Mechanisms of Retinoic Acid Resistance in Acute Promyelocytic Leukemia

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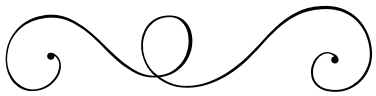
DEDICATION



"Great is the art of beginning, but greater is the art of ending."

Longfellow, Henry Wadsworth

This thesis is dedicated to my parents, Diane and Ross McLeod. Don't worry, I won't quiz you on its contents. You both often go unappreciated because you are so quiet about all the wonderful things you do for your children, but do not think that all the ways you have helped me over the years have gone unnoticed. I can humbly admit that not one experiment would have been done, not one paper would have been written and not one talk would have been given, without your love and support. You taught me to be an independent thinker, you gave me the freedom, both temporal and financial, to pursue my goals and you were the source of never-ending encouragement and positivity. I know that I am an absolute mess but I hope you are as happy and proud to see this finally finished as I am.

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LIST OF ABBREVIATIONS

3C	Chromosome Conformation Capture
3D	Three dimensional
ADP	Adenosine di-phosphate
ALCL	Anaplastic Large Cell Lymphoma
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
ATO	Arsenic trioxide
RA	All Trans Retinoic Acid
B	Zinc finger motifs
bHLH	Basic helix-loop-helix
bps	Base pairs
BRE^d	Downstream TFIIB recognition element
BRE^u	Upstream TFIIB recognition element
BRG1	Brahma-related gene 1
BRM	Brahma
CAT	Chloramphenicol acetyltransferase
CBF	Core binding factor
CC	Coiled-coil domain
CEBPB	CEBPBeta
CEBPE	CEBPEpsilon
CEBPs	CCAAT-enhancer binding proteins
ChIP	Chromatin Immunoprecipitation
CML	Chronic myeloid leukemia
CSNK2	Casein kinase II
CTCF	CCCTC-binding factor
CTD	C-terminal repeat domain

CTD	Carboxy-terminal domain of RNAPII
Dar	Darinaparsin
DBD	DNA-binding domain
DCE	Downstream core element
DN	Dominant negative
DNMTs	DNA methyltransferases
DPE	Downstream promoter element
DRB	5,6-dichlorobenzimidazole 1-β-d-ribofuranoside
DRs	Direct repeats
dsDNA	Double stranded DNA
E1	Activating enzyme
ECL	Enhanced chemiluminescence
ENU	N-ethyl-N-nitrourea
eRNAs	enhancer RNAs
ES	Embryonic stem
FAB	French-American-British cooperative group
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FISH	Fluorescence *in situ* hybridization
GEP	Gene expression profiling
GSCF	Granulocyte colony-stimulating factor
GTFs	General transcription factors
H12	Twelfth helix
H2BK5me	Monomethylation of lysine 5 on histone H2B
H3K14ac	Acetylated lysine 14, histone 3
H3K27me3	Trimethylated lysine 27. histone 3
H3K36me3	Trimethylation of lysine 36 on histone 3
H3K4me1	Monomethylation of histone 3 lysine 4
H3K4me3	Trimethylation of lysine 4 on histone 3

H3K79me3	Trimethylation of lysine 79 on histone 3
H3K9ac	Acetylated lysine 9, histone 3
H3K9me2	Dimethylated lysine 9, histone 3
H3K9me3	Trimethylated lysine 9, histone 3
H4K20me	Monomethylation of lysine 20 on histone 4
HATs	Histone acetyltransferases
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
Hi-C	High-troughput genome sequencing
HMWs	High molecular weight complexes
HREs	Hormone response elements
HSCs/HPCs	Human hematopoietic stem cells/hematopoietic progenitor cells
Inr	Initiatior element
IRES	Internal ribosomal entry site
K	Lysine
kDNA	Kinetoplast DNA
LBD	Ligand binding domain
LCRs	Locus control regions
LIC	Leukemia initiating cell
LSC	Leukemia stem cell
MDS	Myelodysplastic syndromes
miRNA	microRNAs
MLL	Mixed lineage leukemia locus
mRNA(s)	Messenger RNA(s)
MTE	Motif 10 element
NBs	Nuclear bodies
NBT	Nitro-blue-tetrazolium
NDRs	Nucleosome depleted regions

NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NPM	Nucleophosmin
NPMi	NPM inhibitor
NR	Nuclear receptor
PARG	Poly-ADP-ribose-glycohydrolase
PARP	Poly-ADP-ribose polymerase
PAS	Periodic Acid-Schiff
PcG	Polycomb group
PCR	Polymerase Chain Reaction
PDGF	Platelet derived growth factor
PEC	Pre-elongation complex
PIC	Pre-initiation complex
PMA	4α-phorbol 12-myristate 13-acetate
PML	Promyelocytic leukemia protein/gene
PRC2	PcG Repressive Complex 2
PRE	PcG Response Element
PRKC	Protein Kinase C
PRKCD	Protein Kinase C delta
PRKCDKD	PRKCD kinase dead construct
PTMs	Post-translational modifications
qPCR/Q-RT-PCR	Quantitative Real-Time PCR
R	Zinc-finger RING motif
RARA	Retinoic acid receptor alpha
RAREs	Retinoic acid response elements
RNAi	RNA interference
RNAP	RNA polymerase
RNAPII	RNA Polymerase II
RXRs	Retinoid X Receptors

S2	Serine 2
S2P	Phosphorylated serine 2 residue
S5	Serine 5
S5P	Phosphorylated serine 5 residue
S7	Serine 7
SUMO	Small ubiquitin-like modifier
TAFs	TBP-associated factors
TBP	TATA-binding protein
TFs	Transcription factors
TOP2B	Topoisomerase II beta
TP53	Human p53 gene
TSS	Transcription Start Site
UTRs	Untranslated regions
WHO	World Health Organization
XCPEs	X core promoter elements
Y-patches	Pyrimidine patches

ABSTRACT

Acute promyelocytic leukemia (APL) has become a highly curable disease with contemporary treatment, with all-*trans* retinoic acid (RA) serving as the cornerstone of therapy. Interactions between the retinoic acid receptor alpha (RARA) and co-regulators play a key role in coordinating gene transcription and myeloid differentiation. In the majority of APL cases, RARA is fused with the promyelocytic leukemia (*PML*) gene, resulting in the expression of the fusion protein, PML/RARA. Here, we report that Nucleophosmin (NPM) associates with, and negatively modulates, PML/RARA transcriptional activity. Furthermore, increased levels and association of NPM underlies resistance to retinoic acid (RA) in a model APL cell line. The mechanism by which this occurs involves aberrant recruitment of the chromatin remodeler, BRG1. Pharmacological inhibition of NPM was able to circumvent resistance by abrogating BRG1 recruitment, restoring RA-induced gene expression and ultimately, RA-induced differentiation. These results identify a novel mechanism of resistance in APL and provide further insights to the role of NPM in gene regulation and differentiation.

NPM is not the only protein that associates with PML/RARA in our RA-resistant model. Previous work established topoisomerase II beta (TOP2B) as another mediator of resistance. Mirroring NPM, TOP2B is overexpressed in the resistant cell line and abrogation of TOP2B levels results in restoration of RA sensitivity. Studies to determine the mechanism by which TOP2B protein is regulated found that levels of protein kinase C delta (PRKCD) correlated with TOP2B protein expression. Moreover, activation of PRKCD by RA or 4α-phorbol 12-myristate 13-acetate (PMA) led to an increase of TOP2B protein levels. Most notably, in our resistant cells, we observed increased basal phosphorylation levels of threonine 505 on PRKCD, a marker of activation. The combination of RA and PRKCD inhibition was able to overcome the TOP2B repressive effects on RA-target genes and led to an increased expression of the granulocytic differentiation

marker, cd11c. These results suggest that PRKCD regulates TOP2B expression, and a constitutively active PRKCD in the resistant cell line leads to overexpression of TOP2B.

In conclusion, these studies indicate that formation of a stable association between both NPM and TOP2B with PML/RARA is crucial to the development of RA-resistance in our model system. Cumulatively, this work has contributed to an enhanced understanding of the role of NPM and TOP2B in gene regulation and suggests that aberrant recruitment of co-repressor complexes is a general mechanism of transcriptional resistance.

SOMMAIRE

La leucémie aigüe promyélocytaire (LAP) est, de nos jours, une maladie très bien traitée grâce à des thérapies novatrices, dont l'acide tout-*trans*-rétinoïque (AR) est la pierre angulaire. Les interactions entre le récepteur alpha de l'acide rétinoïque (RAAR) et des co-régulateurs jouent un rôle clef dans la coordination de la transcription des gènes et in fine de la différenciation myélocytaire. Dans la majorité des cas de LAP, le gène *RAAR* est fusionné au gène leucémie promyélocytaire (*LPM*), ce qui se traduit par l'expression de la protéine de fusion PML/RAAR. Nous montrons ici que nucléophosmine (NPM) s'associe avec, et module de façon négative, l'activité transcriptionnelle de LPM/RAAR. De plus, l'augmentation des niveaux ainsi que de l'association de NPM sont des éléments sous-jacents de la résistance à l'acide rétinoïque dans une lignée cellulaire de LAP. Le mécanisme par lequel cela se produit implique un recrutement aberrant du facteur de remodelage de chromatine BRG1. L'inhibition pharmacologique de NPM parvient à circonvenir la résistance en abrogeant le recrutement de BRG1, ce qui augmente l'expression des gènes induite par l'AR et ultimement, la différenciation induite par l'AR. Ces résultats identifient un nouveau mécanisme de résistance de la LAP et fournissent de nouvelles perspectives à propos du rôle de NPM dans la régulation des gènes et de la différentiation.

NPM n'est pas la seule protéine qui s'associe avec PML/RARA dans notre modèle résistant à l'AR. Des travaux précédents ont établi la topoisomérase II beta (TOP2B) en tant qu'autre médiatrice de résistance. Tout comme NPM, TOP2B est surexprimée dans la lignée cellulaire résistante et l'abrogation des niveaux de TOP2B se traduit par une restauration de la sensibilité à l'AR. Ainsi, nous avons tenté de déterminer le mécanisme par lequel la protéine TOP2B été régulée dans notre model cellulaire. Nos résultats démontrent que les niveaux de la protéine kinase C delta (PRKCD) corrèlent avec l'expression de la protéine TOP2B. De plus, l'activation de PRKCD par l'AR ou 4α-phorbol 12-myristate

13-acetate (PMA) conduit à une augmentation des niveaux de protéine TOP2B. Notamment, dans des cellules résistantes, nous avons observé une augmentation des niveaux de phosphorylation de la thréonine 505 sur PRKCD, ce qui est une marque d'activation. La combinaison de l'AR avec l'inhibition de PRKCD parvient à surmonter les effets répressifs de TOP2B sur les gènes cibles de l'AR et conduit à l'augmentation de l'expression du marqueur granulocytaire cd11c démontrant une hausse de la differentiation. Ces résultats suggèrent que PRKCD régule l'expression de TOP2B, et qu'une PRKCD constitutivement active dans des cellules résistantes conduit à la surexpression de TOP2B.

En conclusion, ces études indiquent que la formation d'une association stable entre à la fois NPM, TOP2B, et PML/RARA est cruciale pour le développement de la résistance à l'AR dans notre modèle. Globalement, ce projet a contribué à une compréhension approfondie du rôle de NPM et TOP2B dans la régulation des gènes et suggère que le recrutement aberrant de complexes corépresseurs est un mécanisme général de résistance transcriptionnelle.

CHAPTER 1: LITERATURE REVIEW.

1.1 Eukaryotic Transcriptional Regulation

The development, growth, and survival of eukaryotic organisms in response to environmental cues requires the elegant orchestration of approximately twenty thousand protein-coding genes [1]. The regulation of gene expression is therefore an intricate process involving diverse mechanisms ranging from chromatin remodeling to protein stability. However, transcription initiation is the most critical, and therefore highly controlled, step. Initiation of transcription in eukaryotes is governed by two interconnected regulatory components: *cis*-acting DNA elements and the *trans*-acting protein factors that interact with those elements. Furthermore, the chromatin signature, as defined by epigenetic modifications as well as the three-dimensional architecture of the chromatin, influences initiation by governing access of the *trans*-acting factors to these *cis*-elements in a gene- and context-specific manner.

1.1.1 *Cis*-acting elements

In eukaryotes, protein-coding genes are transcribed by RNA polymerase II (RNAPII), and the interplay between many DNA motifs controls the rate and level of production of specific messenger RNAs (mRNAs) by RNAPII. *Cis*-acting elements can be broadly divided into two subsets: (1) a promoter, which is composed of a core promoter and elements in close proximity to the transcription start site (TSS) and (2) distal regulatory elements, which are often located hundreds, if not thousands, of base pairs from the TSS and include enhancers, silencers, insulators and locus control regions (LCRs) (Figure 1.1). However, it is important to remember that the classification of elements that control transcription based on their distance from the TSS actually represents more of a continuous spectrum than distinct groups. Enhancers and LCRs that can stimulate transcription from tens of thousands of base pairs away are at one extreme, while promoter-proximal elements are at the other. Researchers have identified a large

number of control regions that can influence transcription from distances between these two extremes.

1.1.1.1 Promoters

CORE PROMOTERS

The dissection of core promoter components contributes fundamental insights into the mechanisms by which transcription occurs in eukaryotes. The cascade of signaling events that precede the activation of transcription must converge upon the transcription machinery located at the core promoter. Core promoters represent more than simple DNA docking sites for the basal transcription machinery. Rather, the core promoter elements form a complex checkpoint to transcription initiation.

The TSS of a gene is defined as the first nucleotide that is transcribed by RNAPII at the 5' end of a gene. The core promoter is the minimal stretch of a contiguous DNA sequence that is sufficient to direct the accurate initiation of transcription starting at the TSS [2]. However, this reductive definition fails to accurately describe the diversity and complexity of core promoter composition seen across species and even within the same organism's genome.

Core promoter structure can be dichotomized into two major types—focused and dispersed. Historically, promoters were thought to contain a single TSS or a distinct cluster of TSSs over several nucleotides (ie "focused"). However, recent bioinformatics studies have revealed that the majority of strong human RNA polymerase II core promoters exhibit transcription from up to dozens of closely-spaced start sites that are distributed over 50 to 100 nucleotides (ie "dispersed") and are typically found within CpG islands (see Section 1.1.3.1) [3, 4]. However, individual instances of "dispersed" promoters were described long before the genome-wide studies [5, 6]. In vertebrates, dispersed promoters are more common, however, focused promoters are used by a broader range of organisms and are more ancient [7].

A number of core promoter elements have been identified in metazoans and include the following (Figure 1.1):

(1) TATA box: This was the first eukaryotic core promoter element to be identified [8, 9], which is appropriate as it is the most ancient and most widely used motif throughout nature. In fact, the TATA box, and its cognate protein factor the TATA-binding protein (TBP), are conserved from Archaea to humans.

(2) initiator (Inr) element: The initiator motif encompasses the TSS. The Inr is a recognition site for the binding of TFIID.

(3) TFIIB recognition elements (BRE^u and BRE^d): The BRE was originally identified as a TFIIB-binding sequence that is immediately upstream of a subset of TATA boxes [10]. It was subsequently found that TFIIB can bind upstream (BRE^u) or downstream (BRE^d) of the TATA box [11]. Depending on the blend of other elements present in a promoter, the net effect by the BRE^u and BRE^d on activity can either stimulatory or inhibitory [10, 12, 13].

(4) downstream promoter element (DPE): The DPE was identified as a downstream TFIID recognition sequence that is important for basal transcription activity [14]. The DPE is conserved from *Drosophila* to humans, and it functions cooperatively with the Inr, where the spacing between the two elements is critical for optimal transcription [15].

(5) motif 10 element (MTE): The MTE was found through a combination of computational and biochemical studies [16]. Like the DPE, the MTE functions cooperatively with the Inr with a strict Inr-MTE spacing requirement.

(6) downstream core element (DCE): The DCE was originally discovered in the human *HBB* promoter, and it consists of three sub-elements [17]. It also interacts with TFIID, but appears to be distinct from the DPE [18].

(7) X core promoter elements (XCPEs): The XCPE motif is present in about

1% of human core promoters, most of which are TATA-less. XCPE exhibits little activity by itself. Instead, it acts in conjunction with sequence-specific activators, such as NRF1, NF-1, and Sp1 [19].

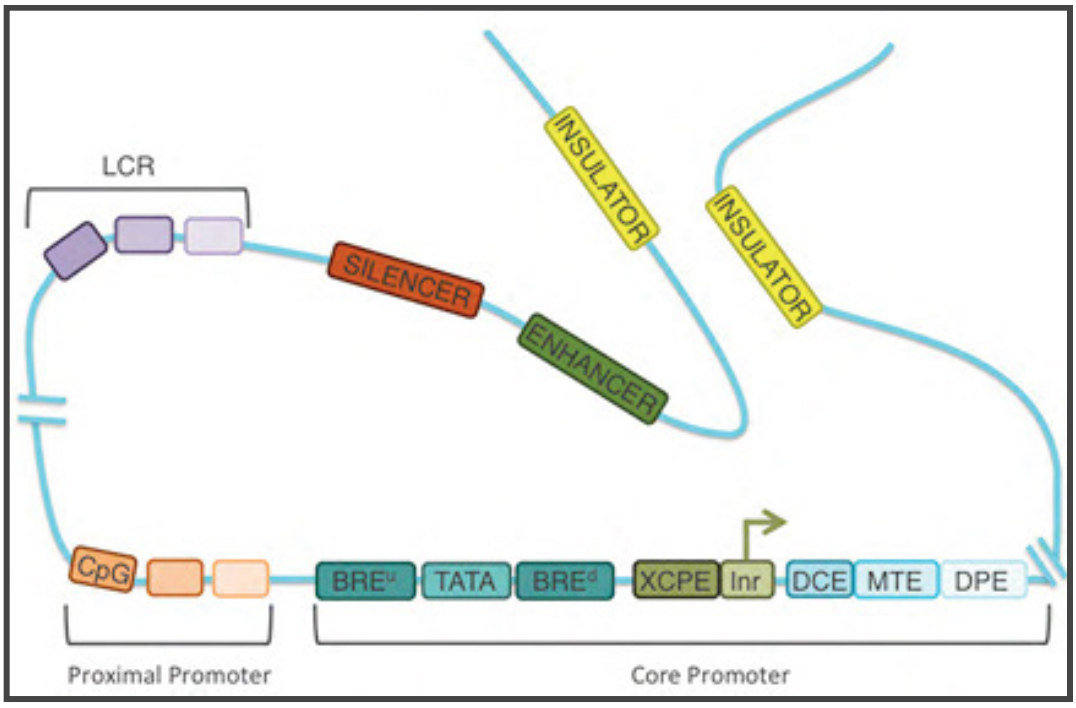

Figure 1.1. Simplified schematic of a typical eukaryotic gene regulatory region, showing common cis-regulatory acting elements.

Importantly, comprehensive analyses of the human genome reveals that there is no universally required element within promoters necessary for promoter activity [20, 21]; rather, each is present in only a subset of core promoters. In the future, it will be important to investigate functional interactions between different core promoter motifs. Along these lines, computational studies have revealed the co-occurrence of various combinations of core promoter motifs. In mammalian cells, the simple model of TATA-driven pre-initiation complex (PIC) assembly is the clear exception rather than the rule, as only an estimated 10-20% [20, 21] of mammalian promoters contain a functional TATA box. Ironically, most of the studies examining fundamental mechanisms of transcriptional regulation have been carried out using promoters that have "focused" start sites, particularly,

TATA-containing promoters. Thus, how the transcriptional machinery recognizes "dispersed" promoters and initiates transcription from multiple, individual TSSs remains poorly characterized. A number of questions remain regarding the mechanisms at play for promoters utilizing multiple start sites: (1) whether individual start sites are specifically driven by definitive core promoter elements or whether a single "loose" element can drive transcription from multiple locations, (2) how transcription from different start sites within a promoter can be differently regulated, (3) which general transcription factors (GTFs) are used for transcription from different start sites at these types of promoters, and (4) whether a stable PIC is formed for transcriptional initiation from each start site. Finally, some core promoters lack all the known core promoter motifs, leading to the question of whether there are other as yet identified sequences responsible for transcriptional activity.

PROXIMAL PROMOTERS

Whereas the core promoter elements support the formation of the large multi-protein complexes that direct RNAPII to begin transcription, proximal promoters contain the *cis*-elements that interact with sequence-specific DNA binding proteins that can alter the rate of transcription. The proximal promoter region is located immediately upstream of the core promoter, approximately 200 to 50 base pairs (bps) from the TSS. This region binds regulatory transcription factors (TFs), which have position specific interactions with the basal factors and RNAPII. Closely spaced transcription factor binding sites can facilitate protein–protein interactions, therefore clustering of protein-binding elements is often a hallmark of proximal promoter regions. Many proximal promoter binding sites are organism specific. For example, the CpG island is a proximal promoter element found in ~60% in human promoters, the MIG1 binding motif site is found in yeast, and Y-patches (pyrimidine patches) are found in the majority of plant promoters.

1.1.1.2 Enhancers

The precise and complex temporal- and tissue-specific expression pattern of genes often requires the deployment of additional *cis*-regulatory elements distal to the promoter. Enhancers were first characterized more than 30 years ago as sequences from the SV40 genome that were capable of increasing the transcription of a cloned *HBB* gene [22]. Enhancers were subsequently discovered in the eukaryotic genome and, as in the viral genome, serve to positively regulate transcription [23, 24]. Enhancers typically span a few hundred base pairs and are composed of tightly grouped clusters of transcription factor binding sites (6- to 20-bp motifs), to which combinations of cell- and lineage-specific *trans*-activating factors bind in sequence-specific manner. They can be located in intergenic regions, introns and exons, or even tens to hundreds of kilobases away from the core promoter. Additionally, these regulatory elements are capable of enhancing transcription independent of both the distance from and orientation relative to the promoter [25]. However, this spatial organization as well as the orientation of the specific *trans*-activating binding sites within an enhancer is crucial to its regulatory function, suggesting independence of distance and orientation can only be applied to the enhancer cluster as a whole.

Enhancer elements can be located some distance from the core promoter where the transcription initiation apparatus is bound. The transcription factors that bind enhancers have been shown to contact those factors which bind promoters upon activation of transcription [26, 27]. These data imply that gene expression is accompanied by the formation of "DNA-loops," which allow for direct contacts between core promoters and enhancers over long distances [28]. Evidence for DNA loop formation during transcription was first described in bacteria and bacteriophage gene expression systems [29, 30]. A number of studies in nuclear organization via Chromosome Conformation Capture (3C) and its related techniques have provided abundant evidence for the "DNA-looping" model in eukaryotic cells as well [31, 32]. The Mediator subunit Med12 and Cohesin have

been suggested to stabilize enhancer-promoter looping [33] for active genes in embryonic stem cells.

It is well established that eukaryotic gene transcription is accompanied by patterns of acetylation and methylation on nucleosomes near promoters (see Section 1.1.3), but high resolution examination of chromatin modification states elsewhere in the human genome found that enhancers are marked by monomethylation of histone H3 lysine 4 (H3K4me1) [34]. In addition to the H3K4me1 enrichment, predicted enhancers are frequently marked by acetylation of H3K27, DNaseI hypersensitivity, and many contain evolutionarily conserved sequences. Unlike promoters and insulators, but similar to p300 binding sites, the histone modification patterns at predicted enhancers are largely cell type-specific [35].

Interestingly, it was recently shown that a fraction of extragenic RNAPII transcription sites overlap with enhancer regions [36-38], and produce bidirectional, non-polyadenylated transcripts. Furthermore, the transcription of these so-called "enhancer RNAs (eRNAs)" positively correlates with the levels of messenger RNA synthesis of the surrounding protein-coding genes [37]. However, what remains unanswered is whether the eRNAs themselves have a biological activity or if RNAPII at enhancers serves to recruit chromatin modifying enzymes and thereby influence enhancer function. Alternatively, if the enhancer-promoter interaction model holds true, then eRNAs could simply be the result of transcription of the wrong DNA sequence.

1.1.1.3 Silencers

Silencers reside at the opposite functional extreme of enhancers. As the name given to these DNA elements implies, silencers serve to negatively regulate gene transcription. However, there is a considerable amount of confusion and lack of consistency with regards to the definition of transcriptional 'silencers [39].' Silencer elements are generally distance and orientation-independent elements (although there are exceptions) that can be situated as the part of a proximal

promoter, a distal enhancer, or at far distances from their target promoters as independent regulatory elements; silencer elements have been found in the introns, exons, and the 5'- and 3'-untranslated regions (UTRs) of their target genes.

Silencers serve as binding sites for repressor proteins, which negatively regulate transcription either alone or in concert with co-repressors. Repressors may cooperate in binding to silencer elements [40] and silencing elements can act synergistically [41]. A number of different models for the mechanism of gene suppression by repressors have been proposed: (1) repressors binding to a silencing element can block the binding of a nearby activator [40], or can directly compete for the same binding site [42], (2) repressors can induce a repressive chromatin environment through the recruitment of histone modifying enzymes or chromatin stabilizing factors and thereby prevent site-specific activators or the GTFs from accessing a promoter [43] or (3) the repressor may block transcription initiation by preventing the assembly of the PIC [44].

The default state of most genes is silenced, therefore a fundamental question in transcriptional regulation is how a gene switches from a silenced to an activated state. The clues for the underlying mechanism for this may be similar to that seen with eRNAs. One well-known silencing element is the Polycomb group (PcG) Response Element (PRE). Findings with PREs in *Drosophila* show that the conversion from a silenced to an activated mode depends on the presence of noncoding transcription across the PRE element [45]. Although the precise mechanism is not understood, the act of transcription is thought to induce chromatin modifications that prevent access of repressive complexes to DNA. Importantly, the PcG Repressive complex 2 (PRC2), which recognizes PREs, has been implicated in the initial steps of leukemogenesis [46].

1.1.1.4 Locus control regions

Locus control regions (LCRs) are operationally defined by their ability to strongly enhance the expression of linked genes in a tissue-specific, position-

independent and copy number-dependent manner. LCRs have been described in many mammalian genes, particularly those involved in hematopoiesis [47-51], indicating that this mode of regulation plays an important role in the control of this developmental process. Locus control regions are just arrays of *cis*-acting elements that function in concert to regulate an entire locus or gene cluster. Each of the elements is bound by a *trans*-acting protein and the integration of all resulting signals defines proper spatial-temporal gene expression. The components of an LCR commonly co-localize to sites of DNase I hypersensitivity in the chromatin of expressing cells.

The LCR was first identified in the human beta-globin locus and this now serves as the paradigm for LCR action [52-54]. While the *HBE1, HGB2*, *HGB1, HBD* and *HBB* genes are under the control of their own promoters, the proximity of each gene to the LCR dictates the timing of that gene's expression during mammalian development. Inverting the order of genes with respect to the LCR interferes with proper gene expression, resulting, for example, in embryonic expression of the adult *HBB* gene and no expression of the *HBE1* gene [55]. The globin LCR lies approximately 6 to 25 kilobases (kbs) upstream of the gene cluster so it has been proposed that this element accomplishes the long-range transcriptional control of its target genes through a DNA looping model, similar to that proposed for enhancers.

1.1.1.5 Insulators

To ensure the normal expression of a gene is not influenced by the surrounding chromatin landscape or regulatory elements, there are specialized genomic sequences that serve as barriers against influences from the neighboring DNA. These regions, called 'insulators,' partition the genome into discrete regulatory domains. Insulators have two main activities: (1) to prevent heterochromatin from encroaching into and silencing neighboring euchromatic regions of the genome or (2) to block communication between enhancers and unrelated promoters [56]. Typically, insulators are approximately 0.5-3 kb in size

and act in a position-dependent and orientation-independent manner.

The CCCTC-binding factor (CTCF) is the only major sequence-specific *trans*-acting factor identified in the establishment of vertebrate insulators [57]. An analysis of human primary fibroblasts estimated the number of CTCF binding sites in potential insulators of the human genome to be over 13 000, with a chromosomal distribution that is strongly correlated with genes [58]. Furthermore, computational and biochemical analyses of these sites defined a core 20-mer CTCF consensus motif, which subsequent studies have confirmed in different mouse, human, and chicken cells [59-63]. A large number of CTCF binding sites are highly conserved across species [64], although there is considerable nucleotide variability within the core binding motif [58] and a substantial number of sites lack the consensus motif altogether [64]. The loss of CTCF binding at a chromatin boundary has been associated with cancer development through aberrant silencing of important tumor suppressor genes [65]. These data highlight the critical role of insulator sequences in maintaining proper gene expression programs and imply that loss of insulator integrity contributes to the pathogenesis of human malignancies.

1.1.2 *Trans*-acting elements

Trans-acting factors are the proteins that bind to *cis*-elements to control gene expression. These factors are actively transported through the cell, and bind to DNA or other proteins to regulate the spatial and temporal dynamics of gene expression. RNA polymerase II is itself a *trans*-acting factor. The additional factors involved in the accurate transcription of genes by RNAPII can be classified into 3 groups: general transcription factors, promoter-specific transcription factors (activators and repressors) and co-activators/co-repressors.

1.1.2.1 RNA polymerase II

The synthesis of RNA from a DNA template, termed transcription, is a highly coordinated process mediated by RNA polymerase. The activity of the central enzyme in this process, RNA polymerase (RNAP), was discovered by

Weiss and Gladstone [66] in 1959. They showed that rat liver nuclei supported RNA synthesis in reaction mixtures containing all four rNTPS. Shortly thereafter, Audrey Stevens [67] reported the same activity in *E. coli,* demonstrating that the enzyme was present in both prokaryotes and eukaryotes. It would take another decade for Roeder and Rutter [68] to isolate three forms (I, II and III) of eukaryotic polymerase based on chromatographic separation from sea urchin embryos.

The distinct functions of the RNAPs remained elusive until Chambon's and Roeder's groups found that the specific activity of each RNA polymerase could be resolved based upon their differential sensitivities to the inhibitor α-amanitin [69-71], a drug isolated from the death cap fungus, *Amanita phalloides.* Using α-amanitin sensitivity assays, RNA polymerase II (RNAPII) was deduced to be the form responsible for transcribing mRNAs [72-74].

The C-terminal repeat domain (CTD) of RNAPII is an unusual sequence arrangement at the end of the largest RNAPII subunit that serves as a flexible binding scaffold for numerous factors. This domain is inherently unstructured, yet evolutionarily conserved among eukaryotes, and consists of tandem copies of the consensus repeat heptad $Y_1S_2P_3T_4S_5P_6S_7$ [75]. The number of repeats increases with the complexity of the organism, explanding from 26 in yeast to 52 in mammals. The CTD is essential for life: cells containing only RNAPII from which two-thirds or more of the repeats have been removed are inviable [76, 77]. While the CTD is indispensable *in vivo*, it is not required for general transcription factor-mediated initiation and RNA synthesis *in vitro* [77-81]. Thus, the CTD is dispensable for the catalytic activity of RNAPII.

Dynamic phosphorylation patterning of the CTD during gene transcription coordinates the recruitment of accessory factors to the elongating polymerase and nascent transcript. The heptapeptide consensus contains five potential phosphoacceptor amino acids (Figure 1.2, top panel), but experimental evidence suggests that the serines at positions 2, 5 and 7 (S2, S5 and S7) are the

predominant sites of phosphorylation.

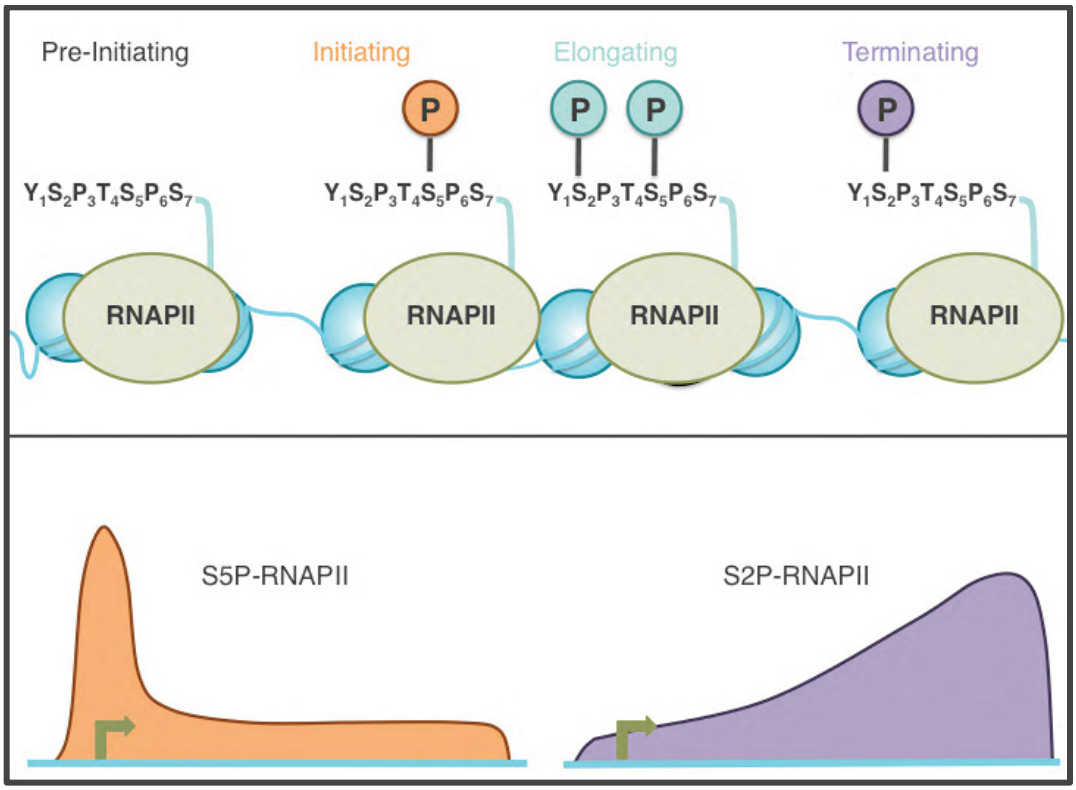

Figure 1.2. Changes in the phosphorylation pattern of the CTD of RNAPII during transcription.

In one transcription cycle there are four phosphorylated states of RNAPII (Figure 1.2, top panel). First, RNAPII with unphosphorylated CTD is recruited to a promoter and assembles with Mediator and the general transcription factors (GTFs). Second, early in the transition from pre-initiation to elongation, the CTD is phosphorylated on S5 residues. Chromatin immunoprecipitation (ChIP) profiling experiments utilizing a monoclonal antibody specific for phosphorylated S5 (S5P) yield a strong S5P signal at the 5′ ends of transcription units [82] which decreases successively towards the 3'end of genes (Figure 1.2, bottom panel). The cyclin-dependent kinase subunit (CDK7 in metazoans, KIN28 in *S. cerevisiae*) of TFIIH phosphorylates both S5 and S7 of the CTD in a Mediator dependent manner [83, 84], which leads to the dissociation of Mediator [85]. CDK8 of the

Mediator complex also phosphorylates S5, but its exact functional role within the CTD phosphorylation cycle remains unclear. First described as a transcriptional repressor, there is now evidence that in certain cellular contexts cdk8 plays a role in gene activation [86, 87]. Although it is thought that S5 phosphorylation (S5P) by CDK7/KIN28 is associated with promoter release [85, 88, 89], chemical inhibition of this kinase does not impair global mRNA synthesis [90, 91]. It is clear, however, that S5P is critical for cap formation at the 5′ end of nascent mRNAs. Third, after initiation, CTD S2 phosphorylation (S2P) rises downstream of the TSS and correlates with the generation of an elongation-proficient RNAPII. The recruitment of kinases during this step is S5P dependent. It has been proposed that this RNAPII represents a third form of phosphorylated polymerase [92], whereby the CTD contains repeats phosphorylated at both S2 and S5 positions. Finally, near the 3′ end of the gene, CTD phosphorylation is dominated by S2P residues (Figure 1.2, bottom panel); this is in agreement with the recruitment of factors and complexes involved in transcription elongation and mRNA splicing [93, 94].

All the amino acids of a heptapeptide repeat can be modified; the tyrosine, threonine and three serines can be modified by phosphorylation, the two prolines can undergo isomerization between *cis*- and *trans*-conformation, and the serine and threonine residues can also be glycosylated [95]. Additionally, 31 of the 52 mammalian CTD repeats diverge from the consensus and the arginine and lysine residues of the non-consensus repeats can be targets of methylation [96] and ubiquitination [97], respectively. These modifications generate an enormous combinatorial potential of RNAPII forms in a living cell in respect to their CTD modification pattern. Similar to what was proposed for the post-translational modification of histones, this has led to the hypothesis of a “CTD code” [82, 98, 99], where combinations of modifications orchestrate the recruitment and interaction of factors with the transcribing RNAPII. One of caveats to understanding the CTD code is that a lot of this work has been done with

antibodies raised against different phosphorylated forms of the CTD in ChIP experiments. However, these antibodies provide no information on the actual number, distribution, and protein occupancy of the individual heptad repeats.

1.1.2.2 Mediator and the general transcription factors

The transcription initiation machinery is an elaborate assembly of proteins that is over 3.5 MDa in size. Collectively, this assembly has been called the pre-initiation complex (PIC). However, due to the discovery that many RNAPII complexes may be "paused" at the promoter, even generating short transcripts of approximately ~20-45 nucleotides [100], the term pre-elongation complex (PEC) has been proposed as a more general descriptor of the factors involved in transcription initiation [101]. Five general transcription factors (GTFs) are necessary and can be sufficient for initiation of transcription by RNA polymerase II in reconstituted *in vitro* systems. These are TFIIB, TFIID, TFIE, TFIIF and TFIIH. The role of TFIIA in transcription initiation has been the subject of much controversy, because its requirement in reconstituted transcription varies from system to system. The requirement for TFIIA can be correlated with the purity of the reconstituted transcription factors, with purer systems being less dependent on TFIIA [102]. Much of this variability can be attributed to the ability of TFIIA to relieve the repressive effects of certain negative factors associated with TFIID that may be present in cruder preparations, a process called anti-repression [103-105].

In addition to these classic GTFs, it is apparent that *in vivo* transcription also requires Mediator, a highly conserved, enormous (1.2 MDa) 26-subunit complex that was originally identified by the Young [106] and Kornberg [107] laboratories as an activity required for transcriptional activation in a reconstituted system from yeast. Dylan Taatjes, while working for Robert Tijian, demonstrated that Mediator exists as not one, as was originally thought, but two different complexes [108]. The larger complex can exist in a free state or in a complex with RNA polymerase II, termed holoenzyme. The smaller complex can reversibly

associate with Mediator and contains the proteins CDK8, cyclin C, MED12, and MED13.

One model of PIC assembly proposes that the GTFs assemble on the core promoter in a stepwise fashion to form a PIC/PEC, which directs RNAPII to the TSS [109]. This conventional model for ordered transcription initiation is characterized by a distinct series of events: (1) recognition of core promoter elements by TFIID, a multi-subunit complex consisting of the TATA-box-binding protein (TBP) and 10-12 tightly bound TBP-associated factors (TAFs), (2) recognition of the TFIID-promoter complex by TFIIB, (3) recruitment of a TFIIF/RNAPII complex, and (4) binding of TFIIE and TFIIH to complete PIC formation. TFIIA can join the complex at any stage after TFIID binding and stabilizes the initiation complex. *In vitro* reconstitution systems of PIC assembly has provided a biochemical model for the sequential pathway of transcription initiation. However, more recently, cryo-electron microscopy snapshots of PIC intermediates during sequential assembly allowed researchers to study the molecular mechanism behind this process at an unprecedented small scale and track the effect of each additional factor on the PIC [110]. This regimented assembly model has been challenged, with the alternative proposal that some, if not all, components of the PIC are pre-assembled *in vivo* [111-113].

1.1.2.3 Sequence specific transcription factors

RNAPII and the GTFs alone are sufficient to drive only very low levels of accurately initiated transcription *in vitro*, a process referred to as basal transcription. A second class of factors, the sequence-specific transcription factors, is needed to stimulate robust transcription of DNA. The history of transcription factor (TF) discovery reads like a veritable Who's Who for transcription enthusiasts. The first detailed mapping of DNA sequences bound by a transcription factor was published in 1978 by Robert Tjian [114], who showed that a protein similar to the SV40 T antigen bound in a sequential manner to tandem recognition sequences. He narrowed the binding site to a region

containing palindromic sequences, a motif now known to be characteristic of many other transcription-factor-binding sites, and he postulated that the protein bound as a multimer. Three years later, Keith Yamamoto and colleagues reported that the glucocorticoid receptor selectively recognizes and binds a 4.5-kb fragment of the mammary tumour virus genome, which they had shown previously to mediate hormone-responsive transcription [115]. Shortly thereafter, Dynan and Tjian isolated the transcriptional activator Sp1 from HeLa cells and showed that it was a promoter-specific factor and located its binding sites upstream of the transcription-initiation site [116].

Over the next few years, Brent and Ptashne and Hope and Struhl demonstrated the modular nature of transcriptional regulators. Virtually all endogenous regulators contain two essential modules, a DNA-binding domain (DBD) and a regulatory domain. Through simple domain swapping experiments, these groups showed that these functional domains could be interchanged [117, 118]. In 1979, the discovery of p53 by the labs of Pierre May, Arnold Levine and Lionel Crawford [119-121] was reported. An exhaustive amount of basic and translational research has been devoted over thirty years to the sequence-specific transcription factor p53. *TP53* (the human p53 gene) is one of the most commonly mutated tumor suppressor genes in human cancer, and as the "guardian of the genome," functions as a node in numerous essential signaling pathways.

The DBD of TFs have a much higher affinity for their target sequences than for other DNA sequences [122, 123]. Transcription factors can be distinguished by the three-dimensional structure of their DNA-binding domain and include the C2H2 zinc finger family (the largest family—[124]), leucine zippers, and helix-loop-helix proteins. These different structural motifs determine the specificity of association with consensus DNA sequences. The activation domain works by (1) increasing PIC formation through a mechanism thought to involve direct interactions with one or more components of the transcription machinery, (2) by promoting a step in the transcription process subsequent to PIC

assembly, such as initiation, elongation or re-initiation or (3) by recruiting activities that modify chromatin structure. Some of the sequence specific transcription factors require the recruitment of co-repressors or co-activators via protein-protein interaction to assist in in the performance of their functions.

Most importantly, unlike prokaryotes, which often use single proteins for transcriptional regulation of a gene, eukaryotic gene expression regulation involves the coordination of many proteins. This combinatorial mechanism integrates diverse signals into the expression of a large number of genes in a complex spatio-temporal pattern by a comparatively low number of protein regulators. Furthermore, a notable property of activators is that they can stimulate transcription synergistically, so that the total of their regulatory activity is greater than the sum of all their individual effects on transcription [125, 126]. This phenomenon has been recently recapitulated with engineered transcription activator-like effector transcription factors (TALE-TFs), which provide a powerful experimental system for better understanding complex gene regulation in mammalian systems [127].

1.1.2.4 Co-activators and Co-repressors

While transcription factors bind directly to DNA, they often work in concert with co-activators or co-repressors. These accessory proteins do not bind DNA directly and instead recognize protein motifs. They exist in multi-protein complexes that dock onto transcription factors and histones, allowing transcription to take place.

Co-activators function in a variety of ways and either contain or recruit the enzymatic activities that alter the chromatin landscape so that it changes from a quiescent state to one permitting active gene transcription. Generally, co-activators can be functionally divided into five classes. One class of proteins covalently modifies histones in ways that opens up the chromatin to grant access to other proteins to the DNA. For example, CBP and p300 are ubiquitous histone acetyltransferases (HATs) that interact with a wide variety of transcription factors

and other proteins [128]. The members of the Mediator complex are considered a second class of co-activators. They form a bridge between transcription factors, the GTFs and RNA polymerase II (see Section 1.1.2.2). Third, protein complexes containing the mammalian BRM or BRG1 (or their yeast homologs SWI/SNF) contain ATP-dependent chromatin remodeling activities that alter the higher order structure and position of nucleosomes. Fourth, a group of co-regulators catalyze post-translational modifications on sequence-specific TFs and components of the general transcription machinery. Lastly, there are co-activators with as yet unknown function.

Not surprisingly, co-repressors have the opposite effects on chromatin structure, essentially making it inaccessible to the binding of transcription factors or resistant to their actions. These proteins (such as NCoR) are often associated with histone deacetylase (HDAC) activity, though other mechanisms for gene silencing clearly exist [128].

1.1.3 Epigenetic mechanisms

Epigenetic regulation refers to stable changes in gene function that occur without alteration of the primary DNA sequence [129]. There are four molecular mechanisms of epigenetic cellular memory at play in mammalian cells. Three of these, DNA methylation, histone modifications, and nucleosome positioning, result in the remodeling of chromatin and therefore regulate transcription. In contrast, RNA interference (RNAi) is a post-transcriptional mechanism and therefore will not be discussed here further (for an excellent review on this topic, see [130]).

"Epigenetics," a term coined by Conrad Hal Waddington during World War II, has evolved from an obscure field of research in plants to having a pivotal role in human development and disease. The importance of the epigenetic landscape in these processes is highlighted by the observation that disruption of the epigenetic machinery gives rise to all typical cancer characteristics, implicating alterations in the epigenome in cancer initiation and progression.

More specifically, anomalous epigenetic changes occur frequently in acute leukemia. The recurring chromosomal translocations seen in Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL) result in the generation of chimeric fusion proteins, and several of these fusion oncoproteins contribute to the development of leukemia partly by disrupting the modification of chromatin, through recruitment of chromatin-modifying coregulators [131]. The emerging link between the enzymes that modify chromatin and the transcription factors that control leukemic cell development and function strongly suggests a prominent role for epigenetic regulation in establishing and maintaining leukemic gene expression programs.

Unlike chromosomal deletions that lead to an irreversible loss of function, gene silencing by epigenetic mechanisms is reversible. This makes "epimutations" attractive drug targets with the goal of restoring the normal epigenetic landscape by pharmacologically inhibiting enzymes of the epigenetic machinery.

1.1.3.1 DNA methylation

Methylation of DNA is catalyzed by DNA methyltransferases (DNMTs), which use S-adenosyl-methionine as a methyl group donor. DNA is methylated on a cytosine base that precedes a guaunosine (referred to as a CpG dinucleotide) [132]. The distribution of CpG dinucleotides in the human genome is not uniform, such that there are regions of DNA enriched for CpGs, known as CpG islands. These islands are located within the promoter regions of approximately 60% of the genes in the human genome. In normal cells, the CpG island cytosines are unmethylated, in contrast to most CpG sites outside of CpG islands, which are methylated. This methylation may help maintain non-coding DNA in a transcriptionally inert state, whereas the unmethylated state of the CpG islands in gene promoters permits active gene transcription. However, the DNA methylation patterns in human cancer cells are considerably perturbed. It was established 30 years ago that cancer cells are globally DNA hypomethylated, and

there is a significant decrease in the genomic methylcytosine content of malignant cells when compared to normal tissues [133, 134]. This hypomethylation of CpGs in the bulk of the cancer-cell genome has the potential to contribute to carcinogenesis by several mechanisms including: fostering chromosome instability; predisposing cells to aberrant chromosome recombination; causing re-expression of growth-promoting imprinted genes; upregulating the expression of retrotransposons (small DNA elements that move from place to place in the genome via an RNA intermediate), endogenous retroviruses and proto-oncogenes; and, interfering with the normal downregulation of certain genes indirectly through hypomethylation of centromeric DNA [135].

Table 1.1. Genes silenced due to hypermethylation in acute leukemia.

Gene	Protein	Type of Leukemia	Protein Function
CALCA	Calcitonin	ALL	Peptide hormone known to participate in calcium and phosphorus metabolism
CDH1	E-Cadherin	ALL/AML	Calcium-dependent cell-cell adhesion protein
CDKN1C	p57(KIP2)	ALL	Cyclin-dependent kinase inhibitor
DAPK	Death-associated protein kinase	ALL/AML	Pro-apoptotic Serine/Threonine kinase
ER	Estrogen Receptor	ALL/AML	DNA binding transcription factor that regulates estrogen-induced gene transcription
HIC1	Hypermethylated in cancer 1 protein	AML	Transcriptional repressor
PTPN6	Protein tyrosine phosphatase, non-receptor type 6 (previously known as SHP1)	ALL/AML	Tyrosine phosphatase
WT1	Wilms' tumor protein	AML	Zinc finger transcription factor
$p15^{INK4b}$	p15	AML	Cyclin-dependent kinase inhibitor
p73	p73	ALL	G1-S cell cycle control

At the same time, cancers present with gains in methylation specifically in promoter-associated CpG islands. CpG island hypermethylation and subsequent

transcriptional inactivity represents an important mechanism in the pathogenesis of neoplasms. This means of tumor suppressor silencing has been shown to occur in both ALL and AML. Genes that have been frequently reported to be hypermethylated in these hematopoietic malignancies are listed in Table 1.1. Aberrant CpG island methylation in these genes leads to transcriptional shut-down and involves the recruitment of methyl-binding proteins and histonedeacetylases (HDACs) to regions near transcription start sites [136, 137].

In both in the laboratory and the clinic, the cytidine analogs 5-aza-2'-deoxycytidine (decitabine) and 5-azacytidine are capable of reactivating tumor suppressor genes silenced by promoter hypermethylation [138]. Incorporation of these agents into DNA leads to subsequent DNA demethylation via their irreversible inhibition of DNMTs. Both agents have proven clinical activity in myelodysplastic syndromes (MDS) [139] and are in trials in acute leukemia.

1.1.3.2 Histone modifications

Histones are proteins that previously had the boring reputation as being the scaffold around which DNA is wrapped in order to package very long DNA strands into the nucleus. They are globular proteins with a flexible N-terminus (ie the tail) that protrudes from the nucleosome [140]. The nucleosome is a fundamental repeating unit of eukaryotic chromatin consists of a nucleosome core particle (147 base pairs of DNA wrapped around a histone octamer containing 2 H2A-H2B dimers and 1 H3-H4 tetramer) plus the region of linker DNA that stretches between nucleosomes. However, histones are now realized to have a more exciting role as important regulators of gene expression. Histone tails, and to a lesser extent, the histone core, undergo a wide spectrum of post-translational modifications, including phosphorylation, acetylation, methylation, ubiquitination, SUMOylation and ADP-ribosylation [141]. The combination of these modifications in a specific genomic region is hypothesized to function as a cipher for chromatin-DNA interactions, forming what is loosely referred to as the, “Histone Code.” Genome-wide studies have revealed that this code leads to a

more “open” or “closed” state of chromatin structure and therefore, to the activation or repression of gene expression.

The best-characterized histone modification is acetylation. Enzymes with histone acetyl transferase (HAT) activity add acetyl groups to the lysine (K) residues of histones, while the acetyl groups are removed by enzymes with histone deacetylase activity (HDACs and sirtuins) [142]. It is proposed that histone acetylation results in a weakened histone-DNA interaction via neutralization of the positive charge of lysine and subsequently provides greater access by transcription factors to chromatin. The acetylation of histone tails reflects a delicate balance between the activities of HATs and HDACs. Slight perturbations in this balance can largely influence gene expression, and restoration of this equilibrium represents a therapeutic strategy in acute leukemia. Aberrant activity of HATs and HDACs, resulting in deregulated gene transcription is a feature of many cancers, including many hematologic malignancies [143]. Treatment with histone deacetylase inhibitors (HDACis) affects the expression of 2-10% of a selective subset of genes, with the ratio of upregulated to downregulated being close to 1:1[144]. Noticeably, normal cells are almost always considerably more resistant than leukemic cells to the effects of HDACis, indicating the pivotal role epigenetic modulation plays in controlling leukemogenesis.

Like histone acetylation, the phosphorylation of histones is highly dynamic. The levels of the modification are controlled by kinases and phosphatases that add and remove the modification, respectively. Phosphate groups are added to the hydroxyl group of serines, threonines and tyrosines and this modification results in the addition of a significant negative charge to the histone, which ultimately affects chromatin structure. Histones are known to be mono- and poly-ADP ribosylated on glutamate and arginine residues but the function of this modification remains unelucidated. Poly-ADP-ribosylation of histones is performed by the poly-ADP-ribose polymerase (PARP) family of

enzymes and reversed by the poly-ADP-ribose-glycohydrolase (PARG) family of enzymes. These enzymes function together to control the levels of poly-ADP ribosylated histones that have been correlated with a relatively relaxed chromatin state. Presumably, this is a consequence, at least in part, of the negative charge that the modification confers to the histone.

Histone methylation mainly occurs on the side chains of lysines and arginines. Unlike the modifications previously discussed, histone methylation does not alter the charge of the histone protein. Furthermore, there is an added level of complexity as lysines may be mono-, di- or tri-methylated, whereas arginines may be mono-, symmetrically or asymmetrically di-methylated.

Acetylation, phosphorylation, poly-ADP-ribosylation and methylation of histone proteins result in relatively small molecular changes to amino-acid side chains. In contrast, ubiquitylation results in a much larger covalent modification. Ubiquitin is a 76-amino acid polypeptide covalently attached to histone lysines via the sequential action of three enzymes, E1-activating, E2-conjugating and E3-ligating. The enzyme complexes determine both substrate specificity (i.e., which lysine is targeted) as well as the degree of ubiquitylation (i.e., either mono- or poly-ubiquitylated). For histones, mono-ubiquitylation seems most relevant. Two well-characterized sites lie within H2A and H2B. H2AK119ub is involved in gene silencing [145, 146], whereas H2BK120ub (H2BK123 in yeast) plays an important role in transcriptional initiation and elongation [147-149]. Similar to ubiquitylation, SUMOylation involves the covalent attachment of small ubiquitin-like modifier (SUMO) molecules to histone lysines via the action of E1, E2 and E3 enzymes. SUMOylation has been detected on all four core histones [150, 151] and seems to function by antagonizing ubiquitylation and acetylation that might otherwise occur on the same lysine side chain. Consequently, it has mainly been associated with repressive functions, but more work is clearly needed to elucidate the molecular mechanism(s) through which SUMOylation exerts its effect on chromatin. Even though ubiquitylation and SUMOylation are large modifications,

they are still highly dynamic. These modifications are removed via the action of de-ubiquitinating and de-SUMOylating enzymes and these activities are important for both gene activity and silencing.

Histone modifications exert their effects via two main mechanisms. The first involves the modification(s) directly influencing the overall structure of chromatin, either over short or long distances. The second involves the modification regulating (either positively or negatively) the binding of effector molecules. Through these two mechanisms, histone modifications ultimately affect gene expression. There are some very general correlations that can be made between a specific mark and the transcriptional status of a gene. For instance, tri-methylation of lysines 4, 36 or 79 on H3 (H3K4me3, H3K36me3 and H3K79me3, respectively), mono-methylation of H4K20 and H2BK5 (H4K20me and H2BK5me), and acetylation of H3K9 and H3K14 (H3K9ac and H3K14ac) correlate with gene activation, whereas di- or tri-methylation of H3K9 (H3K9me2 and H3K9me3) and tri-methylation of H3K27 (H3K27me3) correlate with gene repression [34, 152-155]. Interestingly, in embryonic stem (ES) cells, key developmental genes remain poised for lineage-specific activation or repression as a result of bivalent modifications. H3K4me3 and H3K27me3 are typically opposite in functional meaning, but yet exist concurrently within certain domains [156, 157]. It is important to remember that histone modifications are just as relevant in the regulation of other DNA processes such as repair, replication and recombination.

An extra level of complexity must be considered due to the combinatorial action of the different post-translational modifications. There are multiple outcomes of this cross-talk, which has been hypothesized to fine-tune the final readout [158]. (1) Lysine residues can be acetylated, methylated ubiquitylated and SUMOylated. Therefore, there may be competitive antagonism between modifications if more than one modification pathway is targeting the same lysine. For instance, as stated above, H3K9 can be acetylated in an activating context, or

methylated, which is generally associated with repression. (2) One modification may be dependent upon another. A good example of this is the ubiquitylation of H2BK120 by Rad6/Bre1which directly stimulates di- and tri-methylation of H3K4 by SET [159]. (3) The binding of a protein to a particular modification can be disrupted by an adjacent modification. For example, during the M phase of the cell cycle, H3S10 phosphorylation is sufficient to eject HP1 proteins from their H3K9me3 binding sites [160]. This action has been described as a 'phospho/methyl switch'. This has also been observed with H3K4me3 binding of TFIID, which is decreased by phosphorylation of the adjacent threonine residue (H3T3), and coincides with mitotic inhibition of transcription [161]. Furthermore, the modified amino acids do not have to be directly adjacent to each other. Tony Kouzarides' group demonstrated that H3K4 acetylation reduces Chp1/Clr4 affinity to H3K9me [162]. (4) An enzyme's activity may be affected due to modification of its substrate. (5) Different modifications may also co-operate to recruit specific factors. For example, both TFIID and BPTF bind more strongly to the H3K4me3 mark when it is flanked by acetylation on H3K9 and H3K14 acetylation [163, 164]. Finally, there may also be cross-talk between histone modifications and DNA methylation. This may be cooperative, as in the case of UHRF1 recruitment, or inhibitory, as seen with the lysine demethylase, KDM2A [165].

1.1.3.3 Nucleosome positioning and remodeling

Nucleosomes, unlike transcription factors with DNA binding domains, do not bind exclusively to a specific DNA sequence; therefore nucleosomes were initially believed to provide a universal, nonspecific packaging of genomic DNA. However, it is now accepted that nucleosome distribution across the genome is far from random and that they occupy favored positions. There are two different, but related, terms when discussing nucleosomes. 'Nucleosome positioning' is the stretch of DNA sequence that is wrapped around the nucleosome and can be specified by the genomic location of the nucleosome center. Nucleosome

positioning is a dynamic process, but sequencing-based mapping approaches identify the positions of individual nucleosomes in a single cell at a specific time. 'Nucleosome occupancy', is the percent of the population bound by a nucleosome at a given position.

In living cells nucleosome organization is determined by multiple factors, including the action of chromatin remodelers, competition with sequence-specific DNA-binding proteins, and the DNA sequence preferences of the nucleosomes themselves. Although nucleosomes do not bind to a canonical DNA motif, studies by Kaplan *et al.* [166] and others have suggested that A/T-rich sequences are characteristic of nucleosome depleted regions (NDRs), whereas G/C-rich sequences are enriched for nucleosomes [166, 167].

Highly accessible regions in genomes are identified by preferential restriction endonuclease cleavage, DNase I hypersensitivity and MNase digestion analyses. When combined with next-gen sequencing, these techniques provide a high resolution, genome wide nucleosome map. Such analyses have revealed a distinct contrast between nucleosome density in transcriptionally functional regions (i.e. promoters, enhancers and terminators) and that in transcribed sequences. For example, in *S. cerevisiae*, >90% of the promoters contain stretches of DNA with very low nucleosome occupancy [168]. These nucleosome-depleted regions (NDRs) are on average ~150 bp in length, roughly enough to accommodate a single nucleosome. Nucleosomes surrounding these NDRs vary from the traditional nucleosome core particle, both in terms of composition and stability [169, 170]. Most importantly, when the genome-wide nucleosome density map is aligned with the TSSs of individual genes, nucleosomes in the vicinity of TSSs tend to be located at specific positions. In particular, the −1 and +1 nucleosomes flanking the promoter are located at highly defined positions [168].

Nucleosome occupancy and positioning are important chromatin features critical to transcriptional, and by extension biological, outcomes. In particular,

nucleosomes affect transcription by modulating the accessibility of DNA-binding proteins such as regulatory factors and the transcriptional machinery to DNA. Many functional activator binding sites are located in NDRs. Other factors can access their nucleosomal binding sites, but with a lower binding affinity. Finally, some factors, such as NF-κB p50, can apparently bind to nucleosomal DNA with the same association constant as free DNA by accommodating the bent DNA within the nucleosome [171]. The assembly of RNAPII and PIC on promoters of expressed genes is even more firmly inversely correlated with nucleosome occupancy [172, 173] than activators. In the human genome, nucleosome occupancy immediately upstream of TSSs is decreased in an RNAPII-dependent manner. The transcription initiation complex is bulky and some of its components (such as TBP) severely bend DNA, which could explain why it is incompatible with the nucleosome structure. Indeed, TBP to the TATA sequence was shown to be severely inhibited when this sequence is buried.

In vitro nucleosome assembly experiments with purified histones and naked DNA can recapitulate some, but not all, of the aspects of *in vivo* nucleosome patterning. In particular, nucleosome depletion at promoters and terminators can be reconstituted but the strong positioning of the +1 nucleosome is not observed [174]. These observations argue against the genomic code as being the primary determinant of the position of the +1 nucleosome. However, proper reconstitution of *in vivo* nucleosome positioning pattern can be achieved if a yeast crude extract and ATP are added to purified histones and DNA [175]. Thus, nucleosome positioning, particularly at the 5' end of genes, appears to be driven by ATP-dependent activities. Mammalian SWI/SNF (also called BAF), a complex conserved from yeast to humans, uses the energy of ATP hydrolysis to reposition nucleosomes. In humans the complex contains either of two mutually-exclusive ATPase enzymatic subunits, Brahma (BRM) or Brahma-related gene 1 (BRG1). BRM/BRG1 act as the core catalytic subunit of BAF and facilitate gene activation or repression by displacing nucleosomes. Both are able to participate in

a largely interchangeable manner in chromatin remodeling *in vitro* [176]. These proteins also have important independent roles in the maintenance of embryonic stem cell pluripotency and self-renewal. In proliferating ES cells, the BAF complex is mainly composed of BRG1 and BAF155, however during differentiation, BRM is recruited to replace BRG1 in the BAF complex [177]. More relevant to the work presented herein, SWI/SNF activity, and more specifically, BRG1 activity is required for myeloid differentiation. Expression of an ATPase-deficient form of BRG1 compromises myeloid differentiation and results in a maturational arrest at the promyelocytic/metamyelocytic stage.

1.1.4 3D chromosomal organization

As described in previous sections, the interplay between *cis*-regulatory elements and *trans*-acting factors play a fundamental role in regulating gene expression. Recently, the three dimensional (3D) spatial organization and compartimentalization of the genome within the cell nucleus has been recognized as a higher order of transcriptional regulation. This realization has been fueled by the advent of high-resolution genome-wide maps of histone marks, DNA binding proteins and physical interactions between genomic regions.

1.1.4.1 Chromosome territories and chromatin interactions

Interphase chromosomes are not positioned randomly in the nucleus, but occupy spatially distinct regions within the nuclear architecture, called chromosome territories. The term chromosome territory was first introduced by the German cytologist Theodor Boveri in his elegant studies of blastomere stages of the horse roundworm in 1909 [178]. However, this theory fell out of favor during the mid-twentieth century, mainly because electron microscopic evidence argued for an unraveling of chromosomes in interphase nuclei into intermingling chromatin fibers of 10–30 nm in diameter with no sign of individual chromosomes [179]. It was really the development of three-dimensional (3D) fluorescence *in situ* hybridization (FISH) in combination with light optical serial sectioning of nuclei by laser confocal microscopy and 3D image reconstruction

that was responsible for the resurgence in popularity of this theory. Using these techniques, experiments have demonstrated that each interphase chromosome occupies a distinct territory inside the cell nucleus.

The development of chromosome conformation capture (3C) in combination with high-throughput genome sequencing (Hi-C) [180] allows for the cataloging of the interactions of the whole genome. Data sets via this method have confirmed the existence of chromosome territories, that small gene-rich chromosomes preferentially interact with one another and that the "open" and "closed" chromatin domains throughout the genome occupy different spatial compartments in the nucleus [180]. Even the arms of a chromosome have their own territories. Chromosome arm painting with confocal imaging and 3D reconstruction of the arms of chromosomes 3 and 6 in human lymphocytes showed that the chromosome arms maintained distinct subdomains within the chromosome territory [181]. Additionally, in *Drosophila*, genes that are bound by the Polycomb group proteins (PcG) might be far apart on the chromosome, but meet frequently in the nuclear space. However, these interactions are confinced to the PcG target genes within the same arm of the same chromosome [182]. Generally, heterochromatin and repressed regions mainly localize close to the nuclear periphery, whereas the euchromatin and active regions accumulate at the inner part of the nucleus. This pattern is conserved through evolution from unicellular to multicellular organisms.

1.1.4.2 Transcription factories

There is considerable evidence that transcription does not occur diffusely throughout the nucleus, but rather is concentrated at a number of specialized, discrete sites. The term transcription "factory" was first used in 1993 by Jackson and colleagues [183]. Using confocal microscopy to visualize labeled nascent mRNA transcripts, they found that transcription primarily occurred at 300–500 discrete sites in HeLa cells, rather than being homogeneously distributed throughout the nucleus.

Estimation of the number of transcription factories in a cell nucleus depends upon the species, the cell type and importantly, the experimental method. The majority of data would suggest that the number of factories varies from a few hundred [183, 184] to a few thousand per cell [185, 186]. Estimates of the number of RNAPII molecules per factory range from 4 to 30, and factories are associated with many other molecules involved in transcriptional activation and mRNA processing [186]. There is conflicting evidence about whether transcription factories assemble *de novo* in response to transcriptional demands, or whether they are stable structures whose number in a cell nucleus remains relatively constant. Treatment with either 5,6-dichlorobenzimidazole 1-β-d-ribofuranoside (DRB) to inhibit elongation [187] or heat shock to globally turn off transcription [188] did not alter the number of factories per cell, and RNA polymerase II was still distributed in localized foci [189]. These data suggest that transcription factories exist in the absence of transcription and that they remain unchanged when transcription is interrupted. However, in contrast to the preceding observations, results obtained from live cell studies using GFP-tagged RNA polymerase II have suggested that transcription factories are dynamic and so, in principle, could assemble or disassemble on demand [190, 191].

A simple calculation yielding a low ratio of transcription factories to active genes suggests that genes are likely to share factories. Compelling proof for this possibility has come from a study by Osborne *et. al.* [192] whereby the authors used FISH and 3C assays to show that transcriptionally active genes located megabases apart, either on the same chromosome or on different chromosomes, were frequently spatially associated. Furthermore, there is evidence that related active genes are dynamically organized into shared nuclear sub-compartments. Episomal constructs driven by various promoters resulted in clustering of episomes at a subset of factories dependent on the similarities of their promoters and gene structure; episomes with identical promoters tended to cluster at the same factory, episomes with introns tended to be transcribed at one

set of factories, and episomes without introns tended to be transcribed at another set of factories [193]. This suggests that different types of templates are transcribed in separate factories that specialize in transcribing a certain type of gene or genes.

An intriguing question related to the concept of transcription factories is whether the polymerase molecules within a factory remain stationary to the transcribed DNA, with the DNA then reeled through the factory site. This is in opposition to the conventional view, whereby the comparatively small RNAPII travels down the giant DNA polymer. *In vitro* experiments under artificial conditions (i.e. where the template is immobilized) show RNAPII sliding along DNA [194, 195]. However, indirect evidence of a stationary polymerase model comes from numerous studies involving Br-UTP incorporation [186], 3C analysis [196], and immunofluorescence [197]. Additionally, if the polymerase is indeed stationary, it must be tethered to something. Several lines of evidence suggest that the polymerase could be associated with the nuclear matrix. For example, a proteomic analysis focusing on large fragments of transcriptional factories revealed some structural components, including spectrin, lamins and actin [198] could be involved in securing transcription factories.

In addition to increased transcription efficiency, another potential functional consequence of gene–gene associations at transcription factories could be to inadvertently facilitate the gene translocation events that are frequently seen in hematological malignancies. For example, *Myc* and *Igh* are the most common translocation partners in Burkitt lymphoma, and these two loci frequently co-localize [199] in B cells, at least in part because they share the same transcription factory [200]. A similar argument has been made for translocation events between the mixed lineage leukemia locus (MLL) and the *AF4* and *AF9* genes [201]. Cowell *et. al.* [201] found that *AF4* and *AF9* were more frequently associated with MLL in the same transcription factory compared to several other genes that did not show high translocation frequencies with MLL.

1.2 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) defines a heterogeneous group of malignancies characterized by a rapid increase and accumulation of immature hematopoietic cells (called myeloblasts). The resultant crowding of the bone marrow hinders production of healthy blood cells. As well, the malignant cells spill over into the bloodstream and can spread to other organs of the body. AML is usually characterized by unchecked growth of the malignant cells and early death, if left untreated.

1.2.1 Classification

The diversity of AMLs in terms of phenotype and prognostic factors necessitates a classification system to help select the best therapeutic approach. The French-American-British (FAB) cooperative group initially proposed a classification of AML based on the maturation status of the blast cells in 1976 [202]. The original proposal was revised and expanded in 1985 (Table 1.2) and divides AML into 8 subtypes (M0 to M7) [203]. The 2001 and later, the 2008, World Health Organization (WHO) classification was proposed in an attempt to incorporate new genetic information with the morphologic, cytochemical, immunophenotypic, and clinical information in order to define more clinically relevant disease entities and provide clinicians with a diagnostic algorithm for myeloid neoplasms (Table 1.3) [204].

Table 1.2. French American British (FAB) classification of acute myeloid leukemia.

M0	**AML with no Romanowsky or cytochemical evidence of differentiation**
M1	**Myeloblastic leukemia with little maturation**
M2	**Myeloblastic leukemia with maturation**
M3	**Acute promyelocytic leukemia (APL)**
M3h APL	hypergranular variant
M3v APL	microgranular variant
M4	**Acute myelomonocytic leukemia (AMML)**
M4eo	AMML with dysplastic marrow eosinophils
M5	**Acute monoblastic leukemia (AMoL)**
M5a AMoL	poorly differentiated
M5b AMoL	differentiated
M6	**Erythroleukemia**
M6a	AML with erythroid dysplasia
M6b	Erythroleukemia
M7	**Acute megakaryoblastic leukemia (AMkL)**

Table 1.3. World Health Organization (WHO) classification of acute myeloid leukemia.

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); RUNX1/RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB/MYH11
APL with t(15;17)(q22;q12); PML/RARA
AML with t(9;11)(p22;q23); MLLT3/MLL
AML with t(6;9)(p23;q34); DEK/NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1/EVI1
Provisional entity: AML with mutated NPM1
Provisional entity: AML with CEBPA
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia (Pure erythroid leukemia; Erythroleukemia, erythroid/myeloid)
Acute megakaryoblastic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm
Acute leukemias of ambiguous lineage
Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); BCR/ABL
Mixed phenotype acute leukemia with t(v;11q23); MLL rearranged
Mixed phenotype leukemia, B-myeloid, NOS
Mixed phenotype acute leukemia, T-myeloid, NOS
Provisional entity: Natural killer (NK) cell lymphoblastic leukemia/ lymphoma

1.2.2 Epidemiology and clinical features

An estimated 1 in 254 men and women will be diagnosed with AML during their lifetime, and while this makes AML a relatively rare disease, the overall five-year relative survival for 2003-2009 is only 24.2%. In adults, the median age at diagnosis is approximately 65 years and the male:female incidence ratio is approximately 5:3. This incidence is similar among persons of different races [205].

Patients with AML generally present with vague and non-specific symptoms related to complications of pancytopenia (e.g. anemia, neutropenia, and thrombocytopenia), including weakness and easy fatigability, infections of variable severity, and/or hemorrhagic findings such as gingival bleeding, bruising, or nose-bleeds. The median leukocyte count at diagnosis is approximately 15 x 10^9/L (15,000 cells/microL). The vast majority of patients will have circulating myeloblasts that can be detected on a peripheral smear.

Myeloblasts are immature cells with large nuclei, and relatively little cytoplasm. Myeloblasts are difficult to distinguish visually from lymphoblasts, however the non-lymphoid lineage of AML blasts can be identified by any of the following [206-210]:

(1) The presence of an Auer rod on microscopy. Auer rods are abnormal azurophilic crystalline-like granules that represent the coalescence of primary lysosomal granules of myeloid-precursors.

(2) Cytochemical stains demonstrating positivity for Sudan black B, myeloperoxidase, chloroacetate esterase, nonspecific esterase or Periodic Acid-Schiff (PAS) activites.

(3) Immunochemistry identifying the expression of myeloid antigens such as cd11b, cd33 and cd117.

(4) Specific cytogenetic abnormalities that are seen only in myeloid leukemias.

Bone marrow biopsy and aspiration are also key components in the

diagnosis of AML. The bone marrow is usually hypercellular due to a partial or almost total replacement of the normal cellular components of the marrow by immature or undifferentiated cells. Finally, evolving techniques for the diagnosis and classification of this malignancy include proteomics and gene expression profiling (GEP). GEP in particular has shown great promise for AML [211-214]. Expression of regulatory microRNAs (miRNA) is also being increasingly recognized as an important prognostic tool [215, 216].

1.2.3 Molecular pathology

Leukemia is, in essence, a clonal disorder of deranged and disordered hematopoiesis that results from the acquisition of mutations in hematopoietic progenitors that confer a proliferative and/or survival advantage, and impair hematopoietic differentiation. A variety of factors contribute to the development of acute leukemia. These factors disrupt the delicate balance between self-renewal and differentiation that is characteristic of normal hematopoiesis and lead to the expansion of leukemic precursor cells. Kelly and Gilliland [217] have proposed a model for the development of acute myeloid leukemia, which is also applicable to the development of acute lymphoblastic leukemia (ALL), whereby they hypothesize that these diseases emerge as a consequence of an association between at least two broad classes of mutations (Figure 1.3). Class I, or activating mutations, typically result in the aberrant activation of signal transduction pathways and provide a proliferative and/or survival advantage to hematopoietic progenitors. Class II mutations arrest differentiation as a consequence of loss of function mutations in transcription factors or cofactors that are important for normal hematopoietic differentiation. Class II mutations include fusion proteins resulting from the balanced chromosomal translocations that are often found in hematopoietic malignancies. These chimeric oncoproteins commonly involve transcription factors such as RUNX1 (previously known as AML1) and retinoic acid receptor alpha (RARA), and repress genes implicated in myeloid and lymphoid differentiation.

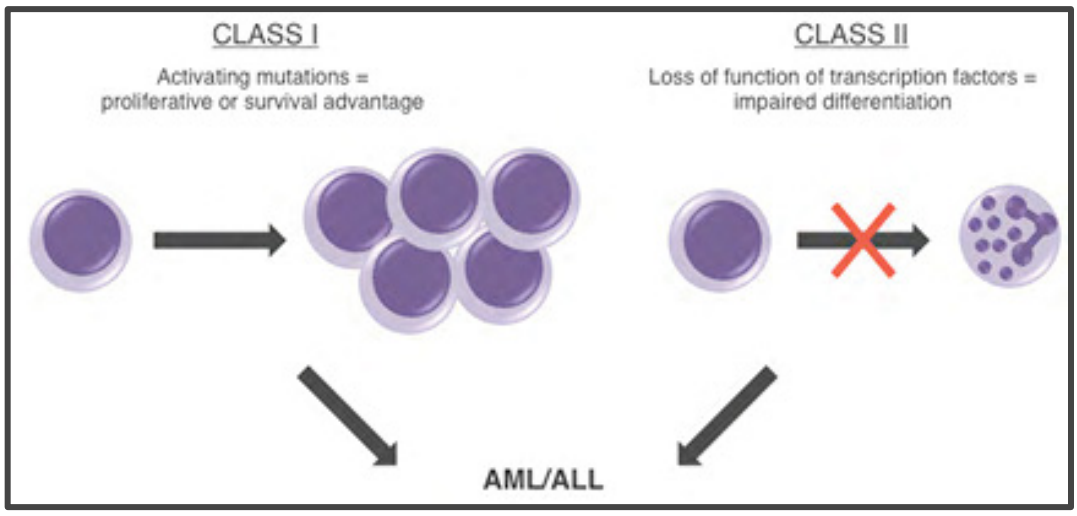

Figure 1.3. The two hit model of acute leukemogenesis. This model hypothesizes that AML and ALL are the consequence of a combination of at least two broad classes of mutations. Class I mutations involve an activating lesion in signaling pathways and confer a proliferative and/or survival advantage to hematopoietic cells. Class II mutations lead to an arrest of lymphoid or myeloid differentiation as a result of loss of function of transcription factors or cofactors that are important for normal hematopoietic differentiation.

The disruptions in hematopoiesis-specific transcription factor (TF) function (Class II mutations) can be subdivided into three different groups based on the normal role of the TF:

(1) Lineage-specific TFs, which play pivotal roles in the differentiation process of a specific lineage such as CEBPA, PU.1, GATA-1 and RARA.

(2) TFs that are expressed in multiple lineages and which function as transcriptional organizers such as the core binding factor (CBF) family and the C2H2 zinc-finger, EVI-1.

(3) TFs that mediate global changes in transcriptional control during tissue development such as the HOX genes.

Despite the hypothesis that transcription factors are the gatekeepers of AML, cumulative evidence from different murine models of leukemia provide convincing support for the necessity of cooperative transforming events (Class I events) in addition to the expression of fusion genes (Class II events) for the

development of acute leukemia. For example, expression of PML/RARA, the product of a gene fusion associated with a subtype of AML, under the control of the Cathepsin G promoter in transgenic mice, results in altered myeloid development, but PML/RARA alone is not sufficient to directly cause AML [218]. Similarly, expression of *RUNX1/CBFA2T1*, another common gene fusion in AML, does not produce acute leukemia in mice until combined with a dose of the chemical mutagenic agent, N-ethyl-N-nitrourea (ENU) [219]. This two-hit phenomenon repeats itself with the other common fusions associated with human leukemia [220, 221]. A study of monozygotic twins who both developed ETV6/RUNX1 positive ALL [222] provides further support for the requirement of secondary genetic hits. The specific genomic breakpoint in fusions is essentially unique to each patient with leukemia, however the twin leukemic DNA in this study shared an identical *ETV6/RUNX1* fusion sequence. The most plausible explanation for this finding is that the chromosome translocation event occurred *in utero*. However, despite possessing identical *ETV6/RUNX1* gene rearrangements in an identical genetic background from birth, the ALL manifested itself only later in life and with differential timing between the twins. Additionally, the concordance rate for ALL in syngeneic twins is estimated at only 5% [223]. Together these data indicate that the intrauterine translocation event is insufficient for clinical leukemia and that a postnatal promotional event also occurs to induce leukemia [224]. Thus, both animal models and clinical observation support the notion that two cooperating genetic insults, leading to a proliferative advantage and to impaired hematopoietic differentiation, are necessary for the clinical manifestation of acute leukemia.

1.2.4 Leukemic stem cells

Cancer is a clonal disease that initiates in a single cell that has accumulated sufficient genetic damage to cause uncontrolled proliferation, and therefore the progeny of this cell make up the tumor [225]. The experimental setting of transplantation, where subsets of tumor cells are transferred from one

organism to another, demonstrates that tumors are functionally heterogeneous, as only a certain subpopulation of cells has the capacity to maintain or reinitiate the tumor. It is not always easy to define which cells within the population have this capability for self-renewal or tumor re-initiation.

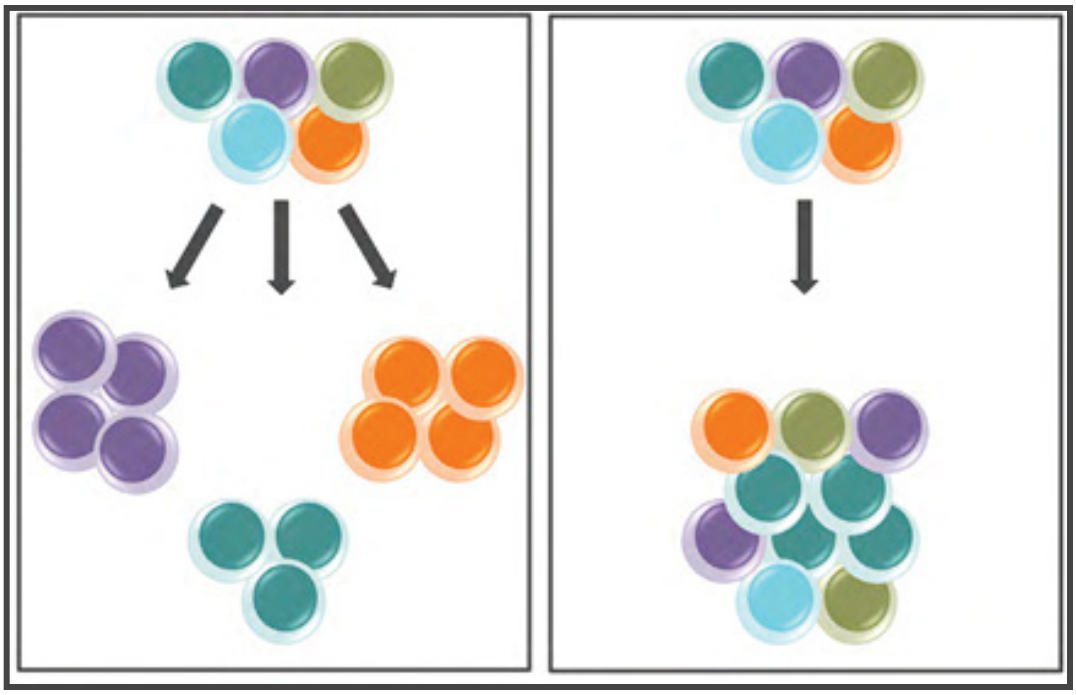

Figure 1.4. Two models accounting for the hetergeneous proliferative potential of leukemic cells. The stochastic model (left panel) assumes that every cell in a tumor is potentially tumor-initiating. Progression is governed by rare, random events. Cells with mutations that acquire growth advantage will dominate over all other cells in the tumor and will originate a new clone. In contrast, the hierarchichal model (right panel) posits that acute leukemia consists of two functionally distinct cell types (1) leukemic stem cells (LSCs, dark blue), which are self-renewing cells with the capacity to intitate, sustain and expand the disease, and (2) non-self renewing progeny cells, derived from LSCs through differentiation, which likely make up the bulk of the tumor and account for disease symptamaology.

Two general, but distinct models have been proposed to account for the heterogeneous proliferative potential of tumor cells (Figure 1.4). The stochastic model proposes that all cells within the tumor are equivalent with respect to their potential to re-initiate the clone, however, any one cell would have a low probability of exhibiting this potential. In contrast, the hierarchical model predicts that re-initiation potential is a property of only a distinct subset of cells within the

tumor, the so-called "cancer stem cell [226]."

There is strong support for the idea that cancer, and in particular leukemia, is a stem cell disease. Persuasive evidence for this concept comes from the discovery that most AML blasts do not proliferate [227, 228] and that only 1-4% of leukemic cells transplanted *in vivo* are able to form spleen colonies. The most compelling evidence for a leukemia stem cell (LSC), or leukemia initiating cell (LIC), has been provided by a Canadian group based at the University of Toronto. In a seminal paper in this field, Lapidot *et. al.* [229] reported that xenograft transplantation of only a few, highly-selected human LSCs into immunodeficient mice was able to recapitulate a leukemic disease that reproduced many features of the AML seen in the original human patients. Subsequently, cell purification based on cell surface markers demonstrated that that the cells capable of initiating human AML in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) were from a rare subpopulation of $CD34^{+}/CD38^{-}$ cells. Normal HSCs share this same $CD34^{+}/CD38^{-}$ phenotype, suggesting that normal primitive cells, rather than committed progenitor cells, are the target for malignant transformation.

Further studies support a multistep process whereby an initial molecular aberration occurs at the level of the stem cell, creating a pre-leukemic LSC. Either these cells or their progenitors then undergo additional oncogenic events that ultimately lead to transformation into overt disease [230, 231]. LSCs themselves also demonstrate self-renewal heterogeneity, with a population of quiescent stem cells capable of endless self-renewal, progenitor populations with more limited potential for self-renewal, and, finally, a population of blasts with no self-renewal potential that is responsible for the clinical manifestations of the disease [232]. This hierarchical arrangement of LSCs mirrors that of hematopoietic stem cells (HSCs) and lends credence to the idea that, in leukemia, the primary target for cell transformation resides within the HSC population.

The concept of the LSC has increasingly important implications for

treatment of acute leukemia. Most conventional chemotherapies and even existing targeted therapies may fail to affect the LSC. This may be attributable to the fact that the majority of the LSC population exists in the G0 phase of the cell cycle, where the absence of DNA synthesis, cell division or any activity related to self-renewal, renders it insensitive to anti-leukemic drugs. The induction of LSCs to enter the cell cycle, where they are then vulnerable to destruction by standard chemotherapy drugs, represents one therapeutic strategy. Indeed, Saito [233] and colleagues demonstrated in a mouse model of human AML that treatment with granulocyte colony-stimulating factor (G-CSF), a cytokine that induces cell cycle entry of hematopoietic stem cells, caused the transplanted leukemia stem cells to proliferate and rendered them susceptible to the chemotherapeutic, cytarabine. This finding provides proof of principle that the eradication of leukemia at its roots can be accomplished by developing treatments focused on pushing LSCs out of their quiecscent state. An analogous approach, in which newly diagnosed AML patients received chemotherapy, with or without G-CSF, showed the addition of G-CSF concurrently with chemotherapy improved disease-free survival [234].

Additionally, HSCs interact with their microenvironment through interactions between the chemokine SDF-1 and its receptor CXCR-4, and by the adhesion molecules VCAM/VLA4 and Angiopoietin-1/TIE-2. The same molecules are also involved in leukemia stem cell–niche interactions, and elevated levels of CXCR4 and VLA4 have been associated with poor response to chemotherapy and unfavorable prognosis in AML [235-237]. Therefore, it may be possible to affect leukemia stem cells directly by targeting the specific molecules mediating LSC-niche interactions. Finally, in addition to nonspecific treatments, such as G-CSF, that manipulate LSC behavior, targeting LSC-specific molecules may represent an effective therapy for AML. For example, specific targeting of the PML/RARA leukemic oncoprotein expressed in LSCs with arsenic trioxide may be the mechanism by which this treatment achieves long-term remission of murine, and possibly human, leukemia [238].

Although not all malignancies fit into the cancer stem cell model, AML in particular is one of the diseases for which there is solid evidence of stem cell–like behavior. Consequently, there is considerable leukemia research devoted to further understanding the unique biologic and molecular properties of LSCs, by comparison with both their non-self-renewing downstream progeny and their normal HSC counterparts.

1.3 Acute Promyelocytic Leukemia

If looking for a mnemonic to remember the relevant facts about acute promyelocytic leukemia (APL), one just has to remember that APL is a disease of A's. It is acute and it is highly sensitive to treatment with antracyclines, all-*trans*-retinoic acid (RA) and arsenic trioxide (ATO). APL is the M3 subtype of acute myeloid leukemia according to the FAB classification system. Although representing only 5-8% of AML cases in adults, APL serves as the paradigm both for understanding the pathogenesis of leukemia and the response to differentiation inducing agents. At the genetic level (with only one known exception), APL is characterized by a specific chromosomal rearrangement between the retinoic acid receptor alpha (*RARA*) on chromosome 17 and a number of partners. The majority of patients (98%) present with the 15;17 translocation, t(15;17), which results in a fusion of *RARA* with the promyelocytic leukemia (*PML*) gene on chromosome 15 [239, 240]. The presence of fusions involving the RARA is without question the central player driving APL and dictating the response of this disease to therapeutic agents. However, beyond this knowledge, the molecular mechanisms that contribute to the complicated pathogenesis and the response to treatment of APL, are not completely defined. As more is understood about this hematological malignancy, there are more opportunities refine and improve treatment based on this knowledge.

1.3.1 Retinoic acid receptor function

Almost three decades have gone by since the cloning of the first nuclear receptor (NR) [241]. Since then, the NR field has exploded with the functional

elaboration of many members of this highly conserved superfamily. Nuclear receptors are one of the most abundant classes of transcriptional regulators in animals and span a vast diversity of biological functions including growth, differentiation, metabolism, reproduction and morphogenesis.

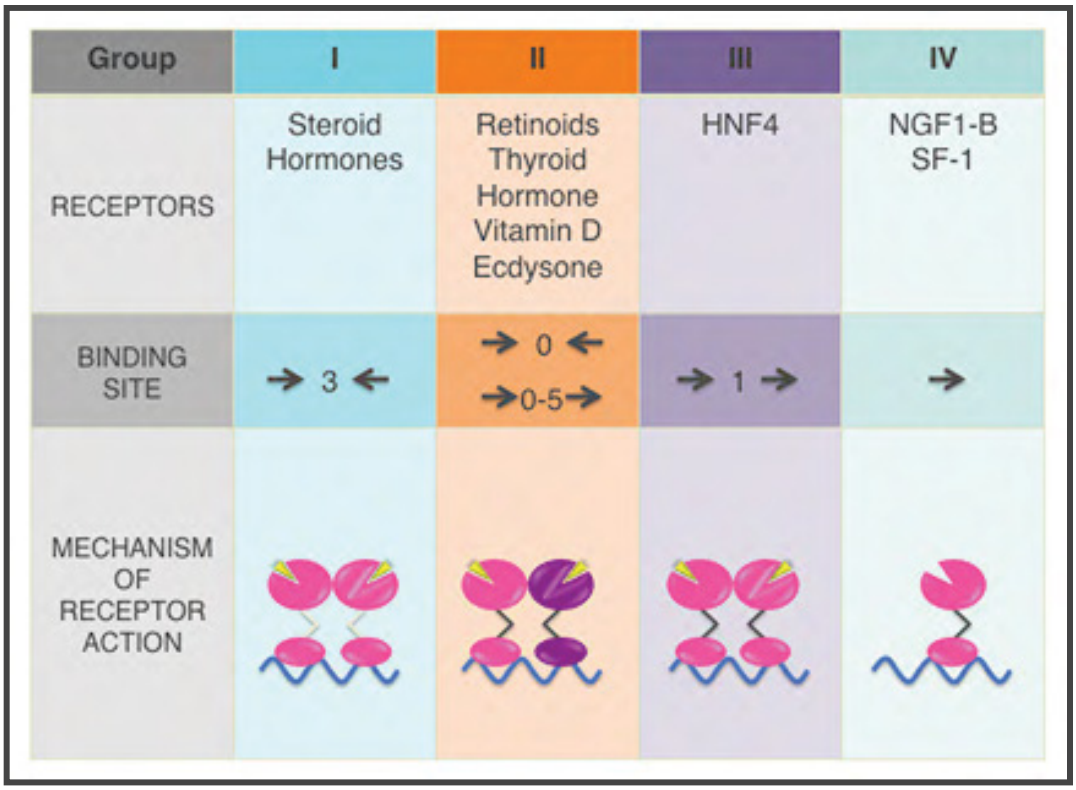

Figure 1.5. Classification of the nuclear receptor superfamily.

The nuclear receptor superfamily can be broadly divided into four groups according to their ligand binding, DNA binding and dimerization properties (Figure 1.5). A prototypical NR (Figure 1.6) consists of a variable N-terminal region (A/B), a highly conserved DNA-binding domain (DBD, region C), a linker region, D, and a conserved E region that contains the ligand binding domain. The F region is absent in RXRs and its function in RARs is still unknown. However, this region is phosphorylated [242, 243] and therefore may serve to modulate the receptor's transactivation function, as demonstrated for the estrogen receptors

[244, 245].

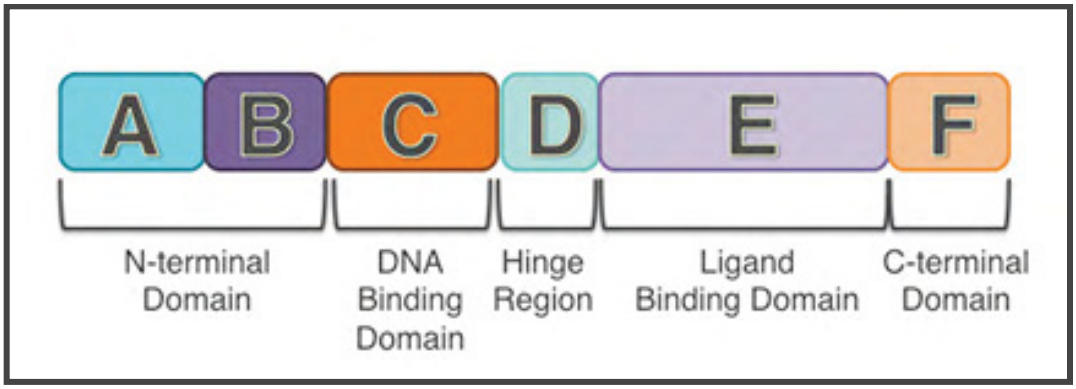

Figure 1.6. Structural organization of nuclear receptors.

RARs, which include separate genes for *RARB* and *RARG* in addition to *RARA*, belong to one major group of the NR superfamily, along with the thyroid hormone and vitamin D receptors. These receptors reside permanently in the nucleus, bound to specific DNA response elements in the regulatory regions of their target genes referred to as hormone response elements (HREs). HREs are bipartite elements that are composed of two hexameric core-site motifs. Recognition of the HRE is mediated by two characteristic zinc finger motifs in the DBD in the N-terminal half of the protein. These receptors also share the property of binding to HREs as a heterodimer with common adapter proteins called retinoid X receptors (RXRs), of which there are also three different genes (*RXRA, RARB, RARG*). The HREs for the NRs that heterodimerize with RXR typically consist of two direct repeats (DRs) of the core hexanucleotide motif. The discriminator for HRE specificity is the spacing of between the DRs, which is two or five for retinoic acid response elements (RAREs) [246]. Additionally, HRE spacers of one nucleotide have specificity for RXR homodimers or in some cases, may heterodimerize with RAR in reverse polarity [247]. The LBD in the E-region of RAR consists of 12 alpha-helices and two beta-strands linked by a series of loops. This three-dimensional structure forms a hydrophobic binding cavity that

can bind all-*trans*-retinoic acid (RA), the ligand for the RARs. The formation of the holo-receptor by entry of RA into the binding pocket causes the mobile twelfth helix (H12) to close the cavity.

In the absence of ligand, the RAR apo-receptor associates with a co-repressor complex and transcription of the associated gene is repressed. Central to this complex is a co-repressor protein (NCoR or SMRT) which directly interacts with each component of the apo-receptor and which recruits other proteins with histone deacetylase (HDAC) activity [248, 249]. Treatment with physiological concentrations of ligand causes a conformational shift resulting in displacement of the co-repressors and a concurrent recruitment of co-activators, leading to initiation of transcription of genes important for stimulating myeloid differentiation and regulating the cell cycle [250, 251]. The core component of the co-activator complex is a p160 protein that recruits proteins with histone acetylase (HAT) activity. Thus, the crux of RARA function is to serve as a sensitive switch to regulate transcription in response to RA by recruiting co-regulatory molecules that subsequently modify the chromatin and contact the basal transcription machinery.

Of course, the actual biological process of transcriptional regulation by the RARs is a highly complex and dynamic process involving variable expression and modifications of a multitude of molecular components that modulate transcriptional activity. First, through expression of different combinations of RXRs and RARs, variations in RAREs, competition for limiting quantities of RXRs, and differences in retinoid ligand ultilization, the retinoid receptor system generates enormous heterogeneity in the response to retinoids in tissue- and cell-specific contexts. Furthermore, at least eight classes of protein modifications have been identified that can affect the interactions and activities of these components. Acetylation of histones, as described earlier, is one representative modification. There is also increasing evidence for the regulatory role of lysine acetylation of many non-histone proteins, including RXRA [252] and p160 [253]. Protein

modification by phosphorylation plays a major role in modulating retinoid-mediated transcription at all levels of the process. There are at least four phosphorylation sites in RARA that are targeted by several different kinases, including the signaling kinases MSK1 [254], PKA [255] and PKC [256]. These and additional kinases also target RARA-associated cofactors [257]. Finally, ubiquitination and SUMOylation of RARA can be regulated by RA and affect stability of the protein and its association with RXR [258].

Thus, RARA is a member of a group of essential proteins that function as ligand-activated transcription factors and as such provide a direct link between signaling molecules and the transcription of select genes, many of which have central effects on cell and tissue growth, differentiation and homeostasis.

1.3.2 Role of PML and PML nuclear bodies

The promyelocytic leukemia protein (PML) is a tumor suppressor ubiquitously expressed in mammalian cells and distinctly localized to punctate nuclear structures known as PML nuclear bodies (NBs). Understanding PML function is an area of intense research because PML is the fusion partner in the majority of APL cases, indicating that this protein contributes to the pathogenesis of this malignancy. Additionally, although PML has been implicated in numerous physiologically important functions, including DNA repair, senescence and apoptosis, its exact biological role remains enigmatic.

Alternative splicing of the PML mRNA leads to generation of several PML isoforms [259]. Structurally, all isoforms of PML contain an amino-terminal RBCC/TRIM motif. This is a tripartite structure that contains a zinc-finger RING motif (R), two additional zinc-finger motifs (B-boxes, B) and an alpha-helical coiled-coil domain (CC). Proteins with RBCC/TRIM motifs can homo- and hetero-multimerize through their coiled-coil domains. Interestingly, this canonical structure is shared by other members of a large gene family [260], two of which can also form oncogenic hybrid proteins as a result of tumor-associated chromosome translocations [261-263].

While the spectrum of PML functions remain cryptic, G.G. Maul's group established that PML is the essential protein for proper NB assembly under physiological conditions [264]. PML nuclear bodies (NBs) are discrete subnuclear structures approximately 0.5 μm in diameter that are closely associated with the nuclear matrix. They are highly dynamic structures to which more than fifty proteins have been found to transiently localize. Current evidence implies that PML NBs are functionally, as well as structurally, heterogeneous, and many biochemical and molecular functions have been ascribed to them. Maul's work showed the dispersal of all other NB-associated proteins in the absence of PML and that NBs could be reconstructed by the introduction of wild-type PML into $PML^{-/-}$ cells.

Most proteins are subjected to post-translational modifications (PTMs), which represent a way to reversibly regulate cellular location and biological activity. PML is post-translationally modified and single PTMs, such as SUMOylation and phosphorylation, are capable of regulating PML function. Moreover, PML can be multiply modified and these modifications can expand PML's functional repertoire by acting combinatorially. Novel PTMs of PML and their consequences are continuously being elucidated. For instance, Hayakawa *et al*. recently demonstrated that PML can be acetylated [265], which is associated with enhanced PML SUMOylation. Accordingly, one of the earliest questions about the mechanism of NB formation concerned the necessity of SUMOylation for the recruitment of PML itself to the NB. Work done by the Maul lab [264] and others [266] found that a SUMOylation deficient mutant did still accumulate in the NBs, suggesting that SUMO modification was not required for PML localization. However, these experiments were not done in a $PML^{-/-}$ background, therefore permitting the possibility that the mutant formed heterodimeric complexes with the endogenous wild-type PML, which then recruited the mutant to the NB. Subsequent reports by others using the same PML mutant in the context of a $PML^{-/-}$ background showed that PML SUMOylation is essential for

PML to localize to the NBs [267]. Additionally, biochemical fractionation experiments demonstrated that only NB-associated PML is modified with SUMO1 [268].

The importance of this protein is highlighted by the pathology associated with PML disruption, as evidenced in the disease APL, and the observation that $PML^{-/-}$ mice are more vulnerable to tumourigenesis [269] and bacterial infection [270]. However, in apparent contradiction to these data, an analysis of a chronic myeloid leukemia (CML) model in mice indicated that greater oncogenicity was associated with higher PML expression [271]. Finally, PML is not essential for viability as $PML^{-/-}$ mice are phenotypically normal under non-stressed conditions [272]. These confounding outcomes are likely related to the highly diverse and complex activity of PML in normal and diseased cells.

1.3.3 Role of PML/RARA in leukemogenesis

APL is characterized by a block in differentiation at the promyelocytic stage of myeloid development. In APL cells, the prevailing view is that leukemic effects of the chimeric protein, PML/RARA, are due to its function as a dominant negative (DN) inhibitor of normal RARA function. The chimera localizes to promoters normally regulated by RARA, and acts as a constitutive transcriptional repressor that interferes with gene expression programs involved in granulocytic differentiation.

There is an abundance of molecular evidence in support of this model. Like RARA, PML/RARA recruits RXR [273, 274] and can bind to RAREs. PML/RARA has been shown to form high molecular weight complexes (HMWs) and the oligomerization domain of PML enhances the capacity of PML/RARA to recruit co-repressors such as NCoR, SMRT [275], HDACs [276, 277], polycomb group proteins and DNA-methylation complexes [278, 279]. Physiological RA concentrations are not sufficient to cause the release of these HMWs from PML/RARA and accordingly, PML/RARA reduces RA-induced activation of many canonical RARA target genes [240]. Furthermore, the introduction of

PML/RARA in human hematopoietic stem cells/hematopoietic progenitor cells (HSCs/HPCs) co-opts the differentiation program and induces a maturation arrest at the promyelocyte stage, however a PML/RARA mutant unable to bind NCoR did not have this maturation block [280]. Additionally, replacement of the coiled-coil domain of PML with the tetramerization domain of p53 was sufficient to recruit NCoR and block differentiation in response to RA [281], suggesting that the contribution of the PML moiety to the fusion might simply be related to complex recruitment. Collectively, these data suggest that repression of the normal RARA transcriptional program represents a key event in APL leukemogenesis.

However, abolishing the normal RARA ligand-dependent transcriptional program alone does not recapitulate the APL disease phenotype. Importantly, transgenic mice expressing an RARA mutant unable to bind ligand do not develop leukemia [282]. Complementary to this, mice expressing a recombinant transgene in which HDAC1, a key RARA co-repressor, was tethered to RARA, were created. Despite the ability of this fusion to heterodimerize with RXR, bind to an RARE consensus sequence and repress transcription of a luciferase construct, none of the resulting mice developed leukemia [283]. These data further indicate that the model whereby PML/RARA's leukemogenic activity is through aberrant recruitment of HDACS and other co-repressors is too simplified.

It has been proposed that PML/RARA exhibits a significant gain of function. Global gene expression analysis after inducible expression of PML/RARA shows the expected down-regulation of differentiation genes, but an equivalent number of genes were upregulated, including genes implicated in the self-renewal of HSCs, such as *LMO1* and *JAG1* [284]. *In vitro* evidence suggests PML/RARA has gained an expanded DNA binding capacity away from canonical RAREs to more widely spaced DRs [274] and can affect the transcription of genes controlled by other nuclear receptors [273, 285]. ChIP-seq analysis in the patient derived cell line NB4 [286] validates this finding and reveals a gain of function in

the DNA binding repertoire for PML/RARA as compared with wild-type RARA [287]. Furthermore, most of these additional PML/RARA binding regions are associated with epigenetic alterations, including histone acetylation and methylation and DNA methylation, indicative of functional significance. Finally, there is evidence for PML/RARA interfering with the function of transcription factors other then RARA. PML/RARA was demonstrated to form a repressive transcription complex with PU.1 [288], which has been shown to be an important TF in normal hematopoiesis and in generation of myeloid leukemia through disruption of its function [289]. An alternative variation on this theme is that the co-repressor complex formed by PML/RARA may indirectly affect the activity of alternative transcription factors by depleting modulating co-factors such as RXRs or HDACs. Accordingly, genetic experiments have demonstrated that PML/RARA binding to RXRA, the universal partner for heterodimeric nuclear receptors, is required for APL development *in vivo* [290]. An indirect mechanism may also apply to the modulation of the AP-1 transcription factor composed of Fos and Jun, which has been suggested to be of central importance in APL pathogenesis [291-293].

One of the signature cytological findings in APL is that PML is delocalized to a myriad of microspeckles throughout the nucleus in APL cells, rather than concentrated in discrete PML nuclear bodies (NBs) [294, 295]. Treatment with all-*trans* retinoic acid or arsenic trioxide (ATO) degrades the aberrant PML/RARA fusion protein and relocalizes NB components [268, 296], linking NB disruption to APL pathogenesis. As previously stated, transcriptional repression through enhanced recruitment of co-repressors to the RARA portion of PML/RARA oligomers has long been the dogma of APL pathogenesis. However, de Thé's group challenged this principle when they demonstrated that without the K160 SUMOylation site within PML, PML/RARA is unable to efficiently induce immortalization or a differentiation block either *ex vivo* or *in vivo* [297]. Importantly, the PML/RARA mutant behaves exactly as PML/RARA in terms of

dimerization, DNA binding and affinity for the SMRT co-repressor, but still fails to significantly impair differentiation of primary hematopoietic progenitors. Furthermore, although PML/RARA-K160R transgenic mice present with myeloproliferation, they never develop typical APL. Collectively, these observations led de Thé's group to postulate that the APL differentiation block is not solely due to RARA-moiety dimerization, but somehow must also involve a PML-moiety K160-SUMOylation dependant mechanism. The model they propose is one whereby the PML moiety of PML/RARA, via its K160 SUMOylation site, acts to recruit the transcriptional repressor Daxx. This, coupled with PML/RARA's ability to strongly recruit the SMRT co-repressor complex, results in a strong repression of RA-target genes, ultimately resulting in APL. Compelling evidence in favor of this model was provided when the fusion of PML/RARA-K160R to the repression domain of Daxx (thereby artificially recreating PML/RARA-Daxx recruitment) recapitulated all the features of cell transformation by the wild-type fusion protein [297]. However, an obvious caveat remains: the importance of the K160 site might not be due to its modification by SUMO but rather due to another post-translational modification, such as ISGylation or acetylation. Finally, the disruption of PML nuclear bodies through expression of the chimeric protein, indicates that the fusion interferes with normal PML function. As mentioned previously, the cellular roles of PML remain poorly understood, however, this protein does seem to have important functions in apoptosis, p53 regulation and senescence.

PML/RARA transgenic mice develop leukemias after a long latency and with a low penetrance (~5 to 30% after 6 to 12 months) [218, 298, 299], demonstrating that while the APL-associated fusion protein induces a pre-leukemic state, other genetic events are necessary for progression to a frank leukemia. This suggests that a second transformative event is required for full neoplastic development. However, these models were done with transgenic mice expressing PML/RARA in early myeloid cells under control of human cathepsin

G regulatory sequences. Westervelt *et. al* [300] created a more "realistic" knock-in mouse model in which a single copy of *PML/RARA* was inserted into the 5'-UTR of the endogenous murine cathepsin G locus. Interestingly, there was a dramatic increase in disease penetrance (90%) without any changes in latency and remarkably, the expression level of PML/RARA in bone marrow cells or APL cells was less than 3% of that measured in the low-penetrance transgenic model. Genetic cross-breeding experiments to select transgenic mice with haploinsufficiency for genes affecting APL differentiation (*PU.1* [301] and *CEBPA* [302]) and/or other APL cell processes (*PML* [303]) demonstrated markedly increased leukemia penetrance, suggesting that endogenous genetic or epigenetic changes that reduce the expression or activity of these molecules could be involved in disease progression. Finally, the co-expression of kinase genes with mutations that augment cell proliferation (activating *FLT3* [304] or *RAS* [305] mutations) had a potent effect, both decreasing latency and increasing penetrance. The presence of these secondary mutations might also explain why APL cannot be cured by differentiation therapy with RA alone, but is highly curable by combinations of RA and cytotoxic chemotherapy.

As mentioned in an earlier section, all leukemia cells are not functionally equal: only very few leukemia cells are able to give rise to a new tumor upon *in vivo* transplantation. In murine APL, leukemia initiating cells (LICs) represent approximately 1% of blasts [306]. The primary leukemia initiating activity of PML/RARA has been associated with little initial change in differentiation, but rather, with the acquisition of an enhanced self-renewal capacity at all levels of the myeloid differentiation hierarchy, although, phenotypically, they are committed myeloid progenitors [302, 306]. Comparison of the gene expression profiles of wild-type murine promyelocyte-enriched populations and promyelocyte-enriched populations from pre-leukemic PML/RARA-expressing mice revealed very little changes in gene expression and no differences in cell phenotype [307]. This contrasts with substantial changes seen in leukemic

promyelocytes. This suggests that the expression signature of APL cells reflects the genetic changes that contributed to progression [307] and not inititation.

1.3.4 Alternative RARA partners

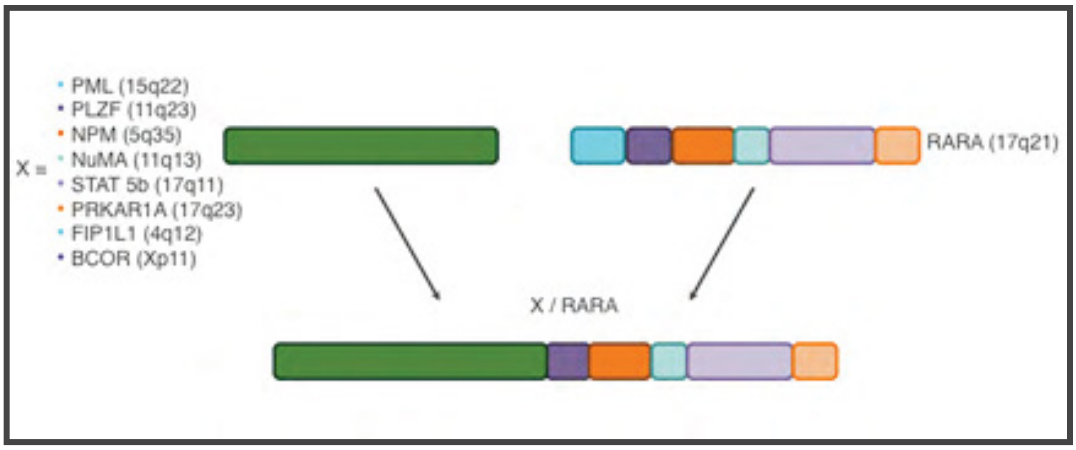

Figure 1.7. RARA fusion partners associated with APL.

To date, nine different chromosomal translocation partners have been identified in patients with APL. All but one of those (the exception being one case report of a *NUP98/RARAG* fusion [308]), involve the *RARA* gene on chromosome 17q21. The generically named *X* gene in the *X/RARA* fusion gene transcript is ligated to the 5'-end of the third exon of RARA. Thus, the RARA moiety lacks only a portion of the A/B region associated with ligand-independent activator function (Figure 1.7). This finding again supports a perturbation in RARA signaling as being the critical factor in the specification of the APL phenotype. The N-terminal fractions contributed by the fusion partners donate additional dimerization domains. These acquired dimerization domains, together with the DNA binding domain of RARA, are required for the oncogenic effect of the fusion proteins and promote formation of chimeric receptor homodimers and provide additional co-repressor binding domains. The resulting X/RARA fusion proteins have been shown to recruit HDACs, NCoR, SMRT, DNMT1, DNMT3A, repressive histone methyltransferases and polycomb group proteins [276, 309-

311]. Furthermore, recruitment of RXR by X/RARA is a consistent requirement for leukemogenicity of all X/RARAs tested [290, 312]. Finally, the fusion protein expressed in APL divides APL clinically into those that respond to RA therapy versus those that are insensitive.

Additionally, some translocations generate the reciprocal *RARA/X* fusion genes which can result in the co-expression of their transcripts in leukemic blasts [313-315]. The presence of the RARA/X fusion is especially important for fusions involving PLZF. PLZF/RARA-positive APL cases are associated with a poorer prognosis than their PML/RARA counterparts and these patients are RA insensitive [316]. Most patients with this fusion express the reciprocal *RARA/PLZF* transcript. Interestingly, one patient that expressed PLZF/RARA without the corresponding RARA/PLZF was responsive to RA therapy [317, 318], implying an important roles in oncogenesis for the co-expressed RARA/X reciprocal fusion proteins [313, 314, 319, 320]. Additionally, the partner proteins in APL have important growth-regulatory roles in normal myeloid cells. It may be that the loss of one allele of the partner protein combined with the effects of the X/RARA or reciprocal RARA/X fusion protein, which compromises normal partner protein function, contribute to the development of leukemia.

1.3.5 Clinical Features of APL and response to RA treatment

The most significant clinical feature of APL is a hemorrhagic diathesis manifested by large hematomas, intracranial hemorrhage or gastrointestinal bleeding. APL is an aggressive disease and the risk of hemorrhage is great, therefore the diagnosis of APL is considered a medical emergency that requires immediate intervention. However, APL has undergone a radical change in perception in the medical community over the last fifty years from being the most malignant form of AML to the most curable.

All-*trans* retinoic acid (RA), a vitamin A derivative, has a selectively potent therapeutic effect in APL patients compared to the other subtypes of acute leukemia. Up until the last year, RA with chemotherapy has been the standard of

care for APL, resulting in cure rates exceeding 80%. RA causes differentiation of the leukemic cells and not cytotoxicity, as observed with conventional chemotherapies, as has been confirmed by a careful analysis of morphology, the emergence of mature granulocytic immunophenotypic markers and the presence of Auer rods in the blast cells [321, 322]. Arsenic trioxide (ATO) therapy has been proven as an effective second-line treatment for APL. Both RA and ATO are considered targeted therapies in APL because they target the leukemic fusion protein. A landmark mouse study showing that ATO was necessary for leukemic initiating cell (LIC) clearance, while RA was necessary for blast differentiation [238], supported the hypothesis that the two agents would be effective when given in combination as frontline therapy. In an exciting advancement in the APL field, results from a Phase III clinical trial showed a 100% complete remission with RA + ATO and a better overall survival with RA–arsenic trioxide as compared with RA–chemotherapy [323]. These results are significant for patients because they mean that this hematological malignancy can be successfully treated with two targeted agents, eliminating the need for cytotoxic chemotherapy. The sensitivity of APL cells to RA therapy is inextricably tied to the presence of fusions containing RARA. However, the molecular mechanisms underlying the response to this differentiation agent are not as simple as they first appear.

Pharmacological doses of RA induce proteolytic degradation of the PML/RARA chimeric protein that is the hallmark of this leukemia. If the mechanism of PML/RARA action were as simple as being a dominant negative transcriptional repressor, then the storybook explanation for the therapeutic efficacy of this agent would be that upon removal of the fusion protein, the normal RARA transcriptional program is restored and granulocytic differentiation proceeds as normal. However, this model is too simplistic.

Most *in vitro* work done in APL has made use of the NB4 cell line. NB4 cells are a t(15;17) positive, patient-derived cell line established in 1991 by Michel Lanotte [286]. RA treatment (1 μM) induces a complete morphological

and functional maturation of this cell line within four days. PML/RARA is catabolized in response to 1 μM RA in NB4 cells in both a proteasome and caspase-dependent manner [324-327]. The kinetics of this catabolism vary slightly, however most experimental evidence points to a down-regulation of the fusion protein occurring within 6 hours of 1 μM RA treatment, with a restoration of normal PML nuclear bodies and a concomitant cell cycle arrest by 12 hours. The degradation is substantial by 24 hours and complete by 48 hours. Both the PML and RARA moieties have been suggested as the targets of degradation. A caspase-3 cleavage site maps to the PML moiety of the fusion, whereas RA directs proteasome-mediated degradation via the RARA portion. Two distinct degradation sites would account for the antagonism of PML/RARA function exhibited by both caspase and proteasome inhibitors [324].

Additionally, evidence has been presented for RA-directed proteolysis via post-translational modification by the interferon-stimulated gene 15 kDa protein (ISG15, termed ISGylation). ISG15 protein belongs to the growing family of ubiquitin-like proteins whose biological functions are actively being pursued. Like ubiquitin, ISG15 is covalently conjugated to target substrates via an enzymatic cascade. Previous research has associated RA-induced differentiation of APL cells with several steps in the ISGylation pathway. The activating enzyme (E1) of the ISG15 conjugating system is the ubiquitin E1-like protein UBE1L [328]. UBE1L is induced in RA-sensitive APL cells but not in those that are resistant to RA-mediated differentiation [329]. Significantly, overexpression of UBE1L triggered PML/RARA degradation and caused a rapid induction of apoptosis in RA-sensitive APL cells. The inverse relationship between PML/RARA and UBE1L protein expression has fueled speculation that RA, via UBE1L, promotes PML/RARA ISGylation and degradation [329]. A direct protein-protein interaction between ISG15 and the PML portion of PML/RARA was shown [330]. However, while the functional consequences of ISGylation remain mysterious, considerable evidence to date indicates that ISG15

does not target proteins for proteasomal degradation [330, 331] and may actually antagonize ubiquitin-mediated protein turnover.

It has been suggested that RA-mediated PML/RARA degradation is not essential for differentiation. Inhibition of PML/RARA caspase-dependent degradation does not prevent RA induced myeloid differentiation [324]. A potential hypothesis in view of this result would be that pharmacological doses of RA could activate PML/RARA dependent transcription. In fact, PML/RARA has been itself shown to act as a ligand-dependent transcriptional activator [332] in an $RAR^{-/-}$ background.

Molecular insights into the differentiation response of APL cells by RA can be gained through transcriptome analysis. Many different studies of gene expression profiling that use cell lines, either NB4, U937 or HL60 [333-336] or APL blasts [337] as models to study RA-induced differentiation in APL have already been published. There are recent studies to suggesting that RA may clear PML/RARA from promoters, thereby restoring wild-type RARA function and subverting the differentiation block. However, the contribution of RA-modulated gene expression to cell differentiation and disease clearance remains ill defined.

Exposure of APL cells to RA is followed by changes in the regulation of many hundreds of genes. Although the models, dosing and timing between these studies vary widely, there are some interesting trends. First, changes in gene expression with RA begin early and continue for up to 3 days. While the early genes represent direct transcriptional targets, those occurring at later time points probably represent secondary targets dependent upon the up-regulation of other proteins. Several targets, such as *ICAM-1* [334] and *ELF4* [335], were validated to be *bona fide* RA transcriptional targets via the use of cycloheximide. Second, approximately equal numbers of genes are down-regulated by RA as are up-regulated. This again occurs early, indicating that the effect is direct [333, 337] and this provides evidence that PML/RARA acts as more than just a transcriptional repressor.

Third, several important functional classes of genes are selectively regulated in response to RA. As the simplified model of PML/RARA function would predict, there is observed up-regulation of several key transcription factors/cofactors involved in hematopoiesis. These include members of the CCAAT-enhancer binding proteins (CEBPs), basic helix-loop-helix (bHLH) [338] and Ets families. Among these, the CEBPs are extremely important. *CEBPA* [339], *CEBPB* [340] and *CEBPE* [341] have all been shown to be up-regulated at early time points after RA exposure. *CEBPE* has classical RARE in its promoter [342] but no canonical RARE sequence has been found in the promoters of *CEBPA* or *CEBPB*. PU.1, an ETS member, is a recognized master transcription regulator of differentiation processes in hematopoietic cells, and, when it is experimentally manipulated to be expressed or not in APL cells, it has the corresponding effects of promoting or inhibiting terminal granulocytic differentiation, respectively [343]. Expression of PML/RARA suppresses PU.1 expression and treatment with RA for at least 12 hours rescues expression. The longer RA time-point indicates that the effect on PU.1 is not direct. The regulated temporal expression of critical transcription factors is the hallmark of blood cell differentiation. Indeed, PU.1 has a CEBPB transcription factor binding site in its promoter, so RA may function to restore CEBPB expression and thereby initiate a cascade culminating in PU.1 expression. Functional classification of common target genes in APL blasts showed that repression of genes involved in stem cell renewal and/or inhibition of cell differentiation are also early events in RA-dependent maturation. Some of the repressed genes classified in this category, including *RUNX1*[344], *HHEX* [345], *ALCAM* [346], *WT1* [347], *ETV6* [348], are involved in multiple hematopoietic stem cell (HSC) functions such as development, maintenance and homing. The down-regulation of these targets suggests that discontinuation of a cell renewal program also represents an important RA function.

Fourth, and most striking, using models that respond to RA but do not express the PML/RARA fusion protein, it becomes evident that some RA-induced changes in gene expression are PML/RARA dependent [333, 335]. Lee *et. al.* [333]compared the RA-response of NB4 cells with that of HL60 cells, which respond to RA-induced differentiation but do not have a chromosomal translocation. Their analysis found that while RA universally induced and repressed genes over a 48-hour time course, there was absolutely no overlap in the gene sets between the two cell lines. This result is recapitulated in the U937PR9 model, a cell line with a transduced and inducible PML/RARA transgene. In U937PR9 cells, many more genes are regulated in response to RA than in control U937 cells containing endogenous RARA. Together, these observations seem to corroborate the view that at least the early transcription response to RA is mediated predominantly by PML/RARA, not RARA.

1.3.6 Molecular mechanisms of resistance to RA

The strongest evidence in support of PML/RARA as the central mediator of RA activity in APL is the observation that clinical relapse from RA therapy is associated with molecular alterations in the RARA ligand-binding domain (LBD) of the fusion protein [349, 350]. Some of these mutations render PML/RARA unable to bind RA and diminish the transcriptional regulation in response to RA [351]. Interestingly, these alterations are exclusive to the *PML/RARA* chimeric gene, and the remaining copy of *RARA* remains wild-type.

In vitro models of RA resistance have been useful for elucidation of not only the mechanisms of RA resistance but also provide insight into the functions of PML/RARA. Table 1.4 presents RA-resistant cell lines that have been derived from the NB4 cell line by various labs. By studying these cell lines, important insights into the contributions of different pathways to RA sensitivity can be gained. As in the clinic, a proportion of resistance to RA *in vitro* is associated with the clonal emergence of cells with mutations in the LBD region of PML/RARA [352-355]. However, the more interesting mechanistic questions

underlying RA response arise in cases of RA resistance in the context of a wild-type PML/RARA. For example, in the NB4-GR007/6 cell line [356], RA resistance was related to constitutive activation of the proteasome and resultant degradation of PML/RARA protein [357]. Sensitivity to RA could be partially restored by inhibition of proteasome activity and fully restored by forced expression of PML/RARA, but not RARA. This provides more evidence that PML/RARA is necessary for the RA-mediated differentiation response. In NB4-MR2 and NB4-MR6 cells, resistance was correlated with the formation of higher molecular weight PML/RARA complexes [358]. Finally, the NB4-LR1 cell line [359] is interesting because it does not mature in terms of morphology, NBT reduction or granulocytic cell-surface marker expression with RA alone, but RA does render these cells competent to maturation triggering by cyclic AMP (cAMP) signaling.

Table 1.4. Nomenclature of resistant cell lines consistent with recommendation by Roussel and Lanotte [360].

Laboratory	Cell Line	PML/RARA Abnormality	References
Dmitrovsky	NB4-DR1	ΔPhe286	Nason-Burchenal *et. al.* (1997); Nason-Burchenal *et. al.* (1998)
	NB4-DR2 NB4-DR3	No known PML/RARA mutation	
Gambacorti	NB4-GR306 NB4-GR007/6	Loss of PML/RARA protein expression	Dermime *et. al.* (1993); Dermime *et. al.* (1995)
Lanotte	NB4-LR1	No known PML/RARA mutation; degrade PML/RARA but do not mature in terms of morphology, NBT reduction or cd11c expression	Ruchaud *et. al.* (1994); Duprez *et. al.* (2000)
	NB4-LR2	Gln411STOP	
Miller	NB4-MR2	Leu398Pro	Rosenauer *et. al.* (1996); Shao *et. al.* (1997)
	NB4-MR2 NB4-MR6	Forms higher weight PML/RARA complexes	
Naoe	NB4-NR1	Pro407Leu	Kitamura *et. al.* (1997)

1.4 Rationale and Objectives

Acute promyelocytic leukemia (APL) provides a model of a malignancy that can be successfully treated by an agent, all-*trans* retinoic acid (RA), targeted towards the product of a chromosomal translocation. Unfortunately APL cells develop resistance *in vivo*, a phenomenon that can be recapitulated *in vitro*. Using a stably RA-resistant cell line we developed from the APL cell line NB4, termed MR2, we have shown that resistance to RA-mediated transcription and differentiation is associated with enhanced binding of higher molecular weight complexes to PML/RARA [358]. Employing a GST pull-down strategy followed by mass spectrometry analysis, we identified eight proteins with an increased association with PML/RARA in MR2 cells. We verified Topoisomerase II Beta (TOP2B) as one of these novel mediators of resistance to RA [361]. TOP2B is highly overexpressed in the MR2 cell line and binds PML/RARA, repressing transcription of RARA target genes and differentiation in response to RA.

In this thesis, we aimed to further elucidate the molecular basis for resistance to RA in the MR2 cell line, which retains wild-type PML/RARA expression [358]. Our specific objectives were as follows:

(1) To investigate the transcriptional defect in response to RA in the MR2 cell line and determine at which stage of transcription that occurs.

(2) To examine the role of Nucleophosmin (NPM), another one of the eight identified proteins and a protein closely linked with leukemogenesis, on RA-induced gene expression.

(3) To define the mechanisms mediating the de-regulation of TOP2B protein levels in the MR2 cell line.

CHAPTER 2: NPM AND BRG1 MEDIATE A NOVEL MECHANISM OF TRANSCRIPTIONAL RESISTANCE TO RA IN ACUTE PROMYELOCYTIC LEUKEMIA.

2.1 Preface

Prior to this study, we identified eight proteins that had increased interaction with PML/RARA in RA-resistant MR2 cells [361]. We followed up that identification with the characterization of the roles of one of those proteins, TOP2B, in mediating the resistance phenotype. In this chapter, we began to characterize the role of another one of the identified proteins, Nucleophosmin (NPM). We elected to follow-up with NPM due to the important role this protein has in Acute Myeloid Leukemia (AML). NPM mutations are the most common genetic change in adult patients with cytogenetically normal AML, with a 53% incidence reported in one study [362]. Furthermore, previous results show a significant reduction in RA-induced transcriptional activity in RA-resistant APL cells, despite the presence of a wild-type PML/RARA [358]. Prior to the work contained in this Chapter, the mechanism by which this blockade in transcription occurs had never been fully addressed. We speculated that both NPM and TOP2B might play a role in that blockage. The purpose of this study was to investigate the mechanisms by which NPM and TOP2B interfere with RA-induced transcriptional activity.

* This chapter contains original, unpublished data.

2.2 Abstract

Perturbation in the transcriptional control of genes driving cellular differentiation is an established paradigm whereby oncogenic fusion proteins promote leukemia. From a retinoic acid (RA) sensitive Acute Promyelocytic Leukemia (APL) cell line, we derived an RA-resistant subclone characterized by a block in transcription initiation, despite maintaining wild-type PML/RARA expression. We uncovered an aberrant interaction between PML/RARA, Nucleophosmin (NPM) and Topoisomerase II Beta (TOP2B). Surprisingly, RA stimulation in these cells results in enhanced chromatin association of the nucleosome remodeler BRG1. Inhibition of NPM or TOP2B abrogated BRG1 recruitment. NPM inhibition and targeting BRG1 restored differentiation when combined with RA. Here, we demonstrate a role for NPM and BRG1 in obstructing RA-differentiation and implicate chromatin remodeling in mediating therapeutic resistance in malignancies. NPM mutations are the most common genetic change in patients with acute leukemia (AML) therefore, importantly, our model may be applicable other more common leukemias driven by an aberrant NPM.

2.3 Introduction

Nuclear hormone receptors are ligand-activated transcription factors that transduce messages carried by signaling molecules into transcriptional responses. The retinoid receptor alpha (*RARA*) gene encodes one of three nuclear retinoid receptor proteins. Whereas the other two have key roles in embryogenesis, RARA finely tunes the differentiation of granulocytes by acting as a transcriptional regulator of genes involved in this program [363].

In the absence of ligand, RARA is bound to DNA along with its partner receptor, the retinoid X receptor (RXR), and co-repressors [249, 364, 365]. Upon binding ligand, retinoid receptors undergo a conformational change, releasing co-repressors such as NCoR and SMRT, and recruiting an arsenal of co-activator proteins that facilitate the recruitment of RNA polymerase II (RNAPII) and the

general transcription factors (GTFs) to the promoter [249, 366, 367]. Several chromatin-remodeling complexes make direct physical interactions with RARA and carry out structural modifications of chromatin to regulate transcription. Importantly, BRG1, the ATPase subunit of the SWI/SNF complex, plays a critical role in differentiation through regulation of gene expression and is required for transactivation by many nuclear receptors, including RARA [368].

The importance of RARA in granulopoiesis is clearly evident in Acute Promyelocytic Leukemia (APL). APL is a form of acute myeloid leukemia (AML) characterized clinically by an accumulation of immature promyelocytes in the bone marrow and peripheral blood, stemming from a blockage in myeloid differentiation [369, 370]. The majority of APL patients respond to the differentiating action of pharmacological concentrations of all-*trans* retinoic acid (RA), a vitamin A derivative. In fact, this treatment was the first example of a successful therapeutic approach inducing differentiation rather than cytotoxicity, and it has since become the prototype for differentiation therapy in cancer. Although treatment with RA alone results in a complete remission, its duration is transient, as a significant proportion of patients relapse and subsequently develop RA resistance *in vivo*, a phenomenon that can be modeled *in vitro* [358, 371].

At the molecular level, APL blasts harbor a chromosomal translocation involving the *RARA* gene located on chromosome 17 [370, 372]. Numerous fusion partners of *RARA* have been identified, but the *PML* gene of chromosome 15 is the most common companion gene. Approximately 95% of affected individuals have the (15;17) translocation, producing the PML/RARA chimera [373]. PML/RARA acts as a dominant negative inhibitor of normal retinoid receptor function. The fusion protein retains standard RARA biochemical properties, in that it still binds DNA at retinoic acid response elements (RAREs), can still heterodimerize with RXR, and binds RA [273, 285, 374, 375]. However, PML/RARA is a much more potent transcriptional repressor than RARA, as it is unresponsive to physiological concentrations of ligand, such that co-repressors are

not released and RA target genes remain unexpressed, resulting in the accumulation of myeloid progenitors [275, 277, 376].

The PML/RARA fusion protein blocks myeloid differentiation by transcriptionally repressing RA target genes and therefore RA-mediated neutrophil maturation. Thus, APL cell lines are a useful model system to study the conversion of transcription factors into oncogenic facilitators in other hematological malignancies. Additionally, *in vitro*-derived RA-resistant cell lines provide clues into the mechanisms of RA resistance in APL. We have previously isolated three RA-resistant subclones from the parental RA-sensitive cell line NB4, denoted MR2, MR4 and MR6 [358]. The molecular basis for resistance to the transactivation function of RA in the MR2 cell line, which retains wild-type PML/RARA expression [358], remains unknown.

We previously reported that resistance to RA-mediated transcription and differentiation in the MR2 cell line is associated with an altered pattern of high-molecular weight complexes binding to PML/RARA [358]. Importantly, we furthered this observation by identifying eight novel members of these complexes. One of these was Nucleophosmin (NPM), a nucleolar protein [377] intimately linked with the development of acute leukemia [378] and another was Topoisomerase II Beta (TOP2B), which we characterized as playing a central role in RA-resistance [361]. NPM plays important roles in the regulation of cell proliferation and apoptosis and is found to be more highly expressed in malignant and proliferating cells than in normal cells [379, 380]. Conversely, NPM expression is down-regulated in cells undergoing differentiation [381, 382]. It remains unclear how cells harboring elevated NPM achieve malignant properties. Here we characterize the novel role of NPM as a transcriptional co-repressor of the PML/RARA oncoprotein and a key mediator of the differentiation block observed in RA-resistant APL cells.

2.4 Materials and Methods

2.4.1 Materials

RPMI 1640 and fetal bovine serum were purchased from Wisent (St Bruno, QC, Canada). All-*trans* RA and the Nucleophosmin inhibitor, NSC 348884, were purchased from Sigma-Aldrich (St Louis, MO, USA) and Axon Medchem BV (Groningen, The Netherlands) respectively. The TOP2B inhibitor ICRF-193 was obtained from Biomol (Plymouth Meeting, PA, USA). Darinaparsin (ZIO-101, S-dimethylarsino-glutathione) was obtained from Ziopharm Oncology (New York, NY, USA).

2.4.2 Cell culture

Derivation of the RA-resistant cell line MR2 from the parental APL cell line NB4 was previously described [358]. All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin (Wisent).

2.4.3 RNA extraction and analysis

mRNA was isolated using the Absolutely RNA® Miniprep kit (Agilent Technologies, Santa Clara, CA, USA) and cDNA was generated from 1μg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Mississauga, ON, Canada). We determined relative mRNA levels, normalized to endogenous 18S rRNA (TaqMan® hs99999901_s1 probe, Applied Biosystems, Carlsbad, CA, USA), using SYBR Green I chemistry in an ABI 7500 Fast Real-time PCR machine (Applied Biosystems). See Supplemental Table 2.1 for primer sequences.

2.4.4 Western blotting

Whole cell lysates were diluted 1:1 with 2 × SDS sample buffer. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, St. Laurent, QC, Canada). After blocking, membranes were probed with the indicated antibodies (Supplemental Table 2.2). The resulting signals were detected using the enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA) system.

2.4.5 Chromatin immunoprecipitation (ChIP)

All ChIP analysis was performed as detailed in Gomes *et al.*[383]. Briefly, NB4 and MR2 cells were grown to ~60% confluency and fixed with 1% formaldehyde. Whole-cell lysates (1 mg per condition) were subjected to ChIP with the indicated antibodies (Supplemental Table 2.2), followed by DNA purification. ChIP-enriched DNA was analyzed with quantitative real-time PCR as detailed in Gomes *et al.* (2006) with the indicated primer sets (Supplemental Table 2.3) [383].

2.4.6 Co-immunoprecipitation

Approximately $7x10^6$ cells were harvested and lysed in IP lysis buffer (20 mM Tris-HCL pH 7.5, 420 mM NaCl, 2 mM $MgCl_2$, 10% Glycerol, 0.5% Glycerol, 0.5% NP-40 and 0.5% Triton-X) completed with protease inhibitors. 2 mg of protein per condition, diluted in 0.5% Tritron-X IP buffer, were pre-cleared for two hours with protein G sepharose, after which the beads were removed. TOP2B or IgG antibody were added overnight at 4°C. Immune complexes were recovered with protein G sepharose, washed 3 times in 0.5% Triton-X IP buffer, once in 0.1% Triton-X IP buffer, then boiled in 2X SDS sample buffer, and subsequently analyzed by Western blot.

2.4.7 shRNA-mediated knockdown

Cell lines stably transduced with shRNAs targeting BRG1 were established using a 24 h polybrene (5 mg/ml) transduction of NB4 and MR2 cells with Mission™ TRC lentiviral particles (Sigma-Aldrich, St. Louis, MO, USA). Approximately $1x10^6$ cells were infected with 200,000 lentiviral units in 2 ml of complete RPMI 1640 media. Cultures were supplemented with additional media on Day 2 and Day 3. At Day 5 post-transduction, the cells were given fresh media supplemented with 1 μg/ml puromycin (Invitrogen, Carlsbad, CA, USA) for selection purposes.

2.4.8 Differentiation assays

Cell surface expression of cd11c (BD Biosciences, Franklin Lakes, NJ, USA) was determined by flow-assisted cell sorting performed according to the antibody

manufacturer's specifications (BD Biosciences) using the FACSCalibur flow cytometer (BD Biosciences). Background staining was controlled using an isotype control PE-conjugated mouse IgG1 kappa (BD Biosciences). For each sample, viable cells were gated, and expression of cd11c surface markers of 1×10^4 cells was evaluated. Nitro-blue-tetrazolium (NBT) reduction assays were performed as previously described [384]. Briefly, 1×10^6 cells were resuspended in an NBT solution consisting of 3 mg NBT, 10 μg of PMA and 1.5 mL of incomplete RPMI 1640 media. The mixture was then incubated at 37°C for 15 minutes and washed with PBS. The fraction of NBT-positive (blue) cells was determined by counting using a hemocytometer.

2.5 Results

2.5.1 Levels of PML/RARA-interacting proteins are elevated in the MR2 resistant cell line, and NPM interacts with PML/RARA only in the resistant cell line.

Previous work using high-performance liquid chromatography analysis of PML/RARA in the RA-resistant MR2 cell line revealed the formation of higher molecular-weight PML/RARA complexes, not evident in the parental NB4 line, which expresses similar levels of wild type RARA and PML/RARA [358]. Using mass spectrometry, we identified eight novel members of these complexes with increased interaction with PML/RARA in the MR2 line compared to that in the NB4 line (Table 2.1). Examination of the expression profile of six of these reveals a strong up-regulation at the protein level in the MR2 versus the NB4 cell line, without a corresponding robust increase in mRNA (Figure 2.1A). This up-regulation is selective, as several other known nuclear receptor co-regulators have the same expression, or even decreased expression, in the MR2 cell line when compared to the NB4 line (Supplemental 2.1A).

We elected to verify the interaction between NPM and PML/RARA, given this protein's central role in a large proportion of adult leukemia [378]. Disruption of the *NPM* gene by translocation is frequently found in human hematopoietic malignancies, and NPM appears to contribute to oncogenesis by activating the

oncogenic potential of the fused protein partner [385, 386]. Moreover, a recent report shows that NPM acts as a transcriptional co-repressor during RA-induced differentiation of HL60 cells [387]. We confirmed the mass spectrometry results by performing a GST-tagged PML/RARA pull-down with untreated NB4 and MR2 nuclear extracts (Figure 2.1B). Strikingly, an association between NPM and PML/RARA is detectable in the MR2 cell line only, and this interaction is mediated through the PML moiety of the fusion.

We previously validated Topoisomerase II Beta (TOP2B) as another aberrantly associated PML/RARA protein in the MR2 cell line [361]. We therefore used endogenous co-immunoprecipitation (Figure 2.1C) to show that NPM and TOP2B also associate. Consistent with the previous results, pull-down with a TOP2B antibody results in pull down of NPM from MR2 cellular extracts only.

2.5.2 NPM localizes to the *CEBPB* gene locus in MR2 cells.

The PML/RARA fusion protein blocks myeloid differentiation by repressing RA target genes and consequently RA-mediated neutrophil maturation. Quantitative real-time PCR (Q-RT-PCR) analyses over an 8 hour RA treatment reveal that mRNA accumulation of four known RARA target genes is clearly induced in NB4 cells by RA, while the same accumulation is not observed in the RA-resistant cell line, MR2 (Figure 2.2A and Supplemental Figure 2.2A).

Two well established RA-target genes, *CEBPbeta (CEBPB)* [340] and *CEBPepsilon (CEBPE) [341]*, encode members of the bZIP CEBP family of transcription factors, and both play a critical role in the differentiation of the myeloid lineage. The mRNA levels of these genes correlate with their respective protein levels, as assessed by Western blotting in both the sensitive and resistant cells (Figure 2.2B).

Normal transcriptional regulation in response to RA requires the coordinated action of RARA and a variety of cofactor complexes [388]. In order to study the exact steps of transcriptional activation in the presence of the

PML/RARA fusion and at which step a transcriptional blockade occurs in the resistant cells, we performed high-resolution quantitative ChIP analysis. This assay enables us to generate detailed maps of protein occupancy on the *CEBPB* locus after stimulating the transactivation process. The schematic in Figure 2.2C shows the most relevant features of this gene including the location of 7 amplicons used for Q-RT-PCR quantification of the ChIP-enriched DNA. Both NB4 and MR2 express similar levels of the fusion protein and initial ChIP profiling of the *CEBPB* locus under basal conditions shows similar recruitment levels and patterns of localization for RARA in both cell lines (Figure 2.2D). However, a dramatically enhanced association of NPM is observed exclusively in the resistant cells (Figure 2.2E).

2.5.3 Differential RNAPII patterning and pre-initiation complex recruitment between RA-sensitive and RA-resistant cells at the *CEBPB* gene in the RA-activated state.

RNAPII activity can be stimulated at various stages of the transcription cycle by the action of myriad regulators of recruitment and post-recruitment steps. *CEBPB* shows little preloaded RNAPII in both the sensitive and resistant cells (Figure 2.3A). Upon transcriptional activation of this gene with RA, the total amount of RNAPII associated with the proximal promoter increases in NB4 cells, an effect not observed in the MR2 cells (Figure 2.3A). This suggests that RA activates this gene, at least in part, by promoting RNAPII recruitment. RNAPII phosphorylation of the Serine 5 and Serine 2 residues (S5P and S2P) of the carboxy-terminal domain (CTD) repeats occurs at post-recruitment steps and is catalyzed by protein kinases in the initiation and elongation complexes, respectively [389]. In the sensitive cells, S5P and S2P patterning on both *CEBPB* and *CEBPE* increases strongly upon activation. S5P, a mark of active transcription initiation is increased upon treatment at the 5' end of the gene. S2P, a mark of actively elongating RNAPII, increases towards the 3' end of the gene

(Figure 2.3B-C and Supplemental Figure 2.3B-C). In contrast, neither of these signals increases in response to RA treatment in the resistant cells.

The failure of the resistant cells to recruit and activate RNAPII in response to RA stimulation led us to examine the recruitment of subunits of the Pre-Initiation Complex (PIC). Interestingly, there is a clear lack of recruitment of CDK7 (Figure 3D), the kinase subunit of the general transcription TFIIH, a key component of the PIC, and which is also the principal S5 kinase [390]. Recruitment of additional components of the PIC, such as TFIIB and TFIIF, is also stimulated by RA in the parental NB4 cell line, but not in the resistant cells (Figures 2.3E and Supplemental 2.3D). Upon activation by ligand, the retinoid receptors interact with and recruit the Mediator co-activator complex, which stimulates RNAPII activity by diverse mechanisms, including positive effects on PIC formation, enhancer-promoter chromatin looping and transcription elongation. The subunit of the Mediator complex that is responsible for interaction with nuclear receptors was identified as MED1 (DRIP205, TRAP220) [391]. We have previously shown that MED1 interacts with the PML/RARA found in NB4 cells in a ligand-dependent manner [367]. Furthermore, the MR2 RA-resistant cell line maintained normal MED1 complexes that interacted with retinoid receptors in a ligand-dependent manner, when assessed by an *in vitro* GST pull-down assay. However, by conducting ChIP tiling analyses, we now show that RA fails to recruit MED1 to the endogenous *CEBPB* promoter in the MR2 cell line (Figure 2.3F). Together, these data demonstrate that the molecular mechanism of RA resistance involves a defect in RNAPII recruitment and activation at the promoter of key differentiation genes.

2.5.4 NPM inhibition restores sensitivity to RA-induced gene transcription and differentiation.

To examine whether NPM overexpression and aberrant interaction with PML/RARA is responsible for the repression of RA-induced gene expression in the MR2 cell line, we treated both NB4 and MR2 cells with the NPM inhibitor,

NSC348884. Treatment with this small molecule inhibitor has been shown to disrupt NPM's higher order structures [392]. Strikingly, in the resistant cells, NPM inhibition, in combination with RA, leads to increases in CDK7 recruitment (Figure 2.4A), S5 phosphorylation (Figure 2.4B) and restored mRNA expression of the RA-target genes (Figure 2.4C and Supplemental 2.4A).

Our data that NPM inhibition overrides the transcriptional repression on RA-target genes led to the hypothesis that this inhibition might also abolish the differentiation block in the MR2 cell line. Treatment of sensitive cells with RA induces expression of the myeloid-specific cell surface marker, cd11c. We first assessed cd11c expression by FACS analysis in NB4 and MR2 cells treated for three days either with RA, NPM inhibitor (NPMi) or the combination (Figure 2.4D). We observe that RA alone and the combination of RA and NPMi are both sufficient to cause increased cd11c cell surface expression in the RA-sensitive NB4 cells. However, in the MR2 cell line, only the combination treatment led to the re-establishment of this differentiation marker.

To confirm that NPM inhibition overcomes the differentiation block in APL cells, we performed a nitro-blue-tetrazolium (NBT) reduction analysis, which assesses terminal granulocytic differentiation [393]. While NPMi alone had little discernible effect on differentiation in either cell line, RA and NPMi co-treatment leads to significant NBT reduction within 5 days (Figure 2.4E). This is consistent with the ChIP analysis, mRNA expression and cd11c data, where only a modest response to treatment was observed in the MR2 cell line in response to 10^{-6} M RA.

2.5.5 RA induces recruitment of the chromatin remodeler BRG1 to the *CEBPB* gene in a TOP2B/NPM-dependent fashion.

In order to activate gene expression, retinoid receptors must override repressive chromatin structures. To this end, ligand-induced conformational changes in the receptors will cause the dissociation of co-repressors and the concomitant recruitment of co-activators with histone modifying and chromatin

remodeling activities necessary for RNAPII recruitment [253]. In the resistant cells, as well as in the sensitive cells, there is an increase in histone H4 acetylation (Figure 2.5A) throughout the gene body as well as in the promoter in response to RA. This indicates that activator-induced histone acetylation is not sufficient for RNAPII activation, an observation also made in other systems [87].

ChIP tiling of the steps upstream of transcription initiation revealed a more robust recruitment of the chromatin-remodeler ATPase subunit BRG1 to the *CEBPB* locus in response to RA-treatment in the resistant cells compared to the sensitive cells (Figure 2.5B). Examination of the BRG1 expression profile discloses a strong up-regulation in MR2 cells compared to NB4 cells (Figure 2.5C, left), without a corresponding biologically significant increase in mRNA (Figure 2.5C, right).

Having shown that TOP2B interacts with both PML/RARA and NPM in the resistant cells, we next used endogenous co-immunoprecipitation (Figure 2.5D) to investigate whether BRG1 and TOP2B associate. Pull down with TOP2B followed by immunoblotting for BRG1 reveals a basal interaction between the two proteins that is enhanced with RA treatment. In agreement with the ChIP analysis, this interaction is limited to the resistant cell line. To examine whether this interaction is necessary for the aberrant recruitment of BRG1 to the *CEBPB* locus in the MR2 cell line, we treated both NB4 and MR2 cells with the TOP2B inhibitor, ICRF. Treatment with this catalytic inhibitor has been shown to induce TOP2B degradation [394]. ChIP analyses performed on cells treated with RA, ICRF or the combination (Figure 2.5E) demonstrated a direct correlation between TOP2B inhibition and reduced BRG1 recruitment in the MR2 cell line. Interestingly, treatment with ICRF in NB4 cells had the opposite effect, enhancing BRG1 recruitment, suggesting a differential functional outcome of BRG1 recruitment between the cell lines. Similar results were obtained when both cell lines were treated with the NPM inhibitor alone or in combination with RA (Figure 2.5F). Together these results indicate that the resistant cell line expressed

elevated levels of BRG1 and that recruitment of BRG1 to the promoter of RA-target genes was dependent on the repressive factors TOP2B and NPM in MR2 cells only. These data suggest NPM, TOP2B and BRG1 cooperate as a repressive complex, effectively suppressing PML/RARA mediated transcription.

2.5.6 BRG1 knockdown and pharmacological targeting of BRG1 with Darinaparsin restores sensitivity to RA-induced gene transcription and differentiation.

We next investigated whether BRG1 functioned as a repressor of RA-induced transcriptional activation in the MR2 cell line. Five stable BRG1 knockdown clones, along with a stable clone expressing a non-targeting shRNA were created in both the NB4 and R2 cell lines. Based on the efficacy of BRG1 knockdown in the MR2 cell line (Supplemental Figure 2.5A), we selected three clones (sh2, sh3 and sh5) with which to continue our investigation.

Q-RT-PCR analysis of RA-target gene expression revealed BRG1 knockdown restored sensitivity to RA treatment in the MR2 cell line, in all three clones tested (Figure 2.6A and Supplemental Figure 2.5B). We assessed cd11c expression by FACS analysis in all NB4 and MR2 clones treated for three days with RA (Supplemental Figure 2.5C). We observe that RA alone and the combination of RA and BRG1 knockdown are both sufficient to cause increased cd11c cell surface expression in the RA-sensitive NB4 cells. However, in the MR2 clones, only the combination of RA with knockdown of BRG1 expression led to the re-establishment of this differentiation marker.

To confirm that BRG1 knockdown overcomes the differentiation block in APL cells, we again performed a nitro-blue-tetrazolium (NBT) reduction analysis. While BRG1 knockdown alone had little discernible effect on differentiation of any of the cell lines, RA and BRG1 co-treatment lead to significant NBT reduction within 5 days (Figure 2.6B). This is consistent with the mRNA expression and cd11c data, where only a modest response to treatment was observed in the MR2 cell line in response to 10^{-6} M RA after 5 days.

Cumulatively, these results indicate that knockdown of BRG1 in resistant cells restored sensitivity to RA-mediated differentiation, from early gene expression to terminal functional capacity. Moreover, these results substantiate the hypothesis that BRG1 acts as a transcriptional repressor in the MR2 cell line.

Darinaparsin (Dar) is a novel arsenical currently in clinical trials as a chemotherapeutic [395]. Previously, our lab demonstrated that Dar treatment of NB4 cells prevents recruitment of BRG1 to the *HMOX1* promoter [395]. We thus investigated if Dar treatment of MR2 cells would restore sensitivity to RA through an analogous mechanism. Q-RT-PCR of cells treated with low doses of Dar in combination with RA showed restored expression of RA-target genes in the MR2 cell line, while neither Dar nor RA alone could achieve this (Figure 2.6C and Supplemental Figure 2.5D). This restored expression of RA target genes was sufficient to induce differentiation in the MR2 cells, as shown by expression of cd11c, again only when treated with the combination (Supplemental Figure 2.5E). Finally, we confirmed the induction of functional differentiation by RA and Dar in the MR2 cell line as assessed by NBT reduction assay (Figure 2.6D). Similar to BRG1 knockdown, NB4 cells were maximally sensitive to RA treatment alone, and neither gene expression, cd11c expression, nor NBT reduction were substantially enhanced by co-treatment with Darinaparsin (Figures 2.6C, 2.6D, Supplemental 2.5D and E). Thus, these results demonstrate that resistance to RA-induced differentiation in the MR2 cell line can be overcome by concomitant administration of Darinaparsin with RA. Furthermore, these results establish a novel function for Darinaparsin in the context of APL cells that are resistant to primary courses of treatment using retinoic acid, and establish a basis for further clinical study.

2.6 Discussion

Previously, we observed in the MR2 cell line that PML/RARA associated with a higher molecular weight complex than that in the NB4 cell line. Subsequent mass spectrometry identified eight proteins that had novel interactions

with PML/RARA. Two of these, TOP2B and NPM, have now been shown to mediate repression in the MR2 cell line, as inhibition of these proteins resulted in a loss of resistance to RA. We thus hypothesize that these interactions represent a novel co-repressor complex that interacts with PML/RARA and represses genes critical for cellular differentiation. We further this by demonstrating that both TOP2B and NPM are necessary for the recruitment of BRG1, and that functional inhibition of these proteins abates BRG1's presence at the *CEBPB* gene in MR2 cells. Similarly, many of the other proteins identified in the MR2 PML/RARA complex (Table 2.1) have been shown to interact with BRG1 both directly and indirectly. For example, SAP130 associates with the corepressor complex mSin3A, which incorporates BRG1 under certain conditions [396]. HNRNPU has been shown to form a complex with BRG1 that is necessary for RNAPII mediated transcriptional activity and interactions between HNRNPC1/C2 and the SWI/SNF complex, which includes BRG1, have also been reported [397, 398]. Most interestingly, PML itself has been shown to mediate recruitment of BRG1 to the *Oct4* promoter [399].

Importantly, our data now define a novel function for NPM as a negative regulator of RA-induced gene regulation and differentiation toward granulocytes. In RA-resistant cells, we show an interaction between PML/RARA, the fusion most commonly underlying APL, and NPM that is mediated through the PML portion of the chimera. The presence of NPM is inversely correlated PIC formation at the *CEBPB* locus, and pharmacological targeting of NPM in combination with RA relieved the inhibition of transcription exerted by this protein.

NPM is inextricably linked to human tumorigenesis. NPM has been found as a fusion partner to RARA in a rare variant of APL, and to the ALK kinase in in Anaplastic Large Cell Lymphoma (ALCL). Strikingly, *NPM1* has also been found to be mutated and aberrantly localized in leukemic blasts in a high proportion of patients with Acute Myeloid Leukemia. NPM has been shown to

enhance the proliferative potential of hematopoietic stem cells (HSCs), and NPM overexpression increases HSC survival upon DNA damage and oxidative stress [400]. Beyond hematological malignancies, NPM is frequently overexpressed in solid tumors of diverse histological origin. Many mechanisms have been proposed for how altered NPM expression contributes to oncogenesis, however, these probably stem from NPM's many proposed roles in very diverse cellular processes. We hypothesize that the over-expressed NPM found in our model might not exclusively locate within the nucleolus, but might be found throughout the nucleus where it now has the opportunity to interact with PML in the altered nuclear architecture of APL cells. This interaction might be mediated by the coiled-coil domains found in both PML and NPM.

Transcriptional profiling by ChIP analysis surprisingly revealed that RA treatment of our resistant cells was associated with enhanced recruitment of BRG1. BRG1 is an ATPase helicase subunit of the SWI/SNF family of proteins that serves to regulate gene expression by altering the chromatin landscape surrounding genes by modulating the position of nucleosomes in relation to DNA [401]. Thus, BRG1 can function both to activate and repress gene expression [402]. We hypothesized that BRG1 mediates resistance to RA-induced differentiation in the MR2 cells. Abrogation of BRG1 activity, either by knockdown, or pharmacological intervention, restored sensitivity to RA as observed by early gene expression, cd11c surface expression, and functional capacity of differentiated cells.

First, our results first implicate BRG1 as a transcriptional repressor in the MR2 cell line. BRG1 as a transcriptional repressor has been demonstrated in other contexts. For example, Ooi *et al.* showed that BRG1 enhanced REST-mediated repression by recruiting and stabilizing binding of this repressor complex to chromatin [403]. BRG1 has also been shown to interact with and recruit DNA methyltransferases, thus promoting gene silencing by DNA methylation [404]. Additionally, BRG1 interacts with corepressive complexes such as

mSin3A/HDAC2/PRMT5, which promote histone tail deacetylation and methylation and subsequent chromatin condensation [405]. Our results are congruent with the above findings and provide a novel setting in which BRG1 serves as a mediator of transcriptional repression. However, whether or not this is dependent upon its nucleosomal repositioning capacity remains a topic of current investigation. Based on the data presented herein, we hypothesize that BRG1 might act to further contribute to a more heterochromatic architecture at the promoters of RA target genes in the MR2 cell line.

Second, we show that the addition of Darinaparsin to RA treatment restores sensitivity in resistant cells. Darinaparsin is a novel organic arsenical that is currently in phase II clinical trials as a chemotherapeutic in the treatment of various malignancies, although its mechanism of action remains largely uncharacterized [406]. Previously, our lab demonstrated that treatment with Darinaparsin prevented recruitment of BRG1 to the *HMOX1* promoter in NB4 cells [395]. Here, we demonstrated that targeted BRG1 knockdown restored sensitivity to RA treatment in resistant cells. However, knockdown is not currently a viable clinical approach, and thus we examined if Darinaparsin's effects on BRG1 would restore RA-sensitivity. Our results demonstrated that only the combination of Darinaparsin and RA could overcome the transcription block in resistant cells, while neither treatment alone was effective. The application of arsenicals in other malignancies has been limited by the high concentrations necessary to achieve cytotoxicity [407-409]. Our results are interesting as they provide a setting in which low-dose treatment with Dar can be used to promote differentiation instead of cytotoxicity, therefore potentially broadening the therapeutic spectrum of arsenic-based compounds in the treatment of cancer.

Cellular mechanisms underlying resistance to chemotherapeutic agents have been carefully studied, as experimental models can be easily generated via *in vitro* selection. Resistance to the differentiating effects of RA in APL cells *in vitro* and *in vivo* is traditionally associated with a mutation in the ligand-binding

domain of the PML/RARA oncogenic fusion protein [355]. In contrast, we report that stable resistance to RA in an acute leukemia cell line, in the context of a wild-type PML/RARA is driven by an aberrant association with a putative co-repressor complex containing NPM and TOP2B leading to recruitment of BRG1 to RA-target genes. The finding that BRG1 activity at gene loci can underlie an oncogenic process such as resistance to treatment opens up opportunities for compounds directed at BRG1, such as Darinaparsin, to be used in combination with chemotherapy to treat adult leukemia patients to maximize cell death or differentiation.

2.7 Acknowledgements

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2.8 Figures

Table 2.1: Identification of proteins associated with GST-PML/RARA in the MR2 cell line.

Protein	Mass (Da)	Ion Score
DNA Topoisomerase II Beta	183,548	493
Replication Factor C Subunit 4	40,170	465
Nucleophosmin	31,090	371
Hetereogeneous Nuclear Ribonucleoproteins C1/C2	32,004	304
60S Acidic Ribosomal Protein P0	34,423	106
Histone Deacetylase Complex Subunit SAP130	136,590	82
U5 Small Nuclear Ribonucleoprotein 40 kDa	39,730	68
Hetereogeneous Nuclear Ribonucleoproteins U	89,631	62

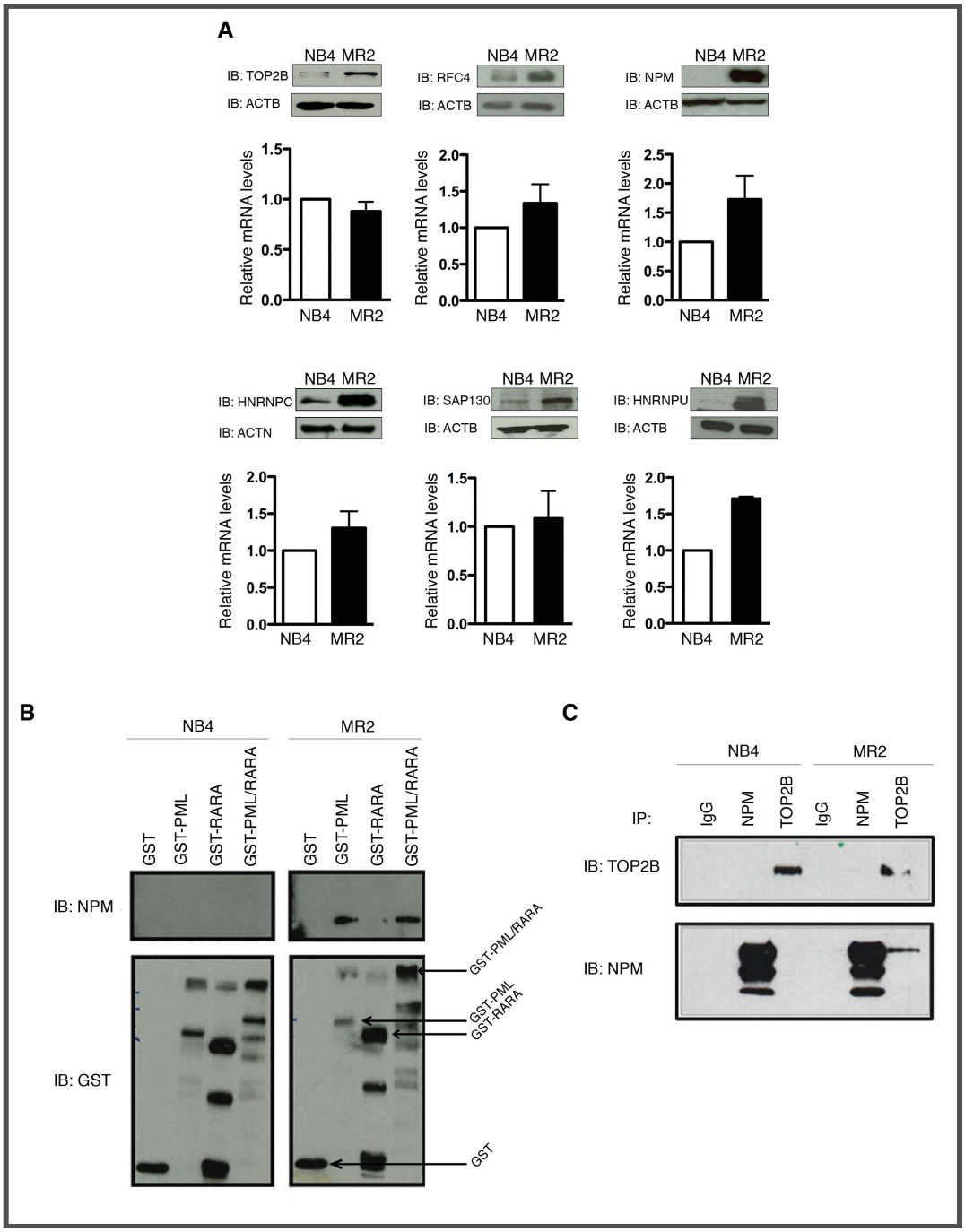

A
NB4 MR2
IB: TOP2B
IB: ACTB
IB: RFC4
IB: NPM
IB: HNRNPC
IB: ACTN
IB: SAP130
IB: HNRNPU
Relative mRNA levels
NB4
MR2
B
NB4
MR2
GST
GST-PML
GST-RARA
GST-PML/RARA
IB: NPM
IB: GST
C
IP:
IgG
NPM
TOP2B
IB: TOP2B
IB: NPM

Figure 2.1. NPM interacts with both PML/RARA and TOPO2B in the MR2 resistant cells only. (A) Differential protein (top panels) and mRNA (bottom panels) levels of six proteins identified as being differentially associated with a GST-PML/RARA fusion in MR2 cells versus NB4 cells. (B) GST pull-down assay defines PML/RARA domains that mediate interaction with NPM. Purified GST, GST-PML, GST-RARA and GST-PML/RARA protein were incubated with nuclear extracts from NB4 and MR2 cells (C) Endogenous co-immunoprecipitation with TOP2B and NPM antibodies followed by immunoblotting indicates an interaction between NPM and TOPO2B solely in MR2 cells.

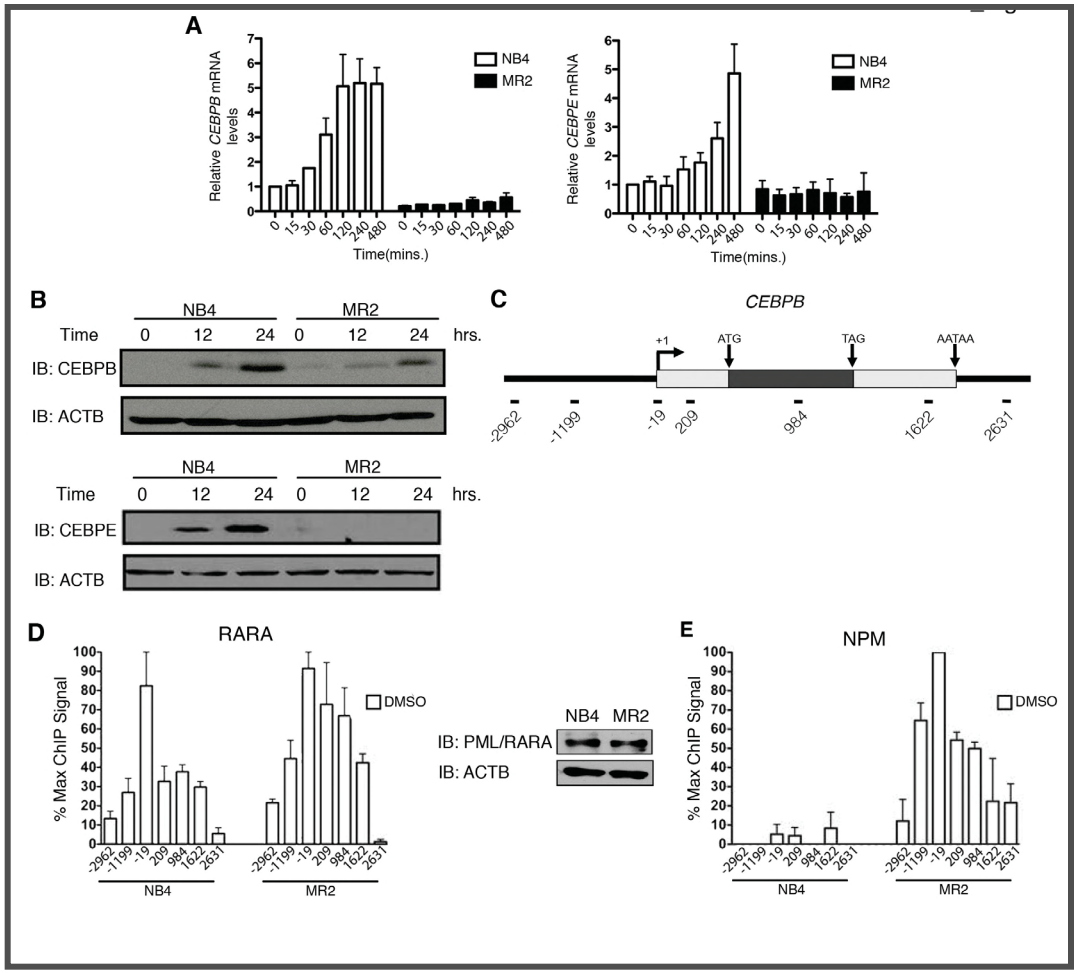

A
Relative CEBPB mRNA levels
NB4
MR2
Time(mins.)
Relative CEBPE mRNA levels
B
NB4
MR2
Time
hrs.
IB: CEBPB
IB: ACTB
IB: CEBPE
C
CEBPB
+1
ATG
TAG
AATAA
D
RARA
% Max ChIP Signal
DMSO
IB: PML/RARA
E
NPM

Figure 2.2. Defective transcriptional activation of RARA target genes upon RA treatment in MR2 versus the NB4 cell line correlates with increased NPM at the promoter. (A) Quantitative real-time PCR analysis of *CEBPbeta* and *CEBPepsilon* mRNA induction following 8 hour RA treatment expressed as fold induction over untreated cells after normalization to 18S rRNA levels. Error bars represent the standard error of the mean. (B) Immunoblot analysis demonstrating differential CEBPB and CEBPE protein expression in response to RA treatment (C) Schematic of the *CEBPbeta* locus indicating the overall gene structure. Amplicons used in real-time PCR quantification of ChIP-enriched DNA are named according to their relative distance (bps) to the transcription start site. *CEBPbeta* is an intronless gene. (D) High-density ChIP tiling of RARA at the *CEBPbeta* locus under basal conditions in NB4 and MR2 cells and immunoblot analysis of PML/RARA expression levels in the two cell lines. (E) High-density ChIP tiling of NPM at the *CEBPbeta* locus under basal conditions in NB4 and MR2 cells.

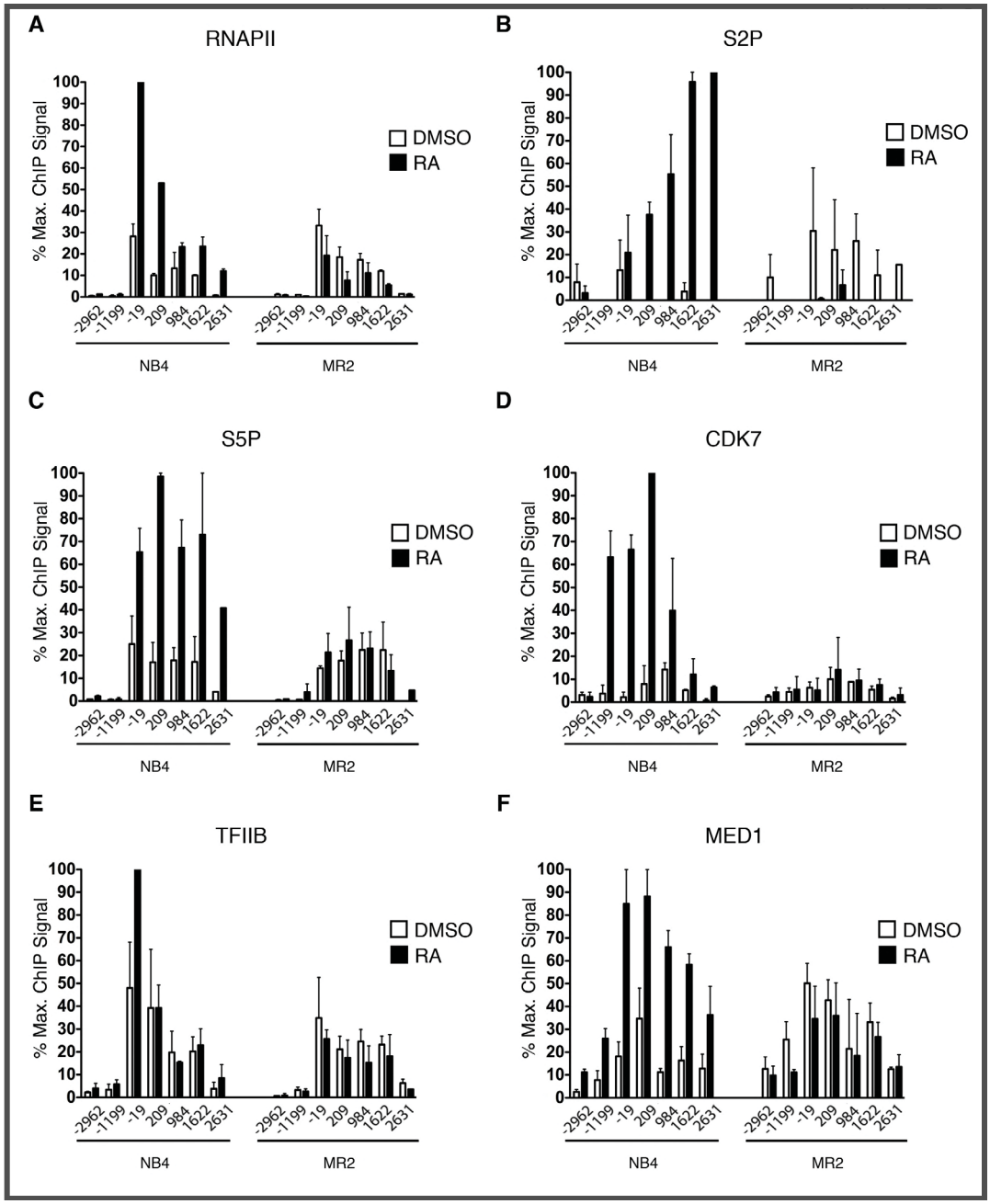

A
RNAPII
B
S2P
C
S5P
D
CDK7
E
TFIIB
F
MED1
% Max. ChIP Signal
DMSO
RA
-2962
-1199
-19
209
984
1622
2631
NB4
MR2

Figure 2.3. Differential recruitment of transcription initiating factors to the *CEBPbeta* gene locus in response to RA treatment. (A-F) High-density ChIP tiling of RNAPII, RNAPII phosphorylation at Serine 2 (S2P), RNAPII phosphorylation at Serine 5 (S5P), CDK7, TFIIB and MED1 at the *CEBPbeta* locus before and after RA treatment (1 hr) of NB4 and MR2 cells.

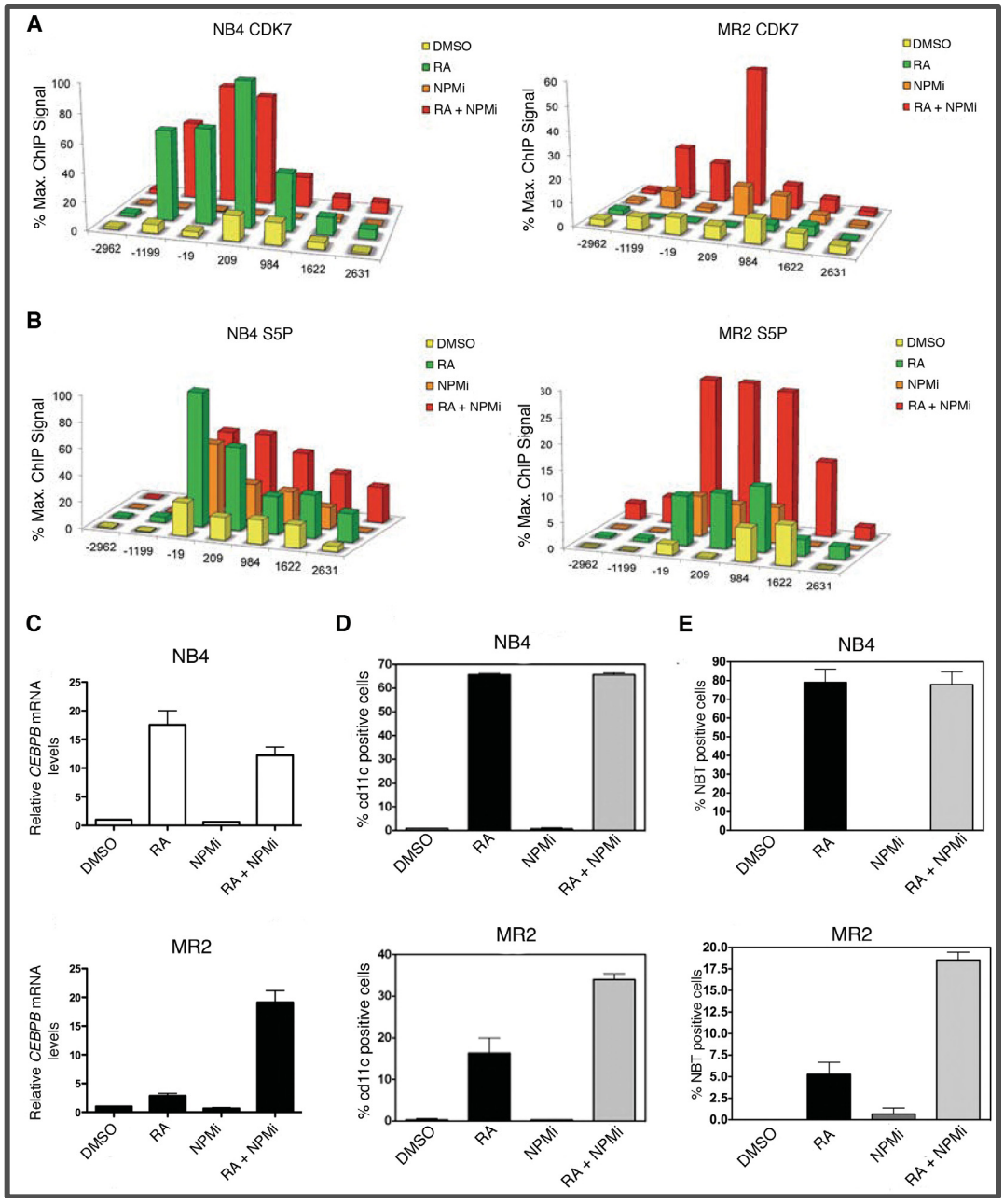

A
NB4 CDK7
% Max. ChIP Signal
DMSO
RA
NPMi
RA + NPMi
-2962
-1199
-19
209
984
1622
2631
MR2 CDK7
B
NB4 S5P
MR2 S5P
C
NB4
Relative CEBPB mRNA levels
DMSO
RA
NPMi
RA + NPMi
MR2
D
NB4
% cd11c positive cells
MR2
E
NB4
% NBT positive cells
MR2

Figure 2.4. Inhibition of NPM restores myeloid lineage differentiation in resistant cells. (A) ChIP analysis was carried out using protein extracts of DMSO, RA (1hr), NPMi (16hr) or NPMi and RA-treated (16 hr + 1hr) MR2 and NB4 cells using an antibody recognizing CDK7. (B) ChIP analysis was carried out using protein extracts of DMSO, RA (1hr), NPMi (16hr) or NPMi and RA-treated (16 hr + 1hr) MR2 and NB4 cells using antibodies against phosphorylated Serine 5 of RNAPII (S5P). (C) Quantitative real-time PCR analysis of *CEBPbeta* mRNA induction following DMSO, RA, NPMi or RA and NPMi treatment. Data is expressed as fold induction over DMSO treated cells and normalized to 18S ribosomal RNA levels. Error bars represent the standard error. (D) Percentages of NB4 and MR2 cells expressing the differentiation marker cd11c in response to 3-day exposure to RA, NPMi, or a combination of both. (E) Results of nitro-blue-tetrazolium reduction assay performed on NB4 and MR2 cells treated with RA, NPMi, or the combination for 5 days.

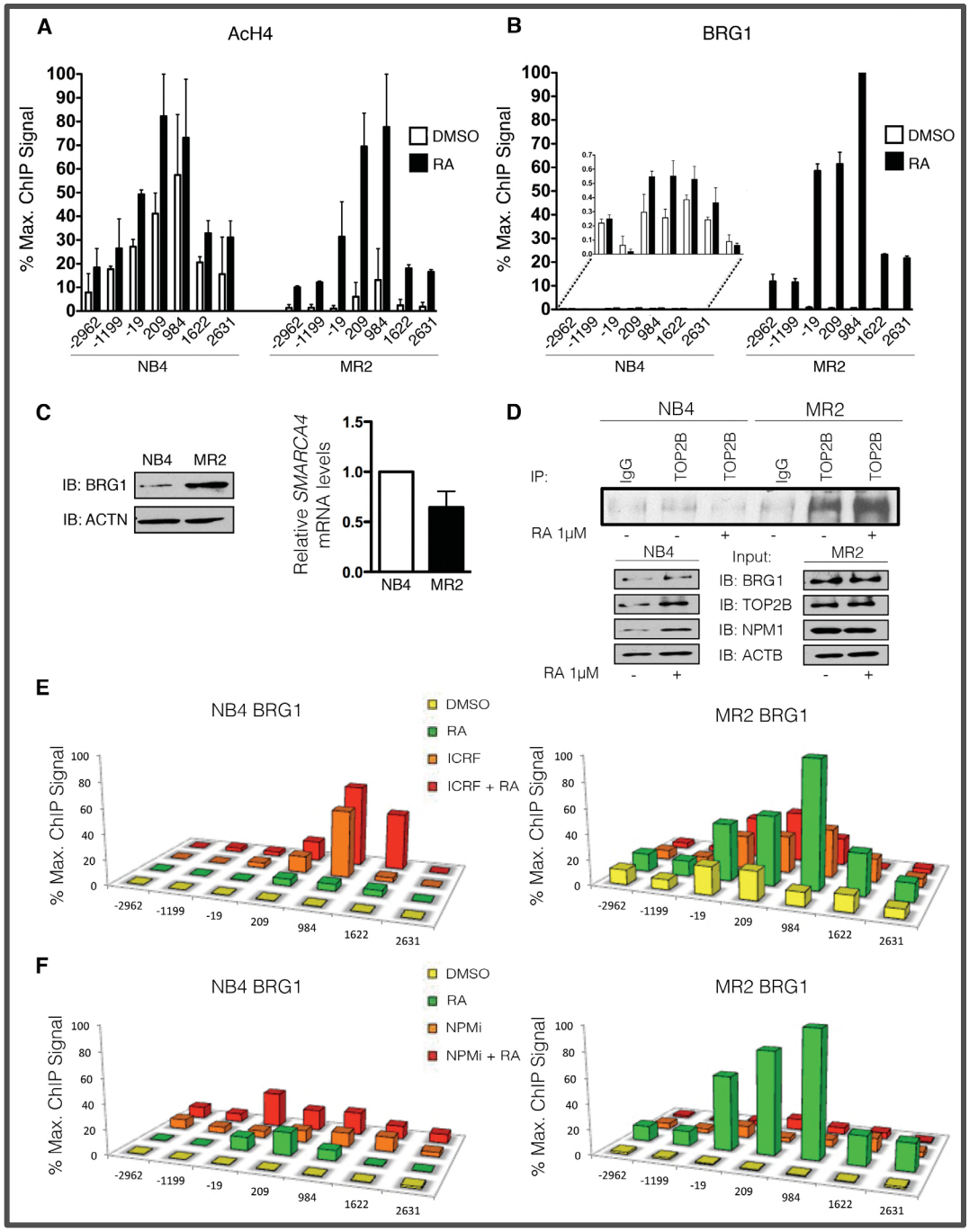

A
AcH4
% Max. ChIP Signal
DMSO
RA
-2962
-1199
-19
209
984
1622
2631
NB4
MR2
B
BRG1
C
NB4
MR2
IB: BRG1
IB: ACTN
Relative SMARCA4 mRNA levels
D
IP:
IgG
TOP2B
RA 1μM
Input:
IB: BRG1
IB: TOP2B
IB: NPM1
IB: ACTB
E
NB4 BRG1
MR2 BRG1
ICRF
ICRF + RA
F
NPMi
NPMi + RA

Figure 2.5. Differential recruitment of BRG1 to the *CEBPbeta* gene locus in response to RA treatment that is NPM and TOP2B dependent. (A) High-density ChIP tiling of acetylated H4 (AcH4) at the *CEBPbeta* locus before and after RA treatment (1 hr) of NB4 and MR2 cells. (C) Immunoblot analysis demonstrating differential basal BRG1 protein expression in NB4 and MR2 cells (left) with no corresponding increase in BRG1 mRNA levels (right). (D) Endogenous co-immunoprecipitation with TOP2B antibody followed by immunoblotting for BRG1 indicates an interaction between BRG1 and TOP2B solely in MR2 cells. (E) High-density ChIP tiling of BRG1 at the *CEBPB* locus after treatment with DMSO, RA (1hr), the TOP2B inhibitor ICRF (overnight), or a combined treatment of ICRF (overnight pretreatment) and RA (1hr) in NB4 and MR2 cells. (F) High-density ChIP tiling of BRG1 at the *CEBPB* locus after treatment with DMSO, RA (1hr), NPM inhibitor (NPMi, overnight), or a combined treatment of NPMi (overnight pretreatment) and RA (1hr) in NB4 and MR2 cells.

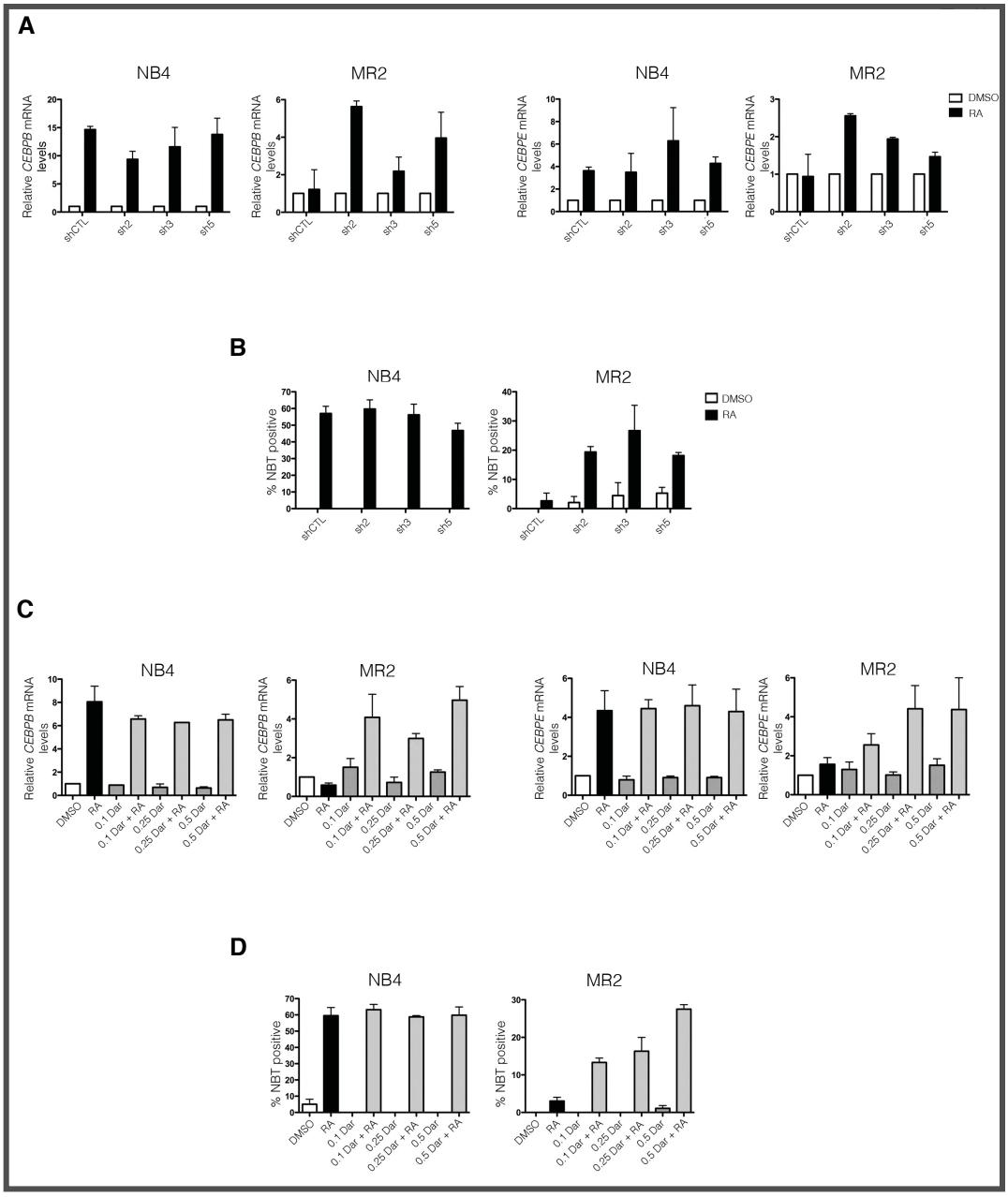
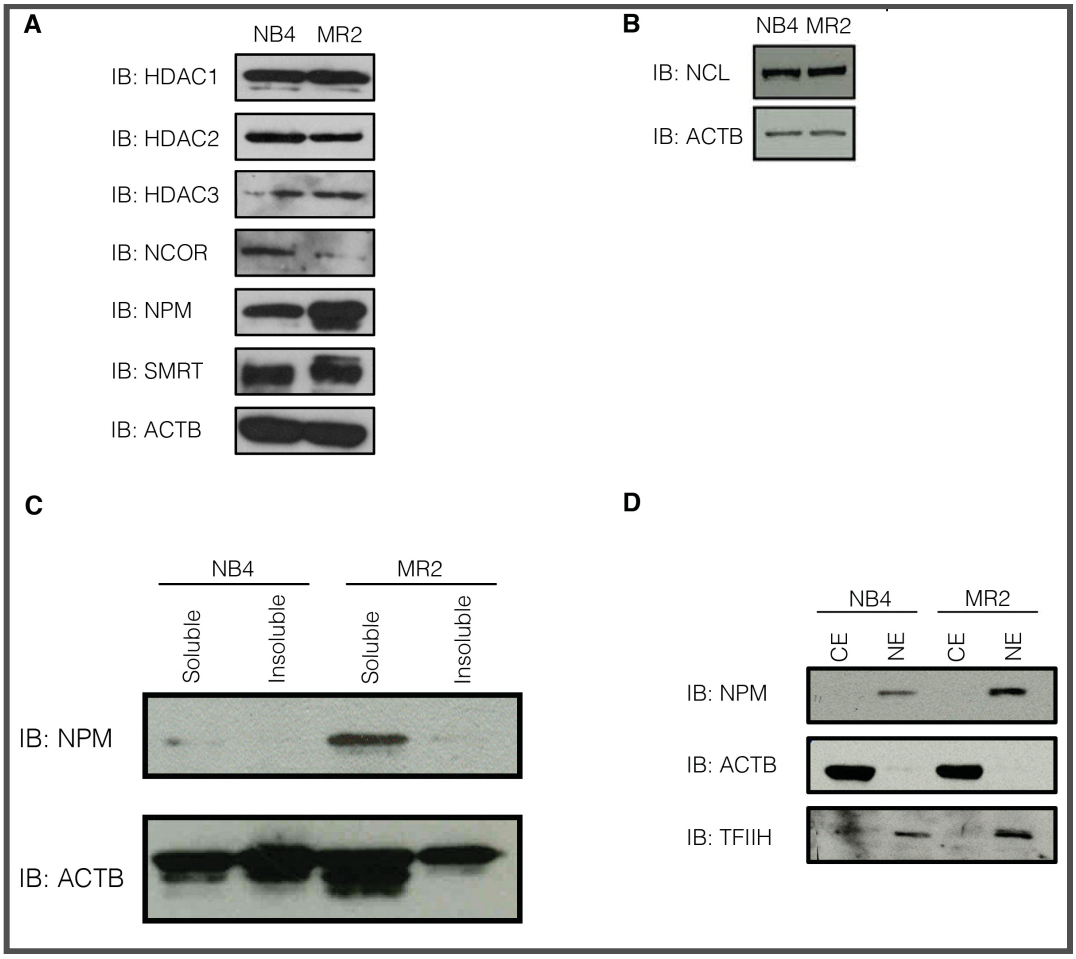
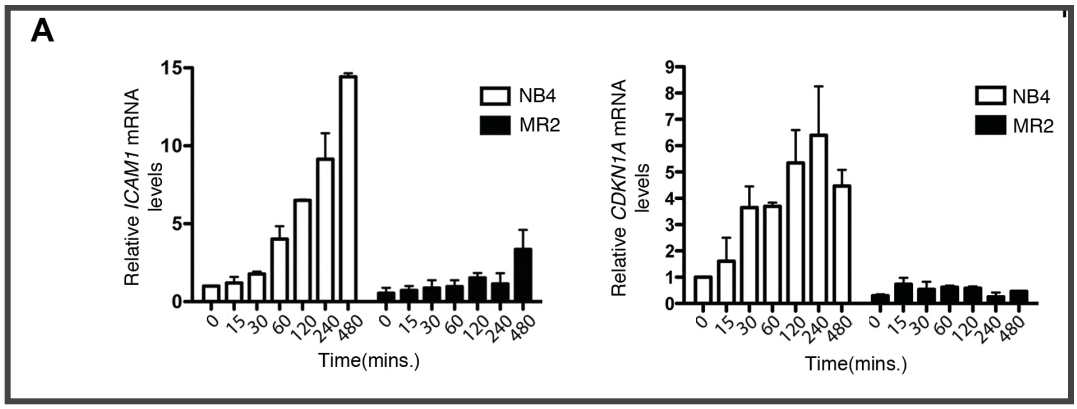

A
NB4
MR2
NB4
MR2
Relative CEBPB mRNA levels
Relative CEBPE mRNA levels
shCTL
sh2
sh3
sh5
DMSO
RA
B
NB4
MR2
% NBT positive
C
NB4
MR2
NB4
MR2
DMSO
RA
0.1 Dar
0.1 Dar + RA
0.25 Dar
0.25 Dar + RA
0.5 Dar
0.5 Dar + RA
D
NB4
MR2
% NBT positive

Figure 2.6. Targeting BRG1 restores sensitivity to RA-induced gene transcription and differentiation. (A) Quantitative real-time PCR analysis of *CEBPB* and *CEBPE* mRNA induction in BRG1 knockdown clones following 8 hour RA treatment expressed as fold induction over untreated cells after normalization to 18S rRNA levels. Error bars represent the standard error. (B) Results of nitro-blue-tetrazolium reduction assay performed on BRG1 knockdown NB4 and MR2 cells treated with RA for 5 days, with retreatment at day 3. (C) Quantitative real-time PCR analysis of *CEBPB* and *CEBPE* mRNA induction following pretreatment with increasing doses of Darinaparsin, with or without subsequent 8 hour RA treatment. Results express fold induction over untreated cells after normalization to 18S rRNA levels. Error bars represent the standard error. (D) Results of nitro-blue-tetrazolium reduction assay performed on NB4 and MR2 cells treated with RA, Darinaparsin, or both for 5 days, with retreatment at day 3.

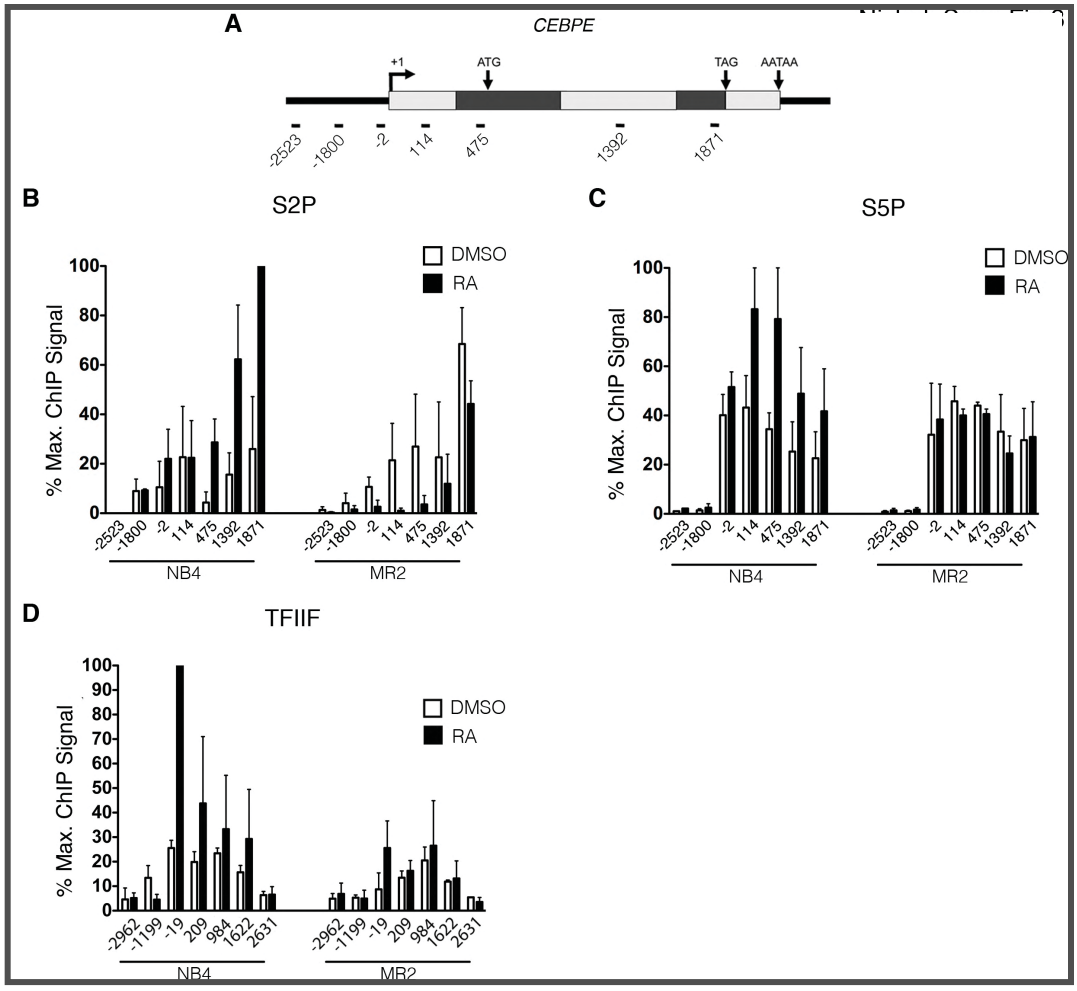
2.9 Supplemental Figures



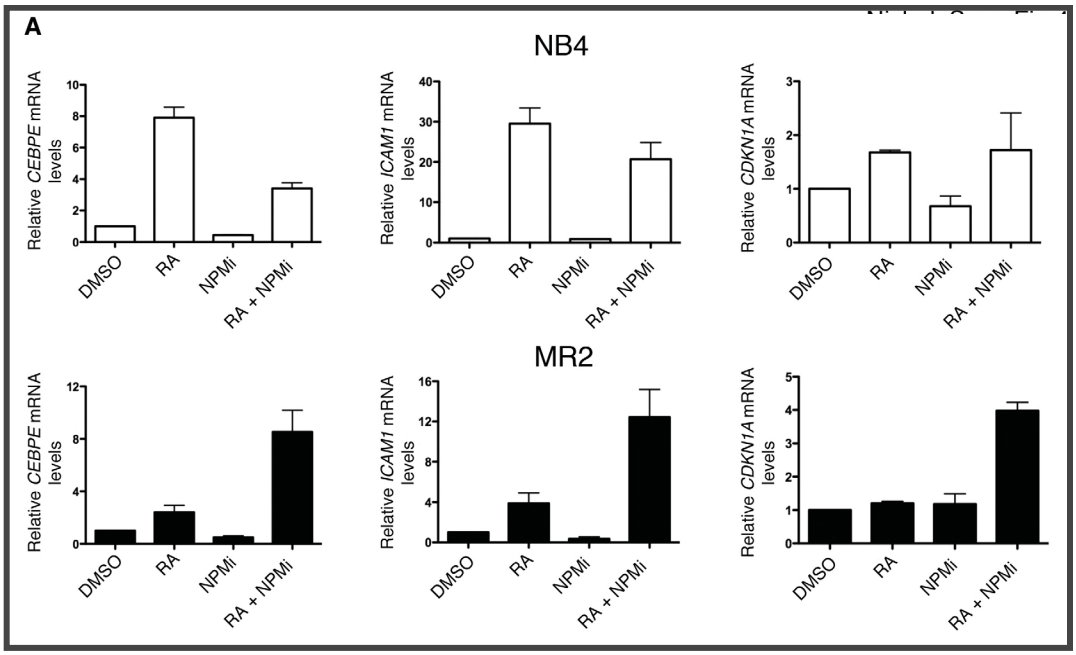
Supplemental Figure 2.1. Overexpression of NPM in MR2 cells is not due to universal protein up-regulation, nor differences in NPM solubility or cellular localization. (A) Immunoblot analysis of several established nuclear receptor co-factors. (B) Immunoblot analysis of the nucleolar protein, Nucelolin (NCL). (C) Immunoblot analysis of NPM expression in both soluble and insoluble cell fractions in both NB4 and MR2 cells. (D) Nuclear-cytoplasmic fractionation of both NB4 and MR2 cells followed by immunoblot analysis for NPM. Beta-Actin (ACTB) and TFIIH serve as cytoplasmic and nuclear fractionation controls, respectively.



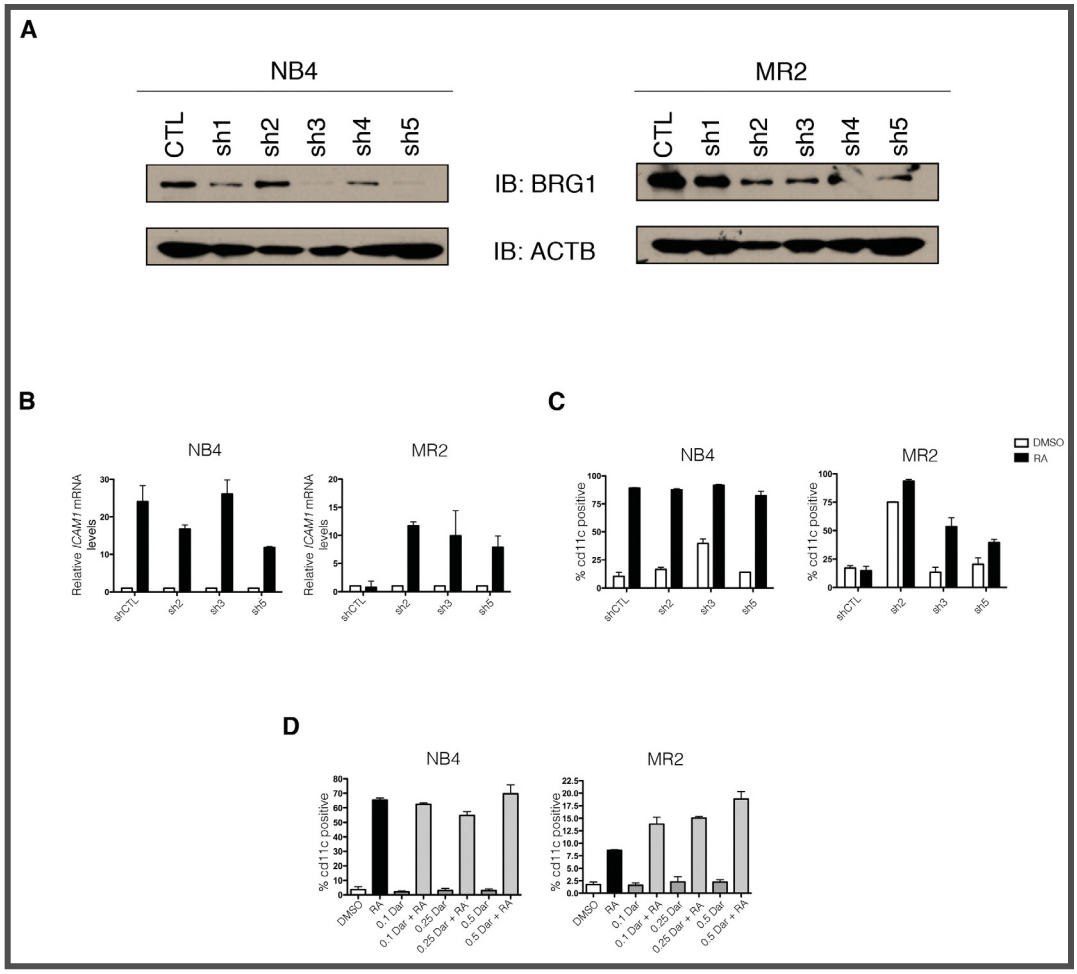
Supplemental Figure 2.2. Defective transcriptional activation of RARA target genes upon RA treatment in MR2 versus the NB4 cell line. (A) Quantitative real-time PCR analysis of *ICAM1* and *CDKN1A* mRNA induction following 8 hour RA treatment expressed as fold induction over untreated cells after normalization to 18S rRNA levels. Error bars represent the standard error of the mean.


A
CEBPE
+1
ATG
TAG
AATAA
-2523
-1800
-2
114
475
1392
1871
B
S2P
C
S5P
DMSO
RA
% Max. ChIP Signal
NB4
MR2
D
TFIIF
-2962
-1199
-19
209
984
1622
2631

Supplemental Figure 2.3. Differential state of RNAPII phosphorylation at the *CEBPepsilon* gene locus in response to RA treatment. (A) Schematic of the *CEBPepsilon* locus indicating the overall gene structure. Amplicons used in real-time PCR quantification of ChIP-enriched DNA are named according to their relative distance (bps) to the transcription start site. (B-C) High-density ChIP tiling of RNAPII phosphorylation at Serine 2 (S2P) and RNAPII phosphorylation at Serine 5 (S5P) at the *CEBPepsilon* locus before and after RA treatment (1 hr) of NB4 and MR2 cells. (D) High-density ChIP tiling of TFIIF at the *CEBPbeta* locus before and after RA treatment (1 hr) of NB4 and MR2 cells.



Supplemental Figure 2.4. Inhibition of NPM restores transcriptional activation in response to RA in the MR2 cell line. (A) Quantitative real-time PCR analysis of *CEBPE*, *ICAM1, and CDKN1A* mRNA induction following DMSO, RA, NPMi or RA and NPMi treatment expressed as fold induction over DMSO cells after normalization to 18S rRNA levels. Error bars represent the standard error of the mean.


A
NB4
MR2
CTL
sh1
sh2
sh3
sh4
sh5
IB: BRG1
IB: ACTB
B
NB4
MR2
Relative ICAM1 mRNA levels
shCTL
sh2
sh3
sh5
C
NB4
MR2
DMSO
RA
% cd11c positive
shCTL
sh2
sh3
sh5
D
NB4
MR2
% cd11c positive
DMSO
RA
0.1 Dar
0.1 Dar + RA
0.25 Dar
0.25 Dar + RA
0.5 Dar
0.5 Dar + RA

Supplemental Figure 2.5. Targeting BRG1 restores sensitivity to RA-induced gene transcription and cell-surface marker expression. (A) Immunoblot analysis to assess efficiency of shRNA knockdown of BRG1 in NB4 and MR2 cells. Cells were transduced with 5 distinct shRNAs and one non-targeting shRNA as control. (B) Quantitative real-time PCR analysis of *ICAM1* mRNA induction in NB4 and MR2 BRG1 knockdown clones following 8-hour RA treatment expressed as fold induction over untreated cells after normalization to 18S rRNA levels. Error bars represent the standard error of the mean. (C) Percentages of BRG1 knockdown NB4 and MR2 cells expressing the differentiation marker cd11c in response to 3-day treatment with RA. (D) Percentages of NB4 and MR2 cells expressing the differentiation marker cd11c in response to 3-day treatment with RA, Darinaparsin, or a combination of the two.

Supplemental Table 2.1: Q-RT-PCR Primers

CDKN1A F	CTGGAGACTCTCAGGGTCGAAA
CDKN1A R	GATTAGGGCTTCCTCTTGGAGAA
CEBPB F	AGAAGACCGTGGACAAGCACA
CEBPB R	CTCCAGGACCTTGTGCTGCGT
CEBPE F	CCAGCCTCTGCGCGTTCTCAA
CEBPE R	CAAGGCTATCTTTGTTCACTGCC
HNRNPC F	TGTGGAGGCAATCTTTTCGA
HNRNPC R	TGATACACGCTGACGTTTCG
HNRNPU F	ATAAGCAACAGGGAGAAAATTAGGTAA
HNRNPU R	GGAGGCAAGGGAAGGATGAG
ICAM1 F	GCAGACAGTGACCATCTACAGCTT
ICAM1 R	CTTCTGAGACCTCTGGCTTCGT
NPM F	CTCTGGAGCGTTCTTTTATC
NPM R	CGGCACGCAAGGTAGGA
RFC4 F	AGATTAGGTGACGGAGCTAAGACTTC
RFC4 R	CCTCAGGTTTCCGCGATACA
SAP130 F	CCTGCATCACATCATGACTACAAA
SAP130 R	GGCCCAGGAGCATTGCT
SMARCA4 F	TGCACACGTGCGTCAAAGGC
SMARCA4 R	GTCTCGCTTGCGCTTCCGTG

Supplemental Table 2.2: Antibodies

Antibody	Company	Catalog #
AcH4	MILLIPORE	06-866
ACTB	SIGMA-ALDRICH	A5441
ACTN	SANTA CRUZ BIOTECHNOLOGY INC.	sc-17829
BRG1 (ChIP)	BETHYL LABS	A300-813A
BRG1 (Immunoblot)	SANTA CRUZ BIOTECHNOLOGY INC.	sc-17796
CDK7	BETHYL LABS	A300-405A
CEBPB	SANTA CRUZ BIOTECHNOLOGY INC.	sc-150
CEBPE	SANTA CRUZ BIOTECHNOLOGY INC.	sc-130029
GST	SANTA CRUZ BIOTECHNOLOGY INC.	sc-549
HDAC1	MILLIPORE	05-614
HDAC2	ZYMED	51-5100
HDAC3	CELL SIGNALLING	3949
HNRNPC	SANTA CRUZ BIOTECHNOLOGY INC.	sc-32308
HNRNPU	SANTA CRUZ BIOTECHNOLOGY INC.	sc-32315
MED1	SANTA CRUZ BIOTECHNOLOGY INC.	sc-5334x
NCL	SANTA CRUZ	sc-56640

	BIOTECHNOLOGY INC.	
NCOR	SANTA CRUZ BIOTECHNOLOGY INC.	sc-8994
NPM (ChIP)	SANTA CRUZ BIOTECHNOLOGY INC.	sc-6013-R
NPM (Immunoblot)	SANTA CRUZ BIOTECHNOLOGY INC.	sc-47725
PML/RARA	ABCAM	ab43152
RFC4	SANTA CRUZ BIOTECHNOLOGY INC.	sc-28301
RNAPII	SANTA CRUZ BIOTECHNOLOGY INC.	sc-9001x
SAP130	SANTA CRUZ BIOTECHNOLOGY INC.	sc-21324
SMRT	ABR	PA1-843
S2P	COVANCE	MMS-129R
S5P	COVANCE	MMS-134R
TFIIB	SANTA CRUZ BIOTECHNOLOGY INC.	sc-225x
TFIIF	SANTA CRUZ BIOTECHNOLOGY INC.	sc-235
TFIIH	SANTA CRUZ BIOTECHNOLOGY INC.	sc-6859
TOP2B (Immunoblot)	BD BIOSCIENCES	611493
TOP2B (IP)	SANTA CRUZ BIOTECHNOLOGY INC.	sc-13059

Supplemental Table 3: ChIP Q-RT-PCR Primers

CEBPB -2962 F	CTGGCCTGCAAGGTCCGAGT
CEBPB -2962 R	ACCTGGGGTCACACAGTGCG
CEBPB -1199 F	CCCACCGTAAGCACAGGGCA
CEBPB -1199R	CCTCACGGGAGGGGGTGGTA
CEBPB -19 F	GTGACGCAGCGGTTGCTACG
CEBPB -19 R	TGGGTCCCCTTCCCAGTCCC
CEBPB +209 F	GGCACCCGCGTTCATGCAA
CEBPB +209 R	GGCAGGGGGAGACATGCTGG
CEBPB +984 F	GCCCTCGCAGGTCAAGAGCA
CEBPB +984 R	TCGCTGTGCTTGTCCACGGT
CEBPB +1622 F	GGGACTGACGCAACCCACGT
CEBPB +1622 R	AGCGATTACTCAGGGCCCGG
CEBPB +2631 F	TGGCTGAGCCCAGGCACAAA
CEBPB +2631 R	GCCGGAAGGGGCAAAGGGAT
CEBPE -2523 F	CTGCTGACCCGGCAGAGCTT
CEBPE -2523 R	AATGGGAGGTGGGGAGGGCA
CEBPE -1800 F	AGACCCCATCCCAGCCAGGT
CEBPE -1800R	TCTCACAGCGCTGGCTCTCG
CEBPE -2 F	CAGAGGAAGGAAAAGGAAGCAGAGCA
CEBPE -2 R	CACCCACTCCTGTGTGGCCT
CEBPE +114 F	ATCGAGAGAGGGCAGGCCCA
CEBPE +114 R	CCCCCACCTGCTCTTGAGGC
CEBPE +475 F	CGGCCCTTTGCCTACCCTCC
CEBPE +475 R	CCCTGGGGTCGTAGCTCCCT
CEBPE +1392 F	GCCCTGGCTGCCGAGATTCC

CEBPE +1392 R	GGCACGGAGAGACGGAGAGG
CEBPE +1871 F	ACTCTGCGGACCCCCATCCT
CEBPE +1871 R	CCCTCTTTGCCACCCCGGTT

CHAPTER 3: TARGETING PRKCD-MEDIATED TOPO-ISOMERASE II BETA OVEREXPRESSION SUBVERTS THE DIFFERENTIATION BLOCK IN A RETINOIC ACID-RESISTANT APL CELL LINE.

3.1 Preface

TOP2B was found to be overexpressed in the MR2 cell line [361]. Furthermore, the increased levels of TOP2B protein were not due to increased gene expression as Q-RT-PCR analysis showed no significant difference in TOP2B mRNA levels between the NB4 and MR2 cell lines. This suggested to us that either protein synthesis or protein stability were the root cause of increased expression of TOP2B. Based on what was known about regulation of TOP2B, we hypothesized that RA may activate specific kinases that lead to increased phosphorylation levels of TOP2B, thereby increasing its stability and decreasing its rate of degradation. In the following chapter, we investigate the mechanism by which TOP2B protein levels are regulated in APL cells.

*The results presented in this chapter were part of an original research article published in: Leukemia. 2010 Apr; 24(4): 729-39 [410].

3.2 Abstract

Retinoic acid (RA) relieves the maturation block in t(15:17) acute promyelocytic leukemia (APL), leading to granulocytic differentiation. However, RA treatment alone invariably results in RA resistance, both *in vivo* and *in vitro*. RA-resistant cell lines have been shown to serve as useful models for elucidation of mechanisms of resistance. Previously, we identified topoisomerase II beta (TOP2B) as a novel mediator of RA-resistance in APL cell lines. Here we show that both TOP2B protein stability and activity is regulated by a member of the protein kinase C (PRKC) family, PRKC delta (PRKCD). Co-treatment with a pharmacologic inhibitor of PRKCD and RA resulted in the induction of an RA responsive reporter construct as well as the endogenous RA target genes, *CEBPE*, *CYP26A1* and *DDX58*. Furthermore, the co-treatment overcame the differentiation block in RA-resistant cells, as assessed by morphological analysis, restoration of PML nuclear bodies, induction of cd11c cell-surface expression and an increase in NBT reduction. Cumulatively, our data suggest a model whereby inhibition of PRKCD decreases TOP2B protein levels, leading to a loss of TOP2B mediated repressive effects on RA-induced transcription and granulocytic differentiation.

3.3 Introduction

Acute promyelocytic leukemia (APL) is a subtype of acute myelogenous leukemia (AML), representing 5-8% of AML cases in adults. At the genetic level, APL is characterized by a specific chromosomal rearrangement between the retinoic acid receptor alpha (*RARA*) and the promyelocytic leukemia (*PML*) genes [239, 240, 411]. The resulting chimeric protein, PML/RARA, acts as a dominant negative inhibitor of normal retinoid receptor function. It locates to promoters normally regulated by RARA, aberrantly recruits co-repressor proteins, and thereby inhibits the RARA-mediated gene expression. On the cellular level, the result is a block in granulocytic differentiation and an accumulation of myeloid progenitors arrested at the promyelocyte stage. APL patients are treated with

therapeutic doses of all-*trans* retinoic acid (RA), a vitamin A derivative that activates RARA and circumvents the differentiation block [412]. Unfortunately, while treatment with RA alone results in a complete remission, the reprieve is not long-lasting, as RA resistance develops *in vivo [413],* a phenomenon that can be modeled *in vitro [414]*.

Acquired mutation in the PML/RARA oncoprotein is one source of RA-resistant APL. Studies on the PML/RARA protein in RA-resistant patient cells or cell lines established that roughly 33% possessed a mutation in the ligand-binding domain (LBD) of the RARA portion of the fusion protein, resulting in a dysfunctional PML-RARA that is unable to respond to pharmacologic doses of RA [349, 350, 415, 416]. However, the mechanisms of acquired resistance in the remaining 67% are undefined. The cells continue to express wild-type PML-RARA, yet are resistant to RA-induced differentiation.

In vitro derived RA-resistant cell lines are useful experimental models for the study of mechanisms of RA-resistance in APL. Our lab has previously isolated three RA-resistant sub-clones from the parental RA-sensitive cell line NB4, denoted MR2, MR4 and MR6 [355, 358]. Consistent with the pattern in other models of RA-resistance, one of these resistant subclones, MR4, contains a mutation in the LBD of PML/RARA, while the other two retain wild-type PML/RARA expression [355]. We have reported that resistance to RA-mediated transcription and differentiation in these cell lines is associated with enhanced binding of corepressor complexes to PML/RARA [358]. We furthered this observation by identifying topoisomerase II beta (TOP2B) as a novel member of these complexes. Additionally, we showed that TOP2B inhibits RA-induced gene expression and granulocytic differentiation by negatively modulating RARA transcriptional activity [361]. Type II topoisomerases catalyze changes in the topological state of DNA by generating transient breaks in double strand DNA (dsDNA) [417, 418]. Due to their DNA cleavage properties, TOP2s serve as target of antineoplastic agents, including DNA damaging agents [419]. Two

isoforms of TOP2 (A and B) exist in mammals. TOP2B is a phosphoprotein in which most of the phosphorylation sites have been mapped to the C-terminal domain and are thought to be mostly serine or threonine residues [420]. Sequence analysis of TOP2 phosphorylation sites revealed 30 possible protein kinase C (PRKC) sites and 40 casein kinase II (CSNK2) sites [417]. However, the functions of TOP2B's phosphorylation sites have not yet been characterized and the identification of possible phosphorylation sites responsible for TOP2B stability has remained elusive.

RA treatment leads to an increase of TOP2B protein levels in hematopoietic cells during differentiation [421]. Interestingly, this correlates with an increase in TOP2B protein stability and phosphorylation levels [421], although the exact mechanism by which RA upregulates and stabilizes TOP2B protein levels in differentiating cells has not been determined. Previously, we demonstrated that increased TOP2B levels mediate RA-resistance by inhibiting RA-induced gene expression and subsequent maturation towards granulocytes in APL cell lines. Consistent with this finding, the RA-resistant MR2 subline has comparatively higher basal TOP2B protein levels [361]. Interestingly, RA leads to increased levels of TOP2B protein in the RA-sensitive NB4 cell line, suggesting the possibility of a negative feedback loop. We speculated that RA may activate specific kinases that lead to increased phosphorylation levels of TOP2B, thereby increasing its stability and decreasing its rate of degradation. Here, we investigate the mechanism by which TOP2B protein levels are regulated in APL cells.

3.4 Materials and Methods

3.4.1 Materials

RPMI 1640 and fetal bovine serum (FBS) were purchased from Wisent (St. Bruno, QC, Canada). All-*trans* retinoic acid (RA) and 4α-phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rottlerin was purchased from Biomol (Plymouth Meeting, PA, USA). The PRKCD pSUPER shRNA-producing plasmid (Oligoengine, Seattle, WA, USA)

was directed against the following target sequence: 5'-AAACTCATGGTTCTTGATGTAGTGG-3'. The PRKCD kinase dead (PRKCDKD) construct was kindly provided by Dr. Trevor J. Biden (Garvan Institute of Medical Research, Sydney, Australia).

3.4.2 Cell culture

Derivation of the RA-resistant cell line MR2 from the parental APL cell line NB4 was previously described [358]. All cell lines were maintained in RPMI-1640 supplemented with 10% FBS.

3.4.3 Western blot analysis

Cell lysates were diluted 1:1 with 2X SDS sample buffer. Proteins were fractionated by electrophoresis on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Mississauga, ON, Canada). Membranes were probed with antibodies against TOP2B (catalogue no. 611493, BD Biosciences, San Diego, CA, USA), PRKCD (catalogue no. 610398, BD Biosciences), PRKCD phospho-threonine 505 (catalogue no. 3974S, Cell Signaling, Beverly, MA, USA) and ACTB (catalogue no. A5441, Sigma-Aldrich). The resulting signals were detected using the enhanced chemiluminescence (ECL; Amersham Pharmacia) system.

3.4.4 Decatenation assay

TOP2B enzymatic activity was assayed by measuring the decatenation of kinetoplast DNA (kDNA). A standard assay using 2 μg nuclear lysates was carried out according to the manufacturer's protocol (TopoGEN, Port Orange, FL, USA). Samples were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide. Reaction products were visualized and photographed on a UV transilluminator using the ChemiGenius^2 imaging system (Syngene, Frederick, MD, USA).

3.4.5 Transient transfections

NB4 cells (1×10^7 cells/transfection) were transfected by electroporation with 5 μg of the reporter plasmid βRARE-tk-CAT, with or without the pTB114 plasmid,

which contains the full-length TOP2B isoform fused to GFP in the pEGFP-C3 vector as previously described. Following electroporation, cells were replenished in media, and grown for 48 hours in the absence or presence of RA. Chloramphenicol acetyltransferase (CAT) activity was measured by means of a modified protocol of the organic diffusion method. The CAT counts were normalized with protein concentration to obtain the relative CAT activity.

3.4.6 RNA extraction and analysis

Total mRNA was isolated using the TRIzol method (Invitrogen, Carlsbad, CA, USA). cDNA was generated from 5 μg total RNA using random primers and SuperScript^{TM} II reverse transcriptase (Invitrogen). *CEBPE*, and *CYP26A1* mRNA levels were assessed by quantitative real time PCR (qPCR) analysis using Power SYBR green master mix (Applied Biosystems, FosterCity, CA, USA) with the following primer sets: *CEBPE*: sense 5'-CGG CTG GCC CCT TAC AC-3', antisense 5'-AGC CGG TAC TCA AGG CTA TCT TT-3'; and *CYP26A1*: sense 5'-GAC ATG CAG GCA CTA AAG CAA T-3', antisense 5'-CAC TGG CCG TGG TTT CGT-3'. cDNA was amplified for *DDX58* using a Taqman hybridization probe and Taqman Fast Master Mix (Applied Biosystems). $\Delta\Delta C_t$ values were normalized with those obtained from the amplification of endogenous *GAPDH* mRNA. qPCR was performed on the 7500 Fast Real-time PCR system (Applied Biosystems) using standard parameters and analyzed using relative quantification with DMSO-treated NB4 cells as the calibrator.

3.4.7 Morphology

Morphological changes in NB4 and MR2 cells were evaluated using conventional light-field microscopy of Giemsa–Wright (Sigma-Aldrich) stained cytosmears.

3.4.8 Immunofluorescence

NB4 and MR2 cells were treated for 18 hours with either 1 μM RA, 1 μM Rottlerin, or the combination. Cytospin preparations were fixed with 4% paraformaldehyde and stained with mouse anti-PML antibody (5E10 antibody

from Dr. R. van Driel, University of Amsterdam, Amsterdam, the Netherlands) as previously described.

3.4.9 Differentiation assays

Cells to be used for fluorescence-activated cell sorting (FACS) analysis of differentiation markers and in nitro-blue-tetrazolium (NBT) reduction assays were seeded at $3x10^4$ cells/mL in 5 mLs media in 6 well plates. Immunofluorescence staining of the cell surface myeloid specific antigen cd11c (PharMingen, Mississauga, ON, Canada) was assessed by flow assisted cell cytometry and performed according to the antibody manufacturer's specifications using the FACSCalibur flow cytometer (BD Biosciences, San Diego, CA, USA). Background staining was controlled using an isotype control PE conjugated mouse IgG1 (PharMingen). In each sample, viable cells were gated, and expression of cd11c surface markers of $1x10^4$ cells was evaluated. NBT assays were performed as previously described.

3.5 Results

3.5.1 Activation of PKCD correlates with increased TOP2B protein levels

A recent study established that protein kinase C delta (PRKCD), a member of the novel subgroup of the PRKC family of serine/threonine kinases, interacted with the TOP2A protein. This association led to increased protein expression of TOP2A and was dependent on the kinase activity of PRKCD. PRKCD is the most abundant PRKC isoform found in hematopoietic cells. It is activated in response to platelet derived growth factor (PDGF), 4α-phorbol 12-myristate 13-acetate (PMA) and RA [422]. It is of interest that RA, implicated in TOP2B stability and phosphorylation [421], can induce activation of PRKCD. As the two isoforms of TOP2 have 70% amino acid sequence identity, we therefore hypothesized that PRKCD may be the kinase responsible for the accumulation of TOP2B protein by RA, and may also cause the higher endogenous TOP2B levels in the MR2 cell line.

We asked whether activation of PRKCD by RA correlates with increased TOP2B levels in both NB4 and MR2 cell lines. By western blot analysis we observe that RA induction of TOP2B protein levels and activation of PRKCD, as measured by phosphorylation at threonine 505 (PRKCD pT505), occur with similar timing (Figure 3.1A). Consistent with previously published data, total PRKCD levels are not significantly different in the two cell lines, and remained the same prior to, and after, RA treatment. Most strikingly, MR2 cells show substantially increased basal levels of PRKCD pT505, when compared to NB4. This finding corresponds to the increased basal levels of TOP2B also seen in this cell line. We then asked whether other activators of PRKCD would have a similar effect. We found that treatment with the PRKC activator PMA for 2 hours increased both PRKCD pT505 and TOP2B levels in both cell lines (Figure 3.1B). Interestingly, PMA and RA activate PRKCD at different time points, 2 hours and 24 hours, respectively. This correlates with the observed timing of increased expression of TOP2B by these 2 agents. These results suggest that the hyperactive PRKCD may mediate the increased levels of TOP2B in the MR2 cell line.

3.5.2 Inhibition of PRKCD leads to decreased TOP2B protein levels

To further test our hypothesis that PRKCD regulates TOP2B levels in NB4 and MR2 cells, we tested the effects of PRKCD inhibition on TOP2B protein levels. Treatment of NB4 and MR2 cells with increasing concentrations of Rottlerin, decreased the levels of TOP2B protein in a dose dependent manner (Figure 3.2A). Rottlerin has been reported to be a selective PRKCD inhibitor [423-425] and has been used in many studies to implicate PRKCD in a variety of cancers, including hematological malignancies. There have been published reports that Rottlerin might have additional effects, and we therefore utilized additional approaches to confirm our initial findings. In accordance with the pharmacological inhibition of PRKCD, targeted knockdown of PRKCD in MR2 cells with shRNA, as well as by expression of a dominant negative inhibitory form of PRKCD (PRKCDKD), resulted in reduction of TOP2B levels (Figures

3.2B and C). In contrast, Go6976, an inhibitor of the conventional PRKC isoforms (A,B,G), had no effect on TOP2B protein levels (Figure 3.2D). This further supports out hypothesis that TOP2B regulation is dependent on the PRKCD isoform.

3.5.3 PRKCD modulatesTOP2B catalytic activity

Based on the evidence that activation of PRKCD regulates TOP2B levels, we next examined the enzymatic consequence of this proposed regulation. Type II topoisomerases alter the topological state of dsDNA by cleaving and religating DNA strands. Previous reports suggested that the decatenation activity of TOP2A is enhanced by phopshorylation by both PRKCD and CSNK2. To monitor the decatenation activity of TOP2B, we assayed its ability to decatenate kDNA, a interlocked network of DNA rings made up of several thousand 2.5-kb monomers and a few larger maxicircles. We incubated the kDNA substrate with nuclear extracts from NB4 and MR2 cells and resolved the products by agarose gel electrophoresis. Here, we observed that decatenation activity was substantially enhanced (Figure 3.2E, lanes 1-4) after treatment with RA. The RA-induced activation is PRKCD-dependent, as inhibiting PRKCD with Rottlerin prevented RA from inducing decatenation activity in the cell line. Interestingly, nuclear extracts from MR2 cells possessed higher basal decatenation activity (Figure 3.2E, lanes 5-8), reflective of their higher TOP2B and activated PRKCD protein levels. Stimulation with RA did not enhance this activity, while Rottlerin treatment decreased it below basal levels.

3.5.4 PRKCD inhibition decreases TOP2B protein stability

We previously determined that there is no significant difference in basal TOP2B mRNA levels in the MR2 cell line versus the NB4 cell line. To determine whether the increased TOP2B protein levels observed in MR2 cells was due to increased protein stability, TOP2B protein levels were measured by immunoblotting following cycloheximide-mediated translation inhibition. Although NB4 cells demonstrated a time-dependent degradation of TOP2B

protein levels starting at 8 hours (Figure 3.3A, lanes 1-4), no detectable degradation was observed in MR2 cells until 24 hours (Figure 3.3B, lanes 1-4).

We next sought to examine whether a hyperactive PRKCD may be responsible for the increased stability of the TOP2B protein in the MR2 cell line. Thus we measured the rate of TOP2B degradation after PRKCD inhibition by treating NB4 and MR2 cells with either the vehicle, DMSO, or Rottlerin. Twenty-four hours after treatment, the cells were incubated with cycloheximide, and, at the indicated times, cell lysates were harvested. Western blot analysis showed increased TOP2B protein degradation upon PRKCD inhibition in both cell lines. In the NB4 cell line we observed a significant decrease of TOP2B after inhibition of PRKCD, as compared to DMSO treatment (Figure 3.3A, lanes 5-8). Importantly, in the MR2 cell line, at 24 hours cycloheximide treatment, we observed an 80% decrease in TOP2B levels when PRKCD is inhibited, compared to only a 20% decrease in the untreated sample (Figure 3.3B, lane 4 versus lane 8). This considerable decrease in TOP2B protein levels at 24 hours suggests that PRKCD contributes to the increased TOP2B stability in the MR2 cell line. Of note, because of the increased levels of TOP2B in the MR2 cell line, a higher concentration of Rottlerin (2 μM) was required for downregulation of TOP2B. Despite decreasing TOP2B protein levels in both cell lines at 24 hours, Rottlerin treatment resulted in an increase in TOP2B transcript at this same time point (Figure 3.3C, first column). Additionally, Actinomycin D time course assays following the 24 hours DMSO or Rottlerin treatment were done to rule out variability in TOP2B mRNA stability (Figure 3.3C). No appreciable decrease in mRNA stability due to treatment with Rottlerin was observed. Surprisingly, chemical inhibition of PRKCD resulted in an increased TOP2B mRNA expression in both the NB4 and MR2 cell lines, while simultaneously decreasing the amount of TOP2B protein, suggesting a possible positive feedback mechanism.

3.5.5 Inhibition of PRKCD relieves TOP2B-mediated repressive effects on RA target genes

We have previously shown that TOP2B overexpression efficiently represses RA-induced gene expression. To test whether PRKCD inhibition could alleviate the repressive effects of increased TOP2B levels on gene expression, we performed transient co-transfections with an RARE-reporter gene and a TOP2B overexpression vector (pTB114) in NB4 cells. RA treatment, in the absence of TOP2B overexpression, strongly induces RARE reporter gene transcription. Upon TOP2B overexpression we observed a repression of RA-induced RARE reporter gene activation (Figure 3.4A). However, the addition of Rottlerin restored induction of the RARE reporter gene by RA.

To confirm that PRKCD inhibition overcomes repression of RA responsive genes, we treated NB4 and MR2 cells with Rottlerin, RA and the combination and measured mRNA levels of the RA target genes, *CEBPE*, *CYP26A1* and *DDX58*. Increased mRNA induction of all three genes was observed in both cell lines with the combination treatment as compared to RA or Rottlerin alone (Figure 3.4B).

3.5.6 Inhibition of PRKCD restores sensitivity to RA-induced differentiation in the MR2 cell line

Our data that PRKCD inhibition overrides the transcriptional repression exerted by TOP2B on RA-target genes led to the hypothesis that this inhibition would then be able to abolish the differentiation block in the MR2 cell line. In normal cells, PML locates to punctuate structures known as PML nuclear bodies, whereas in APL cells PML/RARA expression disrupts these structures and disperses PML into nuclear microspeckles. Treatment with RA reverses this abnormal nuclear localization. We first assessed PML staining by immunofluorescence in NB4 and MR2 cells treated for 18 h either with RA, Rottlerin or the combination (Figure 3.5A). Cells were stained with anti-PML antibodies and counterstained with DAPI to confirm nuclear staining (data not

shown). In the untreated NB4 and MR2 cells, immunofluorescence showed a diffuse PML nuclear staining. We saw that RA alone and the combination of RA and Rottlerin are both sufficient to cause larger PML nuclear bodies to form in the RA-sensitive NB4 cells. However, in the MR2 cell line, only the combination treatment led to the re-establishment of PML nuclear bodies.

To extend and confirm these results, we performed morphologic analysis of both cell lines treated for 5 days. Figure 3.5B demonstrates that the MR2 cells only differentiate after exposure to both RA and Rottlerin. The granulocytic maturation pattern was similar for all the differentiated cells, with polylobular nuclei, chromatin condensation and a decreased nuclear:cytoplasmic ratio.

Next, we examined expression levels of a cell surface marker associated with granulocytic differentiation (Figure 3.5C) after 5-day treatment. As expected, RA alone is enough to stimulate significant cd11c expression in NB4 cells, whereas the same treatment in MR2 cells generates only a modest increase in expression. Additionally, with Rottlerin alone there is a slight increase in cd11c expression in both cell lines. However, only in the presence of both RA and Rottlerin do we observe a robust increase in cd11c expression levels in the MR2 cell line.

As a final measure of the ability of PRKCD inhibition to overcome the differentiation block in APL cells, we performed a NBT reduction analysis. Rottlerin alone had little discernible effect on differentiation in either cell line. Again, the effect of the combination is greater than either agent alone; RA and Rottlerin co-treatment leads to significant NBT reduction within 5 days (Figure 3.5D). This is consistent with the immunofluorescence, morphology and cd11c data, where only a moderate increase in terminal differentiation was observed in the MR2 cell line in response to 10^{-6} M RA after 5 days.

The published report establishing that RA induces PRKCD activation also presents data that inhibition of PRKCD by Rottlerin reversed RA induction of cd11b cell surface expression in the NB4 cell line, suggesting an opposite effect

on differentiation than was found here. We therefore undertook additional experiments to show that the combination of RA and Rottlerin induced differentiation in both the NB4 and MR2 cell lines. The combination treatment was more active in regards to differentiation induction than either agent alone, as evaluated by all four criteria tested. To our knowledge, these data are the first to define PRKCD as a negative regulator of RA-induced gene regulation and differentiation towards granulocytes. We thus conclude that the hyperactive PRKCD in the MR2 cell line leads to enhanced TOP2B levels, which blocks the differentiation pathway towards granulocytes.

3.6 Discussion

The amount of an active protein available to carry out defined physiological functions is a major player in governing cell growth and proliferation. Our studies begin to uncover the mechanisms and ramifications of TOP2B protein regulation. We show that activation of PRKCD, by RA and PMA, leads to increased levels of TOP2B protein and conversely, inhibition of PRKCD correlates with decreased levels of TOP2B. We were the first to report that increased levels of TOP2B protein can mediate resistance to RA in APL cell lines [361]. That observation is now furthered by this report demonstrating that a hyperactive PRKCD is responsible for increased TOP2B levels in the RA-resistant cell line, MR2, and hence, ultimately contributes to RA-resistance.

Both of the human topoisomerase II isoforms, TOP2A and TOP2B, are phosphorylated at several sites, primarily in the C-terminal domain [426-428]. Some specific phosphorylation sites and their functional consequences, have been mapped for TOP2A [429-432], and a few, but not all, of the phosphoacceptor sites are conserved between the A and B isoforms. However, little is currently known about the ramifications of TOP2B phosphorylation. Potential functional roles for site-specific phosphorylation have been hinted at by several groups demonstrating hyper-phosphorylation of TOP2B in doxorubicin-resistant HL-60 cells [433] and phosphorylation during RA-induced differentiation of wild-type HL-60 cells,

which correlated with a slower degradation rate of TOP2B protein [421]. The latter observation is now made more concrete by the research contained herein showing increased protein degradation upon PRKCD inhibition.

There are examples of phosphorylation increasing the metabolic stability, and consequently the activity, of a protein, as has been shown for the transcription factors p53 [434] and c-fos [435]. Our assessment of decatenation ability confirms that TOP2B catalytic activity is considerably enhanced after activation of PRKCD and abrogated after inhibition of PRKCD. These results suggest that TOP2B be introduced into a family of proteins whose functional activity is managed through phosphorylation-dependent stability. However, no increase in total TOP2B phosphorylation after RA treatment or conversely, no decrease with PRKCD inhibition, was observed in ^{32}P labeling experiments (data not shown). We speculate that this might be due to changes in only a few of the many possible TOP2B phospho-acceptor sites, which would not affect the overall phosphorylation signal of the protein. Our ongoing studies aim to identify the specific TOP2B residues of PRKCD-mediated phosphorylation in order to characterize their functional role.

RA has been shown to directly bind the different PKC isoforms, including PRKCD [436]. Given the late timing of PRKCD activation after RA treatment, it is reasonable to hypothesize that RA is not acting directly on PRKCD and that another kinase might lie upstream in the pathway. Several MAP kinases have been previously shown to be involved in the response to RA [437, 438]. In particular, the p38 MAP kinase is phosphorylated and activated by RA treatment in NB4 cells [439] and PRKCD phosphorylation at Thr505 was recently shown to be dependent upon p38 [440]. However, RA activates many complex signalling cascades that may also lie upstream of PRKCD and TOP2B. Indeed, future directions will aim to elucidate more clearly the entire signalling pathway leading to TOP2B regulation.

We previously found that TOP2B is overexpressed in the RA-resistant APL cell line, MR2 [361], and TOP2B has also been found to be overexpressed in acute myeloid leukemia [441] and lymphoma [442] patients. However, mechanistic data on the causes of this upregulation have been lacking. Given that TOP2B is a target of many anticancer agents, its increased expression can enhance the cytotoxic activity of these agents in tumor cells [443-446]. In particular, RA-resistant APL cells with excess TOP2B protein may respond better to treatment with anthracyclines. This hypothesis is supported by data from the clinic; when RA is co-administered with anthracycline-based therapy, APL patients have improved remission and survival rates [447]. On the other hand, in the context of leukemic differentiation, increased levels of TOP2B protein has an inhibitory effect on RA-induced differentiation and apoptosis [420].

It is also of general interest to mention that other RA targets are regulated in a similar manner as TOP2B in cancer cells: the transcription factor SOX9 [448], the myeloid transcription factor CEBPE [340], the growth inhibitory protein IGFBP-3 [449], and the translation repressor 4E-BP2 [450] are all regulated by RA. Specifically, the basal level of expression of these proteins is low, but inducible in RA-sensitive cells, while in some RA-resistant cell lines, the basal level of expression is constitutively high and is only minimally further induced upon RA treatment. It had been shown that transcriptional activation by nuclear receptors requires a signal dependent exchange of co-activators and co-repressors [451, 452]. The de-regulated TOP2B levels may negatively affect RA signalling at the level of co-regulator cycling, either by preventing efficient dissociation and/or degradation of the co-repressor complex or by inhibiting recruitment of the co-activator complexes, even in the presence of pharmacological doses of RA. A similar mechanism has recently been proposed for another repressor of RA-signalling [453]. The possibility does exist that increased TOP2B levels are mere bystanders to the development of resistance. However, we feel that cumulatively our data supports the model where TOP2B is

a more active mediator of resistance, since inhibition of TOP2B, either directly [361] or indirectly via PRKCD, re-establishes sensitivity to RA-induced differentiation.

TOP2B protein levels can have an impact on both sensitivity and resistance to cancer therapeutics. Thus, identifying the mechanisms by which TOP2B levels are regulated may assist in the treatment of leukemia as well as the multitude of cancers that respond to TOP2 poisons. Our findings that RA-resistance in APL cells can be overcome by targeting both the PRKCD and RA pathways may provide a basis for the rational design of novel therapies for not only RA-resistant APL, but other more common leukemias that have an increased TOP2B expression.

3.7 Acknowledgements

We are grateful to Björn D. Kuhl and Filippa Pettersson for critical reading of the manuscript. This work was supported by a grant from the Canadian Institutes of Health Research. W. H. Miller, Jr., is a Chercheur National of Fonds de la Recherche en Santé du Québec. J. Nichol is supported by a student fellowship from the Cole Foundation.

3.8 Figures

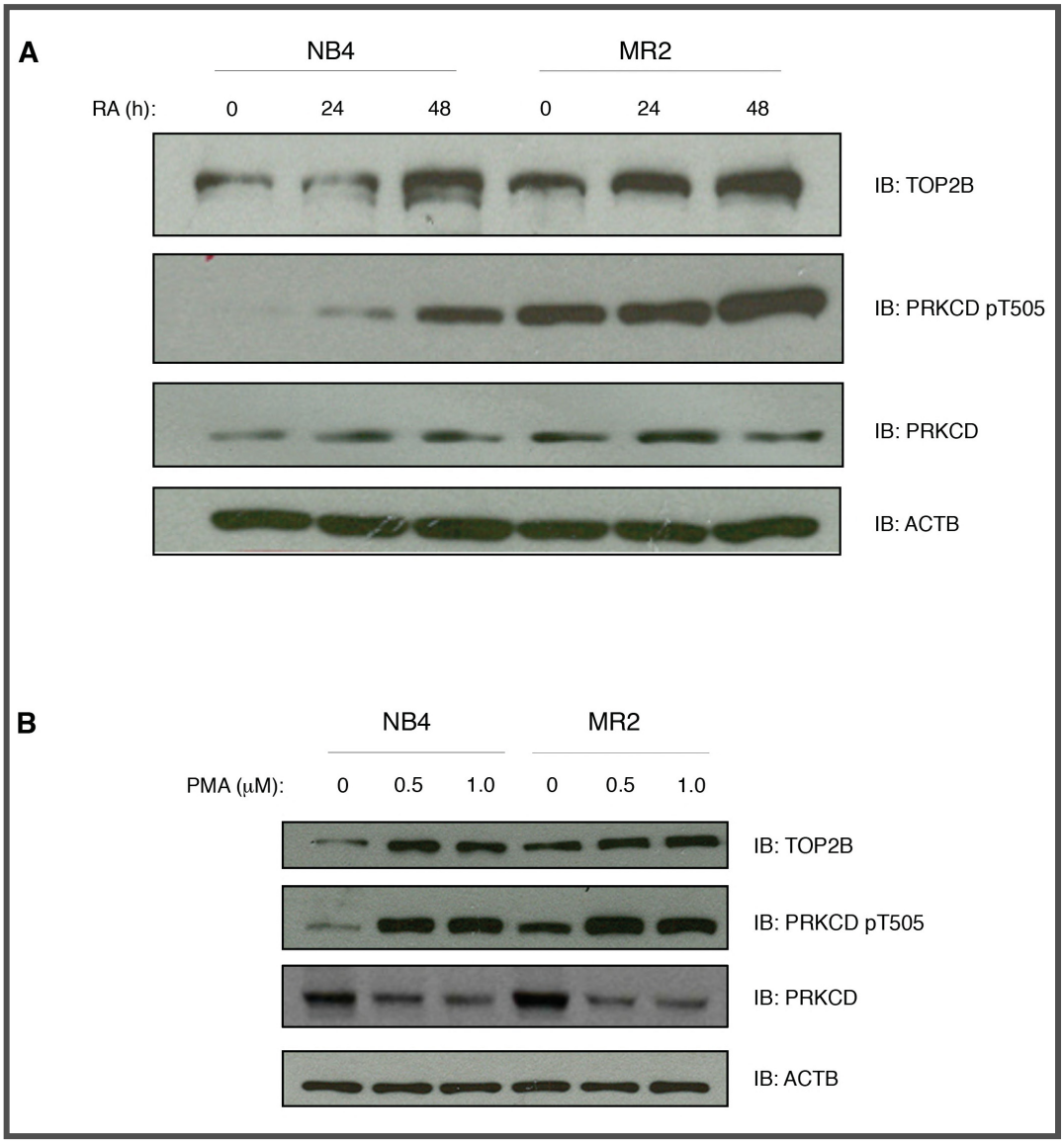

A
NB4
MR2
RA (h):
0
24
48
0
24
48
IB: TOP2B
IB: PRKCD pT505
IB: PRKCD
IB: ACTB
B
NB4
MR2
PMA (μM):
0
0.5
1.0
0
0.5
1.0
IB: TOP2B
IB: PRKCD pT505
IB: PRKCD
IB: ACTB

Figure 3.1. Activation of PRKCD correlates with increased TOP2B levels. Threonine phosphorylation at residue 505 of PRKCD (PRKCD pT505) and an increase in TOP2B protein levels occurred after 24 and 48 hours 1 μM RA (A) and after 2 hours PMA treatment (B). Total nuclear protein was separated on a 10% polyacrylamide gel, blotted onto nitrocellulose and subjected to western blotting using TOP2B, PRKCD and PRKCD pT505 antibodies. ACTB served as the loading controls. Results are representative of 3 experiments.

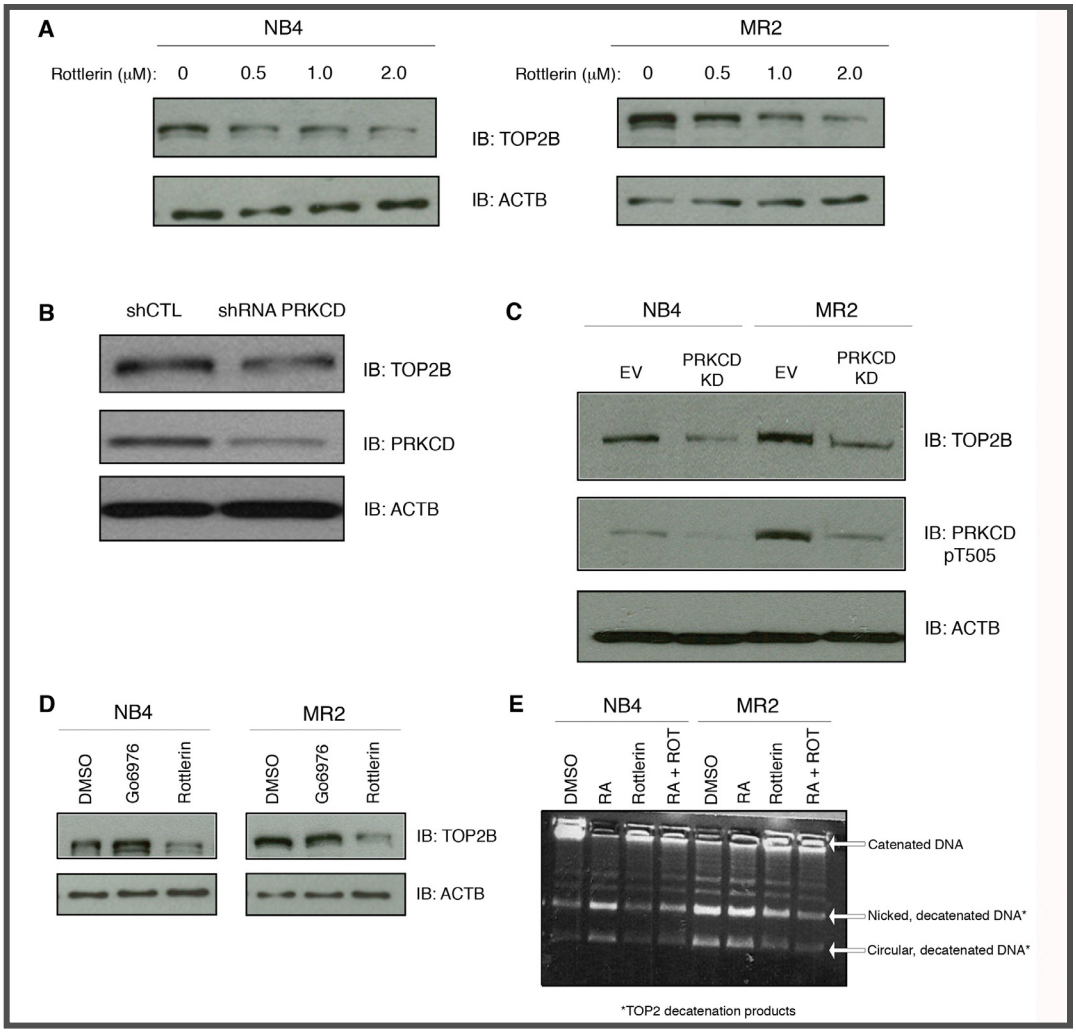

A
NB4
MR2
Rottlerin (μM): 0 0.5 1.0 2.0
Rottlerin (μM): 0 0.5 1.0 2.0
IB: TOP2B
IB: ACTB
B
shCTL
shRNA PRKCD
IB: TOP2B
IB: PRKCD
IB: ACTB
C
NB4
MR2
EV
PRKCD KD
EV
PRKCD KD
IB: TOP2B
IB: PRKCD pT505
IB: ACTB
D
NB4
MR2
DMSO
Go6976
Rottlerin
DMSO
Go6976
Rottlerin
IB: TOP2B
IB: ACTB
E
NB4
MR2
DMSO
RA
Rottlerin
RA + ROT
DMSO
RA
Rottlerin
RA + ROT
Catenated DNA
Nicked, decatenated DNA*
Circular, decatenated DNA*
*TOP2 decatenation products

Figure 3.2. PRKCD regulates TOP2B protein levels and catalytic activity (A) A decrease in TOP2B protein levels with increasing concentrations of Rottlerin was observed in both the NB4 and MR2 cell lines. Total nuclear protein was subjected to western blotting using a TOP2B antibody (B) MR2 cells were transfected with scrambled shRNA or a shRNA against PRKCD. Total levels of TOP2B and PRKCD protein were analyzed by western blotting. (C) The kinase activity of PRKCD is required for TOP2B protein stability. NB4 and MR2 cells were transfected with either an empty vector (EV) or a vector expressing a kinase dead PRKCD (PRKC KD). (D) NB4 and MR2 cells were treated with Go6976 and Rottlerin. To observe a decrease in TOP2B protein levels, total nuclear proteins were subjected to western blotting using TOP2B antibody. ACTB was used as a loading control for (A)-(D). (E) TOP2B-mediated decatenation of kDNA is PRKC-dependent. TOP2B activity was measured by decatenation of kDNA to nicked open circular and relaxed closed circular DNA. A representative image of a 1% agarose gel (ethidium bromide-stained) is shown.

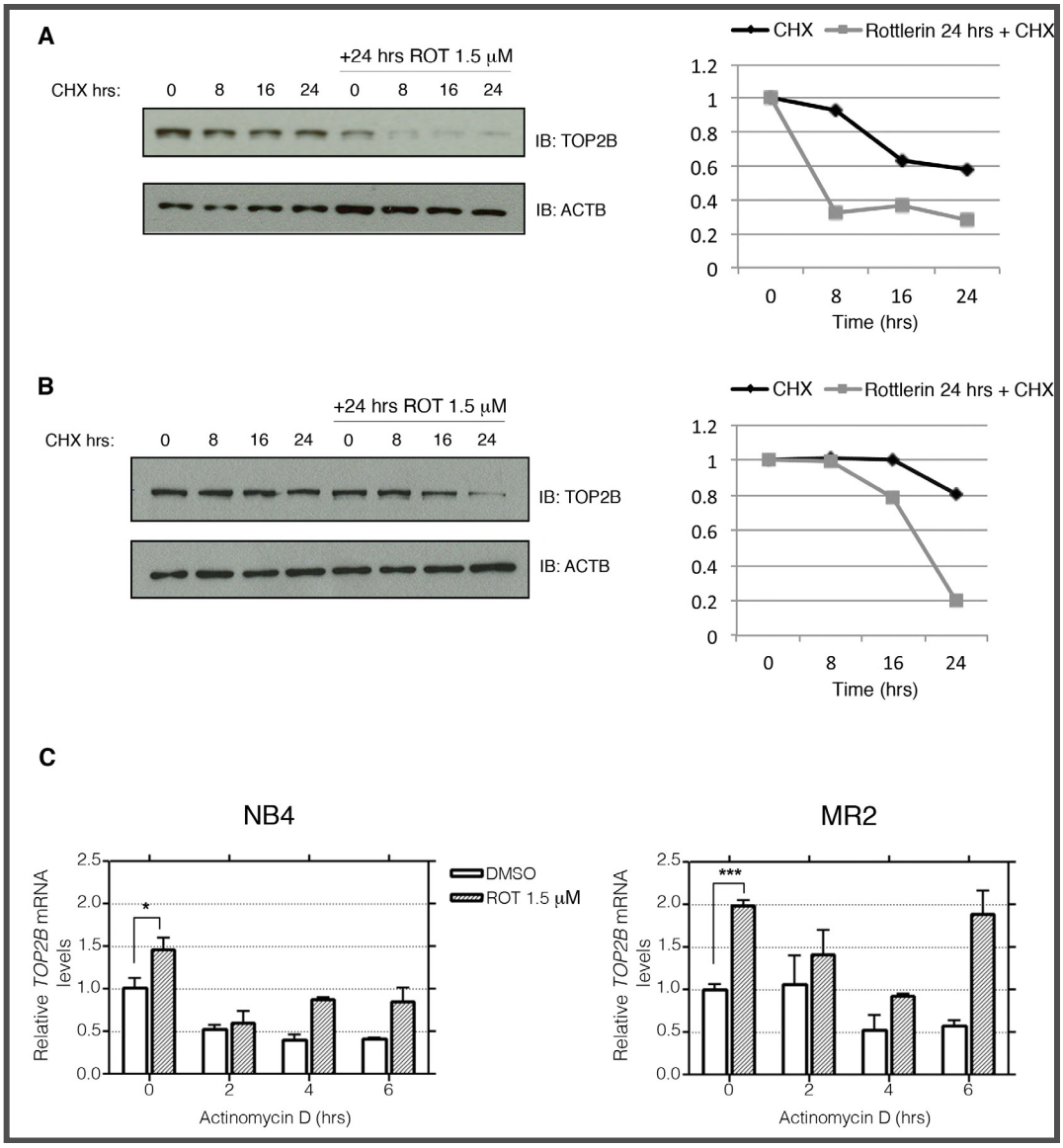

A
+24 hrs ROT 1.5 μM
CHX hrs: 0 8 16 24 0 8 16 24
IB: TOP2B
IB: ACTB
CHX
Rottlerin 24 hrs + CHX
1.2
1
0.8
0.6
0.4
0.2
0
0 8 16 24
Time (hrs)
B
+24 hrs ROT 1.5 μM
CHX hrs: 0 8 16 24 0 8 16 24
IB: TOP2B
IB: ACTB
CHX
Rottlerin 24 hrs + CHX
1.2
1
0.8
0.6
0.4
0.2
0
0 8 16 24
Time (hrs)
C
NB4
MR2
Relative TOP2B mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
*
***
DMSO
ROT 1.5 μM
0 2 4 6
Actinomycin D (hrs)

Figure 3.3. PRKCD regulates the half-life of TOP2B. (A, B) NB4 and MR2 cells were treated with either DMSO or Rottlerin for 24 hours, followed by treatment with cycloheximide (CHX) for 0, 8, 16 and 24 hours. To observe a decrease in TOP2B protein levels, total nuclear proteins were subjected to western blotting using a TOP2B antibody. Densitometry was performed on the western blot analysis, using ACTB as a loading control. Densitometry results were plotted and shown in the graph. Results are representative of 3 experiments. (C) NB4 cells (top panel) or MR2 cells (lower panel) were pre-treated for 24 hours with either DMSO or Rottlerin. Accumulation of *TOP2B* mRNA was analyzed following Actinomycin D treatment for the indicated times. Results shown are representative of 3 experiments. Error bars represent SD. Asterisks indicate significant differences between DMSO-treated cells and Rottlerin treated cells with no Actinomycin D treatment (*, $p < 0.05$; ***, $p < 0.001$).

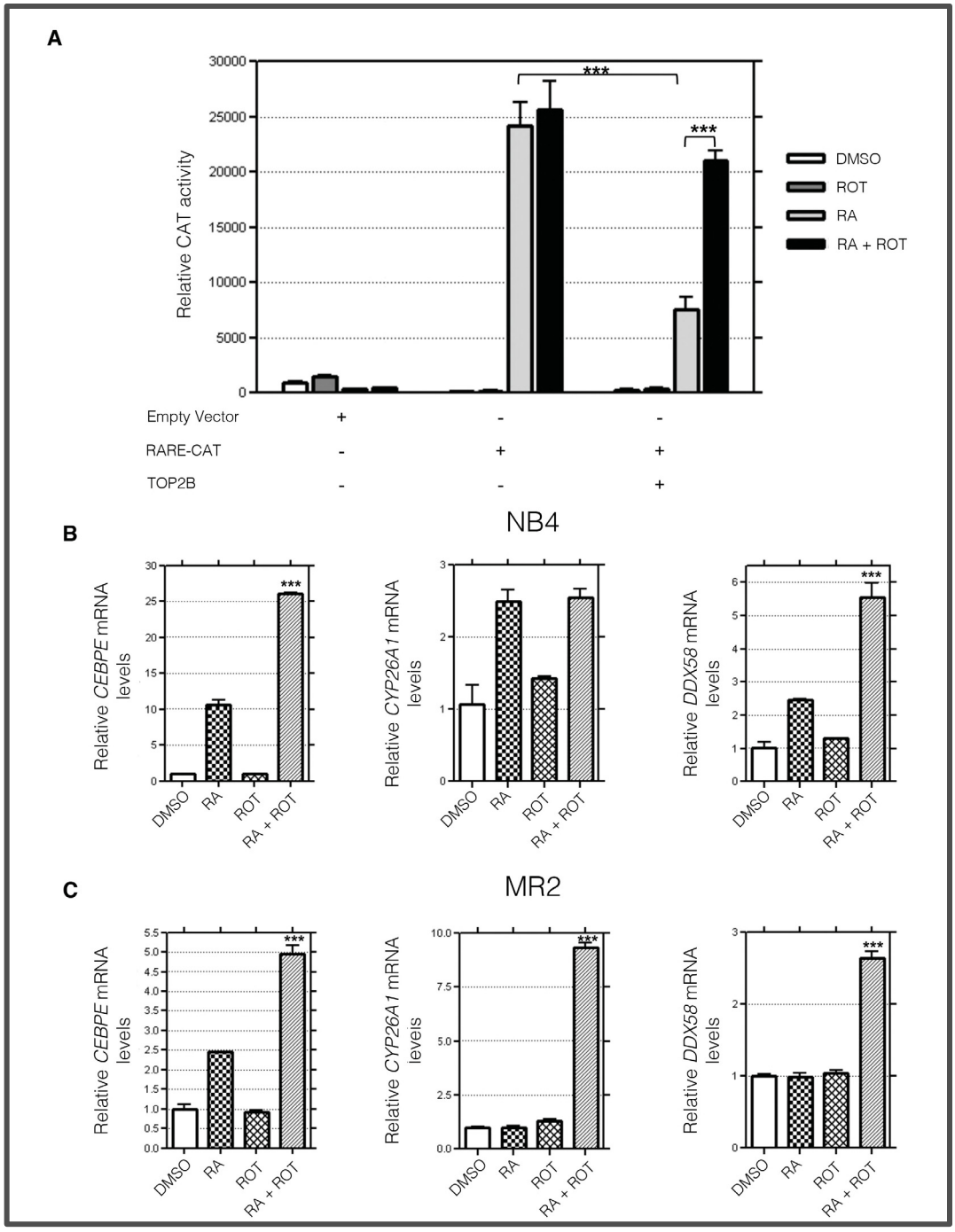

A
Relative CAT activity
30000
25000
20000
15000
10000
5000
0
***
***
DMSO
ROT
RA
RA + ROT
Empty Vector + - -
RARE-CAT - + +
TOP2B - - +
B
NB4
Relative CEBPE mRNA levels
Relative CYP26A1 mRNA levels
Relative DDX58 mRNA levels
DMSO
RA
ROT
RA + ROT
C
MR2
Relative CEBPE mRNA levels
Relative CYP26A1 mRNA levels
Relative DDX58 mRNA levels
DMSO
RA
ROT
RA + ROT

Figure 3.4. Inhibition of PRKCD relieves TOP2B repressive effects on RA target genes. (A) Transiently transfected NB4 cells were electroporated with empty vector, βRARE-tk-CAT and the TOP2B vector (pTB114) alone or in combination. Twenty-four hours after transfection, the cells were treated with RA, Rottlerin (ROT) or the combination for an additional 24 hours. Results shown are representative of 3 experiments. Error bars represent SD. Asterisks indicate significant differences between RA-treated cells with and without overexpression of TOP2B and between TOP2B overexpressing cells treated with RA or the combination of RA and Rottlerin (***, $p < 0.001$). mRNA expression was analyzed in response to treatments with 1 μM RA, 1.5 μM Rottlerin or the combination for *CEBPE*, *CYP26A1* (24 hours) and *DDX58* (72 hours) in NB4 cells (B) or MR2 cells (C). Results shown are representative of 3 experiments. Error bars represent SD. Asterisks indicate significant differences between RA-treated cells and RA plus Rottlerin treated cells (***, $p < 0.001$).

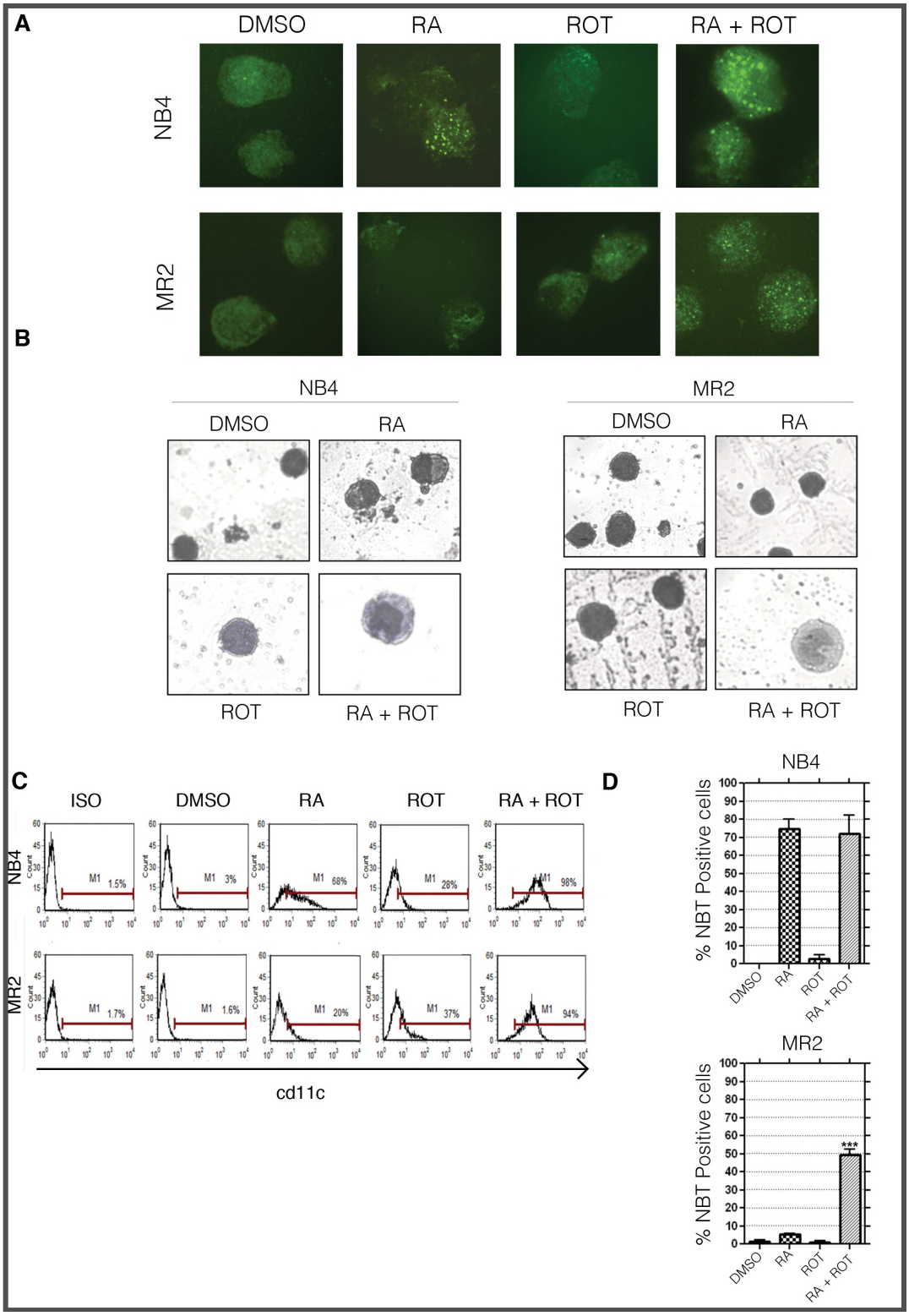

A
DMSO
RA
ROT
RA + ROT
NB4
MR2
B
NB4
MR2
DMSO
RA
ROT
RA + ROT
C
ISO
DMSO
RA
ROT
RA + ROT
NB4
MR2
M1 1.5%
M1 3%
M1 68%
M1 28%
M1 98%
M1 1.7%
M1 1.6%
M1 20%
M1 37%
M1 94%
Count
cd11c
D
NB4
MR2
% NBT Positive cells
DMSO
RA
ROT
RA + ROT
***

Figure 3.5. Inhibition of PRKCD restores RA sensitivity in the NB4-MR2 cell line. (A) NB4 and MR2 cells were treated for 12 h with 1 μM RA, 1 μM Rottlerin (ROT) or the combination and stained for PML and detected with fluorescein isothiocyanate (FITC)-anti-mouse antibodies by immunofluorescence. 4',6-diamidino-2-phenylindole (DAPI) was used to control for nuclear staining (not shown). (B) Morphologic analysis of representative NB4 and MR2 cells treated with Rottlerin and RA for 5 days. Cells were stained with Giemsa-Wright and were viewed at x 100 magnification. (C) Cytofluorimetric analysis of surface marker expression. Percentages of NB4 and MR2 cells expressing cd11c in response to 5 day exposure to 1 μM RA and Rottlerin, or the combination. Results are representative of 1 of 3 experiments performed in triplicate. (D) Results of NBT reduction assay performed on NB4 and MR2 cells treated with RA, Rottlerin or the combination for 5 days. Asterisks indicate significant differences between RA-treated cells and RA plus Rottlerin treated cells (***, $p < 0.001$).

CHAPTER 4: GENERAL DISCUSSION.

There is a long established relationship between structural genomic aberrations, particularly chromosome translocations and inversions, and acute leukemia. Specific cytogenetic abnormalities are often uniquely associated with clinically distinct subsets of acute myeloid leukemia. Identifying the DNA sequences surrounding the chromosomal breakpoints has had a monumental impact on our understanding of leukemogenesis, carcinogenesis in general, and the normal cellular functions of the protein products of the involved genes.

Chromosomal translocations that fuse the retinoic acid receptor (RARA) to any one of a number of translocation partners are the cytogenetic abnormalities that define acute promyelocytic leukemia. Since its discovery in 1990 [454-456], one of these RARA fusions, PML/RARA, has served as the archetypal oncofusion protein for understanding the molecular aspects of oncogenesis. Many mechanisms have been proposed for PML/RARA function, including dominant negative behavior over normal RARA and PML, homodimerization, oligomerization, interaction with RXR, expanded DNA binding affinity and recruitment of a wide spectrum of co-repressors with epigenetic enzyme activities.

Nuclear receptors comprise a family of ligand-dependent transcription factors that includes the retinoic acid receptors. Many co-factors implicated in their transcriptional regulation have been identified [457]. We reported the identification of a putative complex that associates specifically with PML/RARA in an APL cell line resistant to the effects of all-*trans* retinoic acid [361]. The idea of a co-repressor complex particular to RA-resistant PML/RARA is intriguing. We have now validated two of those identified proteins, NPM and TOP2B, as mediating resistance to RA, both at the transcriptional and functional levels. Despite beginning to characterize this PML/RARA complex, fundamental questions remain to be answered. For example, which members are required to

inhibit transcription remain to be established; details of their mode of recruitment are lacking, and little is known about their precise functions in regulating transcription. Two additional proteins of the eight identified, SAP130 and HNRNPU have been shown previously to play a role in transcriptional repression. HNRNPU binds to the glucocorticoid receptor and is involved in transrepression [458] and SAP130 was identified as a member of the mSIN3A co-repressor complex [396]. Manipulation of levels of the remaining members followed by transcriptional and functional studies, similar to the experimental strategies employed with TOP2B and NPM, will be needed to begin to tease out the respective importance and contribution of each member to the complex as a whole.

In Chapter 2, we began to characterize the role of Nucleophosmin. NPM is a very abundant and highly conserved phospho-protein that resides primarily in the nucleolus, although it shuttles rapidly between the nucleus and cytoplasm. By shuttling between these cellular compartments, NPM plays a role in diverse processes (reviewed in [459]), including regulating centrosome duplication, the transport of pre-ribosomal particles and ribosome biogenesis, the maintenance of genomic stability, participation in DNA-repair processes, and the regulation of DNA transcription. GST-pull down studies indicate that NPM interacts with PML/RARA through the PML moiety of the fusion. No known interaction between PML and NPM has thus far been established. Immunofluorescent studies show that PML is largely found in the nucleus associated within 10–30 nuclear bodies [294-296, 460-462]. NPM primarily localizes to the nucleoli of cells, in two to five discreet aggregates [463-465]. The hematological malignancies associated with the mis-localization of these proteins highlight that their proper protein function is highly dependent on intracellular localization. In APL, PML/RARA disrupts PML localization within the nuclear bodies and delocalizes PML in a microspeckled, nuclear pattern [294-296]. In AML, the most common mutations of *NPM1* involve the insertion of 4 basepairs at the C-terminal

portion of the protein. The C-terminal mutations are heterozygous and result in an inability of the protein to perform its usual nuclear-cytoplasmic shuttling function. The cytoplasmic mutant ($NPMc^+$) has been isolated from leukemic blasts [378]. It has been shown that $NPMc^+$ relocalizes ARF to the cytoplasm, thus inhibiting its functional interaction with the p53 negative regulator, Mdm2, and blunting ARF-induced activation of the p53 transcriptional program. Our resistant cells do not have the $NPMc^+$ mutation, though we do find a dramatic overexpression of NPM at the protein level. We therefore hypothesize that this over-expressed NPM might not exclusively locate within the nucleolus, but might be found throughout the nucleus where it now has the opportunity to interact with PML in the altered nuclear architecture of APL cells. This interaction might be mediated by their respective coiled-coil domains. Alternatively, NPM is modified by SUMO [466]. A SUMO binding domain located within exon seven of PML has been reported [467]. This motif enables PML to form non-covalent interactions with other SUMO-modified proteins, including itself. The interaction between PML and NPM might be dependent upon NPM SUMOylation. Mutation of the SUMOylation site within NPM would begin to elucidate the necessity of this post-translational modification in the interaction between NPM and the PML/RARA fusion.

The amount of an active protein available to carry out defined physiological functions is critical in governing cell growth and proliferation. Interestingly TOP2B and NPM, as well as four other complex members, show increased expression at the protein level in the resistant cells versus the sensitive cells. These increased levels are not reflected in the relative mRNA levels. However, mechanistic data on the causes of this up-regulation are only known for TOP2B.

In Chapter 3, we determined that TOP2B protein levels seem to be regulated at the level of protein stability. Our studies begin to uncover the mechanisms and ramifications of TOP2B protein regulation. We show that

activation of PRKCD, by RA and PMA, leads to increased levels of TOP2B protein and conversely, inhibition of PRKCD correlates with decreased levels of TOP2B. That observation is furthered by the data demonstrating that a hyperactive PRKCD is responsible for increased TOP2B levels in the RA-resistant cell line, MR2, and hence, ultimately contributes to RA-resistance. Any potential role of PRKCD signalling in mediating the stability of each of the other seven members of the complex remains to be determined. Like TOP2B, NPM is phosphorylated and its phosphorylation at specific residues is related to its function. For example, NPM phosphorylation at Ser125 by the cell cycle-related Ser/Thr protein kinase, casein kinase 2, serves to not only regulate NPM function during ribosome biosynthesis [468] but also enable NPM to perform its function as a molecular chaperone [469].

The applicability of PRKCD signalling in mediating the up-regulated protein levels may or may not extend beyond TOP2B. Analogous to regulation of DNA transcription, mRNA contains many *cis*-acting sequences which constitute a platform for *trans*-factors to bind and exert regulatory control over mRNA stability and translational efficiency. The 3' untranslated regions of mRNAs (3′ UTRs) often contain microRNA (miRNA) binding sites [470] and/or other regulatory elements, such as AU-rich elements (AREs) [471]. Approximately 50% of human genes have alternative polyadenylation, leading to heterogeneous 3' end formation of transcripts [472]. These differences can influence cell processes, such as proliferation, and therefore have pathological consequences [473]. However, the mechanisms of alternative 3′-UTR processing for specific mRNA populations remain controversial. Some genes, such as *BCL2*, have been described to be translated by an alternative cap-independent mechanism [474]. This mechanism is driven by an internal ribosome entry site (IRES), which allows the expression of the respective gene in specific conditions when general cap-dependent protein synthesis is impaired. These conditions include mitosis as well as hypoxia, apoptosis, viral infection and amino-acid starvation [475]. IRESs are

cis-acting elements, located mainly at the 5′-UTR of the mRNA. They allow initiation of translation by recruitment of the small ribosomal subunit to its secondary RNA structure close to the initiator AUG, independent of the 5′ cap [476, 477]. Although the exact molecular mechanism of IRES-mediated internal initiation of translation is still unclear, most known IRESs depend on the binding of several IRES *trans*-acting factors for efficient initiation of translation. HNRNPC1/C2, another PML/RARA interacting protein we identified as associated with RA-resistance, is one of these IRES *trans*-acting factors [478] and has been previously implicated in vitamin D resistance [479].

Gene expression in eukaryotes is a highly complex and tightly regulated process. RA plays a critical role during the differentiation of myeloid progenitors towards the granulocytic lineage by regulating the transcription of important mediators of the differentiation process. In APL, PML/RARA has been proposed to act as a dominant negative inhibitor of endogenous RARA signaling by repressing the normal RARA gene expression program. In RA-sensitive cells, pharmacological doses of all-trans retinoic acid can relieve this repression. However, in RA-resistant cells, this repression is maintained even with high dose RA treatment. Repression of genes often involves the establishment and maintenance of chromatin in a structure that prevents transcription. ATP-dependent chromatin remodeling activities have more typically been associated with transcriptional activators, but more recent evidence has implied their role in transcriptional repression. Many of these chromatin modifying enzymes do not bind DNA specifically, and while it is possible for chromatin to be modified in a global manner, more often the enzymatic activity is targeted to appropriate genes by site-specific transcription factors via complexes containing multiple proteins.

TOP2B and NPM are capable of interacting directly with PML/RARA but also appear to function as platform proteins for the recruitment of BRG1. One of the central observations made in this thesis is the novel recruitment of BRG1 to PML/RARA target genes in RA-resistant APL cells upon RA-treatment (Figure

4.1). BRG1 can be recruited by both activators and repressors, though the precise role of BRG1 in mechanisms of repression remains unclear. One transcription factor that recruits BRG1 as a co-repressor is the Repressor Element 1-Silencing Transcription factor (REST). BRG1 enhances REST mediated repression at some REST target genes by increasing the interaction of REST with the local chromatin at its binding sites [403]. Furthermore, REST-chromatin interactions, mediated by BRG1, are enhanced following an increase in histone acetylation in a manner dependent on the BRG1 bromodomain. Such a mechanism may be applicable to other transcriptional repressors that utilize BRG1. Our results presented here are consistent with these data. We find an increase in histone H4 acetylation after RA treatment in the resistant cells that may precede co-repressor recruitment. Additionally, ChIP-Seq data from the ENOCDE Consortium displays several REST binding peaks at the *CEBPB* locus. Further studies in our resistant cell line could therefore focus on whether BRG1 is facilitating REST repression at the *CEBPB* locus.

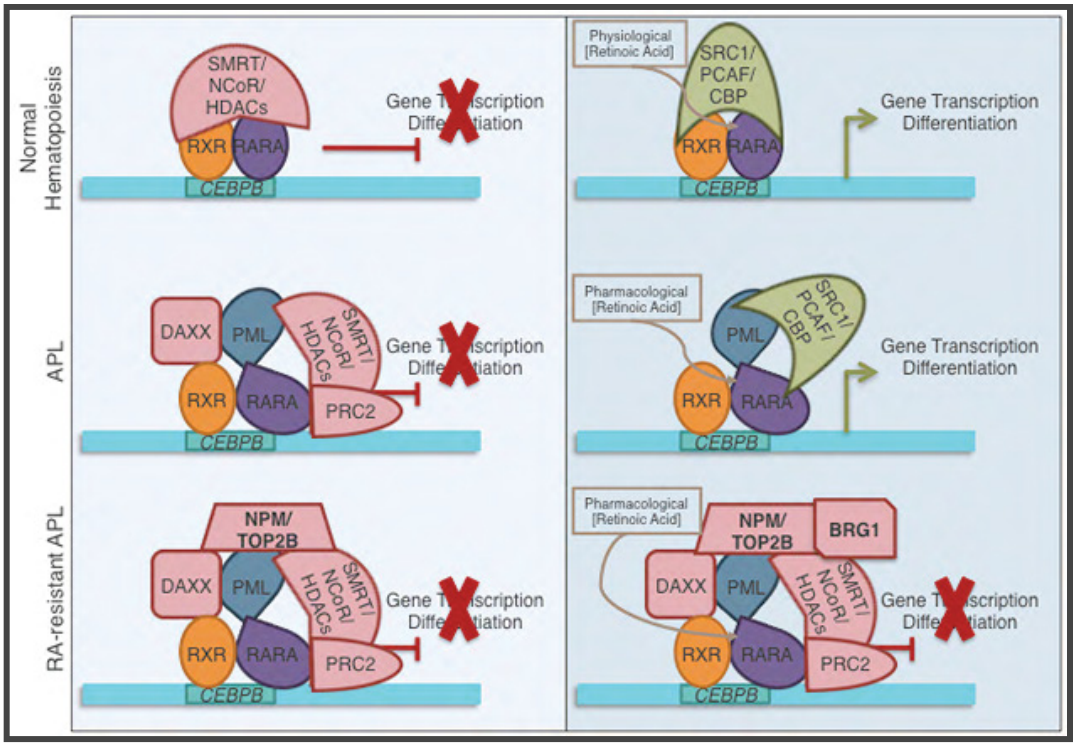


Figure 4.1. Model of RARA and PML/RARA-mediated transcriptional repression and RA-induced activation in the context of normal hematopoiesis, APL and RA-resistant APL.

APL is a subtype of myeloid leukemia characterized by a genetic lesion that leads to a block in granulocytic differentiation at the promyelocyte stage. It is paradoxical that the fusion protein that plays an integral role in the pathogenesis of this disease may also confer sensitivity to all-trans retinoic acid (RA). This is apparent from the inherent insensitivity of APL cells with other RARA fusion proteins such as PLZF/RARA or STAT5B/RARA. Finding novel strategies to induce differentiation of APL cells *in vitro* will hopefully help us overcome RA resistance not only in APL, but also to apply this type of therapy to the other subtypes of AML. This rationale has served as the *raison d'etre* of this thesis project. Understanding the basic mechanisms at play in both RA sensitive and RA insensitive contexts will provide an understanding of the general principles governing transcriptional regulation.

In summary, while the challenge of overcoming the maturation block in APL resistant cells has been met *in vitro*, it will be important to determine the

relevance of our findings to other forms of leukemia. Additionally, while RA and arsenic trioxide (ATO) are now validated as an effective frontline strategy, our understanding of the molecular underpinnings of APL pathology and the response to RA and ATO remains tenuous. Elucidation of these may shed light onto common etiologies for other AML subtypes, particularly those with NPM mutations and fusions, and lead to the development of therapeutic strategies for the less curable forms of this disease.

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APPENDIX A

Contributions of authors:

The candidate performed the majority of the research (design, bench work, analysis and writing) presented in this thesis under the supervision of Dr. Wilson H. Miller, Jr. and with the advice and support of Dr. Joaquin Espinosa.

Chapter 2

Design of the experiments was performed by Jessica Nichol, Dr. Matthew Galbraith and Dr. Joaquin Espinosa. The candidate performed the experiments and was responsible for all the data analysis and interpretation of the data. The candidate wrote the first draft of the manuscript, which was subsequently edited by all co-authors.

Chapter 3

Dr. Suzan McNamara and Jessica Nichol contributed equally to the experimental design and execution and the subsequent interpretation of results. Technical assistance was provided by Hongling Wang. The candidate wrote the first draft of the manuscript, which was subsequently edited and by all co-authors.

APPENDIX B

List of Publications not included in this thesis:

Refereed Papers

1. Nichol JN, Garnier N, and Miller WH Jr., *Triple A therapy: The molecular underpinnings of the unique sensitivity of leukemic promyelocytes to anthracyclines, all-trans retinoic acid and arsenic trioxide.* Balliere's Best Practice in Medicine (invited review, Balliere's Best Practice in Medicine, submitted April 2014).

2. Garnier N, Redstone G, Dahabieh M, Nichol JN, Del Rincon SV, Gu Y, Bohle DS, Sun Y, Conklin DS, Mann KK, Miller WH Jr., *The novel arsenical darinaparsin is transported by cysteine importing systems.* Mol Pharm. 2014 Apr;85(4): 576-85

3. Nichol JN, Petruccelli LA, and Miller WH Jr., *Expanding PML's functional repertoire through post-translational mechanisms.* Front Biosci. 2009 Jan 1; 14: 2293-306.

4. Rousseau C, Nichol JN, Pettersson F, Couture MC, and Miller WH Jr*., ERbeta sensitizes breast cancer cells to retinoic acid: evidence of transcriptional crosstalk.* Mol Cancer Res. 2004 Sep; 2(9): 523-31.

Textbook Chapters

1. Nichol JN, Assouline S, and Miller WH Jr., *Chapter 14: The etiology of acute leukemia.* Neoplastic Diseases of the Blood. Wiernik PH, Goldman JM, Dutcher J, Kyle RA (Eds.). 5th edition: 2013.