RECIPROCAL REGULATION OF MYC AND MICRORNA MIR-308 DURING DROSOPHILA EMBRYOGENESIS

by

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ABSTRACT

KAVEH DANESHVAR. Reciprocal regulation of Myc and microRNA miR-308 during *Drosophila* embryogenesis. (Under the direction of DR. CHRISTINE RICHARDSON)

Myc is a conserved transcription factor with a role in the regulation of genes that are involved in growth and development. The abundance of Myc protein in the cells must be exquisitely controlled to avoid growth abnormalities caused by too much or too little Myc. An intriguing mode of regulation exists in which overabundance of Myc protein triggers a negative feedback regulation that leads to its abundance. In this study, I illustrate a mechanism for dMyc negative feedback regulation in *Drosophila* embryogenesis. I show that *Drosophila* Myc protein (dMyc) binds to the microRNA miR-308 locus and increases its expression. An increase in miR-308 levels leads to destabilization of dMyc mRNA and reduced dMyc protein levels. *In vivo* knockdown of miR-308 confirmed constant regulation of dMyc levels by miR-308 in embryos. My results also show that this regulatory loop is crucial for maintaining appropriate dMyc levels and normal development. Perturbation of the loop, either by elevated miR-308 or elevated dMyc, caused lethality. Combining elevated levels of both, therefore restoring balance between mir-308 and dMyc levels, resulted in suppression of lethality. These results reveal a sensitive feedback mechanism that is crucial to prevent the pathologies caused by abnormal levels of dMyc. Moreover, I show that the cross-regulation of dMyc and miR-308 has a role in regulation of dMyc target genes.

In the second part of this study, I show that dMyc localizes in histone locus bodies during replication. The work that I describe here began with an observation of

unexpected, punctate spots of Myc protein in certain regions of *Drosophila* embryos. I investigated the identity of these puncta and demonstrate that Myc is co-localized with coilin, a marker for Cajal Bodies (CBs), and Lsm11, a marker for Histone Locus bodies (HLBs), in embryos, larvae and ovaries. Using the MPM-2 antibody, I show that Myc's association with HLBs occurs only during replication in both endocycling and mitotic cells. These results reveal a novel role for Myc in replication-dependent histone mRNA production and processing.

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CHAPTER 1: INTRODUCTION

Myc is a transcription factor with an indispensable function in cell proliferation and growth that is conserved among metazoans (Brown, Cole, & Erives, 2008)(de la Cova, Abril, Bellosta, Gallant, & Johnston, 2004). Since its initial discovery as an oncogene (Varmus, 1984), many studies have been conducted on Myc's structure, its molecular mechanism of function, its potency as a stem cell inducer and, finally, it's suitability as a drug target. In this chapter, I will summarize some of the basic and recent studies on the structure and biological function of Myc. I will explain the similarities between mammalian Myc, c-Myc, and its counterpart in *Drosophila* Melanogaster, dMyc, and the suitability of *Drosophila* embryogenesis for studying molecular mechanisms of Myc's function.

This introduction chapter is organized into two sections, corresponding to two separate sets of hypotheses and results. The focus of the first section is on the molecular mechanisms of the events that enable Myc to regulate its own protein levels in a negative feedback manner, and a possible role of non-coding RNAs in these events. The focus of the second section is on the sub-nuclear localization of dMyc in nucleolar bodies such as Cajal bodies and Histone Locus Bodies. In the end of each section, I will explain some of the current unanswered questions, and introduce my hypotheses.

Myc functions as a transcription factor

Regulation of gene expression enables cells to maintain a specific biological program and to respond to internal and external stimuli. Cells can regulate their gene expression at least at four different levels: 1) at the epigenetic level, the accessibility of certain regions of the genome to transcription machinery is variable, depending upon the chromatin and DNA modifications and state of the chromatin (Berger, 2007). 2) At the transcription level, transcription factors can recruit enhancers, blocker or basic transcription factors that ultimately change the transcription of a particular gene (Latchman, 1997). 3) After transcription, the RNA transcript is subject to certain rounds of splicing, editing and modifications that ultimately determine the transcript variant and the availability of that particular RNA for translation (Gott & Emeson, 2000). I addition, non-coding RNAs, such as microRNAs, can interfere with the stability and translation of mRNA transcripts at this stage (Bushati & Cohen, 2007). 4) After translation, proteins are subject to post-translational modifications that can alter the function of the protein or its turnover (Walsh, 2006).

Transcription factors are groups of proteins that modulate, positively or negatively, the transcription of their targets genes by binding to the regulatory sequences of DNA (Latchman, 1997). Transcription factors are categorized based on their structures, their partners, their functions and their target gens. The basic helix-loop-helix (bHLH) family of transcription factors are involved in variety of developmental processes (Massari & Murre, 2000). These groups of transcription factors dimerize with other members of bHLH transcription factors, and together, they can bind to certain

motifs on DNA. Myc belongs to basic helix-loop-helix (bHLH) protein family of transcription factors.

Myc is a DNA-binding transcription factor whose binding site has been found at the promoters of ribosome biogenesis genes in all metazoans (with the exception of nematodes) (Brown et al., 2008). Myc affects transcription by inducing or repressing hundreds, if not thousands, of target genes; it can bind to 11% of all human promoters, with some sites bound in almost all cells and some sites bound only at high Myc levels (Fernandez et al., 2003). In addition to regulation of genes transcribed by RNA Polymerase II, Myc stimulates transcription by RNA Polymerases I and III, helping to promote protein synthesis consistent with its primordial role in ribosome biosynthesis (Brown et al., 2008)(Eilers & Eisenman, 2008)(van Riggelen, Yetil, & Felsher, 2010). 20% of genes regulated by *Drosophila* Myc during development consist of those required for ribosome biogenesis and a further 12% regulate protein synthesis. Accordingly, mutations in RNA Polymerase I induce phenotypes resembling mutations in dmyc, and over-expression of dmyc dramatically increases the size of nucleoli (Grewal, Li, Orian, Eisenman, & Edgar, 2005).

Molecular mechanism of gene regulation by Myc

In a classical model, Myc dimerizes with its partner, Max, and the Myc-Max heterodimer binds to E-boxes (CACGTG, CATGTG and alternative sequences) in the promoter region of target genes (Blackwell et al., 1993; Eilers & Eisenman, 2008). There are different factors and pathways upstream and downstream of this dimerization and binding; however, the focus of this study is on the events downstream to this binding.

Binding of Myc-Max dimer to the E-box triggers the recruitment of other factors needed for enhancing transcription at the neighboring loci. At the chromatin level, it is shown that the binding of Myc to a specific locus recruits histone acetyl transferases (HATs). Addition of acetyl group to histones by HATs changes the state of chromatin form a closed form to a more open form. This chromatin remodeling makes the locus more accessible to general transcription factors and enhancers.

Myc's regulation of its targets occurs mainly at the transcriptional level. This is mainly through recruitment of chromatin remodeling factors and facilitating the recruitment of basic transcription machinery (Cole & Cowling, 2008). Other studies show that Myc can also release cause a pause release in the elongation phase of the transcription (Rahl et al., 2010). It has been shown that Myc's mechanisms of function also extend to regulation of translation and DNA replication (Cole & Cowling, 2008).

Biological function of Myc

The biological effects of gene regulation by Myc are predominantly due to its capability to up-regulate expression of certain groups of genes. Myc up-regulates the expression of the genes that are involved in increase of cell mass (Schmidt, 1999)(Bernard & Eilers, 2006), progression of cell division (Neufeld & Edgar, 1998)(Steiner, Rudolph, Müller, & Eilers, 1996) and increased energy metabolism (Morrish, Neretti, Sedivy, & Hockenbery, 2008). There are only a small number of genes identified as Myc repression targets. These targets are predominantly involved in differentiation and control of cell growth (Zeller et al., 2006). Altogether, Myc regulation of its target genes favors a cell growth and proliferation program.

Null mutations of dmyc in *Drosophila* result in the failure of endoreplication in oogenesis and larval stages, causing lethality in whole animals and infertility in females with germline clones (Maines, Stevens, Tong, & Stein, 2004)(Pierce et al., 2008). Endoreplication is important in larval muscle cells, which are multi-nucleated, and the size of the polyploid nuclei within muscle cells determines the size of the muscle and the size of the entire animal. Greater Myc accumulation in muscle cells leads to larger nuclei and greater overall body size (Demontis & Perrimon, 2009). Larval growth is dependent on dietary amino acid availability, and 51% of gene expression changes that occur upon amino acid starvation overlap gene expression changes that occur in dmyc mutants. A further 60% of gene expression changes that occur upon re-feeding of starved larvae also occur in larvae over-expressing dmyc (Li et al., 2010).

Diploid larval cells mutant for dmyc are smaller than wild type cells and grow poorly, and over-expression of dmyc in these cells increases cell size independently of cell cycle control (Johnston, Prober, Edgar, Eisenman, & Gallant, 1999). In addition to changes in cell size, overexpression of dmyc in clones of imaginal discs confers a growth advantage to cells having greater amounts of dmyc expression than their neighbors. The cells with greater Myc levels induce apoptosis in neighbors expressing less Myc (de la Cova et al., 2004)(Moreno & Basler, 2004). This cellular competition is mediated by secreted factors in a mechanism that is likely important for overall growth regulation (Senoo-Matsuda & Johnston, 2007).

Drosophila Myc (dMyc)

Drosophila Myc gene (dm) has three exons and it codes for dMyc protein that has 717 amino acids. In *Drosophila*, dMyc is required for oogenesis and the proper growth of

Drosophila larvae (Maines et al., 2004; Pierce et al., 2004). Loss of dMyc has been shown to retard the growth of cells and cause lethality (Kappes, Deshpande, Mulvey, Horabin, & Schedl, 2011). Conversely, overexpression of dMyc results in increased size of larvae and organs of adult flies (Johnston et al., 1999).

Despite their moderate 26% amino acid sequence similarity, *Drosophila* dMyc and human c-Myc have significant functional similarities with each other. In fact, c-Myc of vertebrates and dMyc of *Drosophila* can be reciprocally replaced in rescue assays (Trumpp et al., 2001)(Benassayag et al., 2005). Similar to human c-Myc, dMyc dimerizes with Max protein to form a heterodimer with the ability to bind to E-Boxes (P Gallant, 2006). *Drosophila* Max, dMax, has 52% amino acid sequence similarity to human Max and they both have similar genomic organization (P Gallant, 2006). Because of its conserved structure and function and its vast similarity with c-Myc, dMyc as a suitable model for studying molecular mechanisms of gene regulation by Myc.

Drosophila embryogenesis as model to study Myc

It takes about ten days for a single fertilized egg cell to develop into an adult *Drosophila*. The first twenty-four hours of *Drosophila* embryogenesis is called embryogenesis (Ashburner, Golic, & Hawley, 2011). Embryogenesis of *Drosophila* starts with a single fertilized egg cell, and continues with rapid production of thousands of cells in a short period of time. By the middle of embryogenesis, cells start finding their identity according to their differentiation programs. While the growth program continues throughout the development, differentiation program defines the identity of the cells. The balance between the growth and differentiation programs has be subtly controlled in order to prevent developmental abnormalities.

It has been shown that there are vast similarities between growth program during development, and cancer. Certain pathways, transcription factors, non-coding RNAs and epigenetic marks that promote the growth and prevent differentiation, are involved in the tumor growth and metastasis as well. This raises the question that how the same growth promoting factors can regulate the normal development, without promoting tumor. The answer lies in the mechanisms that subtly control these growth promoting pathways and factors.

Myc is shown to be one of the major transcription factors involved in promoting cell proliferation and growth. Myc is also one the four transcription factors that were introduced as inducers of pluripotency in mouse fibroblasts (Takahashi & Yamanaka, 2006). During the *Drosophila* embryogenesis, dMyc levels are relatively high, despite some fluctuations. My colleagues and I have observed that too much or too little dMyc protein can cause lethality during embryogenesis. This shows the indispensible role of dMyc during the rapid proliferation and growth phase, and the importance of its subtle regulation.

Negative feedback regulation of Myc

Certain types of human cancers lack proper regulation of Myc levels resulting in uncontrolled cell proliferation and tumor formation (Eilers & Eisenman, 2008; Meyer & Penn, 2008). Hence, tight control and maintenance of appropriate Myc protein levels is crucial. My colleagues and others have demonstrated that Myc protein utilizes auto-regulation to repress its own transcription in *Drosophila* and rodents (Goodliffe, Wieschaus, & Cole, 2005; Penn, Brooks, Laufer, & Land, 1990). However, it is likely that additional post-transcriptional and post-translational regulatory pathways also

contribute to the critical maintenance of Myc protein levels in cells. Growing evidence has shown that mammalian Myc uses microRNAs to partially regulate its targets (Chang et al., 2008; Lin, Jackson, Guo, Linsley, & Eisenman, 2009; O'Donnell, Wentzel, Zeller, Dang, & Mendell, 2005; Wang et al., 2011; Xiong, Du, & Liang, 2010) as well as its own transcript levels (Liao & Lu, 2011).

MicroRNAs in their role in regulation of gene expression

MicroRNAs are a group of short (21-25 nucleotides) non-coding RNAs involved in negative regulation of gene expression (He & Hannon, 2004). They block the translation of transcripts by binding to their complementary sequences in 3′ untranslated region (3′-UTR) regions of mRNAs (Berezikov, Cuppen, & Plasterk, 2006). In mice and human cell lines, Myc abundance has been shown to be associated with changes in expression patterns of certain microRNAs, which in turn regulate the expression of other genes (Chang et al., 2008; Lin et al., 2009). In addition, Myc's mRNA itself has been shown to be a target of microRNAs in mice and human cell lines (Xiong et al., 2010) and it is also a predicted target of *Drosophila* "dme-miR-2a-1/6/11/13/308" microRNA family (Ruby et al., 2007).

Role of microRNAs in gene regulation by dMyc

The key hypothesis of the first section of this dissertation is that microRNAs have a role in gene regulation by dMyc. Myc protein regulates many genes, but only a small portion of those genes has a binding site for Myc. This shows the possibility of involvement of non-coding RNAs in regulation by Myc. A hypothetical mechanism would be that Myc directly regulates microRNAs and through increasing or decreasing their expression, it indirectly regulates the expression of its target genes at the post-

transcriptional level. This indirect target regulation via microRNAs can be extended to the Myc's transcript itself. Myc possesses a negative feedback regulation mechanism on its own levels. It is possible that microRNAs are part of that mechanism. A possible model would be that Myc binds to the locus of certain microRNAs, increasing their transcription, and those microRNA target the Myc's mRNA.

Localization of dMyc in the nucleus

During the course of carrying out experiments by my colleagues and I, we observed punctate spots of dMyc protein within the embryonic cells. Given the role of Myc protein in growth regulation including the control of the size of nucleoli, I investigated whether Myc puncta corresponded with nucleoli. Experiments shown here demonstrate that Myc largely does not localize to the nucleolus in *Drosophila*, consistent with previous reports of the lack of Myc association with rDNA (Grewal et al., 2005). I investigated the identity of the sub-nuclear puncta of Myc and show that Myc overlaps coilin and Lsm11 in the Histone Locus Body (HLB) of *Drosophila*.

Cajal bodies

Cajal Bodies are organelles within the nucleus where the accumulation and some assembly of snRNPs occurs before mature snRNPs relocate to chromosomes for splicing (Z. F. Nizami, Deryusheva, & Gall, 2010). They often appear adjacent to the nucleolus in *Drosophila*, and have been shown to transiently associate with several different loci in mammalian cells (Liu et al., 2006)(Matera, Izaguire-Sierra, Praveen, & Rajendra, 2009). The signature protein component of Cajal bodies is coilin; homozygous null coilin tissues lack Cajal bodies in *Drosophila*, and coilin knockout mice lack functional Cajal bodies (Liu et al., 2006)(Tucker et al., 2001).

Histone locus bodies

The histone genes of *Drosophila melanogaster* exist as tandemly repeated sets of the canonical histone genes, which are transcribed during S phase of the cell cycle. The resulting replication-dependent histone transcripts lack a poly-A tail, and instead the 3' of histone mRNAs ends in a conserved stem-loop structure. Metazoans share this feature along with the U7 snRNP that binds the stem-loop, which includes proteins SLBP, Lsm10 and Lsm11 (Marzluff, Wagner, & Duronio, 2008). Lsm10 and Lsm11 are required for histone pre-mRNA processing and are found in the Histone Locus Bodies (HLB), a Cajal body-like nuclear body associated with the histone gene locus (Godfrey et al., 2009; Liu et al., 2006). Nascent histone transcripts associate with a Cyclin E/Cdk2 dependent phospho-epitope localized to the HLB (White et al., 2007).

Structure of the dissertation

This dissertation starts with an introduction chapter, the current chapter, and will continue with materials and methods chapter. The results obtained in this research are demonstrated in two separate chapters. In the first results chapter of my dissertation, I will describe my findings about the regulatory relationship of Myc and microRNA 308 (miR-308) in *Drosophila*. I show that miR-308 levels increase in response to elevated levels of dMyc through the physical interaction of dMyc with the miR-308 locus, and dMyc's transcript itself is a target of miR-308. In a miR-308 knockdown study, I show that dMyc levels are under the control of miR-308 during embryogenesis. Furthermore, I show that ectopic expression of miR-308 in embryos can rescue lethality by dMyc overexpression. Finally, through analysis of dMyc's targets and comparing them to the

predicted targets of miR-308, I propose models explaining a concerted gene regulation program by dMyc and miR-308.

In the second results chapter of my dissertation, I will explain my finding of localization of dMyc in histone locus bodies during mitosis. My work includes the use of sequential elimination of markers that led my colleagues and me to the finding that dMyc localizes to histone locus bodies during mitosis. These findings suggest a possible role for dMyc in regulation or processing of the transcripts of the histone genes. I show that Myc associates with all HLBs that contain the same Cyclin E/Cdk2 phospho-epitope, and that Myc does not associate with HLBs of non-replicating cells. Our results reveal a novel role for Myc as a cell-cycle dependent component of HLBs. As such, these results suggest that part of the impact of Myc protein on the success of growth and proliferation may be linked to a role in histone mRNA synthesis and/or processing. Myc's role in this aspect of biology reveals even greater complexity than previously thought for Myc, and it opens new avenues of investigation into the biology of this important protein.

CHAPTER 2: MATERIALS AND METHODS

MicroRNA microarray

0-24 hour old embryos form each genotype were collected on a grape agar plate. Total RNA, including small RNAs, was isolated by miRNAeasy (Qiagen, California, USA). Small RNAs were subject to poly-adenylation and fluorescent labeling using a power labeling kit (Exiqon, Copenhagen, Denmark). Dual-color labeling with common reference method was used. The reference sample was a cocktail of equal amounts of RNA from four different genotypes (Gal4, Gal4 X UAS-dm; UAs-dm, UAS-dm; UAS-dm and wild-type Oregon-R). Reference sample was labeled with Hy5 (red) and other samples were labeled with Hy3 (green). Equal amounts of each sample were mixed with equal amount of the reference sample. Samples were hybridized on Exiqon microRNA microarray version 11, in a automated hybridization chamber (Tecan, Männedorf, Switzerland). Then run protocol was imported form Exiqon website. The experiment was carried out in two biological replicates and four technical replicates on each slide. Images of the microarray slides were taken by a laser scanner (Tecan, Männedorf, Switzerlan). Spot intensities were acquired and normalized by GenePix Pro (Molecular Devices, California, USA), using global normalization method. T-test statistical analysis was carried out by using Differential expression analysis module on Genepattern (Reich et al., 2006).

Affymetrix microarray

Total RNA, was isolated from 0-24 hour old embryos by Trizol (Invitrogen, California, USA), according to manufacture's protocol. Processing and analysis of microarrays were carried out by Expression Analysis (Durham, North Carolina, USA). RNA samples from 3 biological replicates of each group were labeled and hybridized on Affymetrix *Drosophila* Genome 2.0 Array. Data was analyzed using Two-Group Comparisons with Permutation Analysis for Differential Expression (PADE). Data have been deposited at the NCBI Gene Expression Omnibus (GEO) repository (GSE38529).

Drosophila strains and genetics

Flies expressing Gal4 ubiquitously in embryos were used (w[*];P{w[+mW.hs]=GAL4-da.G32}UH1) for all the crosses involving Gal4-UAS system (Fischer et al., 1988). UAS-dmyc flies (P{ry[+t7.2]=hsFLP}22, y[1] w[*]; P{w[+mC]=UAS-dm.Z}42) were used for generation of both doubly homozygous flies. Double balancer flies (w[*]; Kr[If-1]/CyO; D[1]/TM6C, Sb[1] Tb[1]) were used for balancing the heterozygous flies for the generation of doubly homozygous flies. Both of the mentioned stocks were obtained from Bloomington Stock Center (Indiana University, Bloomington, IN, USA). Hs-dm (Heat-shock dMyc flies) were a gift form Peter Gallant.

Molecular cloning

Plasmas needed for the generation of transgenic flies were made on a pUAST backbone. A genomic region of Drosphila containing the gene for miR-308 was amplified using following primers: 5- GCT ATG AAT TCG GTG ATC TTC TTG CCG TTC T-3 and 5- CGA TAC TCG AGG AAT CGT CCT GGA GAA GGT G-3. Primers had an additional 6-neclutide extensions that serve as restriction sites for restriction

enzymes EcoRI and XhoI. After amplification of the genomic region, the product was digested using EcoRI and XhoI (New England Biolabs, Massachusetts, USA). pUAST plasmid was also digested using the same restriction enzymes. The products of the both reactions were run on an agarose gel and were separated and cleaned using a DNA cleanup system (Promega, Wisconsin, USA). Digested products were mixed in 3:1 (insert: plasmid) ratio and were incubated with DNA ligase (Invitorgen, California, USA). The product of the ligation reaction was used for bacterial transformation. Competent "Top10" bacteria (Invitrogen, California, USA) were transfected using heat shock procedure according to manufacturer recommendation. Transfected bacteria were grown in LB agar medium plus ampicillin, as selection marker. 12 colonies were randomly selected for testing the insertion of the miR-308 genomic region into the pUAST plasmid backbone. Using enzymatic digestion, the insertion was confirmed. The construct was sequenced and the insertion and the correct orientation were confirmed.

Site-directed mutagenesis

To generate a pUAST plasmid that carries a mutated form of the miR-308 gene, the original construct was used. Primers expanding the miR-308 region with random changes in the seed sequence was used to amplify the plasmid (Primers: 5- ATATTT TTG TGT TTT GTT TCG TTT TGC AAT CCA AGG ATC CGG ATT ATA CTG TGA GAT GAC CAG CGT G -3 and 5- CAC GCT GGT CAT CTC ACA GTA TAA TCC GGA TCC TTG GAT TGC AAA ACG AAA CAA AAC ACA AAA ATA T-3). Accuprime Pfx (Invitrogen, California, USA) high fidelity DNA polymerase was used for amplification of the entire construct. Amplification reaction was done a PCR machine for 19 cycles. The amplification product was incubated with DpnI (Agilent Technologies,

Colorado, USA) restriction enzyme to digest the parent unmutated construct. The product was used for transformation of bacteria. Bacterial transformation was conducted using the same procedures described above.

Generation of transgenic *Drosophila*

UAS-miR-308 flies were constructed by cloning 300 base pairs of the genomic region of microRNA-308 into the pUAST plasmid (Brand and Perrimon, 1993) and injecting the construct into *Drosophila* embryos (BestGene, California, USA). 10 independent lines were generated, four of which were homozygous for UAS-miR-308. UAS-mutated-miR-308 flies were constructed by site-directed mutagenesis using Accuprime Pfx (Invitrogen, California, USA) on the same UAS-miR-308 construct and injection as explained. All crosses were done in 25°C.

Quantification of miR-308 by qPCR

Total RNA including small RNAs was extracted by miRNeasy mini-kit (Qiagene, California, USA), according to the manufacturer's instruction. Universal cDNA synthesis kit, SYBR green and specific LNA primers (all from Exiqon, Denmark) were used for quantification of miR-308 in ABI 7500 Fast real-time PCR system (Applied Biosystems, California, USA). microRNA miR-1 was used as the reference gene as it showed no significant change in the microarray study. Comparative Ct method was used for analyzing the expression levels and relative fold changes (Schmittgen & Livak, 2008).

Quantitative RT-PCR of mRNAs

Total RNA was isolated using Trizol (Invitrogen, California). Power SYBR® Green RNA-to-CT™ 1-Step Kit and ABI 7500 Fast real-time PCR system (Applied Biosystems, California, USA) were used for quantification of mRNA transcripts.

Comparative Ct method was used for quantification of all mRNA transcripts (Schmittgen and Livak, 2008). Ras was used as internal reference gene for quantification, as described previously (Khan et al., 2009). Q-RT-PCR Primers: dMyc (F: ATG CAC ATC ACC GAT CAC AG, R: TGG GCC ATC TGG AAC TGT AG) dRas (F: ATA TCG GCA CCT ACC GTG AG, R: GT CTT GGC GGA TGT CTC AAT) RpS23 (F: CGT CCT GGA GAA GGT CGG CG, R: ACC TTG AAG CGC ACA CCG GG).

MiR-308 Knockdown

Canton-S (wild-type) embryos were collected 30 minutes after egg deposition and were dechorionated in 50% sodium hypochlorite solution for 2 minutes and were rinsed several times with water. Embryos were desiccated for an appropriate, empirically determined time. Embryos were then injected with a 100uM anti-miRNA-308 LNA inhibitor solution in PBS or PBS alone (control). The sequence of the inhibitor was complementary to the mature sequence of miR-308 (Exiqon, Copenhagen, Denmark). The embryos were allowed to develop at 18°C for 15 hours before collecting them for either RNA or protein extraction.

Chromatin Immuno-precipitation

Approximately 0.5 grams of 0-24 hours embryos were dechroniated and fixed in PBS/Hepatane/formaldehyde and sonicated in SDS-lysis buffer. The EZ-ChIP kit (Millipore, Massachusetts, USA) was used for precipitation and washes. Anti-MycN and anti-dMyc antibodies (SantaCruz Biotechnology, sc-28208 and sc-28207) were used for immuno-precipitation of Myc protein. ChIP primers: ChIP region I: 5’-TGGCGAGATACGGCGGGACA-3’ and 5’-GGTTTGAGTCCAGGGTGGATGAACG-3’ ChIP region II: 5’- GCGAGGCGTCGAGACGTGTT-3’ and 5’-

TCTACAACAGGCAAGCCAAGAGGT-3'. Fibrillarin promoter region: 5'-TTTTACGCACCTGGTTTGCCCA-3' and 5'-CCTCTCCGCCTGGTGTTGAACTT-3'.

Protein extraction and quantification

Protein lysates were prepared by using RIPA buffer (Santa Cruz Biotechnology, California, USA). Flash frozen embryos were homogenized in 500 ul of RIPA buffer by using a sonicator. Debris was separated by centrifuge (12000 g, 10 minutes). Protein samples were stored in -20 C all the time. Protein quantification was done using BCA assay (Thermo Fisher Scientific, Massachusetts, USA).

Immunoblots

8% poly-acrylamide gels were used for separation of whole cell protein extracts. Detection was carried out using rabbit anti-dMyc (Santa Cruz biotechnology, California, USA, Catalog number: 28207) and mouse anti-rabbit HRP. Anti-beta Actin antibody (Abcam, Massachusetts, USA, Catalog number: ab8224) was used as loading control. Blots were quantified with NIH ImageJ software package (http://rsbweb.nih.gov/ij/).

Growth and survival

Less than hour old embryos from each studied genotypes were collected on grape agar plates. Embryos were counted and transferred to new grape agar plates. Growth and development of the animals were assessed after 24, 48, 72 and 96 hours. Fraction of live animals was plotted in Microsoft Excel. Fisher exact test was used for determining the significance of alive to dead ratio for each genotype.

Functional analysis and graphical design

Functional analysis of gene lists was performed by Ontologizer 2.0 (Bauer et al., 2008). Heat map of created by ArcGIS (Esri, California, USA). Schematic structure of

the genes was made using the CLC Genomic Workbench Utility (Aarhus, Denmark). Other graphics and diagrams were made using Microsoft PowerPoint. Images were cropped and adjusted using Adobe Photoshop (California, USA). Graphs were generated using GraphPad PRISM and Microsoft Excel.

Tissue fixation

Embryos were collected and transferred to a metal mesh. Embryos were dechroniated in 100% bleach for 1 minute, followed by washes with deionized water. Embryos were fixed in solution containing 8% paraformaldehyde, 10% PBS and heptane (1:0.1:3 v/v/v). Fixation was performed for 20 minutes on a horizontal shaker. Ovaries were fixed and stained according to Frydman and Spradling (Frydman & Spradling, 2001). Larvae were fixed and stained according to Neufeld, Johnston and Edgar (Neufeld, de la Cruz, Johnston, & Edgar, 1998). Generally, the procedure for larvae fixation was the same as embryos, except that paraformaldehyde was used instead of formaldehyde.

Immunostaining

I used three different Myc antibodies (Santa Cruz Biotechnology, California, USA). Immunostaining of larvae and embryos expressing an RNAi construct specific for dmyc showed the absence of Myc puncta.

Primary antibodies were used at the following concentrations: rabbit anti-Myc 1:500, goat anti-Myc 1:250, mouse anti-fibrillarin 1:1000 (Abcam, Massachusetts, USA), 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, Massachusetts, USA) and mouse MPM-2 1:1000 (Millipore).

To be certain of the validity of the co-localization observed during this study, I obtained the following data from control experiments: single stainings for Myc showed similar puncta; stainings with each primary antibody combined with the wrong secondary showed no staining pattern except for one case, and I eliminated that secondary antibody. Staining with one primary antibody and all secondary antibodies together showed the same patterns as with one secondary alone. For microscopy I used sequential scanning of each channel, ensuring that detection of each fluorophore occurred only with the correct excitation laser.

Microscopy

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 (Figure 1A was obtained on the Zeiss and I did not alter those images following acquisition). Modified images were cropped using Adobe Photoshop.

CHAPTER 3: Reciprocal regulation of dMyc and miR-308

The broad objective of studies explained in this chapter was to investigate a possible role of microRNAs in gene expression regulation by dMyc. dMyc is shown to be involved in regulation of thousands of genes during and after development of *Drosophila*. Despite the conventional theory that Myc regulates its target by binding to their loci, it's shown that a significant number of genes don't necessarily have a binding site for Myc. Based on this, I hypothesized that dMyc regulate specific microRNAs during embryogenesis, through which, it regulates other transcripts. I n this chapter, I first explain how I established an in-vivo gain-of-function system that has elevated levels of dMyc. Then I will explain the experiments that led me to discover the unique role of microRNA miR-308 in feedback regulation of dMyc as well as target regulation.

Ectopic expression of dMyc alters the endogenous expression of dMyc.

The first aim of this study was to identify possible microRNAs that are regulated by dMyc, in a gain-of-function system in which total level of dMyc is higher than normal conditions. However, It is shown that Myc protein in mammals and *Drosophila*, have a negative feedback regulation effect on their own levels. This feedback regulation could possibly serve as a barrier to ectopically increase dMyc levels. In an effort to establish a gain-of-function system for increasing dMyc levels in *Drosophila* embryo, I used an hs-dMyc transgenic *Drosophila* strain. These flies express ectopic dMyc under a heat-shock

promoter. After inducing a 30-minute 37-Celsius heat shock, I collected embryos and extracted total RNA. Using RT-PCR with specific primers, I tested the levels of endogenous, ectopic and total (ectopic + endogenous) dMyc. Results showed that ectopic expression of dMyc in *Drosophila* embryos alters the endogenous levels of dMyc, balancing the total dMyc (Figure 1). Therefore, this model was ruled out for gain-of-function studies in *Drosophila* embryos. Next, I used yeast UAS-Gal4 system to increase the total level of dMyc in embryos. The aim was to generate a transgenic *Drosophila* strain that ia homozygous for two copies of ectopic dMyc on 2^{nd} and 3^{rd} chromosome. I used two different homozygous transgenic *Drosophilae* with a copy of ectopic dMyc on 2^{nd} (+; UAS-dm/UAS-dm) and 3^{rd} (+; +; UAS-dm/UAS-dm) chromosome. I crossed each strain with flies that carry chromosome balancers. The progenies were heterozygous for a copy of dMyc on either second or third chromosome (+; UAS-dm/Cyo and +; +; UAS-dm/TM3sb). By crossing these two flies together, I screened for flies carrying no chromosome balancer/marker (+; UAS-dm/UAS-dm; UAS-dm/UAS-dm). These flies were viable and fertile (Figure 2).

To test if the doubly homozygous flies are able to drive overexpression of dMyc, I crossed these flies to transgenic flies homozygous for Gal4. The progeny of this cross would have both Gal4 and two copies of ectopic dMyc. I tested the levels of transcript and protein of dMyc in the 0-24 hours embryos. Results showed that the two ectopic copies of UAS-dm are enough to overcome the barrier of dMyc's auto-regulatory circuit. dMyc levels were higher than the control (flies having Gal4 only) at the mRNA and protein level (Figure 3). I used this transgenic gain-of-function animal model to test my hypotheses based on the increased levels of dMyc in embryogenesis.

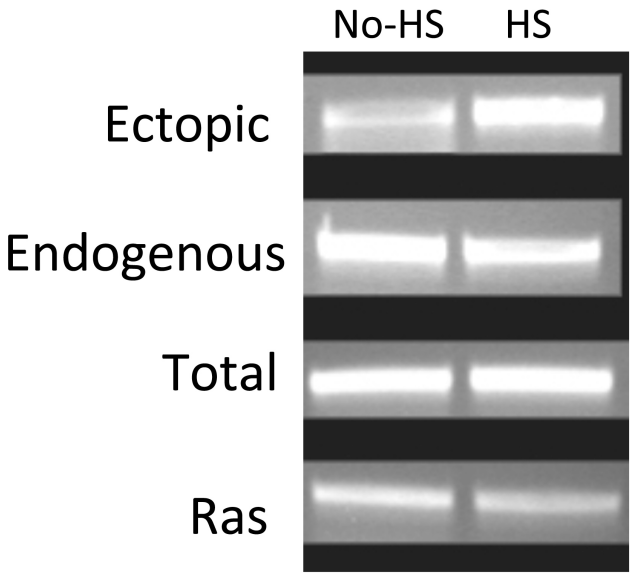


Figure 1. Ectopic dMyc represses endogenous dMyc. Agarose gel electrophoresis shows the RT-PCR product for ectopic (top panel), endogenous (second form the top) and total dMyc (third form the top) from control embryos (no heat shock) on the left and embryos undergone heat shock on the right. The bottom panel shows the expression levels of Ras, as loading control, in control embryos and embryos with ectopic copy of dMyc.

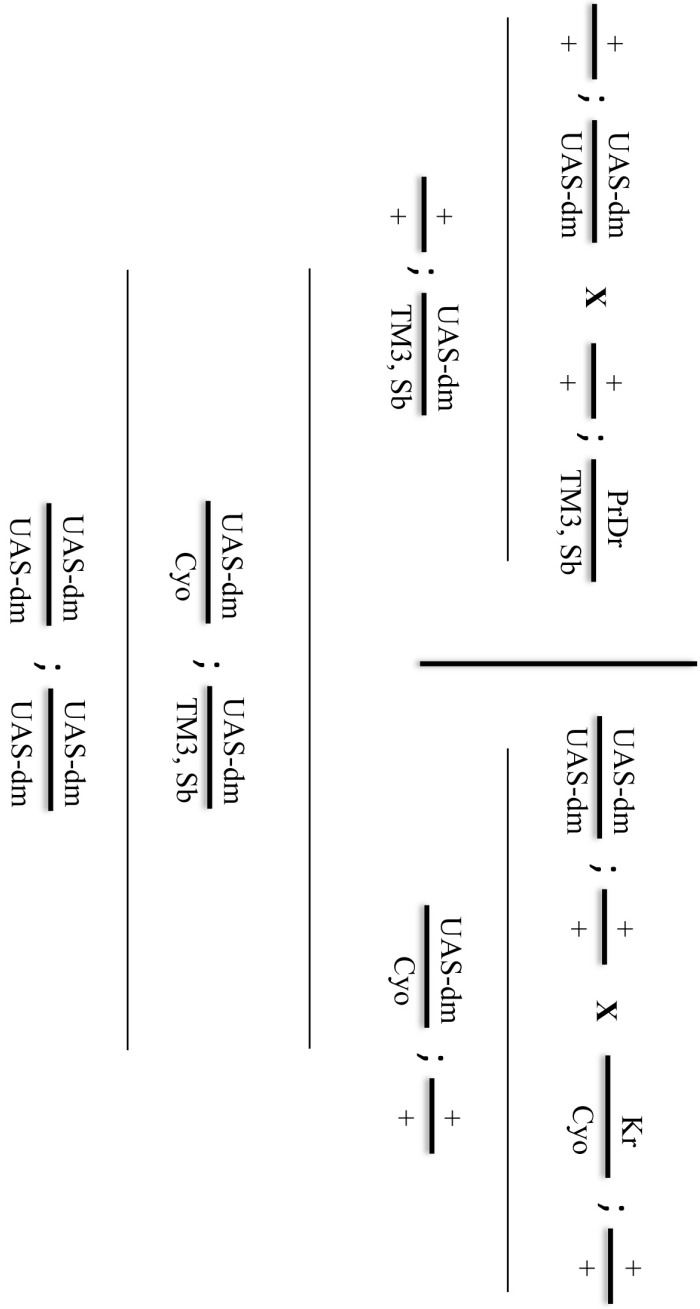


Figure 2. Description of genetic crosses to make UAS-dMyc flies. Flies homozygous for UAS-dm on second and third chromosomes are separately crossed to flies carrying chromosome balancers/markers. The progeny of those separate crosses cross are ultimately crossed together to obtain flies homozygous for UAS-dm on second and third chromosome.

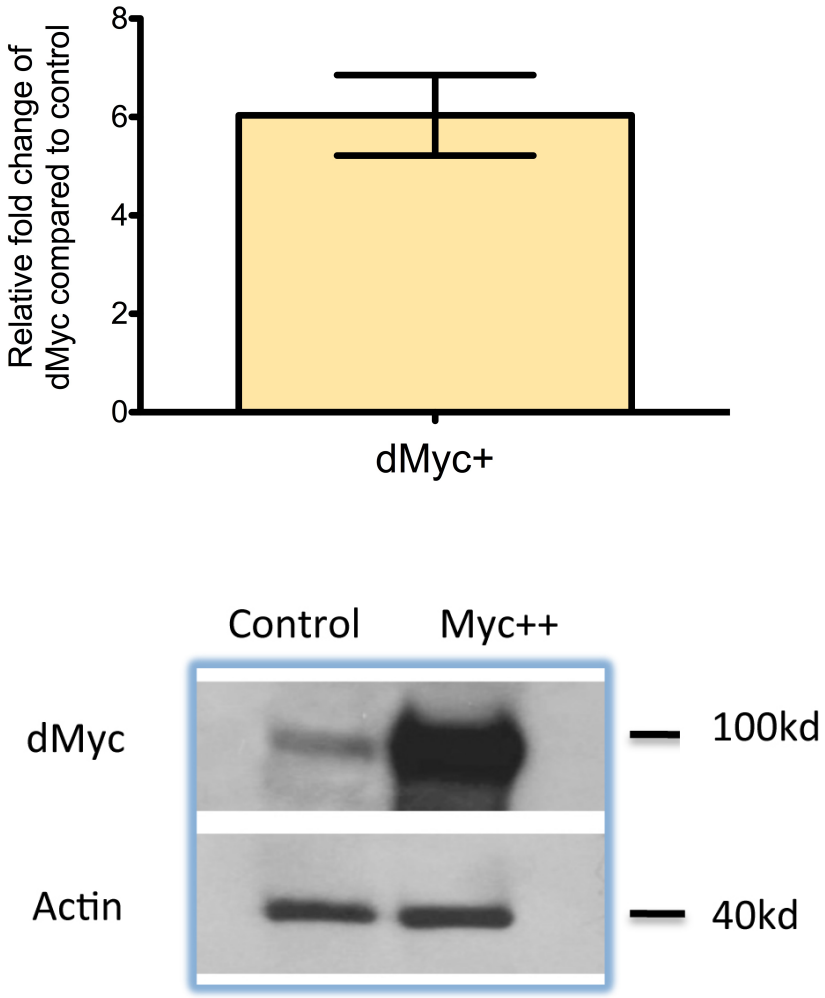


Figure 3. Two copies of UAS-dm are enough to obtain an increase in dMyc levels. Top Panel: Quantitative RT-PCR shows the levels of total dMyc in control embryos (Gal4 only) and embryos with Gla4 and two ectopic copies of dMyc. Relative expression is normalized to Ras. The error bar indicates the standard deviation of three biological replicates (n=3). Bottom panel: western blot with antibody against dMyc shows the total levels of dMyc protein in control embryos (Gal4 only) and embryos with Gal4 and two ectopic copies of dMyc. An antibody against actin is used as to show the protein levels of actin as a loading control.

Elevated levels of dMyc result in an increase in miR-308 levels.

It is generally accepted that direct regulation of gene expression by dMyc occurs through the direct binding of dMyc to E-boxes in the promoter region of target genes (Peter Gallant, 2009). However, many genes that are responsive to dMyc in *Drosophila* lack E-boxes or similar sequences (Goodliffe, Cole, & Wieschaus, 2007). I asked to what extent dMyc indirectly regulates its targets via microRNAs. Using the gain-of-function system explained in the previous section, I increased levels of dMyc in *Drosophila* embryos. The hypothesis for this experiment was that increased levels of dMyc cause differential expression of microRNAs during embryogenesis.

To study the expression of all the known microRNAs in *Drosophila*, I decided to use microarray technique. I used a microRNA microarray to measure the genome-wide expression of microRNAs throughout embryogenesis. The first question was, which microRNA transcripts are present during *Drosophila* embryogenesis. I observed that out of 152 available probes for *Drosophila* microRNAs, 42 microRNAs were expressed in embryos. The second question was if the elevated levels of dMyc cause changes (up or down-regulation) in the expression of microRNAs. I observed that ectopically increased levels of dMyc caused a significant, 1.8-fold (p-value < 0.05, fold change > 1.5) increase in the expression of *Drosophila* microRNA-308 (miR-308), compared to that of control (Figure 4).

To further validate the increase in miR-308 transcript levels, I conducted a quantitative PCR experiment with primers specific to the mature sequence of miR-308. Because of their short length, mature microRNAs cannot be utilized as a template for PCR amplification. Therefor, addition of a poly-A tail and a tag sequence is necessary

before amplification. After addition of poly-A tail by poly-A polymerase enzyme, and addition of a tag according to manufacturer's protocol (Exiqon, Denmark), I amplified the mature sequence of miR-308 in a real-time PCR. Quantitative RT-PCR of miR-308 confirmed 2.9-fold (+/- 0.9, n=3) increase in miR-308 in response to elevated levels of dMyc (Figure 5). Since microRNAs are involved in post-transcriptional regulation of the genes and microRNA miR-308 responds to the elevated levels of the dMyc, this result suggests a possible role for miR-308 in dMyc's regulation of gene expression. This is particularly interesting because not all the known genes that are affected my perturbed levels of Myc have a binding site for Myc.

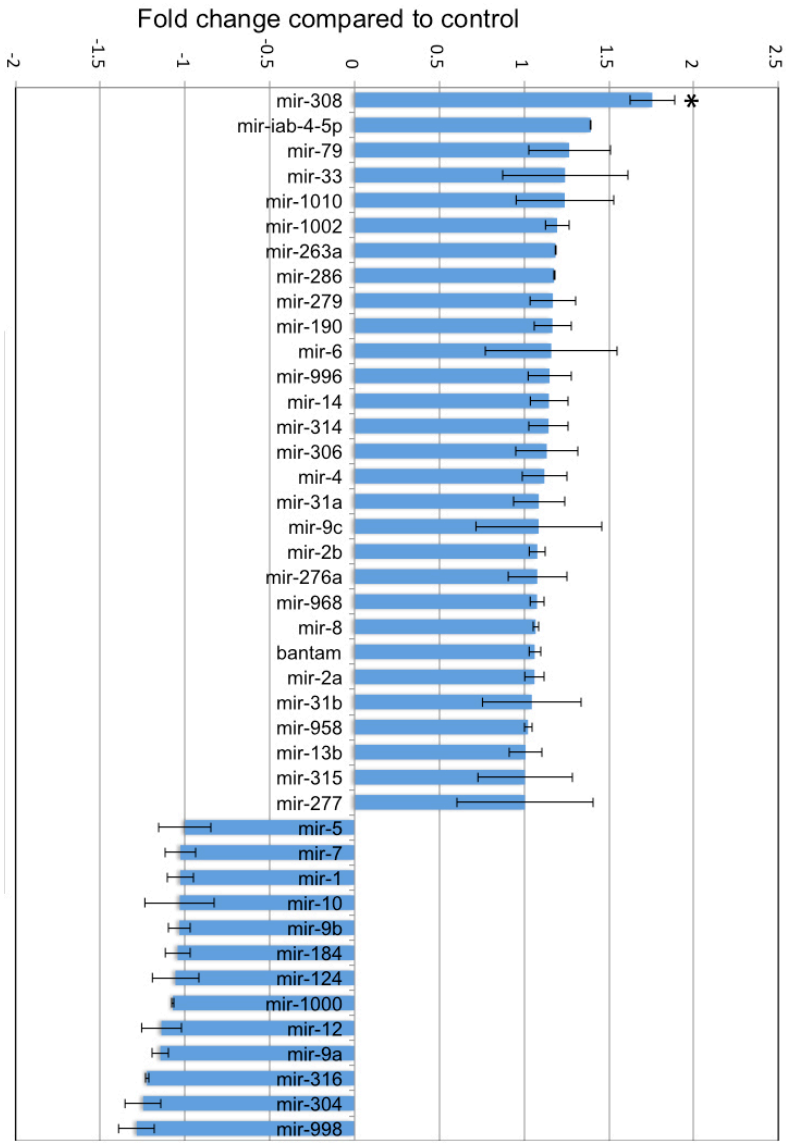


Figure 4. microRNA microarray in control embryos vs. embryos with high dMyc. MicroRNA microarray analysis of expression of microRNAs responding to elevated dMyc protein. X-axis indicates only those microRNAs that are expressed in embryos. Y-axis indicates relative fold change of each microRNA compared to that of control. Data are presented as means ± standard deviation of two independent biological replicates Asterisk represents the significant difference based on t-test (cutoff: P-value < 0.05, fold change > 1.5).

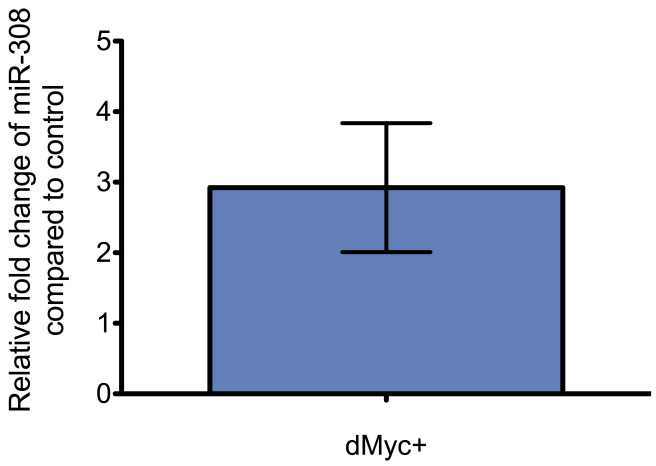


Figure 5. Quantitative PCR validation of of microRNA microarray. Quantitative RT-PCR analysis of miR-308 expression in control vs. embryos with elevated amounts of dMyc, normalized to miR-1. Data are presented as means ± standard deviation of three independent biological replicates.

dMyc physically interacts with miR-308 and its host gene locus

To further elucidate the molecular mechanisms involved in regulation of miR-308 by dMyc, I studied the locus of miR-308 gene. *miR-308* gene is located in the second intron of the *ribosomal protein S23* (*RpS23*) gene. I identified two non-canonical E-boxes (CATGTG) upstream of the miR-308 gene (Figure 6). Since miR-308 levels increases in response to and increase in dMyc protein levels, I asked whether dMyc directly binds to the locus of *miR-308* gene. I carried out chromatin Immunoprecipitation (ChIP) using two different dMyc antibodies. Results show a strong association (5.2-fold enrichment relative to IgG) of dMyc to the intronic region of the *RpS23* gene (Figure 7). I also observed a lower association of dMyc (3.6-fold enrichment compared to IgG) to the upstream region of *RpS23* transcription start site. I used the locus of *Fibrillarin* gene as a positive control because it has multiple canonical E-boxes and is a known target of dMyc (Orian et al., 2003).

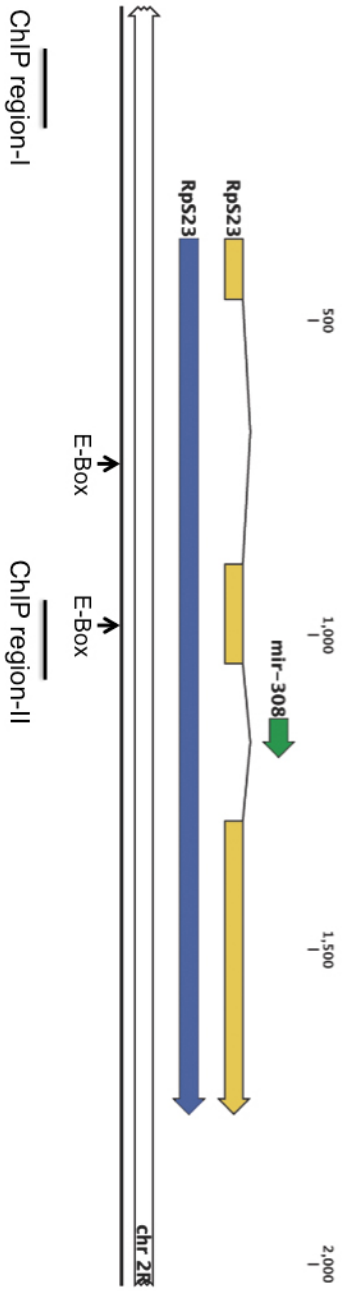


Figure 6. Locus of miR-308 gene in the intronic region of RpS23. *miR-308* locus is shown in the second intron of the *RpS23* gene. Arrows in the upstream region of the miR-308 gene indicate the positions of non-canonical E-boxes (CATGTG).

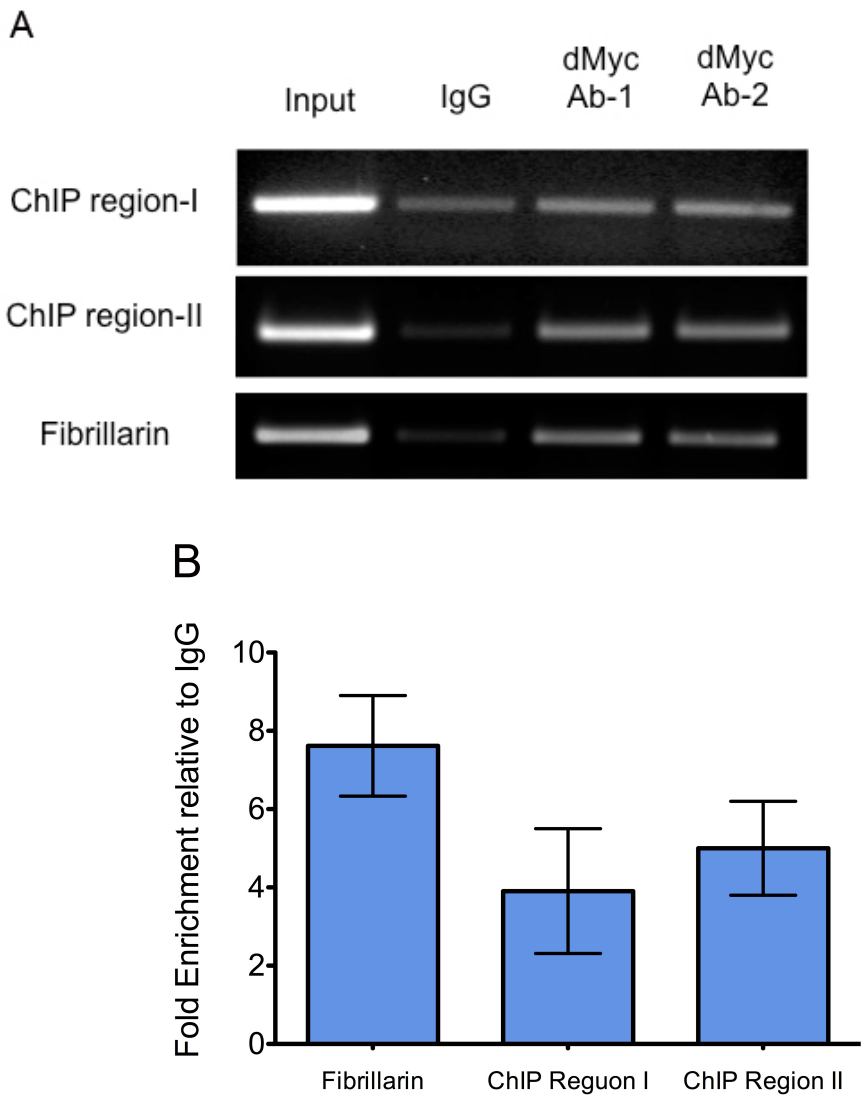


Figure 7. Chromatin immunoprecipitatoion of dMyc at the miR-308 locus. (A) Chromatin immuno-precipitation of dMyc by two different anti-dMyc antibodies and IgG, followed by agarose gel electrophoresis electrophoresis of PCR product. (B) Quantification of ChIP by qPCR. Data is shown as fold enrichment compared to that of the IgG control. Data are presented as means ± standard deviation of three independent biological replicates.

Elevated levels of dMyc result in an increase in RpS23 levels.

It has been shown that intronic microRNAs are co-expressed with their host genes (Baskerville & Bartel, 2005; Truscott, Islam, López-Bigas, & Frolov, 2011). I asked whether dMyc binding to the locus of miR-308 changes the expression of RpS23 as well.

Quantitative RT-PCR analysis showed that the transcript levels of RpS23 increase in response to increased levels of dMyc (Figure 8). Overall, this result suggests that dMyc regulates the expression of miR-308 and its host gene, *RpS23*, by binding to their shared locus.

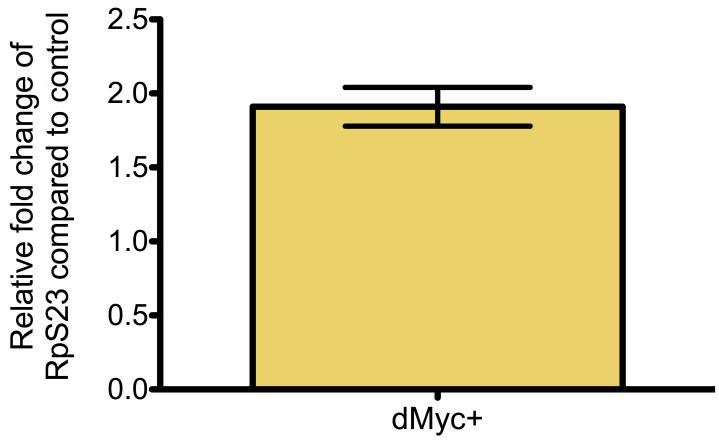


Figure 8. Expression of RpS23 in control versus dMyc+ embryos. Effect of elevated level of Myc on the transcript levels of *RpS23*, the gene that hosts *miR-308*. Data are presented as means ± standard deviation of three independent biological replicates.

MiR-308 belongs to the conserved miR-2a microRNA family

MiR-308 belongs to the *Drosophila* miR-2 conserved family of microRNAs. Members of miR-2 family (miR-2a, miR-6, miR-11, miR-13 and miR-308) are predominantly expressed during embryogenesis and share a vast number of predicted targets based on their common 6 nucleotide seed sequence (5′-AUCACA-3′) (Aravin et al., 2003). Prediction of the targets of the members of this family is based on the complementary sequence alignment of this seed sequence with the 3′-UTR region of the mRNA transcripts. Members of the *Drosophila* miR-2 family are involved in the repression of apoptosis by inhibiting the expression of pro-apoptotic genes grim, hid, reaper and skl (Ge et al., 2011)(Goyal, McCall, Agapite, Hartwieg, & Steller, 2000). A

member of miR-2s family, the genomic sequence of miR-308 stem-loop gene is highly conserved across 10 species. MicroRNA miR-308 is expressed in low amounts during the early and mid stages of *Drosophila* embryogenesis (Figure 9).

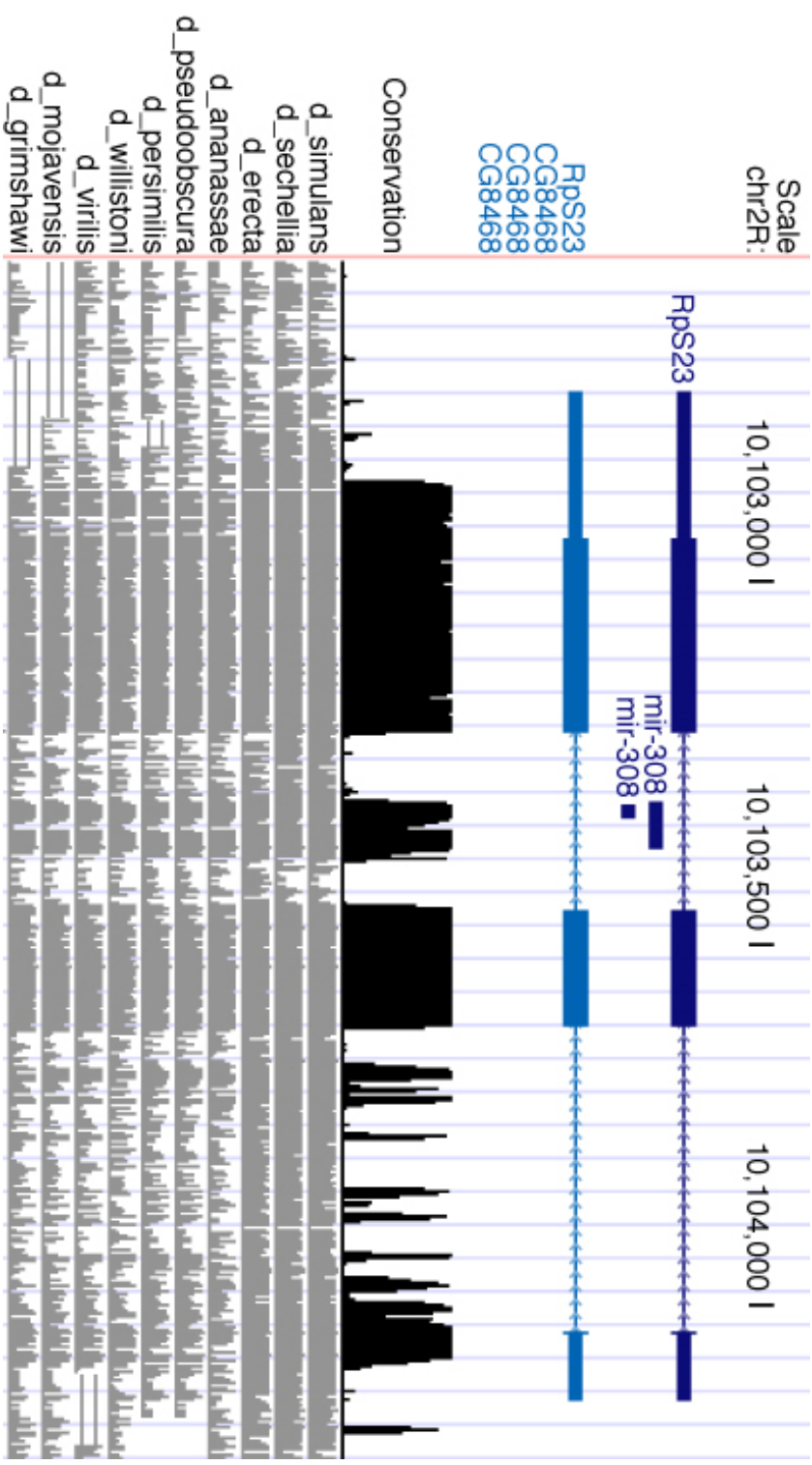


Figure 9. MiR-308 gene is conserved among 11 *Drosophila* species. Locus of the miR-308 gene is shown in the second intron of RpS23 gene, on the second chromosome of *Drosophila* melanogaster. Gray peaks indicate the highly conserved regions in different *Drosophila* species. Diagram is generated by UCSC genome browser.

dMyc's transcript is a predicted target of miR-308

miR-308 belongs to the *Drosophila* miR-2 conserved family of microRNAs. Members of miR-2 family (miR-2a, miR-6, miR-11, miR-13 and miR-308) are predominantly expressed during embryogenesis and share a vast number of predicted targets based on their common 6 nucleotide seed sequence (5′-AUCACA-3′) (Aravin et al., 2003). I identified the predicted targets of mir-308 by using two available algorithms, TargetScanFly (Ruby et al., 2007) and microRNA.org (Enright et al., 2003). Both indicated that a conserved sequence in the 3′-UTR region of dMyc's transcript is a putative target of miR-308 (Figure 10) and other members of the miR-2 microRNA family (Figure 11).

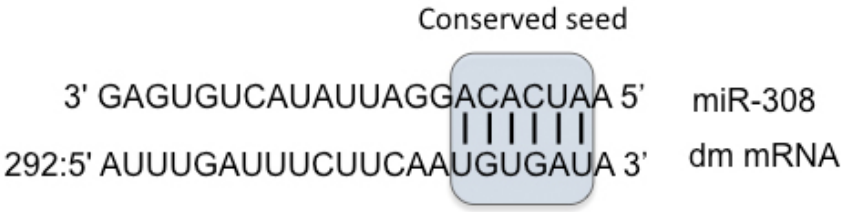
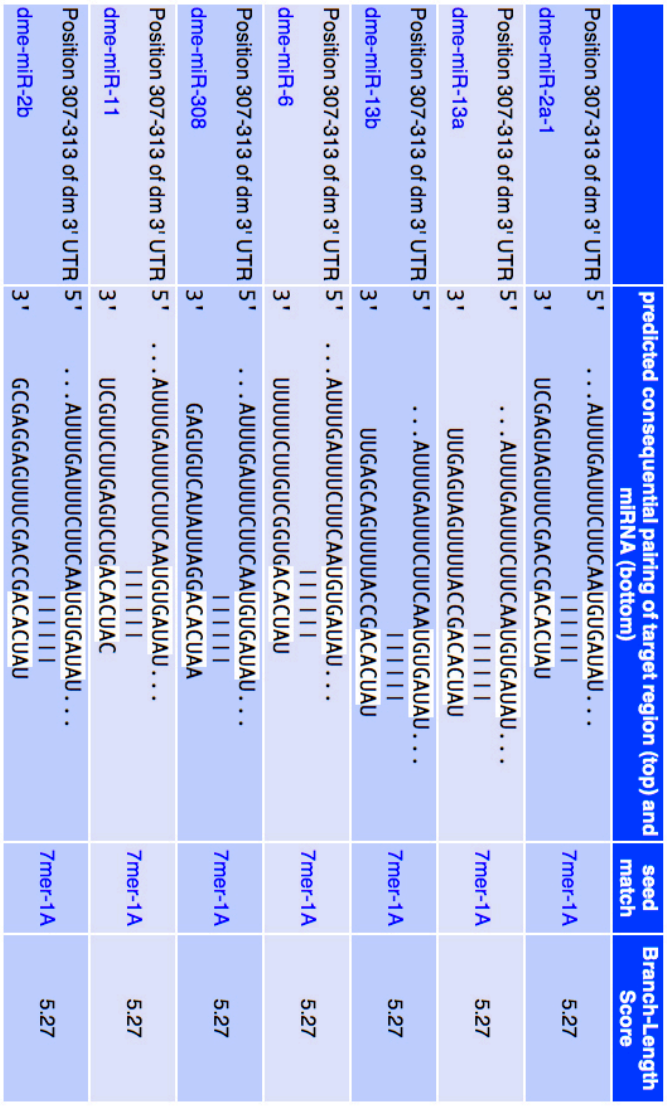


Figure 10. miR-308 has a conserved seed sequence match for dMyc mRNA. The full mature sequence of microRNA miR-308 is shown on the top and part of the untranslated transcript of dMyc is shown at the bottom. The number 292 denotes the number of nucleotide form the last translated nucleotide on the dMyc mRNA. The box and vertical lines indicate the alignment of the complementary seed sequence in the conserved region of the miR-308 and dMyc mRNA.



	predicted consequential pairing of target region (top) and miRNA (bottom)	seed match	Branch-Length Score
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-2a-1	3' UCGAGUAGUUUCGACCGACACUAU	7mer-1A	5.27
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-13a	3' UUGAGUAGUUUUACCGACACUAU	7mer-1A	5.27
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-13b	3' UUGAGCAGUUUUACCGACACUAU	7mer-1A	5.27
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-6	3' UUUUUCUUGUCGGUGACACUAU	7mer-1A	5.27
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-308	3' GAGUGUCAUAUUAGGACACUAA	7mer-1A	5.27
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-11	3' UCGUUCUUGAGUCUGACACUAC	7mer-1A	5.27
Position 307-313 of dm 3' UTR	5' ...AUUUGAUUUCUUCAAUGUGAUAU...		
dme-miR-2b	3' GCGAGGAGUUUCGACCGACACUAU	7mer-1A	5.27

Figure 11. Conservation of miR-2a family seed sequence match with dMyc mRNA. Each row of the table shows one of the miR-2a family members and their conserved sequence seed match for dMyc mRNA. 7mer-1A denotes the type of the seed sequence (6 nucleotide starting with an A). Scores denote the strength of the prediction and are calculated based on conservation and thermodynamic stability of RNA-RNA double helix (Target scan).

Based on the computational prediction, I hypothesized that dMyc's transcript is a target of miR-308 and that miR-308 is part of the negative feedback regulation of dMyc. To test the effect of miR-308 on the expression of dMyc *in vivo*, I chose to use a gain-of-function approach to increase levels of miR-308 in the embryos.

Generation of UAS-miR-308 transgenic *Drosophila*

Eukaryotic microRNAs are transcribed by RNA-Pol-II. Therefore, a system in which the full sequence of the microRNA stem-loop is under a strong RNA-Pol-II promoter can be used for a gain-of-function study. I designed this gain-of-function system by cloning a 300 bp genomic region containing the gene for miR-308, and ligation of this fragment into a pUAST plasmid. pUAST is an efficient tool, based on transposition of a p-element, for integration of a DNA fragment into the germ cells of *Drosophila* embryos (Figure 12)(Figure 13). The generated transgenic *Drosophila* lines express miR-308 under the control of the yeast Gal4 UAS enhancer. As a control, I generated flies expressing a mutated form of miR-308 with a change in the seed sequence from 5'-AUCACA-3' to 5'-GGAUCC-3' (Figure 12). I crossed these flies to those carrying Gal4 and confirmed the over-expression miR-308 and in embryos carrying UAS-miR-308 , but not in embryos carrying mutated-miR-308, by quantitative RT-PCR (Figure 14).

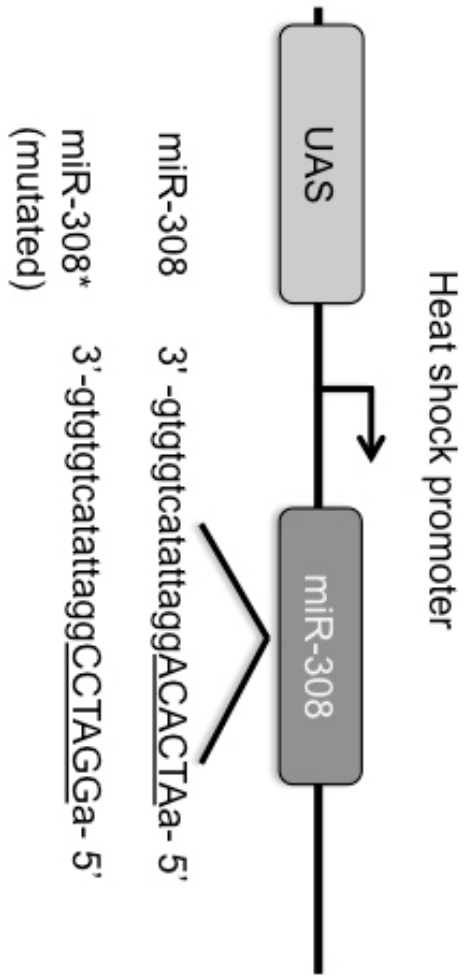


Figure 12. Generation of transgenic *Drosophila* expressing ectopic miR-308. Two transgenic *Drosophila* strains were designed based on the yeast UAS-Gal4 system. The diagram shows the structure of the transgenes miR-308 and mutated miR-308* under the heat shock promoter and UAS enhancer. The mutated miR-308* gene has 5 random mutations in its seed sequence.

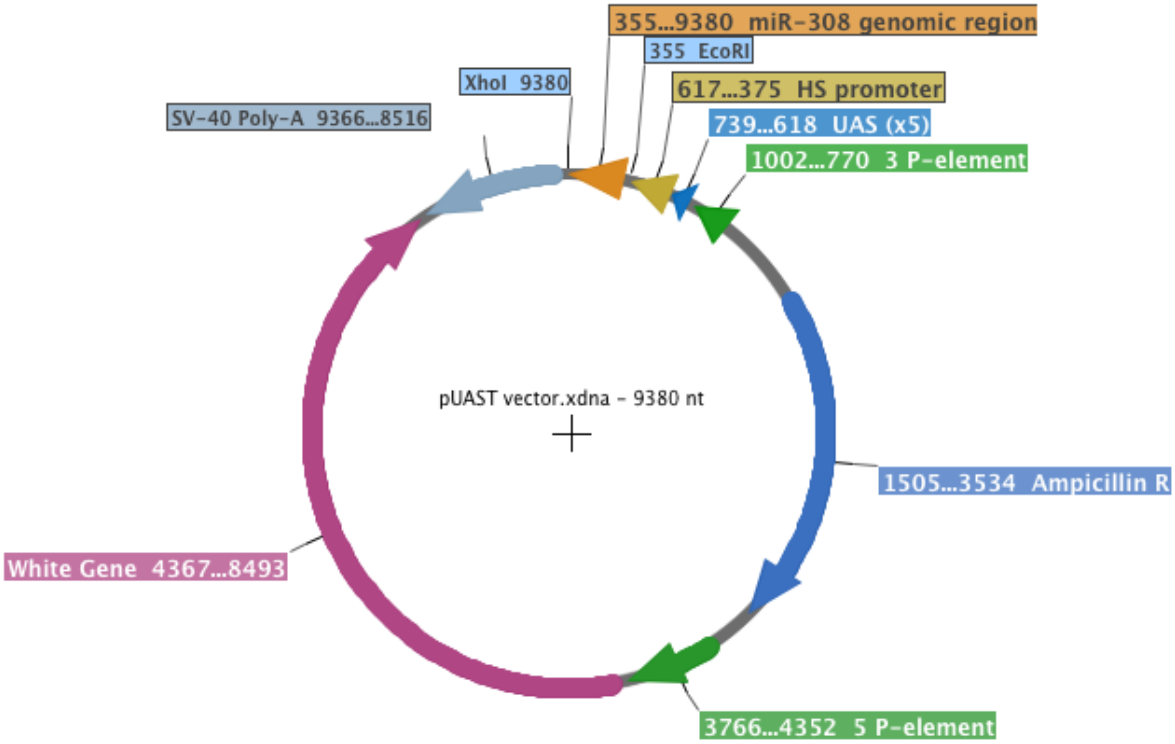


Figure 13. Map of the UAS-miR-308 construct in a pUASt plasmid backbone. The genomic region of the miR-308 or mutated miR-308* (Orange) is digested and ligated into a pUAST plasmid between the EcoRI and XhoI restriction sites, downstream to the heat shock promoter (Yellow) and five UAS enhancer regulatory sequences (Blue), and upstream to a poly-A sequence. The backbone plasma has a bacterial resistance gene (ampicillin) and a gene coding for the White protein that is the red eye marker (Purple). Two p-element sequences are used for integration of the region in between them into the *Drosophila* genome, by the enzyme transposase.

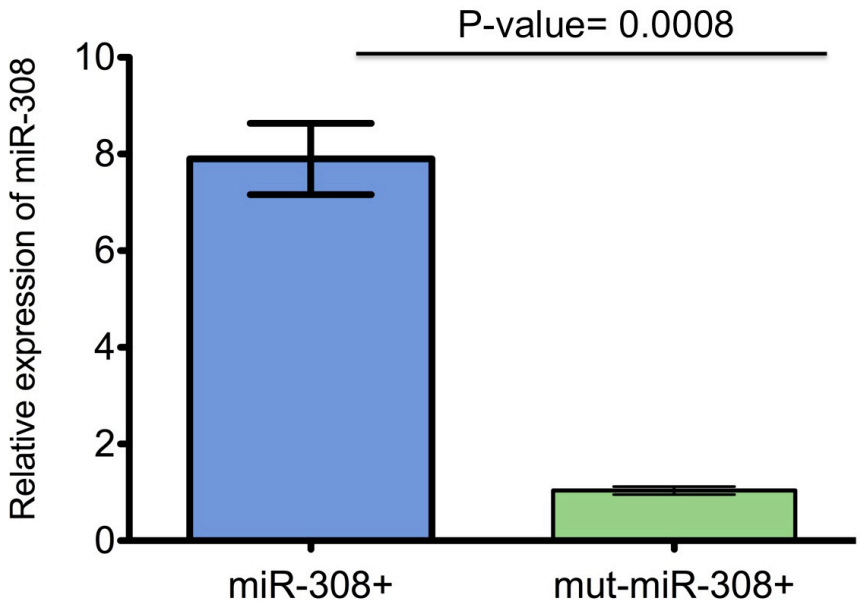


Figure 14. Ectopic expression of miR-308 in transgenic *Drosophila*. Bar chart shows the transcript levels of miR-308 in embryos with background levels (Control), embryos expressing ectopic miR-308 under UAS enhancer (miR-308+) and embryos expressing ectopic mutated miR-308* under the UAS enhancer (mut-miR-308+). Relative expression of miR-308 is normalized to miR-1. P-value is calculated base on student t-test in three independent biological replicates.

MiR-308 overexpression results in reduced dMyc transcript levels

microRNAs regulate the expression of their target genes primarily by inhibition of their translation. However, microRNAs can also destabilize their target mRNA (Baek et al., 2008). I tested whether the ectopic expression of miR-308 results in a decrease in dMyc transcript and protein levels. I collected 0-24 hour embryos from a cross of daughterless-Gal4 females and UAS-miR-308 males, and measured dMyc levels by q-RT-PCR. I observed a modest 14% (+/-5, n=5) reduction of dMyc transcripts in embryos expressing ectopic miR-308 compared to that of control (Figure 15).

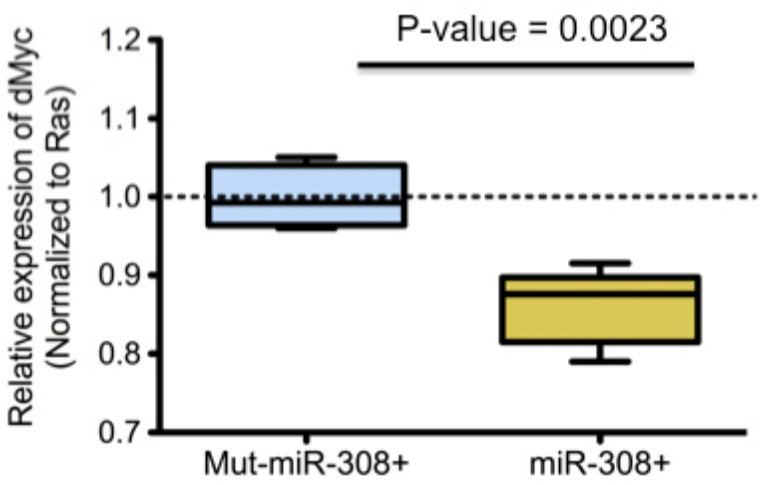


Figure 15. dMyc transcript levels decreases in response to an increase in miR-308 levels. Box and whisker plot shows the levels of dMyc transcript measured by q-RT-PCR in *Drosophila* embryos with elevated levels of mutated miR-308* (Blue) and embryos with elevated levels of miR-308 (Yellow), compared to that of control (dotted line). Relative expression of miR-308 is normalized to Ras. Results are shown as median (middle line) range (the upper and lower limits of the boxes) and standard deviations (error bars). P-value is calculated based on student t-test in four biological replicates.

miR-308 overexpression results in reduced dMyc protein levels.

MicroRNAs are known to repress the translation of their target transcripts by forming a double helix to their matching seed sequence. To test if miR-308 lowers the expression of dMyc protein, I carried out western blot with an antibody specific to dMyc protein. Despite the modest reduction in transcript levels, I observed a substantial 68% decrease in dMyc protein levels in embryos expressing ectopic miR-308, compared to that of control (Figure 16).

MiR-308 overexpression causes lethality in embryos.

I asked whether these animals show reduced survival, potentially caused by the reduction in dMyc protein accumulation. Our survival assay showed that these animals have a reduced survival rate. 19% (+/-9, n=97, P-value=1.1E-15, Fisher exact test) of them survived past 96 hours after egg deposition, compared to 77% (+/-6, n=81) of

control. Together, these data suggest that miR-308 is sufficient to mediate a negative regulatory mechanism controlling dMyc *in vivo*.

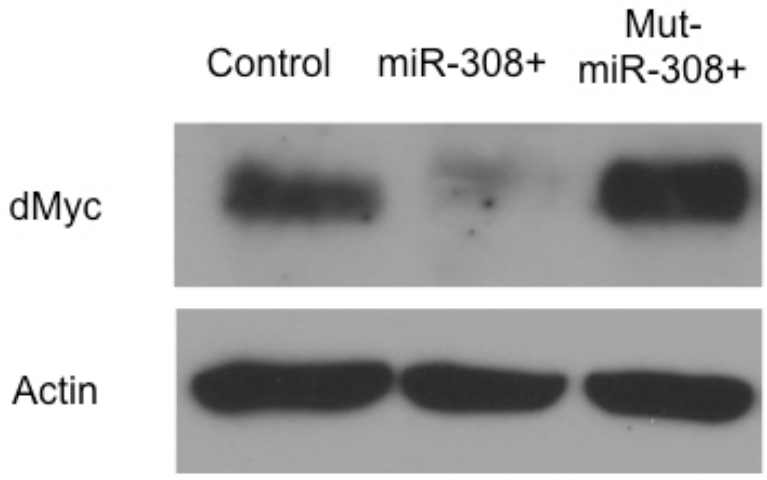


Figure 16. dMyc protein levels decreases in response to an increase in miR-308 levels. Western blot with antibody specific to dMyc (Top panel) shows a decrease in dMyc protein level in response to an increase in elevated levels of miR-308, but not mutated-miR-308. Anti-actin antibody is used as a loading control (Bottom panel).

In vivo knockdown of miR-308 leads to overexpression of dMyc.

To determine whether dMyc transcripts are normally repressed by miR-308, I depleted miR-308 in early embryos by injecting LNA (Locked Nucleic Acids)- modified oligonucleotides complimentary to the sequence of miR-308. LNAs are modified DNAs with higher stability and specificity that can effectively inhibit microRNAs (Ørom, Kauppinen, & Lund, 2006). Wild-type embryos 30 minutes after egg deposition were injected with either a LNA inhibitor against miR-308 or with injection buffer. Quantitative RT-PCR showed that the inhibition of miR-308 causes an 18% (+/-4, n=3) increase in dMyc mRNA levels (Figure 17). Immunoblotting showed a 47% increase in dMyc protein accumulation after inhibition of miR-308, compared to control (Figure 18). These results show that miR-308, despite its low abundance (Aravin et al., 2003),

constantly represses dMyc levels during embryogenesis. These data, taken with our chromatin-IP study demonstrating dMyc binding to the locus of *miR-308* in wild-type embryos, suggests a constant feedback loop between dMyc and miR-308 that limits the accumulation of dMyc protein during embryogenesis. Since other members of miR-2 family share the same seed sequence, it would be interesting to determine whether dMyc is under repression by other members of miR-2 family.

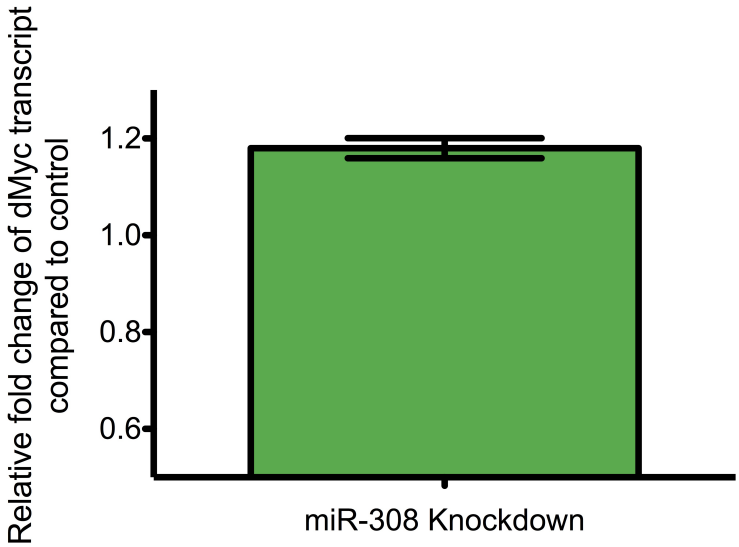


Figure 17. Knockdown of miR-308 causes an increase in dMyc mRNA level. Levels of dMyc transcript in embryos injected with injection buffer (Blue) and embryos injected with anti-miR-308 LNA (Green) measured by q-RT-PCR. The error bar indicates the standard deviation of three biological replicates (n=3).

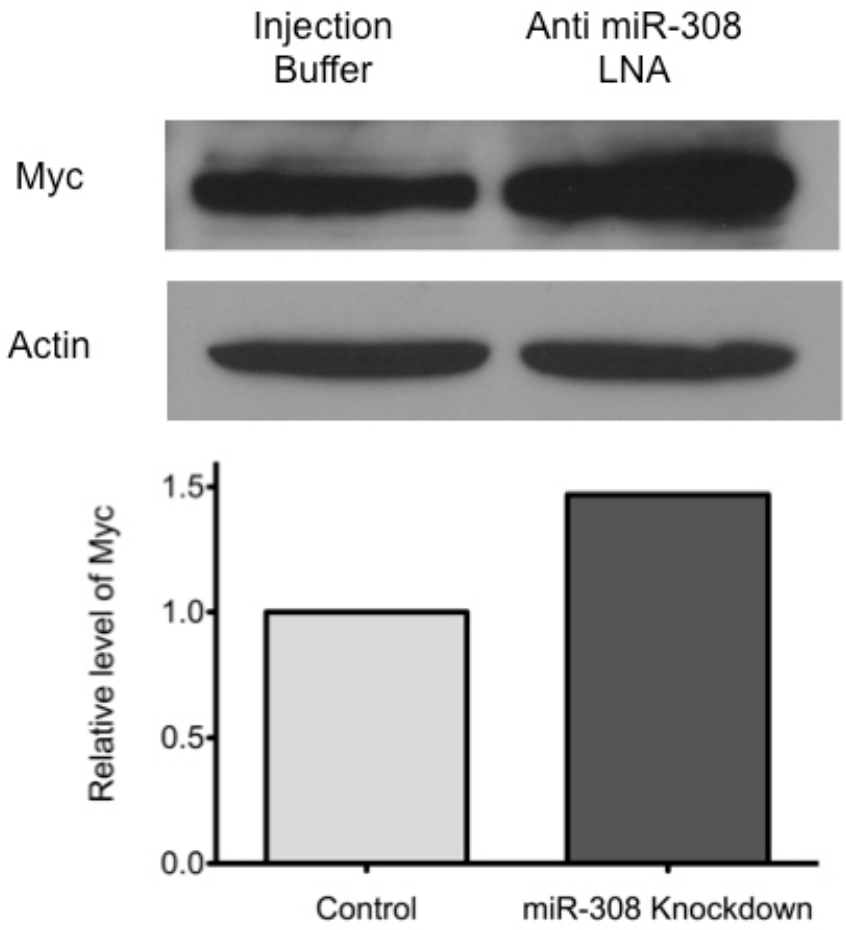


Figure 18. Knockdown of miR-308 causes an increase in dMyc protein level. Western blot with antibody specific to dMyc in in embryos injected with PBS and embryos injected with inhibitor of miR-308. Actin is used as loading control. Bar graph shows the normalized quantification of the western blot bands of only one replicate, using ImageJ software. Bar graph is shown for indicating the difference between observed quantities and has no statistical implication.

MiR-308 can rescue the dMyc overexpression phenotype.

Overexpression of dMyc can cause lethality. It is believed that this lethality is due to the induction of apoptosis by dMyc (Montero, Müller, & Gallant, 2008). In our experiments, dMyc protein levels decrease upon overexpression of miR-308. As previously reported, I also observed that dMyc overexpressing embryos do not survive beyond 72 hours after egg deposition (Khan et al, 2009). I sought to determine whether miR-308 could rescue the dMyc overexpression lethal phenotype. By meiotic

recombination of the two transgenes on third chromosome, I generated flies expressing both dMyc and miR-308 under the control of separate UAS enhancers that are responsive to the Gal4 transcription factor. Doubly transgenic flies were crossed to flies expressing Gal4 protein ubiquitously in embryos (daughterless-Gal4). Immunoblotting showed that dMyc protein accumulation was balanced by the addition of UAS-miR-308; it resembled wild type levels rather than ectopic levels (Figure 19). Consistent with these results, when crossed to Gal4 expressing flies, 40% (+/-12, n=84) of these double UAS transgenic animals survived beyond 72 hours, compared to 0% (n=94, p-value=9.7E-14, Fisher exact test) UAS-dmyc alone and 19% (+/-9, n=97, p-value=4E-4, Fisher exact test) UAS-miR-308 alone (Figure 20). Overall, these results show a role for miR-308 to secure the balanced accumulation of dMyc during development. miR-308's response to elevated dMyc levels shows that this regulation is not passive and is precisely correlated to dMyc's levels.

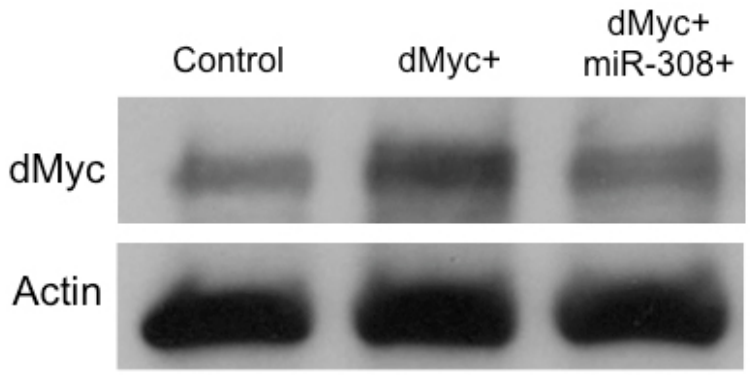


Figure 19. Levels of dMyc protein can be balanced by ectopic expression of miR-308. Western blot showing the dMyc protein level in control embryos, embryos with overabundance of dMyc and embryos with overabundance of both dMyc and miR-308 (Top panel). Actin is used as loading control (Bottom panel).

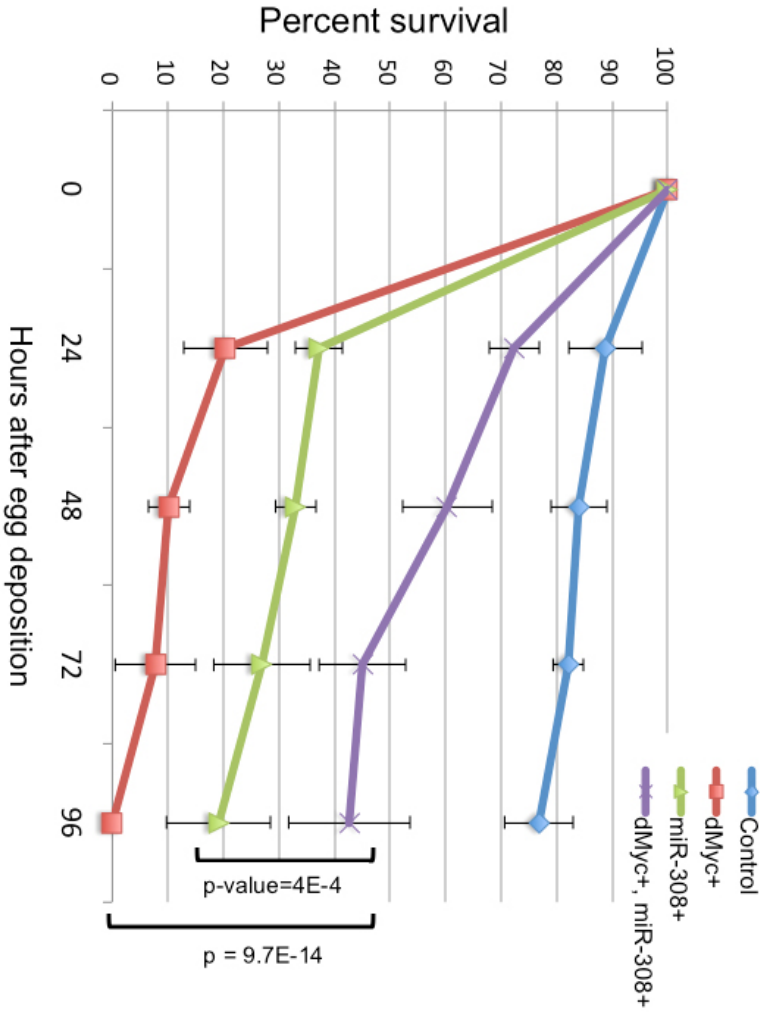


Figure 20. miR-308 can rescue the dMyc+ phenotype. Survival assay of the four genotypes: control (Blue), miR-308 overexpressing animals (Green), dMyc overexpressing animals (Red) and animals having overexpression of dMyc and miR-308 (Purple). Data are presented as means ± standard deviation of three independent replicates. P-values are calculated by Fisher exact test.

Functional relationship between dMyc targets and miR-308 targets.

In an effort to understand the broader significance of the feedback regulation between dMyc and miR-308, I examined the degree to which dMyc's transcriptional regulation of downstream targets may be antagonized by miR-308. I analyzed the targets of dMyc in embryogenesis using whole genome expression profiling. By crossing flies carrying Gal4 to flies carrying two ectopic copies of UAS-dmyc, I obtained embryos with

elevated levels of Myc. Total RNA from this cross and the control wild-type embryos was obtained and analyzed using Affymetrix GeneChip *Drosophila* Genome 2.0 Array. Differentially expressed transcripts were selected ($P<0.05$, FDR= 17%) for downstream functional analysis. Out of 624 affected genes, 607 transcripts were up-regulated and 17 transcripts were down-regulated. I considered the function of up-regulated targets of dMyc. I used a MGSA Byesian network model (Bauer, Gagneur, & Robinson, 2010) in Ontologizer 2.0 software to categorize those predicted targets according to their annotation of biological process (Bauer, Grossmann, Vingron, & Robinson, 2008). This model categorizes a set of genes based on their functional annotation and assigns each category a score that corresponds with the significance of enrichment. Results show that dMyc's targets in embryogenesis are mainly involved in ribosome biogenesis, t-RNA metabolism and RNA modification, consistent with previous reports (Grewal et al., 2005) (Figure 22).

Given our observed inverse relationship between miR-308 and dMyc, I then determined whether predicted targets of miR-308 are functionally related to targets of dMyc in the context of its over-expression. I identified 729 conserved targets of miR-308 using microrna.org tools (Enright et al., 2003), and classified these predicted targets using the method described above. Results show that miR-308 predicted targets are mainly involved in organ morphogenesis and development (Figure 22). This potential role of miR-308 in repression of sensory differentiation and morphogenesis is consistent with dMyc function in promoting a growth and proliferation program. Our analysis suggests that dMyc and miR-308 may have an overlapping role in orchestrating the complex series of events that balance growth and differentiation during development.

Between the identified 624 dMyc targets and 730 miR-308 predicted targets, I identified 26 common targets (Supplementary Fig. 1). This overlap was significantly different from any overlap that could occur randomly by this analysis (P-value<0.008, hypergeometric distribution) (Figure 21). Functional analysis of miR-308 and dMyc's common targets showed a significant enrichment in bristle morphogenesis (Figure 22). The observation that mir-308 may decrease expression of these genes while Myc activates them suggests a specific role for miR-308 in fine-tuning dMyc's function as an inhibitor of morphogenesis.

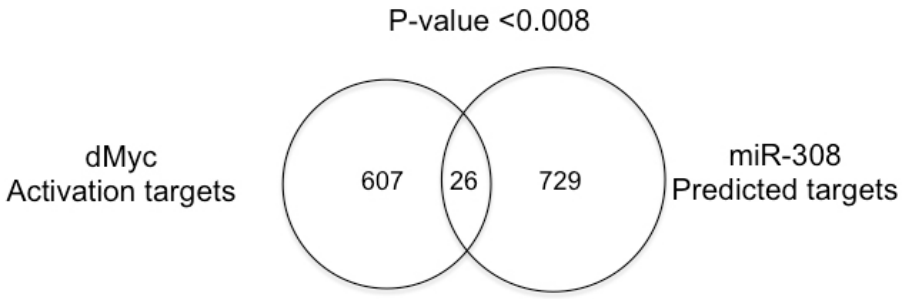


Figure 21. Overlap between dMyc and miR-308 targets. Overlap of dMyc targets and miR-308 predicted targets. P-value shows the statistical analysis of significance of the overlap using hypergeometrical distribution.

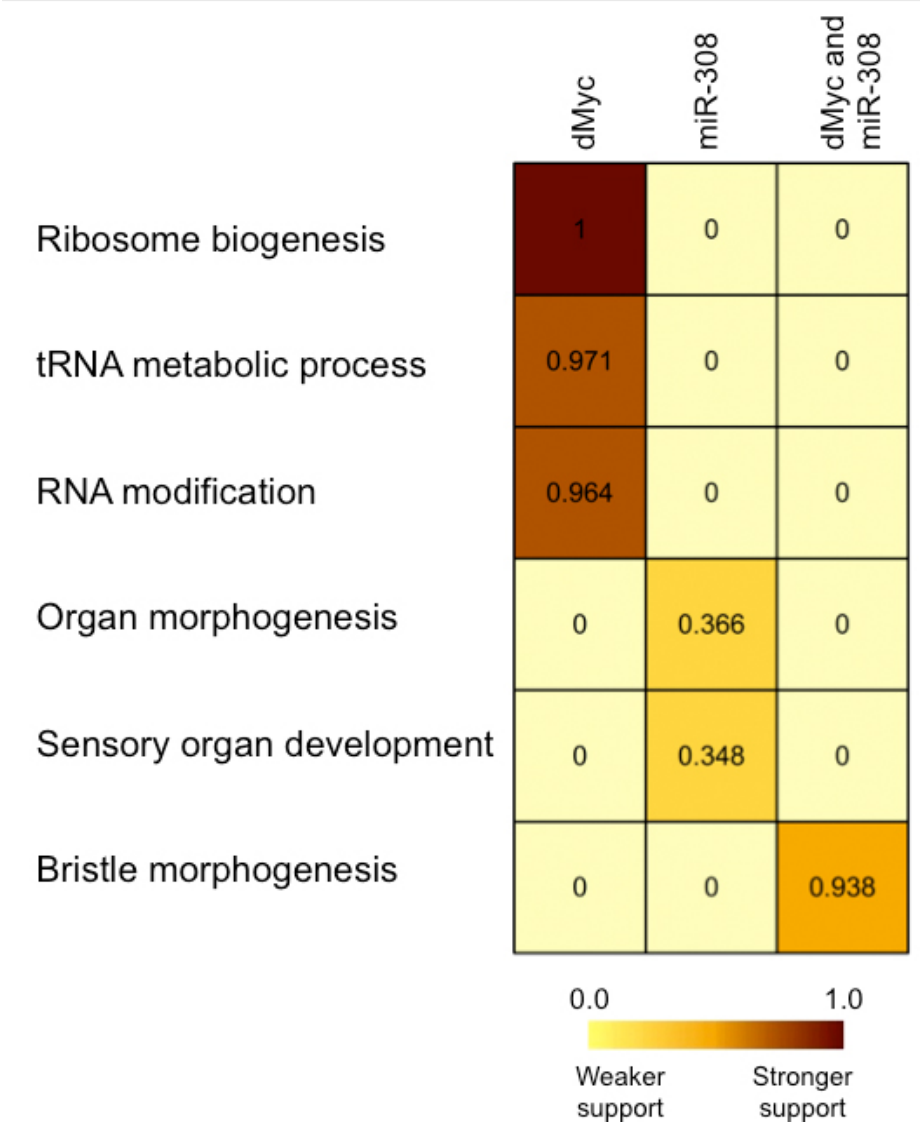


Figure 22. Functional analysis of dMyc and miR-308 targets. Heat map MGSA functional analysis for dMyc up-regulation targets (Left column), miR-308 predicted targets (Middle column) and their common targets (Right column). Yellow color shows a weaker support and dark brown color shows stronger support for enrichment.

CHAPTER 4: LOCALIZATION OF DMYC IN HISTONE LOCUS BODIES

Despite its established role in regulation of transcription by binding to the target loci, new roles are being discovered for Myc. During the antibody stainings to examine Myc protein levels in our lab, I observed clusters of cells containing punctate spots of dMyc. As a transcription factor, the general nuclear localization that I observed in most cells was expected, however I was curious to determine the identity of the dMyc puncta. In this chapter, I will explain a sequence of experiments that led me and my colleagues to discover sub-cellular localization of dMyc protein and propose a possible role for it in transcription and processing of histone transcripts.

dMyc rarely localizes to the nucleolus

Although *in vitro* experiments have not shown dMyc to be associated with ribosomal DNA in *Drosophila*, dMyc abundance correlates with the size and integrity of nucleoli (Marinho, Casares, & Pereira, 2011)(Grewal et al., 2005). Therefore, I began my investigation by double staining ovaries, larval salivary glands and embryos with antibodies specific for dMyc and fibrillarin, a marker for nucleoli.

In ovaries, I observed broad dMyc accumulation with many dMyc puncta located within the nuclei of nurse cells. Myc appeared excluded from nucleoli in both nurse cells and follicle cells. Different from the nurse cells, I observed no dMyc puncta but general nuclear staining of dMyc in follicle cells (Figure 23). In the salivary glands of larvae and

stage 10 embryos, dMyc dMyc localized to the nucleus and was largely excluded from the nucleolus (Figure 24) and (Figure 25).

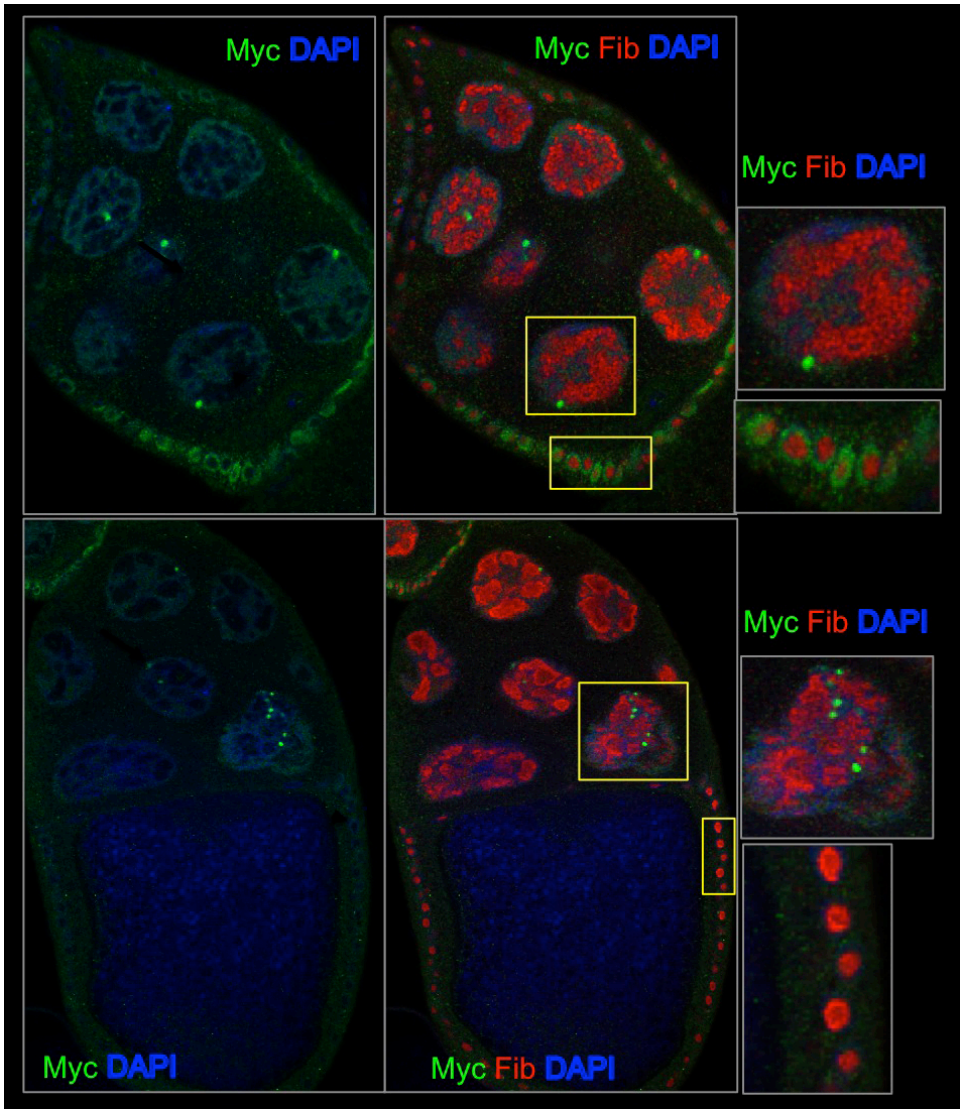


Figure 23. dMyc rarely localizes to nucleolus of follicle cells. dMyc (green), fibrillarin (red) and DAPI label stage 8 (top) and 10 (lower) egg chambers. Arrows label a nurse cell, and arrowheads label a follicle cell.

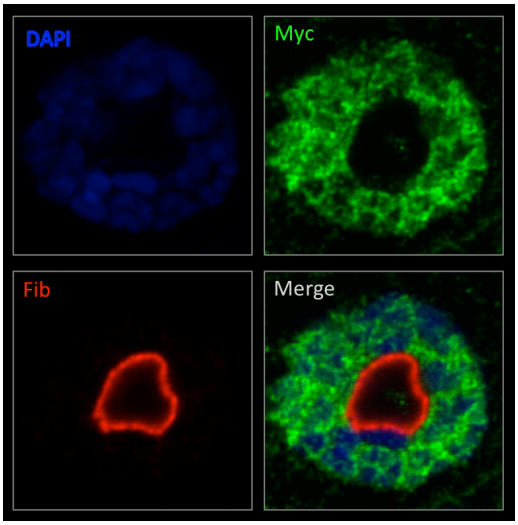


Figure 24. dMyc rarely localizes to nucleolus of salivary gland cells. A larval salivary gland nucleus labeled with DAPI (blue), dMyc (green) and Fibrillarin (red) shows the exclusion of dMyc from the nucleolus.

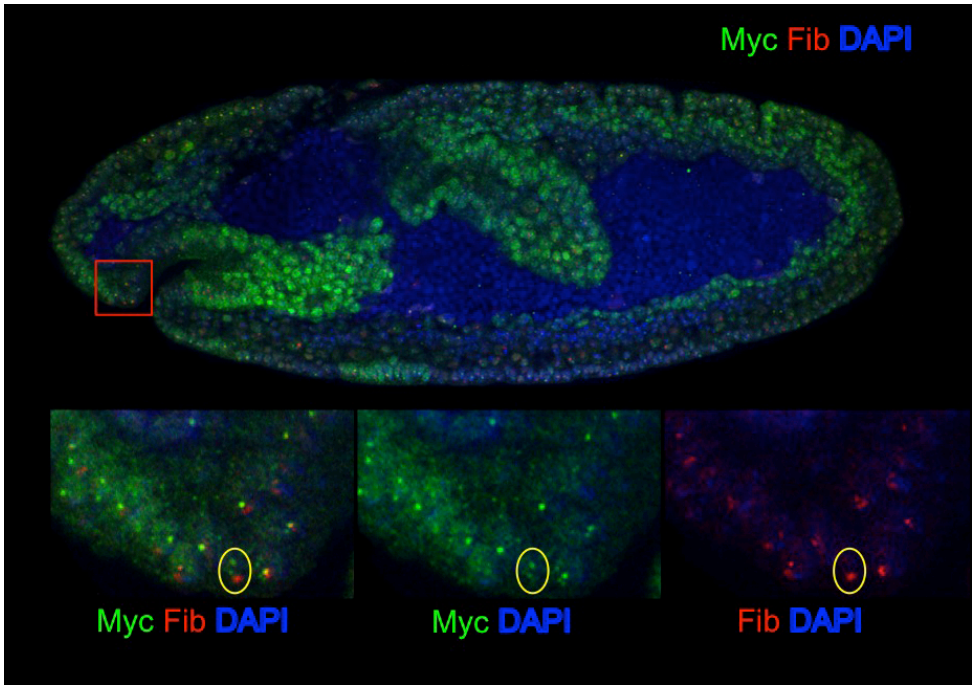


Figure 25. dMyc rarely localizes to nucleolus of embryo cells. A stage 10 embryo labeled with dMyc (green), fibrillarin (red) and DAPI (blue) showing minimal overlap of dMyc and fibrillarin (bottom three panels, note the cell within the yellow circles with a bright fibrillarin domain that lacks dMyc).

Fibrillarin is present in a sub-nuclear organelle known as the Cajal Body (Liu et al., 2006). In embryos, dMyc infrequently overlapped fibrillarin (Figure 1C, at stage 8, 63 dMyc puncta also contained fibrillarin, n=213). The overlap that I observed with dMyc and fibrillarin in both larvae and embryos coincided with the Cajal Body, not the nucleolus.

dMyc protein co-localizes with coilin

Given the presence of fibrillarin in CBs and the minimal overlap of Myc and fibrillarin, I next hypothesized that the Myc puncta were localized to CBs. Therefore, I double stained ovaries, larvae and embryos with anti-Myc and anti-coilin antibodies (a gift from Dr. Joseph Gall).

In salivary glands of third instar larvae, I observed co-localization of dMyc with most of the large coilin bodies (dMyc appeared in 22 out of 27 large coilin bodies) (Figure 26). During embryogenesis, dMyc puncta appeared following the onset of cellularization. In the cellular blastoderm, dMyc puncta almost always overlapped coilin (75 Myc positive CBs, n=78 CBs). In cells of the early postblastoderm mitotic domains, dMyc protein exhibited puncta that overlapped the CBs of those cells (39 Myc positive CBs, n=41, Figure 2B, upper panels) (Figure 27). Later, at approximately stage 11, Myc protein appeared diffuse in cells of the endoderm and the visceral musculature of the mesoderm. In the head regions and ectoderm, Myc protein appeared in puncta that overlapped CBs (32 Myc positive CBs, n=37) (Figure 29).

In ovaries, Myc and coilin localized to the same nuclear bodies in nurse cells. Beyond stage 2 of oogenesis, Myc bodies almost always contained coilin (49 in 50 Myc bodies contained coilin), although less than half of Cajal bodies contained Myc (21 Myc

positive CBs, n=57, Figure 2A). In the germarium and in follicle cells, Myc appeared diffuse and not obviously localized to any coilin-containing body (Figure 28).

These data show that dMyc and coilin co-localize broadly in *Drosophila* tissues, and the localization may correspond to CBs, HLBs or both. I investigated the possibility that the Myc and coilin overlap occurs in HLBs.

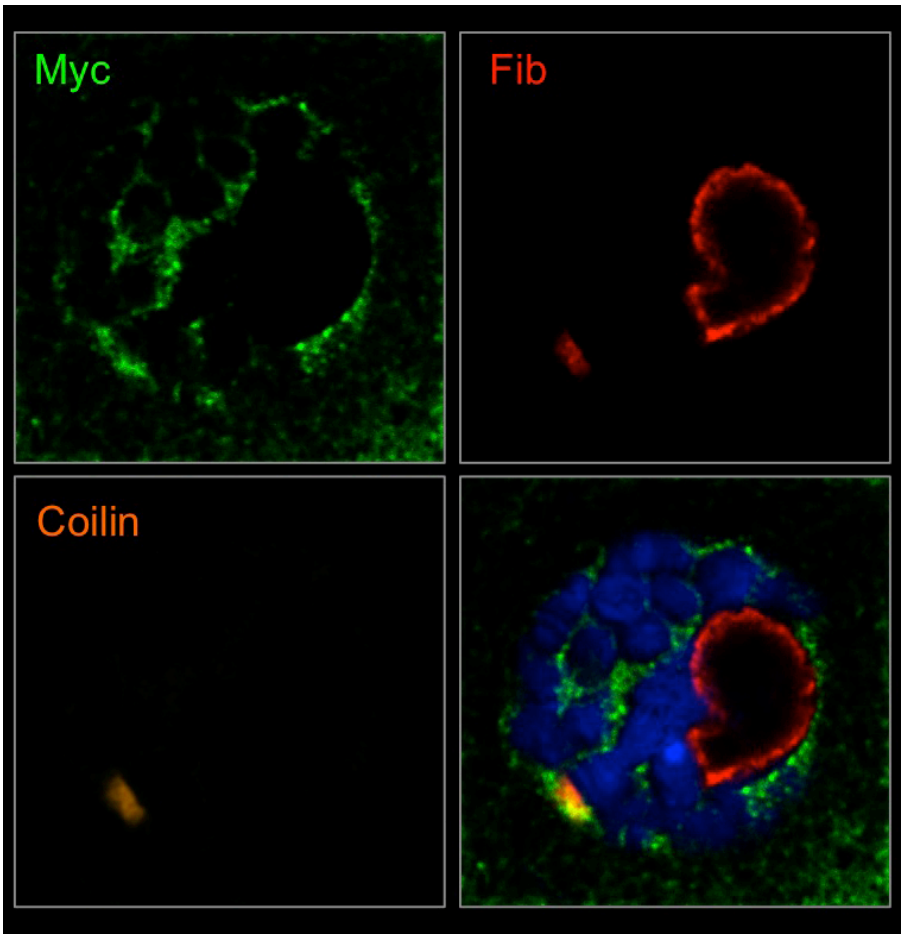


Figure 26. dMyc co-localizes with large coilin bodies in salivary glands. A larval salivary gland cell labeled with dMyc (green), fibrillarin (red), coilin (orange) and DAPI, showing that Myc, fibrillarin and coilin overlap outside of the nucleolus.

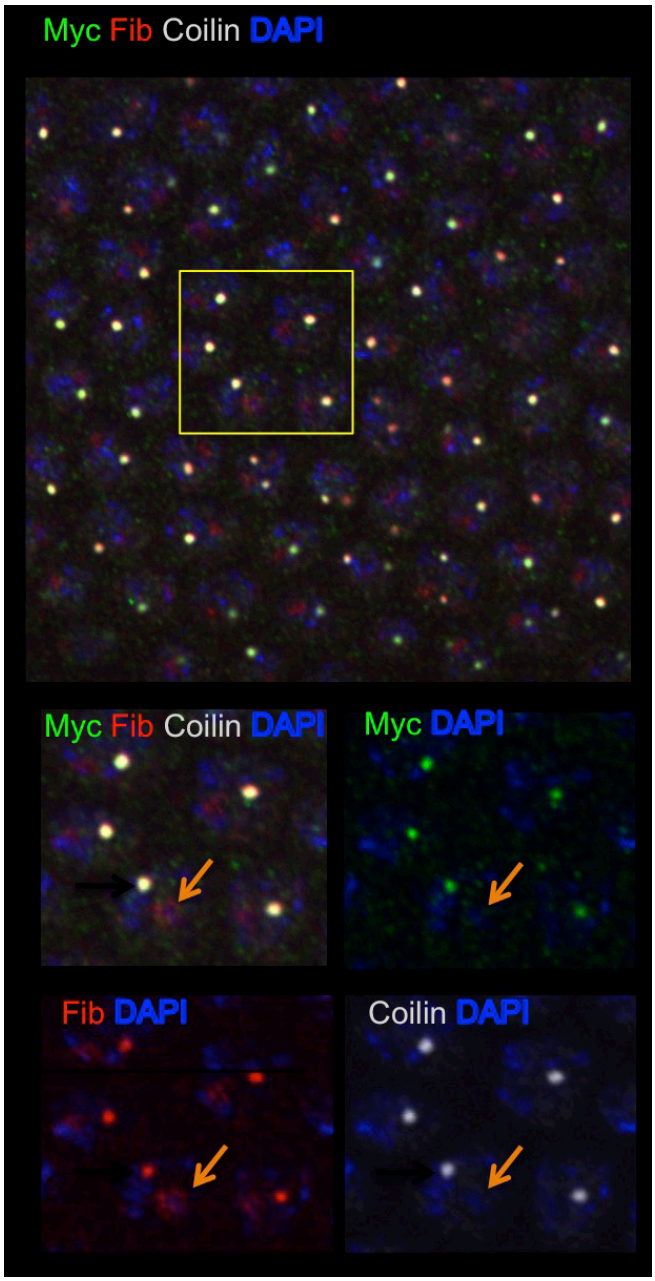


Figure 27. dMyc localizes in most of the coilin bodies in early embryos. A stage 6 embryo labeled with dMyc (green), fibrillarin (red), coilin (white) and DAPI, showing that locations where dMyc and fibrillarin overlap are puncta containing coilin (shown by the white arrow in the higher magnification boxes below). dMyc does not overlap with fibrillarin in the nucleolus (shown by the orange arrow).

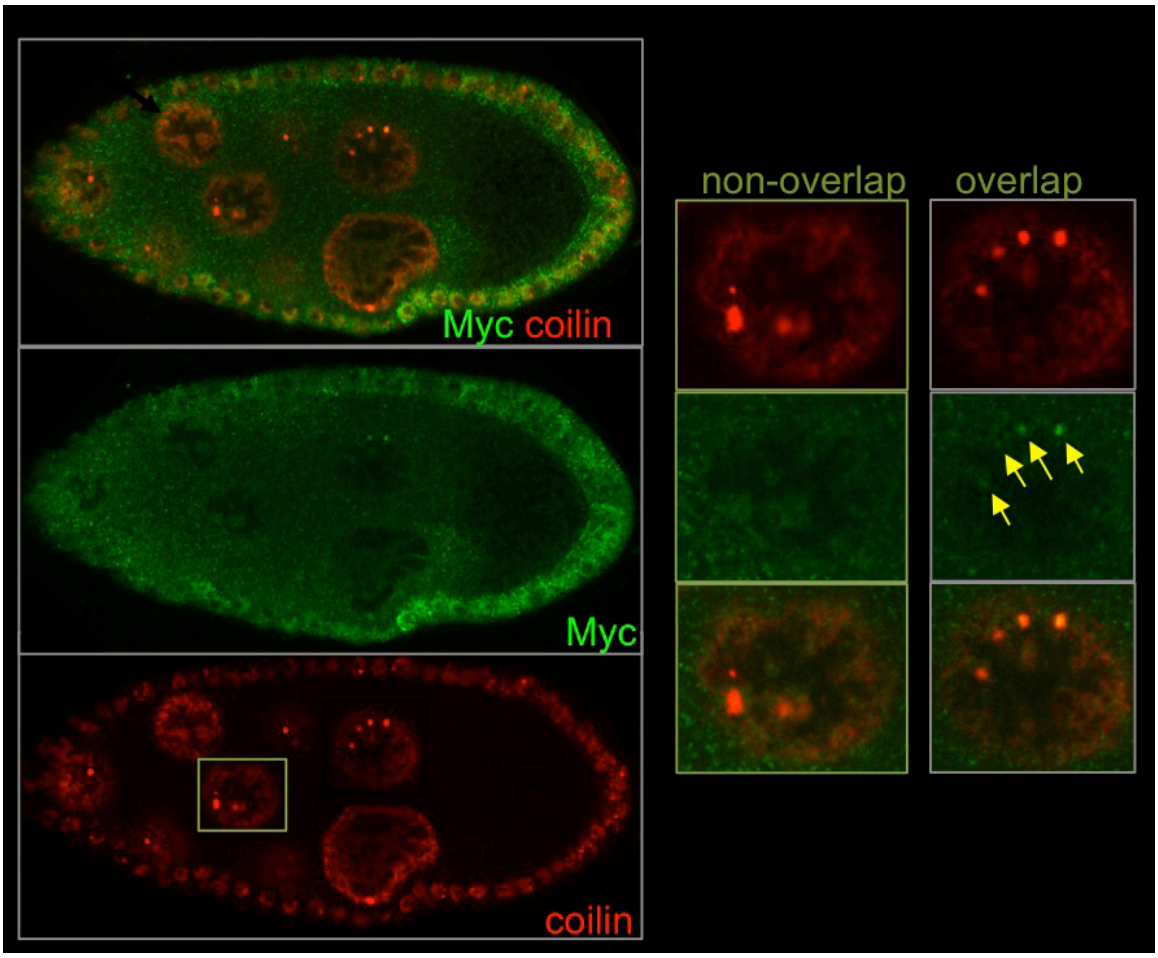


Figure 28. Overlap of dMyc and coilin in the ovaries. A stage 9 egg chamber labeled with Myc (green) and coilin (orange). The light gray arrow points to a nurse cell, and arrowhead points to a follicle cell. A nurse cell lacking overlap of Myc with coilin is shown (nurse cell in the green boxes, panels 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)

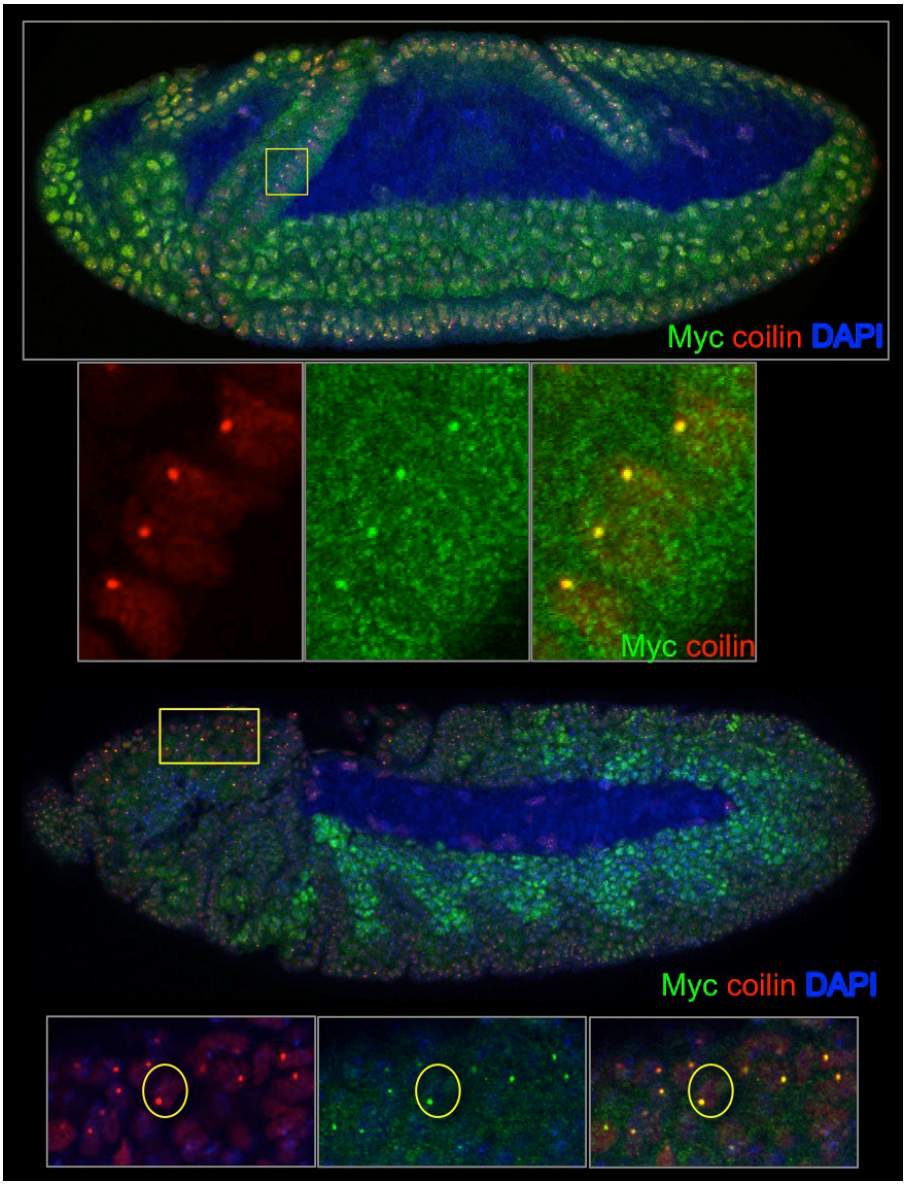


Figure 29. dMyc colocalizes with coilin in stage 8 and 11 embryos. A stage 8 embryo (top) and stage 11 embryo (bottom) labeled with Myc (green), coilin (red) and DAPI (blue). Myc is generally diffuse throughout nuclei at stage 8 except for parts of the cephalic furrow (higher magnification boxes below the top embryo; the region magnified is indicated in the yellow box) and future ectoderm. By stage 11, Myc appears in puncta of the ectoderm and head regions, the latter is shown magnified in the yellow box. These puncta contain coilin.

Myc and coilin localization occurs mainly in HLBs

The Histone Locus Body (HLB) is a similar nuclear body to Cajal Bodies, however it is distinct in that it contains the U7 snRNP and associates with the histone

gene loci in *Drosophila* (Liu et al., 2006)(Z. F. Nizami et al., 2010). HLBs and CBs can reside adjacent to each other or apart from each other, and both contain coilin (Liu et al., 2006)(Liu et al., 2009). Because I observed Myc and coilin together in the nucleus, I investigated whether Myc puncta were HLBs rather than CBs. To visualize HLBs along with coilin, I obtained transgenic flies expressing an HLB marker, Lsm11-EYFP under the control of Gal4 (a gift from Dr. Joseph Gall). Lsm11 is a protein component of the U7 snRNP, which is specific for HLBs (Z. Nizami, Deryusheva, & Gall, 2010). I induced expression of the transgene and triple-stained tissues with anti-Myc, anti-coilin and anti-GFP (embryos). In additional experiments, I stained wild type larvae and ovaries with anti-Lsm11 (also from Dr. Gall), anti-Myc and anti-coilin.

In salivary glands of third instar larvae, immuno-staining against Myc and GFP or Lsm11 showed the localization of Myc to HLBs; 81% of Lsm11 and coilin-containing HLBs included Myc (n=27) (Figure 30). Myc localization to CBs containing coilin but no Lsm11 was less common; Myc appeared in 20% of non-Lsm11 Cajal Bodies, n=29. In *Drosophila* egg chambers, Myc overlapped coilin and Lsm11 in nurse cell nuclei (Figure 31).

Similarly, in embryos, Myc puncta frequently contained both coilin and Lsm11 (Figure 32), suggesting that the majority of embryonic Myc bodies are HLBs (in 221 Myc puncta, 208 also contained both coilin and Lsm11). The degree of Myc and Lsm11 overlap depended on the germ layer and/or region of the embryo. For instance, 86% of HLBs were Myc positive in the ventral ectoderm of stage 10 embryos (n=117), however 13% of HLBs were Myc positive in the endoderm of the posterior midgut at the same stage (n=52).

These data show that Myc occurs in the HLB of both mitotic and endoreplicating cells. However, Myc does not localize to HLBs in all nurse cells of an egg chamber, nor does it localize to all HLBs in the cells of an embryo and larval salivary glands.

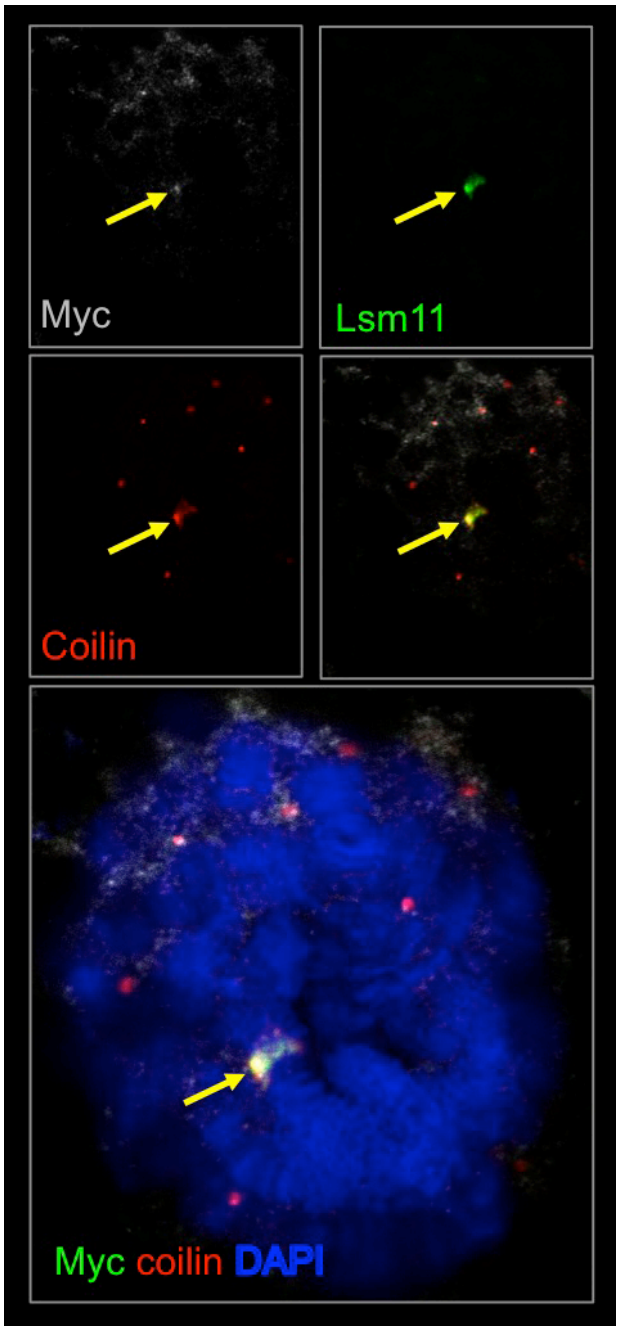


Figure 30. Myc localizes to histone locus bodies in salivary glands. A larval salivary gland nucleus labeled with Myc (white), coilin (red), Lsm11 (green) and DAPI (blue) shows overlap of Myc with coilin in the largest coilin-containing body.

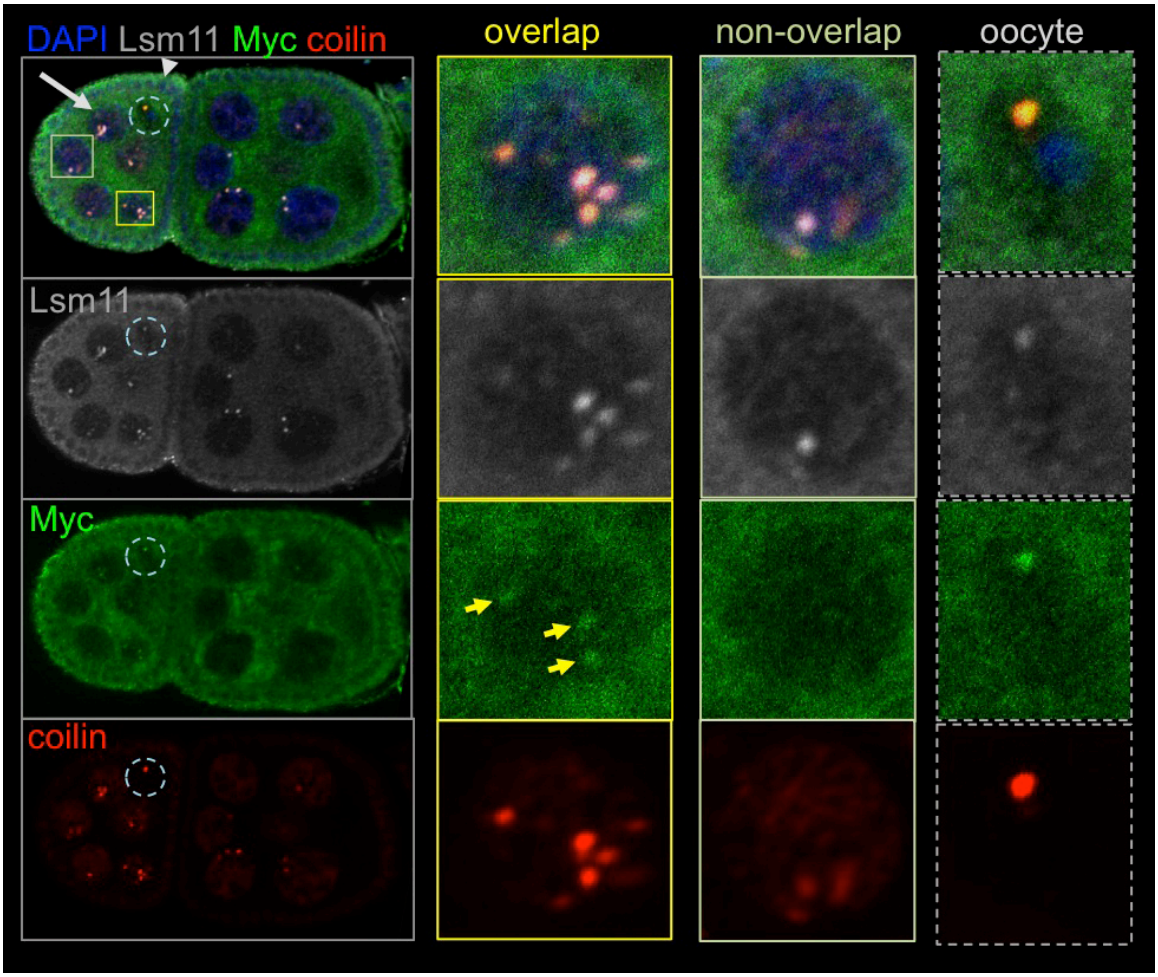


Figure 31. dMyc localizes to HLBs in ovaries. Two egg chambers, ~stages 5-6, labeled as indicated and showing that Myc, coilin and Lsm11 co-localize in nurse cells and the oocyte nucleus (the arrow labels a nurse cell and arrowhead labels a follicle cell; the oocyte nucleus is within the light blue dashed 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).

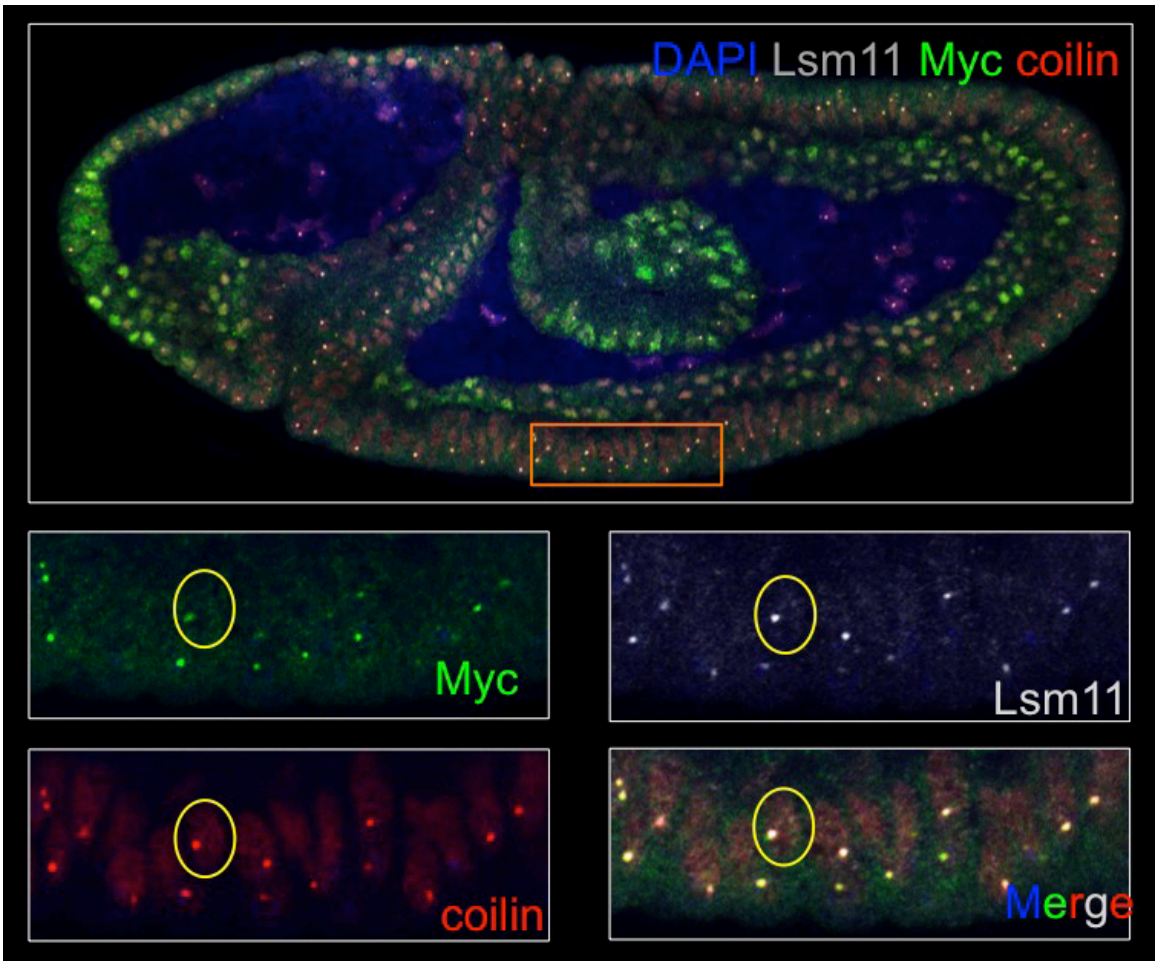


Figure 32. dMyc localizes to HLBs in stage 8-9 embryos. A wild type, stage 8-9 embryo labeled with Myc (green), coilin (red), Lsm11-EYFP (white) and DAPI (blue) showing that dMyc, coilin and Lsm11 co-localize to the majority of the bodies occurring in these embryos (lower panels show the cells in the orange box).

Myc localizes to HLBs only during replication

Because many different cell types showed dMyc in the HLB, however not uniformly within an egg chamber or embryo, I investigated whether dMyc localization to HLBs is cell cycle dependent. I stained embryos, larvae and ovaries with the monoclonal antibody MPM-2, which cross-reacts with phospho-epitopes of mitotic cells in many organisms (Edgar et al., 2001). In *Drosophila* embryos, MPM-2 recognizes the phospho-

epitope of a protein present in HLBs, but only in cells with active Cyclin E/Cdk2 (Frydman & Spradling, 2001).

I first examined all coilin-containing bodies, which may be CBs or HLBs, by staining embryos with MPM-2, anti-coilin and anti-Myc antibodies. dMyc appeared in 100% of the coilin and MPM-2 positive bodies (n=30). dMyc appeared in just 10% of CBs or HLBs lacking MPM-2, n=30 (Figure 33). I 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 (Figure 34), all of which are HLBs later in oogenesis (Liu et al., 2009).

To examine bodies identifiable as HLBs in cells undergoing replication, I stained ovaries and embryos with MPM-2, anti-Lsm11 (or anti-GFP) and anti-dMyc. dMyc and Lsm11 co-localized only in the presence of the MPM-2 epitope. HLBs containing both Lsm11 and MPM-2 were positive for dMyc (Figure 36). HLBs lacking the MPM-2 epitope also lacked dMyc (Figure 34) and (Figure 36). 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) (Figure 35) and (Figure 36).

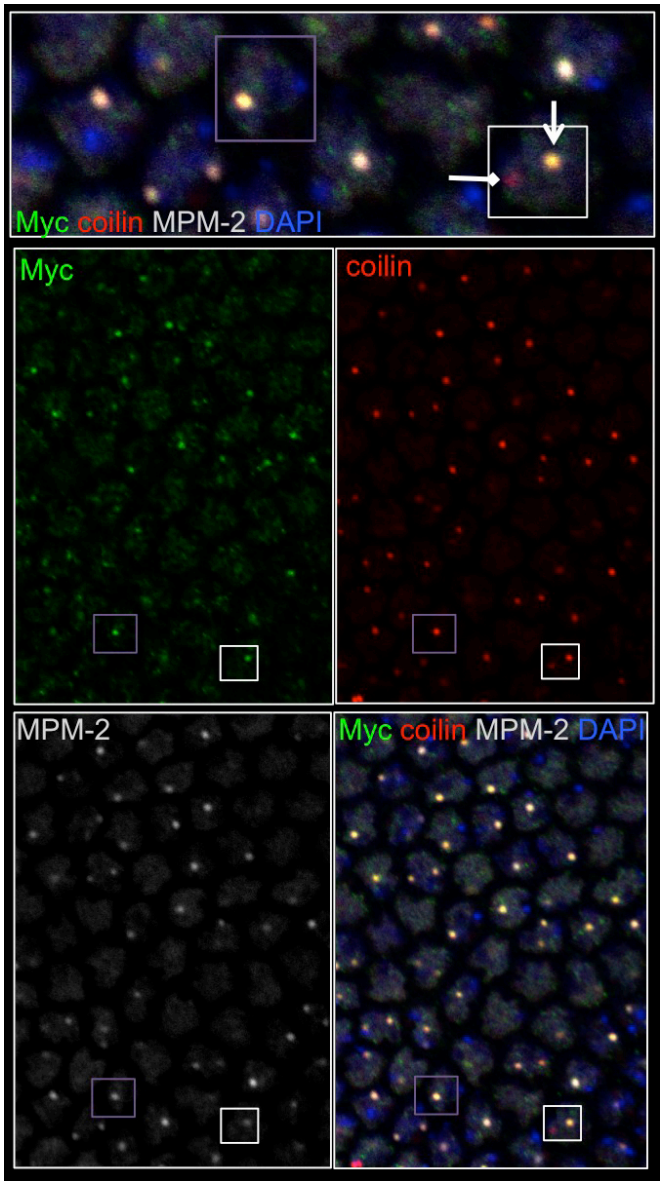


Figure 33. dMyc localizes to MPM2 containing HLBs in gastrulating embryo. The surface of an embryo at the onset of gastrulation, labeled with Myc (green), coilin (red) and MPM-2 (white) and DAPI, with a higher magnification of the merged image above. MPM-2 positive cells are replicating, and the MPM-2 bodies contain Myc and coilin (note cell in the purple boxes). One CB is evident with no MPM-2 or Myc present (in white box, HLB is labeled with an arrow, and the CB is labeled with a diamond-headed arrow).

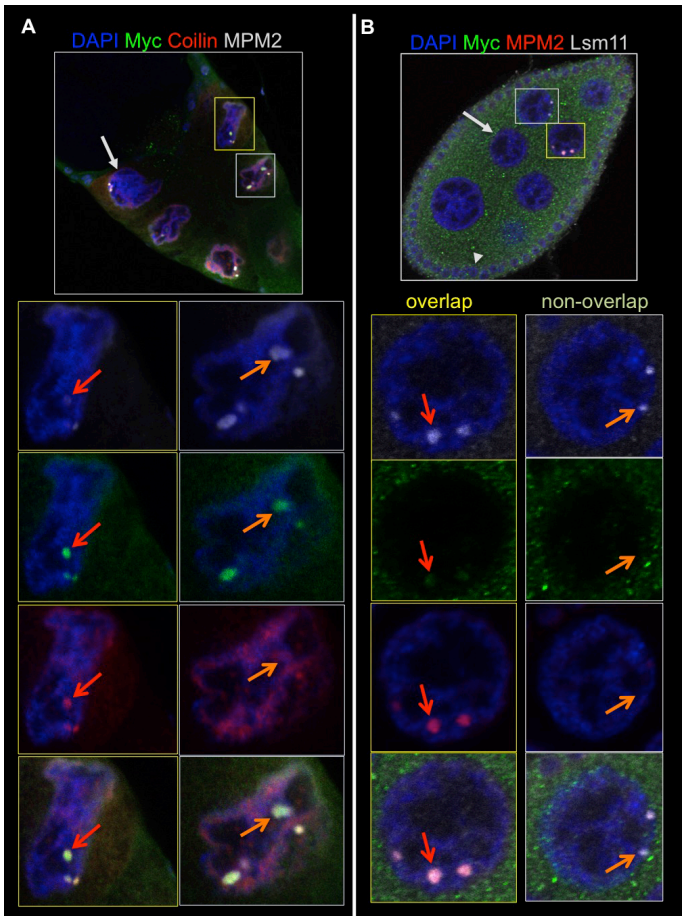


Figure 34. dMyc localizes to MPM2 containing HLBs in ovaries. (A) A stage 10 egg chamber is shown, labeled with Myc (green), coilin (red), MPM-2 (white) and DAPI, and a nurse cell is labeled with the light gray arrow. Myc, coilin and MPM-2 overlap in the HLB of the nurse cell in the yellow boxes, and MPM-2 and Myc overlap although coilin staining is weak in the HLB of the nurse cell in the gray boxes. (B) 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).

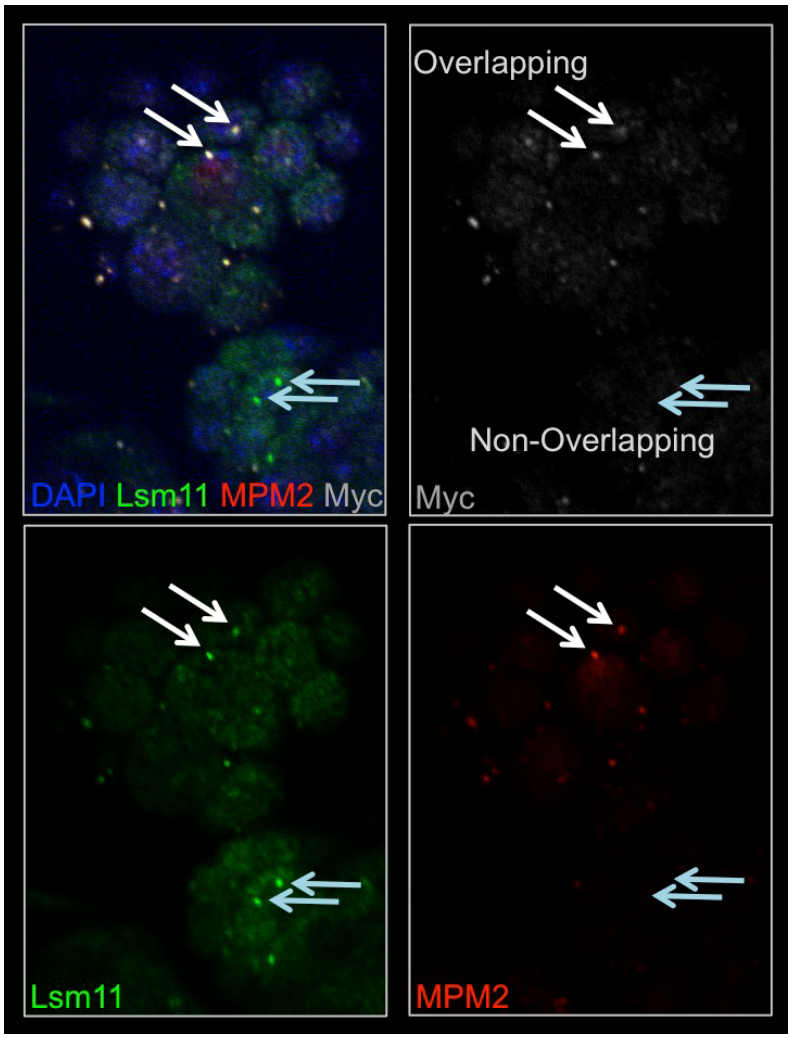


Figure 35. dMyc overlaps LSM11, only in the presence of MPM2, in embryo. A germband-retracted embryo labeled with Myc (white), MPM-2 (red), Lsm11 (green) and DAPI showing that HLBs containing MPM-2 and Lsm11 also contain Myc (white arrows), and HLBs that contain Lsm11 but not MPM-2 do not contain Myc (light blue arrows).

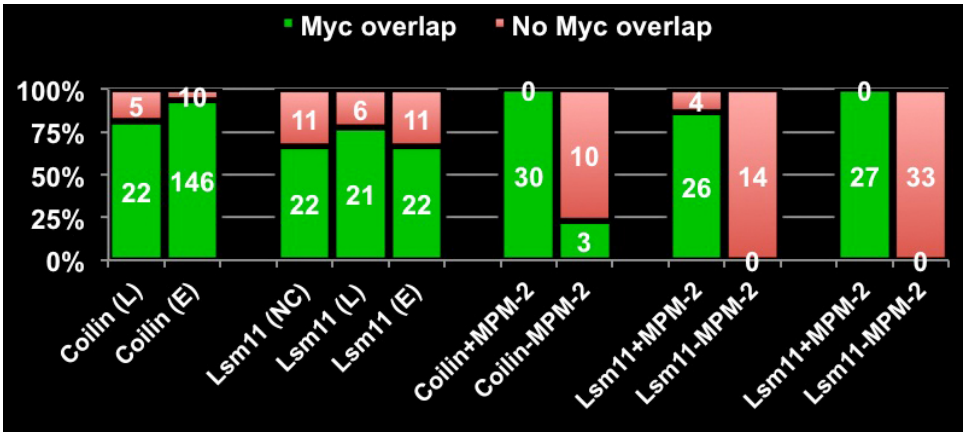


Figure 36. Quantification of dMyc overlap. A chart showing the numbers reported in the text of Myc-overlapping (green bars) and non-Myc-overlapping puncta containing coilin (C), Lsm11 (L), and MPM-2 in embryos (E and the third and fifth sets of bars), larvae (L) and nurse cells (NC and also the fourth set of bars).

CHAPTER 6: DISCUSSION AND FUTURE DIRECTION

In *Caenorhabditis elegans*, microRNAs are shown to participate in feedback circuits with key transcription factors, and these networks have a high capacity for regulation of target genes (Martinez et al., 2008). Here, I report the novel finding of a feedback circuit between dMyc, a key transcription factor in animal biology, and miR-308, a member of the conserved *Drosophila* miR-2 family. My results suggest three models for dMyc and miR-308 interaction (Figure 37). In the first model, supported by our loss-of-function and gain-of-function studies, I show that dMyc and miR-308 are in a direct cross-talk with each other and that miR-308 responds to dMyc levels and regulates dMyc's protein levels. In the second model, I identified common targets between dMyc and miR-308. This model shows a possible role for miR-308 in specific fine-tuning of regulation by dMyc. It would be interesting to determine the extent to which levels of these common targets respond to different proportions of dMyc and miR-308. In the third model, I suggest a functional relationship between dMyc and miR-308 in the determination of a cell growth versus a differentiation program. In this model, I suggest collaboration between dMyc and miR-308 in programming cells into rapid proliferation and cessation of morphogenesis. All together, these results reveal a crucial role for miR-308 in feedback regulation of dMyc, and fine-tuning of target regulation by dMyc during development.

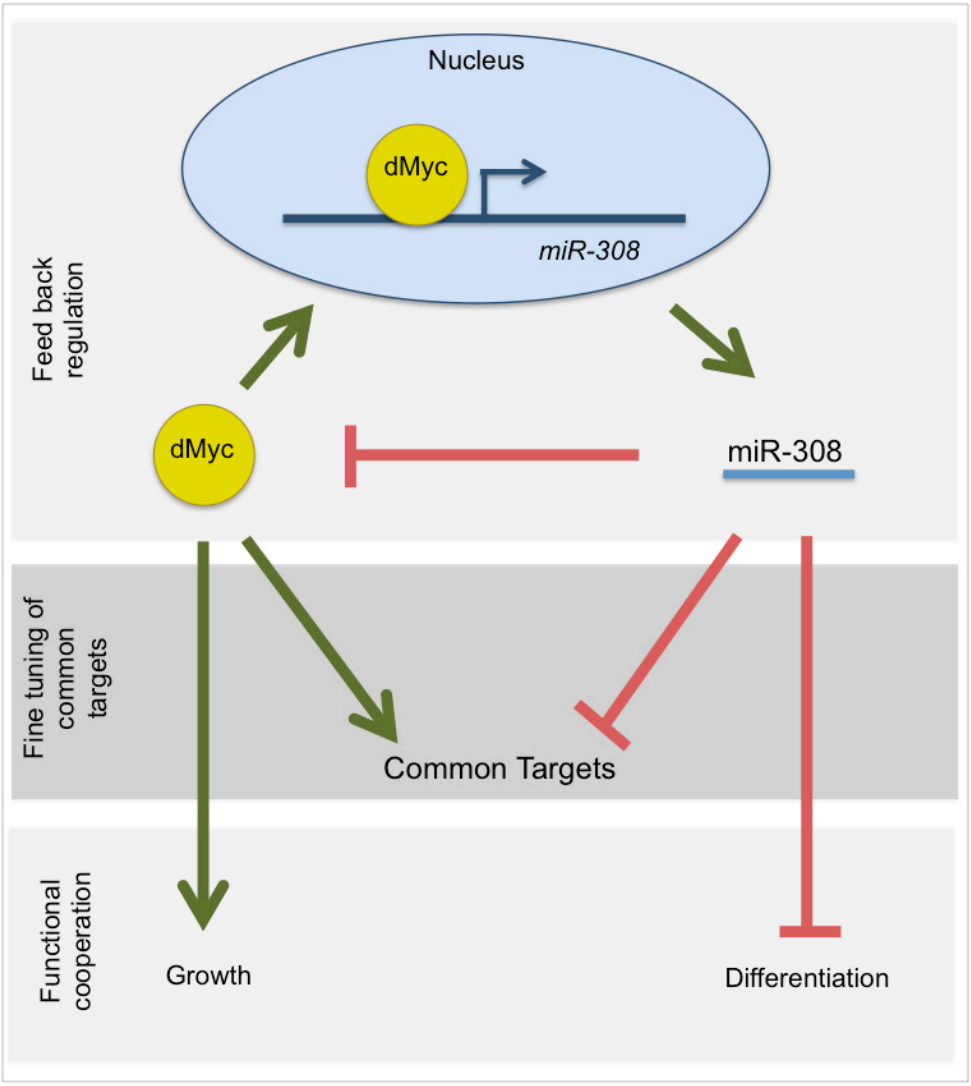


Figure 37. Models of gene regulation by Myc. Three proposed models for the cross-talk of dMyc and miR-308 and its role in feedback regulation (top panel), target regulation (middle panel) and cell proliferation programming (bottom panel).

Localization of dMyc in histone locus bodies

Data obtained form my and my colleague's work show that dMyc is a component of the histone locus bodies along with Lsm11 and the MPM-2 epitope-containing protein during replication, the time at which the canonical histone genes are transcribed (Daneshvar, Khan, & Goodliffe, 2011) (Figure 38). I observed little cell-type specificity of dMyc puncta, since we identified dMyc puncta throughout the embryo and in larval

and ovary tissues. One exception is the lack of Myc puncta in the germarium and follicle cells of the ovary.

What could be the function or consequence of Myc in the HLB? One obvious possibility is that Myc helps boost transcription of the histone genes, consistent with its localization during replication when those genes are transcribed. In human embryonic stem cells and fibroblasts, HLBs contain the U7 snRNP in addition to a histone gene coactivator protein, $p220^{NPAT}$, during mid-late G1 through S phase of the cell cycle (Marzluff et al., 2008). These data suggest that HLBs are capable of histone gene transcription initiation. It is therefore logical to consider that Myc's role in the HLB is related to transcriptional activation. If this is indeed the case, Myc loss-of-function mutants should have decreased histone gene expression. Short-term knock-down of Myc by RNAi should address this question, as long as pleiotropic effects are minimized. The reciprocal should also be informative; over-expression of Myc may induce higher levels of histone gene expression. In our previous experiments expressing ectopic Myc (Goodliffe et al., 2007)(Goodliffe et al., 2005), we have not found dramatic changes in the levels of histone gene transcripts, however. Over-expression of Myc may not lead to increased levels of Myc in HLBs; that would have to be determined before conclusions can be drawn about the effect of elevated Myc on histone gene expression.

In human primary cells as in *Drosophila*, the U7 snRNP localizes to the HLB. In most human cancer cell lines, however, the U7 snRNP often localizes to the Cajal Body rather than the HLB, and therefore an intriguing delocalization of the U7 snRNP occurs in cancerous cells (Matera et al., 2009). Elevated telomerase activity is a hallmark of cancer cells (de Lange, 1994), and Cajal Bodies play a role in telomere length regulation;

human telomerase RNA and the telomerase reverse transcriptase, hTERT, localize to CBs near telomeres during S phase (Matera et al., 2009). Myc protein has been found to bind directly to TRF/PIN2, a DNA binding protein involved in telomere capping and telomerase inhibition. Expression of the TRF/PIN2 interaction domain of Myc, the protein's C-terminus that lacks its trans-activation domain, led to increased telomere length *in vivo* (Kim & Chen, 2007). It would be informative to determine the localization of Myc with respect to HLBs, CBs and TRF/PIN2 in wild type and cancerous cells.

How might Myc be targeted to the HLB? Myc has been shown to be phosphorylated by cyclin E/Cdk2, altering Myc function, at mammalian c-Myc residue Ser-62 (Kupsco, Wu, Marzluff, Thapar, & Duronio, 2006). *Drosophila* Myc is not identical in this region of the protein, Myc Box I, however it does harbor a serine residue at the site next to the Ser-62 orthologous site (Cowling & Cole, 2006). An intriguing hypothesis is that cyclin E/Cdk2 phosphorylation of Myc causes subsequent localization to the HLB. Mutations eliminating potential phosphorylation sites of Myc in *Drosophila* would be informative in addressing this hypothesis. If a Ser-62 to alanine-62 mutant protein is unable to localize to the HLB, then phosphorylation of that site may have a role in Myc's localization to the HLB. Alternatively, ectopic cyclin E expression may drive constitutive localization of Myc in the HLB. We are pursuing this experiment.

Recently, White and colleagues identified several novel components of HLBs in *Drosophila*. Using biochemical and genetic approaches in S2 culture cells, the group identified Spt6 and the *Drosophila* NPAT homolog, Mxc, as novel components of the HLB. Myc was not identified as a component of the HLB in these experiments, but neither were two known components of HLBs: Lsm10 and Lsm11 (White et al., 2011).

Despite this fact, the new knowledge of HLB components, including Myc as described in this study, will help determine the function of HLBs, whose complete set of functions remains unclear.

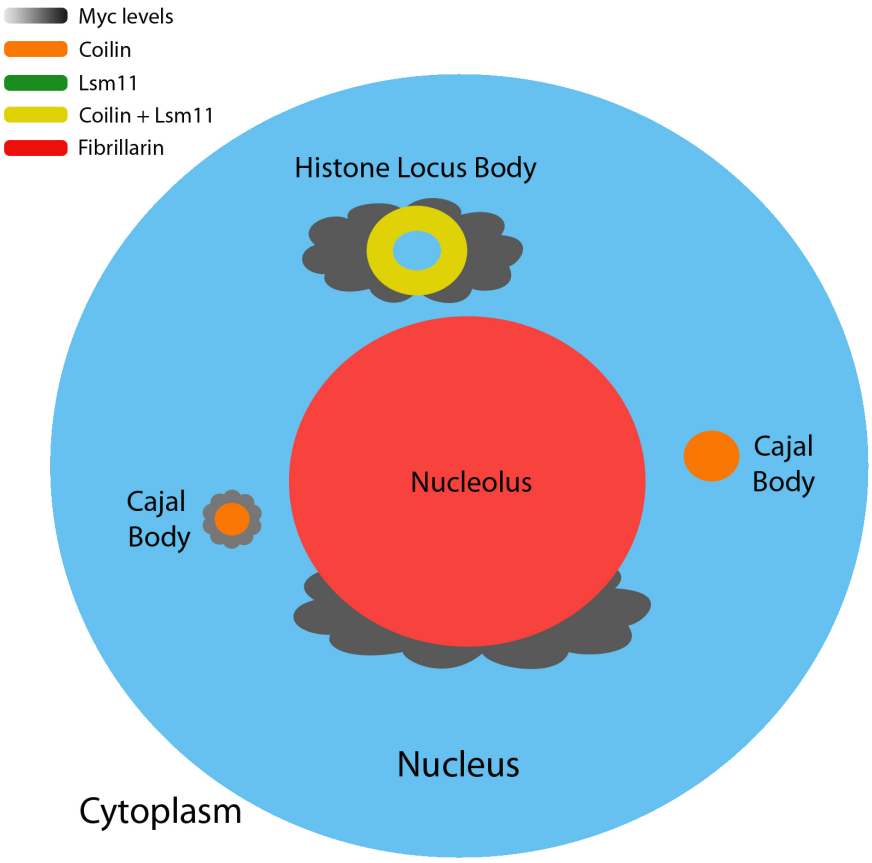


Figure 38. Localization of Myc in histone locus body. Cartoon shows the localization of dMyc in histone locus bodies during replication.

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