

**Structural characterization of the
Polycomb Repressive Complex 1-binding
partner, Ubiquitin Specific Protease 11**

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Declaration

The work presented here was performed by me, unless clearly stated or referred to.

A handwritten signature in dark ink, appearing to read 'Azar Sharif', written in a cursive style.

Azar Sharif

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Abstract

Ubiquitin Specific Protease 11 (USP11), USP4 and USP15 are highly conserved and are characterised by an N-terminal “domain present in ubiquitin specific proteases” (DUSP) and “ubiquitin-like” (UBL) domains. This DUSP-UBL (DU) domain is thought to be involved in substrate recognition. It was shown that USP11 co-purifies with human Polycomb Repressive Complex type 1 (PRC1) and regulates the stability of the E3 ligase component of PRC1 (Maertens *et al*, 2010). PRC1 repress transcription from the *INK4a* tumour suppressor locus. Hence knockdown of USP11 in primary human fibroblasts causes de-repression of INK4a, followed by a senescence-like proliferative arrest.

In this project we aimed to map the interaction between USP11 and PRC1 components (BMI1, RING2, MEL18 and CBX8). We used two methods to investigate their interactions; yeast two-hybrid and *in vitro* pull down. Unexpectedly, we could not confirm a direct interaction between USP11 and any PRC1 component. We hypothesize that the lack of post-translation modifications, the presence of fusion tags and/or the need of a multi-subunit PRC1 complex might be needed to observe a high affinity interaction.

We also aimed to map the interaction between three PRC1 components; RING2, BMI1 and RYBP, with the ultimate aim of solving the X-ray structure of the complex. The main obstacle in this project was to express, extract and purify these proteins at high levels in bacterial culture. Preliminary data suggests that RYBP and BMI1 do not interact directly.

Here we report the 3.6 Å resolution X-ray structure of the human USP11 DU. The sequence linking the DUSP and UBL domains, the DU finger, could not be assigned in the

electron density map due to low resolution. Comparison with the related USP4 DU crystal structure reveals that the structures are mostly conserved.

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I dedicate this thesis to my family.

Mum, you are my heart.

Dad, you are my reason.

Arash, you are my muse.

Without you I would not be who I am now.

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List of Abbreviations

Ade	Adenine
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BSA	Bovine serum albumin
CBX	Chromobox homolog
CDKIs	Cyclin – dependent kinase inhibitors
dATP	Deoxyribo adenosine triphosphate
dCTP	Deoxyribo cytidine triphosphate
DDR	DNA damage response
dGTP	Deoxyribo guanosine triphosphate
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribo nucleotide triphosphate
DSBs	Double-strand breaks
DTT	Dithiothreitol
dTTP	Deoxyribo thymidine triphosphate
DU	DUSP-UBL1 domains
DUBs	Deubiquitinases
DUSP	Domain present in ubiquitin specific proteases
dUTP	Deoxyribo uracil triphosphate
EDTA	Ethylenediaminetetraacetic acid
GST	Glutathione S-transferase
His	Histidine
HRP	Horse radish peroxidase
IPTG	Isopropyl- β -D-thiogalactopyranoside
JAMMs	JAMM/MPN domain-associated metallopeptidases
LB	Luria-Bertani (growth medium)
Leu	Leucine
MCPIP	Monocyte chemotactic protein-induced protein
MIT	Microtubule-interacting and trafficking
OTUs	Ovarian-tumor proteases
PAGE	PAGE Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pc	Polycomb
PcG	Polycomb group
PCR	Polymerase chain reaction
Ph	Polyhomeotic
PIPES	Piperazine-N,N'-bis-2-ethanesulfonic acid
PMSF	Phenylmethanesulphonyl fluoride
pRB	Retinoblastoma proteins
PRC	Polycomb Repressive Complex
PREs	Polycomb response elements
Psc	Posterior sex comb

PTMa	Post-translational modifications
RNA	Ribonucleic acid
RYBP	RING1/YY1-binding protein
Sce	Sex comb extra
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
TAE	Tris-acetate-EDTA
TB	Transformation Buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGS	Tris-Glycine-SDS buffer
Trp	Tryptophan
UBL	Ubiquitin-like
UCHs	Ubiquitin carboxy-terminal hydrolases
USPs	Ubiquitin-specific proteases
WT	Wild type
w/v	Weight/volume
YY1	Yin and Yang 1

Chapter I: Introduction

1.1 Overview of USP11

Post-translational modifications (PTMs) play central roles in modulating the activity of many proteins. These modifications can occur at any time in the protein production and generate an active response system to regulate the cellular microenvironment. Specific amino acid side chains and peptide linkages are often covalently modified by enzymes that attach tags to proteins. PTMs can occur on its own or in combination with post-translational cleavage through a step-wise mechanism of protein maturation or activation.

1.1.1 Deubiquitinases (DUBs)

Two PTMs, ubiquitination and deubiquitination, play a key role in different transduction cascades and in protein stability. Ubiquitination is a substrate specific process of conjugating ubiquitin (as a monomer or as polyubiquitin chains) to proteins, which is catalyzed by three enzymes: E1, a ubiquitin-activating enzyme; E2, a ubiquitin-conjugation enzyme; and E3, a ubiquitin ligase that conjugates the carboxyl terminus of ubiquitin to a lysine residue of the target protein (Hershko *et al*, 1983). Various ubiquitin linkages are formed depending on the lysine residue involved, with different physiological roles. Mono- and poly-ubiquitination of Lys63-linked chains has a role in marking the proteins for degradation via lysosomal pathways. However other, non-proteolytic, cell signalling pathways have also been linked to poly-ubiquitination of Lys63; including pathways involved in DNA damage response (reviewed in Ulrich and

Walden, 2010). Poly-ubiquitin chains where the linkage happens on Lys11, Lys29, or Lys48 are typical signals for proteasomal degradation (Ikeda and Dikic, 2008; Dammer *et al*, 2011).

On the contrary, deubiquitination, which is the removal of ubiquitin from the target proteins, is done by deubiquitinases (DUBs). DUBs interact with the target ubiquitin via various ubiquitin-binding domains; including the zinc finger ubiquitin-specific protease domain, the ubiquitin-interacting motif, and the ubiquitin-associated domain. DUBs also contain ubiquitin-like domains (UBLs) with low sequence homology but similar tertiary structure to ubiquitin. These domains lack the C-terminal Gly-Gly motif and therefore are not cleaved off by the catalytic activity of the DUB (Komander *et al*, 2009).

DUBs have various roles in the cell (Figure 1.1). Genes encode ubiquitin (*UBC*, *UBB*, *UBA52* and *UBA80*) as a linear fusion of multiple copies of ubiquitin with or without ribosomal proteins. DUBs generate free ubiquitin from these fusion protein chains, thus making ubiquitin available in the cell. In addition, DUBs can cleave ubiquitin chains from post-translationally modified proteins. Deubiquitination of these proteins therefore results in stabilization of the protein and reversal of the degradative or non-degradative signals. DUBs can also modify the ubiquitination level of a protein by cleaving some ubiquitin proteins from the chain. The process of the setting and removal of ubiquitin is thought to be a highly regulated process and is both substrate and ubiquitin chain type specific.

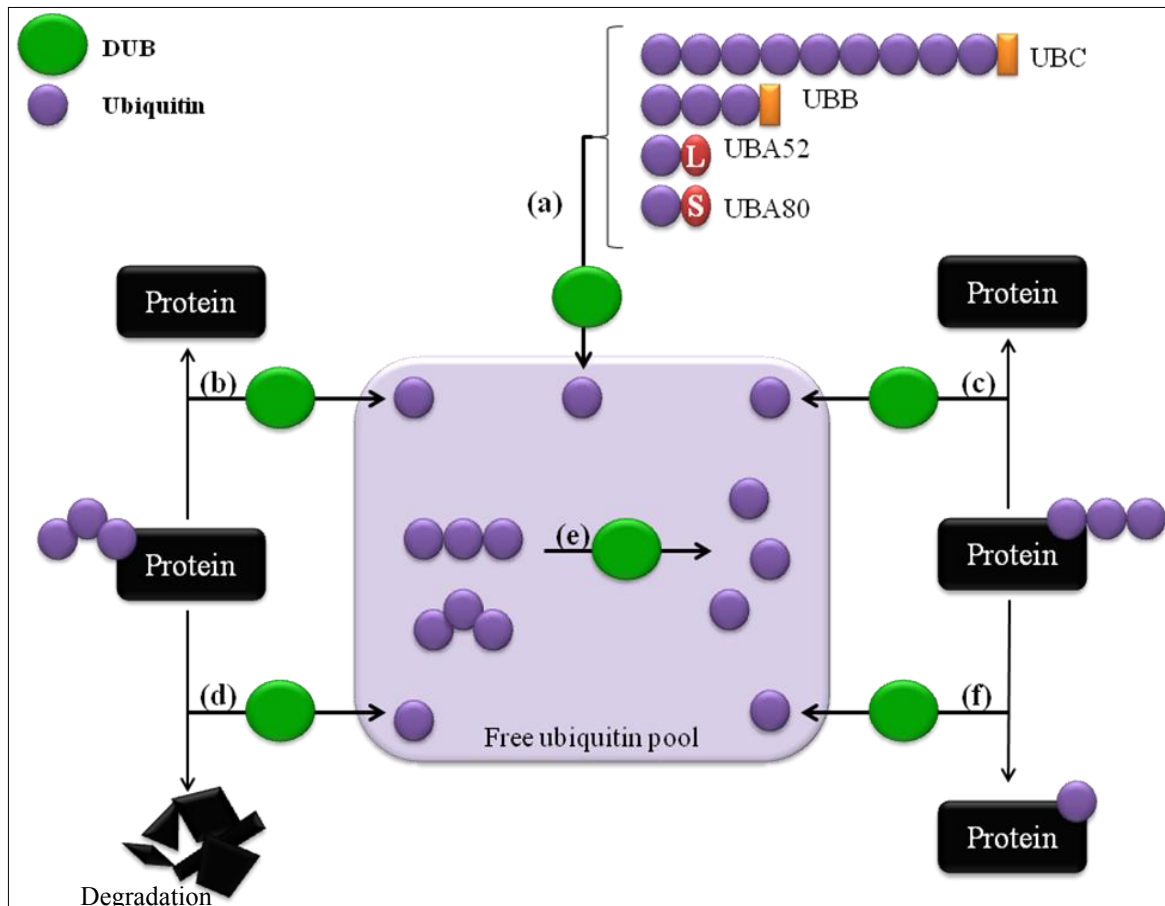


Figure 1.1 Different roles of DUBs.

(a) DUBs cleave off single ubiquitin proteins from polyubiquitin chains. The linear fusion of multiple copies of ubiquitin, which are encoded by four genes; *UBC*, *UBB*, *UBA52* and *UBA80*. Ubiquitin can also be found as a single protein fused with either of two ribosomal proteins, 40S ribosomal protein L40 (L) and 60S ribosomal protein S27a (S). (b) DUBs can prevent protein degradation. (c) DUBs remove a non-degradative ubiquitin signal. (d) DUBs, substrates of the 26S proteasome and lysosomal pathways, are involved in preserving and recycling of ubiquitin, after degradation of ubiquitinated proteins. (e) DUBs add to the ubiquitin pool by generating free ubiquitins from polyubiquitin chains formed by en masse removal from substrates. (f) DUBs are involved in altering ubiquitin signals by editing the ubiquitin chains attached to the substrates. Adapted from Komander *et al*, 2009.

At least 98 DUBs are encoded in the human genome. Based on their sequence and structural similarity, DUBs can be subdivided into six families: ovarian-tumor proteases (OTUs), ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), Machado-Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases (JAMMs) and the monocyte chemotactic protein-induced protein (MCPIP) family (reviewed in Reyes-Turcu *et al*, 2009). There are over 50 USPs in humans, making this family the largest among DUBs.

1.1.2 USPs

USPs are large DUB proteins with a typical Mw of 100 kDa. It is thought that, unlike the UCH enzymes, which are 30-40 kDa in size mostly involved in the recycling of ubiquitin, the domains extending around the catalytic domain of USPs are involved in substrate recognition. All USPs have a conserved papain-like catalytic domain which upon binding to ubiquitin goes through conformational changes (Hu *et al*, 2005; Renatus *et al*, 2006). The catalytic domains combine with other domains, such as TRAF-like (Tumor necrosis factor Receptor-Associated Factor-like), DUSP, UBL or zinc finger, to form various USPs. The roles of some of these domains have been characterised. For example, in 2008, ubiquitin interacting motif was reported to enhance interaction with the substrate in USP25 (Meulmeester *et al*, 2008). It has also been shown that USP8 has a microtubule-interacting and trafficking (MIT) domain involved in localization and function of DUBs (Row *et al*, 2007).

Based on sequence homology, USP11 is most closely related to USP4 and USP15 (Figure 1.2). USP4 was shown to have a role in spliceosome regulation (Song *et al*, 2010). USP15 was shown to stabilize the tumor suppressor protein adenomatous polyposis coli through COP9 signalosome mediated regulation (Zhou *et al*, 2003; Huang *et al*, 2009), regulate activity and stability of the RING-box protein Rbx1 (Hetfeld *et al*, 2005), caspase-3 in Paclitaxel-induced apoptosis pathway (Xu *et al*, 2009) and human papillomavirus type 16 E6 oncoprotein stability (Vos *et al*, 2009). Furthermore, USP15 deubiquitinates I κ B α 14 resulting in down-regulation of the NF- κ B pathway (Schweitzer *et al*, 2007).

The “domain present in ubiquitin specific proteases” (DUSP) is thought to be involved in substrate recognition by USPs. Although no interaction partners are known for the DUSP domain of USP15, USP4 DUSP domain was shown to interact with Sart3 in the mRNA splicing pathway (Song *et al*, 2010). USP4, USP11, and USP15 contain two UBL domains within their sequence. The function of UBL1 domain, immediately following the DUSP domain is unknown. Interestingly, it was postulated that UBL2 domain in USP4 is involved in regulating the catalytic activity of the enzyme (Luna-Vargas *et al*, 2011). Based on this model, the UBL domain partially inhibits the deubiquitinating activity of the USP4 through competitive binding into the ubiquitin binding site of the catalytic domain (Figure 1.3).

Harper and colleagues determined an X-ray structure of the USP15 N-terminal DUSP and UBL1 domains (USP11/DU) (Harper *et al*, 2011). They showed that these two domains are aligned in tandem in an 80 Å elongated arrangement. The DUSP and UBL in USP15 are connected through an intervening β -hairpin structure (DU finger). The UBL

domain in USP15 is unlikely to have an ubiquitin mimicry role, even though it has a similar β -grasp fold to ubiquitin, because of the presence of longer loop regions and different surface characterisations.

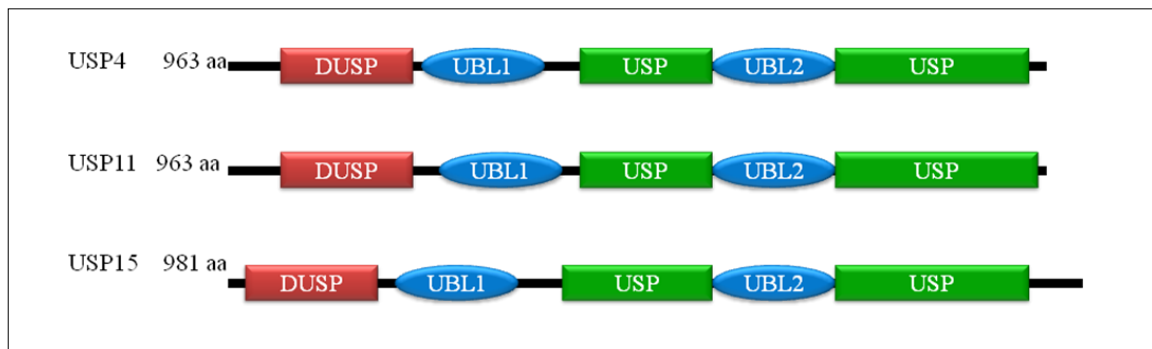


Figure 1.2) Schematic comparisons between USP4, USP11 and USP15 domains.

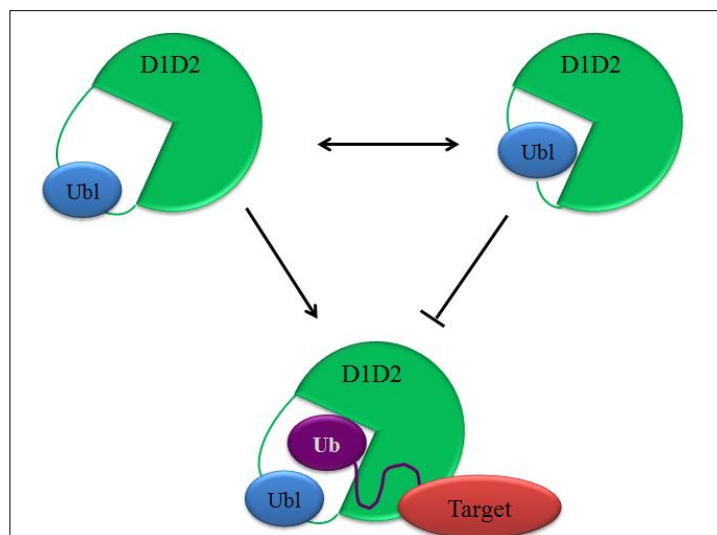


Figure 1.3) Schematic model of USP4 catalytic domain (D1D2) inhibition by the Ubl domain.

D1D2, Ubl and ubiquitin (Ub) are shown in green, blue and purple, respectively.

Adapted from Luna-Vargas *et al*, 2011.

1.1.3 USP11

USP11 was first described as ubiquitin C-terminal hydrolase on the X chromosome (UHX1) (Swanson *et al*, 1996) and was later renamed as USP11 (Baker *et al*, 1999). The *USP11* gene (2763 bp) encodes a 105 kDa protein which contains Cys and His boxes, involved in deubiquitinating activity. USP11 protein is localized mainly in the nucleus (Ideguchi *et al*. 2002). It was shown that USP11 interacts with a Ran-binding protein (RanBPM), involved in microtubule nucleation (Nakamura *et al*, 1998).

In 2008, USP11 was identified as one of cellular targets of Human papillomaviruses (HPV)-16 E7 protein (Lin *et al*, 2008). Both USP11 and HPV-16E7 are localized in the nucleus and are thought to interact there. It was demonstrated that USP11 reduces the levels of ubiquitinated E7 and therefore decreases turnover of the viral protein. Stable E7 regulates the downstream viral genes, and induces degradation of pRb. Conversely, Su *et al*. reported that USP11 could reduce influenza virus replication. It was showed that USP11 interacts with several viral RNA replication complex components; PB2, PA, and NP (Liao *et al*, 2010). These findings can potentially be used in designing antiviral drugs.

USP11 is also thought to influence the TNF α -mediated IKK α -p53 signalling pathway. It was suggested that USP11 modulated levels of IKK α and may therefore be involved in regulating the TNF α -mediated IKK α -p53 signalling pathway (Yamaguchi *et al*, 2007). Further studies showed I κ B α interacts with and is deubiquitinated by USP11. I κ B α degradation results in TNF α -induced NF- κ B nuclear translocation and activation. It was

proposed that the deubiquitination of I κ B α is done by USP11 and USP15 that act in collaboration (Schweitzer *et al*, 2007).

Two de-ubiquitinating enzymes, USP7 and USP11, were co-purified with Polycomb Repressive Complex 1 (PRC1) containing MEL18 and BMI1 from mammalian cells. Both USP7 and USP11 bind to several PRC1 components forming high-molecular-weight complexes. USP11 was shown to remove ubiquitin from PRC1 components, MEL18, RING1, and BMI1, without any significant direct effect on H2A ubiquitination. Importantly, USP7 and USP11 were shown to regulate the stability of the E3 ligase subunit of PRC1 by preventing proteasomal degradation (Maertens *et al*, 2010).

1.2 Overview of Polycomb group proteins

Polycomb group (PcG) proteins are transcriptional repressors that have a crucial role in regulation of epigenetic gene silencing and play a major part in embryogenesis, cellular differentiation and development. Their expression or activity is often deregulated in cancer cells.

1.2.1 PcG protein complexes

The Polycomb group (PcG) proteins were first discovered in *Drosophila melanogaster* as repressor of homeotic (*HOX*) genes; proteins that affect the patterning of the male sex combs. It was initially shown that mutations in the PcG genes results in multiple homeotic transformation. The phenotype of the first mutant was disrupted segmentation in the form of multiple sex combs; therefore it was called *Polycomb* (Sandler *et al*, 1968). Further studies discovered that the Polycomb gene is part of a family, the Polycomb group (Lewis, 1978).

PcG proteins form nuclear complexes which have different biochemical roles, including recognition and establishment of histone PTMs and chromatin compaction. They form chromatin-modifying complexes, called Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2, Figure 1.4), which are involved in transcriptional repression of housekeeping genes, through epigenetic regulation (Bracken *et al*, 2006). The PRC2 complex sets the trimethyl mark on lysine 27 of histone H3 (H3K27me3) and recruits the maintenance

complex PRC1, which in turn ubiquitinates Lysine 119 of histone H2A (uH2A) (Cao and Zhang, 2004; Cao *et al*, 2005; Wang *et al*, 2004).

In fruit flies, PRC1 is composed of stoichiometric amounts of Polycomb (Pc), Posterior sex comb (Psc), Sex comb extra (Sce) and Polyhomeotic (Ph). However, the mammalian PcG system is considerably more complicated, as each of the PRC1 components have multiple orthologs: five PC proteins (CBX2/4/6/7/8), six PSC (MEL18, BMI1, MBLR, RNF159, NSPC1 and RNF3), three PH (HPH1, HPH2 and HPH3) and two SCE (RING1 and RING2) (Reviewed in Gil and Peters, 2006). Of note, it is the PSC/SCE dimer component of PRC1 that catalyzes the ubiquitination of H2A at Lys119 (Cao *et al*, 2005; Buchwald *et al*, 2006).

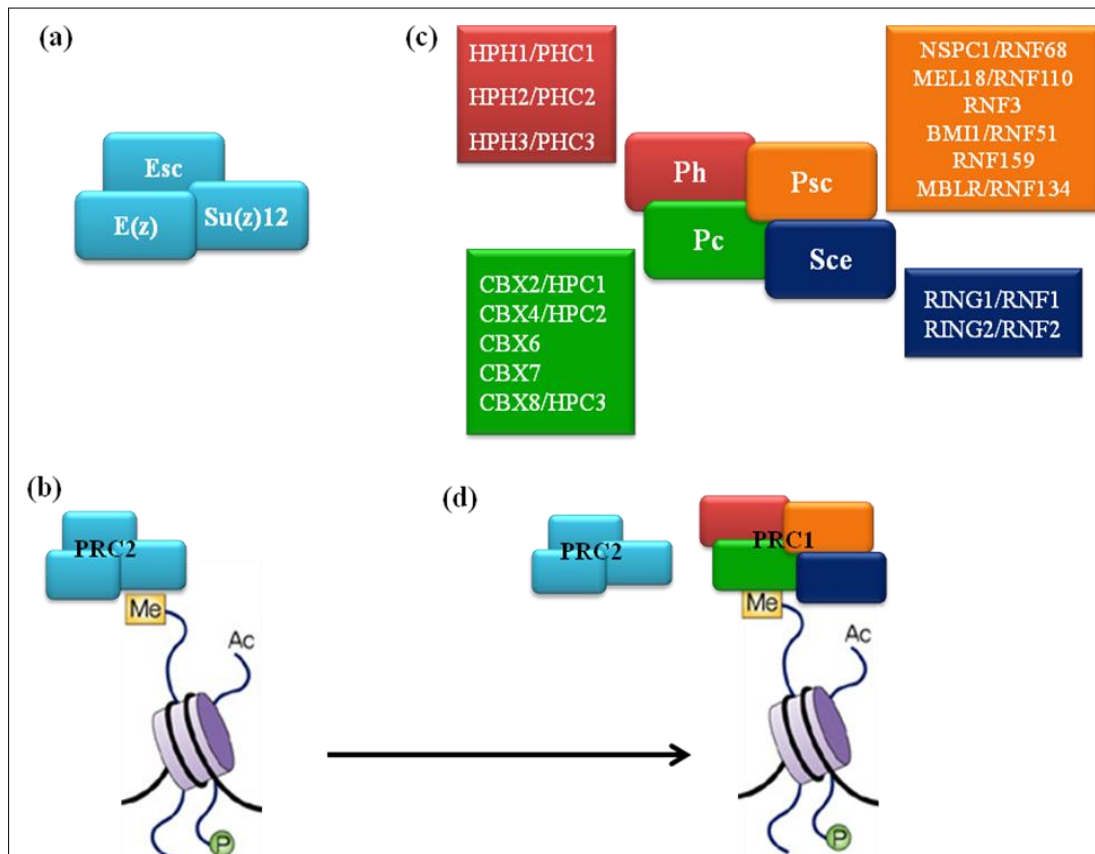


Figure 1.4) The Polycomb group (PcG) proteins.

(a) The PRC2 complex (red) has three core components, Enhancer of Zeste (E(z)), Extra sex combs (Esc) and Suppressor of Zeste-12 (Su(z)12). **(b)** PRC2 methylates histone H3 on lysine 27 (H3K27) catalysed by the histone methyl transferase, E(z) subunit. **(c)** The methylation of H3K27 by PRC2 recruits PRC1 comprised of stoichiometric amounts of Polycomb (Pc), Posterior sex combs (Psc), Polyhomeotic (Ph) and Sex combs extra (Sce) proteins. **(d)** The chromodomain of the Pc protein in PRC1 recognizes the H3K27-methylated chromatin. . PRC1 ubiquitinates Lys119 of histone H2A. These epigenetic changes are heritable and play a major role in the establishment of the body pattern throughout development. Ac, acetylation; Me, methylation; P, phosphorylation. Adapted from Gil and Peters, 2006.

The balance between silencing and activating homeotic genes is held by another group protein called the trithorax group (TrxG) that is shown to activate homeotic genes and together with the PcG proteins form the cellular memory system. In addition to PcG

protein, the heterogeneous group of TrxG proteins have a vital role in the epigenetic regulation of the cell cycle, senescence, DNA damage and stem cell biology (Reviewed in Schuettengruber *et al*, 2011).

1.2.2. PRC1 components

Polycombs: CBX8

PC proteins are characterised by an N-terminal chromobox and a C-terminal C-box involved in protein-protein interaction. The chromobox domain in PC proteins was shown to recognize the H3K27me3 mark set by PRC2, allowing the recruitment of PRC1 to chromatin marked for repression (Fischle *et al*, 2003; Min *et al*, 2003). Chromobox homolog 8 (CBX8) was first discovered in a yeast two-hybrid screen using RING1 as a bait. The C-box of CBX8 and the RING finger of RING1 are required for the interaction between the two proteins (Bardos *et al*, 2000).

Although the exact significance of association is not yet clear, CBX8 is also present in complexes recruited by MLL fusion proteins (Monroe *et al*, 2011; Mueller *et al*, 2007) and essential for MLL-AF9-induced leukemogenesis (Tan *et al*, 2011). Myeloid/lymphoid or mixed-lineage leukemia (MLL) is a histone methyltransferase, involved in transcription activation, which plays a crucial role in early development and hematopoiesis.

CBX8 was shown to downregulate the expression of tumour suppressor proteins, INK4a/ARF (Dietrich *et al*, 2007). Maertens and colleagues in subsequent studies showed that *INK4a/ARF* is regulated by several distinct PRC1 complexes (Maertens *et al*, 2009).

It was suggested that PC proteins are involved in chromatin compaction (Grau *et al*, 2010). It is thought that in PC-less PRC1 complexes, RYBP may substitute the role of PC in chromatin compaction, perhaps via its affinity to DNA (Neira *et al*, 2009). It is unclear whether CBX8 has a PRC1-independent function in transcriptional regulation, similar to certain other PC such as CBX4 (Kerppola, 2009).

Posterior sex combs: *BMI1*

PSC proteins typically contain an N-terminal RING domain followed by a largely unstructured region rich in proline and serine residues. The RING domain of PSC dimerizes with the RING domain of SCE proteins and it is this complex that functions as an E3 ligase to ubiquitinate H2A at Lys119 (Cao *et al*, 2005). Of the six described PSC proteins in mammals, MEL18 and BMI1 (with 71% sequence conservation) are the best characterised.

B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), was first characterised as a cooperating oncogene with the Eμ-*myc* transgene in B cell transformation in mice (van Lohuizen *et al*, 1991). BMI1 is a 36.8-kDa predominantly nuclear protein (van Lohuizen *et al*, 1991). Human *BMI1* gene is located on chromosome 10 (10p11.23) and encodes 326 amino acids.

Bmi1 acts as a transcriptional repressor of the INK4a/ARF locus and therefore inactivates the p16INK4a-pRb and the p14ARF-MDM2-p53 pathways. Hence, Bmi1 is a key player in cell cycle regulation (Jacobs *et al*, 1999).

BMI1 is thought to have a crucial role in self-renewal of stem cells and has been linked to the initiation of cancer (Cui *et al*, 2007) and a number of human malignancies (Balasubramanian *et al*, 2008). Bmi1 is involved in the oncogenesis of the hematopoietic system (Bea *et al*, 2001) and small cell carcinoma in the lung (Vonlanthen *et al*, 2001). Bmi1 overexpression reactivates the human telomerase reverse transcriptase gene in normal fibroblasts and mammary epithelial cells (Dimri *et al*, 2001). Studies show that Bmi1 also adds a tumorigenic capacity in colon cancer (Kim *et al*, 2004a), medulloblastoma (Leung *et al*, 2004), laryngeal cancer (Chen *et al*, 2011), breast cancer (Kim *et al*, 2004b), prostate cancer (Lukacs *et al*, 2010), and pancreatic cancer (Proctor *et al*, 2013). Further studies have revealed that targeting BMI1 by gene therapy stops chemoresistance in cancer cells and therefore enhances the prognosis (Cao *et al*, 2011).

Sex combs extra: RING1 and RING2

RING finger protein genes were first identified in 1991 (Hanson *et al*, 1991), and encode proteins that are the core components of the mammalian Polycomb system; Ring1A/RING1 and Ring1B/RING2 (reviewed in Vidal, 2009). These two proteins, in complex with PSC, form the E3 protein ligase that catalyzes the PcG-dependent ubiquitylation of histone H2A. RING2 has 336 amino acids and is 100% conserved in human and mice.

RING1 and RING2 share 49% amino acid sequence identity and share three conserved regions; the N-terminal RING finger involved in binding PSC proteins and two

conserved regions at the C terminus that form a globular domain involved in homodimerization (Czypionka *et al*, 2007).

Polyhomeotic

Polyhomeotic was first described in *Drosophila* as two virtually identical copies, Ph proximal and Ph distal (Dura *et al*, 1985; Dura *et al*, 1987). Early studies identified evolutionary conserved functional domains in these proteins. Three mammalian PH proteins (850-960 aa) are homologues to, but smaller than, the *Drosophila* Ph proteins. The conserved domains in all Ph proteins are located at the C-terminus in both long and short splice variants; homology domain 1 (HD1), the (Cys)₄-type Zn coordination- or FCS domain, and the homology domain II (HD2) or sterile alpha motif (SAM) domain, thought to be involved in protein-protein interaction.

RYBP

In 1999, a yeast two-hybrid screen for RING1A led to the identification of RING1/YY1-binding protein (RYBP) (Garcia *et al*, 1999). In an effort to characterise functions of different mammalian PRC1 complexes large-scale proteomics was performed on distinct PRC1 complexes isolated from cells. It was found that RYBP (or its homolog YAF2) was present in most complexes, except those containing Sex combs on midleg (SCMs), PC, and PHs (Gao *et al*, 2012). SCM protein is a key transcriptional repressor of PcG. SCMs and PHs both contain a zinc finger motif and are related.

Interestingly, previous studies of PRC1 had not identified RYBP as a genuine PRC1 component (Cao *et al*, 2005; Saurin *et al*, 2001; Maertens *et al*, 2009). In retrospect, technical factors might explain why the identification of RYBP as a PRC1 component in previous studies had been missed. In some of these studies epitope tags were used for PC proteins (Ren and Kerppola, 2011; Saurin *et al*, 2001), PSC (Elderkin *et al*, 2007; Maertens *et al*, 2009) and other PRC1 components (Cao *et al*, 2005; Wang *et al*, 2004). In other studies protein purifications were based on the PRC1 H2A ubiquitination activity assay (Wang *et al*, 2004).

Gao *et al* reported that RYBP increases the E3 ligase activity of PSC/SCE, whilst Tavares *et al* could not discern a change in the enzymatic activity (Gao *et al*, 2012; Tavares *et al*, 2012). It remains to be seen what the exact role is of RYBP in the complex. RYBP and its homolog YAF2 were also shown to co-purify with BCOR, a complex involved in germinal centre formation and possibly apoptosis (Gearhart *et al*, 2006; Sanchez *et al*, 2007).

1.2.3 The interaction between RYBP, BMI1 and RING1/2

BMI1 and RING1/2 contain a RING finger at their N-terminal region. These proteins form a complex both *in vivo* and *in vitro* via their RING fingers (Satijn and Otte, 1999). This complex is involved in transferring the ubiquitin from the E2 to the target by interacting with and repositioning the E2-ubiquitin thiolester closer to the target (Joazeiro and Weissman, 2000). The RING finger of BMI1 plays an important part in the tumorigenic role of the protein (Alkema *et al*, 1997).

The structure of Ring1b/Bmi1 RING-RING complex resolution was determined at 2Å resolution (Buchwald *et al*, 2006). A helix and a long loop are extended from the C-terminus of Bmi1 and N-terminus of Ring1b RING motif, respectively (Figure 1.5). The RING motif has two large Zn₂p-binding loops, a short three-stranded antiparallel β-sheet, a central α-helix and a 3₁₀-helix. The only apparent difference between the two Ring motifs is the presence of three additional residues (amino acids 62 to 64) in Bmi1 following the 3₁₀-helix.

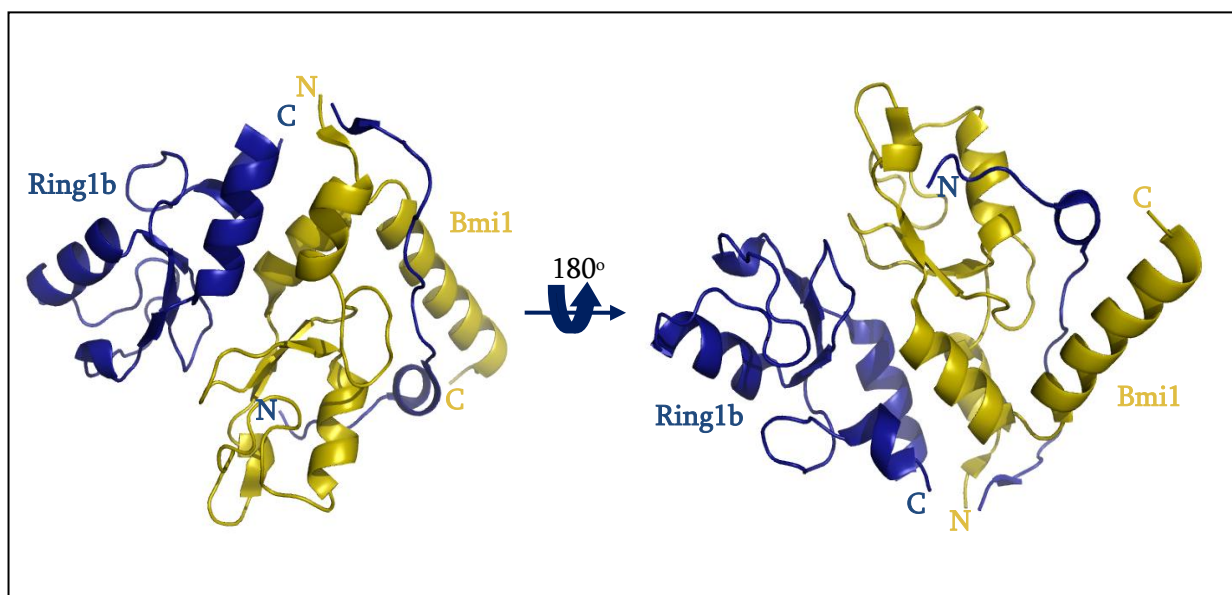


Figure 1.5) Crystal structure of the Ring1b/Bmi1 RING-domain complex.

Cartoon representation of the Ring1b and Bmi1 are shown in blue and yellow, respectively. Adapted from Buchwald *et al*, 2006.

Yeast two-hybrid screening showed that RYBP binds to the C-terminus of RING2 via its C-terminal domain (Garcia *et al*, 1999). Interestingly, PRC1 complexes that contain RYBP do not contain PC. Later studies showed that PC and RYBP bind to the same hydrophobic surface of RING2, despite minimal sequence identity (Wang *et al*, 2010) (Figure 1.6), providing an explanation for the observation of the two distinct PRC1 type complexes.

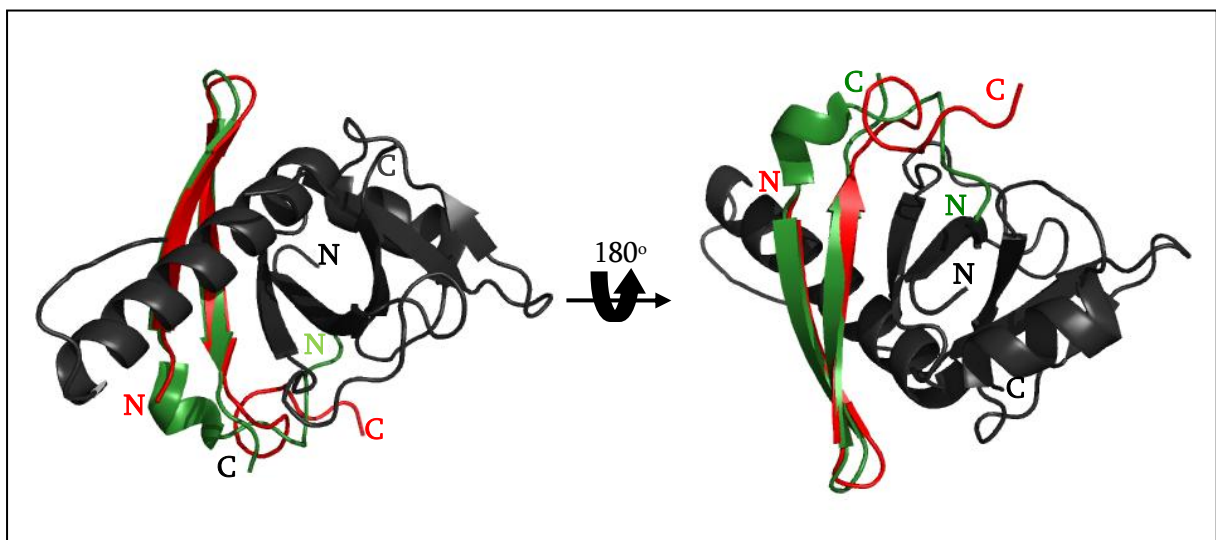


Figure 1.6) Crystal structures of the C-terminal region RING2 in complex RYBP/145 – 179 and the C-box of CBX7.

An overlay of the two interactions is shown. RING2, CBX7 C-box and RYBP/145-179 are shown in black, red and green cartoon representations, respectively. Adapted from Wang *et al*, 2010.

The anti-parallel β sheet in RYBP/158-172 is almost identical to the β sheet in CBX7 C-box, and despite significant differences in the remainder of their structures, RYBP and CBX7 bind to the same RING2 surface (Figure 1.6). These loop structures are formed by the residues at the C-terminus of CBX7 C-box β sheet and at the N-terminus of RYBP β sheet. Therefore the conformation of the two loops is completely different (Figure 1.7). Intriguingly, knockdown of RYBP results in a significant drop in RING2 protein level, suggesting a crucial stabilizing role for RYBP on RING2 (Tavares *et al*, 2012).

1.2.4 Functionally distinct PRC1 family complexes

Recently, a comprehensive proteomic and genomic approach to characterise different mammalian PRC1 complexes was carried out (Gao *et al*, 2012). Using tandem affinity purification of PRC1 components, six distinct complexes were characterised to contain different members of the PSC family; PRC1.1-1.6 (Figure 1.6). Whilst it was originally thought that PRC1 are recruited to chromatin by the recognition of H2K27me3 by the chromo box region in PC, the observation that PRC1 complexes exist without PC proteins, suggests that distinct PRC1 subunit dependent recruitment pathways may be at work. RYBP was found to be part of the PRC1 complexes (PRC1.2 and PRC1.4) that do not contain CBXs, PHCs and SCMs. ChIP-sequence analyses suggested that PRC1 isoforms may have different chromatin targets and biological functions (Gao *et al*, 2012). The impact of different factors in the activity of the PRC1 complexes remains to be determined.

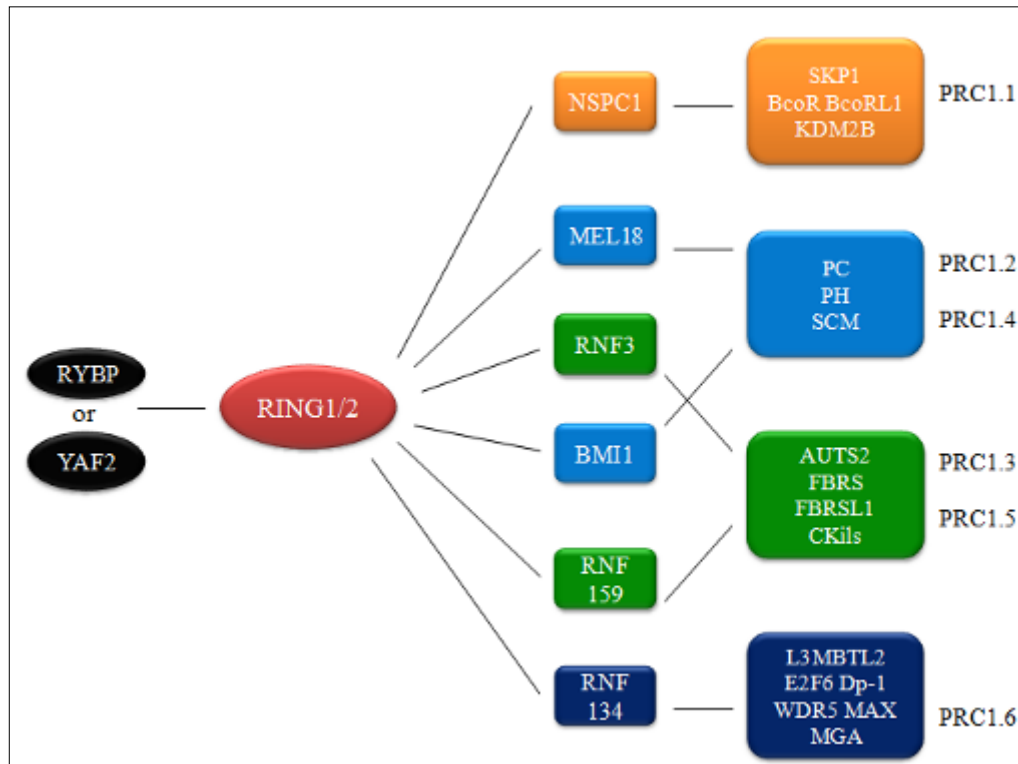


Figure 1.7) Schematic description of PRC1 family complexes.

Adapted from Gao *et al*, 2012.

RYBP- and PC-containing versions of PRC1.4 can be reconstructed *in vitro*, and the RYBP-containing PRC1.4 has distinct stimulatory effect on the enzymatic activity of the RING2-BMI1. It was also shown that the knockdown of RYBP results in the failure of ubiquitination of H2AK119, suggesting that RYBP plays an important role in regulation of RING2-BMI1 activity.

PC proteins were implicated in chromatin compaction (Grau *et al*, 2010). In the absence of PC, RYBP-PRC1 complexes may be directing the chromatin compaction, via the affinity of RYBP to DNA (Neira *et al*, 2009), although the exact mechanisms of this process is unknown.

1.2.5 Recruitment of PRC1 complexes to target loci

The mechanism of PcG proteins recruitment to the chromatin remains enigmatic, since most PcG proteins do not bind DNA directly. PcG proteins interact with chromatin as large protein-protein complexes. In *Drosophila*, *cis*-regulatory DNA elements, dubbed Polycomb Response Elements (PREs) are required for PcG recruitment. These PREs are thought to bridge PcG complexes to target genes by DNA-protein and protein-protein interactions (reviewed in Muller and Kassis, 2006). In contrast to *Drosophila*, mammalian PcG complexes have a broad distribution across the target genes they repress, making it difficult to identify PREs (Boyer *et al*, 2006; Lee *et al*, 2006; Ku *et al*, 2008).

Association with specific DNA binding factors, binding to H3K27me3 and interactions with non-coding RNAs are among several models suggested for the recruitment of PRC1 and PRC2 to their target sites (reviewed in Simon and Kingston, 2009; Schuettengruber and Cavalli, 2009). Yin and Yang 1 (YY1), a ubiquitously expressed DNA binding Zn finger protein is known to function both as a transcriptional activator and repressor. Knock-down of YY1 in mouse myoblasts was shown to result in dislodgement of EZH2 and removal of H3K27me3 from target genes (Caretta *et al*, 2004), suggesting a role for YY1 in PRC2 recruitment. It was generally accepted that PRC1 complexes are recruited to sites enriched with H3K27me3 by a PC protein binding this modified histone via its chromobox. However, the recent discovery of PRC1 complexes lacking the PC component (Gao *et al*, 2012), combined with the observation that in PRC2 null cells PcG target genes are still ubiquitinated at H2AK119 (Tavares *et al*, 2012) suggests the existence of alternative mechanisms of PRC1 recruitment. Indeed, the E3 ligase component of

PRC1 (PSC/SCE) in complex with RYBP can be detected at PcG target genes in PRC2 null cells (Tavares *et al*, 2012). It is possible that YY1 recruits the RYBP-type PRC1 complexes independently of PRC2, via its interaction with RYBP (Wilkinson *et al*, 2010), or that another yet undefined mechanism is at work.

1.2.6 PRC1 components and regulation of p16^{INK4a}

In addition to genes involved in cell fate decisions and pluripotency (Bracken *et al*, 2007; Lee *et al*, 2006), PcG proteins were shown to regulate the *INK4a-ARF-INK4b* tumour suppressor locus (Reviewed in Gil and Peters, 2006; Sharpless, 2005). The locus encodes three proteins, p16^{INK4a}, p14^{ARF}, and p15^{INK4b}, which play key roles in senescence, the state of irreversible growth arrest in response to DNA damage and oncogenic stress. Besides being a front-line defence against oncogenic mutations, senescence sets limits on proliferative lifespan of cells and contributes to ageing (Reviewed in Klauke *et al*, 2011). *INK4a* is frequently mutated, deleted or methylated in human cancers, presumably enabling early tumour cells to escape from oncogene-induced senescence (Ruas and Peters, 1998). Over-expression of PcG proteins (e.g. EZH2 in prostate and BMI1 in breast cancer) have a similar effect (Reviewed in Klauke *et al*, 2011; Schuringa and Vellenga, 2010). This concept was demonstrated in cultured human fibroblasts, where ectopic expression of PRC1 components causes repression of *INK4a* and lifespan extension, whereas shRNA-mediated knockdown results in de-repression of *INK4a* and p16^{INK4a}-dependent growth arrest (Dietrich *et al*, 2007; Gil *et al*, 2004; Itahana *et al*, 2003; Brookes

et al, 2004). Maertens *et al*. showed that multiple variants of the PRC1 complex co-localise on the *INK4a* locus and regulate its expression (Maertens *et al*, 2009). Intriguingly, shRNA-mediated knockdown of USP7 and USP11 causes de-repression of p16^{INK4a} and dislodgement of PRC1 components from the locus, through the increased turnover of chromatin-bound MEL18 and BMI1 (Maertens *et al*, 2010).

1.3 Aims

In 2010 Maertens *et al.* showed that USP11 co-localizes and interacts with PRC1 components. It was shown that GST-USP11 can pull down CBX8, MEL18, BMI1, RING1A and RING1B produced in a cell-free system (Maertens *et al.*, 2010). With the ultimate aim of co-crystallizing USP11 and its binding partners, I set out to investigate the interaction between USP11 and PRC1 components (specifically MEL18, BMI1, RING2, and CBX8) using yeast two hybrid and *in vitro* pull-down reactions.

In 2012 RYBP was classified as a PRC1 component, which is present in the complexes that lack PC (Gao *et al.*, 2012). It appears that RYBP and PC compete for the binding to RING2 (Wang *et al.*, 2010). The binding between BMI1 and RING2 has been reported to happen through their RING domains (Buchwald *et al.*, 2006; Li *et al.*, 2006); it is so far not known whether a direct interaction exists between BMI1 and RYBP. I aimed to map the interaction between RING2, BMI1 and RYBP, to assist with further characterisation of their mechanism in the cell.

The N-terminal region of USP11, composed of a DUSP and UBL domains, is thought to be involved in substrate recognition. This X-ray structure of the DUSP-UBL domain of the homologous USP4 and USP15 proteins was recently reported (Bacik *et al.*, 2009; Harper *et al.*, 2011). The DUSP and UBL domains are linked by a β -hairpin, dubbed the DU finger. Whilst this USP4 fragment seems to dimerize, the homologous USP15 fragment appears to be monomeric. By sequence alignment, the DU finger in USP11 is shorter by three amino acids suggesting a different conformation of the protein in that region. In the last part of

this project I aimed to express and purify USP11 N-terminal domain at high levels to solve its X-ray structure. Further characterisation of the interaction between USP11 and the PRC1 components is potentially crucial in developing anti-cancer drugs that stabilize the PcG proteins in cancerous cells.

Chapter II: Materials and Methods

2.1 DNA constructs for protein expression in bacteria

Plasmids and oligonucleotide primers used in PCR and plasmid constructions are listed in Tables S1 and S2 (Appendix). All restriction enzymes were purchased from New England Biolabs. All plasmids generated in this work were sequence verified.

2.2 DNA plasmid construction

2.2.1 Amplification of DNA by Polymerase chain reaction (PCR)

PCR amplifications were carried out in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Template DNA (50 – 100 ng) was added to a 50 µl PCR mixture containing 250 µM of each deoxyribo-nucleotide triphosphate (dNTP) [deoxyribo-adenosine triphosphate (dATP), deoxyribo-thymidine triphosphate (dTTP), deoxyribo-cytidine triphosphate (dCTP), and deoxyribo-guanosine triphosphate (dGTP)], 100 pmol of each oligonucleotide primer and PfuUltra II fusion hot start DNA polymerase (1 µl) in 1x PfuUltra II reaction buffer (Stratagene) provided with the enzyme. PCR cycling conditions were as follows; one cycle of denaturation at 96 °C for 5 min, 25 cycles of amplification consisting of denaturation for 30 sec at 96 °C, primer annealing for 30 sec at 56 °C, followed by 1 min/kb template extension at 72 °C and ending with incubation at 72 °C for 4 min. PCR products were separated by electrophoresis in 1% (w/v) agarose in TAE

buffer (40 mM Tris base, 20 mM acetic acid, 50 mM ethylenediaminetetraacetic acid (EDTA)). DNA was visualized using GelRed fluorescent stain (Biotium™).

2.2.2 DNA digestion

DNA digestion for plasmid construction

PCR product or plasmid DNA (5 – 10 µg) was mixed with 2 – 4 µl restriction enzyme(s) (40 – 80 U, New England Biolabs) in the presence of reaction buffer provided with each enzyme, made up to a final volume of 50 µl with double distilled water (ddH₂O) and incubated at 37 °C for 3-5 h prior to ligation (Section 2.1.4).

Analytical restriction digest

For restriction analysis of plasmid DNA, 3 µl of plasmid (0.5-1 µg) was mixed with 0.2 µl of each restriction enzyme (4 U) in the presence of compatible reaction buffer provided with the enzymes, made up to a final volume of 20 µl with ddH₂O and incubated at 37 °C for 1 h. Digested product was analysed by electrophoresis through 1 % (w/v) agarose in TAE buffer.

2.2.3 DNA purification from agarose gels

DNA was extracted and purified from agarose gels using the GE Healthcare gel extraction kit according to the manufacturer's instruction. Briefly, 10 µl capture buffer type 3 (provided with kit) was added for each 10 µg agarose gel slice and incubated at 60 °C until

agarose was dissolved. The DNA was bound to the GFX MicroSpin™ (provided) and washed with 500 µl wash buffer type 1 (provided). The DNA was eluted with 30-50 µl of elution buffer type 4 (provided).

2.2.4 DNA Ligation

Digested and gel purified PCR and plasmid DNA fragments were mixed in a 1:1 molar ratio and ligated using 1 µl T4 DNA ligase (1 Weiss U, Invitrogen) in 1x ligase buffer, in a final reaction volume of 10 µl. The reactions were incubated at 16 °C overnight and 1 µl was used to transform competent *Escherichia coli* cells.

2.2.5 Bacterial Transformation

One µl of a ligation product or 50 ng plasmid DNA was added to 50 – 100 µl chemically competent DH5α strain of *E. coli* (Meselson and Yuan, 1968). The bacteria were incubated on ice for 30 min and heat shocked at 42 °C for 30 sec. The samples were cooled on ice for 1 min, supplemented with 100 µl SOC medium (casein enzymatic hydrolysate 20 g/L, yeast extract 5 g/L, NaCl 0.5 g/L, MgSO₄ 2.4 g/L, KCl 0.186 g/L and 0.4% glucose) and incubated at 37 °C for 1 h. The cells were plated onto selective Luria Bertani broth (LB) agar plates supplemented with appropriate antibiotics and incubated overnight at 37 °C to allow formation of colonies.

To isolate plasmid DNA, single colonies were picked to inoculate 5 ml LB cultures supplemented with antibiotics for selection. The cultures were grown overnight at 37 °C and plasmid DNA was purified by small-scale (mini) preparation (Section 2.2.7).

2.2.6 Screening bacterial clones for the intended plasmid constructs by colony PCR

Bacterial colonies were screened directly for the presence of the desired insert by colony PCR. In some cases, purified plasmid DNA was analysed by restriction digestion in order to determine if the desired insert was present (Section 2.2.2).

Ten to twelve bacterial colonies were selected from the culture plate. Each was added to a 20 µl PCR mixture containing 125 µM of each dNTP, 10 pmol of each oligonucleotide primer (one that anneals to the insert and one that anneals to the vector), 0.5 U Taq DNA polymerase (Invitrogen) and 2 mM MgCl₂ made up in 1x PCR buffer provided with the enzyme. Ligation product (0.5 µl) was used as a positive control. A clear area on the culture plate was used as negative control. PCR cycling conditions were as follows; 5 min incubation at 96°C, 35 cycles consisting of denaturation for 30 sec at 96 °C, primer annealing for 30 sec at 52 °C followed by 1 min template extension at 72°C and ending with incubation at 72 °C for 7 min. The products were separated by electrophoresis in 1% agarose.

2.2.7 Small scale preparation of plasmid DNA (miniprep)

A single bacterial colony was used to inoculate 5 ml LB supplemented with the appropriate antibiotics and grown overnight in a shaking incubator at 37°C. Plasmid DNA was purified from the bacterial pellet using miniprep kits (Fermentas). Briefly, bacterial pellet was resuspended in 250 µl of the Resuspension Solution (provided with the kit). To lyse cells 250 µl of Lysing Solution (provided) was added and mixed gently. To neutralize the bacterial lysate, 350 µl of the Neutralization Solution was added and mixed gently. The lysate cleared from the debris by centrifugation at 10,000 *g* was bound to the GeneJET spin column (provided) to capture the DNA plasmid and washed twice with 500 µl of the wash solution (provided) before eluting with 30-50 µl of the elution buffer (provided).

2.2.8 Medium scale preparation of plasmid DNA (midiprep)

A single bacterial colony was used to inoculate a starter culture in 5 ml LB medium, supplemented with appropriate antibiotics for 8 h. The starter culture was used to inoculate a larger-scale bacteria culture in 150 ml selective LB medium at 37 °C over-night with vigorous shaking. The cells were harvested and plasmid DNA was purified from the pellet using the Qiagen HiSpeed Midiprep kit. Briefly, the bacterial pellet was resuspended in 6 ml Buffer P1 (provided) and lysed by addition of 6 ml lysis buffer P2 (provided). The suspension was mixed and incubated for 5 min at room temperature. To neutralize sodium hydroxide present in the lysing buffer, 6 ml of chilled buffer P3

(provided) was added, the suspension was mixed, transferred to a cartridge (provided) and incubated for 15 min. The lysate was cleared from debris by filtering the suspension through a cartridge (provided). The DNA was allowed to bind to equilibrated QIAGEN-tip (provided) by gravity flow. The column was washed with 20 ml of buffer QC (provided) and DNA was eluted with 5 ml of buffer QF (provided). DNA was precipitated by adding 3.5 ml isopropanol and the sample was centrifuged for 30 min at 17,000 *g* at 4 °C. The supernatant was removed and the DNA pellet was washed with 2 ml ice cold 70% ethanol. The DNA pellet was air dried for 5-10 min and dissolved in TE buffer (provided).

2.2.9 Preparation of chemically competent *E. coli*

Chemically competent *E. coli* were prepared using published procedures (Inoue *et al*, 1990). Briefly, bacteria were grown in LB medium at 18 °C until OD₆₀₀ absorbance reached 0.8. The culture was cooled down on ice for 10 min, before harvesting the cells by centrifugation at 18,000 *g* for 1 min. The bacterial pellet was gently resuspended in 30 ml ice-cold transformation (TB) buffer (10 mM piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES) pH 6.7, 15 mM CaCl₂, 250 mM KCl and 55 mM MnCl₂). Resuspended cells were incubated on ice for 10 min. Cells were harvested again and resuspended in 8 ml TB buffer supplemented with 7% dimethyl sulfoxide (DMSO). The cells were incubated on ice for further 10 min, aliquoted and snap-frozen by immersion into liquid nitrogen.

2.2.10 Site-directed mutagenesis

The pCDF-DUET1-USP11/76-286 plasmid was used as a template to obtain three sets of amino acid substitutions in the DU domain of USP11; [E106A/K107A], [K146A/E147A] and [K278A/K279A]. Mutations were introduced by site directed mutagenesis. Fifty ng template plasmid was amplified for 18 cycles after denaturing at 96°C for 2 min. In each cycle the primers were annealed for 1 min at 58 °C and extended for 5 min at 72 °C. The mixture was incubated for further 10 min at 72 °C and cooled to below 37 °C. One µl DpnI (New England BioLabs) was added to the mixture and incubated for 1 h at 37 °C. One µl of DpnI-treated DNA was used to transform competent *E. coli*. The plasmids containing the correct mutation, verified by sequencing were used for protein expression.

2.3 Yeast two-hybrid assay

2.3.1 MATCHMAKER GAL4 Two-Hybrid System 3

Yeast AH109 was transformed with pGADT7 and pGBKT7 by the lithium acetate-mediated method (CLONTECH). Briefly, for small scale transformation of yeast cells, competent cells were prepared by inoculating 50 ml YPDA medium with 2-3 yeast colonies and incubating them at 30 °C for 16-18 h, with vigorous shaking. When OD₆₀₀ of the culture reached 2, 5 ml of the culture was used to inoculate 300 ml YPDA and incubated at 30 °C for 3 h, with vigorous shaking. After harvesting the cells by centrifugation, the cell pellet was washed in 30 ml TE (10x TE buffer: 0.1 M Tris-HCl pH 7.5, 10 mM EDTA) and then resuspended in 1.5 ml of TE buffer supplemented with 0.1

mM lithium acetate. The competent cells were transformed by adding the mixture of DNA-BD/Bait plasmid (0.5 µg), AD/pray plasmid (0.1 µg) and herring testis carrier DNA (10 µg) to 100 µl of yeast competent cells. The mixture was supplemented with 0.6 ml PEG/LiAc solution and mixed by vortexing. The cells were incubated at 30 °C for 30 min with vigorous shaking. Seventy µl DMSO was added to the culture and gently mixed. The cells were incubated at 42 °C for 15 min, then cooled down on ice for 2 min and plated on selective minimal YPDA agar plates.

To select for yeast cells co-transformed with both plasmids, the yeast cells were grown on double drop-out plates (minimal medium missing Leu and Trp), and incubated at 30 °C for 7 days. Four colonies from each transformation were then streaked out onto quadruple drop-out plates (minimal medium missing Leu, Trp, His and Ade) to check for expression of the reporter genes and incubated at 30 °C. Colonies were allowed to form for 7 days before analysis.

2.3.2 Yeast Protein Extraction

Five ml YPDA medium was inoculated with a fresh yeast colony, co-transformed with pGBKT7 and pGADT7-USP11. Untransformed AH109 was used as negative control. The overnight grown pre-culture was used to inoculate 50 ml YPDA medium. Cultures were incubated at 30 °C with vigorous shaking until the OD₆₀₀ reached 0.5. The cultures were cooled down by pouring them into a 100-ml tube, half-filled with ice. Cells were

harvested by centrifugation and washed with ice-cold ddH₂O. The pellet was then snap-frozen in liquid nitrogen and stored at -80 °C.

Cells were thawed by resuspending the pellet in 1 ml pre-warmed cracking buffer (8 M Urea, 5% (w/v) SDS, 40 mM Tris pH 6.8, 0.1 EDTA, 0.4 mg/ml Bromophenol blue) supplemented with 10 µl β-mercaptoethanol, 20 µl protease inhibitor solution and 50 µl PMSF. Glass beads were added to the samples which were heated at 70 °C for 10 min and vortexed for 1 min. The lysate was cleared from debris, boiled for 3 min, and vortexed for 1 min. The resulting lysates were boiled in SDS-PAGE loading buffer for 2 min and proteins were separated by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) electrophoresis. The proteins were detected by Western blotting (section 2.5.3).

2.4 Recombinant protein expression and purification

All, except the first, column purification steps were carried out using an ÄKTA Purifier HPLC instrument (GE Healthcare).

2.4.1 Recombinant protein expression

The recombinant expression plasmid coding for GST- or hexahistidine (His₆-) tagged proteins was transformed into the PC2 [BL21(DE3)ΔendA::TcR, pLysS] or Rosetta-2(DE3)pLacI (Novagen) *E. coli* cells. A colony was used to inoculate LB or Terrific Broth

(TB) medium, supplemented with appropriate antibiotics. The culture was grown to an OD₆₀₀ of 0.9 in LB selective medium, or 2 in TB selective medium, and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 25 °C for 4 h or at 18 °C for 16-20 h. The bacteria were then harvested by centrifugation at 28,000 *g* 4 °C and stored at -80 °C until use.

2.4.2 Affinity purification of GST-tagged recombinant proteins

Proteins required specific extraction conditions. In this section the general purification steps are described. The complete buffer composition is given for each protein in the relevant results chapter. Cells were harvested and lysed in ice-cold buffer A (25 mM Tris-HCl, pH 7.4, different NaCl concentrations) plus 1 mM phenylmethylsulfonyl fluoride (PMSF). Where the Rosetta2(DE3)pLacI strain was used, hen egg white lysozyme was added to aid bacteria lysis. Since the PC2 strain expresses lysozyme, addition of lysozyme was not required when extracting proteins from PC2 cells. Before sonicating the cells on ice, 1% Triton X-100 was added to the resuspended cells. Cell debris was removed from the sonicated samples by centrifugation at 16,000 *g* for 30 min at 4°C. The samples were bound to a column of pre-equilibrated Glutathione Sepharose resin (GE Healthcare) in buffer A. The column was washed with 100ml of buffer A, proteins eluted in buffer A with 20 mM glutathione (pH 7.8) in 1-ml, or 0.5-ml fractions. Fractions enriched with recombinant proteins were identified by SDS PAGE and pooled.

In some cases, the GST-tag was cleaved overnight at 4 °C by incubation with human rhinovirus 14 3C protease (ratio of GST-tagged protein to protease was kept at 50:1 (w/w)) in the presence of 5 mM DTT. The NaCl concentration of the protein was reduced by diluting the protein with 25 mM Tris pH 7.4 and run over an anionic/cationic exchange column (GE Healthcare). Each protein was eluted with a unique linear salt gradient from the ion exchange column. Fractions enriched in recombinant proteins were pooled. Purified proteins were concentrated to the appropriate level and supplemented with 2 mM DTT and 10% glycerol, snap frozen in liquid nitrogen and stored at -80 °C.

2.4.3 Affinity purification of His₆-tagged recombinant proteins

Bacterial pellets were resuspended in ice-cold buffer A (25 mM Tris-HCl pH 7.4, different NaCl concentrations) plus 1mM PMSF and sonicated on ice. Cell debris was removed from the sonicated samples by centrifugation at 16,000 *g* for 30 min at 4 °C. The samples were bound to a column of pre-equilibrated CL-Nickel ChroMatrix resin (Jena Bioscience) in the presence of 10 mM imidazole. The column was washed with 100ml buffer A supplemented with 10 mM imidazole, and proteins were eluted in buffer A with 200 mM imidazole in 1-ml fractions. Fractions containing recombinant protein were pooled. In some cases, the His₆-tag was cleaved overnight at 4 °C by incubating with human rhinovirus 14 3C protease (ratio of His₆-tagged protein to protease was kept at 50:1 (w/w)) in the presence of 5 mM DL-Dithiothreitol (DTT). For His₆-SUMO tagged proteins, the

His6-SUMO tag was removed by addition of His₆-Ulp1 (provided by Dr. G Maertens) in the presence of 5 mM DTT. Protease cleavage was done overnight at 4°C.

2.4.4 Ion exchange and size exclusion chromatography

The salt concentration of the protein eluted from the glutathione sepharose or CL-Nickel ChroMatrix, was reduced by diluting the samples with ice cold 25 mM Tris pH 7.4. Proteins were bound to 5-ml HiTrap Q (anion-exchange) or SP (cation exchange) columns (GE Healthcare). Recombinant protein was eluted with a linear 50-500 mM NaCl gradient in 25 mM Tris-HCl, pH 7.4. Fractions enriched with recombinant protein were pooled. If necessary, further purification was carried out by size exclusion chromatography on a HiLoad 16/60 Superdex-200 column (GE Healthcare). Purified proteins were concentrated to the appropriate level, supplemented with 2 mM DTT and 10% glycerol and snap-frozen in liquid nitrogen and stored at -80°C.

2.5 Protein Analyses

2.5.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

To fractionate protein samples, polyacrylamide gel electrophoresis was used. An 11% resolving gel comprises monomer mix (11% acrylamide, 0.29% bisacrylamide, 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.38 M Tris-HCl, pH 8.8) supplemented with 0.042% (w/v) ammonium persulphate and 0.084% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED). An 11% resolving gel was topped with 4% stacking gel, containing a monomer mix (4% acrylamide, 0.1% bisacrylamide, 0.1% (w/v) SDS and 0.12 M Tris-HCl, pH 6.8) supplemented with 0.046% (w/v) ammonium persulphate and 0.123% (v/v) TEMED.

Protein samples were mixed with Laemmli sample buffer (4x Stock: 8% (w/v) SDS, 40% (v/v) glycerol, 0.01% bromophenol blue, 250 mM Tris-HCl, pH 6.8 supplemented with 25 mM DTT). The proteins were separated by SDS-PAGE (11% gel) run at 180 V in Tris-Glycine-SDS (TGS) buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) (Laemmli, 1970).

2.5.2 Staining PAGE gels with Coomassie dye

In order to visualise the separated proteins by SDS-PAGE, Coomassie Brilliant Blue R250 was used. Gels were soaked and agitated in Coomassie solution (40% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie R250) for more than 2 h. In order to obtain a clear background, the staining solution was replaced with destaining solution (40% (v/v) methanol, 5% (v/v) acetic acid).

2.5.3 Western blotting

To electrotransfer proteins to nitrocellulose membrane (Bio-Rad), the SDS-PAGE gel was blotted at 100 V for 1 h in transfer buffer (25 mM Tris, 192 mM glycine). The membrane was blocked in phosphate buffered saline (PBS) supplemented with 5% (v/v) skimmed milk (Marvel) for 30 min at room temperature with gentle agitation. Primary antibodies (Table 2.1) were diluted in 5% milk/PBST (PBS supplemented with 0.1% Tween-20) and allowed to bind to the membrane for 1 h at room temperature. The membrane was washed with three changes of PBST allowing 10 min for each wash and incubated for 1 h with horse radish peroxidase (HRP)-conjugated secondary antibodies diluted in PBST. After 3-5 washes in PBST, signals were developed using ECL-plus chemiluminescent detection reagents (Pierce).

Antibodies used for Western blotting

Antibodies used in this study are listed in Table 2.1.

Primary Antibody	Secondary Antibody
Anti-USP11 (1:2000) (Maertens <i>et al</i> , 2009)	Anti-Rabbit IgG (1:2000) (GE Healthcare)
Anti-mRing1b (1:500) (Kindly provided by H. Koseki, RIKEN Institute, Japan)	Anti-Mouse IgG (1:2000) (GE Healthcare)
Anti-His ₆ (1:1000) (Qiagen)	Anti-Mouse IgG (1:2000) (GE Healthcare)
HRP conjugated M2 Anti-FLAG (1:2000) (Sigma)	—

Table 2.1) Antibodies used in this study.

2.6 *In vitro* pull down assays

2.6.1 Glutathione-S-transferase pull down assays

The GST-tagged proteins or GST (used as a negative control) were incubated with the desired protein (typically 5 µg each) and 15 µl glutathione sepharose beads (settled bead volume) (Sigma) in 1 ml binding buffer (0.15 M NaCl, 2 mM DTT, 0.02 M Tris pH 7.4, 0.5% NP40, 1 mM PMSF) for 3 h at 4 °C with gentle agitation. Ten µg of bovine serum albumin (BSA, Sigma) was added to reduce non-specific binding. After extensive washes with binding buffer, the bound proteins were eluted by boiling the beads in 30 µl 3x Laemmli buffer. Ten µl was separated on an 11% SDS-PAGE gel. Proteins were visualized by Coomassie staining (Section 2.5.2).

2.6.2 His₆-tag pull down assays

An aliquot of 10-15 µl of CL-Nickel ChroMatrix (settled bead volume) was washed with binding buffer (25 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP40, 1 mM DTT, 10% glycerol and 20 mM imidazole). One ml binding buffer was added to the CL-Nickel ChroMatrix beads as well as 5 µg of either of the His₆-tagged proteins and 10 µg BSA. For the negative control, no His₆-tagged protein fragments were added to the beads. The samples were incubated with the desired protein (5 µg) for 3 h at 4 °C, with gentle rocking. The beads were washed extensively with the binding buffer to remove unbound proteins. Thirty µl 3x Laemmli sample buffer was added to the beads and bound proteins were eluted by

boiling the beads for 3 min. Ten μ l was fractionated on 11% SDS-PAGE gel. Proteins were visualized by Coomassie staining (section 2.5.2).

2.7 Crystallization and structure determination

Purified USP11/76-286 at a concentration of 20 mg/ml was used to set up crystallization trays where equal amounts of USP11/76-286 and precipitant solution were mixed. Initially, sitting drop crystallization screening was done in MRC 96-well crystallization plates (Molecular Dimensions) using several commercial sparse matrix screens; including Crystal screen 1 and 2, PEG/Ion, Index, (Hampton Research), Structure screen I and II, Morpheus, JCSG-plus, PACT premier, ProPlex, Midas, (Molecular Dimensions), Wizard screen I and II, Wizard Cryo I and II, JBScreen Cryo (Jena Biosciences). In each well, 0.2 μ l protein and 0.2 μ l precipitant were mixed using the Mosquito robot, and the plates were incubated at 18 °C in a vibration-free incubator. Formation of precipitates and crystals was examined regularly using a stereo microscope (Leica).

For optimizing the crystallization conditions, hanging drop vapour diffusion crystallization experiments were setup in greased 24- and 48-well plates (Hampton Research) by mixing 1 μ l of protein-DNA complex solution with 1 μ l reservoir solution on a siliconized cover slip (Hampton Research). Crystallization was allowed to occur at 18 °C in a vibration-free incubator.

Intact crystals were dipped into cryoprotectant, using appropriate sized cryoloops (Hampton Research) and snap-frozen by immersion into liquid nitrogen. The crystals were stored in liquid nitrogen until the diffraction experiments were carried out. X-ray diffraction data, collected at beam line I03 of the Diamond Light Source (Oxfordshire, UK) at 100K, were integrated with XDS (Kabsch, 2010), merged and scaled using Scala CCP4 (Collaborative Computational Project, number 4, 1994) of the CCP4 suite. Crystals diffracted to 3.6-Å resolution.

Chapter III: Mapping the interaction between PRC1 components and USP11

Maertens *et al.* described the presence of USP11 in PRC1 complexes purified from human cells (Maertens *et al.*, 2010). In an attempt to characterise further the interaction between USP11 and PRC1 components, we used several *in vitro* approaches, as described below.

3.1 Results

Several plasmids were constructed to express different PRC1 and USP11 protein fragments in bacteria and yeast to investigate the interaction (Table 3.1).

Plasmids (yeast two-hybrid screens)	Plasmids (<i>in vitro</i> pull downs)
pGADT7M-USP11/ Δ DUSP	pGEX-6p-3-BMI1
pGADT7M-USP11/ Δ DUSP Δ DUF	pGEX-6p-3- BMI1/1-109
pGAT7M-USP11/ Δ UCH1	pGEX-6p-3-RING1B/1-331
pGADT7M-USP11/ Δ UCH2	pGEX-6p-3-RING1B/1-159
pGADT7M- USP11/ Δ UCH1&UCH2	pCDF-H6P-Duet1-USP11/76-286

Table 3.1) Plasmid constructs made to express different fragments of USP11 and PRC1 components.

3.1.1 Investigating the interaction by Yeast Two-Hybrid assay

In order to identify the minimal binding regions involved in a direct interaction between USP11 and PRC1 components a yeast two-hybrid assay was used to avoid the need to purify many different USP11 and PRC1 deletion mutants. A direct interaction between different PRC1 core components using yeast two-hybrid had previously been established in AH109 yeast cells (Maertens G., unpublished results).

Constructs were cloned to produce USP11 fused to the activation domain (AD), and selected PRC1 protein components (MEL18-Flag, MEL18/ Δ RING-Flag (residues 121-344), MEL18/RING-Flag (residues 1-121) and BMI1-Flag) fused to the DNA binding domain (DB). Plasmids expressing DB-p53 and AD-SV40 large T antigen (LTag) were co-transformed into yeast AH109 as a positive control, and DB-Lamin C and AD-SV40 LTag as negative control.

In order to eliminate the possibility of detecting false positive results (by activation of the reported genes), plasmids containing either DB-MEL18 or -BMI1 fusion genes were co-transformed with a plasmid expressing only the AD. Likewise, AD-USP11 was co-expressed with a plasmid expressing only the DB domain in yeast.

Only yeast cells which were co-transformed with plasmids containing DB-p53 and AD-LTag formed colonies on the selection media (Table 3.1). This confirmed that the yeast two-hybrid system is efficient.

AH109 cells were then transformed to express AD-USP11 together with DB fused MEL18-Flag, MEL18/ Δ RING-Flag, MEL18/RING-Flag or BMI1-Flag. Unexpectedly, no

yeast colonies formed on the selective medium and therefore no interaction could be confirmed (Table 3.2). Expression of the MEL18- and BMI1-Flag deletion constructs in the yeast cells was previously confirmed by Dr. G. Maertens (unpublished results).

To ensure that USP11 is expressed in the yeast cells Western blot was carried out on the yeast extracts (Figure 3.1). Based on these results the expression level of USP11 was determined to be low.

Bait-DB	Prey-AD	Colony formation
P53	LTA _g	+
Lamin C	LTA _g	-
MEL18-Flag	—	-
MEL18/ Δ RING-Flag	—	-
MEL18/RING-Flag	—	-
BMI1 - Flag	—	-
—	USP11	-
MEL18-Flag	USP11	-
MEL18/ Δ RING-Flag	USP11	-
MEL18/RING-Flag	USP11	-
BMI1 - Flag	USP11	-

Table 3.2) Yeast two-hybrid assay results.

Proteins used in the yeast two-hybrid, either fused with DNA binding domain or activating domain are shown here. (+) indicates that colonies were formed on the selection medium after 7 days incubation, and (-) shows the absence of colonies.

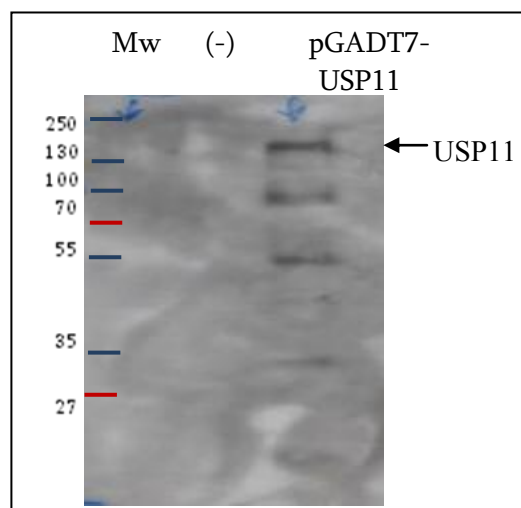


Figure 4.1) Western blot of yeast extracts shows low expression of USP11.

In each lane, 10 μ l of yeast extract was applied to the 11% SDS-PAGE gel.

3.1.2 Investigating the interaction between USP11 and PRC1 by *in vitro* pull down assays

3.1.2.1 Large scale expression and purification of proteins

Full length CBX8-FLAG, His₆-SUMO-tagged USP11/76-963, and His₆-tagged USP11/306-963 were kindly provided by Dr. G. Maertens. His₆-tagged USP11/76-286 was expressed and purified as described in Chapter III. Baculovirus-produced FLAG-mBmi1 and FLAG-mRing1b were kindly provided by Dr. Sarah Elderkin (Babraham Institute, Cambridge).

Constructs were cloned to express GST-tagged BMI1, BMI1/1-109 (RING domain), RING2/1-331 and RING2/1-159 (Buchwald *et al.* 2006). PC2 cells were transformed with each of these plasmids and two litres of LB medium was used to grow the bacterial culture to OD₆₀₀ of 0.8. At this stage the bacteria were induced to express the desired protein at 25

°C for 4 h. The cells were harvested and after clearing the sonicated lysate from debris, the GST-tagged proteins were purified over a Glutathione Sepharose resin (Figure 3.2). A fraction of resin-bound proteins was used to pull down USP11 fragments (Figure 3.4). Purification of untagged-RING2/1-331 is described in Chapter V, section 5.1.1.

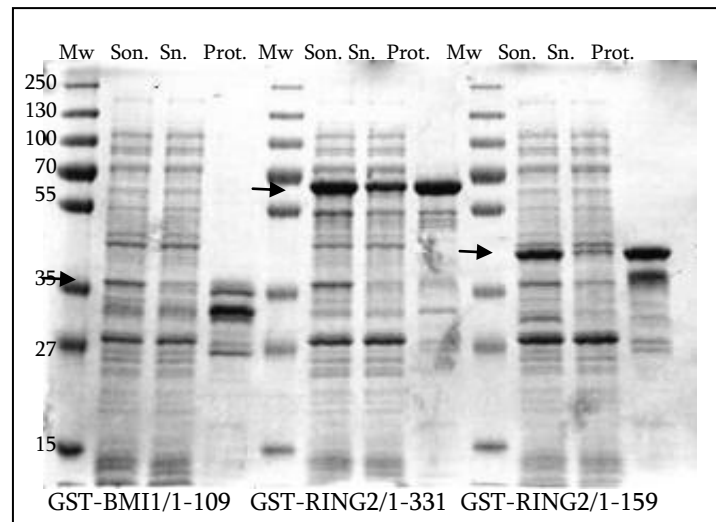


Figure 3.2) Extraction, purification and binding of GST-tagged BMI1/1-109, RING2/1-331 and RING2/1-159.

Samples from sonicated (Son.) lysate, supernatant (Sn.) of cleared lysate were separated by PAGE. Samples from the glutathione sepharose resins bound proteins were also examined to confirm the presence of purified GST-tagged proteins (Prot.). The arrows show migration of GST-tagged BMI1/1-109, RING2/1-331 and RING2/1-159, from left to right respectively.

3.1.2.2 *In vitro* Pull Down assays

Glutathione sepharose to which GST-tagged BMI1/RING, RING2/1-331 or RING2/1-159 were bound (Section 3.1.2.1) was incubated with untagged USP11 or USP11/76-286 (containing the N-terminal DUSP/UBL1 domains). As a negative control, GST bound to

glutathione sepharose was used as a bait to verify that USP11 fragments do not interact with this 28 kDa protein. Pull downs were carried out as described in Chapter II, Section 2.6. Bound proteins were eluted in Laemmli sample buffer and separated by SDS-PAGE. No interaction was detectable with Coomassie staining (Figure 3.3).

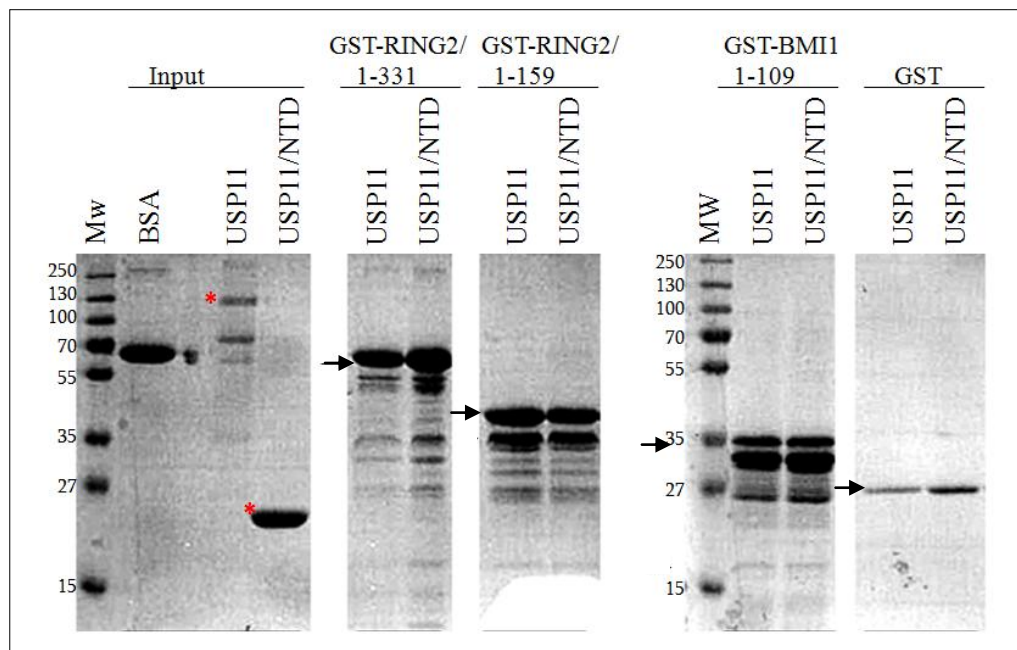


Figure 3.3) USP11 does not interact with GST-fused BMI1/RING and RING2.

Purified recombinant proteins USP11 and USP11/NTD (USP11/76-286) were incubated with resins containing bound GST-RING2/1-331, GST-RING2/1-159, or GST-BMI1/1-109 and then washed extensively. The bound proteins were dissociated and separated by SDS-PAGE. Mw markers are indicated at the left side of each panel. Red stars indicate migration of USP11 and USP11NTD. The Arrows show migration of GST-RING2/1-331, GST-RING2/1-159, and GST-BMI1/1-109 and GST, from left to right, respectively.

In order to confirm that the GST-tag is not interfering with the interaction in the GST pull downs, a reciprocal His₆-tag pull down experiment was carried out. The His₆-USP11/76-963 (provided by Dr. G. Maertens) and His₆-USP11/76-286 were bound to the CL-Nickel ChroMatrix beads. Untagged RING2/1-331 was then used in the pull down. As a negative control, CL-Nickel ChroMatrix beads alone were used. As shown in Figure 3.4a, no interaction could be detected in the SDS-page gel between USP11 fragments and RING2/1-331. Western blotting using an antibody that specifically recognizes RING2 was used to confirm the absence of a specific interaction (Figure 3.4b).

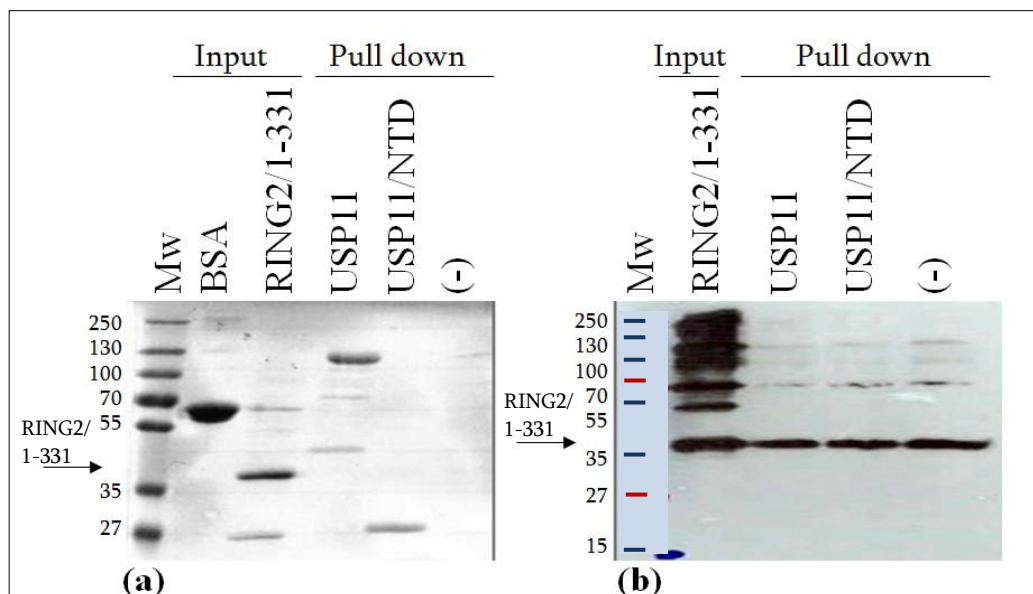


Figure 3.4) Interaction of His₆-USP11/76-963 and His₆-USP11/76-286 with RING2/1-331 analyzed by pull-down experiment.

(a) Purified recombinant protein RING2/1-331 was incubated with resins containing bound His₆-USP11/76-963 and His₆-USP11/76-286(NTD) and then washed extensively. The bound proteins were dissociated and separated by SDS-PAGE gels and stained with Coomassie. Beads alone (-) were used as negative control. **(b)** Western blot detecting RING2/1-331. Mw markers are indicated at the left side of each panel. The arrows show migration of RING2/1-331.

In previous studies full-length USP11 was used in experiments (Maertens *et al*, 2010). We noticed that removal of the first 75 amino acids of the protein increases the expression and, thus, yield of the protein. To test whether these residues are necessary for the interaction, the binding to the same proteins, as reported by Maertens *et al*. in 2010, was tested. Baculovirus produced Flag-tagged mRing1b and mBmi1 (provided by Dr. S. Elderkin) and bacterially expressed and purified CBX8-Flag (provided by Dr. G. Maertens) were tested for binding to His₆-USP11/76-963 and His₆-USP11/76-286. CL-Nickel ChroMatrix beads were used as a negative control. The beads were washed extensively to remove unbound proteins. Western blotting was used to detect interactions, using anti-Flag-HRP antibody (Figure 3.5).

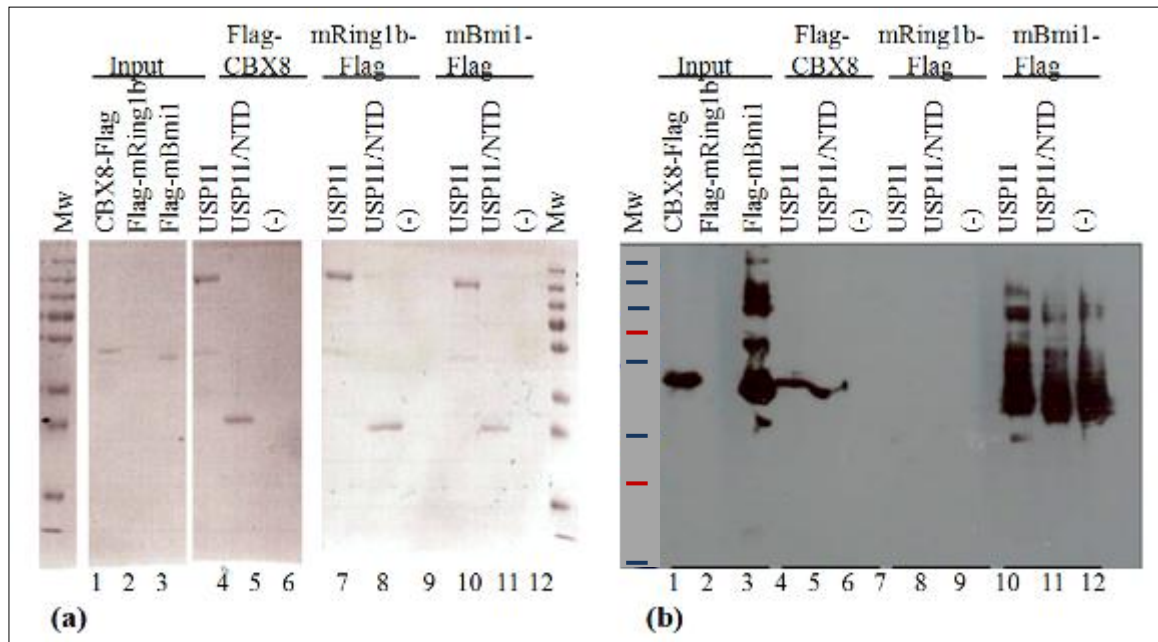


Figure 3.5) His₆-USP11/76-963 and His₆-USP11/76-286 CL-Nickel ChroMatrix pull downs.

Purified recombinant Flag-tagged mRing1b, mBmi1 and CBX8 proteins were incubated with resins containing bound His₆-USP11/76-963 (USP11) and His₆-USP11/76-286 (USP11/NTD) and then washed extensively. The bound proteins were dissociated and separated by SDS-PAGE gel and stained with Coomassie **(a)** or, transferred to nitrocellulose membrane and detected with HRP conjugated anti-Flag antibody **(b)**. Beads alone (-) were used as negative control. Mw markers are indicated at the left side of each panel.

The mBmi1 protein was found co-precipitating with our proteins of interest as well as with the negative control (Figure 3.5b, lanes 10-12). It is known that Zn²⁺-binding RING domains tend to non-specifically bind to metal chelate affinity resins. Therefore, it is not clear whether mBmi1 interacts with the USP11 fragments or not. The amount of baculovirus produced mRing1b protein used in the pull-down was too low, and was not

detectable by Western blot (Figure 3.5b, lanes 7-9). In this experiment it appears that CBX8-Flag interacts with both USP11 fragments. However, a repeat experiment did not confirm pull down of CBX8-Flag with USP11 fragments (Figure 3.6). The signal observed in lanes 4-5 in Figure 3.6b is thus most likely due to the sample of lane 3 that ran over into these lanes.

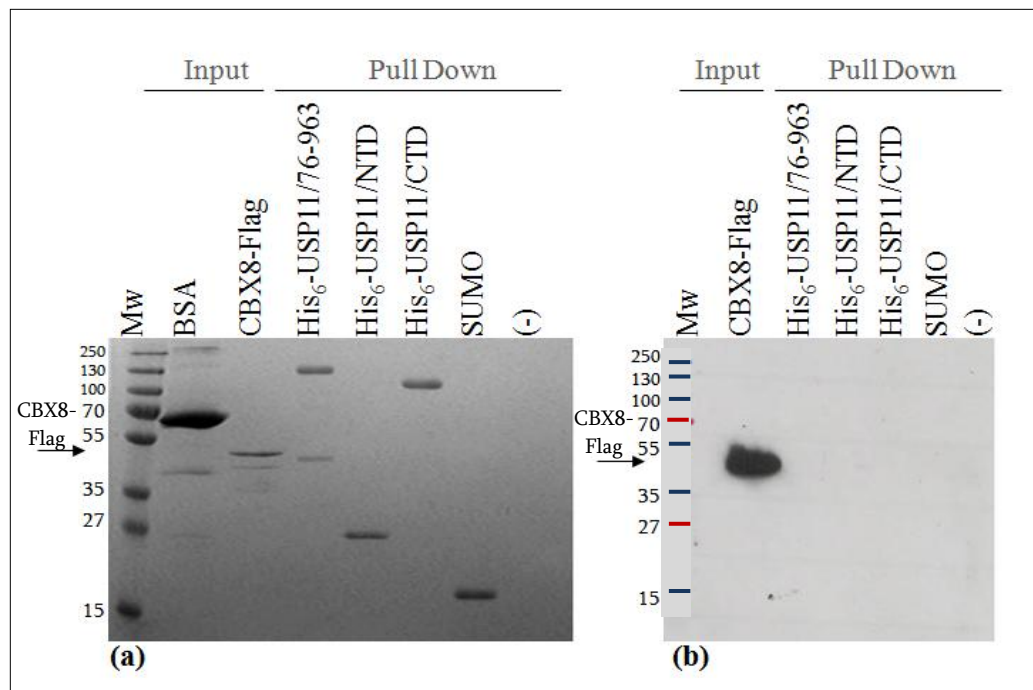


Figure 3.6) His₆-USP11/76-963, His₆-SUMO- USP11/306-963 and His₆-USP11/76-286 CL-Nickel ChroMatrix pull-downs.

Purified recombinant Flag-tagged CBX8 protein was incubated with resins containing bound His₆-USP11/76-963 (Δ USP11), His₆-SUMO- USP11/306-963 (Δ USP11-C) and His₆-USP11/76-286 (Δ USP11-N); washed extensively. The bound proteins were dissociated and separated by SDS-PAGE gel (a), transferred to nitrocellulose membrane and detected with HRP conjugated anti-Flag antibody (b). Beads alone (-) and His₆-SUMO protein were used as negative control. Mw markers are indicated at the left side of each gel. The arrows show migration of CBX8-Flag.

3.2 Discussion

In 2010, USP11 was described to be present in PRC1 complexes isolated from human cells. Interestingly, USP11 regulates the stability of the E3 ligase component of PRC1 (Maertens *et al*, 2010). Due to the high stability of PRC1 complexes in mammalian cells, it may be problematic to identify the direct interactions involved between USP11 and the PRC1 components using immunoprecipitations of deletion fragments. We know that full-length GST-tagged USP11 can pull down CBX8, mMel18, mBmi1 and mRing1b produced in a cell-free system (Maertens *et al*, 2010). In this project we aimed to map the interaction between USP11 and PRC1 components.

A direct interaction between different PRC1 core components using yeast two-hybrid had previously been established. DB-fusions of MEL18 and BMI1 can bind to AD-fused PC, SCE and PH proteins (G. Maertens, unpublished results). Therefore, to determine the minimal binding regions involved in a direct interaction between USP11 and PRC1 components and to circumvent the need to purify different USP11 and PRC1 deletion mutants, we initially used a yeast two-hybrid assay. Contrary to our expectations no interaction could be detected between AD-USP11 and DB fused MEL18-Flag, MEL18/ Δ RING-Flag, MEL18/RING-Flag or BMI1-Flag, in AH109 cells, as no colonies were formed on the plates selecting for expression of the reporter genes.

It is possible that the N-terminal fusions with AD and DB domains could interfere with the interaction of USP11 with PRC1 components. In addition, poor expression of USP11 in yeast might be problematic. Given the negative result of the yeast two-hybrid

experiments, we used pull-down assays in further attempts to detect an interaction between PRC1 components and USP11. Using GST-tagged fusions of PRC1 subunits and untagged USP11 fragments, or His₆-tagged USP11 fragments and untagged PRC1 components, we could not confirm a direct interaction *in vitro*. There are several possible explanations for this observation. (i) The 28 kDa GST-tag fused to the N-terminal RING domains of BMI1 and RING1B could interfere with the interaction. (ii) BMI1 and RING1B are substrates of USP11 and it is known that interactions between enzyme and substrate are weak. (iii) PRC1 components are phosphorylated in mammalian cells (Elderkin *et al*, 2007; Liu *et al*, 2012; Voncken *et al*, 1999); however our recombinant proteins are not phosphorylated. (iv) A strong interaction between USP11 and its substrates could be dependent on their ubiquitination status. Proteins used in our study were not ubiquitinated. (v) Finally, interaction with USP11 may require a complete PRC1 complex.

Maertens *et al*. detected interactions between GST-USP11 and PRC1 components *in vitro*. The interactions described were weak as they could only be detected by Western blot or by autoradiography. Moreover, using ³⁵S labelled *in vitro* translated protein, it was suggested that the RING domains of RING2 and MEL18 (or the homologous BMI1) proteins are involved in the direct interaction with USP11 (Maertens *et al*, 2010). It is, thus, possible that fusion of GST to the N-terminus of BMI1 and RING2 interferes in the interaction with USP11. In the previous report, GST-USP11 was used as bait. GST tag forms stable dimmers, which may increase apparent interaction affinity. Our study used a USP11 deletion fragment where the first 75 amino acids are omitted. This allowed us to

purify high levels of USP11, but it is possible that this region is important for the interaction with PRC1.

In conclusion, we could not confirm strong direct interactions between USP11 fragments and individual PRC1 components. We suggest that residues between amino acid 1-75 might be important for the interaction with PRC1 components, or that more than one PRC1 component and/or post translational modification could be necessary for high affinity interaction with USP11.

Alternatively, in future studies, different protein fragments could be expressed and purified from insect cells to study the interaction between different USP11 and PRC1 fragments using in vitro pull downs. The protein production yield in insect cells is usually high and the proteins are post-translationally modified, often mimicking protein expression in mammalian cells.

Chapter IV: Mapping the interaction between RYBP, BMI1 and RING2

It was shown that RYBP, BMI1 and RING2 can form a complex and are sufficient for H2A ubiquitination (Gao *et al*, 2012). BMI1 and RING2 are known to interact via their RING domains (Buchwald *et al*, 2006). In an attempt to characterise the interaction between these three PRC1 components, we used *in vitro* approaches, as described below.

4.1 Results

Several plasmids were constructed to express different RYBP, BMI1 and RING2 fragments in bacteria to investigate the interaction between them (Table 4.1).

Plasmids	Antibiotic
pET-28a-SUMO-BMI1	Kanamycin
pCFD-H6P-Duet1-BMI1	Spectinomycin
pET-28a-SUMO-RING2/1-331	Kanamycin
pGEX-6p-3-RING2/1-331	Ampicillin
pGEX-6p-1- RYBP	Ampicillin
pCDF-H6P-RYBP	Spectinomycin
pCDF-H6P-RYBP/BMI1	Spectinomycin

Table 4.1) Plasmid constructs made to express RYBP, BMI1 and RING2/1-331 with different tags.

4.1.1 Expression and purification of RYBP, BMI1 and RING2

In order to study the molecular interaction between RYBP, BMI1 and RING2, we searched for the highest level of expression and purification of these proteins in different conditions.

RYBP

In the first attempt, a construct containing His₆-tagged RYBP was cloned and transformed into PC2 cells. The protein expression was induced in a 3 L cell culture at OD₆₀₀ of 0.9 at 25 °C for 4 h. After harvesting the cells, the protein was extracted by resuspending the cells in a buffer containing 25 mM Tris pH 7.4 and 150 mM NaCl. After sonicating the cells a sample was taken to detect the expression level. Another sample was taken after clearing the sonicated lysate from debris to detect the extraction level. Samples were separated by SDS-PAGE gel and stained with Coomassie (Figure4.1).

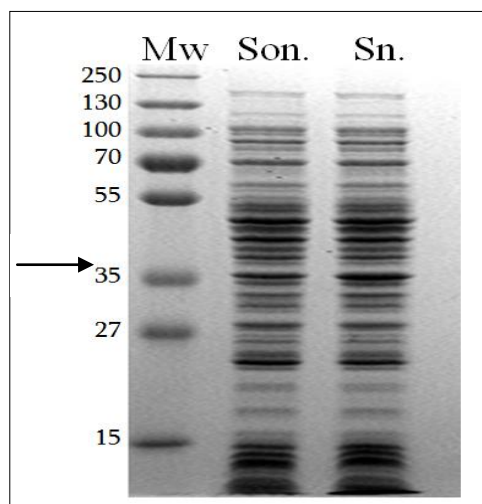


Figure 4.1) His₆-RYBP expression and extraction level.

Samples from sonicated (Son.) cell lysate and its supernatant (Sn.) were separated by SDS-PAGE. Mw marker is indicated at the left side the gel. The arrow indicated the migration of His₆-RYBP.

His₆-RYBP was purified over a CL-Nickel ChroMatrix and eluted with 200 mM imidazole in ten 1-ml fractions. Fractions number 2 - 7 were pooled together and were further purified by cation exchange chromatography on a 5-ml HiTrap Sepharose column (Figure 4.2).

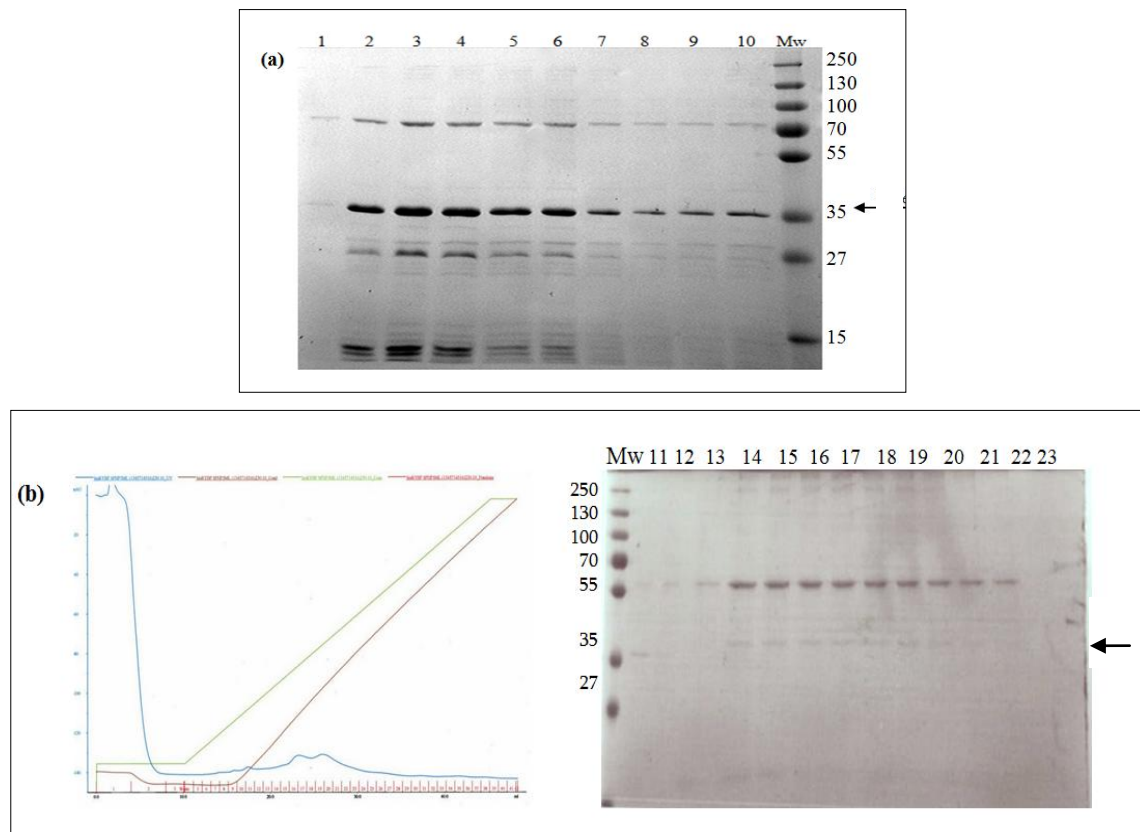


Figure 4.2) His₆-RYBP purification steps.

(a) 10 μ l samples from 10 1-ml fractions (lanes 1 to 10) of CL-Nickel ChroMatrix affinity purification of His₆-RYBP were subjected to SDS-PAGE to detect the protein. (b) Chromatogram showing the purification of the protein on the cation exchange. The eluted protein samples were subjected to SDS-PAGE to identify the peak of the protein. The arrows indicate the migration of His₆-RYBP. The X and Y axes show the UV absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used, starting at 5% and ending at 100%.

A negligible amount of His₆-RYBP was eluted from the cation exchange column, suggesting that the protein binds irreversibly to the column matrix. In order to enhance the expression level, a construct was cloned to express GST-RYBP. It was transformed into PC2 cells. Six l of bacterial culture was induced at OD₆₀₀ of 0.9 for 4 h at 25°C. After harvesting the cells, sequential protein extraction was carried out using buffers containing 25 mM Tris-HCl pH 7.4 and either 150 mM, 250 mM or 500 mM NaCl. Samples were taken after sonicating the resuspended cells and after clearing the sonicated lysate from debris to estimate the level of protein extracted. The samples were analysed by SDS-PAGE and the proteins stained with Coomassie (Figure4.3).

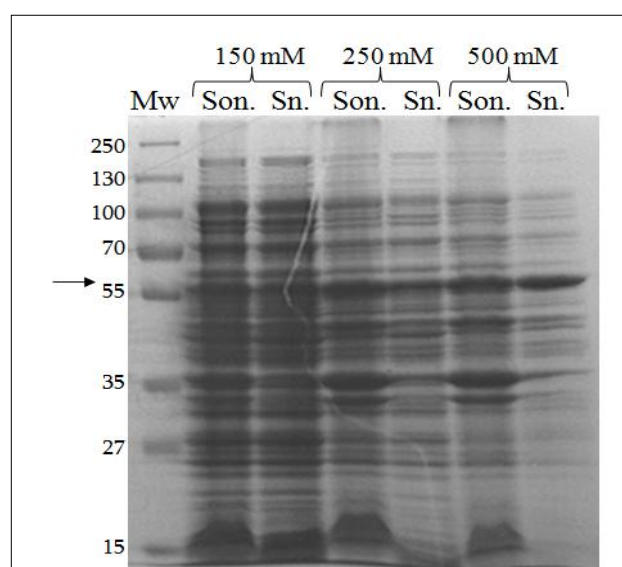


Figure 4.3) GST-RYBP expression and extraction level.

Commassie stained SDS-PAGE gel of sonicated (Son.) and supernatant (Sn.) of the centrifuged cell lysates prepared in different buffers indicate that the extraction condition is best at 500 mM NaCl buffer. The arrow indicates the migration of GST-RYBP.

GST-RYBP extracted with a buffer containing 500 mM NaCl (Figure 4.3) was used to purify the protein further by binding to Glutathione Sepharose resins. The protein was eluted from the resins using 20 mM glutathione in the extraction buffer; in five 0.5 ml fractions (Figure 4.4). The GST-tag was cleaved off from the protein using 3C protease. The samples from pre- and post-protease treatment were fractionated by SDS-PAGE and the gel stained with Coomassie (Figure 4.4).

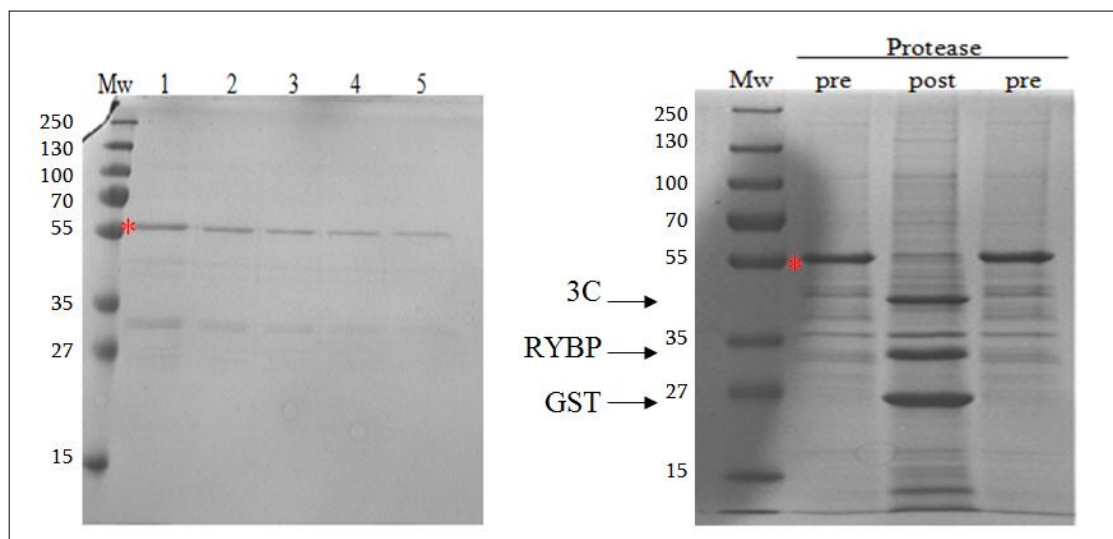


Figure 4.4) Purification of GST-RYBP.

The affinity purification samples were applied to SDS-PAGE gel and Coomassie stained (left panel). The presence of GST-RYBP was confirmed by visualizing the shift in protein size, after cleaving the GST tag with 3C protease (right panel). Red stars show the migration of GST-RYBP.

All the eluted fractions were pooled together and the protein was purified from the glutathione, using dialysis. The protein was concentrated and frozen in liquid nitrogen after adding glycerol to 10% final concentration.

RING2

A construct was cloned to express GST-RING2/1-331 based on published results (Buchwald *et al.* 2006). The plasmid was transformed into PC2 cells. Six litres of the cell culture was induced to express the protein at OD₆₀₀ of 0.9 for 4 h at 25°C. The cells were harvested and resuspended in 25 mM Tris pH 7.4 and 500 mM NaCl extraction buffer. After sonication a sample was taken to estimate the expression level. After clearing the sonicated lysate from debris another sample was taken to measure an approximate extraction level (Figure 4.5).

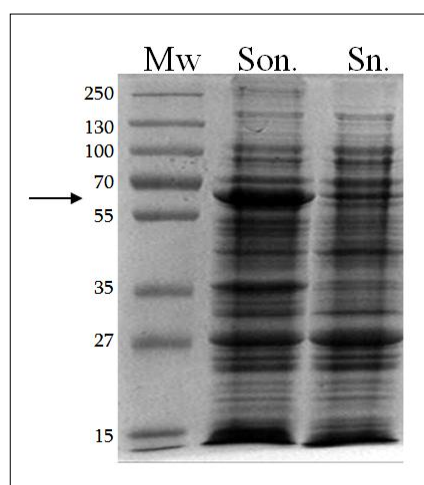


Figure 4.5) GST-RING2/1-331 expression and extraction level.

Commissie blue stained SDS-PAGE gel of sonicated (Son.) and supernatant (Sn.) of the centrifuged sonicated cell lysates. The arrow indicates the migration of GST-RING2/1-331.

As shown in the Figure 4.5 the expression level of the GST-tagged RING2/1-331 is high, however most of the protein remained in the pellet and could not be extracted. Nevertheless, we continued with the extracted protein and purified it by binding to Glutathione Sepharose resin. GST-RING2 was eluted with 20 mM glutathione in ten 1-ml fractions (Figure 4.6a). Fraction number 2 - 6 were pooled together and the GST-tag was cleaved off with 3C protease at 4 °C overnight (Figure 4.6b). The protein was further purified by size exclusion chromatography and samples from the peak fractions were loaded on SDS-PAGE to detect the purified protein (Figure 4.6b). Fraction number 21 to 25 were pooled together, concentrated and snap frozen in liquid nitrogen after adding glycerol to final 10% concentration.

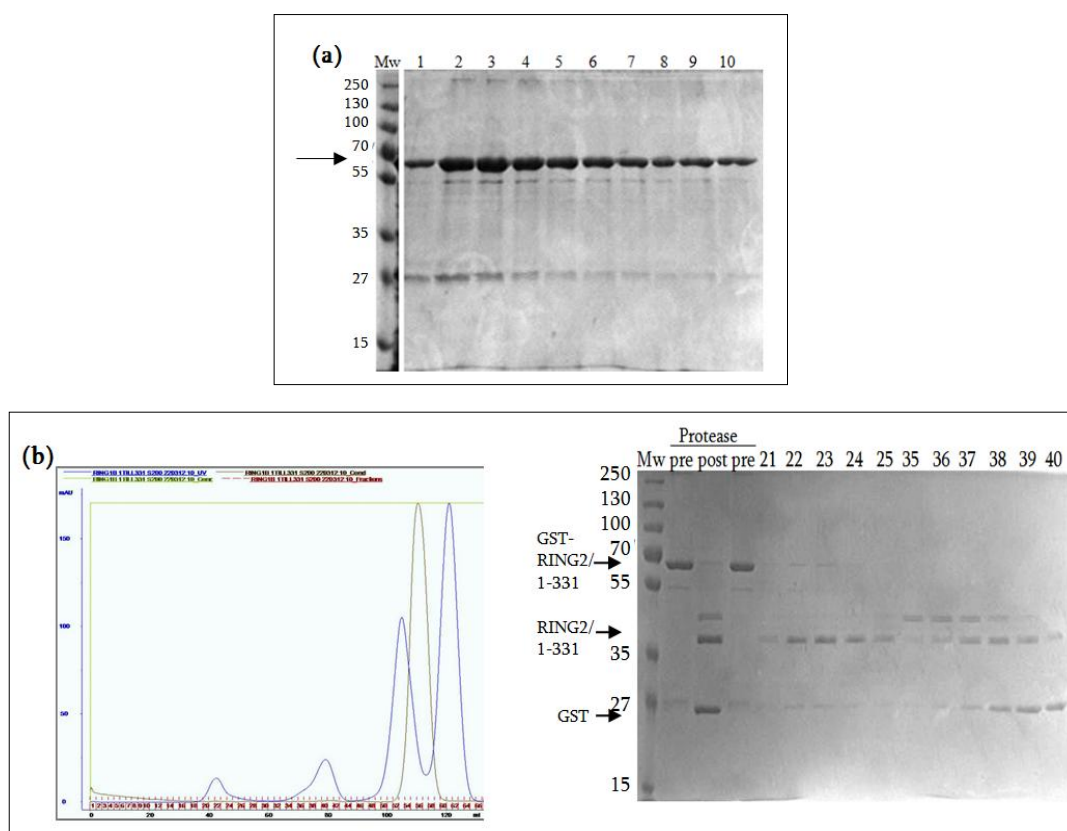


Figure 4.6) RING2/1-331 purification steps.

(a) SDS-PAGE of GST-RING2/1-331 Glutathione Sepharose affinity purification. (b) Chromatogram showing the purification on the size exclusion column of RING2/1-331 after cleaving the GST-tag by protein protease 3C. The SDS-PAGE of the cleaved and un-cleaved protein shows that the protease activity was completed. The eluted protein fractions were subjected to SDS-PAGE to identify the peak of the protein. The eluted fractions were applied to a SDS-PAGE gel and the peak fractions were pooled together and concentrated. The X and Y axes show the UV absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used, 5%

BMI1

Two plasmid constructs were prepared to express untagged-BMI1, His₆-SUMO- BMI1. In addition, construct encoding GST-BMI1 was provided by Dr. G. Maertens.

Firstly, untagged-BMI1 was expressed in 6 L of LB medium for 4 h at 25°C. Cells were harvested and the protein was sequentially extracted with three different buffers, differing in NaCl concentration; 150 mM, 250 mM or 500 mM NaCl. The sonicated lysates were cleared from debris (Figure 4.7). The extraction level was not sufficient to continue the purification.

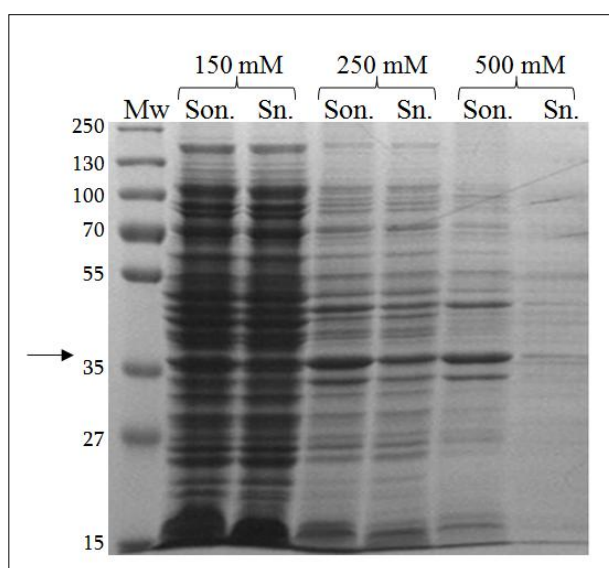


Figure 4.7) BMI1 expression and extraction level.

Commissie stained SDS-PAGE gel of sonicated (Son.) and supernatant (Sn.) of the centrifuged sonicated cell lysates in different buffers [containing 150 mM, 250 mM or 500 mM NaCl]. The arrow indicates the migration of BMI1.

As seen in Figure 4.7 the extraction of the untagged BMI1 is inefficient. To increase the solubility of the protein, the construct expressing His₆-SUMO-BMI1 was transformed into PC2 cells. The protein expression of 6 L cell culture was induced at OD₆₀₀ of 0.9 for 4 h at 25°C. The protein was extracted from the harvested cells in four different buffers; containing either 50 mM, 150 mM, 250 mM or 500 mM NaCl. The sonicated lysate was cleared from debris. The expression and extraction levels were estimated by loading samples of sonicated and cleared lysate on the SDS-PAGE gel (Figure 4.8).

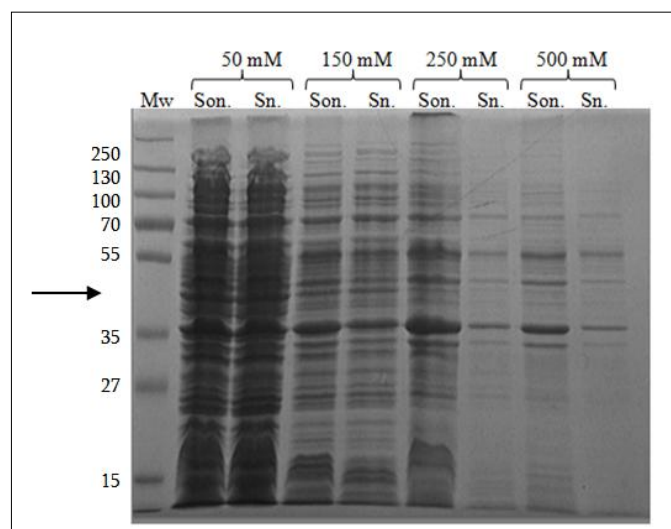


Figure 4.8) His₆- SUMO-BMI1 expression and extraction level.

Commassie blue stained SDS-PAGE gel of sonicated (Son.) and supernatant (Sn.) of the centrifuged sonicated cell lysates in different buffers [containing either 50 mM, 150 mM, 250 mM or 500 mM NaCl. The arrow shows the immigration of His₆- SUMO-BMI1.

Although the expression level is low, the cleared lysate in the buffer containing 50 mM NaCl was chosen for affinity purification on CL-Nickel ChroMatrix. The protein was eluted in 200 mM imidazole in ten 1-ml fractions. A sample from each fraction was separated by SDS-PAGE and proteins were detected by staining with Coomassie (Figure 4.9). His₆-SUMO-BMI1 was either not eluted from the CL-Nickel ChroMatrix or was severely degraded.

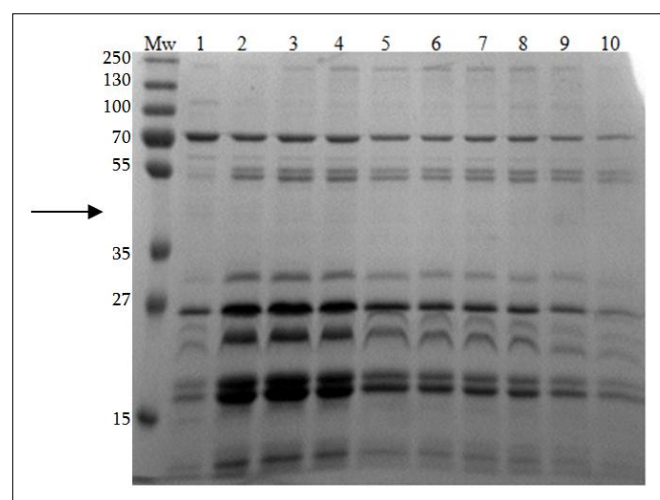


Figure 4.9) Affinity purification of His₆-SUMO-BMI1.

Samples of eluted protein with 200 mM imidazole in ten 1-ml fractions were loaded on SDS-PAGE gel and stained with Coomassie. The arrow shows the immigration of His₆-SUMO-BMI1.

In the next step, we attempted to use a construct for expression of GST-tagged BMI1. Six L of transformed Rosetta 2(DE3)pLacI cell culture in TB medium was induced to express the protein with 0.1% IPTG at 18 °C for 16-18 h. The protein was extracted using the same buffers used in Buchwald *et al.* (2006). The expression and the extraction level can be estimated from the samples taken before and after clearing the lysates from the debris, respectively (Figure 4.10). The GST-tagged protein was purified by binding the

lysate on Glutathione Sepharose column. The protein was eluted in 20 mM glutathione, three times the volume of the resins (Figure 4.10). The efficiency of the elution was estimated by taking a 10 µl sample from the Glutathione Sepharose resins, boiling the beads in 50 µl SDS-loading buffer for 2 min and separating it by SDS-PAGE (Figure 4.10).

The GST-tag was removed from BMI1 by 3C protease. The protein was further purified by cation exchange chromatography on a 5-ml HiTrap SP sepharose column. Fraction 13-18 were pooled, concentrated and snap frozen in liquid nitrogen after adding glycerol to final 10% concentration (Figure 4.10).

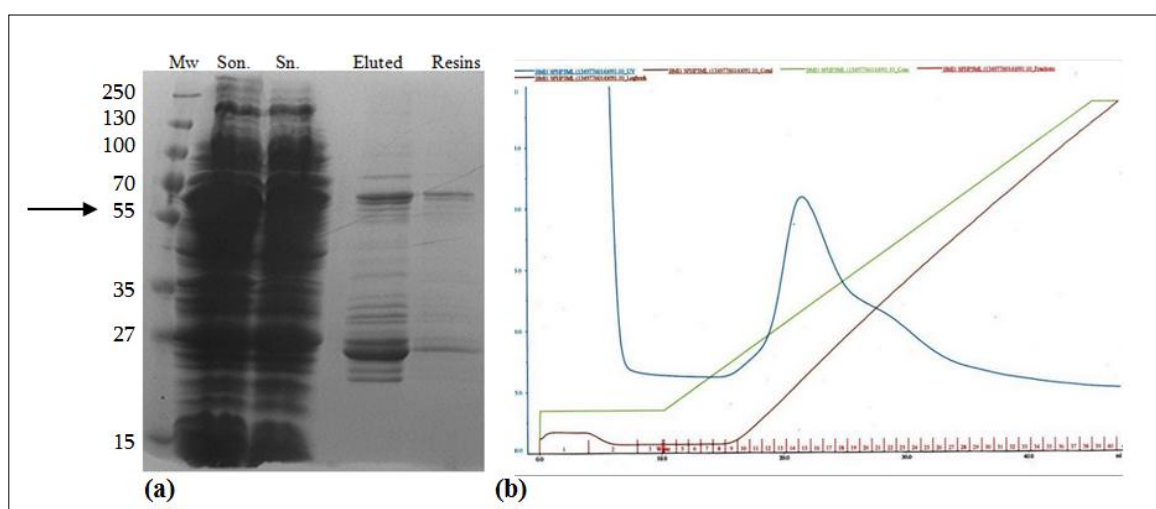


Figure 4.10) Expression, extraction and purification of GST-BMI1.

(a) The expression (son.), extraction (sn.) and elution levels of GST-BMI1 can be estimated. The arrow indicated the migration of GST-BMI1. (b) Further purification was carried out by loading the protein on cation exchange column after cleaving the GST-tag by protein protease 3C. The X and Y axes show the UV absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used, starting at 10% and ending at 100% B,

Co-expression of PRC1 components in bacteria

In many cases, where proteins expressed in *E. coli* are insoluble, co-expression of a binding partner might solubilise the protein and allow for co-purification of a functional protein-protein complex. GST-RING2 is expressed at very high levels in *E. coli* but we could not efficiently extract the protein. We know that RING2 and BMI1 interact, and also that RING2 and RYBP form a complex. With the aim of purifying a hetero-dimeric or -trimeric PRC1 subcomplexes, we co-expressed combinations of RING2 with RYBP and/or BMI1 in *E. coli*.

RING2-RYBP

Compatible plasmid constructs encoding GST-RING2/1-331 and His₆-RYBP were co-transformed and co-expressed in PC2 cells. After cells were harvested from 6 L culture, sequential protein extraction was carried out with buffers containing different concentrations of NaCl; 150 mM, 250 mM, 500 mM. Samples were taken before and after clearing the sonicated lysate from debris and separated by PAGE to examine the expression and extraction levels (Figure 4.11).

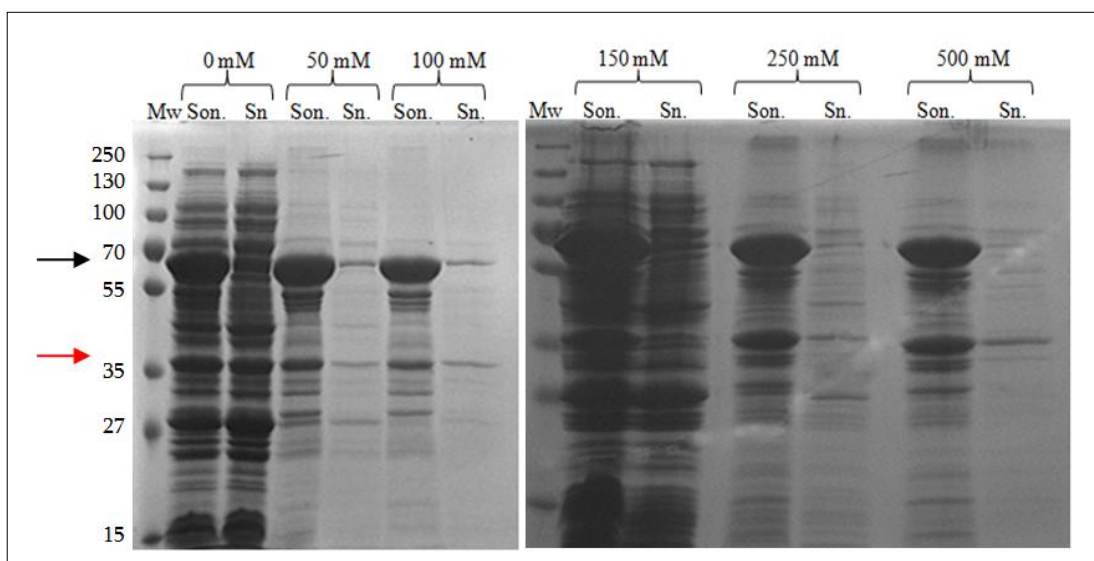


Figure 4.11) Extraction and expression levels of co-expressed GST-RING2 and His₆-RYBP.

The expression (son.) and extraction (sn.) levels of the co-expression in six different extraction conditions were estimated by SDS-PAGE gel. The migration of GST-RING2 and His₆-RYBP are shown with black and red arrows, respectively.

Half of the lysate sonicated in 150 mM NaCl was allowed to bind to Glutathione Sepharose resin. The protein was eluted with sonication buffer supplemented with 20 mM glutathione in ten 0.5-ml fractions. The other half was cleared over CL-Nickel ChroMatrix and eluted with 200 mM imidazole in ten 0.5-ml fractions. Samples from each fraction were separated by SDS-PAGE to detect the best purification method (Figure 4.12). In GST-affinity purification only the GST-RING2 was eluted.

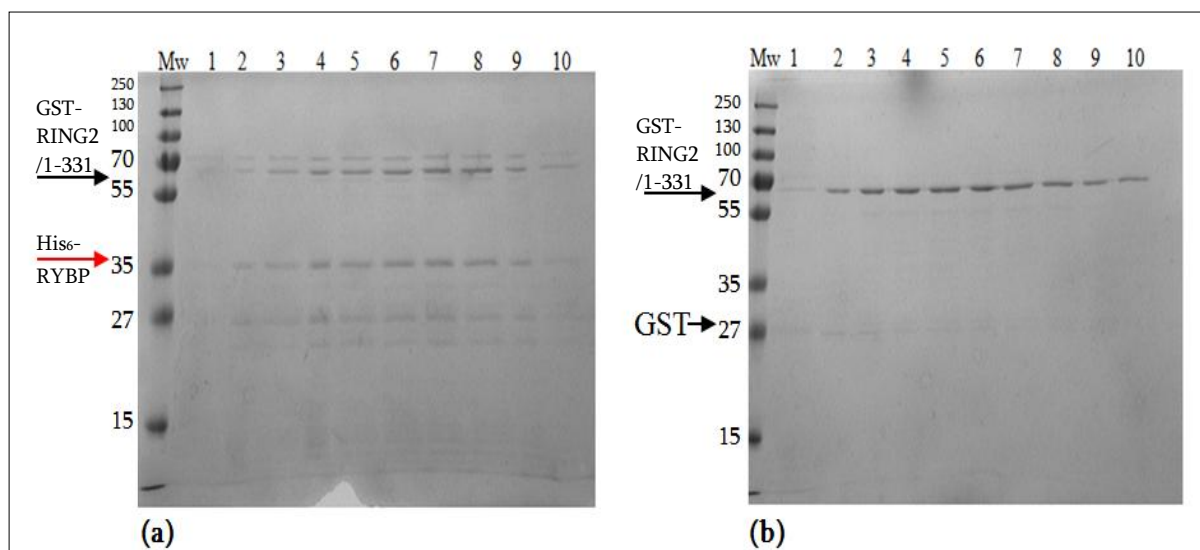


Figure 4.12) Affinity purification of co-expressed GST-RING2/1-331 and His6-RYBP extracted in 150 mM NaCl buffer.

(a) Samples eluted from CL-Nickel ChroMatrix in 10 1-ml fractions were separated by SDS-PAGE. Fractions two to nine, containing only GST-RING2/1-331, were pooled together for further purifications. **(b)** Samples eluted from glutathione Sepharose resins in 10 1-ml fractions were separated by SDS-PAGE. Fractions 3 - 9, containing both proteins were pooled together for further experiments.

GST-RING2/1-331 in fractions 2 - 9 were pooled together for further experiments, concentrated and snap-frozen in liquid nitrogen. His₆-affinity purification allowed us to elute GST-RING2 and His₆-RYBP as complex, albeit at very low yield (Figure 4.12a). Fraction numbers 3 - 9 were pooled together. After removing the tags by 3C protease, the proteins were purified by size exclusion chromatography (Figure 4.13). None of the proteins could be detected in the fractions eluted from the column; examined on Coomassie-stained SDS-PAGE gel.

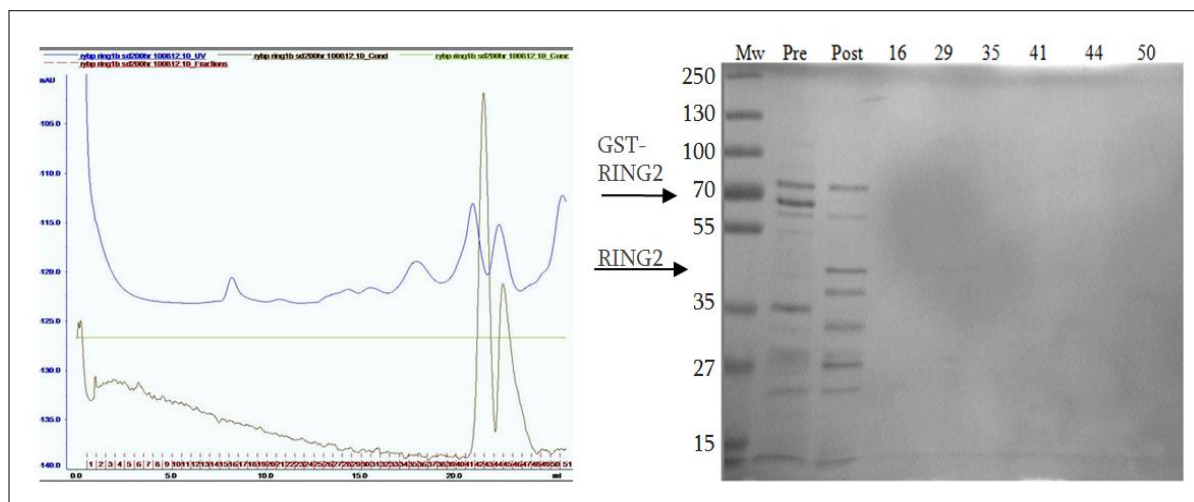


Figure 4.13) Purification of RING2 and RYBP by size exclusion chromatography.

Samples from the peak fractions eluted from size exclusion column were tested on SDS-PAGE gel. Proteins could not be detected. The X and Y axes show the UB absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used, 5%.

In an attempt to increase the solubility of RING2, constructs were made to express His₆-SUMO-RING2, which was co-transformed into PC2 cells with a plasmid expressing His₆-RYBP. Protein expression was induced in 6 L culture at OD₆₀₀ of 0.9 for 4 h at 25°C. Cells were harvested and the proteins were extracted in four different buffers. The expression and the extraction levels were estimated by taking samples from before and after clearing the lysate from debris (Figure 4.14). The expression level of His₆-SUMO-RING2 had drastically decreased in comparison with GST-RING2, co-expressed with His₆-RYBP. The extraction level, compared to GST-RING2/ His₆-RYBP co-expression, had not improved; therefore, the experiment was terminated.

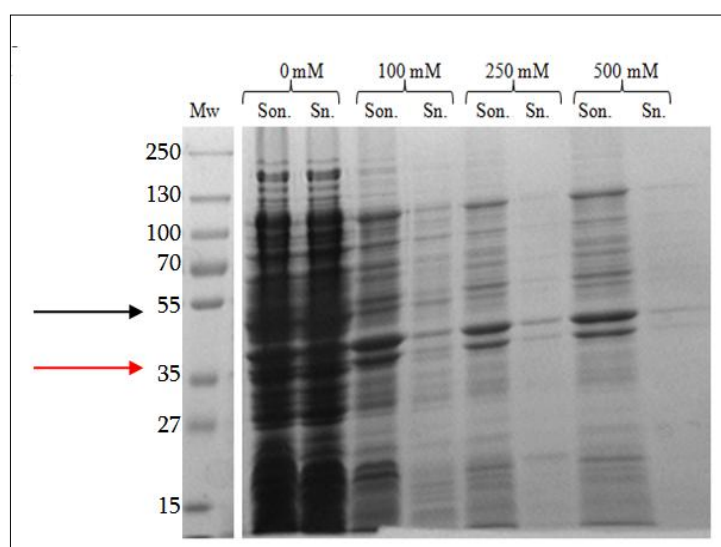


Figure 4.14) Expression level and extraction efficiency of co-expressed His₆-SUMO- RING2 and His₆-RYBP.

Extraction was done in different conditions. His₆-SUMO-RING2 and His₆-RYBP migration is indicated with black and red arrows, respectively.

RING2-BMI1

GST-tagged RING2 and untagged BMI1 were co-transformed into PC2 cells. The protein expression was induced at OD₆₀₀ of 0.9, in 6 L culture, for 4 h at 25°C. The cells were harvested and proteins were extracted in two sequential conditions, using buffers containing either 0 mM or 100 mM NaCl. Samples were taken before and after clearing the lysate from debris, to estimate the extraction and expression levels (Figure 4.15). The extraction level for both proteins was very low; therefore, the experiment was terminated.

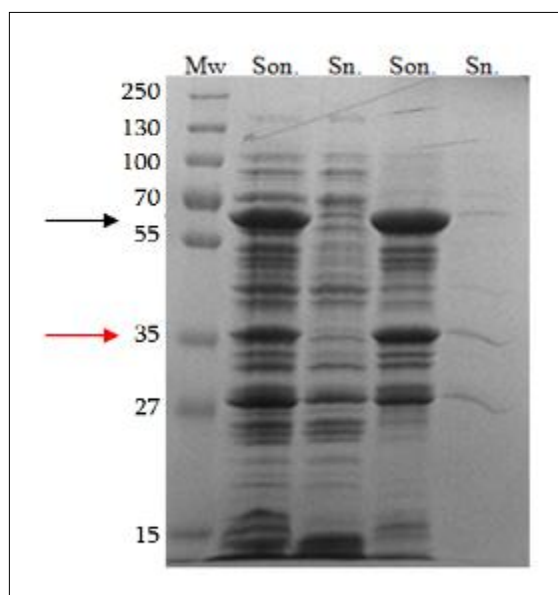


Figure 4.15) Expression level and extraction efficiency of co-expressed GST-RING2 (black arrow) and BMI1 (red arrow).

We also tried the co-expression of His₆-SUMO-RING2 (described above) with untagged BMI1 in the PC2 strain. The protein expression was induced for 4 h at 25°C. The cells were harvested and proteins extracted in four conditions differing in NaCl concentration. The expression and extraction levels were estimated by taking samples before and after clearing the sonicated lysate from debris (Figure 4.16a). The extraction level was shown to be sufficient in 0 mM NaCl extraction buffer and was chosen for further purification by clearing the lysate over CL-Nickel ChroMatrix. The proteins were eluted in ten 1-ml fractions with 200 mM imidazole, visualized on SDS-PAGE gel stained with Coomassie (Figure 4.16b). The protein complex could not be eluted from the affinity purification column. The experiment was terminated.

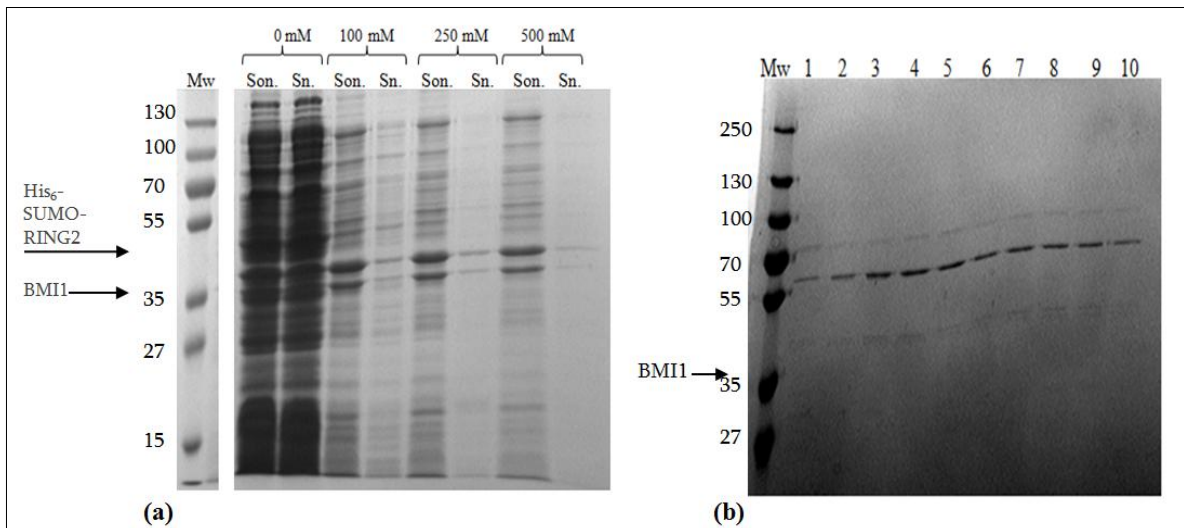


Figure 4.16) Co-expressed His₆-SUMO-RING2 and BMI1.

(a) Expression and Extraction of co-expressed His₆-SUMO-RING2 and BMI1 in different conditions. **(b)** Samples eluted from CL-Nickel ChroMatrix in 10 1-ml fractions were separated by SDS-PAGE.

RING2-BMI1-RYBP

It is clear that although RING2, RYBP and BMI1 can be expressed at relatively high levels, extraction of a dimer was very inefficient suggesting that either the interaction between the two proteins is very weak and they don't form a complex in *E. coli* or that the heterodimeric complexes are insoluble. We hypothesized that maybe a trimeric complex would be more soluble and thus tried co-expression of all three of these subunits in *E. coli*. Expression of proteins was induced in 6 L culture at OD₆₀₀ of 0.9 for 4 h at 25°C. In first instance we co-transformed the PC2 strain with plasmids encoding for GST-RING2, His₆-RYBP and His₆-SUMO-BMI1. These three plasmids each contain a different resistance cassette and co-transformed bacteria can thus be selected by growing the cells in the presence of ampicillin, spectinomycin and kanamycin. After cells were harvested, proteins

were extracted in four conditions differing in NaCl concentration; 0, 100, 250 and 500 mM. The expression and extraction levels were estimated by taking samples from before and after clearing the sonicated lysate from debris, loaded on SDS-PAGE gel and stained with Coomassie (Figure 4.17). GST-RING2 was not significantly eluted in either of the buffers used. His₆-RYBP was eluted most efficiently in extraction buffer containing 500 mM NaCl and BMI1 in 0 mM NaCl. As proteins in form of a complex could not be eluted, the experiment was terminated.

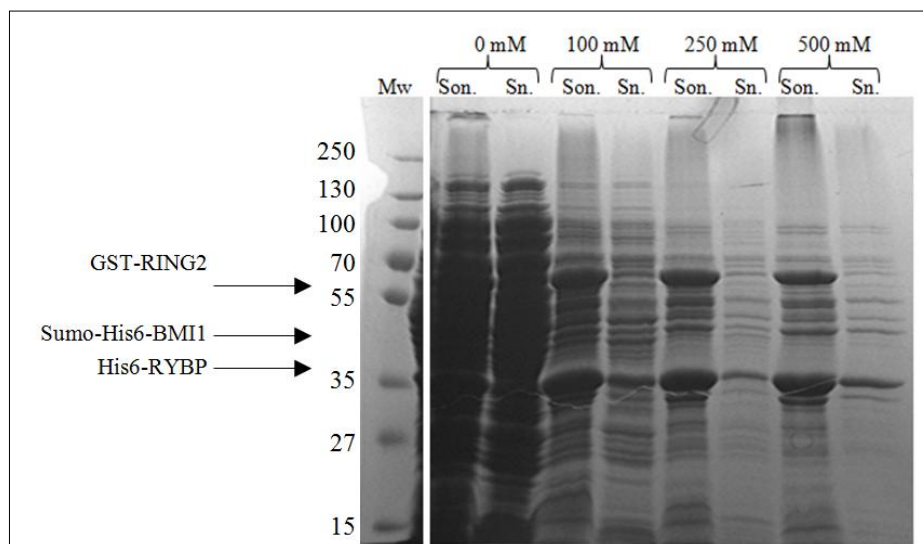


Figure 4.17) Expression and extraction level of GST-RING2, His₆-RYBP and His₆-SUMO-BMI1 co-expression.

Proteins were extracted in four different conditions, to find the best extraction condition. Each protein is best extracted in different condition.

We also tried the combination of His₆-SUMO-RING2 with His₆-RYBP and untagged BMI1. After inducing the protein expression for 4 h, the cells were harvested and underwent the sequential protein extraction procedure. The proteins were extracted under four conditions, differing in NaCl concentration of the extraction buffer; 0 mM, 100 mM, 250 mM and 500 mM. Samples were taken from before and after the sonicated lysate were cleared from debris, to estimate the expression and extractions levels (Figure 4.18a).

Unlike BMI1 which had shown increased expression levels, compared to previous co-expression, the expression levels of RING2 and RYBP had considerably decreased. None of the three proteins could be efficiently extracted, the experiment was therefore terminated.

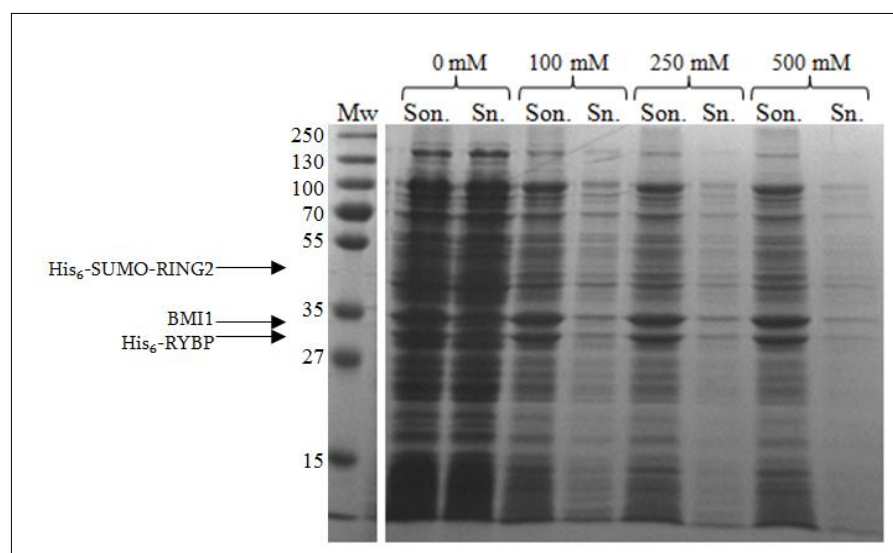


Figure 4.18) Co-expression of His₆-SUMO-RING2, His₆-RYBP.

Expression and extraction of co-expressed His₆-SUMO- RING2, His₆-RYBP and untagged BMI1 in four conditions.

4.1.2 Investigating BMI1-RYBP interaction using pull down assays

The interaction between BMI1 and RYBP was investigated using the GST-pull down assay. It was shown that the RING domain of Bmi1 interacts with the RING domain of Ring1b (Li *et al*, 2006; Buchwald *et al*, 2006). Therefore in this experiment, GST-RING2 was used as positive control and GST as negative control. GST-RYBP, GST-RING2 and GST were mixed separately with BMI1 in binding buffer and complexes were allowed to form by gentle rocking at 4°C. Two hours later, equilibrated glutathione sepharose resin was added to capture the GST-tagged protein and the complexes they may have formed. After 3 h gentle rocking at 4°C, the beads were extensively washed. Bound proteins were eluted by boiling the beads in Laemmli sample buffer and were then separated on SDS-PAGE gels. The gels were stained with Coomassie (Figure 4.19a). As can be seen in lane 2 of Figure 4.19 a, very little BMI1 was used for the pull-down. We thus also ran the samples on gel for Western blot detection with an anti-BMI1 antibody (Figure 4.19b). As expected, BMI1 is recovered with GST-RING2, thus confirming the interaction between these two proteins. The BMI1 was not pulled-down with GST-RYBP since the amount of BMI1 detected by Western blot is similar to background interaction with GST (compare lanes 1 and 2 with lane 3 in panel b) suggesting that BMI1 and RYBP do not interact directly, or minimally do not display a high affinity interaction.

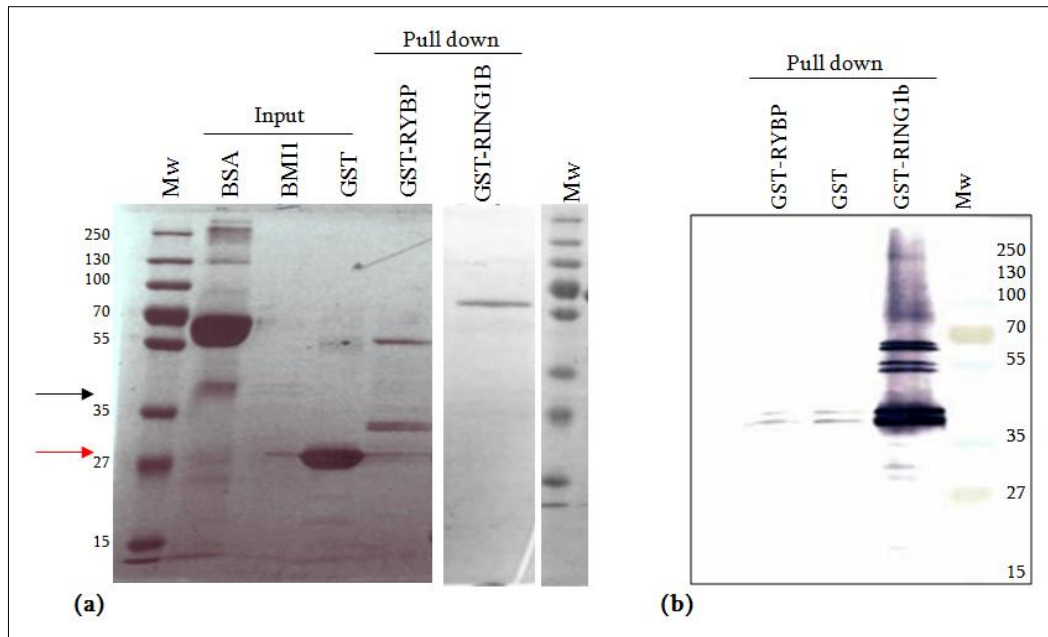


Figure 4.19) GST-RYBP glutathione sepharose pull downs.

Purified BMI1 and GST-RYBP or GST-RING2 (positive control) were mixed with glutathione sepharose resins for 3 h and the beads were washed extensively. Bound proteins were dissociated and separated by SDS-PAGE gel and stained with Coomassie (a), or transferred to a nitrocellulose membrane and detected with anti-BMI1 antibody (b) to detect BMI1 in the pull downs. GST was used as negative control. Black and red arrows indicate migration of BMI1 and GST, respectively.

4.2 Discussion

It was shown that RING2, MEL18 (BMI1 homolog) and RYBP can mono-ubiquitinate H2A on nucleosome substrates *in vitro* (Tavares *et al*, 2012). In this project we aimed to purify three different PRC1 components (BMI1, RING2 and RYBP) and investigate their interactions. We also aimed to form a multi-component PRC1 complex using these three proteins and investigate whether this increases the affinity for USP11.

The main challenge in this project was to optimize the expression and extraction levels in bacteria. For each of these proteins, different tags, bacteria culture mediums, and temperatures were tested to identify the best condition in which bacteria best express the desired protein. In addition, we also used different extraction conditions, but were unsuccessful in purifying the recombinant proteins.

We also tested the possibility of co-expressing different combinations of proteins in bacteria. Co-expression of proteins in the bacteria cell could improve the stability and solubility of the proteins. In some cases the co-transformation increased the expression of one or both proteins compared to when they were expressed separately. However, it was observed that each protein could be extracted from the bacteria in different extraction condition, and none of the proteins formed a stable complex and, thus, could not be purified as a complex.

Even though we could not purify high levels of the PRC1 components, we had enough protein to investigate whether there is a direct interaction between BMI1 and RYBP. Whilst the interaction between RING2 and BMI1 could be confirmed, under the same

conditions we could not observe a direct interaction between BMI1 and RYBP. It is possible that there is no direct interaction between RYBP and BMI1, and the trimeric complex exists by direct interaction of these two proteins with RING2. Alternatively, it is possible that the GST-tag on RYBP sterically interferes with the interaction, a reciprocal pull down, using His₆-tagged BMI1 and untagged RYBP is thus required to confirm our observation.

We can conclude that production of pure recombinant RING2, BMI1 and RYBP at high levels is difficult to achieve in *E. coli*. Currently, studies are being carried out in our group, by Dr. Maertens, to express the proteins in Sf9 insect cells to potentially improve the protein expression level.

Chapter V: Structural characterisation of USP11 N-terminal domain

The X-ray crystal structure of USP15 and USP4 DU has been solved (Harper *et al*, 2011; Bacik *et al*, 2009). In an attempt to characterise the structure of USP11/DU we used a range of USP11 deletion constructs to obtain crystals and determine its structure.

5.1 Results

Several plasmids were made in the effort to solve the structure of USP11 DU (Table 5.1).

Plasmids
pCDF-H6P-Duet1-USP11/1-286
pCDF-H6P-Duet1-USP11/76-286
pCDF-H6P-Duet1-USP11/76-286[E106A/K107A]
pCDF-H6P-Duet1-USP11/76-286[K146A/E147A]
pCDF-H6P-Duet1-USP11/76-286[K278A/K279A]

Table 5.1) Plasmid constructs made to express different fragments of USP11 DU.

These plasmids contain a streptomycin resistant gene.

5.1.1 Expression and purification of USP11 N-terminal fragments.

A construct containing full-length GST-USP11, available in the laboratory and provided by Dr. G. Maertens, was used to express the protein in 6 L TB medium in Rosetta 2(D3)pLacI *E. coli* cells at 16 °C for 20 h. Protein was extracted and eluted in ten 1ml-fractions with 20 mM glutathione (Figure 5.1).

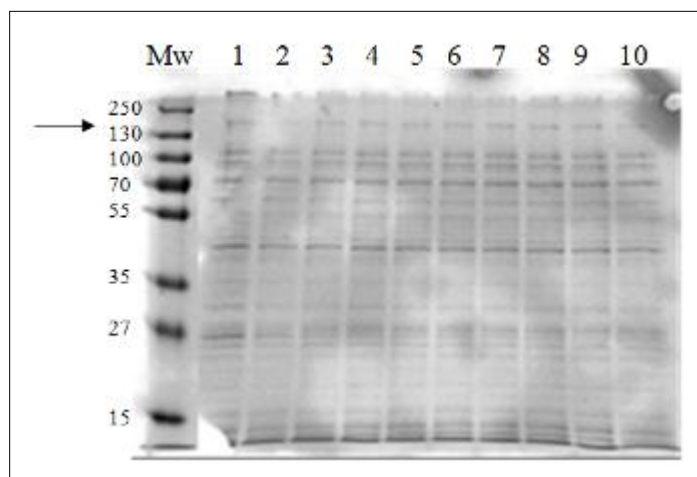


Figure 5.1) Affinity purification of GST-USP11.

Samples of eluted protein were separated by SDS-PAGE. The arrow indicates the migration position of full-length GST-USP11.

Since the large scale expression of full length USP11 resulted in a negligible protein yield, we decided to focus our efforts on expression and purification of fragments of USP11.

In order to determine the X-ray structure of the N-terminal region of USP11, plasmid constructs were cloned encoding this region of USP11 and containing both DUSP (76-195) and UBL1 (196-286) domains. The function and structural significance of the first 76 amino acid is not known. Two constructs encoding His₆-tagged USP11/1-286 and USP11/76-286 gene were kindly provided by Dr. G. Maertens. Each of these constructs was transformed into *E. coli* PC2 strains. Six L LB medium supplemented with the 100 µg/ml spectinomycin (Melford) were used to grow the bacterial culture to OD₆₀₀ of 0.8. The bacteria were induced by adding 0.1% IPTG to the culture and incubating it at 25 °C for 4 h. The cells were harvested and after clearing the sonicated lysate from debris, the His₆-tagged proteins were purified over a CL-Nickel ChroMatrix (Figure 5.2a and 5.3a). The His₆-tag was removed by human rhinovirus 14 3C protease overnight at 4 °C (Figure 5.2b and 5.3b). The cleaved protein was further purified by anion exchange chromatography (Figure 5.2b and 5.3b), followed by size exclusion chromatography (Figure 5.2c and 5.3c).

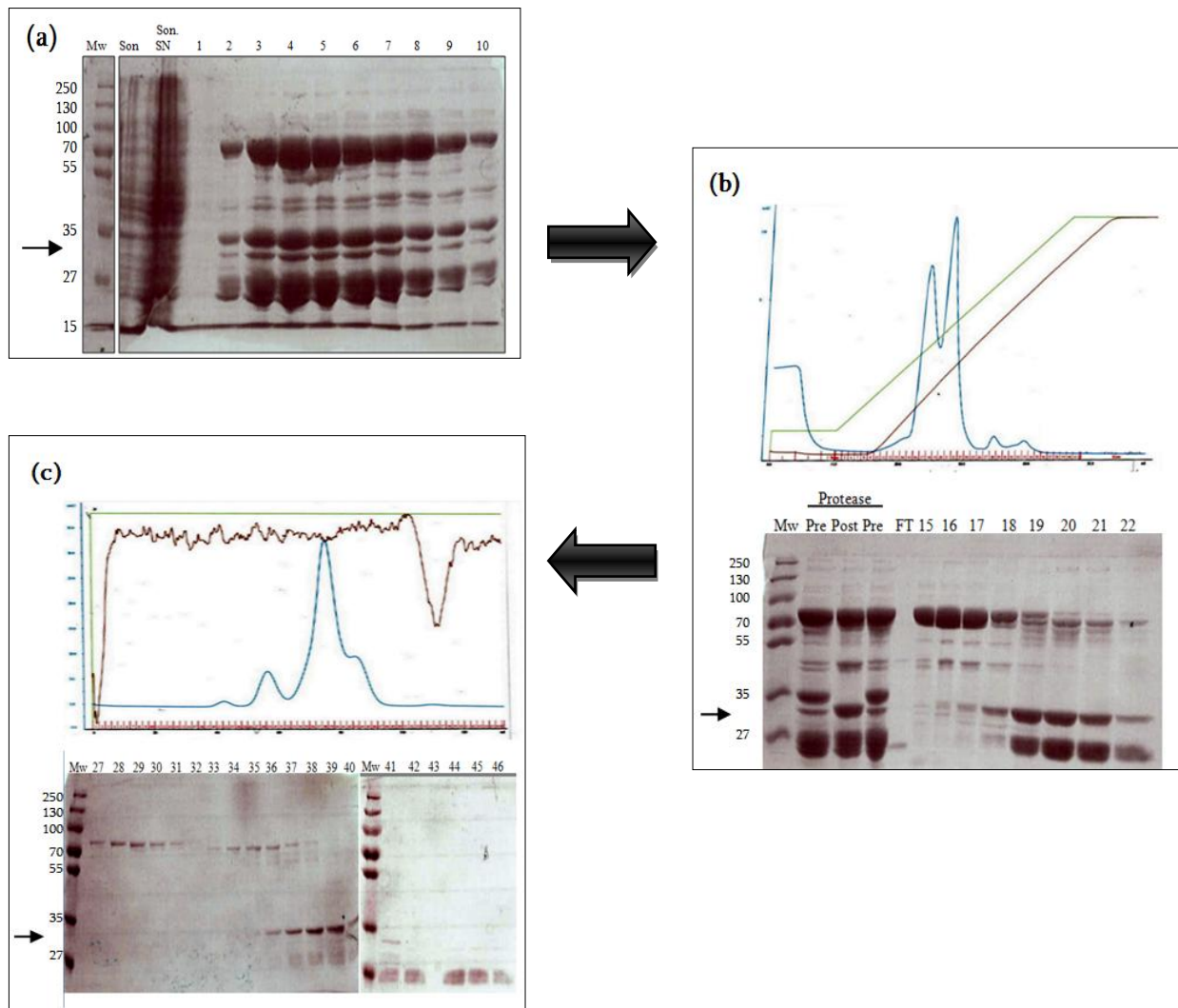


Figure 5.2) His₆-USP11/1-286 purification steps.

(a) SDS-PAGE of His₆-USP11/1-286 CL-Nickel ChroMatrix affinity purification. Arrowhead indicates migration position of His₆-USP11/1-286. **(b)** Chromatogram showing the purification on the anion exchange HiTrap Q column (upper panel) of USP11/1-286 after cleaving the His₆-tag by human rhinovirus 14 3C protease. The protein was eluted with a linear salt gradient, from 15% B to 100% B. The SDS-PAGE of the cleaved and un-cleaved protein shows that the tag was fully removed. The eluted protein fractions were subjected to SDS-PAGE to identify the peak of the protein. Arrowhead indicates migration position of USP11/1-286. **(c)** Purification of the fragment was completed by size exclusion chromatography. The protein was eluted in 20% B. The eluted fractions were separated by SDS-PAGE. Fraction numbers are indicated at the top of the gel, size of the Molecular weight marker is indicated at the left side of the gel. Arrowhead indicates migration position of USP11/1-286. The X and Y axes show the UV absorption at 280 nm and the number of the eluted fraction, respectively. Blue lines denote UV absorption at 280 nm, brown

lines reflect the conductivity of the sample and the green lines indicate the percentage of high salt containing buffer B used.

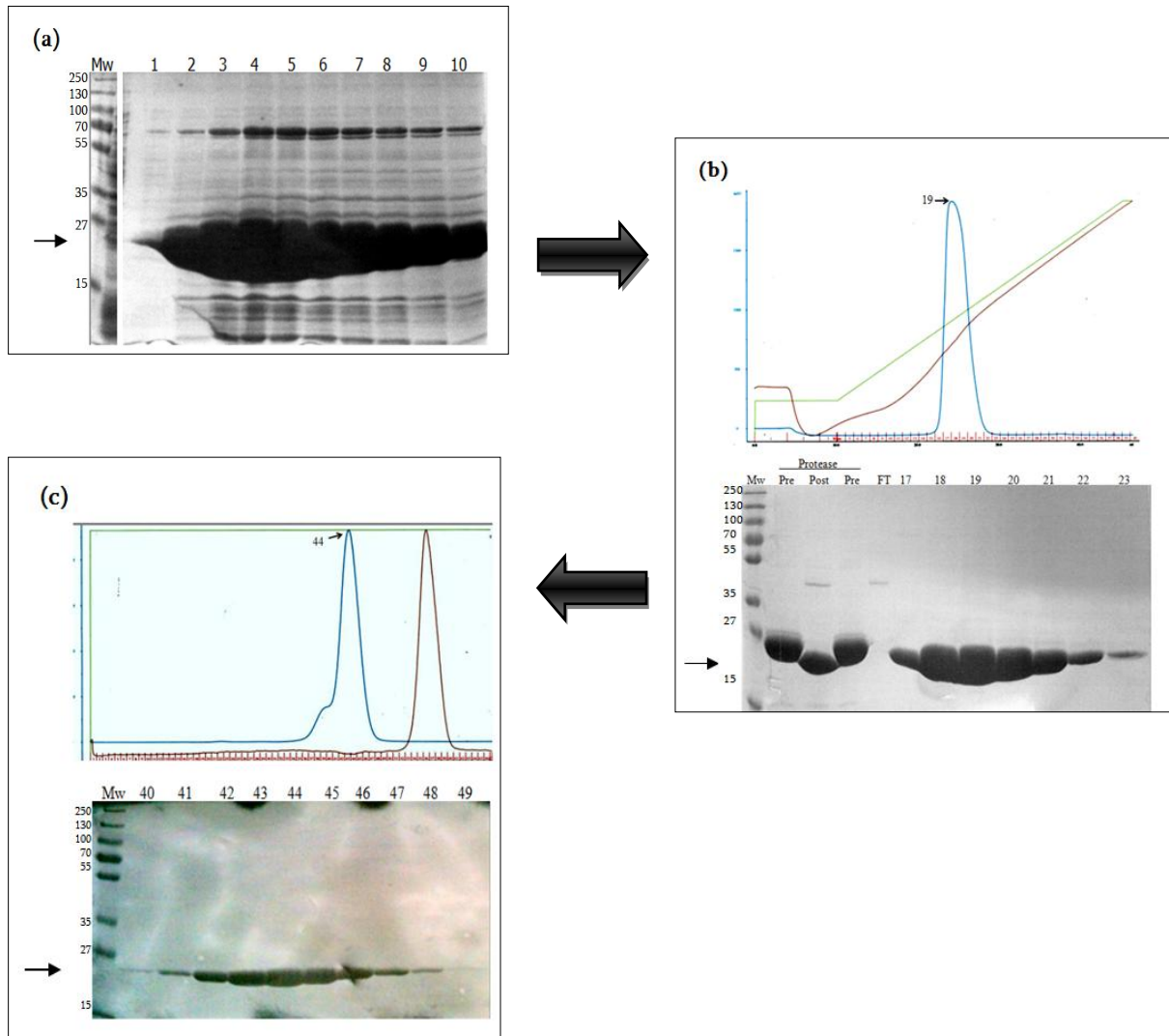


Figure 5.3) His₆-USP11/76-286 Protein purification steps.

(a) SDS-PAGE of His₆-USP11/76-286 CL-Nickel ChroMatrix affinity purification. Arrowhead indicates migration position of His₆-USP11/76-286. **(b)** Chromatogram showing the purification on the anion exchange HisTrap Q column (upper panel) of USP11/76-286 after cleaving the His₆-tag by human rhinovirus 14 3C. The protein was eluted with a linear salt gradient, from 15% B to 100% B. The SDS-PAGE of the cleaved and un-cleaved protein shows that the protease activity was completed. The eluted protein fractions were subjected to SDS-PAGE to identify the peak of the protein. Arrowhead indicates migration position of USP11/76-286. **(c)** Purification of the fragment was completed by size exclusion chromatography. The protein was eluted in 20% B. The eluted fractions were applied to a SDS-PAGE gel and the peak fractions were pooled together and concentrated to 20mg/ml. Arrowhead indicates migration position of USP11/76-286. The X and Y axes show the UB absorption at 280 nm and the number of the eluted fraction, respectively. The

blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample.
The green line indicates the percentage of buffer B used.

5.1.2 Crystallization of USP11/76-286

USP11/1-286 was expressed at lower levels and could not be purified from low-molecular weight contaminants (Figure 5.2). In contrast, USP11/76-286 was expressed at very high levels and the purification was successful (Figure 5.3). Therefore, we focused on crystallisation screening with USP11/76-286 construct.

In the first attempt USP11/76-286 was concentrated to 9 mg/ml and subjected to crystallization screening using 11 different commercially available sparse matrix screens (96 different conditions per plate) in sitting drop format. Crystals were formed in 6 different conditions forming clusters of needles/rods (Figure 5.4). However, most drops on the screens remained clear even after prolonged incubation of crystallization plates, indicating that the construct is highly soluble.

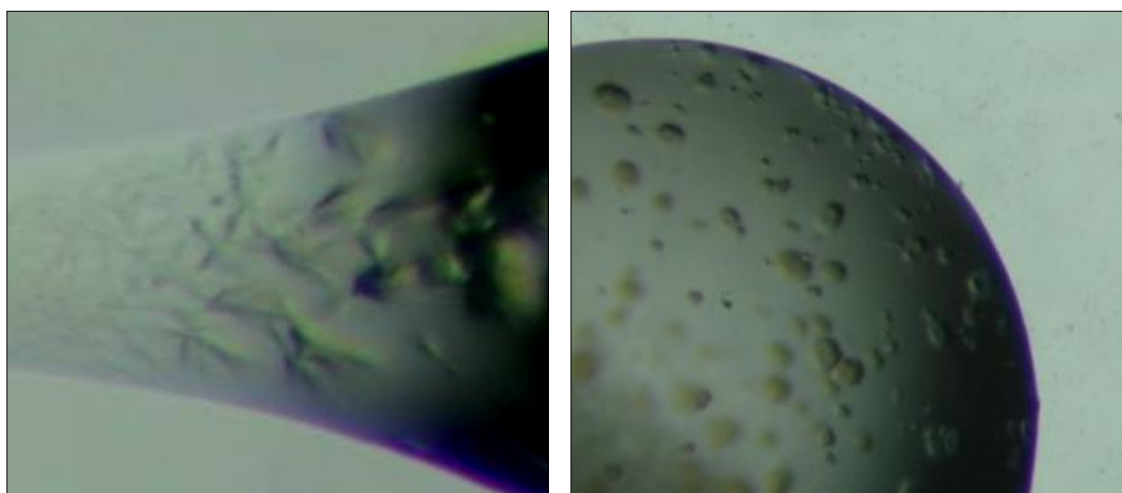


Figure 5.4) USP11/76-286 (9mg/ml) crystals.

Crystals grew into star shaped bundle of needle rods after 7 days at 18°C.

Therefore, in the next step we repeated the crystallization screening using purified USP11/76-286 that was concentrated to 20 mg/ml. We observed crystals shaped in hexagonal rods in a number of crystallization conditions. From the original screening many conditions allowed the protein fragments to form hexagonal rod crystals. These conditions were chosen and used to optimize the crystal-growth conditions; we varied pH, precipitant and buffer concentration, and used an additive screen (Hampton research), to optimize crystallization conditions (Figure 5.5).

The best crystals grew at 18 °C in hanging drops the presence of 13 % (w/v) polyethylene glycol (PEG)-8,000, 0.2 M MgCl₂ and 0.1 M Tris-HCl pH 6.5, within 72 h. For cryoprotection, the crystals were soaked in solution in which crystals were formed, supplemented with PEG-8,000 and glycerol (to a final concentration of 20 % and 15 %, respectively) and snap-frozen by immersion in liquid nitrogen. In total 134 crystals were taken to the beam line. They typically diffracted with a resolution ranging from 4.8 to 8 Å and the best crystal diffracted at 3.6 Å resolution. Data statistics of the crystal used for data collection, calculated by Dr. Maertens and Prof. Cherepanov, are summarized in Table 5.2. The space group was assigned to P6₂22 with unit cell parameters of $a = b = 81.5$ Å, $c = 320.9$ Å (Figure 5.6).

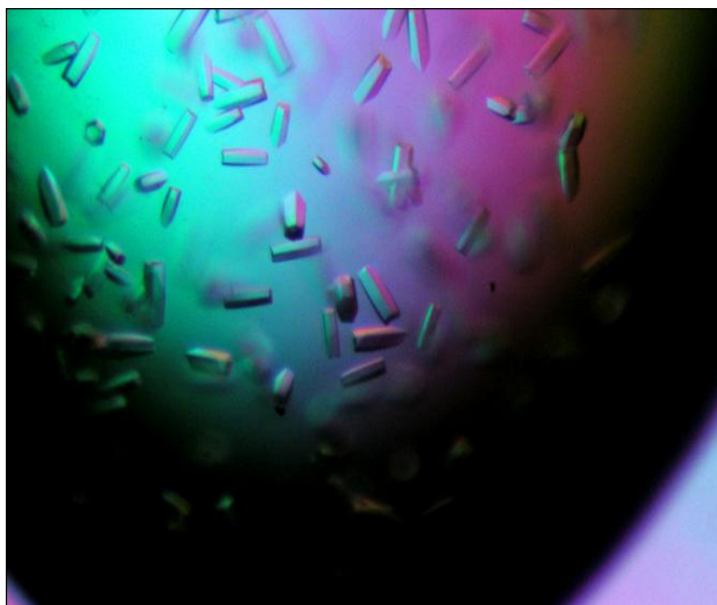


Figure 5.5) USP11/76-286 crystals in the form of a hexagonal rods grown in different conditions using 20 mg/ml protein (eg. 12% PEG8K, 0.2M MgCl₂, 0.1M Tris pH 7.7; or 20% PEG3350, 0.2M Na₂SO₄, 0.1M BTP pH 7.5).

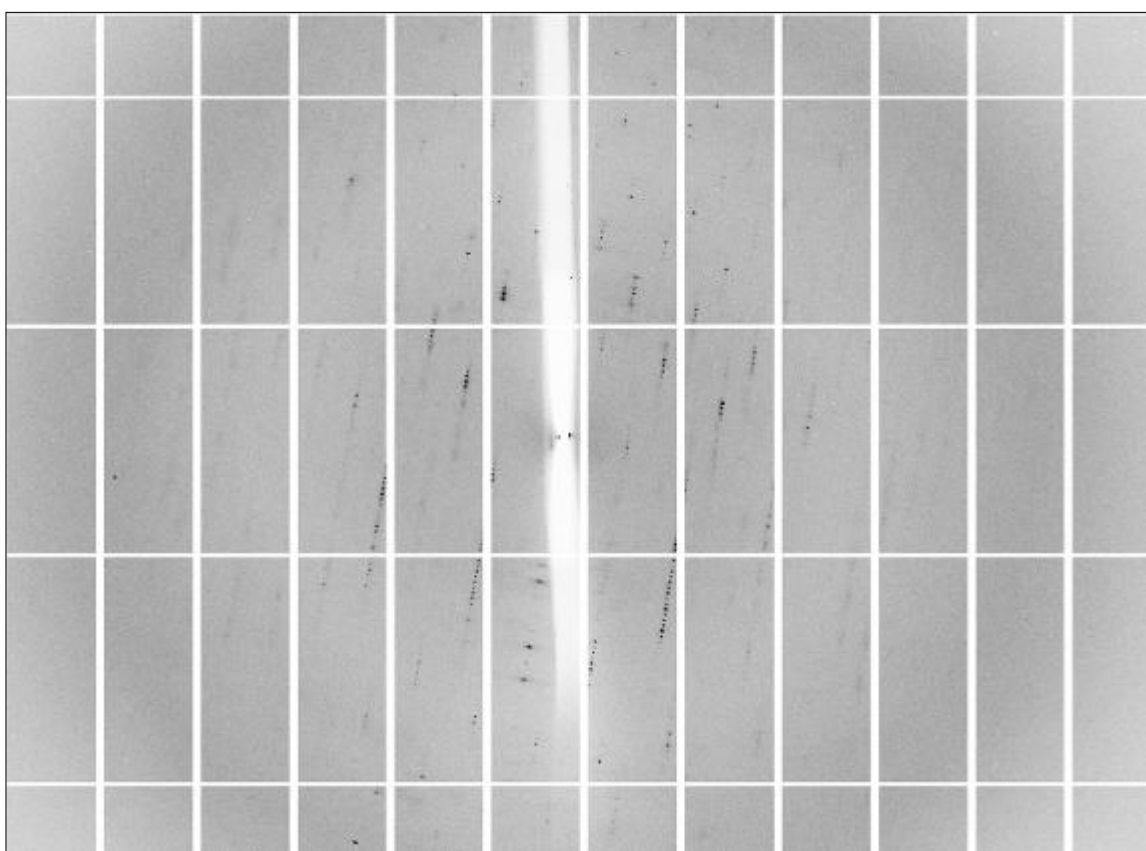


Figure 5.6) An image of diffraction pattern of a USP11/76-286 crystal.

Data collection		Refinement	
Space group	P6 ₂ 22	No. reflections:	
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	82.49, 82.49, 321.36	work	7361
Resolution (Å)	3.60	free	630
R _{merge} (%)	14.1 (176.4)	R _{work} /R _{free} (%)	36.25/40.97
< <i>I</i> / σ (<i>I</i>)>	6.2 (1.1)	No. atoms:	
Completeness (%)	98.4 (99.9)	protein	2524
Redundancy	4.5	Average B (Å ²):	
		protein	74.39
		R.m.s. deviations:	
		bond lengths (Å)	0.005
		bond angles (°)	1.151
		Ramachandran plot (%)	
		favored	95.89
		outliers	1

Table 5.2) X-ray data-collection and refinement statistics. Values in brackets are for the highest resolution shell.

5.1.3 Expression and purification of three mutant forms of USP11/76-286

Amino acids with long flexible side chains might interfere with the packing of the protein into the crystal. Substitution of flexible surface exposed amino acids for Ala residues is known as surface entropy reduction approach. In an attempt to obtain crystal forms of USP11/76-286 diffracting to a higher resolution we used Surface Entropy Reduction prediction (SERp) server version 1.20 (Goldschmidt et al, 2007) to find suitable residues in this construct for mutagenesis. Obtaining the protein structure with higher resolution will help up us to solve the structure with improved details. Subsequently we can compare

the USP11 DU structure with other solved DU structures with enhanced analytical outcome.

At three sites in USP11/76-286, two flexible amino acids are present in close proximity; Lys and Glu. Mutations were introduced in the fragment at each of these sites, separately, to replace Lys and Glu with a small amino acid, Ala. Constructs were cloned encoding for either His₆-USP11/76-286[E106A/K107A], His₆-USP11/76-286[K146A/E147A] or His₆-USP11/76-286[K278A/K279A] and transformed into *E. coli* PC2 cells. Six L LB cultures were induced at 25 °C for 4 h. Cells were harvested and the sonicated lysate was cleared from debris by centrifugation. The His₆-tagged proteins were purified over a CL-Nickel ChroMatrix (Figure 5.7a, 5.8a, 5.9a). The His₆-tag was removed by human rhinovirus 3C protease cleavage overnight at 4°C. The cleaved proteins were further purified by anion exchange chromatography (Figure 5.7b, 5.8b, 5.9b), followed by size exclusion chromatography (Figure 5.7c, 5.8c, 5.9c).

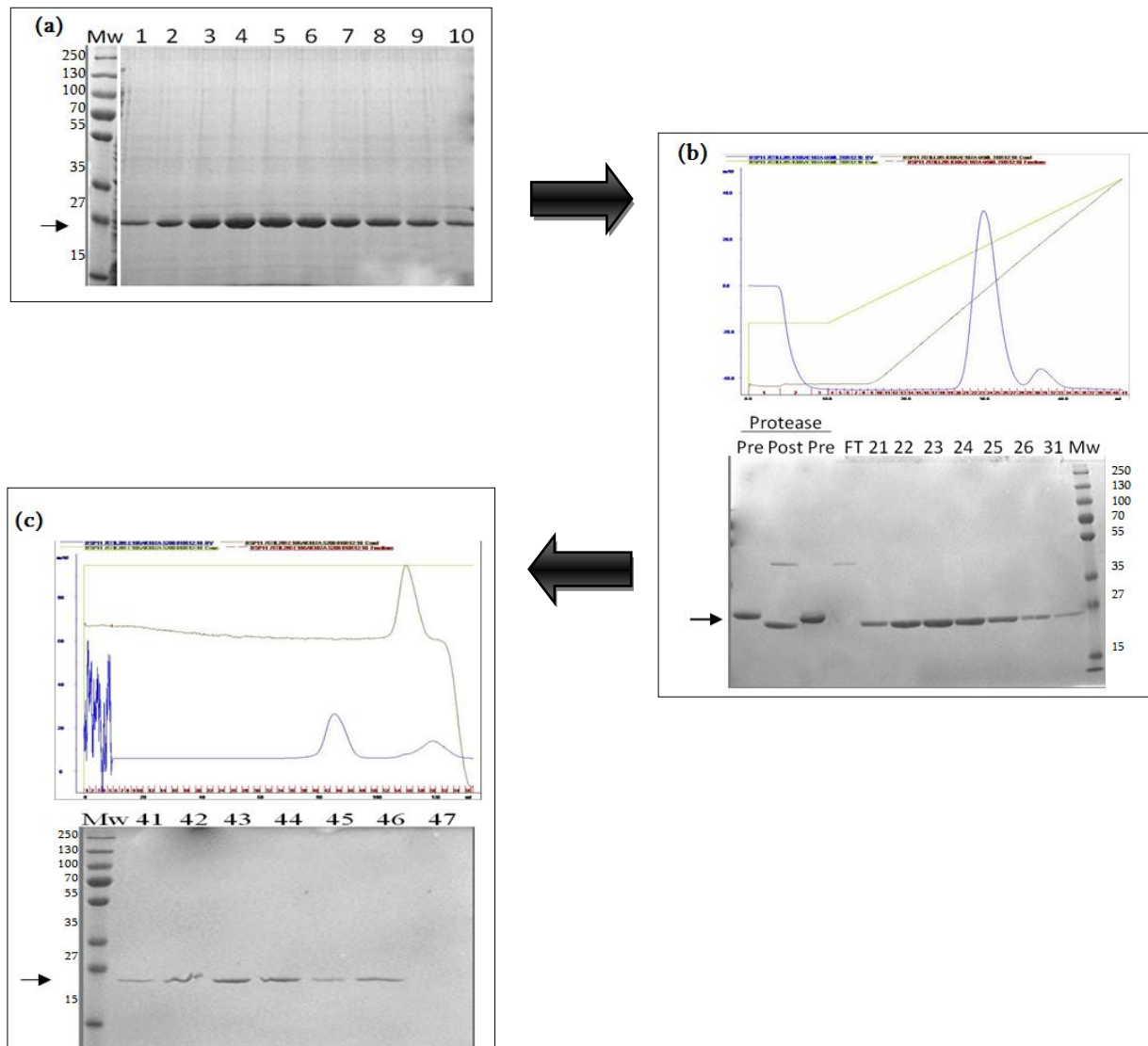


Figure 5.7) His₆-USP11/76-286[E106A/K107A] Protein purification steps.

(a) SDS-PAGE of His₆-USP11/76-286 CL-Nickel ChroMatrix affinity purification. Arrowhead indicates migration position of the tagged protein. (b) Chromatogram showing the purification on the anion exchange column (upper panel) of USP11/76-286 after cleaving the His₆-tag by protein protease human rhinovirus 3C. The protein was eluted with a linear salt gradient, from 15% B to 100% B. The SDS-PAGE of the cleaved and un-cleaved protein shows that the protease activity was completed. The eluted protein fractions were subjected to SDS-PAGE to identify the peak of the protein. (c) Purification of the fragment was completed by size exclusion chromatography. The protein was eluted with 20% B. The eluted fractions were separated on 11% SDS-PAGE gel and the peak fractions were pooled together and concentrated to 6 mg/ml. Arrowhead indicates migration position of the untagged protein in (b) and (c). The X and Y axes show the UB

absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used.

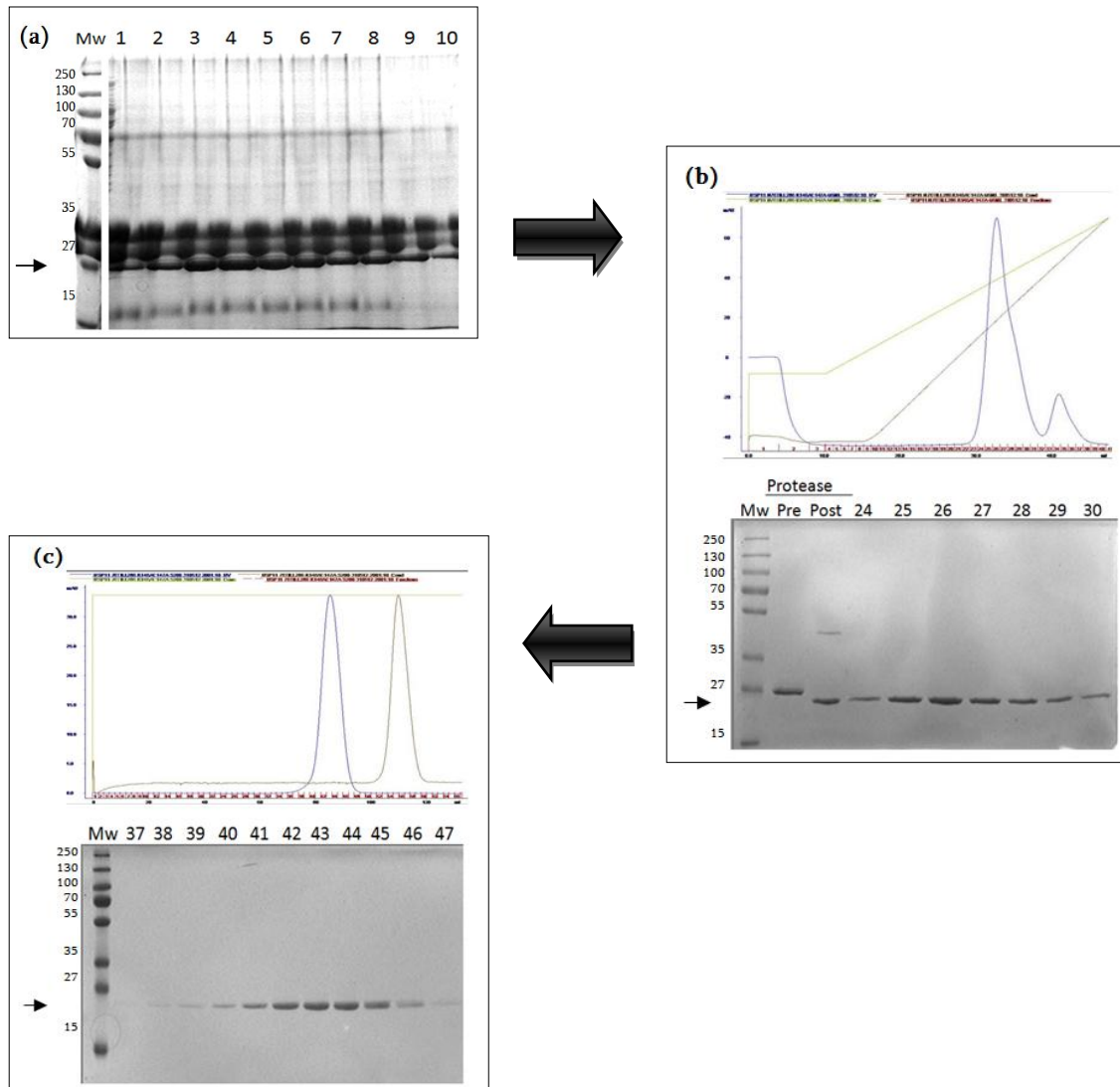


Figure 5.8) His₆-USP11/76-286[K146A/E147A] Protein purification steps.

(a) SDS-PAGE of His₆-USP11/76-286 CL-Nickel ChroMatrix affinity purification. Arrowhead indicates migration position of the tagged protein. (b) Chromatogram showing the purification on the anion exchange column (upper panel) of USP11/76-286 after cleaving the His₆-tag by protein protease human rhinovirus 3C. The protein was eluted with a linear salt gradient, from 15% B to 100% B. The SDS-PAGE of the cleaved and un-cleaved protein shows that the protease activity was completed. The eluted protein fractions were subjected to SDS-PAGE to identify the peak of the protein. (c) Purification of the fragment was completed by size exclusion chromatography. The protein was eluted with 20% B. The eluted fractions were separated on 11% SDS-PAGE gel and the peak fractions were pooled together and concentrated to 5 mg/ml. Arrowhead indicates migration position of the untagged protein in (b) and (c). The X and Y axes show the UB absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes

UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used.

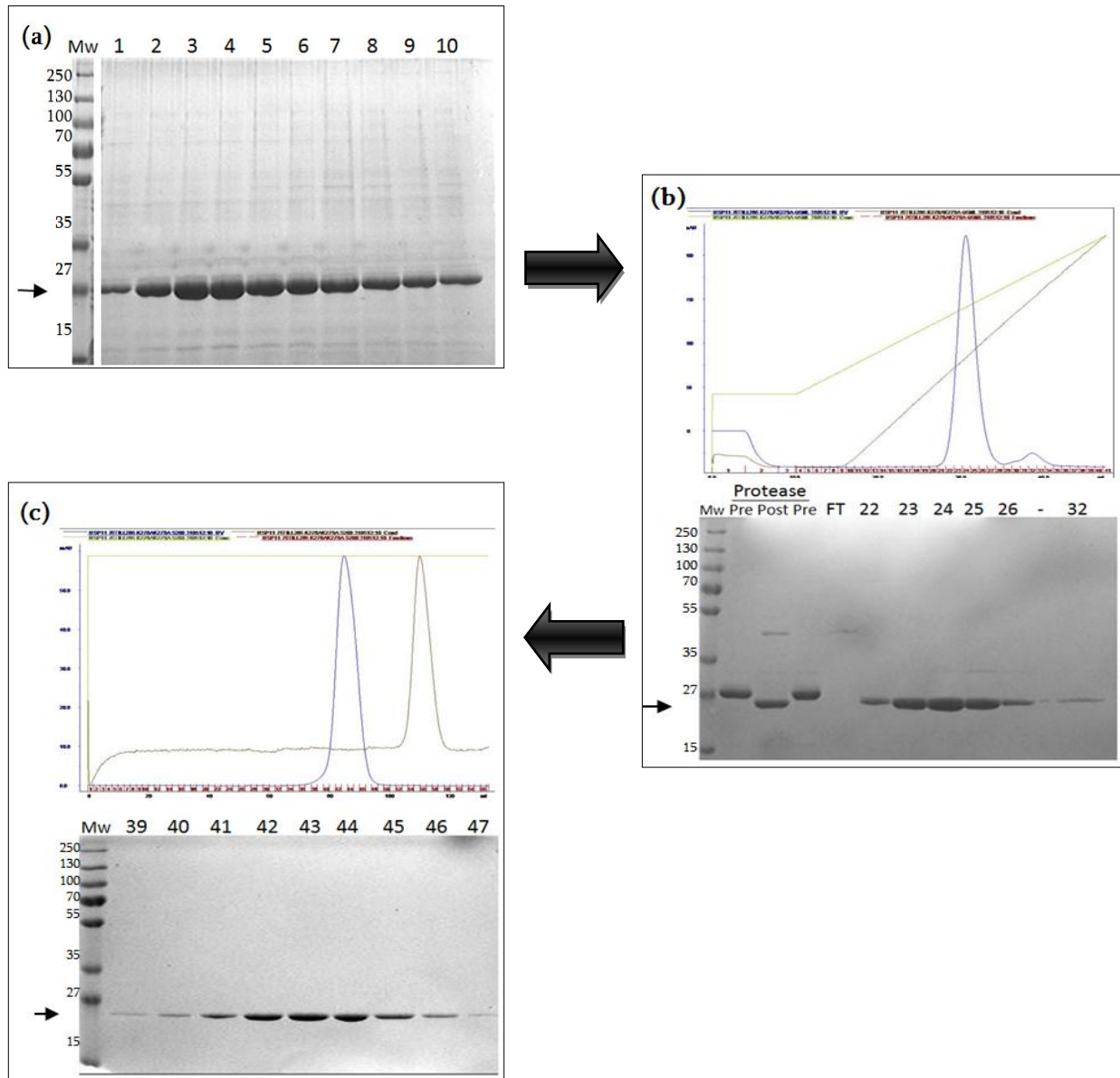


Figure 5.9) His₆-USP11/76-286[K278AK279A] Protein purification steps.

(a) SDS-PAGE of His₆-USP11/76-286 CL-Nickel ChroMatrix affinity purification. (b) Chromatogram showing the purification on the anion exchange column (upper panel) of USP11/76-286 after cleaving the His₆-tag by protein protease human rhinovirus 3C. The protein was eluted with a linear salt gradient, from 15% B to 100% B. The SDS-PAGE of the cleaved and un-cleaved protein shows that the protease activity was completed. The eluted protein fractions were subjected to SDS-PAGE to identify the peak of the protein. (c) Purification of the fragment was completed by size exclusion chromatography. The protein was eluted with 20% B. The eluted fractions were applied to a SDS-PAGE gel and the peak fractions were pooled together and concentrated to 8.5 mg/ml. Arrowhead indicates migration position of the protein. The X and Y

axes show the UB absorption at 280 nm and the number of the eluted fraction, respectively. The blue line denotes UV absorption at 280 nm, the brown line reflects the conductivity of the sample. The green line indicates the percentage of buffer B used.

5.1.4 Crystallization of USP11/76-286[K278A/K279A]

Among the three USP11/76-286 mutants, USP11/76-286[K278A/K279A] was expressed and purified most successfully. USP11/76-286[K278A/K279A] could be concentrated up to 8.5 mg/ml which was used in crystal screening; mutants USP11/76-286[E106A/K107A] and USP11/76-286[K146A/E147A] were less soluble. USP11/76-286[K278A/K279A] was used to screen for an intact crystal form in 11 standard commercially available screening conditions (1:1 ratio), and the crystallization plates were incubated at 18°C. Crystallization was not observed in any of the conditions.

5.1.5 Analysis of the USP11/76-286 structure

The structure of USP11/76-286 was solved by molecular replacement using Phaser (McCoy *et al*, 2007) and refined in Phenix (Adams *et al*, 2010) , by Prof. Cherepanov and Dr. Maertens. The electron density map was visualized using Coot software (Emsley *et al*, 2010) and the structure was analysed with PyMol (<http://www.pymol.org>) (Figure 5.10a), with Dr. Maertens supervision. The USP11/76-286 structure contains two molecules per crystal unit. We used the Protein Interfaces, Surfaces and Assemblies service (PISA) (Krissinel and Henrick, 2007) to calculate the likelihood of the observed dimers in the crystals to be biologically significant. PISA uses physical-chemical models of protein-protein interactions to identify chemically stable complexes in crystal packing. Based on PISA, interface scored 0.000 in Complex Formation Significance Score (CSS) for USP11/76-286. Therefore, USP11/76-286 fragment, unlike the USP4/DU, is not predicted

to form functional dimers. This is consistent with elution of the protein construct from gel filtration columns.

Crystals belonged to the space-group P6₂22. The structure was solved by molecular replacement using the coordinates from Protein Data Bank entry 3JYU (Bacik *et al*, 2009) (Figure 5.10). Indeed, both the DUSP and UBL domains could be identified in our USP11/76-286 crystals using the coordinates of the homologous mouse USP4/NTD structure. The DU finger which links the DUSP and UBL domains in USP15 and USP4 (Harper *et al*. 2011; Bacik *et al*, 2009) could not be assigned in our structures due to the relatively low resolution of our diffraction data. The electron density map of USP11/UBL1 is shown in Figure 5.11, drawn at 1 σ .

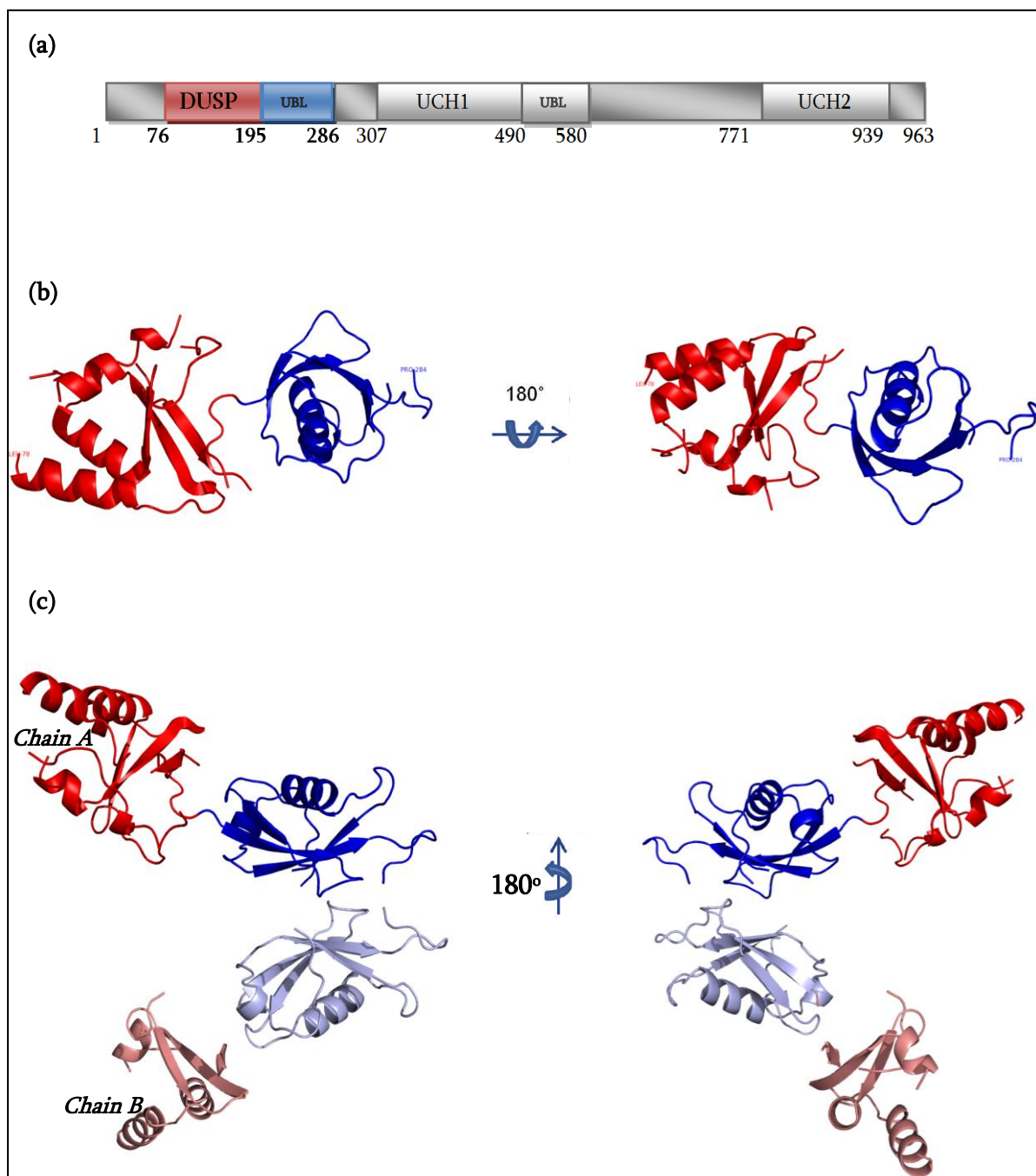


Figure 5.10) USP11 N-terminal domain structure.

(a) Schematic representation of USP11 highlighting the overall predicted domain organization (using the protein homology search tool SMART) and the location of catalytic residues (UCH1 and UCH2). DUSP and UBL1 domains are shown in red and blue respectively. (b) USP11/76-286 DUSP (red) and Ubl1 (blue) domains in cartoon representation. The DU finger β -hairpin structure is not defined because of low resolution. (c) Two parallel dimeric arrangements of the protein present in the crystal packing unit. Chain A is shown in bright red (DUSP) and blue (UBL1) and chain B is shown in faded red (DUSP) and blue (UBL1).

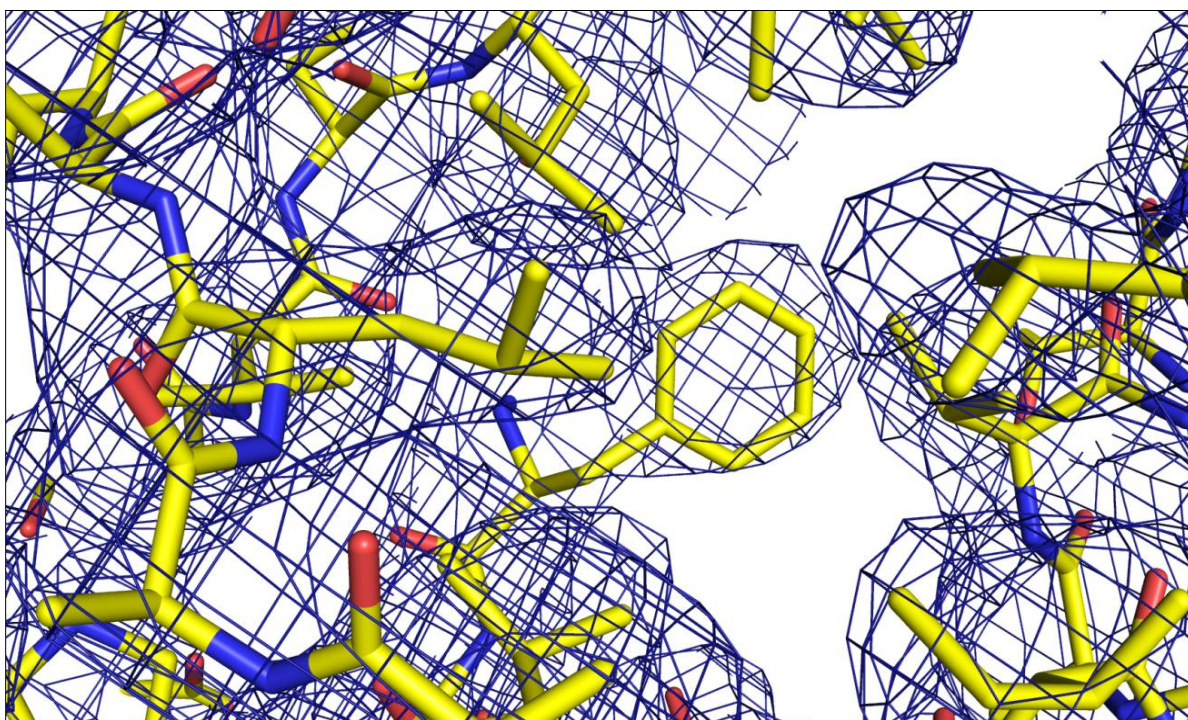


Figure 5.11) Close-up of region in USP11/UBL1 with electron density map drawn at 1 σ .

The cartoon structure of USP11/DU was aligned to the homologous region of the USP4 structure (Figure 5.12). The alignment showed that overall structure of the DUSP-UBL region of USP11 is organized in a similar way to that of USP11. However, there are slight adjustments in relative orientations of the DUSP and UBL domains.



Figure 5.12) Cartoon structure of USP11 N-terminal aligned with USP4 N-terminal domains.

The USP11/NTD is shown in blue and the USP4/NTD is shown in red. DUSP domain of USP4 is shifted away from the USP11; however the Ubl1 domains are more closely aligned.

5.2 Discussion

The crystal structure of USP4/DU and USP15/DU with a resolution of 2.37 Å and 1.50 Å, respectively, has been published (Bacik *et al*, 2009; Harper *et al*, 2011). It was predicted that USP11/DU would have a different configuration from USP15 and USP4, especially at its DU finger (Harper *et al*, 2011). In this project we aimed to solve the crystal structure of a homologous USP11/DU fragment. Expression and purification of USP11/1-286 was difficult since the protein was expressed at low levels, and large amounts of pure protein needed for crystallography were impossible to obtain. The first 76 amino acids, which have no predicted functional or structural significance, were removed from the fragment. It was shown that USP11/76-286 can be produced and purified at high levels and that this protein fragment was highly soluble.

The proteins crystallized as hexagonal rods. The structure of USP11/76-286 was determined by X-ray diffraction analysis and refined to a 3.6 Å resolution. USP11/76-286 and mouse USP4/8-227 share 48% sequence conservation, of which 35.7% of the residues are identical. The coordinates from the PDB entry of mouse USP4/NTD (3JYU) (Bacik *et al*, 2009), was thus used to solve the structure by molecular replacement.

In each crystal unit, two proteins were present; chain A and B. Interestingly, more residues are missing from chain B in electron density maps than chain A, suggesting a more flexible packing of this protein chain within the crystal. The average B factors for chain A and B are 57.5 and 94.8, respectively (Baverage, CCP4 program suite 6.3.0). The region connecting USP11 DUSP and UBL domains could not be built due to low

resolution of X-ray diffraction data. However, phased electron density suggested that it likely adopts a structure similar to that of the DU finger observed in USP4 (Figure 5.12). Our data provide an outline to understand the structure and function of the DU subfamily.

With the aim of increasing the resolution of our X ray data, mutations were introduced that were predicted to have an impact on crystal packing. However, these mutant proteins were not able to crystallize under the conditions tested. After determining the crystal structure of WT USP11/76-286 we were able to investigate the reasons behind lack of crystal formation. It was revealed that K287 mutation into A could potentially result in destabilizing a loop located at the interface of two protein chain in the crystal unit (Figure 5.13).

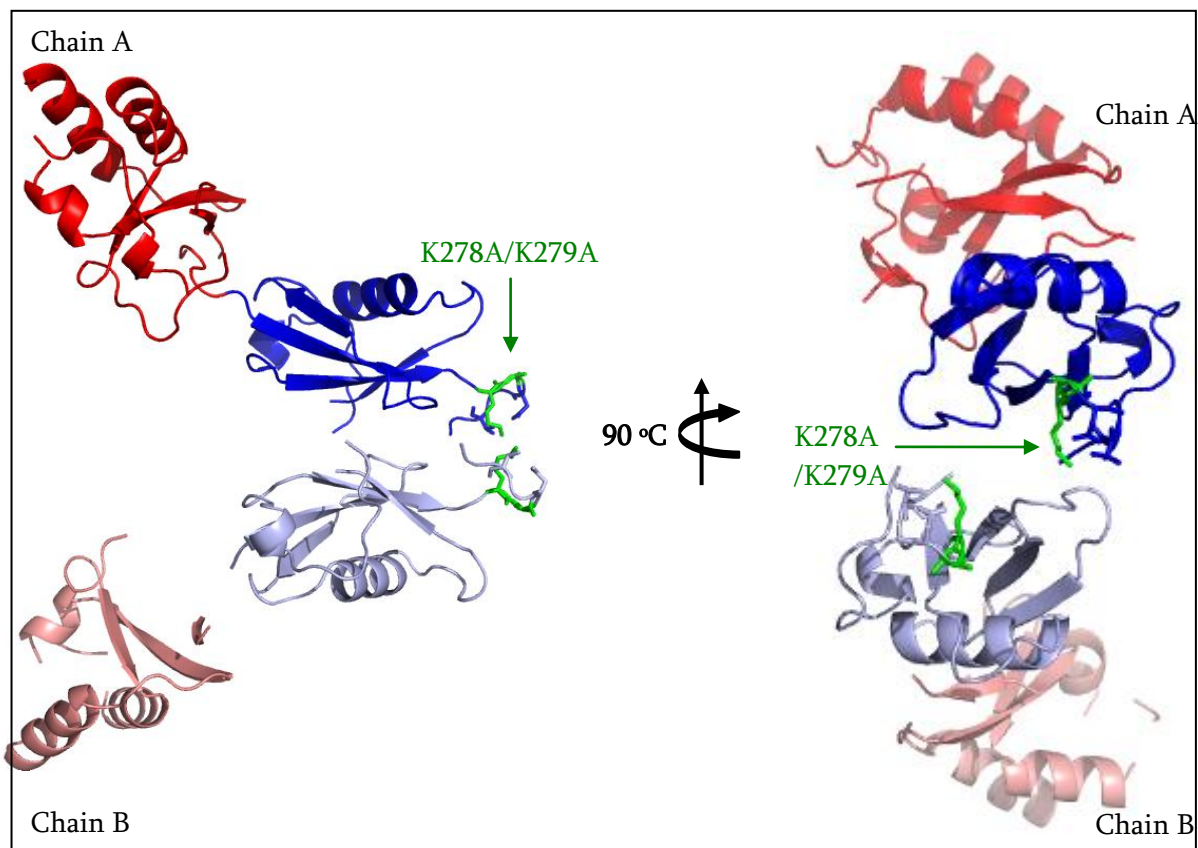


Figure 5.13) Position of K278/K279 mutations in the USP11/76-286 crystal structure.

The cartoon structure of the protein, on the left, is oriented in the same way as Figure 5.10c. The stick structure of the point mutations is coloured green on both chains. The stick structure of residues D280 and T282 are also shown. The side chain of K278 is likely to interact with the side chains of D280 (distance 3.2 Å) and T282 (distance 2.4 Å) via hydrogen bonds. K278 mutation into A would take away these side chains, thus, destabilizes the loop which could obstruct the crystal packing.

Before we obtained a crystal that diffracted at 3.6 Å resolution, we used the PHYRE prediction software (PHYRE Protein Fold Recognition Server version 2.0) to determine whether some flexible residues could be removed from our construct. We designed a truncated fragment of USP11 NTD; USP11/76-278 in which the C-terminal eight residues were removed (data not shown). Unfortunately, this protein fragment did not crystallize. In our crystal structure of USP11/76-286, the C-terminus of the fragment makes important crystal contacts, explaining why further truncations could not form the same crystals.

Conclusions and future work

By sequential chromatin immunoprecipitation, Maertens and colleagues showed that various distinct PRC1 complexes bind simultaneously and interdependently to the same region of the *INK4a/ARF* locus which cause de-repression of p16^{INK4a} and, hence, senescence-associated proliferative arrest (Maertens *et al*, 2009). Further studies showed that USP11 co-purifies with MEL18 and BMI1 complexes from mammalian cells, binds to several PRC1 components and regulates the stability of the E3 ligase subunit of PRC1, preventing their proteasomal degradation. Hence, shRNA-mediated knockdown of USP7 and USP11 causes de-repression of p16^{INK4a} and dislodgement of PRC1 components from the locus, through the increased turnover of chromatin-bound MEL18 and BMI1 (Maertens *et al*, 2010). GST-USP11 can pull down CBX8, mouse Mel18, mBmi1, and mRing1b produced in a cell-free system (Maertens *et al*, 2010).

Oncogene induced senescence is observed in premalignant lesions. Tumour progression requires bypass or escape from senescence (Ruas and Peters, 1998). The PcG proteins are often over-expressed in tumours and are thought to contribute to unlimited cell proliferation typical of cancer cells (Schuring *et al*, 2010; Klauke *et al*, 2011). Reinstating senescence in tumour cells is a desirable outcome in cancer therapy. Further characterisation of the interaction between USP11 and the PRC1 components and, thus, possibly influencing the stability of PcG proteins in the cell with the aim of reinstating senescence, is a first step of a long-term goal to develop an anti-cancer drug.

In this project we aimed to map the interaction between USP11 and PRC1 components. We used two systems to investigate the interaction; yeast two-hybrid and *in vitro* pull down. We did not detect strong interactions between USP11 fragments and individual PRC1 components. In future studies we suggest to use USP11/1-286 in the *in vitro* pull down studies; because residues 1-75 may play an important stabilizing part in the interaction. One of the reasons that we were not able to detect a high affinity USP11-PRC1 interaction *in vitro* might be the lack of post-translational modification. Another likely reason for the apparent lack of interaction between USP11 and PRC1 proteins is that a complete PRC1 complex may be required for the interaction. In future studies, proteins could be expressed in insect cells, where post-translational modifications take place and expression levels could be higher.

In 2006 it was reported that Ring1b and Bmi1 interact directly via their RING domains and the crystal structure of regions involved in the interaction was solved (Buchwald *et al*, 2006). Gao *et al*. identified six distinct PRC1 complexes with different genomic localizations, suggesting that distinct PRC1 subunit dependent recruitment pathways may be at work. It was also shown that RYBP, BMI1 and RING2 can ubiquitinate H2A (Gao *et al*, 2012), independent of H3K27me3 activity of PRC2 (Tavares *et al*, 2012). In this study, we aimed to map the interaction between RING2, BMI1 and RYBP. We tried to purify (or co-purify) these proteins from bacteria culture in high quantity to use in *in vitro* pull down; however, we faced a challenge in identifying the best expression, extraction and purification methods and failed to obtain these protein in high levels. After mapping the interaction regions the ultimate goal was to determine structure of the protein-protein

complexes. This remains a goal of the laboratory and experiments are being carried out to co-express multisubunit PRC1 subcomplexes in insect cells in an attempt to improve the expression levels.

To date, the crystal structure of the DUSP-UBL domains of two proteins closely related to USP11, namely USP4 and USP15, have been solved. In this project we identified a fragment of the amino terminal domain of USP11 that encompasses the DUSP-UBL domains and that can be produced and purified in large quantities: USP11/76-286. We were able to crystallize this fragment under several conditions, performed several rounds of crystallization optimization and screened 134 crystals at synchrotron beam lines. The best crystal diffracted at 3.6-Å resolution, which allowed us to determine the structure of the N-terminal fragment of USP11. Alignment of the USP11 DUSP-UBL with USP4 DUSP-UBL shows that the tertiary structure of these domains is similar. Unfortunately, we could not assign the amino acid residues in the DU finger that links these two domains due to the relatively low resolution of our structure. Our laboratory is currently carrying out experiments to express full length USP11 in insect cells. If USP11 expression is high and the purified protein is intact, we could use the full length protein to form crystals and solve the structure.

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Appendixes

Supplementary Table S1. List of plasmid constructs designed for use in yeast two-hybrid screening. Target DNA was cut from the original plasmids and pasted into pGADT7M, modified version of pGADT7 with multiple cloning sites (Dr. G. Maertens, unpublished results).

Plasmids Construct	Restriction Digestion	Original plasmid
pGADT7M-USP11/ Δ DUSP	AgeI / EcoR1	pQHA- USP11/ Δ DUSP
pGADT7M-USP11/ Δ DUSP Δ DUF	AgeI / EcoR1	pQHA-USP11/ Δ DUSP Δ DUF
pGADT7M-USP11/ Δ UCH1	AgeI / EcoR1	pQHA-USP11/ Δ UCH1
pGADT7M-USP11/ Δ UCH2	AgeI / EcoR1	pQHA-USP11/ Δ UCH2
pGADT7M-USP11/ Δ UCH1&UCH2	AgeI / EcoR1	pQHA-USP11/ Δ UCH1&UCH2

Supplementary Table S2. List of primers designed for plasmid constructs used for *in vitro* pull down studies. The verified PCR products were cloned into pGEX-6P-3, pGEX-6P-1 (GE Healthcare) or pET28a-SUMO (kindly provided by Andre LB Ambrosio, Laboratório Nacional de Biociências - LNBio, CNPEM, Brazil).

Plasmids	Restriction Digestion	Template	Primer (sense)	Primer (anti-sense)
pGEX-6p-3- BMI1/1-109	BamH1 XhoI	pQBMI1-Flag (Maertens <i>et al</i> , 2009)	CCGGCAATTGAT GCATCGAACAAC GAGAATC	CCGGCTCGAGTCAGC CATTGGCAGCATCAG C
pGEX-6p-3-BMI1/1-300	BamH1 SalI	pQBMI1-Flag (Maertens <i>et al</i> , 2009)	CCGGCAATTGAT GCATCGAACAAC GAGAATC	CTGAGTCGACTCAGG GGCTGTTGCTGG
pCFD-H6P-Duet1-BMI1	BglI XhoI	pQBMI1-Flag (Maertens <i>et al</i> , 2009)	CCGGCAATTGAT GCATCGAACAAC GAGAATC	CCGGCTCGAGTCAAC CAGAAGAAGTTGCT GATGACC
pGEX-6p-3-RING2/1-331	BamH1 XhoI	pGM-Flag-RING2 (Maertens <i>et al</i> , 2010)	CCGGGGATCCAT GTCTCAGGCTGT GCAGACAAACGG	CCGGCTCGAGTCAAG GTGCGTAATAAAGTT CCATGG
pGEX-6p-3-RING2/1-159	BamH1 XhoI	pGM-Flag-RING2 (Maertens <i>et al</i> , 2010)	CCGGGGATCCAT GTCTCAGGCTGT GCAGACAAACGG	CCGGCTCGAGTCAGC CTCGCTGCAGTCTGT TC
pGEX-6p-1- RYBP	SalI EcoR1	Hela cDNA Library	GGCCGAATTCAT GACCATGGGCGA CAAGAAG	CCGGGTCGACTCAGA AAGATTCATCATTGA CTGCTG

Supplementary Table S3. List of primers designed for plasmid constructs used for *in vitro* pull down studies. Target DNS was cut from the original plasmids with appropriate restriction digestion enzymes. .

Plasmids	Restriction Digestion	Original Plasmid
pET-28a-SUMO-BMI1/1-300	BamH1 / SalI	pGEX-6p-3-BMI1/1-300
pET-28a-SUMO-BMI1	BamH1 / XhoI	pGEX-6p-3-BMI1
pET-28a-SUMO-RING2/1-331	BamH1 / XhoI	pGEX-6p-3-RING2/1-331
pCDF-H6P-RYBP/BMI1	BamH1 / XhoI	pCDF-H6P-RYBP pCDF-H6P-Duet1-BMI1

Supplementary Table S4. List of primers designed for plasmid constructs used to express USP11 fragments. The verified PCR products were cloned into pCDF-H6P-PP2ACA (Maertens G. unpublished results).

Plasmids	Restriction Digestion	Template	Primer (sense)	Primer (anti-sense)
pCDF-H6P-Duet1-USP11/1-286	SalI EcoR1	pQHA-USP11 ^{wt} (Maertens <i>et al</i> , 2010)	GGCCGAATTCATG GCAGTAGCCCCGC G	GGCCGTCGACTCA TGCGCTGGGCCAA GTGCC
pCDF-H6P-Duet1-USP11/76-286	SalI EcoR1	pQHA-USP11 ^{wt} (Maertens <i>et al</i> , 2010)	GGCCGAATTCCCA GGCCTGGACAGCC	GGCCGTCGACTCA TGCGCTGGGCCAA GTGCC
pCDF-H6P-Duet1-USP11/76-286[E106AK107A]	SalI EcoR1	pCDF-H6P-Duet1-USP11/76-286	CGAAAGCTGGTTC CTTGTGGCGGCGC ACTGGTATAAGCA GTGG	CCACTGCTTATACC AGTGCGCCGCCAC AAGGAACCAGCTT TCG
pCDF-H6P-Duet1-USP11/76-286[K146AE147A]	SalI EcoR1	pCDF-H6P-Duet1-USP11/76-286	GAGATAAACTGGC GCCTCGCGGCGGG ACTGGTGGAAGGC GAGG	CCTCGCCTTCCACC AGTCCCGCCGCGA GGCGCCAGTTTATC TC
pCDF-H6P-Duet1-USP11/76-286[K278AK279A]	SalI EcoR1	pCDF-H6P-Duet1-USP11/76-286	GATCATCATGGAG ACCCGCGCGGCAG ATGGCACTTGGCC CAGC	GCTGGGCCAAGTG CCATCTGCCGCGCG GGTCTCCATGATG ATC