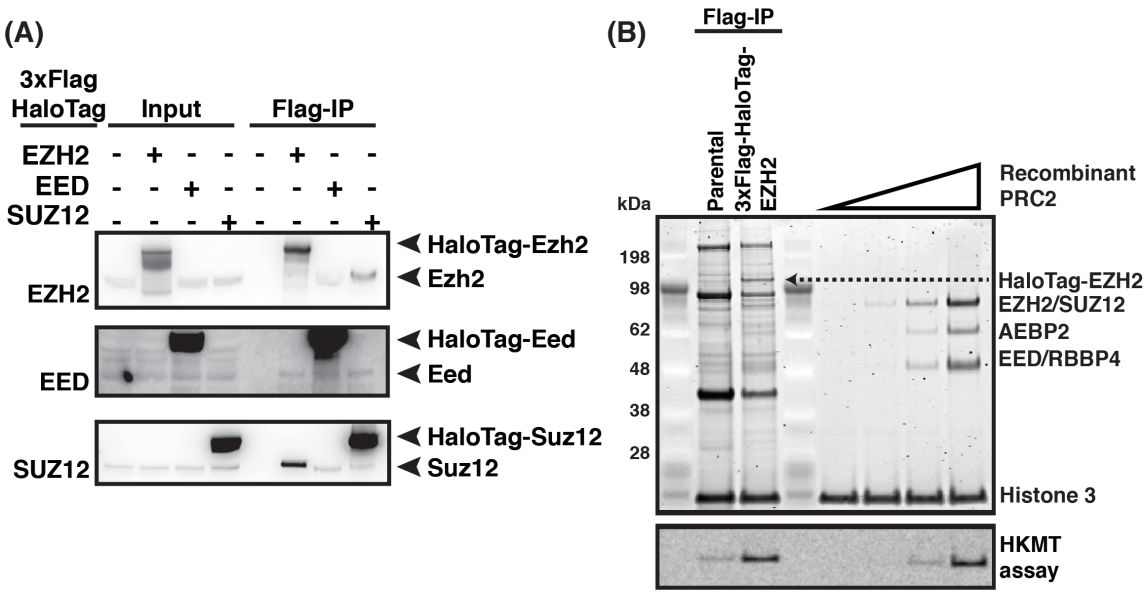
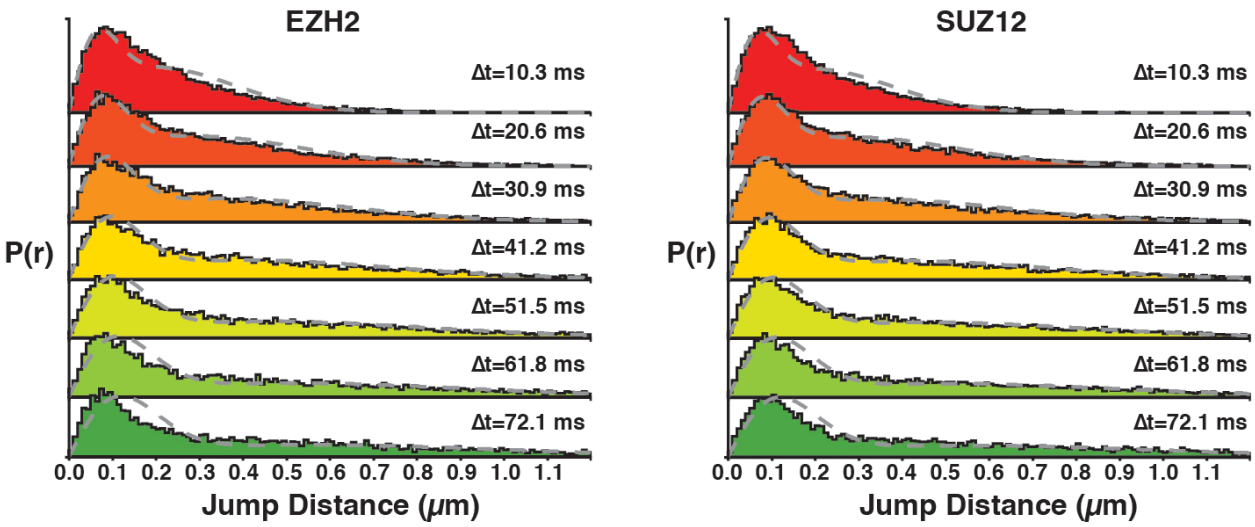
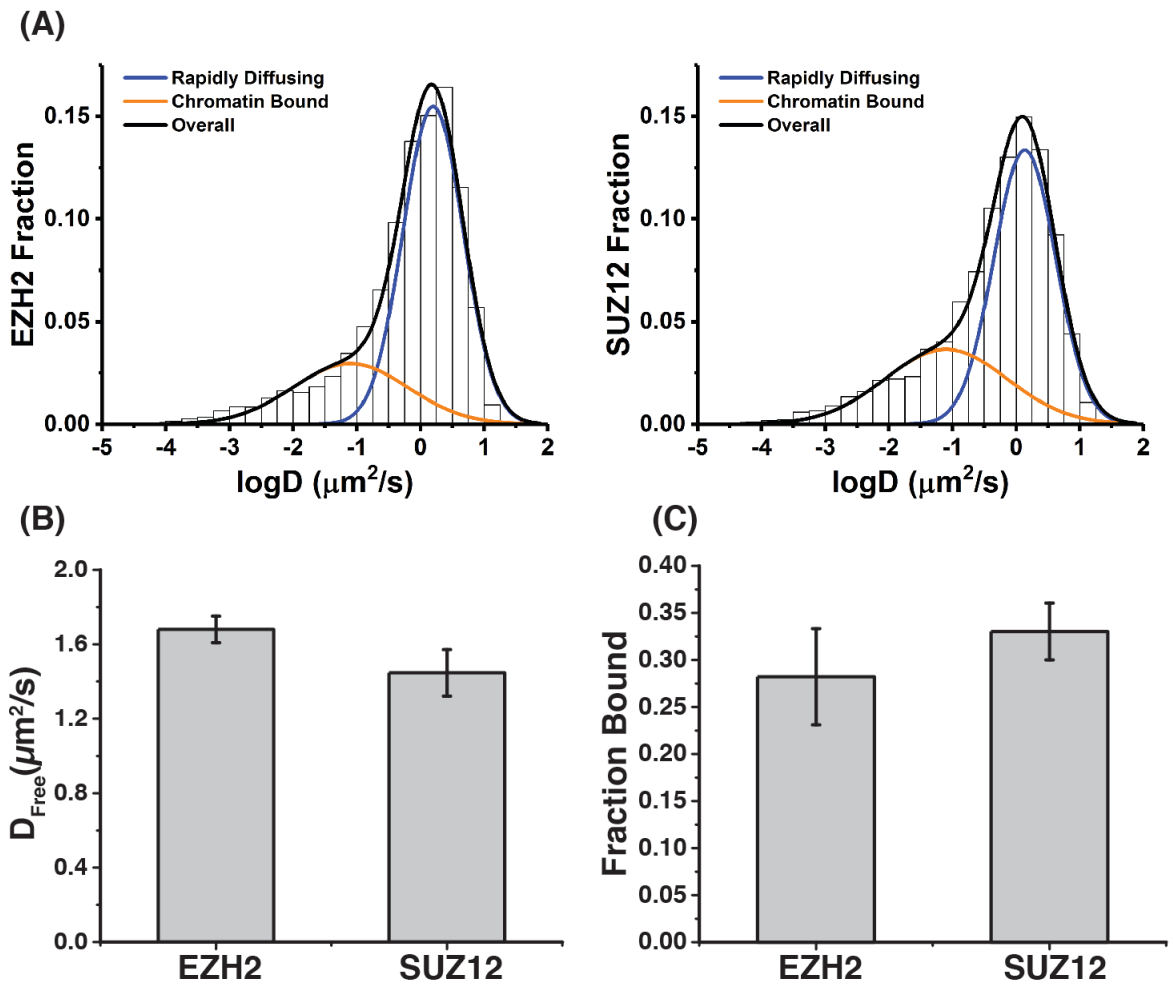


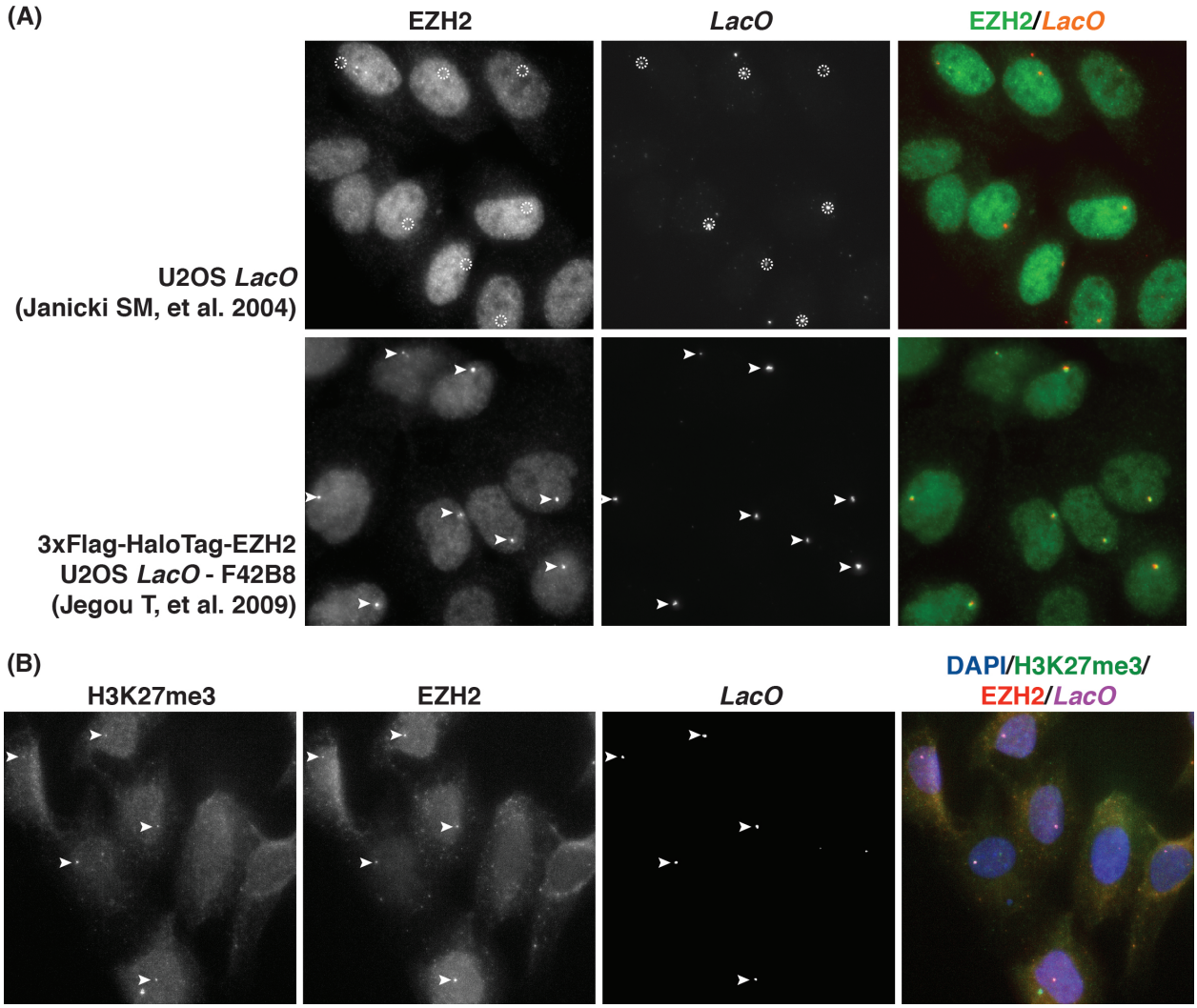
Supplemental Figure 1. The 3xFlag-HaloTag does not disrupt PRC2 assembly or activity. (A) Western blot of PRC2 subunits after αFlag co-immunoprecipitation. PRC2 subunits fused to a 3xFlag-HaloTag were transiently expressed in HEK293T cells. αFlag immunoprecipitation was performed on cell lysate from both un-transfected and transfected cells. Western blots using αEZH2, αEED, and αSUZ12 antibodies were performed on 1% of the input lysate and on 20% of the αFlag immunoprecipitation. The 3xFlag-HaloTag fusion protein and endogenous protein bands are indicated by arrows on the right. (B) αFlag co-immunoprecipitation and activity assay from genome-edited 3xFlag-HaloTag-EZH2 cells. Immunoprecipitation was performed on parental cells and genome-edited 3xFlag-HaloTag-EZH2 cells. A histone lysine methyltransferase (HKMT) assay (bottom), using Histone3 and S-Adenosyl Methionine (C^{14}) as substrates, was performed on the immunoprecipitated samples (left) and recombinantly purified PRC2 from SF9 insect cells (right). The activity assay samples were imaged with SyproRuby protein stain as well as autoradiography.



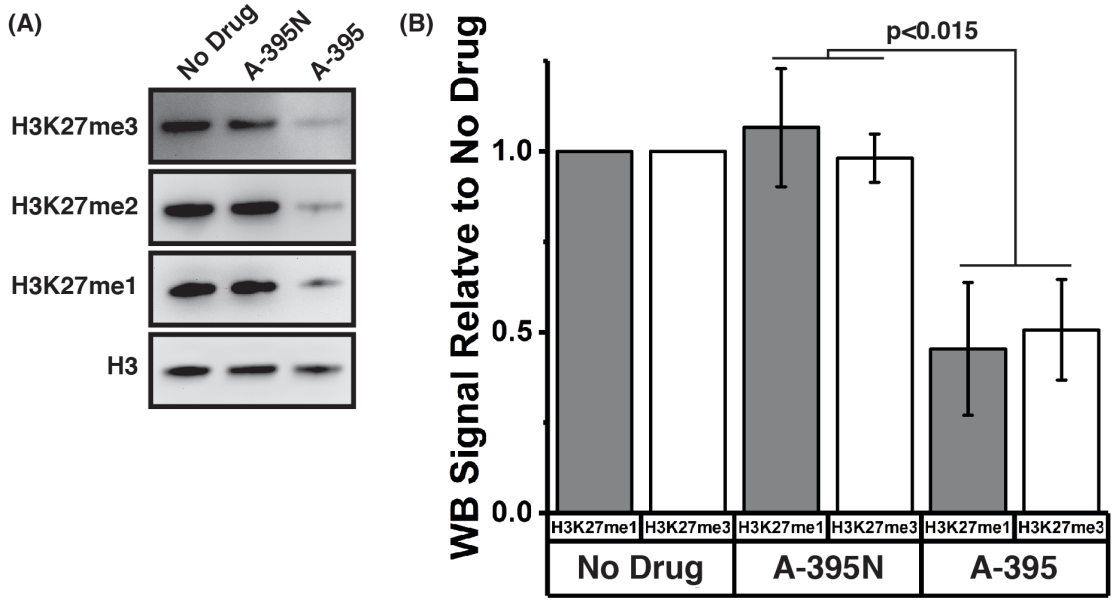
Supplemental Figure 2. Spot-on jump length distribution plots for EZH2 and SUZ12. 3xFlag-HaloTag-EZH2 and 3xFlag-HaloTag-SUZ12 cells were labeled with JF646 and imaged at 97.5 Hz. These plots describe the probability that these proteins will move a certain distance within a given time interval. The time intervals are indicated on the right by Δt ranging from 10.3 msec to 72.1 msec. These plots were fit to a model assuming that both a free and bound population of EZH2 and SUZ12 exist (gray dashed line). This fit was used to extract the D_{Free} and fraction bound values shown in Figure 2.



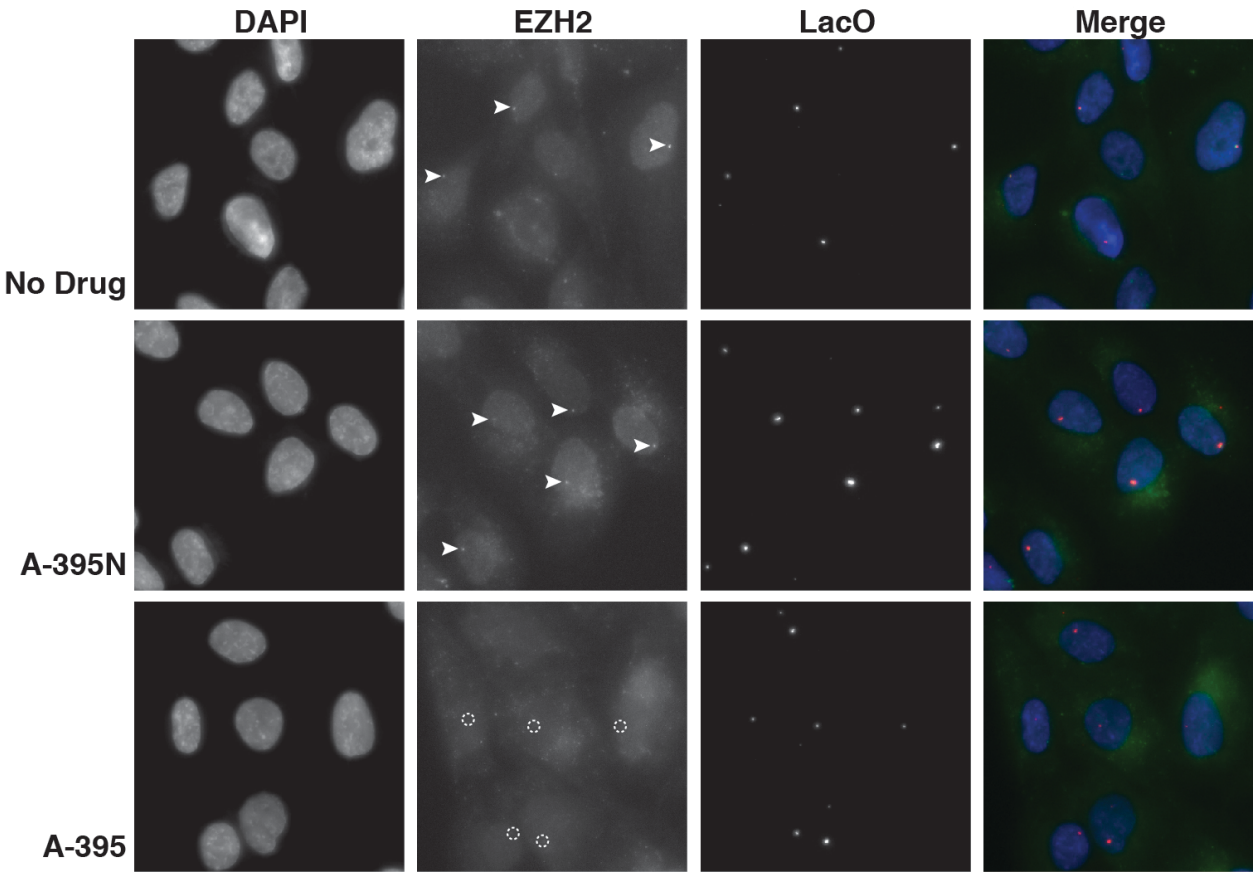
Supplemental Figure 3. Imaging EZH2 and SUZ12 at 46 Hz and analyzing trajectories with MSDanalyzer underestimates D_{Free} and overestimates the fraction bound compared to imaging at 97.5 Hz and using spot-on. (A) Histograms of the distribution of diffusion coefficients for 3xFlag-HaloTag-EZH2 and 3xFlag-HaloTag-SUZ12. 3xFlag-HaloTag-EZH2 or 3xFlag-HaloTag-SUZ12 cells were labeled with JF646 (described in main methods section) and imaged using HiLo illumination (Tokunaga et al. 2008), at 46 Hz, with 20% AOTF. Particles were localized, tracked, and evaluated with SLIMfast and evalSPT. Diffusion coefficient analysis was performed using MSDanalyzer software (Tarantino et al. 2014; Normanno et al. 2015) instead of spot-on. Each histogram was fit using two Gaussian distributions that represent the chromatin-bound and rapidly diffusing fractions. (B) D_{Free} was determined by the center of rapidly diffusing Gaussian. These values are underestimated compared to those shown in Figure 2. (C) Fraction bound was determined by the area under each of the peaks [FractionBound = $A_{chromatin\text{-}bound}/(A_{chromatin\text{-}bound}+A_{rapidly\ diffusing})$]. The fraction bound is also overestimated compared to the fraction bound shown in Figure 2. (Average and SD of N=3 biological replicates. N>12 cells within each biological replicate).



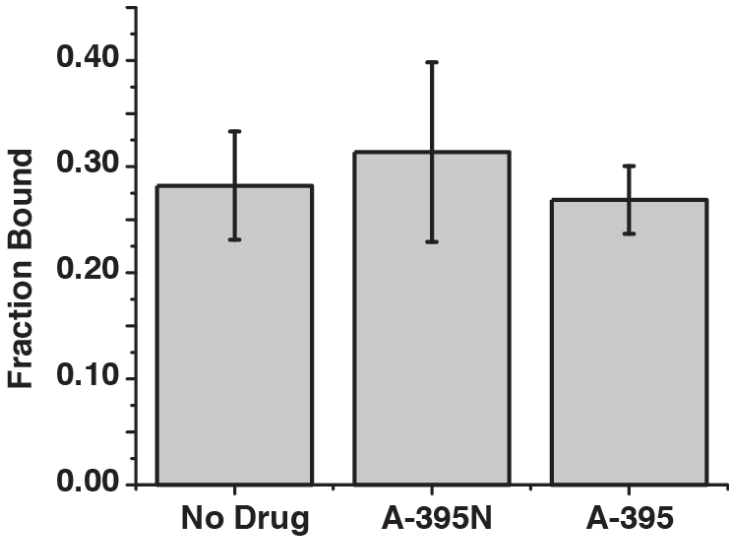
Supplemental Figure 4. EZH2 and H3K27me3 are enriched at the LacO array in the F42B8 cell line. (A) Fixed cell imaging of 3xFlag-HaloTag-EZH2 genome edited cells using different U2OS LacO parental cell backgrounds. Dashed circles indicate regions where EZH2 does not colocalize with the LacO array and arrows indicate colocalization of EZH2 with the LacO array. The parental cell line used for genome-editing is indicated on the left side of the figure. (B) Fixed cell imaging of EZH2 and H3K27me3 in 3xFlag-HaloTag-EZH2 (F42B8) cells. HaloTag-EZH2 was labeled with JF549. Arrows indicate enrichment of EZH2 and H3K27me3 at the LacO array.



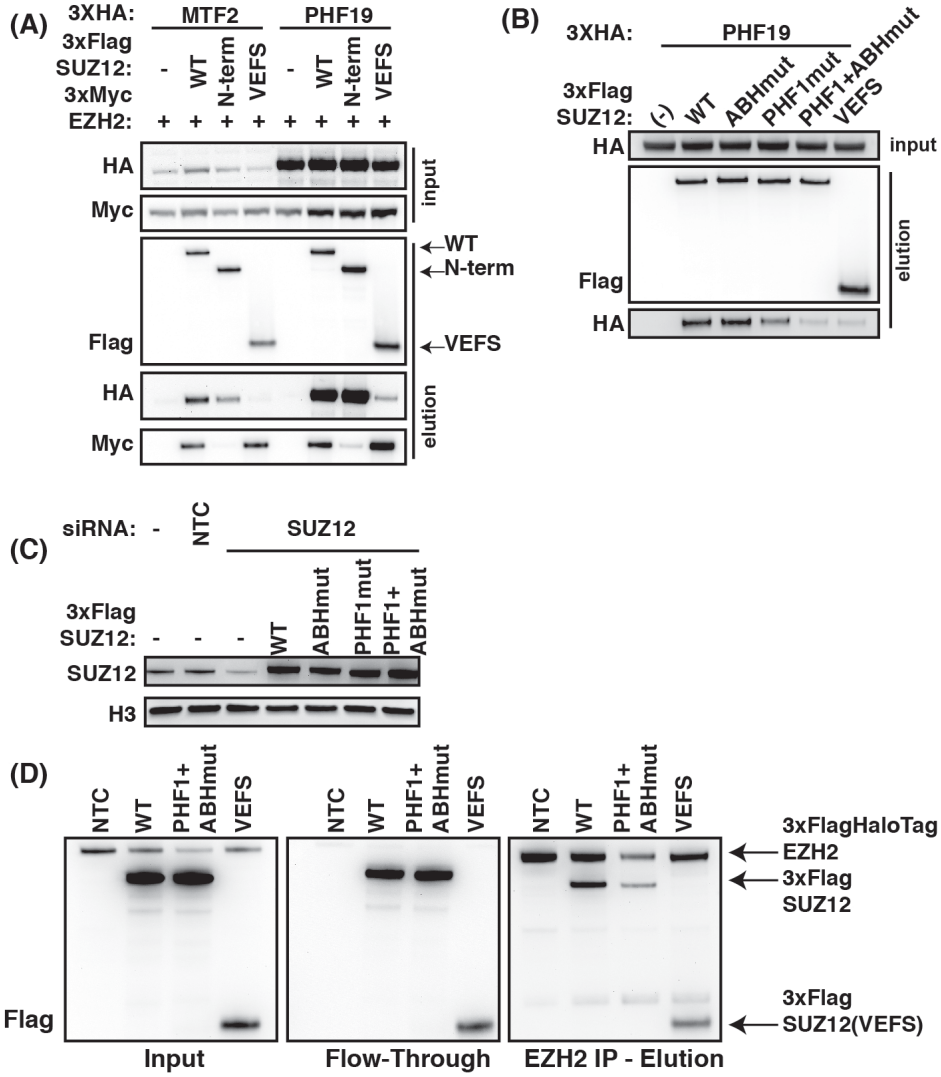
Supplemental Figure 5. A-395 depletes H3K27me1, H3K27me2, and H3K27me3 compared to untreated and A-359N treated cells. (A) Western blot analysis to compare H3K27 methylation levels between untreated, A-395N, and A-395 treated 3xFlag-HaloTag-EZH2 cells. 3xFlag-HaloTag-EZH2 cells were left untreated, or treated with A-395N or A-395 for 72 hours, imaged, and lysed in SDS lysis buffer. Western blots using αH3, αH3K27me1, αH3K27me2, and αH3K27me3 antibodies were performed on each of the samples. (B) Quantification of the H3K27me1 (gray bars) and H3K27me3 (white bars) levels. The western blot signal intensity was normalized to H3 signal and the signals within both treatment conditions were plotted relative to the No Drug condition (average and SD from N=3, biological replicates).



Supplemental Figure 6. Visual comparison of EZH2 colocalization with the LacO array after A-395 treatment. Wider view (compared to Figure 4A) of fixed cell imaging on HaloTag-EZH2 cells that were left untreated, or treated with A-395N or A-395. HaloTag-EZH2 was labeled with JF549. Arrows indicate sites of colocalization of EZH2 with the LacO array and dashed circles indicate lack of colocalization.

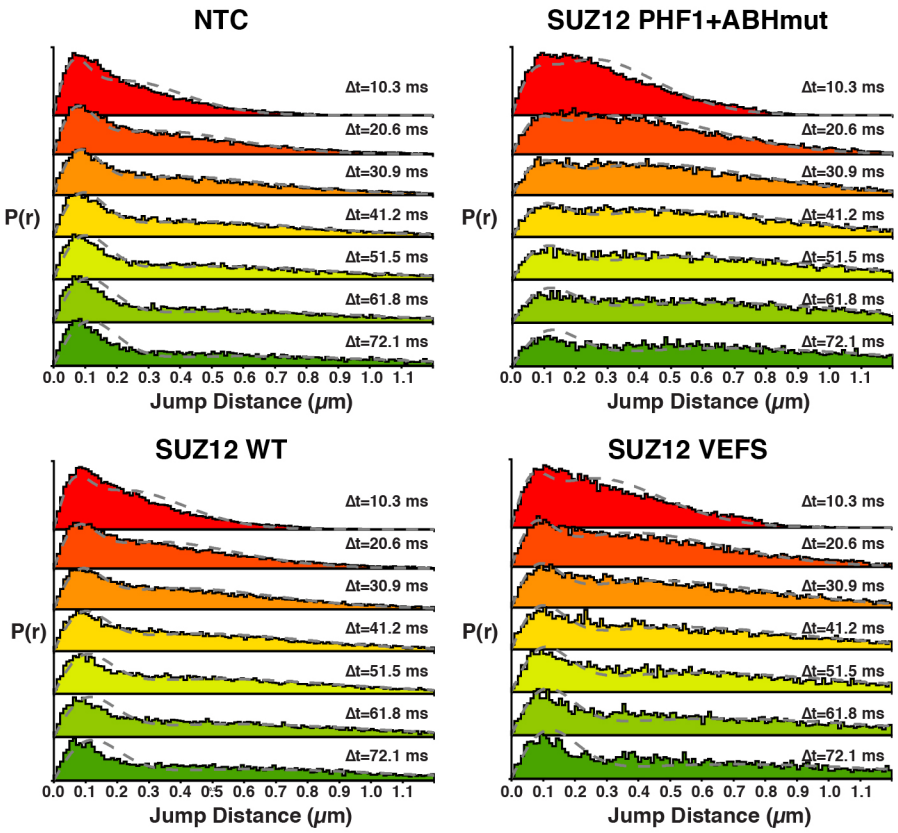


Supplemental Figure 7. The chromatin-bound fraction of 3xFlag-HaloTag-EZH2 in untreated, A-395N or A-395 treated cells. Imaging at 46 Hz and fraction bound analysis was performed as described in supplemental figure 3 in untreated cells and cells that were treated with either A-395N or A-395 for 72 h (average and SD of N=3 biological replicates using >12 cells per replicate). There is no significant difference in the chromatin bound fraction of HaloTag-EZH2 in the presence of the EED inhibitor.



Supplemental Figure 8. MTF2 and PHF19 interact with the N-terminus of SUZ12 and N-terminal SUZ12 mutations do not disrupt the interaction between SUZ12 and 3xFlag-HaloTag-EZH2. (A) αHA, αMyc, and αFlag western blots on input and elution samples from an αFlag immunoprecipitation. 3xFlag-SUZ12, 3xHA-MTF2 or 3xHA-PHF19, and 3xMyc-EZH2 were expressed in HEK293T cells. 3xFlag-SUZ12 was immunoprecipitated, eluted with 3xFlag peptide and all three proteins were detected by western. The N-terminus of SUZ12 is necessary to co-immunoprecipitate MTF2 and PHF19. (B) Western blots using the indicated primary antibody on input and elution samples from an αFlag IP of 3xFlag-SUZ12 mutants. The interaction between SUZ12 and PHF19 is disrupted in the PHF1mut and the ABH+PHF1mut variants of SUZ12. (C) αSUZ12 and αH3 western blots showing SUZ12 knockdown and ectopic expression. HaloTag-EZH2 cells were transfected with siRNA and SUZ12 variants, lysed in SDS loading buffer, and western blot analysis was performed using αSUZ12 and αH3 antibodies. NTC=non-targeting control siRNA; SUZ12 = siRNA to SUZ12 3'UTR. (D) αFlag western blots of input, flow-through, and elution samples following an αEZH2 immunoprecipitation. 3xFlag-HaloTag-

EZH2 cells were transfected with the indicated 3xFlag-SUZ12 variants, a siRNA pool targeting the SUZ12 3'UTR, and nuclear BFP as a transfection marker. NTC refers to cells transfected with a non-targeting pool of siRNA and nuclear BFP. After imaging (Figure 5), cell lysate was subjected to an αEZH2 immunoprecipitation. 3xFlag-SUZ12 mutants associated with EZH2 equivalently compared to 3xFlag-SUZ12 WT.



Supplemental Figure 9. Jump length distributions generated by spot-on of 3xFlag-HaloTag-EZH2 when bound to the indicated SUZ12 variants. These plots describe the probability that 3xFlag-HaloTag-EZH2 will move a certain distance within the indicated time interval.

Supplemental Methods

Flag immunoprecipitation of ectopically expressed 3xFlag-HaloTag- PRC2 subunits (Fig. S1A)

$5.5x10^6$ HEK293T cells were plated on a 10 cm^2 tissue culture dish 17 hours before transfection. Transfection of pcDNA3.1 3xFlag-HaloTag-EZH2, pcDNA3.1 3xFlag-HaloTag-EED, or pcDNA3.1 3xFlag-HaloTag-SUZ12 was performed using 17.5 μg of plasmid and Lipofectamine 2000 (Thermo Fisher #11668019), per manufacturer's protocol. 24 hours after transfection, cells were expanded to a 15 cm tissue culture dish.

48 hours after transfection, cells were trypsin harvested and washed twice with cold 1x PBS (National Diagnostics #CL-253), each time centrifuging at 100 rcf. Cells were lysed in 1000 μl of cold NP-40 lysis buffer (1% Nonidet P 40 Substitute (Sigma-Aldrich #74385), 25 mM Tris pH 7.5, 5% glycerol, 150 mM NaCl, 2.5 mM $MgCl_2$, 1x protease inhibitor cocktail (Thermo Fisher #A32965), 2 mM tris(2-carboxyethyl)phosphine (TCEP) pH 7.0 (Thermo Fisher #20490), 250 units/mL Benzonase (Sigma-Aldrich #E1014)) for 30 min on ice vortexing every 5 min. Cell lysate was clarified by centrifugation at >13,000 rcf at 4°C for 10 min. 900 μL of lysate was bound to 20 μl of pre-equilibrated αFlag affinity resin (Sigma-Aldrich #A2220) resuspended in 100 μl of lysis buffer. The binding was performed for 2 hours at 4°C rotating end-over-end.

Beads were then centrifuged at 100 rcf at room temperature and washed 3x with room-temperature wash buffer (1% Nonidet P 40 Substitute (Sigma-Aldrich #74385), 25 mM Tris pH 7.5, 5% glycerol, 150 mM NaCl, 2.5 mM $MgCl_2$,). The final wash was completely removed and bound proteins were eluted for 30 min at room temperature in 75 μl of Wash buffer supplemented with 150 ng/μl 3xFlag peptide (Sigma-Aldrich #F4799). The elution was spun through paper filter spin cups (Thermo Fisher #PI69700) and resuspended in 25 μl NuPAGE LDS Sample Buffer (4x) (Thermo Fisher Scientific #NP0008) supplemented with 143 mM β-mercaptoethanol (Sigma-Aldrich #M6250). Samples were run on NuPAGE 4-12% gels and Western blot analysis was carried out as detailed in the main methods.

Flag immunoprecipitation on endogenous 3xFlag-HaloTag-EZH2 for HKMT activity assay (Fig. S1B)

$2x10^8$ parental and 3xFlag-HaloTag-EZH2 U2OS cells were trypsin harvested and washed twice with cold 1x PBS (National Diagnostics #CL-253), each time centrifuging at 100 rcf. Cells were resuspended in 2.5x cell volume of cold NP-40 lysis buffer (detailed above) and placed on ice for 30 min. Lysate was clarified by centrifugation at >13,000 rcf for 10 min at 4°C. Clarified lysate was combined with 10 μl of packed and pre-equilibrated αFlag affinity resin (Sigma-Aldrich #A2220) resuspended in 50 μl of lysis buffer and rotated end-over-end at 4°C for 2 hours.

Beads were then washed 4 times with 1 mL of room-temperature wash buffer (detailed above) and 1 time with HKMT assay buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 2 mM 2-mercaptoethanol, 0.1 mg/ml Bovine Serum Albumin, 5% v/v glycerol). The final wash was completely removed and bound proteins were eluted for 30 min at room-temperature in 15 μl of HKMT assay buffer supplemented with 150 ng/μl 3xFlag peptide (Sigma-Aldrich #F4799). The elution was spun through paper filter spin cups (Thermo Fisher #PI69700).

HKMT activity assay (Fig. S1B)

9 μl of the elution from the above immunoprecipitation, or of recombinant PRC2 (purification of recombinant PRC2 is detailed in (Davidovich et al. 2013)), was combined with 1.38 μg of Histone H3.1 (New England BioLabs #M2503) and 24 μM S-[methyl-^{14}C]-adenosylmethionine (PerkinElmer #NEC363050UC), in a final reaction volume of 15 μl of HKMT assay buffer. Final PRC2 concentrations were 7.7, 23, 70, and 210 nM.
The reactions were incubated for 1 hour at 30 ^{o}C.

5 μl of NuPAGE LDS Sample Buffer (4x) supplemented with 143 mM β-mercaptoethanol was added to each sample to stop the reaction and samples were heated to 98^{o}C for 3 min, placed on ice for 2 min, and run at 150 V on a NuPAGE 4-12% Bis-Tris gel. SyproRuby protein stain (Thermo Fisher #S12000) was used on the gel, per manufacturer's protocol, and imaged on a TyphoonTRIO phosphoimager. Gels were then dried and developed with an autoradiography screen and imaged on a TyphoonTRIO phosphoimager.

EZH2 immunoprecipitation (Fig. S8C)

After imaging, cells were trypsin harvested and lysed in cold NP-40 lysis buffer detailed above. The lysate was incubated with αEZH2 (Cell Signaling # 5246) antibody at 4C overnight and then with Protein G agarose beads (EMD Millipore #IP05) at 4C for 1 hour. The agarose beads were then washed 4 times with wash buffer (detailed above) and the protein complexes were eluted by boiling in SDS elution buffer (Thermo Fisher #NP0008). Western blots were carried out as detailed in the main methods section.

Single-particle tracking (Fig. S3)

Single-particle trajectories were generated as previously detailed in (Schmidt et al. 2016). In brief, particles were localized and tracked using MatLab 2011b to run SLIMfast (Serge et al. 2008). The trajectories of the particles were evaluated using evalSPT (Normanno et al. 2015). Diffusion coefficients were obtained by running DiffusionSingle in MatLab 2017 (a gift of Zhe Liu) which uses the MSDanalyzer script (Tarantino et al. 2014). Diffusion coefficient histograms were fit using OriginPro graphing software. Histograms were fit using the minimal number of Gaussian distributions that generated an R^2>0.95. To standardize the fraction of molecules within the chromatin-bound distribution, we constrained the peak of the chromatin-bound population to be centered around logD = -1.1 $\mu m^2/s$ for both proteins.

Parameter	**Diffusion Analysis**
Lag time (ms)	22
λ_{ex} (nm)	647
λ_{em} (nm)	670
Pixel size (nm)	160
Numerical Aperture	1.49
Expected D_{max} ($\mu m^2/s$)	5
Tracklength	>5 frames
Deflation loops	0
Localization error	10^{-6}
Max competitors	1

Blinking (frames)	2

EZH2 and H3K27me3 immunofluorescence (Fig. S4A)

$1.5x10^5$ cells were plated in wells of a 24-well glass imaging dish (Cellvis #P24-1.5H-N) ~ 17 hours before performing immunofluorescence. (Note: In Figure 4A, Figure S4B, and Figure S6 3xFlag-HaloTag-EZH2 was labeled with 500 nM HaloTag JF549 for 5 min and washed with media before the cells were fixed, detailed in (Schmidt et al. 2016) and above. Whereas in Figure S4A, cells were left unlabeled for antibody-based immunofluorescence.)

Cells were fixed in 1x PBS supplemented with 3.7% formaldehyde (Thermo Fisher #BP531500) for 10 min at room temperature. Fixing solution was removed and cells were washed twice with PBS and permeabilized in 1x PBS containing 0.1% Triton X-100 for 5 min at room temperature. Cells were then blocked in blocking buffer (1x PBS, 0.1% Triton X-100, 3% Bovine Serum Albumin) for 30 min at room temperature. Blocking buffer was then removed and cells were incubated overnight at 4^oC with primary antibodies diluted in blocking buffer.

Cells were washed 3 times in 1x PBS and incubated for 1 hour, in the dark at room temperature, with secondary antibody diluted in blocking buffer. Cells were again washed 3 times with 1x PBS and then re-crosslinked in 1x PBS supplemented with 3.7% formaldehyde (Thermo Fisher #BP531500) for 10 min at room temperature in dark. Afterwards, cells were washed 4 times with 1x PBS and the final wash was completely removed. *LacO* DNA FISH was performed as detailed in the main methods.

Antibody	**Dilution**
EZH2 (Cell Signaling # 5246)	1/100
H3K27me3 (Cell Signaling #9733)	1/1600
αRabbit IgG AF488 (Invitrogen #A11034)	1/500

Supplemental western blot antibodies

Antibody	**Dilution**
EED (Santa Cruz Bio #28701)	1/200
H3K27me1 (Active Motif #61015)	1/1000
H3K27me2 (Cell Signaling #9728)	1/1000
SUZ12 (Cell Signaling #3737)	1/1000

Supplemental References

Davidovich C, Zheng L, Goodrich KJ, Cech TR. 2013. Promiscuous RNA binding by Polycomb repressive complex 2. *Nat Struct Mol Biol* **20**: 1250-1257.

Normanno D, Boudarene L, Dugast-Darzacq C, Chen J, Richter C, Proux F, Benichou O, Voituriez R, Darzacq X, Dahan M. 2015. Probing the target search of DNA-binding proteins in mammalian cells using TetR as model searcher. *Nat Commun* **6**: 7357.

Schmidt JC, Zaug AJ, Cech TR. 2016. Live Cell Imaging Reveals the Dynamics of Telomerase Recruitment to Telomeres. *Cell* **166**: 1188-1197 e1189.

Serge A, Bertaux N, Rigneault H, Marguet D. 2008. Dynamic multiple-target tracing to probe spatiotemporal cartography of cell membranes. *Nat Methods* **5**: 687-694.

Tarantino N, Tinevez JY, Crowell EF, Boisson B, Henriques R, Mhlanga M, Agou F, Israel A, Laplantine E. 2014. TNF and IL-1 exhibit distinct ubiquitin requirements for inducing NEMO-IKK supramolecular structures. *J Cell Biol* **204**: 231-245.

Tokunaga M, Imamoto N, Sakata-Sogawa K. 2008. Highly inclined thin illumination enables clear single-molecule imaging in cells. *Nat Methods* **5**: 159-161.