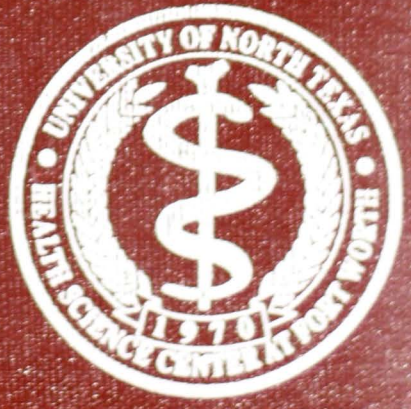
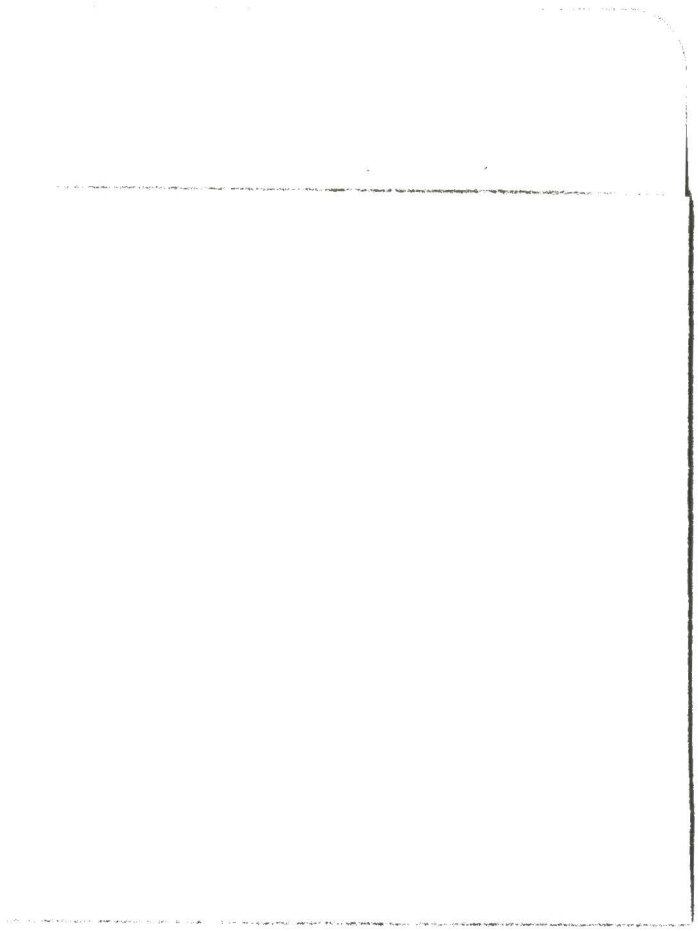

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970



Leslie Don Roberts, **YY1 Mediated Competitive Regulation: A governing principle behind phenotype-specific gene expression in vascular smooth muscle cells.**

Doctor of Philosophy (Biomedical Science), July 2005

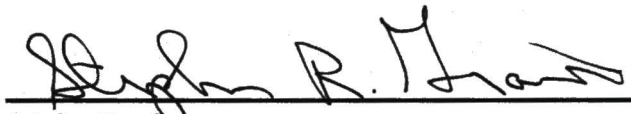
The vascular smooth muscle cell (VSMC) lacks the typical phenotypic restriction that limit most cell-types to expressing a single phenotype, as a result, these cells are uniquely suited to wound repair, as well as, exacerbating several vascular disease-states. While much is known regarding the specific transcription factors that drive phenotype-specific gene expression the mechanisms that regulate the transition between phenotype-specific gene programs remain poorly defined. To further explore these mechanisms, we sought to better understand how VSMCs stably express their default contractile-specific gene program despite the inherent instabilities of their environment. This study explored the regulatory implications of a yet undescribed regulatory domain, that resides with a high-frequency in the promoters of most contractile-specific gene. These domains, which we term dual regulatory domains (DRD), orient the core binding site for the transcriptional repressor Ying Yang-1 in close proximity to, or overlapping with, the core binding site for a variety of transcriptional activators. This study specifically examines the regulatory implications at two DRD where YY1 competes with the transcriptional activators C/EBPβ (C/CAAT-enhancer binding protein beta). Our findings demonstrate: i.) YY1 acts as a dominant, negative, regulator of the smooth muscle myosin heavy chain (SM-MHC) gene promoter; ii.) YY1 binds to, and repressing from, multiple sites within the regulatory context of this promoter; and iii.) The transactivation potential of C/EBPβ competes with transrepressive potential of YY1 for regulatory control over SM-MHC

promoter activity and does so in a stoichiometric-dependent fashion. These findings argue that the relative concentrations of YY1 define the effective dose required of specific transcriptional activators to compete with and override the repressive effect of YY1, and by doing so, directly dictate which genes will be expressed.

YY1 MEDIATED COMPETITIVE REGULATION: A GOVERNING PRINCIPLE BEHIND PHENOTYPE-SPECIFIC GENE EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS

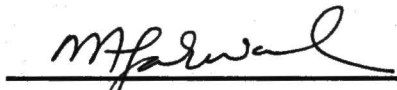
Leslie Don Roberts, B.S.

APPROVED:

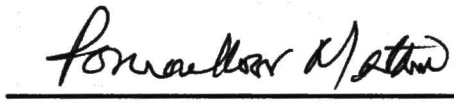


Major Professor

Committee Member

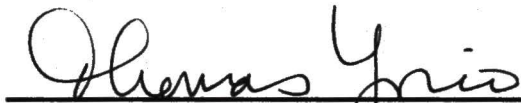


Committee Member

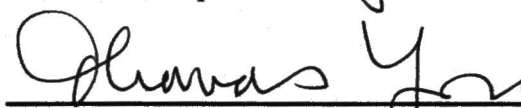


Committee Member

University Member



Chairman, Department of Biomedical Science



Dean, Graduate School of Biomedical Science

YY1 MEDIATED COMPETITIVE REGULATION: A GOVERNING PRINCIPLE BEHIND PHENOTYPE-SPECIFIC GENE EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS

DISSERTATION

Presented to the Graduate Council of the
Graduate School of Biomedical Science

University of North Texas Health Science
Center at Fort Worth

For the Degree of

DOCTOR OF PHILOSOPHY

By

Leslie Don Roberts, B.S.

Fort Worth, Texas

April 2005

ACKNOWLEDGMENTS

I especially thank Dr. Sheryl White for providing the Smooth Muscle Myosin Heavy Chain promoter reporter constructs and Genomatix, Inc. for providing full, temporary, access to its database and software to analyze the numerous promoter sequences required during this study. Without these critical contributions this research would not have been possible. Also, I would like to sincerely thank the members of my doctoral advisory committee, including Dr. Glenn Dillion, Dr. Peter Raven, Dr. Neeraj Agarwal, and Dr. Porunelloor Mathew for their assistance and guidance in the many facets of my doctoral training. I am particularly grateful to Dr. Peter Raven and Dr. Glenn Dillon for their assistance in the preparation of this document. I would also like to thank Dr. Tom Yorio and Dr. Victoria Rudick for their dedication to supporting myself and other students in the department of Biomedical Science. I must extend my greatest gratitude to my major professor Dr. Stephen Grant who generously contributed both extensive time and funding to the successful completion of my research. I am equally grateful to each member of Dr. Grant's laboratory both past and present. In addition, I thank my mother the late Elaine Roberts, I miss her dearly. I also would like to thank my Father Bob Roberts and sisters Kim and Regina Roberts for their continual support in all my endeavors. I deeply appreciate all your efforts.

Original Articles

Roberts LD and Grant SR; *YY1 MEDIATED COMPETITIVE REGULATION: A governing principle behind phenotype-specific gene regulation in vascular smooth muscle cells.* (Manuscript in progress)

Ellis JJ, Valencia TG, Zeng H, Roberts LD, Deaton RA, Grant SR.; *CaM kinase II&C phosphorylation of 14-3-3beta in vascular smooth muscle cells: activation of class II HDAC repression.*: Molecular and Cellular Biochemistry. 2003 Jan;242(1-2):153-61.

Valencia TG, Roberts LD, Zeng H, Grant SR.; *Tetracycline-inducible CaM kinase II silences hypertrophy-sensitive gene expression in rat neonate cardiomyocytes.*: Biochemical Biophysics Research Communications. 2000 Aug 11;274(3):803-10.

Published Abstracts

*2004 FASAB: Transcriptional Regulation During Cell Growth, Differentiation and Development; Cold Spring Harbor, NY.: R*egulating Smooth Muscle Myosin Heavy Chain Promoter Activity Involves Competitive Regulation Via Ying Yang-1 ; Roberts, L. D. and Grant, S. R. - Poster

2003 Systems Biology: Genomic Approaches to Transcriptional Regulation; Cold Spring Harbor, NY.: Ying Yang-1 Defines the Activation Threshold for Smooth Muscle Myosin Heavy Chain Promoter Activation; Roberts, L. D. and Grant, S. R. - Poster

2002 American Heart Association; Keystone, CO.: Competition for the Regulation of the Smooth Muscle Myosin Heavy Chain Promoter: C/EBPβ-mediated activation vs. Ying Yang 1 (YY1)-mediated repression; Roberts, L. D. and Grant, S. R. - Poster

2000 Southwestern Society for Experimental Biology and Medicine; New Orleans, LA.: Ca2+/CaM-Dependent Protein Kinase IIα Negatively Regulates the Induction of Hypertrophic Responses initiated by Ca2+ Dependent Protein Phosphatases and Kinases; Roberts, L. D., Valencia, T. V. and Grant, S. R. - Oral Presentation

1999 American Heart Association Basic Cardiovascular Science; Snowbird, UT.: Tetracycline-Inducible CAM Kinase II Silences Hypertrophy Sensitive Gene Expression; Valencia, T. V., Roberts, L. D., Zeng, H. and Grant, S. R.. - Poster

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

Term	-	Definition
VSMC		Vascular Smooth Muscle Cell
YY1		Ying Yang 1
SRF		Serum Response Factor
SM-MHC		Smooth Muscle Myosin Heavy Chain
DRD		Dual Regulatory Domain
GATA		A transcription factor named for its DNA consensus binding motif
C/EBPβ		C/CAAT enhancer binding protein beta
NFAT		Nuclear factor of activated T-cells
MEF		Myocyte enhancer factor
AP-1 / 2		Activator protein type-1 / type –2
CTF		CAAT-binding transcription factor
SM γ-Actin		Smooth Muscle gamma Actin
CArG box		denotes the nucleotide composition of the SRF binding site
SM22		a smooth muscle specific protein
IL-1β		Interlukin 1 beta
NFκB		Nuclear Factor kappa B
$cPLA_2$		Cytoplasmic Phospholipase type-2
iNOS		Inducible Nitrousoxide Synthase

COX-2	Cyclooxygenase type-2
CAAT	The binding site for CTF
NF-1	Nuclear Factor 1
TEF-1	Transcriptional Enhancing Factor –1
ERK	Extracellular Signal Regulated Kinase
MCP	Monocyte Chemoattractant Protein
p38MAPK	p38 Map Kinase
FGF	Fibroblast Growth Factor
TGFβ	Transforming Growth Factor beta
BMP	Bone Morpogenic protein
Smad	Similar to mothers against decapentaplegic
Ets	Factor family with homology to the Ets-protooncogenes
Rb	Retinoblastoma Protein
SP-1	simian-virus-40-protein-1
Oct-1	Octamer binding factor
Nkx	homobox/homeodomain protein
Phox/Mhox	Paired like homobox
PAC-1	Rat Pulmonary Artery cells
TATAA box	A nucleotide composition that represents the classical transcriptional start site

CHAPTER I

INTRODUCTION

The purpose of this chapter is to introduce what is currently understood regarding vascular smooth muscle cell (VSMC) phenotype maintenance and transitional control. This chapter provides a brief overview of: i.) VSMC phenotypic switching as it relates to wound healing and disease; ii.) mechanisms of VSMC transcriptional regulation; and iii.) how phenotype-specific promoters function. To conclude this chapter will propose a novel mechanism that potentially regulates VSMC phenotype specific gene program and propose experimentation to test this.

VSMC Phenotypic Switching, Wound Healing and Disease

The etiology of chronic hypertension aligns sustained intravascular pressure with VSMC hypertrophy (73, 15, 41). This stimulus drives the overall increase in contractile-specific gene expression via the re-expression of fetal contractile protein isoforms (11, 4). This increase in protein in turn increases the size of the VSMC, and is generally recognized as the classical morphological feature of VSMC hypertrophy (64). VSMC hypertrophy reduces overall vascular plasticity and impedes adequate adjustments of intravascular diameter in response to elevated vascular pressure, thereby, exacerbating an already hypertensive state. In contrast, the etiology of atherosclerosis aligns the phenotypic transition of VSMCs to a proliferative phenotype induced by inflammatory

cytokines (19, 17). Typically, the inflammatory response can be invoked by vascular wall damage or intravascular plaque formation (32). The process of plaque formation evokes an extended immunological response involving the release of many cytokines from the vascular invasion of macrophages, which stimulate VSMCs to migrate to the site of injury (99). This prolonged event results in the excessive accumulation of VSMCs that thickens the vessel wall causing a narrowing of the vessel lumen over time. A similar immunological response is also induced during the more punctuated processes of wound repair (53). In general, this response is favorable when restoring vascular wall thickness after crushing insults that cause extensive cell death which causes vascular wall thinning. Another aberration in smooth muscle phenotype regulation limits the effectiveness of the most meaningful treatment of atherosclerosis, i.e. balloon angioplasty. The de-endothelialization of the intravascular wall, resulting from mechanical insult incurred during the angioplasty process, exposes the VSMCs to the increased mitogen concentrations of the circulating serum. The increased serum mitogen concentration induces VSMC reentry into cell-cycle which results in hyperplastic expansion of VSMC into the vessel lumen (24, 33). As a result 25-40 percent of patients that undergo angioplasty eventually face re-occlusion of the cleared vessel due to the hyperplastic expansion of VSMCs (American Heart Association 1998). Hence, the mechanical insult of balloon angioplasty evokes a typically beneficial wound repair response, which, if left unchecked re-occludes, the vessel lumen. Therefore, the ability of the VSMC to modulate its phenotype can be detrimental, if triggered at an inopportune time.

Mechanisms of VSMC Transcriptional Regulation

The VSCM is unable to derive phenotype stability from the typical means of terminal differentiation. Therefore, it is reasonable to hypothesize that VSMC must derive its phenotypic stability from the precision in which it expresses each of its phenotype-specific gene programs. Phenotypic-specific genes generally exhibit coupled expression in response to similar stimuli and are collectively referenced as members of the same gene program (10, 63). Translating the effects of the various phenotype-specific stimuli are specific groups of transcription factors, whose collective action transmits the precision required to express genes in a phenotype-specific fashion (91, 57, 85). To establish a particular phenotype, the VSMC biases the nuclear representation of key phenotype-specific transcription factors so that the expression of the targeted gene program is overwhelmingly favored. Likewise, when transitioning between the phenotypes the VSMC tips this nuclear regulatory equilibrium in favor of other factors which target the expression of an alternative gene program (85).

Significantly, the regulatory nature of the VSMC nuclear environment is not pristine. For example, the VSMC nucleus retains many more transcription factors, many with opposing phenotype-specificity, than are required for a given phenotype (85). However, these factors are sufficiently underrepresented in the nucleus that their regulatory impact is considered negligible. Yet, their collective presence does constitute a background of signaling static that is potentially disruptive to the nominal expression of the resident gene program. Nevertheless, current understanding suggests that all factors resident in the VSMC nucleus are balanced such that gene expression is guided by the predominant regulatory tendency of this equilibrium.

Theoretically, if this nuclear equilibrium was purely stoichiometric in nature, a fluctuation in the concentration of any transcription factor would alter the regulatory dynamics and thus gene expression profiles, throughout the nucleus. Phenotype-specific gene expression is, however, highly stable despite typical fluctuations in background concentrations of transcription factors that occur as a VSMC adapts to the ebb and flow of daily stimuli. Additionally, very little overlap in phenotype-specific gene expression is witnessed when VSMC transition between phenotypes (80). This suggests that VSMC gene expression is governed by failsafe mechanism(s) that prevent the simultaneous expression of multiple gene programs and buffers the nuclear environment to "normal" fluctuations in background stimuli. These principles reflect those that govern receptor/ligand interactions and argue the regulatory impact of any given transcription factor may be restrained by similar (concentration based) thresholds (49,50). If so, this poses many new and intriguing insights that better explain the existing confusion inherent to the current understanding of transcriptional regulatory dynamics.

Promoter Control

Monitoring and responding to changes in the nuclear regulatory equilibrium is the principle function of a gene's promoter. A gene's promoter is best envisioned as a flexible docking platform that recruits critical transcription factors that, in turn, direct the transcription of a downstream gene. Encoded within the regulatory context of a promoter are two major regions of significance: i.) the basal promoter, that includes the TATAA box; and ii.) various enhancer cassettes, typically many kilobases upstream of the TATAA box. Regulation of the basal promoter and the TATAA box has been well

described (34). Enhancer cassettes, however, have proven much more enigmatic. Enhancer cassettes typically bind phenotype-specific transcriptional enhancers to convey phenotype-specificity to the expression of a downstream gene (101). However, the defining principles of these regions have yet to be clearly elucidated, Thereby, making identification of these regions particularly difficult. Furthermore, phenotype-specificity is not overtly obvious within the regulatory context of typical phenotype-specific promoters. For example, the regulatory context of most promoters consist of highly repetitious and widely diverse transcription factor binding sites, most of which are irrelevant to the proper expression of the downstream gene. It is inescapable, however, that phenotype-specific promoters do selectively express their downstream gene in a highly specific fashion reflecting a higher-order of regulation. Nevertheless, it appears that genes within the same gene program retain equivalent enhancer cassettes (101). This allows the cell to synchronize the regulation of many genes with common stimuli and appears to be the governing principle behind phenotype-specific gene expression.

The Potential Function of the Dual Regulatory Domain

Proper phenotype-specific gene expression is regimented by the association of multiple phenotype-specific transcription factors with explicit recognition binding motifs resident within a gene's promoter. The analysis of five contractile-specific gene promoters identified that each contained numerous binding sites for both contractile and proliferation-specific transcription factors. (Preliminary data) Therefore, denoting the ability of a transcription factor to bind a promoter by the mere existence of it's consensus recognition motif can not explain the precision and stability of gene expression witnessed

"*in vivo*". Interestingly, numerous domains were identified where the binding sites for YY1, a transcriptional repressor, fell in close proximity to or overlapped binding sites for the various phenotypic-specific transcriptional transactivators. (Preliminary data) Importantly, the regulatory topography of these promoter domains provide a potential dual-regulatory platform, whereby many individual transcriptional transactivators would have to compete with YY1 for promoter occupancy. Therefore, we suggest that the relative concentration of YY1 would mandate the minimal effective concentration required for the competitive transcriptional transactivators.

Briefly, YY1 is known to be strictly regulated in a phenotypic specific fashion. Likewise, while the regulatory significance has been elusive, many transcriptional activators are known to compete with YY1 (79, 60, 100, 52, 51, 96,67). Finally, a regulatory role for YY1 has been documented in each of the main phenotypes of the VSMC. (61, 72, 95, 87, 28, 77, 38).

Statement of the Problem

The inability of VSMC to terminally differentiate predisposes the VSMC to unique disease states. Simply stated, there is no clear demarcation that separates the regulatory mechanism that controls VSMC phenotypic switching events as it pertains to wound healing and those that evolve to a disease state. Thus, new therapeutic strategies that capitalize upon stabilizing the VSMC contractile phenotype and promote VSMC resistance to phenotypic alteration will offer the most beneficial therapeutic value. This study proposes to investigate those transcriptional regulatory mechanisms which communicate precision and stability to phenotype-specific gene expression.

Objectives and Delimitations of the Investigation

While studying the regulatory effect of YY1 as it pertains to the regulation of multiple promoters specific to contractile, proliferative and mitotic phenotypes would be valuable it is experimentally impractical within a reasonable (2-year) time period. Therefore, we propose to focus on the smooth muscle myosin heavy chain (SM-MHC) gene promoter. The SM-MHC gene is a prototypic contractile-specific gene in the VSMC. It has been established that –4200 base pairs (bp) of the proximal promoter region plus the first intron is required for the promoter spatiotemporal expression of this gene *in vivo* (44). However, due to the regulatory complexity associated with the length of this promoter fragment, the present investigation will be limited to the first -1621bp of the proximal promoter. Significantly, three CArG-boxes determined to be involved in the proper, *in vivo*, expression of the gene are located within the first -1621bp of this promoter (44). Thereby, conferring regulatory significance to the specific length of the promoter. Within the published -2520bp of the SM-MHC proximal promoter are 11 potential DRDs (Preliminary data). Most of these DRDs are unique with regards to the transcriptional activators that potentially compete with YY1. Likewise, the quantity of work required to investigate each of the DRDs tandem to each transactivator is impractical for the timeframe of this investigation. There are, however, two similar DRDs where YY1 is positioned to compete with the transcriptional transactivator C/EBPβ located at –1563bp and –748bp proximal to the start of transcription. (Preliminary data)

C/EBPβ embodies the ideal transactivator for testing competition with YY1. C/EBPβ has dual functionality in both the contractile and proliferative-specific gene expression (56). Also, C/EBPβ is known to induce SM-MHC promoter activity during the contractile phenotype and during the proliferative phenotype C/EBPβ drives the expression of proliferative-specific genes. Thus, C/EBPβ must some how redirect its regulatory influence between the two phenotypes. Therefore, we intend to identify the mechanism that allows this factor to switch between targeting contractile-specific gene regulation and proliferative-specific gene regulation.

Based upon our model, we contend that elevating YY1 concentrations in VSMC, as is the case during VSMC proliferation (76), will impede the ability of C/EBPβ to induce SM-MHC promoter activation, presumably as a result of YY1 dominant competition. Likewise, elevating the concentration of C/EBPβ above YY1's, as is the case during the contractile phenotype (76), we contend will restore the ability of C/EBPβ to induce the SM-MHC promoter, presumably by restoring the stoichiometry, and thus, the competitive equilibrium between YY1 and C/EBPβ.

We will employ the a series of SM-MHC promoter truncations to explore these two DRDs, these being: i.) –1621bp SM-MHC fragment which retain both the –1563 and –748 DRDs; and ii.) –1249bp fragment which retains only the –748bp DRD; iii.) –602bp fragment that has neither DRDs (preliminary data). We anticipate that the ability to quantify a competitive response from each SM-MHC promoter fragment that will vary depending upon the quantity of DRDs each retains. Likewise, we expect that competition will not be quantifiable in the –602bp SM-MHC promoter fragment that is devoid of DRD. To fully explore the proposed model of competitive regulation in its full context

requires extensive time and resources thus, we have limited the scope of this investigation to three specific aims:

Specific Aim #1: Explore, with three SM-MHC promoter truncations, the extent of YY1 mediated SM-MHC promoter control.

Specific Aim #2: Determine, with three SM-MHC promoter truncations, the ability of YY1 to compete with C/EBPβ at two DRDs namely the –1563bp DRD (YY1:C/EBPβ) and –748bp DRD (YY1:C/EBPβ).

Specific Aim #3: Demonstrate via mutational alteration of the YY1 core consensus sequence binding motif embedded in each DRD, that YY1 binding to these domains is indispensable to it's ability to compete with C/EBPβ.

Hypotheses

We hypothesize that: i.) YY1 acts as a dominant, negative, regulator of SM-MHC; ii.) YY1 binds to and represses at multiple sites within the promoter and these events establish a repressive threshold that governs SM-MHC promoter activity; and iii.) transcriptional activators, whose binding sites lay in close proximity to or overlap with those of YY1, competitively displace YY1 from the DRDs, if sufficiently abundant.

CHAPTER II

REVIEW OF THE LITERATURE

The intent of this chapter is to review the current principles that facilitate VSMC phenotypic maintenance and transitional control. This section will specifically discuss:

i.) The function of several phenotype-specific transcription factors;

ii.) The function of YY1 in phenotype control and gene expression;

iii.) The basics of competitive regulation.

Serum Response Factor (SRF)

Upon the onset of differentiation, many genes, primarily driven by the trans-activator SRF binding its DNA binding sequence, CArG, become up-regulated (69, 92, 54, 42, 8, 81). Briefly, the CArG box represents the SRF consensus binding motif and the majority of smooth muscle specific genes contain at least two of these CArG boxes. (54) It is argued that the relative abundance of SRF concurrent with the co-activators it recruits, such as GATA (named for its DNA binding sequence) and C/EBPβ (C/CAAT enhancer-binding protein beta), enables SRF to have many diverse functions in VSMC (54). Specifically, at the onset of VSMC differentiation, it has been observed that SRF up-regulates: SM-MHC (45, 47, 98); smooth muscle γ-actin (7, 9); calponin (14, 55), SM22 (71, 81); α and β-tropomyosins (80); and α1-integrin (94).

GATA (transcription factor that binds the DNA sequence "gata")

GATA-6 is the principle GATA family member expressed in VSMC (97). As previously stated GATA's involvement in the pro-differentiated ternary complex established its importance to differentiation-specific gene expression in VSMC. A previous study demonstrated, that infection of restenotic lesions, post-balloon angioplasty, with an adenovirus over-expressing GATA-6 significantly reduced lesion formation by promoting VSMC differentiation (48). This finding was further substantiated by observation that over-expression of GATA-6 induced cell-cycle arrest (48). Interestingly, GATA-6 has also been observed functioning as a negative regulator of differentiation-specific gene expression while promoting the proliferative phenotype in VSMC suggesting a dual role for GATA-6 outside the contractile phenotype (97, 40).

C/EBPβ (C/CAAT-enhancer binding protein beta)

Like GATA-6, the inclusion of C/EBPβ in the ternary complex has established its importance to VSMC differentiation-specific gene expression. Interestingly, C/EBPβ in VSMC is rapidly induced upon exposure to inflammatory cytokines such as IL-1β (30). It has been demonstrated that exposure to IL-1β shifts the dimerization strategy away from SRF and GATA and promotes its partnering with p50 subunit of NFκB and Ets transcription factors forming a ternary that favors the expression of inflammatory responsive genes (2). Exposure to inflammatory cytokines stimulate VSMC to express genes such as $cPLA_2$ (12, 2), serum amyloid A1 (36), scavenger receptors 1+2 (56), iNOS (86) and COX2 (56), all of which are considered important to the progression of atherosclerosis. Additionally, it has also been observed that C/EBPβ drives the

expression of α-smooth muscle actin (30), an event previously described to be important to the VSMC contractile phenotype.

Additional Transcription Factors

Briefly, additional factors are known to modulate VSMC transcription, for example, CAAT/binding protein (CTF/NF-1) (1, 13, 31), nuclear factor of activated T-cells (NFAT) (27, 83), myocyte enhancer factor-2 (MEF2) (83) and the less well understood transcriptional enhancer factor-1 (TEF-1) (93), have all been identified. It has previously been observed that both serum and thrombin can up regulate SM-MHC gene expression. This up-regulation appears to be mediated through an ERK dependent activation of CTF/NF-1 (77). Additionally, a connection between the activation of CTF/NF-1 and the Ca^{2+}/calmodulin dependent protein kinase IV (70) has been made. Moreover, the similarity in binding consensus between CTF (CAAT) and C/EBPβ (CCAAT) cannot be ignored. Interestingly, it has also been described that blocking the calcineurin:NFAT pathway impedes progression of neointimal formation in atherosclerotic lesions by impeding the NFAT dependent COX-2 re-expression (43). Furthermore, COX-2 is a component essential to the inflammatory response. Additionally, it has been previously documented that MEF2 does bind and induce SM-MHC expression thus establishing its role in differentiation-specific gene expression (35) Also, MEF2 has demonstrated its ability to have an important role in progression of the inflammatory response by driving the up-regulation of monocyte chemo-attractant protein-1 (MCP-1) in a p38MAPK dependent fashion (84).

The Role of Ying Yang 1 (YY1) in VSMC Phenotypic Control

YY1 is a multifunction transcription factor that has surfaced as a key mediator of phenotypic control. For example: i.) YY1's transcriptional regulatory influence is broad and targets multiple facets of phenotypic regulation (78); and ii.) YY1 is a selective repressor of differentiation-specific gene expression in VSMC and draws from its ability to effect many aspects of transcriptional regulation (78). Aside from its role as a transcriptional regulator, YY1 has clearly demonstrated its ability to directly influence cell-cycle progression, DNA damage repair, tumor suppression and chromatin remodeling(67, 72, 95, 87, 28, 77, 38). YY1 can also incur several post-translational modifications (95, 65, 28, 67, 72, 76).

It is evident that while message pools of YY1 are relatively stable YY1 protein pools change dramatically, principally via proteolytic degradation (68). Recently Calpain, a Ca^{2+} dependent protease, was identified to target YY1 for degradation (90). Importantly, previous studies suggest that Calpain requires a specific Ca^{2+} signature that reflect VSMC that present a contractile phenotype (88). This is further supported by previous studies, which reported YY1 protein pool was diminished upon the onset of differentiation (65). These findings support a model that suggest that by diminishing the overall concentration of YY1, and thus its repressive effect, allows the re-expression of contractile related genes.

Stimulation with transforming growth factor beta (TGFβ), interlukin-1 beta (IL-1β) or fibroblast growth factor (FGF) has been shown to increase YY1 protein pools and enhance its binding to DNA. Additionally, YY1 can selectively antagonize TGFβ induced myocyte differentiation (37). Briefly, both TGFβ and BMP (bone morphogenic

protein) are considered pro-myogenic differentiation factors. Previous studies have described these factors ability to stimulate non-muscle cells to attain muscle-like phenotypes (37). These studies also suggested that YY1 may define threshold levels for TGFβ or BMP signaling by limiting the effect of these cytokines on their downstream targets, the transcription factor Smad (Similar to Mothers Against Decapentaplegic). The Smad transcription factors are known to induce contractile-specific gene expression (37).

As previously described, several factors required for proper regulation of differentiation-specific gene expression (SRF, GATA-6 and C/EBPβ) and those that drive the inflammatory response (NFκB, Ets, C/EBPβ, CTF/NF-1, NFAT, MEF2) have been identified. Interestingly, many of these factors share dual roles in gene programs with opposing phenotype specificities. For example, stimulating "contractile" VSMC with IL-1β alters the nuclear equilibrium such that GATA concentrations fall, SRF concentrations remain constant while YY1 and C/EBPβ become elevated. This change in balance enhances YY1 repression of the SRF driven differentiation-specific gene expression and concurrently diminishes GATA's ability to promote differentiation-specific gene expression. These events parallel increases in C/EBPβ protein pools and the activation of both the p50 subunit of NFκB and Ets transcription factors. The collective actions of, these factors drive the expression of proliferation-specific genes (2, 6, 23).

In summary, VSMCs defaults to a differentiated "contractile" phenotype when YY1 concentrations drop and allow the re-expression of contractile related genes. Exposure to inflammatory cytokines drive an increase in YY1 protein concentrations that repress contractile related genes and promotes proliferation specific gene expression.

Exposure to specific mitogens and cytokines, elevates YY1 protein pools such that it silences contractile-related gene expression and either: i.) promotes the VSMC to re-enters cell cycle; or ii.) permits the expression of proliferations-specific genes.

YY1 and VSMC Transcriptional Regulation

YY1 is known to target differentiated-specific gene expression in myocytes. First, YY1 resides in the VSMC nucleus when it's nuclear equilibrium favors the expression of differentiation-specific genes (39). Second, under these conditions YY1 binds to the promoter of actively expressed contractile-related genes. When bound, YY1 attenuates the activity of these promoters rather than completely silencing them. These findings argue that basal promoter activity can be augmented if the ability of YY1 to bind the promoter is removed. This strongly suggests that YY1 functions to fine-tune gene expression, the full implications of which, however, have yet to be explored.

YY1's ability to represses gene transcription is multifaceted. It has been previously demonstrated that YY1 repress differentiation-specific gene expression in myocytes (39). This is the collective effect of: i.) dependency of differentiation-specific gene expression on CArG boxes, which SRF binds; and ii.) the ability of YY1 to directly compete with SRF for binding. Importantly, the CArG box is a composite sequence that contains two core YY1 binding sites [ATGC**CCAT**ATA**TGG**(A/T)NNT]. This strongly suggest the regulatory dichotomy between these two factor co-evolved. Similarly, the binding site for C/EBPβ contains a degenerate YY1 binding site [(A/G)N(**A**/G)**T**(**G**/T)(A/T/**G**)NG(A/C)AA (A/G/T)NN]. Intriguingly, competition with YY1 has also been described for a wide array of transcriptional regulators whose

similarity in DNA binding sequence is much less overt namely, AP2 (79), SP1 (60), Oct-1, NFAT (100), Ets (52), Nkx (3), Phox/Mhox (51), AP1 (96), and GATA (67). Importantly, many factors (GATA, SRF, CEBP, MEF2, SP-1) previously demonstrated to be competitive with YY1 are indispensable for the proper expression of contractile genes in vascular myocytes. This indicates that these factors may be unique in their ability to displace YY1 in order to de-repress a promoter concurrent to their individual stimulatory function once bound. This argument sets forth the possibility that the ubiquitous transcriptional repressor like YY1 helps prevent alterations in gene expression profiles by restricting the access of transcriptional activators from binding to and altering a promoters activity.

In summary, these previous findings form the basis upon which we delineated the specific aims to be tested. We expect that by establishing the functional role of YY1 and C/EBPβ within the DRD of the SM-MHC promoter we will clarify a model of phenotypic switching which is fundamental to the VSMC expression of a contractile, proliferative and mitotic phenotype.

CHAPTER III

MATERIALS AND METHODS

Cell culture

Rat pulmonary artery cell line (PAC-1) were a generous gift from the Rothman laboratory and has been previously described (74). Initial cultures of PAC-1 were multiplied by cell culture techniques. Aliquots were divided upon receipt of the cells and were stored in liquid nitrogen (1998). Before experimentation began a single aliquot of PAC-1 was grown and divided to generate 15 stock samples that in turn were utilized throughout the course of the experiments. Each stock sample was then thawed and cultured for five experimental passages. The cell cultures lines were disposed of at the end of 15 generations. Cultures used for experimental purposes were grown in 199 media supplemented with 10% FBS and Gentamicin and in 37^{oc} environment of 5% CO_2 with 95% humidity.

Cells were passed by 1:1000 trypsin digestion for 2 minutes, washed with 199 media and collected in a sterile disposable 50ml polystyrene centrifugation screw-cap tube. The cells were then centrifuged in a swinging bucket table-top Beckman TJ-6centrifuge spinning at 4000rpm for 4 minutes. The washing media was removed and cells were resuspended in 11ml of 199 media by gentle pipetting with large bore 10ml disposable pipette until the solution was of equal density and contained no visible

aggregates of cells. To ascertain the quantity of cells in solution, 1ml of the cell resuspension was added to 19ml of an isotonic solution and counted in a cell counter (Coulter Electronics). The number of cells in 0.5ml of cell suspension was determined by measuring changes in electrical resistance as individual cells crossed the threshold between cell suspension and cell counter. Three measurements were made and averaged for accuracy and if readings differed by more then 500 units the stock of cells was further resuspended and then recounted. Calculations of total cell number from measurements were as follows: (((Adv. 3x Coulter measurement) x 2) x 20) = #cells / ml in cell suspension. When these cells reached 80% confluency they were harvested by trypsin digestion. Typically, one T-175 tissue flask yields provided enough cells for twenty 12-well plates with sufficient cells to restore the seed culture within 72hrs.

Cell Plating

Cultures of rat pulmonary artery smooth muscle cells were maintained in a T-175 tissue culture flask complete with 10% Fetal bovine serum + 199 Media + Gentamycin. These cultures were maintained in a Steri-Cult® CO_2 incubator (Forma Scientific©) in an environment of 37^{oC}, 5% CO_2 and 95% humidity. PAC-1 cells were used for experimental purposes between pass 5 and 15. All experiments were conducted in Flacon© 12-well tissue culture dishes (Cat #353043), and plated with $2.2x10^5$ cells per 12-well plate. Preliminary investigations established a seeding density of $2.2x10^5$ cell/12-well cell culture plate to provide the optimal 70-80% cellular confluency in 48 hrs. after seeding. This protocol was calibrated using the cationic lipid Lipofectamine© as the transfection reagent.

Transfection

To ensure consistencies across treatment groups a "master mix" was created for each experimental treatment groups and each promoter reporter. A zero point, for purposes of data normalization between experiments, was set at 1=BASELINE consisting of 30ng promoter reporter + 30ng pSVK3 + 10ng pBluescript. A 60ng DNA dose represented the quantity of DNA in the first experimental group and was used to control for any additional DNA effect. The appropriate empty vector control was also added to each so that the total DNA was constant relative to the corresponding experimental groups. The empty vector controls used were pBluescript to control for the absence of C/EBPb and pSVK3 to control for the absence of YY1.

We used the cationic lipid Lipofectamine® (Invitrogen© Cat#18324-080) according to manufactures recommendation as the transfection agent. Prior to transfection the cells were washed twice with serum free 199 media. After 1hr incubation of transfection reagent with DNA, a 0.5ml of transfection solution was added to each triplicate series and returned to the incubator for 12 hrs. After 12hr incubation of the transfection reagent, cells were rewashed twice with serum free 199 media, and fed with 0.2%FBS + 199media + Gentamycin (1:20,000). Subsequently the cultures were allowed to grow for an additional 24hrs, after which, the samples were harvested by addition of 30ul of 1x reporter lysis buffer (Promega ©) and scraping with a plastic policeman.

Reporter Assay

Single well samples were collected and transferred to individual luminometry cuvettes. Fresh luciferase substrate was reconstituted before each assay and multiple

vials were mixed to ensure consistency during the assay. 100ul of luciferase substrate was added to each cuvette and three measurements of the relative lights emission was recorded within the first 45s after substrate addition. The highest of the three recordings was reported as the relative luciferase activity for that experimental sample.

DNA Preparation and Mutations

Plasmid DNA was isolated by the classical alkaline lysis protocol while super-coiled and circular plasmids were purified twice via cesium chloride ultra-centrifugation. The SM-MHC promoter truncations series (p4200, p1621, p1256, p602) were obtained and have been previously described (Owens et al. 1996). All SM-MHC promoter-reporter plasmid were required transformational and harvested from JM109 (Promega © cat#??). The empty vectors pBluescript and pSVK3 and expression vectors pBluescript:C/EBPβ and pSVK3:YY1 were isolated from DH5α (Promega © cat#??).

We used the Quick Change® site-directed mutagenesis kit to generate the various SM-MHC mutations. The following primers were used and their reverse complements were ordered PAGE purified from Integrated DNA technology Coralville, IA: YY1 (-1563) [5`-CCTCTGGCCTGCAAAATGAGGCTGGGACAGGTTTG-3`], YY1 (-748) [5`-GCATCCTCCAAGTGAAATAACGCCAGTAGCCACCCGCTTTTC-3`]. It is of particular interest that the YY1 (-1563) primer sequence shared >505 homology to three regions of the pGL2basic backbone. Successful mutation at this site required an elevated annealing temperature of 65^{oC}.

Statistics and Data Presentation

The data represented are the average of at least three triplicated experimental sets and reflect at least 9 independent experiments. Data presented in terms of "Relative

Luciferase Activity" denote the raw data collected from the luminometer. Data presented in terms of "Fold Activation" denote the data has been manipulated such that the baseline values were divided out from each experimental group of the same treatment. Where applicable a 2-way ANOVA was conducted to confirm significance between promoters and between treatment. Significance is reported for P values ranging from P=0.05 to P=0.001.

Promoter Analysis

The promoter of smooth muscle γ-actin (AB_000471), smooth muscle α-actin (M35194), smooth muscle myosin heavy chain (NM_002474), and SM22 (D84344) were analyzed via MatInspector© Professional (2001). Stringencies of transcription binding sites was set to 100%core / 95% matrix.

Smooth Muscle Myosin Heavy Chain Promoter

In order to test the regulatory contribution communicated by the DRD's we used the regulatory context of the SM-MHC promoter as an optimal tool to explore the mechanisms that convey stability to the differentiated phenotype at the level of gene expression.

Previously, it had been found that the proximal -4200bp of this promoter plus the first intron of SM-MHC conveyed tissue specificity. Two CArG boxes located between 1317bp and 1055bp have been deemed critical to the proper activation of the promoter. Analysis of the promoter identified 1557 consensus binding motifs representing a wide variety of transcription factors (significance based on: 95% core / 85% matrix identity MatInspector Professional©). Of these sites many YY1 binding clusters were evident, i.e. within the first –2520bp of this promoter twenty-four YY1 binding sites are evident,

twenty of which form 11 paired regions separated by ~525bp. This topography strongly correlates to nucleosomal architecture and has sufficient distance to span two nucleosomal units. In close proximity to or overlapping with the paired YY1 binding sites are bindings sites for factors known to drive the expression differentiation-specific genes. Because it would it would be impractical to explore all the possible sites of competition between YY1 and each factor, we tested competition in terms of the YY1 and C/EBP at two specific DRDs, namely DRD -1563 and DRD -748. It should be noted that, there exist multiple YY1 binding sites independent of these two DRDs. In addition there are numerous independent C/EBP binding sites and it is unknown how many YY1 or C/EBP sites are resident in the full -4200bp promoter. However, close inspection of the promoter fragments identified the : -1621bp fragmented consisted of [12:C/EBP and 11:YY1], the -1249bp fragment consisted of [11:C/EBP and 7:YY1], and the -602bp consisted of [5:C/EBP and 3:YY1]. Thus, shortening the promoter length proportionally diminished the total binding sites for YY1 and C/EBP.

CHAPTER IV

RESULTS

First, it was necessary to establish a treatment that allowed the greatest precision when monitoring the effective range of both C/EBPβ and YY1. We chose a 10-point dose response (0-100ng) that covered the function range of both factors. We then proceeded with the following experimental design: i.e. we sought to hold one factor at a gene dose that reflects ½ it's maximal activity and co-transfect the full effective dose range of the other. The objective was to observe how this alteration in stoichiometry altered the predefined maximal activity of the saturating factor. We anticipated that by elevating cellular concentrations of YY1 we would cause a reduction in the maximal effect of C/EBPβ. Moreover, if C/EBPβ mediated transactivation and YY1 mediated transrepression functioned independently, we would expect each promoter truncation to demonstrate an equivalent response to the same alterations in the nuclear environment, since each promoter truncation is proportionally equivalent in binding sites for C/EBPβ and YY1: i.e. -1621bp SM-MHC [$_{12}$CEBP, $_{11}$YY1] = -1249bp SM-MHC [$_{11}$CEBP, $_{7}$YY1] = -602bp SM-MHC [$_{5}$CEBP, $_{3}$YY1]. If competition is present and reliant upon the DRDs, co-treatment of C/EBPβ and YY1 will affect each promoter differentially and proportionally to the quantity of DRD each retains: -1621bp SM-MHC [2x DRD] >> -1249bp SM-MHC [1x DRD] > -602bp SM-MHC [0x DRD]. Moreover, we anticipated

this mechanism would be present regardless of which factor was increased and which was held constant. Likewise, we also reversed this experimental approach so that the concentration of YY1 was increased relative to a constant 1/2 maximal activating dose of C/EBPβ in order to further establish the functional relevancy of competition occurring at the DRD.

The effects of C/EBPβ mediated transactivation using the Wild-type SM-MHC (-4200bp, -1621bp, -1249bp, -602bp)

The data from these experiments are presented in figures 1A-1D (open circles). As anticipated each promoter fragment demonstrated a dose-dependent increase in activity relative to increasing does of C/EBPβ. Specifically, as the largest of the promoter truncations the -4200bp SM-MHC fragment was the least sensitive to transactivation of C/EBPβ. The -4200bp promoter fragment generated only a 0.65 fold maximal induction in reporter activity. Moreover, this induction was rapidly lost with treatment of C/EBPβ in excess of 40ng (Fig. 1A closed circles).

Treatment with C/EBPβ was ineffective in activating the –1621bp SM-MHC promoter reporter at a dose less than 20ng. The promoter activity increased linearly in the dose range of 30-50ng after which it stabilized in the dose range of 50-80ng. Furthermore, we recorded a decrease in reporter activity in the range of 80-100ng of C/EBPβ gene dose. The -1621bp promoter fragment was most sensitive to the effects of C/EBPβ at a gene dose of 60ng where it generated a 3-fold induction of overall reporter activity (Fig. 1B closed circles).

The -1249bp SM-MHC was sensitive to C/EBPβ stimulation at all doses. This promoter responded with a linear induction in reporter activity in the dose range of 2.5ng - 40ng and was stable between 40ng – 100ng (Fig. 1C closed circles) Maximum sensitivity to elevated C/EBPβ gene dose was observed in the range between 40ng – 100ng, where a 5-fold induction in overall reporter activity was recorded.

Finally, the -602bp SM-MHC promoter reporter demonstrated a linear response to C/EBPβ at each dose tested. The maximal observed sensitivity for the –602bp promoter fragment was 16 fold and occurred at 100ng. (Fig. 1D closed circles).

In summary, these data clearly demonstrate that the sensitivity of each promoter truncation to C/EBPβ was increased as the promoters overall length decreased.

YY1 Repression of the Wild-type SM-MHC Promoters Truncations (-4200bp, -1621bp, -1249bp, -602bp)

The data presented from these experiments are presented in figures 2A-2D (closed squares). As anticipated, each SM-MHC promoter truncation generated a dose-dependent decrease in reporter activity with increased YY1 gene dose. Specifically, treatment of the -4200bp SM-MHC promoter with increasing YY1 gene dose repressed promoter activity in a linear fashion between the range of 2.5ng - 60ng and exhibited a stable, maximal repression between 60ng and 100ng. A maximal repression of 3-fold below baseline activity was recorded for the -4200bp promoter fragment (Fig. 2A closed squares).

Treatment with increasing YY1 gene dose repressed the -1621bp SM-MHC linearly in the range of 5.0-100ng. A maximal 2-fold reduction in basal reporter activity was recorded for the -1621bp promoter fragment at a dose of 100ng (Fig. 2B closed squares).

Under the same conditions, YY1 mediated repression of the -1249bp promoter fragment was biphasic. YY1 generated a stable first phase repression of 1-fold below basal reporter activity in the range of 5-50ng. The second phase was linear in nature between 50-80ng at which point this repression stabilized between 80-100ng. The maximal 1.3 fold repression of -1240bp SM-MHC was recorded at 100ng (Fig. 2C closed squares).

Finally, treatment of the -602bp SM-MHC with increasing YY1 gene dose exhibited a slight dose-dependent repression that was both stable and maximal between the range of 30-100ng. (Fig. 2D closed squares).

In summary, these data demonstrate that the required dose necessary to achieve maximal repression diminishes as promoter length decreases.

The effects of increasing C/EBPβ vs. ½ $_{max}$ YY1 and the response of the Wild-type SM-MHC Promoter Truncations

In order to observe what we contend is competitive regulation between C/EBPβ and YY1 it was necessary to simulate the conditions in which we would expect to observe competition. To this end, we enhanced the intracellular pools of YY1 by ½ it's maximal activity (40ng) and observed how this affected the previously established

maximal activity of C/EBPβ. We observed an attenuation in C/EBPβ mediated maximal promoter reporter in all but the -602bp SM-MHC promoter truncation.

Specifically, in the presence of a ½ maximal repressive dose of YY1, C/EBPβ gene dose in the range of 2.5-5ng was ineffective in stimulating the -4200bp SM-MHC promoter fragment. However, –4200bp SM-MHC promoter reporter activity was linearly increased in the range of 5.0-20ng, and stabilized between the range of 20-40ng. The effect of C/EBPβ gene dose diminished in the range of 40-60ng (Fig. 1A open triangles). Moreover, relative to the previously defined maximal effect of C/EBPβ• for the –4200bp SM-MHC promoter reporter (Fig. 1A closed circles), a ½ maximal repressive dose of YY1 diminished C/EBPβ maximal effect by 53%.

Likewise, treatment with ½ maximal YY1 gene dose dramatically reduced the sensitivity of the -1621bp SM-MHC promoter reporter to C/EBPβ mediated maximal activation. Specifically, in the presence of a ½ maximal repressive dose of YY1, C/EBPβ was ineffective in stimulating reporter activity within the range of 2.5-10ng, it's activation was modest but stable in the range of 20-80ng and diminished at 100ng (Fig. 1B open triangles). Comparatively, treatment with ½ maximal repressive dose of YY1 attenuated the maximal effect of C/EBPβ by 70% of it's previously recorded maximal activity.

Likewise, when the -1249bp SM-MHC promoter fragment was treated under the same conditions C/EBPβ induced reporter activity in a linear fashion between the doses of 2.5-40ng after which C/EBPβ effect stabilized and remained constant between 40-100ng (Fig. 1C open triangles). Compared to the previously established maximal

response of C/EBPβ for this promoter, this treatment attenuated 30% of C/EBPβ's previously recorded maximal activity.

Finally, treatment of the -602bp SM-MHC promoter fragment under the same conditions demonstrated the dose range of 2.5-20ng to be ineffective while 20-100ng generated a dose-dependent increase in reporter activity (Fig. 1D open triangles). While the ½ maximal repressive dose of YY1 clearly had a response on overall promoter behavior C/EBPβ readily restored it's previously documented maximal activity in the range of 50-60ng.

In summary, this data demonstrate that as promoter length decreases the ability of YY1 to impede C/EBPβ mediated transactivation of the promoter fragment also decreases.

The effects of increasing YY1 vs. ½ $_{max}$ C/EBPβ and the response of the Wild-type SM-MHC Promoter Truncations

The previous study demonstrated a strong correlation between promoter length and the ability of YY1 to impede C/EBPβ mediated transactivation. To further test the hypothesis that this was the effect of competition between C/EBPβ and YY1, we inverted our experimental design to observe how increasing C/EBPβ to ½ its maximal activating dose effected the ability of YY1 to repress each of the wild-type SM-MHC promoter fragments. We observed that increasing C/EBPβ gene dose to reflect ½ its maximal activity significantly affected the YY1 mediated dose-dependent repression of each SM-MHC promoter truncation differently.

First, treatment of the -4200bp SM-MHC with increasing dose of YY1 concurrent to a ½ maximal dose of C/EBPβ did not appreciably alter the sensitivity of -4200bp to C/EBPβ. It appeared that YY1 was ineffective in repressing the –4200bp SM-MHC promoter in the dose range of 0-60ng. YY1 modestly repressed this promoter in the dose range of 60-100ng (Fig. 2A open inverted triangles). The maximal recorded repression at 100ng YY1 for the –4200bp SM-MHC promoter truncation was 0.33 fold below the response recorded for C/EBPβ, reflecting ½ its maximal activity. Moreover, when exposed to the same treatment, repression of the –1621bp SM-MHC promoter fragment appeared biphasic. Increased YY1 gene dose in the range of 2.5-20ng was ineffective in repressing the activity of this promoter fragment, where as the YY1 gene dose in the range of 20-60ng repressed the promoter fragment in a linear, dose-dependent fashion after which YY1 repression was stable. YY1's maximal repression was recorded to be 3.3-fold below the ½ maximal activity of C/EBPβ (Fig. 2B open inverted triangles). Similarly, YY1 mediated repression of the -1249bp promoter fragment was biphasic. YY1 repression was initially effective at 5.0ng of gene dose after which it rapidly repressed between 5-20ng, stabilized between 20-60ng and accelerated to its maximal repression of 5-fold below experimental baseline in the range of 60-100ng (Fig. 2C open inverted triangles). Finally, YY1 repressed the –602bp promoter fragment the same at each dose measured. This repression reflected the same magnitude of repression witnessed in the absence of C/EBPβ.

In summary, these data demonstrate that the ability of C/EBPβ to impede YY1 repression is related to promoter length.

Exploring the Dual Regulatory Domains

-1621bp SM-MHC: Wild-type vs. DRD(-1563 YY1^{mut}) vs. DRD (-1563 YY1^{mut}, -748 YY1^{mut})

The previous findings demonstrated two things: i.) the repressive effect of YY1 could effect the activating effect of C/EBPβ and *visa versa* and; ii.) the extent of the effect was dependent upon promoter length. Resident within the -1621bp SM-MHC promoter fragment are two punitive DRDs that would account for these properties. These domains were tested to assert any role they may have had in the previous findings.

As a prelude to the recorded data we generated to mutant forms of the –1621bp promoter truncation. The first, -1621bp SM-MHC [DRD (-1563 YY1^{mut})] and the second -1621bp SM-MHC [DRD (-1563 YY1^{mut}), (-748 YY1^{mut})] mutation of any YY1 binding site, either singularly or in combination, enhanced the unstimulated basal activity of each promoter relative to its wild-type -1621bp SM-MHC counterpart. Importantly, many past studies have documented the removal of YY1 binding sites to augment baseline promoter reporter activity of contractile related gene in VSMC. However, how the augmentation in promoter activity was manifested remained unclear. Three possibilities exist to account for these findings: i.) mutating the YY1 binding site prevented YY1 from binding and repressing; ii.) mutating the YY1 binding site enhanced the binding of a proximal transcription activator or; iii) there were unrecognized variability in between the preparations between the wild-type and mutant promoter reporters. We did two things to circumvent these problems. First, we rigorously standardized each promoter-reporter so it reflected the average response of 30 individual promoter-reporter preparations (see materials and methods). Second, we subtracted out the unstimulated baseline values from

each collected for a given promoter-reporter data point. This normalized each data set to begin at [1] and is expressed as fold activation above each individual promoter-reporters unstimulated baseline. This simplified each data set so they could be directly compared to each other on the same scale. We then tested each mutation under the same experimental guidelines as previously described.

Importantly, two major components tend to make data collected with PAC-1 cells drift; i.) batch difference in the fetal bovine serum and ii.) unknown variables between different cryopreserved cell stocks. Since this section of the experimentation requires that three promoter-reporters are inherently the same to be directly compared to each other, it was necessary to eliminate variables. To do this, all experimentation was completed within the same reanimated PAC-1 cell stock between passage 5 and 15 and sufficient fetal bovine serum was pre-mixed to ensure homogeneity. Moreover, due to the relative importance of the this data we tripled our data set so that each experiment is the collective average of nine independent experiments, each assayed in triplicate thus, every data point represents 27 independent measurements of the reporter activity under its experimental conditions.

-1621bp SM-MHC wild-type: C/EBPβ vs. ½ $_{max}$ YY1

In testing the relevancy of the two DRD we retested the -1621bp wild-type SM-MHC promoter with newly outlined conditions. We recorded a 3.0 fold induction above baseline reporter activity (Fig. 3A closed circles) and a 0.5 fold repression of basal reporter activity (Fig. 3A closed squares). Moreover, co-treatment with ½ maximal repressive dose of YY1 attenuated 68% of the previously recorded maximal

transactivation mediated by C/EBPβ (Fig. 3A open diamonds). These results faithfully reflect previous findings regarding the response of the –1621bp SM-MHC promoter reporter to C/EBPβ and YY1 treatment.

-1621bp SM-MHC / DRD (-1563 YY1^{mut}): C/EBPβ vs. ½ $_{max}$ YY1

Specifically, in order to ascertain the functional relevance of the DRD located -1563bp from the transcriptional start site we mutated the embedded YY1 binding site and treated this modified promoter under the same condition as previously described. We observed the sensitivity of this promoter fragment to C/EBPβ transactivation was enhanced. We recorded a 3.5 fold induction above unstimulated basal reporter activity in response to increasing dose of C/EBPβ (Fig. 3B closed circles) and a stable 0.5 fold reduction in response to the ½ maximal repressive dose of YY1 (Fig. 3B closed squares). Moreover, co-treatment with ½ maximal YY1 attenuated C/EBPβ's previously recorded maximal activity by 40% (Fig. 3B open diamonds).

-1621bp DRD (–1563 YY1^{mut}), (–748 YY1^{mut}): C/EBPβ vs. ½ $_{max}$ YY1

Additionally, in order to ascertain the functional relevance of both DRD's located -1563bp and –748bp we mutated each YY1 binding sites within the –1621bp promoter fragment. We observed the sensitivity of this promoter fragment to C/EBPβ transactivation was further enhanced. We recorded a 4.0 fold induction above unstimulated basal reporter activity in response to increasing dose of C/EBPβ (Fig. 3C open circles) while ½ repressive dose of YY1 remained unchanged at 0.5 fold below

baseline (Fig. 3C closed squares). Moreover, treatment with the ½ maximal repressive dose of YY1 impeded C/EBPβ mediated transactivation of this promoter by 30% (Fig. 3C open inverted triangles).

Comparative Analysis (Wild-type vs. Single Mutant vs. Double Mutant): C/EBPβ vs. ½ $_{max}$ YY1

Next, we extracted all data recorded under conditions when ½ maximal YY1 was co-treated with increasing dose of C/EBPβ and graphed the results relative to all data collected for the wild-type -1621bp SM-MHC promoter fragment (Fig. 4) Increasing C/EBPβ (closed circles) ½ max YY1 (closed squares) and co-treatment (open triangles). Relative to the wild-type -1621bp SM-MHC promoter fragment 68% attenuation in C/EBPβ maximal activity under conditions of co-treatment, mutating the embedded YY1 resident in the DRD (-1563) restored 80% of wild-type activity (open diamonds). Likewise, the 70% attenuation witnessed in C/EBPβ maximal activity witnessed for the -1621bp SM-MHC DRD (-1563 YY1^{mut}) and (-748 YY1^{mut}) was equal to 100% of C/EBPβ maximal transactivation of the wild-type -1621bp SM-MHC promoter fragment (open inverted triangles).

-1621bp SM-MHC wild-type: YY1 vs. ½ $_{max}$ C/EBPβ

Again as previously described all experiments were conducted with in the same cell stock between passages 5-15 and each figure represent 9 independent experiments. We then reversed our experimental design so that YY1 dose was increasing and C/EBPβ

was held at ½ its maximal activating dose and tested the response of the mutated promoter to this treatment. We found a ½ maximal dose of C/EBPβ to transactivated the wild-type -1621bp SM-MHC promoter 4-fold (Fig. 5A open circles). YY1 demonstrated stable repression throughout the full experimental range (5-100ng) of YY1 maximal repression for YY1 was recorded as 0.5 fold below baseline (Fig. 5A closed squares). Co-treatment of increasing dose of YY1 with ½ maximal activating dose of C/EBPβ slowed the rate of YY1 mediated repression such that a linear rate of repression was witnessed between the dosages 10-60ng at which point in time maximal repression was restored and was stable maintained in the dose range of 60-100ng .

-1621bp SM-MHC (-1563 YY1^{mut}): YY1 vs. ½ $_{max}$ C/EBPβ

We treated the -1621bp SM-MHC DRD (-1563 YY1^{mut}) under the same conditions as previously described. Treatment of this promoter with ½ maximal activating dose of C/EBPβ generated a 6-fold induction of reporter activity that was less stable than previously witnessed for the wild-type -1249bp SM-MHC promoter fragment (Fig. 5B closed circles). YY1 demonstrated stable repression throughout the full experimental range (5-100ng) of YY1 maximal repression for YY1 was recorded as 0.5 fold below baseline (Fig. 5B closed squares). Co-treatment of increasing dose of YY1 with ½ maximal activating dose of C/EBPβ slowed the rate of YY1 mediated repression further than previously witnessed for the wild-type -1249bp SM-MHC promoter fragment. A linear rate of repression was recorded between the dosages 10-80ng at

which point in time maximal repression was restored and was stably maintained in the dose range of 80-100ng (Fig. 5B open diamonds).

-1621bp SM-MHC DRD (-1563 YY1^{mut}), (-748 YY1^{mut}): YY1 vs. ½ $_{max}$ C/EBPβ

We treated of the -1621bp SM-MHC DRD (-1563 YY1^{mut}), (-748 YY1^{mut}) under the same conditions as previously described. Treatment of this promoter with ½ maximal activating dose of C/EBPβ generated a 12-fold induction of reporter activity that was less stable than previously witnessed for the wild-type -1621bp SM-MHC promoter fragment (Fig. 5C closed circles). YY1 demonstrated stable repression in the throughout the full experimental dose range (5-100ng) of YY1. Maximal repression for YY1 was recorded as 0.5 fold below baseline (Fig. 5C closed squares). Co-treatment of increasing dose of YY1 with ½ maximal activating dose of C/EBPβ slowed the rate of YY1 mediated repression further than previously witnessed for the -1621bp SM-MHC promoter fragment. A linear rate of repression was recorded for this promoter between the dosages 10-80ng at which point in time maximal repression was restored and was stably maintained in the dose range of 80-100ng (Fig. 5C open inverted triangles).

Comparative Analysis (Wild-type vs. Single Mutant vs. Double Mutant): YY1 vs. ½ $_{max}$ C/EBPβ

Next, we extracted all data recorded under conditions when ½ maximal C/EBPβ was co-treated with increasing dose of YY1 and graphed the results relative to all data collected for the wild-type -1621bp SM-MHC promoter fragment (Fig. 6), ½ max

activating dose C/EBPβ (closed circles) increasing YY1 (closed squares) and co-treatment (open triangles). We then compared the rate at which YY1 restored its maximal repression relative to its dose. It was observed that relative to the wild-type -1621bp SM-MHC promoter fragment, the rate at which both the -1621bp SM-MHC DRD (-1563 $YY1^{mut}$) and -1621bp SM-MHC DRD (-1563 $YY1^{mut}$), (-748 $YY1^{mut}$) restored YY1 maximal repression was significantly impeded. Specifically, while the wild-type achieve maximal repression at 60ng of YY1, the -1621bp SM-MHC DRD (-1563 $YY1^{mut}$) required the full 100ng of YY1 to fully restore maximal repression relative to the wild-type (open diamonds). Likewise, relative to the wild-type the -1621bp SM-MHC DRD (-1563 $YY1^{mut}$), (-748 $YY1^{mut}$) YY1 at doses less than 60ng did not appreciably differ than those recorded for the wild-type response to a ½ maximal activating dose of C/EBPβ. Maximal repression was restored for this reporter at the dose of 100ng (open inverted triangles).

-1249bp SM-MHC: Wild-type vs. DRD (-748 $YY1^{mut}$)

To further test our hypothesis we recapitulated the previous study with the –1249 SM-MHC promoter reporter which contained only the DRD (-748). While the previous study demonstrated a clear regulatory effect communicated by both DRDs, we continued this study to observe the behavior of a single DRD under conditions of competition. To assess any regulatory effect communicated by YY1 from this site we mutated the YY1 binding consensus embedded within the DRD (-748). We then tested the effect of mutating the YY1 binding site in altering the regulatory profile of this promoter fragment. Treatment of this promoter fragment occurred under the same conditions as

previously outlined for the –1621bp SM-MHC promoter fragment. As stated before each experimental series was conducted within the same PAC-1 subculture between passages 5-15 and shared the same fetal bovine serum used in the previous -1621bp SM-MHC experiments.

-1249bp SM-MHC Wild-Type: C/EBPβ vs. ½ max YY1

The wild-type -1249bp SM-MHC promoter truncation, treated with increasing dose of C/EBPβ, generated a dose-dependent increase in reporter activity that was maximal at 80ng and recorded as a 2-fold induction above unstimulated basal activity (Fig. 7A closed circles). Likewise, the ½ maximal repressive dose of YY1 generated a moderately stable 0.5-fold repression (Fig. 7A closed boxes). The dose-dependent increase in reporter activity previously recorded was impeded by 30% in the presence of ½, maximal repressive dose of YY1(Fig 7A open triangles). This is in agreement with original findings regarding the behavior of the -1240bp SM-MHC promoter fragment (Fig 2C open triangles).

-1249bp SM-MHC DRD (-748 $YY1^{mut}$): C/EBPβ vs. ½ max YY1

When treated with increasing dose of C/EBPβ, the –1249bp SM-MHC DRD (-748) generated a dose-dependent increase in reporter activity that was linear in nature. The maximal recorded activity of this promoter was at 100ng and recorded as a 10-fold induction above unstimulated basal activity (Fig. 7B closed circles). Likewise, the ½ maximal repressive dose of YY1 generated a moderately stable 0.5-fold repression (Fig.

7B closed boxes). The dose-dependent increase in reporter activity previously recorded was impeded by 10% in the presence of ½ maximal repressive dose of YY1 (Fig. 7B open triangles).

Comparative Analysis Wild-type vs. DRD (-748): YY1 vs. ½ $_{max}$ C/EBPβ

Next, we extracted all data recorded under conditions when increasing dose of C/EBPβ was co-treated with ½ maximal repressive dose of YY1 and graphed the results relative to all data collected for the wild-type -1249bp SM-MHC promoter fragment (Fig. 8), increasing dose of C/EBPβ (closed circles) ½ maximal repressive dose of YY1 (closed squares) and co-treatment (open triangles). We then observed the effect mutating the embedded YY1 binding site within the DRD (-748) had in altering this promoters response relative to wild-type. Comparing effect of the mutation made in the YY1 binding site embedded in the DRD (-748) relative to the wild-type promoter revealed a substantial relinquishment of YY1 negative regulatory control. Specifically, the 90% restoration in C/EBPβ maximal activity as witnessed from the -1249bp SM-MHC DRD (-748 YY1^{mut}) translated in to a comparative 200% increase above the maximal activity elicited by C/EBPβ previously recorded for the wild-type -1249bp SM-MHC promoter truncation.

-1249bp SM-MHC Wild-Type: YY1 vs. ½ max C/EBPβ

We then reversed the experimental design and recorded how this promoter responded to a ½ maximal activating dose of C/EBPβ, as well as increasing dose of YY1

and finally the co-treatment of increasing dose of YY1 and ½ maximal activating dose of C/EBPβ. We witnessed for the wild-type –1249bp SM-MHC promoter truncation a stable 2.1 fold activation above basal reporter activity in response to ½ maximal C/EBPβ (Fig. 9A closed circles). Likewise, we recorded a dose-dependent repression of reporter activity in response to increasing dose of YY1. This occurred in a linear fashion with the maximal repression recorded for YY1 to occur at 100ng (Fig 9A closed squares). A ½ maximal activating dose of C/EBPβ slowed this rate of repression with maximal repression occurring at 100ng.

-1249bp SM-MHC DRD (-748 YY1^{mut}): YY1 vs. ½ max C/EBPβ

Next we treated the -1249bp SM-MHC DRD (-748 YY1^{mut}) as previously described. We recorded very unstable results for treatments containing a ½ maximal activating dose of C/EBPβ, its tendency however, was 4-fold in nature. (Fig. 9B closed circles). YY1 repression however was very stable and appeared maximal at each dose (Fig. 9B closed squares). As witnessed for treatment with C/EBPβ alone, the results for co-treatment with increasing dose of YY1 were very unstable and tended to be associated with the values recorded for -1249bp SM-MHC DRD (-749 YY1^{mut}) treated with ½ maximal activating dose of C/EBPβ (Fig. 9B open diamonds).

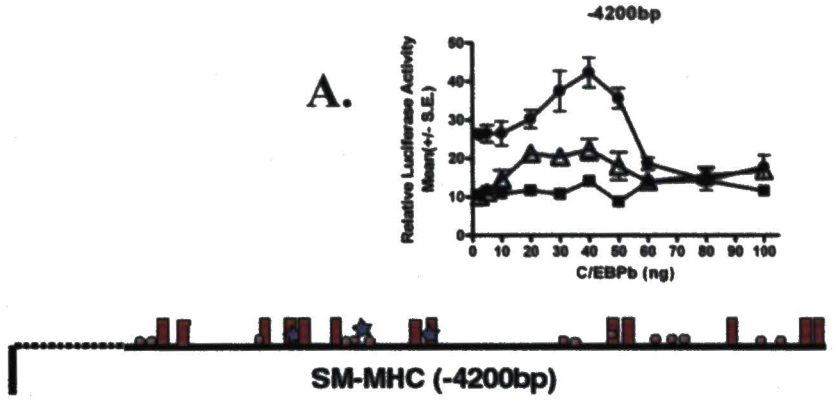
Comparative Analysis (Wild-type vs. DRD (-748 YY1^{mut})): YY1 vs. ½ $_{max}$ C/EBPβ

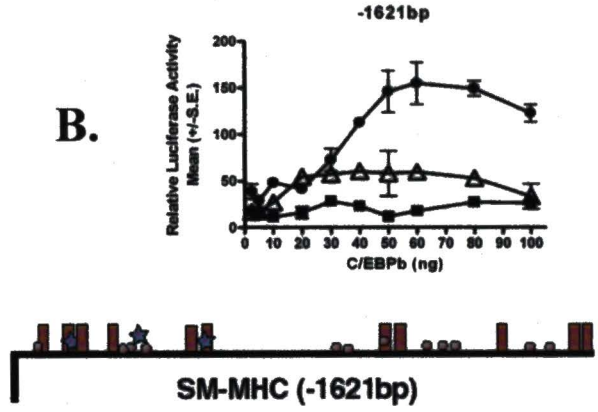
We then extracted all data recorded under conditions when ½ maximal C/EBPβ was co-treated with increasing dose of YY1 and graphed the results relative to all data

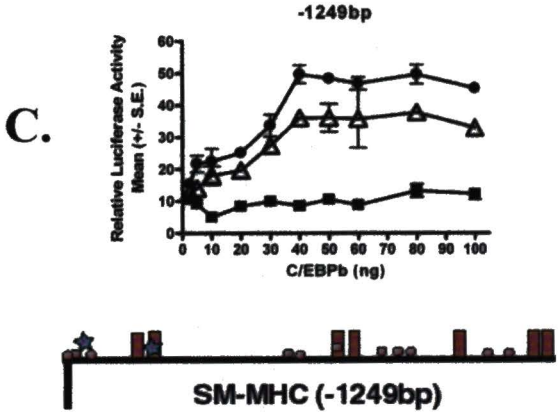
collected for the wild-type -1249bp SM-MHC promoter fragment (Fig. 10), ½ max activating dose C/EBPβ (closed circles) increasing YY1 (closed squares) and co-treatment (open triangles). We then compared the rate at which YY1 restored its maximal repression relative to its dose. It was observed that relative to the wild-type -1249bp SM-MHC promoter fragment, the rate at which both the -1249bp SM-MHC DRD (-748 $YY1^{mut}$) restored YY1 maximal repression was significantly impeded. Specifically, while the wild-type achieve maximal repression at 100ng of YY1 the -1249bp SM-MHC DRD (-748 $YY1^{mut}$), despite the full 100ng of YY1, did not fully restore maximal repression relative to the wild-type (open diamonds). Likewise, compared to wild-type this rate of repression strongly match those values recorded for ½ maximal activation mediated by C/EBPβ with regards to the wild-type promoter.

Figure 1: Testing the competitive regulatory influence between C/EBPβ and YY1 relative to the wild-type SM-MHC (-4200bp, -1621bp, -1249bp, -602bp).

PAC-1 cells were transiently transfected with 30ng of a luciferase based promoter-reporter plasmid under the explicit control of one of four truncations of the SM-MHC promoter: (A) -4200bp SM-MHC, (B)-1621bp SM-MHC, (C) -1249bpSM-MHC or (D) – 602bpSM-MHC. Each promoter-reporter was then treated with increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pBluescript:C/EBPβ (closed circles) and 40ng of the empty vector pSVK3 empty vector, 40ng of pSVK3:YY1 and increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of the empty vector pBluescript (closed squares) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pBlusecript:C/EBPβ and 40ng pSVK3:YY1 (open triangles). Data represent average luciferase activity as determined by luminometry.


A.
-4200bp
Relative Luciferase Activity Mean(+/- S.E.)
C/EBPb (ng)
SM-MHC (-4200bp)


B.
-1621bp
Relative Luciferase Activity Mean (+/-S.E.)
C/EBPb (ng)
SM-MHC (-1621bp)


C.
-1249bp
Relative Luciferase Activity Mean (+/- S.E.)
C/EBPb (ng)
SM-MHC (-1249bp)

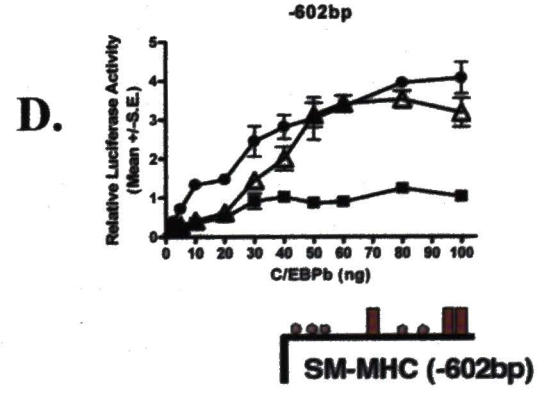
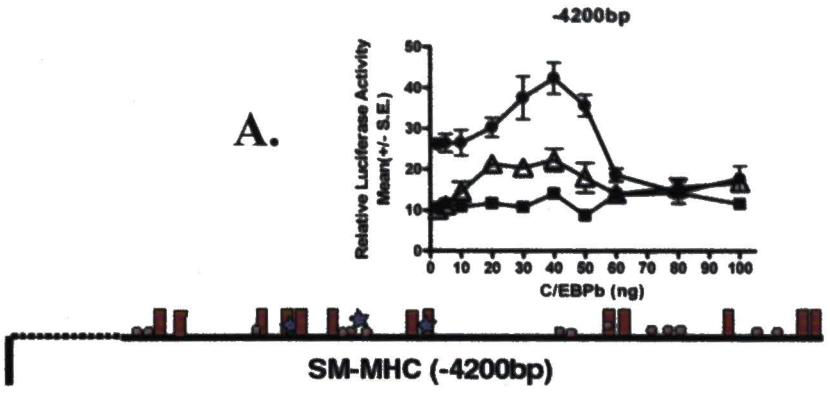
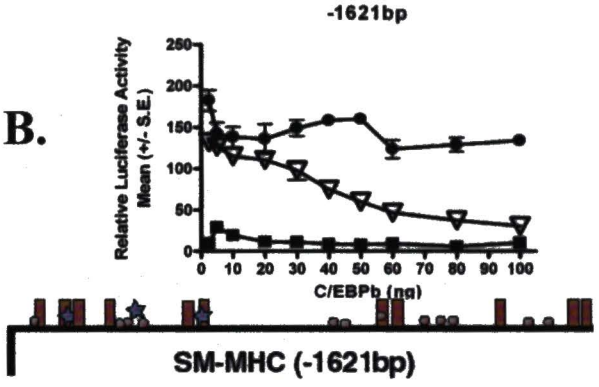
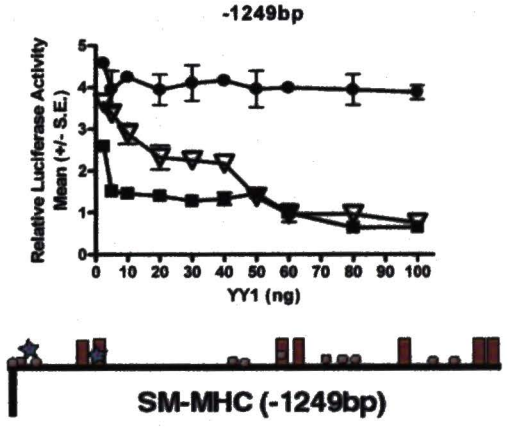

D.
-602bp
Relative Luciferase Activity (Mean +/-S.E.)
C/EBPb (ng)
SM-MHC (-602bp)

Figure 2: Testing the competitive regulatory influence between C/EBPβ and YY1 relative to the wild-type SM-MHC (-4200bp, -1621bp, -1249bp, -602bp).

PAC-1 cells were transiently transfected with 30ng of a luciferase based promoter-reporter plasmid under the explicit control of one of four truncations of the SM-MHC promoter: (A) -4200bp SM-MHC, (B)-1621bp SM-MHC, (C) -1249bpSM-MHC or (D) – 602bpSM-MHC. Each promoter-reporter was then treated 40ng of pBluescript:C/EBPβ and increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of the empty vector pSVK3 empty vector (closed circles) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pSVK3:YY1 and 40ng of the empty vector pBluescript (closed squares) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pSVK3:YY1 and 40ng pBluescript:C/EBPβ (open triangles). 48hrs post-transfection luciferase activity was determined by luminometry.


A.
-4200bp
Relative Luciferase Activity
Mean(+/- S.E.)
C/EBPb (ng)
SM-MHC (-4200bp)


B.
-1621bp
Relative Luciferase Activity
Mean (+/- S.E.)
C/EBPb (ng)
SM-MHC (-1621bp)


C.
-1249bp
Relative Luciferase Activity
Mean (+/- S.E.)
YY1 (ng)
SM-MHC (-1249bp)

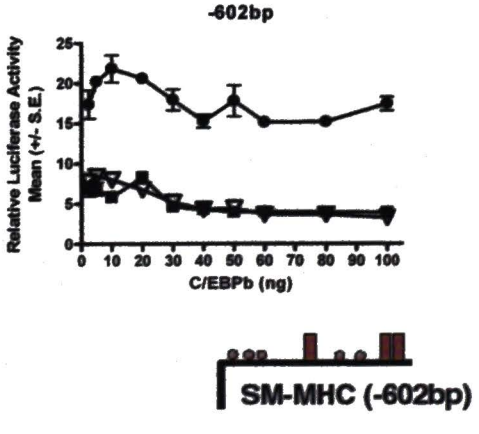

D.
-602bp
Relative Luciferase Activity
Mean (+/- S.E.)
C/EBPb (ng)
SM-MHC (-602bp)

Figure 3: Testing the competitive regulatory influence between C/EBPβ and YY1 relative to the –1621bp SM-MHC: Wild-type vs. DRD (-1563 $YY1^{mut}$) vs. DRD (-1563 $YY1^{mut}$, -748 $YY1^{mut}$)

PAC-1 cells were transiently transfected with 30ng of a luciferase based promoter-reporter plasmid under the explicit control of one of three variations of the -1621bp SM-MHC promoter fragment. SM-MHC promoter: (A.) Wild-Type, (B.) DRD (-1563 $YY1^{mut}$), or (C.) DRD (-1563 $YY1^{mut}$, -748 $YY1^{mut}$) Each promoter-reporter was then treated with increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pBluescript:C/EBPβ (closed circles) and 40ng of the empty vector pSVK3 empty vector, 40ng of pSVK3:YY1 and increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of the empty vector pBluescript (closed squares) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pBlusecript:C/EBPβ and 40ng pSVK3:YY1 (open triangles). Fold activity above baseline. Baseline was removed via the Base/Value method.

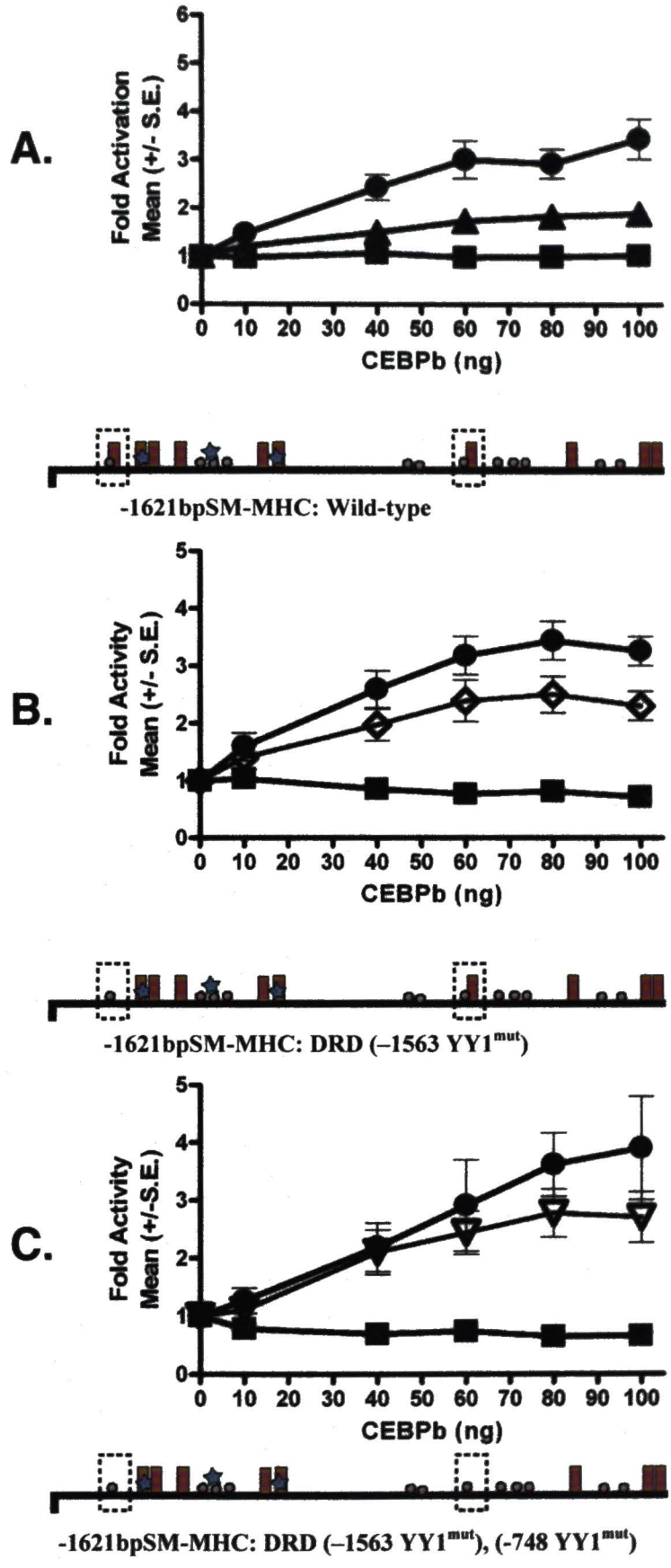
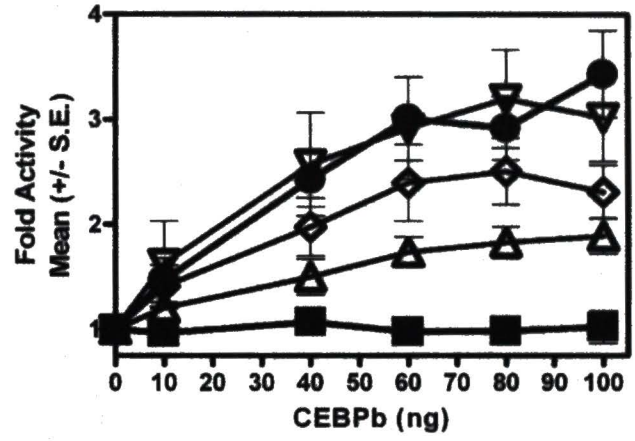

A.
Fold Activation Mean (+/- S.E.)
0 1 2 3 4 5 6
0 10 20 30 40 50 60 70 80 90 100
CEBPb (ng)
-1621bpSM-MHC: Wild-type
B.
Fold Activity Mean (+/- S.E.)
0 1 2 3 4 5
0 10 20 30 40 50 60 70 80 90 100
CEBPb (ng)
-1621bpSM-MHC: DRD (–1563 YY1mut)
C.
Fold Activity Mean (+/-S.E.)
0 1 2 3 4 5
0 10 20 30 40 50 60 70 80 90 100
CEBPb (ng)
-1621bpSM-MHC: DRD (–1563 YY1mut), (-748 YY1mut)

Figure 4: Comparative Analysis: Wild-type vs. DRD (-1563 YY1^{mut}) vs. DRD (-1563 YY1^{mut}, -748 YY1^{mut})

Data representing the various -1621 SM-MHC promoter(s) tested under condition of competition was extracted and graphed together with recorded measurement for the wild-type response to increasing C/EBPβ (closed circles), ½ max YY1 (closed squares) or co-treatment relative to the wild-type (open triangles), –1621p SM-MHC DRD (-1563 YY1^{mut}) (open diamonds) or –1621p SM-MHC DRD (-1563 YY1^{mut}, -748 YY1^{mut}) (open inverted diamonds).



-1621bpSM-MHC: Wild-type

-1621bpSM-MHC: DRD (–1563 $YY1^{mut}$)

-1621bpSM-MHC: DRD (–1563 $YY1^{mut}$), (-748 $YY1^{mut}$)

Figure 5: Testing the competitive regulatory influence between C/EBPβ and YY1 relative to the –1621bp SM-MHC: Wild-type vs. DRD (-1563 YY1^{mut}) vs. DRD (-1563 YY1^{mut}, -748 YY1^{mut})

PAC-1 cells were transiently transfected with 30ng of a luciferase based promoter-reporter plasmid under the explicit control of one of four truncations of the SM-MHC promoter: (A) wild-type, (B) DRD (-1563 YY1^{mut}), (C) DRD (-1563 YY1^{mut}, -748 YY1^{mut}). Each promoter-reporter was then treated 40ng of pBluescript:C/EBPβ and increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of the empty vector pSVK3 empty vector (closed circles) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pSVK3:YY1 and 40ng of the empty vector pBluescript (closed squares) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pSVK3:YY1 and 40ng pBluescript:C/EBPβ (open triangles), –1621p SM-MHC DRD (-1563 YY1^{mut}) (open diamonds) or –1621p SM-MHC DRD (-1563 YY1^{mut}, -748 YY1^{mut}) (open inverted diamonds).

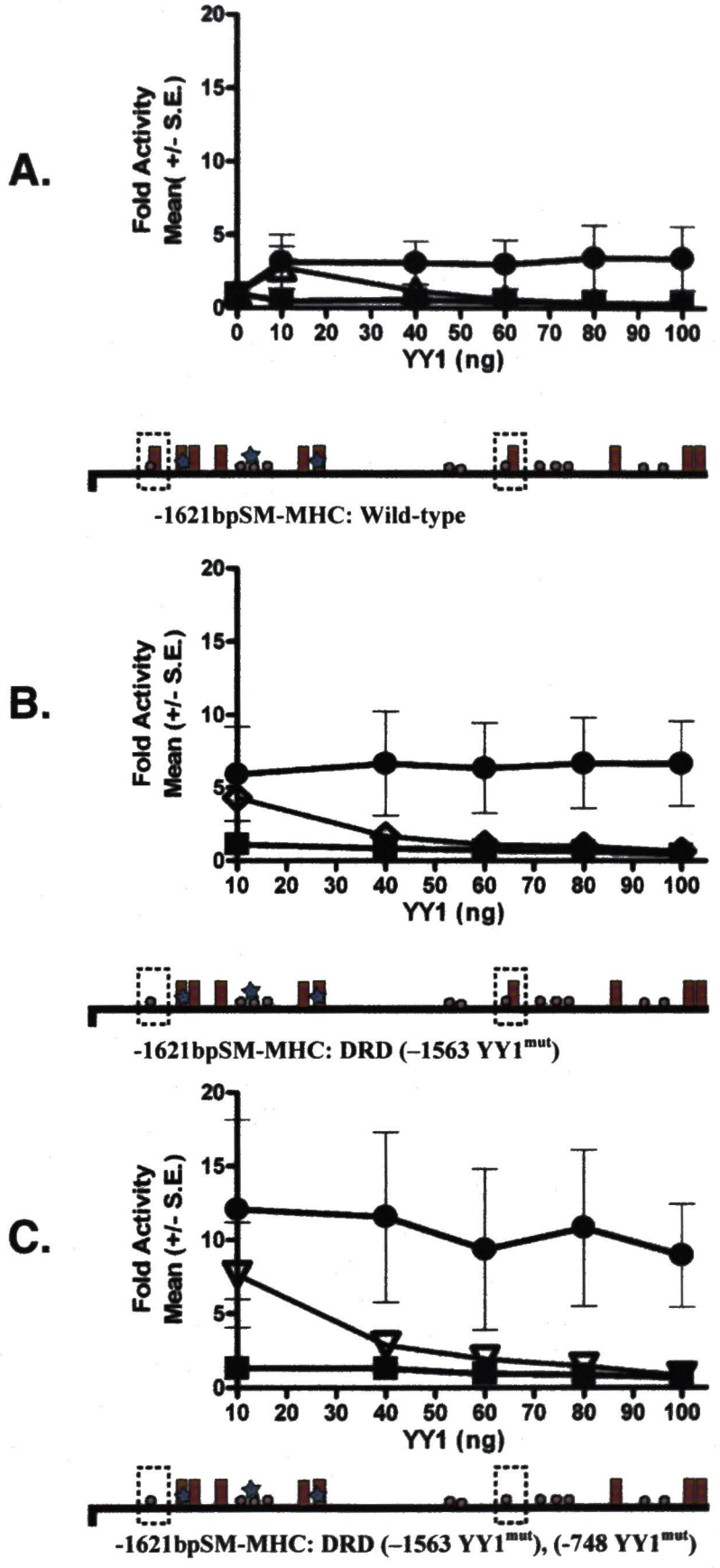
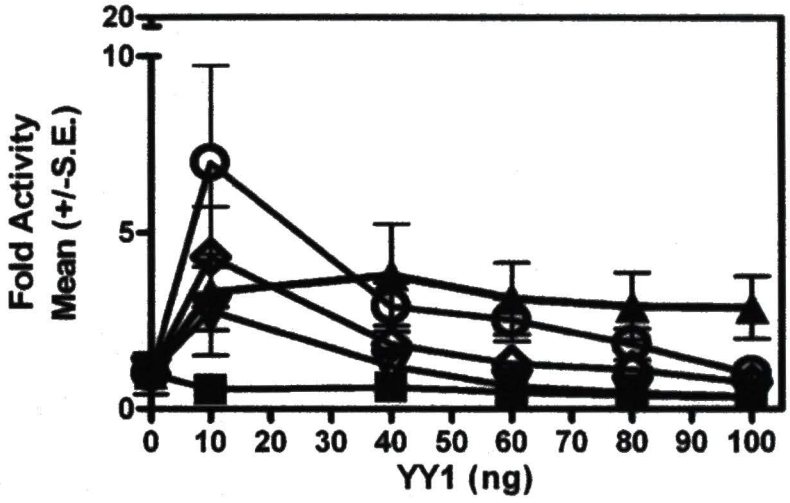

A.
Fold Activity
Mean(+/- S.E.)
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
YY1 (ng)
-1621bpSM-MHC: Wild-type
B.
Fold Activity
Mean (+/- S.E.)
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
YY1 (ng)
-1621bpSM-MHC: DRD (–1563 YY1mut)
C.
Fold Activity
Mean (+/- S.E.)
20
15
10
5
0
10 20 30 40 50 60 70 80 90 100
YY1 (ng)
-1621bpSM-MHC: DRD (–1563 YY1mut), (-748 YY1mut)

Figure 6: Comparative Analysis: Wild-type vs. DRD (-1563 YY1^{mut}) vs. DRD (-1563 YY1^{mut}, -748 YY1^{mut})

Data representing the various -1621 SM-MHC promoter(s) tested under condition of competition was extracted and graphed together with recorded measurement for the wild-type response to increasing C/EBPβ (closed circles), ½ max YY1 (closed squares) or co-treatment relative to the wild-type (open triangles), –1621p SM-MHC DRD (-1563 YY1^{mut}) (open diamonds) or –1621p SM-MHC DRD (-1563 YY1^{mut}, -748 YY1^{mut}) (open inverted diamonds).



-1621bpSM-MHC: Wild-type

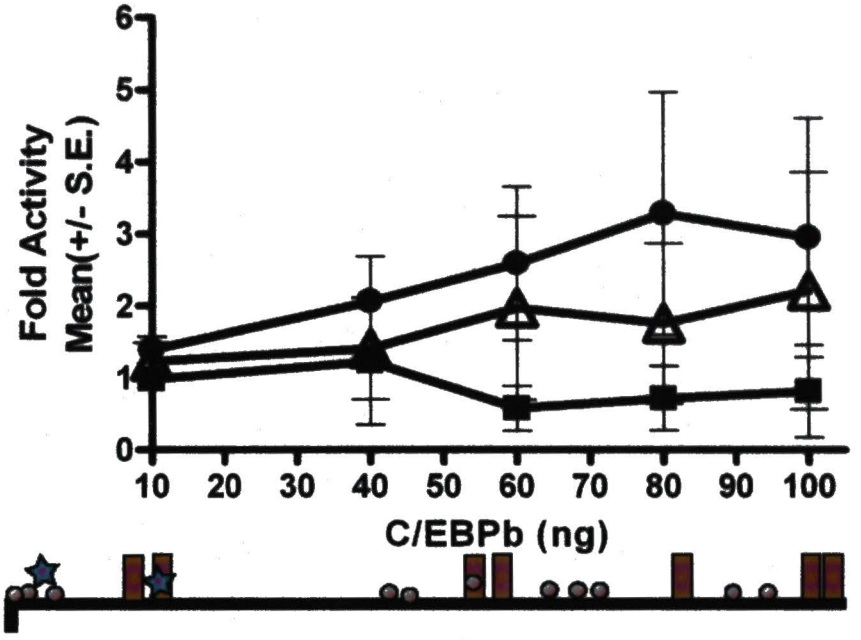
-1621bpSM-MHC: DRD (–1563 $YY1^{mut}$)

-1621bpSM-MHC: DRD (–1563 $YY1^{mut}$), (-748 $YY1^{mut}$)

Figure 7: Testing the competitive regulatory influence between C/EBPβ and YY1 relative to the –1249bp SM-MHC: Wild-type vs. DRD (-748 $YY1^{mut}$)

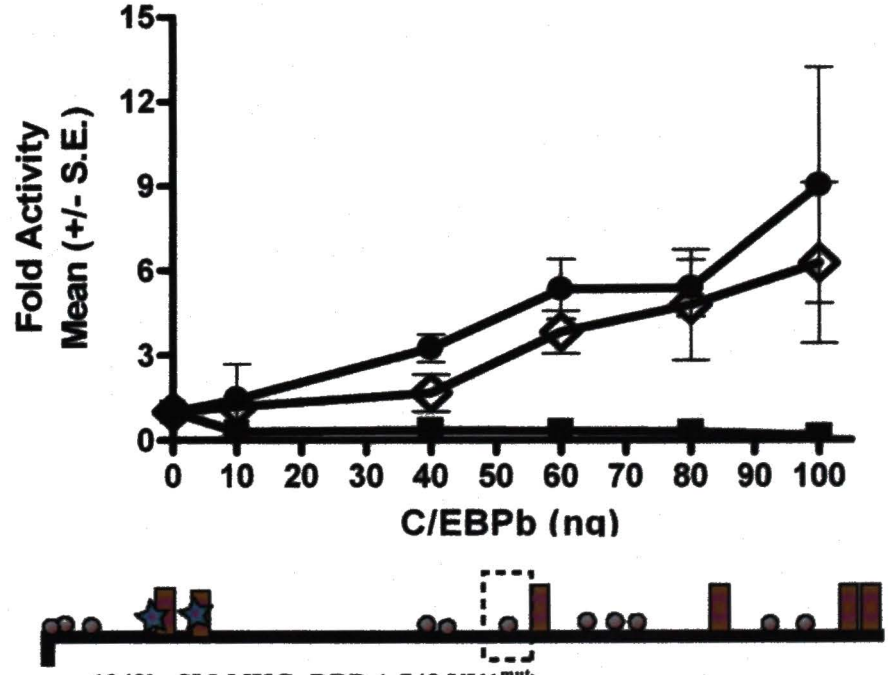
PAC-1 cells were transiently transfected with 30ng of a luciferase based promoter-reporter plasmid under the explicit control of one of three variations of the -1249bp SM-MHC promoter fragment. SM-MHC promoter: (A.) Wild-Type, (B.) DRD (-748 $YY1^{mut}$) Each promoter-reporter was then treated with increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pBluescript:C/EBPβ (closed circles) and 40ng of the empty vector pSVK3 empty vector, 40ng of pSVK3:YY1 and increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of the empty vector pBluescript (closed squares) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pBluescript:C/EBPβ and 40ng pSVK3:YY1 (open triangles). Fold activity above baseline. Baseline was removed via the Base/Value method.

A.



-1249bpSM-MHC: Wild-type

B.



-1249bpSM-MHC: DRD (–748 YY1^{mut})

Figure 8: Comparative Analysis: Wild-type vs. DRD (-748 YY1^{mut})

Data representing the –1249 SM-MHC promoter tested under condition of competition was extracted and graphed together with recorded measurement for the wild-type response to increasing C/EBPβ (closed circles), ½ max YY1 (closed squares) or co-treatment relative to the wild-type (open triangles) or –1249bp SM-MHC DRD (-748 YY1^{mut}) (open diamonds).

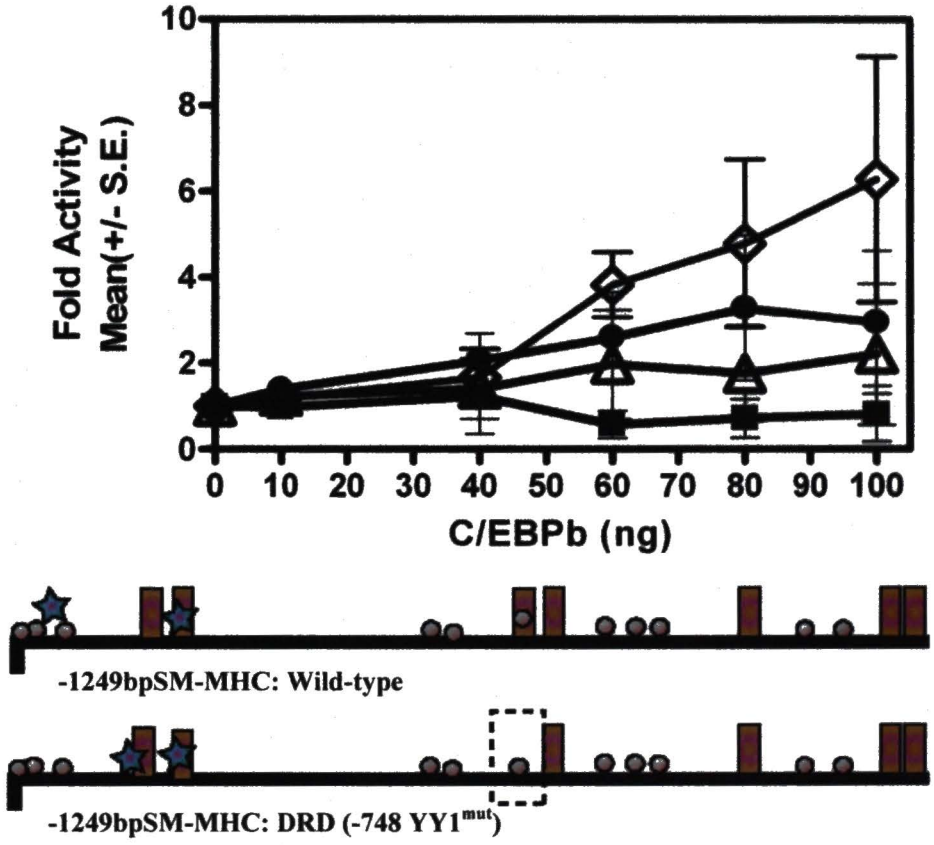
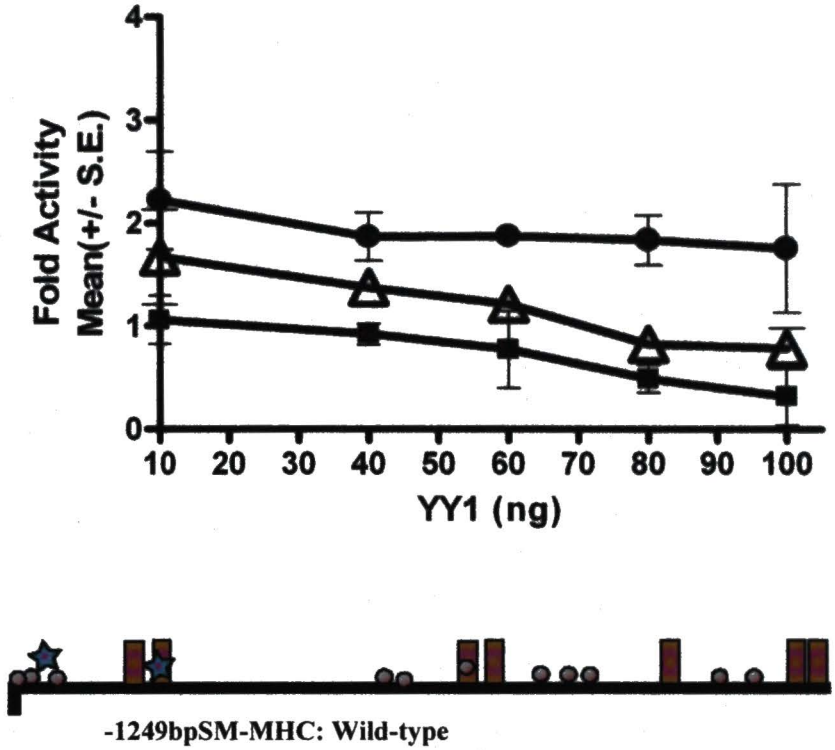

Fold Activity
Mean(+/- S.E.)
10
8
6
4
2
0
0 10 20 30 40 50 60 70 80 90 100
C/EBPb (ng)
-1249bpSM-MHC: Wild-type
-1249bpSM-MHC: DRD (-748 YY1mut)

Figure 9: Testing the competitive regulatory influence between C/EBPβ and YY1 relative to the –1249bp SM-MHC: Wild-type vs. DRD (-748 YY1^{mut})

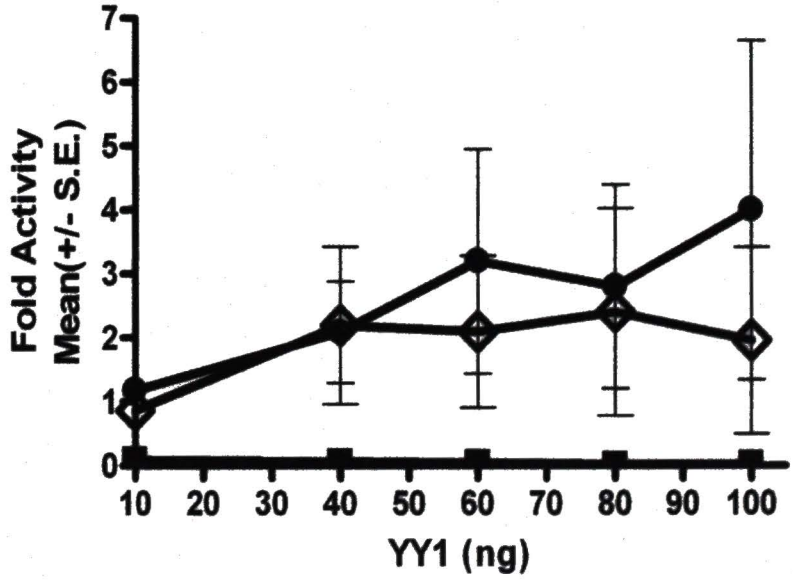
PAC-1 cells were transiently transfected with 30ng of a luciferase based promoter-reporter plasmid under the explicit control of one of four truncations of the SM-MHC promoter: (A) wild-type, (B) DRD (-748 YY1^{mut}). Each promoter-reporter was then treated 40ng of pBluescript:C/EBPβ and increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of the empty vector pSVK3 empty vector (closed circles) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pSVK3:YY1 and 40ng of the empty vector pBluescript (closed squares) or increasing dose (2.5ng, 5ng, 10ng, 20ng, 30ng, 40ng, 50ng, 60ng, 80ng, 100ng) of pSVK3:YY1 and 40ng pBluescript : C/EBPβ (open triangles), –1249p SM-MHC DRD (-748 YY1^{mut}) (open diamonds)

A.



-1249bpSM-MHC: Wild-type

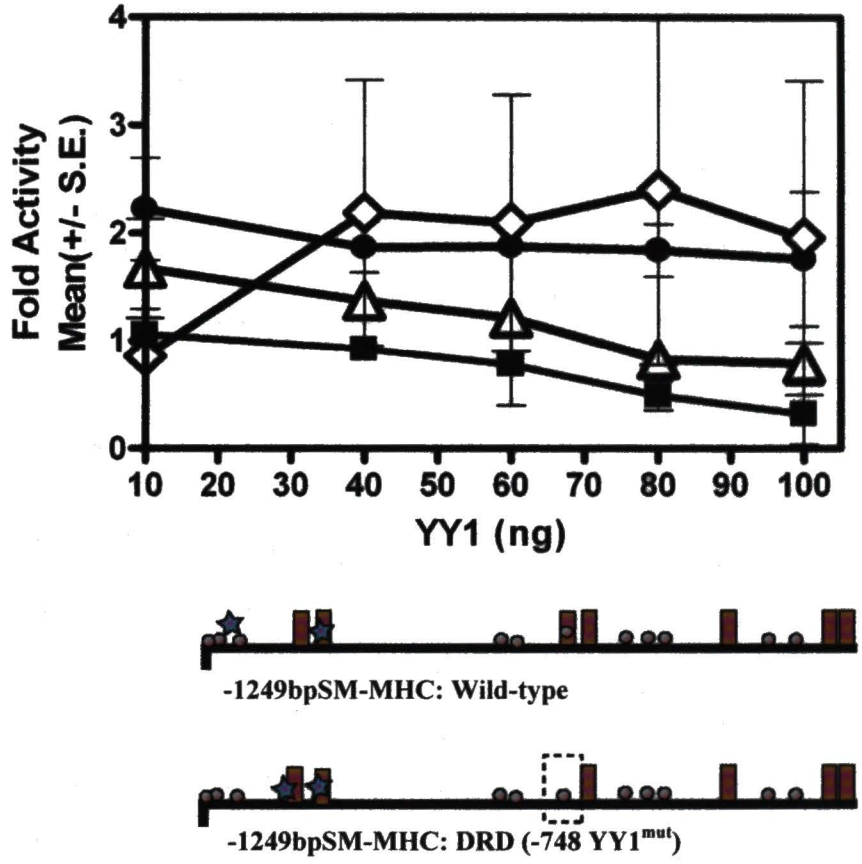
B.



-1249bpSM-MHC: DRD (-748 $YY1^{mut}$)

Figure 10. Comparative Analysis: Wild-type vs. DRD (-748 YY1^{mut})

Data representing the various -1249 SM-MHC promoter(s) tested under condition of competition was extracted and graphed together with recorded measurement for the wild-type response to increasing C/EBPβ (closed circles), ½ max YY1 (closed squares) or co-treatment relative to the wild-type (open triangles), –1249p SM-MHC DRD (-748 YY1^{mut}) (open diamonds).


Fold Activity
Mean(+/- S.E.)
0
1
2
3
4
10
20
30
40
50
60
70
80
90
100
YY1 (ng)
-1249bpSM-MHC: Wild-type
-1249bpSM-MHC: DRD (-748 YY1mut)

CHAPTER IV

DISCUSSION

This study was designed to explore possible mechanisms that control VSMC gene program switching which results in phenotype alteration. This study tested the hypothesis that the repressive action of YY1 regimented C/EBPβ mediated activation of SM-MHC promoter activity by competitive regulation at two discreet sites. The findings demonstrate that: 1.) a distinct competitive regulatory influence exists between YY1 and C/EBPβ from at least two distinct domains; 2.) the overall output of SM-MHC promoter, while modifiable by C/EBPβ, is dominantly regulated by YY1; and 3.) YY1's negative regulatory influence over C/EBPβ increased with promoter length increased, arguing additional YY1 binding sites not specifically explored in this study. While these data support our fundamental hypothesis this chapter will proceed by discussing: i.) the possible means by which YY1 communicates its regulatory influence; ii.) similarities and to previously defined mechanism and the implications of those mechanism and; iii.) the limitations of the current study.

Transcriptional Repression

There are two principle modalities of transcriptional repression, long-range and short-range. Long-range repression is denoted as a repressive influence that spans kilobases of DNA (59, 5). Short-range repression is denoted as a repressive influence that occurs within 1-100bp to directly impede the activity of proximal transcriptional activators (21, 22, 26). It is known that YY1 recruits class-I histone deacetyltransferases (HDAC) as co-repressors (87, 25). Class–I HDAC's are known to function as long-range repressors (18, 66). The mode of YY1 repression has yet to be entirely delineated but the findings of the present investigation strongly suggest for the first time that YY1 function as a short-range repressor.

Short-range repression is well studied in *Drosophila* (21, 22, 26). Several means of short range repression have been suggested: i.) masking; ii.) squelching; and iii.) competition. Masking denotes the ability of a repressor to mask the binding site of a proximal transcriptional activator (29). Squelching denotes the binding of a repressor proximal to a transcriptional activator and blocking the interaction of its transactivation domain with the transcriptional complex (16, 89). Competition denotes when a transcriptional repressor and a transcriptional activator compete for overlapping binding sites (75, 46, 62). We contend our data support a model of competition. The data of the present investigation demonstrate the titratable regulatory influence between C/EBPβ and YY1. We contend this would not be evident, if the means of YY1 repression were masking or squelching, since these modalities predict a discreet on/off event that functions autonomously of the stoichiometry between two factors.

We also extend the possibility that competition between YY1 and a multitude of transcriptional activators may occur. For example, the consensus binding sequence of SRF retains two core YY1 binding sites making it highly likely that YY1 competes with SRF whenever both factors are present. The implications of this possibility are significant since SRF is unique in its specificity to muscle-specific gene expression and YY1 is a specific repressor of muscle-specific gene expression (39, 51, 52). While we did not study SRF explicitly we did explore YY1 mediated competitive regulation with C/EBPβ at two locations within the SM-MHC gene promoter. Importantly, the proximity of the C/EBPβ binding site and the YY1 binding sites differed. These core binding sites for these factors were separated by a single base pair in the DRD(-1563) while the DRD(-748) were separated by 9bp (unpublished data). Both, the proximity of these sites and the probability of steric hindrances argue against co-occupancy at of these domains. Likewise, YY1 is known to deform DNA causing sharp bends away from itself making it unlikely both factors co-occupy these sites (58).

The findings of the present investigation also suggest that YY1 competitive regulation provides repression on multiple-tiers of regulatory control based on its relative concentration. We interpret this in terms of the ability to restore the basal activity of the SM-MHC promoter with varying concentration of YY1 that in turn is dependant upon promoter length. We contend the biphasic repressive response can be separated as follows: i.) the first phase represents competition with elevated C/EBPβ until the effects of C/EBPβ transactivation is silenced and SM-MHC promoter activity is restored to its originally defined basal activity; and ii.) the second phase of repression is likely the result

of further YY1 loading at additional sites to compete with factors, not explicitly investigated in this study, most likely accountable for the basal activity of the promoter.

The Implications of, and Similarities to, Mechanisms Previously Published

Eukaryotes are an aggregation of numerous specialized cell types working together in a concerted effort that benefit the entire organism. Eukaryotes arise from a common progenitor and, each specialized cell is the product of strictly controlled gene expression and numerous cell divisions. These processes follow a strictly controlled, pre-programmed series of phenotypic switching events that make each new cell-type slightly more specialized than the last. This process concludes after each cell attains its terminal adult phenotype where it conducts it's specialized function for the duration of its existence. Unlike most cells, the adult vascular smooth muscle cell maturation process does not restrict these cells to expressing a single terminal phenotype. While these cells typically express a default "contractile" phenotype, the lack of phenotypic restriction allows these cells to stably present alternative phenotypes by expressing one of three phenotype-specific gene programs.

Significant information exists regarding specific factors that drive the expression of VSMC phenotype-specific genes. However, very little is known as to how the VSMC regulates the transition between it's clearly demarcated expression of different gene programs. Clarification can be found in the way *Drosophila melanogaster* regulates distinct patterns of gene expression via a concentration gradient of the transcription factor "Dorsal". This transcription factor is responsible for the dorsal-ventral (DV) patterning in the *Drosophila* embryo and the regulatory control governing the handling of multiple

gene programs (102) and shares a striking resemblance to VSMC phenotype-specific gene program switching.

The transcription factor Dorsal exhibits a nuclear gradient patterning of distribution through out the *Drosophila* embryo and this gradient controls DV patterning and gastrulation by regulating a variety of target genes in a concentration dependent fashion (104). Briefly, nearly 50 genes exhibiting localized expression patterns along the DV axis have been identified, it is estimated that 30 of the 50 genes correspond to direct transcriptional targets of the Dorsal gradient, of which, enhancers for 18 of these genes have been delineated (Markstein, m 2004). These 18 enhancers targeted by Dorsal can be further separated into at least three functional categories: i.) Type-1 enhancers respond to the peak concentration of Dorsal in the ventral regions of the embryo (104); ii.) Type II enhancers respond to the intermediate concentrations of Dorsal in the ventral regions of the presumptive neurogenic ectoderm (103, 105); and iii.) Type-III enhancers are regulated by the lowest concentrations of Dorsal throughout the neurogenic ectoderm (106). Interestingly, while FGF, EGF (Epidermal Growth Factor) and TGFβ each stimulated Dorsal mediated transcription, the Type-I enhancers function to restrict FGF signaling whereas, Type-II enhancers trigger EGF signaling and the Type-III produce a broad TGFβ signaling gradient. Likewise, the genes targeted by Dorsal retain different quantities and type of these enhancements which dictate the threshold responsiveness of each gene (110, 103, 105). Moreover, this pattern of gene expression has clearly defined boundaries evident in the gene expression patterning driven by the Dorsal gradient. Recently, it has been determined that, while the expression pattern of these many genes are driven by a common stimuli, they are spatially restricted by the localized effects of

transcriptional repressors (102). Thus, while all Dorsal responsive gene were capable of being expressed, it was the transcriptional repressors that dictated which genes were expressed.

The regulation of spatially restricted gene expression in *Drosophila* by transcriptional repressors shares striking similarities to the regulatory mechanism that prevent VSMC from expressing more than one phenotype-specific gene program at any given time and provide clarity for how VSMC can tolerate fluctuations in environmental stimuli, known to drive phenotype-switching *in vitro,* without significantly altering the expression profile of the resident gene program *in vivo.*

Currently, it is known that a multitude of transcription factors exist in the nuclear compartment of VSMC in a phenotype-independent fashion. It is unknown, however, how a VSMC differentiates between the many pools of phenotype-specific transcription factors. Currently, it is argued that VSMC selectively express a phenotypic-specific gene program by biasing the nuclear representation of the transcription activators that preferentially target those genes for expression and each phenotypic-specific gene program is target by a distinct subset of transcription factors. (1, 13, 91, 27, 83, 93, 35, 30) We know now that the phenotype-specificity of many factors are actually directed by the concerted efforts of a group of transcription factors. For example, the SRF:GATA:CEBPβ ternary that drives contractile gene expression while the CEBPβ:Ets:NFκB appears to drive the expression of proliferation specific genes (2). It has been fairly well delineated that SRF most likely directs the phenotype specificity to the members of its ternary complex and thus, is considered the limiting factor for contractile-specific gene expression (54). Importantly, SRF has strictly controlled protein

concentrations and if significantly diminished, indirectly negates the regulatory impact of C/EBPβ and GATA (54). As previously identified, an opposing regulatory dynamic exists between YY1 and SRF (3, 39, 51, 52) apparently by virtue of similarities in binding site sequence. We report here for the first time that an additional transcriptional activator that drives contractile-specific gene expression competes with YY1, namely C/EBPβ.

Importantly, SRF is present at sufficient concentrations in the VSMC nucleus to be detectable during each of the phenotypes that the VSMC present (7). As stated before, most if not all contractile-related genes are driven by at least two SRF binding events and the most prototypical of contractile-specific gene markers, smooth muscle myosin heavy chain, is strictly expressed only when the VSMC presents a contractile phenotype (47, 45, 44). Since contractile-specific gene expression does not appear to be leaky, explaining away a clear demarcation in these genes' expression patterns by the diminished probability of their expression seems insufficient. Similar findings have been witnessed by the local effect of spatially restricted transcriptional repressors in *Drosophila* (20).

Furthermore, the VSMC selectively engages the precise expression of numerous gene programs in order to present distinctly different cellular phenotypes. Likewise, while phenotype-switching during development typically occurs in a linearly directed fashion, the predominate VSMC phenotype switching occurs in a circular fashion such that these cells can return to previous expressed phenotypes. Correlating the regulatory control governing gene program switching in *Drosophila* suggest, that while this process may be triggered by extracellular stimuli, the local effects of a transcriptional repressors directs the ability of each gene program to be expressed. Therefore, we contend that it is

reasonable to correlate VSMC gene program switch as similar to the developmentally regulated gene programs of *Drosophila*. Collectively these findings with those of the present investigation argues that as phenotype altering stimuli fluctuates extracellularly, it is not registered as significant until the downstream transcription factors attain sufficient concentration to out compete YY1, otherwise, YY1 prevents the transcription factors from associating with the promoter. Moreover, a strong correlation exist between elevations in YY1 protein pool concentrations and: i.) the repression of contractile related genes; ii.) the induction of proliferation-specific genes; as well as, iii.) entry into cell-cycle. Our data clearly support elevated concentrations of YY1 as being sufficient to repress SM-MHC promoter activity.

Potential Limitations:

It is recognized that these investigations were accomplished despite several limitations that may result in some differences in the interpretation of the observed data. For example, measuring the regulatory behavior of a episomally regulated and expressed promoter-reporter construct have inherent limitations to the applicability of data collected from them.

First, promoter-reporters are regulated outside the control of chromosomal DNA. The regulatory influence conveyed from chromosomal DNA is highly profound and poorly understood. Interpreting data gathered from the behavior of promoter-reporters needs caution and the understanding that greater regulatory influences may be communicated from chromosomally expressed genes. Likewise, the actual measurement of these studies is the relative activity of the luciferase reporter gene product. It is

accepted that correlating relative luciferase activity is a reasonable reflection of overall luciferase transcription driven by the relative activity of the attached promoter. While we contend this to be a reasonable measurement of promoter activity, we must volunteer the possibility that modification to total luciferase production can be effected by means other than that of transcription, for example protein turnover or translational regulation which could possibly skew the observed data.

Second, the treatment we are employing to test SM-MHC response is in the form of a gene dose. However, the overall change in protein concentrations for C/EBPβ and YY1 were not confirmed by western blot due to the technical limitation of transient transfection. Therefore, much of the data interpretation is based on the widely accepted view that the constitutive overexpression of these expression vectors do elevate intracellular protein pools of expressed gene products.

Third, while this study strongly argues a competitive displacement model between C/EBPβ and YY1 it did not positively delineate the binding activities between these two factors.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTION

In conclusion, our findings support a model whereby the concentration of YY1 essentially defines the effective concentration a competitive transcriptional activator must attain in order to influence promoter activation. We contend this type of model address many obscure realities known regarding VSMC transcriptional regulation. This model provides an explanation as to why VSMC cells retain a nuclear constituent of transcription factors not involved in the expression of the resident gene program. Likewise, this model explains how fluctuations in the various pools of transcription factors can occur without altering the overall transcriptional dynamic of the cell. Furthermore, this model establishes discreet boundaries, which if breeched allow a distinct alteration in gene expression profiles without the necessity of completely exhausting the nuclear environment of all irrelevant transcription factors.

It should be noted that this study was limited to only one transcription activator, namely C/EBPβ and requires further study on additional factors, yet, the regulatory significance of C/EBPβ is substantial in that C/EBPβ must redirect its transactivation potential between the expression of two mutually exclusive gene programs. The model explains that if YY1 concentrations are overly abundant SRF dependent gene expression

is silenced, thus, the association of C/EBPβ with the SRF:GATA ternary is likely not to associate with the promoter of contractile related genes. However, the ternary thought to drive the expression of proliferation-specific genes is composed of transcription factors whose immediate DNA binding consensus shares no significant similarity to YY1's thus, likely not as extensively under the competitive control of YY1.

Much remains to be elucidated to fully define the mechanism of competitive regulation. Most of this work needs to focus on the proteolytic degradation of YY1. Significant information exist that suggest a proteolytic fragment of YY1, rather than the full length protein, is responsible for regulating gene expression during the contractile phenotype. Likewise, the mechanisms previously defined to target YY1 for proteolytic degradation are Ca^{+2} sensitive proteases. This is intriguing since many contend that Ca^{2+} mismanagement is the root of phenotypic malfunction involved in most heart and vascular disease.

In closing, it is known that the initialization and progression of hypertrophic disease states in both the heart and vasculature arises from the re-expression of fetal contractile driven by elevations in key transcription factors that preferentially target embryonic genes for re-expression. Interestingly, the binding recognition sequences for these factors (NFAT, MEF, GATA, Nkx) share the least similarities with YY1. It is intriguing to postulate that the re-expression of embryonic genes targeted by these factors occurs because each factor, according to our model, is potentially regulated outside of YY1's competitive regulatory control and thus, may be expressed autonomously from the negative regulatory supervision of YY1.

REFERENCES

1. Andreani, M., et al., *Transcriptional regulation of inflammatory secreted phospholipases A(2).* Biochim Biophys Acta, 2000. **1488**(1-2): p. 149-58.

2. Antonio, V., et al., *Transcriptional regulation of the rat type IIA phospholipase A2 gene by cAMP and interleukin-1beta in vascular smooth muscle cells: interplay of the CCAAT/enhancer binding protein (C/EBP), nuclear factor-kappaB and Ets transcription factors.* Biochem J, 2002. **368**(Pt 2): p. 415-24.

3. □asutani, H., et al., *Autonomously binding protein detected on ets box of c-fos serum response element in proliferating cells.* Gene Expr, 1999. **8**(1): p. 33-42.

4. Bar, H., et al., *Upregulation of embryonic transcription factors in right ventricular hypertrophy.* Basic Res Cardiol, 2003. **98**(5): p. 285-94.

5. Barolo, S. and M. Levine, *hairy mediates dominant repression in the Drosophila embryo.* Embo J, 1997. **16**(10): p. 2883-91.

6. Becker, C., et al., *Regulation of IL-12 p40 promoter activity in primary human monocytes: roles of NF-kappaB, CCAAT/enhancer-binding protein beta, and PU.1 and identification of a novel repressor element (GA-12) that responds to IL-4 and prostaglandin E(2).* J Immunol, 2001. **167**(5): p. 2608-18.

7. Browning, C.L., et al., *The developmentally regulated expression of serum response factor plays a key role in the control of smooth muscle-specific genes.* Dev Biol, 1998. **194**(1): p. 18-37.

8. Camoretti-Mercado, B., et al., *Physiological control of smooth muscle-specific gene expression through regulated nuclear translocation of serum response factor.* J Biol Chem, 2000. **275**(39): p. 30387-93.

9. Carson, J.A., et al., *Smooth muscle gamma-actin promoter regulation by RhoA and serum response factor signaling.* Biochim Biophys Acta, 2003. **1628**(2): p. 133-9.

10. Chen, W.T., J.M. Chen, and S.C. Mueller, *Coupled expression and colocalization of 140K cell adhesion molecules, fibronectin, and laminin during morphogenesis and cytodifferentiation of chick lung cells.* J Cell Biol, 1986. **103**(3): p. 1073-90.

11. Chien, R., *Signaling mechanisms for the activation of an embryonic gene program during the hypertrophy of cardiac ventricular muscle.* Basic Res Cardiol, 1992. **87 Suppl 2**: p. 49-58.

12. Couturier, C., et al., *Protein kinase A-dependent stimulation of rat type II secreted phospholipase A(2) gene transcription involves C/EBP-beta and -delta in vascular smooth muscle cells.* Arterioscler Thromb Vasc Biol, 2000. **20**(12): p. 2559-65.

13. Degterev, A. and J.A. Foster, *The role of NF-1 factors in regulation of elastin gene transcription.* Matrix Biol, 1999. **18**(3): p. 295-307.

14. Du, K.L., et al., *Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation.* Mol Cell Biol, 2003. **23**(7): p. 2425-37.

15. Dzau, V.J. and G.H. Gibbons, *Cell biology of vascular hypertrophy in systemic hypertension.* Am J Cardiol, 1988. **62**(11): p. 30G-35G.

16. Farmer, G., et al., *Transcriptional repression by p53 involves molecular interactions distinct from those with the TATA box binding protein.* Nucleic Acids Res, 1996. **24**(21): p. 4281-8.

17. Fischer-Dzoga, K., *Cellular proliferation, cellular death and atherosclerosis.* Artery, 1979. **5**(3): p. 222-36.

18. Forsberg, E.C. and E.H. Bresnick, *Histone acetylation beyond promoters: long-range acetylation patterns in the chromatin world.* Bioessays, 2001. **23**(9): p. 820-30.

19. Geary, R.L., et al., *Time course of cellular proliferation, intimal hyperplasia, and remodeling following angioplasty in monkeys with established atherosclerosis. A nonhuman primate model of restenosis.* Arterioscler Thromb Vasc Biol, 1996. **16**(1): p. 34-43.

20. Goltsev, Y., et al., *Different combinations of gap repressors for common stripes in Anopheles and Drosophila embryos.* Dev Biol, 2004. **275**(2): p. 435-46.

21. Gray, S. and M. Levine, *Short-range transcriptional repressors mediate both quenching and direct repression within complex loci in Drosophila.* Genes Dev, 1996. **10**(6): p. 700-10.

22. Gray, S., P. Szymanski, and M. Levine, *Short-range repression permits multiple enhancers to function autonomously within a complex promoter.* Genes Dev, 1994. **8**(15): p. 1829-38.

23. Grove, M. and M. Plumb, *C/EBP, NF-kappa B, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1 alpha immediate-early gene.* Mol Cell Biol, 1993. **13**(9): p. 5276-89.

24. Harker, L.A., *Role of platelets and thrombosis in mechanisms of acute occlusion and restenosis after angioplasty.* Am J Cardiol, 1987. **60**(3): p. 20B-28B.

25. He, G. and D.M. Margolis, *Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat.* Mol Cell Biol, 2002. **22**(9): p. 2965-73.

26. Hewitt, G.F., et al., *Transcriptional repression by the Drosophila giant protein: cis element positioning provides an alternative means of interpreting an effector gradient.* Development, 1999. **126**(6): p. 1201-10.

27. Hill-Eubanks, D.C., et al., *NFAT regulation in smooth muscle.* Trends Cardiovasc Med, 2003. **13**(2): p. 56-62.

28. Hiromura, M., et al., *YY1 is regulated by O-linked N-acetylglucosaminylation (O-glcNAcylation).* J Biol Chem, 2003. **278**(16): p. 14046-52.

29. Hsieh, J.J. and S.D. Hayward, *Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2.* Science, 1995. **268**(5210): p. 560-3.

30. Hu, B., et al., *CCAAT/enhancer-binding protein beta isoforms and the regulation of alpha-smooth muscle actin gene expression by IL-1 beta.* J Immunol, 2004. **173**(7): p. 4661-8.

31. Ikegawa, R., et al., *Evidence for pepstatin-sensitive conversion of porcine big endothelin-1 to endothelin-1 by the endothelial cell extract.* Biochem Biophys Res Commun, 1990. **167**(2): p. 860-6.

32. Ip, J.H., et al., *Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation.* J Am Coll Cardiol, 1990. **15**(7): p. 1667-87.

33. Ip, J.H., et al., *The role of platelets, thrombin and hyperplasia in restenosis after coronary angioplasty.* J Am Coll Cardiol, 1991. **17**(6 Suppl B): p. 77B-88B.

34. Kahn, J.D., *Topological effects of the TATA box binding protein on minicircle DNA and a possible thermodynamic linkage to chromatin remodeling.* Biochemistry, 2000. **39**(13): p. 3520-4.

35. Katoh, Y., et al., *MEF2B is a component of a smooth muscle-specific complex that binds an A/T-rich element important for smooth muscle myosin heavy chain gene expression.* J Biol Chem, 1998. **273**(3): p. 1511-8.

36. Kumon, Y., et al., *Transcriptional regulation of serum amyloid A1 gene expression in human aortic smooth muscle cells involves CCAAT/enhancer binding proteins (C/EBP) and is distinct from HepG2 cells.* Scand J Immunol, 2002. **56**(5): p. 504-11.

37. Kurisaki, K., et al., *Nuclear factor YY1 inhibits transforming growth factor beta- and bone morphogenetic protein-induced cell differentiation.* Mol Cell Biol, 2003. **23**(13): p. 4494-510.

38. Lee, J.S., et al., *Relief of YY1 transcriptional repression by adenovirus E1A is mediated by E1A-associated protein p300.* Genes Dev, 1995. **9**(10): p. 1188-98.

39. Lee, T.C., Y. Shi, and R.J. Schwartz, *Displacement of BrdUrd-induced YY1 by serum response factor activates skeletal alpha-actin transcription in embryonic myoblasts.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9814-8.

40. Lepore, J.J., et al., *GATA-6 regulates genes promoting synthetic functions in vascular smooth muscle cells.* Arterioscler Thromb Vasc Biol, 2005. **25**(2): p. 309-14.

41. Lever, A.F., *Slow pressor mechanisms in hypertension: a role for hypertrophy of resistance vessels?* J Hypertens, 1986. **4**(5): p. 515-24.

42. Lilly, B., E.N. Olson, and M.C. Beckerle, *Identification of a CArG box-dependent enhancer within the cysteine-rich protein 1 gene that directs expression in arterial but not venous or visceral smooth muscle cells.* Dev Biol, 2001. **240**(2): p. 531-47.

43. Liu, Z., et al., *Blockade of nuclear factor of activated T cells activation signaling suppresses balloon injury-induced neointima formation in a rat carotid artery model.* J Biol Chem, 2005.

44. Madsen, C.S., et al., *Smooth muscle-specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5'-flanking and first intronic DNA sequence.* Circ Res, 1998. **82**(8): p. 908-17.

45. Madsen, C.S., C.P. Regan, and G.K. Owens, *Interaction of CArG elements and a GC-rich repressor element in transcriptional regulation of the smooth muscle myosin heavy chain gene in vascular smooth muscle cells.* J Biol Chem, 1997. **272**(47): p. 29842-51.

46. Mailly, F., et al., *The human cut homeodomain protein can repress gene expression by two distinct mechanisms: active repression and competition for binding site occupancy.* Mol Cell Biol, 1996. **16**(10): p. 5346-57.

47. Manabe, I. and G.K. Owens, *CArG elements control smooth muscle subtype-specific expression of smooth muscle myosin in vivo.* J Clin Invest, 2001. **107**(7): p. 823-34.

48. Mano, T., et al., *Reversal of GATA-6 downregulation promotes smooth muscle differentiation and inhibits intimal hyperplasia in balloon-injured rat carotid artery.* Circ Res, 1999. **84**(6): p. 647-54.

49. Manukhin, B.N., *[Analysis ligand-receptor interactions from the molecular to the organism levels].* Ross Fiziol Zh Im I M Sechenova, 2000. **86**(9): p. 1220-32.

50. Manukhin, B.N., et al., *[Quantitative analysis of ligand-receptor interactions in physiological experiments].* Ross Fiziol Zh Im I M Sechenova, 1998. **84**(10): p. 1049-60.

51. Martin, K.A., et al., *A competitive mechanism of CArG element regulation by YY1 and SRF: implications for assessment of Phox1/MHox transcription factor interactions at CArG elements.* DNA Cell Biol, 1997. **16**(5): p. 653-61.

52. Masutani, H., et al., *Autonomously binding protein detected on ets box of c-fos serum response element in proliferating cells.* Gene Expr, 1999. **8**(1): p. 33-42.

53. McCaffrey, T.A., *TGF-betas and TGF-beta receptors in atherosclerosis.* Cytokine Growth Factor Rev, 2000. **11**(1-2): p. 103-14.

54. Miano, J.M., *Serum response factor: toggling between disparate programs of gene expression.* J Mol Cell Cardiol, 2003. **35**(6): p. 577-93.

55. Miano, J.M., et al., *Serum response factor-dependent regulation of the smooth muscle calponin gene.* J Biol Chem, 2000. **275**(13): p. 9814-22.

56. Mietus-Snyder, M., C.K. Glass, and R.E. Pitas, *Transcriptional activation of scavenger receptor expression in human smooth muscle cells requires AP-1/c-Jun and C/EBPbeta: both AP-1 binding and JNK activation are induced by phorbol esters and oxidative stress.* Arterioscler Thromb Vasc Biol, 1998. **18**(9): p. 1440-9.

57. Nagai, R., K. Kowase, and M. Kurabayashi, *Transcriptional regulation of smooth muscle phenotypic modulation.* Ann N Y Acad Sci, 2000. **902**: p. 214-22; discussion 222-3.

58. Natesan, S., et al., *Transcriptional squelching re-examined.* Nature, 1997. **390**(6658): p. 349-50.

59. Nibu, Y., H. Zhang, and M. Levine, *Local action of long-range repressors in the Drosophila embryo.* Embo J, 2001. **20**(9): p. 2246-53.

60. Nunez, B.S. and W.V. Vedeckis, *Characterization of promoter 1B in the human glucocorticoid receptor gene.* Mol Cell Endocrinol, 2002. **189**(1-2): p. 191-9.

61. Oei, S.L. and Y. Shi, *Poly(ADP-ribosyl)ation of transcription factor Yin Yang 1 under conditions of DNA damage.* Biochem Biophys Res Commun, 2001. **285**(1): p. 27-31.

62. Ohkuma, Y., et al., *Engrailed, a homeodomain protein, can repress in vitro transcription by competition with the TATA box-binding protein transcription factor IID.* Proc Natl Acad Sci U S A, 1990. **87**(6): p. 2289-93.

63. Ostrom, R.S., et al., *Localization of adenylyl cyclase isoforms and G protein-coupled receptors in vascular smooth muscle cells: expression in caveolin-rich and noncaveolin domains.* Mol Pharmacol, 2002. **62**(5): p. 983-92.

64. Owens, G.K., P.S. Rabinovitch, and S.M. Schwartz, *Smooth muscle cell hypertrophy versus hyperplasia in hypertension.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7759-63.

65. Patten, M., W.E. Hartogensis, and C.S. Long, *Interleukin-1beta is a negative transcriptional regulator of alpha1-adrenergic induced gene expression in cultured cardiac myocytes.* J Biol Chem, 1996. **271**(35): p. 21134-41.

66. Perrod, S. and S.M. Gasser, *Long-range silencing and position effects at telomeres and centromeres: parallels and differences.* Cell Mol Life Sci, 2003. **60**(11): p. 2303-18.

67. Peters, B., et al., *Protein-DNA interactions in the epsilon-globin gene silencer.* J Biol Chem, 1993. **268**(5): p. 3430-7.

68. Pizzorno, M.C., *Nuclear cathepsin B-like protease cleaves transcription factor YY1 in differentiated cells.* Biochim Biophys Acta, 2001. **1536**(1): p. 31-42.

69. Proweller, A., W.S. Pear, and M.S. Parmacek, *Notch signaling represses myocardin-induced smooth muscle cell differentiation.* J Biol Chem, 2005. **280**(10): p. 8994-9004.

70. Qin, C., et al., *Estrogen up-regulation of p53 gene expression in MCF-7 breast cancer cells is mediated by calmodulin kinase IV-dependent activation of a nuclear factor kappaB/CCAAT-binding transcription factor-1 complex.* Mol Endocrinol, 2002. **16**(8): p. 1793-809.

71. Qiu, P., X.H. Feng, and L. Li, *Interaction of Smad3 and SRF-associated complex mediates TGF-beta1 signals to regulate SM22 transcription during myofibroblast differentiation.* J Mol Cell Cardiol, 2003. **35**(12): p. 1407-20.

72. Rezai-Zadeh, N., et al., *Targeted recruitment of a histone H4-specific methyltransferase by the transcription factor YY1.* Genes Dev, 2003. **17**(8): p. 1019-29.

73. Rosendorff, C., *Vascular hypertrophy in hypertension: role of the renin-angiotensin system.* Mt Sinai J Med, 1998. **65**(2): p. 108-17.

74. Rothman A, K.T., Taubman MB, Berk BC, Smith CWJ, Nadal-Ginard B, *Development and Characterization of a Cloned Rat Pulmonary Arterial Smooth Muscle Cell Line That Maintains Differentiated Properties Through Multiple Subcultures.* Circulation, 1992. **86**: p. 1977-1986.

75. Saller, E. and M. Bienz, *Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription.* EMBO Rep, 2001. **2**(4): p. 298-305.

76. Santiago, F.S., et al., *Induction of the transcriptional repressor Yin Yang-1 by vascular cell injury. Autocrine/paracrine role of endogenous fibroblast growth factor-2.* J Biol Chem, 2001. **276**(44): p. 41143-9.

77. Schlisio, S., et al., *Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function.* Embo J, 2002. **21**(21): p. 5775-86.

78. Shi, Y., J.S. Lee, and K.M. Galvin, *Everything you have ever wanted to know about Yin Yang 1.* Biochim Biophys Acta, 1997. **1332**(2): p. F49-66.

79. Shi, Z., et al., *YY1 is involved in RANKL-induced transcription of the tartrate-resistant acid phosphatase gene in osteoclast differentiation.* Gene, 2004. **343**: p. 117-26.

80. Sobue, K., K. Hayashi, and W. Nishida, *Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation.* Mol Cell Biochem, 1999. **190**(1-2): p. 105-18.

81. Solway, J., et al., *Structure and expression of a smooth muscle cell-specific gene, SM22 alpha.* J Biol Chem, 1995. **270**(22): p. 13460-9.

82. Stathopoulos, A. and M. Levine, *Dorsal gradient networks in the Drosophila embryo.* Dev Biol, 2002. **246**(1): p. 57-67.

83. Suzuki, E., et al., *Angiotensin II induces myocyte enhancer factor 2- and calcineurin/nuclear factor of activated T cell-dependent transcriptional activation in vascular myocytes.* Circ Res, 2002. **90**(9): p. 1004-11.

84. Suzuki, E., et al., *Myocyte enhancer factor 2 mediates vascular inflammation via the p38-dependent pathway.* Circ Res, 2004. **95**(1): p. 42-9.

85. Suzuki, T., R. Nagai, and Y. Yazaki, *Mechanisms of transcriptional regulation of gene expression in smooth muscle cells.* Circ Res, 1998. **82**(12): p. 1238-42.

86. Teng, X., et al., *C/EBP-beta mediates iNOS induction by hypoxia in rat pulmonary microvascular smooth muscle cells.* Circ Res, 2002. **90**(2): p. 125-7.

87. Thomas, M.J. and E. Seto, *Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key?* Gene, 1999. **236**(2): p. 197-208.

88. Tompa, P., R. Toth-Boconadi, and P. Friedrich, *Frequency decoding of fast calcium oscillations by calpain.* Cell Calcium, 2001. **29**(3): p. 161-70.

89. Um, M., C. Li, and J.L. Manley, *The transcriptional repressor even-skipped interacts directly with TATA-binding protein.* Mol Cell Biol, 1995. **15**(9): p. 5007-16.

90. Walowitz, J.L., et al., *Proteolytic regulation of the zinc finger transcription factor YY1, a repressor of muscle-restricted gene expression.* J Biol Chem, 1998. **273**(12): p. 6656-61.

91. Walsh, K. and A. Takahashi, *Transcriptional regulation of vascular smooth muscle cell phenotype.* Z Kardiol, 2001. **90 Suppl 3**: p. 12-6.

92. Wang, L., et al., *Mechanical strain inhibits airway smooth muscle gene transcription via protein kinase C signaling.* Am J Respir Cell Mol Biol, 2004. **31**(1): p. 54-61.

93. Wang, S.X., et al., *Cell cycle-mediated regulation of smooth muscle alpha-actin gene transcription in fibroblasts and vascular smooth muscle cells involves multiple adenovirus E1A-interacting cofactors.* J Biol Chem, 2005. **280**(7): p. 6204-14.

94. Wei, L., et al., *beta1 integrin and organized actin filaments facilitate cardiomyocyte-specific RhoA-dependent activation of the skeletal alpha-actin promoter.* Faseb J, 2001. **15**(3): p. 785-96.

95. Yao, Y.L., W.M. Yang, and E. Seto, *Regulation of transcription factor YY1 by acetylation and deacetylation.* Mol Cell Biol, 2001. **21**(17): p. 5979-91.

96. Ye, J., et al., *The nuclear factor YY1 suppresses the human gamma interferon promoter through two mechanisms: inhibition of AP1 binding and activation of a silencer element.* Mol Cell Biol, 1996. **16**(9): p. 4744-53.

97. Yin, F. and B.P. Herring, *GATA-6 can act as a positive or negative regulator of smooth muscle-specific gene expression.* J Biol Chem, 2005. **280**(6): p. 4745-52.

98. Yoshida, T., et al., *Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes.* Circ Res, 2003. **92**(8): p. 856-64.

99. Young, J.L., P. Libby, and U. Schonbeck, *Cytokines in the pathogenesis of atherosclerosis.* Thromb Haemost, 2002. **88**(4): p. 554-67.

100. Zabel, M.D., et al., *Yin Yang 1, Oct1, and NFAT-4 form repeating, cyclosporin-sensitive regulatory modules within the murine CD21 intronic control region.* J Immunol, 2002. **168**(7): p. 3341-50.

101. Bagga, R., J.A. Armstrong, and B.M. Emerson, *Role of chromatin structure and distal enhancers in tissue-specific transcriptional regulation in vitro.* Cold Spring Harb Symp Quant Biol, 1998. **63**: p. 569-76.

102. Huang, A.M., J. Rusch, and M. Levine, *An anteroposterior Dorsal gradient in the Drosophila embryo.* Genes Dev, 1997. **11**(15): p. 1963-73.

103. Ip, Y.T., et al., *The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the Drosophila embryo.* Genes Dev, 1992. **6**(9): p. 1728-39.

104. Markstein, M., et al., *A regulatory code for neurogenic gene expression in the Drosophila embryo.* Development, 2004. **131**(10): p. 2387-94.

105. Papatsenko, D. and M. Levine, *Quantitative analysis of binding motifs mediating diverse spatial readouts of the Dorsal gradient in the Drosophila embryo.* Proc Natl Acad Sci U S A, 2005. **102**(14): p. 4966-71.

106. Podos, S.D. and E.L. Ferguson, *Morphogen gradients: new insights from DPP.* Trends Genet, 1999. **15**(10): p. 396-402.

107. Stathopoulos, A. and M. Levine, *Dorsal gradient networks in the Drosophila embryo.* Dev Biol, 2002. **246**(1): p. 57-67.

108. Stathopoulos, A., et al., *pyramus and thisbe: FGF genes that pattern the mesoderm of Drosophila embryos.* Genes Dev, 2004. **18**(6): p. 687-99.

109. Stathopoulos, A. and M. Levine, *Whole-genome analysis of Drosophila gastrulation.* Curr Opin Genet Dev, 2004. **14**(5): p. 477-84.

110. Jiang, J. and M. Levine, *Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen.* Cell, 1993. **72**(5): p. 741-52.