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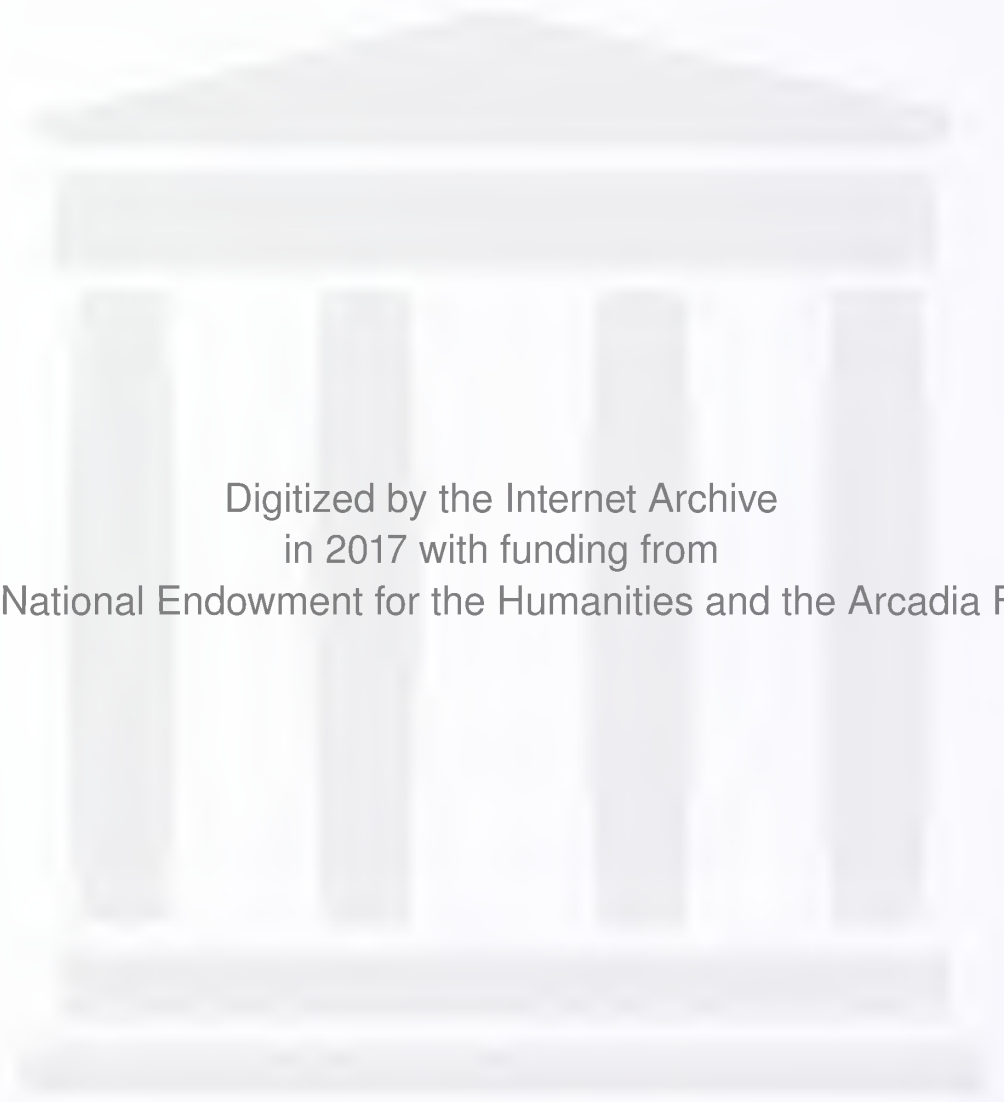

# The Role of CCAAT Displacement Protein in Neutrophil-Specific Gene Expression

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Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

By

Aimee Elizabeth Lee

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THE ROLE OF CCAAT DISPLACEMENT PROTEIN IN NEUTROPHIL-SPECIFIC GENE EXPRESSION. Aimee Lee, Arati Khanna-Gupta and Nancy Berliner. Section of Hematology, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.

CCAAT displacement protein (CDP) is a highly conserved, ubiquitously expressed homeodomain protein with extensive homology to the Drosophila *cut* protein. CDP contains three conserved DNA-binding repeats called *cut* repeats, as well as a conserved homeodomain sequence. CDP is a transcriptional repressor of several developmentally regulated genes including gp91-phox, CCAAT enhancer binding protein epsilon (C/EBPε), and its downstream targets the neutrophil secondary granule proteins (SGPs), including lactoferrin (LF). We have previously shown that CDP binds to and represses both the C/EBPε and LF gene promoters, thereby preventing expression of SGPs both directly and indirectly. CDP overexpression represses expression of SGPs in 32Dcl3 cells, a murine myeloid cell line that undergoes differentiation in response to G-CSF stimulation. Several attempts at generating a CDP knockout mouse have been undertaken, but all have produced incomplete knockouts. I generated short hairpin RNA (shRNA) constructs to knock down CDP in 32Dwt18 cells, which contain a chimeric G-CSF receptor linking the intracellular domain of the G-CSF receptor with the extracellular component of the erythropoietin (EPO) receptor, and differentiate in response to EPO. CDP repression in clones expressing the shRNA for CDP appears to correlate with increased LF expression in uninduced cells. Control clones do not express LF until induced with EPO for several days. The knockdown of CDP does not appear to affect the expression of C/EBPε, suggesting that LF expression reflects direct modulation of CDP binding to its promoter and is not an indirect effect of increased C/EBPε expression. This suggests that CDP can function as the sole negative regulatory element for LF gene expression, and that relief of CDP repression can increase LF expression independent of positive regulatory factors.

**Acknowledgements**

I would like to thank Dr. Nancy Berliner for her support, advice and encyclopedic knowledge over the past 5 years. This project could not have begun without the help of the entire Berliner laboratory, including Arati Khanna-Gupta, Terry Zibello, Peter Gaines, and Hong Sun, as well as the fellows, residents and medical students in the laboratory.

I would also like to thank the Yale University Department of Internal Medicine, especially the Section of Hematology, for their support over the years.

I am obliged to the Howard Hughes Medical Institute for providing funding for my year of research. Through this year, I have learned tools I hope to carry throughout a career in academic medicine. After my first year of medical school, my introduction to both the Berliner laboratory and research at Yale was funded by the Office of Student Research. I am especially indebted to Donna Carranzo for organizing my funding throughout medical school.

I owe a great deal of thanks to my family and friends. Thank you for being there, especially when I needed it the most.

**Table of Contents**

## Introduction

Mature cells in all hematopoietic lineages are required for proper functioning of the adult mammal. The generation of mature neutrophils from hematopoietic stem cell (HSC) precursors is a process which is still incompletely understood. The basic model for formation of mature blood products from HSCs involves the regeneration of HSCs, as well as formation of precursors for other hematopoietic lineages. As a hematopoietic cell differentiates, it loses proliferative potential and gains specific characteristics of endstage mature cells. The HSC gives rise to both the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). The CMP further differentiates into the myeloid erythroid progenitor (MEP), which gives rise to megakaryocytes, basophils and eosinophils, and the granulocyte/macrophage progenitor (GMP), which gives rise to both monocyte and granulocyte precursors.

To ensure a correct number of mature cells in each lineage, hematopoiesis is tightly regulated by many different cytokines and transcription factors (1,2). Granulocytes and monocytes originate from a common progenitor cell, but the precise mechanism by which the terminal products of maturation are formed is still unclear. Although granulocytes and monocytes share several transcription factors with overlapping functions, other factors will tip the balance in favor of producing either granulocytes or monocytes (reviewed in 1). PU.1, Sp1 and the CCAAT enhancer binding protein (C/EBP) family of transcription factors are shared transcription factors. Maf and Jun family members may favor the development of the monocyte lineage, while higher levels of C/EBPs could

direct the formation of neutrophil granulocyte precursors. The terminal maturation of neutrophils requires PU.1, C/EBPs (especially C/EBPε), Sp1 and retinoic acid receptors (RARs). The milieu is further enhanced by the presence of a variety of cytokines, or colony stimulating factors (CSFs) (reviewed in 2). In myeloid lineages, GM-CSF furthers the development of clones of granulocytes and macrophages, M-CSF stimulates production of macrophages, and G-CSF induces granulocyte formation. Interleukin-3 (IL-3) can stimulate development of eosinophils, mast cells, megakaryocytes and erythroid cells in addition to producing macrophages and granulocytes.

Granulocyte differentiation can be followed morphologically through phenotypic changes in the neutrophil nucleus, and biochemically through changes in cytoplasmic granule expression (Figure 1). Condensation of chromatin eventually transforms the immature myeloblast nucleus into the multilobed nucleus of a mature neutrophil. Biochemically, the promyelocyte is characterized by expression of primary "nonspecific" granules which contain myeloperoxidase (MPO). The transition between promyelocyte and myelocyte is marked by expression of secondary "specific" granule proteins (SGPs) such as lactoferrin (LF), neutrophil collagenase (NC), neutrophil gelatinase (NG), and transcobalmin 1 (TC1) (3). Although expression of SGP mRNA and protein is coordinately upregulated at the promyelocyte to myelocyte transition, the genes themselves are physically unlinked (4). This suggests a coordinated and stage-specific upregulation presumably induced by common transcription factors that result in SGP gene expression.

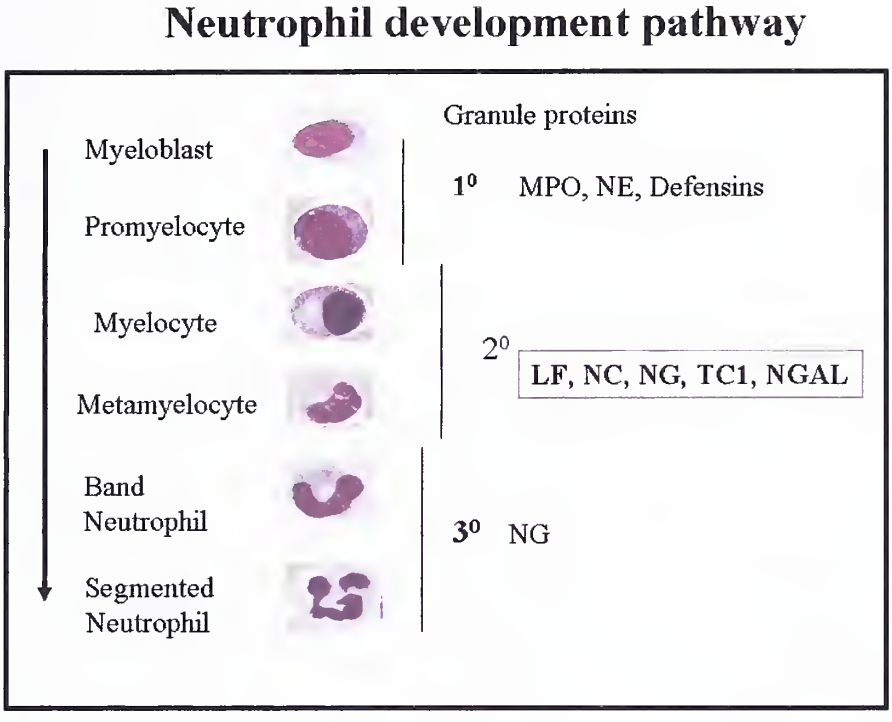


**Figure 1. Neutrophil Maturation.** Neutrophil maturation proceeds from the immature myeloblast to the segmented neutrophil. The morphological changes are paralleled by changes in granule protein expression. Figure courtesy of Arati Khanna-Gupta.

Granule proteins formed at the same stage in development will sort into the same granules (5). One can then define the neutrophil developmental stage by the biochemical contents of the granules. Expression of SGPs serves as a marker for terminal myeloid differentiation (6). C/EBP epsilon (C/EBPε) contributes to the morphological and functional differentiation of neutrophils by upregulating SGP expression. Clinically, absence of SGPs or abnormal SGP expression is found in acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), and specific granule deficiency (SGD). All are associated with disruptions in normal myeloid differentiation.

In previous studies, our laboratory has demonstrated that a known negative regulator of transcription, CCAAT displacement protein (CDP) binds to the LF promoter and represses its transcription (7). In further studies, it was shown that CDP not only exerts these direct effects on the LF promoter, but also indirectly represses SGP expression by repressing C/EBPε (8). The overexpression of CDP inhibits expression of SGP genes in 32Dcl3 cells, an IL-3 dependent murine myeloid cell line that undergoes differentiation in response to IL-3 withdrawal and G-CSF stimulation (9). Moreover, LF is not expressed in NB4 cells, a human acute promyelocytic cell line which contains the t(15;17) PML-RARα translocation. While CDP binding to the LF promoter is abolished in 32Dcl3 cells coincident with LF expression, CDP has been found to remain bound to the LF promoter in NB4 cells after morphological differentiation following ATRA induction. This suggests that decreased CDP binding to the LF promoter is necessary and permissive for LF gene expression.

CDP is a highly conserved, ubiquitously expressed homeodomain protein with extensive homology to the Drosophila *cut* protein (reviewed in 10). The protein contains three highly conserved DNA-binding repeats called *cut* repeats (CR1-3), as well as a conserved homeodomain (HD) area (11) (Figure 2), which mediate its DNA binding capacity. CDP is a 350 kb gene with 33 exons (12) which maps to chromosome 7q22 (13). Rearrangements in 7q22 have been seen in AML, MDS and uterine leiomyomas, suggesting that CDP may function as a tumor suppressor gene (reviewed in 10). However, this hypothesis remains unproven.


**Figure 2. Schematic drawing of CDP.** CDP contains 3 *cut* repeats (CR) as well as a homeodomain region (HD). These 4 conserved areas mediate DNA binding activity.

CDP is a negative regulator of several developmentally regulated myeloid genes, including gp91-phox, a component of the neutrophil respiratory burst NADPH oxidase complex (11). Interestingly, gp91-phox is expressed earlier than SGPs in myeloid differentiation. The mechanism by which CDP mediates this temporal gene regulation is currently unknown. Recent studies have shown that CDP cannot bind DNA as a monomer and that several splice isoforms of CDP exist (14). Different combinations of CR1-3 and HD, as well as variations in splicing, may account for the differential effects of CDP. Phosphorylation and recruitment of histone deacetylases have also been proposed as mechanisms contributing to the variety in CDP function (15,16).

Our laboratory has previously demonstrated that CDP binds to and represses both the C/EBPε and LF promoters (7, 8). Overexpression of CDP leads to repression of SGPs in mouse and human cell lines without inhibiting morphological differentiation. Due to the size of the CDP gene several attempts at generating a CDP knockout mouse have been unsuccessful, generating partial knockouts each time. The ΔCR1 mouse, in which the first *cut* repeat has been deleted, exhibits a mild phenotype consisting of curly vibrissae,

wavy hair, and a high degree of pup loss most likely due to feeding difficulties (17). ΔCR3HD, displays partial neonatal lethality with survivors surrendering to a wasting disease within 2-3 weeks. Interestingly, they also display myeloid hyperplasia and lymphoid apoptosis (18).

As generating a complete knockout of CDP has proven to be a difficult task, we decided to employ a PCR-based RNA interference (RNAi) strategy to attempt to knock-down CDP in myeloid cells. RNAi harnesses the cell's inherent ability to degrade RNA from exogenous sources in a targeted manner. Several methods are available to produce RNAi in mammalian cells, including synthesized small interfering RNAs (siRNAs), in vitro RNAseIII processed dsRNA, and short hairpin RNAs (shRNAs). I chose to use a vector-based shRNA strategy for several reasons: 1) the shRNA fragments are relatively simple to create and clone into appropriate vectors, 2) this strategy is more cost-effective than chemically synthesized siRNAs, 3) a vector-based strategy allows for the generation of stable cell lines, and 4) this method was previously successful in hematopoietic cell lines. A myeloid cell line in which CDP is knocked-down will provide valuable and more complete information on the role CDP plays in the developing neutrophil.

**Hypothesis and Specific Aims**

The aim of this project is to characterize and define the role of CDP in myeloid differentiation using normal and leukemic cells as models. I proposed to perform a functional knockout of mouse CDP by utilizing short hairpin RNAs (shRNAs) to degrade CDP mRNA, thus preventing the formation of a final protein product. I hypothesize that, as SGP expression occurs at the same stage in differentiation yet the genes themselves are physically unlinked, SGP expression is regulated by shared transcription factors that direct the coordinate expression of SGPs during normal myeloid differentiation. I further hypothesize that one of these critical transcription factors is CDP. As transcriptional regulation of granulopoiesis is disrupted in AML, SGD and MDS, CDP must play an integral role in myeloid differentiation. The ultimate goal of this project will be to analyze the CDP knock down cells using a myeloid specific cDNA microarray generated in the Berliner Lab in collaboration with Dr. Arch Perkins (Dept of Pathology, Yale University School of Medicine). These analyses will shed light on all genes regulated by CDP during myeloid differentiation, and will provide valuable and more complete information on the role CDP plays in the developing neutrophil.

## Methods

*Short Hairpin RNA (shRNA) vector construction.* shRNA constructs were generated as previously described by Hannon (19, 20). Briefly, the group uses a PCR-based method to create generate vector-based RNA interference. A web based RNA oligonucleotide retriever was developed by the group, and interested researchers enter a nucleic acid accession number for the target gene. The web based program then designs an oligonucleotide which best targets future degradation of mRNA. shRNA design does not correlate with the location of *cut* repeats or HD (Figure 3).

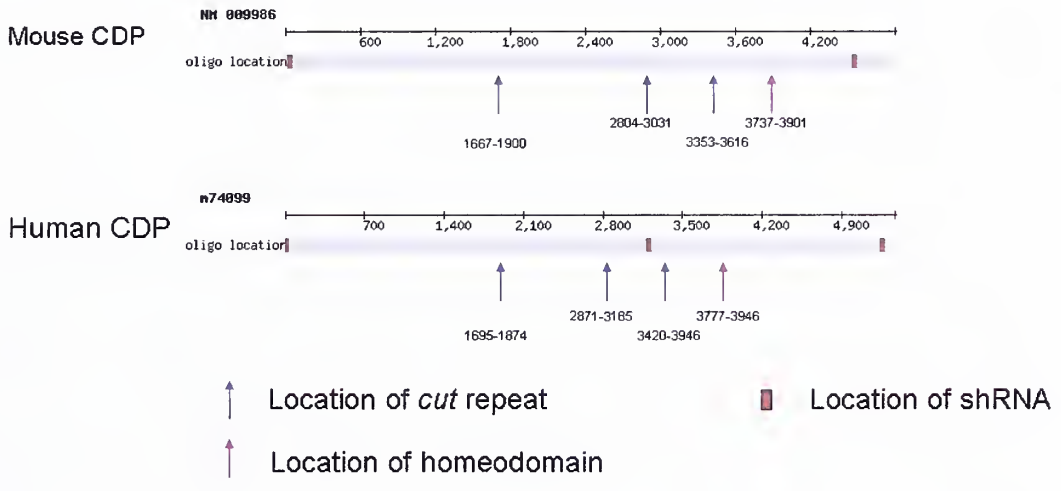


***Figure 3.*** **Location of shRNA constructs in relation to *cut* repeats and homeodomain.** shRNA constructs are targeted to the 5' and 3' ends of each gene. An additional site in the middle of the gene is targeted in the human CDP shRNA construct. None of the regions targeted by shRNA are known DNA-binding regions.

Instead, the hairpin is designed in the most efficient location for RNAi degradation of mRNA. Oligonucleotides were made by the Oligonucleotide Synthesis Laboratory (Department of Pathology, Yale University) then used as the forward 5' PCR primer, while another oligonucleotide which contains the U6 promoter sequence is used as the 3'

primer (Figure 4). PCR with DMSO using the pGEM-Zeo-U6 vector as template was performed, then the PCR fragment was cloned using TOPO cloning (Invitrogen, Carlsbad, CA) for use in transient transfections. For stable transfections, the PCR fragment was subcloned into the GFP-containing MigR1 vector (Figure 5).

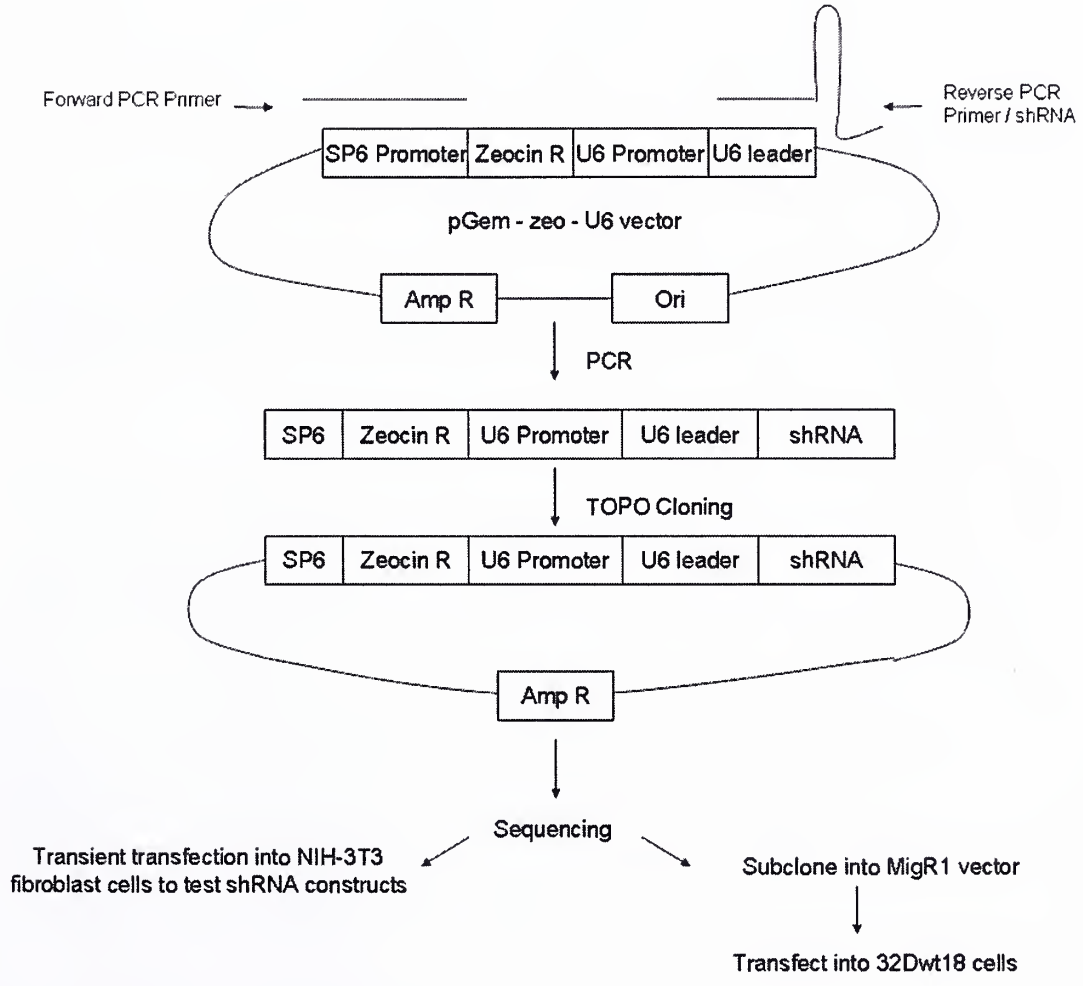


**Figure 4. shRNA Construction.** As outlined in the text, forward and reverse PCR primers were developed based on the Hannon method. PCR fragments containing the shRNA were first cloned into the TA vector and subcloned into MigR1.

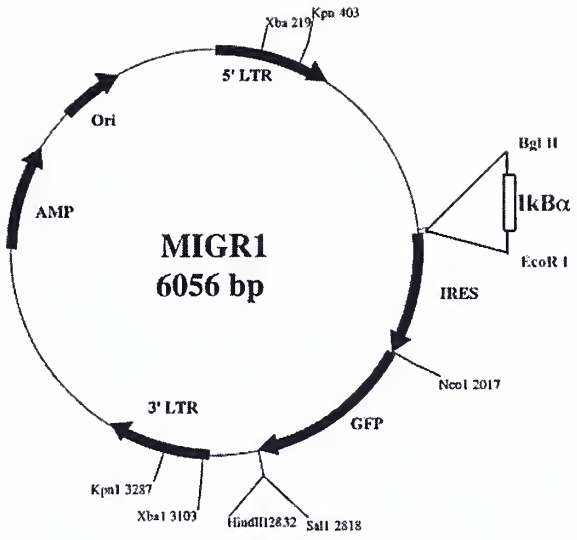


**Figure 5. MigR1 vector.** The MigR1 vector is 6056 bp in size and contains genes for ampicillin resistance as well as GFP driven by an IRES. The shRNA PCR fragment was cloned into the EcoR I and Bgl II restriction sites.

*Tissue culture.* NIH3T3 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Calf Serum (FCS, Gemini Bio-products). 32Dwt18 cells were maintained in Iscove's Modified Dulbecco Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS, Gemini Bio-products) and 10% WEHI-3D-conditioned medium. All growth media were supplemented with 5U/mL penicillin, 5μg/mL streptomycin sulfate and 2mM L-glutamine.

*Transient transfection of NIH3T3 cells.* NIH3T3 cells were plated at a density of 1.5 x $10^6$ cells per 10 cm plate the day prior to transfection in DMEM with 10% FCS. The morning of transfection, cells were washed once with PBS, and then incubated in Optimem (Gibco). 10μg DNA was added to a mixture of 3μL Fugene 6 Transfection

Reagent (Roche, Indianapolis, IN) and Optimem to a total volume of 100μL. Cells were incubated overnight in Optimem and DNA mixture, and then medium was changed to DMEM the following DNA. Cells were harvested 72 hours after transfection.

*Stable transfection of 32Dwt18 cells.* Exponentially growing 32Dwt18 cells ($1.0 \times 10^7$) were washed twice with PBS and resuspended in 180μL HEPES-buffered saline. 10μg of DNA was transfected by electroporation. The electroporated cells were resuspended in IMDM supplemented with 10% WEHI-3D-conditioned medium and 10% FBS.

*Flow cytometry.* $7 \times 10^6$ 32Dwt18 cells were washed in PBS and resuspended in IMDM supplemented with 10% WEHI-3D-conditioned medium and 2.5% FBS with propidium iodide to a final concentration of 2μg/mL. Cells were sorted by Mr. Rocco Carbone (Yale Cancer Center shared facility) and collected in 4 mL IMDM supplemented with 10% WEHI-3D-conditioned medium and 10% FBS.

*Induction of differentiation.* Stably transfected 32Dwt18 cells were collected in growth medium (GM) for day 0 samples, or induced to differentiate in induction medium (IM) IMDM supplemented with 10% FBS without 10% WEHI-3D-conditioned medium, and addition of 1U/mL EPO for 4 days. Media was replaced every 3 days during the induction procedure, and cell densities were maintained at or below $1.0 \times 10^6$ cells/mL. All growth media were supplemented with 5U/mL penicillin, 5μg/mL streptomycin sulfate and 2mM L-glutamine.

*Cytospins.* 1.0 x $10^4$ cells were centrifuged at low speed onto a microscope slide and stained with Wright-Geimsa staining.

*Growth Curve Analysis.* 1 x $10^5$ cells were grown in regular growth medium, induced to differentiate with EPO, or grown in IMDM without WEHI-3D-conditioned medium. Cell densities were maintained at or below 1.0 x $10^6$ cells/mL. Cells were counted daily using a hemacytometer (Reichert, Buffalo, NY) with Trypan Blue Stain 0.4% (Gibco).

*Isolation of total RNA.* 5-10 x $10^6$ cells were pelleted at 1250 rpm and then homogenized in 1 mL TRIzol reagent (Invitrogen). Resuspended cells were incubated for 5 minutes at room temperature then 0.2 mL chloroform was added per 1 mL TRIzol reagent. Samples were vigorously shaken and allowed to stand at room temperature for 2 minutes. Samples were then centrifuged at 12,000 RPM at 4°C for 15 minutes. The aqueous phase was transferred to a new tube and 0.5 mL isopropanol added, then incubated at room temperature for 10 minutes. Samples were centrifuged at 12,000 RPM at 4°C for 10-15min, supernatant removed and washed once with 75% EtOH. The RNA pellet was resuspended in DEPC-$H_2O$ and stored at -70°C until used.

*Polymerase Chain Reaction (PCR).* 100 ng of total RNA was mixed with 50 mg of oligo dT, denatured at 65°C for 10 minutes, and mixed with reverse transcription buffer, dNTPs, RNAsin, and dithiothreitol (DTT) in a final volume of 50 μL. Reactions were then incubated with reverse transcriptase for 1 hour at 37°C. 1 μL of the resultant cDNA

was then subjected to 30-40 cycles of PCR under standard conditions with 100 ng of appropriate primers and 1 to 3 U Taq Polymerase.

*Preparation of nuclear extracts.* $1.0 \times 10^7$ cells were centrifuged at 4°C. Cells were washed twice in ice-cold phosphate buffered saline (PBS) and once in Buffer A (10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF). Cells were then lysed following a 5-minute incubation on ice in Buffer A with 0.1% Nonidet P-40, and then centrifuged at 4°C for 15 minutes to recover nuclei. Nuclei were then lysed in high-salt Buffer C (20 mmol/L HEPES-KOH (pH 7.9), 10% glycerol, 420 mmol/L NaCl, 10 mmol/L KCl, 0.2 mmol/L EDTA, and 0.5 mmol/L DTT) and nuclear extracts were recovered by centrifugation for 15 minutes at 4°C. Aliquots of nuclear protein were analyzed for total protein concentration, then frozen immediately and stored at -70°C until used.

*Preparation of whole cell extracts.* $1 \times 10^7$ cells were centrifuged at 4°C. Cells were washed once with 4°C phosphate buffered saline (PBS) and resuspended in 100 µL RIPA Lysis Buffer (Upstate Biotechnology, Lake Placid, NY) with protease inhibitor. Cells were incubated on ice for 30 minutes, then centrifuged at 10,000 x g for 10 minutes in 4°C. The supernatant was transferred to a new tube, analyzed by spectrophotometry for concentration, and frozen immediately on dry ice. Samples were maintained at -70°C until used.

*Western Blot.* 15 μg protein were separated on a 4-12% Bis-Tris Gel (Invitrogen) and transferred to PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Membranes were blocked in TST (20mM Tris [ph 7.5], 150 mL NaCl, 0.1% Tween-20) with 5% nonfat dry milk for 1 hour at 25°C. For detection of CDP, a mouse monoclonal antibody was incubated with the membrane in TST/5% milk overnight at 4°C at a concentration of 1:1000. Rabbit anti-mouse secondary antibody was applied at a concentration of 1:3000 and signals were detected using the enhanced chemiluminescent technique (Boehringer Mannheim).

*Northern Blot.* 10 μg total RNA was separated on a 1% denaturing gel, transferred to nitrocellulose filters, and hybridized to $^{32}$P-labelled cDNA fragments at 42°C in 50% formamide. The filters were washed at in high stringency 0.1% SDS and 0.1X SSC at 55°C and autographed. Blots were probed with a 600-bp mouse LF probe cloned in our laboratory.

*Quantitative PCR (Q-PCR).* cDNA was prepared as above and adjusted to concentration of 10 ng/μL. 2 μL sample cDNA was incubated with 100ng of appropriate primers and 25 μL iQ SYBR Green Supermix (Bio-Rad) to a total volume of 50μL. PCR was performed on a Bio-Rad Thermocycler at appropriate annealing conditions for a total of 40 cycles.

**Results**

CDP shRNA constructs were first tested for their ability to reduce CDP levels by transient transfection in fibroblast cell lines. NIH-3T3 cells are murine fibroblast cells that express little endogenous CDP in whole cell lysates. Cotransfections of CDP expression plasmids with two different shRNA constructs successfully decreased expression of ectopically expressed CDP as demonstrated by both western blot and RT-PCR (Figure 6). I have found that the shRNA at the 5' end of the RNA transcript (shRNA 1) is the most efficient at knocking down gene expression by RT-PCR. However, by western blot it appears that the shRNA located at the 3' end of the RNA transcript was more efficient at CDP knock-down. The combination of the two plasmids appears to be even more efficient at decreasing CDP levels. Three human CDP shRNA constructs were also tested in HeLa cells with similar results (data not shown).

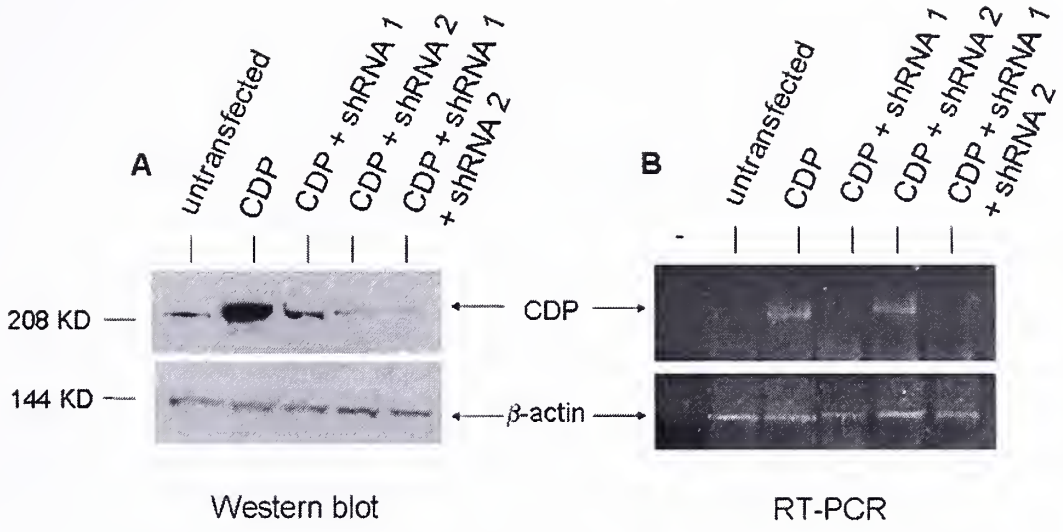


***Figure 6.*** **Western blot and RT-PCR demonstrate decrease in CDP expression with shRNA constructs in NIH 3T3 cells.** NIH 3T3 cells were transiently transfected with murine CDP expression plasmid alone, and CDP in combination with shRNA vectors. (A) Western blot of transfected NIH 3T3 cells shows decreased CDP expression with shRNA vectors. (B) RT-PCR also shows decreased levels of CDP expression in cells containing shRNA 1.

Although many cell lines have been shown to express endogenous CDP, it is found at very low levels in whole cell extracts, which is characteristic of transcription factors. To assess the expression of endogenous CDP in human and mouse myeloid cells, I performed a western blot using NB4 whole cell extracts, as well as whole cell extracts and nuclear extracts of 32D cells (Figure 7). NB4 cells show endogenous expression of CDP on western blot with whole cell extracts. CDP is undetectable in whole cell lysates of 32D cells, but is easily detected in nuclear extracts.

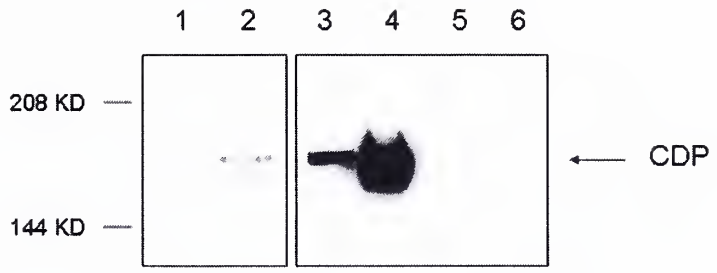


***Figure 7.*** **Western blot of NB4 and 32D cells for CDP.** Lane 1 NB4 whole cell extracts uninduced. Lane 2 NB4 whole cell extract induced with ATRA day 3. Lane 3 32D nuclear extract uninduced. Lane 4 32D nuclear extract induced with G-CSF day 4. Lane 5 32D whole cell extract uninduced. Lane 6 32D whole cell extract induced with G-CSF day 4.

A high degree of cell death is seen after induction of 32Dcl3 cells, reflecting stochastic variation in the level of G-CSF expression. To alleviate this problem, 32Dwt18 cells have been previously generated. These cells express a chimeric G-CSF receptor with the intracellular domain of the G-CSF linked to the extracellular erythropoietin binding domain of the erythropoietin receptor. As these cells exhibit much less cell death on differentiation with erythropoietin, I used 32Dwt18 cells to generate stable cell lines.

After generating a pooled population of CDP shRNA expressing cells, I assessed the growth characteristics of control and CDP shRNA containing 32Dwt18 cells by cell counting (Figure 8). 32Dwt18 cells expressing the shRNA 1 construct do not have different growth characteristics than those containing the empty MigR1 vector. Cells in regular GM continue to grow at an exponential pace, while cells induced to differentiate

with EPO in IM have a longer doubling time. Those cells that were maintained in IM without IL-3 did not grow, and were too few to count after 3 days.

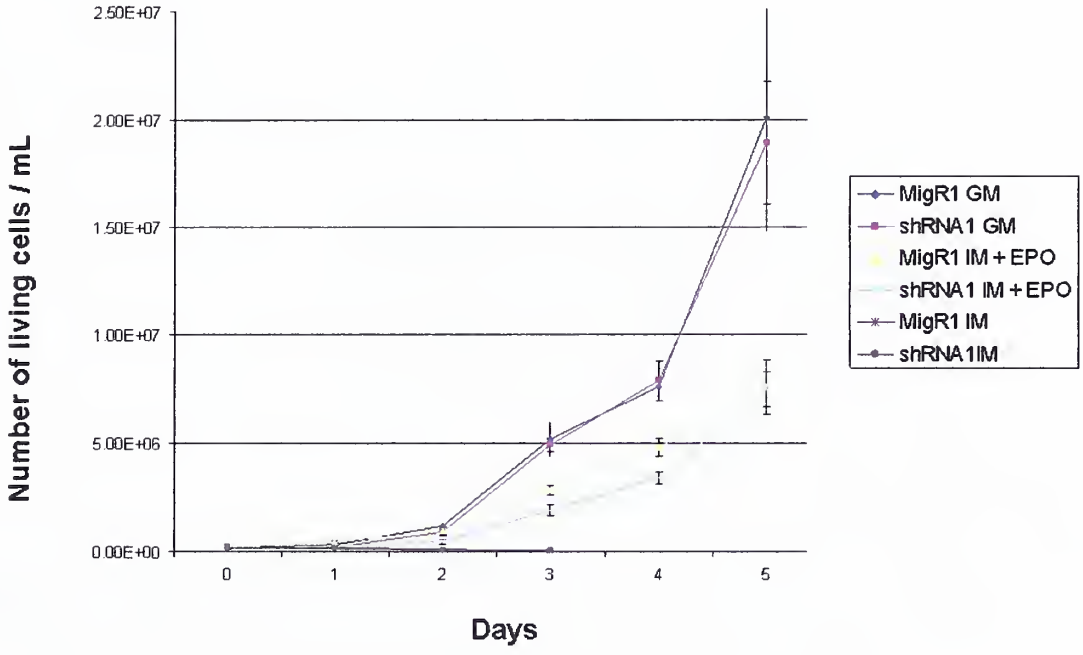


***Figure 8.*** **Growth curve of 32Dwt18 cells transfected with MigR1 vector alone versus shRNA 1.** 32Dwt18 cells are an IL-3 dependent cell line which undergoes differentiation along the neutrophil lineage upon induction with EPO in the absence of IL-3. Cells transfected with shRNA remain IL-3 dependent, as evidenced by lack of growth in IL-3 deficient IM. Transfected cells show no significant differences in growth characteristics in GM and upon induction with EPO.

After cells were sorted twice for GFP, I performed RT-PCR to look at LF in uninduced and induced cells. After 22 cycles of PCR, lactoferrin remained undetectable in uninduced cells containing the MigR1 vector (Figure 9). MigR1 cells then showed

normal upregulation of LF following induction with EPO. However, the cells containing CDP shRNA did show expression of LF at day 0.

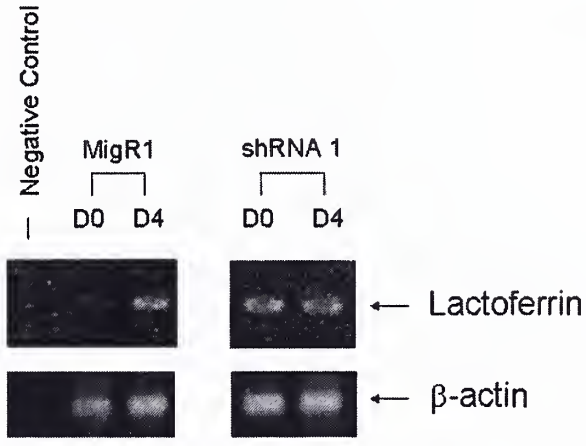


***Figure 9.*** **RT-PCR demonstrates LF expression at day 0 in 32Dwt18 cells expressing shRNA 1.** 32Dwt18 cells are induced to differentiate with EPO and are shown at day 0 and day 4. Expression of LF at day 0 is not seen in cells expressing MigR1 vector alone, but is seen in cells expressing shRNA 1 construct. Both cells express LF following induction with EPO.

To further quantify this difference in LF expression in day 0 cells, I performed a northern blot on the same cells. Again, LF expression was absent in uninduced cells containing MigR1 alone (Figure 10). LF was normally upregulated in these cells by day 4. However, in CDP shRNA cells LF expression was again seen in day 0 cells. Densitometry analysis was then performed, and demonstrated a 14-fold increase in LF expression at day 0. Quantitative expression of LF at day 4 was unchanged.

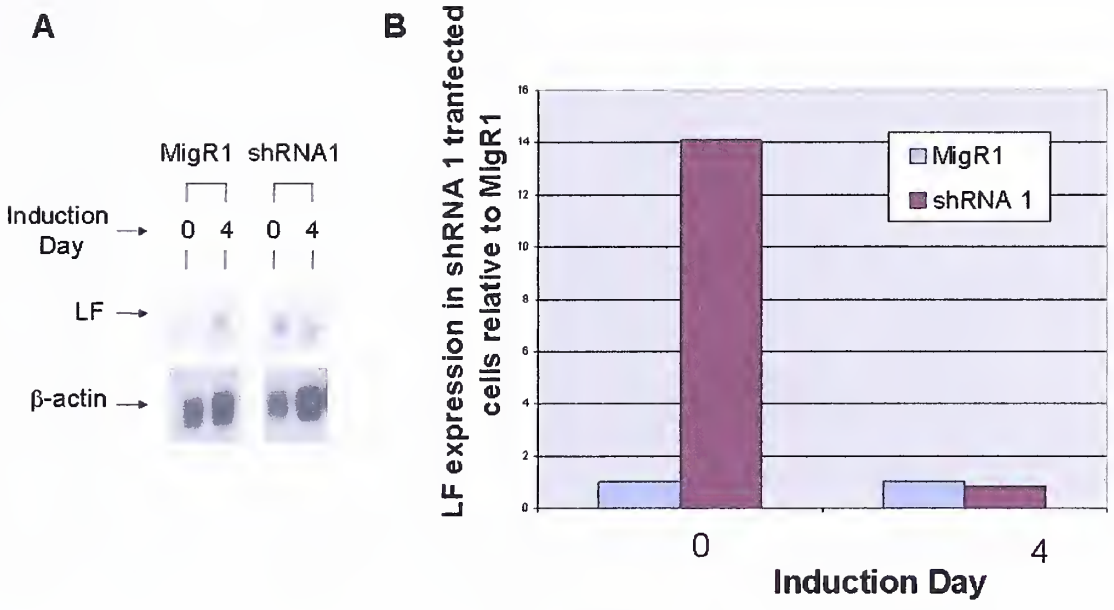


***Figure 10.*** **Northern analysis demonstrates increased LF expression at day 0.** (A) RNA extracts from wt18 cells transfected with MigR1 and shRNA1 were analyzed by northern blot. LF expression is seen at day 0 in cells expressing shRNA, but not in cells expressing MigR1 vector alone. (B) Densitometry analysis of northern blot shows a 14-fold increase in LF expression at day 0 in shRNA expressing cells relative to MigR1. Relative expression of LF at day 4 remains unchanged.

To characterize the effect of CDP knock-down in other CDP-regulated genes I performed Q-PCR of cDNAs from MigR1 and CDP shRNA cell lines for CDP, LF, C/EBPε, and gp91-phox. Q-PCR demonstrated a 3.9-fold decrease in CDP expression with approximately a 12-fold increase in LF expression (Figure 11). Expression of C/EBPε and gp91-phox was unchanged following CDP knock-down.

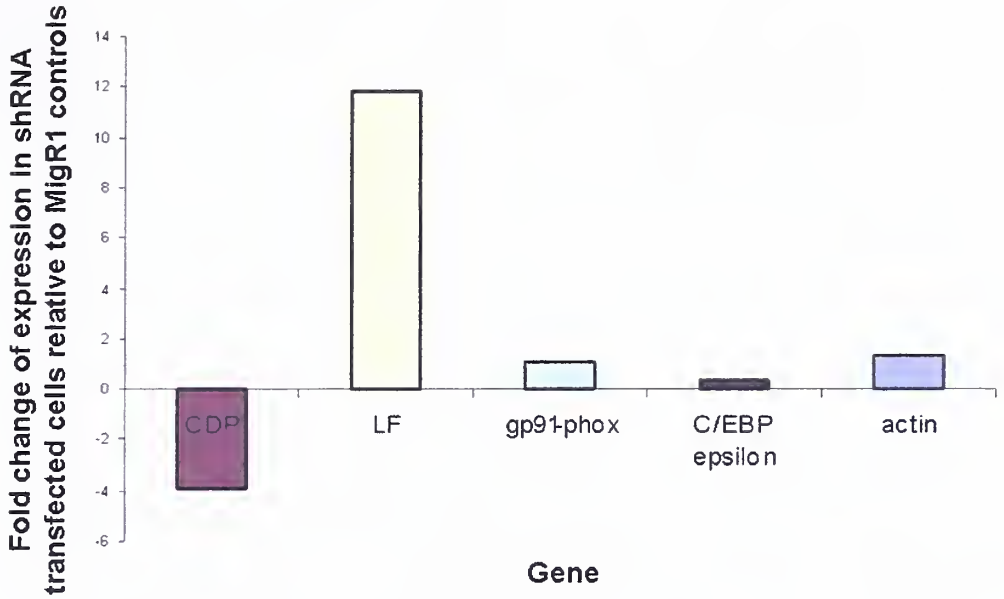


*Figure 11.* **Q-PCR demonstrates decreased CDP expression and increased LF expression in shRNA clone relative to MigR1 controls.** Q-PCR confirms a 3.9-fold decrease in CDP expression in shRNA expressing cells relative to MigR1 cells at day 0. LF expression is also increased 11.8-fold relative to MigR1. Expression of other CDP-regulated genes such as gp91-phox and C/EBP epsilon remains unchanged.

## Discussion

*Generation of cell lines*

I employed both positive and negative selection strategies in an attempt to create stable cell lines expressing CDP shRNA. I performed cotransfections of my shRNA constructs in the MigR1 vector (shRNA 1, shRNA 2, and a combination of shRNA 1 + 2) with empty pBabe-puro vector in a 10:1 ratio, and selected these cells in puromycin to generate stable cell lines. However, given that 32Dwt18 cells have a very low transfection efficiency, on initial inspection of these cells I saw very few GFP-expressing cells. In addition, after initial high rates of cell death upon addition of puromycin, the transfected cells did not regrow.

The shRNA PCR fragment itself also contains a gene for zeocin resistance (Figure 4). Again, 32Dwt18 cells transfected with shRNA constructs did not regrow following addition of zeocin. In conversations with other groups who used the same technique to generate shRNA constructs for various target genes, they have similarly identified difficulties in selecting with zeocin. Possible difficulties with the zeocin resistance gene include inadequate expression or modification of the gene locus which do not allow resistance to zeocin.

Since the shRNA construct is in the GFP-containing MigR1 vector, we performed flow cytometry sorting for GFP expression as an alternative to antibiotic selection. This demonstrated the very low transfection efficiency of 32Dwt18 cells, since only 0.35-

0.55% of cells had GFP expression by flow cytometry. GFP may be expressed at low levels undetectable by microscopy, or the U6 shRNA promoter may compete with the LTR promoter which drives GFP expression. The latter is unlikely, since the shRNA is driven from a RNA polymerase III promoter and GFP expression is driven from a RNA polymerase II promoter (see Figure 5). This positive selection for GFP was the most fruitful method to produce cells stably expressing CDP shRNA. The second sort by flow cytometry yielded adequate purity to allow analysis of the cell lines, as approximately 70% of the cells were then GFP positive. The third flow cytometry sort generated cells that were 99.97% GFP positive, allowing for generation of the final data. During all FACS analysis, strict gating criteria were used in order to maximize the number of GFP positive cells. This strict criterion allows for the possibility that even more cells were GFP positive, albeit at lower levels of expression.

*Cell Growth*

After cells were sorted for the third time, they continued to grow normally for approximately 2 weeks, then died rapidly. After repeating flow cytometry several times, the cells continued to die when they were approximately 99% GFP positive. Cells grown in the same growth medium and incubator that were not sorted a third time, and were thus approximately 70% GFP positive, continued to maintain normal doubling times and growth characteristics. These cells remained approximately 70% GFP positive after a period of growth in culture, suggesting that GFP negative cells do not outgrow those expressing GFP. A trans-effect whereby the GFP negative cells support growth of GFP positive cells is not likely, as CDP is not known to have any extracellular effects. One

hypothesis is that a minimal amount of CDP is essential for normal growth and cell division. When no CDP is available to the cell, it will cease growth. Since CDP is known to have many roles within the cell and at different times in differentiation, this could mean that CDP is essential for proper cell growth and division.

In addition to generating a pooled population of cells expressing the CDP shRNA construct, a monoclonal population was generated by sorting single cells into wells on 96-well plates. This was performed after the second enrichment for GFP. Single cell clones were successfully generated, and several clones were expanded. After Q-PCR testing of these clonal cell lines, it was apparent that some cell lines repressed CDP expression better than others. Q-PCR analysis of these clones showed that they were also the most efficient at upregulating LF expression. However, after approximately one week in culture, clones with the lowest levels of CDP ceased to grow and began to die. Data from these cell clones and from cell pools discussed above suggest that CDP may indeed have an impact on cell growth. Very low or absent levels of CDP expression may contribute to an apoptotic signal within the cell. Alternatively, high level expression of CDP-regulated genes such as LF or other yet unknown factors may negatively influence the growth curve of cells lacking CDP. These possibilities are being actively investigated in the laboratory.

As it has been suggested that CDP may be a tumor suppressor gene product, I performed cell counts to generate a growth curve for cells containing CDP shRNA and MigR1 alone. Since this was impossible to perform on the clonal cell populations with the

lowest levels of CDP, I performed the analysis on pooled cell populations after the second enrichment for GFP. No significant differences in growth characteristics were seen between the two cell types. Cells in growth medium continued to divide exponentially while the growth of those induced to differentiate exhibited a plateau in growth. This was expected, as cells lose proliferative capacity as they differentiate. In addition, CDP shRNA containing cells remain IL-3 dependent. If CDP were indeed a tumor suppressor, one possible effect of repressing CDP could be that this IL-3 dependent cell line could become independent of IL-3. Our results do not support this hypothesis, as cells quickly died in the absence of IL-3. The theory that CDP is a tumor suppressor is not supported here, although much more data are required to fully analyze this potential role. However, this is potentially at odds with the data that show growth inhibition in both clonal and pooled cell populations with highly effective CDP knock-down. Although the knock-down of CDP did not change initial growth characteristics of the cells, the possibility exists that cells cannot grow and divide for a long period of time without CDP. In addition, intermediate levels of CDP expression may be adequate to permit cell growth while allowing LF expression.

*Effect of CDP knock-down*

CDP is known to be expressed in actively dividing cells, and is thought to repress the expression of genes required in terminally differentiated cells. Although preliminary studies did not suggest that CDP overexpression affects morphologic differentiation, the effect of loss of CDP expression remains unknown. Cytospins of uninduced and induced cells did not show any morphological difference between control cells and CDP knock-

down cells. It is likely that morphological differentiation and chromatin condensation is controlled by different mechanisms than biochemical maturation.

Western blot data showing decreased CDP expression in cells containing CDP shRNA is supported by Q-PCR data. One single cell clone containing shRNA 1 indicated a 4-fold decrease in CDP expression. Other single-cell clones also confirmed this data, albeit with varying levels of CDP knock-down. RNAi is known to generate clones with varying levels of expression. As previously mentioned, cells with the highest level of CDP knock-down did not grow long enough to expand for complete analysis. For this reason, I tested many clones before picking a few which best repressed CDP expression while maintaining adequate cell growth.

The RT-PCR data show that LF is undetectable in control MigR1 clones after 22 cycles of PCR, and is expressed at day 4 following induction. This is expected, as immature wild-type myeloid cells do not express SGPs until they are induced to differentiate. SGP expression can normally be seen following induction with a differentiating agent. However, in the CDP shRNA cell clones we can see expression of LF at day 0. This suggests that CDP may be the major repressor of LF expression in maturing granulocytes.

Although CDP knock-down has the effect of upregulating LF expression at day 0, the expression of LF at day 4 of induction was unchanged from control cells. This was a somewhat unexpected result, since it has been clearly shown that C/EBPε, a transcription factor expressed in myeloid progenitors beyond the promyelocyte stage, is an important

inducer of SGP expression. This result could be accounted for in a few ways. The main transactivator of LF expression, C/EBPε, is expressed prior to induction of differentiation. If CDP is indeed the sole repressor of LF, then one could postulate that releasing the cell from CDP regulation following induction of differentiation may put the cell at maximum producing capacity for production of LF. If this were the case, LF expression in day 4 control cells would be at its maximum level. Indeed, CDP knock-down cells may also be at their maximum production capacity in their uninduced state.

Another reason we may detect similar levels of LF expression in day 4 cells is that the sensitivity for detecting these high levels of expression may be low. Densitometry is not sensitive for detecting high levels of expression, as the blot may appear overexposed. It may be necessary to examine a less exposed blot to reevaluate this comparison. However, Q-PCR has the ability to detect more subtle changes in expression, and we can confirm the data by Q-PCR.

CDP shRNA single cell clones were also tested for expression of other CDP regulated proteins, such C/EBPε and gp91-phox. No changes in expression of C/EBPε and gp91-phox were seen in these cell lines. This may suggest that genes expressed earlier in development may have a more complex mechanism of negative regulation. As CDP will still be expressed in the cell up to the point of LF expression, these genes may require the removal of an additional negative regulator to allow their expression. CDP is also a complex protein, with a variety of mechanisms of action. Different combinations of *cut* repeats and the homeodomain, in addition to the recruitment of histone deacetylaces and

phosphorylation, have been proposed as possible mechanisms of action. While it appears by both PCR and western blot that we are able to knock-down the entire protein, it may in fact be that we are knocking down only the part of the protein responsible for repressing LF expression. Alternatively, the small amount of CDP remaining in the cell may have a higher affinity for the early promoters. This change in affinity may be determined by the post-translational modifications discussed above.

*Human Cell Lines*

I also generated three shRNA constructs targeting the human CDP gene, with the goal of expression in NB4 cells. NB4 are a human cell line which contains the t(15:17) PML-RARα translocation. These cells do not express SGPs following induction to differentiate, and CDP has been found to remain bound to the LF promoter following induction. However, morphological differentiation is not affected. We have previously hypothesized that one of the changes associated with leukemic transformation is a disruption of the post-translational modification of CDP, as reflected in the failure to release CDP binding to the LF promoter upon induced maturation. We hypothesized that removal of CDP would permit expression of LF and the other SGPs in this cell line. However, we were unable to generate even a pooled cell population of human CDP shRNA-containing NB4 cells. NB4 cells were appropriately transfected and sorted for GFP expression. These cells were expanded following the first enrichment for GFP, but following the second selection, cells ceased to proliferate. This was attempted several times by several different members of the laboratory without success. We may attempt to generate these cells using antibiotic selection, or by cloning very early in methylcellulose.

However, CDP expression in these cells may be required for cell growth and we may have to explore an alternative approach for analyzing the role of CDP in NB4 cells.

*Conclusions*

Although the role of human CDP knock-down has yet to be explored, I have successfully shown that both human and murine CDP shRNA constructs are effective at knocking down CDP expression in fibroblast cell lines. Expression of LF can be seen by RT-PCR, Q-PCR and northern blot in uninduced 32Dwt18 cells containing shRNA constructs, suggesting that CDP plays a critical role in the negative regulation of LF. However, expression of other CDP-regulated genes, such as gp91-phox and C/EBPε remains unchanged. This suggests that LF expression reflects the direct modulation of CDP binding to its promoter, not an indirect effect of C/EBPε expression. The data on the role of CDP in cell growth have not been fully explored. Data from pools of transfected cells does not seem to support the theory that CDP may function as a tumor suppressor, yet low or absent levels of CDP seem to contribute to a cell death phenotype in both 32Dwt18 and NB4 cells. The results suggest that CDP can function as the sole negative regulatory element for LF gene expression, and that relief of CDP repression can increase LF expression independent of increased binding of positive regulatory factors.

**Future Directions**

To elucidate some of the more subtle differences in gene expression, we will perform cDNA microarray analysis using a myeloid specific cDNA microarray generated in the Berliner Lab in collaboration with Dr. Arch Perkins (Dept of Pathology, Yale University School of Medicine). The results of this microarray will shed light on all genes regulated by CDP during myeloid differentiation, and will provide valuable and more complete information on the role CDP plays in the developing neutrophil. From here, we can further direct our studies on neutrophil-specific gene expression.

Human NB4 cells, which harbor the t(15;17) PML-RARα translocation, do not express SGPs even after induction to differentiate with ATRA. However, morphological differentiation is not affected. In these cells, CDP has been found to continually bind to the LF promoter following morphological differentiation. I have generated three human CDP shRNA constructs and tested them for CDP knock-down in HeLa cells. However, we have encountered difficulty in placing these constructs in NB4 cells. Stable NB4 cell lines expressing these constructs need to be generated, and a similar analysis of NB4 CDP shRNA cell lines will be conducted. We hypothesize that the knock-down of CDP in these NB4 cells will allow expression of LF as well as other SGPs.

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