Supplemental Materials

Supplemental Figures
Figure S1. Additional analysis of Mediator occupancy in AML cells, related to Figure 1.
(A) Comparison of tag counts of BRD4 ChIP-seq and MED1 ChIP-seq at 10,604 MED1 peaks in the RN2 genome.

(B-G) ChIP-qPCR analysis of BRD4, MED1, MED12, MED23, or control IgG at the Myc locus in RN2 cells following 2 hr treatment with DMSO or with 500 nM JQ1.

(H) Comparison of fold change in tag counts of BRD4 vs. MED1 ChIP-seq at 10,604 MED1 peaks in the genome after 2 hr 500 nM JQ1 treatment.
Figure S2. Additional data analyzing JQ1-sensitive MED1 peaks, related to Figure 2.

(A) Stratification of the 200 most JQ1-sensitive MED1 peaks based on whether or not they overlap with a super-enhancer (as defined in Figure 1C). Minimum required overlap was 1 bp.

(B) Average fold change in FPKM after 2 hr 500 nM JQ1 treatment for genes associated with the 200 most JQ1-sensitive Brd4 peaks (left) and for genes associated with the 200 most JQ1-sensitive MED1 peaks (right). The numbers in parentheses represent the number of genes matched to the class of peaks indicated. The p value is the result of a Mann-Whitney test.

(C) ChIP-seq meta-profiles for the hematopoietic ETS TFs ERG and FLI1 at 200 JQ1-sensitive MED1 peaks or the remaining 10,404 MED1 peaks.

(D) Motif counts at the 200 most JQ1-sensitive MED1 peak cores or the remaining 10,404 MED1 peak cores. Peak cores were defined as the 400bp surrounding the summits of the indicated peaks. Motif counts were performed using the FIMO algorithm (Grant et al., 2011).
Figure S3. Additional data characterizing phenotypes of Mediator subunit knockdown, related to Figure 3.
(A) qRT-PCR analysis to test knockdown efficiency of Mediator subunit shRNAs in RN2 cells. Relative mRNA levels of the indicated Mediator subunits following 48 hr treatment of RN2 cells with doxycycline to induce expression of the indicated shRNA from the TRMPV-Neo vector. Values were normalized to Gapdh expression within each sample and to shRen values across samples. Data are presented as mean ± SEM; n=3.

(B) Negative-selection screening in iMEF cells using the indicated shRNAs (LMN vector) chosen from the screen in (Figure 3A). GFP+/shRNA+ percentages were normalized to values taken on day 2 and tracked for 16 days. Data are presented as mean ± SEM; n=3.

(C) Relative mRNA levels of the indicated Mediator subunit following expression of the indicated haripins in iMEF cells.

(D) Relative mRNA levels of the indicated Mediator kinase following knockdown of Cdk8 or Cdk19 with the indicated shRNA (LMN vector) in RN2 cells. Data are presented as mean ± SEM; n=2-3.

(E) Negative-selection competition assay of double shRNA targeting of Cdk8 and Cdk19. Plotted is the percentage of GFP+/mCherry+ cells over time following transduction of RN2 cells with the indicated pair of shRNAs. mCherry-containing constructs (LMN-Cherry) were introduced first, followed by the GFP-containing constructs (LMN vector). Data are presented as mean ± SEM; n=3.
Figure S4. Additional data characterizing transcriptional effects of MED8 knockdown, related to Figure 4.

(A) Fold change in FPKM values for 8,201 genes expressed in AML (defined by FPKM>5 in shRen sample) following 48 hr of doxycycline-induced shRNA expression. Two independent Med8 shRNAs were evaluated and the reported fold change values for each gene are the average value of the two hairpins. The genes are ranked in order of increasing fold change. The numbers in parentheses represent the fold change expression rank of the indicated genes.

(B) Gene Set Enrichment Analysis (GSEA) performed on MED8 RNA-Seq data using 10,379 gene sets, including all gene sets in the Molecular Signatures Database. Signatures are plotted by their Normalized Enrichment Scores and FDR q-values as reported by GSEA.

(C) Scatter plots comparing fold change in FPKM values from RNA-Seq data sets of shRen vs. shMed8, shMed12, or shMed23. 8,122 genes expressed (FPKM>5) in all shRen samples were used in this analysis.

(D) Table of normalized enrichment scores of the indicated gene sets, as reported by GSEA, for RNA-Seq studies following shMed8, shMed12, shMed23, or 6 h 500 nM JQ1 treatment.
Figure S5. Additional data characterizing the impact of Mediator subunit knockdown on BRD4 genome occupancy, related to Figure 5.
(A) Fold change in occupancy of BRD4 at 10,604 MED1-defined peaks in AML following 48 hr treatment with doxycycline to induce expression of a MED12 shRNA. The peaks are ranked in order of increasing fold change. Colored dots highlight the indicated loci in the genome.

(B) Box plot showing fold change of BRD4 occupancy 48 hr treatment with doxycycline to induce a Med12 shRNA at 200 JQ1-sensitive MED1 peaks or at 10,404 remaining peaks in the genome. *** represents a p value < 0.0001, the result of a Mann-Whitney test.

(C) ChIP-qPCR analysis of BRD4 at the Myc locus following 48 hr treatment with doxycycline to induce a Med12 shRNA. TRMPV-Neo vector was used.

(D) Fold change in occupancy of BRD4 at 10,604 MED1 peaks in AML following 48 hr treatment with doxycycline to induce expression of a Med23 shRNA. The peaks are ranked in order of increasing fold change. Colored dots highlight the indicated loci in the genome.

(E) Box plot showing fold change of BRD4 occupancy after 48 hr treatment with doxycycline to induce a Med23 shRNA at 200 JQ1-sensitive MED1 peaks or at 10,404 remaining peaks in the genome. *** represents a p value < 0.0001, the result of a Mann-Whitney test.
Supplemental Tables

Table S1 - Top 200 JQ1-sensitive MED1 High Confidence Regions, related to Figure 2

Table S2 - MED1 region gene assignments, related to Figure 2

Table S3 - shRNA sequences used in this study, related to Figure 3

Table S4 - Primers used for ChIP-qPCR and qRT-PCR in this study, related to Figure 5 and Figures S1, S3, and S5
Supplemental Experimental Procedures

Cell culture
RN2 cells were derived as previously described (Zuber et al., 2011) and were cultured in RPMI-1640 with 10% FBS and Penicillin/streptomycin. iMEF cells, Plat-E cells, and 293T cells were cultured in DMEM with 10% FBS and penicillin/streptomycin. Transfections were carried out using Calcium phosphate or PEI transfection methods.

shRNA screen
LMN-shRNAs vectors were individually packaged in retroviruses using the Plat E cell line using calcium phosphate transfection methods. A murine MLL-AF9/NrasG12D (RN2) or immortalized fibroblast (iMEF) cell line was infected with these viruses in a one-by-one manner. GFP percentage, corresponding to the percent of cells that were infected with an shRNA, was tracked using a Guava EasyCyte (Millipore). The loss of GFP over time corresponds to the loss of the shRNA from the population (in favor of uninfected cells), and thus is a suitable measure of the toxicity of an shRNA. The sequences of the shRNAs used in this study can be found in Table S3.

RT-qPCR
Total RNA was extracted from cells using TRIzol (Invitrogen) according to the manufacturer’s instructions. Isolating RNA was treated with DNase I to eliminate contaminating genomic DNA. SuperScript III reverse transcriptase was used to synthesize cDNA according to the manufacturer’s protocol. Results were quantified by qPCR performed using SYBR green on an ABI 7900HT Fast Real-Time PCR machine, and normalized to Gapdh. Primers used for analysis can be found in Table S4.

Antibodies for ChIP
BRD4: Bethyl A301-985 (2µg/IP)
MED1: Bethyl A300-793A (2µg/IP)
MED12: Bethyl A300-774 (5µg/IP)
MED23: Bethyl A300-423A (5µg/IP)
CDK9: SC-484(1.6µg/IP)
PolII: Ab5408 (2µg/IP)

ChIP-qPCR
5-20 million RN2 cells were crosslinked in 1% formaldehyde for 20 minutes and quenched for 10 minutes in 0.125M Glycine. Nuclear lysates were sonicated in 20-million cell batches using a BioRuptor water bath sonicator. Sonicated chromatin was pre-cleared using rabbit IgG and agarose beads followed by addition of the antibody and beads (BRD4 and MED1) or was incubated with the relevant antibody for two hours followed by addition of magnetic beads (MED12, MED23, CDK9, PolII) and overnight incubation. After extensive washing, crosslinks were reversed overnight at 65°C. DNA was treated with RNase and proteinase K and purified using the QIAGEN PCR purification kit and analyzed using an ABI 7900HT Fast Real-Time PCR machine and SYBR green. Primers used for analysis can be found in Table S4.

ChIP-Seq
120 million RN2 cells were crosslinked in 1% formaldehyde for 20 minutes and quenched for 10 minutes in 0.125M Glycine. Nuclear lysates were sonicated in 20-million cell batches using a BioRuptor water bath sonicator. Sonicated chromatin was pre-cleared using rabbit IgG and agarose beads or was directly incubated with the BRD4 antibody for two hours followed by addition of magnetic beads (BRD4 ChIP-seq in MED12/23 knockdown conditions). For both magnetic and
agarose bead ChIP conditions, IP with the relevant antibodies was done overnight at 4°C with rotation. After extensive washing as previously described (Steger et al., 2008), crosslinks were reversed overnight at 65°C. DNA was treated with RNase and proteinase K and purified using the QIAGEN PCR purification kit. Libraries were constructed using the TruSeq ChIP Sample Prep kit from Illumina. Libraries underwent a final amplification step of 15 PCR cycles and were analyzed using a Bioanalyzer with a High Sensitivity chip (Agilent). Libraries were single-end sequenced on a HiSeq2000 with reads of 50bp.

**ChIP-seq analysis**

1. Previously Published data
   a. 5,135 high confidence BRD4 peaks were previously defined (Roe et al., 2015).
   b. The following RN2 ChIP-seq datasets from (Roe et al., 2015) were used:
      i. C/EBPα, C/EBPβ, MYB, ERG, FL11, PU.1 BRD4
   c. For density plots, these 5,135 high-confidence BRD4 peaks were used.
      i. Sequencing reads from each ChIP-seq dataset used were binned in 500 20bp bins around the MACS-defined summit of these 5,135 BRD4 peaks. These resulting matrices were converted into a heatmap using Java TreeView 1.1.6r4.

2. Peak calling for new ChIP-seq datasets
   a. For all new ChIP-seq datasets, 50bp sequence reads were mapped to mm9 using the BOWTIE algorithm. To call peaks, the MACS algorithm version 1.4.2 was used.
   b. To define a set of high-confidence MED1 peaks in the AML genome, the MACS output for each of the three MED1 (untreated, DMSO, and DMSO) replicates was intersected as follows:
      i. MACS-called peaks were filtered to retain only those peaks with a FDR value ≤ 1 and a fold-enrichment over input of at least 10.
      ii. The intervals of each dataset were intersected sequentially (minimum overlap 1bp) to retain only those peaks that appeared in all datasets being intersected.
      iii. The result was a set of 10,604 high-confidence MED1 peaks. Overlapping these peak boundaries with the areas ±2kb around RefSeq transcription start sites for mm9 resulted in a distribution of 6328 promoter regions and 4276 putative enhancer regions. A list of the 200 most JQ1-sensitive MED1 regions and their fold change across the average of two data sets is available in Table S1.

3. Identifying MED1 super enhancers
   a. As described before (Loven et al., 2013), the ROSE algorithm was used to stitch together any of the 10,604 MED1 peaks that occurred within 12.5kb of each other. The recommended TSS-exclusion zone of ±2.5kb was used. From the resulting 4,056 stitched regions, 178 super enhancers were called by ROSE.

4. Defining JQ1-sensitivity at MED1 peaks
   a. For all subsequent analyses, including calculation of fold change after JQ1 treatment and analyses of occupancy at subsets of these 10,604 peaks, BOWTIE-mapped bam files were filtered to remove redundant reads.
   b. To calculate the fold change of Mediator (and BRD4) following JQ1 treatment, ChIP-seq reads that mapped into high-confidence MED1 intervals (minimum overlap 1bp) were counted in both replicates of the DMSO and JQ1 datasets. Normalization to total number of unique mapped reads was applied. A fold change of MED1 at each peak was then calculated by dividing the number of reads at each peak in the JQ1 sample by the number of reads at each peak in the corresponding DMSO sample. The fold change across both replicates was averaged and this average was used to plot the fold changes and to identify the 200 most sensitive MED1 regions.
c. To calculate the fold change at Super Enhancers, the same method was used as in (b) but using the stitched intervals supplied by ROSE.

d. Additionally, these ROSE-stitched intervals were intersected with the 200 most sensitive MED1 unstitched peaks (minimum overlap 1bp). This resulted in 75 super enhancers being defined as “sensitive” and 103 super enhancers defined as “insensitive” to JQ1.

5. Associating MED1 peaks with genes

a. The GREAT algorithm (McLean et al., 2010) defines “gene neighborhoods” in several ways. The default method is -1kb from a gene body to +5kb from the end of a gene body, plus an extension of up to 1Mb until the next gene neighborhood. Peaks falling within these gene neighborhoods are then attributed to these genes. The default GREAT definition of gene neighborhoods was used for the results presented here. Using the alternative gene neighborhood definition (two-nearest genes) produced similar results. We supplied GREAT with the 10,604 MED1 regions as well as the 178 stitched super-enhancer regions for gene assignments.

b. To improve accuracy of gene assignment, the genes assigned to the 200-sensitive MED1 peaks and to the 178 super enhancers were manually corrected in the following ways when possible:

i. If a peak overlapped with an expressed gene and GREAT did not assign it to that gene, the overlapping expressed gene was used instead.

ii. If GREAT assigned a peak to a gene that is not expressed in RN2 cells (FPKM < 5), the following was applied:
   1. If GREAT also assigned a second possible gene that was expressed, this gene was used
   2. If GREAT did not assign a second gene OR if the second gene was also not expressed, the nearest expressed gene was used.

iii. The Myc enhancers, which we have previously characterized as looping to the Myc gene in RN2 cells (Shi et al., 2013), were assigned to Myc instead of the nearby (unexpressed) Gsdmc.

iv. If none of the above criteria could be met, the peak was left unassigned to a gene.

v. Genes assigned to 200 JQ1-sensitive MED1 peaks and to super-enhancers can be found in Table S2.

6. Calculating the fold change in gene expression as was done using the RN2 DMSO vs RN2 6 h JQ1 RNA-seq from Roe et al, 2015. “All expressed genes” refers to all genes with FPKM > 5 in this data set. “JQ1 Insensitive MED1 Peaks” refers to all genes assigned to the 10,404 MED1 peaks not included in the set of 200 most JQ1-sensitive MED1 peaks. If a gene was associated with multiple peaks (e.g., Myc enhancers corresponding to Myc), only one instance of that gene was counted.

**RNA-seq**

**RNA isolation**

Cells were lysed in 1mL TRIzol reagent and incubated for 3 minutes at room temperature. 200µL Chloroform was added and samples were incubated for 10 minutes at room temperature. After 15 minutes of centrifugation, supernatants were added to 500µL isopropanol. RNA was precipitated and used for library construction.

**Library construction**

The Illumina TruSeq sample prep kit v2 was used.

2µg of RNA was poly-A selected and enzymatically fragmented. cDNA was synthesized using Super Script II master mix (Life Technologies), followed by end repair, A tailing, and adapter ligation.

Libraries were single-end sequenced on a HiSeq2000 machine, 50bp reads.
RNA-seq Data analysis
Reads were trimmed for quality purposes and mapped to the mm9 genome using Tophat. Differentially expressed genes were identified with Cuffdiff, and structural RNAs were masked. Genes with an FPKM value of greater than 5 were used for analysis. Average fold change of two independent hairpins per gene (shMed12, shMed23, or shMed8) versus the average of two independent replicates of shRen were used to calculate the fold change for these experiments. Gene set enrichment analysis was performed using the preranked GSEA option for RNA-seq. To the library of 10,348 MSigDB gene sets, additional gene sets were added, including those defined in RN2 cells based on Brd4 or Myb shRNAs, or based on JQ1 treatment.

Flow Cytometry
RN2 cells transduced with doxycycline-inducible TRMPV-Neo shRNAs were treated with 1 µg/mL doxycycline for 4 days to induce shRNA expression. Cells were incubated in APC-cKit or –Mac1 antibodies (BioLegend) at a 1:200 dilution in FACS buffer for 1 hour at 4°C. Cells were washed three times in FACS buffer and analyzed on an LSRII. Analysis was done with FlowJo.

May-Grünwald/Giemsa staining
RN2 cells transduced with doxycycline-inducible TRMPV-Neo shRNAs were treated with doxycycline for 4 days to induce shRNA expression. 500,000 cells/mL were resuspended in FACS buffer and spun onto glass slides using a Cytospin 2 Centrifuge at 500rpm for 5 min. Staining was done using May-Grünwald and Giemsa solutions (Sigma) according to manufacturer’s protocols. Images were acquired on a Zeiss Observer Microscope at 40x and Auto-Color corrected.


Supplemental References


