et al. 2017). This longer binding may result from the incorporation of CBX1/3 or L3MBTL2 in PCGF6-PRC1 complexes, both of which can bind to histone modifications, or through H2AK119ub1 binding by RYBP. Interestingly, a binding time of 50 s is on the same scale as the rate at which vPRC1 complexes may be required to deposit H2AK119ub1 to maintain constant levels of H2AK119ub1 (Section 4.2.5). It is unclear whether PRC1 can deposit H2AK119ub1 more than once with a single chromatin binding event, and therefore stable binding beyond the time it takes to perform catalysis may not be necessary, while less stable binding might reduce its ability to deposit ubiquitylation.

PCGF3 accounts for a small fraction of the total pool of PCGF proteins in mESCs. Little is known about how PCGF3/5-PRC1 complexes interact with chromatin, outside of specific protein-protein interactions that occur in the context of X-chromosome inactivation (Pintacuda et al. 2017). On autosomes, the main function of these complexes appears to be depositing H2AK119ub1 genome-wide, regardless of context (Fursova et al. 2019). There is also some evidence that these complexes may be recruited to a small number of sites by the transcription factor USF1 (Scelfo et al. 2019). Consistent with a role in maintaining H2AK119ub1 genome-wide and weak enrichment at Polycomb-repressed promoters (Scelfo et al. 2019), PCGF3 is largely dynamic, having only a small chromatin-bound fraction, of which a small proportion is engaged in stable binding. This relative lack of stable binding may therefore allow PCGF3/5-PRC1 to bind and modify nucleosomes across the genome via a ‘hit and run’ mechanism.

PCGF1 also accounts for only a small fraction of total vPRC1 complexes. Surprisingly, unlike other vPRC1 subunits, PCGF1 has a higher bound fraction than RING1B and a similarly long stable binding time. This may result from the unique incorporation into PCGF1-PRC1 of KDM2B, which can bind to non-methylated CpG dinucleotides through its ZF-CXXC domain and targets PCGF1-PRC1 to CpG islands (Farcas et al. 2012; Wu et al. 2013; He et al. 2013; Blackledge et al. 2014). PCGF1-PRC1 is responsible for the majority of H2AK119ub1 at Polycomb target sites and also contributes to deposition of this modification throughout the genome (Fursova et al. 2019). Besides stabilisation of binding at CGIs by KDM2B, it is unclear how else PCGF1-PRC1 might achieve longer stable binding times compared to other vPRC1 complexes.

Overall, this characterisation of PRC1 complex dynamics and abundance is consistent with prior experiments investigating the determinants of PRC1 chromatin binding by ChIP. Removal of PRC2 and therefore H3K27me3, on which cPRC1 chromatin binding is thought to depend, was found to result in a substantial loss of RING1B occupancy (Tavares et al. 2012), suggesting that cPRC1 is responsible for the majority of RING1B chromatin binding. This would be the expected observation based on my data, given that PCGF2 and CBX7, which are cPRC1 subunits, are highly abundant relative to other PRC1 subunits and have more stable binding than all other subunits besides the less abundant PCGF1. Removal of PCGF2 and PCGF4 also results in a similarly dramatic reduction in RING1B occupancy in ChIP, while removal of the less abundant vPRC1-exclusive PCGF proteins does not result in a reduction of a similar size unless all four are removed together (Fursova et al. 2019). However, removal of all vPRC1-exclusive PCGF proteins also results in a major loss of H3K27me3 at PRC1-bound sites, and therefore, even in the case of the vPRC1-specific complex deletion, the loss of RING1B occupancy as observed by ChIP may largely result from loss of cPRC1 chromatin binding via H3K27me3.

Quantification of the number of molecules of PRC1 subunits revealed similar nuclear concentrations of RING1B in mESCs to the previously characterised concentrations of PC in *Drosophila* embryos (Steffen et al. 2013) and sensory organ precursor cells (Fonseca et al. 2012), at approximately 100 nM. Measurement of concentrations of subunits exclusive to different PRC1 complexes, however, indicated that PCGF1 and PCGF3, which are responsible for the majority of H2AK119ub1 deposition (Fursova et al. 2019), are present at considerably lower concentrations (≈ 10 nM). Furthermore, while the concentration of RING1B is similar to that of *Drosophila* PC, the concentration of nucleosome substrates is 10-fold higher in mESCs (Section 4.2.5 and Steffen

et al. 2012). In mESCs, therefore, a relatively much smaller pool of vPRC1 complexes appear to be capable of maintaining global ubiquitylation and Polycomb-mediated gene repression (Fursova et al. 2019), although quantification of the *Drosophila* homologues of vPRC1 subunits has not been performed, and may yet reveal similarities in this aspect too.

Combining my quantification of PRC1 subunit abundances with SPT measurements of PRC1 binding and existing knowledge of the Polycomb repressive system further allowed me to construct a loosely quantitative model of PRC1 dynamics. These measurements revealed that the number of bound PRC1 complexes is similar to or lower than the number genes repressed by PRC1 in mESCs (Fursova et al. 2019; Blackledge et al. 2019). Furthermore, H2AK119ub1 is known to be deposited by PRC1 at low levels throughout the genome (Fursova et al. 2019), indicating that PRC1 binding is not merely limited to target promoters and further reducing the number of complexes likely to be occupying repressed genes at any given time. This low occupancy makes it difficult to imagine that these complexes could directly repress transcription through binding to promoters. Instead, it would seem more likely that a small number of PRC1 complexes achieve repression through their ubiquitin E3 ligase activity, which is in line with recent reports that Polycomb-mediated repression depends both on the more catalytically active vPRC1 complexes (Fursova et al. 2019) and PRC1 catalytic activity itself (Blackledge et al. 2019; Tamburri et al. 2019). Although evidence directly linking the major product of PRC1 catalytic activity, H2AK119ub1, to repression of transcription is still lacking, it is considerably more abundant than the complex which deposits it and is also enriched at promoters of repressed genes.

Interestingly, combining prior measurements of nucleosome spacing (Teif et al. 2012), genome size (Mouse Genome Sequencing Consortium 2002), and the number of H2AK119ub1-modified histones (Fursova et al. 2019; Albright et al. 1979; Matsui et al. 1979) indicates that H2AK119ub1-modified histones likely outnumber PRC1 complexes by at least 100-fold in mESCs. H2AK119ub1 has been measured previously to have a relatively short decay half life of 90 minutes (Seale 1981), longer than histone acetylation, but shorter than phosphorylation and methylation (Chestier and Yaniv 1979; Jackson et al. 1975; Zee et al. 2009). Therefore, to maintain H2AK119ub1 at a constant level, each vPRC1 complex would be required to catalyse approximately one ubiquitylation reaction each minute. Although comparing measured rates of catalysis for ubiquitin E3 ligases *in vitro* to this *in vivo* estimate is difficult due to the use of non-physiological conditions in *in vitro* assays and presumably much higher target search times *in*

vivo, this rate at least is on the same order of magnitude as previously reported apparent second order rate constants for vPRC1 complexes *in vitro* (Taherbhoy et al. 2015). By building on this initial quantitative characterisation of the Polycomb system, it may therefore be possible to begin to develop quantitative models for the function of the mammalian Polycomb system, similar to work developing models of the *Drosophila* Polycomb system (Fonseca et al. 2012; Steffen et al. 2013).

Live-cell dynamics measurements of individual PRC1 subunits revealed that different PRC1 complexes show broadly similar diffusing and binding behaviours, although there are significant differences between complexes which may result from their individual modes of binding to chromatin. Because individual PRC1 complexes are present in different proportions, these modes of binding will contribute to the overall dynamics of PRC1 to different extents. Therefore, having characterised the dynamics of each PRC1 complex, I aimed to perturb individual chromatin binding modalities to measure their contribution to PRC1 dynamics.

POLYCOMB-DEPENDENT HISTONE MODIFICATIONS CONTRIBUTE TO POLYCOMB COMPLEX CHROMATIN BINDING

5.1 Introduction

Both Polycomb complexes, PRC1 and PRC2, have histone-modifying activity. PRC1 catalyses the monoubiquitylation of H2AK119 (Wang et al. 2004a), while PRC2 catalyses mono-, di- and trimethylation of H3K27 (Cao et al. 2002). How these modifications are involved in Polycomb-mediated gene repression remains an area of active research, although PRC1 catalytic activity has been shown to be a key driver of repression, suggesting that H2AK119ub1 might play a central role (Blackledge et al. 2019; Tamburri et al. 2019).

Regardless of their direct contribution to gene repression, both H3K27me3 and H2AK119ub1 can be bound by PRC1 and PRC2. cPRC1 complexes incorporate CBX proteins, which bind to H3K27me3 (Cao et al. 2002; Bernstein et al. 2006; Morey et al. 2012; Zhen et al. 2016), while vPRC1 complexes bind to H2AK119ub1 through RYBP/YAF2 (Arrigoni et al. 2006; Almeida et al. 2017). Similarly, PRC2 complexes bind H2AK119ub1 through JARID2, which is incorporated into a subset of PRC2 complexes (Cooper et al. 2014; Kalb et al. 2014; Cooper et al. 2016; Grijzenhout et al. 2016), while H3K27me3 is bound by the core PRC2 subunit EED, which allosterically activates the methyltransferase activity of the complex (Hansen et al. 2008; Margueron et al. 2009; Jiao and Liu 2015; Justin et al. 2016; Brooun et al. 2016). Both modifications, therefore, may contribute to how PRC1 and PRC2 engage with chromatin.

The roles of both H2AK119ub1 and H3K27me3 in Polycomb protein chromatin binding have previously been investigated using ChIP approaches. Removal of PRC2 and loss of H3K27me3 has been found to cause a major decrease of RING1B chromatin occupancy as measured by ChIP (Schoeftner et al. 2006; Boyer et al. 2006; Leeb et al. 2010; Tavares et al. 2012). H3K27me3 appears to contribute modestly to PRC2 chromatin binding. H3K27me3 does not appear to

enhance binding PRC2 binding to chromatin *in vitro* (Wang et al. 2017a), while inhibition of H3K27 methylation resulted in only a small reduction in stable PRC2 chromatin binding as measured by SPT (Youmans et al. 2018). Conditional removal of PRC1 catalytic activity causes a major decrease in chromatin occupancy by PRC2, as well as a smaller decrease in chromatin occupancy of PRC1 (Blackledge et al. 2019). Reduction in PRC1 occupancy in these experiments may be accounted for by the reduction in H3K27me3 at the same sites due to reduced PRC2 binding, and therefore loss of cPRC1 binding.

Because of the limitations inherent to ChIP approaches in studying chromatin binding (Section 1.3.1), I wanted to investigate the effect of loss of Polycomb-dependent histone modifications on PRC1 and PRC2 chromatin binding using live cell imaging and SPT. Furthermore, I aimed to define how binding to these modifications contributes to the dynamics I had previously observed for PRC1 subunits.

5.2 Results

5.2.1 Generation of endogenous HaloTag cell lines in a PRC1 conditional catalytic point mutant background

I aimed to measure the contribution made by H2AK119ub1 to the interactions of Polycomb complexes with chromatin by removing H2AK119ub1 in mESCs and measuring changes in the dynamics of Polycomb proteins using SPT. To do this, I took advantage of a previously described cell line in which a PRC1 conditional catalytic point mutant (CPM) system has been engineered (PRC1^{CPM}, Blackledge et al. 2019). In this cell line, the catalytic activity of PRC1 complexes is removed by mutating key residues in RING1B and its paralogue RING1A which allow it to interact with an E2 ubiquitin-conjugating enzyme (Buchwald et al. 2006; Tsuboi et al. 2018; Blackledge et al. 2019). This interaction is essential to enable RING1B to transfer the E2-conjugated ubiquitin onto H2AK119. These cells were generated by engineering the endogenous *Ring1b* gene to contain a copy of the exon encoding the E2 interaction domain with I53A and D56K mutations antisense to the wild type exon. Both exons are flanked by inverted double LoxP/Lox2272 sites and the cells also express tamoxifen-inducible CRE recombinase. On tamoxifen treatment, the CRE recombinase inverts the LoxP/Lox2272-flanked region, causing the cell to change from expressing wild type RING1B to the catalytically inactive RING1B^{I53A/D56K} mutant. These cells also have

equivalent I50A and D53K mutations constitutively engineered into the endogenous *Ring1a* gene. Therefore, in these cells, it is possible to completely remove PRC1 ubiquitin E3 ligase activity, and therefore H2AK119ub1, following tamoxifen treatment (Figure 5.1 A and Blackledge et al. 2019).

I introduced endogenous HaloTag fusions of Polycomb subunits into the PRC1^{CPM} cell line with the aim of measuring the contribution of PRC1 catalytic activity to their dynamics. I chose to tag RING1B as described above (Section 3.2.1) and the PRC2 core subunit SUZ12 in order to determine how each Polycomb complex is affected by removal of PRC1 catalytic activity. I generated an endogenous N-terminal HaloTag fusion of SUZ12 similar to a fusion employed previously (Youmans et al. 2018). I confirmed that tagging was homozygous and expression of the tagged protein was similar to wild type for both proteins by western blotting (Figure 5.1 B and C), and that the HaloTag was functional and the tagged protein localised normally within the nucleus by fluorescence microscopy (Figure 5.1 D). I further confirmed that tamoxifen treatment caused a loss of H2AK119ub1 in both cell lines, while bulk H3K27me3, deposited by PRC2, was largely unaffected by tamoxifen treatment (Figure 5.1 E and F). As described previously for the N-terminal HaloTag fusion of SUZ12 (Youmans et al. 2018), H3K27me3 levels were reduced relative to those in RING1B-HT PRC1^{CPM} cells. The decreased H3K27me3 levels were unlikely to affect the dynamics of the tagged SUZ12, however, as complete removal of H3K27me3 using a PRC2 inhibitor was found to only slightly reduce the stability of PRC2 binding (Youmans et al. 2018). I therefore was able to generate cell lines in which I could conditionally remove PRC1 catalytic activity and measure PRC1 and PRC2 dynamics and subnuclear localisation in live-cell imaging experiments.

5.2.2 Removal of PRC1 catalytic activity has modest effects on Polycomb complex chromatin binding

To begin characterising the contribution of PRC1 catalytic activity to Polycomb complex chromatin binding, I first applied 65 Hz SPT and analysis using Spot-On as described above (Section 3.2.2) to measure the bound fractions of RING1B and SUZ12 before and after tamoxifen treatment in PRC1^{CPM} cells. In untreated cells, RING1B had a bound fraction similar to my previous measurement for RING1B (Figure 3.2), while the bound fraction of SUZ12 was slightly lower, though comparable to those of other Polycomb proteins I have measured (Figure 5.2 A

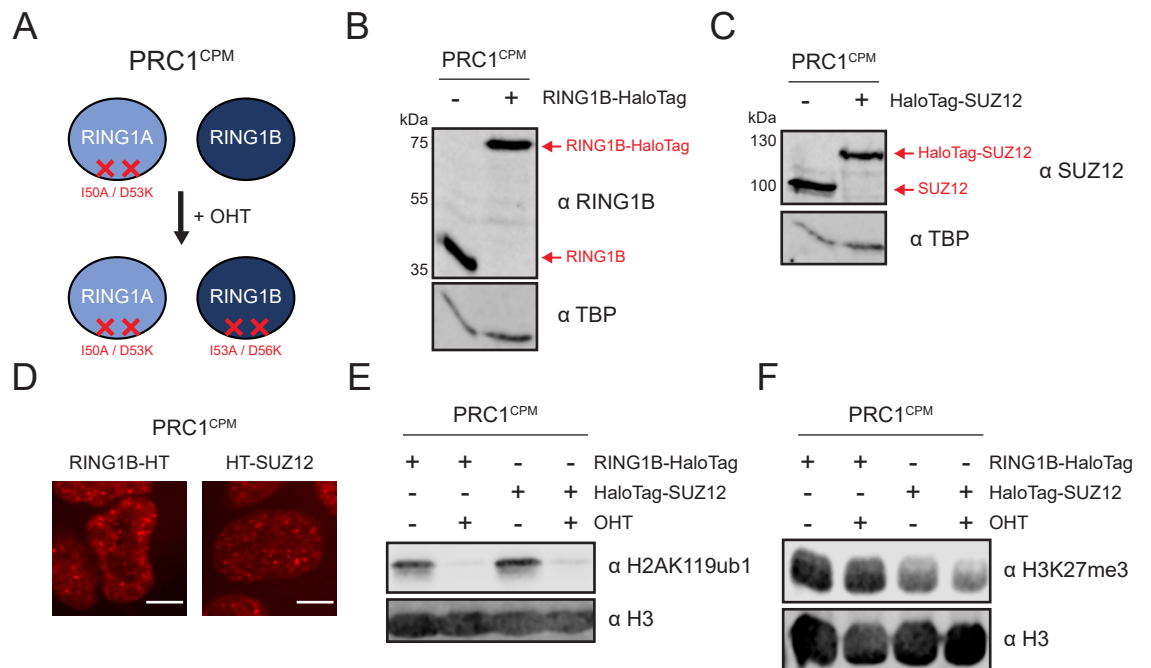


Figure 5.1: Generation of HaloTag cell lines for PRC1 and PRC2 in a PRC1^{CPM} background

(A) A schematic of the PRC1^{CPM} cell line. RING1A is constitutively mutated (RING1A^{I50A/D53K}) and addition of OHT causes expression of catalytically inactive RING1B (RING1B^{I53A/D56K}) in place of wild type RING1B.

(B) Western blot showing homozygous tagging of RING1B (37.6 kDa) with the HaloTag (34.5 kDa) to give a 72.1 kDa fusion protein (RING1B-HaloTag) in a PRC1^{CPM} background. TBP is a loading control.

(C) Western blot showing homozygous tagging of SUZ12 (83.0 kDa) with the HaloTag (34.5 kDa) to give a 117.5 kDa fusion protein (HaloTag-SUZ12) in a PRC1^{CPM} background. TBP is a loading control.

(D) Single Z-slice of nuclei of tagged cells labelled with Halo-JF₅₄₉ to confirm HaloTag function and subnuclear localisation. Scale bar = 5 μm.

(E) Western blot showing levels of H2AK119ub1 in both RING1B-HT PRC1^{CPM} and HT-SUZ12 PRC1^{CPM} cells before and after 72 h OHT treatment. Histone H3 is included as a loading control.

(F) Western blot showing levels of H3K27me3 in both RING1B-HT PRC1^{CPM} and HT-SUZ12 PRC1^{CPM} cells before and after 72 h OHT treatment. Histone H3 is included as a loading control.

and Chapter 4). Surprisingly, the bound fraction of RING1B was not affected by removal of PRC1 catalytic activity, while the bound fraction of SUZ12 was modestly reduced. A reduced bound fraction suggests that PRC2 binding may be less frequent (i.e. reduced k_{on}) or less stable (increased k_{off}) in the absence of PRC1 catalytic activity.

I next measured the survival times (Section 3.2.3) of each protein in the presence and absence of tamoxifen to determine whether binding stability of each Polycomb complex was affected by removal of PRC1 catalytic activity. Both RING1B and SUZ12 had similar proportions of stably bound molecules in untreated cells, and, as I observed previously for RING1B (Section 3.2.3), stable binding was longer than 100 s, beyond what I was able accurately measure (Figure 5.2 B and C). Following removal of PRC1 catalytic activity, the fraction of RING1B molecules which bound stably was modestly reduced, while SUZ12 was unaffected (Figure 5.2 B). There was no measurable change in the binding time for either protein, as both remained beyond the limit of accurate measurement (Figure 5.2 C). Together, these results suggest that, although the number of bound PRC1 complexes is not affected, fewer bound complexes engage in stable binding events in the absence of PRC1 catalytic activity and H2AK119ub1. Conversely, the probability of PRC2 binding to chromatin is reduced following removal of PRC1 catalytic activity, while its binding stability appears to be unaffected, indicating that complexes which do bind are stabilised by other means.

To investigate changes in PRC1 and PRC2 dynamics in the absence of PRC1 catalytic activity in further detail, I analysed tracks for RING1B and SUZ12 in PRC1^{CPM} cells using vbSPT as described previously (Section 4.2.2). This revealed that each protein produced a distinct transition probability network which was largely consistent in structure in the presence and absence of tamoxifen (Figure 5.3). Both proteins exhibit a decrease in the fraction of steps belonging to the two states with the lowest D^* , reflecting decreased chromatin binding. For SUZ12, this was consistent with the decreased bound fraction observed in the Spot-On analysis. Additionally, there was no change in the time that molecules remained in these states (Figure 5.3 B, dwell times), in line with the lack of observed changes to SUZ12 stable binding (Figure 5.2 B and C).

However, for RING1B, I observed a decrease in both the fraction of molecules in the least diffusing states and the dwell time in these states (Figure 5.3 A). This decreased dwell time, which is consistent with the reduced fraction of stably bound RING1B molecules (Figure 5.2 B), likely

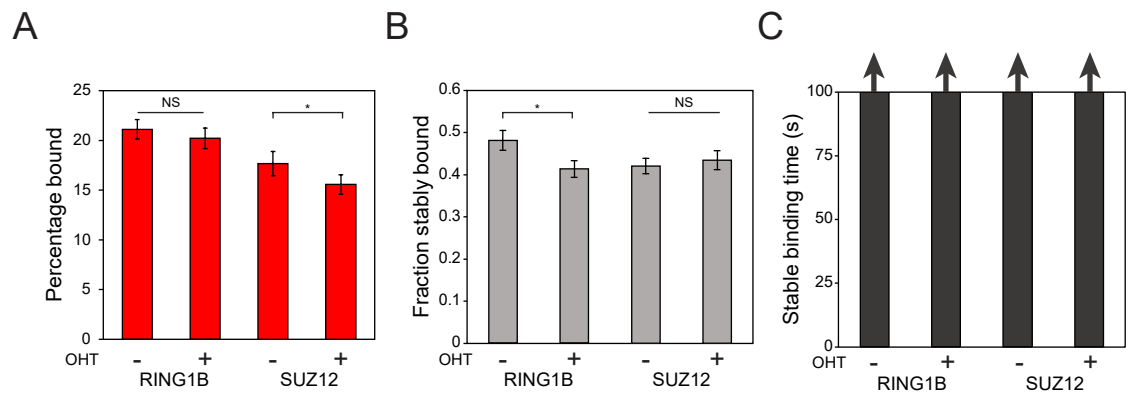


Figure 5.2: Removal of PRC1 catalytic activity reduces the bound fraction of SUZ12 and destabilises RING1B binding

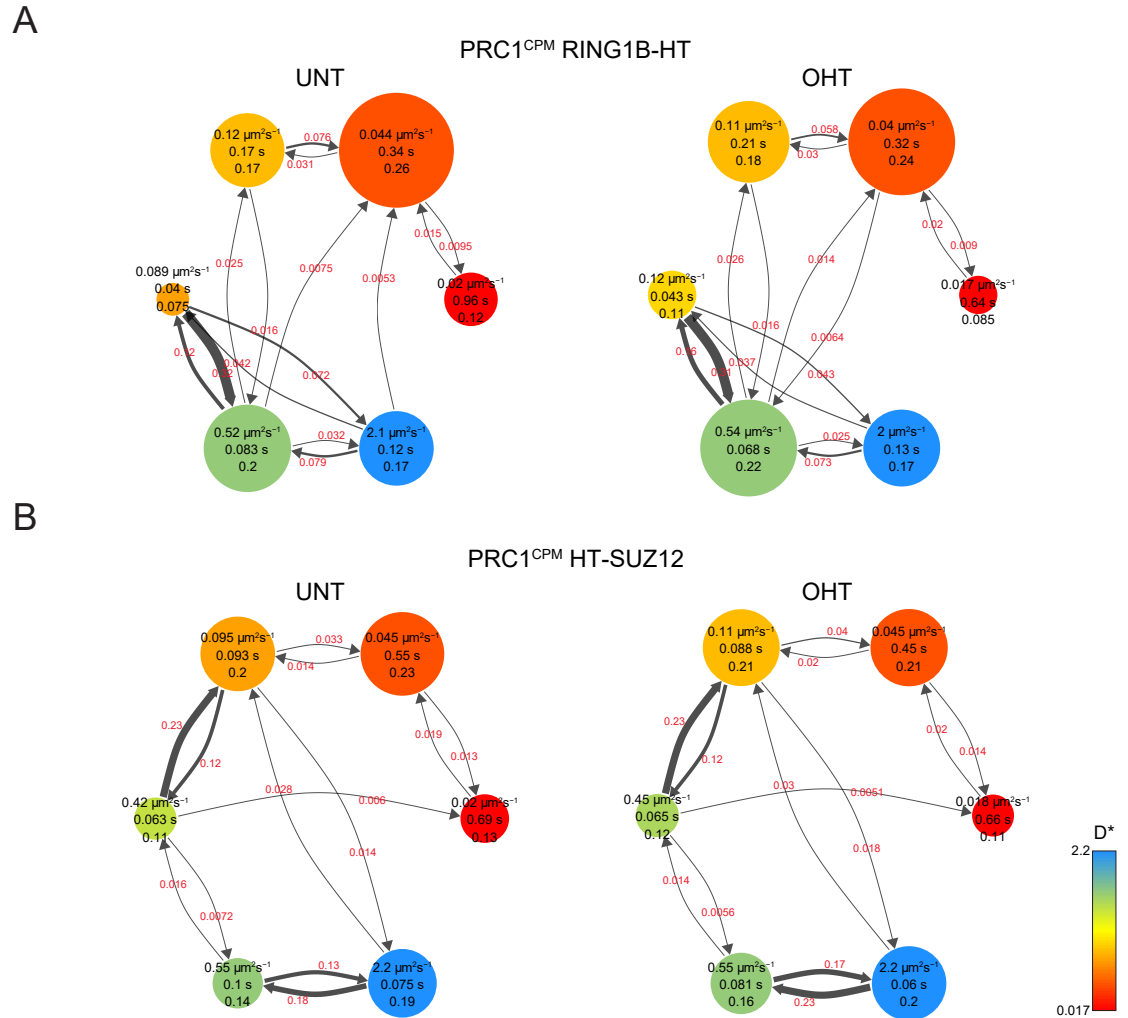
(A) Bar chart showing percentages of molecules in the bound state for RING1B and SUZ12 in PRC1^{CPM} cells before (UNT) and after 72 h tamoxifen treatment (OHT), as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >50 cells. * indicates $p < 0.05$ as calculated by Student's t test. NS = not significant.

(B) Bar chart showing the fraction of RING1B and SUZ12 molecules in PRC1^{CPM} cells exhibiting stable binding in 0.5 s tracking experiments before (UNT) and after 72 h tamoxifen treatment (OHT). Error bars indicate standard error of the mean for >20 cells. * indicates $p < 0.05$ as calculated by Student's t test. NS = not significant.

(C) Bar chart showing stable binding times for RING1B and SUZ12 in PRC1^{CPM} cells before (UNT) and after 72 h tamoxifen treatment (OHT), in 0.5 s tracking experiments. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Survival times were measured from >20 cells.

explains the reduction in bound fractions in vbSPT which is not observed in the Spot-On analysis (Figure 5.2 A). While vbSPT considers all steps in all tracks of sufficient length, Spot-On is limited to the first 13 steps (or 195 ms) of each track. As a result, Spot-On is less influenced by longer tracks, which are predominately bound, but is not able to observe that bound sections of tracks were substantially reduced in length, from 960 ms to 640 ms, as measured by vbSPT. This decrease in length will have the effect of reducing the number of steps with small diffusion coefficients when considering all steps, as in the vbSPT analysis, but is not sufficient to significantly decrease the fraction of bound molecules as measured by Spot-On.

Thus, removal of PRC1 catalytic activity reduces the frequency of PRC2 chromatin binding without affecting the stability of this binding, while reducing the stability but not frequency of PRC1 chromatin binding. This may suggest that binding to H2AK119ub1 is responsible for allowing PRC2 to bind more frequently to sites enriched with PRC1, while acting to stabilise PRC1 at the same sites.



5.2.3 Correct subnuclear localisation of Polycomb complexes requires PRC1 catalytic activity

Because I observed changes in PRC1 and PRC2 chromatin binding on removal of PRC1 catalytic activity, and both PRC1 and PRC2 occupancy at RING1B peaks was observed to be disrupted in ChIP under the same conditions (Blackledge et al. 2019; Tamburri et al. 2019), I wanted to examine whether the subnuclear localisation of PRC1 and PRC2. Polycomb bodies are enriched for bound PRC1 (Sections 3.2.4 and 4.2.2) and are thought to contain strong Polycomb targets (Isono et al. 2013). Because removal of PRC1 catalytic activity has been found to disrupt the occupancy of both PRC1 and PRC2 at Polycomb targets, enrichment of the complexes at Polycomb bodies might be expected reduced under the same conditions.

I therefore analysed enrichment of RING1B and SUZ12 at Polycomb bodies in PRC1^{CPM} cells before and after tamoxifen treatment. Localisation at Polycomb bodies of both proteins was visibly disrupted (Figure 5.4 A), with a more dramatic disruption of SUZ12 localisation. Importantly, this did not result from any change in the protein levels between tamoxifen-treated and untreated cells (Figure 5.4 B). Counting the number of Polycomb bodies revealed a decrease in the number of foci for both proteins, from a mean of 94 to 72 for RING1B, and from 120 to 57 for SUZ12 (Figure 5.4 C). Protein enrichment in Polycomb bodies over the rest of the nucleus for both RING1B and SUZ12 was also reduced (Figure 5.4 D), consistent with a loss of preference for binding these sites. By dividing the Polycomb bodies into four groups, with boundaries defined by separating bodies in untreated cells into quartiles by either mean fluorescence intensity or volume, I found that, in tamoxifen treated cells, only Polycomb bodies of the lowest fluorescence intensity remained visible for SUZ12, while the effect on RING1B was less severe, with a loss of only the brightest foci (Figure 5.4 E). Furthermore, Polycomb bodies of larger volumes were lost to a greater extent for both proteins, indicating an overall decrease in Polycomb body volume (Figure 5.4 F). PRC1 catalytic activity therefore is a major factor in Polycomb protein enrichment at Polycomb bodies, particularly for PRC2, which exhibited a dramatic loss in localisation to these nuclear foci.

5.2.4 Generation of a system to rapidly deplete PRC2

In addition to binding to H2AK119ub1, both PRC1 and PRC2 are known to bind to H3K27me3, which is deposited by PRC2. Binding of H3K27me3 by PRC2 has been found to have a relatively

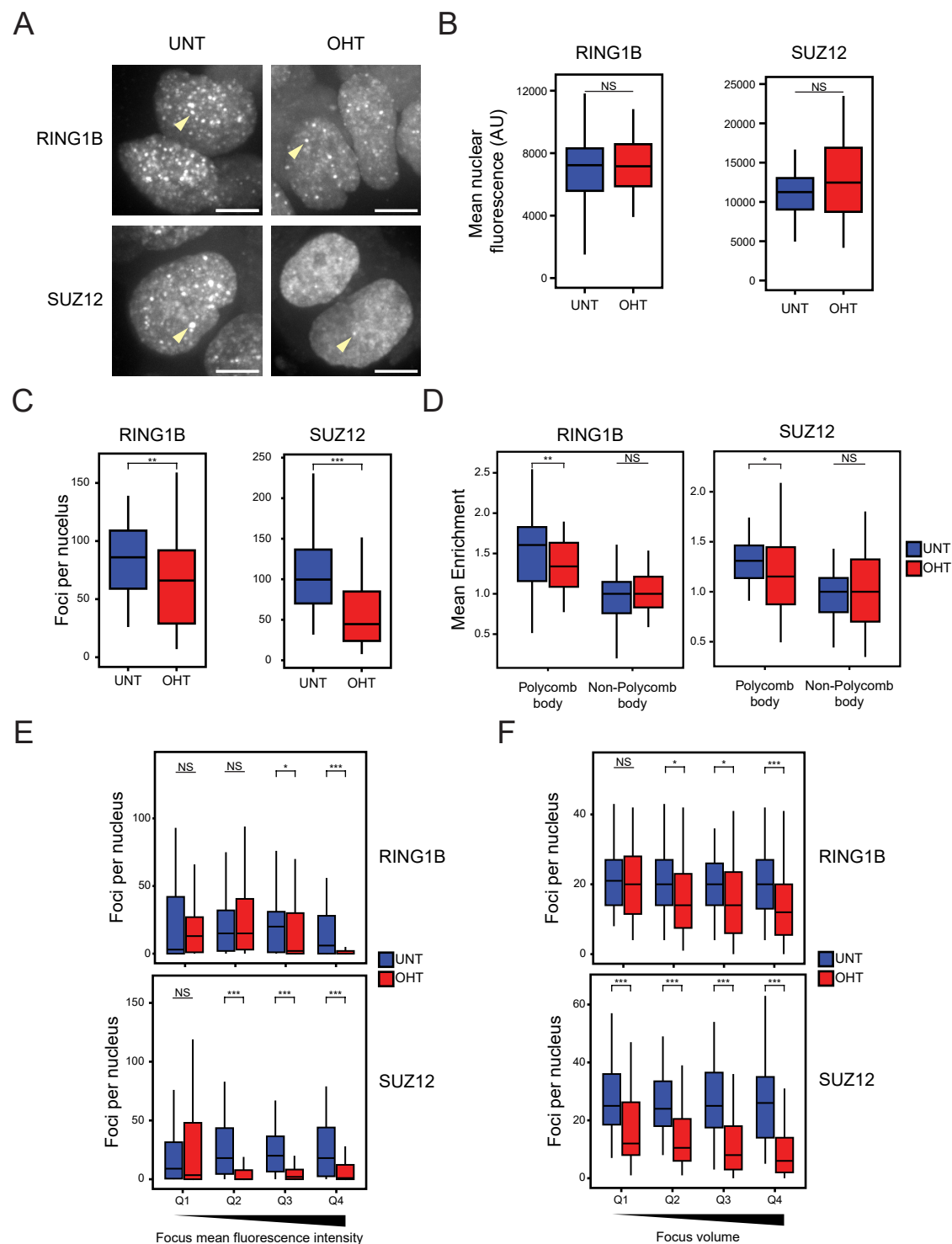


Figure 5.4: PRC1 catalytic activity is required for normal subnuclear localisation of Polycomb proteins

(A) Maximum intensity projections of RING1B-HT-JF₅₄₉ PRC1^{CPM} and JF₅₄₉-HT-SUZ12 PRC1^{CPM} ESCs before (UNT) and after OHT treatment (OHT). Example nuclear foci (Polycomb bodies) are indicated by arrowheads. Scale bar is 5 μ m.

(B) Box plots comparing mean nuclear fluorescence for RING1B and SUZ12 in PRC1^{CPM} ESCs before (UNT, blue) and after OHT treatment (OHT, red). n > 50 cells per condition.

(C) Box plots comparing the number of nuclear foci observed for RING1B and SUZ12 in PRC1^{CPM} ESCs before (UNT, blue) and after OHT treatment (OHT, red). n > 50 cells per condition.

(D) Boxplot comparing the enrichment of fluorescence intensity in Polycomb bodies relative to the remaining nuclear volume for RING1B and SUZ12 in PRC1^{CPM} ESCs before (UNT, blue) and after OHT treatment (OHT, red). Enrichment is calculated from the mean fluorescence intensity per unit volume of all Polycomb bodies in each cell compared to the remaining nuclear volume (non-Polycomb body), normalised to the median non-Polycomb fluorescence. n > 50 cells per condition.

(E) Box plots comparing number of nuclear foci counted for RING1B and SUZ12 in PRC1^{CPM} ESCs before (UNT, blue) and after OHT treatment (OHT, red), with foci divided into quartiles based on focus mean intensities in untreated cells. n > 50 cells per condition.

(F) Box plots comparing number of nuclear foci counted for RING1B and SUZ12 in PRC1^{CPM} ESCs before (UNT, blue) and after OHT treatment (OHT, red), with foci divided into quartiles based on focus volumes in untreated cells. n > 50 cells per condition.

small contribution to PRC2 dynamics (Youmans et al. 2018), despite being important for the catalytic activity of the complex (Margueron et al. 2009; Jiao and Liu 2015). Removal of PRC2 and H3K27me3 also results in the loss of a large proportion of PRC1 occupancy in ChIP experiments (Schoeftner et al. 2006; Boyer et al. 2006; Leeb et al. 2010; Tavares et al. 2012), and appears to be responsible for the majority of chromatin binding by cPRC1 subunit CBX7 (Cao et al. 2002; Bernstein et al. 2006; Morey et al. 2012; Zhen et al. 2016). Because cPRC1 appears to make up a major proportion of PRC1 complexes in mESCs (Section 4.2.4), I wanted to test the contribution of H3K27me3 to the dynamics of total PRC1, cPRC1 and vPRC1. I therefore sought to develop cell lines in which I could rapidly deplete PRC2, resulting in a loss of H3K27me3, and then characterise PRC1 dynamics.

To achieve this, I implemented the dTAG system into PRC1-HaloTag fusion cell lines I had previously generated (Nabet et al. 2018). This system uses a modified FKBP tag (FKBP12^{F36V}) which binds to bifunctional molecule (dTAG-13) containing both a bumped FKBP ligand and a ligand for the CRBN E3 ubiquitin ligase. Binding of the ligand to the tagged protein thereby recruits CRBN, which polyubiquitylates the protein, targeting it for degradation (Figure 5.5 A). I used CRISPR/Cas9-mediated genome editing to introduce the FKBP12^{F36V} sequence at the N-terminus of *Suz12*, a core PRC2 subunit. It is known that the histone methylation activity of PRC2 is dependent on interaction of the enzymatic EZH subunit with SUZ12, and therefore depletion of SUZ12 using the dTAG system would result in a loss of H3K27me3 (Cao and Zhang 2004; Jiao

and Liu 2015).

I engineered this SUZ12 degron system into three cell lines: RING1B-HT PRC1^{CPM}, PCGF2-HT, and HT-PCGF6. Measuring the effect of PRC2 depletion on RING1B dynamics would indicate the effect on the complex as a whole, and the addition of the dTAG system in a PRC1^{CPM} background would allow me to simultaneously remove both PRC1 catalytic activity and PRC2. PCGF2 dynamics represent mostly cPRC1 complexes, on which I hypothesised there would be a larger effect than on PRC1 as a whole, as this subset of complexes bind H3K27me3. Finally, I included PCGF6 as a control for a PRC1 subunit I did not expect to be directly affected by loss of PRC2 and H3K27me3, as PCGF6-PRC1 has no known mechanism for binding H3K27me3.

I confirmed that the FKBP12^{F36V} tag was introduced homozygously in each cell line by western blotting (5.5 B). Treatment with dTAG-13 resulted in a complete loss of protein observable by western blot within one hour, and this depletion was maintained in longer treatments. H3K27me3 was lost from cells more slowly, and so I opted to use a 96 h dTAG-13 treatment for live cell imaging experiments to allow for sufficient loss of H3K27me3 (Figure 5.5 C). Bulk H2AK119ub1 was unaffected by this treatment as measured by western blot (Figure 5.5 C), in line with previous observations (Tavares et al. 2012; Fursova et al. 2019). I was therefore able to generate cell lines in which I could rapidly degrade SUZ12 (henceforth referred to as dTAG-SUZ12), leading to a loss of H3K27me3, allowing me to investigate the contribution of this histone modification to PRC1 dynamics.

5.2.5 H3K27me3 contributes to cPRC1 chromatin binding

Having generated dTAG-SUZ12 cell lines with endogenous PRC1-HaloTag fusions, I performed SPT experiments to measure the dynamics of PRC1 before and after dTAG-13 treatment and measure the contribution of H3K27me3 to PRC1 dynamics and chromatin binding. Analysis of 65 Hz SPT using Spot-On revealed that the bound fractions for both RING1B and PCGF2 were significantly reduced, likely due to a loss of binding to H3K27me3 (Figure 5.6 A). PCGF6, however, was unaffected as expected, given that PCGF6-PRC1 complexes are not thought to bind H3K27me3. Measurement of the fraction of stably bound molecules and stable binding times for RING1B, PCGF2 and PCGF6 following depletion of H3K27me3 did not reveal significant changes for any of the three proteins (Figure 5.6 B and C). This suggests that H3K27me3 functions to increase the frequency of PRC1 binding to chromatin (increasing k_{on}) rather than to stabilise it

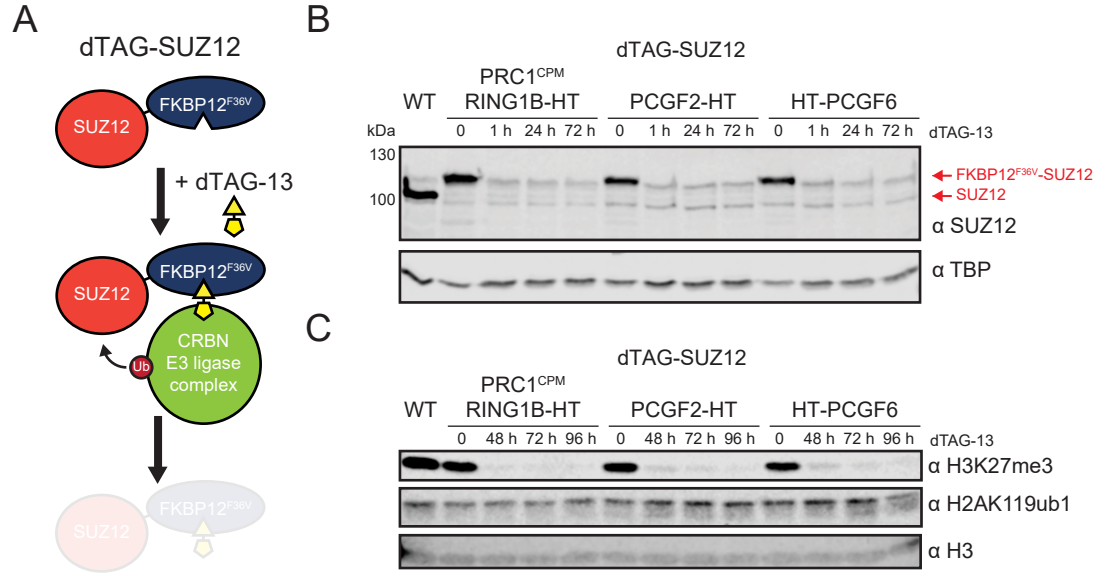


Figure 5.5: The dTAG system allows for rapid degradation of SUZ12 and depletion of H3K27me3

(A) A schematic of the dTAG system applied to SUZ12. Treatment of cells containing the FKBP12^{F36V}-SUZ12 fusion protein with dTAG-13 leads to recruitment of the CRBN E3 ubiquitin ligase, which polyubiquitylates the target protein leading to its degradation.

(B) Western blots showing homozygous tagging of SUZ12 (83.0 kDa) with FKBP12^{F36V} (13.0 kDa) at N-terminus and treatment with dTAG-13, resulting in degradation of the tagged protein. TBP is a loading control. WT indicates untargeted mESCs.

(C) Western blots showing H3K27me3 and H2AK119ub1 in dTAG-SUZ12 cells before and after treatment with dTAG-13. H3 is a loading control. WT indicates untargeted mESCs.

(decreasing k_{off}).

Analysing SPT tracks using vbSPT for RING1B, PCGF2 and PCGF6 before and after SUZ12 and H3K27me3 depletion revealed a change in transition probability network structure for both RING1B and PCGF2, while PCGF6 was again unaffected (Figure 5.7 A - C). The transition probability networks of RING1B and PCGF2 are similar both before and after dTAG-13 treatment, undergoing similar changes on loss of PRC2 (Figure 5.7 A and B). Both proteins appear to lose a substantial proportion of steps in the slow diffusing state with $D^* \approx 0.14 \mu\text{m}^2 \text{s}^{-1}$. This may represent a loss of short binding events ($<15 \text{ ms}$) which appear to lead to slow diffusion. These short ($<100 \text{ ms}$) sections of tracks are classified as slow by vbSPT, because short-lived binding events will only affect one or two consecutive steps each, but these steps are likely classified as bound by Spot-On, which does not consider them in context. Loss of short binding events would therefore account for the observed loss of slow diffusing fraction in vbSPT and bound fraction in Spot-On analysis. The lack of change in the two diffusing states with the lowest D^* values in vbSPT analysis is consistent with the unchanged stable binding I observed (Figure 5.6 B and C). Together, SPT analysis of PRC1 dynamics in dTAG-SUZ12 cells indicates that PRC2 functions

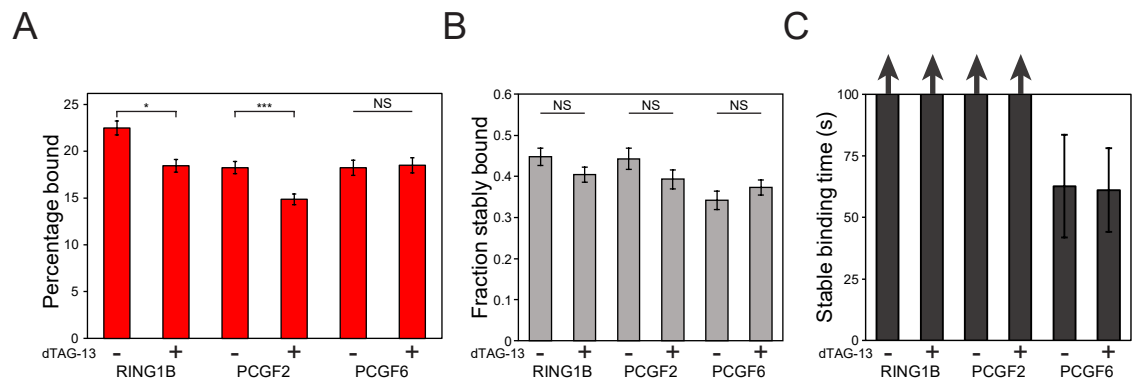


Figure 5.6: Removal of H3K27me3 decreases cPRC1 binding to chromatin

(A) Bar chart showing percentages of molecules in the bound state for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 cells before (UNT) and after 96 h dTAG-13 treatment (dTAG-13), as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >60 cells. * indicates $p < 0.05$ as calculated by Student's t test. NS = not significant.

(B) Bar chart showing the fraction of RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 cells exhibiting stable binding in 0.5 s tracking experiments before (UNT) and after 96 h dTAG-13 treatment (dTAG-13). Error bars indicate standard error of the mean for >20 cells. * indicates $p < 0.05$ as calculated by Student's t test. NS = not significant.

(C) Bar chart showing stable binding times for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 cells before (UNT) and after 96 h dTAG-13 treatment (dTAG-13) in 0.5 s tracking experiments. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Survival times were measured from >20 cells. Error bars indicate standard error of the mean.

to increase the frequency of cPRC1 chromatin binding, while binding is stabilised through other means. The dynamics of vPRC1 subunit PCGF6 appear to be unaffected by removal of PRC2.

5.2.6 H3K27me3 contributes modestly to the formation of PRC1 nuclear foci

Depletion of SUZ12 and H3K27me3 reduced chromatin binding of subunits of cPRC1 complexes (RING1B and PCGF2), but not that of PCGF6, which forms only vPRC1 complexes. cPRC1 has been proposed as the driver of formation of Polycomb bodies through self association of cPRC1 PHC subunits (Isono et al. 2013), and, therefore, I investigated whether disruption of cPRC1 chromatin binding by removal of H3K27me3 would affect Polycomb localisation at these sites using live cell fluorescence microscopy (Figure 5.8 A).

Analysing the nuclear fluorescence revealed, surprisingly, that the amount of RING1B, PCGF2 and PCGF6 protein was modestly but reproducibly increased following depletion of SUZ12 (Figure 5.8 B). This may suggest the existence of an undescribed mechanism for compensating for loss of PRC2 by increased expression of PRC1, or that PRC2 normally slightly represses PRC1 gene expression or controls PRC1 protein levels. Despite the increase in the expression of RING1B and PCGF2, the absolute number of bound molecules in these cells, based on a combination of fluorescence intensity and the bound fraction measured by SPT, was still reduced

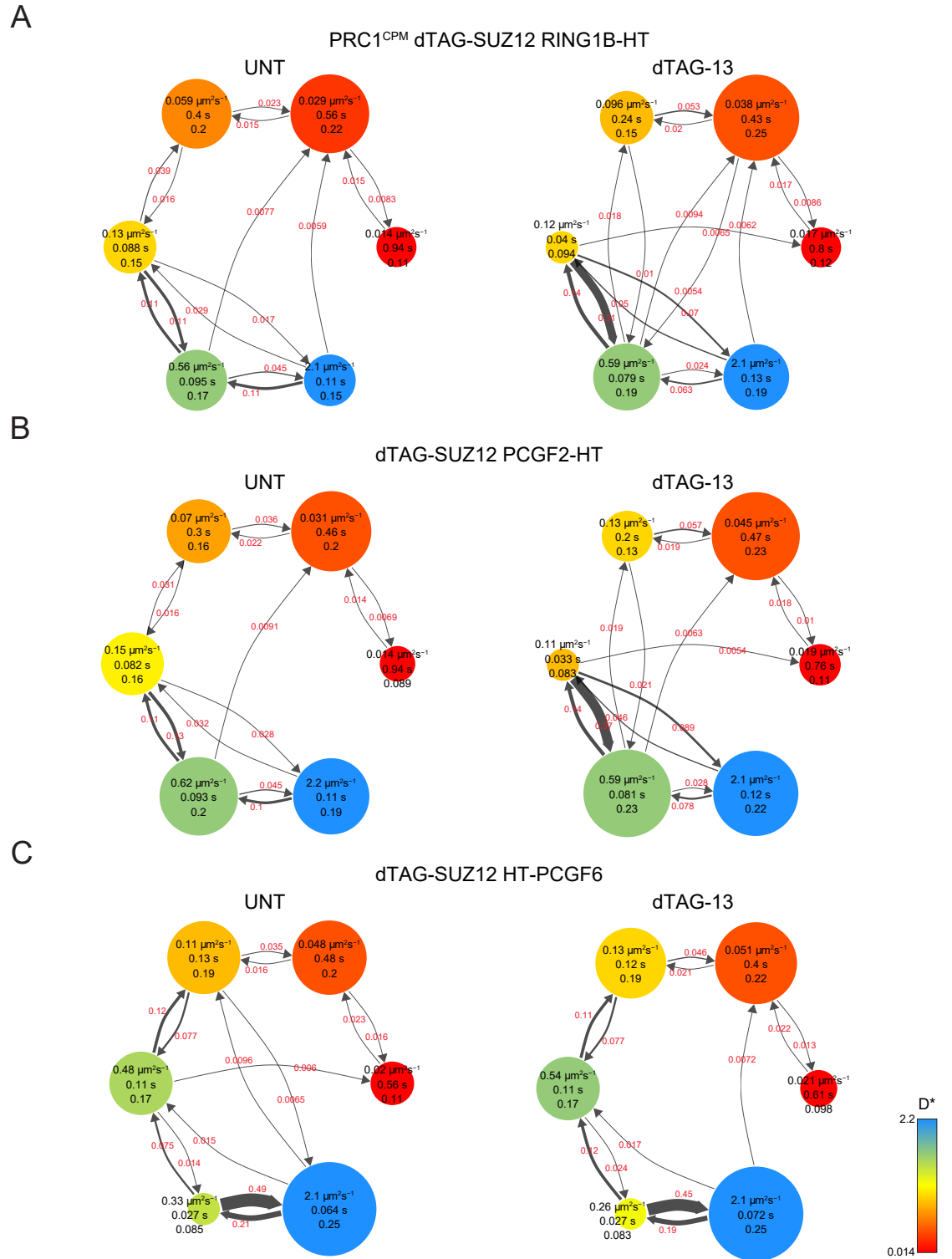


Figure 5.7: Depletion of SUZ12 and H3K27me3 alters the transition probability network of cPRC1 subunits
(A) - (C) Transition probability networks showing vbSPT outputs for (A) RING1B, (B) PCGF2, and (C) PCGF6 in dTAG-SUZ12 cells before (UNT) and after 96 h dTAG-13 treatment (dTAG-13). Diffusive states are represented as nodes. Node colour is scaled to apparent diffusion coefficient (D^* , $\mu\text{m}^2 \text{s}^{-1}$), and node size is scaled to the fraction of molecules in the state. Each state is labelled with its corresponding apparent diffusion coefficient, dwell time, and fraction of molecules in the state. Arrows between nodes indicate state transitions, and red numbers and arrow weight indicate the transition probability. Transitions with probability < 0.005 are not shown.

(a loss of approximately 460 molecules for RING1B and 410 for PCGF2), while the number of bound molecules of PCGF6 increased (a gain of 270). It is possible, however, that to some degree, the decreased bound fraction arises from reduced binding site availability due to the increased number of proteins.

Although expression of PRC1 subunits was increased, analysis of the observed Polycomb bodies revealed a decrease in the number of foci visible for PCGF2 from a mean of 144 to 107 per cell, while the number of foci visible for RING1B or PCGF6 was not strongly reduced, consistent with a loss of only cPRC1 binding (Figure 5.8 C). The lack of complete loss of foci for PCGF2 suggests that enrichment of PCGF2 in Polycomb bodies is not solely dependent on cPRC1 binding to H3K27me3. Curiously, RING1B and PCGF2 showed a slight upward trend in enrichment of protein in Polycomb bodies despite diminished chromatin binding (Figure 5.8 D). This might be the product of increased cPRC1 levels driving increased enrichment of cPRC1 at these sites through self interaction.

As would be anticipated given higher protein levels, RING1B, PCGF2 and PCGF6 Polycomb bodies were brighter following depletion of SUZ12 (Figure 5.8 E), while the distribution of volumes for visible foci was largely unaltered, indicating no change in the mean volumes of the Polycomb bodies which remain (Figure 5.8 F). Together, these results indicate that, while depletion of SUZ12 reduces the number of sites of PCGF2 enrichment, PRC2 and H3K27me3 cannot be the only factor driving the stability of Polycomb nuclear foci. Furthermore, the lack of any effect on PCGF6 foci suggests that recruitment to these sites for PCGF6-PRC1 complexes is independent of cPRC1 and H3K27me3.

5.2.7 Simultaneous depletion of SUZ12 and removal of PRC1 catalytic activity does not further reduce PRC1 chromatin binding

Having tested the contributions of PRC1 catalytic activity and PRC2 to PRC1 dynamics and chromatin binding individually, I wanted to test whether simultaneous removal of both factors would cause a greater effect. I therefore treated my previously generated RING1B-HT PRC1^{CPM} dTAG-SUZ12 with both tamoxifen and dTAG-13, causing conversion of RING1B to the catalytically inactive RING1B^{I53A/D56K} mutant along with degradation of SUZ12 and performed SPT to determine the effect on RING1B chromatin binding (Figure 5.9 A). Unexpectedly, the bound fraction of RING1B was only slightly reduced compared to untreated cells, with

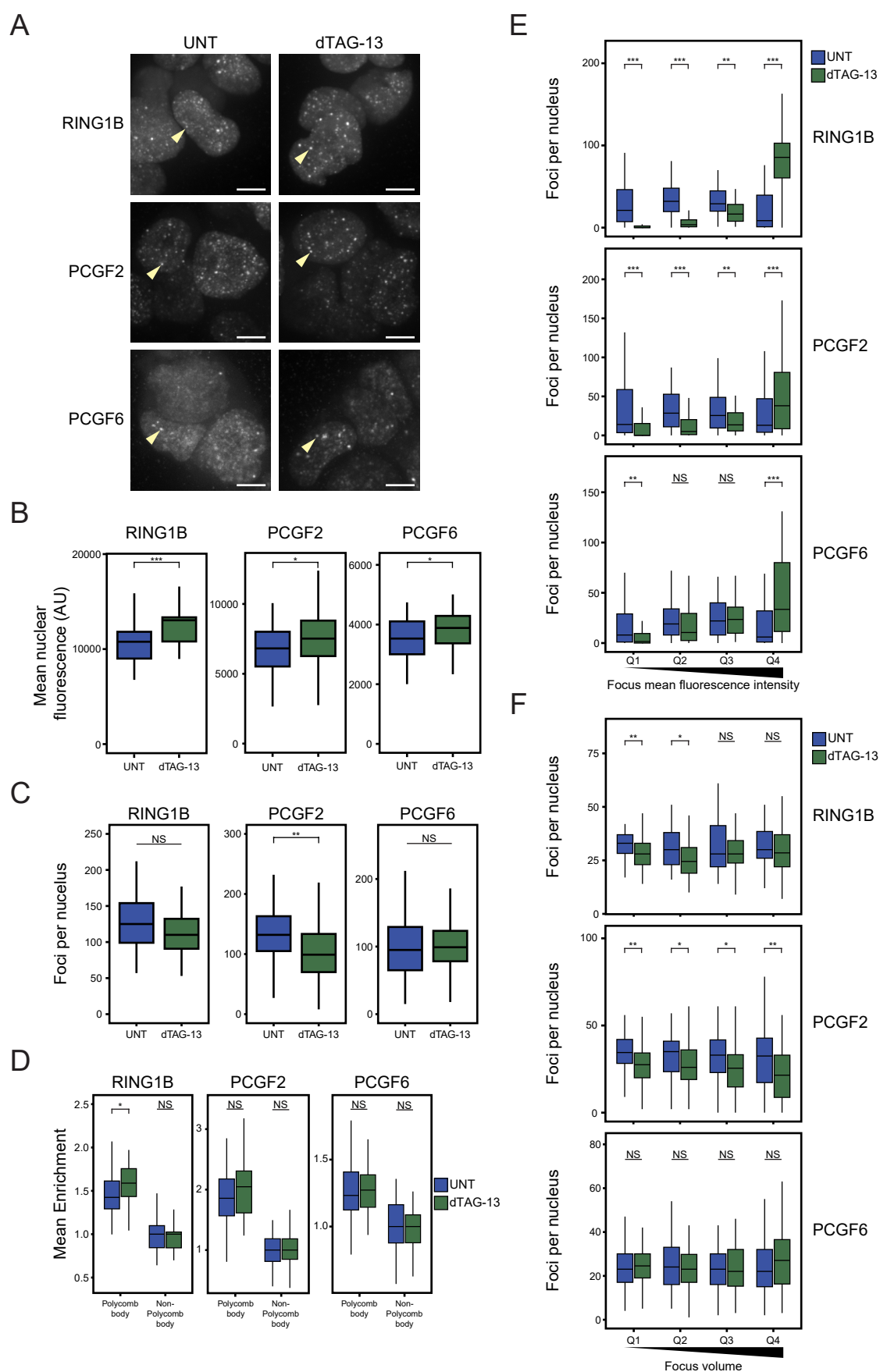


Figure 5.8: Depletion of SUZ12 modestly affects cPRC1 localisation to Polycomb bodies

(A) Maximum intensity projections of RING1B-HT-JF₅₄₉ PRC1^{CPM} dTAG-SUZ12, PCGF2-HT-JF₅₄₉ dTAG-SUZ12 and JF₅₄₉-HT-PCGF6 dTAG-SUZ12 ESCs before (UNT) and after dTAG-13 treatment (dTAG-13). Example nuclear foci (Polycomb bodies) are indicated by arrowheads. Scale bar is 5 μ m.

(B) Box plots comparing mean nuclear fluorescence for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 treatment (dTAG-13, green). $n > 50$ cells per condition.

(C) Box plots comparing the number of nuclear foci observed for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 treatment (dTAG-13, green). $n > 50$ cells per condition.

(D) Boxplot comparing the enrichment of fluorescence intensity in Polycomb bodies relative to the remaining nuclear volume for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 treatment (dTAG-13, green). Enrichment is calculated from the mean fluorescence intensity per unit volume of all Polycomb bodies in each cell compared to the remaining nuclear volume (non-Polycomb body), normalised to the median non-Polycomb fluorescence. $n > 50$ cells per condition.

(E) Box plots comparing number of nuclear foci counted for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 treatment (dTAG-13, green), with foci divided into quartiles based on focus mean intensities in untreated cells. $n > 50$ cells per condition.

(F) Box plots comparing number of nuclear foci counted for RING1B, PCGF2 and PCGF6 in dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 treatment (dTAG-13, green), with foci divided into quartiles based on focus volumes in untreated cells. $n > 50$ cells per condition.

a magnitude of decrease smaller than that for cells treated with dTAG alone (Figure 5.9 B). Measurement of stable binding revealed no significant change in the fraction of molecules which were bound stably, or any measurable change in the binding time (Figure 5.9 C and D).

Analysis of tracks of RING1B molecules from cells following depletion of SUZ12 and removal of PRC1 catalytic activity using vbSPT revealed a similarly small effect on the transition probability network for RING1B (Figure 5.10). The major observable change was a decrease in the proportion of steps in states with the lowest D^* values and a corresponding increase in the proportion in states with faster diffusion, recapitulating the observed decrease in bound fraction from the Spot-On analysis. There was no major change to the dwell time of any states, consistent with the observed lack of change in binding times (Figure 5.9 C and D). Notably, the perturbation of the network structure I observed in the dTAG-13-only treatment did not occur following treatment with both dTAG-13 and tamoxifen.

Together, SPT approaches reveal that PRC1 catalytic activity and H2AK119ub1, and PRC2 and H3K27me3 do not appear to have an additive effect on PRC1 dynamics and chromatin binding. In fact, simultaneous removal of PRC1 catalytic activity and functional PRC2 appears to have a more subtle effect on PRC1 dynamics than either one individually. This may result from the distinct contributions each activity makes to PRC1 dynamics. While loss of PRC1 catalytic activity reduces the stability of PRC1 interactions with chromatin, loss of PRC2 and H3K27me3 reduces the frequency of short-lived interactions. Simultaneous loss of both transient and stable binding

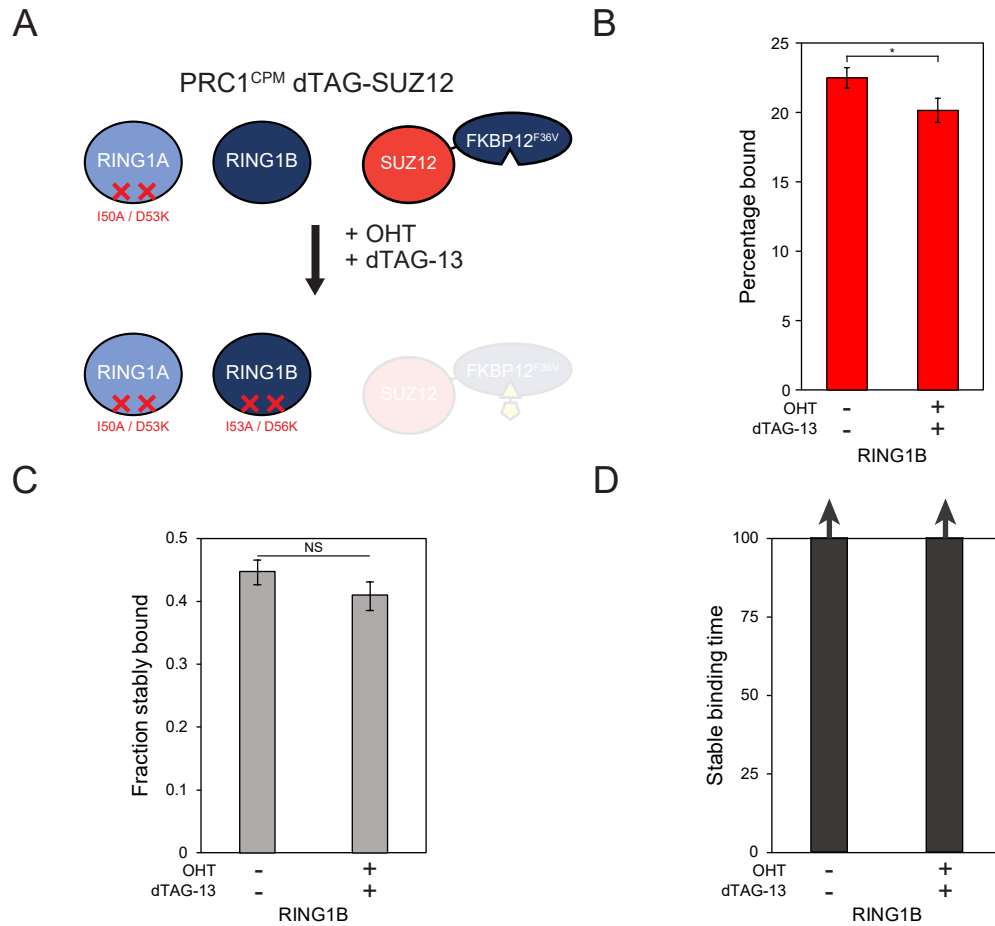


Figure 5.9: Removal of H3K27me3 decreases cPRC1 binding to chromatin

(A) Schematic for treatment of PRC1^{CPM} dTAG-SUZ12 with both tamoxifen (OHT) and dTAG13.

(B) Bar chart showing percentages of molecules in the bound state for RING1B in PRC1^{CPM} dTAG-SUZ12 cells before (UNT) and after 96 h dTAG-13 treatment (dTAG-13) and 72 h tamoxifen treatment (OHT), as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >60 cells. * indicates $p < 0.05$ as calculated by Student's t test. NS = not significant.

(C) Bar chart showing the fraction of RING1B in PRC1^{CPM} dTAG-SUZ12 cells exhibiting stable binding in 0.5 s tracking experiments before (UNT) and after 96 h dTAG-13 treatment (dTAG-13) and 72 h tamoxifen treatment (OHT). Error bars indicate standard error of the mean for >20 cells. * indicates $p < 0.05$ as calculated by Student's t test. NS = not significant.

(D) Bar chart showing stable binding times for RING1B in PRC1^{CPM} dTAG-SUZ12 cells before (UNT) and after 96 h dTAG-13 treatment (dTAG-13) and 72 h tamoxifen treatment (OHT) in 0.5 s tracking experiments. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Survival times were measured from >20 cells.

may therefore be reflected as a small net change in overall behaviour.

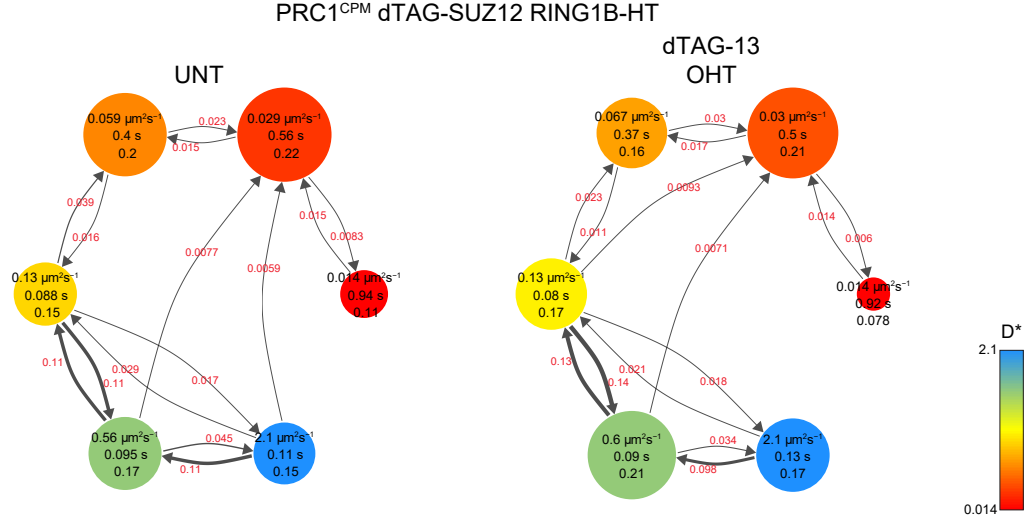


Figure 5.10: Simultaneous removal of PRC1 catalytic activity and depletion of PRC2 only modestly reduces RING1B binding in vbSPT analysis

Transition probability network showing vbSPT outputs for RING1B in PRC1^{CPM} dTAG-SUZ12 cells before (UNT) and after 96 h dTAG-13 treatment (dTAG-13) and 72 h tamoxifen treatment (OHT). Diffusive states are represented as nodes. Node colour is scaled to apparent diffusion coefficient (D^* , $\mu\text{m}^2 \text{s}^{-1}$), and node size is scaled to the fraction of molecules in the state. Each state is labelled with its corresponding apparent diffusion coefficient, dwell time, and fraction of molecules in the state. Arrows between nodes indicate state transitions, and red numbers and arrow weight indicate the transition probability. Transitions with probability <0.005 are not shown.

5.2.8 Both PRC1 catalytic activity and PRC2 contribute to PRC1 occupancy at Polycomb bodies

While binding of H3K27me3 and H2AK119ub1 is present in mutually exclusive PRC1 complexes, and these binding modalities appear to make independent contributions to PRC1 chromatin binding, strong Polycomb targets are enriched for both histone modifications. I hypothesised, therefore, that PRC1 binding and enrichment at these sites may be dependent on both histone modifications. Given that Polycomb bodies are proposed to represent strong Polycomb targets (Isono et al. 2013), I analysed RING1B subnuclear localisation in cells following removal of PRC1 catalytic activity and H2AK119ub1, and depletion of SUZ12 and H3K27me3 (Figure 5.11 A). Interestingly, RING1B expression was not increased following treatment with both tamoxifen and dTAG-13 as it was in cells treated with dTAG-13 only (Figure 5.11 B). This suggests that the mechanism resulting in increased PRC1 levels is not active in the absence of PRC1 catalytic activity.

Despite the modest effect on PRC1 dynamics following removal of PRC1 catalytic activity and SUZ12 depletion, the number of visible foci in dTAG-13 and tamoxifen-treated cells was strongly

reduced, from a mean of 145 to 80 (Figure 5.11 C). Enrichment of RING1B in Polycomb bodies relative to the rest of the nucleus was unchanged following dTAG-13 and tamoxifen treatment (Figure 5.11 D). This is likely because foci with intermediate brightness became less abundant, while the numbers of both the most and least bright foci were unchanged, resulting in a similar mean brightness and therefore enrichment of foci in total (Figure 5.11 E). This suggests that the most enriched sites are able to maintain enrichment of Polycomb proteins, while sites with weaker enrichment are more strongly affected, either shifting to a class with lower mean intensity or no longer being visible at all. Average Polycomb body volume was also slightly reduced, with a preferential loss of Polycomb bodies from higher volume quartiles (Figure 5.11 F).

PRC1 catalytic activity and PRC2 are therefore responsible for a large part of PRC1 recruitment to Polycomb bodies, suggesting that maintenance of PRC1 binding at many of these sites is dependent on the activity of both Polycomb complexes.

5.3 Summary and discussion

Here I have performed SPT and analysed the localisation of Polycomb proteins with a view to understanding how histone modifications deposited by both PRC1 and PRC2 affect Polycomb dynamics and interactions with chromatin. To achieve this, I employed two systems which enabled me to conditionally remove PRC1 catalytic activity (PRC1^{CPM}) and deplete a core subunit of PRC2, SUZ12 (dTAG-SUZ12), respectively.

While each perturbation results in the loss of the histone modification deposited by their respective complex, it is not possible to state definitively that any effects observed derive directly from the loss of histone modifications. While histone H2A is the major target of PRC1 ubiquitylation, it is possible that the complex also modifies other proteins which may be involved in regulating PRC1 and PRC2 chromatin occupancy. Furthermore, loss of PRC1 catalytic activity has been shown to result in a dramatic reduction in H3K27me3 at peaks of RING1B occupancy in ChIP experiments (Blackledge et al. 2019), although bulk levels of H3K27me3 are not reduced. However, I did not observe the same reduction in bound fraction for PRC1 in the absence of PRC1 catalytic activity as occurred in SUZ12-depleted cells. Similarly, H3K27me3 has been described to only slightly stabilise PRC2 chromatin binding (Youmans et al. 2018), while I observed no effect on SUZ12 chromatin binding stability in absence of PRC1 catalytic activity. Therefore, because the changes

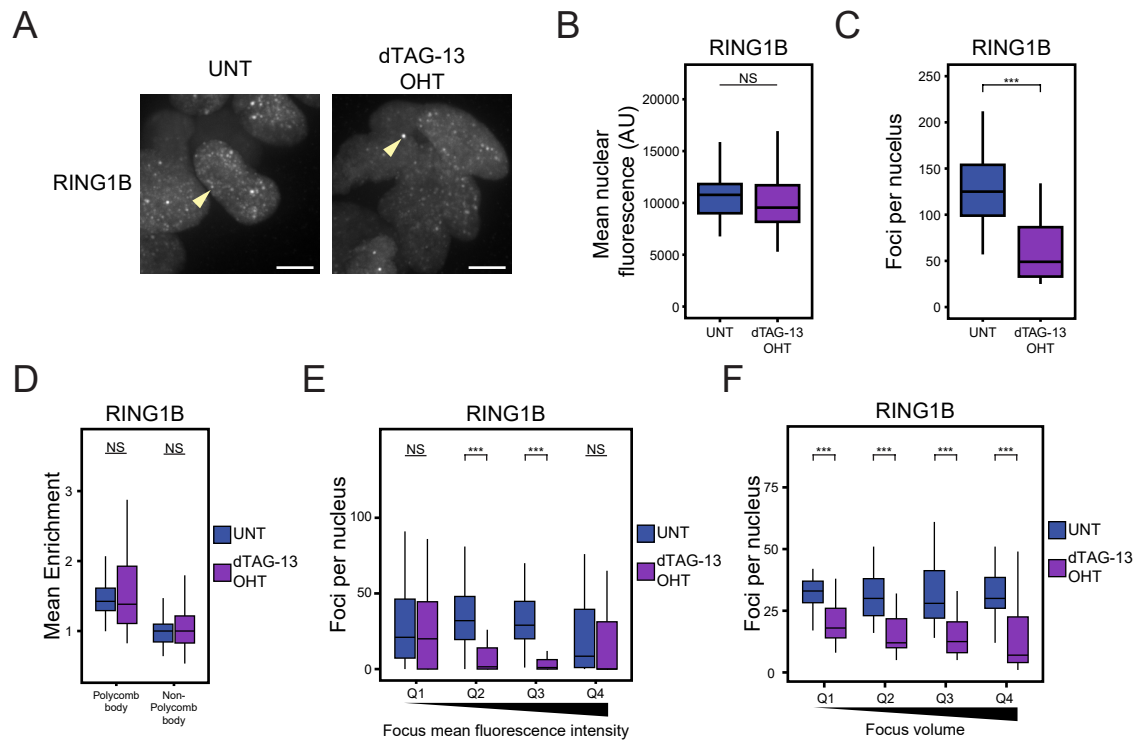


Figure 5.11: PRC1 catalytic activity and PRC2 contribute together to PRC1 enrichment at Polycomb bodies//
(A) Maximum intensity projections of RING1B-HT-JF₅₄₉ PRC1^{CPM} dTAG-SUZ12 ESCs before (UNT) and after dTAG-13 and tamoxifen treatment (dTAG-13 and OHT). Example nuclear foci (Polycomb bodies) are indicated by arrowheads. Scale bar is 5 μ m.
(B) Box plots comparing mean nuclear fluorescence for RING1B in PRC1^{CPM} dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 and tamoxifen treatment (dTAG-13 and OHT, purple). $n > 50$ cells per condition.
(C) Box plots comparing the number of nuclear foci observed for RING1B in PRC1^{CPM} dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 and tamoxifen treatment (dTAG-13 and OHT, purple). $n > 50$ cells per condition.
(D) Boxplot comparing the relative mean fluorescence signal per unit volume of all Polycomb bodies in each cell compared to the remaining nuclear volume, normalised to the median non-Polycomb fluorescence, for RING1B in PRC1^{CPM} dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 and tamoxifen treatment (dTAG-13 and OHT, purple). $n > 50$ cells per condition.
(E) Box plots comparing number of nuclear foci counted for RING1B in PRC1^{CPM} dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 and tamoxifen treatment (dTAG-13 and OHT, purple), with foci divided into quartiles based on focus mean intensities in untreated cells. $n > 50$ cells per condition.
(F) Box plots comparing number of nuclear foci counted for RING1B in PRC1^{CPM} dTAG-SUZ12 ESCs before (UNT, blue) and after dTAG-13 and tamoxifen treatment (dTAG-13 and OHT, purple), with foci divided into quartiles based on focus volumes in untreated cells. $n > 50$ cells per condition.

I have observed in chromatin binding are different under conditions of loss of PRC1 catalytic activity and depletion of SUZ12, it seems unlikely that the loss of H3K27me3 at RING1B peaks in the absence of PRC1 catalytic activity is the cause of the changes in chromatin binding in PRC1^{CPM} cells.

Depletion of functional PRC2 through degradation of SUZ12 may also result in loss of PRC2 activities besides methylation of H3K27 which could also affect PRC1 dynamics. Further, loss of the activity of either Polycomb complex may have effects on the chromatin environment which alter Polycomb protein binding. It has been reported previously that, while Polycomb complexes do not modify chromatin accessibility, PRC1 is responsible for elevated nucleosome occupancy at Polycomb-occupied promoters (King et al. 2018), and removal of PRC1 catalytic activity may therefore cause a change in nucleosome occupancy and Polycomb chromatin binding. However, the different effects on PRC1 and PRC2 in the absence of PRC1 catalytic activity, and lack of any change to PCGF6, which does not bind H3K27me3, in the absence of functional PRC2 would appear to contradict the existence of some general mechanism by which chromatin binding is altered, as more consistent changes to chromatin binding between proteins might be expected. It is likely, therefore, although not conclusive, that the changes I observed in Polycomb protein dynamics and chromatin binding result from a loss of binding to H3K27me3 and H2AK119ub1, functionalities which have been described for both complexes (Zhen et al. 2016; Arrigoni et al. 2006; Cooper et al. 2016; Hansen et al. 2008; Margueron et al. 2009).

Interestingly, the contributions I and others have observed to PRC1 and PRC2 binding by Polycomb-mediated histone modifications appear to mirror each other. Each complex appears to rely on the modification deposited by the other complex for increasing the frequency at which it binds to chromatin, as I observed for PCGF2 and RING1B in dTAG-SUZ12 cells (Section 5.2.5), and for SUZ12 in PRC1^{CPM} cells (Section 5.2.2). This was also previously described for PRC2 in cells expressing mutant SUZ12 which prevents association of PRC2 with the AEBP2 and JARID2 subunits, which recognise H2AK119ub1 (Youmans et al. 2018; Cooper et al. 2016). Recognition by each complex of their own modification, however, seems to instead stabilise chromatin binding (Section 5.2.2 and Youmans et al. 2018). This suggests a system in which cPRC1, which binds H3K27me3 through CBX proteins, and PRC2 complexes containing JARID2, which bind H2AK119ub1, use these modifications to bind more frequently to sites in the genome which are already occupied by the other complex. Meanwhile, vPRC1 complexes, which bind

H2AK119ub1 through RYBP/YAF2 and deposit the same modification, and PRC2, which binds H3K27me3 through EED, use these modifications to stabilise binding events which are initiated through other binding modalities, promoting their catalytic activity at sites where the modifications are already present to generate a positive feedback loop. A key difference between PRC1 and PRC2, however, is that, while binding to H3K27me3 and H2AK119ub1 cannot occur together in the same PRC1 complex, as CBX proteins and RYBP/YAF2 are mutually exclusive, JARID2-containing PRC2 complexes will also contain EED, which binds H3K27me3. PRC2, therefore, would appear to be better suited to a role in maintaining Polycomb-mediated modifications through positive feedback.

Although both H2AK119ub1 and H3K27me3 are present throughout the genome (Jung et al. 2010; Ferrari et al. 2014; Lee et al. 2015; Fursova et al. 2019), PRC1 and PRC2 must be targeted to specific sites to repress transcription and enrich these sites with H2AK119ub1 and H3K27me3. Enrichment of these histone modifications may provide additional binding sites and stabilise binding of each complex at these sites. H2AK119ub1 and H3K27me3 may therefore have a role in the maintenance of Polycomb enrichment following establishment of repression. Because H2AK119ub1 and H3K27me3 affect the binding of both PRC1 and PRC2, both histone modifications likely contribute to this maintenance function. This would explain the larger reduction in Polycomb body localisation I observe following removal of both histone modifications, given that these sites appear to represent strong Polycomb targets with high levels of both histone modifications. It is worth noting, however, that these sites of enrichment persist in the absence of both modifications, albeit in reduced numbers. This suggests that other chromatin binding mechanisms are able to enrich cPRC1 complexes at these sites and produce the association of PHC proteins which is required for the existence of foci (Isono et al. 2013), for example, through proposed DNA binding by CBX7 (Zhen et al. 2016).

One surprising feature of the changes I observed in binding of Polycomb proteins in the absence of PRC1- or PRC2-mediated histone modifications was that the apparent magnitude of the changes was much smaller than might be expected from ChIP-seq data under the same conditions (Tavares et al. 2012; Blackledge et al. 2019). This is likely explainable by a combination of two features of ChIP experiments and their analysis. First, analysis of ChIP experiments typically focusses exclusively on peaks of enrichment of either bound protein or histone modifications. For core Polycomb subunits and histone modifications, there are usually considered to be up to

approximately 10,000 genomic sites with peaks of enrichment, with approximately 2000 high-occupancy sites. However, both H2AK119ub1 and H3K27me3 are deposited more broadly than the peaks of PRC1 and PRC2 ChIP occupancy (Oksuz et al. 2018; Fursova et al. 2019), indicating that these complexes can bind outside of peaks. Because binding occurs broadly across the genome, binding to enriched sites will therefore account for a small proportion of total binding, meaning that perturbations which more strongly affect enriched sites, as I have proposed is the case for removal of these histone modifications, are likely to have modest effects on observed binding overall. Conversely, the focus of ChIP-seq analysis on these more strongly affected enriched sites would indicate a larger effect on binding, producing a disparity between the two techniques. Second, the use of crosslinking, which is heavily dependent on binding time (Schmiedeberg et al. 2009), may further amplify the effect of changes in binding stability, resulting in more dramatic changes in occupancy than observed in SPT experiments.

The fact that stable PCGF2 chromatin binding was not lost following removal of SUZ12 and H3K27me3 was surprising, as H3K27me3 binding by CBX proteins is thought to be the major mechanism by which cPRC1 complexes are recruited to chromatin. However, vbSPT indicated that the reduction in bound fraction observed for both PCGF2 and RING1B in the absence of H3K27me3 might result from a loss of shorter binding events (Section 5.2.5). This suggests that cPRC1 H3K27me3 binding may function as a mechanism for increasing the probability of transiently interacting with chromatin, rather than the mechanism by which these interactions can be stabilised, consistent with a previous study which suggested that the *Drosophila* CBX homologue, PC, binds dynamically to chromatin through H3K27me3 (Ringrose et al. 2004). The remaining stable binding might be explained by DNA binding by CBX7, which has been proposed previously (Zhen et al. 2016), or through the direct interaction between RING1B-PCGF2 and the nucleosome acidic patch (McGinty et al. 2014). PCGF2 can also form variant complexes containing RYBP/YAF2, which bind to H2AK119ub1. This interaction would presumably account for a small fraction of stable PCGF2 binding, if any, since the effect on RING1B binding stability in the absence of H2AK119ub1 was modest. Further work will therefore be required to discover the other determinants of PCGF2 chromatin binding.

Measurement of PRC1 and PRC2 dynamics and subnuclear localisation in live cells therefore revealed that PRC1-deposited H2AK119ub1 promotes chromatin binding by PRC2, while PRC2-deposited H3K27me3 promotes cPRC1 chromatin binding. H2AK119ub1 further stabilises

chromatin binding of PRC1. These modifications are clearly important for stabilising and maintaining Polycomb occupancy at Polycomb-repressed genes, however, they cannot account for the initial recruitment of Polycomb complexes to sites which are not yet enriched with the modifications. In addition to binding to histone modifications, PRC1 complexes also incorporate subunits which recognise and bind to features of DNA, and these mechanisms of binding may be involved in this initiating role. In chapter 6, I will investigate the contributions of PRC1 complexes with DNA binding to PRC1 dynamics.

INVESTIGATING THE CONTRIBUTION OF DNA BINDING TO PRC1 DYNAMICS

6.1 Introduction

DNA binding is thought to encompass a central mechanism by which PRC1 complexes interact with chromatin. In *Drosophila*, where Polycomb was first identified, Polycomb complexes are recruited to DNA regulatory elements called PREs which are bound by a range of sequence-specific transcription factors (Section 1.2.7 and Fauvarque and Dura 1993; Müller and Bienz 1991; Simon et al. 1993). Mammalian PRC1 complexes also incorporate proteins with a range of DNA-binding activities, some of which are sequence-specific, as in *Drosophila*, while others are specific for more generic features of DNA.

Subunits which bind DNA have been described for all of the major forms of PRC1 complexes present in mESCs. PCGF1-PRC1 incorporates the ZF-CXXC protein KDM2B, which binds to nonmethylated CpG dinucleotides (Farcas et al. 2012; Wu et al. 2013; He et al. 2013; Blackledge et al. 2014). This protein has been implicated as a major factor determining PCGF1-PRC1 chromatin binding at CGIs (Blackledge et al. 2014), which overlap around 70% of promoters in mESCs (Long et al. 2013a). Furthermore, it has been proposed that this DNA binding might allow PRC1 to sample promoters to identify sites where Polycomb chromatin domains can be established to repress transcription (Klose et al. 2013).

DNA binding is also thought to be one of the primary drivers underpinning PCGF6-PRC1 chromatin occupancy (Endoh et al. 2017; Stielow et al. 2018; Scelfo et al. 2019). PCGF6-PRC1 complexes incorporate the sequence-specific transcription factors MGA/MAX and E2F6 (Gao et al. 2012; Kloet et al. 2016; Hauri et al. 2016), and are found to be enriched at sites where the binding motifs for these transcription factors are present (Endoh et al. 2017; Stielow et al. 2018; Scelfo et al. 2019).

A mechanism for DNA binding in PCGF2-PRC1 complexes has been described, via the chromodomain and an AT-hook-like motif in the CBX7 subunit (Senthilkumar and Mishra 2009;

Zhen et al. 2016). PCGF3 has also recently been described to interact with the sequence-specific transcription factor USF1, which appears to drive its recruitment to a small number of genomic sites in mESCs (Scelfo et al. 2019). Because both of these mechanisms for PRC1 binding of DNA have been relatively recently described, their importance in and contribution to PRC1 function is not yet well understood.

Therefore, since the DNA binding mechanisms of PCGF1- and PCGF6-PRC1 complexes have been well defined, and these complexes are important for Polycomb-mediated gene repression, I wanted to investigate the contributions of DNA binding to PRC1 dynamics.

6.2 Results

6.2.1 Generation of endogenous HaloTag cell lines in which the KDM2B DNA binding domain can be removed

I observed previously that PCGF1 exhibited different dynamic behaviours to other vPRC1 subunits, having a larger bound fraction and more stable binding than either PCGF3 or PCGF6 (Section 4.2.3). Because nonmethylated CpG dinucleotide binding by KDM2B is both the central mechanism by which PCGF1-PRC1 complexes interact with CGI chromatin based on ChIP experiments (Blackledge et al. 2014), and unique to PCGF1-PRC1 complexes (Gearhart et al. 2006; Sanchez et al. 2007; Gao et al. 2012; Farcas et al. 2012), I hypothesised that the differences in PCGF1 dynamics relative to other vPRC1 subunits might arise from this mode of binding. In order to determine whether KDM2B DNA binding is responsible for the higher bound fraction and more stable binding of PCGF1 compared to other PCGF proteins and RING1B, and to measure the contribution of KDM2B DNA binding to overall PRC1 dynamics, I generated cell lines in which I could remove KDM2B DNA binding and measure RING1B and PCGF1 dynamics.

In order to disrupt KDM2B DNA binding, I took advantage of a previously described cell line in which the exon of *Kdm2b* encoding the ZF-CXXC domain responsible for DNA binding is flanked by loxP sequences, and which express tamoxifen-inducible CRE recombinase (*Kdm2b-CXXC^{fl/fl}*). Treatment of the cells with tamoxifen thus causes removal of the exon encoding the ZF-CXXC domain (Figure 6.1 A and Blackledge et al. 2014). The resulting spliced transcript is still translated in frame, and produces KDM2B protein which interacts with PCGF1-PRC1 as normal, despite lacking the ZF-CXXC domain (Figure 6.1 B and Blackledge et al. 2014). Removal of ZF-CXXC

domain of KDM2B results in loss of KDM2B occupancy at CGI target sites as measured by ChIP (Figure 6.1 C and Blackledge et al. 2014).

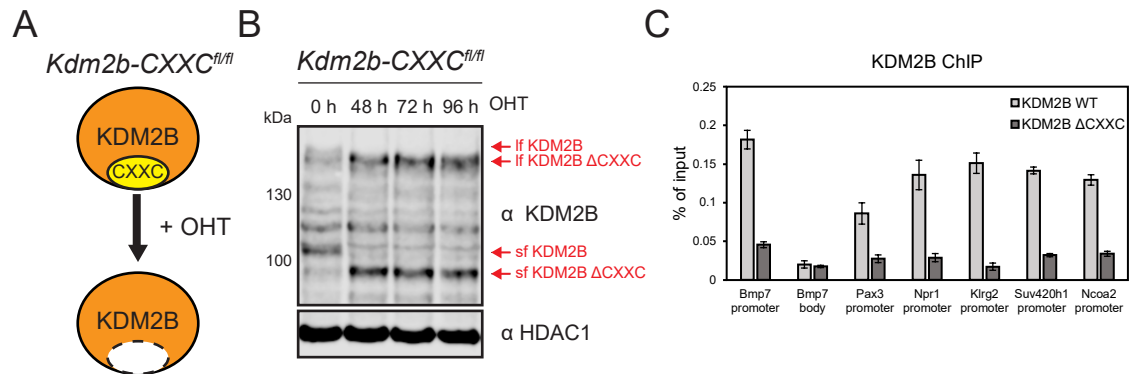


Figure 6.1: KDM2B DNA binding can be disrupted without perturbing protein expression

(A) Schematic of the *Kdm2b-CXXC^{fl/fl}* system. Treatment with tamoxifen (OHT) causes removal of the exon encoding the DNA-binding ZF-CXXC domain of KDM2B, resulting in expression of protein lacking the domain.

(B) Western blot of KDM2B showing a decrease in size of both the short (sf, 88 kDa to 80 kDa) and long (lf, 145 kDa to 137 kDa) isoforms of KDM2B following tamoxifen treatment and removal of the ZF-CXXC encoding exon. HDAC1 is included as a loading control.

(C) KDM2B ChIP-qPCR analysis in *Kdm2b-CXXC^{fl/fl}* cells before (KDM2B WT) and after (KDM2B Δ CXXC) tamoxifen treatment to confirm that KDM2B is lost from chromatin after ZF-CXXC domain removal. qPCR primers for promoters of nonmethylated CpG island genes to which KDM2B binds, and one gene body (*Bmp7* body) to which KDM2B does not bind, were used to measure percent of input recovered. Error bars indicate the standard error of the mean of three biological replicates.

To measure the dynamics of RING1B and PCGF1 following removal of the ZF-CXXC domain of KDM2B, I introduced the HaloTag endogenously into *Ring1b* or *Pcgf1* in *Kdm2b-CXXC^{fl/fl}* cells as previously described (Sections 3.2.1 and 4.2.1). I confirmed that each protein was homozygously tagged and that tamoxifen treatment functioned normally by western blot (Figure 6.2 A - C), and that the tagged protein localised as normal by fluorescence microscopy (Figure 6.2 D). I was, therefore, able to generate cell lines in which I could disrupt KDM2B DNA binding and measure RING1B and PCGF1 dynamics. Subnuclear localisation of RING1B and PCGF1 in these cells appeared to be unchanged following tamoxifen treatment (Figure 6.2 D - G), although weak PCGF1 signal due to its relatively low expression made quantitative assessment of its subnuclear localisation challenging.

6.2.2 Removal of the KDM2B DNA-binding domain disrupts PCGF1 dynamics but not binding

To characterise the effect on total PRC1 and PCGF1 dynamics following disruption of KDM2B DNA binding, I first measured the bound fractions of each protein by SPT. As expected, given the relatively small amount of PCGF1 relative to total RING1B, there was no change to the bound

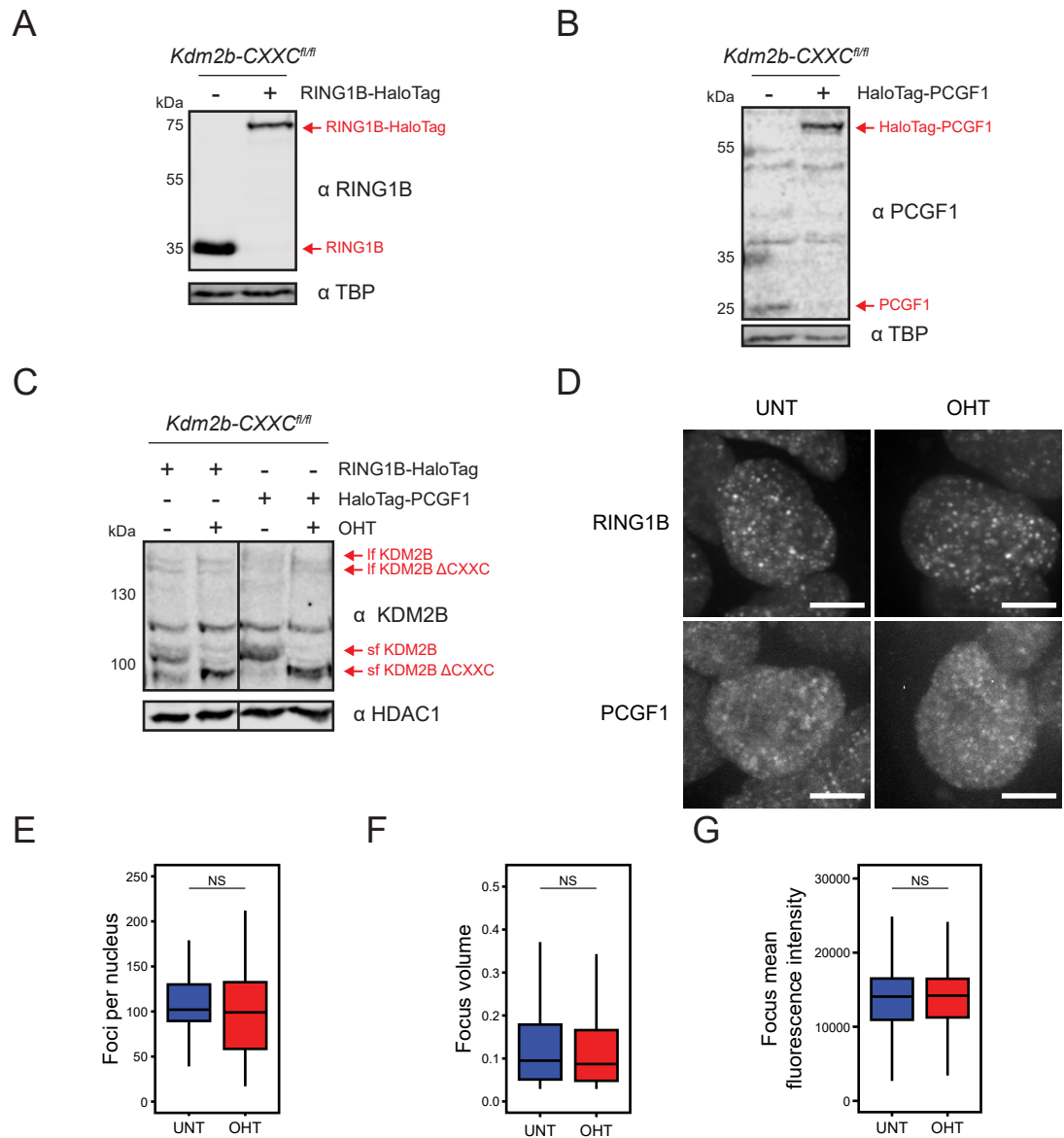


Figure 6.2: Generation of HaloTag fusions of RING1B and PCGF1 in *Kdm2b-CXXC^{fl/fl}* cells

(A) Western blot showing homozygous tagging of RING1B (37.6 kDa) with the HaloTag (34.5 kDa) to give a 72.1 kDa fusion protein (RING1B-HT) in *Kdm2b-CXXC^{fl/fl}* cells. TBP is a loading control.

(B) Western blot showing homozygous tagging of PCGF1 (29.2 kDa) with the HaloTag (34.5 kDa) to give a 63.7 kDa fusion protein (HT-PCGF1) cells *Kdm2b-CXXC^{fl/fl}*. TBP is a loading control.

(C) Western blot of KDM2B showing a decrease in size of both the short (sf, 88 kDa to 80 kDa) and long (lf, 145 kDa to 137 kDa) isoforms of KDM2B following tamoxifen treatment and removal of the ZF-CXXC encoding exon in HaloTag fusion cell lines. HDAC1 is included as a loading control.

(D) Maximum intensity projections of RING1B-HT-JF₅₄₉ and JF₅₄₉-HT-PCGF1 *Kdm2b-CXXC^{fl/fl}* ESCs before (UNT) and after OHT treatment (OHT). Scale bar is 5 μm.

(E) Box plots comparing the number of nuclear foci observed for RING1B in RING1B-HT *Kdm2b-CXXC^{fl/fl}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, blue).

(F) Box plots comparing the volume of nuclear foci observed for RING1B in RING1B-HT *Kdm2b-CXXC^{fl/fl}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, blue).

(G) Box plots comparing the mean fluorescence intensity of nuclear foci observed for RING1B in RING1B-HT *Kdm2b-CXXC^{fl/fl}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, blue). n > 40 cells per condition.

fraction of RING1B (Figure 6.3 A). Surprisingly, however, given the major role DNA binding by KDM2B is thought to have in PCGF1 targeting to CGIs, the bound fraction of PCGF1 was also unchanged. Measurement of the fraction of stably bound protein and binding time also indicated that stable binding of PCGF1 and RING1B was not disrupted (Figure 6.3 B and C).

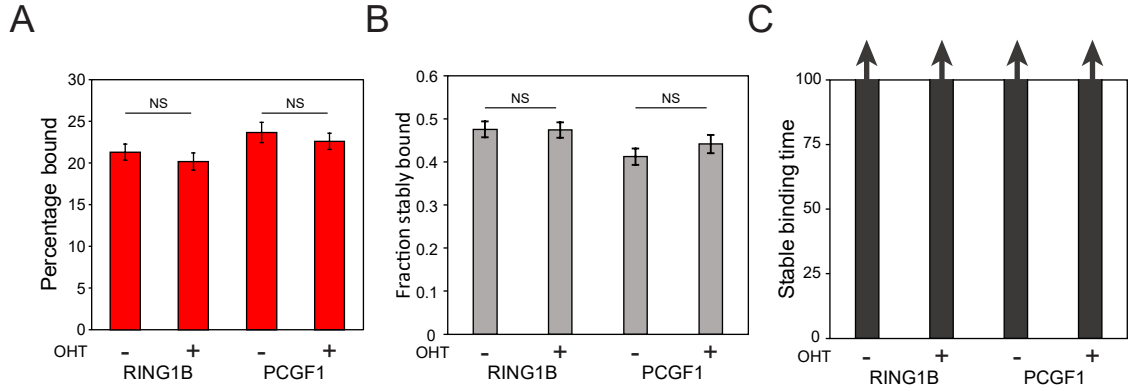


Figure 6.3: KDM2B DNA binding is not required for PCGF1 stable chromatin binding

(A) Bar chart showing percentages of molecules in the bound state for RING1B and PCGF1 in *Kdm2b-CXXC^{fl/fl}* cells before (UNT) and after 72 h tamoxifen treatment (OHT), as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >50 cells. NS = p value not significant as calculated by Student's t test.

(B) Bar chart showing the fraction of RING1B and PCGF1 molecules in *Kdm2b-CXXC^{fl/fl}* cells exhibiting stable binding in 0.5 s tracking experiments before (UNT) and after 72 h tamoxifen treatment (OHT). Error bars indicate standard error of the mean for >20 cells. NS = p value not significant as calculated by Student's t test.

(C) Bar chart showing stable binding times for RING1B and PCGF1 in *Kdm2b-CXXC^{fl/fl}* cells before (UNT) and after 72 h tamoxifen treatment (OHT), in 0.5 s tracking experiments. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Survival times were measured from >20 cells.

I further analysed my tracking data from *Kdm2b-CXXC^{fl/fl}* cells using vbSPT in order to determine whether there was any change in RING1B or PCGF1 dynamics which could not be observed in the overall bound fraction and stable binding. vbSPT analysis indicated very little change in RING1B dynamics, although there was a slight decrease in the fraction of steps belonging to the slow diffusing state with $D^* = 0.1 \mu\text{m}^2 \text{s}^{-1}$ (Figure 6.4 A). Conversely, PCGF1 exhibited a change in overall network structure affecting the slowly diffusing states, with no effect on the bound states (Figure 6.4 B). This may suggest that KDM2B DNA binding contributes to brief interactions of PCGF1 with chromatin below the temporal resolution of the SPT approach, but is dispensable for more stable PCGF1 chromatin binding. The resemblance between the transition network for PCGF1 tamoxifen-treated cells and RING1B suggests that PCGF1 and RING1B have relatively similar dynamics following disruption of KDM2B DNA binding. Together, SPT of PCGF1 in the absence of KDM2B DNA binding yielded a surprising result that suggests PCGF1 chromatin binding is not wholly dependent on KDM2B and may have some binding which is common to other PRC1 complexes.

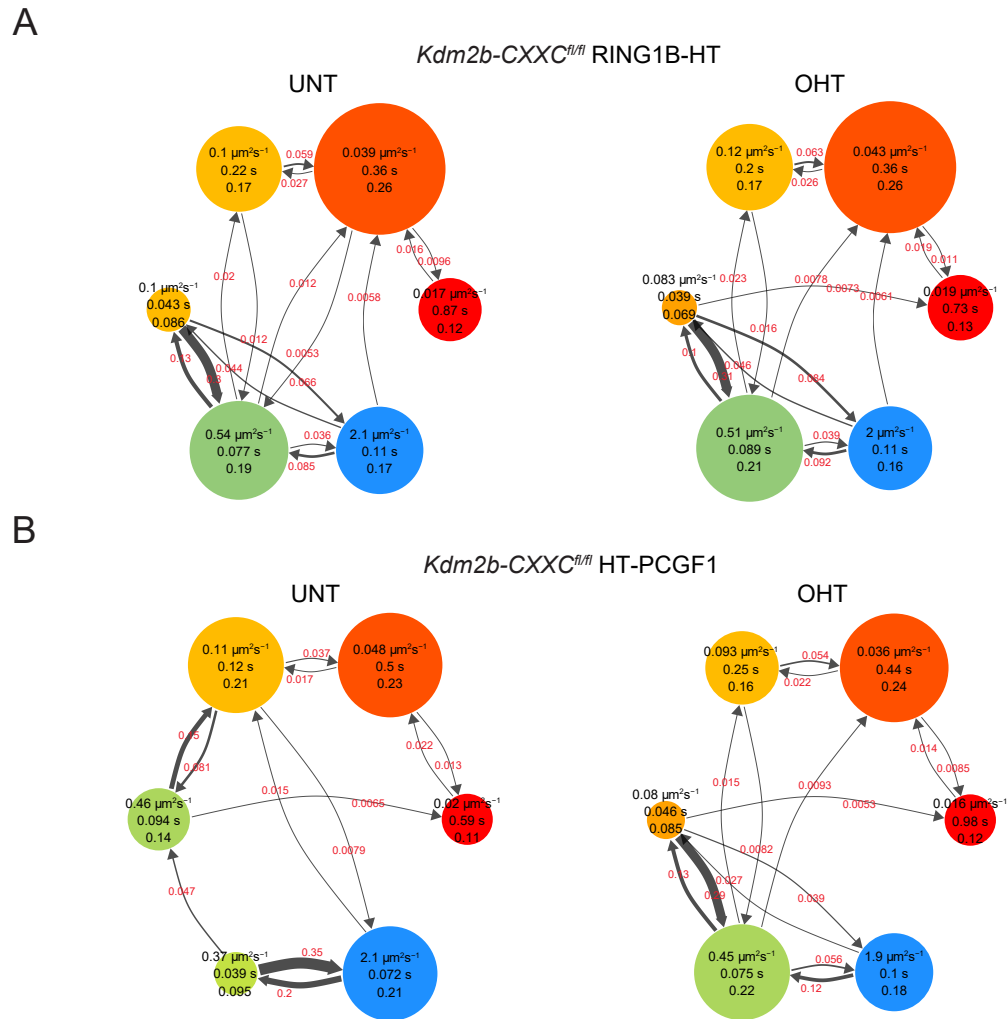


Figure 6.4: Loss of KDM2B DNA binding affects the slow diffusing behaviour of PCGF1-PRC1

(A) - (B) Transition probability networks showing vbSPT outputs for (A) RING1B, and (B) PCGF1 in *Kdm2b-CXXC^{fl/fl}* cells before (UNT) and after 72 h tamoxifen treatment (OHT). Diffusive states are represented as nodes. Node colour is scaled to apparent diffusion coefficient (D^* , $\mu\text{m}^2 \text{s}^{-1}$), and node size is scaled to the fraction of molecules in the state. Each state is labelled with its corresponding apparent diffusion coefficient, dwell time, and fraction of molecules in the state. Arrows between nodes indicate state transitions, and red numbers and arrow weight indicate the transition probability. Transitions with probability < 0.005 are not shown.

The study which originally described the *Kdm2b-CXXC^{fl/fl}* system reported a loss of both KDM2B and PCGF1 occupancy in ChIP following removal of the KDM2B ZF-CXXC domain (Blackledge et al. 2014). KDM2B occupancy was measured genome-wide by ChIP-seq, while PCGF1 occupancy was only investigated at a subset of gene promoters by ChIP-qPCR. Notably, this subset consisted of sites with strong losses of RING1B, while many sites did not lose RING1B occupancy, with the lowest reductions in RING1B occupancy occurring at the sites with the most RING1B. I hypothesised, therefore, that PCGF1 binding might be maintained by some other mechanism at strong Polycomb target sites where RING1B occupancy was not reduced. To test this, I performed ChIP-qPCR on PCGF1 in *Kdm2b-CXXC^{fl/fl}* cells before and after tamoxifen treatment. I measured PCGF1 occupancy at two of the sites that were previously shown to lose RING1B occupancy (*Npr1* and *Klrg2* promoters), as well as four strong Polycomb target sites (*HoxA7*, *Bcor*, *Pax3* and *Bmp7* promoters), and a gene body where PCGF1 should not bind (Figure 6.5). Although PCGF1 occupancy was reduced at the weaker Polycomb target sites as previously described, it was only partially reduced or remained the same at the stronger sites. Taken together, SPT and ChIP-qPCR of PCGF1 strongly suggest the existence of an alternate mechanism by which PCGF1 interacts with chromatin in addition to DNA binding by KDM2B.

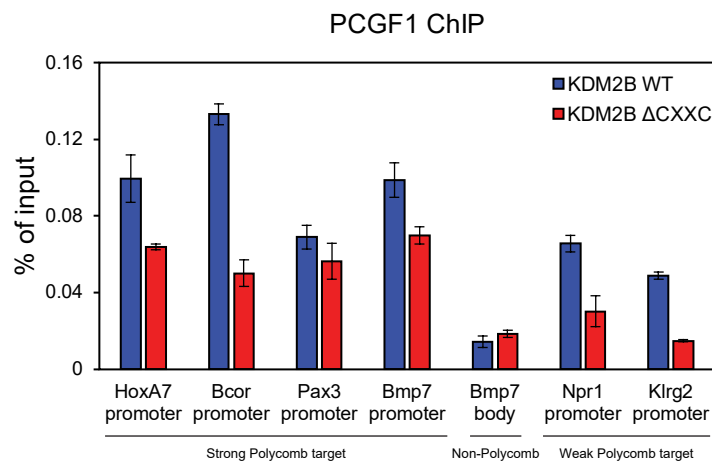


Figure 6.5: PCGF1 chromatin binding is not abolished by disruption of KDM2B DNA binding

PCGF1 ChIP-qPCR analysis in *Kdm2b-CXXC^{fl/fl}* cells before (KDM2B WT) and after (KDM2B ΔCXXC) tamoxifen treatment. qPCR primers for promoters of strong Polycomb target sites (*HoxA7*, *Bcor*, *Pax3* and *Bmp7* promoters), a gene body which is not a Polycomb target (*Bmp7* body) and weak Polycomb target sites (*Npr1* and *Klrg2* promoters) were used to measure percent of input recovered. Error bars indicate the standard error of the mean of three biological replicates.

6.2.3 Generating a cell line in which to measure the contribution of PCGF6 to PRC1 complex dynamics

DNA-binding transcription factors, in particular the MGA/MAX heterodimer and E2F6, are thought to be the major factors through which PCGF6-PRC1 complexes interact with chromatin (Endoh et al. 2017; Stielow et al. 2018; Scelfo et al. 2019). I previously observed that PCGF6 has different dynamics to RING1B, displaying shorter stable binding and a slightly smaller fraction of stably bound molecules (Section 4.2.3). Because PCGF6 has been found to interact with sites independently of RING1B by ChIP (Endoh et al. 2017; Scelfo et al. 2019), I wanted to determine whether the differences in dynamics I observed were due to differences between PCGF6-PRC1 complexes and other PRC1 complexes, or whether these dynamics in part represented PCGF6 not in complex with PRC1. To this end, I employed a previously described cell line in which all PCGF proteins besides PCGF6 can be removed, leaving only PCGF6-PRC1 complexes intact (Fursova et al. 2019). In these cells, PCGF4, which is not expressed in mESCs, is constitutively deleted, while PCGF1/2/3/5 can be conditionally knocked out (*Pcgf1/2/3/5^{fl/fl} Pcgf4^{-/-}*) (Figure 6.6 A and Fursova et al. 2019).

I introduced a C-terminal HaloTag into the endogenous alleles of *Ring1b* in *Pcgf1/2/3/5^{fl/fl} Pcgf4^{-/-}* cells as described above, and confirmed RING1B was homozygously tagged by western blotting (Figure 6.6 B). Although RING1B levels appeared reduced relative to the untagged protein by western blot, quantification of fluorescence intensity of the tagged protein by fluorescence microscopy indicated that RING1B levels were only slightly lower than in my previously generated RING1B-HT cell line (Figure 6.6 C). This difference may arise as a result of the two cell lines being generated from parental cell lines which were independently derived from mice. I further confirmed that the HaloTag was functional and that the tagged protein localised normally by fluorescence microscopy (Figure 6.6 D). After confirming the tagged protein functioned normally, I ensured that tamoxifen treatment resulted in loss of PCGF1/2/3/5 protein by western blot (Figure 6.6 E). Consistent with previous observations following removal of PCGF1/2/3/5 (Fursova et al. 2019), levels of RING1B also appeared to be reduced.

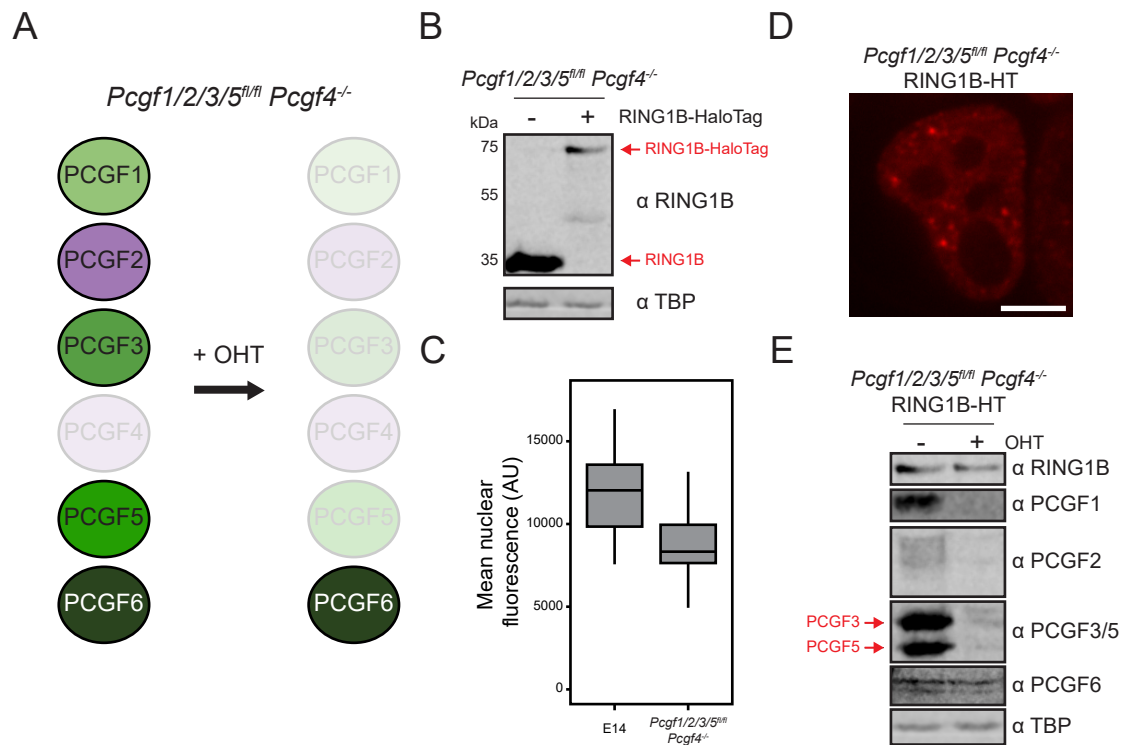


Figure 6.6: Generation of a *Pcgl1/2/3/5^{fl/fl} Pcgl4^{-/-}* cell line in which PRC1 dynamics can be measured

(A) Schematic of the *Pcgl1/2/3/5^{fl/fl} Pcgl4^{-/-}* system. Untreated cells have *Pcgl4* constitutively deleted. Treatment with tamoxifen (OHT) causes deletion of *Pcgl1/2/3/5*, resulting in expression of only PCGF6.

(B) Western blot showing homozygous tagging of RING1B (37.6 kDa) with the HaloTag (34.5 kDa) to give a 72.1 kDa fusion protein (RING1B-HT) in *Pcgl1/2/3/5^{fl/fl} Pcgl4^{-/-}* cells. TBP is a loading control.

(C) Box plots comparing mean nuclear fluorescence for RING1B in RING1B-HT E14 cells and *Pcgl1/2/3/5^{fl/fl} Pcgl4^{-/-}* cells. $n > 50$ cells per condition.

(D) Single Z-slice of nuclei of tagged *Pcgl1/2/3/5^{fl/fl} Pcgl4^{-/-}* cells labelled with Halo-JF₅₄₉ to confirm HaloTag function and subnuclear localisation. Scale bar = 5 μ m.

(E) Western blot showing loss of PCGF1/2/3/5 protein following tamoxifen treatment. TBP is included as a loading control.

6.2.4 RING1B stable binding is dependent on PCGF1/2/3/5

I began characterising RING1B dynamics in the absence of PCGF1/2/3/5 by measuring the bound fraction of RING1B by SPT. The bound fraction of RING1B was not significantly reduced following tamoxifen treatment, but did appear more similar to the bound fraction I measured previously for PCGF6 (Figure 6.7 A). Analysis of RING1B stable binding revealed that the fraction of RING1B molecules which bound stably was not significantly different after tamoxifen treatment and similar to that of PCGF6 (Figure 6.7 B). However, the stable binding time of RING1B was reduced following tamoxifen treatment, matching that of PCGF6 (Figure 6.7 C). This suggests that the stable binding over 100 s I observed for RING1B in untreated cells is dependent on PCGF1/2/3/5.

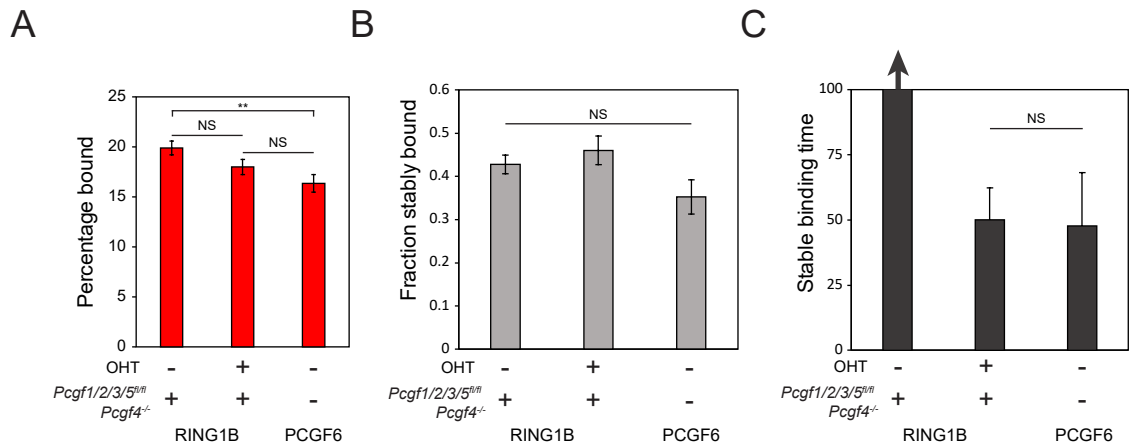


Figure 6.7: RING1B binding stability is reduced in the absence of PCGF1/2/3/5

(A) Bar chart showing percentages of molecules in the bound state for RING1B in *Pcgf1/2/3/5^{fl/fl} Pcgf4^{-/-}* cells before (UNT) and after 72 h tamoxifen treatment (OHT) and for PCGF6 in E14 mESCs, as determined from SPT data using Spot-On. Error bars indicate standard error of the mean for >50 cells. ** indicates $p < 0.005$ as calculated by Student's t test. NS = not significant.

(B) Bar chart showing the fraction of RING1B molecules in *Pcgf1/2/3/5^{fl/fl} Pcgf4^{-/-}* cells before (UNT) and after 72 h tamoxifen treatment (OHT) and for PCGF6 molecules in E14 mESCs exhibiting stable binding in 0.5 s tracking experiments. Error bars indicate standard error of the mean for >20 cells. NS = p value not significant as calculated by Student's t test.

(C) Bar chart showing stable binding times for RING1B in *Pcgf1/2/3/5^{fl/fl} Pcgf4^{-/-}* cells before (UNT) and after 72 h tamoxifen treatment (OHT) and for PCGF6 in E14 mESCs, in 0.5 s tracking experiments. Arrow and bar extending beyond the upper limit of the plot indicate stable binding which approached or exceeded the photobleaching time, rendering accurate estimation of binding time impossible. Survival times were measured from >20 cells. NS = p value not significant as calculated by Student's t test.

I further analysed my SPT data using vbSPT in order to determine whether RING1B dynamics would more closely resemble those of PCGF6 following removal of PCGF1/2/3/5. Consistent with the lack of a large change in the fractions of both bound protein and stably bound molecules, there was little change in the transition probability network for RING1B following tamoxifen treatment, and it did not resemble the network I had observed for PCGF6 (Figure 6.8 and 4.7).

Interestingly, the D^* values for the diffusing states of RING1B appeared to be broadly increased following tamoxifen treatment, indicating faster diffusion. The features of a particle which govern rate of diffusion in a constant medium are the size and mass of the particle, and these increased D^* values therefore suggest that the size of complexes in which RING1B is incorporated decreases following removal of PCGF1/2/3/5. Together, the increased rate of diffusion and lack of change to resemble the transition probability network PCGF6 suggest that while the stabilisation of RING1B chromatin binding may depend on PCGF1/2/3/5, the overall dynamics of RING1B in their absence remain different to those of PCGF6. This may be due to diffusion of free RING1B, while formation of PCGF6-PRC1 is required for stable binding, or because RING1B-independent PCGF6 complexes contribute to PCGF6 dynamic behaviour.

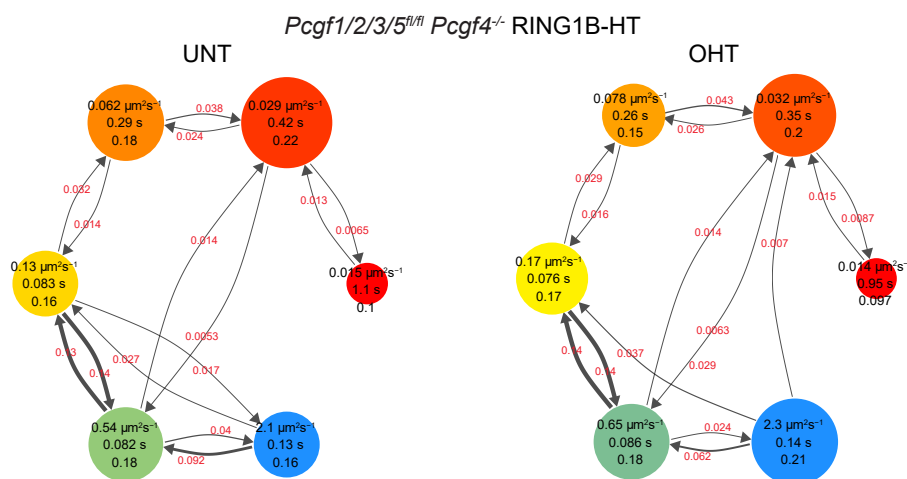


Figure 6.8: RING1B diffusion is faster but otherwise unchanged in the absence of PCGF1/2/3/5

Transition probability networks showing vbSPT outputs for RING1B in *Pcgl1/2/3/5^{fl/fl} Pcgl4^{-/-}* cells before (UNT) and after 72 h tamoxifen treatment (OHT). Diffusive states are represented as nodes. Node colour is scaled to apparent diffusion coefficient (D^* , $\mu\text{m}^2 \text{s}^{-1}$), and node size is scaled to the fraction of molecules in the state. Each state is labelled with its corresponding apparent diffusion coefficient, dwell time, and fraction of molecules in the state. Arrows between nodes indicate state transitions, and red numbers and arrow weight indicate the transition probability. Transitions with probability < 0.005 are not shown.

6.2.5 Removal of PCGF1/2/3/5 results in abnormal RING1B subnuclear localisation

Formation of Polycomb bodies in mESCs is known to depend on PHC proteins, which are subunits of cPRC1 complexes (Isono et al. 2013). I had previously observed that disruption of cPRC1 chromatin binding by depletion of H3K27me3 led to a reduction in the number of visible Polycomb bodies (Sections 5.2.6 and 5.2.8). I therefore wanted to test whether Polycomb body formation was further disrupted in cells following removal of PCGF1/2/3/5, which lack intact

cPRC1 complexes due to the absence of PCGF2/4. Examining the subnuclear localisation of RING1B after removal of PCGF1/2/3/5 revealed a substantial change in appearance. Cells lacking PCGF1/2/3/5 generally had fewer than 10 bright foci per nucleus, while RING1B normally forms tens of foci that are easily visible (Figure 6.9 A).

RING1B protein levels were reduced following tamoxifen treatment, as measured by mean fluorescence intensity of JF₅₄₉-labelled nuclei (Figure 6.9 B). The fraction of remaining RING1B is only somewhat higher than the quantity of PCGF6 relative to RING1B I measured in wild type HT-PCGF6 cells (Figure 4.8). This reduction in the levels of RING1B may occur due to destabilisation or other downregulation of expression of the protein as a result of loss of PCGF proteins with which to dimerise.

As expected, the number of Polycomb bodies per nucleus was reduced following removal of PCGF1/2/3/5, although a complete loss of nuclear foci did not occur (Figure 6.9 C). Besides the small number of very bright foci, approximately 50 less bright foci were also present. Overall, the foci that remained were less enriched relative to the rest of the nucleus (Figure 6.9 D) and also were typically of smaller volume (Figure 6.9 E). Some degree of enrichment at these sites therefore persists, albeit weakly, in the absence of PCGF1/2/3/5, suggesting that PCGF6-PRC1 can independently be enriched at some genomic sites at sufficient levels to appear as nuclear foci.

6.3 Summary and discussion

Here I have investigated the roles played by complexes with subunits which bind DNA in the dynamics of PRC1-chromatin interactions. I have used a system in which I can conditionally remove the DNA-binding ZF-CXXC domain of KDM2B, a PCGF1-PRC1 subunit (Gearhart et al. 2006; Sanchez et al. 2007; Gao et al. 2012; Farcas et al. 2012; Wu et al. 2013; He et al. 2013; Blackledge et al. 2014), to measure the effect on the dynamics of RING1B and PCGF1, and a system in which I can remove all PCGF proteins except PCGF6, which interacts with DNA-binding transcription factors such as MGA/MAX and E2F6 (Gao et al. 2012; Kloet et al. 2016; Hauri et al. 2016), to measure the effects on RING1B dynamics. Collectively, these experiments indicate that RING1B stable binding does not arise from DNA binding by either KDM2B or the transcription factors with which PCGF6 interacts. A lack of highly stable residence on chromatin through DNA binding alone may be advantageous for the Polycomb system. The sites to which

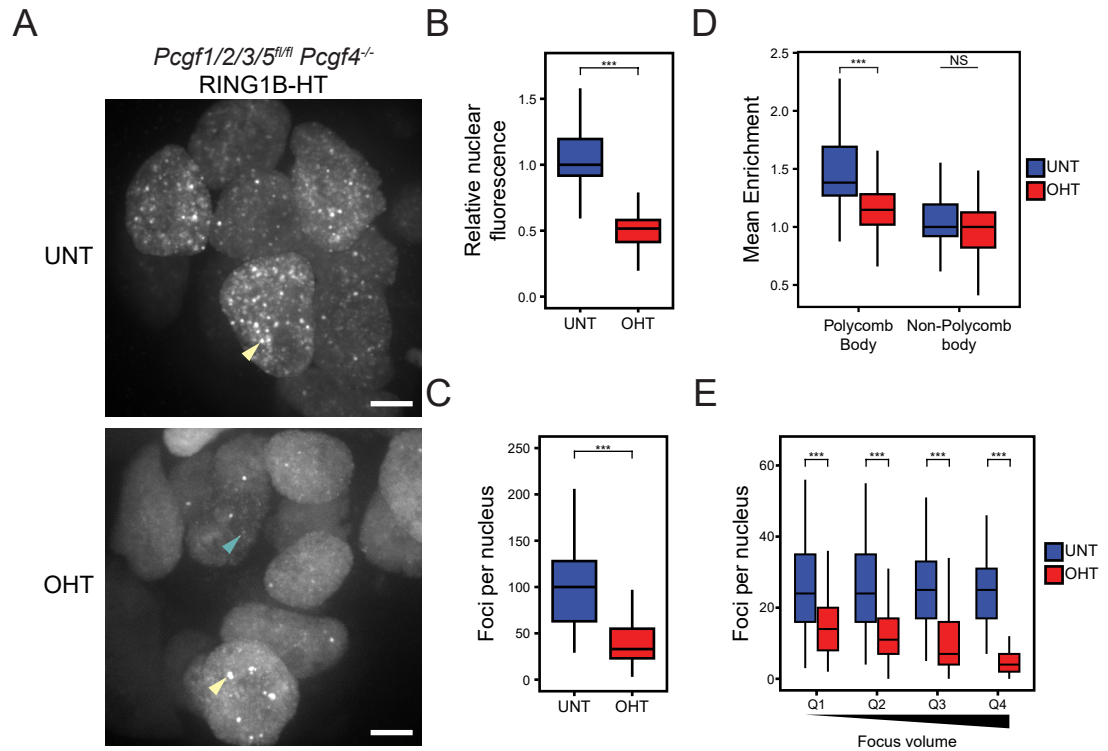


Figure 6.9: Correct RING1B subnuclear localisation depends on PCGF1/2/3/5

(A) Maximum intensity projections of RING1B-HT-JF₅₄₉ *Pcgl/2/3/5^{fl/fl} Pcgf4^{-/-}* ESCs before (UNT) and after tamoxifen treatment (OHT). Example bright nuclear foci (Polycomb bodies) are indicated by yellow arrowheads, while a weak nuclear focus in tamoxifen treated cells is indicated by a blue arrow. Scale bar is 5 μ m.

(B) Box plots comparing mean nuclear fluorescence for RING1B in *Pcgl/2/3/5^{fl/fl} Pcgf4^{-/-}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, red). $n > 50$ cells per condition.

(C) Box plots comparing the number of nuclear foci observed for RING1B in *Pcgl/2/3/5^{fl/fl} Pcgf4^{-/-}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, blue). $n > 50$ cells per condition.

(D) Boxplot comparing the relative mean fluorescence signal per unit volume of all Polycomb bodies in each cell compared to the remaining nuclear volume, normalised to the median non-Polycomb fluorescence, for RING1B in *Pcgl/2/3/5^{fl/fl} Pcgf4^{-/-}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, blue). $n > 50$ cells per condition.

(E) Box plots comparing number of nuclear foci counted for RING1B in *Pcgl/2/3/5^{fl/fl} Pcgf4^{-/-}* ESCs before (UNT, blue) and after tamoxifen treatment (OHT, blue), with foci divided into quartiles based on focus volumes in untreated cells. $n > 50$ cells per condition.

KDM2B and PCGF6-PRC1-incorporated transcription factors are known to bind outnumber the genes repressed by the Polycomb system (Long et al. 2013a; Stielow et al. 2018) and, therefore, promoting highly stable binding at all of these sites would likely be counterproductive. Instead, by producing the observed shorter binding events, which may then be stabilised by other binding activities at suitable sites, binding to DNA could allow PRC1 complexes to ‘sample’ this large number of genomic sites as previously proposed (Klose et al. 2013). These experiments therefore provide evidence in favour of a potential method to identify target sites and establish Polycomb chromatin domains through DNA binding.

Removal of the KDM2B DNA-binding domain resulted in little effect on the dynamics of total PRC1, as measured via RING1B. This was expected, given that PCGF1 accounts for a small fraction of the total pool of PCGF proteins (Figure 4.8), and should account for only a small fraction of PRC1 complexes that would be affected. A minimal effect of this perturbation on RING1B binding is consistent with previous ChIP experiments in which RING1B occupancy was predominately reduced at sites with lower initial RING1B occupancy (Blackledge et al. 2014). These sites, which have lower enrichment for Polycomb proteins and often lack fully established Polycomb domains, are therefore more dependent on KDM2B DNA binding, but likely represent a small fraction of overall RING1B binding.

Surprisingly, given that KDM2B DNA binding is thought to be the central mechanism by which PCGF1 is targeted to CGIs, PCGF1 chromatin binding also only modestly affected. Although PCGF1 occupancy was lost at weak Polycomb targets (Blackledge et al. 2014), I observed that the bound fraction of PCGF1 was not reduced, and that PCGF1 occupancy was less reduced at strong Polycomb targets (Figures 6.3 and 6.5). This indicates stable PCGF1 binding depends on an additional chromatin binding mechanism besides KDM2B DNA binding. Currently, only three other mechanisms are known through which PCGF1 might interact with chromatin: binding to H2AK119ub1 through RYBP/YAF2 (Arrigoni et al. 2006; Almeida et al. 2017), nucleosome binding by the RING1B/PCGF1 dimer (Bentley et al. 2011; McGinty et al. 2014; Leung et al. 2014), or KDM2B histone binding through its JmjC demethylase domain (He et al. 2008). Binding by other domains of KDM2B is unlikely to explain remaining PCGF1 binding, given that KDM2B occupancy is lost at all sites. Binding to H2AK119ub1 through RYBP/YAF2 and binding of the nucleosome acidic patch by the PCGF1-RING1B dimer are also inherent to PCGF3- and PCGF6-PRC1 complexes, which do not exhibit the same extent of stable binding (Figure 4.6), suggesting

that these modes of binding are also not likely to be responsible for the remaining stable PCGF1 binding. Beyond these known mechanisms for chromatin interaction, it is currently unclear what could account for the remaining PCGF1 binding.

Analysis of RING1B binding following the removal of PCGF1/2/3/5 indicated that the highly stable chromatin binding observed for RING1B depends on these PCGF proteins, as the stability of RING1B binding was strongly reduced, matching that of PCGF6. This result was expected, given that this highly stable binding was only observed for PCGF1 and PCGF2 (Figures 4.3 and 4.6), and suggests that only PCGF1 and PCGF2 complexes are capable of achieving this extent of stable chromatin binding. Although the stable binding of RING1B following removal of PCGF1/2/3/5 resembled that of PCGF6, RING1B dynamics overall remained different to those of PCGF6 (Figures 4.7 and 6.8). This suggests that RING1B and PCGF6 may diffuse independently as well as in complex together. For RING1B, this would be consistent with the existence of free RING1B in tamoxifen treated cells. Although RING1B levels are reduced, they remain higher than the levels of PCGF6 measured in wild type cells (Figures 4.8 and 6.9 B), indicating that some RING1B outside of complexes with PCGF6 may exist. Furthermore, RING1B diffusing states have higher apparent diffusion coefficients, indicating that it is part of a smaller context or diffusing alone (Figure 6.8). The existence of PCGF6 complexes independent of RING1B has also been suggested previously, based on occupancy separate and functions that appear to be independent of RING1B (Endoh et al. 2017; Scelfo et al. 2019). Because RING1B stable binding after removal of PCGF1/2/3/5 was similar to that of PCGF6, this may suggest that while one or both proteins can diffuse independently, RING1B must interact with PCGF6 in order to bind to chromatin.

Although localisation of RING1B to nuclear foci was strongly reduced following removal of PCGF1/2/3/5, it was not completely abolished (Figure 6.9). The majority of remaining foci were much less enriched relative to the rest of the nucleus and were smaller in volume. This is consistent with previous observations that mutation or deletion of PHC2, a cPRC1 subunit implicated in Polycomb body formation, disrupted all strong Polycomb bodies, resulting in a granular distribution of Polycomb proteins (Isono et al. 2013). PHC2 appears to function in this role through the capability of its SAM domain to polymerise (Kim et al. 2002; Isono et al. 2013), and it is therefore possible that the strong foci represent large sites that are compacted or multiple sites that are brought into contact with each other through the activity of cPRC1 and PHC2, and are

therefore lost after removal of PCGF1/2/3/5. Conversely, the weaker foci may represent single loci with high Polycomb binding, as these remain following loss of cPRC1 complexes. Interestingly, a small number of very bright RING1B foci remained after removal of PCGF1/2/3/5 in most nuclei. These foci might therefore represent one or a few very strong sites of PCGF6 chromatin binding.

Both the persistence of stable PCGF1 chromatin binding in the absence of KDM2B DNA binding and the maintenance of almost normal RING1B dynamics following removal of PCGF1/2/3/5 suggest gaps remain in our understanding of how PRC1 complexes interact with chromatin. Further work is therefore required to expand our understanding of how PRC1 complexes interact with chromatin through complete dissection of the factors which contribute to PRC1 chromatin binding. Going forward, my work suggests that use of a combination of approaches which measure occupancy at genomic loci (such as ChIP) and live cell dynamics approaches, as described here, will be essential in developing our understanding of PRC1 chromatin binding.

CONCLUSIONS AND FUTURE DIRECTIONS

The mechanisms by which Polycomb proteins interact with chromatin in mammals have been extensively described using approaches like ChIP which map genomic occupancy, but relatively little work has been done to quantitatively describe the dynamics of these interactions. Thus far, quantitative studies on mammalian Polycomb protein dynamics have focussed on PRC2 and the CBX subunits of PRC1 (Zhen et al. 2016; Tatavosian et al. 2018a; Youmans et al. 2018), while less quantitative techniques like FRAP have been applied to a wider range of Polycomb proteins (Ren et al. 2008; Vandenbunder et al. 2014; Almeida et al. 2017). Furthermore, all but one of these studies (Youmans et al. 2018), have used exogenously expressed tagged proteins. Therefore, the dynamics of distinct PRC1 complexes and PRC1 dynamics as a whole remain poorly understood. In this thesis, I have characterised the dynamics of a broad range of PRC1 subunits, and investigated how perturbation of their proposed targeting mechanisms affects their dynamics and chromatin binding. Questions about the Polycomb system, such as how often proteins bind to target sites and in what number, cannot be addressed by genomic approaches alone. Therefore, a combination of these genomic approaches and quantitative approaches like SPT, quantification of protein abundance, and measurement of subnuclear localisation will provide new mechanistic insight into the function of the Polycomb system.

I discovered that PRC1 subunits have relatively small bound fractions, with around half of bound molecules having a short residence time (Chapters 3 and 4). This suggests that the observations from prior ChIP experiments have focussed narrowly on only the small proportion of molecules which are stably and frequently bound at target gene promoters, as these will be preferentially captured. In addition to their dynamic natures, PRC1 subunits are of relatively low number in the mESC nucleus compared to both the number of genes the complex has a role in repressing and the total number of available nucleosome substrates across the genome (Sections 4.2.4 and 4.2.5). This highly dynamic nature may enable a limited number of complexes to function both at target gene promoters and broadly across the genome. Although it has been known that Polycomb complexes modify histones both at target gene promoters and across the genome (Albright et al. 1979; Matsui

et al. 1979; Fursova et al. 2019; Jung et al. 2010; Ferrari et al. 2014; Lee et al. 2015), interactions away from target gene promoters, which appear to account for a large proportion of Polycomb interactions with chromatin, have largely been missed by ChIP approaches. This is likely the result of population averaging of the binding of a small number of molecules in each nucleus, and the low probability of a binding event occurring at any single genomic locus without specific enrichment. My findings therefore suggest that the majority of PRC1 complexes engage dynamically with chromatin, with more dynamic behaviour (PCGF3, PCGF6, RYBP) correlating with subunits of complexes involved in depositing H2AK119ub1 genome-wide (Fursova et al. 2019). Whether this dynamic nature is required to repress pervasive transcription through deposition of H2AK119ub1, as has been proposed for PRC2 and H3K27me2 (Lee et al. 2015), or whether broad deposition of the modification is a by-product of continuous sampling of the genome to identify target genes remains to be seen.

Interestingly, despite their overall dynamic nature, PRC1 proteins which displayed stable chromatin binding had average binding times longer than many other characterised nuclear proteins. These stable binding events for RING1B and PCGF2 were not disrupted by removal of PRC2 despite losses in their bound fraction (Section 5.2.5) and previously characterised loss of enrichment at Polycomb targets by ChIP (Tavares et al. 2012). This suggests stable PRC1 binding does not necessarily always correspond to a site with high PRC1 occupancy in ChIP. It remains to be seen whether these long binding events are necessary to achieve catalysis or correspond to the structural roles these complexes are proposed to play. Determining their functional relevance will require integration of approaches that disrupt and can measure the loss of this stable binding, coupled with a capacity to measure the effects this disruption has on the deposition of H2AK119ub1 and Polycomb-mediated structure.

cPRC1 subunits are less dynamic than vPRC1 subunits, having longer binding times and larger fractions of bound protein than vPRC1, with the exception of PCGF1 (Sections 4.2.2 and 4.2.3). cPRC1 complexes, however, make minimal contributions to H2AK119ub1 deposition and transcriptional repression by PRC1 in mESCs, while vPRC1 plays the major role in Polycomb system function (Fursova et al. 2019). The most stable and abundant PRC1 binding therefore appears to correspond to complexes which do not contribute to gene repression in mESCs (Section 4.2.4). cPRC1 complexes are implicated in PRC1-mediated structuring of chromatin (Eskeland et al. 2010; Grau et al. 2011; Isono et al. 2013), and Polycomb-mediated chromatin structure

changes during differentiation are linked to changes in transcription (Noordermeer et al. 2011; Narendra et al. 2015; Narendra et al. 2016; Kundu et al. 2017). cPRC1 complexes, despite not contributing to transcription in mESCs, may be performing a key role in maintaining these structures for future differentiation. This is similar to the requirement for PRC2 in differentiation of mESCs (Pasini et al. 2004; Peng et al. 2009; Leeb et al. 2010; Landeira et al. 2010; Pasini et al. 2010; Walker et al. 2010; Ballaré et al. 2012), despite its apparently small contribution to Polycomb-mediated repression in these cells (Tavares et al. 2012; Riising et al. 2014). Indeed, these observations might be linked, as H3K27me3 deposition by PRC2 is important for cPRC1 chromatin binding via CBX proteins (Zhen et al. 2016 and section 5.2.5). Although PCGF2 stable chromatin binding was not dependent on the presence of PRC2 and H3K27me3, PRC2 may function to enrich this stable binding at Polycomb target gene promoters. Enrichment of cPRC1 at these sites may be required to ensure the correct chromatin structure required for regulation of their transcription through differentiation.

The effects that removal of PRC2 and of PRC1 catalytic activity have on Polycomb protein chromatin binding has been examined in ChIP experiments (Tavares et al. 2012; Blackledge et al. 2019). Although I observed reductions in bound fraction and binding stability, these changes were small compared to the decrease in occupancy observed in ChIP (Chapter 5). The results of ChIP experiments alone might therefore be misleading, or provide an incomplete picture of what is occurring, due to their focus on sites of enrichment. In the case of SUZ12, loss of PRC2 binding to H2AK119ub1 might be sufficient to prevent PRC2 from being correctly enriched at target sites, while not disrupting interactions with chromatin more broadly. The fact that bulk H3K27me3 is not appreciably reduced in the absence of PRC1 catalytic activity (Section 5.2.1) or when all of the major known targeting subunits of the complex are removed (Højfeldt et al. 2019; Healy et al. 2019), suggests this is the case. Although an equivalent experiment removing all PRC1 targeting subunits has not yet been done, PRC1 also deposits H2AK119ub1 across the genome, suggesting that it too can interact with chromatin without specificity. Currently, the only described means of removing these genome-wide modifications involve removal of catalytic activity or subunits central to catalysis, indicating that we do not yet know how to disrupt the mechanisms that mediate this global activity. Deposition of histone modifications globally may therefore occur through an uncharacterised interaction mechanism, or, more likely, through the inherent ability of PRC1 and PRC2 to bind to nucleosomes as part of their basic catalytic function. Determining the contribution

of these putative generic interactions with chromatin to Polycomb protein dynamics will therefore be important to furthering our understanding of how these complexes interact with chromatin.

Although one prior study examined a small number of strong Polycomb targets and found that they localised within Polycomb bodies in mESCs (Isono et al. 2013), evidence that these foci represent either single Polycomb target gene promoters or clusters thereof is still lacking. However, I observed that removal of PRC1 catalytic activity caused a reduction in Polycomb protein localisation within Polycomb bodies similar to the reduction in Polycomb protein occupancy at target gene promoters observed by ChIP, despite only small effects on Polycomb protein dynamics (Section 5.4 and Blackledge et al. 2019). Furthermore, simultaneous depletion of PRC2 and removal of PRC1 catalytic activity had an additive effect on disrupting PRC1 localisation within Polycomb bodies but not on its overall dynamics (Section 5.11). Together, these results strongly suggest that genomic sites within Polycomb bodies are enriched for both H2AK119ub1 and H3K27me3, and therefore represent Polycomb target genes. Whether all Polycomb bodies also represent clusters of Polycomb target genes in mESCs, as previously indicated for a small number of genes (Isono et al. 2013), remains unclear and will require the study of a broader set of Polycomb targets. Interestingly, depletion of cohesin, which appears to antagonise long range interactions between Polycomb chromatin domains, did not result in an observable change in localisation within Polycomb bodies (Rhodes et al. 2019). This may indicate that Polycomb bodies more frequently contain only single target genes, or that interactions within foci are generally shorter range, as these interactions were less strongly enhanced by cohesin depletion. More direct experiments will be required to confirm or refute these ideas, and would ideally measure interaction frequencies directly through imaging target genes in live cells.

Disruption of KDM2B DNA binding revealed that it was dispensable for normal binding frequency and stability of PCGF1, having only a small effect on the slow diffusing population of molecules (Section 6.2.2). Although DNA binding by KDM2B has been demonstrated to be essential for normal mouse development, and its disruption results in loss of RING1B and SUZ12 occupancy at a proportion of target sites (Blackledge et al. 2014), it is clearly not the only mechanism by which PCGF1 can interact with chromatin. Furthermore, PCGF1 enrichment at a number of strong Polycomb targets appears to remain after disruption of KDM2B DNA binding, while it is lost from weaker targets, suggesting that the complex may also possess an uncharacterised additional mechanism for interacting with strong Polycomb targets (Section 6.2.2).

Based on experiments removing KDM2B DNA binding, PRC1 catalytic activity and PRC2, it is clear that we do not yet understand which mechanisms of PRC1 interaction with chromatin are responsible for the more stable chromatin binding I have observed for RING1B, PCGF1, PCGF2 and CBX7. This stable binding does not appear to be dependent on binding of H3K27me3, H2AK119ub1, or DNA, by KDM2B, despite these representing the major characterised binding mechanisms for PCGF2/4- and PCGF1-PRC1 complexes. Following removal of all PCGF proteins except PCGF6, however, I observed a large reduction in RING1B chromatin binding stability. This indicates that RING1B stable chromatin binding is dependent on PCGF1/2/3/5, and most likely on PCGF1/2, given that these subunits display the most stable chromatin binding. This more stable chromatin binding is likely to be either an intrinsic property of these PCGF proteins, perhaps through nucleosome binding, or through other PRC1 subunits specifically recruited by these PCGF proteins. Further work will be required to elucidate what features of PCGF1- and PCGF2-PRC1 complexes stabilise their binding relative to other PRC1 complexes.

While disruptions of individual mechanisms of Polycomb chromatin binding may be sufficient to completely, or near-completely disrupt Polycomb protein enrichment at Polycomb target genes as measured by ChIP (for example Tavares et al. 2012; Blackledge et al. 2014; Blackledge et al. 2019), none of these perturbations were sufficient to abolish all chromatin binding as measured by SPT in live cells. Because Polycomb complexes typically incorporate multiple mechanisms for interacting with chromatin, future work investigating the contributions of different binding mechanisms will require combinatorial perturbations in order to investigate their behaviour in live cells. Furthermore, while these experiments have been performed in mESCs, evidence indicates that the composition, expression and importance of different Polycomb complexes varies according to cell type (Ren et al. 2008; Morey et al. 2012; O’Loghlen et al. 2012; Kloet et al. 2016). Changes to the composition of the Polycomb system as cells differentiate may cause alterations in how different mechanisms contribute to Polycomb protein chromatin binding, as well as to how the Polycomb system achieves repression of target genes. Similar quantitative characterisations of the Polycomb system in more differentiated cell types will therefore be necessary for a full understanding of how PRC1 and PRC2 operate with different system parameters. Further advances in our understanding on the Polycomb system may also be enabled by comparison of quantitative measurements in mammalian systems with measurements already performed in *Drosophila* (Ficz et al. 2005; Fonseca et al. 2012; Steffen et al. 2013), as well as implementation of similar direct

approaches for measuring protein dynamics in *Drosophila* and other species. These approaches may reveal previously unrealised differences and similarities between the Polycomb system in different organisms, and yield new insight into how the Polycomb system functions.

Thus far, the majority of studies quantitatively measuring the dynamics of nuclear proteins by SPT have focussed on transcription factors, with relatively few addressing the dynamics of chromatin-modifying proteins and complexes. In general, Polycomb proteins appear to bind more stably than transcription factors, while being more dynamic than structural proteins like histones and cohesin (Section 3.3). It is possible that investigation of the dynamics of other chromatin-modifying complexes will reveal both similar dynamics to Polycomb complexes and similarly intertwined contributions to those dynamics resulting from multiple mechanisms of interacting with chromatin. Developing a detailed, quantitative understanding of the relatively well studied Polycomb system may therefore allow it to serve as a paradigm for understanding the dynamics and chromatin binding of other chromatin-modifying complexes.

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