KAP1 represses differentiation-inducible genes in embryonic stem cells through cooperative binding with PRC1 and derepresses pluripotency-associated genes

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Inventory of Supplemental Information

Supplemental Fig.s Page

Supplemental Tables

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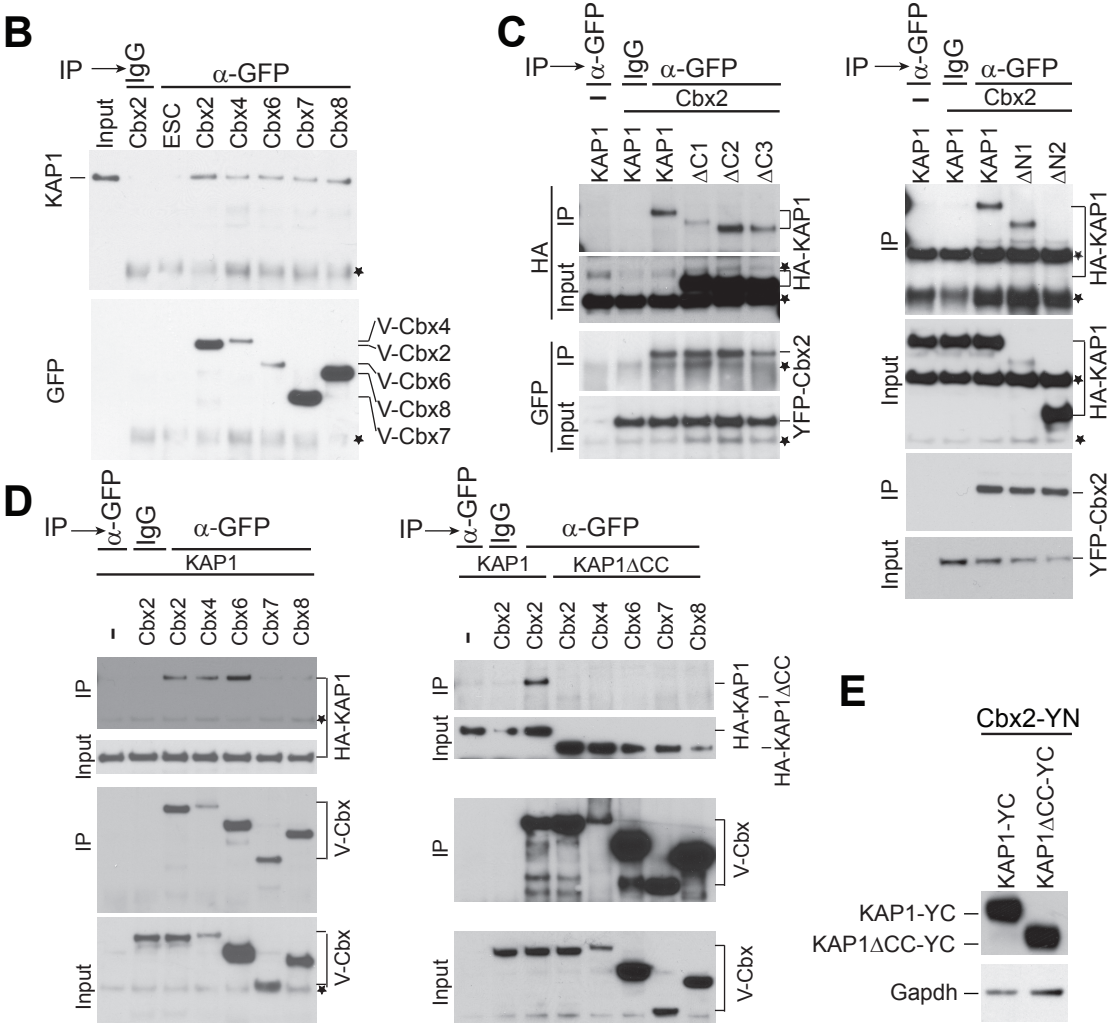


Fig. S1. Characterization of KAP1 interactions with PRC1

(A) KAP1 peptides identified by LC-MS/MS analysis of complexes purified in association with Cbx family proteins from ES cells. Proteins that formed complexes with Cbx2 and Cbx7 were purified from ES cells by sequential affinity chromatography (1). The sequences of peptides generated by protease digestion of the co-purified proteins were determined by LC-MS/MS analysis

Results: 18 unique peptides corresponding to the sequence of KAP1 were detected in the fraction purified in association with Cbx 2 and 17 unique peptides were detected in the fraction purified in association with Cbx7. The sequences of peptides that correspond to the KAP1 protein are indicated in boldface and underlining in the amino acid sequence of KAP1. The peptide sequences identified by LC-MS/MS encompassed 39% of the complete KAP1 sequence.

(B) Endogenous KAP1 co-precipitation with different Cbx family proteins. Extracts of ES cells that stably expressed the Cbx family proteins indicated above the lanes fused to Venus (2) were incubated with anti-GFP antibodies or control IgG. The immunoprecipitates were analyzed by immunoblotting using anti-KAP1 as well as anti-GFP antibodies. ESC: parental ES cells. 5% of the total cell extracts were analyzed in parallel (Input). * indicates cross-reactive bands.

Results: KAP1 co-precipitated with each of the CBX family fusion proteins that were expressed in ES cells. Differences in the levels of expression of different Cbx proteins could influence the relative efficiencies of KAP1 co-precipitation. Whereas KAP1 formed complexes with all PRC1 subunits examined in ES cells, these represent a small subset of all known PRC1 subunits (3).

(C) Co-precipitation of KAP1 deletion derivatives with Cbx2. The KAP1 deletion derivatives indicated above the lanes (see diagrams in Fig. 1D) fused to N-terminal HA were expressed transiently with YFP-Cbx2 in HEK293T cells. The cell extracts were incubated with anti-GFP or control (IgG) antibodies. The immunoprecipitates were analyzed by immunoblotting using anti-HA (upper blots) and anti-GFP (lower blots) antibodies. 5% of the cell extracts were analyzed in parallel (Input). * indicates cross-

reactive bands.

Results: All of the KAP1 deletion derivatives that contained the coiled-coil domain co-precipitated with Cbx2. All of the KAP1 deletion derivatives in which the coiled-coil domain was deleted were precipitated with significantly lower or undetectable efficiencies. Deletion of the C-terminal half of the coiled-coil domain of KAP1 reduced, but did not eliminate its co-precipitation with Cbx2 (Fig. 1E, $\Delta CC^{1/2}$). The coiled-coil domain was therefore required for KAP1 co-precipitation with Cbx2 when they were ectopically expressed in HEK293T cells.

(D) Comparison of intact KAP1 and KAP1ΔCC interactions with different Cbx family proteins. Intact KAP1 or KAP1ΔCC fused to an N-terminal HA epitope was expressed transiently with the Cbx family proteins indicated above the lanes fused to Venus in HEK293T cells. The cell extracts were incubated with anti-GFP (α-GFP) or control (IgG) antibodies. The immunoprecipitates were analyzed by immunoblotting using anti-HA (upper blots) and anti-GFP (lower blots) antibodies. 5% of the cell extracts were analyzed in parallel (Input). * indicates cross-reactive bands.

Results: KAP1 co-precipitated with Cbx proteins that were transiently expressed in HEK293T cells with different effciencies. The differences in the efficiencies of KAP1 co-precipitation with different Cbx proteins from ES cells versus HEK293T cells could be due to differences in the levels of Cbx protein expression.

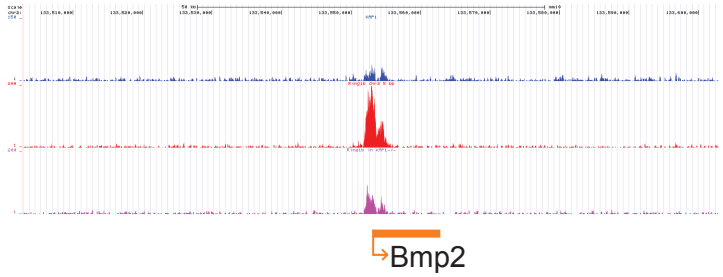
(E) Comparison of the levels of intact KAP1 and KAP1ΔCC BiFC fusion protein expression. Extracts from HEK293T cells that were transiently transfected with plasmids encoding the fusion proteins indicated above the lanes were analyzed by immunoblotting using anti-KAP1 (upper blot) and anti-GAPDH (lower blot) antibodies.

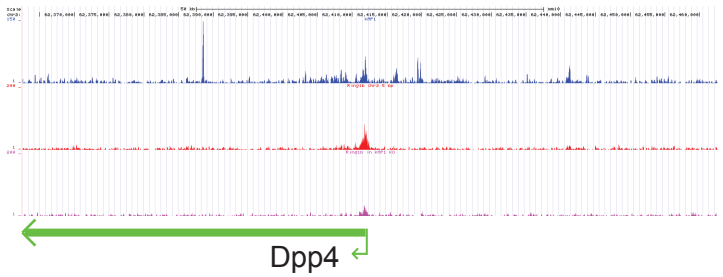
Results: Intact KAP1 and KAP1ΔCC fusion proteins were expressed at comparable levels.

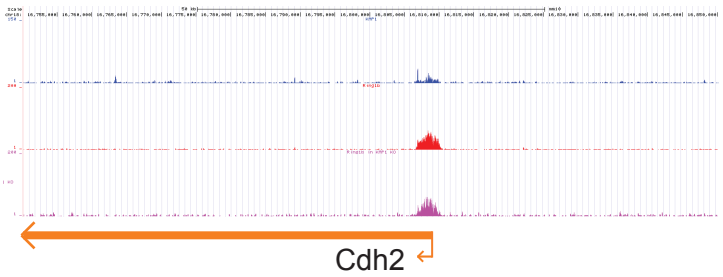
A

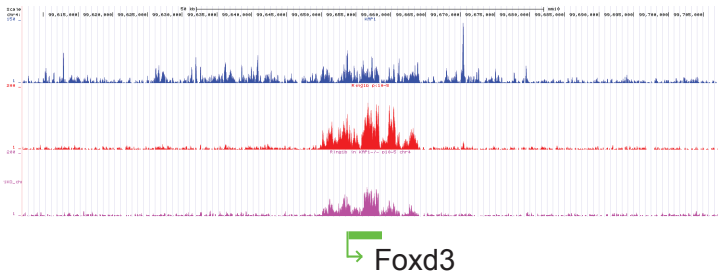
KAP1-repressed
Differentiation-inducible

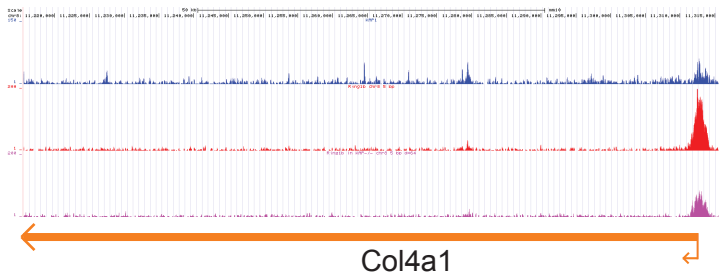
KAP1-derepressed
Pluripotency-associated

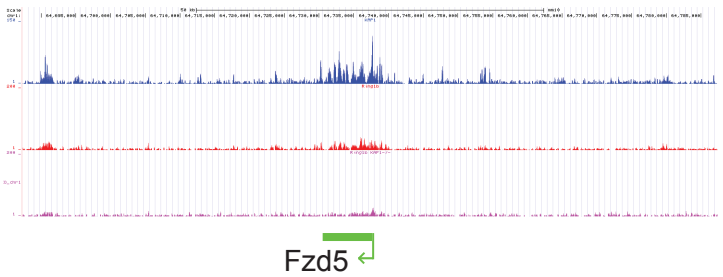


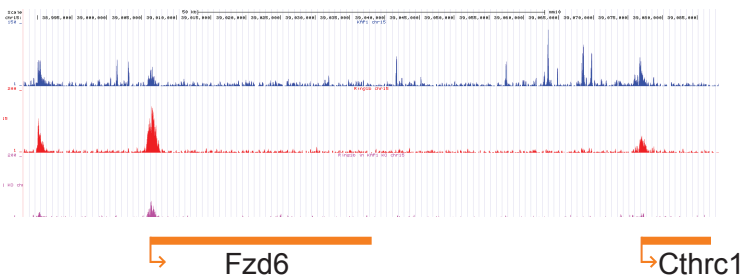


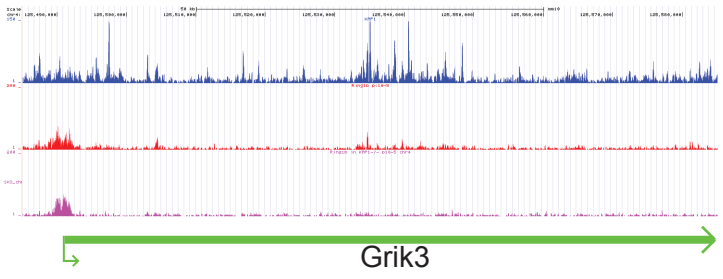


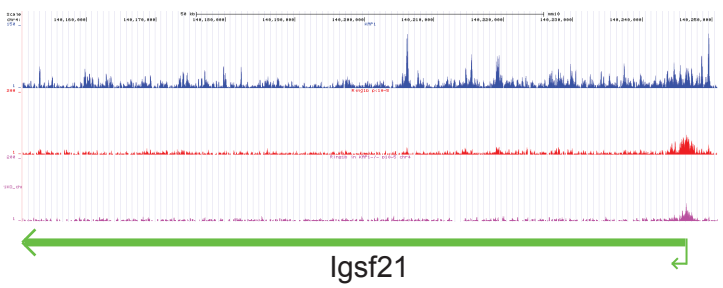


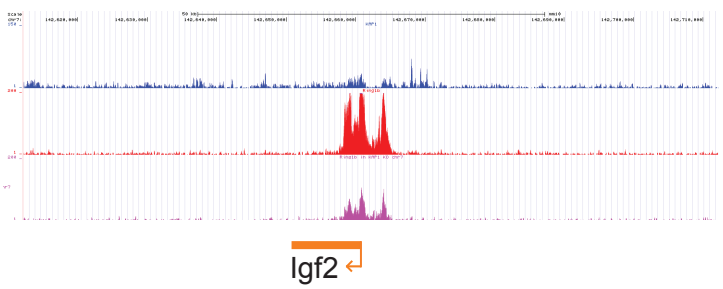


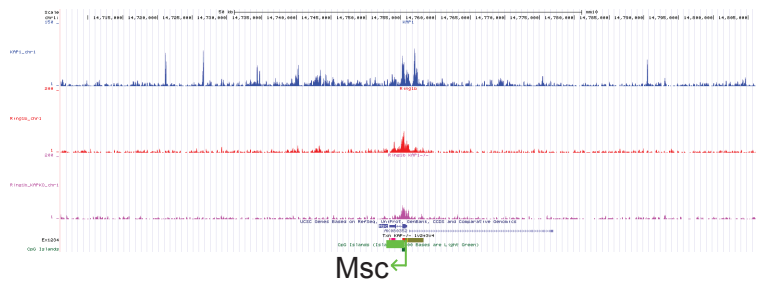












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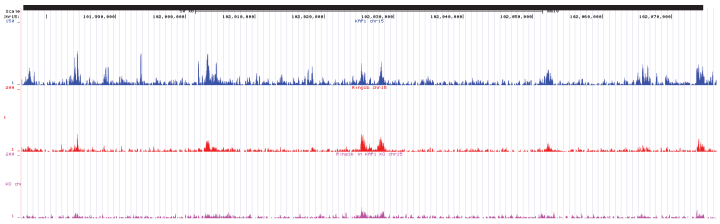
Differentiation-inducible



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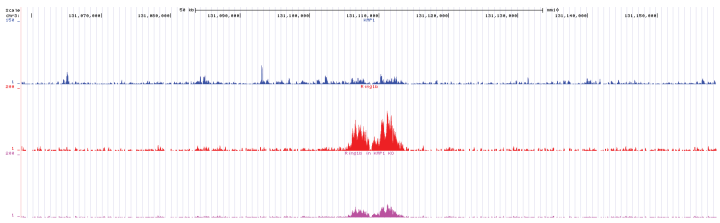
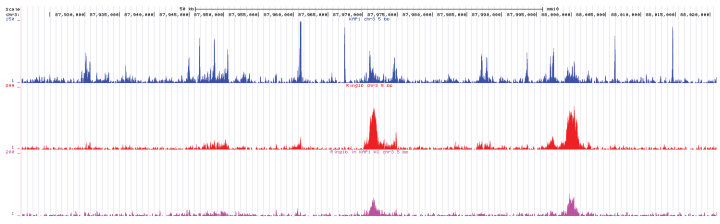


Lef1

Lmx1a



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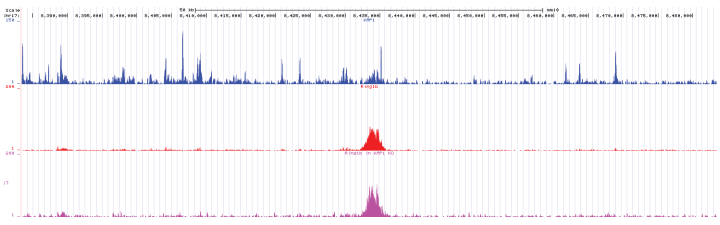

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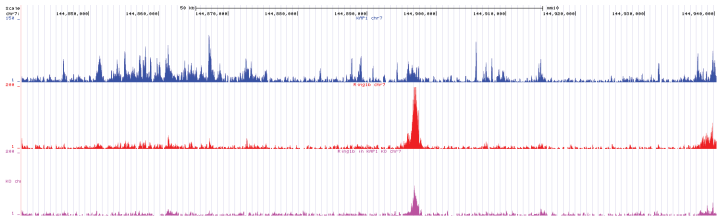
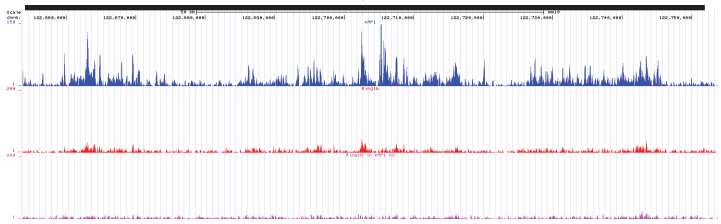


Fgf4 Fgf15

Pluripotency-associated



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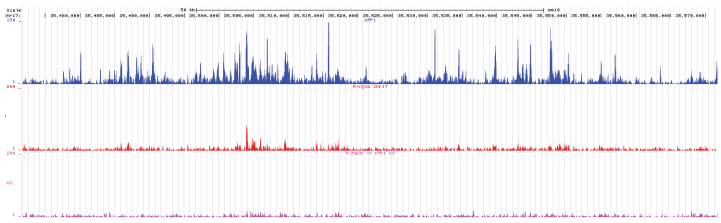

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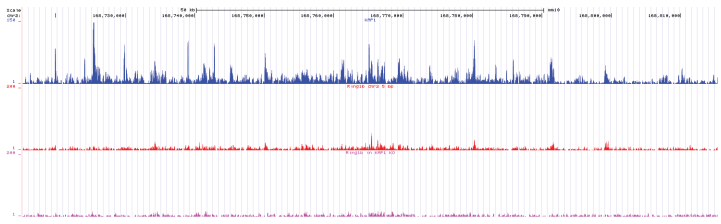

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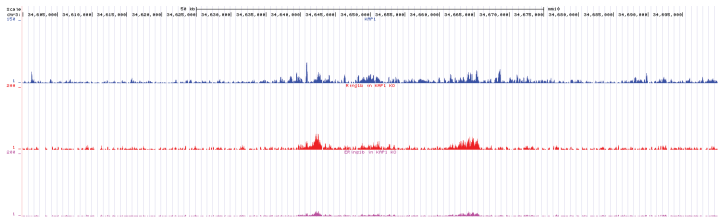

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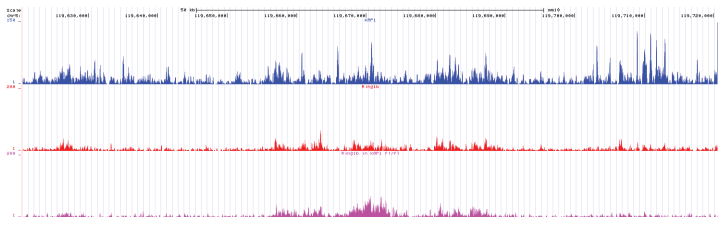

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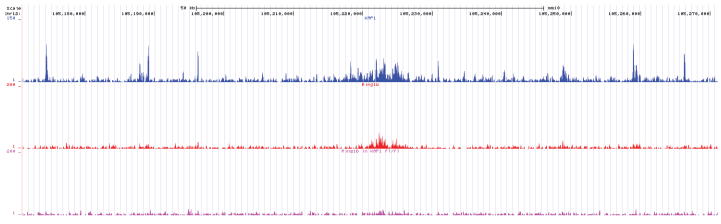

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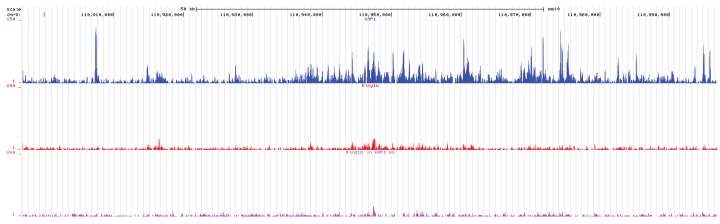
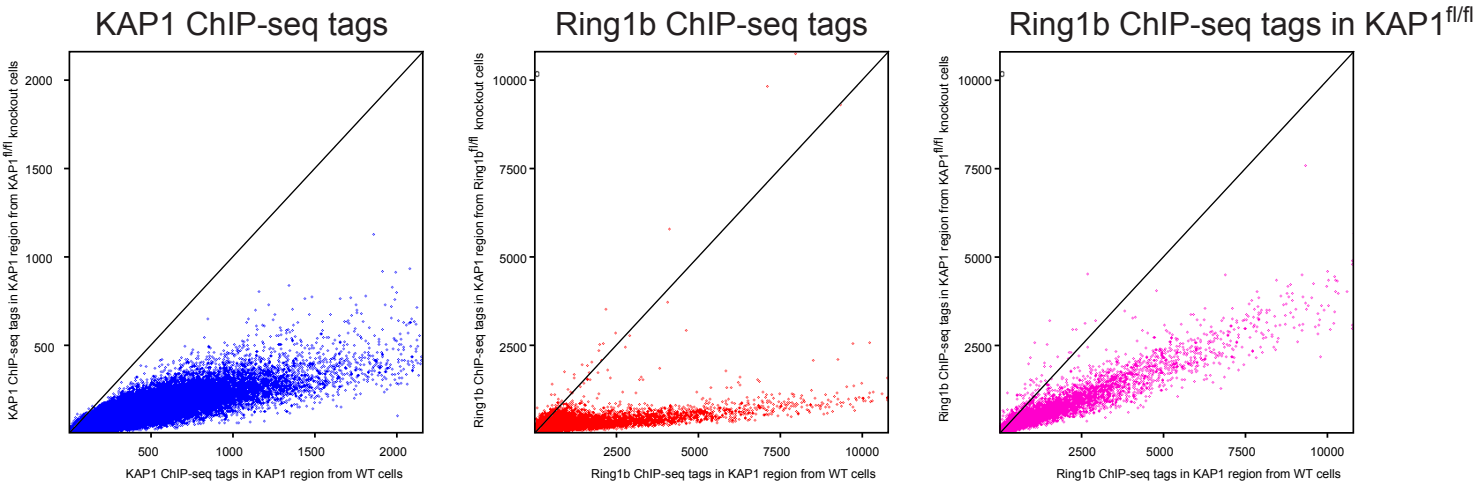


Tdgf1

B

KAP1 binding regions



Ring1b binding regions

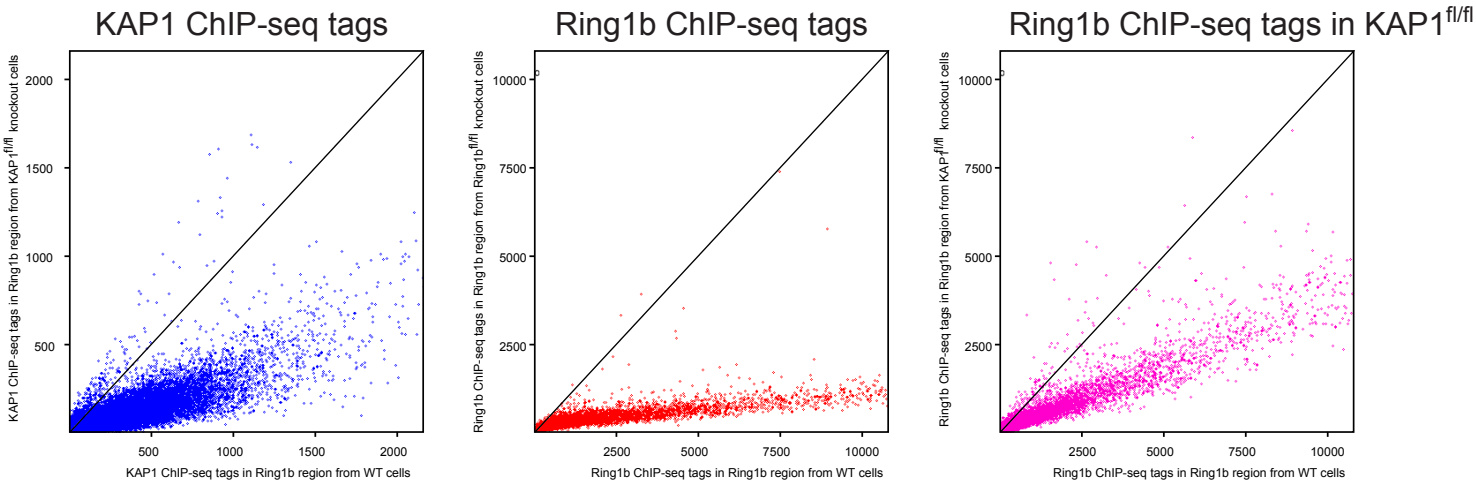


Fig. S2. Distributions of KAP1 and Ring1b ChIP-seq tags at differentiation-inducible *versus* pluripotency-associated genes and validation of KAP1 and Ring1b binding to the same regions.

(A) ChIP-seq tag distributions at selected differentiation-inducible (left panels) and pluripotency-associated (right panels) genes. The distributions of KAP1 (blue), Ring1b (red) and Ring1b in *KAP1*$^{fl/fl}$ knockout (magenta) ChIP-seq tags are shown in genomic regions spanning the genes indicated below the tracks. Selected differentiation-inducible (orange) and pluripotency-associated (green) are shown. The ChIP-seq tag tracks are shown using the same scales encompassing 100,000 base pairs at each locus The height of the tracks corresponds to 150 ChIP-seq tags for the KAP1 track and 200 ChIP-seq tags for both Ring1b tracks.

Results: The KAP1 and Ring1b ChIP-seq tags at differentiation-inducible genes (left panels) were concentrated near the transcription start sites and the level of Ring1b ChIP-seq tags was typically higher than the level of KAP1 ChIP-seq tags. At pluripotency-associated genes, KAP1 ChIP-seq tags were distributed throughout transcribed and flanking sequences and were generally more abundant than the Ring1b ChIP-seq tags.

Most of the genes whose transcription changed upon *KAP1*$^{fl/fl}$ knockout were bound by both KAP1 and Ring1b. 16% of the differentiation-inducible genes and 2% of the pluripotency-associated genes contained no KAP1 or Ring1b binding regions. These could be regulated indirectly, or they could represent false negatives since the conditional *KAP1*$^{fl/fl}$ and *Ring1b*$^{fl/fl}$ knockout cells that were used as negative controls had residual KAP1 and Ring1b binding. Many differentiation-inducible promoters were bound predominantly by Ring1b, whereas a majority of pluripotency-associated promoters were bound predominantly by KAP1.

(B) Analysis of KAP1 ChIP-seq tag enrichment in Ring1b binding regions and vice versa. The ChIP-seq tag counts for KAP1 in *KAP1*$^{fl/fl}$ knockout (blue), Ring1b in *Ring1b*$^{fl/fl}$ knockout (red) and Ring1b in *KAP1*$^{fl/fl}$ knockout (magenta) ChIP-seq tags were plotted as a function of the respective ChIP-seq tag counts from wild type (WT)

cells. The upper row of panels shows ChIP-seq tag counts in KAP1 binding regions. The lower row of panels shows ChIP-seq tag counts in Ring1b binding regions. The diagonal lines show the predicted ChIP-seq tag counts if *KAP1*$^{fl/fl}$ and *Ring1b*$^{fl/fl}$ knockout caused no changes in ChIP-seq tag levels (i.e. *KAP1*$^{fl/fl}$ and *Ring1b*$^{fl/fl}$ knockout have no effect on ChIP-seq tag counts).

Results: The KAP1 ChIP-seq tag counts in Ring1b binding regions and the Ring1b ChIP-seq tags in KAP1 binding regions reflected specific KAP1 and Ring1b binding since their levels were reduced in *KAP1*$^{fl/fl}$ knockout and in *Ring1b*$^{fl/fl}$ knockout cells, respectively. The Ring1b ChIP-seq tag counts decreased at a large majority of Ring1b and KAP1 binding regions upon *KAP1*$^{fl/fl}$ knockout, but they increased at a small number of binding regions.

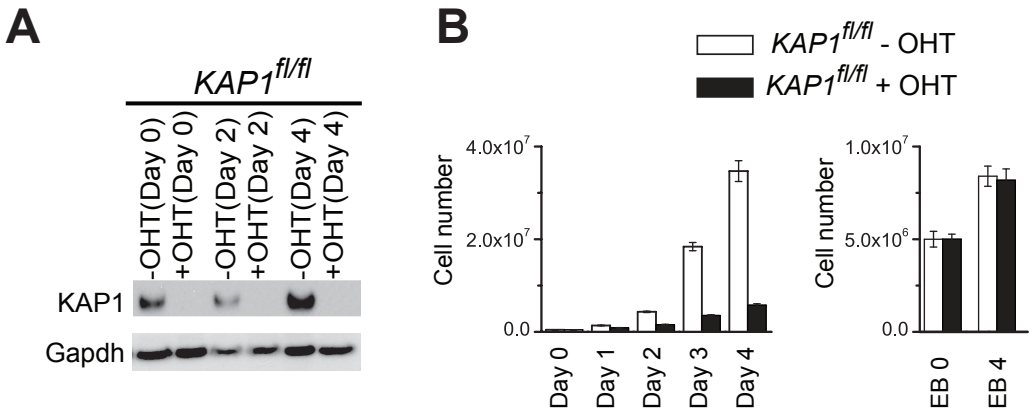

A
KAP1fl/fl
-OHT(Day 0)
+OHT(Day 0)
-OHT(Day 2)
+OHT(Day 2)
-OHT(Day 4)
+OHT(Day 4)
KAP1
Gapdh
B
KAP1fl/fl - OHT
KAP1fl/fl + OHT
Cell number
4.0x10^7
2.0x10^7
0.0
Day 0
Day 1
Day 2
Day 3
Day 4
Cell number
1.0x10^7
5.0x10^6
0.0
EB 0
EB 4

Fig. S3. Effects of *KAP1*$^{fl/fl}$ knockout during embryoid body formation on the level of KAP1 expression and on cell proliferation

(A) Effects of *KAP1*$^{fl/fl}$ knockout during embryoid body formation on the levels of KAP1. The level of KAP1 was measured by immunoblotting extracts from cells treated with tamoxifen (+OHT) either concurrent with the induction of differentiation (Day 0) or on Day 2 or Day 4 after the induction of differentiation (tamoxifen was removed after two days). The embryoid bodies were harvested and KAP1 expression was analyzed 8 days after the inducition of differentiation.

Results: KAP1 was depleted with similar efficiencies in ES cells and during embryoid body formation.

(B) Effects of KAP1 depletion before and after the induction of embryoid body differentiation on cell proliferation. The number of cells was counted in cultures that were either untreated (−OHT; open bars) or treated (+OHT; solid bars) with tamoxifen before (left graph) and after (right graph) the induction of embryoid body differentiation. The tamoxifen was removed 24 hours after treatment.

Results: KAP1 depletion reduced ES cell promiferation, but had little effect on cell proliferation during embryoid body formation.

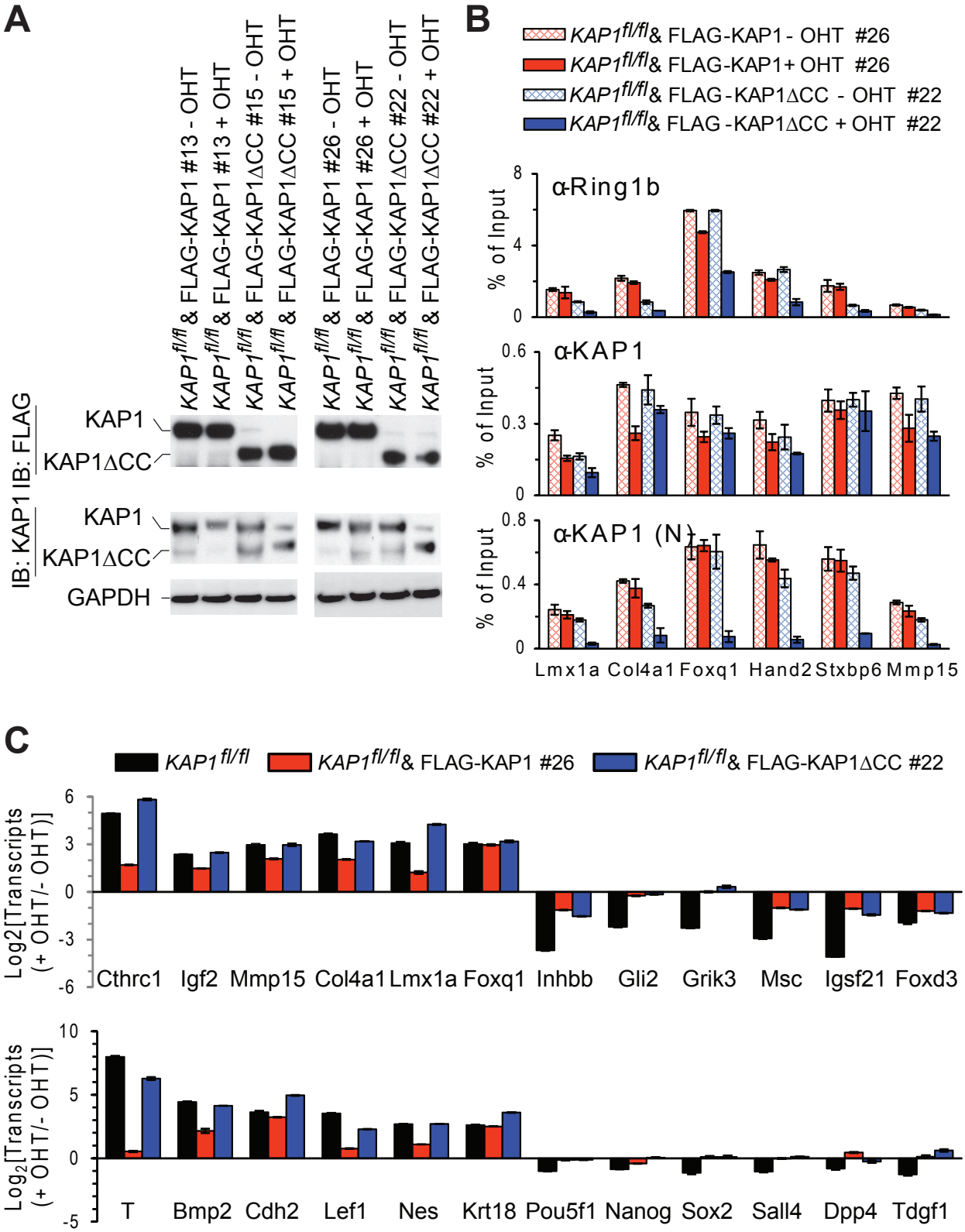

A
KAP1fl/fl & FLAG-KAP1 #13 - OHT
KAP1fl/fl & FLAG-KAP1 #13 + OHT
KAP1fl/fl & FLAG-KAP1ΔCC #15 - OHT
KAP1fl/fl & FLAG-KAP1ΔCC #15 + OHT
KAP1fl/fl & FLAG-KAP1 #26 - OHT
KAP1fl/fl & FLAG-KAP1 #26 + OHT
KAP1fl/fl & FLAG-KAP1ΔCC #22 - OHT
KAP1fl/fl & FLAG-KAP1ΔCC #22 + OHT
IB: FLAG
KAP1
KAP1ΔCC
IB: KAP1
KAP1
KAP1ΔCC
GAPDH
B
KAP1fl/fl & FLAG-KAP1 - OHT #26
KAP1fl/fl & FLAG-KAP1 + OHT #26
KAP1fl/fl & FLAG-KAP1ΔCC - OHT #22
KAP1fl/fl & FLAG-KAP1ΔCC + OHT #22
α-Ring1b
% of Input
α-KAP1
α-KAP1 (N)
Lmx1a Col4a1 Foxq1 Hand2 Stxbp6 Mmp15
C
KAP1fl/fl
KAP1fl/fl & FLAG-KAP1 #26
KAP1fl/fl & FLAG-KAP1ΔCC #22
Log2[Transcripts (+ OHT/- OHT)]
Cthrc1 Igf2 Mmp15 Col4a1 Lmx1a Foxq1 Inhbb Gli2 Grik3 Msc Igsf21 Foxd3
Log2[Transcripts (+ OHT/- OHT)]
T Bmp2 Cdh2 Lef1 Nes Krt18 Pou5f1 Nanog Sox2 Sall4 Dpp4 Tdgf1

Fig. S4. Effects of stably expressed intact KAP1 and KAP1ΔCC on PRC1 binding and on transcription at differentiation-inducible *versus* pluripotency-associated promoters upon *KAP1*$^{fl/fl}$ knockout (related to Fig. 6)

(A) Comparison of the levels of intact KAP1 and KAP1ΔCC expression in *KAP1*$^{fl/fl}$ cells untreated (-OHT) or treated (+OHT) with tamoxifen for 24 h followed by 48 h without tamoxifen. We generated *KAP1*$^{fl/fl}$ ES cell lines that stably expressed intact KAP1 or KAP1 lacking the coiled-coil domain that was required for interaction with PRC1 (KAP1ΔCC). Extracts of cells cultured in parallel with those used in the experiments shown in Fig.s 6A and S6B were analyzed by immunoblotting using the antibodies indicated to the left of the images. Note the difference in mobilities between endogenous KAP1 and KAP1 fused to the FLAG epitope.

Results: KAP1 and KAP1ΔCC were expressed at levels comparable to endogenous KAP1 and to each other in several independent cell lines. Endogenous KAP1 was depleted with similar efficiencies in cells that expressed intact KAP1 and KAP1ΔCC. The differences in the effects of intact KAP1 *versus* KAP1ΔCC expression on PRC1 binding and on transcription at differentiation-inducible genes were therefore not due to differences between the levels of KAP1 and KAP1ΔCC expression or in endogenous KAP1 depletion in these cells.

(B) Effects of intact KAP1 and KAP1ΔCC expression in *KAP1*$^{fl/fl}$ knockout cells on Ring1b binding at the promoters indicated below the bars. The levels of Ring1b (α-Ring1b), total KAP1 (α-KAP1) and full-length KAP1 [α-KAP1 (N) does not recognize KAP1ΔCC] binding were measured at the promoters indicated below the bar graphs. The levels were compared in *KAP1*$^{fl/fl}$ cells that expressed no ectopic KAP1 (white and black), intact KAP1 (red; clone #26) or KAP1ΔCC (blue; clone #22) and that were cultured in the absence (-OHT, open and checkered bars) or in the presence (+OHT, solid bars) of tamoxifen. The ChIP-qPCR signals were normalized using anti-H3 ChIP. The data shown represent the means and standard deviations of three replicate qPCR measurements and are representative of two experiments the were performed using different stable cell lines.

Results: Intact KAP1 and KAP1ΔCC expression in ES cells had distinct effects on Ring1b binding at differentiation-inducible genes before and after endogenous *KAP1*$^{fl/fl}$ knockout. Endogenous KAP1 binding was depleted with similar efficiencies in cells that expressed intact KAP1 and KAP1ΔCC upon *KAP1*$^{fl/fl}$ knockout, indicating that the differences in PRC1 binding and transcription were not due to differences in endogenous KAP1 binding.

(C) Effects of intact KAP1 and KAP1ΔCC expression in *KAP1*$^{fl/fl}$ knockout cells on the transcription of differentiation-inducible versus pluripotency-associated genes. The changes in transcription of the genes indicated below the bars upon *KAP1*$^{fl/fl}$ cells knockout (+OHT/-OHT) were compared between *KAP1*$^{fl/fl}$ cells that expressed no ectopic KAP1 (black bars), intact KAP1 (red bars; clone #26) or KAP1ΔCC (blue bars; clone #22). The transcript levels were normalized by the levels of Gapdh transcripts. The data shown represent the means and standard deviations of three replicate qPCR measurements and are representative of two experiments the were performed using different stable cell lines.

Results: We investigated the roles of the interaction between KAP1 and PRC1 in regulation of differentiation-inducible *versus* pluripotency-associated gene transcription. We compared the levels of these transcripts in *KAP1*$^{fl/fl}$ cells that expressed either intact KAP1 or KAP1ΔCC before and after endogenous *KAP1*$^{fl/fl}$ knockout. Intact KAP1 expression repressed the differentiation-inducible and activated the pluripotency-associated genes examined. KAP1ΔCC expression did not repress the differentiation-inducible genes whose transcription increased upon *KAP1*$^{fl/fl}$ knockout. In contrast, KAP1ΔCC expression activated transcription of the pluripotency-associated genes whose transcription decreased upon *KAP1*$^{fl/fl}$ knockout. The coiled-coil domain of KAP1 was therefore required for the repression of differentiation-inducible gene transcription, but not for the activation of pluripotency-associated gene transcription.

Supporting Table 1. Changes in transcript levels upon KAP1$^{fl/fl}$ knockout and upon embryoid body formation

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Supporting Table 2. Characterization of ChIP-seq tag libraries

Library designation	Ring1b	*Ring1b*$^{fl/fl}$ knockout	Ring1b in *KAP1*$^{fl/fl}$ knockout	KAP1	*KAP1*$^{fl/fl}$ knockout
ES cell line	*Ring1b*$^{fl/fl}$	*Ring1b*$^{fl/fl}$	*KAP1*$^{fl/fl}$	*KAP1*$^{fl/fl}$	*KAP1*$^{fl/fl}$
Treatment	+Ethanol	+Tamoxifen	+Tamoxifen	+Ethanol	+Tamoxifen
ChIP antibody	anti-Ring1b	anti-Ring1b	anti-Ring1b	anti-KAP1	anti-KAP1
DNA fragment size peak (bp)	185	177	183	174	177
Mapped ChIP-seq tags	146573447	135028510	132033045	152548205	134162121
MACS shift model (d)	61		59	69	
Binding regions (p<10e-5)	13516		5562	82750	

Supporting Table 3. Genes bound by KAP1 and Ring1b identified by ChIP-seq analysis

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This table is provided as a separate file in Microsoft Excel format

Supporting Table 4. Promoters bound by KAP1 and Ring1b identified by ChIP-seq analysis

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This table is provided as a separate file in Microsoft Excel format

Supporting Table 5. Sequences of primers used for ChIP-qPCR analyses

Gene	Left primer (5'-3')	Right primer (5'-3')
Adamts8	tctctggcacagtgaatggagaac	tgtggctggatggtgaactcct
Bhlhe22	ccttcagtgcggaagctct	tgcctgcatgaggatgtagt
Bmi1	tcctgaagtgacaaagacaagg	taactcgccagcctgacc
Col4a1	cctgatgggacagaactttggtca	agcatcgtcatcttagcaccctga
Cthrc1	aactcaggagttgcggtctc	gcaggttgacagtggtcaga
Cyp1b1	gcatcttgcccagaaagcta	ggctttaccctgggattgag
Efna5	agccactgcctccttcttc	gagcggacgccaaataaata
Fgf15	tgccctgcgcctgcaacttgagt	tccactttctcgccatgacacctc
Fgf4	tacctgctgggcctcaaa	tgtccctcgtgtctgcgtgca
Foxd3	aggaaggctggtgaagtgag	ggtcctcgacgcttctctac
Foxq1	tcaacgagtacctcatgggcaagt	tgacgaaacagtcgttgagcgaga
Fzd1	ccatttgtgtcaatccctca	gcctagaaacccgttggag
Fzd6	ctgtagaaccggccgaag	aagcctattaactcggggagtc
Gapdh	atcctgtaggccaggtgatg	aggctcaagggcttttaagg
Gli2	tacagaggacgcggagga	gcagtccctaaggacaccag
Grik3	ggggaggacaaagttccaa	cgtatccaggttccctagcc
Hand2	tcctcaaaagcaagacaggag	agcttgcaacttcgaaggaa
Igf2	caagtggattaattatacgctttctg	agaggcgggtaggctcac
Igsf21	cctatcgcagccaaccag	tctgggctgtaataggatgctc
Inhbb	gtcggtgccatttatccatc	gccacttttgctacctcgaa
Krt18	cggaactcctgttctggtct	ggtggagcgagttgtgaag
Lhx1	tgggcagtctgacacgcacac	aagacgccaagagctcaggattc
Lmx1a	taagttgcaggagcaagtgggtga	tcggatctgcaccgtaagtgatga
Meis1	tgggataagcctagcagacaa	cttcaggtgttcgttgttgg
Mmp15	tatccctcgcctccagtcatttagc	ctcgtcactcgtaaagcccacatt
Msc	agccagagaaagcgagactg	cggtggacatcgcctatt
Ntrk2	gggagtgaagctgtgatcct	ccgcatcatcttctcttattcc
Ppm1l	acggacgaagtgaagaccatcgtt	agcacatcaagtcctccaagacga
RLTR4-MM-int	cttgggtgaaaggacagaggaaggtcgag	ataaaatcagggctcttaggaggtgaacac
Sox1	acaggaacggagacttcgag	ccactttctgggtctgaagc
Stxbp6	ctgggagcctttgcaagcaagtat	tcctccttcccgcttcactcattt

Supporting Table 6. Sequences of primers used for RT-qPCR analyses

Gene	Left primer (5'-3')	Right primer (5'-3')
Adamts8	agaggaatgtccaccaaacg	gtgtggttgtaggcattatatttctc
Bhlhe22	ccttcagtgcggaagctct	tgcctgcatgaggatgtagt
Bmi1	aaaccagaccactcctgaaca	tcttcttctcttcatctcatttttga
Bmp2	gatctttaccggctccagtct	tgggatgttctccagatgttc
Cdh2	ggtggaggagaagaagaccag	ggcatcaggctccacagtat
Col4a1	ttaaaggactccagggaccac	cccactgagcctgtcacac
Cthrc1	gtgaagcaaaaagcgctga	tgctggtccttgtagacacatt
Cyp1b1	acatccccaagaatacggtc	tagacagttcctcaccgatg
Dpp4	gagcgcagaagagccgaagga	acacattgggggtaggaaca
Efna5	gccaggccgagagtatttc	caggaccttcttccgttgtc
Fgf15	tgccctgcgcctgcaacttgagt	tccactttctcgccatgacacctc
Fgf4	tacctgctgggcctcaaa	tgtccctcgtgtctgcgtgca
Foxd3	aggaaggctggtgaagtgag	ggtcctcgacgcttctctac
Foxq1	ggcctcgaagactcctttct	cccgctacttttgaggatagg
Fzd1	acatcgcgtacaaccagacc	gcgtcctcctgattcgtg
Fzd6	ttaagcgaaaccgcaagc	ttggaaatgaccttcagccta
Gapdh	gccaaaagggtcatcatctc	cacacccatcacaaacatgg
Gli2	tacagaggacgcggagga	gcagtccctaaggacaccag
Grik3	cagaggattcacttccatgaca	tacaacacccagtgccaact
Hand2	ccgacaccaaactctccaa	gatccatgaggtaggcgatg
Igf2	caagtggattaattatacgctttctg	agaggcgggtaggctcac
Igsf21	cctatcgcagccaaccag	tctgggctgtaataggatgctc
Inhbb	gatcatcagctttgcagagaca	tgccttcattagagacgaagaa
KAP1(mouse&human)	accagccaaccagcggaaatgtga	gagtcggtagccagctgatgca
KAP1(mouse)	cggaaatgtgagcgtgttctc	cggtagccagctgatgcaa
Krt18	agatgacaccaacatcacaagg	cttccagaccttggacttcct
Lef1	ccgtcagatgtcaactccaagcaa	acccgtgatgggataaacaggct
Lhx1	tgggcagtctgacacgcacac	aagacgccaagagctcaggattc
Lmx1a	cagcaacaggaccaacagaa	cccactaccatttgtctgagc
Meis1	gacgctttaaagagagataaagatgc	catttctcaaaaatcagtgctaaga
Mmp15	ctaaagacgccgaagtgtacg	gctggggtaggtagccataga
Msc	agctttccaaactggacacg	gtccagagaccacgaatgg
Nanog	cctccagcagatgcaagaactc	cttcaaccactggtttttctgcc
Nes	tcccttagtctggaagtggcta	ggtgtctgcaagcgagagtt
Ntrk2	tgccgagtgctacaacctct	gcgtccttcagcgtcttc
Pou5f1	gctcaccctgggcgttctc	ggccgcagcttacacatgttc
Ppm1l	ccagctgaaggaaaggaaga	tggctaggattccctggac
Sall4	tcaccacgaaaggcaacctgaagg	cattcaggacgctggtgtactggtt

Supporting Table 6 cont. Sequences of primers used for RT-qPCR analyses

Gene	Left primer (5'-3')	Right primer (5'-3')
Sox1	acaggaacggagacttcgag	ccactttctgggtctgaagc
Sox2	caggagaaccccaagatgcacaa	attaagctcctgggtcgcaag
Stxbp6	agtttattaactgccaatccaaaatta	tgcactggtcacactatcagc
T/Brachyury	gctctaaggaaccaccggtcatc	atgggactgcagcatggacag
Tbx3	cagcactcgacctgtgaaaa	attggctctttgacgctttc
Tcl1	acgcgtttctcatgctcac	gaaaattgcaatgccagtcc
Tdgf1	cgcaactgtgaacatgatgttcgc	gaaaggaagcttttcaggtcac

Supplemental Materials and Methods

Cell lines

KAP1$^{fl/fl}$ ES cells were provided by Didier Trono (School of Life Sciences, Ecole Polytechnique Fe´de´rale de Lausanne (EPFL), Lausanne, Switzerland) and were cultured as described (4). *Ring1a*$^{-/-}$*;Ring1b*$^{fl/fl}$*;Rosa26::CreERT2* (5), *Ring1b*$^{fl/fl}$*;Rosa26::CreERT2* (5), *Bmi1*$^{-/-}$*Mel18*$^{-/-}$ (6), *Cbx2*$^{-/-}$ (7), *Cbx2#1, Cbx4#11, Cbx6#7, Cbx7#3 and Cbx8#1* ES cell lines were described previously (2). *Cbx7*$^{-/-}$ ES cell lines were isolated from blastocyst embryos of *Cbx7*$^{tm1b/tm1b}$ mice generated by Cre-mediated recombination of *Cbx7*$^{tm1a/tm1a}$ mice obtained from the Welcome Trust Sanger Institute. *P*$_{TRE}$*-shMga* ES cells that conditionally expressed an shRNA targeting Mga were prepared by stable transfection of pTripZ that expressed an shRNA targeting Mga into pGK mouse ES cells. *KAP1*$^{fl/fl}$ *& KAP1* and *KAP1*$^{fl/fl}$ *& KAP1*Δ*CC* ES cells lines were generated by transfection of plasmids encoding intact KAP1 or KAP1ΔCC into *KAP1*$^{fl/fl}$ ES cells and selection for stable expression using G418. The levels of KAP1 and KAP1ΔCC proteins were determined by immunoblotting using anti-KAP1 and anti-FLAG antibodies. HEK293T cells were obtained from ATCC.

Plasmid expression vectors

The GFP-HA-KAP1 expression vector containing the sequence encoding EGFP and the HA epitope fused to the N-terminus of the human KAP1 coding sequence under the control of the CMV promoter in the pEGFP-C1 vector was provided by Dr. Yael Ziv (The David and Inez Myers Laboratory for Cancer Genetics, Tel Aviv University) (8). The FLAG-HA-KAP1 expression vector was prepared by substituting the sequence encoding GFP by the FLAG epitope. Deletions in the FLAG-HA-KAP1 coding sequence were as follows: ΔC1, deletion of amino acids 785-836; ΔC2, deletion of amino acids (737-836); ΔC3, deletion of amino acids (615-836); ΔN1, deletion of amino acids (1-135); ΔN2, deletion of amino acids (1-381); ΔB1, deletion of amino acids (136-203); Δ(B1B2), deletion of amino acids (136-250); $\Delta CC^{1/2}$, deletion of amino acids (318-381); ΔCC, deletion of amino acids (251-381); Δ(B2CC), deletion of amino acids (204-381); ΔPxVxL, deletion of amino acids (381-615).

The KAP1 expression vectors for BiFC analysis were constructed by replacing the sequence encoding EGFP in GFP-HA-KAP1 by the sequence encoding the C-terminal 155 amino acids of the yellow fluorescent protein (YC). The expression vectors encoding full-length YFP or the N-terminal 172 amino acids of YFP (YN) fused to the N-termini of human CBX family proteins were described previously (2).

BiFC analysis of KAP interactions with Cbx family proteins

Twenty-four hours before transfection, HEK293T cells were seeded at 30% confluence in cover glass chambers (Nalge Nunc International). Cells were co-transfected with 0.25 µg plasmids expressing VN-Cbx family proteins, 0.25 µg plasmids expressing YC-KAP1, and 0.5 µg pCDNA3(+) using 3 µL of Fugene 6, by following the manufacturer's protocol (Roche). Twenty-four hours after transfection, the medium was changed to DMEM lacking phenol red (Invitrogen). The epifluorescence images were acquired using an Olympus IX81 inverted fluorescence microscope equipped with a 60× water objective (numerical aperture, 1.2) and a Hamamatsu ER II charge-coupled device camera. BiFC fluorescence was excited using a 500/20 nm excitation filter and emission was detected using a 535/30 nm filter.

Flow cytometry analysis of the specificity of BiFC complex formation

HEK293T cells were co-transfected in six-well plates with 0.5 µg plasmids expressing YN-Cbx family proteins, 0.5 µg plasmids expressing YC-KAP1 and mutants, and 0.1 µg Jun-CFP using 3.3 µl of FuGene6 as described above. Twenty-four hours after transfection, HEK293T cells were harvested using the citrate solution (135 mM KCl, 15 mM sodium citrate). Half of the cells were collected for determination of protein level by western blotting. The remaining cells were fixed with 3.7% formaldehyde for 10 min at room temperature, washed with PBS, and re-suspended in 250 µL of PBS containing 0.8% BSA. The fluorescence intensities were analyzed using a Beckton Dickinson FACSCanto^{TM} II system. The BiFC and CFP signals were excited using 488-nm and 407-nm laser lines, respectively. The correction factors used to compensate for the cross-talk between YFP and CFP acquisition channels were determined using cells that expressed YFP and CFP separately. Threshold values were set to exclude 99% of non-

transfected cells. The mean fluorescence intensities of the BiFC complexes were normalized by the mean fluorescence intensities of co-expressed CFP fusions.

Antibodies

The antibodies are described in or were obtained from the following sources: anti-KAP1 (Abcam, ab10484); anti-Ring1b (MBL, D139-3). anti-Mel18 (Santa Cruz, sc-10744); anti-Cbx2 (9); anti-Cbx7 (Abcam, ab21873) was used for endogenous IP in Fig. 1B and ChIP in Fig.s 5 and 6; anti-Cbx7 (9) was used for ChIP in Fig. 2; anti-Mga was raised against an Mga fragment fused to GST; anti-L3MBTL2 (10); Max (C-17, Santa Cruz, sc-197); E2F6 (Abcam, ab53061); anti-FLAG (Sigma, F3165); anti-Gapdh (Abcam, ab9484); anti-GFP (FitzGerald, 20R-GR011); anti-PanH3 (Abcam, ab1791); anti-H3K27Me3 (Upstate, 07-449); anti-H3K9Me3 (Upstate, 07-523); anti-H3K4Me3 (Millipore, 04-745).

Immunoprecipitation and immunoblotting

For investigation of KAP1 and PRC1 subunit interactions, ES cell nuclear extracts were prepared by isolating nuclei from the wild type and mutant ES cells indicated and re-suspending them in nuclear extraction buffer (20 mM Tris-HCl, pH 7.5, 0.05% NP-40, 350 mM NaCl, 0.25 mM EDTA, 20% Glycerol, 0.1 mM Na_3VO_4, 0.1 mM PMSF, protein inhibitor cocktail tablet (Roche, 1 tablet in 7 ml of lysis buffer)). The suspension was rotated for 30 min at 4°C, and centrifuged at 12,000g for 30 min at 4°C. The extract was pre-cleared using protein G agarose beads.

To analyzed interactions among endogenous proteins, 1 ml nuclear extract was incubated with the antibodies or antisera indicated at 4°C overnight and 50 µL protein G beads were added and rotated for an additional 2 hours. The bound immunoprecipitated complexes were washed 5 times with buffer (50 mM Tris-HCl, pH 7.9, 0.1% NP-40, 150 mM NaCl, 0.25 mM EDTA, 0.1 mM PMSF). The proteins were resolved using NuPAGE 4-12% Bis-Tris Gel (Invitrogen) and were transferred to Immobilon membrane (Millipore). Specific proteins were detected using the antibodies indicated and ECL Plus (Amersham). The membranes were exposed to Hyperfilm (Amersham) and the films were scanned using a flatbed scanner.

To map the regions of KAP1 required for interaction with Cbx2, 10 µg of YFP-Cbx2 and 10 µg of FLAG-HA-KAP1 or mutants were transiently transfected into HEK293T cells grown on a 10-cm plate to 50% confluence using Fugene6 according to manufacturer instructions. 36–48 h after the transfection the cells were lysed in 1 ml of lysis buffer (20mM HEPES at pH 7.9, 150 mM NaCl, 0.25 mM EDTA, 0.1 mM PMSF, 0.5 % NP-40, 0.1 mM Na_3VO_4, 20% glycerol, and protein inhibitor cocktail tablet (Roche, 1 tablet in 7 ml of lysis buffer)). 1 ml of the cell extract was incubated with 5 µg of anti-GFP antibodies at 4°C overnight. The immune complexes were captured on 50 µL protein G beads beads and were washed five times with buffer (50 mM Tris-HCl, pH 7.9, 0.1% NP-40, 150 mM NaCl, 0.25 mM EDTA, 0.1 mM PMSF). The proteins were analyzed by immunoblotting using the antibodies indicated as described above. To investigate KAP1 interaction with different Cbx family proteins, the same procedure was used.

To analyze the specificity of the interaction between the coiled coil region of KAP1 and PRC1 subunits, nuclear extracts were prepared from ES cells that stably expressed FLAG-HA-KAP1 or FLAG-HA-KAP1ΔCC as described above. The 1 ml nuclear extract was pre-cleared using protein G agarose beads, and incubated with anti-FLAG M2 beads (Sigma, A2220) at 4°C overnight. The immunoprecipitated complexes were washed 5 times with 50 mM Tris-HCl, pH 7.9, 0.1% NP-40, 150 mM NaCl, 0.25 mM EDTA, 0.1 mM PMSF. The proteins were analyzed by immunoblotting using the antibodies indicated as described above.

Analysis of transcript levels by RT-qPCR

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using 1µg of total RNA using the Reverse Transcription System(Promega) with random primers according to the instructions provided by the manufacturer. qPCR was performed using Light Cycler 480 SYBR Green I Master (Roche) in a Light Cycler 480 II (Roche). Triplicate or quadruplicate PCR reactions were carried out for each sample. All transcript levels were normalized by the levels of Gapdh transcripts in the same samples. The sequences of the primers used for qPCR are shown in Table S6.

Microarray analysis of transcript levels

Wild type and *KAP1*$^{fl/fl}$ ES cells were treated with tamoxifen for 24 h followed by 48 h culture without tamoxifen. Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). The integrity of RNA was analyzed by RNA 6000 Nano Bioanalyzer. The transcripts were reverse transcribed and the labeled cDNAs were analyzed by hybridization to the Affymetrix Mouse Gene ST 1.1 array following the manufacturer's instructions. The changes in hybridization signals were quantified using software provided by Affymetrix.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed by fixing the cells with 1.42% formaldehyde for 15 min and washing them sequentially with buffer LB1 (50 mM Hepes-KOH, pH 7.9, 140mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) and LB2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1.5 mM EDTA) and LB3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1.5 mM EDTA, 0.1% Na-Deoxycholate, 0.5% Na-Lauroylsarcosine). The chromatin was fragmented using Bioruptor (Diagenode) to produce chromatin fragments containing 100-1,000 bp DNA. After pre-incubation with protein G beads, antibodies (anti-KAP1, 5 µg; anti-Ring1b, 3 µg; anti-Cbx7, 10µl; anti-Mel18, 5 µg; anti-H3K27Me3, 5 µg; anti-H3K27Me9, 5 µg; anti-H3K27Me4, 5 µg; anti-H3, 5 µg) were added to 1 ml of fragmented chromatin and the precipitates were extensively washed. The immunoprecipitated DNA was quantified using LightCycler 480 SYBR Green I Master (Roche) with a LightCycler 480 II (Roche). Triplicate or quadruplicate PCR reactions were carried out for each sample. The efficiencies of chromatin immunoprecipitation were quantified relative to a standard curve prepared using input chromatin. The efficiencies of all ChIP assays were normalized by the efficiencies of ChIP using anti-H3 antibodies of the same samples performed in parallel. The sequences of the primers used for ChIP analysis are shown in Table S5.

ChIP-Seq analysis

Kap1$^{fl/fl}$ and *Ring1b*$^{fl/fl}$ ES cells were treated with tamoxifen or ethanol for 48 hours and cultured an additional 48 hours without tamoxifen or ethanol. The number of cells in each culture was estimated by counting cells cultured in parallel under identical conditions. The cells were fixed using formaldehyde at a final concentration of 1.2% for 10 min at room temperature. Formaldehyde fixation was terminated by adding glycine to a final concentration of 125 mM for 10 min. The fixed cells were washed with cold PBS, collected by scraping and harvested by centrifugation at 2,000g for 5 min at 4°C. 3 X10^7 cells were suspended in 1 ml LB1 (50 mM Hepes-KOH, pH 7.9, 140mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100) and were rocked at 4°C for 10 min. Cells were pelleted by centrifugation at 1,350g at 4°C for 5 min. The pellets were suspended in 1 ml LB2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1.5 mM EDTA) and rocked at room temperature for 10 min. After centrifugation, the pellets were suspended in 1 ml of LB3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1.5 mM EDTA, 0.1% Na-Deoxycholate, 0.5% Na-Lauroylsarcosine). The chromatin was sonicated until a size distribution with a maximum at 200 bp was reached using a Bioruptor (Diagenode). After sonication, Triton X-100 was added to a concentration of 1%. The mixture was centrifuged at 20,000g for 10 min at 4°C. To the lysates, BSA and tRNA were added to obtain the final concentrations of 1 mg/ml and 0.25 mg/ml, respectively. After pre-clearing with agarose beads, antibodies (anti-KAP1, 5 µg, anti-Ring1b, 3 µg, and anti-H3, 5 µg [served as a control to test for any variability in preparation of ChIP samples and construction of sequencing libraries]) were added to 1 ml of fragmented chromatin and the mixtures were rotate at 4 °C for overnight. Agarose beads were added to the mixture and the reaction was rotated at 4 °C for an additional 3 h. The beads were washed 5 times with 1 ml of RIPA buffer (50 mM Hepes-KOH, pH 7.9, 0.5 M LiCl, 0.5 M EDTA, 1.0% NP-40, 0.7% Na-Deoxycholate). After transferring the beads to a new tube, they were washed once with 1 ml of TE buffer containing 50 mM NaCl. The immunoprecipitated chromatin was eluted with elution buffer (50 mM Tris-HCl, pH8.0, 10 mM EDTA, 1.0% SDS) and the cross-links were reversed by addition of RNase A and Proteinase K at final concentrations of 0.2 µg/ml each. After phenol: chloroform

extraction, the DNAs were precipitated and dissolved in Qiagen Buffer EB (10 mM Tris•HCl, pH8.5).

The sequencing libraries were constructed by following the instructions provided by Illumina. Briefly, the immunoprecipitated DNA ends were repaired. After dA addition and adaptor ligation, gel electrophoresis was performed and DNAs ranging in size from approximately 100-300 bp were recovered. The recovered DNAs were subjected to PCR amplification using primers provided by Illumina. After purification, the variability of the entire process for preparing samples was tested by qPCR quantification of DNA precipitated by H3. The levels of DNA precipitated by anti-H3 antibody from tamoxifen-treated cells were the same as those precipitated from ethanol-treated cells, suggesting that preparation of the ChIP samples and construction of libraries did not introduce variability into the analysis. The DNA concentrations of the libraries were quantified using a Qubit 2.0 Fluorometer and the size distributions were determined using a Agilent 2100 Bioanalyzer. The DNA library was sequenced by Illumina Genome Analyzer at the University of Michigan Medical School DNA Sequencing Core Facility.

Bioinformatic analysis of ChIP-seq data

The ChIP-seq tags were mapped to the mouse genome (mm10) using Bowtie2 with default settings. The tags were also mapped to mouse genome (mm9) with similar results. The coverage of tags in genes whose transcription increased or decreased upon *KAP1*$^{fl/fl}$ knockout as well as in annotated promoters was determined using ngs.plot.r v. 1.64. The regions with significant enrichment of KAP1 and Ring1b ChIP-seq tags were determined using MACS14 with default settings ($p<10^{-5}$). The overlap between genes and promoters bound by KAP1 and Ring1b as well as those that whose transcription increased or decreased upon *KAP1*$^{fl/fl}$ knockout were determined using Bedtools v. 2.16.2. Promoters were defined as sequences within 1 kb from a transcription start site annotated in RefSeq Genes as of 2013-05-28. Genes were defined as RefSeq Genes together with 1 kb of flanking sequences. Overlapping promoters and genes were merged. The distributions of ChIP-seq tags were plotted using the UCSC genome browser.

Differentiation of mouse ES cells

Embryoid bodies (EB) were formed by seeding 2.0×10^6 ES cells into 10 cm BD Falcon™ Petri Dishes made of polystyrene (Bacteriological Petri Dish, Cat. 351029) in ES cell medium without LIF. Tamoxifen (1 µM, from Sigma) was added to the medium at different times as indicated in the Fig. legends for a period of two days. The medium was changed every two days. Embryoid bodies were collected at 4 and 8 days after inducing differentiation and transcript levels were quantified as described.

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