Characterization of an immunodeficiency-associated EZH2 variant

Francisco Perez de los Santos[1], Lily Beck[1,2], Emily DeCurtis[1,3], April M. Griffin[1], Maggie M. Balas[1,3], Charlotte Marchioni[1,3], Attila Kumanovics[7], Matthew R. G. Taylor[4], Kimberly R. Jordan[5], Charles H. Kirkpatrick[#, 6], and Aaron M. Johnson*[1,2,3]

[1]Department of Biochemistry and Molecular Genetics
[2]Structural Biology, Biochemistry, and Biophysics Program
[3]Molecular Biology Program
[4]Division of Adult Clinical Genetics, Department of Medicine
[5]Department of Immunology and Microbiology
[6]Division of Allergy and Clinical Immunology, Department of Medicine
University of Colorado – Anschutz Medical Campus
[7]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester
*Corresponding Author
[#]Deceased

Abstract

Regulation of gene expression is central to the development of immune cells and their ability to respond to infection. As part of a clinical evaluation, we identified two sisters with recurrent infections, hypogammaglobulinemia, and memory B cell deficiency, diagnosed as common variable immunodeficiency. Whole exome sequencing identified a heterozygous variant (Leu50Ser, L50S) in a conserved region of EZH2, the catalytic subunit of the epigenetic gene repressor Polycomb Repressive Complex 2 (PRC2). EZH2-catalyzed histone H3 lysine 27 methylation (H3K27me) in bulk was not overall significantly disrupted by this variant, in patient samples or cell lines expressing EZH2-L50S. EZH2-L50S protein is expressed similar to wild-type and can form PRC2. However, we find that specific genomic regions that normally have high wild-type levels of H3K27me3 are deficient in the L50S context, particularly around gene promoters. EZH2-L50S is still recruited to these sites, but is not as active. Using recombinant purified PRC2, we determine that L50S affects methylation of nucleosomes and disrupts allosteric stimulation that normally amplifies H3K27me3, consistent with the location of L50 in the allosteric regulatory region of PRC2. Thus, variation of EZH2 L50, occurring at low frequency in the population may interfere with normal B cell gene expression patterns, contributing to immunodeficiency. This study has implications for genetic variation in PRC2 in the general population.

43 **Introduction**

44 Epigenetic regulation of gene expression by chromatin-based mechanisms is essential to development
45 and cell differentiation in response to stimuli in adult tissues of the body. One major pathway for chromatin
46 regulation is through the Polycomb group proteins that induce the formation of facultative
47 heterochromatin[1]. Polycomb proteins exist in two primary complexes, polycomb repressive complex 1
48 and 2 (PRC1 and PRC2). Both complexes have histone modification activity, both can be recruited to
49 chromatin by separate mechanisms to trigger facultative heterochromatic gene repression, and each can
50 promote the other's activity. PRC2 is solely responsible for catalyzing histone H3 lysine 27 (H3K27)
51 methylation via the histone methyltransferase subunit EZH2, or its paralog EZH1 in some contexts.
52 H3K27 methylation triggers heterochromatin formation as a preferred binding site for multiple "reader"
53 subunits of PRC1, which can support chromatin compaction and gene repression. PRC2 is essential in
54 early development, both to initially silence cell type-specific genes and also to turn off stem cell genes
55 during differentiation. Later, in adult tissues PRC2 functions in two capacities: 1) maintaining previously-
56 established polycomb repressed regions of the genome. This activity is carried out primarily by EZH1-
57 containing PRC2, which is specialized for this function, rather than establishing new repression; 2) In a
58 subset of tissues that go through major differentiation and proliferation programs, EZH2 is induced and
59 EZH2-containing PRC2 is an important regulator. For example, both B cell differentiation and function
60 require EZH2[2-5]. Loss of EZH2 leads to impaired development of B cells[2]. Activated mature B cells also
61 express high levels of EZH2 and loss of EZH2 leads to functionally deficient memory B cells, including a
62 decrease in germinal centers and antibody secreting cells and failing to generate a robust recall
63 response[4-6]. On the other hand, increased EZH2 function in B cells due to somatic gain-of-function
64 mutations leads to B cell lymphomas[7].

65 The histone modification that PRC2 catalyzes, H3K27 methylation, not only promotes PRC1
66 activity, but it also is an allosteric stimulator of PRC2's own activity[8]. This allosteric activation occurs
67 through the PRC2 subunit EED, which, together with EZH2 and SUZ12, form the core subunits of PRC2[9].
68 EED can bind to tri-methylated H3K27 and this binding is transmitted to the active site of EZH2 to increase

69 methylation of additional H3K27[10]. This mechanism is essential for all PRC2 activity, since mutation of
70 the EED pocket that binds methylated H3K27 and disruption of the allosteric network result in severe loss
71 of H3K27 methylation[8,10,11]. The allostery between EZH2 and EED is disrupted in the developmental
72 skeletal overgrowth of Weaver Syndrome, where mutations in both genes are found[12-14].

73 Here, we present a study that began with treatment of a pair of siblings who developed antibody
74 deficiency and B cell loss during adult years. Whole exome sequencing disclosed that both have a
75 heterozygous variant in EZH2, c.149T>C, present at 1 in ~10-20,000 individuals, which will result in a
76 substitution of leucine 50 to serine. In the remaining PBMC population, which is depleted of B cells, bulk
77 H3K27 methylation is not significantly different from unaffected samples. Introduction of the L50S
78 mutation into EZH2 in cells demonstrated stable L50S protein which was able to form PRC2. EZH2-L50S
79 transgene expression led to a minor defect in bulk H3K27 tri-methylation levels. Genome-wide
80 determination of H3K27me3 levels by CUT&Tag revealed that regions with normally high wild-type
81 H3K27me3 levels were depleted in the EZH2-L50S context, though EZH2-L50S was still recruited to
82 these regions similar to wild-type. Using purified PRC2 in biochemical assays, we found a decrease in
83 nucleosome methylation and allosteric stimulation with EZH2-L50S compared to wild-type. Overall, our
84 study shows that a genetic variant of EZH2 associated with reduced B cell function in adulthood has a
85 hypomorphic molecular phenotype.

86

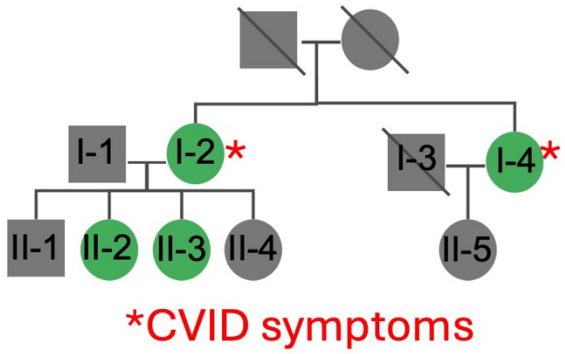
87 **Results**

88 ***Association of the L50S variant of EZH2 with a B cell immunodeficiency***

89 In the course of the clinical practice of the late Dr. Kirkpatrick, a 38-year-old woman (I-2 in Figure 1A)
90 was referred for evaluation of repeated respiratory infections including pneumonia. Later, her sister (I-4)
91 was referred for evaluation of chronic hives and, over ~5 years of follow up, began to have recurrent
92 infections, including infectious sinusitis and bronchitis. The sisters were evaluated to have
93 hypogammaglobulinemia, a severe defect in antibody production (Figure 1B) and were given the

diagnosis of “common variable immune deficiency” (CVID). Both sisters were treated with immune globulin replacement therapy which has been beneficial. Prior to treatment, response after immunization was measured for three vaccines. Responses to *tetanus* toxoid and *H. influenza* b conjugate vaccines, were both well below the expected >=4-fold increase in titer in sera collected >=four weeks after immunizations. The pneumococcal vaccine (Pneumovax-23) response was also below normal, with 0 out of 14 (I-2) and 2 out of 14 (I-4) serotypes eliciting a >=4-fold response, whereas an expected normal response is at least 11 out of 14 (Figure 1C). Mononuclear cells were separated from heparinized whole blood by density gradient centrifugation and stained for lymphocyte type for general profiling (Figure 1D). All values for T-cells were in the normal range. The most striking abnormality observed was very low numbers of CD27+ memory B cells. These findings suggested that maturation of the peripheral B cell population had not progressed beyond the “naïve” (IgM+/IgD+CD27-) stage or there was a depletion of memory B cells.

A



B

Immunoglobulin Levels

	I-1	I-2	I-4
Age/Sex	53M	38F*	36F*
IgG	954	267	414
IgA	324	14	38
IgM	146	15	42

C

Vaccine Response

Vaccine		I-2	I-4
Tetanus (IU/ml)	Pre- Post-	0.45 0.38	2.66 1.9
H. Inf b (ug/ml)	Pre- Post-	n.d. 3.9	1.3 1.13
Pneumo (ug/ml)	Pre- Post-	all< 0.44 all< 0.44	all< 0.44 2/14

D

Leukocyte Profile

Cell type	I-2	I-4	Normal Range
CD3+ T	936	1388	708-2790
CD4+ T	494	994	425-1860
CD8+ T	416	252	216-1292
CD16+/56+ NK	39	n.d.	84-1064
CD19+ B	299	50	90-1170
IgD+/CD27- B	291	42.5	30-924
IgD+/CD27+ B	4.5	5.3	7.2-410
IgD-/CD27+ B	3.8	1.7	7.2-490

Figure 1. Identification of a variant in EZH2 associated with B cell immunodeficiency. A) Family tree with CVID patients identified, along with EZH2 variant status (heterozygous) indicated by green shading. B) Ig profiling at the time of diagnosis for patients. C) Vaccine challenge antibody titer response, pre- and post-vaccination. Vaccines: *tetanus* toxoid and *H. influenza* b conjugate vaccines and

111 pneumococcal vaccine (Pneumovax-23). D) Flow cytometry profiling of leukocyte type. For B-D, Red
112 indicates below normal levels.
113

114 Exome sequencing and subsequent Sanger sequencing was performed within the family cohort, which
115 identified two variants of interest, heterozygous in both sisters, one in the EZH2 gene and one in
116 UNC13D. Certain UNC13D variants are associated with autoimmune lymphoproliferative disease
117 (ALPS)[15] and hematophagocytic lymphohistiocytosis (HLH). The particular UNC13D variant, Val781Ile,
118 has a high estimated heterozygous frequency in the general population (1 in ~250 individuals, per
119 gnomAD[16]) and is variably classified in ClinVar as "likely benign" and "uncertain significance"[17], none of
120 the family members have had clinical disorders that resemble ALPS or HLH, and
121 hypogammaglobulinemia is not generally observed in HLH. The EZH2 variant, NM_004456.5 (EZH2):
122 c.149>C (p. Leu50Ser) has for some time been categorized as 'likely pathogenic' based on its association
123 with a patient with Weaver Syndrome[18], an overgrowth condition frequently caused by mutations in
124 subunits of the Polycomb Repressive Complex 2[19], of which EZH2 is the catalytic subunit. However, in
125 the midst of our study, this variant has been re-classified as being of 'conflicting significance' with the
126 increase in available genome sequences from individuals (see below for more discussion) and lack of
127 any functional studies (prior to the current study). EZH2 is a known driver of B cell development[2,20] and
128 maturation[4,21]. Based on this, we hypothesized that the L50S variant may have reduced function, resulting
129 in an eventual adult onset of B cell deficiency.
130

131 ***EZH2 L50S is a rare variant in a conserved protein sequence located in the EED binding domain***
132

133 Mapping of the L50 position of EZH2 onto the cryo-EM structure of PRC2[22,23] identified the residue to be
134 in the EED binding domain within a long alpha helix that spans the "back side" of EED, opposite the
135 pocket that binds methylated H3K27 for allosteric stimulation of the complex (Figure 2A,B). Leucine 50
136 is positioned more on the outward face of the helix, rather than buried in the interface with EED, though
137 this varies subtly in different structures. Sequence alignment of EZH2 protein with vertebrates and *D.*
138 *melanogaster* revealed that L50 is highly-conserved across nearly all mammals and many vertebrates

139 and in a region with at least three flanking amino acids on either side conserved to zebrafish (Figure 2C,
140 Supplemental Figure 1 for full alignment). However, *D. melanogaster* does not have a similar region. We
141 noted that there are some species where this position differs from leucine, which may help explain the
142 tolerance for L50S.
143
144 With the high conservation among vertebrates, we next asked what the extent of this variation was in the
145 human population. Our initial identification of the L50S association with the patients occurred prior to the
146 large influx of individual exome and genome sequences that was made available through repositories
147 such as gnomAD[16]. With the release of gnomAD v4, greater evidence of L50S in the human population
148 was revealed, suggesting that L50S is a rare, but significant variant, with an allele frequency of ~$7x10^{-5}$,
149 estimating it in the top 10 most frequent missense alleles of EZH2 present in the human population, with
150 no homozygotes reported. A much lower occurrence of L50V is also reported in the gnomAD database.
151 With an estimated 1 in ~10,000-20,000 individuals harboring this variant, according to all current UK
152 Biobank[24] and AllofUs[25] data, we were motivated to further characterize whether there may be an altered
153 function of this EZH2 L50S variant that has a significant frequency in the human population.
154
155 ***EZH2 L50S variant blood samples do not show reduced bulk histone methylation***
156
157 Since EZH2 is a methyltransferase primarily for histone H3 lysine 27, we used peripheral blood
158 mononuclear (PBMC) samples from one of the affected sisters to characterize total H3K27 methylation.
159 Histones were extracted by the high salt method according to standard protocol[26]. Quantification of the
160 three states of histone methylation, normalized to total histone H3, demonstrated no significant loss in
161 histone methylation in the PBMC sample compared to a control hayfever patient (Figure 2E). Additional
162 experiments using flow cytometry with permeabilization to profile H3K27me3 confirmed that no significant
163 change in histone methylation was observed in the patient sample across leukocytes and specifically in
164 the remaining B cells (Figure 2F, Supplementary Figures 2,3). We concluded from this that either the
165 EZH2 variant L50S does not affect histone methylation, or that the context of the patient samples was

166 not optimal to address this. We noted that since B cell maturation requires high EZH2 activity, and the
167 patient samples had abnormally very low B cell count (19 cell/μL at sample collection, years later than in
168 Figure 1, ~5 times below minimum normal range), that the blood samples may be depleted for the very
169 cell type most affected. Due to these low B cell numbers in the patient blood, it has been challenging to
170 pursue more directed questions of the activity of the variant. In light of this, we wished to establish a
171 system to study the EZH2-L50S variant more directly and with the capacity for a wider series of
172 experimental approaches.

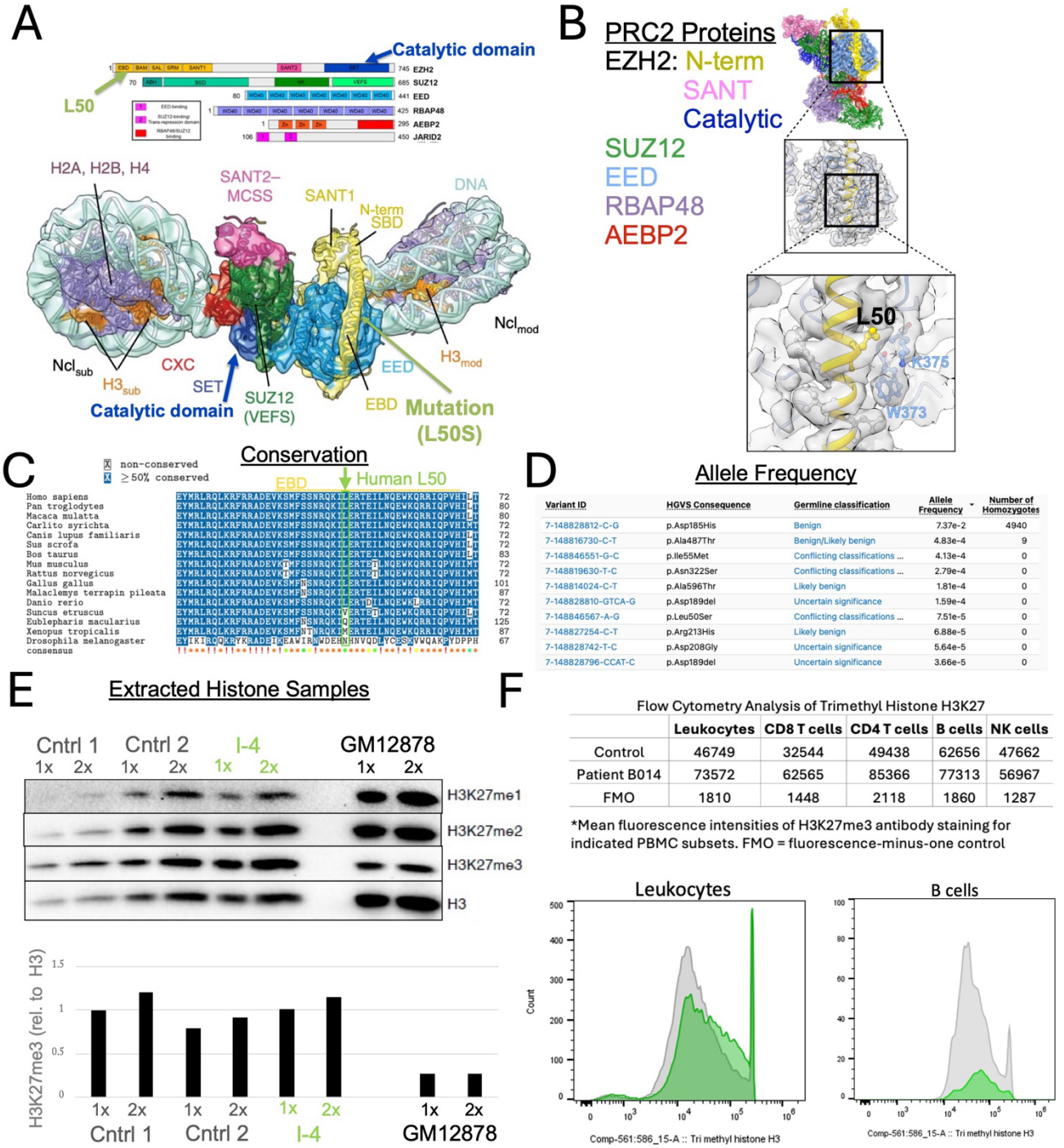


Figure 2. EZH2-L50S structural, genetic, and patient histone sample analysis. A,B) Location of L50 relative to the primary and tertiary structure of PRC2 bound in between two nucleosomes. Adapted from[23,27]. C) EZH2 protein sequence region alignment across vertebrates, highlighting conservation of L50 and divergence in certain species, including non-vertebrate *D. melanogaster*. Consensus symbol key: ! = 16/16; orange * = 15/16; yellow * = 14/16; green * = less than 14/16. D) Data from the gnomAD database, indicating the top 10 most frequent EZH2 alleles, including L50S. E) Extracted histone samples from control or I-4 patient PBMCs or a B cell line(GM12878) analyzed by western for histone H3K27 methylation or total histone H3. F) Leukocyte flowcytometry analysis of Control 2(gray) or I-4

182 patient(green) to quantify cell types and simultaneously H3K27me3 levels. (*Bottom)* Histogram of all
183 leukocytes (*left*) and B cells (*right*). FMO is a control with all antibodies except for H3K27me3.
184
185
186 ***An inducible system to study EZH2-L50S demonstrates stable variant protein and a shift in***
187 ***histone methylation pattern***

188

189 To further study the EZH2 L50S variant, we constructed multiple clones of an inducible cell line that
190 expresses wild-type (WT) or L50S EZH2 transgene, introduced at a specific genetic 'safe harbor' locus
191 via Cre-mediated recombination[28]. Briefly, HEK 293T cell line was a previously-engineered to include a
192 selectable marker with LoxP sites flanking it[29]. We introduced into this cell line a plasmid carrying a FLAG
193 epitope-tagged EZH2, WT or L50S, and a different selectable marker, flanked by LoxP sites, transfected
194 with a plasmid expressing Cre recombinase and stable cell lines were selected with antibiotic (Figure
195 3A). We first characterized the expression of EZH2 in these clones and observed reproducible similar
196 expression patterns for WT and L50S EZH2 transgene, which can be distinguished from endogenous
197 EZH2 by size, the transgene is FLAG-tagged, and also with specific western probing for the FLAG epitope
198 (Supplemental Figure 4). Wild-type and L50S EZH2 were both expressed at similar levels, demonstrating
199 that the L50S variant does not destabilize the protein (Figure 3B). Interestingly, we noted that induction
200 of transgene expression led to near complete reduction of the endogenous EZH2, apparently by a post-
201 translational mechanism that we have yet to uncover and do not focus on further in the current study. In
202 effect, due to the transition from endogenous to transgene EZH2 protein, we were able to obtain a
203 'pseudo-homozygous' context to study the EZH2 L50S protein, in the absence of nearly all WT
204 endogenous EZH2.

205

206 We took advantage of the transgene predominance to first determine the levels of H3K27 methylation in
207 WT and L50S contexts. Western analysis of H3K27 di- and tri-methylation demonstrated a shift in signal
208 normalized to the total histone H3, where the L50S transgene generated a lower level of H3K27
209 trimethylation in most lines, followed by a higher level to H3K27 di-methylation. While there was variation
210 among the clones and the trend was not statistically significant by general p-value standards, this shift in

signal suggested that EZH2-L50S may be affecting some small population of sites in the genome, reducing the levels of H3K27 trimethylation.

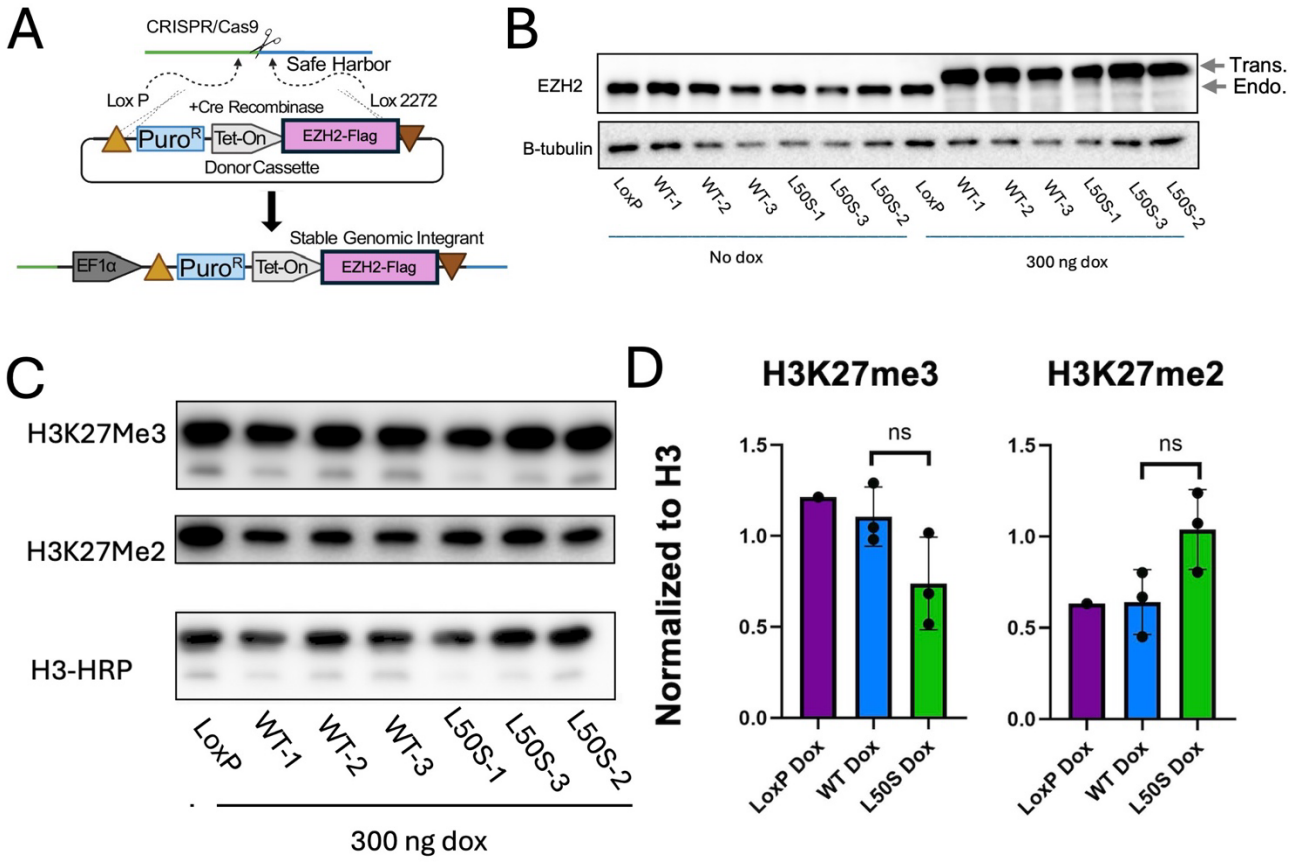


Figure 3. EZH2-L50S is stable and expression leads to only minor H3K27 methylation changes. A) Schematic of the safe harbor locus transgene insertion strategy, adapted from[28]. B) Transgene cell line tests of EZH2 induction via western analysis. Endogenous EZH2 protein (Endo.) and transgene FLAG-EZH2 protein (Trans.) indicated. C,D) Western analysis of histone methylation with EZH2 transgene induction with quantification in D).

CUT&Tag epigenomic profiling reveals regions of H3K27me3 loss in the EZH2 L50S context

To analyze effects of EZH2-L50S compared to wild-type across the genome, we used Cleavage Under Targets and Tagmentation (CUT&Tag)[30], an antibody-tethered method for probing epigenomic state in situ with isolated whole nuclei. H3K27me3 CUT&Tag was performed with LoxP parental cell line or cell lines with wild-type or L50S EZH2 induced as above (Figure 4A). Libraries from H3K27me3 CUT&Tag samples that were generated and sequenced all demonstrated prominent mononucleosome-sized fragments (Supplemental Figure 5), which were used for further analysis. Combined peaks for WT

229 samples were called and the window around the peak summits analyzed. While all L50S samples
230 demonstrated enrichment of reads within the majority of WT peak regions (Supplemental Figure 6), the
231 overall signal was reduced (Figure 4B). Differential enrichment analysis was performed on the WT versus
232 L50S samples, identifying a small set of peaks that were higher in WT (WT>L50S), while the majority
233 were unchanged (WT=L50S) (Figure 4C, Supplemental Files 1 and 2). We noted that the WT>L50S
234 peaks tended to be those with higher intensity within all peaks, whereas the WT=L50S peaks tended to
235 be average to low intensity (Figure 4D). The fact that no significant WT<L50S peaks were called suggests
236 a high likelihood for L50S to negatively affect H3K27me3 (Figure 4E). We compared the locations of the
237 differential peaks to annotations of gene features including promoter proximity (Figure 4F,G). WT>L50S
238 peaks were highly enriched near promoters, with more than ~75% overlapping within the window from
239 7kb up or downstream of a promoter (Figure 4F). Gene Ontology analysis of WT>L50S genes
240 demonstrated enrichment for neuronal biological processes (Supplemental File 3), consistent with
241 neuronal genes normally being repressed in HEK293 cells[31]. Conversely, WT=L50S peaks displayed a
242 distribution pattern similar to shuffling the peaks. WT>L50S peaks were roughly evenly balanced in their
243 propensity to be up or downstream of the TSS with a slight bias towards downstream (Figure 4G). Since
244 the majority of peaks were unchanged, the CUT&Tag analysis helped confirm that the overall H3K27me3
245 levels are not majorly affected by L50S. We were thus able to highlight specific loci, mainly at highly
246 methylated promoter regions in WT context, that lost significant H3K27me3 in the L50S context.
247

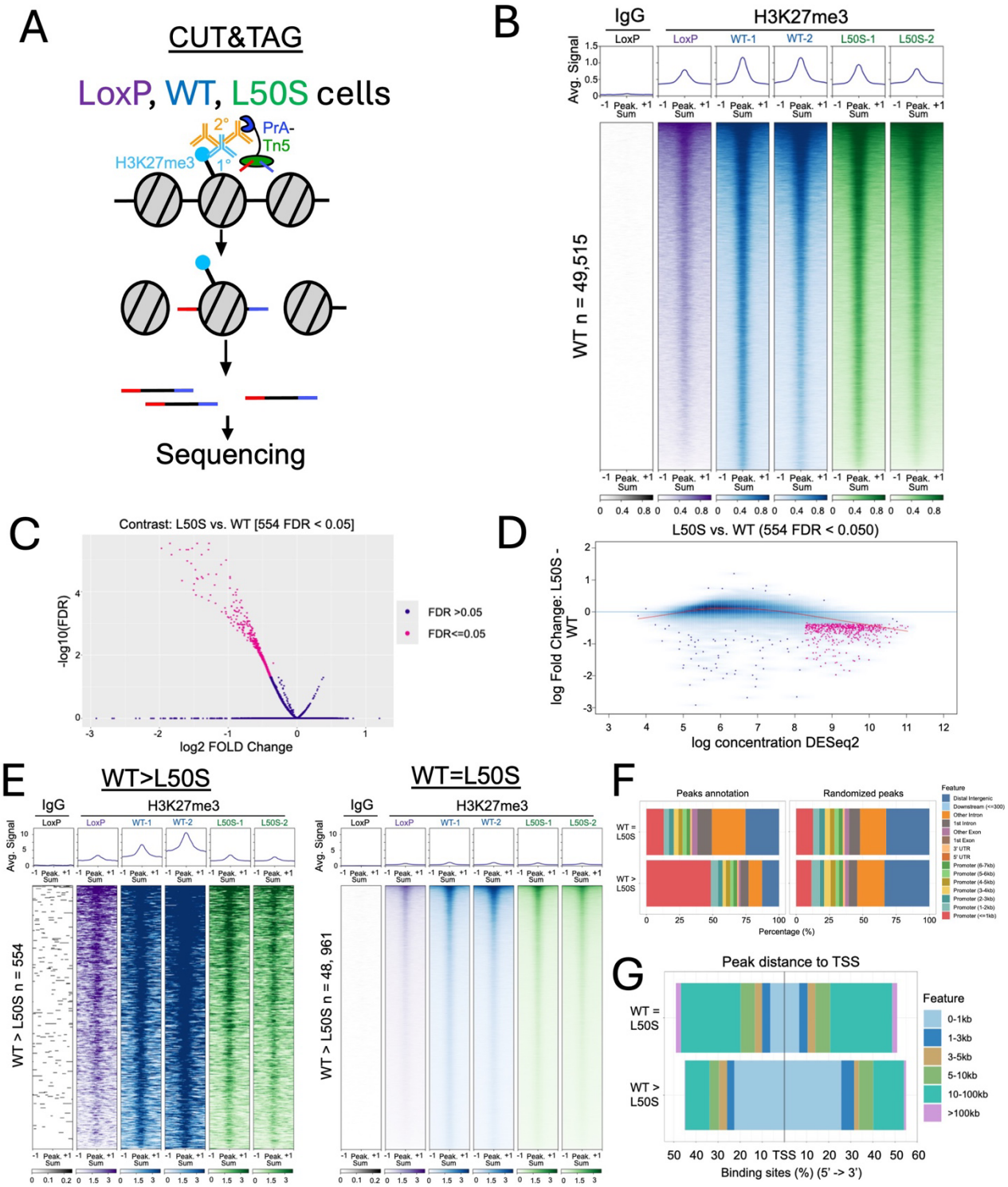


Figure 4. Epigenomic profiling of H3K27me3 in EZH2-L50S expressing cells. A) Schematic for the CUT&Tag experiments performed. B) Heatmaps of CUT&Tag for H3K27me3 (or IgG, far left) highlighting the peak regions identified in the wild-type transgene context, +/- 1 kilobases from peak summit. C) Volcano plot for differential H3K27me3 across the WT and L50S samples and regions in B). Highlighted in magenta are regions that are significantly different between the two types of samples. D) Plot of signal intensity vs. fold-change difference between WT and L50S samples, significant differences in magenta. E) Heatmaps broken out into classes of loci, WT>L50S and WT=L50S. F) Genomic annotation of groups

256 from E) and a set of the same sized windows randomly distributed across the genome as a reference.
257 G) Distance of the peak summits from E,F) to the nearest TSS, indicating relative position, upstream or
258 downstream.
259

260

261 ***EZH-L50S is still recruited to promoters yet is deficient at H3K27 methylation there***

262

263 Since the majority of changes in the WT>L50S H3K27me3 analysis were near promoter regions, we
264 focused in on those for further analysis (Figure 5A). To gain further insight into the mechanism for
265 H3K27me3 loss in those regions, we profiled WT and L50S FLAG-tagged EZH2 by CUT&Tag. We
266 reasoned that either PRC2 with EZH2-L50S was deficient in recruitment to regions of high WT activity or
267 that the L50S complex, once recruited, was intrinsically less active at those regions where WT can
268 operate robustly. Somewhat surprisingly, we found that EZH2-L50S was recruited at a similar, if
269 somewhat higher, level to the promoters with significant WT H3K27me3. Specifically at regions that were
270 significantly lower in H3K27me3 in L50S compared to WT, we observed no decrease, even a slight
271 increase, in recruitment of EZH2-L50S and presumably the full PRC2 complex. To confirm that the FLAG
272 antibody used to identify binding sites of WT or L50S was specific to EZH2, we performed CUT&Tag with
273 an EZH2 antibody in WT cells and observed highly similar enrichment to the FLAG-tagged WT EZH2 that
274 was profiled by FLAG antibody (Figure 5A).

275

276 We separated the WT>L50S promoter windows into quartiles, based on differential H3K27me3 (Figure
277 5B). In the top quartile, whereas WT H3K27me3 signal is strong directly over the peak of EZH2, L50S
278 H3K27me3 signal is depleted, even as a strong peak of EZH2-L50S itself is observed. Individual
279 examples of this epigenomic profiling, randomly selected from the top quartile of WT>L50S promoter
280 regions are shown (Figure 5C). Profiles of individual loci are representative of the high WT and low L50S
281 H3K27me3 signal and demonstrate EZH2 recruitment in both WT and L50S contexts. Since the overall
282 levels of H3K27me3 in bulk are not statistically different in L50S, we asked whether there was a
283 compensation of methylation outside of the differential regions. To do this we analyzed overall read
284 counts in three categories that cover all genomic coordinates: intergenic, gene body, and promoter-

285 proximal regions (Figure 5D). We observed higher signal for L50S in intergenic and gene body regions
286 than WT, which may explain overall levels of H3K27me3 being only mildly affected by the variant. This
287 result is consistent with a higher proportion of unique L50S H3K27me3 peaks than WT peaks and more
288 EZH2-L50S peaks outside of the shared WT/L50S peaks (Supplemental Figure 6).
289

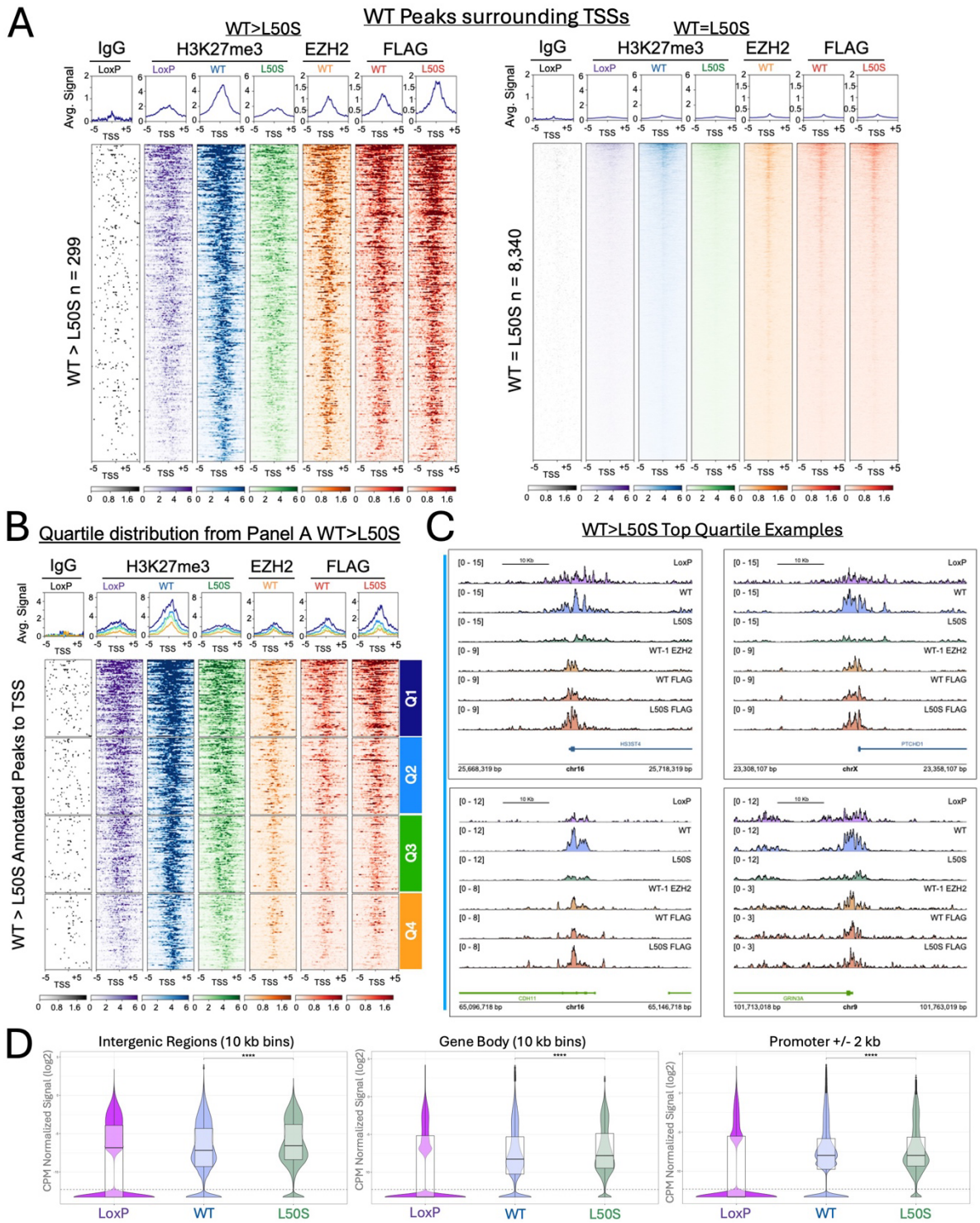


Figure 5. EZH2-L50S is recruited to regions that it cannot properly methylate. A) Heatmaps centered on peaks from Figure 4A that occur within 2 kb of a TSS. Included are combined profiles of H3K27me3 CUT&Tag as well as CUT&Tag for EZH2 and FLAG epitopes, for indicated cell line contexts, +/- 5 kilobases from the TSS. B) Quartile separation of WT>L50S H3K27me3 peaks form A). C) Genome browser tracks from randomly chosen loci from Quartile 1 in B). D) Analysis of H3K27me3 CUT&Tag reads across three classes of genomic region, intergenic, gene body, or promoter proximal. Statistical significance between the indicated samples is shown (Wilcoxon's test); ****p<0.0001.

299 ***EZH2-L50S assembles into PRC2 but is deficient in histone methylation activity toward***
300 ***nucleosomes***
301
302 After identifying a deficiency in the ability of EZH2-L50S to perform histone H3 lysine 27 tri-methylation
303 at regions of high wild-type activity, we next wished to further characterize why this EZH2 variant has
304 lower activity in cells. We first tested whether the L50S mutation in EZH2 disrupted a key subunit
305 interaction within the core of PRC2. While we had already determined that EZH2-L50S could be recruited
306 to PRC2 targets, equally to the WT subunit (Figure 5A), it was still possible that the reduced activity after
307 recruitment was due to a key core subunit that was missing from the L50S complex. We performed co-
308 immunoprecipitation with the FLAG antibody for either WT or L50S EZH2 and probed by western blotting
309 for the core PRC2 subunits SUZ12 and EED. In both cases, both WT and L50S associated with an equal
310 amount of each subunit (Figure 6A), suggesting that the EZH2-L50S variant does not disrupt core PRC2
311 interactions. This is consistent with the normal recruitment profile for EZH2-L50S (Figure 5A), since
312 SUZ12 is crucial for EZH2 recruitment[32]. The fact that EED can still associate with EZH2-L50S is
313 important, since the L50 position lies in the EED-binding domain helix of EZH2 (Figure 2A,B). Our results
314 demonstrate that the L50S variant does not lead to a major loss of interaction with the core PRC2
315 subunits.
316
317 To continue to directly characterize the EZH2-L50S variant in PRC2, we used an established baculovirus
318 expression and purification system[33] to isolate purified human PRC2. Subunit stoichiometry of the L50S
319 version was identical to WT (Figure 6B). We first tested the general histone methylation ability of the
320 purified complex with pure recombinant histones, lacking any modifications, a sample of human histone
321 octamer that exists as H3/H4 tetramers and H2A/H2B dimers under the PRC2 reaction conditions. Under
322 these conditions, we observed no significant difference in activity between WT and L50S complexes
323 (Figure 6C). Next, we tested the methylation activity on nucleosomes, a more complex substrate that,
324 through its assembly, requires a specific context to promote histone methylation, especially di- and tri-
325 methylation of H3K27[34]. Incubation of L50S with a nucleosome containing a linker DNA sequence

326 produced a lower amount of H3K27 methylation (both di- and tri-methylation), roughly 25% compared to
327 WT PRC2 (Figure 6D, “No peptide”). We next asked whether the PRC2 activity that is stimulated
328 allosterically by binding of the EED subunit to a methylated peptide may be affected in the L50S context,
329 since leucine 50 is in the EED-binding domain of EZH2. We titrated a synthetic trimethylated peptide into
330 the nucleosome histone methyltransferase assay and observed a deficiency in the ability of PRC2-L50S
331 to allosterically respond (Figure 6D).
332
333 Our results identify a hypomorphic EZH2 variant, L50S, in a family with B cell immunodeficiency. PRC2-
334 L50S can assemble and bind promoters, but its activity is weak at promoter regions where wild-type
335 PRC2 is most active, whereas L50S has higher propensity to methylate in a distributive manner in non-
336 promoter regions (Figure 6E). PRC2-L50S is less active on nucleosomes and does not respond normally
337 to allosteric stimulation that is mediated through EED.
338

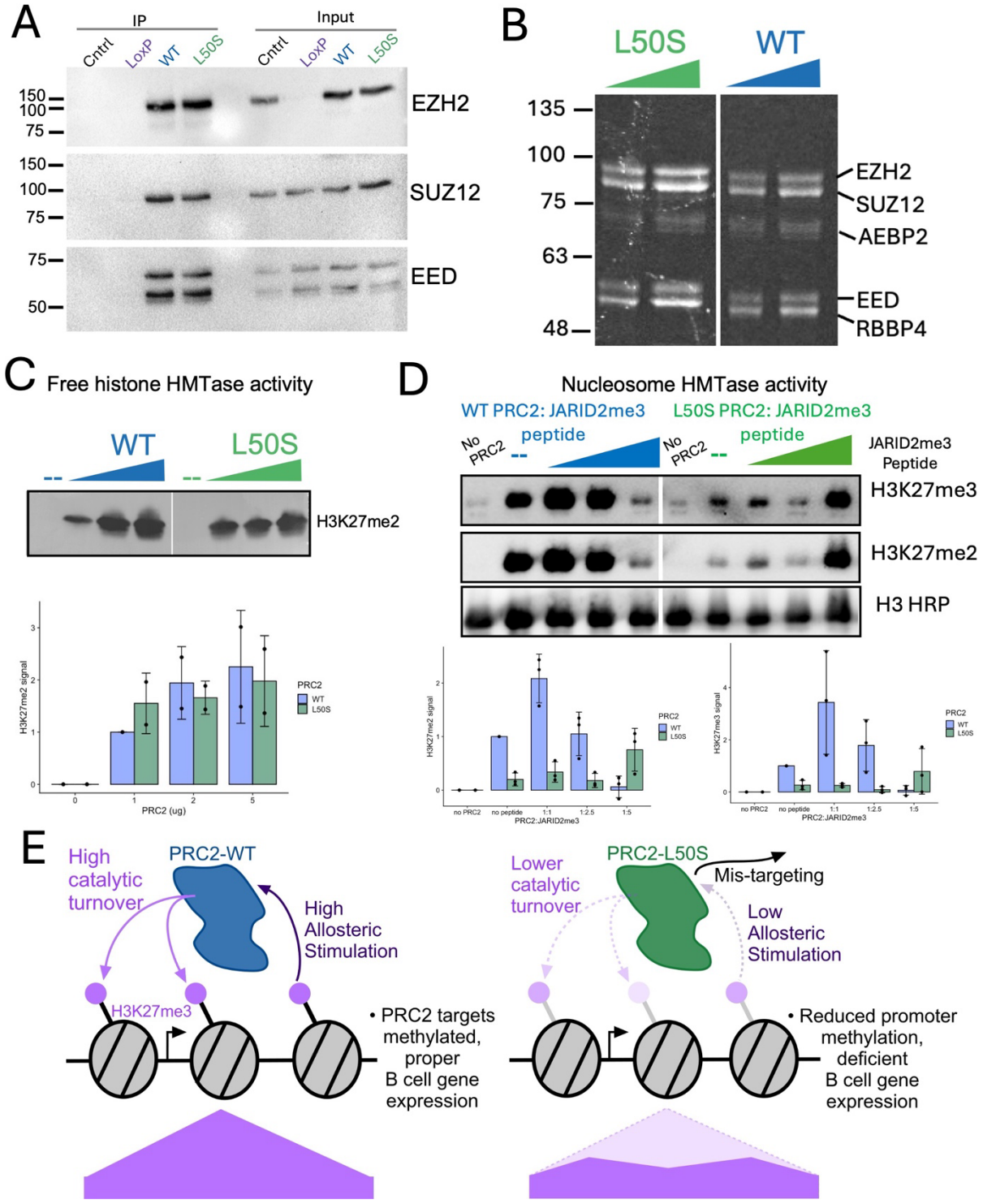


Figure 6. EZH2-L50S leads to lower PRC2 activity and allosteric stimulation on nucleosomes. A) Co-immunoprecipitation experiments using anti-FLAG antibody, with indicated cell lines. Western analysis performed for EZH2, SUZ12, and EED. Control indicates identical IP conditions with no FLAG antibody using the WT cell line. B) SDS-PAGE and Coomassie-stained, recombinant, baculovirus-expressed and purified five-subunit PRC2 complex with EZH2-WT or -L50S. C) Histone methyltransferase (HMTase) assays with recombinant PRC2 using free histone samples. H3K27me2 western and quantification shown. D) Histone methyltransferase (HMTase) assays with recombinant PRC2 using a nucleosome substrate with increasing titration of a stimulatory methylated peptide

corresponding to a region of JARID2. E) Model of EZH2-L50S effect on promoter-proximal H3K27 methylation. The variant lowers PRC2 catalytic turnover and dynamics of the complex at promoter chromatin, in part by lowering the ability of allosteric stimulation by H3K27 methylation itself. Flanking chromatin, methylated by PRC2.2, is less affected. PRC2-L50S also exhibits some mis-targeting away from promoter regions, where it displays a diffuse methylation pattern.

Discussion

While EZH2 expression has been known for some time to be induced during B cell activation and EZH2 knockout disrupts B cell function during an immune response[4,21], our work connects a human genetic context in a B cell immunodeficiency with a hypomorphic variant in EZH2. The adult-onset loss of B cells is likely buffered earlier in life by the wild-type EZH2 allele and the fact that L50S is not a complete loss of function or a dominant negative, nor does it directly affect the PRC2 active site. In fact, our examination of overall histone methylation levels detected only minor differences and only in the transgene context (Figures 2 and 3). This same observation is seen in PRC2 accessory protein knockouts, where even the triple knockout of all PCLs, JARID2 and AEBP2, which drastically affects H3K27me3 at PRC2 targets, has minimal effect on bulk H3K27 methylation[35]. Despite a general maintenance of overall histone methylation, we see explicit deficiency of L50S to support wild-type level H3K27me3 at primarily promoter regions where wild-type PRC2 activity is very high (Figure 5A). Surprisingly, EZH2-L50S is recruited to a higher level than wild-type to these affected regions, suggesting that L50S leads to PRC2 getting “stuck”, but low in activity. Our biochemical analysis demonstrates a loss of nucleosome methylation and EED allosteric stimulation, suggesting that this could lead to improper B cell gene expression (Figure 6E). Overall, this work sheds new light on the regulation or PRC2 and highlights a new player in the population genetics of immunity.

The pattern of H3K27me3 at promoters that are deficient for methylation in the L50S context is reminiscent of H3K27me3 in a knockout for all PRC2.1 activity (PCL 1-3, triple KO), observed in previous work[35,36], with a trough observed where the peak intensity is seen in WT. In this background, PRC2.2 remains to maintain the “arms” of the methylation peak. PRC2.2 is distinguished by incorporation of AEBP2 and JARID2 accessory factors. The methylated peptide used in our characterization of purified

379 EZH2-L50S (Figure 6D) is derived from the region of JARID2 that is normally methylated by EZH2 and
380 which can promote allosteric stimulation of EZH2 when JARID2 associates in PRC2.2[37]. This mechanism
381 operates in a similar manner to allosteric stimulation when the histone H3K27me3 tail binds to EED[8].
382 While the allosteric stimulation of the peptide, provided in trans, is affected in the L50S context, at high
383 concentrations, the peptide begins to stimulate PRC2-L50S (Figure 6D). When full-length JARID2 forms
384 a stable complex with PRC2, this peptide is likely present at high local concentration to provide some
385 level of allosteric stimulation, which may help explain why the H3K27me3 pattern in the L50S context
386 appears similar to the pattern when PRC2.2 is active, but PRC2.1 is not. The accessory factors for
387 PRC2.1, the PCLs, are DNA binding proteins which are thought to help recruit/stabilize PRC2.1 to the
388 regions that gain highest H3K27me3[35]. Despite the histone methylation pattern similarity to PCL KO, we
389 do not observe a loss of EZH2 recruitment, and presumably the full PRC2 is bound even at the regions
390 that lose the most H3K27me3. This behavior of normal/higher PRC2 recruitment, yet lower H3K27
391 methylation, more closely resembles mutants that disrupt the protein interaction network that promotes
392 allosteric stimulation from EED methylated peptide binding to higher EZH2 activity[10]. Currently, we
393 interpret the L50S context as potentially a combination effect on PRC2.1 mechanism and disruption of
394 allosteric activation, where both could contribute to the molecular outcome. This suggests a de-coupling
395 of the PCL-dependent mechanism, favoring JARID2-dependent activity. Interestingly, JARID2 was found
396 to be down-regulated by a microRNA in B cells during germinal center formation[38], which prevents
397 JARID2-mediated apoptosis. We predict that EZH2-L50S may tip the balance toward JARID2, which may
398 then lead to B cell apoptosis instead of expansion in germinal centers. This would help explain how EZH2
399 is required for B cell expansion in GCs[4], perhaps through a PRC2.1-dominant mechanism. This finding
400 is consistent with a role for PCL3 (PHF19) from PRC2.1 in GC B cell expansion and production of
401 antibody-secreting cells[39]. This model is also supported by recent work demonstrating that loss of JARID2
402 promotes B cell output while loss of EZH2 reduces B cell differentiation and exit from the bone marrow[40].
403
404 In conclusion, our work highlights a human genetic context associated with B cell dysfunction and loss,
405 resulting in CVID, based on a hypomorphic variant that affects the Polycomb system. The CVID patients

406 developed CVID in adulthood, suggesting that EZH2-L50S, as a heterozygous genetic context, does not
407 affect human development as Weaver Syndrome does. This is likely due to the compensation of the wild-
408 type allele and the fact that EZH2-L50S is not as severe a loss of function as Weaver Syndrome
409 mutations, nor does it act as a dominant negative as WS mutations can[41]. With the significant prevalence
410 of this rare variant in the human population, inclusion of EZH2 testing in immunodeficiencies should be
411 considered, and future work is needed to determine if other factors may interact to determine the clinical
412 outcome of those that bear EZH2-L50S.
413
414
415
416
417 **Materials and Methods**
418
419 **Patient testing in the clinic**
420 The clinical samples were collected according to institutional guidelines and with the approval of the
421 Colorado Multiple Institutional Review Board. Profiles for IgG, IgM and IgA and were measured by
422 standard clinical laboratory methods. Immunization tests were done with commercially available,
423 FDA-approved vaccines. Pneumovax-23 (Merck), Hemophilus b conjugate (GSK) and tetanus toxoid
424 (& Diptheria) (Sanofi Pasteur) were given in the standard doses. Serum for antibody titers and
425 measurements of antibody avidity were collected prior to immunization and approximately 4 weeks
426 after immunizations. These vaccines were selected because the antibody response to 1) (tetanus)
427 is T-cell dependent; 2) (pnuemovax-23) is T-cell independent; and 3) (hemophilus b conjugate)
428 depends on T-cell help. After the blood was clotted, serum was collected from centrifuged samples
429 and stored at -80°C. Titers of antibodies in serum pairs were assayed together using commercial
430 multiplex bead assays as specified by the manufacturers. Expected “normal” responses for the
431 purposes of this study, as suggested in the Practice Parameter for the Diagnosis and Management
432 of Primary Immunodeficiency[42], are ≥ 4-fold increases in antibody titers after immunizations with

433 tetanus toxoid and Hemophilus influenza b conjugate. Expected results after immunization with 23-
434 valent pneumococcal vaccine are either a ≥4-fold increase in titer or attainment of a titer of 1.3 μg/ml
435 or greater for at least 70% of the serotypes that are assayed[42]. Lymphocyte populations and sub-
436 populations were quantitated from heparinized venous blood that had been centrifuged on Ficoll-
437 Paque gradients. The mononuclear cells were tagged with selected monoclonal antibodies and
438 quantitated by flow cytometry.

439
440

441 **Patient genetic sequencing**

442
443 The clinical samples were collected according to institutional guidelines and with the approval of the
444 Colorado Multiple Institutional Review Board. Patient PBMC samples from Ficoll-Paque gradient were
445 subjected to standard DNA extraction protocols and prepared for Illumina sequencing library. Whole
446 genome sequencing at 125X coverage was performed by the University of Colorado Comprehensive
447 Cancer Center Genomics Core Facility.

448

449 The freeze 9 genotype call set is produced by the Freeze 8 variant calling pipeline performed by the
450 TOPMed Informatics Research Center (Center for Statistical Genetics, University of Michigan, Hyun Min
451 Kang, Jonathon LeFaive and Gonçalo Abecasis). The software tools in this version of the pipeline are
452 available on github at https://github.com/statgen/topmed_variant_calling. The following description refers
453 to specific components of the pipeline. These variant calling software tools are under continuous
454 development; updated versions can be accessed at http://github.com/atks/vt or
455 http://github.com/hyunminkang/apigenome.

456
457 The GotCloud pipeline detects variant sites and calls genotypes for a list of samples with aligned
458 sequence reads. Specifically, the pipeline for freeze 8 consists of the following six key steps: Sample
459 quality control, Variant detection, Variant consolidation, Genotype and feature collection, Inference of
460 nuclear pedigree, Variant filtering. These procedures have been integrated into the current release of the
461 GotCloud software package at https://github.com/statgen/topmed_variant_calling.

462

463 **Flow Cytometry**

464 Cryopreserved PBMC were thawed in 1 x Phosphate Buffered Saline (PBS) containing 1% Fetal Bovine
465 Serum (FBS) and 0.1% Sodium Azide and blocked for 10 min at room temperature in 1 x PBS containing
466 10% human serum and 10% mouse serum. After washing, cells were incubated for 30 min at 4°C with
467 antibodies specific for CD3, CD4, CD8, CD11b, CD11c, CD14, CD15, CD16, CD19, CD45, CD56, CD66,
468 and HLADR. Cells were washed and permeabilized with Transcription Factor Buffer Set (BD Pharmingen)
469 and incubated for 1 h at 4°C with antibodies specific for tri methyl histone H3. Samples were collected on
470 an LSR Fortessa X-20 Cell Analyzer (BD Biosciences) and analyzed with FlowJo v10 software.

471

472 **Protein alignment**

473

474 EZH2 orthologs protein sequences from the indicated species were obtained from the NCBI database.
475 Sequence alignment was performed using msa package in R[43] that provides an interface for Clustal W[44].

476 **Plasmids**

477 Wild-type and L50S EZH2 coding sequences were cloned into pRD-RIPE [29] to be used in Cre/Lox
478 generation of cell lines (see below).

479 **Cell culture and cell lines**

480 Parental LoxP HEK293T[29] and derived cell lines were routinely grown in Dulbecco's Modification of
481 Eagle's Medium 1x with 4.5 g/L glucose, L-glutamine and sodium pyruvate (Corning, DMEM1x). DMEM
482 1x was supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Avantor Seradigm), 100 IU/mL Penicillin/
483 and 100 µg/mL Streptomycin (Gibco). Blasticidin (Gibco) 300 ng/mL and Puromycin 3 µg/mL (Sigma-
484 Aldrich) were added to the growth media when needed.

485 All LoxP-containing cell lines followed the same protocol for cassette switching, as described[28]. Cells
486 were co- transfected with cassette plasmid with 1% (wt/wt) of a Cre encoding plasmid (pBT140, addgene
487 #27493). Donor plasmids with transgenes have a tetracycline transactivator construct, as well. Plasmids
488 were transfected with Lipofectamine LTX reagent (Thermo Fisher Scientific 5338030) and Opti-MEM
489 (Thermo Fisher Scientific 31985070) following the manufacturer's protocol for 24 hr. After 24 hr in non-
490 selective medium, puromycin-resistant colonies were expanded in growth medium supplemented with 5
491 µg/mL puromycin. Three independent WT and L50S cell lines were produced through this method (WT-
492 1, -2, 3; L50S-1, -2, -3).

493

494 **Immunoblotting**

495 Cells were grown as described above in 10-cm dishes, washed once with 1X PBS, treated with 1 ml of
496 0.25% trypsin (Corning), pelleted, and supernatant was discarded. Approximately 1 x 10^6 cells were then
497 homogenized as described[45] with 200 µL of TOPEX+ buffer (50mM Tris-HCl pH7.5,300 mM NaCl, 0.5%
498 Triton X-100, 1% SDS, 1mM DTT, 1X Complete EDTA-free Protease Inhibitor (Roche Applied Science),
499 33.33 U/mL Benzonase Nuclease HC (EMD-Millipore). Samples were then incubated for 20 min on ice,
500 and then centrifuged for 20 min, 4ºC at 13,000 rpm. Supernatant was transferred to a fresh tube, and
501 protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo-Scientific).
502 Samples were run on SDS-PAGE gels, transferred to nitrocellulose or PVDF membranes, and blocked
503 with TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% milk for 30 min at room
504 temperature. Membranes were then incubated in TBST with 3% BSA containing one of the following
505 primary antibodies: H3K27me3 (9733, Cell Signaling Technology) with a dilution of 1:5,000 during 1 hour
506 at room temperature; H3K27me2 (9728, Cell Signaling Technology) with a dilution of 1:5,000 during
507 overnight at 4ºC; H3K27me1 (61016, Active Motif) with a dilution of 1:1,000 during overnight at 4ºC; H3-
508 HRP (21054, Abcam) with a dilution of 1:100,000 during 1 hour at room temperature; EZH2 (5246, Cell
509 Signaling Technology) with a dilution of 1:2,000 during overnight at 4ºC; FLAG (F1804, Sigma-Aldrich)

510 with a dilution of 1:400 during overnight at 4ºC; SUZ12 (D39F6, Cell Signaling Technology) with a dilution
511 of 1:500 during overnight at 4ºC; EED (85322, Cell Signaling Technology) with a dilution of 1:1000 during
512 overnight at 4ºC; B-tubulin (Y1061, UBPBio) with a dilution of 1:1,000 during overnight at 4ºC.
513 Membranes were then washed 3 times for 5 min and incubated in TBST with 3% BSA containing the
514 corresponding anti-rabbit or anti-mouse secondary HRP-conjugated antibody (1706516 or 1705046,
515 BIORAD) with a dilution of 1:20,000 during 1 hour at room temperature. Membranes were then washed
516 3 times for 5 min with TBST and visualized using chemiluminescence Western Chemiluminescent HRP
517 Substrate (WBKLS0500, Immobilon).

518

519 **CUT&Tag**

520 H3K27me3, EZH2, and FLAG signals were profiled using the CUTANA Direct-to-PCR CUT&Tag Protocol
521 (V2) from EpiCypher. Briefly, cells were grown under doxycycline inducible conditions (300 ng/mL) for 8
522 days, harvested with 0.25% Trypsin-EDTA solution (Corning, 25-053-cl) and counted in Countess Cell
523 Counting Chamber Slides (Invitrogen, C10228) using 0.4% Trypan Blue Solution (Gibco, 15252061).

524 To extract nuclei, a total of 1 x 10^6 cells were washed in 1 mL 1x PBS, then 1.1 x 10^5 cells were centrifuged
525 and resuspended in 100 µL cold Nuclei Extraction Buffer (20 mM HEPES-KOH pH 7.9, 10 mM KCl, 0.1%
526 Triton x-100, 20% glycerol, 0.5 mM spermidine and 1x Complete protease inhibitor(Millipore-Sigma). To
527 bind nuclei to CUTANA Concanavalin A Beads (EpiCypher, 21-1401), nuclei samples were centrifuged,
528 resuspended in 105 µL of cold Nuclei Extraction Buffer, and 100 µL of sample were transferred into a
529 200 µL tube containing 10 µL of activated Concanavalin A Beads. To bind primary antibody to target
530 regions, bound nuclei on Concanavalin A Bead samples were centrifuged, resuspended in 50 µL of cold
531 Antibody 150 Buffer (20 mM HEPES-KOH pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 1x protease
532 inhibitor, 0.01% digitonin, 2 mM EDTA), then specific antibodies were added to the samples and
533 incubated overnight at 4 ºC. Antibodies used: 1 µL of H3K27me3 (9733, Cell Signaling Technology), 1

534 µL of EZH2 (5246, Cell Signaling), 1 µL of anti-FLAG (F1804, Sigma-Aldrich) or 0.5 µL IgG (13-0042,
535 Epicypher).

536 To bind secondary antibody to primary antibody, 0.5 µL of matching secondary antibody, anti-rabbit (13-
537 0047, Epicypher) or anti-mouse (13-0048, Epicypher), were diluted in 50 µL of Digitonin 150 Buffer (20
538 mM HEPES-KOH pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 1x protease inhibitor, 0.01% digitonin),
539 added to the corresponding primary antibody, and samples were then incubated at room temperature for
540 30 min. To bind CUTANA pAG-Tn5 (EpiCypher, 15-1017) to secondary antibody, samples were washed
541 two times with 200 µL of cold Digitonin 150 Buffer, resuspended in 50 µL of cold Digitonin 300 Buffer (20
542 mM HEPES-KOH pH 7.5, 300 mM NaCl, 0.5 mM spermidine, 1x protease inhibitor and 0.01% digitonin),
543 2.5 µL of pAG-Tn5 was added to each sample, samples were then incubated for 1 hr at room temperature.

544 To perform Tn5 tagmentation over chromatin, samples were washed two times with 200 uL of cold
545 Digitonin 300 Buffer, resuspended in 50 µL of Tagmentation Reaction Buffer (20 mM HEPES pH 7.5, 300
546 mM NaCl, 0.5 mM spermidine, 1x protease inhibitor, 10 mM MgCl2, and 0.01% digitonin), and incubated
547 for 1 hr at 37 ºC. To stop the tagmentation reaction and perform release of tagmented chromatin into
548 solution, samples were placed on a magnet and supernatant was discarded. Tagmented chromatin
549 bound to magnetic beads was then resuspended in 50 µL of equilibrated to room temperature Pre-Wash
550 150 Buffer (20 mM HEPES-KOH pH 7.5, 150 mM NaCl). Magnetic beads were placed back on a magnet,
551 supernatant was discarded, and resuspended in 5 µL SDS Release Buffer (10 mM TAPS pH 8.5 and
552 0.1% SDS). Tagmented chromatin was released by incubation for 1 hr at 58ºC and quenched using 15
553 uL of SDS Quench Buffer (0.67% Triton x-100).

554 To perform library amplification, 2 µL of i5 universal primer (EpiCypher), 2 µL i7 barcoded primer
555 (EpiCypher) and 25 µL NEBNext High Fidelity 2x PCR Master Mix (NEB, M0541S) were added to
556 tagmented chromatin samples, the following PCR conditions were used: 5 min at 58°C, 5 min at 72°C,
557 45 sec at 98°C, 16 – 19 cycles of 15 sec at 98°C, 10 sec at 60°C and final extension of 1 min at 72°C.
558 Remaining oligos and undesired PCR products were cleaned up using 1.3X High Prep (MAGBIO,

559 AC60005) magnetic beads following manufacturer recommendations. Amplified libraries were washed
560 away from magnetic beads in nuclease free water. To check for library concentration and fragment size
561 distribution, Qubit 1X dsDNA High Sensitivity Assay Kit (Invitrogen, Q33231) and High Sensitivity D1000
562 ScreenTape Assay for TapeStation (Agilent, 5067-5584) were used. Libraries were paired-end
563 sequenced by Novogene or University of Colorado Cancer Center Genomics Core services.

564

565 **CUT&Tag data analysis**

566 Data analysis was performed using certain approaches described previously[46]. Initial data pre-processing
567 was done using Cutadapt (Martin, 2011): Illumina adaptors were removed from sequencing reads, the
568 reads were then trimmed to a maximum of 140 bp, and shorter reads than 35 bp were discarded.

569 Trimmed Read 1 and Read 2 alignment to human genome version hg38 was done using Bowtie2[47] with
570 the following parameters, *--local --very-sensitive-local --no-unal –no-mixed --no-discordant --I 10 --X*
571 *2000*. Duplicated reads were removed using the following Samtools[48] commands, *samtools sort -n*
572 *<FILE.sam>* | *samtools fixmate -m - -* | *samtools sort -* | *samtools markdup -s -r - <FILE.out.sam>*. Aligned
573 reads in sam format were processed to the bam format using the following commands, *samtools view -b*
574 *-F 0x04 <FILE.out.sam> > <FILE.out.bam>*. Read length size distribution was analyzed using the
575 command *bamPEFragmentSize* from deepTools2[49], then fragments within a size from 100 bp to 300 bp
576 were sieved using *alignmentSieve*, and used in the following analysis.

577 Biological and/or technical sieved replicates were merged using the following command *samtools merge*
578 *-n <FILE.merged.out.sieved.bam> <FILE1.out.sieved.bam> <FILE2.out.sieved.bam>*. CUT&Tag peaks
579 were called using SEACR[50] with the following arguments *-c 0.05 -n norm -m stringent*. Bigwig files were
580 generated using *bamCoverage*[49] with the following arguments *--normalizeUsing CPM --*
581 *effectiveGenomeSize 2913022398 --extendReads –binSize 10 --blackListFilename <BLACKLIST.file>*.
582 Differential peak analysis was done using DiffBind (http://bioconductor.org)[51]. Peak summits were re-

583 centered and re-sized to +/- 1 Kb using *dba.counts(summits = 2000),* blacklisted regions were excluded
584 from the analysis using *dba.blacklist()*, data was normalized to library size using the argument
585 *DBA_NORM_LIB*, and only reads in peaks were used for the differential binding calculation using the
586 argument *DBA_LIBSIZE_PEAKREADS*. To generate a differential analysis report of all re-centered
587 binding sites, the command *dba.report()* was used. MA plots and volcano plots were generated using the
588 commands *dba.plotMA()* and *dba.plotVolcano()*, respectively. "WT>L50S" peaks with a FDR < 0.05, as
589 well as WT=L50S" peaks were filtered out from the differential analysis report, then annotated to Gencode
590 annotation v47[52] using ChIPseeker[53]. No "WT<L50S" peaks with a FDR < 0.05 were identified. Annotation
591 bar plot and distance to TSS plot were done using *plotAnnoBar()* and *plotDistToTSS()* functions,
592 respectively. Annotated peaks within +/- 2 Kb to TSSs were filtered, gene coordinates were obtained and
593 the corresponding bed files were generated using homemade R scripts. Heatmaps for scored regions
594 under re-centered peak summits +/- 5 Kb as well as TSS associated peaks were generated using
595 *plotHeatmap* function from deeptools[49]. Genome browser visualization plots were done using
596 *Plotgardener*[54].

597 **Code availability**

598 Code available upon request.

599 **Data availability**

600 GEO submission availability forthcoming.

601 **Immunoprecipitation**

602 10 µg of FLAG antibody (F1804, Sigma-Aldrich) was pre-bound to 200 µL Dynabeads Protein G (Thermo
603 Fisher Scientific, 10004D) and resuspended in Lysis Buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-
604 40, 2 mM EDTA pH 8.0 and 1X Complete Protease Inhibitor from Millipore-Sigma). To extract protein
605 complexes, trypsinized cell samples were washed twice with 1X PBS, centrifuged, and proteins were
606 extracted with 1.5 volume more than cell pellet of Lysis Buffer. Protein extracts were then incubated at

607 4ºC for 60 min with rotation and centrifuged at 13,000 rpms in a microfuge for 15 min at 4ºC.
608 Immunoprecipitation was performed by mixing 1 mg/ml of protein extract diluted in Lysis Buffer with 200
609 µL of bead/FLAG antibody suspension and incubated overnight at 4ºC with rotation. Immunoprecipitated
610 proteins were then washed three times with Lysis Buffer on a magnet and resuspended in 50 µl 1X SDS
611 buffer. SDS-PAGE and immunoblotting was performed as described above.

612 **Histone methyltransferase assays**

613 HMTase assays were performed in a total volume of 20 µL containing methyltransferase buffer (50 mM
614 Tris (pH 8.8), 2 mM $MgCl_2$, 20% Triton-X, and 1 mM TCEP) with 100 µM *S*-adenosylmethionine (New
615 England Biolabs), 914 nM histone octamer, and recombinant human WT and L50S PRC2 complexes
616 titrated at points of 0, 150, 310, and 770 nM. The reaction mixture was incubated for 30 min at room
617 temperature and stopped by adding 5 µL of 5X SDS loading dye containing BME. After HMT reactions,
618 samples were incubated for 5 min at 95°C and separated on SDS-PAGE gels. Gels were then subjected
619 to wet transfer (30% MeOH transfer buffer) of histones to 0.45-µm polyvinylidene difluoride membranes
620 (Millipore), and protein was detected by Western blot analysis using primary αRb H3K27me2 antibody
621 (Cell Signaling Technology, 9728S, D18C8), secondary antibody (Bio-Rad, #1705046), and H3-HRP
622 (horseradish peroxidase) (Abcam, #ab21054).
623
624 HMTase assays were performed in a reaction buffer containing methyltransferase buffer (10mM HEPES
625 NaOH (pH 7.5), 2.5 mM $MgCl_2$, 4% glycerol, 0.25 mM EDTA and 0.1 mM DTT). This was used to bring
626 the final reaction volume up to 15 µL, with 99.3 µM *S*-adenosylmethionine (New England Biolabs), 601
627 nM JARID2, a titration of JARID2me3 peptide from 648nM to 64.9uM, 160 nM nucleosome, and 570 nM
628 recombinant human PRC2 complexes under the following conditions. Nucleosomes were reconstituted
629 according to[55]. The DNA template is a version of the 601 DNA sequence with 38 bp upstream linker and
630 a small downstream 11 bp linker with a sticky end, 196 bp total. The reaction mixture was incubated for
631 30 min at 30°C and stopped by adding 3.75 µl of 5X SDS loading dye. After HMTase reactions, samples
632 were incubated for 5 min at 95°C and separated by SDS-PAGE. Gels were then subjected to wet transfer

(30% MeOH transfer buffer) of histones to 0.45-µm polyvinylidene difluoride membranes (Millipore), and protein was detected by western blot analysis using primary αRb H3K27me3 antibody (Cell Signaling Technology, 9733S, C36B11), H3K27me2 antibody (Cell Signaling Technology, 9728S, D18C8), secondary antibody (Bio-Rad, #1705046), and H3-HRP (horseradish peroxidase) (Abcam, #ab21054).

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