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KDM2B recruitment of the Polycomb group complex, PRC1.1, requires cooperation between PCGF1 and BCORL1

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SUMMARY

KDM2B recruits H2A-ubiquitinating activity of a non-canonical Polycomb Repression Complex 1 (PRC1.1) to CpG islands, facilitating gene repression. We investigated the molecular basis of recruitment using in vitro assembly assays to identify minimal components, subcomplexes and domains required for recruitment. A minimal four-component PRC1.1 complex can be assembled by combining two separately isolated subcomplexes: the DNA binding KDM2B/SKP1 heterodimer and the heterodimer of BCORL1 and the core PRC1.1 component PCGF1. The crystal structure of the KDM2B/SKP1/BCORL1/PCGF1 complex illustrates the crucial role played by the PCGF1/BCORL1 heterodimer. The BCORL1 PUFD domain positions residues preceding the RAWUL domain of PCGF1 to create an extended interface for interaction with KDM2B, which is unique to the PCGF1-containing PRC1.1 complex. The structure also suggests how KDM2B might simultaneously function in PRC1.1 and an SCF ubiquitin ligase complex and the possible molecular consequences of BCOR PUFD internal tandem duplications found in pediatric kidney and brain tumors.

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AUTHOR CONTRIBUTIONS

S.J.W. and C.A.K. conceptualized and designed the methodology with help from M.D.G. and V.J.B. S.J.W., A.B.T., D.R.N., D.J.H., A.K.R., J.A.A., O.J.L. and C.A.K. carried out the investigation. B.D. and P.J.H. provided resources. B.D. also performed formal analysis of the AUC data. S.J.W., M.D.G., V.J.B and C.A.K. wrote the manuscript. C.A.K. supervised the entire study with help from M.D.G. and V.J.B.

The authors declare no conflicts of interest.

INTRODUCTION

KDM2B (also called FBXL10, NDY1, and JHDM1B) is a JmjC domain containing H3K36me2 demethylase that plays diverse roles in cancer. It has been characterized as a candidate tumor suppressor gene in murine studies where lymphomas stemmed from the homozygous disruption of *Kdm2b* (Suzuki, et al., 2006). Conversely, KDM2B overexpression can also promote tumorigenesis (He, et al., 2011; Kottakis, et al., 2014; Tzatsos, et al., 2013). Furthermore, *KDM2B* mutations that produce a truncated protein have been observed in diffuse large B-cell lymphomas, though the precise tumorigenic role of truncated *KDM2B* remains unclear (Pasqualucci, et al., 2011).

KDM2B can function within a multi-protein assembly that includes members of the PRC1 family of developmental regulatory proteins (Simon and Kingston, 2013). While PRC1 was originally identified in *Drosophila* as having four core proteins (Pc, Ph, Sce and Psc) (Francis, et al., 2001; Shao, et al., 1999), subsequent studies have revealed a variety of deviations for both *Drosophila* (Lagarou, et al., 2008) and mammalian PRC1s (Gao, et al., 2012) whereby functionally distinct, non-canonical versions exist that lack some of the core proteins. A key player in defining the composition of mammalian non-canonical PRC1s is the Psc ortholog of which there are six paralogs in mammals, PCGF1-6 (Gao, et al., 2012). For example, the non-canonical PRC1.1 houses the PRC1 core proteins PCGF1 (also called NSPC1), RING1B/RING1A, the H2A ubiquitin ligase, along with non-Polycomb group (PcG) proteins including KDM2B and BCOR (or its close homolog BCORL1) (Farcas, et al., 2012; Gao, et al., 2012; Gearhart, et al., 2006; Oliviero, et al., 2015).

Central and largely unresolved issues are the molecular and structural bases by which the PRC1 variants are able to bind to specific genomic loci. In *Drosophila*, site specific transcription factors play an important role in recruiting PRCs to Polycomb responsive elements. A recent study revealed a network of sterile alpha motif mediated protein-protein interactions of *Drosophila* PcG proteins results in the recruitment of canonical PRC1 to sites bound by Pleiohomeotic, a protein that houses a specific DNA binding domain (Frey, et al., 2016). In mammalian cells, the contribution of site specific DNA binding factors in targeting of canonical and non-canonical PRC1s has been described in only a limited number of instances (reviewed in (Blackledge, et al., 2015) and (Entrevan, et al., 2016)). A more widespread targeting mechanism for non-canonical PRC1.1 involves KDM2B (Boulard, et al., 2015; Farcas, et al., 2012; He, et al., 2013; Wu, et al., 2013). In embryonic stem cells KDM2B, via its ZF-CxxC domain, binds to non-methylated CpG islands (CGIs) throughout the genome and contributes to stable PRC1.1 recruitment at a subset of CGIs. Once targeted, PRC1.1 functions as a ubiquitin ligase to attach ubiquitin onto lysine 119 of histone H2A (H2AK119ub). Knockdown of KDM2B results in reduced PRC1.1 recruitment and H2A ubiquitylation (Boulard, et al., 2015; Farcas, et al., 2012; He, et al., 2013; Wu, et al., 2013). Additional mechanisms likely contribute to the formation of stable Polycomb chromatin domains (Blackledge, et al., 2015; Entrevan, et al., 2016).

We sought to understand the structural basis of KDM2B recruitment of PRC1.1 components. Using recombinant, bacterially expressed proteins to assemble minimized versions of PRC1.1 allowed precise control of the assembly without the influence of external eukaryotic

factors. This led to the identification of the specific protein regions responsible for the KDM2B/PRC1.1 interaction and the crystal structure determination of the core of PRC1.1. Our studies highlight the important role of the RAWUL domain in selectively forming functionally distinct PRC1 complexes. Additionally, our structure allows modeling of the potential role played by KDM2B in simultaneously functioning within an H2A and protein ubiquitin ligase complexes, and for predicting the molecular consequence of internal tandem duplications (ITDs) that occur within the PUFD domain of BCOR that are associated with pediatric kidney and brain tumors (Roy, et al., 2015; Sturm, et al., 2016; Ueno-Yokohata, et al., 2015).

RESULTS

In vitro assembly of non-canonical PRC1.1 by uniting separately isolated subcomplexes

In ESCs, KDM2B localizes to non-methylated CGIs with or without the recruitment of RING1B and H2A ubiqutinating activity (Boulard, et al., 2015; Farcas, et al., 2012; He, et al., 2013; Wu, et al., 2013). We reasoned that assembly of a complete PRC1.1 complex at a subset of KDM2B targets in vivo may proceed via association of two subcomplexes: one containing the DNA binding activity (KDM2B/SKP1) and the other containing the PRC1.1 H2A ubiquitin ligase activity. To test this idea we attempted to in vitro assemble a five component complex by combining separately isolated KDM2B/SKP1 and RING1B/PCGF1/BCORL1 subcomplexes. The KDM2B Fbox-LRR region (residues 1059–1336, henceforth referred to as KDM2B) (Figure. 1A) was used because PCGF1 affinity purifications fail to enrich KDM2B lacking Fbox-LRR (M.D.G. and V.J.B., unpublished). Additionally, when the Fbox-LRR is deleted from KDM2B, it fails to bind PcG proteins PCGF1 and RING1B (Wu, et al., 2013). The Fbox proteins in the SCF family of ubiquitin ligases bind directly to SKP1 (Zheng, et al., 2002). We were able to isolate bacterially expressed KDM2B only when co-expressed and co-purified with SKP1, which together form a 40 kDa complex. For the H2A ubiquitin ligase activity containing PRC1.1 subcomplex, we isolated an 85 kDa heterotrimeric complex that included full-length RING1B, the region encompassing the conserved RING finger and RAWUL domains of PCGF1 (residues 35–255), and BCORL1 PUFD (1594–1711), the direct binding partner to the PCGF1 RAWUL domain (Junco, et al., 2013) (Figure 1A). We demonstrated the assembly of the 125 kDa RING1B/PCGF1/BCORL1/KDM2B/SKP1 complex using sedimentation velocity analytical ultracentifugation (SV AUC) (Figure 1B). The van Holde-Weischet (vHW) plot of SV AUC experiment shows both RING1B/PCGF1/BCORL1 (~4 s) and KDM2B/SKP1 (~3.4 s) complexes matching what would be expected for a 1:1:1 and a 1:1 stoichiometry, respectively. When combined, the resulting vHW plot shows a larger s-value distribution than either of the individual subcomplexes indicating assembly of the heteropentamer. We conclude that the core of the non-canonical PRC1.1 can be assembled using a mechanism that likely occurs in cells by combining separately isolated and stable KDM2B/SKP1 and PRC1.1 subcomplexes.

Identification of minimum requirements for KDM2B/SKP1 and RING1B/PCGF1/BCORL1 interaction

We next performed a deletion analysis to more precisely map the proteins and regions responsible for the direct interactions required to unite KDM2B/SKP1 and RING1B/

PCGF1/BCORL1. Assembly of the two subcomplexes into a larger complex was again monitored using SV AUC (Figure 2A). By removing RING1B from the PcG complex used in the heteropentamer assembly shown in Figure 1B, we found that RING1B is not required for assembly with KDM2B as just the PCGF1/BCORL1 dimer was sufficient to assemble with KDM2B/SKP1 (Figure 2A, top left panel). Subsequent deletion experiments revealed that the PCGF1 RAWUL domain (167–255) in complex with BCORL1 PUFD domain was unable to assemble with KDM2B/SKP1 (Figure 2A, bottom right panel). By extension, this result also indicates that the BCORL1 PUFD alone is unable to bind KDM2B. A PCGF1 construct that includes 17 residues N-terminal to the RAWUL (150–255), however, was necessary and sufficient for heterotetramer assembly. The equilibrium dissociation constant (K_D), measured using isothermal calorimetry (ITC), between the PCGF1 150–255/BCORL1 PUFD dimer and KDM2B/SKP1 dimer was measured to be 2.8×10^{-6} M (Figure 2B). An important conclusion from these results, as noted above, is that BCORL1 PUFD alone is unable to associate with KDM2B/SKP1. As PCGF1 is unstable in the absence of BCORL1, we were unable to isolate just PCGF1 to test whether PCGF1 alone can bind KDM2B/SKP1. These results also do not eliminate the possibility that PCGF1 residues 150–166 is the minimum region required for KDM2B binding. However, as discussed below, this appears not to be true. Rather, these and subsequent results strongly suggest that the dimer of PCGF1 and BCORL1 is required for binding KDM2B.

Heterotetramer crystal structure reveals PCGF1/BCORL1 dimer is required for KDM2B interaction

The identified minimum protein regions required for heterotetramer assembly (Figure 2B: PCGF1 150–255, BCOR PUFD (1594–1711), KDM2B 1059–1336, and full-length SKP1) were used to crystallize and determine the 2.55 Å structure of this complex (Table S1, Figure 3). The requirement for PCGF1 and BCORL1 dimerization prior to assembly with KDM2B can be explained by the observation that the RAWUL/PUFD dimer interface straddles the KDM2B LRR with residues from both PCGF1 and BCORL1 providing key KDM2B contacts (Figure 3A, Figure S1). Interestingly, an alignment of KDM2B aromatic residues on the edge of alternating LRRs demarcate the landing site for PCGF1/BCORL1 (Figure 3B). Positioning of the RAWUL/PUFD dimer on the KDM2B LRR places the N-terminus of the PCGF1 RAWUL in close proximity to KDM2B thus explaining the need for additional residues N-terminal to the RAWUL for stable interaction. Notably, Tyr 163, a residue preceding the PCGF1 RAWUL, works in concert with BCORL1 PUFD N-terminal residue Phe 1596, through an aromatic ring stacking interaction, to contact KDM2B (Figure 3C). BCORL1 Leu 1665 is another example of the cooperativity between BCORL1 and PCGF1 utilized in binding KDM2B. The previously determined structure of the dimer between the PCGF1 RAWUL and BCORL1 PUFD (Junco, et al., 2013) had shown BCORL1 Leu 1665 inserted into a hydrophobic pocket on PCGF1 RAWUL called the Leu cage. Mutating Leu 1665, however, did not disrupt RAWUL/PUFD dimerization suggesting little contribution to the binding energy of dimerization (Junco, et al., 2013). The heterotetramer structure, however, reveals the role of Leu 1665 is perhaps less to stabilize PCGF1 RAWUL/BCORL1 PUFD dimerization and more so to position the adjacent residue, BCORL1 Gln 1664, in a conformation favorable for contacting KDM2B (Figure 3D). Assembly studies using a Ni^{2+} pulldown assays corroborated the importance of BCORL1

Leu 1665 and other residues observed to mediate assembly (Figure 3E). In sum, the structure supports a hierarchical assembly model where PCGF1 RAWUL/BCORL1 PUFD dimerization is required to associate with KDM2B, which neither individual component can do alone.

In addition to its involvement in the non-canonical PRC1.1 H2A ubiquitin ligase complex, the presence of an Fbox domain within KDM2B raises the possibility that it can also function within a different ubiquitin ligase complex targeting proteins for degradation. CUL1, like KDM2B, binds directly to SKP1 and is a key component of the SCF (SKP1-CULLIN-Fbox) family of ubiquitin ligases (Zheng, et al., 2002). Proteomic studies did not identify CUL1 as part of PRC1.1 (Farcas, et al., 2012; Gao, et al., 2012; Gearhart, et al., 2006). Also, KDM2B co-immunoprecipiation (co-IP) in different cell lines failed to detect CUL1 while successfully detecting Skp2, another Fbox containing protein (Koyama-Nasu, et al., 2007). A recent study, however, did show involvement of KDM2B as part of a SCF ubiquitin ligase complex, able to co-IP with CUL1 and target c-Fos for ubiquitination and subsequent degradation (Han, et al., 2016). Overlaying CUL1/SKP1 structure (Zheng, et al., 2002) over the heterotetramer structure allowed us to assess whether it would be sterically feasible to have both KDM2B and CUL1 simultaneously bind SKP1. This analysis revealed that both CUL1 and KDM2B can easily co-exist on separate binding patches on the surface of SKP1 while avoiding any steric clashes with any other components (Figure S2). Moreover, the LRR of KDM2B and the five-helix repeats of CUL1 are in the same vicinity to possibly co-interact with the substrate. This analysis reveals that it is quite possible for KDM2B to be simultaneously involved in both the PRC1 H2A and SCF ubiquitin ligase activities.

PCGF3 does not associate with KDM2B in vitro

We next wondered if the PCGF1/BCORL1 dimerization required to assemble with KDM2B also extends to PCGF3. Like PCGF1, PCGF3 RAWUL dimerizes with the PUFDs of both BCOR and BCORL1 (Junco, et al., 2013). The ability of PCGF3 to associate with KDM2B, however, is questionable. In 293 cells, immunoprecipitation of PCGF3 yielded signals for KDM2B though they were weaker than those observed for PCGF1 (Gao, et al., 2012). In mouse ESCs, KDM2B purification identified only PCGF1 and not PCGF3 even though PCGF3 is present as indicated by its co-purification with RING1B in the same cells (Farcas, et al., 2012). To determine if PCGF3 could assemble with KDM2B like PCGF1, we performed Ni^{2+} pulldown assays utilizing a region of PCGF3 (132–242) that is equivalent to the region of PCGF1 (150–255) required to bind KDM2B (Figure S1). The assembled components eluted from the Ni^{2+} resin reveal that the three PCGF proteins (PCGF1 RAWUL alone (167–255), PCGF1 150–255 and PCGF3 132–242) were all capable of binding BCORL1 PUFD while only PCGF1 150–255 assembles with KDM2B/SKP1 (Figure 4). The ability of PCGF3 to bind BCORL1 serves as an important internal control as it indicates that PCGF3 is indeed folded and able to perform binding functions. We conclude that PCGF3 is unable to associate with KDM2B utilizing the same binding mode as PCGF1. The possibility of PCGF3 binding KDM2B via an alternative binding mode awaits further investigation.

DISCUSSION

We present the molecular basis of the assembly of PRC1.1 responsible for the recruitment of the H2A ubiquitin ligase activity to KDM2B bound CGIs. The assembly and structural studies suggest a hierarchical assembly whereby PCGF1 must interact with the PUFD domain of BCORL1 and can exist as its own subcomplex able to associate with KDM2B. These results highlight the important role the PCGF RAWULs play in the assembly of the functionally distinct PRC1 family of complexes. Not only do the RAWULs bind selectively to different proteins (Junco, et al., 2013), it appears that dimerization with their primary binding partner can create additional binding surfaces to provide an additional layer of selectivity in binding other proteins to ultimately form distinct PRC1s.

The studies presented here are focused on the PUFD of BCORL1. Given the similarity between BCOR and BCORL1 in their domain structures (Figure 1) and PUFD sequences (Figure S1) and structure (Junco, et al., 2013), our findings serve as a guide to extend our observations to BCOR. For example, the results presented here can be used to help predict the consequence of the BCOR PUFD in-frame, internal tandem duplications (ITDs) that have recently been found to occur in pediatric clear cell sarcoma of the kidney (Roy, et al., 2015; Ueno-Yokohata, et al., 2015) and a newly identified class of genetic alterations observed in primitive neuroectodermal tumors of the CNS (CNS-PNET) (Sturm, et al., 2016). Mapping the location of the ITDs on the RAWUL/PUFD dimer structure (Figure S3) (Junco, et al., 2013) suggests that the ITDs have the potential to preserve the three dimensional fold of the PUFD core. The core of the PUFD being the region that excludes both PUFD termini strands and which also shares homology to the Dachshund homology domain (Junco, et al., 2013). In contrast, the ITDs insert residues into either the N- or C-terminal beta strand of the PUFD that extend beyond the core (Figure S3) and are disordered in the monomer (based on NMR relaxation data, S.J.W. and C.A.K. unpublished). Upon binding the RAWUL, the two strands become ordered to augment the PCGF1 RAWUL beta sheet (Junco, et al., 2013). BCOR ITDs are presumed to affect critical contacts between the PUFD and the PCGF1 RAWUL domains and are likely to alter PCGF1 binding affinity. The assembly studies presented here suggest that the association between BCOR and KDM2B may also be disrupted by the ITDs which would have implications for the recruitment of ubiquitin ligase activity to unmethylated CGIs. A preserved core of the PUFD domain present in BCOR ITDs may represent hypomorphic alleles that are phenotypically distinct from the presumed loss-of-function mutations observed in myeloid malignancies (Damm, et al., 2013). Additional experiments will be required to test these speculations. Nevertheless, our results do provide a foundation for understanding the molecular basis of genetic alterations that can lead to disease and require consideration for any rational therapeutic development strategies.

EXPERIMENTAL PROCEDURES

Protein purification

Proteins were expressed in BL21 Gold (DE3) cells (Stratagene) pre-transformed with the pRARE plasmid (Novagen). Proteins were purified by resuspending bacterial cells in a 50 mM Tris (pH 8.0), 200 mM NaCl, 10 mM imidazole (pH 7.5), 1 mM PMSF, 5–10 mM β-

mercaptoethanol, and 5% glycerol solution (using 10mL of buffer for 1 L of bacterial culture). Cells were lysed by sonication and purified using Ni^{2+} affinity chromatography. The eluted protein was digested with TEV to remove the N-terminal sequence (unless otherwise noted) followed by a second Ni^{2+} affinity chromatography purification in which the desired protein was collected. Proteins were further purified by ion exchange chromatography and gel filtration.

Analytical ultracentrifugation

The KDM2B Fbox+LRR region (residues 1059–1336) containing an N-terminal hexahistidine tag and a TEV cleavage site and SKP1 (residues 2–163) was co-expressed from pCDFDuet-1. The BCORL1 PUFD was co-expressed in a modified pET-30a vector (Novagen) with an METR leader sequence along with various PCGF1 constructs (35–255, 130–255, 141–255, 150–255, 159–255. 167–255) in pET-3c housing a hexahistidine tag and TEV cleavage site. For the RING1B/BCORL1/PCGF1 complex, all three components were co-expressed: RING1B with a leader sequence that includes a hexahistidine tag and a TEV cleavage site from a modified pET-3c; the BCORL1 and PCGF1 from a dicistronic pET-30a housing the sequences noted above. Experiments were conducted using a Beckman Optima XL-I analytical ultracentrifuge, scanned in intensity mode at 280 nm, 20°C, 35 krpm in standard two-channel epon centerpieces (Beckman-Coulter). Ultrascan III (version 2.0 release 1498, January 2013) was used to analyze the data.

Isothermal titration calorimetry

The KDM2B/SKP1 and PCGF1/BCORL1 complexes were prepared separately then placed into 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM TCEP. Titration experiments were performed using 30 μM of the KDM2B/SKP1 complex in the cell while injecting 10 μL of a 300 μM stock solution of the PCGF1/BCORL1 into the cell. Experiments were performed at 25 °C. Data was analyzed and the figure prepared using Origin 7.

X-ray crystallography

BCORL1 PUFD/PCGF1 150–255 and the KDM2B/SKP1 dimers were isolated separately, incorporating selenomethionine following a previously published protocol (Junco, et al., 2013). After initial purifications of the separate dimers they were combined and allowed to form the heterotetramer overnight at 4 °C. The heterotetram er was further purified using gel filtration chromatography. Proteins were crystallized in 100mM HEPES (pH 7.25), 10% 2-methy-2,4-pentanediol and 100 mM NaCl using hanging-drop diffusion. Additional details are available in Table S1. The PDB ID code for the heterotetramer structure is 5JH5.

Ni^{2+} pulldown assay

For Figure 3E, bacterially expressed lysates from cells expressing all four components of the heterotetramer (bottom gels) was loaded onto Ni^{2+} sepharose beads. The beads were washed then eluted with a 500 mM imidazole solution (top gels). For Figure 4, PCGF1 or PCGF3 proteins were co-expressed with just the BCORL1 PUFD. In order to account for the differential protein expression levels and to control the amount of the PCGF(6His)/BCORL1 dimer immobilized on the Ni^{2+} resin, different volumes of the bacterial lysates were used to

obtain the level of bound PCGF(6His)/BCORL1 shown on the gel. 10×10^{-6} M purified KS complex was introduced to the resin, washed then eluted with 500 mM imidazole.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- In vitro assembly of PRC1.1 core is achieved by combining two separate subcomplexes
- PCGF1/BCORL1 cooperation is required for association with KDM2B/SKP1
- PCGF1 RAWUL domain binding selectivity is critical for assembling distinct PRC1s
- Structure provides foundation for assessing BCOR PUFD ITDs in brain and kidney tumors

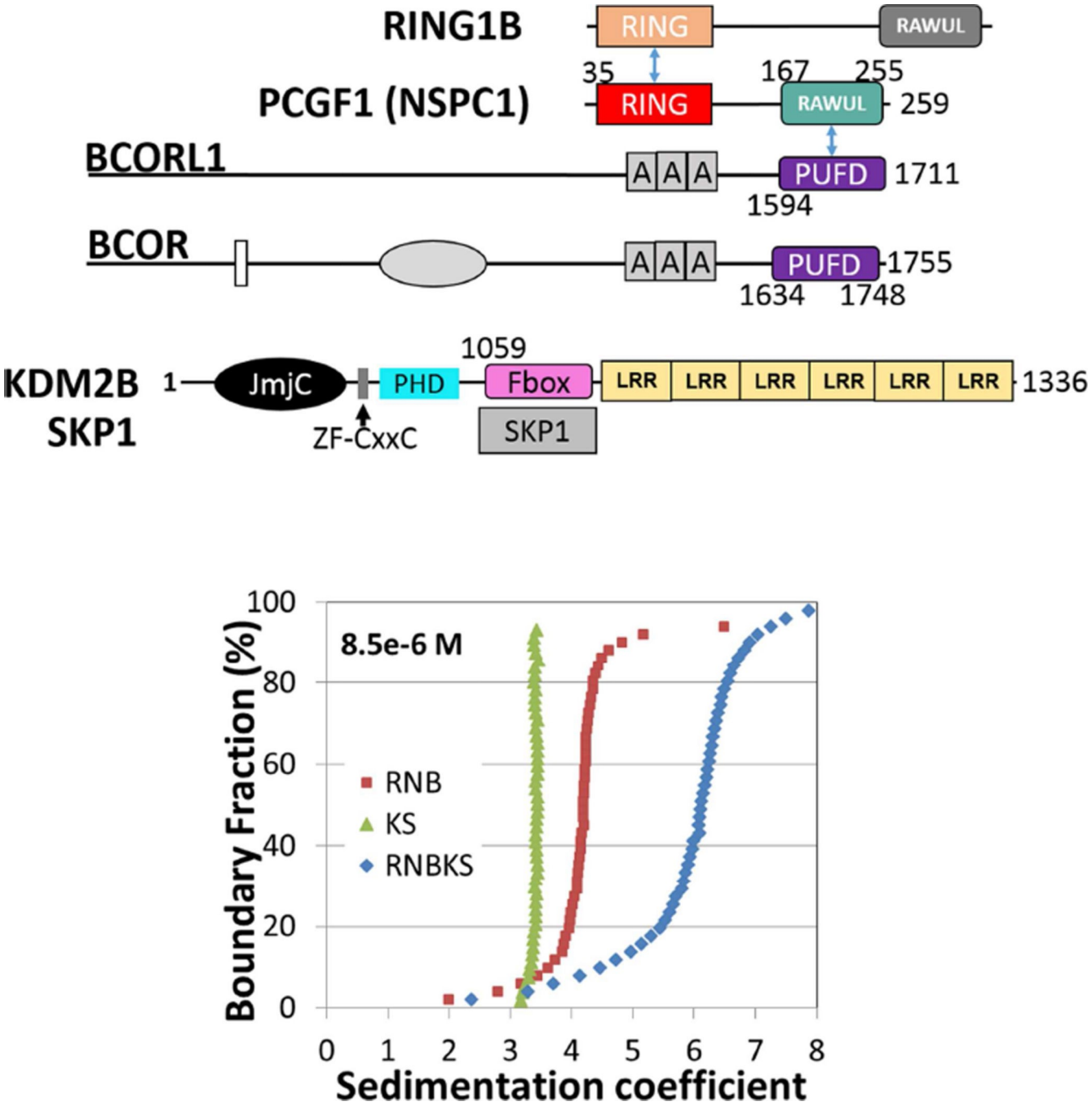

Figure 1. In vitro assembly of the core of PRC1.1

(A) Domain structures of PRC1.1 proteins. The numbering for BCORL1 is for NP_068765. BCOR domains: box is BCL6 interaction region, oval is MMLT1/3 interaction region, and A is an ankyrin repeat. KDM2B domains: JmjcC H3 K36 demethylase domain, ZF-CxxC is the zinc finger non-methylated DNA binding domain, PHD is a domain that binds methylated histones, and LRR is the leucine-rich repeat domain. The blue arrows indicate domains involved in direct protein-protein interactions. Sequences for the structured domains used in the crystal structure determination are shown in Figure S1.

(B) van Holde-Weischet (vHW) plots of the PcG subcomplex (RPB) composed of RING1B, PCGF1 35–255, and BCORL1 PUFD (1594–1711); the KDM2B 1059–1336/SKP1 (KS) dimer; and RNB combined with KS. All samples were prepared to 8.5×10^{-6} M concentration.

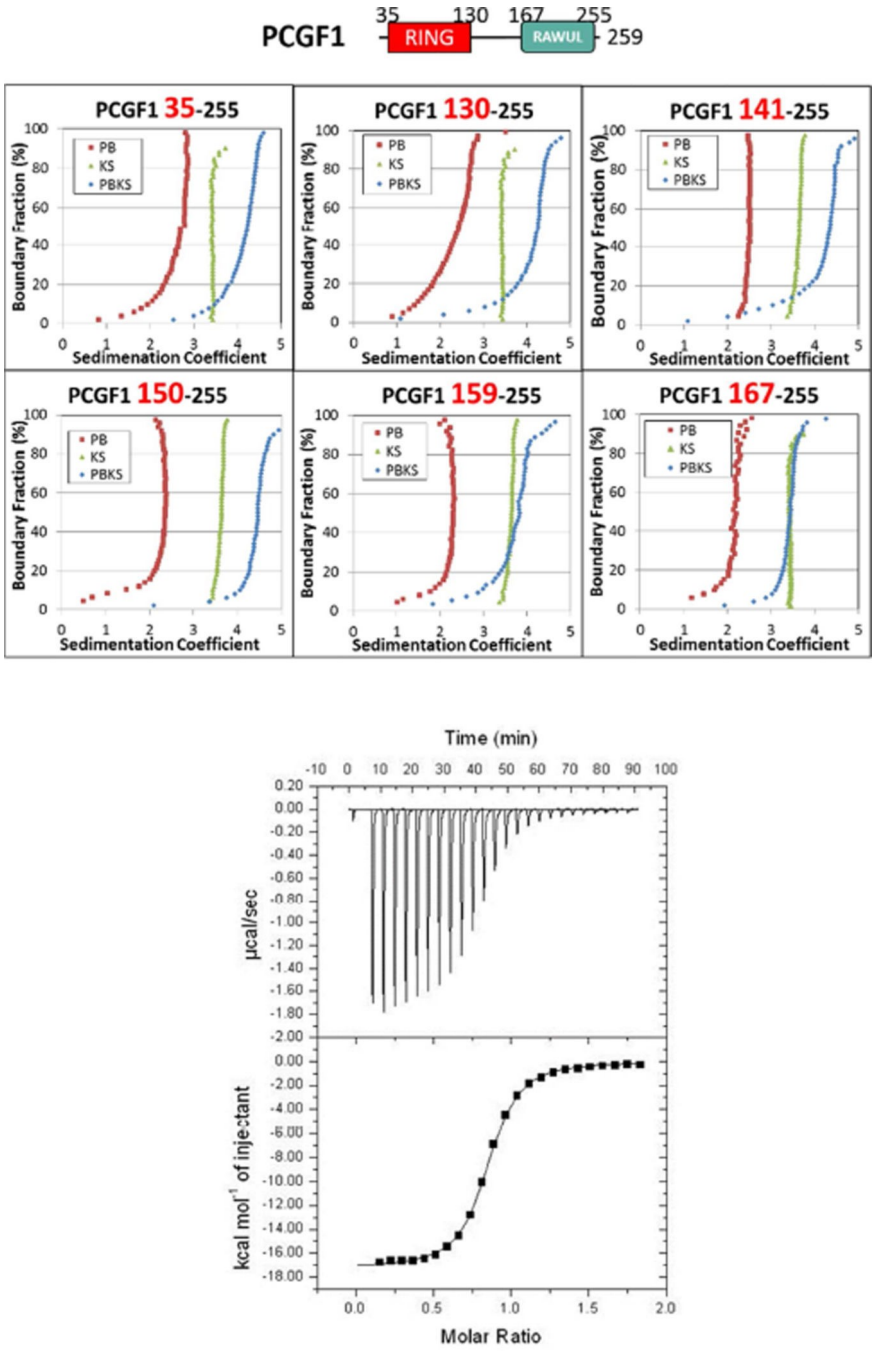

Figure 2. Residues N-terminal to PCGF1 RAWUL is required for assembly

(A) vHW plot of the SV AUC experiments using BCORL1 PUFD in complex with different PCGF1 proteins (PB) combined with KDM2B Fbox+LRR (1059–1336)/SKP1 dimer (KS). Domain structure of PCGF1 with numbered boundary locations of the structured domains are shown. The regions of PCGF1 used in the experiment are indicated at the top of each plot. All samples were prepared to 8.5×10^{-6} M concentration.

(B) Isothermal calorimetry titration between PCGF1 150–255/BCORL1 PUFD dimer with the KS dimer. K_D was measured to be $2.8 \pm 1.0\times10^{-6}$ M.

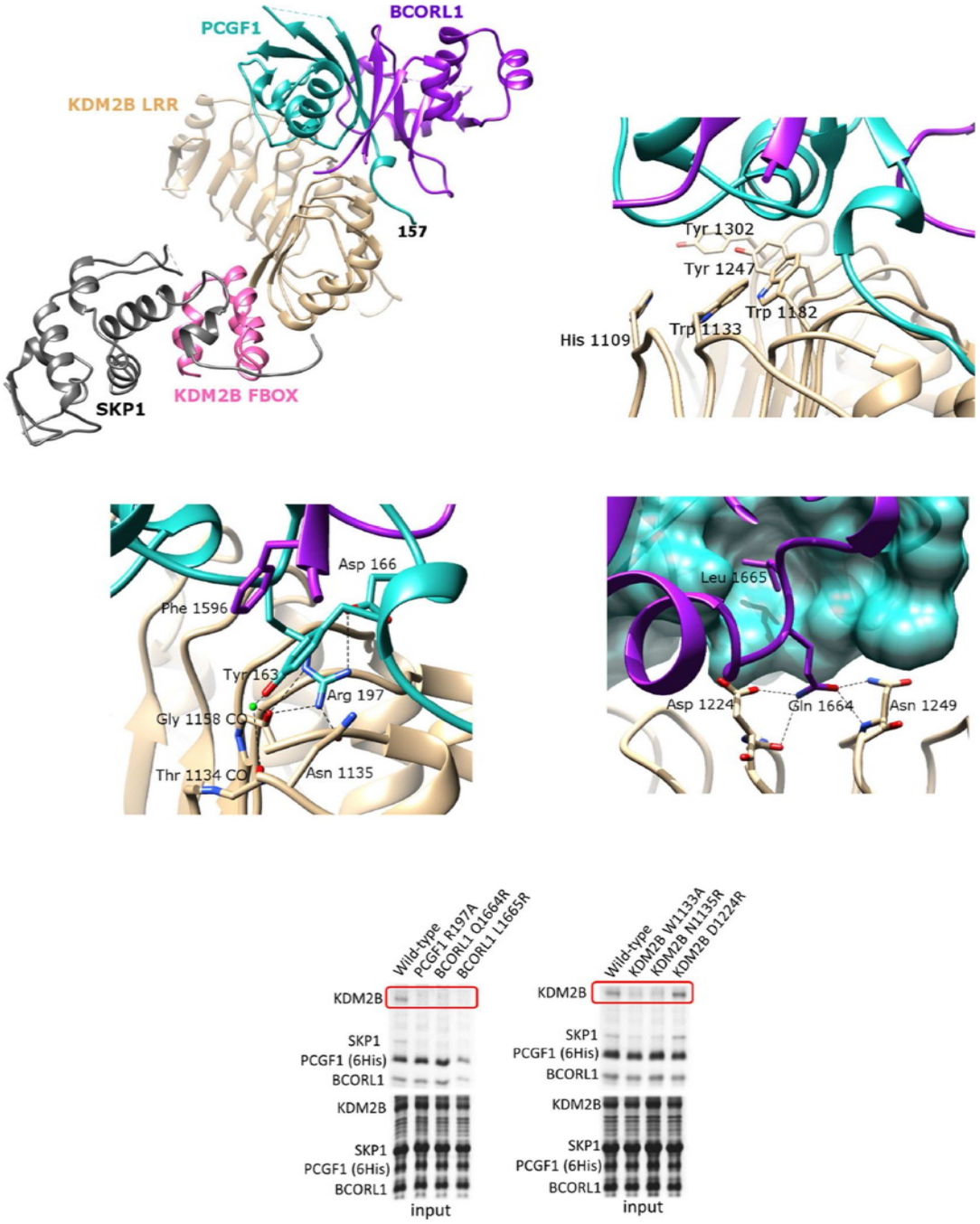

Figure 3. Crystal structure of KDM2B/SKP1/PCGF1/BCORL1

(A) The asymmetric unit of the crystal structure showing PCGF1 (150–255, sea green), BCORL1 PUFD (1594–1711, purple), KDM2B (1059–1336, gold), and SKP1 (gray). Sequences of proteins and contacts made by the residues are in Figure S1. The most N-terminal PCGF1 residue that could be modeled was 157, which is indicated.

(B) Alternating KDM2B LRRs align aromatic residues that demarcate the PCGF1/BCORL1 binding site.

(C) and (D) Examples of hierarchical assembly showing cooperativity between the PCGF1 RAWUL N-terminal extension and the BCORL1 PUFD N-terminal residues in binding KDM2B (C) and the role of BCORL1 Leu 1665 binding the Leu cage of PCGF1 and allowing the proper positioning of Gln 1664 (D).

(E) Corroboration of the structure using Ni^{2+} affinity pulldown assay. Approximately 5% of the bacterially expressed lysate from cells expressing all four components of the heterotetramer (left, bottom gel) was loaded onto Ni^{2+} sepharose beads, washed and eluted. Presence of KDM2B (red box) indicates binding to the immobilized hexahistine tagged PCGF1. See also Figure S2 for the overlay of the heterotetramer to the SCF-ub ligase complex.

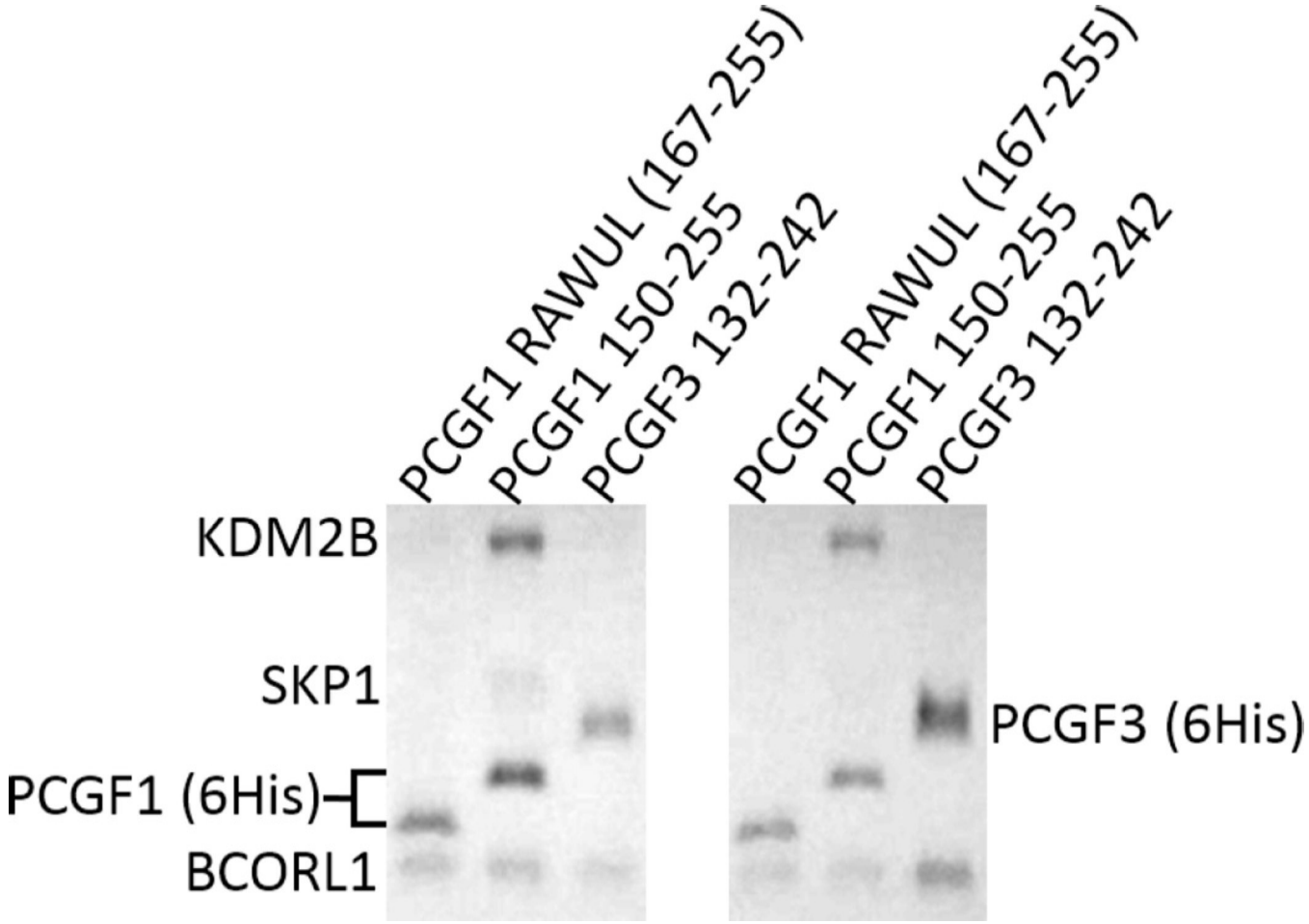

Figure 4. Only PCGF1 binds KDM2B

Differential amounts of bacterial lysates of the hexahistidine tagged PCGF proteins co-expressed with BCORL1 PUFD were used to immobilize the shown amounts of the PCGF(6His)/BCORL1 dimer on the Ni^{2+} resin. 10×10^{-6} M of purified KDM2B/SKP1 was introduced to the resin, washed, all contents eluted, then detected on a Coomassie stained SDS PAGE. The right gel shows a larger amount of immobilized PCGF3/BCORL1 compared to PCGF1/BCORL1 to assure that the lack of KDM2B binding was not due to lower amounts of the PCGF3/BCORL1 used in the left gel. See Figure S1 for the sequences of the proteins.