Hsa-mir183/EGR1-mediated regulation of E2F1 is required for CML stem/progenitor cell survival

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Running title: ***Hsa-mir183/EGR1/E2F1 in CML stem cells***

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Key points

1. hsa-mir183/EGR1/E2F1 is a novel and critical factor for CML SPC survival
2. E2F1 plays a pivotal role in regulating CML SPC proliferation status

Abstract

Chronic myeloid leukemia (CML) stem/progenitor cells (SPC) express a transcriptional program characteristic of proliferation, yet can achieve and maintain quiescence. Understanding the mechanisms by which leukemic SPC maintain quiescence will help to clarify how they persist during long-term targeted treatment. We have identified a novel BCR-ABL1 protein kinase dependent pathway mediated by the up-regulation of *hsa-mir183*, the down-regulation of its direct target EGR1 and, as a consequence, up-regulation of E2F1. We show here that inhibition of *hsa-mir183* reduced proliferation and impaired colony formation of CML SPC. Downstream of this, inhibition of *E2F1* also reduced proliferation of CML SPC, leading to p53-mediated apoptosis. In addition, we demonstrate that E2F1 plays a pivotal role in regulating CML SPC proliferation status. Thus, for the first time, we highlight the mechanism of *hsa-mir183*/EGR1-mediated E2F1 regulation and demonstrate this axis as a novel, critical factor for CML SPC survival, offering new insights into leukemic stem cell eradication.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease of hemopoietic stem cell (HSC) origin resulting from the chromosomal translocation t(9;22)(q34;q11) that gives rise to the fusion gene, *BCR-ABL1*. ABL tyrosine kinase inhibitors (TKI) lead to long-term remission in the majority of CML patients, but approximately 50% of cases relapse when treatment is discontinued, as TKI are unable to fully eradicate quiescent stem/progenitor cells (SPC),[1-9] despite being able to inhibit BCR-ABL signaling in these cells.[6,9,10] Investigations have demonstrated that TKI-resistant SPC in patients show reduced levels of *BCR-ABL1* expression as compared to baseline, and exhibit a more primitive, quiescent transcriptional signature that becomes predominant over time in response to TKI therapy.[11-14] It is not currently known whether this quiescent signature is driven by CML SPC intrinsic signaling by the microenvironment, or by a combination of both.

One possible candidate for cell intrinsic regulation of the quiescent CML phenotype is the transcription factor E2F1, which regulates cell proliferation by activating genes important for G1-S-phase progression.[15] In mice, deletion of *E2f1* resulted in increased T-cell numbers,[16,17] whilst combined loss of *E2f1/2/3* affected mature hemopoietic cell proliferation[18,19] and survival of the myeloid lineage,[16,20] yet no effect was demonstrated on HSC function.[21,22] In CML, E2F3 was shown to be important for disease initiation[23] and silencing of E2F1 in K562 cells or $CD34^+$ cells led to activation of PP2A and BCR-ABL1 suppression.[24] In mouse fibroblasts, triple inactivation of *E2f1/2/3* led to p53 activation and cell cycle arrest,[25] while deletion of *p53* restored E2F1 transcriptional activity.[26] In recent work we have demonstrated that p53 acts as a key signaling hub to maintain survival of CML SPC.[27]

As BCR-ABL had previously been shown to modulate miRNA levels,[28] to gain insight into how CML SPCs maintain quiescence, we investigated novel and publicly deposited mRNA/microRNA (miRNA) transcriptomic datasets derived from primitive human CML SPC.[27,29-34] Here we show that in CML SPC, the cancer-related miRNA *hsa-mir183* is highly expressed in a BCR-ABL1-dependent manner and hypothesize that this miRNA deregulates specific SPC intrinsic mechanisms. Interestingly, it has been reported that *hsa-mir183* targets *EGR1,* a member of the immediate early response transcription factor family that regulates proliferation and mobilization of normal SPC. The genetic network regulated by EGR1 responsible for stem cell division and migration has not been entirely elucidated. Interestingly, EGR1 loss has been shown to promote development of BCR-ABL1-mediated leukemia, while constitutive Egr1 is able to override leukemia conferred by deregulated E2F1 leukaemic cells.[35,36,37] Furthermore, it has recently been shown that genetic deletion of *Egr1* accelerates BCR-ABL1-driven CML.[38] Here, we identify a novel CML-specific pathway in which BCR-ABL1 protein kinase regulates *hsa-mir183*-mediated inhibition of EGR1 leading to up-regulation of E2F1. In addition, we investigate the role that *hsa-mir183/EGR1*-mediated E2F1 expression plays in priming proliferation in CML SPC.

Methods

Cell isolation and culture. Fresh leukapheresis or peripheral blood (PB) samples were from patients (informed consent) with chronic phase (CP) CML at diagnosis or non-CML donors (defined as normal). Samples were enriched for $CD34^+$, Pyronin Y^-(PY) Hoechst$^-$ (Ho), $CD34^+38^+$ and $CD34^+38^-$ populations as described.[4] Dual-fluorescence in situ hybridization (D-FISH) was performed as previously described.[39] Where indicated, cells were treated with imatinib, dasatinib, nilotinib (Selleckchem, UK) or colcemid. Colony forming assays (CFC) in methylcellulose medium (H4434, M3434, StemCell Technologies, Canada) were performed as described.[40]

RNA extraction and Q-PCR. RNA Extraction was performed using the RNeasy Mini Kit (Qiagen, UK); cDNA was synthesized using the High-Capacity cDNA RT Kit (Applied Biosystems, UK), Direct one-step Q-PCR (Invitrogen, UK), cells to CT kit for miRNA (Ambion, UK), or the Power SYBR Green Cells-to-C_t Kit (Ambion/Lifetech, UK). Q-PCR was performed on the ABI7900 (Applied Biosystems) or Fluidigm platforms (Fluidigm Corporation, USA).

Microarray. The CML versus normal microarray data were obtained from ArrayExpress (E-MTAB-2508, Affymetrix Human Genome U133A) and describe gene expression in quiescent CML/normal $CD34^+PY^-Ho^-$ cells. Differential expression was calculated using Rank Products (RP, false discovery rate (FDR) calculated using 1000 permutations).[41]

For the TKI treatment data, cells were treated for 8 hours (hrs) ($CD34^+38^-$) or 7 days (d) ($CD34^+$) with 5uM imatinib, 150nM dasatinib or 5uM nilotinib (no growth factors). cDNA from viable cells (7d only) was hybridized to Affymetrix Human Gene 1.0 ST arrays (E-MTAB-2594) and normalized using RMA.[42] Differentially expressed genes were identified using a paired-sample Limma analysis[43] with pooled TKI treatments and a significance threshold of FDR≤0.05 (Benjamini-Hochberg multiple testing correction was

applied).[44]

For miRNA data, cDNA was hybridized to a miRNA chip based on Sanger miRBase Release 14 (LC Sciences, Texas) and normalized using a LOWESS (Locally-weighted Regression) method on the background-subtracted data. Raw data are publicly available via ArrayExpress (accession E-MTAB-3220) and normalized data are provided in Table S1. The resulting miRNA data were analyzed using LIMMA.[43]

Enrichment analysis. A PANTHER Enrichment Test (release 20141219) was used to identify enrichment of Gene Ontology biological process terms (release 20150111) in the list of genes identified as differentially expressed in CML versus normal cells using RP (Table S2). The Bonferroni correction was applied to the p-values to account for multiple testing. The GSEA analysis was carried out using GSEA (2-2.2.2) as obtained from the Broad Institute (gsea2-2.2.2.jar, http://software.broadinstitute.org/gsea/index.jsp);[45] q values were calculated using 10,000 permutations of the phenotype label. The hypergeometric distribution was used to calculate enrichment statistics.

Comparison of distributions. One sided Kolmogorov-Smirnov tests (using ks.test in the base R stats package) were carried out to identify positive shifts in distribution for a gene set, as compared to background. 10,000 random subsamplings of the transcriptomic data were used to generate the expected null distribution for the Kolmogorov-Smirnov statistic for calculation of FDRs.

Western blotting. Western blotting was performed as described elsewhere.[46] Antibodies used were c-ABL1, GAPDH and Tubulin, (Cell Signaling, UK); E2F1, (Upstate, USA); p53-Do-1 (Santa Cruz, Germany).

Fluorescence activated cell sorting, flow cytometry and imaging analysis. Cells were stained with 7-AAD (Becton Dickinson, UK), Zombie Aqua (Biolegend), CellTrace

Violet Cell Proliferation Kit (Invitrogen), and DAPI (Sigma Aldrich) according to manufacturer's instructions. For intracellular analysis, cells were fixed and permeabilized using 'Fix and Perm' (Merck Chemicals Ltd, UK) or the Fixation/Permeabilization Solution Kit (Becton Dickinson, UK). Primary antibodies were phospho-p53-Ser15, p27 (R and D Systems, UK), p21 (Santa Cruz Biotech), phospho-AKT-T308, phospho-STAT5-Y694, BCL2, Ki-67, Annexin-V, CD34 and CD38 (Becton Dickinson). $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells were isolated as previously described.[27]

Retinoblastoma (Rb) phosphorylation was measured with Cellomic Phospho-Rb activation kit (ThermoScientific, MA) according to manufacturer's instructions and analyzed by Operetta High Content Imaging System (Operetta, PerkinElmer UK).

Gene knock-down. *E2F1* (sh-E2F1, sh-E2F1-1 and sh-E2F1-2) and scrambled shRNAs were sub-cloned into the pLKO.1 GFP vector. Lentiviral infection was carried out as described elsewhere.[47] Custom Mirzip lentiviral system *(*PGK promoter) and mirzip scrambled vector (Cambridge Bioscience Ltd, UK) were used. *E2F1*-siRNA, -siRNA1, -siRNA2; *p53*-siRNA, -siRNA1; *EGR1*-siRNA; scrambled siRNA; scrambled siRNA1 (100nM) from Ambion.

Luciferase Assay. *EGR1* cDNA was amplified from human mononuclear cells. The 3'untranslated region (UTR) sequence containing the *hsa-mir183* binding site or a mutated binding site were cloned into the pmirGLO vector (Dual Glo Luciferase Assay System, Promega, UK). KCL22 cells were co-transfected with pmirGLO vector containing wild type (WT) or mutated (MUT) *EGR1* sequence, and 50mM *hsa-mir183* mimic or scrambled control (Integrated DNA Technologies, Germany) by electroporation. Luminescence was measured after 48hrs using a Glomax 20/20 Luminometer (Promega).

Mice and bone marrow (BM) transplantation. *E2f1*$^{-/-}$ mice were obtained from Jackson Laboratories. BM transplants were performed by tail vein injection into lethally irradiated (two doses of 4.25 Gy) SJL C57/B6 CD45.1 recipients. 1000 LSK cells were transplanted from *E2f1*$^{+/+}$ and *E2f1*$^{-/-}$ donors (CD45.2^{+}) alongside 2x10^{5} unfractionated BM support cells from SJL CD45.1 mice and kept on Baytril antibiotic for 2 weeks. Peripheral blood was analyzed at 4, 8, 12 and 16 weeks after transplantation and BM was analyzed at 16 weeks post-transplant. Population analysed were: LSK; LT-HSC, (long term haemopoietic stem cells); ST-HSC (short term haemopoietic stem cells); MMP1 (multipotent progenitors 1); MMP2 (multipotent progenitors 2); GMP (granulocyte/macrophage progenitors); MEP (megakaryocyte/erythrocyte progenitors); CMP (Common myeloid progenitors); myeloid cells; B-Cells.

For secondary transplantations, 2,000 CD45.2^{+} LSK cells from primary recipients were transplanted with 2x10^{5} CD45.1^{+} support cells into CD45.1^{+} irradiated recipient mice. Monoclonal antibodies against CD45.1 and CD45.2 were used. For C-kit^{+} (CD117^{+}) cell isolation, BM cells were passed through MACS separation columns (Miltenyi Biotec, UK) using manufacturer's instructions. C-kit enriched cells were isolated using FACS Aria cell sorter (Becton Dickinson).

Statistics

Statistical analyses were performed using the Student's t test. A threshold of $P<.05$ was defined as statistically significant (*). Levels of $P<.01$ (**) and $P<.001$ (***) were taken to be highly statistically significant.

Study approval

All animal experiments were carried out according to UK Home Office regulations.

Results

CML SPC are predominantly quiescent despite expressing active BCR-ABL1

BCR-ABL1 confers a proliferative advantage to primitive CML cells.[32] To investigate cell cycle status, SPC ($CD34^+38^-$) and more mature cells ($CD34^+38^+$) were isolated from primary normal and CML CP samples (Figure 1Ai). As previously reported, CML SPC expressed high levels of BCR-ABL1 (Figure 1Aii) when compared to more mature cells prior to treatment.[40,48,49] Despite this, CML SPC showed no significant difference in the percentage of quiescent cells (Figure 1Bi-ii, replicates shown in Figure S1A). Similarly, mRNA levels of the cell cycle inhibitors *p21, p27*, *p57* and protein levels of p21 and p27 did not differ between normal and CML SPC (Figure S1B-C). The levels of phospho-AKT, phospho-STAT5 and BCL2, proteins downstream of BCR-ABL1, were higher in CML than normal SPC (Figure 1C), showing that, despite being quiescent at the functional level, BCR-ABL1 kinase activity and transcriptional machinery are active, suggesting that CML SPC are "primed to proliferate", as previously reported.[50] Consistent with the published literature,[6,9,10] BCR-ABL1 activity is affected by treatment with TKI in SPCs, as evidenced by a decrease in the levels of its downstream targets phospho-STAT5 and phospho-AKT (Figure S1D). While CML $CD34^+38^-$ SPC showed lower levels of BCL2 and less phosphorylation of AKT and STAT5 compared to mature CML $CD34^+38^+$ cells, these levels were still higher than in the equivalent population of normal cells (Figure 1C).

BCR-ABL1 regulates *hsa-mir183* and *EGR1* in CML SPC

BCR-ABL1 modulates the expression of miRNAs,[28] therefore to elaborate networks that may regulate SPC quiescence, we performed global miRNA expression profiling in normal and CML SPC. Several miRNAs were differentially expressed, including *hsa-*

mir183 which was up-regulated in CML (Figure 2A). Validation by OncoMir array confirmed that *hsa-mir183* was significantly up-regulated by 38-fold in CML versus normal SPC (Figure 2Bi-ii). The up-regulation of *hsa-mir183* was BCR-ABL1 kinase dependent as its expression decreased upon treatment with TKIs (Figure 2C). Published evidence,[35,36] together with computational target prediction by the miRWalk database,[51] indicated that *hsa-mir183* targets and down-regulates *EGR1,* which plays a role in normal SPC proliferation and development of BCR-ABL1-mediated leukemia. As predicted, *EGR1* mRNA levels were significantly lower in CML versus normal SPC (Figure 2Di). This effect appeared to be BCR-ABL dependent as primary CML $CD34^+$ cells demonstrated a significant increase in *EGR1* expression following long term exposure to TKI ($p=0.01$; Figure 2Dii). To further confirm that the regulation of EGR1 was *hsa-mir183* dependent*, hsa-mir183* expression was knocked-down in CML SPC using a GFP-lentiviral-based anti-miRNA. GFP^+ SPC were analyzed for the level of the *EGR1* transcripts, the expression of which was rescued by *hsa-mir183* knockdown (Figure 2E). In addition, we found that SPC with *hsa-mir183* knock-down proliferated less than control cells (Figure 2F) and generated fewer colonies in CFC assays (Figure 2G).

To prove direct binding between *hsa-mir183* and *EGR1* in CML cells, we generated oligos complementary to the *EGR1* 3'UTR (WT) encompassing the binding site for *hsa-mir183* (Figure S1F). As a control, a mutated version of the binding site was designed by inserting a BamHI restriction site. Both oligos were transfected into CML KCL22 cells, together with either 50nM *hsa-mir183* mimic or a negative control. After 48hrs, cell lysates showed a significant decrease in luciferase signal in the cells containing the WT *EGR1* sequence and the *hsa-mir183* mimic, however there was no effect in the cells containing *EGR1* MUT sequence (Figure 2H), confirming that *hsa-mir183* binds directly

to the predicted sequence within *EGR1* to prevent its transcription. In summary, these data support a model where BCR-ABL1-induced *hsa-mir183* leads to a decrease of *EGR1*.

E2F1 signaling pathway is deregulated in CML SPC

To investigate how this hsa-mir183/EGR1 axis relates to transcriptional control of the cell cycle, specifically in relation to *E2F1*, we have performed *in silico* and *in vitro* assays. Comparison of previously published transcriptomic data from quiescent (G0) normal and CML cells, defined as $CD34^{+}$ $PY^{-}Ho^{-}$ (ArrayExpress accession E-MTAB-2508)[32] by principal component analysis (PCA) demonstrates clear separation of CML and normal samples using E2F1 target gene expression data (Figure 3Ai; targets extracted from the MetaCoreKB, Table S3). Overall, these E2F1 targets exhibit a statistically significant positive shift (i.e. towards up-regulation) with respect to differential expression when comparing primary quiescent CML SPC to quiescent normal SPC[32] (Figure S2Ai; D=0.18, $p=2.06x10^{-36}$, Kolmogorov-Smirnov test). Random resampling of these data demonstrated that this difference is unlikely to occur by chance (Figure S2Aii; false discovery rate (FDR)<0.0001, 10,000 iterations). This statistically significant positive shift for E2F1 targets was also observed in a second, complementary dataset of primary CML and normal quiescent cells (GEO accession GSE24739)[29] (Figure S2Bi; D=0.11, $p=8.90x10^{-13}$, Kolmogorov-Smirnov test); here, again, a difference of the same magnitude or greater is very unlikely to occur by chance (Figure S2Bii; FDR<0.0001, 10,000 iterations).

Cell cycle related Gene Ontology[52] terms were significantly over-represented in the list of differentially expressed genes[32,41] (Table S2). E2F1 target genes identified as significantly differentially up-regulated in the first CML versus normal G0 transcriptional

dataset (ArrayExpress accession E-MTAB-2508) are summarised in Figure 3Aii; these same genes were shown to be significantly up-regulated in the second CML/normal G0 dataset by GSEA[45] (Figure S2C; NES=1.40, p=0.05). Interestingly, although *E2F1*, *E2F2* and *E2F3* share a high degree of functional redundancy, *E2F2* and *E2F3* were not differentially expressed between normal and CML cells (Figure S2D). Taken together, these *in silico* results suggest that E2F1 transcriptional targets are up-regulated in quiescent CML SPC as compared to quiescent normal SPC.

To validate these *in silico* findings, we next performed *in vitro* functional experiments. Up-regulation of *E2F1* mRNA in CML SPC was confirmed by Q-PCR (Figure 4A). As expected, given that Rb protein phosphorylation (i.e. Rb inactivation) often coincides with E2F1 up-regulation,[53] phospho-Rb (inactive) was high in CML SPC (Figure 4B, raw data provided in Table S4). The higher activity of E2F1 in CML SPC, in terms of cell cycle regulation, was confirmed by expression of key downstream genes and additional E2F1 targets (Figure 4C). Similar to the bulk SPC data (Figure 4A-C), CML and normal G0 SPC ($CD34^{+}38^{-}PY^{-}Ho$) showed up-regulation of *E2F1* and its downstream genes (Figure S3A-C).

To determine whether *hsa-mir183*/*EGR1* were involved in *E2F1* regulation of the cell cycle, we again knocked-down *hsa-mir183* and observed a decrease in *E2F1* and an increase in *p21* mRNA levels, suggesting that the up-regulation of E2F1 was mediated by *hsa-mir183*, presumably through *EGR1* (Figure 3B). To confirm this we, knocked-down *EGR1* using siRNA which indeed caused an increase in *E2F1* and *CDK1* mRNA levels compared to control (Figure 3C).

These data therefore support a model whereby BCR-ABL1-induced *hsa-mir183* expression leads to a decrease of *EGR1* expression, which in turn results in the up-regulation of E2F1 activity in CML SPC (Figure 3D).

E2F1 regulation is dependent on BCR-ABL1 kinase activity

To investigate whether *E2F1* regulation in CML SPC was BCR-ABL1 kinase dependent as found for *hsa-mir183* (Figure 2), we compared transcriptional data for SPC ±TKI (8hrs) and $CD34^+$ cells ±TKI (7d) (Figure 4D). *BCR-ABL1* positive (confirmed by D-FISH) cells were treated, sorted again for viable cells, and analyzed using Affymetrix Human Gene 1.0 ST chips. Analysis revealed a limited effect on gene expression at 8hrs (49 differentially expressed genes, FDR=0.05), but an extensive effect (n≈20,000, FDR=0.05) at 7d; expression of *E2F1* (Figure 4Di-ii) and most of its downstream targets (Figure S4) were significantly decreased. To determine whether the BCR-ABL1 driven regulation of E2F1 in the SPC of patients was sensitive to TKI therapy *in vivo*, we interrogated transcriptomic data from CML $CD34^+$ cells harvested from six patients, before and at 7d after imatinib treatment (GEO accession GSE12211).[30] We observed that significantly more of the E2F1 targets were down-regulated by TKI *in vivo* than expected by chance (29 of 54, $q=6.48x10^{-24}$). To confirm that these effects were the result of BCR-ABL1 kinase inhibition rather than an enrichment for resistant leukemic cells, CML SPC were treated for 7d with dasatinib, washed and cultured for a further three days without drug; levels of *E2F1* and *CDK1* were restored following wash-out (Figure 4E). Similar behavior was seen in other representative E2F1 target genes (Figure S5A). Taken together, these data suggest that *E2F1* regulation in CML SPC is BCR-ABL1 kinase dependent. Interestingly, E2F1 target genes were similarly deregulated in primary $CD34^+$ CML versus normal PB samples, for both TKI-responders (R) and TKI-non-responders (NR) (GEO accession GSE14671):[33] random sampling demonstrated that E2F1 targets were significantly more correlated across TKI-R and NR than we would expect by chance ($r=0.70$, $q<0.001$) (Figure S5B). The same result was found for aggressive and indolent CML samples (ArrayExpress accession E-MIMR-

17) (r=0.73, q<0.001) (Figure S5C).[34] Together, these analyses indicate that the *E2F1* dependent deregulation observed in our unselected CML samples is also present in TKI-NR and aggressive CML phenotypes, and that targeting this deregulation may have wide clinical scope.

E2F1 is dispensable for normal SPC survival

Before considering the *hsa-mir-183*/EGR1/E2F1 axis for therapeutic targeting in CML SPC we first wished to confirm that targeting E2F1 would not be detrimental to normal SPC homeostasis using *E2f1*$^{-/-}$ mice.[17] The reconstitution capacity of normal SPC lacking *E2f1* was tested by transplanting *E2f1*$^{-/-}$ and WT CD45.2^{+} LSK cells into lethally irradiated syngeneic CD45.1^{+} recipients. Cells of both genotypes contributed equally to long-term hemopoiesis following primary and secondary transplantation (Figure 5Ai-ii). Full characterization of the BM of primary and secondary transplanted mice showed minimal differences in the reconstitution potential of WT and *E2f1*$^{-/-}$ LSK cells in terms of long and short-term stem cells, various progenitor populations and mature myeloid and B-cells (Figure S6A-B). Finally, *E2f1*$^{-/-}$ (CD45.2^{+}) and *E2f1* WT (CD45.1^{+}) BM cells were transplanted in ratios of 9:1, 1:1 and 1:9 into lethally irradiated syngeneic CD45.1^{+} recipient mice to test long-term reconstitution primary ability of mutant versus WT cells. Analysis of PB at 4, 8, 12 and 16 weeks post-transplant showed that *E2f1*$^{-/-}$ cells exhibited comparable reconstitution ability to WT cells (Figure 5Bi-ii). Together these data suggest that loss of E2F1 has no detrimental effect on normal SPC maintenance and function.

E2F1 is required for CML SPC survival *in vitro*

We next asked whether increased activity of E2F1 in CML SPC led to an increased dependency on E2F1 for survival. *E2F1* was knocked-down in normal and CML SPC using a GFP-lentiviral shRNA. The $GFP^+CD34^+38^-$ cells showed a significant decrease in *E2F1* expression in both normal and CML cells, and increase in *p21* mRNA in CML (Figure 6A-B). In CFC assays, the number of colonies derived from CML, but not normal, SPC was significantly decreased, suggesting that *E2F1* is required for CML SPC colony forming potential (Figure 6Ci-iii). Furthermore, knockdown of *E2F1* inhibited proliferation of CML SPC to a similar extent as the colcemid control (Figure 6D). *E2F1* depletion also induced a significant increase in cell death in CML, but not in normal SPC, as indicated by an increased percentage of Annexin-V^+ cells (Figure 6E). These data suggest that E2F1 is required for CML SPC survival and proliferation *in vitro*. The efficacy of the knock-down system and the subsequent effects in primary CML cells using was corroborated using different sets of shRNAs (Figure S7A-B).

p53 is known to mediate cell cycle arrest and apoptosis in primary mouse fibroblasts with triple inactivation of *E2f1/2/3.*[25] Given this and the increase in *p21* mRNA levels we observed following E2F1 knockdown, we investigated whether the cell cycle arrest and apoptosis upon depletion of *E2F1* in CML SPC was mediated by p53 activation. CML SPC were transfected with an *E2F1*-specific siRNA or control sequence. While the level of E2F1 was decreased, the level of p53 protein was unchanged (Figure S8Ai). However, an increase in Serine 15 (Ser15) phosphorylation on p53, a crucial site for the induction of cell cycle arrest,[54] was observed in SPC transduced with *E2F1* siRNA compared to control (Figure S8Aii), suggesting that a decrease in *E2F1* resulted in activation of p53. The efficacy of the knock-down system in primary $CD34^+$ CML cells was confirmed using different sets of siRNA (Figure S8B).

To determine whether the changes in apoptosis in *E2F1* knock-down CML SPC were the direct result of increased p53 activity, we performed knock-downs of *E2F1* and *p53*, separately and in combination, with the double knock-down resulting in reduced *E2F1* on a background of low *p53* (expression of *Puma* and *CDK1* were measured as representative indicators of p53 and E2F1 activity, respectively) (Figure S8C). p53 activity was higher upon *E2F1* knock-down, while E2F1 activity was higher upon *p53* knock-down. In the combination, no changes were seen in either the p53 or E2F1 downstream targets. Three days after transfection, apoptosis levels were significantly higher in the *E2F1* knock-down, whereas there was no difference in the combination relative to control (Figure S8D). These data imply that the apoptosis and inhibition of proliferation arising from E2F1 knock-down in CML SPCs is mediated by p53.

Discussion

The oncogenic role of the E2F family members has previously been reported.[53,55-57] E2F3 has been shown to play a role in controlling BCR-ABL1-driven leukemogenesis *in vitro* and *in vivo*,[23] while *in vitro* studies have indicated that E2F1 regulates the cell cycle of BCR-ABL1 positive cell lines.[58,59] Here, we have employed primary patient-derived cells to demonstrate that E2F1 is required for the survival of CML but not normal SPC *in vitro*, and that E2F1 is regulated in CML SPC via the BCR-ABL1/*hsa-mir183*/EGR1 axis. In CML SPC, E2F1 was the effector of a novel signaling pathway mediated by up-regulation of *hsa-mir183* and inhibition of its direct target *EGR1*. Indeed, recent evidence demonstrates that leukemia development is accelerated in the absence of EGR1 in a CML mouse model.[38] E2F1 up-regulation was BCR-ABL1 kinase dependent and its inhibition led to a decrease in colony forming potential, cell cycle arrest and

induction of p53-mediated cell death, suggesting the dependency of leukemic SPC on E2F1.

Our data provide mechanistic insight into how CML SPC death induced by E2F1 inhibition may be mediated by p53. We have previously shown that the apoptotic activity of p53 is down-regulated in leukemic stem cells[27] and here we have demonstrated that its activity is increased upon inhibition of E2F1 in CML SPC (by showing that increased cell death in *E2F1* knock-down CML SPC was associated with post-transcriptional modification of Ser15 on p53). To investigate the functional link between E2F1 and p53 in CML SPC, we hypothesized that cell death due to *E2F1* knock-down might be rescued by simultaneous knock-down of *p53*. While these data suggest that knock-down of *p53* may be sufficient to ameliorate cell death of *E2F1* deficient CML SPC, further work will be required to definitively prove the relevance of this mechanism is CML.

Despite E2F1 up-regulation being BCR-ABL dependent, recent work in single CML SPCs has demonstrated that E2F1 signalling and proliferation-associated gene expression are active in a subpopulation of BCR-ABL$^+$ cells that persist following TKI treatment[14]. This suggests that therapeutic intervention (via therapeutic targeting of E2F1 or the other components of our model), could address a pressing unmet clinical need for CML patients.

Of relevance to designing a novel therapy for CML, our work indicated that normal SPC were not affected by inhibition of the E2F1 pathway, suggesting a potential therapeutic window and CML specificity. Although our studies suggest that inhibition of E2F1 itself may represent a promising therapeutic target, transcription factors are notoriously challenging in drug development and alternative routes to target the BCR-ABL1/hsa-

mir183/EGR1/E2F1 axis may prove to be more tractable. Future investigations will explore the therapeutic potential of the BCR-ABL1/hsa-mir183/EGR1/E2F1 axis in CML.

Acknowledgments

We would like to dedicate this manuscript to the memory of Prof. Tessa Holyoake, an inspirational, generous and enthusiastic clinician and scientist.

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Authorship

FP and LEMH designed and performed research, analyzed data, and wrote the manuscript. LP, MMS, LJ, CJC and AS performed experiments and reviewed the manuscript. MTS, SAA, AH, GVH, MAO, RB, GL, KRK and ADW provided material, interpreted data, and reviewed the manuscript. TLH designed the study, analyzed data, and wrote the manuscript.

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Figure legends

Figure 1. CML SPC are predominantly quiescent. (A) (i) Gating strategy for Normal and CML SPC enriched for $CD34^{+}38^{-}$ and $CD34^{+}38^{+}$ cells. (ii) Level of BCR-ABL1 and ABL measured in $CD34^{+}38^{-}$ and $CD34^{+}38^{+}$ cells. (B) (i) Representative dot plots showing the cell cycle phases in normal and CML SPC measured by Ki-67/7-AAD staining. N=1 representative sample shown. (ii) Percentage of normal (n=4) and CML (n=4) SPC in G0 cell cycle phase. (C) Phosphorylation of AKT and STAT5 and level of BCL2 measured by FACS in CML $CD34^{+}38^{-}$ and $CD34^{+}38^{+}$cells. (*P<.05; **P<.01).

Figure 2. CML SPC show regulation of the *hsa-mir183*/EGR1 axis. (A) Genome-wide miRNA expression for normal and CML SPC (N=5 biological replicates). (B) (i) Heatmap for the OncoMir MiRNA Q-PCR Array in SPC showing statistically significant regulation of miRNAs between normal and CML SPC. (ii) Q-PCR for *hsa-mir183* regulation in CML versus normal SPC. (C) CML SPC treated for 24hrs with dasatinib (DAS, 150nM) and nilotinib (NIL, 1uM) and analysis by Q-PCR for *hsa-mir183*. ND=no drug. (D) (i) mRNA level of the *hsa-mir183* target gene *EGR1* in normal and CML $CD34^{+}38^{-}$ cells. (ii) CML $CD34^{+}$ cells treated for 7 days with imatinib (IM, 5μM) and live cells analysed by Q-PCR for *EGR1* expression (N=6). ND=no drug. (E) *hsa-mir183* knock-down GFP^{+} CML SPC sorted and analyzed for *EGR1* mRNA level by Q-PCR. Mir zip-scrambled vector was used as negative control. (F) Representative plot for cell divisions analyzed in *hsa-mir183* knock-down GFP^{+} CML SPC using Cell Trace Violet staining. Colcemid treatment used to visualize undivided cells. (G) CFC analysis carried out in *hsa-mir183* knock-down CML SPC. (H) Luciferase assay showing binding between of *hsa-mir183* and *EGR1* in KCL22 cells. Cells transfected with olio's containing the *EGR1* 3'UTR (along with the binding site for *hsa-mir183*, wild type, WT)

or a mutant version (MUT) together with *hsa-mir183* mimic or scrambled negative control. Each experiment had N=3 biological replicates; *P<.05; **P<.01; *** P <.001.

Figure 3. CML SPC show deregulation of E2F1 signaling networks. (A) (i) Analysis of mRNA screen for $CD34^{+}PY^{-}Ho^{-}$ normal (N=2 biological replicates) and CML (N=5 biological replicates) cells. PCA reduced the high dimensional dataset to 3 dimensions using E2F1 targets as obtained from MetaCoreKB (targets listed in Table S3). Each sample is represented as a dot; CML and normal samples are colored red and black respectively. 3D ellipses are drawn around each set of samples (CML or normal) using the mean and covariance of that set, to represent each sample type's 95% confidence region. Axes labels indicate the % variability accounted for by each principal component. (ii) The network of BCR-ABL1, *E2F1* and *E2F1* targets, significantly deregulated in CML is shown. Node color indicates transcriptional deregulation in CML versus normal G0 cells (ArrayExpress accession E-MTAB-2508), with red/green indicating up-/down-regulation respectively; color intensity indicates the extent of the deregulation (as indicated by the color bar). (B) Levels of E2F1 and p21 mRNA measured by Q-PCR in *hsa-mir183* knock-down CML SPC. (C) Levels of EGR1, E2F1 and CDK1 mRNA measured by Q-PCR after knocking down EGR1 by siRNA in CML SPC. (D) Schematic summary of the hsa-mir183/EGR1-mediated regulation of E2F1. (Each experiment had N=3 biological replicates;* P<.05.)

Figure 4. *E2F1* regulation in CML SPC by BCR-ABL1. (A) Nuclear levels of *E2F1* mRNA measured in normal and CML $CD34^{+}38^{-}$ and $CD34^{+}38^{+}$ cells by Q-PCR. (B) Phosphorylation level of Rb measured in normal and CML SPC by a high-content screening-based platform. (C) mRNA levels of E2F1 downstream genes measured in

normal and CML $CD34^+38^-$ and $CD34^+38^+$ cells by Q-PCR. (D) (i) Heatmap showing regulation of *E2F1* and its signaling (yellow: up-regulation; blue: down-regulation, see color bar) in CML SPC after treatment with TKI (values for imatinib, dasatinib and nilotinib pooled together) for 7d (treated columns indicated by "+"). (ii) *E2F1* response to TKI treatment for 8hrs and 7d. (E) *E2F1* and *CDK1* mRNA levels from CML SPC treated for with dasatinib (DAS, 150nM) or washed out and cultured for further 3d (DAS w/o). •= outliers as calculated using Tukey's method.[24] Each experiment had N=3 biological replicates. *P <.05; ** P <.01; *** P <.001).

Figure 5. Normal *E2f1*$^{-/-}$ SPC cells retain functionality. (A) *E2f1*$^{-/-}$ or WT BM LSK cells transplanted into lethally irradiated $CD45.1^+$ WT recipient mice (n=5) for long-term reconstitution (i). 16 weeks post-transplantation, $CD45.2^+$ LSK WT or *E2f1*$^{-/-}$ cells purified from harvested BM and transplanted into lethally irradiated $CD45.1^+$ WT secondary recipient mice (N=4/5) (ii). The engraftment ability of *E2f1*$^{+/+}$ WT (black) and *E2f1*$^{-/-}$ (grey) LSK cells was assessed by the percentage of $CD45.2^+/CD45.1^-$ cells in PB at weeks 4, 8, 12 and 16 post primary and secondary transplantation. (B) (i) Schematic representation of experimental design. *E2f1*$^{-/-}$ ($CD45.2^+$) and *E2f1*$^{+/+}$ WT BM cells ($CD45.1^+$) were transplanted in ratios of 9:1, 1:1 and 1:9 into lethally irradiated (7Gy) $CD45.1^+$ WT recipient mice (N=5) for long-term reconstitution. (ii) Percentages of $CD45.2^+$ *E2f1*$^{-/-}$ donor versus recipient cells ($CD45.1^+$) at weeks 4, 8, 12 and 16 post-transplant. NS: not significant with *P>.05*.

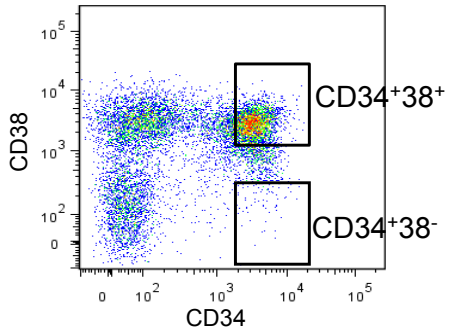
Figure 6. *E2F1* knock-down induces a block in proliferation and increased cell death in CML SPC. mRNA levels of (A) *E2F1* and (B) *p21* by Q-PCR in E2F1 knock-down GFP^+ normal and CML SPC. Transfection with scrambled vector was used as

negative control. (C) CFC analysis to measure colony forming ability following *E2F1* knock-down in (i) CML and (ii) normal SPC. (iii) Representative images for CFC from normal and CML SPC upon *E2F1* knock-down. (D) Representative histogram of cell divisions by CellTrace Violet staining in *E2F1* knock-down normal and CML SPC. Colcemid treatment was used to visualize undivided cells. (E) Percentage of early apoptosis indicated by Annexin-V^+/7-AAD^- cells. Each experiment had N=3 biological replicates. *P <.05; ** P <.01.

Figure 1

A

i



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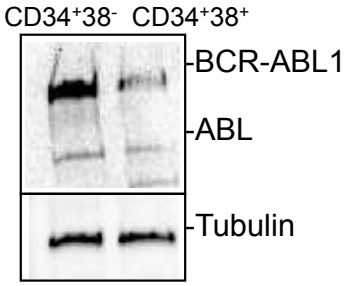
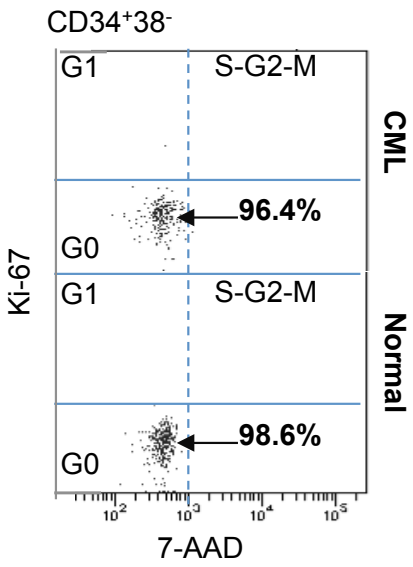


B

i



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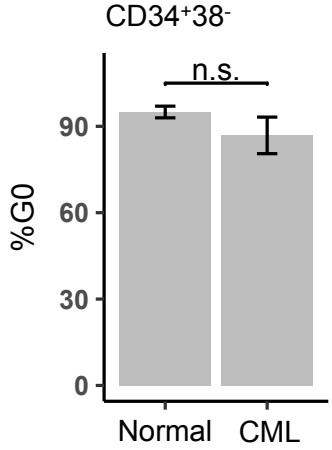

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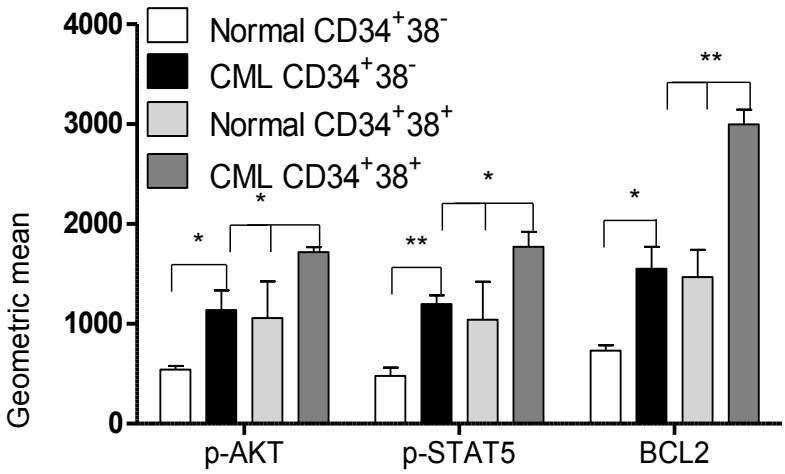
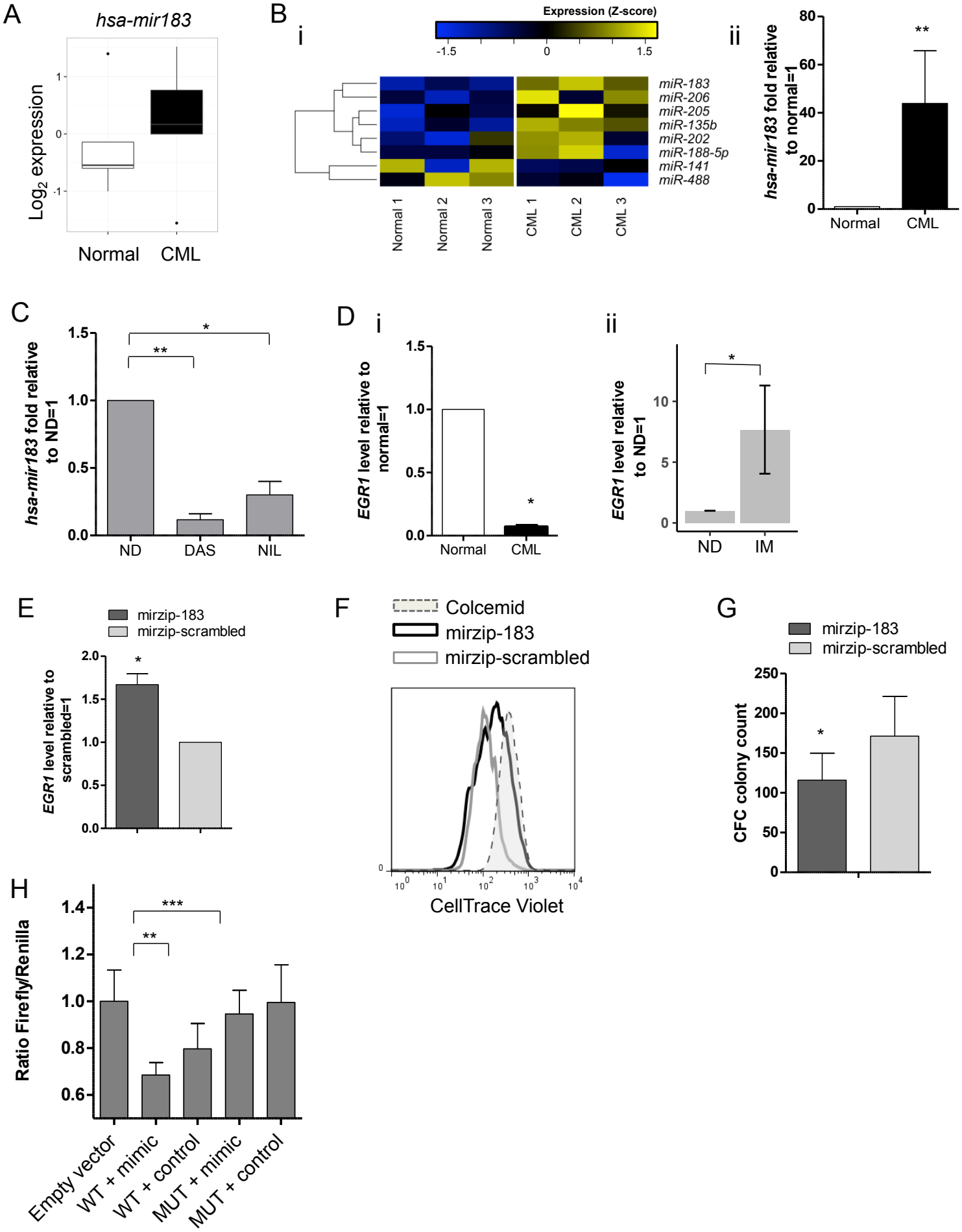


Figure 2


A
hsa-mir183
Log2 expression
Normal
CML
B
i
Expression (Z-score)
-1.5
0
1.5
miR-183
miR-206
miR-205
miR-135b
miR-202
miR-188-5p
miR-141
miR-488
Normal 1
Normal 2
Normal 3
CML 1
CML 2
CML 3
ii
hsa-mir183 fold relative to normal=1
Normal
CML
C
hsa-mir183 fold relative to ND=1
ND
DAS
NIL
D
i
EGR1 level relative to normal=1
Normal
CML
ii
EGR1 level relative to ND=1
ND
IM
E
mirzip-183
mirzip-scrambled
EGR1 level relative to scrambled=1
F
Colcemid
mirzip-183
mirzip-scrambled
CellTrace Violet
G
mirzip-183
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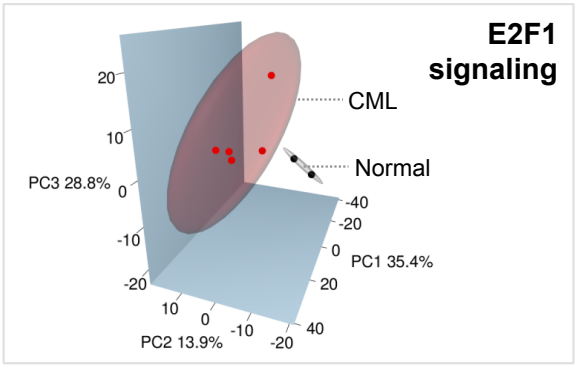
MUT + mimic
MUT + control

Figure 3

A i



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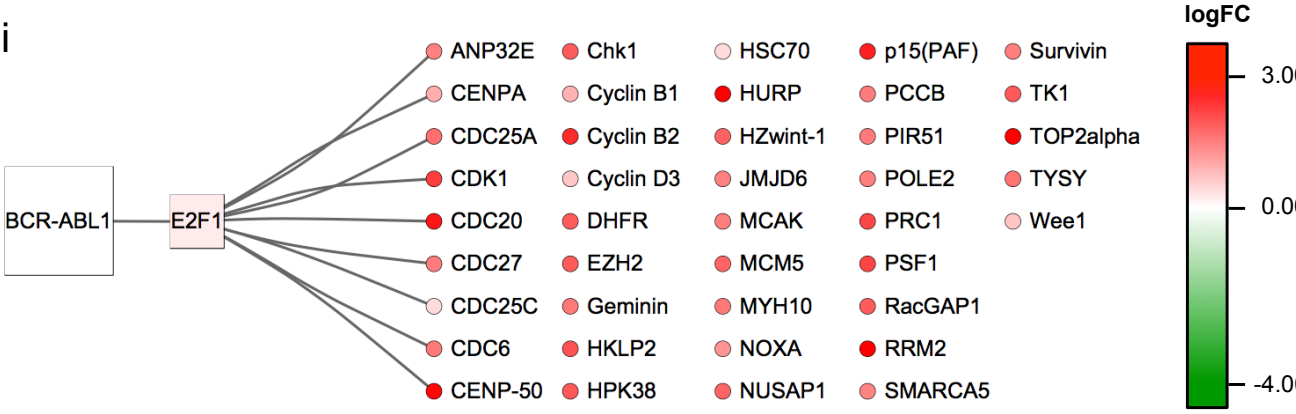

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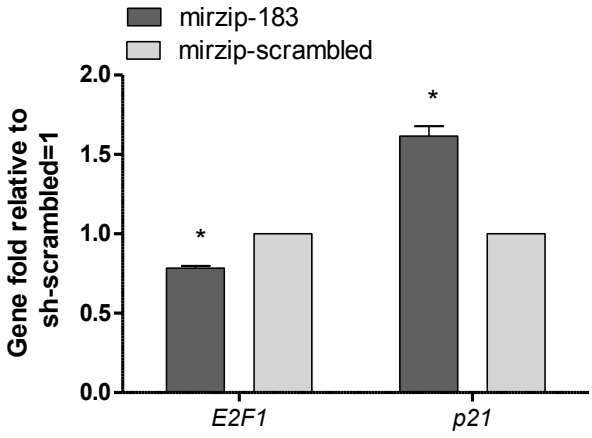

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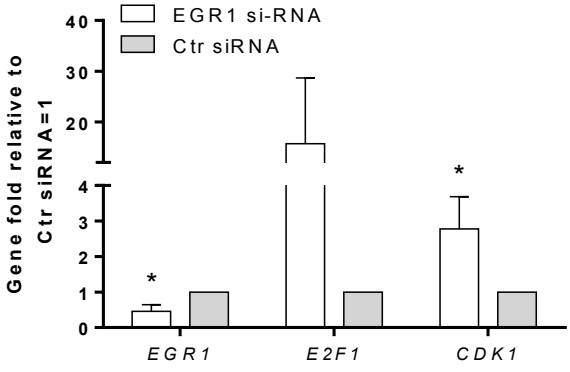

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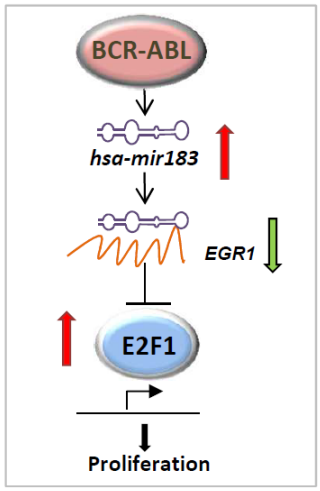


Figure 4

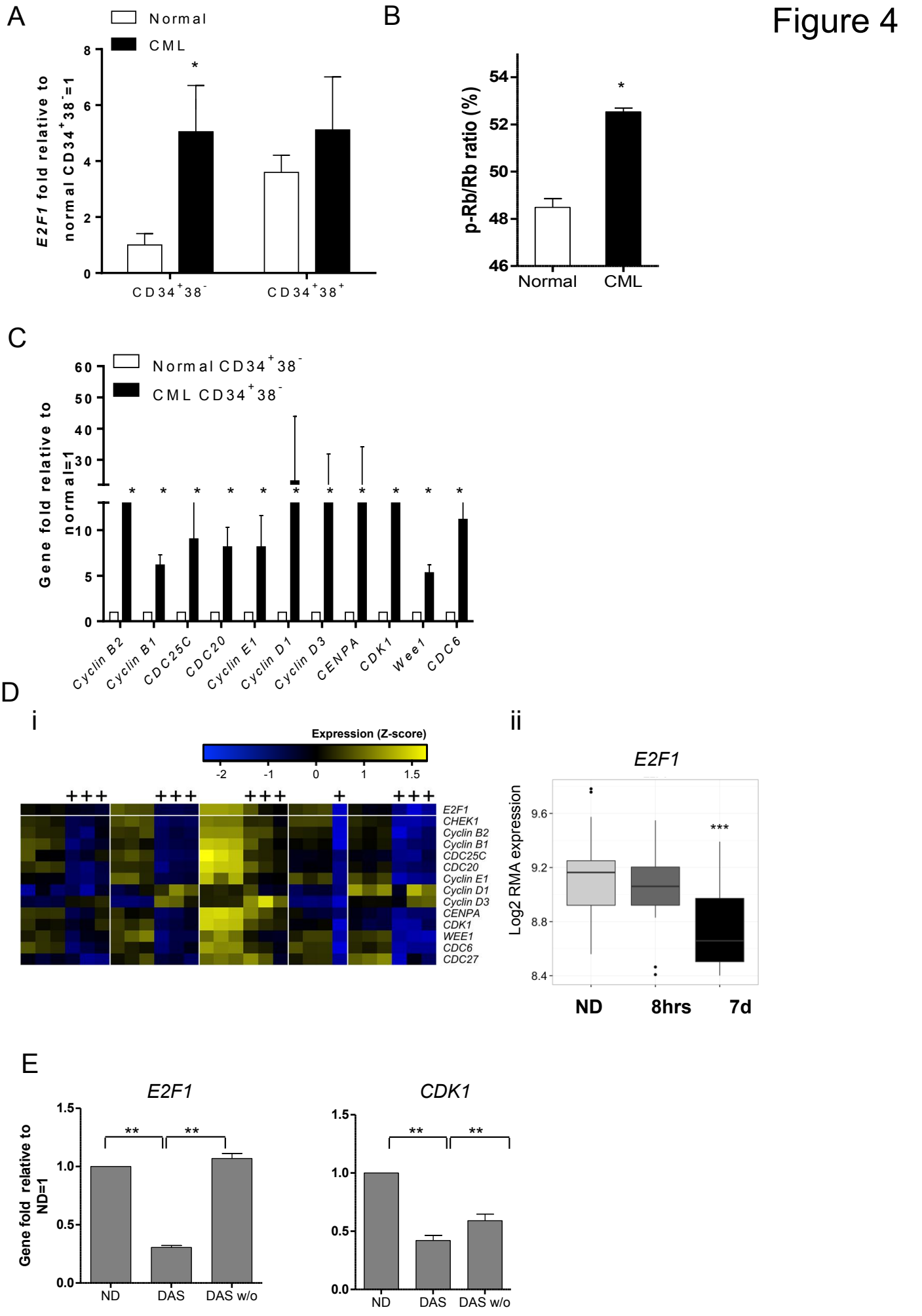
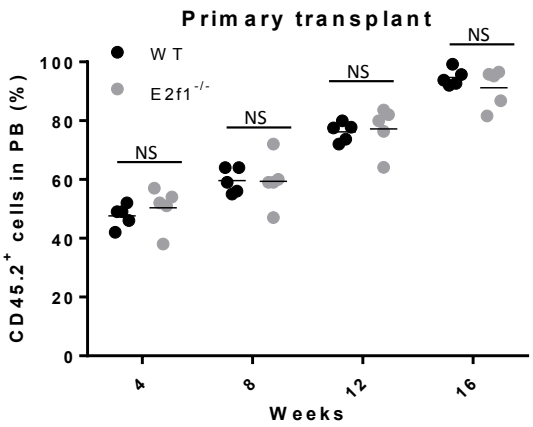

A
Normal
CML
E2F1 fold relative to normal CD34+38-=1
CD34+38-
CD34+38+
B
p-Rb/Rb ratio (%)
Normal
CML
C
Normal CD34+38-
CML CD34+38-
Gene fold relative to normal=1
Cyclin B2
Cyclin B1
CDC25C
CDC20
Cyclin E1
Cyclin D1
Cyclin D3
CENPA
CDK1
Wee1
CDC6
D
i
Expression (Z-score)
E2F1
CHEK1
Cyclin B2
Cyclin B1
CDC25C
CDC20
Cyclin E1
Cyclin D1
Cyclin D3
CENPA
CDK1
WEE1
CDC6
CDC27
ii
E2F1
Log2 RMA expression
ND
8hrs
7d
E
E2F1
CDK1
Gene fold relative to ND=1
ND
DAS
DAS w/o

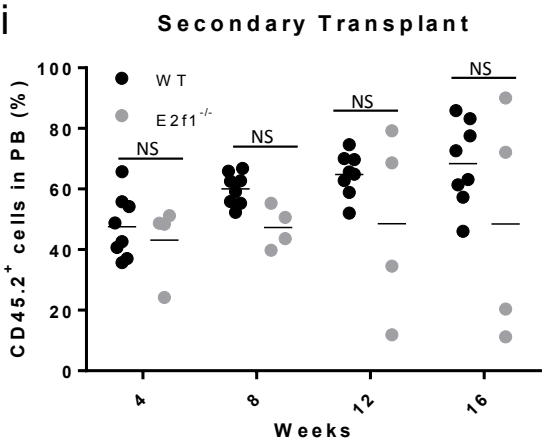
Figure 5

A

i

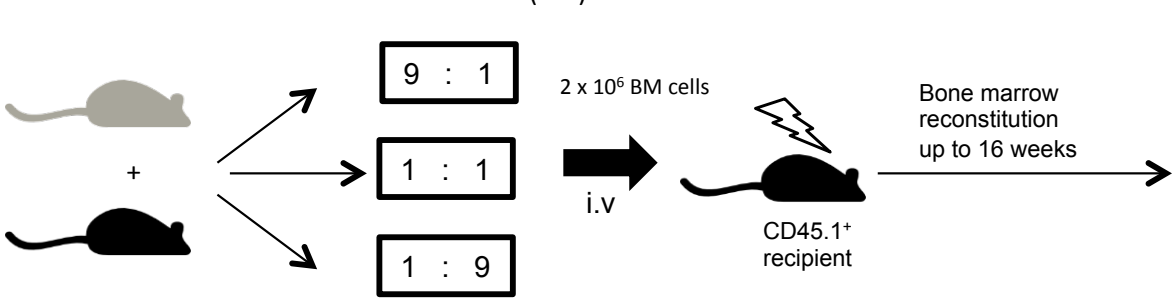


ii



B

i



ii

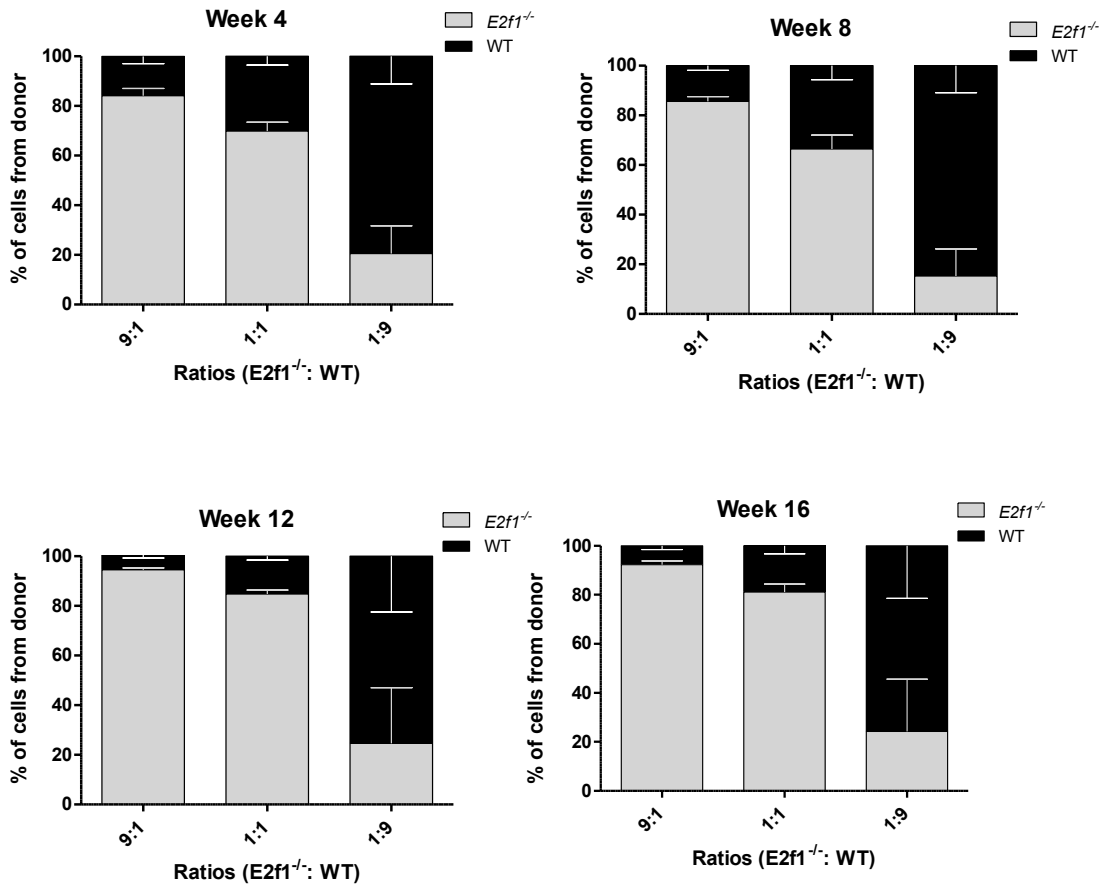
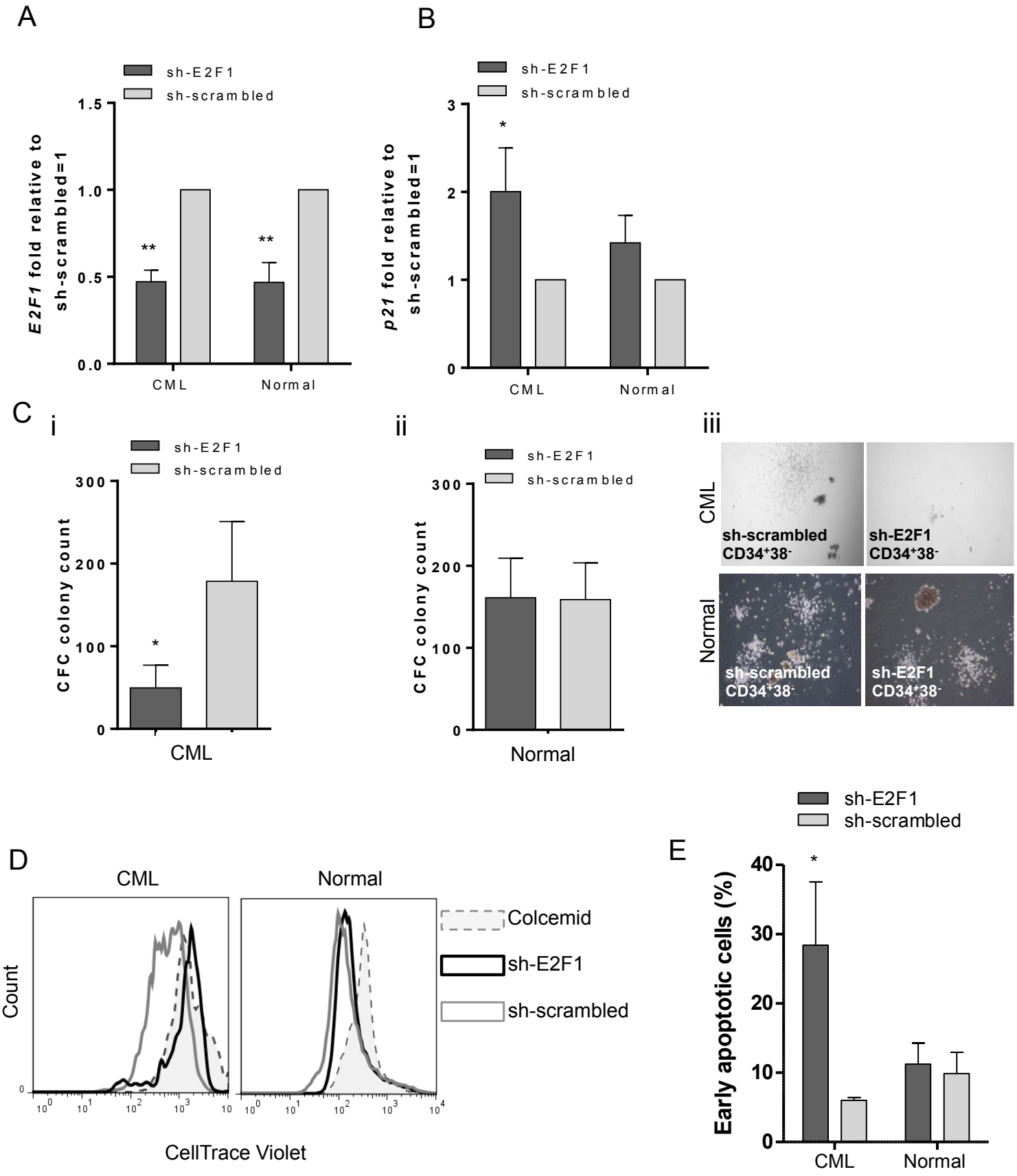


Figure 6


A
sh-E2F1
sh-scrambled
E2F1 fold relative to sh-scrambled=1
1.5
1.0
0.5
0.0
**
**
CML
Normal
B
sh-E2F1
sh-scrambled
p21 fold relative to sh-scrambled=1
3
2
1
0
*
CML
Normal
C
i
sh-E2F1
sh-scrambled
CFC colony count
300
200
100
0
*
CML
ii
sh-E2F1
sh-scrambled
CFC colony count
300
200
100
0
Normal
iii
CML
Normal
sh-scrambled CD34+38-
sh-E2F1 CD34+38-
sh-scrambled CD34+38-
sh-E2F1 CD34+38-
D
CML
Normal
Count
0
Colcemid
sh-E2F1
sh-scrambled
CellTrace Violet
E
sh-E2F1
sh-scrambled
Early apoptotic cells (%)
40
30
20
10
0
*
CML
Normal