E2F1-3 Are Critical for Myeloid Development

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Running Title: E2F1-3 in Myeloid Development
Abstract
Hematopoietic development involves the coordinated activity of differentiation and cell cycle regulators. In current models of mammalian cell cycle control, E2f activators (E2f1, E2f2 and E2f3) are portrayed as the ultimate transcriptional effectors that commit cells to enter and progress through S-phase. Using conditional gene knockout strategies we show that E2f1-3 are not required for the proliferation of early myeloid progenitors. Rather, these E2fs are critical for cell survival and proliferation at two distinct steps of myeloid development. First, E2f1-3 are required as transcriptional repressors for the survival of CD11b+ myeloid progenitors and then they are required as activators for the proliferation of CD11b+ macrophages. In bone marrow macrophages (BMMs), we show that E2f1-3 respond to CSF1-Myc mitogenic signals and serve to activate E2f target genes and promote their proliferation. Together, these findings expose dual functions for E2f1-3 at distinct stages of myeloid development in vivo, first as repressors in cell survival and then as activators in cell proliferation. In summary, this work places E2f1-3 in a specific signaling cascade that is critical for myeloid development in vivo.

Introduction
Molecular events regulating hematopoiesis involve coordinated expression of components that regulate the cell cycle (1). Cyclins and cyclin-dependent kinases (cdk) are crucial for the maintenance of hematopoietic stem cells (HSCs) (2,3). The combined ablation of all three D-type cyclins or their catalytic partners, cdk4 and cdk6, results in a decrease in HSCs proliferation (4,5). Conversely, inhibitors of cdks restrain the ability of HSCs to proliferate. Indeed, mice deficient for p21\textsuperscript{Cip1} or p27\textsuperscript{Kip1} have excessive HSC proliferation, rendering HSC pools sensitive to exhaustion (2,6). Moreover, mice lacking Bmi-1, a repressor of the cdk inhibitor p16\textsuperscript{Ink4a}, show a significant impairment in HSC self-renewal (7).

One consequence of mitogen mediated activation of cdk activity is the phosphorylation of the retinoblastoma (Rb) tumor suppressor and its two related pocket proteins, p107 and p130 (8). These phosphorylation events lead to the release of E2fs from Rb-E2f complexes and the accumulation of E2f transcriptional activity late in G\textsubscript{1} to maximally activate the expression of a wide range of E2f-target genes (9). Ablation of murine Rb in adult HSCs leads to increased expression of E2f targets and enhanced proliferation of myeloid lineages, which is significantly exacerbated by the additional loss of p107 and p130 (10,11). In this classic paradigm of cell cycle control, the three activator E2fs (E2f1, E2f2 and E2f3) are invariably viewed as the final effectors of a transcriptional program that commit cells to enter S phase (12). As cells progress through S phase, E2f1-3 protein levels decrease and E2f repressors (E2f4, E2f5, E2f6, E2f7 and E2f8) reload on E2f target promoters and downregulate their expression (13-15). The coordinated oscillatory activation and repression of E2f target gene expression is believed to promote safe passage of cells through cell cycle transitions and provide ample points of control for monitoring appropriate cell proliferation.

Analysis of individual E2f knockout mice has revealed important roles for E2f1-3 in hematopoiesis. For example, E2f1\textsuperscript{-/-} mice have slightly increased number of T cells in the thymus due to impaired apoptosis (16) and E2f1-3 mice have defects during erythroid cell maturation and develop autoimmune diseases late in life due to enhanced proliferation of effector/memory T lymphocytes (17). Combined loss of E2f1 and E2f2 leads to impaired maturation of red blood cells, a condition that resembles megaloblastic anemia in humans, and also leads to decreased B-cell differentiation beyond the pre B-cell stage (18). Hematopoiesis has not been carefully evaluated in E2f3\textsuperscript{-/-} mice, partly due to their increased morbidity (19-21). Given the
importance of E2f activators in the control of cell proliferation, it is peculiar that mice lacking each of these E2fs have relatively normal cell cycles. One explanation for the absence of proliferative defects in these single or double E2f knockout mice is the unprecedented functional redundancy that exists among E2f family members during development (20).

Colony stimulating factor 1 (CSF-1) is a mitogenic ligand required for myeloid cell survival, proliferation and differentiation (22). Original work by Stanley and colleagues demonstrated that binding of CSF-1 to its cognate receptor (CSF-1R) leads to the activation of the Ras/Raf/MAPK pathway and induction of c-Myc expression (23-25). Like E2f, c-Myc is a key transcription factor that stimulates cell growth and cell cycle progression (26). Subsequent studies showed that expression of a mutant form of the CSF-1R lacking the ability to transmit a proliferative signal (CSF-1RY809F) failed to induce c-Myc and cyclin D1 in response to CSF-1 stimulation (27,28). Importantly, enforced expression of these two components were shown to rescue CSF1-mediated proliferation of CSF-1R<sup>Y809F</sup> expressing cells (25,27). More recently, Trumpp and colleagues demonstrated that conditional deletion of c-Myc results in the accumulation of HSCs in the bone marrow and depletion of hematopoietic lineages (29,30). A link between c-Myc and E2fs has long been speculated to be critical in how these two transcription factor families regulate cell proliferation. Earlier cell culture studies had suggested an intimate relationship linking c-Myc to the regulation of E2fs (31-34), however in vivo evidence in support of this remains conspicuously absent.

We exploited the well-defined signaling pathways of hematopoietic differentiation to rigorously examine the in vivo role of E2f1-3 in cell proliferation. Loss of Rb results in myeloproliferation and the fact that E2f1-3 are regulated by Rb (10). We therefore wanted to investigate whether ablation of E2f1-3 would similarly affect cells of the myeloid lineage. Using Mx-cre transgenic mice (35) and a conditional allele of E2f3 (<sup>E2f1</sup>-/-<sup>E2f2</sup>-/-<sup>E2f3</sup><sup>f/f</sup>) we show that E2f1-3 are dispensable for HSC proliferation, but are instead required at two distinct steps of myeloid development. We show that E2f1-3 are required for the survival of myeloid progenitors and subsequently, for the proliferation of committed bone marrow macrophages (BMMs). Interestingly, the survival role of E2fs in myeloid progenitors is associated with a function for these E2fs in transcriptional repression, and the proliferation role in committed BMMs is associated with their function in transcriptional activation. These studies expose and contextualize the dual roles for E2f1-3 in transcription repression and activation in vivo by casting these factors during discrete stages of myeloid differentiation in a defined physiological signaling cascade involving CSF-1 and Myc.

**Experimental Procedures**

**Mice**

E2f<sup>1/-</sup>, E2f<sup>2/-</sup> and c-Myc<sup>fl</sup> mice were a gift from Michael Greenberg, Stuart Orkins and Andreas Trumpp respectively. Generation of E2f3<sup>f/f</sup> mouse has been described previously (21). Eight to ten-week old mice received five injections of 250ug of polyinosine-polycytidine (pIpC) every alternate day and mice were sacrificed 24 hrs after the last injection. All the experimental as well as control mice were injected with pIpC. Adaptive transfer experiments were performed using cells isolated from BM of E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3<sup>f/fl</sup> and Mx-cre;E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3<sup>f/fl</sup> mice and injected into lethally irradiated wild type mice via tail vein. Mice were maintained on antibiotic water for five weeks after the transplant, followed by 5 injections of pIpC as described above. Primers sequences for genotyping are listed in the Supplementary Figure S-5.

**Flow Cytometry**

Bone marrow cells isolated from the femurs of mice were analyzed by FACS. Briefly, single cell suspensions were prepared from the bone marrow, were counted and stained with a panel of fluorochrome conjugated antibodies to determine number of cells in different hematopoietic lineages. For GMP analysis the cells were stained as Lin<sup>-</sup>IL-7Rα Sca-1<sup>-</sup>...
Kit+CD34+FcγRII/III<sup>high</sup>. After staining the cells were analyzed using FACS Calibur using Cell Quest Pro software (Becton Dickinson, BD). Mice were injected with BrdU one hour before harvesting of BM and stained with FITC–BrdU antibody using the Cytofix/Cytoperm kit (BD-552598). For analysis of apoptosis BM cells were stained with FITC-annexin V antibody (BD-556547). BM cells were stained with FITC-conjugated CD11b antibody for sorting CD11b<sup>+</sup> myeloid cell from the BM using FACS Aria system.

Microarray analysis
BM samples were isolated as described previously. Four independent samples from each genetic group were used for gene expression analysis by Affymetrix microarray. RNA was isolated using TRIzol reagent. RNA was then subjected to purification and processed for hybridization to Affymetrix Mouse Genome 430 2.0 Arrays. Genes that increased or decreased at least 1.5-fold (p<0.001) in Mx-cre samples relative to control samples were used to generate the heatmaps illustrated in Figure 2A. Expression values were normalized and log transformed using RMAExpress. RMA normalized data was analyzed using BRB-ArrayTools 3.7.0.

Real-Time RT-PCR
RNA was isolated using Trizol reagent from CD11b<sup>+</sup> cells and MEFs harvested at the indicated time point. To determine the expression of E2Fs in different hematopoietic lineages within the bone marrow, cells from bone marrow were isolated, then stained with lineage specific antibodies (Cd11b, Ter119 and B220) and sorted using FACS. Real time PCR was then performed on RNA isolated from the sorted cells. Reverse transcription of total RNA was performed using Superscript III reverse transcriptase (Invitrogen) and RNase Inhibitor (Roche) according to the manufacturer’s protocol. Real-time PCR was then performed using a BioRad iCycler and reactions were performed in triplicate and relative amounts of cDNA were normalized to RPL4.

ChIP Assays
For ChIP assays, briefly, harvested CD11b<sup>+</sup> cells, BMMs and MEFs were crosslinked and chromatin was sonicated to an average size of 200-1000 bp. Lysates were subsequently pre-cleared with Salmon Sperm DNA/Protein G agarose slurry. Antibodies specific to α-c-Myc (2 ug, SC-262), α -E2F4 (2ug, SC-1082) or α -E2F3 (2ug, SC-878) were then added to each sample and incubated overnight at 4°C. Antibody-protein-DNA complexes were recovered by addition of 60 ul of Salmon Sperm DNA/Protein G agarose slurry and incubation for 1h at 4°C. Following extensive washing, the complexes were eluted and de-crosslinked at 65°C for 4h. Finally, samples were treated with Proteinase K (Roche) and Rnase A (Roche) and purified through Qiagick columns (Qiagen). Real-time PCR quantification of immunoprecipitated DNA was performed using the BioRad iCycler machine with primers specific for the indicated promoter regions. Primer sequences are listed in Figure S5.

Isolation and Culture of BMM
Bone marrow cells isolated were grown in RPMI media containing 50ng/ml of recombinant human CSF-1 (RDI-3025, Fritzgerald) and penicillin–streptomycin antibiotics (Invitrogen) on bacteriological plastic plates for five days. For BrdU incorporation and RT-PCR assays, BMM were synchronized by incubation in RPMI with 0.2% FBS for 20h. Cells were stimulated by the addition of RPMI supplemented with 50ng/ml of recombinant human CSF-1 and harvested at the indicated time points. BMM isolated from E2f1<sup>+/+</sup>2<sup>/+</sup>3<sup>+/+</sup> and E2f1<sup>−/−</sup>2<sup>−/−</sup>3<sup>−/−</sup> were infected with vector or cre-retroviruses.

Cell Culture and Retroviral Infection
NIH-3T3 cells expressing the wild type (T56) and mutant (809) human CSF-1R receptor were used in the study (27). Primary MEFs were made from E13.5 embryos using standard methods. The c-Myc<sup>+/−</sup> cell lines were established using the NIH-3T9 protocol (21). All cells were grown in DMEM with 15% fetal bovine serum. For the production of retrovirus, the full-length cDNAs for CSF-1R,
cre recombinase and a myc-tagged E2f3a were sub-cloned into their respective pBabe-retroviral vectors. High titer retroviruses were produced by transient transfection of retroviral constructs into the Phoenix-Eco packaging cell line as described previously (36). MEFs were infected by incubating the cells with the supernatants containing 4ug/ml of Sequabrene (Sigma) from the transfected cells. Subsequent to infection, cells were grown in selection media containing either 2.5 ug/ml puromycin (Sigma) or 400ug/ml hygromycin (Roche) or both, for 3-5 days. For BrdU incorporation and RT-PCR experiments, sub-confluent MEFs were synchronized by incubation in DMEM with 0.2% FBS or serum free media for 72 hours. Cells were then stimulated to proliferate by the addition of DMEM supplemented with 15% FBS or 100ng/ml of recombinant human CSF-1 and harvested at the indicated time points.

**Proliferation and BrdU Assays**

Colony formation assays were performed by plating 500 cells per 100mm dish. The colonies were fixed with 70% ethanol and stained with 5mg/ml crystal violet in 20% methanol. Colonies from three separate plates at the appropriate density were counted and the mean and standard deviation from one representative experiment is reported unless otherwise stated. For BrdU incorporation assays, serum or CSF-1 stimulated cells were incubated with 50uM BrdU for the indicated time and subsequently fixed with methanol and acetic acid (1:1). Cells were stained with anti-BrdU antibody (Ab-3, Oncogene) as previously described (37) and counter-stained with 4,6-diamidino-2-phenylinole (DAPI). A total of 500 DAPI positive nuclei were scored for each time point.

**Western Blot**

Immunoblot analyses were performed by standard procedures using ECL reagents as described by the manufacturer (Amersham Biosciences). The following commercial antibodies were used: α -E2F1 (SC-193, Santa Cruz), α -E2F2 (SC-633, Santa Cruz), α -E2F3 (SC-878, Santa Cruz) and α -tubulin (T-9026, Sigma).

**Promoter luciferase assays**

NIH-3T3 cell expressing the wild type CSF-1R, mutant CSR-IR (809) receptors and c-Mycfl/fl cells were transfected with the E2f3a and E2f3b luciferase expression vectors, together with renilla as an internal control, as previously described (32). After transfection, cells were brought to quiescence by serum starvation. Cells were stimulated by the addition of 15% serum or 100ng/ml of CSF-1. Cells were harvested at various times after stimulation, and luciferase activity was measured using a luminometer.

**Results**

**E2f1-3 are essential for myeloid cell survival and development**

To explore the potential role for the E2f1-3 transcription factors in hematopoietic development we first examined their expression in the bone marrow of wild type mice. Quantitative RT-PCR assays shown in Figure 1A illustrate the relative expression of E2f1, E2f2, E2f3a and E2f3b in mouse embryo fibroblasts (MEFs) and lymphoid, erythroid and myeloid lineages. This analysis showed that E2f1, E2f2 and E2f3a are highly expressed in all three hematopoietic lineages and that E2f3b expression is significantly restricted to the myeloid lineage. We used null alleles of E2f1 (E2f1−/−) and E2f2 (E2f2−/−), and a conditional allele of E2f3 (E2f3f/f) together with the established Mx-cre transgene to examine the individual and combined roles of the three activator E2fs in vivo. Southern blot and PCR assays showed the efficient Mx-cre mediated deletion of E2f3f/f in bone marrow (BM) cells isolated from polyinosine-polycytidine (pIpC) injected Mx-cre;E2f3f/f and Mx-cre;E2f1−/−E2f3f/f mice (Figure 1B and 1C). The ablation of any single or double combination of activator E2fs had little impact on the total cellularity of bone marrow (BM) or individual hematopoietic cell lineages (Figure 1D and Supplementary Figure S1A). Because of the high degree of functional redundancy among E2fs during embryonic development (20), we examined the consequences of ablating the entire set of activator E2fs (Mx-cre;E2f1−/−E2f2−/−E2f3f/f).
Ablation of \(E2f1-3\) resulted in a 4-fold reduction of BM cells and a corresponding decrease in the number of CD11b\(^+\) myeloid cells (Figure 1D and Supplementary Figure S1B). Consistent with a specific defect in the myeloid differentiation cascade, we observed an accumulation of granulocytic macrophage progenitors (GMPs) (Figure 1D). Whereas PCR and southern blot analysis had shown a near-complete deletion of \(E2f3^{f/f}\) in \(Mx-cre;E2f3^{f/f}\) and \(Mx-cre;E2f1^-/-E2f3^{f/f}\) mice, cells isolated from \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) were only partially deleted (~40%) (Figure 1B), indicating a selection against the expansion of cells lacking these three E2fs.

Given the strict requirement for \(E2f1-3\) in the proliferation of MEFs (21), we analyzed the proliferation of \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) myeloid cells. Surprisingly, we observed a higher percentage of CD11b\(^+\) \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) cells in S phase when compared to all the other groups of mice, as determined by BrdU incorporation and flow cytometry (Figure 1E-1H). In contrast, Annexin V staining revealed massive apoptosis of CD11b\(^+\) cells in \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) mice (Figure 1I and 1J). Transplantation of BM from control and \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) mice into lethally irradiated wild type mice, followed by the standard pIpC injection regimen, resulted in a similar increase of apoptosis and reduction of myeloid lineages in triply deleted mice (Supplementary Figure S2). Collectively, these results suggest that \(E2f1-3\) are dispensable for mammalian cell proliferation \(in\) \(vivo\) and are instead required for the survival of myeloid cells through a cell-autonomous mechanism.

**E2f1-3 represses E2f target genes \(in\) \(vivo\)**

To explore the underlying cause for the observed apoptosis in \(E2f1-3\) deficient myeloid cells, we compared global gene expression profiles in control (\(Mx-cre;E2f1^-/-\)) and \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) CD11b\(^+\) sorted cells. We used an unbiased method similar to Gene Set Enrichment Analysis (38) to identify genes that were differential expressed between the two groups. From the ~45K probes in Affymetrix oligo-arrays, 248 genes were upregulated and 323 were downregulated >1.5-fold (\(p<0.001\)) in the \(Mx-cre;E2f1^-/-E2f2^-/-E2f3^{f/f}\) samples (Figure 2A). Gene ontology pathways revealed that most of the differentially expressed genes identified by this method are involved in DNA replication, mitosis and nucleotide metabolism. Approximately 45 of the 248 upregulated genes have been reported to be E2f responsive and interestingly 66% of these (30/45) have been implicated in promoting the G1/S transition (Figure 2B and Supplementary Table 1), consistent with increased DNA replication in \(E2f1-3\) deficient myeloid cells. Of the 323 downregulated genes, expression of 69 genes has been shown to be E2f responsive and almost half of this group (29/69) have been implicated to be involved in G2/M (Figure 2B, Supplementary Table 1 and 2). Quantitative RT-PCR confirmed the changes in the expression of G1/S and G2/M related target genes (Figure 2C). The majority of promoters in these two classes of G1/S and G2/M related gene targets contained E2f consensus binding sites that were conserved between mouse and human (Figure 2D and 2E). Chromatin immunoprecipitation (ChIP) assays on sorted wild type CD11b\(^+\) cells showed that E2f3 was specifically loaded onto the proximal promoters of both the G1/S and G2/M gene targets (Figure 2F and Supplementary Figure S3A-S3B). Parallel ChIP assays performed on sorted CD11b\(^+\) myeloid cells derived from \(E2f3a^-/-\) and \(E2f3b^-/-\) mice suggested that both isoforms participate in the loading of E2f3 on promoters (Supplementary Figure S3C-S3D).

We then investigated why loss of \(E2f1-3\) led to an upregulation of G1/S genes but a downregulation of G2/M genes. The upregulation of G1/S genes is consistent with the recent demonstration that E2f1-3 function as repressors in the differentiating retina and small intestine of mice (39,40). We hypothesized that the downregulation of G2/M targets in triply deleted myeloid cells may be due to the overcompensation by other ‘professional’ E2f repressors. Expression of the known E2F repressors (\(E2f4\), \(E2f5\), \(E2f6\), \(E2f7\) and \(E2f8\)) was not significantly changed in \(E2f1-3\) deficient CD11b\(^+\) myeloid cells (Supplementary Figure S3E). While E2f4
Expression was not changed in E2f1-3 deleted BM cells it remained possible that there might have been a redistribution of E2f4 protein to E2f1-3 target promoters. Indeed, previous studies in E2f1-3 deleted MEFs have shown that E2f4 is inappropriately recruited to the promoters of E2f1-3 target genes and contributes to cell cycle exit in these cells (41). We thus tested this hypothesis by performing E2f4 ChIP on both G1/S and G2/M targets, using E2f4-specific antibodies on sorted CD11b+ cells derived from control and Mx-cre;E2f1-/-E2f2-/-E2f3f/f mice. These assays showed a significant increase in the specific loading of E2f4 onto G2/M target promoters of E2f1-3 deficient CD11b+ cells (Figure 2G). It would appear that a deficiency of E2f1-3 in myeloid cells can be overcome by the compensatory loading of E2f4 to selected target promoters (G2/M targets), leading to their silencing. How E2f4 is recruited to G2/M regulated promoters but not to G1/S-promoters is not yet clear, but may involve specific interactions with other factors that co-regulate these G2/M targets, and lead to their permanent or more profound repression. We suggest that the upregulation of G1/S genes and downregulation of G2/M genes produce conflicting signals that force E2f1-3 deficient cells to replicate DNA and inhibit mitosis, leading to the accumulation of cells in S phase and the initiation of a massive apoptotic response. From these data we conclude that contrary to their established function in cell culture systems, E2f1-3 function in vivo to repress E2f target gene expression.

E2f1-3 are required for the CSF-1 induced proliferation of BMMs

Because Mx-cre mediated inactivation of E2f1-3 caused an almost complete depletion of the CD11b+ lineage, subsequent differentiation steps potentially regulated by these E2fs could not be investigated further. We thus exploited the ability to differentiate BM cells in culture by the addition of the CSF-1 ligand in order to explore the functions of E2f1-3 in terminally differentiated macrophages (BMMs) (42). To this end, BM cells isolated from the femur of wild type mice were cultured in media containing CSF-1, allowing the expansion of Cd11b+ cells into differentiated BMMs (43). We then examined the ability of growth factor starved BMMs to proliferation in response to re-stimulation with CSF-1. As expected, quantitative RT-PCR assays showed an immediate induction of c-Myc expression that was followed by a marked increase in E2f1, E2f2 and E2f3a expression, a corresponding induction of classic G1/S-regulated E2f target genes (Figure 3A) and the entry of BMMs into S-phase (Figure 3B). The levels of E2f3b mRNA did not change in response to CSF-1, consistent with its known cell cycle-independent expression pattern (37). The introduction of a cre-expressing retrovirus into E2f1-/-E2f2-/-E2f3f/f BMMs resulted in the efficient deletion of the E2f3f/f allele (Figure 3D) and a 3-fold reduction in BrdU incorporation (Figure 3E-3F). Moreover, bright images of E2f1-/-E2f2-/-E2f3f/f deleted BMMs appeared normal, without any effect on cell survival (Figure 3C and data not shown); cre-expression had no effect on BMMs containing a wild type E2f3 allele. These results suggest that proliferation of committed BMMs require E2f1-3 for their ability to activate gene expression and promote their expansion in response to CSF-1 mediated signals.

E2f3a is a downstream target of c-Myc required for cell proliferation

c-Myc is a critical component of the CSF-1 signaling cascade that is necessary for cell proliferation (27). More recent studies have suggested an intimate relationship between c-Myc and the regulation of E2fs (32), however mechanistic details linking the two transcriptional networks remain unknown. To determine whether the CSF-1 induction of E2f1, E2f2 and E2f3a is mediated by c-Myc, we generated and analyzed c-Myc floxed (Mycf/f) BMM. We infected Mycf/f BMM with control- or cre-retroviruses, synchronized in G0 and then re-stimulated with CSF-1. PCR-genotyping confirmed the efficient cre-mediated deletion of the conditional c-Mycf/f alleles (Supplementary Figure S4A). Quantitative RT-PCR analysis showed that the typical CSF-1 mediated induction of E2f1, E2f2 and E2f3a expression which was
abrogated in c-Myc deleted BMM (Figure 4A). To further corroborate our findings in BMM we performed similar experiments in c-Myc floxed mouse embryo fibroblasts (MEFs) overexpressing human CSF-1R (c-Myc<sup>f/f</sup>-CSF-1R), which are responsive to recombinant human-derived CSF-1 but not endogenous murine CSF-1. This cell system can recapitulate molecular events that follow activation of the CSF-1R and can be exploited to elucidate molecular details of these events. In contrast to control-treatment, ablation of c-Myc impaired the ability of cells to enter S-phase in response to either serum or CSF-1 (Supplementary Figure S4B). Similarly, no induction of E2f1, E2f2, and E2f3a expression could be observed in c-Myc deleted cells following CSF-1 stimulation (Figure 4B).

Previous studies have shown that the E2f3 locus plays a particularly important role in the control of cell proliferation and we thus focused our subsequent analysis on the regulation and function of E2f3a (21). To determine whether E2f3a is regulated by c-Myc at the transcriptional level, we analyzed the activity of E2f3a-promoter reporter constructs in c-Myc<sup>f/f</sup>-CSF-1R cells infected with control or cre-expressing retroviruses. These assays revealed that the serum or CSF-1 mediated increase in E2f3a promoter activity was abolished by the cre-mediated deletion of c-Myc (Supplementary Figure S4C). We then addressed whether enforced expression of E2f3a could restore the proliferation of c-Myc-deleted MEFs. Interestingly, over-expression of E2f3a restored the ability of c-Myc-deleted cells to enter S phase in response to CSF-1 but failed to support mitosis and the continued proliferation of these cells as determined by colony formation assays (Supplementary Figure S5A-S5D).

ChIP assays were then used to determine whether c-Myc could directly regulate the expression of E2f3a in BMMs. The E2f3a promoter contains three E-box elements (Myc sites I-III) that can serve as potential binding sites for c-Myc (32). ChIP assays were performed using Myc-specific antibodies and immunoprecipitated DNA was amplified with primers flanking the E-box sites on the E2f3a promoter (Figure 4C). As a positive control, we also assayed the nucleolin promoter, an established direct target of c-Myc (44). The results from these ChIP assays showed that c-Myc in BMMs was specifically recruited to Myc I-III sites on the E2f3a promoter, but not to flanking irrelevant sequences (‘No site’) in response to CSF-1 (Figure 4C). Together, these results substantiate our findings that c-Myc is required for the expression of E2f1-3 and is directly involved in CSF-1 mediated activation of E2f3a.

**E2f1-3 are indispensable for cell growth**

To evaluate the role of E2f1-3 in CSF-1 mediated cellular proliferation, we used a similar strategy as described above to generate E2f3<sup>f/f</sup> and E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3<sup>f/f</sup> cells overexpressing human CSF-1R (E2f3<sup>f/f</sup>-CSF-1R and E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3<sup>f/f</sup>-CSF-1R, respectively). These cells were infected with control- or cre-retroviruses as before, synchronized by serum deprivation and then stimulated by the addition of either serum or CSF-1 to the media. While the cre-mediated inactivation of E2f3 only marginally reduced the ability of cells to enter S phase, the combined loss of E2f1-3 severely compromised S phase entry in response to either serum or CSF-1 (Figure 5A and 5C). Consistent with a defect in S phase entry, E2f1-3 triply-deleted cells failed to form colonies (Figure 5B and 5D). The few colonies that arose in cre-expressing E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3<sup>f/f</sup>-CSF-1R cells retained at least one intact copy of the E2f3<sup>f</sup> allele (Figure 5E). Importantly, the normal serum or CSF-1 mediated induction of c-Myc expression, which occurred ~4 hours post-stimulation, was not affected by the loss of E2f1-3 (Figure 5F). From these results we suggest that E2f1-3 are essential components of the CSF-1 mitogenic signaling pathway that function downstream of c-Myc.

**E2f1-3 are direct transcriptional target of c-Myc**

To further explore how CSF1 mediated signaling leads to the activation of E2f3a we utilized mouse NIH-3T3 cell lines expressing either wild type (T56) or mutant (Y809F) forms of the human CSF-1R receptor that lack...
the ability to activate the expression of c-Myc and transmit CSF-1 mediated proliferative signals (28). As previously shown, both T56 and mutant Y809F cells entered S-phase in response to serum addition, but only T56 3T3 cells responded to CSF-1 (Figure 6A). Quantitative RT-PCR and Western blot analysis showed that the CSF-1-induced entry of T56 cells into the cell cycle corresponded with the early induction of c-Myc and the subsequent increase of E2f1, E2f2 and E2f3a expression as well as in the increase of E2f target gene expression (Figure 6C). In contrast, mutant Y809F cells failed to activate the expression of c-Myc, E2f1, E2f2 and E2f3a and classic E2f target genes in response to CSF-1 (Figures 6B - 6D). The expression of E2f3b and other E2f family members were unaffected by CSF-1 stimulation in either T56 or Y809F cells (Figure 6C and 6D; and data not shown).

The above results raised the possibility that the induction of E2f1, E2f2 and E2f3a by CSF-1 may be directly dependent on c-Myc function. To test this possibility we analyzed a series of E2f3a-promoter reporter constructs in both T56 and Y809F cells. CSF-1 stimulation activated the E2f3a reporter in T56 cells but not in Y809F mutant cells (Figure 7A). The induction of the E2f3a reporter in T56 cells was specific since neither serum nor CSF-1 had any effect on E2f3b-reporter activity. These results suggest that the CSF-1 mediated induction of E2f3a mRNA occurs at the transcriptional level. To test for c-Myc binding, we performed ChIP assays in T56 and Y809F cells using c-Myc-specific antibodies. The results from these ChIP assays showed that c-Myc was loaded on Myc I-III sites on the E2f3a promoter in response to both serum and CSF-1 in T56 cells (Figure 7B and 7C). In contrast, no recruitment of c-Myc was observed in Y809F cells following CSF-1 stimulation, consistent with the absence of c-Myc induction in these cells. These results recapitulate our earlier findings in BMMs suggesting that the CSF-1 mediated activation of E2f3a involves the binding of c-Myc to the E2f3a promoter.

Discussion

In this study we evaluated the role of E2fs in hematopoietic development in vivo by analyzing the consequences of conditionally ablating the entire subclass of activator E2fs in HSCs. We show that E2f1-3 regulates cell survival and proliferation at distinct stages during myeloid development through their dual ability to repress and activate transcription, respectively. During early stages of myeloid development, E2f1-3 are dispensable for the proliferation of CD11b+ cells but are instead required for their survival. Once myeloid precursor cells commit to the macrophage lineage, E2f1-3 provide cells with the ability to respond to CSF-1 signals and allow their expansion prior to exit into the periphery. In summary, these findings contextualize the role of E2f1-3 in cell survival and proliferation to specific developmental stages during the life-span of a myeloid cell.

The coordinated expression and activity of cell cycle regulators are crucial for proper hematopoietic development (1,3,45). HSCs lacking c-Myc accumulate due to their inability to properly differentiate and give rise to hematopoietic lineages (30). Mice lacking D-type cyclins or all three-interphase cdks (cdk2, cdk4 and cdk6) die by mid-late gestation due to defective hematopoiesis attributed to decreased expansion of HSCs (4,5). In contrast, conditional inactivation of Rb in the hematopoietic system leads to myeloproliferation, but interestingly, Rb deficient HSCs and progenitor cells did not display any detectable change in the expression of cell cycle-related genes (10). Here, we show that E2f1-3 are not required for HSC proliferation and differentiation in vivo, but rather are required for the survival of Cd11b+ myeloid cells. The antagonistic phenotypes observed in myeloid cells deficient for either Rb/p107/p130 or E2f1-3 suggest that myeloid development is tightly regulated by the Rb-E2f pathway.

In the textbook model of mammalian cell cycle regulation, the E2f1-3 proteins are viewed as the ultimate effectors downstream of the cdk/Rb pathway that activate a gene expression program that is critical for S phase
entry and cell cycle progression (46). Consistent with this view, loss of E2f1-3 in mouse embryo fibroblasts results in a decrease in the expression of a broad group of E2f targets and a complete cell cycle arrest (21). However, emerging data from mouse and other model organisms indicate that E2f function in vivo is complex and likely not restricted to the control of cell cycle progression (46). This study provides additional evidence that E2f1-3, while dispensable for the proliferation of the majority of hematopoietic lineages, are necessary for myeloid cell survival. Global gene expression analysis of E2f1-3 deficient myeloid cells revealed increased expression of G1/S-related E2f target genes but decreased expression of G2/M-related targets. We speculate that this conflict in G1/S and G2/M gene expression programs forces E2f1-3 deficient myeloid cells to inappropriately initiate DNA replication and accumulate in G2/M, resulting in the induction of apoptotic cell death. The molecular signals eliciting this apoptosis remain to be discovered.

In contrast to the rigid view that E2f1-3 proteins function as transcriptional activators, recent in vivo studies demonstrate that these ‘activators’ can also function as ‘repressors’ in specific developmental contexts (40). For instance, in stem cells of the retina and small intestine, E2f1-3 adhere to their canonical role as transcriptional activators whose function is inhibited through association with Rb (40). But in differentiating epithelial cells of the same tissues, E2f1-3 function in association with Rb as transcriptional repressors (39). From the analysis of tissues deleted for Rb, E2f1-3 or Rb and E2f1-3, it was suggested that phosphorylation of the Rb protein is the molecular event that switch E2f1-3 from functioning as activators in dividing progenitors to repressors in differentiating cells (39). Consistent with studies in the retina and small intestine (39,40), our results in the hematopoietic system of mice reveal that E2f1-3 function as activators or repressors of G1/S regulated genes in a manner that is dependent on the differentiation stage of the cell. Unlike in the small intestine, retina and lens, E2f1-3 in myeloid progenitors appear to play a role in the regulation of both G1/S and G2/M genes.

Mice carrying mutant alleles of C/EBPa that lack the ability to bind E2fs fail to support granulocyte differentiation, suggesting that repression of E2f targets by C/EBPa-E2f complexes is essential for myeloid development (47). In the murine liver, C/EBPa is part of a protein complex containing Rb-E2f4 that binds and represses E2F-responsive gene promoters (48), raising the possibility that in E2f1-3 deficient myeloid cells, C/EBPa and Rb-E2f4 complexes might jointly contribute to the observed overcompensatory repression of G2/M targets in these cells.

As Cd11b+ myeloid precursor cells commit to the macrophage lineage, E2f1-3 provides these cells with the ability to expand in response to CSF-1. CSF-1 signaling is complex and difficult to study in vivo. We therefore used the MEFs and NIH 3T3 cells expressing the wild type and mutant (Y809F) forms of human CSF-1R receptor to study mechanism of E2F regulation in CSF-1 signaling pathway and to explore Myc-E2F connection. Using mutant forms of the CSF-1R and conditional alleles of c-Myc, we show that this ability to expand requires the c-Myc mediated activation of E2f1-3 expression. Engagement of the CSF-1R by its ligand leads to the accumulation of c-Myc protein and it’s recruitment to canonical Myc-binding sites on the E2f3a promoter. The resulting activation of E2f3a expression provide BMMs with the necessary levels of E2f proteins to activate E2f targets and force cells to enter S phase, divide and expand prior to exiting into the peripheral organs. In this context of acute growth factor stimulation, like in previous cell culture studies, E2fs conform to their classical role as transcriptional activators that are rate limiting for cell proliferation.

In summary, our results provide compelling evidence that E2f1-3 are essential for the survival of dividing Cd11b+ precursor cells and the proliferation of terminally differentiated BMMs. Only in the latter circumstance, E2f1-3 assume the role of classic transcription activators to stimulate cellular proliferation.
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Author Contribution
P.T., N.S. and R.O. performed experiments and collected data. C.P. and A.R. technically assisted with experiments. P.T. and G.L. designed experiments, analyzed data and wrote the paper. M.F.R. and M.O. contributed key reagents.
The authors declare no competing financial interests.

Figure Legends:

Figure 1. *E2f1-3 are essential for myeloid development*. Analysis of BM cells isolated from 8-10 week old mice two weeks after pIpC treatment. (A) Real time PCR analysis of expression of *E2f1*, *E2f2*, *E2f3a* and *E2f3b* in FACS sorted cells of different hematopoietic lineages. Bone marrow cells were isolated and stained with lineage specific antibodies (Cd11b, Ter119 and B220) and sorted by FACS. Real time PCR was then performed on RNA isolated from these sorted cells. (B) Southern blot on genomic DNA isolated from BM of Mx-Cre *E2f3*+/−, *E2f1*−/−*E2f3*+/− and *E2f1*+/−*E2f2*+/−*E2f3*+/− mice. A minimum of three samples were analyzed from each genetic group. Two representative samples analyzed from each genetic group are shown. (C) *E2f3* PCR genotyping on genomic DNA isolated from BM of Mx-Cre *E2f3*+/−, *E2f1*−/−*E2f3*+/− and *E2f1*+/−*E2f2*+/−*E2f3*+/− mice. (D) Analysis of cells isolated from femur of mice of the indicated genotype showing bone marrow cellularity (p<0.0003), number of granulocyte-macrophage progenitors (GMPs) (p<0.018) and number of CD11b+ myeloid cells (p<0.0002). Values are mean ± SD. (E) FACS profile of CD11b+ myeloid cells stained with α-BrdU antibody to determine the number of cells in S-phase. (F) Quantification of CD11b+ BrdU positive cells, values are mean ± SD (p<0.0008). (G and H) Analysis of DNA content in CD11b+ cells by PI staining, values are mean ± SD (p<0.003). (I) Representative FACS profile of myeloid cells stained with apoptotic marker annexin V. (J) Quantification of CD11b+ apoptotic cells, values are mean ± SD (p<0.005).

Figure 2. Ablation of *E2f1-3* in BM leads to upregulation of G1/S and downregulation of G2/M genes in Cd11b+myeloid cells. (A) Heatmap showing hierarchical clustering analysis of genes differentially expressed (p<0.001) between Mx-cre;*E2f1*−/−*E2f2*−/−*E2f3*+/− (Mx-cre) and *E2f1*−/− (control). Four independent samples were analyzed from each genetic group. (B) Scatter plots comparing E2F target genes between Mx-cre and *E2f1*−/− (control). Blue dots indicate total number of genes that were changed. Red dots indicate G1/S and S-phase target genes that are up regulated and/or G2/M target genes that are down regulated more than 1.5 fold. (C) Quantitative real-time PCR was performed to compare the relative expression of selected G1/S (top panel) and G2/M (bottom panel) E2F-target genes in control and Mx-cre. The number on the y-axis represents four independent samples from each genetic group. (D) Pie diagram illustrates that majority of E2F target genes are involved G1/S regulation are upregulated in Mx-cre;*E2f1*−/−*E2f2*−/−*E2f3*+/− myeloid cells (left panel). The figure shows percentage of E2F sites conserved between
the human and mouse promoter (right panel). (E) Pie diagram illustrates that majority of down-regulated genes in Mx-cre;E2f1−/−E2f2−/−E2f3f/f (Mx-cre) myeloid cells are E2F targets involved in G1/S and G2/M regulation (right panel). (F) ChIP assay showing E2F3 recruitment on promoters of G1/S genes in wild type myeloid cells. (G) ChIP assay in E2f1−/− (closed bars) and Mx-cre;E2f1−/−E2f2−/−E2f3f/f (open bars) myeloid cells showing E2F4 loading on promoters of G1/S and G2/M genes.

Figure 3. E2f1-3 are required for BMM proliferation. (A) RT-PCR analysis of E2Fs and E2F-target genes on BMM at indicated time points. The values on the y-axis represent fold induction. (B) Bone marrow derived macrophages (BMM) were plated at a density of 1x10⁵ cells/60-mm plate. Cells were grown in a medium containing 50ng/ml CSF-1 and were harvested and counted every 24 h for 4 days (left panel). Cells were harvested for BrdU incorporation. Cells were stained with anti-BrdU antibody and counterstained with DAPI, and BrdU-positive cells were counted as described under "Materials and Methods" (right panel). (C) Bright field images of vector- or cre-treated E2f1−/−E2f2−/−E2f3f/f BMM at 10x magnification. (D) E2f3 PCR genotyping on genomic DNA isolated from cre treated E2f1−/−E2f2−/−E2f3f/f BMM. (E) FACS analysis of BrdU−Cd11b+ cells of indicated genotypes. (F) BMM from E2f1+/+2+/+3+/+ and E2f1−/−2−/−3f/f mice were infected with either vector- or cre- retroviruses. BMM were co-stained with Cd11b and BrdU and analyzed by FACS. The graph depicts percent Cd11b+ cells that are also positive for BrdU+, values are mean ± SD (p<0.006).

Figure 4. c-Myc is required for the activation of E2f1, E2f2 and E2f3a. c-Mycf/f BMM or c-Mycf/f-CSF1R cells were infected with either the control- or cre- retroviruses and serum starved. Quiescent cells were then re-stimulated with media containing serum or CSF-1 and assessed by RT-PCR analysis for target gene expression at the indicated time points. Total RNA was used to measure the expression of the indicated E2F-target genes from (A) c-Mycf/f-BMM and (B) c-Mycf/f-CSF1R cells. The y-axis represents the average fold induction in gene expression, where the level at the 0 hour time point is equal to one. (C) ChIP assays on lysates from wild type BMM using antibodies for c-myc. Immunoprecipitated DNA was measured by real-time PCR using primers flanking the myc sites on the E2f3a promoter. Results are shown as percent of total input at the indicated time upon stimulation of quiescent BMMs with CSF-1. RT-PCR was performed in triplicate and cycle numbers were normalized to 1% of the input DNA.

Figure 5. E2F1-3 are important for cellular proliferation. The E2f3f/f-CSF-1R and E2f1−/−E2f2−/−E2f3f/f–CSF-1R cell lines were infected with either control- or cre- expressing retroviruses and then used for the following assays. (A) BrdU incorporation in E2f3f/f-CSF-1R cell line. Quiescent MEFs with the indicated genotypes were re-stimulated with media containing serum or CSF-1 and assessed for BrdU incorporation at the indicated time points. A total of 500 DAPI-stained nuclei from each cell line were counted, and the percent positive cells for BrdU incorporation are shown. (B) E2f3f/f-CSF-1R MEFs were plated for colony formation assay. Values shown have been corrected for deletion of E2f3 by colony PCR. (C) BrdU incorporation of E2f1−/−E2f2−/−E2f3f/f-CSF1R cell line. The graph shows the percent positive cells for BrdU incorporation. (D) E2f1−/−E2f2−/−E2f3f/f-CSF-1R MEFs were plated for colony formation assay. Values shown have been corrected for deletion of E2f3 by colony PCR. (E) E2f3 PCR genotyping on genomic DNA from the cre-infected E2f3f/f-CSF1R and E2f1−/−E2f2−/−E2f3f/f-CSF-1R colonies grown in the presence of CSF-1. (F) Bar graphs showing fold change in c-Myc expression in control and cre-treated E2f1−/−E2f2−/−E2f3f/f-CSF1R after stimulation with serum (left panel) or CSF-1 (right panel).

Figure 6. CSF-1-specific mitogenic signal leads to the activation of E2f1, E2f2 and E2f3a. (A) BrdU incorporation (right panels) in serum (red) and CSF-1 (blue) stimulated T56 (left panels) and 809 (right panels) NIH-3T3 cells. Quiescent MEFs with the indicated genotypes
were stimulated with media containing serum or CSF-1 and assessed for BrdU incorporation at the indicated time points as described in "Materials and Methods". (B-C) RT-PCR analysis of c-myc, E2F’s and E2F target gene expression. Total RNA was harvested from MEFs treated as in (A) and was used to measure of expression E2F-target genes as described in "Materials and Methods". The y-axis represents the average fold induction in gene expression, where the level at the 0 hour time point is equal to one. (D) Cellular lysates from serum or CSF-1 stimulated cells were used for a Western blot probed with the E2F1, E2F2 and E2F3 antibodies.

Figure 7. The E2f3a promoter, but not the E2f3b promoter is activated by c-Myc. (A) NIH-3T3 cells were transfected with either the E2f3a- or E2f3b-luciferase plasmid, along with thymidine kinase renilla luciferase construct as an internal control. Transfected cells were incubated in low-serum and then stimulated with either serum (red) or CSF-1 (blue). Cells were harvested at the indicated time points and luciferase activity was measured. (B) Schematic representation of the E2f3a promoter showing myc binding sites. (C) ChIP assays on cell lysates from synchronized T56 and 809 MEFs using antibodies for c-myc. Results are shown as percent of total input at the indicated time upon stimulation of quiescent MEFs with serum or CSF-1. RT-PCR was performed in triplicate and cycle numbers were normalized to 1% of the input DNA.
References

1. Weissman, I. (2000) *Cell* **100**, 157-168

2. Cheng, T., Rodrigues, N., Shen, H., Yang, Y., Dombkowski, D., Sykes, M., and Scadden, D. (2000) *Science* **287**, 1804-1808

3. Passegué, E., Wagers, A., Giuriato, S., Anderson, W., and Weissman, I. (2005) *J Exp Med* **202**, 1599-1611

4. Kozar, K., Ciemerych, M., Rebel, V., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R., Akashi, K., and Sicinski, P. (2004) *Cell* **118**, 477-491

5. Malumbres, M., Sotillo, R., Santamaria, D., Galán, J., Cerezo, A., Ortega, S., Dubus, P., and Barbacid, M. (2004) *Cell* **118**, 493-504

6. Cheng, T., Rodrigues, N., Dombkowski, D., Stier, S., and Scadden, D. (2000) *Nat Med* **6**, 1235-1240

7. Park, I., Qian, D., Kiel, M., Becker, M., Pihalja, M., Weissman, I., Morrison, S., and Clarke, M. (2003) *Nature* **423**, 302-305

8. Burkhart, D., and Sage, J. (2008) *Nat Rev Cancer* **8**, 671-682

9. Nevins, J. (2001) *Hum Mol Genet* **10**, 699-703

10. Walkley, C., Shea, J., Sims, N., Purton, L., and Orkin, S. (2007) *Cell* **129**, 1081-1095

11. Viatour, P., Somervaille, T., Venkatasubrahmanyam, S., Kogan, S., McLaughlin, M., Weissman, I., Butte, A., Passegué, E., and Sage, J. (2008) *Cell Stem Cell* **3**, 416-428

12. Trimarchi, J., and Lees, J. (2002) *Nat Rev Mol Cell Biol* **3**, 11-20

13. Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M., and Nevins, J. (2001) *Mol Cell Biol* **21**, 4684-4699

14. Zhu, W., Giangrande, P., and Nevins, J. (2004) *EMBO J* **23**, 4615-4626

15. Giangrande, P., Zhu, W., Schlisio, S., Sun, X., Mori, S., Gaubatz, S., and Nevins, J. (2004) *Genes Dev* **18**, 2941-2951

16. Field, S., Tsai, F., Kuo, F., Zubiaga, A., Kaelin, W. J., Livingston, D., Orkin, S., and Greenberg, M. (1996) *Cell* **85**, 549-561

17. Murga, M., Fernández-Capetillo, O., Field, S., Moreno, B., Borlado, L., Fujiwara, Y., Balomenos, D., Vicario, A., Carrera, A., Orkin, S., Greenberg, M., and Zubiaga, A. (2001) *Immunology* **15**, 959-970

18. Li, F., Zhu, J., Hogan, C., and DeGregori, J. (2003) *Mol Cell Biol* **23**, 3607-3622
19. Humbert, P., Verona, R., Trimarchi, J., Rogers, C., Dandapani, S., and Lees, J. (2000) *Genes Dev* **14**, 690-703

20. Tsai, S., Opavsky, R., Sharma, N., Wu, L., Naidu, S., Nolan, E., Feria-Arias, E., Timmers, C., Opavska, J., de Bruin, A., Chong, J., Trikha, P., Fernandez, S., Stromberg, P., Rosol, T., and Leone, G. (2008) *Nature* **454**, 1137-1141

21. Wu, L., Timmers, C., Maiti, B., Saavedra, H., Sang, L., Chong, G., Nuckolls, F., Giangrande, P., Wright, F., Field, S., Greenberg, M., Orkin, S., Nevins, J., Robinson, M., and Leone, G. (2001) *Nature* **414**, 457-462

22. Stanley, E., Guilbert, L., Tushinski, R., and Bartelmez, S. (1983) *J Cell Biochem* **21**, 151-159

23. Matsushime, H., Roussel, M., Ashmun, R., and Sherr, C. (1991) *Cell* **65**, 701-713

24. Sherr, C., Rettenmier, C., Sacca, R., Roussel, M., Look, A., and Stanley, E. (1985) *Cell* **41**, 665-676

25. Roussel, M., Theodoras, A., Pagano, M., and Sherr, C. (1995) *Proc Natl Acad Sci U S A* **92**, 6837-6841

26. Secombe, J., Pierce, S., and Eisenman, R. (2004) *Cell* **117**, 153-156

27. Roussel, M., Cleveland, J., Shurtleff, S., and Sherr, C. (1991) *Nature* **353**, 361-363

28. Roussel, M. (1994) *J Cell Sci Suppl* **18**, 105-108

29. Laurenti, E., Varnum-Finney, B., Wilson, A., Ferrero, I., Blanco-Bose, W., Ehninger, A., Knoepfler, P., Cheng, P., MacDonald, H., Eisenman, R., Bernstein, I., and Trumpp, A. (2008) *Cell Stem Cell* **3**, 611-624

30. Wilson, A., Murphy, M., Oskarsson, T., Kaloulis, K., Bettes, M., Oser, G., Pasche, A., Knabenhans, C., Macdonald, H., and Trumpp, A. (2004) *Genes Dev* **18**, 2747-2763

31. Leone, G., Sears, R., Huang, E., Rempel, R., Nuckolls, F., Park, C., Giangrande, P., Wu, L., Saavedra, H., Field, S., Thompson, M., Yang, H., Fujiwara, Y., Greenberg, M., Orkin, S., Smith, C., and Nevins, J. (2001) *Mol Cell* **8**, 105-113

32. Adams, M., Sears, R., Nuckolls, F., Leone, G., and Nevins, J. (2000) *Mol Cell Biol* **20**, 3633-3639

33. Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D., and Amati, B. (1996) *EMBO J* **15**, 6595-6604

34. Müller, D., Bouchard, C., Rudolph, B., Steiner, P., Stuckmann, I., Saffrich, R., Ansorge, W., Huttner, W., and Eilers, M. (1997) *Oncogene* **15**, 2561-2576

35. Kühn, R., Schwenk, F., Aguet, M., and Rajewsky, K. (1995) *Science* **269**, 1427-1429

36. Pear, W., Nolan, G., Scott, M., and Baltimore, D. (1993) *Proc Natl Acad Sci U S A* **90**, 8392-8396
37. Leone, G., Nuckolls, F., Ishida, S., Adams, M., Sears, R., Jakoi, L., Miron, A., and Nevins, J. (2000) *Mol Cell Biol* **20**, 3626-3632

38. Mootha, V., Lindgren, C., Eriksson, K., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstråle, M., Laurila, E., Houlistis, N., Daly, M., Patterson, N., Mesirov, J., Golub, T., Tamayo, P., Spiegelman, B., Lander, E., Hirschhorn, J., Altshuler, D., and Groop, L. (2003) *Nat Genet* **34**, 267-273

39. Chen, D., Pacal, M., Wenzel, P., Knoepfler, P., Leone, G., and Bremner, R. (2009) *Nature* **462**, 925-929

40. Chong, J., Wenzel, P., Sáenz-Robles, M., Nair, V., Ferrey, A., Hagan, J., Gomez, Y., Sharma, N., Chen, H., Ouseph, M., Wang, S., Trikha, P., Culp, B., Mezache, L., Winton, D., Sansom, O., Chen, D., Bremner, R., Cantalupo, P., Robinson, M., Pipas, J., and Leone, G. (2009) *Nature* **462**, 930-934

41. Timmers, C., Sharma, N., Opavsky, R., Maiti, B., Wu, L., Wu, J., Orringer, D., Trikha, P., Saavedra, H., and Leone, G. (2007) *Mol Cell Biol* **27**, 65-78

42. Himes, S., Cronau, S., Mulford, C., and Hume, D. (2005) *Oncogene* **24**, 5278-5286

43. Tushinski, R., and Stanley, E. (1985) *J Cell Physiol* **122**, 221-228

44. Greasley, P., Bonnard, C., and Amati, B. (2000) *Nucleic Acids Res* **28**, 446-453

45. Malumbres, M., and Barbacid, M. (2009) *Nat Rev Cancer* **9**, 153-166

46. Chen, H., Tsai, S., and Leone, G. (2009) *Nat Rev Cancer* **9**, 785-797

47. Porse, B., Pedersen TA, Xu, X., Lindberg, B., Wewer, U., Friis-Hansen, L., and Nerlov, C. (2001) *Cell* **107**, 247-258

48. Iakova, P., Awad, S., and Timchenko, N. (2003) *Cell* **113**, 495-506
A. Relative gene expression

B. Western blots

C. Mx-cre

D. Total no. of cells (10^6)

E. E2f1 / E2f2 / E2f3

F. BrdU

G. DNA Content

H. Cell Cycle

I. Annexin V

J. Annexin V
A) c-Myc<sup>fl/fl</sup> BMM

- **E2f1**
  - CSF-1
  - Fold induction in gene expression
  - 0, 14, 0, 14
  - Cre: hrs

- **E2f2**
  - Fold induction in gene expression
  - 0, 14

- **E2f3a**
  - Fold induction in gene expression
  - 0, 14

- **E2f3b**
  - Fold induction in gene expression
  - 0, 14

B) 3T3 c-Myc<sup>fl/fl</sup> – CSF1R

- **E2f2**
  - 0, 18

- **E2f3a**
  - 0, 18

- **E2f3b**
  - 0, 18

C) 

- **MYC**
  - nucleolin
  - Myc I
  - Myc II
  - Myc II+III
  - No site

- % of total input DNA

- 0, 2, 4

- 0, 2, 4

- 0, 2, 4

- 0, 2, 4: hrs
A

**E2f1-Cre**

| Serum | CSF1 |
|-------|------|
| -     | -    |
| 0     | 18   |
| +     | 0    |
| +     | 18   |

% BrdU positive cells

[Graph showing % BrdU positive cells for Serum and CSF1 conditions for E2f1-Cre]

B

**E2f1-Cre**

| Serum | CSF1 |
|-------|------|
| -     | -    |
| 0     | 18   |
| +     | 0    |
| +     | 18   |

% Colony formation

[Graph showing % Colony formation for Serum and CSF1 conditions for E2f1-Cre]

C

**E2f1-Cre**

| Serum | CSF1 |
|-------|------|
| -     | -    |
| 0     | 18   |
| +     | 0    |
| +     | 18   |

% BrdU positive cells

[Graph showing % BrdU positive cells for Serum and CSF1 conditions for E2f1-Cre]

D

**E2f1-Cre**

| Serum | CSF1 |
|-------|------|
| -     | -    |
| 0     | 18   |
| +     | 0    |
| +     | 18   |

% Colony formation

[Graph showing % Colony formation for Serum and CSF1 conditions for E2f1-Cre]

E

**E2f1-Cre**

| Serum | CSF1 |
|-------|------|
| -     | -    |
| 0     | 18   |
| +     | 0    |
| +     | 18   |

% BrdU positive cells

[Graph showing % BrdU positive cells for Serum and CSF1 conditions for E2f1-Cre]

F

**E2f1-Cre**

| Serum | CSF1 |
|-------|------|
| -     | -    |
| 0     | 18   |
| +     | 0    |
| +     | 18   |

% Colony formation

[Graph showing % Colony formation for Serum and CSF1 conditions for E2f1-Cre]
A  

**E2f3a promoter:**

- **T56 3T3**
- **809 3T3**

![](image)

**Relative luciferase activity**

- Hours post-stimulation: 0, 1, 2, 4, 12, 14, 16
- Serum (red bars) vs. CSF1 (blue bars)

---

B  

**E2f3a promoter:**

- MYC I
- MYC II
- MYC III
- No site

---

C  

**Anti c-MYC ChIP:**

- **T56 3T3**
- **809 3T3**

![](image)

**% of total input DNA**

- Serum (red bars) vs. CSF1 (blue bars)

---

**Nucleolin**

- MYC I
- MYC II & III
- No site

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**Trikha Fig 7**
E2F1-3 Are Critical for Myeloid Development
Prashant Trikha, Nidhi Sharma, Rene Opavsky, Andreas Reyes, Clarissa Pena, Michael C. Ostrowski, Martine F. Roussel and Gustavo Leone

J. Biol. Chem. published online November 28, 2010

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