Novel Small-RNA Mediated Gene-Regulatory Mechanisms for Long-term Memory

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ABSTRACT

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Memory storage and memory-related synaptic plasticity rely on precise spatiotemporal regulation of gene expression. To explore the role of small RNAs in memory-related synaptic plasticity we carried out massive parallel sequencing to profile the small RNAs of *Aplysia*. We identified 170 distinct 21-23 nt sized miRNAs, 13 of which were novel and specific to *Aplysia*. Nine miRNAs were brain-enriched, and several of these were rapidly down-regulated by transient exposure to serotonin, a modulatory neurotransmitter released during learning. Two abundant, and conserved brain-specific miRNAs, miR-124 and miR-22 were exclusively present pre-synaptically in a sensory-motor synapse where they constrain synaptic facilitation through regulation of the transcriptional factor CREB1 and translation factor CPEB respectively. We therefore provide the first evidence that a modulatory neurotransmitter important for learning can regulate the levels of small RNAs and present a novel role for miR-124 and miR-22 in long-term plasticity of synapses in the mature nervous system. While mining the small RNA libraries for miRNAs, we discovered an unexpected and abundant expression in brain of a 28-nt sized class of piRNAs, which had been thought to be germ-line specific. These piRNAs have unique biogenesis patterns and predominant nuclear localization. Moreover, we find that whereas miRNAs are down-regulated by exposure to serotonin, piRNAs are up-regulated. Importantly, we find that the piwi/piRNA complex facilitates serotonin-dependent methylation of a conserved CpG island in the promoter of CREB2, the major inhibitory constraint of memory in *Aplysia*, leading to the persistence of long-term synaptic facilitation. Taken together, these findings provide a new serotonin-dependent, bidirectional, small-RNA mediated gene regulatory mechanism during plasticity where miRNAs provide translational control and piRNAs provide long-lasting transcriptional control for the persistence of memory.

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Chapter 1

The Neural Basis of Memory

The human brain has an extraordinary ability to acquire information through learning, and to store and recall that information through a process we call memory. These seemingly simple brain processes allow us to accumulate personal experiences over a lifetime, giving meaning to our individuality, relationships and ambitions, and giving purpose to our daily existence. It is not surprising therefore, that over the centuries, philosophers and scientists alike have been drawn towards unraveling the mysteries of the human brain and its role in memory processes.

The early Greek scholars (circa 300 BCE) could not agree upon whether cognition and memory were made possible by the heart or the brain; Aristotle for example, was a major proponent of the heart being the seat of our cognition and soul. Subsequent clinical observations that lesions of the brain, not the heart, provided damage to cognitive processes, revealed that the brain may indeed be responsible for guiding our memories and defining our personalities. Through careful dissections and anatomical observations of the brain, Galen (circa 150 CE) first put forth a unified model of the brain, which was so compelling that it became the prevailing idea of brain function until the medieval and renaissance periods (circa 1600 CE). In this model, the fluid filled ventricles of the brain were the primary conveyors of information, while the convoluted mass of flesh surrounding the ventricles were thought to exist simply to provide structural support (Figure 1.1).

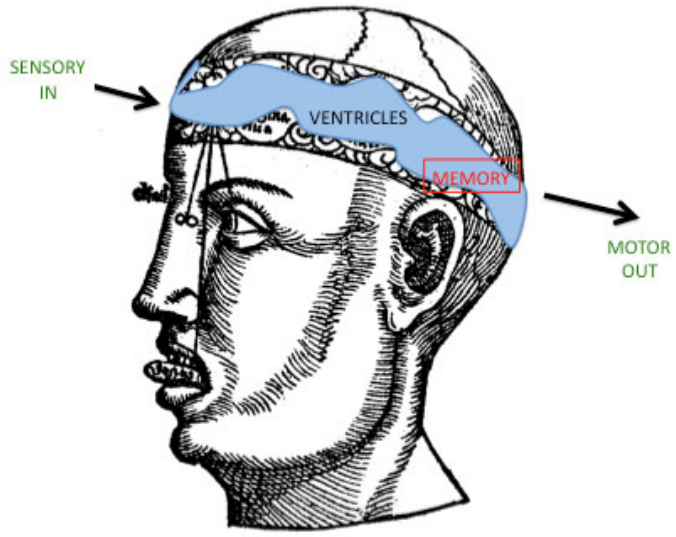

Figure 1.1: Ventricle-centered view of the medieval and renaissance brain

The anterior-most "first room" (lateral ventricles) received sensory information and was also the area of fanciful imaginations that arose from sensory experience. These were then transferred to the "second room" (third ventricle) where conscious processing of that information occurred, such as judgement and decision-making. This information were then transferred to the posterior-most "third and final room" (fourth ventricle) where it was stored in memory for the long term and available for later recall. Over time, through lesions studies and autopsies from clinical cases, it became obvious that the tissue surrounding the ventricles were the most computationally useful part of the brain.

The work of two neurologists in the 1800's first highlighted the localized, and often lateralized, function of the human brain. Paul Broca and Carl Wernicke described patients who either couldn't produce or understand speech, respectively. They conducted autopsies of their patients after their deaths and described particular areas of the brain, now named after them, that were responsible for the observed language deficiencies: Broca's area for speech production and Wernicke's area for speech comprehension. Subsequent patient studies revealed a remarkably consistent localization and left-hemisphere lateralization of these areas,

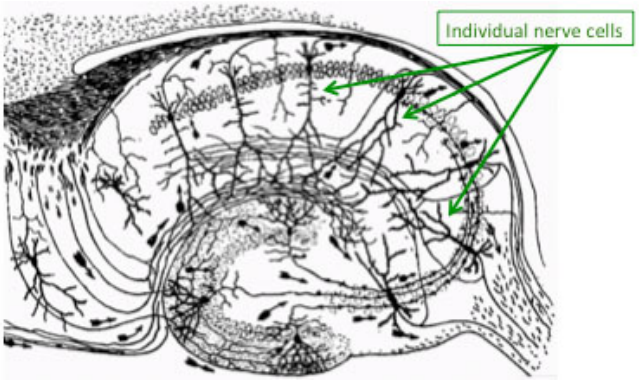

Figure 1.2: Cajal's drawing detailing the cellular nature of the human hippocampus

giving rise to the idea of localized functions in the brain.

A revolution in the modern understanding of the cellular contribution to memory came at the turn of the 20th century with the work of Santiago Ramon y Cajal, who is considered the father of neuroscience. In 1894, he put forth the "neuron doctrine." While Golgi and others before him had thought of the brain as a sheet-like syncitium of cells, Cajal was the first to suggest that the billions of individual cells were completely separate from one another while still in communication with each other, at their points of contact, later called "the synapse" by Charles Sherrington ([Ramon y Cajal, 1894])(Figure 1.2). His revolutionary observations were made possible by his use of newborn animals, where individual neurons were easier to identify because they were fewer in number and more sparsely situated. Furthermore, his use of the Golgi staining method in brain sections was fortuitous as this silver stain method only stains an occasional neuron, but does so in its entirety, so as to highlight the features of a single nerve cell amidst the complex web of nerve arrangements in the brain. Cajal noticed that as an animal developed from a new-born into an adult, it's brain did not significantly add new neurons, and he therefore proposed that memories are not stored in new neurons, but rather, in connections between existing nerve cells ([Ramon y Cajal, 1894]). This prescient idea has garnered experimental support over the years and has come to dominate our present day thinking of how memories are encoded and retrieved.

How can the strength of connections between neurons subserve memories? This question

alone has inspired decades of research by both experimentalists and theoreticians to propose models that can explain how networks of neurons store information and mediate memory. Jerzy Konorski was one of the first to attempt to build on Cajal's cellular connectionist approach to memory, and he proposed that sensory stimuli could produce either a transient excitability change in neurons, or an enduring plastic change in their connections, an idea he termed synaptic plasticity. In the early 1960s, Spencer and Thompson and Kandel and Tauc introduced neural analogues of learning. Although their experiments were conducted with arbitrary cells and non-physiologic inputs and outputs, they could begin to explain how synapses "remember" the transient nature of an electrical stimulus even while the stimulus is no longer present. It was not until the early 1970's, however, that attempts were made to study synaptic plasticity in the context of real behavior, with a clear intention of causally relating changes in neural activity and synaptic plasticity with behavior. Much of this work was spear-headed by Eric Kandel through his work in the marine mollusk *Aplysia*. As a direct test of the Cajal-Konorski ideas, Kandel and Tauc described heterosynaptic facilitation in *Aplysia* neurons, a process whereby activity in one pathway causes an enduring enhancement of synaptic activity in another ([Kandel and Tauc, 1964]), and that this processes underlies sensitization of gill withdrawal, a learned behavior ([Castellucci *et al.*, 1970]). The subsequent characterization of the molecular steps responsible for mediating sensitization of gill withdrawal in *Aplysia* marked a major transition in our understanding of memory processes. In addition to work in *Aplysia*, there were several other invertebrate and vertebrate systems that were paramount in progressing the biology of memory such as the spinal cord habituation in frog, escape reflex in crayfish, and olfactory learning in the fly ([Spencer *et al.*, 1966; Krasne, 1969; Alkon, 1974; Quinn *et al.*, 1974]).

Another, non-heterosynaptic, mechanism of synaptic plasticity was proposed by Donald Hebb. He suggested that when one neuron A repeatedly and persistently stimulates another neuron B, then the strength of their connection is enhanced such that neuron A can now more easily fire neuron B, thus holding a memory of their association ([Hebb, 1949]). Inherent in Hebb's theory for associative learning was the idea that a single cell could be involved

in multiple memory associations. He hypothesized the existence of cell assemblies, where individual cells dynamically changed their loyalties from one assembly or trace into another depending on time and context. In 1973, Bliss and Lomo reported the discovery of an enduring form of synaptic plasticity in Rabbit Hippocampus ([Bliss and Lomo, 1973]). They observed that delivery of a high frequency train of stimuli to pre-synaptic fibers prior to a single pre-synaptic pulse caused a stronger and more enduring EPSP in the post-synaptic cell, than if the single pre-synaptic pulse was applied by itself. Bliss and Lomo, however, seemed to be unaware of Hebb's hypotheses, and therefore, it wasn't until the work of Wigstrom and Gustafsson in the 1980's, that the coincident pre- and post- synaptic activity characteristic of hippocampal LTP came to be recognized as a Hebbian process for associative learning ([Wigstrom and Gustafsson, 1985]). These initial studies on LTP spawned an avalanche of studies and it is now the most studied experimental model of memory. The strength of LTP as a model for the study of memory lies in 1. its robustness and applicability to almost any brain area and experimental model and 2. that LTP could account for several of the properties one sees in how memories are encoded: through specificity, associativity, and persistence of the observed synaptic plasticity.

Since mechanisms of synaptic plasticity and LTP were observable throughout the brain, the question emerged as to whether memories were distributed in the brain or whether there were certain "memory centers" responsible for storage and retrieval of information. Concurrent with anatomical and physiological studies, clinical studies of lesions in the brain of human subjects were critical in highlighting the role of the hippocampus in memory, especially in processing declarative memories, which are memories for facts and knowledge that can be consciously recalled. The earliest and most influential lesion study came from a classic report published in 1957 by William Scoville and Brenda Milner in which they provided dramatic demonstration of the importance of the human hippocampus in learning and memory ([Scoville and Milner, 1957]). They described a patient, Henry Molaison, better known as H.M., who was not able to form new long-term declarative memories after undergoing bilateral resection of his hippocampus for treatment of intractable epilepsy. While the surgery

was successful in that it cured him of his epilepsy, he tragically was unable to remember any new person or event since his surgery. He was even unable to recognize his care-takers and investigators, and those who had worked with him on a regular basis for decades after the time of his surgery. Despite his severe inability to commit declarative memories to long-term memory, Milner noticed to her surprise, that he was able to learn and commit to long-term memory certain tasks that required, what we now call, implicit or procedural memory. For instance, he was able to learn to draw a figure by looking at its reflection in a mirror (an ordinarily difficult motor task), and although by the last trial he was able to perform the task with ease, he consciously denied ever having previously performed the task in the past. This clinical observation, together with those of Larry Squire and others formed a body of evidence to show that memory is not a unitary process, and instead that there are multiple memory systems, the major distinction occurring between those memories that are explicit (factual, requiring the hippocampus, and consciously recalled) with those that are implicit (procedural, independent of the hippocampus, and unconscious). In addition to laying out the existence of multiple memory systems, subsequent research on HM brought to light several other important properties of human memory: since his memory deficits were described as anterograde amnesia, his declarative memories prior to the surgery were primarily intact, suggesting that after a critical period, even declarative memories are distributed to the cortex and become independent of the hippocampus; and similarly, his adequate performance in cognitive tasks suggested that his short-term and working memory were well- intact and that those too are independent of the hippocampus.

By the mid 1970's there was a convergence of ideas from clinical, physiological, and anatomical studies to believe that explicit memories were routed through the mammalian hippocampus. The study of the molecular and physiological mechanisms underlying memory storage, however, became more tractable through the study of implicit memory in invertebrates, with much of this work being spear-headed by Eric Kandel through his work on the Marine Mollusk *Aplysia*. I will use the remainder of this chapter to focus on the major findings from *Aplysia* in the later decades of the 20th Century, primarily to serve as an

introduction for my research in this thesis which has been conducted entirely in *Aplysia*, and also because the mechanisms for synaptic plasticity underlying learning and memory in *Aplysia* have been found in other invertebrate and mammalian systems.

Since the neurons of *Aplysia* were relatively large (upto 1mm in diameter), few in number (the nervous system having on the order of 10^4 neurons compared with humans having 10^{11}), with stereotyped location allowing identification from one animal to another, it provided a nice system for mapping out the locations of individual neurons, identifying their properties, and importantly, locating sites within neural circuits that were modified by learning and memory. The sensory-motor circuit seen in Figure 1.3. governs the gill-withdrawal reflex of *Aplysia*, and the weakening or strengthening of this synapse occurs during habituation or sensitization of the gill respectively. A single tap at the siphon elicits a normal baseline response in the sensory neuron that is communicated through to the motor neuron, causing a withdrawal of the gill. Repeated taps of the siphon causes habituation of the sensory neuron, a decrease in transmitter release across the sensory-motor synapse, a weakening of synaptic strength, and a corresponding attenuation of gill withdrawal ([Castellucci and Kandel, 1974]). Sensitization, on the other hand, by pairing the siphon tap with a single noxious stimulus to the tail, activates interneurons from the tail that release serotonin (5HT) at the sensory-motor synapse, causing a local increase in cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) levels in the nerve terminals of sensory neurons, leading to an increase in transmitter release across the sensory-motor synapse, a corresponding enhancement in synaptic strength, and an exaggerated gill withdrawal response ([Castellucci and Kandel, 1976]). Taken together, these early studies on habituation and sensitization provide direct evidence in support of Cajal, Konorski, and Hebb's theories that synaptic connections are not immutable, but rather can be modified by learning. Furthermore, it became evident that a single synapse could be modified in opposing directions, that it could store more than one memory, and importantly, that there need not be dedicated memory neurons in the brain, and instead that the ability for memory is built into the existing neural architecture of the brain that may otherwise be specialized for sensory, motor, and cognitive

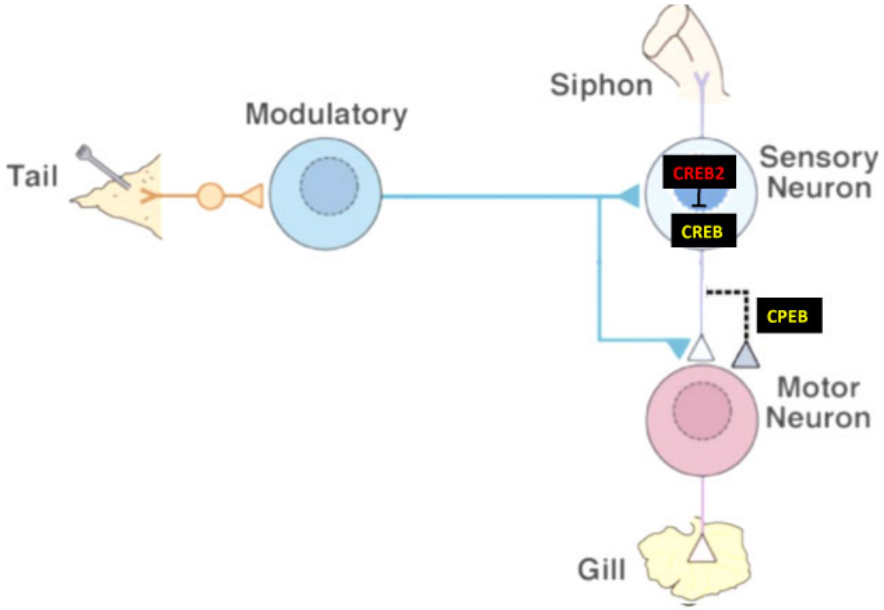

Figure 1.3: Circuity underlying sensitization of the gill-withdrawal reflex of *Aplysia*

processes.

Another breakthrough in our understanding of synaptic plasticity as a mechanism for learning and memory occurred with the realization that a single training leading to changes in transmitter levels at the sensory-motor synapse lead only to short-term changes in the gill-withdrawal behavior. Subsequent studies found that repeated spaced training, such as repeated shocks to the tail, were required to form long-term memories ([Carew *et al.*, 1972; Pinsker *et al.*, 1973]), reflected by long term changes in the strength of the sensory-motor synapse that required 1. new protein synthesis and 2. the growth of new stable connections between the sensory and motor neuron. The earliest clues for the role of protein synthesis in converting short-term to long-term memory came from studies in which inhibitors of transcription were sufficient to prevent long-term memories ([Agranoff *et al.*, 1966; Roberts and Flexner, 1969; Squire and Barondes, 1970]). Subsequent studies in *Aplysia* showed that with repeated training, 5HT activates cAMP, which activates and allows the catalytic subunit of PKA to move from the synapse to the nucleus of the sensory neuron where it activates a transcription factor CREB-1 (cAMP response element binding protein), which in

turn activates gene products that are sent back to the synapse to facilitate the growth and stability of new synaptic connections ([Glanzman *et al.*, 1989; Dash *et al.*, 1990; Bacskai *et al.*, 1993; Alberini *et al.*, 1994; Hegde *et al.*, 1997]). The subsequent discovery of a transcriptional inhibitor, CREB-2, which inhibits CREB1 and blocks the growth of new synaptic connections, revealed the important and general principle that, at steady-state, a class of memory suppressor genes act coordinately to constrain the transfer of short term memories into long-term memories ([Abel *et al.*, 1998; Yin *et al.*, 1994]). These are the constraints that have to be overcome to varying degrees, by repeated training, to create and maintain long-term memories.

How are the growth of new connections stabilized for the long-term? Since short-term memories are initially formed in a synapse-specific way, there followed a natural assumption that long-term memories were stored and stabilized in the same synapses in which they were formed, and therefore, were also synapse-specific. But if CREB-dependent transcription is necessary for long-term memory, then how are newly synthesized gene products from the nucleus specifically used at only certain activated synapses, and not at all the synapses? One idea that was entertained was that the gene products from the nucleus are specifically sent only to the activated synapses. Meanwhile, Uwe Frey, in the lab of Richard Morris, and Kelsey Martin in the lab of Eric Kandel, independently tested the idea that gene products are sent to all synapses, but that only those synapses that are tagged can capture and use them effectively. They indeed found that tagged synapses can capture the long-term memory process more easily, giving rise to the exploration of synaptic tagging and local protein synthesis as a major field of study ([Frey and Morris, 1997; Martin *et al.*, 1997]). Several non-mutually exclusive models have since been proposed to explain how local protein synthesis at the synapse can maintain a new memory state in the face of constant molecular turnover. One compelling idea is that a local translation factor, CPEB (cytoplasmic polyadenylation element binding protein) may have prion like properties, in that it can exist in two states, one as a monomeric non-functional form, and the other as an activated, 5HT-dependent polymeric form that when aggregated, is self-perpetuating, resistant to degradation, and

can remain persistently active, perhaps for the lifetime of the memory ([Si *et al.*, 2003a; Si *et al.*, 2003b; Si *et al.*, 2010]). Another mechanism proposes that a locally active protein kinase (PKCζ) is made such that it has a very long half-life, and therefore resistant to rapid turnover, allowing for long-term synaptic activity, perhaps for the duration of a memory. Blocking CPEB activity has been shown to interfere with the maintenance, but not the induction, of long-term synaptic facilitation, and even more fascinating, blocking PKCζ activity can interfere with memories in behaving animals even days or weeks after they have been formed ([Sacktor, 2008; Serrano *et al.*, 2008]). Ongoing experiments in the lab suggest that CPEB and PKCζ interact with each other to provide a molecular machine for persistent states of activity at a synapse. Other molecules that could serve as a synaptic tag and maintain persistent changes in synaptic strength are cycling receptors, such as the AMPARs, catalytically persistent kinases like CAMKII, and cytoskeletal structures, like actin, each of which can contribute either structurally or functionally to the maintenance of a new synaptic state ([Lisman, 1985; Lisman *et al.*, 2002; Hayer and Bhalla, 2005]). Finally, the emerging field of small non-coding RNA biology reveals an attractive role for small RNAs as local mediators in the induction and maintenance of synaptic plasticity, the details of which are introduced in the next chapter, and the results of which are described in chapters 3 and 4 of this thesis. Cell-wide (intrinsic) changes in excitability during memory are also discussed, and more specifically, I suggest that small-RNA mediated epigenetic regulation could be an essential component of long-lasting memories, the details of which are described in chapter 5 of this thesis.

In this thesis, I describe a set of studies on the multiple roles for small RNAs in the transcriptional and translational control of synaptic plasticity during memory storage. I describe the role of two brain-specific miRNAs, miR-124 and miR-22, in constraining serotonin-dependent long-term facilitation through regulation of the transcription factor CREB and the translation factor CPEB respectively. In addition, I describe the first demonstration of the existence of piRNAs in neurons, and a role for the piwi/piRNA complex in mediating serotonin-dependent methylation and silencing of the transcriptional repressor CREB2, the

major inhibitory constraint of memory in *Aplysia*, leading to the persistence of long-term synaptic facilitation.

Chapter 2

The History of Small RNAs and their Role in Memory-Related Synaptic Plasticity

A series of puzzling scientific observations during the early 90's led eventually to the discovery of RNAi (RNA interference) in 1998, one of the most conserved and robust gene regulatory mechanisms to be found. The story began perhaps in 1990 with two scientists, Carolyn Napoli and Richard Jorgensen, who were attempting to over-express the purple pigment, chalcone synthase, in petunias through introduction of a transgene. However, rather than obtaining purple petunias, they ended up with white petunias, leading them to suggest that the transgene had "cosuppressed" the endogenous gene, through a mechanism that was unclear at the time ([Napoli *et al.*, 1990]). A few years later, Roman and Macino described yet another instance where an attempt to over-express a gene in fungi via plasmid transformation caused not only an inability to express the exogenous gene, but also the endogenous gene ([Romano and Macino, 1992]). Meanwhile, experiments from plant virologists suggested that a virally infected plant shows only infection in some of its leaves, but will develop immunity to the virus in its other leaves, and furthermore, that resistance can also occur in plants containing any transgene that shares homology with the infecting

virus. In 1995, Guo and Kemphues described, in animal models, a seemingly unrelated but equally puzzling observation: In their gene knockdown experiments, antisense RNA and sense RNA were equally effective in silencing expression of the target mRNA ([Guo and Kemphues, 1995]). Since antisense RNA was thought to effect gene silencing by hybridization and subsequent degradation of the target RNA, it was unclear why the control sense RNA would also lead to gene silencing. The final link to all of these seemingly disparate observations came with the seminal 1998 study by Fire and Mello in which they provided an explanation for the "previously reported silencing of endogenous genes by cosuppression, quelling, virally induced gene silencing, and also sense RNA." Fire and Mello hypothesized, in an attempt to explain the findings of Guo and Kemphues, that their single stranded sense and antisense RNA preparations were each contaminated by double stranded RNA (dsRNA) from the activity of bacteriophage RNA polymerases during the preparation. By extensively purifying the sense and antisense preparations, they found that each alone was significantly less effective in knockdown of the target gene, when compared to dsRNA targeting of the same gene ([Fire *et al.*, 1998]). Indeed the common thread in all of the previously observed mysteries was the surprisingly potent gene silencing that occurred as a result of dsRNA being introduced into the system, whether it was through a transgene, a virus, or contamination in single stranded RNA preps. Thus the dsRNA mediated gene silencing came to be termed RNAi. This study by Fire and Mello established an entirely new conceptual framework for the effects of RNA on gene silencing, and spawned an avalanche of research dedicated to unraveling the mechanistic details of RNAi, while also simultaneously catalyzing the emergence of the biology of small non-coding RNAs .

How did dsRNA effect gene silencing? Assuming that the dsRNA must first unwind, allowing the antisense strand to bind the target sense mRNA, Hamilton and Baulcombe searched for evidence of full length antisense RNA but were not able to detect any ([Hamilton and Baulcombe, 1999]). Since the silencing effects of dsRNA were so potent and further since the antisense strand was not detectable, It was soon hypothesized that RNAi was mediated by a stable intermediate. In 2000, the Hannon and Bartel labs purified and fractioned RNA

from dsRNA transfected drosophila cells and discovered that the dsRNA had been effectively chopped into small 21-23 nt RNA species, siRNAs, which were the stable intermediates effecting the gene silencing ([Hammond *et al.*, 2000; Zamore *et al.*, 2000]). To further prove the point, the Tuschl lab showed definitely that short chemically synthesized 21-23 nt siRNAs, when provided with 2nt 3'overhangs and incubated with target luciferase mRNA, caused cleavage of the target mRNA ([Elbashir *et al.*, 2001b]). Further experiments from the Tuschl lab emphasized that these modified siRNAs were capable of silencing both heterologous and endogenous genes, not just in invertebrates, but also in mammalian cells ([Elbashir *et al.*, 2001a]). Finally, to determine how the introduction of siRNA leads eventually to gene silencing, Tuschl and colleagues devised a beautiful experiment to identify the components of the RISC (RNA induced silencing complex). They conjugated the 3' ends of their siRNAs to biotin and co-immunoprecipitated the siRNA and associated protein complex via biotin. Purification of the complex by size and weight and corresponding mass spectrometry of the conspicuous 100 KDa band led to the first identification of the family of argonaute proteins responsible for RNAi ([Martinez *et al.*, 2002]). These argonaute proteins were shown to be able to have cleavage activity on their targets ([Liu *et al.*, 2004]) and, in complex with dicer ([Gregory *et al.*, 2005]) and TRBP serve, serve as the minimal RISC necessary to effect gene silencing in every species identified to date with RNAi.

In the wake of these discoveries in RNAi, emerged the field of small non-coding RNA biology. Its initial excitement came from the revelation of a vast and unexplored set of conserved small RNA genes whose hijacking and shared use of the RNAi machinery facilitated gene regulation at unprecedented specificity and complexity. In 1993, Victor Ambros and colleagues discovered through genetic analysis in *C. elegans* that a gene product from the lin-4 locus is responsible for regulating lin-14 protein levels for the specification of development timing in the worm. When they mapped the lin-4 locus, they found to their surprise that the locus was not a conventional gene in that it did not lead to a protein product. Rather, it produced a small 22-nt non-coding RNA that had been processed from a hairpin precursor ([Lee *et al.*, 1993]). At the same time, the Ruvkun lab discovered that the lin-14 3'UTR

had regulatory regions sharing sequence complementarity to the lin-4 short RNA identified by Ambros ([Wightman *et al.*, 1993]). With back to back papers in CELL, they together identified the first microRNA-mRNA target interaction. At that time, however, there was no evidence of conservation of lin-4 in other species, and therefore no reason to believe that such a non-coding regulatory RNA was a widespread mechanism for gene regulation in other species. But the discovery RNAi, in the meantime, offered a plausible mechanism for how processed short double stranded RNA species could effect gene silencing. In 2000, the Ruvkun lab identified the second miRNA, let-7, which also came from a double stranded precursor hairpin structure, and base-paired with the 3'UTR of its target mRNA to silence it ([Reinhart *et al.*, 2000]). It wasn't until the landmark discovery by the Ruvkun lab showing that let-7 was perfectly conserved throughout the animal phyla (we are now in the post-genome era), that it became clear there must be other similar small RNAs in other species, and that the regulatory of lin-4 and let-7 may just indeed be a hugely generalizable phenomenon ([Pasquinelli *et al.*, 2000]). In a race to discover the entire family of small RNAs, the Ambros, Bartel and Tuschl labs published back to back papers in Science in 2001, revealing hundreds of miRNAs that emerged from size-fractioned cDNA libraries of extracts from flies to humans, revealing a larger than expected gene family having millions of years of conservation ([Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001]).

During the first decade of the 20th century, there was a tremendous explosion in scientific studies pertaining to the functional role of these newly discovered family of miRNAs. Their initial roles appeared mostly during development, in specifying cell fates, however, soon, they were implicated in the regulation of various processes including nervous system function and synaptic plasticity. Since learning-related synaptic plasticity requires sophisticated mRNA sequestration and spatio-temporal regulation at nuclear, axonal, and synaptic compartments, miRNAs seemed ideally suited to serve as negative translational regulators of synaptic plasticity. Moreover, their ability to form autoregulatory loops ([Rybak *et al.*, 2008; Johnston and Hobert, 426]) suggests their potential involvement in either homeostatic or

switch-like events during various phases of synaptic plasticity, an inherently multi-stable phenomenon. Several studies have demonstrated the involvement of brain-specific miRNAs in synapse formation and of miRNA ribonucleoprotein complexes (miRNPs) in controlling local protein synthesis associated with stable memory ([Schratt, 2009]). These findings encouraged me to explore systematically the miRNA population of the *Aplysia* central nervous system to understand their functions during learning-related synaptic plasticity. In Chapter 3, I discuss my systematic characterization of the miRNAs of the *Aplysia* central nervous system, and in Chapter 4 I discuss the specific functional and mechanistic characterization of two brain-specific miRNAs, miR-124 and miR-22, and their roles in constraining synaptic facilitation, through regulation of the transcription factor, CREB and the translation factor, CPEB respectively. I was able, for the first time, to provide evidence that miRNAs are modulated by learning-related neurotransmitters, and to show that changes in miRNA levels are causally related to changes in synaptic strength, for which we provided a direct mechanism.

The discovery of a large class of miRNAs in 2001, was followed by the discovery of various other classes of small RNAs, including rasiRNAs, tasiRNAs, endogenous siRNAs, and finally piRNAs in 2006 ([Aravin *et al.*, 2006; Girard *et al.*, 2006]). Of these, piRNAs are the largest class, and are amply present in animals gonads, though their function remains mysterious. Though we were not searching for piRNAs, we unexpectedly discovered the presence of a huge population of piRNAs in *Aplysia* neurons and further investigation provided a rich function during synaptic plasticity. I introduce piRNAs in Chapter 5 and also describe our functional characterization of piRNAs as epigenetic regulators of memory-related synaptic plasticity in *Aplysia* neurons. I was able to provide the first evidence for the abundant presence of piRNAs outside the gonads, in neurons, and to highlight their functional role in silencing promoters during synaptic plasticity and therefore in effecting long term changes in gene expression that may underlie the persistence of memory.

Chapter 3

Characterizing the miRNA profile of *Aplysia*

3.1 Introduction

miRNAs are a class of conserved, 20 to 23 nucleotide (nt) non-coding RNAs that depend on the RNAi machinery for maturation and function, and are able to mediate cleavage or translational repression of their target mRNAs by preferentially binding to their 3' UTRs ([Filipowicz *et al.*, 2008; Bartel, 2009]). Discovery of the first miRNAs in *C. elegans* led to an understanding of their regulatory role in cell lineage specification ([Lee *et al.*, 1993; Wightman *et al.*, 1993; Reinhart *et al.*, 2000]). The subsequent development of methods for the large-scale identification of miRNAs ([Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001]) and the resulting functional studies revealed that miRNAs control many other cellular functions including proliferation, metabolism, apoptosis, immunity and more recently, neuronal growth and plasticity. To obtain a more complete inventory of small RNAs that may have a role in learning-related synaptic plasticity, I carried out massive parallel sequencing to profile the small RNAs of *Aplysia californica*. My collaborators and I identified 170 distinct miRNAs, 13 of which were novel and specific to *Aplysia*. Nine miRNAs were brain-enriched, and several of these were rapidly down-regulated by transient exposure

to serotonin, a modulatory neurotransmitter released during learning. Further characterization of the brain-enriched miRNAs revealed two brain-specific miRNAs, miR-124 and miR-22, that were exclusively present pre-synaptically in a sensory-motor synapse where they constrain serotonin-induced synaptic facilitation through regulation of the transcriptional factor CREB. I therefore present direct evidence that a modulatory neurotransmitter important for learning can regulate the levels of small RNAs and present a novel role for miR-124 in long-term plasticity of synapses in the mature nervous system.

3.2 Results

3.2.1 Evolutionary Analysis of *Aplysia* miRNAs

I prepared small RNA cDNA libraries from isolated central nervous system (CNS), and from the whole animal with CNS removed. Within the CNS, we also generated small RNA libraries from dissected abdominal and pleural ganglia. The libraries from the whole animals and CNS were sequenced using 454 sequencing technology yielding a total of about 250,000 sequence-reads for each library. The abdominal and pleural libraries were sequenced by traditional Sanger sequencing until approximately 2000 reads were collected for each library.

Because we lacked an assembled genome, my collaborator Rob Sheridan and I first built an *Aplysia*-specific annotation database to distinguish miRNAs from turnover of abundant and conserved non-coding RNAs such as rRNAs, tRNAs, or snRNAs. The total content of rRNAs, tRNAs and snRNAs taken together varied between 5 and 25% per library (Supplementary Table 1). To be considered a miRNA the residual sequences needed to satisfy the following three criteria: (1) Precise 5' end processing: Length variants of members of a sequence family preferentially aligned to the 5' end; (2) Fold-back precursor structure: A fold-back structure had to be identified comprising a genomic fragment retrieved from unassembled trace archives; (3) Cloning of the miR* sequence: Since double-stranded miR/miR* processing intermediates are assembled in an asymmetric fashion, capture of miR* that generates short 3' overhangs when paired to the mature miRNA is further evidence for prototypical

miRNA biogenesis.

The *Aplysia* genome trace sequence archives do not yet cover the full *Aplysia* genome, we therefore still considered certain sequences as miRNAs, even if they did not map to the trace sequences, if we could map them to miRNA precursors annotated in other species. We identified 170 distinct miRNAs in *Aplysia*, of which 157 were orthologs to known miRNAs in other species and 13 were specific to *Aplysia*. The new discoveries are catalogued in Table 3.1. All miRNAs are catalogued in Supplementary Table 2. 60 sequences that were abundantly cloned and that demonstrated good 5' processing, but that were neither conserved nor mapped, were designated miRNA candidates (Supplementary Table 3). The evidence for each miRNA is summarized in the following interactive website:
http://cbio.mskcc.org/ sheridan/aplysia/aplysia_candidate_table_2008_11_11.html.
The overall abundance of miRNAs in the small RNA libraries ranged from 50 to 80%, consistent with the miRNA content in small RNA libraries prepared from other species ([Aravin *et al.*, 2003; Landgraf *et al.*, 2007]).

A phylogenetic analysis of the *Aplysia* transcriptome revealed that *Aplysia* is closer in evolutionary distance to the vertebrates than are *C. elegans* and *D. melanogaster* ([Moroz *et al.*, 2006]). Rob and I similarly find that *Aplysia* miRNAs more closely resemble vertebrate miRNAs both in sequence similarity of individual genes and in the abundance of shared miRNA genes. We grouped the 170 distinct *Aplysia* miRNAs into 103 miRNA gene families based primarily on seed sequence similarity, of which 41 families are conserved specifically in vertebrates, whereas only 13 map specifically to invertebrates (Figure 3.1A). When we fit the observed miRNA gene gains and losses onto various phylogenetic trees, we find that our data best fits a model where *Aplysia* is a very ancient ancestor to the invertebrates, lies outside the *D. melanogaster*/*C. elegans* clade, and more directly straddles the invertebrate and vertebrate lineages (Figure 3.1B). A salient feature in support of this model is the presence of 46 miRNAs that are preserved in vertebrates and *Aplysia*, but subsequently lost in *D. melanogaster* and *C. elegans* (Figure 3.1B). Taken together, these findings illustrate that *Aplysia* miRNAs are ancient and well conserved, with relatively few losses or gains of genes,

mature miRNA	mature miRNA sequence	number	star sequence
name	sequence	of reads	cloned
miR-100001	UGCCAUUUUUAUCAGUCACUGUG	17379	+
miR-100053	UGCCCUAUCCGUCAGGAACUGU	2169	+
miR-100097	UCAGCAGUUGUACCACUGAUUUGA	634	+
miR-100098	UGAGACAGUGUGUCCUCCCUUG	493	-
miR-100102-5p	AUUUGGCACUUGUGGAAUAAUCG	285	+
miR-100106	CAUCUACCUAUCCUUCUUCUUC	221	-
miR-100060	CUUGGCACUGGCGGAAUAGUCAC	174	-
miR-100102-3p	AUUAUACACCGGUGCCAAAU	151	+
miR-100072	UUACCCUGGAGAACCGAGCGUGU	125	-
miR-100070	GAAGCGGGUGCUCUUAUUU	109	-
miR-100090	UAUCCGCUCACAAUUCCCC	102	-
miR-100087	UUGUGACCGUUAUAAUGGGCAUU	75	-
miR-100091	AGCGGUGAUAUUUUUGUCUGGC	69	-

Table 3.1: New miRNAs discovered in A. *californica*

All sequences shown here had a mapping to the genome with a recognizable precursor hairpin structure, and good 5 processing of mature sequence length variants. The predominantly cloned sequence is given, along with the number of sequence reads, including its predominant length variants, and an indication of whether the star sequence was (+) or was not (-) cloned.

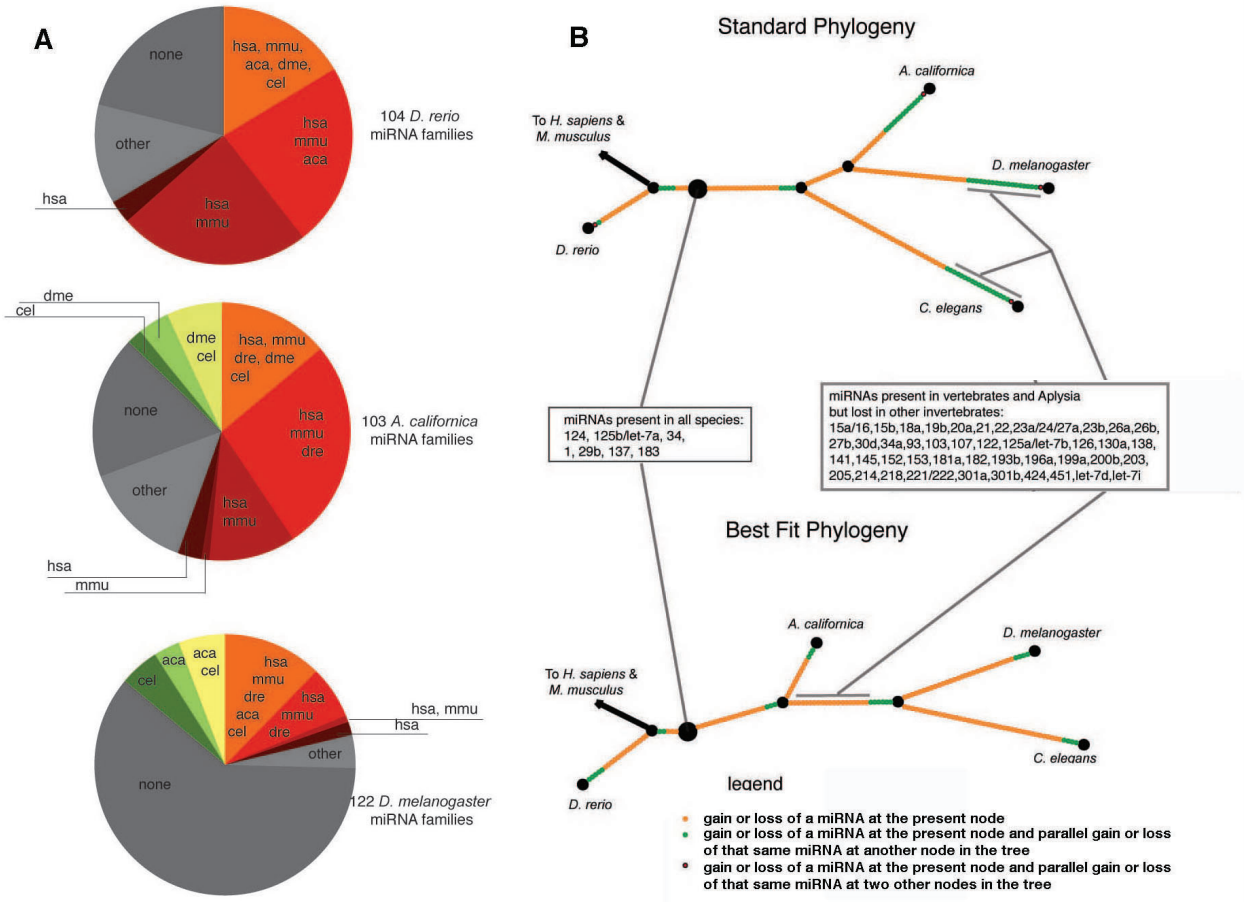

Figure 3.1: *Aplysia* miRNAs are more similar to vertebrate than invertebrate miRNAs A. The fraction of miRNA families in a given species that bear homology relationships with miRNA families in other species. Homology with vertebrates are displayed in shades of red, invertebrates in yellow and green. Absence of conservation, or mixed conservation patterns (other) are displayed in shades of gray. B. The evolutionary relationship of the miRNAs in 5 species, as understood through gain and loss events, is mapped onto both a standard phylogeny (based on rRNA distances) and an alternate phylogeny (based on best fit of our data).

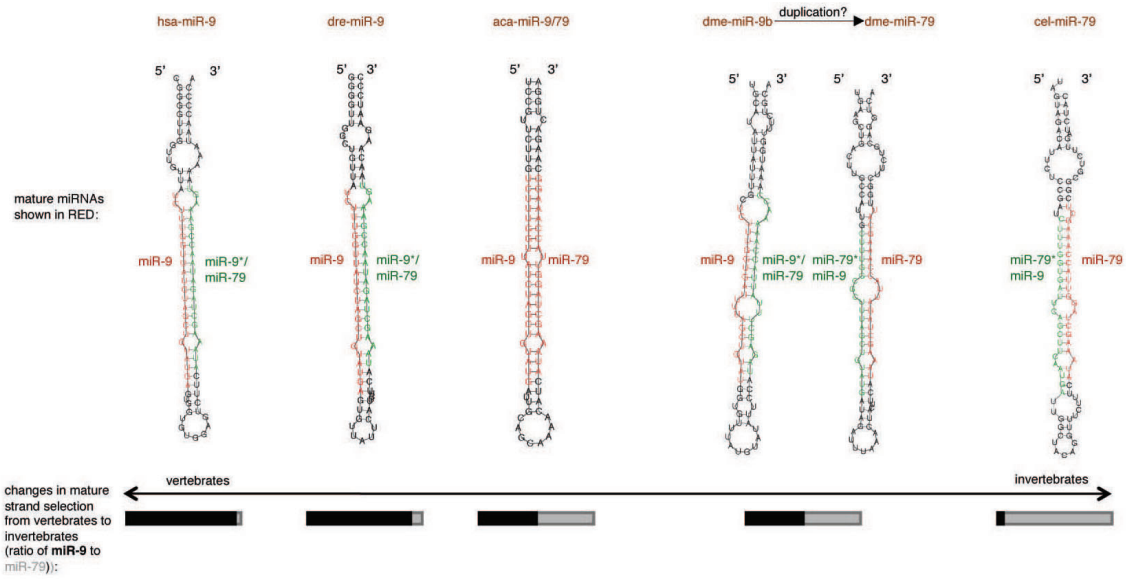

Figure 3.2: Evolution of the miR-9/-79 gene family

miR-9 and miR-79, although thought to be distinct miRNAs emerging from separate loci, are in fact star sequences of each other. Here we show that miR-9 is preferentially expressed in vertebrates, while miR-79 is preferentially expressed in invertebrates. *Aplysia*, however, expresses both in equal proportions in 3-p/5-p fashion.

which makes it a distinctive model organism among invertebrates that shares important genomic similarities with vertebrates and mammals.

We observed one striking aspect of evolutionary history regarding the miR-9/79 gene family. The invertebrate-specific miR-79, and the vertebrate- and Drosophila-specific miR-9 are expressed in equal proportions in *Aplysia*, and are in fact star sequences of each other, which are sequences found on opposite strands of the same precursor hairpin (Figure 3.2). It is likely, then, that *Aplysia* mir-9/79 is a single gene that displays symmetric maturation patterns for both strands, whereas in other species, the gene has duplicated to give rise to multiple gene copies with asymmetric strand preference producing either miR-79 in other invertebrates or miR-9 in vertebrates.

3.2.2 Cellular and Sub-cellular Distribution of Brain-Enriched miRNAs

Deep sequencing revealed the expression of over 100 distinct miRNA genes expressed in the *Aplysia* CNS. The miRNAs comprising the top 95% of clones are shown in order of their abundance in (Figure 3.3 with their enrichment in the CNS versus the rest of the body.

Nine miRNAs are either brain-specific or brain-enriched by cloning (Figure 3.3, and confirmed with Northern blotting in Figure 3.4), three of which are miRNAs unique to *Aplysia*. In general, there was good overlap of the miRNAs of Aplyisa CNS with the miRNAs of the human and rodent brain but notable exceptions include the high abundance and brain enrichment of miR-22c (Figure 3.4), miR-184 (Figure 3.4), miR-34b, and miR-190, where studies in other species have not found CNS- enrichment for these miRNAs ([Chen *et al.*, 2005; Ruby *et al.*, 2007; Landgraf *et al.*, 2007]). The multicopy cistrons of miR-1/133a and miR-206/133b, which are muscle-specific in vertebrates and *D. melanogaster*, were abundantly expressed in *Aplysia* CNS. Finally, the low expression of miR-9 and the complete absence of miR-128 in *Aplysia* CNS is noteworthy because both are highly abundant, and brain-specific, in vertebrates.

To learn which miRNAs might function in a compartment-specific manner, I developed a protocol (described in methods) for in situ hybridization of miRNAs in *Aplysia* using synthetic DNA probes. A functional circuit containing a sensory and motor neuron from *Aplysia* ganglia was dissected and placed in co-culture, and then examined by in situ hybridization for localization patterns of various miRNAs (Table 3.2). I found that miR-124 stained much more intensely in the sensory neuron compared with the motor neuron (Figure 3.5A), and a 4 nt mismatch containing control probe showed no signal. I also consistently observed both a perinuclear density for mir-124, as well as punctuate staining in the processes (Figure 3.5B). Further in situ hybridization studies of the more abundant miRNAs in CNS revealed several other miRNAs (such as miRs 22c, 125c, let-7a, 100, and 8b) that were specifically expressed in the sensory neuron compared to the motor neuron, and some that were enriched in either the cell body alone (miR-1) or in the neurite processes alone (miR-100001) (Figure 3.5). The

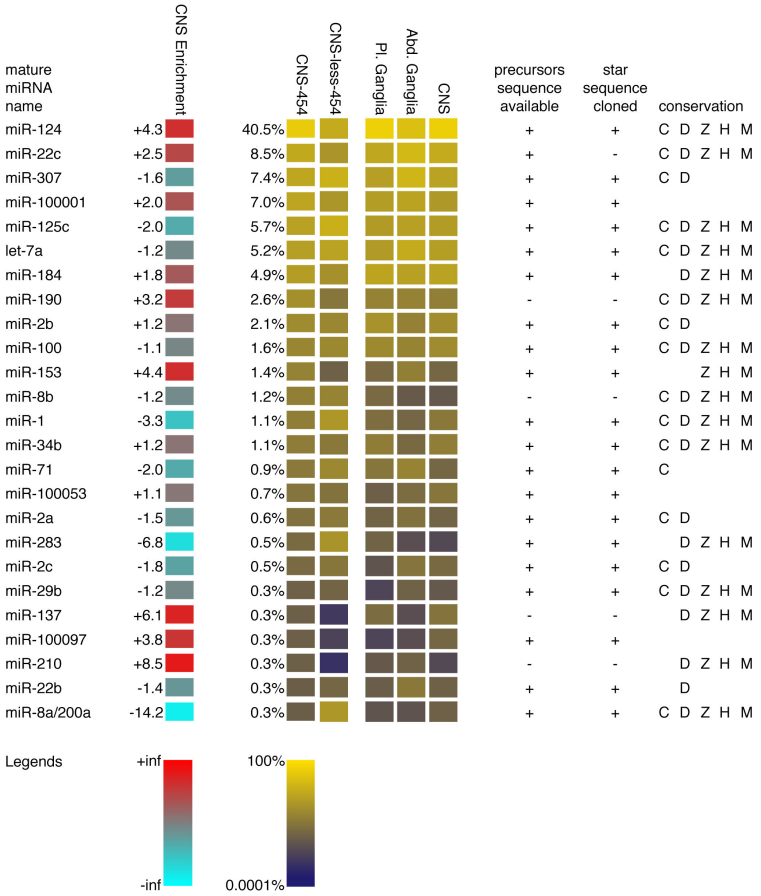

Figure 3.3: The abundant miRNAs observed in *Aplysia* CNS

The top 95% of miRNA clone content from the CNS library is shown, along with enrichment in the brain as compared to the whole animal, distribution in abdominal and pleural ganglia, the existence or absence of a precursor in the genome together with cloning evidence for its star sequence, and finally homology relationships to *H. sapiens* (H), *M. musculus* (M), *D. rerio* (Z), *D. melanogaster* (D), and *C. elegans* (C).

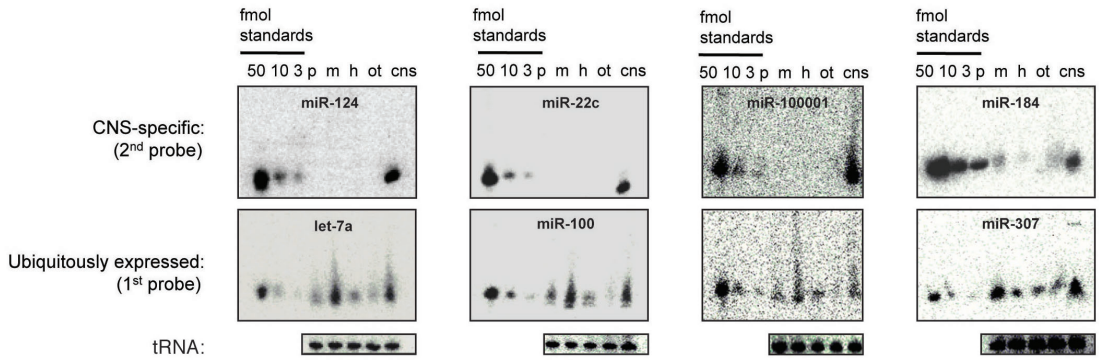

Figure 3.4: miRNA tissue specificity

Expression of 8 different mature miRNAs across various tissues including (p) hepatopancreas, (m) muscle, (h) heart, (ot) ovotestis, and (cns) central nervous system. Detection of synthetic miRNAs loaded on the far left of the blots at a concentration of 50 fmol, 10 fmol, and 3 fmol serve as positive controls. tRNA bands are shown to control for equal loading of samples.

differential expression of miRNAs between sensory and motor neurons is also apparent from miRNA clone frequencies of abdominal versus pleural ganglia, the latter of which contain many more sensory neurons.

3.2.3 Serotonin Regulates miRNA Levels

miR-124 has been shown to be important during neuronal differentiation and in specifying neuronal identity ([Lim *et al.*, 2005; Makeyev *et al.*, 2007; Visvanathan *et al.*, 21; Cheng *et al.*, 2009]). My finding that miR-124 is relatively absent in the motor neuron of a sensory-motor co-culture highlights the possiblity that miR-124 may not be present in all neurons and may have functions in addition to maintaining neural identity. I therefore asked: might miR-124 be regulated by synaptic activity? Specifically, I wanted to know whether it might be modulated by serotonin.

I found, by Northern analysis, that within one hour of exposure to five spaced pulses of serotonin the levels of miR-124 were consistently reduced two-fold (Figure 3.6A). These

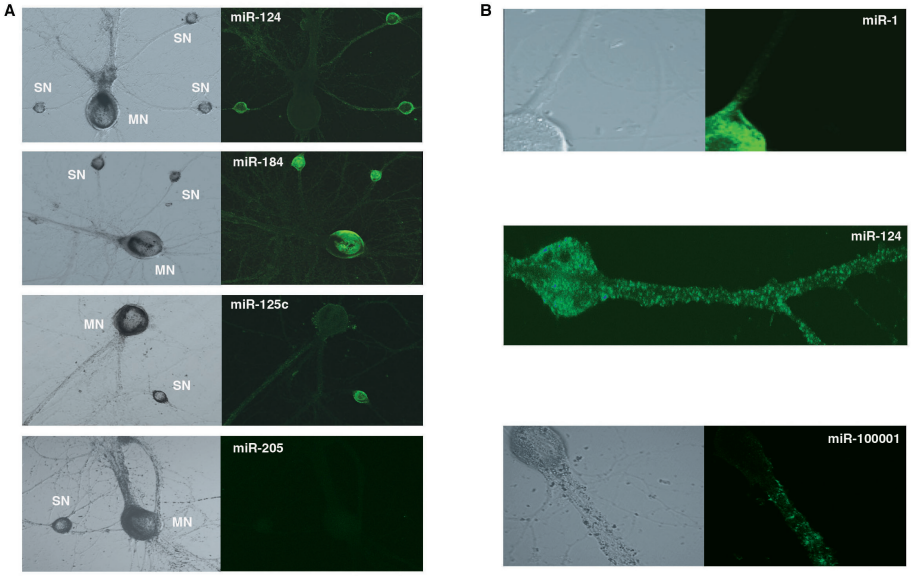

Figure 3.5: miRNA cell-specificity & sub-cellular distribution

A. Projection images of 10x confocally acquired images from 1 μm slices through a sensory (SN) motor (MN) co-culture in situ hybridized with DNA probes complementary to the mature miRNA sequence. As a negative control, cells were probed for mir-205, which is not expressed in *Aplysia* neurons, and therefore show no staining. B. Projection images of 40x confocally acquired images showing an example of a miRNA that is primarily found in the cell body (miR-1), primarily in the cell process (miR-100001), and in both compartments (miR-124).

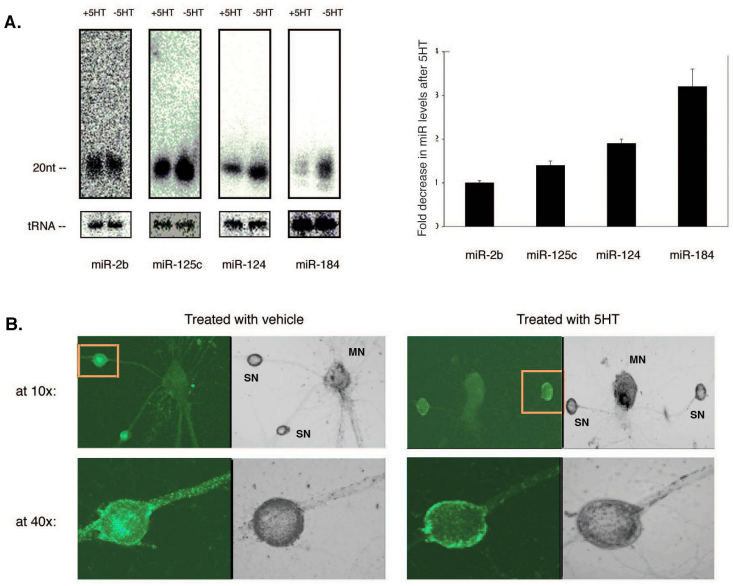

Figure 3.6: miRNAs are rapidly down-regulated by serotonin

A. Mature miRNA levels in untreated CNS (-5HT) and CNS treated with five spaced pulses of serotonin (+5HT). Blots were re-probed for tRNA to monitor equal loading of samples. Changes in miRNA levels are quantified and presented as a mean of 6 independent trails ± S.D. B. In situ hybridization experiments in sensory-motor co-cultures show that exposure to 5 pulses of 5HT causes a significant reduction of miR-124 levels in sensory neurons.

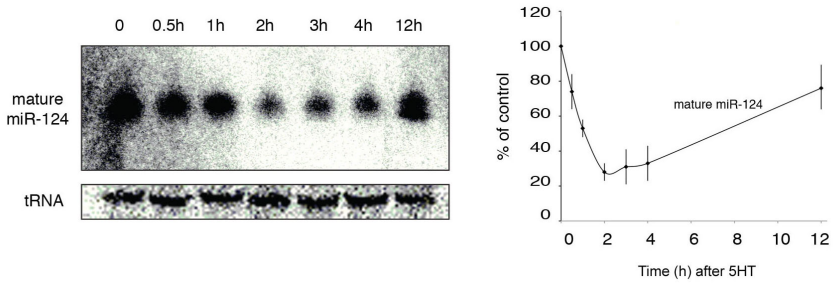

Figure 3.7: Time course of mature miR-124 levels after exposure to 5HT
Mature miR-124 levels in CNS in control cells (0) and in CNS at 30 minutes (0.5 h), 1, 2, 3, 4, and 12 hours after treatment with 5HT. The blots are re-probed for tRNA to control for equal loading of samples. The data are quantified in the right panel and presented as a mean of 4 independent trials ± S.D.

findings were corroborated by in situ hybridization analysis, which also showed a drop in miR-124 levels in both the sensory neuron cell body and neurite processes within one hour after washout from five pulses of serotonin (Figure 3.6B). No change in miR-124 levels was observed in cells treated with just one pulse of serotonin (Supplementary Figure 1). To determine how long it takes for miR-124 levels to return to baseline after exposure to five pulses of 5HT, I performed a time course analysis and found that miR-124 levels continue to drop even two hours after 5HT treatment, but then slowly re-accumulate, returning to baseline by 12 hours (Figure 3.7).

To better understand the mechanism underlying the serotonin-induced regulation of miR-124, I tested whether the miR-124 precursor levels were also affected by 5HT, and found by real time PCR, that pre-miR-124 levels remained unaffected in sensory neurons after exposure to five pulses of 5HT (Figure 3.8A). This indicated that the regulation of miR-124 occurs downstream to the biogenesis of the precursor species, either at the level of the RNase III Drosha processing or turnover of the Argonaute-bound miRNA complex. Since 5HT is known to activate several downstream signaling pathways, including PKA ([Castellucci *et al.*, 1980]), MAPK ([Martin *et al.*, 1997]), PKC ([Sacktor and Schwartz, 1990]), and

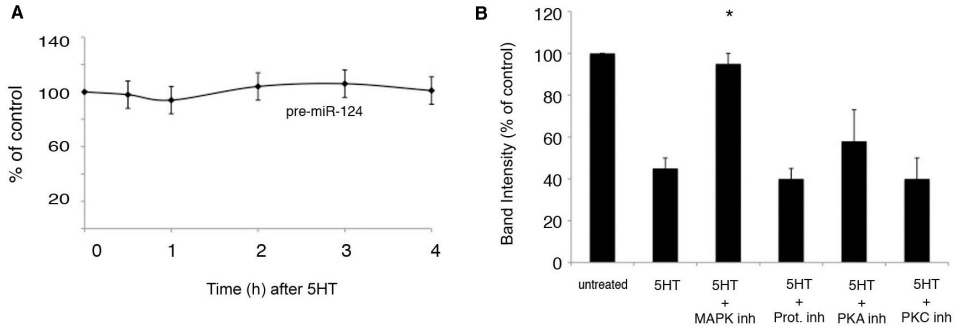

Figure 3.8: Serotonin-dependent down-regulation of miR-124 is MAPK dependent
A. Real time PCR data showing miR-124 precursor levels at 0, 0.5, 1, 2, and 4 hours after treatment with 5 pulses of 5HT. Data are shown as a mean of 6 independent trials ± S.D. B. CNS were treated with 10 μM, in L-15, of each of the indicated inhibitors for 30 minutes prior to treatment with 5 pulses of 5HT. Following 1.5 hours after washout from 5HT and the inhibitors, total RNA was extracted, northern blotted, and probed for miR-124. Levels of miR-124 are given as mean band intensity from Northern blots and the data are presented here as a mean of 4 independent trials ± S.D.

the proteasome ([Hegde *et al.*, 1997]), I applied inhibitors of each of these molecules, in the presence of 5HT, to determine which, if any, contributes most to the regulation of miR-124. I found, by Northern analysis, that a MAPK inhibitor (U0126) almost fully abolished the 5HT-induced down-regulation of miR-124. By contrast, inhibitors of PKC (Bisindolylmaleimide), and the proteasome (MG-132) had no effect, and a PKA inhibitor (KT5720) showed a modest, but insignificant, attenuation of the 5HT-induced miR-124 effect (Figure 3.8B).

In screening other miRNAs for serotonin dependent regulation, I found one miRNA (miR-184) that had an even more pronounced, 3-fold, reduction, and others that either showed modest (miR-125c) or no (miR-2b) regulation by serotonin (Figure 3.6A).

This is the first demonstration that a synaptic neurotransmitter can regulate miRNA levels. In the case of miR-124 I find that this occurs rapidly, is sustained for many hours, occurs through MAPK signaling, and affects only the mature miRNA levels, not the precursor

form. The ability of serotonin to modulate miR-124 levels is of specific interest because its previously known function in neuronal differentiation suggested constitutive expression in mature neurons to maintain neuronal identity.

3.3 Discussion

In mining the miRNAs of *Aplysia* by deep sequencing, I provide what is perhaps the most comprehensive catalogue to date of the miRNA population in a central nervous system. The well-conserved nature of these miRNAs encourages their study in other nervous systems. In *Aplysia* it specifically allows study of miRNAs at the level of single cells and single synapses in processes ranging from neuronal development to synapse formation, stabilization, and plasticity. As more organisms are mined for their miRNAs, we are likely to gain a better understanding of how ancient and diverse miRNA gene families are, and what constraints they face during evolution.

In this initial study, I describe 170 distinct miRNAs present in *Aplysia*, of which 13 were previously unknown. Recent studies ([Lu *et al.*, 2008; Liu *et al.*, 2008a; Grimson *et al.*, 2008]) indicate that miRNA evolution has been dynamic and that most species have undergone dramatic changes in their miRNA gene content characterized by greater than normal rates of gene loss, gain, and duplication events. The miRNAs of *Aplysia*, however, appear to be particularly stable relative to its last common ancestor. *Aplysia* has gained only 13 miRNAs from its shared ancestry with vertebrates (though this number is likely to increase as the *Aplysia* genome coverage improves), and preserves over 40 miRNAs that are subsequently lost in *D. melanogaster* and *C. elegans*. Interestingly, the abundant miRNAs in *Aplysia* CNS appear to be as well conserved in invertebrates as vertebrates (Figure 3.3, whereas the whole animal miRNA population in *Aplysia* (Figure 3.1 has a significant enrichment of shared vertebrate miRNAs, compared with invertebrate miRNAs, and many of these, such as miR-15/16, miR-145, and miR-221 are abundant and have important function in mammals. The similarity of genes between *Aplysia* and vertebrate systems is not entirely due to loss of genes

in *C. elegans* and *D. melanogaster*. An analysis of well-conserved miRNAs shows that the vertebrate homolog is often more similar in sequence identity to the *Aplysia* homolog than it is to the homologs of *C. elegans* and *D. melanogaster*. The underlying similarity between *Aplysia* miRNAs and vertebrate miRNAs may also correlate with similarity in targets and in function, therefore strengthening the ability to use *Aplysia* as a model to understand the role of miRNAs in mammalian and even human neural function.

Expression analysis in cultured neurons of sensorimotor synapses revealed several miRNAs that were localized to distinct cells and sub-cellular compartments. Of the miRNAs that were screened, the striking enrichment of miR-124 in the sensory neuron with relative absence of expression in the motor neuron was most surprising. Earlier studies of miR-124 found that it had a ubiquitous and constitutive expression pattern in most neuronal cell types of the mammalian brain, which together with its lack of expression in progenitor cells, suggested a primary role for miR-124 in specifying and maintaining neuronal cell identity. My studies of miR-124 in *Aplysia* revealed that, in addition to being non-uniformly expressed in adult neurons, miR-124 is rapidly and robustly regulated by the neurotransmitter serotonin, indicating additional roles for miR-124 in mature neurons. Several other miRNAs showed a similar down-regulation by serotonin, suggesting a general mechanism by which synaptic activity might relieve negative constraints on gene expression during learning-related plasticity.

My finding that small RNAs can be regulated by conventional neurotransmitters extends further the scope of neurotransmitter actions. Neurotransmitters were first appreciated in the context of their ability to (1) regulate gating of ion channels, and subsequently to (2) covalently modify protein substrates by activating second messenger pathways. Subsequently, it was found that transmitters (3) also regulate transcription (reviewed in [Kandel, 2001]). I now describe a fourth function of neurotransmitter action, the regulation of small RNAs. These considerations raise the further question: How are the miRNAs regulated by serotonin? Recent studies have uncovered two major stages of regulation during miRNA biogenesis, one at the Drosha cleavage step that converts the primary transcript into a

miRNA precursor, and the second at the Dicer cleavage step that converts the precursor to the mature form ([Obernosterer *et al.*, 2006; Heo *et al.*, 2008; Michlewski *et al.*, 2008; Viswanathan *et al.*, 2008]). The ability of serotonin to selectively affect mature miR-124 levels, without affecting its precursor, argues for regulation during Dicer processing, or during RISC incorporation and stabilization by Argonaute, or even perhaps is the result of passive turnover of the miRNA in response to increased turnover of their target mRNAs.

The study by Ashraf et al. 2006 showed learning-dependent changes in RISC, and that this was dependent on the proteasome. In light of their finding, I reasoned that the proteasome may also be involved in the serotonin regulation of miR-124, especially since changes in miRNA levels are rapid and may mean enhanced degradation rather than impeded maturation. However, I found that inhibiting the proteasome had no effect on the serotonin-induced down-regulation of miR-124. Instead, I did observe that a MAPK inhibitor almost fully abolished the ability of serotonin to regulate miR-124. MAPK is one of the major signaling molecules downstream of serotonin that is known to activate CREB ([Martin *et al.*, 1997; Impey *et al.*, 1998]), and my data would suggest that one way it does so is by relieving miR-124 inhibition of CREB. The dissection of the precise mechanism by which MAPK down-regulates miR-124 will require first an understanding of the MAPK substrates in the RNAi pathway, and then an exploration of how phosphorylation events, say on Dicer or Argonaute, may lead to the destabilization of the mature miRNAs.

mature miRNA	number of reads	CNS-enrichment	sensory neuron	motor neuron	cell body	cell process
miR-124	88,678	++	+		+	+
miR-307	28,561		+	+		+
miR-125c	24,803		+		+	+
miR-22c	20,405	+	+		+	+
let-7a	17,452		+		+	+
miR-184	12,629	+	+	+	+	+
miR-100001	8,690	+	+	+	+	+
miR-1	6,593		+	+	+	
miR-2b	6,128		+	+	+	+
miR-100	5,339		+		+	+
miR-8b	4,040		+		+	+
miR-153	2,951	++	+	+	+	+

Table 3.2: Summary of miRNA expression patterns in an *Aplysia* sensory-motor co-culture as assessed by in situ hybridization.

For each miRNA, the table indicates whether it was enriched (++), present (+), or absent ( ), in the cell types and compartments listed. The table is sorted in miRNAs of decreasing abundance in the CNS, and their clone frequencies are listed.

Chapter 4

Functional Analysis of aca-miR-124 and aca-miR-22 as Inhibitory Constraints on Synaptic Facilitation

4.1 Introduction

In *Aplysia*, both short-term memory lasting minutes and long-term memory lasting days have been well characterized in the gill-withdrawal reflex in response to sensitization, a simple form of learned fear (reviewed in [Kandel, 2001]). In an *Aplysia* sensory-motor culture system ([Montarolo *et al.*, 1986]), delivery of one pulse of serotonin (5HT), a modulatory neurotransmitter released in the intact animal by sensitizing stimuli, elicits PKA-dependent short-term facilitation lasting minutes. By contrast, five spaced pulses of serotonin cause both PKA and MAPK to translocate to the nucleus ([Martin *et al.*, 1997b]), thereby releasing inhibition by the repressor CREB2 and activating CREB-dependent transcription, leading to long-term synaptic facilitation and growth of new synaptic connections. Thus in sensitization, as in many other forms of learning, nuclear activation of CREB is an important component of a general switch that converts short-term into long-term plasticity in both vertebrates and invertebrates ([Dash *et al.*, 1990; Barco *et al.*, 2002]). In addition,

studies on both the gill-withdrawal reflex and the mammalian hippocampus has delineated the importance of local protein synthesis at the synapse, mediated by the persistent activity of CPEB and PKCζ ([Si *et al.*, 2003a; Serrano *et al.*, 2008]) in sustaining synapse activity independent from the distant cell body (reviewed by [Sutton and Schuman, 2006; Martin and Zukin, 2006]). Indeed, communication between the nucleus and the synapse, via the shuttling of mRNA and proteins by kinesin motors, serves as still another critical regulatory point in the induction of long-term facilitation ([Puthanveettil *et al.*, 2008]).

Given the high degree of spatiotemporal regulation required for long-term synaptic plasticity, and given that some brain-enriched miRNAs are localized to specific sub-cellular compartments and respond rapidly to external stimuli and neurotransmitter activity, I searched functionally to identify a mechanism and functional role for miRNAs in the regulation of synaptic plasticity. I found in the previous chapter that the most highly abundant, well-conserved, brain-specific miRNA, *Aplysia* miR-124, is specific to the pre-synaptic sensory neuron where it is rapidly down-regulated by serotonin. In the absence of 5HT, I provide evidence in this chapter for the function role of miR-124 as an inhibitory constraint on synaptic plasticity and long-term facilitation through the regulation of CREB, the transcriptional switch critical for converting short- to long-term facilitation. I also provide unpublished data on the role of brain-specific miR-22 in regulating CPEB and PKCζ and in constraining synaptic facilitation.

4.2 Results

4.2.1 miR-124 constrains long-term synaptic facilitation

To determine the physiological relevance of the 5-HT-induced changes in miR-124 levels, my collaborator Ferdinando Fiumara altered miR-124 levels in sensory neurons and analyzed the effect on the 5-HT-induced long-term facilitation (LTF) of synapses between the sensory and motor neuron (Figure 4.1A, B). Injection of a duplex miR-124 mimic (Dharmacon, Inc.), designed to increase the levels of miR-124 in sensory neurons, caused a significant

reduction in LTF as measured at 24 and 48 hrs after exposure to five pulses of 5-HT (n=10), when compared to un-injected controls in the same co-culture (n=9, $F(1,17) = 5.27$, $p < 0.05$, two-way ANOVA with one repeated measure (time); Figure 4.1D, E). Conversely, injection of the single stranded antisense miR-124 inhibitor (Dharmacon, Inc.), designed to reduce the levels of miR-124, caused a significant increase in synaptic facilitation of the 5HT treated synapses (n=14) with respect to untreated controls (n=14) as measured at 24 and 48 hrs ($F(1,26) = 4.70$, $p < 0.04$, two-way ANOVA with one repeated measure; Figure 4.1J, K). Control experiments with the injection of scrambled miR mimic (n=16) and scrambled miR inhibitor (n=16) did not show significant changes in LTF ($p > 0.05$, two-way ANOVA with one repeated measure; Figure 4.1G, H, M, N). The observed differences in LTF among the different treatments were not due to differences in the basal strength of the synaptic connections in the different experimental groups (Figure 4.1F, I, L, O). In situ hybridization confirmed that the miR-124 mimics and inhibitors were able to increase or decrease respectively the levels of miR-124 in sensory neurons (Figure 4.1C).

To further support these observations, we also performed physiological experiments using an alternative knockdown method. To inhibit miR-124, we bath-applied antisense 2-O-methyl-oligoribonucleotides conjugated with the peptide penetratin (Qbiogene, Inc.). The penetratin-conjugated inhibitor (200 nM) is capable of crossing the membrane of cultured *Aplysia* neurons and of inducing inhibition of miR-124, as determined by in situ hybridization (Figure 4.2P, also shown for inhibition in whole ganglia by Northern blotting in Figure 4.3). To induce a significant inhibition, we incubated cells with the penetratin-conjugated inhibitor for 24 hrs before testing the basal amplitude of the EPSP and applying five pulses of 5-HT. The experiments with penetratin-conjugated miR-124 inhibitor confirmed that LTF was significantly enhanced following inhibition of miR-124 ($+127.1 \pm 16.36$, n=9) as compared to controls treated with penetratin-conjugated to a miR-194 inhibitor ($+67.35 \pm 18.18$, n=9, $p < 0.01$, Newman-Keuls post-hoc test after two-way ANOVA; Figure 4.2R). The inhibition of miR-124, within these temporal limits, did not affect basal synaptic transmission while interfering with 5HT induced LTF (Figure 4.2Q).

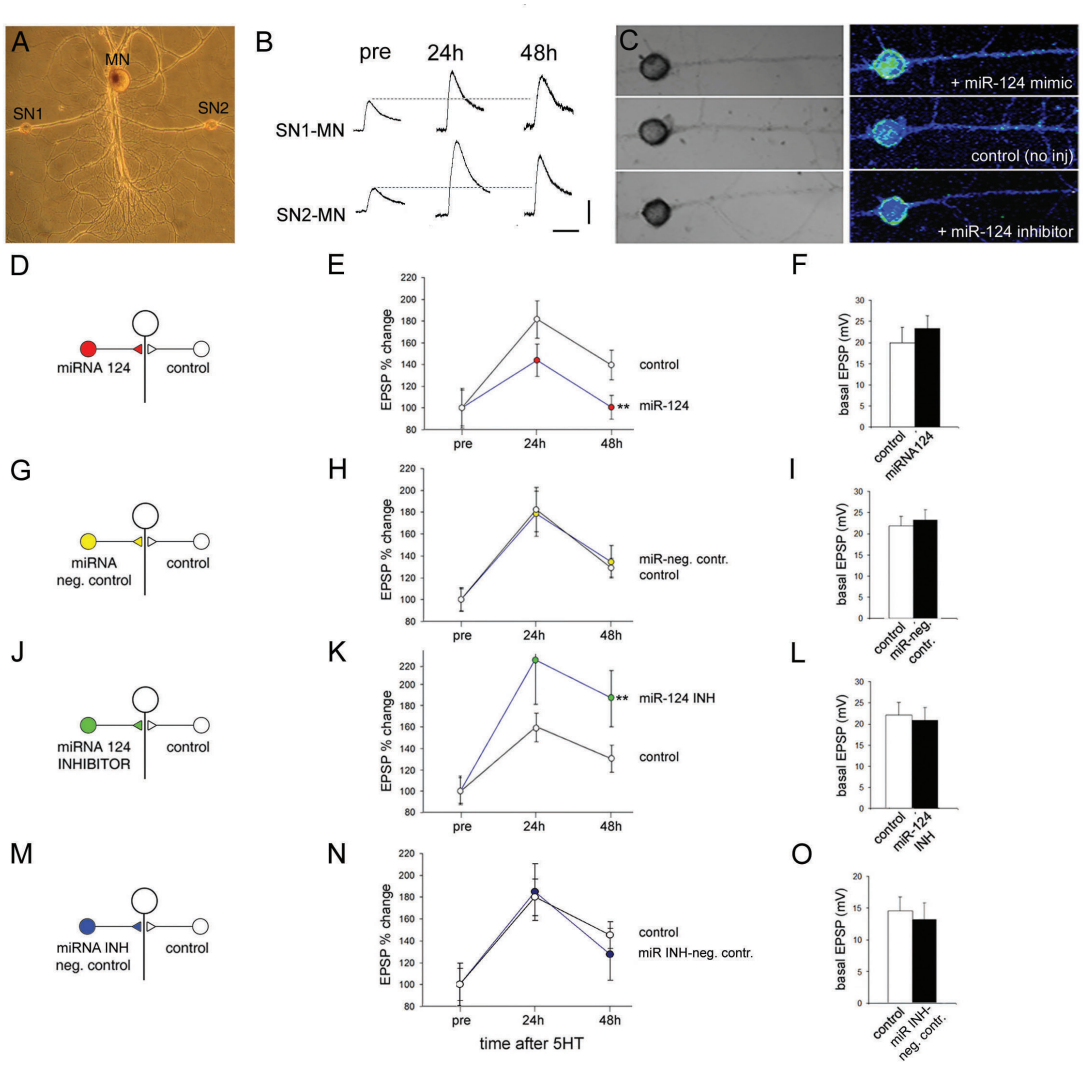

Figure 4.1: miR-124 constrains serotonin-dependent synaptic facilitation

A. Phase contrast micrograph of the experimental model. B. Representative EPSPs in motor neurons after stimulation of the sensory neuron. C. In situ hybridized miR-124 levels in sensory neurons injected with miR-124 mimic or inhibitor. D, G, J, M. One sensory neuron, per co-culture, was injected with miR-124 mimic (D), mimic negative control (G), inhibitor (J), or inhibitor negative control (M), the other was left untreated. E, H, K, N. Percentage change in EPSP at 24 and 48 hrs after 5HT treatment with respect to pretreatment values. F, I, L, O. Average EPSPs measured before 5HT treatment to control for changes in basal synaptic strength.

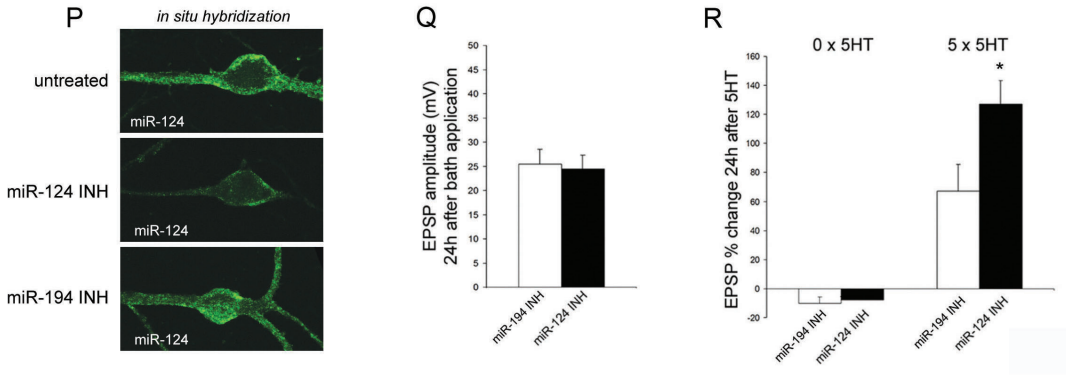

Figure 4.2: miR-124 knockdown enhances serotonin-dependent synaptic facilitation P. Penetratin conjugated antisense inhibitors show a significant reduction in endogenous miR-124 levels, as compared with untreated cells, or cells treated with a control miR-194 antisense inhibitor. Q. Mean amplitude of EPSPs measured at sensorimotor synapses 24 hrs after the bath application of either a penetratin-conjugated miR-124 inhibitor (200 nM) or a control penetratin-conjugated miR-194. R. Average percentage synaptic facilitation measured at 24 hrs after treatment with either 0 or 5 serotonin pulses in cultures that had been pre-incubated with either the penetratin-conjugated miR-124 inhibitor or the control penetratin-conjugated miR-194 inhibitor.

4.2.2 miR-124 regulates CREB1

How does the down-regulation of miR-124 lead to long-term facilitation? To find potential targets of miR-124, I screened many genes relevant to plasticity and known to be regulated by serotonin, for increases in expression levels after inhibition of miR-124. De-sheathed pleural ganglia were incubated in antisense 2-O-methyl-oligoribonucleotides conjugated with penetratin to inhibit miR-124 (confirmed by Northern blot Figure 4.3), following which total protein was extracted and Western blotted. I found that inhibition of miR-124 led to a robust up-regulation in the *Aplysia* pleural ganglia of CREB1, the activator of transcription required for long-term facilitation (Figure 4.4A). This is consistent with the fact that not only the protein ([Bartsch *et al.*, 1998]) but also the CREB1 mRNA levels are up-regulated by serotonin ([Liu *et al.*, 2008b]). I also found several genes, whose expression levels were unaffected by miR-124 inhibition (Figure 4.4B). Specifically, CREB2, the repressor that antagonizes CREB1 in synaptic depression, was unaffected by miR-124.

To be certain that miR-124 acts through CREB, I asked whether inhibition of miR-124 might affect the regulation of genes downstream to CREB. I observed that all three known immediate response genes, induced by serotonin in a CREB- dependent manner, increased in their level of protein and in their level of transcript (Figure 4.4) after inhibition of miR-124. These three genes are: 1) ubiquitin C-terminal hydrolase (UCH) ([Hegde *et al.*, 1997]), 2) CAAT enhancer binding protein (C/EBP) ([Alberini *et al.*, 1994]), and 3) kinesin heavy chain (KHC) ([Puthanveettil *et al.*, 2008]). The increased protein and mRNA levels of these three genes were specific, because control antisense inhibitor did not alter levels of UCH, C/EBP, or KHC, and moreover, inhibition of miR-124 did not affect other plasticity related genes such as MAPK, neurexin, and tubulin (Figure 4.4). The observed induction of protein levels of UCH, C/EBP, and KHC by inhibition of miR-124 was further enhanced by exposure to 5HT (Supplementary Figure 2). This suggests that the miRNA inhibition is just one of perhaps many parallel 5HT-mediated events that converge to activate CREB and its immediate early genes.

A conserved putative target site (Supplementary Figure 3) for miR-124 in the CREB1

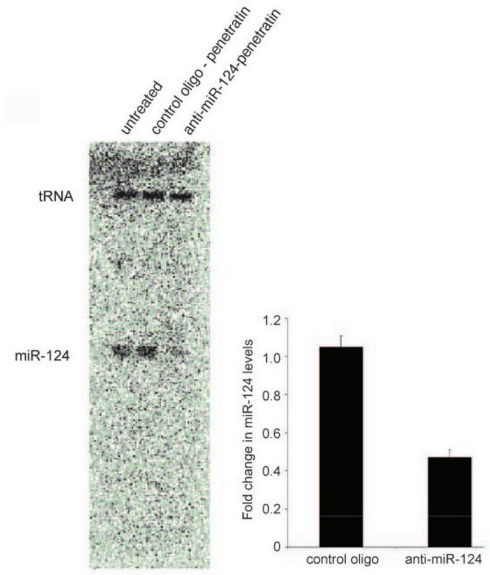

Figure 4.3: Penetratin antisense conjugates efficiently knockdown miRNAs

Cells treated with either 2-O-methyl oligonucleotides antisense to miR-124 conjugated to penetratin, or with 2-O-methyl control oligonucleotides antisense to miR-194, or with vehicle alone were RNA extracted, blotted and probed for miR-124. Level of knockdown is quantified by taking the mean % reduction of antisense miR-124 as compared to antisense miR-194 over 4 independent trials ± S.D.

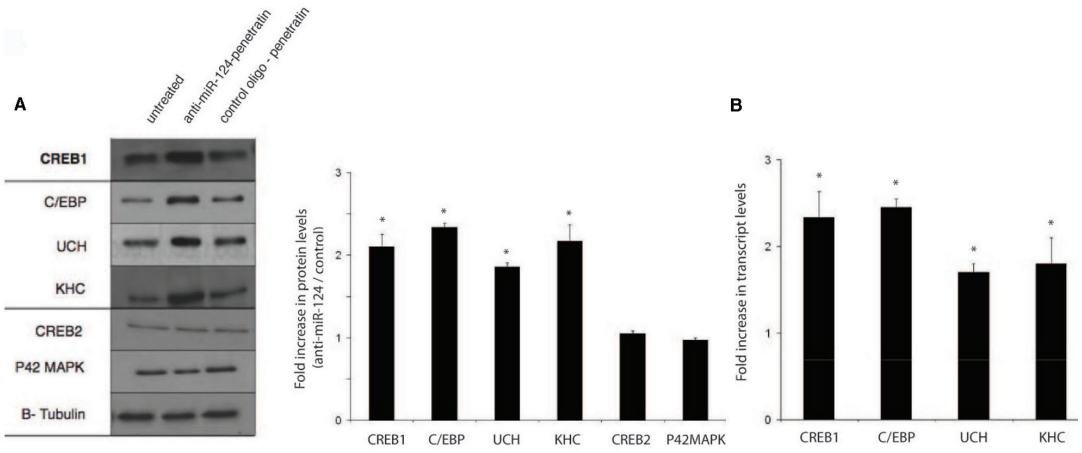

Figure 4.4: miR-124 regulates levels of CREB and its immediate early genes

Fold increase in protein and transcript levels of CREB, KHC, UCH, and C/EBP after inhibition of miR-124, as detected by western blot and real time PCR. Proteins downstream to CREB (KHC, UCH, and C/EBP) have significantly increased transcript levels, whereas a transcript not known to be an immediate early gene of CREB, neurexin shows no such increase. Protein levels were normalized to tubluin, transcript levels were normalized to GAPDH, and data are presented as a mean of 5 independent trials ± S.D.

3UTR of vertebrates and mammals indicated that miR-124 might directly bind and inhibit the translation of CREB1 mRNA. To determine whether miR-124 directly binds and regulates *Aplysia* CREB, I cloned the full length 3UTR of *Aplysia* CREB1 and found a putative miR-124 binding site (9-mer seed + GU Wobble) near the poly A signal (Figure 4.5). To test whether this site is functional, I examined the effect of miR-124 over-expression, on a luciferase reporter fused to the CREB1 3UTR. I found that miR-124 over-expression was able to repress the expression of the luciferase reporter by 45% ($p < .01$) when carrying the wildtype UTR, but had no significant effect on the reporter when the seed of the miR-124 binding site in the UTR was disrupted by a 2 nt mismatch, or when the reporter was fused to a truncated form of the UTR that no longer contained the miR-124 binding site (Figure 4.5). In addition, the over-expression of an unrelated miRNA, let-7, had no significant effect on the reporter construct carrying the full length CREB1 UTR (Figure 4.5). As a positive control, an siRNA targeting the luciferase gene was able to repress luciferase activity by 80% (Figure 4.5). These data indicate that miR-124 directly regulates *Aplysia* CREB1 by binding to its UTR near the poly A signal.

While cloning the *Aplysia* CREB1 3UTR, I discovered a novel and previously uncharacterized isoform of CREB in *Aplysia*, which differs from the canonical CREB1 in its last exon and 3UTR (Supplementary Figure 5). This newly identified CREB isoform (which I term CREB1d) also bore a putative miR-124 target site, but showed no direct regulation by miR-124 on luciferase reporter assays (Supplementary Figure 4). The lack of regulation could be because the seed of this site is weak (six-mer seed + GU wobble), or because this site is in the ORF, which is considered to be functionally weaker than sites in the UTR ([Bartel, 2009]).

CREB1 is a transcription factor that acts as a switch to convert short-term, protein-synthesis-independent facilitation (requiring one pulse of 5HT), into long-term, protein-synthesis dependent facilitation (requiring five pulses of 5HT). Therefore, neurons that over-express CREB1 require only one pulse, rather than five pulses, of 5HT for the induction of LTF ([Bartsch *et al.*, 1998]). If CREB1 were indeed regulated in vivo by miR-124, the in-

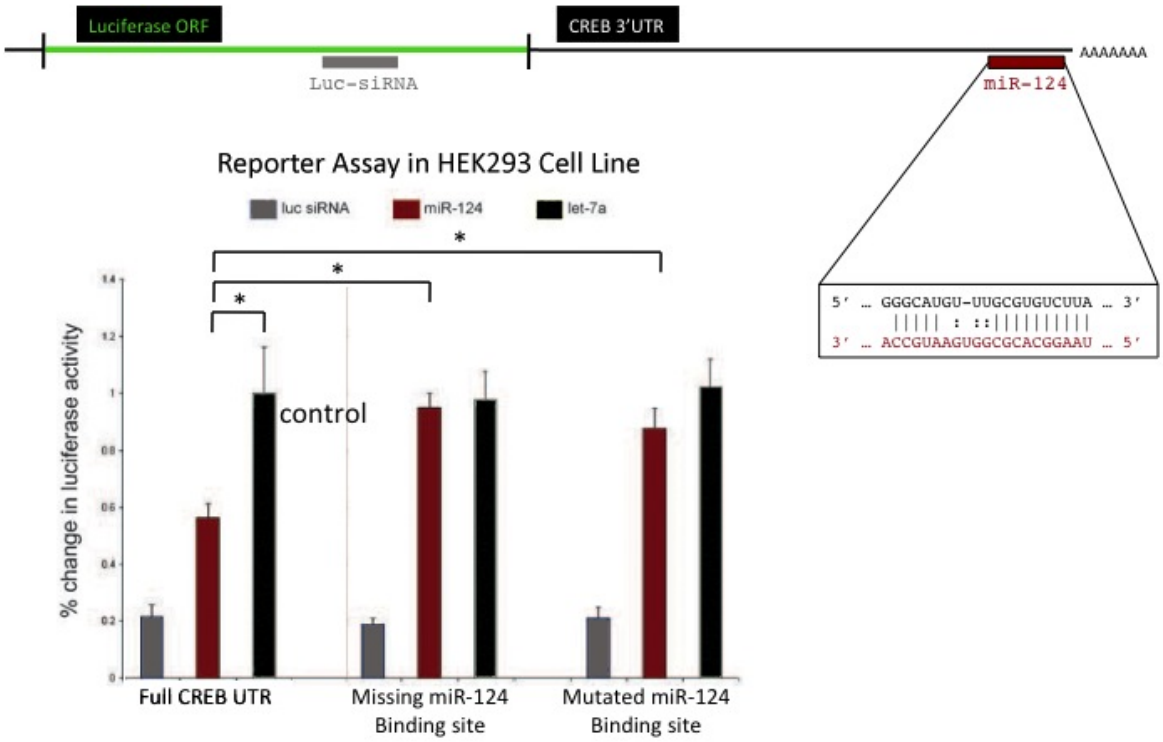

Figure 4.5: miR-124 directly binds and translationally represses CREB

A luciferase reporter (100ng) bearing the CREB UTR (full CREB UTR) is repressed by 45% when co-transfected with miR-124 duplex (5pmol) in HEK293 cells. The same reporter, when co-transfected with let-7, shows no significant change in expression levels. Luciferase reporters bearing the CREB UTR with a 2nt mutation in the miR-124 binding site (mutated CREB UTR), and a truncated CREB UTR that is missing the entire miR-124 binding site (truncated CREB UTR) are not significantly affected by co-transfection with miR-124 duplexes. An siRNA directed against the luciferase firefly gene (luc siRNA), a positive control, was able to repress all constructs containing the firefly gene by 80%. Each data point is expressed as a ratio of renilla to firefly activity, normalized to the change in luciferase activity when plasmids are transfected alone without miR duplexes. Data are shown as a mean of 8 independent trials ± S.D.

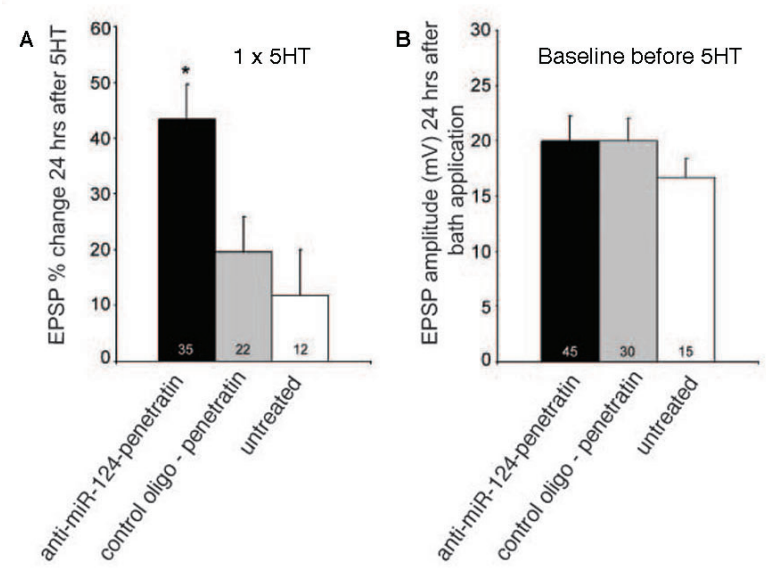

Figure 4.6: miR-124 knockdown cause LTF with Just 1 pulse of serotonin

Average percentage synaptic facilitation measured at 24 hrs after treatment with a single pulse of serotonin in cultures that had been pre-incubated with either the penetratin-conjugated miR-124 inhibitor or the control miR-194 inhibitor, as well as of untreated controls. The observed differences in facilitation between groups were not due to differences in the basal strength of synapses.

hibition of miR-124 in sensory neurons, through its enhancement of CREB1, should require fewer pulses of 5HT to cause LTF. Indeed, in response to even a single pulse of 5-HT cells treated with miR-124 inhibitor showed a significant level of facilitation after 24 hrs (+42.66 ± 6.18, n=35; comparable to that observed with CREB over-expression in [Bartsch *et al.*, 1998]) with respect to a control miR-194 inhibitor (+20.57 ± 6.37, n=22, $p < 0.04$, Newman-Keuls post-hoc test after two-way ANOVA; Figure 4.6A) and with respect to control cells treated with vehicle alone (+11.77 ± 8.18, n=12, $p < 0.01$, Newman-Keuls post-hoc test after one-way ANOVA). The observed differences in the facilitation between treated and untreated groups were not due to differences in the basal strength of the synaptic connections as tested before 5HT application (Figure 4.6B). Together with the previous observations, these data support the idea that the 5-HT-dependent down-regulation of miR-124, by allowing an increase in the levels of CREB and CREB-dependent transcription, is an important component of a switch that converts short-term to long-term synaptic plasticity.

4.2.3 miR-22 constrains long-term synaptic facilitation through CPEB

After miR-124, the second most highly abundant neuronal miRNA in *Aplysia* is miR-22 and the results described below are obtained through joint efforts from myself and my collaborator, Ferdinando Fiumara. From my initial screen on localization and responsiveness to 5HT, in Chapter 3, I found that miR-22 is brain specific (3.4), rapidly down-regulated by 5HT (3.6), and preferentially expressed in the sensory neurons of a the sensory-motor synapse of *Aplysia* (4.7).

To determine whether it has a role in the functional regulation of synaptic plasticity, Ferdinando again performed electrophysiological experiments by perturbing miR-22 levels in sensory neurons and determining the resulting change in synaptic efficacy. Over-expression of miR-22, like that of miR-124, produced approximately 50% reduction in long-term facilitation as observed at both 24 h and 48 h after 5HT, while knockdown of miR-22 enhanced LTF by almost 2-fold (4.8).

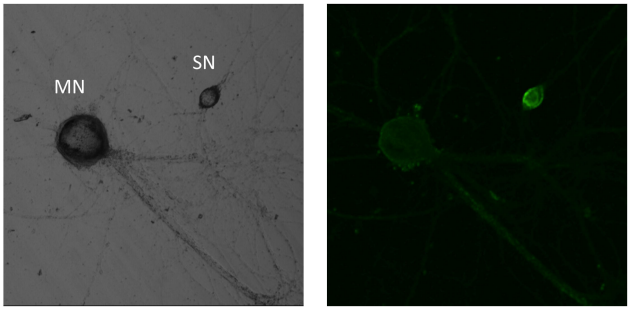

Figure 4.7: aca-miR-22 is preferentially present in sensory neurons of a sensory-motor synapse

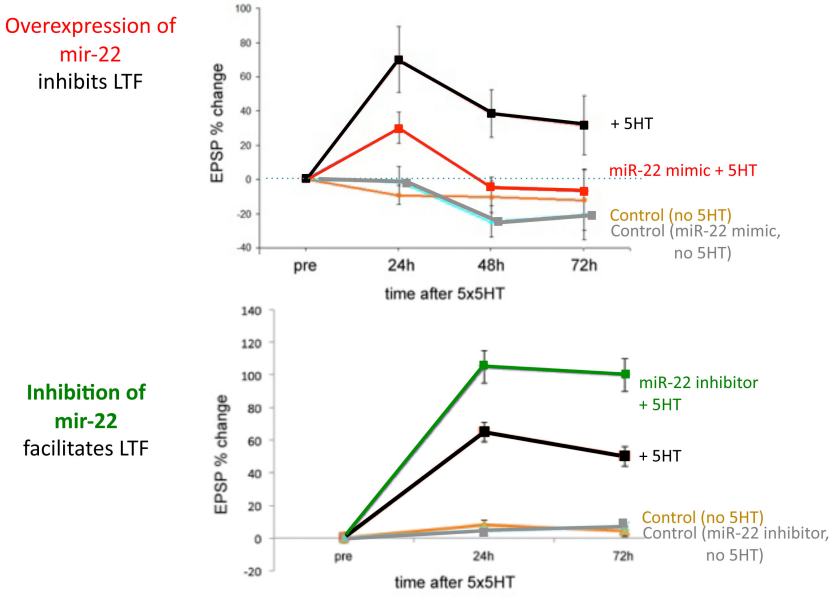

Figure 4.8: aca-miR-22 constrains serotonin-induced long term facilitation

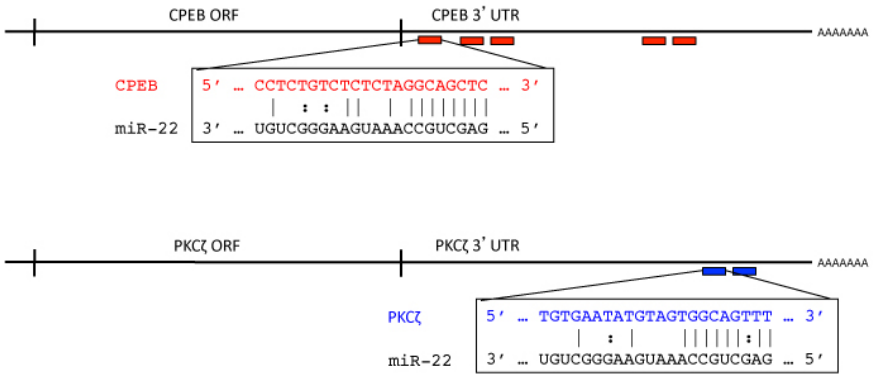

Figure 4.9: miR-22 has putative binding sites on the 3'UTR of CPEB and PKCζ

I next wanted to address what its potential targets may be, and began by searching the 3'UTR of plasticity-related genes in *Aplysia* for cognate binding sites for miR-22. I found, unexpectedly, that both CPEB (Cytoplasmic Polyadenylation Element Binding Protein) and PKCζ (Protein Kinase C - zeta) had several predicted miR-22 binding sites, with at least one in each case being a strong candidate (4.9). The potential regulation of both CPEB and PKCζ by miR-22 is of great significance for several reasons: Kausik Si and Eric Kandel first described in 2003 that the local translation factor, CPEB, has prion-like properties and that its ability to be self-sustaining and persistently active could form the basis for the maintenance of long-term memories ([Si *et al.*, 2003a]). Independent work from the Saktor lab described another persistently active molecule, PKCζ, in the maintenance of long-term memories ([Serrano *et al.*, 2008]). As an integration of the two aforementioned studies, recent work in the Kandel lab suggests that CPEB and PKCζ mutually activate each other in a persistently active feedback loop that may be responsible for maintaining long-lasting memories. The potential regulation of both CPEB and PKCζ by miR-22, therefore, would provide further independent evidence that the two molecules do indeed interact in a self-sustaining feedback unit.

To experimentally confirm that miR-22 functionally regulates CPEB and PKCζ, I incubated de-sheathed pleural ganglia in inhibitors of miR-22, or control inhibitors (miR-25), or left ganglia completely untreated, and after 24 h harvested protein from all samples and

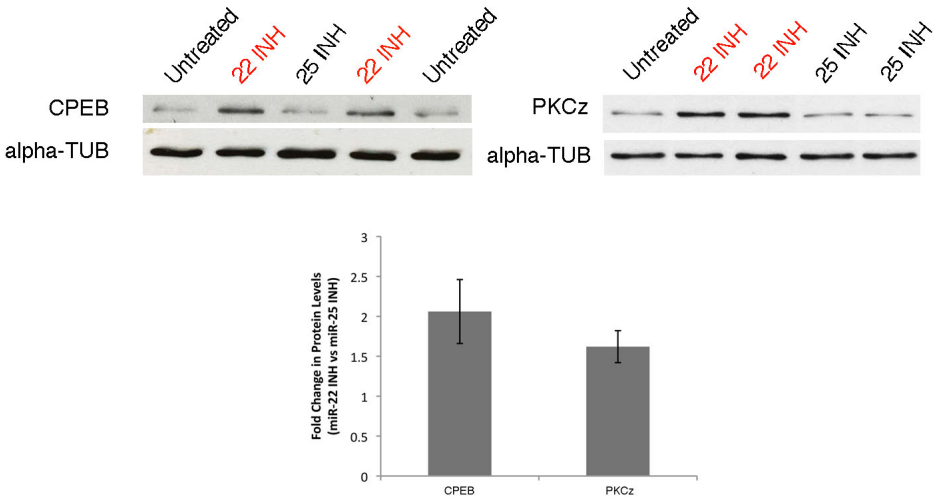

Figure 4.10: Inhibition of miR-22 increases both CPEB and PKCζ expression levels

western blotted them. In samples treated with miR-22 inhibitors, there was a consistent up-regulation of CPEB and PKCζ levels as compared with miR-25 treated samples and with untreated samples (4.10).

The coordinated regulation of both CPEB and PKCζ by miR-22 could be a result of miR-22 directly targeting each individually, or by miR-22 targeting one directly and affecting the other indirectly. Experiments in the lab are currently underway to distinguish between these two possibilities. Further investigations of the regulatory role of miR-22 could highlight the role of both CPEB and PKCζ in functioning as a self-sustaining molecular circuit at synapses that drives the maintenance of memories.

4.3 Discussion

I find that both miR-124 and miR-22 serve as negative constraints on serotonin-induced long-term facilitation, since increased or decreased miRNA levels in sensory neurons leads to a significant inhibition or enhancement respectively of synaptic facilitation. In particular, the inhibition of miR-124 confers to sensori-motor synapses a greater sensitivity for serotonin,

since just one pulse of serotonin is sufficient to cause long-term facilitation. These physiology data also suggest that miR-124 inhibition is just one of many 5HT-mediated events that activates CREB to induce long-term facilitation, since the inhibition of miR-124 alone, in the absence of 5HT, does not lead to long-term facilitation. Therefore, while the observed effects of the miR-124 manipulations on LTF are of a significant magnitude, it is likely that these effects would be even greater if there were a coordinated manipulation of several miRNAs that act together in parallel pathways during synaptic plasticity. The observation that miR-124 and miR-22 levels affect facilitation both at 24 and 48 hrs after exposure to spaced pulses of serotonin suggests that miR-124 and miR-22 regulation are required not only for the induction phase, but that they are also critical for the maintenance phase of synaptic facilitation. Since miR-124 and miR-22 levels return back to baseline within 12 hours after exposure to serotonin, the initial drop in miRNA levels during this time window appears to be sufficient enough to up-regulate the relevant transcripts to allow for facilitation for up to 48 hours after exposure to serotonin. Indeed, the up-regulation of many plasticity related transcripts are transient and fall into this initial time-window. The data also suggest that miR-124 and miR-22 do not significantly affect or contribute to serotonin-independent processes such as basal and constitutive synaptic activity. However, since all of our experiments were conducted on several day old cultures, at which point the cells and synapses are fully mature and stable, our studies leave open the possibility that miR-124 and miR-22 contribute to serotonin-independent processes in immature neurons such as neurite out-growth and synapse formation.

The negative constraint that miR-124 imposes on synaptic facilitation is mediated, at least in part, by its direct regulation of CREB. The fact that miR-124 inhibition significantly and specifically increases CREB1 levels, along with immediate downstream genes such as UCH, C/EBP and KHC, that miR-124 serotonin kinetics parallels the CREB1 serotonin kinetics, and that miR-124 inhibition can provide the switch necessary to convert short-term facilitation into long-term facilitation, all strongly support the conclusion that miR-124 can tightly control CREB and CREB-mediated signaling during plasticity. CREB has been

extensively studied over the years for its regulation by kinase dependent post-translational modifications, such as phosphorylation by PKA and MAPK. The present study, however, is one of the first to address post-transcriptional regulation of CREB. While this additional level of regulation might appear redundant, for example by paralleling the function of CREB2, it is likely that miR-124 inhibition allows for more rapid and transient control over CREB expression, as well as the opportunity for CREB to be drawn into various distinct downstream pathways once activated. I also noticed that CREB, in turn, may be able to regulate miR-124 expression levels since there are several putative CREB binding sites in the presumed promoter region upstream of the *Aplysia* mir-124 gene (Supplementary Figure 6). Although *Aplysia* and mammalian systems have clear differences in the complexities of their CNS, and also even in the types of neurotransmitters used during long-term memory processes, the underlying calcium induced signaling pathways (including cAMP, PKA, MAPK, and CREB) and their functions are very much shared (reviewed by [Kandel, 2001]). It is therefore very likely that miR-124 is activity-regulated in the mammalian hippocampus, and regulates CREB in much the same way as observed here, especially in light of the fact that the mammalian CREB1 UTR bears a conserved miR-124 target site as predicted by targetscan ([Lewis *et al.*, 2003]), which was recently confirmed as a site directly bound by Argonaute in mouse brain ([Chi *et al.*, 2009]).

In summary, I have identified a comprehensive set of brain-enriched miRNAs in *Aplysia*, many of which can be regulated by the neuromodulator serotonin, signifying potential roles in learning-related synaptic plasticity. Specifically, I demonstrated that brain-specific miR-124 and miR-22 respond to serotonin by de-repressing CREB and CPEB respectively and enhancing serotonin-dependent long-term facilitation. This initial study compels the exploration of how neuromodulators act through small RNAs during various forms of plasticity and whether some act locally at synapses. The likelihood of a coordinated set of miRNAs combinatorially regulating events at the synapse makes possible a new and rich layer of computational complexity that could be responsible for the emergence of discrete and long-lasting states of activity at the synapse.

Chapter 5

Identification and Functional Analysis of piRNAs as Epigenetic Regulators of Synaptic Plasticity

5.1 Introduction

The lifetime of many human memories run on the order of years, whereas the RNA or protein molecules that may subserve these memory traces are thought to turnover on the order of days ([Price *et al.*, 2010]). Several hypotheses have been proposed to explain how memories can remain stable in the face of constant molecular turnover: 1. Prion-like proteins at synapses can adopt active, stable, and self-perpetuating conformations that preclude turnover of the protein ([Si *et al.*, 2003a; Bailey *et al.*, 2004; Si *et al.*, 2010]), 2. Auto-regulatory and positive feedback loops within protein networks can allow persistent enzymatic activity of proteins or newly synthesized protein to take the place of existing protein machinery without loss in state and function ([Lisman, 1985; Lisman *et al.*, 2002; Hayer and Bhalla, 2005; Sacktor, 2008]), and 3. Perhaps most tantalizing, is the possibility that epigenetic mechanisms such DNA methylation can alter gene-expression and thus the intrinsic properties of neurons in a long-term fashion, perhaps on the order of years ([Crick,

1984; Davis and Squire, ; Weaver *et al.*, 2004; Miller and Sweatt, 2007; Miller *et al.*, 2010; Feng *et al.*, 2010]).

Small regulatory non-coding RNAs have been found to effect long-lasting changes in cellular phenotypes during development, through both their involvement in auto-regulatory feedback loops ([Hobert, 2008; Rybak *et al.*, 2008; Krol *et al.*, 2010b]), as well as in the transcriptional and epigenetic regulation of gene-expression ([Wassenegger, 2005; Yin and Lin, 2007; Saito and Siomi, 2010]). To better understand the regulatory roles of miRNAs during long-term memory, I previously generated a miRNA library from the *Aplysia* central nervous system and characterized the role of a brain-specific miRNA, aca-miR-124, in constraining long-term synaptic facilitation through repression of the transcriptional activator CREB1 ([Rajasethupathy *et al.*, 2009]). In the process of mining and characterizing miRNAs from *Aplysia* CNS, my collaborator Tom Tuschl made the unexpected discovery of the existence of neuronally expressed piRNAs. piRNAs (piwi-interacting RNAs) are a class of 26-32 nt small RNAs that are thought to have germline restricted expression ([Aravin *et al.*, 2006; Girard *et al.*, 2006]). Unlike any other class of non-coding RNA, piRNAs are generated from long genomic clusters that are syntenic, but not necessarily sequence-conserved, across species ([Farazi *et al.*, 2008]). piRNAs stably associate with piwi proteins and while the function of the resulting RNP complex is still not fully understood, some studies point to a possible role in the epigenetic regulation of transposable elements in the germline through de-novo DNA methylation ([Aravin *et al.*, 2007; Brennecke *et al.*, 2008; Kuramochi-Miyagawa *et al.*, 2008]). Specifically, mice lacking one or more of its piwi homologs were shown to have substantial de-methylation and de-repression of transposable elements targeted by germline piRNAs.

Here we find that *Aplysia* piRNAs are broadly expressed outside of the ovotestes, and are amply present in neurons. These piRNAs are abundant, have unique biogenesis patterns, associate with a neuronal piwi protein, and are distinctly regulated by neuromodulators important for learning and memory. By analogy to their role in germline, we find that the piwi/piRNA complex in neurons can methylate target genes, but in this case it targets a

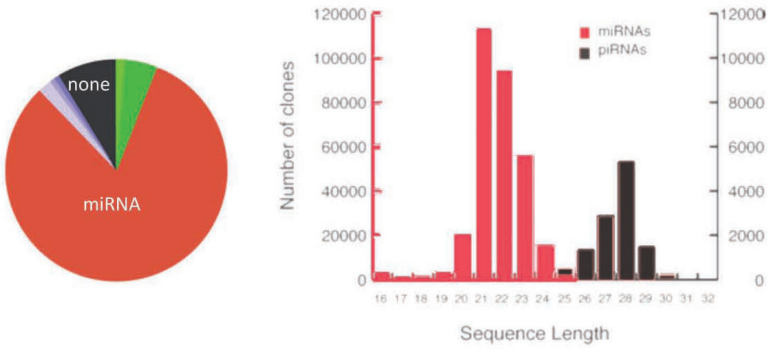

Figure 5.1: Two classes of small RNAs in *Aplysia* CNS
A size histogram of the cloned small RNAs revealed two populations, and further characterization confirmed the new class of sequences (shown in black) to be piRNAs.

critical plasticity-related gene and transcriptional repressor of memory, CREB2 ([Bartsch *et al.*, 1995]), and methylates its promoter by first accessing its nascent transcript. The DNA methylation of CREB2 by the piwi/piRNA complex provides a mechanism by which transient external stimuli can cause long lasting changes in the gene-expression of neurons involved in long-term memory storage.

5.2 Results and Discussion

5.2.1 Identification of neuronal piRNAs in *Aplysia* that stably associate with Piwi in nuclear compartments.

My previous generation of a small RNA library from *Aplysia* CNS resulted in the majority of read sequences being mapped as miRNAs, with a minority of reads (20 %) that mapped to the *Aplysia* genome but could not be annotated (Figure 5.1).

Further examination of these non-annotated small RNA sequences revealed the unexpected presence in brain of another distinct class of small RNAs characterized by a pre-

dominant length of 28 nt and a strong preference for 5' U (Figure 5.1 5.2B). When these sequences were mapped to the unassembled genome trace files followed by the assembly of larger contigs comprising these regions, my collaborator Rob Sheridan identified clusters containing additional sequences with the same features, revealing a pattern characteristic of mammalian piRNAs (see methods for further annotation details). We identified 372 distinct piRNA clusters (Scaffold coordinates given in Supplementary Table 5), of which a portion of one representative cluster is shown (Figure 5.2B). *Aplysia* piRNAs exhibit unusual biogenesis patterns (Betel et al. 2007, Kim et al. 2009) in that within a cluster of piRNA reads, one or a few individual piRNAs were cloned hundreds of times more frequently than surrounding piRNAs in the same cluster (Figure 5.2B). This piRNA biogenesis pattern leads to an accumulation of specific piRNAs similar in read frequencies to miRNAs (Supplementary Table 6). To more comprehensively survey piRNA expression in the *Aplysia* CNS, as well as other tissues, both in the juvenile animal as well as in the adult, my collaborator Sebastian Frey generated anew 10 different small RNA cDNA libraries using barcoded adapters and subjected the libraries to deep sequencing using the Illumina platform. Of the sequences that were annotated, the piRNA content per library averaged 15 %, compared with the miRNA content which averaged 60 % (Supplementary Tables 4 and 5).

Because piRNAs are preferentially expressed in germline cells in both vertebrates and invertebrates, I anticipated gonad-specific expression in *Aplysia*. Although I found the overall piRNA content (and piRNA to miRNA ratio) to be highest in the ovotestes (Supplementary Table 4), there are several abundant piRNAs that are selectively enriched in the CNS (Figure 5.2C). To confirm the cloning data, abundant piRNAs originating from 2 distinct clusters were analyzed by quantitative northern blots and detected in brain, as well as in ovotestis and heart, but to a lesser extent in other organs such as muscle or hepatopancreas (Figure 5.3).

Consistent with piRNA expression in the CNS, I was also able to clone the full-length cDNA for the 964 kDa Piwi protein from the CNS. The sequences of these clones are homologous to vertebrate piwi proteins and have conserved PAZ and Piwi domains. The *Aplysia*

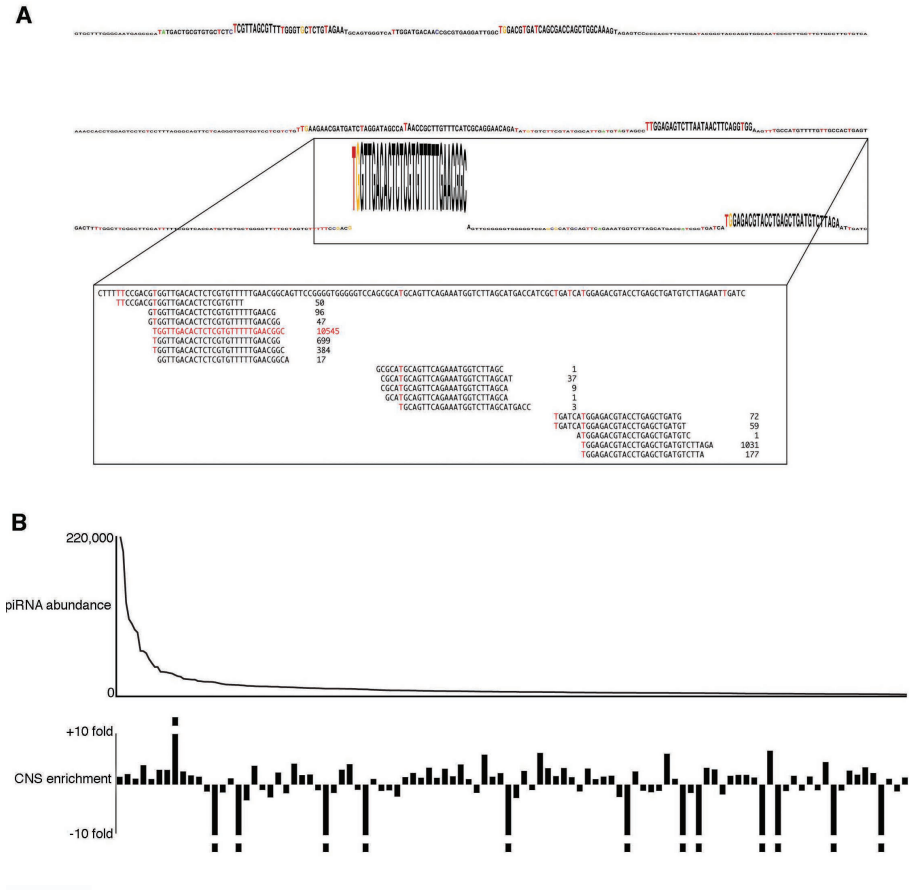

Figure 5.2: One representative piRNA cluster and enrichment of piRNAs in CNS

A. One continuous genomic region of *Aplysia* encoding a piRNA cluster. A representative 600 bp region within the full 21 kilobase cluster is shown here. This cluster generates several thousand piRNAs, with one piRNA peak region cloned orders of magnitude more abundantly than the surrounding piRNAs as reflected in this diagram where the height of the nucleotide bases is proportional to its clone frequency. The clones mapping to the peak piRNA are shown in the inset and U(T) bias start sites are indicated in red. B. The top 100 piRNAs are plotted on the x-axis in decreasing order of abundance, and their enrichment in CNS is shown as a positive deflection along the y-axis.

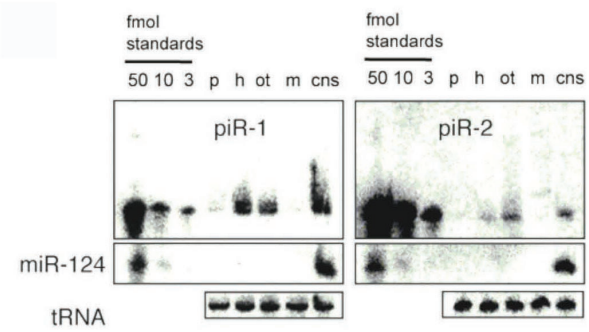

Figure 5.3: Presence of piRNAs in Brain as detected by Northern Blot

Two abundant piRNA sequences are shown probed by Northern blot. These two piRNAs are reflective of several of the abundant piRNAs that are detected in brain (cns), but also in ovotestis (ot) and heart (h). Detection of synthetic piRNAs loaded on the far left of the blots, at a concentration of 50 fmol, 10 fmol, and 3 fmol serve as positive controls and allow quantitation. A re-probing of aca-miR-124 showing brain specificity is shown for each blot, and finally a re-probing of tRNA to control for equal loading of samples.

piwi protein is much more closely related to piwi proteins, by homology, than to argonaute proteins of other species, and within the piwi family, more closely related to vertebrate than invertebrate piwis (Figure 5.4A), as is often the case with *Aplysia* proteins. I generated a polyclonal antibody for the *Aplysia* piwi protein that detects the induced recombinant protein as well as the protein in *Aplysia* neural extracts as a single band (Figure 5.4B). To determine whether that the piwi protein stably interacts with piRNAs, I immunoprecipitated piwi from neural extracts (Figure 5.4C) and extracted the RNAs from the piwi complex. When blots of the RNAs from the Piwi IP and Argonaute (Ago) IP were probed for a piRNA (aca-piR-1) and miRNA (aca-miR-22), the piRNA was detected only in piwi immunoprecipitates whereas the miRNA was detected only in Ago immunoprecipitates (Figure 5.4D). I further found that RNA from neural extracts after piwi knockdown (with 2-O-methyl antisense oligoribonucleotides) are depleted in piRNAs when compared to control extracts, with no detectable change in the levels of other non-coding RNAs, such as miRNAs, or tRNA (Figure 5.4E). These experiments demonstrate that there are indeed two distinct classes of small RNAs in *Aplysia* CNS, miRNAs and piRNAs, each of which associates with its respective Ago and Piwi protein.

To better understand the sub-cellular localization of piwi and piRNAs in *Aplysia*, I first separated neural protein and RNA extracts into nuclear and cytoplasmic fractions, and probed for the piwi protein on western blots and piRNAs on northern blots. I loaded equal volume of nuclear and cytoplasmic fractions to reflect their proportionate volumes in the cell. Effective fractionation was confirmed by the presence of GAPDH only in cytoplasmic compartments, and Histone H3 in nuclear compartments. I detected the Piwi protein primarily in the nuclear compartment (Figure 5.5B). Consistent with this finding, over-expression of GFP tagged piwi in *Aplysia* sensory neurons shows a predominant nuclear localization of the piwi protein (Figure 5.5A). A northern blot comparing small RNA content in the nuclear and cytoplasmic fractions with total unfractioned RNA also revealed that the piRNAs are primarily nuclear, whereas the miRNAs are primarily cytoplasmic (Figure 5.5C). Taken together, both piwi and piRNAs in *Aplysia* neurons have predominant nuclear localization

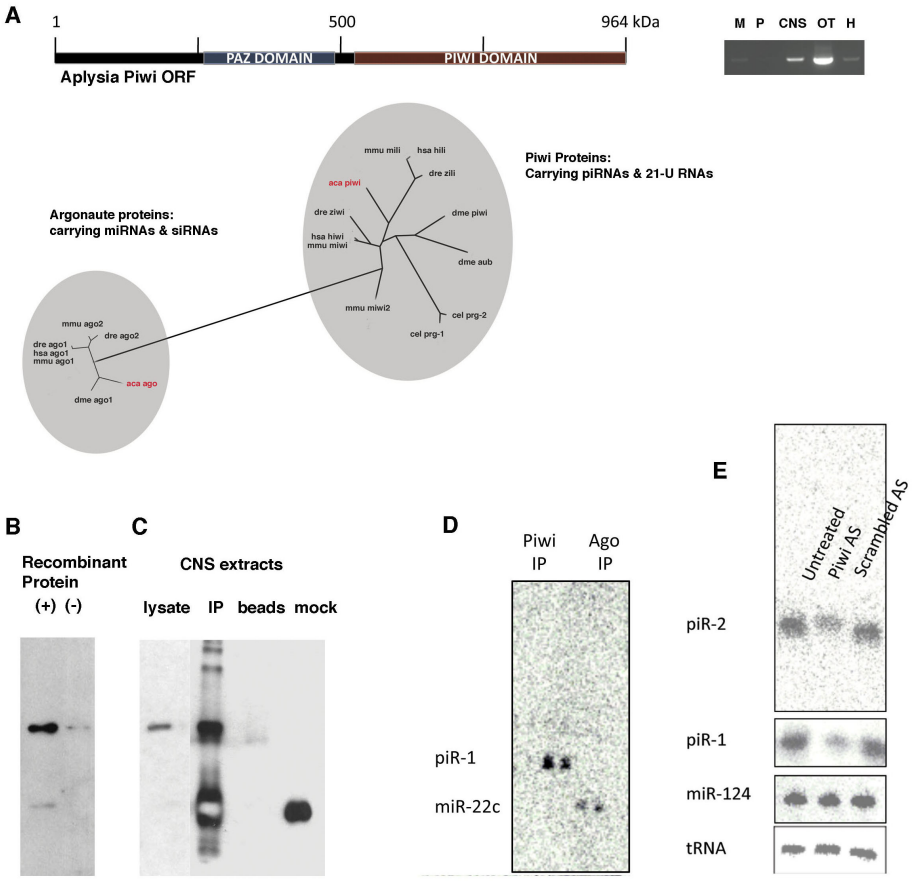

Figure 5.4: Apysia Piwi is neuronally expressed and stably associates with piRNAs A. The full length 964 kDa *Aplysia* piwi has conserved PAZ and PIWI domains, and clusters more closely with piwi than argonaute proteins of other species. B. A polyclonal antibody generated against the C-terminal of the *Aplysia* piwi protein recognizes induced recombinant protein at 130 kDa. C. The antibody also recognizes piwi protein from *Aplysia* neural extracts and specifically IPs the protein as a single band at 130 kDa. D. RNA from piwi IP and ago IP were northern blotted and probed for a piRNA and a miRNA. The piRNA is only detected in the piwi IP while the miRNA is only detected in the argonaute IP. E. *Aplysia* sensory neurons were treated with piwi antisense, scrambled oligoribonucleotides, or left untreated, and the total RNA was extracted, northern blotted, and probed for specific small RNAs. The piwi knockdown samples had a specific depletion in piRNAs but no change in miRNA.

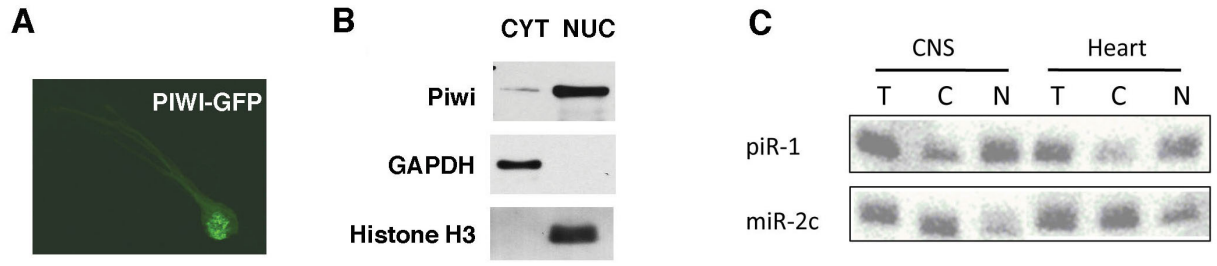

Figure 5.5: *Aplysia* piwi is neuronally expressed

A. Over-expression of *Aplysia* Piwi protein with GFP tagged at the C-terminus reveals a nuclear localization of piwi in sensory neurons. B. Nuclear (NUC)/ Cytoplasmic (CYT) fractionation of neuronal proteins followed by western blot revealed a nuclear localization for piwi protein. GAPDH is detected only in the cytoplasmic fraction and Histone H3 is detected only in the nuclear fraction, confirming effectiveness fractionation. C. Nuclear (N) / Cytoplasmic (C) fractionation of total (T) RNA followed by northern blot revealed a nuclear enrichment of piRNA, compared with a cytoplasmic enrichment of miRNA.

suggesting a nuclear function for the piwi/piRNA complex.

5.2.2 Piwi/piRNA complexes enhance memory-related synaptic plasticity by regulating the transcriptional repressor, CREB2.

To determine whether piRNAs have a regulatory role in memory-related synaptic plasticity, I screened some of the abundant neuronal piRNAs for changes in expression levels upon exposure to serotonin (5HT), a neuromodulator important for learning and memory. A subset of the selected piRNAs was significantly up-regulated (Figure 5.6). aca-piR-4 and aca-piR-15 are examples of piRNAs that were robustly induced by 5HT. The former was transiently induced while the latter had a more delayed but persistent activation. The increase in piRNA expression in response to 5HT was particularly interesting in comparison with the activity of miRNAs, which by contrast, were rapidly down-regulated in neurons

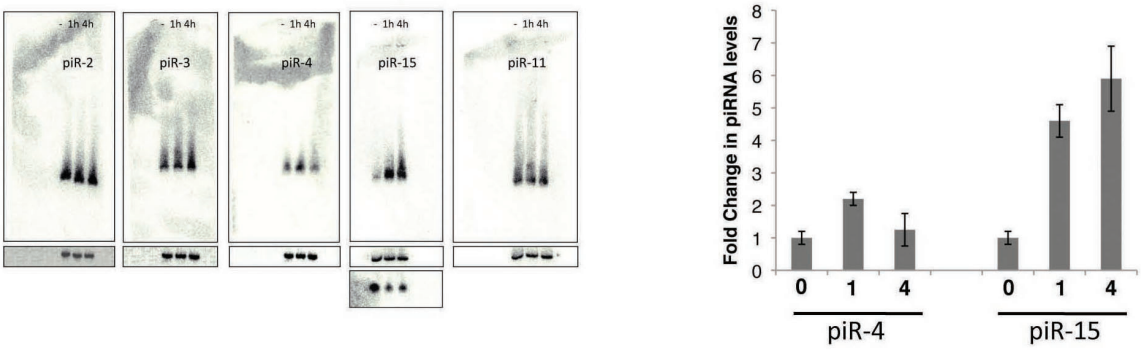

Figure 5.6: piRNAs are up-regulated by serotonin

A. *Aplysia* CNS were treated either with vehicle (-) or with 5 x 5HT and RNA was extracted 1 h and 4 h later and northern blotted. Some piRNAs had no response to 5HT (aca-piR-2, -3, -11) wile those that did were uniformly up-regulated by 5HT (the two shown here are aca-piR-4, and -15). The miRNAs (aca-miR-124 shown here), by contrast, are down-regulated by 5HT. tRNA bands are shown to control for equal loading of samples. Changes in piRNA levels are quantified in (B) and are presented as a mean of 4 independent trials ± S.D.

in response to neuromodulators and to neuronal activity, both in *Aplysia* as well as in mammalian systems ([Rajasethupathy *et al.*, 2009; Krol *et al.*, 2010a]). These observations suggest that the two classes of small RNAs in the *Aplysia* CNS could exercise coordinated bi-directional activity of their targets during memory-related synaptic plasticity.

To better understand the functional relevance of these 5HT-induced piRNAs, my collaborator Igor Antonov and I explored their role in memory-related synaptic plasticity in cultured neurons in response to 5HT. The co-cultures used in these experiments consisted of two sensory neurons that each synapse on a single target motor neuron. We first depleted piwi (and consequently its associated piRNA population) from sensory neurons that form synapses with motor neurons in culture, and assayed for changes in the strength of the sensory-motor synapse. We injected an antisense 2-O-methyl oligoribonucleotide to piwi in one sensory neuron of the co-culture while the other sensory neuron was left unmodified as an internal control. In each case electrical activity was recorded in the motor neuron

after exposure to 5HT to determine the change in baseline synaptic transmission and in memory-related long-term facilitation (LTF) at these synapses. We found that knockdown of piwi significantly impaired long-term facilitation (LTF) as measured at 24 and 48 h after exposure to five pulses of 5HT (n=34), when compared with uninjected controls in the same co-culture (n=37; $F(3,95)=13.63$; $p<0.001$ repeated measures ANOVA; $p<0.02$ and $p<0.04$ at 24 and 48h, respectively, Newman-Keuls post hoc test; Figure 5.7A). The observed differences between the two groups were not due to differences in the basal strength of the synaptic connections. We confirmed the efficacy of piwi knockdown by western blotting (Figure 5.8A), and also confirmed that the piwi knockdown specifically prevented the accumulation of mature piRNAs (Figure 5.4E). Control experiments with the injection of scrambled antisense 2-O-methyl oligoribonucleotides did not show changes in LTF (n=23, scrambled AS vs n=9, 5x5-HT; $p > 0.05$ at both 24 and 48h, Newman-Keuls post hoc test; Figure 5.7B). We next determined whether over-expression of piwi had the opposite effect. Over-expression of Piwi-GFP (n=22) caused a significant enhancement of 5HT-dependent long-term synaptic facilitation with respect to untreated controls (n=40) as measured at 24 and 48 h ($F(2,78)=44.04$; $p<0.001$ repeated measures ANOVA; $p<0.001$ Newman-Keuls post hoc test at both 24 and 48h; Figure 5.7C). Taken together, we conclude that 5HT induces the activity of piwi-associated piRNAs, which in turn act to enhance LTF.

To identify genes through which piwi might act to enhance 5HT-dependent long-term facilitation, I screened many plasticity-related genes for changes in expression levels after knockdown of piwi. Desheathed pleural ganglia were incubated in antisense 2-O-methyl oligoribonucleotides conjugated with penetratin to inhibit piwi (confirmed by Western blot Figure 5.8A), and total protein was extracted and Western blots prepared and probed with specific antibodies. I found that inhibition of piwi led to a reproducible two-fold up-regulation of the transcriptional repressor, CREB2, when compared to neurons treated with scrambled control 2O-methyl oligoribonucleotides. This effect was specific to CREB2, as piwi inhibition had no effect on several other plasticity-related genes such as C/EBP and CPEB (Figure 5.8A). The observed increase in CREB2 protein levels was supported by an even

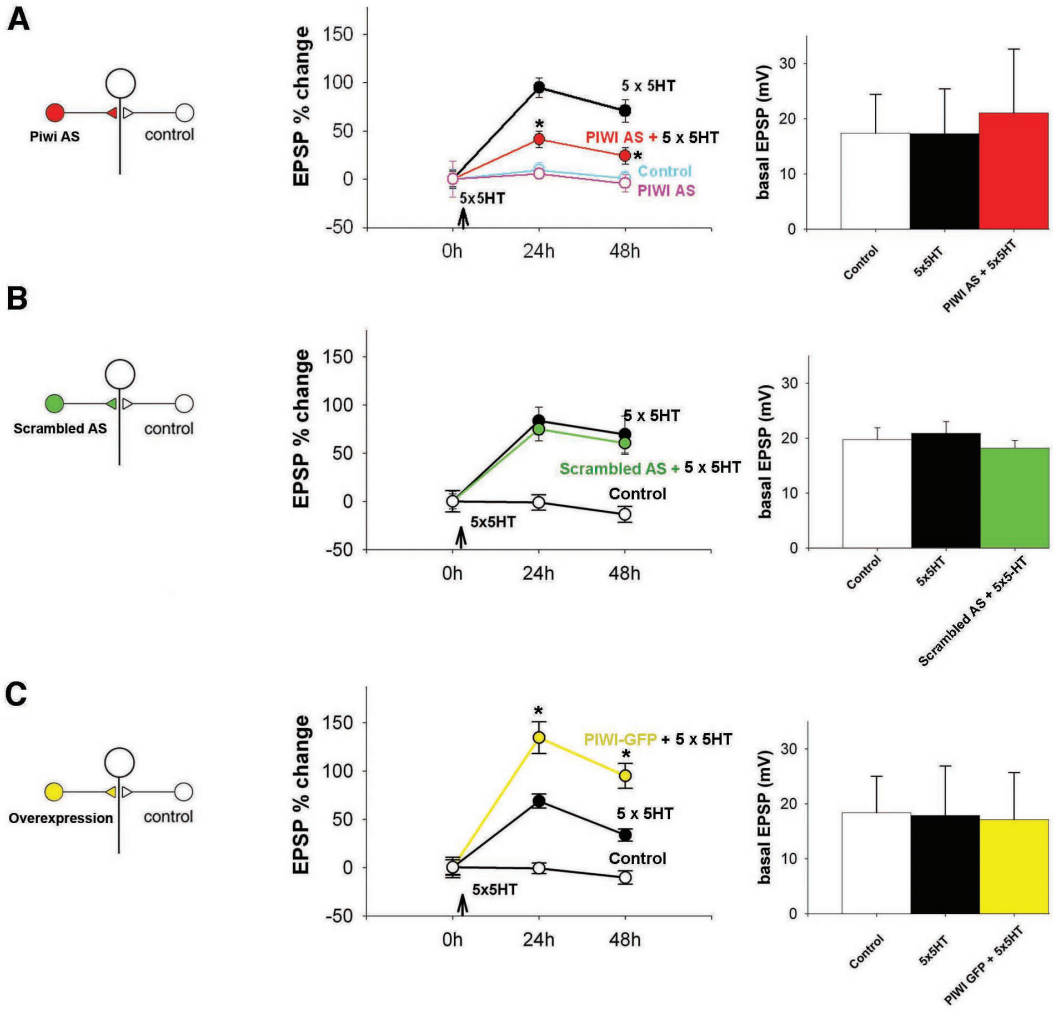

Figure 5.7: piwi enhances serotonin-dependent long-term synaptic facilitation

A, B, C. Graphs reporting the percentage change in EPSP amplitude measured at 24 hrs and 48 hrs after 5x5HT application with respect to pretreatment values in the different experimental groups. A Schematic representation is also shown for each experiment indicating which treatments were applied to the sensorimotor co-cultures for electrophysiological experiments. In each co-culture, one of the two sensory neurons was injected either with 5 M piwi antisense (C), scrambled negative control (D), or piwi-GFP (E), whereas the other sensory neuron was left untreated as a control. In all cases, the observed changes in LTF are not related to differences in basal synaptic strength.

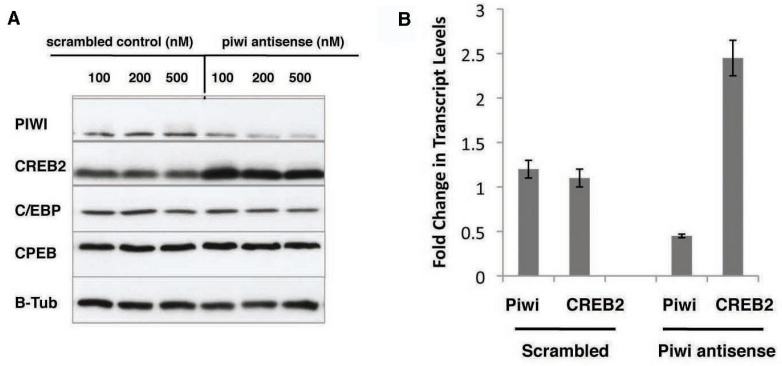

Figure 5.8: piwi regulates CREB2 expression levels transcriptionally

A. *Aplysia* sensory neurons incubated with an oligoribonucleotide antisense to piwi and linked to a diffusible peptide penetration show significant reduction in piwi protein levels when compared with neurons incubated with scrambled control oligoribonucleotides. Knockdown of piwi causes a robust up-regulation of CREB2, which is specific as there is no significant change in expression levels of C/EBP or CPEB (effects quantified as a mean of 4 independent trials ± S.D). B. Real Time PCR experiments show that knockdown of piwi produces a significant increase in CREB2 RNA levels suggesting that piwi regulates CREB2 transcriptionally.

greater increase in CREB2 mRNA levels (Figure 5.8B). Thus, the piwi-dependent increase in CREB2 protein appears to result from action either at the level of RNA transcription or mRNA stability. Our earlier observation that piwi is found primarily in the nucleus suggests that it may act at the level of transcription.

5.2.3 CREB2 is methylated at its promoter in response to 5HT induced synaptic plasticity

To gain insight into the mechanism of CREB2 regulation by piwi, I asked whether 5HT acts on CREB2 at the level of transcription. Earlier studies followed CREB2 expression levels up to 3-4 h after exposure to 5HT, and in this time-frame, no change in CREB2 was noted

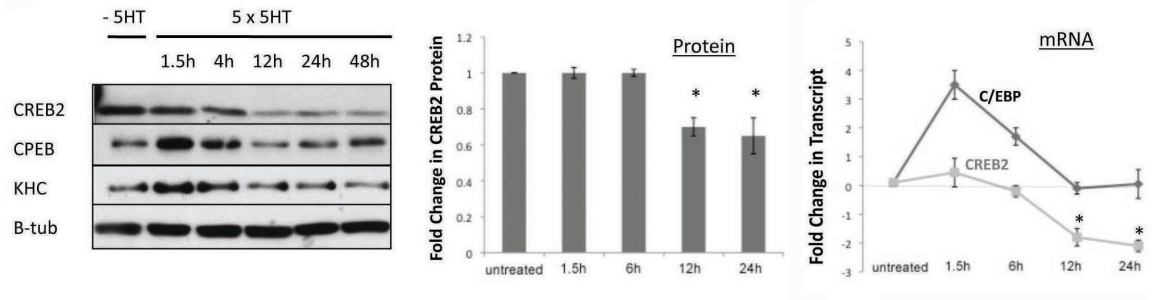

Figure 5.9: serotonin causes a persistent depression in CREB2 mRNA and protein levels A. *Aplysia* sensory neurons were either treated with vehicle or 5HT and protein was subsequently extracted at 1.5, 4, 12, 24, and 48 hours after 5HT. CREB2 levels drop at 12 hours and are persistently depressed even 48 h afterward without returning to baseline. Re-probing for CPEB and KHC (immediate early genes) shows a transient up-regulation with a return to baseline. Probing for tubulin is shown to control for equal loading of samples. This exact time course was run only once, but a similar time course is shown quantified in the next panel as a mean of 3 independent trials ± S.D. B. Real time PCR experiments showing that CREB2 RNA levels are persistently and even more robustly down-regulated after exposure to 5HT. The 5HT-dependent early induction of C/EBP mRNA (a known immediate early gene) from the same preparation is shown as a positive control.

([Bartsch *et al.*, 1995]). I therefore monitored the levels of CREB2 for days after the initial exposure to 5HT and noticed that CREB2 protein levels begin to drop at 12 h and continue to remain low for up to 48 h with no rebound to the initial base line level of expression (Figure 5.9A). At the protein level, the reduction in CREB2 levels was modest, but the effect was more pronounced at the mRNA level (Figure 5.9B). This long-lasting effect on both the CREB2 protein and RNA levels indicates that a stable 5HT-dependent repressive state is established. Since piwi and piRNAs have known roles in epigenetic regulation in the germline through DNA methylation, I asked whether CREB2 also is being regulated by piwi through methylation at its promoter.

The *Aplysia* DNA methyltransferase (DNMT) is well expressed in neurons ([Moroz *et al.*,

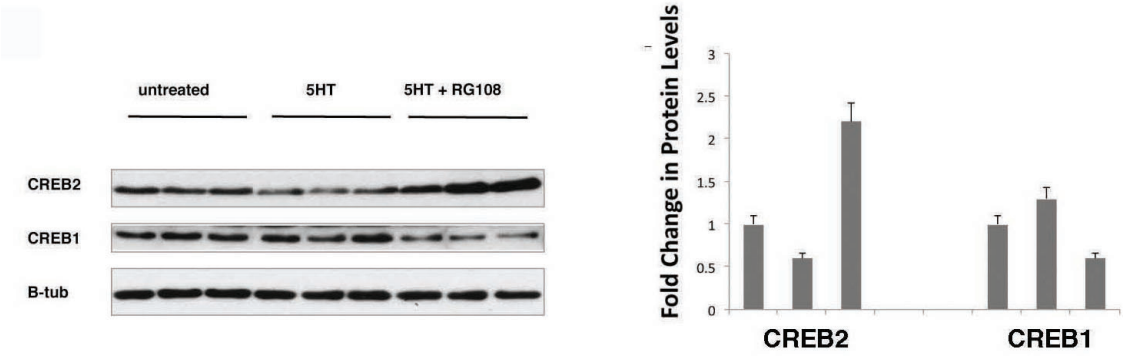

Figure 5.10: DNMT inhibitors prevent serotonin-induced down-regulation of CREB2 Three independent experiments each, of neurons treated with vehicle, 5HT, or 5HT in the presence of a DNA methyltransferase inhibitor (RG108) are shown where the proteins were extracted 12 h later and western blotted. CREB2 protein levels are significantly down-regulated in response to 5HT, and this effect is abolished and reversed in the presence of RG108. A concomitant opposite effect is seen for CREB1, where protein levels are decreased as would be expected from the antagonistic effects of CREB2.

2006; Moroz and Kohn, 2010]) and its enzymatically active domain is highly conserved among the vertebrate homologs of DNMTs. I therefore inhibited ApDNMT enzymatic activity chronically in neurons with the DNMT inhibitor RG108 and observed a strong increase in CREB2 levels. To determine if DNMT activity on CREB2 was dependent on 5HT, I applied RG108 to neurons in the presence of 5HT and found that 12 h later, the 5HT-dependent persistent down-regulation of CREB2 was abolished (Figure 5.10).

These effects of RG108 appear to be specific to CREB2 since there was no significant up-regulation of CREB1 levels. In fact, a modest down-regulation was apparent. To determine whether the effects of DNMT inhibition on CREB2 levels were functionally important during memory-related plasticity, Igor again performed electrophysiological experiments on sensory-motor co-cultures, in the absence and presence of RG108. Remarkably, bath application of the inhibitor RG108 (n=38) almost fully abolished 5HT-dependent long-term facilitation with respect to controls (n=37), as measured at both 24 and 48 h ($F(3,100)=12.86$; $p<0.001$

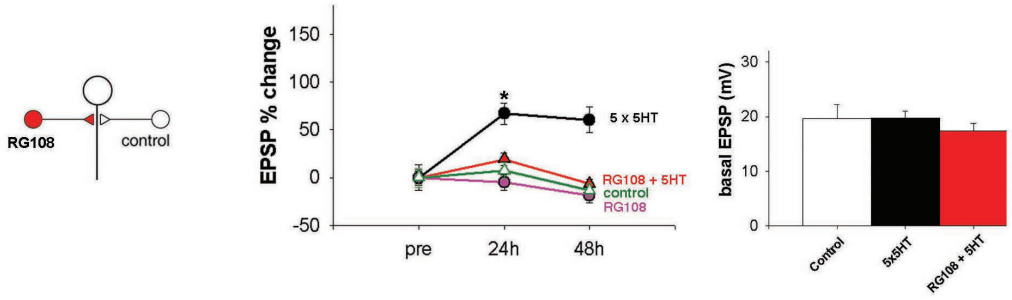

Figure 5.11: DNMT inhibitors abolish serotonin-dependent synaptic facilitation Electrophysiology experiment reporting percentage change in EPSP amplitude measured at 24 h and 48 h after 5x5HT with respect to pre-treatment values for neurons treated with RG108, as compared to control population. The inhibitor was confirmed to not be toxic to the cells as application of the inhibitor alone in the absence of 5HT had no effect on the baseline strength of the synapses.

repeated measures ANOVA; $p<0.03$ and $p<0.02$ at 24 and 48h, respectively, Newman-Keuls post hoc test; Figure 5.11). The effect of RG108 was entirely dependent on 5HT, as the application of RG108 alone, in the absence of 5HT had no effect on the baseline activity of the cells (Figure 5.11).

To determine if DNMT acted indirectly on CREB2 or whether it directly methylated the promoter of CREB2, I examined the promoter region of CREB2 for possible CpG islands. I found two predicted CpG islands, one that is proximal to the translational start site (200 bp upstream of the first ATG) and which encompasses a CRE-binding elment and TATA-binding site, and the other that is distal (700 bp upstream of the first ATG) (Figure 5.12A). I also noticed that the promoter of ATF4, the human homolog of CREB2, also contains a conserved CpG island (http://genome.ucsc.edu). To test whether either of the predicted CpG island was functional, I extracted genomic DNA and treated it with bisulfite. This procedure allows recognition of methylated bases in DNA ([Callinan and AP, 2006]), because bisulfite converts all genomic cytosine residues to uridine excepting the methylated cytosines, which are inert to bisulfite treatment. By scoring the C to T conversion rates of genomic

DNA after bisulfite treatment, one can determine the fraction of DNA that exists in the methylated versus un-methylated state. I first asked whether methylation specific primers (MSP -designed to detect only the methylated copies of genomic CREB2) have a differential ability to amplify genomic DNA from cells that either have, or have not, been treated with 5HT. I found that exposure to 5HT dramatically increases the methylated fraction of the proximal CpG island but not the distal CpG island (Figure 5.12A). I next designed both USPs (un-methylated specific primers, designed to detect only the unmethylated copies of genomic DNA) and MSPs for the promoter regions of CREB2 and CREB1 to compare the fractional representation of the methylated and unmethylated states of the CpG islands at baseline and after exposure to 5HT. I found that in the basal state the CREB2 promoter exists in both methylated and un-methylated forms, but 12 h after exposure to 5HT, the promoter is almost entirely in the methylated form, and in the presence of DNMT inhibitors, the promoter is almost entirely in the un-methylated form (Figure 5.12B). This pattern of methylation of the CREB2 promoter is in direct contrast to the CREB1 promoter, which exists almost entirely in the un-methylated form at baseline, remains un-methylated after exposure to 5HT, and again remains un-methylated in the presence of DNMT inhibitors (Figure 5.12B).

To more quantitatively measure the methylated and un-methylated fraction of the CREB2 promoter, I designed primers that lie outside the CpG island and amplified the region in between by pyro-sequencing, which scores the C to T conversion of genomic DNA (1 - % Methylation) in real time. I found that the CREB2 promoter exists in a 50 % methylated form at baseline, which is striking particularly when compared with the promoters of CREB1 and PKA-R, which display little to no methyation (Figure 5.13). This finding suggests that the CREB2 promoter is dynamically regulated by methylation and that its methylation state at baseline may reflect experience. After exposure to 5HT, every CpG site within the CpG island of CREB2 has increased methylation, with those at the beginning and end of the CpG island showing the most significant increase (Figure 5.13). Extraction and bisulfite treatment of genomic DNA after exposure to DNMT inhibitors prevents, as

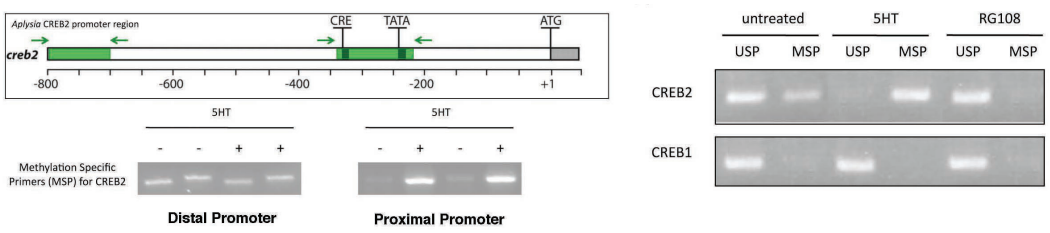

Figure 5.12: Serotonin-dependent methylation of CREB2 as seen by PCR

A. The genomic locus for *Aplysia* CREB2 is shown. Areas in green are predicted CpG islands. Methylation specific primers (MSP) designed to detect methylation at the distal CpG island shows no change in methylation patterns within 12 h after exposure to 5HT. MSPs designed for the proximal CpG island detect higher levels of methylation in 5HT treated samples (+) compared with controls (-). B. Using MSPs (methylation specific primers) and USPs (un-methylation specific primers) I detect the ratio of the methylated to the un-methylated form of the CREB2 promoter under baseline conditions, and compared with 5HT and RG108 treated samples. CREB2 promoter shifts entirely to the methylated form with exposure to 5HT and back to the un-methylated form with DNMT inhibitor RG108. CREB1 always exists in the un-methylated form.

expected, the 5HT-induced increase in methylation and drops methylation levels to below baseline (Figure 5.13). Taken together, these data reveal that 5HT causes direct methylation of the proximal CpG island in the CREB2 promoter, and that this methylation leads to a long-term and persistent down-regulation of CREB2 RNA and protein levels and the resultant persistence of memory-related synaptic plasticity.

5.2.4 Piwi/piRNA complexes control the methylation state of the CREB2 promoter

Given that that piwi is regulating CREB2 at the transcriptional level (Figure 5.8), I asked whether piwi was required for the observed serotonin-dependent methylation of CREB2 in neurons. I inhibited piwi in sensory neurons and extracted the genomic DNA after exposure to 5HT. Following bisulfite treatment, I scored the % methylation by pyrosequenceing and found that inhibition of piwi completely abolished the serotonin-dependent increase in methylation at the promoter (Figure 5.14A). The reversal in methylation patterns was most significant at the beginning and ends of the CpG island consistent with the observation that the same sequence areas were most sensitive to serotonin (Figure 5.14A). To determine which piRNA mediates this effect, I searched the CREB2 locus for potential piRNA bindings sites and identified four well-expressed candidate piRNAs that had good complimentary to the promoter, 5UTR, and initial coding segment of the CREB2 mRNA (Figure 5.14B).

Through a series of knockdown experiments using 2-O-methyl oligoribonucleotides specific to each of the four piRNAs, I observed that one piRNA, aca-piR-F had the strongest effect on CREB2 expression. Knockdown of aca-piR-F, but not aca-piR-A, C, or D, increased the baseline levels of CREB2, both at the protein and RNA level, demonstrating that aca-piR-F is a transcriptional regulator of CREB2 (Figure 5.15C). If aca-piR-F were indeed mediating the observed 5HT-dependent methylation effects of CREB2, then aca-piR-F should be regulated by 5HT on a similar time course. I followed aca-piR-F levels with exposure to 5HT as a function of time, and noticed a slightly delayed, but persistent and robust up-regulation of aca-piR-F that peaked at 3-4 hours before drop-

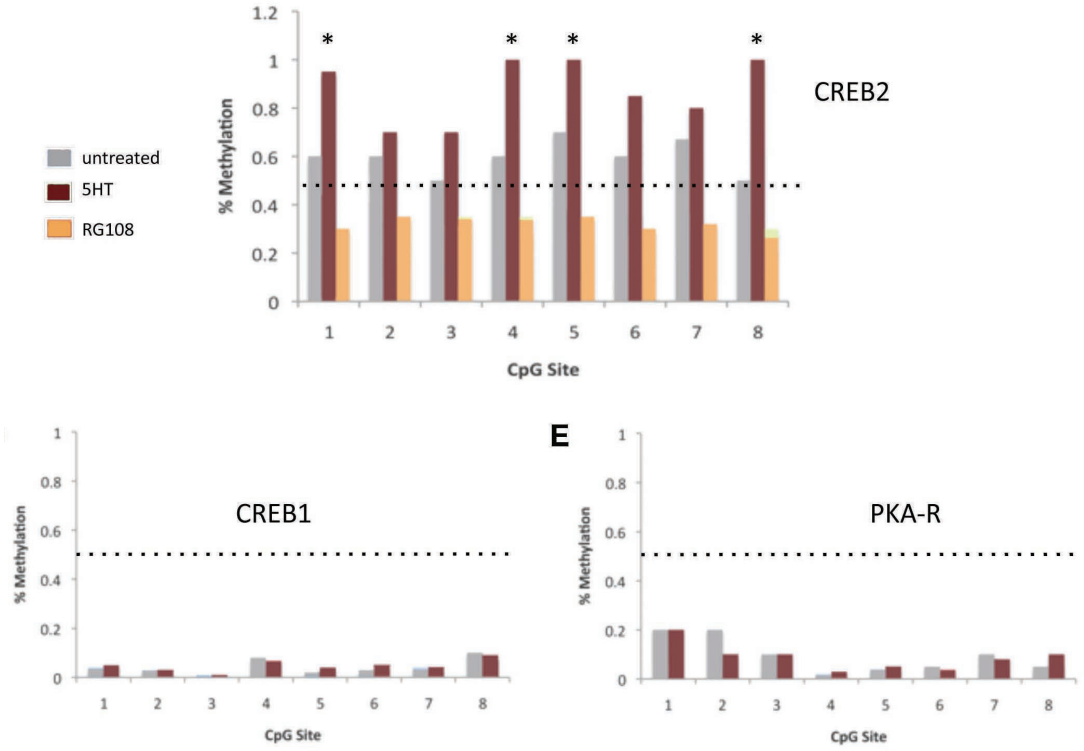

Figure 5.13: Serotonin-dependent methylation of CREB2 as seen by pyrosequencing High-throughput, real-time, pyrosequencing of the CREB2 promoter region shows a significant baseline level of methylation (gray) at individual CpG sites that is then robustly up-regulated with exposure to 5HT (maroon) and this effect is abolished in the presence of the DNMT inhibitor RG108 (orange). Effects are quantified as the mean of four independent trials ± S.D. These effects are specific to CREB2 as neither the CREB1 or PKA-R promoters show significant baseline methylation or any serotonin-dependent changes in methylation status.

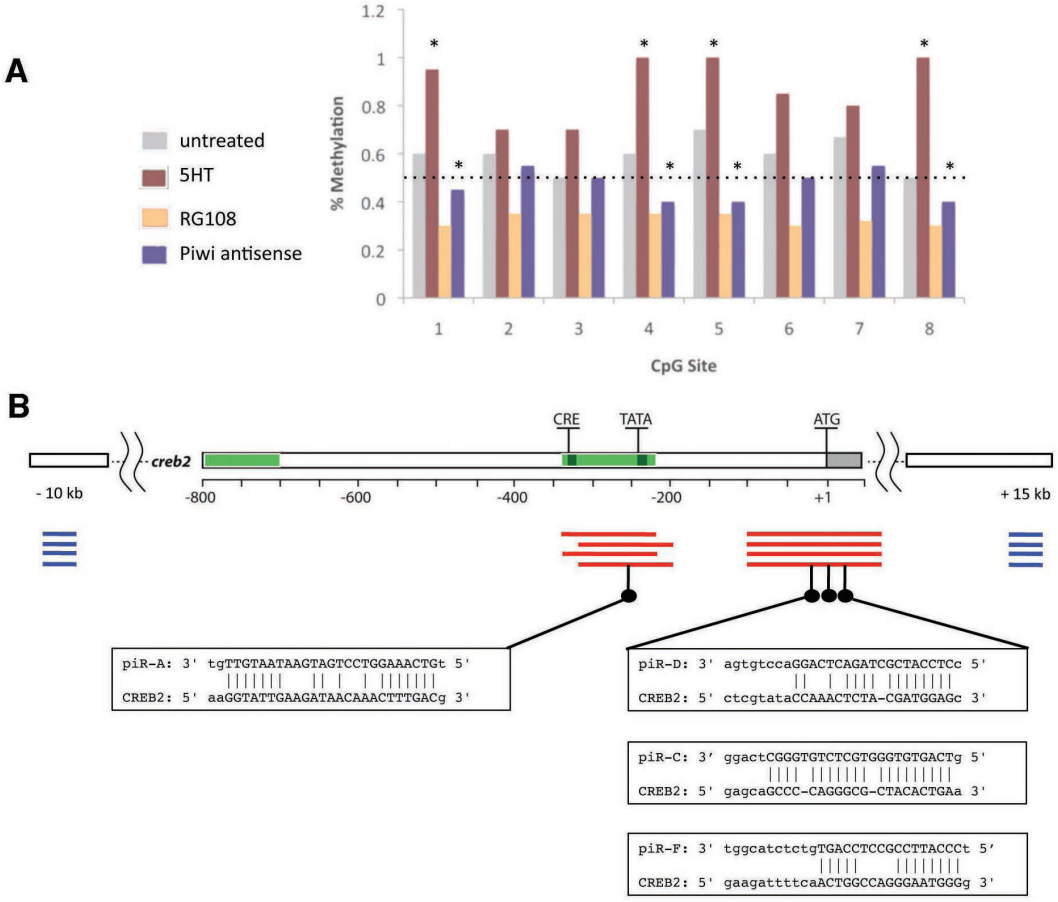

Figure 5.14: Piwi mediates serotonin-dependent methylation of the CREB2 promoter A. High-throughput, real-time, pyrosequencing of the CREB2 promoter region shows increased methylation in response to 5HT (maroon) which is fully reversed when 5HT is applied in the presence a piwi inhibitor (blue). The results are quantified as a mean of 4 independent trials ± S.D. B. A diagram of the CREB2 genomic locus. The CpG island is marked in green and the translational ATG start site is indicated. In red are ESTs mapping to this locus showing bi-directional transcription at the promoter and a precise 5'UTR start site for the CREB2 mRNA. In blue are piRNA clusters being generated upstream and downstream of the CREB2 locus. piRNAs abundantly generated in trans with potential target sites to transcribed regions (ESTs) from this locus are shown.

ping back to baseline at 12 hours (Figure 5.15D). This time course is consistent with the observed drop in CREB2 RNA levels, which begin between at 6 hours after exposure to 5HT. Since the binding site for aca-piR-F lies near the translational start site of CREB2, I propose a model in which the piwi/piRNA complex, through aca-piR-F, binds the nascent CREB2 transcript, thereby bringing it within close proximity for regulation of the CREB2 promoter during 5HT dependent long-term memory (Figure 5.15E). Transcriptional control of gene-expression through complementary base pairing of a small RNA with a nascent mRNA transcript has been previously discovered, first in the exciting work from S. pombe and more recently from a study in *C. elegans* ([Verdel *et al.*, 2004; Guang *et al.*, 2010]).

The discovery that piRNAs exist outside the germline, in several major organs of *Aplysia*, but significantly in the nervous system, suggests much broader roles for piRNAs than has been previously appreciated. In addition to their presence and in certain cases enrichment in neurons, *Aplysia* piRNAs are unique from those previously described in that they derive from hotspots in the genome where they are abundantly expressed amongst a background of other piRNAs in the same cluster that are uniformly low in their expression levels. Importantly, several of the abundant piRNAs are regulated by neuromodulators suggesting functions in learning and memory. While notable exceptions exist (ref), I and others have previously found a rapid turn-over of several neuronal miRNAs in response to neuromodulators and neuronal activity (ref), which contrasts the observed slow but more enduring up-regulation of the few neuronal piRNAs addressed in this study. Additionally, while aca-miR-124 (Rajasethupathy et al. 2009) and aca-miR-22 (In Preparation) constrain serotonin dependent long-term facilitation, piwi-dependent piR-F enhances it. We currently have very few cases with which to draw generalizable conclusions, but future large-scale studies of small RNA function in neurons may highlight the possible existence of two distinct classes of small RNAs that are bi-directionally regulated by neuromodulators, that act in a coordinated reciprocal fashion on a distinct and functionally segregated population of targets, to effect either facilitation or constraint, on memory related synaptic plasticity (Figure 5.16).

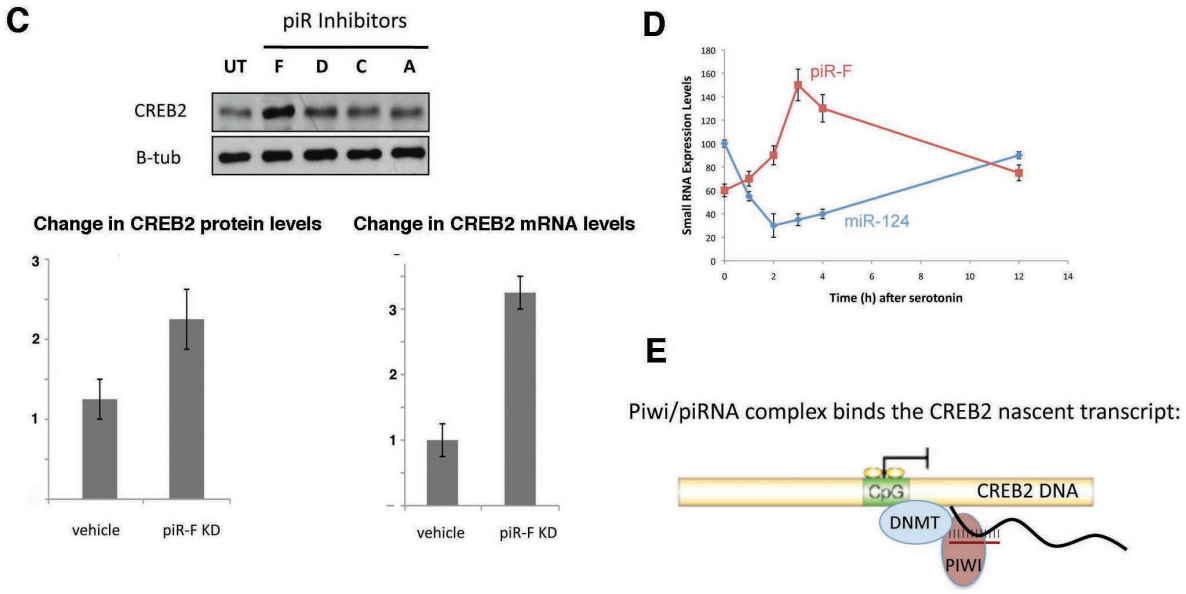

Figure 5.15: Piwi mediates CREB2 methylation through piR-F

C. Inhibitors of aca-piR-A, -C, -D, and -F were applied to *Aplysia* sensory neurons for one day, after which protein and RNA were extracted and analyzed by western blot and real time PCR. Inhibition of aca-piR-F caused a significant up-regulation of CREB2 protein and RNA levels when compared to untreated cells, or those treated with inhibitors of aca-piR-A, -C, or -D. Results are quantified and shown as a mean of 3 independent trails ± S.D. D. The time course of aca-piR-F after the initial exposure to 5HT (time 0 h). The previously described time course of aca-miR-124 is shown for comparison.E. The data suggest a model where the guide piRNA targets the nascent CREB2 transcript, which is in close proximity to access and regulate the promoter.

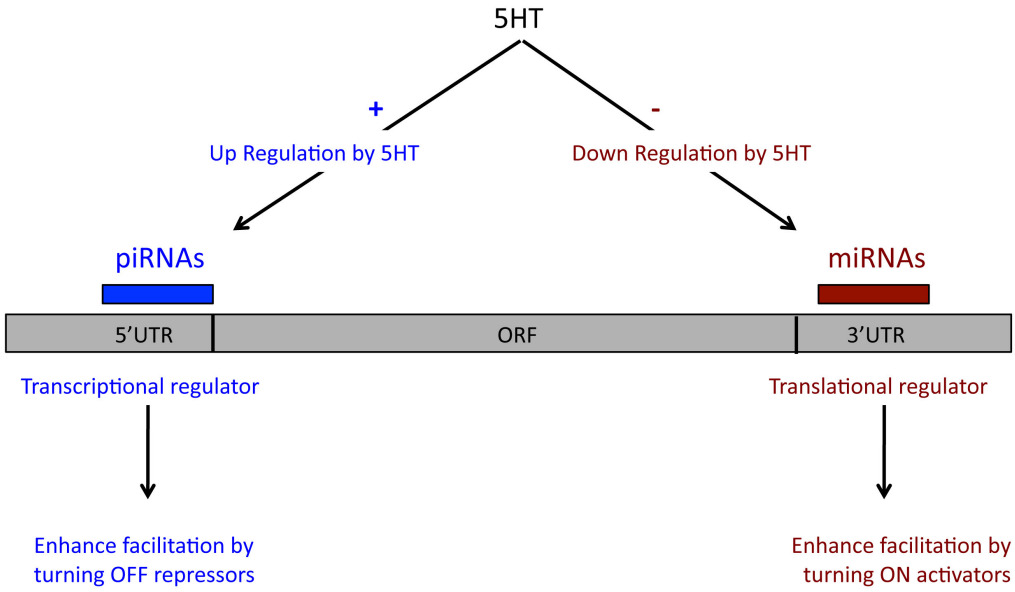

Figure 5.16: A working model: The integrative action of small-RNAs during synaptic plasticity

Further studies would also benefit from a genome wide analysis of piRNA/piwi occupied promoter regions during serotonin-mediated synaptic plasticity to gain a complete picture of the epigenetic landscape during memory. One attractive possibility is that piRNAs are directed only toward inhibitors of plasticity, and that with each repeated train of stimulus (or pulse of serotonin, or behavioral training) the promoters of more inhibitory genes are silenced, such that eventually the cell is maximally primed and excitable, allowing for the strongest associative memories.

The regulation of CREB2 by DNA methylation has important consequences for the activity-dependent long-term changes in cell-wide properties, such as the intrinsic excitability, of the neuron. This is an attractive explanation for how neurons translate transient stimuli into stable internal representations, and is consistent with several earlier studies that elegantly show the role of epigenetic regulation in memory (12-14). Our data also suggests that each neuron may have a different baseline level of CREB2 expression, which reflects its experience and immediate history, and this would be consistent with earlier observations

showing variations in baseline levels of CREB1 across populations of neurons in the amygdala ([Han *et al.*, 2007]). Since CREB2 is antagonistic to CREB1, long-lasting changes in CREB2 levels could set up a CREB1 distribution in neuronal cells, based on experience, that can dictate which neurons are already holding a memory trace and which neurons are readily drawn into new memory traces ([Han *et al.*, 2007; Won and Silva, 2008]). The likelihood that CREB2 set-points within a neuron can dictate its functional capacity for both memory and flexibility is further supported by a ubiquitin ligase over-expressing mouse model in the lab that is phenotypically much smarter than wild-type mice, and these mice show significantly reduced baseline CREB2 expression when compared with wildtype mice (personal communication).

In summary, I show that piwi/piRNAs control the activity dependent epigenetic regulation of the transcription factor CREB2, which may be an important and general mechanism of small RNA mediated long-lasting regulation of gene-expression in neurons that underlies long-term memory storage. This initial study compels the exploration of a genome-wide approach toward understanding the extent of piRNA-mediate epigenetic regulation in neurons during learning and memory.

Chapter 6

Conclusion and Future Directions

Small RNAs, both miRNAs and piRNAs, have great potential to produce switch-like, persistent, change in gene expression that underly one or another step in the long-term memory process. miRNAs can do so through feedback loops in protein networks, while piRNAs can do so through promoter silencing and long-term transcriptional repression. When it comes to appreciating small RNA biology and its relation to the nervous system, we are still at the very tip of the ice-berg. Despite its potential, much confusion remains to be lifted before we have a unified understanding of the coordinated function for these hundreds of small RNAs.

The first puzzling observation is that most miRNAs effect very small, often undetectable cellular or behavioral phenotypes ([Miska *et al.*, 2007]). Furthermore, the miRNA targeting rules have been vague and have plagued the field since its inception. While the seed, or first eight nucleotides of the miRNA, do appear to be important for 3'UTR target recognition by base-complementarity, as evidence by structural ([Wang *et al.*, 2008]) and functional ([Lim *et al.*, 2005; Lewis *et al.*, 2005]) studies, it is neither necessary nor sufficient ([Didiano and Hobert, 2006]). Some miRNAs bind their target mRNA in the coding region, or even in the 5'UTR, and several bind with imperfect seed pairing ([Wightman *et al.*, 1993; Reinhart *et al.*, 2000; Brennecke *et al.*, 2005]). It was also initially thought that conservation of sequence of the miRNA and predicted target were reliable predictors of function, but this has proven not to be the case. Several miRNAs function robustly in one particular species

or system, but lack conserved function in other species ([Giraldez *et al.*, 2006; Farh *et al.*, 2005; Sood *et al.*, 2006]). The ease with which new hairpin precursors can evolve and therefore the ease with which species-specific miRNA functionalities evolve, has complicated the construction of simple rules for miRNA targeting and function. It therefore appears that at the moment the exceptions outnumber the rules in miRNA biology. Of the hundreds of miRNA genes identified, how do we begin to focus on the important players and how do we identify generalizable themes in miRNA based gene regulation?

In the context of the nervous system, and specifically of memory-related synaptic plasticity, there are a few good starting points. A first step involves the identification of miRNAs that are regulated by activity. I and others find that only a small subset of the entire family of miRNAs respond robustly to neuromodulators and to neuronal activity ([Rajasethupathy *et al.*, 2009; Krol *et al.*, 2010a]). Focusing the initial functional analysis on these miRNAs helped ensure the likelihood of a strong phenotype for the physiology of synaptic plasticity. Secondly, it appears that those miRNAs that target a critical node of a pathway, where signaling converges or where kinetic rate limiting steps occur, are more likely to be responsible for strong physiological phenotypes. For instance, miR-124 regulation of CREB seemed from the outset to yield a strong knockdown phenotype since there is significant signaling convergence at transcription factors, and CREB in particular has an important and conserved roles in mediating the switch from short-to long term memory. These activity-dependent miRNAs may also lead us to the identification of those miRNAs that target multiple genes of a common pathway, and therefore serve as master regulators for a given physiology. This idea was first convincingly documented by [Giraldez *et al.*, 2006] when they found coordinated control of hundreds of targets by a single miRNA family is used to control temporal identity. In my study, I found that a brain-specific miRNA, miR-22, functionally represses the transcripts of both the atypical kinase, PKCζ, as well as the translation factor CPEB (unpublished data). Not only are PKCζ and CPEB part of a common regulatory pathway for synaptic plasticity, but these are the only two molecules that have been shown to become resistant to degradation in an activity-dependent way, and therefore capable of contributing

to persistent synaptic activity that underlies long-term memory. More importantly, my collaborators in the lab and I have growing evidence that the two proteins interact functionally in a feedback loop, setting up a bistable state, where the switching between states could perhaps be dictated by the common miRNA target. This attractive, and perhaps generalizable, mechanism for miRNAs providing switches to the existing feedback architecture of protein networks was not an a priori expectation but rather a fortuitous discovery that the same miRNA targets two independent genes belonging to the same pathway. Such motifs could be more easily discovered through computational and experimental analyses of the role of miRNAs in regulating an entire pathway rather than a single gene. More global analyses of miRNA regulation in the context of signaling pathways could provide conserved miRNA regulatory motifs that may not be evident otherwise. Therefore, while an approach based on finding the targets and function of individual miRNAs and piRNAs, as presented in this thesis, is valuable in gaining insights into function, the field would benefit greatly from taking a systems approach to small RNA biology.

piRNA biology is even more at its infancy than miRNA biology, largely due to the vast number and heterogeneity of piRNAs, and lack of a good endogenous or cell culture system to study the biochemical and functional properties of these piwi associated RNAs. *Aplysia* offers an attractive system for the study of piRNA biology, especially as it pertains to memory-related synaptic plasticity. The piRNAs in *Aplysia* are similar to mammalian piRNAs in that they are mostly not repeat associated, but rather, map to unique regions of the genome. Furthermore, piRNAs in *Aplysia* are abundant in several organs apart from the gonads, and have a significant presence in neurons, comparable to the expression levels of miRNAs. The presence of a single dominant piRNA within each piRNA cluster offers clues to their biogenesis, which still remains a mystery, as well as to which piRNAs may be most functionally relevant. I find that some piRNAs respond to neurotransmitter and neuronal activity by a rapid and sharp increase in expression levels, and that one particular piRNA silences the promoter of a transcriptional inhibitor during synaptic plasticity in an activity-dependent way.

In future work one will need to focus on the mechanism of piRNA and piwi directed methylation. piRNA mediated methylation has been observed in many systems (albeit not in neurons until now) including fruity fly, mice, and now the seaslug, however, the exact mechanism remains unclear. Piwi is likely a central player in recruiting and effecting the methylation, so future experiments directed toward more exhaustively characterizing its associated protein complexes could prove informative. A more direct mechanistic understanding could open the door to a generalizable understanding of piRNA mediated regulation during synaptic plasticity. One pressing future experiment would be to perform a genome wide analysis of piRNA/piwi occupied promoter regions during serotonin-mediated synaptic plasticity to gain a complete picture of the epigenetic landscape during memory in *Aplysia*. One attractive possibility is that piRNAs are directed only toward inhibitors of plasticity, and that with each repeated train of stimulus (or pulse of serotonin, or behavioral training) the promoters of more inhibitory genes are silenced, such that eventually the cell is maximally excitable and its synapses are maximally facilitated. I believe that future studies on piwi mediated transcriptional control will be exciting, but there is no reason to believe that piRNAs alone are responsible for epigenetic regulation during plasticity. Irrespective of their biogenesis properties, small RNAs confer versatile sequence specificity to mechanisms of gene regulation, and therefore, any small RNA that evolves a functionality for its guide protein to recruit methylation elements to the target promoter could prove equally effective. It is possible therefore, that one of the many rapidly multiplying classes of nuclear small RNAs take over the same task in other species.

Although the findings in this thesis are limited to the function of a single piRNA, and there are likely to be many others with the same or different targets, it does highlight the possibility of large-scale epigenetic regulation during synaptic plasticity, which has broad implications for mechanisms of learning and memory. The role of epigenetic modifications in differentiated cells, especially in adult neurons, has been controversial. The concern being that changes in gene expression should be permanent during development, but not in adult neurons since the plastic nature of synaptic connections, by definition, requires bi-

direction and reversible changes in gene expression. The identification of both functional DNA methyltransferases and DNA demethylases in adult neurons, moreover, brought forth the possibility that epigenetic changes in the adult brain may not necessarily be permanent, but may simply be long-lasting, and more permanent than the other known modifications so far described. Subsequent studies have identified individual gene loci that are methylated in response to neurotransmitter activity, though the time course of onset and persistence of methylation are unclear and would be a useful further area of research. Our study is the first to provide electrophysiological evidence that lack of DNMT enzymes causally affects, by completely abolishing, synaptic facilitation that underlies long-term memory.

Epigenetic modifications caused by DNMTs provide an ideal mechanism for transient stimuli to be translated into stable long-term internal representations within the cell, through long-term changes in gene-expression. It begs the obvious question however, as to how transcriptional, and therefore cell-wide changes in neuronal excitability (intrinsic plasticity), could effectively mediate synapse-specific excitability changes (synaptic plasticity)? As discussions previously have emphasized, it is likely that both forms of plasticity co-exist such that one can fine-tune the other, but it is also possible, that the two exist entirely independently in certain contexts. While synaptic plasticity affords orders of magnitude more computational power, intrinsic plasticity has the advantage of priming memories and allowing for robust generalized learning where the same association rules are applicable to experiential learning in various contexts. Whereas synaptic plasticity is ideal for storage of explicit memories requiring attention to detail, intrinsic plasticity may be more ideal for storing implicit and associative memories as observed throughout the CNS of invertebrate systems and specified cortical areas of vertebrate systems. Since human life is characterized by a great deal of habit-formation and repetition based associative learning, the use of intrinsic plasticity throughout much of the neural architecture of the human brain may turn out to be an efficient method for memory storage. Early studies from the Kandel lab have demonstrated that serotonin can give rise to both synaptic and intrinsic plasticity, and further that intrinsic plasticity may be important for processes of recall. Furthermore, work in the 1980's

by Brons & Woody and Alkon & Rasmussen offered preliminary experimental and theoretical model for the role of intrinsic plasticity in memory storage ([Brons and Woody, 1980; Alkon and Rasmussen, 1988]). More recently, the Johnston, Linden, and Turrigiano labs, among others, have consistently shown that behavioral memory and/or LTP can trigger activity-dependent changes in the intrinsic plasticity of neurons ([Turrigiano *et al.*, 1994; Zhang and Linden, 2003; Frick *et al.*, 2004]). Despite these studies, there still exists a general paucity of studies devoted to mechanisms of intrinsic plasticity when compared with the overwhelming interest in mechanisms of synaptic plasticity. My own studies regarding the role of piRNAs in epigenetic regulation, together with the early studies in intrinsic plasticity, compel me to believe we may benefit in the future by dedicating more effort toward understanding how intrinsic plasticity interfaces with synaptic plasticity, primes associative memories, and facilitates recall.

Given our discussion of piRNA induced epigenetic (long-lasting) changes, as well as the role of persistently active molecules such as CPEB and PKCζ in effecting long-term cellular and synaptic changes, another natural question emerges: Do stable cellular and synaptic states (whether induced or at base-line) require persistently active molecules? In theory, a stable protein network is created whenever the kinetic rates of synthesis and degradation are balanced such that the network can produce a stable output for a given input. Therefore, in principle, we should be able to move a protein network from one stable state (from baseline perhaps) to another (potentiated or depressed) by adjusting the kinetic rates of sources and sinks, which may inevitably lead to molecules that are more persistent than they were before, but perhaps not necessarily permanently persistent.

Over the last decades, there have been many molecular candidates that have "information storage" capacity - for instance, second messengers such as cAMP were intriguing because they could transduce a very transient calcium influx into a longer-lasting cellular signal by dispersing the information to many downstream signals. Then there was the discovery of kinases that had some level of persistent activity - PKA early on and more recently PKCζ, when cleaved of their regulatory domains, can prolong their magnitude and dura-

tion of catalytic activity; CaMKII - when phosphorylated, becomes auto-catalytic for an extended time even in the absence of calcium and calmodulin; PKCα by anchoring to synaptic membranes, increases its effective concentration and local activity for a greater duration of time. Long-term stable changes, however, need not occur through persistently active enzymes: transcriptional switches (CREB1 and CREB2), translational switches (CPEB) and cytoskeletal switches (actin anchoring) are equally effective in translating short term events into long-term events. Each of these above mentioned switches have some level of persistent activity but vary in their duration. Therefore, as a cell receives information, the initial responders to calcium transients begin a cascade of events where persistent molecules pass the baton to progressively slower and more robust switches, until eventually the final switch is read out through enzymatic, structural, or gene-expression changes. In the end, while a protein network may not necessarily require permanently persistent molecules, it might still be a compelling mechanism for reaching stable states and it will be exciting to observe whether CPEB and PKCζ may form the final switch or whether their activity may be further transduced to more enduring switches.

Chapter 7

Methods

Small RNA Cloning and Annotation

All animals were obtained from the NIH/University of Miami National Resource for *Aplysia*. Prior to dissection, animals were anesthetized by injection of isotonic MgCl2 (337 mM) at a volume of 50% to 60% of their body weight. RNA was isolated from dissected tissue according to the standard Trizol (Invitrogen) protocol, with additional extractions with acidic phenol:chloroform:isoamyl alcohol, and finally again with chloroform before precipitation in 3 volumes of ethanol. Starting RNA amounts for each library were as follows: whole animal 250 μg; CNS 90 μg; pleural ganglia 45 μg; abdominal ganglia 90 μg. Small RNA cloning was performed as described ([Hafner *et al.*, 2008]) with the exception that libraries in Chapter 5 were prepared using barcoded adapters and the Illumina platform was used for sequencing. Pre-adenylated 3adapters were used, along with a truncated T4 RNA ligase, Rnl2 ([Ho *et al.*, 2004]) to avoid circularization of the microRNAs during 3 adapter ligation. 5 adapter ligation was carried out at standard conditions with T4 RNA ligase (Fermentas Life Sciences) in the presence of ATP. The adapter sequences were as follows: 3 adapter - AppTTTAACCCGGCACCCTC; 5 adapter ATCGTaggcaccugaaa. After both ligation steps, and following RT-PCR, the markers were removed from the samples by PmeI digest. The samples were again PCR amplified, concatenated, and then cloned into the commercially available TOPO 2.1 vector as described ([Hafner *et al.*, 2008]). A total of about 250,000

reads each were obtained for the whole animal and CNS libraries by 454 sequencing (454 Life Sciences, Connecticut, USA) and 15,000 reads for each Solexa library (Rockefeller Sequencing Core, New York, NY). Traditional Sanger sequencing was used (Columbia Genome Center, New York, USA) to obtain approximately 15000, 20000, and 30000 reads each from the abdominal, pleural, and CNS libraries respectively.

Small RNA sequences obtained from tissue libraries were extracted and assigned to annotation categories using a similar procedure and similar computing pipeline as described ([Landgraf *et al.*, 2007]). For categorizing small RNA sequences, A database of annotated RNA sequences was assembled from several public sources:
GenBank (http://www.ncbi.nih.gov/Genbank/index.html) for the inclusion of rRNA, tRNA, sn/snoRNA, scRNA, and mRNA from *C. elegans*,*D. melanogaster*, *A. californica*, and *H. sapiens*; a database of *Aplysia* ESTs (http://aplysia.cu-genome.org/html) as of Jan 2007; a tRNA dataset GtRNA (http://www.lowelad.ucsc.edu); an sn/snoRNA dataset from the small RNA database previously hosted at (http://mbcr.bcm.tmc.edu); and a database of miRNAs (http://microrna.sanger.ac.uk/sequences/index.shtml; release version 11.0, Aug 2008). Since *Aplysia* lacks an annotated genome, we assembled genomic contigs comprising tRNA and rRNA regions and added these sequences to the annotation database. These contigs were assembled from sequencing traces available in the NCBI trace archive (http://trace.ncbi.nlm.nih.gov). For assembly of tRNA contigs, all annotated tRNAs from other species were obtained, and mapped to the *Aplysia* trace archives to form contigs around matched regions. These were then aligned to full length tRNAs from other species and regions of high similarity were assigned as *Aplysia* tRNA. Assembly of rRNA was done similarly using the sequence U13369.1 (Human ribosomal DNA complete repeating unit) as a scaffold against which the contig was assembled.

Sequencing data (from both 454 and Sanger sequencing) were scanned to locate adapter sequence boundaries using the wu-blast (http://blast.wustl.edu) alignment program, and an in-house alignment program previously developed in collaboration with Mihaela Zavolan (http://www.biozentrum.unibas.ch/zavolan). In locating the adapters, the tolerance for mis-

matches or gaps was either 2 or 3, depending on the length of the adapter. Extracted small RNA sequences that were shorter than 16 nt or longer than 41 nt, or that contained more than one indeterminate base, were excluded from our analysis. Extracted small RNAs with a low degree of complexity were assigned their own annotation category and not further considered. Other extracted small RNAs were aligned to the annotation database, finding the best degree of alignment match up to a maximum of 3 mismatches or gaps. For most annotation categories, antisense alignments to elements in the annotation database were ignored. The small RNA sequences were assigned to whichever category they matched best, but in the case where a sequence had equally good matches to several annotation categories, priority was given in the following order: rRNA, tRNA, sn/snoRNA, miRNA, miRNA candidate, piRNA, miscRNA, mRNA, and E. coli.

Aplysia sequences that mapped a miRNA from another species, or sequences that did not map to any annotation category, were considered a miRNA if its length variants aligned well to the 5 end, it had a map to the *Aplysia* trace archives and a foldback structure, and if the star sequence was cloned. The precursor sequence for these newly annotated miRNAs were then added to the annotation database as *Aplysia* miRNAs. In cases where some sequences were annotated as miRNA without having a precursor (assigned miRNA through orthology or processing patterns), the mature sequence was given flanking bases and entered into the annotation database as an *Aplysia* miRNA. The remaining sequences were again aligned to the updated annotation database, and this iterative process continued until an exhaustive search of potential miRNAs was catalogued.

piRNA candidates were chosen based on their length (between 26 and 33 nt), a 5' terminal U, and their property of clustering together in a contig. To assess their clustering potential piRNA candidates were aligned to Aplysia genome trace sequences. Trace sequences were formed into groups that shared common matching piRNA candidates, and the trace groups containing at least 3 distinct piRNA candidates were assembled into contigs using Phred/Phrap. The contigs that contained at least 10,000 total reads, with at least a 60% U start bias, and no more than 1000bp interval between individual piRNAs were defined as

piRNA clusters. 372 such piRNA clusters were generated. All small RNA sequences were mapped to these clusters and any clone that mapped perfectly or within one mismatch to a piRNA cluster was annotated as a piRNA. All procedures pertaining to sequence extraction, annotation, contig assembly, building of miRNA precursors, families, and orthology tables are further discussed in supplemental methods.

In Situ Hybridization

To prepare cultured neurons for in situ hybridization, cells were fixed in 4% paraformaldehyde in artificial seawater for 15 minutes at room temperature, and then washed with PBS. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and then endogenous peroxidase activity was quenched using 3% H2O2 for 20 minutes, following which a 10 minute acetylation step was performed, all at room temperature, with quick 1x PBS washes between each step. Pre-hybridization was carried out for 1 hr at 42C in 50% formamide, 5x SSC, 5x Denhardts solution, and 0.1 mg/ml each of salmon sperm DNA and yeast tRNA. Hybridization was then carried out for 4 hrs, at 42C, with 60ng of 3end-labeled digoxigenin (DIG) probe per 150 μl of hybridization solution. The first wash was done using 5x SSC for 20 minutes at 42C, and two subsequent washes were done with 0.5x SSC for 10 minutes each at the same temperature. The probes were then blocked for 1 hr in 10% (in TBS) heat inactivated sheep serum at room temperature, incubated in 1:1000 dilution of anti-DIG-POD antibody (roche) in 1% sheep serum (in TBS) overnight at 4C, then labeled for detection with TSA-Plus FITC system (PerkinElmer) according to the manufacturers instructions.

Pharmacological treatment, Northern and Western blot analysis

Whole CNS or pleural ganglia were dissected in ice-cold sea water, de-sheathed, and kept in L-15 supplemented with glutamine for 24 hrs at 18C. Serotonin treatment was performed with 5 pulses of 10 μM 5HT for five minutes each at 20 minute intervals. All drug treatments were done at a concentration of 10 μM in L-15 and bath applied to CNS for thirty minutes prior to treatment with five pulses of 5HT. The inhibitors used in this study are as follows: KT5720 (PKA inhibitor, Calbiochem), U0126 (MAPK inhibitor, Sigma), MG-132 (Protea-

some inhibitor, Sigma), and Bisindolylmaleimide (PKC inhibitor, Calbiochem). Inhibition of miRNAs, piRNAs, and kiwi was carried out using penetratin conjugated 2-O-methyl antisense oligonucleotides. These oligonucleotides were ordered (Dharmacon, Inc.) with 5 thiol modification and incubated, with equimolar concentrations of activated Penetratin (Qbiogene, Inc. PENA0500) featuring an N-terminal pyridyl disulfide functional group, for 15 min at 65C, then 1 hour at 37C. The penetratin conjugated antisense oligonucleotides were checked for conjugation efficiency by Coomassie staining on 17% polyacrylamide gels, and knockdown efficiency by Northern blot. 150 μl of 200 nM penetratin conjugated oligonucleotides were then applied to de-sheathed pleural ganglia in Eppendorf tubes, for a minimum of 4 hrs before washout, and kept in L-15 with glutamine for a minimum of 24 hrs before harvesting RNA or protein. Antisense sequence used for piwi knockdown: Piwi-AS: GGUCGGGUUGAUCACCACAACUAG Antisense sequences used for piRNA knockdowns: piR-A: ACAACATTATTCATCAGGACCTTTGACA piR-C: CCTGAGCCCACA-GAGCACCCACACTGAC piR-D: TCACAGGTCCTGAGTCTAGCGATGGAGGA piR-F: ACCGTAGAGACACTGGAGGCGGAATGGGA

Northern blot analysis was performed as described ([Landgraf *et al.*, 2007]). Between 20 and 40 μg of total RNA was loaded per lane, the probes were 5'32P-radiolabeled 21- or 22- nt oligodeoxynucleotides complementary to the predominantly cloned miRNA sequence, and the hybridization was done at 42C. To monitor equal loading of total RNA, the blots were reprobed with:
5'-TGGAGGGGACACCTGGGTTCGA-3' to detect tRNA.

For Western blotting, protein was isolated from de-sheathed pleural ganglia by incubating and rotating in SDS-urea lysis buffer (50 μl per 2 pleural ganglia) for 15 minutes at room temperature followed by centrifugation at 13000 RPM for 10 minutes, and collection of the supernatant. Protein samples were then quantified using the BCA kit (Pierce Biotechnology) and 15 μg were loaded for Western blot analysis. The following commercial antibodies were used: CREB1 (New England Biolabs) 1:1000, MAPK (Cell Signaling Technology) 1:1000, C/EBP (Santa Cruz Biotechnology, Inc.) 1:1000, UCH (Biomolecules)

1:1000, Tubulin (Sigma-Aldrich) 1:10000. KHC and CREB2 were rabbit polyclonal antibodies raised in the laboratory. Following incubation with primary antibodies, a 1:10000 dilution of either anti-rabbit or anti-mouse antiserum was used to detect protein bands by chemiluminescence (Amersham Biosciences).

Quantitative Real Time PCR

Ganglia were dissected, maintained, and treated as described above. RNA was isolated according to the traditional Trizol (Invitrogen) method. After the isopropanol precipitation, the pellet was washed with 70% ethanol, and converted into cDNA using random hexamer priming and Superscript III (Invitrogen Life Sciences). Primers were selected using the Primer Express software (Applied Biosystems) and chosen to ensure no significant amplification of DNA. The primer sequences were as follows:
CREB1: TCTCGGAAACGGGAATTACG; TTCCCTGGCTGCCTCTCTATT.
CREB2: GCCAGAACATGTCATCATGG; CCTCCCCCTTCTTCTTCATC.
KHC: GTTCGGCCTCTGAATCAGTCA; TTGAGAACAAACTTGCTGCCA.
C/EBP: GCCCCCTACTCCACAAAGTCT; CTGGCCCTCTTATCCACGTACT.
UCH: GTACATGCCCTGGCGAACA; CTTTGCAGCATCGAAGGGA.
NRX: ACCCTCCAGATCGACGCTG; TGGGCTTGTTTGCCTGTTG.
Pre-miR-124: CCCATTTGTGTTCACTGTGTG; ACCGCGTGCCTTAATAGTGT.
GAPDH: GCCTACACCGAGGACGATGT; GGCGGTGTCTCCCTTAAAGTC.

DNA Methylation Assays

DNA purification (DNA mini kit; Qiagen) was performed on *Aplysia* sensory neuron clusters. Purified DNA was then processed for bisulfite modification (Epitect Bisulfite Kit; Qiagen). Quantitative PCR was used to determine the DNA methylation status of the CREB2 and CREB1 genes. Methylation-specific PCR primers were designed using the Methprimer software (available at www.urogene.org/methprimer/). Primer sequences are as follows:
Detection of unmethylated CREB2 (USP):
GTTTTAAATATTTTTGTGTGAATTTATTGAA
ATCAAAACACAATAAAATCAAACACTAATC

Detection of methylated CREB2 (MSP):
TATTTTCGTGTGAATTTATCGAAAAT
CCGTCCAATAAAAAAACGAAATAACCGT
Detection of unmethylated CREB1 (USP):
GGTATTAAGGTTTGAAAAGTTTTGTG
CTCAATTAACCTCATAACAATCAAT
Detection of methylated CREB1 (MSP):
GGTATTAAGGTTTGAAAAGTTTTGC
CAATTAACGTCCTAACGATCGAT

For quantitative methylation analysis through real-time PCR and pyrosequencing of the CREB2, CREB1, and PKA-R promoters, we used the Sequenom massArray facility at Cornell (http://vivo.cornell.edu/display/SequenomMassARRAY) and all primers were designed using the epidesigner software
(available at http://www.epidesigner.com/). Primer sequences are as follows:
CREB2: aggaagagagAGGTGGTTTATTATTTTTTATGTTTG;
cagtaatacgactcactatagggagaaggctCTCCAAAAATCCAACTCCATC
CREB1: aggaagagagTTGTATATTTTGGATTTATGATAAGTTG;
cagtaatacgactcactatagggagaaggctCAAATAACCAAACCATAACTTTAACC
PKA-R: aggaagagagAAAGTTTTGTTTTTTTGATTGGTTT;
cagtaatacgactcactatagggagaaggctACTATTTCACAAATAATTTCTACTCACA

Reporter Assays

HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) in 96-well plates (250,000 cells/well) at 50% confluency with 100 ng psicheck-2 dual promoter plasmid (Promega), with renilla bearing the synthetic UTRs, and firefly serving as the internal transfection control. Cells were simultaneously transfected with or without 5pmol miRNA duplex. Firefly and Renilla luciferase activities were measured 36 hrs after transfection with Dual-luciferase assay (Promega).

Cell Culture, Injections, and Electrophysiology

Cell cultures of *Aplysia* neurons were prepared as previously reported ([Montarolo *et al.*, 1986]). For intracellular injections, miRNA mimic and inhibitors (Dharmacon, Inc.) were re-suspended in nuclease-free water (Ambion, Inc.) to obtain a final concentration of 5.0-5.5 M, aliquoted and stored at 20 C. piwi antisense oligoribonucleotide (5 uM) was used for knockdown of piwi, or a piwi-GFP expression vector (1 g/ μl) was used for over-expression of piwi. Details of the piwi antisense are given above. To generate the pNEX-apPiwi-eGFP, the apPiwi ORF was PCR-amplified from cDNA and subcloned into a pNEX3-eGFP vector (Kaang, 1996) modified by the insertion of a Gateway Destination cassette (Invitrogen) within the polylinker (pNEX3-eGFP-DEST vector). Before injection, each solution was combined with 10% v/v 2M KCl and 5% v/v of a saturated fast green solution to monitor the intracellular injection under both electrophysiological and visual control. For the injections, 2-5 l of each solution were loaded into the tip of beveled sharp glass microelectrodes. After impalement, sensory neurons were injected by 7-10 pressure pulses (1-10 psi; 300-500 ms) delivered through a pneumatic picopump (PV820; WPI) under electrophysiological and visual control. The cells were then transferred for 24 hrs at 18C and subsequently tested electrophysiologically after adding 0.5-1 ml of fresh L-15 into the culture dish.

Part I

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Part II

Appendices

Figure Captions for Supplementary Data

Supplemental Figure 1. One pulse of 5HT has no effect on miR-124 levels.

Northern blot showing mature miR-124 levels in untreated CNS (untreated) and CNS treated with one pulse of 5HT (1 x 5HT). Each lane was loaded with 20ug of total RNA and the gel was stained with ethidium bromide (EtBr) to monitor equal loading of samples.

Supplemental Figure 2. Inhibition of miR-124 causes enhanced expression of immediate early genes when combined with 5HT treatment, than when done alone.

Western blots showing increased levels of kinesin heavy chain (KHC), ubiquitin c-terminal hydrolase (UCH), and C/EBP (C/AAT enhancer binding protein), all immediate early genes of CREB, when miR-124 inhibition is combined with 5HT treatment (miR-124 INH +5HT) than when miR-124 is inhibited alone (miR-124 INH). A representative tubulin blot is shown to control for loading.

Supplemental Figure 3. CREB 3'UTR has a conserved miR-124 target site.

miR-124 (shown in red) has a predicted target site in the mouse and human CREB UTR with a perfect 7-mer seed. The *Aplysia* site is different in that the seed region contains a GU wobble, but there is extensive base-pairing both within and outside the seed region.

Supplemental Figure 4. A non-functional isoform-specific miR-124 target site in CREB1d ORF.

The miR-124 target site in the *Aplysia* CREB1d ORF is shown, along with the constructs used for the following reporter assay (there is no miR-124 site in the CREB1a ORF). A luciferase reporter (100ng) bearing the CREB1d ORF is not significantly affected by co-transfection with miR-124 duplex (5pmol) in HEK293 cells. As controls, a luciferase reporter bearing a CREB1a ORF was also unaffected by miR-124, and an siRNA directed against all luciferase (luc siRNA) was able to repress all firefly-luciferase containing constructs by 80%. Each data point is expressed as a ratio of renilla to firefly activity, normalized to the change in luciferase activity when plasmids are transfected alone without miR duplexes. Data is shown as a mean of 4 independent trials ± S.D.

Supplemental Figure 5. A novel isoform for *Aplysia* CREB1 – CREB1d.

An assembled *Aplysia* genomic contig for the CREB1 isoforms, including the exon and intron organization, are shown. Due to a large repetitive region the genomic assembly of

the CREB1 region is divided into two contigs. The three previously characterized CREB1a/b/c isoforms (Bartsch et al. 1998), represented in the orange track, span exons II-VII (the 5' UTR exon I is not shown and is shared by all isoforms; exons III and IV are not labeled). The newly identified isoform CREB1d differs from the other isoforms in the 3' end where a portion of the intronic region between exon VI and VII is transcribed, a new 3'UTR is generated, and exon VII is excluded. The predicted (and confirmed) miR-124 target site for CREB1a is shown. The predicted (but non-functional) miR-124 target site for CREB1d lies at position 764, and this site is not found in CREB1a/b/c isoforms.

Supplemental Figure 6. A genomic contig for miR-124 shows putative CREB binding sites.

Predicted CREB binding sites upstream of the mir-124 gene are shown. Predicted CREB binding sites are marked as green bars with their contig coordinates (prediction by the JASPAR database). Shown in blue is an EST transcript expressed in the *Aplysia* CNS.

Supplementary Table Captions

Supplemental Table 1. Small RNA composition of the cDNA libraries used in this study.

The whole animal and CNS libraries were sequenced using 454 technology, while the CNS_sanger, pleural ganglia, and abdominal ganglia libraries were sequenced by the traditional Sanger method. The numbers indicate clone frequencies. rRNA; ribosomal RNA; tRNA, transfer RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; miscRNA, other non-coding RNAs; mRNA, messenger RNA; none, sequences that either did not map to the trace archives, or mapped without known annotation; low_complexity, sequences that had 2, 3, or 4 nt repeat structures. 2% of the none annotated sequences that did not map to the trace archives mapped instead to the *E. coli* genome. Total clone frequencies for miRNAs and miRNA candidates are listed separately.

Supplemental Table 2. Summary of the miRNA profile of Aplyisa.

The miRNA profile was constructed by aligning small RNA clones annotated with category miRNA to the identified miRNA precursor sequences. If a miRNA matched more than one precursor, the counts were divided equally among matched precursors. The miRNA clone frequencies from the five libraries are given: 1) CNS and 2) whole animal libraries done through 454, and the 3) CNS, 4) pleural ganglia, and 5) abdominal ganglia libraries done through Sanger sequencing. The second column indicates whether the sequence derives from the 3p or 5p arm of the precursor. The miRNA sequences listed show the predominantly cloned sequences. An asterix (*) indicates sequences that pair to the predominantly cloned mature miRNA. A carrot (^) indicates

sequences that were assigned a new name, but which bear some family relationship, as assessed by seed sequence similarity, to know miRNA genes.

Supplemental Table 3. Summary of the miRNA candidates in *Aplysia*.

These sequences did not have any match to the (incomplete) *Aplysia* trace archives, but had good 5' processing and therefore are catalogued in this table as miRNA candidates. The numbers indicate the clone frequency across the various libraries.

Supplemental Table 4. Small RNA composition of the cDNA libraries used in this study.

The adult and juvenile libraries across five different tissues were created using barcoded adapters and sequenced on the Illumina platform. One additional adult CNS library was sequenced using one full Solexa run. And the two 454 sequenced libraries from Rajasethupathy et al. are shown for comparison. The numbers indicate clone frequencies. rRNA; ribosomal RNA; tRNA, transfer RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; miscRNA, other non-coding RNAs; mRNA, messenger RNA; none, sequences that either did not map to the trace archives, or mapped without known annotation; low_complexity, sequences that had 2, 3, or 4 nt repeat structures. A portion of the sequences that did not map to *Aplysia* genome assembly mapped instead to the *E. coli* genome.

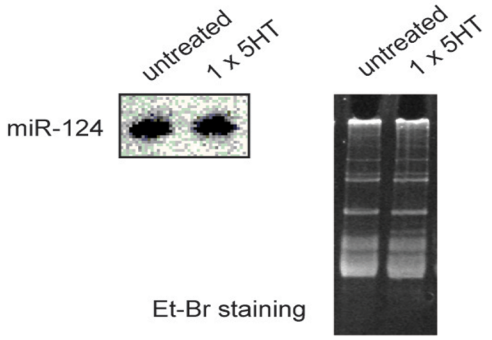
Supplemental Table 5. piRNA expression profile of *Aplyisa*.

The piRNA profile was constructed by aligning small RNA clones to the annotated piRNA clusters. Any sequence that mapped within a piRNA cluster was annotated as a piRNA. The read frequencies of the variants of individual piRNAs were grouped together to report total read count in each library. The last column provides a scaffold location in the *Aplysia* genome assembly to which the piRNA mapped. Only the top 500 cloned sequences of the total ~16,000 are shown here.

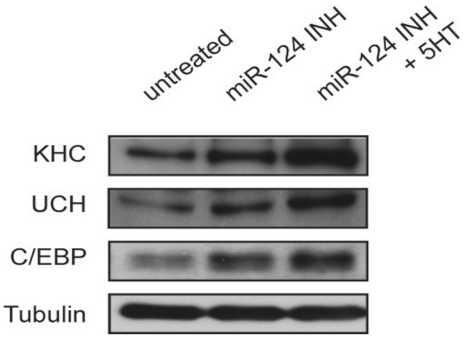
Supplemental Table 6. Top 75 miRNAs and piRNAs listed in order of abundance.

The highly expressed miRNAs and piRNAs are listed in order of clone frequency. piRNAs (highlighted in green) are comparable in abundance to miRNAs based on their clone frequencies.

Supplemental Figure 1.



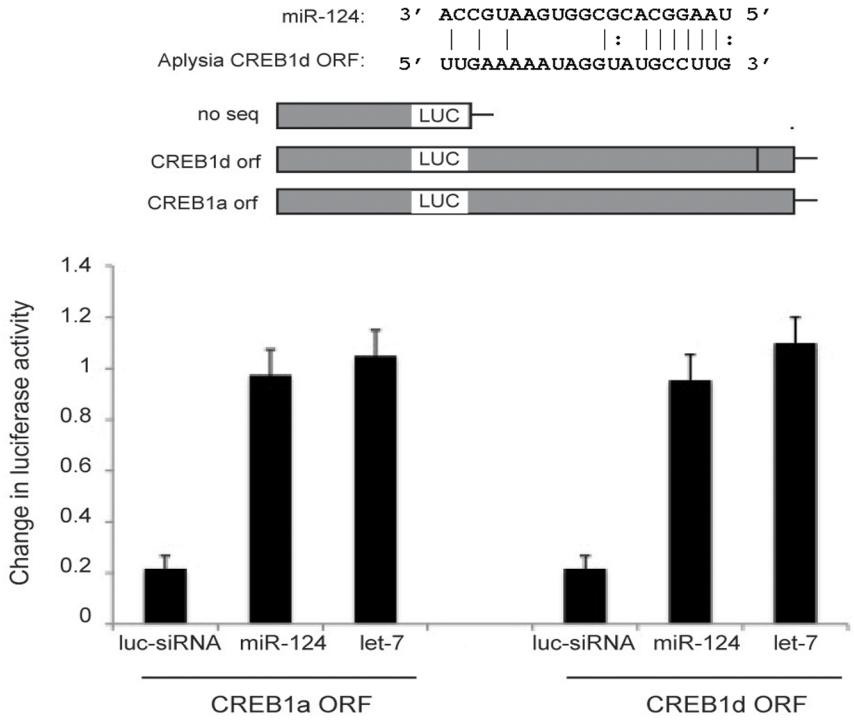
Supplemental Figure 2.



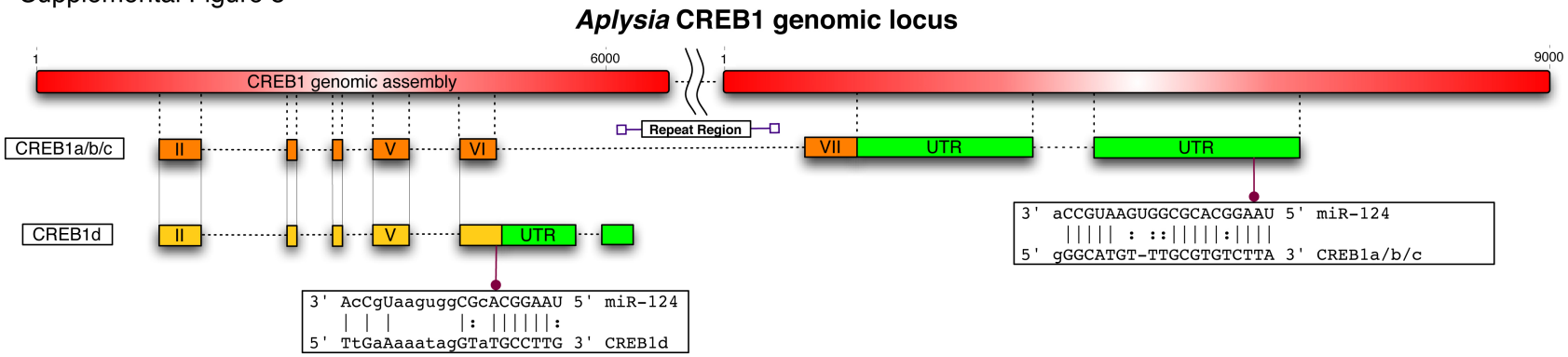
Supplemental Figure 3.

```
5′ ...CUGAGCUCCUUGAUUGCCUUA... 3′   HUMAN CREB1 3′UTR
5′ ...CUGAGCUCCUUGAUUGCCUUA... 3′   MOUSE CREB1 3′UTR
         | : || ::|  |||||||
3′ ...ACCGUAAGUGGCGCACGGAAU... 5′   miR-124
        ||||| : ::||||||:||||
5′ ...GGGCAUGU-UUGCGUGUCUUA... 3′   APLYSIA CREB1 3′UTR
```

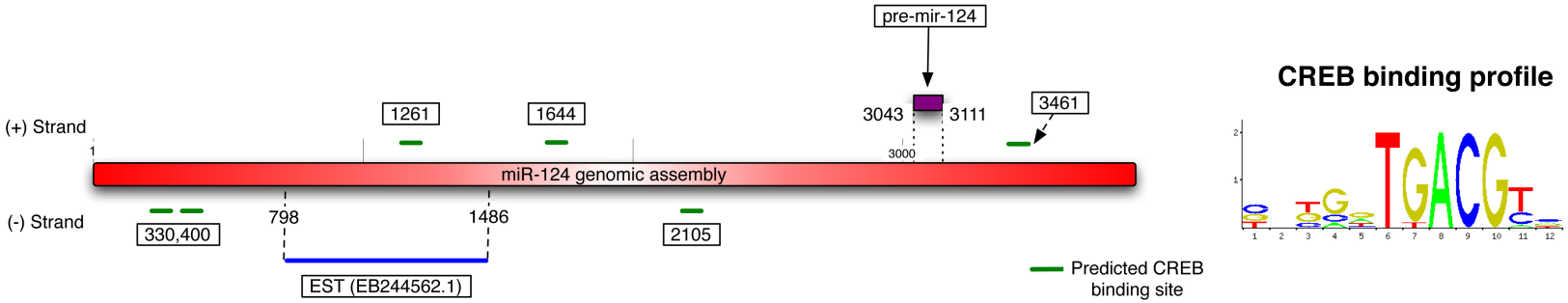
Supplemental Figure 4.



Supplemental Figure 5



Supplemental Figure 6



Supplementary Table 1: small RNA composition of cDNA libraries, Round 1

Sequence type	CNS-less-454	CNS-454	CNS	Pl. Ganglia	Abd. Ganglia	Total
rRNA	84338	2489	12	27	33	86899
tRNA	8518	10874	53	107	27	19579
sn/sno-RNA	605	18	0	0	0	623
miRNA	119866	182786	989	2390	1528	307559
miRNAcandidate	2883	4589	22	39	40	7573
piRNA	4143	10799	53	100	8	15103
miscRNA	80	5	0	1	0	86
mRNA	8519	1526	12	29	1	10087
e_coli	6440	1565	29	70	27	8131
none	19435	8724	144	235	108	28646
low_complexity	153	8	1	1	0	163
Total	254980	223383	1315	2999	1772	484449

Supplementary Table 2: miRNA Profile

precursor	arm	mature	form	most matched sequence	ext	len(min, max)	copies	CNS-less-454	CNS-454	CNS	Pl. Ganglia	Abd. Ganglia
aca-mir-124_gnl_ti_1856773791_-_68_88_extend3p	3p	aca-miR-124	miRNA	UAAGGCACGCGGUGAAUGCCA		21(16 25)	88678	11208	75365	490	1233	382
aca-mir-124_gnl_ti_1856773791_-_68_88_extend3p	5p	<unknown>	miRNA*	GUGUUCACUGUGUGAGCCUUGGU		23(22 25)	23	6	17	0	0	0
aca-mir-307_gnl_ti_1856253838_-_471_491_extend3p	3p	aca-miR-307	miRNA	UCACAACCUGAUUGAAUGAGG		21(16 27)	28561	14368	13772	63	131	227
aca-mir-307_gnl_ti_1856253838_-_471_491_extend3p	5p	<unknown>	miRNA*	CCUUUUUCAUCGGGUUGUGUUG		22(18 23)	48	23	25	0	0	0
aca-mir-125c_contiginputset27.fa.Contig1_+_779_800_extend3p	5p	aca-miR-125c	miRNA	UCCCUGAGACCAUAAUUUGUGC		22(16 26)	24803.5	13914.5	10648	46	107	88
aca-mir-125c_contiginputset27.fa.Contig1_+_779_800_extend3p	3p	<unknown>	miRNA*	CCAGGUUGUGGUCUUAGGAACG		22(21 25)	241	161	77	0	2	1
aca-mir-22c_contiginputset10.fa.Contig1_-_630_651_extend3p	3p	aca-miR-22c	miRNA	GAGCUGCCAAAUGAAGGGCUGU		22(16 27)	20405	4046.5	15806.5	93	186	273
aca-mir-22c_contiginputset10.fa.Contig1_-_630_651_extend3p	5p	<unknown>	miRNA*	CGCUCCUCCUUUGGCUAGCUAUCC	~	24(24 24)	1	0	1	0	0	0
aca-let-7a_contiginputset31.fa.Contig1_+_523_543_extend3p	5p	aca-let-7a	miRNA	UGAGGUAGUAGGUUGUAUUGU		21(16 28)	17451.5	7419	9731.5	52	105.5	143.5
aca-let-7a_contiginputset31.fa.Contig1_+_523_543_extend3p	3p	<unknown>	miRNA*	CAGUACAAUCUGCUAGCUUUCC		22(21 23)	175	160	15	0	0	0
aca-mir-184_contiginputset3.fa.Contig1_+_508_529_extend5p	3p	aca-miR-184	miRNA	UGGACGGAGAACUGAUAAGGGC		22(17 28)	12629	3219	9098	63	162	87
aca-mir-184_contiginputset3.fa.Contig1_+_508_529_extend5p	5p	<unknown>	miRNA*	CCUCAUCACUUGUCCGUCCGGU		22(16 24)	156	51	104	0	0	1
aca-mir-100001_contiginputset42.fa.Contig1_-_366_388_extend5p	5p	aca-miR-100001	miRNA	UGCCAUUUUUAUCAGUCACUGUG		23(16 26)	8689.5	2051.5	6509	19.5	59	50.5
aca-mir-100001_contiginputset42.fa.Contig1_-_366_388_extend5p	3p	<unknown>	miRNA*	CGGGGCUGGUGUGAAUGGCUUA		22(22 22)	4	2	2	0	0	0
aca-mir-100001_contiginputset42.fa.Contig2_+_431_453_extend3p	5p	aca-miR-100001	miRNA	UGCCAUUUUUAUCAGUCACUGUG		23(16 26)	8689.5	2051.5	6509	19.5	59	50.5
aca-mir-100001_contiginputset42.fa.Contig2_+_431_453_extend3p	3p	<unknown>	miRNA*	CGGGGCUGGUGUGAAUGGCUUA		22(22 22)	4	2	2	0	0	0
aca-mir-1_contiginputset9.fa.Contig1_+_728_749_extend5p	3p	aca-miR-1	miRNA	UGGAAUGUAAAGAAGUAUGUAU		22(16 26)	6593	4448	2117	9	13	6
aca-mir-1_contiginputset9.fa.Contig1_+_728_749_extend5p	5p	<unknown>	miRNA*	ACAUACUUCUUUGCUAUCCCAUA		23(17 24)	25	23	2	0	0	0
aca-mir-2b_contiginputset48.fa.Contig1_-_1426_1448_extend3p	3p	aca-miR-2b	miRNA	UAUCACAGCCUGCUUGGAUCAGU		23(16 27)	6127.5	2102	3911.5	23	70	21
aca-mir-2b_contiginputset48.fa.Contig1_-_1426_1448_extend3p	5p	<unknown>	miRNA*	CUGGCCAGGUGGUUGCGAUGUG		22(21 22)	52	12	40	0	0	0
aca-mir-190	5p	aca-miR-190	miRNA	AGAUAUGUUUGAUAUAUUUGGUG		23(16 26)	5842	960	4814	12	34	22
aca-mir-100_gnl_ti_1159794745_+_268_289_extend3p	5p	aca-miR-100	miRNA	AACCCGUAGAACCGAACUUGUG		22(16 25)	5339	2191.5	3055.5	21	47	24
aca-mir-100_gnl_ti_1159794745_+_268_289_extend3p	3p	<unknown>	miRNA*	CAAGUUUGCCUCUACGGGAGC		21(21 24)	87	24	60	1	0	2
aca-mir-283_contiginputset18.fa.Contig1_-_848_869_extend5p	5p	aca-miR-283	miRNA	UAAUAUCAGCUGGUAAUCCUGA		22(16 26)	4623	3756	856	1	8	2
aca-mir-283_contiginputset18.fa.Contig1_-_848_869_extend5p	3p	<unknown>	miRNA*	CAGGAGGCCGGCUGAUGUUACA		22(22 24)	29	23	6	0	0	0
aca-mir-8b	5p	aca-miR-8b	miRNA	UAAUGCUGUCAGGUAAAGAUGUC		23(16 26)	4040.5	1751.5	2272.5	2	11	3.5
aca-mir-71_contiginputset48.fa.Contig1_-_1762_1782_extend5p	5p	aca-miR-71	miRNA	UGAAAGACAUGGGUAGUGAGA		21(16 26)	3958	2202	1710	4	19	23
aca-mir-71_contiginputset48.fa.Contig1_-_1762_1782_extend5p	3p	<unknown>	miRNA*	GCUCACUACUCUGUCUUUUGCA		22(20 23)	27	23	4	0	0	0
aca-mir-279_contiginputset7.fa.Contig1_-_493_513_extend3p	3p	aca-miR-279	miRNA	UGACUAGAUCCACACUCAUCC		21(18 27)	3473	3467	6	0	0	0
aca-mir-279_contiginputset7.fa.Contig1_-_493_513_extend3p	5p	<unknown>	miRNA*	UGUGUGUGUGUGUGCA	~	16(16 16)	1	1	0	0	0	0
aca-mir-133_contiginputset32.fa.Contig1_-_286_307_extend3p	3p	aca-miR-133	miRNA	UUGGUCCCCUUCAAUCAGUUGU		22(17 25)	3247	2850	391	2	3	1

aca-mir-133_contiginputset32.fa.Contig1_-_286_307_extend3p	5p	<unknown>	miRNA*	AGCUGGUUGAACACGGGCCAAAU	~	23(23 23)	1	1	0	0	0	0
aca-mir-750_contiginputset16.fa.Contig1_+_620_641_extend5p	3p	aca-miR-750	miRNA	CCAGAUCUAACUCUUCCAGCUC		22(16 26)	3179	3041	134	0	3	1
aca-mir-750_contiginputset16.fa.Contig1_+_620_641_extend5p	5p	<unknown>	miRNA*	CGUUGGAAGAUUGGAUCUCAGC	~	22(19 22)	15	15	0	0	0	0
aca-mir-34b_contiginputset15.fa.Contig1_+_535_556_extend3p	5p	aca-miR-34b	miRNA	UGGCAGUGUGGUUAGCUGGUUG		22(16 26)	3050.5	1048	1957.5	11	27	7
aca-mir-34b_contiginputset15.fa.Contig1_+_535_556_extend3p	3p	<unknown>	miRNA*	ACCACUAUCCGCAUUGCCACGA		22(16 23)	57	30	27	0	0	0
aca-mir-153_contiginputset1.fa.Contig1_+_841_862_extend5p	3p	aca-miR-153	miRNA	UUGCAUAGUCACAAAAGUGAUC		22(17 26)	2951	368	2549	4	11	19
aca-mir-153_contiginputset1.fa.Contig1_+_841_862_extend5p	5p	<unknown>	miRNA*	CAGCUUUUGUGAUUUAGCAAUU		22(22 22)	2	0	2	0	0	0
aca-mir-8a_200a_contiginputset19.fa.Contig1_-_536_558_extend3p	3p	aca-miR-8a/200a	miRNA	UAAUACUGUCAGGUAAAGAUGUC		23(16 27)	2458	2210	243.25	1.5	2	1.25
aca-mir-8a_200a_contiginputset19.fa.Contig1_-_536_558_extend3p	5p	<unknown>	miRNA*	CGUCUUACCUAGCAGCAUUGGA		22(21 22)	8.5	6	2.5	0	0	0
aca-mir-8a_200a_contiginputset19.fa.Contig2_+_789_811_extend5p	3p	aca-miR-8a/200a	miRNA	UAAUACUGUCAGGUAAAGAUGUC		23(16 27)	2458	2210	243.25	1.5	2	1.25
aca-mir-8a_200a_contiginputset19.fa.Contig2_+_789_811_extend5p	5p	<unknown>	miRNA*	CGUCUUACCUAGCAGCAUUGGA		22(21 22)	8.5	6	2.5	0	0	0
aca-mir-100053_gnl_ti_1159522646_-_499_520_extend5p	5p	aca-miR-100053	miRNA	UGCCCUAUCCGUCAGGAACUGU		22(16 25)	2169	776	1370	8	7	8
aca-mir-100053_gnl_ti_1159522646_-_499_520_extend5p	3p	<unknown>	miRNA*	AGUGUCUGUCGGAGCGGCCACA		22(20 25)	25	4	21	0	0	0
aca-mir-2c_contiginputset48.fa.Contig1_-_588_610_extend3p	3p	aca-miR-2c	miRNA	UAUCACAGCCAGCUUUGAUGACA		23(17 25)	1836.75	968.333333	848.4166 67	4.5	4	11.5
aca-mir-2c_contiginputset48.fa.Contig1_-_588_610_extend3p	5p	<unknown>	miRNA*	ACGUCAAGGCGGUUGUGAUGUG		22(20 23)	8	0	8	0	0	0
aca-mir-755_gnl_ti_1803022211_-_657_679_extend5p	3p	aca-miR-755-3p	3p	UGAGAUUCAACUCCUCCAACUGC	~	23(18 26)	1786	1748	38	0	0	0
aca-mir-755_gnl_ti_1803022211_-_657_679_extend5p	5p	aca-miR-755-5p	5p	AGUGGAGAGAGUUUUAUCUCAUC		23(19 25)	389	385	4	0	0	0
aca-mir-10_contiginputset47.fa.Contig1_-_671_694_extend5p	5p	aca-miR-10	miRNA	UACCCUGUAGAUAUCCGAAUUUGU		24(17 26)	1702	1507	189	2	3	1
aca-mir-10_contiginputset47.fa.Contig1_-_671_694_extend5p	3p	<unknown>	miRNA*	AAAUUCGUAUCUGCGUGGUAUU		22(22 23)	5	5	0	0	0	0
aca-mir-2a_contiginputset48.fa.Contig1_-_1102_1124_extend3p	3p	aca-miR-2a	miRNA	UAUCACAGCCAGCUUUGAUGAGCU		24(17 27)	1517.16667	769.416667	734.9166 67	2.25	5.5	5.08333333
aca-mir-2a_contiginputset48.fa.Contig1_-_1102_1124_extend3p	5p	<unknown>	miRNA*	CACGUCAGAGUGACUGUGAUUUG		23(23 23)	10	10	0	0	0	0
aca-mir-2d_contiginputset48.fa.Contig1_-_839_861_extend3p	3p	aca-miR-2d	miRNA	UAUCACAGUCAGCUUUGAUGAGC		23(18 30)	1514.33333	1040	462.3333 33	2	4	6
aca-mir-315_contiginputset40.fa.Contig1_+_714_736_extend3p	5p	aca-miR-315	miRNA	UUUUGAUUGUUGCUCAGAAAGCC		23(16 26)	1504	1468	36	0	0	0
aca-mir-315_contiginputset40.fa.Contig1_+_714_736_extend3p	3p	<unknown>	miRNA*	CGCUCGAGCCACAAUCAAACAG		22(19 22)	11	11	0	0	0	0
aca-mir-317	5p	aca-miR-317	miRNA	UGAACACAGCUGGUGGUAUCU		21(16 23)	1401	995	401	0	3	2
aca-mir-100096a_contiginputset48.fa.Contig1_-_1588_1610_extend3p	3p	aca-miR-100096a	miRNA	UCACAGCCAGCUUUGAUGAGCG		22(18 27)	1132.08333	688.333333	436.4166 67	0	4	3.33333333
aca-mir-29b_contiginputset4.fa.Contig1_-_334_355_extend3p	3p	aca-miR-29b	miRNA	UAGCACCAUUUGAAAUCAGUGC		22(16 25)	995	421	565	2	2	5
aca-mir-29b_contiginputset4.fa.Contig1_-_334_355_extend3p	5p	<unknown>	miRNA*	CUGGUCUCGAGUGGUGGAUA		20(20 22)	9	2	7	0	0	0
aca-mir-22b_gnl_ti_1864243534_+_243_265_extend5p	3p	aca-miR-22b	miRNA	AGCUGCCUGAUGAAGAGCUGUCC		23(16 28)	965	453.5	488.5	3	6	14
aca-mir-22b_gnl_ti_1864243534_+_243_265_extend5p	5p	<unknown>	miRNA*	CGGCUCUUCACCUGGUAGACUUG		23(16 25)	48	9	39	0	0	0
aca-let-7b	5p	aca-let-7b	miRNA	UGAGGUAGUAGGUUGUGUGGUU		22(16 25)	924	887.5	35.5	1	0	0
aca-bantam_contiginputset24.fa.Contig1_+_718_739_extend5p	3p	aca-bantam	miRNA	UGAGAUCAUUGUGAAAACUGAU		22(16 25)	901	415	475	0	1	10
aca-bantam_contiginputset24.fa.Contig1_+_718_739_extend5p	5p	<unknown>	miRNA*	CUGGUUUUCACAGUGAUUUGCCAGA		25(19 27)	51	0	51	0	0	0
aca-mir-2a_contiginputset48.fa.Contig1_-_1588_1610_extend3p	3p	aca-miR-2a	miRNA	UAUCACAGCCAGCUUUGAUGAGC		23(17 27)	759.583333	322.083333	429	1.25	2.5	4.75

aca-mir-100097_contiginputset20.fa.Contig1_+_609_632_extend5p	3p	aca-miR-100097	miRNA	UCAGCAGUUGUACCACUGAUUUGA		24(16 27)	634	91	535	4	2	2
aca-mir-100097_contiginputset20.fa.Contig1_+_609_632_extend5p	5p	<unknown>	miRNA*	CGUCAGUGGCCAAUUGCUCUGGUA		24(22 25)	19	1	18	0	0	0
aca-mir-137	5p	aca-miR-137	miRNA	UAUUGCUUGAGAAUACACGUA		21(16 24)	629	58	550	7	12	2
aca-mir-9_79_contiginputset12.fa.Contig1_+_504_524_extend5p	3p	aca-miR-9/79-3p	3p	AUAAAGCUAGGUUACCAAAGG		21(16 24)	592	459	133	0	0	0
aca-mir-9_79_contiginputset12.fa.Contig1_+_504_524_extend5p	5p	aca-miR-9/79-5p	5p	UCUUUGGUUAUCUAGCUGUAUG		22(16 25)	443	388	55	0	0	0
aca-mir-210	5p	aca-miR-210	miRNA	UUGUGCGUGAGACAGCGACC		20(17 21)	579	40	528	1	5	5
aca-mir-277_gnl_ti_1813892569_+_377_399_extend5p	3p	aca-miR-277	miRNA	UAAAUGCAUUAUCUGGUAUCUGA		23(19 25)	503	495	8	0	0	0
aca-mir-100098_gnl_ti_1816092611_+_412_433_extend5p	3p	aca-miR-100098	miRNA	UGAGACAGUGUGUCCUCCCUUG	~	22(18 23)	493	301	190.5	0.5	0	1
aca-mir-93	5p	aca-miR-93	miRNA	CAAAGUGCUGUUCGUGCAGGUAG		23(16 24)	476	465	11	0	0	0
aca-mir-375	5p	aca-miR-375	miRNA	UUUGUUCGUUCGGCUCGCGUU		21(18 23)	476	475	1	0	0	0
aca-mir-23a	5p	aca-miR-23a	miRNA	AUCACAUUGCCAGGGAUUUCC		21(19 23)	470.5	466.5	4	0	0	0
aca-mir-278	5p	aca-miR-278	miRNA	UCGGUGGGACUUUCGUUCGUUU		22(19 23)	435	432	3	0	0	0
aca-mir-30c	5p	aca-miR-30c	miRNA	UAAACAUCCUACACUCUCAG		20(16 22)	389	388	1	0	0	0
aca-mir-27a	5p	aca-miR-27a	miRNA	UUCACAGUGGCUAAGUUCCGC		21(17 23)	376.5	356	19.5	0	1	0
aca-mir-20	5p	aca-miR-20	miRNA	UAAAGUGCUUAUAGUGCAG		19(16 21)	367	364	3	0	0	0
aca-mir-92b	5p	aca-miR-92b	miRNA	AAUUGCACUAAUCCCGGCCUGC		22(16 24)	358.5	273.5	85	0	0	0
aca-mir-18	5p	aca-miR-18	miRNA	UAAGGUGCAUCUAGUGCAGAUAG		23(17 25)	316	315	1	0	0	0
aca-mir-16	5p	aca-miR-16	miRNA	UAGCAGCACGUAAAUAUUGGCG		22(17 24)	311	289	20	1	1	0
aca-mir-92a_contiginputset46.fa.Contig1_+_596_617_extend5p	3p	aca-miR-92a	miRNA	GAUUGCACUUCUACCGGCCUAC		22(16 24)	291	179.5	110.5	0	0.5	0.5
aca-mir-92a_contiginputset46.fa.Contig2_+_471_492_extend5p	3p	aca-miR-92a	miRNA	GAUUGCACUUCUACCGGCCUAC		22(16 24)	291	179.5	110.5	0	0.5	0.5
aca-mir-100102_contiginputset0.fa.Contig1_+_271_293_extend3p	5p	aca-miR-100102-5p	5p	AUUUGGCACUUGUGGAAUAAUCG		23(16 25)	285	279	6	0	0	0
aca-mir-100102_contiginputset0.fa.Contig1_+_271_293_extend3p	3p	aca-miR-100102-3p	3p	AUUAUACACCGGUGCCAAAU		20(19 24)	151	111	40	0	0	0
aca-mir-281_contiginputset11.fa.Contig1_+_290_310_extend5p	3p	aca-miR-281-3p	3p	UGUCAUGGAGUUGCUCUCUUU		21(16 23)	274	257	17	0	0	0
aca-mir-281_contiginputset11.fa.Contig1_+_290_310_extend5p	5p	aca-miR-281-5p	5p	AAGGGAGCAUCCGUCGACAGU		21(19 25)	111	98	13	0	0	0
aca-let-7f	5p	aca-let-7f	miRNA	UGAGGUAGUAGGUUGUAUAGUU	~	22(17 23)	256.5	237	19	0	0	0.5
aca-mir-100106_gnl_ti_1820396829_+_585_607_extend5p	3p	aca-miR-100106	miRNA	CAUCUACCUAUCCUUCUUCUUC	~	22(19 23)	221	89	124	0	2	6
aca-mir-34a	5p	aca-miR-34a	miRNA	UGGCAGUGUCUUAGCUGGUUGUU		23(18 25)	210.5	201	9.5	0	0	0
aca-mir-252b_contiginputset50.fa.Contig1_+_456_476_extend3p	5p	aca-miR-252b	miRNA	CUAAGUACUGGUGCCGCGGGA		21(20 24)	206	172	34	0	0	0
aca-mir-26a	5p	aca-miR-26a	miRNA	UUCAAGUAAUCCAGGAUAGGCU		22(18 24)	200	197	3	0	0	0
aca-mir-193b_gnl_ti_1805816615_+_696_715_extend5p	3p	aca-miR-193b	miRNA	UACUGGCCUUCAAAAUCCCA	~	20(17 21)	191	185	5	1	0	0
aca-mir-23b	5p	aca-miR-23b	miRNA	AUCACAUUGCCAGGGAUUACC		21(17 23)	182.5	177.5	5	0	0	0
aca-mir-100060_contiginputset38.fa.Contig1_+_740_762_extend3p	5p	aca-miR-100060	miRNA	CUUGGCACUGGCGGAAUAGUCAC		23(19 25)	174	143	31	0	0	0
aca-mir-7_contiginputset36.fa.Contig1_-_897_920_extend5p	5p	aca-miR-7	miRNA	UGGAAGACUAGUGAUUUAGUUGUU		24(17 26)	166	109	55	0	1	1
aca-mir-7_contiginputset36.fa.Contig1_-_897_920_extend5p	3p	<unknown>	miRNA*	CAAUUAAUCACAAUCUUCUAUGA		23(22 24)	7	7	0	0	0	0

aca-mir-324	5p	aca-miR-324	miRNA	CCACUGCCCCAGGUGCUGCUGG		22(17 25)	163	150	12	0	1	0
aca-mir-24	5p	aca-miR-24	miRNA	UGGCUCAGUUCAGCAGGAACAGU		23(17 24)	149	149	0	0	0	0
aca-mir-122	5p	aca-miR-122	miRNA	UGGAGUGUGACAAUGGUGUUUG		22(20 24)	142	70	64	1	7	0
aca-mir-103	5p	aca-miR-103	miRNA	AGCAGCAUUGUACAGGGCUAUG		22(16 23)	130.5	125.5	5	0	0	0
aca-mir-100072_gnl_ti_1811851617_+_253_275_extend3p	5p	aca-miR-100072	miRNA	UUACCCUGGAGAACCGAGCGUGU		23(22 28)	125	114	11	0	0	0
aca-let-7i	5p	aca-let-7i	miRNA	UGAGGUAGUAGUUUGUGCUGU		21(20 23)	124.5	119.5	5	0	0	0
aca-mir-250b_contiginputset5.fa.Contig1_+_489_510_extend5p	3p	aca-miR-250b	miRNA	UAUCACAGCCAACAGAUGGGCU		22(19 25)	117	46.5	70	0	0.5	0
aca-mir-250b_contiginputset5.fa.Contig2_-_592_613_extend3p	3p	aca-miR-250b	miRNA	UAUCACAGCCAACAGAUGGGCU		22(19 25)	117	46.5	70	0	0.5	0
aca-mir-106b	5p	aca-miR-106b	miRNA	UAAAGUGCUGACAGUGCAG		19(16 21)	116	115	1	0	0	0
aca-mir-365b_contiginputset53.fa.Contig1_+_297_317_extend5p	3p	aca-miR-365b	miRNA	UAAUGCCCCCACUAACCCUAA		21(18 25)	115	108	7	0	0	0
aca-mir-365b_contiginputset53.fa.Contig1_+_297_317_extend5p	5p	<unknown>	miRNA*	UGGGGUUAUUGUCGGCAUUGCA		22(20 22)	2	2	0	0	0	0
aca-mir-125b	5p	aca-miR-125b	miRNA	UCCCUGAGACCCUAACUUGUGA		22(20 24)	112.5	105.5	7	0	0	0
aca-mir-100070_gnl_ti_1149853407_+_168_186_extend5p	3p	aca-miR-100070	miRNA	GAAGCGGGUGCUCUUAUUU	~	19(16 20)	109	108	1	0	0	0
aca-mir-196a	5p	aca-miR-196a	miRNA	UAGGUAGUUUCAUGUUGUUGGG		22(21 24)	105	103	2	0	0	0
aca-mir-100090_contiginputset35.fa.Contig1_+_415_433_extend5p	3p	aca-miR-100090	miRNA	UAUCCGCUCACAAUUCCCC		19(16 22)	102	90	12	0	0	0
aca-mir-235_gnl_ti_1154236445_+_258_281_extend5p	3p	aca-miR-235	miRNA	UAUUGCACUUUCCAUGGCCUUC		22(20 24)	101	99	2	0	0	0
aca-mir-203	5p	aca-miR-203	miRNA	UGAAAUGUUUAGGACCACUAGU		22(19 23)	99	98	1	0	0	0
aca-mir-107	5p	aca-miR-107	miRNA	AGCAGCAUUGUACAGGGCUAUGA	~	23(16 23)	98.5	97.5	1	0	0	0
aca-mir-15a	5p	aca-miR-15a	miRNA	UAGCAGCACAUAAUGGUUUGUG		22(20 24)	97	87	10	0	0	0
aca-mir-130a	5p	aca-miR-130a	miRNA	CAGUGCAAUGUUAAAAGGGCAU		22(17 24)	84	78	6	0	0	0
aca-mir-99b	5p	aca-miR-99b	miRNA	CACCCGUAGAACCGACCUUGCG		22(20 23)	83	77.5	3.5	2	0	0
aca-mir-13_contiginputset33.fa.Contig2_-_1348_1370_extend3p	3p	aca-miR-13	miRNA	UAUCACAGUAAUUUAGAUGGGCU		23(21 25)	76	54.5	21	0.5	0	0
aca-mir-13_contiginputset33.fa.Contig1_-_1392_1414_extend3p	3p	aca-miR-13	miRNA	UAUCACAGUAAUUUAGAUGGGCU		23(21 25)	76	54.5	21	0.5	0	0
aca-mir-7d	5p	aca-miR-7d	miRNA	AGAGGUAGUAGGUUGCAUAGUU		22(20 23)	75.3333333	75.3333333	0	0	0	0
aca-mir-100087_contiginputset43.fa.Contig1_+_443_465_extend3p	5p	aca-miR-100087	miRNA	UUGUGACCGUUAUAAUGGGCAUU		23(20 25)	75	70	5	0	0	0
aca-mir-221	5p	aca-miR-221	miRNA	AGCUACAUUGUCUGCUGGGUUUC		23(16 24)	74	74	0	0	0	0
aca-mir-486	5p	aca-miR-486	miRNA	UCCUGUACUGAGCUGCCCCGAG		22(19 24)	70	67	3	0	0	0
aca-mir-27b	5p	aca-miR-27b	miRNA	UUCACAGUGGCUAAGUUCUGC		21(19 23)	69.5	63	6.5	0	0	0
aca-mir-100091_gnl_ti_1161037233_-_93_114_extend5p	5p	aca-miR-100091	miRNA	AGCGGUGAUAUUUUUGUCUGGC		22(20 24)	69	9	59	0	1	0
aca-mir-100091_gnl_ti_1161037233_-_93_114_extend5p	3p	<unknown>	miRNA*	UAGGACAAAACUAUCACCGAUU	~	22(19 22)	2.5	0	2.5	0	0	0
aca-mir-145	5p	aca-miR-145	miRNA	GUCCAGUUUUCCCAGGAAUCCC		22(21 24)	66	66	0	0	0	0
aca-mir-126	5p	aca-miR-126	miRNA	UCGUACCGUGAGUAAUAAUGCG		22(19 23)	66	65	1	0	0	0
aca-mir-200b	5p	aca-miR-200b	miRNA	UAAUACUGCCUGGUAAUGAUGAC		23(21 24)	62	62	0	0	0	0
aca-mir-98	5p	aca-miR-98	miRNA	UGAGGUAGUAAGUUGUAUUGUU		22(17 24)	60.1666667	32.6666667	27	0	0.5	0

aca-mir-252a	5p	aca-miR-252a	miRNA	AUAAGUAGUGGUGCCGCAGGUA		22(19 24)	60	60	0	0	0	0
aca-mir-143	5p	aca-miR-143	miRNA	UGAGAUGAAGCACUGUAGCUC		21(19 23)	57	56	1	0	0	0
aca-mir-100111_contiginputset22.fa.Contig1_+_783_804_extend5p	5p	<unknown>	5p	UGAGUAUUACUUCAGGUUACUGA	~	23(16 23)	55	53	2	0	0	0
aca-mir-100111_contiginputset22.fa.Contig1_+_783_804_extend5p	3p	aca-miR-100111	3p	AGUACCUAAUGUGAUAUUCUCA		22(20 22)	48	45	3	0	0	0
aca-mir-205	5p	aca-miR-205	miRNA	UCCUUCAUUCCACCGGAGUCUG		22(20 23)	54	54	0	0	0	0
aca-mir-19b	5p	aca-miR-19b	miRNA	UGUGCAAAUCCAUGCAAAACUG		22(18 23)	52.5	52.5	0	0	0	0
aca-mir-15b	5p	aca-miR-15b	miRNA	UAGCAGCACAUCAUGGUUUACA		22(17 22)	50	49	1	0	0	0
aca-mir-26b	5p	aca-miR-26b	miRNA	UUCAAGUAAUUCAGGAUAGGUU		22(19 23)	48	47	1	0	0	0
aca-mir-424_322	5p	aca-miR-424/322	miRNA	CAGCAGCAAUUCAUGUUUUGA		21(17 23)	47	32	13	0	2	0
aca-mir-100096b_contiginputset33.fa.Contig1_-_767_786_extend3p	3p	aca-miR-100096b	miRNA	UCACAGCCAGUUUGAUGAGC		20(20 24)	45.2916667	29.9166667	14.7083333	0	0.5	0.16666667
aca-mir-100096b_contiginputset33.fa.Contig1_-_767_786_extend3p	5p	<unknown>	miRNA*	GCUUGUCAAUCUGGAUGUGCUG		22(22 22)	1.5	0	1.5	0	0	0
aca-mir-100096b_contiginputset33.fa.Contig2_-_723_742_extend3p	3p	aca-miR-100096b	miRNA	UCACAGCCAGUUUGAUGAGC		20(20 24)	45.2916667	29.9166667	14.7083333	0	0.5	0.16666667
aca-mir-100096b_contiginputset33.fa.Contig2_-_723_742_extend3p	5p	<unknown>	miRNA*	GCUUGUCAAUCUGGAUGUGCUG		22(22 22)	1.5	0	1.5	0	0	0
aca-mir-574	5p	aca-miR-574	miRNA	CACGCUCAUGCACACACCCACA		22(20 22)	45	41	4	0	0	0
aca-mir-21	5p	aca-miR-21	miRNA	UAGCUUAUCAGACUGAUGUUGA		22(21 24)	43	37	6	0	0	0
aca-mir-199a	5p	aca-miR-199a	miRNA	CCCAGUGUUCAGACUACCUGUUC		23(20 24)	41	18	22	0	1	0
aca-mir-151	5p	aca-miR-151	miRNA	UCGAGGAGCUCACAGUCUAGU		21(20 22)	39	37	1	1	0	0
aca-mir-100088_contiginputset33.fa.Contig2_-_1835_1856_extend3p	3p	aca-miR-100088	miRNA	UAUCACAAUCAUUUCUGGGGUG		22(19 24)	38.5	20.5	17	0	1	0
aca-mir-100088_contiginputset33.fa.Contig1_-_1882_1903_extend3p	3p	aca-miR-100088	miRNA	UAUCACAAUCAUUUCUGGGGUG		22(19 24)	38.5	20.5	17	0	1	0
aca-mir-214	5p	aca-miR-214	miRNA	ACAGCAGGCACAGACAGGCAGU		22(19 23)	38	36	1	0	1	0
aca-mir-125a	5p	aca-miR-125a	miRNA	UCCCUGAGACCCUUUAACCUGUG		23(21 25)	37	32	5	0	0	0
aca-mir-365a	5p	aca-miR-365a	miRNA	UAAUGCCCCUAAAAAUCCUUAU		22(20 24)	37	37	0	0	0	0
aca-mir-22a	5p	aca-miR-22a	miRNA	AAGCUGCCAGUUGAAGAACUGU		22(19 22)	37	37	0	0	0	0
aca-mir-152	5p	aca-miR-152	miRNA	UCAGUGCAUGACAGAACUUGGG		22(21 24)	37	34	3	0	0	0
aca-mir-301a	5p	aca-miR-301a	miRNA	CAGUGCAAUAGUAUUGUCAAAG		22(16 23)	37	37	0	0	0	0
aca-mir-72_31b_contiginputset37.fa.Contig1_+_192_214_extend3p	5p	aca-miR-72/31b	miRNA	AGGCAAGAUGUUGGCAUAGCUGA		23(18 24)	36	32.5	3.5	0	0	0
aca-mir-72_31b_contiginputset37.fa.Contig1_+_192_214_extend3p	3p	<unknown>	miRNA*	AGCUGUGUCAUAUGUUGCCAAU		22(22 22)	3	3	0	0	0	0
aca-mir-250a_contiginputset5.fa.Contig1_+_48_69_extend5p	3p	aca-miR-250a	miRNA	AAUCACAGUCAAUAGAUGGGCA		22(20 23)	36	28	8	0	0	0
aca-mir-72_31b_contiginputset37.fa.Contig2_+_146_168_extend3p	5p	aca-miR-72/31b	miRNA	AGGCAAGAUGUUGGCAUAGCUGA		23(18 24)	36	32.5	3.5	0	0	0
aca-mir-72_31b_contiginputset37.fa.Contig2_+_146_168_extend3p	3p	<unknown>	miRNA*	AGCUGUGUCAUAUGUUGCCAAU		22(22 22)	3	3	0	0	0	0
aca-mir-250a_contiginputset5.fa.Contig2_-_1033_1054_extend3p	3p	aca-miR-250a	miRNA	AAUCACAGUCAAUAGAUGGGCA		22(20 23)	36	28	8	0	0	0
aca-mir-31a	5p	aca-miR-31a	miRNA	AGGCAAGAUGCUGGCAUAGCUG		22(19 23)	36	36	0	0	0	0
aca-mir-30d	5p	aca-miR-30d	miRNA	UGUAAACAUCCCCGACUGGAAGC		23(18 24)	35	35	0	0	0	0
aca-mir-138	5p	aca-miR-138	miRNA	AGCUGGUGUUGUGAAUCAGGCCG		23(21 23)	34	34	0	0	0	0

aca-mir-222	5p	aca-miR-222	miRNA	AGCUACAUCUGGCUACUGGGU		21(17 23)	33	33	0	0	0	0
aca-mir-219_contiginputset52.fa.Contig1_+_435_455_extend3p	5p	aca-miR-219	5p	UGAUUGUCCAAACGCAAUUCU		21(20 24)	32	30	2	0	0	0
aca-mir-219_contiginputset52.fa.Contig1_+_435_455_extend3p	3p	<unknown>	3p	AGAACUGUGUAUGGACAUCAG		21(21 22)	19	19	0	0	0	0
aca-mir-196b	5p	aca-miR-196b	miRNA	UAGGUAGUUUCCUGUUGUUGGG		22(17 24)	31	31	0	0	0	0
aca-mir-263_contiginputset25.fa.Contig1_+_584_606_extend3p	5p	aca-miR-263	miRNA	AAUGGCACUGGUAGAAUUCACGG		23(18 23)	30	7	22	0	1	0
aca-mir-263_contiginputset25.fa.Contig1_+_584_606_extend3p	3p	<unknown>	miRNA*	GUGUUCUUCCGGUGGCAUCCA		21(21 21)	4	0	4	0	0	0
aca-mir-33_gnl_ti_1163625215_+_125_145_extend3p	5p	aca-miR-33	miRNA	GUGCAUUGUAGUUGCAUUGCG		21(16 24)	25	14	11	0	0	0
aca-mir-33_gnl_ti_1163625215_+_125_145_extend3p	3p	<unknown>	miRNA*	CAGUGCAUCUGCAGUGCAAUCA	~	22(22 22)	2	0	2	0	0	0
aca-mir-218	5p	aca-miR-218	miRNA	UUGUGCUUGAUCUAACCAUGUG		22(21 23)	25	25	0	0	0	0
aca-mir-652	5p	aca-miR-652	miRNA	AAUGGCGCCACUAGGGUUGUG		21(17 23)	24	24	0	0	0	0
aca-mir-30a	5p	aca-miR-30a	miRNA	CUUUCAGUCGGAUGUUUGCAGC		22(22 22)	23	23	0	0	0	0
aca-mir-451	5p	aca-miR-451	miRNA	AAACCGUUACCAUUACUGAGU		21(21 22)	23	23	0	0	0	0
aca-mir-141	5p	aca-miR-141	miRNA	UAACACUGUCUGGUAAAGAUGG		22(21 23)	22.5	22.5	0	0	0	0
aca-mir-191	5p	aca-miR-191	miRNA	CAACGGAAUCCCAAAAGCAG		20(19 22)	22	20	2	0	0	0
aca-mir-509	5p	aca-miR-509	miRNA	UACUGCAGACGUGGCAAUCAUG		22(21 23)	19	9	9	1	0	0
aca-mir-25	5p	aca-miR-25	miRNA	CAUUGCACUUGUCUCGGUCUG		21(16 22)	18.5	18.5	0	0	0	0
aca-mir-182	5p	aca-miR-182	miRNA	UUUGGCAAUGGUAGAACUCACACU		24(19 26)	18	18	0	0	0	0
aca-mir-224	5p	aca-miR-224	miRNA	CAAGUCACUAGUGGUUCCGUUUAG		24(22 24)	18	18	0	0	0	0
aca-mir-197	5p	aca-miR-197	miRNA	UUCACCACCUUCUCCACCCAGC		22(18 23)	18	18	0	0	0	0
aca-mir-148a	5p	aca-miR-148a	miRNA	UCAGUGCACUACAGAACUUUGU		22(21 23)	17	15	2	0	0	0
aca-mir-505	5p	aca-miR-505	miRNA	CGUCAACACUUGCUGGUUUCCUCU		24(22 24)	16	16	0	0	0	0
aca-mir-181a	5p	aca-miR-181a	miRNA	AACAUUCAACGCUGUCGGUGAGU		23(22 23)	16	16	0	0	0	0
aca-mir-301b	5p	aca-miR-301b	miRNA	CAGUGCAAUGAUAUUGUCAAAGC		23(21 23)	16	16	0	0	0	0
aca-mir-766	5p	aca-miR-766	miRNA	ACUCCAGCCCCACAGCCUCAG		21(17 22)	16	16	0	0	0	0
aca-mir-455	5p	aca-miR-455	miRNA	UAUGUGCCUUUGGACUACAUCG		22(22 24)	13	13	0	0	0	0
aca-mir-330	5p	aca-miR-330	miRNA	CAAAGCACACGGCCUGCAG		19(16 20)	11	11	0	0	0	0
aca-mir-328	5p	aca-miR-328	miRNA	CUGGCCCUCUCUGCCCUUCCGU		22(21 23)	10	10	0	0	0	0
aca-mir-508	5p	aca-miR-508	miRNA	UGAUUGUAGCCUUUUGGAGUAG		22(22 23)	10	10	0	0	0	0
aca-mir-193a_gnl_ti_1816120363_+_789_807_extend3p	5p	aca-miR-193a	miRNA	AACUGGCCUACAAAGUCCCA	~	20(20 21)	7	7	0	0	0	0
aca-mir-100112_gnl_ti_1181921049_+_475_493_extend5p	3p	aca-miR-100112	miRNA	CGGGAGGCAGAGGUUGCAG	~	19(19 19)	2.5	2.5	0	0	0	0
aca-mir-100113_contiginputset2.fa.Contig1_-_58_77_extend3p	3p	aca-miR-100113	miRNA	UCAGAGUUAGAUGAUGCUGU		20(20 22)	5	5	0	0	0	0
aca-mir-100113_contiginputset2.fa.Contig2_-_84_103_extend3p	3p	aca-miR-100113	miRNA	UCAGAGUUAGAUGAUGCUGU		20(20 22)	2.5	2.5	0	0	0	0

Supplementary Table 3: miRNA candidate list

precursor	arm	mature	form	most matched sequence	ext	len(min, max)	copies	CNS-less-454	CNS-454	CNS	Pl. Ganglia	Abd. Ganglia
aca-mircandidate-4	5p	aca-miRcandidate-4	miRNA	UUCGUUGUCGUCGAAACCUGCC		22(16 25)	3653	712	2876	13	22	30
aca-mircandidate-5	5p	aca-miRcandidate-5	miRNA	UAGGGCAAAACUAUCACCGAUU		22(17 23)	1149.5	84	1043.5	4	9	9
aca-mircandidate-6	5p	aca-miRcandidate-6	miRNA	CCUGGUCCACAUGGGUCGGA		20(18 21)	755	753	2	0	0	0
aca-mircandidate-18	5p	aca-miRcandidate-18	miRNA	UGAGACAGUGCGUCCUCCCUCA		22(18 23)	438	362	75.5	0.5	0	0
aca-mircandidate-19	5p	aca-miRcandidate-19	miRNA	GAGGUGUUUCAAAGGCGCUAC		21(19 23)	343	14	321	2	5	1
aca-mircandidate-8	5p	aca-miRcandidate-8	miRNA	GGGGAGUGUUUGCCACGGUGGUG		23(22 25)	159	6	150	0	3	0
aca-mircandidate-9	5p	aca-miRcandidate-9	miRNA	GUUCAAAUCCUCUACAGCGUACCA		24(17 24)	131	131	0	0	0	0
aca-mircandidate-7	5p	aca-miRcandidate-7	miRNA	CUUGUGCGUGUCACAGUAGC		20(20 21)	127	119	8	0	0	0
aca-mircandidate-57	5p	aca-miRcandidate-57	miRNA	UAUUAUGCUGCUAUUCACGAGA		22(20 22)	117	107	10	0	0	0
aca-mircandidate-10	5p	aca-miRcandidate-10	miRNA	GAGGUCCGACGGCAGUGGUAUA		22(16 23)	108	16	89	3	0	0
aca-mircandidate-11	5p	aca-miRcandidate-11	miRNA	GUUCAAAUCUUGUAUCCGGCGCCA		24(17 25)	107	104	3	0	0	0
aca-mircandidate-16	5p	aca-miRcandidate-16	miRNA	GUUCGAAUCCUGCCACGAUCACCA		24(18 25)	94	93	1	0	0	0
aca-mircandidate-13	5p	aca-miRcandidate-13	miRNA	AUGGAACCUUUUAACUUUGGCU		22(20 23)	91	90	1	0	0	0
aca-mircandidate-12	5p	aca-miRcandidate-12	miRNA	UUAGGGCCCUGGCUCCAUCUCC		22(16 23)	89	88	1	0	0	0
aca-mircandidate-14	5p	aca-miRcandidate-14	miRNA	AAUGAGGUCCGUAACGCGAUCGGC		24(16 26)	84	75	9	0	0	0
aca-mircandidate-15	5p	aca-miRcandidate-15	miRNA	UAGGGUACUACGUGUGUUACACA		23(20 25)	84	56	28	0	0	0
aca-mircandidate-17	5p	aca-miRcandidate-17	miRNA	UGGAACUCACACAUGCUUGG		20(20 21)	57	57	0	0	0	0
aca-mircandidate-56	5p	aca-miRcandidate-56	miRNA	GGCUGGUCCGAUGGUAGUGGG		21(19 22)	51	50	1	0	0	0
aca-mircandidate-67	5p	aca-miRcandidate-67	miRNA	UGAUUGGUACGUCUGUGGGUAA		22(20 23)	22	17	5	0	0	0
aca-mircandidate-23	5p	aca-miRcandidate-23	miRNA	CCGUGCAAUGAUGAAAGGGCAG		22(21 22)	16	16	0	0	0	0
aca-mircandidate-33	5p	aca-miRcandidate-33	miRNA	AUGUGCAAAUCUAUGCAAAACUGA	~	24(22 24)	15.5	15.5	0	0	0	0
aca-mircandidate-39	5p	aca-miRcandidate-39	miRNA	UUAUAAUACAACCUGAUAAGUG		22(16 22)	14	14	0	0	0	0
aca-mircandidate-55	5p	aca-miRcandidate-55	miRNA	AGGAAGCCCUGGAGGGGCUGGAGG		24(16 24)	12	12	0	0	0	0
aca-mircandidate-47	5p	aca-miRcandidate-47	miRNA	UCAGGCUCAGUCCCCUCCCGAA		22(20 22)	11	11	0	0	0	0
aca-mircandidate-44	5p	aca-miRcandidate-44	miRNA	CAUCGGGAAUGUCGUGUCCGC		21(21 22)	11	2	7	1	1	0
aca-mircandidate-50	5p	aca-miRcandidate-50	miRNA	UUCACAGGGAGGUGUCAUUUAUG		23(19 23)	10	4	5	0	1	0
aca-mircandidate-46	5p	aca-miRcandidate-46	miRNA	UAGUGCAAUAUUGCUUAUAGGG		22(20 23)	10	10	0	0	0	0
aca-mircandidate-49	5p	aca-miRcandidate-49	miRNA	GACCCUGGUCUGCACUCUAUC		21(20 21)	10	10	0	0	0	0
aca-mircandidate-24	5p	aca-miRcandidate-24	miRNA	UAACAGUCUACAGCCAUGGUCA		22(19 22)	10	6	4	0	0	0
aca-mircandidate-27	5p	aca-miRcandidate-27	miRNA	UCAGUGCAUCACAGAACUUUGU		22(22 24)	9	9	0	0	0	0
aca-mircandidate-34	5p	aca-miRcandidate-34	miRNA	UAACACUGUCUGGUAACGAUGU		22(21 22)	9	9	0	0	0	0

aca-mircandidate-22	5p	aca-miRcandidate-22	miRNA	UCACAGUGAACCGGUCUCUUAAGC	24(21 24)	8	7	1	0	0	0
aca-mircandidate-51	5p	aca-miRcandidate-51	miRNA	CAUGCCUUGAGUGUAGGACCGU	22(21 22)	8	7	1	0	0	0
aca-mircandidate-30	5p	aca-miRcandidate-30	miRNA	UAUGGCACUGGUAGAAUUCACU	22(22 22)	8	8	0	0	0	0
aca-mircandidate-40	5p	aca-miRcandidate-40	miRNA	ACUGGACUUGGAGUCAGAAGG	21(21 21)	8	8	0	0	0	0
aca-mircandidate-53	5p	aca-miRcandidate-53	miRNA	UCCGAGCCUGGGUCUCCCUCUUA	23(18 23)	8	8	0	0	0	0
aca-mircandidate-62	5p	aca-miRcandidate-62	miRNA	CUUCCGCAACCAUUUAUGAUGUGUGC	26(26 26)	8	8	0	0	0	0
aca-mircandidate-43	5p	aca-miRcandidate-43	miRNA	CGCCUGCGACUCGGUCCCAG	20(20 21)	7	7	0	0	0	0
aca-mircandidate-37	5p	aca-miRcandidate-37	miRNA	CUAGGUAUGGUCCCAGGGAUCC	22(22 22)	7	7	0	0	0	0
aca-mircandidate-21	5p	aca-miRcandidate-21	miRNA	AAGGAGCUUACAAUCUAGCUG	21(20 24)	7	7	0	0	0	0
aca-mircandidate-52	5p	aca-miRcandidate-52	miRNA	UCGGGGAUCAUCAUGUCACGAG	22(22 22)	7	7	0	0	0	0
aca-mircandidate-29	5p	aca-miRcandidate-29	miRNA	GUAUGGACAUCAUCGAGAU	19(19 19)	6	6	0	0	0	0
aca-mircandidate-36	5p	aca-miRcandidate-36	miRNA	CCUCUGGGCCCUUCCUCCAGU	21(21 22)	6	6	0	0	0	0
aca-mircandidate-20	5p	aca-miRcandidate-20	miRNA	UGCGGGGCUAGGGCUAACAG	20(19 22)	6	6	0	0	0	0
aca-mircandidate-35	5p	aca-miRcandidate-35	miRNA	UGUCAGUUUGUCAAAUACCCCAU	23(23 23)	6	6	0	0	0	0
aca-mircandidate-3	5p	aca-miRcandidate-3	miRNA	UGAGGGGCAGAGACGAGACUUU	22(20 24)	6	6	0	0	0	0
aca-mircandidate-26	5p	aca-miRcandidate-26	miRNA	UGAGAACUGAAUUCCAUAGGCUG	23(23 24)	6	2	4	0	0	0
aca-mircandidate-54	5p	aca-miRcandidate-54	miRNA	GGAUUACAGGGAUGAGCCACCG	22(22 22)	6	6	0	0	0	0
aca-mircandidate-48	5p	aca-miRcandidate-48	miRNA	AAUGCACCCGGGCAAGGAUUCUG	23(23 23)	6	6	0	0	0	0
aca-mircandidate-45	5p	aca-miRcandidate-45	miRNA	UGGCAGUGUAUUGUUAGCUGGU	22(22 22)	6	6	0	0	0	0
aca-mircandidate-28	5p	aca-miRcandidate-28	miRNA	UCUCCCAACCCUUGUACCAGUG	22(21 22)	5	4	1	0	0	0
aca-mircandidate-25	5p	aca-miRcandidate-25	miRNA	UCUACAGUGCACGUGUCUCCA	21(21 23)	5	5	0	0	0	0
aca-mircandidate-42	5p	aca-miRcandidate-42	miRNA	AGAUCAGAAGGUGAUUGUGGCU	22(22 22)	5	5	0	0	0	0
aca-mircandidate-1	5p	aca-miRcandidate-1	miRNA	UGUAACAGCAACUCCAUGUGG	21(16 21)	5	4	1	0	0	0
aca-mircandidate-38	5p	aca-miRcandidate-38	miRNA	UUAUCAGAAUCUCCAGGGGUAC	22(22 23)	5	5	0	0	0	0
aca-mircandidate-2	5p	aca-miRcandidate-2	miRNA	UUCCCUUUGCCAUCCUUCGCCU	22(21 22)	5	5	0	0	0	0
aca-mircandidate-31	5p	aca-miRcandidate-31	miRNA	CAAAGAAUUCUCCUUUUGGGCUU	23(23 24)	5	5	0	0	0	0
aca-mircandidate-41	5p	aca-miRcandidate-41	miRNA	UGGUAGACUAUGGAACGUAGG	21(21 22)	4	2	2	0	0	0
aca-mircandidate-70	5p	aca-miRcandidate-70	miRNA	AACAAACAUGGUGCACUUCUU	21(21 22)	4	4	0	0	0	0
aca-mircandidate-68	5p	aca-miRcandidate-68	miRNA	UAGGUUAUCCGUGUUGCCUUCG	22(22 22)	3	3	0	0	0	0
aca-mircandidate-71	5p	aca-miRcandidate-71	miRNA	AUCAACAGACAUUAAUUGGGCGC	23(22 23)	3	3	0	0	0	0
aca-mircandidate-66	5p	aca-miRcandidate-66	miRNA	UUUGGCACUAGCACAUUUUUGC	22(18 22)	3	3	0	0	0	0
aca-mircandidate-32	5p	aca-miRcandidate-32	miRNA	CCCAGUGUUUAGACUAUCUGUU	22(21 23)	3	2	1	0	0	0
aca-mircandidate-65	5p	aca-miRcandidate-65	miRNA	GUAAGGCACCCUUCUGAGUAGA	22(22 22)	3	3	0	0	0	0
aca-mircandidate-72	5p	aca-miRcandidate-72	miRNA	UCGGAUCCGUCUGAGCUUGG	20(20 20)	2	0	2	0	0	0

aca-mircandidate-64	5p	aca-miRcandidate-64	miRNA	AAUCGUACAGGGUCAUCCACU	21(21 21)	2	2	0	0	0	0
aca-mircandidate-60	5p	aca-miRcandidate-60	miRNA	UAUGCAAGGGCAAGCUCUCUG	21(21 21)	1	0	1	0	0	0
aca-mircandidate-69	5p	aca-miRcandidate-69	miRNA	UAUGCCUGCUGACCAUCACC	20(20 20)	1	1	0	0	0	0
aca-mircandidate-59	5p	aca-miRcandidate-59	miRNA	AUCAUGAUGGGCUCCUCGGUG	21(21 21)	1	0	1	0	0	0
aca-mircandidate-74	5p	aca-miRcandidate-74	miRNA	CCAUUGCAUAUCGGAGUUG	19(19 19)	1	1	0	0	0	0
aca-mircandidate-58	5p	aca-miRcandidate-58	miRNA	GCCUGCUGGGGUGGAACCUGG	21(21 21)	1	0	1	0	0	0
aca-mircandidate-75	5p	aca-miRcandidate-75	miRNA	UGUGACUGGUUGACCAGAGGGG	22(22 22)	1	0	1	0	0	0
aca-mircandidate-63	5p	aca-miRcandidate-63	miRNA	AAUGUUGCUCGGUGAACCCCU	21(21 21)	1	1	0	0	0	0
aca-mircandidate-61	5p	aca-miRcandidate-61	miRNA	UGAAGGUCUACUGUGUGCCAGU	22(22 22)	1	0	1	0	0	0
aca-mircandidate-73	5p	aca-miRcandidate-73	miRNA	UACUCAGGAGAGUGGCAAUCA	21(21 21)	1	1	0	0	0	0

Supplementary Table 4: Small RNA composition of cDNA libraries, Round 2

category	Ad_CNS	J_CNS	Ad_OT	J_OT	Ad_Musc	J_Musc	Ad_H	J_H	Ad_L	J_L	CNS_Sol	CNS_454	whole_454	total
miRNA	574937	347308	137473	119682	520226	437821	556817	230210	68378	49633	16086597	175345	114627	19419054
None	377392	335211	525837	652066	436043	291903	413922	208915	351837	117802	3072673	6494	9966	6800061
piRNA	126504	78050	243867	366461	134501	71044	115539	45031	19566	12343	2310847	6233	2343	3532329
rRNA	275987	272269	21547	137783	167953	195667	215721	132341	56635	9047	105568	1601	59616	1651735
tRNA	48803	10800	63260	57035	18914	23138	160249	5893	3799	1523	109509	3594	4851	511368
mRNA	2766	2158	4355	4400	3986	1967	8525	1295	4249	596	13240	234	517	48288
SnoRNA	306	156	3966	4426	225	110	759	31	25	15	896	3	316	11234
e_coli	142	309	145	263	220	1168	460	286	135	73	2102	3	134	5440
low_cmplx	54	24	721	39	38	21	62	15	10	4	738	3	110	1839
miscRNA	10	4	48	10	6	6	5	8	11	13	0	5	36	162
Total	1406901	1046289	1001219	1342165	1282112	1022845	1472059	624025	504645	191049	21702170	193515	192516	31981510

Solexa bar coded libraries:

Ad_CNS: Aplysia Adult CNS
Ad_OT: Aplysia Adult Ovotestes
Ad_Musc: Aplysia Adult Muscle
Ad_H: Aplysia Adult Heart
Ad_L: Aplysia Adult Liver
J_CNS: Aplysia Juvenile CNS
J_OT: Aplysia Juvenile Ovotestes
J_Muscle: Aplysia Juvenile Muscle
J_H: Aplysia Juvenile Heart
J_L: Aplysia Juvenile Liver

Solexa single run:
CNS_Sol: Aplysia Adult CNS

454 libraries:
CNS_454: Aplysia Adult CNS
Wh_454: Aplysia Adult Whole animal minus CNS

Supplementary Table 5: piRNA expression profile – top 500

seq	total	Ad_CNS	J_CNS	Ad_OT	J_OT	Ad_Musc	J_Musc	Ad_H	J_H	Ad_L	J_L	CNS_Sol	CNS_454	Wh_454	Scaffold Location
TAGAAGCTTGATTTGTAGCATTTAAGGTT	241980	18358	10956	10859	3990	49431	7069	11559	8691	2299	333	118324	72	39	_aplCal1_2321_14968_33608
CGACATTATTTCAACTGCTGAGGCAGTCCT	69753	10	1338	579	1199	1	3	2	2	1	0	66618	0	0	_aplCal1_2299_30198_72073
TCCATCCGGTTCTCGAGAAGTTCTGCTTT	67538	3722	898	1895	747	618	588	1660	174	186	79	56837	121	13	_aplCal1_1895_51672_71846
TAGAATAATTTCCAGGACGTAGGAATTGT	38639	1611	1341	1448	2255	1662	1849	2221	1441	1034	529	23208	21	19	_aplCal1_610_113720_122340
TACGTCACATTGGTCCTGACCTATTGGTG	37125	228	67	4	25	105	24	414	15	7	3	36210	23	0	_aplCal1_1036_138128_154149
TTTTGTTGTGAAAATATGCTGACATGTTT	36803	3977	2771	3047	1273	1770	1725	4489	964	1035	1739	13984	6	23	_aplCal1_2339_7934_26341
TAACATCTGATACCGGTGACGGGAGAGG	36082	428	0	168	1	167	0	216	5	5	0	35092	0	0	_aplCal1_1712_18148_35054
TCAAATCTGGTCCTGGTTGCAAAAGCTCC	33296	264	0	19	1	65	0	71	1	6	0	32869	0	0	_aplCal1_2299_30198_72073
TACCAAGTACGGTGCCCGAACGGTGGCTGGT	29614	503	358	93	89	1700	81	592	150	34	12	25996	0	6	_aplCal1_1036_128796_134511
TGAATTGTCTTGTATGTTTAGTAGTGT	27186	1008	455	473	477	1479	929	1042	522	1326	379	18927	46	123	_aplCal1_2339_7934_26341
TCCAAGTCGAGCAATCCTTGCTGAAGGCT	24839	742	709	27	238	442	538	843	615	18	41	20613	3	10	_aplCal1_694_58829_61351
TTGATGATTTCGGGCAGCACCTCTGCATG	24496	161	8	305	274	109	66	85	117	9	20	23340	2	0	_aplCal1_155_244249_252418
GACCAAGTACGATGCCTGAACGGTGGCT	24070	569	460	53	288	55	104	261	75	18	4	22115	66	2	_aplCal1_2491_12726_20630
TAAACTATCGCAGTATTTGACCACAGGCC	22009	140	59	357	156	100	21	80	108	45	4	20933	5	1	_aplCal1_1667_1422_36545
TGTAACTGAGGACATCATACTTTACTCTGAT	21114	99	240	13	36	179	299	102	116	60	155	19815	0	0	_aplCal1_379_63480_78689
ATCGAGAACATTGATAATAGTGAAGTTG	20515	424	4	212	4	581	4	364	2	95	2	18823	0	0	_aplCal1_2299_30198_72073
TGTCGTTGGACCTGTAAAACACAGGGTCA	20315	94	3	5	3	2	0	1	1	0	0	20206	0	0	_aplCal1_1712_18148_35054
TCACCCTGACATGTGTGTTTCTCTCACCC	20229	235	134	56	96	306	231	260	79	154	66	18567	19	26	_aplCal1_2339_7934_26341
TAGAATTGGAATATGGATCCTCCATAGAGGT	19471	408	364	640	456	195	422	99	133	35	51	16664	4	0	_aplCal1_5_620223_635068
TGGAGAGATGAAAAGTTCAACTTTTGGGC	18418	407	127	52	66	154	34	144	83	32	26	17289	4	0	_aplCal1_1895_51672_71846
TAAATATTGTACTGCGAAAGGCGAGGAGC	18018	520	521	14	147	574	285	1462	309	23	3	14158	2	0	_aplCal1_98_137957_177330
GACCAAGTATGATGCCAAAACGGTGGCTG	17540	578	170	42	88	111	17	180	122	44	1	16159	25	3	_aplCal1_2299_30198_72073
TGCCTTTGATGACAACCTACATGCTGATG	16315	13	58	5	11	31	52	37	23	12	12	16057	4	0	_aplCal1_1280_88301_92322
AAACACATCATCCTTCTGCTGAGGGCCG	16146	228	1	172	1	62	0	134	5	11	0	15532	0	0	_aplCal1_77_95376_114859
TTGCCTGAAGACATCATACACAACTCTGATA	15630	55	108	12	28	89	97	53	65	32	54	15037	0	0	_aplCal1_379_63480_78689
TAGGTGTAGCAAAGTATACCTCATTAGGTG	15030	110	44	159	122	122	1	2	83	0	0	14383	4	0	_aplCal1_1667_1422_36545
TGGTTGACACTCTCGTGTTTTTGAACGGC	14752	306	364	263	89	43	115	403	70	30	11	13042	16	0	_aplCal1_2082_38884_60606
AAAATCACGTCGACTTTCGACAACAGTGC	13917	174	0	115	6	109	1	99	2	2	0	13409	0	0	_aplCal1_1712_18148_35054
TCCATCCGGTCCTCGAGAAGTTCTGCTTT	13632	664	298	294	439	106	107	311	212	35	116	10586	444	20	_aplCal1_1895_51672_71846
TACCAGAGCCGGCTGTAATGGTTCAGCCCCG	12681	763	187	215	110	765	225	322	143	170	24	9684	69	4	_aplCal1_610_113720_122340
TTTGCGAGCCCTTCTGCGATGCAGAATAT	11741	23	3	32	1	4	0	47	0	3	1	11627	0	0	_aplCal1_77_95376_114859
TTTGCGAGCCCTTCTGCGATGTAGAATAT	10644	40	7	33	2	10	0	49	3	0	1	10499	0	0	_aplCal1_412_231367_237094
TCACACATTTGTTGACAGCATCTCGGTC	10484	1102	1	88	24	239	173	788	2	21	12	8034	0	0	_aplCal1_645_48662_56779

TCAAAGGTCAGGATGACCGTGTTGGTTT	10457	159	114	193	118	297	39	171	40	87	29	9199	5	6	_aplCal1_0_1461183_1467458
TTGTTATATTTCAACATACTGATGGTGC	10314	55	1	6	1	42	0	53	0	8	0	10128	20	0	_aplCal1_1895_51672_71846
TAGTTACTACAGTTGGGCTTGCAAGTGT	10214	160	10	146	226	148	54	145	11	23	12	9249	21	9	_aplCal1_1667_1422_36545
TACCAAGTAAGATGCCCGAACGGTGGCTG	9920	72	36	341	111	66	9	57	22	3	2	9186	15	0	_aplCal1_1_567304_570505
TCCATCCGGTCCTCGAGAGGTTCTGCTTT	9911	495	141	427	112	104	140	332	2	46	1	8002	109	0	_aplCal1_1895_51672_71846
TCATGAAGGAACCGTCTTAATCCCGAGC	9852	112	327	256	589	244	179	246	53	3	37	7805	1	0	_aplCal1_1667_1422_36545
TCAAATCTGGTCCTGGTTGCGAAAGCTCC	9566	73	0	8	0	10	0	27	0	1	0	9447	0	0	_aplCal1_2299_30198_72073
TGGTATCGTTCGGAAGTACGTGGAATAC	9533	629	1027	35	365	304	702	435	451	273	251	4933	69	59	_aplCal1_0_1461183_1467458
TACTTTTCTAATAAAAGCCTCTGGATG	9400	0	79	4	8	0	16	0	57	0	1	9225	3	7	_aplCal1_39_566527_578980
TCGTAGTTGATCAAGTCCCTCAGTGATG	9367	230	104	88	92	156	2	190	33	19	0	8452	1	0	_aplCal1_101_532920_559602
CGTCATTATTTCAACTGCTGAGGCAGTCCT	9314	3	100	78	83	0	0	0	0	0	0	9050	0	0	_aplCal1_2299_30198_72073
TTCGAAGAACAGACTCAAAGGAGTCGTCA	9203	80	27	85	112	10	13	22	11	1	2	8840	0	0	_aplCal1_2358_50666_58753
TTCGGCTGATGCCTTTGATGACAAC	8935	18	51	7	16	37	42	58	28	15	36	8624	3	0	_aplCal1_1280_88301_92322
TACAATTCAGGCCTGGAAGTTTCTTGGAT	8815	657	632	135	231	252	141	392	334	31	23	5957	26	4	_aplCal1_523_215863_271832
TAGCTAAGTGTTTCTTGCCCGTATCGCAC	8754	556	97	7	35	421	201	248	51	11	10	6954	111	52	_aplCal1_1036_128796_134511
TTGCAAAGGAGACAATCTCACGTTGTTC	8649	454	3	211	38	326	406	1665	2	97	339	5108	0	0	_aplCal1_2321_35916_43532
TCCTTGGTTGAGAACGACTGCCCATTGTGAC	8328	341	98	26	176	848	543	250	91	12	3	5932	3	5	_aplCal1_1036_138128_154149
TTACATTGTGAGTGACGTGACGCGTCCACC	8212	223	51	169	60	173	39	189	30	21	26	7231	0	0	_aplCal1_393_78915_104964
TGTTGGGTTGTGCTCGATCTTGGAGCGCT	8108	66	0	7	0	31	0	42	0	2	0	7960	0	0	_aplCal1_190_74320_82083
TGTTCTTAATGGAAGGCTCATTTAAGCGC	7923	78	0	29	0	19	0	49	2	3	0	7743	0	0	_aplCal1_2299_30198_72073
TCGTAGTTGATCAAGTTCCTCGGTGATG	7813	239	79	119	125	193	0	180	22	13	1	6835	4	3	_aplCal1_101_532920_559602
TTCTAAGAATACTAATGTACTGGTCAGGC	7730	341	0	146	0	396	0	177	0	22	0	6648	0	0	_aplCal1_412_231367_237094
TGGCAATTTGGGCAGCAAGGATGCCCAGT	7682	181	30	12	48	113	23	164	23	3	3	7082	0	0	_aplCal1_1036_128796_134511
TGAGCGGAATATACTGCTTGAACAGCAAGCG	7551	720	145	380	79	137	178	218	59	6	15	5614	0	0	_aplCal1_492_75504_92774
TGTTAAAAAGGTCACAAAGCCATTAGGGTCT	7366	92	0	168	2	36	143	225	0	40	4	6656	0	0	_aplCal1_2321_35916_43532
TTCAGCTAAGGAAGTGACTCTAGTCTCAGG	7256	193	137	569	805	84	189	117	211	46	24	4877	4	0	_aplCal1_1667_1422_36545
TGCAGATTCCTCCAAATACCCACCGGTCT	7218	13	10	32	19	2	1	19	5	1	0	7116	0	0	_aplCal1_101_532920_559602
CACTTTCGTCGATTTCTACAACTTGTCC	7052	3	0	4	6	0	0	0	0	0	0	7039	0	0	_aplCal1_2653_34608_42410
TGGAAGCTGCCATAATCTGCCACATGCCC	6932	43	32	31	64	18	35	7	1	3	1	6692	5	0	_aplCal1_523_215863_271832
TGAATTGTCCTGTATGTTTAGTAGTGTGT	6858	237	110	182	94	378	468	202	241	260	118	4539	24	5	_aplCal1_2339_7934_26341
TGAAGGAAAGGTGATTGCGGCATCCTGCT	6806	261	537	43	438	434	424	308	176	41	111	4012	17	4	_aplCal1_523_215863_271832
TCCGAATTGACGAACGTAGAAGTTTGAGA	6535	3231	74	111	157	272	101	290	112	5	1	2179	2	0	_aplCal1_77_359166_370796
TGAGTTGTCCTGTATGTTTAGTAGTGT	6507	419	239	148	278	332	413	365	310	550	254	3088	92	19	_aplCal1_2339_7934_26341
TCGACAGACCTGTACCCGCCTGGGGCAT	6248	73	0	31	13	65	0	58	7	1	0	6000	0	0	_aplCal1_147_26539_30349
TCCATCCGGTCCTCGAGAAGTTCTGATTT	6124	636	174	387	120	214	133	277	0	67	0	4098	13	5	_aplCal1_1895_51672_71846
TGCCAAATCCATTCTTATAGTCTTTCCACC	6055	360	133	27	51	950	185	211	43	13	2	4051	18	11	_aplCal1_1036_138128_154149

TAGGATAGTCGCCGTCACACTCAGGTGACCC	6052	25	41	65	151	11	26	11	19	17	20	5612	45	9	_aplCal1_697_50289_68955
TTCTGTAGCCTCGCTTTCATAGCCTTGTGC	5972	0	27	14	32	0	31	0	1	0	0	5867	0	0	_aplCal1_2299_30198_72073
TGGTGAGTCTGATGTTTTGTACGTTCGCA	5941	37	99	33	79	49	33	261	311	0	46	4783	201	9	_aplCal1_2321_14968_33608
TACCAAGTACGGTGCCCGAACGGTGGCT	5894	160	35	26	16	267	10	249	18	7	1	5077	27	1	_aplCal1_1036_128796_13451
TGGCAATTGGGGCAACAAGGACTAGCCT	5756	216	412	79	351	40	110	274	72	39	11	3851	262	39	_aplCal1_0_1461183_1467458
TCAGTGTAGTCTCTCTTAGCAGCGAAGTC	5743	100	12	18	45	95	90	106	28	10	0	5239	0	0	_aplCal1_98_137957_177330
TCACCCTGACATGTGTGTTTCCCTCACGCT	5663	68	33	25	28	75	59	89	6	58	20	5174	2	26	_aplCal1_2339_7934_26341
AAGAAAACCACCGCTCGTACTTGAATGTTT	5612	229	219	125	188	400	192	361	101	30	22	3744	1	0	_aplCal1_2082_38884_60606
TTGATGCAGTCCCTGGGATCATTCCCAGT	5583	84	25	0	58	121	1	120	0	19	0	5155	0	0	_aplCal1_455_224202_242097
TTTTGTAAACCCATCTGATGTTGAGATT	5519	544	112	144	47	510	162	383	64	58	37	3456	0	2	_aplCal1_2321_14968_33608
TCATAAACGTATTGGGCCAAGGGCTACCA	5513	3	5	75	262	0	8	10	6	0	2	5142	0	0	_aplCal1_5_620223_635068
TAGGAGCATAGACAAAACATTGAAGTTTT	5414	8	8	2	9	4	0	5	4	0	2	5372	0	0	_aplCal1_77_95376_114859
TAACGTAGAAGCTGTCTGTCAAGCAGAAC	5368	218	7	928	33	638	711	411	34	17	27	2344	0	0	_aplCal1_155_244249_252418
TCTCTCTCAAGGCATTTTCCTGTGGGCC	5340	33	5	11	31	17	6	22	2	11	0	5197	1	4	_aplCal1_2082_38884_60606
TCGATCCTCAGTTCGTATCTCCCTCAGTG	5320	175	109	16	66	858	372	120	49	3	3	3478	9	62	_aplCal1_1036_138128_154149
TCGGCATCGCGACTGACTATGGCCAAACT	5191	1359	424	22	51	956	4	1632	337	19	1	386	0	0	_aplCal1_12_1070314_1075598
TGCACGTCTTGTAACATGCGCAGAATTT	5180	284	61	115	66	51	42	153	67	5	0	4336	0	0	_aplCal1_1895_51672_71846
TACCTACGTGCCACACATCACTGAACCA	5163	92	48	13	22	58	55	64	44	20	12	4697	28	10	_aplCal1_2339_7934_26341
TGACGAAAGTGACATCTACCCTTGAACC	5148	74	2	71	2	70	0	91	10	1	0	4827	0	0	_aplCal1_1712_18148_35054
TAGAACATTGCACAGTCAAGAAGATTGC	5086	1	0	24	6	0	2	0	34	0	0	5019	0	0	_aplCal1_1117_24553_42207
GACGTCACATCTGAATTTACCAATGGGGT	5073	526	423	17	30	162	53	383	83	16	3	3375	2	0	_aplCal1_1036_138128_154149
TGGTTTGATGAAAAACAATGTCTGAAC	5065	15	36	2	11	18	36	22	19	1	3	4897	0	5	_aplCal1_126_96595_100798
TCAACAAAGTGCCGACCGTAGAGTCTTA	4992	53	33	142	14	81	40	44	32	22	2	4478	48	3	_aplCal1_671_220110_228807
TCACTGAATAATCGAAGATCACGCTTGG	4943	109	97	66	102	19	2	26	24	5	6	4487	0	0	_aplCal1_77_95376_114859
TAGATCTAGAAGTTGTAGCTCCCGAGCT	4837	30	34	7	27	1	1	0	0	1	0	4736	0	0	_aplCal1_60_254223_258249
TTATTGGAAGAAGGGTTGCAGCAGTTCT	4831	57	7	25	13	81	6	44	0	3	1	4594	0	0	_aplCal1_2653_34608_42410
TAGGATTTCTTCCTTGACAGAGAATTCGC	4801	315	180	128	190	58	46	254	101	16	25	3488	0	0	_aplCal1_610_113720_122340
ATTCATGAAGATATCATAATTGTCTCTGATA	4645	5	33	3	6	28	19	25	10	3	2	4511	0	0	_aplCal1_379_63480_78689
TCCCATCCTGCCTACAGCGTCTCTACGGT	4549	75	22	30	21	26	14	35	5	1	0	4284	33	3	_aplCal1_101_532920_559602
TGGGCTACTCTCCCAAAGGCACGATCAGC	4517	198	3	127	4	67	0	98	5	4	0	4011	0	0	_aplCal1_77_95376_114859
TGTAGTCTTCCATACACCACACGTGTATGT	4506	16	482	1	22	0	578	0	106	0	239	3056	0	6	_aplCal1_2339_7934_26341
TGTAAATCGAATGCTTCTTTGCAGGCGTG	4497	289	37	3	124	127	0	253	0	29	0	3635	0	0	_aplCal1_420_43790_46689
TAACATCAATGTCAGACTCAGCATCAAGC	4461	220	0	40	7	277	0	75	0	66	1	3774	1	0	_aplCal1_714_24215_35038
TGCTCTTTACCAGTATAGATCATGAGGGC	4356	434	0	96	11	254	0	231	14	3	0	3312	1	0	_aplCal1_1712_18148_35054
TGGAGAATATCACACCGGACAGATGGAAT	4347	396	2	2171	110	236	75	420	8	9	2	918	0	0	_aplCal1_2321_35916_43532
TCAAACAGCCAGTGACTGTCCCAGTAGTC	4316	212	0	168	2	262	0	219	11	6	0	3435	1	0	_aplCal1_1712_18148_35054

TGAACCTGGATTGTGAGTGACTTGCGGGT	4315	249	2	192	104	182	53	521	12	17	11	2972	0	0	_aplCal1_101_532920_559602
TTTTCAGCGACTGGTGAGATAGTCACCGAA	4250	23	5	34	31	0	0	2	2	0	2	4151	0	0	_aplCal1_697_50289_68955
TCCAAGTTGGGCAATCCTTACTAAAGGCT	4179	60	66	64	257	7	64	47	14	1	1	3588	2	8	_aplCal1_0_1461183_1467458
TCACACTGACGGTACTTCAGATTCACTA	4146	191	228	122	171	675	412	537	89	205	88	1407	6	15	_aplCal1_2339_7934_26341
TTTAATGTGCTGTTTGGCATTGTATGCCTT	4135	5	36	2	13	5	0	3	1	1	0	4069	0	0	_aplCal1_2299_30198_72073
TTCTAAGAATATTAATGTACTGGTCAGGC	4053	144	96	91	41	149	1	70	58	11	17	3375	0	0	_aplCal1_77_95376_114859
CGACATTATTTCAACTGCTGAGGC	4044	6	282	94	158	1	2	3	0	1	0	3497	0	0	_aplCal1_2299_30198_72073
TCCAACATCTCCAGTTGCTGTGAACGTC	4004	344	144	79	73	123	41	98	37	44	1	2999	9	12	_aplCal1_2339_7934_26341
TAGAAGTTGTAGCTCCCGAGCTAGGCCA	4003	96	18	42	67	4	1	1	3	2	1	3767	1	0	_aplCal1_439_201156_223269
TTGTTTGATGAGAAACAATGTCTGAAC	3957	2	9	1	0	11	16	20	19	0	1	3870	0	8	_aplCal1_149_161811_162817
TTGTTGTGACTAGCTCAACTCGAGGTCG	3904	36	44	33	1	26	2	17	1	3	0	3732	2	7	_aplCal1_178_206973_213522
TTGATTGATATGATTTGAGCAAACTCGGC	3857	74	210	588	851	77	122	339	278	36	20	1262	0	0	_aplCal1_2321_14968_33608
TACGAAGAGTGTGCAACTGAGTGCTGACC	3845	0	1	533	2887	0	24	0	1	1	0	398	0	0	_aplCal1_2304_47457_69575
TCCGACATTGCCTTTTCTCTTTACAAAGGCT	3832	75	56	38	31	85	4	59	6	10	0	3454	9	5	_aplCal1_1972_39301_75265
TGCGCACTTGACAAGGAGTACACCAGAGG	3821	101	599	21	145	14	140	57	135	0	4	2605	0	0	_aplCal1_0_1461183_1467458
TATCGTTCGGAAGTACGTGGAATACTGCC	3751	126	74	2	3014	34	14	8	4	3	0	472	0	0	_aplCal1_0_1461183_1467458
TGTTAGCTCAGAAAACACTTGGTTTTCAGA	3726	0	0	703	475	0	1	1	2	3	0	2536	0	5	_aplCal1_2304_47457_69575
TAGTGAAAAGTTGTTGTTGTTGGCGTAGT	3699	137	202	12	56	464	865	106	58	4	2	1789	0	4	_aplCal1_1036_138128_154149
TTTGTATTTCCACCTGCCTTTGAAGCCG	3691	38	11	3	52	30	0	25	1	6	0	3525	0	0	_aplCal1_420_43790_46689
TACAACAATGTCACATCATTAAAGAGAGC	3659	10	0	22	5	11	5	51	0	5	0	3550	0	0	_aplCal1_645_48662_56779
TGAACCTGGATTGTGAGTGACTTGCGGTC	3652	167	5	75	57	139	96	291	5	7	7	2803	0	0	_aplCal1_645_48662_56779
TGAGTTGTCCTGTATGTTTAGTAATGTGT	3647	144	159	93	216	263	594	278	292	627	182	674	108	17	_aplCal1_2339_7934_26341
TATCTGGTTGGTTCATGAAGGAACCGTCT	3624	11	2	0	4	3	1	0	0	1	0	3602	0	0	_aplCal1_1667_1422_36545
TGCGACTTTTCGTGCTGTGTACAAGCACTCA	3587	2	11	16	25	1	0	1	1	0	0	3530	0	0	_aplCal1_283_154778_166233
TCAGTTCCTTCGTTTTTGTCTGGTTTTCC	3577	172	104	77	249	144	101	146	47	82	11	2436	1	7	_aplCal1_439_201156_223269
TTCGTACTTTACACTCTTGGATTGCGCG	3535	218	44	6	113	197	54	129	28	40	5	2697	4	0	_aplCal1_436_270311_282387
TATTCATCCAAGACTTGCACAGGCTC	3449	28	24	7	13	9	3	20	13	3	1	3326	2	0	_aplCal1_178_206973_213522
TCCTTGGTTGAGAACGACTGGCCATTGT	3435	646	124	173	87	542	180	165	53	12	2	1326	70	55	_aplCal1_1036_138128_154149
CAGAAAACTGAGGATCTTCATGAAACGATC	3374	167	3	27	35	779	0	1107	14	17	3	1222	0	0	_aplCal1_77_95376_114859
TGTTTATTTGTGGTTCCTGATTTTGGTC	3364	1	6	40	51	0	0	0	3	0	2	3261	0	0	_aplCal1_1057_159778_180055
TCTGTTACCTTCATTGATTGCACTGGGC	3364	159	1	108	12	81	1	96	2	16	0	2888	0	0	_aplCal1_2299_30198_72073
TGGTATAACGTGGCAACAAGATTGCCCTT	3355	9	2	53	38	11	2	2	5	1	1	3231	0	0	_aplCal1_1637_9142_22678
TCTTTTTCTGGGAGCTCATGTTGGCGC	3348	128	19	238	36	125	44	83	86	8	20	2558	0	3	_aplCal1_393_78915_104964
TGTATTCTTGGATTGCGCGTTCTTGGAT	3345	69	29	9	34	177	16	34	7	6	2	2961	1	0	_aplCal1_1036_128796_134511
TCTAAGAGGAAAACCTGGGCCAAAGAGG	3335	1	1	1912	1362	1	10	0	2	0	0	46	0	0	_aplCal1_0_1461183_1467458
TGTCGGATGAAATATTCTGCAAAAATGTC	3319	224	64	64	60	31	22	43	19	1	0	2791	0	0	_aplCal1_8571_1_5208

TTTCAGAATAAGTACGAGACTGTTCTCT	3306	20	29	61	22	5	16	36	7	2	4	3104	0	0	_aplCal1_697_50289_68955
TTCTCGATGAGGGTAGTGCACCGCAGAC	3276	333	0	114	6	78	1	234	15	3	0	2491	1	0	_aplCal1_1712_18148_35054
TGTCGTCACGGACGTCCTCTAGAGGTACG	3232	18	20	4	34	3	6	9	1	1	1	3135	0	0	_aplCal1_2358_50666_58753
TGAATGAGTTGTACGTGTCCGCGATGACC	3202	169	4	133	44	230	2	141	25	3	0	2437	14	0	_aplCal1_147_26539_30349
TGTATATGTGTCCAGTACATAACCACTGTCC	3181	11	7	18	16	18	13	14	3	4	0	3077	0	0	_aplCal1_446_312983_330760
ATTTTGTTGTGAAAATATGCTGACATGTT	3178	75	418	82	155	128	1288	294	298	66	230	144	0	0	_aplCal1_2339_7934_26341
TATACCTTTTTTCTGTGGTACAAGTTCATT	3123	325	73	91	79	941	238	547	78	46	21	681	0	3	_aplCal1_2321_14968_33608
GCAGAACGTACCGTTGAAGGATTGGTAGC	3120	83	56	24	44	72	70	81	11	13	2	2656	8	0	_aplCal1_1972_39301_75265
TAATTTATCTTGGCTCATGCTGTGCAACC	3067	262	139	90	80	186	27	280	9	41	3	1950	0	0	_aplCal1_98_137957_177330
GCAGAATGTTGTTTTGGATAATGGACA	3062	351	178	49	56	155	144	303	69	8	1	1748	0	0	_aplCal1_98_137957_177330
TGGGTACAGAGACATCTCTGCTGAAGCT	3052	8	13	7	28	6	9	5	1	0	0	2975	0	0	_aplCal1_523_215863_271832
TGTTGACGTCTGTTTCCTGTTGTACAGGATG	3042	143	6	13	22	16	0	31	2	3	0	2806	0	0	_aplCal1_1032_9624_27540
TAAGACACTGCTCATTCCCCTACAGGCA	3035	2	0	95	291	0	4	0	0	0	0	2643	0	0	_aplCal1_2304_47457_69575
TCCAAGATTGAAGTCCGCCTTGAAGACA	3033	407	30	66	85	55	0	320	15	28	9	2018	0	0	_aplCal1_77_95376_114859
TTGAGAACGACTGGCCATTGTGACAAGCC	3015	77	193	4	12	334	177	38	45	1	3	2130	1	0	_aplCal1_1036_138128_154149
TCTTTTTCATCTCAGCTTTCACTCGACT	3013	120	0	43	9	27	0	30	0	9	0	2775	0	0	_aplCal1_2299_30198_72073
TAAGAACTTTCATAATAAGCATAGGATT	3003	428	0	4	36	605	72	616	217	28	0	997	0	0	_aplCal1_2261_59004_71749
TATTTCTACAACCTTTGCTCTTGCTGT	3001	50	18	37	2	40	35	52	2	0	0	2764	0	1	_aplCal1_98_137957_177330
TTGGATCCAAGTCGAGCAATCCTTGCTGA	2989	154	54	2	22	104	67	205	20	4	8	2349	0	0	_aplCal1_1036_128796_134511
TGTGAAAGCGGTAATTTTTATAAGTCAGC	2977	162	138	103	91	142	235	257	14	11	1	1813	7	3	_aplCal1_610_113720_122340
TGGTTTCAAGTTCAAGAGCCTGTATGTC	2949	353	0	111	7	119	0	82	1	6	0	2270	0	0	_aplCal1_2299_30198_72073
AAATGACCCCAAAGATGTAAAGATATAGC	2933	178	0	93	2	123	0	188	0	25	0	2324	0	0	_aplCal1_77_95376_114859
TCCCATCCCGCATACAGCGTCTCTACGGT	2914	72	25	36	49	29	12	44	2	3	0	2633	5	4	_aplCal1_101_532920_559602
TCCTTGCTGAAGGCTGTATGCTGGCCGTG	2899	0	14	15	69	1	0	0	7	0	1	2792	0	0	_aplCal1_2299_30198_72073
TGTACCAGAGCCGGCTGTAATGGTTTAGCC	2872	314	91	130	24	520	71	156	46	188	1	1240	85	6	_aplCal1_610_113720_122340
TGAAGAATTCTGATCTGGTCAGCGGGTCAAA	2869	96	25	6	51	63	0	46	0	6	0	2576	0	0	_aplCal1_420_43790_46689
TACCGACCGTGAACCAGCCACGCGTTC	2863	35	20	8	19	32	42	97	10	5	1	2581	11	2	_aplCal1_610_113720_122340
TAGTAAGCACTTCATAATCTACGATGGG	2862	40	52	16	27	43	71	40	66	6	0	2292	187	22	_aplCal1_2082_38884_60606
CTTGATTTGTAGCATTTAAGGTTACGCC	2831	172	81	6	38	376	150	54	116	9	15	1814	0	0	_aplCal1_2321_14968_33608
TACCTACGTGCCACACATCACTGGACTAC	2828	49	9	8	8	21	20	38	14	15	3	2633	5	5	_aplCal1_2339_7934_26341
TATTTTTCGACCGTTGATCGCTCTTGCGC	2801	60	12	21	36	57	5	71	6	8	2	2519	4	0	_aplCal1_366_175072_190841
TTATTGGAAGAAGAGTCGCAGCAGTTCT	2772	77	11	60	9	85	0	48	4	5	4	2468	1	0	_aplCal1_101_532920_559602
TATTTCTACATAAGAAAGAGCATTTCCGG	2767	79	371	149	52	41	67	51	18	9	2	1928	0	0	_aplCal1_2299_30198_72073
TGTTCTAACTGATTGTAGATGCGTTTGGC	2761	81	57	58	169	34	18	42	8	16	2	2276	0	0	_aplCal1_10_358654_362785
TAAAGTCAAACCTCAGTTCGTATCTCCCT	2760	447	40	33	36	267	32	1161	108	4	1	624	6	1	_aplCal1_1036_138128_154149
TCAGAAATTCAGAGACGGGGATTTTGCT	2759	166	0	182	6	218	12	522	0	12	0	1641	0	0	_aplCal1_2321_35916_43532

TGTTTCTACTATCAGCCGGAAATGTGTT	2751	396	187	181	346	185	200	275	42	19	28	892	0	0	_aplCal1_523_215863_271832
TAACACTCACATGATCGCCAACAGGCTT	2746	49	0	37	4	6	0	13	0	9	0	2627	1	0	_aplCal1_371_102376_107690
TGCTTCTGGTCCCACAATTGAACGCACAG	2740	2	418	101	200	0	218	0	59	0	7	1735	0	0	_aplCal1_2299_30198_72073
AACAAGGCAGAGACGTTTGACAAAAGAAC	2740	1	200	133	93	1	273	0	3	0	2	2034	0	0	_aplCal1_2299_30198_72073
TTTAATGAGTCAAGGAGAACAAGTGTCC	2739	155	1	184	14	226	131	178	14	33	22	1781	0	0	_aplCal1_645_48662_56779
TGATAGCAATGAAGTGATTGACAAGGGC	2726	0	0	1	3	0	0	0	15	0	0	2707	0	0	_aplCal1_1117_24553_42207
TGACAATGCCATGGTGTTCACTGAACCGTC	2724	75	0	60	24	28	1	38	0	0	0	2498	0	0	_aplCal1_2299_30198_72073
TAGGAACCCGGTCACTTTTCTGATGAGGT	2706	0	0	658	2036	0	1	0	1	0	2	8	0	0	_aplCal1_2304_47457_69575
TGGGTTCTTTCAGAAAGCATGCACTGCAC	2681	132	29	79	1	122	1	68	24	19	4	2202	0	0	_aplCal1_393_78915_104964
TCTCTCTCAAGGCGTTTTCCTGTGGGCC	2669	18	4	6	10	7	2	8	1	5	0	2606	2	0	_aplCal1_2082_38884_60606
CACATGATGAACCAATTACGACGACGACC	2627	117	2	17	0	26	0	24	0	6	0	2427	0	8	_aplCal1_1743_4810_24833
TAACGTGGTTGCATGTACTGCACATGCCA	2624	13	38	0	82	2	9	19	4	1	0	2456	0	0	_aplCal1_195_419710_423701
TCGTGGCTTCCTGTGCTACCTTGGCGTA	2621	14	15	0	27	3	8	4	4	1	1	2542	2	0	_aplCal1_0_1461183_1467458
TCTAGAGGTACGTATGTGTCACTCGCGG	2620	57	683	28	14	18	22	20	27	1	1	1700	49	0	_aplCal1_2358_50666_58753
TCGTAGTTGATCAAGGCCCTCAGTGATG	2613	30	10	15	13	13	0	23	1	1	0	2501	1	5	_aplCal1_101_532920_559602
TTTTATAGCACCCTACCAAGCACAGGCCT	2607	177	32	18	6	46	11	112	7	17	2	2179	0	0	_aplCal1_492_75504_92774
TTATGGATAGACAAACTGAGAAATACATC	2594	5	2	11	9	10	2	13	12	3	0	2527	0	0	_aplCal1_2339_7934_26341
TTTTGAACTCCTGACTGTGATTCACAGTGT	2584	29	38	7	8	7	21	17	25	1	4	2426	1	0	_aplCal1_98_137957_177330
AGGAAACCCAAGGATCGTTGAAGCGACGGT	2575	132	0	5	3	9	0	97	1	1	1	2326	0	0	_aplCal1_714_24215_35038
TAGAAGGACTTGCCGACCTCGTGGCTTT	2574	1	0	12	1	1	0	0	0	0	0	2559	0	0	_aplCal1_6_196087_201571
TGTGTTTGTACTGGAGGCATCCCTCTCTT	2571	24	17	1	15	7	6	15	0	0	0	2486	0	0	_aplCal1_988_231977_255942
TGGGGATGTATCCCAGAACTTGGGGGTT	2567	3	55	15	103	1	14	8	20	0	6	2337	0	5	_aplCal1_439_201156_223269
TCTGCGTAGACATTCTGACACACCTAGAG	2535	60	92	22	229	18	112	94	38	39	7	1824	0	0	_aplCal1_10_358654_362785
TCAGGAGGTTTACACGTTTCGTGTCGTG	2523	57	1	12	2	167	1	26	32	3	1	2216	5	0	_aplCal1_147_26539_30349
TTGAAGAGGATCACTTGGAAAGTGAGTCT	2515	204	1	396	8	494	5	170	63	89	0	1085	0	0	_aplCal1_7_1016346_1020018
TTGGTGTAAATCGAATGCTTCTTTGCAGG	2512	28	27	5	35	4	0	8	0	13	0	2391	1	0	_aplCal1_420_43790_46689
TGCTCAGGAGGTTTACACGTTTCGTGTCG	2503	19	0	35	2	4	0	31	2	0	0	2410	0	0	_aplCal1_147_26539_30349
TCTGGACTGATGCGGTCTCGAAGGCATA	2501	78	4	4	4	2	1	19	0	1	0	2388	0	0	_aplCal1_1972_39301_75265
TGTAGCAATGTCGTTCGTGGTGACAAGG	2462	1	188	64	13	0	9	0	0	0	1	2186	0	0	_aplCal1_2299_30198_72073
CGCAGCTCCAGTCGGGGAAAAGCTTTGATC	2459	149	228	130	518	24	165	165	166	10	2	899	0	3	_aplCal1_2321_14968_33608
TGTAAGATCACCGGCGACACTCACCCCTG	2441	47	66	8	11	51	59	51	14	2	6	2119	7	0	_aplCal1_98_137957_177330
TGTATTCCTTGTGCATACTCTCTGTATGCCT	2439	19	0	0	0	1	0	1	1	0	1	2416	0	0	_aplCal1_1712_18148_35054
TGATATAGGATGAAGGCTTTCAGAAGTCC	2426	14	6	55	103	8	7	15	7	1	1	2209	0	0	_aplCal1_1216_28641_44559
TTGGAACCCGGTCACTTTTCTGATGAGGT	2419	0	0	512	1895	0	5	0	1	0	2	4	0	0	_aplCal1_2304_47457_69575
TTTTTGCTCTGCTGTGCCTCGAGCTCAGTT	2414	13	0	60	1	3	8	8	0	0	0	2321	0	0	_aplCal1_101_532920_559602
AATGGAGTTGTCCTATCTAGCATTTCTTC	2392	152	40	31	178	69	9	67	1	5	0	1835	5	0	_aplCal1_2339_7934_26341

TAGGTTCTCAACACAGCGTATCAGCTTG	2374	7	1	32	29	5	18	9	2	0	1	2270	0	0	_aplCal1_645_48662_56779
GACGTCACATCTGTATTTGCCAATGGGTA	2373	257	143	18	24	69	39	426	44	6	1	1329	17	0	_aplCal1_1036_138128_154142
TCACCCTGACATGTGTCTTTCCCACACCC	2372	75	28	10	43	48	65	75	9	49	17	1906	34	13	_aplCal1_2339_7934_26341
TCCAGACTTTTCTTCGGACTGAAGCTCA	2363	38	0	1	0	1	0	5	0	0	0	2318	0	0	_aplCal1_1712_18148_35054
TCTAGAGGTATGTATGTGTCACTCGCACA	2362	114	187	22	39	52	76	59	147	4	3	1653	6	0	_aplCal1_2358_50666_58753
TATCCAAACATCTCTTGATCAGGGGTCT	2358	0	4	0	11	0	3	0	0	0	1	2338	1	0	_aplCal1_101_532920_559602
TTCAGTCTCTGTTGCGCACTTGACAAGG	2353	42	17	18	4	32	47	29	10	5	16	2133	0	0	_aplCal1_737_233617_250062
GACAAACGTAGCTGCTGAGAGGTACAGT	2351	209	2	230	255	434	39	404	3	19	15	741	0	0	_aplCal1_645_48662_56779
TGTTTTTGCTCTGCTGTGCCTCGAGCTC	2349	13	0	11	1	15	11	13	0	8	0	2276	1	0	_aplCal1_155_244249_252418
TAGAGCTCCATTTTCTTCACCCTGGTCT	2339	10	2	0	1	1	2	2	2	1	0	2315	3	0	_aplCal1_2339_7934_26341
TGGGGATATGTCATATAATATGGTGGCA	2336	17	1	0	3	0	1	2	0	0	0	2312	0	0	_aplCal1_98_137957_177330
TGCATCTCCCGTCTTGAACAGATTCAGATC	2331	281	56	147	132	55	42	169	10	10	1	1428	0	0	_aplCal1_6_108252_113930
ACGAACGGACGAATCTCTTTGAAGAGATATT	2322	108	3	201	0	120	3	291	34	37	0	1525	0	0	_aplCal1_7_1016346_1020018
TCAAACGTATCACGGAGTCGGCCAAGCGG	2321	79	1	54	35	130	77	151	1	24	9	1760	0	0	_aplCal1_155_244249_252418
TACAAATCAGCCTGAGCAACATTAAAAGAC	2320	1	449	2	240	3	69	8	299	2	28	1218	1	0	_aplCal1_2299_30198_72073
TCCTTGTTGAGGAACCATATGTCCGTGAC	2299	104	0	30	7	61	0	56	0	9	0	2032	0	0	_aplCal1_2299_30198_72073
TCCGAGAGTGTTTACAGTGTTGTATGCT	2293	83	74	91	20	42	28	88	20	12	5	1785	42	3	_aplCal1_1972_39301_75265
TTTACACCTGACTTCATCCTGCTTCAGCCG	2291	19	20	14	13	13	7	7	0	1	0	2192	1	4	_aplCal1_523_215863_271832
TTGTGAAGTTCTGATTGTGATCCCTTGT	2276	73	81	36	70	34	12	43	21	30	8	1866	1	1	_aplCal1_697_50289_68955
TATAGTCTCTGTTGTACTCACTTGTACTA	2267	7	5	9	26	4	0	11	0	0	1	2204	0	0	_aplCal1_195_419710_423701
TGTTAATGCATGCGAGAGTTTTGATCCTT	2259	159	114	105	71	122	44	287	11	15	0	1331	0	0	_aplCal1_98_137957_177330
TAATAGTTGTCCGTGTACAAAATGTACCCTT	2241	49	4	10	6	2	0	4	0	3	1	2157	5	0	_aplCal1_48_678521_693149
TGGTATCGTTCGAACGTGCATTGAATAC	2235	41	20	43	12	24	95	33	13	7	9	1918	20	0	_aplCal1_1712_18148_35054
TGATAATAGCTTGGTCTCTAAGGACCCTGT	2225	30	44	38	41	40	77	2	0	3	1	1949	0	0	_aplCal1_523_215863_271832
TACATAATCATCAGTGGTTTTGAAAATGC	2225	351	28	33	9	296	38	255	11	15	0	1188	1	0	_aplCal1_2321_14968_33608
TGTAGTCCTCTACACACCACACGTGTATGT	2213	64	42	24	31	39	55	58	6	30	22	1834	0	8	_aplCal1_2339_7934_26341
TTTTCTTCGGACTGAAGCTCATGGCTGT	2211	7	0	71	1	4	0	22	0	0	0	2106	0	0	_aplCal1_1712_18148_35054
TGTAGTCCTCTATACACCACACGTGTGTGTT	2190	80	42	10	23	22	45	27	7	5	9	1919	1	0	_aplCal1_2339_7934_26341
TAGCTTTTGATGCAGTCCCTGGGATCA	2146	9	5	3	8	0	0	0	0	0	0	2121	0	0	_aplCal1_455_224202_242097
TGAACATCGTCTTCATGGTGCTCAGCGGC	2114	23	237	81	147	90	238	27	46	8	3	1214	0	0	_aplCal1_10_358654
TAGGCTGTGATTAACGGTCAAATGTTGGT	2105	144	100	150	191	38	57	44	22	4	4	1350	1	0	_aplCal1_1972_39301_75265
TTCGCAACAAGATCCACTGTTTGTGGGTGTC	2102	12	13	0	31	4	2	6	3	0	0	2031	0	0	_aplCal1_195_419710_423701
TGGTCAATAACTGCCCTGCTGTCTGCACG	2084	30	35	20	21	15	18	28	61	12	2	1837	5	0	_aplCal1_610_113720_122340
TCCCATTCCGCCTCCAGTGTCTCTACGGT	2083	32	22	8	13	15	17	11	3	1	0	1947	14	0	_aplCal1_101_532920_559602
CAATTCTGCTTGGAATACGACATCCTCTC	2077	58	0	18	4	70	12	60	0	3	0	1852	0	0	_aplCal1_2321_35916_43532
CATAAGCACCCTCATCTCCAGCTGGGAC	2040	88	0	1	1	17	0	39	0	2	0	1892	0	0	_aplCal1_2299_30198_72073

TGGGTGTTGGTTGGGATGTTTCCCAGTGC	2035	0	16	219	202	6	11	12	60	4	4	1500	1	0	_aplCal1_2321_14968_33608
TATAGCGATATCATGCTCTTCTCTTGACT	2033	88	14	3	5	6	1	61	0	1	0	1854	0	0	_aplCal1_98_137957_177330
TACGATGTGGAGACTTGATATAGGTAATC	2017	154	7	76	7	403	103	414	2	0	6	845	0	0	_aplCal1_455_224202_242097
TCTTTTCGGTAGACCCAGCTGTGGCAGG	2011	9	27	106	69	13	15	9	13	12	3	1735	0	0	_aplCal1_101_532920_559602
TAGAATTTTGCTGCAGCTATTGAGAGGTT	2010	7	41	13	22	2	14	6	5	1	3	1896	0	0	_aplCal1_283_154778_166233
TACACATCTCCGACTCTGCACTGCACGG	2004	77	61	93	19	69	7	154	16	3	5	1500	0	0	_aplCal1_471_221309_226546
TTCTGTAAAATTGCTATAGTCCAGCTTTT	1983	29	6	14	29	4	3	15	2	4	2	1871	0	4	_aplCal1_697_50289_68955
TGCTGCCTCCACATGAATTCAACTAGATC	1968	18	7	5	4	1	4	2	0	0	0	1927	0	0	_aplCal1_1972_39301_75265
TGGATTTGAAGTCTCCAAGATCATGTGG	1965	57	2	74	12	70	67	51	6	17	4	1605	0	0	_aplCal1_2321_35916_43532
TGGAGACGTACCTGAGCTGATGTCTTAGA	1956	94	30	26	76	127	105	97	136	4	2	1259	0	0	_aplCal1_2082_38884_60606
GATGACAATGCCATGGTGTTCACTGAACC	1944	120	1	64	2	98	0	57	1	1	0	1600	0	0	_aplCal1_2299_30198_72073
TGTATTATCTTCAGGTTCACCGAGCAAAC	1939	3	43	3	31	0	41	0	36	0	0	1782	0	0	_aplCal1_39_566527_578980
TCCTCCATCGCTAGACTCAGGACCTGTGA	1924	71	45	3	10	1292	71	15	2	7	3	370	19	16	_aplCal1_1036_138128_154149
TAGGATTTCATTTTTTCCAACCAGGAAGA	1924	0	209	12	696	0	513	1	198	0	268	27	0	0	_aplCal1_101_532920_559602
TCTGATGTTGTCTTTGTCTTTACGACGCT	1905	16	21	12	25	26	14	15	60	2	44	1670	0	0	_aplCal1_523_215863_271832
TTTGACGTCTCACATTCAGCCAGCCAATC	1902	3	0	370	654	0	6	0	3	0	0	862	0	4	_aplCal1_2304_47457_69575
TGCTTTATGAACGGAAAACCACTGGTCA	1901	7	0	9	24	0	0	0	1	1	0	1859	0	0	_aplCal1_697_50289_68955
TGAAAACGTTACACAGATAACCTGTTGC	1900	277	29	11	116	257	0	313	0	44	0	853	0	0	_aplCal1_455_224202_242097
TCACATAGCCGTTTGTGTTTAAAGATGC	1897	61	10	36	1	12	1	44	3	11	0	1693	2	23	_aplCal1_523_215863_271832
TAGTGTCGTGAAAAGGGTGTATCCCACCT	1897	0	57	4	225	0	1	3	8	0	0	1599	0	0	_aplCal1_53_340648_376011
TCCAACTAAGTGACGGATTATGAAAATCT	1894	81	86	121	365	89	215	346	96	11	3	481	0	0	_aplCal1_53_340648_376011
TTCTTTCTATGGCTAAGCCAATCCCAGA	1878	54	11	25	31	26	19	56	7	21	0	1625	1	2	_aplCal1_2082_38884_60606
TGCAGTCTTGATTGAGTTGGTCTGTCGGC	1872	0	3	871	945	3	8	16	2	0	0	24	0	0	_aplCal1_1895_51672_71846
TAATCACATTTCTCACTTTGAAGAGGGG	1858	54	0	12	4	27	0	23	1	5	0	1731	1	0	_aplCal1_714_24215_35038
TGAAGATAACGTAGAAGCTGTCTGTCAAGC	1852	33	111	26	1001	29	37	11	13	3	58	530	0	0	_aplCal1_155_244249_252418
TGCGTCTTCATTGCCGCTGATGGCCA	1849	33	3	4	6	3	3	4	1	1	0	1786	5	0	_aplCal1_1565_34739_40201
TTCAGCTAAGGAAGTGACTCTAGTCTC	1848	13	5	77	45	2	2	2	3	0	2	1697	0	0	_aplCal1_1667_1422_36545
TATGTATTGTTTGCACACAAATGCACAGC	1846	79	0	31	6	54	3	57	1	4	2	1607	2	0	_aplCal1_714_24215_35038
TACATCCAGAGCACAAAACAGTATCAGC	1837	124	167	28	48	32	31	111	10	3	13	1268	2	0	_aplCal1_523_215863_271832
CACAAGATCCTTGGGCATTCTAACCCTGTT	1829	30	0	12	5	29	0	23	0	2	0	1728	0	0	_aplCal1_77_95376_114859
TGATATAACGTGGCAACAAGATTGCCCTT	1823	10	0	32	1	11	0	3	0	2	1	1763	0	0	_aplCal1_714_24215_35038
TTGCATCCGTACATAGTCATCAGAGGTCT	1822	0	10	1	19	0	5	0	5	0	0	1781	1	0	_aplCal1_39_566527_578980
AGCATTCACCATGCAGAGGTCGATCAGAT	1816	3	12	16	8	0	0	0	0	0	0	1777	0	0	_aplCal1_258_136483_156496
TATAATGTAGTTGGAGACCCTCTCCGCCT	1813	0	350	3	458	1	97	0	323	0	23	557	1	0	_aplCal1_101_532920_559602
TGCTTCAAGGTTTGAGATGTACTGGCTG	1810	122	15	81	44	121	163	147	12	10	17	1078	0	0	_aplCal1_2321_35916_43532
TAAGACACAACAAAGAAGTACTTTGAAGCC	1801	0	128	610	1000	0	2	0	0	1	60	0	0	0	_aplCal1_101_532920_559602

TTGGATTGATGACGTTCTTTAATGACTC	1790	22	59	30	35	17	100	5	104	5	0	1412	1	0	_aplCal1_446_312983_330760
TTGTTCTCACTGTATTTTCGAAAGGCTT	1781	300	39	23	17	28	37	192	27	5	1	1112	0	0	_aplCal1_98_137957_177330
TGCATGTCATTGACAATTATCTCATTCA	1764	74	52	43	109	62	63	85	13	10	2	1243	0	8	_aplCal1_1667_1422_36545
TACCAAGTAAGATGCCCGAATGGTAGCTG	1760	53	14	75	103	55	22	38	9	2	0	1375	7	7	_aplCal1_1667_1422_36545
TCTAGGAAAAGACCGAAAAACTGAGAGG	1756	67	27	31	19	67	2	49	1	2	0	1490	1	0	_aplCal1_492_75504_92774
TGAAAAAGGTGGACCATGTCTTCAGAAAG	1755	62	4	70	62	117	362	110	22	7	10	929	0	0	_aplCal1_1599_68017_76308
TAATATAAAACTAAAACAGCATCAAGGG	1749	73	0	8	5	15	0	34	0	5	0	1609	0	0	_aplCal1_2299_30198_72073
TGAACGAAGAGAGAGTCTCGTCTGATTC	1748	192	4	259	10	201	447	202	3	9	54	367	0	0	_aplCal1_155_244249_252418
TAGTTGATCAAGTCCCTCAGTGATGTTC	1747	76	19	134	145	112	4	2	87	0	0	1160	5	3	_aplCal1_101_532920_559602
TAGATGTTCCTGTTCACGTTGGTTGGATC	1731	35	35	102	51	116	12	27	123	13	4	1212	1	0	_aplCal1_1210_56333_70614
TGTTTGATTGCCACTTCACTTTTCCGTCA	1721	129	0	84	32	95	90	108	8	13	6	1154	2	0	_aplCal1_645_48662_56779
TTGTCGACAGACCTGTACCCGCCTGGGGC	1709	23	0	8	9	34	0	10	0	1	0	1624	0	0	_aplCal1_147_26539_30349
TGTAAAGGAGTAGTCCTGTATAACAGGAGGG	1704	82	38	52	103	71	16	79	66	14	11	1172	0	0	_aplCal1_283_154778_166233
TAGAACATTAAATTGTCTTTCCTACTGTGCC	1703	76	29	8	25	36	6	207	1	0	0	1313	1	1	_aplCal1_98_137957_177330
AAAAGTGGACAAAATCATGATTAAACCATT	1697	146	35	22	3	109	195	312	34	3	0	838	0	0	_aplCal1_98_137957_177330
TCCTAGTCAGTTCATATGTAGACATATGGGG	1688	5	9	31	26	6	1	1	0	3	0	1606	0	0	_aplCal1_190_74320_82083
TGAAGAAACCAAAATGGAAGGGTCAGCTT	1668	243	43	123	386	120	409	102	111	6	50	75	0	0	_aplCal1_1599_68017_76308
CAACAACGTTTCTCTTAAGCCACCTATTG	1667	2	184	2	195	3	197	4	7	0	0	1073	0	0	_aplCal1_2299_30198_72073
TTTGCTTGCAGGGACTTCCTTCTGTCCAAA	1656	12	5	9	48	4	6	17	4	1	3	1547	0	0	_aplCal1_1295_28499_46057
TAACACTGATTAAATCGCTATTTGGGGC	1655	125	19	62	40	59	8	92	0	12	3	1233	2	0	_aplCal1_1295_28499_46057
TCAAACCTCAGTTCGTATCTCCCTGAGT	1653	151	8	7	8	421	49	27	11	7	0	949	9	6	_aplCal1_1036_138128_154149
TAAGAGAATTAACCCCACAGTTCGGGCAT	1630	15	190	19	94	18	62	22	1	3	1	1205	0	0	_aplCal1_523_215863_271832
TTTAAGCTCACATACGTATCGGTTTACAGG	1627	87	14	49	130	64	16	36	18	39	4	1165	4	1	_aplCal1_1295_28499_46057
TTCTTGTCCTCTACAAGCTTTCTAAGGGC	1621	42	1	6	7	12	1	40	1	0	0	1511	0	0	_aplCal1_55_253441_264685
TGGACATCTTTGATAACAGACTCCTGTTC	1620	0	0	0	0	1	0	0	5	0	0	1614	0	0	_aplCal1_1117_24553_42207
TCTCAGTACCACTCGCTATATTCTCTGCCC	1620	0	0	35	62	0	0	0	0	0	0	1523	0	0	_aplCal1_2304_15568_27364
TGATTTCGGCGAAGTGCACTTTGACCAG	1619	73	122	40	62	26	69	64	22	4	4	1130	3	0	_aplCal1_10_358654_362785
TGATAGAATTCCAACAAGTCTCTGACGAC	1619	40	18	46	81	9	0	6	1	5	3	1410	0	0	_aplCal1_190_74320_82083
TACTTTGTTGTAAAGAGCGTAGGCAGAGG	1618	64	0	47	14	35	236	71	5	5	1	1140	0	0	_aplCal1_2321_35916_43532
TGTGATAATATGCGGTCATTCCATGCCGAC	1616	25	36	2	5	35	43	73	21	0	0	1374	2	0	_aplCal1_988_231977_255942
TCAGAGCATGCAAAAATGTCATGCGACG	1611	200	179	24	28	7	34	71	91	0	4	961	12	0	_aplCal1_98_137957_177330
TAAATCTCAGGCGTCCCCTCCACGGACAGAC	1607	49	0	9	8	28	0	47	0	6	0	1460	0	0	_aplCal1_77_95376_114859
TGGTCAATAACCGCCCTACTGTCTGCTCG	1599	91	39	38	57	42	71	45	26	20	15	1112	30	13	_aplCal1_610_113720_122340
TCCGCTGTAAATAAGCCCGGTTGCGTTCC	1599	117	32	30	7	210	107	153	47	14	10	871	0	1	_aplCal1_2261_59004_71749
AAGAAGCTTGACTTATAGCAGTCAAAGTT	1583	6	152	5	639	8	78	25	85	1	5	579	0	0	_aplCal1_39_566527_578980
TATGAGTCTGCCTATTTTTGAATTTGCGT	1580	10	30	20	29	27	8	21	50	0	5	1369	10	1	_aplCal1_610_113720_122340

TCCCATCCCGCCTCCAGTGTCTCTACGGT	1578	23	11	15	13	6	3	9	2	1	0	1490	5	0	_aplCal1_101_532920_559602
TTATTCTCTTGTATCTCTGCGAGATGTC	1576	7	0	1	1	0	0	0	1	0	0	1566	0	0	_aplCal1_361_225129_237092
TAGTTCTTCATCAGCTGTTCAAAATGG	1575	110	0	119	4	35	0	43	5	7	0	1251	1	0	_aplCal1_1712_18148_35054
TGCACGTCTTGTTCTGGGCACTACAA	1570	128	19	25	35	20	14	17	1	0	0	1310	1	0	_aplCal1_1895_51672_71846
TACAAACTTTTGGGCCAGAACTGGTTCGCT	1569	17	4	9	12	8	7	13	0	1	2	1496	0	0	_aplCal1_5028_1_8811
TCATTGAGGTAAAGACGACTGTGGAGG	1558	4	86	13	249	0	85	0	56	0	1	1064	0	0	_aplCal1_2299_30198_72073
TGGTCTATGTACCAGAGCCGGCTGTAAT	1555	42	38	2	25	1	33	8	9	5	7	1332	51	2	_aplCal1_610_113720_122340
TGCATGCAGATCTGATGAAGTCTTGGGTT	1539	75	74	18	138	46	20	83	160	10	4	902	8	1	_aplCal1_523_215863_271832
TTTCCTCCATGCTGCAGCCCTTTAGATC	1536	6	9	2	0	0	0	0	0	0	1	1518	0	0	_aplCal1_0_1461183_1467458
TTATTCTCGAGGAAAGTACGCGTTTAGATC	1534	93	22	115	106	61	62	197	16	17	2	842	1	0	_aplCal1_610_113720_122340
TCGCACCGAAGTTGAATTCAGCAACTCT	1528	1	212	0	11	0	9	0	16	0	1	1278	0	0	_aplCal1_2082_38884_60606
TCATTCAAGAATTTCCTCCGGGAAATGAAC	1527	10	3	5	0	5	2	0	0	0	0	1502	0	0	_aplCal1_98_137957_177330
TGTTCTTAAAGTTTTCTTCGCCGTGGTA	1525	78	0	68	12	77	0	81	0	25	0	1184	0	0	_aplCal1_645_48662_56779
TGGAATGTAGGCTGAGGCTTTTTTTAAAA	1524	22	118	236	177	162	187	86	63	3	14	456	0	0	_aplCal1_523_215863_271832
AAGTATACCTCATTAGGTGTTTTGCCGAT	1522	4	0	2	3	0	5	8	0	0	0	1493	3	4	_aplCal1_1667_1422_36545
TCTTGGTAGGCTATTACGAGGCAAGGCT	1516	168	2	77	4	104	26	131	2	14	0	988	0	0	_aplCal1_2321_35916_43532
TCAACGAAGTGCCGACCGTAGAGTCTTA	1516	23	13	71	44	19	34	9	13	3	7	1280	0	0	_aplCal1_455_224202_242097
TAGGAATGTATCCCAGATTATGTCTTAGT	1516	28	94	481	338	53	235	27	23	4	17	216	0	0	_aplCal1_2321_14968_33608
TCCACAAAGAATATATGGAAAAAGGAGC	1512	93	0	12	41	23	0	52	4	3	1	1278	0	5	_aplCal1_77_95376_114859
TCGATGTTGCAGACTCAGGTTTTTCAGCT	1507	167	0	10	10	7	4	24	1	0	0	1284	0	0	_aplCal1_961_84400_111904
TTCTGCCCGTTGCCTCTTTAAGAGACTCC	1503	7	0	0	2	1	3	6	0	0	0	1483	1	0	_aplCal1_610_113720_122340
TGGTAATATAAAACAGCATCAAGGGACA	1498	152	0	11	2	13	2	40	1	7	0	1270	0	0	_aplCal1_2299_30198_72073
TCTGAAAGGGATTTTTGTAGGTTTGAAATC	1496	0	1	606	888	0	1	0	0	0	0	0	0	0	_aplCal1_2304_15568_27364
ACACTTGTAAGGCTTTTCTCCT	1496	172	188	128	167	140	156	157	90	40	50	197	1	10	_aplCal1_366_175072_190841
TTCTATTGGTTGTAGACTTGTTTTGCTG	1489	1	1	528	784	0	6	0	3	0	0	166	0	0	_aplCal1_2304_47457_69575
TTCTGCAGGTTCTATTCTGGCACCTCCACC	1482	17	0	15	1	3	1	2	0	1	1	1441	0	0	_aplCal1_1972_39301_75265
TAAAAGAATCACTTTGGCATAACCTGCCAGC	1470	0	1	29	23	0	4	19	21	2	0	1371	0	0	_aplCal1_77_359166_370796
TGATTGTCAACTTTTAGAACCTTTCGGTC	1468	7	6	7	6	9	2	7	3	0	0	1421	0	0	_aplCal1_1210_56333_70614
CGGCAAATCAGACGATATAGGAAAGCAGC	1468	7	4	2	0	1212	89	3	4	1	0	146	0	0	_aplCal1_1036_94615_110603
TCAGTTATCAGTTGTCAGAACAAGTATG	1465	28	5	7	4	8	16	24	26	2	1	1340	4	0	_aplCal1_77_359166_370796
TGTAGTCTCTCTTAGCAGCGAAGTCTTC	1463	17	78	6	18	3	11	23	4	1	0	1301	1	0	_aplCal1_98_137957_177330
TGGTTGTCCAGAGCAGCAGCAATTGCACA	1456	66	0	89	4	41	0	143	1	0	1	1111	0	0	_aplCal1_1667_1422_36545
TAGAATGTGAACAGATCGTAAACAATGC	1450	32	128	149	101	36	96	9	7	4	2	885	1	0	_aplCal1_446_312983_330760
TTGTTGACTGCAGCTCGGTCTGTCAAGG	1443	8	0	5	0	8	4	2	0	1	0	1415	0	0	_aplCal1_645_48662_56779
TTTTGCGTAGCACACAACACAGGCAAGGC	1428	28	15	0	5	0	0	0	0	0	0	1380	0	0	_aplCal1_439_201156_223269
TGCACGTCCTGTAGTGTGCGCAGAATTT	1428	162	44	50	80	38	24	57	46	8	0	917	2	0	_aplCal1_2339_7934_26341

CATCAACTGCACAGCATGTGCTGAAAA	1427	0	7	0	5	0	2	0	1	0	0	1412	0	0	_aplCal1_2299_30198_72073
TCTCGCTTGTCTACAAACTTTTGGGCCA	1424	67	5	13	10	22	0	22	0	8	0	1276	1	0	_aplCal1_492_75504_92774
TGGCGAAGTACCTTGAGCGATGGCTCTCT	1407	2	1	85	80	0	1	0	0	0	0	1237	1	0	_aplCal1_697_50289_68955
TAGTTCAGTTTGTTGAGCCAGGGTAGATC	1406	45	58	5	98	75	0	98	1	15	0	1010	0	1	_aplCal1_455_224202_242097
TCGTGATAGGGTGTTTTGCTTCCTGGTTT	1402	0	0	250	867	0	2	0	0	0	2	281	0	0	_aplCal1_2304_47457_69575
TAGGAATGTATCCCAGAACCTGTCTTAGA	1396	3	4	590	602	7	9	9	5	2	0	165	0	0	_aplCal1_12_1070314_1075598
TGGTCAATAACCGCCATACTGTCTACCCG	1391	122	31	10	17	240	84	119	45	5	0	618	91	9	_aplCal1_1036_128796_134511
TTTTCAATGTGATTCGTGCATGCACCCGT	1383	40	104	26	20	18	8	7	14	0	1	1143	0	2	_aplCal1_2653_34608_42410
TGTTGATGTCTGTTTCCTGTTGTACAGGATT	1379	11	0	3	2	3	0	4	2	2	0	1352	0	0	_aplCal1_740_112786_124340
TTATTATGAAGTCAACTACCCTCCTGGTG	1362	66	0	48	1	72	1	32	28	9	0	1105	0	0	_aplCal1_7_1016346_1020018
TTCAGTCTTTCGGCATGCCACTTAGTGT	1359	27	0	7	0	3	0	2	0	0	0	1320	0	0	_aplCal1_2299_30198_72073
TACGTGTCCGCGATGACCGGTTTGTCGAC	1357	81	2	24	7	22	0	20	0	0	0	1200	1	0	_aplCal1_147_26539_30349
TGAAAACCAAATGCATCCACTTGAAGAAC	1351	74	66	12	25	253	66	97	108	11	5	634	0	0	_aplCal1_366_175072_190841
TTGTTCTATTTTCCGTCATCCAGGCGACT	1349	24	13	1	27	3	8	18	4	3	2	1246	0	0	_aplCal1_366_175072_190841
TCGGAAAGTACCACACTCTCGCTTATTTC	1345	91	34	36	169	50	50	51	7	13	7	834	0	3	_aplCal1_1972_39301_75265
TCGACGAGATGGCTTTCTGAAAGGCGGC	1345	12	1	133	40	7	22	150	0	4	5	971	0	0	_aplCal1_101_532920_559602
TTGTCGGCATCTTGAACGGTTCGTTCATGAA	1343	9	0	16	5	0	42	5	1	0	0	1265	0	0	_aplCal1_645_48662_56779
TACAAACTTTTGGGCCAGAAGTGGTTCGCT	1338	64	17	38	34	12	16	42	0	7	4	1104	0	0	_aplCal1_613_149832_164946
TGAATTTGTTTGGGTTTCTGTTTCCTCG	1330	12	26	13	35	14	86	10	19	0	0	1112	3	0	_aplCal1_10_358654_362785
TCCAATTGAAACGGCAATGCTGAGCCTGT	1328	0	0	1	8	0	0	0	0	0	0	1318	1	0	_aplCal1_1117_24553_42207
TTTTTGTTCTCTTCACGGCAAGAGCGG	1327	34	0	31	8	69	67	42	2	10	5	1059	0	0	_aplCal1_155_244249_252418
TTTTATAGCACCGTACCAAGCACAGGCCT	1327	47	3	8	4	19	2	39	1	7	2	1195	0	0	_aplCal1_1216_28641_44559
TTTCTCTAAACCAAACCAGAAATTGCCGG	1321	103	160	15	12	162	81	57	1	1	0	720	9	0	_aplCal1_1036_138128_154149
TTATACTTTTTAGACGAACTGGGGCGAT	1318	0	24	0	26	0	23	0	26	0	0	1219	0	0	_aplCal1_39_566527_578980
TCTTGCATCCGTACATAGTCATCAGAGG	1316	0	93	0	56	0	13	0	46	0	0	1104	4	0	_aplCal1_39_566527_578980
TCGTTCTGTTCCATTCTTTGAAATGGGGG	1315	0	3	0	5	0	0	2	1	0	0	1304	0	0	_aplCal1_98_137957_177330
CAATATGAAACGTCTTGAATCAGACAAA	1313	16	9	14	90	2	4	3	6	0	0	1169	0	0	_aplCal1_523_215863_271832
TGCTACGTGGCTGTGGTGCCTCTGCCTG	1309	0	0	4	3	0	0	0	0	0	0	1292	0	10	_aplCal1_439_201156_223269
TGGAGACGTACCGTAGGCCATGCCTCTCA	1308	53	7	18	5	7	7	18	11	1	4	1169	6	2	_aplCal1_697_50289_68955
TCCTTTAGGGCAGTTCTCACGGTGGTGGT	1307	0	9	0	10	0	3	0	11	0	3	1264	7	0	_aplCal1_2082_38884_60606
TCACTGAAAAGAAGCGTGCCTTTCCTCA	1307	60	0	3	0	27	0	30	0	11	0	1175	1	0	_aplCal1_2299_30198_72073
TCAGACAAGTCAATGGTAATCAGCACCCA	1306	30	285	15	70	10	188	20	54	0	8	625	1	0	_aplCal1_1667_1422_36545
TGCGTCTTTTGTACTGGAGATGGCCACG	1302	201	34	42	59	243	104	346	16	14	5	238	0	0	_aplCal1_12_688185_696854
TGGTAAGAGCATCTCATGGCTGACTGGTG	1300	0	0	50	73	0	0	0	0	0	0	1177	0	0	_aplCal1_2304_47457_69575
TAGTTACGAGATGCTCGGTGAAACTCTT	1299	100	4	224	4	52	0	106	9	2	0	797	1	0	_aplCal1_147_26539_30349
TCATCTTCTGAATATCTGGGGCATTCTCT	1296	9	1	8	7	6	4	7	2	1	0	1251	0	0	_aplCal1_446_312983_330760

AAAGCTCCCATCCCTGATAGATACATCC	1295	106	32	68	33	48	23	60	18	14	2	890	1	0	_aplCal1_1972_39301_75265
TTCTTGTCTCTGCTACTCCAATGGCATGC	1287	1	1	2	10	1	2	5	0	1	0	1264	0	0	_aplCal1_10_358654_362785
TGGGTTTCGGATGATGCCACTTCTGCAGC	1275	26	7	77	80	25	3	52	1	2	3	999	0	0	_aplCal1_2299_30198_72073
TGACGTGGTTGCATGTACTGCACATGCCA	1275	3	2	0	5	4	1	7	1	0	0	1252	0	0	_aplCal1_1055_183474_193567
TCATTCAACTTTATTGTCTTTGAAAGGTC	1272	65	21	28	40	32	2	55	3	4	0	1018	0	4	_aplCal1_523_215863_271832
TGGAAGCACTCACTCCTGTAGAAGGAGCA	1247	1	6	3	247	0	762	2	4	0	0	222	0	0	_aplCal1_471_221309_226546
TGTTACGGATTTTCTCTGAAGAGGTGAG	1245	0	0	11	26	0	1	4	3	0	0	1200	0	0	_aplCal1_5_620223_635068
TGATCTACGAAGGCTGACATCTTCTGCTC	1244	30	25	25	56	14	5	19	6	6	2	1056	0	0	_aplCal1_10_358654_362785
TAATAAGAACTTAAGTCCTTCACCGTAC	1243	25	31	12	468	30	28	8	0	14	0	627	0	0	_aplCal1_523_215863_271832
TAGTCTATGGCATTAGCTACTGAAGAGC	1240	7	52	35	245	6	0	12	5	0	2	868	0	8	_aplCal1_1210_56333_70614
TAGAATTATTGTGTTCGTTTCTAGTGT	1239	32	17	102	47	139	45	87	17	4	2	735	10	2	_aplCal1_68_527628_533547
TGAACAGGAACCAGGACTTCTTCATGAAGT	1235	42	132	170	262	33	143	41	98	22	183	103	5	1	_aplCal1_1405_25102_40112
TGAACAGAGATGTCCTCATTCTGCAGGTC	1234	33	6	241	146	174	112	112	27	35	2	344	2	0	_aplCal1_155_244249_252418
TCTCTGTTGCGCACTTGACAAGGAGTA	1234	17	4	5	6	5	5	6	2	0	0	1184	0	0	_aplCal1_737_233617_250062
AACGAGATCCTAGGGCATTCCAACCCTGTT	1233	26	0	4	6	15	0	32	0	2	0	1147	1	0	_aplCal1_190_74320_82083
TTCTTGAAACAGGTGGTTGTCCAGAGCAG	1227	7	4	6	16	3	20	0	11	0	3	1153	0	4	_aplCal1_1667_1422_36545
CAAAGAAATTGAAGCTCCATCGGACAGG	1226	181	73	83	0	198	6	149	0	7	0	529	0	0	_aplCal1_393_78915_104964
TGGACATTCGTGGGAATGTATCCCAAA	1225	2	1	3	5	0	0	0	0	0	0	1214	0	0	_aplCal1_2299_30198_72073
TGTGTCTTCATCACTGCTGCTAGCAGCTGGC	1220	33	3	0	3	0	0	0	0	0	0	1181	0	0	_aplCal1_439_201156_223269
TGTTTTGACTGTAGCTTATGAAGCTGGC	1219	33	16	13	19	8	18	9	9	1	1	1084	2	6	_aplCal1_1210_56333_70614
TCAACAGACAAATCTGTCTGGAGGGTAGC	1219	16	1	55	10	23	7	12	2	11	2	1080	0	0	_aplCal1_155_244249_252418
CACCGAATCCATTCTTATAGTCTTTCCATC	1215	121	15	20	54	316	90	44	2	2	0	549	0	2	_aplCal1_1036_138128_154149
TAATAAGAACTTAGTTCCTTCACCGAACT	1205	54	4	7	468	14	19	7	0	3	0	628	0	1	_aplCal1_439_201156_223269
TAAGAAATTCTAAGAAAGGCTGCTGCTTT	1205	4	5	60	59	16	7	11	1	0	5	1037	0	0	_aplCal1_1972_39301_75265
TCGAAACATTTCCTGCAACCAAAGCAGAGC	1198	1	27	75	223	0	18	5	34	0	5	810	0	0	_aplCal1_2299_30198_72073
TGGTATTGCTTTCTCTGCCACTCTGCGAC	1188	3	5	9	9	4	45	2	0	6	0	1105	0	0	_aplCal1_523_215863_271832
TCGACATCTTCGGTTGTTTCCACTCCATC	1188	60	45	33	162	43	65	40	14	8	0	717	1	0	_aplCal1_446_312983_330760
TCAAACAAGTGTTCGTGCGCCATTTGGTTT	1188	0	5	1	16	0	6	0	2	0	0	1156	2	0	_aplCal1_39_566527_578980
TAGTTTTCAGCAAAATCCATTGTCAGAAC	1185	206	10	40	9	87	28	145	38	10	1	605	0	6	_aplCal1_471_221309_226546
TGTTATGCTTAATTTTTTTCTGTTGTCT	1184	150	29	42	78	65	53	78	37	9	1	641	0	1	_aplCal1_439_201156_223269
TGAAACTCAGACATGTTGGCATTGACCG	1184	5	45	35	7	0	12	0	1	2	1	1076	0	0	_aplCal1_101_532920_559602
CTGAATTGTATCTCCCTTGCACAGAGT	1184	81	25	46	90	23	46	47	12	7	2	800	0	5	_aplCal1_1484_21639_27657
TATTTCGGTAGGTACCTTCAGTATAAGGG	1179	18	66	89	157	9	59	15	35	6	10	709	3	3	_aplCal1_98_137957_177330
TTGGAGAGTCTTAATAACTTCAGGTGG	1178	0	7	0	23	0	3	0	5	0	0	1140	0	0	_aplCal1_2082_38884_60606
TGCAGTTGAGCAGAACCAGGTCCTTCCCG	1178	29	1	103	34	76	50	99	2	8	9	767	0	0	_aplCal1_645_48662_56779
TGAAGGATAAGACACTGCTCATTCCCCT	1177	1	0	308	367	1	1	2	1	0	0	496	0	0	_aplCal1_2304_15568_27364

CTTCCATACACCACACGTGTATGTA	1175	0	8	0	2	0	12	0	2	0	5	1146	0	0	_aplCal1_2339_7934_26341
TAGCTAAGTGTTCCTTGCCCGTATCGCAG	1170	77	12	14	9	29	58	54	29	7	1	860	3	17	_aplCal1_610_113720_122340
TAAACCAAACTCCTGTTTGCAGCAGGCCG	1169	37	0	5	0	0	0	9	0	0	0	1118	0	0	_aplCal1_2299_30198_72073
TCGGCACTTCCACAGGACACTGCTGT	1161	1	0	44	566	0	2	0	0	0	2	546	0	0	_aplCal1_2304_15568_27364
AGACTTACATGGAATACCGCCGAAGGTC	1156	13	4	81	15	6	3	6	0	1	0	1027	0	0	_aplCal1_11_198663_200992
TTTTTGTTGTAAAAATATGCTGACATGTT	1152	190	50	38	34	61	28	168	17	64	18	481	0	3	_aplCal1_2339_7934_26341
TATTATTTCTTCTATTATTTACTCGAAC	1151	83	43	52	35	80	58	131	25	25	14	601	0	4	_aplCal1_610_113720_122340
TGAGAATTGCCAAGAACCTGAAGAATTC	1149	35	102	23	227	39	6	48	6	1	0	662	0	0	_aplCal1_420_43790_46689
TGTAAACGGAAAATTCACCTTCAATGGGAT	1145	5	2	239	133	0	1	0	0	6	2	757	0	0	_aplCal1_190_74320_82083
TAGTTGATCAAGTTCCTCGGTGATGTTC	1144	60	13	109	157	89	2	1	19	1	0	690	3	0	_aplCal1_101_532920_559602
TTTCTGCCAACTACCGTCAAAGGAGACAT	1143	79	11	9	16	5	2	17	3	1	0	1000	0	0	_aplCal1_2082_38884_60606
TTCACTCTTGAAACCGTGGAAGCACTCAC	1142	141	61	9	4	193	14	213	6	18	2	480	0	1	_aplCal1_471_221309_226546
TGGACTTTGACTTCAGCAGAAGGCGAGTG	1139	289	26	116	112	116	2	376	13	50	14	24	1	0	_aplCal1_1210_56333_70614
TCGCTTACGGCATTCAGTTCATTATAGA	1136	141	29	20	10	76	2	118	28	24	12	676	0	0	_aplCal1_77_95376_114859
TGAGTTTTTTGTTCTCTGCACGGCAAG	1129	80	2	60	21	67	126	239	3	35	10	486	0	0	_aplCal1_155_244249_252418
TGAGTAACACGGTGTTGACTTACAGGAGC	1128	81	0	2	2	33	1	190	99	8	0	712	0	0	_aplCal1_7_1016346_1020018
TAGAATTGGAATATGAATCCTCCATATAGT	1124	164	26	130	149	190	133	19	7	1	5	300	0	0	_aplCal1_53_318395_339530
TATTTGTCGGAGCACGAGAAAGTGCGTCC	1122	9	6	6	3	2	2	1	2	0	1	1090	0	0	_aplCal1_283_154778_166233
TCATAACTGGCTTTCAGGGCGTCCACTGT	1114	57	90	2	26	193	208	18	12	1	3	480	22	2	_aplCal1_1036_138128_154149
GACCAAGTACGATGCCCGAACGGTGGCTGGA	1109	2	1	16	4	1	1	1	0	0	1	1082	0	0	_aplCal1_0_1461183_1467458
TGCTCGGATGACCAGAGCTGTGCCAGGGC	1105	1	5	505	430	26	18	19	27	2	0	72	0	0	_aplCal1_6_515672_521685
TGCGCGTTCTTGGATCCAAGTTGGGCAAT	1096	14	1	3	4	1	0	8	0	0	0	1065	0	0	_aplCal1_129_530819_548585
TCTGACCTGTCTGACTGACCTCAGCGTG	1096	0	0	68	152	0	1	0	0	0	0	875	0	0	_aplCal1_2304_15568_27364
TCAAGCTAATGACGTCACATCTGTATTTGC	1096	59	5	2	1	19	5	163	23	1	0	818	0	0	_aplCal1_1036_138128_154149
TATGGTTGATTCTCCAAGCTCTCTGCCT	1095	2	0	3	10	0	0	1	0	0	0	1079	0	0	_aplCal1_1667_1422_36545
TCCGACATTGCCTTTTCTCTTTACAAAG	1091	88	30	8	11	36	7	29	9	2	2	852	10	7	_aplCal1_1972_39301_75265
TAGGAAACGTCAATGTCCAGGACACCATT	1087	120	87	34	238	145	163	96	13	18	10	163	0	0	_aplCal1_55_253441_264685
TGATAAGATCAAAAGACCGTACACTCTCC	1085	32	15	16	45	58	74	10	0	13	0	822	0	0	_aplCal1_523_215863_271832
TACTTCTTCTATGTCAAGACATTGCACG	1085	39	11	3	4	7	8	104	35	1	1	870	0	2	_aplCal1_98_137957_177330
TACGCGAACCACTGGATTACCGTGTTCT	1080	93	11	2	0	5	0	2	0	0	0	967	0	0	_aplCal1_2299_30198_72073
TTACCACCTCCCGTAGAGTCTGACCTTC	1078	42	40	9	28	31	46	29	12	14	9	807	5	6	_aplCal1_610_113720_122340
TAATCACATTTCTCACTTTGAAGAGGGGATC	1078	82	1	7	14	14	1	51	1	9	0	898	0	0	_aplCal1_714_24215_35038
AGTTAGTTCAGTCCTGTTGTTGTAGGCT	1076	0	0	1048	27	0	0	0	0	1	0	0	0	0	_aplCal1_2653_1_6026
CGAAATCTGAAACTTTGCCTCTGGGAAGC	1074	37	10	2	6	0	8	11	0	3	0	997	0	0	_aplCal1_48_678521_693149
TGTAGTCGTGAAGTAGTCATTCAAGAGGT	1072	21	48	6	21	11	10	16	2	1	0	934	2	0	_aplCal1_2369_34957_57029
GTAGAAGTTTGAGATGTGACGTAGCACC	1072	27	10	10	55	14	6	35	4	3	0	908	0	0	_aplCal1_77_359166_370796

TGGAGCTCAATTTAGTACTGTAGTGTGC	1070	8	19	6	1	2	4	12	16	0	1	989	12	0	_aplCal1_2339_7934_26341
TTTTAAAGAAGTCTTTTAGCCACTGTTT	1054	8	4	57	36	11	1	5	3	4	0	925	0	0	_aplCal1_1284_120846_136482
TCATCGATCGCTTTGTCCTGACCAGGTTC	1053	1	0	16	7	0	11	0	3	1	0	1014	0	0	_aplCal1_492_75504_92774
TGAGTAACCTCGTGTGATCGCTCGGGGTT	1051	0	31	10	9	0	14	1	24	0	0	956	1	5	_aplCal1_39_566527_578980
TACGGATCTGCGTAGTGTACGTCTTGCC	1048	168	2	7	12	59	1	12	0	9	0	778	0	0	_aplCal1_155_244249_252418
TGAGACGATAAAGGAGATTGTCCTCCTTC	1044	10	36	8	51	7	26	10	0	8	0	886	1	1	_aplCal1_523_215863_271832
TCGCCACAATTTCCTCCATGCTGCAGCCC	1043	13	11	4	2	5	1	3	3	1	0	1000	0	0	_aplCal1_0_1461183_1467458
CGACTGTAATATGCAGCACCAATGGGGG	1041	11	5	5	1	2	0	4	0	0	1	1012	0	0	_aplCal1_77_95376_114859
TGAAGAAGTTGACTCGAAATGTGCTGCA	1038	19	12	5	4	3	6	25	2	0	2	945	14	1	_aplCal1_446_272304_285124
TGACTACGGCAAACTGCTTTATAGTGGCCT	1037	38	18	12	52	18	7	13	6	4	0	867	2	0	_aplCal1_178_206973_213522
TGCTTTTTCTAAACTCAGCATGAGTGAGT	1029	0	14	0	18	0	13	0	4	0	2	978	0	0	_aplCal1_101_532920_559602
TGTTCTTGATGGAAGGCTCATTTAAGCGC	1028	56	0	3	2	36	0	16	0	0	0	915	0	0	_aplCal1_2299_30198_72073
TACGAATCTGCGTAGTGTACGTCTTGCC	1028	175	0	15	13	60	139	20	0	13	3	590	0	0	_aplCal1_2321_35916_43532
TTTCTGTTTGGCACTTCTCGCCCGCTCT	1027	16	0	12	6	45	10	26	0	11	2	899	0	0	_aplCal1_155_244249_252418
TACCGACCGTGAACCAGCCACACATTCA	1026	47	20	9	27	53	55	76	15	12	4	674	27	7	_aplCal1_610_113720_122340
TTTTCCTTTTGAAGTTTGTTTGCTCGGC	1019	13	48	63	76	27	31	44	35	5	2	675	0	0	_aplCal1_12_1070314_1075598
TAACTGATTGTAGATGCGTTTGGCTAT	1014	26	50	12	53	10	17	4	2	0	0	840	0	0	_aplCal1_10_358654_362785
TGCTTCTGGTCCCACAATTGAACTCAAAGT	1013	0	367	0	303	0	85	0	51	0	8	199	0	0	_aplCal1_2299_30198_72073
TCCTTGTGGAAACTAGCAAAACACAGTCC	1011	18	44	9	26	11	31	11	11	0	0	850	0	0	_aplCal1_2299_30198_72073
TGGCTTTAAATCGTCTCTTGGCATTTTCC	1004	23	22	10	71	2	13	29	8	2	1	823	0	0	_aplCal1_1057_159778_180055
TTTGTCTATTCTGCAACTCCCTGAAGAGC	1003	1	22	13	60	0	1	1	4	0	0	901	0	0	_aplCal1_455_224202_242097
TGAATGAACACACCTCACGGAAGTACGC	1003	60	33	19	91	5	13	37	9	16	0	717	0	3	_aplCal1_1057_159778_180055
TGGACGTGATCAGCGACCAGCTGGCAAAGT	1002	0	146	0	41	0	40	0	53	0	20	702	0	0	_aplCal1_2082_38884_60606
CAAGTTAGGAGAAAATTTTGCCATCTGGTT	1001	7	8	2	3	5	1	1	12	0	1	961	0	0	_aplCal1_283_154778_166233
TGAAACTCCAAGAGAAGAGAAGTTCAACA	997	386	21	58	31	44	26	93	3	5	0	330	0	0	_aplCal1_2299_30198_72073
TGAAAGGGAACAGTCCTTGACAGCATTTC	982	40	36	7	36	15	35	38	11	18	12	734	0	0	_aplCal1_1895_51672_71846
TCCACGATGGCTCGAGACGTATCTTCAGC	978	16	22	88	56	7	19	59	15	31	2	661	2	0	_aplCal1_446_272304_285124
TATGGAATGCCGAAAGCCTTGCTTGCTCC	977	13	2	27	50	6	1	5	0	6	0	863	4	0	_aplCal1_1284_120846_136482
TGAATAGTTTGAGTTCCCTTAACTGGCTG	973	16	32	13	36	10	61	8	0	5	0	792	0	0	_aplCal1_523_215863_271832
TGACGAAAGTGACATCTGCCCTTGAACC	972	122	0	146	0	119	0	124	2	2	0	457	0	0	_aplCal1_147_26539_30349
TACAAGTCAGCCTGAGCAACATTAAAAGAC	971	3	105	2	74	1	88	3	138	0	8	548	1	0	_aplCal1_2299_30198_72073
TAGATCAATGGGCAAAATATGTGGGTGC	970	1	3	38	35	1	1	33	12	1	0	842	3	0	_aplCal1_98_137957_177330
TGCGAATAGTGTTTGCTGTCAGCCTGCCA	967	20	64	8	65	14	7	24	10	18	8	710	11	8	_aplCal1_446_272304_285124
TTATTCTCGAGGAGAGTACGCGTTTAGTT	965	106	24	45	55	58	14	90	14	13	7	521	0	18	_aplCal1_610_113720_122340

Supplementary Table 6. Top 75 miRNAs and piRNAs listed in order of abundance (clone frequency)

sequence	category	total
TCACAACCTGATTGAATGAGGACT	miRNA	7542197
TGGAATGTAAAGAAGTATGTAT	miRNA	1769359
TGAGATCATTGTGAAAACTGATT	miRNA	1640802
AGATATGTTTGATATATTTGGTGA	miRNA	990330
TATCACAGTCAGCTTTGATGAGCT	miRNA	817119
TGAGGTAGTAGGTTGTATTGTT	miRNA	630287
GAGCTGCCAAATGAAGGGCTGT	miRNA	622430
TCAGCAGTTGTACCACTGATTTGA	miRNA	568766
TGCCATTTTTATCAGTCACTGTGA	miRNA	555317
TGGACGGAGAACTGATAAGGGCA	miRNA	536113
TACCCTGTAGATATCCGAATTTGT	miRNA	418245
TGAAAGACATGGGTAGTGAGATG	miRNA	391268
AACCCGTAGAACCGAACTTGT	miRNA	264636
TATCACAGCCTGCTTGGATCAGT	miRNA	244991
TAGAAGCTTGATTTGTAGCATTTAAGGTT	piRNA	241980
TATCACAGCCAGCTTTGATGAGCT	miRNA	208641
TCCCTGAGACCATAATTTGTGC	miRNA	204011
TGGAAGACTAGTGATTTAGTTGTT	miRNA	152877
TAATGCTGTCAGGTAAAGATGTCA	miRNA	146360
TGGACGGAGAACTGATAAGGGCT	miRNA	142872
TAATATCAGCTGGTAATCCTGAGT	miRNA	141954
AGCTGCCTGATGAAGAGCTGTCC	miRNA	141799
TTCGTTGTCGTCGAAACCTGCCT	miRNA	132851
TATCACAGCCAGCTTTGATGACA	miRNA	127664
TAAGGCACGCGGTGAATGCCA	miRNA	125750
TAATACTGTCAGGTAAAGATGTC	miRNA	83510
CGACATTATTTCAACTGCTGAGGCAGTCCT	piRNA	69753
TCCATCCGGTTCTCGAGAAGTTCTGCTTT	piRNA	67538
TTGGTCCCCTTCAATCAGTTGT	miRNA	63301
CCAGATCTAACTCTTCCAGCTCA	miRNA	53133
TTGCATAGTCACAAAAGTGATC	miRNA	51774
GTGAGCAAAGTTTCAGGTGTAT	miRNA	48836
TAGAATAATTTCCAGGACGTAGGAATTGT	piRNA	38639
TACGTCACATTGGTCCTGACCTATTGGTG	piRNA	37125
TTTTGTTGTGAAAATATGCTGACATGTTT	piRNA	36803
TAACATCTGATACCGGTGACGGGAGAGG	piRNA	36082
CGGCTCTTCACCTGGTAGACTTG	miRNA	35761
TGCCCTATCCGTCAGGAACTGT	miRNA	33834
TCAAATCTGGTCCTGGTTGCAAAAGCTCC	piRNA	33296
TGAAAGACATGGGTAGTGAGATT	miRNA	31362
TGAGATTCAACTCCTCCAACTGC	miRNA	29748
TACCAAGTACGGTGCCCGAACGGTGGCTGGT	piRNA	29614

TATCACAGTAATTTAGATGGGCT	miRNA	27872
TGAATTGTCTTGTATGTTTAGTAGTGT	piRNA	27186
TGGACGGAGAACTGATAAGGGCTT	miRNA	26716
TCCAAGTCGAGCAATCCTTGCTGAAGGCT	piRNA	24839
TTGATGATTTCGGGCAGCACCTCTGCATG	piRNA	24496
GACCAAGTACGATGCCTGAACGGTGGCT	piRNA	24070
TAAACTATCGCAGTATTTGACCACAGGCC	piRNA	22009
TGTAACTGAGGACATCATACTTTACTCTGAT	piRNA	21114
TGGCAGTGTGGTTAGCTGGTTGT	miRNA	21107
ATCGAGAACATTGATAATAGTGAAGTTG	piRNA	20515
TGTCGTTGGACCTGTAAAACACAGGGTCA	piRNA	20315
TCACCCTGACATGTGTGTTTCTCTCACCC	piRNA	20229
TAGAATTGGAATATGGATCCTCCATAGAGGT	piRNA	19471
CTTGGCACTGGCGGAATAGTCAC	miRNA	18807
TGGAGAGATGAAAAGTTCAACTTTTGGGC	piRNA	18418
TAAATATTGTACTGCGAAAGGCGAGGAGC	piRNA	18018
GACCAAGTATGATGCCAAAACGGTGGCTG	piRNA	17540
TGCCTTTGATGACAACCTACATGCTGATG	piRNA	16315
AAACACATCATCCTTCTGCTGAGGGCCG	piRNA	16146
TTGCCTGAAGACATCATACACAACTCTGATA	piRNA	15630
TAGGTGTAGCAAAGTATACCTCATTAGGTG	piRNA	15030
ATTTGGCACTTGTGGAATAATCG	miRNA	14838
TGGTTGACACTCTCGTGTTTTTGAACGGC	piRNA	14752
TAAGGCACGCGGTGAATGCCAAGA	miRNA	14608
TCTTTGGTTATCTAGCTGTATGA	miRNA	14304
AAAATCACGTCGACTTTCGACAACAGTGC	piRNA	13917
CTGGTTTTCACAGTGATTTGCCAGA	miRNA	13741
TCCATCCGGTCCTCGAGAAGTTCTGCTTT	piRNA	13632
TACCAGAGCCGGCTGTAATGGTTCAGCCCCG	piRNA	12681
TCACAGCCAGCTTTGATGAGCG	miRNA	12204
TATCACAGCCAACAGATGGGCT	miRNA	11860
TTTGCGAGCCCTTCTGCGATGCAGAATAT	piRNA	11741
TTTTGATTGTTGCTCAGAAAGCC	miRNA	11390
TGAACACAGCTGGTGGTATCT	miRNA	11294
TATTGCTTGAGAATACACGTAA	miRNA	10918