IDENTIFICATION OF A MINIMAL CIS-ELEMENT AND COGNATE TRANS-FACTORS REQUIRED FOR THE REGULATION OF *RAC2* GENE EXPRESSION DURING K562 CELL DIFFERENTIATION

Rajarajeswari Muthukrishnan

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology,
Indiana University

December 2008

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

David G. Skalnik Ph.D, Chair

B. Paul Herring Ph.D

Doctoral Committee

Simon J. Rhodes Ph.D

Ronald C. Wek Ph.D

Date of Defense
October, 1, 2008

I dedicate this work to my loving parents Muthukrishnan and Lakshmi, my caring brother Karthik, my amazing husband Suresh and my adorable son Charan

ACKNOWLEDGEMENTS

It is a pleasure to thank all the people who made this thesis possible. I would like to express my deep and sincere gratitude to my supervisor, Dr. David G. Skalnik for his invaluable support, encouragement, supervision and useful suggestions throughout this research work. Most of all, I would like to thank him for his patience and understanding during the tough times of my research. As a result my research life was enjoyable and rewarding. I would like to thank each member of my committee – Dr. Ronald C. Wek, Dr. Simon J. Rhodes and Dr. B. Paul Herring for their support, guidance, wise advices and critical comments during my graduate research. I am greatly indebted to my undergraduate vice-chancellor late Dr. Rajammal P. Devadoss for the opportunity to choose biochemistry for my carreer. I would have been lost without her help and support. I would like thank my masters mentor Dr. S. Shanmugasundram, undergrad teachers Dr. Padma, Dr. Jeyanthi, Dr. Saroja for their guidance, support and inspiration.

I am thankful to my lab colleagues Dr. Jeong Heon Lee, Courtney M. Tate, Erika Dobrota, Dr. Jill S. Butler, Suzanne Young and Hitesh Kathuria for their friendship and for providing a stimulating and fun environment for research. I truly enjoyed working with them. I am especially grateful to Dr. Jeong Heon Lee for his time and critical suggestions during my research.

I appreciate the help of other labs in Wells Center of Pediatric Research for their support. My special thanks to everyone in Kelley lab for their friendship. I would like to thank Field lab, Dinauer lab and Conway lab for providing technical help for my

experiments anytime I walked in. I am also grateful to the office staffs in the Biochemistry and Pediatrics departments for providing administrative help anytime.

I wish to thank my incredible group of friends – Dr. Sirisha Asuri, Judy Rose James, Sirisha Pocha Reddy, Sulochana devi Baskaran for helping me get through the difficult times, and for all the emotional support, friendship, and caring they provided. I also want to thank my friends Nanda Kumar and Krupakar for their friendship and support in helping me with my decision to do Ph.D abroad. I am grateful to my wonderful group of undergraduate friends Hema Ishwarya, Sathya priya and Uma for their love, friendship and support.

Lastly, and most importantly, I wish to thank my parents, B. Muthukrishnan and Lakshmi Muthukrishnan for their unconditional love and support through out my life. They always trusted and accepted me for what I am and constantly encouraged me to aim high and work hard. They have worked hard to provide me with the best in everything. I am truly blessed to have such wonderful parents and they mean the world to me. I am grateful to my wonderful brother and friend M. Karthikeyan and his family, whose constant encouragement, guidance and love I have relied throughout my life. Nothing of this would have been possible without the love, caring and support of my amazing husband and best friend Suresh Annangudi. He encouraged and guided me through my tough times in Ph.D. He taught me to learn from negative results during research and enjoy the science in it. He constantly reminds me about the beautiful and fun filled life besides science. My special thanks to my adorable son Charan annangudi who was born right after my thesis defense.

ABSTRACT

Rajarajeswari Muthukrishnan

IDENTIFICATION OF A MINIMAL CIS-ELEMENT AND COGNATE TRANS-FACTORS REQUIRED FOR THE REGULATION OF *RAC2* GENE EXPRESSION DURING K562 CELL DIFFERENTIATION

This dissertation examines the molecular mechanisms regulating *Rac2* gene expression during cell differentiation and identification of a minimal cis-element required for the induction of *Rac2* gene expression during K562 cell differentiation. The Rho family GTPase Rac2 is expressed in hematopoietic cell lineages and is further up-regulated upon terminal myeloid cell differentiation. Rac2 plays an important role in many hematopoietic cellular functions, such as neutrophil chemotaxis, superoxide production, cytoskeletal reorganization, and stem cell adhesion. Despite the crucial role of Rac2 in blood cell function, little is known about the mechanisms of *Rac2* gene regulation during blood cell differentiation. Previous studies from the Skalnik lab determined that a human *Rac2* gene fragment containing the 1.6 kb upstream and 8 kb downstream sequence directs lineage-specific expression of Rac2 in transgenic mice. In addition, epigenetic modifications such as DNA methylation also play important roles in the lineage-specific expression of Rac2.

The current study investigated the molecular mechanisms regulating human *Rac2* gene expression during cell differentiation using chemically induced megakaryocytic differentiation of the human chronic myelogenous leukemia cell line K562 as the model system. Phorbol 12-myristate 13-acetate (PMA) stimulation of K562 cells resulted in

increased *Rac2* mRNA expression as analyzed by real time-polymerase chain reaction (RT-PCR). Luciferase reporter gene assays revealed that increased transcriptional activity of the *Rac2* gene is mediated by the *Rac2* promoter region. Nested 5'- deletions of the promoter region identified a critical regulatory region between -4223 bp and -4008 bp upstream of the transcription start site. Super shift and chromatin immunoprecipitation assays indicated binding by the transcription factor AP1 to three distinct binding sites within the 135 bp minimal regulatory region. PMA stimulation of K562 cells led to extensive changes in chromatin structure, including increased histone H3 acetylation, within the 135 bp *Rac2* cis-element.

These findings provide evidence for the interplay between epigenetic modifications, transcription factors and cis-acting regulatory elements within the *Rac2* gene promoter region to regulate Rac2 expression during K562 cell differentiation.

David G. Skalnik, Ph.D
Committee Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AP1	activator protein 1
ATF	activating transcription factors
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BAF	Brg1-associated factors
Bp	base pair
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CMV	Cytomegalovirus
CPM	counts per minute
CSF	colony stimulating factor
C_t	threshold cycle
CTP	cytidine triphosphate
CTD	C-terminal domain
ºC	degree centigrade
DEPC	diethyl pyrocarbonate
Dd	double distilled water
DMSO	dimethyl sulphonic acid
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	dna methyltransferase
DTT	Dithiothreitol
DUB	de-ubiquitinating enzyme

EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
ES cells	embryonic stem cells
EtOH	Ethanol
GM-CSF	granulocyte-macrophage colony stimulating factor
GTP	guanosine triphosphate
H	hour(s)
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	N-2-hydroxythylpiperazine-N'-2-ethanesulfonic acid
HEX1/HEXO1	human exonuclease I
HMT	histone methyltransferase
HP1	heterochromatin protein 1
K	lysine
Kb	kilobases
LB broth	luria-Bertani broth
LCR	locus control region
Luc	luciferase
mRNA	messenger RNA
MBD	methyl CpG binding domain
MeCP	methyl CpG binding protein
MNase	micrococcal nuclease
Mg	milligram

Min	minute(s)
mM	millimolar
M	molar
NADPH	nicotinamide adenine dinucleotide phosphate
ng	nanogram
nt	nucleotide
NuRD	nucleosome remodeling deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HSC/P	hematopoietic stem cells/Progenitor cells
ONPG	ortho-nitrophenyl-β-galactoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDD	phorbol-12, 13- didecanoate
PMSF	phenylmethylsulfonyl fluoride
PMA	phorbol 12-myristate 13-acetate
Pol II	RNA polymerase II
PSA	prostate specific antigen
RAC2	Ras related C3 botulinum toxin substrate
RNA	ribonucleic acid
RPA	RNase protection assay
RT	room temperature
RT-PCR	Real-time-Polymerase chain reaction
ROS	reactive oxygen species
s	second(s)

SAM	S-adenosyl-L-methionine
SCF	stem cell factor
SDS	sodium dodecyl sulfate
TAF	TBP-associated factor
TB	Terrific broth
TBE	tris-borate EDTA buffer
TBP	TATA box binding protein
TRE	TPA response element
TSA	trichostatin A
UTP	uridine triphosphate
V	Volts
XIC	X-inactivation center

INTRODUCTION

I. Transcriptional regulation of genes

Genomic DNA is the ultimate template of our heredity. In spite of the completion of the human genome project, many challenges remain in understanding the regulation of genetic information. Human cells contain 20,000-25,000 genes, which include housekeeping genes, genes expressed during cell differentiation, genes constitutively expressed in differentiated cells and genes expressed upon stimulation. Coordination of gene expression in eukaryotes is intricate as cis-acting DNA sequences can be located tens of thousands of base pairs away from the transcription start site. Therefore dynamic interplay between the cis-acting elements and DNA binding proteins is needed for proper regulation of these genes. Regulatory elements of a gene include the basal promoter, enhancers, silencers, insulators and locus control regions (LCRs). These elements are recognized by sequence-specific DNA binding proteins.

Promoters are located immediately 5' to the start of each gene and contain binding sites for transcription factors. RNA polymerase begins transcription at the start site of the gene denoted as nucleotide +1. The basal elements of the promoter include the TATA box and the initiator. Some, but not all eukaryotic promoters, contain a TATA box that has a consensus sequence (TATAa/tAa/t) that is positioned close to the transcription start site. An initiator is often found centered at the transcription start site and has a consensus sequence YYANa/TYY, where Y denote a pyrimidine and N is any base. These elements facilitate melting or unwinding of DNA during RNA polymerase loading. These two elements constitute the core promoter to allow accurate initiation of

transcription. The factors that are required for the proper recruitment of RNA polymerase onto the promoter allows transcription by the basal transcription machinery.

The basal transcription factors needed for the loading of RNA polymerase to the promoter include TATA binding protein (TBP), TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH. Binding of TBP to the TATA box is the first step in the assembly of the basal transcriptional apparatus. TBP along with some associated factors binds to the promoter as a complex referred to as TFIID. TFIIA then binds to TBP and stabilizes its interaction with the DNA. Binding of TBP to TATA box distorts the DNA and allows binding of TFIIB that provides the platform for the recruitment of RNA polymerase II (Pol II). Pol II is found associated with a factor called TFIIF, a helicase that is involved in the melting of the DNA. TFIIH has kinase and helicase activity. The kinase activity of this factor is required for the phosphorylation of the C-terminus domain (CTD) of Pol II that consists of repeats of the amino acid sequence Y-S-P-T-S-P-S (Spangler, Wang et al. 2001). The CTD phosphorylation of Pol II plays an important role in transcription initiation and elongation. Pol II with unphosphorylated CTD binds to the start site prior to transcription initiation and this interaction is lost when CTD is phosphorylated. Phosphorylation of CTD at serine 5 by TFIIH facilitates promoter clearance of RNA polymerase during transcription initiation (Jones, Phatnani et al. 2004). In addition to the basal transcription factors, transcription factors such Sp1 and NF1 can bind to the proximal promoter region and help in the loading of Pol II (Yang 1998; Phatnani and Greenleaf 2006). Proximal promoter elements consist of additional binding sites for transcription factors and are sensitive to their precise location. Apart from the promoter region, various other

regulatory regions are also present throughout the gene locus to facilitate gene transcription.

Enhancers are position and orientation independent DNA elements that may be located upstream or downstream of a gene, within a gene, or thousands of base pairs away from the start site of the gene. Enhancers contain binding sites for transcription factors that communicate with the basal transcription machinery to increase the rate of transcription of the targeted gene. Enhancer sites have been proposed to interact with the basal transcriptional apparatus by mechanisms inducing DNA looping, scanning or chromatin remodeling.

DNA looping permits transcription factors bound to enhancer sites to contact other proteins in the basal promoter (Bulger and Groudine 2002). In the scanning model, Pol II binds to the enhancer and scans along the DNA until it reaches the promoter (Blackwood and Kadonaga 1998). In the chromatin remodeling model, factors that bind the enhancer can propagate a change in the chromatin structure that facilitates recruitment of the basal transcription machinery (Ward, Hernandez-Hoyos et al. 1998). Many enhancer elements in higher eukaryotes activate gene transcription in a tissue or differentiation specific manner. This is achieved in two ways. An enhancer can act in a specific manner if the activator that binds to it is present in only some types of cells. Alternatively, if a tissue-specific repressor can bind to a silencer element located near the enhancer element, making the enhancer inaccessible to its transcription factor. Silencers are regulatory regions like enhancers. However, binding of transcription factors to these elements facilitate repression of gene expression.

Boundary or insulator elements are regions that create autonomous regulatory domains within a genome (Kuhn and Geyer 2003). Efficient enhancer and silencer action require mechanisms that facilitate their interaction with specific promoters. Insulators can lie between the regulatory elements of genes and prevent inappropriate action of these elements on neighbouring genes (Bondarenko, Liu et al. 2003). The nucleotide sequence CCCTC is present in all insulators discovered so far, and this sequence can bind the protein CTCF (CCCTC binding factor). Insulators prevent the spread of the chromatin domain between two loci (Bell, West et al. 2001).

LCRs are elements that are present several kilobases away from the gene, but exert their effect on transcription by establishing and maintaining an open chromatin configuration at the locus, which facilitates expression of all genes present in a cluster (Festenstein, Tolaini et al. 1996). Most LCRs are DNase I hypersensitive and they may also have enhancer activity. These elements were first identified in the *β-globin* gene locus and are critical for the regulation of the cluster (Caterina, Ryan et al. 1991).

Introns are parts of primary transcripts encoded in DNA that are removed by splicing during pre-RNA processing. It was believed that these non-coding sequences were junk, meaning they had no function in an organism and could be ignored. But, in recent years, mounting evidence suggests that this is not the case. Although they are non-coding, there is strong evidence that many introns function as regulators of transcription. The *SOX9, RNS2, β1 tubulin, c-fms, and ADH* genes are examples of genes carrying regulatory elements in their intronic regions (Köhler 1996; McKenzie 1996; Tiffany 1996; Himes 2001; Morishita 2001).

II. Chromatin Structure

Chromatin is a complex of DNA and protein that makes up chromosomes. Nucleosomes constitute the fundamental repeating unit of chromatin and are interconnected by linker DNA. Nucleosomes are further folded through a series of successively higher ordered structures, which form chromosomes. This compaction provides physical packaging needed to fit the large eukaryotic genomes into the nucleus, and provide for an additional level of control needed for the regulation of gene expression (Caterino and Hayes 2007).

Histones are basic proteins and are the major protein component present in chromatin. The nucleosome core particle consists of approximately 147 base pairs of DNA wrapped twice around a histone octamer consisting of 2 copies each of the core histones H2A, H2B, H3, and H4. Linker histone H1 is involved in the compaction of chromatin by binding to the linker DNA. Thus a nucleosome without the linker histone H1 resembles beads on a string of DNA. The tight interaction of DNA with histone proteins protects them against micrococcal nuclease activity in experimental tests. Nucleosomes can prevent binding of RNA polymerase or transcription factors to regulatory regions at inappropriate times, thereby providing control over gene expression. These packaged regulatory regions undergo remodeling during gene activation to provide access to DNA binding factors. The two major chromatin structures found in eukaryotes are euchromatin and heterochromatin.

1. Heterochromatin

Heterochromatin is a tightly packed form of DNA and is characteristic of transcriptionally repressed genes and repetitive DNA. Genetically inactive satellite sequences, centromeres, and telomeres are all heterochromatic. A whole chromosome can even be maintained in a heterochromatic state as seen in the case of X chromosome inactivation.

X chromosome inactivation is a process by which one of the two X chromosomes in the cells of female mammals is randomly inactivated early in development, so that females with two X chromosomes do not have twice as many gene products as males who possess only a single copy of the X chromosome. The X-inactivation center (XIC) on the X chromosome contains sequences that are necessary and sufficient to cause X-inactivation. The XIC contains a non-translated RNA gene called *Xist* that is involved in X-inactivation by coating the chromosome to be inactivated.

Heterochromatin plays an important role in gene regulation by rendering the DNA inaccessible to DNA binding factors. The two forms of heterochromatin include constitutive heterochromatin and facultative heterochromatin. Some regions of DNA, such as centromeres that are tightly packed in all cells resulting in poor expression, is known as constitutive heterochromatin. Facultative heterochromatin are regions of DNA that are euchromatic in some cells and heterochromatic in other. This type of chromatin structure is associated with differentiation- or tissue-specific genes. The family of heterochromatin protein 1 (HP1) is usually associated with heterochromatin, heightening tight packaging of DNA by interacting with histone proteins (Cheutin, McNairn et al. 2003).

2. Euchromatin

Euchromatin is a lightly packed form of DNA and is characteristic of transcriptionally active genes. The less compact structure of DNA allows access to DNA binding factors and RNA polymerase, thereby permitting transcription. The main example of euchromatin includes house keeping genes that need to be expressed all the time.

III. Epigenetic regulation of genes

Epigenetics is the study of heritable changes in gene expression brought about by the chemical marks that are added to the DNA or chromatin proteins without any changes in the nucleotide sequences (Wolffe and Matzke 1999). Epigenetic modifications communicate with the promoter sequences and transcription factors to control when and where genes are expressed. Epigenetic processes modifications include cytosine methylation of genomic DNA and covalent modification of histone proteins. They participate in establishing specific chromatin states, which dictate heritable patterns of gene expression. Thus epigenetic modifications serve as a rich source of regulation in addition to the genetic instruction written in the genetic code itself (Fuks, Burgers et al. 2000). Epigenetic regulation can be short term or long term.

Short term epigenetic regulation involves transient control of differentiation-specific genes that shows flexible patterns of gene expression (Cavalli 2006). For example, the Homeobox (*Hox*), and distal-less homeobox (*Dlx*) gene families are required during later stages of cell differentiation and are therefore repressed in undifferentiated stem cells (Lee, Jenner et al. 2006). On the other hand, genes required

for maintaining pluripotency in stem cells, such as transcription factors OCT4 and NANOG (Hattori, Imao et al. 2007), are repressed upon differentiation of stem cells. Long term epigenetic regulation involves silencing of transposons and imprinted genes that are stably maintained in a repressed state by epigenetic marks for many cell divisions.

1. DNA methylation

CpG islands are genomic regions that are at least 200 bp, with greater than 50% GC content. CpG islands are enriched at the promoters and transcription start sites of house keeping genes. DNA methylation is an epigenetic modification that occurs in the context of the CpG dinucleotide and is associated with heterochromatin. It involves addition of a methyl group to the carbon 5 position of the cytosine ring from the methyl donor S-adenosyl-L-methionine (SAM). The cytosine residue in the complementary CpG is also methylated symmetrically (Ballestar and Wolffe 2001). Around 60-90% of all CpG dinucleotides are methylated in mammals. The group of enzymes that catalyzes this reaction is DNA methyltransferases. These enzymes are responsible for establishing DNA methylation during development and for its propagation to new strands during replication. DNA methylation affects genome functions by inhibiting gene transcription.

All diploid organisms inherit two copies of autosomal chromosomes, one from each parent, during fertilization. Expression occurs from both alleles for a vast majority of the genes. However, some genes are repressed depending on its parental origin. These genes are called imprinted genes. DNA methylation plays an indispensable role during

two major epigenetic phenomena found in mammals, genomic imprinting and X-chromosome inactivation.

Cytosine methylation alters local chromatin structure by interaction with other epigenetic modifications such as histone protein modifications. In addition, it also works by changing or blocking the protein-DNA interactions required for gene expression. Methylation is known to block binding of transcription factors such as AP-2, c-Myc/Myn, E2F, Sp1 and NF-κB to their target regions (Tate and Bird 1993).

DNA methylation plays an important role in the regulation of tissue-specific gene expression, such as the *Globin* gene cluster (Goren, Simchen et al. 2006), *Rac2* (Ladd, Butler et al. 2004), *Shank3* (Beri, Tonna et al. 2007), and the Killer immunoglobulin-like receptors (*KIR*) (Chan, Kurago et al. 2003). DNA methylation is also found in the downstream transcribed regions of a gene (Intragenic CpG methylation), where it may play a role to decrease the efficiency of elongation by inducing formation of a compact chromatin structure (Lorincz, Dickerson et al. 2004). Intragenic CpG methylation also prevents the activation of transposable elements and maintains these parasitic elements in a silent state. In bacteria, methylation is part of the system that protects the host against bacteriophage infection (Wolffe and Matzke 1999).

(i) DNA methyltransferases and demethylases

De novo methylation and maintenance methylation are two distinct processes required for the establishment and inheritance of cytosine methylation patterns. Both these processes are essential for embryonic development beyond gastrulation. The enzymes that carry out these functions are classified into three groups: de novo methyltransferases, which includes the Dnmt3 family (Dnmt3a and Dnmt3b), the maintenance DNA methyltransferase Dnmt1, and Dnmt2.

De novo methyltransferases (Dnmt3 family)

This group of enzymes catalyzes the addition of a methyl group to CpG base pairs, resulting in the creation of a new hemi-methylated CpG. De novo methylation activity is detected predominantly in early embryos and it plays an important role in organizing and compartmentalizing the genome during tissue differentiation (Okano, Bell et al. 1999). Methyltransferases also play a crucial role in the inactivation of X chromosomes in mammals (Plath, Mlynarczyk-Evans et al. 2002; Okamoto, Otte et al. 2004) and genomic imprinting (Li, Beard et al. 1993; Li, Beard et al. 1993) in mammals. The Dnmt3 family consists of two genes, *Dnmt3a* and *Dnmt3b*. These genes are highly expressed in undifferentiated embryonic stem (ES) cells and are down-regulated after differentiation. Inactivation of both *Dnmt3a* and *Dnmt3b* disrupts de novo methylation in ES cells and de novo methylation occurs genome wide during early development (Okano, Bell et al. 1999). In addition, Dnmt3a and Dnmt3b exhibit distinct functions during development. Dnmt3b is important during early development and methylates a broad range of target sequences, whereas Dnmt3a is critical during late development,

methylating particular set of sequences. For example, Dnmt3b specifically methylates the minor satellite repeats in centromeres of embryos. Dnmt3a and Dnmt3b are transcriptional repressors that localize to heterochromatin regions (Bachman, Rountree et al. 2001). Dnmt3a binds histone deacetylase 1 (HDAC1), acting as a transcriptional co-repressor with deacetylase activity by associating with the DNA-binding transcriptional repressor RP58 (Fuks, Burgers et al. 2001). Dnmt3b interacts with the chromatin remodeling enzyme Hsnf2h and HDACs 1 and 2 to repress transcription (Geiman, Sankpal et al. 2004).

Maintenance methyltransferase (Dnmt1 family)

Dnmt1 was the first eukaryotic methyltransferase to be purified and its cDNA cloned. Dnmt1 is the most abundant DNA methyltransferase in somatic cells (Robertson, Uzvolgyi et al. 1999). Dnmt1is highly expressed during S phase (Robertson, Keyomarsi et al. 2000) and is targeted to sites of DNA replication in mammalian nuclei (Leonhardt, Page et al. 1992). Consistent with its expression pattern, this maintenance methylase shows specificity towards hemi-methylated DNA. This form of modified DNA is the immediate product of replication. Disruption of the *Dnmt1* gene in mice results in several interesting phenotypes, such as severe demethylation, biallelic expression of most imprinted genes such as *H19* and *Kcnq1ot1* (Li, Beard et al. 1993), activation of all X chromosomes due to the demethylation of *Xist* (Beard, Li et al. 1995), and an increase in rates of loss of heterozygosity because of mitotic recombination (Li, Bestor et al. 1992). *Dnmt1* is the only gene known to be required for the repression of transposons in mammalian somatic cells (Damelin and Bestor 2007).

DNMT2

Dnmt2 contains all the sequence motifs diagnostic of DNA (cytosine-5)-methyltransferases, but lacks the large N-terminal regulatory domain common to other eukaryotic methyltransferases (Yoder and Bestor 1998). Dnmt2 is ubiquitously expressed and shows an expression pattern similar to Dnmt1. The gene is well conserved among eukaryotes, including mammals, *Arabidopsis thaliana, Xenopus laevis, Drosophila melanogaster* (Gowher, Leismann et al. 2000) and *Danio rerio,* which contain methylated genomes, but also in organisms lacking detectable cytosine methylation, such as yeast. The yeast *pmt1* gene belongs to Dnmt2 family but is enzymatically inactive due to amino acid change at a potentially catalytic site (Pinarbasi, Elliott et al. 1996). Human Dnmt2 does not catalyze methylation of DNA, but instead methylates a small RNA- $tRNA^{Asp}$ specifically cytosine-38 in the anticodon loop (Goll, Kirpekar et al. 2006).

(ii) Cytosine Demethylase

A genome wide demethylation occurs early in development, and specific sites in imprinted genes escape demethylation, maintaining a methylated state throughout pre-implantation development (Kafri, Gao et al. 1993). Demethylation is also found during cell differentiation and in cancer. DNA demethylation can occur by two possible mechanisms. The first mechanism involves passive demethylation, when methylation is not maintained during DNA replication, and the second mechanism involves active demethylation which is the the removal of methylation catalyzed by a demethylase. The identity of the demethylase activity is controversial. The DNA demethylase MBD2b has been reported to catalyze the hydrolytic removal of methyl residues from methyl cytosine

in DNA and to demethylate both hemi-methylated and fully methylated DNA (Bhattacharya, Ramchandani et al. 1999). It is a member of a conserved family of MBD (methyl CpG-binding domain) proteins (Lewis, Meehan et al. 1992). The processivity of this enzyme helps in the removal of methyl groups from DNA without damaging the DNA, thus maintaining the integrity of the genome (Cervoni, Bhattacharya et al. 1999). However, this finding has not been reproduced by other laboratories to date. 5-methyl cytosine DNA glycosylase is also an important candidate for a demethylase in vivo (Zhu, Benjamin et al. 2001).

(iii) Methyl CpG binding proteins

Methyl CpG binding proteins play critical roles in deciphering epigenetic methylation patterns by mediating interactions between DNA methylation, histone modifications and other chromatin components. They are also required to hinder DNA-binding activities of several transcription factors such as E2F, CREB, AP2, and cMyb. The five major family members of this group of proteins include MeCP2, MBD1, MBD2, MBD3 and MBD4. All these proteins share a conserved DNA-binding domain (MBD). The *MeCP2* gene is located on the X chromosome and MeCP2 is a transcriptional repressor with abundant binding sites in chromatin (Nan, Campoy et al. 1997). This gene is mutated in patients with Rett syndrome, a neuro developmental disorder characterized by loss of speech, autism, ataxia, and mental retardation. MeCP2 null mice show loss of normal expression of several imprinted genes, such as *Dlx5, Dlx6 Ube3A,* and *Gabrb3* (Horike 2005; Samaco, Hogart et al. 2005). MeCP2 mediates transcription repression by interacting with other chromatin remodeling complexes. MeCP2 associates with the co-

repressor molecule Sin3a to maintain the repressed state of the *Bdnf* gene (Martinowich, Hattori et al. 2003).

MBD1 interacts with the histone H3 lysine 9 (H3K9) methyltransferase enzyme SETDB1, coupling DNA methylation and histone modification. Depletion of MBD1 results in hypomethylation on histone H3K9 and reactivates the normally silenced gene *p53BP2* (Tumor protein p53 binding protein 2) (Sarraf and Stancheva 2004). MBD2-deficient mice are viable, but lack the MeCP1 complex, and therefore show defective repression of methylated promoters. MBD2 is the key silencer of IL4 expression in naïve T cells and is displaced by the transcription factor GATA3 upon differentiation to allow IL4 expression (Hutchins, Mullen et al. 2002). MBD3 is required for proper embryonic development, as shown by knock out studies. In contrast to other Methy CpG binding proteins, MBD4 is known for its role in DNA repair and as a transcriptional repressor.

2. Histone protein modifications

The histone tails are subject to a variety of post-translational modifications, including acetylation, phosphorylation, methylation, ubiqutination and ADP-ribosylation (Van Holde 1989). These modifications are site-specific, interdependent and can induce distinct organization of the chromatin.

(i) Histone methylation

Methylation of histones occurs on histones H3, H4, and H2B (Nakayama, Rice et al. 2001) and can occur at multiple residues. Defined methylation patterns are related to distinct functional readouts of chromosomal DNA (Peters 2005). They have been shown

to play important roles in several genomic functions, including heterochromatin formation, X chromosome inactivation, and epigenetic gene regulation at euchromatic and heterochromatic positions, and therefore have extensive implications for proliferation, cell-type differentiation, development, gene expression, genome stability and cancer. Histone methylation does not influence the net charge of the histone tails and as a result does not alter the interaction of the histone tails with the DNA. Instead, methylated histones act as a recognition template for effector proteins to influence transcription (Byvoet, Shepherd et al. 1972). The major methylation sites within the histone tails are the basic amino acid residues lysine and arginine (Santos-Rosa, Schneider et al. 2002). Arginine can be either mono- or di-methylated, while lysine can be mono-, di-, or tri-methylated (Santos-Rosa, Schneider et al. 2002).

Histone methylation occurs on lysine residues 4, 9, 27, 36 and 79 in H3 and on position 20 in H4 (Nakayama, Rice et al. 2001). Methylation of histone H3K4 and H3K36 are associated with activation of transcriptional activity, and histone H3K9 and H3K27 methylation are associated with repressive chromatin. H3K79 methylation has been associated with both transcriptional activation (Im, Park et al. 2003) and repression (Wood, Schneider et al. 2005). Histone H3K36 methylation has been linked to efficient elongation. Histone H3K4 can be mono-, di- or tri-methylated and are present on all autosomes and in both the promoter and the coding regions of active genes (Santos-Rosa, Schneider et al. 2002). Significant levels of di- and tri-methyl H3K4 are mark of gene activity in eukaryotes, although di-methylated H3K4 is present in some repressed genes. The methionine-regulated *MET16* gene shows tri-methylation of H3K4 only when this gene is active, whereas di-methylation of H3K4 is seen even in the repressed state. This

result shows that di-methylated H3K4 may play a role in determining a transcriptionally permissive state, and the tri-methylated state may allow for an active chromatin configuration (Santos-Rosa, Schneider et al. 2002). The levels of di- and tri-methylation are very low in 3' transcribed regions of genes, suggesting that these modifications are not absolutely required for passage of Pol II (Schneider, Bannister et al. 2004).

Accumulation of histone H3K9 methylation is seen at the inactive X-chromosome (Boggs, Cheung et al. 2002), and its presence has been linked to heterochromatic subdomains and gene silencing (Saccani, Pantano et al. 2001; Santos-Rosa, Schneider et al. 2002). H3K9 methylation creates a binding site for the heterochromatic HP1 proteins that is recruited by the tumor suppressor retinoblastoma protein (pRb) and thus helps in the induction and propagation of heterochromatic subdomains (Jenuwein 2001).

Histone methyltransferases (HMTases) are a major group of enzymes that catalyze the transfer of methyl groups to histone proteins. This class of protein methyltransferases is characterized by the presence of one invariant protein motif, the SET domain, which has the potential to methylate lysine in the N-terminal tail of histones. There are about ten different SET-domain containing protein families which include more than 70 gene sequences in mammals (Jenuwein 2001). The mammalian homologue of the *Drosophila* position-effect variegation (PEV) modifier Su(var)3-9, Su(var)39h1 and Su(var)39h2 were the first identified proteins with histone methyltransferase activity. Su(var)39h enzymes are selective for histone H3K9 methylation. In addition to the SET domain that is crucial for catalytic activity, Su(var)39 proteins also contain a chromodomain which is found in chromatin related proteins and a cysteine rich domain. H3K9 methylation creates a binding site for the heterochromatic

HP1 proteins that form complexes with Suv39h HMTases. *Suv39h* knock-out mice show impaired viability and reduced genomic stability, and display increased risk for B-cell lymphoma (Peters, O'Carroll et al. 2001). Thus HMTases serve as important epigenetic regulators during development. *G9a* is another SET-domain protein containing a dual HMTase which methylates histone H3 at the K9 and K27 positions (Tachibana, Sugimoto et al. 2001).

The other group of histone methyltransferases that catalyzes methylation of histone H3 at K4 position is SET-9/SET-7 (Wang, Cao et al. 2001). These enzymes lack the SET-domain associated cysteine-rich regions found in Suv39 proteins, suggesting that these regions may provide for the substrate specificity for SET domains. SET-1 in *Saccharomyces cerevisiae* is a histone H3K4 methyltransferase, but mediates gene silencing (Briggs, Bryk et al. 2001), and the SET-2 methyltransferase shows selectivity for histone H3K36 methylation and also medites gene repression (Strahl, Grant et al. 2002). The SET-7 methyltransferase selectively methylates histone H4 at the K20 position (Nishioka, Rice et al. 2002).

(ii) Histone arginine methylation

Arginine methylation occurs on either or both of the two terminal guanidino nitrogen atoms (Aletta, Cimato et al. 1998). The five distinct classes of arginine methyltransferases include PRMT1, PRMT2/HRMT1L1 (Scott, Kyriakou et al. 1998), PRMT3 (Tang, Gary et al. 1998), CARM1 (Chen, Ma et al. 1999), and JBP1 (Pollack, Kotenko et al. 1999). CARM1 can cooperate with p160 co-activators to enhance the ability of nuclear receptors to activate transcription (Chen, Ma et al. 1999). CARM1 and

PRMT1 are recruited to the promoter through contact with the p160 coactivator to methylate histone H3 and H4, respectively (Koh, Chen et al. 2001).

(iii) Histone demethylases

Histone demethylases catalyze the removal of methyl groups on histone lysine and arginine residues. The two major groups of histone lysine demethylases identified so far include lysine-specific demethylases 1 (LSD1) and jumonji C (JmjC) family proteins. Peptidyl arginine deiminase 4 (PAD4/PAD14) catalyzes demethylation of monomethyl arginine. LSD1 catalyzdemethylation of H3K4me1/2, but not tri-methylated H3K4 (Shi, Lan et al. 2004). LSD1 contains a C-terminal amine oxidase like (AOL) domain, which creates a catalytic center for substrate binding, and an N-terminal SWIRM domain, which is important for the stability of LSD1. The third domain is the tower domain that regulates the catalytic activity of LSD1 (Chen, Yang et al. 2006; Stavropoulos, Blobel et al. 2006). LSD1 is often found as a multiprotein complex with HDAC1/2, corepressor CoREST, and a PHD domain containing protein BHC80 (Shi, Lan et al. 2004; Lee, Wynder et al. 2006). Thus, this complex catalyzes histone deacetylation and demethylation. LSD1 also associates with a complex containing MLL1, a histone H3K4 methyltransferase (Nakamura, Mori et al. 2002). This suggests a balance between methylated and unmethylated H3K4 for the regulation of gene transcription. LSD1 is required for ES cell lineage determination and differentiation. JmjC family proteins include JHDM1, JHDM2, and JMJD2 subfamilies. JHDM1 demethylates mono and dimethylated H3K36 (Tsukada, Fang et al. 2006). JMJD2 proteins catalyze demethylation of trimethylated histone H3-K9/36 (Whetstine, Nottke et al. 2006). JmjC

proteins can associate with several other proteins such as HDAC 1/2/3, nuclear hormone receptors and histone methyltransferases such as MLL2/3/4 (Cloos, Christensen et al. 2008).

(iv) Histone acetylation

Histone acetylation is the best characterized N-terminal modification that occurs post-translationally on the ε-NH^{3+} groups of the conserved lysine residues in histone tails. Histone acetylation is a reversible process and is involved in the promoter-specific activation of genes. Histone acetyltransferases are the group of enzymes that catalyze the transfer of an acetyl moiety from acetyl coenzyme A to the ε-NH^{3+} groups of the lysine residues. Acetylation neutralizes the positive charge of histones and results in a negative charge of the modified lysine residue, causing a decreased interaction between the histone and DNA. This allosteric change in nucleosome conformation renders the DNA more accessible to the transcriptional machinery (Gu, Filippi et al. 2003). Around 46% of histone acetylation is sufficient to prevent compaction of chromatin and stimulate traversing of Pol II during transcription.

HAT activities are grouped into two general classes based on their suspected cellular origin and functions. Cytoplasmic, B-type HATs catalyze acetylation events linked to the transport of newly synthesized histones from the cytoplasm to the nucleus for deposition onto newly replicated DNA. Conversely, nuclear A-type HATs likely catalyze transcription-related acetylation events (Brownell, Zhou et al. 1996). Most of the HATs characterized have previously identified function in transcription regulation. Gcn5, the first HAT to be characterized, is a transcriptional activator in yeast (Brownell, Zhou

et al. 1996). The TAF130/250 histone acetylase (Mizzen et al. 1996) is a subunit of the TFIID complex of the basal transcription machinery and hence is associated with all promoters during transcriptional initiation. The p300/CBP histone acetylase is a mammalian coactivator that binds directly to numerous activating transcription factors such as AP1 (Bannister, Oehler et al. 1995), myb (Dai, Akimaru et al. 1996), and transcriptional activators such as MITF. Some of the other coactivators with HAT activity include PCAF, Esa1, NuA4, and steroid receptor coactivators. Transcription factors can recruit HATs. In addition to histone acetylation, gene regulation by HATs also involve acetylation of transcription factors such as p53, GATA-1, GATA-3, and basal transcription factors such as TFIIE and TFIIF (Soutoglou, Katrakili et al. 2000).

The reversible process of deacetylation is brought about by a group of enzymes called histone deacetylases (HDACs) that catalyze the removal of acetyl groups and reestablish the positive charge on the histone. The four classes of HDACs include Class I (HDAC1, HDAC2, HDAC3, HDAC8), Class II (HDAC4, HDAC5, HDAC6, HDAC7A, HDAC9, HDAC10), Class III (Homologs of Sir2 in the yeast *Saccharomyces cerevisiae)* and Class IV (HDAC11). Histone deacetylase complexes are targeted to cytosine-methylated promoters by methyl CpG binding protein MeCP2 (Ng and Bird 1999), Recruitment of HDAC to the methylated promoter maintains these regions in a hypo-acetylated state, inhibiting transcription in the process (Eden 1998). In addition, repressive transcription factors, such as Mad, can also facilitate recruitment of HDAC to genomic targets. HDAC complexes can repress transcription, even in the absence of histone acetylation. This is mediated by the interaction of N-CoR and mSin3A, the components of HDAC complex, with the preinitiation complex, therefore interfering with

the transcription machinery. Disruption of HDACs has been linked to a wide variety of human cancers. Trichostatin A (TSA) is a potent inhibitor of HDACs that can result in hyperacetylation of histones and thus activate transcription of genes (Marks, Richon et al. 2001).

(v) Histone phosphorylation

Phosphorylation of histones is prevalent in histone H1 and H3. Histone H1 is phosphorylated on serine/threonine residues on its N- and C- terminal domains, while H3 is phosphorylated on serine/threonine residues on its N-terminal domain. Histone H3 phosphorylation favors transcriptional activation and is implicated in the activation of early response genes such as *c-Fos* and *c-Jun* (Clayton, Rose et al. 2000). Protein phosphatase 1 appears to be the histone H3 phosphatase and two kinases of the Aurora/AIK family, Aurora-A and Aurora-B, have been identified as the histone H3 kinase (Crosio, Fimia et al. 2002). A balance of protein phosphatase and kinase activities is needed to maintain steady state levels of protein phosphorylation (Murnion, Adams et al. 2001). Histone H3S10 phosphorylation is required for mitotic chromosome condensation and segregation during the cell division (Hendzel, Wei et al. 1997; Wei, Mizzen et al. 1998). Histone phosphorylation is associated with histone acetylation during transcriptional activation. Phosphorylated histone H3S10 binds GCN5 preferentially and acetylates the same histone H3 N-terminal tail at the Lys14 position and thus induces transcription.

(vi) Histone Ubiquitination and Sumolyation

Histone H2A was the first identified ubiquitinated histone (Goldknopf, Taylor et al. 1975) and its ubiquitination site has been mapped to the highly conserved residue, Lys 119 (Nickel, Allis et al. 1989). This modification exists mostly in a mono-ubiquitinated form. In addition to H2A, H2B is also ubiquitinated (West and Bonner 1980). Histone ubiqutination is also a dynamic process involving ubiqutination and deubiqutination. Polycomb protein complex Ring1A/B-Bmi1 has been identified as the major E3 ligase targeting H2A, linking uH2A to gene silencing and tumor development (Wang, Wang et al. 2004). The identified mammalian H2A de-ubiquitinating enzymes (DUBs) include members of two distinct protease families, 2A-DUB and USP (Ubiquitin Specific Protease) (Nicassio, Corrado et al. 2007; Zhu, Zhou et al. 2007). Ubiquitination of histones plays a role in both transcriptional activation (Henry, Wyce et al. 2003) and repression (Dover, Schneider et al. 2002).

Similar to ubiquitination, sumolyation of histones requires E1 activating (SAE1/SAE2) and E2 conjugating (UBC9) enzymes, and it competes with histone acetylation or ubiquitination (Shiio and Eisenman 2003). This modification thus favors repression of transcription. Histone sumolyation and acetylation sites extensively overlap, and mutation of sumolyated histones, or the enzymes needed for this process such as Ubc9, lead to marked increase of histone acetylation (Nathan, Ingvarsdottir et al. 2006).

3. ATP-dependent chromatin remodeling

ATP-dependent chromatin remodeling involves mobilization and repositioning of nucleosomes by sliding of nucleosomes along the path of the DNA, transfer of a histone

octamer from a nucleosome to a separate DNA template, or generation of super helical torsion in DNA. These alterations in the position of the nucleosomes with respect to the DNA sequences around them, increases or reduces the accessibility of a site for transcription factors and thus may lead to transcriptional activation or repression. The enzymes that catalyze this process are specialized factors that use the energy of ATP hydrolysis to bring about structural alterations in nucleosomes. They are multi-subunit complexes with an ATPase as the catalytic center.

The three families of ATP-dependent chromatin remodeling factors include the SWI/SNF2, Mi-2/CHD, and the ISWI complexes. The compositions and biochemical mode of action of these complexes are distinct and therefore bring about changes in nucleosome structure by mediating distinct functions. SNF2 subfamily complexes are very large ~2 MDa assemblies composed of 10-15 different polypeptides. The distinguishing feature of these complexes is the presence of a bromodomain (Martens and Winston 2003) that can bind acetylated histone H3 or H4 (Dhalluin, Carlson et al. 1999). The SNF2 complexes can mediate displacement of histones from one DNA template to a separate segment of DNA (jumping), creating energetically unfavorable disruption of histone octamer-DNA contacts (Lorch, Zhang et al. 1999). Thus these complexes catalyze disassembly of nucleosomes by mediating translocation of nucleosomes by ~80 bp. In humans, the Brg1-associated factors (BAF) complex is related to SWI/SNF (Wang 2003).

On the other hand, ISWI subfamily members such as ACF, CHRAC, RSF and NURF are smaller (~0.5 MDa), with 2-5 components and promote the assembly of nucleosomes. NURF and CHRAC cause sliding of nucleosomes by causing transient

alteration in the weak interactions between histones and DNA (Hamiche, Sandaltzopoulos et al. 1999; Langst, Bonte et al. 1999). Thus, these complexes mediate modest repositioning of nucleosomes by ~10 bp. Amidst these differences, both these complexes can cause structural changes in nucleosomes by mediating super helical torsion in DNA or chromatin, which can in turn affect DNA-histone interactions or the interaction of other proteins, such as the transcription repressors with the DNA (Gavin and Simpson 1997).

The Mi-2 group of enzymes are characterized by a chromodomain (Delmas, Stokes et al. 1993) that can bind methylated DNA and histone tails. Nucleosome-remodeling deacetylase (NuRD) complexes in humans consist of the Mi-2 ATPase subunit, which possess both chromatin remodeling and histone deacetylase activities. This Mi-2 group of chromatin remodeling complexes functions in the repression of gene expression. The Ikaros complex, a transcription factor important for normal B- and T-cell development, interacts with NuRD complexes in lymphoid and erythroid cells (Kim, Sif et al. 1999). In erythroid cells, they function in the repression of *γ-globin* gene expression at the time of the switch from fetal to adult globin production (O'Neill, Schoetz et al. 2000).

4. Interplay between epigenetic modifications and transcription machinery

RNA polymerase holoenzyme interacts with transcription factors or epigenetic factors, such as DNA and/or histone modifications, to form a large network that participates in the transcription process (Kadonaga 1998). An unanswered question regarding transcriptional activity is whether epigenetic modifications facilitate

recruitment of transcription factors or vice versa. Evidence is available in favor of both ideas.

Cooperative interaction between epigenetic modifications is important in providing additional control over genomic regulation. A variety of inactive or active chromatin marks communicate with each other to establish and maintain the transcriptional activity of a gene. DNA methylation, histone deacetylation, histone H3K9 methylation and binding of HP1 are some of the common inactive chromatin marks that mediate gene repression. *Dim-5*, a SET-domain gene with histone H3K9 methyltransferase activity, directs DNA methylation in *Neurospora crassa* (Tamaru and Selker 2001). Mutation of histone H3K9 methyltransferase activity resulted in reduced DNA methylation. Additional evidence showing the effect of histone methylation on DNA methylation invalues the interaction of Dnmt1 and MeCP2 with HDAC. HDACs from deacetylated chromatin interacts with Dnmt1 and MeCP2, which facilitates establishment and maintenance of cytosine methylation. This interaction thus prepares the H3 amino terminus for substrate recognition by HMTases. SuVar, a histone H3K9 methyltransferase, can interact with MeCP2 along with HP1, resulting in compact chromatin. Transcription factors and repressors can also interact with these proteins to repress gene expression. E2F-6, a member of the E2F-family of transcription factors co-exists in a protein complex with HP1 and SUV39H1, histone H3K9 methyltransferase (Zhang and Reinberg 2001). Repression by YY1, a transcriptional repressor, is mediated by interaction with HDAC2 (Yang, Inouye et al. 1996).

Similarly, enzymes that catalyze establishment of active chromatin marks, such as HATs and histone H3K4 methyltransferases also communicate with transcription factors.

In fact, as discussed earlier, many of the HATs identified so far are previously characterized coactivators, such as GCN5 (Georgakopoulos, Gounalaki et al. 1995; Brownell, Zhou et al. 1996) and p300/CBP. p300/CBP links activators to the transcription machinery at promoters regulated by nuclear hormone receptors and the oncoprotein E1A (Chakravarti, LaMorte et al. 1996).

IV. Hematopoiesis

Hematopoiesis is a remarkable process that involves formation and development of mature blood cell components, starting from a relatively small stem cell compartment. The whole process is regulated by several cytokines (Fig 1). In a developing embryo, formation of blood cell formation starts in blood islands present in the yolk sac. During development, the blood formation shifts to liver, spleen and lymph nodes (Palis and Yoder 2001). Bone marrow and spleen carries out the entire task of blood cell development after birth. The three lineages of blood cells include erythroid, lymphoid, and myeloid lineages. Erythroid cells consist of the red blood cells, which facilitates oxygenization. The lymphoid lineage consists of T cells and B cells that play important roles in the adaptive immune system. The myeloid cells include granulocytes, megakaryocytes and macrophages.

The process of cell differentiation and lineage commitment is achieved by a complex coordination of signal transduction pathways and cell-type specific gene activation (Kluger, Lian et al. 2004). The precise mechanisms activating or repressing gene transcription required for development are brought about by various cis- and trans-acting regulatory elements (Orkin 1995). In addition to various lineage-specific or

differentiation-specific transcription factors, epigenetic factors also play a crucial role in this procees (Rice, Hormaeche et al. 2007). Thus, hematopoiesis has served as an important model for understanding mammalian gene regulation. Research on identifying the molecular mechanisms controlling cell type-specific gene expression is a fruitful approach to understand hematopoietic development.

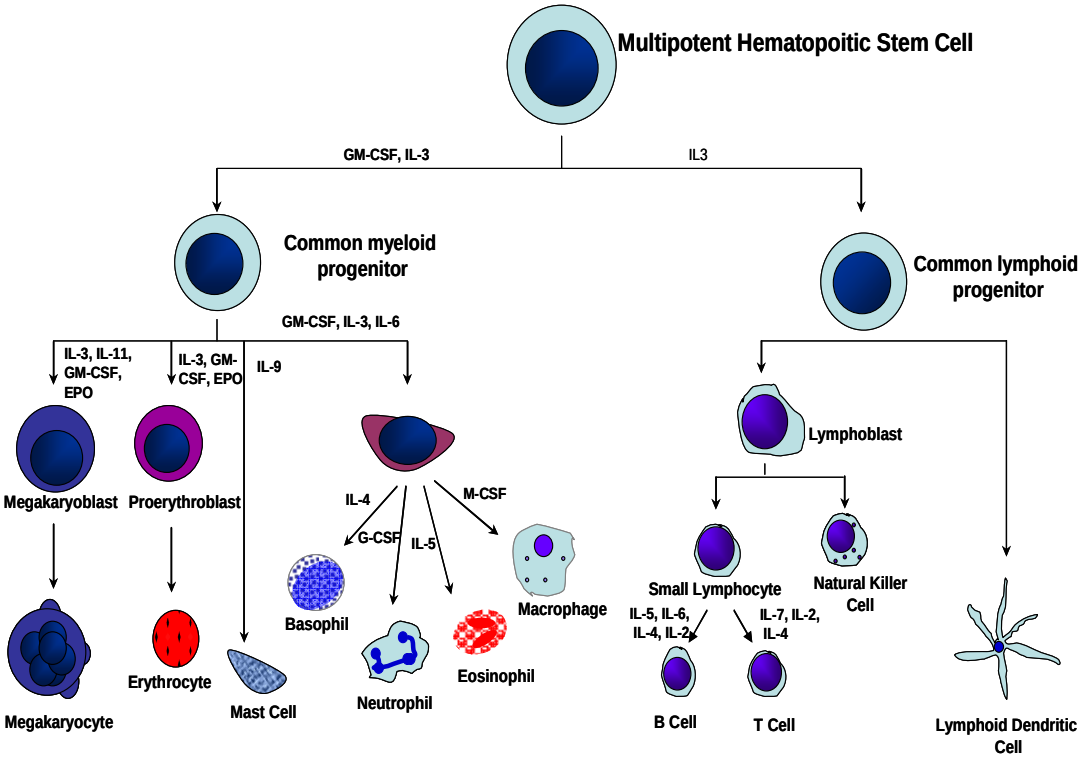


Figure 1. A diagram that shows the development of different blood cells from hematopoietic stem cell to mature cells and the cytokines involved in the regulation of differentiation.

V. Transcription factors and epigenetic regulation in hematopoiesis

Renewal or commitment of hematopoietic stem cells (HSC) is triggered by the differential expression of lineage-specific transcription factors. These factors can bind to the regulatory regions of genes, such as promoters, or LCRs to mediate cell type-specific gene expression. Transcription factors can also render their effect by activating or repressing the transcriptional pathway of another transcription factor. Though several transcription factors control commitment of HSCs to lymphoid or myeloid lineages, not all factors are specific to hematopoietic lineages. Critical transcriptional regulators such as PU.1, C/EBP, c-Myb, Sp1, AML1 and GATA-1 play central roles in determining myeloid lineage commitment (Skalnik 2002). Over-expression of transcription factor c-Myb induces differentiation of K562 cells to the granulocytic/monocytic lineage (Valledor, Borras et al. 1998). A separate set of transcription factors mediates differentiation of lymphoid cells. The Ikaros complex plays an important role in the development of all lymphoid lineages (Georgopoulos, Moore et al. 1992). The transcription factor Pax5 plays an important role in the commitment to B cell lineage.

Inhibition or change in signaling pathways that activate differentiation can lead to a switch in lineages. For example, Notch1 signaling promotes commitment of common lymphoid progenitors to thymocytes while inhibiting B cell production (Wilson, MacDonald et al. 2001). Inhibition of protein kinase C suppresses megakaryocytic differentiation and induces erythroid differentiation in human erythroleukemia (HEL) cells (Hong, Martin et al. 1996).

The expression of transcription factors required for the differentiation of hematopoietic lineages can be regulated by epigenetic modifications, such as cytosine

methylation. PU.1 is highly expressed in differentiated B cells, but not in T cells, correlating with the methylation status of the *PU.1* gene promoter (Amaravadi and Klemsz 1999). GATA3 plays important roles in the maturation of CD4 cells and displays reduced cytosine methylation in CD4+ cells compared to CD34+, CD8+, T and B cells (Wilson, Makar et al. 2005). Epigenetic modifications can also influence hematopoietic differentiation by inhibiting or favoring binding of transcription factors. MeCP2 exists in a complex with the transcriptional co-repressor Sin3A and HDACs which can block the ability of PU.1 to activate transcription through its cognate binding site (Bird, 2002; Suzuki et al. 2003). In addition, transcription factors also regulate specific gene expression patterns during hematopoiesis by recruiting HAT or HDAC complexes to the promoters of target genes. Transcriptional activation of the *β-globin* locus during erythropoiesis is achieved by recruitment of CBP/p300, a histone acetyltransferase, by GATA-1 (Hung, Lau et al. 1999). On the other hand, CBP-mediated histone acetylation is inhibited by transcription factor PU.1 (Hong, Kim et al. 2002). Thus, CBP also interacts with transcription factors of opposing functions, such as GATA-1 and PU.1, to regulate cell cycle arrest and cell cycle progression during hematopoiesis.

VI. Cell lines as model systems for hematopoiesis

The technical difficulties, and the scarcity of purified populations of multi- or pluripotent progenitors cells, has slowed down the understanding of lineage determination and intracellular events during hematopoiesis. Mature cells comprise a very small percentage of bone marrow cells, and maintenance of them in tissue culture is very difficult. Isolation of mature cells requires biological, biochemical, and molecular

analysis of purified populations of each of the hematopoietic subpopulations and thus is a very laborious process. Hematopoietic development begins with the response of an early progenitor cell to microenvironmental influences. The entire process is promoted by soluble regulatory cytokines or growth factors, each activating distinct and in some cases overlapping pathways and targets (Zhu and Emerson 2002). The most common growth factors that stimulate blood cell formation include stem cell factor (SCF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte CSF (G-CSF) and macrophage CSF (M-CSF) (Kelley, Graham et al. 1999). These factors activate signal transduction pathways that change expression of transcription factors that in turn activate genes that determines the differentiation of blood cells (Fortunel, Hatzfeld et al. 2000).

Multipotent hematopoietic cell lines contain mixed populations of committed progenitor like and immature cells, which are inducible with variety of chemical stimuli and thus develop or mature into particular hematopoietic lineage based on the stimulus used. These differentiation processes require further examination of the intracellular events, such as signaling pathways and gene expression, and serve as an excellent mimic of hematopoietic differentiation. Some of the multipotent cell lines used for these studies includes HEL, MEG-01, Dami, CMK, and K562.

Human K562 cells are one such multipotent hematopoietic cell line initially derived from a patient with chronic myelogenous leukemia (Lozzio and Lozzio 1975). Undifferentiated K562 cells express surface markers characteristics of megakaryocytic, erythroid, and monocytic lineages. Hemin, aphidicolin, or 1-β-D-arabinofuranosylcytosine and sodium butyrate treatment commits K562 cells towards an erythroid lineage (Rutherford, Clegg et al. 1981; Delgado, Lerga et al. 1995). PMA

induces differentiation along the megakaryocytic lineage, with an accompanying increase in expression of megakaryocytic markers, and a concominant decrease in the expression of markers of monocyte and erythroid lineages (Tetteroo, Massaro et al. 1984). Some of the early and specific markers for examining the megakaryocyte specific gene expression include glycoprotein IIb/IIIa (GPIIb/IIIa), glycoprotein Ib, thrombospondin (Fukuda 1981; Long, Heffner et al. 1990). PMA treatment leads to the induction of transcription factors such as AP1, and egr-1 that further facilitates cell-specific expression of genes required for differentiation. PMA stimulation of K562 cells leads to the activation of the protein kinase C and further downstream activation of the Mitogen activated protein kinase (MAPK) to modulate megakaryocytic differentiation (Herrera, Hubbell et al. 1998). Activation of the MAPK pathway alone is necessary and sufficient to cause megakaryocytic differentiation of K562 cells in the absence of PMA stimulation (Whalen, Galasinski et al. 1997).

VII. Rho GTPases

Rho GTPases belong to the Ras super family, which has been divided into six families: Ras, Rho, Arf, Sar, Ran and Rab (Bourne, Sanders et al. 1990). Rho GTPases are molecular switches that cycle between two combinatorial states: a GTP bound active state and a GDP bound inactive state. At least 23 Rho-related proteins have been identified in humans, but the best characterized members of this family include RhoA, Rac and Cdc42. Members of Rho GTPases are critical for several aspects of cell behavior, such as cell proliferation, apoptosis, adhesion (Huang, Alessandrini et al. 1993; Laudanna, Campbell et al. 1996), motility, differentiation (Erickson, Galletta et al. 1997),

gene expression (Huang, Alessandrini et al. 1993), and vesicular trafficking (Brown, O'Sullivan et al. 1998) and have therefore gained much attention among cell biologists.

1. Rac GTPase

The three isoforms of Rac GTPases include Rac1, Rac2 and Rac3 which share greater than 80% sequence identities. The greatest divergence among Rac1, Rac2, and Rac3 occurs at the C-terminal at residues 180-192, which are also hypervariable regions in the Ras proteins. The identified differences between these isoforms in their C-terminal ends may explain differences in subcellular localization and/or binding to specific regulatory molecules (Tao, Filippi et al. 2002).

The three Rac GTPases differ in their expression pattern. Rac1 is ubiquitously expressed (Didsbury, Weber et al. 1989; Moll, Sansig et al. 1991) while Rac3 is also widely expressed with higher expression in brain (Haataja, Groffen et al. 1997; Bolis, Corbetta et al. 2003). By contrast, Rac2 expression is restricted to hematopoietic lineages (Didsbury, Weber et al. 1989; Shirsat, Pignolo et al. 1990). *Rac2* mRNA displays relative myeloid tissue selectivity and showed a further increase upon differentiation of HL-60 and U937 cells to neutrophil-like and monocyte-like morphology, respectively (Didsbury, Weber et al. 1989). Though expressed highly in hematopoietic cells, recent reports additionally show expression of Rac2 in endothelial cells (Wang, Rebert et al. 2007) and activated vascular smooth muscle cells (Tian and Autieri 2007).

2. Functions of Rac2

Neutrophil chemotaxis and cell migration

Neutrophils are chemotactic cells that respond with directed motility to signals like bacterial peptides and chemokines. A broad lamellipodia at the leading edge aids in the locomotion of neutrophils. Regulation of cytoskeletal dynamics by Rac2 is critical for orchestrating cell movement and for normal cell orientation and polarization of neutrophils. *Rac2* knock out neutrophils exhibit severe impairment in the direction and speed of movement (Roberts, Kim et al. 1999). Patients carrying a naturally-occurring dominant-negative *Rac2* mutation (Asp57→Asn) exhibit reduced neutrophil migration and suffer from severe recurrent infections. This is the first case of disease due to mutation in a Rho GTPase, which results in human phagocyte immunodeficiency syndrome (Williams, Tao et al. 2000). In addition, Rac2 is required for macrophage accumulation during peritoneal inflammation (Pradip, Peng et al. 2003).

Superoxide production

Phagocytic leukocytes play major roles in the host immune response and respond to pathogens by producing reactive oxygen species (ROS) through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Castro and Freeman 2001). The NADPH oxidases are a group of plasma membrane-associated enzymes that catalyze the production of reactive oxidants such as free radicals by the one-electron reduction of oxygen, using NADPH as the electron donor. The five components of this complex include a membrane bound cytochrome b_{558}, composed of $gp91^{phox}$, $p22^{phox}$, and the cytosolic components $p47^{phox}$ and $p67^{phox}$ (Chanock, el Benna et al. 1994; DeLeo and

Quinn 1996). The GTP-binding protein Rac2 is required for the regulation of NADPH oxidase activity and induces the assembly of this complex (Knaus, Heyworth et al. 1991). *Rac2* knock out mice have neutrophils whose superoxide production is reduced 4-fold in response to PMA stimulation (Haataja, Groffen et al. 1997). Patients suffering from a phagocyte immunodeficiency syndrome due to nonfunctional Rac2 have neutrophils impaired in the ability to generate free radicals and they are predisposed to poor wound healing and life threatening bacterial infections (Gu, Jia et al. 2001). Infection due to the bacterial pathogen *Anaplasma phagocytophila* results in the downregulation of $gp91^{phox}$ and *Rac2* gene expression, thereby leading to defective superoxide production of the host granulocyte (Carlyon, Chan et al. 2002).

Stem cell adhesion

Regulatory events such as adhesion and localization of hematopoietic cells play critical roles in maintaining the homeostasis of blood cell production (Tan, Yazicioglu et al. 2003). *Rac2* knock out hematopoietic stem cells/progenitor cells (HSC/P) exhibits defective integrin mediated cell adhesion and enhanced mobilization (Yang, Atkinson et al. 2001). In addition, *Rac2* knock out cells also showed defect in cortical F-actin assembly, an important function required for cell migration and adhesion and therefore exhibited reduced actin based membrane protrusion (Weston and Stankovic 2004). Thus, Rac2 plays a critical role in controlling cytoskeletal changes that are important for blood cell formation and function (Gu, Filippi et al. 2003).

Blood cell development

Rac2 plays important roles in the development and regulation of B and T lymphocytes. Rac2 shares an important role with Rac1 in the production of common lymphoid progenitors. B cell development in the bone marrow and spleen proceeds through a series of differentiation stages. *Rac2*-deficient mice display peripheral blood B lymphocytosis and marked reductions in peritoneal cavity B-1a lymphocytes (Croker, Tarlinton et al. 2002). Rac2 regulates the survival and proliferation signals during T-cell development. *Rac1/Rac2* knockout T-cells exhibit variety of defects such as suppression of T-cell receptor mediated proliferation and IL2 production (Guo, Cancelas et al. 2008). They are also defective in receptor clustering during T-cell stimulation and Th1 differentiation and show perturbed T-cell distribution and chemotaxis (Yu, Leitenberg et al. 2001).

VIII. Rac2 gene regulation

The human *Rac2* gene spans a 18 kb segment in chromosome 22q1 and consists of 6 exons and a seventh noncoding exon containing the 3' noncoding sequences ending with a polyadenylation signal (Didsbury, Weber et al. 1989). The proximal human *Rac2* gene promoter is GC-rich and lacks consensus TATA and CCAAT boxes (Ladd, Butler et al. 2004). Therefore, multiple transcription initiation sites are present in the human and murine *Rac2* genes, upstream of their translation initiation site (Ou, Pollock et al. 1999). The major transcription initiation site of the human *Rac2* gene is located 134 bp upstream of the translation initiation site. The human *Rac2* promoter region contains consensus-binding sites for Sp1, Ets/PU.1, and MZF-1 transcription factors that have been implicated in myeloid-cell restricted gene expression. Transcription factors Sp1 and Sp3

bind within the -74 to -58 murine *Rac2* promoter (Ou, Pollock et al. 1999). Transcription factor Sp1 plays an important role in the regulation of *Rac2* promoter activity, as deletion of -216 to -30 bp of human Rac2 upstream region, which contains multiple SP1 sites, led to a severe decrease in the promoter activity (Ladd, Butler et al. 2004).

The regulatory elements required for the lineage-specific expression of Rac2 have been studied in cell lines and transgenic mice. Both the murine and human *Rac2* gene promoters exhibit strong but promiscuous promoter activity following stable or transient transfection into cell lines (Ou, Pollock et al. 1999; Ladd, Butler et al. 2004). Promoter deletion analysis in cell lines identified cell type-specific cis-elements between -4358 bp and -216 bp of the human *Rac2* gene promoter region. The human *Rac2* gene, including 1.6 kb of upstream sequence and 8 kb of downstream sequence, contains cis-elements sufficient to restrict Rac2 expression to hematopoietic tissues in transgenic mice (Ladd, Butler et al. 2004).

DNA methylation was also found to be important for restricting human Rac2 expression to hematopoietic cells. The Rac2 5'- and 3'- flanking sequences exhibited increased cytosine methylation in non-expressing cell lines. In addition, treatment of Rac2 non-expressing HEK293 cells with 5-Aza-dC, an inhibitor of Dnmt induced R*ac2* gene expression. This showed the importance of DNA cytosine methylation in regulating the lineage-specific expression of Rac2 (Ladd, Butler et al. 2004).

IX. Focus of the dissertation

This dissertation investigates the molecular mechanisms regulating *Rac2* gene expression during hematopoeitic cell differentiation. The hematopoietic-specific expression of Rac2 has previously been investigated in cell lines and transgenic mice. The 4.5 kb human *Rac2* gene promoter region showed strong but promiscuous acitivity in cell lines. Hematopoietic-specific regulatory elements lie within the human *Rac2* gene locus including the 1.6 kb upstream and 8 kb downstream sequence. The importance of DNA methylation in the hematopoietic-specific expression of Rac2 has also been studied (Ladd, Butler et al. 2004). However, regulatory elements required for the modulation of *Rac2* gene expression during terminal hematopoietic cell differentiation remain to be identified.

Identification of the cis-element(s) and transcription factors that regulate *Rac2* gene expression during terminal cell differentiation will be important in determining the regulatory factors required for differentiation-specific expression of Rac2. Determining the role of epigenetic modifications in this process is important to understand the communication between transcription factor(s) and chromatin structure to control *Rac2* gene expression. Thus, the focus of this dissertation work is to identify the regulatory elements of the *Rac2* gene and to understand the interplay between these factors to provide an added level of control for *Rac2* gene expression during cell differentiation. This analysis may provide a better understanding of the complexity in balancing tissue-specific and differentiation-specific regulation to bring about an appropriate pattern of *Rac2* gene expression.

METHODS

I. Cell Culture

K562 human chronic myelogenous leukemia cells were grown in suspension in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 5% fetal clone III serum (Hyclone, Logan, UT), 0.2 mM glutamate and 50 units/ml penicillin/streptomycin at 37ºC and 5% CO_2. Megakaryoblastic differentiation of K562 cells was induced with 100 nM phorbol myristate acetate (PMA) (Sigma Chemical Co, St Louis, MO) dissolved in dimethyl sulfoxide (DMSO). Treatment with 100 nM phorbol 12, 13-didecanoate (PDD) (Sigma Chemical Co, St Louis, MO) dissolved in DMSO was used as a negative control.

II. Nuclear Extract Preparation (Dignam Protocol)

K562 cells were grown to approximately 1×10^6 cells/ml and treated with DMSO or PMA for 24 h. The cells were scraped off the plate and collected by centrifugation at 500 x g for 5 minutes (min) at 4ºC. Cell pellets were washed with ice cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2PO_4 and 1.4 mM KH_2PO_4) and pelleted by centrifugation at 500 x g for 5 min. The volume of cell pellet was taken as 1 volume. The cell pellets were resuspended with 5 volumes of ice cold buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES] pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing freshly added protease inhibitor cocktail (0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM leupeptin, 5 μg/ml aprotinin and 5 μg/ml pepstatin), and incubated on ice for 10 min for swelling. The cells were then pelleted by centrifugation at 500 x g for 10 min at 4ºC. The cell pellets were

then suspended in 2 volumes of ice cold buffer A containing protease inhibitors and 5% NP-40. The cells were then lysed by 10 strokes of a dounce homogenizer. The homogenized solution was then collected by centrifugation at 500 x g for 10 min at 4ºC. The cell pellets were resuspended in 250 µl of buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid [EDTA]) containing freshly added protease inhibitor cocktail and incubated on ice for 15 min. The cells were then collected by centrifugation for 20 min at 12000 x g at 4ºC. The supernatant containing nuclear extract was collected and stored in aliquots at -80ºC. The concentration of total nuclear proteins was assayed by Bradford assay (Bradford 1976) before use.

III. Preparation of DNA Probes for Binding Assays

1. Annealing of complementary oligonucleotides

Equal moles of single-stranded oligonucleotides of complementary sequence were mixed in 0.2 M NaCl and boiled at 95ºC for 5 min. The oligonucleotides were then annealed by allowing them to cool slowly to room temperature overnight.

2. Labeling and purification of oligonucleotides

Double-stranded oligonucleotides were labeled with T4 polynucleotide kinase. Sixty nanogram of double stranded oligonucleotide was mixed with 2 µl 10X kinase buffer, 1 µl T4 polynucleotide kinase, and 5 µl of γ-^{32}P ATP. The reaction mixture was incubated at 37ºC for 1 h. The labeled probed was mixed with 1/5 volume of 5X DNA loading buffer and resolved by electrophoresis using an 8% native polyacrylamide gel in 0.5X

TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA) at 200 V for approximately 1 h. The wet gel was covered with plastic wrap and exposed to X-ray film in the dark room for 30 seconds (s). The band corresponding to probe was excised and cut into small pieces and transferred to the upper chamber of a spin-X tube contining 300 µl of elution buffer (0.5 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA pH 8.0 and 0.1 % sodium dodecyl sulfate [SDS]). The probe was eluted by incubation in a 37ºC water bath for 4 h and separated from gel pieces by centrifugation at 6000 x g for 5 min. Another 150 µl of elution buffer was added to wash the gel pieces and the probe was reeluted by centrifugation at 6000 x g for 5 min. The eluted probe was treated with an equal volume of 1:1 phenol/chloroform and subjected to centrifugation at 12,000 x g for 2 min. The aqueous phase was transferred to a new microfuge tube and precipitated with a 1/10 volume of 3 M sodium acetate, 1 µl of 10 µg/µl tRNA and 2 volumes of 100% ice cold ethanol (EtOH). The solution was allowed to stay at -20ºC for 1 h and pelleted by centrifugation at 12,000 x g for 10 min and washed with 500 µl ice cold 70% ethanol. The pellet was then allowed to air dry and dissolved in 100 µl of double distilled (dd) H_2O. Two microliters of purified probe was used for scintillation counting.

3. Purification of labeled probe with Sephadex G-200 spin columns

Sephadex G-200 oligonucleotide spin columns were mixed well for equal distribution of beads, followed by centrifugation at 4000 x g for 1 min. The labeled probe was mixed with 30 µl of Tris-EDTA buffer and loaded onto G200 oligo spin column followed by centrifugation at 4000 x g for 4 min. Two microliters of purified probe was used for scintillation counting.

IV. Electophoretic Mobility Shift Assay

The binding mixture containing 5-10 μg of nuclear extract was incubated on ice with 2 μl of binding buffer (10 mM Tris pH 7.5, 50 mM K-glutamate, 5 mM $MgCl_2$, 1 mM DTT (freshly added), 1 mM EDTA, 5% glycerol) and 0.5 mM poly dI:dC and incubated on ice for 30 min. For competition assays, 60 ng of non-radioactive oligonucleotide was added to the mixture and incubated on ice for 30 min. 1×10^4 counts per minutes (CPM) labeled oligonucleotide probe was added to each reaction mixture and incubated on ice for 30 min. For super shift analysis, 2-3 μg of antibody directed against unmodified c-Jun (Active Motif, Carlsbad, CA) or c-Fos antibody (Santa Cruz Biotechnologies, Santa Cruz, California) or normal rabbit serum (Santa Cruz) was added followed by incubation at room temperature for 40 min. Two microliters of DNA loading buffer was added to the mixture and separated on a 5% native polyacrylamide gel in 0.5X TRIS-borate EDTA (TBE) at 250 V for 2 h on ice or in the cold room. The gels were dried by vacuum and exposed to Kodak (Rochester, NY) Hyperfilm at -80ºC with an intensifying screen.

The oligonucleotides used for EMSA are listed in Table 1.

Table 1. Double Stranded Oligonucleotides used in EMSA

Sequences of oligonucleotides corresponding to the 135 bp *Rac2* promoter regulatory region are indicated. The sense strand of each double stranded oligonucleotide is listed with AP1 transcription factor binding sites underlined and in bold italics and mutated bases shown in bold.

Oligos	Sequence
Oligo 1	5'– gttgttaca***tgtgtca***tggtgtgtctgtccttt 3'
Oligo 2	5'– gtgtctgtccctttattagtgagtagtgtttt 3'
Oligo 3	5'– tgagtagtgtcccaccgcagggatgtgtcattt 3'
Oligo 4	5'–gga***tgtgtca***catttggttattcatttagtttt 3'
Oligo 5	5'– ttcatttagtcccatcctcttttgactacattt 3'
Oligo 6	5'– tttgactacactttgcaaactcactgaatcttt 3'
Oligo 7	5'– tcact***gaatcaca***gaaccacgaggcctctgttt 3'
AP1 control oligo	5'- gcttgatga***tgactca***gccggaa 3'
AP1 mutant oligo	5' – cgcttga**tgacttg**gccggaa 3'

V. Construction of Plasmids

1. Construction of 5'- deletion constructs of the 4.5 kb human *Rac2* promoter

5'- deletion constructs of the 4.5 kb human *Rac2* promoter were sub-cloned into the pGL3-Basic reporter gene vector (Promega, Madison, WI). Truncations of the 4.5 kb human *Rac2* promoter were created using restriction enzymes *StuI* at -4223 bp, *AflIII* at -4088 bp, *HindIII* at -3824 bp and *KpnI* at -1100 bp. Two additional constructs were made by sub-cloning polymerase chain reaction (PCR) amplified fragments spanning -3500 bp to +134 bp and -30 to +134 bp into pGL3-Basic. PCR primers used for the generation of these constructs are described in Table 2. A schematic representation of the constructs is shown in Figure 2.

2. Construction of the EF1α-luc and -30bp+135-luc constructs

The *EF1α* promoter from plasmid pEF/PAC (kindly provided by Dr. Mary Dinauer, Indiana University) was digested using restriction enzymes NotI and HindIII and sub-cloned into the pGL3-Basic luciferase reporter gene vector (Promega, Madison, WI). The NotI end of EF1α was made blunt by Klenow filling and sub-cloned into pGL3-Basic. A schematic representation of the construct is shown in Figure 3A. The 135 bp region between -4223 bp to -4088 bp was PCR amplified, followed by restriction enzyme digestion with *KpnI* and *SacI* and sub-cloned upstream of the -30 to +134 bp region in pGL3-Basic vector. This construct will be referred to as -30bp+135-luc construct. A schematic representation of the construct is shown in Figure 3B.

3. Construction of the c-Jun expression vector

The full length human c-Jun cDNA from retroviral vector pMIEG3 (kindly provided by Dr. Alexander Dent, Indiana University) was digested with restriction enzymes *NotI* and *XhoI* and sub-cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). A schematic representation of the construct is shown in Figure 4. The full length human c-Fos cDNA in pCMV6-XL5 expression vector was purchased from Origene Technologies (Rockville, MD).

Table 2. PCR primers used for the generation of -3500 bp and -30 bp *Rac2* promoter deletion constructs

Sequences of oligonucleotide used to generate PCR products corresponding to human *Rac2* promoter fragments. Each oligonucleotide sequence contains a restriction enzyme linker at the 5'- end. The numbers in primer indicate the nucleotide positions within the human *Rac2* promoter. The restriction enzyme sites are shown in bold.

Construct	**Primer**	**Sequence**
-3500 bp – luc	-3.5 kb SacI sense	5'-c**gagctc**gggaggttccatgggtgcat-3'
	Basic XhoI antisense	5'-cc**gctcgag**cggtgtcggtggtgacagctcag-3'
-30 bp – luc	-30 MluI sense	5'- cc**acgcg**tcgaccgtggggagagcagc-3'
	+134 XhoI antisense	5'-**gctcgag**cgtgtccggagcctggagag-3'

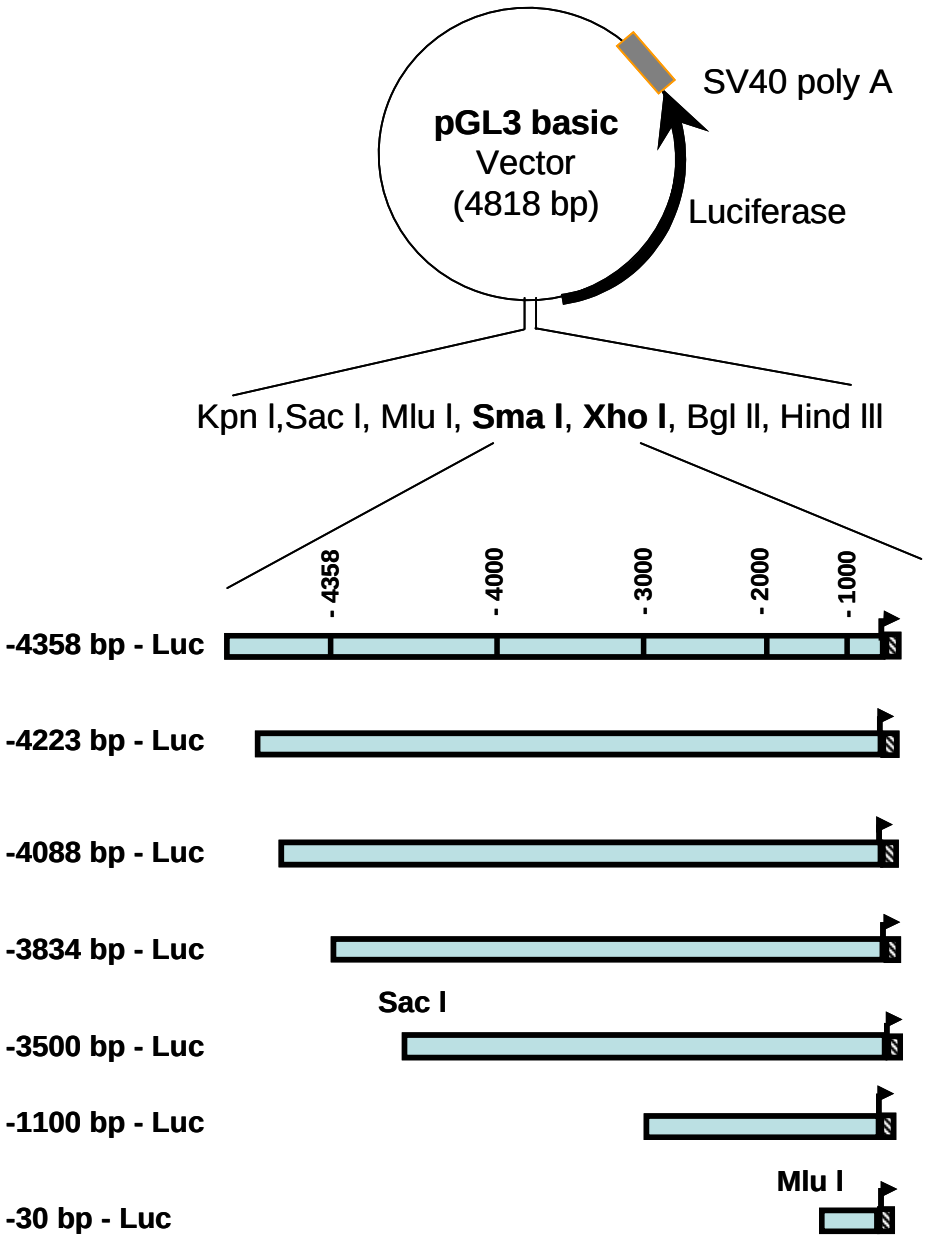
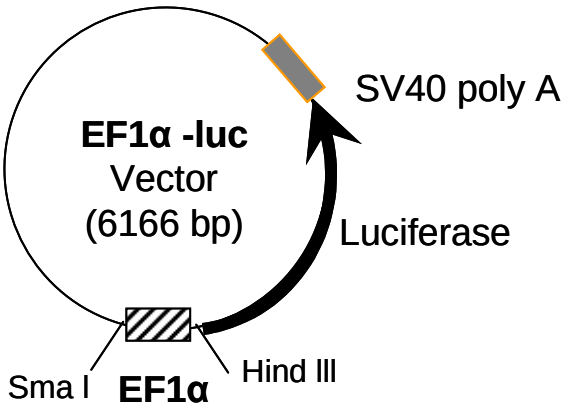


Figure 2. Schematic representation of the 5'- deletion constructs of the 4.5 kb human *Rac2* promoter in pGL3-Basic vector. Various lengths of 5'- deletion constructs of the 4.5 kb human *Rac2* promoter obtained by restriction enzyme digestion or PCR amplification were sub-cloned into pGL3-Basic reporter gene vector. All constructs extend to +134 bp of *Rac2* (denoted by the diagonally shaded box). Arrows indicate the +1 transcription start site.

A.



B.

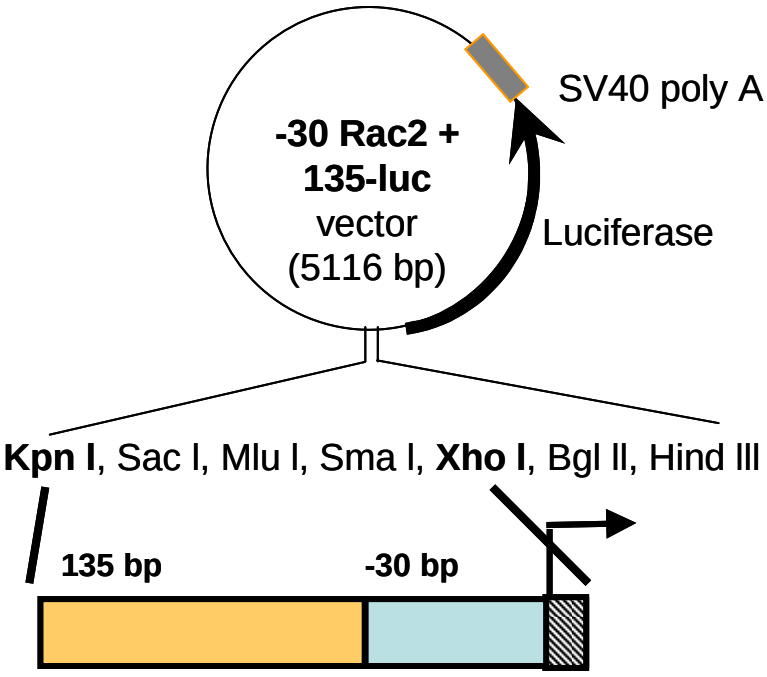


Figure 3. Schematic representation of the *EF1α*/luc, Core Rac2+135/luc constructs.

A) The *EF1α* promoter from plasmid pEF1α/PAC sub-cloned into the pGL3-Basic vector. B) PCR amplified 135 bp region between -4223 bp to -4088 bp of the *Rac2* promoter sub-cloned upstream of the -30 to +134 bp core *Rac2* promoter region in pGL3-Basic reporter gene vector.

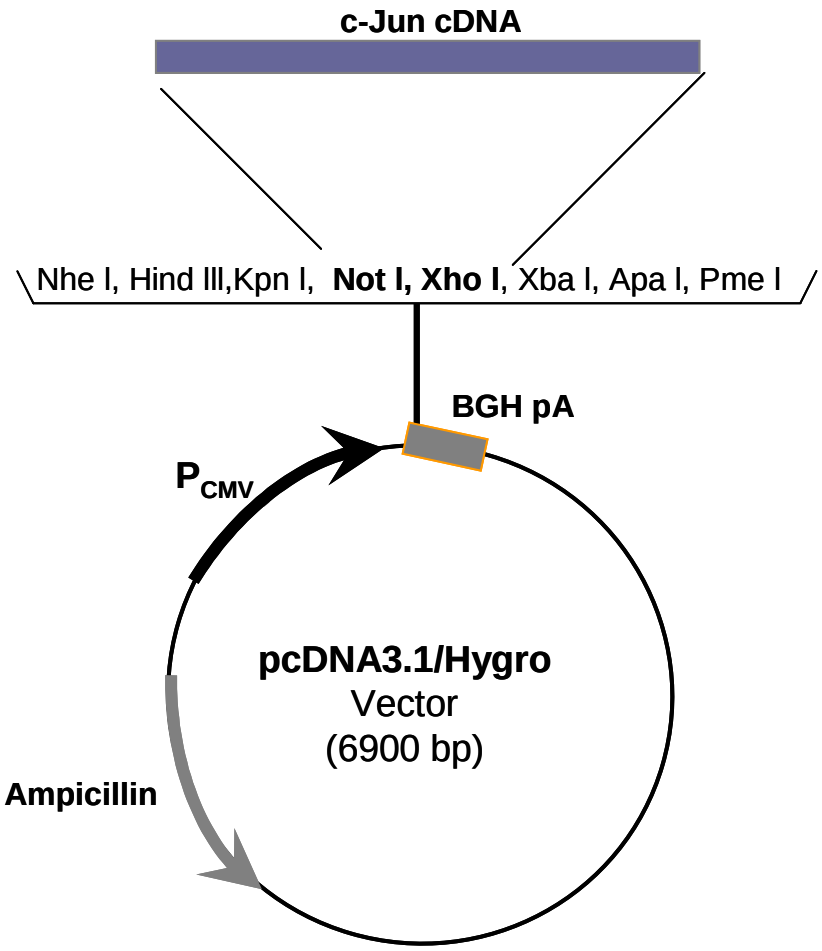


Figure 4. Schematic representation of pcDNA 3.1 vector containing full length human c-Jun cDNA. The human cJun cDNA from the MIEG3-cJun retroviral vector was sub-cloned into pcDNA3.1/Hygro vector.

VI. Purification of plasmid constructs

1. Small scale purification of plasmid constructs

Small scale purifications of plasmid DNA were performed using the miniprep kit from Sigma. Transformed bacterial colonies were inoculated into 1.5 ml of Luria-Bertani Broth (LB) supplemented with ampicillin (50 μg/ml) and incubated overnight at 37ºC. The overnight culture was transferred to a 1.5 ml microcentrifuge tube and collected by centrifugation at 12,000 x g for 1-2 min. The supernatant was discarded, and the pellets were resuspended in 200 μl of ice-cold resuspension solution containing RNAase A provided in the kit. The cell suspension was then mixed with 200 μl of lysis solution and mixed by gentle inversion for 5-6 times. After incubation at room temperature for 5 min, 300 μl of neutralization solution was added and mixed by gentle inversion for 5-6 times. The bacterial lysate was collected by centrifugation at 12,000 x g for 10 min. Meanwhile, the spin columns provided in the kit were prepared by addition of 500 μl column preparation solution, followed by centrifugation at 12,000 x g for 1 min. The cleared bacterial lysate was then transferred into the prepared spin column for binding and centrifugation was done at 12,000 x g for 1 min. The spin columns were washed with 700 μl of wash solution by centrifugation at 12,000 x g for 1 min. Any residual wash solution was removed from the column and plasmid DNA was eluted by addition of 100 μl ddH_2O.

2. Large scale purification of plasmid constructs

Large scale purifications of plasmid DNA were performed using the maxiprep kit from Qiagen. A singly colony from a freshly streaked plate was used to inoculate 2 ml of

ampicillin (50 μg/ml) LB and grown for 8 h at 37ºC. The 2 ml culture was used to inoculate 250 ml of ampicillin (50 μg/ml) LB and grown overnight at 37ºC. The following day, the bacterial cultures were transferred into clean Nalgene tubes and the pellets were collected by centrifugation at 9000 rpm for 15 min at 4ºC. The supernatant was discarded and the pellet was resuspended using 10 ml of buffer P1 containing RNase A. Ten milliliter of buffer P2-lysis blue was added to each tube and the solution turns blue upon mixing by rapid inversion of the tubes. After incubation at room temperature for 5 min, 15 ml of ice-cold buffer P3 was added and mixed 5-7 times by rapid inversion. The bacterial lysates were immediately transferred to a Qia-filter maxi cartridge and incubated at room temperature for 10 min. During incubation, the Hispeed maxi tip was prepared by placing on a compatible tube and allowed to equilibrate by addition of 10 ml of buffer QBT. After incubation, cleared bacterial lysates were allowed to flow through the equilibrated cartridges by inserting a plunger into the syringe containing the bacterial lysates after removal of the cap. The columns were then washed with 60 ml of buffer QC. After washing, the DNA was eluted into clean 15 ml tubes by addition of 10 ml of buffer QF. The DNA was precipitated by addition of 15 ml isopropanol and incubated at room temperature for 10 min. The DNA was bound to a column by addition of the 25 ml solution into 30 ml syringe containing column. The column was washed with 2 ml of ice cold 70% EtOH and the column was transferred to a 5 ml syringe. The DNA was eluted from the column by addition of 500 μl ddH_2O and re eluted with another 500 μl ddH_2O. The DNA was stored at -20ºC until use.

VII. Quantitative Real-time PCR

Total RNA was extracted from K562 cells using Tri-reagent (Molecular Research Center, Cincinnati, OH). Two hundred nanograms of RNA was reverse transcribed using Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). In addition to RNA, the mixture contained 0.1 μg of random primers (Roche, Mannheim, Germany), and 5 mM dNTPs to a total of twelve microliter. The mixture was incubated at 65ºC for 5 min and immediately plunged into ice. The solution was collected by vortexing followed by addition of 4 μl of 5X strand buffer, 2 μl of 0.1 mM DTT and 1 μl of RNase OUT (Roche) and incubated at room temperature for 2 min. One microliter of superscript II reverse transcriptase was added to make up the final volume of the reaction to 20 μl and incubated at room temperature for 10 min and 42ºC for 50 min. Finally, the enzyme was inactivated by incubation at 80ºC for 15 min. The cDNA was stored at -20ºC until use.

All real-time PCR reactions were performed in a 25 μl mixture containing 20 ng of cDNA preparation (2 μl), SYBR-Green I core reagent, including AmpliTaq-GOLD polymerase (Applied Biosystems, Foster City, CA), 0.4 μM forward primer and 0.4 μM reverse primer. Real-time PCR analysis was done for human *Rac2* mRNA and human *actin* mRNA, and relative quantification was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was carried out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Human *Rac2* transcript levels were normalized to human *actin* transcript levels from the same preparations of cDNA. The PCR primers used for the quantification of human *Rac2* and human *actin* transcripts levels are listed in Table 3.

Table 3. Sequence of oligonucleotide used for real-time PCR analysis

Sequences of oligonucleotides used to quantify the relative transcript levels of human *Rac2* and human *actin*.

Gene	**Primer**	**Sequence**
Human Rac2	hRac2 full forward	5'-ccaccgtgtttgacaactattca-3'
	hRac2 full Reverse	5'-ccacagccccaggttcac-3'
Human Actin	hActin full forward	5'-ctcactgagcgaggctacagct-3'
	hActin full Reverse	5'-ttgatgtcgcgcacgattt-3'

VII. Flow Cytometric Analysis

DMSO, PMA or PDD treated K562 cells were scraped off the plate and detached by pipetting in 1X PBS on ice and washed twice with 1X PBS. Viable cells were counted by trypan blue exclusion, and 1×10^5cells per tube were resuspended in 1X PBS and labeled with PE-conjugated CD41 antibody (kindly provided by Dr. Mary Dinauer, Indiana University). Mouse IgG2A (kindly provided by Dr. Edward F. Srour, Indiana University) was used as an isotype control. Flow cytometry was performed on a Becton-Dickinson FACScan Analyzer. Histograms are based on the analysis of 10,000 cells.

VIII. Site-directed Mutagenesis

Mutagenesis of the putative AP1 binding sites was performed using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with minor modifications. Oligonucleotides needed for mutagenesis were synthesized by Operon Biotechnologies Inc (Huntsville, AL). The following considerations were made in selecting primers. Both strands of mutagenic primers contained the mutation and anneal to the same sequence on opposite strands of the plasmid. In addition the mutation was designed to be in the middle of the primer with around 15 bases of correct sequence on both sides. The PCR reaction mixture contained 5 µl of 10X reaction buffer (provided in the kit), 10 ng of plasmid in which the mutation needs to be incorporated, 125 ng of primer 1 and primer 2 (corresponding to both strands), 1 µl of dNTP mix and 1 µl of pfu ultra high fidelity DNA polymerase (provided in the kit). The PCR mixture was overlayed with a drop of mineral oil. The PCR cycling parameters are described in Table 4.

Table 4. Cycling parameters used for the mutagenesis of AP1 sites in the -30+135 *Rac2* promoter construct.

Segment	Cycle	Temperature	Time
1	1	95ºC	30 sec
2	20	95ºC	30 sec
		55ºC	1 min
		68ºC	6 min

.

The amplified products were digested with 2 μl of *DpnI* and incubated at 37ºC for more than 5 h to ensure complete digestion of the parental nonmutated supercoiled DNA. Following digestion, 1 μl of the digested plasmids DNA was transformed into XL1-Blue super competent cells or α-select silver efficiency competent cells. The plates were incubated at 37ºC for overnight. The transformants were then confirmed for the insertion of mutation by sequencing (DNA sequencing core facility, Indiana University School of Medicine). AP1 mutations were incorporated in the 135 bp *Rac2* promoter region sub-cloned upstream of the -30 bp *Rac2* core promoter in pGL3-Basic reporter gene vector. The primers used for site-directed mutagenesis are described in Table 5.

Table 5. Oligonucleotides used for PCR generation of mutated AP1 sites in the -30+135 bp *Rac2* promoter construct

Mutation of the AP1 binding sites in the 135 bp region between -4223 bp and -4088 bp of the human *Rac2* promoter region was done with the Quickchange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the following sense and antisense oligonucleotide primers. The sequence corresponding to AP1 binding sites that are mutated is indicated in bold italics.

Oligo	Primer	Sequence
Oligo 1	Primer 1	5'- ccctgtctgtgtggt***gttcgag***accccgagcgcgcag -3'
	Primer 2	5'- ctgcgcgctcggggt***ctcgaac***accacacagacaggg -3'
Oligo 4	Primer 1	5'- tgaataaccaaatg***caagctc***tccctgcggtgggac -3'
	Primer 2	5'- gtcccaccgcaggga***gagcttg***catttggttattca -3'
Oligo 7	Primer 1	5'- ggcggagcaccaaga***cctcgaac***cactcaaacgtttcac -3'
	Primer 2	5'- gtgaaacgtttgagtg***gttcgagg***tcttggtgctccgcc -3'

IX. Chromatin Immunoprecipitation Assay

Chromatin preparation and immunoprecipitation were performed with a ChIP kit (Millipore, Bedford, MA) with minor modifications. K562 cells treated with PMA or DMSO were collected by centrifugation at 1200 rpm for 5 min at 4ºC. The cells were counted after collection to ensure the amount of lysis solution to be added to have equal amount of starting material in all samples. Protein-DNA crosslinking was achieved by incubating cells (2×10^6 cells/reaction) with 1% formaldehyde for 10 min at 37ºC. Cross-linking was blocked by adding glycine to a final concentration of 0.125 M and incubating at room temperature for 5 min. The cells were then pelleted with ice-cold PBS by centrifugation for 5 min at 2000 rpm at 4ºC. Cells were lysed by addition of pre-warmed SDS lysis buffer (200 μl/$1x10^6$ cells) (provided in the kit) and incubated for 10 min on ice. One milliliter of lysate containing DNA crosslinked to proteins was sonicated to an average length of 0.2-1.5 kb (checked by running on gel) with 8 sets of 15 sec pulses. This standardized sonicated condition was then used in all ChIP assays. The sonicated lysates were then cleared by centrifugation and diluted 10-fold with ChIP dilution buffer provided in the kit. One percent of the diluted lysate was reserved as an input sample. The diluted lysate was pre-cleared with salmon sperm DNA/Protein A agarose slurry (provided in the kit) for 1 hr at 4ºC with agitation. The agarose was removed by brief centrifugation and the supernatant was used for immunoprecipitation of chromatin with antibodies for Pol II (Santa Cruz Biotechnology), c-Jun (Santa Cruz), c-Fos (Santa Cruz), acetylated histone H3 (Millipore, Bedford, MA), dimethylated histone H3K9 (Millipore) or normal rabbit serum (Santa Cruz) by incubating overnight with antibodies at 4ºC with rotation. The antibody/DNA complexes were collected using Protein A agarose slurry by

incubating for 1 h at 4ºC with rotation. The agarose was pelleted to remove the unbound, non-specific DNA. The slurry containing the DNA/antibody complex was washed for 5 min with each of low salt immune complex, high salt immune complex, LiCl immune complex wash buffers (provided in the kit) and twice with TE buffer before elution. After washing, the DNA/protein complex was eluted using freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). The eluate was reverse crosslinked using 5 M NaCl by incubating at 65ºC for at least 5 h. Reverse crosslinked eluates were treated with 2 µl of proteinase K (10 mg/ml) to denature proteins, followed by purification of the DNA using a high pure PCR product purification kit (Roche, Mannheim, Germany). Immunoprecipitated DNA was analyzed by standard PCR.

The PCR primers used for the analysis of immunoprecipiated DNA are listed in Table 6.

Table 6. Oligonucleotides used for PCR amplification of the immunoprecipitated DNA

Sequences of oligonucleotides corresponding to the 135 bp human *Rac2* promoter region between -4223 bp and -4088 bp, the 129 bp intragenic region (+15 kb) of the *Rac2* locus and 210 bp intragenic region (+3.5 kb) of the *actin* gene locus.

Gene Region	**Primer**	**Sequence**
135 bp *Rac2* promoter region	Rac2 135bp Forward	5'-actttgcgttttctaggatttcac-3'
	Rac2 135bp Reverse	5'-caagcattgtctatcaatggcac-3'
+15 kb *Rac2* intragenic region	Rac2+15kb Forward	5'-ttcacttcagggaaactgtgc-3'
	Rac2+15kb Reverse	5'-gcacagatgaggagaaaaggc-3'
+3.5 kb *actin* intragenic region	Actin+3.5kb Forward	5'-ggtgatagcattgctttcgtg-3'
	Actin+3.5kb Reverse	5'-gtctcaagtcagtctacaggt-3'

X. Nuclease Accessibility Assay

Nuclei were isolated from K562 cells treated with DMSO or 100 nM PMA using nuclei preparation buffer (15 mM Tris-HCl pH 7.5, 0.3 M sucrose, 60 mM KCl, 2 mM ethylene glycol tetraacetic acid ([EGTA]), 15 mM NaCl, and 5 mM $MgCl_2$) containing 5% NP-40. Further washes were performed in nuclei digestion buffer without NP-40 and samples were stored in nuclei preparation buffer at -80ºC until used. Aliquots of nuclei (1×10^6) were incubated with 100 U of Nuclease S7 (Roche) in nuclei digestion buffer (60 mM $CaCl_2$, 750 mM NaCl) for 20 min at 37ºC, and the reactions were terminated by the addition of stop buffer (10 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K). Genomic DNA was purified using the high pure PCR product purification kit (Roche). The 135 bp Rac2 regulatory region was PCR amplified and the PCR product intensities were determined using ImageJ software. The amount of PCR product amplified from nuclease S7 digested samples was normalized to the amount of PCR product amplified from undigested DNA.

XI. Transient Transfection

K562 human chronic myelogenous leukemia cells were grown to a density of 500,000 cells per ml. The cells were harvested by centrifugation in 50 ml conical tubes and resuspended at a density of 1×10^7 K562 cells per 300 μl complete medium. For transient transfection of *Rac2* promoter constructs followed by PMA treatment of K562 cells, the cells were mixed with 1.6 nmoles of Rac2 constructs plus pcDNA3.1 for a total of 5 μg DNA. Since 1.6 nmoles of the largest construct is 5 μg of DNA and 1.6 nmoles of the smallest construct is 2.5 μg of DNA, various amounts of pcDNA3.1 plasmid was added

to each sample so that each contained 5 μg of total DNA. For AP1 over expression studies, the cells were mixed with 0.5 μg of cytomegalovirus (CMV)-β-galactosidase plasmid, 2.5 μg of -30+135 *Rac2* promoter construct and 8 nmoles of AP1 expression vectors or empty vectors plus empty pBluescript SK+ vector for a total of 40 μg DNA.

Three hundred microliter aliquots of cells were placed into 4 mm electroporation cuvettes (Midwest Scientific) along with an appropriate concentration of plasmid constructs. The cuvettes were vortexed briefly prior to electroporation with a voltage of 220 V and capacitance of 950 μF. Electroporated cells were divided into two samples and one sample was treated with 100 nM PMA and the other with DMSO. Cells were harvested at 16-24 h post-transfection and lysed for reporter gene assays.

XII. Reporter Gene Assays

Luciferase reporter gene assays were performed using the luciferase reporter assay system (Promega, Madison, WI, USA) as per the manufacturer's instruction. Transfected cells were scraped off the plate and collected by centrifugation at 1200 rpm for 5 min. Pelleted cells were washed with 1 ml of 1X ice-cold PBS followed by centrifugation at 8000 rpm for 4 min. The cells were mixed 100 μl of 1X Lysis buffer (Promega) and incubated at room temperature for 15 min. The cell lysate was then collected by centrifugation at 12,000 rpm for 4 min and the supernatant was transferred to a fresh 1.5 ml microcentrifuge tube for assay. Twenty microliter of lysate was used per luciferase reaction with 100 μl of luciferase assay substrate and the light units were read using a Lumat LB 9501 chemiluminometer (EG & G Berthold, Wildbad, Germany). The assays were performed in triplicate for each experiment.

β-galactosidase activity was measured by colorimetric analysis and was used to standardize the luciferase activity between samples to correct for variable transfection efficiency. Thirty microliters of cell lysate prepared using promega reporter lysis buffer was added to 270 µl of β-galactosidase assay buffer containing 200 µl of 0.1 M $NaPO_4$, 66 µl ortho-nitrophenyl-β-galactoside [ONPG] and 3 µl of 100X Mg^{2+} (0.1 M $MgCl_2$ and 4.5 M β-mercaptoethanol). The mixture was vortexed and incubated at 37ºC until a change in color to pale yellow was observed. The assay was stopped by the addition of 1M Na_2CO_3 and the activity was measured at OD_{420}. Lysate from untreated K562 cells treated as above was used as the blank.

XIII. RNA Isolation

RNA isolation by isopropyl alcohol precipitation

Total RNA was isolated from K562 cell lines using the Tri-reagent solution (Molecular Research Center, Cincinnati, OH). Six hundred microliters of Tri-reagent per 5×10^6 cells was added. After thorough mixing, the solution was incubated at room temperature for 5 min. Two hundred microliters of chloroform was added to each tube and mixed thoroughly by vortexing and incubated at room temperature for 10 min, followed by centrifugation at 14,000 x g for 15 min in cold. The aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube and the RNA was precipitated by addition of 0.6 volumes of isopropanol. The RNA was pelleted at 14,000 x g at 4ºC for 10 min and the pellet was washed with 500 µl of 75% EtOH in diethyl pyrocarbonate (DEPC)-treated H_2O. The pelleted RNA was air-dried for few min and then resuspended in appropriate volumes of DEPC-treated H_2O.

XIV. *In vitro* Transcription

Antisense RNA transcripts were generated from human *Rac2* riboprobes and were linearized with the restriction enzyme *NotI*. The commercially derived 18S rRNA template (Ambion, Austin, TX) was already linear. The human *Rac2* riboprobe was generated at a specific activity of approximately 4×10^9 cpm/μg, and the 18S riboprobe was generated with lower specific activity, 4×10^6 cpm/μg, to compensate for the greater abundance of the 18S transcript. The transcription reaction occurred in a 20 μl reaction mixture containing 1 μg of DNA template, with a final concentration of the 1X transcription buffer (MAXIscript™ Kit, Ambion), 0.5 mM each of ATP, CTP and GTP, 3.125 μM of α-^{32}p UTP (Perkin Elmer), and 30 to 60 units of T7 RNA polymerase. The reaction was incubated for 10 min at 37ºC. Following incubation, the DNA template was degraded by addition of 2 units of DNaseI and incubating the reaction for 15 min at 37ºC. The labeled probe was mixed with equal volume of gel loading buffer II (Ambion) and separated on a 8 M urea/5% polyacrylamide gel at 300 V for 45 min in 1X TBE. The wet gel was exposed to X-ray film for approximately 1 min in the dark room. The band corresponding to full length probe was excised, broken into pieces and transferred into a 1.5 ml RNase-free microcentrifuge tube containing 400 μl of probe elution buffer (Ambion) and incubated at 37ºC for 4 h. The eluted probe for transferred to a new microcentrifuge tube and 2 μl of probe was used for scintillation counting to determine the amount of radioactive label.

The 18S riboprobe was generated with a very low specific activity (5×10^4 cpm/μg) with the ratio of labeled to unlabeled UTP of 1:160,000. The reaction was incubated for 3 h at 37ºC. The DNA template was degraded by adding 2 units of DNaseI

and incubating the reaction for 15 min at 37ºC. The unincorporated labeled nucleotides were removed by NucAway™ Spin Columns (Ambion) as per the manufacturer's protocol. Two microliters of eluted probe was used for scintillation counting for counting the radioactive probe. Commercial RNA marker RNA century™ marker template was generated as per the manufacture's protocol (Ambion). The RNA Century molecular weight markers provided size reference for 100 to 500 nucleotides.

XV. RNase Protection Assay

RNase protection assays (RPA) were performed on 10 μg total RNA using the RNase protection assay kit (Ambion) according to the manufacturer's instructions. Radiolabeled RNA probes were generated from human and murine *Rac2* cDNAs (Ladd et al. 2004) and 18S rRNA (Ambion, Austin, TX), using the MAXIscript in vitro transcription kit (Ambion). The labeled human *Rac2* and 18S rRNA riboprobes were hybridized with 10 μg of total RNA isolated from K562 cell lines. Two additional tubes containing yeast RNA were used as control for probe integrity and function of RNases. In addition the mixture contained 10 μl of Hybridization III buffer (Ambion). The tubes were heated to 94ºC for 2-3 min and then incubated at 42ºC for 18 h to allow the probe to hybridize to its complement in the sample RNA. The unprotected single-stranded RNA was digested using a 1:50 dilution of RNase A/T1 mix in the 150 μl of RNase Digestion III buffer (Ambion) and incubating the samples at 37ºC for 30 min. One of the yeast RNA tubes was also treated with RNase mix to verify that the probe was not protected in the absence of homologous sequence and thus provides a positive control for the RNases. The other tube containing yeast RNA was treated with 150 μl of RNase Digestion III buffer without

RNases as a control for the integrity of the probe. The samples were precipitated using 225 µl of RNase Inactivation/Precipitation III Solution (Ambion). The samples were incubated at -20ºC for at least 15 min, followed by centrifugation at 14,000 x g for 15 min at 4ºC. After discarding the supernatant, the pellets were air-dried for few minutes. Two microliter of gel loading buffer (Ambion) was added to each tube and the samples were incubated at 94ºC for 3 min. The sampled were separated on an 8 M urea/5% polyacrylamide gel at 1800 V for approximately 2 h in 1X TBE. The gel was transferred to 3 mm Whatman filter paper, covered with plastic wrap and exposed to X-ray film at -80ºC.

XVI. Genomic DNA Isolation

The cells were scraped off the plate, followed by centrifugation at 3000 x g for 5 min. The pellets were washed with 1X PBS and 600 µl of DNA lysis buffer (10 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS) was added for lysis reaction. After lysis, the cell lysates were incubated with 3 µl of 20 mg/ml RNase A (Roche Scientific) for 15-30 min. Two hundred microliter of 7.5 M ammonium acetate was added for the precipitation of proteins. The samples were vortexed for 20 sec and incubated on ice for at least 5 min, followed by centrifugation at 14,000 x g for 10 min for the removal of proteins and cellular debris. Precipitation of genomic DNA was carried out by addition of 600 µl of isopropanol to the samples and mixed gently at least 25 times. The DNA was pelleted by centrifugation at 14,000 x g for 10 min at 4ºC. DNA pellets were washed with 500 µl of ice-cold EtOH and air dried. The pellets were then suspended in appropriate volume of ddH_2O.

XVII. Western blot

Whole cell extracts were isolated from K562 cells stimulated with PMA using SDS lysis buffer. The extracts were sonicated for 1 min followed by centrifugation to collect the supernatant. The concentration of proteins in each extract was determined by Bradford assay (BioRad, Richmond, CA). Equal amounts of cell lysate were run on SDS-polyacrylamide gels and transferred to nitrocellulose. Immunoblotting was performed using 1:10,000 anti- c-Jun antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The filter was washed with Tris-HCl buffered saline with 0.1 % tween 20 (TBST), and incubated with anti-rabbit horse radish peroxidase antibodies (Amersham Pharmacia Biotech, Amersham, UK). After further washing, antibody bound to the filter was visualized using the ECL detection reagents (Amersham Pharmacia Biotech, Amersham, UK).

XVIII. Trichostatin A treatment of K562 cells

Around 1×10^7 K562 cells were transfected with AP1 expression vectors for 24 h before treatment. Untransfected or transfected cells were then treated with 300 ng/ml of HDAC inhibitor trichostatin A (TSA) or equal amount of DMSO (vehicle control) for 5 h.

RESULTS

I. *Rac2* gene expression increases upon PMA stimulation and megakaryocytic differentiation of K562 cells

Treatment of K562 cells with PMA results in changes of cell morphology and acquisition of various cell surface markers unique to platelets and megakaryocytes (Burger, Zutter et al. 1992; Racke, Lewandowska et al. 1997). Following PMA treatment K562 cells became adherent (Fig 5A), indicating increased cell surface adhesion due to increased integrin expression (Whalen, Galasinski et al. 1997). These effects were not seen following treatment with PDD, an inactive analog of PMA, or with DMSO vehicle (Fig 5A). Further studies were performed to examine expression of platelet surface glycoprotein IIb/IIIa (CD41 antigen), an early marker of megakaryocyte differentiation (Burger, Zutter et al. 1992). Flow cytometric analysis showed increased CD41 expression upon 24 h PMA treatment of K562 cells compared to the DMSO or PDD treated cells (Fig 5B). *Rac2* gene expression was previously reported to be up-regulated upon terminal myeloid cell differentiation (Didsbury, Weber et al. 1989). *Rac2* mRNA levels during K562 cell differentiation was examined by quantitative real-time PCR analysis (Fig 6A). PMA stimulation led to about 5-fold increase in Rac2 mRNA levels compared to that of DMSO-treated cells. PDD treated cells did not show significant changes in *Rac2* gene expression (Fig 6A). Induction of *Rac2* gene expression was further confirmed by RNase protection assay, which showed a 6-fold increase in *Rac2* mRNA levels upon PMA stimulation of K562 cells (Fig 6B).

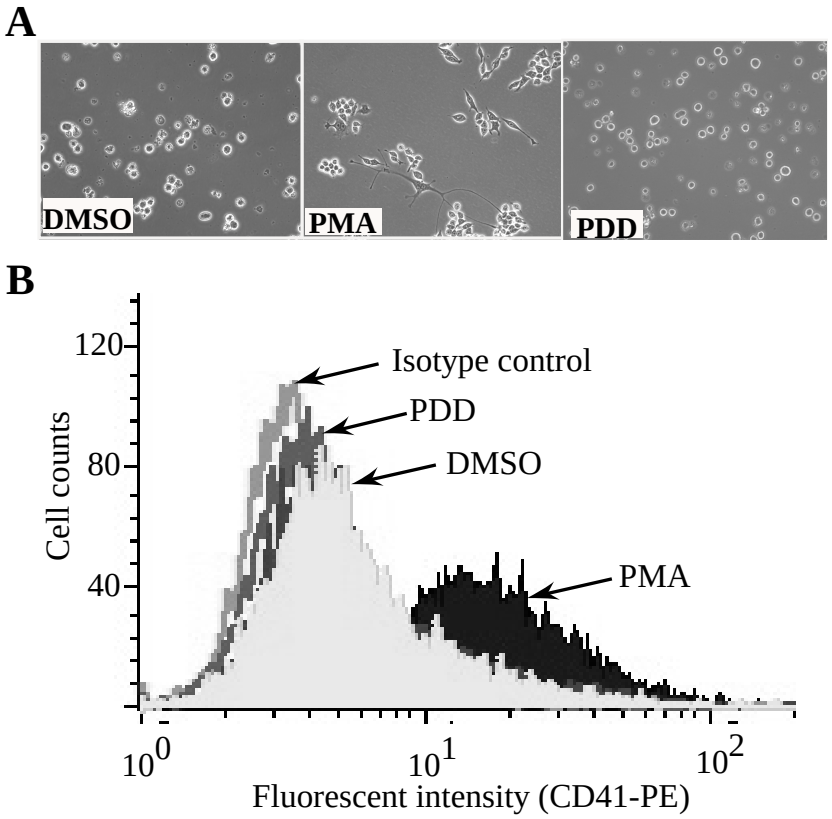


Figure 5. *Rac2* mRNA levels are induced upon PMA stimulation of K562 cells.

A) Light microscopy analysis of K562 cells grown for three days in medium containing DMSO, 100 nM PMA, or 100 nM PDD. Magnification 20X.

B) K562 cells were grown in medium containing DMSO, 100 nM PMA, or 100 nM PDD for 24 h. Surface staining of human CD41 was performed using PE-conjugated antibodies and analyzed by flow cytometry. Mouse IgG2A was used as an isotype control.

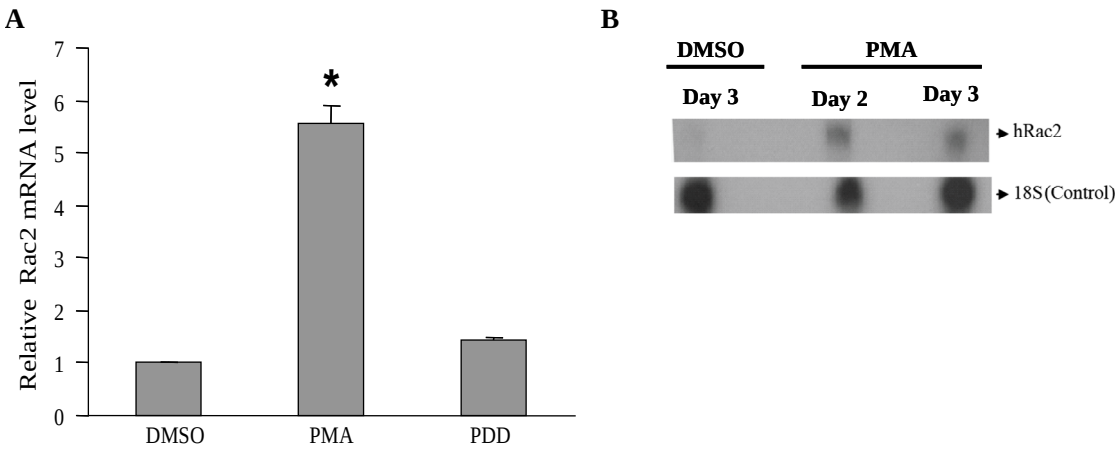


Figure 6. *Rac2 mRNA* levels are induced upon PMA stimulation and megakaryocytic differentiation of K562 cells.

A) Quantitative real-time PCR analysis was performed to quantify *Rac2 mRNA* levels in K562 cells treated with DMSO, 100 nM PMA, or 100 nM PDD for 24 h. ΔC_t (threshold cycle) values of *Rac2* mRNA were normalized to ΔC_t values of human *actin* mRNA from the same cDNA preparations. Normalized *Rac2* transcript levels in K562 cells treated with PMA and PDD were then calibrated against the *Rac2* transcript level in DMSO treated samples. The level of *Rac2* mRNA in DMSO treated cells was set at 1. The data represent a summary of three independent experiments. Error bars indicate the standard error, and the asterisk indicates a *P* value of <0.05 compared to DMSO control.

B) RNase protection assays were performed as described in the Methods using 10 μg of RNA isolated from K562 cells treated with DMSO or 100 nM PMA for two or three days. The riboprobe for human *Rac2* mRNA was generated at a specific activity of 4×10^9 cpm/μg, while the 18S riboprobe was generated at a specific activity of 2.5×10^6 cpm/μg. The protected fragments for the human *Rac2* and *18S* riboprobe are 362 nt and 80 nt, respectively.

II. Transcription of the *Rac2* gene increases upon PMA stimulation

Quantitative real-time PCR analysis showed a time dependent increase in *Rac2* mRNA levels following PMA stimulation of K562 cells for 12 and 24 h (Fig 7A). To determine if the increase in *Rac2* mRNA levels is due to increased transcription, the coding region of the *Rac2* gene was examined for enrichment of RNA polymerase II by chromatin immunoprecipitation analysis (Sandoval, Rodriguez et al. 2004). Treatment of K562 cells with PMA resulted in a progressive enrichment of RNA polymerase II within the coding region of the *Rac2* gene, indicating an increase in the rate of transcription (Fig 7B). No enrichment of RNA polymerase II within the human *actin* gene was observed upon PMA treatment of K562 cells.

III. PMA responsive regulatory cis-elements reside within the 4.5 kb proximal *Rac2* gene promoter

A luciferase reporter plasmid carrying 4.5 kb of the human *Rac2* gene proximal promoter was transiently transfected into K562 cells. Following transfection the cells were split into two populations. One sample was treated with PMA for 24 h and the other with DMSO. PMA stimulation resulted in a 5-fold increase in reporter gene expression compared to that of the DMSO treated samples (Fig 8), similar to the induction of endogenous *Rac2* mRNA levels upon PMA treatment of K562 cells (Fig 6A). An *SV40* promoter luciferase reporter gene vector was used as a positive control and the *EF1α* promoter in pGL3/luciferase reporter gene vector was used as a negative control. These data demonstrate that DNA regulatory elements sufficient to direct PMA responsive *Rac2* gene expression reside within the proximal 4.5 kb *Rac2* gene promoter region.

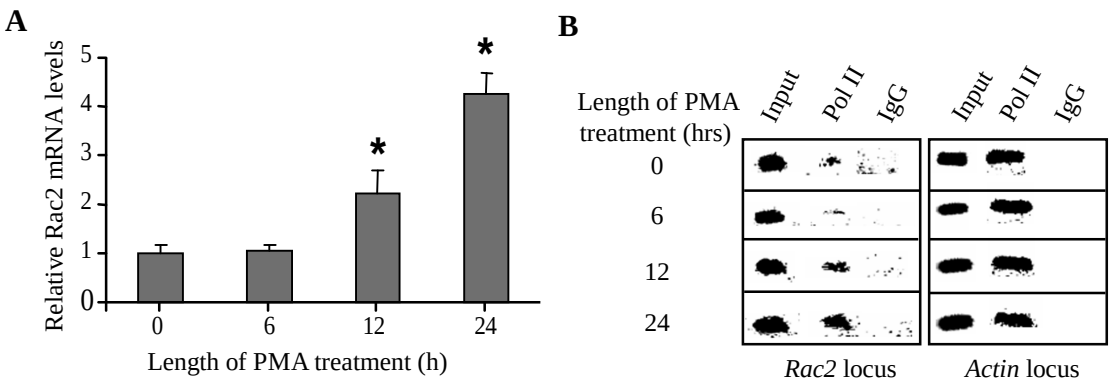


Figure 7. Transcription of the *Rac2* gene increases upon PMA stimulation.

A) *Rac2* mRNA levels from K562 cells stimulated with 100 nM PMA for 0, 6, 12 or 24 h were analyzed by quantitative real-time PCR analysis. ΔC_t values of *Rac2* mRNA were normalized to ΔC_t values of *actin* mRNA from the same cDNA preparations. The data represent a summary of at least three independent experiments. Error bars indicate the standard error. *P* values were determined by a standard *t* test, and asterisks indicate a *P* value of <0.05 compared to the 0 h time point.

B) Chromatin was isolated from K562 cells treated with PMA for various time periods and immunoprecipitated using anti-RNA polymerase II antibody or normal rabbit serum. PCR analysis of genomic DNA extracted from input samples (1% of the diluted lysate) and the immunoprecipitates using amplicons from a 200 bp intragenic region (+4 kb) of the *Rac2* gene locus and a 200 bp intragenic region (+4 kb) of the *Actin* gene locus. The figure shown is representative of at least three independent experiments.

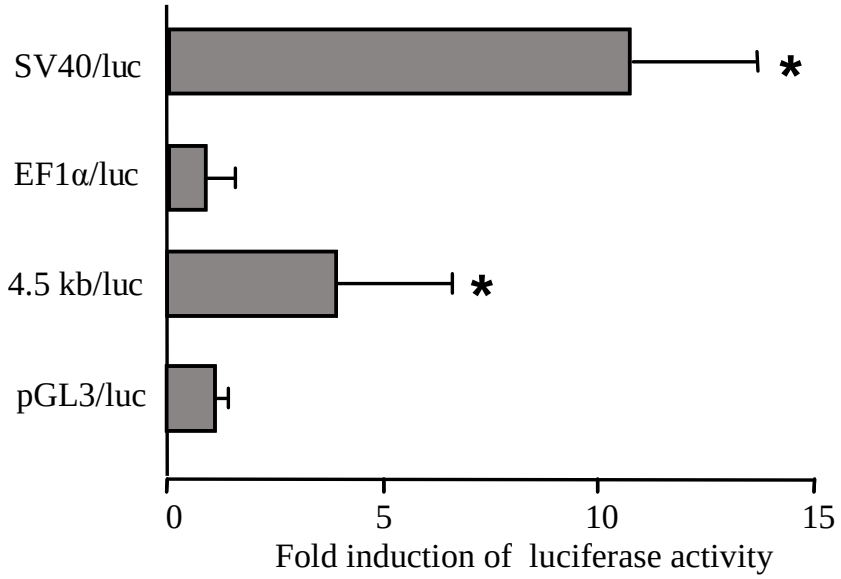


Figure 8. The 4.5 kb proximal *Rac2* promoter contains PMA-responsive cis-elements.

A pGL3/luc (luciferase) vector or pGL3/luciferase vector containing the 4.5 kb proximal *Rac2* gene promoter, *EF1α* promoter, or *SV40* promoter were transfected into K562 cells. After transfection the cells were divided into two samples, and one sample was treated with 100 nM PMA and the other with DMSO for 24 h. The cells were harvested and luciferase activity was determined. The difference in luciferase activity of the PMA treated samples compared to the DMSO controls was calculated and represented as fold induction of luciferase units. Results represent normalized mean ± standard error and are representative of at least six independent experiments with multiple plasmid preparations, performed in triplicate. *P* values were determined by a standard *t* test, and asterisks indicate *P* values of <0.05 relative to the empty pGL3/luciferase vector.

IV. A 135 bp region within the 4.5 kb proximal *Rac2* gene promoter is necessary and sufficient for the induction of transcription upon PMA stimulation

A series of 5'- deletions of the 4.5 kb *Rac2* promoter were generated to identify the cis-elements necessary for PMA induction of *Rac2* gene expression in K562 cells (Fig 9). Deletion of the promoter to -4223 bp did not alter the level of reporter gene induction compared to that of the 4.5 kb construct. However, deletion of the 135 bp promoter region between -4223 bp and -4088 bp led to a loss of the PMA induced promoter activity (Fig 9). The basal unstimulated transcriptional activity of these constructs did not show any significant variation (Fig 11).

The 135 bp *Rac2* promoter region (-4223 to -4088 bp) was sub-cloned upstream of the -30 to +134 bp *Rac2* core promoter in the pGL3/luciferase reporter gene vector (referred to as -30+135/luciferase) to test if this regulatory region is sufficient to serve as a PMA responsive cis-element. Transient transfection of this construct showed a significant 25-fold increase in the induction of reporter gene expression compared to that of the -30 bp/luciferase construct (Fig 10). These results demonstrate that the 135 bp regulatory region is sufficient to direct increased transcriptional activity of the *Rac2* gene promoter upon PMA stimulation of K562 cells.

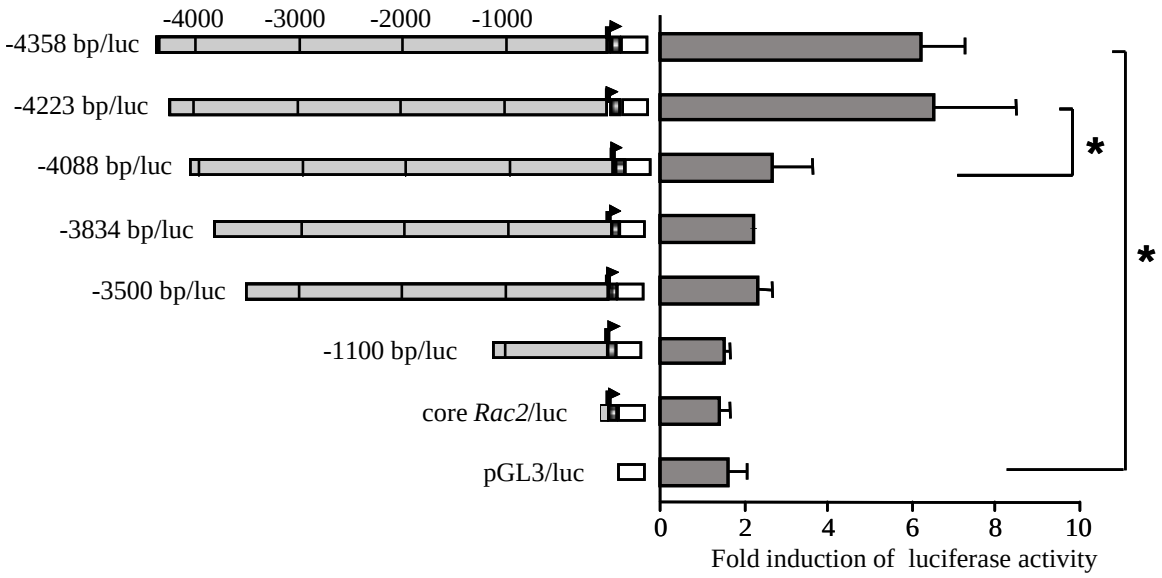


Figure 9. The 135 bp region between -4223 bp and -4088 bp of the *Rac2* gene promoter is necessary for PMA-responsive transcription.

Various lengths of 5'- deletion constructs of the 4.5 kb human *Rac2* gene promoter obtained by restriction enzyme digestion or PCR amplification were sub-cloned into pGL3/luciferase reporter gene vector. All constructs extend to +134 bp of the *Rac2* gene (denoted by the shaded box). Arrows indicate the transcription start site at the +1 position. Plasmids containing *Rac2* promoter deletion constructs were transiently transfected into K562 cells followed by partitioning into two samples. One was treated with 100 nM PMA and other with DMSO for 24 h. The fold difference in luciferase activity of the PMA treated samples compared to the DMSO treated samples is presented. Results represent normalized mean ± standard error and are representative of at least six independent experiments performed in triplicate and asterisks indicate *P* values of <0.05.

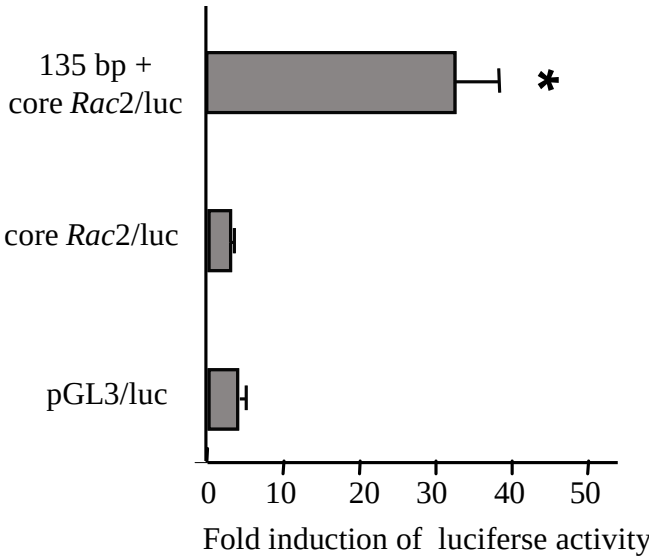


Figure 10. The 135 bp regulatory cis-element of the *Rac2* promoter is sufficient for PMA-responsive transcription.

The pGL3/luciferase vector containing the 135 bp region between -4223 bp and -4088 bp of the *Rac2* gene promoter region upstream of the -30 bp *Rac2* core promoter in pGL3/luciferase reporter gene vector, -30 bp *Rac2* core promoter construct in pGL3/luciferase reporter gene vector or the pGL3/luciferase reporter gene vector was transiently transfected into K562 cells followed by partitioning into two samples. One was treated with 100 nM PMA and other with DMSO for 24 h. The fold difference in luciferase activity of the PMA treated samples over DMSO is presented. Results represent normalized mean ± standard error and are representative of at least six independent experiments performed in triplicate and asterisk indicate *P* values of <0.05 compared to -30 bp/luciferase construct.

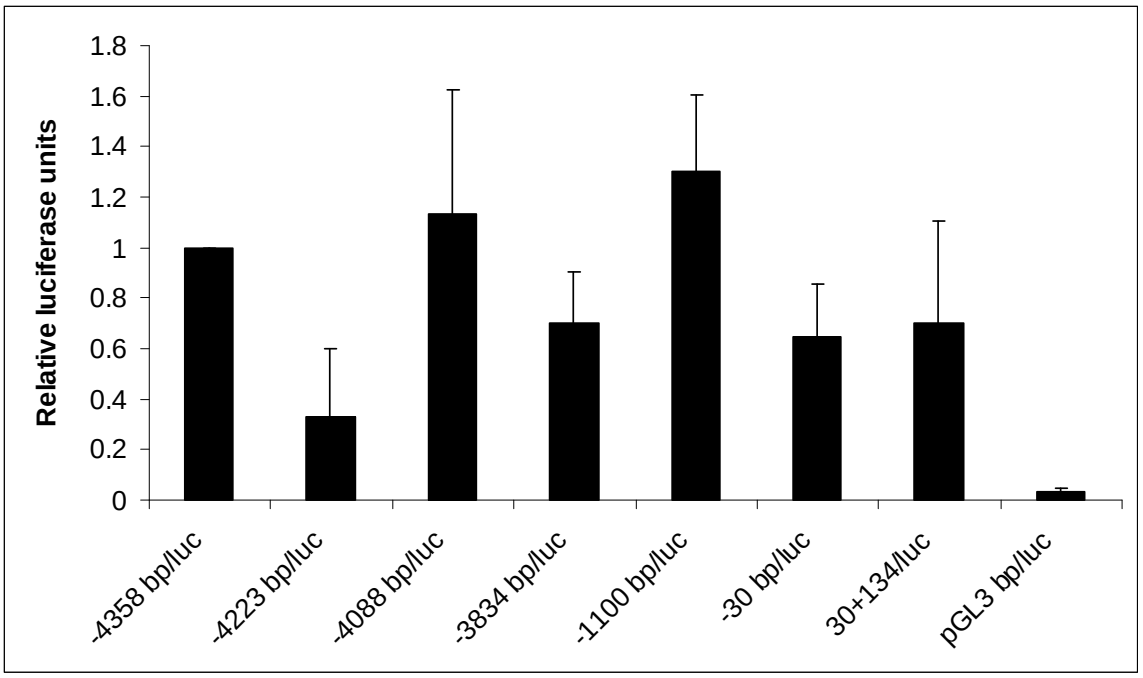


Figure 11. *Rac2* promoter deletion constructs do not show changes in basal luiferase units in unstimulated K562 cells.

Various lengths of 5'- deletion constructs of the 4.5 kb human *Rac2* gene promoter sub-cloned into pGL3/luciferase reporter gene vector were transiently transfected into K562 cells. Twenty four hours after transfection, the cells were harvested and luciferase and β-galactosidase activities were determined. After normalization with β-galactosidase activity, the fold difference in luciferase units relative to -4358 bp/luciferase construct was calculated and represented as relative luciferase units. Results represent normalized mean ± standard error and are representative of at least three independent experiments performed in duplicate.

V. Identification of PMA responsive DNA – binding proteins that interact with the 135 bp *Rac2* gene regulatory region

EMSA was performed with seven overlapping oligo probes that span the 135 bp *Rac2* gene regulatory region to detect PMA induced DNA-binding proteins (Fig 12A). Of the seven probes, Oligo probes 1, 4 and 7 revealed a DNA-protein complex that becomes more intense following stimulation with PMA. Although nuclear proteins bind to all the other oligonucleotide probes, no difference in protein binding was seen for oligo probes 2, 3, 5 or 6 when comparing extracts from PMA stimulated versus unstimulated cells (Fig 12B). In addition, the PMA responsive protein binding seen with oligo probes 1, 4 and 7 were of similar mobility when run on the same gel (Fig 12C), suggesting that a common protein/transcription factor binding site may be present at three distinct sites within the 135 bp *Rac2* regulatory region.

Competition experiments were performed to further test the presence of common transcription factor binding site in oligo probes 1, 4 and 7. The PMA responsive mobility shifts observed with oligo probes 1, 4 and 7 were effectively and specifically competed away using unlabeled oligo 1, 4 or 7 (Fig 13). This result showed that the 135 bp *Rac2* gene promoter regions shares a common protein binding site at three distict positions. Database analysis using the TRANSFAC transcription factor search analysis program (Wingender, Dietze et al. 1996; Wingender, Karas et al. 1997) revealed putative AP1 binding sites within oligo probes 1, 4 and 7 (Fig 12A). AP1 transcription factors are known to up-regulate transcription of genes containing the TPA response element (TRE; 5'-TGAG/CTCA-3) (Angel, Baumann et al. 1987; Angel, Imagawa et al. 1987). AP1 consists of homo- or heterodimers of Jun, Fos or ATF proteins (Bohmann, Bos et al.

1987; Hess, Angel et al. 2004). In addition, Jun and Fos proteins are also early response genes that play important roles in myeloid cell differentiation (Lord, Abdollahi et al. 1993; Shafarenko, Amanullah et al. 2004). To determine if the common transcription factor binding site within the 135 bp region corresponds to AP1, unlabeled AP1 control oligos known to bind AP1 (Ignatov and Keath 2002) were added to the EMSA reaction as competitors. Non-radiolabeled AP1 oligos effectively disrupted the PMA responsive protein binding seen at all three sites (Fig 13). Addition of non-radiolabeled mutant AP1 oligo, that fails to bind AP1 (Zutter, Painter et al. 1999; Lai and Cheng 2002), did not disrupt the PMA responsive protein binding (Fig 13). Furthermore, addition of nuclear extracts from the PMA-treated-cells to the control probe containing AP1 site produced a shift of the same mobility (Fig 13). This showed the presence of AP1 binding sites at three distinct locations within the 135 bp *Rac2* regulatory region. Super shift analysis was done to further test the binding of AP1 protein complex to the putative sites present in oligo probes 1, 4 and 7. Lastly, super shift analysis with anti c-Jun antibody added to the mixture resulted in the dissociation of protein-DNA complex and hence showed decrease in protein binding (Fig 14). Addition of anti c-Fos antibody also resulted in diminished protein binding or super shift (Fig 14). These results revealed that AP1 complexes containing c-Jun and c-Fos as binding partners interacts at three distinct sites within the 135 bp *Rac2* gene regulatory region.

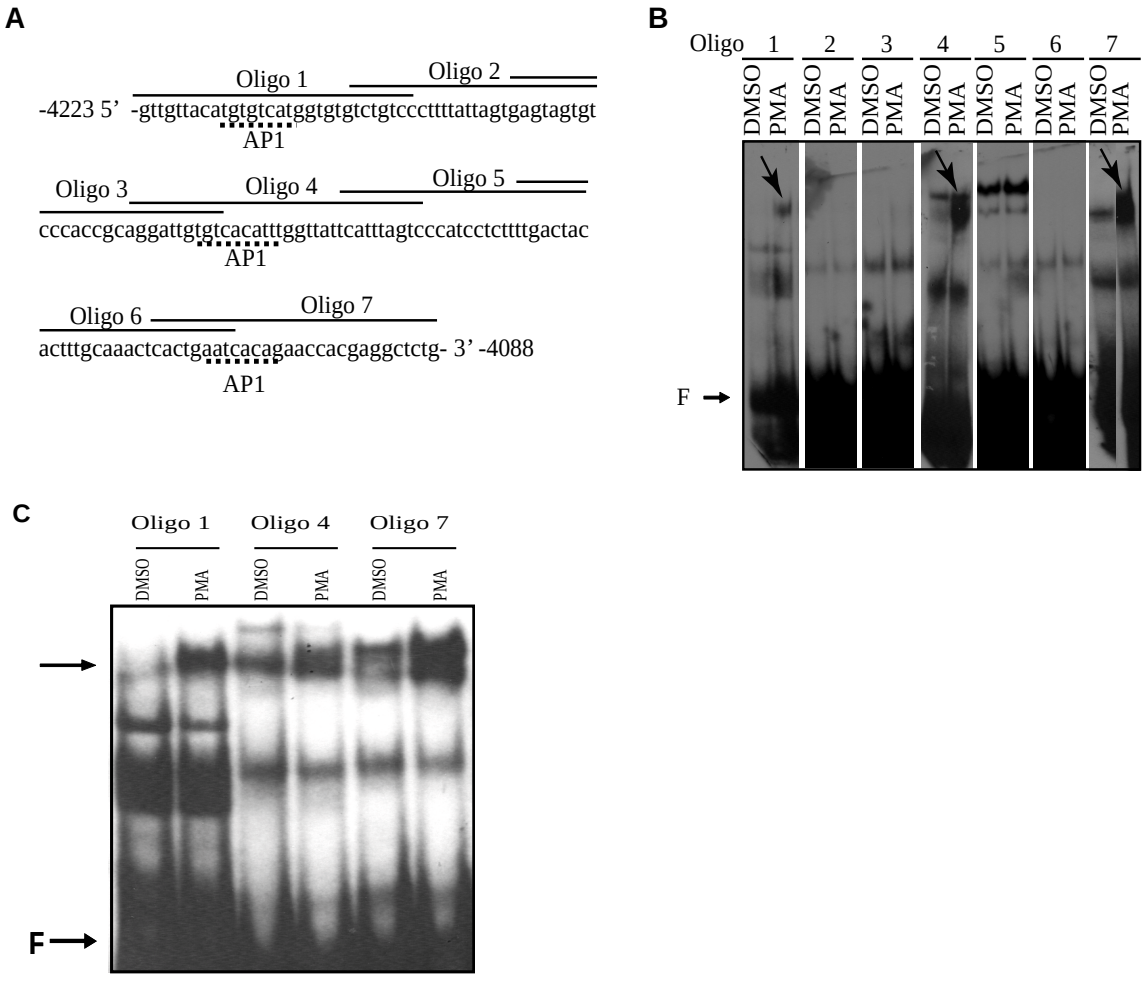


Figure 12. Identification of three protein binding sites within the 135 bp *Rac2* gene promoter region.

A) Schematic representation of the 135 bp region between -4223 bp and -4088 bp of the *Rac2* gene promoter region. The positions of seven overlapping oligo probes are indicated. Three putative AP1 binding sites identified by TRANSFAC database analysis are indicated by dotted lines.

B) EMSA was performed using seven overlapping oligo probes (1-7) as outlined in the methods. Labeled probes were incubated with 5 μg of nuclear extract isolated from K562 cells treated for 24 h with DMSO or 100 nM PMA. DNA-protein complexes that are induced by PMA treatment are indicated by arrows and “F” denotes free probe.
C) EMSA was performed using oligos 1, 4 or 7 as outlined in the methods and run on the same gel. Labeled probes were incubated with 5 μg of nuclear extract isolated from K562 cells treated for 24 h with DMSO or 100 nM PMA. DNA-protein complexes of similar size that are induced by PMA treatment in all three oligos are indicated by arrow. “F” denotes free probe.

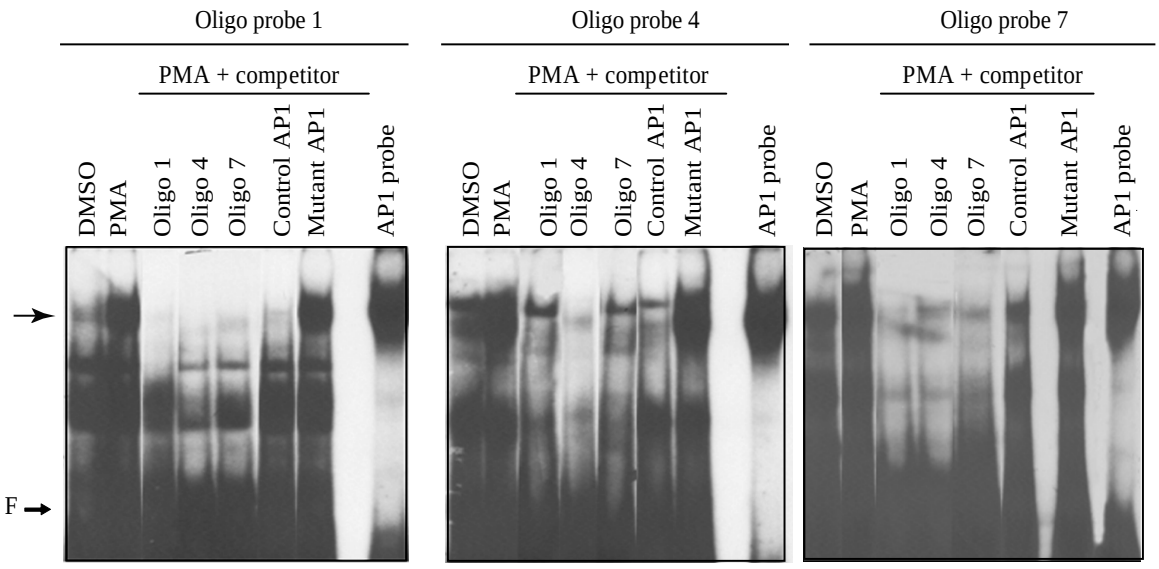


Figure 13. Identification of the common transcription factor binding site within the 135 bp *Rac2* regulatory region.

Oligo 1, 4 or 7 probes were incubated with 5 μg of nuclear extract isolated from K562 cells treated for 24 h with DMSO or 100 nM PMA. Competition experiments were done by mixing a 70-fold excess of non-radiolabeled Oligo 1, Oligo 4, Oligo 7, AP1 control oligo, or AP1 mutant oligo with the PMA-treated samples. “Control” denotes AP1 control oligonucleotide probe incubated with 5 μg of nuclear extract from K562 cells treated with 100 nM PMA. DNA-protein complexes that are induced by PMA treatment are indicated by the arrow and “F” denotes free probe.

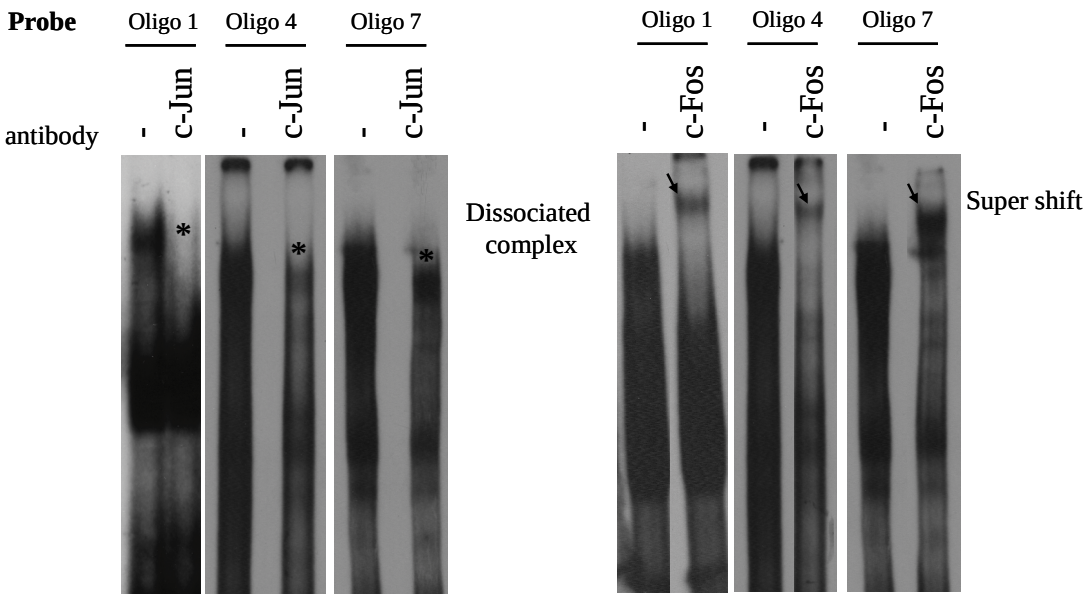


Figure 14. c-Jun and c-Fos are AP1 components that interact with the 135 bp *Rac2* gene promoter region.

Super shift analysis was performed by mixing labeled Oligos 1, 4 or 7 with 5 µg of nuclear extract isolated from K562 cells treated with 100 nM PMA for 24 h along with antibodies against c-Jun or c-Fos. Arrows indicate the super shifted c-Fos/AP1 protein complex and * denotes the absence or decrease in the intensity of the oligo/nuclear-protein complex due to the interference of c-Jun or c-Fos antibody with the oligo/nuclear-protein complex formation.

VI. AP1 binds to the 135 bp *Rac2* gene regulatory region *in vivo*

Chromatin immunoprecipitation assays were conducted to assess AP1 binding to the *Rac2* gene promoter *in vivo*. Crosslinked and sheared chromatin prepared from unstimulated K562 cells and K562 cells stimulated with PMA for 24 h was immunoprecipiated with c-Jun or c-Fos antibodies. Figure 15 shows that both c-Jun and c-Fos efficiently bind to the 135 bp region *in vivo* upon PMA treatment of K562 cells. Binding of AP1 to the 135 bp region was not detected in DMSO treated K562 cells. The interaction of AP1 to the *Rac2* gene promoter is specific, as no enrichment of AP1 was observed within the body of the *Rac2* gene (+15 kb) that lacks putative AP1 binding sites (Fig 15).

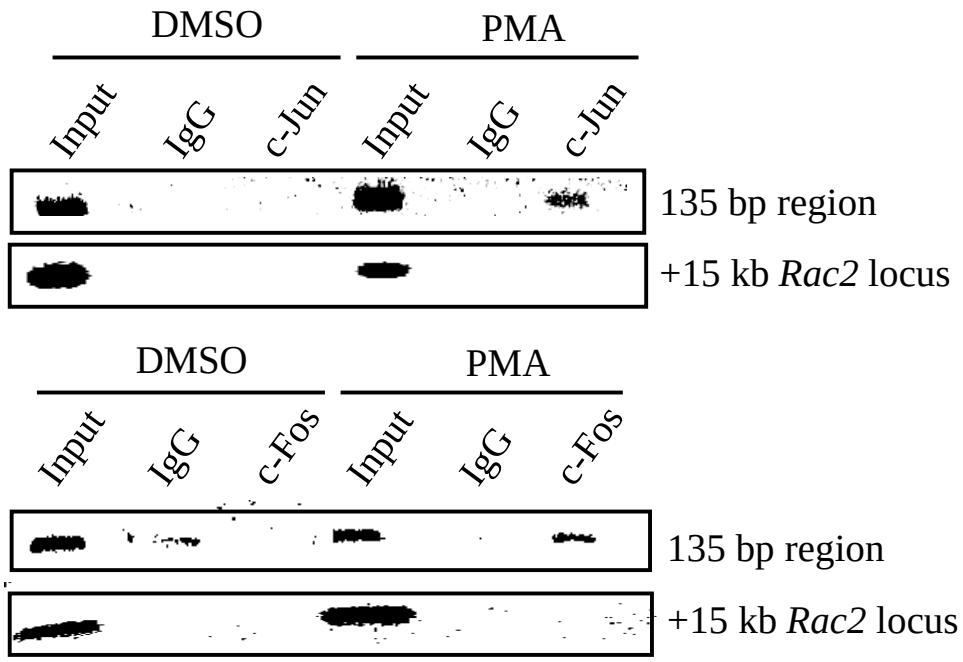


Figure 15. **AP1 binds to the 135 bp region between -4223 and -4088 bp of the *Rac2* gene promoter region *in vivo*.**

Chromatin was prepared from K562 cells treated for 24 h with DMSO or 100 nM PMA. ChIP assays were performed using antibodies against c-Jun, c-Fos, or normal rabbit serum. The figure shows the results of the PCR analysis of genomic DNA extracted from input samples and the immunoprecipitates using amplicons from the 135 bp region between -4223 and -4088 bp of the *Rac2* gene promoter region and a 175 bp intragenic region (+15 kb) of the *Rac2* locus. The figure shown is representative of at least three independent experiments.

VII. All three AP1 sites within the 135 bp region are critical for *Rac2* gene promoter activity upon PMA stimulation

Each of the three AP1 sites in the -30+135/luciferase construct was individually mutated by site directed mutagenesis. Transient transfection of these constructs followed by a 24 h PMA stimulation of K562 cells showed that individual ablation of any of the AP1 sites in the 135 bp region led to a significant decrease in reporter gene activity compared to that of the wild type parental construct. Triple mutation of all three AP1 sites completely abolished induction of reporter gene activity (Fig 16A), showing that all three AP1 sites in the 135 bp region are important for the transcriptional activity of the *Rac2* gene promoter following PMA stimulation. The luciferase activity of these constructs in unstimulated K562 cells did not change relative to the -30bp/luciferase construct (Fig 16B).

VIII. Trans-activation of *Rac2* gene promoter activity by AP1 transcription factors

To test if AP1 transactivates the *Rac2* promoter, K562 cells were co-transfected with the -30+135/luciferase construct and AP1 expression vectors. c-Jun proteins can form stable homo dimers but bind DNA less effectively than c-Jun/c-Fos heterodimers (Bakiri, Matsuo et al. 2002). In addition, c-Jun homodimers can serve as co-activators of transcription (Grondin, Lefrancois et al. 2007). Overexpression of c-Jun alone did not transactivate *Rac2* promoter activity (Fig 17A). On the other hand c-Fos proteins can only form heterodimers with c-Jun and cannot form homodimers to activate transcription (Milde-Langosch 2005). Cotransfection of a c-Fos expression construct also did not transactivate *Rac2* promoter activity, as expected. Cotransfection of both c-Jun and c-Fos

expression vectors led to a significant 6.5-fold increase in reporter gene expression compared to cells cotransfected with empty vector (Fig 17A). These results demonstrated that the heterodimer of c-Jun and c-Fos activates *Rac2* promoter activity in the absence of PMA stimulation.

Additional studies were performed to test if AP1 induces expression of the endogenous *Rac2* gene in the absence of PMA stimulation of K562 cells. Quantitative real-time PCR analysis was carried out to examine endogenous *Rac2* gene expression in K562 cells cotransfected with c-Jun and/or c-Fos expression vectors for twenty four hours. Results showed that neither c-Jun, c-Fos nor the addition of c-Jun and c-Fos induced expression of the endogenous *Rac2* gene in the absence of PMA stimulation of K562 cells (Fig 17B). Over expression of c-Jun and c-Fos proteins in transfected K562 cells were confirmed by western blot analysis (Fig 17C). Expression of c-Jun was observed only in K562 cells transfected with the pcDNA/c-Jun expression vector. c-Fos expression was observed only in K562 cells transfected with PCMV6-XL5/c-Fos expression vector. Because exogenous AP1 transactivates transiently transfected *Rac2* promoter constructs but does not induce expression of the endogenous *Rac2* gene, changes in chromatin structure may be an additional factor required for the induction of *Rac2* gene expression during PMA stimulated K562 cell differentiation. Unlike the endogenous *Rac2* mRNA levels that is increased only after 6 h following PMA treatment (Fig 7A), AP1 expression is induced within one hour of PMA treatment (Eriksson, Arminen et al. 2005) and can also bind to oligo 1, 4 and 7 within one hour of PMA treatment (Fig 18). So we hypothesized that changes may occur in the chromatin structure of the *Rac2* gene locus during K562 cell differentiation to permit AP1 binding.

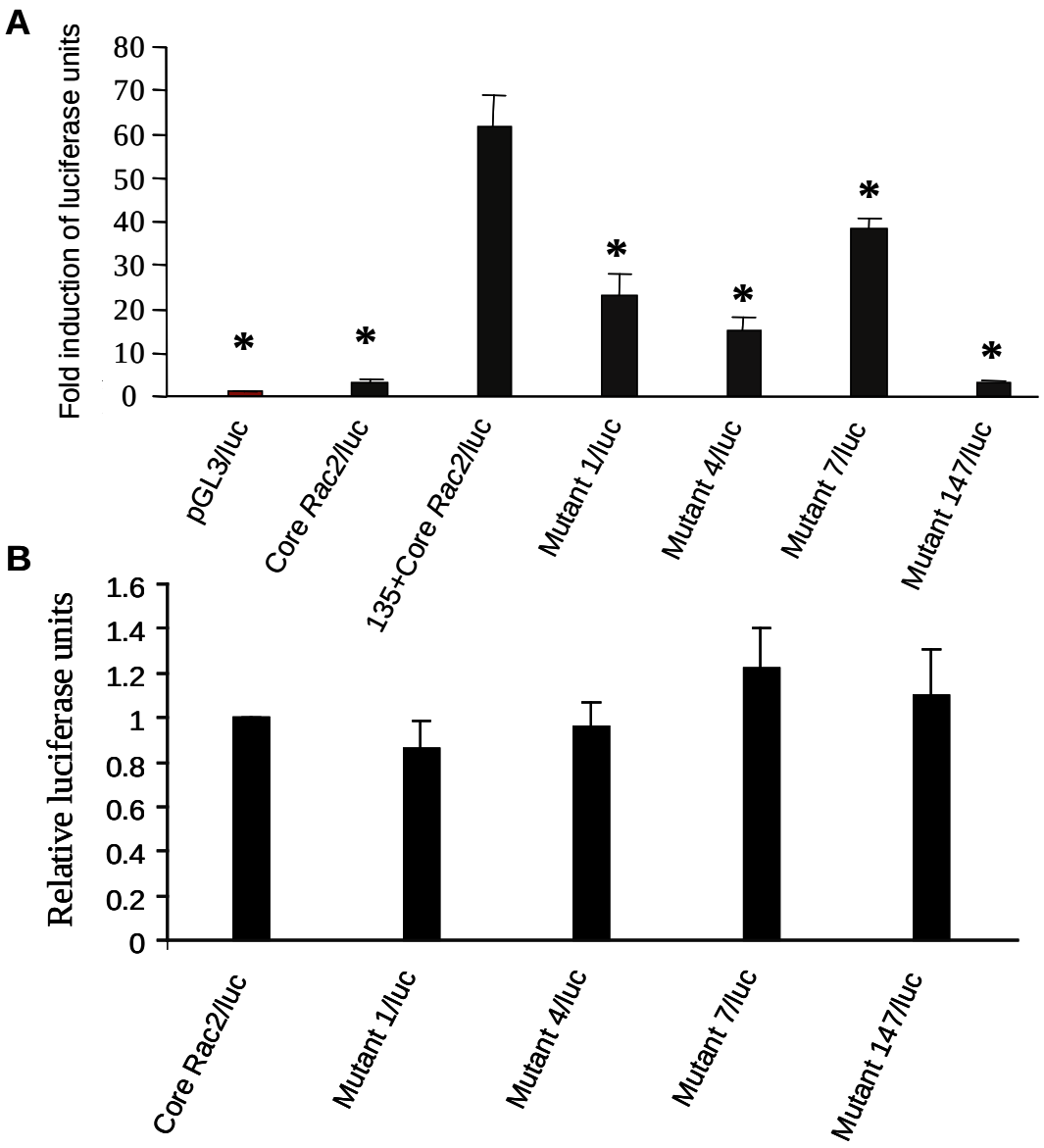


Figure 16. Functional activity of AP1 binding sites within the 135 bp *Rac2* regulatory region.

A) Single (referred to as Mutant 1, Mutant 4, Mutant 7) or triple mutations (referred to as Mutant 147) of the AP1 sites in the 135 bp region between -4223 bp and -4088 bp *Rac2* gene promoter region or the wild type 135 bp region sub-cloned upstream of the -30 bp *Rac2* gene core promoter construct in pGL3/luciferase vector were made by site-directed mutagenesis and transiently transfected into K562 cells. After transfection the cells were

divided into two samples and one was treated with 100 nM PMA and other with DMSO for 24 h. The cells were harvested and the relative luciferase activity was determined. The difference in luciferase activity of the PMA treated samples compared to DMSO treated samples was calculated and represented as fold luciferase units. Results represent normalized mean ± standard error and are representative of at least six independent experiments performed in triplicate. *P* values were determined by a standard *t* test, and the asterisks indicate *P* values of <0.05 compared to 135+core Rac2/luciferase construct. B) The -30 bp Core *Rac2* promoter/luciferase construct or the single or triple mutants of the AP1 sites in the 135 bp region sub-cloned upstream of the -30 bp *Rac2* gene core promoter construct in pGL3/luciferase vector were transiently transfected into K562 cells. Twenty four hours after transfection, the cells were harvested and luciferase and β-galactosidase activities were determined. After normalization with β-galactosidase activity, the fold difference in luciferase units relative to -30 bp/luciferase construct was calculated and represented as relative luciferase units. Results represent normalized mean ± standard error and are representative of at least three independent experiments performed in duplicate.

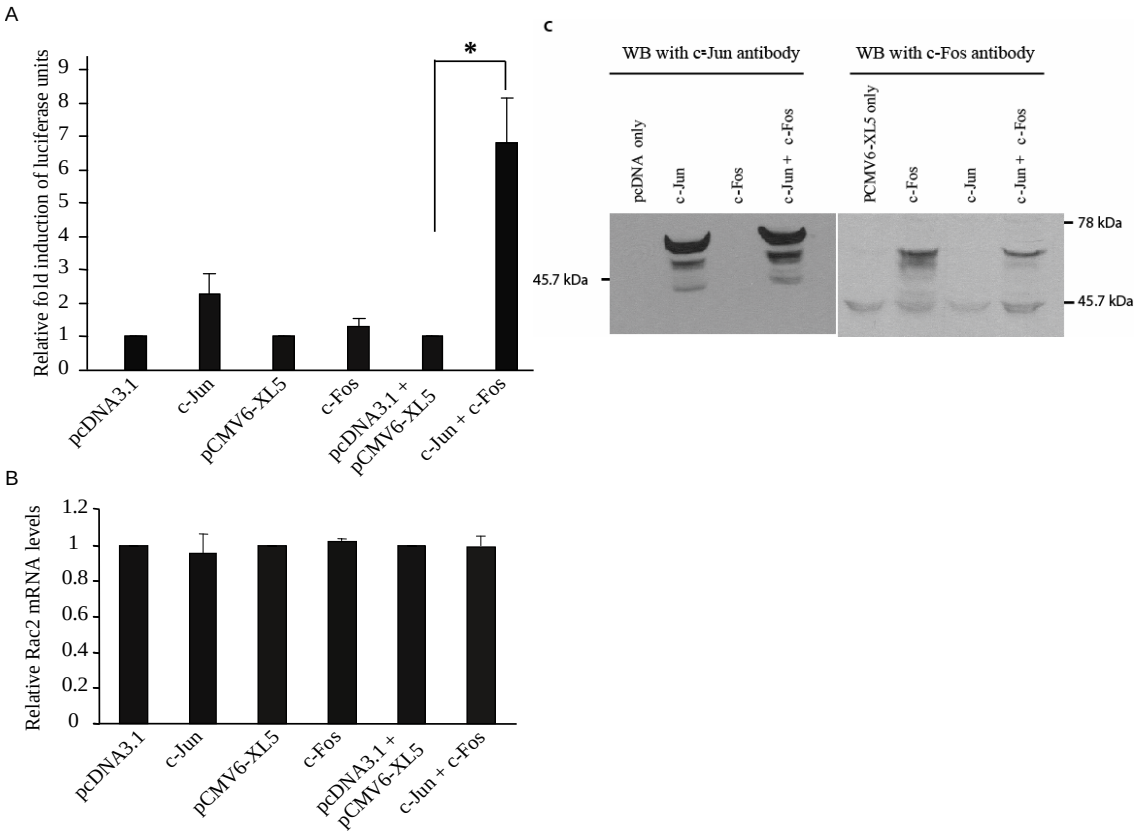


Figure 17. Trans-activation of *Rac2* promoter activity by AP1 proteins.

A) K562 cells were co-transfected with plasmid containing 135 bp between -4223 and -4088 bp *Rac2* gene promoter upstream of the -30 bp Rac2 core promoter in the pGL3-luciferase vector, c-Jun and/or c-Fos expression vectors and CMV-β-galactosidase vector for normalization of transfection efficiency. Twenty four hours after transfection, the cells were harvested and luciferase and β-galactosidase activities were determined. After normalization with β-galactosidase activity, the fold difference in luciferase units of cells transfected with c-Jun and/or c-Fos expression vector compared to the empty vector was calculated and represented as relative fold induction of luciferase units. Results represent normalized mean ± standard error and are representative of at least three independent

experiments performed in duplicate and asterisk indicates a *P* value of <0.05 compared to pcDNA + PCMV6-XL5.

B) Quantitative real-time PCR analysis was performed to quantify *Rac2 mRNA* levels in K562 cells transfected with c-Jun and/or c-Fos expression vectors for 24 h. ΔC_t values of *Rac2 mRNA* were normalized to ΔC_t values of *actin* mRNA from the same cDNA preparations. Normalized *Rac2* transcript level in K562 cells transfected with c-Jun and/or c-Fos expression vectors were then calibrated against the empty vectors. The data shown are representative of two separate experiments.

C) Twenty micrograms of whole cell extracts from K562 cells transfected with empty vector, c-Jun and/or c-Fos expression vectors for 24 h were subjected to western blot analysis. Proteins were separated by 5% SDS-PAGE, blotted onto nitrocellulose membrane and detected with polyclonal c-Jun or c-Fos antibody. Arrows indicate c-Jun or c-Fos expression.

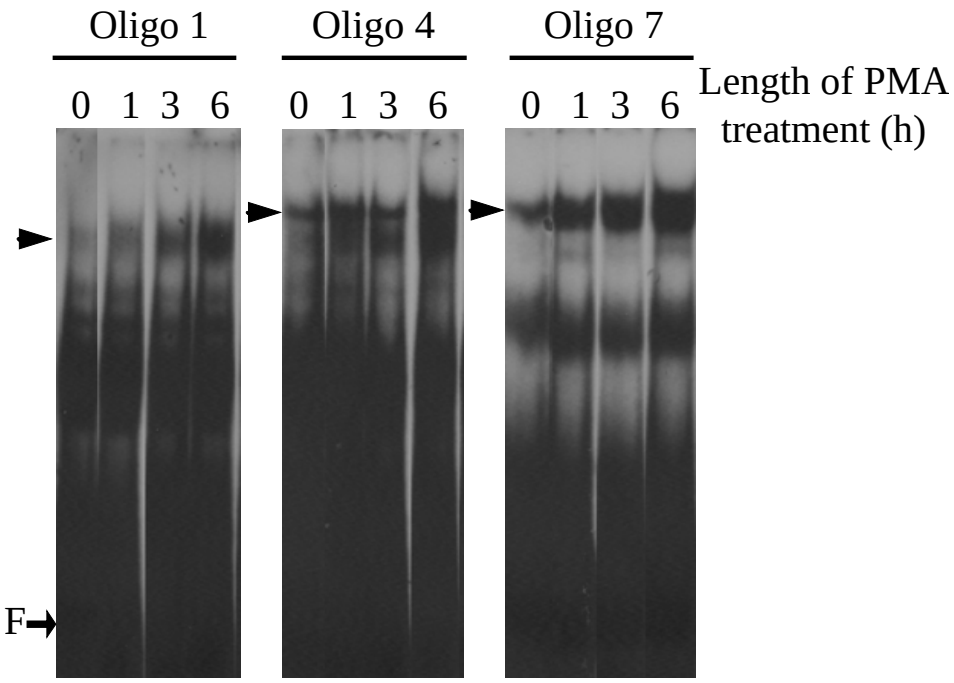


Figure 18. Kinetics of AP1 binding to the 135 bp *Rac2* gene promoter region following PMA induction.

EMSA was performed using oligos 1, 4 or 7 probes as outlined in the methods. Labeled probes were incubated with 5 μg of nuclear extract isolated from K562 cells treated for 0, 1, 3 or 6 h with 100 nM PMA. DNA-protein complexes that are induced by PMA treatment in all three oligos are indicated by arrows. “F” denotes free probe.

IX. PMA stimulation induces chromatin remodeling at the 135 bp *Rac2* gene regulatory region

The functional organization of chromosomes into euchromatin and heterochromatin domains has been associated with distinct post-translational modifications of the histone tails, such as acetylation, phosphorylation and methylation (Cheung, Allis et al. 2000; Jenuwein and Allis 2001). Histone modifications such as histone acetylation and histone H3K4 methylation accompany gene transcription and are required for tissue-specific and context dependent induction of many genes (Bottardi, Aumont et al. 2003; Thomas, Gao et al. 2005; Ekici, Hohl et al. 2008; Witcher, Pettersson et al. 2008). To determine if histone modifications change within the 135 bp *Rac2* regulatory region upon PMA stimulation of K562 cells for 24 h, chromatin immunoprecipitation assays were performed using antibodies specific for histone H3K9 dimethylation, a marker of inactive chromatin (Nakayama, Rice et al. 2001; Peters, Mermoud et al. 2002; Tamaru, Zhang et al. 2003) and histone H3K9/14 acetylation, a marker of active chromatin (Jenuwein and Allis 2001; Roh, Cuddapah et al. 2005). Immunoprecipitated genomic DNA enriched for these modifications was then analyzed by PCR for the presence of the 135 bp *Rac2* regulatory region. PMA stimulated K562 cells showed a marked decrease of histone H3K9 dimethylation compared to that of the unstimulated cells within the 135 bp *Rac2* gene promoter region. On the other hand, unstimulated K562 cells showed no or little histone acetylation at this genomic site. Upon PMA stimulation of K562 cells there was a striking increase of histone acetylation within the 135 bp region (Fig 19A).

Chromatin modifications associated with transcriptionally active DNA, such as histone acetylation, recruit other chromatin remodeling complexes, thereby changing the physical structure of the chromatin to a more open form (Struhl 1998; Sterner and Berger 2000; Gorisch, Wachsmuth et al. 2005). Nuclease sensitivity assays were performed to assess chromatin accessibility within the 135 bp *Rac2* gene regulatory region. Intact nuclei isolated from K562 cells stimulated with PMA or DMSO for 24 h were treated with Micrococcal nuclease (MNase/Nuclease S7), which preferentially digests DNA in more relaxed configuration compared to compact DNA. Results showed that the 135 bp *Rac2* gene regulatory region from PMA-stimulated K562 cells showed less intense band and thus is more accessible to MNase compared to DMSO-treated cells (Fig 19B). This indicates that PMA stimulation of K562 cells leads to a more relaxed chromatin structure within the 135 bp *Rac2* gene regulatory locus.

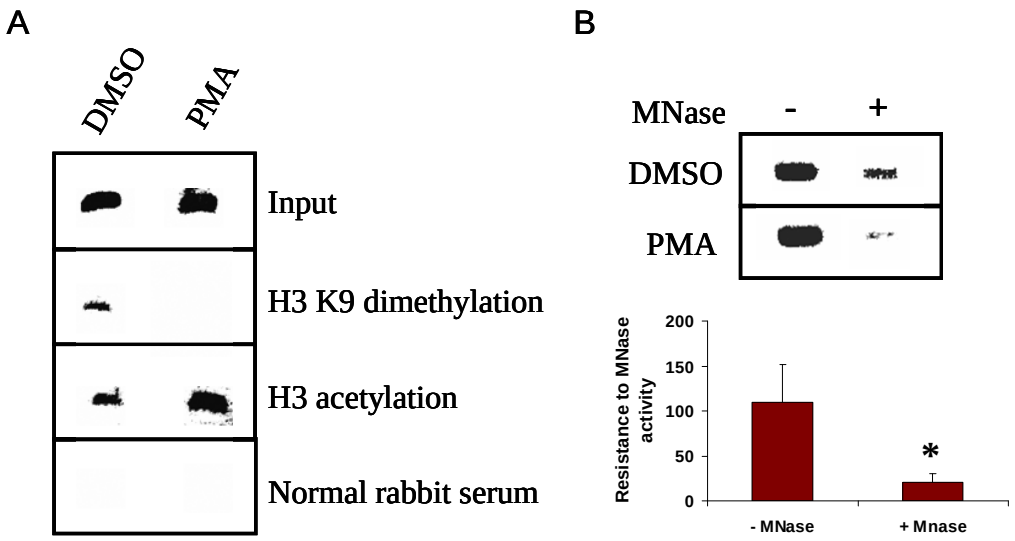


Figure 19. Histone modifications and chromatin remodeling of the 135 bp *Rac2* region upon PMA stimulation of K562 cells.

A) Chromatin was prepared from K562 cells treated with DMSO or 100 nm PMA for 24 h and subjected to ChIP analysis with antibodies against acetylated histone H3, dimethylated histone H3K9, or normal rabbit serum. The figure show the results of analysis of genomic DNA extracted from input samples and the immunoprecipitates using amplicons from the 135 bp region between -4223 bp and -4088 bp of the *Rac2* gene promoter. The data are representative of two independent experiments.

B) Intact nuclei were isolated from K562 cells treated with DMSO or 100 nM PMA for 24 h as described in the Methods and subjected to treatment with 100 U of MNase for 30 min. Genomic DNA was extracted and the 135 bp region between -4223 and -4088 bp of the *Rac2* gene promoter region was PCR amplified to assess chromatin accessibility. The data are representative of three independent experiments. The graph represents the ratio of the densitometric readings of PCR product intensity of samples digested with or without MNase to the intensity of the PCR product from undigested samples.

X. Concurrent binding of AP1 and chromatin remodeling at the 135 bp *Rac2* gene regulatory region

To determine the kinetics at which histone H3 acetylation and transcription factor binding occur within the 135 bp *Rac2* regulatory region, ChIP assays were done using antibodies against c-Jun and histone H3 acetylation. K562 cells were treated with PMA for various time periods. As shown in Figure 17A, both histone acetylation and binding of c-Jun to the 135 bp *Rac2* regulatory locus starts to appear by 5 h of PMA treatment and are markedly increased by 9 h of PMA treatment (Fig 20A). The kinetics of chromatin remodeling of the 135 bp *Rac2* regulatory locus was similarly analyzed by nuclease sensitivity assay. Intact nuclei isolated from K562 cells treated with PMA for various time periods were digested with MNase. PCR probing for the 135 bp *Rac2* regulatory region indicate that though the locus starts to become hypersensitive to the nuclease by 5 h of PMA treatment, a marked decrease in the intensity of the band was seen at 9 and 24 h of PMA treatment (Fig 20B). Enrichment of histone acetylation (Fig 20A), chromatin remodeling (Fig 20B) and c-Jun (Fig 20A) by 9 h of PMA treatment indicate concurrent eoccurrence of all three events in the 135 bp *Rac2* gene regulatory region. In addition, the pattern of c-Jun binding and chromatin remodeling within the 135 bp *Rac2* gene regulatory region fits well with the kinetics of *Rac2* gene transcription (Fig 7A). This indicates cross-talk between these events to mediate *Rac2* gene transcription upon PMA stimulation of K562 cells.

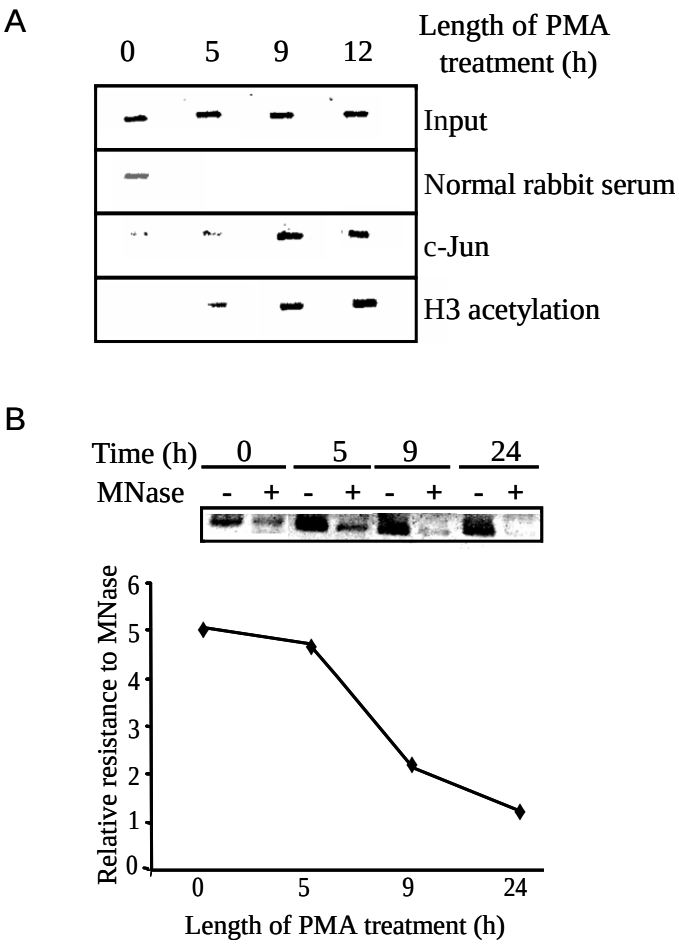


Figure 20. Concurrent binding of AP1 and chromatin remodeling in the 135 bp *Rac2* gene promoter region.

A) Chromatin was prepared from K562 cells treated 100 nM PMA for 0, 5, 9 and 12 h and subjected to ChIP analysis with antibodies against c-Jun, acetylated histone H3, dimethylated histone H3K9, or normal rabbit serum. The figure shows the results of PCR analysis of genomic DNA extracted from input samples and the immunoprecipitates using amplicons from the 135 bp region between -4223 bp and -4088 bp of the *Rac2* promoter. The data are representative of three independent experiments.

B) Intact nuclei were isolated from K562 cells treated with 100 nM PMA for 0, 5, 9 and 24 h as described in Methods and subjected to treatment with 100 U of MNase (nuclease

S7). Genomic DNA was extracted and probed for the 135 bp region between -4223 and -4088 bp *Rac2* gene promoter region to assess chromatin remodeling by PCR. The data are representative of two separate experiments. The graph represents the densitometric analysis of PCR product intensity of samples digested with MNase to the intensity of the PCR product from undigested samples, for each time period.

XI. Histone H3 acetylation is not sufficient to permit induction of the endogenous *Rac2* gene in the presence of AP1

To determine if histone acetylation within the 135 bp *Rac2* gene promoter region is sufficient to activate endogenous *Rac2* gene expression in the presence of AP1, unstimulated K562 cells were treated trichostatin (TSA), a general deacetylase inhibitor. TSA treatment of K562 cells results in bulk acetylation of histones H3 and H4 (Gui and Dean 2001). To test if histone acetylation changes within the 135 bp *Rac2* regulatory region upon TSA treatment of K562 cells, chromatin immunoprecipitation assays were performed using antibodies specific for histone H3 acetylation. Analysis of the immunoprecipitated genomic DNA enriched for this modification showed a marked increase of histone H3 acetylation within the 135 bp *Rac2* gene promoter region similar to the PMA stimulated K562 cells (Fig 21A). After confirming the appearance of histone acetylation within the 135 bp *Rac2* gene promoter region, endogenous *Rac2* gene expression was examined by quantitative real-time PCR analysis. Endogenous *Rac2* gene expression was not induced by TSA treatment of K562 cells (Fig 21B). As AP1 is not expressed in unstimulated K562 cells, K562 cells were cotransfected with c-Jun and c-Fos expression vectors followed by TSA treatment for 5 h. No changes in endogenous *Rac2* transcript levels of K562 cells were observed upon overexpression of AP1 and TSA treatment. Thus histone H3 acetylation and the presence of AP1 are not sufficient to activate the endogenous *Rac2* gene.

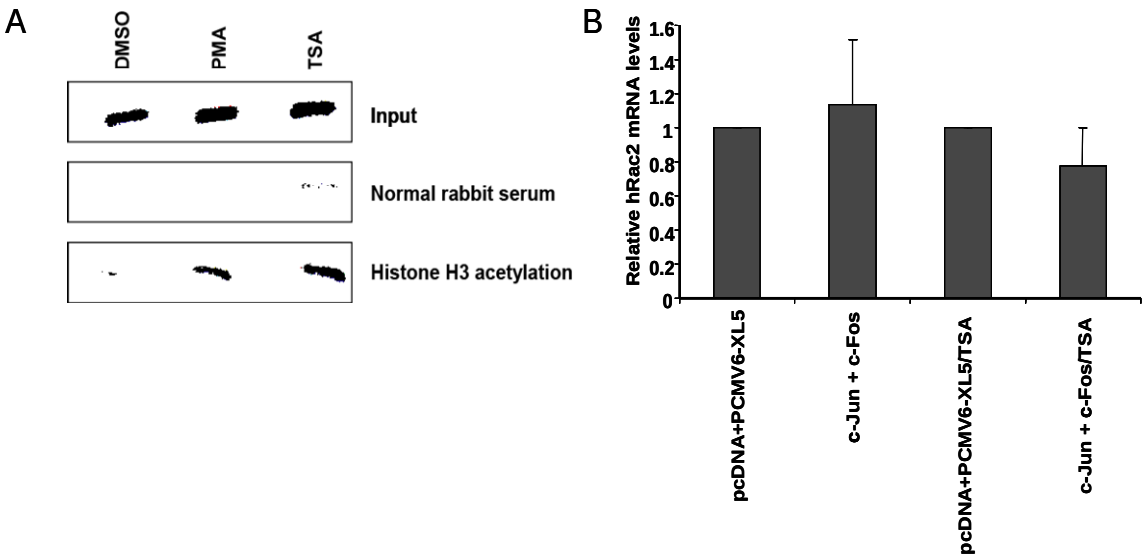


Figure 21. Increased histone H3 acetylation is not sufficient to induce the endogenous *Rac2* gene in the presence of AP1.

A) Chromatin was prepared from K562 cells treated with DMSO or 100 nm PMA for 24 h or 300 ng/ml TSA for 5 h and subjected to ChIP analysis with acetylated histone H3 antibody or normal rabbit serum. The figure show the results of analysis of genomic DNA extracted from input samples and the immunoprecipitates using amplicons from the 135 bp region between -4223 bp and -4088 bp of the *Rac2* promoter. The data are representative of two independent experiments.

B) K562 cells were transfected with empty vector and c-Jun/c-Fos expression vectors for 24 h. After transfection, the cells were treated with DMSO or 300 ng/ml TSA for 5 h. Quantitative real-time PCR analysis was performed to quantify *Rac2* mRNA levels in transfected K562 cells. ΔC_t values of *Rac2* mRNA were normalized to ΔC_t values of *Actin* mRNA from the same cDNA preparations. Normalized *Rac2* transcript level in K562 cells treated with PMA or TSA were calibrated against DMSO treated and K562

cells transfected with c-Jun and c-Fos expression vectors were calibrated against the empty vectors. The data shown are representative of two separate experiments.

DISCUSSION

I. *Rac2* gene expression in PMA-stimulated K562 cells

The objective of this study was to investigate the genetic and epigenetic regulation of the *Rac2* gene during myeloid cell differentiation. Because isolation and maintenance of mature myeloid cells from bone marrow cells is difficult, the pluripotent cell line K562 was used as the model system in this study. Undifferentiated K562 cells express surface markers characteristic of megakaryocytic, erythroid, and myeloid lineages and chemical stimulation of these cells induce lineage-specific terminal differentiation based on the stimulus used. PMA stimulation of K562 cells induces its differentiation into megakaryocytes (Fig 2).

Rac2 is expressed in undifferentiated K562 cells (Ladd, Butler et al. 2004), but PMA stimulation of K562 cells led to a further time-dependent induction of *Rac2* gene expression (Fig 7A). The increased recruitment of Pol II to the coding region of the *Rac2* gene upon PMA stimulation of K562 cells strongly suggests the regulation of *Rac2* gene expression to be primarily exercised at the transcriptional level (Fig 7B). Early response genes such as *c-Jun, c-Fos, and TNFα* (Yeh, Ambudkar et al. 1992; Karin, Liu et al. 1997; Sullivan, Reddy et al. 2007) exhibit rapid transcription minutes after PMA stimulation. Unlike these genes, *Rac2* is induced after 6-12 h of PMA stimulation (Fig 7A) showing that *Rac2* gene expression is affected as a secondary response to PMA treatment. Similar to Rac2, transcription of inflammatory mediators such as IL-6 and IL-12 are delayed following PMA stimulation (Sullivan, Reddy et al. 2007). Early and late response genes rely on similar transcription factors (NF-κB, AP1, and C/EBP) to activate transcription. But unlike the promoters of early response genes that are immediately

accessible to transcription factors, the physical structure of secondary response genes must be modified to allow transcription factor binding (Saccani, Pantano et al. 2001).

II. Identification of the cis-element sufficient for PMA-induced *Rac2* promoter activity

Transient transfection studies identified PMA-responsive elements within the 4.5 kb human *Rac2* gene promoter region (Fig 8). The regulatory elements required for the stage-specific expression of many genes, such as α_{IIb} *Integrin*, *p21* and *glycoprotein IIb*, reside within their proximal promoter regions. 5'- deletion analysis of the 4.5 kb *Rac2* promoter construct identified a 135 bp novel regulatory element between the -4.2 kb to -4 kb *Rac2* gene promoter region that is necessary and sufficient for the transcriptional activity of the *Rac2* promoter following PMA stimulation (Figs 9, 10). Deletion of the 135 bp region led to complete abolishment of PMA-stimulated *Rac2* promoter activity, showing that all regulatory elements required for transcriptional activity of the *Rac2* promoter during PMA stimulation of K562 cells lie within this minimal regulatory region. The 135 bp *Rac2* gene region induced much higher expression of the construct when present upstream of the -30 bp *Rac2* core promoter (Fig 10). This indicates that the regulatory elements present within this region can act independent of its distance from the core promoter. In addition, there may be some stimulus-specific repressive cis-element(s) between the -4.0 kb and -30 bp *Rac2* gene promoter region. Repression could be brought about by factors that bind downstream of the 135 bp region to interact with the basal transcription machinery. For example, phosphorylated Dr1 proteins interact with TBP to reduce gene transcription (Kraus, Inostroza et al. 1994). On the other hand,

repressors might function by interfering with the activity of the nuclear protein(s) that bind the 135 bp *Rac2* gene promoter region. For example, the factors Id and IκB repress binding of NF-κB to their DNA binding sites (Almawi and Melemedjian 2002). Even repetitive DNA sequences sequester DNA binding proteins and can act as negative regulators of transcriptional activity (Liu, Wu et al. 2007). Furthermore, it will be interesting to know if the transcriptional activity conferred by the 135 bp region is specific to the *Rac2* promoter region or if it can function upstream of a heterologous promoter.

III. The AP1 transcription factor is required for *Rac2* gene expression upon PMA stimulation of K562 cells

The regulatory sites within the 135 bp *Rac2* promoter region have been localized to three AP1 consensus binding sites (Figs 12, 13). The Jun and Fos families of proteins, collectively referred to as AP1 (Curran and Franza 1988), share a highly homologous domain composed of a basic segment and a leucine zipper (Gentz, Rauscher et al. 1989). They are expressed ubiquitously, but may be differentially expressed in specific cell types (Ney, Sorrentino et al. 1990). Upon stimulation, regulation of AP1 activity occurs by activating transcription of these genes as well as through phosphorylation of existing Jun and Fos proteins at specific serine and threonine residues. Members of the Jun family bind with high affinity to DNA as a homodimeric or heterodimeric complex with members of Fos family (Halazonetis, Georgopoulos et al. 1988) and each dimer binds to a single AP1 site. Though the EMSA studies demonstrated that Fos/Jun heterodimers specifically bind to these sites (Fig 14), these results do not rule out the possibility that

Jun homodimers or other AP1 family members can also bind this site. It is possible that Fos/Jun heterodimers and Jun homodimers bind to and/or compete for the AP1 consensus sites. Which dimer binds in vivo may be determined in part by the levels of each factor, which in turn may depend on the differentiation state of the cell, the cell type or by the different binding affinities observed for various AP1 dimers. Jun/Fos heterodimers exhibit a greater binding activity and are more stable than Jun/Jun homodimers. In addition, c-Jun and c-Fos proteins play a critical role in myeloid cell differentiation (Lord, Abdollahi et al. 1993; Shafarenko, Amanullah et al. 2004).

Furthermore, all the three AP1 binding sites are present close to each other within the 135 bp *Rac2* gene regulatory region (Fig 12) and lie in the distal region of the *Rac2* gene promoter. Close spacing of AP1 sites has been shown to allow formation of a highly active complex that favors formation of a loop structure to bring an enhancer element in close proximity to the transcription initiation complex (Ney, Sorrentino et al. 1990; Chytil, Peterson et al. 1998; Chinenov and Kerppola 2001). It is interesting that AP1 factors have been shown to induce DNA bending (Kerppola and Curran 1991). AP1-induced changes in DNA structure may promote cooperative binding of factors to nearby sites and mediate interaction between several other regulatory proteins (Kerppola and Curran 1991). A tandem pair of AP1 sites found within the human *β-globin* dominant control region functions as an inducible enhancer in erythroid cells (Ney, Sorrentino et al. 1990). In addition, DNA bending due to enrichment of AP1 binding to the 135 bp region may bring distal AP1 sites within closer proximity to the basal transcription machinery (Chinenov and Kerppola 2001).

The data presented here demonstrates a requirement for all three AP1 binding sites for maximal *Rac2* gene transcription. Individual ablation of AP1 sites within the 135 bp *Rac2* gene promoter region did not completely abolish promoter activity upon PMA stimulation but still led to a significant decrease in promoter activity (Fig 16A). This may be due to a requirement for a critical concentration or localization of AP1 to alter the structure of the DNA to facilitate transcription (Shivaswamy, Bhinge et al. 2008). In addition, increased recruitment of AP1 to all three sites within the *Rac2* gene promoter region may facilitate adoption of a flexible structure by bending of DNA as described earlier (Kerppola 1998), thereby augmenting recruitment of RNA-pol II. Similar enrichment of RNA-pol II by AP1 increases transcription of *ccl2,* a chemokine gene (Wolter, Doerrie et al. 2008). Over-expression of c-Jun alone was not sufficient to activate transcription of the *Rac2* promoter construct (Fig 17A). This may be due to the weak binding affinity of c-Jun homodimers for AP1 binding sites in the 135 bp region. On the other hand, over-expression of c-Jun and c-Fos proteins can form hetero-dimers with stronger DNA binding activity and resulted in the induction of -30+135/luciferase construct. Though over-expressed AP1 proteins led to a significant increase in the activity of the -30+135/luciferase construct, the activity was much lower than the PMA-stimulated constructs (Fig 17A, Fig 10). This may be a consequence of altered stability of the dimers and DNA binding properties between the over-expressed and endogenous AP1 proteins. In addition, the reduced phosphorylation status of over-expressed AP1 proteins due to lower ERK/JNK activity in unstimulated K562 cells (Eriksson, Arminen et al. 2005) may affect its trans-activation potential. Transient transfection experiments represent an imperfect approximation of the role of these elements, as the ability of

transcription factors to activate transcription is highly dependent on the surrounding DNA region and the environment of surrounding binding sites. Interestingly, c-Jun/c-Fos heterodimers were not able to induce endogenous *Rac2* gene expression in the absence of PMA stimulation (Fig 17B). Thus, the chromatin structure of the *Rac2* promoter may provide additional control over the regulation of Rac2 during cell differentiation by regulating transcription factor access to cis-regulatory elements (Paranjape, Kamakaka et al. 1994; Hashimshony, Zhang et al. 2003; Bhaumik, Smith et al. 2007).

IV. Changes in chromatin structure are required for the induction of *Rac2* gene expression upon PMA stimulation

Major reorganization of chromatin structure, including changes in histone modifications and DNA methylation, are features of early embryonic development and are fundamental components of gene regulation and cellular differentiation. Cytosine methylation has previously been shown to play an important role in the lineage-specific expression of *Rac2*. The 5'- and 3'- flanking sequences of the *Rac2* gene locus exhibit decreased cytosine methylation in Rac2-expressing cells (Ladd, Butler et al. 2004). Treatment with 5-aza-2'-deoxycytidine, an inhibitor of DNA methyltransferase, led to the loss of cytosine methylation and expression of Rac2 in non-hematopoietic cells. Thus, cytosine methylation plays a dominant role in the tissue-specific expression of Rac2 (Ladd, Butler et al. 2004). Furthermore, covalent modifications of histones provide the molecular code required for changes in chromatin structure and for recruitment of transcription factors required for gene transcription during development (Strahl and Allis 2000). IL6 promoter is hyper acetylated before NF-κB recruitment, thereby serving as a

marker for changes in the promoter structure to activate transcription (Saccani, Pantano et al. 2001). Therefore, epigenetic changes during cell differentiation may initiate further structural alterations within the 135 bp *Rac2* regulatory locus to accommodate AP1.

A decrease in histone H3K9 dimethylation, an inactive chromatin mark, and an increase in histone H3 acetylation, an active chromatin mark, observed within the 135 bp *Rac2* gene regulatory region (Fig 19A) show that histone modifications that are characteristics of transcriptionally competent genes are acquired in the 135 bp *Rac2* gene locus upon PMA stimulation of K562 cells. Gene expression changes due to global induction of histone acetylation are characteristic of early and late differentiation programs and the acetyl marks in the chromatin can stimulate transcription by communicating with transcription factors or other chromatin remodeling proteins (Tse, Sera et al. 1998; Verdone, Caserta et al. 2005). Histone acetylation is important for enhancer-mediated transcriptional activation of several genes, such as ε-globin (Gui and Dean 2001), c-myc (Madisen, Krumm et al. 1998) and β-interferon (Parekh and Maniatis 1999). Interestingly, HATs, such as GCN5 and CBP/P300, are coactivators of transcription (Kuo, Zhou et al. 1998; Berger 1999). Additionally, p300/CBP also interacts with AP1 to stimulate transcription (Bannister, Oehler et al. 1995; Parekh and Maniatis 1999). Acetylated chromatin can also influence gene expression by interaction with SWI/SNF chromatin remodeling complexes (Agalioti, Lomvardas et al. 2000) that modulate chromatin structure and provide access to transcription factors. On the other hand, histone H3K9 methylation is a hallmark of inactive chromatin and facilitates repression of gene expression during development (Nakayama, Rice et al. 2001; Tachibana, Sugimoto et al. 2002). Histone H3K9 methylation is enriched in the hypo-

acetylated 135 bp *Rac2* gene promoter region in unstimulated K562 cells. Histone H3K9 methyl marks can be recognized by the chromodomain of HP1 which participates in heterochromatin formation, to maintain that locus in a compact form (Stewart, Li et al. 2005). Upon PMA stimulation, loss of histone H3K9 methylation may be required to permit formation of relaxed chromatin around the 135 bp *Rac2* gene promoter region.

Changes in the chromatin structure of the 135 bp *Rac2* gene promoter region were shown by increased sensitivity of this region to MNase upon PMA treatment (Fig 19B). In addition, changes in chromatin structure of regulatory elements during development in response to histone modifications has been shown for several genes including ε-globin (Gui and Dean 2001) and TNFα (Sullivan, Reddy et al. 2007). The chromatin structure of the TNF-α locus changes from heterochromatin to euchromatin in a progressive fashion, reaching euchromatin slightly after myeloid cell differentiation (Sullivan, Reddy et al. 2007). Therefore, histone modifications during cell differentiation may impose changes in the physical structure of the *Rac2* promoter and thereby aid changes in the transcriptional activity of the gene. Kinetics of c-Jun binding, histone H3 acetylation and changes in the chromatin structure (Fig 20 A, B) showed concurrent occurrence of all three events on to the 135 bp *Rac2* regulatory region upon PMA stimulation of K562 cells for atleast 9 h. It is possible that *Rac2* gene expression is affected by cooperative interactions between epigenetic modifications and AP1 transcription factor. However the precise integration level of this cross talk is still not known. HATs such as CBP/p300 can interact with c-Jun to recruit AP1 to the binding sites. On the other hand, alteration in the chromatin structure of the 135 bp *Rac2* regulatory locus in stimulated K562 cells may also permit access to AP1 that further

induces *Rac2* gene transcription. Enriched binding of c-Jun to the 135 bp *Rac2* region is observed after 9 h of PMA stimulation. It is interesting that enriched recruitment of Pol II to the *Rac2* gene coding region was also observed by 12 h of PMA stimulation. This suggests that binding of AP1 to the 135 bp *Rac2* gene promoter region can then induce *Rac2* gene expression by augmenting recruitment of basal transcription machinery.

TSA is a general inhibitor of HDACs that result in elevated histone acetylation and is associated with increased gene transcription. Interestingly, TSA treatment of unstimulated K562 cells in the presence of AP1 is not sufficient to induce endogenous *Rac2* gene expression (Fig 21B). This effect may not be due to the absence of histone acetylation as a three to five-fold increase in total acetylated histone H3 and H4 has been reported upon TSA treatment of K562 cells (Gui and Dean 2001). In addition, TSA treatment of unstimulated K562 cells also resulted in a marked increase in the histone acetylation of the 135 bp *Rac2* gene promoter region (Fig 21A). These observations suggest that, although histone acetylation may contribute to the regulation of *Rac2* gene transcription during PMA stimulation, some second essential signal required for the recruitment of AP1 may be missing. It has been shown that while TSA can promote formation of histone acetylation, which leads to drastic changes in gene expression during development, it cannot induce lineage-specific differentiation programs in ES cells (McCool, Xu et al. 2007). Furthermore, global deacetylation by TSA led to inhibition of ES cell differentiation (Lee, Hart et al. 2004). Therefore, in addition to histone acetylation and AP1 transcription factors, some additional regulatory events or histone modifications gained during PMA stimulation of K562 cells may be necessary for endogenous *Rac2* gene expression. Absence of histone H3K9 methylation, a transcription

repressive mark, was also observed in the 135 bp *Rac2* gene promoter region, upon PMA stimulation of K562 cells (Fig 19A). Therefore it is also possible that, in addition to the appearance of the active chromatin marks, the repressive histone modifications that are already present in unstimulated cells must be removed to alter gene transcription. Combination of these signals may be required to alter chromatin structure of the *Rac2* gene locus. Inaddition, as mentioned earlier, the post-translational modification status of the over-expressed AP1 can also affect it's binding to the hyper-acetylated 135 bp *Rac2* gene region.

V. Interplay of transcription factors and epigenetic modifications in *Rac2* gene regulation

This study illustrates the complexity of *Rac2* gene regulation during cell differentiation**.** The analysis of AP1-mediated *Rac2* gene expression in this study provides evidence on its combinatorial potential to act with epigenetic modifications and mediate specific gene control during differentiation. This provides evidence for cooperative interactions between regulatory factors to mediate stimulus-dependent gene expression. It is interesting that two waves of epigenetic changes occur within the *Rac2* locus. The first wave, loss of cytosine methylation occurs when tissue specificity is conferred. Loss of cytosine methylation and subsequent Sp1 binding are critical for the tissue-specific expression of Rac2, but Rac2 was not expressed upon TSA treatment of non-expressing cells (Ladd, Butler et al. 2004). This suggests that changes in histone acetylation alone may not influence the tissue-specific expression of Rac2. The second wave of histone modifications that may modulates the chromatin structure of Rac2, and

binding of transcription factor AP1, occurs in response to external signals during differentiation. Thus AP1 and Sp1 may mediate the complex pattern of tissue-specific and stimulus-dependent regulation of *Rac2* gene expression by coordinating with epigenetic modifications that change the physical structure of chromatin at the *Rac2* locus.

VI. Future Directions

Future studies can aim at dissecting out the communication between various histone protein modifications and chromatin structure to accommodate AP1 and thereby provide an added perspective on the complexity of stage-specific expression of Rac2. Treatment of unstimulated K562 cells with TSA along with over-expression of AP1 proteins did not induce endogenous *Rac2* gene expression (Fig 21B). Changes in the chromatin structure and c-Jun binding to the 135 bp *Rac2* regulatory region under these conditions should be confirmed. Binding of c-Jun to the 135 bp region upon TSA treatment will need evaluation of the phosphorylation status of c-Jun. Phosphorylation of c-Jun at Ser-73 and Ser-63, located within its transactivation domain is essential for its ability to activate transcription. Therefore, changes in endogenous *Rac2* gene expression can be monitored upon overexpression of c-Jun with mutation of serine 63 and serine 73 residues to glutamic acid or aspartic acid (pseudophosphorylation) that can mimic the effects of the addition of a phosphate group.

Lack of alterations in the structure of the 135 bp *Rac2* region upon TSA treatment would suggest that a second signal is needed, in addition to histone acetylation, to induce permissiveness of the 135 bp *Rac2* gene locus. This also suggests that the repressive

effects of non-permissive chromatin cannot be completely countered by AP1, indicating the dominance of the chromatin effects. The possibilities presented in the "Discussion" section can be tested. One is to determine if the 135 bp *Rac2* promoter region has bivalent chromatin marks. Bivalent chromatin marks represents the simultaneous presence of both repressive and active histone marks in the same chromatin. The possible role of histone H3K9 methylation as a bivalent chromatin mark along with histone acetylation to modulate *Rac2* gene expression needs to be evaluated. Histone H3K9 methyl marks may be present with low level of histone acetyl marks in unstimulated cells. Upon stimulation, there is a marked increase in the acetylation of histone H3 at the 135 bp *Rac2* gene regulatory region (Fig 19). In addition, it is possible that removal of histone H3K9 methyl marks also ensure alterations in the chromatin locus. This can be evaluated by looking at the kinetics of histone H3K9 methylation across the 135 bp *Rac2* gene promoter region by ChIP analysis. Individual ChIP analysis for each modification may represent two subpopulations with distinct histone code changes. Therefore, the simultaneous presence of both marks can be examined by sequential ChIP analysis that will only retain chromatin containing both the modifications.

Additionally, the presence of various other histone modifications known to regulate gene expression can be examined within the 135 bp *Rac2* gene promoter region to have a much broader view of the histone code changes that can influence *Rac2* gene expression upon PMA stimulation. For example, histone H3K27 methylation is another repressive chromatin mark found in genes induced upon differentiation and histone

H3K4 methylation is an active chromatin mark that plays an important role in regulating the transcriptional activity of many genes. In addition, histone H3K4 methylation is present in permissive chromatin regions along with histone acetylation.

If removal of histone H3K9 methylation proves to be the second signal required for the permissiveness of the 135 bp *Rac2* regulatory region to interact with AP1, it will be interesting to look at the recruitment of histone demethylases of H3K9 methylation such as LSD1, JHDM1A and JHDM2A, to the 135 bp *Rac2* gene regulatory region upon PMA stimulation of K562 cells. In addition, as histone H3K9 methylation modulates chromatin structure through its interaction with heterochromatin protein-1 (HP-1), the recruitment of HP-1 to the 135 bp *Rac2* gene regulatory region in unstimulated and stimulated K562 cells can also be tested by ChIP analysis. Histone protein modifications can also bring about changes in chromatin structure by recruiting chromatin remodeling complexes. It will be interesting to characterize the recruitment of these enzyme complexes to the 135 bp *Rac2* gene regulatory regions upon PMA stimulation of K562 cells.

In addition, to gain further understanding of the mechanism by which the 135 bp *Rac2* gene regulatory region and AP1 activate transcription of *Rac2* upon PMA stimulation of K562 cells, it will be interesting to look at the recruitment of Pol II to the 135 bp region and the kinetics of its recruitment by ChIP analysis. Enrichment of RNA-polymerase to the androgen responsive elements in the enhancer region of the prostate-specific antigen (*PSA*) gene resulted in the formation of an activation complex by DNA looping (Shang, Myers et al. 2002). In a similar way, presence of RNA-polymerase in the 135 bp region along with AP1 would explain the looping mechanism by which the distal

AP1 sites may act on the promoter to induce *Rac2* gene expression upon stimulation of K562 cells. DNA cytosine methylation plays an important role in regulating the hematopoietic-specific expression of Rac2 (Ladd, Butler et al. 2004). It will be interesting to analyze the changes in the cytosine methylation pattern of the three CpG motifs within the 135 bp *Rac2* gene regulatory region upon PMA stimulation of K562 cells by bisulfite sequencing. On the other hand, though the importance of DNA methylation in the hematopoietic-specific expression of Rac2 has been delineated, the role of histone protein modifications has not yet been characterized. Therefore, characterization of the histone code changes required for the tissue-specific expression of Rac2 can provide a clear picture on the complexity of *Rac2* gene regulation and the interplay between epigenetic modifications to regulate appropriate tissue-specific and stage-specific expression of Rac2.

Upon characterization of the molecular events required for *Rac2* gene expression during myeloid cell differentiation, confirmation of these findings can be done in primary cells. The results strongly suggest the role of epigenetic control to effectively modulate *Rac2* gene expression. Although, leukemic cell lines offer great advantages with respect to handling and maintenance, especially with the type of experiments described in this dissertation which involves transfection with various constructs and examination of histone code changes, there is a clear possibility of altered epigenetic control in these malignant cell lines. Induced differentiation of primary cells to the myeloid lineage may offer a better system to corroborate these findings.

REFERENCES

Agalioti, T., S. Lomvardas, et al. (2000). "Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter." Cell **103**(4): 667-78.

Aletta, J. M., T. R. Cimato, et al. (1998). "Protein methylation: a signal event in post-translational modification." Trends Biochem Sci **23**(3): 89-91.

Almawi, W. Y. and O. K. Melemedjian (2002). "Negative regulation of nuclear factor-kappaB activation and function by glucocorticoids." J Mol Endocrinol **28**(2): 69-78.

Amaravadi, L. and M. J. Klemsz (1999). "DNA methylation and chromatin structure regulate PU.1 expression." DNA Cell Biol **18**(12): 875-84.

Angel, P., I. Baumann, et al. (1987). "12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region." Mol Cell Biol **7**(6): 2256-66.

Angel, P., M. Imagawa, et al. (1987). "Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor." Cell **49**(6): 729-39.

Bachman, K. E., M. R. Rountree, et al. (2001). "Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin." J Biol Chem **276**(34): 32282-7.

Bakiri, L., K. Matsuo, et al. (2002). "Promoter specificity and biological activity of tethered AP-1 dimers." Mol Cell Biol **22**(13): 4952-64.

Ballestar, E. and A. P. Wolffe (2001). "Methyl-CpG-binding proteins. Targeting specific gene repression." Eur J Biochem **268**(1): 1-6.

Bannister, A. J., T. Oehler, et al. (1995). "Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation in vivo and CBP binding in vitro." Oncogene **11**(12): 2509-14.

Beard, C., E. Li, et al. (1995). "Loss of methylation activates Xist in somatic but not in embryonic cells." Genes Dev **9**(19): 2325-34.

Bell, A. C., A. G. West, et al. (2001). "Insulators and boundaries: versatile regulatory elements in the eukaryotic." Science **291**(5503): 447-50.

Berger, S. L. (1999). "Gene activation by histone and factor acetyltransferases." Curr Opin Cell Biol **11**(3): 336-41.

Beri, S., N. Tonna, et al. (2007). "DNA methylation regulates tissue-specific expression of Shank3." J Neurochem **101**(5): 1380-91.

Bhattacharya, S. K., S. Ramchandani, et al. (1999). "A mammalian protein with specific demethylase activity for mCpG DNA." Nature **397**(6720): 579-83.

Bhaumik, S. R., E. Smith, et al. (2007). "Covalent modifications of histones during development and disease pathogenesis." Nat Struct Mol Biol **14**(11): 1008-16.

Blackwood, E. M. and J. T. Kadonaga (1998). "Going the distance: a current view of enhancer action." Science **281**(5373): 60-3.

Boggs, B. A., P. Cheung, et al. (2002). "Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes." Nat Genet **30**(1): 73-6.

Bohmann, D., T. J. Bos, et al. (1987). "Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1." Science **238**(4832): 1386-92.
Bolis, A., S. Corbetta, et al. (2003). "Differential distribution of Rac1 and Rac3 GTPases in the developing mouse brain: implications for a role of Rac3 in Purkinje cell differentiation." Eur J Neurosci **18**(9): 2417-24.
Bondarenko, V. A., Y. V. Liu, et al. (2003). "Communication over a large distance: enhancers and insulators." Biochem Cell Biol **81**(3): 241-51.
Bottardi, S., A. Aumont, et al. (2003). "Developmental stage-specific epigenetic control of human beta-globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation." Blood **102**(12): 3989-97.
Bourne, H. R., D. A. Sanders, et al. (1990). "The GTPase superfamily: a conserved switch for diverse cell functions." Nature **348**(6297): 125-32.
Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.
Briggs, S. D., M. Bryk, et al. (2001). "Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae." Genes Dev **15**(24): 3286-95.
Brown, A. M., A. J. O'Sullivan, et al. (1998). "Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42." Mol Biol Cell **9**(5): 1053-63.
Brownell, J. E., J. Zhou, et al. (1996). "Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation." Cell **84**(6): 843-51.
Bulger, M. and M. Groudine (2002). "TRAPping enhancer function." Nat Genet **32**(4): 555-6.
Burger, S. R., M. M. Zutter, et al. (1992). "Induced cell surface expression of functional alpha 2 beta 1 integrin during megakaryocytic differentiation of K562 leukemic cells." Exp Cell Res **202**(1): 28-35.
Byvoet, P., G. R. Shepherd, et al. (1972). "The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells." Arch Biochem Biophys **148**(2): 558-67.
Carlyon, J. A., W. T. Chan, et al. (2002). "Repression of rac2 mRNA expression by Anaplasma phagocytophila is essential to the inhibition of superoxide production and bacterial proliferation." J Immunol. **169**(12): 7009-7018.
Castro, L. and B. A. Freeman (2001). "Reactive oxygen species in human health and disease." Nutrition **17**(2): 161, 163-5.
Caterina, J. J., T. M. Ryan, et al. (1991). "Human beta-globin locus control region: analysis of the 5' DNase I hypersensitive site HS 2 in transgenic mice." Proc Natl Acad Sci U S A **88**(5): 1626-30.
Caterino, T. L. and J. J. Hayes (2007). "Chromatin structure depends on what's in the nucleosome's pocket." Nat Struct Mol Biol **14**(11): 1056-8.
Cavalli, G. (2006). "Chromatin and epigenetics in development: blending cellular memory with cell fate plasticity." Development **133**(11): 2089-94.

Cervoni, N., S. Bhattacharya, et al. (1999). "DNA demethylase is a processive enzyme." J Biol Chem **274**(13): 8363-6.
Chakravarti, D., V. J. LaMorte, et al. (1996). "Role of CBP/P300 in nuclear receptor signalling." Nature **383**(6595): 99-103.
Chan, H. W., Z. B. Kurago, et al. (2003). "DNA methylation maintains allele-specific KIR gene expression in human natural killer cells." J Exp Med **197**(2): 245-55.
Chanock, S. J., J. el Benna, et al. (1994). "The respiratory burst oxidase." J Biol Chem **269**(40): 24519-22.
Chen, D., H. Ma, et al. (1999). "Regulation of transcription by a protein methyltransferase." Science **284**(5423): 2174-7.
Chen, Y., Y. Yang, et al. (2006). "Crystal structure of human histone lysine-specific demethylase 1 (LSD1)." Proc Natl Acad Sci U S A **103**(38): 13956-61.
Cheung, P., C. D. Allis, et al. (2000). "Signaling to chromatin through histone modifications." Cell **103**(2): 263-71.
Cheutin, T., A. J. McNairn, et al. (2003). "Maintenance of stable heterochromatin domains by dynamic HP1 binding." Science **299**(5607): 721-5.
Chinenov, Y. and T. K. Kerppola (2001). "Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity." Oncogene **20**(19): 2438-52.
Chytil, M., B. R. Peterson, et al. (1998). "The orientation of the AP-1 heterodimer on DNA strongly affects transcriptional potency." Proc Natl Acad Sci U S A **95**(24): 14076-81.
Clayton, A. L., S. Rose, et al. (2000). "Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation." Embo J **19**(14): 3714-26.
Cloos, P. A., J. Christensen, et al. (2008). "Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease." Genes Dev **22**(9): 1115-40.
Croker, B. A., D. M. Tarlinton, et al. (2002). "The Rac2 guanosine triphosphatase regulates B lymphocyte antigen receptor responses and chemotaxis and is required for establishment of B-1a and marginal zone B lymphocytes." J Immunol **168**(7): 3376-86.
Crosio, C., G. M. Fimia, et al. (2002). "Mitotic phosphorylation of histone H3: Spatio-temporal regulation by mammalian aurora kinases." Molecular and Cellular biology **22**: 874-885.
Curran, T. and B. R. Franza, Jr. (1988). "Fos and Jun: the AP-1 connection." Cell **55**(3): 395-7.
Dai, P., H. Akimaru, et al. (1996). "CBP as a transcriptional coactivator of c-Myb." Genes Dev **10**(5): 528-40.
Damelin, M. and T. H. Bestor (2007). "Biological functions of DNA methyltransferase 1 require its methyltransferase activity." Mol Cell Biol **27**(11): 3891-9.
DeLeo, F. R. and M. T. Quinn (1996). "Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins." J Leukoc Biol **60**(6): 677-91.
Delgado, M. D., A. Lerga, et al. (1995). "Differential regulation of Max and role of c-Myc during erythroid and myelomonocytic differentiation of K562 cells." Oncogene **10**(8): 1659-65.

Delmas, V., D. G. Stokes, et al. (1993). "A mammalian DNA-binding protein that contains a chromodomain and an SNF2/SWI2-like helicase domain." Proc Natl Acad Sci U S A **90**(6): 2414-8.
Dhalluin, C., J. E. Carlson, et al. (1999). "Structure and ligand of a histone acetyltransferase bromodomain." Nature **399**(6735): 491-6.
Didsbury, J., R. F. Weber, et al. (1989). "rac, a novel ras-related family of proteins that are botulinum toxin substrates." J Biol Chem **264**(28): 16378-82.
Dover, J., J. Schneider, et al. (2002). "Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6." J Biol Chem **277**(32): 28368-71.
Eden, S., Hashimshony, T., Keshet, I., Cedar, H., and Thorne, A.W. (1998). "DNA methylation models histone acetylation." Nature **394**: 842.
Ekici, M., M. Hohl, et al. (2008). "Transcription of genes encoding synaptic vesicle proteins in human neural stem cells: chromatin accessibility, histone methylation pattern, and the essential role of rest." J Biol Chem **283**(14): 9257-68.
Erickson, M. R., B. J. Galletta, et al. (1997). "Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization." J Cell Biol **138**(3): 589-603.
Eriksson, M., L. Arminen, et al. (2005). "AP-1 regulates alpha2beta1 integrin expression by ERK-dependent signals during megakaryocytic differentiation of K562 cells." Exp Cell Res **304**(1): 175-86.
Festenstein, R., M. Tolaini, et al. (1996). "Locus control region function and heterochromatin-induced position effect variegation." Science **271**(5252): 1123-5.
Fortunel, N. O., A. Hatzfeld, et al. (2000). "Transforming growth factor-beta: pleiotropic role in the regulation of hematopoiesis." Blood **96**(6): 2022-36.
Fuks, F., W. A. Burgers, et al. (2000). "DNA methyltransferase Dnmt1 associates with histone deacetylase activity." Nat Genet **24**(1): 88-91.
Fuks, F., W. A. Burgers, et al. (2001). "Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription." Embo J **20**(10): 2536-44.
Fukuda, M. (1981). "Tumor-promoting phorbol diester-induced specific changes in cell surface glycoprotein profile of K562 human leukemic cells." Cancer Res **41**(11 Pt 1): 4621-8.
Gavin, I. M. and R. T. Simpson (1997). "Interplay of yeast global transcriptional regulators Ssn6p-Tup1p and Swi-Snf and their effect on chromatin structure." Embo J **16**(20): 6263-71.
Geiman, T. M., U. T. Sankpal, et al. (2004). "DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system." Biochem Biophys Res Commun **318**(2): 544-55.
Gentz, R., F. J. Rauscher, 3rd, et al. (1989). "Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains." Science **243**(4899): 1695-9.
Georgakopoulos, T., N. Gounalaki, et al. (1995). "Genetic evidence for the interaction of the yeast transcriptional co-activator proteins GCN5 and ADA2." Mol Gen Genet **246**(6): 723-8.
Georgopoulos, K., D. D. Moore, et al. (1992). "Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment." Science **258**(5083): 808-12.

Goldknopf, I. L., C. W. Taylor, et al. (1975). "Isolation and characterization of protein A24, a "histone-like" non-histone chromosomal protein." J Biol Chem **250**(18): 7182-7.
Goll, M. G., F. Kirpekar, et al. (2006). "Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2." Science **311**(5759): 395-8.
Goren, A., G. Simchen, et al. (2006). "Fine tuning of globin gene expression by DNA methylation." PLoS ONE **1**: e46.
Gorisch, S. M., M. Wachsmuth, et al. (2005). "Histone acetylation increases chromatin accessibility." J Cell Sci **118**(Pt 24): 5825-34.
Gowher, H., O. Leismann, et al. (2000). "DNA of Drosophila melanogaster contains 5-methylcytosine." Embo J **19**(24): 6918-23.
Grondin, B., M. Lefrancois, et al. (2007). "c-Jun homodimers can function as a context-specific coactivator." Mol Cell Biol **27**(8): 2919-33.
Gu, Y., M. D. Filippi, et al. (2003). "Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases." Science **302**(5644): 445-9.
Gu, Y., B. Jia, et al. (2001). "Biochemical and biological characterization of a human Rac2 GTPase mutant associated with phagocytic immunodeficiency." J Biol Chem **276**(19): 15929-38.
Gui, C. Y. and A. Dean (2001). "Acetylation of a specific promoter nucleosome accompanies activation of the epsilon-globin gene by beta-globin locus control region HS2." Mol Cell Biol **21**(4): 1155-63.
Guo, F., J. A. Cancelas, et al. (2008). "Rac GTPase isoforms, Rac1 and Rac2, play a redundant and crucial role in T-cell development." Blood.
Haataja, L., J. Groffen, et al. (1997). "Characterization of RAC3, a novel member of the Rho family." J Biol Chem **272**(33): 20384-8.
Halazonetis, T. D., K. Georgopoulos, et al. (1988). "c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities." Cell **55**(5): 917-24.
Hamiche, A., R. Sandaltzopoulos, et al. (1999). "ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF." Cell **97**(7): 833-42.
Hashimshony, T., J. Zhang, et al. (2003). "The role of DNA methylation in setting up chromatin structure during development." Nat Genet **34**(2): 187-92.
Hattori, N., Y. Imao, et al. (2007). "Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells." Genes Cells **12**(3): 387-96.
Hendzel, M. J., Y. Wei, et al. (1997). "Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation." Chromosoma **106**(6): 348-60.
Henry, K. W., A. Wyce, et al. (2003). "Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8." Genes Dev **17**(21): 2648-63.
Herrera, R., S. Hubbell, et al. (1998). "A role for the MEK/MAPK pathway in PMA-induced cell cycle arrest: modulation of megakaryocytic differentiation of K562 cells." Exp Cell Res **238**(2): 407-14.
Hess, J., P. Angel, et al. (2004). "AP-1 subunits: quarrel and harmony among siblings." J Cell Sci **117**(Pt 25): 5965-73.

Himes, S. R., Tagoh, H., Goonetilleke, N., Sasmono, T., Oceandy, D., Clark, R., Bonifer, C., and Hume, D.A (2001). "A highly conserved c-fms gene intronic element controls macrophage-specific and regulated expression." Journal of Leukocyte Biology. **70**: 812-820.

Hong, W., A. Y. Kim, et al. (2002). "Inhibition of CBP-mediated protein acetylation by the Ets family oncoprotein PU.1." Mol Cell Biol **22**(11): 3729-43.

Hong, Y., J. F. Martin, et al. (1996). "Inhibition of protein kinase C suppresses megakaryocytic differentiation and stimulates erythroid differentiation in HEL cells." Blood **87**(1): 123-31.

Horike, S. (2005). "[How the methyl-CpG binding protein-related epigenetic disease turns on the genes that produce its symptoms]." Tanpakushitsu Kakusan Koso **50**(8): 978-84.

Huang, W., A. Alessandrini, et al. (1993). "Raf-1 forms a stable complex with Mek1 and activates Mek1 by serine phosphorylation." Proc Natl Acad Sci U S A **90**(23): 10947-51.

Hung, H. L., J. Lau, et al. (1999). "CREB-Binding protein acetylates hematopoietic transcription factor GATA-1 at functionally important sites." Mol Cell Biol **19**(5): 3496-505.

Hutchins, A. S., A. C. Mullen, et al. (2002). "Gene silencing quantitatively controls the function of a developmental trans-activator." Mol Cell **10**(1): 81-91.

Ignatov, A. and E. J. Keath (2002). "Gel shift assay of nuclear extracts from Histoplasma capsulatum demonstrates the presence of several DNA binding proteins." Infect Immun **70**(4): 2238-41.

Im, H., C. Park, et al. (2003). "Dynamic regulation of histone H3 methylated at lysine 79 within a tissue-specific chromatin domain." J Biol Chem **278**(20): 18346-52.

Jenuwein, T. (2001). "Re-SET-ting heterochromatin by histone methyltransferases." Trends Cell Biol **11**(6): 266-73.

Jenuwein, T. and C. D. Allis (2001). "Translating the histone code." Science **293**(5532): 1074-80.

Jones, J. C., H. P. Phatnani, et al. (2004). "C-terminal repeat domain kinase I phosphorylates Ser2 and Ser5 of RNA polymerase II C-terminal domain repeats." J Biol Chem **279**(24): 24957-64.

Kadonaga, J. T. (1998). "Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines." Cell **92**(3): 307-13.

Kafri, T., X. Gao, et al. (1993). "Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo." Proc Natl Acad Sci U S A **90**(22): 10558-62.

Karin, M., Z. Liu, et al. (1997). "AP-1 function and regulation." Curr Opin Cell Biol **9**(2): 240-6.

Kelley, T. W., M. M. Graham, et al. (1999). "Macrophage colony-stimulating factor promotes cell survival through Akt/protein kinase B." J Biol Chem **274**(37): 26393-8.

Kerppola, T. K. (1998). "Transcriptional cooperativity: bending over backwards and doing the flip." Structure **6**(5): 549-54.

Kerppola, T. K. and T. Curran (1991). "DNA bending by Fos and Jun: the flexible hinge model." Science **254**(5035): 1210-4.

Kerppola, T. K. and T. Curran (1991). "Fos-Jun heterodimers and Jun homodimers bend DNA in opposite orientations: implications for transcription factor cooperativity." Cell **66**(2): 317-26.
Kim, J., S. Sif, et al. (1999). "Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes." Immunity **10**(3): 345-55.
Kluger, Y., Z. Lian, et al. (2004). "A panorama of lineage-specific transcription in hematopoiesis." Bioessays **26**(12): 1276-87.
Knaus, U. G., P. G. Heyworth, et al. (1991). "Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2." Science **254**(5037): 1512-5.
Koh, S. S., D. Chen, et al. (2001). "Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities." J Biol Chem **276**(2): 1089-98.
Köhler, J., Schäfer-Preuss, S., and Buttgereit, D. (1996). "Related enhancers in the intron of the ß1 tubulin gene of Drosophila melanogaster are essential for maternal and CNS-specific expression during embryogenesis." Nucleic Acids Research **24**: 2543-2550.
Kraus, V. B., J. A. Inostroza, et al. (1994). "Interaction of the Dr1 inhibitory factor with the TATA binding protein is disrupted by adenovirus E1A." Proc Natl Acad Sci U S A **91**(14): 6279-82.
Kuhn, E. J. and P. K. Geyer (2003). "Genomic insulators: connecting properties to mechanism." Curr Opin Cell Biol **15**(3): 259-65.
Kuo, M. H., J. Zhou, et al. (1998). "Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo." Genes Dev **12**(5): 627-39.
Ladd, P. D., J. S. Butler, et al. (2004). "Identification of a genomic fragment that directs hematopoietic-specific expression of Rac2 and analysis of the DNA methylation profile of the gene locus." Gene **341**: 323-33.
Lai, C. F. and S. L. Cheng (2002). "Signal transductions induced by bone morphogenetic protein-2 and transforming growth factor-beta in normal human osteoblastic cells." J Biol Chem **277**(18): 15514-22.
Langst, G., E. J. Bonte, et al. (1999). "Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer." Cell **97**(7): 843-52.
Laudanna, C., J. J. Campbell, et al. (1996). "Role of Rho in chemoattractant-activated leukocyte adhesion through integrins." Science **271**(5251): 981-3.
Lee, J. H., S. R. Hart, et al. (2004). "Histone deacetylase activity is required for embryonic stem cell differentiation." Genesis **38**(1): 32-8.
Lee, M. G., C. Wynder, et al. (2006). "Functional interplay between histone demethylase and deacetylase enzymes." Mol Cell Biol **26**(17): 6395-402.
Lee, T. I., R. G. Jenner, et al. (2006). "Control of developmental regulators by Polycomb in human embryonic stem cells." Cell **125**(2): 301-13.
Leonhardt, H., A. W. Page, et al. (1992). "A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei." Cell **71**(5): 865-73.
Lewis, J. D., R. R. Meehan, et al. (1992). "Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA." Cell **69**(6): 905-14.

Li, E., C. Beard, et al. (1993). "DNA methylation, genomic imprinting, and mammalian development." Cold Spring Harb Symp Quant Biol **58**: 297-305.
Li, E., C. Beard, et al. (1993). "Role for DNA methylation in genomic imprinting." Nature **366**(6453): 362-5.
Li, E., T. H. Bestor, et al. (1992). "Targeted mutation of the DNA methyltransferase gene results in embryonic lethality." Cell **69**(6): 915-26.
Liu, X., B. Wu, et al. (2007). "Functional sequestration of transcription factor activity by repetitive DNA." J Biol Chem **282**(29): 20868-76.
Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-8.
Long, M. W., C. H. Heffner, et al. (1990). "Regulation of megakaryocyte phenotype in human erythroleukemia cells." J Clin Invest **85**(4): 1072-84.
Lorch, Y., M. Zhang, et al. (1999). "Histone octamer transfer by a chromatin-remodeling complex." Cell **96**(3): 389-92.
Lord, K. A., A. Abdollahi, et al. (1993). "Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation." Mol Cell Biol **13**(2): 841-51.
Lorincz, M. C., D. R. Dickerson, et al. (2004). "Intragenic DNA methylation alters chromatin structure and elongation efficiency in mammalian cells." Nat Struct Mol Biol **11**(11): 1068-75.
Lozzio, C. B. and B. B. Lozzio (1975). "Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome." Blood **45**(3): 321-34.
Madisen, L., A. Krumm, et al. (1998). "The immunoglobulin heavy chain locus control region increases histone acetylation along linked c-myc genes." Mol Cell Biol **18**(11): 6281-92.
Marks, P. A., V. M. Richon, et al. (2001). "Histone deacetylase inhibitors as new cancer drugs." Curr Opin Oncol **13**(6): 477-83.
Martens, J. A. and F. Winston (2003). "Recent advances in understanding chromatin remodeling by Swi/Snf complexes." Curr Opin Genet Dev **13**(2): 136-42.
Martinowich, K., D. Hattori, et al. (2003). "DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation." Science **302**(5646): 890-3.
McCool, K. W., X. Xu, et al. (2007). "The role of histone acetylation in regulating early gene expression patterns during early embryonic stem cell differentiation." J Biol Chem **282**(9): 6696-706.
McKenzie, R. a. B., M.D. (1996). "The two small introns of the Drosophila affinidisjuncta Adh gene are required for normal transcription." Nucleic Acids Research **24**: 3635-3642.
Milde-Langosch, K. (2005). "The Fos family of transcription factors and their role in tumourigenesis." Eur J Cancer **41**(16): 2449-61.
Moll, J., G. Sansig, et al. (1991). "The murine rac1 gene: cDNA cloning, tissue distribution and regulated expression of rac1 mRNA by disassembly of actin microfilaments." Oncogene **6**(5): 863-6.

Morishita, M., Kishino, T., Furukawa, K., Yonekura, A., Miyazaki, Y., Kanematsu, T., Yamashita, S., and Tsukazaki, T. (2001). "A 30-base-pair element in the first intron of SOX9 acts as an enhancer in ATDC5." Biochemical and Biophysical Research Communications **288**: 347-355.

Murnion, M. E., R. R. Adams, et al. (2001). "Chromatin-associated protein phosphatase 1 regulates aurora-B and histone H3 phosphorylation." J Biol Chem **276**(28): 26656-65.

Nakamura, T., T. Mori, et al. (2002). "ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation." Mol Cell **10**(5): 1119-28.

Nakayama, J., J. C. Rice, et al. (2001). "Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly." Science **292**(5514): 110-3.

Nan, X., F. J. Campoy, et al. (1997). "MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin." Cell **88**(4): 471-81.

Nathan, D., K. Ingvarsdottir, et al. (2006). "Histone sumoylation is a negative regulator in Saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications." Genes Dev **20**(8): 966-76.

Ney, P. A., B. P. Sorrentino, et al. (1990). "Tandem AP-1-binding sites within the human beta-globin dominant control region function as an inducible enhancer in erythroid cells." Genes Dev **4**(6): 993-1006.

Nicassio, F., N. Corrado, et al. (2007). "Human USP3 is a chromatin modifier required for S phase progression and genome stability." Curr Biol **17**(22): 1972-7.

Nickel, B. E., C. D. Allis, et al. (1989). "Ubiquitinated histone H2B is preferentially located in transcriptionally active chromatin." Biochemistry **28**(3): 958-63.

Nishioka, K., J. C. Rice, et al. (2002). "PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin." Mol Cell **9**(6): 1201-13.

O'Neill, D. W., S. S. Schoetz, et al. (2000). "An ikaros-containing chromatin-remodeling complex in adult-type erythroid cells." Mol Cell Biol **20**(20): 7572-82.

Okamoto, I., A. P. Otte, et al. (2004). "Epigenetic dynamics of imprinted X inactivation during early mouse development." Science **303**(5658): 644-9.

Okano, M., D. W. Bell, et al. (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell **99**(3): 247-57.

Orkin, S. H. (1995). "Transcription factors and hematopoietic development." J Biol Chem **270**(10): 4955-8.

Ou, X., J. Pollock, et al. (1999). "Identification and functional characterization of the murine Rac2 gene promoter." DNA Cell Biol **18**(3): 253-63.

Palis, J. and M. C. Yoder (2001). "Yolk-sac hematopoiesis: the first blood cells of mouse and man." Exp Hematol **29**(8): 927-36.

Paranjape, S. M., R. T. Kamakaka, et al. (1994). "Role of chromatin structure in the regulation of transcription by RNA polymerase II." Annu Rev Biochem **63**: 265-97.

Parekh, B. S. and T. Maniatis (1999). "Virus infection leads to localized hyperacetylation of histones H3 and H4 at the IFN-beta promoter." Mol Cell **3**(1): 125-9.

Peters, A., and Schubeler, D. (2005). "Methylation of histones: playing memory with DNA " Current Opinion in Cell Biology **17**: 230-238.
Peters, A. H., J. E. Mermoud, et al. (2002). "Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin." Nat Genet **30**(1): 77-80.
Peters, A. H., D. O'Carroll, et al. (2001). "Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability." Cell **107**(3): 323-37.
Phatnani, H. P. and A. L. Greenleaf (2006). "Phosphorylation and functions of the RNA polymerase II CTD." Genes Dev **20**(21): 2922-36.
Pinarbasi, E., J. Elliott, et al. (1996). "Activation of a yeast pseudo DNA methyltransferase by deletion of a single amino acid." J Mol Biol **257**(4): 804-13.
Plath, K., S. Mlynarczyk-Evans, et al. (2002). "Xist RNA and the mechanism of X chromosome inactivation." Annu Rev Genet **36**: 233-78.
Pollack, B. P., S. V. Kotenko, et al. (1999). "The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity." J Biol Chem **274**(44): 31531-42.
Pradip, D., X. Peng, et al. (2003). "Rac2 specificity in macrophage integrin signaling: potential role for Syk kinase." J Biol Chem **278**(43): 41661-9.
Racke, F. K., K. Lewandowska, et al. (1997). "Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells." J Biol Chem **272**(37): 23366-70.
Rice, K. L., I. Hormaeche, et al. (2007). "Epigenetic regulation of normal and malignant hematopoiesis." Oncogene **26**(47): 6697-714.
Roberts, A. W., C. Kim, et al. (1999). "Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense." Immunity **10**(2): 183-96.
Robertson, K. D., K. Keyomarsi, et al. (2000). "Differential mRNA expression of the human DNA methyltransferases (DNMTs) 1, 3a and 3b during the G(0)/G(1) to S phase transition in normal and tumor cells." Nucleic Acids Res **28**(10): 2108-13.
Robertson, K. D., E. Uzvolgyi, et al. (1999). "The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors." Nucleic Acids Res **27**(11): 2291-8.
Roh, T. Y., S. Cuddapah, et al. (2005). "Active chromatin domains are defined by acetylation islands revealed by genome-wide mapping." Genes Dev **19**(5): 542-52.
Rutherford, T., J. B. Clegg, et al. (1981). "Embryonic erythroid differentiation in the human leukemic cell line K562." Proc Natl Acad Sci U S A **78**(1): 348-52.
Saccani, S., S. Pantano, et al. (2001). "Two waves of nuclear factor kappaB recruitment to target promoters." J Exp Med **193**(12): 1351-9.
Samaco, R. C., A. Hogart, et al. (2005). "Epigenetic overlap in autism-spectrum neurodevelopmental disorders: MECP2 deficiency causes reduced expression of UBE3A and GABRB3." Hum Mol Genet **14**(4): 483-92.
Sandoval, J., J. L. Rodriguez, et al. (2004). "RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription." Nucleic Acids Res **32**(11): e88.
Santos-Rosa, H., R. Schneider, et al. (2002). "Active genes are tri-methylated at K4 of histone H3." Nature **419**(6905): 407-11.

Sarraf, S. A. and I. Stancheva (2004). "Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly." Mol Cell **15**(4): 595-605.
Schneider, R., A. J. Bannister, et al. (2004). "Histone H3 lysine 4 methylation patterns in higher eukaryotic genes." Nat Cell Biol **6**(1): 73-7.
Scott, H. S., D. S. Kyriakou, et al. (1998). "Characterization of a novel gene, C21orf2, on human chromosome 21q22.3 and its exclusion as the APECED gene by mutation analysis." Genomics **47**(1): 64-70.
Shafarenko, M., A. Amanullah, et al. (2004). "Fos modulates myeloid cell survival and differentiation and partially abrogates the c-Myc block in terminal myeloid differentiation." Blood **103**(11): 4259-67.
Shang, Y., M. Myers, et al. (2002). "Formation of the androgen receptor transcription complex." Mol Cell **9**(3): 601-10.
Shi, Y., F. Lan, et al. (2004). "Histone demethylation mediated by the nuclear amine oxidase homolog LSD1." Cell **119**(7): 941-53.
Shiio, Y. and R. N. Eisenman (2003). "Histone sumoylation is associated with transcriptional repression." Proc Natl Acad Sci U S A **100**(23): 13225-30.
Shirsat, N. V., R. J. Pignolo, et al. (1990). "A member of the ras gene superfamily is expressed specifically in T, B and myeloid hemopoietic cells." Oncogene **5**(5): 769-72.
Shivaswamy, S., A. Bhinge, et al. (2008). "Dynamic remodeling of individual nucleosomes across a eukaryotic genome in response to transcriptional perturbation." PLoS Biol **6**(3): e65.
Skalnik, D. G. (2002). "Transcriptional mechanisms regulating myeloid-specific genes." Gene **284**(1-2): 1-21.
Soutoglou, E., N. Katrakili, et al. (2000). "Acetylation regulates transcription factor activity at multiple levels." Mol Cell **5**(4): 745-51.
Spangler, L., X. Wang, et al. (2001). "TFIIH action in transcription initiation and promoter escape requires distinct regions of downstream promoter DNA." Proc Natl Acad Sci U S A **98**(10): 5544-9.
Stavropoulos, P., G. Blobel, et al. (2006). "Crystal structure and mechanism of human lysine-specific demethylase-1." Nat Struct Mol Biol **13**(7): 626-32.
Sterner, D. E. and S. L. Berger (2000). "Acetylation of histones and transcription-related factors." Microbiol Mol Biol Rev **64**(2): 435-59.
Stewart, M. D., J. Li, et al. (2005). "Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment." Mol Cell Biol **25**(7): 2525-38.
Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-5.
Strahl, B. D., P. A. Grant, et al. (2002). "Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression." Mol Cell Biol **22**(5): 1298-306.
Struhl, K. (1998). "Histone acetylation and transcriptional regulatory mechanisms." Genes Dev **12**(5): 599-606.
Sullivan, K. E., A. B. Reddy, et al. (2007). "Epigenetic regulation of tumor necrosis factor alpha." Mol Cell Biol **27**(14): 5147-60.

Tachibana, M., K. Sugimoto, et al. (2001). "Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3." J Biol Chem **276**(27): 25309-17.

Tachibana, M., K. Sugimoto, et al. (2002). "G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis." Genes Dev **16**(14): 1779-91.

Tamaru, H. and E. U. Selker (2001). "A histone H3 methyltransferase controls DNA methylation in Neurospora crassa." Nature **414**(6861): 277-83.

Tamaru, H., X. Zhang, et al. (2003). "Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa." Nat Genet **34**(1): 75-9.

Tan, B. L., M. N. Yazicioglu, et al. (2003). "Genetic evidence for convergence of c-Kit- and alpha4 integrin-mediated signals on class IA PI-3kinase and the Rac pathway in regulating integrin-directed migration in mast cells." Blood **101**(12): 4725-32.

Tang, J., J. D. Gary, et al. (1998). "PRMT 3, a type I protein arginine N-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation." J Biol Chem **273**(27): 16935-45.

Tao, W., M. D. Filippi, et al. (2002). "The TRQQKRP motif located near the C-terminus of Rac2 is essential for Rac2 biologic functions and intracellular localization." Blood **100**(5): 1679-88.

Tate, P. H. and A. P. Bird (1993). "Effects of DNA methylation on DNA-binding proteins and gene expression." Curr Opin Genet Dev **3**(2): 226-31.

Tetteroo, P. A., F. Massaro, et al. (1984). "Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells." Leuk Res **8**(2): 197-206.

Thomas, R. M., L. Gao, et al. (2005). "Signals from CD28 induce stable epigenetic modification of the IL-2 promoter." J Immunol **174**(8): 4639-46.

Tian, Y. and M. V. Autieri (2007). "Cytokine expression and AIF-1-mediated activation of Rac2 in vascular smooth muscle cells: a role for Rac2 in VSMC activation." Am J Physiol Cell Physiol **292**(2): C841-9.

Tiffany, H. L., Handen, J.S., and Rosenberg, H.F. (1996). "Enhanced expression of the eosinophil-derived neurotoxin ribonuclease (RNS2) gene requires interaction between the promoter and intron." The Journal of Biological Chemistry **271**: 12387-12393.

Tse, C., T. Sera, et al. (1998). "Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III." Mol Cell Biol **18**(8): 4629-38.

Tsukada, Y., J. Fang, et al. (2006). "Histone demethylation by a family of JmjC domain-containing proteins." Nature **439**(7078): 811-6.

Valledor, A. F., F. E. Borras, et al. (1998). "Transcription factors that regulate monocyte/macrophage differentiation." J Leukoc Biol **63**(4): 405-17.

Van Holde, K. E. (1989). Chromatin, Springer Press, New York. .

Verdone, L., M. Caserta, et al. (2005). "Role of histone acetylation in the control of gene expression." Biochem Cell Biol **83**(3): 344-53.

Wang, H., R. Cao, et al. (2001). "Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase." Mol Cell **8**(6): 1207-17.

Wang, H., L. Wang, et al. (2004). "Role of histone H2A ubiquitination in Polycomb silencing." Nature **431**(7010): 873-8.
Wang, W. (2003). "The SWI/SNF family of ATP-dependent chromatin remodelers: similar mechanisms for diverse functions." Curr Top Microbiol Immunol **274**: 143-69.
Wang, Y., N. A. Rebert, et al. (2007). "Rac2 is expressed by pulmonary endothelial cells." FASEB Journal **21**: 871-79.
Ward, S. B., G. Hernandez-Hoyos, et al. (1998). "Chromatin remodeling of the interleukin-2 gene: distinct alterations in the proximal versus distal enhancer regions." Nucleic Acids Res **26**(12): 2923-34.
Wei, Y., C. A. Mizzen, et al. (1998). "Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena." Proc Natl Acad Sci U S A **95**(13): 7480-4.
West, M. H. and W. M. Bonner (1980). "Histone 2B can be modified by the attachment of ubiquitin." Nucleic Acids Res **8**(20): 4671-80.
Weston, V. J. and T. Stankovic (2004). "Rac1 and Rac2 GTPases in haematopoiesis." Bioessays **26**(3): 221-4.
Whalen, A. M., S. C. Galasinski, et al. (1997). "Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase kinase." Mol Cell Biol **17**(4): 1947-58.
Whetstine, J. R., A. Nottke, et al. (2006). "Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases." Cell **125**(3): 467-81.
Williams, D. A., W. Tao, et al. (2000). "Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency." Blood **96**(5): 1646-54.
Wilson, A., H. R. MacDonald, et al. (2001). "Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus." J Exp Med **194**(7): 1003-12.
Wilson, C. B., K. W. Makar, et al. (2005). "DNA methylation and the expanding epigenetics of T cell lineage commitment." Semin Immunol **17**(2): 105-19.
Wingender, E., P. Dietze, et al. (1996). "TRANSFAC: a database on transcription factors and their DNA binding sites." Nucleic Acids Res **24**(1): 238-41.
Wingender, E., H. Karas, et al. (1997). "TRANSFAC database as a bridge between sequence data libraries and biological function." Pac Symp Biocomput: 477-85.
Witcher, M., F. Pettersson, et al. (2008). "Retinoic acid modulates chromatin to potentiate tumor necrosis factor alpha signaling on the DIF2 promoter." Nucleic Acids Res **36**(2): 435-43.
Wolffe, A. P. and M. A. Matzke (1999). "Epigenetics: regulation through repression." Science **286**(5439): 481-6.
Wolter, S., A. Doerrie, et al. (2008). "c-Jun controls histone modifications, NF-kappaB recruitment, and RNA polymerase II function to activate the ccl2 gene." Mol Cell Biol **28**(13): 4407-23.
Wood, A., J. Schneider, et al. (2005). "Cross-talking histones: implications for the regulation of gene expression and DNA repair." Biochem Cell Biol **83**(4): 460-7.
Yang, F. C., S. J. Atkinson, et al. (2001). "Rac and Cdc42 GTPases control hematopoietic stem cell shape, adhesion, migration, and mobilization." Proc Natl Acad Sci U S A **98**(10): 5614-8.

Yang, V. W. (1998). "Eukaryotic transcription factors: identification, characterization and functions." J Nutr **128**(11): 2045-51.
Yang, W. M., C. Inouye, et al. (1996). "Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3." Proc Natl Acad Sci U S A **93**(23): 12845-50.
Yeh, C. K., I. S. Ambudkar, et al. (1992). "Differential expression of early response genes, c-jun, c-fos, and jun B, in A5 cells." Am J Physiol **263**(6 Pt 1): G934-8.
Yoder, J. A. and T. H. Bestor (1998). "A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast." Hum Mol Genet **7**(2): 279-84.
Yu, H., D. Leitenberg, et al. (2001). "Deficiency of small GTPase Rac2 affects T cell activation." J Exp Med **194**(7): 915-26.
Zhang, Y. and D. Reinberg (2001). "Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails." Genes Dev **15**(18): 2343-60.
Zhu, B., D. Benjamin, et al. (2001). "Overexpression of 5-methylcytosine DNA glycosylase in human embryonic kidney cells EcR293 demethylates the promoter of a hormone-regulated reporter gene." Proc Natl Acad Sci U S A **98**(9): 5031-6.
Zhu, J. and S. G. Emerson (2002). "Hematopoietic cytokines, transcription factors and lineage commitment." Oncogene **21**(21): 3295-313.
Zhu, P., W. Zhou, et al. (2007). "A histone H2A deubiquitinase complex coordinating histone acetylation and H1 dissociation in transcriptional regulation." Mol Cell **27**(4): 609-21.
Zutter, M. M., A. D. Painter, et al. (1999). "The Megakaryocyte/Platelet-specific enhancer of the alpha2beta1 integrin gene: two tandem AP1 sites and the mitogen-activated protein kinase signaling cascade." Blood **93**(5): 1600-11.