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Cell of Origin in AML: Susceptibility to MN1-Induced Transformation Is Regulated by the MEIS1/AbdB-like HOX Protein Complex

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SUMMARY

Pathways defining susceptibility of normal cells to oncogenic transformation may be valuable therapeutic targets. We characterized the cell of origin and its critical pathways in MN1-induced leukemias. Common myeloid (CMP) but not granulocyte-macrophage progenitors (GMP) could be transformed by MN1. Complementation studies of CMP-signature genes in GMPs demonstrated that MN1-leukemogenicity required the MEIS1/AbdB-like HOX-protein complex. ChIP-sequencing identified common target genes of MN1 and MEIS1 and demonstrated identical binding sites for a large proportion of their chromatin targets. Transcriptional repression of MEIS1 targets in established MN1 leukemias demonstrated antileukemic activity. As MN1 relies on but cannot activate expression of MEIS1/AbdB-like HOX proteins, transcriptional activity of these genes determines cellular susceptibility to MN1-induced transformation and may represent a promising therapeutic target.

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive neoplastic disease that is characterized by enhanced proliferation, blocked differentiation, and dysregulated apoptosis. AML has been recognized as heterogeneous on morphologic, cytogenetic, and molecular levels. Fusion genes, acquired mutations, and dysregulated gene expression have been identified as leukemogenic events in AML patients. Not only is heterogeneity recognized between patients, but also between AML cells from one patient. Studies showing that only a small proportion of AML cells were clonogenic in culture (Buick et al., 1977) and that only a small fraction of AML blood blasts could transfer disease to immune-deficient mice (Bonnet and Dick, 1997; Lapidot et al.,

Significance

The cancer stem cell concept postulates a hierarchical organization of tumors with cancer stem cells residing at the top of this hierarchy. Whether cancer stem cells derive from normal stem cells or whether committed cells reacquire stem cell properties during transformation remains elusive. Moreover, pathways determining cellular susceptibility to transformation have not been characterized. Employing single cell transformation of prospectively isolated hematopoietic stem and progenitor cells, we identify a narrow window of transformation susceptibility during hematopoietic differentiation. Transformation susceptibility required the activity of a well-characterized self-renewal pathway. Inhibition of this pathway demonstrated antileukemic activity, suggesting that transformation susceptibility may be a therapeutic target in a wide range of malignancies.

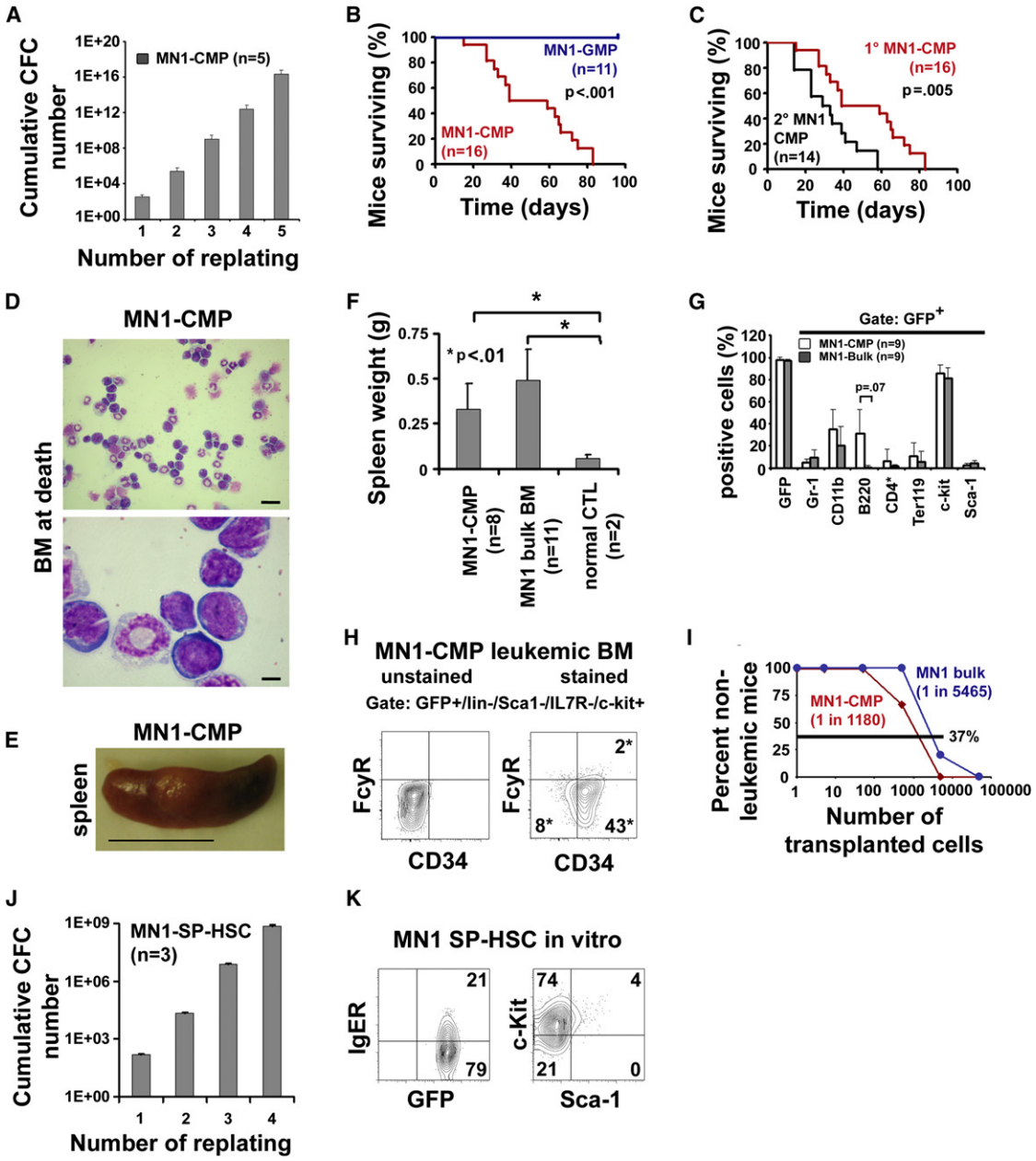

Figure 1. CMPs, but Not GMPs, Are Susceptible to MN1-Induced Transformation

(A) Cumulative CFC yield is shown for an initial plating of 1000 MN1-expressing cells derived from a single cell sorted CMP (mean ± SD of 5 independent experiments; single cell sorted CMPs were transduced with MN1 and 5 independent proliferating clones were evaluated in duplicate CFC assays).

(B) Survival analysis of mice transplanted with MN1-transduced cells derived from single cell sorted CMPs (n = 16 mice from 4 single cell-infected CMP clones) and GMPs (n = 11 mice from 3 single cell-infected GMP clones and 3 bulk infected, sorted GMPs [130, 4000, and 10,000 sorted GMPs]).

(C) Secondary transplantation analysis. Bone marrow of primary leukemic MN1-CMP mice was transplanted into secondary mice (n = 14 from 5 primary mice), and survival times were compared to primary transplants.

(D) Representative Wright-Giemsa stained cytospin preparation of bone marrow cells from leukemic mice (scale bars represent 50 μm in the upper panel and 10 μm in the lower panel).

(E) Spleen from a moribund leukemic mouse transplanted with MN1-CMP cells (scale bar, 1 cm).

(F) Average spleen weight of leukemic MN1-CMP mice and mice transplanted with bulk infected bone marrow compared to mice transplanted with normal bone marrow cells (mean ± SD).

(G) Immunophenotype of leukemic MN1-CMP compared to bulk infected bone marrow cells from moribund mice (mean ± SD). Expression of markers other than B220 were not significantly different.

*In MN1-bulk cells, percentage of cells positive for CD4 *and* CD8 is shown.

(H) Myeloid progenitor-cell immunophenotype of leukemic bone marrow cells from a MN1-CMP mouse.

*Frequencies in gates are percentages from all viable cells. Cells with CMP phenotype fall within the lower right quadrant.

1994) provided evidence for a hierarchical cellular organization of human AML.

AML cells that can initiate leukemia in xenotransplant models are operationally defined as leukemia-initiating cells (LIC). Since AML LICs and normal human hematopoietic stem cells (HSC) were characterized by a CD34+ CD38− surface phenotype (Bonnet and Dick, 1997; Miyamoto et al., 2000), it was specu lated that AML LICs originate from HSCs (Passegue et al., 2003). However, additional studies in chronic myeloid leukemia patient samples or mouse models of human leukemia identified LICs with immunophenotypic characteristics of myeloid or even lymphoid progenitor cells (Deshpande et al., 2006; Jamieson et al., 2004; Kirstetter et al., 2008; Somervaille and Cleary, 2006). These studies suggested that leukemic transformation may also occur at the level of progenitor cells by conferring self-renewal properties to committed progenitor cells. However, the phenotype of LICs in established leukemias may be deter mined by the oncogenic event rather than reflecting the pheno type of the originating cell, and thus the phenotype of LICs may not be indicative of the cell of origin. Other studies therefore prospectively isolated hematopoietic stem and progenitor cells and retrovirally introduced oncogenes in these cells to identify possible cells of origin in these leukemias. The fusion oncopro teins MLL-ENL, MLL-AF9, and MOZ-TIF2 that are found in human AML patients have the capacity to transform prospec tively isolated common myeloid progenitors (CMP) and/or granu locyte-macrophage progenitors (GMP) (Chen et al., 2008a; Coz zio et al., 2003; Huntly et al., 2004; Krivtsov et al., 2006), demon strating that CMPs, in addition to HSCs, can be cells of origin in leukemic transformation. These studies concluded that leukemia-associated oncogenes confer self-renewal properties to committed hematopoietic progenitors to allow unlimited expansion of the leukemic clone. However, the cellular and molecular characteristics of normal hematopoietic cells that confer susceptibility to transformation remain elusive.

Two types of observations suggest that intrinsic properties of normal cells may play a critical role in transformation. First, there is no evidence that terminally differentiated cells can be trans-formed by leukemia-associated oncogenes. Second, like committed progenitors, transformation-susceptible cells retain extensive, though limited, self-renewal potential. We hypothe-sized that the ability of an oncogene to transform normal cells may be largely determined by the transcriptome and epigenome of the susceptible cell. By focusing on transformation-suscep-tible cells, it may be possible to identify oncogene-independent pathways that can be targeted in a wide range of leukemias, largely independent of the transforming event.

The *MN1* gene was identified as a fusion partner of *TEL*, an *ETS* transcription factor, in patients with AML or myelodysplastic syndrome containing the translocation t(12;22)(p13;q11) (Buijs et al., 1995). Gene expression analysis of *MN1* in a large number of human AML patients with normal cytogenetics showed that high expression of *MN1* is an independent poor prognostic marker (Heuser et al., 2006; Langer et al., 2009). Functional studies have demonstrated that overexpression of MN1 alone produces an aggressive myeloid malignancy (Carella et al., 2007; Heuser et al., 2007). MN1 has also been shown to induce resistance to the differentiation-inducing agent ATRA both in vitro and in AML patients (Heuser et al., 2007). The *MN1* locus was identified as a common insertion site in insertional mutagenesis screens, and functional collaboration of MN1 and NUP98-HOXD13 (Slape et al., 2007), AML1 (Watanabe-Okochi et al., 2008), CBFB/MYH11 (Carella et al., 2007), CALM-AF10 (Caudell et al., 2010), HOXA9 (Heuser et al., 2009a), and MLL-ENL (Liu et al., 2010) has been proven or suggested. Functional studies of MN1 in human cells also point to a critical role of MN1 in leukemogenesis, as overexpression of MN1 in human CD34+ cord blood cells dramat-ically enhanced the proliferative potential of these cells (Kandilci and Grosveld, 2009), and knockdown of MN1 in human leukemia cell lines reduced their proliferation (Liu et al., 2010).

The molecular mechanisms by which MN1 exerts its effects are largely unknown. Whether MN1 primarily transforms HSCs or myeloid progenitor cells is not known. We chose the MN1 model of leukemia to characterize the variation in susceptibility to transformation within normal hematopoietic cell populations and to identify the genetic program(s) that determine suscepti-bility to MN1-induced transformation.

RESULTS

CMPs but Not GMPs Are Susceptible to MN1-Induced Transformation

To identify the cell of origin in murine MN1 leukemias, we em-ployed retroviral transduction of single cell-sorted hematopoietic progenitor cells (Figure S1A, available online). Single HSCs, CMPs, GMPs, MEPs, or mature myeloid cells were fluores-cence-activated cell sorted (FACS) according to previously established immunophenotypes into wells of a 96-well plate (Table S1), which had been coated by irradiated viral producer cells producing MN1IRESeGFP virus. Sorting of progenitor pop-ulations from 10- to 12-week old mice yielded cell purities greater than 90% and demonstrated the expected enrichment in colony-forming units and differentiation potential of CMPs, GMPs, and MEPs into myeloid and erythroid, myeloid, or prefer-entially erythroid lineages, respectively (Figures S1B–S1D).

Transplantation of 1.5–2.2 × 10^4 sorted CMPs, GMPs, or MEPs into irradiated recipient mice (n = 3) resulted, as expected, in only transient engraftment (data not shown). Of 768 single sorted CMPs and GMPs, 11% and 1% of the clones, respec-tively, proliferated over a period of 6 days to form a microscopi-cally visible cell mass (Table S2). Only CMP clones, but not GMP clones, were immortalized by MN1 in vitro and induced leuke-mias in vivo (Figure 1). MN1-transduced CMP clones could be serially replated in CFC assays up to and beyond the fifth

(I) Limiting-dilution transplantation analysis to determine the leukemia-initiating cell frequency in MN1-CMP compared to bulk infected bone marrow cells (6 different cell doses [range 1–50,000 cells], 3–4 mice per cell dose).

(J) Cumulative CFC yield is shown for an initial plating of 1000 MN1-expressing cells derived from a single cell sorted SP-HSC (mean ± SD of 3 independent experiments).

(K) Immunophenotype of MN1 SP-HSC cells cultured in vitro. The GFP gate indicates that all cells are transduced with MN1. See also Figure S1 and Tables S1 and S2.

replating and rapidly induced transplantable leukemias in mice (Figures 1A, 1B, and 1C). MN1-CMP leukemias showed a high proportion of myeloid blasts in bone marrow (Figure 1D), infiltrated spleen (Figures 1E and 1F), and liver (data not shown) and had an immature immunophenotype (high c-kit expression) with some expression of myeloid markers Gr-1 and CD11b, similar to MN1 leukemias generated by transduction of 5-FU treated bulk bone marrow cells (Figure 1G). In contrast to MN1-bulk leukemias, expression of the B cell marker B220 was increased in MN1-CMP leukemias. Strikingly, the CMP phenotype was highly preserved in MN1-CMP cells that were cultured in vitro for 49 days (Figure S1E) and in leukemic bone marrow cells from diseased mice (Figure 1H). Limiting-dilution analysis using in vitro cultured MN1-CMP and MN1-bulk cells showed that the LIC frequency was similar in both models (MN1-CMP, 1 LIC in 1180 cells; MN1-bulk, 1 LIC in 5464 cells). Thus, leukemias derived from bulk infections and from sorted CMPs share most characteristics, suggesting that CMPs constitute the main target population for MN1-induced transformation.

To further investigate whether GMPs are susceptible to MN1-induced transformation, GMPs were sorted and 150–4000 bulk cells were transduced with MN1 (12 independent experiments). Transduction efficiency ranged from 35%–48% (Figure S1F). None of these cultures yielded colonies in CFC assays or engrafted lethally irradiated mice (Table S2 and Figure 1B). Single cell sorting of MEPs and granulocytes and infection with MN1 did not induce significant proliferation of clones (Table S1). Single cell sorting of 89 HSCs using two different sorting strategies (see Table S1) yielded 32 highly proliferating clones. Sixteen clones were positive for MN1, and transduction efficiency was 68%–98% at day 10 after transduction. These cells proliferated as immortalized cell lines in vitro, as demonstrated by their capacity to serially replate in CFC assays (Figure 1J). In suspension culture, most cells expressed high levels of c-kit and were negative for the mast cell marker IgER (Figure 1K). Surprisingly, transplantation of MN1-transduced side population (SP−) HSCs from 6 clones into 14 mice resulted in only transient engraftment in peripheral blood (6% ± 2.6% at 7 weeks), and no leukemias developed up to 26 weeks after transplantation. In summary, we show at the single cell level that CMPs, but not GMPs or HSCs, are susceptible to MN1-induced transformation.

Normal CMPs and Leukemic MN1 Cells Share a Common Gene Expression Signature

To identify differences between CMPs and GMPs that may explain the different susceptibilities to transformation, we compared gene expression profiles of CMPs and GMPs to expression profiles of bone marrow cells from MN1 leukemic mice (GFP+) and mature myeloid bone marrow cells (Gr1+/CD11b+) from healthy mice. Two hundred and sixty-two probe sets corresponding to 205 genes were significantly differentially expressed both between CMPs versus GMPs and MN1 versus Gr-1+/CD11b+ cells (Table S3). Seventy-five percent of differentially regulated genes in CMPs compared to GMPs were regulated in the same direction in MN1 cells compared to mature myeloid cells (Figure 2A and Figures S2A and S2B). The 40 most highly expressed overlapping genes included *Meis1*, *Flt3*, and *Mef2c* (Figure 2B). Gene expression profiles of MN1 and mature myeloid cells also showed that two other genes that are important for hematopoietic stem cell self-renewal, *Hoxa9* and *Hoxa10*, were upregulated in MN1 cells. This was confirmed by quantitative RT-PCR comparing MN1 transformed with freshly isolated total bone marrow cells (Figure S2C). Gene expression of *Meis1*, *Flt3*, *HoxA9*, and *HoxA10* was significantly downregulated in GMPs compared to CMPs or HSCs (stem and progenitor cells defined by the LSK phenotype) as measured by Affymetrix gene expression profiles (Figure 2C).

When we evaluated endogenous *Mn1* gene expression in murine hematopoietic stem/progenitor and mature cell populations by quantitative RT-PCR, we found that *Mn1* expression was elevated in HSCs (defined by the LSKFLT3-negative phenotype), lymphoid-primed multipotent progenitors (LMPPs), CMPs, and MEPs but was dramatically downregulated in GMPs and mature cells of different hematopoietic lineages (Figure 2D). Interestingly, this expression pattern was almost identical for *Meis1* (Figure 2D). Whereas expression of *HoxA9* was similar in HSCs, CMPs, and GMPs, *HoxA10* expression was much lower in CMPs, GMPs and more mature cells than in HSCs (Figure 2D).

Overexpression of MEIS1 Renders GMPs Susceptible to MN1-Induced Transformation

Based on the above data, we hypothesized that the CMP-specific gene expression signature determined susceptibility to MN1-induced transformation and that reactivation of this signature in GMPs may render GMPs susceptible to MN1. GMPs were sorted and cotransduced with MN1 expressed from the SFFV promoter and either a control vector or selected genes of the CMP/MN1 signature (MEIS1, FLT3, HOXA9, HOXA10, RBPMS, GATA2, PLEK, FYN, or NRIP1). Cells were subsequently plated in CFC media and evaluated for serial replating capacity in vitro and, in instances where colonies were formed, for leukemia induction in vivo (Figure S3A). Transduction of GMPs with MN1 and a control vector did not induce colony growth in CFC assays (n = 12, Table S2, Figures 3A and 3B). Also, GMPs transduced with MEIS1 and a control vector did not produce colonies (n = 4, Figures 3A and 3B). Strikingly, however, cotransduction of MN1 and MEIS1 in GMPs induced colony growth in 16 of 22 experiments (Table S2, Figure 3A). Double-transduced cells could be replated up to and beyond the eighth replating (Figure 3B). This result was also obtained when MN1 was expressed from the MSCV promoter. All cells from the first CFC plating were positive for both MN1 and MEIS1, as demonstrated by FACS analysis (Figure 3C). The cells grew as packed colonies; 65% of cells had macrophage morphology, while 28% of cells had blast-like morphology (Figure 3D). The cells were negative for the mast cell marker IgER and expressed markers typical of MN1 cells (high proportion of c-kit and Flt3, low proportion of CD11b and Gr-1 expressing cells; Figure 3E).

Cotransduction of MN1 and HOXA9 also immortalized GMPs in 5 of 5 experiments (Figures 3A and 3F). However, HOXA9 with a control vector also immortalized GMPs, and thus not all cells were double transduced with MN1 (Figure 3C). MN1+HOXA9 and CTL+HOXA9 GMPs had a similar CFC output during the first two replatings (Figure 3F). MN1+HOXA9 transduced GMPs grew as packed colonies in which 50% of cells had blast-like morphology (Figure 3D), whereas CTL+HOXA9

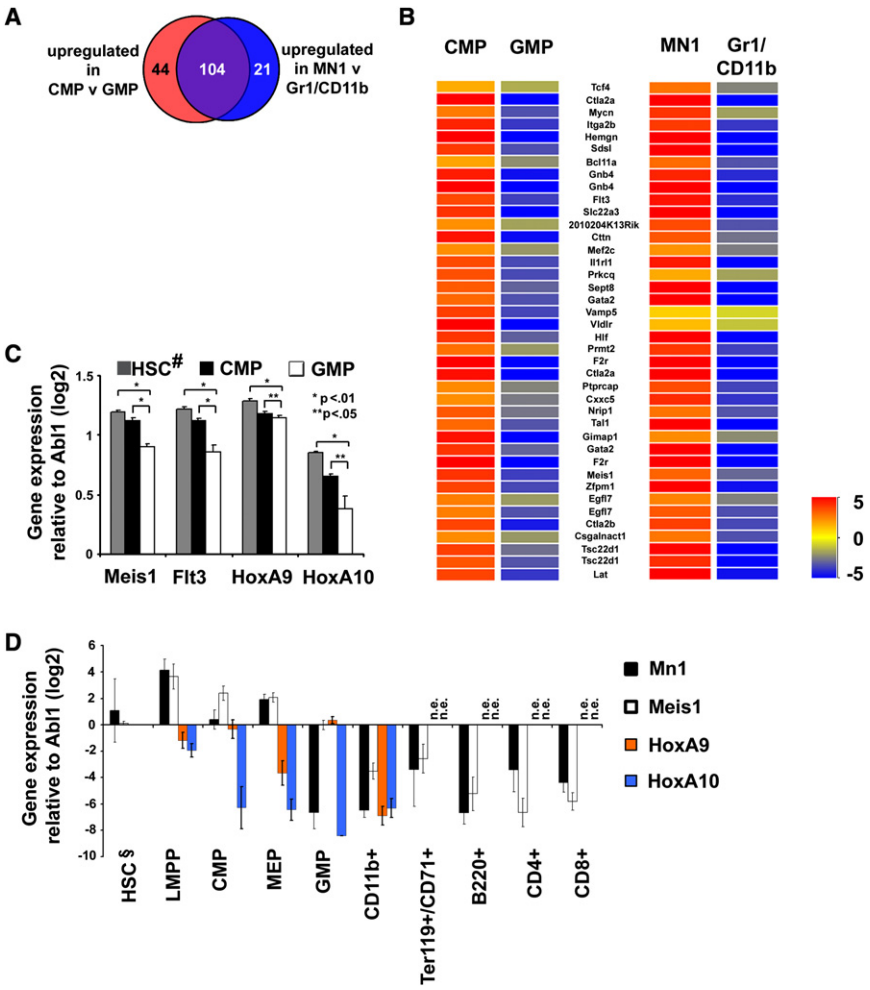

Figure 2. Normal CMPs and Leukemic MN1 Cells Share a Common Gene Expression Signature

(A) Venn diagram of genes upregulated in CMPs compared to GMPs and in leukemic MN1 bone marrow cells compared to normal Gr1+/CD11b+ bone marrow cells.

(B) Heatmap of top 40 overexpressed genes in CMPs compared to GMPs (n = 4, mean expression) that were also upregulated in MN1 leukemic bone marrow cells when compared to normal Gr1+/CD11b+ cells (n = 2, mean expression).

(C) Gene expression of *Meis1, Flt3, HoxA9*, and *HoxA10* measured on Affymetrix microarrays and normalized to expression of *Abl1* (n = 4, mean ± SD).

(D) Gene expression of endogenous *Mn1*, *Meis1*, *HoxA9*, and *HoxA10* in hematopoietic progenitor and mature cell populations relative to *Abl1* expression measured by quantitative RT-PCR. Data was normalized to the first measurement in HSCs (n = 3, mean ± SD; n.e., not expressed). See also Figure S2 and Table S3.

#Enriched for HSCs by sorting lin-/Sca-1+/c-Kit+ cells.

§Enriched for HSCs by sorting lin-/Sca-1+/c-Kit+/Flt3- cells.

GMPs grew more loosely (data not shown). Cotransduction of MN1 and HOXA10 immortalized GMPs in 5 of 5 experiments up to and beyond the fourth CFC replating, whereas HOXA10 with a control vector could not immortalize GMPs (Figures 3A and 3G). All cells from the first CFC plating were positive for HOXA10, demonstrating the requirement of HOXA10 for MN1-induced transformation of GMPs (Figure 3C). Approximately 70% of these cells had a blast-like morphology (Figure 3D). Cotransduction of GMPs with FLT3, RBPMS, GATA2, PLEK, FYN, or NRIP1 (see Figure S3B for expression levels) with a control vector or with MN1 did not induce any colony formation in CFCs (Figure 3A).

We next evaluated whether GMPs immortalized by MN1 and MEIS1 could induce leukemia in mice. MN1/MEIS1-GMPs from six independent experiments harvested from the first or up to the fifth CFC plating were intravenously injected into lethally irradiated mice at cell doses ranging from 5×10^4 to 2×10^6 cells per mouse. None of 27 mice showed long-term engraftment or developed leukemia (Figure 3H). Similarly, MN1/HOXA9-GMP cells also failed to engraft mice when 5×10^4 to 2.6×10^5 cells were transplanted (Figure 3H). MN1/HOXA10-GMP cells from three independent experiments harvested after the first or second CFC replating induced leukemia in two mice transplanted with 7.6×10^5 cells after 65 and 69 days, whereas no engraftment was found in mice transplanted with 1×10^5 cells (Figure 3H). MN1/HOXA10-GMP leukemias were characterized by large blast-like cells (Figure S3C), high c-Kit expression, and lack of expression of lineage markers (Figure S3D). The LIC frequency in these cells was calculated as 1 LIC in 6.14×10^5 cells. These results suggested that transformation of GMPs by MN1 in combination with MEIS1 or HOXA9/A10 was less efficient than transformation of CMPs by MN1 alone. We evaluated whether defects in homing of MN1/MEIS1-GMPs could explain the lack of engraftment by transplanting $0.5–1 \times 10^6$ cells intrafemorally into lethally irradiated mice. However, no long-term engraftment was noted in these mice (n = 4, data not shown).

We next evaluated whether MN1/MEIS1-GMP cells could be complemented by other genes of the CMP signature. MN1/MEIS1-GMP cells were transduced with a control vector or with HOXA9, HOXA10, GATA2, RBPMS, PLEK, FYN, NRIP1, VEGFC, or PROCR (also known as EPCR) and transplanted into lethally irradiated mice. Only mice receiving transplants with MN1/MEIS1-GMP cells transduced with HOXA9 or HOXA10 showed engraftment in peripheral blood at 4 weeks, whereas all other mice did not show engraftment after 4 and 8 weeks (Figure 4A). Mice receiving transplants of MN1/MEIS1-GMP cells transduced with HOXA9 or HOXA10 rapidly succumbed to leukemia with a similar disease latency documented for MN1-CMP leukemic mice (Figure 4B). Immunophenotyping of mice demonstrated a myeloid phenotype (CD11b+, Gr-1+) with overexpression of c-kit in these leukemias

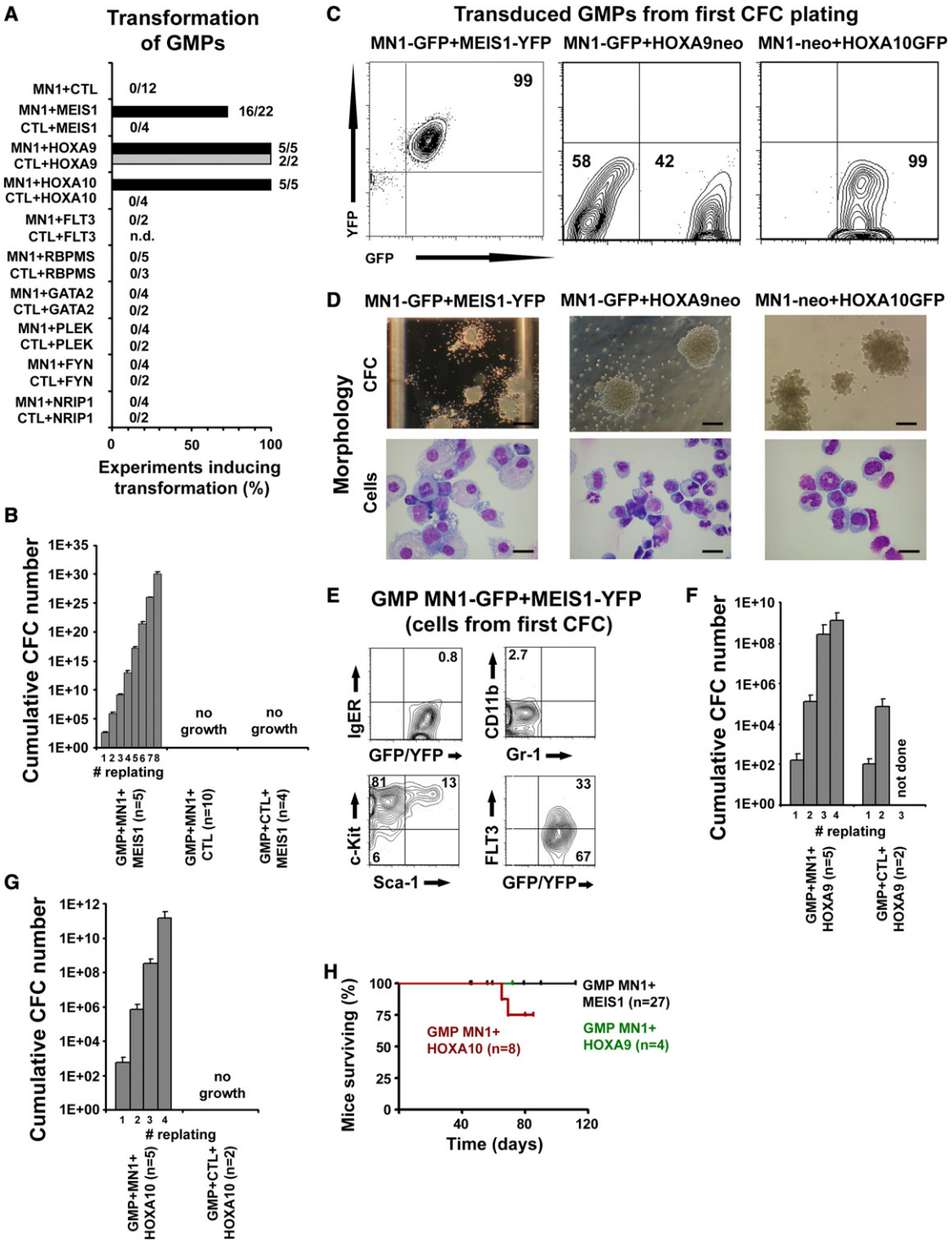

Figure 3. Overexpression of MEIS1 Renders GMPs Susceptible to MN1-Induced Transformation

(A) Effect of cotransduction of MN1 and a gene selected from the CMP signature on GMPs. The number of experiments and the frequency of transformation of GMPs, i.e., in vitro proliferation, is given.

(B) Sorted GMPs (150–4000) were retrovirally transduced for 2 days, cultured in suspension for 3 days, and plated in CFC media. Cumulative CFC yield is shown (n = 4–10 independent experiments, mean ± SD).

(C) Transduction efficiency of GMPs cotransduced with MN1 and MEIS1, MN1 and HOXA9, or MN1 and HOXA10, evaluated at the end of the first CFC plating.

(D) Morphology of CFCs and corresponding cells of GMPs cotransduced with MN1 and MEIS1, MN1 and HOXA9, or MN1 and HOXA10, evaluated at the end of the first CFC plating. Scale bars represent 100 μm (upper row) and 10 μm (lower row).

(E) Immunophenotype of leukemic GMPs immortalized by MN1 and MEIS1 cotransduction. Cells were analyzed at the end of the first CFC plating (mean ± SD).

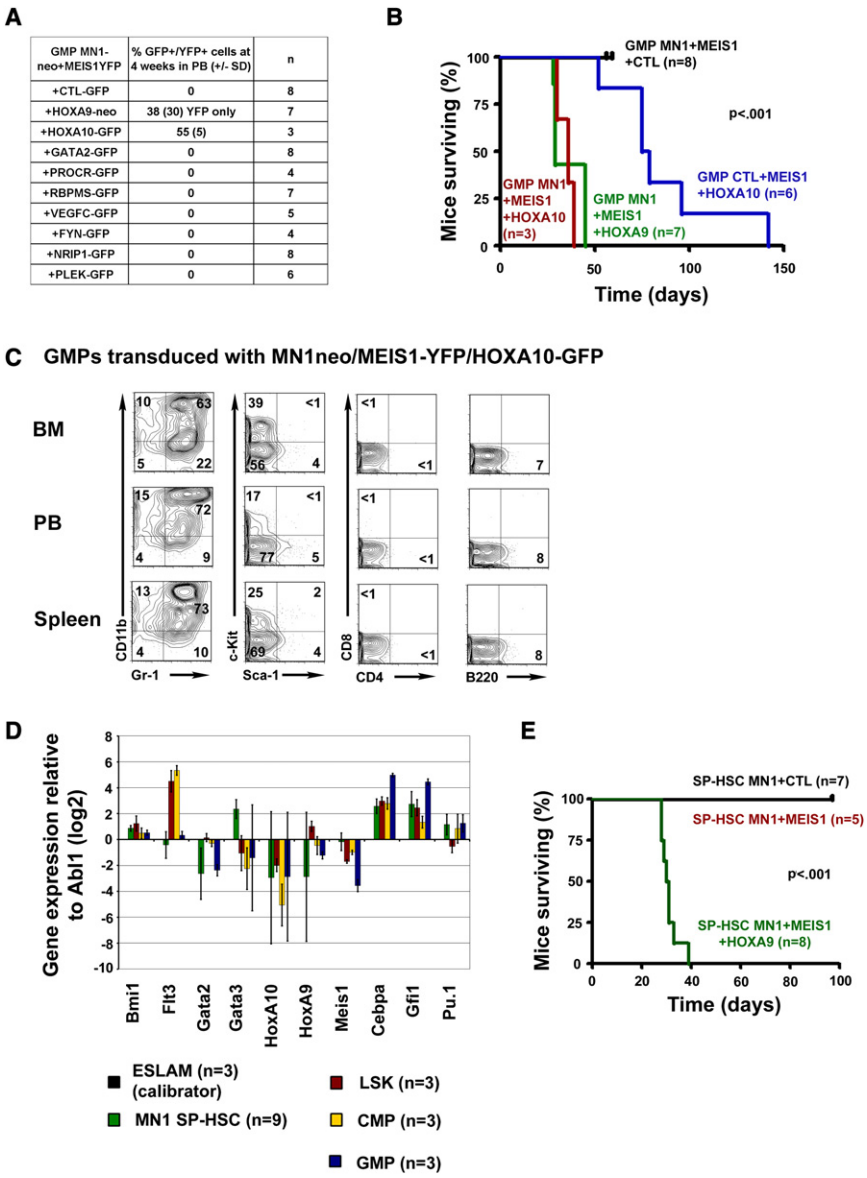

Figure 4. Complementation of MN1/MEIS1-GMPs and of MN1 SP-HSCs with an Additional Gene of the CMP Signature

(A) Effect of transduction of MN1/MEIS1-GMPs with genes of the CMP signature on engraftment levels in peripheral blood 4 weeks after transplantation (mean ± SD).

(B) Survival analysis of mice receiving transplants of MN1/MEIS1-GMPs transduced with a control vector (n = 8), HOXA9 (n = 7), or HOXA10 (n = 3).

(C) Immunophenotype of a leukemic mouse at death that received a transplant of MN1/MEIS1-GMPs transduced with HOXA10. Numbers are percent of MEIS1-YFP/HOXA10-GFP-positive cells.

(D) Gene expression of hematopoietic transcription factors in sorted ESLAM cells, MN1 SP-HSCs, CMPs, and GMPs relative to *Abl1*. Expression in ESLAM cells was used to calibrate the data in the other cell types (mean ± SD).

(E) Survival analysis of mice receiving transplants of MN1 SP-HSCs transduced with a control vector (n = 7), MEIS1 (n = 7), or MEIS1+HOXA9 (n = 8).

(Figure 4C). As a control, we transduced GMP cells with a control vector, MEIS1, and HOXA10. Whereas one mouse died from leukemia after 52 days, the other mice remained healthy for at least 70 days (Figure 4B).

To investigate whether MN1 SP-HSC cells retained a similar gene expression profile compared to HSCs, we measured expression levels of genes previously associated with self-renewal and myeloid differentiation in HSCs, MN1 SP-HSCs, LSK cells, CMPs, and GMPs. *Meis1, Flt3*, and *Bmi1* were expressed at similar levels in MN1 SP-HSCs as in HSCs, whereas *HoxA9, HoxA10*, and *Gata2* were expressed at lower levels, possibly explaining the lack of engraftment potential of MN1 SP-HSCs (Figure 4D). Therefore, we also performed complementation experiments with MEIS1 and HOXA9 in MN1 SP-HSCs. Cotransduction of MEIS1 into these cells did not enhance engraftment or development of leukemia. However, cotransduction of MEIS1 and HOXA9 induced leukemia in mice with a very short latency ($p < 0.001$, Figure 4E). In summary, overexpression of MEIS1 or HOXA10 in GMPs can render GMPs susceptible to MN1-induced in vitro immortalization, but not in vivo transformation. HOXA9 overexpression in GMPs is sufficient for in vitro immortalization, but even the combined expression of HOXA9 and MN1 in GMPs does not induce leukemias in vivo. Coexpression of MN1, MEIS1, and one of the AbdB-like HOX proteins HOXA9 or HOXA10 in GMPs readily induces leukemia in mice. Finally, MN1 SP-HSCs have low expression of HOXA9 and HOXA10 compared to HSCs, and upregulation of MEIS1 and HOXA9 restores leukemic activity of these cells in vivo.

MN1 and MEIS1 Co-Occupy Select Promoter Regions of Putative Direct Target Genes

It is well known that AbdB-like HOX proteins (HOXA9 to HOXA13) directly interact with MEIS1 to enhance their DNA binding specificity (Shen et al., 1997). Based on our findings that MN1 requires

(F) Cumulative CFC yield is shown for GMPs transduced with MN1 and HOXA9 or HOXA9 and a control vector (n = 2–5 independent experiments, mean ± SD).

(G) Cumulative CFC yield is shown for GMPs transduced with MN1 and HOXA10 or HOXA10 and a control vector (n = 2–5 independent experiments, mean ± SD).

(H) Survival analysis of mice receiving transplants of cells derived from GMPs that were transduced with MN1 and MEIS1 (n = 27), MN1 and HOXA9 (n = 4), or MN1 and HOXA10 (n = 8) and plated in CFCs. See also Figure S3.

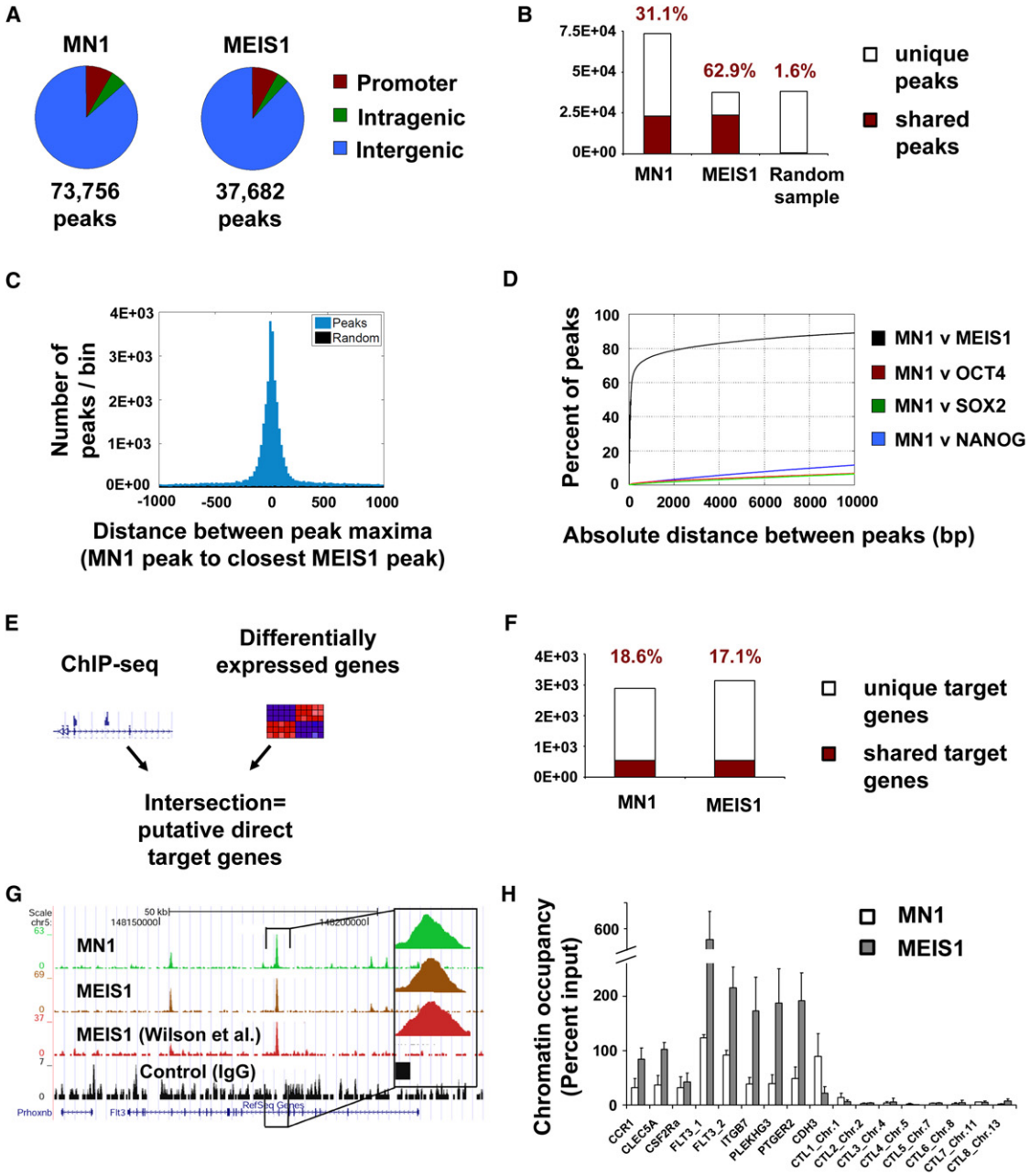

Figure 5. MN1 and MEIS1 Co-Occupy Promoter Regions of Putative Direct Target Genes

Results of ChIP-Sequencing using an anti-HA antibody in MN1-HA leukemic cells or MEIS1-HA+ND13 leukemic cells and comparison with published data using anti-MEIS1 and IgG control antibodies in HPC-7 cells.

(A) Total number of peaks for MN1 and MEIS1 libraries and distribution of peaks in promoter, intragenic, and intergenic regions.

(B) Proportion of chromatin peaks that are shared by MN1 and MEIS1 (at least 50% overlapping sequence). The expected overlap of chromatin peaks of a random sample containing 37,682 peaks with MN1 peaks is shown for comparison.

(C) Histogram showing the distance between peak maxima of MN1 peaks to their closest MEIS1 peak, indicating that MN1 binding sites are highly enriched at MEIS1 binding sites. The distance of random peaks to their closest MEIS1 peak shows no enrichment.

(D) The distance between peak maxima of MN1 peaks to their closest MEIS1, OCT4, SOX2, or NANOG peak is plotted against the percentage of peaks having that distance to their neighboring peaks.

(E) Algorithm to identify putatively direct target genes of MN1 and MEIS1. Differentially expressed genes in MN1 leukemia bone marrow compared to normal Gr-1+/CD11b+ bone marrow cells or in MEIS1-HA+ND13 leukemic compared to CTL+ND13 nonleukemic cells were identified (≥2-fold). Chromatin peaks of MN1 or MEIS1 that fell within ± 5 kb of the genomic extents of differentially expressed genes were selected, and identical peaks and target genes of MN1 and MEIS1 datasets were identified.

(F) Number and proportion of putatively direct target genes that are unique or shared between MN1 and MEIS1.

MEIS1 and an AbdB-like HOX protein for full transformation of GMPs, we hypothesized that MN1 may rely on interaction with the MEIS1/HOX protein complex. To investigate whether MN1 and MEIS1 co-occupy similar target genes, we performed chromatin immunoprepitation (ChIP)-sequencing on MEIS1-HA+ND13 and MN1-HA leukemic cells using an anti-HA antibody. These data were complemented by ChIP-sequencing data using an anti-MEIS1 antibody and a control IgG antibody in HPC-7 cells, published previously (Wilson et al., 2010).

Sequencing yielded more than 15 million mappable reads per sample. Using a threshold of height 5 (MN1) or 6 (MEIS1), 73,756 and 37,682 enriched ChIP-seq regions (referred to as ''peaks,'' from genome browser visualizations) were identified for MN1 and MEIS1, respectively, of which most were located in intergenic regions (Figure 5A). Of all MN1 peaks, 31.1% had at least 50% sequence overlap with MEIS1 peaks, and 62.9% of MEIS1 peaks overlapped with MN1 peaks, whereas the expected overlap of a random sample is 1.6% (Figure 5B). Peaks shared by MN1 and MEIS1 that were within regulatory regions of genes, defined as 5 kb proximally or distally of the genomic location of Refseq genes, were associated with 1975 *targeted* genes (whereas ''targeted gene'' refers to a gene having an MN1 or MEIS1 peak within its regulatory region). Plotting the distance of the peak maxima of MN1 peaks to their closest MEIS1 peak showed that a large number of MN1 peaks directly overlapped MEIS1 peaks (Figure 5C). Seventy-five percent of MN1 peaks were located within 1000 bp of the next MEIS1 peak, whereas only 1%–2% of MN1 peaks were located within 1000 bp of the next OCT4, SOX2, or NANOG peaks determined in a previous study in mouse embryonic stem cells (Chen et al., 2008b) (Figure 5D).

We next identified MN1-*regulated* genes (n = 3480) by comparing gene expression profiles of bone marrow cells from MN1 leukemic mice and mature myeloid bone marrow cells (Gr1+/CD11b+) from healthy mice. Similarly, MEIS1-*regulated* genes (n = 7252) were identified by comparing gene expression profiles of MEIS1-HA+ND13 transduced leukemic cells with CTL+ND13 transduced nonleukemic bone marrow cells (Argiropoulos et al., 2008). Eight hundred and fifty-eight genes were regulated by MN1 and MEIS1. To identify directly targeted and regulated genes of MN1 and MEIS1, peaks from ChIP-Seq were intersected with regulatory regions of differentially expressed genes (Figure 5E, Tables S4–S6). Compared to Gr1+CD11b+ cells, approximately half of the MN1 targeted and regulated genes were upregulated, while the other half was downregulated. Approximately one-quarter of MEIS1 targeted and regulated genes were upregulated, whereas the other genes were downregulated. The majority of common MN1 and MEIS1 target genes were regulated in the same direction (82.5%, 72% down, 10.5% up); 18.6% and 17.1% of genes targeted and regulated by MN1 or MEIS1, respectively, were regulated and targeted by both MN1 and MEIS1 (n = 537 genes, Figure 5F). The location of chromatin peaks of MN1, MEIS1, or the peaks shared by MN1 and MEIS1 within regulated genes are provided in Tables S7–S9.

Gene set enrichment analysis was performed with MN1 targeted and regulated genes or their overlap with MEIS1 targeted and regulated genes using the Gene Ontology (collection C5) and curated gene sets (collection C2, http://www.broadinstitute.org/gsea/msigdb/index.jsp). The most highly enriched gene sets were ''hematopoiesis related transcription factors'' and several AML and HOXA-related gene sets. We found fewer significantly depleted gene sets, and they had lower normalized enrichment scores than enriched gene sets. Several depleted gene sets were related to apoptosis.

To validate the ChIP-Seq results, we compared our MEIS1 library with published ChIP-Seq data in which an anti-MEIS1 antibody was used (Wilson et al., 2010). Comparison of the two datasets showed a very high degree of overlap (56.4% of the peaks identified by Wilson et al. were also found in our dataset). As shown for the FLT3 gene, the peaks directly matched each other (Figure 5G). An IgG control ChIP-Seq data set published by Wilson et al. (2010) confirmed the specificity of MN1 and MEIS1 peaks (Figure 5G). Representative chromatin peaks were also validated by qPCR looking at target regions of both MN1 and MEIS1 or MN1 alone (Cdh3 is only targeted by MN1) and control regions not targeted by the two proteins. Whereas all randomly selected chromatin regions showed significant enrichment for both MN1 and MEIS1 (except the Cdh3 gene for MEIS1), no enrichment was found in the control regions (Figure 5H). In summary, these data identify putative direct target genes of MN1 and MEIS1, and show co-occupancy of MN1 and MEIS1 at 18.6% and 17.1% of their respective target genes.

A Dominant-Negative Form of MEIS1 Demonstrates Antileukemic Activity in Established MN1 Leukemias

Next, we evaluated the role of Meis1 for maintenance of established MN1 leukemias. Recently, we demonstrated that an engineered fusion protein of the repressive domain of M33 to MEIS1 (M33MEIS1) confers transcriptional repression to MEIS1 target genes that are otherwise upregulated in normal and malignant hematopoiesis (Argiropoulos et al., 2010). Transduction of M33MEIS1 or a control vector in MN1 leukemic cells resulted in a reduction of CFCs at the end of the first plating (Figure 6A). Whereas CTL-transduced MN1 cells retained the CTL vector during CFC culture, the majority of M33MEIS1 transduced cells were lost during CFC culture (Figure 6B). Double-transduced MN1+CTL or MN1+M33MEIS1 expressing cells, which had a double transduction rate of 55%–95%, with or without sorting, were injected into lethally irradiated recipient mice (5×10^4 to 7.5×10^5 cells per mouse). Six weeks after transplantation, approximately 70% of peripheral blood cells from mice receiving MN1+CTL transduced cells were positive for MN1 and the CTL vector, whereas 18% were positive for MN1 only. Strikingly,

(G) UCSC genome browser visualization of MN1, MEIS1, and control sequencing tracks at the FLT3 locus showing exactly matching peaks for MN1 and MEIS1 from anti-HA antibody and from anti-MEIS1 antibody immunoprecipitations (Wilson et al., 2010). The insert shows an enlarged view of the main peak in the FLT3 region (corresponding to FLT3-1 in Figure 5G).

(H) Validation of selected ChIP-Seq peaks shared by MN1 and MEIS1, targeted only by MN1 (CDH3), and not targeted by either MN1 or MEIS1 (CTL). Chromatin-immunoprecipitates of MN1 or MEIS1 from MN1-HA or MEIS1-HA+ND13 leukemic cells, respectively, were quantified by qPCR and compared to input DNA (n = 3, mean ± SD). See also Tables S4, S5, S6, S7, S8 and S9.

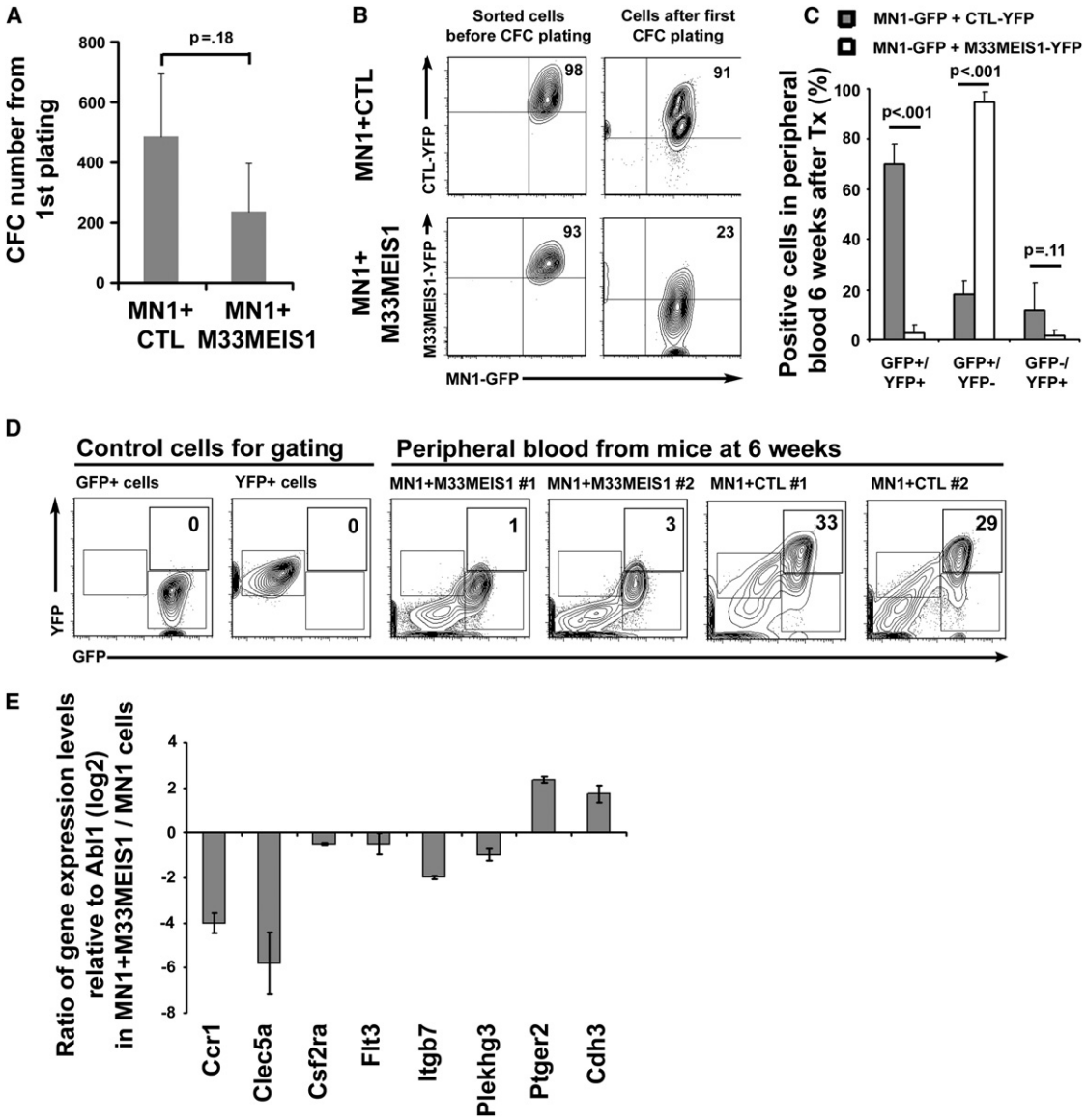

Figure 6. A Dominant-Negative Form of MEIS1 Demonstrates Antileukemic Activity in Established MN1 Leukemias

(A) Number of colonies from first plating of MN1 cells transduced with a control vector or M33MEIS1 (n = 3, mean ± SD).

(B) Proportion of double-transduced cells before and after the first CFC plating. MN1+CTL transduced cells retained expression of the control vector, whereas the proportion of cells expressing M33MEIS1 greatly decreased during CFC culture.

(C) Engraftment of MN1 cells transduced with CTL (n = 5) or M33MEIS1 (n = 4) in recipient mice 6 weeks after transplantation. The proportion of double- or single-transduced donor-derived cells in peripheral blood is shown (mean ± SD).

(D) Representative FACS blots from peripheral blood of mice receiving transplants of MN1 cells transduced with CTL or M33MEIS1 are shown.

(E) Gene expression of target genes of MN1 and MEIS1 (Cdh3 is only targeted by MN1) in MN1 and MN1+M33MEIS1 cells relative to Abl1 (log2). MN1 cells were used to calibrate the data in the other cell type (mean ± SD, n = 3).

only 2.7% of peripheral blood cells from mice receiving MN1+M33MEIS1-transduced cells expressed MN1 and M33MEIS1, whereas 95% of cells only expressed MN1 ($p < 0.001$, Figure 6C). Representative FACS blots from two mice per group are shown in Figure 6D. Gene expression analysis of validated targets of MN1 and/or MEIS1 in MN1 or MN1+M33MEIS1 cells confirmed the repressive effect of M33MEIS1 on several of its target genes. Of note, Cdh3, which is not targeted by MEIS1, was not repressed by M33MEIS1. These data suggest that M33-MEIS1 inhibits proliferation by interfering with MEIS1 transcriptional activity and, so, prevents engraftment of MN1 leukemias in mice.

DISCUSSION

Oncogenic transformation is generally regarded as a multistep process and it has been difficult to recapitulate this process in a step-wise order. A critical question is whether leukemias arise

from hematopoietic stem cells that have partially differentiated but maintain their self-renewal potential or whether self-renewal is reactivated in more differentiated cells, thus giving rise to leukemia stem cells. We used the MN1 model of leukemia to address this question, as constitutive activation of a single gene, MN1, induces AML by enhancing self-renewal and by blocking differentiation through distinct functions of the gene (Heuser et al., 2007). We identified CMPs, but not GMPs, as the cells of origin in MN1 leukemias. Susceptibility to MN1 was determined by the activity of MEIS1 and AbdB-like HOX protein complexes. We show that MN1 and MEIS1 colocalize at the chromatin level and that expression of a dominant-negative form of MEIS1 abolishes leukemogenicity of established MN1 leukemias.

Prospective isolation and transduction of single cells demonstrated susceptibility of CMPs (but not GMPs, MEPs, or more differentiated myeloid cells) to MN1-induced transformation. Our approach of directly infecting single cell-sorted hematopoietic progenitor cells, thus preventing interaction of hematopoietic cells by cell-cell contact, has not been reported previously. Wang et al. used a similar approach, where a bulk of sorted progenitor cells was infected and transduced cells were subsequently single cell sorted into wells of a 96-well plate (Wang et al., 2010). We investigated whether earlier stages of the hematopoietic hierarchy could also be transformed by MN1. Although HSCs were sorted to a high purity and showed a striking proliferative potential in suspension culture, MN1-transduced HSCs did not induce leukemia in mice. Only contransduction of MEIS1 and HOXA9 restored the leukemic potential in vivo. Expression analysis suggested that *Meis1* expression levels were similar in MN1 SP-HSCs and sorted HSCs, but *HoxA9* and *HoxA10* levels were reduced. It remains unclear why expression of MN in SP-HSCs cannot sustain expression of HOXA genes, but additional factors that are differentially expressed between HSCs and CMPs likely play a role. Our retroviral approach may have been insufficient to target the HSC at its earliest stage. Interestingly, transduction of sorted LMPPs with MN1 rapidly induced leukemias in mice (data not shown). Thus, the spectrum of susceptible cells to MN1-induced transformation is the CMP and multipotent progenitor cell stage, but it may not extend to the most primitive HSC stage.

We determined the LIC frequency of MN1 leukemias derived from bulk infected bone marrow cells and from single cell sorted CMPs transduced with MN1. In both cases, transduced cells were cultured for 3 weeks and the LIC frequency was determined from these cultured cells. The LIC frequency in MN1-bulk cells was very similar to that in MN1-CMPs. It is thus interesting that MN1 leukemias have a fairly constant LIC frequency under in vitro culture conditions, apparently independent of the cell of origin. A recent study characterized the phenotype of LICs in human AML and found LICs in both LMPP-like and GMP-like populations. However, whether these populations were also cells of origin in these patients can not be addressed in primary disease (Goardon et al., 2011).

Prospective isolation and transduction of single sorted GMPs with MN1 did not immortalize cells in vitro. Because technical issues cannot be excluded in single cell sorting and infection, we sorted GMPs and infected up to 4000 GMPs in bulk cell culture. Again, no immortalized cell line could be derived. Cotransduction of MN1 with MEIS1, HOXA9, or HOXA10, but with none of the other genes tested, resulted in immortalized cell lines in vitro. Interestingly, MN1/MEIS1 cotransduced GMPs did not engraft mice. Similar findings were recently reported for HOXA9/MEIS1 cotransduced GMPs that were immortalized in vitro but could not engraft mice (Wang et al., 2010). In contrast, the fusion oncogenes MLL-ENL (Cozzio et al., 2003), MLL-AF9 (Krivtsov et al., 2006), and MOZ-TIF2 (Huntly et al., 2004) have been shown to fully transform GMPs, giving rise to leukemias in vivo. Our results indicate that HOXA9 is the only natural gene reported thus far that can immortalize GMPs in vitro. Full transformation of GMPs to leukemia by MN1 required an AbdB-like HOX gene in addition to MEIS1. In summary, we generated a model in which we could track the requirement of several genes and pathways that determine transformation susceptibility in hematopoietic progenitor cells.

MEIS1 is a cofactor of AbdB-like HOX proteins and directly interacts with HOXA9, HOXA10, and other AbdB-like HOX proteins (Shen et al., 1999). It is well established that AbdB-like HOX proteins stabilize DNA binding of MEIS1 and enhance transcriptional activation of its target genes (Shen et al., 1997). Our results demonstrate that MN1 requires active MEIS1 and AbdB-like HOX gene expression for transformation. Activation of only one factor of this complex in GMPs is sufficient for in vitro immortalization, but not for in vivo transformation. Importantly, our data suggest that MN1 can only maintain MEIS1 or an AbdB-like HOX gene signature in CMPs, not induce expression of MEIS1 or AbdB-like HOX genes in GMPs. We have previously shown that constitutive activation of HOXA9 to MN1-transformed cells dramatically enhanced the frequency and self-renewal of leukemia stem cells (Heuser et al., 2009a), supporting the role of AbdB-like HOX proteins as cooperating transcription factors for MN1. The MLL fusion proteins MLL-ENL and MLL-AF9 can fully transform GMPs when retrovirally expressed at high levels (Chen et al., 2008a; Cozzio et al., 2003; Krivtsov et al., 2006), in part by activating MEIS1 and HOXA9 expression (Zeisig et al., 2004). However, Wang et al. (2010) recently described that constitutive expression of MEIS1 and HOXA9 in GMPs cannot fully transform GMPs. They found that β-catenin expression, an activator of the WNT pathway and a target gene of MLL-AF9, in HOXA9/MEIS1-transduced GMPs readily transforms these cells in vivo (Wang et al., 2010). It can be speculated that MN1 might be an activator of the WNT pathway collaborating with HOXA9/MEIS1 in GMP transformation. However, previous data on the downstream targets of MN1 did not establish a link between MN1 and the WNT pathway. Besides, gene expression profiling studies of murine leukemias have not revealed MN1 as a target gene of MLL fusions (Chen et al., 2008a; Somervaille et al., 2009). Thus, it is possible that MN1 complements HOXA9/MEIS1 transformation of GMPs independently of the WNT pathway, representing an additional pathway collaborating with HOXA9/MEIS1 in GMPs.

In our study, MN1 was expressed at high levels in CMPs but downregulated in GMPs, and single cell infection of CMPs by MN1 arrested the cells phenotypically at the CMP stage. In contrast to a previous report that found the highest MN1 expression levels in GMPs (Carella et al., 2007), these data suggest that MN1 negatively regulates the transition from CMPs to GMPs in normal hematopoiesis. ChIP-sequencing analysis showed that MN1 and MEIS1 strikingly co-occupy regulatory regions of their

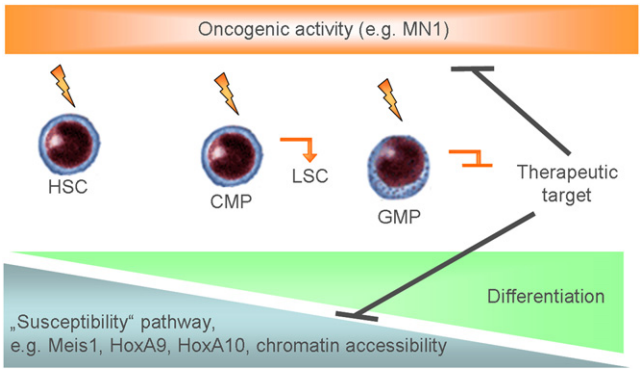

Figure 7. Schematic Highlighting the Interplay of a Constitutively Active Oncogenic Pathway and Variable Stages of Cellular Susceptibility to Transformation

The "susceptibility pathway" may represent a therapeutic target in multiple leukemic entities, whereas the oncogenic pathway represents a therapeutic target specific for the dysregulated pathway.

target genes. Previous studies have shown that maintenance of an active chromatin state of MEIS1 loci was critical for self-renewal of normal and leukemia stem cells (Wong et al., 2007); thus, we tested whether transcriptional repression of MEIS1 loci may reduce leukemogenicity of established MN1 leukemias. We fused the repressive domain of M33, a member of the Polycomb group, to MEIS1. This fusion has been shown to facilitate Polycomb group complex formation at direct MEIS1 target genes and to result in transcriptional repression of these genes (Argiropoulos et al., 2010). Interestingly, leukemogenic activity of MN1 was abolished by the M33MEIS1-fusion protein, confirming that a functional MEIS1 is critical for MN1 function.

MEIS1, HOXA9, and HOXA10 have been extensively characterized as genes of the self-renewal signature in normal and malignant hematopoiesis (Krivtsov and Armstrong, 2007). The aim of the present study was to identify pathways that determine transformation susceptibility to MN1. Our results highlight the critical role of the normal cell in oncogenic transformation. In our model, the oncogene has a constant level of activity, while the activity of the susceptibility-regulating pathway declines with differentiation of the target cell (Figure 7). Susceptibility to the oncogene in our study was determined by the well-characterized self-renewal genes MEIS1, HOXA9, and HOXA10. We suggest that the susceptibility pathway represents an independent therapeutic target besides the transforming event that induces the malignancy. Inhibition of this pathway, e.g., by transcriptional repression of target loci, as exemplified by M33-MEIS1, appears to be a promising strategy for the treatment of AML, and targeting this self-renewal pathway may be effective in a wide range of leukemias.

EXPERIMENTAL PROCEDURES

A detailed description of experimental procedures can be found in the Supplemental Information.

Retroviral Vectors and Vector Production

Retroviral vectors for expression of MN1 (Heuser et al., 2007), MEIS1 (Pineault et al., 2003), M33MEIS1 (Argiropoulos et al., 2010), FLT3(Palmqvist et al., 2006), HOXA9 (Kroon et al., 1998), HOXA10 (Buske et al., 2001), and ND13 (Pineault et al., 2003) have been described previously. MN1 was subcloned in the MSCVPGKneo vector, and an HA-tag was cloned to the C terminus of MN1 in the pSF91-MN1-IRESeGFP vector. Gateway cloning was used to clone human cDNAs of RBPMS, GATA2, PLEK, FYN, NRIP1, VEGFC, and PROCR upstream of IRESeGFP in expression vector pSF91IRESeGFP-RfA-EXPR.

FACS Sorting of Hematopoietic Progenitor Cells

Freshly isolated cells from murine bone marrow were used for fluorescence activated cell sorting (FACS) of hematopoietic progenitor or differentiated cell populations. For sorting of single progenitor cells, we first enriched the cells by sorting c-kit expressing cells. Cells were then stained as detailed in Table S1 and as described before (Akashi et al., 2000; Heuser et al., 2007), and single cells were deposited into wells of a round-bottom 96-well plate. Sorting of other populations was performed as detailed in the Supplemental Information.

Transduction and Culture Conditions of Single Cell Sorted Hematopoietic Progenitor Cells

One day before transduction was initiated, GP+E86 viral producer cells were irradiated with 4000 cGy, and plated in wells of a round-bottom 96-well plate containing 100 μl medium at a density of 3.3×10^4 cells per well. Single cell deposition into wells of a test plate was visually confirmed before single progenitor cells were directly sorted into wells of a 96-well plate. Cells were cocultured for 48 hr in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 10 ng/mL human interleukin-6 (hIL-6), 6 ng/mL murine interleukin-3 (mIL-3), and 20 ng/mL murine stem cell factor (mSCF; all from StemCell Technologies, Inc., Vancouver, Canada). After 48 hr, cells were replated in an empty 96-well plate and 100 μl fresh medium were added. When cells proliferated to a microscopically visible cell mass, they were replated and used in CFC assays or mouse transplantation.

Mice and Retroviral Infection of Primary Bone Marrow Cells and Bone Marrow Transplantation

C57BL/6J mice were bred and maintained in the animal research center of the British Columbia Cancer Agency as approved by the University of British Columbia Animal Care Committee. Primary mouse bone marrow cells were transduced after prestimulation for 48 hr, as previously described (Heuser et al., 2009b). Briefly, cells were cocultured with irradiated viral producer cells for 48 hr in the presence of 5 μg/mL protamine sulfate (Sigma-Aldrich) and then plated in CFC media or directly transplanted into lethally irradiated syngeneic recipient mice that were exposed to a single dose of 750 cGy total-body irradiation accompanied by a life-sparing dose of 1×10^5 freshly isolated bone marrow cells from syngeneic mice.

Gene Expression Profiling and Gene Set Enrichment Analysis

MN1 leukemia cells and Gr-1+/CD11b+ bone marrow cells were hybridized to the Affymetrix GeneChip Mouse430 2.0 (43.000 probes) microarray (n = 2) according to the manufacturer's instructions. Experiments were performed at British Columbia Genome Sciences Centre, Vancouver, Canada. Gene expression data can be found at the Gene Expression Omnibus database (GEO accession number GSE22923). Gene expression profiling of CMPs and GMPs using Affymetrix GeneChip Mouse430A array (22,690 probes) has been described elsewhere (Krivtsov et al., 2006). Gene expression profiling of MEIS1+ND13 and CTL+ND13 cells using Affymetrix GeneChip Mouse430 array (43.000 probes) has been described previously (Argiropoulos et al., 2008).

The gene set enrichment analysis software (Subramanian et al., 2005) (http://www.broad.mit.edu/gsea/index.jsp) was used to compare enrichment of curated gene sets (dataset C2) and Gene Ontology gene sets (dataset C5, available from the Molecular Signature database v2.5 [Subramanian et al., 2005]).

ChIP Assay and ChIP-Sequencing

Chromatin immunoprecipitation (ChIP) assays were performed using sequential cross-linking with disuccinimidyl glutarate (Sigma) and formaldehyde followed by immunoprecipitation (see Supplemental Experimental Procedures) using an anti-HA antibody (Abcam ab9110). qPCR was performed to verify

enrichment, and Illumina DNA library construction for next generation sequencing was performed as described previously (Robertson et al., 2007). Cluster generation and 50 bp sequencing on the Illumina Genome Analyzer IIx (Illumina, San Diego, CA) was performed following the manufacturer's instructions. Details about library construction, processing of ChIP-Seq data, and validation by ChIP-qPCR can be found in the Supplemental Information.

Statistical Analysis

Pairwise comparisons were performed by Student's t test for continuous variables and by chi-square test for categorical variables. The two-sided level of significance was set at p values less than 0.05. Comparison of survival curves were performed using the log-rank test.

ACCESSION NUMBER

MN1-gene expression data can be found at the Gene Expression Omnibus database (GEO accession number GSE22923).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, three figures, and nine tables and can be found with this article online at doi:10.1016/j.ccr.2011.06.020.

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