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New genes in the *let-7* miRNA regulatory network in *C. elegans*

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by

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ABSTRACT OF THE THESIS

New genes in the *let-7* miRNA regulatory network in *C. elegans*

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MicroRNAs (miRNA) are small non-coding RNAs that function in the regulation of post-transcriptional gene expression. MiRNAs work by binding to complementary sites of target mRNAs to suppress these genes. In *C. elegans,* the *let-7* miRNA promotes terminal differentiation. Mutations in the *let-7* gene result in abnormal cellular division, which is likely related to the cellular proliferation phenotype of human tumors that lose *let-7* expression (Bussing et al., 2008). To decipher how *let-7* controls cellular processes it is necessary to discover new targets. The Pasquinelli lab performed a genome-wide screen to discover new genes that are regulated by *let-7*. Genetic RNA expression and

computational analyses led to the discovery of twenty-three suppressors of *let*-7 phenotypes, from which five genes were proven to be associated with RISC in a *let*-7 dependent manner, including the new targets *T27D12.1, prmt-1,* and *opt-2* (also known as *pept-1*). Targets of *let-7* have been shown to actually regulate *let-7* themselves through feedback loops in *C. elegans* (Hammell et al., 2009) (Roush and Slack, 2009). Thus I further analyzed the role of the suppressors of *let*-7 phenotypes and demonstrated five genes to be regulators of *let-7*, including *nduf-7, T25G3.3, T08B2.8, hbl-1,* and *prmt-1*. The protein arginine methyltransferase, PRMT-1, appears to both positively regulate the expression of *let-7* and also be a direct target of negative regulation by *let-7*, forming a novel feedback loop.

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate gene expression when their 21-22 nucleotide mature sequence imperfectly base pairs to a target mRNA. MiRNAs control a number of different biological pathways including embryogenesis, neuronal differentiation, Hox-mediated development and hematopoiesis. The miRNA *let-7* plays a key role in tumorigenesis, as it is important for causing cellular differentiation (Bussing et al., 2008).

miRNA Biogenesis

RNA polymerase II transcribes miRNAs in the nucleus to create the primary miRNA (Figure 1) (Kim et al., 2009). The primary miRNA is then processed into the 60-70 nucleotide precursor hairpin by the RNase III endonuclease Drosha, in a complex with Pasha. Another RNase III Dicer processes precursor into the 21-22 nucleotide mature miRNA. The mature sequence is then loaded into an RNA induced silencing complex (RISC), where the miRNA can imperfectly base pair to target mRNA 3'UTRs to down regulate gene expression. RISC includes Argonaute proteins, Argonaute-like proteins (ALG-1) in *C. elegans*, that interact with target mRNAs through guidance by miRNAs.

Since miRNA recognition is not perfectly complementary, they can affect many targets, thus it is important to have biogenesis highly regulated. The expression of the *let*-7 miRNA is regulated at the transcription level by *hbl-1* (Roush and Slack, 2009). *C. elegans* progress through four larval stages, (L1-

L4), before transitioning into adulthood. Gene expression at each of these stages is highly regulated to ensure proper development. HBL-1 is a repressing transcription factor that inhibits cell signaling seen in late larval stages (L3 and L4) and in adulthood. *let-7* is needed to properly transition from L4-to-adulthood and thus should not be expressed early in development. The *let-7* promoter has HBL-1 binding sites that contribute to regulation as seen through reporter analysis with mutated binding sites. HBL-1 is present in the L2-to-L3 transition and prevents *let-7* from being transcribed by directly binding to the *let-7* promoter. Down-regulation of HBL-1 in the L3 stage, permits transcription of *let-7* in some tissues.

let-7 expression is further regulated post-transcriptionally by the cold shock domain protein LIN-28 (Lehrbach et al., 2009, Van Wynsberghe et al., 2011). LIN-28 is a highly conserved protein known to be partially involved in maintaining cells in an undifferentiated state. *lin-28* activity negatively suppresses *let-7* in the early larval stages, L1 and L2, and preserves undifferentiated seam cells. The LIN-28 protein is detected through Western Blots at the L1 and L2 stages of the worm, but a severe decrease in LIN-28 is seen at L3 coinciding with the appearance of mature *let-7*. Immunoprecipitation assays show LIN-28 is able to bind to the *let-7* gene, as well as the primary *let-7*. Primary *let-7* is processed by Drosha co-transcriptionally to form precursor *let-7.* Thus, LIN-28 co-transcriptionally prevents precursor and mature *let-7* accumulation by preventing primary *let-7* from being processed by Drosha.

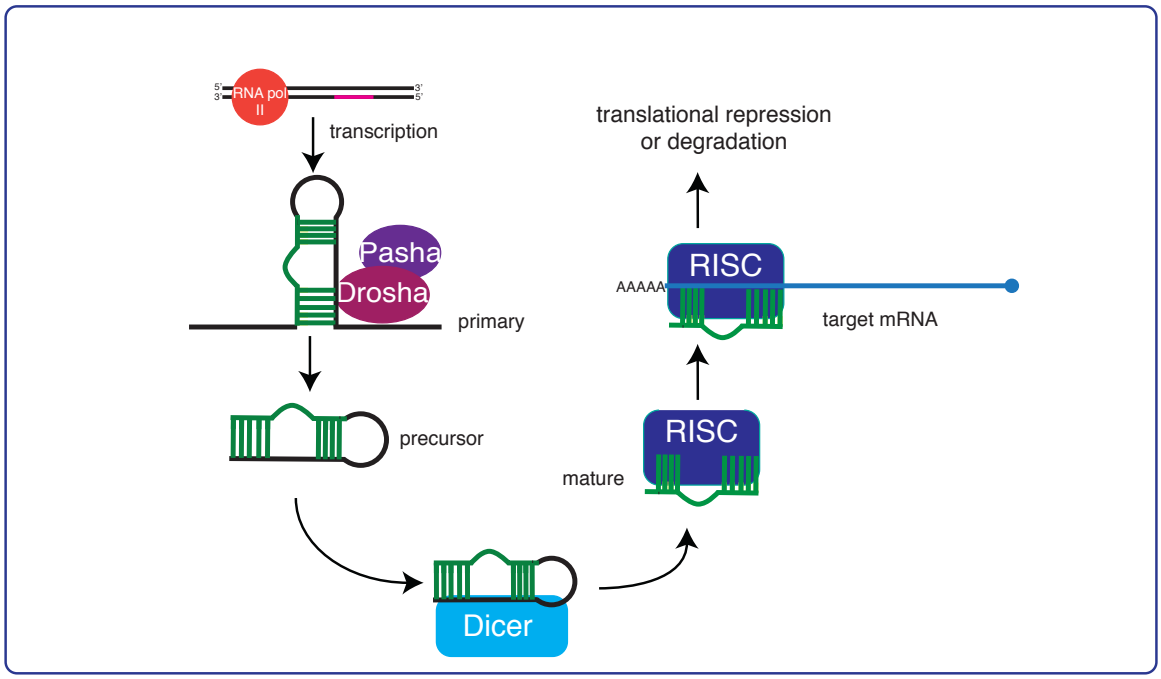

Figure 1: miRNA Biogenesis
This diagram depicts miRNA biogenesis. Primary miRNA is transcribed by RNA polymerase II. The precursor, hairpin, structure is then cleaved out by RNase III Drosha in a complex with Pasha. Precursor miRNA is cleaved by RNase III Dicer to form a single stranded 21-22 nucleotide mature miRNA. This mature miRNA is loaded into RISC where the miRNA can bind to target mRNA and regulate gene expression through translational repression or mRNA degradation.

Regulation of Gene Expression by miRNAs

MiRNAs guide RISC to target mRNAs to cause down regulation of target gene expression. While miRNA binding does not require perfect base pairing, it is traditionally thought that nucleotides 2-7 on the 5' end of the miRNA, known as the seed sequence, should perfectly base pair for proper target recognition (Bartel, 2009). However, perfect base pairing of the 3' end of the miRNA to its target is able to compensate for mismatches in the seed sequence. MiRNAs usually bind to the 3'UTR of target mRNAs and efficiency of target recognition can be affected by the location and nucleotide composition of miRNA binding sites in the 3' UTR. However recently, miRNA binding sites have also been found in coding regions of mRNAs and may also contribute to posttranscriptional regulation (Fang and Rajewsky, 2011).

MiRNAs bind to their target mRNAs leading to repression of protein expression through several different pathways. miRNAs have been shown to affect translational efficiency at four different steps in translation (Eulalio et al., 2008). Evidence for the inhibition of translation initiation and elongation has been seen, as well as premature termination of translation, and cotranslational degradation of proteins. Until recently, translational repression was thought to be the only mechanism of down-regulating gene expression in animals by miRNAs. However, the loss of expression of target genes can also be attributed to mRNA decay. It has been shown that knockdown of proteins in the miRNA pathway leads to increased levels of mRNAs that are known miRNA targets (Bagga et al.,

2005). Furthermore, overexpression of miRNAs in mammalian cells caused target mRNA levels to decrease as seen via microarray analysis (Lim et al., 2005). mRNA degradation is a result of deadenylation and decapping of target mRNAs through miRNA association (Eulalio et al., 2008).

The Role of *let-7* in *C. elegans* as a Heterochronic Gene

let-7 was first discovered in *C. elegans* and since then has been defined as a heterochronic switch gene important for developmental changes in the transition from larval to adult stages (Reinhart et al., 2000). The heterochronic pathway is responsible for regulating temporally restricted events. These genes control regulatory pathways in order to ensure an accurate sequence of events occurs as the worm develops through each larval stage to an adult. A mutation in a heterochronic gene causes temporal transformation, resulting in precocious or retarded development of specific cell types. *let-7* was discovered to be part of the heterochronic pathway through a genetic screen in *C. elegans* looking for mutant genes that could suppress the synthetic sterile phenotype of heterochronic mutant worms *lin-14(n179)* and *egl-35(n694)*. The *let-7(n2853)* worms were able to suppress the phenotype and placed *let-7* in the heterochronic pathway. The *let-7(n2853)* worms have a mutation in the seed sequence, changing a G to an A which results in a severe knockdown of *let-7* levels. This causes *let-7(n2853)* worms to have a lethal temperature-sensitive phenotype where they burst through their vulva at the larval to adult transition.

In *C. elegans* during each larval stage the seam cells, hypodermal skin cells, divide similarly to the way stem cells divide: the cell divides to give a differentiated daughter cell, and the other cell continues self-renewing until the transition into adulthood (Sulston and Horvitz, 1977). When the L4-to-adult transition occurs, the self-renewing cell terminally differentiates to form the alae, a cuticle structure along the side of the worms. However, in *let-7(n2853)* worms a retarded phenotype is seen in the transition from L4-to-adult (Reinhart et al., 2000). In the retarded phenotype, cells continue to divide and enter an extra larval stage, L5, before differentiating and the alae is formed improperly. When *let-7* is not expressed in high enough levels, overproliferation of seam cells occurs as these cells fail to properly differentiate.

Transgenic worm strains with the *let-7* promoter fused to GFP have been used to study spatial expression of *let-7* (Johnson et al., 2003). GFP was expressed in most somatic tissue including seam cells, vulva precursor cells, intestinal cells, muscle cells, the pharynx and neurons. Only seam cell expression was shown to be temporally regulated at the transcriptional level in these reporter worms. However, mature *let-7* is known to be temporally regulated with expression beginning at the L3 stage and continuing into adulthood (Reinhart et al., 2000). This expression pattern is similar to the expression seen in *D. melanogaster* where *let-7* is undetectable until a late larval stage and continues into adulthood (Pasquinelli et al., 2000). This data is consistent with *let-7* playing a role in determining a cell's decision to differentiate

and no longer having the ability to self-renew.

let-7 is subject to a complicated biogenesis regulation that involves many genes known to be in the heterochronic pathway. *lin-28* and *hbl-1* are both part of the heterochronic pathway early on in development and negatively regulate *let-7* expression (Van Wynsberghe et al., 2011) (Roush and Slack, 2009). *lin-28(n719)* mutant worms are able to partially suppress the retarded alae phenotype of the null mutant, *let-7(mn112)* (Reinhart et al., 2000). *let-7* mutant phenotypes are also suppressed by direct targets, such as *lin-41* (Slack et al., 2000). *lin-41* mutants have precocious terminal differentiation of seam cells, thus when crossed to a *let-7(mn112)* worm the *let-7* retarded phenotype is suppressed (Reinhart et al., 2000). When *lin-41* is overexpressed in worms, retarded phenotypes are seen at the L4-to-adult transition similarly to a *let-7* mutant phenotype (Slack et al., 2000). Thus, the heterochronic defects seen in *let-7* mutants may be due to overexpression of its target genes.

A Conserved Role for *let-7* in Regulating Cellular Differentiation

The *let-7* miRNA is conserved across bilaterian phylogeny (Pasquinelli et al., 2000). The *let-7* transcript is detectable in *C. elegans, D. melanogaster, D. Rerio, H. elegans, P. sibogae, H. Sapiens* and other species. A developmentally staged northern blot of *D. melanogaster* shows *let-7* is first detectable at late larval stages into adulthood, illustrating the function of *let-7* in defining a cell's adult fate. Northern blotting reveals that *let-7* expression levels differ among tissues in *H. Sapiens*, with bone marrow having the least prevalent levels of *let-7,*

possibly due to the abundance of stem cells that would not express *let-7*. (Pasquinelli et al., 2000). Thus *let-7* is conserved across species to help prevent a stem-cell like state, further illuminating its role as a tumor suppressor.

The *let-7* miRNA is a tumor suppressor and functions by down-regulating genes that would otherwise enable cell proliferation, including RAS, MYC, HMGA2, and proteins involved in the cell cycle (Bussing et al., 2008). Loss of *let-7* is implicated in lung cancer cells, most likely because RAS activity is no longer being properly regulated. A study in breast cancer cell lines derived from tumor initiating cells (cancer stem cells) showed significant depletion of *let-7* levels. When *let-7* was transfected into the cell line, a decrease in the ability of the cells to self-renew and form tumors in immunodeficient mice was seen (Yu et al., 2007). The role of *let-7* in lung cancer has also been studied in immunodeficient mice (Trang et al., 2010). Human lung cancer cells were injected into the mice leading to the development of tumors. Intratumoral injections of *let-7* into the mice proved to cause necrosis within the tumor and halted further tumor growth. These observations are in agreement with the role of *let-7* as an important regulator of cellular differentiation and proliferation during development since the loss of *let-7* leads to uncontrolled cell growth and subsequent tumor formation.

Identification of Targets of miRNA Regulation

To fully understand how *let-7* plays such a crucial role in the cell, it is necessary to discover and study mRNA targets. Many labs use genome wide

screens to find possible miRNA targets. Microarray analysis of model animals with a mutated miRNA allows for possible targets to be identified as having higher mRNA levels. Using RNAi to screen for genes that can suppress miRNA mutant phenotypes is another high throughput way of testing for possible targets and led to the discovery of the *let-7* target *daf-12* (Grosshans et al., 2005). Immunoprecipitation assays for proteins that are a part of RISC, such as AIN-1 and ALG-1, can lead to the discovery of new targets (Zhang et al., 2007, Zisoulis et al., 2010). RNA Immunoprecipitation (RIP) and Crosslinking Immunoprecipation (CLIP) are techniques that can be used to immunoprecipitate RNA that is bound by ALG-1 through the use of an ALG-1 antibody. In RIP, the intact mRNA transcript is isolated and converted into cDNA from which PCR for a gene of interest can be performed to determine possible miRNA regulation. While in CLIP analysis only the RNA protected by ALG-1 is isolated before it can be deep sequenced and mapped to the *C. elegans* genome giving potential targets of miRNAs. The CLIP technique allows for identification of the specific ALG-1 binding sites on the potential target mRNAs. Computational analysis has also been widely used and is based on Watson-Crick base pairing to search for predicted targets (Bartel, 2009). Most miRNA target prediction programs search for near perfect binding of the seed sequence, because it is thought to be important for target recognition.

Since *C. elegans* has several known *let-7* mutant alleles, these worm strains can be used to find novel targets through genetic analysis. Shaun Hunter,

a former PhD student in the Pasquinelli lab, performed a microarray screen to discover genes mis-regulated in *let-7* mutants. In these arrays, he looked for genes upregulated in *let-7(n2853)* worms compared to N2, wild-type, worms. These genes were then screened using RNAi for molecules that suppress the *let-7* mutant phenotypes. He found 23 genes that suppressed a *let-7* mutant phenotype, and I was able to verify five of them as likely direct targets of *let-7*.

Accumulating evidence has suggested that feedback loops are a common miRNA regulatory mechanism. As previously discussed, *let-7* is regulated by *hbl-1* (Roush and Slack, 2009), additionally *hbl-1* is regulated by *let-7* family members (Abrahante et al., 2003) (Abbott et al., 2005). *hbl-1* mutants have precocious heterochronic phenotypes, which is commonly seen in targets of *let-7* (Abrahante et al., 2003). The 3' UTR of *hbl-1* is necessary for temporal regulation of *hbl-1* and includes *let-7* complementary sites. Mutations in the *let-7* family members causes *hbl-1* reporter constructs to be mis-regulated, making *hbl-1* a likely target (Abbott et al., 2005). Together this places *hbl-1* in a negative feedback loop with *let-7* family members. *hbl-1* is just one example of *let-7* targets regulating *let-7* as *daf-12* and *myc* have also been shown to affect *let-7* expression through feedback loops in worms and mammalian cells, respectively (Hammell et al., 2009) (Bussing et al., 2008). To determine if any of the twenty-three suppressors of *let-7* phenotypes may actually be regulating *let-7* levels, I performed multiple phenotypic RNAi screens and Northern analysis. Through this analysis I was able to discover five new regulators of *let-7*. I have found a novel

feedback loop between *let-7* and *prmt-1*. *prmt-1* may prove to play an important role in tumorigenesis since knockdown of *prmt-1* causes a decrease in *let-7*, a common occurrence in tumor cells.

Results: Chapter 1

Genome wide screen to identify possible *let-7* targets

Microarray analysis can detect small changes in gene expression that may occur from mRNA decay through miRNA targeting. Therefore Dr. Shaun Hunter performed a genome wide microarray screen to identify direct targets of *let-7* through comparison of N2 and *let-7(n2853)* worms. *let-7(n2853)* mutant worms produce less mature *let-7* then N2 worms, and therefore targets of *let-7* would be up-regulated in these strains. Six independent experiments were performed comparing N2 and *let-7(n2853)* RNA from L4 worms, on Affymetrix arrays. 2216 genes were shown to be significantly up-regulated in the mutant strain.

In order to find direct targets rather than genes that are simply downstream of *let-7*, the up-regulated genes were then filtered through a second microarray screen. Microarray data was compared between *lin-29* mutants and *let-7* mutants, as *lin-29* is downstream of *let-7* and direct targets of *let-7* should not be up-regulated in *lin-29* worms. 206 genes were found to be up-regulated in *let-7* mutants compared to N2 and *lin-29*. Computational analysis was also performed by our collaborator, Dr. Gene Yeo, to identify which genes could partially base pair to the mature *let-7* sequence. He identified two conserved 6-mers that are complementary to *let-7* to be enriched in the 3' UTR of genes up-regulated in *let-7(n2853)*. 150 genes contained at least one of the 6-mers. PicTar and TargetScan were also used to predict targets giving a total of 163 genes with *let-7* complementarity.

Combining the candidates from each of these filters gave 348 unique

genes to test for functional interaction with *let-7*. In order to test for genetic interaction with *let-7*, RNAi was performed by Dr. Hunter to search for suppression of *let-7* phenotypes. *let-7* mutants have heterochronic defects in which seam cells do not properly differentiate and continue to divide. N2 worms have sixteen visible seam cell nuclei, while *let-7* mutants have extra seam cells because the cells continue to divide until L5 (Reinhart et al., 2000) (Hayes et al., 2006). This retarded phenotype causes the vulva to form improperly, leading to *let-7* mutants rupturing through their vulva. Sixteen genes were found to suppress the vulva bursting phenotype of *let-7(mn112)* worms when the genes were knocked down by RNAi. Ten genes were able to suppress the extra seam cell nuclei phenotype of *let-7(n2853)* worms, giving a total of twenty-three genes that were able to suppress one or both *let-7* phenotypes (Table 1). These genes were placed in the let-7 pathway but more analysis was needed to test if these genes might be direct targets of *let-7.*

Targets are associated with ALG-1 in a *let-7* dependent manner

To determine which possible targets were regulated by the *let-7* miRNA, I searched for ALG-1 binding sites in the twenty-three suppressors. ALG-1 binding sites were discovered through CLIP of L4 staged N2 and *alg-1* mutant worms (Zisoulis et al., 2010). In CLIP analysis only the ALG-1 bound region of mRNA Is isolated, so the exact site targeted on an mRNA can be identified. ALG-1 is in the RISC complex and binds target mRNA through miRNA base-pairing. Thus any RNA associated with ALG-1 is likely regulated by a miRNA. The RNA was

deep sequenced and cross-referenced with the *C. elegans* genome. I found that eight of the twenty-three genes had significant ALG-1 binding sites and thus were being regulated by a miRNA (Table 1). This included known *let-7* targets such as *daf-12*, and *lin-41*, as well as *hbl-1*, which is a known target of the *let-7* sisters. ALG-1 binding sites were found in the 3'UTR, which is typically thought to be where miRNAs bind to and regulate mRNAs; however significant sites were also seen in introns and exons of these genes.

To prove *let-7* is responsible for the interaction of ALG-1 with these genes, I needed to analyze their association with ALG-1 in N2 and *let-7(n2853)* worms. Dr. Zisoulis performed RIP using an ALG-1 antibody on L4 staged N2 and *let-7(n2853)* worms. He isolated the RNA associated with ALG-1 and performed RT-PCR. I then performed semi-quantitative PCR on the cDNA for each of the possible targets with an ALG-1 binding site (Figure 2a). When comparing the two strains, the N2 worms should have higher levels of *let-7* targets in the IP if *let-7* is responsible for these RNAs being associated with the RISC complex. IgG was used as a negative control, and I verified detection of the possible targets at L4 through the input from worm lysates. Four independent RIPs were analyzed, and the target being enriched in the N2 IP at least 2/4 times was considered to be likely dependent on *let-7* for ALG-1 association. The known targets *lin-41* and *daf-12* both tested positive as targets through this experimental technique. *fos-1* was used as a negative control as it did not have any significant CLIP hits, nor did *fos-1* amplify from the IP's in either worm strain. *lin-14* was also used as a

negative control because it is a known target of *lin-4*, and thus there should be no significant changes in *lin-4* interaction with ALG-1 between the two strains. *daf-9* and *adt-2* had significant CLIP hits but could not be verified as targets through the RIP analysis. *adt-2* had similar levels in the N2 and *let-7(n2853)* mutant strains suggesting it may be targeted by a different miRNA. Three novel targets were identified: *prmt-1, opt-2,* and *T27D12.1*. They were all enriched in the N2 IP compared to the *let-7* IP, and are therefore associated with ALG-1 in a *let-7* dependent manner.

To further investigate the effects of *let-7* on expression of the newly identified targets, qPCR was performed in three independent experiments using RNA extractions comparing L4 staged N2 and *let-7(n2853)* worms (Figure 2b). If regulation is at the level of mRNA degradation, then target levels should be higher in *let-7(n2853)* worms then N2 worms, as *let-7* is not present to down-regulate these targets. This is the case for the positive control *lin-41*, which was up-regulated four fold in *let-7(n2853)*. Similarly, *prmt-1* was highly up-regulated in *let-7(n2853)* and is likely regulated by mRNA decay. While *T27D12.1* and *opt-2* were up-regulated in the *let-7* mutant, the difference from N2 worms was not even two fold. These genes may be mainly regulated at the translational level, rather then through mRNA decay. A western blot comparing *T27D12.1* and *opt-2* protein levels in N2 and *let-7(n2853)* worms could confirm this theory.

Further computational analysis of the new targets was performed to show dependence on *let-7* for ALG-1 association. RNA hybrid, a miRNA target

prediction tool, was used to search for *let-7* complementary sites (LCS) within the ALG-1 binding sites of the target genes (Figure 2c). *T27D12.1* and *opt-2* both had ALG-1 binding sites in their exons that contained predicted LCSs. The *let-7* seed sequence perfectly base paired to these new targets, strongly suggesting they are regulated by *let-7*. *prmt-1* has an ALG-1 binding site in the 3'UTR that also overlapped with a site that substantially base pairs with *let-7*.

Genetic analysis has proven to be a useful tool in detecting genes that are potential targets of *let-7*. Microarray analysis was used to detect genes that are mis-regulated in *let-7* mutants, and RNAi phenotypic screens proved genetic interaction with *let-7* for twenty-three of the genes. I have further analyzed the genes with ALG-1 binding sites because they are likely targets of a miRNA. Five genes are associated with ALG-1 in a *let-7* dependent manner, including three novel potential targets *T27D12.1, opt-2*, and *prmt-1*.

Table 1: Potential direct targets of *let-7* identified.
An RNAi screen was performed for over 300 genes that were shown via microarray analysis to be mis-regulated in *let-7* mutant worms (increase in *let-7*). The RNAi screen sought to identify genes that could suppress *let-7* mutant phenotypes. The *let-7* phenotypes suppressed include extra seam cell nuclei (S) and vulva bursting (B). ALG-1 binding sites were identified through CLIP analysis indicating direct miRNA targeting, these genes are highlighted in yellow. ALG-1 binding sites were found in exons (ex), introns (in), and 3'UTRs.

Gene	Sequence Name	Increase in *let-7*	Phenotype Suppressed	Gene Description	ALG-1 Binding Site
daf-12	F11A1.3	2.1	BS	nuclear hormone receptor	1 in, 2 3'UTR
lin-41	C12C8.3	2.8	BS	Ring finger-B Box-Coiled coil	1 ex, 1 3'UTR
opt-2	K0437.2	1.15	BS	oligopeptide transporter family member	1 ex
nhr-25	F11C1.6	1.71	B	nuclear hormone receptor	
hbl-1	F13D11.2	1.23	B	zinc-finger transcription factor	3 3'UTR
adt-2	F08C6.1	1.07	B	ADAMTS family	1 ex, 1 3'UTR
	T08B2.8	1.04	B	mitochondrial ribosomal protein L23	
	C26E6.6	1.13	B	Ribosomal protein L3	
lin-11	ZC247.3	1.1	B	homeodomain transcription factor	
sox-1	C32E12.5	1.49	B	HMG-box transcription factor	
sox-2	K08A8.2	1.11	B	HMG-box transcription factor	
fos-1	F29G9.4	1.21	B	Fos bZip transcription factor ortholog	
	T25G3.3	1.15	B	ortholog of *S cerevisiae* NMD3	
rha-2	C06E1.10	1.16	B	DEAH RNA helicase	
	Y39B6A.33	1.08	B	tumor suppressor gene	
	F42A8.1	1.48	B	unknown function	
	ZK1236.1	1.13	S	Elongation factor-type GTP-binding protein	
	W10D5.2	1.04	S	NADH-ubiquinone oxidoreductase	
sor-1	ZK1236.3	1.1	S	SOp-2 Related (ectopic expression of Hox genes)	
daf-9	T13C5.1	1.61	S	Cytochrome P450 from CYP2 subfamily	1 3'UTR
	Y113G7B.17	1.19	S	Protein arginine N-methyltransferase family	1 ex, 1 3'UTR
	T27D12.1	1.31	S	Permease of the major facilitator superfamily	1 ex, 1 3'UTR
clec-51	B0218.6	1.13	S	C-type Lectin family member	

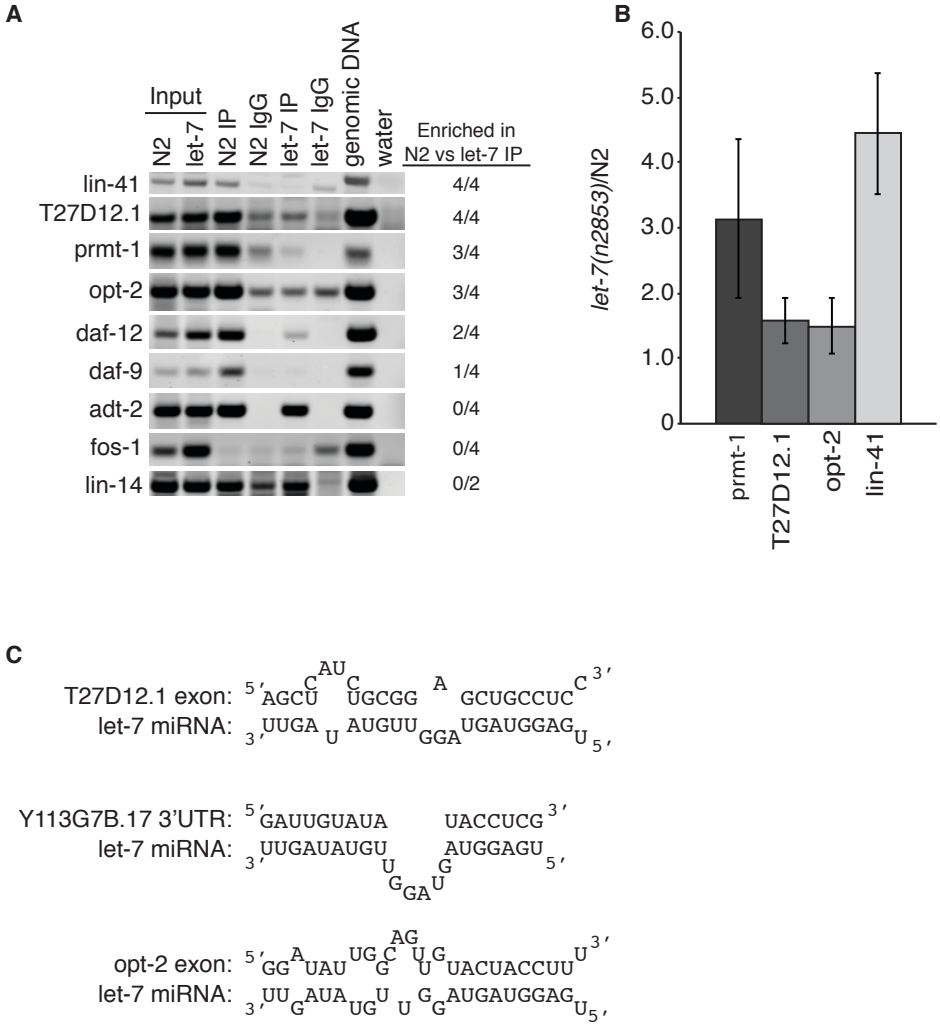

Figure 2: New direct targets of *let-7* miRNA

(a) Sequences in the indicated genes were detected by semi-quantitative PCR of cDNA from ALG-1 immunoprecipitation assays from L4 staged N2 and *let-7(n2853)* strains. Enrichment in the N2 compared to *let-7* IP from 4 independent experiments shows dependence on *let-7* miRNA for interaction with ALG-1. Three new targets were identified, *T27D12.1, prmt-1,* and *opt-2*.

(b) qPCR analysis of N2 and *let-7(n2853)* cDNA from L4 staged worms (n=3). Targets were normalized to 18S ribosomal RNA. New targets of *let-7* are shown to be upregulated in mutants compared to N2, as well as the *lin-41* control.

(c) *let-7* complementary sites (LCS) are present in each of the newly identified targets. Each LCS is within an ALG-1 binding site.

Results: Chapter 2

Investigation of feedback regulation by *let-7* targets

Feedback regulation on *let-7* by its target genes has previously been seen by *daf-12* and *hbl-1* (Hammell et al., 2009) (Roush and Slack, 2008). The nuclear hormone receptor, *daf-12,* can positively or negatively regulate *let-7* family miRNAs depending on the presence of its ligand (Hammell et al., 2009). While *hbl-1,* a transcription factor, negatively regulates *let-7* expression (Roush and Slack, 2008). Thus, *let-7* feedback regulation can have positive or negative effects on *let-7* expression. In order to study possible feedback regulation on *let-7,* I have used the *lin-28(n719)* mutant worm strain. LIN-28 suppresses the processing of primary *let-7* to precursor *let-7*, preventing precocious expression of *let-7* in early larval stages (Van Wynsberghe et al., 2011). Loss of function mutations in *lin-28(n719)* result in a partially penetrant temperature-sensitive protruding multiple vulva (pmuv) phenotype that is dependent on *let-7* levels. The *lin-28(n719)* pmuv phenotype is expressed in ~67% of the population, and the remaining worms express one protruding vulva (pvul). When *lin-28(n719)* worms are crossed to *let-7(mn112)* null mutant worms, the pmuv phenotype is no longer observed and 100% of the population expresses the pvul phenotype. This loss of phenotype coinciding with the loss of *let-7* indicates that the pmuv phenotype is dependent on *let-7* levels. (Pasquinelli, unpublished data).

A phenotypic RNAi screen was performed against the twenty-three *let-7* candidate targets to identify potential regulators of *let-7* activity. Since the pmuv phenotype is thought to be dependent on *let-7* levels, I screened for significant

changes in the penetrance of pmuv in the population. Thus, I hypothesized that a decrease in the pmuv phenotype would correlate with a decrease in *let-7*, while an increase would mean an increase in *let-7* levels. Five independent experiments were performed, in which the L4 staged *lin-28(n719)* worms were scored for the percentage of pmuv. Vector, a plasmid with no insertion for targeting, was used as a control. *lin-46* and *lin-42* were also used as controls, as they are known to affect *lin-28* phenotypes when mutated. *lin-46* decreases the pmuv phenotype, and was seen to be significantly lower than the vector control (Pepper et al., 2004). *lin-42* does not enhance the pmuv phenotype but actually enhances lethality of the worms later in development (Pasquinelli, unpublished data). Nine of the twenty-three potential *let-7* targets were shown to significantly change the pmuv phenotype (Figure 3). RNAi of five of the genes significantly suppressed the pmuv phenotype, as compared to vector control, while five of the genes enhanced the pmuv phenotype. *lin-41* did not significantly alter the pmuv phenotype; however, it did cause a bursting phenotype and was therefore moved on for further analysis. These genes may have affected the pmuv phenotype by altering *let-7* levels. To ensure the changes were specifically due to regulation of *let-7*, a secondary RNAi screen was performed.

Discovery of *let-7* regulation by potential targets

A secondary screen was performed to narrow down the candidates that may be able to affect *let-7* activity. *lin-28(n719)*;*let-7(mn112)* worms were used because they are null for *let-7*, therefore no changes in phenotype should occur if

their previous pmuv phenotype changes were due to alterations of *let-7*. RNAi was again used against the potential *let-7* interactors and the L4 worms were scored for percentage of pvul (Figure 4). *lin-41* and *nhr-25* showed a significant decrease in the pvul phenotype, and only about 1% of the population expressed pvul. *lin-41* and *nhr-25* must have changed the vulva phenotypes independently from *let-7* in these RNAi screens and were not further analyzed. The other genes did not change phenotypes significantly and were still considered possible regulators of *let-7*. In order to verify actual regulation of *let-7,* PAGE northerns were performed to measure mature *let-7* levels directly.

The phenotypic screens narrowed down the possible regulators of *let-7* from twenty-three to eleven candidates. However, northern analysis was needed to confirm actual regulation through directly measuring *let-7* levels. In *lin-28(n719)* worms mature *let-7* is detectable as early as 10 hours, at the L1 stage, while in N2 worms *let-7* is not seen until L3, at 24 hours. I chose to look for changes in *let-7* levels at 24 hours since this is when *let*-7 is detectable in N2 worms. L3 staged *lin-28(n719)* worms were grown under RNAi conditions for each of the candidate genes. The worms were collected and RNA extractions were performed. RNA was analyzed on a PAGE northern and probed for mature *let-7* and 5.8S ribosomal RNA as a loading control as its expression is relatively constant. Mature *let-7* was measured for each candidate and normalized to vector control, set to 1. Five of the genes were able to significantly change mature *let-7* levels (Figure 5). *hbl-1*, a known regulator of *let-7*, showed a fold

increase in *let-7* of 1.54±0.07. *T25G3.3* and *nduf-7* both showed a significant increase in mature *let-7* levels under RNAi, while *prmt-1* and *T08B2.8* showed a decrease. Thus, four novel regulators of *let-7* were discovered in a *lin-28(n719)* background at a later larval stage in *C. elegans*.

prmt-1 was further analyzed at an earlier stage in worm development, L1, when *let-7* is first detectable in *lin-28(n719)* worms. *lin-28(n719)* worms were grown on RNAi for either *prmt-1* or vector before being collected for a double RNA extraction. The RNA was analyzed via PAGE northern using probes for mature *let-7* and 5.8S was used as a loading control. *prmt-1* had significantly lower levels of mature *let-7* compared to vector control in *lin-28(n719)* worms (Figure 6).

Twenty-three genes were identified as being able to suppress *let-7* phenotypes, and therefore have some genetic interaction with *let-7*. Phenotypic RNAi screens implicated eleven genes as possibly regulating *let-7* activity. Five of these genes proved to be significantly altering mature *let-7* levels in a late larval stage, including the known *let-7* regulator *hbl-1*. RNAi of *prmt-1* showed a significant decrease in *let-7* during multiple stages in *lin-28(n719)* worm development. *prmt-1* was also proven to be a likely target of *let-7*, placing *prmt-1* and *let-7* in a novel feedback loop.

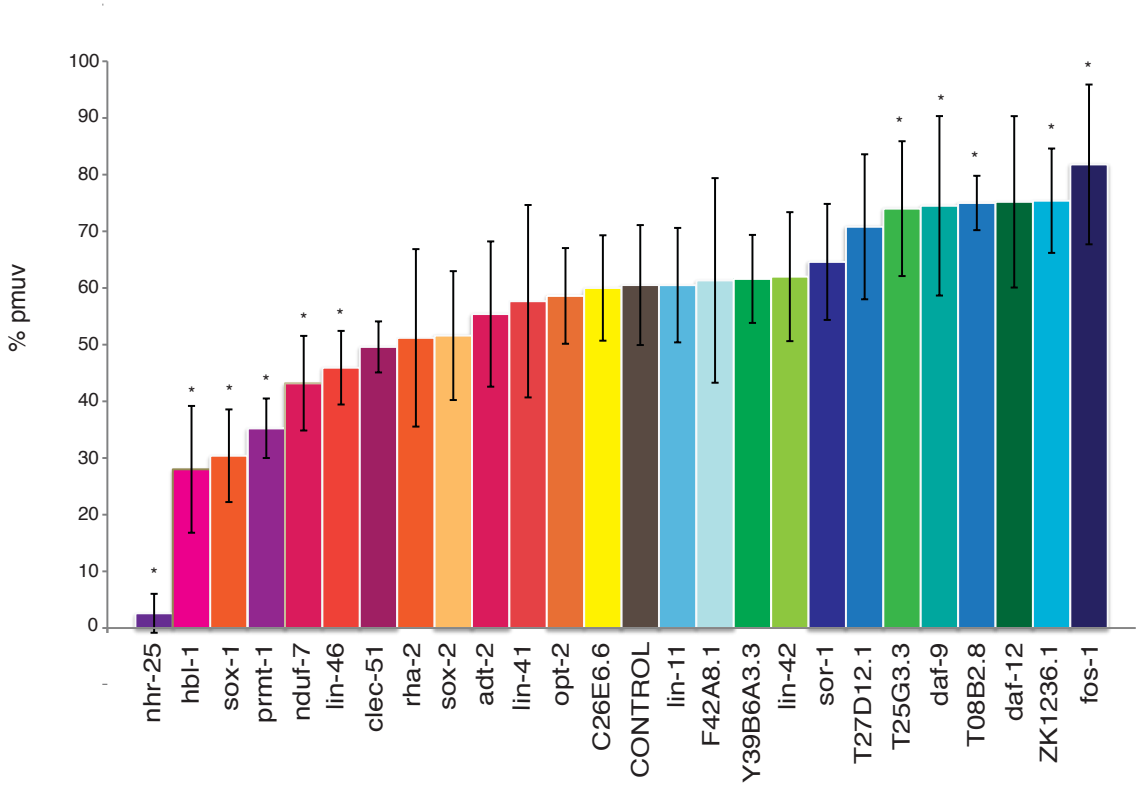

Figure 3: Primary screen identifies potential regulators of *let-7*
Results of an RNAi phenotypic screen of *lin-28(n719)* worms detecting changes in protruding multi-vulva (pmuv). 50-100 *lin-28(n719)* worms were grown to L4 under RNAi conditions for each specific gene (x-axis) and scored for percentage of pmuv (y-axis). The bar graphs represent the average percent of pmuv worms as determined from n=5. Error bars represent the standard deviation from the mean and the * point to clones that resulted in significant enhancement or suppression in the % of pmuv worms when compared to control (empty vector), *P<0.05.

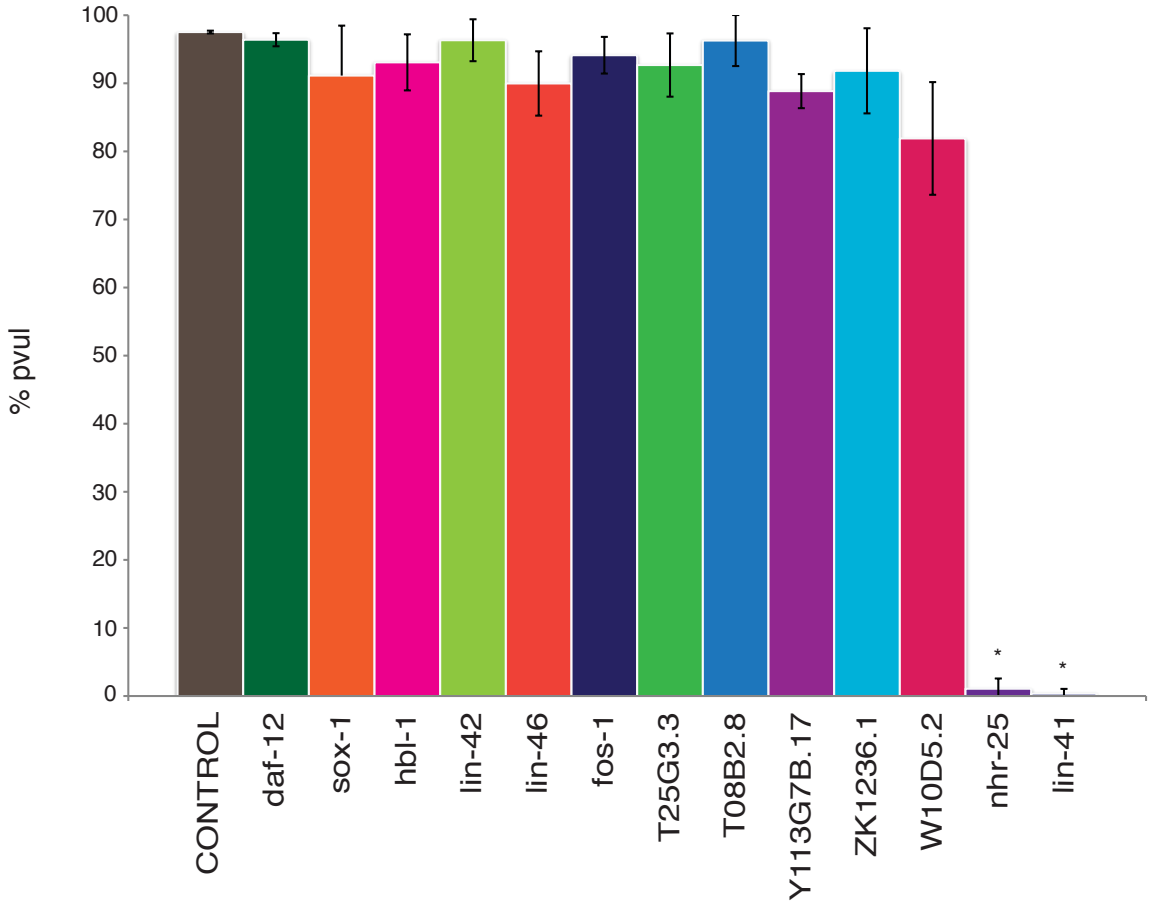

Figure 4: Secondary screen shows dependence on *let-7*
Results of an RNAi Phenotypic Screen of *lin-28(n719);let-7(mn112)* worms detecting changes in protruding vulva (pvul). 50-100 *lin-28(n719);let-7(mn112)* worms were grown to L4 under RNAi conditions for each gene (x-axis), and scored for the percentage of pvul (y-axis). The bar graph represents the average percent of pvul worms as determined from n=4. Error bars represent the standard deviation from the mean and the * points to clones that have a significant suppression of the pvul phenotype when compared to control (empty vector), *P<0.05

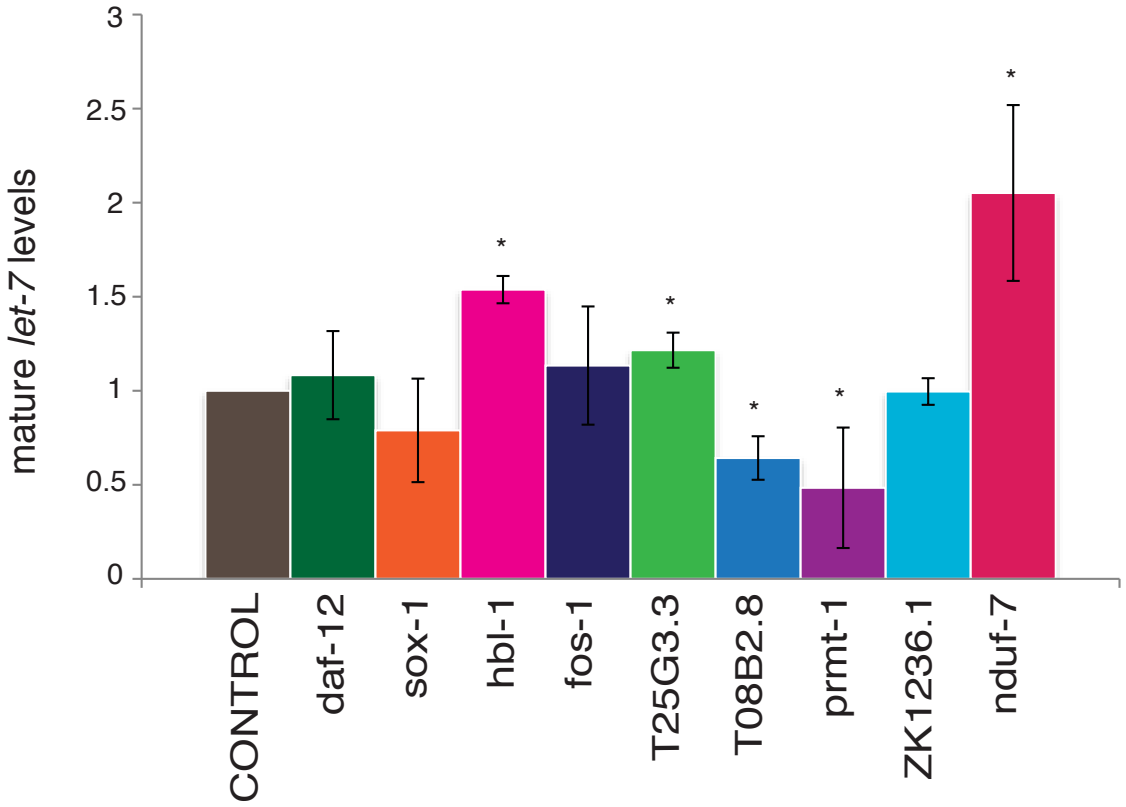

Figure 5: Northern analysis verifies regulators of *let-7*
PAGE northern analysis of mature *let-7* levels from RNA of L3 *lin-28(n719)* worms grown under RNAi of each candidate gene (x-axis). RNA samples were analyzed for mature *let-7* and normalized to 5.8S ribosomal, with vector control set to 1. The bar graph represents the average amount of mature *let-7* from n=3. Error bars represent the standard deviation from the mean and the * point to clones that resulted in a significant difference in mature let-7 levels, *P<0.05.

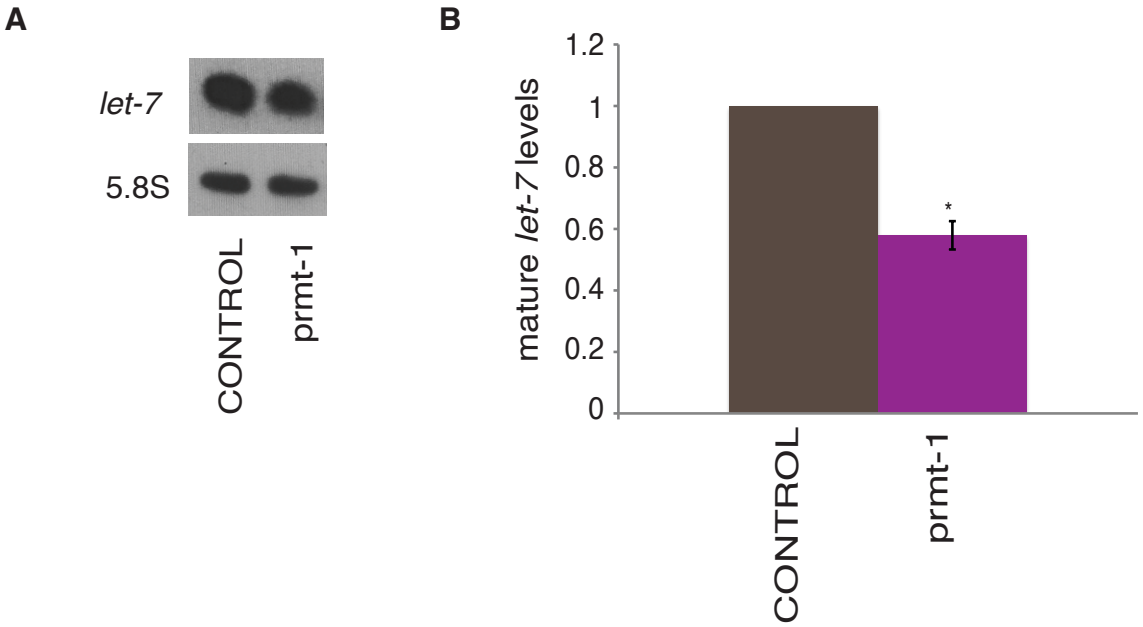

Figure 6: Northern analysis of the effects on *let-7* under *prmt-1* RNAi

(a) Representative PAGE northern of L1 staged *lin-28(n719)* worms under RNAi of control or *prmt-1*.

(b) RNA samples were analyzed for mature *let-7* and normalized to 5.8S ribosomal, with vector control set to 1. The bar graph represents the average amount of mature *let-7* from n=3. Error bars represent the standard deviation from the mean and the * point to clones that resulted in significant difference in mature let-7 levels, *P<0.05.

Discussion

MiRNAs are an abundant class of gene regulators in the cell, and because they do not require perfect binding, each miRNA has many targets. To fully understand the role they play in different pathways it is necessary to find their mRNA targets. I was able to able to find five likely targets of *let-7* through immunoprecipitation and computational analysis of twenty-three genes that suppress *let-7* phenotypes under RNAi. This included 2 known targets, *lin-41* and *daf-12*, and 3 novel targets, *prmt-1, opt-2,* and *T27D12.1*. The other suppressors of *let-7* phenotypes were not confirmed or discarded as being targets, but some were implicated as being regulators of *let-7*. *let-7* is highly regulated since it plays such an important role in causing cellular differentiation, and I was able to find four novel genes that affect *let-7* levels when knocked down by RNAi in *lin-28(n719)* worms. *T25G3.3* and *T08B2.8* increased the presence of mature *let-7* under RNAi, while *nduf-7* and *prmt-1* decreased mature *let-7* levels. *prmt-1* is not only regulated by *let-7*, but also is able to regulate *let-7* through a negative feedback loop.

Discovery of novel *let-7* targets

opt-2, also known as *pept-1*, is a member of the peptide transport family, and is homologous to the human protein OPT-2. PEPT proteins are able to transport peptides, as well as antibiotics, enzyme inhibitors, and anti tumor agents making them highly applicable for medicinal purposes (Rubio-Aliaga et al., 200). *opt-2* was able to suppress both *let-7* phenotypes under RNAi and was associated with ALG-1 in a *let-7* dependent manner. OPT-2 is expressed in

the intestinal tract of *C. elegans* facilitating the uptake of di- and tripeptides (Nehrke, 2003). Reporters of *let-7* are also expressed in the intestine (Johnson et al., 2003) (Kai, unpublished data) giving *let-7* access to regulate *opt-2*. However, temporal expression of *opt-2* is not consistent with being regulated by *let-7*. Through RT-PCR mRNA levels were measured at different stages in development, and L3-L4 worms showed higher expression levels of *opt-2* then did L1-L2 worms (Fei et al., 1994). This is not what is to be expected for a target of *let-7*, although it should be noted that microarray analysis and qPCR showed higher levels of *opt-2* mRNA in *let-7* mutants compared to N2 worms.

T27D12.1 has not been studied in *C. elegans* and little is known about the gene. It is predicted to be part of the major facilitator superfamily as a membrane transport protein. Expression patterns of this gene are unknown and there is no current literature on its function in *C. elegans*. However, it has been part of microarray and RNAi screens (Niwa et al., 2009). In a study looking for *hbl-1* targets, a transgenic worm was created that overexpressed *hbl-1* under heat shock treatment. After heat shock treatment, the worms were subject to microarray analysis, in which *T27D12.1* was found to be down-regulated. RNAi analysis of genes that were affected by *hbl-1* overexpression was performed, but *T27D12.1* was unable to alter any *hbl-1* phenotypes.

PRMT-1 is a homolog of the human protein arginine methyltransferase. In mammalian cells, PRMT1 is a major contributor to methylation of H4 Arg-3, leading to transcriptional activation (Strahl et al., 2001) (Wang et al., 2001). More

recently, PRMT1 has been shown to methylate arginine residues on other proteins in mammalian cells and *C. elegans* (Yamagata et al., 2008) (Takahashi et al., 2011). FOXO/DAF-16 was found to be methylated by PRMT1 to prevent phosphorylation of DAF-16 by AKT. In *C. elegans,* transcriptional reporters of *prmt-1* have shown it to be expressed in several tissues, including the intestine, pharynx, vulva muscle, and neurons (Takahashi et al., 2011) (Hunt-Newbury et al., 2007). *let-7* is also present in these cell types, and expression of GFP was shown to decrease as the worms moved into the later larval stages. This supports *prmt-1* being a target of *let-7* as it is down-regulated when *let-7* is expressed in *C. elegans*.

Discovery of novel *let-7* regulators

prmt-1 was also proven to be a regulator of *let-7* levels, along with *nduf-7*, *T25G3.3*, and *T08B2.8*. *T25G3.3* and *T08B2.8* both increased the pmuv phenotype in *lin-28(n719)* worms under RNAi conditions, which I theorized was an indication of their ability to increase *let-7* levels. While this was true for *T25G3.3*, *T08B2.8* actually showed a decrease in *let-7*. A similar result was seen for the genes that decreased the pmuv phenotype; *prmt-1* decreased *let-7* levels as predicted, while *nduf-7* increased *let-7* levels. However, upon gonad staging of *lin-28(n719)* worms I found higher concentrations of RNAi of *nduf-7* caused the most severe developmental delays. Thus the worm vulvas might not have fully developed by 48 hours when they were scored.

T25G3.3 is an ortholog of NMD3 in *S. cerevisiae*, and is thought to play a

role in transport of large ribosomal subunits out of the nucleus. *T08B2.8* is thought to be a mitochondrial ribosomal protein. Neither of these genes has been studied in *C. elegans* and their expression patterns are unknown. *nduf-7* is an ortholog to the human *nduf-7*, NADH-ubiquinone oxidoreductase Fe-S protein 7. When *nduf-7* is mutated it causes Leigh syndrome, a neurometabolic disorder that eventually causes death. In *C. elegans nduf-7* is expressed in neurons, muscle tissue, and the pharynx (Hunt-Newbury et al., 2007), which overlaps with *let-7* tissue expression (Johnson et al., 2003). None of these genes have been studied in worms and further experiments will need to be performed in order to determine how and why they may regulate *let-7*.

A negative feedback loop by a *let-7* target

I have shown that *prmt-1* is both regulated by and able to regulate *let-7*, resulting in a negative feedback loop. In a *lin-28(n719)* worm, *prmt-1* RNAi caused down-regulation of *let-7* at early and late larval stages. Therefore in wildtype conditions of *prmt-1, prmt-1* is positively regulating *let-7*, and increases mature *let-7* levels. I predict *prmt-1* could be altering *let-7* levels by indirectly increasing transcription of primary *let-7*. RNAi was able to affect mature and precursor *let-7* (data not shown), and due to previous evidence of the role of *prmt-1* in the cell, transcription is likely to be affected. PRMT-1 has been shown to methylate histones in human tissue culture, and proteins in *C. elegans*. PRMT-1 could be activating transcription through histone methylation, although its role in histone modifications has not yet been studied in *C. elegans*. In *C.*

elegans there is evidence of alterations to a transcription factor by PRMT-1, and this function may be conserved to other proteins that transcriptionally regulate *let-7*.

Future Directions

In the future I hope to validate *prmt-1* as a target of *let-7* through transgenic worms with a reporter construct containing GFP::3'UTR of *prmt*-1 with and without the LCS. I have also obtained a *prmt-1* mutant worm strain (VC2597), to verify the effects on *let-7* seen through RNAi experiments. To determine how *prmt-1* is affecting *let-7* levels I will need to perform a developmental time course comparing N2 worms to the mutants. Analyzing primary, precursor, and mature *let-7* levels at multiple stages in worm development will determine at which point in the miRNA pathway *prmt-1* is able to affect *let-7*. RNAi of the other genes regulating *let-7* should also be performed at an early larval stage to determine if *let-7* is affected throughout development. RNAi of the candidate genes should also be performed on N2 worms to verify if the effects on *let-7* are still seen. Transgenic worm strains containing GFP reporter constructs for the *let-7* regulators would be created to determine expression patterns and narrow down how let-7 is affected, as they have not yet been well-characterized. In summary, I hope my studies will verify six novel *let-7* interactors contributing a deeper understanding to the role *let-7* plays in the cell.

Materials and Methods

***C. elegans* RNA immunoprecipitation (RIP)**

Synchronized starved L1 N2 and *let-7(n2853)* worms were grown at 25°C for 29 hours before being cross-linked by UV treatment. Equal amounts of lysates were precleared before immunoprecipitation with the appropriate antibody and protein G Dynabeads (Invitrogen). Immunoprecipitated material associated with the beads was subjected to protein degradation and RNA extraction before RT-PCR with random primers.

Semi-quantitative PCR

PCR was performed with GoTaq polymerase (Promega) and 10 uM of each forward and reverse primer was used. 1 ul of a 1:10 dilution of RIP cDNA was used for analysis. Primers are listed in Table 2.

qPCR.

RNA was collected from N2 and *let-7(n2853)* worms grown at 25°C for 28 hours before RT-PCR was performed. qPCR was performed on cDNA with SYBR green (Applied Biosystems) and 10 uM of each forward and reverse primer on an ABI Prism 7000 real time PCR machine. Primers are listed in Table 2 and most were the same used for semi-quantitatve PCR.

Worm Culture Conditions.

Worms were cultured at 25°C and synchronized by standard hypochlorite treatment. Development was initiated by plating arrested L1 hatchlings on NGM plates seeded with OP50 bacteria or RNAi bacteria on RNAi plates.

RNAi conditions and scoring.

50-100 arrested L1 hatchlings were plated on RNAi plates and scored for vulva phenotypes at 48hr (25°C) in the adult worms. Suppression/Enhancement was determined by a Ttest comparing worms on each RNAi vector to those on the empty L4440 control vector grown on the same day.

Northern Blot Analysis.

RNAi was performed for candidate gene and empty L4440 control vector. To detect miRNAs, northern analyses were performed by separating 15 µg of RNA in 11% denaturing polyacrylamide gels and transferred to nylon membranes Radiolabeled DNA probe was generated from the IDT StarFire Kit for *let-7* (AACTATACAACCTACTACCTCA) and the 5.8S probe is a PCR-generated DNA probe (A479 + A480) radiolabeled using Prime-It II Random Primer Labeling Kit (Stratagene). Each was hybridized to the blot at different times in 5× SSC, 7% SDS, 0.02 m sodium phosphate, 1× Denhardt's solution for approximately 12 h at 50°C and then washed in 3× SSC, 5% SDS, 0.025 m sodium phosphate, 10× Denhardt's solution at 50°C. Blots were then analyzed with ImageQuant software (Molecular Dynamics). Significant changes were determined by a Ttest comparing worms on each RNAi vector to those on the empty L4440 control vector grown on the same day.

Table 2: Primers used for semi-quantitative PCR, qPCR, and Northern Probe

Target	Lab Designation	Sequence
daf-12	A484 A2647	GCCATGCCTTCAATACCTCAC TGATAGTGGCTATTTCCAAGTTGA
lin-41	A2203 A2204	ACATGTTTCTGGGCGATAGG CGTGCTGTTGGCTACTTCAA
adt-2	A2666 A2667	GCAACTGAAAATGGAGCACA GCGCTGTCCGGTAACAAATA
opt-2	A2650 A2651	GCCAGGATATTGGCAGTTGT TCCGGTCAATGCATAGATGA
fos-1	A2437 A2652	TCAATTCTATGCCACCACCA TTTCCGAACGACCGTGTAAT
daf-9	A2674 A2675	GATTACTGCTCCGCCAAAAC TTGGTAAAGCTTGATGGGAGA
T27D12.1	A2655 A2656	CAACACATTGGCTTTTGTCG ACTGCTTGGAAAGCACTGGT
Y113G7B.17	A2672 A2673	TGGAGTACCGAGCAGAGTGA TTTTGCCCCCAATTTTTACA
lin-14	A426 A449	GGGGCCAAATATCTATATCCAAAGTAG ATGAGAACAATGACGACAAATAGG
lin-41(qPCR)	A261 A262	TTGCAGCAATCGATGAAGACAAC AGTGGGCCATGTGCCAAGAATAG
18S (qPCR)	A839 A2145	GCGTACGGCTCATTAGAGCAGATATCAC GCAGACACCCTTTCCGGATAGA
5.8S probe	A479 A480	CTAGCTTCAGCGATGGATCGGTTGC GAACCAGACGTACCAACTGGAGGCCC

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