Interplay of Myc and Max with Epigenetic Regulator Bmi1

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ABSTRACT

The polycomb group protein Bmi1 is an epigenetic regulator essential for the proliferation of many types of cancers. By impeding the expression of the tumor suppressor p53, Bmi1 is able to prevent apoptosis and senescence. c-Myc, a prominent oncogene, cooperates with Bmi1 to stimulate cellular transformation and tumorigenesis. Further investigation of the basic biological interplay between Bmi1 and c-Myc is crucial for our understanding of their tumorigenic ability. In my project I demonstrated that c-Myc and Bmi1 directly interact with each other and form nuclear foci. Overexpression of Max, a known partner of Myc, disrupts the Bmi1 and c-Myc interaction and prevents the formation of nuclear foci. Similar results were obtained with another member of the Myc family, L-Myc. Additionally, I found that HDAC3interacts and co-localizes with Myc. HDAC3 also forms nuclear foci with Bmi1 and the addition of Max abrogates this interaction. In addition to the well-established role of Bmi1 as an epigenetic regulator, it has been recently shown that Bmi1 is part of an E3 ubiquitin-ligase complex, known as the Bmi1/RING1A or B complex. This complex controlsthe stability of many proteins. I showed that Bmi1 induces an L-Myc ubiquitination which in turn causes the degradation of L-Myc. This data proposes a novel regulatory mechanism for the stability of the Myc oncogenes. The results of this thesis provide new insight into the basic biochemical interplay ofBmi1 with Myc and Max.

RÉSUMÉ

La protéinede groupe polycomb Bmi1 est essentielle pour la prolifération de nombreux types de cancers. En freinant l'expression du suppresseur de tumeur p53, Bmi1 est capable de prévenir l'apoptose et la sénescence. c-Myc, une autre oncogène, s'associe à Bmi1 pour stimuler la transformation et la tumorigenèse. Une enquête plus approfondie de l'interaction biologique fondamentale entre Bmi1 et c-Myc est crucial pour notre compréhension de leur capacité à promouvoir la tumorigène. Dans mon projet, j'ai démontré que c-Myc et Bmi1 interagissent directement et forment des foyers nucléaires. La surexpression de Max, un partenaire connu de Myc, perturbe l'interaction entre Bmi1 et c-Myc et empêche la formation de foyers nucléaires. Des résultats similaires ont été obtenus avec un autre membre de la famille Myc, L-Myc. En outre, j'ai constaté que HDAC3 interagi et se co-localise avec Myc. HDAC3 forme aussi des foyers nucléaires avec Bmi1 et l'ajout de Max abroge cette interaction. En plus du rôle bien établi de Bmi1 comme un régulateur épigénétique, il a été démontré récemment que Bmi1 fait partie d'une ubiquitine-ligase E3 complexe, connu sous le nom complexe Bmi1/RING1A ou B. Ce complexe contrôle la stabilité de nombreuses protéines. J'ai démontré que Bmi1 induit l'ubiquitination de L-Myc qui à son tour provoque la dégradation de celle-ci. Ces données proposent un nouveau mécanisme de réglementation pour la stabilité des oncogènes Myc.

Les résultats de cette thèse fournissent un nouvel éclairage sur l'interaction biochimique de Bmi1 avec Myc et Max.

PREFACE

I have chosen to write a traditional style thesis according to McGill University Guidelines for Thesis Preparation.

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PUBLICATIONS

1. Kim G., **Gocevski G.**, Wu CJ, and Yang XJ (2010-11). Dietary, metabolic and potentially environmental modulation of the lysine acetylation machinery. *Int J Cell Biol.*v.2010; 2010: 632739

2. Tahmasebi S, Savage P, **Gocevski G.**, Xiao L, and Yang XJ (2012). Differential regulation of somatic cell reprogramming and adipocyte differentiation by sumoylation. *J Biol Chem*, under revision.

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CONTRIBUTIONS OF OTHERS

Dr. Soroush Tahmasebi

- First discoveredc-Myc and Bmi1nuclear foci.
- Made the following expression vectors: Flagc-Myc Mutants and HA-Max

Linya You

- Prepared the following expression vector Flag L-Myc.

Nadine Pelletier

- Engineered the following expression vector GFP-Bmi1.

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Regulating the stability of L-Myc by Bmi1

LIST OF ABBREVIATIONS

[^{35}S]	Radioactive sulphur
5-AZA-dC	5-aza-2-deoxycytidine
aa	Amino acid
ADP	Adenosine diphosphate
Akt	Also known as Protein Kinase B
Amp	Amperage
ANOVA	Analysis of variance
Arf	ADP Ribosylation Factors
Arf-Bp1	ARF-binding protein 1
Arg	Arginine
B/Cdk1	B/cyclin-dependent kinases
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
bHLHZIP	Basic helix -loop-helix-zipper
Bmi1	B-cell-specific Moloney murine leukemia virus integration site 1
BR	Basic region
BSA	Bovine serum albumin
CBP	CREB-binding protein
CBX#	Chromobox homologue #= 2 or 4 or 8
CDC25A	Cell division cycle 25A
CDK	Cyclin-dependent kinase
CDKN2#	Cyclin-dependent kinase inhibitor 2 #= A or B

cDMEM	Complete DMEM
C/EBP#	CCAAT-enhancer-binding proteins #=β
CGI	CpG island
Chip	Chromatin immunoprecipitation
CKI	Cyclin-dependent kinase inhibitor
CKII	Casein kinase 2
CNS	Central nervous system
C-Raf	V-raf-1 murine leukemia viral oncogene homolog 1
Cyst	Cystein
Cyt c	Cytochrome c
DBD	DNA binding domain
DM	Drosophila melanogaster
DMC	Double minute chromosomes
DMEM	Dulbecco's modified Eagle's medium
DNMT	DNA methyltransferase
E4F1	E4F transcription factor 1
E2F#	E2 promoter binding factor #= 1 or 2 or 3
E-Box	DNA sequence of CACGTG
EDTA	Ethylenediaminetetraacetic acid
EED	Embryonic ectoderm development
EMYCS	E2F c-MYC Specific
EZH2	Enhancer of zeste homolog 2
Fbw7	F-box/WD repeat-containing protein 7
FBS	Fetal bovine serum
FLAG	DYKDDDDK amino acids
Gadd#	Growth arrest and DNA damage #= 34 or 45 or 153

GAS1	Growth arrest-specific protein 1
GATA-1	GATA binding protein 1
GCN5	Amino-acid-synthesis protein-5
GFP	Green fluorescent protein
Glut	Glutamine
GNAT	GCN5-related N-acetyltransferase
GSK-3β	Glycogen synthase kinase 3β
H2AK119Ub1	Mono-ubiquitinylation of histone H2A at lysine 119
H3K27me3	Tri-methylation of lysine 27 on histone H3
HA	Haemagglutinating
HAT	Histone acetyltransferase
HAUSP	Herpesvirus-associated ubiquitin-specific protease
HBP1	HMG-box transcription factor 1
HCl	Hydrogen chloride
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitors
HDM	Histone demethylase
He1-N1	ELAV-like protein
HectH9	Homologous to E6AP carboxyl terminus homologous protein 9
HEK293	Human embryonic kidney 293
HIPK2	Homeodomain interacting protein kinase 2
HL-60	Human promyelocytic leukemia cells
HLB	Histone locus bodies
HLH	Helix-loop-helix
HLH-LZ	Helix-loop-helix-leucine zipper
HMT	Histone methyltransferase
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1

Hox	Homeobox
HPH#	Human polyhomeotic #= 1 or 2
HRP	Horseradish peroxidase
HSR	Homogenous staining regions
hTERT	Human Telomerase Reverse Transcriptase
HuD	ELAV-like protein 4
Huwe1	HECT, UBA and WWE domain containing 1
ID2	Inhibitor of DNA binding 2
IE4F1	Inhibitor of E4F1
Ig	Immunoglobulin
IL-1β	Interleukin 1β
INK4#	**IN**hibitors of CD**K4** #= A or B
Inr	Initiator
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thio-galactoside
IRES	Internal ribosome entry segment
ITAF	IRES trans acting factors
JmjC	Jumonji domain-containing
Kbp	Kilo basepairs
KCl	Potassium chloride
kDa	Kilodalton
L11	Ribosomal protein **L11**
LB	Lysogeny broth
LCR	Locus control region
LDH-A	Lactate dehydrogenase-A
LiCl	Lithium chloride
LSD1	Lysine-specific demethylase 1

LZ	Leucine zipper
Lys	Lysine
Mad#	Max dimerization proteins #= 1 or 3 or 4
MAPKAP	Mitogen-activated protein kinase-activated protein
Max	Myc-associated factor X
MB#	Myc homology boxes #= I or II or III or IV
MBD	Methyl-binding domain
MBLR	Mel18 and Bmi1-like RING finger protein
MBP	Maltose binding protein
MC29	Avian myelocytomatosis virus
MDM2	Mouse double minute 2
Med	Medi**i**ator
Mel18	Also called PCGF2
MG132	Cell permeable proteosome inhibitor
MGA	Max gene associated
$MgCl_2$	Magnesium chloride
MLX	Max-like protein X
MNT	Max binding protein
MXD#	Max dimerization proteins #= 1 or 2 or 3 or 4
Mxi1	Max interactor 1
mGCN5	Mammalian GCN5
miR17-92	MicroRNA cluster 17-92
Miz-1	Myc-interacting zinc fingerprotein 1
MLL	11q23
Mule	Mcl-1 ubiquitin ligase E3
Myc	Myelocytomatosis
MYCNOT	MYCN Overlap Transcript

MYST	**M**OZ, **Y**bf2/Sas3, **S**as2, and **T**ip60
NaF	Sodium fluoride
Na_3VO_4	Sodium orthovanadate
NB	Nuclear bodies
NLS	Nuclear localization sequence
N-Myc$^{\Delta 1b}$	one exon 1b-spliced N-Myc mRNA
NP-40	Nonidet P-40
NSC	Neural stem cell
OA	Okadaic acid
Oct-1	POU2F1
ORF	Open Reading Frame
p300	EP300 or E1A binding protein p300
Pax5	Paired box 5
PBS	Phosphate buffered saline
PBS-T	PBS-Tween 20
Pc	Polycomb
PCAF	P300/CBP-associated factor
PcG	Polycomb group
PCGF2	Polycomb group ring finger 2
PCR	Polymerase chain reaction
PEST sequence	Peptide stretch rich in proline, glutamic acid, serine, and threonine
PH	Polyhomeotic
PHC#	Polyhomeotic-like protein#= 1 or 2 or 3
PI3K	Phosphoinositide-3-kinase
PIASXβ	Protein inhibitor of activated STAT Xβ
PIC	Pre-initiation complex
PKC	Protein kinase C

pKSII	Phagemid pBluescript II KS
PML	Progressive multifocal leukoencephalopathy
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
POU2F1	POU domain, class 2, transcription factor 1
PP2A	Protein phosphatase 2A
PRC#	Polycomb repressive complex #= 1 or 2
P/S	Penicillin/streptomycin
PSC	Posterior sex combs
P-TEFb	Positive transcription elongation factor b
PTB-1	Polypyrimidine tract-binding protein 1
PTM	Posttranslational modification
RB	Retinoblastoma
RBBP	Retinoblastoma binding protein
RING1#	Ring finger protein 1 #= A or B
RNAP II	RNA polymerase II
RNF2	Ring finger protein 2
ROS	Reactive oxygen species
S#	Serine #= amino acid number
SA-β-gal	Senescence associated beta-gal
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET	**S**U (Z) 3-9, **E**ZH2, **T**rithorax
Shh	Sonic hedgehog
SID	Share related repression domains
SIN3	Swi-independent 3

SIRT1	Sirtuin 1
Skp2	S-phase kinase-associated protein 2
Smad	SMA/MAD
Sp1	Specificity Protein 1
SPM	**S**CM, **P**H, **M**BT domain
SPOP	Speckle-type POZ protein
Suz12	Suppressor of zeste homologue 12
SWI/SNF	SWItch/Sucrose NonFermentable
T#	Threonine #= amino acid number
TF	Transcription factors
TFII#	Transcription factor II #= A or B or D or E or F or H
TGF-β	Transforming growth factor beta
TIE	TGF-β inhibitory element
TIP60	Tat-interactive protein 60
TNT	Transcription/translation
TPA	12-O-tetradecanoyl phorbol-13-acetate
Top2α	Topoisomerase 2α
TRRAP	transactivation/transformation-associated protein
TrxG	Trithorax group
TSA	Trichostatin A
Tyro	Tyrosine
Ub	ubiquitin
UTR	Untranslated region
WT	Wild type
WT1	Wilm's tumor suppressor protein 1
YB-1	Y-box binding protein 1
ZNF134	Zinc fingerprotein 134

Chapter I

Literature Review

1.0 Gene regulation

Genetic material providescells with a blueprint for all requisite cellular structures and processes. In order to coordinate essential cellular functions, including growth and division, temporal and spatial regulation of gene expression through transcriptional regulatory mechanisms is crucial.. Repression and silencing are two examples of these regulatory mechanisms.

1.1Repression versus Silencing

Gene repression is characterized as a transient interruption ingene transcription mediated by the attachment of a DNA-binding protein, called a repressor, to an operator of a gene promoter[1]. This temporary transcription block is nullifiedby either preventing the transcription of the regulatory gene or throughthe induction of an inducer[1]. Gene silencing, on the other hand, is an epigenetic mechanism that operates on both the transcriptional as well as the post-transcriptional level and causes long lasting gene inactivation [1].

1.2Epigenetic regulation

Epigenetics refers to all processes thatcause analteration in geneexpression[2]. These modifications, which do not involve a change in the underlying genetic sequence, may remain throughout a cell's life span and can be inheritedby subsequent generations[2]. DNA methylation or histone PTM are two examples of such epigenetic changes [3].

1.2.1 DNA methylation

DNA methylation is a PTM catalyzed by DNMTs[4]. This covalent modification is characterized by the addition of a methyl group by S-adenosyl Methionine to the 5' position of cytosine found at CpG dinucleotides[3]. CpG dinucleotides can be found scattered throughout the genome as well as in dense clusters called CGIs. Usually, CGIs appear at the 5'end of promoters and average 1000 base pairs[5-7]. DNA methylation of CGIs at gene promotersis commonly associated with the long lastingsilencing of gene expression [3]. Moreover, methylation of CGIs near gene promoters allows forthe recruitment of MBD proteins, which in turn prevents the binding of TFs[7]. MBD proteins have been identified as members of a large transcriptional co-repressor complex known to include HDACs[3].

1.2.2 Histone PTMs

Histone PTMs are covalent modifications occurring on specific histone aa residues[8].These epigenetic modifications generate a code that directs the cellular machinery toeither promote or inhibit gene transcription by regulating the DNA-histone interaction.This biological method of information retention has been termed the histone code [1]. Acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation are some of the PTMsthat modify histones [9]. Acetylation of histones H3 and H4 is the most thoroughly studied PTM. H3 and H4 lysine residues are subject to acetylation by HATs and HDACs and methylation by HMTs and HDMs [9].

1.2.2.1 HATs and HDACs

The cellular histone acetylation profileis the result ofsignals stemming from HATs and HDACs. HATs are responsible for adding acetyl residues, whereas HDACs remove these same moieties.

HATs are generally divided into 2 categories: the cytoplasmic HATs and the nuclear HATs[10, 11]. Nuclear HATsregulate gene transcriptionby directly altering chromatin-bound histones, whereas cytoplasmic HATs modify histones and non-histones posttranslationally before they assemble into nucleosomes [12, 13]. Generally, histone acetylation is considered to be a mark of transcriptional activation.Although five HAT families have been identified,GNAT, p300, and MYST have been studied mostextensively[3, 14]. As a whole, these super complexeshave been shown to play a role in cellular processes, including cell cycle progression, cell fate determination,and development [15].

HDACs are responsible for the removal of acetyl moieties on histone Lys residues. Generally, these processes instigate transcriptional repression. Numerous cellular pathwaysinvolved in signal transduction, apoptosis, cell cycle regulation, and cell growth are affected by these enzymes [16]. HDACs are capable of deacetylating histone as well as non-histone proteins[17]. Through their deacetylase domain, HDACs are also able to induce PTMs on TFs and Chaperone proteins [3].

1.2.2.2HMTs and HDMs

Similar to acetylation, histone methylation also acts as a method of regulating gene expression. The addition of up to three methyl groups on a specific histone aa, enables HMTs to prompt either transcriptional activation or repression[18]. For example, H3 methylation at Lys 4 and Lys 36 is commonly linked to active gene expression, whereasgene silencing usually ensues if Lys 9 and Lys 27 are di or tri methylated[18]. Interestingly, histone methylating enzymes usually contain a SET domain[19]. LikeHAT/HDAC coupling, HDMs reverse the process catalyzedby HMTs.Recently, two HDMs families have been discovered, including LSD1 and JmjC[3]. Similar to HATs and HDACs, HMTs and HDMs have also been identified as members of large protein complexes.

1.3PcG and TrxG proteins

In 1978, a group of Drosophila Melanogaster mutants called Pc were shown to develop legs in the place of antennas [20]. These abnormalities were caused by the inability of the Polycomb group (PcG) family of proteins to repress a Hox gene cluster[20]. Mechanistically, PcG proteins trigger the compaction of chromatin therebyhindering the activity of transcriptional machinery[21].These mutants were also instrumental in identifying another class of mutants called TrxG proteins, which were shown to function as PcG proteinantagonists[22].

Both PcG and TrxG proteins play a role in determining the methylation status of histones[20]. To this effect, PcG proteins have been characterized as initiators of histone

modifications responsible for mediating the repression of transcription, while TrxG proteins have been shown to set the stage for histone modifications that lead to transcriptional activation[20]. Interestingly, both of these protein complexes are able to facilitate the acetylation of histones by directly or indirectly recruiting other proteins.To this effect, PRC complexes have been purified together with HDACs, includingSIRT1 and HDAC2. Likewise, the HAT MYST1 has been identified in a TrxG protein complex containing MLL [23, 24]. Additionally, not only can acetylation directly activate transcription but it also indirectly prevents the formation of the repressive H3K27me3 signal [20]. For instance, TrxG can directly activate transcription, by recruiting HATs,as well asprevent the establishment of H3K27me3 repressive marks near gene promotersaddedby PcG proteins [20]. Generally, these complexes seem to be responsible for tightly controlling cellular histone methylation andacetylation profiles.

1.3.1 PRC1

PcG proteins can be grouped into two general complexes called PRC1 and PRC2 (Fig. 1A). The first multi-subunit complex,PRC1, is responsible for recognizing the initial histone code deposited by PRC2 [25].In turn, the PRC2-inducedmono- di- or tri-methylation of H3K27 is responsible for maintaining chromatin in a transcriptional inactive state[25].However, PRC1 has recently been shown to interact with chromatinwithout PRC2 [20, 26]. Additionally, genome wide analysis demonstratingthe chromatin occupancy state of PRC1 and PRC2 indicate thatthese protein complexes do not bind to identical regions[20, 27].

The cellular PRC responsemay be modified by PTMs(Fig. 2). Accordingly,the phosphorylation of Bmi1 by MAPKAP kinase 3 at residues S251, S253, and S255 has been shown to causea changein PRC1 chromatin association [28].Moreover, several PTMsidentified in both PRC complexes have been shown to significantlyperturb normal cellular physiology[29]. Responses to these PTMs, ranging from amplified PRC activity to transcriptional repression,have allowed the PRC complexes to produce a tailored physiological response based on upstream signal integration.Furthermore, the array of interaction possibilities amongst the diverse PRC membersenablescells togenerateunique protein complexes [25]. The various mammalian PRC1 complexes (Fig. 1B) can beassembledwith the differential paring of the following proteins:CBX2, CBX4, CBX8, PHC1, PHC2, PHC3, Bmi1, RING1 or RNF2[20, 30]. Interestingly, Bmi1 possess a RING domain that is necessary for its oncogenic activity as well as its nuclear shuttling ability[14].

1.3.2Bmi1:A member of the PRC1 complex

Bmi1was initially established as an oncogene that cooperates with Myc in lymphomagenesis[31].Since its discovery, alternative names such as Polycomb group ring finger 4 (PCGF4) and Ring finger protein 51 (RNF51)have been ascribed to this protein[32]. The Drosophila Melanogaster homologue of Bmi1, PSC,was initially described as a protein whichregulates the body segments of these fliesby upholding the repression of homeotic genes[20].Currently,a large body of knowledge exists regardingthe structural and functional features of Bmi1.

1.3.2.1Domain and structure

The Bmi1gene is foundon human chromosome 10 at segmentation p11.23. Its entire nucleotide sequence is composed of 9 introns and 10 exons located on a nucleotide stretch of 10.04kbp[32]. The 3.2 kbp mRNA of Bmi1 encodes a 326 aa protein whose molecular weight is 36.9kDa(Fig. 1C) [33]. Although Bmi1 does not possess any enzymatic activity, it exerts its action by regulating the activityof the PRC1 complex [34]. A potential NLS located at aa 81-95 allowsBmi1 to shuttle between the nucleus and cytoplasm [35, 36]. The N-terminal region of this protein has a Cyst-rich RING zinc finger domain at aa 18 to 57,which binds two atoms of zinc and plays a key role in the ubiquitination process[37].Throughtheir direct interaction with E2 conjugating enzymes, RING finger domains facilitate the transfer of the ubiquitin tail found on E2 to target proteins[38].Interestingly, a natural variant of Bmi1 has a Cyst to Tyrsubstitution located within itsRING finger domain at aa 18 [37]. Functionally, thissubstitution is responsible for decreasingBmi1 protein levels and causing a tremendous increase in its cellular ubiquitination [39].

In hematopoietic stem cells Bmi1 interacts with an inhibitor of proliferation through a site located at aa164-228 ofBmi1 [40].Additionally, Bmi1 has been shown to interact with vertebrate homology domain I and II containing proteinscalled HPH1 and HPH2[41].Thefunctionalhomology domains ofHPH1 and HPH2 are required for Bmi1 interaction[41].Two ring finger domain-containingproteins, RING1A and B, have also been identified as Bmi1interaction partners.These ubiquitin ligases alter proteins

throughthe process of ubiquitination. Additionally, RING1A interacts with Bmi1 and causes the degradation of Top2α [42].

1.3.2.2Cellular function

Bmi1 has been demonstrated to play an important role in several cellular events. To this effect, the transcriptional silencing of CDKN2A/B, E3 ubiquitin ligase activity, andcell proliferation inhibitionhaveall been associatedwith Bmi1activity[35].

1.3.2.2.1 Transcriptional silencing of CDKN2A/B

Bmi1 allows cells to evade senescence by transcriptionally repressing 3 prominent tumor suppressors encoded by the genes CDKN2A and CDKN2B;located on human chromosome 9p21 [20]. Transcripts of CDKN2A give rise to the tumor suppressors INK4A and Arf, whereas CDKN2B transcripts generate INK4B [21]. INK4A/B transcripts are known as CKIs. These CKIs obstruct CDK4-mediated RBphosphorylation [21]. Hypophosphorylated RB can no longer activate E2F thereby hindering the cell cycle progression and initiating cellular senescence [21]. Additionally, ArfinhibitsMDM2thus preventing the degradation of p53. Increasingcellular p53 levels leads tothe activation of various gene targets responsible for cell cycle arrest and apoptosis[33]. Bmi1 is also able to preserve adult NSCs through its repression of INK4A and Arf [43, 44]. Recently, Bmi1 has been shown to bind and regulate the p21 response in Shh postnatal cerebellar granule progenitors [45].

1.3.2.2.2 E3 Ubiquitin Ligase

Bmi1 and RING1B both contain a RING domain and are membersof the PRC1 complex [46]. RING1B is an ubiquitin ligase that can monoubiquitinate and auto-polyubiquitinate its own H2A histone [46]. Interestingly, Bmi1 can induce the mono-ubiquitination activity of RING1B and limit its ability to auto-polyubiquitinate[47]. It has been previously postulated that RING1B may need to be auto-polyubiquitylation in order to cause the monoubiquitylation of H2AK119Ub1,a process required for the transcriptional repression caused by PcG proteins [46-48].Interestingly, Bmi1 also activates another E3 ubiquitin ligase called RING1A[49]. The ablation of RING1A has been shown to cause a global decrease in cellular H2A ubiquitination levels, resulting amongst other things in an increase in Hox gene activity[49]. Recently, the E3 ubiquitin ligase complex Bmi1/RING1A was shown to control the half-life of Top2α by regulating its proteasomal degradation [42].

1.3.2.2.3 Proliferation inhibitor

E4F1, a proliferation inhibitor, has recently been shown to directly interact with Bmi1 [35]. Thisinteraction regulates the proliferative ability of hematopoietic cells. Accordingly, decreasing E4F1 levels in Bmi1 null hematopoietic cells salvages the hematopoietic compartment for up to 3 months [35]. Classical downstream targets of Bmi1 (INK4A, Arf and p53)aredispensable for the functional interaction betweenBmi1 and E4F1[35] .

1.3.2.3PTM-mediated Bmi1 regulation

Activated MAPKAP kinase 3 phosphorylates Bmi1 causing itto dissociatefrom the chromatin [47, 50]. The loss of Bmi1-bound chromatin results in the reactivation of CDKN2A [28]. As it currently stands, the functional outcomes of MAPKAP kinase 3 induced phosphorylation have yet to be elucidated.

1.3.2.4 Cancer

Bmi1 was initially identifiedas a protein cooperating with Myc in lymphomagenesis[31, 51].Presently, Bmi1 is known to be strongly expressed in a variety of hematological malignancies [35, 52-56](mantle cell lymphoma, myeloid dysplastic syndrome, chronic myeloid leukemia and acute myeloid leukemia) and solid tumors [44, 57-63](ovarian cancer, bladder cancer, squamous cell carcinoma, prostate cancer, breast carcinomas, lung cancer and pancreatic cancer).

1.3.2.5Development

Bmi1 null mice demonstratesevere but progressive defects in the liver, bone marrow, spleen and thymus [64]. The premature senescence of progenitor cells in the hematopoietic compartment is thought to be the cause of theseabnormalities[40]. Supporting this claim is the accumulation of the senescence marker SA-β-gal in these cells [40]. Additionally, significant patterning defects withinthe molecular and granular layers of the cerebellum as well as Purkinje cells anomalieshave been observed [64]. Other neurological anomalies of Bmi1 -/- mice includea decrease in brain size observed 14 days postpartum as well as an ataxic gate that is accompanied by episodes of epileptic

seizures [65]. Severe anterior-posterior skeletal abnormalities are also observed in Bmi1 null mice [64]. The source of this mismatch is thought to stem fromthe irregular expression of the Hox genes[64].

1.4Myc

29 years have passed sincethe sequence of Myc was first identified in the MC29 retrovirus [66]. Since then, Myc has been extensively studied. This oncogeneis induced by a plethora of growth factors and is essential forcell proliferation[67]. Accordingly, it is not surprising to learn that approximately 70 % of human tumors express abnormal levels of Myc and that if itsexpression is suppressed, tumors generally tend to regress [67, 68]. Furthermore, Myc has the capacity toupregulate or represses $1/10^{th}$of the human genome [68]. To this effect, cells have devised and implemented an extensive array of regulatory mechanisms to keep this transforming gene in check.

1.4.1Domain and Structure

Mammals havefive different Myc paralogues, includingc-Myc, N-Myc, L-Myc, S-Myc and B-Myc[36, 69]. These genes produceproteinsof variablesizes and domaincomposition (Fig. 3A-D). Moreover, Myc paralogues are of variable length and are situated at different chromosomal locations(Fig. 3A-D).Nevertheless, they all share three conserved structural domains, includingMyc Boxes (MBs), a Transactivation domain (TAD), and a DNA binding domain (DBD) (Fig. 3).The DBDis comprised ofthe Basic region (BR) and the helix-loop-helix-leucine-zipper (LH-LZ) region. The BR is associated with the specific binding of canonical and non-canonical Myc E-boxes, which

have a core consensus sequence of 5'-CACGTG-3'[66]. The HLH-LZ region, on the other hand, is essential for the interaction of Myc with its partner Max[70]. Both the BR and the HLH-LZ regions are vital for the full transformation capability of Myc in primary and immortalized cells lines[66].

MBs are conserved segments of the Myc protein that share structural and functional similarities amongst family members [71]. These boxes are bound by specific protein partners and are subject to PTMs at particular aa residues (Fig. 4). The TADis conserved in allmembers and contains MBI and MBII (Fig. 3).

MBI regulates the turnover of Myc and is essential for the maximal transcriptional activity of this oncogene [72, 73].MBII, on the other hand,appearsto be the keyfunctional segment of the TADdue to its ability toactivategene transcription at various DNA binding regions[68].MBIIIa/b and MBIV are located between the TAD and DBD domains. MBIIIa is partially responsible for Myc's ability to repress gene activity and induce apoptosis[74, 75]. Recruitment of HDAC3 by MBIIIa leads to the transcriptional silencing of the ID2 and Gadd153 promoters[74]. The HDAC3-Myc interaction appearsnecessary for the ability of Myc to induce apoptosis [74]. Although the MBIIIb domain is highly conserved, no function has been assigned toit as of yet. MBIVhas been shown to modulate several cellular processes. To this effect, it is responsible for controlling G2 arrest, promoting apoptosis and affecting Myc'sDNA binding ability [76]. Moreover, MBIV has also been shown to facilitatethis oncogene'spropensity to transform cells [76].

1.4.2 Control of Mycfunction

1.4.2.1 Deregulation

Myc studies have permitted scientists to uncover 3 novel mechanisms of oncogenic activation which have been used to characterize and classify a variety of other prominent oncogenes[66]. These mechanisms include insertional mutation, chromosome translocation and gene amplification [66].

1.4.2.2 Regulation of Myc function

The expression of Myc appears tobe regulated through a variety of mechanisms, including transcriptional control, mRNA turnover, protein expression,protein degradation,and PTM.

Transcriptional control

Groundbreaking research exploringthe transcriptional and post-transcriptional regulation of eukaryotic genes has previously examined these regulatory mechanism at theMyclocus[66, 77]. Although this locus has been identified as a convergence node for various signaling pathways, research pertaining to LCR function as well as enhancesome assembly is currently insufficient to fullyunderstandthe transcriptional regulation of the Myc gene [77].

c-Myc

c-Myc auto-suppresses its own promoter by heterodimerizing with Max during the initiation of transcription [77]. This dose-dependent suppression occurs on the P2

promoter,one of its 4 promoters, by a mechanism that implicates the Inr elements and the E2F-binding site[77, 78]. However, repression of the Myc promoter by the E2F-RB complexat the E2F-binding site does not suppress the expression of the Myc gene during the exit of quiescence[79]. Instead,a non-E2F complex called EMYCS contacts the DNA at distinct but overlapping residues on the E2F-binding site[79]. The DNA binding component of the EMYCS complexweighs105kDa and appears to be responsible forthe transcriptional transactivation of the c-Myc promoter [79].The auto-regulatorymechanism exhibitedby c-Myc also extends to the rest of the family. To this effect,N-Myc and L-Myc are also capable ofrepressing their own expression[77]. An extensive list of TF, and their partners, have been shown to directly bind to the c-Myc promoter[77] (Fig. 4). For instance, the following list of TF have been shown to engage the Myc promoter *in vivo:*estrogen receptors, androgen receptor, GATA-1, C/EBPβ, p53 and p73[77]. One member of the PcG protein family, Mel 18, has been shown to repress the c-Myc promoter[77]. It appears thatMel18 induces c-Myc repression during cellular senescence by downregulating endogenous c-Myc mRNA and protein expression[80, 81]. Although the precise mechanics underlyingthis interplay are unclear, it has beensuggested that Mel 18 may bind to the 0.8 kbp Mel 18 responsive region found on the c-Myc promoter [77].

N-Myc

In contrast to c-Myc, N-Myc has only one major promoter that can be activate at multiple initiation sites [82]. The first 3 E2F TF, all of which function as activators, are able to bind to specific consensus sequences on the N-Myc promoter and engendergene transcription [82]. TGF-β binds to the TIE binding site which flanks the N-Myc promoter

E2F binding site [83]. Interestingly, the transcriptional repression of the N-Myc promoter caused by its interaction withTGF-β/TIE might also stem from itsinterplaywithE2F-4[82]. The N-Myc promoter also possesses a GC rich region called the CT-Box which can be bound by SP1 [83]. Moreover, a portion of the CT-box overlaps with the TIE. Due to the proximity of the E2F binding site and the CT-box, various studies have shown that both of these sites are able to activate or repress the N-Myc promoter [83]. A variety of other TF such as Pax5, WT1, and HBP1 also regulate N-Myc promoter-related transcriptional processes [82].Interestingly, a major negative cis-regulatory element located on the N-Myc gene has been identified at the 3' end of exon and intron one[83].

L-Myc

The L-Myc gene is located on chromosome 1p32 and is composed of 3 exons and 2 introns [84, 85]. This gene has a single transcriptional start site located at its5' end which has not,at present, been shown to respond to any peptide hormones or cell proliferation inducers [84].

mRNA and Protein expression

c-Myc

Due to its potent oncogenic properties, c-Myc's mRNA and proteins levels are tightly regulated throughdiverse cellular processes.Accordingly, this oncogene is normallyexpressed at low levels and hasa half-life of 20-30 minutes [77]. Regulatory processes at the translational and mRNA processing levels are essential for gauging the expression demand forc-Myc in cells. For example, the 5'UTR is responsible for maintaining the expression of c-Myc mRNA in pathological conditions such as apoptosis,

genotoxic stress and viral infections[86].The IRES induced translation of c-Myc mRNA requires both initiation factors and ITAFs[86]. Moreover, ITAFsused by c-Myc mRNA includeYB-1, p54, PTB-1, and hnRNPA1[86].It is interesting to note, thatproliferating cells can raise their c-Myc mRNA levels up to 40 timesthat of quiescent cells. However, during cellular arrest and differentiation the mRNA levels are maintained at 10 % of their previous value [77, 87]. Importantly, a constant mitogenic stimulation is required to maintain the expression of c-Myc in order to prevent anti-proliferative signals as well as differentiation cues [77].

N-Myc

The transcription of the N-Myc gene is a complexprocess that involvesthe alternative splicing of its 3 exons (exon1a/b, exon 2, and exon3) to generate 2 distinct mRNA forms[88]. Although 3 possible mRNA forms maybe generated, only the full length N-Myc and the N-Myc$^{\Delta 1b}$ are transcribed [88].While both mRNA have a half-life of 15 min, the translational efficiency and the protein concentration generated from equivalent amounts of mRNAis greater with N-Myc$^{\Delta 1b}$ [88, 89]. Interestingly, N-Myc$^{\Delta 1b}$mRNA can be translatedinto two different proteins, including N-Myc$^{\Delta 1b}$ and MYCNOT. This duality occurs as a resultof the presence of two ORFs located at its 5' end[88]. Furthermore, an IRES sequence located on the 5'UTR of the N-Myc mRNA has been shown to increase its half-life, initiate translation processes, and its cytoplasmic shuttling [82].Additionally, the stability of N-Myc mRNA can be affected by the binding of ELAV-like proteins (p40, HuD and He1-N1)at the 3'UTR[82, 90].

L-Myc

Similarly to N-Myc and c-Myc, the L-Myc gene is composed of 3 exons and 2 introns which can be transcribed into two different mRNAs, including the long form (exon 1- intron 1- exon 2 –exon 3 or exon1- exon 2 –exon 3) and the short form (exon 1 – exon 2 and a fragment of intron 2) [91]. The long and short form,a 364 and 206 aa protein respectively, are generated by alternative splicing anddifferential polyadenylation [92]. Moreover, the short mRNA is more stable with a half-live of 120-180 min, whereas the long form has a half-life of only 45-90 min [91]. Interestingly, the long form generates 3 proteins with masses of 60 kDa, 61kDa, and 66 kDa, whereas the short form generates 34 kDa and 37 kDa products[91]. Presently, the functions of these various L-Myc proteins are unknown.Recently, however, it was shownthat the short L-Myc mRNA contains an IRES sequence located on the 5'UTR upstream of its initiator codon [93].

Protein degradation

Mycturnover is mediated by the proteasome which recognizes and targets ubiquitinated proteins for degradation[94]. Currently, the specific ubiquitinated Lys residues of Myc have yet to be identified. However, a Myc mutant lacking Lys-143, Lys-157, Lys-275, Lys-317, Lys-323, and Lys-371has been shown to exhibit less ubiquitination mediated by HectH9, a ubiquitin ligase capable of binding both N-Myc and c-Myc [94-96].

Presently, the three key regions responsiblefor Mycturnover include MBI, MBII, and MBIIIa/PEST[94]. Of these three domains, degradation mediated by MBI has been the most thoroughly documented and is currently the most wellunderstood [94]. In Burkitt's lymphoma, a mutation at aa T58 causes a substantial decrease in the

ubiquitination of c-Myc [72]. In WT c-Myc and N-Myc proteins, GSK3β is responsible for phosphorylating T58,which in turn initiates Fbw7-mediated ubiquitination and degradation[97]. However, in order to initiate the Fbw7degradation cascade, S62 must be phosphorylated [98]. The proteins responsible for these phospho-priming events are the mitotic cyclin B/Cdk1 complex in N-Myc and Map-Kinase, in the case of c-Myc [99, 100].

MBII, on the other hand, is bound by Skp2, an F-box ubiquitin ligase [101].It has been demonstrated that a functional MBII domain is required for Skp2-Myc interaction [94]. Interestingly, although both Skp2 and Fbw7 have been shown to simultaneously regulate the turnover of Myc independently, these regulatory mechanisms appear to be additive [94].

Very little is currently known regarding themechanisms governing the ubiquitination of MBIIIa and its adjacent PEST sequence.It is interesting to note, however, that deletion of either MBIIIa, PEST or both of these sequences leads to an increase in Myc stability and an elevation in ubiquitinated Myc proteins levels[94].As of yet, no account of L-Myc ubiquitination has been reported.

Post-Translational Modification (PTM)

c-Myc

The c-Myc protein is the most extensively studied member of itsfamily in terms of PTM. As stated earlier, c-Myc isphosphorylated at T58 by GSK-3β and at S62 by MAP-Kinase. Interestingly, an O-linked glycosylation site at T58 has been previously reported. This site has been established as a mutational hot spot in many lymphomas[102]. N6-acetyl-Lys moieties are added at aa 143, 148, 157, 275, 317, 323, and 371by PCAF [103].

Acetylation of c-Myc by either mGCN5/PCAF or TIP60 has been shown toincrease proteinstability[104]. It has also been demonstrated that CKII phosphorylates c-Myc at its C-terminal acidic region [105].C-Raf, a widely known Serine/Threonine kinase, involved in the Ras signaling pathway, binds to c-Myc and phosphorylates residue T8 *in vitro*[36, 106].

N-Myc

The N-Myc protein can be phosphorylated by CKII attwo distinct residues, S261& S263, inside itscentral acidic region [107]. As stated earlier, N-Myc can be phosphorylated byGSK-3β at T58 and at S62 by the cyclin B/Cdk1complex [36, 94]. These phosphorylation events are responsible for mediating N-Myc turnover.

L-Myc

The L-Myc protein mayappear as three distinct phosphorylated bands when subjected to SDS-PAGE gel electrophoresis. This phosphorylation isquicklyapparent whencells are treated with PKC activators or inhibitors of S/T protein phosphatases [108]. The N-terminal S38 and S42 residues of L-Myc have been identified asvital targets for PKC-dependent phosphorylation. Likewise, GSK-3β has also been shown to phosphorylate these target sites*in vitro*[109].

1.4.2.3 Myc:As a transcriptional activator and repressor

Myc is implicated in various cellular processes, including cell proliferation, cell growth, apoptosis, stem cell self-renewal and differentiation [110]. Initially believed to act solely as a transcriptional facilitatorenabling the activation of its target genes

throughMax-dependent interaction, Myc has recently been shown to also function as a transcriptional repressor [111, 112].

Transcriptional activator

Microarray studies have demonstrated that Myc is able to transactivate a large portion of its gene targets by a factor of 2 [113]. E-Boxes bound by the Myc-Max heterodimeric complex via their bHLH-LZ domain cause a weak transcriptional activation of their target genes [114]. It is currently believed that Myc recruits acetyltransferase multi-complexes such as TRRAPtoacetylatehistones H3 and H4[66, 71, 115].Loosely packed chromatin, due to the acetylation of specific histones,in turn facilitates the recruitment of chromatin remodeler complexessuch as GCN5 and SWI/SNF;makingthe target loci more accessible to transcriptional machinery [116]. RNA polymerase II, a transcriptional machinery component, isresponsible for the transcription of most Myc target genes. In this respect, Mycis able torelease RNA polymerase II pausedat its target promoters by binding to its CTD kinase throughits TADand promotetranscriptional elongation [117, 118]. Additionally, this oncogene facilitatesthe initiation of translation by promotingmRNA maturation through the methylation of the 5'cap [68].

Transcriptional repressor

The current body of knowledge pertaining tothe mechanism of transcriptional repression is meager.Myc has recently been shown to sustain cellular proliferation by repressing the activation of cell cycle genes such as GAS1, p15, p21, p27, and Gadd-34, -45, -153 [111, 119].Currently, two mechanisms of Myc-induced transcriptionalrepression have been proposed. The first mechanism involves binding of the Myc-Max

heterodimeric complex to Inr sequences of their targetgene promoters[120]. Once bound, the C-terminal domain of Myc interacts with Miz-1 or other transcriptional activators and prevents transcriptional initiation[120]. The other mechanism does not involve the formation of the Myc-Max complex but rather entails an interaction between Myc and the Sp1 TF or the Sp1/Smad complex[120]. The central region of the Myc protein interacts with Sp1 or Sp1/Smad and inhibits Sp1-mediated transcriptional activation [120]. Presently, additional research is required to identify which Sp1 promoters are repressed by Myc [120].

1.4.3 Myc in cellular transformation

Myc is a multi-functional protein that influences a variety of cellular compartments. These cellular modules tightly regulate the activity of Myc by interacting with specific segments of the Myc protein. Importantly, MBs of Myc contributedistinctlyand/or additively to its biological function as well as its ability to stimulate neoplastic transformation (Fig. 4)[66]. Although Myc is involved invarious cellular functions, it strongly influences pathways implicated in cell growth, cell cycle progression, andapoptosis[66].

Cell growth

Activated Myc allows cells to obtain, directly or indirectly, most of the components necessary for growth. Upon mitogenic stimulation, Myc will directly modulate protein biogenesis by stimulating the transcription of genes controlled byRNA polymerase I, II, and III[110]. Myc is also able to increase protein translation by directly influencingtranslation machinery and ribosomal proteins [110]. Cellular metabolism,

more specificallymitochondrial biogenesis and function,isconsiderably impactedby Myc. For example, Myc is able to upregulate LDH-A protein levels resulting in increasedlactate production [121]. Moreover, Myc has also been shown to increase the availability of cellular nucleic acids and aa in order to meet the augmented demand stemming fromcell growth [110]. The sum total of all Myc functions enablescellsto have a constant supply ofall ingredients necessaryforsustained cell growth.

Cell cycleprogression

Transformed or proliferating cells not only require nutrients and signaling cuesto sustain growth but must also possess the capacity to divide. Myc is able to decrease the time that cells spend in G1 as well as help proliferating cells progress to S phase [122].While many genes are activated by Myc, few target loci are repressed by this oncogene. For instance, Myc can simultaneously inhibit and promotespecific components essential for cell cycle progression [123]. Myc activates cell cycle progression genes such as cyclin (D1, D2, E1, A2), CDK4, CDC25A, E2F1, and E2F2, yetinhibits CDKIs and cell cycle checkpoint genes, including p15, p21, and p27[66, 124-126]. Cells expressing this oncogenein a deregulated fashion usually gain a proliferative advantaged over neighboring cells if they are able to ward off the apoptotic response.

Apoptosis

Myc expressing cells are more inclined to undergo apoptosis [127]. This response is accentuated when the expression of Myc is deregulated and cellsare devoid of survival factors [66]. Programmed cell death can be provoked by Myc through multiple pathways.

For example, abnormal Myc expression may lead to the transcriptional upregulation of Arf whichhas been shown to induce a p53-dependent apoptotic response [66, 128]. In othercases, Myc may interact with Miz-1 and repress p21 thereby strengtheningthe p53 response [66, 111].Myc mayalso cooperate with Bmi1 to alter the p53-MDM2-Arf pathway and induce tumorigenesis [51, 129]. Additionally, Myc may play a role in altering the balance between pro-apoptotic and anti-apoptotic proteins [66]. To this effect, the anti-apoptotic proteins Bcl-2 and Bcl-xL are indirectly blocked by Myc, whereas pro-apoptotic protein such as BAX are activated by this oncogene[130, 131].Through both of these mechanisms Myc has been shown toshift cells towards an apoptotic response by causing the release of Cyt C which activates effector caspases and triggerscell death [66].

1.5 MAX: a transcription factor

1.5.1 Domain and Structure

The Max protein is encoded by a gene located on human chromosome 14 at segmentation 14q23and spanning9.63 Kb[36]. Its mRNA encodes three proteins derived from alternative splicing. The first of these variants is a long 160 aa protein, the second is missing aa13 to 21, and the last is comprised uniquely of aa99 to 160[132].Nevertheless, additional isoforms may exist that have yet to be uncovered.It is interesting to note that the Max has an NLS located within aa 152 to 156[36]. Furthermore, its N-terminal region has a BR at aa 24 and is followed by a HLH and a LZ motif at aa 39-75 and 81-102, respectively(Fig. 5)[36].

1.5.2 Cellular function

Although Maxmay function as aweak transcriptional repressor, results from several studies have suggested transcriptionalneutrality[133, 134]. Current literature suggests that Max predominantlyfunctions as a cofactor required for other proteins to bind DNA [134].Importantly, Max also appears essential for many Myc-related biological functions[67, 135, 136].For example, the p53 promoter possesses anE-Box transcriptional regulatory sequence recognized by DNA binding proteins containing a bHLHZIP motif, including Myc and Max[137]. Moreover, it has been shown that the transactivation of the human p53 promoter by the heterodimer Myc/Maxleads to the activation of the p53 promoter resulting increased p53 protein levels[138]. Additionally, a cluster of bHLHZIP proteins, including MNT, MXD1-4, and MGA also require Max as a cofactor to exert their biological functions[134, 139, 140]. These Myc-related bHLHZIP proteins function as transcriptional repressors of Myc [134].For example, Myc-Max complexes were ejected and substituted by the MXD1-Max heterodimeric complex at hTERT and cyclin D2 promoters [139, 141]. Interestingly, these transcriptional repressors are only able to bind DNA in the presence of Max [139]. Furthermore, MXD1 and MXD4 can form heterodimers with another bHLHZIP protein called MLX [142]. As a result of this interplay, MLX is able to modulate Max-dependent interaction (Fig. 6)[134].

1.5.3 Regulation

In contrast with its highly regulated partners, Max is constantly transcribed and translated, especially during phases of cell cycle entry [134, 143]. While Max has a $T^{1/2}$ of over 24 hrs,itspartners' $T^{1/2}$ isaround 30 min [134]. Interestingly, Max levels may play

a limiting role in some situations, including those in which c-Myc levels are high[134, 144].

As opposed to its other partners, Max can form homodimers and bind to E-box sequencesonly if its N-terminal S2 and/or S11 are not phosphorylated [145, 146]. Presently,the signaling pathways implicated in regulatingMax phosphorylation have yet to be elucidated in the physiological context [134]. Interestingly, phosphorylation of Max does not alter the DNA binding ability of the Myc-Max heterodimeric complex [134, 147]. In addition to phosphorylation, Lys-57, Lys-144 and Lys-145 of the Max protein have been identified as acetylation target sitesin mammalian cells[148]. To this effect, HDACi and HAT such as p300 have been shown to engender Max acetylation[148]. Interestingly, Max shuttles backto the cytoplasm when all three of its Lysresidues are replaced with Glut[148]. Deletion oftheNLS, which includes Lys-144 and Lys-145, does not affect its nuclear localization. The nuclear localization of Max and its suppression of Myc-dependent transactivation are modulated when its Lys residues are substituted with Glut but not Arg residues [148].

1.5.4Knockout mice

Max deficient mice are embryonically lethal at E6.5-E7.5, which is earlier than that observed for c-Myc and N-Myc deficient mice [149, 150].It is hypothesized that thedevelopmental arrest in embryonic and extra-embryonic tissuesstems from decreased cell proliferation [150]. It is interesting to note, however, that the maternal stores of Max may be able rescue the early phenotype of Max deficient mice and also mask certain roles of Myc duringthe early stages of development [150].

1.6 Issues to be addressed in this thesis

Bmi1 and c-Myc oncogenes play synergistic roles in murine lymphomagenesis, however the mechanisms underlying this interaction arepoorly understood. It is known that the INK4a–Arf tumor suppressor locus is a critical downstream target of the PRC transcriptional repressor Bmi1. Others have shown that part of Myc's ability to induce apoptosis depends on the induction of Arf. Based on this relationship, it was found that down-regulation of INK4a–Arf by Bmi1 underlies its ability to cooperate with Myc in tumorigenesis. Aformer PhD student in our laboratory discovered nuclear foci in HEK293-cells transiently expressing both Bmi1 and c-Myc. I thus hypothesized that c-Myc physically and functionally interacts with Bmi1 in order to regulate diverse cellular process. This thesis aims to address the nature of this interaction as well asinvestigate how this phenomenon translates to other Myc family members.

1.7 Figures and Legends

Figure 1

From PRC complexes to Bmi1.

(A) Pictorial representation of both PRC complexes. The spatial organization of the PRC subunits represents their interaction network.

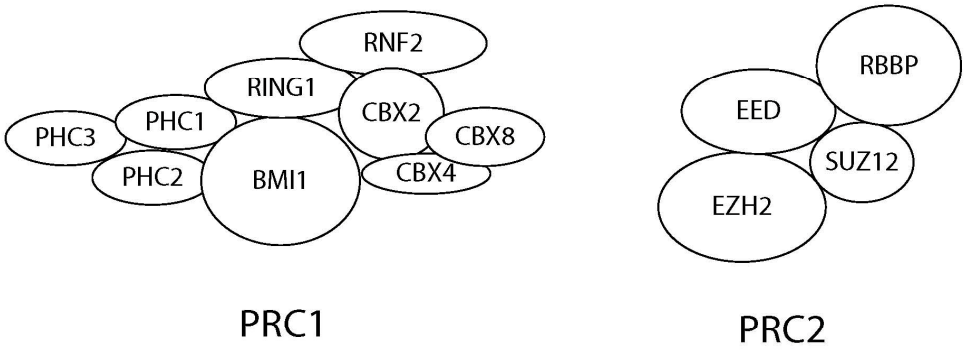
(B) Tabular representation of PRC1's characteristics. Drosophila Melanogaster and Human homologues are shown along with their individual subunit domains and associated functional activities.

(C) Diagram depicting Bmi1 protein structure. Aa residues are assigned to protein size, RINGdomain, NLS, and IE4F.

Figure 1Aistaken and adapted from figure 2found in[47]
Figure 1Bistaken and adapted from table 1 found in [47]

Figure 1

A



B

Complex	Drosophila melanogaster	Human	Domain	Activity
PRC1	PC	CBX2, CBX4, CBX8	Chromodomain	Binds H3K27me3
	PH	PHC1, PHC2, PHC3	Zink finger and SPM	Required for silencing
	RING	RING1 and RNF2	RING zink finger	Ubiquitin ligase
	PSC	Bmi1, PCGF2, ZNF134	Zink finger and SPM	Protein-Protein interaction

C

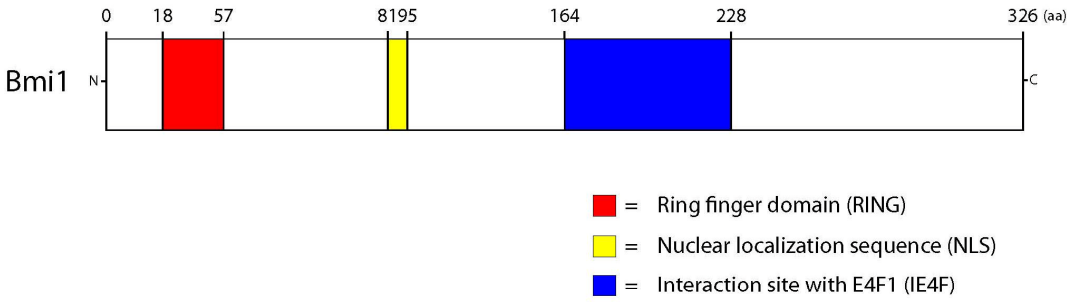


Figure 2

PTMs of the PRC complexes.

List of PRC subunits and their associated PTMs in the biological setting. More specifically, these PTMs are associated with the interacting proteinresponsible for the modification, the residues modified and the biological outcome resulting from these PTM.

This illustration is adapted fromTable 2 of [47]

N.B. interrogation marks represent potential functional capability

Figure 2

PRCs		PTM	Residues	Interacting protein	Function
PRC1	BMI1	Ubiquitination	Unknown	CULLIN3-SPOP	X chromosome inactivation ?
		Phosphorylation	S251, S253, S255	MAPKAP kinase 3	PRC1 chromatin association
	MEL18	Phosphorylation	Unknown	PKC	Allow Ub. Transfer to H2A
	MBLR	Phosphorylation	S32	CDK7	Unknown
	RING1B	Ubiquitination	K112	Itself	PRC1 activity
	CBX4	Sumoylation	K490	Unknown	Transcriptional repression
		Phosphorylation	Mainly T495	HIPK2	Transcriptional repression
PRC2	EZH2	Sumoylation	Unknown	Unknown	Unknown
		Phosphorylation	S21	PI3K-Akt	H3K27me3 levels
	SUZ12	Sumoylation	Mainly K75	PIASXβ	Unknown
	EED	Phosphorylation	Unknown	Unknown	PRC2 stability ?

Figure 3

Structural features of the Myc family.

(A) Domain characterization of the Myc family of proteins by comparative sequence analysis using UniProtKB. c-Myc is utilized as the template for the comparison. Aa residues are assigned to protein size, transactivation region, MB, NLS, BR, and HLH-LZ.

(B) Pictorial representation of the human Myc family of proteins.

(C) Chromosomal position and gene span of the Myc family in mouse and humans.

(D) ClustalW2 cladogram tree showing the evolutionary relationship between various human andmouse Myc members.

Figure 3

A

Myc protein family characteristic										
Myc gene family	Protein size	Transactivation domain	MBI	MBI I	MBIIa	MBIIIb	MBIV	NLS	BR	HLH-LZ
C-Myc	439	1 - 143	44 - 63	128 - 143	188 - 199	259 - 270	304 - 324	320 - 328	355 - 369	370 - 439
N-Myc	464	1 - 125	46 - 65	110 - 125	186 - 197	274 - 285	327 - 349	345 - 353	381 - 394	395 - 454
L1-Myc	364	1 - 103	24 - 43	89 - 103	N/A	N/A	N/A	263 - 270	282 - 294	295 - 361
S-Myc	431	1 - 126	44 - 63	111 - 126	N/A	N/A	N/A	N/A	349 - 361	362 - 421
B-Myc	170	1 - 134	39 - 57	119 - 134	N/A	N/A	N/A	N/A	N/A	N/A

B

C

Myc genes	Chromosome location		Gene length in Base Pairs (BP)	
	Human	Mouse	Human	Mouse
C-Myc	Chr 8q24.21	Chr 15qD	5366	2825
N-Myc	Chr 2p24.1	Chr 12qA1.1	6774	5743
L1-Myc	Chr 1p34.2	Chr 4qD2.2	6592	6381
S-Myc	N/A	Chr XqA1.1	N/A	2361
B-Myc	N/A	Chr 2qA3	N/A	840

D

Figure 4

Facets of Myc MBs.

(A) Representation of the biological and transformational functions associated with MB.

(B) PTMs found at MB sites on c-Myc, N-Myc,and L-Myc.

(C) Pictorial representation of the diverse binding partners of Myc.c-Myc is utilized as the template for the comparison.

Figure 4Aistaken and adaptedfromtable 1 and box 2 found in[134]

Figure 4Cistaken and adapted fromfigure 3 found in [71]

Figure 4

A

Myc Homology Boxes (MB)	Associated Functions	
	Myc Biology	Myc Transformation
MB I	• Controls the turnover of Myc proteins	• Essential for 1° REF co-Transformation with activated RAS. • Deletion mutants are able to transform Rat-1A cells.
MBII	Responsible for most of the biological functions of Myc: • Full transcriptional activation and repression • Binding of Coactivators • Null MBII inhibit most of the Myc phenotypes	• Domain essential in transforming REFs and Rat-1a cells. • Region of interation with TRRAP and other cofactors involved in transformation.
MBIIIa	• Functions broadly to attenuate Myc's apoptotic response • Implicated in the repression of transcription	• Region essential for Rat-1A transformation. • Shows intermediate transformation potential compared to wild type
MBIIIb		• Conserved domain with no assigned function
MBIV	• Affects Myc's DNA binding ability • Required for apoptosis • Hyperactive for G2 arrest	• Required for focus formation in Rat-1A and RK3E cells. • Dispensable for REF co-transformation focus and Rat-1A soft agar assays

Figure 4

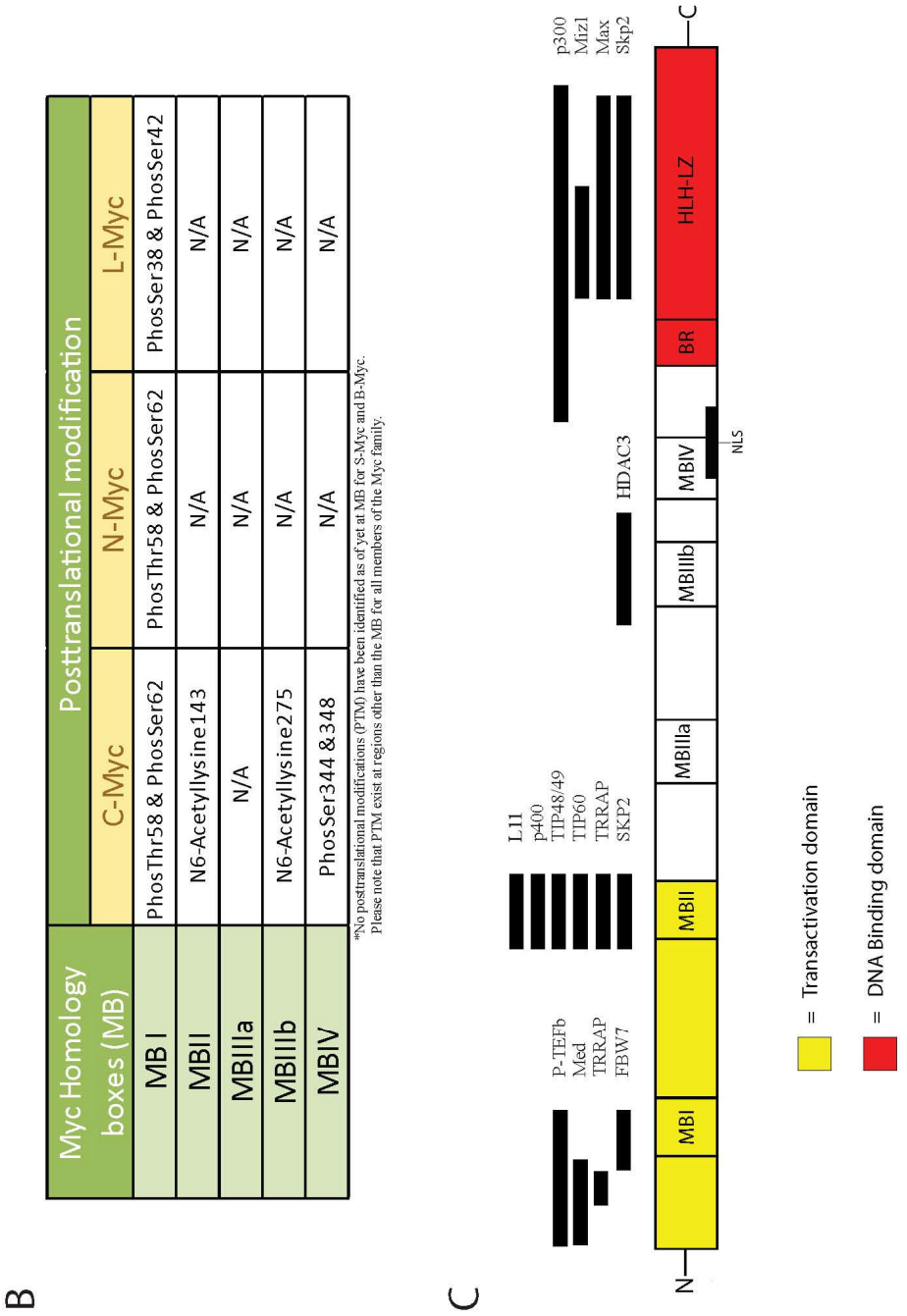

B
Myc Homology boxes (MB)
Posttranslational modification
C-Myc
N-Myc
L-Myc
MB I
PhosThr58 & PhosSer62
PhosThr58 & PhosSer62
PhosSer38 & PhosSer42
MBII
N6-Acetyllysine143
N/A
N/A
MBIIIa
N/A
N/A
N/A
MBIIIb
N6-Acetyllysine275
N/A
N/A
MBIV
PhosSer344 &348
N/A
N/A
*No posttranslational modifications (PTM) have been identified as of yet at MB for S-Myc and B-Myc.
Please note that PTM exist at regions other than the MB for all members of the Myc family.
C
P-TEFb
Med
TRRAP
FBW7
L11
p400
TIP48/49
TIP60
TRRAP
SKP2
HDAC3
p300
Miz1
Max
Skp2
N
MBI
MBII
MBIIIa
MBIIIb
MBIV
NLS
BR
HLH-LZ
C
= Transactivation domain
= DNA Binding domain

Figure 5

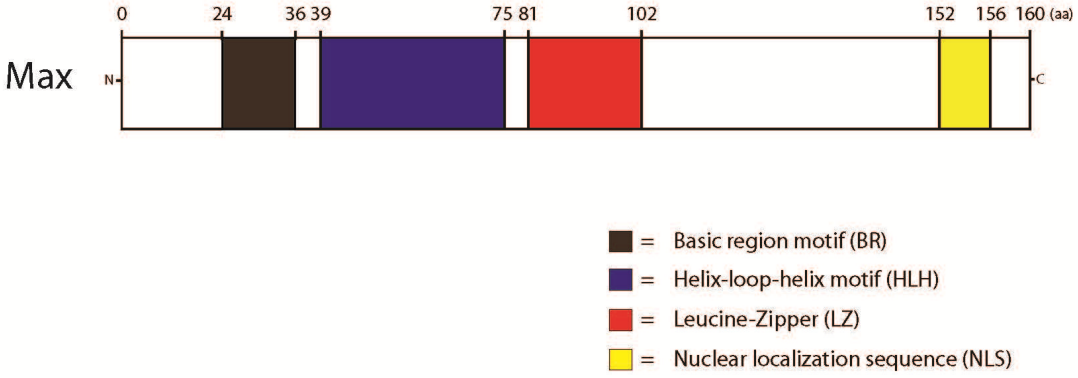
Max structure and PTM

(A) Pictorial representation of the human Max protein. Aa residues are assigned to protein size, BR, HLH-LZ, and NLS.

(B) Listof known Max PTMs.

Figure 5

A



B

Posttranslational modification	
Modification	Residue
PhosphoSerine	2 & 11
PhosphoTyrosine	123
N6-acetyllysine	66, 153, and 154

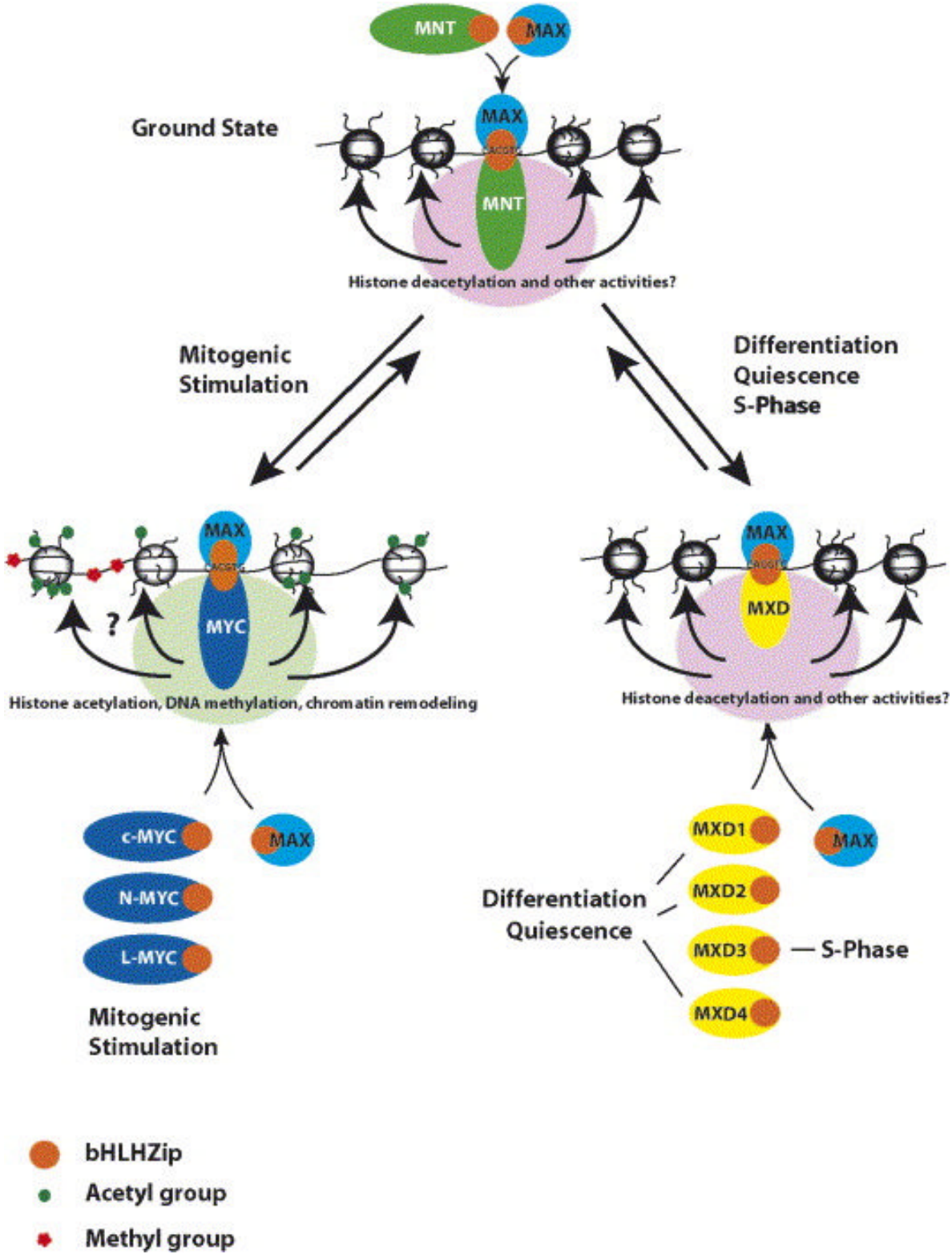
Figure 6

The Max network

The Max protein iscontinuouslyexpressed within the cell.As a result of this constant expression as well as its ability to interact with a wide variety of proteins, Max possesses the ability to modulate the transcriptional activity of its target promoters. For example, the MNT and MXD families both interact with Max and with SIN3A and SIN3B corepressors. These corepressors in turn cooperate with HDACs to cause chromatin compaction thereby preventing basal transcriptional machinery from accessing the promoter.Alternatively, although theMyc-Max complex has beenshown to cause, in certain circumstance, the transcriptional repression of certain promoters, ittypicallyengendersthe transcriptional activation of itstarget promoter.

Figure 6 is adapted from figure 1 found in[134]

Figure 6


MNT
MAX
Ground State
MAX
MNT
Histone deacetylation and other activities?
Mitogenic
Stimulation
Differentiation
Quiescence
S-Phase
MAX
?
MYC
Histone acetylation, DNA methylation, chromatin remodeling
MAX
MXD
Histone deacetylation and other activities?
c-MYC
N-MYC
L-MYC
MAX
Mitogenic
Stimulation
MXD1
MXD2
MXD3
MXD4
MAX
Differentiation
Quiescence
— S-Phase
bHLHZip
Acetyl group
Methyl group

Chapter II

Interaction betweenMyc andthe epigenetic regulator Bmi1

1.Introduction

PcG proteins are responsible for repressingthe transcription of various genes implicated in cell fatedetermination and cell proliferation. PRC1 and PRC2, generalPcG multiprotein complexes,haverecently been identified.Bmi1, a critical component of PRC1, plays an important role in the maintenance of Hox gene expression and in polycomb-mediated gene silencing. Furthermore, Bmi1facilitates RING1BE3 ligase activity,which in turn leads to the mono-ubiquitination ofLys-119 onhistone H2A[47].Nuclear structures, called PcG bodies, located near pericentric heterochromatin have also been associated with Bmi1[151]. Although the function of these bodies has not yet been defined, it is well established that pericentric heterochromatin is involved inmediating appropriatechromosomal segregation [151]. c-Myc has also been linked, with its partner Max,to another type of nuclear structure called a PML body[152]. Functionally, these bodies are known to mediate protein sumoylation and PTMs[153]. They also harbor ubiquitin, HAUSPand other elements required for the proteasomal degradation of proteins[152, 153]. Accordingly,PML bodies maybe linked to c-MycPTM or turnover. This notion is supportedby the finding that c-Myc and Max accumulate in the nucleolus following MG132 treatment [152].In HeLa cells, Myc has been shown to aggregatein PML bodies even in the absence ofMG132 [152]. Furthermore, the PML protein, which recruits TF and cofactors to the NB, has also been shown to physically interact with Myc. Interestingly, the PML-Myc interaction increases upon proteasomal inhibition[154].Additionally, Myc localizes to specific nuclear bodies with GSK3 followingT58 phosphorylation [155]. Recently, Myc was identified in HLB with Collin during the replication of endocycling and mitotic Drosophila cells[156]. HLB are well

characterized centers for histone mRNA processing and transcription [156].Due to the novelty of these foci, additionalstudies should be performed in order touncoverthe mechanisms underlyingBmi1 andMyc nuclear foci formation.

Bmi1 and Myc oncogenessynergistically cause tumorigenesis and cellular transformation [51]. Cells expressing Myc gain a proliferative advantage at the expense of apoptotic susceptibility [51]. However, deregulated Bmi1 levels upregulates Arf thereby preventing the Myc induced programmed cell deathresponse [51]. Other mechanisms underlyingthe Bmi1-Mycinterplay remain to be elucidated and require further investigation.

Our laboratory previously identified nuclear foci in HEK293 cells transfectedwith Bmi1 and c-Myc.Here I show that Bmi1 can form nuclear foci withL-Myc,another member ofthe Myc family.Using a variety of *in vitro* and *in vivo* techniques, I demonstratethat Bmi1 physicallyinteracts with the DBDof c-Myc. It is interesting to note that Myc's MBII may also be required for Bmi1 interaction and focus formation.Additional experimentationshould aim to shed light on this issue. Furthermore, byintroducingMax,I was able to block Bmi1-Myc interaction under certain physiologic conditions. Moreover,Max-mediates its disruption in a dose and Myctype dependent manner.

HDACiare known to repressgenes involved in growth inhibition as well as tumor suppressors[157]. As Bmi1 hasbeen identified as a potentialHDACi target andMyc may possess the ability to recruit HDAC3,it can be postulated that arelationshipmay exist betweenthese proteins[74]. I have shown thatHDAC 3 co-localizeswithBmi1-Myc nuclear

focibut only when Max is not overexpressed. These findings suggesta novel molecular relationship involvingBmi1, Myc and Max.

2. Materials and Methods

Plasmid constructs. cDNAs of human Bmi1, c-Myc, L-Myc, and Maxwere purchased from Open Biosystems, and mammalian expression plasmids containing Flag or HA tags were generated using the pcDNA3.1 vector (Invitrogen). GFP and mCherry constructs were derived from pEGFP-C2 (Invitrogen) and pmCherry-1 vectors (Clontech), respectively. Constructs were generated according to instructions from the manufacturer. HA-tagged ubiquitin plasmid was a gift from Wing SS.

Cell culture.HEK293cells were maintained in DMEM (Invitrogen) and were supplemented with 10% FBS (Sigma), and 1% P/S (Invitrogen).

Antibodies. The following antibodies were used: anti-Flag (Sigma, F3165), anti-HA (BabCO/Covance), anti-Mouse HRP IgG (Amersham, NA93IV), goat anti-Rabbit IgG (Fisher, AP307FMI), anti-GFP (Santa Cruz, SC-8334).

Cell transfection. For western blotting as well as IPs, 0.04 X 10^6 and 0.4 X 10^6 HEK293 cells were plated per well of a12 well plate and 10 cm dish, respectively. Transfections were performed with 3 µl or 20 µl Superfect (Qiagen) and 1.5 µg or 10 µg total DNA, respectively. For fluorescence microscopy, 0.04 X 10^6 HEK293 cells were plated per well of a 12 well plate. 12 well plate HEK293 cell transfections were performed with 3 µl Superfect (Qiagen) and 1.5 µg total plasmid DNA. Total plasmid DNA concentration was kept constant with pKSII (+) (Stratagene) supplementation. Transfected cells were incubated for 3 hrs at 37°C. Afterwards, wells/dishes were washed with 1 X PBS and new cDMEM (DMEM with 10 % FBS and 1 % P/S) was added to each

well/dish (1ml per well of a 12 well plate and 10ml in each 10cm dish). Experiments were performed 24-48 hrs post-transfection.

Co-immunoprecipitation. To analyze Bmi1 and Max binding to c-Myc (wild type or ΔI or ΔI-II or ΔI-III) or L-Myc,GFP-Bmi1 and HA-Max expression plasmids were transfected along with Flag tagged c-Myc (wild type or ΔI or ΔI-II or ΔI-III) or Flag tagged L-Myc in HEK293 cells. Transfections were performed in 10 cm plates using 20 µl Superfect and 10 µg of total DNA. Transfections were performed as specified by the manufacturer. 48 hrs post-transfection, cells were washed twice with cold 1 X PBS and lysed in buffer K ([20 mM] sodium phosphate[pH= 7.0], [150 mM]KCl, [30 mM] sodium pyrophosphate, 0.1 % NP-40, [5 mM] EDTA, [10 mM]NaF, [0.1 mM] Na_3VO_4, and the protease inhibitors leupeptin, aprotinin, pepstatin, and PMSF). Cell extracts were subjected to affinity purification on M2 agarose beads (Sigma) as instructed by the manufacturer. 200 µl extracts were added to 20 µl of pre-washed, with 200 µl buffer K,M2 agarose beads (Sigma) and rotated at 4°C for 2 hrs. Following 3 washes with 250 µl buffer K, bound proteins were eluted with 2 µl Flag peptide (Sigma) in 40 µl buffer K. Eluded proteins were stored at -80°C.

Immunoblotting. After adding 3 X SDS sample buffer, whole cell extracts and IP extracts were boiled for 5 min and then separated on a 12 % SDS-PAGE gelfor 90 min with a constant voltage of 120. Gels were transferred to a nitrocellulose membrane for 2 hrsat 4°C with a constant current of 200 mAmp. Membranes were then blocked in PBS-T (1 X PBS and 0.15 % tween-20) with 5% milk for 1 hr at room temperature and then incubated overnight at 4°C with 2 µl of their corresponding primary antibodies (anti-HA (Covance), anti-Flag (Sigma), anti-GFP (Santa Cruz)) in 5 ml PBS-T/5 % milk.

Membranes were washed with PBS-T (3 X 10 min), and then incubated with1 µl of their appropriate secondary antibodies for 1 hr at room temperature in 5 ml PBS-T/5 % milk. Following a 3 X 10 min washing procedurewith PBS-T, membranes were incubated for 5 min in a 4 ml Supersignal enhanced chemiluminescent solution mix (Pierce). Membranes were developed on chemiluminescent films in a dark room and visualized there. Developed films were then scanned and processed with Adobe Photoshop and Illustrator.

Bacterial protein extraction.MBPtagged c-Myc expression plasmids were grown overnight at 37□in DH5α cells on ampicilin resistant LB plates. Picked colonies were incubated in a 5ml LB-ampicilin (100 mg/ml) solution and shaken overnight a 37□. 2ml of bacterial culture was added to a 200ml LB-ampicilin flask and grown to 0.4λ absorbance after which2ml of [0.1M] IPTG was added. Following 2 hrs of inoculation in LB-ampicilin media supplemented with 2 ml of 0.2% glucose, cultures were collected, centrifuged andwashedwith 1 X PBS 2 times. Pellets were then resuspended in buffer K ([20 mM] sodium phosphate, [pH= 7.0], [150mM]KCl, [20 mM] sodium pyrophosphate, 0.1% NP-40, [5 mM] EDTA, [10 mM]NaF, [0.1 mM] Na_3VO_4, and the protease inhibitors leupeptin, aprotinin, pepstatin, and PMSF) or buffer B ([20 mM]Tris-HCl [pH= 8.0], 10% glycerol, [5 mM] $MgCl_2$, 0.1% NP-40, and the protease inhibitors leupeptin, aprotinin, pepstatin, and PMSF) containing [0.5M]KCl and sonicated(VirSonic 100apparatus set at strength 5) 6 times each, on ice,with 1 min intervals in between. Sonicated whole cell extracts were then centrifuged at 4°C for 10 mins and stored at -80°C.

***In vitro* transcription and translation.** 1ng of HA-Bmi1 expression plasmid was used as template DNA with 1 µl of 40 µg/µl RNsin® Ribonuclease Inhibitor, 2 µl of TNT

Reaction Buffer, 25 µl of TNT Rabbit Reticulocyte lysate, 1 µl of 1 mM minus Methionine Amino Acid mixture, 1 µl of TNT® RNA polymerase and [^{35}S] Methionine. The reagents were mixed in a 0.5 ml eppendorf tube and then placed in a 30□ incubator for 90 mins. The [^{35}S] radioactively labeled HA-Bmi1proteinswere stored at -80°C.

MBP pull down assay. 5µl of MBP-tagged c-Myc (wild type or ΔI or ΔI-III) whole cell extractsand [^{35}S] radioactively labeled HA-Bmi1proteinswere added to 20 µl (pre-washed with 200 µl buffer B)amylose resin with200 µl buffer B and 5% BSA. Following a2 hr rotation at 4°C, resin mixtures were washed 3times with buffer B.Proteins werethen eluted in40 µl buffer B following a 30 mins rotation at 4°C with 10% maltose. After adding 3X SDS loading buffer, samples were boiled for 5 min and separatedona 12 % SDS-PAGE gel for 90 min with a constant voltage of 120. SDS Gelswere soaked in 0.1%R250 coomassie blue solution for 20 minat room temperature and destained with a destaining solution (450ml ethanol, 100ml acetic acid, and 450 ml H_20) for 60 mins. Gels were then washed 4 times for 30 mins with distilled water and dried for 50 min. Autoradiography on a chemiluminescent film was performed to detect theradioactive signal emitted by the [^{35}S] radioactively labeled HA-Bmi1.

Statistical analysis. For experiments with more than two conditions, one-way ANOVAs were performed with a Bonferroni multiple comparison test. For experiments with two conditions, unpaired two-tailed *t*-tests were performed. $P<0.05$ was considered statistically significant.

3. Results

Subcellular Bmi1 and Myc colocalization. Bmi1 and c-Myc synergistically induce transformation and tumorigenesis by preventingapoptosisthrough aMyc dependent upregulation of Arf. However, little else is known about their interconnectivity. A former PhD student visualized in live cells using green fluorescence microscopyGFPnuclear foci inHEK293 cells transfected with the expression plasmids GFP-Bmi1 and Flag tagged c-Myc. Irepeated his experiment and reconfirmed his findings (Fig. 1A). To investigate if other Myc family members displayedsimilar nuclear structures,Itransfected the expression plasmid for GFP-Bmi1in HEK293 cells with Flag tagged L-Myc (Fig. 1A).The L-Myc and Bmi1 nuclear foci that were observed with the aid of a fluorescent microscope exhibitedsimilar morphological features as those observed with Bmi1 and c-Myc. Thus,I concluded that Bmi1-Myc nuclear foci are not featuresexclusive to c-Myc.

MBs are important for the biological activity of Myc proteins. To examine which MBis essential for the formation of these nuclear foci, various Flag tagged c-Myc MB mutant expression plasmids were generated (Fig. 1B). HEK293 cells were transfected with expression plasmids encoding GFP-Bmi1 together with Flag tagged c-Myc mutants (ΔI. ΔI-II or ΔI-III) (Fig.1C). Nuclear foci were observedin live cells using green fluorescent microscopy for ΔIbut not with ΔI-II or ΔI-III. ThereforeMBIis not necessaryfor the formation of Bmi1 and c-Myc nuclear foci.

Bmi1 and c-Myc physically interact *in vitro* and *in vivo*.The observation that Bmi1 can form nuclear foci with c-Myc prompted me to examine whether Bmi1 directly interacts with Myc to form these nuclear structures. To address this question, an *in vitro*

transcription and translation assaywas performed toassess Bmi1'sability to interact with c-Myc *in vitro*(Fig. 2A& B).Radioactively labeled Bmi1 was mixed with bacterial cell extracts expressing MBP tagged c-Myc(WT, ΔI or ΔI-III) andwas then pulled down. MBP proteins were detected on aR250 Coomassive Blue stained SDS-PAGE gel(Fig. 2B) and autoradiography was performed to spot the radioactively labeledBmi1 proteins (Fig. 2A). Bmi1 was able tophysically bind to all MBP tagged c-Myc proteins implying that MBs are not essential for the Bmi1 and c-Myc interaction.However, residual endogenous proteins left behind in the whole cell extractsmayor may nothave interacted with the radioactively labeled Bmi1 proteins.To further investigate this interaction from another perspective, I performed an immunoprecipitation experiment toexaminewhether I would be able to detect the interaction of Bmi1 with various c-Myc proteins. Accordingly, I co-expressed GFP-Bmi1 along with Flag tagged c-Myc (WT, ΔI, ΔI-II or ΔI-III)in HEK293 cells and performed western blotting with antibodies against Flag and GFP (Fig. 2B). Bmi1 was effectively immunoprecipitated with all c-Myc proteins.However, Bmi1 weakly interacted with ΔI-III. Taken together,these resultsimplythat Bmi1 directly interacts with c-Myc and that c-Myc'sDBD could serve as a docking site forBmi1.

Bmi1 associates with the DBDand MBII of c-Myc. Max is a transcription cofactor that binds to the HLH-LZ domain of Myc proteins. To assess whether Max can affect the formation of Bmi1 and c-Myc nuclear foci, I transfected HEK293 cells with GFP-Bmi1 together with mCherry-Max and Flag tagged c-Myc(Fig. 3A-B). Strikingly, the addition of Max prevents the formation ofany Bmi1 and c-Myc nuclear foci. To validate these results from another angle, HEK293 cells were transfected withthe expression plasmids encoding GFP-Bmi1 as well as mCherry tagged c-Myc and HA-Max

(Fig 3C-D). As shown in figure 3A, the addition of Maxalmost completely disruptsc-Myc-Bmi1 nuclear foci. Taken together, these results indicate that Max is able to prevent the formation of Bmi1 and c-Myc nuclear foci. I therefore hypothesize that Max and Bmi1competeto bind c-Myc's HLH-LZ domain. To address this conjecture, an immunoprecipitation experiment was performed to detect if Max is able to outcompete Bmi1 frombinding to c-Myc. For this I co-expressed in HEK293 cells the expression plasmids encoding GFP-Bmi1along with HA-Max and Flag tagged c-Myc and performed western blotting with antibodies against GFP, Flag, and HA(Fig. 4A).Although Maxseems to be able tocompletely outcompeteBmi1 frombinding toc-Myc,anti-GFP protein levels in Fig.4A are very weak and make the interpretation of thisexperiment difficult. Additionally, the experiment was only performed once and must be repeated at least two more times beforeany conclusion canbe drawn. Our laboratory is currently addressing these experimental setbacks.Next I sought to investigatethe ability of Max to dislodge Bmi1 from the various c-Myc mutants (ΔI,ΔI-II or ΔI-III) (Fig. 4B). As it turns out, Max does not permit Bmi1 to bind the c-Myc ΔI-II and ΔI-IIImutants. However, Bmi1 can bind,to a lesser extent,ΔI in the presence of Max.Taken together, these results indicate that Max and Bmi1 compete to bind to c-Myc's DBDand possibly MB II.These experiments were only performed once and must be repeated at least two more times beforeany conclusion canbe drawn. Additionally, more experiments are needed to understand why the loss of MB I weakly affects the binding of Bmi1 to Myc in the presence of Max.

Max mediated Myc-Bmi1 nuclear foci disruption is dose dependent.Although my previous experiments suggest that Max disrupts Bmi1 and Myc nuclear foci, the

extent of thisdisruption has not beenaddressed.Therefore, Isought to investigate whatimpactadding different concentrations of Max might have on the formation of Bmi1-Myc nuclear foci. Specifically, the GFP-Bmi1expression plasmid was transfected along with the expression plasmidsencoding Flag tagged Myc (L-, c-,ΔI,ΔI-II or ΔI-III) and HA-Max, which were added in increasing concentrations(Fig. 5A and B).Concomitantly with the results observed in Fig. 4, ΔI and L-Myc wereonly partially disrupted in their ability to form nuclear foci. On the other hand, c-Myc and Bmi1 nuclear foci were severely affected following the addition of 100ng of the expression plasmid Max.As expected, c-Myc nuclear foci fall close to 0% when Max levels reach 300ngbut L-Myc nuclear foci levels decrease by only half;with 30% nuclear foci still present at 300ng of Max. ΔI levels like L-Myc decrease by half but still remain relatively high with 40% nuclear foci visible following the addition of 300 ng of Max. ΔI-II and ΔI-III mutants' focilevels plunge to 0% after reaching 200ng of Max. Importantly,although in Fig. 5B certain columns are not statistically significant, the overall tendency of the diagrams isnot affected. Members of the laboratory are currently addressing these problems. Therefore, it can be said that the difference in Max-mediated disruption of Bmi1-Myc nuclear foci is dose dependent and Myc type dependent.

HDAC 3 encourages Bmi1-Myc nuclear foci. HDACi are known to triggerthe inactivation of repressors located on growth inhibitory genes and tumor suppressors [158].These regulatory genes are normally silenced by PcG proteins[158]. It was recently reported that Bmi1 expression could be downregulated by HDACi[158].Moreover, Myc is able to recruit HDAC3with MBIII and cause the repression of gene activity[74]. To investigate the possibility ofHDAC3recruitmentto Bmi1-Myc nuclear foci, HEK293

cellswere transfected with the expression plasmids encoding GFP-Bmi1, mCherry-HDAC3, HA-Max, and Flag tagged L-Myc or c-Myc (Fig. 6A). With the aid of a fluorescence microscope, HDAC3 was detected at c-Myc and L-Myc nuclear foci. Interestingly, the displacement of HDAC3 from Bmi1 and L-Myc nuclear foci mediated by Maxwasonly partialwhereas a complete loss wasobserved in the case ofc-Myc. To gaina better understanding of HDAC's rolein the formation of these nuclear foci, Flag tagged c-Myc or L-Myc and GFP-Bmi1 were transfected in HEK293cellsand treated with 25 ng of TSA, an HDACi (Fig 6B). A significant drop in the formation of nuclear foci was noted,especially for L-Myc. Taken together these results indicate the possibility that HDAC3is involved in the formation ofthese Bmi1-Myc nuclear foci.

4. Discussion

Bmi1 and Myc make nuclear foci. The expression of Myc is commonly deregulated in many human neoplasias. Studies from transgenic animals strongly support the conclusion that the deregulation of Myc induces tumorigenesis.Myctransgenic mice have identifiedBmi1, a proto-oncogenic member of the PRC1 complex, as a gene which is able tocooperate with c-Mycat the inceptionof B-cell lymphomas[51]. Moreover, transgenic Bmi1-Myc mice have been shown to die fromaggressive leukemia as newborns[159].Whereas an*in vivo* cooperation has been established between these two oncogenes, the molecular basis for theirsynergisticaction remainsvaguedue toscarce knowledge of their basic biological mechanics.Unpublished data from our laboratory demonstrated that nuclear foci are generatedby Bmi1 and c-Myc.To gain insights into this interplay, I reconfirmed that indeed both proteins are able to co-localizeand form nuclear foci (Fig. 1). Furthermore, I demonstrated that these nuclear foci are not unique to c-Myc but extend to other members of the Myc family(Fig. 1).I thenwent on to showthat these two proteins can directly bind to each other *in vitro*and *in vivo*(Fig. 2). In order to strengthen the *in vitro* and *in vivo* studies, I would need to track Max protein levels in whole cell extracts. Additionally, these experiments will need to be duplicated. Future graduate students in our laboratory will be addressing theseimportant controls.However, from these preliminary results I am able to conclude that Bmi1 directly interactswith Mycandwhile doing so,formsnuclear foci.

Bmi1 physically interacts with the DBD and MBII of c-Myc. To identify the Myc-Bmi1 interaction site, c-Myc MB mutant expression vectors were generated (Fig 1). I was able to determine that MBIwas not required for the formation of Bmi1 and c-Myc

nuclear foci(Fig. 1). By performing *in vitro*and *in vivo* experiments, I detected Bmi1 bound to all c-Myc mutants (Fig. 2 and 4). These experiments will need to be duplicated several more times in order to drawa definitive conclusion. However, these preliminary results suggest that Bmi1 bindsto theDBDof c-Myc.This hypothesis was directlytested by usingMax, a protein able to bind to the DBD of Myc, with Bmi1 and Myc. I observedthe disappearance of Myc-Bmi1 nuclear focifollowingthe addition of Max (Fig. 3). Quantifying the degreeof foci loss revealed an almost 100% deficit (Fig 3). I thenexaminedMax'sability to modulate the binding of Bmi1 to Myc (Fig. 4). Maxwas able to out-competeBmi1 in terms ofc-Myc binding.Due to antibody problems in the laboratory, the anti-GFP protein levels in Fig.4A are very weak and make the interpretation of thisexperiment difficult. A graduate student in our laboratory is currently addressing this experimental setback.On the other hand, c-Myc mutants and Bmi1were differentially affected by the addition of Max(Fig. 4). ΔIwasstill able to bind Bmi1 followingthe addition of Max, but to a lower extent. However, a complete loss in the binding ability of Bmi1 withΔI-IIorΔI-III was noted followingthe addition of Max.These experiments will need to be duplicated so that a valid inference can be made. However, fromthese preliminary results I can conclude that Bmi1 is able to physically interact withthe DBD and possibly MBII of c-Myc. This model would seem plausible, as it appears tobe consistent with other Myc/chromatin remodeler interactions. To further verify Bmi1's ability to bind MBII, future graduate students would need to generate a c-Myc ΔBHLH-LZ mutant anddo an immunoprecipitation.

Bmi1 physically interacts with L-Myc. I demonstrated earlier that L-Myc canformnuclear foci with Bmi1 (Fig. 1A). An immunoprecipitation experiment was

performed in order to examine the ability of Max to affect the binding of Bmi1 with L-Myc (Fig. 4). Similar to ΔI,L-Mycdisplays a decrease in Bmi1 binding followingthe addition of Max (Fig. 4).Therefore, it appears that the binding of Bmi1to c-Myc or L-Myc is differentially affected upon adding Max. This may indicate that the Max protein could bind to different regions of the Myc proteins, including MBII. Further experimentationis required to validate this conclusion.

Max is insufficient to completely disrupt the Bmi1 and L-Myc interaction. I demonstrated earlier that L-Myc can make nuclear foci with Bmi1 (Fig. 1A). An immunoprecipitation experiment was performed in order to investigate to what extent the addition of Max can block the interaction between L-Myc and Bmi1. To test this hypothesis, HEK293 cells were transfected with expression plasmids for GFP-Bmi1along with HA-Max and Flag tagged L-Myc and western blotting was performed with antibodies against GFP, Flag, and HA (Fig. 4C). Similar to ΔI, Max cannot completely disrupt Bmi1's ability to interact with L-Myc but can reduce it. Surprisingly, an L-Myc band shift,suggesting the addition of a PTM, can be observed when overexpressing Max with L-Myc. At the present time, additional experiments are required to conclude anything regardingthe nature and function of these L-Myc bands. More experiments and conclusions are presented in the following section of this thesis.

The Max mediated Myc-Bmi1 nuclear foci disruption is dose dependent. To determine the levels of Maxneeded to dislodge Bmi1 from Myc, Bmi1 as well as Max, added in a graded fashion, wereused in combination with various Myc's(Fig. 5). In concordance with previous results,ΔI and L-Myc retained their ability to form nuclear foci whereasc-Myc,ΔI-IIand ΔI-IIIlost their foci forming ability following the addition of

200ng of Max expression plasmid. I then evaluated, in percentage, the loss of nuclear foci at each graded addition of Max(Fig. 5). It permitted me to conclude that L-Myc is least affected by the addition of Max followed by ΔI. c-Myc, ΔI-II, and ΔI-III all displayed a complete or almost complete loss ofnuclear foci. Although certain columns in Fig. 5B are not statistically significant, the overall tendency of the diagrams isnot affected. Future graduate students will address these problems. In conclusion, these results permitted me to deduce that distinct Myc-Bmi1 combinations are differentially affected by the overexpressionof Maxat various concentrations.

HDACs promotethe formation of Bmi1-Myc nuclear foci. HDACi are known silencing targets of PcG proteins and PRCs[160]. The ability of HDACi to inhibit Bmi1 as well as the ability of Myc to recruit HDAC3 through MBIII indicatesthat these proteins maybe linked[74, 160]. I discovered thatHDAC3 can co-localize to Bmi1-Myc nuclear foci (Fig 6). Followingthe addition of Max, this co-localization is partially lostwith L-Myc but is completely absentin the case ofc-Myc. Interestingly, TSAtreated L-Myc nuclear foci are disruptedto a greater extent than those of c-Myc (Fig 6).These preliminary results indicate that HDAC3 maybe requiredforthe biological organization of these Bmi1-Myc nuclear foci. However, more tests are required before any conclusions can be drawn.

In Fig. 7, Ipropose a model for the mechanistic functioning of these nuclear foci.In the model, Myc combines with PRC1, which is attached to repressive histonemarks like H3K27me3 by PC,through Bmi1 by virtue of Myc's DBD or MBII. At the same time, Myc recruits HDAC3 viaMBIII and causes the deacetylation of histones resulting inchromatin compaction. However, uponthe arrival of Max, the complex

dissociates enabling Myc-Maxto bind the promoters of these repressed genes and in turn lead totheir transcriptional activation. PRC1 can either remain bound or dissociate fromH3K27me3 marks. To validate this model I would needto ensurethat Myc can be recruited to H3K27me3 bound PRC1 complexes.This may be examined eitherdirectly byimmunofluorescence visualization using H3K27me3 antibodies merged with Myc and Bmi1 antibodies or indirectly by visualizingthe regression of nuclear foci following 5-AZA-dC treatment,a DNA methylation inhibitor. *In vivo*complex validation can be done by overexpressing Bmi1 and Mycin cell lines and then performinga Chip on Chip.If and once targets have been identified,quantitative PCR of target promoterscan be done in order to assess their activity. These markers can then be used to assess Myc and Bmi1's ability to impact lymphomatic cancers.

5. Figure Legends and Figures

Figure 1. Subcellular colocalization of Bmi1 and c-Myc. (A) HEK293 cells were transiently transfected with expression plasmids encoding GFP-Bmi1 and Flag tagged c-Myc or L-Myc. Livegreen fluorescence microscopy was performed to monitor subcellular localization of GFP-Bmi1. The experiment was repeated on three separate occasions. (B) Schematicrepresentation of c-Myc and tree different deletion mutants (C). Shown atthe right is where nuclear foci were observed when c-Myc or its deletion mutants were co-expressed with Bmi1. (C) HEK293 cells were transiently transfected with expression plasmids encoding GFP-Bmi1 and Flag tagged c-Myc mutants as indicated. GFP-Bmi1 localization was determined by the use of livegreen fluorescence microscopy. The following experiment has been repeated on three separate occasions.

Figure 1

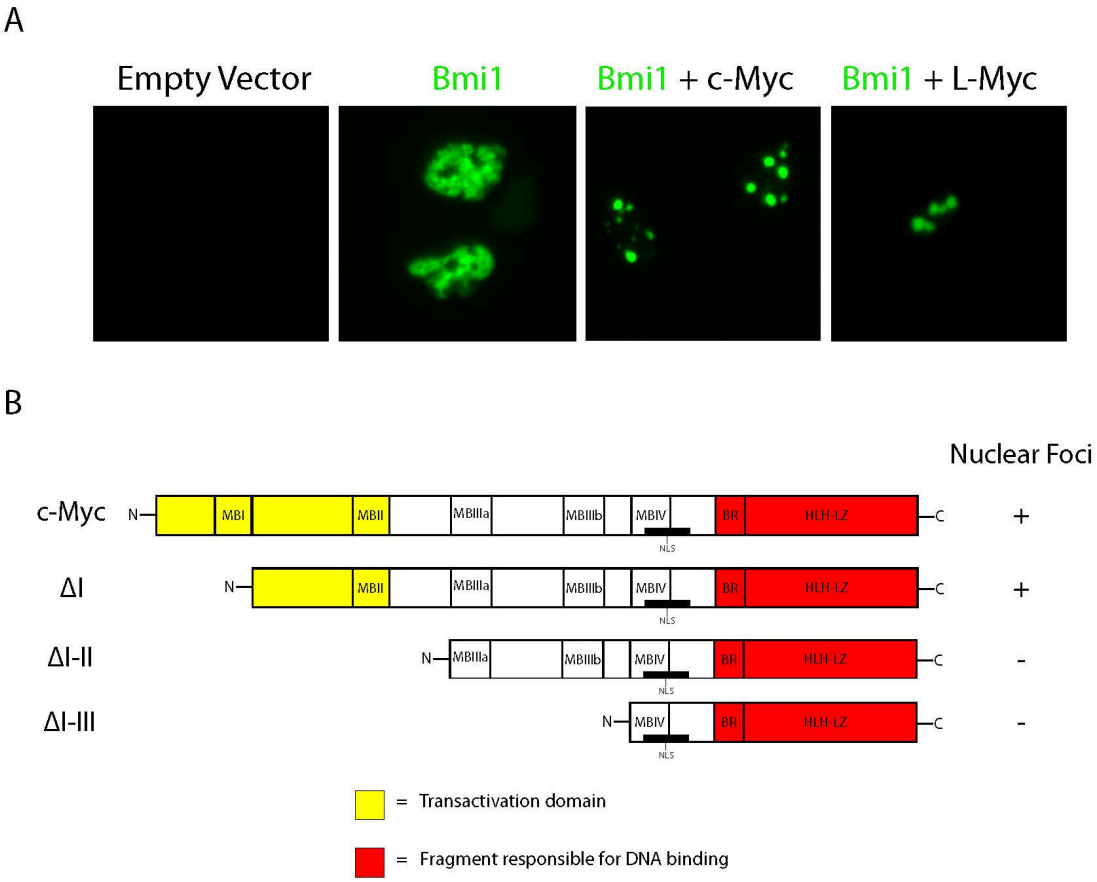

A
Empty Vector
Bmi1
Bmi1 + c-Myc
Bmi1 + L-Myc
B
Nuclear Foci
c-Myc
ΔI
ΔI-II
ΔI-III
N
C
MBI
MBII
MBIIIa
MBIIIb
MBIV
NLS
BR
HLH-LZ
+
+
-
-
= Transactivation domain
= Fragment responsible for DNA binding
C
Bmi1 + ΔI
Bmi1 + ΔI-ΔII
Bmi1 + ΔI-ΔIII

Figure 2. Interactionbetween Bmi1 and c-Myc*in vitro* and *in vivo*.(A-B) Bmi1 was synthesized *in vitro*in rabbit reticulocyte in the presence of [^{35}S]methionine. [^{35}S]labeled Bmi1 was mixed with Amylose resin immobilized MBP-tagged c-Myc proteins. After extensive washing, the bound protein mixture was eluded with maltose for SDS-PAGE and subsequent Coomassie staining (B) and autoradiography (A). The experiment was repeated twice. (C) HEK293 cells were transiently transfected with expression plasmids encoding Flag tagged c-Myc, deletion mutants (Fig 1B), and GFP-Bmi1 as indicated. Forty-eight hours later, cells were harvested in buffer K and Flag-tagged proteins were immunoprecipitated on M2 agarose beads and eluded with Flag peptide. Whole cell extracts (Input: 20μl) and immunoprecipitates (IP: 20μl) were separated by SDS-PAGE for immunoblotting with antibodies directed against the indicated proteins. The experiment was repeated twice.

Figure 2

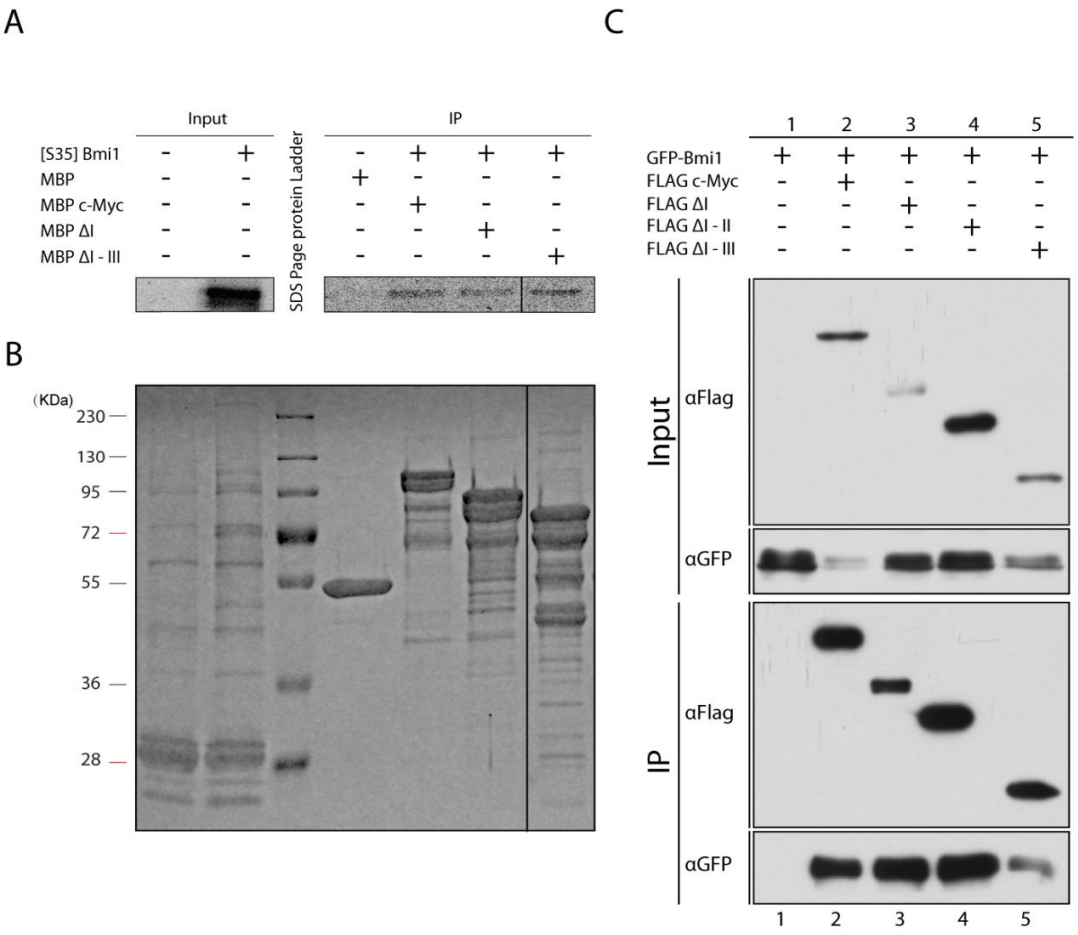
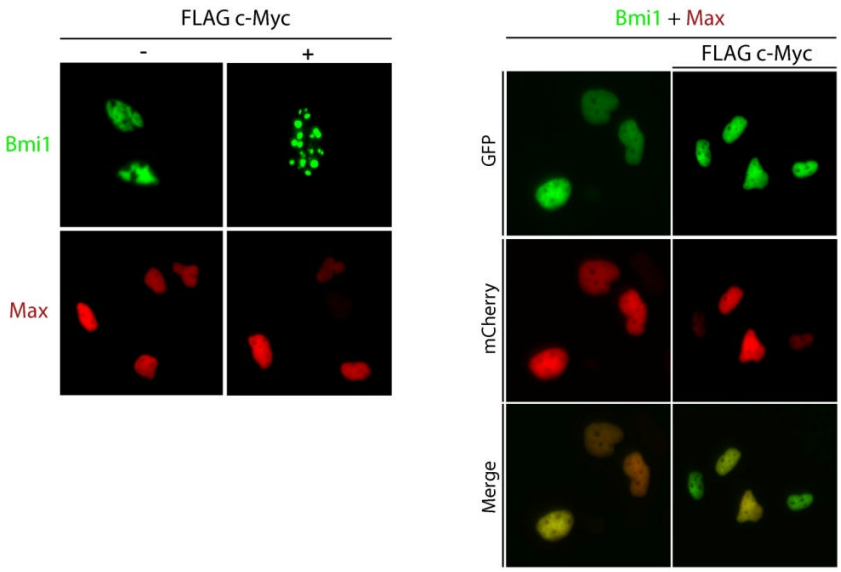

A
Input
IP
[S35] Bmi1
MBP
MBP c-Myc
MBP ΔI
MBP ΔI - III
SDS Page protein Ladder
B
(KDa)
230
130
95
72
55
36
28
C
1 2 3 4 5
GFP-Bmi1
FLAG c-Myc
FLAG ΔI
FLAG ΔI - II
FLAG ΔI - III
Input
αFlag
αGFP
IP
αFlag
αGFP
1 2 3 4 5

Figure 3. Max inhibits formation of c-Myc-Bmi1 nuclear foci. (A) HEK293 cells were transiently transfected with the expression plasmid encoding GFP-Bmi1, together with those for Flag tagged c-Myc and mCherry-Max as indicated. GFP-Bmi1 and mCherry-Max were visualized via green and red fluorescence microscopy, respectively. The experiment was repeated 3 times. (B) Quantification of (A). At least 100 cells were counted for each condition for 3 independent experiments. ***Indicates $p<.0001$. (C) HEK293 cells were transiently transfected with the expression plasmid encoding GFP-Bmi1, together with those mCherry tagged c-Myc and HA-Max as indicated. Green and red fluorescence microscopy was performed to monitor the subcellular localization of GFP-Bmi1 and mCherry-tagged c-Myc, respectively. The experiment was repeated 3 times. (D) Quantification of (C). At least 100 cells were counted for each condition for 3 independent experiments. ***Indicates $p<.0001$.

Figure 3

A


FLAG c-Myc
-
+
Bmi1
Max
Bmi1 + Max
FLAG c-Myc
GFP
mCherry
Merge

B

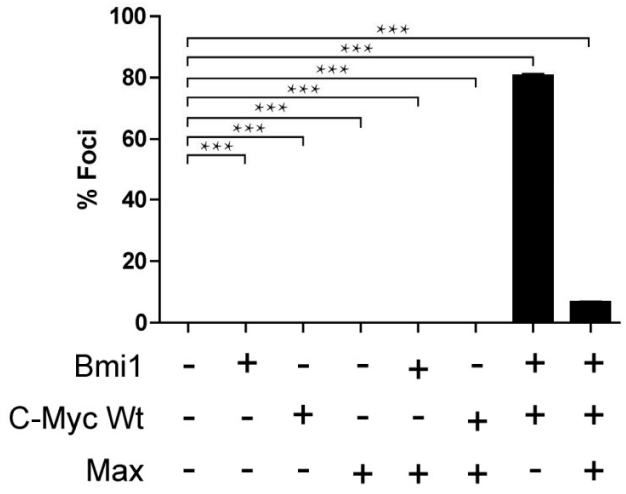
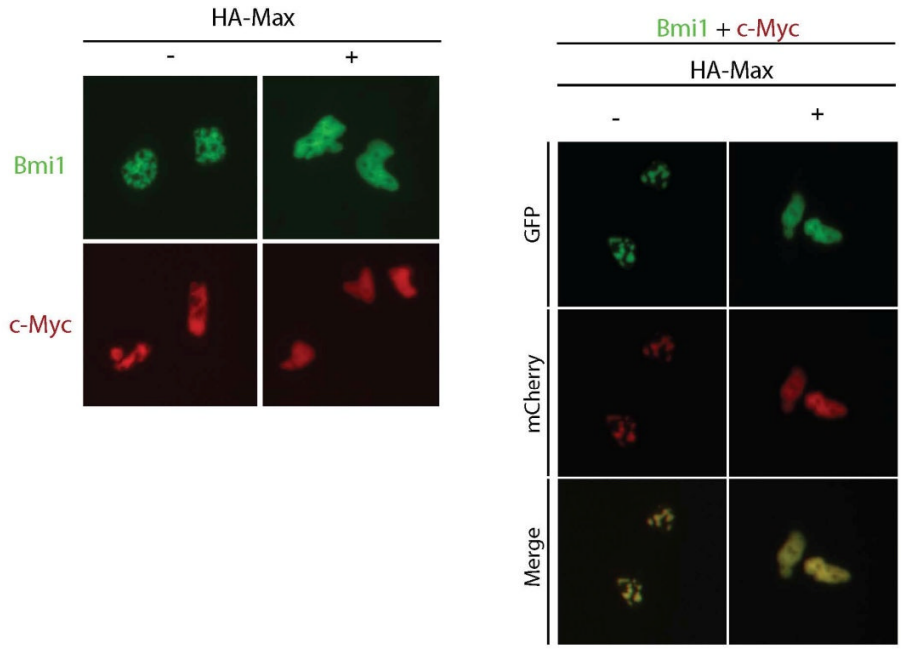

100
80
60
40
20
0
% Foci
***
Bmi1 - + - - + - + +
C-Myc Wt - - + - - + + +
Max - - - + + + - +

Figure 3

C


HA-Max
-
+
Bmi1
c-Myc
Bmi1 + c-Myc
HA-Max
-
+
GFP
mCherry
Merge

D

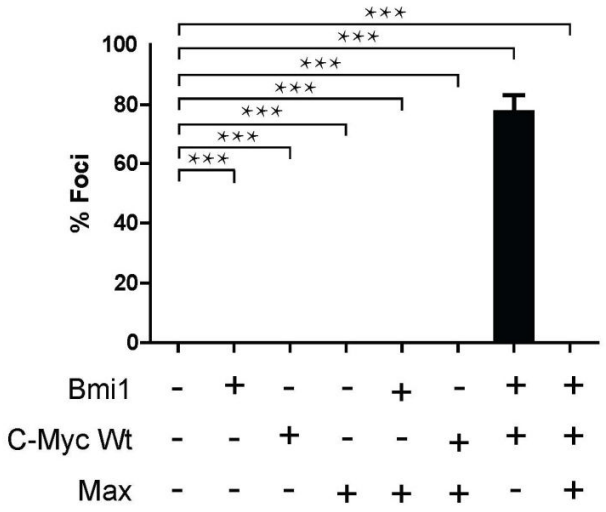

***
***
***
***
***
***
***
100
80
60
40
20
0
% Foci
Bmi1 - + - - + - + +
C-Myc Wt - - + - - + + +
Max - - - + + + - +

Figure 4. Differential capabilities of Max and Bmi1 to bind Myc proteins. (A) HEK293 cells were transiently transfected with expression plasmids encoding Flag tagged c-Myc and GFP-Bmi1. Forty-eight hours later, cells were harvested in buffer K and Flag-tagged proteins were immunoprecipitated on M2 agarose beads and eluded with Flag peptide. Whole cell extracts (Input: 20μl) and immunoprecipitates (IP: 20μl) were separated by SDS-PAGE, and immunoblotting was performed with antibodies against the indicated proteins. The experiment was repeated on one occasion. (B) As in (A)except that Flag tagged c-Myc was substituted with ΔI (lane 1, 6-8), ΔI-II (lane 2, 9-11) or ΔI-III (lane 3, 12-14). The experiment was repeated on one occasion. (C) As in (A)except that Flag tagged L-Myc was substituted withFlag tagged c-Myc. The experiment was repeated twice.

Figure 4

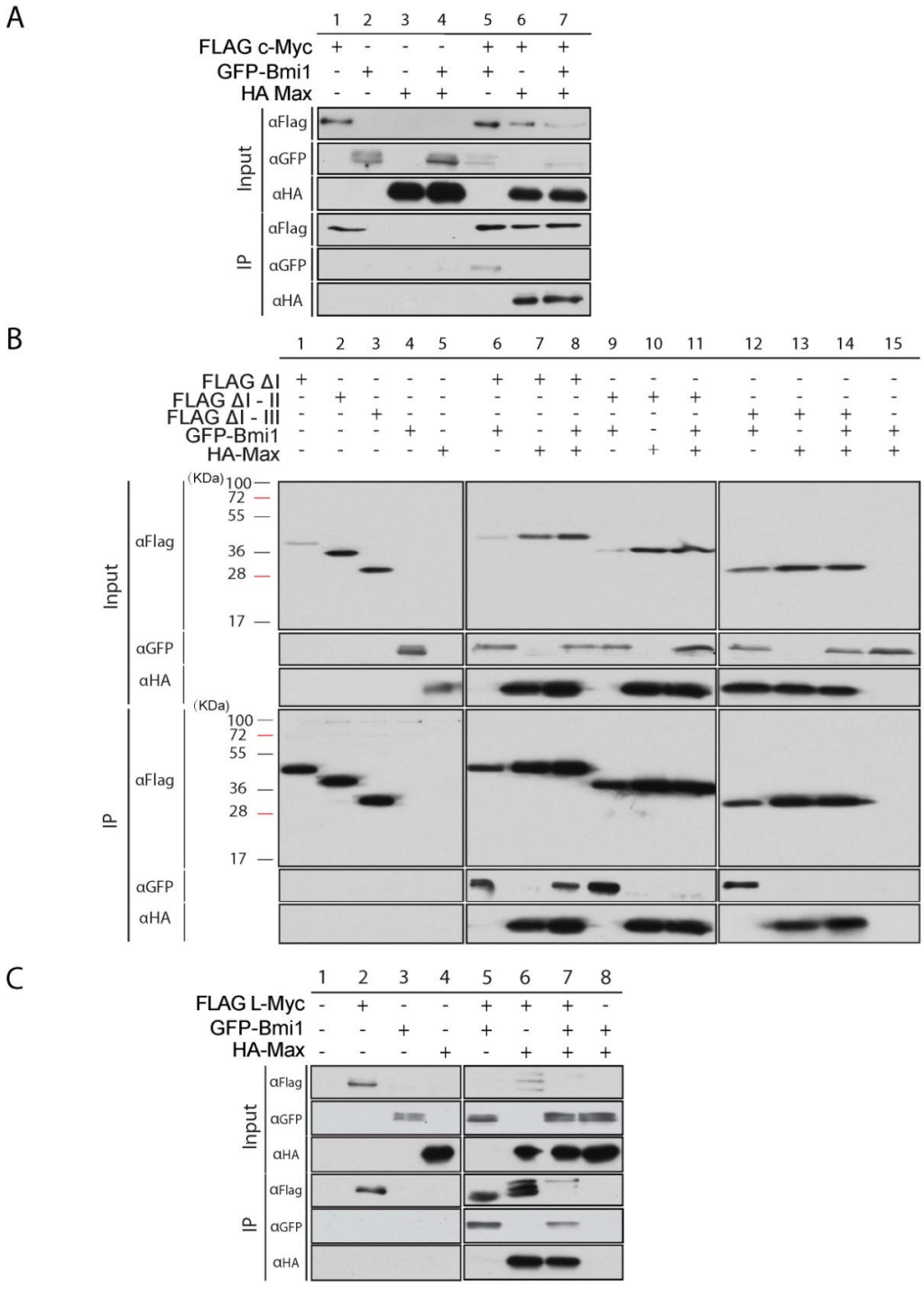

A
1 2 3 4 5 6 7
FLAG c-Myc + - - - + + +
GFP-Bmi1 - + - + + - +
HA Max - - + + - + +
Input
IP
αFlag
αGFP
αHA
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
FLAG ΔI + - - - - + + + - - - - - - -
FLAG ΔI - II - + - - - - - - + + + - - - -
FLAG ΔI - III - - + - - - - - - - - + + + -
GFP-Bmi1 - - - + - + - + + - + + - + + +
HA-Max - - - - + - + + - + + - + + +
(KDa)
100
72
55
36
28
17
Input
IP
αFlag
αGFP
αHA
C
1 2 3 4 5 6 7 8
FLAG L-Myc - + - - + + + -
GFP-Bmi1 - - + - + - + +
HA-Max - - - + - + + +
Input
IP
αFlag
αGFP
αHA

Figure 5. Max disrupts Myc-Bmi1 nuclear foci formation in a dose-dependent manner.(A)HEK293 cells were transiently transfected with a GFP-Bmi1 expression plasmid, along with Flag tagged c-Myc (wild type, ΔI, ΔI-II or ΔI-III) or L-Myc. In addition, increasingconcentrations of HA-Max expression plasmid (20–300ng) were co-transfected. 24-48 hours post-transfection, GFP-Bmi1 was visualized via live green fluorescence microscopy.The experiment was repeated 3 times. (B) Quantification of (A) at different Max concentrations. At least 100 cells were counted for each condition for 3 independent experiments. *** Indicates $p< 0.001$, ** indicates $p< 0.05$, * indicates $p< 0.1$. All values in (B) starting from 0 ng of Max are compared to the first lane.

Figure 5

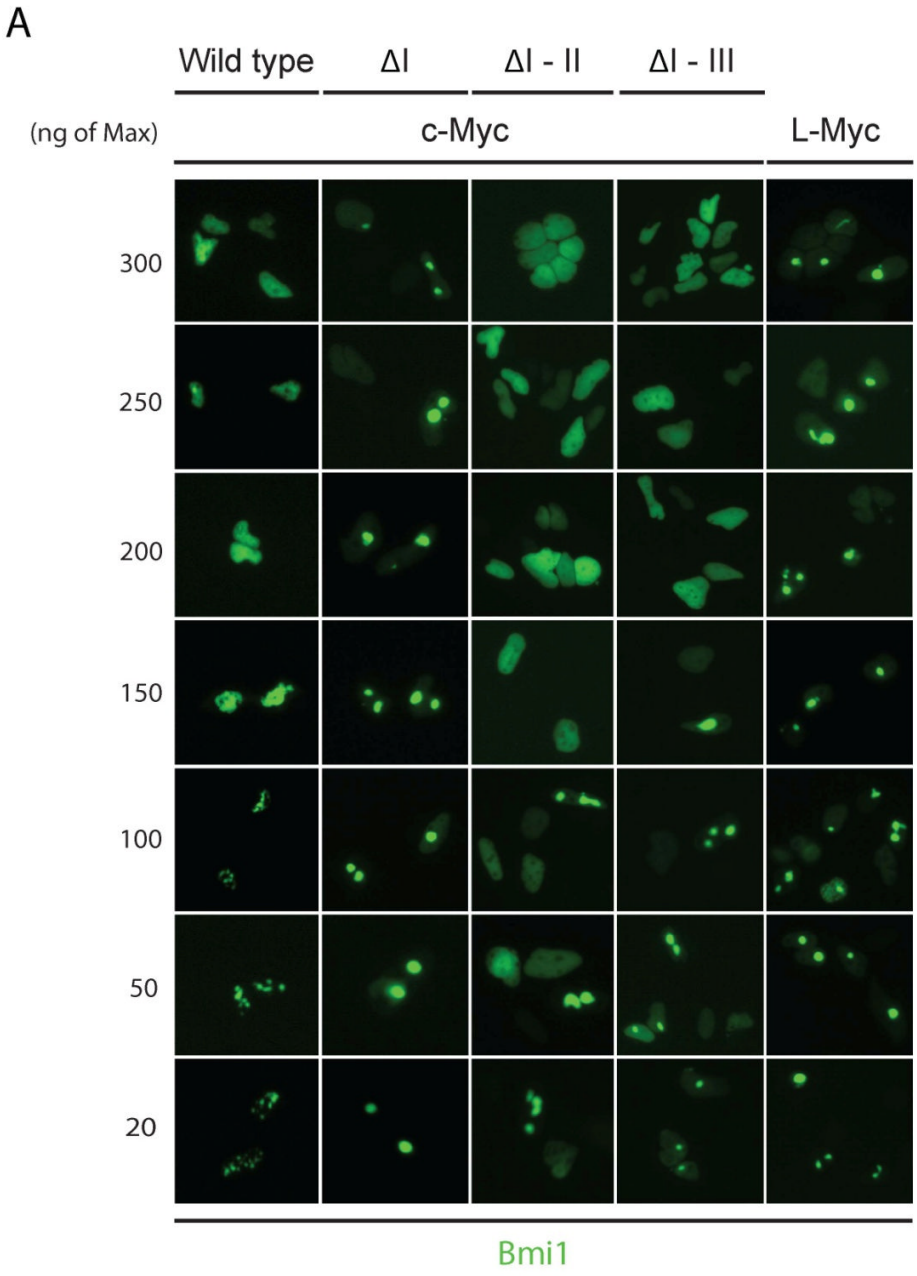

A
Wild type
ΔI
ΔI - II
ΔI - III
(ng of Max)
c-Myc
L-Myc
300
250
200
150
100
50
20
Bmi1

Figure 5

B

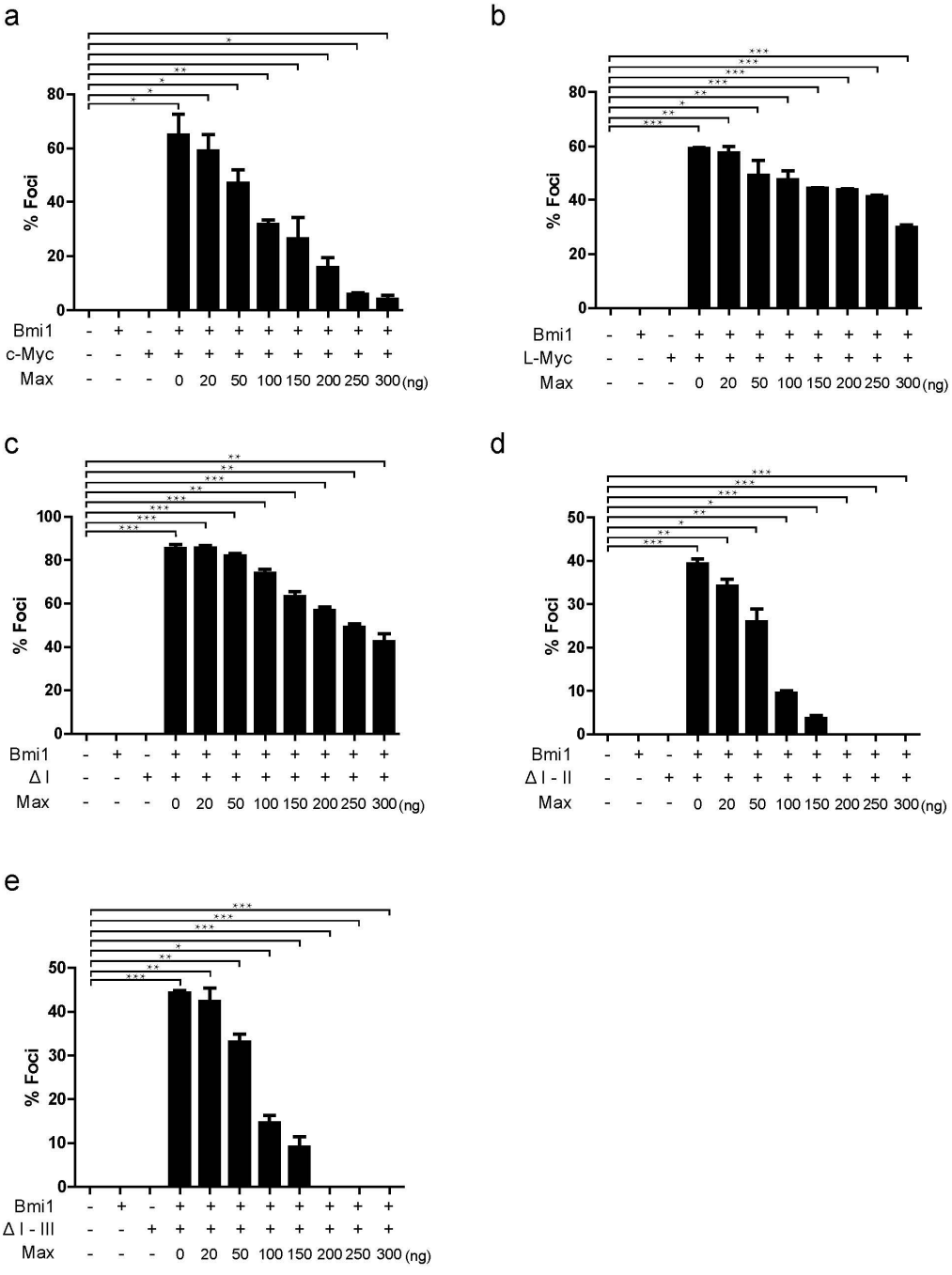
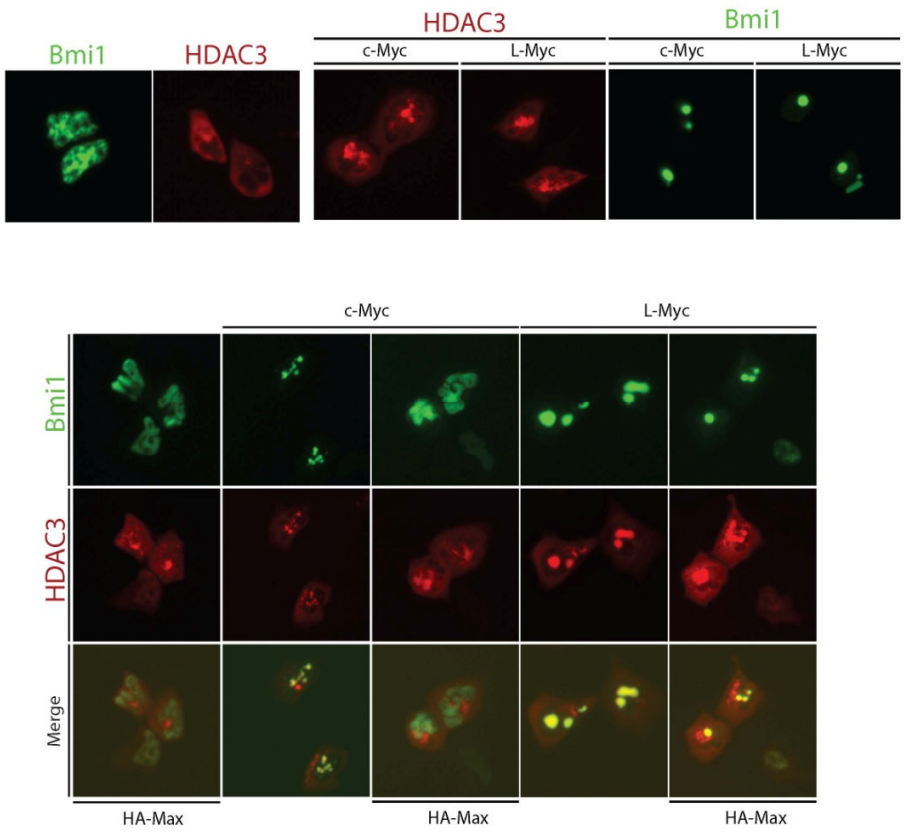

a
% Foci
Bmi1
c-Myc
Max
0 20 50 100 150 200 250 300 (ng)
b
% Foci
Bmi1
L-Myc
Max
0 20 50 100 150 200 250 300 (ng)
c
% Foci
Bmi1
Δ I
Max
0 20 50 100 150 200 250 300 (ng)
d
% Foci
Bmi1
Δ I - II
Max
0 20 50 100 150 200 250 300 (ng)
e
% Foci
Bmi1
Δ I - III
Max
0 20 50 100 150 200 250 300 (ng)

Figure 6.HDAC3promotes the formationof Bmi1-Myc nuclear foci.(A) HEK293 cells were transiently transfected with expression plasmids encoding GFP-Bmi1, mCherry-HDAC3 and, HA-Max,along with either Flag tagged c-Myc or L-Myc. GFP-Bmi1 and mCherry-HDAC3 were visualized using green and red fluorescence microscopy, respectively. The experiment was repeated on two separate occasions. (B) HEK293 cells were transiently transfected with an expression plasmid for GFP-Bmi1, together with either Flag tagged c-Myc or L-Myc. Cells were treated with the HDAC inhibitor TSA (25ng/ml) for 24 h before GFP-Bmi1 and mCherry-HDAC3 localization was observed by green and red fluorescence microscopy. For quantification of (B) at least 100 cells were counted for each condition for 3 independent experiments. *** Indicates $p<.0001$ vs. adjacent bar at the left, * indicates $p<.05$ vs. adjacent bar at the left.

Figure 6

A


Bmi1
HDAC3
HDAC3
c-Myc
L-Myc
Bmi1
c-Myc
L-Myc
c-Myc
L-Myc
Bmi1
HDAC3
Merge
HA-Max
HA-Max
HA-Max

B

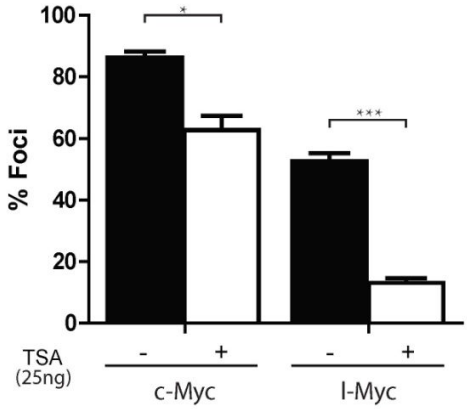
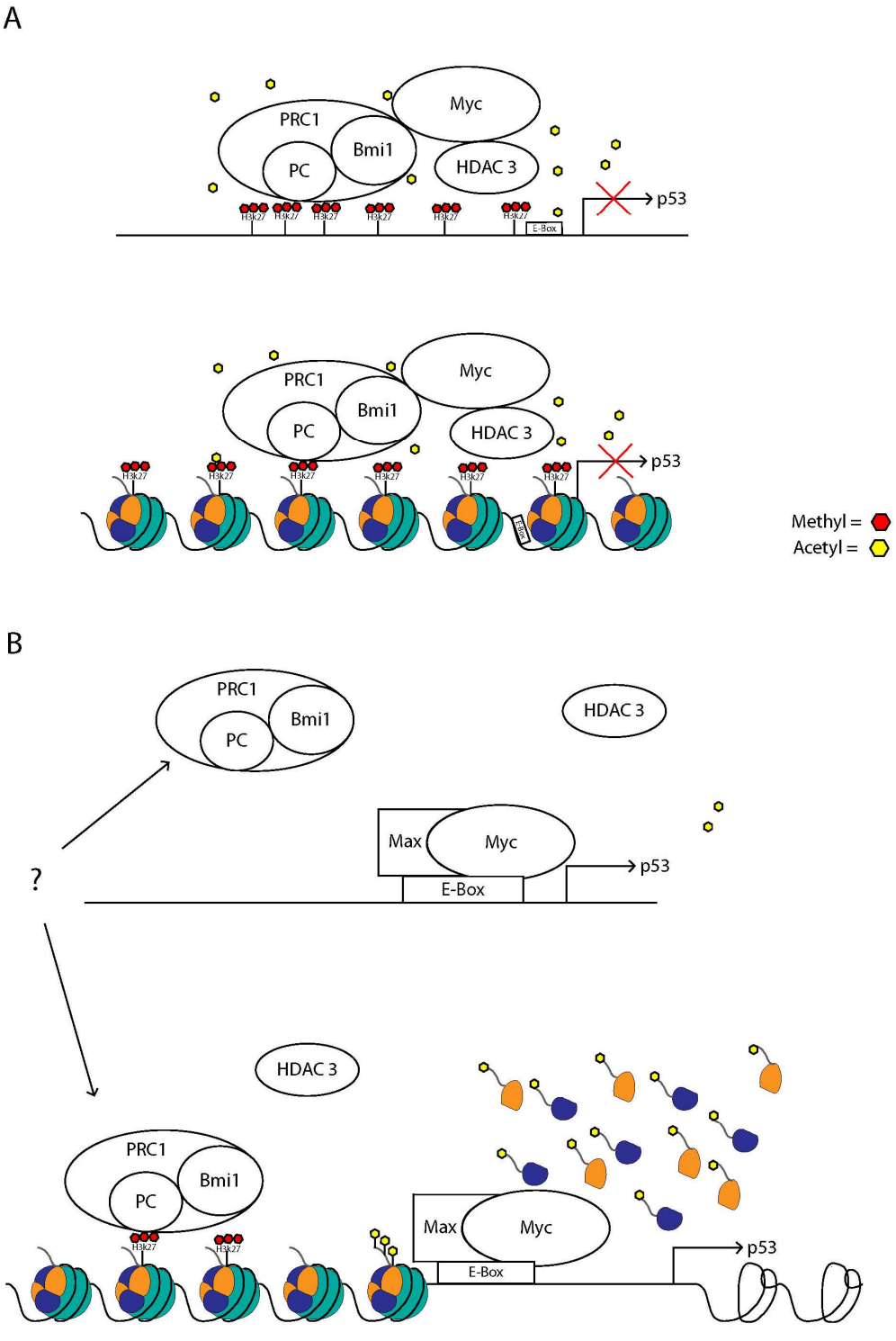

100
80
60
40
20
0
% Foci
*
***
TSA
(25ng)
-
+
-
+
c-Myc
l-Myc

Figure 7. Hypothetical transcriptional interplay betweenMyc, Bmi1 and Max.(A)PRC1 bound to H3k27me3 repressive marks recruits Myc to DNA viaBmi1. HDAC3 is recruited to Bmi1-Myc nuclear foci by Myc; leading to DNA compaction. (B) Max binds to Myc and causes a break in the Myc-PRC1 interaction. The newly formed Myc-Max complex is now able to promote the transcription of the repressed gene. PRC1 either remains bound to the H3k27me3 histone marks or dissociates from them and relocates elsewhere on the DNA.

Figure 7


A
PRC1
Bmi1
PC
Myc
HDAC 3
p53
H3k27
E-Box
Methyl =
Acetyl =
B
PRC1
Bmi1
PC
HDAC 3
?
Max
Myc
E-Box
p53

Chapter III

Regulating the stability of L-Myc through its interaction withBmi1

1. Introduction

In my previous study, I demonstratedthat Bmi1 and Myc interact directly with each other. I further demonstrate that the overexpression of Max and L-Myc leads to the formation of an L-Myc band shift that suggests the addition of a PTM. Interestingly, GSK3β has been shown to cause the phosphorylation of L-Myc on S38 and S42 in a PKC-dependent manner [109]. However, I show that the inhibition of GSK3β has no effect on thisMax-induced L-Myc band shift.

Following T58 and S62 phosphorylation, c-Myc and N-Myc proteins are polyubiquitinated and become targets for degradation [94]. Recently, an E3 ubiquitin ligase complex called Bmi1/RING1A was shown to dictatethe half-life of Top2α by regulating its proteasomal degradation [42].I demonstrate that Bmi1 can induce L-Myc ubiquitination as long as Max is not overexpressed. It is also interesting to note that L-Myc protein levels drop as a consequence of this ubiquitination. The following data suggests a novelregulatory mechanism for the stability of Myc oncogenes.

2. Materials and Methods

Plasmid constructs. cDNAs of human Bmi1, c-Myc, L-Myc, and Max were purchased from Open Biosystems, and mammalian expression plasmids containing Flag or HA tags were generated using the pcDNA3.1 vector (Invitrogen). GFP and mCherry constructs were derived from pEGFP-C2 (Invitrogen) and pmCherry-1 vector (Clontech), respectively. Constructs were generated according to instructions from the manufacturer. The HA-tagged ubiquitin plasmid was a gift from Wing SS.

Cell culture. HEK293 cells were maintained in DMEM (Invitrogen) and were supplemented with 10 % FBS (Sigma), and 1 % P/S (Invitrogen).

Antibodies. The following antibodies were used: anti-Flag (Sigma, F3165), anti-HA (BabCO/Covance), anti-Mouse HRP IgG (Amersham, NA93IV), goat anti-Rabbit IgG (Fisher, AP307FMI), anti-GFP (Santa Cruz, SC-8334).

Cell transfection. For western blotting as well as IPs, 0.04 X 10^6 and 0.4 X 10^6 HEK293 cells were plated per well of a 12 well plate and 10 cm dish, respectively. Transfections were performed with 3 µl or 20 µl Superfect (Qiagen) and 1.5 µg or 10 µg total DNA, respectively. For fluorescence microscopy, 0.04 X 10^6 HEK293 cells were plated per well of a 12 well plate. 12 well plate HEK293 cell transfections were performed with 3 µl Superfect (Qiagen) and 1.5 µg total plasmid DNA. Total plasmid DNA concentration was kept constant with pKSII (+) (Stratagene) supplementation. Transfected cells were incubated for 3 hrs at 37 °C. Afterwards, wells/dishes were washed with 1 X PBS and new cDMEM (DMEM with 10 % FBS and 1 % P/S) was added to each well/dish (1 ml per well of a 12 well plate and 10 ml in each 10 cm dish). Experiments were performed 24-48 hrs post-transfection.

Co-immunoprecipitation. To analyze Bmi1 and Max binding to c-Myc (wild type or ΔI or ΔI-II or ΔI-III) or L-Myc, GFP-Bmi1 and HA-Max expression plasmids were transfected along with Flag tagged c-Myc (wild type or ΔI or ΔI-II or ΔI-III) or Flag tagged L-Myc in HEK293 cells. Transfections were performed in 10 cm plates using 20 µl Superfect and 10 µg of total DNA. Transfections were performed as specified by the manufacturer. 48 hrs post-transfection, cells were washed twice with cold 1 X PBS and lysed in buffer K ([20 mM] sodium phosphate [pH= 7.0], [150 mM] KCl, [30 mM] sodium pyrophosphate, 0.1 % NP-40, [5 mM] EDTA, [10 mM] NaF, [0.1 mM] Na_3VO_4, and the protease inhibitors leupeptin, aprotinin, pepstatin, and PMSF). Cell extracts were subjected to affinity purification on M2 agarose beads (Sigma) as instructed by the manufacturer. 200 µl extracts were added to 20 µl of pre-washed, with 200 µl buffer K, M2 agarose beads (Sigma) and rotated at 4 °C for 2 hrs. Following 3 washes with 250 µl buffer K, bound proteins were eluted with 2 µl Flag peptide (Sigma) in 40 µl buffer K. Eluded proteins were stored at -80°C.

Immunoblotting. After adding 3 X SDS sample buffer, whole cell extracts and IP extracts were boiled for 5 min and then separated on a 12 % SDS-PAGE gel for 90 min with a constant voltage of 120. Gels were transferred to a nitrocellulose membrane for 2 hrs at 4 °C with a constant current of 200 mAmp. Membranes were then blocked in PBS-T (1 X PBS and 0.15 % tween-20) with 5 % milk for 1 hr at room temperature and then incubated overnight at 4 °C with 2 µl of their corresponding primary antibodies (anti-HA (Covance), anti-Flag (Sigma), anti-GFP (Santa Cruz)) in 5 ml PBS-T/5 % milk. Membranes were washed with PBS-T (3 X 10 min), and then incubated with 1 µl of their appropriate secondary antibodies for 1 hr at room temperature in 5 ml PBS-T/5 % milk.

Following a 3 X 10 min wash with PBS-T, membranes were incubated for 5 min in a 4 ml Supersignal enhanced chemiluminescent solution mix (Pierce). Membranes were developed on chemiluminescent films in a dark room and visualized there. Developed films were then scanned and processed with Adobe Photoshop and Illustrator.

Ubiquitination assay. To analyze the Ubiquitin binding ability of L-Myc, expression plasmids for Flag L-Myc were transfected in HEK293 cells along with constructs expressing GFP-Bmi1, HA-Max, and HA-Ubiquitin. Transfections were performed in 10 cm dishes with 20 µl Superfect and 10 µg of total DNA. Forty-eight hrs post-transfection, cells were washed 2 times with 1 X PBS and lysed with a 300 µl solution of buffer S ([15 mM] Tris-HCl [pH= 6.7], 0.5 % SDS, 3 % glycerol, 0.8 X PBS, 4 % NP-40, 0.1 % mercaptoethanol, [25 mM] N-ethylmaleimide, and the protease inhibitors leupeptin, aprotinin, pepstatin, and PMSF). Extracts were scrapped from the plates and sonicated 3 times, 10s/each, with VirSonic 100 set at strength 5. For affinity purification with Flag-tagged proteins, 200 µl of extracts were added to 20 µl of M2 agarose beads pre-washed with 200 µl of buffer R (1 X PBS, 5 % NP-40, 1 % mercaptoethanol, and the protease inhibitors leupeptin, aprotinin, pepstatin, and PMSF) and rotated for 2 hrs at 4 °C. Following 3 washes with buffer R, bound proteins were eluted with 2 µl Flag peptide (Sigma) in 40 µl buffer R. Eluded proteins were stored at -80°C.

3. Results

Max-induces an L-Myc PTM *in vivo.* The DBD permits L-Myc to bind both Max and DNA simultaneously. In Fig 4C of the previous chapter, I discovered a Max-induced L-Myc band shift which mayeither be a PTM, an alternative L-Myc splice product or something else. Interestingly, phosphorylation of the L-Myc oncoprotein is modified when under the effect of TPA by way of a rapid signal transduction mechanism interconnected with PKC [108]. This PTM is quickly amplified following TPA treatment but can also be inhibited with the use of a S/T protein phosphatase like OA [108]. Under the assumptionthat the Max-induced L-Myc band shift observed in Fig 4C was indicative of phosphorylation, an immunoprecipitation was performed subsequent to treating cells with the PP2A inhibitor OA (100μM) 1 hr prior to harvesting. Flag tagged L-Myc and HA-Max expression plasmids were co-expressed inHEK293 cells and western blotting with antibodies against Flag and HA were used to detect these proteins (Fig 1A). No effect was observed on the L-Myc band shift following OA treatment. However, anti-Flag input levels are very weak and make the interpretation of thisexperiment difficult. The experiment was performed twice and needs to be repeatedagainin order to arrive ata definitive conclusion. Our laboratory is currently addressing these experimental caveats. Furthermore, the N-terminal S38 and S42 of L-Myc are necessary for its phosphorylation[109]. These serines can be phosphorylated *in vitro* by GSK3β. To investigate if GSK3β is the cause of the band shift, an immunoprecipitation was performed after cells were treated with 30mM LiCl and 50nM Rapamycin 48 hrs prior toharvesting (Fig. 1B). Specifically, Flag tagged L-Myc and HA-Max expression plasmids were co-expressed in HEK293 cells and western blotting with antibodies against

Flag and HA were used to detect these proteins. No effect on the L-Myc band shift was observed following these treatments. Therefore, these L-Myc band shifts are indicative of either PTM initiated by an unidentified protein complex, L-Myc splice variants or something else.

Max hinders the Bmi1-induced polyubiquitylation of L-Myc.c-Myc or N-Myc are known to undergo degradation following phosphorylation of S62 and T58. A ubiquitination assay was performed to examine whether this process occurs subsequent tothe Maxinduced L-Myc band shift (Fig. 2). Specifically, the Flag tagged L-Myc expression plasmid was transfected in HEK293 cells along with GFP-Bmi1, HA-Max and HA-Ubiquitin. Western blotting was performed on transiently transfected cells and antibodies against GFP, Flag, HA, and tubulin were used to detectprotein levels. Surprisingly, an increase in L-Myc ubiquitination was noted upon the addition of Bmi1, though the phenotype was reversed to its original statefollowingthe overexpressionof Max. Interestingly, the decrease in L-Myc protein levels correlatesinversely with the increase in L-Myc ubiquitination, as long as Max isnot overexpressed. Taken together, these results imply that Bmi1 is able to induce the ubiquitination of L-Myc.

4. Discussion

Bmi1 and Max modulateL-Mycubiquitination. Quantitative regulation of oncogene and tumor suppressor protein levels must be tightly regulated in normal cells.These proteinsare often controlled by PTM. The L-Myc oncoprotein is modified by TPA through signal transduction mechanisms involving PKC [108].Italso been shown that the N-terminal S38 and S42 residues of L-Myc are both necessary for its phosphorylation and furthermore can be phosphorylated *in vitro* by GSK-3β [108]. My results indicate that when Max is overexpressed with L-Myc, an L-Myc band shift appears (Fig. 4 of previous chapter), possibly indicative of the addition of a PTM, thatis independent ofGSK3β activity (Fig. 1). These Max-induced L-Myc band shifts resemble phosphorylation events phenotypically reminiscent of a TPA induced L-Myc phosphorylation [108]. OA treated cells revealed that this PTM is unaffected by one of thePP2A inhibitors(Fig. 1). However, other phosphatase inhibitors could be used to insure that these Max-induced band shifts are not PTMs. I have additionallyuncovered a Bmi1-mediated increase in L-Myc protein ubiquitination and, possibly, degradation (Fig. 2). Interestingly, following Maxoverexpression, the L-Myc's protein levels as well as its ubiquitination state are restored back to normal. Additional experiments are needed to confirm this experimental inference.

A simplistic model for the turnover of L-Myc is proposed based on my results (Fig 3). This model proposes that Max stabilizes L-Myc and prevents Bmi1-induced ubiquitination. Interestingly, an E3 ubiquitin ligase called Bmi1/RING1A is able to control the proteasomal degradation of Top2α [42]. It is possible that the degradation of

L-Myc exemplified in my thesis occurs through the same process as described by the Bmi1/RING1A group. To validate this hypothesis, we would need to acquire the Bmi1/RING1A inhibitor developed by the aforementioned research group and try it in mymodel. Additionally,it would be interesting to see if and how the transcriptions of CDKN2 genes are affected by this differential interaction and to what extent these genes impact the well known target of Bmi1, p53.

Bmi1 appears to have a dual role with regards to its interaction with Myc. On the one hand, Bmi1 seems to recruit Myc to the PRC1 complex. However, the Bmi1 Ring E3 complex also targets Myc for degradation. Further experiments should be performed to further validatethese theories.

5. Figure Legends and Figures

Figure 1. Max induces L-Myc modification independent of GSK3β. (A) HEK293 cells were transiently transfected with expression plasmids encoding Flag tagged L-Myc and HA-Max. Forty-eight hours later, cells were treated with the PP2A inhibitor OA (100μM) for 60 min. Cells were then harvested in buffer K and Flag-tagged proteins were immunoprecipitated on M2 agarose beads and eluded with Flag peptide. Whole cell extracts (Input: 20μl) and immunoprecipitates (IP: 20μl)) were separated by SDS-PAGE and immunoblotting was performed with antibodies against the indicated proteins. The experiment was repeated twice. (B) As in (A) except cells were treated with 30 mM LiCl or 50 nM Rapamycin for forty-eight hours before they were harvest.The LiCl experiment was repeated on one occasion and the Rapamycin experiment was repeated twice.

Figure 1

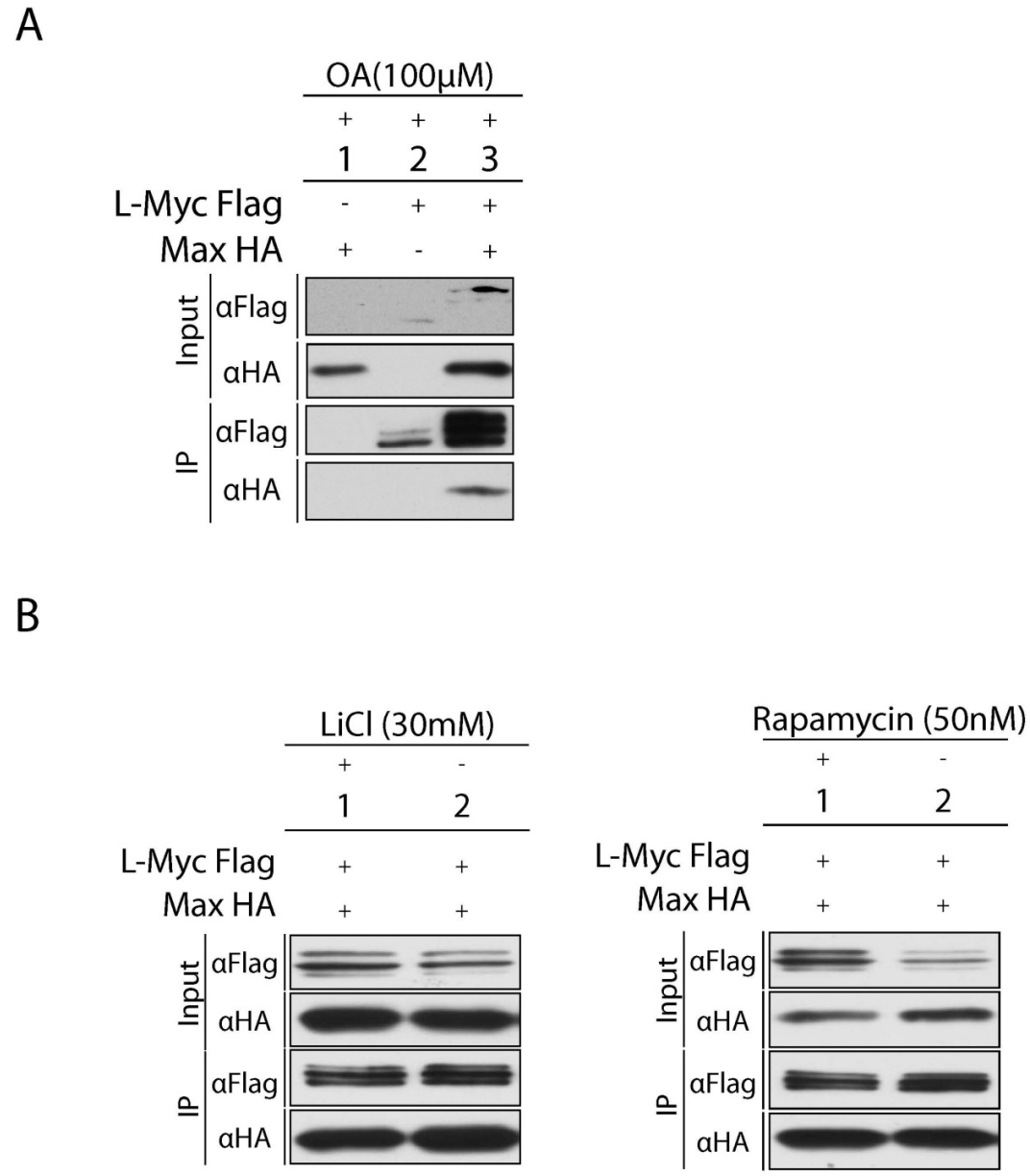

A
OA(100μM)
+ + +
1 2 3
L-Myc Flag - + +
Max HA + - +
Input
αFlag
αHA
IP
αFlag
αHA
B
LiCl (30mM)
+ -
1 2
L-Myc Flag + +
Max HA + +
Input
αFlag
αHA
IP
αFlag
αHA
Rapamycin (50nM)
+ -
1 2
L-Myc Flag + +
Max HA + +
Input
αFlag
αHA
IP
αFlag
αHA

Figure 2. Theeffect of Bmi1 and Max on the ubiquitination state of L-Myc. HEK293 cells were transiently transfected with expression plasmids encoding Flag tagged L-Myc, together with GFP-Bmi1 and HA-Max. Forty-eight hours later, cells were harvested in buffer S and Flag-tagged proteins were immunoprecipitated on M2 agarose beads and eluded in buffer R with Flag peptide. Whole cell extracts (Input: 20µl) and immunoprecipitates (IP: 20µl) were separated by SDS-PAGE and immunoblotting was performed with antibodies against GFP, HA, Flag and α-tubulin as indicated. The experiment was repeated twice.

Figure 2

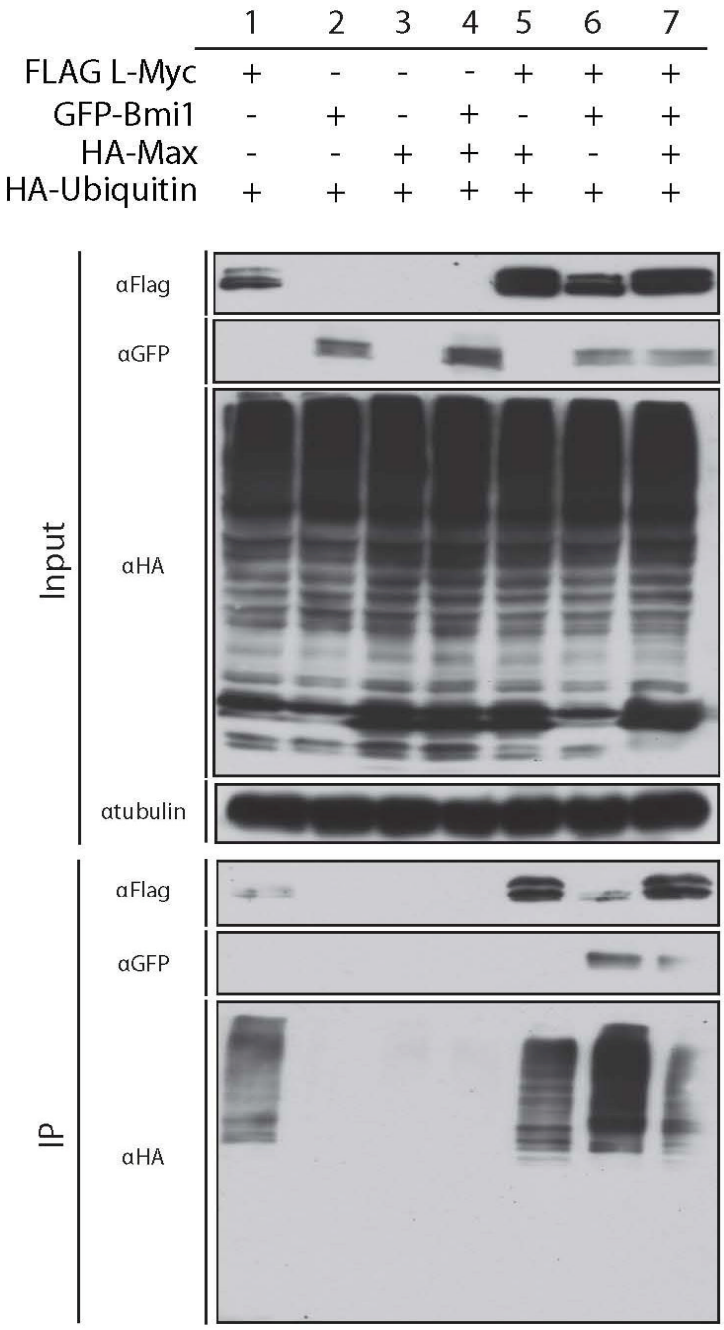
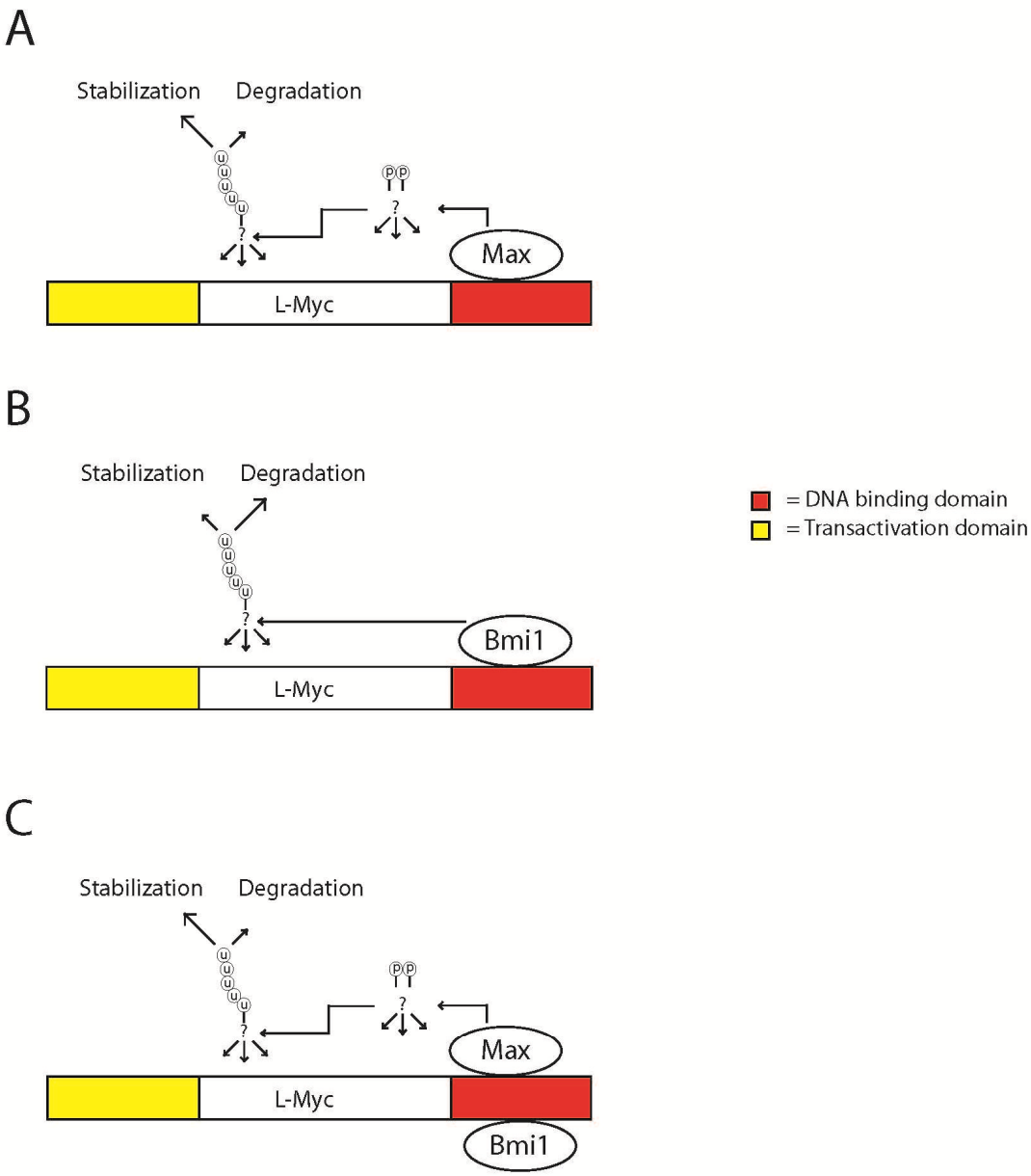

1 2 3 4 5 6 7
FLAG L-Myc + - - - + + +
GFP-Bmi1 - + - + - + +
HA-Max - - + + + - +
HA-Ubiquitin + + + + + + +
Input
αFlag
αGFP
αHA
αtubulin
IP
αFlag
αGFP
αHA

Figure 3. Model illustrating the effect of Bmi1 and Max on L-Myc protein stability.(A) Cartoon demonstratingthe effect ofMax and L-Myc protein interaction. (B) Cartoon demonstrating the effect of Bmi1 andL-Myc proteininteraction. (C) Cartoon depictingthe mechanism by which Max inhibits Bmi1-induced L-Myc ubiquitination.

Figure 3


A
Stabilization
Degradation
Max
L-Myc
B
Stabilization
Degradation
Bmi1
L-Myc
= DNA binding domain
= Transactivation domain
C
Stabilization
Degradation
Max
L-Myc
Bmi1

References

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Chapter III

MAJOR CONTRIBUTIONS TO THE SCIENCE FORUM

1. Overexpression of Myc and Bmi1 causes the formation of nuclear foci *in vivo*.
2. Direct physical interaction between Myc and Bmi1 involving Myc's DBD and MBII,*in vivo* and *in vitro*.
3. Overexpression of Max disruptsBmi1-Myc nuclear foci.
4. *In vivo* induction of L-Myc PTMby Max.
5. Bmi1-induced L-Myc ubiquitination is reversed upon the overexpressionof Max.
6. Bmi1 modulates L-Myc protein levels *in vivo.*
7. HDAC3 is recruited to Bmi1-Myc nuclear foci *in vivo*.