REGULATION OF MYC AND ITS LOCALIZATION TO HISTONE LOCUS BODY IN DROSOPHILA

by

Abid Khan

A dissertation submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in
Biology

Charlotte

2012

Approved by:

_____
Dr. Julie Goodliffe

_____
Dr. Matthew Parrow

_____
Dr. Christine Richardson

_____
Dr. Pinku Mukherjee

_____
Dr. John Bender

ABSTRACT

ABID KHAN. Regulation of Myc and its localization to Histone Locus Body in *Drosophila.* (Under the direction of DR. JULIE GOODLIFFE)

Myc is a transcriptional factor required for normal growth and development in vertebrates and invertebrates alike. Loss of function mutations in Myc can cause embryonic lethality in mammals and larval death in flies whereas an increase in its activity can lead to tumorigenesis. Therefore, proper regulation of Myc is very important to ensure normal development. Regulation of Myc occurs by several context specific mechanisms. One such mechanism is the negative feedback autoregulation of Myc and this mechanism is lost in all tumorigenic cell lines. Like its mammalian homolog, the *Drosophila* Myc (dMyc) undergoes autoregulation in the presence of an ectopic myc gene leading to a Myc null phenotype. Polycomb (Pc), a chromatin binding repressor is required for Myc autoregulation. Upon Pc knockdown, levels of Su(z)2, a Pc group related protein increase significantly, suggesting that Pc represses Su(z)2. We show here that ectopic Su(z)2 can interfere with Myc autorepression and restore endogenous Myc levels as well as rescue larval lethality caused due to Myc autorepression. Su(z)2 does not however, affect general repression by Myc suggesting that repression of myc locus occurs by a different mechanism. During this study we observed that Myc protein forms distinct puncta in certain tissues. Upon investigating we found that these Myc "spots" localize to sub-nuclear organelles known as Histone Locus Body (HLB). HLBs are histone pre-mRNA processing centers formed at histone gene locus. We show here that Myc localizes to the HLBs only during S phase. Since hisones are transcribed only during S phase we hypothesize that Myc aids in histone transcription, a novel role for Myc.

DEDICATION

I dedicate my doctoral dissertation to my parents for their love, support, the relentless persistence and determination which ensured my academic success and also because of the countless sacrifices they made throughout their life to ensure that my siblings and I had the best of life.

I would also like to dedicate this accomplishment to Mr. Faiyaz Ali and his family for their unconditional love and support throughout the period of my doctoral work. I feel blessed to have met them five years ago and since then my love and respect for them has only increased. They opened their doors and hearts to me and made me feel like family. It is not possible to express in words the deepest gratitude I feel towards them. I carry a huge burden of debt of their love that can never be repaid.

ACKNOWLEDGEMENT

I would like to acknowledge and thank all those people who made this doctoral dissertation possible. I express my deepest and sincere gratitude to my mentor Dr. Julie Goodliffe for her guidance, motivation and especially for granting me the freedom to express myself and develop as an independent researcher. My gratitude also goes to my committee members Dr. Matthew Parrow, Dr. Christine Richardson, Dr. Pinku Mukherjee and Dr. John Bender.

I would also like to thank my family members Samreen Hamid and Ambreen Hamid for the emotional support and all my friends and colleagues at UNC, Charlotte especially, Hana Alkahlout, Rita Bagwe, Bhawana Bariar, Kaveh Daneshvar, Sritama Nath and Pushti Patel for making graduate school fun and memorable.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Drosophila Myc (dMyc), like its mammalian homolog, c-Myc, is a transcriptional factor belonging to the basic helix-loop-helix-zipper (bHLHZ) family of proteins. It binds to and regulates the expression of several genes required for normal development. Its target genes are involved in important cellular functions such as protein synthesis, metabolism, riobosomal biogenesis, growth and proliferation to. Hence, deregulation in Myc activity can lead to a wide variety of human cancers (Secombe, Pierce et al. 2004; Eilers and Eisenman 2008). In this section we will discuss the molecular basis of dMyc activity, its regulation during development and the physiological consequence of deregulation of its expression.

1.1 Myc as a transcriptional factor:

Myc protein consists broadly of two major domains: the C terminal containing a dimerization/DNA binding domain required for DNA binding via heterodimerization with its partner Max and the N terminus transactivation domain. Deletion of the dimerization domain causes loss of its biological activity suggesting that binding to DNA via dimerization with Max is essential for its function (Stone, de Lange et al. 1987; Dang, McGuire et al. 1989; Blackwood, Lüscher et al. 1992). The Myc-Max heterodimer recognizes and binds to a consensus sequence CACGTG known as the E box at the promoter of its target gene. The transactivation domain (TAD) of Myc is required for the transcriptional activation of its target gene (Amati, Dalton et al. 1992). Unlike Myc, Max

lacks a TAD but is required by Myc for sequence specific DNA binding. Although the Myc-Max dimer is a relatively weak activator of transcription, the TAD coupled to a GAL4 DNA binding domain can activate transcription 20-200 fold higher (Brough, Hofmann et al. 1995).

As a transcriptional activator, Myc-Max dimer interacts with other cofactors such as TRRAP, which then recruits histone acetyl transferases (HAT) such as GCN5 (McMahon, Van Buskirk et al. 1998; McMahon, Wood et al. 2000). The HAT acetylates the histones at the promoter leading to the transcriptional activation of the target gene. Myc is also known to associate with other cofactors such as Tip60 Lid/Rbp2 H3-K4 demethylase, HectH9 ubiquitin ligase, etc., required to modify chromatin to aid in gene activation (Adhikary and Eilers 2005; Cole and Nikiforov 2006; Secombe, Li et al. 2007).

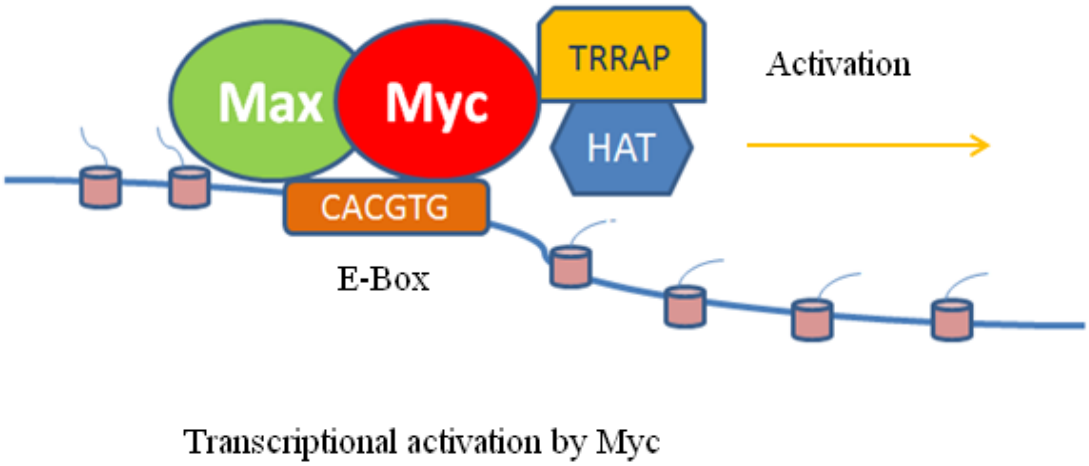


Figure 1: A schematic representation of the mechanism of transcriptional activation by Myc.

On the contrary, transcriptional repression by Myc is rather indirect. Mycis known to bind and inhibit the function of the transcription factor Miz1, leading to the

repression of Miz1 activated targets such as p15 and certain Cdk inhibitors(Staller, Peukert et al. 2001).

In contrast to Myc, Max can associate with other bHLHZ proteins known as the Mxd protein family and Mnt. Unlike Myc-Max dimers which cause transcriptional activation, the Mxd-Max heterodimer binds to E box and represses transcription by associating with the Sin3 corepressor complex which consists of histone deacetylases (HDACs)(Gallant, Shiio et al. 1996; Schreiber-Agus and DePinho 1998). Mnt behaves asantagonist to Myc in developmental context (Loo, Secombe et al. 2005). Conditional deletion of Mnt in breast epithelium causes tumorigenesis and cells depleted for Mnt by RNAi phenocopy cells that overexpress Myc (Hurlin, Zhou et al. 2003; Hooker and Hurlin 2006). In *Drosophila* Mnt mutation can partially rescue Myc null mutant phenotype(Pierce, Yost et al. 2008).

1.2 Biological Functions of Myc:

Myc is essential for normal growth and development in vertebrates and invertebrates alike with the exception of nematodes. Mice that are homozygous null for c-myc do not survive past 9.5 days of embryogenesis (Davis, Wims et al. 1993). Flies with a hypomorphic mutation have abnormally small body size whereas a null mutation causes larval lethality(Johnston, Prober et al. 1999). Myc was recently identified as one of the four key factors required to induce pluripotency in a somatic cell to produce an embryonic stem cell like state, suggesting that Myc is very important for early embryogenesis (Takahashi and Yamanaka 2006).

As a transcription factor Myc controls the expression of a large number of genes. It is shown to be associated with 10%-15% of genomic loci in both mammals and

Drosophila (Fernandez, Frank et al. 2003; Orian, van Steensel et al. 2003). The bound loci represent genes with a broad range of functions including ribosome biosynthesis, protein translation, metabolism, and cell cycle regulation.

Similar to mammalian Myc, *Drosophila* Myc (dMyc) is a positive regulator of growth and proliferation. It is capable of transforming primary mammalian cells and rescuing the proliferation defects in c-myc null fibroblasts (Schreiber-Agus, Stein et al. 1997). In the wing imaginal discs of *Drosophila,* dmyc mutation causes smaller cell size without affecting the cell cycle distribution. Overexpression of dmyc resulted in larger cells without any change in cell division rate (Johnston, Prober et al. 1999). The regulation of cellular growth without changes in cell division rate suggests that Myc regulates components of cell growth machinery. This is achieved in part by regulating genes involved in ribosome biogenesis, protein translation and metabolism (Orian, van Steensel et al. 2003).

Most of the genes regulated by Myc are transcribed by RNA polymerase II but there is evidence that Myc can induce activation of genes transcribed by RNA polymerase I and III (Grewal, Li et al. 2005; Steiger, Furrer et al. 2008). The genes transcribed by RNA polymerase I and III make up the translation machinery (rRNAs and t-RNA). While c-Myc is known to bind to rDNA loci and activate transcription of ribosomal RNA, dMyc does not physically occupy these loci but is required for the induction of these genes in an RNA polymerase I dependent manner (Grandori, Gomez-Roman et al. 2005; Grewal, Li et al. 2005).

Recently it has been shown that Myc acts downstream of target of rapamycin (TOR) and insulin receptor (InR) pathways to promote cell growth in a nutrient

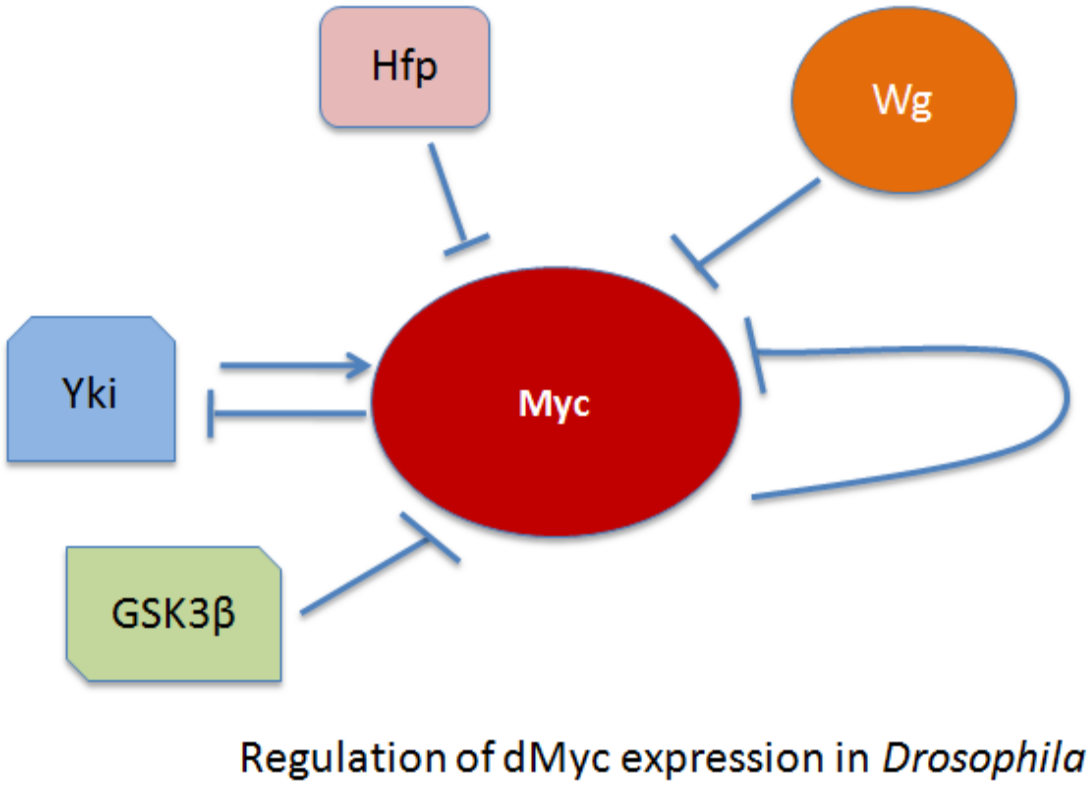
availability dependent manner (Teleman, Hietakangas et al. 2008; Li, Edgar et al. 2010). Myc is also required for endoreplication in larval salivary gland cells (Pierce, Yost et al. 2004). These cells can reach ploidy of up to 1000n and make up most of the larval mass. Myc is known to regulate cell cycle progression in the endoreplicating cells. Mutations in dmyc cause cells to stall in G1 whereas overexpression can accelerate the G1-S transition (Maines, Stevens et al. 2004; Pierce, Yost et al. 2004). Although Myc is not essential for cell cycle progression, it is thought that Myc increases the frequency of S phase in endoreplicating cells by increasing the stability and hence activity of CycE/Cdk2.

One of the unique functions of Myc, which is not common to other growth factors, is induction of cell competition. It is a phenomenon by which cells with high levels of Myc protein out compete and kill the neighboring cells with relatively low levels of Myc. The cells overexpressing Myc can grow faster than their wildtype neighboring cells which eventually undergo apoptosis and are eliminated (de la Cova, Abril et al. 2004; Moreno and Basler 2004). It is thought that cell competition could contribute to an overall organ size control mechanism.

1.3 Regulation of dMyc expression:

Although Myc is widely studied for its role in growth and proliferation, surprisingly there is not much data about the regulation of dmyc gene in comparison to the function of the protein. This is probably because regulation of dmyc is a highly dynamic and a very context specific process that involves different regulators in a spatiotemporal dependent manner. In the growing wing imaginal discs, Myc is expressed throughout the organ but is later lost in the zone of non-proliferating cells (ZNC) due to the activity of wingless (Wg). Wg represses dmyc expression in the ZNC (Johnston and

Edgar 1998; Johnston, Prober et al. 1999). The inhibition of dmyc by Wg could be through half-pint (Hfp), a homolog of human FBP interacting repressor (FIR), a pre-mRNA splicing factor also called PUF60. Hfp mutants have elevated dmyc transcripts



Regulation of dMyc expression in *Drosophila*

and ZNC fails to form(Quinn, Dickins et al. 2004).The Hippo pathway is also known to

Figure 2: Regulation of dMyc expression at the transcript and protein level. Arrow denotes activation and the perpendicular lines denote repression. Hfp, Wg and GSK3β repress Myc whereas Yki activates dMyc expression

regulate dmyc expression in the growing wing. Yorkie (Yki), the downstream effector of the pathway and Myc work in negative feedback regulatory mechanism. Yki, directly activates dmyc transcription, whereas high levels of Myc repress Yki in a transcriptional and post-transcriptional mechanism (Neto-Silva, de Beco et al. 2010; Stocker 2011). The

dMyc protein has a short half-life and its stability can be regulated by GSK3β which phosphorylates Myc, marking it for degradation (Bellosta and Gallant 2010).

1.4 Myc Autoregulation:

Myc expression is vital during the proliferative period of a cell, whereas when cells start to differentiate, Myc levels start to drop accordingly and remain at a basal level after terminal differentiation. This is important to prevent uncontrolled proliferation of cells and to ensure normal differentiation during development. Understanding how cells achieve a tightly orchestrated temporal Myc expression program is important to understand Myc induced tumorigenesis. Culture cells with a constitutively active ectopic c-myc gene undergo an auotoregulatory mechanism and switch off the expression of the endogenous myc gene. The expression is inhibited at the transcriptional initiation step and requires additional trans-acting cofactors (Penn, Brooks et al. 1990). The extent of the endogenous c-myc silencing was found to be proportional to the Myc protein levels in the cells. Interestingly, all tumorigenic cell lines with an ectopic myc gene were defective in autoregulation, whereas cell lines with intact Myc autorepression mechanism were not tumorigenic when implanted into nude mice (F Grignani 1990; Facchini, Chen et al. 1997).

Like its mammalian homolog, the *Drosophila* myc gene also undergoes autorepression in the presence of an ectopic myc gene. Interestingly, a chromatin binding repressor Polycomb (Pc) was identified in a screen performed to isolate repressors of dmyc transcription. *In vivo* experiments demonstrated that Pc is required for dmyc autorepression and also for Myc induced transcriptional repression of other loci (Goodliffe, Wieschaus et al. 2005). Upon Pc knockdown, dmyc autorepression is

abrogated and 73% of dmyc repression targets are derepressed, suggesting that in general, Myc induced repression of its own locus as well as other repression targets requires the presence of Pc. If Pc is involved directly in repressing these loci, then depletion of other Polycomb Group (PcG) members that are required for Pc activity should produce similar if not same results as with Pc knockdown. However, the knockdown of Pho, a protein required to recruit Pc to the repression targets, does not affect Myc autorepressioin (Goodliffe, Wieschaus et al. 2005). Therefore, we hypothesize that the role of Pc in dmyc autorepression is rather indirect. Pc is required to repress another gene(s) which can interfere with dmyc expression. In this study we investigate the role of Pc and a PcG related protein, Su(z)2 in Myc autoregulation during embryogenesis.

1.5 Polycomb Group (PcG):

PcG is a multi-protein transcriptional repressive complex required for the maintenance of cellular transcriptional memory throughout development. PcG proteins are best known for their role in the spatial regulation of Hox genes, demonstrated by the fact that mutations in PcG members can cause homeotic transformations (Kennison 1995; Beuchle, Struhl et al. 2001). In addition to Hox gene regulation, PcG is known for its role in cell cycle control, cancer, X-inactivation, cell fate decisions and stem cell differentiation (Wang, Mager et al. 2001; Sparmann and van Lohuizen 2006; Pasini, Bracken et al. 2007).

1.6 Recruitment of PcG complexes:

PcG proteins work in two separate multi-protein complexes: Polycomb repressive complex 1 (PRC1), which contains Polycomb (Pc), Polyhomeotic (PH), Posterior sex combs (PSC) and RING (Francis, Saurin et al. 2001; Saurin, Shao et al. 2001). Polycomb

Repressive Complex 2 (PRC2) which contains extra sex combs (ESC), Enhancer-of-zesteE(Z), the suppressorof position effect variegation (PEV) SU(var)12(Brown, Mucci et al. 1998; Muller, Hart et al. 2002; Brown, Fritsch et al. 2003).A third complex, PhoRC, has been identified which contains the sequence specific DNA binding protein Pleiohomeotic (Pho) which is required to recruit other PcG components (Klymenko, Papp et al. 2006).

PcG proteins are recruited to specific DNA sequences known as the Polycomb response elements (PRE) (Ringrose, Rehmsmeier et al. 2003). PREs can be located anywhere from the proximal promoter to a few kilobases (kb) away from the transcription start site (TSS). These elements contain sequences recognized by different transcription factors such as Pho, Pho-l, and GAGA factor that then recruit other PcG proteins (Ringrose and Paro 2007). Upon binding to the PRE, Pho then recruits other members of the PRC2 complex.E(z), a component of PRC2, contains a SET domain that catalyzes the di and tri-methylation of histone H3 lysine 27(H3K27) at the locus. Pc contains a chromodomain which recognizes and binds to methylated H3K27.

Although this mechanism seems logical, PcG recruitment is much more complex than this. First, Pho is not only able to recruit PRC2 but can also bind PRC1 components like Pc and Ph in-vitro (Mohd-Sarip, Cleard et al. 2005). Also, Pho binding sites alone are insufficient in recruiting PcG proteins to DNA in vivo. In Drosophila, Pho mutants are lethal in late developmental stage, and in mutant salivary glands lacking Pho, PcG proteins occupy most of their target loci on polytene chromosomes suggesting that recruitment PcG complexes can be mediated by other proteins in the absence of Pho(Brown, Fritsch et al. 2003).

1.7 Mechanism of transcriptional repression by PcG complex:

Although there is a great deal of evidence regarding the biochemical properties of the PcG complexes, there isn't a unanimous mechanism of transcriptional repression *in vivo*. PcG complexes have been shown to act in multiple ways to induce transcriptional repression. Firstly, the *Drosophila* PRC1 complex is shown to induce chromatin compaction *in vitro* (Francis, Kingston et al. 2004), however, there is no evidence of such compaction *in vivo.* PcG proteins do not localize to DNA dense regions in plants or animals, suggesting that PcG complexes do not induce compaction *in vivo*and that repression is caused by a mechanism other than chromatin compaction (Köhler and Villar 2008).Second, the role of mono-ubiquitination of histone H2A lysine 119 (H2AK119) inPRC1 mediated repression is not well understood. Loss of this ubiquitination marks causes depression of PcG targets(Schwartz and Pirrotta 2007). It is suggested that ubiquitination limits the processivity of RNA Pol II, thereby causing transcriptional inhibition. PcG binding negatively correlates with RNA Pol II binding; however many PcG targets are expressed even when bound by PcG complex (Bracken, Dietrich et al. 2006; Schwartz, Kahn et al. 2006). This suggests that RNA Pol II occupancy does not affect transcriptional repression. In another study it has been shown that PcG does not affect RNA Pol II binding but rather interferes with the transcriptional initiation (Dellino, Schwartz et al. 2004). Another possibility is that the presence of H3K27Me3 marks inhibits the deposition of activation marks at these loci; however, in embryonic stem cells, many genes contain activation as well as repression marks, known as "bivalent domains" (Bernstein, Mikkelsen et al. 2006). These genes are bound by RNA Pol II and held in check by ubiquitination of H2A and poised for either activation or

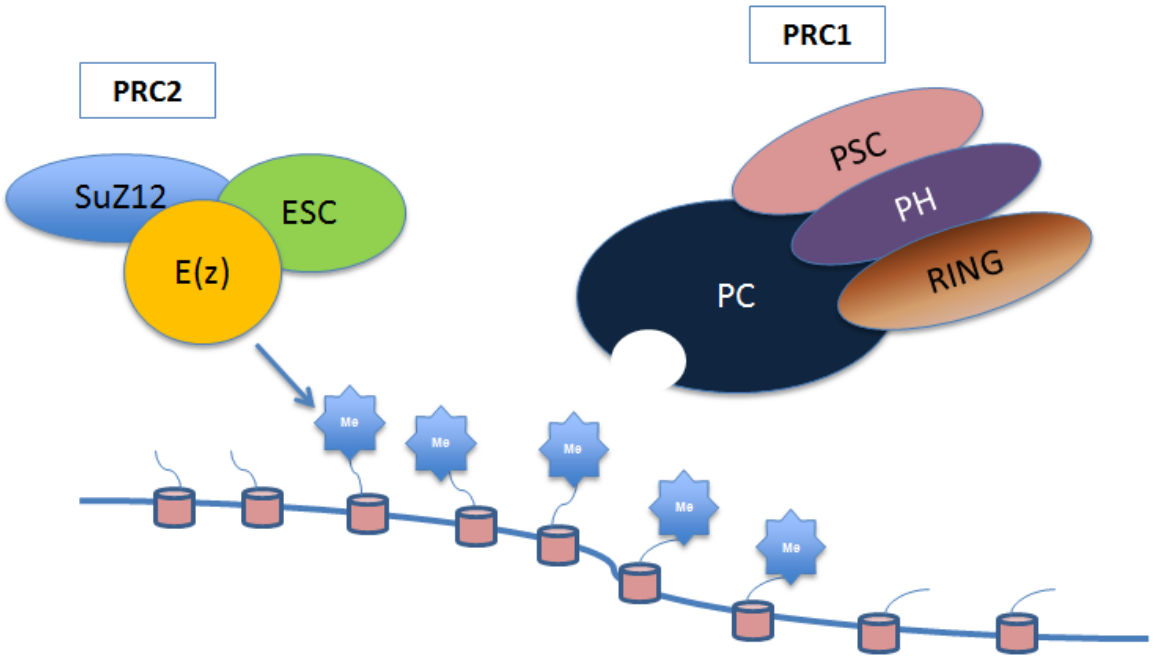


Figure 3: A schematic of the PcG complexes and their mode of action in transcriptional repression

repression depending on the context(Stock, Giadrossi et al. 2007). Lastly, there is growing evidence demonstrating the role of RNA interference (RNAi) machinery in PcG mediated repression. Studies have shown that the RNAi pathway is required for pairing-sensitive-silencing (PSS), a phenomenon by which PREs tend to pair up from different regions of the same chromosome or from different chromosomes. In contrast however, a recent study shows that insulators and not PREs are required for long distance interactions between Pc targets(Li, Müller et al. 2011). RNAi machinery does not affect PcG recruitment but rather the establishment and/or maintenance of long distance contacts between PREs (Grimaud, Bantignies et al. 2006).

1.8 Supressor of Zeste 2 [Su(z)2]:

Su(z)2 is a transcriptional repressor and a PcG related protein, homologous to PSC, a member of PRC1 complex. Overexpression of either of these genes causes abnormalities in sensory bristle development (Brunk, Martin et al. 1991; Sharp, Martin et al. 1994) suggesting a common functional relationship. At the molecular level, Su(z)2 shares a 200 amino acid homology, known as homology region (HR domain) with PSC in which they are 37% identical, and a large (more than 1100 amino acids) C-terminal region which lacks sequence identity but has similar amino acid contents (similar amino acids region, SAACR)(Brunk, Martin et al. 1991). The HR domain is also similar to the HR domains of two mammalian proteins, Mel-18 and Bmi-1. Bmi-1 is a PcG protein in mammals which co-operates with Myc in tumorigenesis,(van Lohuizen, Verbeek et al. 1991) whereas Mel-18 acts as a tumor suppressor by repressing Bmi-1 and Myc (Guo, Datta et al. 2007).

The HR domain provides for the sequence specific DNA binding of these proteins and is evolutionarily conserved, whereas the SAACR domain is responsible for transcriptional repression. When targeted to a reporter gene promoter in mammalian cells, LexA fusion constructs of SAACR domain of Su(z)2 can be strong transcriptional repressors (Bunker and Kingston 1994). Although Su(z)2 is not part of the PcG complexes, it shares common targets with PSC, suggesting a degree of functional redundancy. Deletion of the Psc-Su(z)2 locus causes hyperproliferation (Classen, Bunker et al. 2009) in imaginal discs but deletion of either of them in mitotic clones has no phenotype, suggesting that they can compensate for each other (Beuchle, Struhl et al. 2001). Through a series of biochemical experiments, it has been shown that PSC and

Su(z)2 are functional homologs and that Su(z)2 can replace PSC in a functional PRC1 complex *in-vitro* (Lo, Ahuja et al. 2009). In addition to this, it can inhibit Swi/Snf mediated chromatin remodeling and cause chromatin compaction like other PcG proteins.

1.9 Cajal Body and Histone Locus Body:

Cajal Bodies (CB) are named after the Nobel laureate Ramon y Cajal who discovered them as small round body in the nuclei of nerve cells and called them accessory bodies. Coilin was identified to be a signature marker of these bodies (Andrade, Chan et al. 1991). Although coilin is an important component of the CB, the biochemical functions of the protein are not well understood. It interacts with Lsm10 and Lsm11, components of the U7snRNPs (small nuclear Ribonuclearprotein), suggesting a role in RNA processing (Nizami, Deryusheva et al. 2010). Coilin is required for CB formation. Homozygous coilin knockout mice die as embryos; the adults that do survive have fertility and fecundity defects (Walker, Tian et al. 2009). In *Drosophila* however, homozygous mutants are perfectly viable with no phenotypic defects. Cells of these adult flies, lack CBs (Liu, Murphy et al. 2006; Liu, Wu et al. 2009).

Histone Locus Bodies (HLB) were discovered in *Xenopus laevis* oocytes and were thought to be a subset of CBs. Recently, Liu and others showed, using two different probes (U85sca (CB-specific RNA) and U7snRNP) thought to be specific to CB, that the U7snRNP localizes to multiple foci. Surprisingly, they found that the U85sca and U7snRNA label two distinct nuclear bodies. The U85sca containing organelle was designated as CB because of the presence of other CB specific markers whereas the U7snRNP containing body was named HLB because of its association with the histone

gene locus (Liu, Murphy et al. 2006). HLBs were also identified later in cultured mammalian cells (Ghule, Dominski et al. 2008).

HLBs and CBs can lie very closely to or even touch one another in many nuclei and coilin is found in both CBs and HLBs. (Daneshvar, Khan et al. 2011). The main function of HLB is replication dependent histone gene transcription and processing of histone pre-mRNA(White, Leslie et al. 2007). The U7 snRNP forms an important part of the HLB and is required for histone pre-mRNA processing. The histone mRNA, unlike other mRNAs, is not polyadenylated at its 3' end and also does not have introns. The 3' carries a stem loop extension that is cleaved prior to export to the cytoplasm. The endonucleolytic cleavage requires the U7snRNA which binds to the histone downstream element and stem-loop binding protein (SLBP) via Lsm10 and Lsm11, two protein components of U7snRNPs (Godfrey, Kupsco et al. 2006; Marzluff, Wagner et al. 2008; Godfrey, White et al. 2009). Many other proteins have been recently detected in the HLBs including FLASH, a protein required for histone pre-mRNA processing, NPAT, a transcription factor involved in histone gene expression, and symplekin, stem-loop binding protein (SLBP).

Using the MPM2 antibody that recognizes a phospho epitope in HLB foci in cells undergoing S phase or cells with active Cyclin E/Cdk2, White and others identified two novel components of HLBs in *Drosophila* and showed that HLB assembly occurs through a hierarchical assembly process. Mxc, a homolog of the mammalian NPAT was identified to be essential for the HLB assembly along with Spt6, a transcription elongation factor(White, Burch et al. 2011). Mxc is required for histone gene transcription and processing. Although HLBs can form independent of histone gene

transcription, the integrity of such HLBs is compromised and without histone mRNA, the primitive HLBs formed at the histone locus soon disintegrate. Therefore, histone mRNA is required for the proper assembly and function of HLBs.

CHAPTER 2: MATERIALS AND METHODS

2.1 Fly Stocks, husbandry and Genetics:

w[*]; P{w[+mW, hs]=Gal4-da.G32}UH1

w[*]; P{w[+mW, hs]=Gal4-arm.S}11

w[1118]; P{w[+mC]=UAS-dm.Z}132

P{ry[+t7.2]=hsFLP}22, y[1] w[*]; P{w[+mC]=UAS-dm.Z}42

P{XP}Su(z)2d01221

w[*]; Su(z)2XP; UAS dmyc

Oregon-R

UAS-Lsm11-EYFP

w^{1118}; Df(2L)ED1196, P{3'.RS5+3.3'}ED1196/SM6a

UAS-let-7/GFP

Stocks were maintained at 18^{o}C. Expression of transgene was induced by crossing to a Gal4 driver.

2.2 RNA isolation:

Embryos were collected on grape agar plates with yeast. Embryos were dechorionated with 50% sodium hypochlorite solution for 2 minutes and washed with water several times. Total RNA was isolated using TRIzol (Invitrogen). Embryos were homogenized in TRIzol and phenol-chloroform was added to separate the aqueous solution from the organic. RNA was precipitated using sodium acetate and ethanol. RNA samples DNAse

treated and tested for quality and concentration using NanoDrop spectrophotometer prior to RT-PCR.

2.3 RT-PCR:

For semi-quantitative RT-PCR, we used AccessQuick RT-PCR system (Promega) to amplify target transcripts from RNA, and quantified band intensities using Quantity 1 (Bio-Rad). PCR cycles were minimized to examine expression changes within the linear range (24–25 cycles, depending on the primer set). For all experiments, RNA was extracted from 0-21 hrs of embryo collection. Endogenous dmyc expression was assayed using a primer set that amplifies the 5′ untranslated region (UTR) of dmyc, which is absent in the UAS dmyc transgene. To amplify ectopic myc expression, we used a primer that binds to the 9E10 epitope tag present on the transgenic transcript. All experiments were done in biological triplicates, with no more than 25 PCR cycles. For qRT-PCR, we used Power SYBR® Green RNA-to-CT™ 1-Step Kit and ABI 7500 Fast real-time PCR system (Applied Biosystems, California, USA) to quantify mRNA transcripts. Comparative Ct method was used for quantification of all mRNA transcripts. Ras64B was used as internal reference gene for quantification.

2.4 Chromatin Immunoprecipitation (ChIP):

ChIP was performed as described previously (Goodliffe et al., 2005). Briefly, a collection of 0-24 hours old embryos were dechroniated in bleach and fixed in 3.7% formaldehyde in PBS/Heptane and sonicated in SDS-lysis buffer. The EZ-ChIP kit (Millipore, Massachusetts, USA) was used for precipitation and washes. Antibodies were used at a concentration of 1:100. Precipitated DNA was used as template for PCR amplification using GoTaq Hot-Start polymerase (Promega), and band intensities were quantified using Quantity 1 (Bio-Rad). Anti-H3K27-3Me is obtained from Millipore, 07-449. Anti-MycN antibodies (SantaCruz Biotechnology, sc-28208)

2.5 Tissue fixation and Immunostaining:

Embryos were fixed in formaldehyde/PBS, and stained using 1:500 concentration of antibody in PBS/0.1% Triton X-100/5% BSA. Other tissues were also fixed in formaldehyde/PBS; permeabilized and stained in TritonX/PBS/BSA. For the validation of the co-localization spots we performed several control experiments. We stained Myc primary with the right secondary; Myc primary with individual wrong secondary antibody; Myc primary with all secondary antibodies. We eliminated the secondary antibody that showed cross-reactivity to Myc primary. For microscopy we used sequential scanning of each channel, ensuring that detection of each fluorophore occurred only with the correct excitation laser.

2.6 Antibodies used:

Primary antibodies were used at the following concentrations: rabbit anti-Myc 1:500, goat anti-Myc 1:250, mouse anti-fibrillarin 1:1000 (abcam), guinea pig anti-coilin 1:2000 (ovaries, embryos, the antibody was a gift from Joseph Gall) and 1:500 (larvae), rabbit anti-Lsm11 1:2000 (ovaries and embryos, gift from Joseph Gall) and 1:500 (larvae), mouse anti-GFP 1:500 (Covance), chicken anti-GFP (abcam) and mouse MPM-2 1:1000 (Millipore).

2.7 Epifluorescence Microscopy:

Embryos were mounted in SlowFade Gold with DAPI (Invitrogen), and imaged using a Motic BA400 compound microscope, Lumen 200 Illumination Systems epi-fluorescence, Spot Cooled CCD monochrome camera and software. We photographed all embryos with identical bulb intensity and acquisition settings

2.8 Confocal Micscroscopy:

Images were generated using a Zeiss LSM 710 or Olympus FluoView FV1000 confocal microscope. Images were acquired such that there were no saturated pixels, with minimal offset. Modifications to images were minor, and limited to gamma adjustment and contrast adjustments within the Olympus FV1000 software. Modified images were cropped using Adobe Photoshop.

2.9 Northern Blotting for miRNA:

Total RNA was extracted using TRIzol (Invitrogen). Approximately 10μg of total RNA was heated at 90^{o}C for 3 mins and electropheretically separated through a 15% urea-polyacrilamide gel at 125V for 2hr. RNA was transferred electrophoretically to a nitrocellulose membrane at 4^{o}C for 2 hr. RNA was crosslinked by UV radiation and the membrane was baked at 80^{o}C for 30 min. Probe for let-7 was designed for the mature sequence. Let-7antisense probe was radiolabeled by incorporation of [α^{-32P}]dATP 6000 Ci/mmol as recommended bythe vendor. Membrane was hybridized with the let-7 probe for 24 h at 42°C in 7%SDS, 0.2MNa2PO4, pH 7.2, and washed twice with 2X SSPE 0.1% SDS, and once with 1X SSPE 0.1% SDS, and 0.5X SSPE0.1% SDS at 42°C. The radioactive signals of *let-7* transcripts were quantified by using a PhosphorImager.

CHAPTER 3: SU(Z)2 ANTAGONIZES MYC AUTOREPRESSION

3.1 Su(z)2 upregulation results in loss of autorepression by Myc

As previously discussed, the myc gene undergoes autoregulation under higher Myc protein levels. In *Drosophila* embryos, ectopic expression of dmyc leads to a general widespread repression of nearly 200 genes including the *Drosophila* myc (dmyc) gene. Polycomb (Pc) is required for this repression; upon Polycomb depletion by RNAi, autorepression by myc is abrogated leading to restoration of normal levels of myc transcripts (Goodliffe, Wieschaus et al. 2005). One explanation for the possible role of Polycomb is that it is directly involved in the repression of the dmyc gene. Since Polycomb is a known chromatin binding repressor, we hypothesized that Polycomb physically occupies the dmyc locus and leads to its repression under high Myc protein levels. However, this hypothesis is inconsistent with some of our previous data indicating that Pho, the protein required for the physical targeting of Polycomb to chromatin, is dispensable for Myc autorepression. If Polycomb directly represses the dmyc gene, then our Pho RNAi data should have been identical to the Polycomb RNAi data. Since this was not the case, we considered an alternate hypothesis.

We hypothesized that the role of Polycomb in myc autorepression is largely indirect; in that, it is required to repress a certain target(s) that can interfere with the mechanism of myc autorepression. If our hypothesis is true then it follows that Polycomb

RNAi leads to the loss of Polycomb protein, followed by the derepression of a certain gene(s) product, whose increased abundance can then abrogate myc autorepression. Therefore, our Polycomb RNAi microarray data should show us global gene expression changes from which we can identify the possible candidate genes that could be involved in the myc autorepression pathway.

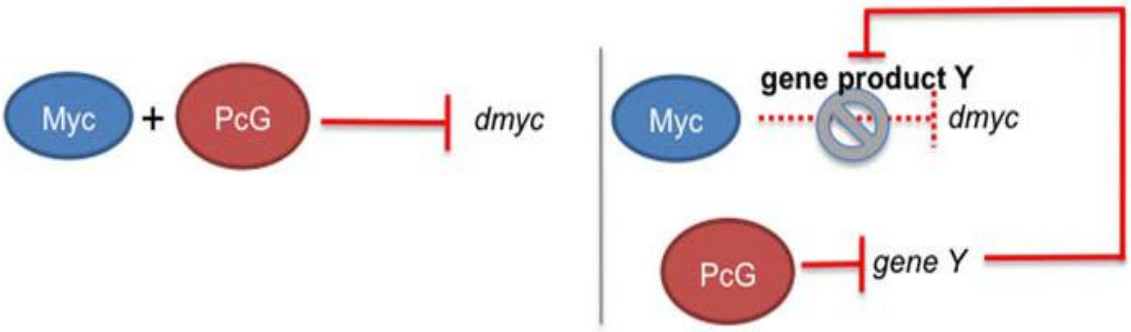


Figure 4: A schematic representation of the hypothesis for mechanism of Pc mediated Myc autorepression. Pc is required to repress gene Y which can interfere and abrogate Myc autorepression

Examining our previous microarray gene expression data, we looked at expression changes in some key developmental regulators that are known to be involved in gene regulation either functioning directly as a transcription factor or as a part of a multi-protein complex. We looked at genes belonging to the Trithorax Group (Trx) and Polycomb Group (PcG). Of the 18 genes we analyzed, only one gene, Suppressor of Zeste 2 (Su(z)2) showed a significant increase in the expression upon Pc RNAi (Figure 5). Levels of Su(z)2 transcripts increased 4 fold in the Pc RNAi embryos compared to the wild type. This suggests that Polycomb represses Su(z)2, which is consistent with data from other published reports (Ali and Bender 2004; Classen, Bunker et al. 2009).

Su(z)2 is a homolog of another PcG protein called PSC. It shares a 200 amino acid homology with PSC and two other mammalian proteins Bmi-1 and Mel-18 (Brunk, Martin et al. 1991; van Lohuizen, Frasch et al. 1991). Functionally, Su(z)2 is known to be

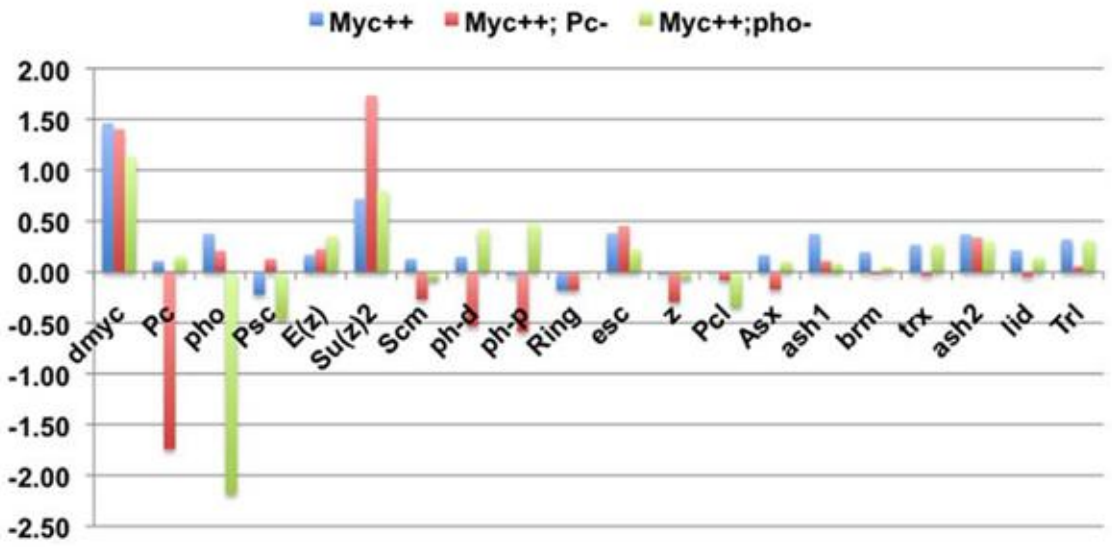


Figure 5: A candidate Y gene: Log ratios of gene expression of select PcG and Trx group genes compared to wild type (Gal4) are shown here in embryos with ectopic Myc(blue), ectopic Myc plus Pc RNAi (red) and ectopic Myc plus Pho RNAi (green). Su(z)2 levels increase significantly in embryos with ectopic Myc plus Pc RNAi compared to Myc++ embryos and hence was chosen as a candidate Y gene for further experiments.

a potent transcriptional repressor (Brunk, Martin et al. 1991; Brunk, Martin et al. 1991; Bunker and Kingston 1994; Sharp, Abramova et al. 1997). Our finding was intriguing, because we did not expect a transcriptional repressor to be involved in suppressing repression. Although we were surprised to find Su(z)2 to be the only candidate gene, we pursued the possibility that Su(z)2 could be involved in the pathway.

Based on our data we hypothesized that Su(z)2 disrupts Myc autorepression and Polycomb is required to repress Su(z)2. A schematic representation of the hypothesis is depicted in Figure 4. To test the above hypothesis, we obtained flies from the Exelixis collection that have an XP insertion at the endogenous Su(z)2 gene (referred to as Su(z)2XP from here on) which leads to its overexpression under the influence of a Gal4 driver. We combined homozygous Su(z)2XP flies on second chromosome with flies homozygous for ectopic dmyc on third, whose expression is also driven by a Gal4 driver (see Materials and Methods). We obtained embryos that express either ectopic dMyc alone or ectopic Su(z)2 alone or both ectopic Myc and Su(z)2 under the control of armadillo-Gal4 (arm-Gal4).

In embryos expressing ectopic dmyc, the levels of endogenous dmyc transcripts reduced dramatically compared to that of wild type embryos as seen by RT-PCR. These data are consistent with our previous microarray data on embryos expressing ectopic myc. In embryos overexpressing both ectopic Su(z)2 and dmyc, the levels of the endogenous myc increase significantly ($P = 0.036$) and are comparable to the levels in wild type embryos. This suggests that Su(z)2 clearly interferes with Myc autorepression. As seen in Figure 1.3, the levels of endogenous Myc are slightly higher in embryos overexpressing both ectopic Su(z)2 and ectopic dmyc compared to that of wild type.

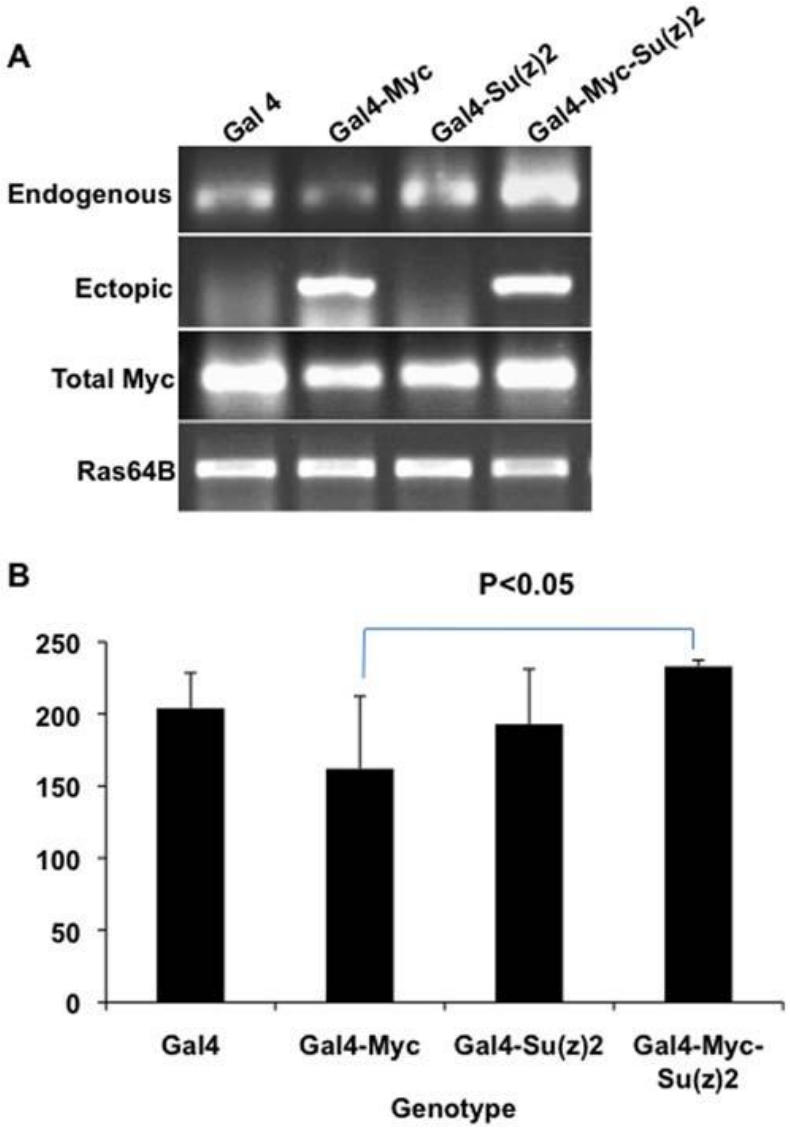
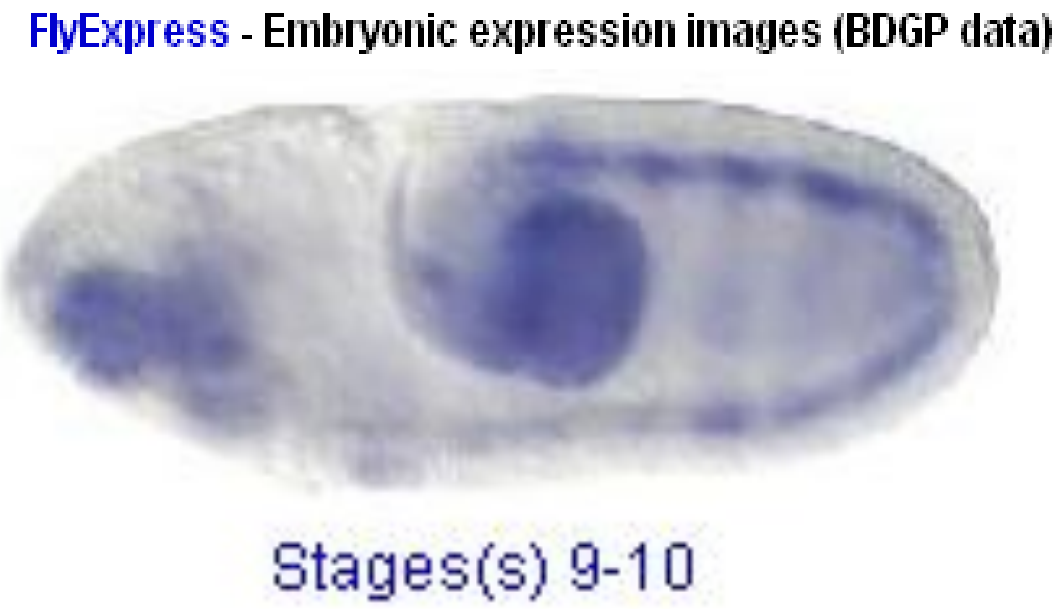


Figure 6: Su(z)2 upregulation results in loss of autorepression by Myc. A) RT-PCR analysis of endogenous, ectopic and total Myc expression in embryos of four different genotypes, which are indicated above each lane (Gal4 = arm-Gal4, Gal4-Myc = armGal4; UAS dmyc, Gal4-Su(z)2 = arm-Gal4; Su(z)2XP, Gal4-Myc-Su(z)2 = armGal4;Su(z)2XP; UAS dmyc). A 0–21 hour collection of embryos was used for RNA isolation and for all subsequent assays. Ras64B was used as a loading control. B) A chartshowing endogenous dmyc expression, the average of biological triplicates is plotted with standard deviations indicated for four genotypes of embryos. Expression was quantified using quantity 1 (Bio-Rad). The blue line denotes a statistically significant change inendogenous dmyc levels from Gal4-Myc to Gal4-Myc-Su(z)2.

We reason that the combination of ectopic Su(z)2 and dmyc is leading to the induction of the endogenous dmyc in addition to the alleviation of the autorepression. This can also be seen in the embryos expressing ectopic Su(z)2 alone. The levels of dmyc transcripts in these embryos are again slightly higher than those of wild type. We interpret these results to mean that wild type cells undergo some degree of auto-repression, which is reduced in the presence of Su(z)2, leading to increased dmyc expression. Therefore, Su(z)2 alone seems to be sufficient to alleviate the endogenous myc autorepression or to induce Myc expression through an unknown mechanism. These data demonstrate the importance of the regulation of Myc regulation, more specifically, dmyc autorepression during embryogenesis and the role of Su(z)2 in this pathway.

Next, we looked at the dMyc protein expression levels in these embryos to support our RT-PCR data. We obtained an antibody from Santa Cruz Biotech that targets dMyc. We tested the antibody for its specificity (Figure 7) and found that the dMyc protein expression pattern was identical to the fluorescent in-situ hybridization (FISH) pattern of dmyc transcripts in wild type embryos (FlyFISH). We used this antibody to test for dMyc expression levels in embryos of the four genotypes as shown in Figure 8 (armGal4; armGal4—UASdmyc; armGal4—Su(z)2XP; armGal4—UASdmyc,Su(z)2XP). Embryos expressing ectopic Myc have dramatic reduction in overall Myc protein levels, whereas embryos expressing both ectopic Myc and Su(z)2 look identical to wild type with regard to Myc levels. These data are consistent with our RT-PCR data showing dmyc autorepression both at the transcriptional as well as translational level.



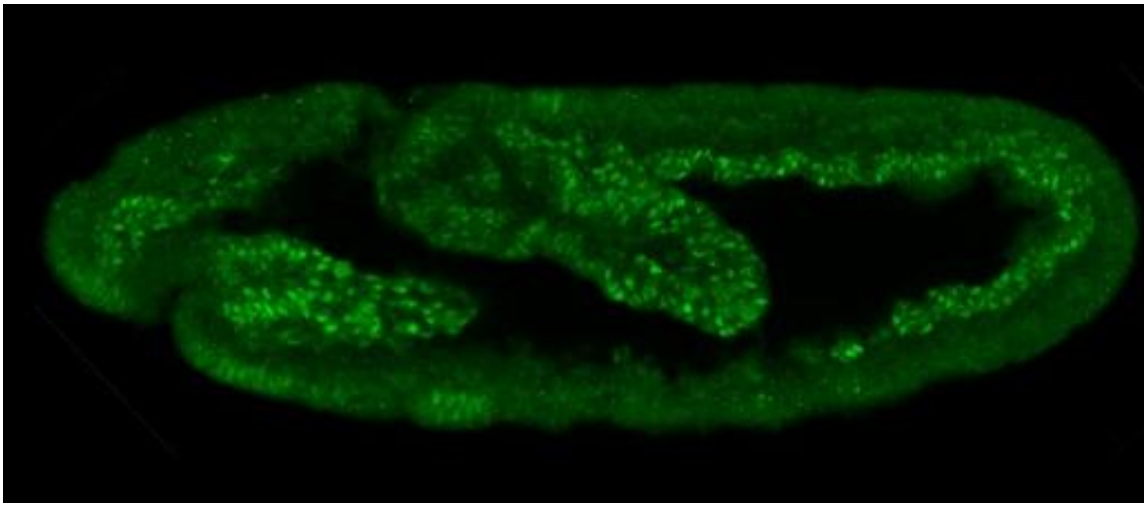


Figure 7: dMyc antibody validation: Wild type embryo stained for Myc protein (green) exhibits a staining pattern identical to in-situ hybridization of dmyc transcripts in similarly staged embryo (courtesy: FlyExpress).

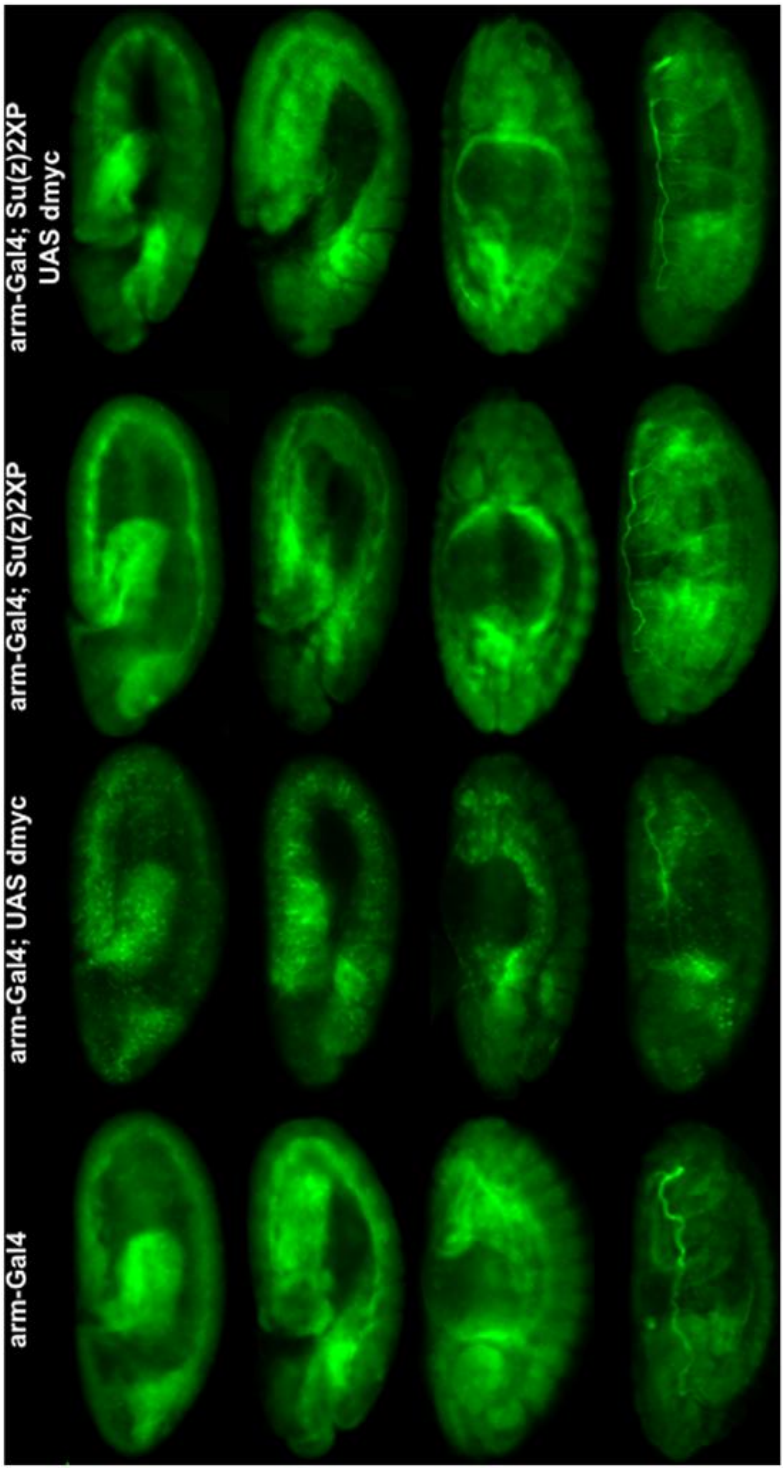


Figure 8: dmyc auto-repression reduces total Myc protein levels compared to wild type, which is rescued by combining ectopic Myc with ectopic Su(z)2 expression. Similarly aged embryos of four genotypes are beside one another, with the genotypes of embryos indicated above each column. Anti-Myc staining is green.

In addition to the reduction in Myc protein levels, we observed certain other physical and morphological abnormalities in the embryos expressing ectopic Myc. These embryos have an aberrant Myc expression pattern which is very different from wild type embryos (Figure 9). When observed closely, we could discern that the Myc protein expression pattern is highly localized with certain few cells having a lot of Myc whereas most of the surrounding cells either lacking or showing very faint signal. One explanation could be that the Myc signal seen in these embryos is emanating mostly from the ectopic Myc. The arm-Gal4 is a weak driver of expression; as a result, the ectopic Myc levels are very low compared to wild type endogenous Myc levels. This small amount of ectopic Myc is enough to drive autorepression of endogenous Myc to such a drastic level that the embryos expressing the ectopic gene experience an almost complete Myc knockdown effect. What is left in these embryos is only the ectopic Myc, but its levels and distribution differ greatly from wild type.

As a consequence of the changes in Myc levels and distribution upon Mycauto-repression, we observed that most of the embryos do not survive embryogenesis. Therefore we used a different driver, Gal4-da.G32, because it allowed more embryos to hatch into larvae than the arm-Gal4 driver. With Gal4-da.G32 we were able to see many embryos hatch into larvae, but these larvae were very small and they failed to survive past 4 days (Figure 10). They behave like wandering third instar larvae but their size is comparable to that of first instar. These larvae demonstrate a classic Myc hypomorphic phenotype and resemble larvae with Myc knockdown mutation(Pierce, Yost et al. 2008).

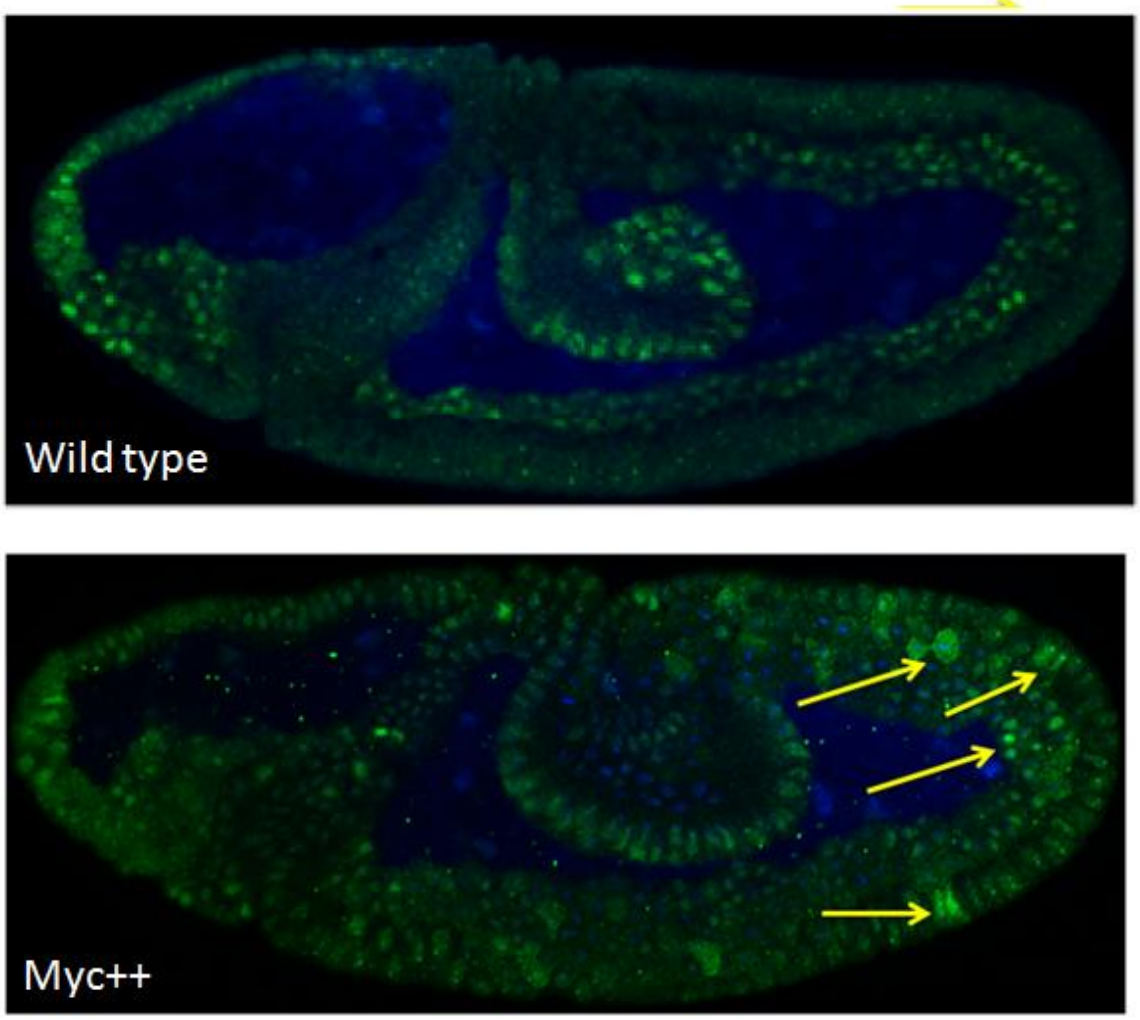


Figure 9: Aberrant Myc expression pattern and morphology of Myc++ embryos: Similar staged wild type and Myc++ (expressing ectopic Myc) embryos are shown. Myc ++ embryos clearly have an aberrant expression pattern compared to the wild type embryos. Arrows mark clusters of cells with unusually high levels of Myc.

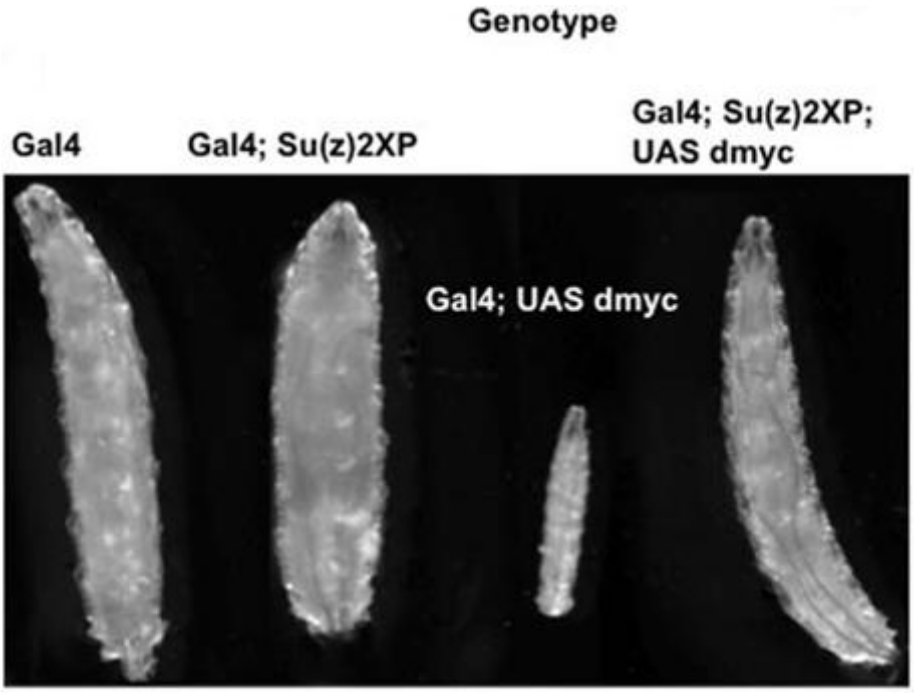


Figure 10: Consequence of Myc autorepression:
Living larvae of the genotypes shown, all grown at low density, aged 4 days after egg laying at room temperature, and photographed simultaneously

In contrast, the embryos expressing both ectopic Myc and Su(z)2 were perfectly viable and phenotypically normal. Ectopic Su(z)2 completely rescued the Myc knockout phenotype by abrogating Myc autorepression. Taken together, the molecular and the phenotypical data demonstrate the effect of the strong Myc autorepression mechanism and the role of Su(z)2 in rescuing this phenotype.

3.2 General repression by Myc is maintained or possibly enhanced by Su(z)2

Myc is primarily a transcriptional activator but it is also known to repress certain targets. We wanted to test whether ectopic Su(z)2 can interfere with Myc's ability to repress genes other than dmyc. We looked at 6 such targets (Cyp6a8, CG31274, Obp56a, CG12868, CG31445, JhI-26) that are known to be repressed during embryogenesis by Myc(Goodliffe, Cole et al. 2007). In embryos with ectopic Myc, these targets were significantly repressed compared to wild type, as shown in Fig 1.6. Although the overall Myc levels in these embryos drops due to autorepression, we reason that the repression of these targets by Myc occurs either before or during dmyc autorepression. These targets remain repressed in embryos that express both ectopic Su(z)2 and Myc, suggesting that Su(z)2 does not affect Myc's ability to repress its targets.

Surprisingly, these genes were also repressed in embryos expressing ectopic Su(z)2 alone. One possible explanation is that, ectopic Su(z)2 disrupts the endogenous myc autorepression leading to slightly higher Myc levels in these embryos compared to wild type. This increase in Myc levels could be responsible for the repression of these genes. Alternatively, ectopic Su(z)2 could also be directly responsible for the repression of these genes. Taken together, our results suggest that Su(z)2 disrupts autorepression by Myc but does not affect Myc's ability to repress its targets.

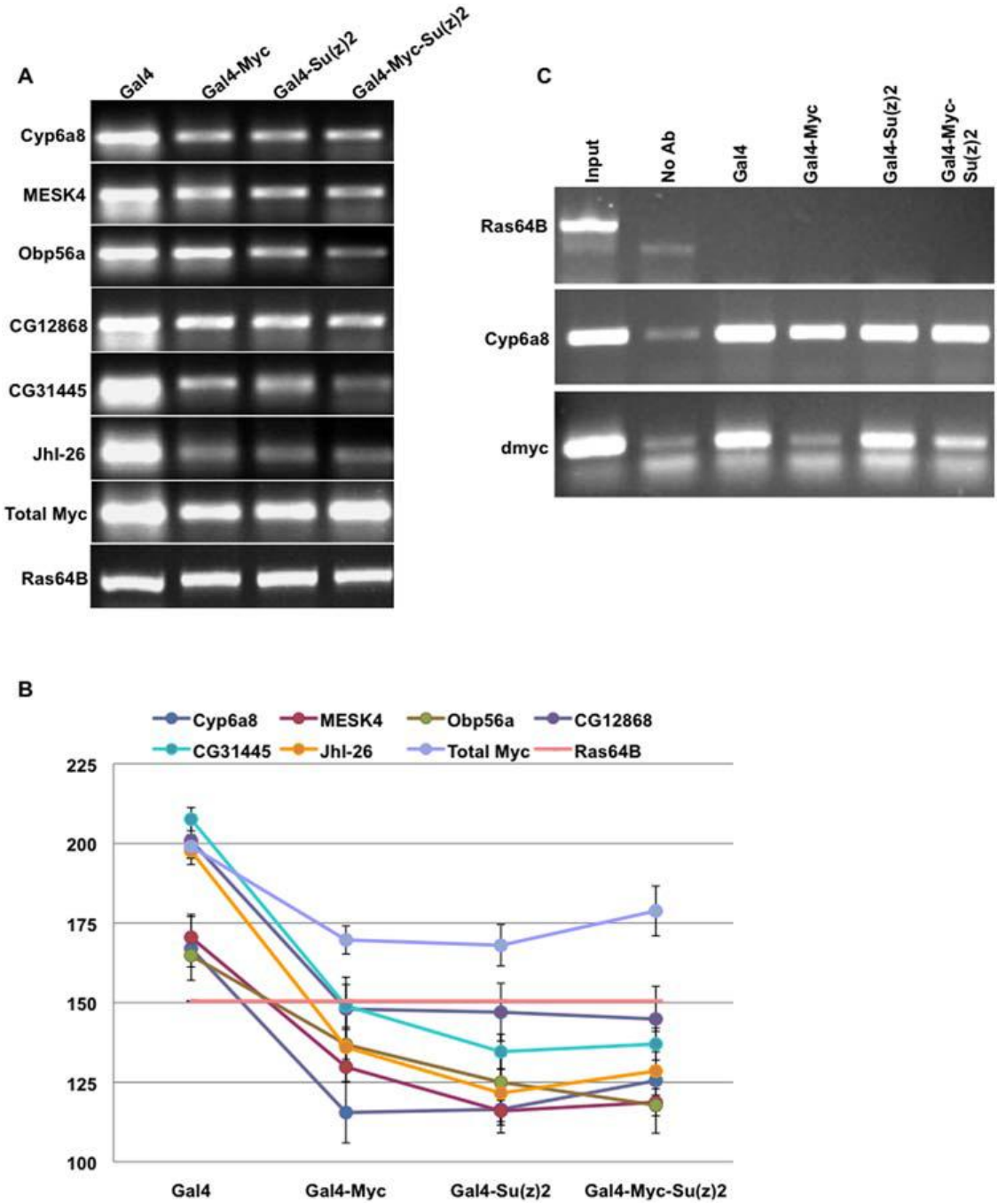


Figure 11: Ectopic Su(z)2 does not interfere with Myc repression of targets other than dmyc. A) RT-PCR data showing the expression of six Myc targets of repression in four genotypes, as indicated above each column of bands. Total dmyc expression and Ras64B expression, a loading and RNA level control, are shown in the bottom two panels. B) The average band intensities indicating levels of expression and relative standard deviation are plotted for the 8 genes shown in A. Genotypes are indicated along the X axis, and the Y axis shows band intensities as quantified by Quantity 1 (Bio-Rad). C) ChIP results showing H3K27 tri-methylation of one of the 8 genes shown in A, in the 4 genotypes of the experiment. Ras64B is a negative control.

Histone modifications are known to cause or maintain specific transcriptional states of genes. Specific covalent modifications are attributed to either activation or repression. Trimethylation of lysine 27 on Histone H3 (H3K27me3) is a well defined mark of repression which is recognized by PcG complexes and is also known to be found at Myc repression targets (Goodliffe, Wieschaus et al. 2005; Goodliffe, Cole et al. 2007). We wanted to test whether Su(z)2 influenced the histone methylation status at the Myc repression targets. We performed Chromatin Immunoprecipitation (ChIP) to test for the enrichment of H3K27me3 mark at the above mentioned Myc repression targets. Surprisingly there was no difference in the methylation levels at the 6 genes in all four genotypes. Figure 1.7 shows the histone methylation levels at Cyp6a8 (one of the six repression targets) and dmyc. We were also surprised to find reduced histone methylation levels at dmyc in embryos expressing ectopic Myc. This result was contrary to the autorepressiondata seen in Figure 1.3 and 1.4. These data suggest that this embryonic chromatin modification, histone H3 trimethylation of lysine 27, does not mediate the transcriptional state of Myc repression targets.

3.3 Activation by Myc is enhanced by Su(z)2:

As shown earlier, ectopic Su(z)2 abrogates Myc autorepression, leading to restoration of overall Myc levels and consequently rescuing the Myc knockdown phenotype in larvae. We were interested in determining the basis for this rescue by ectopic Su(z)2. We wanted to test whether ectopic Su(z)2 restores the normal Myc transcriptional activity by eliminating autorepression. We tested activation by Myc using the same system in all four genotypes. We looked at three known Myc targets of

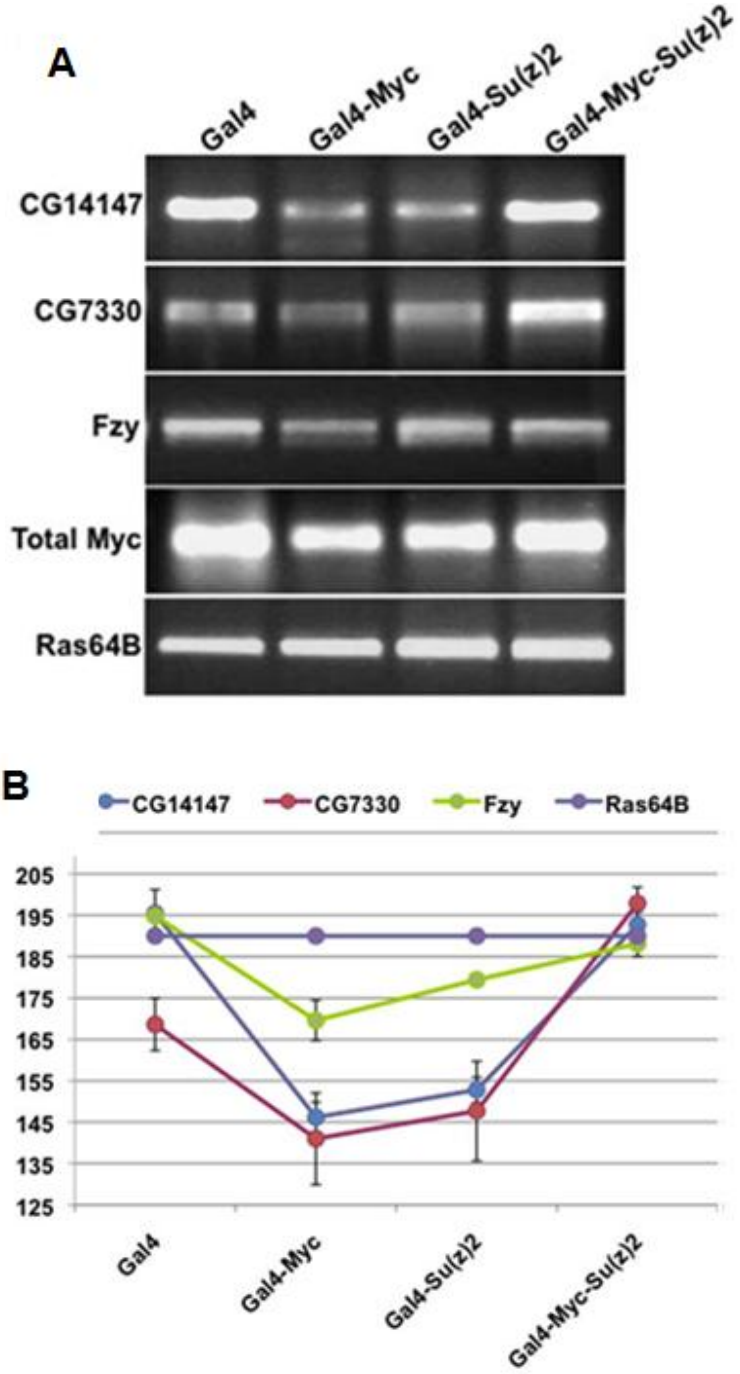


Figure 12: Increased Su(z)2 provides for activation of Myc targets, and a reduction in their H3K27 tri-methylation. A) RT-PCR analysis showing expression of three Myc activation targets (indicated on the left side of the gel pictures) in embryos of 4 different genotypes (indicated above the lanes). B) Average band intensities (Quantity 1) of biological triplicates are plotted on the right, with relative standard deviations indicated.

activation (CG14147, CG7330, Fzy) (Goodliffe, Cole et al. 2007). In embryos expressing ectopic Myc, the expression of all three targets is lower than that of wild type because the overall levels of Myc are lower than those of the wild type embryos (Figure 1.8). Interestingly, in embryos expressing both ectopic Su(z)2 and Myc, the levels of all three genes are higher and similar to the levels in wild type embryos. This effect likely occurs because the Myc levels in these embryos are also comparable to those in wild type embryos due to the lack of Myc autorepression.The results are also consistent with these targets being Myc responsive; they are activated under normal Myc levels and de-activated under low Myc condition. This result helps explain the molecular reason behind the rescue of the Myc knockdown phenotype. These data suggest that ectopic Su(z)2 leads to the restoration of endogenous Myc levels that are sufficient enough to perform its normal transcriptional activity during embryogenesis.

3.4 Ectopic Su(z)2 alters histone H3 lysine 27 methylation at Myc activation targets:

Embryonic Myc activation targets are known to be marked by histone H3 lysine 27 trimethylation(Goodliffe, Cole et al. 2007). We were interested to see whether ectopic Su(z)2 can alter the histone modifications at Myc target genes leading to their activation by Myc. We performed Chromatin Immunoprecipitation (ChIP) using antibodies against H3K27-Me3 to test for the histone methylation status at five known Myc activation targets (CG14147,CG7330, Cyp309a2, SamDC and 128up). All of these targets are known to be Myc responsive (Goodliffe, Wieschaus et al. 2005; Goodliffe, Cole et al. 2007); SamDC and 128up are known to have Myc bound at their promoters (Orian, van Steensel et al. 2003). Keeping conditions identical to our RT-PCR experiments, we

performed ChIP on embryos aged 0-21 hrs. As shown in Figure 1.9, in embryos expressing ectopic Myc alone, 4 out of 5 of these targets had higher H3K27

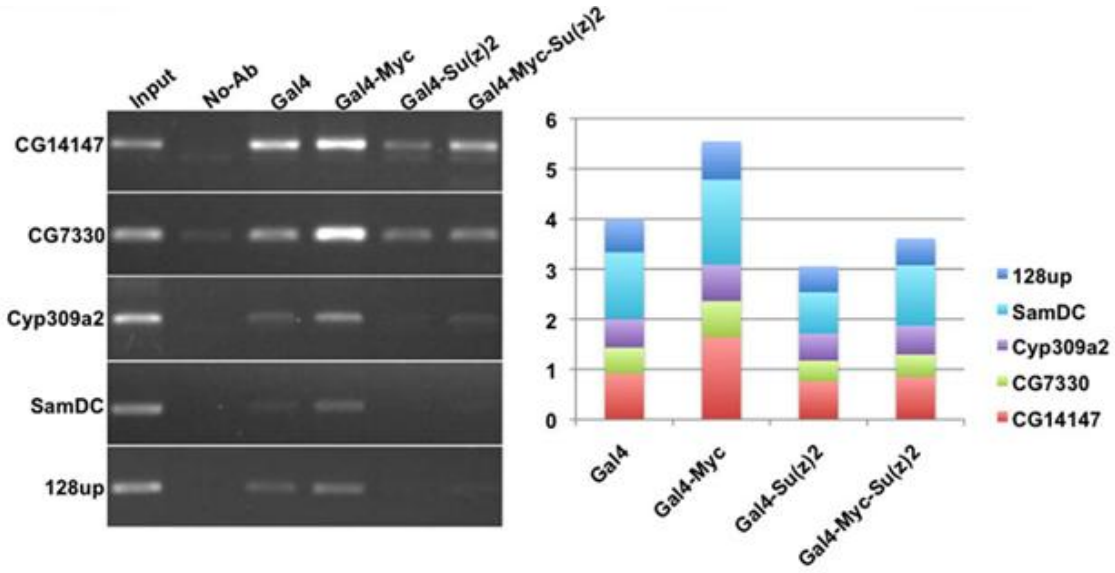


Figure 13: Su(z)2 alters histone methylation at Myc targets: ChIP assay showing histone H3K27 tri-methylation at five Myc activation targets (indicated to the left of the gel pictures) in embryos of genotypes indicated on top. Data shown on the left are plotted in a stacked column chart; the y-axis is the density of each PCR product divided by the density of the input sample PCR product, and the values for each gene are stacked together for each of the four genotypes.

trimethylation compared to wild type, consistent with their deactivation seen in figure 1.8. Embryos expressing both ectopic Su(z)2 and Myc, show reduced H3K27 methylation at these targets similar to that in wild type embryos. Interestingly, embryos expressing ectopic Su(z)2 alone showed reduced histone methylation compared to wild type at 3 out of 5 of these targets. These data are consistent with our RT-PCR data showing the expression levels of Myc activation targets. The data also correlate well with the Myc levels in these four genotypes, suggesting that low Myc levels influence increased methylation of these targets leading to deactivation and conversely, higher Myc levels cause activation of these targets through a reduction in the histone methylation.

3.5 Discussion:

Su(z)2 is a chromatin binding repressor that belongs to Polycomb Group related complexes. It is a functional homolog of PSC, a PcG protein present in the PRC1 complex (Lo, Ahuja et al. 2009). Su(z)2 and PSC are known to share a set of common targets, exhibiting a degree of evolutionary redundancy in their function. This is evident by the abnormal bristle phenotype caused by the overexpression of either of these genes(Brunk, Martin et al. 1991; Sharp, Martin et al. 1994). PSC and Su(z)2 are also known to co-localize at many loci on polytene chromosome (Rastelli, Chan et al. 1993; Sharp, Abramova et al. 1997). Deletion of the PSC-Su(z)2 region causes hyper-proliferation in the wing imaginal disc whereas clones homozygous mutant for either of the two genes are normal, suggesting again the redundancy in the function of these two genes (Beuchle, Struhl et al. 2001).

Recently, it has been shown that Su(z)2 co-precipitates with the components of the PRC1 complex. Also, the authors demonstrated that Su(z)2 can replace PSC in a

functional PRC1 complex *in-vitro* but Su(z)2 has a lower DNA binding affinity than PSC(Lo, Ahuja et al. 2009). Taken together, these findings may provide a rationale for the observed role of Su(z)2 in the abrogation of Myc autorepression. One hypothesis is that ectopic Su(z)2 can replace PSC in the PRC1 complex, thereby rendering the complex ineffective. This hypothesis may only hold true if the PcG complexes are directly involved in the repression of the dmyc locus. In embryos with ectopic Su(z)2, the high levels of Su(z)2 protein could compete with and replace the endogenous PSC from the PRC1 complex. Although, PSC and Su(z)2 can be redundant, the high levels of Su(z)2 protein in these embryos could possibly produce many non-functional complexes with other PcG components which could interfere or compete with the functional complexes. Since Su(z)2 has lower DNA binding affinity, the PRC1 complex with Su(z)2 could be ineffective in the repression of the dmyc locus in embryos with ectopic Myc and Su(z)2. This hypothesis could also help explain the fact that although Polycomb is required for Myc autorepression, ectopic Su(z)2 can eliminate Myc autorepression even in the presence of Polycomb.

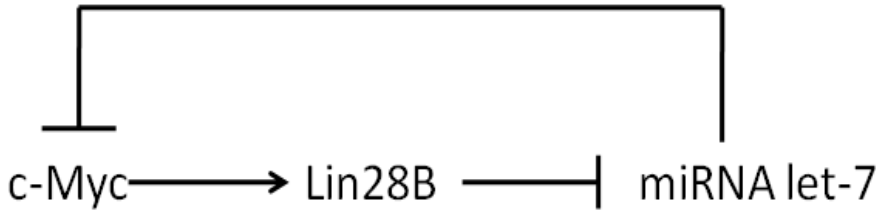
Our results show that ectopic Su(z)2 can derepress Myc but does not affect repression by Myc, suggesting that Su(z)2 works in different mechanisms in these two situations. It is interesting to note that a repressor is interfering with Myc repression. Therefore we have not ruled out the possibility that Su(z)2 could be involved in a more indirect manner. It is possible that Su(z)2 represses another gene(s) that could be involved in the regulation of Myc transcription and that Polycomb is required to maintain low levels of endogenous Su(z)2 during early embryogenesis.

Su(z)2 has two mammalian homologs: Bmi-1 and Mel-18(van Lohuizen, Frasch et al. 1991; Sharp, Abramova et al. 1997). Bmi-1 and Mel-18 share the same homology region with PSC and Su(z)2, suggesting that they belong to an evolutionary gene family with similar molecular functions. However, Bmi-1 and Mel-18 are known to exhibit opposite roles in the progression of cancer. Mel-18 acts as a tumor suppressor by repressing c-Myc and Bmi-1 whereas Bmi-1 is known as a proto-oncogene (Guo, Zeng et al. 2007; Wiederschain, Chen et al. 2007). Bmi-1 was isolated as a collaborator of Myc in tumorigenesis (Haupt, Alexander et al. 1991; van Lohuizen, Verbeek et al. 1991). Our findings demonstrate an interesting function for Su(z)2 whereby it can upregulate Myc transcription suggesting a potentially co-operating role in Myc induced growth and proliferation. However, Su(z)2 has also been shown to be a tumor suppressor gene (Classen, Bunker et al. 2009).Knowing the conflicting roles of Su(z)2, it becomes imperative to ask one important question here: Is Su(z)2 a functional homolog of Bmi-1 or Mel-18? It would be interesting to pursue further experiments to determine the precise role of Su(z)2 during development. One hypothesis is that the roles of PSC and Su(z)2 are very context specific. Su(z)2 behaves largely as a tumor suppressor by regulating the expression of proto-oncogenes, suggesting that it is similar to Mel-18 but Su(z)2 can also switch roles to promote growth under special conditions as seen in our experiments. For future studies, it would very be interesting to study the broader evolutionary context of this functional dimorphism exhibited by Su(z)2.

CHAPTER 4: ROLE OF LET-7 miRNA IN REGULATIONOF DMYC

4.1: Let-7 miRNA could be required for Myc autorepression:

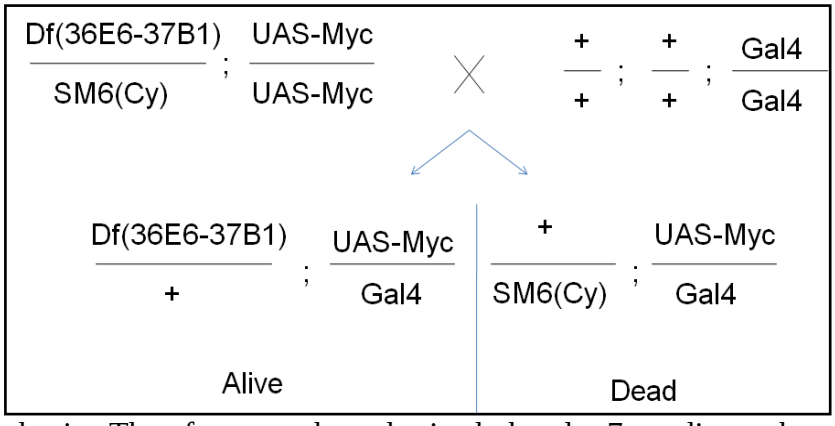
Overexpression of c-Myc causes tumorigenesis via a widespread repression of miRNAs, along with the upregulation of many other targets. The let-7 family was identified as one of the groups of miRNAs repressed by c-Myc(Chang, Yu et al. 2008; Chang, Zeitels et al. 2009). However, c-Myc does not repress let-7 miRNAs transcriptionally, but rather it inhibits their maturation by a post-transcriptional mechanism that involves the activation of Lin28B, an RNA binding protein(Chang, Zeitels et al. 2009).Lin28B is directly activated by c-Myc, and is responsible for the inhibition of miRNA biogenesis. Lin28B inhibits the Drosha and Dicer mediated processing of let-7 miRNA by binding to the let-7 primary transcript (Newman, Thomson et al. 2008; Rybak, Fuchs et al. 2008; Viswanathan, Daley et al. 2008). Interestingly, the



let-7 family of miRNAs is known to be strong tumor suppressor, inhibiting the translation of many oncogenes, one of which is c-Myc (Esquela-Kerscher, Trang et al. 2008; Roush and Slack 2008; He, Chen et al. 2010). This is a positive feedback mechanism by which c-Myc activates Lin28B that blocks the processing of let-7 and consequently prevents the

down-regulation of c-Myc by let-7.

Drosophila has a single let-7 gene which is known to be important for metamorphosis from larval to pupal stages (Caygill and Johnston 2008). Let-7 is induced by the expression of ecdysone, a steroid hormone required for metamorphosis (Sempere, Dubrovsky et al. 2002).The timing of let-7 expression is critical to ensure normal development. It's expression begins during the late third instar larval stage and continues during the pupal stages, reaching a peak on the second day of the pupal life (Sempere, Dubrovsky et al. 2002). Interestingly, the expression of dMyc inversely correlates with the expression of let-7(modENCODE). dMyc expression peaks at larval L1 stage and drops dramatically during late L3 stage. The levels continue to remain low throughout the pupal and adult life. Like its mammalian homolog, it is possible that *Drosophila*let-7 could repress dMyc. The high levels of dMyc during the larval stages could act as a systemic signal to trigger the process of metamorphosis by inducing let-7. The induction of let-7 in turn, represses dMyc, slowing growth and proliferation and initiating



metamorphosis. Therefore, we hypothesized that let-7 mediates the process of Myc autorepression in our model.

To test the hypothesis, we performed a preliminary genetic cross with flies heterozygous for a deficiency spanning the let-7 locus. Along with let-7, the locus

contains few other annotated genes. Nevertheless, we went ahead to perform a preliminary test. If let-7 is required for Myc autorepression, then deletion of let-7 should abrogate autorepression leading to the rescue of the autorepression phenotype. The cross is illustrated above. We combined flies heterozygous for the deficiency with homozygous UAS-dMyc on the third chromosome. We then crossed these flies to flies carrying the da-Gal4 driver. All embryos resulting from this cross express ectopic dMyc. As shown in the schematic above, the cross yielded two classes of progeny. Only flies with the Df chromosome survived whereas none of the embryos with the Cy chromosome survived past larval L1 stage. The surviving flies express both endogenous and ectopic Myc, as seen by RT-PCR (Figure 14). As a consequence of ectopic dMyc expression, the embryos undergo autorepression and as shown in our earlier experiments, these embryos do not survive past the larval stages. However, embryos that are heterozygous for the deficiency are perfectly viable. They progress through normal life cycle to reach adulthood without any discernable phenotypic abnormalities. On the contrary, embryos with the SM6 balancer chromosome, which has a dominant marker (Cy), exhibited the autorepression phenotype and died as 2nd instar larvae. These data suggest that let-7 could be required to induce Myc autorepression. However, since the deficiency encompasses other genes along with let-7, we cannot be certain that let-7 is the only gene responsible for this rescue.

4.2: Let-7 miRNA is upregulated in embryos undergoing Myc autorepression:

In the embryos expressing ectopic Myc, the levels of dMyc are higher than a threshold that is normal for embryogenesis. The sum total of endogenous Myc and ectopic Myc could potentially mimic the levels found during the L3 stage. We

hypothesize that this abundance of Myc triggers the premature expression of let-7 during embryogenesis and consequently, leads to the down-regulation of endogenous Myc. However, the untimely and uncontrolled expression of let-7 leads to such a dramatic knockdown of Myc that the organism fails to progress through normal development due to the lack of endogenous Myc. To test this hypothesis we looked at the expression of let-7 miRNA by Northern Blot analysis. Our results indicate that let-7 miRNA is expressed in embryos with ectopic Myc but not in wild type embryos, as expected. Let-7 transcript levels are absentin wild type embryos (negative control), elevated in embryos with ectopic Myc, and highin the two positive controls: let-7 overexpressing embryos and adult flies (Figure 15A).

4.3 let-7 does not repress Myc unlike its mammalian homolog:

To test whether let-7 miRNA represses dMyc, we obtained transgenic flies that contain a UAS-let-7 sequence on the second chromosome (a gift from Dr. Laura Johnston). To induce the ectopic expression of let-7 we crossed the UAS-let7 flies to flies with the da-Gal4 driver. The expression of the let-7 miRNA in the resulting embryos was validated by Northern Blot as seen in Figure 15. Initial q-PCR analysis revealed that dMyc is down-regulated in embryos overexpressing let-7 (Figure 15B). The expression of Myc levels in this experiment were normalized to Ras64B, a control gene used for normalizing in all of our previous experiments.

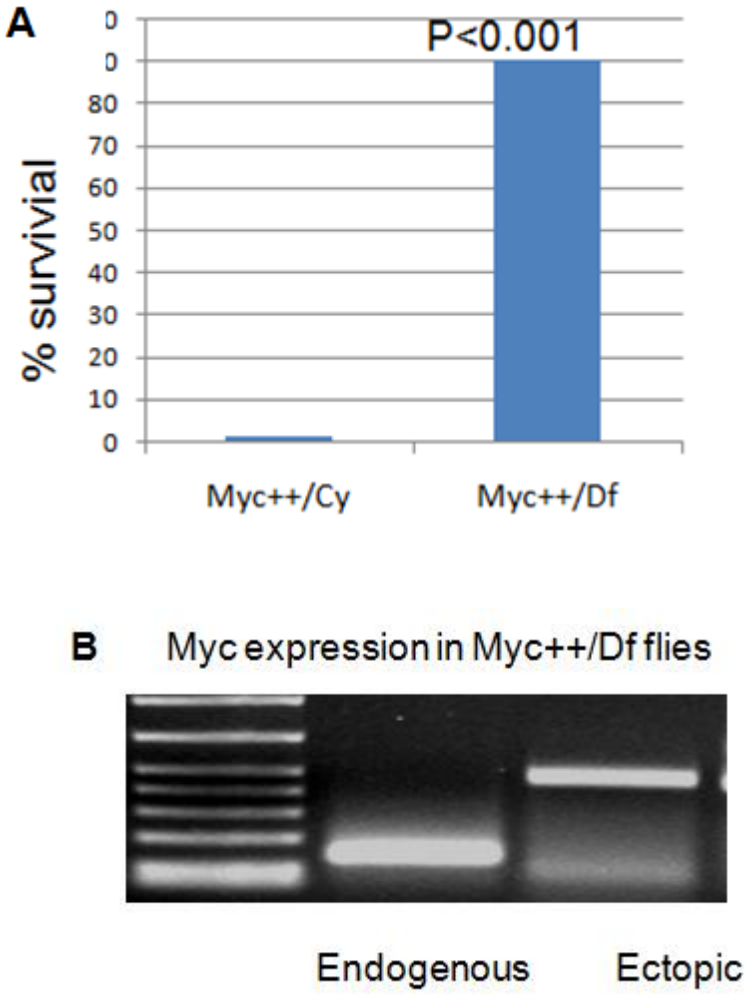


Figure 14: Df(36E6-37B1) lacking the let-7 gene rescues Myc autorepression phenotype: A) Percentage of surviving progeny from the cross. 0 embryos carrying the Cy chromosome survived whereas 90% of embryos with the Df chromosome survived to adulthood (n=30). Student t-test shows $p<0.001$. B) RT PCR showing expression of endogenous and ectopic Myc in surviving flies with the Df chromosome.

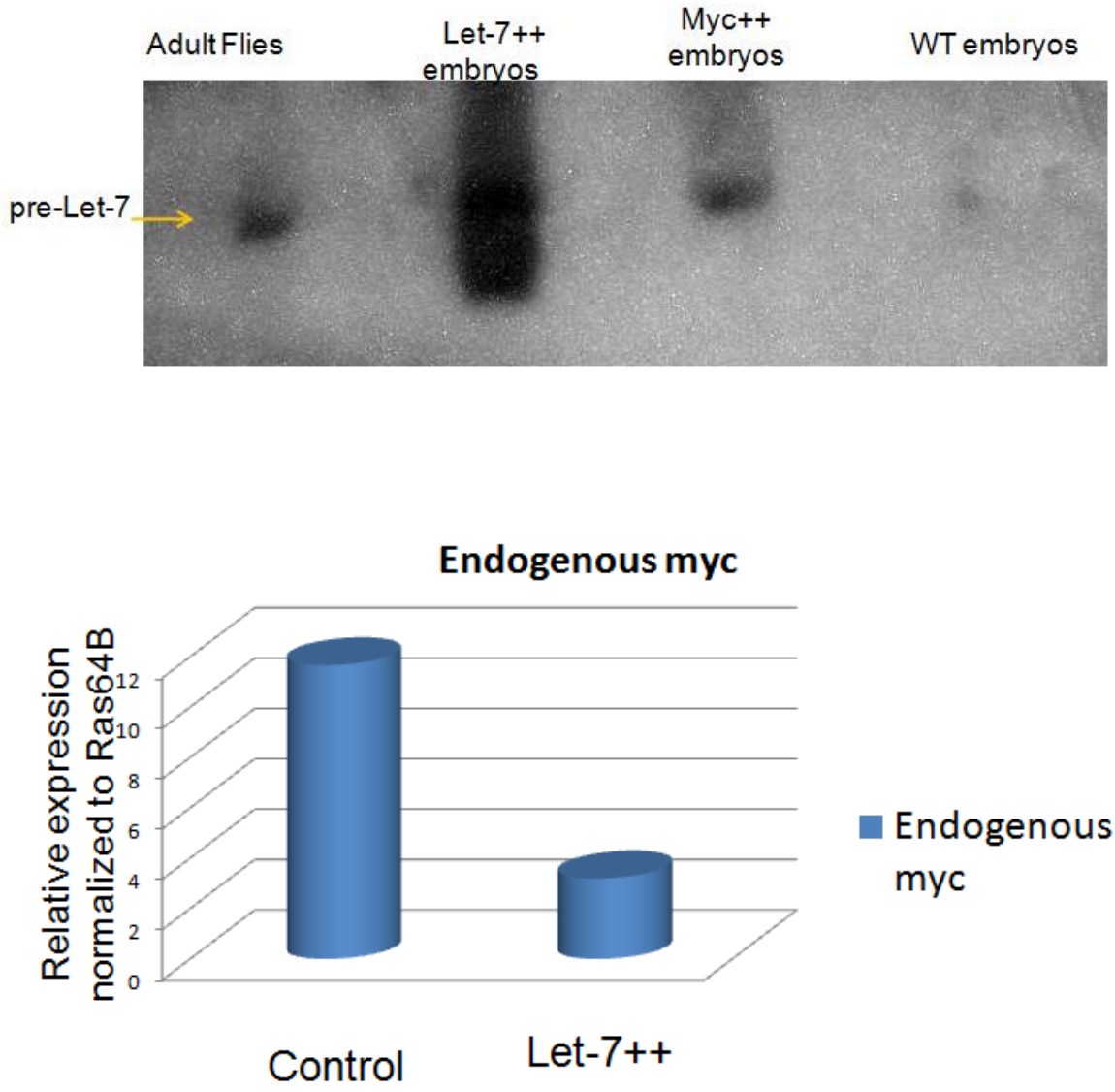


Figure 15: Let-7 expression in embryos undergoing autorepression: A) Northern Blot analysis for small RNA showing let-7 miRNA expression in positive controls (adult flies, let-7 overexpressing embryos), wild type embryos (negative control) and embryos with ectopic Myc (Myc++). B) q-RT PCR analysis showing relative quantification of endogenous Myc expression in wild-type (control) and let-7 overexpressing embryos (let-7++). The expression of Myc was normalized to Ras 64B.

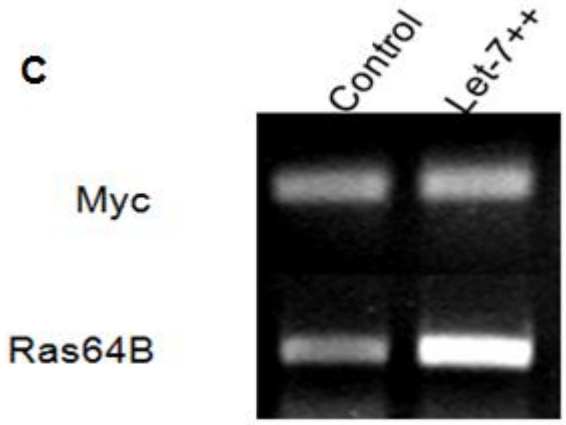


Figure 15C: Ras64B is upregulated in let-7++ embryos: RT PCR analysis showing Ras64B, used as a normalizing gene in the Qrt-PCR experiment in B, is upregulated in Let-7++ embryos whereas Myc levels do not change.

The expression of Ras64B did not change in any of our previous experiments. However, gel electrophoresis analysis following conventional RT-PCR showed that the levels of Ras64B were elevated in embryos overexpressing let-7 (Figure 15C). We repeated this experiment numerous times by quantifying and normalizing the total RNA content in each sample and found that Ras64B was significantly higher in let-7++ embryos compared to wild type. These data nullify the q-PCR results we obtained earlier since the Myc levels were normalized to Ras64B. The Myc levels do not change in let-7++ embryos compared to wild type embryos Therefore, we found that *Drosohpila* let-7 miRNA, unlike its mammalian homolog, does not repress dMyc.

4.4 Discussion:

Regulation of Myc is critical to ensure normal growth and development. One way to achieve this precise regulation is by employing miRNAs that ensure the fine-tuning of Myc levels based on the developmental context. MiRNAs are a class of genes that help regulate protein concentration of a gene product in the cell by binding to and preventing the translation of its mRNA. Let-7 is an evolutionarily conserved gene family known to initiate differentiation, metamorphosis and inhibit proliferation(Johnson, Esquela-Kerscher et al. 2007; Caygill and Johnston 2008; Sokol, Xu et al. 2008). In humans, the let-7 family consists of ten different isoforms of which let-7a is known to inhibit Myc translation. Overexpression of Myc leads to direct transactivation of Lin28B which inhibits the maturation of let-7a miRNA. This positive feedback loop as depicted in the figure earlier demonstrates a mechanism by which Myc levels can be controlled during cellular growth. Unlike mammals, *Drosophila* has a single let-7 gene whose onset of expression coincides with the decline of dMyc expression during development. Also, let-7 is required for differentiation and metamorphosis, both of which are inhibited by Myc. Therefore, we thought it is logical tohypothesize that a similar mechanism of Myc regulation could occur during *Drosophila* embryogenesis and development. However, our data suggests that let-7 miRNA does not repress dMyc regardless of the developmental context. The decline in Myc expression during late L3 stages and coincidentally the onset of let-7 expression could be mutually exclusive phenomena regulated by different mechanisms.

One interesting observation that can be made from our results is the induction of let-7 in the ectopic Myc expressing embryos. These embryos undergo autorepression and

consequently have very low levels of endogenous Myc. It is intriguing to note that let-7 is being induced in these embryos since let-7 is only expressed after the L2 stage. One possible explanation for this premature expression could be due to the dramatic decrease in endogenous Myc. Contrary to our initial hypothesis that the induction of let-7 in the autorepression embryos is due to the ectopic overexpression of Myc, we postulate that let-7 induction is a consequence of Myc autorepression and not a cause. The decrease in endogenous Myc causes a systemic trigger in the organism to stop proliferation and initiate differentiation and as a consequence, leads to the induction of let-7 miRNA.

Another interesting observation is the phenotypic rescue obtained using the Df(36E6-37B1) deficiency flies. The cytological locus 36E6-37B1 is a 600MBregion which encompasses 30 other annotated genes. It is possible that any one of these genes, other than let-7, could be involved in the Myc autorepression pathway. Performing an RNAi screen for individual genes could help narrow down potential candidate genes involved in Myc regulation. Alternatively, two or more of these genes could act coordinately in a direct or indirect manner to regulate Myc expression. In any case, we have identified a list of 30 genes that are required for regulation of Myc levels.

We hypothesize that there could be many other factors that are required for the regulation of Myc levels during embryogenesis. One way to test this hypothesis is by performing a genome wide genetic screen using the *Drosophila* deficiency kit which has about 400 fly lines with overlapping deficiencies spanning the entire genome.

CHAPTER 5: MYC LOCALIZES TO HISTONE LOCUS BODIES

5.1 Myc rarely localizes to nucleolus

During our preliminary antibody staining experiments to observe Myc expression in embryos, we noticed distinct Myc spots in certain tissues (Figure 16). We were curious about the identity of these puncta. Myc protein binds to the ribosomal DNA and stimulates the transcription of rRNA genes by RNA polymerase I in mammalian cells (Arabi, Wu et al. 2005; Grandori, Gomez-Roman et al. 2005). Unlike its mammalian homolog, dMyc is not found to be present at the rRNA genes (Grewal, Li et al. 2005), however high Myc levels are correlated with the increased size of nucleolus (Marinho, Casares et al. 2011). Nonetheless we began investigating the identity of these spots by double staining different tissues with antibodies against Myc and Fibrillarin, a nucleolar marker (Figure 17).

Myc was largely excluded from the nucleolus in all the tissues we tested. In the nurse cells of the ovaries, we observed many Myc spots in the nuclei but these rarely overlapped with fibrillarin, suggesting that Myc is not present in nucleolus (Figure 17B). In the follicle cell nuclei, we failed to observe any distinct Myc spots, although we did observe general Myc staining. Similarly Myc was largely excluded from the nucleolus in the salivary gland nuclei. However, in embryos, we did observe Myc overlap with Fibrillarin in a few cells (Figure 17 A), although in most cells, the spots of Myc and Fibrillarin were clearly distinct.

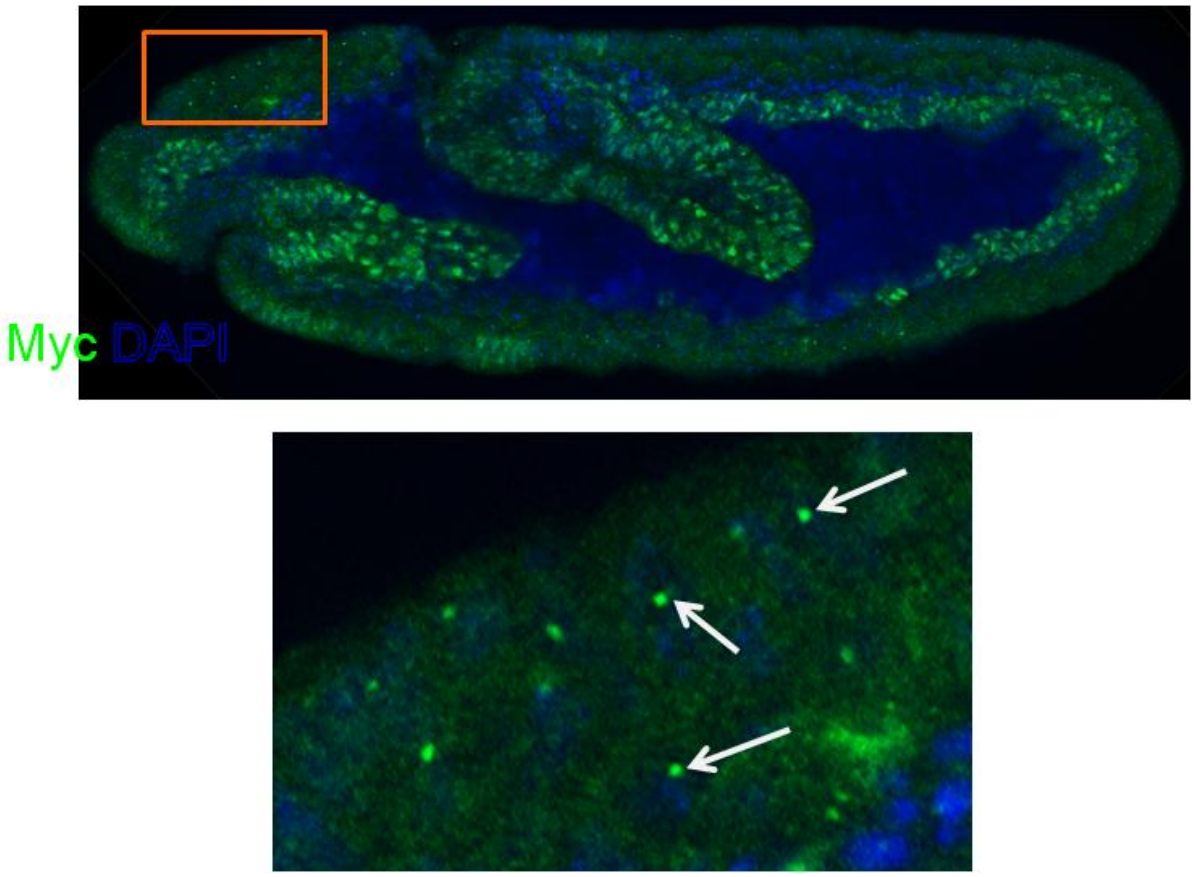


Figure 16: Myc protein forms distinct puncta in certain tissues. Stage 10/11 embryoimmunostained for Myc (green) and Dapi (blue). Bottom panel is a zoomed in picture of the orange box in the head region of the embryo. The arrows point at the Myc spots in the cells of the head region.

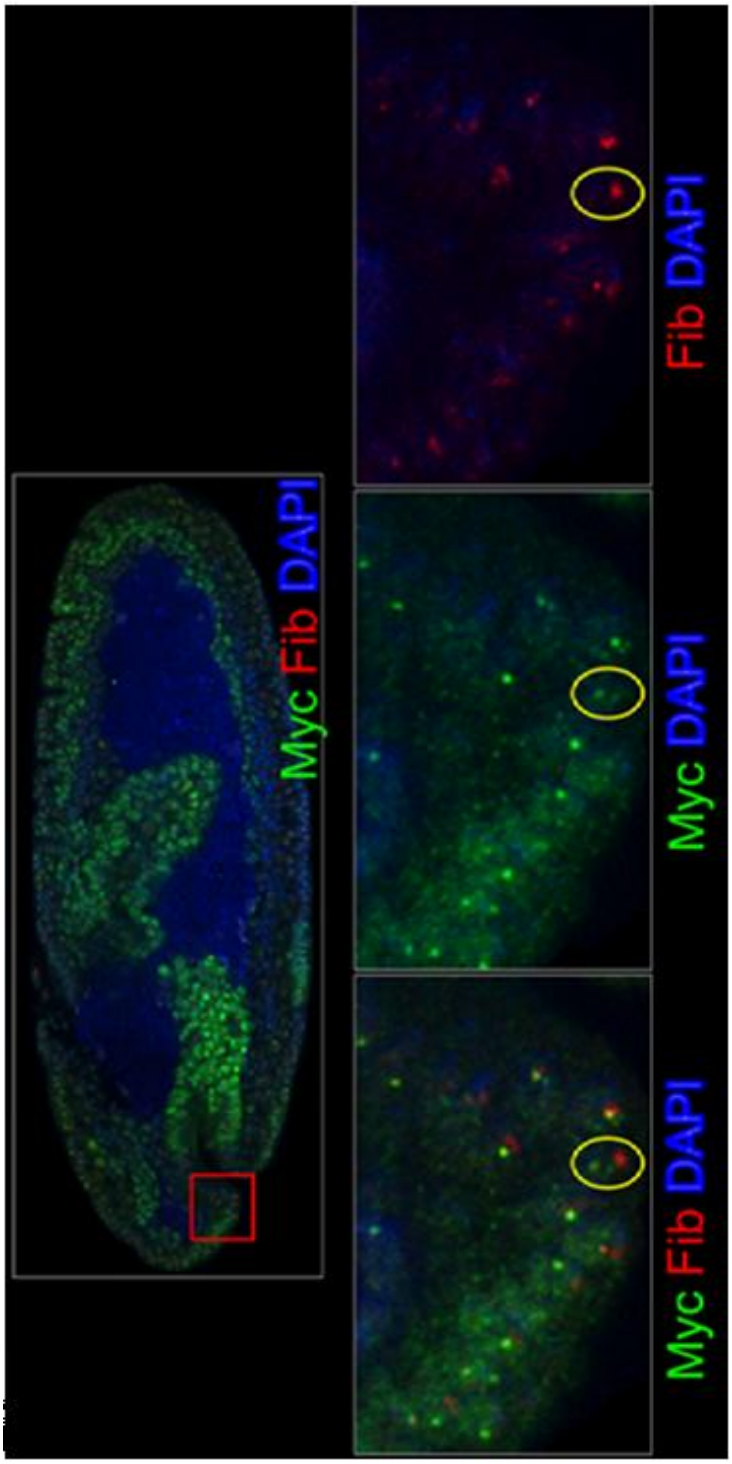


Figure 17A: Myc rarely localize to nucleolus: A stage 10 embryo labeled with Myc (green), fibrillarin (red) and DAPI (blue) showing minimal overlap of Myc and fibrillarin (bottom three panels, note the cell within the yellow circles with a bright fibrillarin domain that lacks Myc).

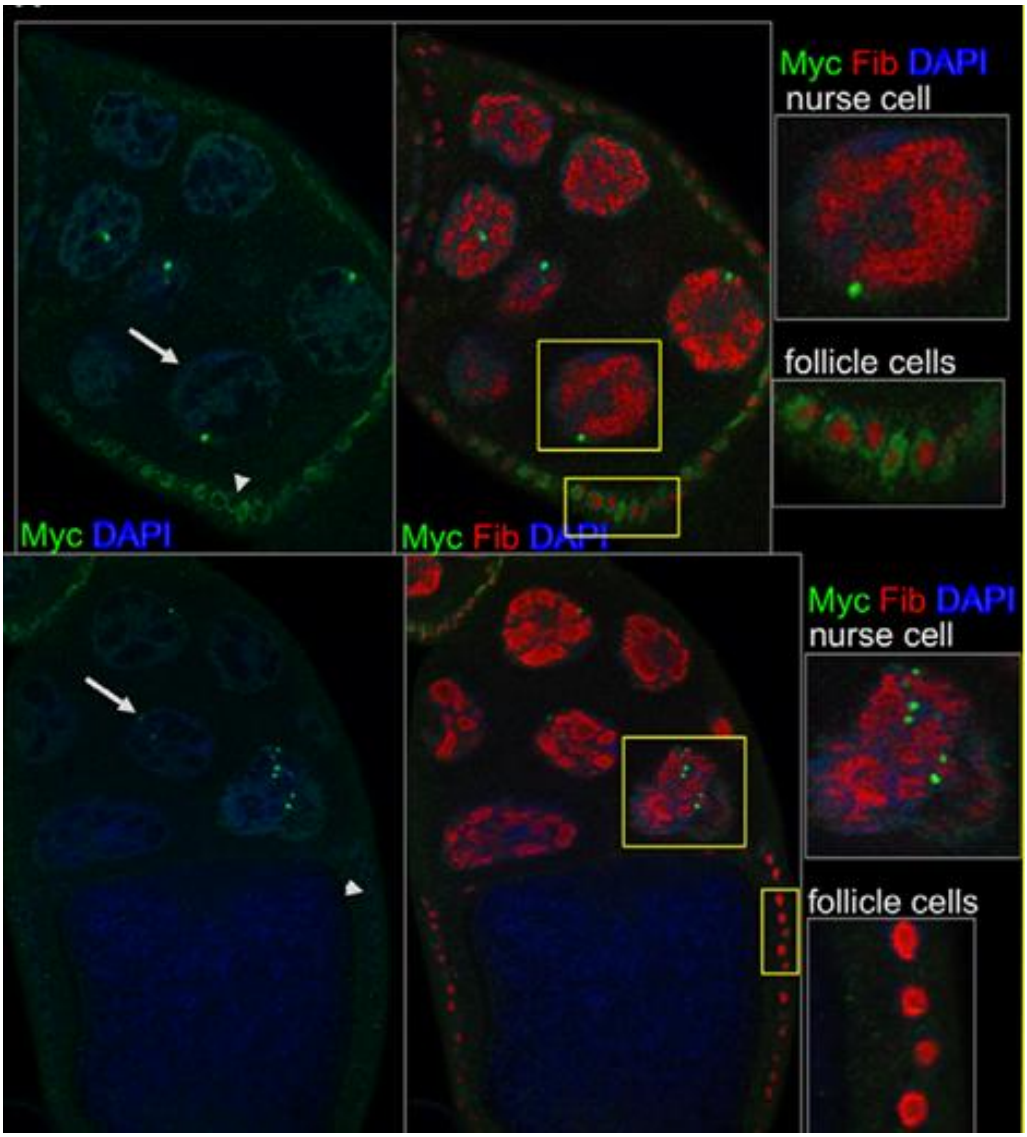


Figure 17B: Myc rarely localizes to nucleolus: Myc (green), fibrillarin (red) and DAPI label stage 8 (top) and 10 (lower) egg chambers. Arrows label a nurse cell, and arrowheads label a follicle cell.

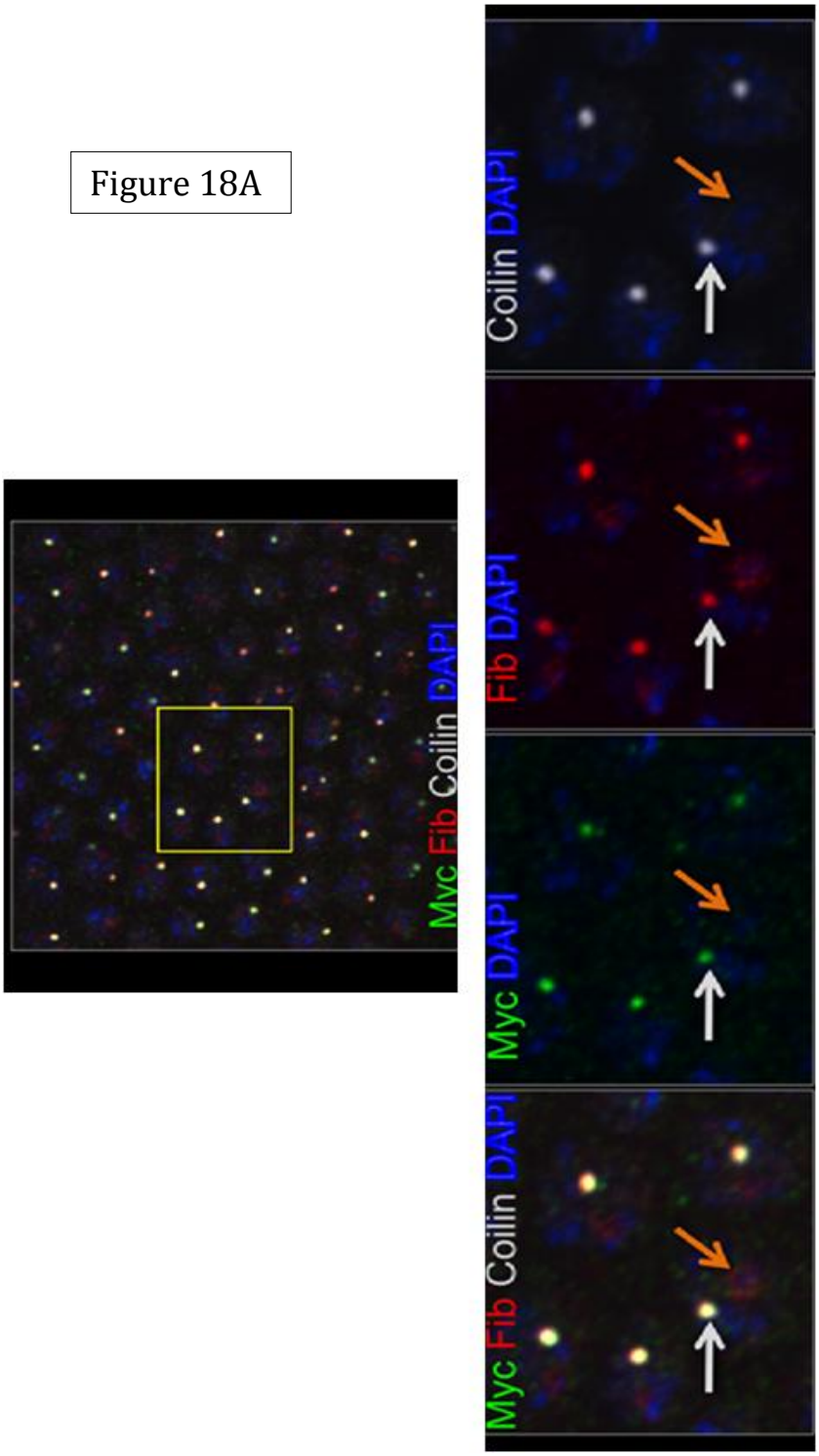
Although Fibrillarin is a strong nucleolar marker, it is also known to be present in sub-nuclear organelles called Cajal Bodies (Liu, Murphy et al. 2006). The overlap observed with Myc and Fibrillain in embryos and to a certain extent in salivary glands coincided with Cajal Bodies and not the nucleolus (see below).

5.2 Myc co-localizes with Coilin

Cajal Bodies (CB) are dynamic sub-nuclear organelles that are involved in the assembly and processing of small nuclear ribonucleoproteins (snRNPs) before they are exported to chromosomes for splicing (Nizami, Deryusheva et al. 2010). They are transient and dynamic bodies frequently occurring adjacent to the nucleolus. They are identified by the presence of a signature protein called coilin. Coilin is required for the nucleation and integrity of the CBs. In *Drosophila* and *Arabidopsis*, coilin null tissues lack CBs. Coilin knockout mice have serious fecundity and fertility defects and are semi-lethal(Liu, Wu et al. 2009; Walker, Tian et al. 2009). Cultured cells from coilin-knockout mice lack functional CBs and show the presence of "residual" bodies containing some of the components of CBs (Tucker, Berciano et al. 2001). Since we saw a minimal overlap of Myc with fibrillarin, we hypothesized that Myc localizes to the CBs. We double stained tissues with antibodies against Myc and coilin.

In nurse cells of ovaries, Myc and coilin localized to the same bodies. Myc spots almost always contained coilin (49 of 50 Myc bodies contained coilin), although less than half of Cajal Bodies contained Myc (21 of 57 CBs contained Myc). In follicle cells of the ovary, we did not see any Myc spots. In salivary gland nuclei, Myc spots colocalized with most of the large coilin bodies (22 of 27 coilin bodies).

Figure 18A


Myc Fib Coilin DAPI
Coilin DAPI
Fib DAPI
Myc DAPI
Myc Fib Coilin DAPI

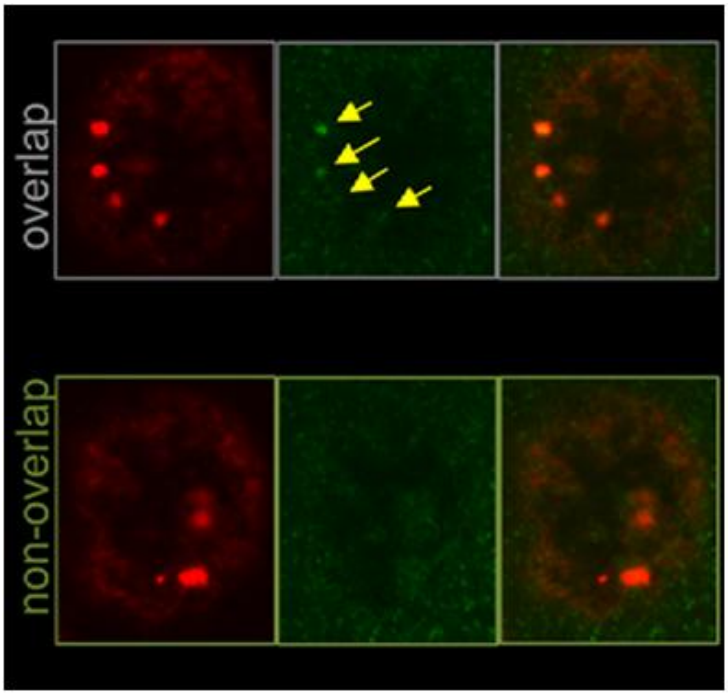


Figure 18B

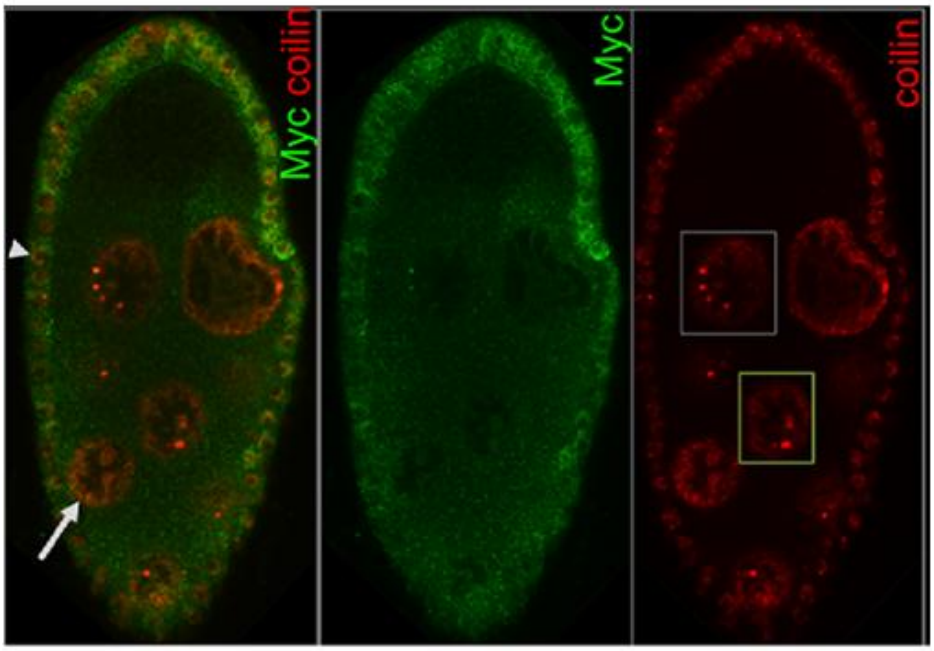


Figure 18: Myc co-localizes with coilin: A) A stage 6 embryo labeled with Myc (green), fibrillarin (red), coilin (white) and DAPI, showing that locations where Myc and fibrillarin overlap are punctacontainingcoilin (shown by the white arrow in the higher magnification boxes below). Myc does not overlap with fibrillarin in the nucleolus (shown by the orange arrow). B)A stage 9 egg chamber labeled with Myc (green) and coilin (orange). The light gray arrow points to a nurse cell, and arrowhead points to a follicle cell. A nurse cell lacking overlap of Myc with coilin is shown (nurse cell in the green boxes, panels below and left), and a nurse cell with Myc and coilin containing puncta is also shown (nurse cell in the light gray boxes, panels below and right).

In embryos, Myc puncta start to appear post-cellularization, and following their occurance in the cellular blastoderm, Myc puncta always overlapped with coilin (75 Myc positive CBs, n=78). Myc puncta were also found to overlap coilin in the cells of postblastoderm mitotic domains (39 Myc positive CBs, n=41 CB). During later stages, Myc appeared diffuse in most cells of endoderm and we could not discern any Myc spots. However, in the cells of ectoderm and the head region, Myc puncta were seen to overlap coilin (32 Myc positive CBs, n=37CB).These data indicate that Myc protein appears in a coilin containing body; it could be either a Cajal Body or a Histone Locus Body (HLB) of which coilin is also a component. Next we tested whether Myc and coilin are components of HLBs or CBs.

5.3 Myc and Coilin localization occurs mainly in HLBs

The HLB is a dynamic sub-nuclear organelle similar to the Cajal body but is always associated with the histone gene loci and hence the name, Histone Locus Body. It is marked by the presence of U7snRNP which is required for the processing of histone Mrna(Godfrey, Kupsco et al. 2006; Nizami, Deryusheva et al. 2010). CBs and HLBs can reside next to each other and both can contain coilin. Since we found Myc puncta positive for coilin, we investigated if these pucta were HLBs rather than CBs. We obtained transgenic flies expressing an HLB marker, Lsm11-EYFP (gift from Dr. Gall) under the control of Gal4. Lsm11 is the protein component of U7snRNP and is specific for HLBs (Godfrey, White et al. 2009). We induced the expression of Lsm11-EYFP and triple stained embryos with anti-gfp, anti-Myc and anti-coilin. We also stained wild type larvae and ovaries with anti-Lsm11 (gift from Dr. Gall), anti-Myc and anti-coilin.

A

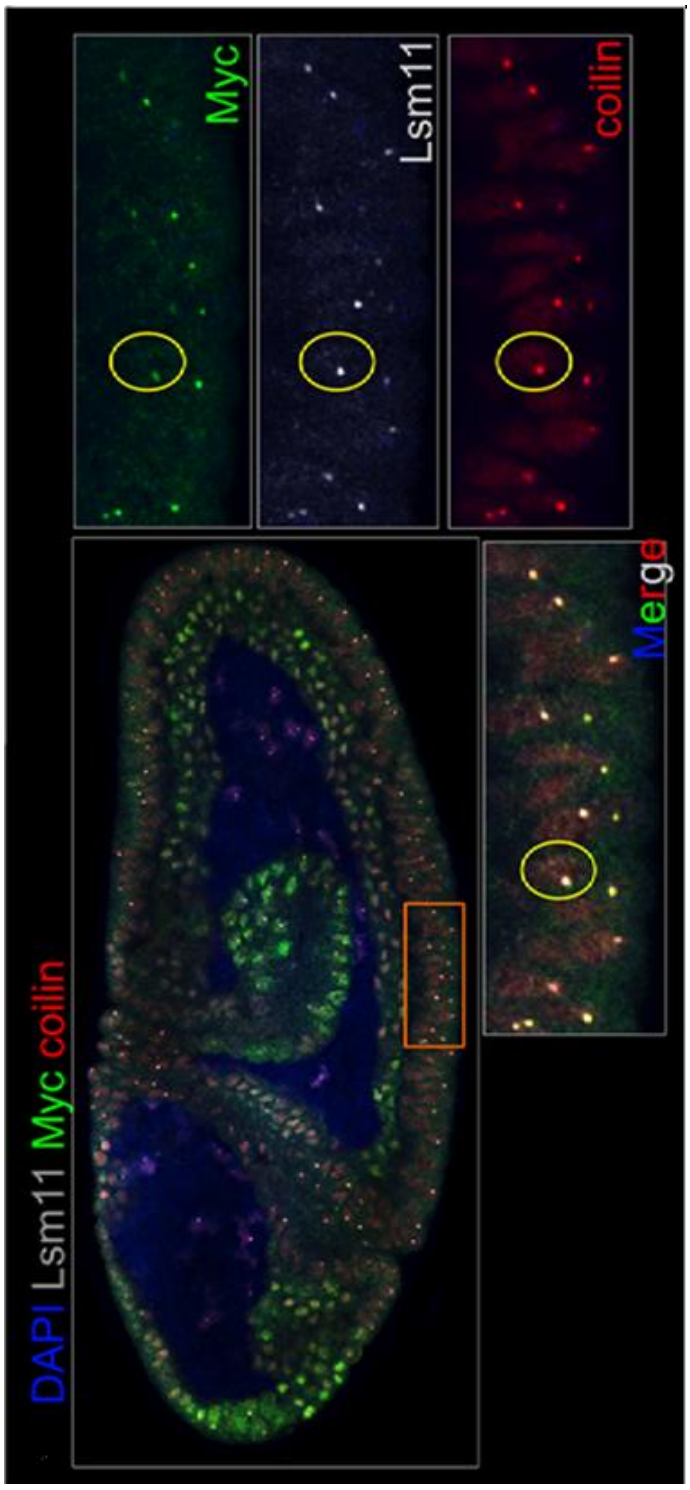


Figure 19A: Myc and coilin overlap occurs only in HLBs: A wild type, stage 8-9 embryo labeled with Myc (green), coilin (red), Lsm11-EYFP (white) and DAPI (blue) showing that Myc, coilin and Lsm11 co-localize to the majority of the bodies occurring in these embryos (lower panels show the cells in the orange box).

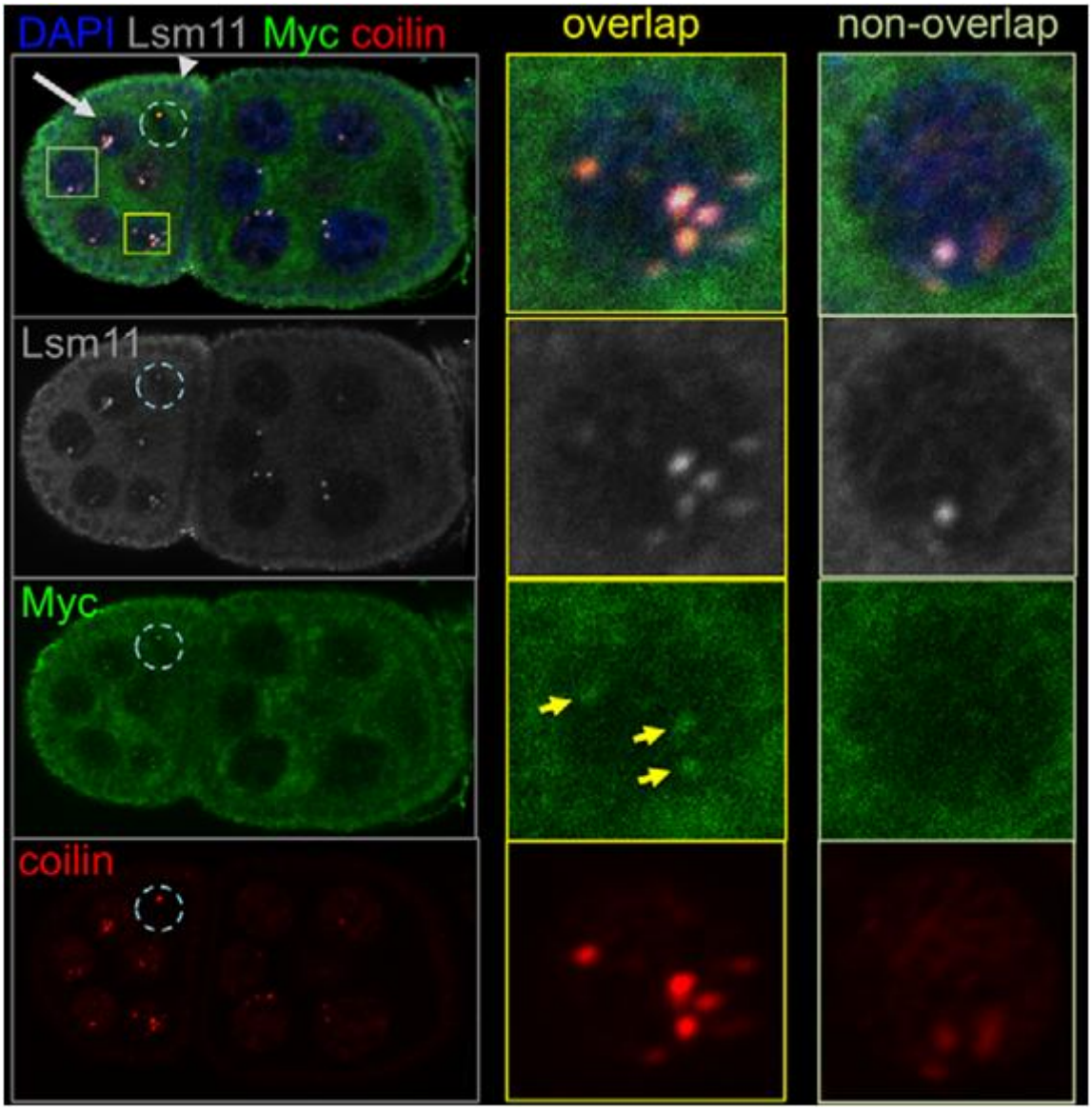


Figure 19B: Myc and coilin overlap occurs only in HLBs: Two egg chambers, ,stages 5-6, labeled as indicated and showing that Myc, coilin and Lsm11 co-localize in nurse cells and the oocyte nucleus (the arrow labels a nurse cell and arrowhead labels a follicle cell; the oocyte nucleus is within the light blue dashed circle). The panels on the right show a nurse cell with Myc, coilin and Lsm11 in the same bodies; a nurse cell lacking Myc in a coilin-Lsm11 body; the oocyte with all three co-localized (right panels).

In nurse cell nuclei, Myc overlapped with coilin and Lsm11. Immunostaining salivary glands with anit-Myc and anti-GFP or anti-Lsm11 showed Myc localization to HLBs. 81% of Lsm11 and coilin positive HLBs contained Myc (n=27). Myc was rarely seen in bodies positive for coilin but lacking Lsm11. Only 20% of non-Lsm11 CBs contained Myc. These data suggest that Myc is only present in HLBs. In embryos, Myc puncta were almost always positive for Lsm11 and coilin suggesting that majority of embryonic bodies are HLBs (in 221 Myc punta 208 also contained Lsm11 and coilin).

5.4 Myc localizes to HLBs only during replication:

Because many different cell types showed Myc in the HLB, however not uniformly within an egg chamber or embryo, we investigated whether Myc localization to HLBs is cell cycle dependent. We stained embryos, larvae and ovaries with the monoclonal antibody MPM-2, which cross-reacts with phosphoepitopes of mitotic cells in many organisms. In Drosophila embryos, MPM-2 recognizes the 59 hosphor-epitope of a protein present in HLBs, but only in cells with active Cyclin E/Cdk2. We first examined all coilin-containing bodies, which may be CBs or HLBs, by staining embryos with MPM-2, anti-coilin and anti-Myc antibodies. Myc appeared in 100% of the coilin and MPM-2 positive bodies, n= 30. Myc appeared in just 10% of CBs or HLBs lacking MPM-2, n =30. We found similar results in ovaries, that Myc overlapped MPM-2 in all nurse cells containing puncta positive for MPM-2 (n = 30 nurse cells), and overlap with coilin was limited to MPM-2 positive bodies (all of which are HLBs later in oogenesis.

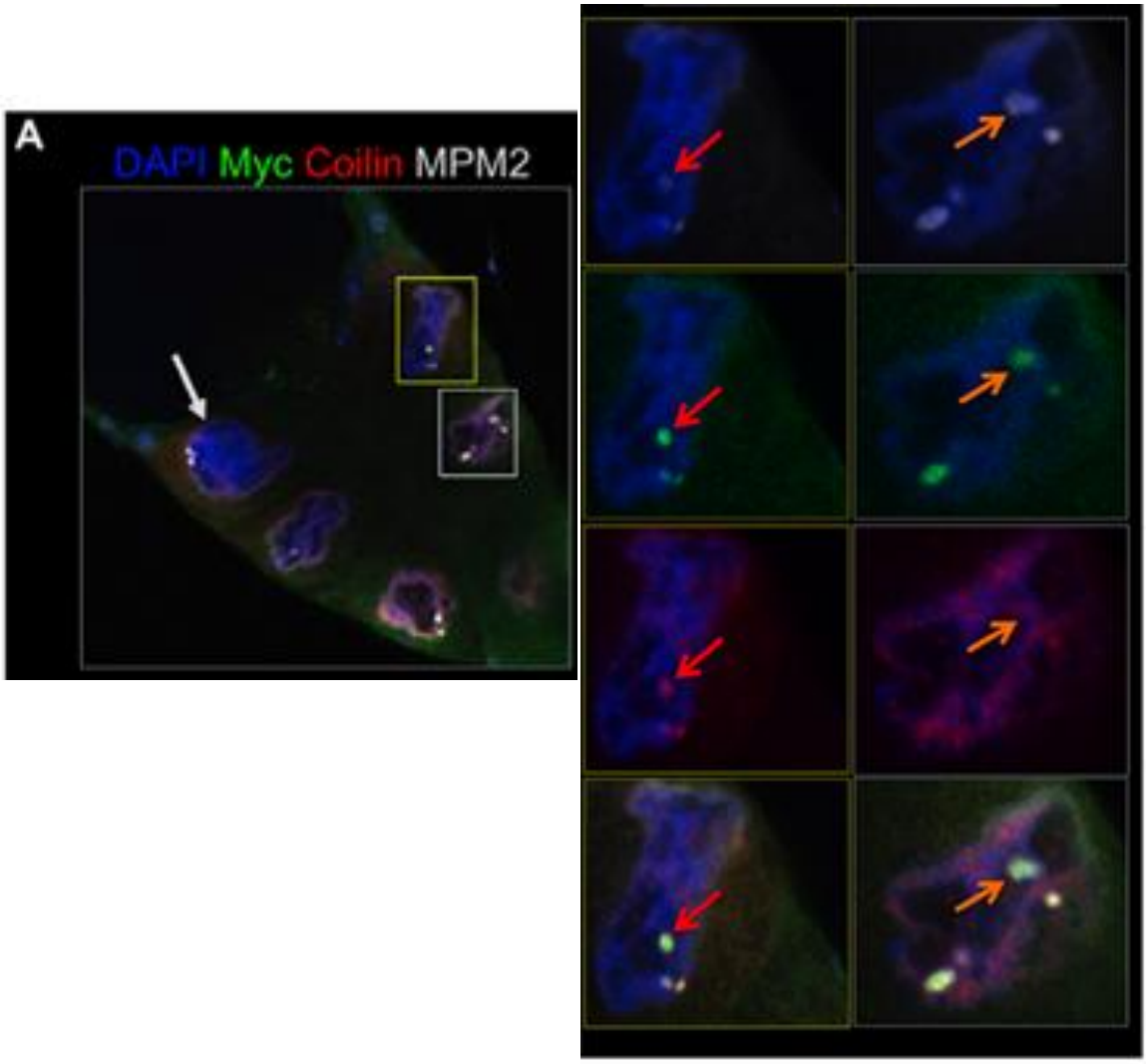


Figure 20: Myc localizes to HLBs in replicating cells. A) A stage 10 egg chamber is shown, labeled with Myc (green), coilin (red), MPM-2 (white) and DAPI, and a nurse cell is labeled with the light gray arrow. Myc, coilin and MPM-2 overlap in the HLB of the nurse cell in the yellow boxes, and MPM-2 and Myc overlap although coilin staining is weak in the HLB of the nurse cell in the gray boxes.

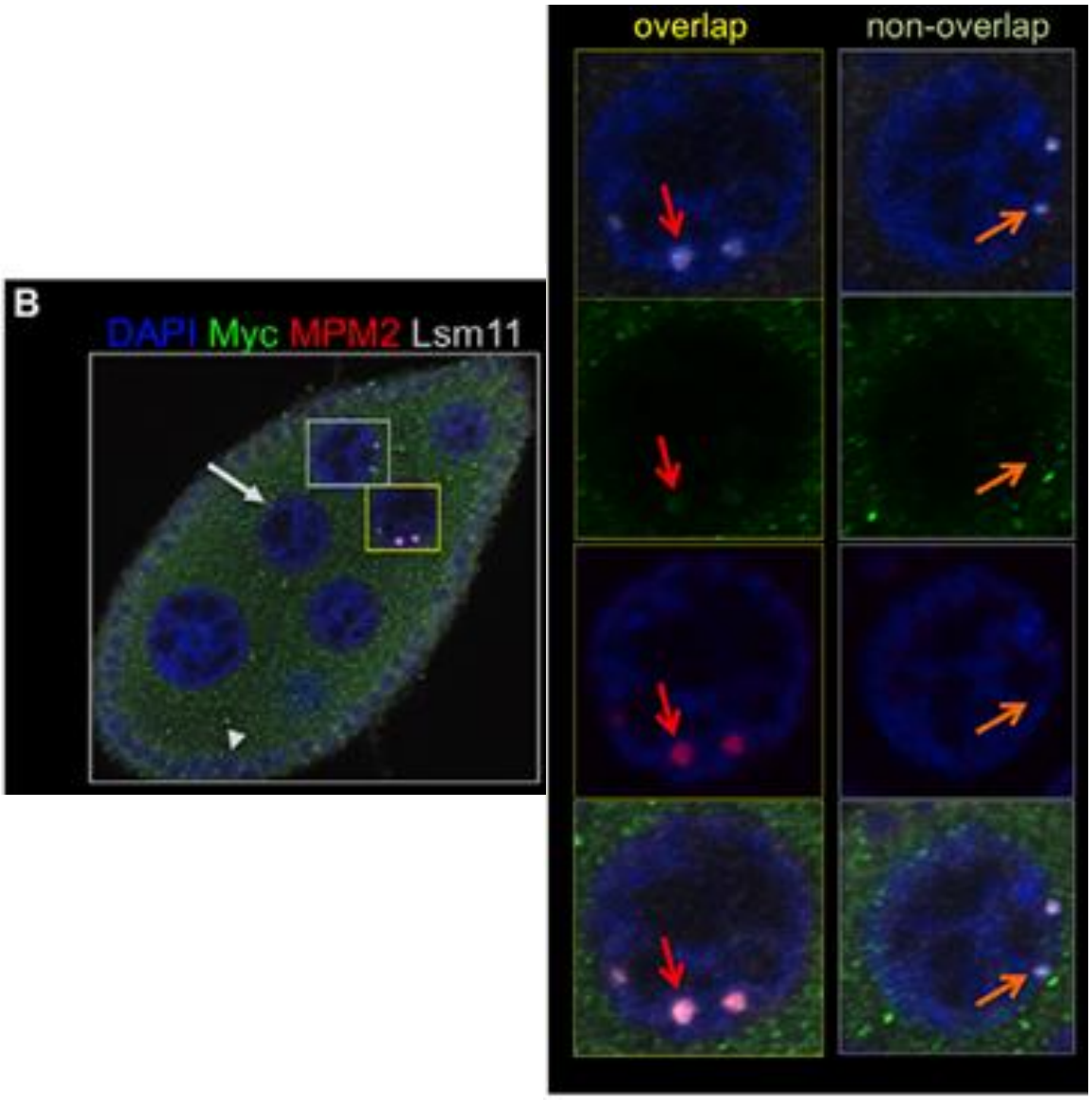


Figure 20B) A stage 8 egg chamber, labeled with Myc (green), MPM-2 (red), Lsm-11 (white) and DAPI shows that Myc puncta are the HLBs of replicating nurse cells. A nurse cell is shown with MPM-2 positive HLBs (cell in yellow boxes and magnified below), and Myc appears in those HLBs. A nurse cell is shown with Lsm11, non-MPM-2 staining HLBs, and Myc is absent (cell in light gray boxes, magnified below in the right-most panels).

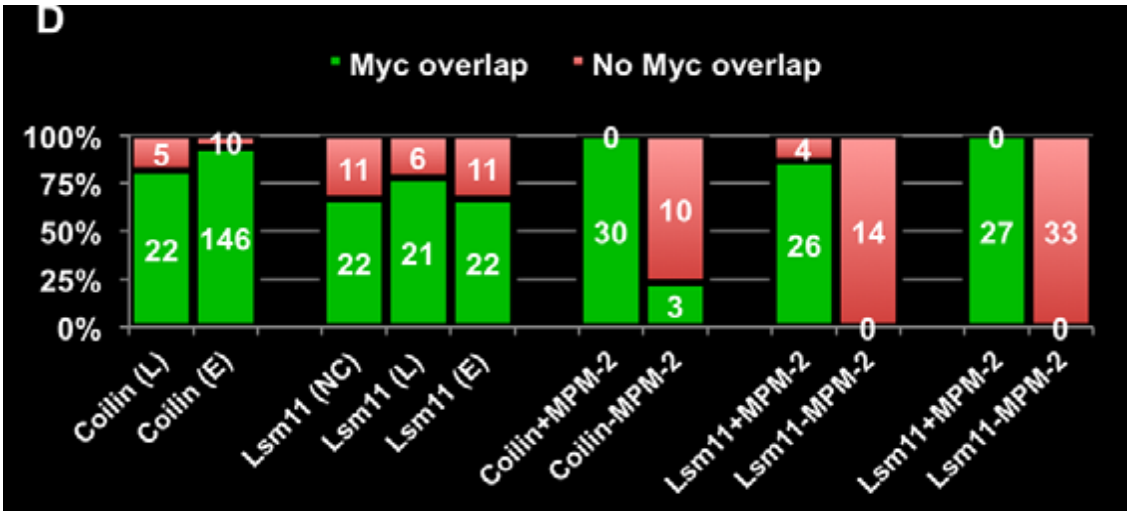


Figure 21: Quantification of Myc foci: A chart showing the numbers reported in the text of Myc-overlapping (green bars) and non-Myc-overlapping puncta containing coilin (C), Lsm11 (L), and MPM-2 in embryos (E and the third and fifth sets of bars), larvae (L) and nurse cells (NC and also the fourth set of bars).

To examine bodies identifiable as HLBs in cells undergoing replication, we stained ovaries and embryos with MPM-2, anti- Lsm11 (or anti-GFP) and anti-Myc. Myc and Lsm11 co-localized only in the presence of the MPM-2 epitope. HLBs containing bothLsm11 and MPM-2 were positive for Myc. HLBs lacking the MPM-2 epitope also lacked Myc. In embryos, replicating cells were identified with MPM-2 positive HLBs, and those bodies always included Myc (n= 27). Myc was never observed in MPM-2 negative HLBs, n= 33.

Discussion:

In our study we find that Myc localizes to HLBs. What is the function of Myc in HLB? Since Myc is a transcription factor it is logical to assume that it is required for histone gene transcription. However, with the discovery of Mxc in HLB, does it mean that Myc assumes a secondary role in transcription or is Myc involved in processing? Could it be possible that there are multiple tissue specific transcription factors required for histone gene transcription? White and others have shown that Mxc is required for HLB assembly and function in a cell-autonomous manner. So what then is the role of Myc in HLB? Is Myc required for HLB assembly? We conducted some preliminary experiments to address this question. From the initial results, it seems that Myc is not required for HLB formation. We tried transient knockdown of Myc in larval salivary glands and embryos by crossing flies with a UAS-MycRNAi construct to a hsGal4 driver; however, the Myc knockdown model was found to be highly inconsistent and hence we are not certain of the conclusion. A better system would be to use S2 cells and induce transient Myc RNAi and assess HLB formation and also histone gene transcription. This system can also be used to assay for histone mRNA processing as well. This would give us a better picture if Myc is involved in transcription or processing or both.

Another possible avenue of investigation would be the mode of Myc localization to HLB during S phase. Does dMyc get phosphorylated by a G1/S specific kinase to facilitate its localization to HLB? Although, c-Myc is known to be phosphorylated at S62 by CycE/Cdk2, this residue is not conserved in dMyc. It is possible that there could be other sites that are phosphorylated by Cdk2 during G1/S transition and hence it causes localization to HLB.

CHAPTER 6: SIGNIFICANCE AND FUTURE DIRECTION

In this chapter I will attempt to give the reader a measure of the overall significance of both my projects and a general perspective of how this study could be helpful in advancing the prevailing understanding of these fields. Both the projects probe the basic functions of Myc as a transcription factor. While the HLB project merits further investigation based on the sheer novelty of the findings, the autorepression project bears great potential in identification of factors involved in regulation of Myc expression in normal development and tumorigenesis. A general concern is the translation potential of these findings in the *Drosophila* model to the mammalian model. Functional studies in *Drosophila* have been shown to be able to translate into human model because of the evolutionary conservation of the biological activity of important genes such as Myc. Hence, my work here could be used as a template for further investigation both in *Drosophila* as well as human cell culture models.

6.1 Myc Autorepression: Significance:

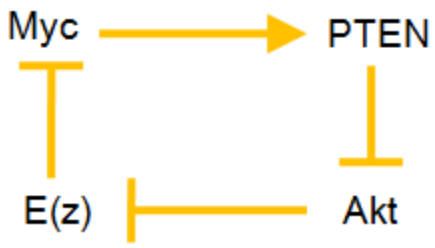
In this study I found that Myc autorepression occurs in the cells expressing an ectopic Myc gene resulting in the down regulation of the endogenous dmyc locus. This phenomenon requires Pc; since Pc represses Su(z)2, knockdown of Pc results in upregulation of Su(z)2 and the abundance of Su(z)2 leads to disruption of Myc autorepression via an unknown mechanism. In tumorigenic mammalian cells, Myc

autorepression is lost, leading to increased Myc protein levels that can cause increased proliferation, transformation and immortalization of cells. Therefore, this project is significant because of the identification of a factor that can cause disruption of Myc autorepression and increase Myc expression. However, the precise mechanism or the factors that contribute to the induction and loss of Myc autorepression in tumorigenic cells is still unknown.

One hypothesis is that because of ectopic Myc expression, the Myc protein levels reach a critical concentration that leads to the activation of a gene(s) or pathway that is a

direct repressor of the endogenous Myc locus. This could be achieved either at transcriptional level or post-transciptional level, depending on the nature of the gene activated. High levels of Myc can induce a miRNA that has a seed sequence complementary to endogenous Myc. Therefore, activation of such a miRNA could lead to downregulation of dmyc transcripts and protein. One such miRNA has been identified in *Drosophila*. The miRNA-308 is a direct target of Myc and is only induced at high Myc protein levels (Kaveh Daneshvar, personal communication). This miRNA has been shown to bind to endogenous Myc transcripts and inhibit translation to a great extent. However, overexpression of the miRNA alone in embryos does not phenocopy Myc autorepression, suggesting that repression mechanism by the miRNA is very modest and is not sufficient to create a Myc null phenotype.

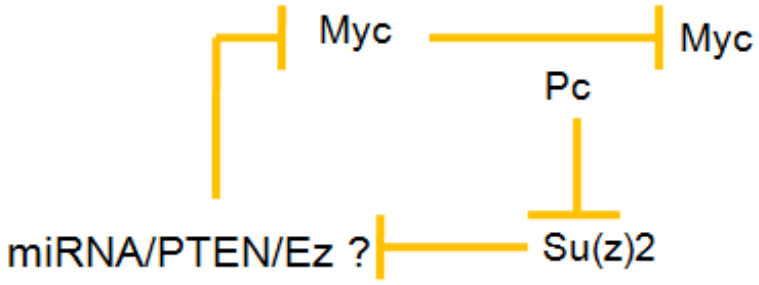
On the other hand, this could be possible if high Myc levels activate a gene or a pathway that can lead to transcriptional silencing of the endogenous dmyc locus. One such pathway has recently been identified that could shed some light on the autorepression mechanism (Kaur, M and Cole, MD 2012). The authors noticed that Myc



repression targets, including the endogenous Myc promoter, have elevated H3K27Me3 mark. Upon investigation, they found that this was due to increased stability of the histone methyltransferase Ezh2. The stability of Ezh2 is regulated by Akt by phosphorylation of a conserved serine 21 residue. Akt is inhibited by phosphorylation at serine 473 by PTEN. The authors have shown that upon ectopic Myc expression, Myc transactivates PTEN, a bona fide Myc activation target. PTEN causes deactivation of the Akt kinase by phosphorylating Akt at S473, thereby leading to increased Ezh2 protein which methylates H3K27 at Myc repression targets including the myc promoter leading to the transcriptional downregulation of these genes.

To understand how Myc autorepression is lost in cells that have an ectopic myc gene or how the expression of Myc is maintained at low levels in a terminally differentiated cell, we have to identify the pathway(s) that control Myc expression. In the study mentioned above, the authors have shown that the PTEN/Akt pathway is activated ectopic Myc gene expression. In my study I have identified Su(z)2 as a component of an

unknown pathway that can prevent the repression of dmyc locus. Since Su(z)2 is a transcriptional repressor, it is logical to propose that Su(z)2 represses a repressor ("X") of



Myc. Su(z)2 could repress a direct transcriptional repressor of Myc or act more upstream of the pathway that has many intermediaries that have a cumulative effect of Myc repression. In that case, the "X" here could represent more than one gene. Based on the new evidence, a new hypothesis is that Su(z)2 represses PTEN or any component of that pathway resulting in depletion of Ez protein levels that cause H3K27 demethylation at dmyc locus. Our results show that in the presence of ectopic Su(z)2, Myc activation targets have low levels of H3K27 trimethylation. This could be due to a decrease in Ez activity which suggests that excess Su(z)2 inhibits the PTEN/Akt/Ez pathway. Alternatively, Su(z)2 could also repress miRNA-308 which regulates Myc post-transcriptionaly.

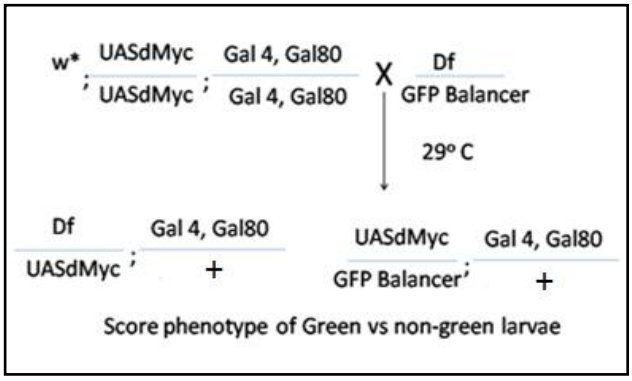
Su(z)2 has been shown to be a strong suppressor of growth and proliferation via the inhibition of the Jak-Stat pathway. However, in the context of Myc autorepression, I have found that Su(z)2 promotes growth via the depression of dmyc gene. These conflicting roles could be due to the difference in developmental context. The two mammalian homologs of Su(z)2, Mel-18 and Bmi-1, have opposite roles in tumorigenesis.

While Mel-18 is a tumor suppressor known to repress c-myc, Bmi-1 is a direct transactivation target of Myc known to cooperate in Myc induced tumorigenesis. However, neither of these genes has been shown to induce Myc expression either in a cancer model or c-myc autorepression model. Which of these two homologs is Su(z)2 most similar to in terms of its role in the regulation of Myc? To address this question I propose some future experiments that could help us understand the evolutionary function of Su(z)2 and its mammalian homologs in regulation of Myc.

6.2 Future direction:

To understand the role of Su(z)2 in Myc autorepression and tumorigenesis, it would be interesting to see if it can abrogate c-myc autorepression in mammalian cell culture model. If overexpression of Su(z)2 under the c-myc autorepression genetic background model leads to depression of the c-myc locus then that would suggest that this pathway is evolutionarily conserved. By overexpressing either Bmi-1 or Mel-18 under the same genetic background and analyzing depression of c-myc as a readout, we could infer if Su(z)2 acts as Mel-18 or Bmi-1in this context.

If the autorepression pathway in conserved then we could use our genetic model in flies because of the simplicity of the genome to identify factors involved in the



pathway. One way to identify new factors in a pathway is by performing a genetic screen to look for modifiers of the phenotype caused due to the genetic background of the model. I propose to conduct a genetic screen by making use of the deficiency kit available through the stock center.As shown in the figure we will cross flies carrying the ectopic myc gene to flies that are heterozygous for a deficiency (Df) on either the 2^{nd} or 3^{rd} chromosome. The deficiency is a deletion that can range from a few kilobases to a few megabases on one of the homologous chromosomes. These deletions are mapped to their precise cytological and sequence coordinates. The fly stock center at Bloomington maintains fly lines that have overlapping deletions that span entire chromosomes.In order to control the expression of the ectopic myc gene we will make use of the Gal4-Gal80 system. Upon shifting to 29^0C, the embryos exhibit mycautorepression phenotype that results in larval lethality due to the induction of the ectopic myc gene. We will cross these flies to the flies that are heterozygous for a deficiency (Df) and transfer them to 29^oC incubator. Flies with the Df also have a GFP balancer chromosome. As shown in the figure this cross will yield two categories of progeny;

1) carrying the deficiency (Df) chromosome along with a copy of ectopic myc ---(test group---rescue?) and

2) a copy of ectopic myc and GFP balancer chromosome. (control group—autorepression phenotype)

I propose to use all the deficiencies in the 2nd and 3rd chromosome available through the Bloomington stock center.

6.3 Myc in HLB: Significance:

In my study I have shown that Myc localizes to HLBs during replication or S phase. This is a novel finding with regard to Myc's biological activity. Although, we're not yet certain of Myc's role in HLBs during replication, a rational hypothesis is that it is involved in transcription of the replication dependent histone genes. Histone genes are transcribed and processed during S phase. During replication, a cell requires new histones to incorporate into the duplicated genome. Proper transcription and processing of histones is essential to maintain genomic integrity during cell division. This project is significant because of its novelty and its implication in maintenance of genetic integrity. However, many questions still remain unanswered with regard to the overall impact of this finding. Some of the important ones are concerning the role of Myc in histone transcription and processing. Is Myc required for histone transcription or processing or both? Does Myc directly activate histone transcription or is it required only to modify chromatin by recruiting other cofactors? How is Myc localization to HLB coordinated? Does Myc require phosphorylation by a kinase to facilitate its localization? c-Myc is known to be a Cdk2 substrate for phosphorylation. Does dMyc also get phosphorylated by Cdk2? Alternatively, it is also possible that Myc has no role in histone gene transcription or processing and that its localization to HLB is a means to sequester the nuclear Myc protein to HLB during replication and mitosis.

6.4 Myc in HLB: Future Direction:

To address some of the questions mentioned above I propose some follow up studies that would be helpful in understanding the importance of Myc in HLBs. First, to

understand the role of Myc in HLB, it is essential to analyze histone gene transcription in Myc mutants or Myc RNAi background. The histone locus is a cluster of repeats of a

Single unit of the 5KB *Drosophila* histone gene repeat

5KB region that contains the core histone genes. The H3/H4 gene pair is separated by a 300nt region, as in the H2A/H2B gene pair. The intergenic region between H3 and H4 contains information necessary for HLB formation and histone gene transcription of both H3/H4 and H2A/H2B gene pairs (Robert Duronio, personal communication). Therefore, the 300nt between H3 and H4 must contain sequences that are recongnized by specific transcription factors. It would be interesting to see if this region has an E-box. Myc binding to this region can be tested by performing a ChIP experiment. If Myc binds to this region, then it can be inferred that it does aid in histone transcription. Does Myc play a role in histone mRNA processing? A simple northern blot analysis for any of the histones can answer this question. Using probes for mature, processed histone H3 mRNA we can test this is wild type and Myc mutant background to check if histone processing is affected upon Myc knockdown. How is Myc localization to HLB during S phase achieved? Does Myc get phosphorylated by a kinase? To address this, we have to

conduct mutational analysis by expressing deletion mutants of Myc in cells and test for its localization to HLBs as readout. Once we identify a deletion mutant that fails in localization, we can narrow down the putative phosphorylation sites in that domain. By

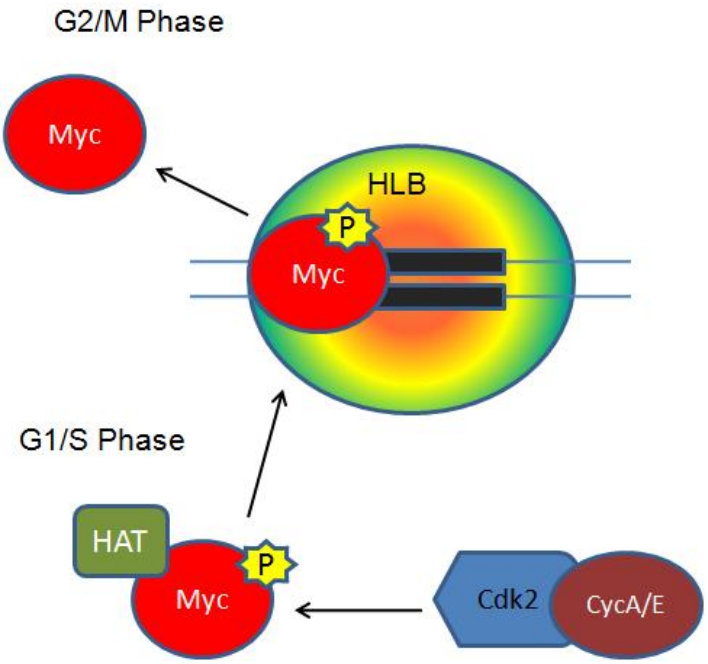


Figure 22: Mechanism of Myc localization to HLB: The cartoon depicts possible mechanism of Myc localization to HLB during S Phase.

mutating individual amino acid residues in that domain, we can test for the localization of full length Myc to HLBs. This can tells us definitively as to which amino acids get phosphorylated.

My findings in both these projects have far reaching implications with respect to Myc function in maintenance of genomic integrity and regulation of Myc induced growth and proliferation. With the discovery of Myc in HLB we have shown that Myc plays many important and diverse roles in a cell and hence it is a critical player in controlling vital cellular processes.

REFERENCES

Adhikary, S. and M. Eilers (2005). "Transcriptional regulation and transformation by Myc proteins." Nat Rev Mol Cell Biol**6**(8): 635-645.

Ali, J. Y. and W. Bender (2004). "Cross-regulation among the polycomb group genes in Drosophila melanogaster." Mol Cell Biol**24**(17): 7737-7747.

Amati, B., S. Dalton, et al. (1992). "Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max." Nature**359**(6394): 423-426.

Andrade, L. E., E. K. Chan, et al. (1991). "Human autoantibody to a novel protein of the nuclear coiled body: immunological characterization and cDNA cloning of p80-coilin." The Journal of Experimental Medicine**173**(6): 1407-1419.

Arabi, A., S. Wu, et al. (2005). "c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription." Nat Cell Biol**7**(3): 303-310.

Bellosta, P. and P. Gallant (2010). "Myc Function in Drosophila." Genes Cancer**1**(6): 542-546.

Bernstein, B. E., T. S. Mikkelsen, et al. (2006). "A bivalent chromatin structure marks key developmental genes in embryonic stem cells." Cell**125**(2): 315-326.

Beuchle, D., G. Struhl, et al. (2001). "Polycomb group proteins and heritable silencing of Drosophila Hox genes." Development**128**(6): 993-1004.

Blackwood, E. M., B. Lüscher, et al. (1992). "Myc and Max associate in vivo." Genes & Development**6**(1): 71-80.

Bracken, A. P., N. Dietrich, et al. (2006). "Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions." Genes Dev**20**(9): 1123-1136.

Brough, D. E., T. J. Hofmann, et al. (1995). "An essential domain of the c-myc protein interacts with a nuclear factor that is also required for E1A-mediated transformation." Molecular and Cellular Biology**15**(3): 1536-1544.

Brown, J. L., C. Fritsch, et al. (2003). "The Drosophila pho-like gene encodes a YY1-related DNA binding protein that is redundant with pleiohomeotic in homeotic gene silencing." Development**130**(2): 285-294.

Brown, J. L., D. Mucci, et al. (1998). "The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1." Mol Cell**1**(7): 1057-1064.

Brunk, B. P., E. C. Martin, et al. (1991). "Drosophila genes Posterior Sex Combs and Suppressor two of zeste encode proteins with homology to the murine bmi-1 oncogene." Nature**353**(6342): 351-353.

Brunk, B. P., E. C. Martin, et al. (1991). "Drosophila genes Posterior Sex Combs and Suppressor two of zeste encode proteins with homology to the murine bmi-1 oncogene." Nature**353**(6342): 351-353.

Brunk, B. P., E. C. Martin, et al. (1991). "Molecular genetics of the Posterior sex combs/Suppressor 2 of zeste region of Drosophila: aberrant expression of the Suppressor 2 of zeste gene results in abnormal bristle development." Genetics**128**(1): 119-132.

Bunker, C. A. and R. E. Kingston (1994). "Transcriptional repression by Drosophila and mammalian Polycomb group proteins in transfected mammalian cells." Mol Cell Biol**14**(3): 1721-1732.

Caygill, E. E. and L. A. Johnston (2008). "Temporal Regulation of Metamorphic Processes in Drosophila by the let-7 and miR-125 Heterochronic MicroRNAs." Current Biology**18**(13): 943-950.

Chang, T. C., D. Yu, et al. (2008). "Widespread microRNA repression by Myc contributes to tumorigenesis." Nat Genet**40**(1): 43-50.

Chang, T. C., L. R. Zeitels, et al. (2009). "Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation." Proc Natl Acad Sci U S A**106**(9): 3384-3389.

Classen, A.-K., B. D. Bunker, et al. (2009). "A tumor suppressor activity of Drosophila Polycomb genes mediated by JAK-STAT signaling." Nat Genet**41**(10): 1150-1155.

Cole, M. D. and M. A. Nikiforov (2006). "Transcriptional activation by the Myc oncoprotein." Curr Top Microbiol Immunol**302**: 33-50.

Daneshvar, K., A. Khan, et al. (2011). "Myc Localizes to Histone Locus Bodies during Replication in Drosophila." PLoS ONE**6**(8): e23928.

Dang, C. V., M. McGuire, et al. (1989). "Involvement of the 'leucine zipper' region in the oligomerization and transforming activity of human c-myc protein." Nature**337**(6208): 664-666.

Davis, A. C., M. Wims, et al. (1993). "A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice." Genes Dev**7**(4): 671-682.

de la Cova, C., M. Abril, et al. (2004). "Drosophila myc regulates organ size by inducing cell competition." Cell**117**(1): 107-116.

Dellino, G. I., Y. B. Schwartz, et al. (2004). "Polycomb silencing blocks transcription initiation." Mol Cell**13**(6): 887-893.

Eilers, M. and R. N. Eisenman (2008). "Myc's broad reach." Genes Dev**22**(20): 2755-2766.

Esquela-Kerscher, A., P. Trang, et al. (2008). "The let-7 microRNA reduces tumor growth in mouse models of lung cancer." Cell Cycle**7**(6): 759-764.

F Grignani, L. L., G Inghirami, L Sternas, K Cechova, and R Dalla-Favera (1990). "Negative autoregulation of c-myc gene expression is inactivated in transformed cells." EMBO J.**December; 9(12)**: 3913–3922.

Facchini, L. M., S. Chen, et al. (1997). "The Myc negative autoregulation mechanism requires Myc-Max association and involves the c-Myc P2 minimal." Mol. Cell. Biol.**17**: 100-114.

Fernandez, P. C., S. R. Frank, et al. (2003). "Genomic targets of the human c-Myc protein." Genes Dev**17**(9): 1115-1129.

Francis, N. J., R. E. Kingston, et al. (2004). "Chromatin compaction by a polycomb group protein complex." Science**306**(5701): 1574-1577.

Francis, N. J., A. J. Saurin, et al. (2001). "Reconstitution of a functional core polycomb repressive complex." Mol Cell**8**(3): 545-556.

Gallant, P., Y. Shiio, et al. (1996). "Myc and Max homologs in Drosophila." Science**274**(5292): 1523-1527.

Ghule, P. N., Z. Dominski, et al. (2008). "Staged assembly of histone gene expression machinery at subnuclear foci in the abbreviated cell cycle of human embryonic stem cells." Proc Natl Acad Sci U S A**105**(44): 16964-16969.

Godfrey, A. C., J. M. Kupsco, et al. (2006). "U7 snRNA mutations in Drosophila block histone pre-mRNA processing and disrupt oogenesis." RNA**12**(3): 396-409.

Godfrey, A. C., A. E. White, et al. (2009). "The Drosophila U7 snRNP proteins Lsm10 and Lsm11 are required for histone pre-mRNA processing and play an essential role in development." RNA**15**(9): 1661-1672.

Goodliffe, J., M. Cole, et al. (2007). "Coordinated regulation of Myc trans-activation targets by Polycomb and the Trithorax group protein Ash1." BMC Molecular Biology**8**(1): 40.

Goodliffe, J. M., M. D. Cole, et al. (2007). "Coordinated regulation of Myc trans-activation targets by Polycomb and the Trithorax group protein Ash1." BMC Molecular Biology**8**(1): 40.

Goodliffe, J. M., E. Wieschaus, et al. (2005). "Polycomb mediates Myc autorepression and its transcriptional control of many loci in Drosophila." Genes & Development**19**(24): 2941-2946.

Grandori, C., N. Gomez-Roman, et al. (2005). "c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I." Nat Cell Biol**7**(3): 311-318.

Grewal, S. S., L. Li, et al. (2005). "Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development." Nat Cell Biol**7**(3): 295-302.

Grimaud, C., F. Bantignies, et al. (2006). "RNAi Components Are Required for Nuclear Clustering of Polycomb Group Response Elements." Cell**124**(5): 957-971.

Guo, W.-J., M.-S. Zeng, et al. (2007). "Mel-18 Acts as a Tumor Suppressor by Repressing Bmi-1 Expression and Down-regulating Akt Activity in Breast Cancer Cells." Cancer Research**67**(11): 5083-5089.

Guo, W. J., S. Datta, et al. (2007). "Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins." Mol Biol Cell**18**(2): 536-546.

Haupt, Y., W. S. Alexander, et al. (1991). "Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice." Cell**65**(5): 753-763.

He, X.-y., J.-x. Chen, et al. (2010). "The let-7a microRNA protects from growth of lung carcinoma by suppression of k-Ras and c-Myc in nude mice." Journal of Cancer Research and Clinical Oncology**136**(7): 1023-1028.

Hooker, C. W. and P. J. Hurlin (2006). "Of Myc and Mnt." Journal of Cell Science**119**(2): 208-216.

Hurlin, P. J., Z.-Q. Zhou, et al. (2003). "Deletion of Mnt leads to disrupted cell cycle control and tumorigenesis." EMBO J**22**(18): 4584-4596.

Johnson, C. D., A. Esquela-Kerscher, et al. (2007). "The let-7 MicroRNA Represses Cell Proliferation Pathways in Human Cells." Cancer Research**67**(16): 7713-7722.

Johnston, L. A. and B. A. Edgar (1998). "Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing." Nature**394**(6688): 82-84.

Johnston, L. A., D. A. Prober, et al. (1999). "Drosophila myc regulates cellular growth during development." Cell**98**(6): 779-790.

Kennison, J. A. (1995). "The Polycomb and Trithorax Group Proteins of Drosophila: Trans-Regulators of Homeotic Gene Function." Annual Review of Genetics**29**(1): 289-303.

Klymenko, T., B. Papp, et al. (2006). "A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities." Genes & Development**20**(9): 1110-1122.

Köhler, C. and C. B. R. Villar (2008). "Programming of gene expression by Polycomb group proteins." Trends in cell biology**18**(5): 236-243.

Li, H.-B., M. Müller, et al. (2011). "Insulators, Not Polycomb Response Elements, Are Required for Long-Range Interactions between Polycomb Targets in Drosophila melanogaster." Molecular and Cellular Biology**31**(4): 616-625.

Li, L., B. A. Edgar, et al. (2010). "Nutritional control of gene expression in Drosophila larvae via TOR, Myc and a novel cis-regulatory element." BMC Cell Biol**11**: 7.

Liu, J. L., C. Murphy, et al. (2006). "The Drosophila melanogaster Cajal body." J Cell Biol**172**(6): 875-884.

Liu, J. L., Z. Wu, et al. (2009). "Coilin is essential for Cajal body organization in Drosophila melanogaster." Mol Biol Cell**20**(6): 1661-1670.

Lo, S. M., N. K. Ahuja, et al. (2009). "Polycomb Group Protein Suppressor 2 of Zeste Is a Functional Homolog of Posterior Sex Combs." Mol. Cell. Biol.**29**(2): 515-525.

Lo, S. M., N. K. Ahuja, et al. (2009). "Polycomb group protein Suppressor 2 of zeste is a functional homolog of Posterior Sex Combs." Mol Cell Biol**29**(2): 515-525.

Loo, L. W. M., J. Secombe, et al. (2005). "The Transcriptional Repressor dMnt Is a Regulator of Growth in Drosophila melanogaster." Molecular and Cellular Biology**25**(16): 7078-7091.

Maines, J. Z., L. M. Stevens, et al. (2004). "Drosophila dMyc is required for ovary cell growth and endoreplication." Development**131**(4): 775-786.

Marinho, J., F. Casares, et al. (2011). "The Drosophila Nol12 homologue viriato is a dMyc target that regulates nucleolar architecture and is required for dMyc-stimulated cell growth." Development**138**(2): 349-357.

Marzluff, W. F., E. J. Wagner, et al. (2008). "Metabolism and regulation of canonical histone mRNAs: life without a poly(A) tail." Nat Rev Genet**9**(11): 843-854.

McMahon, S. B., H. A. Van Buskirk, et al. (1998). "The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins." Cell**94**(3): 363-374.

McMahon, S. B., M. A. Wood, et al. (2000). "The Essential Cofactor TRRAP Recruits the Histone Acetyltransferase hGCN5 to c-Myc." Molecular and Cellular Biology**20**(2): 556-562.

Mohd-Sarip, A., F. Cleard, et al. (2005). "Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex." Genes Dev**19**(15): 1755-1760.

Moreno, E. and K. Basler (2004). "dMyc transforms cells into super-competitors." Cell**117**(1): 117-129.

Muller, J., C. M. Hart, et al. (2002). "Histone methyltransferase activity of a Drosophila Polycomb group repressor complex." Cell**111**(2): 197-208.

Neto-Silva, R. M., S. de Beco, et al. (2010). "Evidence for a Growth-Stabilizing Regulatory Feedback Mechanism between Myc and Yorkie, the Drosophila Homolog of Yap." Developmental cell**19**(4): 507-520.

Newman, M. A., J. M. Thomson, et al. (2008). "Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing." RNA**14**(8): 1539-1549.

Nizami, Z., S. Deryusheva, et al. (2010). "The Cajal body and histone locus body." Cold Spring Harb Perspect Biol**2**(7): a000653.

Orian, A., B. van Steensel, et al. (2003). "Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network." Genes Dev**17**(9): 1101-1114.

Pasini, D., A. P. Bracken, et al. (2007). "The Polycomb Group Protein Suz12 Is Required for Embryonic Stem Cell Differentiation." Molecular and Cellular Biology**27**(10): 3769-3779.

Penn, L. J., M. W. Brooks, et al. (1990). "Negative autoregulation of c-myc transcription." Embo J**9**(4): 1113-1121.

Pierce, S. B., C. Yost, et al. (2008). "Drosophila growth and development in the absence of dMyc and dMnt." Dev Biol**315**(2): 303-316.

Pierce, S. B., C. Yost, et al. (2004). "dMyc is required for larval growth and endoreplication in Drosophila." Development**131**(10): 2317-2327.

Quinn, L. M., R. A. Dickins, et al. (2004). "Drosophila Hfp negatively regulates dmyc and stg to inhibit cell proliferation." Development**131**(6): 1411-1423.

Rastelli, L., C. S. Chan, et al. (1993). "Related chromosome binding sites for zeste, suppressors of zeste and Polycomb group proteins in Drosophila and their dependence on Enhancer of zeste function." Embo J**12**(4): 1513-1522.

Ringrose, L. and R. Paro (2007). "Polycomb/Trithorax response elements and epigenetic memory of cell identity." Development**134**(2): 223-232.

Ringrose, L., M. Rehmsmeier, et al. (2003). "Genome-wide prediction of Polycomb/Trithorax response elements in Drosophila melanogaster." Dev Cell**5**(5): 759-771.

Roush, S. and F. J. Slack (2008). "The let-7 family of microRNAs." Trends in cell biology**18**(10): 505-516.

Rybak, A., H. Fuchs, et al. (2008). "A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment." Nat Cell Biol**10**(8): 987-993.

Saurin, A. J., Z. Shao, et al. (2001). "A Drosophila Polycomb group complex includes Zeste and dTAFII proteins." Nature**412**(6847): 655-660.

Schreiber-Agus, N. and R. A. DePinho (1998). "Repression by the Mad(Mxi1)-Sin3 complex." BioEssays**20**(10): 808-818.

Schreiber-Agus, N., D. Stein, et al. (1997). "Drosophila Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype." Proc Natl Acad Sci U S A**94**(4): 1235-1240.

Schwartz, Y. B., T. G. Kahn, et al. (2006). "Genome-wide analysis of Polycomb targets in Drosophila melanogaster." Nat Genet**38**(6): 700-705.

Schwartz, Y. B. and V. Pirrotta (2007). "Polycomb silencing mechanisms and the management of genomic programmes." Nat Rev Genet**8**(1): 9-22.

Secombe, J., L. Li, et al. (2007). "The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth." Genes Dev**21**(5): 537-551.

Secombe, J., S. B. Pierce, et al. (2004). "Myc: a weapon of mass destruction." Cell**117**(2): 153-156.

Sempere, L. F., E. B. Dubrovsky, et al. (2002). "The Expression of the let-7 Small Regulatory RNA Is Controlled by Ecdysone during Metamorphosis in Drosophila melanogaster." Developmental Biology**244**(1): 170-179.

Sharp, E. J., N. A. Abramova, et al. (1997). "The conserved HR domain of the Drosophila suppressor 2 of zeste [Su(z)2] and murine bmi-1 proteins constitutes a locus-specific chromosome binding domain." Chromosoma**106**(2): 70-80.

Sharp, E. J., E. C. Martin, et al. (1994). "Directed overexpression of suppressor 2 of zeste and Posterior Sex Combs results in bristle abnormalities in Drosophila melanogaster." Dev Biol**161**(2): 379-392.

Sokol, N. S., P. Xu, et al. (2008). "Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis." Genes & Development**22**(12): 1591-1596.

Sparmann, A. and M. van Lohuizen (2006). "Polycomb silencers control cell fate, development and cancer." Nat Rev Cancer**6**(11): 846-856.

Staller, P., K. Peukert, et al. (2001). "Repression of p15INK4b expression by Myc through association with Miz-1." Nat Cell Biol**3**(4): 392-399.

Steiger, D., M. Furrer, et al. (2008). "Max-independent functions of Myc in Drosophila melanogaster." Nat Genet.

Stock, J. K., S. Giadrossi, et al. (2007). "Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells." Nat Cell Biol**9**(12): 1428-1435.

Stocker, H. (2011). "Growth Control: Myc and Yorkie Get Connected." Current Biology**21**(1): R37-R39.

Stone, J., T. de Lange, et al. (1987). "Definition of regions in human c-myc that are involved in transformation and nuclear localization." Molecular and Cellular Biology**7**(5): 1697-1709.

Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell**126**(4): 663-676.

Teleman, A. A., V. Hietakangas, et al. (2008). "Nutritional Control of Protein Biosynthetic Capacity by Insulin via Myc in Drosophila." Cell Metabolism**7**(1): 21-32.

Tucker, K. E., M. T. Berciano, et al. (2001). "Residual Cajal bodies in coilin knockout mice fail to recruit Sm snRNPs and SMN, the spinal muscular atrophy gene product." J Cell Biol**154**(2): 293-307.

van Lohuizen, M., M. Frasch, et al. (1991). "Sequence similarity between the mammalian bmi-1 proto-oncogene and the Drosophila regulatory genes Psc and Su(z)2." Nature**353**(6342): 353-355.

van Lohuizen, M., S. Verbeek, et al. (1991). "Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging." Cell**65**(5): 737-752.

Viswanathan, S. R., G. Q. Daley, et al. (2008). "Selective Blockade of MicroRNA Processing by Lin28." Science**320**(5872): 97-100.

Walker, M. P., L. Tian, et al. (2009). "Reduced viability, fertility and fecundity in mice lacking the cajal body marker protein, coilin." PLoS ONE**4**(7): e6171.

Wang, J., J. Mager, et al. (2001). "Imprinted X inactivation maintained by a mouse Polycomb group gene." Nat Genet**28**(4): 371-375.

White, A. E., B. D. Burch, et al. (2011). "Drosophila histone locus bodies form by hierarchical recruitment of components." The Journal of Cell Biology**193**(4): 677-694.

White, A. E., M. E. Leslie, et al. (2007). "Developmental and cell cycle regulation of the Drosophila histone locus body." Mol Biol Cell**18**(7): 2491-2502.

Wiederschain, D., L. Chen, et al. (2007). "Contribution of polycomb homologues Bmi-1 and Mel-18 to medulloblastoma pathogenesis." Mol Cell Biol**27**(13): 4968-4979.