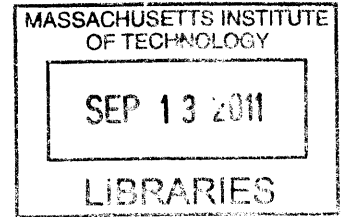MicroRNA Targeting in *Mus musculus* and *Caenorhabditis elegans*

by

Ginamarie N. Lafkas

B.S., Biology
Mount St. Mary's College, 2003

SUBMITTED TO THE DEPARTMENT OF BIOLOGY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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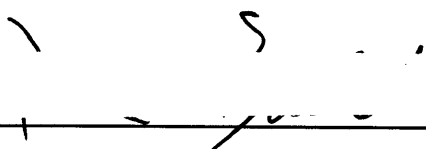AT THE
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MicroRNA Targeting in *Mus musculus* and *Caenorhabditis elegans*

by

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Submitted to the Department of Biology
In Partial Fulfillment of the Requirements for the Degree of
Masters of Science in Biology

ABSTRACT

MicroRNAs (miRNAs) are small, approximately 22 nucleotide RNAs that regulate gene expression post-transcriptionally by base-pairing to complementary sites in the target mRNA. The first miRNA, *lin-4*, was discovered in 1993 in *Caenorhabditis elegans*; since then hundreds of miRNAs have been identified in *C. elegans*, *Drosophila melanogaster*, plants, mouse, and humans, where they approach a number equivalent to 1-2% of the protein-coding genes. With the exception of plants, miRNAs most commonly regulate targets by imperfectly pairing to 3' untranslated regions (UTRs), leading to translational repression or mRNA destabilization.

The microRNA miR-196 is encoded at three paralogous locations in the *HoxA, B,* and *C* clusters in mammals and has conserved complementarity to the 3'UTRs of *Hoxb8, Hoxc8,* and *Hoxa7*; in particular, miR-196 has complete complementarity to *Hoxb8* with the exception of a single G:U wobble. In 2004, Yekta et al., were able to detect RNA fragments diagnostic of miR-196-directed cleavage of *Hoxb8 transcript in* mouse embryos, and cell culture experiments showed down-regulation of *Hoxb8, Hoxc8, Hoxd8,* and *Hoxa7.*

To address the biological significance of miR-196 mediated repression of *Hox* genes *in vivo*, we attempted to generate targeted transgenic mice for the *Hoxa7, b8* and *c8* genes. These mice would allow us to study the resulting phenotypic and molecular consequences and determine the impact this regulation has on establishment of the anterior-posterior axis in the developing embryo as well as its role in defining the expression boundaries of the individual *Hox* genes *in vivo*.

The *lin-4* miRNA plays a role in regulating the heterochronic genes involved in larval development in *C. elegans*. We used luciferase assays to test the efficacy of the seven proposed *lin-4* binding sites in the *lin-14* 3'UTR and to determine if *lin-4* is capable of recognizing and mediating repression through them. The wild-type *lin-14* 3'UTR was compared with mutant UTRs in which the *lin-4* target sites were mutated. We determined that the three canonical 8mer sites are functional, as expected, and that at least one of the four additional sites is also recognized by *lin-4* and contributes to the overall repression of the *lin-14* 3'UTR.

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The role of miR-196 in regulating the *Hox* cluster

Abstract

To further characterize the role of miR-196 in regulating the expression of *Hoxb8, Hoxc8,* and *Hoxa7*, we attempted to generate targeted transgenic mice with mutant miR-196 sites that would prevent miRNA-mediated regulation of these targets. Targeting vectors were constructed using recombineering for each of these three genes. To disrupt target recognition by miR-196, nucleotides that complement the 3rd, 5th, and 7th nucleotides in the seed of miR-196 were mutated in the 3'UTR's of each *Hox* gene construct. All three constructs were verified by sequencing and diagnostic restriction digests, then linearized and submitted to the Rippel Mouse ES Cell & Transgenics Facility. Genomic DNA from embryonic stem cells that survived selection were screened by Southern Blot to check for homologous recombination and single integration at each of the three *Hox* genes.

Abbreviations

miRNA, microRNA; UTR, untranslated region; ES cells, embryonic stem cells; nt,

nucleotide, kb, kilobases; neo, neomycin

Introduction

The regulation of gene expression is critical for the viability of all living systems. There are many stages at which the expression of a gene can be modulated, including modifications of the DNA, control of transcription and translation, changes in mRNA processing or stability, and post-translational modifications. It is the ability to control the timing, level, and location of gene expression that allows for cellular differentiation (how cells become specialized), morphogenesis and adaptability of an organism over time. When any of these factors is disrupted, as in the case of transgenic mice that are either null for or overexpress a given *Hox* gene, embryos can exhibit gross morphological defects ranging from homeotic transformations in vertebrae to craniofacial abnormalities (Krumlauf, 1994). For example, mice homozygous for a loss-of-function *Hoxb8* allele, a member of the *Hox* family of transcription factors important for axis formation in embryos, suffer from defects in rib formation, degeneration of spinal ganglia and excessive grooming behaviors after birth (Greer and Capecchi, 2002; van den Akker et al., 1999). In addition, ectopic expression of *Hoxb8* can lead to complete forelimb duplications, persistence of the first spinal ganglion, posterior transformations of lower cervical vertebrae and embryonic lethality in a homozygous state (Fanarraga et al., 1997). Therefore, determining all the mechanisms by which developmental genes are regulated is not only valuable for a clear understanding how development proceeds in its wild-type state but also for accurately assessing what leads to diseased states. The *Hox* clusters

provide a good model for studying the intricacy of developmental gene regulation.

Animal microRNAs are ~22 nt non-coding RNAs that regulate gene expression post-transcriptionally through base-pairing to their target mRNA (Lai, 2003). The first miRNA, *lin-4*, discovered in *C. elegans* plays a role in regulating the heterochronic genes involved in larval development (Lee et al., 1993); since then, through deep sequencing, computational and genetic methods, hundreds of miRNAs have been identified in plants and animals, where they play roles in regulating genes involved in processes from developmental patterning, to signaling pathways and even cancer (Lagos-Quintana et al., 2003; Lee and Ambros, 2001; Lewis et al., 2003; Lim et al., 2003; Stark et al., 2005). miRNAs are derived from polymerase II transcripts known as the primary miRNAs (pri-miRNAs) (Lee et al., 2004). The mature miRNA, capable of silencing targets, is generated by two key processing steps: 1) cleavage by Drosha, a nuclear Ribonuclease III (RNase III), to produce the precursor miRNA (pre-miRNA), which is exported out of the nucleus and 2) cleavage by Dicer, a cytoplasmic RNase III, to produce a ~22 nt miRNA duplex (Bohnsack et al., 2004; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Lee et al., 2003; Lund et al., 2004; Yi et al., 2003). One strand of the miRNA is loaded into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) and the other, known as the star strand, is degraded (Khvorova et al., 2003; Schwarz et al., 2003). miRNAs recognize and repress messages by base-pairing to targets with complementarity primarily to the 5' region of the miRNA

known as the seed (nucleotides 2-7) (Lewis et al., 2003). There are four types of

target sites that contribute to miRNA-mediated repression, an 8mer, a 7mer-m8,

a 7mer-A1 and a 6mer, with the 8mer typically yielding the most robust

repression (Figure 1) (Bartel, 2009; Grimson et al., 2007). Most targets in

metazoans are repressed through some mechanism of translational inhibition or

mRNA destabilization, but when there is extensive complementarity the miRNA

can cause cleavage of the target (Bartel, 2004; Guo et al., 2010; Liu et al., 2005;

Maroney et al., 2006; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et

al., 2006; Pillai et al., 2005; Thermann and Hentze, 2007). In 2004, Yekta et al.,

reported that *Hoxb8* has one site in its 3'UTR with near perfect complementarity

to miR-196 and went on to demonstrate that, in mouse, miR-196 directs cleavage

of *Hoxb8* (Yekta et al., 2004). This is one of the rare targets in mammals known

to be cleaved by a miRNA (Shin et al., 2010).

The three miR-196 genes, miR-196a-1, miR-196a-2, and miR-196b, all

map to locations in three of the four *Hox* clusters, *HoxA*, *HoxB*, and *HoxC*

respectively, with the relative position of each gene being evolutionarily

conserved in vertebrates (Lagos-Quintana et al., 2003; Lim et al., 2003). The

miR-196 miRNAs have target sites in many of the *Hox* genes that are positioned

more anteriorly to the miR-196 loci (Figure 2) (Yekta et al., 2008). Cleavage of

Hoxb8 by miR-196 is detectable in mouse embryos and luciferase reporter

assays and cell culture assays for *Hoxa7*, *Hoxc8*, and *Hoxd8* demonstrated that

a metazoan miRNA is capable of mediating posttranscriptional repression of

target mRNAs. Sensor transgene studies conducted in mice indicate that miR-

196 is present in the posterior trunk (Figure 3) (Mansfield et al., 2004).

Interestingly, the anterior end of miR-196 expression in these mice is similar to

the posterior boundary of *Hoxb8* expression, suggesting that miR-196 may act to

prevent *Hoxb8* expression in this posterior region. Limb specific conditional

Dicer knockouts mice showed that ectopic Retinoic Acid (RA) expression, which

normally induces *Hoxb8* expression only in the anterior forelimb, could induce

expression of *Hoxb8* mRNA in the hindlimb. However, this induction was not

possible in wild-type mice. Additionally, misexpression of miR-196 in chick

embryos not only reduces *Hoxb8* expression throughout the embryo but prevents

RA soaked beads implanted into the forelimb from inducing *Hoxb8* expression

(Hornstein et al., 2005). While it is known that miR-196 is expressed in the

developing embryo, directs cleavage of *Hoxb8* mRNA in the hindlimb, and is

capable of repressing translation of other *Hox* genes in reporter assays, the

developmental importance of this regulation is not known.

In the last 5 years, the global impact of miRNA-mediated repression has

been addressed in part by the generation of various miRNA knockout mice. The

mice from these different studies display phenotypes with varying levels of

severity. The miR-155 knockouts have compromised immune systems that

result in defects in adaptive immunity and autoimmunity, loss of miR-1-2 can

result in lethal heart defects, and miR-208 knockout mice suffer from heart

defects when subjected to cardiac stress (Rodriguez et al., 2007; Thai et al.,

2007; van Rooij et al., 2007; Zhao et al., 2007). While all of these knockouts

provide insight into the role of miRNA-mediated regulation, the identification of

the miRNA target genes responsible for the defects has been a difficult task, as one miRNA can regulate many targets. Generating transgenic mice, in which one gene target of a miRNA is mutated to avoid regulation, provides a system for analyzing the individual relationship between a miRNA and its target. The *Hox* clusters are ideal for this experiment because a single cluster contains multiple genes regulated by miR-196, potentially though different mechanisms. By mutating the miR-196 binding sites in the 3'UTR of a particular *Hox* gene, and not the miRNA itself, we can assess the role of miR-196 as a regulator of the *Hox* clusters by characterizing the phenotypic and molecular consequences that result when this mechanism of regulation is lost.

We attempted to generate targeted transgenic mice in order to address the role that miR-196 plays in regulating specific genes in the *Hox* cluster and the impact this regulation has on establishment of the AP axis in the embryo as well as its role in defining the expression boundaries of the individual *Hox* genes *in vivo*.

Materials and Methods

Generating Targeting Constructs for Hox genes

Three targeting vectors, one for each of the *Hox* genes mentioned

previously, were made using recombineering established by Liu et al., (Liu et al.,

2003). The 3'UTRs were first cloned into a pBluescript vector for mutagenesis

before recombination into the final targeting vector. There are five miR-196 sites

in the *Hoxa7* 3'UTR, one site in *Hoxb8*, and six sites in *Hoxc8* (Figure 4). In each

construct, all miR-196 seed sites in the 3' UTRs were mutated using the

Stratagene QuikChange Multi Site-Directed Mutagenesis kit using mutant

primers (IDT). To disrupt target recognition by miR-196, nucleotides that

complement the 3rd, 5th, and 7th nucleotides in the seed of miR-196 were mutated

in the 3'UTR's of each *Hox* gene changing the seed site from $^{5'}ACUACCUA^{3'}$ to

$^{5'}AGUCCGUA^{3'}$ (an 8mer site as an example). In all three constructs, the

introduced mutations either created or removed a restriction enzyme recognition

sequence that could be used for allelic identification in later assays. Once the 3'

UTR's had all miR-196 binding sites mutated, they were recombined into

targeting vectors containing a Diphtheria Toxin (DTA) driven by the

phoshoglycerate kinase (PGK) promoter for negative selection in embryonic stem

(ES) cells. After the initial recombination was confirmed by sequencing, a PGK-

neomycin transferase (neo) cassette was recombined into the vectors

downstream of the 3'UTR mutations for positive selection (Figure 5). After each

step in the cloning process, the vectors were checked by restriction digest,

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polymerase chain reaction (PCR), and sequencing to ensure accurate construction.

Targeting and Screening of Hox Targeting Constructs

Completed constructs were linearized and electroporated into C57BL/6 ES cells. Several hundreds of neo resistant ES cell clones were selected for each *Hox* gene. Screening for positive clones was first carried out by Southern blot analysis with a probe sequence outside of the long arm and the neo probe, to identify correct homologous recombinants and to exclude clones with multiple integrations, respectively. The same ES cell clones were also screened by PCR with a primer residing outside of the short arm and a primer within the neo cassette.

Preparing ES Cell DNA and Probes for Southern Screening

Genomic DNA from *Hoxa7*, *c8*, and *b8* clones were digested overnight, the digests were loaded into a 0.7% agarose gel, run overnight at 4°C and the DNA was transferred from the gel to a Nytran SuPerCharge membrane overnight using the Whatman TurboBlotter Alkaline Transfer System (Whatman). The long arm probes for each targeting construct were generated using PCR on genomic mouse DNA, gel purified and radiolabeled with [α-^{32}P]dCTP using the Rediprime II DNA Labeling System (Amersham). The labeled probes were purified from unincorporated label using illustra MicroSpin G-50 columns (GE Healthcare).

Southern Blot Analysis

Following transfer, the DNA was fixed to the membranes by UV crosslinking at 254 nm for one minute using a Stratalinker (Stratagene Cloning

Systems, La Jolla, California, USA). The membranes were pre-hybridized in 30 ml of Quick Hyb solution (Stratagene) for one hour at 60°C. Purified probe and salmon sperm DNA were boiled for 5 min and added directly to the hybridization buffer. The membranes were hybridized with probes rotating overnight at 60°C. Following hybridization the membranes were washed and exposed overnight at room temperature.

Results and Discussion

In HeLa cell reporter assays, miR-196 is capable of down regulating *Hoxa7, Hoxb8, Hoxc8* and *Hoxd8*. *Hoxb8* has a near perfect complementarity to miR-196 with the exception of a single G:U wobble and miR-196-directed cleavage of *Hoxb8* has been detected in total RNA from day 15 to 17 mouse embryos (Yekta et al., 2004). Although it is clear that miR-196 is capable of and does regulate members of the *Hox* gene family, the biological significance of this regulation remains unknown. To address the regulatory significance of miR-196, we attempted to generate targeted transgenic mice for three of the *Hox* family members, *Hoxa7, b8 and c8* to study the molecular and phenotypic consequences that loss of miRNA regulation has on the developing mouse embryo.

All three of the targeting constructs for the *Hoxa7, b8* and *c8* genes were submitted to the Rippel Mouse ES Cell & Transgenics Facility at MIT to generate targeted transgenic mice. If properly targeted these mice would possess one allele for the previously mentioned genes with a 3'UTR containing mutant miR-

196 binding sites thus preventing miR-196 mediated repression of that allele in the developing mouse embryo. The first round of targeting yielded 503 clones for *Hoxa7*, 354 for *Hoxb8* and 476 for *Hoxc8* for screening via Southern Blot. A positive clone from screening *Hoxa7* clones with a long arm probe would yield 2 bands: the wild-type band at 11.5 kilobases (kb) and a band at 9.5 kb representing the mutant recombinant band (Figure 6). A positive clone from a *Hoxb8* screening with a probe recognizing the long arm would yield a wild-type band at 12.1 kb and a band at 9.7 kb representing the mutant recombinant band (Figure 7). A positive clone from screening *Hoxc8* with the long arm probe would yield an 11.1 kb wild-type band and a 9.3 kb mutant recombinant band (Figure 8). The first round of southern screening yielded no positive recombinants for any of the three targeted loci. The constructs were resubmitted three more times, twice to the Rippel Mouse ES Cell & Transgenics Facility and once to Harvard Medical School's Brigham & Women's Hospital Transgenic Core Facility. In addition to resubmitting the constructs, the electroporations were switched from using C57BL/6 ES cells to V6.5 ES cells, a 129/B6 F1 hybrid line whose cells are supposed to be highly robust. A total of 6574 clones were screened across all three constructs with no positive recombinants.

Although targeting did not work for the three *Hox* genes attempted here, there are a few known parameters that could be changed in an attempt to increase the chances of homologous recombination in the ES cell and subsequent survival of that cell through the selection process. The goal in generating a construct for targeted transgenics is to minimize disruption of gene

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function especially to the surrounding genes. The frequency of homologous recombination depends on the degree of sequence match and the length of the matching sequence (Hasty et al., 1991; te Riele et al., 1992). Some studies suggest that a minimum of 7 kb of perfectly matched DNA sequence should be present for homologous recombination to occur at a practical frequency. For the targeting constructs in this study, there was approximately 3.5 kb of perfect sequence match before the first UTR mutation. Perhaps if the amount of perfect homology of the long arm (where the UTR mutations were placed) was increased upstream of the mutations, targeting efficiency would also increase.

A second possible factor affecting targeting is the presence of the neo cassette. To prevent long term disruption of gene expression in the targeted region, loxP sites flank the neo gene to allow for excision of the cassette by expression of Cre recombinase; however, the PGK-neo cassette can have unintended consequences on the expression of the targeted and adjacent genes (Meyers et al., 1998; Nagy et al., 1998; Olson et al., 1996). For example, the presence of the neo cassette in the targeted *Hoxa2* locus led to knockdown and premature expression in a number of the *Hox* genes surrounding the neo cassette resulting in death of mice before 3 weeks of age (Ren et al., 2002). Although these effects were not seen until after birth for the *Hoxa2* mice, it is possible that the presence or placement of the neo cassette had a negative effect on the ES cell due to targeting of the three loci described here.

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Acknowledgements

Figure Legends

Figure 1. Types of miRNA target sites. The four types of target sites present in a mRNA that contribute to miRNA-mediated repression: A 6mer complements nucleotides 2-6 in the miRNA seed, a 7mer-m8 has perfect complementarity to nucleotides 2-8 in the miRNA, the 7mer-A1 complements the miRNA seed and has an A at position 1, and an 8mer complements nucleotides 2-8 in the miRNA and has A opposite position 1 of the miRNA.

Figure 2. Genomic organization of the mammalian *Hox* clusters. The four chromosomes encoding the mammalian *Hox* clusters. The different colored arrows indicate paralogous genes from one of the 13 groups. The direction of arrowheads indicates direction of transcription, small blue and green arrowheads show the location of the miRNAs embedded in the clusters. Dashed red lines are repression based on bioinformatics and cell culture assays. Solid red lines indicate miRNA-mediated repression confirmed *in vivo*, by cell culture assay and bioinformatics.

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Figure 3. MiR-196 expression pattern mimics *Hox* expression in the developing mouse embryo. Sensor transgene study and *in situ* hybridization for *Hoxb8* in the developing mouse embryo. (d) *lacZ* expression from a sensor transgene containing miR-196 binding sites. *lacZ* expression is downregulated in the posterior trunk and tailbud. (e) Expression of *Hoxb8* detected by *in situ* hybridization. *Hoxb8* is downregulated in the region where miR-196 is expressed.

Figure 4. miR-196 target site mutations. The 3'UTRs of the 3 *Hox* genes mutated to prevent miR-196-mediated repression. *Hoxb8* has one site with almost complete complementarity, *Hoxc8* has six miR-196 sites, including a site of extended complementarity and *Hoxa7* has five. The red nucleotides indicate the positions in the *Hox* 3'UTRs that were mutated in the targeting constructs.

Figure 5. The final targeting constructs for the three *Hox* genes in this study. All three vectors contain a PGK-DTA marker for negative selection located downstream of the short arm and a PGK-neo cassette for positive selection located between the short and long arms. The 3'UTR mutations are located in the long arm for all three constructs.

Figure 6. Screening for *Hoxa7* recombinants. Southern blot analysis on genomic DNA from a series of ES cells that survived electroporation of *Hoxa7* targeting construct and selection. The first lane was loaded with a digested BAC DNA as a positive control, followed by a 1kb+ ladder and experimental samples. The red

arrows indicate where the wild-type band and recombinant band should appear on the membrane and their expected sizes.

Figure 7. Screening for *Hoxb8* recombinants. Southern blot analysis on genomic DNA from a series of ES cells that survived electroporation of *Hoxb8* targeting construct and selection. The first lane was loaded with a 1kb+ ladder, followed by the experimental samples. The red arrows indicate where the wild-type band and recombinant band should appear on the membrane and their expected sizes.
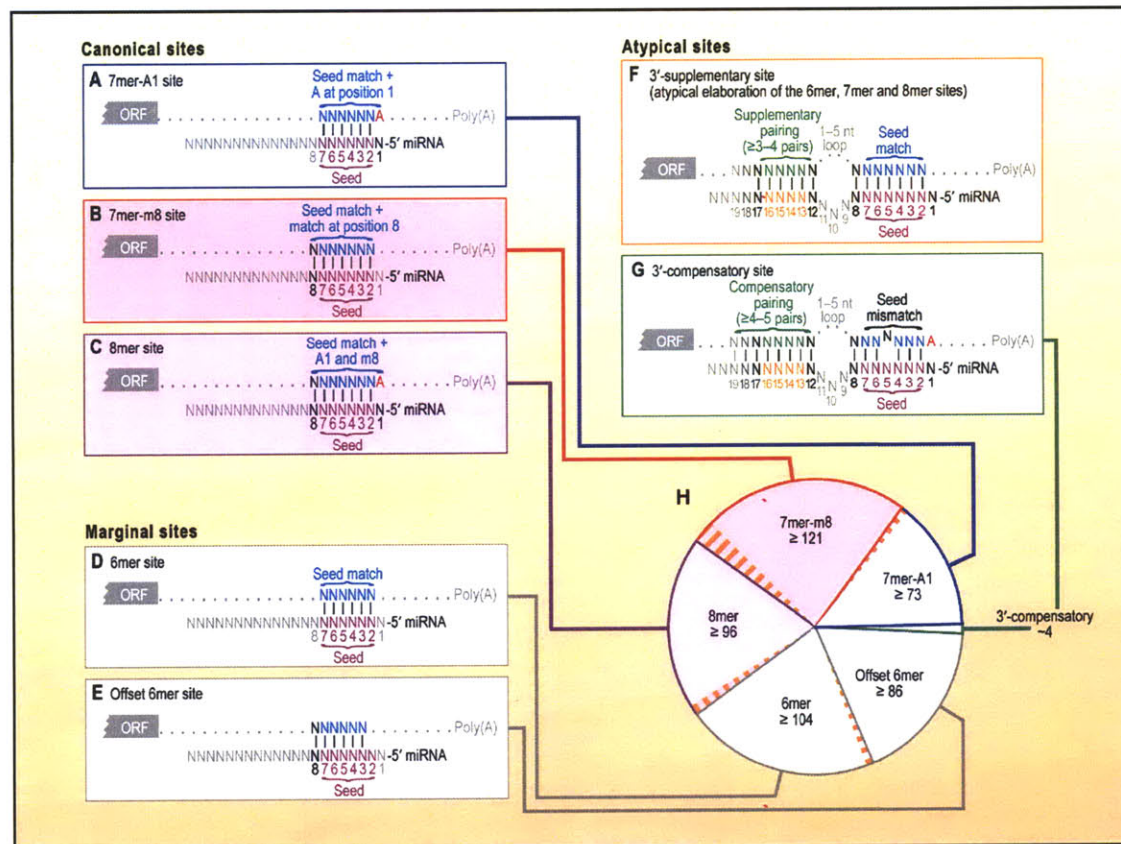
Figure 8. Screening for *Hoxc8* recombinants. Southern blot analysis on genomic DNA from a series of ES cells that survived electroporation of *Hoxc8* targeting construct and selection. The first lane was loaded with a digested BAC DNA as a positive control, followed by a 1kb+ ladder and the experimental samples. The red arrows indicate where the wild-type band and recombinant band should appear on the membrane and their expected sizes.

Figure 1

Bartel, D.P. 2009

Figure 2
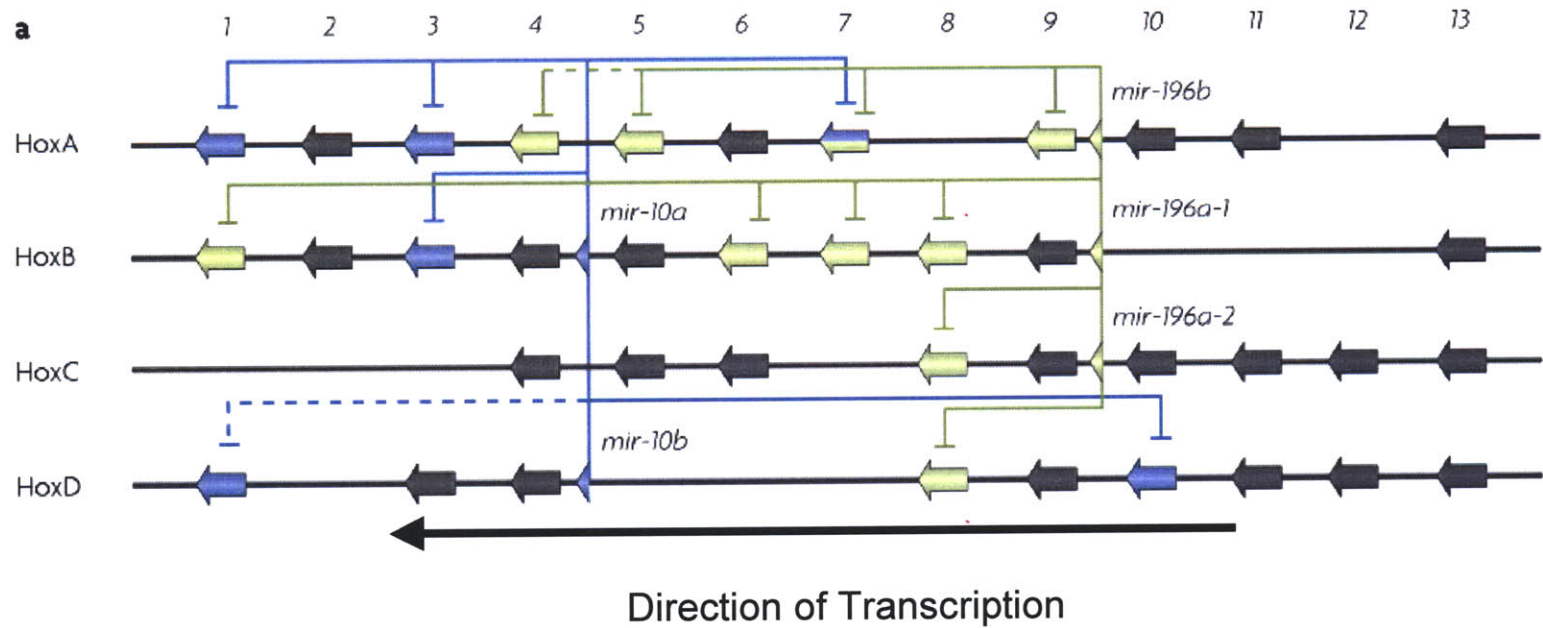
Yekta, S., et al., 2008

Figure 3

Posterior expression boundary of miR-196 sensor transgene

Posterior expression boundary of *hoxb8* mRNA
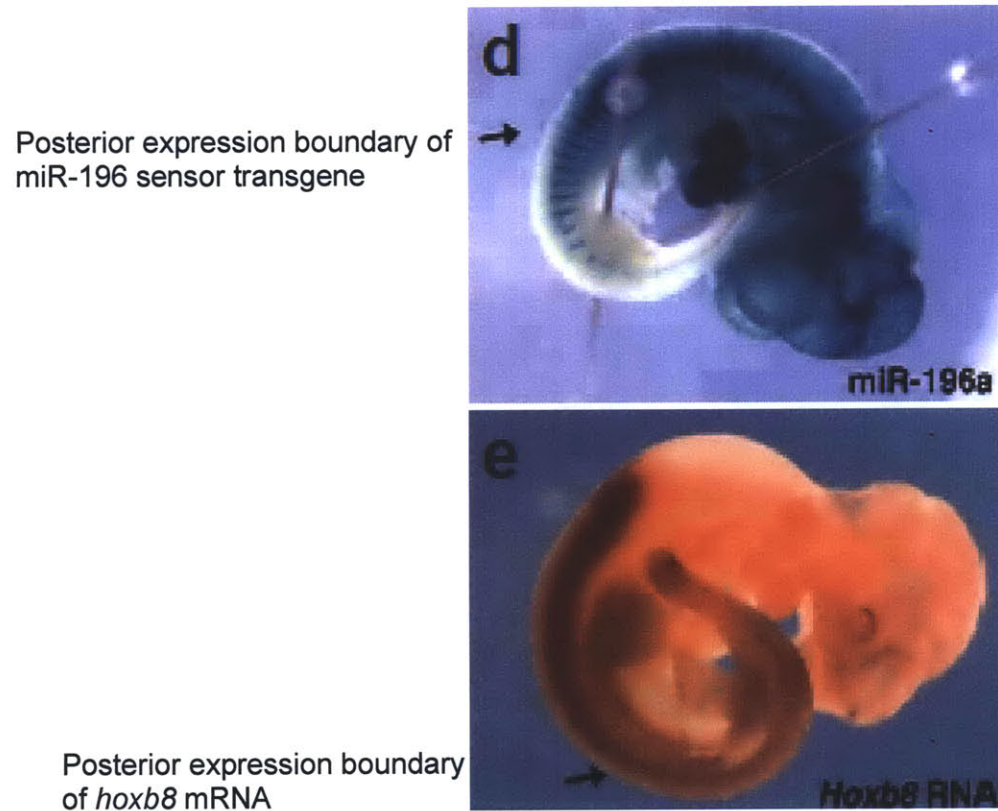
Mansfield, J.H., et al., 2004

Figure 4

Target Site Mutations

5′ CCCAACAACAUGCAAGUCCGUA 3′
|||||||||||| || | || **b8 site**
3′ GGGUUGUUGUACUUUGAUGGAU 5′

5′ CCCAACAAC–UGCGAGUCCGUA 3′
||||||||| || | || **c8 site**
3′ GGGUUGUUG ACUUUGAUGGAU 5′
U

5′ AGUCCGUA 3′
| | | || **8mer site**
3′ UGAUGGAU

5′ AGUCCGU 3′
| | | | **7mer-m8 site**
3′ UGAUGGAU

5′ GUCCGUA 3′
| | | **7mer-A1 site**
3′ UGAUGGAU

Legend:
■ = Extended Complementarity
▨ = Some Extended Complementarity
■ = 8mer
■ = 7mer-m8
■ = 7mer-A1
■ = Hox ORF

Figure 5

Hoxb8 Targeting Vector
17025 bp

ampR
PGK-DTA-bpA
Neo Casette
HoxB8 5' UTR
HoxB8 ORF
HoxB8
miR-196 site
HoxB8 3' UTR
pA Signal

Hoxc8 Targeting Vector
16018 bp

ampR
PGK-DTA-bpA
Neo
C8 exon1
C8 Intron
C8 Exon2
196 7mer
HoxC8
196 7mer-1A
1968mer
1968mer
196Extended
polyA
1967mer-m8

Hoxa7 Targeting Vector
16009 bp

ampR
PGK-DTA-bpA
Neo
A7 Exon
A7 Intron
HoxA7
A7 Exon2
1st polyA
1968mer
1968mer
2nd polyA
1967mer-m8
1968mer
1968mer

Figure 6

BAC Ladder

WT →

Mut →

Expected Band Size: Wild-type = 11.5 kb
Mutant = 9.6 kb

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Figure 7

Ladder

WT →

Mut →
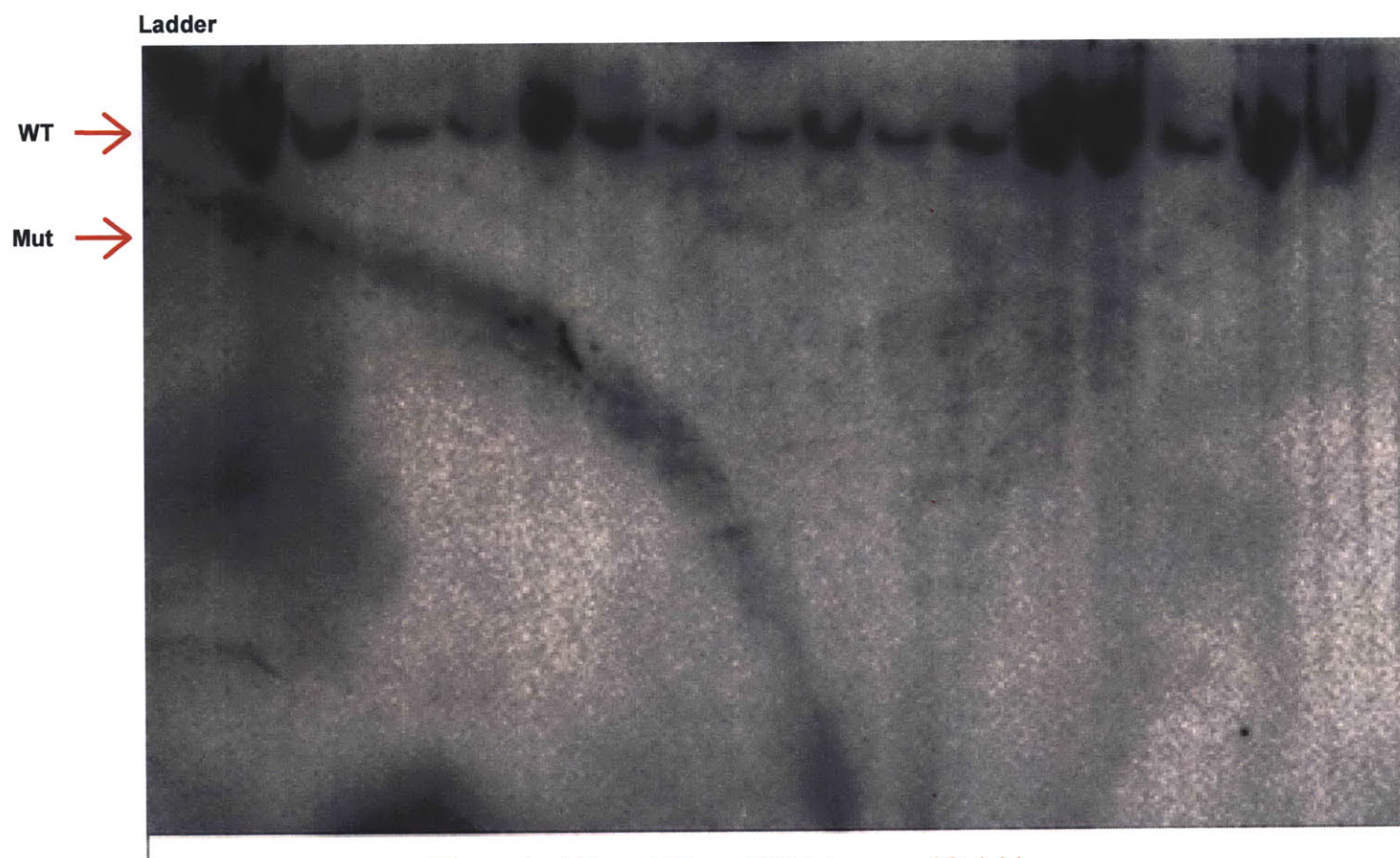
Expected Band Size: Wild-type = 12.1 kb
Mutant = 9.7 kb

28

Figure 8

BAC Ladder

WT →

Mut →
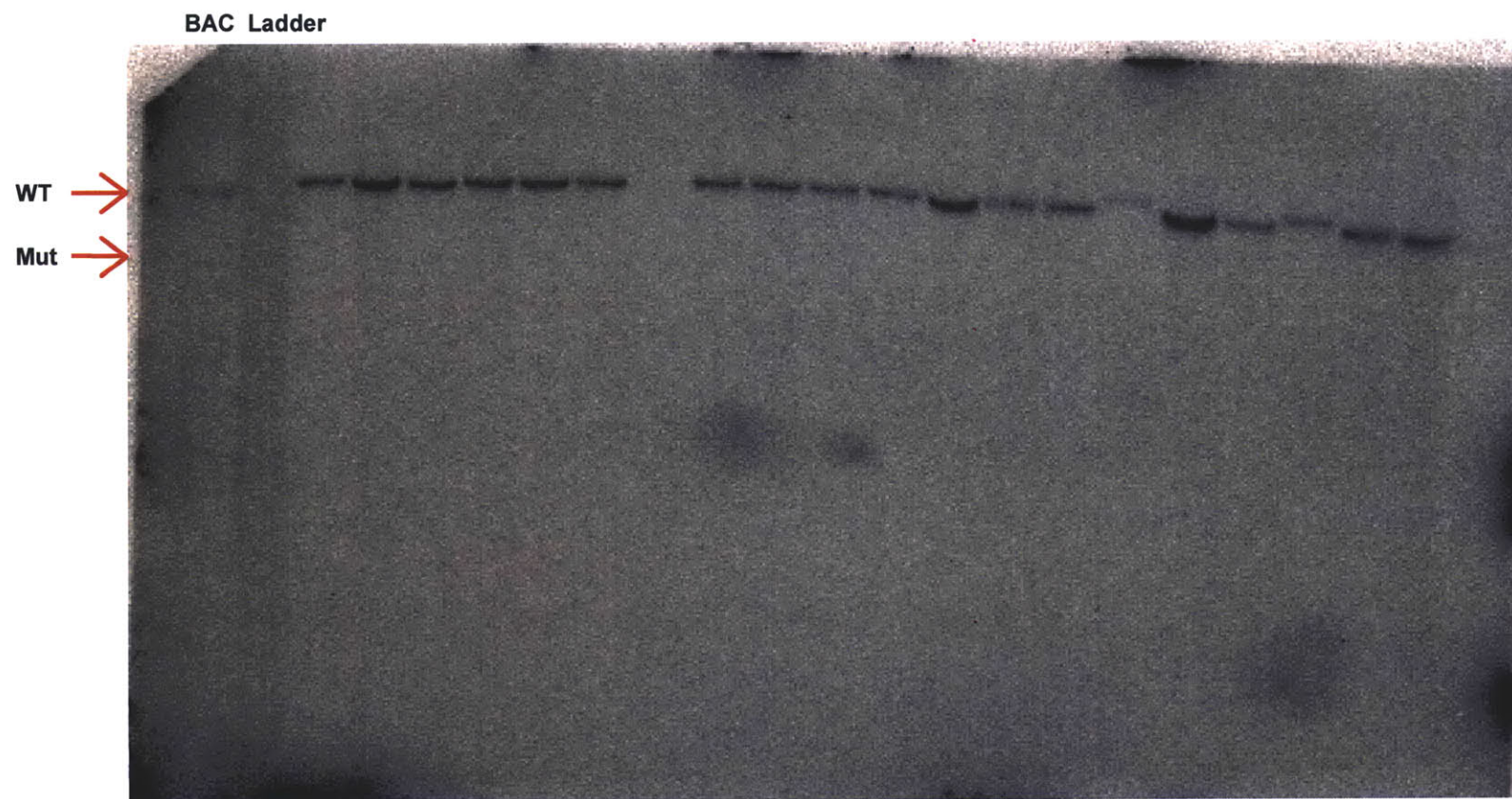
Expected Band Size: Wild-type = 11.1 kb
Mutant = 9.3 kb

Regulation of *lin-14* by the *lin-4* microRNA

Abstract

The first example of a microRNA regulating a target mRNA was found in *C. elegans*. The 3′UTR of *lin-14* mRNA contains seven conserved sites originally proposed to be recognized by the *lin-4* RNA. Three of these sites are now recognized as canonical 8mer sites, while three of the remaining four are offset 6mer sites, which typically mediate only marginal repression. We sought to determine if the *lin-4* miRNA is capable of recognizing all seven sites and mediating repression of *lin-14 mRNA* through them. We used luciferase reporter assays containing the *lin-14* 3′UTR to compare the wild-type UTR with various forms of a UTR in which the *lin-4* seed sites were mutated. We determined that at least one of the three 8mer sites is functional, as expected, and at least one of the four additional sites is also recognized by *lin-4* and contributes to the overall repression of the *lin-14* 3′UTR.

Abbreviations

miRNA, microRNA; UTR, untranslated region; nt, nucleotide; bp, basepair;

Introduction

The *lin-4* and *let-7* genes, the first two miRNAs discovered, were identified in genetic studies by mapping mutant *Caenorhabditis elegans* loci. Both genes encode small RNAs ~22nt long that derive from a hairpin precursor (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). Mutant *lin-4* nematodes exhibit numerous post-embryonic cell-lineage reiterations with diverse phenotypic consequences, including malformation of the vulva, altered body shape, and extra larval molts (Chalfie et al., 1981; Horvitz and Sulston, 1980). *let-7* controls the L4-to-adult transition in *C. elegans* development and is expressed at the late L3 and early L4 stages in the hypodermal seam cells (Johnson et al., 2003). In *let-7* mutants, nematodes fail to execute the L4-to-adult transition, reiterating larval fates and die from bursting of the vulva (Reinhart et al., 2000).

Through the methods of molecular cloning and sequencing of cDNAs generated from small RNA gene products in *C. elegans*, *Drosophila* and human cells the number of miRNA genes increased dramatically (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). All of these genes express small RNAs that were derived from larger hairpin precursors. Continued sequencing efforts have expanded the miRNA gene number in nematodes, flies and vertebrates, and also identified large numbers of miRNA genes in planarians and plants (Landgraf et al., 2007; Mourelatos et al., 2002; Palakodeti et al., 2006; Reinhart and Bartel, 2002; Ruby et al., 2006).

lin-14 is a heterochronic gene required for specifying the division timings of

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a specific group of cells during postembryonic development; this specification

relies on a temporal gradient of Lin-14 protein expression in the developing worm

(Ruvkun and Giusto, 1989). *lin-14* gain-of-function (gf) mutants cause

reiterations of early cell fates at later stages-specify stages; it was subsequently

found that the gf mutations resulted from deletions in the 3'UTR of *lin-14*,

suggesting that the 3'UTR was responsible for the temporal regulation

(Wightman et al., 1991). It was noted that *lin-4* loss-of-function(lf) mutants

phenocopy *lin-14*(gf) mutants and result in the presence of Lin-14 protein at

stages later than L1, indicating that *lin-4* is a negative regulator of *lin-14* (Arasu et

al., 1991; Chalfie et al., 1981; Lee et al., 1993). In 1991, it was determined that

the *lin-14* 3'UTR alone was able to impart the temporal regulation on another

unrelated gene, and this regulation was lost in lin-4(lf) mutant background

(Wightman et al., 1993). The region responsible for regulation was mapped to an

802 bp region in the 3'UTR of *lin-14* that contains seven sites that are

complementary to *lin-4* (Figure 1). Although the initial papers included two

additional 5' nucleotides that we now know are not present in the mature *lin-4*

miRNA (Lau et al., 2001), three of the seven conserved regions are considered

to be canonical 8mer sites for *lin-4* (Grimson et al., 2007). Three additional sites

belong to a new miRNA site type discovered by Friedman et al. (2009). It is

referred to as the offset 6mer (Figure 2) and matches nucleotides 3–8 of the

miRNA (Friedman et al., 2009). A single offset 6mer site can contribute to

miRNA-meditated mRNA destabilization similar to that seen for a canonical 6mer

(Friedman et al., 2009). The last site (site 6 in Figure 1) is referred to as a

mismatched site and is not capable of forming a canonical miRNA interaction with the *lin-4* miRNA.

Despite all that is known now about miRNA targeting in worms, the sites for *lin-4* in the *lin-14* 3'UTR have never been tested for functionality. The aim of this study is to begin to establish how many of the seven originally proposed *lin-4* binding sites are capable of contributing to miRNA-mediated repression through the *lin-4* miRNA.

Materials and Methods

Generating lin-14 luciferase constructs

The *lin-14* 3'UTR was PCR amplified from *C. elegans* genomic DNA and cloned into pBluscript vector for mutagenesis. In each construct all *lin-4* seed sites in the 3'UTRs were mutated using the Stratagene QuikChange Multi Site-Directed Mutagenesis kit with mutant primers (IDT). All mutagenesis was verified by sequencing before transferring to the expression vector. The fully mutagenized UTRs were PCR amplified and subcloned into the pIS1 renilla luciferase reporter vector for study in cell culture. Constructs were verified by restriction digest analysis and sequencing. Four *lin-14* constructs were created for reporter assays: wild-type, 8mer site mutant (only three 8mer sites were mutated), offset 6mer/bulge mutant (only the three offset and one mismatched site were mutated), complete mutant (all sites were mutated); all seed site mutations are the same for each site in every construct and correspond to nucleotides 3rd, 5th and 7th in the *lin-4* miRNA (Figure 3).

Cell Culture Assays

HEK293 cells (ATCC) were plated in 24-well plates (~0.5 x 10$_5$ cells / well) and transfected 24 hours(h) later using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Sigma) with 20 ng of the firefly luciferase control reporter plasmid pISO (Grimson et al., 2007), 20 ng of renilla luciferase reporter plasmid, and 25 nM miRNA duplex per well. Cells were harvested 24h post-transfection. Luciferase activities were measured using dual-luciferase assays (Promega), as described by the manufacturer. Seven biological replicates, each with three technical replicates, were performed. Renilla activity was first normalized to firefly activity to control for transfection efficiency, and then normalized values were analyzed as described previously (Grimson et al., 2007). For mutant constructs, firefly normalized renilla values were normalized to the geometric mean of values from the reporter in which the miRNA sites were mutated. To control for variability in plasmid preparation, the value plotted for each construct was the geometric mean of normalized renilla values from transfections with the cognate miRNA divided by that from transfections with the non-cognate miRNA, miR-124.

Results and Discussion

We aimed to test the efficacy of the seven *lin-4* target sites in the *lin-14* 3'UTR, particularly the three conserved offset 6mer sites and one mismatched site by using luciferase reporter assays. We fused renilla luciferase to the following four versions of the *lin-14* 3'UTR: the wild-type UTR contained seven *lin-4* sites (three 8mer, three offset 6mer, one mismatched site), the mutant 8mer

(three 8mer mutants, three wild-type offset 6mer and one wild-type mismatched sites), the offset/mismatch mutant (three 8mer wild-type, three offset 6mer and one mismatch mutant sites) and full mutant (all sites mutated), respectively. Each construct was transfected with the following miRNAs individually: miR-124 (non-cognate), *lin-4* (cognate), mutant *lin-4* (compensatory mutation made in miRNA to recognize mutant sites in the *lin-14* 3'UTR). Repression by *lin-4* was evaluated by comparing normalized luciferase values from cells cotransfected with *lin-4* to those with miR-124, a non-cognate miRNA.

For the wild-type *lin-14* UTR tested, we observed significant and robust repression by *lin-4*. This repression was lost when the non-cognate or compensatory miRNAs were cotransfected or when all seven *lin-4* sites were mutated. Interestingly, when the three 8mer sites were mutated, leaving only the three offset 6mer and one mismatched sites to mediate *lin-4* mediated repression we still saw a 2 – 4.5 fold repression over the fully mutated UTR, indicating that at least one of the remaining sites are in fact functional miRNA target sites and contribute to the overall repression of the *lin-14* 3'UTR by the *lin-4* miRNA (Figure 4). Friedman et al. (2009), have previously shown that offset 6mers are capable of mediating repression similar to the level seen for canonical 6mers matching the seed site. The enhanced level of repression observed for the offset sites could be attributed to a few factors: first, the sheer number of offset and mismatched sites that together account for over half of the proposed *lin-4* complementary sites in the *lin-14* 3'UTR. It would be interesting to go through and delete single offset 6mer sites as well as combinations of a few to determine

if some sites have a greater effect on repression or if there is a minimum number of offset sites needed to see this type of robust repression. Secondly, the first two offset sites are 11 bp apart and may act cooperatively (Grimson et al., 2007) and thus yield a greater level of repression through *lin-4*. The experiments described here also do not test the functionality of the single mismatched site on its own; so it is unclear if that site contributes to *lin-4* mediated repression. In addition to the two offset 6mer sites, there are two more pairs of sites that may act cooperatively: the first 8mer and third offset 6mer are 11 bp apart, and the third offset 6mer and the second 8mer site are 12 bp apart (Figure 5). However, the observation that repression was observed in the absence of the 8mer sites rules out the possibility that activity of the offset sites was merely due to cooperative action with nearby 8mer sites.

Acknowledgements

Figure Legends

Figure 1. Originally proposed *lin-4* binding sites in the *lin-14 3'UTR.* (A) A representation of the *lin-14* 3'UTR in *C. elegans.* The black shaded bars represent the seven *lin-4* binding sites present in the UTR . In addition to the sites being numbered they are also labeled as bulged or non-bulged. The bulge refers to the bulged C, which is no longer thought to be present now that the correct sequence of *lin-4* has been determined and has been shown to be two nucleotides shorter at its 5' end (Lau et al., 2001). (B) The predicted *lin-4-lin-14* duplexes from Wightman et al., *lin-14* is the top sequence in the duplex, and *lin-4* is the bottom.

Figure 2. The offset 6mer site and its targeting efficiency. (A) The four canonical sites that match the seed of a miRNA. The new site (grey) identified by Friedman et al., is called the offset 6mer and contains 6 contiguous matches to nucleotides 3–8 of the miRNA. (B) Cumulative distribution changes for transcripts with exactly one of the denoted sites in their 3'UTR. All results except the offset 6mer come from previous microarray data tracking mRNA destabilization after transfection of 11 miRNAs (Grimson et al. 2007).

Figure 3. *lin-4* mediated repression of the *lin-14* 3'UTR. Reporters included the luciferase ORF followed by the *lin-14* 3'UTR or a mutated variant of this UTR noted along the x-axis. Fold repression was calculated relative to that of the non-cognate miRNA, miR-124. Plotted are the normalized values, with error bars

representing the third largest and third smallest values.

Figure 4. *lin-4* target site mutations. The complete *lin-14* 3'UTR sequence. The seven target sites are underlined and labeled according to the type of site. The mutations to the *lin-4* site are in red. The first two offset 6mers are 11 bp apart and may be capable of functioning cooperatively. Also the first 8mer is separated from the third offset site by 22 bp followed by and additional 8mer 12 bp downstream allowing for these three site to potentially act cooperatively as well.

Figure 1

A

end ORF

lin-14 3' UTR

polyadenylation site

Bulged C

Non-bulged C

1 2   4   6

3 5   7

200 nt

lin-4 binding sites

B

lin-4 sites

1
CCUCAUGCUCUCAGGAAC
GAGUGUGAGAGUCCUUG

3
CUCGCAUUU   CUCAGGGAAC
GAGUGUGAA   GAGUCCCUUG

5
UUCUACCUCAGGGAAC
GAGGUGGAGUCCCUUG

7
ACUCACAACCAACUCAGGGAC
GAGUGU   GAGUCCCUUG

2
lin-14 ACAUUCAAAACUCAGGAAU
lin-4   UGUGAGU   GAGUCCCUUG

4
UCUCAUUGAACUCAGGAAU
GAGUG   GAGUCCCUUG

6
GCUUAUGU AAAAUCAGGAAC
GAGUGUGA   AGUCCUUG

Wightman, B. et al., 1993

41

Figure 2

A

B

Seed match

Offset 6mer site . . . NNNNNN . . . .
. . . . NNNNNN . . . .
7mer-A1 site . . . . . . NNNNNNA . . . .
7mer-m8 site . . . . . NNNNNNN . . . .
8mer site . . . . NNNNNNNA . . . .

NNNNNNNNNNNNNNNNNNNNNN-5' miRNA
8 7 6 5 4 3 2 1

Seed

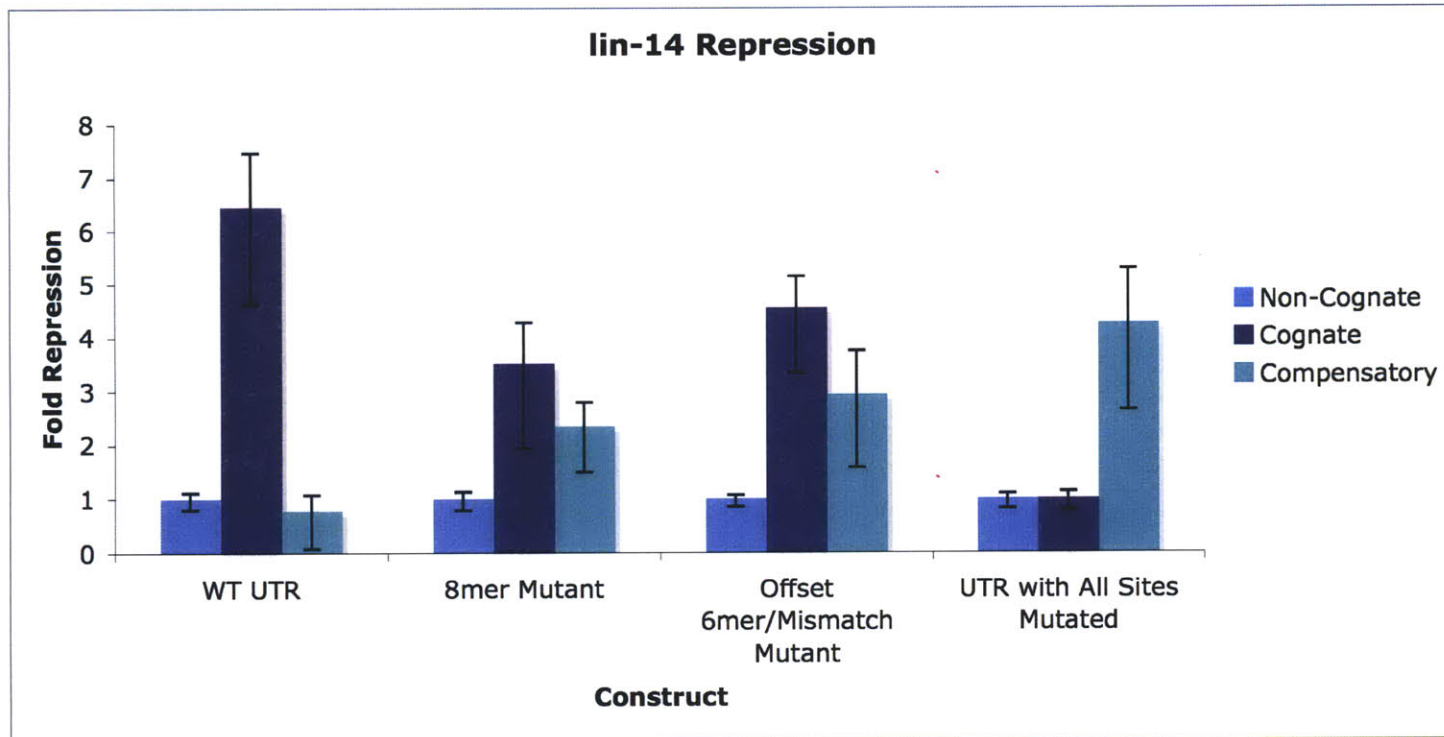Friedman, R.C., et al., 2009

Figure 3

Figure 4

lin-14 3'UTR

aatgccaatttttcgagtcatccttcgggcaatgttcattacactttctctctgttgtacttgagcatgtttcaatttcaatcacaaatgcctttttt

ggagagaattgaaggcaaaaccaaactaaattgagtttggtggaaattgaaattgcaacatgctcaattcattttgttttttttcttttaactatatgg

atgccacgctggattgactcttccgtacactcacgctcattccaaattatccccatctgaccccggactgttttatccaaacctatactgatgtatat

tgtatgcggcatgttatttttttcataaccacaagcatttagcgctttcggcttcagatttctaatcgctgtttctttactgctttgccctttttttcta

acttctgaatttcgttattatgcaacaattctacctcaatttttttgtatctttccccaattctagttgacattattggttttgattgttttcctagc

tctgaaatcccacaactaggcgccactgaattgtatctttcattcccactttcgtttttcgcacattgtagtatctctcaattaggaaacacttgaaa

Offset 6mer
acctctattgcttaggattgatgagagatattgctttttcctgcactcactttacctttgtctcacctcaaaaattgct<u>cccggg</u>aacattcaaaa<u>cc</u>

Offset 6mer
<u>cggg</u>aatttgtcaccttggtctctcatcatatatcttacctcttgtcacacccccatccccagtctcaagttcatttcttactttgtaactccgttt

8mer   Offset 6mer
agtgcgcccaattctcgtcattttgattacactctcttttaatccaa<u>cccgga</u>gaaccaatttttttttctcattgaa<u>cccggg</u>aatttcttctac<u>ccc</u>

8mer
<u>gga</u>gaacctacctcatccactttttcagttgtttggggccaaatatctatatccaaagtagtagtctacaatttagtattttattattacctcccgccg

Bulge site
ttttagcttttaatgttaaaa<u>ccaaga</u>acttttgaaaatgatcttcacctcattcagaagcaaaaatcaggcattttccaaagattttgaaaacacat

8mer
aaacctccttccaagtcaaaactcacaaccaa<u>cccgga</u>gacctttttcttacttctgtatcacaaaaatgattatatttctgatgaatgttgctctgt

cataaatcaatttatttcttttgaaccgaaagccgaaatgtgttttttcctatttgtcgtcattgttctcatccaccccaacttcctctcattattgaa

gtttctcatgtattctacctcatttattattgttttttttttgttcaaaatgtcaatgtgacaaaacaaattgcccgtaatgtctgaaactctgttctt

tcctatgtaactgtatacgaaaaattcccccgttaatttttaatatgtattctgtgtcccatctttgccccagtttatttttgtacccctgtgagcg

ttcaaattacctcagcatccaacaatctttccatcttaacatccatcccattgtacctctgaatcttgcttcgcttacctcgtaacaaatatatttt

atcggcttaaaacctaataaatcatttaccag

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