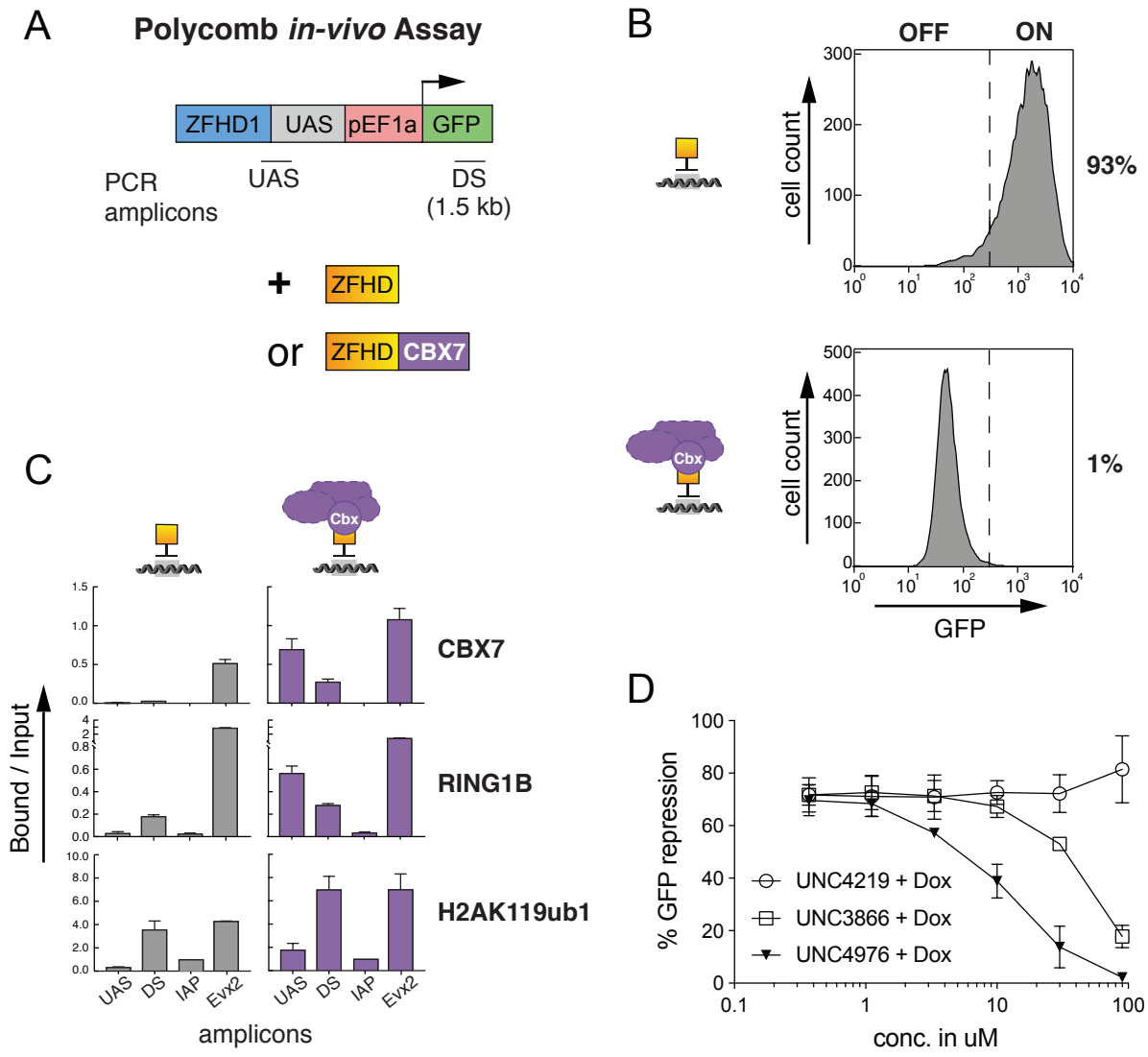


Figure 1. Design and readout of Polycomb in-vivo Assay. Related to Figure 1. A. An array of DNA binding sites including 12 x ZFHD1 and 4 x Gal4 UAS are located upstream of an EF1alpha promoter controlling constitutive expression of a GFP reporter gene. 12 x ZFHD1 DNA binding sites facilitate recruitment of ZFHD1 alone or as a chimeric fusion with human CBX7. **B.** Flow cytometry histogram shows GFP expression in response to expression of ZFHD1 alone (top) or ZFHD1-CBX7 fusion (bottom). Percentage indicates fraction of GFP-positive reporter mESCs. **C.** ChIP-qPCR analysis shows relative enrichment (Bound/Input) of CBX7, RING1B and H2AK119ub1 in response to ZFHD1 alone or ZFHD1-CBX7 tethering. ChIP-qPCR for H2AK119ub1 is normalized to IAP. IAP serves as negative control and Evx2 as positive control. **D.** Small molecule antagonism of propagation of Polycomb-mediated silencing in a separate TetOFF reporter cell line (Moussa et al., 2019). Treatment with control compound (UNC4219) does not impair maintenance of CBX7-induced GFP reporter gene silencing. In contrast, heritable silencing after Dox-induced TetR-CBX7 release is decreased in response to UNC3866 or UNC4976. Percentage of cells maintaining GFP silencing after six days of Dox treatment in response to increasing concentrations of CBX7 antagonists (UNC3866, UNC4976) or control compound (UNC4219). Data in **C** and **D** are presented as mean ± SD from two replicate experiments.

Isothermal Titration Calorimetry (ITC) Affinity Data		
Protein	**UNC3866 ITC K_d (μM)**	**UNC4976 ITC K_d (μM)**
CBX2	1.800 ± 0.210 (n=2)	1.665 ± 0.177 (n=2)
CBX4	0.094 ± 0.017 (n=2)	0.062 ± 0.004 (n=2)
CBX6	0.610 ± 0.008 (n=2)	0.561 ± 0.175 (n=2)
CBX7	0.097 ± 0.003 (n=2)	0.059 ± 0.018 (n=4)
CBX8	1.200 ± 0.021 (n=2)	n.d.*
CDYL2	0.850 ± 0.076 (n=2)	0.459 ± 0.123 (n=2)

Table 1. ITC Data Comparison for UNC3866 and UNC4976. Related to Figure 1. Data are presented as mean ± SD from at least two replicate experiments (n), *not stable in presence of DMSO required for UNC4976 solubilization.

Surface Plasmon Resonance (SPR) Kinetic Data		
CBX2		
	Residence Time ($1/k_d$) (s)	K_d (nM)
UNC4195	53.2	1020.0
UNC5355	34.4	1750.0
CBX4		
	Residence Time ($1/k_d$) (s)	K_d (nM)
UNC4195	46.7	28.3
UNC5355	50.3	26.7
CBX6		
	Residence Time ($1/k_d$) (s)	K_d (nM)
UNC4195	14.5	642.0
UNC5355	15.5	543.0
CBX7		
	Residence Time ($1/k_d$) (s)	K_d (nM)
UNC4195	56.2	42.0
UNC5355	46.1	53.8
CBX8		
	Residence Time ($1/k_d$) (s)	K_d (nM)
UNC4195	28.0	415.0
UNC5355	27.4	458.0
CDYL2		
	Residence Time ($1/k_d$) (s)	K_d (nM)
UNC4195	8.3	698.0
UNC5355	14.2	415.0

Table 2. SPR Data Comparison for biotinylated UNC3866 (UNC4195) and UNC4976 (UNC5355). Related to Figure 1. For each of the six chromodomains tested, residence time is reported as the inverse of the off-rate (k_d) and kinetic dissociation constants (K_d's) are reported and calculated from both kinetic on-rates (k_a) and off-rates (k_d) ($K_d = k_d/k_a$).

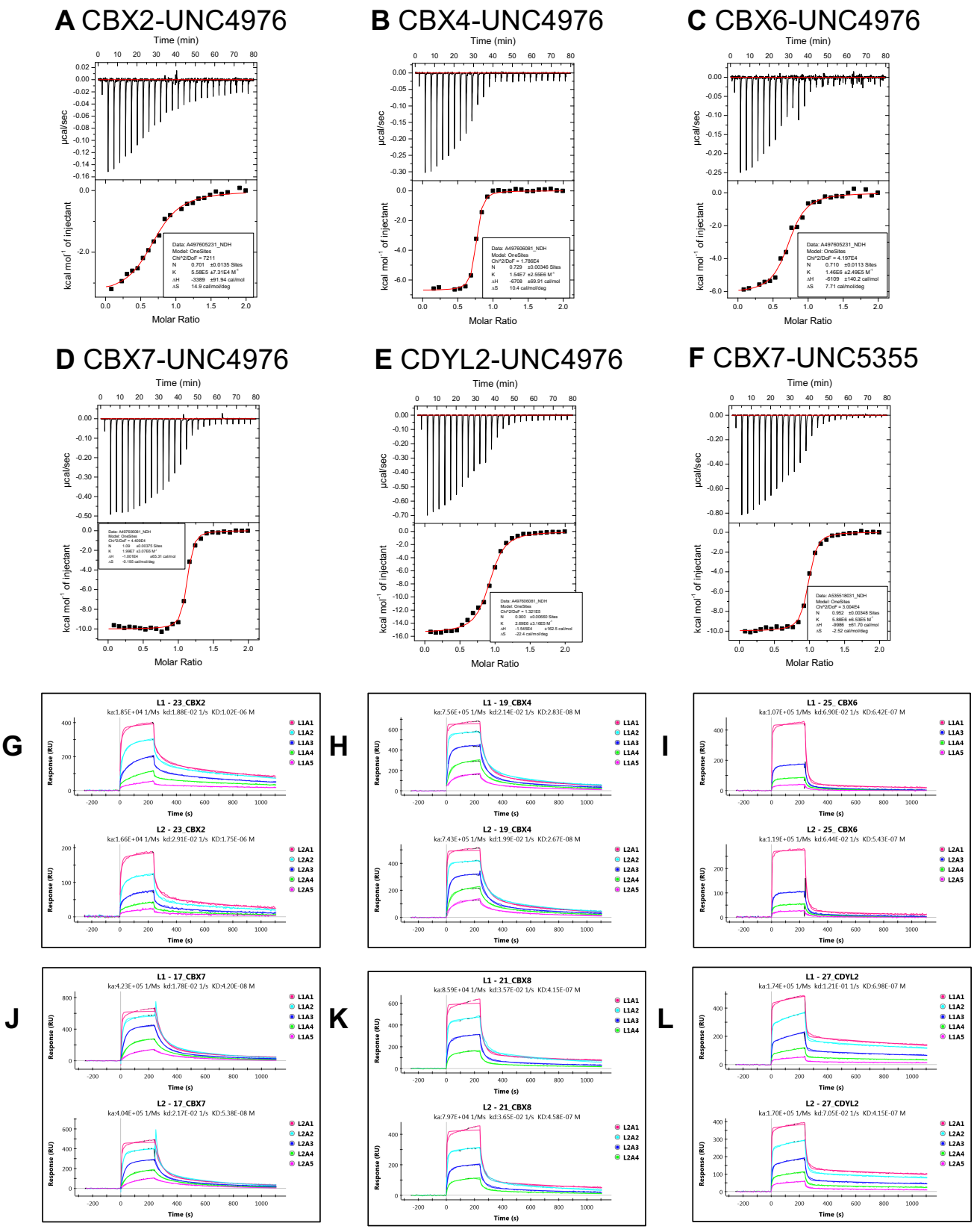
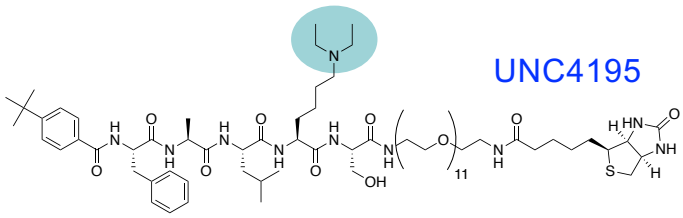
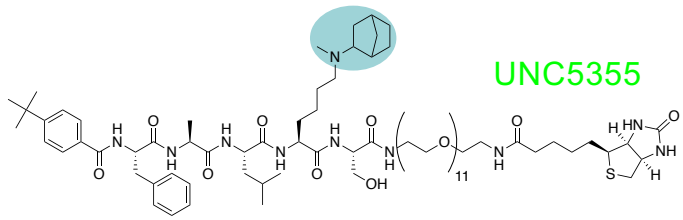

A CBX2-UNC4976
B CBX4-UNC4976
C CBX6-UNC4976
D CBX7-UNC4976
E CDYL2-UNC4976
F CBX7-UNC5355
Time (min)
µcal/sec
kcal mol⁻¹ of injectant
Molar Ratio
G
H
I
J
K
L
L1 - 23_CBX2
L2 - 23_CBX2
L1 - 19_CBX4
L2 - 19_CBX4
L1 - 25_ CBX6
L2 - 25_ CBX6
L1 - 17_CBX7
L2 - 17_CBX7
L1 - 21_CBX8
L2 - 21_CBX8
L1 - 27_CDYL2
L2 - 27_CDYL2
Response (RU)
Time (s)
L1A1
L1A2
L1A3
L1A4
L1A5
L2A1
L2A2
L2A3
L2A4
L2A5

Figure 2. Representative Data from Biophysical Experiments. Related to Figure 1. A-F. Representative Isothermal Titration Calorimetry (ITC) curves for all chromodomains tested with UNC4976 (**A-E**), and CBX7 tested with UNC5355 (**F**). **G-L.** Surface Plasmon Resonance (SPR) Sensorgrams from Kinetic Experiments. Sensorgrams for CBX2 (**G**), CBX4 (**H**), CBX6 (**I**), CBX7 (**J**), CBX8 (**K**), and CDYL2 (**L**) are shown. For each protein, UNC4195 data is shown on the top sensorgram and UNC5355 data is shown on the bottom sensorgram.

A





B

Biomatik Full Array 3

TUDOR	TUDOR	TUDOR	TUDOR	TUDOR	TUDOR+PHD	PHD
A1) 53BP1(1-2) **A2)** 53BP1(1-2)* **A3)** TDRD1-2 **A4)** TDRD1-3 **A5)** TDRD1-4 **A6)** TDRD2 **A7)** TDRD3	**B1)** TDRD4-1 **B2)** TDRD4-3 **B3)** TDRD4-4 **B4)** TDRD5 **B5)** TDRD6-5 **B6)** TDRD6-6 **B7)** TDRD6-7	**C1)** TDRD8 **C2)** TDRD9 **C3)** TDRD10 **C4)** TDRD11 **C5)** TDRD12-1 **C6)** ARID4A **C7)** ARID4B	**D1)** AKAP1 **D2)** LBR **D3)** PHF1 **D4)** PHF19 **D5)** PHF20 **D6)** PHF20-2 **D7)** PHF20L1	**E1)** SETDB1 **E2)** SMN **E3)** SMN* **E4)** SPF30 **E5)** UHRF1 **E6)** ZGPAT **E7)** JMJD2A(1-2)	**F1)** JMJD2B **F2)** JMJD2C **F3)** MTF2 **F4)** PHF1* **TUDOR** **F5)** Pombe1* **F6)** Spindlin1* **F7)** TDRKH*	**G1)** ING2 **G2)** TAF3 **G3)** TRIM24* **G4)** CHD5(1-2)* **CHROMO+PHD** **G5)** CHD3 **G6)** CHD4 **G7)** CHD5
CHROMO	**CHROMO**	**CHROMO**	**CHROMO**	**AGENET**	**IDCL**	**BROMO**
H1) ARID4A **H2)** ARID4B **H3)** CBX2 **H4)** CBX4 **H5)** CBX6 **H6)** CBX7 **H7)** CBX8	**I1)** HP1α/CBX5 **I2)** HP1β **I3)** HP1γ/CBX3 **I4)** CDY1 **I5)** CDYL1b **I6)** CDYL2 **I7)** MSL3	**J1)** CHD1 **J2)** CHD2 **J3)** CHD6 **J4)** CHD7 **J5)** CHD8 **J6)** CHD9 **J7)** TIP60	**K1)** MPP8 **K2)** MRG15 **K3)** MYST1/MOF **K4)** SMARCC1 **K5)** SMARCC2 **K6)** SUV39H1 **K7)** SUV39H2	**L1)** FMR1 **L2)** FXR1 **L3)** FXR2 **ANK** **L4)** GLP **L5)** IκB FL² **BAH** **L6)** ORC1	**M1)** PCNA **MBT** **M2)** L3MBTL1(1-3) **M3)** L3MBTL1(1-3)* **M4)** L3MBTL3 **PWWP** **M5)** DNMT3A² **M6)** NSD1 PWWP+PHD² **Zf-CW** **M7)** ZCWPW1	**N1)** SP140* **N2)** BRD4(1)* **N3)** WDR9(1-2)* **N4)** SMAP* **HEAT** **N5)** N-CAPD3(1-4) **N6)** N-CAPD3(5-8)

C

Methyl Domain Readers Array - BFA5

TUDOR	TUDOR	TUDOR	TUDOR	TUDOR	TUDOR+PHD	PHD	TUDOR	YTH
A1) 53BP1(1-2) [NP_005648.1] A2) 53BP1(1-2) [NP_005648.1] * A3) TDRD1-2 [NP_ 942090.1] A4) TDRD1-3 [NP_942090.1] A5) TDRD1-4 [NP_942090.1] A6) TDRD2 [NP_ 006853.2] A7) TDRD3 [NP_ 110421.1]	B1) TDRD4-1/RNF17 [NP_ 112567.2] B2) TDRD4-3/RNF17 [NP_ 112567.2] B3) TDRD4-4/RNF17 [NP_ 112567.2] B4) TDRD5 [NP_ 001186014.1] B5) TDRD6-5 [NP_ 001161831.1] B6) TDRD6-6 [NP_ 001161831.1] B7) TDRD6-7 [NP_ 001161831.1]	C1) TDRD8 [NP_ 001274744.1] C2) TDRD9 [NP_ 694591.2] C3) TDRD10 [NP_ 001091945.1] C4) TDRD11/SND1/p100TSN [NP_ 055205.2] C5) TDRD12-1 [NP_ 001104292.1] C6) ARID4A [NP_ 002883.3] C7) ARID4B [NP_ 001193723.1]	D1) AKAP1 [NP_ 003479.1] D2) blank D3) PHF1 [NM_ 002636.4] D4) PHF19 [NM_ 001286840.1] D5) PHF20 [NP_ 057520.2] D6) PHF20-2 [NP_ 057520.2] D7) PHF20L1 [NP_ 8571 02.4]	E1) SETDB1 E2) SMN [NP_ 000335.1] E3) SMN [NP_ 075012] * E4) SPF30/SMNDC1 [NP_ 005862.1] E5) UHRF1 [NP_ 001041666.1] E6) ZGPAT [NP_ 115916.3] E7) JMJD2A(1-2) [NP_ 055478.2]	F1) JMJD2B/KDM4B [AA144293.1] F2) JMJD2C/KDM4C [NP_ 055876.2] F3) MTF2 [NP_ 001157864.1] F4) PHF1 [NP_ 002627] * TUDOR F5) Pombe1 [CAA22823] * F6) Spindlin1 * F7) TDRKH **	G1) ING2 [NP_ 001555.1] G2) TAF3 [NP_ 114129.1] G3) TRIM24 PHD+BRD ** G4) blank CHROMO+PHD G5) CHD3 [NP_ 1005271.2] G6) CHD4 [NP_ 001264.2] G7) CHD5 [NP_ 056372.1]	H1) SGF29 [NP_ 612423.1] H2) SPIN1 [NP_ 006708] H3) TDRD3/TDRKH [NP_ 006853.2] H4) LIN9 [NP_ 775106.2] H5) CYB5R FL [NP_ 085056.2] H6) FKBP12 FL [AAA58476] CHNER H7) MeCP2 **	I1) YTHDC1 [Q96MU7] I2) YTHDC2 [Q9H6S0] I3) YTHDF1 [Q9BYJ9] I4) YTHDF2 [Q9Y5A9] I5) YTHDF3 [Q7Z739]
CHROMO J1) ARID4A [NP_ 002883.3] J2) ARID4B [NP_ 057458.4] J3) CBX2 [NP_ 005180.1] J4) CBX4 [NP_ 003646.2] J5) CBX6 [NP_ 055107.3] J6) CBX7 [NP_ 783640.1] J7) CBX8 [NP_ 065700.1]	CHROMO K1) HP1α/CBX5 [NP_ 001120793.1] K2) HP1β [NP_ 006796.1] K3) HP1γ/CBX3 [NP_ 036207.2] K4) CDY1 [NP_ 733841.1] K5) CDYL1b [AAI08725.1] K6) CDYL2 [BAC04720.1] K7) MSL3 [NP_ 523353.2]	CHROMO L1) CHD1 [NP_ 001261.2] L2) CHD2 [NP_ 001262.3] L3) CHD6 [NP_ 115597.3] L4) CHD7 [NP_ 060250.2] L5) CHD8 [NP_ 116410.1] L6) CHD9 [NP_ 079410.4] L7) TIP60 [NP_ 874369.1]	CHROMO M1) MPP8 [NP_ 059990.2] M2) MRG15 [NP_ 006782.1] M3) MYST1/MOF [NP_ 892003.2] M4) SMARCC1 [NP_ 003065.3] M5) SMARCC2 [NP_ 003066.2] M6) SUV39H1 [NP_ 003164.1] M7) SUV39H2 [NP_ 1180353.1]	AGENET N1) FMR1 [NP_ 002015.1] N2) FXR1 [NP_ 005078.2] N3) FXR2 [NP_ 004451.2] ANK N4) GLP [NP_ 079033.4] N5) IκB FL *² BAH N6) ORC1 [NP_ 001177747.1] AGENET+KH N7) FMRP [Q06787.1]	IDCL O1) PCNA [NP_ 872590.1] MBT O2) L3MBTL1(1-3) [NP_ 056293.4] O3) L3MBTL1(1-3) [NP_ 056293]* O4) L3MBTL3 [NP_ 115814.1] PWWP O5) DNMT3A *² O6) NSD1 PWWP+PHD [Q96L73]² O7) mBS95/ZYMND11 PHD +BROMO+PWWP *²	BROMO P1) SP140 [NP_ 009168] * P2) BRD4(1) P3) WDR9(1-2) [Q9NSI6] * P4) SMAP [NP_ 899203] * HEAT P5) N-CAPD3(1-8) [NP_ 056076.1] P6) N-CAPD3(5-8) [NP_ 056076.1] Zf-CW P7) ZCWPW1 [NP_ 060454]	YEATS Q1) YEATS4/GAS41 [NP_ 006521.1] Q2) ENL/MLLT1 [NP_ 005925] Q3) AF9/MLLT3 [NP_ 004520.2] Q4) YEATS2 [NP_ 060493]	HORMA R1) HORMAD1 [Q86X24] R2) HORMAD2 [Q8N7B1] R3) MAD2L1 [Q13257] R4) MAD2L2 [Q9UI95] R5) hMAD2 WT (134) [NM_ 002358.3] ** R6) hMAD2 R133E/Q134A (133) **

Figure 3. Reader Domain Protein Microarray Selectivity Data Comparison with Biotinylated Compounds. Related to Figure 1. A. Protein microarray data for UNC4195 (top) and UNC5355 (bottom) with bound proteins highlighted in teal and yellow boxes. **B.** Complete list of histone reader domains evaluated on the protein microarray screened against UNC4195. Mapping of individual reader domains is described by the legend in the top right corner. **C.** Complete list of histone reader domains evaluated on the protein microarray screened against UNC5355. Mapping of individual reader domains is described by the legend in the top right corner. Boxes A-G and J-P are identical to the microarray described in (**B**), additional domains screened against UNC5355 but not UNC4195 are described in boxes H, I, Q and R.

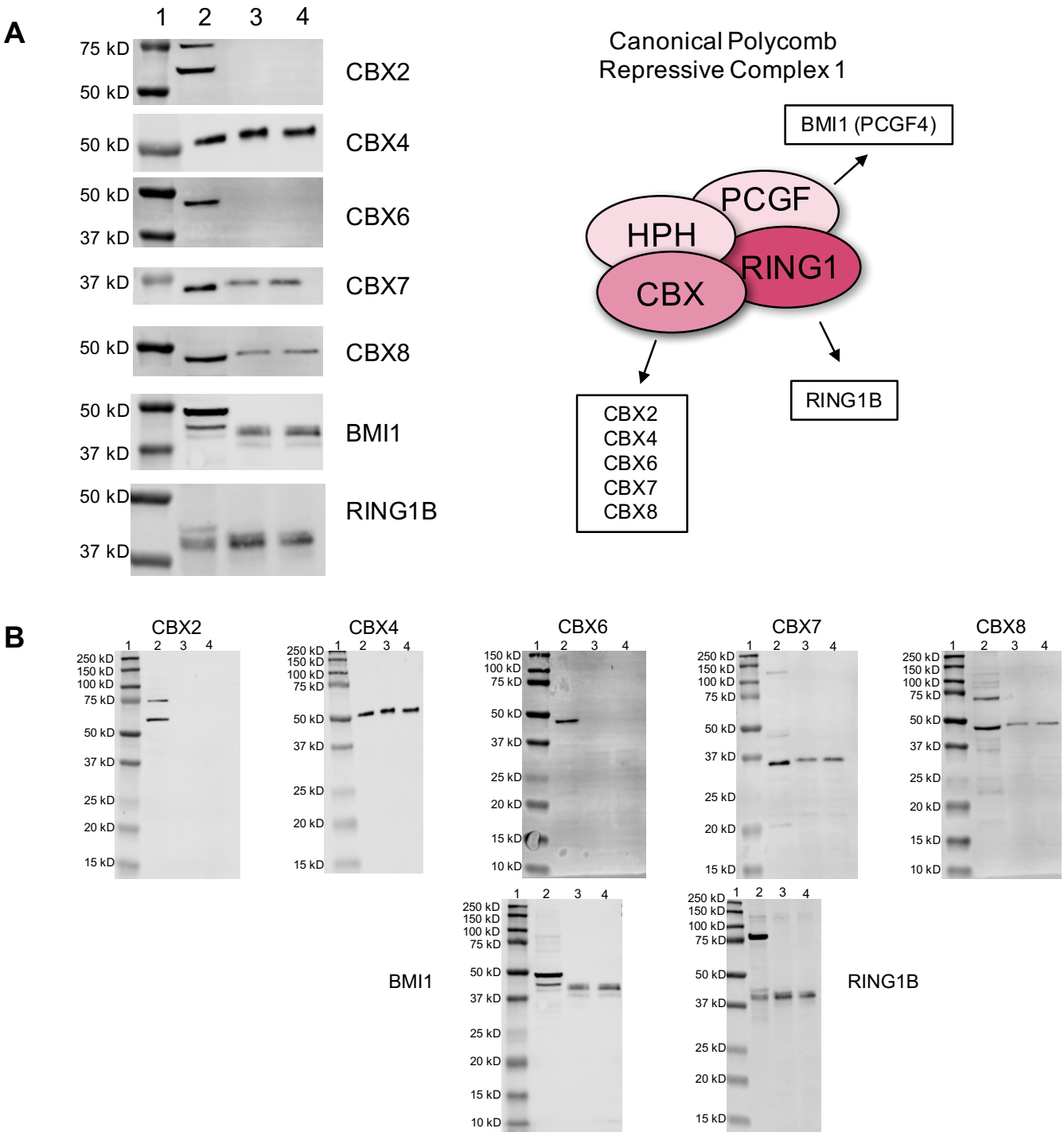


Figure 4. PC3 Lysate Pulldown Experiments with UNC4195 and UNC5355 Biotinylated Ligands. Related to Figure 1. A. (Left) Cropped blots highlighting bands of interest from PC3 lysate pulldowns with biotinylated ligands. (Right) Canonical PRC1 complex, with immunoblotted subunits listed. **B.** Uncropped western blots for individual proteins. For all blots in **A** and **B**, Lane 1= BioRad Precision Plus Protein Dual Color Standards (#1610374), Lane 2= PC3 Lysate Input, Lane 3= UNC4195 pulldown, and Lane 4= UNC5355 pulldown. Western blots shown are representative of three replicate experiments.

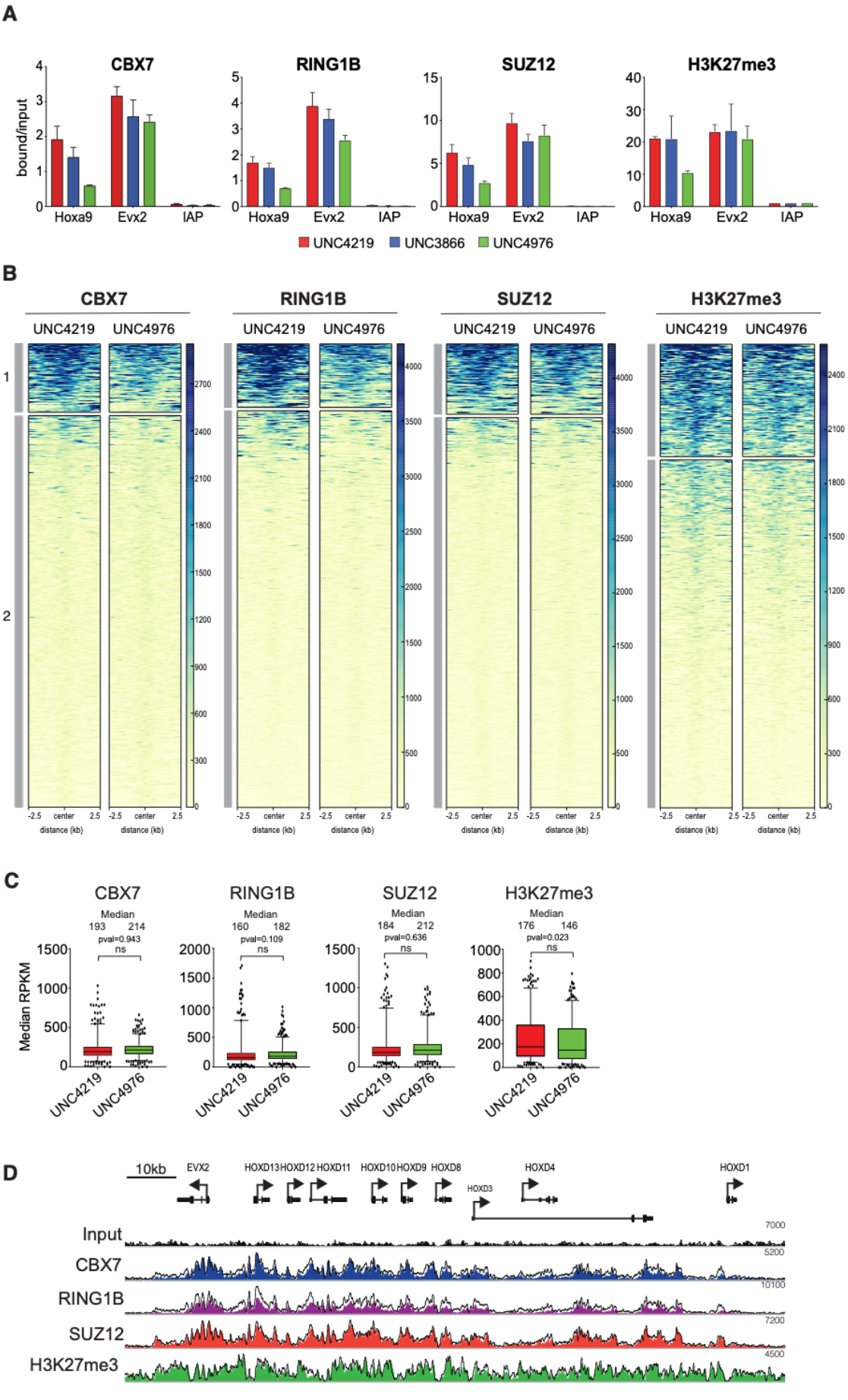

A
CBX7
RING1B
SUZ12
H3K27me3
bound/input
Hoxa9
Evx2
IAP
UNC4219
UNC3866
UNC4976
B
CBX7
RING1B
SUZ12
H3K27me3
UNC4219
UNC4976
distance (kb)
C
CBX7
RING1B
SUZ12
H3K27me3
Median
pval=0.943
pval=0.109
pval=0.636
pval=0.023
ns
Median RPKM
UNC4219
UNC4976
D
10kb
EVX2
HOXD13
HOXD12
HOXD11
HOXD10
HOXD9
HOXD8
HOXD4
HOXD3
HOXD1
Input
CBX7
RING1B
SUZ12
H3K27me3

Figure 5. UNC4976 efficiently displaces CBX-containing PRC1 from Polycomb target genes. Related to Figure 3. A. ChIP-qPCR analysis shows relative enrichment of CBX7, RING1B, SUZ12 and H3K27me3 at selected genes in mES cells treated for 4hrs with 20 μM of control compound UNC4219 (red), UNC3866 (blue) or UNC4976 (green). IAP serves as negative control. Analysis of H3K27me3 is normalized to IAP. Data are presented as mean ± SD from at least two replicate experiments. **B.** Heat maps showing enrichment of capture ChIP-seq for CBX7, RING1B, SUZ12 and H3K27me3 in mES cells treated for 4hrs with 20 μM of control compound UNC4219 or UNC4976. Signal is centered around peaks +/- 2.5 kb and plotted as FPKM normalized mapped reads and separated into “high” (1) and “low” (2) clusters. **C.** Boxplots of heat maps from capture ChIP-seq (**B**) show median RPKM normalized mapped reads of CBX7, RING1B, SUZ12 and H3K27me3 in “low” cluster (2). Statistical significance was calculated using unpaired t-test (pval<0.01). **D.** Screenshots of capture ChIP-seq enrichment at HoxD cluster for RING1B, CBX7, SUZ12 and H3K27me3. Capture ChIP-seq signal of control UNC4219 treatment is shown as an outlined line and UNC4976-treated capture ChIP-seq samples are shown in filled color tracks. All data in A-D are representative of at least two independent biological replicates.

ChIP-qPCR Primer Sequences		
Gene	**Forward Sequence**	**Reverse Sequence**
HoxA9	GGATAATCGCCTAGGTGTGACTTAG	CATCTCTTCTTGCCTCTCTGGG
Evx2	CGCAGCCCATCATTAAGAC	CGGACAAACTGGAGAACCTC
IAP	CTCCATGTGCTCTGCCTTCC	CCCCGTCCCTTTTTTAGGAGA

Table 3. ChIP-qPCR Primer Sequences. Related to Figure 3. Primer sequences for select Polycomb target genes used for ChIP-qPCR.

Capture-ChIP-seq Capture Probe Sequences						
Baits mm9 G11D genome			25 regions	genomic size		Polycomb target genes
chr1	4460681	4511256	**Sox17**	51	kb	y
chr1	17995977	18046157	**Intergenic Crisp4**	50	kb	m
chr2	74450084	74651064	**Hoxd cluster**	201	kb	y
chr2	105488775	105539252	**Pax6**	50	kb	y
chr2	153432137	153522909	**Commd7&Dnmt3b**	91	kb	n
chr3	34537459	34568988	**Sox2**	32	kb	n
chr3	87931198	88031358	**Mef2d**	100	kb	y
chr3	127332802	127342995	**Neurog2**	10	kb	y
chr4	88905858	88962573	**Cdkn2a&b&Gm12610**	57	kb	y
chr4	124294578	124395096	**Pou3f1**	101	kb	y
chr5	98563339	98814815	**Prdm8&Fgf5&1700007G11Rik**	251	kb	y
chr5	120094791	120194420	**Tbx3**	100	kb	n
chr6	122642090	122677539	**Nanog**	35	kb	n
chr7	110957144	111008233	**Hbb locus**	51	kb	n
chr7	137983359	138081333	**Plekha1&Htra1**	98	kb	n/m
chr7	149758083	149858985	**Igf2&H19**	101	kb	y
chr8	44361316	44426512	**Zfp42**	65	kb	n
chr9	89592758	89602918	**Tmed3**	10	kb	n
chr11	118870869	118961636	**Cbx2/4/8**	91	kb	y
chr12	87761185	87877500	**Esrrb**	116	kb	y
chr14	49256167	49306303	**Otx2**	50	kb	y
chr15	78819673	78905009	**G11D locus**	85	kb	n
chr16	91199329	91300463	**Olig1&2**	101	kb	y
chr17	35623948	35666758	**Oct4/Pouf51 locus**	43	kb	n
chr17	50414245	50444405	**Dazl**	30	kb	n

Table 4. Capture-ChIP-seq Probe Sequences. Related to Figure 3. Probe sequences for select Polycomb target genes used for Capture-ChIP-seq.

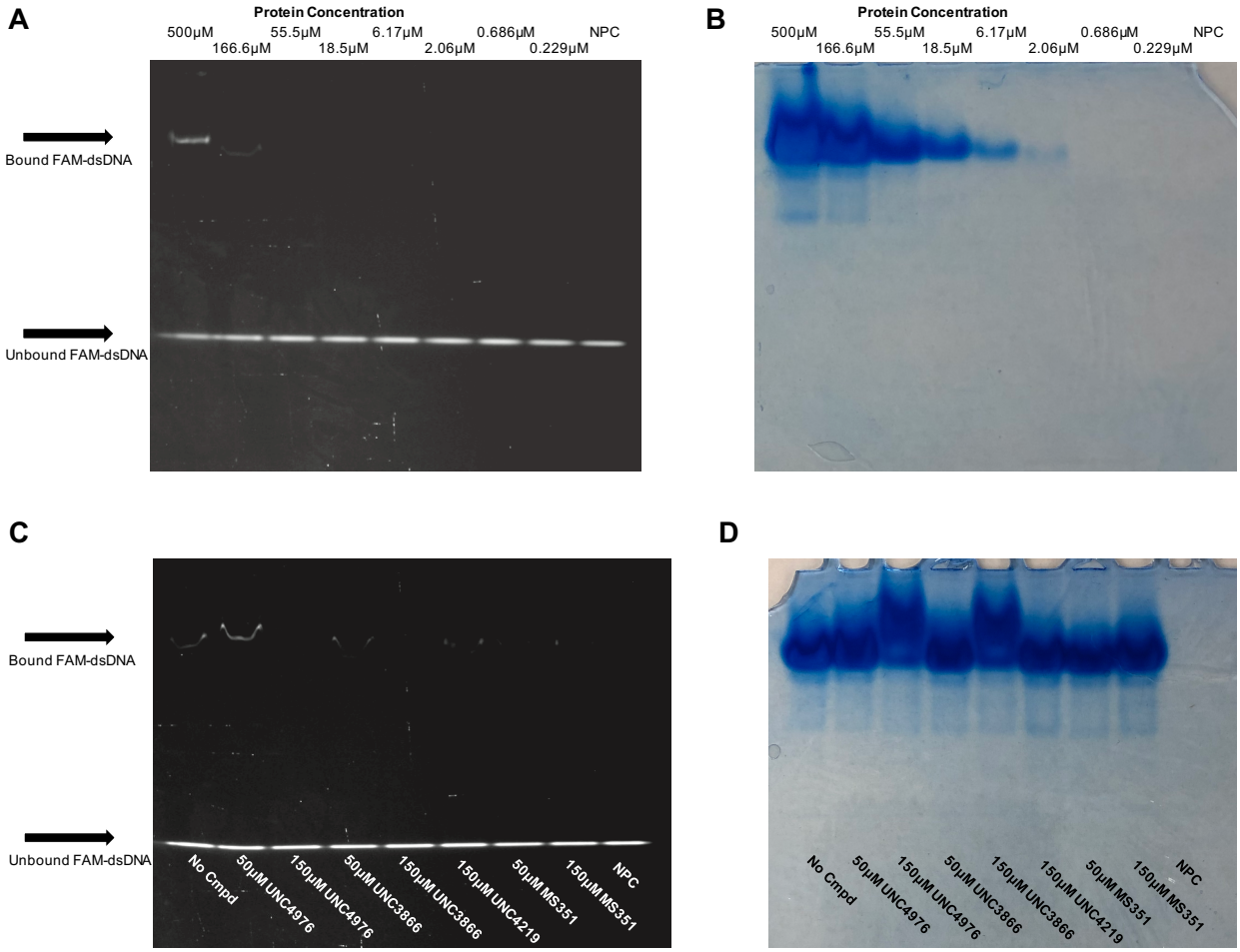


Figure 6. Electrophoretic Mobility Shift Assay (EMSA). Related to Figure 4. A. Fluorescent image of EMSA protein titration of CBX7 chromodomain with 100nM of FAM-dsDNA, demonstrating that CBX7 weakly binds FAM-dsDNA at 166 μM. **B.** Follow up Coomassie staining of Tris-Glycine gel from **A**, demonstrating protein titration of CBX7 chromodomain. **C.** Fluorescent image of EMSA demonstrating that UNC4976 enhances the ability of CBX7 chromodomain to bind to FAM-dsDNA at a 1:3 ratio of compound to protein. All compounds were tested at either 1:3 or 1:1 ratios of compound to protein. **D.** Follow up Coomassie staining of Tris-Glycine gel from **C**, demonstrating that only UNC4976 and UNC3866 bind to CBX7 chromodomain as indicated by band shift. Images are representative of three replicate experiments.

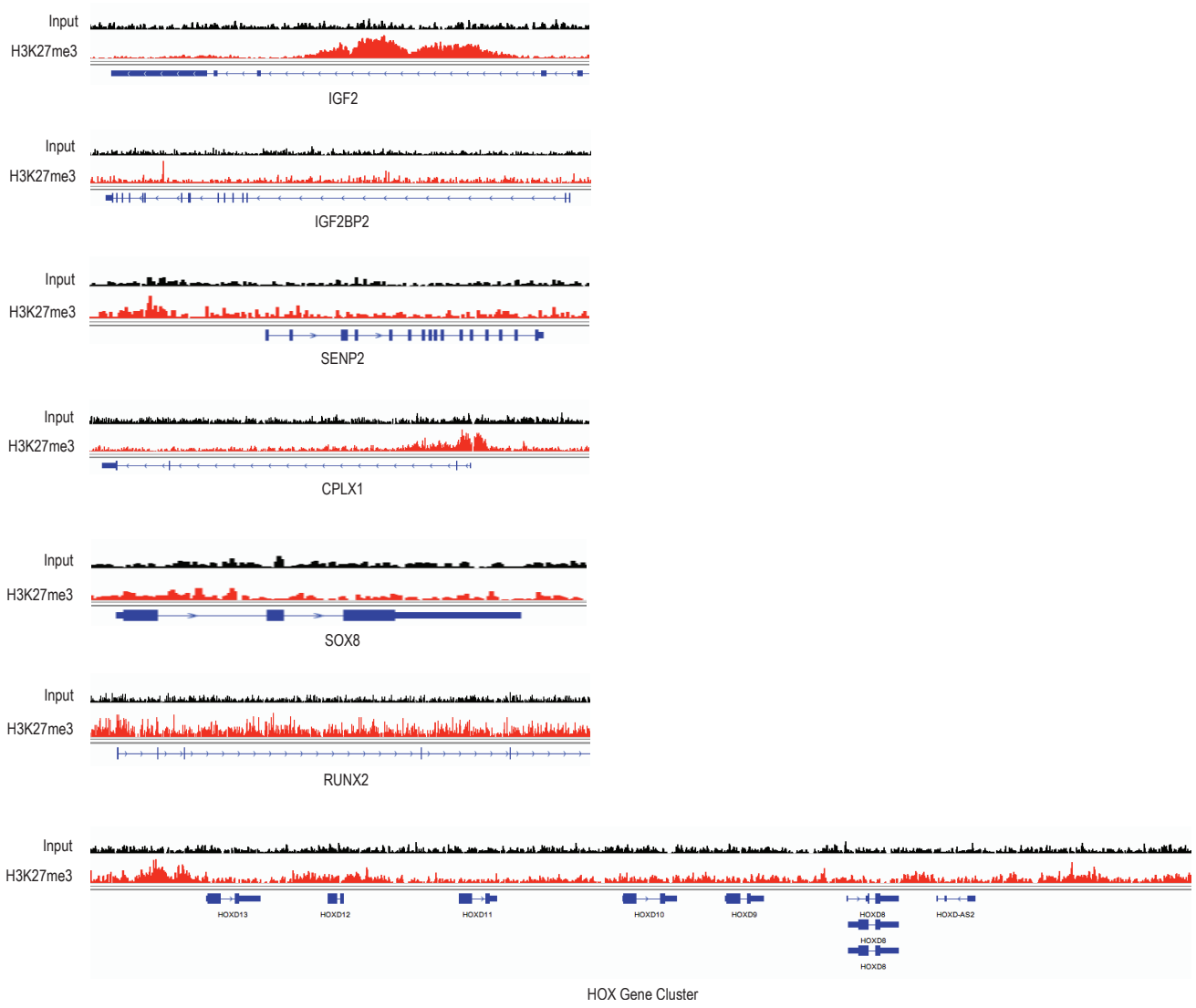


Figure 7. HEK293 Cells H3K27me3 ChIP-seq Data. Related to Figure 6. IGV Views showing H3K27me3 ChIP-seq signals from HEK293 cells (GEO: GSE133391) identifying PRC1 targets that were examined by RT-qPCR.

HEK293 RT-qPCR Primer Sequences		
Gene	**Forward Sequence**	**Reverse Sequence**
GAPDH	AACATCATCCCTGCCTCTACTGG	GTTTTTCTAGACGGCAGGTCAGG
HOXA1	CCAGCCACCAAGAAGCCTGT	CCAGTTCCGTGAGCTGCTTG
HOXA4	CGTGGTGTACCCCTGGATGA	AAGACCTGCTGCCGGGTGTA
HOXC13	TCATCCCCGTCGAAGGCTAC	TGTAGGGCACGCGTTTCTTG
HOXD9	GGCTGTTCGCTGAAGGAGGA	TCTCCAGCTCAAGCGTCTGGT
CPLX1	GTGTGAGTTCTGACCCCTGG	TCTGGGTCCTTCTCCTCGTC
SOX8	GAGCTTGGCAACCGAAAACC	TGCTTCCCAAACCCCAAACT
RUNX2	CGCCTCACAAACAACCACAG	GACTCTGTTGGTCTCGGTGG
CEBPD	CAACCAGGAGATGCAGCAGA	CAGCTGCTTGAAGAACTGCC
IGF2	AGTCGTGGCTCTCCATCTTG	CAGGCACAGGTGACATTCAG
IGFBP2	AAAACGGAGAGTGCTTGGGT	AGCAAGAAGGAGCAGGTGTG

Table 5. RT-qPCR Primer Sequences. Related to Figure 6. Primer sequences for select Polycomb target genes used for RT-qPCR in HEK293 cells.