MicroRNA miR-308 regulates dMyc through a negative feedback loop in Drosophila

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Introduction

Myc is a transcription factor with a primary role in promoting cell proliferation and growth. Myc belongs to the basic helix-loop-helix (bHLH) protein family together with Max. The Myc-Max heterodimer binds to E-boxes (CACGTG, CATGTG, and alternative sequences) in the promoter region of target genes (Eilers and Eisenman, 2008; Blackwell et al., 1993). The biological effects of Myc are predominantly due to the up-regulation of genes that are involved in an increase of cell mass (Schmidt, 1999; Eilers and Eisenman, 2008), progression of cell division (Bouchard et al., 1998) and increased energy metabolism (Morrish et al., 2008). Myc’s regulation of its targets occurs mainly at the transcriptional level. However, it has been shown that Myc’s mechanisms of function also extend to regulation of translation and DNA replication (Cole and Cowling, 2008).

In Drosophila, Myc (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 (Pierce et al., 2008). Conversely, overexpression of dMyc results in increased size of larvae and organs of adult flies (Johnston et al., 1999).

Certain types of human cancers lack proper regulation of Myc levels resulting in uncontrolled cell proliferation and tumor formation (Eilers and Eisenman, 2008; Meyer and Penn, 2008). Hence, tight control and maintenance of appropriate Myc protein levels is crucial.

We and others have demonstrated that Myc protein utilizes auto-regulation to repress its own transcription in Drosophila and rodents (Penn et al., 1990; Goodliffe et al., 2005). Moreover, additional post-transcriptional and post-translational regulatory pathways contribute to the critical maintenance of Myc protein levels in cells (Salghetti et al., 1999; Gregory and Hann, 2000). Growing evidence has shown that mammalian Myc uses microRNAs to partially regulate its targets (Wang et al., 2011; O’Donnell et al., 2005; Chang et al., 2008; Lin et al., 2009; Xiong et al., 2010) as well as its own transcript levels (Kress et al., 2011; Liao and Lu, 2011).

MicroRNAs are a group of short (21–25 nucleotides) non-coding RNA involved in negative regulation of gene expression (He and Hannon, 2004). They block the translation of transcripts by binding to their complementary sequences in 3’ untranslated region (3’-UTR) regions of mRNAs (Berezikov et al., 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).

In this study, we illustrate the regulatory relationship of Myc and microRNA 308 (miR-308) in Drosophila. We 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, we show that dMyc levels are under the control of miR-308 during embryogenesis. Furthermore, ectopic expression of miR-308 in embryos can rescue lethality and prevent the pathologies caused by abnormal levels of dMyc.

Summary

The abundance of Myc protein must be exquisitely controlled to avoid growth abnormalities caused by too much or too little Myc. An intriguing mode of regulation exists in which Myc protein itself leads to reduction in its abundance. We show here that dMyc binds to the miR-308 locus and increases its expression. Using our gain-of-function approach, we show that an increase in miR-308 causes a destabilization of dMyc mRNA and reduced dMyc protein levels. In vivo knockdown of miR-308 confirmed the regulation of dMyc levels in embryos. 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 lower apoptotic activity and suppression of lethality. These results reveal a sensitive feedback mechanism that is crucial to prevent the pathologies caused by abnormal levels of dMyc.

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Key words: dMyc, miR-308, Drosophila melanogaster, Embryogenesis, Growth
suppress apoptotic activity caused by dMyc overexpression. Finally, through analysis of dMyc’s targets and comparing them to the predicted targets of miR-308, we propose models explaining a concerted gene regulation program by dMyc and miR-308.

Materials and Methods

miR-308 microarray

Total RNA, including small RNAs, was isolated from 0–24 hour old embryos 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 to hybridize the samples on Exiqon microRNA microarray version 11. The experiment was carried out in two biological replicates and four technical replicates on each slide. Spot intensities were acquired and normalized by GenePix Pro (Molecular Devices, California, USA). T-test statistical analysis was carried out by using Differential expression analysis module on Genepaint (Reich et al., 2006).

Drosophila strains and genetics

Flies expressing Gal4 ubiquitously in embryos were used (w*; P[w+]; TM6B, Tb) for all the crosses involving yeast Gal4-UAS system (Fischer et al., 1998). UAS-dm-myc flies (P[w+]; hsFLP22, y[1] w[+]; P[w+]; UAS-dm-Z/42) were used for ectopic expression of dMyc and generation of doubly homozygous flies. Both of the mentioned stocks were obtained from Bloomington Stock Center (Indiana University, Bloomington, IN, USA). For dMyc-knockdown study, we used a transgenic Drosophila expressing RNAs for dMyc, under the control of UAS (y[1]; y[1]; P[y+/w+] P[w+]; TRIP.JF01762;attP2). We crossed these flies to flies expressing Gal4 ubiquitously in embryos, as explained above. 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. One of the homozygous transgenic lines (transgenic line no. 10) that has UAS-miR-308 construct on the 3rd chromosome was used for all the experiments involving the ectopic expression of the miR-308. UAS-miR-308 flies were constructed by site-directed mutagenesis using Accuprime Pfx (Invitrogen, California, USA). One of the homozygous transgenic lines (transgenic line no. 10) that has UAS-miR-308 construct on the 3rd chromosome was used for all the experiments involving the ectopic expression of UAS-miR-308. All crosses were done in 25°C.

Quantification of miR-308 by qPCR

Total RNA was isolated using Trizol (Invitrogen, California, USA), according to the manufacturer’s instruction. Universal cDNA synthesis kit (Exiqon, Denmark) was used to make cDNA form mature sequence of microRNAs. Briefly, mature microRNAs were poly-adenylated and CDNA were synthesized by using a poly-T primer with a 3’ degenerate anchor and a 5’ universal tag. Amplification was carried out using a microRNA-specific primer, a universal primer and SYBR green (all from Exiqon, Denmark) in an ABI 7500 Fast real-time PCR system (Applied Biosystems, California, USA). MicroRNA miR-1 was used as the internal reference gene as it showed no significant change in our microRNA microarray study. Comparative Ct method was used for analyzing the expression levels and relative fold changes (Schmittgen and Livak, 2008).

Quantitative RT-PCR of mRNAs

Total RNA was isolated using Trizol (Invitrogen, California, USA). 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 andLivak, 2008). Ras was used as internal reference gene for quantification, as described previously (Khan et al., 2009). Q-RT-PCR Primers: dMyc (F: ATGCCACATCCGCGCGTCA, R: TGGGCGACTCTGGAACCTTAG); dRas (F: ATCCGCGACCCCGCAG, R: GTCCTGAATGCACCCCGCAG); RpS23 (F: GTCTCCGGAAGAGGTCCGCGC, R: ACCTTGAACGGCAGCACCACCGG).

miR-308 knockdown

For each independent experiment, approximately 200 wild-type (Canton-S) 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 5% egg volume as described (Kennerdell and Carthew, 1998). Injection solutions: 100 nM anti-miRNA-308 LNA inhibitor solution (Exiqon, Copenhagen, Denmark) (sequence of the inhibitor: CTCACGATTAATCTCGTGG) in PBS or PBS alone (control). The embryos were allowed to develop at 18°C for 15 hours before collecting them for either RNA or protein extraction.

Chromatin immuno-preciaptation

Approximately 0.5 grams of 0–24 hours embryos were dechorionated and fixed PHS/heparin/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 (Santa Cruz Biotechnology, sc-20280 and sc-28207) were used for immuno-preciaptation of dMyc protein. ChIP primers: ChIP region I: 5’-TGGCCGATACGCGGCGA-3’ and 5’-GTTTGTAGCCAGGGGATG-3’; ChIP region II: 5’-GGGAGGCTCAGAGGCGTT-3’ and 5’-CTTCA-CAACAGGCGACCAGAGGTTG-3’; ChIP region III: 5’-TACGAACTCGGCATG-3’ and 5’-GCCCTAAGCCACTGAGC-3’. Fibrillarin promoter region: 5’-TTTCTTCCGCTGGTGGATCA-3’ and 5’-CTCTTCCGCTGGTGGATCA-3’. Immunobots

8% poly-acylamide gels were used for separation of whole cell protein extracts. Detection of dMyc was carried out using rabbit anti-dMyc (Santa Cruz biotechnology, California, USA, cat. no. 28207) and mouse anti-rabbit HRP. Detection of Fibrillarin was carried out using mouse anti-Fibrillarin (Abcam, Cambridge, USA, cat. no. ab5456) and rabbit anti-mouse HRP. Detection of dMyc was carried out using rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, USA, cat. no. 9661) and mouse and rabbit-HRP. Anti-beta Actin antibody (Abcam, Massachusetts, USA, cat. no. ab8224) was used as loading control. Blots were quantified with NIH Image software package (http://rsweb.nih.gov/ij/).

Growth and survival

Zero to 1 hour old embryos from each genotype were collected on grape agar plates and were counted and transferred to new plates. Survivals of the hatched larvae were assessed after 24, 48, 72 and 96 hours by observing their movement in response to mechanical stimulation. Fraction of the live animals was plotted in Microsoft Excel.

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.

Results

Elevated levels of dMyc results in an increase in miR-308 levels

Regulation of gene expression by dMyc occurs through the direct binding of dMyc to E-boxes in the promoter region of target genes (Gallant, 2009). However, many genes that are responsive to dMyc in Drosophila lack E-boxes or similar sequences (Goodliffe et al., 2007). We asked to what extent dMyc indirectly regulates its targets via microRNAs. Using a yeast Gal4-UAS system, we increased levels of dMyc in Drosophila embryos. We collected 0–24 hour embryos from a cross of daughterless-Gal4 females (w*; P[Gal4-da.G32]/UH1) that express excessive Gal4 protein ubiquitously in embryos from a cross of daughterless-Gal4 females (w*; P[Gal4-da.G32]/UH1) that express excessive Gal4 protein ubiquitously in embryos. Out of 152 available probes for dMyc and miR-308 expression (PADE). Data have been deposited at the NCBI Gene Expression Omnibus (GEO) repository (Accession number: GSE38529).
to that of control (Fig. 1A). Quantitative RT-PCR of miR-308 confirmed 2.9-fold ± 0.9 (mean ± s.d., n = 3) increase in miR-308 in response to elevated levels of dMyc (Fig. 1B). This result suggests a possible role for miR-308 in dMyc’s regulation of gene expression.

dMyc physically interacts with miR-308 and its host gene locus miR-308 gene is located in the second intron of the ribosomal protein S23 (RpS23) gene. We identified two non-canonical E-boxes (CATGTG) upstream of the miR-308 gene (Fig. 2A). We asked whether dMyc directly binds to the locus of miR-308 gene. We carried out chromatin Immunoprecipitation (ChIP) using two different dMyc antibodies in wild-type (Canton-S) embryos, and results show a strong association (5.2-fold enrichment relative to IgG) of dMyc to the intronic region of the RpS23 gene. We also observed a lower association of dMyc (3.6-fold enrichment compared to IgG) to the upstream region of RpS23 transcription start site and no association to the downstream region of RpS23 gene (Fig. 2B,C). We 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).

It has been shown that intronic microRNAs are co-expressed with their host genes (Baskerville and Bartel, 2005; Truscott et al., 2011). We 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 (Fig. 2D). Overall, this result suggests that dMyc regulates the expression of miR-308 and its host gene, RpS23, by binding to their shared locus. However, we did not determine whether dMyc binding to the intronic region of RpS23 gene promotes transcription of miR-308, independent of RpS23 transcript.

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). We 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 and other members of the miR-2 microRNA family (Fig. 3A).

Based on the computational prediction, we 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, we generated transgenic Drosophila lines that express miR-308 under the control of a tubulin promoter (Fig. 3B).
of the yeast Gal4 UAS enhancer. As a control, we generated flies expressing a mutated form of miR-308 with a change in the seed sequence from 5'-AUCACA-3' to 5'-GGAUCC-3' (Fig. 3B). We crossed these flies to flies expressing Gal4 and confirmed the expression of ectopic miR-308 and mutated-miR-308 in embryos by quantitative RT-PCR (Fig. 3C).

miR-308 overexpression results in reduced dMyc transcript and protein 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). We tested whether the ectopic expression of miR-308 leads to a decrease in dMyc transcript and protein levels. We collected 0–24 hour embryos from a cross of daughterless-Gal4 females and UAS-miR-308 males, and measured dMyc levels by qRT-PCR and Western blotting. We observed a modest 14%±5 (mean ± s.d., n=5) reduction of dMyc transcripts in embryos expressing ectopic miR-308 compared to that of control (Fig. 3D). Despite the modest reduction in transcript levels, we observed a substantial 68%±10 (mean ± s.d., n=3) decrease in dMyc protein levels in embryos expressing ectopic miR-308, compared to that of control (Fig. 3E). We 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 (mean ± s.d., n=97, P-value=1.1E−15, Fisher exact test) of them survived past 96 hours after egg deposition, compared to 77%±6 (mean ± s.d., n=81) of control. Together, these data suggest that miR-308 is sufficient to mediate a negative regulatory mechanism controlling dMyc in vivo. While our miR-308 and mutated-miR-308 overexpression models suggest that the 5’-seed sequence of miR-308 is crucial for this regulation, we were not able to conclude that this regulation is a result of direct interaction between miR-308 and dMyc mRNA. Further reporter gene assays, e.g. luciferase assays, on the 3’- UTR region of dMyc mRNA should be carried out to test the direct regulation.

miR-308 overexpression results in reduced Fibrillarin protein level

We asked whether the effect of miR-308 overexpression on the dMyc protein could alter the expression of dMyc targets. We selected fibrillarin as an indicator of dMyc activity. Fibrillarin is a marker for the nucleolus and nucleolar bodies (Daneshvar et al., 2011) and it is involved in biogenesis of ribosomal RNA (Tollervey et al., 1993). Both in Drosophila and vertebrates, fibrillarin has been shown to be a transcriptional target of the Myc protein (Orian et al., 2003; Coller et al., 2000). First, we asked whether a reduction in dMyc levels would alter the levels of fibrillarin in Drosophila embryos. We used transgenic Drosophila embryos with a dMyc-RNAi construct under the control UAS. We crossed these flies with flies expressing Gal4 and collected embryos that were 0–24 hours old for protein
We tested the efficiency of the dMyc-RNAi model and observed reduced dMyc levels compared to the control embryos (Fig. 4A). In a western blot analysis using an anti-fibrillarin antibody, we observed that reduced dMyc levels caused a reduction in the abundance of fibrillarin. If a reduction in dMyc levels causes a decrease in fibrillarin levels, the overexpression of miR-308 should have the same effect on fibrillarin. Our western blot analysis showed a reduction in the abundance of fibrillarin in embryos in response to the overexpression of miR-308 (Fig. 4B).

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

To determine whether dMyc transcripts are normally repressed by miR-308, we 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 et al., 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 (mean ± s.d., n=3) increase in dMyc mRNA levels (Fig. 5A). Immunoblotting showed an increase in dMyc protein accumulation after inhibition of miR-308, compared to control (Fig. 5B).

These results show that miR-308, despite its low abundance (Aravin et al., 2003), can repress 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, suggest a 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.

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 et al., 2008). In our experiments, dMyc protein levels decrease upon overexpression of miR-308. As previously reported, we also observed that dMyc overexpressing embryos do not survive beyond 72 hours after egg deposition (Khan et al., 2009). We sought to determine whether miR-308 could rescue the dMyc overexpression lethal phenotype. By meiotic recombination of the two transgenes on third chromosome, we 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 (Fig. 6A). Consistent with these results, when crossed to Gal4 expressing flies, 40%±12 (mean ± s.d., n=84, three independent crosses) of these double UAS transgenic animals survived beyond 72 hours, compared to 0% (n=94, three independent crosses, p-value=9.7E−14, Fisher exact test) UAS-dmyc alone and 19%±9 (mean ± s.d., n=97, three independent crosses) p-value=4E−4, Fisher exact test) UAS-miR-308 alone (Fig. 6B).

Although the Myc protein has been shown to promote proliferation and growth, the ectopic expression of Myc in normal cells does not cause overgrowth and cancer. It has been shown that overexpression of Myc in normal cells without background mutations triggers apoptosis (Pelengaris et al., 2002; Montero et al., 2008). We asked whether the overexpression of dMyc in Drosophila embryos causes an increase in apoptotic activity, and if this activity can be suppressed by the addition of ectopic miR-308. We used an anti-cleaved caspase-3 antibody to measure apoptotic activities in the embryos. Anti-cleaved human caspase-3 recognizes the initiator caspase Dronc in Drosophila (Fan and Bergmann, 2010). Dronc is the ortholog of the human initiator caspase-9. After receiving upstream apoptotic signals, Dronc undergoes autocatalytic cleavage to become fully processed and activated. Processing of Dronc in the presence of...
Dark, the homolog of mammalian Apaf-1, results in the production of three major fragments: a large fragment, small fragment and pro-domain (Yan et al., 2006; Muro et al., 2004). Our western blot analysis shows that an increase in dMyc levels in *Drosophila* embryos causes an increase in the total level of the Dronc protein and its processed fragments. Consistent with our survival assay, dMyc-triggered apoptosis is balanced by additional ectopic expression of miR-308.

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.

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, we examined the degree to which dMyc’s transcriptional regulation of downstream targets may be antagonized by miR-308. We analyzed the targets of dMyc in embryogenesis using whole genome expression profiling. By crossing flies expressing Gal4 to flies carrying UAS-dmyc, we 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 that were expressed in both groups 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. We considered the function of up-regulated targets of dMyc. We used a MGSA Byesian network model (Bauer et al., 2010) in Ontologizer 2.0 software to categorize those predicted targets according to their annotation of biological process (Bauer et al., 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. A score closer to 1.0 shows a stronger support for the enrichment. Results show that dMyc’s targets in embryogenesis are mainly involved in ribosome biogenesis, t-RNA metabolism and RNA modification (Cutoff=0.5), consistent with previous reports (Grewal et al., 2005) (Fig. 7A).

Given our observed inverse relationship between miR-308 and dMyc, we then determined whether predicted targets of miR-308 are functionally related to targets of dMyc in the context of its over-expression. We 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 (Fig. 7A). However, the score of this enrichment was below the cutoff of 0.5, which probably reflects the difficulty in determining enrichment of a functional category in a large and broad list of target genes. 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 coordinating the complex series of events that balance growth and differentiation during development.

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**Fig. 7.** dMyc up-regulation targets have overlaps with predicted targets of miR-308. (A) Heat map of MGSA functional analysis for dMyc up-regulation targets, miR-308 predicted targets and their common targets. The MGSA model categorizes a set of genes based on their functional annotation and assigns each category a score (between zero and one) that corresponds with the significance of enrichment. A higher score means a stronger support for that enrichment. A cutoff score of 0.5 is considered to call a functional category significant, i.e. a level at which a gene set is estimated to be more likely to be related to certain functional category. In the heat map, a dark brown color corresponds with a higher score and shows stronger support. (B) Overlap of dMyc targets and miR-308 predicted targets. P-value shows the statistical analysis of significance of the overlap using hypergeometrical distribution. (C) List of common genes between dMyc over-expression targets and miR-308 predicted repression targets.
Between the identified 624 dMyc up-regulated targets and 730 predicted targets of miR-308, we identified 26 common targets (Fig. 7B,C). This overlap was significantly different from any overlap that could occur randomly by this analysis (P-value<0.008, hypergeometric distribution) (Fig. 7B). Functional analysis of miR-308 and dMyc’s common targets showed a significant enrichment in bristle morphogenesis (Fig. 7B). The observation that miR-308 may decrease expression of these genes while dMyc activates them suggests a specific role for miR-308 in fine-tuning dMyc’s function as an inhibitor of morphogenesis.

Discussion

Transcription factors and microRNAs are both trans-acting regulators of gene expression, which can alter the expression of their many target genes at different levels, the former at the transcriptional level and the latter at the post-transcriptional level (Hobert, 2004). While transcription factors and microRNA can regulate their targets independently of each other, new findings suggest that they can cooperate in regulating each other and their target genes. In humans, transcription factors and microRNA can form various regulation motifs such as feedback and feed-forward loops that ultimately contribute to the gene regulation programs (Chen et al., 2011). In Caenorhabditis elegans, microRNAs are shown to participate in feedback circuits with key transcription factors, and these networks have a high capacity for the regulation of target genes (Martinez et al., 2008).

Here, we 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. Our results suggest three models for dMyc and miR-308 interaction (Fig. 8).

In the first model, supported by our loss-of-function and gain-of-function studies, we showed that dMyc and miR-308 are in cross-talk with each other and that miR-308 responds to dMyc levels and regulates dMyc’s protein levels (Fig. 8, top panel).

In the second model, we identified common targets between dMyc and miR-308. This model shows a possible role for miR-308 in the regulation of target genes. In humans, transcription factors and microRNA can form various regulation motifs such as feedback and feed-forward loops that ultimately contribute to the gene regulation programs (Chen et al., 2011). In Caenorhabditis elegans, microRNAs are shown to participate in feedback circuits with key transcription factors, and these networks have a high capacity for the regulation of target genes (Martinez et al., 2008).

In the third model, we suggest three models for the cross-talk of dMyc and miR-308 and its role in feedback regulation of dMyc (Fig. 8, middle panel) and growth and differentiation programming (bottom panel).

Three proposed models for the cross-talk of dMyc and miR-308 (Fig. 8, bottom panel). Functional analysis of miR-308 and dMyc’s common targets showed a significant enrichment in bristle morphogenesis (Fig. 7B). The observation that miR-308 may decrease expression of these genes while dMyc activates them suggests a specific role for miR-308 in fine-tuning dMyc’s function as an inhibitor of morphogenesis.

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Competing Interests

The authors have no competing interests to declare.

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