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C/EBPα regulated microRNA-34a targets E2F3 during granulopoiesis and is downregulated in AML with *CEBPA* mutations

Running title: miR-34a is downregulated in C/EBPα mutant AML

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Abstract

The transcription factor CCAAT Enhancer Binding Protein alpha (C/EBPα) is crucial for granulopoiesis and is deregulated by various mechanisms in acute myeloid leukemia (AML). Mutations in the *CEBPA* gene are reported in 10% of human patients with AML. Even though the C/EBPα-mutants are known to display distinct biological function during leukemogenesis, the molecular basis for this subtype of AML remains elusive. We have recently showed the significance of deregulation of C/EBPα regulated microRNA (miR) in AML. In this study, we report that miR-34a is a novel target of C/EBPα in granulopoiesis. During granulopoiesis miR-34a targets E2F3 and blocks myeloid cell proliferation. Analysis of AML samples with *CEBPA* mutations revealed a lower expression of miR-34a and elevated levels of E2F3 as well as E2F1, a transcriptional target of E2F3. Manipulation of miR-34a re-programmes granulocytic differentiation of AML blast cells with *CEBPA* mutations. These results define miR-34a as a novel therapeutic target in AML with *CEBPA* mutations.

Introduction

Acute myeloid leukemia (AML) is characterized by gene mutations, chromosomal aberrations, and epigenetic modifications [1]. Transcription factors have been discovered to be key targets of mutation in AML [2]. CCAAT Enhancer Binding Protein alpha (C/EBPα) is one of the major regulators in granulopoiesis [2]. During granulopoiesis, C/EBPα regulates differentiation at multiple steps, including the transition from the common myeloid progenitor (CMP) to the granulocytic-macrophage progenitor (GMP) [3]. A growing number of studies indicate that C/EBPα is downregulated by various mechanisms in AML, suggesting C/EBPα is a myeloid tumor suppressor [4]. Mutations in the *CEBPA* gene are present in around 10% of AML cases [5]. Reported mutations of *CEBPA* include frame-shift mutations at the N-terminus which result in

the truncated form of C/EBPα (C/EBPα–p30), as well as point mutations at C-terminus [5]. These mutations result in a protein which fails to induce granulopoiesis [6] and has the potential to induce leukemia in mouse models [7,8].

C/EBPα induces myeloid differentiation via two major steps – upregulation of myeloid-specific genes necessary for granulocytic maturation, and inhibition of myeloid cell proliferation [2,9]. Loss of one of these functions results in a block of granulocytic differentiation. Different mechanisms have been reported for C/EBPα-mediated inhibition of cell cycle machinery [4,5]. During granulopoiesis, inhibition of E2F members has been shown as a unique mechanism through which C/EBPα inhibits cell cycle progression [2,5]. Interestingly, loss of C/EBPα-mediated E2F inhibition has been shown to be instrumental in the leukemic transformation process in AML with *CEBPA* mutations [7]. We have recently shown that C/EBPα targets E2F1 via miR-223 and that this pathway is deregulated in different subtypes of AML [10]. We have also reported that mutated C/EBPα (C/EBPα-p30) co-operates with E2F1 to block granulocyte differentiation in AML with *CEBPA* mutations [11]. Given the importance of deregulation of the C/EBPα–E2F pathway in AML, understanding the mechanism of regulation of E2F activity by C/EBPα is critical in the development novel therapeutic agents in AML.

microRNAs (miRNAs) function as key regulators of gene expression programmes [12]. microRNAs control various tumor suppressors and oncogenes, thereby contributing major roles in different steps of carcinogenesis [13]. microRNA-34a (miR-34a) is a widely expressed microRNA and is regulated by the tumor suppressor p53 [14]. miR-34a is downregulated in a variety of tumors [14]. These findings suggest that miR-34a acts as a tumor suppressor in various tissues. miR-34a expression correlates with *CEBPA* mutations in AML [15]. However, there has been no report which shows any specific function of miR-34a in granulopoiesis. We investigated the role of miR-34a in granulopoiesis and in AML with *CEBPA* mutations. Here we report that

C/EBPα directly regulates miR-34a during granulopoiesis. miR-34a blocks myeloid cell cycle progression by inhibiting E2F3. Interestingly, miR-34a was observed to be down-regulated in AML samples with *CEBPA* mutations. We also observed that E2F3 protein levels, as well as protein levels of E2F1, a major transcriptional target of E2F3, were elevated in AML samples with *CEBPA* mutations. Taken together, our study provides evidence that deregulation of the C/EBPα-miR-34a-E2F3 axis forms the molecular basis for AML with *CEBPA* mutations.

Methods

Patient samples

AML blast cells were obtained from the Children's Oncology Group (COG) Myeloid Reference Bank at Fred Hutchinson Cancer Research Center, United States; University Hospital of Munich, Germany; University of Lille Medical School, France and University Hospital of Münster, Germany. The study protocols used for AML patient sample collection were approved by the ethics committees of the participating centers. All patients provided written informed consent in accordance with the Declaration of Helsinki. Mononuclear cells from bone marrow were enriched by Ficoll gradient centrifugation. Human umbilical cord blood samples were collected after full term delivery with informed consent of the mothers from University Hospital of Halle, Germany. Hematopoietic CD34+ cells were isolated from cord blood samples using CD34+ selection kit as described [16]. After isolation of CD34+cells, percentage of cells positive for CD34 antigen was analysed by FACS analysis using PE-conjugated mouse anti-human CD34 antibody (BD Bioscience) and found to be around 82%.

Molecular Analysis

All AML samples were karyotyped in accordance with the guidelines of the International System for Human Cytogenetic Nomenclature as described [17]. Mutation screening was performed using a polymerase chain reaction amplification of coding region of *CEBPA*, as described [18].

Cell cultures

K562-C/EBPα-p42-ER, K562-C/EBPα-p30-ER, K562-C/EBPα-BRM2-ER and K562-ER cells were maintained in RPMI 1640 without phenol red supplemented with 10% charcoal treated fetal bovine serum, 1% penicillin-streptomycin and 2 μg/ml puromycin [6]. Kasumi-6 cells were cultured in RPMI 1640 supplemented with 20% fetal bovine serum, 1% penicillin-streptomycin and 2 ng/ml GM-CSF [19]; AML blast cells were cultured in IMDM supplemented with 20% fetal bovine serum, 1% penicillin-streptomycin and 20 mM HEPES; NB4 cells were cultured in RPMI supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin.

For differentiation experiments, CD34+ cells were seeded at 1X 10^5/ml in IMDM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and cultured for 16 days in the presence of a cytokine cocktail: Days 1-5: rhSCF (50 ng/ml), FLT3L (100 ng/ml), IL-3 (5 ng/ml), GM-CSF (5 ng/ml), and G-CSF (30 ng/ml); Days 5-8: IL-3 (5 ng/ml) and G-CSF(30 ng/ml); Days: 8-16: G-CSF(30 ng/ml). On days 5, 8, 11, and 14, cells were harvested, washed, and expanded with fresh media supplemented with cytokines.

For differentiation of K562-C/EBPα-ER cell lines, cells ($1x10^6$) were induced with 5μM β-estradiol (Sigma) dissolved in ethanol. NB4 cells ($1x10^6$) were induced by the addition of 1μM of retinoic acid (Sigma) dissolved in DMSO.

RNA isolation and miRNA detection

Total RNA from cells was extracted by using the Trizol Method. The miRNA quantification was done with the TaqMan miRNA Detection Kit (Applied Biosystems) using 100ng of RNA in a

Rotor-Gene RG-3000 cycler (Corbett Research, Australia) by the comparative C_T method using U6 expression for normalization. Corresponding RT and PCR primers for miRNA-34a, miR-223, miR-181a and U6 were obtained from Applied Biosystems. All reactions were performed in triplicate.

DNA constructs and cloning

The luciferase vector containing wild type and mutant E2F3 3'UTR has been published [20]. miR-34a promoter #1 (PM-34a-K1/2) was published before [21]. miR-34a promoter constructs (#2 to #5) were amplified from human genomic DNA and cloned into KpnI and XhoI digested pGL3 basic vector. Primer sequences are provided in supplemental Table 1. miR-34a promoter construct #6 was generated using site directed mutagenesis of the miR-34a promoter construct #4 vector by using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Primer sequences are provided in supplemental Table 1.

Lentiviral vector expressing miR-34a was reported before [21]. miR-34a-pCDNA construct has been published [22].

Luciferase assays

To test whether miR-34a directly targets E2F3, Kasumi-6 cells were transiently transfected with 0.7 μg of the E2F3 3'UTR reporter constructs (wild type and mutant), 0.1 μg of Renilla construct and 0.2 μg miR-34a-pCDNA by Effectene transfection reagent (Qiagen) as described by the manufacturer. Firefly luciferase activities from the promoter constructs and Renilla luciferase activity from the internal control plasmid were determined 12 and 24 hour after transfection using the Dual-Luciferase Reporter Assay System (Promega). Values were normalized by using Renilla luciferase.

To find the regulation of miR-34a promoter by C/EBPα, Kasumi-6 cells were transiently transfected with 0.7 μg of the miR-34a promoter constructs (pri-miR-34a promoters #1 to #6), 0.1

μg of Renilla construct and 0.2 μg control or C/EBPα-pCDNA3 vector by Effectene transfection reagent. Promoter activity was done as described before.

Lentiviral transfections

Lentiviral transfections were done as reported before [21].

Cell cycle analysis

For cell cycle analysis, Kasumi-6 cells were transfected with control or miR-34a-pCDNA vectors. 2 days later, cells were fixed in cold ethanol, washed, re-suspended in PBS containing 50 μg/ml Propidium iodide (PI) and 50 μg/ml RNAase A, and analysed by flow cytometry on a FACScan (Becton Dickinson).

Chromatin Immunoprecipitation

The crosslinking of proteins to DNA was accomplished by the addition of 1% formaldehyde for 10 minutes to cultured cells at 37°C. After sonication, the chromatin was immunoprecipitated with 5 μg of anti-C/EBPα and anti-IgG (Santacruz) antibodies at 4°C overnight. Primer sequences are provided in supplemental Table 2.

Cell Differentiation

Cell differentiation was assessed by light microscopy morphological examination of Wright-Giemsa stained cytospins; quantitative real-time PCR (Q-RT-PCR) for G-CSFR and M-CSFR; and FACS analysis using PE-conjugated mouse anti-human CD11b, CD14 and CD41 antibodies (BD Bioscience) as described [11].

Annexin-V assay

The Annexin-V assay was carried out in conjunction with 7-AAD staining according to the manufacture protocol (BD Pharmingen).

Statistical analysis

We used Student's t-tests to determine the statistical significance of experimental results. A *P* value of 0.05 or less was considered significant. The results were represented as the mean ± s.d from three independent experiments.

Results

C/EBPα-p42 upregulates miR-34a during granulopoiesis

Recent work has shown that miR-34a expression correlates with *CEBPA* mutations in AML [15]. To assess the role of miR-34a in granulopoiesis, we analysed expression levels of this microRNA during differentiation of cord blood derived CD34+ hematopoietic progenitor cells (see Methods for details). CD34+ cells were cultured in the presence of a sequential cytokine cocktail which has been shown to induce granulopoiesis [16]. We observed granulocytic differentiation of CD34+ cells as evaluated by morphology and granulocytic marker (Supplemental figure 1). miR-34a expression was analysed by real-time polymerase chain reaction (RT-PCR). We observed a gradual increase of miR-34a expression during granulocytic differentiation and maturation (Figure 1A). We also analysed the expression levels of miR-223, a microRNA which has been shown to be upregulated; and miR-181a, which has been shown not to be regulated during granulopoiesis [23]. As reported before, we found that miR-223 expression increases during granulocytic differentiation without any upregulation in the expression levels of miR-181a (Figure 1 B, C). To further understand the regulation of miR-34a during granulopoiesis, we analysed its expression during retinoic acid induced differentiation of NB4 cells. As shown in Figure 1D, we observed that retinoic acid is able to induce the expression of miR-34a. Interestingly, the miR-34a levels return to basal levels after 2 days. This is similar to miR-223 levels in NB4 cells during RA induced granulopoieisis[10]. This suggests that during RA induced

differentiation, myeloid microRNAs are upregulated only at earlier time points. In order to understand the regulation of miR-34a by C/EBPα proteins, we used an inducible cell line model K562-C/EBPα-ER. We selected this cell line since these cells do not have endogenous C/EBPα and have been reported as a good model for granulocytic differentiation in the context of C/EBPα proteins [6]. K562-C/EBPα-ER cell lines were established by stably transfecting K562 cells with a plasmid encoding an estrogen inducible C/EBPα (p42 form as well as mutants) estrogen receptor fusion protein [6]. We observed that β-estradiol treatment of K562-C/EBPα-p42-ER cells induces granulocytic differentiation (Supplemental figure 2). Our data suggest that induction of C/EBPα leads to upregulation of miR-34a expression in K562-C/EBPα-p42-ER cells (Figure 1E, top). The induction of miR-34a was found to be high at 6 hours, which returns to basal levels at later time points. We have observed a similar pattern of regulation of miR-223 in K562-C/EBPα-p42-ER cells[10]. C/EBPα induction is able to differentiate K562-C/EBPα-p42-ER cells in a very short time intervals (4 days)[6] . So, this pattern of expression of miR-34a could be associated with C/EBPα expression since C/EBPα is not expressed at terminal stages of granulopoiesis. We did not observe any upregulation of miR-34a in the control cell line, K562-ER (Figure 1E, bottom). *CEBPA* mutations comprise N-terminal frame shift mutations and C-terminal point mutations (Supplemental figure 3). We next investigated how these mutants regulate miR-34a. We observed that these two mutants (N-terminal mutant, K562-C/EBPα-p30-ER and C-terminal mutant, K562-C/EBPα-BRM2-ER) fail to upregulate miR-34a (Figure 1F). These data suggest that upregulation of miR-34a during granulopoiesis is a specific function of C/EBPα-p42.

To better understand the direct regulation of miR-34a by C/EBPα-p42, we next examined putative C/EBPα binding sites in the promoter region of miR-34a using Transcription Element Search Software (www.cbil.upenn.edu/tess). We found several C/EBPα-p42 binding sites in the upstream region as well as in the intronic region of miR-34a (data not shown). Interestingly, the

C/EBPα-p42 binding site in the first intronic region of miR-34a was found to be conserved in the human, mouse and rat (Figure 1G). To identify the C/EBPα-p42 binding site necessary for miR-34a expression, we cloned several genomic fragments of miR-34a into a promoter-less luciferase reporter plasmid. The different constructs were co-transfected with C/EBPα-p42 in Kasumi-6 cells, a myeloid leukemia cell line established from the bone marrow cells of a patient having AML with *CEBPA* mutation [19]. Analysis of luciferase activity in these cells showed that C/EBPα is able to transactivate miR-34a (miR-34a#1 to miR-34a#4, Figure 1H and Figure S4; miR-34a#1, Figure S5). Removal of the C/EBPα-p42 binding site by truncation or mutation abolished transcriptional activity (Figure 1H and Figure S4, #5 and #6). We conclude that C/EBPα-p42 binding site located in the first intron of miR-34a as the critical binding site for C/EBPα-p42. Since p53 regulates miR-34a through a distal element [24], we analysed miR-34a transactivation by C/EBPα-p42 in this region. We did not observe any transactivation of miR-34a by C/EBPα-p42 in this region (data not shown).

We next investigated whether C/EBPα-p42 directly binds to the miR-34a element, which we found to be necessary for C/EBPα-p42 regulation in promoter assays. To answer this, we performed chromatin immunoprecipitation (ChIP) experiments in K562-C/EBPα-p42-ER cells. Cells were treated with β-estradiol for various time points and chromatin fragments were immunoprecipitated with an anti-C/EBPα antibody. DNA from the immunoprecipitates were amplified by PCR using primers (oligos#1) located near C/EBPα binding site as well as primers located further upstream of the transcription start site (oligos#2). C/EBPα-p42 was found associated at the region which encompasses oligos#1 (Figure 1I). We found that C/EBPα binding at miR-34a promoter is high at 4 and 6 hours, which correlates with miR-34a expression levels in the same cell line (Figure 1E, top). We did not find any binding of C/EBPα-p42 when genomic region upstream of the transcription start site (oligos#2) were PCR amplified (Figure 1I). Taken

together, these data suggest that C/EBPα-p42 interacts with miR-34a genomic region in vivo and that this binding correlates with the expression of miR-34a.

E2F3 is a direct target of miR-34a during granulopoiesis.

Even though E2F3 has been shown to be a target of miR-34a [25], there has been no report which delineates how miR-34a regulates E2F3 during granulopoiesis. miR-34a binding site in E2F3 is located in the 3'UTR region and is conserved in humans, mice, and rats (Figure 2A, B). Since inhibition of E2F activity is a major pathway for myeloid cell proliferation by C/EBPα-p42 [2,5], we hypothesized that E2F3 is the most significant target of miR-34a in granulopoiesis. To find whether E2F3 is a potential target of miR-34a during granulopoiesis, we conducted luciferase reporter assays in Kasumi-6 cells with construct having E2F3 3` untranslated region (UTR) of the E2F3 gene with a putative miR-34a binding site (E2F3 3'UTR WT, Figure 2C). A control vector containing a mutated miR-34a binding site was also generated (E2F3 3'UTR mut). Overexpression of miR-34a leads to decrease in luciferase activity of the E2F3 3'UTR (Figure 2D). miR-34a did not affect the reporter activity of the vector containing mutated miR-34a binding site, suggesting miR-34a specific inhibition of E2F3 (Figure 2D). To validate E2F3 as a target of miR-34a, we analysed E2F3 protein levels during overexpression of miR-34a in Kasumi-6 cells. miR-34a overexpression leads to marked downregulation of E2F3 protein levels (Figure 2E). Since C/EBPα-p42 directly regulates miR-34a (Figure 1) and miR-34a targets E2F3, we analysed the potential of C/EBPα proteins in regulating E2F3 protein levels. Induction of C/EBPα-p42 is able to downregulate E2F3 protein levels (Figure 2F). The finding that E2F3 levels are downregulated at earlier time points correlates with our finding that miR-34a is upregulated at earlier time points in K562-C/EBPα-p42-ER cells (Figure 1E, top). Meanwhile mutants of C/EBPα (C/EBPα-p30 and C/EBPα-BRM2) fail to downregulate E2F3 protein levels

(Figure 2F). Collectively, these data suggest that miR-34a regulation by C/EBPα-p42 plays a critical role in the regulation of E2F3 protein levels during granulopoiesis.

miR-34a is downregulated in AML with *CEBPA* mutations

Since miR-34a is directly regulated by C/EBPα-p42, we hypothesized that this microRNA could be downregulated in AML with *CEBPA* mutations. To understand the regulation of miR-34a in AML, the expression levels of miR-34a was quantified in diagnostic samples of AML samples. Genetic and morphological features of AML samples used are shown in Table 1. We found a significant downregulation of miR-34a expression in AML samples with *CEBPA* mutations in comparison to AML samples without *CEBPA* mutations (Figure 3A). These results confirm the significance of miR-34a regulation during granulopoiesis and in AML with *CEBPA* mutations. *CEBPA* gene mutations in AML are reported as mono-allelic and bi-allelic and characterised by distinct gene expression patterns [26]. However we did not observe any correlation between distinct *CEBPA* mutations and downregulation of miR-34a in patient samples with *CEBPA* mutations. Expression levels of miR-34a were found to be lowest in CD34+ cells (Figure 3A).

Since E2F3 is a potential target of miR-34a (Figure 2) and miR-34a is downregulated in AML with *CEBPA* mutations (Figure 3A), we hypothesized that E2F3 is overexpressed in AML with *CEBPA* mutations. We analysed E2F3 protein levels by western blot analysis in AML samples. We observed that E2F3 protein is overexpressed in AML samples with *CEBPA* mutations (Figure 3B). This shows an inverse correlation between miR-34a and E2F3 in AML with *CEBPA* mutations. Since E2F3 is demonstrated to regulate E2F1 gene at transcriptional level [27], we hypothesized that overexpression of E2F1 might occur as a consequence of miR-34a downregulation in AML with *CEBPA* mutations. To verify this we analysed E2F1 protein by western blot analysis in AML samples. We observed overexpression of E2F1 protein (Figure 3B) in AML samples with *CEBPA* mutations.

miR-34a inhibits myeloid cell proliferation

C/EBPα-p42 has been shown to inhibit cell proliferation [28,29]. Since miR-34a is regulated by C/EBPα-p42 during granulopoiesis (Figure 1) and miR-34a targets E2F3 (Figure 2), we hypothesized that miR-34a could regulate myeloid proliferation. To answer this, we overexpressed miR-34a in Kasumi-6 cells. We observed that miR-34a overexpression in Kasumi-6 cells inhibited myeloid cell proliferation (Figure 3C). We also observed that miR-34a can impede myeloid cell proliferation in U-937 cells (Figure S6). To better understand the regulation of myeloid cell proliferation by miR-34a, we analysed cell cycle profiles during miR-34a overexpression in Kasumi-6 cells. miR-34a was overexpressed in Kasumi-6 cells and cell cycle profile was analysed 2 days after miR-34a overexpression by propidium iodide staining. We found that miR-34a is able to block transition from G0/G1 to S phase of cell cycle progression as shown by accumulation of cells in the G0/G1 phase and reduction of cells in the S phase during miR-34a overexpression (Figure 3D, E). It is important to mention that C/EBPα-p42 displays a similar effect in the regulation of cell proliferation [29]. Our data suggest that miR-34a could have a major role in the inhibition of myeloid cell cycle machinery by C/EBPα-p42. Taken together, these findings show that miR-34a acts as a tumor suppressor in AML with *CEBPA* mutations.

Overexpression of miR-34a in AML blast cells leads to granulopoiesis.

Since our data indicate that miR-34a is a major player during granulopoiesis which is downregulated in AML with *CEBPA* mutations, we hypothesized that overexpression of miR-34a could induce differentiation in AML blast cells isolated from AML patients with *CEBPA* mutations. To verify this, we overexpressed miR-34a via lentiviral vectors as reported before [21] in primary blast cells isolated from with AML patients with *CEBPA* mutations (14 and 11 of Table 1). As determined by GFP expression, efficient transduction with both control and miR-34a lentiviral was obtained in AML blast cells (49%-50% and 56%-61%, respectively, data not

shown). The overexpression of miR-34a resulted in granulocytic differentiation of AML blast cells as assessed by morphology and by the increased expression of myeloid markers such as CD11b and G-CSFR (Figure 4 A- D). We did not observe any upregulation in the expression levels of CD14, M-CSFR and CD41 (Figure 4 E, F and data not shown), suggesting miR-34a overexpression specifically leads to granulocytic differentiation in AML blast cells with *CEBPA* mutations. Since miR-34a has been shown to induce apoptosis[14], we analysed the ability of miR-34a in apoptosis induction in myeloid cells. Kasumi-6 cells were transfected with control or miR-34a vector and analysed apoptosis 24 hours later. Our data shows that miR-34a did not induce apoptosis in myeloid Kasumi-6 cells (Figure S7). Taken together, our data suggests that miR-34a expression has major functions in the myeloid differentiation and manipulation of miR-34a levels can re-establish myeloid differentiation in primary AML blast cells with *CEBPA* mutations.

Discussion

An emerging number of studies show the complex and heterogeneous picture of the development of acute myeloid leukemia. Recent studies suggest that simple loss of a critical regulator is not the direct cause for AML; rather, it is the modulation of functions of such critical players that plays an important role in leukemogenesis. Deregulation of C/EBPα function has been shown to be a major event in several different steps of leukemogenesis [2,4]. Most of the patients having *CEBPA* mutations carry mutations in both alleles – one allele with N-terminal mutation and one with C-terminal mutation. However, homozygous C- or N-terminal mutations are reported rarely in AML [30]. This suggests that both mutations are needed for the development of leukemia. Interestingly, the N-terminal *CEBPA* mutation has been reported to pre-dispose the development of the C-terminal mutation [31]. Also, patients with *CEBPA* mutations retain the same mutation signature at the time of diagnosis and relapse [32,33]. This suggests that *CEBPA* mutations

are early events during leukemogenesis. Even though the complete absence of C/EBPα has been shown to result in block of granulocytic differentiation in mouse models, these animals do not develop leukemia [34]. This indicates that complete loss of C/EBPα function cannot contribute to the development of AML. Rather specific modulation of basal C/EBPα function is necessary for leukemia development. Recent reports show that C/EBPα-mutant proteins can induce leukemia and possess different leukemogenic properties which are instrumental in the development of AML [7,8]. However, despite the relevance of C/EBPα mutants in the induction of AML, little is known how these mutations affect myeloid cell cycle machinery, which could have profound functions in myeloid cell cycle progression, differentiation, and development of AML. The complex nature of *CEBPA* mutations in AML indicates the necessity to elucidate the molecular mechanisms behind different stages of leukemogenesis.

It is becoming clear that deregulation of C/EBPα-mediated myeloid cell cycle regulation plays a crucial role in the development of AML [8,7]. During granulopoiesis, C/EBPα inhibits E2F activity through its C-terminal domain, which mediates direct protein-protein interaction with E2F, as well as its N-terminal domain which mediates E2F repression [6,35-37]. Disruption of any of these functions of C/EBPα results in accumulation of myeloid progenitors and block of granulocytic differentiation [6,35,36]. Interestingly, the major mutations reported for C/EBPα are distributed in the N- and C- terminal domains, signifying the relevance of these domains of C/EBPα in leukemogenesis. These C/EBPα mutants fail to induce granulopoiesis and are defective in inhibiting myeloid cell proliferation [6]. The N-terminal mutant, C/EBPα-p30, possesses the lineage commitment function of C/EBPα [7]. However, this mutant form fails to control proliferation of myeloid progenitor cells and induces AML [7]. The C-terminal mutant increases the proliferation of long term hematopoietic stem cells (LT-HSC), which results in expansion of

premalignant HSC and induces AML [8]. These studies show the importance of proper regulation of myeloid progenitor proliferation as the turning point in leukemogenesis.

Our data show that C/EBPα mutants fail to induce miR-34a (Figure 1F) and miR-34a is down-regulated in AML with *CEBPA* mutations (Figure 3A). Since E2F3 is a target of miR-34a (Figure 2) and miR-34a controls myeloid proliferation (Figure 3C-E), lack of miR-34 results in accumulation of E2F3 and E2F1 (Figure 3A,B). This suggests that lack of miR-34a could result in the aberrant proliferation of myeloid progenitors. This could be one explanation for the increased HSC expansion observed in *CEBPA* mutation. Further studies are needed to show the relationship between miR-34a deregulation and the expansion of the myeloid progenitor pool in leukemogenesis in the setting of *CEBPA* mutation. Emerging evidence indicates that leukemia-initiating mutations happen in hematopoietic stem cells (HSC) and result in a population of preleukemic HSCs or preleukemic committed progenitors which have aberrant self-renewal capacity [38]. Loss of C/EBPα results in enhanced self-renewal of HSC and overexpression of Bmi-1[3]. Bmi-1 has been shown to increase self-renewal and enhanced repopulating activity of HSCs [39]. Interestingly, *BMI1* is a target gene of E2F1 and is upregulated in certain tumors [40]. These findings show the relevance of our finding that E2F1 as well as E2F3 are overexpressed in AML with *CEBPA* mutation. Whether E2F3 or E2F1 overexpression during *CEBPA* mutations can confer self-renewal to HSCs or committed progenitors awaits further experiments.

E2F-family transcription factors are important players in cell cycle progression and cancer [41]. They are comprised of 8 members, of which E2F1-E2F3 are considered transcriptional activators and E2F4-E2F8 are considered transcriptional repressors [41]. E2F activators have been shown to regulate genes necessary for G1 to S phase transition and DNA replication [41]. E2F3 has been shown to have a crucial role in cell proliferation [42]. The E2F3 transgenic mouse shows development of spontaneous skin tumors [43]. Since E2F3 is one of the key regulators of the *E2F1*

gene [27], overexpression of E2F3 could also affect E2F1 status in the cell. The E2F1 transgenic mouse displays impaired terminal differentiation of megakaryocytes, which results in thrombocytopenia [44]. Deregulated E2F3 and E2F1 expression is reported in a variety of cancers including lung, breast, ovary and prostate [41]. Also, E2F1 transgenic mice are predisposed to the development of spontaneous tumors [45]. Amplification of either the E2F1 or the E2F3 gene locus has been demonstrated as a frequent event in different cancers [41]. All these findings suggest that deregulation of E2F3 as well as E2F1 could play major roles in the pathogenesis of various tumors. Distinct E2F members have been shown to play important roles in lymphomagenesis [46]. E2F1 has been shown to block granulopoiesis [47]. We have recently shown that E2F1 cooperates with C/EBPα-p30 in upregulating PIN1, an oncoprotein upregulated in AML with *CEBPA* mutations [11]. We have also reported that E2F1 transcriptionaly represses miR-223, a key microRNA regulated by C/EBPα during granulopoiesis [10]. E2F1 regulates c-Myc, which has been shown to block C/EBPα [48]. All these findings together with our data showing that E2F3 and E2F1 are overexpressed in AML with *CEBPA* mutation (Figure 3B), underlines deregulation of E2F function by C/EBPα as instrumental in AML with *CEBPA* mutations.

miR-223 is another microRNA involved in granulopoiesis [23]. We have shown that this C/EBPα-regulated microRNA could target E2F1 during granulopoiesis [10]. How ever, *miR-223* null animals did not display a complete block of differentiation [49]. This suggests other microRNAs could be compensating partially for miR-223 function during the absence of miR-223. Further experiments using genetic models are needed to show the relative contribution of microRNAs including miR-223 and miR-34a in granulopoiesis. miR-34a is a well characterized microRNA in various tissues and is controlled by the tumor suppressor p53 [14]. miR-34a is downregulated in a variety of tumors including neuroblastoma, lung cancer, colorectal cancer [14]. Even though this microRNA has been shown to be involved in chronic lymphoid leukemia [50] and megakaryocytic

development [21], little is known about this microRNA in granulopoiesis. Our study reveals novel insights into the function of miR-34a in granulopoiesis. In summary, we propose a model in which miR-34a plays a critical role in regulating the myeloid differentiation programme orchestrated by C/EBPα (Figure 5, top). This could result in downregulation of E2F3, which in turn inhibits myeloid cell proliferation, resulting in granulopoiesis. In AML with *CEBPA* mutations, loss of function of C/EBPα results in a block of miR-34a regulation and lack of inhibition of E2F3, which results in increased proliferation of myeloid progenitors (Figure 5, bottom). Our data demonstrate deregulation of the C/EBPα-miR-34a-E2F3 axis as a critical phenomenon in AML with *CEBPA* mutations. Manipulation of miR-34a could offer novel treatment strategies in AML with *CEBPA* mutations.

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Authorship

Contribution: J.A.P. and P.S.P. performed experiments and analysed the data; P.A.H., C.P., O.N., S.M., S.B. and C.M.T. provided AML patient samples; P.S.P., V.D., P.A.H., C.P., C.M.T. and D.G.T. commented on the manuscript; M.C. discussed the data; J.A.P. wrote the manuscript and designed the research; G.B. supervised the work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Table 1. Genetic and morphologic characteristics of AML patient samples used for microRNA-34a analysis and Western blot analysis.

Patient No.	Morphology by FAB	% Blasts in bone marrow	Karyotype	*CEBPA* mutation status	Age (years)	WBC count (10E9/L)	*FLT3* mutation status
1	M1	95	Normal	Nil	70	12.3	Positive
2	M2	60	Normal	Nil	60	1.8	Negative
3	M4	80	Normal	Nil	79	27.0	Negative
4	M5	95	Normal	Nil	35	174.5	Positive
5	M4	90	Normal	Double	45	105.0	Negative
6	M2	85	Normal	Double	64	759.0	Positive
7	M2	90	Normal	Double	63	308.0	Negative
8	M4	55	del(12)(p11p12),t(17;18)(q24;,q11)[20]	Single	35	16.4	Negative
9	M2	55	Normal	Double	56	60.9	Negative
10	M1	81	Normal	Double	12	29.5	Negative
11	M2	65	Normal	Double	16	9.1	Negative
12	M2	72	Normal	Single	51	45.0	Negative
13	Unclassified	86	Normal	Double	9	37.3	Negative
14	M2	47	Normal	Double	6	19.8	Negative
15	M2	81	Normal	Double	5	23.4	Negative
16	M1	94	Normal	Double	12	160.6	Negative
17	M4	58	Normal	Double	14	122.7	Negative
18	M1	70	Normal	Double	11	20.4	Negative
19	Unknown	Unknown	Normal	Double	34	32.0	Positive
20	M2	85	Normal	Double	48	72.0	Negative
21	M1	85	Normal	Double	61	20.1	Negative
22	M2	58	Normal	Single	72	4.9	Negative
23	M1	Unknown	Normal	Single	73	34.3	Positive
24	M2	72	Normal	Single	51	45.0	Negative
25	M2	49	Normal	Double	31	4.3	Negative

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Figure Legends

Figure 1. C/EBPα-p42 regulates miR-34a during granulopoiesis

(A-C) Hematopoietic CD34+ cells were cultured as discussed in Methods. Total RNA was isolated at different time points and analysed by quantitative real time RT-PCR with oligos for miR-34a (A), miR-223 (B) and miR-181a (C). Data are represented as mean ± s.d. from three independent experiments. * $P < 0.05$.

(D) NB4 cells were induced with retinoic acid (1 μM) for respective time points. Total RNA was analysed by quantitative real time RT-PCR with oligos for miR-34a. Data are represented as mean ± s.d. from three independent experiments. * $P < 0.05$.

(E) K562-C/EBPα-p42-ER cells and K562-ER cells were induced with ß-estradiol (5 μM) for respective time points. Total RNA was analysed by quantitative real time RT-PCR with oligos for miR-34a. Data are represented as mean ± s.d. from three independent experiments. * $P < 0.05$.

(F) K562-C/EBPα-p30-ER cells and K562-C/EBPα-BRM2-ER cells were induced with ß-estradiol (5 μM) for respective time points. Total RNA was analysed by quantitative real time RT-PCR with oligos for miR-34a. Data are represented as mean ± s.d. from three independent experiments.

(G) Schematic representation of the miR-34a genomic region and phylogenic conservation of genomic region in the first intron of miR-34a in human, mouse and rat. The conserved region is shown by sequences in grey box and C/EBPα site is shown in bold letters.

(H) Luciferase reporter assays were performed in Kasumi-6 cells using indicated reporters and C/EBPα. Cells were transfected with corresponding firefly luciferase vectors, Renilla luciferase reporter construct as control vector or C/EBPα-pCDNA3 vector. Luciferase activity was measured 24 hours later. Bars represent promoter activity for the corresponding vectors. Data are represented as mean ± s.d. from three independent experiments. * $P < 0.05$.

(I) Chromatin derived from K562-C/EBPα-p42-ER cells was immunoprecipitated with anti-anti-C/EBPα and IgG antibodies. Recovered DNA was PCR amplified with primers specific for C/EBPα binding amplicon (oligos#1) and the non binding amplicon (oligos#2).

Figure 2. E2F3 is a direct target of miR-34a during granulopoiesis.

(A) Schematic representation of miR-34a binding site in the human E2F3 3'UTR. The numbers (+2730 to +2736) represents the nucleotides relative to the termination codon of human E2F3. (B) Conservation of miR-34a binding site in E2F3 3'UTR in human, mouse and rat genomes.

(C) Sequences of predicted miR-34a binding site of E2F3.

(D) Luciferase assays in Kasumi-6 cells transfected with E2F3 3'UTR constructs (wild type and mutant) and miR-34a-pCDNA. Bars represent luciferase activity for the corresponding vectors. Data are represented as mean ± s.d. from three independent experiments. * $P < 0.05$.

(E) Kasumi-6 cells were transfected with control and miR-34a-pCDNA vectors. Total protein was analysed by Western blot analysis with anti-E2F3 antibody. Values below the gel image indicate the E2F3 protein levels normalized to β-tubulin.

(F) K562-C/EBPα-p42-ER, K562-ER, K562-C/EBPα-p30-ER and K562-C/EBPα-BRM2-ER cells were induced with ß-estradiol (5 μM) for respective time points. Total protein was analysed by Western blot analysis with anti-E2F3 antibody. Values below the gel image indicate the E2F3 protein levels normalized to β-tubulin.

Figure 3. miR-34a functions as a Tumor Suppressor in AML with *CEBPA* mutations

(A) Quantitative real time RT-PCR for miR-34a was carried out using bone marrow cells derived from AML patients. Values were normalized with U6. (AML-NK: AML with normal karyotype; CB: cord blood; PB: peripheral blood). Data are represented as mean from three experiments.

(B) Western blot analysis for E2F3 and E2F1 were carried out using bone marrow cells derived from AML patients. Values below the gel image indicate the E2F3 and E2F1 protein levels normalized to Actin.

(C) Growth curve of Kasumi-6 cells transfected with control or miR-34a.pCDNA vectors. Data are represented as mean ± s.d. from three independent experiments.

(D) Flow cytometry of propidium iodide stained Kasumi-6 cells transfected with control or miR-34a.pCDNA vectors from a representative experiment.

(E) Cell cycle profile of Kasumi-6 cells two days after transfection with control or miR-34a.pCDNA vectors. Data are represented as mean ± s.d. from three independent experiments.

* $P < 0.05$; ** $P < 0.001$.

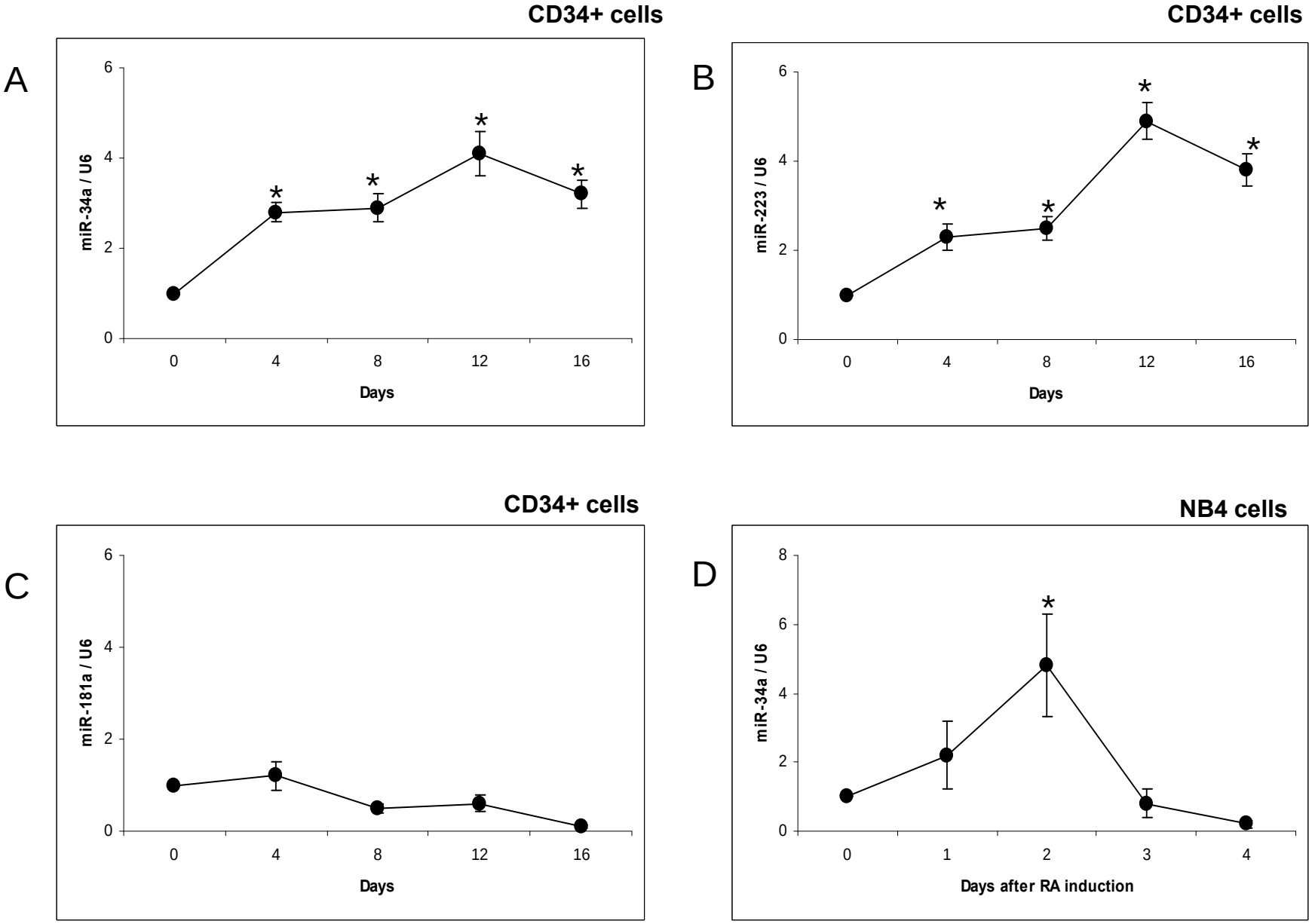
Figure 4. Overexpression of miR-34a in AML blast cells leads to granulopoiesis.

Bone marrow cells derived from AML patients with *CEBPA* mutation (14 and 11 of Table 1) were transfected with control or miR-34a lenti viral vectors. Cells were cultured for 6 days and collected for morphological, immunophenotypic, and myeloid marker expression analysis. (A) Morphologic analysis by light microscopy of Wright Giemsa stained AML blast cells. (B) miR-34a expression levels in blast cells transfected with lenti viral miR-34a vector in comparison with control vector as analysed by Real-time RT-PCR analysis. (C, E) CD11b (C) and CD14 (E) expression levels in blast cells transfected with lenti viral miR-34a vector in comparison with control vector as analysed FACS analysis. (D, F) G-CSFR (D) and M-CSFR (F) expression levels in blast cells transfected with lenti viral miR-34a vector in comparison with control vector as analysed by Real-time RT-PCR analysis. Data are represented as mean ± s.d. from three independent experiments. * $P < 0.05$.

Figure 5. Schematic representation of regulation of granulopoiesis and AML by C/EBPα-miR-34a-E2F3 axis. During granulopoiesis (top panel), C/EBPα transactivates miR-34a, which in turn leads to E2F3 repression and inhibition of cell cycle progression resulting in myeloid differentiation. During *CEBPA* mutations in AML (bottom panel), low activity of C/EBPα fails to

transactivate miR-34a, which results in lack of E2F3 inhibition. Overexpressed E2F3 together with E2F1 could accelerate myeloid cell cycle progression and results in block of granulocytic differentiation.

Figure 1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C
CD34+ cells
miR-181a / U6
Days
D
NB4 cells
miR-34a / U6
Days after RA induction

Figure 1

E

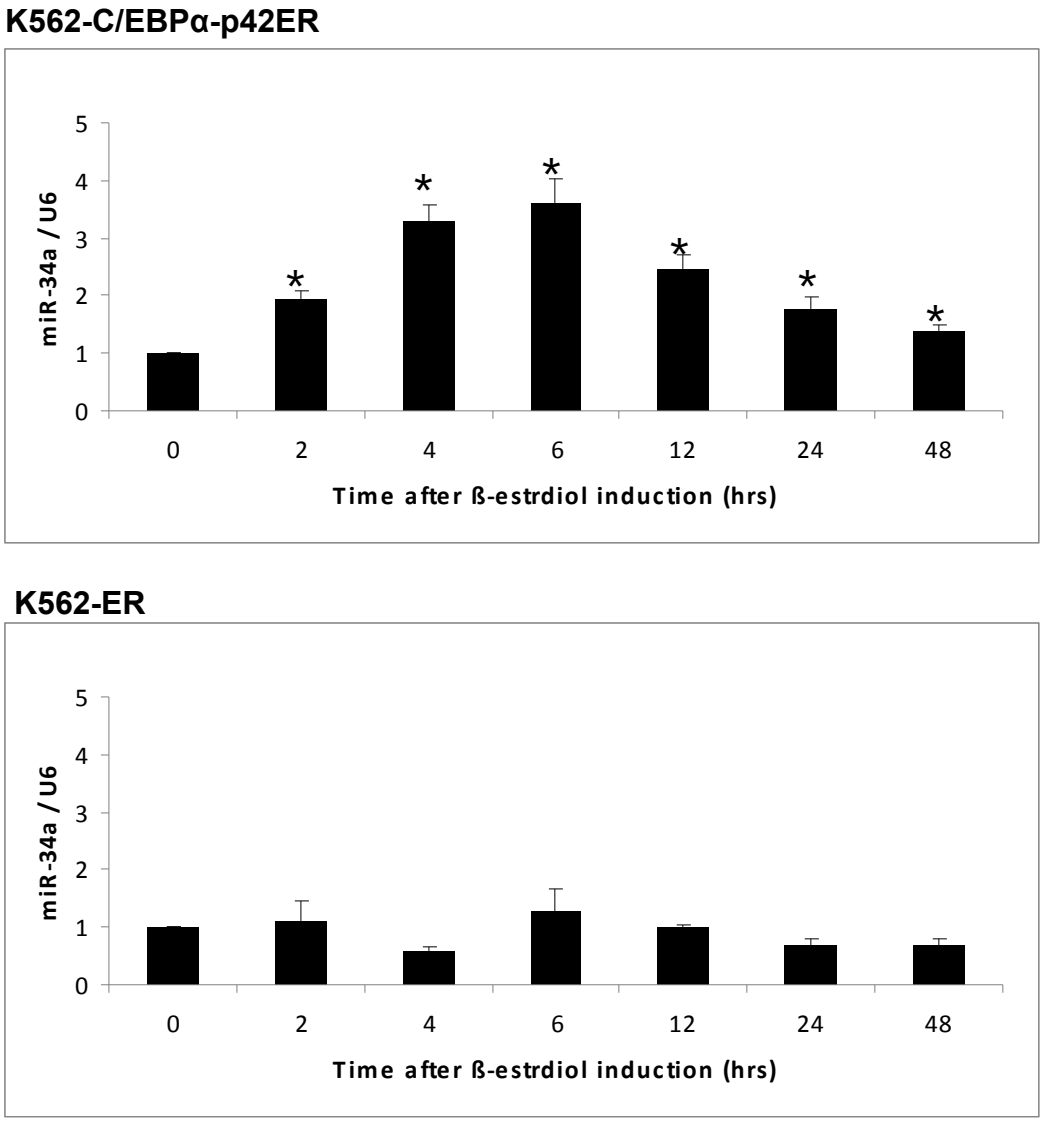
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Figure 1

F

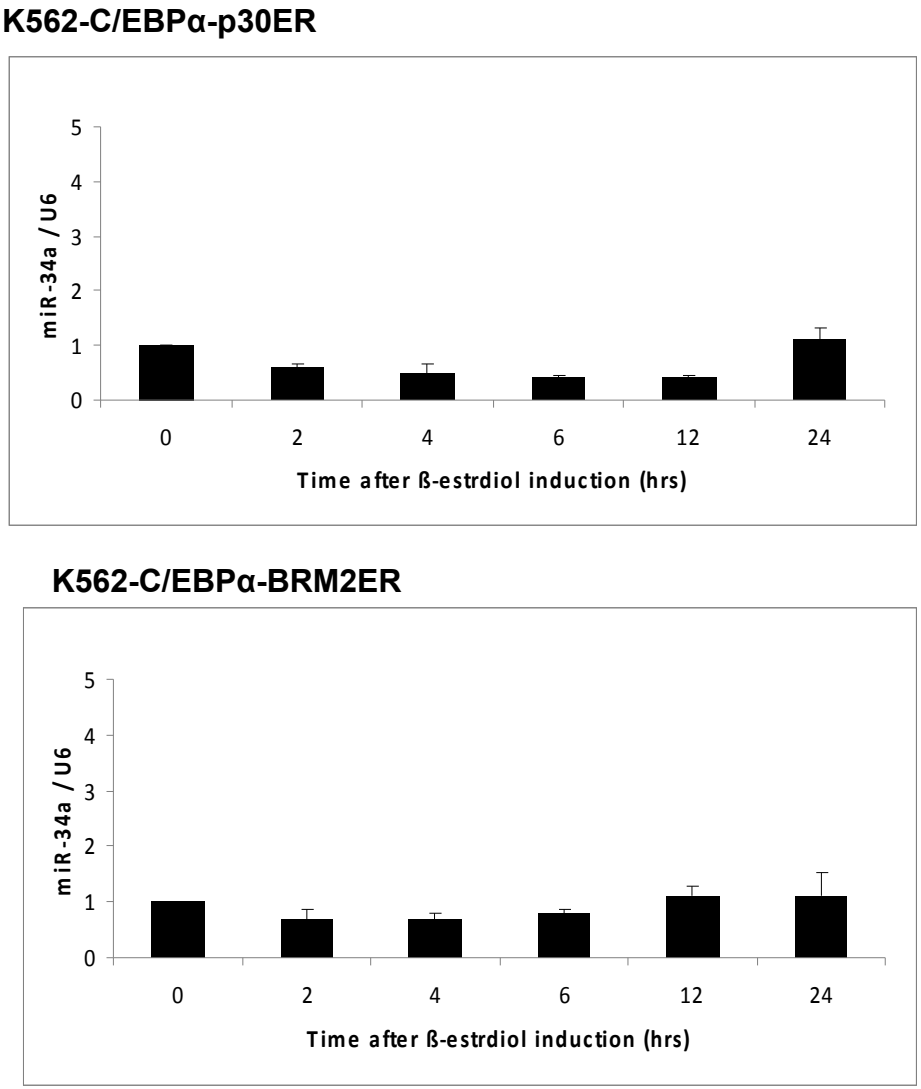
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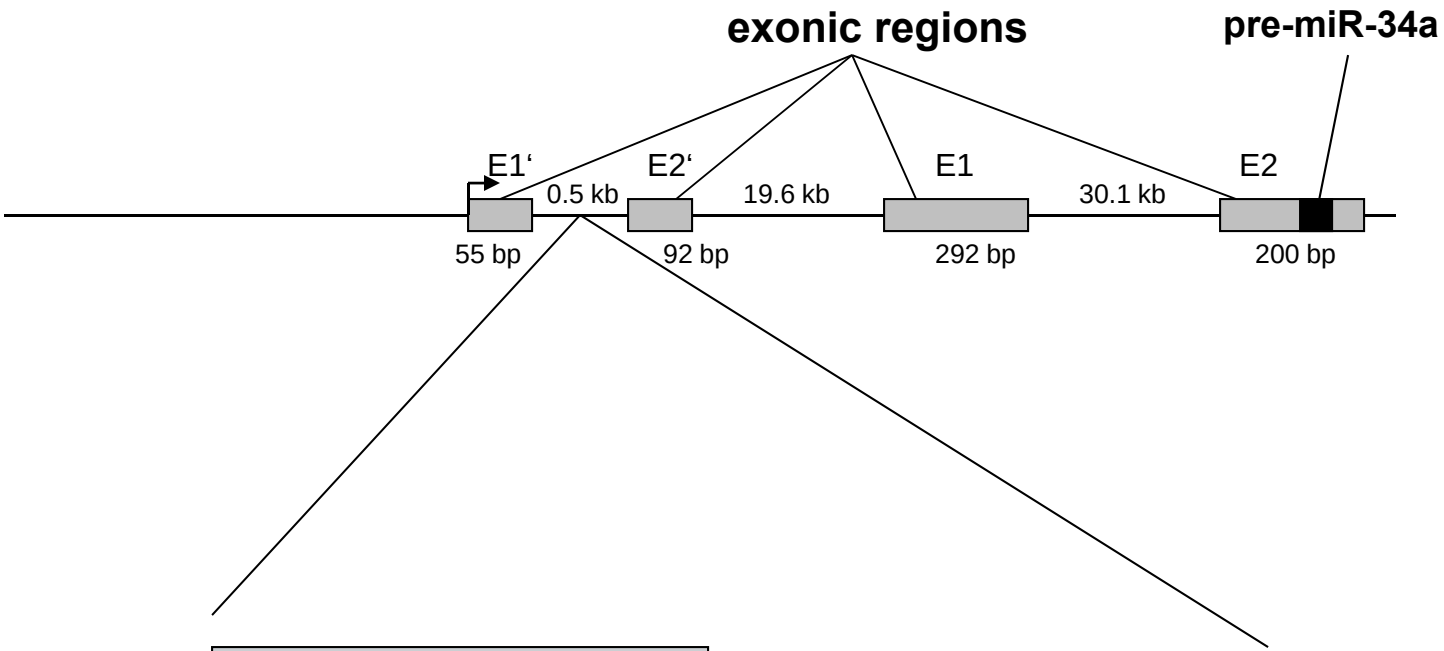
Figure 1

G



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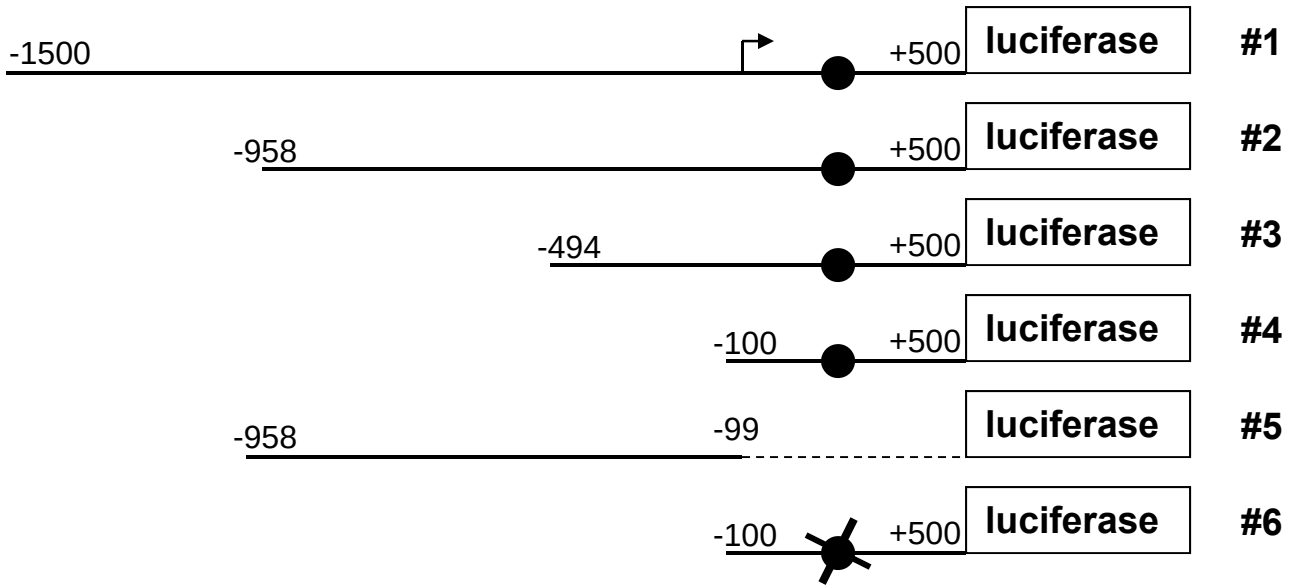
M.musculus AAAACTACTCC**ATTGC**AGTCTCCGCTGCAGTCC
R.norvegicus CAAACTACTCC**ATTGC**GGTCTTCGCTGCAGTCC

Figure 1

H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luciferase
#6

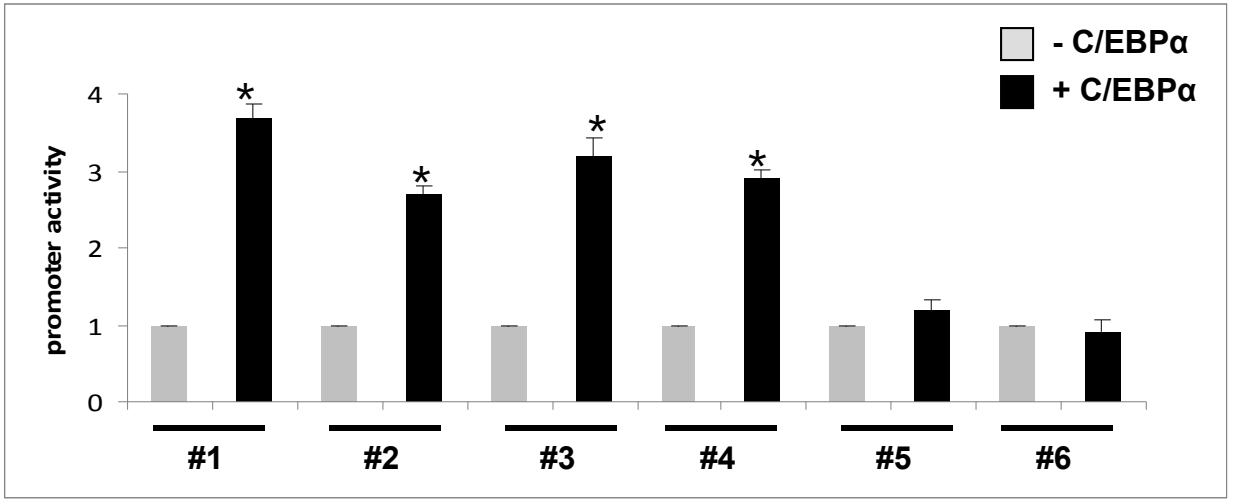
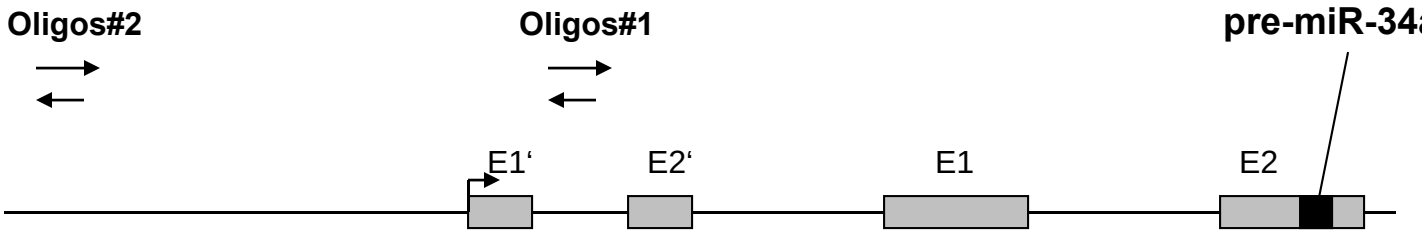

- C/EBPα
+ C/EBPα
promoter activity
0
1
2
3
4
*
*
*
*
#1
#2
#3
#4
#5
#6

Figure 1


Oligos#2
Oligos#1
pre-miR-34a
E1‘
E2‘
E1
E2

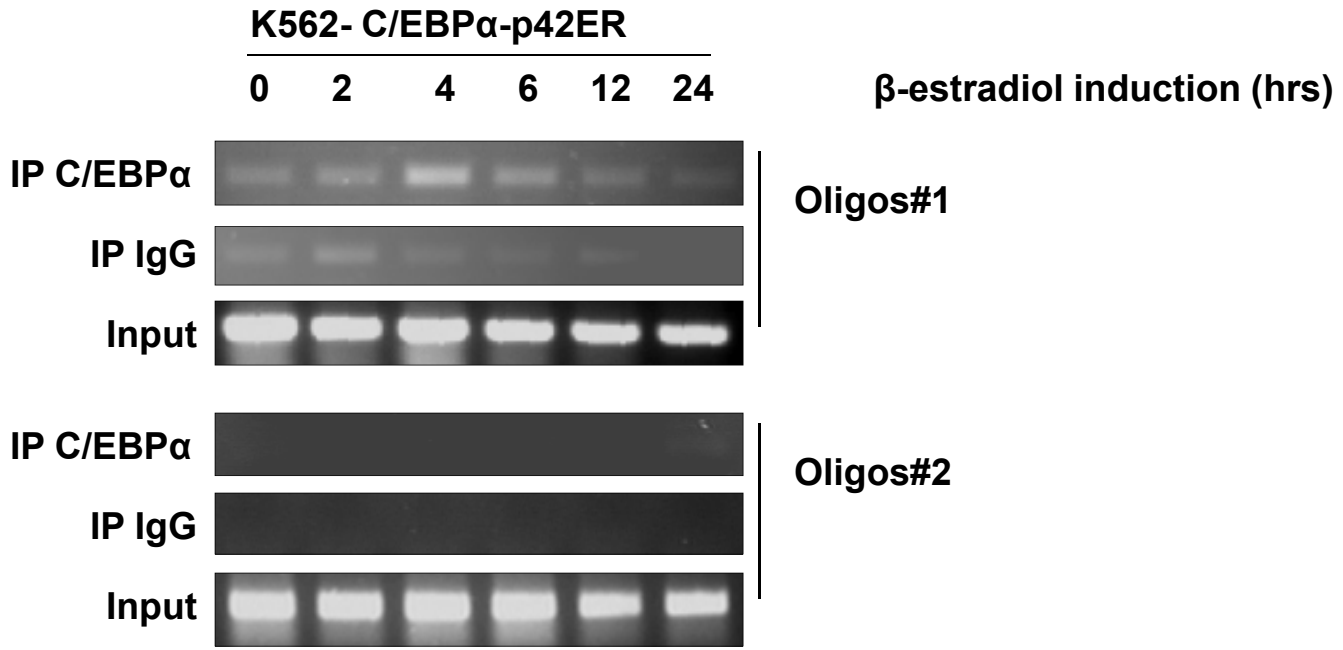

K562- C/EBPα-p42ER
0 2 4 6 12 24
β-estradiol induction (hrs)
IP C/EBPα
IP IgG
Input
Oligos#1
IP C/EBPα
IP IgG
Input
Oligos#2

Figure 2

A

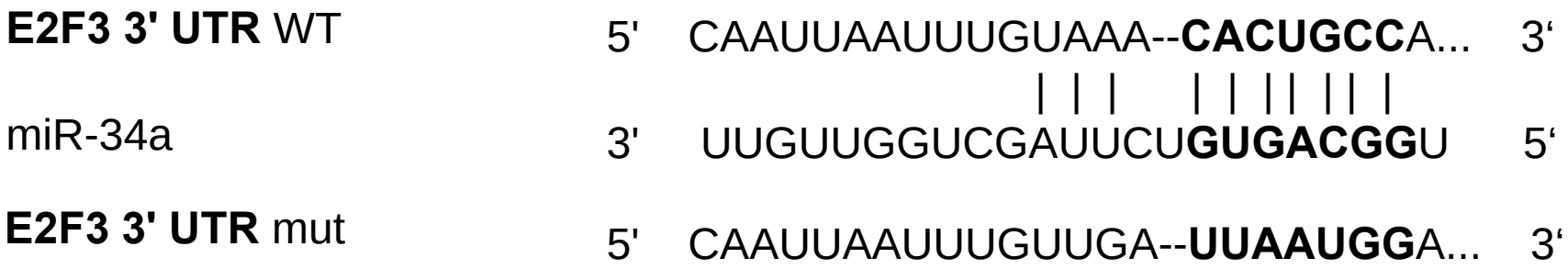
B

H.sapiens	UUAAUUUGUAAA**CACUGCCA**GAAUACUUUCU
M.musculus	UUCAUUUGUAAA**CACUGCCA**GAAUAUUUUCU
R.norvegicus	UUCAGUUGUAAA**CACUGCCA**GAAUAUUUUCU

miR-34a site

Figure 2

C


E2F3 3' UTR WT
5' CAAUUAAUUUGUAAA--CACUGCCA... 3'
miR-34a
3' UUGUUGGUCGAUUCUGUGACGGU 5'
E2F3 3' UTR mut
5' CAAUUAAUUUGUUGA--UUAAUGGA... 3'

D

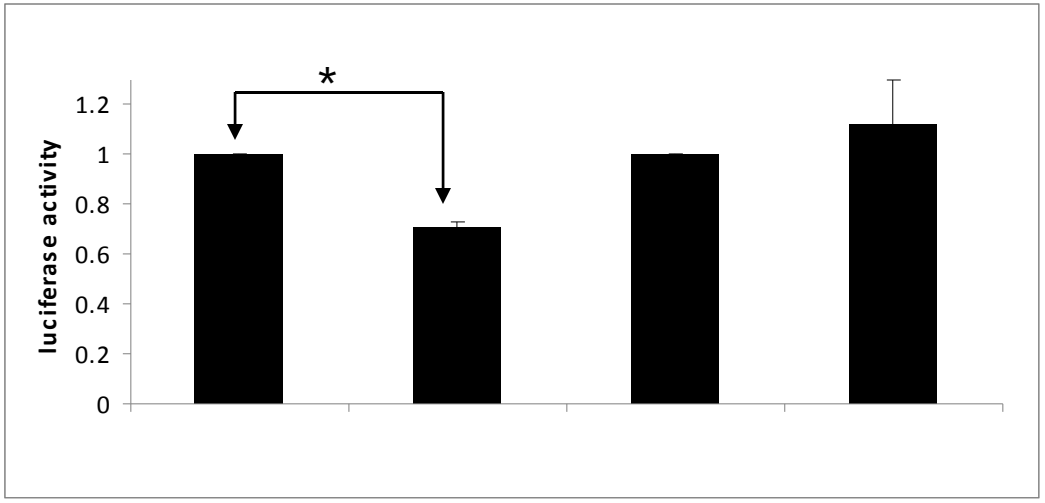

*
luciferase activity
1.2
1
0.8
0.6
0.4
0.2
0
E2F3 3'UTR WT + +
E2F3 3'UTR mut + +
miR-34a-pCDNA + +

Figure 2

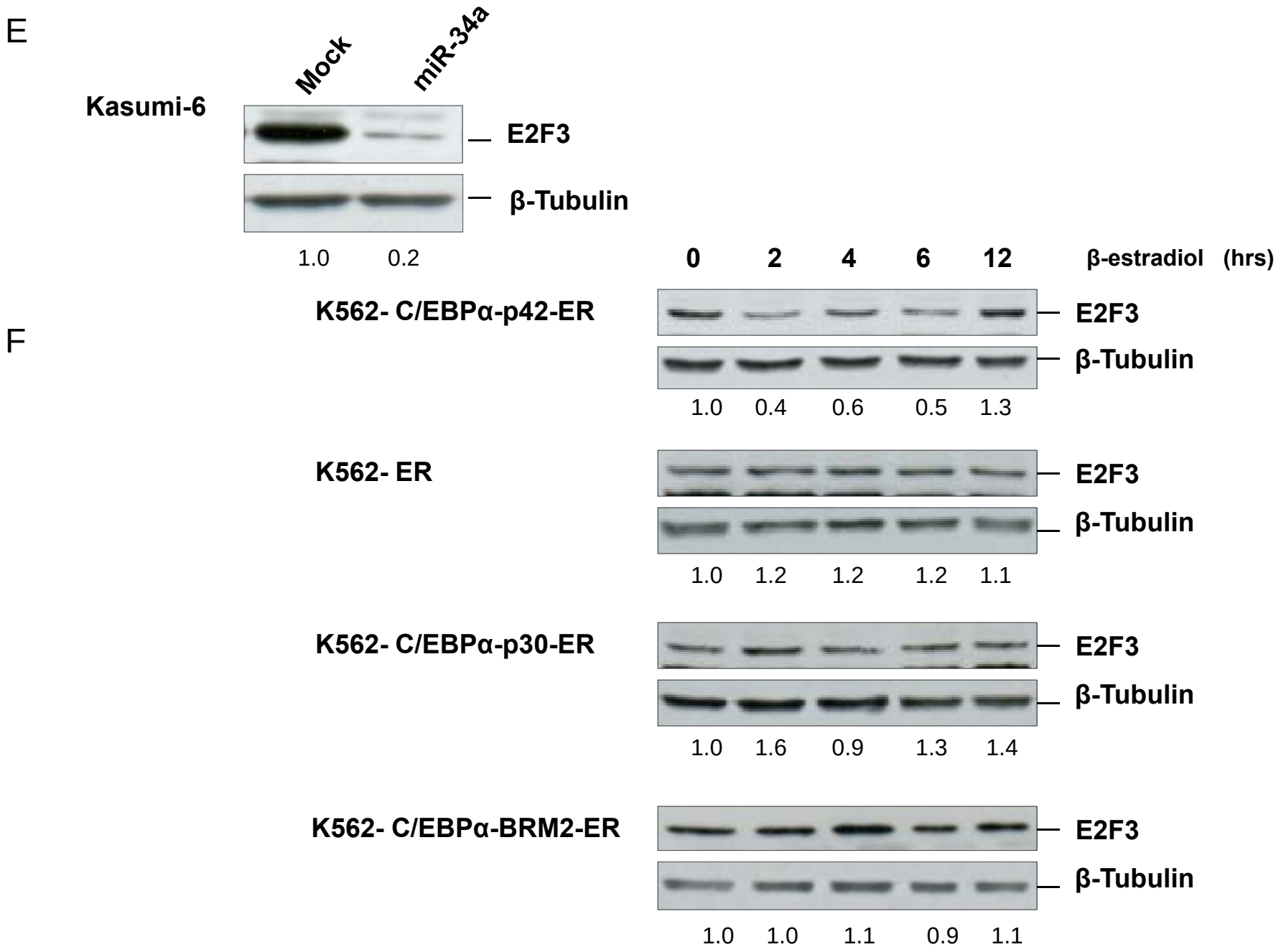

E
Mock
miR-34a
Kasumi-6
E2F3
β-Tubulin
1.0
0.2
F
0
2
4
6
12
β-estradiol (hrs)
K562- C/EBPα-p42-ER
E2F3
β-Tubulin
1.0
0.4
0.6
0.5
1.3
K562- ER
E2F3
β-Tubulin
1.0
1.2
1.2
1.2
1.1
K562- C/EBPα-p30-ER
E2F3
β-Tubulin
1.0
1.6
0.9
1.3
1.4
K562- C/EBPα-BRM2-ER
E2F3
β-Tubulin
1.0
1.0
1.1
0.9
1.1

Figure 3

A

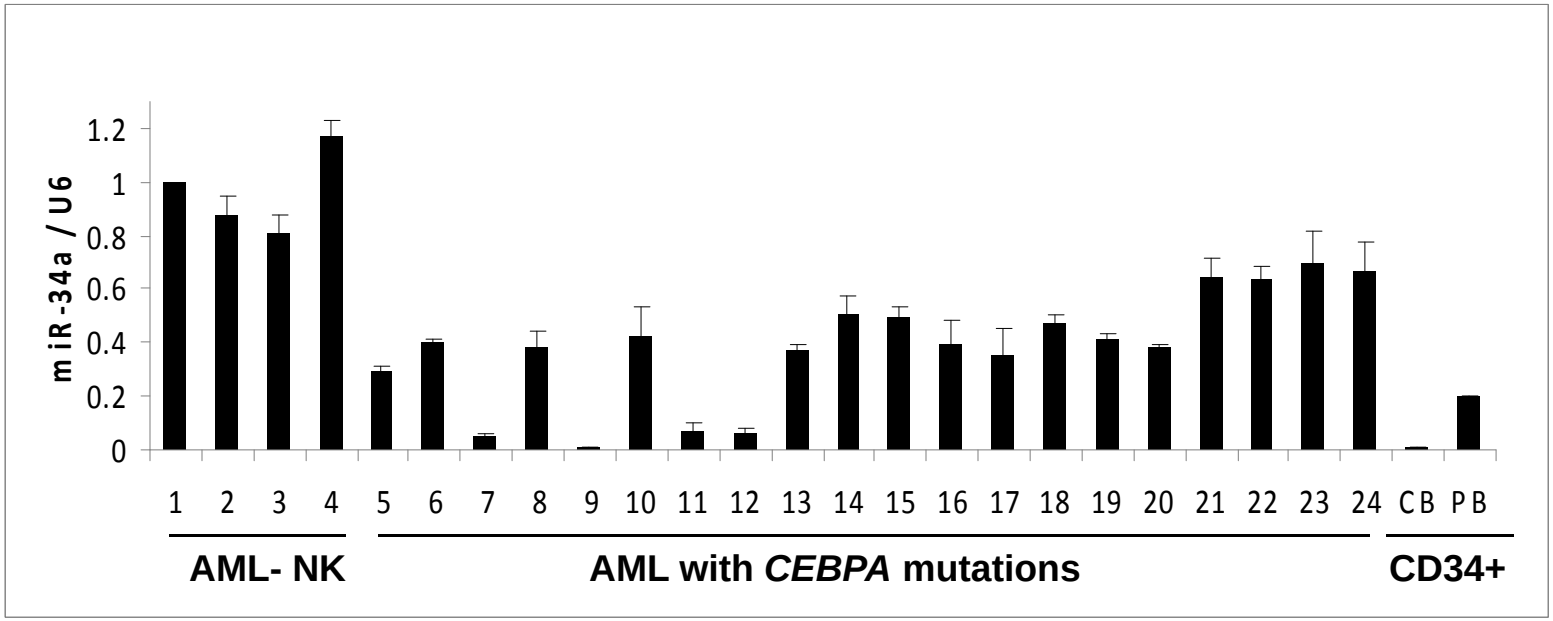

miR-34a / U6
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 CB PB
AML- NK
AML with CEBPA mutations
CD34+

Figure 3

B

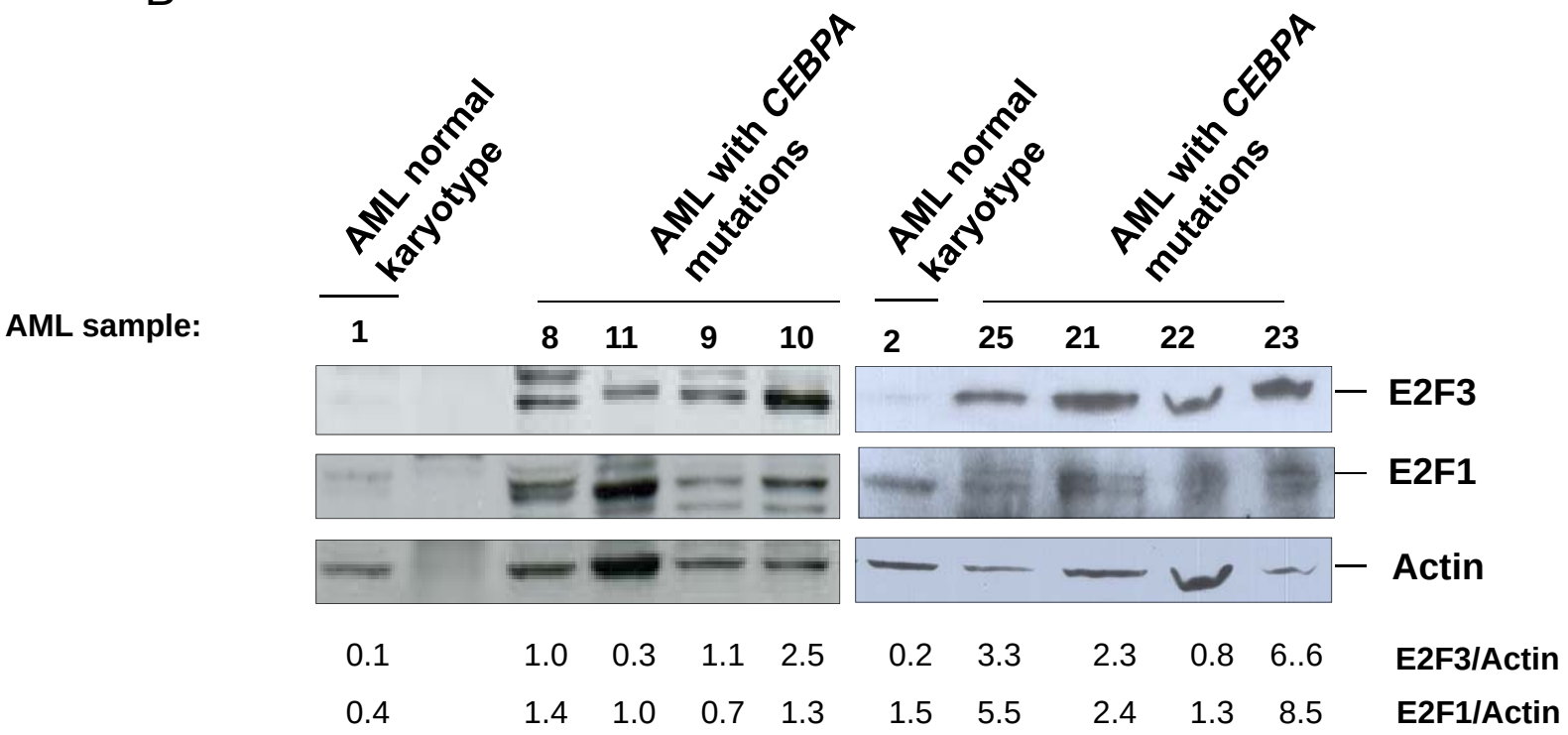
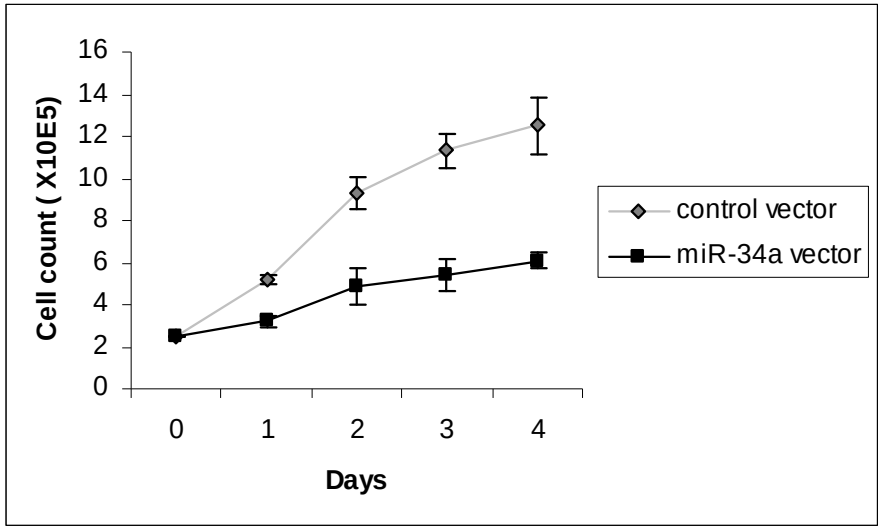

AML normal karyotype
AML with CEBPA mutations
AML normal karyotype
AML with CEBPA mutations
AML sample:
1
8
11
9
10
2
25
21
22
23
E2F3
E2F1
Actin
0.1
1.0
0.3
1.1
2.5
0.2
3.3
2.3
0.8
6..6
E2F3/Actin
0.4
1.4
1.0
0.7
1.3
1.5
5.5
2.4
1.3
8.5
E2F1/Actin

Figure 3

C


Cell count (X10E5)
16
14
12
10
8
6
4
2
0
0
1
2
3
4
Days
control vector
miR-34a vector

D

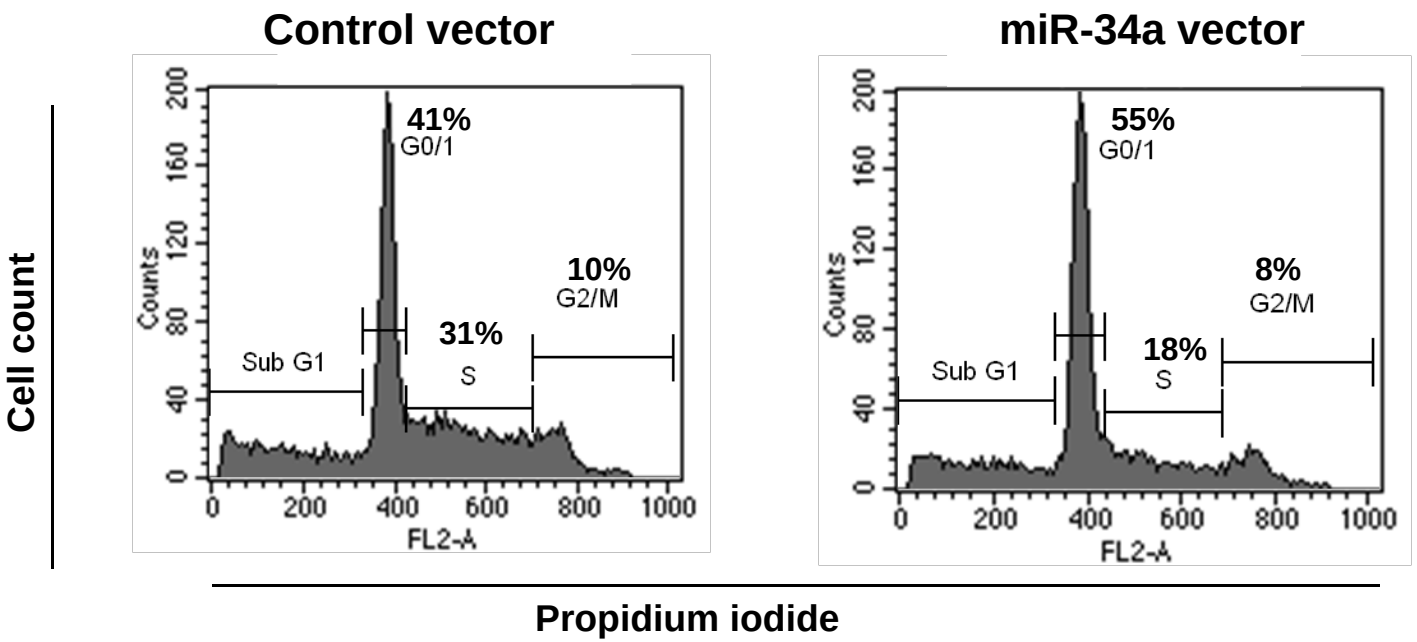

Control vector
miR-34a vector
Cell count
Counts
41%
G0/1
10%
G2/M
31%
S
Sub G1
55%
G0/1
8%
G2/M
18%
S
Sub G1
FL2-A
Propidium iodide

Figure 3

E

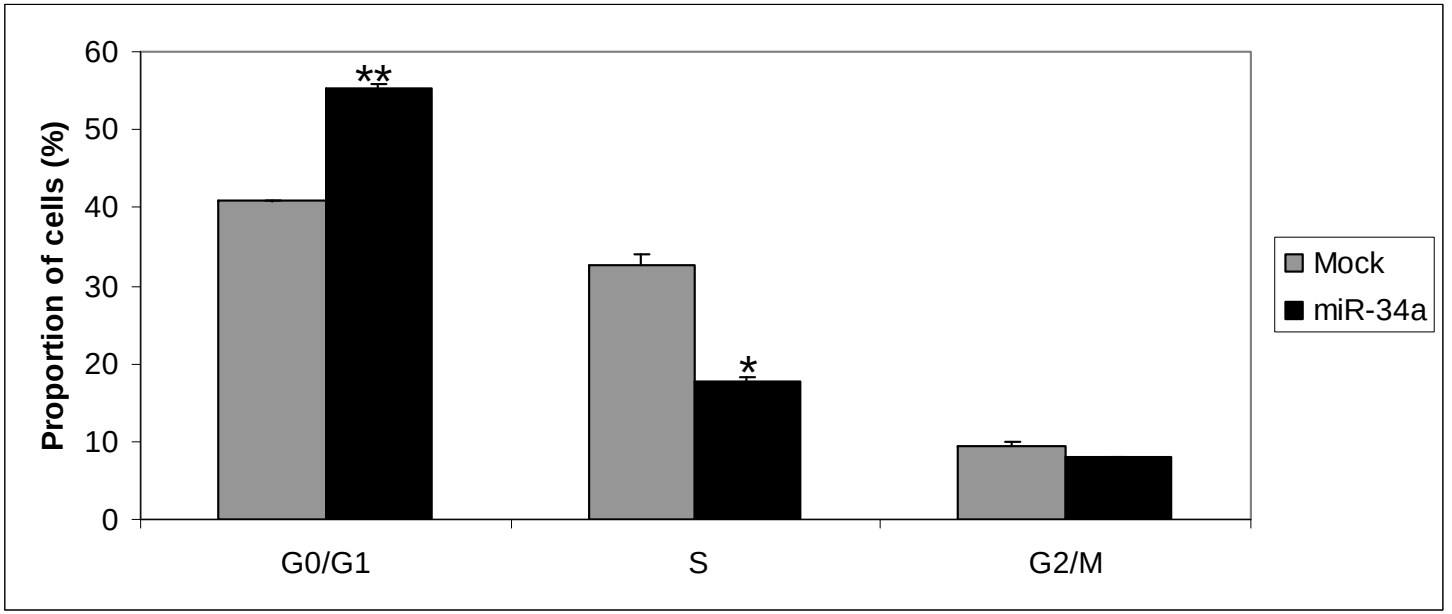


Figure 4

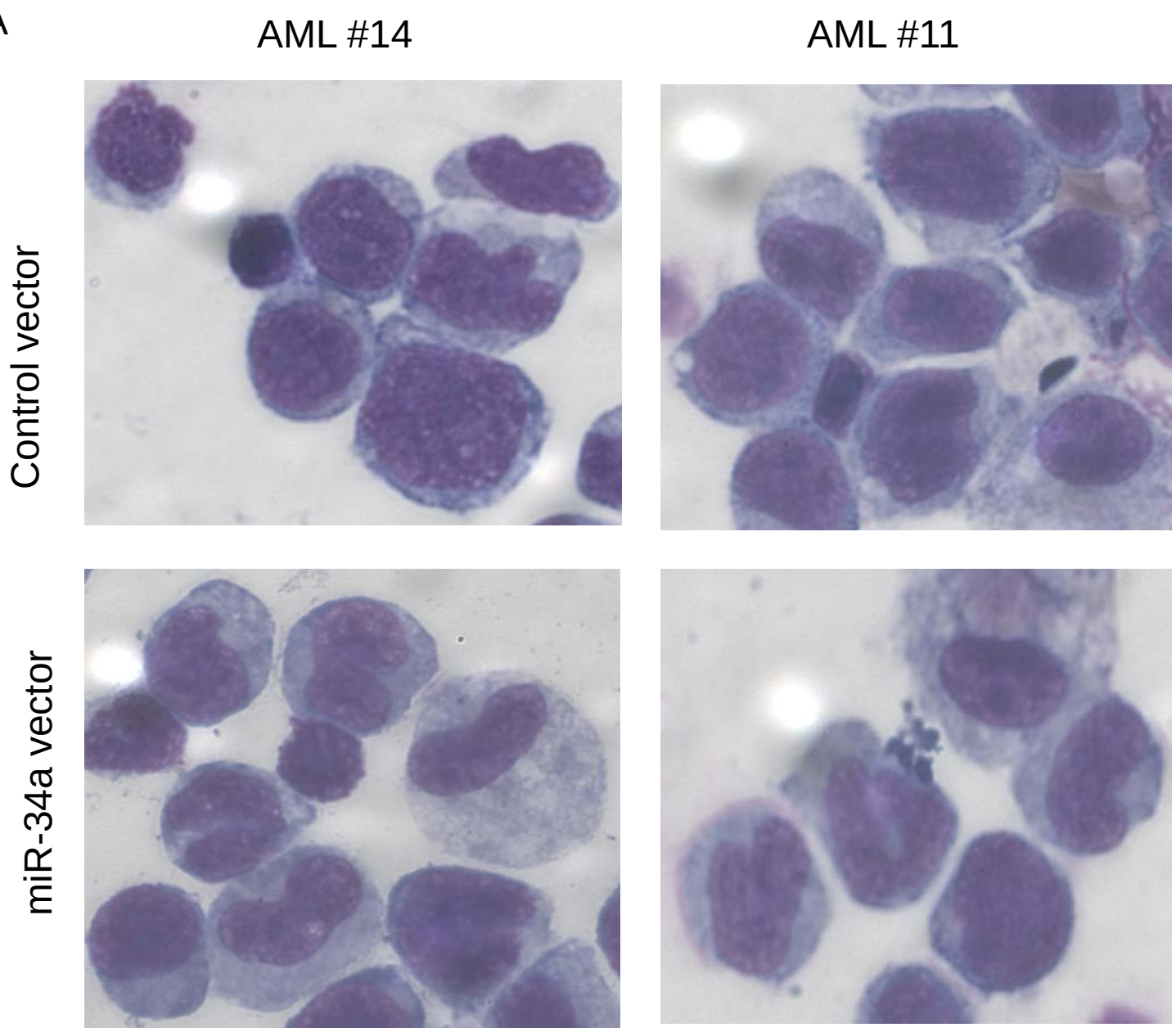
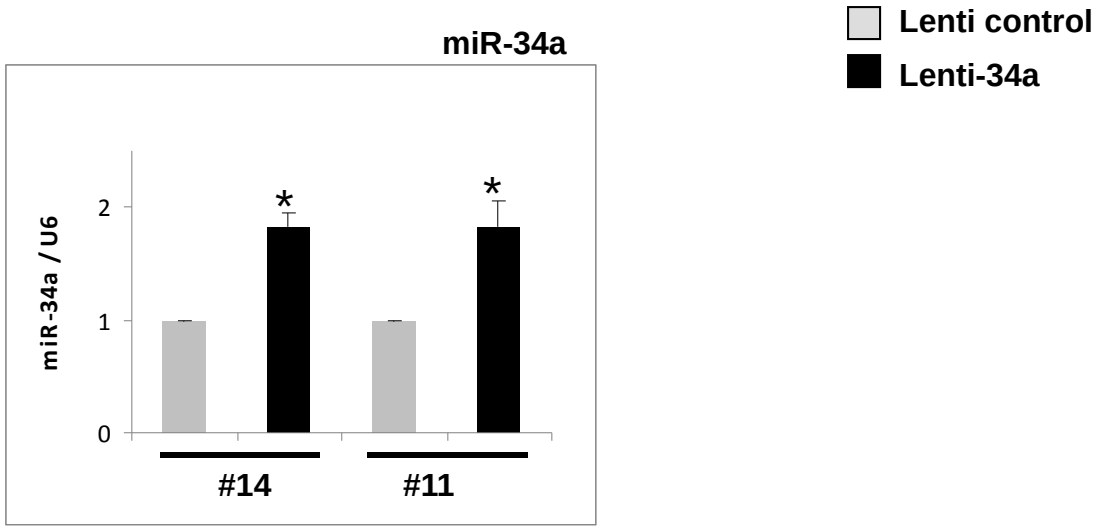
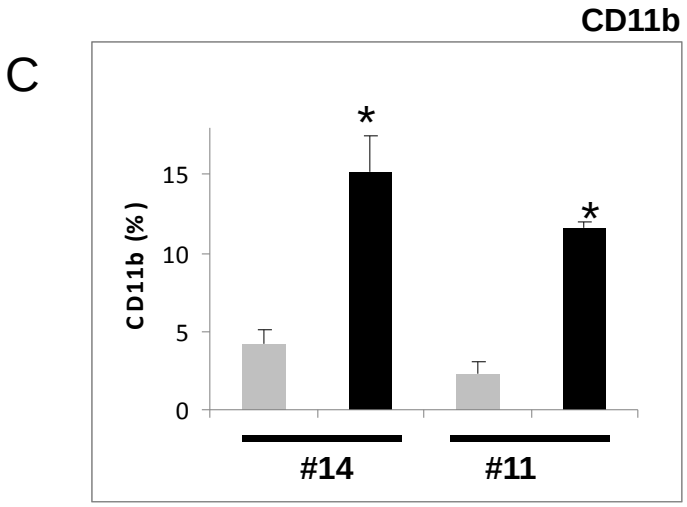


Figure 4


Lenti control
Lenti-34a
B
miR-34a
miR-34a / U6
2
1
0
*
*
#14
#11


C
CD11b
CD11b (%)
15
10
5
0
*
*
#14
#11

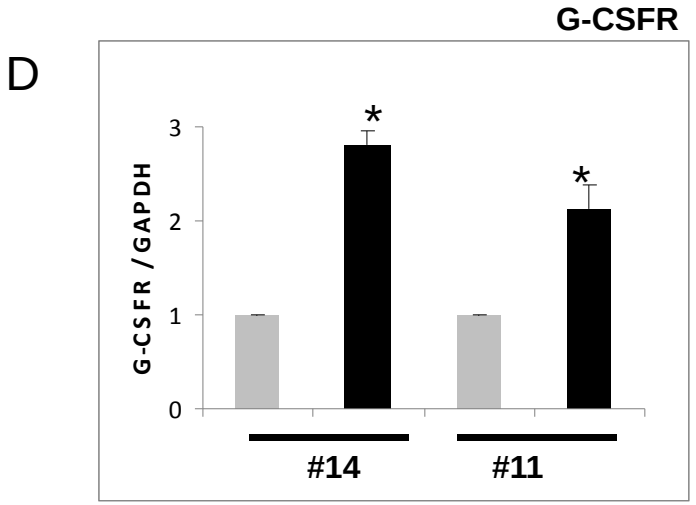

D
G-CSFR
G-CSFR /GAPDH
3
2
1
0
*
*
#14
#11

Figure 4

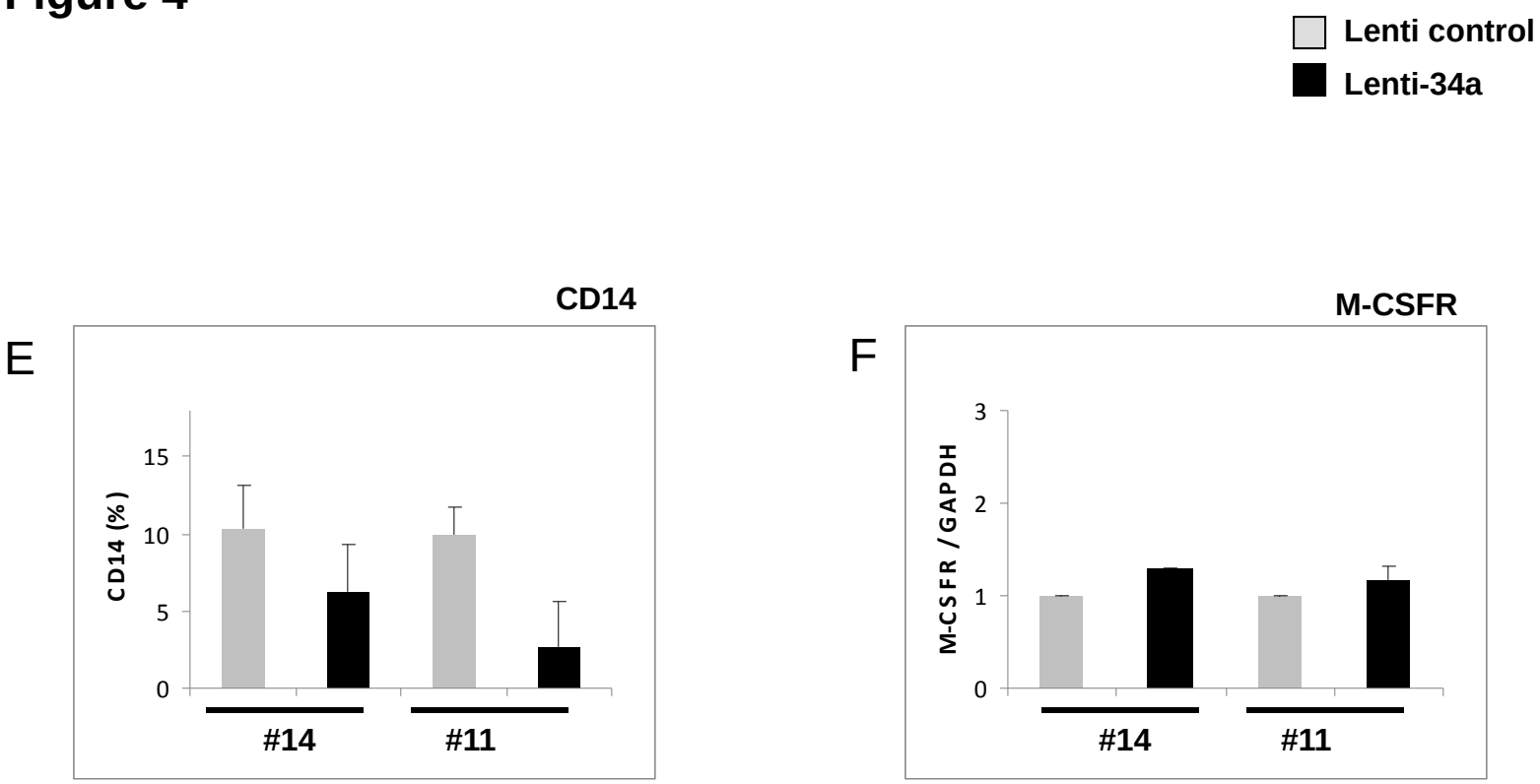
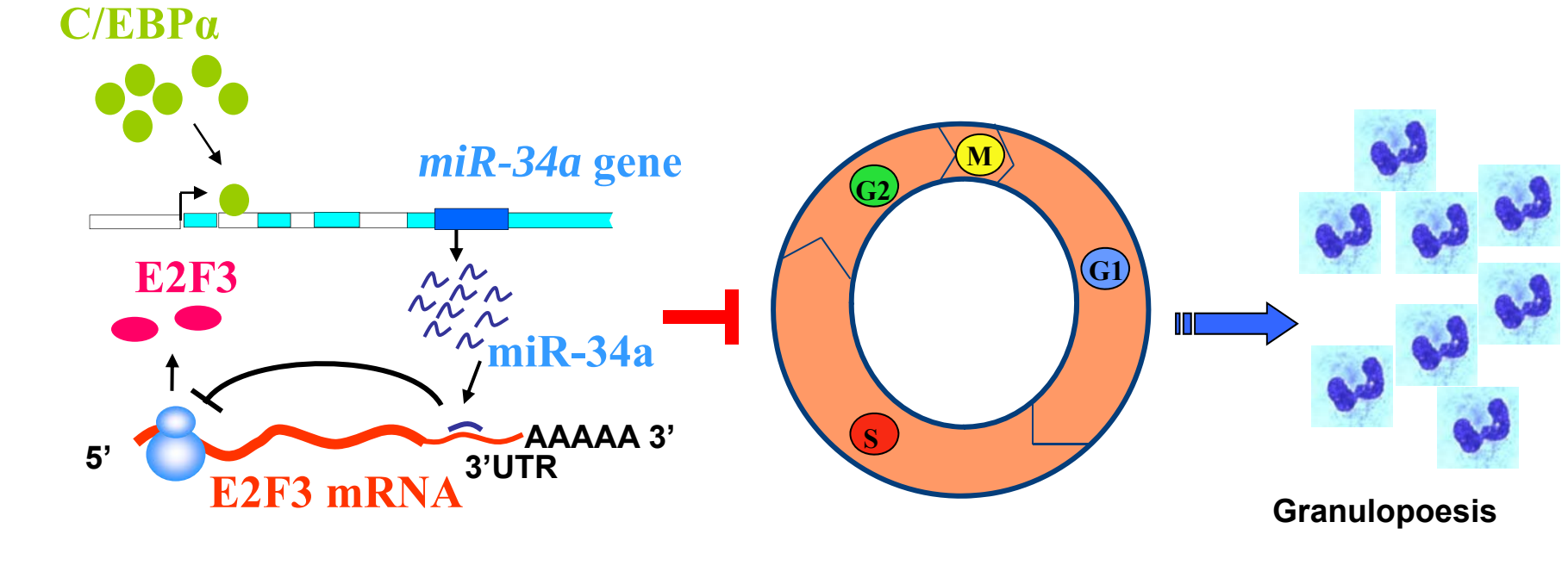
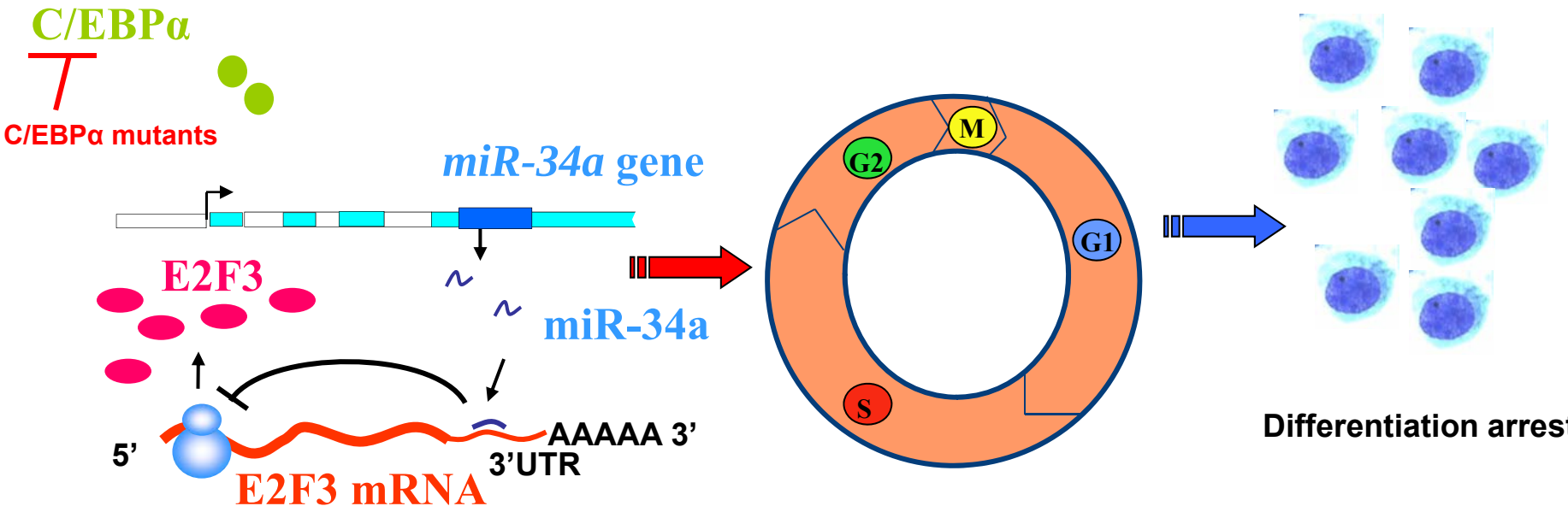

Lenti control
Lenti-34a
E
CD14
CD14 (%)
15
10
5
0
#14
#11
F
M-CSFR
M-CSFR /GAPDH
3
2
1
0
#14
#11

Figure 5


C/EBPα
miR-34a gene
E2F3
miR-34a
5'
AAAAA 3'
3'UTR
E2F3 mRNA
M
G2
G1
S
Granulopoesis


C/EBPα
C/EBPα mutants
miR-34a gene
E2F3
miR-34a
5'
AAAAA 3'
3'UTR
E2F3 mRNA
M
G2
G1
S
Differentiation arrest