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Supplemental Methods

Mice. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital Boston. $Ezh2^{fl}$, $Nkx2\text{-}5^{Lz}$, TNT-Cre, and $Nkx2\text{-}5^{Cre}$ alleles were described previously [1-4]. Construction of the Eed^{fl} allele will be described elsewhere. The mice were on a mixed C57BL6/J and 129 genetic background. Noon of the day of a vaginal plug was defined as day E0.5.

Echocardiography was performed with a VisualSonics Vevo2100. M-mode images were acquired from awake mice and used to calculate fractional shortening, left ventricular end diastolic diameter, left ventricular end systolic diameter, and posterior left ventricular wall thickness.

Histology. For H&E staining , Masson's trichrome staining, and morphometry, embryos were fixed in 4% PFA on ice, then dehydrated, paraffin embedded and sectioned. For morphometric measurements, we measured transverse sections at the level of the atrioventricular valves. Wall thickness was calculated as the ventricular compact myocardial thickness divided by its outer circumference. Trabecular and myocardial area were measured in Adobe Photoshop after selection of image areas with myocardial color range.

For cryosectioning, fixed embryos were cryoprotected in 30% sucrose, then frozen in OCT and sectioned. Immunostaining was performed with antibodies listed in Supplementary Table 1. Primary antibodies were visualized by staining with Alexa-conjugated secondary antibodies (Invitrogen) and imaged on an Olympus FV1000 confocal microscope.

Gene Expression. Total RNA was isolated from embryos using the RNeasy kit (Qiagen) with on column DNase digestion. Quantitative reverse transcription PCR (qRTPCR) was performed using the ABI Power Sybr Green PCR Master Mix (Applied Biosystems). Gene expression was normalized to GAPDH. Primers are provided in Supplementary Table 2.

Genome-wide expression profiling was performed on E12.5 heart apex by RNA-seq, using a modification of a protocol described previously.[5]. Steps for library normalization were omitted. Briefly, polyadenylated RNA was isolated from total RNA using two rounds of selection on oligo-dT Dynabeads (Invitrogen). RNA was converted to cDNA with Superscript III (Invitrogen) and random hexamer primers. After RNaseH treatment and Pol I catalyzed second strand cDNA synthesis, DNA end repair performed with the End-It DNA end-Repair Kit (Epicentre). DNA was then A-tailed with Exo(-) Klenow (NEB), and adapters were ligated using T4 quick ligase. Fragments 150-300 bp were size-selected by agarose gel electrophoresis. Recovered DNA was PCR amplified using Phusion DNA polymerase (NEB), multiplexing PCR primer 1.0, and one indexed primer. The library was quantitated using the QuantIT DNA quantitation kit (Invitrogen). Library quality control was performed using an

Agilent Bioanalyzer. 50 nt paired end reads were obtained using an Illumina HiSeq 2000. Primer sequences are provided in Supplementary Table 2.

RNA-seq data was mapped to the genome using Bowtie[6]. Expression was measured as the reads per one thousand bases per million total reads. Differential expression was determined as described using exact tests between two negative binomial groups[7], and false discovery rate was calculated using the Benjamini and Hochberg method. RNA-seq data are freely available through the NIH-funded Cardiovascular Development Consortium server (http://b2b.hci.utah.edu). Login as guest.

Chromatin Immunoprecipitation. The ChIP protocol has been described in detail.[8]. Briefly, ventricular heart apex was collected and immediately fixed in 1% v/v formaldehyde at room temperature for 7 minutes. Formaldehyde was neutralized with 2.5 M glycine. Tissue was homogenized with a T10 homogenizer (IKA). Cells were then placed in hypotonic lysis buffer and dounce homogenized. DNA was then fragmented in a Misonix 4000 sonicator to an average size of 150-300 bp. Chromatin was ten precleared with protein A Dynabeads (Invitrogen), then immunoprecipitated with specific antibody (Online Table I) and protein A Dynabeads. After extensive washing, complexes were eluted and crosslinks reversed in 1% SDS, 50 mM Tris 8.1, and 10 mM EDTA at 70°C. DNA was purified using the QIAquick PCR purification kit (Qiagen). For quantitation by PCR, locus-specific primers (Online Table II) were used for real time PCR with the ABI Power Sybr Green Master Mix.

To build ChIP-seq libraries, we used the standard Illumina protocols with the NEB Next DNA sample preparation kit and primers listed in Online Table II. Libraries were sequenced (50 nt single end) on an Illumina HiSeq 2000. Sequencing reads were aligned with Bowtie[6], and peaks were identified with Sole-Search [9] with default parameters. ChIP-seq data are freely available from the Cardiovascular Development Consortium server as described above.

Genomic intervals were combined and annotated using the completeMOTIFs pipeline[10]. GO term analysis was performed using the GOstats R library[11], implemented in completeMOTIFs.

Statistics. qRTPCR results were expressed as mean ± SEM, while ChIP-qPCR results were displayed as mean ± SD. Two group comparisions were performed with Student's t-test with $P<0.05$ taken as statistically significant.

Supplemental References

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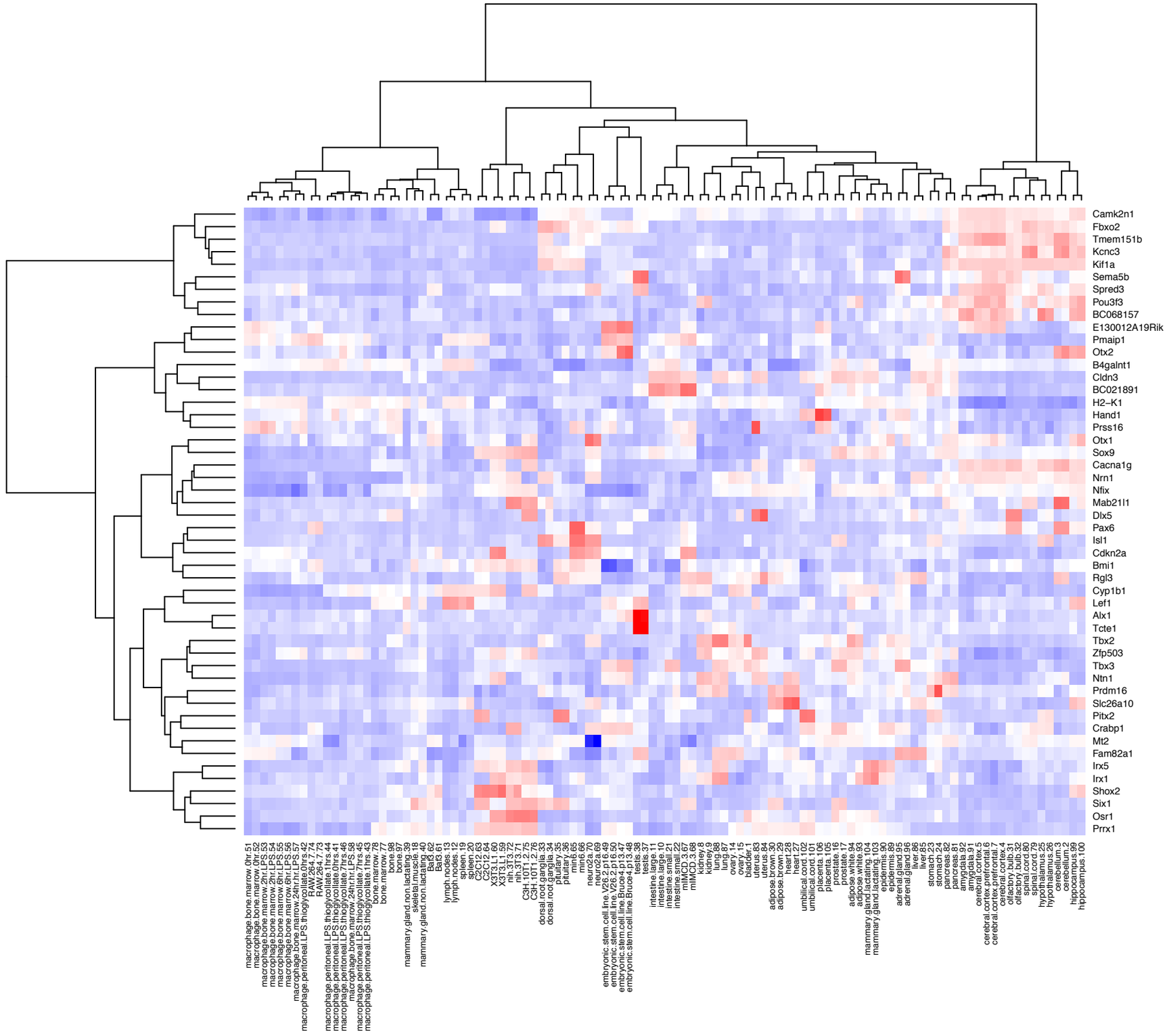
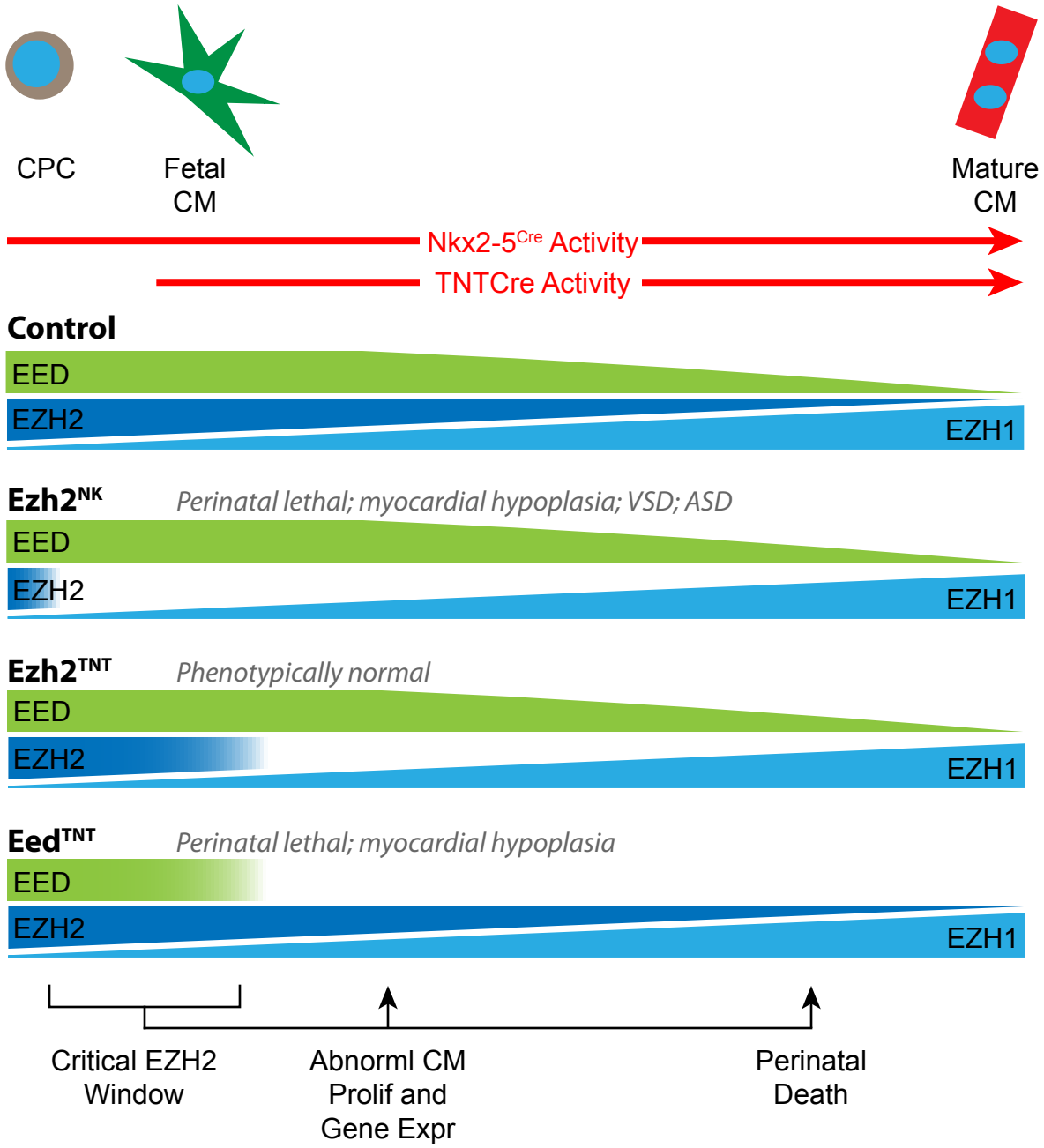


Online Figure I. Heat map showing expression of EZH2-repressed genes in multiple tissues. This is an expanded version of Figure 5B showing gene, sample labels and biological duplicates from GSE15998.



Online Figure 2. PRC2 loss of function models. PRC2 subunits EED and EZH2 were inactivated by Nkx2-5Cre and TNTCre. While Nkx2-5Cre is active in cardiac progenitor cells and cardiomyocytes, TNTCre is restricted to cardiomyocytes. Ezh1 and Ezh2 are partially functionally redundant, and Ezh1 expression increases with cardiomyocyte maturation. Eed is another essential PRC2 subunit, with a single mammalian isoform. PRC2 is required in the TNTCre recombination domain, since EedTNT mutants also develop lethal heart defects. Lack of phenotype in Ezh2TNT is likely due to Ezh1 functional redundancy. Lethal cardiac malformations in Ezh2NK reveal a window where Ezh2 activity is required in cardiac progenitors or early cardiomyocytes. Interestingly, abnormal CM proliferation and gene expression are detectable in Ezh2NK mutants even at later heart stages where Ezh2 is no longer essential. This suggests that heart development cannot recover early perturbations of the chromatin landscape resulting from early Ezh2 inactivation.

Online Table I. Antibodies used in this study.

Antibody	Application	Source	Cat No
EZH2	WB	Cell Signaling	#4905
EZH2	IF	Active Motif	39103
EZH2	ChIP	Millipore	17-662
SUZ12	WB	Active Motif	39357
EED	WB	Reinberg lab	Margueron et al., 2009
H3K27me3	ChIP, IF	Millipore	17-622
phosphohistone H3	IF	Millipore	06-570
TNNI3	IF	Abcam	ab56357
ISL1	IF	Developmental Studies Hybridoma Bank	39.4D5
Tnni3	IF	Abcam	Ab56357
Myl7	IF	Santa Cruz Biotechnology	sc-66967

Online Table II. Oligonucleotides used in this study.

ChIP-qPCR primers

primer ID	Forward	Reverse	ChIP use
Intergenic Control	ATTTTGTGCTGCATAACCTCCT	TAGCAACATCCTAAGCTGGACA	Negative control
Actb	CGTATTAGGTCCATCTTGAGAGTACACAGTATT	GCCATTGAGGCGTGATCGTAGC	Reference
Myh6-1	GAAGTGAGAAATGGGTGGAAAG	CGTCTTGGTTTATCTTGGCTCT	Gata4, Ezh2, H3K27me3
Myh6-2	ATGGGCAGATAGAGGAGAGACA	CAGTTGTTCAACTCACCCTTCA	Gata4, Ezh2, H3K27me3
Myh6-3	AGGAACACTCTCCCTGCTACC	CCTTGGGGAGACACCATATTAC	Gata4, Ezh2, H3K27me3
Myh6-4	ATATGTCACTGCCTGGTTCTCA	AAGCTGACCCAATGTTCTCAGT	Gata4, Ezh2, H3K27me3
Myh6-5	ATCTTGAGGCTCTACCACCAGT	AAGGAGATGTGTGGAGAAGTCC	Gata4, Ezh2, H3K27me3
Ink4b	CGACGGGAGGCAGGTTTTgc	CAATCTAGTGCCGAGGGATGTT	Ezh2, H3K27me3
Ink4a	GTCCGATCCTTTAGCGCTGTT	AGCCCGGACTACAGAAGAGATG	Ezh2, H3K27me3
Six1	CTCTCGTTCTTGTGCAGGTG	TTTTACGCAAGAGCAAGTGG	Ezh2, H3K27me3
Pax6	CTAATCTGCCGAGCTGAACC	GCAGGCGCTAACTTTCCTTA	Ezh2, H3K27me3
Isl1	ACTCAGCTCCATCGCCATT	CCGGGGCTGAAATATGATAA	Ezh2, H3K27me3
Hcn4	AGCGGCTCTACAGCCTTCC	TCACCATCCTCTTCCTCGTC	Ezh2, H3K27me3
Myl7	GGTGTGGCTGGTCTCTTGTT	CCTCTGGGTGATAAGGCTGA	Ezh2, H3K27me3
Tnnt2	TCAAGAGGGACAGCTGGTTT	AACAAGTACCCCACGCCATA	Ezh2, H3K27me3
Neurog1	TGGTCTCCTGAGTGATGTCG	GCCGTACTTAAGGGGTCCTG	Ezh2, H3K27me3

RT-qPCR primers

Gene	Forward	Reverse	Species
Bmi1	TTTATGCAGCTCACCCGTC	TTTCCGATCCAATCTGCTCTG	mouse
Bmp10	ACAAATTCGCCACAGACCG	GAGGGATAGACACATTGAAGAGG	mouse
Hcn4	GATTATCCACCCCTACAGTGAC	ACCACATTGAAGACGATCCAG	mouse
Ink4a	GTGTGCATGACGTGCGGG	GCAGTTCGAATCTGCACCGTAG	mouse
Ink4b	CCCTGTGAACTGAAAATGCAGA	TGTCGAGCTGGAGGTGACTTC	mouse
Isl1	CAGCAACCCAACGACAAAAC	GTCACTCAGTACTTTCCAGGG	mouse
Mlc2a	ATCAACTTCACCGTCTTCCTC	ACTCTTCCTTGTTCACCACC	mouse
Myh6	ACGGTGACCATAAAGGAGGA	TGTCCTCGA TCTTGTCGAAC	mouse
Myh7	GCCCTTTGACCTCAAGAAAG	CTTCACAGTCACCGTCTTGC	mouse
Neurog1	ATCCCCTTTTCTCCTTTCCTG	CTTCAGCCAGTTCCCCATC	mouse
Pax6	CCCTCACCAACACGTACAG	TCATAACTCCGCCCATTCAC	mouse
Serca2	CATCTGCTTGTCCATGTCACTT	CGGTGTGATCTGGAAAATGAG	mouse
Six1	TTAAGAACCGGAGGCAAAGAG	CTTCTGAGCTGGACATGAGC	mouse
Tbx2	CACAAACTGAAGCTGACCAAC	GAAGACATAGGTGCGGAAGG	mouse
Tbx3	AGCCAACGATATCCTGAAACTG	GTGTCTCGAAAACCCTTTGC	mouse
Nppa	GGCCATATTGGAGCAAATCCTGTG	CATGACCTCATCTTCTACCGGCAT	mouse
Tnnt2	GTAGAGGACACCAAACCCAAG	GAGTCTGTAGCTCATTCAGGTC	mouse
Gapdh	4308313, Applied Biosystems		
Ezh1	AATATGGGAGCAAAGGCTCTGTATGTG	AATATGGGAGCAAAGGCTCTGTATGTC	mouse
Ezh2	TTACTGCTGGCACCGTCTGATGTG	TGTCTGCTTCATCCTGAGAAATAATC	mouse

ChIP-seq and RNA-seq library construction primers

PE adapter 1	5' phosphate-GATCGGAAGAGCACACGTCT
PE adapter 2	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Multiplexing PCR Primer 1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
Pr_index_1 (lowercase=index)	CAAGCAGAAGACGGCATACGAGATcgtgatGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Pr_index_4 (lowercase=index)	CAAGCAGAAGACGGCATACGAGATtggtcaGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Pr_index_6 (lowercase=index)	CAAGCAGAAGACGGCATACGAGATattggcGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Online Table VI. Transcriptional modules with genes over-represented among genes upregulated in Ezh2NK.

Module	Nominal P-value	Corrected P-value (Holm)	Module GO	Module Regulators	Overlap	Upregulated only	Module only	Neither
ME1	2.67E-022	2.35E-020	neuron development	Dlx5,Isl1,Pou3f3	220	746	1783	13980
ME12	1.41E-009	1.22E-007	cell adhesion	Irx5,Osr1,Pitx2,Prrx1,Six1,Tbx2,Tbx3	49	917	284	15479
ME25	6.95E-009	5.98E-007	cell adhesion	Irx5,Osr1,Prrx1,Six1,Tbx3	33	933	153	15610

The columns **Overlap**, **Upregulated only**, **Module only**, and **Neither** indicate, respectively, the number of genes in common between the upregulated genes and the module, the number of genes that are upregulated but not in the module, the number of genes that are in the module and not upregulated, and the number of genes that are not upregulated and not in the module. Only genes in common to the RNA-seq data and the Affymetrix 430 2.0 platforms were used in the analysis.