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**THE BIOLOGICAL FUNCTION OF INTERACTING PARTNERS OF ZXD FAMILY  
PROTEINS**

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at the

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May, 2009

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## **DEDICATION**

To my mentor, Dr. Joseph D. Fontes and my elder sister, Hemalatha Koneni

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My endless thanks and appreciation go to Dr. Joseph Fontes, my mentor for his guidance through this program. He is extremely focused; highly motivated scientist at the same time a very good person. He will remain my role model. I consider myself fortunate to have begun my training under his supervision.

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# **THE BIOLOGICAL FUNCTION OF INTERACTING PARTNERS OF ZXD FAMILY PROTEINS**

**Rupa V. Koeneni**

## **Abstract**

We have identified several protein-protein interactions amongst the members of the zinc finger, X-linked (ZXD) family of proteins (ZXDA, ZXDB, ZXDC and ZXDC2) that have important roles in the regulation of major histocompatibility complex class II and several myeloid-specific genes. The ZXDC and ZXDA proteins share significant nucleotide sequence homology and each contain ten C<sub>2</sub>H<sub>2</sub> zinc fingers and a transcription activation domain. In addition, ZXDC has a C-terminal region that is necessary to interact with a key cofactor, CIITA (Class II transactivator) and activate Major histocompatibility complex class II and class I (MHC II and MHC I respectively) genes.

In chapter II we demonstrate that the ZXDC and ZXDA proteins can self-associate, as well as, hetero-associate. Moreover, in vitro studies in our lab revealed that the association of ZXDC with ZXDA is necessary and self-association of neither protein was sufficient to interact with CIITA and thereby activate MHC II gene transcription. In addition to CIITA, we found that ZXDC interacted with two other components of the regulatory factor X complex, namely, RFX5 and RFX-ANK (RFX having ankyrin repeats) which are components of the MHC II enhanceosome. The RFX heterotrimeric complex consisting of RFX5, RFX-AP (RFX-associated protein), RFX-ANK binds to the



conserved X1 box of MHC II promoter. The necessity for RFX complex in MHC II gene regulation is underscored by bare lymphocyte syndrome, a genetic disorder which results from mutation in one of the RFX proteins. Our results support for a role of ZXDC-ZXDA heterocomplex to mediate interactions with components of the MHC II enhanceosome thereby enhancing the stability of the complex as a mechanism of activating MHC II gene transcription.

Interestingly, the ZXD family of proteins seems to have a broader role in gene regulation. In chapter III we demonstrate that the ZXDC protein interacts and represses the transcriptional activities of two myeloid transcription factors, namely, purine box binding protein PU.1 and Growth factor independent-1 (Gfi-1). The *MYC* gene promoter, a known target of Gfi-1, was also found to be bound by ZXDC protein. These results suggest that ZXDC may affect PU.1 and Gfi-1 transcriptional activities and/or recruitment at their target gene promoters suggesting that it may have a functional role in the differentiation of myeloid progenitor cells into specific lineages. We found that ZXDC enhanced PU.1 protein stability whose expression levels are critical for hematopoietic cell lineage decisions and development. We also demonstrated that ZXDC activated the transcription of the early growth response-2 (*EGR2*) gene whose expression is associated with monocyte-specific gene expression and repression of granulocyte genes. Our results strongly suggest that ZXDC, by affecting PU.1 and Gfi-1 recruitment and transcriptional activities at their target gene promoters, and by increasing Egr-2 expression, has an important regulatory role in monopoiesis.

# TABLE OF CONTENTS

<b>ABSTRACT</b> .....	vii
<b>LIST OF FIGURES</b> .....	xi
<b>LIST OF TABLES</b> .....	xiv
<b>LIST OF ABBREVIATIONS</b> .....	xv

## CHAPTER

### I. BACKGROUND AND INTRODUCTION

Major Histocompatibility Complex Class II genes: Structure and Function.....	1
Transcriptional Regulation of MHC II Genes.....	2
MHC II Enhanceosome.....	6
Class II Transactivator: Structure and Function.....	10
Role of ZXDC and ZXDA Proteins in MHC II Gene Regulation.....	13
C <sub>2</sub> H <sub>2</sub> Zinc fingers in Protein-Protein Interactions.....	14
Role of Transcription Factors in Myeloid Development and Leukemogenesis.....	20
PU.1 and its Interacting Partners: Role in Myeloid Development and Differentiation.....	23
Growth Factor Independent-1 in neutrophil differentiation.....	27
Dissertation Goals.....	31

### II. PROTEIN-PROTEIN INTERACTIONS OF ZXDC,

## **ZXDA: ROLE IN MHC CLASS II GENE REGULATION**

Abstract.....	33
Introduction.....	34
Materials and Methods.....	39
Results.....	43
Discussion.....	55
Literature Cited for Chapter II.....	59

## **III. THE INTERACTION OF THE ZXDC PROTEIN WITH HEMATOPOIETIC TRANSCRIPTION FACTORS, PU.1 AND GFI-1: A ROLE IN MYELOPOIESIS**

Abstract.....	65
Introduction.....	66
Materials and methods.....	71
Results.....	79
Discussion.....	103
Literature Cited for Chapter III.....	108

## **IV. DISCUSSION..... 117**

Literature Cited for Chapter IV .....	130
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## **GENERAL REFERENCES..... 140**

# LIST OF FIGURES

## Figure

<b>1.1</b>	Genetic organization of the distribution of MHC class II genes on human chromosome .....	3
<b>1.2</b>	Structure of MHC II molecule .....	4
<b>1.3</b>	Antigen presentation for T-cell activation .....	5
<b>1.4</b>	Schematic representation of the conserved upstream sequences in the promoter of MHC II genes .....	7
<b>1.5</b>	Schematic representation of the MHC II enhanceosome .....	9
<b>1.6</b>	Schematic representation of the various domains of the CIITA protein .....	12
<b>1.7</b>	Schematic representation of the domain structure of the ZXDC protein .....	15
<b>1.8</b>	Schematic representation of the domain structure of the ZXDA protein .....	16
<b>1.9</b>	Structure of a C <sub>2</sub> H <sub>2</sub> type zinc finger .....	17
<b>1.10</b>	Schematic representation of the domain structure of the PU.1 protein and its interacting partners .....	26
<b>1.11</b>	Schematic representation of the domain structure of Growth factor independent-1 .....	29
<b>1.12</b>	Defects in neutrophil differentiation and development upon loss of function mutations of key transcription factors .....	30

<b>2.1</b>	Schematic representation of ZXDC, ZXDA, ZXDB representing the regions of homology in various domains .....	37
<b>2.2</b>	ZXDC and ZXDA proteins self-associate .....	44
<b>2.3</b>	Zinc fingers of ZXDA are necessary to interact with ZXDC .....	46
<b>2.4</b>	Zinc fingers of ZXDC are necessary to interact with ZXDA .....	48
<b>2.5</b>	ZXDC interacts with RFX5 protein .....	50
<b>2.6</b>	ZXDC co-immunoprecipitated with RFXANK .....	52
<b>2.7</b>	ZXDC does not interact with RFXAP .....	54
<b>3.1</b>	A model for PU.1 and Gfi-1 mediated transcriptional regulation of granulocyte and monocyte lineage commitment and maturation .....	69
<b>3.2</b>	ZXDC and ZXDA co-immunoprecipitate with PU.1 .....	80
<b>3.3</b>	Zinc fingers of ZXDC mediate interaction with PU.1 .....	82
<b>3.4</b>	ZXDC interacts with the region of PU.1 containing the activation domain .....	85
<b>3.5</b>	ZXDC and ZXDA repressed PU.1 transcriptional activity .....	87
<b>3.6</b>	ZXDC has a role in PU.1 protein stabilization .....	90
<b>3.7</b>	ZXDC and ZXDA co-immunoprecipitate with Gfi-1 .....	92
<b>3.8</b>	Zinc fingers of ZXDC mediate ZXDC-Gfi-1 interaction .....	94
<b>3.9</b>	ZXDC repressed Gfi-1 transcriptional activity in a dose-dependent manner .....	96
<b>3.10</b>	Chromatin immunoprecipitation (ChIP) analysis in HEK293 cells demonstrating the presence of ZXDC along with Gfi-1	

	at the c-Myc locus .....	98
<b>3.11</b>	ZXDC activates Egr-2 gene transcription .....	100
<b>3.12</b>	ZXDC enhanced Egr-2 and Egr-4 mRNA expression levels .....	102

## LIST OF TABLES

### Table

<b>1.1</b>	Amino acid sequences of ten C <sub>2</sub> H <sub>2</sub> type zinc fingers of ZXDC protein .....	19
<b>1.2</b>	Examples of transcription factor mutations and their effects in AML patients.....	22

## LIST OF ABBREVIATIONS

APC	Antigen presenting cell
AML	Acute myeloid leukemia
AML1	Acute myeloid leukemia protein 1
AMKL	Acute megakaryoblastic leukemia
BSAP	B-cell specific activator protein
BLSII	Bare lymphocyte syndrome type II
CIITA	Class II transactivator
CDKI	Cyclin dependent kinase inhibitor
C/EBP $\alpha$	CCAAT/ enhancer binding protein $\alpha$
C/EBP $\beta$	CCAAT/enhancer binding protein $\beta$
C/EBP $\epsilon$	CCAAT/enhancer binding protein $\epsilon$
ChIP	Chromatin immunoprecipitation
CMP	Common myeloid progenitor
Co-IP	Co-immunoprecipitation
CREB	Cyclic AMP responsive element binding protein
CUS	Conserved upstream sequence
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EGR	Early growth response proteins
ETO	Eight 21 corepressor
FAB	French American British system of classification
FBS	Fetal bovine serum



FLT3	FMS related tyrosine kinase 3
FOG	Friend of GATA
GATA1	GATA binding protein 1
GATA2	GATA binding protein 2
G-CSF	Granulocyte colony stimulating factor
G-CSFR	Granulocyte colony stimulating factor receptor
Gfi-1	Growth factor independent-1
GM-CSF	Granulocyte-monocyte colony stimulating factor
GMP	Granulocyte monocyte progenitor
GR	Glucocorticoid receptor
HA	Haemagglutinin
HDAC	Histone deacetylase
HLA	Human leukocyte antigen
HOX	Homeobox
HSP90	Heat shock protein 90
HSC	Hematopoietic stem cell
IA	Iodoacetamide
IB	Immunoblot
ICSBP	Interferon consensus sequence binding protein
IFN $\alpha$	Interferon alpha
IFN $\beta$	Interferon beta
IFN $\gamma$	Interferon gamma
IP	Immunoprecipitation

IRF	Interferon regulatory factor
ITD	Internal tandem duplication
IL-2	Interleukin 2
LMP	Lymphoid myeloid progenitor
LRR	Leucine rich repeat
MCSF	Macrophage colony stimulating factor
MCSFR	Macrophage colony stimulating factor receptor
MEP	Megakaryocyte erythroid progenitor
MHC II/ MHC class II	Major histocompatibility complex class II
miRNA	Micro-RNA
MLL	Mixed lineage leukemia
MoMLV	Moloney murine leukemia virus
MPP	Multipotential progenitor
MYH11	Myosine heavy chain 11
NEM	N-ethylmaleimide
NF- $\kappa$ B	Nuclear factor kappa B
PML	Promyelocytic leukemia
PMSF	Phenyl methyl sulfonyl fluoride
P/S/T	Proline/serine/threonine rich domain
PU.1	Purine box binding protein coded by Spi-1 gene
PVDF	Polyvinylidene difluoride
RAR $\alpha$	Retinoic acid receptor $\alpha$
RFXANK	Regulatory factor X with ankyrin repeats

RFXAP	Regulatory factor X associated protein
RUNX1	Runt related transcription factor 1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SELEX	Systemic evolution of ligands by exponential enrichment
SNAG	Snail/Gfi1 domain
SPI1	Spleen focus forming virus (SFFV) proviral integration
SUMO	Small ubiquitin like modifier
TAD	Transcriptional activation domain
TCR	T cell receptor
TGF $\beta$	Transforming growth factor $\beta$
VDR	Vitamin D receptor
WB	Western blot
ZXDA	Zinc finger X linked duplicated family member A
ZXDB	Zinc finger X linked duplicated family member B
ZXDC	Zinc finger X linked duplicated family member C

# CHAPTER I

## BACKGROUND AND INTRODUCTION

### Major histocompatibility complex class II genes - Structure and Function

The expression of major histocompatibility complex class II (MHC II) genes is important for adaptive immune response. MHC II molecules are cell surface glycoproteins that are expressed on professional antigen presenting cells (APC) such as B cells, dendritic cells, macrophages and thymic epithelial cells and are inducible by interferon gamma ( $\text{IFN}\gamma$ ) on most other cell types. These proteins are encoded on the short arm of chromosome 6 (6p.21.32) (Trowsdale, 1993) and the genes encoding these proteins are called Human leukocyte antigens (HLA). HLA-DR, HLA-DP and HLA-DQ are the three MHC II isotypes in humans. Fig. 1.1 depicts a schematic representation of the organization of MHC II genes on the short arm of chromosome 6.

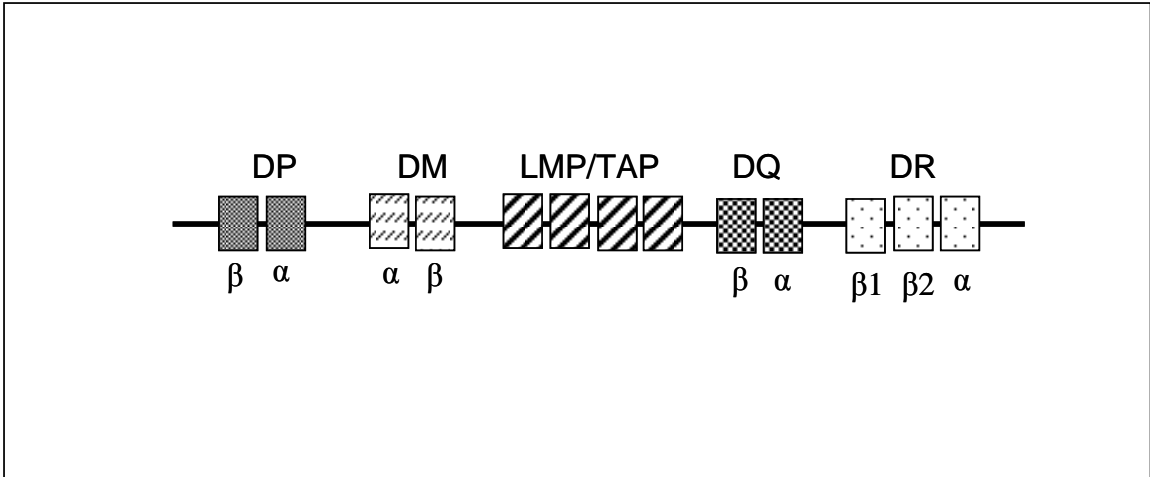
MHC class II is a heterodimer composed of an alpha and a beta chain. The C-terminal transmembrane domains of the alpha and beta chains anchor to the cell membrane and their N-terminal regions form a cleft like structure on the cell surface to present antigenic peptides to the T cell receptor (TCR) of  $\text{CD4}^+$  T helper cells (Fig. 1.2). Based on the MHC II peptide-TCR interaction (Fig. 1.3), it will result in either positive or

negative selection of the CD4<sup>+</sup> T cell population that will determine the selection of the TCR of the mature CD4<sup>+</sup> T cell population (Viret and Janeway, Jr., 1999). This MHC II peptide-TCR interaction is necessary for initiation, maintenance and regulation of antigen specific immune response (Cresswell, 1994; Waldburger et al., 2000). The life span of the CD4<sup>+</sup> T cell population in the periphery is also dependent on this interaction (Viret and Janeway, Jr., 1999).

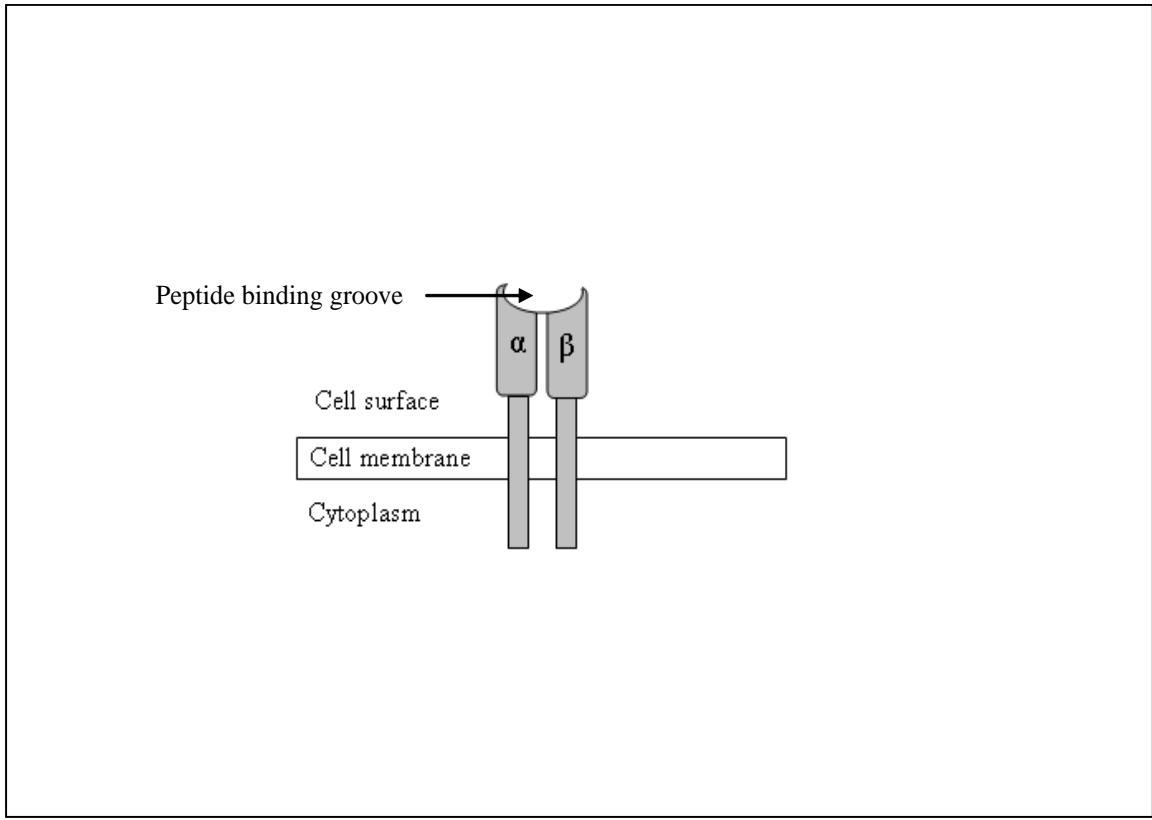
Tight regulation of MHC II gene expression is critical. For example, lack of MHC II expression leads to a severe immune deficient condition called Bare Lymphocyte Syndrome type II (BLS II) (Touraine and Marseglia, 1992), whereas, over-expression of MHC II genes can result in autoimmunity caused by their inappropriate expression (Bottazzo et al., 1986a; Bottazzo et al., 1986b).

### **Transcriptional regulation of MHC II genes**

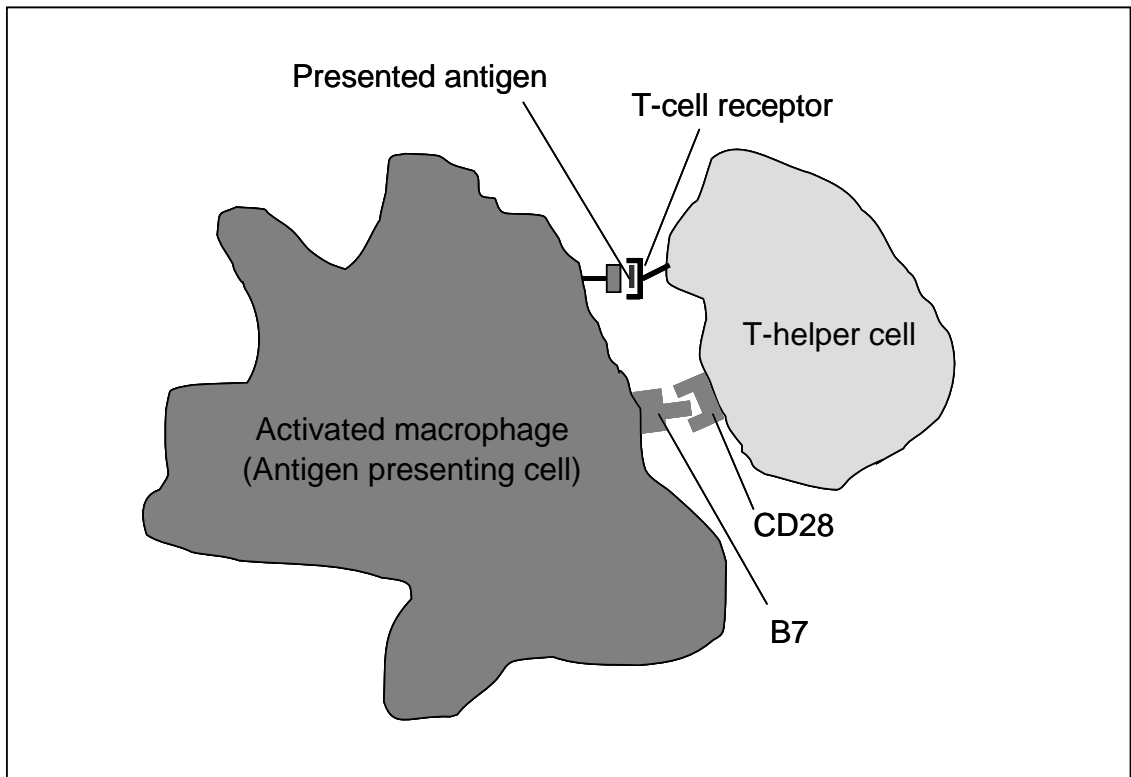
MHC II genes are tightly regulated at the transcriptional level (Mach et al., 1996; Benoist et al., 1990; Glimcher et al., 1992; Ting et al., 1993; Boss et al., 1997). These genes are expressed either in a constitutive or inducible manner (Mach et al., 1996; Benoist et al., 1990; Glimcher et al., 1992; Ting et al., 1993; Boss et al., 1997). Constitutive expression of MHC II genes occurs on professional antigen presenting cells, namely, B lymphocytes, macrophages and dendritic cells, whereas, they can be induced in most cell types by IFN $\gamma$  (Drozina et al., 2005). However, transforming growth factor  $\beta$  (TGF  $\beta$ ), interleukin-1 (IL-1), interferon  $\alpha$  (IFN  $\alpha$ ) inhibit MHC II expression by IFN $\gamma$  (reviewed in Waldburger et al., 2000).



**FIGURE 1.1 Genetic organization of the distribution of MHC class II genes on human chromosome 6:** The distribution of MHC II gene isotypes, HLA-DP, DQ, DR on human chromosome 6 is shown above. Each isotype has distinct  $\alpha$  and  $\beta$  encoding genes. LMP/TAP genes code for proteins necessary for processing antigen and HLA-DM gene code for proteins necessary for antigen presentation of MHC class II molecules.



**FIGURE 1.2 Structure of MHC II molecule:** MHC II molecules are cell surface glycoproteins that are expressed on the surface of professional antigen presenting cells. The protein consisting of an alpha and beta subunit forms a cleft like structure on the cell surface for peptide binding and antigen presentation.



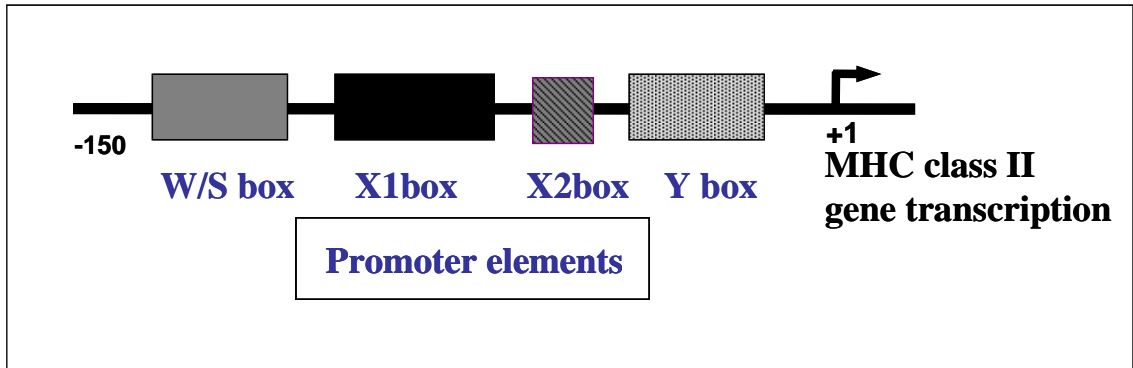
**FIGURE 1.3 Antigen presentation for T-cell activation:** In order for T cells to be activated, they must recognize the antigen presented by the antigen presenting cell (APC). The APC processes and presents peptide antigens to the T-cell receptor (TCR) of the helper T cell. Co stimulation usually involves additional proteins like B7 and CD28 on the surface of APC and T-helper cells respectively (reviewed in Peggs et al., 2008). The MHC II peptide complex-TCR interaction is necessary for initiation, maintenance and regulation of immune responses.



A set of conserved promoter elements (namely, W/S, X1, X2 and Y boxes in a 5' to 3' orientation; Fig. 1.4) are located within 150 bps upstream of the transcription start site that are necessary and sufficient for both constitutive and inducible expression of MHC II proteins. The position, orientation and spacing among the conserved cis-elements are critical for MHC II expression (reviewed in Waldburger et al., 2000). Similar arrangement of cis-elements is seen in the promoters of invariant chain (Ii) and HLA-DM genes (Ting et al., 1997; Westerheide et al., 1997; Brown et al., 1991; Brown et al., 1993; Tai et al., 1999) which code for proteins necessary for intracellular trafficking and peptide loading of MHC II molecules (Busch et al., 1996; Cresswell et al., 1996). Recently, additional X-box like sequences in MHC II region were identified that function as locus control regions (Gomez et al., 2005).

### **MHC II enhanceosome**

The promoter of MHC II genes consist of a set of conserved upstream sequences (CUS), namely, W/S, X1, X2 and Y boxes (5' to 3' orientation). These promoter elements are necessary and sufficient for the transcriptional regulation and expression of these genes in a constitutive and inducible manner (Benoist et al., 1990; Glimcher et al., 1992). The position and orientation of these promoter elements with respect to each other is critical for their function. A set of DNA binding proteins, namely, regulatory factor X (RFX) heterotrimeric complex, cyclic AMP response element binding (CREB) protein and nuclear factor Y (NFY) complex bind to the conserved X1, X2 and Y boxes respectively. The RFX complex, composed of RFX5 (Steimle et al., 1995), RFXAP (RFX associated protein) (Villard et al., 1997) and RFXANK (RFX with ankyrin repeats)

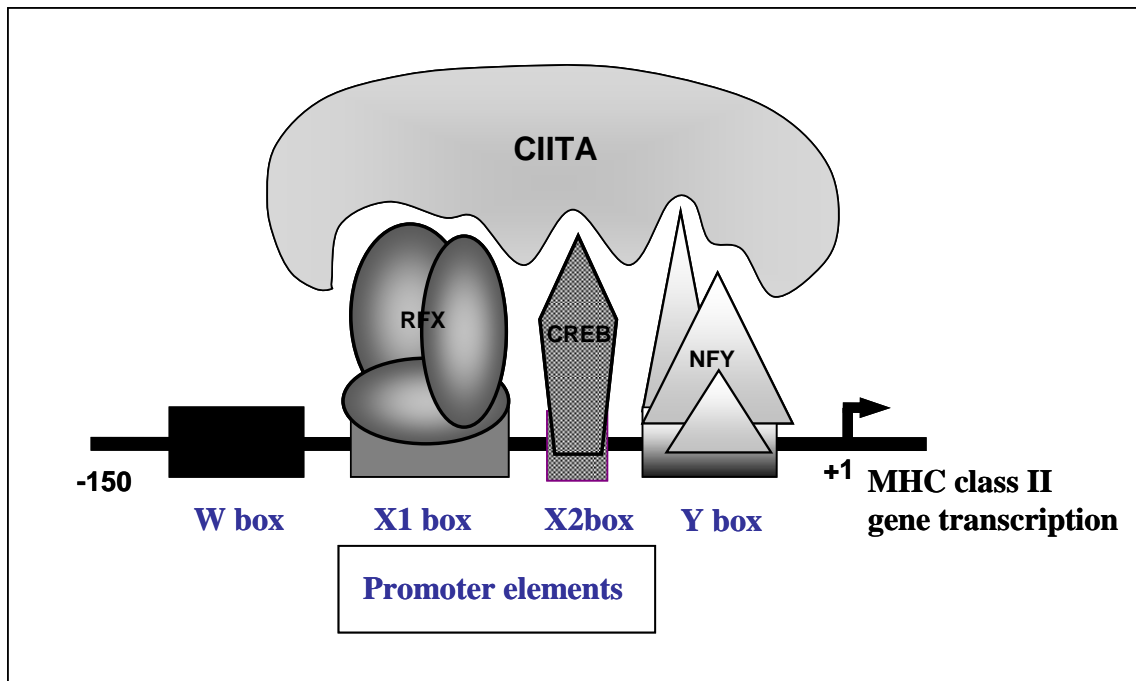


**FIGURE 1.4 Schematic representation of the conserved upstream sequences in the promoter of MHC II genes:** The W/S, X1, X2 and Y box represent the conserved promoter elements located within 150 bps upstream of the transcription start site of the MHC II promoter. The orientation and spacing among these elements with respect to each other is critical for proper MHC II gene regulation. These elements are necessary and sufficient for both constitutive and inducible expression of MHC II genes.

(Masternak et al., 1998) binds to the X1 box as a heterotrimeric complex (DeSandro et al., 2000). The X2 box is bound by cyclic AMP responsive element binding (CREB) protein (Moreno et al., 1999). The NFY complex composed of NFY-A, NFY-B and NFY-C proteins binds to the Y box (Mantovani, 1999). The W/S box is critical for MHC II expression, but the identity of the protein or complex that binds there is not yet known (Kara and Glimcher, 1991). These DNA binding proteins are ubiquitously distributed & though their expression is necessary, it is not sufficient for MHC II expression.

The expression of MHC II genes in a given cell type is mainly determined by a transcriptional cofactor named class II transactivator (CIITA; Steimle et al., 1993). The CIITA protein does not bind DNA directly; instead it is recruited to MHC II promoter through co-operative interactions with the DNA bound factors (Masternak et al., 2000). CIITA also recruits chromatin remodeling factors and histone modifying enzymes and through synergistic interactions with the DNA bound factors forms a three-dimensional higher order complex which is termed the MHC II enhanceosome (Masternak et al., 2000; Waldburger et al., 2000; Jabrane-Ferrat et al., 2003; Spilianakis et al., 2003). Fig. 1.5 demonstrates a schematic representation of the components of the MHC II enhanceosome.

Enhanceosomes are higher order complexes and are held through multiple protein-protein and protein-DNA interactions that function in a co-operative manner to activate target genes. Specific enhanceosomes regulate several other eukaryotic genes, for example, Interferon  $\beta$  (IFN  $\beta$ ), T-cell receptor  $\alpha$  chain and interleukin-6 (IL-6) (Merika and Thanos, 2001). In the case of MHC II enhanceosome, there exist cooperative interactions between the DNA bound factors (Westerheide and Boss, 1999;



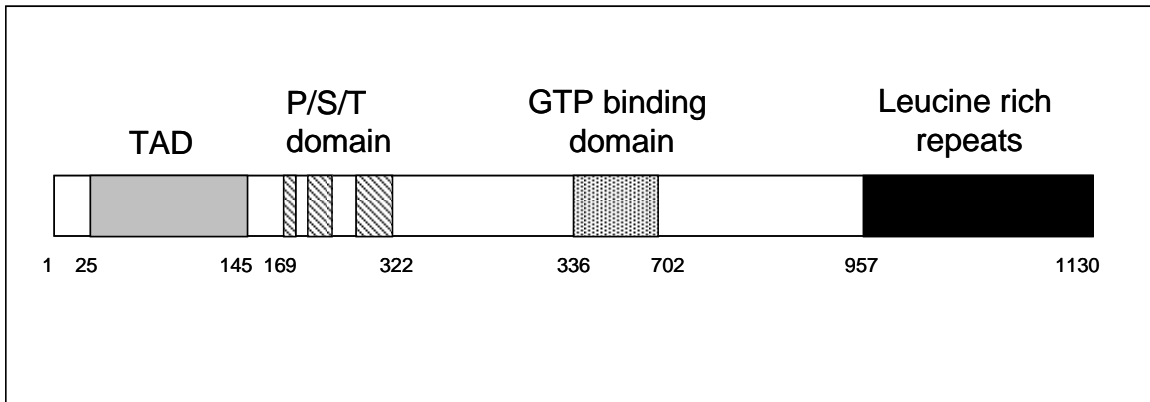
**FIGURE 1.5 Schematic representation of the MHC II enhanceosome:** This figure demonstrates a schematic representation of various cis-elements and trans-acting factors that constitute components of the MHC II enhanceosome. The RFX heterotrimeric complex consisting of RFX5, RFX-AP and RFX-ANK binds to the X1 box, NFY complex composed of NFY-A, B, C binds to the Y box, whereas, CREB protein binds to the X2 box. The factor binding to the W/S box is critical for the recruitment of CIITA, though the identity of this protein is not known. The CIITA protein does not bind DNA directly and is recruited to the MHC II promoter by the DNA bound factors.

Caretti et al., 2000). The individual protein-protein or protein-DNA interactions are weak, but, multiple interactions are capable of recruiting the key transcriptional co-activator, CIITA (Masternak et al., 2000). There are several mechanisms of enhanceosome dependent transcriptional synergy. Some of these include modification of chromatin structure for the recruitment of preinitiation complex, enabling nucleosome modification and enhancing the stability of enhanceosome (reviewed in Merika and Thanos, 2001; Carey, 1998).

### **Class II transactivator - Structure and Function**

CIITA belongs to the CATERPILLER family of proteins which is so named for the various conserved domains-namely CARD domain, transcriptional enhancer, purine binding, pyrin domain and lots of leucine rich repeats (Harton et al., 2002). It is a large protein consisting of 1130 amino acids. Fig. 1.6 is a schematic representation of the various domains of the CIITA protein. The N-terminal region (amino acids 25-145) is rich in acidic amino acids and functions as a transcription activation domain (TAD; Riley et al., 1995). Next to this, is a region rich in proline, serine, threonine (P/S/T) residues. Phosphorylation of the P/S/T domain is required for oligomerization and accumulation of the protein (Tosi et al., 2002). Following the P/S/T domain are three GTP binding motifs that are necessary for nuclear import and self-association (Chin et al., 1997; Harton et al., 1999; Linhoff et al., 2001; Raval et al., 2003). The C-terminal portion of the protein (amino acids 957-1130) has several leucine rich repeats which have a role in protein-protein interactions, nuclear translocation and self-association of CIITA protein (Hake et al., 2000; Kretsovali et al., 2001; Sisk et al., 2001; Towey et al., 2002; Sisk et al., 2003; Linhoff et al., 2001; Harton et al., 2002). CIITA plays an important role in MHC II

enhanceosome assembly. In addition to DNA bound factors, CIITA also interacts with subunits of general transcription machinery (hTAF<sub>II</sub>32, hTAF<sub>II</sub>70, hTAF<sub>II</sub>250; Fontes et al., 1997; Mahanta et al., 1997) and chromatin modifying enzymes (CBP, P300, pCAF; Kretsovali et al., 1998; Fontes et al., 1999; Spilianakis et al., 2000) that have important roles in activating MHC II gene transcription. In order to better understand the molecular mechanisms of CIITA mediated MHC II gene activation, our lab performed yeast-two hybrid assay of human spleen cDNA library with the C-terminal leucine rich repeats of the CIITA protein, as one of its important roles is to mediate protein-protein interactions (Kobe and Kajava, 2001). Of the several positive clones, one was a poorly characterized cDNA coding for a novel zinc finger protein, named as Zinc finger, X-linked, duplicated family member C (ZXDC). ZXDC protein has ten C<sub>2</sub>H<sub>2</sub> type zinc fingers towards the amino terminus, followed by a transcription activation domain and a C-terminal region that mediates interaction with CIITA (Fig. 1.7; Alkandari et al., 2007a). ZXDC protein was so named because of its amino acid sequence homology with two other zinc finger proteins, namely, Zinc finger, X-linked, duplicated family members A and B (ZXDA and ZXDB respectively; Fig. 1.8 demonstrates a schematic representation of the domain structure of the ZXDA protein). Studies in our lab demonstrated that the ZXDC and ZXDA proteins cooperated with CIITA in the activation of MHC II gene transcription, whereas, knockdown of ZXDC or ZXDA reduced the ability of CIITA to activate MHC II genes without affecting CIITA expression.



**FIGURE 1.6 Schematic representation of the various domains of the CIITA protein:**

The CIITA is a 1130 amino acid protein composed of an N-terminal activation domain (amino acids 25-145) rich in acidic amino acids, followed by Proline/Serine/Threonine (P/S/T) domain, GTP binding domain (amino acids 336-702) and C-terminal leucine rich repeat region (amino acids 957-1130).

## **Role of ZXDC and ZXDA proteins in MHC II gene regulation**

Our lab performed yeast-two hybrid assay using the CIITA C-terminal 807 amino acid fragment as bait and identified a novel protein named Zinc finger, X linked, duplicated family member C (ZXDC). The ZXDC protein contained ten multiple adjacent C<sub>2</sub>H<sub>2</sub> type zinc fingers followed by transcription activation domain (TAD) and a C-terminal CIITA binding region. Fig. 1.7 depicts a schematic representation of the various domains of the ZXDC protein. Studies in our lab demonstrated that ZXDC cooperated with CIITA to activate MHC II and MHC I genes, whereas, knock-down of ZXDC reduced MHC II expression by CIITA (Al-Kandari et al., 2007a). In the same report, the presence of ZXDC at MHC II gene promoter in Raji cells, a B cell line that constitutively expresses MHC II genes was shown. ZXDC protein was so named because of its amino acid sequence homology with two other zinc finger proteins, namely, Zinc finger, X-linked, duplicated family members A and B (ZXDA and ZXDB respectively; Fig. 1.8 demonstrates a schematic representation of the domain structure of the ZXDA protein). The ZXDA and ZXDB proteins were identified during X-inactivation studies (Greig et al., 1993). Studies in our lab demonstrated that ZXDA also cooperated with CIITA resulting in synergistic activation of MHC II genes (Al-Kandari et al., 2000b). This was an interesting observation since ZXDA lacked the CIITA binding region, but still cooperated with CIITA suggesting that other interacting partners could mediate ZXDA-CIITA interaction. In the same report, it was demonstrated that the ZXDC and ZXDA proteins can self-associate as well as interact with each other and 'in vitro' studies demonstrated that only the ZXDC-ZXDA hetero-complex but neither protein by itself were found to interact with CIITA. Supporting this, silencing the expression of ZXDC or

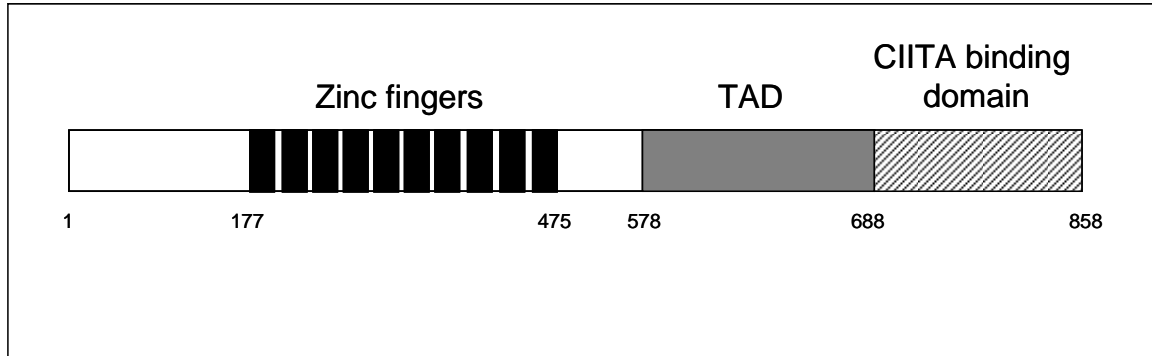


ZXDA reduced MHC II expression by CIITA similar to that of knock-down of both ZXDC and ZXDA demonstrating their cooperative role in MHC II gene activation (Al-Kandari et al., 2000b). In addition, ZXDC protein was found to be present at the MHC II promoter in HeLa cells prior to and after IFN $\gamma$  treatment (Alkandari et al., 2000b).

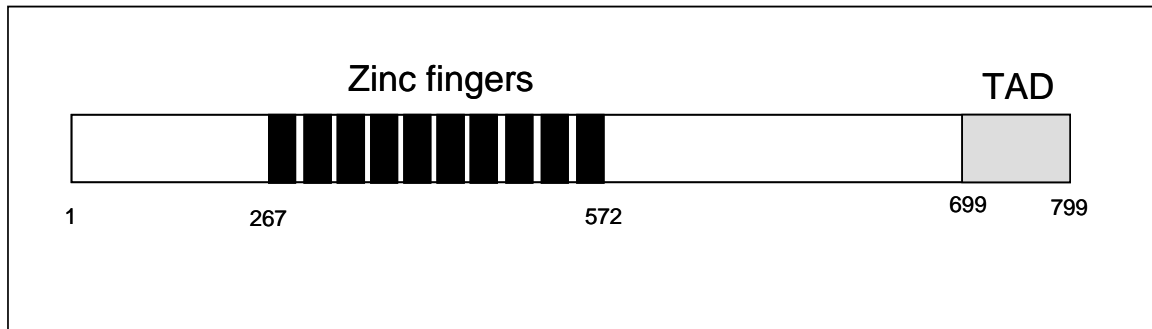
### **C<sub>2</sub>H<sub>2</sub> zinc fingers in protein-protein interactions**

Zinc fingers were first identified as conserved motifs present in the DNA binding protein TFIIIA. A C<sub>2</sub>H<sub>2</sub> zinc finger is also called the TFIIIA or Kruppel-like finger and consists of 20-30 amino acids with a secondary structure formed by tetrahedral binding of Zn<sup>2+</sup> to two cysteine and two histidine residues (Klug and Schwabe, 1995; Pabo et al., 2001; Laity et al., 2001). It consists of two  $\beta$  strands and an  $\alpha$  helix and is generally described as X<sub>2</sub>CX<sub>2-4</sub>CX<sub>12</sub>HX<sub>2-8</sub>H where 'X' is any amino acid and the numbers represent the spacing among the conserved residues (Fig 1.9). The structure of zinc fingers mainly depends on the nature and spacing between the conserved amino acid residues that bind to the Zn<sup>2+</sup> (Mackay et al., 1998). In classical zinc fingers, the zinc atom is coordinated in either a Cys-Cys-His-His or Cis-Cis-His-Cys manner. The C<sub>2</sub>H<sub>2</sub> zinc finger proteins are the largest among the zinc finger family of proteins and were found to have roles in development, differentiation and tumor suppression.

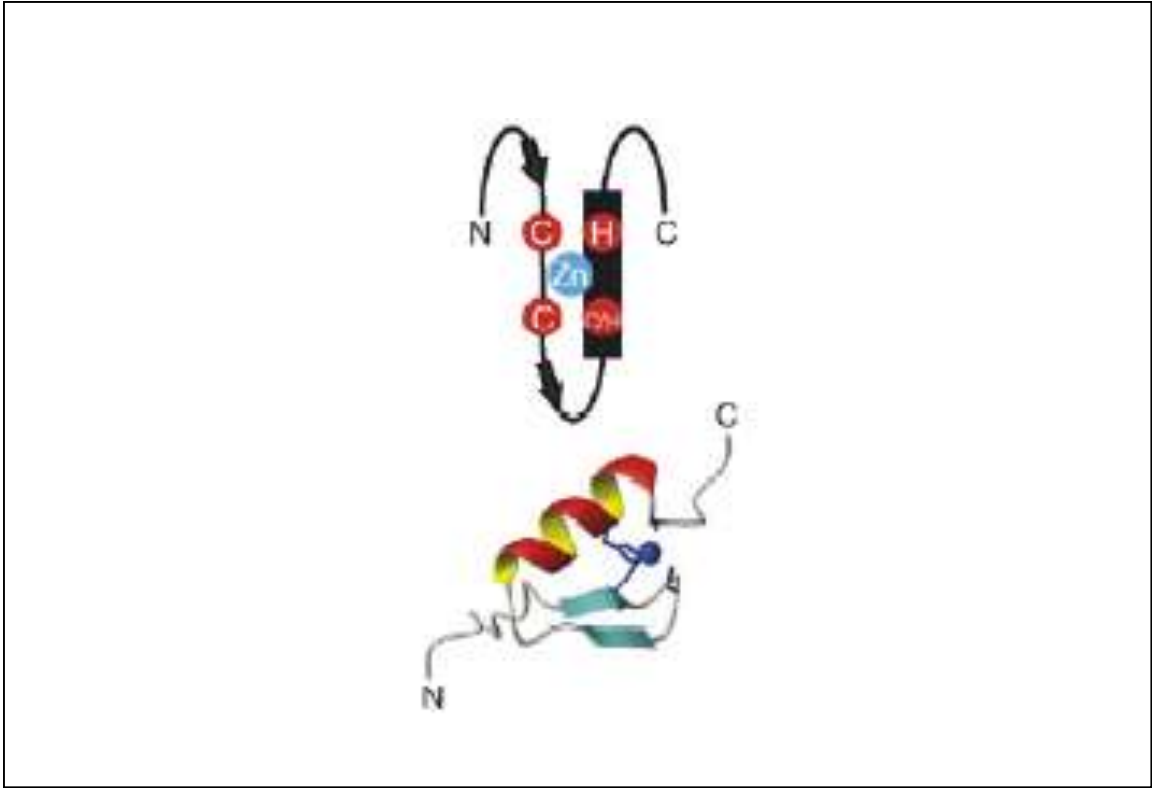
Based on the number and the arrangement of C<sub>2</sub>H<sub>2</sub> zinc fingers in proteins, they are classified into four groups (a) single C<sub>2</sub>H<sub>2</sub> (b) triple C<sub>2</sub>H<sub>2</sub> (c) multiple adjacent C<sub>2</sub>H<sub>2</sub> and (d) separated-paired C<sub>2</sub>H<sub>2</sub> zinc finger proteins (Iuchi et al., 2001). The multiple zinc fingers are generally arranged in tandem with short peptide sequences between them called "linkers". The ZXD family proteins, ZXDC, ZXDA and ZXDB proteins have ten



**FIGURE 1.7 Schematic representation of the domain structure of the ZXDC protein:** ZXDC is a 858 amino acid protein consisting of ten C<sub>2</sub>H<sub>2</sub> type zinc fingers towards the N-terminus (between amino acids 177-475), followed by transcriptional activation domain (TAD; amino acids 578-688) and a C-terminal domain that is necessary for interaction with the CIITA protein (amino acids 688-858).



**FIGURE 1.8 Schematic representation of the domain structure of the ZXDA protein:** ZXDA is a 799 amino acid protein consisting of ten C<sub>2</sub>H<sub>2</sub> type zinc fingers towards the N-terminus of the protein (between amino acids 267-572) and a transcriptional activation domain (TAD; amino acids 699-799) at the C-terminus.



**FIGURE 1.9 Structure of a C<sub>2</sub>H<sub>2</sub> type zinc finger:** A C<sub>2</sub>H<sub>2</sub> zinc finger consists of two  $\beta$  strands and an  $\alpha$  helix and is made of 20-30 amino acids with a secondary structure formed by tetrahedral binding of Zn<sup>2+</sup> to two cysteine and two histidine residues. It is generally represented as X<sub>2</sub>CX<sub>2-4</sub>CX<sub>12</sub>HX<sub>2-8</sub>H where X is any amino acid and the numbers represent spacing among the conserved residues. The structure of a zinc finger mainly depends on the type and spacing among the conserved residues that bind to the Zn<sup>2+</sup> (Ref: Gamsjaeger R et al., 2007).

multiple adjacent C<sub>2</sub>H<sub>2</sub> type zinc fingers, whereas, the ZXDC2 protein, an mRNA variant of ZXDC, lacks the first three fingers that are conserved in other members of the family.

Table 1.1 shows the amino acid sequences of the ten zinc fingers in ZXDC protein.

Although zinc fingers (ZnFs) are majorly known to have roles in DNA binding, there are several examples of zinc finger proteins that have functional roles mediated by protein-protein interactions (Mackay et al., 1998; Sun et al., 1996; Morgan et al., 1997; Merika et al., 1995; Tsai et al., 1998) and by RNA binding (Brown, 2005; Hall, 2005; Lee et al., 2006; Lu et al., 2003). Many zinc finger proteins undergo homo- and/or hetero-dimerization by hydrophobic interactions or by finger-finger binding. Dimerization of these proteins was found to modulate their DNA binding activity and to regulate the expression of target genes. In addition, the relative levels of homo- and hetero-dimers available and the expression levels of the proteins involved modulates their activity that result in important biological effects (Morgan et al., 1997). Also, in many proteins having multiple zinc fingers, some zinc fingers mediate DNA binding, whereas, others are involved in selective protein-protein interactions (Kelley et al., 1998; Tsai et al., 1998; Matsuzawa-Watanabe et al., 2003) which play a role in several regulatory functions.

An example of the multi-functional nature of zinc fingers is the friend of GATA-1 (FOG) protein, a zinc finger transcription factor containing nine classical zinc fingers. Five of these fingers mediate protein-protein interaction with the N-terminal GATA type ZnF of the transcription factor GATA-1. The recruitment of FOG by GATA-1 at GATA-1 target gene promoters is found to be necessary for erythro- and megakaryopoiesis in mammals (Tsang et al., 1998). In another example, transcription factors of the Ikaros

177	C	P	E	P	Q	C	A	L	A	F	A	K	K	H	Q	L	K	V	H	L	L	T	H	
210	C	P	L	E	G	C	G	W	A	F	T	T	S	Y	K	L	K	R	H	L	Q	S	H	
240	C	P	V	G	G	C	G	K	K	F	T	T	V	Y	N	L	K	A	H	M	K	G	H	
270	C	E	V	--	C	A	E	R	F	P	T	H	A	K	L	S	S	H	Q	R	S	H	H	
299	C	D	F	P	G	C	E	K	T	F	I	T	V	S	A	L	F	S	H	N	R	A	H	
330	C	S	F	P	G	C	S	K	Q	Y	D	K	A	C	R	L	K	I	H	L	R	S	H	
360	C	D	S	D	S	C	G	W	T	F	T	S	M	S	K	L	L	R	H	R	R	K	H	
389	C	P	V	E	G	C	G	K	S	F	T	R	A	E	H	L	K	G	H	S	I	T	H	
419	C	P	V	E	G	C	C	A	R	F	S	A	R	S	S	L	Y	I	H	S	K	K	H	
452	C	P	V	S	T	C	N	R	L	F	T	S	K	H	S	M	K	A	H	M	V	R	Q	H

**TABLE 1.1 Amino acid sequences of ten C<sub>2</sub>H<sub>2</sub> type zinc fingers of ZXDC protein:**

The amino acid sequences of ten Cys-Cys:His-His zinc fingers of the ZXDC protein is shown in the table above (Alkandari et al., 2007a)

family have two clusters of zinc fingers - one at the N-terminus and another at the C-terminus. The N-terminal cluster was found to have a role in DNA binding and the C-terminal cluster mediates interactions with itself, as well as, with other members of the family and these interactions were found to have a central role in lymphoid development (Kelley et al., 1998; McCarty et al., 2003). However, it should be noted that direct gene regulation mediated by zinc fingers is not the only regulatory mechanism by zinc finger proteins. There are examples of zinc finger proteins that have biological roles that are mediated by protein-protein interactions. An example is the Escherichia coli heat shock protein, DnaJ (contains two CCCC zinc fingers) that binds to unfolded proteins and helps in their folding in the cell (reviewed in Mackay et al., 1998).

### **Role of transcription factors in myeloid development and leukemogenesis**

Transcription factors play a very important role in hematopoiesis (Blau, 1991; Shivdasani, 1996; Orkin, 1995). The differentiation and development of hematopoietic stem cells into mature myeloid cells, namely, granulocytes and monocytes is known as myelopoiesis. Transcription factors play an important role in the differentiation of hematopoietic stem cells into multipotential progenitors (MPP; granulocyte-monocyte progenitors [GMP], in this case) which in turn give rise to mature granulocytes or monocytes (Friedman, 2007). The MPP have lost complete self-renewal activity but express receptors for certain growth factors and have the potential to develop into specific hematopoietic cell lineages that can develop into mature blood cells. Our study focuses on transcription factors involved in myeloid development and differentiation.

The key role of transcription factors in myeloid differentiation and leukemogenesis has been reviewed vastly in the past few years (Friedman, 2007;

Rosenbauer and Tenen, 2007; Tenen, 2003; Laslo et al., 2006). Studies have demonstrated that cross-talk among several key transcription factors, their expression and activity is critical for determining hematopoietic cell lineage decisions and differentiation (Orkin, 2000). Such regulatory mechanisms are important for normal myeloid development. For example, Acute myeloid leukemia (AML) is a condition in which there is a block in the myeloid differentiation process resulting in uncontrolled proliferation of immature myeloid progenitors. One of the major causes of this group of myeloid leukemias is mutation or dysregulation of certain key transcription factors involved in myeloid development and differentiation. Some examples of transcription factor mutations in patients with AML are listed in table 1.2. Identification and characterization of these factors and understanding their regulatory roles is very important for the discovery of novel therapeutic targets for intervention for the treatment of various types of cancers (Tenen, 2003).

In the determination of hematopoietic cell lineage decisions, several external (eg. growth factors, stroma) or internal factors or both may play a role (Metcalf, 1993; Roberts, 1988; Fairbairn, 1993; Just, 1993). In either or both cases, the interplay among several cell-specific and ubiquitous transcription factors (which mostly results in the expression of growth factors or their receptors) and the net result of this cross-talk seem to play a crucial role for determining hematopoietic cell fates (Tenen et al., 1997). In addition, several chromosomal rearrangements in different types of myeloid leukemias are at sites encoding transcription factors (Rabbitts et al., 1994), knock-out of key



Transcription factor	Mutations and effects	Frequency in AML	FAB system subtype
RUNX1-ETO (t(8;21))	RUNX1 DNA-binding domain fused to the transcriptional corepressor ETO; downregulates expression or activity of PU.1, C/EBP $\alpha$ and RUNX1	12–15%	M2
CBF $\beta$ -MYH11 (inv16)	Inversion of breaks in chromosome 16; joins CBF $\beta$ with the myosin gene MYH11	8–10%	M4 <sub>Eo</sub>
PML-RAR $\alpha$ (t(15;17))	PML gene fused to RAR $\alpha$ ; blocks myeloid transcription factors (such as C/EBP $\alpha$ and PU.1)	6–7%	M3
MLL fusions (t11q23)	MLL gene fused with one of 30 distinct genes encoding partner proteins; believed to dysregulate HOX genes	4–7%	Diverse pattern of myeloid and lymphoid leukaemias
C/EBP $\alpha$	Amino-terminal dominant negative; carboxy-terminal loss of DNA binding	7–9%	M1, M2 (most), M4 (rare)
GATA1	Amino-terminal dominant negative	Nearly 100% in AMKL associated with Down's syndrome	M7 with Down's syndrome
PU.1	Mutations decrease heterodimer formation and DNA binding*; PU.1 activity downregulated by RUNX1-ETO, PML-RAR $\alpha$ and FLT3-ITD	<7%	M0, M4, M5, M6
RUNX1	Missense, nonsense or frameshift mutations (often biallelic); clustered within the runt domain	9%	M0 (most)

**Table 1.2 Examples of transcription factor mutations and their effects in AML patients:** The table above lists transcription factors that are often mutated and their effects in patients with acute myeloid leukemia (AML). AMKL: acute megakaryoblastic leukemia; CBF $\beta$ : core binding factor  $\beta$ ; C/EBP $\alpha$ : CCAAT/ enhancer binding protein  $\alpha$ ; FAB: French-American-British classification of AML that is based on the assessment of the stage of differentiation of blasts; FLT3: FMS-related tyrosine kinase 3; GATA1: GATA binding protein 1; HOX: homeobox; ITD: internal tandem duplication; MLL: mixed lineage leukemia; MYH11: myosin heavy chain 11; PML: promyelocytic leukemia; PU.1: transcription factor encoded by Spi-1; RAR $\alpha$ : retinoic acid receptor  $\alpha$ ; RUNX1: runt-related transcription factor 1. References: Miyoshi et al., 1991; Liu et al., 1993; de The et al., 1990; Chang et al., 1992; Goddard et al., 1991; Kakizuka et al., 1991; Alcalay et al., 1991; Pandolfi et al., 1991; Ayton et al., 2001; Nerlov et al., 2004; Crispino et al., 2005; Cilloni et al., 2003; Wechsler et al., 2002; Crispino et al., 2005; Osato et al., 1999. Table 1.2 has been taken from a review article written by Rosenbauer and Tenen, 2007.

transcription factors in animal models resulted in a block of myeloid cell differentiation (Zhang et al., 1997) and several of the genes that determine the differentiation steps encode transcription factors (Rosenbauer and Tenen, 2007). Taken together, studies demonstrate for the critical role of transcription factors at different stages during myeloid development and differentiation.

### **PU.1 and its interacting partners - Role in myeloid development and differentiation**

PU.1 is a product of spleen focus forming virus (SFFV) proviral integration site 1 (Spi1) oncogene whose expression is enhanced in murine erythroleukemia (Klemsz et al., 1990; Moreau-Gachelin et al, 1988; Paul et al., 1991). Deregulation of PU.1 results in an increase in erythroid self-renewal capacity resulting in a block in differentiation. PU.1 is mainly expressed in monocytes, B lymphocytes, early T lymphocytes and granulocytes (Galson et al., 1993; Hromas et al., 1993). It belongs to the Ets family of transcription factors and recognizes a purine rich core sequence (5' GAGGAA 3'; PU box & hence the name) to regulate target genes (Klemsz et al., 1990). It has been demonstrated that the expression levels of PU.1 are important in the determination of hematopoietic cell lineages, for example, high levels of PU.1 induce monocytic differentiation, whereas, low levels favor granulocyte development (Dahl et al., 2003; Laslo et al., 2006). On the other hand, PU.1<sup>-/-</sup> mice die at late gestational stage and the embryos show a defect in the development of monocytes, lymphocytes and granulocytes (Scott et al., 1994).

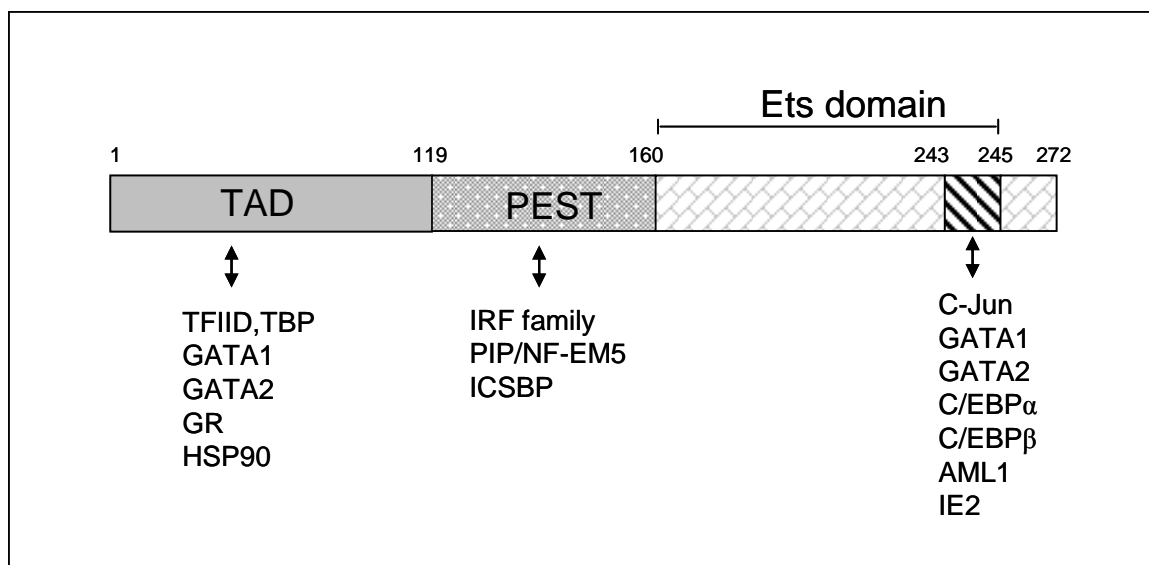
The transcription factor PU.1 is a 272 amino acid protein with an N-terminal activation domain and C-terminal DNA binding Ets domain (Fig. 1.10). The activation domain region is known to interact with several regulatory proteins (Hagemeier et al.,

1993; Klemsz et al., 1990; Klemsz et al., 1996), whereas, the DNA binding domain is a winged helix-turn-helix motif that recognizes the PU.1 core sequence to regulate target genes (Kodandapani et al., 1996). Figure 1.10 depicts a schematic representation of various domains of the PU.1 protein along with their interacting partners (reviewed in Tenen, 2003).

Several myeloid target genes are activated by PU.1. These include activation of macrophage colony stimulating factor (M-CSF) receptor (Zhang et al., 1994; Reddy et al., 1994), granulocyte-macrophage colony stimulating factor (GM-CSF) receptor  $\alpha$  (Hohaus et al., 1995) and granulocyte colony stimulating factor receptor (G-CSF) receptor promoters (Smith et al., 1996). In addition, PU.1 regulates most of the genes involved in the development of multipotential myeloid progenitors into myeloid lineages and for their further differentiation into mature blood cells (DeKoter et al., 1998; Anderson et al., 1999). Hence, PU.1 is considered the “master regulator” of myelopoiesis. Interestingly, PU.1 is also autoregulated (Okuno et al., 2005) like several other hematopoietic transcription factors and such regulation was found to be necessary for their irreversible differentiation and commitment process.

Hematopoietic cell lineages are determined by cell-type specific and stage specific expression of transcription factors which are regulated at either transcriptional level, post-transcriptional level and/or through selective protein-protein interactions (Rosenbauer and Tenen, 2007). First, regulation of transcription factors at the transcriptional level may include a complex set of cis-regulatory elements and trans-acting factors, for example, AML1 (also called RUNX1) binds to the upstream regulatory sequences of *SPI1* and regulates PU.1 expression both positively and negatively in a

lineage specific manner (Huang et al., 2008). Second, an example for regulation at the post-transcriptional level is the C/EBP $\alpha$  mRNA which gives rise to a 42 kDa protein and by the use of an alternate translation initiation site gives rise to a 30 kDa dominant negative isoform. The relative levels of these two isoforms determine the function of C/EBP $\alpha$  protein (Calkhoven et al., 2000). In addition, phosphorylation of C/EBP $\alpha$  on serine 21 results in C/EBP $\alpha$  inactivation that occurs during macrophage differentiation (Ross et al., 2004), whereas, phosphorylation of serine 248 of C/EBP $\alpha$  results in enhanced binding of this protein to G-CSFR promoter promoting granulocytic differentiation (Behre et al., 2002). Third, there are several examples for the regulation of hematopoietic transcription factors through protein-protein interactions which result in either co-operative or antagonistic effects that is essential for their optimal activity and tight regulation. Examples of such interacting partners for PU.1 include GATA binding protein 1 (GATA1; Shivdasani et al., 1996; Orkin et al., 2000), TBP (TATA binding protein; Hagemeyer et al., 1993), CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ; Reddy et al., 2002), Growth factor independence-1 (GFI-1; Dahl et al., 2007), B-cell specific activator protein (BSAP; Maitra and Atchison, 2000), Interferon regulatory factor-8 (IRF-8; Tsujimura et al., 2002) and Ski (Ueki et al., 2008). Protein-protein interactions mainly result in either cooperative or antagonistic effects that are necessary to create imbalances in gene expression program for the development and differentiation of an immature multipotential progenitor into a specific cell lineage type.



Note: PU.1 also interacts with HMGI/Y, SSRP, MKP1, CBP. But, the binding sites are not yet mapped.

**FIGURE 1.10 Schematic representation of the domain structure of the PU.1 protein and its interacting partners:** PU.1 protein is composed of 272 amino acids consisting of N-terminal transcription activation domain (TAD; amino acids 1-119), followed by a region rich in Proline, Serine, Threonine residues (PEST; amino acids 119-160) and a C-terminal Ets domain (between amino acids 160-245) that is necessary for DNA binding. The interaction of PU.1 with several hematopoietic transcription factors has been mapped. The TAD of the protein interacts with the general transcription factors, TFIID, TBP; GATA binding proteins, GATA1, GATA2, Glucocorticoid receptor (GR) and the heat shock protein (HSP90). The PEST domain of the protein interacts with the Interferon regulatory factor (IRF) family, Interferon consensus sequence binding protein (ICSBP). The C-terminal Ets domain of the protein interacts with c-Jun, GATA1, GATA2, CCAAT enhancer binding proteins (C/EBP $\alpha$ , C/EBP $\beta$ ), acute myeloid leukemia protein 1 (AML1). References: Reddy et al., 2002; Dahl et al., 2007; Huang et al., 2007; Rekhtman et al., 1999; Vangala et al., 2003; Bassuk and Leiden, 1995; Aittomaki et al., 2004; Gauthier et al., 1993; Wara-aswapati et al., 1999.

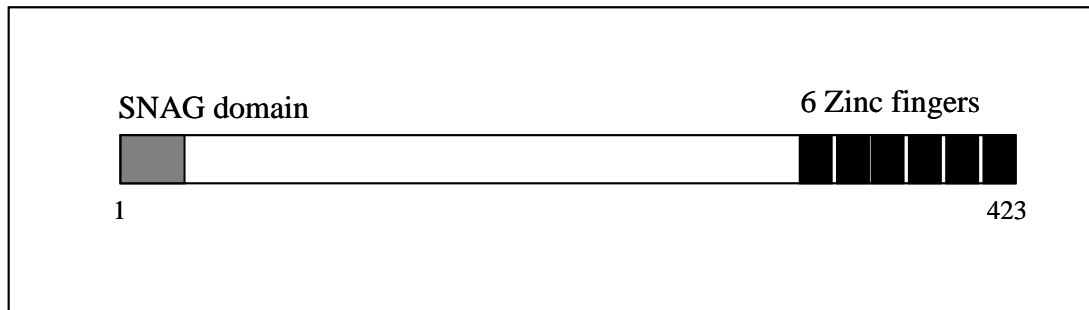
## **Growth factor independent-1 in neutrophil differentiation**

Growth factor independent-1 (GFI-1) was identified as the site of Moloney murine leukemia viral (MoMLV) integration resulting in its over-expression and causing interleukin-2 (IL-2) growth independence in T cell lines (Gilks et al., 1993; Akagi et al., 2004; Zornig et al., 1996). GFI-1 is a transcriptional repressor that is indispensable for neutrophil differentiation and maturation (Hock et al., 2003). GFI-1 is mainly expressed in common lymphoid progenitor, developing B and T lymphocytes, hematopoietic stem cells, granulocyte-monocyte progenitor and mature granulocytes (Zeng et al., 2004; Yucel et al., 2004). *Gfi1*<sup>-/-</sup> mice show a defect in neutrophil differentiation demonstrating proliferation of immature myeloid progenitors that share characteristics of both macrophages (Mac<sup>+</sup>) and neutrophils (Gr1<sup>+</sup>) (Hock et al., 2003; Karsunky et al., 2002). In addition, these mice were highly susceptible to infections from gram-positive bacteria. Studies have also shown that GFI-1 not only promotes neutrophil development and differentiation, but also antagonizes macrophage specific genes (Dahl et al., 2007). For example, GFI-1 interacts with PU.1 and represses its transcriptional activity resulting in the inhibition of expression of macrophage specific genes (Dahl et al., 2007; Hock et al., 2006). A mutation in the *GFI1* gene that inhibited GFI-1 transcriptional repression activity was identified in patients with a type of chronic neutropenia (Person et al., 2003). Another important role of GFI-1 is to maintain hematopoietic stem cell self-renewal capacity (Hock et al., 2004; Zeng et al., 2004).

GFI-1 is a 55kDa nuclear protein that contains an N-terminal SNAG repressor domain and six C<sub>2</sub>H<sub>2</sub> type zinc fingers at the C-terminus (Zweidler-McKay et al., 1996; Grimes et al., 1996; Gilks et al., 1993). Schematic representation of the various domains

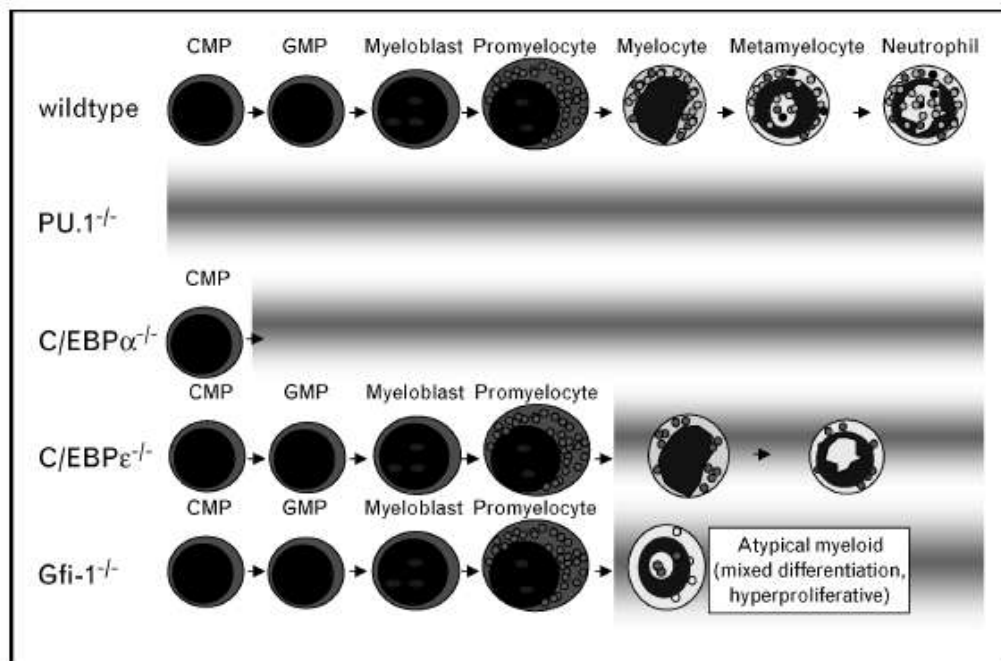
of GFI-1 protein is shown in Fig. 1.11. GFI-1 recognizes a DNA core sequence “AATC” to repress target genes and is a position and orientation independent transcriptional repressor. It has been identified that the repressive effect of this protein is through recruitment of histone deacetylases (HDACs), G9a histone lysine methyl transferase and/or eight 21 corepressor (ETO) (Montoya-Durango et al., 2008; McGhee et al., 2003).

The development of mature neutrophils occurs in a step-wise manner through gene regulation and characteristic granules that determine their function (Borregaard et al., 2001). The promyelocyte stage is characterized by the expression of myeloperoxidase and neutrophil elastase and the expression of primary granule proteins (peroxidase positive), with maturation to myelocyte stage secondary granules (peroxidase negative, lactoferrin positive) are synthesized and later tertiary granules (gelatinase containing) are produced at the metamyelocyte stage. Figure 1.12 depicts the defects in neutrophil development and differentiation that occurs through loss of function mutations of critical transcription factors that are involved in this process (Hock and Orkin, 2006).



**FIGURE 1.11 Schematic representation of the domain structure of Growth factor independent-1 (GFI-1):** GFI-1 is a 423 amino acid protein consisting of an amino-terminal 20 amino acid SNAG repression domain necessary for transcriptional repression and six C<sub>2</sub>H<sub>2</sub> type zinc fingers at the carboxy terminus necessary for DNA binding.





**FIGURE 1.12 Defects in neutrophil differentiation and development upon loss of function mutations of key transcription factors:** Normal myeloid differentiation includes development of the granulocyte monocyte progenitors (GMP) from common myeloid progenitors (CMP). The GMP further differentiates into myeloblast, promyelocyte, myelocyte, metamyelocytes and ultimately into mature neutrophil based on the synthesis of characteristic primary, secondary and tertiary granule proteins. Disruption of PU.1 results in early arrest before common myeloid progenitors. Loss of C/EBPα results in a defect in the differentiation of granulocyte monocyte progenitors from common myeloid progenitors. Loss of C/EBPε results in a defect in terminal neutrophil differentiation. Disruption of GFI-1 results in the proliferation and accumulation of atypical myeloid progenitors that bear characteristics of both monocytes and granulocytes (Ref. Hock et al., 2006).

## **Dissertation goals**

In chapter II, we demonstrate the mechanism of ZXDC mediated activation of MHC II genes. We found that ZXDC and ZXDA proteins can self-associate and that the zinc finger region is necessary to mediate their interaction. We also report that ZXDC interacted with components of the MHC II enhanceosome. We demonstrated by co-immunoprecipitation and Western analysis that ZXDC interacted with RFX5 and RFX-ANK, but not, RFX-AP of the RFX heterotrimeric complex that binds to the X1 box of the conserved upstream sequence of MHC II gene promoter. The ZXDA protein, however, was not found to interact with RFX5. Previous findings demonstrated that ZXDC interacted with MHC II master regulator, CIITA (Al-kandari et al., 2000a). Our model supports for a role of ZXDC-ZXDA hetero-complex to mediate protein-protein interactions with components of the MHC II enhanceosome resulting in an enhancement of the stability of the complex to activate MHC II gene transcription. There are examples for enhanceosome dependent transcriptional activation in eukaryotic genes (reviewed in Carey, 1998; Merika and Thanos, 2001). However, further studies are necessary to confirm the regulatory mechanism of ZXDC-ZXDA complex in the activation of MHC II gene transcription.

In chapter III of this dissertation, our findings strongly suggest a role for ZXD family proteins in myeloid development and differentiation. We observed that ZXDC and ZXDA proteins interacted with the myeloid transcription factors PU.1 and GFI-1. We were able to broadly map ZXDC-PU.1 interaction, the N-terminal region of PU.1 containing the activation domain interacted with the zinc finger region of ZXDC. We found that ZXDC and ZXDA proteins repressed PU.1 transcriptional activity by about

75% and 50% respectively. Interestingly, ZXDC was found to stabilize PU.1 protein expression. Studies have demonstrated that the expression levels of PU.1 are critical for hematopoietic cell lineage decisions, for example, high levels of PU.1 favor monocyte development and low levels promote granulocyte development and differentiation (Dahl et al., 2003; Laslo et al., 2006). In our studies, ZXDC and ZXDA proteins were also found to interact with GFI-1, a zinc finger transcriptional repressor necessary for neutrophil differentiation and maturation. ZXDC was found to repress GFI-1 transcriptional activity in a dose-dependent manner. Chromatin immunoprecipitation experiments demonstrated the presence of ZXDC along with GFI-1 at the *c-myc* promoter that was earlier found to be a direct target of GFI-1 (Duan et al., 2003). These findings suggest that ZXDC may have a role in affecting the recruitment and/or transcriptional activities of PU.1 and GFI-1 at their target gene promoters, especially, following differentiation of hematopoietic progenitor cells.

Importantly, we found that ZXDC is required for the transcription of the early growth response-2 (*EGR-2*) gene. Earlier reports demonstrated that *EGR-2* promotes macrophage development by activating several macrophage specific target genes, as well as, by repressing certain genes necessary for granulocyte development and differentiation (Laslo et al., 2006). In the same report it was also demonstrated that GFI-1 and *EGR-2* function in a counter antagonistic manner. Our findings suggest that induction of *EGR-2* by ZXDC is an important mechanism for the activation of macrophage specific genes and for the repression of genes required for granulocyte development. Our goal is to confirm the regulatory role of ZXDC protein in monocyte development and differentiation.

## **CHAPTER II**

# **PROTEIN-PROTEIN INTERACTIONS OF ZXDC, ZXDA: ROLE IN MHC II GENE REGULATION**

### **Abstract**

The Zinc finger, X-linked, Duplicated family members, C, A, B, C2 (ZXDC, ZXDA, ZXDB, ZXDC2 respectively) constitute the ZXD family of proteins. In our studies, we have found several protein-protein interactions amongst the ZXD family members to have important role in the activation of Major histocompatibility complex class II (MHC II) genes. The ZXDC and ZXDA proteins share significant nucleotide sequence homology and each contain ten C<sub>2</sub>H<sub>2</sub> zinc fingers and a transcription activation domain (Al-Kandari et al., 2007b). In addition, ZXDC has a C-terminal region that is necessary to interact with a key cofactor, Class II transactivator (CIITA) and activate Major histocompatibility complex class II and class I (MHC II and MHC I respectively) genes (Al-Kandari et al., 2007a). ZXDC and ZXDA proteins were found to self-associate, as well as, hetero-associate and their interactions are mediated by the zinc fingers of both proteins. Moreover, in vitro studies in our lab revealed that the association of ZXDC with ZXDA is necessary and self-association of neither protein was

sufficient to interact with CIITA and thereby activate MHC II gene transcription (Al-Kandari et al., 2007b). In addition to CIITA, we found that ZXDC interacted with two Regulatory factor X (RFX) proteins, namely, RFX5 and RFX-ANK (RFX having ankyrin repeats) which are components of the MHC II enhanceosome. The RFX heterotrimeric complex consisting of RFX5, RFX-AP (RFX-associated protein), RFX-ANK binds to the X1 box of MHC II promoter (Burd et al., 2004). The necessity for RFX complex in MHC II gene regulation is underscored by bare lymphocyte syndrome type II, a genetic disorder which results from mutation in one of the RFX proteins (Reith and Mach, 2001). Our findings support for a role of ZXDC-ZXDA heterocomplex to mediate interactions with components of the MHC II enhanceosome thereby enhancing the stability of the complex as a mechanism of activating MHC II gene transcription.

## **Introduction**

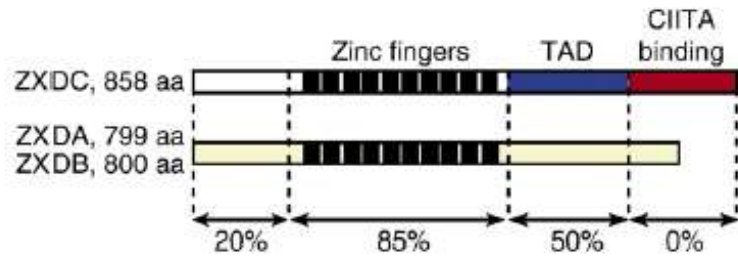
Major histocompatibility complex class II (MHC II) genes express cell surface glycoproteins that play a central role in antigen presentation. These proteins are expressed on the surface of thymic epithelial cells and professional antigen presenting cells namely B cells, macrophages and dendritic cells and can be induced in most other cell types by treatment with cytokines such as interferon  $\gamma$  (Drozina et al., 2005). MHC II proteins present antigenic peptides to T-cell receptors (TCR) of CD4<sup>+</sup> T cells (reviewed in Vyas et al., 2008). Absence of MHC II expression results in a severe immunological disorder known as bare lymphocyte syndrome II (BLS II; Reith and Mach, 2001), whereas, it's over expression results in autoimmune diseases (Klein et al., 1993). Therefore, tight control of MHC II expression is critical for a normal humoral immune response.

MHC II genes are mainly regulated at the transcriptional level (Reith et al., 2001; Ting et al., 2002). The promoter of MHC II genes consist of a set of conserved regions termed as S (or W box), X1, X2 and Y elements respectively which are necessary for their expression in a constitutive and inducible manner (Boss et al., 2003; Nekrep et al., 2003). A set of DNA binding proteins, namely, regulatory factor X complex (RFX), cyclic AMP response element binding protein (CREB), nuclear factor Y complex (NFY) bind to the X1, X2, Y boxes respectively. Although the above proteins are ubiquitously distributed in most cell types, the expression of MHC II genes is determined by the key regulatory co-factor called class II trans activator (CIITA) (Ting et al., 2002; LeibundGut-Landmann et al., 2004).

CIITA belongs to a family of proteins termed the CATERPILLER family named for the set of domains namely, CARD domain, transcriptional enhancer, purine binding, pyrin domain and lots of leucine rich repeats conserved amongst its members (Harton et al., 2002). Several proteins interact with CIITA which fall in the classes of (i) DNA binding proteins, namely, CREB and the components of the RFX and NFY complexes, (ii) general transcription factors, namely, TFIID and p-Tefb, (iii) histone modifying proteins and (iv) proteins involved in chromatin remodeling (Moreno et al., 1999; Kretsovali et al., 1998; Desandro et al., 2000; Hake et al., 2000; Scholl et al., 1997; Zhu et al., 2000; Fontes et al., 1997; Kanazawa et al., 2000; Wright et al., 2006).

Our lab was interested in identifying novel CIITA interacting proteins as it can provide a better understanding of the molecular mechanisms of CIITA mediated MHC II gene activation. Yeast two-hybrid screen was performed with the leucine rich repeats (C-terminal 807 amino acids) of CIITA as one of its major functions is to mediate protein-

protein interactions (Kobe and Kajava, 2001) and identified a cDNA coding for a novel zinc finger protein named Zinc finger, X-linked, duplicated family member C (ZXDC; Al-Kandari et al., 2007a). It is named based on nucleotide homology with two other proteins namely, ZXDA and ZXDB. ZXDC is a 858 amino acid protein with ten multiple adjacent C<sub>2</sub>H<sub>2</sub> type zinc fingers, transcription activation domain (TAD) and a C-terminal region that interacts with the CIITA protein (Fig. 2.1, Al-Kandari et al., 2007b). ZXDA lacks the C-terminal CIITA binding region present in ZXDC. Figure 2.1 illustrates a diagrammatic representation of ZXDC, ZXDA and ZXDB along with the regions of homology between the two proteins. Taken together, ZXDC, ZXDA, ZXDB and an mRNA variant of ZXDC, ZXDC2 constitute the ZXD family.



**Figure 2.1: Schematic representation of ZXDC, ZXDA, ZXDB representing the regions of percentages of homology in various domains.** The black rectangles represent the 10 zinc fingers of ZXDC or ZXDA, TAD: Transcriptional activation domain, CIITA binding is the region of ZXDC that binds to CIITA (Al-Kandari et al., 2007b).



In many zinc finger proteins, the zinc fingers (ZnFs) are involved in macromolecular interactions. Zinc fingers are generally involved in DNA binding, RNA binding and/or selective protein-protein interactions that play a significant role in the regulation of target genes (McCarty et al., 2003; Sun et al., 1996; Hata et al., 2000; Tsai et al., 1998). In addition, there are several examples of proteins with multiple adjacent zinc fingers, for example, TFIIIA, WT-1, and TRA-1 where the ZnFs mediate both DNA and RNA binding (Cassiday et al., 2002). Such macromolecular interactions of zinc fingers play a crucial role in the regulation of target genes. These studies reveal the importance of complex regulatory mechanisms involved in gene activation.

In our studies, we found several protein-protein interactions amongst the ZXDC family members. Co-immunoprecipitation and Western analysis demonstrated that ZXDC and ZXDA proteins associate with themselves as well as with each other (Al-Kandari et al., 2007b). Moreover, *in vitro* studies in our lab revealed that the association of ZXDC with ZXDA is necessary and self-association of neither protein was sufficient to interact with CIITA and thereby activate MHC II gene transcription (Al-Kandari et al., 2007b). We found that ZXDC and ZXDA showed a modest increase and cooperated with CIITA demonstrating a synergistic effect in the activation of MHC class II and MHC class I gene transcription. (Al-Kandari et al., 2007a). Knockdown of ZXDC or ZXDA using siRNA reduced MHC II expression almost 70% by CIITA without affecting the expression of CIITA (Al-Kandari et al., 2007b). Using chromatin immunoprecipitation, we found ZXDC at the promoter of MHC II genes in Raji cells, a B cell line that constitutively express MHC II, and in HeLa cells prior to and after IFN $\gamma$  treatment (Al-Kandari et al., 2007b).

In subsequent experiments, we found that ZXDC also interacts with two other components of the MHC II enhanceosome, namely, RFX5 (Fig. 2.5) and RFX-ANK (Fig. 2.6). The RFX heterotrimeric complex consisting of RFX5, RFX-AP (RFX-associated protein), RFX-ANK (RFX having ankyrin repeats) binds to the X1 box of MHC II promoter (Burd et al., 2004). The necessity for RFX complex in MHC II gene regulation is underscored by a genetic disorder named bare lymphocyte syndrome type II, which results from mutation in one of the RFX proteins (Reith and Mach, 2001).

In the studies presented here we show several protein-protein interactions of the ZXD family members. Co-immunoprecipitation experiments demonstrated self-association of ZXDC and ZXDA proteins and that the zinc fingers of these proteins mediate their interaction. We were also able to demonstrate that ZXDC interacts with RFX5 and RFX-ANK which are components of the MHC II enhanceosome complex.

## **Materials and methods**

**Cell culture and transfection:** Human embryonic kidney 293 (HEK293, ATCC no. CRL-1573) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5g/L glucose, antibiotics and 10% fetal calf serum (FBS). Cells were maintained at 37°C in a humidified air atmosphere and 5% CO<sub>2</sub>. Cells were transfected using Lipofectamine 2000 reagent (InVitrogen, Inc., Carlsbad, CA). Briefly, 10<sup>6</sup> cells were plated in a 6-well tissue culture dish 24 h prior to transfection. For all transfections, 10µl of Lipofectamine 2000 reagent was mixed with a total of 4 µg plasmid DNA and added to the cells

according to the manufacturer's instruction. When necessary, empty vector was used as a stuffer to maintain a total of 4 µg of plasmid DNA.

**Plasmids:** The plasmids that express myc-RFX5 (pCR3-myc-RFX5), Flag-RFXANK (pcDNA3-Flag-RFXANK), HA-RFXAP (pEF-HA-RFXAP) were previously described (Nekrep et al., 2001). The plasmid pCMV-Flag-ZXDA was created by subcloning ZXDA cDNA from pBluescript-ZXDA (clone ID:30347480) into pCMV-Flag vector using *EcoR1* and *BamH1* restriction sites. The plasmid pcDNA3.1-Myc was obtained by annealing Myc-tag oligos and cloned into pcDNA3.1 vector using *HindIII* and *BamH1* restriction sites. The plasmid pCMV-Myc-ZXDA was created by PCR amplifying ZXDA cDNA using pCMV-Flag-ZXDA as a template and cloned into pcDNA3-Myc vector using *BamH1* and *EcoR1* restriction sites. To obtain pCMV-Myc-ZXDA $\Delta$ N-terminus (ZXDA construct with deletion of amino acids 1-266), the required construct was PCR amplified from pCMV-Flag-ZXDA and cloned into pcDNA3-Myc vector using *BamH1* and *EcoR1* restriction sites. The plasmid pCMV-Myc-ZXDA $\Delta$ ZnFs (ZXDA construct with deletion of amino acids 267-573) was created in pcDNA3-Myc-ZXDA using Quikchange II XL-site directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The plasmid pCMV-Myc-ZXDA $\Delta$ C-terminus (ZXDA construct with deletion of amino acids 574-799) was created by PCR amplifying the required fragment from pCMV-Flag-ZXDA and cloned into pcDNA3-Myc vector using *BamH1* and *EcoR1* restriction sites. The plasmid pFlag-CMV-5a-ZXDC was created by PCR amplifying ZXDC cDNA using pCMV-SPORT6-ZXDC as a template and cloned into pFlag-CMV-5a vector using *HindIII* and *BamH1* restriction sites. The plasmids pCMV-3xFlag-ZXDC $\Delta$ N-terminus (ZXDC construct with deletion of amino acids 1-176)

and pCMV-3xFlag-ZXDC $\Delta$ C-terminus (ZXDC construct with deletion of amino acids 472-858) were generated by PCR amplifying appropriate regions of ZXDC cDNA and cloned into pcDNA3-3xFlag vector (an N-terminal fusion vector containing three copies of the Flag epitope) using *Bam*H1 and *Xba*1 restriction sites.

**Co-immunoprecipitation:** HEK293 cells ( $10^6$ ) cells were transfected with the indicated plasmids. Forty-eight hours after transfection, cells were lysed in 500  $\mu$ l of lysis buffer (10mM Tris pH:8, 300mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 25mM N-ethyl maleimide, 10mM iodoacetamide, 2mM Phenyl methyl sulphonyl fluoride and 1X EDTA-free protease inhibitors. The lysate was cleared by centrifugation at 13,000 rpm for 15 min at 4°C. About 10% of the lysate was taken as ‘input control’ to check the expression of the plasmids. The remaining lysate was incubated with 4  $\mu$ l of ANTI-FLAG M2 monoclonal antibody (Sigma, MO) for 3 h at 4°C. The lysate-antibody mix was next incubated with 50  $\mu$ l of protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.) at 4°C. The beads were washed four times with lysis buffer, boiled with Laemmli buffer and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8% polyacrylamide). The resolved samples were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Inc.) using transfer buffer (25mM Tris-pH8.3, 192mM glycine, 20% methanol). Blots were blocked for one hour at room temperature in 1XTBST wash buffer (50mM Tris-pH 8, 150mM NaCl, 0.1% Tween-20) containing 5% non-fat milk and 10% donar bovine serum. Blots were probed with the indicated primary antibody followed by horse radish peroxidase (HRP)-conjugated secondary antibody (SantaCruz Biotechnology, Inc) in wash buffer containing 3% non-fat milk and 10% donor bovine

serum. Detection was done using ECL plus (Amersham, GE Healthcare) and data were analyzed using Typhoon-9410 (GE Healthcare, Piscataway, NJ).

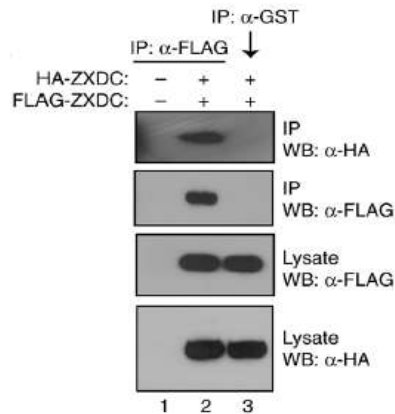
**Antibodies:** For co-immunoprecipitation and Western analysis, the following antibodies were used: Anti-FLAG M2 (1mg/ml, SIGMA, MO), anti-HA 12CA5 (0.4mg/ml, Roche Inc, IN), anti-myc 9E10 (0.4mg/ml, Roche Inc, IN), anti-GST (200 $\mu$ g/ml, Santa Cruz, CA). The affinity purified antibody against the first twenty amino acids of ZXDC was generated by Bethyl laboratories Inc (Montgomery, TX; Al-Kandari et al., 2007a). The HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (200 $\mu$ g/ml) were obtained Santa Cruz Biotech (Santa Cruz, CA).

## Results

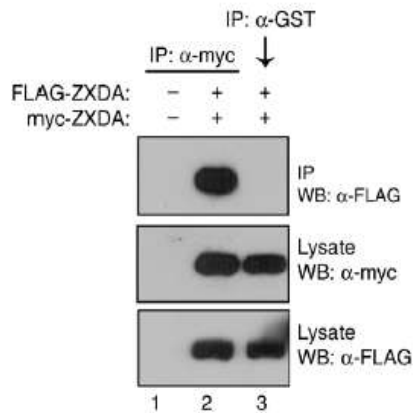
### Self-association of ZXDC and ZXDA proteins:

The ZXDC and ZXDA proteins are zinc finger transcription factors each containing ten C<sub>2</sub>H<sub>2</sub> type zinc fingers (ZnFs). The C<sub>2</sub>H<sub>2</sub> type zinc fingers are generally involved in DNA binding, RNA binding, selective protein-protein interactions or a combination of more than one macromolecular interaction (McCarty et al., 2003; Sun et al., 1996; Hata et al., 2000; Tsai et al., 1998; Wang et al., 2001; Cassiday et al., 2002). Although nucleic acid binding by ZnFs was the primary focus for many years, more recently the importance of protein-protein interactions mediated by ZnFs has come to the fore (reviewed in Gamsjaeger et al., 2007; Brayer et al., 2008a, 2008b).

We were interested to find if ZXDC and ZXDA proteins interacted, and if the interaction was mediated by the ten zinc fingers present in each protein. HEK293 cells were transfected with plasmids that express the indicated proteins (Fig. 2.2). Forty-eight hours after transfection, Co-immunoprecipitation (co-IP) and Western analysis were performed with the indicated antibodies. Our results demonstrated self-association of ZXDC (Fig. 2.2A) and ZXDA (Fig. 2.2B) proteins. Co-IP with a non-specific antibody ( $\alpha$ -GST) served as a negative control (Fig. 2.2 A&B, lane 3). The blots also included lysate controls to check the expression of the proteins.



A.



B.

**Figure 2.2 ZXDC and ZXDA proteins self-associate. A. Self-association of ZXDC:**

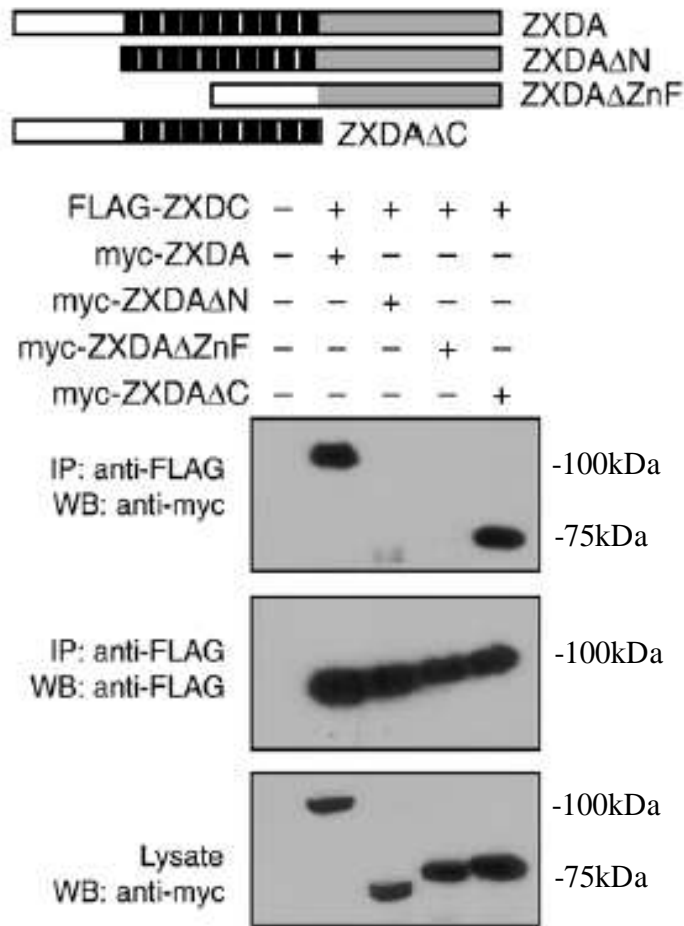
HEK293 cells were transfected with plasmids that express HA- and FLAG-tagged ZXDC. Forty-eight hours post-transfection, co-immunoprecipitation (co-IP) was performed with anti-FLAG (Sigma, MO) followed by Western analysis with anti-HA (Roche, IN). Co-IP with a non-specific antibody ( $\alpha$ -GST was included as a negative control.

**B. Self-association of ZXDA** HEK293 cells were co-transfected with plasmids that express FLAG and myc-tagged ZXDA. Forty-eight hours post-transfection, co-IP and Western analysis were performed using anti-myc (Sigma, MO) and anti-FLAG respectively (Al-Kandari et al., 2007b).

### **The zinc fingers of ZXDA mediate interaction with ZXDC**

We wanted to map the region of ZXDA that is necessary for interaction with ZXDC. Therefore, we made deletions in the N terminus or the C terminus of the ZXDA protein. A schematic representation of the deletion constructs is depicted in Fig. 2.3 (top panel, the black rectangles represent ten zinc fingers of the protein). Our results demonstrated that deletion of the amino terminus significantly affected its interaction with ZXDC (Fig. 2.3, lane 3), whereas, deletion of the zinc finger region of ZXDA completely abolished its interaction with ZXDC (Fig. 2.3, lane 4) demonstrating that the zinc fingers of ZXDA mediate ZXDA-ZXDC interaction. However, the amino terminus of ZXDA also contributes significantly for interacting with ZXDC.

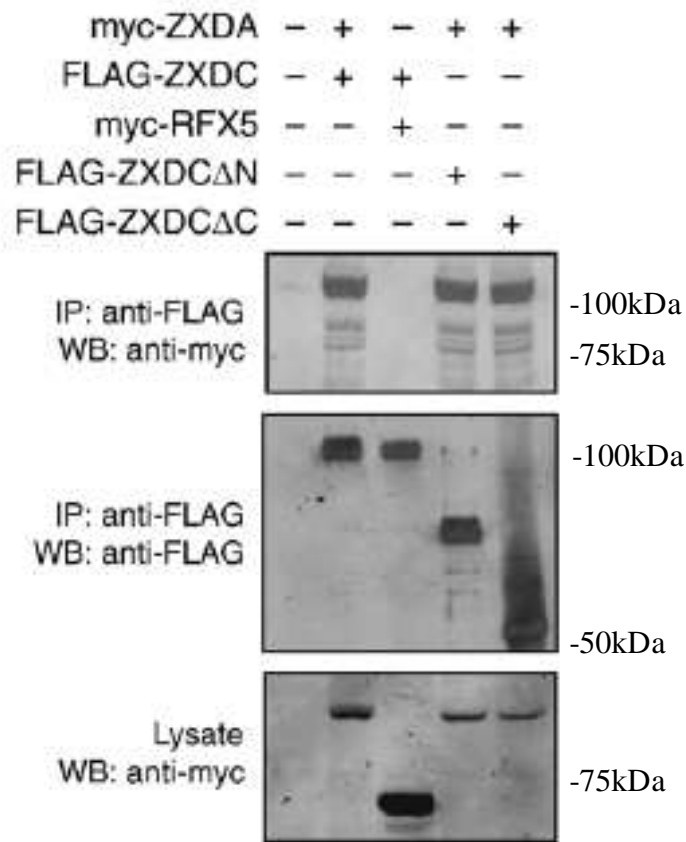




**Figure 2.3 Zinc-fingers of ZXDA are necessary to interact with ZXDC.** Myc-tagged ZXDA constructs with N-terminal, zinc finger region or C-terminal deletions were made (schematic representation of the ZXDA deletion constructs are shown in the top panel). HEK293 cells were co-transfected with plasmids that express each of the ZXDA deletion constructs and full-length FLAG-tagged ZXDC. Co-IP and Western analysis were performed with anti-FLAG (Sigma, MO) and anti-myc (Roche, IN) respectively, demonstrating that the zinc finger region of ZXDA is necessary to associate with ZXDC, though deletion of the N-terminus reduced the interaction significantly. Appropriate controls were included (Al-Kandari et al., 2007).

## **The Zinc fingers of ZXDC are necessary for interaction with ZXDA**

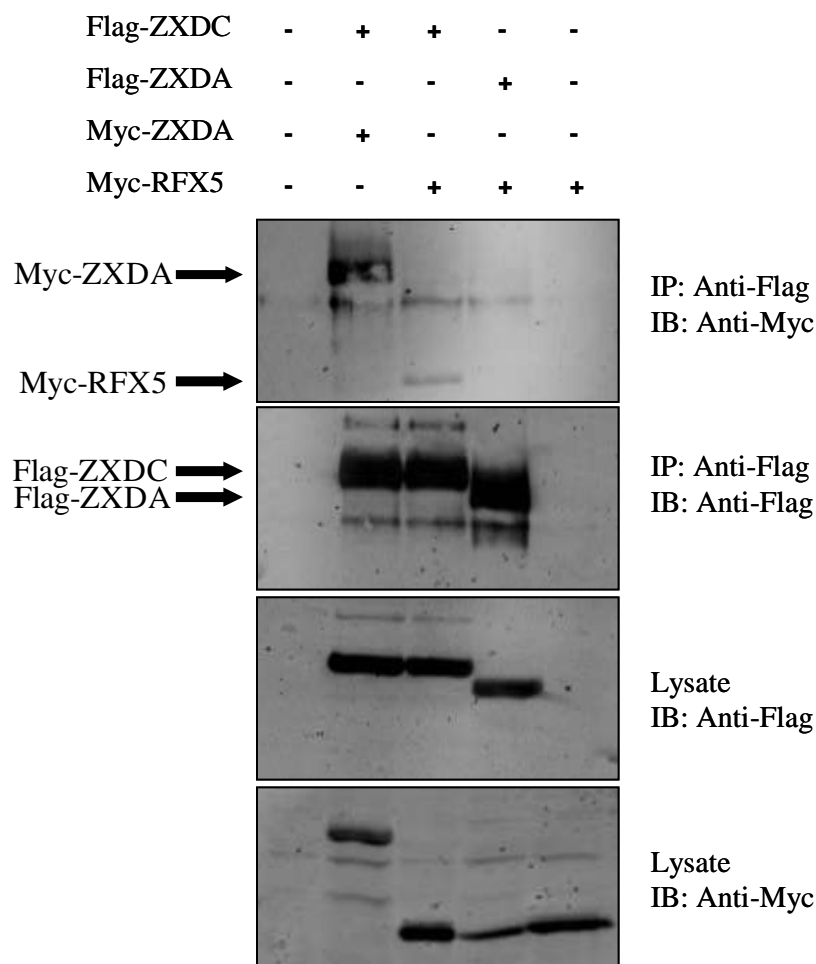
In order to determine the ZXDC domain necessary for interaction with ZXDA, deletion of the N terminus or C terminus of ZXDC with Flag tag were made similar to as depicted in Fig. 2.3 (top panel). Co-IP assays demonstrated that deletion of the N-terminus or C-terminus of the ZXDC protein didn't affect its interaction with ZXDA (Fig. 2.4, top panel, lanes 4 and 5) suggesting that the zinc fingers of ZXDC are necessary to mediate interaction with ZXDA. We were not able to demonstrate which Zinc fingers of ZXDC are involved as deletion of all or some of the zinc fingers resulted in destabilization of the protein (data not presented).



**Figure 2.4 Zinc-fingers of ZXDC are necessary to interact with ZXDA.** FLAG tagged ZXDC constructs with N-terminal or C-terminal deletion were made. HEK293 cells were co-transfected with plasmids that express each of the deletion constructs and full-length myc-tagged ZXDA. Co-IP and Western analysis were performed with anti-FLAG (Sigma, MO) and anti-myc (Roche, IN) respectively. Our results suggest that the zinc fingers of ZXDC mediate interaction with ZXDA (Al-Kandari et al., 2007b).

### **ZXDC, but not ZXDA interacts with RFX5 protein**

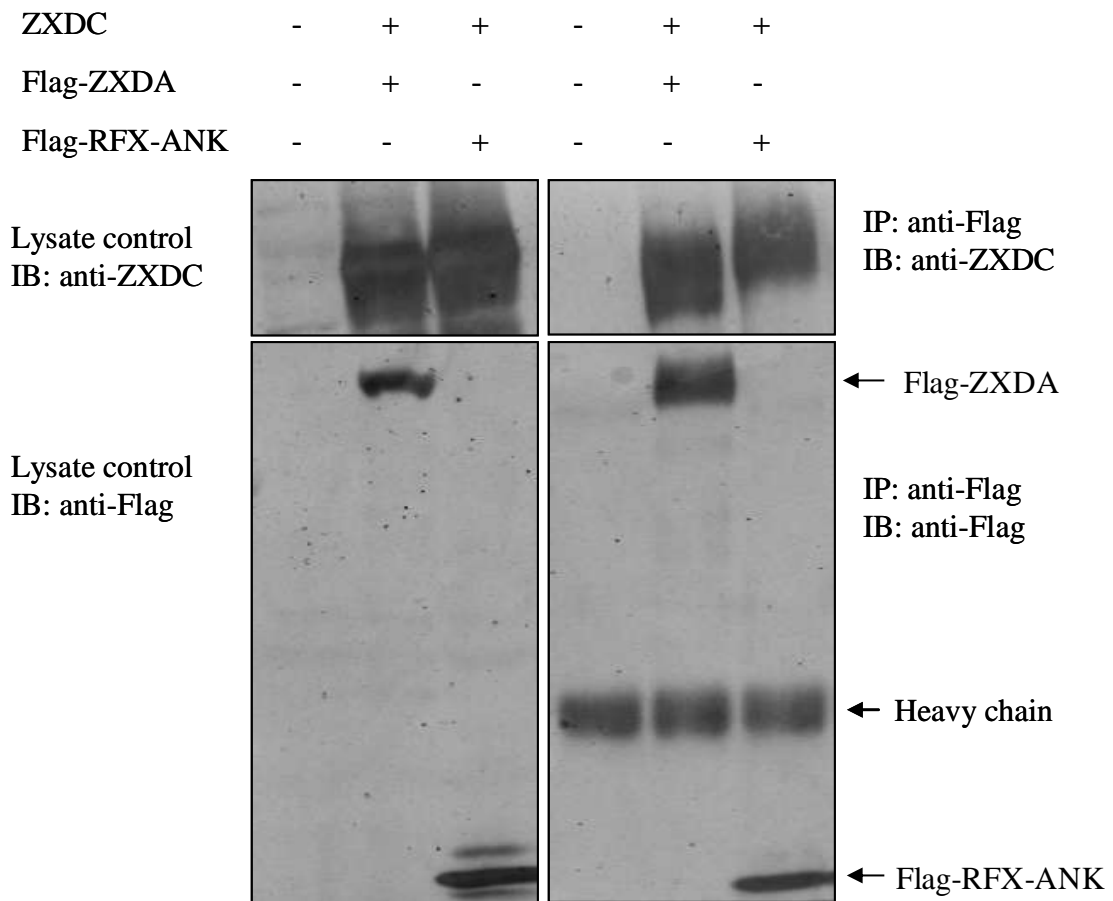
Work by Wafa Al-Kandari in our laboratory established that the ZXDC-ZXDA complex was necessary for binding CIITA. However, we wanted to test whether ZXDC interacted with other components of the MHC II enhanceosome, indicating that additional regulatory mechanisms for ZXDC mediated MHC II gene activation. To find if ZXDC or ZXDA interact with RFX5, HEK293 cells were transfected with plasmids that express Flag-ZXDC or Flag-ZXDA and myc-RFX5. Co-IP with anti-Flag and Western analysis with anti-myc demonstrated that RFX5 interacts with ZXDC (Fig. 2.5, lane 3), but not ZXDA (Fig. 2.5, lane 4). The interaction of ZXDC-ZXDA proteins was used as a positive control (Fig. 2.5, lane 2), whereas, co-IP in the absence of either Flag-tagged proteins was used as a negative control (Fig. 2.5, lane 5). The slower migrating band in Flag-ZXDC lanes represents the sumoylated ZXDC (Jambunathan and Fontes, 2007).



**Figure 2.5 ZXDC interacts with RFX5 protein.** HEK293 cells were co-transfected with the indicated plasmids. Forty-eight hours post-transfection, co-IP and Western analysis were performed with anti-Flag (Sigma, MO) and anti-Myc (Roche, IN) respectively. This experiment demonstrated that ZXDC interacts with RFX5, which binds to the X-box of MHC II promoter along with RFX-AP and RFX-ANK as a heterotrimeric complex. On the other hand, ZXDA did not interact with RFX5. Co-IP of myc-ZXDA by Flag-ZXDC served as a positive control.

### **ZXDC also interacts with RFX-ANK protein of the RFX heterotrimeric complex**

Since ZXDC interacted with RFX5, we wanted to know if ZXDC also associates with other proteins of the regulatory factor X complex, namely, RFX-ANK and RFX-AP. HEK293 cells were transfected with plasmids that express ZXDC and Flag-ZXDA or Flag-RFXANK. Forty-eight hours post-transfection, co-immunoprecipitation was performed with anti-Flag and Western analysis with RFXANK protein (Fig. 2.6, top panel, lane 6). Co-IP of ZXDC with Flag-ZXDA was used as a positive control (Fig. 2.6, top panel, lane 5). Our results suggest that ZXDC, by interacting with components of the MHC II enhanceosome, may stabilize the complex as a mechanism of ZXDC-ZXDA mediated MHC II gene activation.

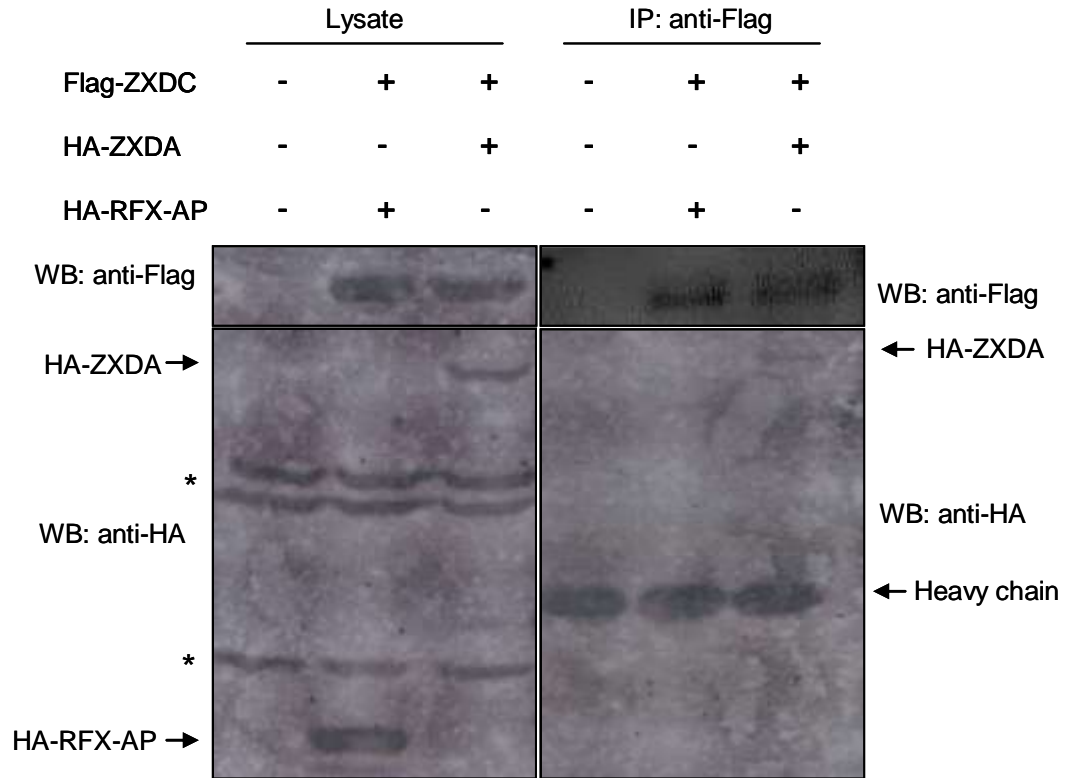


**Figure 2.6 ZXDC co-immunoprecipitated with RFXANK.** HEK293 cells were co-transfected with plasmids that express ZXDC and Flag-ZXDA or Flag-RFXANK. Forty-eight hours post-transfection, co-IP was performed with anti-Flag and Western analysis with anti-ZXDC. Co-IP of ZXDC by Flag-ZXDA was used as a positive control. Appropriate lysate controls were included.

### **ZXDC does not interact with RFX-AP**

The finding that ZXDC interacted with RFX5 and RFX-ANK led us to ask if it is also capable of interacting with RFX-AP. HEK293 cells were transfected with plasmids that express Flag-ZXDC and HA-ZXDA or HA-RFXAP. Forty-eight hours post-transfection, cells were lysed in lysis buffer and Co-IP was performed with anti-Flag and Western analysis with anti-HA. Our results demonstrated that ZXDC does not interact with RFX-AP (Fig. 2.7, bottom panel, lane 5). Co-IP of HA-ZXDA by Flag-ZXDC was used as a positive control (Fig. 2.7, bottom panel, lane 6). Our results demonstrated that ZXDC however, does not interact with RFX-AP (Fig. 2.7, bottom panel, lane 5) of the RFX complex that binds to the X1 promoter element of MHC II genes.





\* Non-specific band

**Figure 2.7 ZXDC does not interact with RFXAP.** HEK293 cells were co-transfected with plasmids that express Flag-ZXDC and HA-RFXAP or HA-ZXDA. Forty-eight hours post-transfection, co-IP and Western analysis was performed with anti-Flag and anti-HA respectively. This demonstrated that ZXDC does not interact with RFXAP, suggesting for the specificity of interaction of ZXDC with other members of the RFX complex, namely, RFX5 and RFXANK. Hetero-association of ZXDC with ZXDA was used as a positive control.

## Discussion

Data presented in this chapter demonstrate selective protein-protein interactions between ZXDC family members, ZXDC and ZXDA and between ZXDC and components of the MHC II enhanceosome. We demonstrated self-association of ZXDC and ZXDA proteins and that the zinc fingers of both proteins mediate their interaction. We found that ZXDC interacted with RFX5 and RFXANK, but not RFX-AP (Figs. 2.5, 2.6, 2.7), which are components of the RFX heterotrimeric complex that binds to the conserved X1 box of the MHC II promoter (Masternak et al., 1998; Steimle et al., 1995; Villard et al., 1997).

The results presented here along with previous findings suggest a possible regulatory role for ZXDC-ZXDA complex in stabilizing MHC II enhanceosome complex resulting in the activation of MHC II gene transcription. Given the important role of C<sub>2</sub>H<sub>2</sub> type zinc fingers in DNA binding, we tried extensively to demonstrate direct binding of ZXDC to MHC II promoter region, especially the W box, but we couldn't find. Since chromatin immunoprecipitation experiments demonstrated that ZXDC is present at MHC II promoters, the most likely interpretation is that ZXDC is recruited to MHC II promoters indirectly, via protein-protein interactions. Our data support this notion. We were able to demonstrate that ZXDC selectively interacted with components of MHC II enhanceosome, specifically, CIITA (Al kandari et al., 2007a), RFX5 (Fig. 2.5), RFXANK (Fig. 2.6) and possibly some other components of the MHC II enhanceosome suggesting a role in stabilizing the enhanceosome complex as a mechanism of MHC II gene activation.

Studies in our lab revealed the role of a novel regulatory factor, ZXDC, in the activation of MHC II gene transcription. The C-terminus of ZXDC was found to be the region interacting with CIITA (Al-Kandari et al., 2007a). Knock-down of ZXDC by siRNA in HEK293 cells showed significant reduction of MHC class II gene expression (Al-Kandari et al., 2007a). Though ZXDA lacked the CIITA binding region, it showed a synergistic effect in the transcription of MHC class II genes by CIITA suggesting that it might cooperate with other proteins to regulate MHC II genes (Al-Kandari et al., 2007b). We were able to demonstrate self- (Fig. 2.2; Al-Kandari et al., 2007b) and hetero-association (Al-Kandari et al., 2007b) of ZXDC and ZXDA proteins. Previous findings demonstrated that ZXDC requires ZXDA to interact with CIITA ‘in vitro’ suggesting an important role of hetero-association (Al-Kandari et al., 2007b). We were able to broadly demonstrate that zinc fingers of ZXDA are necessary to interact with ZXDC and vice-versa (Figs. 2.3, 2.4; Al-Kandari et al., 2007b). We were not able to identify which zinc fingers of ZXDC and ZXDA mediate protein-protein interaction, as deletions of some zinc fingers resulted in destabilization of the protein (data not presented).

It was shown that re-expressing ZXDC following knock-down of endogenous ZXDC by RNA silencing results in partial rescue of MHC class II gene expression by CIITA (Al-Kandari et al., 2007a). Transient knockdown of ZXDC or ZXDA resulted in significant repression of MHC II gene expression. This decrease was not significantly different from knock-down of both ZXDC and ZXDA suggesting for the requirement of both proteins for MHC II gene regulation (Al-kandari et al., 2007b). We observed the presence of ZXDC at the MHC II promoter prior to and after IFN  $\gamma$  treatment (Al-Kandari et al., 2007a). Given the important role of C<sub>2</sub>H<sub>2</sub> type zinc fingers in

macromolecular interactions, they are generally involved in DNA binding, RNA binding or selective protein-protein interactions that play a significant role in the regulation of target genes (McCarty et al., 2003; Sun et al., 1996; Hata et al., 2000; Tsai et al., 1998; Wang et al., 2001). Dr. Srikarthika Jambunathan in our lab identified that ZXDC preferentially binds to a purine rich sequence, AGGGT/A by SELEX assay. The presence of this sequence at the HLA-DRA promoter suggests that it might be a potential binding site for ZXDC. However, the data overall support the binding of the ZXDA-ZXDC complex to MHC II promoters via protein-protein interactions with RFX5, RFXANK and CIITA.

Although, zinc fingers were primarily known for its role in DNA binding (Wolfe et al., 2000), increasing evidence supports for its role in selective protein-protein interactions (reviewed in Gamsjaeger et al., 2007; Brayer et al., 2008a, 2008b). Our study suggests for a role of zinc fingers in protein-protein interactions among ZXD family members (Figs. 2.3, 2.4). Interestingly, we observed that ZXDC interacts with RFX5 (Fig. 2.5) and RFXANK proteins (Fig. 2.6) by co-immunoprecipitation experiments. This could be a possible mechanism for the recruitment of ZXDC along with ZXDA at MHC II promoter. ZXDA alone, however, did not co-immunoprecipitate with RFX5 (Fig. 2.5). The RFX complex consisting of RFX5, RFX-AP, RFX-ANK binds to the X box as a heterotrimeric complex (Masternak et al., 1998; Steimle et al., 1995; Villard et al., 1997; Burd et al., 2004). ZXDC, however, did not interact with RFX-AP (Fig. 2.7) suggesting that these protein-protein interactions are very specific. It is interesting to know if ZXDC is recruited to the promoter as a hetero-dimer along with ZXDA or if ZXDA is recruited later during gene activation. From our findings, we

suggest that the interaction of ZXDC with several proteins that form the MHC II enhanceosome, for example, RFX5, RFX-ANK, CIITA stabilizes the enhanceosome complex resulting in the activation of MHC II gene transcription.

In the case of MHC II enhanceosome, the individual protein-protein or protein-DNA interactions are weak, but, multiple interactions are capable of recruiting the key transcriptional co-activator, CIITA (Masternak et al., 2000). Some other examples of eukaryotic genes regulated by enhanceosomes include Interferon  $\beta$  (IFN  $\beta$ ), T-cell receptor  $\alpha$  chain and interleukin-6 (IL-6) genes (reviewed in Merika et al., 2001; Panne, 2008). There are several mechanisms of enhanceosome dependent transcriptional synergy. Some of these include modification of chromatin structure for the recruitment of preinitiation complex, enabling nucleosome modification and enhancing the stability of enhanceosome (reviewed in Merika et al., 2001; Carey, 1998; Panne, 2008).

At present, we do not know the exact mechanism how ZXDC-ZXDA complex activates MHC II gene transcription. It is possible that ZXDC-ZXDA complex through its interaction with CIITA, RFX5, RFXANK and possibly other components (CREB, NFY or chromatin remodeling factors) might stabilize the enhanceosome complex resulting in gene activation. This suggests as a potential mechanism for ZXDC-ZXDA recruitment at the class II promoter and resulting in the activation of MHC II genes.

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**CHAPTER III**

**THE INTERACTION OF ZXDC PROTEIN WITH THE  
HEMATOPOIETIC TRANSCRIPTION FACTORS, PU.1  
AND GFI-1: A ROLE IN MYELOPOIESIS**

**Abstract**

The ZXDC family of proteins seems to have a broader role in gene regulation, beyond MHC II gene transcription. We have found that ZXDC interacts and represses the transcriptional activities of two myeloid transcription factors, namely, purine box binding protein, PU.1 and growth factor independent-1 (GFI-1). Our studies also demonstrated that the binding of ZXDC protein to PU.1, stabilizes the PU.1 protein. The *MYC* gene promoter, a known target of GFI-1, was found to be bound by the ZXDC protein. These results demonstrate that ZXDC affects PU.1 and GFI-1 transcriptional activities and/or recruitment at their target gene promoters. Preliminary results by our lab suggest that ZXDC favors monocyte development. Specifically, ZXDC activates the transcription of the Egr-2 (Early growth response-2) gene. The expression of Egr-2 is associated with monocyte specific gene expression and repression of granulocyte genes

thus favoring monoopoiesis (Laslo et al., 2006; Krishnaraju et al., 2001). Previous studies by others have demonstrated that GFI-1 and Egr-2 function in an antagonistic manner (Laslo et al., 2006). We propose a model where ZXDC, by affecting PU.1 and GFI-1 recruitment and transcriptional activities at their target gene promoters, and by activating Egr-2 activity and expression, has an important regulatory role in monoopoiesis.

## **Introduction**

A large network of several ubiquitous and tissue specific transcription factors play an important role in the differentiation and development of hematopoietic cells (reviewed in Rosenbauer et al., 2007; Friedman, 2007). Mutation of the genes encoding several of these proteins or misregulation of their expression was found to contribute to leukemia (Rosenbauer et al., 2004, 2006, 2007; Huang et al., 2008; Orkin, 2000; Tenen, 2003, Table 1.2). The transcription factor PU.1/Spi-1 is considered the master regulator for several myeloid specific genes. PU.1 was first identified as the genomic locus rearranged by the spleen focus forming virus proviral insertion (Spi-1) in many erythroid leukemias (Moreau-Gachelin et al., 1988).

The expression of PU.1 is restricted to hematopoietic cells (except mature T cells), especially, in all myeloid lineages, B-cell lineages, developing T cells, a subtype of Th2 cells and hematopoietic stem cells (Kastner and Chan, 2008). PU.1 is a transcriptional activator that belongs to the Ets family and contains an N-terminal activation domain and C-terminal basic helix-turn-helix motif necessary for DNA binding and binds to the purine-rich sequence (5' GAGGAA 3') to regulate target gene expression (reviewed in Friedman, 2007).

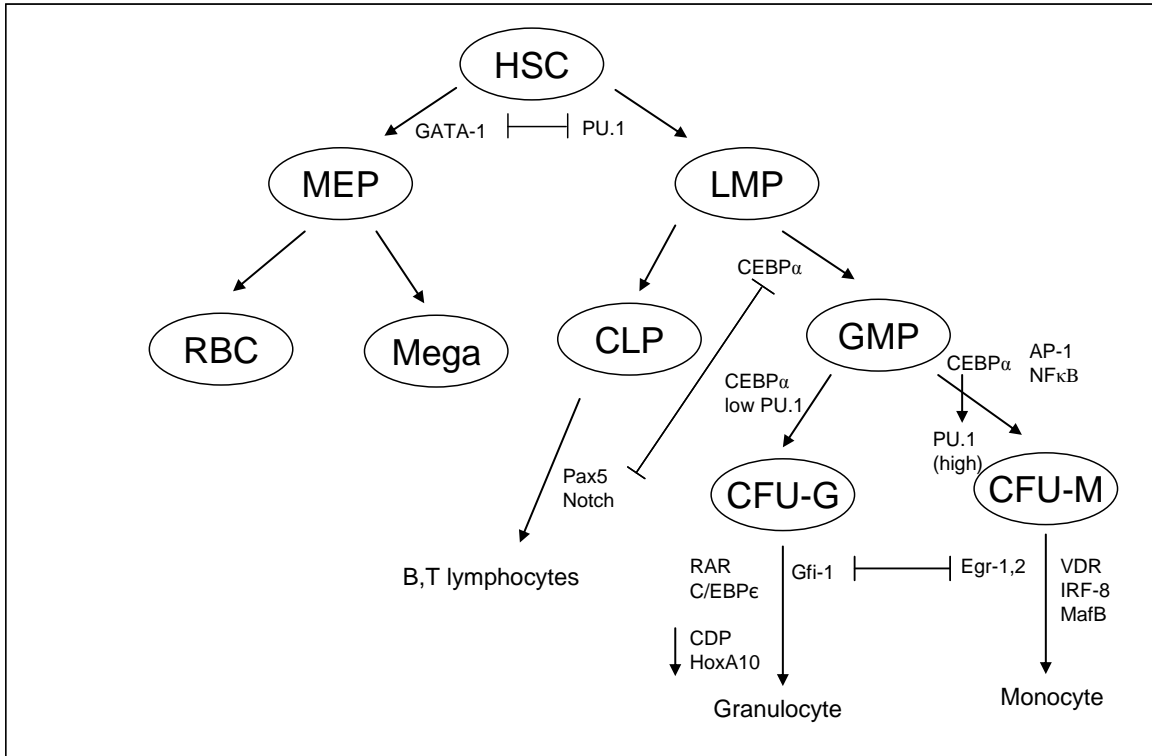
The interaction of PU.1 with several transcription factors such as GATA-1, CCAAT/enhancer binding protein  $\alpha$  (C/EBP  $\alpha$ ), growth factor independent-1 (GFI-1), B cell-specific activator protein and Ski is critical for determining hematopoietic cell fates by regulating the expression of lineage specific genes (Nerlov et al., 2000; Zhang et al., 1999; Reddy et al., 2002; Maitra and Atchison, 2000; Dahl et al., 2007; Ueki et al., 2008). Interestingly, all the above factors inhibit the ability of PU.1 to transactivate its target genes at different stages of differentiation and development. The expression level of PU.1 was found to be very important for lineage decisions. In myeloid progenitor cells, high levels of PU.1 have been reported to favor monopoiesis, whereas, low levels favor granulopoiesis (Dahl et al., 2003; Laslo et al., 2006). Growth factor independent 1 (GFI-1) was first identified as the gene activated by the insertion of Moloney murine leukemia virus in rat T cell lymphoma cell line that acquired interleukin-2 growth independence (Gilks et al., 1993). GFI-1 is expressed mainly in common lymphoid progenitors, developing B- and T-lymphocytes, granulocyte-monocyte progenitor, granulocytes, hematopoietic stem cells (Zeng et al., 2004; Yucel et al., 2004). GFI-1 also has critical role in hematopoietic stem cell maintenance (reviewed in Duan and Horwitz, 2005). GFI-1 is a transcriptional repressor containing an N-terminal repression domain called the SNAG (Snail/Gfi1) domain and six C-terminal zinc fingers required for DNA binding (Grimes et al., 1996; Zweidler-Mckay et al., 1996). The sequence TAAATCAC(A/T)GCA (the core sequence is underlined) was identified as the GFI-1 binding site (Zweidler-Mckay et al., 1996).

GFI-1 opposes gene activation by PU.1, an activity found to be necessary for myeloid progenitors to differentiate into granulocytes (Dahl et al., 2007). GFI-1 knock-

out mice and mutations in GFI-1 in humans that inhibit the ability of GFI-1 to repress PU.1 were found to result in neutropenia (Hock et al, 2003; Person et al., 2003). In this condition, the myeloid progenitors show a block in differentiation and exhibit characteristics of both granulocytes and monocytes. Previous studies demonstrated that GFI-1 and Egr-2/Nab2 function in a counter antagonistic manner to drive myeloid progenitors into neutrophils or macrophages (Laslo et al., 2006). Such antagonistic interplay between several lineage determinants was found to be critical for resolving mixed lineage states (Orkin et al., 2000). Figure 3.1 outlines the role of PU.1 and GFI-1 along with other critical transcription factors necessary for determining granulocyte or monocyte cell lineage decisions and development.

In the studies presented here, we show that ZXDC interacted with PU.1 and GFI-1 and the zinc fingers of ZXDC mediate these protein-protein interactions. ZXDC repressed the ability of GFI-1 to activate transcription in a dose-dependent manner and activated the Egr-2 promoter. The *Myc* promoter, a known target of GFI-1 was also found to be a direct target of ZXDC as demonstrated by chromatin immunoprecipitation experiments in HEK293 cells.

We also found that ZXDC has a role in PU.1 protein stabilization. Earlier studies by others demonstrated that the expression levels of PU.1 are critical for determining hematopoietic cell lineage decisions and its deregulated expression can result in leukemias and lymphomas (DeKoter et al., 2000, 2007; Anderson et al., 2002; Dahl et al., 2003; Cook et al., 2004; Rosenbauer et al., 2004, 2006; Huang et al., 2008; Kastner et al., 2008). Therefore, it is important to study the molecular mechanism of ZXDC mediated PU.1 protein stabilization. Preliminary results in HL-60 cells by the over-expression or



**Figure 3.1 A model for PU.1 and GFI-1 mediated transcriptional regulation of granulocyte and monocyte lineage commitment and maturation:** The figure outlines the role of important transcription factors involved in monocyte and granulocyte differentiation and development. Hematopoietic stem cell (HSC) differentiates into lymphoid-myeloid progenitor (LMP) directed by PU.1 or into megakaryocyte erythroid progenitor (MEP) directed by GATA-binding protein 1 (GATA-1). Cross-inhibition between PU.1 and GATA-1 determines lymphoid-myeloid or erythroid lineages. Similar cross-inhibition occurs between C/EBP $\alpha$  and lymphoid factors Pax5 and Notch to direct lymphoid or granulocyte-monocyte lineages. MEPs give rise to erythrocytes (RBC) or megakaryocytes (mega). High levels of PU.1 direct granulocyte-monocyte progenitor (GMP) to monocytic lineage. C/EBP $\alpha$  induction of PU.1 enhances PU.1 activity. AP-1



and NF- $\kappa$ B may also have positive roles in this pathway. For GMP to differentiate into granulocyte lineage requires C/EBP $\alpha$  and reduced PU.1 levels. Vitamin D receptor (VDR), interferon regulatory factor-8 (IRF-8), mafB, early growth response proteins-1,2 (Egr-1, Egr-2) direct monocytic maturation, whereas, retinoic acid receptors (RARs) and C/EBP $\epsilon$  direct granulocytic differentiation. Loss of CDP- and HoxA10-repressive activities; and GFI-1 mediated repression is necessary for granulocyte differentiation. Cross-inhibition between GFI-1 and Egr-1,2 maintains GMP decisions. This schematic diagram (Fig. 3.1) was adapted from a review article written by Friedman, 2007.

silencing of ZXDC followed by induction with monocytic differentiation agent (PMA) demonstrated that ZXDC favors monocyte development. Taken together, our findings suggest that ZXDC has a regulatory role by stabilizing PU.1 protein expression and/or by affecting GFI-1 and PU.1 transcriptional activities and their recruitment at their target gene promoters following differentiation of hematopoietic progenitor cells. In addition, activation of the Egr-2 promoter activity and expression by ZXDC, supports the notion that ZXDC favors monopoiesis. The results presented in this chapter strongly suggest an important role for ZXDC in monocyte development and differentiation.

## **Materials and methods**

**Cell culture and Transfections:** Human embryonic kidney 293 (HEK293; ATCC No. CRL-1573) cells and HeLa cells were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 4mM L-glutamine, 1.5g/L sodium bicarbonate, 4.5g/L glucose, antibiotics and 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified air atmosphere and 5% CO<sub>2</sub>. HEK293 cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Inc., Carlsbad, CA). Briefly, 2x10<sup>5</sup> cells were plated in a 24-well plate 24 h prior to transfection. For all transfections, 2 µl of Lipofectamine 2000 was mixed with a total of 800 ng plasmid DNA and added to the cells according to the manufacturer's instructions. HeLa cells were plated at 10<sup>5</sup> cells in a 6-well tissue culture dish 24 h prior to transfection. These cells were transfected with TransPass HeLa transfection reagent (New England Biolabs, MA). For all transfections, 3 µg plasmid DNA was mixed with 3 µl TransPass HeLa transfection reagent. When necessary, empty vector was used as a stuffer to keep a total of 3µg plasmid DNA.

**Luciferase assay:** For luciferase reporter experiments, all transfections included plasmid for *Renilla* luciferase reporter driven by the herpes simplex virus-thymidine kinase promoter (phRL-TK-*Renilla*) to serve as an internal control. Forty-eight hours after transfection, luciferase assay was performed using the Dual luciferase kit (Promega, Inc., Madison, WI) according to the manufacturer's instructions. All firefly luciferase values were normalized to the amounts of *Renilla* luciferase expression and relative fold increase were calculated. Mean values of three independent transfections are shown and error bars represent the standard error of the mean.

**Plasmids:** The plasmid pCMV5a-Flag-ZXDC was created by PCR amplifying ZXDC cDNA (GenBank accession No. AL553476) and cloned using *HindIII* and *BamHI* restriction sites of pCMV5a-Flag vector (Sigma, MO). The plasmids, pCMV-3xFlag-ZXDC, pCMV-3xFlag-ZXDC $\Delta$ N-terminus (ZXDC construct with deletion of amino acids 1-176) and pCMV-3xFlag-ZXDC $\Delta$ C-terminus (ZXDC construct with deletion of amino acids 472-858) was created by PCR amplifying appropriate regions of ZXDC cDNA and cloned into pcDNA3-3xFlag vector (an N-terminal fusion vector containing three copies of the Flag epitope) using *BamHI* and *XbaI* restriction sites. The plasmid pCMV-Flag-ZXDA was created by subcloning ZXDA cDNA from pBluescript-ZXDA (clone ID:30347480) into pCMV-Flag vector using *EcoRI* and *BamHI* restriction sites. The full-length human PU.1 cDNA (isolated from THP-1 cell line; pcDNA3.1-ZEO-hPU.1) and myc-tagged PU.1 plasmids with deletions of the activation domain (pCMV/myc/cyto-PU.1 $\Delta$ A) or the DNA binding Ets domain (pCMV/myc/cyto-PU.1 $\Delta$ E) were gifts from Dr. Christophe Nicot (Reference: Datta et al., 2006). The plasmid

pcDNA3.1-myc-PU.1 was obtained by PCR amplifying hPU.1 cDNA using the following primers and cloned into pcDNA3.1-myc vector using *EcoR*I and *Xho*I restriction sites.

Forward primer: 5' GATGAATTCCCTTACAGGCGTGCAAAATGG 3'

Reverse primer: 5' GATCTCGAGTCAGTGGGGCGGGTGGCGCCG 3'

The PU.1 reporter plasmid (pTK-3xPU.1-luciferase) containing three PU.1 binding sites upstream of minimal thymidine kinase promoter driving the luciferase gene was also a gift from Dr. Christophe Nicot. The expression plasmid for rat Growth factor independent-1 (GFI-1) was a gift from Dr. Marshall Horwitz. The Gfi reporter plasmid (pT81-4xGfi-luciferase) was created by annealing the following oligos containing 4xGfi binding sites (CACACCAAATCACTGC) and cloned upstream of minimal thymidine kinase promoter containing pT81 luciferase reporter plasmid (Addgene plasmid 11783) using *Bam*HI and *Hind*III restriction sites at 5' and 3' ends respectively.

Gfi oligo 1: 5'GATCCCACACCAAATCACTGCCACACCAAATCACTGCCACACC  
AAATCACTGCCACACCAAATCACTGCA 3';

Gfi oligo 2: 5' AGCTTGCAGTGATTTGGTGTGGCAGTGATTTGGTGTGGCAGTG  
ATTTGGTGTGGCAGTGATTTGGTGTGG 3'.

The plasmid pRL $\Delta$ CMV was used as an internal control for Egr-2 luciferase assays. This plasmid was generated by the removal of the CMV promoter in pRL-CMV vector (Promega) using restriction enzymes *Bgl*III and *Pst* I followed by blunting of the ends with DNA polymerase I (Klenow). The blunt ends were ligated using T4 DNA ligase. The expression plasmid for *Renilla* luciferase (pRG-TK-*Renilla*) was purchased (Promega, Madison, WI). The plasmid pEgr2-luciferase reporter is generated by cloning

a 922 base pair amplification product containing Egr2 promoter into pGL3basic using *NheI* and *HindIII* restriction sites. The following primers were used for PCR amplification:

Pegr2-1: 5' AAGCTAGCATTCTCTCTCGAAGCTCCC 3'

Pegr2-2: 5' GGAAGCTTTGTCTATTTGCCACTGAC 3'

**Antibodies:** For co-immunoprecipitation and Western analysis, the following antibodies were used: Anti-FLAG M2 (1mg/ml, SIGMA, MO), anti-HA 12CA5 (0.4mg/ml, Roche Inc, IN), anti-myc 9E10 (0.4mg/ml, Roche Inc, IN), anti-GST (200µg/ml, Santa Cruz, CA), anti-beta actin (Santa Cruz, CA). The HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (200µg/ml) were obtained Santa Cruz Biotech (Santa Cruz, CA).

**Co-immunoprecipitation and Western analysis:** HEK293 cells ( $10^6$ ) cells were transfected with the indicated plasmids. Forty-eight hours after transfection, cells were lysed in 500 µl of lysis buffer (10mM Tris pH:8, 300mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 25mM N-ethyl maleimide, 10mM iodoacetamide, 2mM Phenyl methyl sulphonyl fluoride and 1X EDTA-free protease inhibitors. A phosphatase inhibitor, Sodium fluoride at 50 mM concentration was included in the lysis buffer to stabilize phosphorylated PU.1 when needed. Following sonication, the lysate was cleared by centrifugation at 13,000 rpm for 15 min at 4°C. About 10% of the lysate was taken as 'input control' to check the expression of the plasmids. The remaining lysate was incubated with 4 µl of ANTI-FLAG M2 monoclonal antibody (Sigma, MO) for 3 h at 4°C. The lysate-antibody mix was next incubated with 50 µl of protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.) at 4°C. The beads were washed four times with lysis buffer, boiled with Laemmli buffer (100mM Tris-pH 6.8, 2%

$\beta$ -mercaptoethanol, 4% SDS, 20% glycerol and 0.02% w/v bromophenol blue) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 8% polyacrylamide). The resolved samples were transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore, Inc.) using transfer buffer (25mM Tris-pH8.3, 192mM glycine, 20% methanol). Blots were blocked for one hour at room temperature in 1XTBST wash buffer (50mM Tris-pH 8, 150mM NaCl, 0.1% Tween-20) containing 5% non-fat milk and 10% donor bovine serum. Blots were probed with the indicated primary antibody followed by horse radish peroxidase (HRP)-conjugated secondary antibody (SantaCruz Biotechnology, Inc) in wash buffer containing 3% non-fat milk and 10% donor bovine serum. Detection was done using ECL plus (Amersham, GE Healthcare) and data were analyzed using Typhoon-9410 (GE Healthcare, Piscataway, NJ).

**Cycloheximide treatment:** To check if ZXDC has a role in PU.1 protein stabilization, HEK293 cells were transfected with plasmids that express myc-PU.1 or along with Flag-ZXDC. Twenty-four hours after transfection, cells were either untreated or treated with 100 $\mu$ g/ml Cycloheximide (Sigma) to inhibit protein synthesis. Cells were harvested at different time points (1h, 2h, 4h, 8h, 24h) following cycloheximide treatment. Cells were lysed in lysis buffer (10mM Tris pH:8, 300mM sodium chloride, 1% Triton X-100, 0.1% sodium dodecyl sulphate, 25mM N-ethyl maleimide, 10mM iodoacetamide, 2mM Phenyl methyl sulphonyl fluoride and 1X EDTA-free protease inhibitors, 50mM sodium fluoride), protein concentration of the cell lysates was determined using Bio-rad protein quantification assay and 50 $\mu$ g of total protein was analyzed by Western analysis.

**Chromatin immunoprecipitation assay:** HEK293 cells ( $6 \times 10^6$ ) were plated in a 10 cm tissue culture dish 24 hours prior to transfection. The cells were transfected with 6  $\mu\text{g}$  each of ZXDC and GFI-1 expression plasmids (pCMV5a-Flag-ZXDC and pCS2-myc-GFI-1). Forty-eight hours post-transfection, cells were fixed in 1% formaldehyde (SIGMA, MO) for 10 minutes at room temperature. The cross-linking was quenched by the addition of glycine (SIGMA, MO) at a concentration of 0.125M for five minutes at room temperature. The cells were washed with ice-cold PBS and scraped from tissue culture plate and transferred to a microcentrifuge tube and collected by centrifuging at 700xg for 4 minutes at 4°C. The cell pellet was resuspended in 500  $\mu\text{l}$  of SDS-lysis buffer (50mM Tris, pH 8.1, 1% SDS, 10mM EDTA). 2  $\mu\text{l}$  of RNaseA (0.5 mg/ml stock) was added to the cell lysate and resuspended on ice for 10 minutes. The cell lysate was sonicated on ice to generate soluble chromatin with DNA fragments of lengths less than 3kb. The sheared chromatin was cleared by centrifuging at 13000xg, 4°C, for 15 minutes. The cleared chromatin was transferred to a 15ml tube and diluted (1:10) with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl-pH 8.1, 167mM NaCl). The diluted chromatin was pre-cleared with rabbit IgG for one hour at 4°C, followed by incubation with 60 $\mu\text{l}$  protein G agarose beads (Upstate Inc., Temecula, CA) for another hour. The beads were collected by centrifuging at 1000xg for 3 minutes at 4°C. The pre-cleared chromatin was transferred to a fresh tube and 1% of it was set aside as input control. The remaining fraction was aliquoted into separate microcentrifuge tubes and incubated overnight with 5  $\mu\text{g}$  of anti-Flag (Affinity Bioreagents, CO), anti-myc (Roche Inc., IN), anti-RNA polymerase II (abcam In., MA) or non-specific IgG (abcam In., MA). Following incubation with antibody, the sheared

chromatin was incubated with 40  $\mu$ l of Protein G Dynabeads (Invitrogen Inc., CA) for an additional hour at 4°C. The beads were separated using a magnetic separator and washed with the following buffers by mixing (with inversion) the beads in each buffer for 5 minutes at 4°C- Low-salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), High-salt immune complex wash buffer (0.1% SDS, 1% TritonX-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholic acid, 1mM EDTA, 10mM Tris, pH 8.1) and washed twice with TE buffer (10mM Tris-HCl, pH 8, 1mM EDTA). The chromatin complex was eluted with 400  $\mu$ l of elution buffer (1% SDS, 0.1M NaHCO<sub>3</sub>). The DNA-protein crosslinking was reversed by adding 16  $\mu$ l of 5M NaCl to 400  $\mu$ l of the eluate followed by incubation at 65°C for four hours. The protein was degraded by the addition of 6  $\mu$ l of Proteinase K (10 mg/ml stock) (Invitrogen Inc., CA) and incubated at 45°C overnight. The DNA was purified using the PCR purification kit (Qiagen, CA) according to the manufacturer's protocol. The following primers for Myc and beta-actin (negative control, Nakazawa et al., 2007) were used with 2 $\mu$ l DNA and 2X Power SYBR green PCR Mastermix (Applied Biosystems, CO) for real-time PCR analysis using IQ5 real-time PCR detection system (BioRad Inc., CA).

Myc forward primer: 5'GAAGGTATCCAATCCAGATAGCTGTGC 3'

Myc reverse primer: 5' GAGCGTGGGATGTTAGTGTAGATAGGG 3'

beta-actin forward primer: 5' CATCACCATTGGCAATGAGC 3'

beta-actin reverse primer: 5' CATACTCCTGCTTGCTGATC 3'

**RNA preparation and Semiquantitative RT-PCR analysis:** Total RNA was isolated with TRIzol reagent (InVitrogen, Inc.) as described previously (Chomczynski et al.,



1993). cDNA was synthesized with QuantiTect Reverse transcription kit (Qiagen) from 1µg of total RNA. The relative expression levels of Egr-2, Egr-4, ZXDC or beta-2-microglobulin (control) were determined by PCR amplification using Platinum PCR SuperMix (Invitrogen Inc., CA) and the following gene specific primers. The PCR products were resolved by 8% poly acrylamide gel electrophoresis with ethidium bromide staining and data were analyzed using Typhoon scanner (GE Healthcare, Piscataway, NJ).

Egr-2 forward primer: 5' GTAAGCCCTTTCCCTGCCCACTG 3'

Egr-2 reverse primer: 5' GGTCCCTCGCTGCCTCCACTG 3'

Egr-4 forward primer: 5' TGCTCCACCTTAGCGAGTTT 3'

Egr-4 reverse primer: 5' CAAAGCCCAGCTCAAGAAGT 3'

ZXDC forward primer: 5' CAGCAAGAAGTACTGATTACCGAGC 3'

ZXDC reverse primer: 5' GCATGGAAATGGGGAGGACTCA 3'

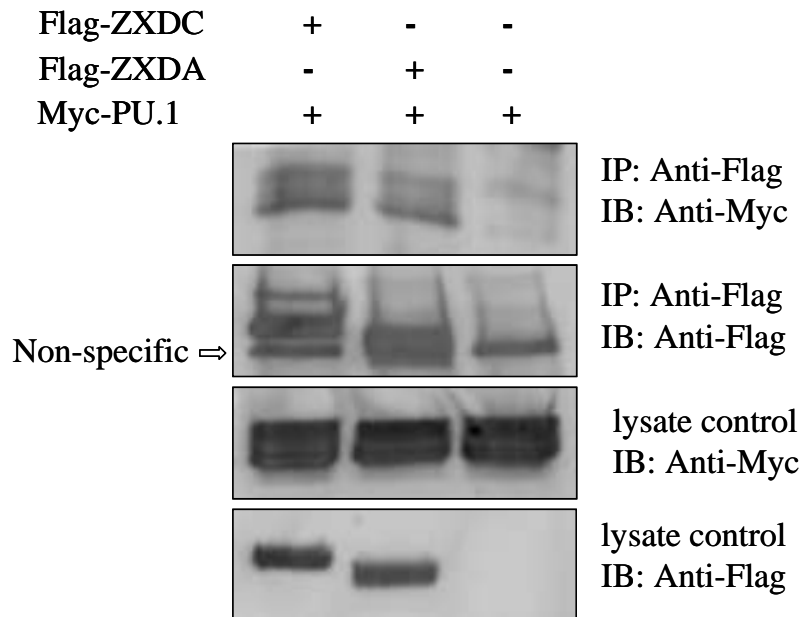
β2 microglobulin forward primer: 5' GTGCTCGCGCTACTCTCTCT 3'

β2 microglobulin reverse primer: 5' TCTCTGCTGGATGACGTGAG 3'

## Results

### **ZXDC and ZXDA proteins interact with the myeloid transcription factor, PU.1**

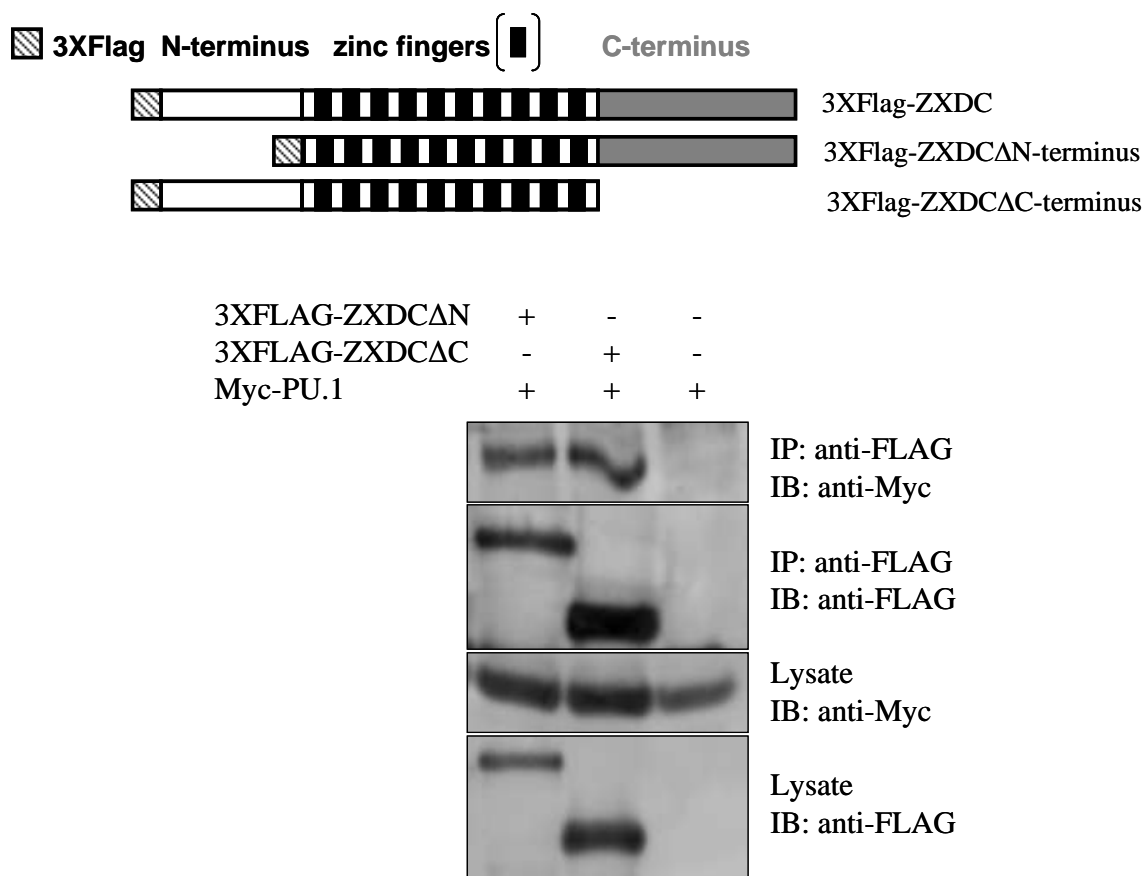
It was previously reported that ZXDC was cloned in a yeast two hybrid assay with the GFI-1 protein (Duan et al., 2007). During experiments to confirm this finding, we also found that ZXDC could interact with the hematopoietic transcription factor PU.1, by co-immunoprecipitation assay (Fig. 3.2). HEK293 cells do not express PU.1 and were co-transfected with expression plasmids for myc-PU.1 and Flag-ZXDC or Flag-ZXDA. Co-immunoprecipitation (co-IP) with anti-Flag and Western blot with anti-myc demonstrated that both ZXDC and ZXDA proteins interacted with PU.1 *in vivo*. As a negative control for co-IP, HEK293 cells were transfected with only the myc-PU.1 expression plasmid (Fig. 3.2, lane 3). A non-specific band was detected in the absence of either Flag-tagged protein (Fig. 3.2; panel 2, lane 3). The slower migrating band in myc-PU.1 lanes represent the phosphorylated PU.1 (reviewed in Sylvia and Fenton, 2000), whereas, the slower migrating band in the ZXDC lane represents the sumoylated form of the ZXDC protein (Jambunathan and Fontes, 2007).



**FIGURE 3.2 ZXDC/ZXDA co-immunoprecipitate with PU.1:** HEK293 cells were co-transfected with pcDNA3.1-myc-PU.1 and pFlag-CMV-5a-ZXDC or pCMV-Flag-ZXDA. Forty-eight hours after transfection, the cell lysate was subjected to co-IP with anti-Flag followed by Western analysis with anti-Myc. The figure includes appropriate controls. The slower migrating band in Myc-PU.1 lanes represent the phosphorylated form of PU.1, where as, the slower migrating band in Flag-ZXDC lane (IP&IB with anti-Flag) represents the sumoylated form of ZXDC.

### **The zinc fingers of ZXDC mediate interaction with PU.1**

To map the region of ZXDC necessary for interaction with PU.1 protein, an N-terminal (ZXDC construct with deletion of amino acids 1-176) or C-terminal deletion (ZXDC construct with deletion of amino acids 472-858) was made in the ZXDC protein (Fig.3.3, top panel). Deletion of the N-terminus or the C-terminus did not affect interaction with PU.1 (Fig. 3.3) suggesting that the zinc finger region of the ZXDC protein mediates its interaction with PU.1. We were not able to finely map which zinc fingers are involved as deletion of all or some zinc fingers of ZXDC resulted in destabilization of the protein (data not presented).

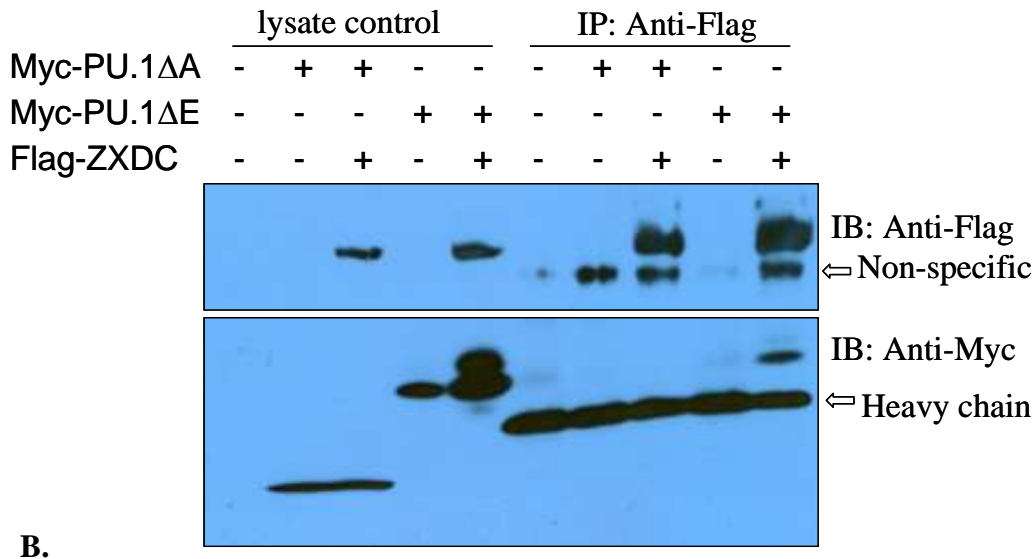
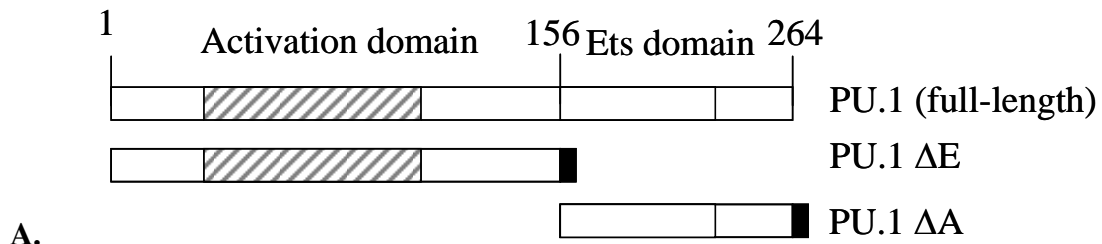


**FIGURE 3.3 Zinc fingers of ZXDC mediate interaction with PU.1.** Schematic representation of ZXDC deletion constructs with deletion of the N-terminus or the C-terminus of ZXDC is shown in the top panel. HEK293 cells were transfected with plasmids that express 3X Flag-ZXDC deletion constructs with deletion of the N-terminus or C-terminus and myc-PU.1. Co-IP was performed with anti-Flag and Western analysis was performed with anti-Myc. Our results demonstrate that deletion of N-terminus or C-terminus of ZXDC did not affect its interaction with PU.1 (Fig. 3.3, lanes 1 & 2, top panel) suggesting that the zinc fingers of ZXDC are necessary to mediate ZXDC-PU.1 interaction. We were not able to demonstrate which zinc fingers are necessary as deletion of some or all zinc fingers resulted in destabilization of the protein (data not presented).

## **The region of PU.1 containing the activation domain is necessary for interaction with ZXDC**

Several key regulatory proteins interact with distinct domains of the PU.1 protein. For example, the amino terminal transcription activation domain of PU.1 interacts with TFIID, TBP, GATA1, GATA2, glucocorticoid receptor and heat shock protein, HSP90 whereas, the PEST domain of PU.1 interacts with Interferon regulatory factor family of proteins (Reddy et al., 2002; Dahl et al. 2007; Huang et al., 2007; Rekhtman et al., 1999; Vangala et al., 2003; Bassuk and Leiden, 1995; Aittomaki et al., 2004; Gauthier et al., 1993; Wara-aswapati et al., 1999). The PU.1 DNA binding, Ets domain interacts with c-Jun, GATA1, GATA2, CCAAT enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) and CCAAT enhancer binding protein  $\beta$  (C/EBP $\beta$ ), acute myeloid leukemia 1 (reviewed in Tenen, 2003). This knowledge can help us understand the regulatory mechanisms of such protein-protein interactions. For example, the C-terminal zinc finger of GATA1 protein competes with the basic region of c-Jun for interaction with the Ets domain of PU.1 (Zhang et al., 1999, Liew et al., 2006) and this is a potential mechanism to prevent myeloid gene activation in erythroid cells. In another example, the leucine zipper of C/EBP $\alpha$  competes with c-Jun for interaction with PU.1 in the absence of DNA (Reddy et al., 2002), whereas, in the presence of DNA, it cooperates and enhances PU.1 activity to activate myeloid promoters (Oelgeschlager et al., 1996). We wanted to identify the PU.1 domain that is necessary for interaction with ZXDC as it can give an idea about the functional consequences of PU.1-ZXDC interaction. To determine the PU.1 domain necessary for interaction with ZXDC, expression plasmids for myc-PU.1 $\Delta$ A (deletion of the activation domain of PU.1) or myc-PU.1 $\Delta$ E (deletion of the Ets domain of PU.1; Fig.

3.4A) and Flag-ZXDC were transfected into HEK293 cells. Co-immunoprecipitation with anti-Flag and Western analysis with anti-myc demonstrated that deletion of the region containing the activation domain of the PU.1 protein inhibits its interaction with ZXDC (Fig. 3.4B).

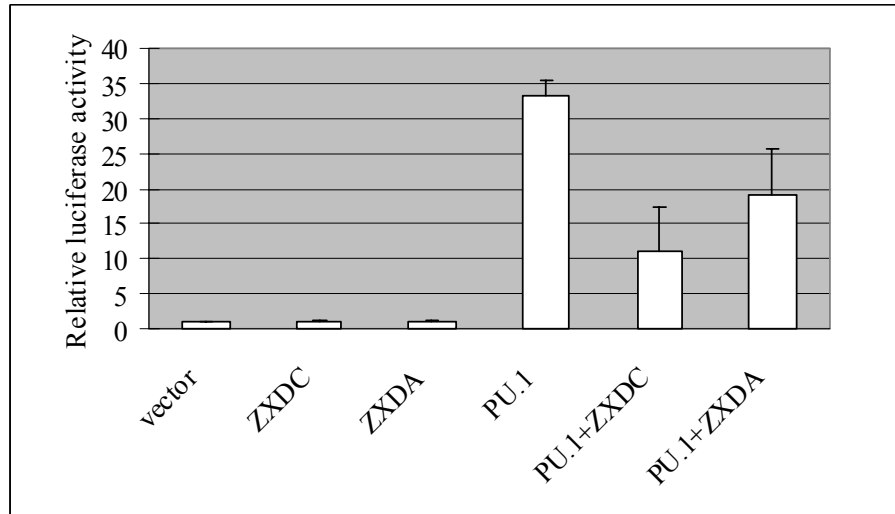


**FIGURE 3.4 A. Schematic representation of the full-length human PU.1 and its deletion constructs.** PU.1 ΔE lacks the DNA binding Ets domain and PU.1ΔA lacks the activation domain of PU.1. The black rectangle represents the Myc epitope at the C-terminus. **B. ZXDC interacts with the region of PU.1 containing the activation domain:** HEK293 cells were co-transfected with PU.1ΔE-myc (myc-tagged PU.1 with deletion of the Ets domain) or PU.1ΔA-myc (myc-tagged PU.1 with deletion of the Activation domain) alone or along with pFlag-CMV-5a-ZXDC. Forty-eight hours post-transfection, co-IP was performed with anti-Flag and Western analysis with anti-Myc. All samples included 10% of the lysate taken prior to co-IP as input control.

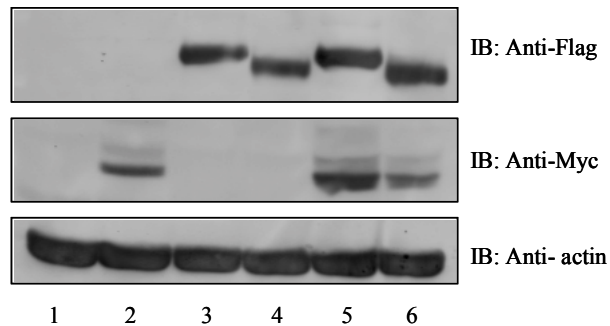


## **ZXDC and ZXDA proteins repressed PU.1 transcriptional activity**

PU.1 is a myeloid master regulator that is necessary for transactivation of several myeloid specific genes (Krysinska et al., 2007, Scott et al., 1994). The interaction of ZXDC and ZXDA proteins with PU.1 was found to have a functional role as they repressed activation of a PU.1-responsive reporter plasmid by about 75% and 50% respectively (Fig. 3.5). Luciferase reporter assay in HeLa cells transfected with plasmids expressing the indicated proteins and a luciferase reporter containing three PU.1 binding sites upstream of a minimal thymidine kinase promoter demonstrated that ZXDC and ZXDA proteins have a repressive effect on PU.1 transcriptional activation. We were not surprised by ZXDA functioning similar to ZXDC, that is having a similar repressive effect on PU.1 activity, since ZXDC and ZXDA proteins share significant amino acid sequence homology especially in the zinc finger region (Alkandari et al., 2007b). It is possible that ZXDC represses PU.1 activity by preventing its DNA binding, but, this mechanism is less likely as ZXDC does not interact with PU.1 Ets domain and therefore may not compete with this region of PU.1 for DNA binding. The bottom panel is a Western control to confirm that the co-expression of ZXDC or ZXDA does not reduce PU.1 expression. Interestingly, we observed enhanced PU.1 protein levels specifically when ZXDC (Fig. 3.5, lane 5) but not ZXDA (Fig. 3.5, lane 6) was over-expressed along with PU.1.



Myc-PU.1	-	+	-	-	+	+
Flag-ZXDC	-	-	+	-	+	-
Flag-ZXDA	-	-	-	+	-	+

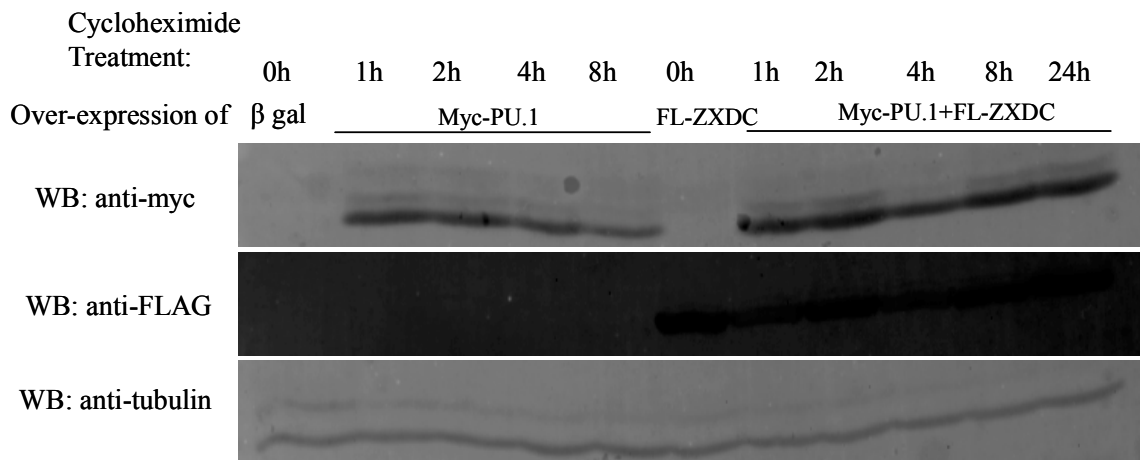


**FIGURE 3.5: ZXDC and ZXDA repressed PU.1 transcriptional activity.** HeLa cells were plated at  $10^5$  cells in 6-well plate. After 24 hours, cells were co-transfected with pcDNA3.1-myc-PU.1, pFlag-CMV-5a-ZXDC, pCMV-Flag-ZXDA plasmids as indicated and the PU.1 reporter plasmid, pTK-PU.1-3X-luciferase containing three PU.1 binding sites upstream of minimal thymidine kinase promoter driving the luciferase gene. Forty-eight hours after transfection, Luciferase assay was performed. ZXDC and ZXDA were found to repress the transcriptional activity of PU.1 by 75% and 50% respectively. The

bottom panel in Fig. 3.5 is a Western control to check for the expression of proteins for the above luciferase assay.

### **ZXDC has a role in PU.1 protein stabilization**

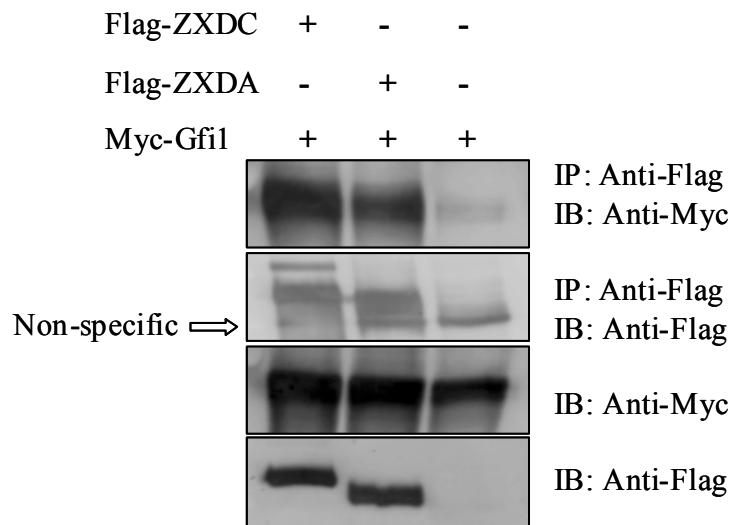
The expression level of PU.1 was found to have an important role in determining hematopoietic cell fate and lineage decisions (Moreau-Gachelin et al., 1988; DeKoter et al., 2000; Anderson et al., 2002; Dahl et al., 2003; Cook et al., 2004; Rosenbauer et al., 2004, 2006; Huang et al., 2008). In order to determine if ZXDC stabilizes PU.1 protein expression, HEK293 cells were co-transfected with expression plasmids for myc-PU.1 by itself or with Flag-ZXDC. Twenty-four hours later, the cells were treated with cycloheximide, and harvest at time intervals to determine if over-expression of ZXDC affected PU.1 protein stability. Our results demonstrated significant decrease in PU.1 protein expression levels by 8h in cells expressing PU.1 alone (Fig. 3.6, lane 5). On the other hand, over-expression of ZXDC stabilized PU.1 expression, the levels remaining high even after 24h of cycloheximide treatment. At present, we do not know the mechanism of ZXDC mediated PU.1 protein stabilization and further studies are needed.



**FIGURE 3.6 ZXDC has a role in PU.1 protein stabilization:** HEK293 cells were transfected with the indicated plasmids. Twenty-four hours post-transfection, cells were either untreated or treated with 100  $\mu$ g/ml cycloheximide for the indicated time points and harvested. Co-transfection of ZXDC was found to have a role in PU.1 protein stabilization.

## **ZXDC and ZXDA proteins also interact with the transcriptional repressor, Growth factor independent-1**

Growth factor independent-1 (GFI-1) is a transcriptional repressor that is required for granulocyte development (Hock et al., 2003). GFI-1 was found to antagonize PU.1 activity to inhibit macrophage differentiation and promote granulocyte development (Dahl et al., 2007). Previous studies by others have shown ZXDC as an interacting partner of GFI-1 by yeast-two hybrid assay (Duan et al., 2007). In order to determine if ZXDC and GFI-1 proteins interact *in vivo*, co-immunoprecipitation assays were performed. Fig. 3.7 demonstrates ZXDC-GFI-1 and ZXDA-GFI-1 interactions. As a negative control for co-IP, HEK293 cells were transfected with only myc-GFI-1 expression plasmid (Fig. 3.7, lane 3). Detection of non-specific bands with anti-Flag occurred in the absence of either Flag-tagged proteins (Fig. 3.7; panel 2, lane 3).

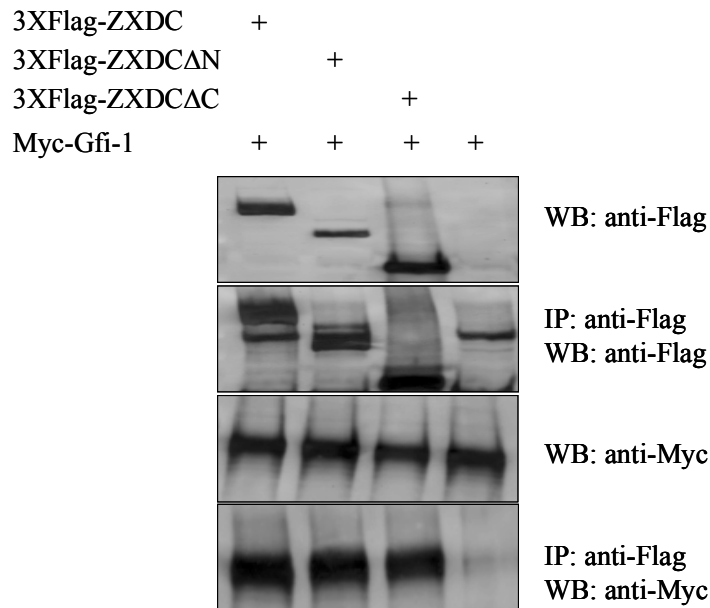
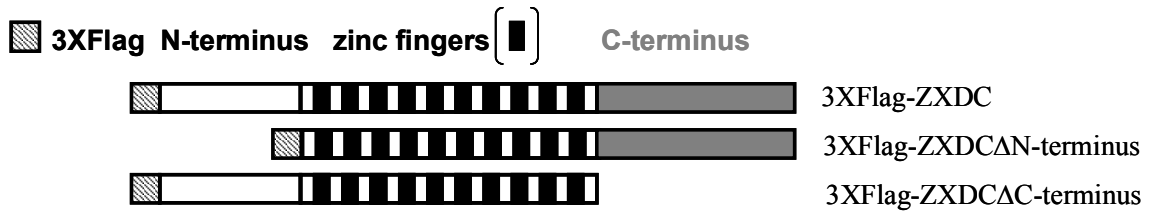


**FIGURE 3.7 ZXDC/ZXDA co-immunoprecipitate with GFI-1:** HEK293 cells were co-transfected with pCS2-myc-Gfi1 and pFlag-CMV-5a-ZXDC or pCMV-Flag-ZXDA. Forty-eight hours post-transfection, the lysate was subjected to co-IP with anti-Flag and Western analysis with anti-Myc. Our results demonstrate that both ZXDC and ZXDA proteins interact with GFI-1 (Fig. 3.7, lanes 1 & 2, top panel). Co-IP in the absence of either Flag-tagged protein was used as a negative control. The bottom two panels show 10% of the lysate taken prior to co-IP as input control.

### **The zinc finger region of ZXDC mediates its interaction with GFI-1**

To map the region of ZXDC necessary for interaction with GFI-1, co-immunoprecipitation assays were performed in HEK293 cells using ZXDC deletion constructs (Fig. 3.8, top panel). Our results demonstrated that deletion of the N-terminal or the C-terminal regions of ZXDC did not affect its interaction with GFI-1 (Fig. 3.8, lanes 2 & 3, bottom panel) suggesting that the zinc fingers of ZXDC are necessary to associate with GFI-1 protein. Co-IP of GFI-1 with full-length ZXDC was used as a positive control (Fig. 3.8, lane 1, bottom panel), whereas, co-IP in the absence of either Flag-tagged proteins was used as a negative control (Fig. 3.8, lane 4, bottom panel)

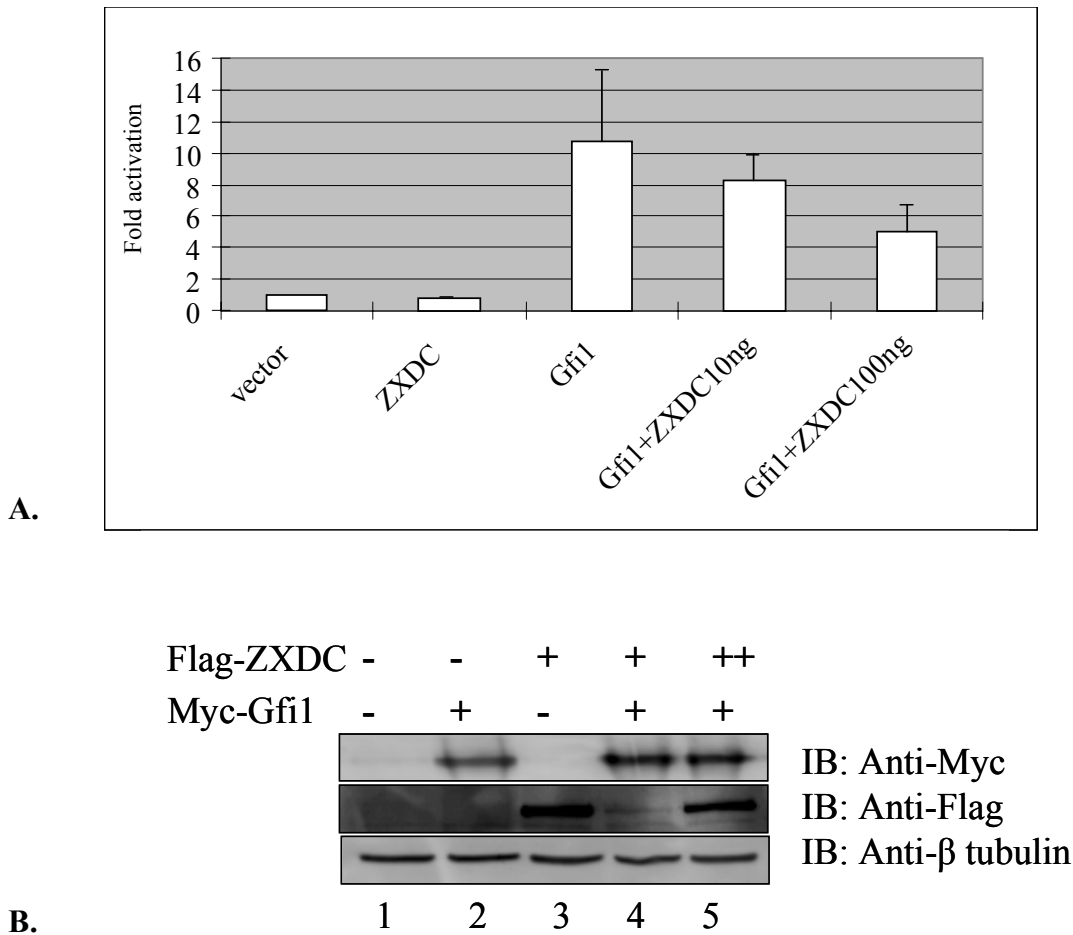




**FIGURE 3.8 Zinc fingers of ZXDC mediate ZXDC-GFI-1 interaction.** HEK293 cells were co-transfected with the indicated plasmids. Forty-eight hours post-transfection co-IP was performed with anti-Flag and Western analysis with anti-Myc. This experiment demonstrated that deletion of the N-terminus or the C-terminus of ZXDC does not affect its interaction with GFI-1 (lanes 2 & 3, bottom panel) suggesting that the zinc fingers of ZXDC are necessary to interact with GFI-1.

### **ZXDC represses the transcriptional activity of GFI-1 in a dose-dependent manner**

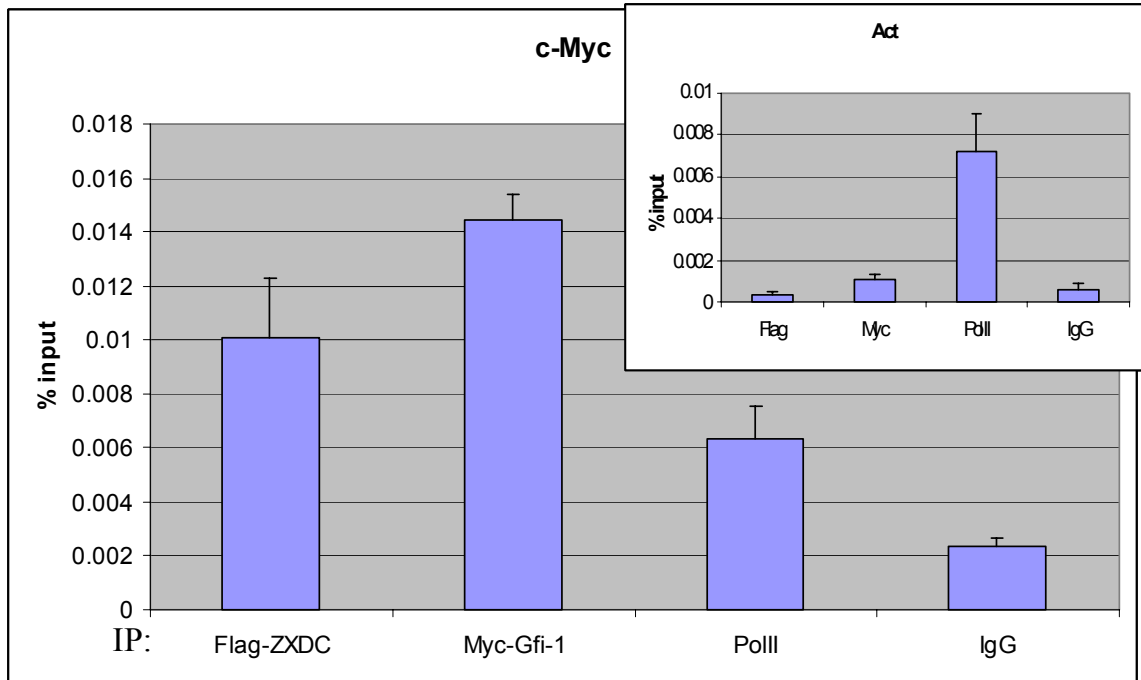
We were interested to determine if the ZXDC-GFI-1 interaction has an effect on GFI-1 transcriptional activity. Fig. 3.9 demonstrates that ZXDC represses the transcription of a GFI-1 responsive reporter plasmid (Fig. 3.9, top panel) without affecting GFI-1 protein expression (Fig. 3.9, bottom panel). This information is important as it leads to further questions such as: Does ZXDC affect GFI-1 recruitment at its target promoters and/or expression of known GFI-1 target genes during hematopoietic cell differentiation? Does ZXDC regulate microRNAs miR-21 and miR-196, known targets of GFI-1 (Chinavenmeni et al., 2009) that are necessary for normal myelopoiesis?



**FIGURE 3.9 A. ZXDC repressed Gfi1 transcriptional activity in a dose-dependent manner.** HEK293 cells were plated at  $2 \times 10^5$  cells in 24-well plate. Cells were co-transfected with 100ng of pT81-Gfi 4X-luciferase and 400ng of pCS2-myc-Gfi1 or increasing amounts (10ng and 100ng) of pFlag-CMV-5a-ZXDC plasmids as indicated. All transfections included 10ng of pHRG-TK-*Renilla* plasmid to serve as internal control. Forty-eight hours after transfection, relative luciferase activity was measured and fold increase calculated. **B.** Western control showing the expression levels of the indicated proteins for the above luciferase experiment.

### **ZXDC is present at the Myc promoter, a known target of GFI-1**

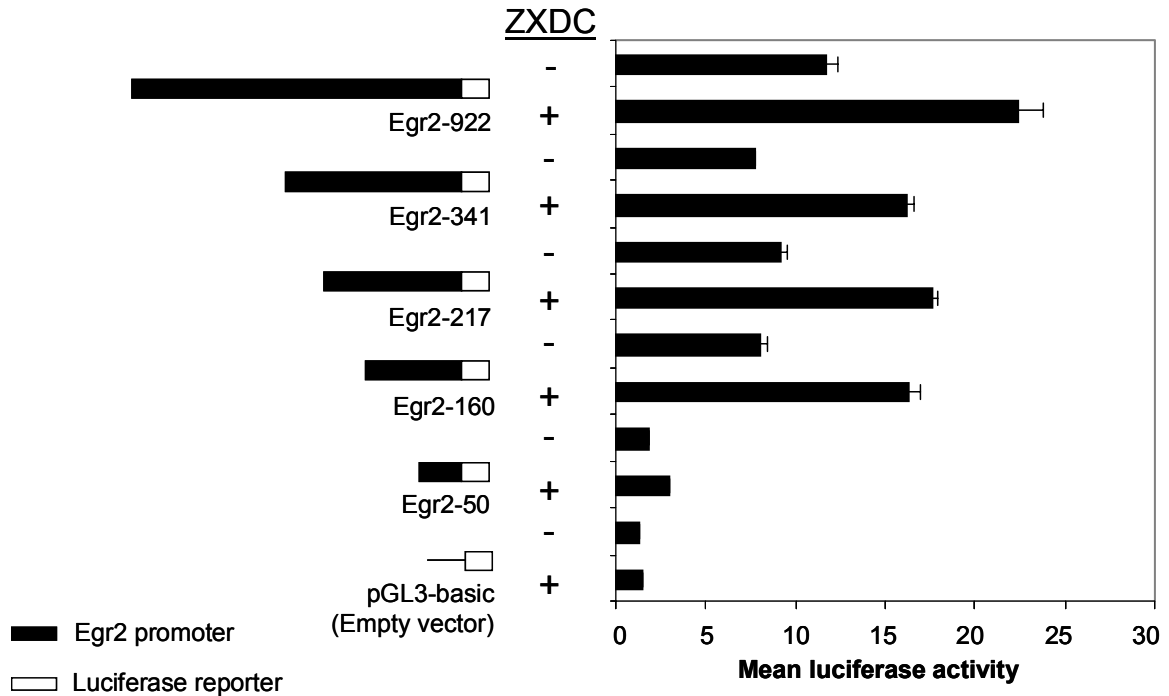
The interaction of ZXDC with GFI-1 and its repressive affect on GFI-1 transcriptional activity (Figs. 3.7, 3.9) suggested that it may have a role in GFI-1 recruitment and/or its activity at its target genes. Therefore, we wanted to find if ZXDC is present along with GFI-1 at the promoters of known GFI-1 target genes. We performed chromatin immunoprecipitation experiments in HEK293 cells over-expressed with Flag-ZXDC and myc-GFI-1 proteins. Using primers specific for the c-myc or beta-actin (negative control) promoters, real-time PCR analysis of the immunoprecipitated DNA with anti-Flag, anti-myc or anti-PolIII was performed. Our results demonstrated an enrichment of ZXDC and GFI-1 proteins along with RNA polymerase II significantly over the background (immunoprecipitation with IgG) (Fig. 3.10). These results also eliminate the possibility that the repressive effect of ZXDC on GFI-1 transcriptional activity is mediated by somehow sequestering the protein and preventing GFI-1 DNA binding.



**FIGURE 3.10 Chromatin immunoprecipitation (ChIP) analysis in HEK293 cells demonstrating the presence of ZXDC along with GFI-1 at the cMyc locus.** HEK293 cells were co-transfected with pFlag-CMV-5a-ZXDC and pCS2-myc-GFI-1. Forty-eight hours post-transfection, ChIP analysis was performed with equal amounts of chromatin using antibodies specific for Flag, Myc, PolII or non-specific serum (IgG) followed by quantitative Real-time PCR analysis of the eluted DNA with primers specific for the promoters of c-Myc or Actin (inset). Results are the average of three independent experiments and error bars represent standard error of the mean (SEM).

### **ZXDC activates Egr-2 promoter activity**

Microarray analysis by the over expression of ZXDC in HEK293 cells identified early growth response-2 (Egr-2) and early growth response-4 (Egr-4) as candidate genes that are upregulated (O. Galkin and J.D. Fontes, unpublished observation). We were interested to study this further as early growth response (Egr) proteins were found to have a role in monopoiesis and at the same time repress granulocyte-specific genes (Laslo et al., 2006). In addition, Egr-2/Nab-2 and GFI-1 proteins were found to function in a counter-antagonistic manner (Laslo et al., 2006). To determine if ZXDC activates the Egr-2 gene at the transcriptional level, we performed reporter assays in HEK293 cells transfected with a luciferase reporter driven by the Egr-2 promoter without or with over-expression of ZXDC. We observed that over-expression of ZXDC activates Egr-2 promoter activity modestly, but in a consistent manner. This modest increase could be because there is already endogenous ZXDC available in these cells. In order to determine to which region of the Egr-2 promoter ZXDC binds, we created various deletions in the Egr-2 promoter-driving the luciferase reporter gene. An Egr-2 promoter plasmid containing only 50 base pairs upstream of the transcription start site significantly reduced the promoter activity, although, the relative activation of this promoter fragment by ZXDC was similar to the full-length promoter. However, further studies are required to determine and precisely map ZXDC binding to the Egr-2 promoter region.

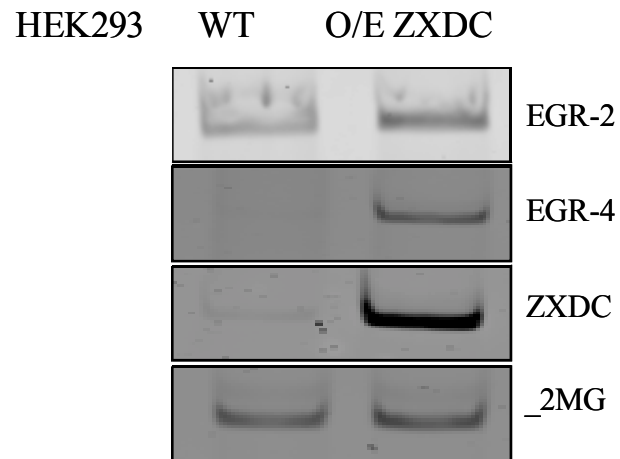


**FIGURE 3.11 ZXDC activates Egr-2 gene transcription.** Schematic representation of the Egr2 promoter as well as various deletions of the promoter driving the luciferase reporter is shown on the left in Fig. 3.11. HEK293 cells were co-transfected with a luciferase reporter driven by Egr-2 promoter (Egr2-L) or with various deletions of the Egr2 promoter or pGL3basic (control backbone plasmid) without or with pFlag-CMV-5a-ZXDC (ZC) along with *Renilla* reporter plasmid (pRLΔCMV, internal control). Forty-eight hours after transfection, Firefly and Renilla luciferase reporter levels were measured. ZXDC showed about a two-fold activation of the EGR2 promoter consistently. Luciferase reporter data in Fig. 3.11 is from three independent experiments and error bars represent the standard error of the mean (SEM).

### **Over-expression of ZXDC enhances Egr-2 and Egr-4 mRNA expression levels**

Since we previously observed modest activation of Egr-2 promoter activity by ZXDC (Fig. 3.11), we also wanted to check the transcript levels of early growth response genes (Egr-2 and Egr-4). HEK293 cells were transfected with plasmids that express  $\beta$ -gal or ZXDC. After forty-eight hours, total RNA was isolated and the mRNA levels of Egr-2, Egr-4, ZXDC and  $\beta$ 2 microglobulin (control) were analyzed by semi-quantitative RT-PCR analysis. The PCR products were analyzed by polyacrylamide gel electrophoresis followed by ethidium bromide-stained agarose gels. This experiment demonstrated that ZXDC enhanced Egr-2 and Egr-4 mRNA expression levels when ZXDC is over-expressed (Fig. 3.12, compare lanes 1 & 2, top panels).





**FIGURE 3.12 ZXDC enhanced Egr-2 and Egr-4 mRNA expression levels.**

Expression of EGR2 and EGR-4 transcript levels is enhanced in the presence of ZXDC. HEK293 cells are either transfected with pCMV-SPORT6-βgal (stuffer, WT) or pFlag-CMV-5a-ZXDC (O/E ZXDC). Forty-eight hours after transfection, total RNA was isolated and semi-quantitative RT-PCR analysis was performed using primers specific for the indicated transcripts with β2microglobulin as control. The samples were run on 8% native polyacrylamide gel followed by staining with ethidium bromide.

## Discussion

A complex interplay involving regulatory cross-talk among several cell-type specific and stage specific transcription factors and their expression levels are critical for the determination of hematopoietic cell fates and lineage decisions (Friedman, 2007; Rosenbauer and Tenen, 2007; Laslo et al., 2006; Orkin., 2000). The transcription factor PU.1 belongs to the ets family of transcription factors and is considered the “master regulator” of myelopoiesis, as it plays a key role in the regulation of several myeloid specific genes. For example, it directs hematopoietic stem cells to the lymphoid-myeloid progenitor and based on its expression levels and interaction with several key transcription factors, is critical for hematopoietic cell-lineage specification. Preliminary studies in our lab suggest that the ZXDC family of proteins play a key role in myeloid development and differentiation.

In the studies presented here, we show that ZXDC and ZXDA proteins physically interact with PU.1 (Fig. 3.2). We found that the zinc finger region of ZXDC interacted with the N-terminal region of PU.1 containing the activation domain (Figs. 3.3 & 3.4). This interaction was found to be functionally significant as ZXDC and ZXDA proteins repressed the transcriptional activity of PU.1 by 75% and 50% respectively (Fig. 3.5). The interaction of PU.1 with several transcription factors, such as, GATA-1, CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ), Growth factor independent-1 (GFI-1), B cell-specific activator protein and Ski was shown to be critical for lineage determination and development of hematopoietic cells (Nerlov et al., 2000; Zhang et al., 1999; Reddy et al., 2002; Maitra and Atchison, 2000; Dahl et al., 2007; Ueki et al., 2008). Interestingly, all the above proteins inhibit the ability of PU.1 to transactivate its target genes at

different stages during hematopoietic differentiation and development. Such cross-talk among hematopoietic regulators is critical for proper cell lineage specification and development. For example, the interaction of GATA proteins with PU.1 that function in a cross-antagonistic manner is crucial for hematopoietic stem cells to commit to erythroid or myeloid lineages respectively (Zhang et al., 1999). Given the vital role of such tight regulation of PU.1, it is important to study the mechanism and the functional significance of the ability of ZXDC and ZXDA to repress PU.1 transactivation during myeloid differentiation.

In our studies, we observed that ZXDC has a role in PU.1 protein stabilization (Fig. 3.6). Previous reports demonstrated that the expression levels of PU.1 are critical for hematopoietic cell lineage specification and that deregulation of its expression can result in leukemias and lymphomas (Moreau-Gachelin et al., 1988; DeKoter et al., 2000; Anderson et al., 2002; Dahl et al., 2003; Cook et al., 2004; Rosenbauer et al., 2004, 2006; Huang et al., 2008). The regulation of PU.1 gene by a proximal promoter (Chen et al., 1995) and upstream regulatory element (Li et al., 2001; Rosenbauer et al., 2004; Okuno et al., 2005) has been well characterized. Interestingly, PU.1 expression was also found to be regulated by sense and antisense RNAs driven by intronic promoters (Ebralidze et al., 2008). Here, we demonstrate that ectopic expression of ZXDC along with PU.1 resulted in PU.1 protein stabilization in HEK293 cells that do not express endogenous PU.1. At present, we do not know if ZXDC has such a role by inhibiting proteasome mediated degradation of PU.1 and if so, identification of the E3 ubiquitin ligase is important to understand the molecular mechanism involved. It is also critical to find if signaling mechanism/s are involved in this process.

We have also found that ZXDC interacted with Growth factor independent-1 (GFI-1), a transcriptional repressor necessary for the granulocyte-monocyte progenitor to differentiate along the granulocyte/neutrophil lineage (Dahl et al., 2007). GFI-1 is a zinc finger transcriptional repressor containing an N-terminal SNAG repression domain and a C-terminal zinc-finger DNA binding domain that binds to a consensus DNA element containing the core sequence AATC (McKay et al., 1996). GFI-1 promotes granulopoiesis by interacting and inhibiting PU.1 mediated transactivation resulting in the repression of monocyte-specific genes (Dahl et al., 2007). Interestingly, ZXDC was found to repress the transcriptional activity of GFI-1 in a dose-dependent manner (Fig. 3.9) suggesting that it may have a role in the lineage specification of granulocyte-monocyte progenitors. It is possible that ZXDC sequesters GFI-1 preventing its ability to bind DNA to mediate this repression. However, chromatin immunoprecipitation experiments in HEK293 cells demonstrate that ZXDC is present at the c-myc promoter (Fig. 3.10) along with GFI-1 that was earlier shown to be a direct target of GFI-1 (Duan et al., 2003). It would be interesting to study further how ZXDC is recruited and how it affects recruitment of GFI-1 at its target gene promoters especially following differentiation in myeloid progenitors.

Several studies demonstrated the critical role of microRNAs in hematopoietic differentiation and leukemogenesis (reviewed in Garzon and Croce, 2008). Recent studies have shown an important role for GFI-1 in the regulation of micro-RNAs, miR-21 (a positive regulator of monopoiesis) and miR-196b (a negative regulator of granulopoiesis) for normal myelopoiesis (Chinavenmeni et al., 2009). In the same report it was demonstrated that GFI1-mutant severe congenital neutropenic patient and Gfi-/-

mice showed deregulated expression of miR-21 and miR-196b. Since ZXDC was found to affect GFI-1 transcriptional activity, it is important to find if it also affects the expression and regulation of miR-21, miR-196b, as well as, several other known GFI-1 target miRNAs, namely, miR-302b and miR-489 (Chinavenmeni et al., 2009). It is also known that GFI-1 plays an important role in hematopoietic stem cell renewal, given the repressive effect of ZXDC on GFI-1, it would be interesting to study how ZXDC functions in this scenario.

We found that ZXDC activated Egr-2 promoter activity as well as the mRNA expression of EGR-2 and EGR-4 (Figs. 3.11 and 3.12). Interestingly, we found a modest increase in the activation of Egr-2 promoter activity, where as, a much higher mRNA expression levels by ZXDC in HEK293 cells (Figs. 3.11 and 3.12). This could be explained by the reporter plasmid lacking important sequences necessary for regulation of the Egr-2 gene, or perhaps the chromatin structure of the plasmid not mimicking that of the endogenous gene, altering its regulation. Alternatively, it is possible that ZXDC may regulate Egr-2 at the post-transcriptional level, perhaps by stabilizing Egr-2 mRNA; however, we do not have data at present to demonstrate this. EGR-2/NAB2 was found to be necessary for the transcriptional activation of several monocyte specific genes and also for the repression of granulocyte specific genes (Friedman, 2007). A possible mechanistic role for ZXDC in maintaining granulocyte-monocyte cell lineage decisions of myeloid progenitors could be that ZXDC by activating Egr-2 promoter activity and/or expression may thereby facilitate EGR-2 activity resulting in further activation of monocyte specific genes, as well as, for the repression of genes necessary for granulocyte development. Supporting above findings, preliminary FACS results by lentiviral

mediated over-expression of ZXDC in HL-60 cells (a pro-myeloblast cell line), demonstrated an enhanced level of monocytic cell surface marker, CD14+ (data not presented), although, further experiments are necessary to confirm above results. In addition, previous studies demonstrated that GFI-1 and Egr-2/Nab2 function in a cross-antagonistic manner to maintain granulocyte-monocyte progenitor developmental decisions (Laslo et al., 2006).

It is interesting how ZXDC functions as a transcriptional activator of certain genes (e.g. MHC II, Egr-2) whereas; it repressed transcription of promoters responsive to PU.1 and GFI-1. Protein-protein interaction with co-activators or co-repressors is a potential mechanism for such dual regulatory functions. For example, Egr-1 & Egr-2 are zinc finger transcription factors that can activate or repress transcription by binding to a consensus DNA sequence (Crosby et al., 1991). It would be interesting to study how ZXDC functions by repressing both PU.1 (pro-monocyte) and GFI-1 (pro-granulocyte) activity. It could be that ZXDC functions at different stages of differentiation. It is possible that ZXDC along with primary lineage determinants (PU.1 and C/EBP $\alpha$ ) are necessary for fine tuning gene expression at different stages of myeloid differentiation, ultimately determining monocyte or granulocyte cell lineage decisions. There are several possibilities for the regulatory role of ZXDC and other members of this family in myeloid differentiation and development, however, further studies are needed to confirm the molecular mechanisms involved.

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## **CHAPTER IV**

### **DISCUSSION**

The goal of my dissertation was to demonstrate the functional role of several protein-protein interactions amongst two members of the zinc finger, X-linked (ZXD) family of proteins, namely, ZXDC and ZXDA that have important roles in the regulation of (a) major histocompatibility complex class II (MHC class II) and (b) several myeloid-specific genes.

In chapter II, we demonstrated self- and hetero-association of ZXDC and ZXDA proteins and that the zinc fingers of both proteins mediated their interaction. In addition, ZXDC was found to interact with two regulatory factor X (RFX) proteins, namely, RFX5 and RFX-ANK (RFX with ankyrin repeats), but not RFX-AP (RFX associated protein) (Figs. 2.5, 2.6, 2.7) which are components of the MHC class II enhanceosome.

The expression of Major histocompatibility complex class II (MHC class II) proteins is important for adaptive immune response (Ting and Trowsdale, 2002) and is tightly regulated at the transcriptional level (Boss and Jensen, 2003; Van den elsen et al., 2004). The transacting factors regulatory factor X (RFX) complex, cyclic AMP response



element binding protein (CREB), and nuclear factor Y (NFY) complex bind to a set of conserved cis-elements, namely, the W/S, X1, X2, Y boxes (from 5' to 3' orientation) respectively at the promoters of MHC class II genes (Ting and Trowsdale, 2002; Nekrep et al., 2003). The cis- and trans-acting factors result in the formation of a three dimensional, higher order nucleoprotein complex called the "MHC class II enhanceosome" (Masternak et al., 2000, Waldburger et al., 2000; Jabrane-Ferrat et al., 2003). However, these genes are turned "on" or "off" based on the presence or absence of the key transcriptional cofactor, the class II transactivator (CIITA; Steimle et al., 1993; Reith and Mach, 2001). Hence, many studies focused on the mechanisms of CIITA mediated transcriptional activation of MHC class II genes.

Studies in our lab identified a novel CIITA binding protein, zinc finger, X-linked, duplicated family member C (ZXDC; Al-Kandari et al., 2000a). ZXDC is so named because of its amino acid sequence homology with two other zinc finger proteins, namely, zinc finger, X-linked, duplicated family members A and B (ZXDA and ZXDB respectively) (Al-Kandari et al., 2007a). Each of the ZXDC and ZXDA proteins contain ten C<sub>2</sub>H<sub>2</sub> zinc fingers and transcription activation domain. The ZXDC protein, in addition, has a C-terminal region that is necessary to interact with CIITA (Al-Kandari et al., 2007a). Recent reports demonstrated that ZXDC activated MHC class II and MHC class I gene transcription, whereas, knock-down of ZXDC by siRNA in HEK293 cells showed significant reduction of MHC class II gene expression (Al-Kandari et al., 2007a). Although ZXDA lacked the CIITA binding region, it showed a synergistic effect in the transcriptional activation of MHC class II genes by CIITA (Al-Kandari et al., 2007b).

suggesting that interactions of ZXDA with other proteins may provide a role for such regulatory mechanism.

At the time I started in the lab, we wanted to address the mechanism of ZXDC mediated activation of MHC class II gene transcription. The ZXDC and ZXDA proteins each contained ten multiple adjacent C<sub>2</sub>H<sub>2</sub> type zinc fingers. The role of C<sub>2</sub>H<sub>2</sub> zinc fingers is in macromolecular interactions, including protein-DNA, protein-RNA and protein-protein interactions (McCarty et al., 2003; Sun et al., 1996; Hata et al., 2000; Tsai et al., 1998; Wang et al., 2001; Cassiday et al., 2002). We were able to demonstrate self- and hetero-association of ZXDC and ZXDA proteins and that the zinc fingers of both proteins mediate their interaction. We were not able to identify which zinc fingers of ZXDC and ZXDA mediate these protein-protein interactions, as deletions of some zinc fingers resulted in destabilization of the protein (data not presented). Interestingly, in vitro protein-protein interaction assays demonstrated that only a hetero-complex of ZXDC and ZXDA but neither protein by itself was sufficient to interact with CIITA (Al-Kandari et al., 2007b). Supporting this, knock-down of ZXDC or ZXDA resulted in significant repression of MHC class II gene expression by CIITA. This decrease was not significantly different from knock-down of both ZXDC and ZXDA suggesting for the requirement of both proteins for MHC class II gene regulation (Al-kandari et al., 2007b). The ability of ZXDC and ZXDA proteins to associate with themselves as well as with each other gives rise to complexes with different combinations of functionally active protein-protein (ZXDC-ZXDA) or protein-DNA interactions. For the latter, it is very important that the nucleotide sequences to which ZXDC binds be identified.

ZXDC was found to be present at the MHC class II promoter in Raji cells, a B cell line that constitutively expresses MHC class II proteins and in HeLa cells prior to and after IFN $\gamma$  treatment (Al-Kandari et al., 2007a, 2007b). Given the important role of C<sub>2</sub>H<sub>2</sub> type zinc fingers in DNA binding, we tried extensively to demonstrate direct binding of ZXDC to MHC class II promoter region, especially the W box, but we could not find. Dr. Srikarthika Jambunathan in our lab identified that ZXDC preferentially binds to a purine rich sequence, AGGGT/A using an in vitro screening assay. However, further studies are necessary to investigate the aspects of ZXDC-DNA interaction.

Although nucleic acid binding by zinc fingers was the primary focus for many years, more recently the importance of protein-protein interactions mediated by zinc fingers has come to the fore (Gamsjaeger et al., 2007; Brayer et al., 2008a, 2008b). Since chromatin immunoprecipitation experiments demonstrated that ZXDC is present at MHC class II promoters, the most likely interpretation is that ZXDC is recruited to the MHC class II promoters indirectly, via protein-protein interactions. Our data support this notion. We were able to demonstrate that ZXDC selectively interacted with components of MHC class II enhanceosome, specifically, CIITA (Al kandari et al., 2007a), RFX5 (Fig. 2.5), RFXANK (Fig. 2.6) and possibly other components of the MHC class II enhanceosome. The results presented here along with previous findings suggest a possible role for ZXDC-ZXDA complex in stabilizing MHC II enhanceosome complex suggesting a mechanism of MHC class II gene activation.

The interaction of ZXDC with the components of the MHC class II enhanceosome could be a possible mechanism for the recruitment of ZXDC along with ZXDA at the MHC class II promoter. ZXDA, however, was not found to interact with

RFX5 and neither was ZXDC protein capable of associating with RFX-AP suggesting specificity for these protein-protein interactions. Mechanistically, an unanswered question is whether ZXDC is recruited to the promoter as a hetero-dimer along with ZXDA or if ZXDA is recruited later during gene activation. Similarly, if further studies could demonstrate the interaction of ZXDC with one or more other components of the MHC class II enhanceosome, namely, CREB or proteins constituting the NFY complex, it would strengthen our model of ZXDC stabilizing the MHC II enhanceosome. The assembly of transcription factors requires precise positioning of the DNA binding sites to form the nucleoprotein complex, enhanceosome. Even in the case of MHC class II enhanceosome, exact spacing and orientation of the conserved W/S, X1, X2 and Y promoter elements is critical for the recruitment and binding of the transacting factors (RFX complex, CREB, NFY complex) and the entire nucleoprotein complex functions as a single unit to regulate MHC class II gene transcription (Jabrane-Ferrat et al., 1996, 2002; Nekrep et al., 2000; Hake et al., 2000; Masternak et al., 2000; Zhu et al., 2000). There are several mechanisms of enhanceosome dependent transcriptional synergy. The individual protein-protein or protein-DNA interactions may be weak, but multiple cooperative interactions result in the formation of a powerful transcriptional activation machine as a result of enhanced stability of the complex (Merika and Thanos, 2001). It is also possible that the three-dimensional structure of the enhanceosome creates optimal interaction surface of the activation domains of CIITA and ZXDC proteins to interact with the general transcription machinery or chromatin remodeling factors for gene activation. An example of such a mechanism is found at  $\alpha$ 1-AT promoter between HNF-1 $\alpha$  and HNF-4 $\alpha$  which recruits TFIID and CBP/PCAF, hBrm respectively (Cosma et al.,

2002). Some other examples of eukaryotic genes regulated by enhanceosomes include interferon- $\beta$  gene, interleukin-6 gene, interleukin-2 receptor  $\alpha$  chain gene (reviewed in Panne, 2008; Carey, 1998; Merika and Thanos, 2001).

The mechanism of ZXDC-ZXDA mediated activation of MHC II genes is not very clear at present. A probable mechanistic role for this hetero-complex in MHC II gene activation is through interaction with CIITA, RFX5 and RFXANK resulting in stabilizing the MHC II enhanceosome. Further studies demonstrating protein-protein interactions of ZXDC or ZXDA with any other components of the MHC II enhanceosome, namely, CREB protein, components of the NFY complex or chromatin remodeling factors will further strengthen this model. However, further study is necessary to know the exact regulatory mechanism by which the ZXDC-ZXDA complex activates MHC class II gene transcription.

In chapter III, we found that ZXDC interacted with two hematopoietic transcription factors, namely, purine rich box-1 (PU.1) protein and growth factor independent-1 (GFI-1). This interaction was found to have a functional role as ZXDC repressed the transcriptional activities of PU.1 and GFI-1 proteins in reporter assays. Chromatin immunoprecipitation experiments demonstrated that ZXDC is present along with GFI-1 at the *Myc* locus, a known target of GFI-1. Our studies also revealed that ZXDC activated the transcription of *Egr-2* gene whose expression is related with the activation of monocytic target genes and repression of granulocytic target genes. We also found that ZXDC enhanced PU.1 protein expression, whose levels are important for determining hematopoietic cell lineage decisions. Our studies probably demonstrate that ZXDC may affect the transcriptional activities and/or recruitment of PU.1 and GFI-1 at

their target gene promoters especially following differentiation of myeloid progenitors. In addition, it is also possible that ZXDC by activating Egr-2 transcription and expression while repressing GFI-1 transcriptional activity may have a role in fine tuning differentiation of myeloid progenitors into monocytic lineage. However, further study is necessary to confirm the regulatory role of ZXDC in monopoiesis.

The interplay and cross-talk among several ubiquitous and cell-type specific transcription factors and their relative expression levels are important to regulate target genes necessary for cell fate and lineage decisions (Friedman, 2007; Rosenbauer and Tenen, 2007; Laslo et al., 2006; Orkin., 2000). Our results demonstrated interaction of ZXDC and ZXDA proteins with two hematopoietic transcription factors, namely, purine box binding protein, PU.1 (Fig. 3.2) and growth factor independent-1 (GFI-1; Fig. 3.7). Purine-rich box-1 (PU.1) protein belongs to the Ets family of transcription factors and is encoded by the spleen focus forming virus proviral integration site-1 (*sfpi1*) gene (McKercher et al., 1996; Dahl et al., 2003; Laslo et al., 2006; Huang et al., 2007a; Yang et al., 2000; Reddy et al., 2002; Rekhtman et al., 1999, 2003; DeKoter and Singh, 2000). PU.1 plays a central role in hematopoiesis as it is necessary for myeloid differentiation, B and T cell development, hematopoietic stem cell maintenance and erythropoiesis (Kastner & Chan, 2008). GFI-1 is a zinc finger transcriptional repressor that is indispensable for neutrophil differentiation and development (Hock et al., 2003). It contains an N-terminal SNAG repression domain and six C-terminal zinc fingers necessary for DNA binding (Grimes et al., 1996; Zweidler-Mckay et al., 1996).

Our studies revealed that ZXDC and ZXDA proteins interacted with the hematopoietic transcription factor PU.1 (Fig. 3.2). The interaction of ZXDC or ZXDA

proteins with PU.1 resulted in repression of PU.1 transcriptional activation by about 75% and 50% respectively (Fig. 3.5). The zinc finger domain of ZXDC was found to be necessary to mediate interaction with the N-terminal region of PU.1 containing the activation domain (Figs. 3.3 & 3.4). There are several examples of transcription factors that interact with distinct domains of the PU.1 protein. Some of these examples include GATA binding protein-1 (GATA-1), CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ), growth factor independent-1 (GFI-1), B-cell specific activator protein (BSAP) (Nerlov et al., 2000; Zhang et al., 1999; Reddy et al., 2002; Maitra and Atchison, 2000; Dahl et al., 2007; Ueki et al., 2008). The interaction of PU.1 with these transcription factors at different stages of hematopoietic differentiation was found to be critical for cell fate/lineage determination and development. It is important to note that each of the transcription factors mentioned above interacts with and represses PU.1 transcriptional activation at specific stages during differentiation. Such interplay among transcription factors often has several regulatory roles. For example, GATA (GATA-1 & GATA-2) and PU.1 proteins function in a cross-antagonistic manner for the determination of erythroid or myeloid cell lineages respectively (Zhang et al., 1999). Given the critical role for interacting partners of PU.1, it is important to address the mechanism and the functional significance of the ability of ZXDC and ZXDA proteins to repress PU.1 transactivation during myeloid differentiation. The ability of both ZXDC and ZXDA proteins to interact with and repress PU.1 transactivation ability lead us to asking further questions: Do they function cooperatively as a homo- or hetero-complex similar to what we saw with interaction with the cofactor CIITA (Al-Kandari et al., 2007b) or if they can compensate for each other functionally. Although, ZXDC and ZXDA proteins share

significant amino acid sequence homology, there are regions that are very distinct for each of them (Al-Kandari et al., 2007b) and hence, it is important to address this.

We have found that ZXDC stabilized PU.1 protein expression levels (Fig. 3.6). This is an important observation since PU.1 protein expression levels are correlated to specifying hematopoietic cell lineage decisions and that deregulation resulting in even slight decrease in its expression levels can result in leukemia (Moreau-Gachelin et al., 1988; DeKoter et al., 2000; Anderson et al., 2002; Dahl et al., 2003; Cook et al., 2004; Rosenbauer et al., 2004, 2006; Huang et al., 2008). At the transcriptional level, the expression of PU.1 is regulated by a proximal promoter (Chen et al., 1995) and an upstream regulatory element located 14 kilo bases upstream of the transcription start site (Li et al., 2001; Rosenbauer et al., 2004; Okuno et al., 2005). At the post-transcriptional level, PU.1 expression is regulated by sense and anti-sense RNAs driven by intronic promoters (Ebraldize et al., 2008). In our studies, we found that over-expression of ZXDC resulted in stabilizing PU.1 protein expression. We found less than two-fold increase in PU.1 mRNA levels upon ZXDC over-expression (data not presented). At present, it is not very clear the mechanism of ZXDC mediated PU.1 protein stabilization. It is possible that ZXDC has such a role by inhibiting PU.1 degradation by the proteasome mediated pathway. However, further study is necessary to address this.

Growth factor independent-1 is a zinc finger transcriptional repressor that is necessary for granulocyte differentiation. GFI-1 inhibits monocytic genes in granulocytes by direct interaction with PU.1 and inhibiting its transcriptional activation. Studies by others also identified ZXDC as one of Growth factor independent-1 interacting partners by yeast-two-hybrid assay (Duan et al., 2007). Our results confirmed



ZXDC-GFI-1 interaction and found that ZXDC repressed GFI-1 transcriptional activity in a dose-dependent manner. At present, we do not know the mechanism of ZXDC mediated repression of GFI-1 transcriptional activity. Since ZXDC was first identified as an interacting partner of GFI-1 in a yeast-two hybrid screen using the C-terminal DNA binding zinc fingers of GFI-1 as a bait, it is possible that ZXDC may sequester GFI-1 by binding to its C-terminal zinc fingers thereby preventing GFI-1 DNA binding. However, chromatin immunoprecipitation experiments in HEK293 cells revealed that ZXDC is present along with GFI-1 at the *Myc* locus, a known target of GFI-1. However, further study is necessary to address if ZXDC affects the transcriptional activity or recruitment of GFI-1 at its target gene promoters during differentiation of myeloid progenitors. Importantly, GFI-1 was found to cooperate with an Ets family transcription factor, ETS1 in the repression of *Bax* which is a pro-apoptotic gene (Nakazawa et al., 2007). Since ZXDC was found to repress GFI-1 transcriptional activity and since both proteins are present at the promoter of *Myc*, which is involved in cell cycle regulation, it would be interesting to find if ZXDC affects apoptosis. It is also important to address if the interaction of ZXDA with GFI-1 is functionally significant.

Recent studies highlight an important role for microRNAs in hematopoiesis (reviewed in Garzon and Croce, 2008). GFI-1 was found to play an important role in the regulated expression of microRNAs, miR-21 and miR-196b that is necessary for normal hematopoiesis (Chinavenmeni et al., 2009). In the same study it was revealed that a mutation in the GFI1 gene described in a patient with severe congenital neutropenic patient and *Gfi*<sup>-/-</sup> mice showed deregulated expression of miR-21 and miR-196b. Based

on the results presented in chapter III and in the paragraph above, it is important to find if ZXDC regulates any of GFI-1 target micro-RNAs.

Microarray analysis identified Egr-2 and Egr-4 as candidate genes that are upregulated upon ZXDC over expression in HEK293 cells (Galkin, O., Fontes, J.D., unpublished observation). Further studies confirmed that ZXDC activates the transcription and expression of Egr-2 gene (Figs. 3.11 and 3.12). To confirm direct regulation of Egr-2 gene by ZXDC, it is important to find the effect of ZXDC knock-down on Egr-2 expression. We expect to see a decrease in the promoter activity and expression of Egr-2 following knock-down of endogenous ZXDC. Alternatively, it is also possible that the ZXDA protein can compensate in which case we may not see a significant decrease in Egr-2 expression levels.

A complex of EGR-2 and the transcriptional co-regulator NAB2 was found to be necessary for the transcriptional activation of several monocyte specific genes and also for the repression of granulocyte specific genes (Laslo et al., 2006). A possible mechanism of ZXDC in granulocyte-monocyte cell lineage decision could be by activation of Egr-2 expression, resulting in transcription of monocyte-specific genes, and repression of granulocyte-specific genes. Supporting this model, preliminary data for over-expression of ZXDC in HL-60 cells (a pro-myeloblast cell line), demonstrated an enhanced level of monocytic cell surface marker (CD14) expression (data not presented). Further experiments are necessary to confirm these preliminary data.

In addition, previous studies demonstrated that GFI-1 and Egr-2/Nab2 function in a cross-antagonistic manner to maintain granulocyte-monocyte progenitor developmental

decisions (Laslo et al., 2006). Although, GFI-1 was found to regulate its target genes by direct DNA binding, a recent report demonstrated that GFI-1 is recruited through Miz-1 to repress CDKN2B gene that encodes the cyclin dependent kinase inhibitor (CDKI), P15<sup>INK4B</sup> (Basu et al., 2009). Interestingly, CDKN2B was found to be a gene that was activated 3.5X when ZXDC was over-expressed in HEK293 cells as demonstrated by microarray analysis (Galkin, O and Fontes, J.D., data not presented). Potentially, a similar mechanism exists for GFI-1 repressing ZXDC activation of Egr-2.

Taken together, our studies demonstrate a dual regulatory role for ZXDC. We found that ZXDC functions in the transcriptional activation of MHC II and Egr-2 genes, whereas, functions in the transcriptional repression of promoters responsive to PU.1 and GFI-1. There are many examples of transcription factors that have such dual regulatory roles. For example, Egr-1 & Egr-2 are transcription factors whose expression is associated with the activation of monocyte target genes and repression of granulocyte target genes thus promoting monocyte development and differentiation. Additional examples of such dichotomous transcriptional regulators include WT1 which can be converted from an activator to a repressor by interaction with a soluble nuclear factor, MDM2 which functions as a co repressor of p53 function, but as a coactivator for the function of E2F, the nuclear hormone receptors that are converted from transcriptional repressors to activators upon interaction with ligand (Holstege et al., 1998; Wang et al., 1995; McKay et al., 1999; Martin et al., 1995). Studies have demonstrated an important role for GFI-1 (Hock et al., 2004; Zeng et al., 2004; reviewed in Duan and Horwitz, 2005) and PU.1 (reviewed in Kastner and Chan, 2008) in hematopoietic stem cell

maintenance and hence it is important to address if ZXDC has a functional role in this scenario.

Our studies probably demonstrate that ZXDC by activating Egr-2 gene transcription and expression, while simultaneously repressing GFI-1 transcriptional activity may favor myeloid progenitors to differentiate along the monocytic lineage, while simultaneously repressing granulocyte target genes. It is also possible that ZXDC has a functional role in fine tuning differentiation of myeloid progenitors by interacting with the primary cell fate determinants, PU.1 and C/EBP $\alpha$  at different stages of hematopoietic differentiation. In this regard it is important to check if ZXDC interacts with C/EBP $\alpha$  for fine tuning differentiation of myeloid progenitors into macrophage or granulocyte lineages (reviewed in Rosenbauer and Tenen, 2007). Further study is necessary to confirm the regulatory roles of ZXDC and other members of the ZXD family proteins in hematopoiesis.

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