UNIVERSITY OF CALIFORNIA, SAN DIEGO

Gene expression and evolution

A dissertation submitted in partial satisfaction of the requirements for

the degree Doctor of Philosophy

in

Biology

by

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University of California, San Diego

2010

DEDICATION

To my father who showed me the wonder that is the natural world

To my mother who taught me to love and be loved

EPIGRAPH

Somewhere, something incredible
is waiting to be known.

Carl Sagan

If we knew what it was we were doing
it would not be called research.

Albert Einstein

I'm sciencing as fast as I can!

Professor Farnsworth

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PUBLICATIONS

Kosman, D., Mizutani, C. M., Lemons, D., Cox, W. G., McGinnis, W. and Bier, E. (2004). Multiplex detection of RNA expression in Drosophila embryos. *Science* **305**, 846.

Kuhnert, F., Campagnolo, L., Xiong, J. W., Lemons, D., Fitch, M. J., Zou, Z., Kiosses, W. B., Gardner, H. and Stuhlmann, H. (2005). Dosage-dependent requirement for mouse Vezf1 in vascular system development. *Dev Biol* **283**, 140-56.

Pearson, J. C., Lemons, D. and McGinnis, W. (2005). Modulating Hox gene functions during animal body patterning. *Nat Rev Genet* **6**, 893-904.

Lemons, D. and McGinnis, W. (2006). Genomic evolution of Hox gene clusters. *Science* **313**, 1918-22.

King, N., Westbrook, M. J., Young, S. L., Kuo, A., Abedin, M., Chapman, J., Fairclough, S., Hellsten, U., Isogai, Y., Letunic, I., Marr, M., Pincus, D., Putnam, N., Rokas, A., Wright, K. J., Zuzow, R., Dirks, W., Good, M., Goodstein, D., Lemons, D., Li, W., Lyons, J. B., Morris, A., Nichols, S., Richter, D. J., Salamov, A., Sequencing, J. G., Bork, P., Lim, W. A., Manning, G., Miller, W. T., McGinnis, W., Shapiro, H., Tjian, R., Grigoriev, I. V., and Rokhsar, D. (2008). The genome of the choanoflagellate Monosiga brevicollis and the origin of metazoans. *Nature* **451**, 783-8.

Pare, A., Lemons, D., Kosman, D., Beaver, W., Freund, Y. and McGinnis, W. (2009). Visualization of individual Scr mRNAs during Drosophila embryogenesis yields evidence for transcriptional bursting. *Curr Biol* **19**, 2037-42.

Lemons, D., Sun, X., Zou, Z., Campagnolo, L., Xiong, J., Kuhnert, F., English, M. A., Licht, J. D., Stuhlmann, H. (submitted) Vascular Endothelial Zinc Finger 1 (VEZF1) is an endothelial transcriptional transactivator that displays sequence-specific DNA binding to the IL-3, FLK-1, and FLT-1 promoters.

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ABSTRACT OF THE DISSERTATION

Gene expression and evolution

by

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The control of the spatial expression of gene products is one of the most important and complex processes contributing to the embryogenesis of metazoans. This control can be achieved through many levels of regulation. Transcription factors such as

homeodomain proteins are important regulators of the phenotypic output of cells in which they are expressed. The Hox genes are a genomic cluster of homeodomain containing master control proteins, each of which is expressed in, and controls the morphology of a specific anteroposterior domain. While transcription factors have long been known to be an important type of regulatory molecule, recent discoveries have shown that there is always more out there for biology to teach us. The discovery of microRNAs has led to a whole new paradigm, in which the expression of many genes is controlled by the activity of tiny RNAs that were once invisible to researchers.

The Hox complexes of bilaterian animals typically contain a few microRNAs, but nearly all of them have the *mir-10* gene. This ancient regulatory molecule is conserved in its sequence, genomic position, and to some extent its spatial expression over evolutionary time scales. In *Drosophila melanogaster*, this gene produces two mature microRNA products, each of which has strong complementarity to conserved sequences in Hox genes. These microRNAs are also expressed in patterns that are complementary to these potential target genes. Despite these correlations, disruption of the normal expression of the *mir-10* gene does not strongly effect the expression of these genes, but may instead function to canalize their expression.

Appendages are structures common to a wide variety of animal body plans. When compared between distantly related animals there are no obvious structural characteristics which suggest they are homologous, yet they are patterned by similar transcription factors. Since these structures are not likely ancestral, their seemingly conserved patterning mechanism may have been acquired through independent co-option of a pre-existing network of genes for a similar purpose. The core genes responsible for patterning

the proximodistal axis of appendages are also expressed in domains of the anterior neurectoderm that are conserved among distantly related animals and function to pattern the anteroposterior axis of the head. This ancient gene network was likely co-opted in order to pattern the axis of appendages during the early evolution of bilaterian animals.

The advent of multicellularity has provided our planet with a rich variety of organisms. It is unknown what was necessary to allow single celled organisms to give rise eventually to the people that would research them, but there are many proteins that are assumed to be important for this process. Multicellular organisms are composed of many different types of cells, and homeodomain transcription factors are often involved in the specification of these cell types. The nearest relatives to animals are choanoflagellates, which are simple single celled organisms that sometimes exist in colonies. Although most animals have a specific set of homeodomain containing genes, most these are either evolutionary novelties specific to animals or have been secondarily lost in choanoflagellates. For this reason it is unknown what role homeodomain proteins played in the process of multicellularization that gave rise to animals, but further studies may provide exciting answers.

Chapter I

Evolution, expression and function of the Hox complex miRNAs

miR-10 and miR-10*

INTRODUCTION

MicroRNAs (miRNAs) are a recently discovered class of regulatory molecule that has dramatically enhanced the standard view of gene regulation. These ~22-nucleotide RNAs are made from transcripts that are generated by RNA polymerase II in a two-step cleavage process that is catalyzed by the nuclear RNaseIII Drosha and the cytoplasmic RNaseIII Dicer (Bartel, 2004; Pasquinelli et al., 2005). After processing, the mature miRNA is packaged into a functional RNA-Induced Silencing Complex (RISC). The RISC can catalyze precise endonucleolytic cleavage of target RNAs if they have perfect or near perfect complementarity to RISC-bound miRNAs, or reduce protein production by unknown post-transcriptional mechanisms if there is imperfect complementarity. Considering the large number of identified miRNAs, few validated *in vivo* targets are known at present. However, there are indications that Hox genes might be an important class of miRNA targets.

For some time, there has been evidence for post-transcriptional regulation of Hox gene expression. Mouse HOXB4 protein and *Hoxb4* transcript patterns of accumulation were similar in somites 7–13 and in the posterior hindbrain of developing embryos, but transcripts were also detected in the posterior neural tube, whereas protein was not (Brend et al., 2003). In addition, there is evidence for post-transcriptional regulation of *HOXC6* in the chick embryonic hindlimb (Nelson et al., 1996). There is also evidence that post-transcriptional regulation of the *Sex combs reduced* (*Scr*) ortholog in the crustacean *Porcellio scaber* might be involved in modification of the first thoracic segment to produce gnathal appendages as opposed to walking legs (Abzhanov and Kaufman, 1999). It is unknown whether these discrepancies between transcript and

protein expression patterns are due to miRNA regulation or other mechanisms such as localized protein instability.

Although a few Hox genes are post-transcriptionally regulated by unknown mechanisms, many Hox genes have been predicted *in silico* to be direct targets of miRNAs in both vertebrates (Lewis et al., 2003) and invertebrates (Enright et al., 2003). On the basis of partial complementarity between miRNA sequence and 3'UTR sequences, *Drosophila melanogaster Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B* transcripts are proposed targets of miRNA regulation (Enright et al., 2003). If true, this indicates that miRNA regulation of Hox protein expression, at least in flies, is the rule rather than the exception.

One validated Hox miRNA target is the protein-encoding Hox transcript from mouse *Hoxb8*. The *Hoxb8* transcript contains a near perfect miR-196 target site in its 3'UTR, and analysis of mouse embryonic RACE clones showed that some of the *Hoxb8* transcripts are cleaved endonucleolytically at the tenth nucleotide of the miRNA target site (Mansfield et al., 2004; Yekta et al., 2004), which is typical for sites with perfect complementarity to miRNA regulators. This target site is conserved in vertebrate *Hoxb8* genes, but it is not known what influence this miRNA-mediated regulation exerts on vertebrate axial morphology.

In addition to miR-196, other miRNAs that are encoded within Hox gene clusters might also be regulators of Hox protein expression. The *mir-10* gene is phylogenetically conserved and is present in a conserved position in both insect and vertebrate Hox clusters, between the *Dfd*/*Hox4* and *Scr*/*Hox5* genes. In mammals, the *mir-10a* gene maps adjacent to *Hoxb4*, and the *mir-10b* gene is adjacent to *Hoxd4*. Additionally, at least one

other miRNA gene maps among the posterior Hox genes in both insect and vertebrate genomes, but the *Drosophila mir-iab-4* and vertebrate *mir-196* miRNA genes do not seem to be structural orthologs, as they have only fragmentary sequence identity. Although not as clear cut as the case for *Hoxb8* regulation, evidence is accumulating that the Hox miRNAs are regulators of Hox genes (Bender, 2008; McGlinn et al., 2009; Ronshaugen et al., 2005; Stark et al., 2008; Woltering and Durston, 2008).

The spatial expression patterns of Hox-cluster miRNAs suggest that they have roles in A–P axial patterning. One way to detect the expression domains of miRNAs is to use ubiquitously transcribed reporter transgenes with 3' UTRs that contain sequences perfectly complementary to putative miRNA trans-regulators (miRNA sensors). The body region in which reporter protein expression is abolished is inferred to represent the expression pattern of the miRNA that is complementary to the target site (Brennecke et al., 2003). This strategy has allowed a rough mapping of spatial domains of miR-10a and miR-196 activity in mouse embryos (Mansfield et al., 2004). The miR-10 sensor indicates that miR-10 activity is present in regions of the posterior thorax and abdomen, but not in the head or tail bud. This is similar to the expression pattern of the adjacent gene, *Hoxb4*, which is consistent with the possibility that they are coordinately regulated (Mansfield et al., 2004). One of the limitations of current 'sensor' studies has been the difficulty of interpreting the 'negative' patterns at cellular resolution.

The primary transcripts of miRNAs are typically much longer than the mature miRNAs (Pasquinelli et al., 2005), and *in situ* hybridization conditions have been developed that allow the detection of these nascent transcripts at their chromosomal sites of transcription. This method was used to detect the *Drosophila pri-mir-1*0 expression

pattern in early embryos (Kosman et al., 2004). The *pri-mir-10* transcripts are detected in

a Hox-like pattern that spans the future thoracic and abdominal segments in blastoderm-

stage embryos. Another method to detect miRNA expression patterns involves the use of

LNA oligonucleotide probes (Valoczi et al., 2004). This allowed the *in situ* localization

of a large subset of zebrafish mature miRNA transcripts (Wienholds et al., 2005),

including those of miR-10a, miR-10b and miR-196a. All three of the Hox miRNAs are

detected in zebrafish embryos in Hox-like patterns in the developing nerve cord and

trunk, with the miR-10a and miR-10b expression borders being anterior to that of miR-

196a. A comparison of the three studies that localized miR-10 expression in mouse,

zebrafish and *Drosophila* embryos indicates that its Hox-like expression pattern in the

posterior head, thoracic and abdominal primordia is conserved in many animal embryos

(Kosman et al., 2004; Mansfield et al., 2004; Wienholds et al., 2005).

Why have *mir-10*, and perhaps *mir-196/iab-4*, genes been conserved in their

specific genomic positions within Hox clusters since the deuterostome–protostome

divergence? It might be that, like the protein-coding Hox-cluster genes, they rely on

regulatory mechanisms such as shared enhancers and long-range Polycomb–Trithorax-

mediated repression and activation (Duboule, 1998; Duboule and Deschamps, 2004;

Ringrose and Paro, 2004). It has been suggested that miRNAs are derived from inverted

duplications of the 3' UTRs of future target genes (Allen et al., 2004). It is possible that

this is the case for the Hox miRNAs, especially considering that they likely target

adjacent Hox-protein-coding genes.

It remains to be seen what significance the *mir-10*, *mir-196* or *mir-iab-4* genes

might have for regulating developmental patterning during bilaterian embryogenesis. It

will be exciting to discover the roles of these highly conserved miRNA genes that have

been residents of animal Hox clusters for at least 550 million years. It is conceivable that

the gain or loss of Hox miRNA target sites, or changes in spatio-temporal expression of

Hox-targeting miRNAs could be an important mode of Hox gene regulation in animal

development and evolution.

RESULTS

Evolution of miR-10

MicroRNAs have been found in multiple branches of the eukaryotic tree of life, but no individual miRNA sequence has been discovered to be shared between animals and plants. The proteins necessary for the function of miRNAs are found in the genomes of most eukaryotes. However, these proteins have other ancestral functions, and it is likely that miRNAs evolved separately in animals and plants by independent co-option of the common machinery present in the genomes of these organisms for processing double stranded RNA and utilization of small single stranded guide RNAs for post-transcriptional regulation (Cerutti and Casas-Mollano, 2006).

Although no pan-eukaryotic miRNAs exist there are many miRNAs which are shared between the deep branches of animal phylogeny (Wheeler et al., 2009). One of the well conserved animal miRNAs is miR-10 which has been found to be produced in both deuterostome and protostome model organisms (Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Lim et al., 2003). Other than being conserved broadly in animals, this miRNA is interesting because it was found to be present in a homologous position between Hox4 and Hox5 orthologs in the Hox complex in both *Drosophila* and vertebrates.

A survey of publicly available genome data revealed that miR-10 sequences are present in the genomes of nearly all bilaterian animals that have had their genomes sequenced (Fig. 1). A single miR-10 sequence is found in all arthropods that have available genome sequence including a crustacean (*Daphnia pulex*), two chelicerates (*Tetranychus urticae*, and *Ixodes scapularis*) and many insects (Fig. 1 - section 1) and the

21 base pair sequence (ACCCTGTAGATCCGAATTTGT) is conserved in all of these animals. Sequences that correspond to miR-10 miRNAs are additionally found in the genomes of many lophotrochozoans (Fig. 1 - section 2) and these animals often have multiple miR-10-related miRNAs with multiple nucleotide changes or insertions in the mature miRNA sequence (Fig. 1 - section 8). Non-vertebrate deuterostomes contain a single miR-10 sequence (Fig. 1 - bottom of section 3) although no miR-10 ortholog is found in available urochordate genome sequences. All vertebrates have at least two miR-10 sequences in their genomes with miR-10a (most similar to arthropod miR-10 of the deuterostome miRNAs) present in the HoxB complex (HoxBb in fish), and miR-10b located in the HoxD complex. The sequence of the mature miR-10a is identical to arthropod miR-10 (Fig. 1 - section 3), although the 5' and 3' ends are shifted in mature sequences of some animals (Griffiths-Jones et al., 2006). The miR-10b sequences are identical to that of miR-10a except for a single nucleotide change in the center of the mature miRNA (Fig. 1 - section 4). The ray finned fish (*Actinopterygii*) have additional miR-10 sequences due to their secondary genome duplication following the divergence with the lineage leading to tetrapods. The HoxBb clusters of most fish retain the ancestral miR-10a sequence, while the HoxBa clusters typically contain miR-10c (Fig. 1 - section 6) which has diverged with a single nucleotide change that is shared between all fish and must have occurred shortly after the genome duplication. Fish HoxDa clusters retain the ancestral miR-10b (miR-10b-1) sequence (Fig. 1 - section 4), while HoxDb clusters contain miR-10d sequences (Fig. 1 - section 5) which differ by a single nucleotide from miR-10b. Fish HoxC clusters additionally contain miR-10b (miR-10b-2) sequences (Fig. 1 - section 4). The salmon (*Salmo salar*) genome has undergone an additional recent

genome duplication and contains at least 7 miR-10 sequences in its various Hox clusters (Mungpakdee et al., 2008). Coelacanth (*Latimeria menadoensis*), frog (*Xenopus tropicalis*), and lizard (*Anolis carolinensis*) genomes also have miR-10 sequences in their HoxC clusters which was lost in birds and mammals. This sequence in frog and lizard differs from that in coelacanth by one nucleotide (Fig. 1 - section 7), a change which likely occurred prior to the first appearance of terrestrial vertebrates. Some animal genomes contain additional miR-10 sequences (Fig. 1 - section 8) that are not direct orthologs of any of the aforementioned sequences but are derived duplicate miRNAs.

The miR-10 sequences present in assembled genomes seem to be present in the Hox complex and almost invariably between the Hox4 and Hox5 orthologous genes (data not shown). Genome sequence assemblies for many species from multiple major insect clades all showed a similar genomic location of miR-10 and it is likely that this should be case for most insects. A number of vertebrate genomes have been assembled and a similar case is found in all of these animals. Available genome sequence from a few ascidians suggests that they have lost the miR-10 miRNA. These animals have either completely, or at least partially, disrupted Hox complexes, and so it is possible that the loss of a coherent Hox complex may coincide with a lack of necessity for regulation by and subsequent loss of miR-10. In order to test this possibility, the genomic location of miR-10 sequences was examined in animals for which extensive whole genome shotgun data was publicly available that had not yet been assembled. Prior to this, no Lophotrochozoan genome or non-insect Arthropod Hox genomes had been assembled.

The sequences for all of the orthologous Hox homeodomains were collected following BLAST searches of the *Lottia gigantea* (limpet), *Capitella* (polychaete worm),

and *Daphnia pulex* (crustacean) whole genome shotgun data from the NCBI trace archives (data not shown). These sequences were then used as primers for the Tracinator program (Rebeiz and Posakony, unpublished) which iteratively BLASTs the sequence ends against the genomic trace database, collects the best hits, and assembles them into a larger contig. The contigs that were output from this analysis were then combined with some additional alignment by hand to create local assemblies of the Hox complex (if present) of each animal.

In these three cases complete Hox complexes were found with preserved co-linear gene order and no intervening genes (Fig. 2) similar to the presumed ancestral state of the Hox complex. The *Capitella* sequence data did not allow for a full assembly connecting the continuous sequences between the Hox8 and Hox9 orthologs. However, the ends of each half of the complex contained no additional non-Hox genes and further sequencing would presumable allow for a full assembly of this complex. Additional non-insect protostome Hox complex assemblies were attempted, but high levels of repetitive sequence or low coverage prevented these from being successful (data not shown).

In each of these three animals, miR-10 sequences were found between the Hox4 and Hox5 orthologs and in the same transcriptional direction (Fig. 2A-C). The *Daphnia* Hox complex contains sequences for *mir-iab-4* and *mir-iab-4-as* hairpins in positions similar to that of insect Hox complexes (Fig. 2A), but these miRNA genes may be restricted to Arthropod lineages as they were not found in either *Lottia* or *Capitella* Hox complexes or genome sequences. In both *Daphnia* and *Capitella* a second miR-10-related hairpin sequence was found between the Hox3 and Hox4 orthologs in the opposite orientation to Hox gene transcription (Fig. 2A-B), which has apparently been lost in

Lottia. The 5' arms of these hairpins are very similar to miR-10, but their 3' arms contain

sequences orthologous to insect miR-993 (data not shown). This miRNA hairpin is found

in many Arthropod genomes, and although there is extensive similarity to miR-10, this

miRNA mainly produces mature miRNA from the 3' arm of the hairpin in developing

Drosophila (Ruby et al., 2007). However, it is unknown if this is the case in all

protostomes and it may produce a miR-10-related mature miRNA from the 5' of the

hairpin or possibly miRNAs from both arms in some animals. There is a conserved

Lophotrochozoan miR-10-related hairpin found between Hox8 and Hox9 orthologs in

both *Lottia* and *Capitella* Hox complexes (Fig. 2B-C). The miRNA sequence is not

identical between the two animals (data not shown), but due to shared location and

conserved sequence differences from the ancestral miR-10 they are assumed to be

orthologous. In addition to these sequences, the *Capitella* Hox complex contains two

additional miR-10-related hairpins. One of these is near the 3' end of the Hox7 ortholog,

and the other is in the same intergenic interval as miR-10 but transcribed in the opposite

direction, likely a result of a local inverted duplication (Fig. 2B).

The data from Lophotrochozoan Hox complexes, as well as genome data from

numerous other bilaterian animals, indicate that the presence of a coherent Hox cluster

always corresponds with the presence of a miR-10 gene between the Hox4 and Hox5

orthologs. However, there are a number of genomes which contain sequences for miR-10

hairpins which do not have coherent co-linear Hox complexes. The purple sea urchin

(*Strongylocentrotus purpuratus*) genome has a Hox complex in which the gene order has

been dramatically altered from the ancestral state and has lost the Hox4 ortholog

(Cameron et al., 2006). However, this complex does contain a miR-10 gene near the

Hox3 ortholog, which may be ancestral despite the reorganization of the complex and

loss of Hox4. The parasitic platyhelminth worm *Schistosoma mansoni* has a miR-10 gene

which has multiple nucleotide changes in the mature sequence (Fig. 1 - section 2). While

Schistosoma does not have a full complement of Hox genes, some of the remaining genes

are found in small clusters in its genome (Koziol et al., 2009). The miR-10 sequence is

found in a region containing Hox4 and Hox2 orthologs, to the 5' of the *Schistosoma*

Hox4 ortholog. Although *Schistosoma* does not have a Hox5 ortholog (Koziol et al.,

2009) this is a similar genome location to the ancestral linkage. One of the two miR-10

genes in the platyhelminth flatworm *Schmidtea mediterranea* (Fig. 1 - section 2) is found

closely linked to a derived homeodomain gene that is not clearly orthologous to any

typical animal homeodomain gene (data not shown) and may either be residual ancestral

Hox linkage or just a coincidence. The leech *Helobdella robusta* has a derived

complement of Hox genes including duplicated and absent genes, which exist in five

separate mini-clusters (data not shown). The miR-10 gene in this genome resides in one

of these mini-clusters, which contains Hox5 and Hox7 orthologs. Its position near the 3'

end of the Hox7 ortholog and sequence are similar to the *Capitella* miR-10-related gene

in this genomic position and likely is a direct orthologs of this miRNA hairpin. This

means that this miR-10 duplicate must have arisen sometime prior to evolution of the

major annelid classes and that the ancestral bilaterian miR-10 ortholog was lost in the

lineage leading to *Helobdella*. Together the genomic positions of miR-10 in animals

which do not have coherent Hox complexes, suggest that while is not necessary to

maintain a coherent cluster in order to retain the miR-10 gene, linkage to the Hox genes

is typically retained, although none of these animals (other than possibly *Schmidtea*) have completely lost Hox gene clustering.

The miR-10 miRNA seems to be common to all bilaterian animals (ignoring secondary loss in a few lineages), but the origin of this miRNA is unknown. Although it has been claimed that miR-10 is found in cnidarians (Prochnik et al., 2007; Sempere et al., 2006) no conclusive evidence has shown this to be the case and definitive miR-10 hairpin sequences are not found in available cnidarian genomes. There are no miRNAs shared between sponges and higher animals, but miR-100 is conserved in cnidarians and bilaterians. This miRNA sequence is similar in sequence to miR-10 (Fig. 3) and it is likely that miR-10 was derived from miR-100, and as cnidarians do not have the full complement of Hox genes found in bilaterian genomes or true Hox complexes it is likely that miR-10 appeared prior to or during the formation of the first true Hox complex.

Other miR-10 related miRNAs are found in the genomes of many animals. Although nematodes genomes do not have miR-10 sequences, they do have a miRNA which is likely derived from miR-10. The 5' half of the nematode miR-57 mature sequence is identical to miR-10 and additional sequence similarity is found in sequences throughout the hairpins of these two miRNAs (Fig. 3). miR-99 is a vertebrate miRNA which is derived from and very similar to miR-100 (Fig. 3). miR-125 is a bilaterian miRNA, which likely also derived from a miR-100 duplicate as it is found to be clustered with miR-100 in many animals (Sokol et al., 2008). miR-993 is a miRNA found in the Hox complex of all arthropods (data not shown) and at least one lophotrochozoan (*Capitella*) (Fig. 2B) between the Hox3 and Hox4 orthologs and is transcribed in the opposite direction of Hox gene and miR-10 transcription. The 5' arm of this hairpin

contains sequences that are very similar to miR-10 (Fig. 3) and likely was created during
and inverted duplication event in an ancient Hox complex prior to the split between
ecdysozoans and lophotrochozoans.

The most likely path of sequence evolution starts with the novel appearance of
miR-100 prior to the evolution of bilaterian animals. This miRNA then gave rise to miR-
10 and miR-125 through duplication and subsequent sequence alterations early in
bilaterian evolution. Subsequently, in protostomes a miR-10 a local inverted duplication
the Hox complex gave rise to miR-993. In the nematode lineage, one of these became
miR-57 through sequence changes while the other was lost. It is not possible to make the
determination of which scenario is more likely because nematodes do not have a coherent
Hox complex or full complement of Hox genes, and so linkage to the Hox3-Hox4
interval or Hox4-Hox5 interval can be determined. It is possible that additional
sequencing of cnidarians may show that miR-10 was present in early animal evolution, in
which case it would be possible for miR-10 to be the progenitor animal miRNA, and not
miR-100. The data from this section is summarized in Figure 4.

Expression of *Drosophila mir-10* transcripts

miRNAs are initially transcribed as PolII primary transcripts which contain a
hairpin structure that is excised by the nuclear RNaseIII enzyme Drosha, exported to the
cytoplasm by Exportin-5, and then the internal loop is cleaved by the cytoplasmic
RNaseIII enzyme Dicer1 (Ambros, 2003). In order to determine the primary transcript
that produces miR-10 (*pri-miR-10*), 5' and 3' RACE were performed on a cDNA library
created from polyA RNA from a 0-24 hr embryo collection. The 3' RACE products

reveal the polyA cleavage site to be about 700 bp downstream of the hairpin sequence (Fig. 5A). 5' RACE gave multiple products, the longest of which revealed exon-intron boundaries and a putative transcriptional start site about 6.8 kb upstream of the hairpin (Fig. 5A). The sequence just upstream of the 5' end of the RACE products contains a likely basal promoter sequence containing INR, DPE, and MTE elements (Butler and Kadonaga, 2002; Lim et al., 2004). These motifs are common to many promoters and are typically found at discrete intervals relative to each other. These motifs in the upstream sequence of miR-10 are found to conform to the sequence consensus and spacing requirements of a proper PolII promoter. This basal promoter structure is also conserved in amongst *Drosophilids* (Fig. 5C), further suggesting its likelihood as the *miR-10* promoter. Together these results indicate that at least one *pri-miR-10* transcript in *Drosophila melanogaster* spans an approximately 7.5 kb region of the chromosome between *Scr* and *Dfd* with a single approximately 5.5 kb intron (Fig. 5A), although secondary transcripts are possible.

To help determine the function of any gene it is important to know in which tissues it is expressed through development. In order to determine the expression pattern of miR-10, *in situ* hybridizations were performed using a probe antisense to *pri-miR-10* sequence and analyzed its expression throughout embryogenesis. miR-10 primary transcripts are first expressed during the blastoderm stage of embryogenesis. At this point *pri-miR-10* is expressed in a broad band which corresponds to the "trunk" of the embryo and is reminiscent of the types of patterns seen with gap gene or Hox gene expression (Fig. 6A). The expression pattern at this stage is largely complementary to that of *Hunchback*, suggestive of a possible negative regulatory interaction. However,

expression of *pri-miR-10* was unaltered when examined in embryos mutant for the

Hunchback gene (data not shown). It is likely that the pattern of *pri-miR-10* in early

embryos is regulated by some combination of maternally deposited morphogens and

early gap genes, although no other possibilities were tested. The miR-10 primary

transcript is also expressed in early embryos in the cells of the yolk (data not shown).

During late blastoderm development and the beginning of gastrulation the expression of

pri-miR-10 becomes downregulated in a subset of cells, taking on a striped appearance,

which is similar to, but not as refined as that of the pair rule genes (Fig. 6B). The stripes

of *pri-miR-10* at these stages are in approximate register with that of the Hox complex

gene *fushi tarazu* (data not shown), which in *Drosophila* has taken on a patterning role

dissimilar to its ancestral Hox gene function. Shortly after the beginning of gastrulation,

the transcription of *pri-miR-10* appears to shut off and then re-initiates during the early

stages of germband elongation in an entirely different pattern. As germband elongation

proceeds, *pri-miR-10* transcription is initiated in the anal pad and large intestine

primordia and in ventral neurectoderm of the trunk segments (Fig. 6C). At this stage *pri-miR-10* is expressed in developing neuroblasts as visualized in embryos stained for *miR-10* and *hunchback* protein (Fig. 6D). After germband retraction *pri-miR-10* is transcribed

in the anal pads and large intestine as well as a band in the central midgut (Fig. 6E). In

the large intestine, transcription is restricted to the endoderm as seen by triple staining for

pri-miR-10, *en*, and *Dpp*, which are expressed in endodermal cells but not mesodermal

tissue (Fig. 6F). In the central midgut *pri-miR-10* is also endoderm specific as seen in

embryos doubly stained for *miR-10* and *Dpp*, which is exclusively in the mesoderm (Fig.

6G). In late embryogenesis *pri-miR-10* staining can be seen in Malpighian tubules (Fig.

6H), as well as in the ventral nerve cord of the central nervous system (CNS) (Fig. 6I). The anteroposterior extent of *pri-miR-10* expression in the CNS is reminiscent of its blastoderm expression pattern. The most anterior expression seen just posterior to the sub-esophageal ganglion, and extends posteriorly to near the terminal end of the CNS (Fig. 6J). It is possible that this pattern of expression in the CNS may be conserved in all bilaterians as a similar expression pattern is seen in zebrafish embryos (Wienholds et al., 2005).

Expression of primary transcript products

After a pre-miRNA is cleaved by Dicer, one strand of the resulting ~22nt dsRNA is packaged into an RNA Induced Silencing Complex (RISC). As research on miRNAs progressed it became clear that, for a subset of miRNAs, either strand of this dsRNA duplex can enter into RISC. The strand which becomes active mature miRNA is determined by the relative stability of the ends of the RNA duplex, where the strand with the 5' end that has a lower binding energy should be packaged more often into RISC (Schwarz et al., 2003). This type of analysis predicts that there is no biochemical reason why miR-10 should be preferentially packaged into RISC over the opposite strand (miR-10*), and in fact suggest that miR-10* may actually be packaged into RISC preferentially over miR-10.

Both miR-10 (Aravin et al., 2003 Aug; Lagos-Quintana et al., 2001) and miR-10* (Lagos-Quintana et al., 2001) were cloned in early efforts to determine the miR-10 complement of the *Drosophila* genome. However, the data from these studies was not enough to statistically determine the relative abundance of either strand for most miRNA

duplexes. Due to the presence of miR-10 sequences in most other animals the possibility

of a second miRNA from this locus was largely ignored. Although the miR-10 sequence

from the 5' arm of the hairpin is well conserved in animals the sequence from the 3' arm

of the hairpin is not particularly well conserved. The conservation that does exist among

disparate animal clades (Fig. 1) may be due mostly to the necessity for complementary

bases which allow formation of a hairpin capable of being processed by the miRNA

machinery. The situation is very different if only arthropods are examined, where both

miR-10 and miR-10* sequences are conserved (Fig. 7A). In deuterostomes only the miR-

10 sequence is completely conserved and the 3' arms of these hairpins have limited

conservation (Fig. 7B). Although the putative "seed" sequence of the potential

deuterostome miR-10* is conserved, free energy calculations suggest that it is unlikely to

be packaged into RISC at appreciable levels (data not shown).

In order to confirm the prediction that both strands of the miR-10 duplex are

present as mature miRNAs in *Drosophila*, northern blot analysis on RNA from multiple

embryonic stages was performed using antisense probes complementary to each arm of

the hairpin. Both miR-10 and miR-10* are present in embryos, with levels increasing

throughout embryogenesis and miR-10* is found at significantly higher levels at all

stages (Fig. 8A). In addition to Northern analysis of miRNAs, it has been demonstrated

that mature miRNAs can be detected by *in situ* hybridization by using modified oligos

composed of LNA nucleotides (Wienholds et al., 2005). Both miR-10 (Fig. 8B) and miR-

10* (Fig. 8C) can be detected during embryogenesis utilizing this technique and the

expression pattern of each is found to be largely similar to that of the primary transcript

(compare Fig. 8B to Fig. 5E) but is found exclusively in the cytoplasm (Fig. 8D).

Identification of putative targets

One way to elucidate the function of miRNAs in the tissues where they are expressed is through the identification of their *in vivo* target genes. Due to the conserved location of the *miR-10* gene in animal Hox complexes, and the potential for miRNAs to have been spawned by and target nearby genes (Allen et al., 2004), it seemed likely that miR-10 and miR-10* might be regulators of Hox gene expression. Simple sequence motif searches of the 3' UTR sequences of *Drosophila* Hox genes resulted in identification of potential target sites of varying strength.

The best putative target site for miR-10 was found in the 3'UTR of the *Sex combs reduced* gene (*Scr*). This sequence would likely form a duplex with miR-10 primed RISC. Although pairing with the 5' "seed" sequence of miR-10 is not as extensive as a prototypical miRNA-mRNA pairing (a continuous helix of base pairs 3-7 as opposed to the prototypical "seed" sequence of base pairs 2-8), there is potential for a long continuous helix formed with the 3' end of the miRNA (bases 11-22) (Fig. 9). Analysis of the putative 3'UTR sequences of other insects indicates that this sequence is found to be conserved among nearly all species examined (Fig. 9). This site is likely to be orthologous and have been maintained since the evolutionary split between the major insect groups, as the regions of potential pairing are maintained. In most of the non-*Drosophilid* insects there are additional sequences in the "seed" region which would provide added strength to the potential interaction. Additionally, the sequence surrounding the putative target site in these UTRs is not well conserved even among the relatively closely related *Drosophilids* suggesting this conservation has been selected for

(data not shown). The development of algorithms which attempt to predict miRNA target sites have been developed that have had varying success at prediction of true positive miRNA-mRNA interactions (Alexiou et al., 2009). Computational prediction of genome wide target sites in the 3'UTRs of all *Drosophila* genes (data not shown) using the RNAHybrid program (Rehmsmeier et al., 2004), along with the conservation among arthropods, suggests that the sequence identified in the 3'UTR of *Scr* is likely the best putative target of miR-10.

Computational prediction of genome wide targets (data not shown) identified a putative target site for miR-10* in the 3'UTR of the Hox gene *Abdominal-B* (*Abd-B*) (Fig. 10). Similar to the putative target site for miR-10 in the *Scr* 3'UTR, the putative miR-10* target site in *Abd-B* contains long potential continuous helix pairings. Additionally, potential target sites are found in the putative 3'UTR sequences of most arthropod *Abd-B* orthologs (Fig. 10), although these are not preserved in their pairing structure as well as the miR-10 target sites in *Scr* orthologs. These target sites are also conserved within a block of non-conserved UTR sequence (data not shown), similar to what was observed in *Scr*. This is one of the top potential target sites for miR-10* in the *Drosophila* genome and the only one which has such a high level of evolutionary conservation.

Functional analysis of miR-10

There are no published examples of post-transcriptional regulation of *Scr* during *Drosophila* embryogenesis. However, there is apparent post-transcriptional regulation of the *Porcelio scaber Scr* ortholog in the maxilliped segment during embryogenesis

(Abzhanov and Kaufman, 1999). miRNAs are may function in a number of ways to downregulate their target genes. Most initial studies suggested that miRNA targeted genes were downregulated by inhibition of protein production without affecting the levels of cytoplasmic mRNA. The expression of mRNA and protein products from the *Scr* gene overlap, for the most part, in all tissues during embryogenesis. However, in germband extended embryos, there is field of cells in the ventral T1 segment which transcribes *Scr* and contains low level accumulation of cytoplasmic mRNA, but that never exhibits any detectable protein accumulation (Fig. 11). During this stage *pri-miR-10* is not actively transcribed in all of these cells, but it was possible that miR-10 produced from early transcripts would be present in these cells. LNA antisense oligo staining for mature miR-10 did not detect appreciable levels of miRNA in these cells at this stage. Additionally, *in situ* analysis of the expression of sequences 5' of the miR-10 hairpin compared to sequences to the 3' suggests that full length *pri-miR-10* transcripts are not made in early blastoderm embryo ectoderm (data not shown) and that this early expression is likely from an alternate transcript from this genomic region. Taken together these data suggest that, although *Scr* is likely post-transcriptionally regulated in these cells, it is not through the activity of miR-10.

Further research on the function of miRNAs suggested that, RISC packaged miRNAs sequester target RNAs to cytoplasmic P-bodies (Liu et al., 2005) and that target RNAs may be degraded without having perfect complementarity to the target RNA (Lai et al., 1998). If this is the mode of activity of regulation of *Scr* by miR-10, then transcript pattern and levels would be altered in embryos ectopically expression or lacking miR-10. Available chromosomal deletions which lack miR-10 sequences are also deficient for *Scr*,

precluding the possibility of ascertaining the change in *Scr* expression in embryos lacking miR-10.

In order to determine the potential change in expression in embryos ectopically expressing miR-10, UAS transgene expression constructs were created. These transgenic flies allow expression of *pri-miR-10* under the control of a variety of spatially and temporally specific GAL4 drivers. This transgene can produce significant amounts of mature miRNA, as visualized by antisense LNA oligo staining in embryos containing both the UAS-miR-10 transgene as well as a *prd*-GAL4 transgene (Fig. 12A).

Ectopic expression of miR-10 by nearly ubiquitous GAL4 drivers results in death during early larval development and noticeable cuticle phenotypes in the anterior of the early larvae. The transgenic larvae die typically during the first instar and occasionally do not hatch, but none survive to adulthood. These larvae have malformations of their anterior cuticle which appear as cuticular breaks near the mouthhooks (compare Fig. 12C-D with Fig. 12B). Although these cuticle phenotypes are reproducible, they do not correlate with any known *Scr* mutant phenotype. Typical cuticle phenotypes seen in *Scr* mutants include reduction of the T1 beard structure, but this is not seen in animals ectopically expressing *pri-miR-10* (data not shown).

Scr and *pri-miR-10* are expressed in largely complementary patterns in both early embryos (Fig. 13D-F) and late stage CNS (Fig. 13A-C). This correlation would make sense if miR-10 is negatively regulating *Scr*, but ectopic expression of *pri-miR-10* does not corroborate this interaction. The *en*-GAL4 driver expresses in a small number of cells which overlap the *Scr* expression pattern. When *pri-miR-10* is expressed under the control of *en*-GAL4, these cells have no detectable decrease in SCR protein levels when

compared to wildtype controls (compare Fig. 13 C-D with A-B). Additionally, embryos ectopically expressing *pri-miR-10* from a variety of other GAL4 drivers do not exhibit detectable changes in either pattern or levels of *Scr* transcript or protein accumulation (data not shown).

In order to test the functionality of the putative miR-10 target site in *Scr*, constructs were created attaching the *Scr* 3'UTR sequence to a luciferase gene. The sequence of the putative miR-10 target site was then mutated in order to disrupt the potential pairing between miR-10 and *Scr* 3'UTR sequences (Fig. 15). These constructs were transfected into S2 cells and the expression levels of luciferase were analyzed either with or without co-transfection of a second transgene expressing *pri-miR-10*. These experiments showed either no significant change in expression of luciferase production or were inconclusive in their results.

Functional analysis of miR-10*

The putative target site for miR-10* in the 3'UTR of *Abd-B* is predicted to form an extremely stable duplex and is comparable in structure and extent of binding of some of the best characterized miRNA-mRNA interactions. In combination with the conservation of target sites in other insect *Abd-B* orthologs, this is highly suggestive that this interaction is likely to be biologically important.

During embryogenesis miR-10 and *Abd-B* are expressed in mostly non-overlapping regions. While *Abd-B* is expressed in the majority of the posterior ectoderm, it is completely excluded from the developing anal pads, where miR-10* is strongly expressed (Fig. 13A-C and G-I). *Abd-B* is also expressed throughout later embryogenesis

in the visceral mesoderm surrounding the large intestine of the hindgut, whereas miR-10* is found in the endodermal cells of this region (Fig. 13J). This complementarity is suggestive of either unidirectional or mutual negative regulatory interactions Embryos which are mutant for *Abd-B* gene products do not show altered expression of *pri-miR-10* (data not shown). Conversely embryos which are deficient for the *mir-10* gene, and thus lacking miR-10*, do not exhibit ectopic expression of *Abd-B* mRNA or protein in either the large intestine endoderm or in the anal pads, suggesting that miR-10* does not regulate *Abd-B* in these tissues (data not shown).

Although transcription is largely complementary in most tissues, in the ventral nerve cord of later stage embryos, both *pri-miR-10* and *Abd-B* are transcribed in parasegments 10-14 (Fig. 13A-C and G-I). The *Abd-B* gene produces multiple transcript variants which code for two different proteins. Of these, the r-type protein (shorter protein with regulatory function) is produced in the CNS only in the most posterior regions (PS14) whereas the m-type protein (longer protein with morphogenetic function) is expressed in more anterior regions of the CNS (PS13-10). *Abd-B* may utilize either of two polyA signals to terminate transcripts, the longer of which contains the putative miR-10* target site. The majority of *Abd-B* expression domains only show expression of the shorter 3'UTR, but the CNS displays expression of at least the longer version of 3'UTR if not a combination of both in parasegments 10-14. Since the domain of *pri-miR-10* transcription extends into the posterior of the CNS but appears to decrease toward the extreme posterior (Fig. 5I, J), it is possible that miR-10* may be downregulating the expression of r-type ABD-B in more anterior parasegments of the CNS, functioning as a protein type switch. To test this possibility, *Abd-B*^{D14} (an allele mutant specifically for

ABD-B m-type protein) was recombined with *Df(3R)CP1*. This strain can produce

embryos which are deficient for both miR-10* and ABD-B m-type protein. Analysis of

ABD-B protein expression shows no ectopic expression of ABD-B r-type protein in this

mutant background (Fig. 14L). This suggests that the longer 3'UTRs found in

parasegments 10-13 belong exclusively to transcripts encoding ABD-B m-type protein.

To test the possibility that miR-10* is regulating *Abd-B* expression in the CNS in

general, transheterozygote *Df(3R)CP1*: *Df(3R)LIN* embryos deficient for miR-10 were

analyzed for alterations in *Abd-B* transcript and protein levels in parasegments 10-14 of

the CNS. The staging of the transheterozygote embryos is difficult due to altered

morphology caused by the lack of genes other than miR-10. However, no significant

differences were seen in the expression levels or anterior extent of expression of either

Abd-B transcripts or protein (Fig. 14K) when compared to wildtype embryos (Fig. 14I).

Ectopic expression of *pri-miR-10* in the CNS either through ubiquitous overexpression

(Fig. 14J) or in a subset of cells (Fig. 14G-H) failed to result in noticeable changes in

levels of ABD-B protein compared to wildtype (Compare to Fig. 14E, F, and I).

DISCUSSION

The miR-10 gene is one of the most ancient animal miRNA genes. It is likely to have developed from an even more ancient miRNA, early in bilaterian evolution. Subsequently it has been duplicated, deleted, and altered many times in many lineages, resulting in a large family of related miRNAs. Most of these are found in the Hox complexes of bilaterian animals but have unknown functions. The *Drosophila mir-10* gene is expressed in the form of a large primary transcript from the Hox complex, and its mature miRNA products have strong evolutionarily conserved putative target sequences in nearby Hox genes. Expression of the primary transcript and its products occurs in multiple tissues and in a mostly complementary pattern to that of the putative target genes *Abd-B* and *Scr*. However, embryos lacking the *mir-10* gene or ectopically expressing the primary transcript do not show observable alterations in the expression of these Hox genes. Despite this, ubiquitous overexpression of *pri-mir-10* results in cuticle malformations and early larval death, but the root cause of this is still unknown. The *mir-10* gene miRNAs may have many targets which are unidentified that may be downregulated resulting in this phenotype.

It is possible that the evolutionarily conserved function of Hox miRNAs is to downregulate Hox proteins in sub-regions of the nerve chord. This possibility is raised by the conserved expression of miR-10 in the CNS of *Drosophila* and zebrafish (Wienholds et al., 2005). Members of the other vertebrate Hox miRNA family, miR-196, are also expressed in the CNS of zebrafish (Wienholds et al., 2005), and it is possible that *mir-iab-4* (not orthologous by sequence, but orthologous by chromosomal position in the Hox complex) also has expression in the CNS. It may be that the main function of the *mir-10*

locus miRNAs is to refine the expression of Hox proteins in the CNS to provide specific

identities to the regions where this downregulation is occurring. It is possible that the

combinatorial activity of multiple miRNAs acting on Hox mRNAs might be required for

proper downregulation and this may explain the lack of regulation seen when *mir-10*

gene products are absent or overexpressed. It is also possible that these miRNAs serve to

canalize expression of their target genes in order to achieve robust, reproducible, and

precise levels and spatial limits of expression in order to maintain a reproducible

phenotypic output (Wu et al., 2009).

Chapter I, in part, contains material previously published in Pearson, J. C.,

Lemons, D. and McGinnis, W. (2005). Modulating Hox gene functions during animal

body patterning. *Nat Rev Genet* **6**, 893-904, and material being prepared for submission

for publication, Lemons, D., Pare, A., McGinnis, W.

MATERIALS AND METHODS

MicroRNA sequences were obtained using BLAST searches of NCBI sequence databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were created using BioEdit software (Hall, 1999). Hox complex assemblies were created using the Tracinator program (Rebeiz and Posakony, unpublished). MicroRNA precursor folding diagrams were created with Mfold (Zuker, 2003). Cuticle preparations were performed as in Bergson and McGinnis, 1990. Northern blots were performed as in Pasquinelli et al., 2003. Standard *in situ* hybridizations and immunohistochemistry were performed as in Kosman et al., 2004. SCR and ABD-B antibodies were obtained from the Developmental Studies Hybridoma Bank, and the EN antibody was a gift from Patrick O'Farrell. LNA *in situ* hybridizations were performed as in Kloosterman et al., 2006.

Figure 1. Alignment of available *mir-10* hairpin sequences. Sequence alignment starting at nucleotide 1 of vertebrate miR-10 mature miRNA (*Drosophila* miR-10 starts at nucleotide 2 of alignment). Highlighted bases are 90% conserved among all sequences. Section divisions in far right column divide the alignment into 1 - Arthropod miR-10, 2 - Lophotrochozoan miR-10, 3 - vertebrate miR-10a (miR-10 in non-vertebrate deuterostomes), 4 - vertebrate miR-10b, 5 - fish miR-10d, 6 - fish miR-10c, 7 - miR-10e, 8 - duplicated orphan miR-10. Special indications: *Acal** - nucleotides 13-14 (TA insertion) removed from this sequence in order to fit alignment; // - nucleotides have been removed starting at these locations to fit alignment. Organism abbreviations are as follows: *Dmel - Drosophila melanogaster, Dsec - Drosophila sechellia, Dsim - Drosophila simulans, Dyak - Drosophila yakuba, Dere - Drosophila erecta, Dana - Drosophila ananassae, Dpse - Drosophila pseudoobscura, Dper - Drosophila persimilis, Dmir - Drosophila miranda, Dvir - Drosophila virilus, Dmoj - Drosophila mojavensis, Dwil - Drosophila willistoni, Dgri - Drosophila grimshawi, Gmor - Glossina morsitans, Mdes - Mayetiola destructor, Agam - Anopheles gambiae, Aaeg - Aedes aegypti, Cpip - Culex pipiens quinquefasciatus, Bmor - Bombyx mori, Tcas - Tribolium castaneum, Amel - Apis mellifera, Nvit - Nasonia vitripennis, Nlon - Nasonia longicornis, Rpro - Rhodnius prolixus, Nlug - Nilaparvata lugens, Phum - Pediculus Humanus, Apis - Acyrthosiphum pisum, Lmig - Locusta migratoria, Dpul - Daphnia pulex, Isca - Ixodes scapularis, Turt - Tetranychus urticae, Vdes - Varroa destructor, Acal - Aplysia californica, Lgig - Lottia gigantea, Cap - Capitella, Hrob - Helobdella robusta, Smed - Schmidtea mediterranea, Sman - Schistosoma mansoni, Emul - Echinococcus multilocularis, Hsap - Homo sapiens, Ppan - Pan paniscus, Ptro - Pan troglodytes, Ggor - Gorilla gorilla, Ppyg - Pongo pygmaeus, Pabe - Pongo abelii, Pham - Papio hamadryas, Mmul - Macaca mulatta, Nleu - Nomascus leucogenys, Slab - Saguinus labiatus, Ageo - Ateles geoffroyi, Cjac - Callithrix jacchus, Tsyr - Tarsius syrichta, Ogar - Otolemur garnettii, Mmur - Microcebus murinus, Tbel - Tupaia belangeri, Mmus - Mus musculus, Mmol - Mus musculus molossinus, Dord - Dipodomys ordii, Rnor - Rattus norvegicus, Cpor - Cavia porcellus, Opri - Ochotona princeps, Stri - Spermophilus tridecemlineatus, Sara - Sorex araneus, Ocun - Oryctolagus cuniculus, Eeur - Erinaceus europaeus, Pvam - Pteropus vampyrus, Mluc - Myotis lucifugus, Cper - Carollia perspicillata, Cfam - Canis familiaris, Aliu - Ailuropoda melanoleuca, Ttru - Tursiops truncatus, Fcat - Felis catus, Btau - Bos Taurus, Bbub - Bubalus bublais, Ecab - Equus caballus, Sscr - Sus scrofa, Dnov - Dasypus novemcinctus, Lafr - Loxodonta africana, Emax - Elephas maximus, Etel - Echinops telfairi, Mdom - Monodelphis domestica, Meug - Macropus eugenii, Oana - Ornithorhynchus anatinus, Tacu - Tachyglossus aculeatus, Tgut - Taeniopygia guttata, Ggal - Gallus gallus, Acar - Anolis carolinensis, Xtro - Xenopus tropicalis, Lmen - Latimeria menadoensis, Amia - Amia calva, Pbic - Polypterus bichir, Drer - Danio rerio, Cmil - Callorhinchus milii, Ccon - Copadichromis conophorus, Abur - Astatotilapia burtoni, Mcon - Mchenga conophoros, Tnig - Tetraodon nigroviridis, Trub - Takifugu rubripes, Snep - Sphoeroides nephelus, Blan - Branchiostoma lanceolatum, Bflo - Branchiostoma floridae, Skow - Saccoglossus kowalevskii, Spur - Strongylocentrotus purpuratus, Afra - Allocentrotus fragilis, Mnem - Macaca nemestrina, Rfer - Rhinolophus ferrumequinum, Chof - Choloepus hoffmanni, Pman - Peromyscus maniculatus, Vvic - Vicugna vicugna, Lpac - Lama pacos, Pcap - Procavia capensis, Olat - Oryzias latipes, Ssal - Salmo salar, Hfra - Heterodontus francisci, Gacu - Gasterosteus aculeatus, Omyk - Oncorhynchus mykiss, Mamb - Megalobrama amblycephala, Ipun - Ictalurus punctatus, Onil - Oreochromis niloticus.*

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Dmel   TACCCTGTAGATCCGAATTTGTTTTATACTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT =
Dsec   TACCCTGTAGATCCGAATTTGTTTTATACTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dsim   TACCCTGTAGATCCGAATTTGTTTTATACTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dyak   TACCCTGTAGATCCGAATTTGTTTTATACTA---GTTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dere   TACCCTGTAGATCCGAATTTGTTTTATACTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dana   TACCCTGTAGATCCGAATTTGTTTTATACTA---GTTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dpse   TACCCTGTAGATCCGAATTTGTTTTACATTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dper   TACCCTGTAGATCCGAATTTGTTTTACATTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dmir   TACCCTGTAGATCCGAATTTGTTTTACATTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT 1
Dvir   CACCCTGTAGATCCGAATTTGTTGTACACTA---GTTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dmoj   TACCCTGTAGATCCGAATTTGTTGTACCATA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dwil   TACCCTGTAGATCCGAATTTGTTTTATACTAG--TTTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Dgri   TACCCTGTAGATCCGAATTTGTTGTACACTA---GCTTTAAGGACAAATTCGGTTCTAGAGAGGTTT |
Gmor   TACCCTGTAGATCCGAATTTGTTTTGAGCT------TTAAACGACAAATTCGGTTCTAGAGAGGTTT |
Mdes   TACCCTGTAGATCCGAATTTGTTTGACATAAG-TTAAAATACGACAAATTCGGTTCTAGAGAGGTTT |
Agam   CACCCTGTAGATCCGAATTTGTTTGAATTTA----TATTAATAACAAATTCGGTTCTAGAGAGGTTT |
Aaeg   CACCCTGTAGATCCGAATTTGTTTGAATATT---TAACAAGCGACAAATTCGGTTCTAGAGAGGTTT 1
Cpip   TACCCTGTAGATCCGAATTTGTTTGAATATA----AACAAGCGACAAATTCGGTTCTAGAGAGGTTT |
Bmor   TACCCTGTAGATCCGAATTTGTTTGAAGT--------GAGGCGACAAATTCGGTTCTAGAGAGGTTT |
Tcas   TACCCTGTAGATCCGAATTTGTTTGAAAT--------CAGGCGACAAATTCGGTTCTAGAGAGGTTT |
Amel   TACCCTGTAGATCCGAATTTGTTTGATAA--------GAGGCGACAAATTCGGTTCTAGAGAGGTTT |
Nvit   TACCCTGTAGATCCGAATTTGTTTGAAGT--------CAGGCGACAAATTCGGTTCTAGAGAGGTTT |
Nlon   TACCCTGTAGATCCGAATTTGTTTGAAGT--------CAGGCGACAAATTCGGTTCTAGAGAGGTTT |
Rpro   TACCCTGTAGATCCGAATTTGTTTGATAC--------AGTGCGACAAATTCGGTTCTAGAGAGGTTT |
Nlug   TACCCTGTAGATCCGAATTTGTTTGACAT--------AAGGCGACAAATTCGGTTCTAGAGAGGTTT 1
Phum   TACCCTGTAGATCCGAATTTGTTTGATAT--------CAAACGACAAATTCGGTTCTAGAGAGGTTT |
Apis   TACCCTGTAGATCCGAATTTGTTTGAAAA--------ACGGCGACAAATTCGGTTCTAGAGAGGTTT |
Lmig   TACCCTGTAGATCCGAATTTGTTTGACAT--------AGTGCGACAAATTCGGTTCTAGAGAGGTTT |
Dpul   TACCCTGTAGATCCGAATTTGTAGTTGCA-------TCACAAGACAAATTCGGTTCTAGAGAGGTTC |
Isca   TACCCTGTAGATCCGAATTTGTCTGCACC--------AAGACTACAAATTCGGTTCTAGAGAGGTTT |
Turt   CACCCTGTAGATCCGAATTTGTCAGAAAATT----GATTTGCCACGAATTCGGCTCTAGAGAGGTTT |
Vdes   TACCCTGTAGATCCGAATTTAATGTGCTGTTA//ACCGGCTAACGTAAATTCGGCTCTAGAGAGGTCG =
Acal*  TACCCTGTAGATCCGAATTTGTGTGCTGAA---------TTTCACAAATTCGTATCTGCGTGGTATT =
Lgig   TACCCTGTAGATCCGAATTTGTGTATGAA-------ACAATCTACAAATTCGTTTTTGCGGGTGATT |
Cap    TACCCTGTAGTTCCGGATTTGTCTTGAATCTC-AGCAAAGCAAACAAATTCGATTCGCTCGGGTTTC |
Hrob   TACCCTGTAGACCCGAGCTTGTTTTTAAACG//TTTATTAGTAACAAGACGGGTTTAAAGGGTGTAC 2
Smed   AACCCTGTAGATCCGAGTTTGATTAATGCCA//AGCATTTCAATCAAAATCGAATCTTCAAGGTGAA |
Sman   AACCCTGTAGATCCGAGTTTGGATGCAG---------TCAGATGCAAATTCGAGTCTATAAGGAAAA |
Emul   CACCCTGTAGACCCGAGTTTGAGTGCCTAGC//GTACAGACTCCCAAGCTCGTGTCTTCAAGGAATC =
Hsap   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAATTCGTATCTAGGGGAATAT =
Ppan   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACATTCGTATCTAGGGGAATAT |
Ptro   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ggor   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ppyg   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Pabe   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Pham   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Mmul   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Nleu   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT 3
Slab   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ageo   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Cjac   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Tsyr   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ogar   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Mmur   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Tbel   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Mmus   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT 3
Mmol   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Dord   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Rnor   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Cpor   TACCCTGTAGGTCCGAATTTGTGTAAGGAAT---TTTATGGTCACAAATTCGTATCTAGGGGAATAT |
Opri   TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
```

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Stri  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Sara  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ocun  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT 3
Eeur  TACCCTGTAGATCCGAATTTGTGTTAAGAAAT--TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Pvam  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Mluc  TACCCTGTAGATCCGAATTTGTGTAAGAAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Cper  TACCCTGTAGATCCGAATTTGTGTAAGAAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Cfam  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ailu  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Ttru  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Fcat  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT 3
Btau  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGATCACAAATTCGTATCTAGGGGAATAT |
Bbub  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGATCACAAATTCGTATCTAGGGGAATAT |
Ecab  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Sscr  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Dnov  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Lafr  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGATCACAAATTCGTATCTAGGGGAATAT |
Emax  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGATCACAAATTCGTATCTAGGGGAATAT |
Etel  TACCCTGTAGATCCGAATTTGTGTAAGGAGT---TTTGTGATCACAAATTCGTATCTAGGGGAATAT 3
Mdom  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Meug  TACCCTGTAGATCCGAATTTGTGTAAGGAAT---TTTGTGGTCACAAATTCGTATCTAGGGGAATAT |
Oana  TACCCTGTAGATCCGAATTTGTGTAAGGAGT---TTCGTGGTCACAAATTCGTATCTAGGGGAATAT |
Tacu  TACCCTGTAGATCCGAATTTGTGTAAGGAGT---TTCGTGGTCACAAATTCGTATCTAGGGGAATAT |
Tgut  TACCCTGTAGATCCGAATTTGTGTAAAAGGA---AGTTGGGTCACAAATTCGTATCTAGGGGAATAT |
Ggal  TACCCTGTAGATCCGAATTTGTGTAAAAGGAA----GTTGGGTCACAAATTCGTATCTAGGGGAATAT |
Acar  TACCCTGTAGATCCGAATTTGTGTAAAAGAA----ACTGCGTCACAAATTCGCGTCTAGGGGAATAT |
Xtro  TACCCTGTAGATCCGAATTTGTGTGAGCGCA----ATCATATCACAAATTCGTGTCTGGGCGGATAT 3
Lmen  TACCCTGTAGATCCGAATTTGTGTGAACAGA---TTTGTGGTCACAAATTCGTGTCTAGGGGAGTAT |
Amia  TACCCTGTAGATCCGAATTTGTGTGAAAAGT---CACACGGTCACAAATTCGTATCTAGGGGAGTAT |
Pbic  TACCCTGTAGATCCGAATTTGTGTGAACAGA---GAAGCGGTCACAAATTCGTATCTAGGGGAGTAT |
Drer  TACCCTGTAGATCCGAATTTGTGTGAATA-------TACAGTCGCAAATTCGTGTCTTGGGGAATAT |
Cmil  TACCCTGTAGATCCGAATTTGTGTTGACAAA---ATTGTGGTCGCAAATTCGTATCTAGGGGAGTAT |
Ccon  CACCCTGTAGATCCGAATTTGTGTAAATACA---GCAGCAGCCACAAATTCGCATCTTGGGGAGTAT |
Abur  CACCCTGTAGATCCGAATTTGTGTAAATACA---GCAGCAGCCACAAATTCGCATCTTGGGGAGTAT |
Mcon  CACCCTGTAGATCCGAATTTGTGTAAATACA---GCAGCAGCCACAAATTCGCATCTTGGGGAGTAT 3
Tnig  CACCCTGTAGATCCGAATTTGTGTAAACATG---ACGGAAGCTGCAAATTCGGGTCTTGGGGAGGAT |
Trub  CACCCTGTAGATCCGAATTTGTGTAAACATG---ACAGACGCTGCAAATTCGGGTCTTGGGGAGGAT |
Snep  CACCCTGTAGATCCGAATTTGTGTGACA----------GAAGTGCAAATTCGCATCTTGGGGAGGAT |
Blan  TACCCTGTAGATCCGAATTTGTGTGAGGTA-----CCCACGTCACAAATTCGTTTCTGCAGGTATAA |
Bflo  TACCCTGTAGATCCGAATTTGTGTGAGGTC------CCAAGTCACAAATTCGTTTCTGCAGGTATAA |
Skow  TACCCTGTAGATCCGAATTTGTGGGTACGT------ACATTTTCACGAATTCGTTTCTACCGGTGACA |
Spur  AACCCTGTAGATCCGAATTTGTGTCCGAGTTT//CTGGTAGTCACAGATTCGTATCTCTGGGTAACT |
Afra  AACCCTGTAGATCCGAATTTGTGTCCGAGTTT//GGTATAGTCACAGATTCGTGTCTCTGGGTAACT =
Hsap  TACCCTGTAGAACCGAATTTGTGTGGTATCC----GTATAGTCACAGATTCGATTCTAGGGGAATAT =
Ppan  TACCCTGTAGAACCGAATTTGTGTGGTATCC----GTATAGTCACAGATTCGATTCTAGGGGAATAT |
Ptro  TACCCTGTAGAACCGAATTTGTGTGGTATCC----GTATAGTCACAGATTCGATTCTAGGGGAATAT |
Ggor  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Ppyg  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Pabe  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Pham  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Mmul  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Mnem  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT 4
Nleu  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Cjac  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Mmur  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACGTAGTCACAGATTCGATTCTAGGGGAATAT |
Ogar  TACCCTGTAGAACCGAATTTGTGTGATATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Tbel  TACCCTGTAGAACCGAATTTGTGTGGTATCC----GCATAGTCACAGATTCGATTCTAGGGGAATAT |
Pvam  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Mluc  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Cper  TACCCTGTAGAACCGAATTTGTGTGGTATCG----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
```

Figure 1. continued

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Rfer  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Chof  TACCCTGTAGAACCGAATTTGTGTGGTATTC----ACATAGTCACAGATTCGATTCTAGGGGAATAT 4
Stri  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Mmus  TACCCTGTAGAACCGAATTTGTGTGGTACCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Rnor  TACCCTGTAGAACCGAATTTGTGTGGTACCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Dord  TACCCTGTAGAACCGAATTTGTGTGGTATC-----CACATGTCACAGATTCGATTCTAGGGGAATAT |
Pman  TACCCTGTAGAACCGAATTTGTGTGGTACCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Cpor  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Opri  TACCCTGTAGAACCGAATTTGTGTGGTGTCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Ocun  TACCCTGTAGAACCGAATTTGTGTGGTATCT----ACATAGTCACAGATTCGATTCTAGGGGAATAT 4
Eeur  TACCCTGTAGAACCGAATTTGTGTGGTCTCC----TCATAGTCACAGATTCGATTCTAGGGGAATAT |
Cfam  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Fcat  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCATAGATTCGATTCTAGGGGAATAT |
Ailu  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Ttru  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACGTAGTCACAGATTCGATTCTAGGGGAATAT |
Btau  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ATGTAGTCACAGATTCGATTCTAGGGGAATAT |
Ecab  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Sscr  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT 4
Vvic  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Lpac  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Lafr  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACGTAGTCACAGATTCGATTCTAGGGGAATAT |
Pcap  TACCCTGTAGAACCGAATTTGTGTGGTATCC----ACGTAGTCACAGATTCGATTCTAGGGGAATAT |
Etel  TACCCTGTAGAACCGAATTTGTGTGGTATCC----GCATAGTCACAGATTCGATTCTAGGGGAATAT |
Dnov  TACCCTGTAGAACCGAATTTGTGTGGTAGTC----ACACAGTCACAGATTCGATTCTAGGGGAATAT |
Mdom  TACCCTGTAGAACCGAATTTGTGTGGTATTT----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Meug  TACCCTGTAGAACCGAATTTGTGTGGTATTC----ACATAGTCACAGATTCGATTCTAGGGGAATAT 4
Oana  TACCCTGTAGAACCGAATTTGTGTGGTATTC----ACATAGTCACAGATTCGATTCTAGGGGAATAT |
Tgut  TACCCTGTAGAACCGAATTTGTGTGATATTC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Ggal  TACCCTGTAGAACCGAATTTGTGTGATATTC----ATATAGTCACAGATTCGATTCTAGGGGAATAT |
Acar  TACCCTGTAGAACCGAATTTGTGTGGTATTT----ACACAGTCACAGATTCGATTCTAGGGGAATAT |
Xtro  TACCCTGTAGAACCGAATTTGTGTGGTTCG------TACAGTCACAGATTCGATTCTAGGGGGATAT |
Lmen  TACCCTGTAGAACCGAATTTGTGTGATGTTA----TCAGAATCACAGATTCAATTCTAGGGGAGTAT |
Drer  TACCCTGTAGAACCGAATTTGTGTGAAAAAA----TAACATTCACAGATTCGATTCTAGGGGAGTAT |
Abur  TACCCTGTAGAACCGAATTTGTGTGATGAAA----CCAGATTCACAGATTCGATTCTAGGGGAGTAT 4
Olat  TACCCTGTAGAACCGAATTTGTGTGAAGAAC----GCTCAATCACAGATTCGATTCTAGGGGAGTAT |
Ssal  TACCCTGTAGAACCGAATTTGTGTGATAAAA----CAACAGTCACAGATTCGATTCTAGGGGAGTAT |
Ssal  TACCCTGTAGAACCGAATTTGTGTGATAAA------ACAAGTCACAGATTCGATTCTAGGGGAGTAT |
Cmil  TACCCTGTAGAACCGAATTTGTGTGATGT-------TTCGGTCACAGATTCGTTTTTAGGGGAGTAT |
Trub  TACCCTGTAGAACCGAATTTGTGTGATGGCG----TCAAAGTCACAGATTCGATTCTAGGGGAGTAT |
Tnig  TACCCTGTAGAACCGAATTTGTGTGATCAAG----TCACAGTCACAGATTCGATTCTAGGGGAGTAT |
Hfra  TACCCTGTAGAACCGAATTTGTGTGATGTT-------TCGGTCACAGATTCGTTTCTAGGGGAGTCT 4
Gacu  TACCCTGTAGAACCGAATTTGTGTGATGATA----CCATAATCACAAATTCGATTCTAGGGGAGTAT |
Cmil  TACCCTGTAGAACCGAATTTGTGTTAGCAGT----TTGTAATCACAAATTCGTCTCTGAGGGAGTAT |
Lmen  TACCCTGTAGAACCGAATTTGTGTGAGCTCT----CGATAATCACAAATTCGTCTCTAGGGGAGTAT |
Ccon  TACCCTGTAGAACCGAATTTGTGTGAAGTAC----AAGCAGTCGCAAATACGTCTCTACAGGAATAC |
Abur  TACCCTGTAGAACCGAATTTGTGTGAAGTAC----AAGCAGTCGCAAATACGTCTCTACAGGAATAC |
Mcon  TACCCTGTAGAACCGAATTTGTGTGAAGTAC----AAGCAGTCGCAAATACGTCTCTACAGGAATAC |
Gacu  TACCCTGTAGAACCGAATTTGTGTGAAATAC----AAACAGTCGCAAATAGGTCTCTGCAGGAATAC |
Drer  TACCCTGTAGAACCGAATTTGTGTCCAAAAC---ATCAAAATCGCAAATACGTCTCTACAGGAATAC 4
Olat  TACCCTGTAGAACCGAATTTGTGTGGAGTAC----AAGCATTCGCAAATACGTCTCTACAGGAATAC |
Tnig  TACCCTGTAGAACCGAATTTGTGTGAGTTC-----AGACAGTCAAGTACGTCTCTACAGGAATAC |
Trub  TACCCTGTAGAACCGAATTTGTGTGAGTTCC----AGACAGTCGCAAGTACGTCTCTACAGGAATAC |
Ssal  TACCCTGTAGAACCGAATTTGTGTTAATAGA----TAACAATCGCAAGTACGTCTCTACAGGAATAC |
Omyk  CACCCTGTAGAACCGAATTTGTGTTTACAAT----TAACAACCGCAAATTCGCCTCTACGGGAATAT |
Ssal  CACCCTGTAGAACCGAATTTGTGTTTACAAT----TAACAACCGCAAATTCGCCTCTACGGGAATAT |
Ssal  CACCCTGTAGAACCGAATTTGTGTTAACAAT----ACGCAACCGCAAATTCGCCTCTACGGGAATAC =
Drer  TACCCTGTAGAACCGAATGTGTGTTTACACA----GCAAATTCACAGATTCGGTTTTAGGGGAGTAT =
Gacu  TACCCTGTAGAACCGAATGTGTGTGGAGGTG----CCCCGGTCACAGATTGGGTTCTAGGGGAGTCT |
Olat  TACCCTGTAGAACCGAATGTGTGTGGAGCTG----CCTCAGTCACAGATTGGGTTCTAGGGGAGTCT 5
Abur  TACCCTGTAGAACCGAATGTGTGTGGAGCTG----CCTCAGTCACAGATTGGGTTCTTGGGGAGTCT |
```

Figure 1. continued

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Trub   TACCCTGTAGAACCGAATGTGTGTGCAGCTG----ACTTGATCACAGATTGGGTTCTAGGGGAGTCT 5
Tnig   TACCCTGTAGAACCGAATGTGTGTGCAGCTG----ACTTGATCACAGATTGGGTTCTAGGGGAGTCT |
Snep   TACCCTGTAGAACCAAATGTGTGTGCAGCTG----ACTTGATCACAGATTGGGTTCTAGGGGAGTCT =
Drer   TACCCTGTAGATCCGGATTTGTGTAAACAGA---CGCACAGTCACAAATTCGTATCTAGGGGAGTAT =
Mamb   TACCCTGTAGATCCGGATTTGTGTAAACAGA---CGCACAGTCACAAATTCGTATCTAGGGGAGTAT |
Ipun   TACCCTGTAGATCCGGATTTGTGTGAACAGA---CACACAGTCACAAATTCGTGTCTAGGGGAGTAT |
Ssal   TACCCTGTAGATCCGGATTTGTGTGAACAGA---CTCACGGTCACAAATTCGTGTCTAAGGGAGTAT |
Ssal   TACCCTGTAGATCCGGATTTGTGTGAACAGA---CTCACGGTCACAAATTCATATCTAAGGGAGTAT 6
Gacu   TACCCTGTAGATCCGGATTTGTGTAGAAATCATTAAAATAATCACAAATTCGCTTCTAGGGGAGTAT |
Onil   TACCCTGTAGATCCGGATTTGTGTAAAAATCATTAATACAATCACAAATTCGCTTCTAGGGGAGTAT |
Trub   TACCCTGTAGATCCGGATTTGTGTAAAAATCATTAAAGCAATCACAAATTCGCTTCTAGGGGAGTAT |
Tnig   TACCCTGTAGATCCGGATTTGTGTAACGATCATTAAAGCAATCACAAATTCGCTTCTAGGGGAGTAT |
Olat   TACCCTGTAGATCCGGATTTGTGTGACGGTCGATGAAACAATCACAAATTCGCTTCTAGGGGAGTAT =
Acar   TACCCTGTAGAATCGAATTTGTGTGAGCAC------CTCAATCACAAATTCGTCTCTAGGGGAGTAT 7
Xtro   CACCCTGTAGAATCGAATTTGTGTGAGTT-------CTGAACCACAGATTCGTCTCTAGGGGGGTAT 7
Bflo   TACCCTGTAGATCCGGACTTGTGGTCACT-------TCATCACACAAATCCGATTTACAGGATAGGC =
Bflo   AACCCTGTGGATCCGATCTTGTGTCGCTAT------AAAAACCACAAGGTGGGACTGACAGGTTCAT |
Blan   AACCCTGTGGATCCGATCTTGTGTCGCTAT------AAAAACCGCAAGGTGGGATCGACAGGTTCAT |
Smed   AACCCTGTAGATCCGAGTTACATGGT-------------TTTTCCAATTCGAATCTCGGGGAATTA |
Lgig   TACCCTGTAGTACCGAGCAAGTGGACAATT-----TTGAAAAACACTGTTCGCTACTGGGGGTCAAA 8
Cap    TACCCTGTCGAACCGAGCGAGTGAGAAAT--------TTACCCACCCCTCAGTTTGCAGGGTACAAG |
Cap    AACCCTGTGGATCCGGGTTTGATTTCAATC------TTTGTAAACAAACTCGATTTCACGGGGAGTG |
Cap    TACCCTGTAGACCCGGGTTTATGTGTTCATGT--TTAACTTACAGAAGCTCGTTCTACAGGTATAT |
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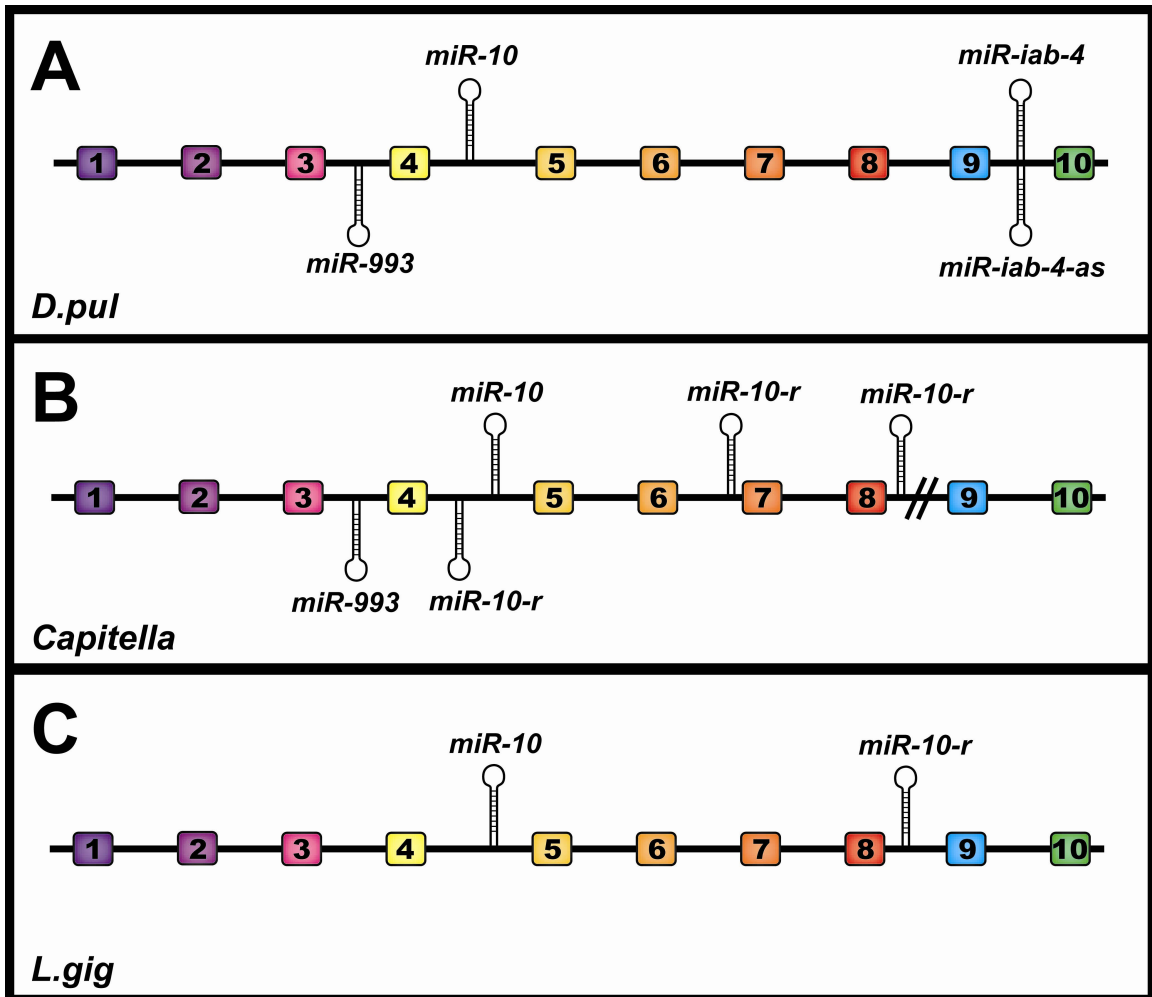
Figure 1. continued

Figure 2. Schematic representations of Hox complexes and their miRNAs. Transcriptional direction of Hox genes is from right to left in all cases. Hairpins on top are transcribed from right to left. Hairpins on bottom are transcribed from left to right. (A) *Daphnia pulex* Hox complex and locations of *miR-10*, *miR-993*, *miR-iab-4*, and *miR-iab-4-as* hairpins. (B) *Capitella* Hox complex and locations of *miR-10*, *miR-993*, and 3 *miR-10-related* (*miR-10-r*) hairpins. // - indicates lack of sequence data to definitively determine linkage. (C) *Lottia gigantea* Hox complex and locations of *miR-10* and a *miR-10-related* (*miR-10-r*) hairpin.

Figure 3. Alignment of hairpin sequences for miR-10 and related miRNAs. Sequence alignment starting at nucleotide 1 of vertebrate miR-10 mature miRNA (*Drosophila* miR-10 starts at nucleotide 2 of alignment). Highlighted bases are 60% conserved among all sequences. Sequences are as follows: *Dm - Drosophila melanogaster*, *Mm - Mus musculus*, *Dr - Danio rerio*, *Ac - Anolis carolinensis*, *Tc - Tribolium castaneum*, *Ce - Caenorhabditis elegans*, *Nv - Nematostella vectensis*.
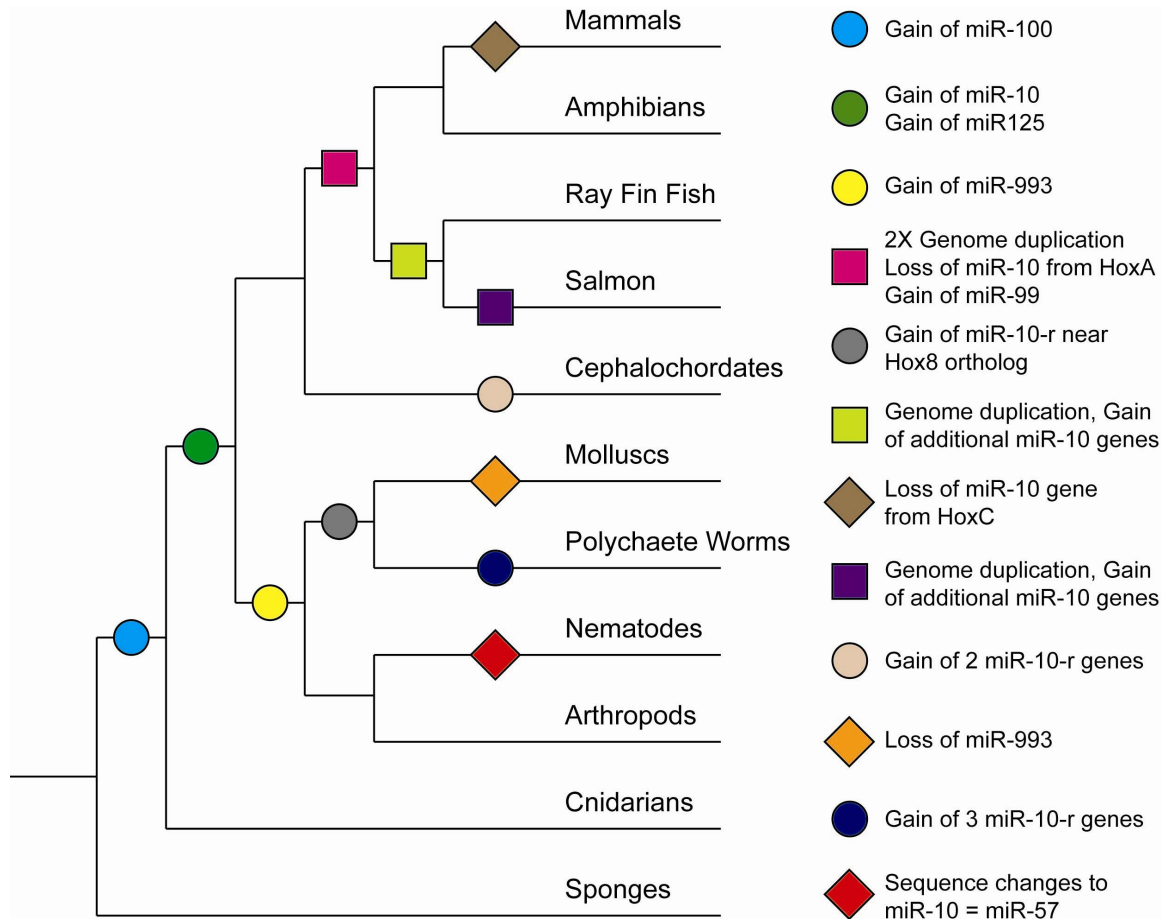
Figure 4. Evolution of miR-10 and related miRNAs. Phylogenetic tree showing gain and loss of miR-10 and related miRNAs. Colored squares indicate genome duplication events and consequently duplicated Hox complexes and miR-10 genes. Colored circles indicate gains of miR-10 and related miRNAs. Colored diamonds indicate loss of specific miRNAs.
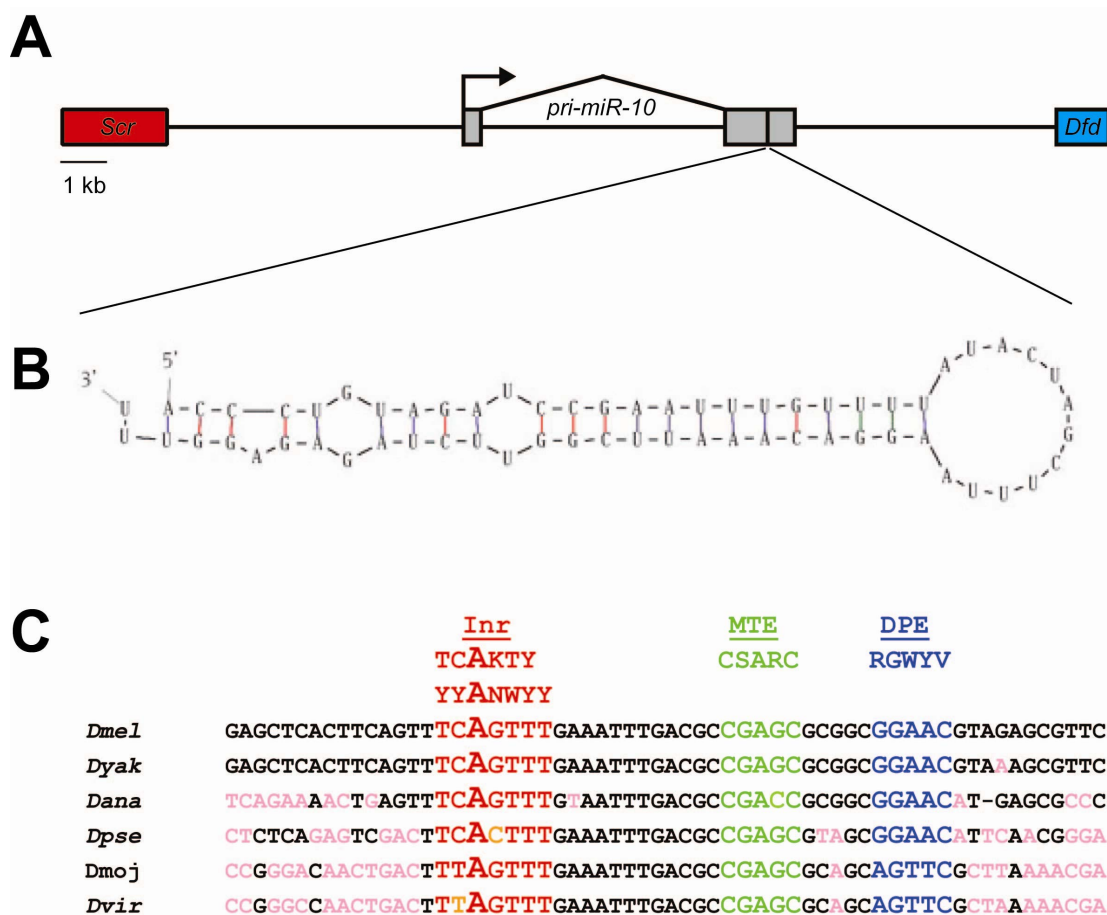
Figure 5. miR-10 precursor molecules and regulation. (A) Diagram of *pri-miR-10* genomic structure determined by 5' and 3' RACE. Indicated as red and blue boxes are the terminal exons of *Scr* and *Dfd* respectively, and the black bar denotes the position of the pre-miRNA hairpin. **(B)** *pre-miR-10* hairpin which is excised from the primary transcript**. (C)** Alignment of basal promoter regions of *pri-miR-10* from six *Drosophila* species. Pink shaded residues are not conserved with *Dmel*. Highlighted in red, green and blue are promoter motifs Initiator (Inr), Motif Ten Element (MTE), and Downstream Promoter Element (DPE) respectively. Consensus sequences for motifs are given above alignment and lighter shaded nucleotides indicate lack of conformity to consensus. Organism abbreviations are as in Figure 1.
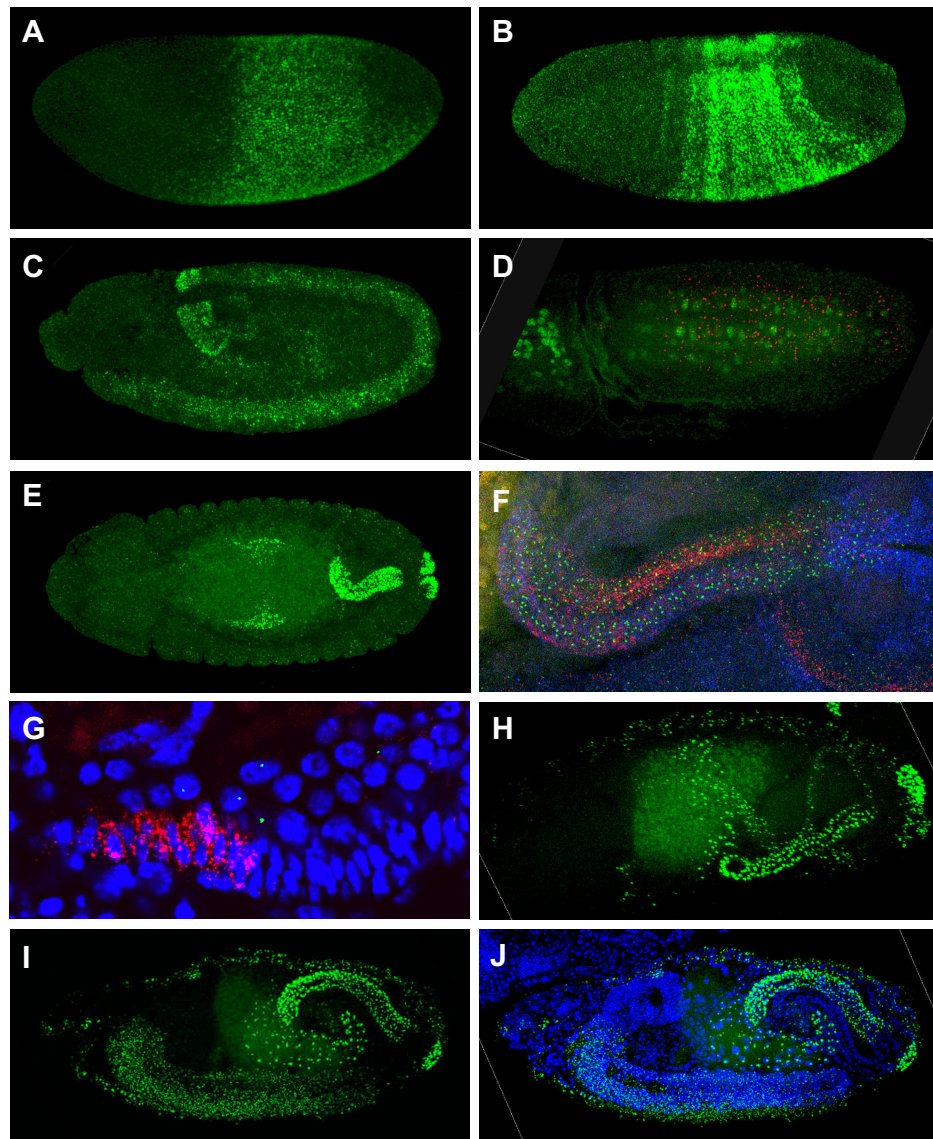
Figure 6. *pri-miR-10 in situ* hybridization through embryogenesis. *pri-miR-10* antisense probe shows spatially localized staining at all stages after the beginning of cellularization. *pri-miR-10* is expressed in an early "gap" pattern (A). In later blastoderm stages *pri-miR-10* develops a "pair rule" pattern (B). In the later stages of embryogenesis *pri-miR-10* transcription occurs in anal pad and large intestine primordia (C,E) as well as ventral ectoderm (C,D) and regions of midgut (E). Large intestine and midgut expression is endoderm specific (F, and G respectively). Staining is also seen in late stage embryos in Malpighian tubules (H) and in the CNS (I). CNS staining has an anterior boundary near the sub-esophageal ganglion and extends to near the posterior end of the ventral nerve chord (J). *pri-miR-10* staining is in green, *Dpp* is in red (F,G), blue is either *en* (F) or DAPI (G,J).

Figure 7. Conservation of mature miRNA sequences in miR-10 hairpins. Highlighted and outlined sequences are 90% conserve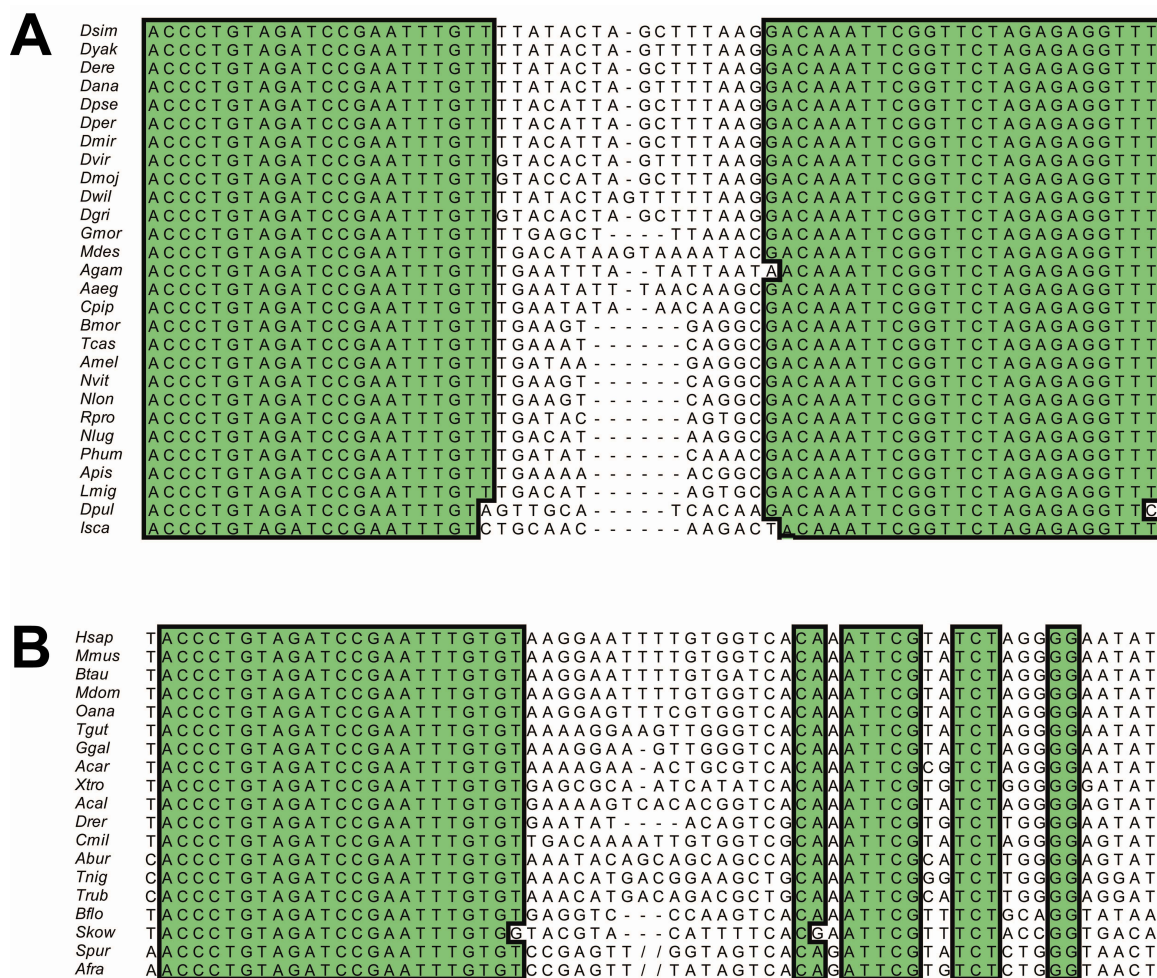d in each alignment. **(A)** Both arms of Arthropod miR-10 hairpins which contribute to the Dicer cleavage product are conserved. **(B)** In deuterostomes only the miR-10 sequence is conserved.

Figure 8. Both miR-10 and miR-10* are expressed as mature miRNAs during *Drosophila* embryogenesis. (A) Northern blots of total RNA purified from *Drosophila* embryos at various developmental stages using ^{32}P end labeled antisense oligo probes against miR-10 and miR-10*. Time course shows both miR-10 and miR-10* are expressed at very low levels in blastoderm embryos and during early germ band extension (0-4 hrs) and progressively increase in abundance throughout embryogenesis. miR-10* is found at much higher levels throughout embryogenesis. **(B)** Dorsal view of a stage 14 *Drosophila* embryo stained for mature miR-10 by LNA *in situ* hybridization. **(C)** Lateral view of a stage 14 *Drosophila* embryo stained for mature miR-10* by LNA *in situ* hybridization. Both miRNAs show identical patterns and their expression at this stage matches that of *pri-miR-10* transcripts (compare to Fig. 5E). **(D)** Close up of anal pad cells showing cytoplasmic localization of miR-10* staining.

A anti-miR-10 probe   anti-miR-10* probe

0-4 hrs | 4-8 hrs | 8-12 hrs | 12-16 hrs | 0-4 hrs | 4-8 hrs | 8-12 hrs | 12-16 hrs

B

miR-10

C

miR-10*

D

miR-10*
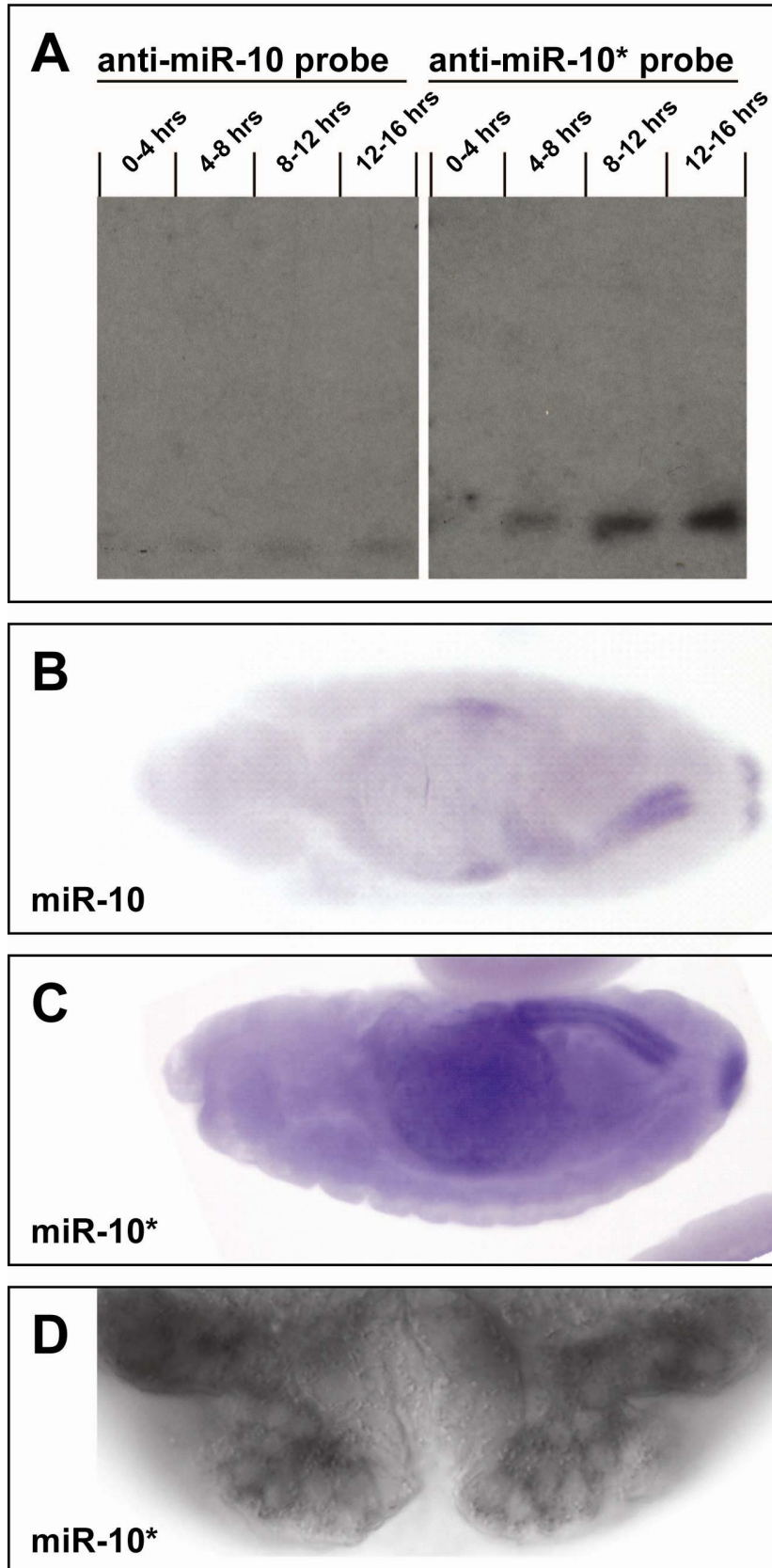
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Dsim    5' AACAAAUUCGGAAGAUAAACAGGAA 3'
Dsec    5' AACAAAUUCGGAAGAUAAACAGGAA 3'
Dyak    5' AACAAAUUCGGAAUAUAAACAGGAA 3'
Dere    5' AACAAAUUCGGAAGAUAAACAGGAA 3'
Dana    5' AACAAAUUCGGAAGCUAAACAGGAA 3'
Dpse    5' AACAAAUUCGGAAGUCAAACAGGAA 3'
Dper    5' AACAAAUUCGGAAGUCAAACAGGAA 3'
Dmir    5' AACAAAUUCGGAAGUCAAACAGGAA 3'
Dwil    5' AACAAAUUCGGAAAACAAACAGGGA 3'
Dvir    5' AACAAAUUCGGAGGAUAAACAGGAA 3'
Dmoj    5' AACAAAUUCGGAGAUGAAACAGGGA 3'
Dgri    5' AACAAAUUCGGAGGACAAACAGGAA 3'
Gmor    5' AACAAAUUCGGGU---GCACAGGGA 3'
Ppap    5' AACAAAUUCGGAGA-AAAACAGGGC 3'
Mdes    5' AACAAAUUCGGAU---CGACAGGAU 3'
Aaeg    5' GACAAAUUCGGAUU--ACACAGGGA 3'
Cpip    5' AGCAAAUUCGGAUU--ACACAGGGA 3'
Agam    5' AGCAAAUUCGGACU-CGUACAGGGC 3'
Bmor    5' AACAAAUUCGGAAU-UGUACAGGGA 3'
Tcas    5' AACAAGUUCGGAAC-UAUACAGGGA 3'
Amel    5' GGCCAAUUCGGAAG-AAUACAGGGA 3'
Nvit    5' AGCAAAUUCGGAAG-AAUACAGGGA 3'
Ngir    5' AGCAAAUUCGGAAG-AAUACAGGGA 3'
Nlon    5' AGCAAAUUCGGAAG-AAUACAGGGA 3'
Phum    5' AACAAAUUCGGAAA-UAUGCAGGGA 3'
           ||||||||||||     ||||||||
miR-10  3' UUGUUUAAGCCUA---GAUGUCCCA 5'
```

Figure 9. miR-10 has evolutionarily conserved target sites in the 3'UTRs of insect *Sex combs reduced* (*Scr*) orthologs. Alignment of conserved sequences found in the 3'UTRs (or 3' of the stop codon in putative UTR sequence) of *Scr* genes in insects and complementarity to mature miR-10 sequence. All species have at least 12 nucleotides of complementarity to the 3' end of miR-10 and 5 or more nucleotides of complementarity in the "seed" region near the 5' end. Highlighted in blue is the conserved region which can pair with miR-10. Dark blue indicates G-U base pairing. Green highlights additional regions of pairing in some species. Bases that are not highlighted would not contribute to pairing between miRNA and mRNA. Species abbreviations are as in Figure 1, except *Ngir - Nasonia giraulti*, and *Ppap - Phlebotomus papatasi*.
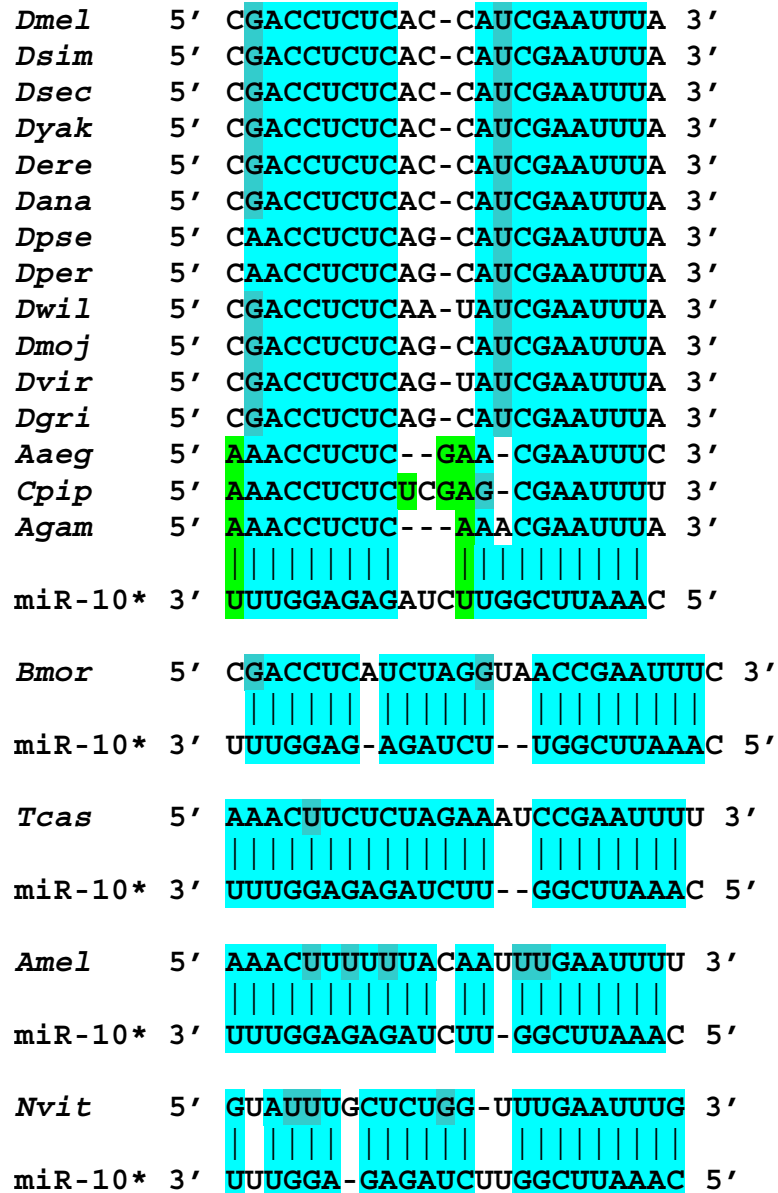
```
Dmel     5' CGACCUCUCAC-CAUCGAAUUUA 3'
Dsim     5' CGACCUCUCAC-CAUCGAAUUUA 3'
Dsec     5' CGACCUCUCAC-CAUCGAAUUUA 3'
Dyak     5' CGACCUCUCAC-CAUCGAAUUUA 3'
Dere     5' CGACCUCUCAC-CAUCGAAUUUA 3'
Dana     5' CGACCUCUCAC-CAUCGAAUUUA 3'
Dpse     5' CAACCUCUCAG-CAUCGAAUUUA 3'
Dper     5' CAACCUCUCAG-CAUCGAAUUUA 3'
Dwil     5' CGACCUCUCAA-UAUCGAAUUUA 3'
Dmoj     5' CGACCUCUCAG-CAUCGAAUUUA 3'
Dvir     5' CGACCUCUCAG-UAUCGAAUUUA 3'
Dgri     5' CGACCUCUCAG-CAUCGAAUUUA 3'
Aaeg     5' AAACCUCUC--GAA-CGAAUUUC 3'
Cpip     5' AAACCUCUCUCGAG-CGAAUUUU 3'
Agam     5' AAACCUCUC---AAACGAAUUUA 3'
            |||||||||   ||||||||||
miR-10*  3' UUUGGAGAGAUCUUGGCUUAAAC 5'


Bmor     5' CGACCUCAUCUAGGUAACCGAAUUUC 3'
            ||||||| |||||| |||||||||
miR-10*  3' UUUGGAG-AGAUCU--UGGCUUAAAC 5'


Tcas     5' AAACUUCUCUAGAAAUCCGAAUUUU 3'
            ||||||||||||||  |||||||
miR-10*  3' UUUGGAGAGAUCUU--GGCUUAAAC 5'


Amel     5' AAACUUUUUUACAAUUUGAAUUUU 3'
            |||||||||||| || ||||||||
miR-10*  3' UUUGGAGAGAUCUU-GGCUUAAAC 5'


Nvit     5' GUAUUUGCUCUGG-UUUGAAUUUG 3'
            | ||||| |||||| ||||||||||
miR-10*  3' UUUGGA-GAGAUCUUGGCUUAAAC 5'
```

**Figure 10. miR-10* has evolutionarily conserved target sites in the 3'UTRs of insect
Abdominal-B (*Abd-B*) orthologs.** Alignment of conserved sequences found in the 3'UTRs (or 3'
of the stop codon in putative UTR sequence) of *Abd-B* genes in insects and complementarity to
mature miR-10* sequence. Potential target-miRNA pairings are not equivalent in structure, but all
species have large blocks of complementarity to both 5' and 3' ends of the miR-10* sequence.
Highlighted in blue is the conserved region which can pair with miR-10. Dark blue indicates G-U
base pairing. Green highlights additional regions of pairing in some species. Bases that are not
highlighted would not contribute to pairing between miRNA and mRNA. Species abbreviations
are as in Figure 1.

44
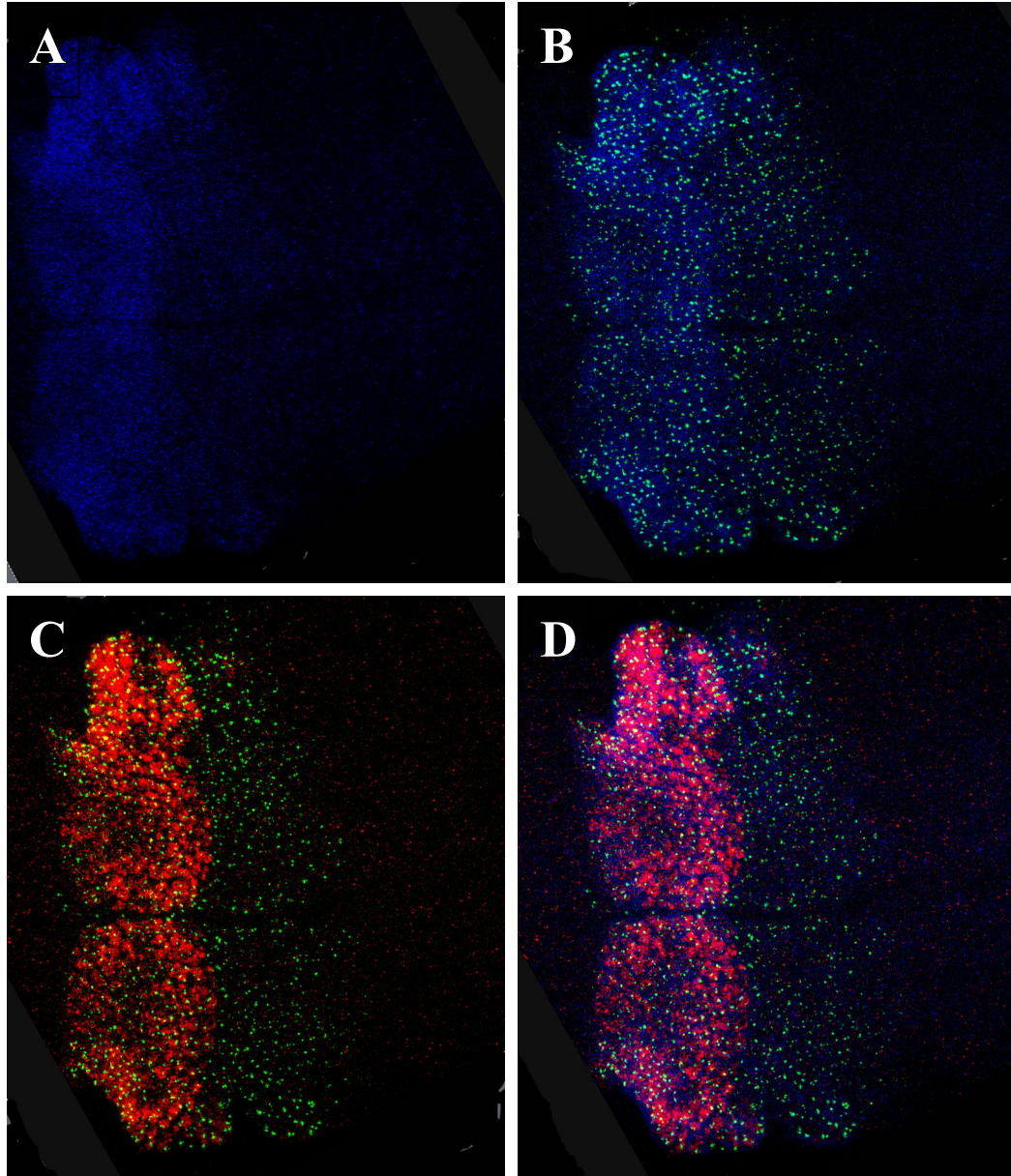
Figure 11. *Scr* protein and cytoplasmic transcript accumulation are downregulated in ventral T1. The pattern of *Scr* cytoplasmic transcript accumulation in the ectoderm includes all of the labial segment as well as lateral regions of the first thoracic segment **(A)**. The cells which are transcribing *Scr* (seen with intron probe – green in B) include all of the ventral and lateral domains of both the labial segment and T1. Scr protein accumulation does not occur in all cells which are transcribing *Scr* **(C)** but coincides completely with those cells that show cytoplasmic transcript accumulation **(D)**.

Figure 12. Mature miRNA is produced from transgenes and results in aberrant phenotypes.
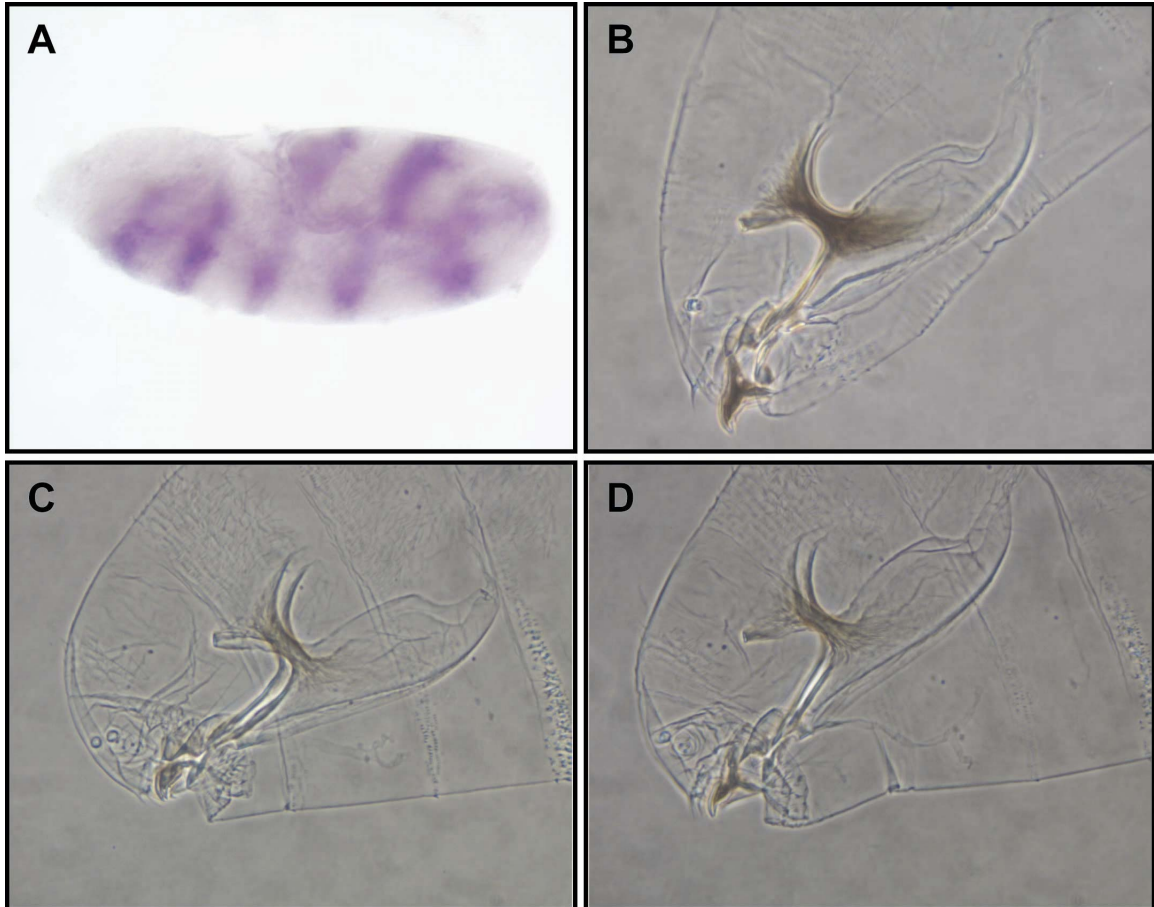When expressed from a *prd*-GAL4 driver, miR-10* is detectable in the *prd* pattern at high levels in a germband extended embryo **(A)**. When expressed from an Actin-GAL4 **(C)** or *da*-GAL4 driver **(D)** malformations are noticeable in the head cuticle when compared to wild type **(B)**.

Figure 13. *pri-miR-10* is expressed in a pattern complementary to predicted targets. Images of *in situ* hybridizations of *pri-miR-10* (green) *Scr* (red) and *Abd-B* (blue) at various stages of development. **(A)** *pri-miR-10* at stage 15. **(B)** *Scr* and *Abd-B* in same embryo as (A). **(C)** Overlay of (A) and (B). **(D)** *pri-miR-10* and **(E)** *Scr* in a blastoderm stage embryo. **(F)** Overlay of (D) and (E). **(G-I)** *pri-miR-10* and *Abd-B* expression in the posterior of the extended germband of a stage 11 embryo. Note overlapping expression in posterior neurectoderm. **(J)** Field of cells from the large intestine of the hindgut of a stage 15 embryo. *pri-miR-10* is expressed in the endodermal cells, while *Abd-B* is expressed in the visceral mesoderm surrounding the gut.
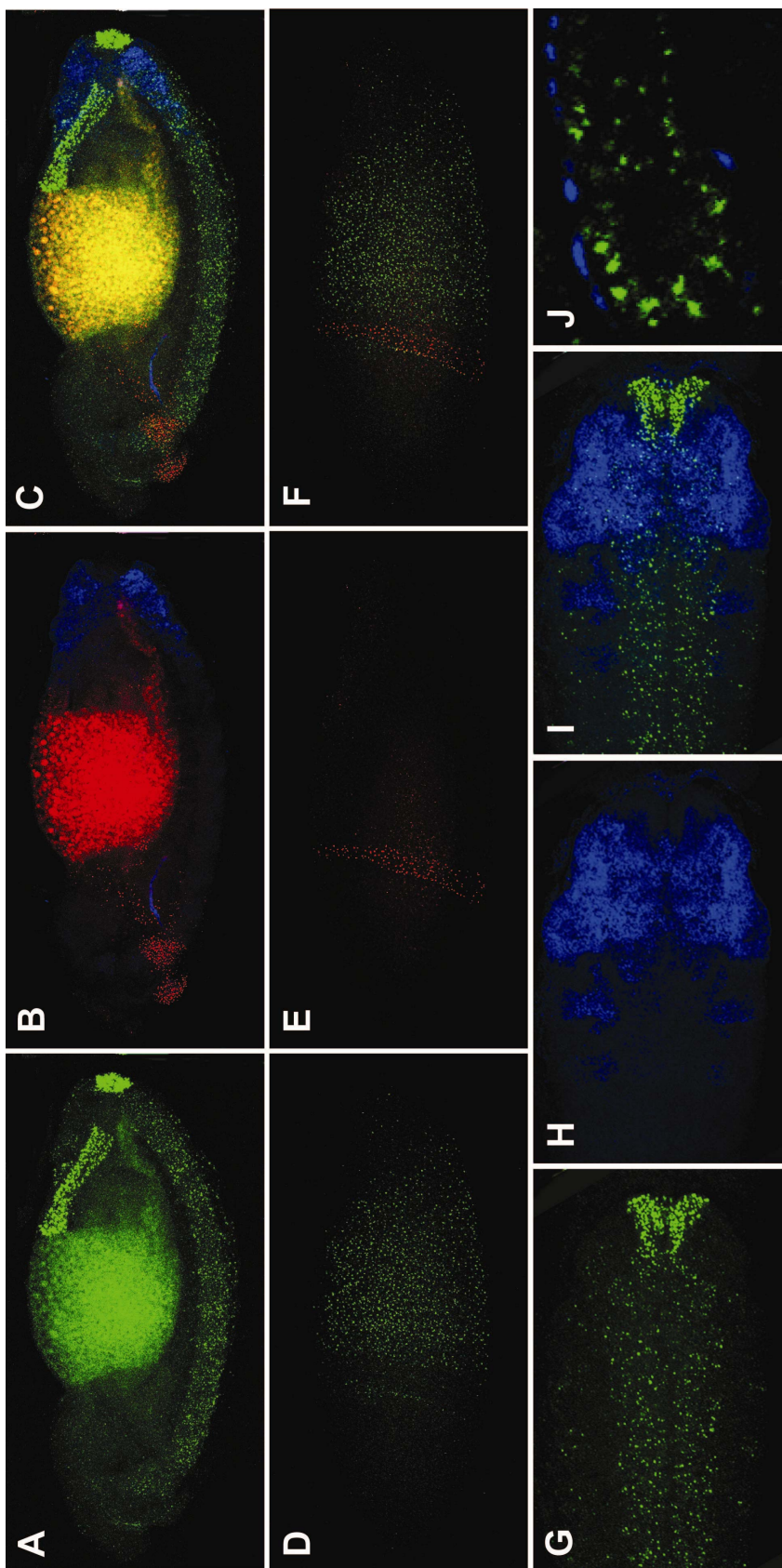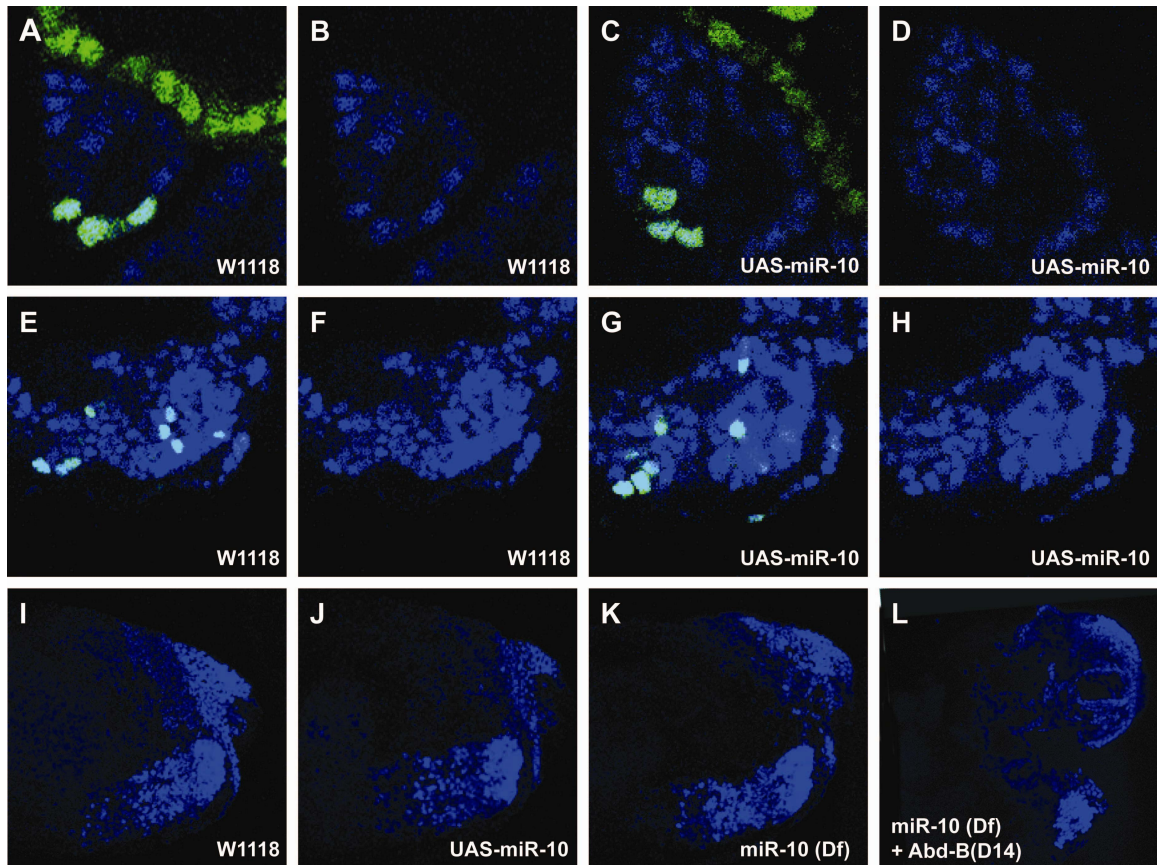
Figure 14. Overexpression or lack of *pri-miR-10* has no observable effect on putative target protein expression. Embryos expressing UAS-*pri-miR-10* under the control of *en*-GAL4 **(C,D,G,H)** exhibit no significant decrease in SCR protein **(A-D)** or ABD-B protein **(E-H)** in cells expressing ectopic miRNA (marked with anti-*en* in green) versus control cells in wild type (W1118) embryos **(A,B,E,F)**. No significant change in ABD-B protein **(I-L)** levels is seen in the CNS of W1118 **(I)** compared to embryos ubiquitously expressing UAS-*pri-miR-10* under the control of *da*-GAL4 **(J)**, or embryos deficient for the miR-10 locus ((*Df3R*)*CP1*). Embryos mutant for the *Abd-B*-m specific transcripts (*Abd-B^{D14}*) and deficient for miR-10 do not exhibit ectopic r-type ABD-B protein **(L)**.

```
Scr 3'UTR       5' ...TGAAGAACAAATTCGGAAGATAAACAGGAAGTAAAAT... 3'
                         ||||||||||||         |||||
miR-10              3'  UUGUUUAAGCCUA---GAUGUCCCA 5'
                         ||||| ||||||      || |
Scr mut UTR     5' ...TGAAGAACAACTTCGGAAGATAACCACGAAGTAAAAT... 3'



Abd-B 3'UTR     5' ...UCGCAAGCGACCUCUCACC-AUCGAAUUUAUUCAUAG... 3'
                          :||||||||     |:||||||||
miR-10              3'   UUUGGAGAGAUCAUGGCUUAAAC 5'
                          :|| ||||     |:| || ||
Abd-B mut UTR   5' ...UCGCAAGCGACGUCUCACC-AUCAAACUUAUUCAUAG... 3'
```
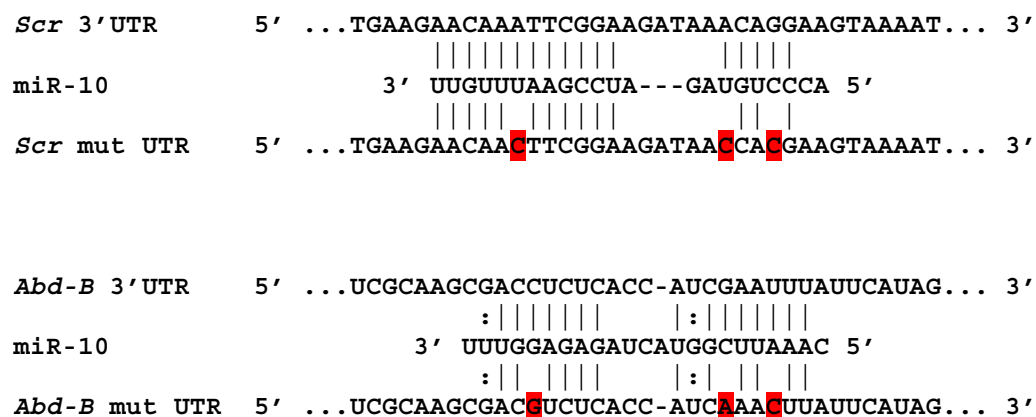
Figure 15. Target site mutations for luciferase experiments. Alignment of *Scr* and *Abd-B* 3'UTR sequences with miR-10 or miR-10* respectively. Vertical bars indicate typical base pairs and colons indicate G-U base pairing. Highlighted in red are point mutations which disrupt continuous helix paring between mRNA and miRNA particularly in "seed" regions.

Chapter II

Co-option of an ancestral anterior head patterning gene network for proximodistal patterning of appendages in early bilaterian evolution

INTRODUCTION

The enormous diversity of extant animal forms is a testament to the power of evolution, and much of this diversity has been achieved through the emergence of novel morphological traits. The origin of novel morphological traits is an extremely important issue in biology, and a frequent source of this novelty is co-option of pre-existing gene networks for new purposes (Carroll et al., 2008). Appendages, such as limbs, fins and antennae, are structures common to many animal body plans which must have arisen at least once, and probably multiple times, in lineages which lacked appendages. We provide evidence that appendage proximodistal patterning genes are expressed in similar registers in the anterior embryonic neurectoderm of *Drosophila melanogaster* and *Saccoglossus kowalevskii* (a hemichordate). These results, in concert with existing expression data from a variety of other animals suggest that a pre-existing gene network for anterior head patterning was co-opted for patterning of the proximodistal axis of appendages of bilaterian animals.

Since the advent of molecular biology, many morphological traits that are shared between disparate animal clades have been found to be controlled by conserved underlying gene networks (Bier, 1997; Holley et al., 1995; McGinnis and Krumlauf, 1992; Olson, 2006; Silver and Rebay, 2005). Morphological novelty, on the other hand, involves the evolution of new traits that are often patterned by co-opted genes or gene networks that originally performed other developmental functions. For example, eye spots on butterfly wings are patterned through the re-deployment, in small foci corresponding to the eye spots, of a gene regulatory network that also controls the growth and pattern of the entire insect wing (Keys et al., 1999). Another example is seen in the

redeployment of a few Hox genes to pattern the paired appendages of vertebrates; these genes having been co-opted from an ancestral role in patterning posterior structures on the main body axis of chordates (McGinnis and Krumlauf, 1992; Zakany and Duboule, 2007). In this study we wished to explore the origins of the proximodistal appendage patterning network.

The patterning of *Drosophila* appendages is a well studied system of proximodistal axis specification. Although numerous genes participate in patterning of *Drosophila* appendages, there is a core network which is responsible for establishing the gross morphological divisions. The gene pair *buttonhead* (*btd*):*D-Sp1*, and the genes *Distal-less* (*Dll*), *dachshund* (*dac*), and *homothorax* (*hth*), exist in an interacting network that regulates the growth and boundaries of the distal, medial, and proximal appendage domains (Kojima, 2004). We refer to these genes as the core proximodistal appendage patterning network.

In early *Drosophila* embryos, *hth* and *btd* are both expressed in the appendage primordia. *btd*:*D-Sp1* (a recently duplicated and diverged gene pair of *Sp8* orthologs in *Drosophila*), are required for the activation of *Dll* in embryonic thoracic appendage primordia (Estella et al., 2003). As the domains of *Dll* expressing cells expand, *hth* becomes excluded from a subset of these cells in response to repression by *Dll* (Bolinger and Boekhoff-Falk, 2005). Cells from these primordia go on to form the larval Keilin's organs and imaginal discs.

Early in leg imaginal disc development, as in the embryonic appendage primordia, cells are divided into two major domains by a central cluster of *Dll* expressing cells surrounded by *hth* expressing cells (Abu-Shaar and Mann, 1998; Wu and Cohen,

1999). As development progresses, *dac* expression comes on in a medial region of leg discs (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). Small regions of overlapping expression of *Dll*, *dac*, and *hth* exist in later leg disc development, although the genes also exhibit mutually repressive interactions in some cells (Abu-Shaar and Mann, 1998; Dong et al., 2001; Wu and Cohen, 1999). Although the regulatory relationships between *btd*:*D-Sp1* and other core appendage patterning genes in the imaginal discs are unknown, the *btd* gene is expressed in a disc domain that overlaps with *hth*, *dac* and *Dll*, but is excluded from the most distal and most proximal cells (Estella et al., 2003). The developing *Drosophila* antenna has a slightly different imaginal disc expression profile than that of the leg, with the medial *dac* domain being smaller and *hth* expression overlapping that of both *dac* and *Dll* (Dong et al., 2001). However, expression domains of these genes still roughly correspond to the same proximodistal fates in both developing leg and antennae (Dong et al., 2001), and similarly ordered and overlapping expression domains of the core genes are conserved in many developing arthropod appendages (Angelini and Kaufman, 2005; Beermann et al., 2004; Schaeper et al., 2009).

Investigation of genes underlying proximodistal development of vertebrate appendages has revealed that, despite structural dissimilarity to arthropod appendages, they develop under the control of genetic networks that include orthologs of *Drosophila btd*:*D-Sp1*, *Dll*, *dac*, and *hth* genes (Pueyo and Couso, 2005). Vertebrate *Sp8* genes are expressed in evolutionarily conserved patterns in distal ectoderm of limb buds, and knockdown of *Sp8* function in chick results in defects of limb outgrowth and patterning (Kawakami et al., 2004). *Dlx* family genes (*Dlx1*, *2*, *5* & *6*; *Dll* orthologs) are also expressed in distal ectoderm of mouse limb buds, and *Dlx5*:*Dlx6* double mutants have

distal limb defects (Kraus and Lufkin, 2006; Panganiban and Rubenstein, 2002). *Dach1*

(a *dac* ortholog) is expressed in a complex pattern in developing mouse limb buds, with a

transient stage when expression is limited to anterior-medial limb bud cells (Davis et al.,

1999; Hammond et al., 1998). *Meis1* (a vertebrate *hth* ortholog) is expressed in the

proximal regions of vertebrate limb buds, and required for the normal development of the

proximal domain of chick appendages (Mercader et al., 1999).

Available fossil data from the Pre-Cambrian does not allow us to be sure of the

body plan of the last common ancestor of vertebrates and arthropods (Valentine, 2004).

However, a synthesis of comparative morphology suggests that it either existed with

rudimentary appendages or lacked them entirely (Shubin et al., 1997). The appendages of

disparate extant bilaterian groups almost certainly evolved independently in multiple

lineages subsequent to their divergence from a common ancestor which lacked

appendages (Shubin et al., 1997). If animal appendages are not derived from a common

ancestral appendage, the involvement of a common gene network in proximodistal

patterning could be due to random convergence of the same set of genes to pattern non-

homologous appendages, or independent co-option of the same gene network which

functioned to pattern an ancestral structure shared by both vertebrates and arthropods

(Davidson and Erwin, 2006; Panganiban et al., 1997; Tabin et al., 1999). Involvement of

a gene network in essential developmental roles (e.g. insect wing patterning) may make

the regulatory interactions within the network resistant to change (Davidson and Erwin,

2006). This does not, however, preclude redeployment of such a patterning network using

different genetic inputs and outputs, which could then contribute to novel morphological

structures (Davidson and Erwin, 2006), such as butterfly wing eye spots (Keys et al., 1999).

It has been previously proposed that lateral appendages might have originated through the co-option of a pre-existing group of genes, including *Dll*, that controlled a rudimentary appendage-like outgrowth in the ancestor of vertebrates and arthropods (Tabin et al., 1999). It has also been proposed that the appendages of vertebrates and arthropods might be modified duplicates of the entire anteroposterior body axis (Minelli, 2000). This proposal is based in part on an ancestral role of Hox genes in patterning the main body axis, and the involvement of a subset of Hox genes in patterning the proximal-distal axis of vertebrate appendages (Zakany and Duboule, 2007). Hox genes do not have similar expression patterns in vertebrate and arthropod appendages, so for this and other reasons the model that the entire anteroposterior body axis patterning system is redeployed in most animal appendages (Minelli, 2000) is not well supported in our opinion. Our proposition relates an ancient conserved network for patterning the anterior neurectoderm of animals to the proximodistal patterning of bilateral animal appendages.

A survey of previous research provides data from a few different animal groups on expression patterns of the core proximodistal appendage patterning genes in various tissues. We noticed that these genes, as well as other genes that are part of the proximodistal appendage patterning network in *Drosophila*, such as *aristaless* (*al*), *apterous* (*ap*), and *BarH1*, are expressed in discrete domains in the anterior embryonic neurectoderm of many chordates and arthropods (Appendix). We considered the hypothesis that a shared anteroposterior expression regimen of these genes in head neurectoderm might be common in bilateral animals. We wished to evaluate this

hypothesis by testing the relative expression patterns of core appendage patterning genes in the anterior neurectoderm of *Drosophila* embryos, as well as in embryos of *Saccoglossus*, a basal deuterostome that lacks bilateral appendages.

RESULTS

Genes of the core proximodistal appendage patterning network are expressed in a

spatially and temporally complex manner during *Drosophila* development. However, in

the anterior neurectoderm of *Drosophila* embryos, these genes are expressed in a clear

anteroposterior order. We determined the relative expression patterns of *Dll*, *dac*, *hth*, and

btd using combinatorial in situ hybridizations, and analyzed their relative expression

patterns in germband extended (stage 11) embryos (Fig. 16b-e). At this stage the

procephalic neurectoderm has been divided into anterior, central, and posterior

protocerebral areas, and a more posterior deuterocerebral area, with the most anterior

cells being those flanking the dorsal midline of the procephalon and posterior cells

located more ventrolaterally (Younossi-Hartenstein et al., 1996) (Fig. 16a). *btd*:*D-Sp1*

and *Dll* are transcribed in overlapping patches covering most of the anterior protocerebral

neurectoderm (Fig. 16b-c). The domain of *dac* transcription is mainly in central and

posterior protocerebral neurectoderm, with small regions of overlap with *Dll* and *btd*:*D-*

Sp1 (Fig. 16d). *hth* transcripts are largely absent in anterior neurectoderm (Fig. 16e), and

are completely excluded from domains which transcribe *Dll* and *btd*:*D-Sp1* (compare to

Fig. 16c). The posterior protocerebral region contains cells which transcribe both *hth* and

dac, but the majority of *hth* transcription is found in the deuterocerebrum and more

posterior neurectoderm (compare Figs. 16d and 16e). Taken together, these data reveal an

expression order of *btd*:*D-Sp1* and *Dll* in the most anterior neurectodermal cells, *dac* in

medial cells, and *hth* in posterior cells, with small zones of overlap at the borders of the

three major domains (Fig. 16f).

Using in situ hybridization, we also tested the expression patterns of the orthologous genes in *Saccoglossus* embryos ranging from gastrula to early gill slit stages. In post gastrulae, *Sp8* (the *btd*/*Sp1* ortholog) is transcribed at high levels in the anterior third of the embryonic neurectoderm, and to a lesser degree in multiple medial to posterior stripes (Fig. 17a). In embryos at the one gill slit stage, expression is similar with the major expression confined to the proboscis (Fig. 17b). The *Saccoglossus dac* ortholog (*Dach*) is transcribed at high levels in a neurectodermal stripe just anterior to the collar, and at low levels throughout most of the rest of the embryo, both in post gastrula (Fig. 17c) and one gill slit stage embryos (Fig. 17d). *Meis*, the ortholog of *hth*, is transcribed at high levels in a broad band in the trunk neurectoderm, as well as in two dorsal patches - one just anterior to the collar; the other in the posterior trunk (Fig. 17e-f). The *Saccoglossus Lhx2/9* gene, orthologous to *apterous*, is transcribed throughout the proboscis neurectoderm in late gastrulae (Fig. 17g) and then becomes restricted mainly to a strong stripe just anterior to the collar (Fig. 17h). Along with previously documented expression patterns for the *Saccoglossus BarH1* and *Dlx* orthologs (Lowe et al., 2003) we provide an expression model (Fig. 17i) summarizing the transcription domains of all of these genes.

DISCUSSION

Based on the above data and previously published work (Appendix), we estimated the ancestral expression domains of the anteroposterior head gene network, and compared them to the approximate domains of the proximodistal appendage gene network in the developing *Drosophila* leg (Fig 18). Our proposal posits that a "head-appendage" network, consisting of the *btd*/*Sp8*, *Dll*/*Dlx*, *dac*/*Dach*, *hth*/*Meis* genes (and likely other genes, some of which are shown in Figure 18), was present in a bilaterian ancestor that lacked appendages, where the network functioned to pattern the anteroposterior head axis. The evolution of this network may even have contributed to the process of cephalization in early animals. Subsequently, this network was co-opted to pattern the proximodistal axis of bilateral appendages through modification of input and output connections.

After a network for anteroposterior head patterning had been co-opted for proximodistal appendage patterning, it could be used specify and diversify the pattern of many body wall outgrowths (e.g. sensory structures, locomotory appendages, external genitalia, feeding appendages, etc.) through changes in the network. These could include variations in the regulatory relationships and expression patterns of the core appendage patterning genes, as well as further modifications of input and output connections (Dong et al., 2001). Consistent with this theory, inputs into this network during *Drosophila* appendage formation, such as *Dpp* and *wg*, are not conserved in this role among insects (Angelini and Kaufman, 2005). At least part of the same network has apparently been co-opted for the development of beetle horns (Moczek et al., 2006; Moczek and Rose, 2009), an appendage-like body wall outgrowth, long after the evolutionary advent of bilateral

appendages. It will be interesting to study the expression pattern of the head-appendage patterning genes in other branches of the evolutionary tree, especially in cnidarians, acoel flatworms, and lophotrochozoans.

Chapter II contains material submitted for publication in Lemons, D., Fritzenwanker, J. F., Gerhart, J., Lowe, C. J., McGinnis, W. Co-option of an anteroposterior head axis gene network for proximodistal patterning of appendages in early bilaterian evolution.

MATERIALS AND METHODS

Drosophila in situ hybridizations were performed as in Kosman et al., 2004. *btd* antisense probes were made from a 2.6 kb genomic fragment starting 49 bp 5' of the coding region. *Dll* antisense probes were made from a 1.4 kb EcoRI cDNA fragment (Cohen et al., 1989). *dac* antisense probes were made from a genomic PCR fragment cloned into pCRII (Invitrogen), the primers for the *dac* fragment were: 5' AAGCAAAGTATAGAACGGATTAGCA 3'; 5' TCCAACGAATCTTTCACTTCG 3'. *Saccoglossus in situ* hybridizations were performed as in Lowe et al., 2004. Antisense probes for *Sp8*, *Lhx2/9*, *Dach*, and *Meis* were made from *Saccoglossus kowalevskii* cDNAs (Freeman et al., 2008), accession numbers NM_001168189, NM_001164971, NM_001164944, and GU384871 respectively.
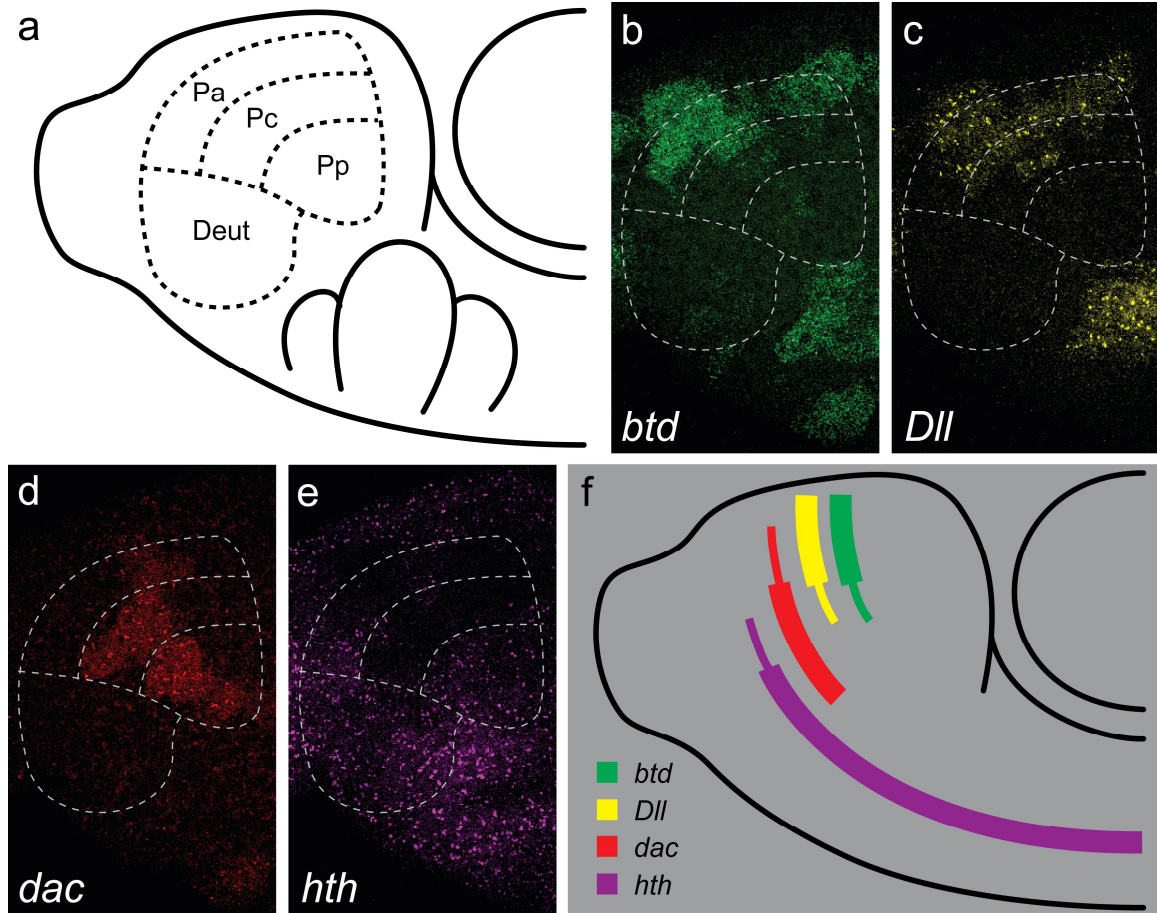
Figure 16. Expression of core appendage network genes in anterior neurectoderm in *Drosophila* embryos. Data are presented as maximum projections of confocal sections through embryonic procephalic neurectoderm of a stage 11 *Drosophila* embryo. **(A)** Schematic of the procephalic region of a stage 11 *Drosophila* embryo displaying subdivisions of the neurectoderm. Indicated are anterior, central, and posterior protocerebral (Pa, Pc, and Pp respectively) and deuterocerebral regions (adapted from Younossi-Hartenstein et al., 1996). **(B)** *btd* is transcribed mainly in the anterior protocerebral neurectoderm with a small region of expression in central regions. Also seen are antennal and maxillary segment expression outside the neurectoderm. The *D-Sp1* transcription pattern is the same as *btd* in this region (data not shown). **(C)** *Dll* is transcribed in nearly the same neurectodermal pattern as *btd* at this stage. **(D)** *dac* is transcribed mainly in central and posterior protocerebral neurectodermal cells and overlaps with the posterior expression of *Dll* and *btd*. **(E)** *hth* is expressed in posterior protocerebral cells and deuterocerebral cells, overlapping with *dac* expression in its posterior expression domain. **(F)** Diagram indicating relative expression domains of *Dll*, *btd*, *dac*, and *hth* in the procephalic neurectoderm.
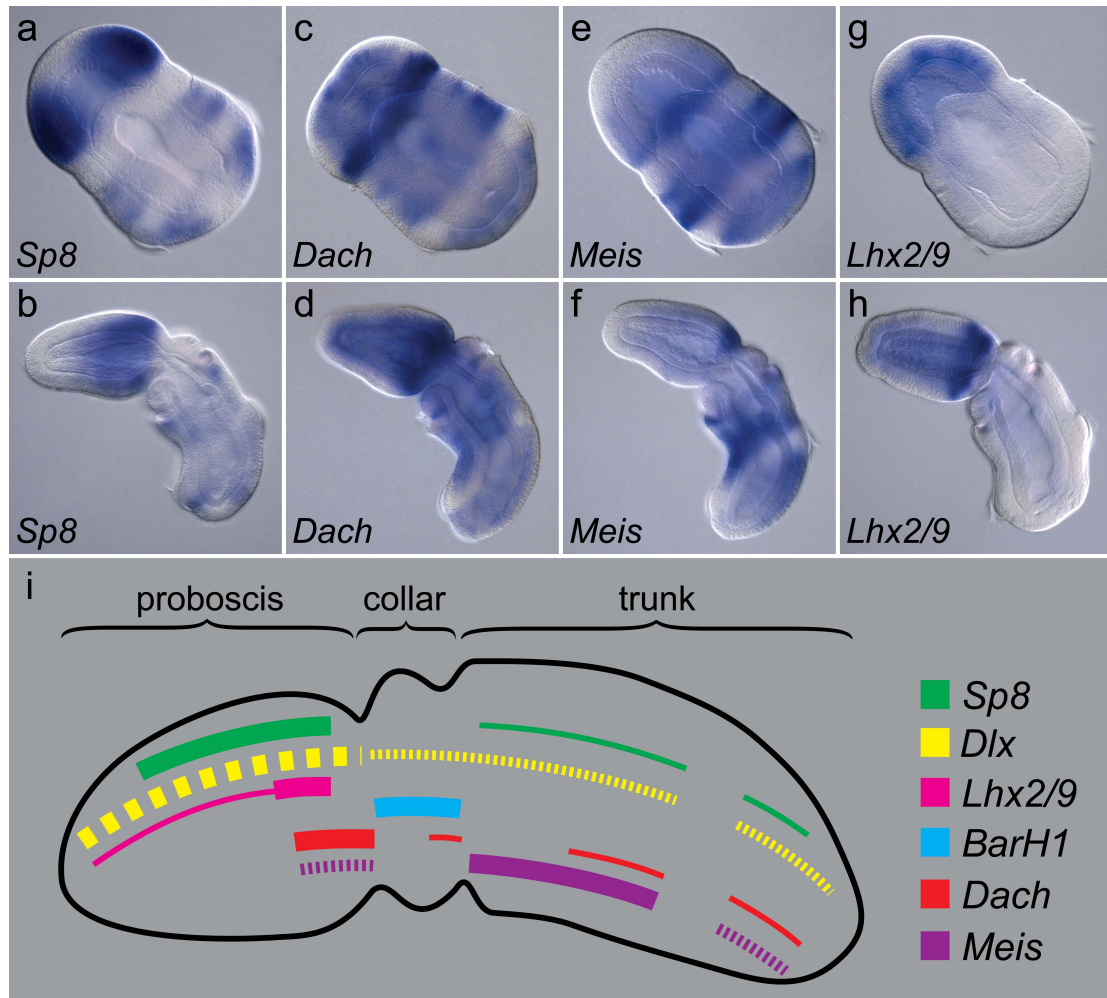
Figure 17. Expression of core network genes in *Saccoglossus* embryos by *in situ* in post gastrula (A,C,E, and G) and one gill slit stages (B,D,F, and H). Data are presented as saggital optical sections with anterior to the upper right of each panel. **(A,B)** High levels of *Sp8* transcripts could be detected in two broad lateral patches in the proboscis. Expression is absent from both the dorsal and ventral midlines and the most anterior region of the proboscis. *Sp8* transcripts are additionally detected at low levels in two broad lateral stripes in the trunk ectoderm with both dorsal and ventral midlines and ciliated band free of expression. **(C,D)** *Dach* is expressed at high levels in an ectodermal stripe just anterior to the collar. Additional low level ectodermal expression is detected throughout much of the embryo. **(E,F)** *Meis* is expressed strongly in the trunk ectoderm excluding the ciliated band and at the base of the proboscis at early developmental stages, which then subsequently refines to a strong dorsal domain. **(G,H)** At post gastrula stage *Lhx2/9* is expressed throughout the proboscis ectoderm. By one gill slit stage expression becomes restricted mainly to a strong stripe at the base of the proboscis. **(I)** A schematic combining these data with previously published expression data for *Dlx* and *BarH1* (Lowe et al., 2003) indicating the relative levels and anteroposterior extents of neurectodermal expression of the appendage network genes. Dashed lines indicate expression in a subset of cells for the indicated anteroposterior domain.
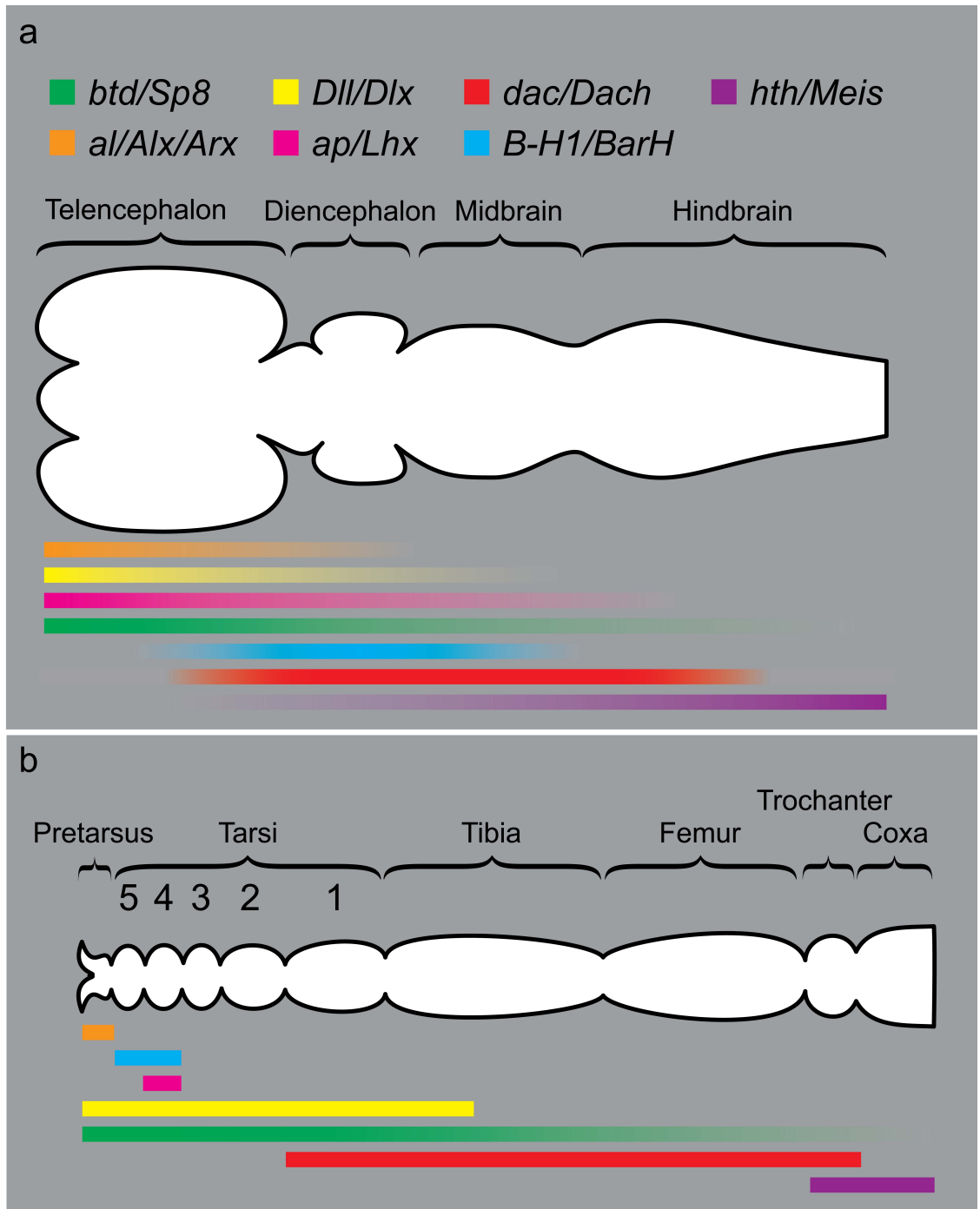
Figure 18. Schematic diagram comparing expression of core network genes in limbs and anterior neurectoderm. (A) Estimated ancestral expression patterns in embryonic anterior neurectoderm (based on conserved domains of expression) is displayed on a generalized diagram of a chordate brain. **(B)** Expression of proximodistal appendage patterning genes is displayed on a diagram of an adult *Drosophila* leg (adapted from Kojima, 2004).

Chapter III

Evolution of homeodomain proteins in early and pre-metazoans

Introduction

Metazoan animals comprise a monophyletic group of multicellular organisms which arose from a unicellular ancestor long before the appearance of the typical animal body forms seen in Cambrian fossil deposits. The transition from unicellularity to multicellularity has occurred multiple times in the ancient past in the ancestors of plants, fungi and animals. Development of a multicellular mode of existence likely allowed for greater adaptability and utilization of resources providing advantages for newly developed phyla to exploit underutilized niches. The multicellularization that gave rise to the metazoans was an extremely important event, which provided the basis for a group of organisms which have evolved to live in nearly every ecosystem. However, little is known about how this multicellularization occurred or what molecules were involved in the process.

There are a number of classes of proteins that would likely be essential to the process of multicellularization. Cell adhesion proteins and extracellular matrix components would allow for the construction of rudimentary multicellular structure. However, an indistinct mass of cells does not make a functioning multicellular organism. True multicellular organisms (as opposed to colonial forms of unicellular organisms) have multiple cell types which perform different functions. In order to accomplish this there must be a mechanism for cells to communicate with each other and respond through changes in cell type or cell function. Signal transduction pathways allow for cell to cell communication, and transcription factors provide proper responses to signals by altering the expression of batteries of downstream effector genes.

Transcription factors are ubiquitous proteins found in all organisms which provide control over when genes activated or repressed. However, homeodomain transcription factors comprise an ancient family restricted to eukaryotes. The homeodomain protein family is distinctive in part due to its size and diversity in multicellular organisms, but principally because most homeodomain proteins assign developmental fates to individual cells, or to groups of adjacent cells in developing multicellular organisms. Most eukaryotes encode one or more genes of the non-TALE homeodomain superclass, with 60 amino acid homeodomains; as well as one or more genes in the TALE superclass, which usually encode 63 amino acid homeodomains (Burglin, 1997; Derelle et al., 2007). In multicellular animals, the non-TALE and TALE superclasses have both duplicated and diverged into many classes and subclasses, often with hundreds of homeodomain genes per genome.

In order to determine what the genetic toolkit of the first animals was comprised of, it is necessary to compare both a variety of animal genomes as well as allied outgroups such as fungi, which together with animals form the *Opisthokonta*. While complete genomes for many fungal and eumetazoan animals exist, the addition of genomic sequence from intermediate species will make elucidation of the process of multicellularization more feasible. The need for these data, in part, has led to the whole genome sequencing of the choanoflagellate *Monosiga brevicollis* and the sponge *Amphimedon queenslandica* (formerly known as *Reniera*). Sponges are the earliest branching metazoan animals and have extremely simple bodies and a low number of cell types when compared to higher metazoans. Choanoflagellates are the nearest outgroup to metazoans and exist either as colonies or single cells which, interestingly, closely

resemble one type of cell found in sponges. It is thought that a unicellular organism similar to a choanoflagellate, or perhaps a particular species of choanoflagellate, was the ancestor of all animals. While it is formally possible that the common ancestor of both fungi and animals was a multicellular organism, this is not thought to be likely and as such the choanoflagellates occupy a unique phylogenetic position which may provide many answers about our simple origins. As part of the genome project for the choanoflagellate *Monosiga brevicollis*, the homeodomain encoding genes in its genome were analyzed as well as those from the recently sequenced species of sponge, *Amphimedon queenslandica*.

RESULTS

Analysis of the demosponge *Amphimedon queenslandica* genomic sequence indicates that it contains at least 31 homeodomain genes. Sponges have simple body plans when compared to other animals and do not exhibit the complex anteroposterior diversification seen in many of the higher animals. As such, it is not surprising that the sponge genome does not contain Hox genes. However, a number of sequences for animal specific types of homeodomain proteins of the ANTP superclass (a diverse group of non-TALE class homeodomain genes) including one each of the BARH, TLX, MSH, BSH, and HEX class genes as well as four NK class genes (data not shown). Additionally, *Amphimedon* has sequences for seven PRD class homeodomains, three LIM class homeodomains, and four POU class homeodomains, all of which are non-TALE class type homeodomain subclasses (Figs. 19). TALE class homeodomains found in the sponge include one each of MEIS, PBX, and SIX class genes as well as five IRO class genes.

Only two homeodomain genes are detected in the *Monosiga brevicollis* genome, both of which group with the MEIS subclass of TALE homeodomains as determined by Bayesian phylogenetic analysis and characteristic C-terminal amino acid residues (Figs. 19 and 20). Although the genome of the last common ancestor of opisthokonts (fungi, choanozoans, and animals) encoded both non-TALE and TALE homeodomain genes (Derelle et al., 2007), the *Monosiga* genome only encodes TALE class homeodomain genes.

DISCUSSION

The analysis of homeodomains from choanoflagellates and sponges, in comparison with human and various fungal homeodomains, indicates that the last common ancestor of the opisthokont lineage had a definitive MEIS class gene (Fig. 20 - red branch). However, none of the other TALE class homeodomains and non-TALE class homeodomains of fungi definitively group with any of the specific sub-classes found in animals (Fig. 20). This would suggest that, while the opisthokont ancestor had both TALE and non-TALE type homeodomain genes that, most of these genes have diverged significantly since the division between animals and fungi, with each group developing its own characteristic sub-classes.

Sponges have at least one gene of many of the major sub-classes of animal homeodomain genes, indicating that these classes are common to all animals and arose either early in animal diversification or prior to the evolution of the first metazoan. Further amplification and diversification of homeodomain genes occurred in the lineage leading to bilaterian animals, and most of the homeodomain types found in triploblast animals, including the HOX subclass, must have been present in the last common ancestor of cnidarians and bilaterians (Ryan et al., 2006).

The lineage leading to *Monosiga* apparently lost all non-TALE homeodomain genes. It is unexpected that none of the other animal specific classes of homeodomain genes are found in this genome, but it is possible that the non-colony forming choanoflagellate *Monosiga* has evolved a simplified genome in an adaptation to its exclusively unicellular existence. It will be interesting to see if this genomic state is common to all choanoflagellates or if this is a derived state found in non-colony forming

species. Ongoing sequencing of a colony forming species of choanoflagellate (*Proterospongia*) may provide further insights into the evolution of homeodomain genes in the lineage leading to animals, as well as the evolution of multicellularity in general.

Chapter III contains material previously published in King, N., Westbrook, M. J., Young, S. L., Kuo, A., Abedin, M., Chapman, J., Fairclough, S., Hellsten, U., Isogai, Y., Letunic, I., Marr, M., Pincus, D., Putnam, N., Rokas, A., Wright, K. J., Zuzow, R., Dirks, W., Good, M., Goodstein, D., Lemons, D., Li, W., Lyons, J. B., Morris, A., Nichols, S., Richter, D. J., Salamov, A., Sequencing, J. G., Bork, P., Lim, W. A., Manning, G., Miller, W. T., McGinnis, W., Shapiro, H., Tjian, R., Grigoriev, I. V., and Rokhsar, D. (2008). The genome of the choanoflagellate Monosiga brevicollis and the origin of metazoans. *Nature* 451, 783-8.

MATERIALS AND METHODS

Phylogenetic analysis was performed on aligned sequences with Mr. Bayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) run with mixed amino acid and inverse gamma settings for 3 million iterations with a burnin of 75000. The unrooted dendrogram was made using FigTree (Andrew Rambaut, http://tree.bio.ed.ac.uk/). *Monosiga brevicollis* sequences were found by BLAST of the assembled genome at JGI. *Homo sapiens* sequences obtained from the NCBI homeodomain resource. Sponge sequences were found by BLAST of the *Reniera* (*Amphimedon queenslandica*) trace data from the NCBI trace archives. Fungal sequences were obtained from the Broad Institute (*Aspergillus nidulans*, *Coprinus cinerea*, *Cryptococcus neoformans*, *Neurospora crassa*, *Rhizopus oryzae*) and JGI (*Aspergillus niger*, *Laccaria bicolor*, *Phanerochaete chrysosporium*, *Phycomyces blakesleeanus*).

Figure 19. Alignment of Homeodomains from various opisthokonts. Highlighted bases are 30% conserved amongst all sequences. Amino acid positions given at the top of the alignment are based on sequence numbering in TALE superclass homeodomains. *Homo sapiens* sequences are labeled with HUMAN and include gene name labels. Sponge (*Amphimedon queenslandica* - formerly known as *Reniera*) sequences are labeled with Ren and include labels of homeodomain family grouping based on phylogenetic analysis in Figure 2. Fungal sequences are labeled with species abbreviations (*A.nid - Aspergillus nidulans*; *C.cin - Coprinus cinerea*; *C.neo - Cryptococcus neoformans*; *N.cra - Neurospora crassa*; *R.ory - Rhizopus oryzae*; *A.nig - Aspergillus niger*; *L.bic - Laccaria bicolor*; *P.chr - Phanerochaete chrysosporium*; *P.bla - Phycomyces blakesleeanus*).

```
                          10        20        30        40        50        60
                 ....|....|....|....|....|....|....|....|....|....|....|....|....
HESX_HUMAN       GRRPRTAFTQNQIEVLENVF---RVNCYPGIDIREDLAQKLNLEEDRIQIWFQNRRAKLKRSH
PMXA_HUMAN       QRRIRTTFTSAQLKELERVF---AETHYPDIYTREELALKIDLTEARVQVWFQNRRAKFRKQE
PMX1_HUMAN       QRRNRTTFNSSQLQALERVF---ERTHYPDAFVREDLARRVNLTEARVQVWFQNRRAKFRRNE
OTX1_HUMAN       QRRERTTFTRSQLDVLEALF---AKTRYPDIFMREEVALKINLPESRVQVWFKNRRAKCRQQQ
CRT1_HUMAN       KRRHRTTFTSLQLEELEKVF---QKTHYPDVYVREQLALRTELTEARVQVWFQNRRAKWRKRE
PRH1_HUMAN       RRRHRTTFSPVQLEQLESAF---GRNQYPDIWARESLARDTGLSEARIQVWFQNRRAKQRKQE
PIX1_HUMAN       QRRQRTHFTSQQLQELEATF---QRNRYPDMSMREEIAVWTNLTEPRVRVWFKNRRAKWRKRE
GSC_HUMAN        KRRHRTIFTDEQLEALENLF---QETKYPDVGTREQLARKVHLREEKVEVWFKNRRAKWRRQK
PAX6_HUMAN       LQRNRTSFTQEQIEALEKEF---ERTHYPDVFARERLAAKIDLPEARIQVWFSNRRAKWRREE
Ren_prd1         QRRHRTNFTSHQLEELEKAF---EKTRYPDVFMREELAMKISLTEARVQVWFQNRRAKWRKAE
Ren_prd2         SKRNRTTFTAHQLDELEMIF---RQTHYPDVLLREKLAQRIGLPESRVQVWFQNRRAKWRKRE
Ren_prd3         KRRYRTTFTSFQLRELEKAF---ERTHYPDVFTREDLANRVELTEARVQVWFQNRRAKWRKKE
Ren_prd4         QRRFRTTFTSYQLQELEAAF---AKTHYPDVFMREDLALRINLTEARVQVWFQNRRAKWRRAQ
Ren_prd5         PKRTRTAYSNSQLDQLELIF---ATTHYPDVFTREDLSRRLGIREDRIQVWFQNRRARFRKQE
Ren_prd6         IKKKRMTYTKQQKDALESYF---YQDSYPDTQARENMSEALGITPEKVQVWFQNRRAKCRKRE
Ren_prd7         PKKTRTQFSPKQLVYLEECF---LKNRFPSAKERESIAEELDLTTQHIQVWFQNRRAKHRRKS
LHX2_HUMAN       TKRMRTSFKHHQLRTMKSYF---AINHNPDAKDLKQLAQKTGLTKRVLQVWFQNARAKFRRNL
LH61_HUMAN       AKRARTSFTAEQLQVMQAQF---AQDNNPDAQTLQKLADMTGLSRRVIQVWFQNCRARHKKHT
ISL1_HUMAN       TTRVRTVLNEKQLHTLRTCY---AANPRPDALMKEQLVEMTGLSPRVIRVWFQNRRCKDKKRS
LHX3_HUMAN       AKRPRTTITAKQLETLKSAY---NTSPKPARHVREQLSSETGLDMRVVQVWFQNRRAKEKRLK
LMXB_HUMAN       PKRPRTILTTQQRRAFKASF---EVSSKPCRKVRELTLAAETGLSVRVVQVWFQNQRAKMKKLA
Ren_LIM1         KGKTRTSINPKQLIVLQATY---EKEPRPSRSMREELAAQTGLTAKVIQVWFQNRRSKDKKDG
Ren_LIM2         QPRIRTVLTEQQLQTLRSVY---QTNPRPDALLKEQLCELTGLSPRVIRVWFQNRRCKDKKAL
Ren_LIM3         QKRPRTTISQKQLDLLKTAY---CVSPKPSRHVRQELSDKTGLDMRVVQVWFQNKRAKDKRTK
OCT6_HUMAN       KRKKRTSIEVGVKGALESHF---LKCPKPSAHEITGLADSLQLEKEVVRVWFCNRRQKEKRMT
PO61_HUMAN       KRKRRTSFTPQAIEALNAYF---EKNPLPTGQEITEIAKELNYDREVVRVWFCNRRQTLKNTS
BR3A_HUMAN       KKRKRTSIAAPEKRSLEAYF---AVQPRPSSEKIAAIAEKLDLKKNVVRVWFCNQRQKQKRMK
OC3A_HUMAN       RKRKRTSIENRVRGNLENLF---LQCPKPTLQQISHIAQQLGLEKDVVRVWFCNRRQKGKRSS
Ren_POU1         HRKRRTTIGMSAKERLEQHF---QVQPKPSSSDITKVADSLNLDKEVIRVWFCNRRQREKRVR
Ren_POU2         RRRRRTAIPVQTKKQLLKEF---ENNPKPSVKALKALAEKLGIRFEVVRVWFCNKRAKKKAGK
Ren_POU3         KRKGRTAISVQTKKQLLKEF---ENDPKPSPKDLKAISEKLGIGFEVVRVWFCNKRAKRKAGK
Ren_POU4         KRKKRVVYTPHALSILNKYF---LKEPRPNRQIIEMVAEELDLLPEEVRVWFCNKRQKYKTSN
A.nid1           KNNKRQRATQDQLVLLEMEF---NKNPTPTAATRERIAQEINMTERSVQIWFQNRRAKIKMLA
N.cra1           KNQKRQRATQDQLTTLEMEF---NKNPTPTATVRERIAEEINMTERSVQIWFQNRRAKIKLLA
R.ory1           STKRRTHLSTEQVSLLESSF---NENSLPDSAVRSRLAQELSVTERTVQIWFQNRRAKEKKIK
P.bla3           AKPKRKRISPDQFRVLSDLF---EKTDTPNYELRERMAGRLNMTNREVQVWFQNRRAKATRAK
R.ory8           IRPKRKRITPNQLEVLTSIF---ERTKTPNYQLREHTAKELNMTNREVQVWFQNRRAKLNRKR
R.ory2           RTRKRTRATPEQLAILEKSF---NVNPSPNSRVREQLSLQLGMTERSIQIWFQNRRAKVKNQT
P.bla1           QPRKRTRASPEQLGILEKTF---NINPSPNNRVREQLSQQLSMSERSIQIWFQNRRAKVKNIA
R.ory3           PVRKRTRATADQLSVLEDTF---AMNVSPNSKLRKQLAEQLQMSERSIQIWFQNRRAKVKHMQ
R.ory4           DTKKRTRVTPGQLAILEETF---SMTATPDSKLRKQLAERLKMPERSIQIWFQNRRAKVKMLQ
L.bic3           EKRKRSRVTQEQLVHLEQYF---KADRCPTATRRREISEQLGMQERQTQIWFQNRRAKAKLQE
P.chr3           EQKKRGRVTPEQLAVLEAIF---AANRSPNAVRRKEISEQLGMTERQTQIWFQNRRAKEKHAG
R.ory5           EIKHRRRTSRAQLKVLEESF---SENPKPNATVRRILAQQLDMTPRGVQIWFQNRRAKAKLLR
R.ory6           ETKHRRRTSRGQVKILEKAF---HDNPKPNGRARERLAESLSMSPRGVQIWFQNRRAKANQQ
L.bic1           EVKHRKRTTSAQLKVLETVF---KRDTKPNASLRTELAAQLDMTARGVQVWFQNRRAKEKVKA
R.ory7           IKAKRKRASPSQLYILNQVF---QQTCFPSTELRIELGKRLGMSPRTVQIWFQNKRQSTRTKE
A.nid3           ARQKRRRTSPEDYAILEAEY---QRNPKPDKISRASIVSRVSLGEKEVQIWFQNRRQNDRRKS
N.cra3           PKGKRKRTTAKDKAILEAAY---NANPKPDKAARQDIVNRVSLNEKEVQIWFQNRRQNDRRKS
A.nid2           ENLSRPRLTKEQVETLEAQF---QAHPKPSSNVKRQLAQQTHLSLPRVANWFQNRRAKQQK
N.cra2           QTEPKPRLAKDEVELLEREF---AKNPKPNTSLKRELAEQMGVEVPRINNWFQNRRAKEKQMR
P.bla2           FHKKRMMLKPYQYKVLQDHF---SANPKPDARVYIDIASRLNVSITKIKNWFQNRRAKARKDK
P.bla4           KIKNRRRFSATEAALERRY---AEEQSPSQHVLQGLADQMSTPRKTITTWFQNRRAKYKRRS
P.bla5           EIKHRHRFSTSELELLEELY---RRHPRPSSSEKKAMAAKLDTTPGRVQVLQNRRAKERKAQ
R.ory9           PIKQRRRFSLEEAQFLEMEY---NNNPSPTQDKIQQIASKINSPRKVVTTWFQNRRAKNRRRS
R.ory10          PIRPRKRFTSNQIHLLEMEY---MKSDHPSRETKETLANQFKTSIRRIQIWFQNRRAKEKRGE
R.ory11          VARRRMRTSKEEMAVLDEYY---RKNPNPNQEEKKEIANLLKMGTKNVHFWFQNRRAKENKKK
A.nig1           KKMKRFRLTHNQTRFLMSEF---TRQAHPDAAHRERLSKEIGLTPRQVQVWFQNRRAKLKRLT
N.cra4           RKMKRFRLTHQQTRFLMSEF---AKQPHPDAAHRERLSREIGLSPRQVQVWFQNRRAKIKRLT
```

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C.neo3        QVKHRRRTTPEQLKVLEFWY---DINPKPDNQLREQLAAQLGMTKRNVQVWFQNRRAKMKGLA
C.neo4        FKSPRKRTNDVQLAMLSEVF---RRTQYPSTEERDELAKQLGMTSRSVQIWFQNRRRAVKVDQ
P.chr2        EKKPRHRMTDKQLERLEALY---QQDTHPTREQKQALGEEVGMDTRTVTVWFQNRRQLSKKNT
C.neo1        KMSPRKRFTIPQLQILEVQW---SNDISPPKVDRQRLAMWMGTRTKHVNIWFQNRRQYEKKVH
C.neo2        GCKVRRRFTKRELEALEVLW---SIAKSPSKYERQRLGAWLGVKTKHITVWFQNRRQEEKRYS
L.bic2        IRKKRKRVDAAQLKVLNETY---NRTAFPSTEERHTLAKALDMSARGVQIWFQNKRQSARQTN
C.cin1        SRRTRKRFTNTQLTMLENLF---HQTSHPSREEREAVAKAGQMEIKSVTIWFQNKRQTERKSQ
P.chr1        PKKPRHRHSAFQLAALNELY---ERDEHPPLEERTSLAERLGMEVKTVNAWFQNKRASTKKRS
P.chr4        VSYGRRRMQPEQLQALQTLY---DANTHPTKAQRMQLARELDLDLKSVNVWYQNKRRSMKKKL
P.bla6        IAKRRPRTTPEQSRILNTHF---ARNPVPSKNEIKLIAREVKIKPRSTHFWYQNKRASVKREG
CUT1_HUMAN    LKKPRVVLAPEEKEALKRAY---QQKPYPSPKTIEDLATQLNLKTSTVINWFHNYRSRIRREL
SIX1_HUMAN    GEETSYCFKEKSRGVLREWY---AHNPYPSPREKRELAEATGLTTTQVSNWFKNRRQRDRAAE
SIX3_HUMAN    GEQKTHCFKERTRSLLREWY---LQDPYPNPSKKRELAQATGLPTQVGNWFKNRRQRDRAAA
Ren_SIX       GEETSYCFKEKSRVVLRQWY---TKNAYPSPREKRQLAEQTGLITTQVSNWFKNRRQRDRAAE
PBX1_HUMAN    ARRRKRRNFNKQATEILNEYFYSHLSNPYPSEEAKEELAKKCGITVSQVSNWFGNKRIRYKKNI
Ren_PBX       ITRTRPVLTRNSLKVLEEWYECHLDHPYPTASQVEWLAQVSSLNTEQVKKWFGNKRSRSKNTR
IRX2_HUMAN    DPAYRKNATRDATATLKAWLNEHRKNPYPTKGEKIMLAIITKMTLTQVSTWFANARRRLKKEN
Ren_IRO1      SAAGSITRRMRNTAVLVKWIEDHQSNPYPTKAEKQYLAYYSGMNMTQLSTWFANARRRIKKIG
Ren_IRO2      VQLASSRRRRRDATHLIEWLDLHQGNPYPTRVEKEQLVVISGMNFKQLNDWFANARRNIRKVG
Ren_IRX3      EKGSSSPGSWRNTDVLALWITEHLQLPYPGKVEKQYLCFYSNMSMKQVSTYFANARR------
Ren_IRO4      CSNDMEARGSEGYKTSGEVVGAHQTNPYPTKAEKECLAECCGMSVKQLCTWFSNSRRQIRKLG
Ren_IRO5      YDSPRYKLTPERAIPLIKWFEEHKDHPYPSRHEKMLLCQSTQLTFTQVSTWFANARRRMKK--
TGIF_HUMAN    KRRRRGNLPKESVQILRDWLYEHRYNAYPSEQEKALLSQQTHLSTLQVCNWFINARRRLLPDM
MEI1_HUMAN    RHKKRGIFPKVATNIMRAWLFQHLTHPYPSEEQKKQLAQDTGLTILQVNNWFINARRRIVQPM
Ren_MEIS      TGKKREKTSPASQKLLKEWLFSHSRCPYPTEDDKQNLCRMTGLSLQQLNNWFINARRRILPQK
Mbrev_MEIS1   SRHCTKRFASSSIDTLKEWLFAHTDRPYPTDQDKTELMQQTGLDLMQINNWFINARRRLLVKV
Mbrev_MEIS2   NTGGRNNMPHEVTSRLKEWFFAHTSHPYPSEQKKRELASQCDLTLQQINNWFINARRRLLNRP
A.nid4        NRRRRGNLPKPVTEILKAWFHAHLDHPYPSEEDKQMLMSRTGLTINQISNWFINARRRHLPAL
N.cra5        KNKRRGNLPKEVTEKLYAWLYGHLNHPYPTEDEKQKMMRETNMQMNQISNWFINARRRKVPLL
P.bla7        KKRRRGNLPREVTEFLKHWLIQHKAHPYPSEKEKGDLACRTGLTVNQISNWFINARRRILQPM
L.bic4        PQRRKRGKLPKETTDYLKAWLHRHSDHPYPSEDEKKQLCHATGLSMSQVSNWMINARRRILAPA
N.cra6        ATKVNNRFSRESIKILKNWLSIHQKHPYPNDEEKEMLQKQTGLSKTQITGWLANARRRGKVM
A.nid5        ARKSSSRLSREAVRILKAWLNDHSDHPYPTEEEKEELKLRTGLKRTQITNWLANARRRGKIRP
A.nid6        DSKESKQFVRKGARVLRDWFYQNEHCPYPSEEEKARLAAETGFSRQRISTWFANARRHKQQK
```

Figure 19. continued
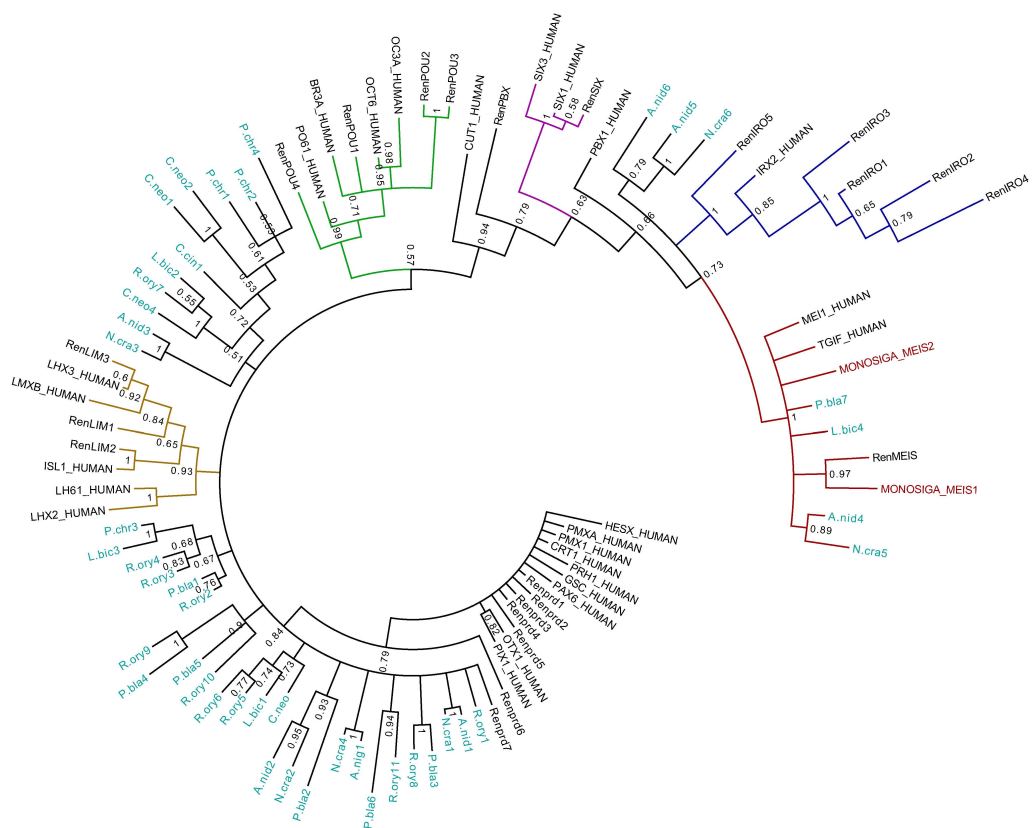
Figure 20. Phylogenetic tree of opisthokont homeodomains. An unrooted dendrogram of selected opisthokont homeodomains determined by Bayesian phylogenetic analysis. Nodes are labeled with posterior probabilities. Fungal gene labels are in light blue and those from *Monosiga* are labeled in red. Branches leading to MEIS class genes are highlighted in red, IRO class in dark blue, SIX class in purple, POU class in green, and LIM class in orange.

APPENDIX

Developmental expression and functional data for genes of the proximodistal appendage patterning network in appendages and anterior neurectoderm

Dll/Dlx

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *Dll* in distal domains of leg and antennal imaginal discs with a small proximal domain in developing legs. Distal leg and antennal truncations and defects in mutants (Abu-Shaar and Mann, 1998; Dong et al., 2001; Panganiban and Rubenstein, 2002; Wu and Cohen, 1999).

Anterior neurectoderm:
Dll expression (using a *Dll-lacZ* reporter line) in a complex pattern of the larval brain in the optic lobe (Kaphingst and Kunes, 1994). *Dll* has also been found to be expressed in anterior parts of the late embryonic brain, including glial cells in the protocerebrum, as well as other clusters of brain cells in the protocerebrum and deuterocerebrum, but not in tritocerebrum and posterior deuterocerebrum (Panganiban and Rubenstein, 2002; Sprecher et al., 2007).

Other:
Unknown

Beetle (*Tribolium castaneum*)

Appendage:
Expression of *Dll* starts prior to appendage budding and is in medial and distal domains of early leg buds and is downregulated later in development in medial domains as *dac* expression increases. This results in similar pattern to *Drosophila* where *Dll* in expressed in distal domains with secondary proximomedial stripe and partial overlap with *dac*. Fused segments and other distal appendage defects in mutants (Beermann et al., 2004; Beermann et al., 2001; Prpic et al., 2001).

Anterior neurectoderm:
Dll expression in the embryonic procephalon (Beermann et al., 2001).

Other:
Dll expression in small groups of PNS cells (Beermann et al., 2001).

Milkweed bug (*Oncopeltus fasciatus*)

Appendage:
Expression of *Dll* starts before appendage budding begins. As the buds develop expression becomes restricted to distal regions with a small stripe more proximally developing later in thoracic limbs reminiscent of the *Drosophila* leg imaginal disc expression patterns. Distal expansion of *dac* and *hth* expression domains with absent or defective distal appendage structures in RNAi embryos (Angelini and Kaufman, 2004).

Anterior neurectoderm:
Dll expression in the procephalon during embryogenesis (Rogers et al., 2002).

Other:
Dll expression in pleuropodial glands (Angelini and Kaufman, 2004).

Cricket (*Gryllus bimaculatus*)

Appendage:
Expression of *Dll* in distal domains of developing legs. Early expression mostly complementary to *hth* comprising medial and distal domains. After robust *dac* expression appears in medial regions *Dll* is restricted to distal regions and a secondary domain at the border of *hth* and *dac* expression. Partially overlapping expression with *dac* and *hth* reminiscent of expression in *Drosophila* legs (Inoue et al., 2002).

Anterior neurectoderm:
Dll expression in the procephalon during embryogenesis (Rogers et al., 2002).

Other:
Unknown

Millipede (*Glomeris marginata*)

Appendage:

Expression of *Dll* in the center of appendage domains before appendage budding and in distal domains of developing appendages. (Prpic and Tautz, 2003).

Anterior neurectoderm:
Dll expression in embryonic brain (Prpic and Tautz, 2003).

Other:
Dll expression in anal valves (Prpic and Tautz, 2003).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
Expression of *Dlx2*, *Dlx5*, and *Dlx6* in distal limb domains including AER cells with altered limb patterning and distal limb defects in *Dlx5/6* mutant mice (Panganiban and Rubenstein, 2002; Robledo et al., 2002).

Anterior neurectoderm:
Dlx1, *Dlx2*, *Dlx5*, and *Dlx6* are expressed in domains in the telencephalon and diencephalon of developing embryos. *Dlx5/6* mutants have anterior head defects including exencephaly (Panganiban and Rubenstein, 2002; Robledo et al., 2002).

Other:
Expression of *Dlx* genes in neural crest, branchial arches, olfactory placode, otic placode, optic cup, and retina (Panganiban and Rubenstein, 2002).

Chick (*Gallus gallus*)

Appendage:
Expression of *Dlx3* and *Dlx5* starts early in limb bud development in distal cells including the AER (Ferrari et al., 1995; Pera and Kessel, 1999).

Anterior neurectoderm:
Dlx orthologs expressed in various domains in developing telencephalon and diencephalon (Borghjid and Siddiqui, 2000; Fernandez et al., 1998; Pera and Kessel, 1999; Puelles et al., 2000; Zhu and Bendall, 2006).

Other:
Expression of *Dlx3* in olfactory placode, otic placode, branchial arches and posterior tailbud (Pera and Kessel, 1999).

Frog (*Xenopus laevis*)

Appendage:
Unknown

Anterior neurectoderm:
Expression of *X-dll3* and *X-dll4* in telencephalon and parts of the diencephalon during development (Papalopulu and Kintner, 1993).

Other:
Expression of *X-dll3* and *X-dll4* in neural crest and cement gland. Expression of *X-dll3* in olfactory placode and otic placode. Expression of *X-dll4* in developing eye (Papalopulu and Kintner, 1993).

Zebrafish (*Danio rerio*)

Appendage:
Expression of all 8 *Dlx* genes in apical ectodermal cells of pectoral fin buds as well as in the medial fin fold (Akimenko et al., 1994; Ellies et al., 1997; Zerucha and Ekker, 2000).

Anterior neurectoderm:
Expression of 5 *Dlx* orthologs in telencephalic and diencephalic domains during development (Akimenko et al., 1994; Ellies et al., 1997; Zerucha and Ekker, 2000).

Other:
Many, but not all, of the *Dlx* orthologs are expressed in olfactory placode, otic vesicle, and visceral arches (Akimenko et al., 1994; Ellies et al., 1997; Zerucha and Ekker, 2000).

Others

Tunicate (*Ciona intestinalis*)

Appendage:
N/A

Anterior neurectoderm:
Expression of *Dlx* in anterior neurectoderm in tailbud stage embryos (Harafuji et al., 2002).

Amphioxus (*Branchiostoma floridae*)

Appendage:
N/A

Anterior neurectoderm:
Dlx expression in neural plate and anterior ectoderm in neurula stage embryos in cells that will become part of the anterior cerebral vesicle in later stage embryos (Holland et al., 1996).

Other:
Dlx expression is fairly broad in the dorsal ectoderm of early embryos (Holland et al., 1996).

btd/*Sp8*

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *btd*:*Sp1* (*Drosophila Sp8* class genes) throughout entire appendage primordia in early embryogenesis and remaining throughout embryogenesis (Wimmer et al., 1996). During imaginal disc development expression is seen in most cells except the most proximal and most distal cells (Estella et al., 2003). Keilin's organs are lost or defective, and *Dll* expression is lost in antennal and thoracic appendage primordia in mutants (Estella et al., 2003).

Anterior neurectoderm:
Btd and *Sp1* expression in anterior procephalic neurectoderm starting in early embryogenesis (Wimmer et al., 1996).

Other:
Strong stripe of *btd* expression in intercalary/mandibular region of blastoderm embryos, where it functions as a gap gene (Cohen and Jurgens, 1990). Later expression in subsets of PNS and ventral nerve cord cells (Wimmer et al., 1996).

Beetle (*Tribolium castaneum*)

Appendage:
Expression of *Sp8* starts prior to formation of appendage buds, and in early limb buds is fairly uniform. As limb bud development progresses expression becomes

refined into multiple medial and distal stripes with the most proximal stripe being just proximal to the *Dll* expression domain. Appendage defects including missing or fused segments in RNAi embryos (Beermann et al., 2004).

Anterior neurectoderm:
Sp8 expression seen in head lobes (Beermann et al., 2004).

Other:
Unknown

Milkweed bug (*Oncopeltus fasciatus*)

Appendage:
Expression of *Sp8/9* is broad during earliest stages of appendage bud development encompassing the entirety of the buds. In later stages expression is restricted to two separate distal domains in the antennae and multiple stripes from proximal to distal in the legs. Fused segments and other appendage malformations seen in RNAi embryos with all appendages being shorter (Schaeper et al., 2009).

Anterior neurectoderm:
Sp8 is expressed in anterior neurectoderm in cells of the protocerebrum and cells that will give rise to the optic center (Schaeper et al., 2009).

Other:
Sp8 is expressed in a subset of cells in the ventral nerve cord (Schaeper et al., 2009).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
Expression of *Sp8* in limb buds and high levels in AER cells. Expression in the telencephalon. Loss of distal limb structures in mutants (Bell et al., 2003; Treichel et al., 2003).

Anterior neurectoderm:
Expression of *Sp8* in telencephalon, olfactory placode, and midbrain-hindbrain border. Forebrain developmental defects, exencephaly, and patterning defects in mutants (Bell et al., 2003; Treichel et al., 2003; Zembrzycki et al., 2007).

Other:

Expression of *Sp8* in neural tube, genital tubercle, nasal processes, and spinal cord (Bell et al., 2003; Treichel et al., 2003).

Chick (*Gallus gallus*)

Appendage:
Expression of *Sp8* in early limb field becoming confined to distal regions later in development with strong expression in the AER. Missing or malformed distal limb structures when dominant negative *Sp8* is expressed (Kawakami et al., 2004).

Anterior neurectoderm:
Expression of *Sp8* in anterior telencephalon and at the midbrain-hindbrain boundary (Kawakami et al., 2004).

Other:
Expression of *Sp8* in the neural tube (Kawakami et al., 2004).

Zebrafish (*Danio rerio*)

Appendage:
Expression of *Sp8* in distal regions of developing pectoral fins (apical fold). Reduced pectoral fin outgrowth in morpholino injected embryos (Kawakami et al., 2004).

Anterior neurectoderm:
Expression of *Sp8* in forebrain and at the midbrain-hindbrain boundary (Kawakami et al., 2004).

Other:
Expression of *Sp8* in the neural tube, neural keel, and tail bud (Kawakami et al., 2004).

Others

Tunicate (*Ciona intestinalis*)

Appendage:
N/A

Anterior neurectoderm:

Expression of *Sp8* in the anteriormost neurectoderm in tailbud stage embryos, likely corresponding to palps (Imai et al., 2004).

dac/Dach

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *dac* in medial cells of developing leg and antennal imaginal discs (Abu-Shaar and Mann, 1998; Dong et al., 2001; Lecuit and Cohen, 1997; Wu and Cohen, 1999). Shortened and fused medial segments in mutant legs (Mardon et al., 1994).

Anterior neurectoderm:
Expression of *dac* starts around stage 9 in medial protocerebral neurectoderm in the embryonic procephalon with expression expanding more posteriorly by stage 10. These cells will give rise to parts of the mushroom body and nearby brain (Noveen et al., 2000).

Other:
Expression of *dac* in the developing eye, and in many cells of the ventral nerve cord in late embryonic stages (Mardon et al., 1994).

Beetle (*Tribolium castaneum*)

Appendage:
Strong expression of *dac* begins proximally in early appendage budding which as the appendage buds grow corresponds to medial domains. Later in embryonic appendage development minor proximal domains appear (Beermann et al., 2004; Prpic et al., 2001). The major domains of *dac* expression overlap with the proximal region of *Dll* expression (Prpic et al., 2001).

Anterior neurectoderm:
Expression of *dac* starting in early embryonic development in multiple anterior head ectoderm domains (possibly future mushroom body) (Prpic et al., 2001).

Other:
Expression of *dac* in embryonic dorsal mesoderm, Malpighian tubules, posterior gut, a subset of cells in the ventral nerve cord, and eye primordia (Prpic et al., 2001).

Milkweed bug (*Oncopeltus fasciatus*)

Appendage:
Expression of *dac* in medial segments of developing legs and antennae with more broad expression in mandibular and maxillary appendages. Medial expansion of *Dll* expression domain in thoracic limbs and absent or defective medial appendage structures in *dac* RNAi embryos (Angelini and Kaufman, 2004).

Anterior neurectoderm:
Expression of *dac* seen in CNS (extent difficult to determine) (Angelini and Kaufman, 2004).

Other:
Expression of *dac* in embryonic eye domain (Angelini and Kaufman, 2004).

Cricket (*Gryllus bimaculatus*)

Appendage:
Strong expression of *dac* in medial domain starting in early leg development. In later embryonic stages expression corresponds to distal femur, tibia, and proximal tarsus. Partially overlapping expression with *dll* and *hth* reminiscent of expression in *Drosophila* legs (Inoue et al., 2002).

Anterior neurectoderm:
Expression of *dac* in early head neurectoderm and persisting during development in a subset of cells in the protocerebrum and deuterocerebrum. This expression likely corresponds to expression domain seen later in adult brains (Inoue et al., 2004).

Other:
Expression of *dac* in embryonic eye primordia starting in early development (Inoue et al., 2004).

Millipede (*Glomeris marginata*)

Appendage:
Expression of *dac* in rings around the center of appendage domains before appendage budding and in medial domains of developing appendages with lower level proximal expression and distal PNS cell expression starting in later limb development. (Prpic and Tautz, 2003).

Anterior neurectoderm:

Expression of *dac* in subset of protocerebrum and deuterocerebrum (Prpic and Tautz, 2003).

Other:
Expression of *dac* in anal valves, heart primordium, and a subset of cells in the ventral nerve cord (Prpic and Tautz, 2003).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
Expression of *Dach1* in limb buds begins around E11 in anterior and posterior medial domains, and by E14.5 is found in distal and medial regions of the limb bud (Caubit et al., 1999; Davis et al., 1999; Hammond et al., 1998). Expression of *Dach2* is largely similar to that of *Dach1* in limb buds (Davis et al., 2001).

Anterior neurectoderm:
Expression is dynamic in the developing CNS with strongest expression in the telencephalon and near the midbrain/hindbrain boundary (Caubit et al., 1999; Davis et al., 1999). Expression of *Dach2* is largely similar to that of *Dach1* except that *Dach2* is expressed in a subset of the telencephalon pattern of *Dach1* (Davis et al., 2001).

Other:
Expression in neural tube, eye, otic pit, trunk mesoderm, branchial arches, and mammary gland primordia (Caubit et al., 1999; Davis et al., 1999; Davis et al., 2001; Hammond et al., 1998).

Chick (*Gallus gallus*)

Appendage:
Expression in the developing limb buds, mainly in proximal and medial regions and strongest in the anterior, but also expressed in the AER (Heanue et al., 2002). Derepression of *Meis2* in limb buds when dominant negative *Dach* is expressed (Kida et al., 2004).

Anterior neurectoderm:
Expression of *Dach1* is dynamically expressed in many tissues of the developing brain with highest levels in the telencephalon and midbrain (Heanue et al., 2002).

Other:

Expression of *Dach1* in branchial arches, neural tube, somites, nasal placode, eye/retina, and large intestine (Heanue et al., 2002).

Zebrafish (*Danio rerio*)

Appendage:
Expression of all three *dach* genes in pectoral fin buds with *dachB* and *dachC* displaying somewhat distal expression and *dachA* expressed in anterior and posterior limb bud regions similar to mouse *Dach* (Hammond et al., 2002).

Anterior neurectoderm:
The three *Dach* orthologs display dynamic expression in the brain during development with expression seen in fore, mid and hindbrain at various points in development (Hammond et al., 2002).

Other:
Expression of *dachA* in the eye/retina and somites, *dachB* in the lateral line system, *dachC* in the pronephros and neural crest, and all three genes in the otic vesicle (Hammond et al., 2002).

Others

Tunicate (*Ciona intestinalis*)

Appendage:
N/A

Anterior neurectoderm:
Dach is expressed in the nerve cord just posterior to the head-tail border in tailbud stage embryos (Mazet et al., 2005).

Other:
Dach expression at the tailbud stage is detected in the buccal cavity, in head mesenchyme. At larval stages, Dach is expressed in mesenchyme associated with the palps as well as in posterior mesenchyme (Mazet et al., 2005).

Amphioxus (*Branchiostoma floridae*)

Appendage:
N/A

Anterior neurectoderm:

Expression of *Dach* in posterior cells of the cerebral vesicle as well as multiple more posterior neural domains (Candiani et al., 2003).

Other:
Expression of *Dach* in paraxial mesoderm in gastrula stage embryos corresponding to somites and pharynx endoderm in later stages. Expression also in the developing frontal eye and ventrolateral neurons in the nerve cord (Candiani et al., 2003).

hth/*Meis*

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *hth* starts early in embryogenesis and covers most of the blastoderm stage embryo except the anterior and posterior termini. In later embryonic stages *hth* expression disappears from many of the cells of the appendage primordia (Kurant et al., 1998; Rieckhof et al., 1997). In leg imaginal discs, *hth* is expressed in proximal cells, and *hth* mutants exhibit proximal leg defects (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999).

Anterior neurectoderm:
Expression of *hth* in posterior head neurectoderm but largely excluded from anterior procephalon (Kurant et al., 1998).

Other:
Expression of *hth* is very broad and includes most of trunk ectoderm, gastric caeca, midgut endoderm, eye imaginal disc, and ventral nerve cord (Kurant et al., 1998; Rieckhof et al., 1997).

Beetle (*Tribolium castaneum*)

Appendage:
Expression of *hth* in proximal cells of early limb buds and, as development progresses, in an additional small stripe of medial cells (Prpic et al., 2003).

Anterior neurectoderm:
Extent of *hth* expression not clear, but appears to be absent in anteriormost cells (Prpic et al., 2003).

Other:
Expression of *hth* in a large proportion of the body except for the distal limbs and anterior head (Prpic et al., 2003).

Milkweed bug (*Oncopeltus fasciatus*)

Appendage:
Expression of *hth* orthologs in body wall and proximal regions of developing appendages of *Oncopeltus* embryos. Absent or fused proximal appendage structures in *hth* RNAi embryos (Angelini and Kaufman, 2004).

Anterior neurectoderm:
Expression of *hth* seen in most of the developing head except for the anteriormost region (Angelini and Kaufman, 2004).

Other:
Expression of *hth* throughout most of the embryo except distal limbs and anterior head (Angelini and Kaufman, 2004).

Cricket (*Gryllus bimaculatus*)

Appendage:
Expression of *hth* in proximal regions of limb buds. Partially overlapping expression with *dll* and *dac* reminiscent of expression in *Drosophila* legs (Inoue et al., 2002).

Anterior neurectoderm:
Unknown

Other:
Unknown

Millipede (*Glomeris marginata*)

Appendage:
Expression of *hth* in developing appendages is proximal and medial in legs and relatively uniform in others, but *exd* is only expressed proximally. (Prpic and Tautz, 2003).

Anterior neurectoderm:
Expression of *hth* in subset of protocerebrum and deuterocerebrum (Prpic and Tautz, 2003).

Other:
Expression of *hth* in lateral plates and many other tissues (Prpic and Tautz, 2003).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
Expression of *Meis1* and *Meis2* in body wall and proximal regions of developing mouse limb buds (Mercader et al., 1999).

Anterior neurectoderm:
Expression of *Meis1* and *Meis2* in overlapping but distinct domains throughout much of the hindbrain and midbrain (Zhang et al., 2002).

Other:
Expression of *Meis1* and *Meis2* in the trunk mesoderm, developing eyes, and nasal placode (Mercader et al., 1999).

Chick (*Gallus gallus*)

Appendage:
Expression of *Meis1* and *Meis2* in body wall and proximal regions of developing chick limb buds and proximalization of limb structures in *Meis* overexpression embryos (Capdevila et al., 1999; Mercader et al., 1999).

Anterior neurectoderm:
Strong expression of *Meis1* and *Meis2* in the midbrain with *Meis1* expressed in subdomains of the diencephalon (hindbrain not analyzed) (Ferran et al., 2007).

Other:
Expression of *Meis1* and *Meis2* in the trunk mesoderm (Mercader et al., 1999).

Frog (*Xenopus laevis*)

Appendage:
Unknown

Anterior neurectoderm:
Expression of *Meis1* early in the midbrain primordia with low level expression in the neural plate. Later in development expression is in separate domains in the

midbrain and in the hindbrain (Maeda et al., 2001). Expression of *Meis3* in hindbrain in developing *Xenopus* embryos. When *Meis* is ectopically expressed, there are missing anterior structures, inhibited anterior neural marker expression and ectopic posterior neural marker expression (Salzberg et al., 1999).

Other:
Expression of *Meis1* in the optic cup, branchial arches, otic vesicle, olfactory placode, and spinal cord (Maeda et al., 2001).

Zebrafish (*Danio rerio*)

Appendage:
Expression of *meis1.1* in proximal cells of pectoral fin buds in later developmental stages (Waskiewicz et al., 2001).

Anterior neurectoderm:
Expression of *meis2* during early embryogenesis in the hindbrain primordia. Strong hindbrain expression persists throughout development and small domains in the telencephalon and midbrain appear during somitogenesis (Biemar et al., 2001; Zerucha and Prince, 2001). Expression of *meis1.1* and *meis2.2* in similar but distinct domains in forebrain, midbrain, and hindbrain over many developmental stages, with *meis3.1* restricted to the hindbrain (Waskiewicz et al., 2001).

Other:
Expression of *meis2* in spinal cord (stronger more anteriorly), somites, lateral mesoderm, heart tube, pharyngeal arches, and retina (Biemar et al., 2001; Zerucha and Prince, 2001). Expression of *meis1.1* in retina and *meis1.1*, *meis2.2*, and *meis3.1* expression in the spinal cord (Waskiewicz et al., 2001).

Others

Tunicate (*Ciona intestinalis*)

Appendage:
N/A

Anterior neurectoderm:
Expression of *Meis* comprises the posterior half of the ectoderm and sensory vesicle primordia during neurula stages. During tailbud stages expression is in the ventral anterior sensory vesicle and posterior head ectoderm (Imai et al., 2004).

Other:

Expression of *Meis* in anterior tail ectoderm during tailbud stages (Imai et al., 2004).

al/Alx/Arx

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *al* in embryonic appendage primordia and in distal most cells of leg and antennal discs as well as in cells which contribute to body wall/proximal most appendage (Campbell et al., 1993; Schneitz et al., 1993). Loss of distal most structures in legs and antennae of *al* mutants (Schneitz et al., 1993). Loss of *al* expression in *Dll* mutant leg discs (Campbell and Tomlinson, 1998). Reciprocal repression between *al* and *BarH1* in leg discs (Kojima et al., 2000).

Anterior neurectoderm:
Expression of *al* in anterior neurectoderm not observed in early embryos (Schneitz et al., 1993).

Other:
Expression of *al* in histoblasts, dorsal ectoderm, stomodaeum, and cells lining the amnioserosa in stage 11 embryos (Schneitz et al., 1993).

Beetle (*Tribolium castaneum*)

Appendage:
Expression of *al* at the distal tips of developing appendages in *Tribolium* with secondary proximal domain near the body wall and later in development an additional domain in a medial position. Missing or aberrant distal most structures of antennae and legs in *al* RNAi treated embryos (Beermann and Schroder, 2004).

Anterior neurectoderm:
No *al* expression detected

Other:
Expression of *al* in ventral histoblasts and dorsal ectoderm (Beermann and Schroder, 2004).

Cricket (*Gryllus bimaculatus*)

Appendage:
Expression of *al* is very similar to that of *Tribolium*. Expression starts in distal tips of early appendage buds as well as a secondary proximal domain (coxopodite) in many appendages, with additional medial domains developing later in embryogenesis in leg buds (Miyawaki et al., 2002).

Anterior neurectoderm:
Expression of *al* not detected

Other:
Expression of *al* in embryonic hindgut (Miyawaki et al., 2002).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
Expression of *Alx4* in anterior of early (E10.5) embryonic limb buds but by E15.5 expression is only in the distal tips of the forming digits (Qu et al., 1997).

Anterior neurectoderm:
Expression of *Arx* throughout much of the telencephalon and parts of the diencephalon (Miura et al., 1997).

Other:
Expression of *Alx-4* in embryos in anterior craniofacial mesenchyme and first branchial arch (Qu et al., 1997). Expression of *Arx* in optic areas, somites, and floor plate (Miura et al., 1997).

Chick (*Gallus gallus*)

Appendage:
Expression of *Alx4* broadly in early limb buds, becoming confined to mainly anterior proximal cells as development progresses (Takahashi et al., 1998).

Anterior neurectoderm:
Expression of *Arx* in dynamic domains of the telencephalon and diencephalon and reduced expression in *Dlx1/2* mutant embryos (Cobos et al., 2005).

Other:
Unknown

Frog (*Xenopus laevis*)

Appendage:
Unknown

Anterior neurectoderm:
Expression of *Arx* in dynamic domains of the telencephalon and diencephalon and forebrain defects in morpholino injected embryos (Seufert et al., 2005).

Other:
Expression of *Arx* in the floor plate (Seufert et al., 2005).

Zebrafish (*Danio rerio*)

Appendage:
Unknown

Anterior neurectoderm:
Expression of *Arx* in dynamic domains of the telencephalon and diencephalon (Miura et al., 1997).

Other:
Expression of *Arx* in somites and floor plate (Miura et al., 1997).

ap/Lhx2/9

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *ap* in fourth tarsal segment during pupal leg development (Cohen et al., 1992; Pueyo et al., 2000), and *ap* mutant flies are missing the fourth tarsal segment (Pueyo et al., 2000).

Anterior neurectoderm:
Expression of *ap* in the embryonic anterior neurectoderm/brain starting at stage 11 (Cohen et al., 1992).

Other:
Expression of *ap* in body wall muscle, pharynx, optic lobe, and a subset of cells in the ventral nerve cord (Rincon-Limas et al., 1999).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
Expression of *Lhx2* and *Lhx9* in distal regions of developing limb buds starting at E10 except for the AER (Retaux et al., 1999; Rincon-Limas et al., 1999). Severe malformations and truncations of distal limb structures in *Lhx2*/*Lhx9* double mutants and altered gene expression of limb patterning genes (e.g. FGF10) (Tzchori et al., 2009).

Anterior neurectoderm:
Expression of *Lhx2* and *Lhx9* in the developing brain starting at E10.5 in distinct but overlapping patterns in the telencephalon and diencephalon with additional expression seen in both midbrain and hindbrain (Retaux et al., 1999; Rincon-Limas et al., 1999). Forebrain malformations in *Lhx2* mutants (Porter et al., 1997). Patterning defects in telencephalon of *Lhx2* mutants (Bulchand et al., 2001).

Other:
Expression of *Lhx9* in gonad, pancreas, spinal cord and liver at E11.5 (Retaux et al., 1999). Expression of *Lhx2* in retina, olfactory epithelium, and neural tube (Rincon-Limas et al., 1999).

Chick (*Gallus gallus*)

Appendage:
Expression of *Lhx2* in distal regions of developing chick limb buds (but not the AER) starting at the beginning of limb bud development and continuing throughout limb development. Expression of dominant negative *Lhx2* results in truncated limbs and misexpression of limb patterning genes (Rodriguez-Esteban et al., 1998).

Anterior neurectoderm:
Expression of *Lhx2* in anterior CNS (Rodriguez-Esteban et al., 1998).

Other:
Expression of *Lhx2* in eye and nasal epithelia and spinal cord (Rodriguez-Esteban et al., 1998).

Zebrafish (*Danio rerio*)

Appendage:
Expression of *Lhx2* in developing pectoral fin buds, widespread early (26hpf) and later restricted to posterior regions (48hpf) (Seth et al., 2006).

Anterior neurectoderm:
Expression of *Lhx2* and *Lhx9* in forebrain of early embryos. Strong expression of *Lhx2* throughout development in the telencephalon and diencephalon with small segmentally repeated domains in the mid and hindbrain. *Lhx9* is expressed in a very similar pattern but is only in a subset of the telencephalon cells that *Lhx2* is found in (Ando et al., 2005). Forebrain axon guidance defects and altered forebrain patterning gene expression (e.g. *Dlx2*) seen in *Lhx2* mutants (Seth et al., 2006).

Other:
Expression of *Lhx2* in the optic vesicle (Seth et al., 2006).

B-H1/BarH

Arthropods

Fly (*Drosophila melanogaster*)

Appendage:
Expression of *BarH1* (*B-H1*) in cells corresponding to tarsal segment 4 in early 3^{rd} instar leg discs and expression in tarsal segments 4 and 5 during late 3^{rd} instar leg disc and pupal leg development. Fusion of tarsal segments in mutants. Expression in distal region (but not the most distal cells) of antennal discs and loss of distal structures in mutants. Reciprocal repression between *BarH1* and *dac* and between *BarH1* and *al*. Activation of *BarH1* by *Dll*. Activation of *ap* by *BarH1* (Kojima et al., 2000).

Anterior neurectoderm:
Expression of *BarH1* in anterior and central procephalic neurectoderm starting around stage 12 and continued expression in the brain in late stage embryos (Higashijima et al., 1992).

Other:
Expression in segment specific patterns in the PNS and posterior CNS and in developing eyes (Higashijima et al., 1992).

Vertebrates

Mouse (*Mus musculus*)

Appendage:
No expression detected for *Barhl1* and unknown for *Barhl2*.

Anterior neurectoderm:
Expression of *Barhl1* and *Barhl2* in the diencephalon starting at E9.5, and at later stages in the midbrain and hindbrain (Bulfone et al., 2000; Mo et al., 2004). *Barhl2* is expressed in the telencephalon starting at E14.5 (Mo et al., 2004).

Other:
Expression in spinal cord (both), inner ear (*Barhl1*), and retina (*Barhl2*) (Bulfone et al., 2000; Mo et al., 2004).

Frog (*Xenopus laevis*)

Appendage:
Unknown

Anterior neurectoderm:
Expression of two BarH genes (*XBH1* and *XBH2*) in separate domains of the diencephalon of postneurula stage embryos with lower level domains in the midbrain and hindbrain appearing later in development (Patterson et al., 2000).

Other:
Expression of *XBH1* in the developing retina (Patterson et al., 2000).

Zebrafish (*Danio rerio*)

Appendage:
Unknown

Anterior neurectoderm:
Expression of *barhl2* in diencephalon primordia of early embryos. Expression of the three BarH genes (*barhl1.1*, *barhl1.2* and *barhl2*) in partially overlapping domains of the diencephalon after 14 somite stage. All three are found in largely similar midbrain and hindbrain domains by 48hpf, with *barhl2* also being expressed in the telencephalon during middle embryonic stages (Colombo et al., 2006).

Other:
Expression of all three BarH genes in developing retina (Colombo et al., 2006).

REFERENCES

Abu-Shaar, M. and Mann, R. S. (1998). Generation of multiple antagonistic domains along the proximodistal axis during Drosophila leg development. *Development* **125**, 3821-30.

Abzhanov, A. and Kaufman, T. C. (1999). Novel regulation of the homeotic gene Scr associated with a crustacean leg-to-maxilliped appendage transformation. *Development* **126**, 1121-8.

Akimenko, M. A., Ekker, M., Wegner, J., Lin, W. and Westerfield, M. (1994). Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. *J Neurosci* **14**, 3475-86.

Alexiou, P., Maragkakis, M., Papadopoulos, G. L., Reczko, M. and Hatzigeorgiou, A. G. (2009). Lost in translation: an assessment and perspective for computational microRNA target identification. *Bioinformatics* **25**, 3049-55.

Allen, E., Xie, Z., Gustafson, A. M., Sung, G. H., Spatafora, J. W. and Carrington, J. C. (2004). Evolution of microRNA genes by inverted duplication of target gene sequences in Arabidopsis thaliana. *Nat Genet* **36**, 1282-90.

Ambros, V. (2003). MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* **113**, 673 - 6.

Ando, H., Kobayashi, M., Tsubokawa, T., Uyemura, K., Furuta, T. and Okamoto, H. (2005). Lhx2 mediates the activity of Six3 in zebrafish forebrain growth. *Dev Biol* **287**, 456-68.

Angelini, D. R. and Kaufman, T. C. (2004). Functional analyses in the hemipteran Oncopeltus fasciatus reveal conserved and derived aspects of appendage patterning in insects. *Dev Biol* **271**, 306-21.

Angelini, D. R. and Kaufman, T. C. (2005). Insect appendages and comparative ontogenetics. *Dev Biol* **286**, 57-77.

Aravin, A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J. and Tuschl, T. (2003 Aug). The small RNA profile during Drosophila melanogaster development. *Dev Cell* **5**, 337 - 50.

Bartel, D. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281 - 97.

Beermann, A., Aranda, M. and Schroder, R. (2004). The Sp8 zinc-finger transcription factor is involved in allometric growth of the limbs in the beetle Tribolium castaneum. *Development* **131**, 733-42.

Beermann, A., Jay, D. G., Beeman, R. W., Hulskamp, M., Tautz, D. and Jurgens, G. (2001). The Short antennae gene of Tribolium is required for limb development and encodes the orthologue of the Drosophila Distal-less protein. *Development* **128**, 287-97.

Beermann, A. and Schroder, R. (2004). Functional stability of the aristaless gene in appendage tip formation during evolution. *Dev Genes Evol* **214**, 303-8.

Bell, S. M., Schreiner, C. M., Waclaw, R. R., Campbell, K., Potter, S. S. and Scott, W. J. (2003). Sp8 is crucial for limb outgrowth and neuropore closure. *Proc Natl Acad Sci U S A* **100**, 12195-200.

Bender, W. (2008). MicroRNAs in the Drosophila bithorax complex. *Genes Dev* **22**, 14-9.

Bergson, C. and McGinnis, W. (1990). An autoregulatory enhancer element of the Drosophila homeotic gene Deformed. *Embo J* **9**, 4287-97.

Biemar, F., Devos, N., Martial, J. A., Driever, W. and Peers, B. (2001). Cloning and expression of the TALE superclass homeobox Meis2 gene during zebrafish embryonic development. *Mech Dev* **109**, 427-31.

Bier, E. (1997). Anti-neural-inhibition: a conserved mechanism for neural induction. *Cell* **89**, 681-4.

Bolinger, R. A. and Boekhoff-Falk, G. (2005). Distal-less functions in subdividing the Drosophila thoracic limb primordium. *Dev Dyn* **232**, 801-16.

Borghjid, S. and Siddiqui, M. A. (2000). Chick homeobox gene cDlx expression demarcates the forebrain anlage, indicating the onset of forebrain regional specification at gastrulation. *Dev Neurosci* **22**, 183-96.

Brend, T., Gilthorpe, J., Summerbell, D. and Rigby, P. W. (2003). Multiple levels of transcriptional and post-transcriptional regulation are required to define the domain of Hoxb4 expression. *Development* **130**, 2717-28.

Brennecke, J., Hipfner, D., Stark, A., Russell, R. and Cohen, S. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113**, 25 - 36.

Bulchand, S., Grove, E. A., Porter, F. D. and Tole, S. (2001). LIM-homeodomain gene Lhx2 regulates the formation of the cortical hem. *Mech Dev* **100**, 165-75.

Bulfone, A., Menguzzato, E., Broccoli, V., Marchitiello, A., Gattuso, C., Mariani, M., Consalez, G. G., Martinez, S., Ballabio, A. and Banfi, S. (2000). Barhl1, a gene belonging to a new subfamily of mammalian homeobox genes, is expressed in migrating neurons of the CNS. *Hum Mol Genet* **9**, 1443-52.

Burglin, T. R. (1997). Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res* **25**, 4173-80.

Butler, J. E. and Kadonaga, J. T. (2002). The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev* **16**, 2583-92.

Cameron, R. A., Rowen, L., Nesbitt, R., Bloom, S., Rast, J. P., Berney, K., Arenas-Mena, C., Martinez, P., Lucas, S., Richardson, P. M. et al. (2006). Unusual gene order and organization of the sea urchin hox cluster. *J Exp Zool B Mol Dev Evol* **306**, 45-58.

Campbell, G. and Tomlinson, A. (1998). The roles of the homeobox genes aristaless and Distal-less in patterning the legs and wings of Drosophila. *Development* **125**, 4483-93.

Campbell, G., Weaver, T. and Tomlinson, A. (1993). Axis specification in the developing Drosophila appendage: the role of wingless, decapentaplegic, and the homeobox gene aristaless. *Cell* **74**, 1113-23.

Candiani, S., Kreslova, J., Benes, V., Oliveri, D., Castagnola, P., Pestarino, M. and Kozmik, Z. (2003). Cloning and developmental expression of amphioxus Dachschund. *Gene Expr Patterns* **3**, 65-9.

Capdevila, J., Tsukui, T., Rodriquez Esteban, C., Zappavigna, V. and Izpisua Belmonte, J. C. (1999). Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol Cell* **4**, 839-49.

Carroll, S. B., Grenier, J. K. and Weatherbee, S. D. (2008). From DNA to diversity : molecular genetics and the evolution of animal design. Malden, Mass. u.a.: Blackwell.

Caubit, X., Thangarajah, R., Theil, T., Wirth, J., Nothwang, H. G., Ruther, U. and Krauss, S. (1999). Mouse Dac, a novel nuclear factor with homology to Drosophila dachshund shows a dynamic expression in the neural crest, the eye, the neocortex, and the limb bud. *Dev Dyn* **214**, 66-80.

Cerutti, H. and Casas-Mollano, J. A. (2006). On the origin and functions of RNA-mediated silencing: from protists to man. *Curr Genet* **50**, 81-99.

Cobos, I., Broccoli, V. and Rubenstein, J. L. (2005). The vertebrate ortholog of Aristaless is regulated by Dlx genes in the developing forebrain. *J Comp Neurol* **483**, 292-303.

Cohen, B., McGuffin, M. E., Pfeifle, C., Segal, D. and Cohen, S. M. (1992). apterous, a gene required for imaginal disc development in Drosophila encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev* **6**, 715-29.

Cohen, S. M., Bronner, G., Kuttner, F., Jurgens, G. and Jackle, H. (1989). Distal-less encodes a homoeodomain protein required for limb development in Drosophila. *Nature* **338**, 432-4.

Cohen, S. M. and Jurgens, G. (1990). Mediation of Drosophila head development by gap-like segmentation genes. *Nature* **346**, 482-5.

Colombo, A., Reig, G., Mione, M. and Concha, M. L. (2006). Zebrafish BarH-like genes define discrete neural domains in the early embryo. *Gene Expr Patterns* **6**, 347-52.

Davidson, E. H. and Erwin, D. H. (2006). Gene regulatory networks and the evolution of animal body plans. *Science* **311**, 796-800.

Davis, R. J., Shen, W., Heanue, T. A. and Mardon, G. (1999). Mouse Dach, a homologue of Drosophila dachshund, is expressed in the developing retina, brain and limbs. *Dev Genes Evol* **209**, 526-36.

Davis, R. J., Shen, W., Sandler, Y. I., Heanue, T. A. and Mardon, G. (2001). Characterization of mouse Dach2, a homologue of Drosophila dachshund. *Mech Dev* **102**, 169-79.

Derelle, R., Lopez, P., Guyader, H. L. and Manuel, M. (2007). Homeodomain proteins belong to the ancestral molecular toolkit of eukaryotes. *Evol Dev* **9**, 212-9.

Dong, P. D., Chu, J. and Panganiban, G. (2001). Proximodistal domain specification and interactions in developing Drosophila appendages. *Development* **128**, 2365-72.

Duboule, D. (1998). Vertebrate hox gene regulation: clustering and/or colinearity? *Curr Opin Genet Dev* **8**, 514-8.

Duboule, D. and Deschamps, J. (2004). Colinearity loops out. *Dev Cell* **6**, 738-40.

Ellies, D. L., Stock, D. W., Hatch, G., Giroux, G., Weiss, K. M. and Ekker, M. (1997). Relationship between the genomic organization and the overlapping embryonic expression patterns of the zebrafish dlx genes. *Genomics* **45**, 580-90.

Enright, A., John, B., Gaul, U., Tuschl, T., Sander, C. and Marks, D. (2003). MicroRNA targets in Drosophila. *Genome Biol* **5**, R1.

Estella, C., Rieckhof, G., Calleja, M. and Morata, G. (2003). The role of buttonhead and Sp1 in the development of the ventral imaginal discs of Drosophila. *Development* **130**, 5929-41.

Fernald, R. D. (2006). Casting a genetic light on the evolution of eyes. *Science* **313**, 1914-8.

Fernandez, A. S., Pieau, C., Reperant, J., Boncinelli, E. and Wassef, M. (1998). Expression of the Emx-1 and Dlx-1 homeobox genes define three molecularly distinct domains in the telencephalon of mouse, chick, turtle and frog embryos: implications for the evolution of telencephalic subdivisions in amniotes. *Development* **125**, 2099-111.

Ferran, J. L., Sanchez-Arrones, L., Sandoval, J. E. and Puelles, L. (2007). A model of early molecular regionalization in the chicken embryonic pretectum. *J Comp Neurol* **505**, 379-403.

Ferrari, D., Sumoy, L., Gannon, J., Sun, H., Brown, A. M., Upholt, W. B. and Kosher, R. A. (1995). The expression pattern of the Distal-less homeobox-containing gene Dlx-5 in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. *Mech Dev* **52**, 257-64.

Freeman, R. M., Jr., Wu, M., Cordonnier-Pratt, M. M., Pratt, L. H., Gruber, C. E., Smith, M., Lander, E. S., Stange-Thomann, N., Lowe, C. J., Gerhart, J. et al. (2008). cDNA sequences for transcription factors and signaling proteins of the hemichordate Saccoglossus kowalevskii: efficacy of the expressed sequence tag (EST) approach for evolutionary and developmental studies of a new organism. *Biol Bull* **214**, 284-302.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A. and Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* **34**, D140-4.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41**, 95–98.

Hammond, K. L., Hanson, I. M., Brown, A. G., Lettice, L. A. and Hill, R. E. (1998). Mammalian and Drosophila dachshund genes are related to the Ski proto-oncogene and are expressed in eye and limb. *Mech Dev* **74**, 121-31.

Hammond, K. L., Hill, R. E., Whitfield, T. T. and Currie, P. D. (2002). Isolation of three zebrafish dachshund homologues and their expression in sensory organs, the central nervous system and pectoral fin buds. *Mech Dev* **112**, 183-9.

Harafuji, N., Keys, D. N. and Levine, M. (2002). Genome-wide identification of tissue-specific enhancers in the Ciona tadpole. *Proc Natl Acad Sci U S A* **99**, 6802-5.

Heanue, T. A., Davis, R. J., Rowitch, D. H., Kispert, A., McMahon, A. P., Mardon, G. and Tabin, C. J. (2002). Dach1, a vertebrate homologue of Drosophila dachshund, is expressed in the developing eye and ear of both chick and mouse and is regulated independently of Pax and Eya genes. *Mech Dev* **111**, 75-87.

Higashijima, S., Michiue, T., Emori, Y. and Saigo, K. (1992). Subtype determination of Drosophila embryonic external sensory organs by redundant homeo box genes BarH1 and BarH2. *Genes Dev* **6**, 1005-18.

Holland, N. D., Panganiban, G., Henyey, E. L. and Holland, L. Z. (1996). Sequence and developmental expression of AmphiDll, an amphioxus Distal-less gene transcribed in the ectoderm, epidermis and nervous system: insights into evolution of craniate forebrain and neural crest. *Development* **122**, 2911-20.

Holley, S. A., Jackson, P. D., Sasai, Y., Lu, B., De Robertis, E. M., Hoffmann, F. M. and Ferguson, E. L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. *Nature* **376**, 249-53.

Huelsenbeck, J. P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754-5.

Imai, K. S., Hino, K., Yagi, K., Satoh, N. and Satou, Y. (2004). Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. *Development* **131**, 4047-58.

Inoue, Y., Mito, T., Miyawaki, K., Matsushima, K., Shinmyo, Y., Heanue, T. A., Mardon, G., Ohuchi, H. and Noji, S. (2002). Correlation of expression patterns of homothorax, dachshund, and Distal-less with the proximodistal segmentation of the cricket leg bud. *Mech Dev* **113**, 141-8.

Inoue, Y., Miyawaki, K., Terasawa, T., Matsushima, K., Shinmyo, Y., Niwa, N., Mito, T., Ohuchi, H. and Noji, S. (2004). Expression patterns of dachshund during head development of Gryllus bimaculatus (cricket). *Gene Expr Patterns* **4**, 725-31.

Kaphingst, K. and Kunes, S. (1994). Pattern formation in the visual centers of the Drosophila brain: wingless acts via decapentaplegic to specify the dorsoventral axis. *Cell* **78**, 437-48.

Kawakami, Y., Esteban, C. R., Matsui, T., Rodriguez-Leon, J., Kato, S. and Izpisua Belmonte, J. C. (2004). Sp8 and Sp9, two closely related buttonhead-like transcription factors, regulate Fgf8 expression and limb outgrowth in vertebrate embryos. *Development* **131**, 4763-74.

Keys, D. N., Lewis, D. L., Selegue, J. E., Pearson, B. J., Goodrich, L. V., Johnson, R. L., Gates, J., Scott, M. P. and Carroll, S. B. (1999). Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. *Science* **283**, 532-4.

Kida, Y., Maeda, Y., Shiraishi, T., Suzuki, T. and Ogura, T. (2004). Chick Dach1 interacts with the Smad complex and Sin3a to control AER formation and limb development along the proximodistal axis. *Development* **131**, 4179-87.

Kloosterman, W. P., Wienholds, E., de Bruijn, E., Kauppinen, S. and Plasterk, R. H. (2006). In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* **3**, 27-9.

Kojima, T. (2004). The mechanism of Drosophila leg development along the proximodistal axis. *Dev Growth Differ* **46**, 115-29.

Kojima, T., Sato, M. and Saigo, K. (2000). Formation and specification of distal leg segments in Drosophila by dual Bar homeobox genes, BarH1 and BarH2. *Development* **127**, 769-78.

Kosman, D., Mizutani, C. M., Lemons, D., Cox, W. G., McGinnis, W. and Bier, E. (2004). Multiplex detection of RNA expression in Drosophila embryos. *Science* **305**, 846.

Koziol, U., Lalanne, A. I. and Castillo, E. (2009). Hox genes in the parasitic platyhelminthes Mesocestoides corti, Echinococcus multilocularis, and Schistosoma mansoni: evidence for a reduced Hox complement. *Biochem Genet* **47**, 100-16.

Kraus, P. and Lufkin, T. (2006). Dlx homeobox gene control of mammalian limb and craniofacial development. *Am J Med Genet A* **140**, 1366-74.

Kurant, E., Pai, C. Y., Sharf, R., Halachmi, N., Sun, Y. H. and Salzberg, A. (1998). Dorsotonals/homothorax, the Drosophila homologue of meis1, interacts with extradenticle in patterning of the embryonic PNS. *Development* **125**, 1037-48.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853 - 8.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A. and Tuschl, T. (2003). New microRNAs from mouse and human. *RNA* **9**, 175 - 9.

Lai, E. C., Burks, C. and Posakony, J. W. (1998). The K box, a conserved 3' UTR sequence motif, negatively regulates accumulation of enhancer of split complex transcripts. *Development* **125**, 4077-88.

Lecuit, T. and Cohen, S. M. (1997). Proximal-distal axis formation in the Drosophila leg. *Nature* **388**, 139-45.

Lewis, B., Shih, I., Jones-Rhoades, M., Bartel, D. and Burge, C. (2003). Prediction of mammalian microRNA targets. *Cell* **115**, 787 - 98.

Lim, C. Y., Santoso, B., Boulay, T., Dong, E., Ohler, U. and Kadonaga, J. T. (2004). The MTE, a new core promoter element for transcription by RNA polymerase II. *Genes Dev* **18**, 1606-17.

Lim, L., Glasner, M., Yekta, S., Burge, C. and Bartel, D. (2003). Vertebrate microRNA genes. *Science* **299**, 1540.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J. and Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* **7**, 719-23.

Lowe, C. J., Tagawa, K., Humphreys, T., Kirschner, M. and Gerhart, J. (2004). Hemichordate embryos: procurement, culture, and basic methods. *Methods Cell Biol* **74**, 171-94.

Lowe, C. J., Wu, M., Salic, A., Evans, L., Lander, E., Stange-Thomann, N., Gruber, C. E., Gerhart, J. and Kirschner, M. (2003). Anteroposterior patterning in hemichordates and the origins of the chordate nervous system. *Cell* **113**, 853-65.

Maeda, R., Mood, K., Jones, T. L., Aruga, J., Buchberg, A. M. and Daar, I. O. (2001). Xmeis1, a protooncogene involved in specifying neural crest cell fate in Xenopus embryos. *Oncogene* **20**, 1329-42.

Mansfield, J. H., Harfe, B. D., Nissen, R., Obenauer, J., Srineel, J., Chaudhuri, A., Farzan-Kashani, R., Zuker, M., Pasquinelli, A. E., Ruvkun, G. et al. (2004). MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. *Nat Genet* **36**, 1079-83.

Mardon, G., Solomon, N. M. and Rubin, G. M. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. *Development* **120**, 3473-86.

Mazet, F., Hutt, J. A., Milloz, J., Millard, J., Graham, A. and Shimeld, S. M. (2005). Molecular evidence from Ciona intestinalis for the evolutionary origin of vertebrate sensory placodes. *Dev Biol* **282**, 494-508.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

McGlinn, E., Yekta, S., Mansfield, J. H., Soutschek, J., Bartel, D. P. and Tabin, C. J. (2009). In ovo application of antagomiRs indicates a role for miR-196 in patterning the chick axial skeleton through Hox gene regulation. *Proc Natl Acad Sci U S A* **106**, 18610-5.

Mercader, N., Leonardo, E., Azpiazu, N., Serrano, A., Morata, G., Martinez, C. and Torres, M. (1999). Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature* **402**, 425-9.

Minelli, A. (2000). Limbs and tail as evolutionarily diverging duplicates of the main body axis. *Evol Dev* **2**, 157-65.

Miura, H., Yanazawa, M., Kato, K. and Kitamura, K. (1997). Expression of a novel aristaless related homeobox gene 'Arx' in the vertebrate telencephalon, diencephalon and floor plate. *Mech Dev* **65**, 99-109.

Miyawaki, K., Inoue, Y., Mito, T., Fujimoto, T., Matsushima, K., Shinmyo, Y., Ohuchi, H. and Noji, S. (2002). Expression patterns of aristaless in developing appendages of Gryllus bimaculatus (cricket). *Mech Dev* **113**, 181-4.

Mo, Z., Li, S., Yang, X. and Xiang, M. (2004). Role of the Barhl2 homeobox gene in the specification of glycinergic amacrine cells. *Development* **131**, 1607-18.

Moczek, A. P., Rose, D., Sewell, W. and Kesselring, B. R. (2006). Conservation, innovation, and the evolution of horned beetle diversity. *Dev Genes Evol* **216**, 655-65.

Moczek, A. P. and Rose, D. J. (2009). Differential recruitment of limb patterning genes during development and diversification of beetle horns. *Proc Natl Acad Sci U S A* **106**, 8992-7.

Mungpakdee, S., Seo, H. C., Angotzi, A. R., Dong, X., Akalin, A. and Chourrout, D. (2008). Differential evolution of the 13 Atlantic salmon Hox clusters. *Mol Biol Evol* **25**, 1333-43.

Nelson, C. E., Morgan, B. A., Burke, A. C., Laufer, E., DiMambro, E., Murtaugh, L. C., Gonzales, E., Tessarollo, L., Parada, L. F. and Tabin, C. (1996). Analysis of Hox gene expression in the chick limb bud. *Development* **122**, 1449-66.

Noveen, A., Daniel, A. and Hartenstein, V. (2000). Early development of the Drosophila mushroom body: the roles of eyeless and dachshund. *Development* **127**, 3475-88.

Olson, E. N. (2006). Gene regulatory networks in the evolution and development of the heart. *Science* **313**, 1922-7.

Panganiban, G., Irvine, S. M., Lowe, C., Roehl, H., Corley, L. S., Sherbon, B., Grenier, J. K., Fallon, J. F., Kimble, J., Walker, M. et al. (1997). The origin and evolution of animal appendages. *Proc Natl Acad Sci U S A* **94**, 5162-6.

Panganiban, G. and Rubenstein, J. L. (2002). Developmental functions of the Distal-less/Dlx homeobox genes. *Development* **129**, 4371-86.

Papalopulu, N. and Kintner, C. (1993). Xenopus Distal-less related homeobox genes are expressed in the developing forebrain and are induced by planar signals. *Development* **117**, 961-75.

Pasquinelli, A. E., McCoy, A., Jimenez, E., Salo, E., Ruvkun, G., Martindale, M. Q. and Baguna, J. (2003). Expression of the 22 nucleotide let-7 heterochronic RNA throughout the Metazoa: a role in life history evolution? *Evol Dev* **5**, 372-8.

Pasquinelli, A. E., Hunter, S. and Bracht, J. (2005). MicroRNAs: a developing story. *Curr Opin Genet Dev* **15**, 200-5.

Patterson, K. D., Cleaver, O., Gerber, W. V., White, F. G. and Krieg, P. A. (2000). Distinct expression patterns for two Xenopus Bar homeobox genes. *Dev Genes Evol* **210**, 140-4.

Pera, E. and Kessel, M. (1999). Expression of DLX3 in chick embryos. *Mech Dev* **89**, 189-93.

Porter, F. D., Drago, J., Xu, Y., Cheema, S. S., Wassif, C., Huang, S. P., Lee, E., Grinberg, A., Massalas, J. S., Bodine, D. et al. (1997). Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development* **124**, 2935-44.

Prochnik, S. E., Rokhsar, D. S. and Aboobaker, A. A. (2007). Evidence for a microRNA expansion in the bilaterian ancestor. *Dev Genes Evol* **217**, 73-7.

Prpic, N. M., Janssen, R., Wigand, B., Klingler, M. and Damen, W. G. (2003). Gene expression in spider appendages reveals reversal of exd/hth spatial specificity, altered leg gap gene dynamics, and suggests divergent distal morphogen signaling. *Dev Biol* **264**, 119-40.

Prpic, N. M. and Tautz, D. (2003). The expression of the proximodistal axis patterning genes Distal-less and dachshund in the appendages of Glomeris marginata (Myriapoda: Diplopoda) suggests a special role of these genes in patterning the head appendages. *Dev Biol* **260**, 97-112.

Prpic, N. M., Wigand, B., Damen, W. G. and Klingler, M. (2001). Expression of dachshund in wild-type and Distal-less mutant Tribolium corroborates serial homologies in insect appendages. *Dev Genes Evol* **211**, 467-77.

Puelles, L., Kuwana, E., Puelles, E., Bulfone, A., Shimamura, K., Keleher, J., Smiga, S. and Rubenstein, J. L. (2000). Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. *J Comp Neurol* **424**, 409-38.

Pueyo, J. I. and Couso, J. P. (2005). Parallels between the proximal-distal development of vertebrate and arthropod appendages: homology without an ancestor? *Curr Opin Genet Dev* **15**, 439-46.

Pueyo, J. I., Galindo, M. I., Bishop, S. A. and Couso, J. P. (2000). Proximal-distal leg development in Drosophila requires the apterous gene and the Lim1 homologue dlim1. *Development* **127**, 5391-402.

Qu, S., Li, L. and Wisdom, R. (1997). Alx-4: cDNA cloning and characterization of a novel paired-type homeodomain protein. *Gene* **203**, 217-23.

Rehmsmeier, M., Steffen, P., Hochsmann, M. and Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. *Rna* **10**, 1507-17.

Retaux, S., Rogard, M., Bach, I., Failli, V. and Besson, M. J. (1999). Lhx9: a novel LIM-homeodomain gene expressed in the developing forebrain. *J Neurosci* **19**, 783-93.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of extradenticle requires homothorax, which encodes an extradenticle-related homeodomain protein. *Cell* **91**, 171-83.

Rincon-Limas, D. E., Lu, C. H., Canal, I., Calleja, M., Rodriguez-Esteban, C., Izpisua-Belmonte, J. C. and Botas, J. (1999). Conservation of the expression and function of apterous orthologs in Drosophila and mammals. *Proc Natl Acad Sci U S A* **96**, 2165-70.

Ringrose, L. and Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. *Annu Rev Genet* **38**, 413-43.

Robledo, R. F., Rajan, L., Li, X. and Lufkin, T. (2002). The Dlx5 and Dlx6 homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. *Genes Dev* **16**, 1089-101.

Rodriguez-Esteban, C., Schwabe, J. W., Pena, J. D., Rincon-Limas, D. E., Magallon, J., Botas, J. and Izpisua Belmonte, J. C. (1998). Lhx2, a vertebrate homologue of apterous, regulates vertebrate limb outgrowth. *Development* **125**, 3925-34.

Rogers, B. T., Peterson, M. D. and Kaufman, T. C. (2002). The development and evolution of insect mouthparts as revealed by the expression patterns of gnathocephalic genes. *Evol Dev* **4**, 96-110.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-4.

Ronshaugen, M., Biemar, F., Piel, J., Levine, M. and Lai, E. C. (2005). The Drosophila microRNA iab-4 causes a dominant homeotic transformation of halteres to wings. *Genes Dev* **19**, 2947-52.

Ruby, J. G., Stark, A., Johnston, W. K., Kellis, M., Bartel, D. P. and Lai, E. C. (2007). Evolution, biogenesis, expression, and target predictions of a substantially expanded set of Drosophila microRNAs. *Genome Res* **17**, 1850-64.

Ryan, J. F., Burton, P. M., Mazza, M. E., Kwong, G. K., Mullikin, J. C. and Finnerty, J. R. (2006). The cnidarian-bilaterian ancestor possessed at least 56 homeoboxes: evidence from the starlet sea anemone, Nematostella vectensis. *Genome Biol* **7**, R64.

Salzberg, A., Elias, S., Nachaliel, N., Bonstein, L., Henig, C. and Frank, D. (1999). A Meis family protein caudalizes neural cell fates in Xenopus. *Mech Dev* **80**, 3-13.

Schaeper, N. D., Prpic, N. M. and Wimmer, E. A. (2009). A conserved function of the zinc finger transcription factor Sp8/9 in allometric appendage growth in the milkweed bug Oncopeltus fasciatus. *Dev Genes Evol*.

Schneitz, K., Spielmann, P. and Noll, M. (1993). Molecular genetics of aristaless, a prd-type homeo box gene involved in the morphogenesis of proximal and distal pattern elements in a subset of appendages in Drosophila. *Genes Dev* **7**, 114-29.

Schwarz, D., Hutvágner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199 - 208.

Sempere, L. F., Cole, C. N., McPeek, M. A. and Peterson, K. J. (2006). The phylogenetic distribution of metazoan microRNAs: insights into evolutionary complexity and constraint. *J Exp Zool B Mol Dev Evol* **306**, 575-88.

Seth, A., Culverwell, J., Walkowicz, M., Toro, S., Rick, J. M., Neuhauss, S. C., Varga, Z. M. and Karlstrom, R. O. (2006). belladonna/(Ihx2) is required for neural patterning and midline axon guidance in the zebrafish forebrain. *Development* **133**, 725-35.

Seufert, D. W., Prescott, N. L. and El-Hodiri, H. M. (2005). Xenopus aristaless-related homeobox (xARX) gene product functions as both a transcriptional activator and repressor in forebrain development. *Dev Dyn* **232**, 313-24.

Shubin, N., Tabin, C. and Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. *Nature* **388**, 639-48.

Silver, S. J. and Rebay, I. (2005). Signaling circuitries in development: insights from the retinal determination gene network. *Development* **132**, 3-13.

Sokol, N. S., Xu, P., Jan, Y. N. and Ambros, V. (2008). Drosophila let-7 microRNA is required for remodeling of the neuromusculature during metamorphosis. *Genes Dev* **22**, 1591-6.

Sprecher, S. G., Reichert, H. and Hartenstein, V. (2007). Gene expression patterns in primary neuronal clusters of the Drosophila embryonic brain. *Gene Expr Patterns* **7**, 584-95.

Stark, A., Bushati, N., Jan, C. H., Kheradpour, P., Hodges, E., Brennecke, J., Bartel, D. P., Cohen, S. M. and Kellis, M. (2008). A single Hox locus in Drosophila produces functional microRNAs from opposite DNA strands. *Genes Dev* **22**, 8-13.

Tabin, C., Carroll, S. and Panganiban, G. (1999). Out on a Limb: Parallels in Vertebrate and Invertebrate Limb Patterning and the Origin of Appendages. *American Zoologist* **39**, 650-663.

Takahashi, M., Tamura, K., Buscher, D., Masuya, H., Yonei-Tamura, S., Matsumoto, K., Naitoh-Matsuo, M., Takeuchi, J., Ogura, K., Shiroishi, T. et al. (1998). The role of Alx-4 in the establishment of anteroposterior polarity during vertebrate limb development. *Development* **125**, 4417-25.

Treichel, D., Schock, F., Jackle, H., Gruss, P. and Mansouri, A. (2003). mBtd is required to maintain signaling during murine limb development. *Genes Dev* **17**, 2630-5.

Tzchori, I., Day, T. F., Carolan, P. J., Zhao, Y., Wassif, C. A., Li, L., Lewandoski, M., Gorivodsky, M., Love, P. E., Porter, F. D. et al. (2009). LIM homeobox transcription factors integrate signaling events that control three-dimensional limb patterning and growth. *Development* **136**, 1375-85.

Valentine, J. W. (2004). On the origin of phyla. Chicago: University of Chicago Press.

Valoczi, A., Hornyik, C., Varga, N., Burgyan, J., Kauppinen, S. and Havelda, Z. (2004). Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res* **32**, e175.

Waskiewicz, A. J., Rikhof, H. A., Hernandez, R. E. and Moens, C. B. (2001). Zebrafish Meis functions to stabilize Pbx proteins and regulate hindbrain patterning. *Development* **128**, 4139-51.

Wheeler, B. M., Heimberg, A. M., Moy, V. N., Sperling, E. A., Holstein, T. W., Heber, S. and Peterson, K. J. (2009). The deep evolution of metazoan microRNAs. *Evol Dev* **11**, 50-68.

Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S. and Plasterk, R. H. (2005). MicroRNA expression in zebrafish embryonic development. *Science* **309**, 310-1.

Wimmer, E. A., Frommer, G., Purnell, B. A. and Jackle, H. (1996). buttonhead and D-Sp1: a novel Drosophila gene pair. *Mech Dev* **59**, 53-62.

Woltering, J. M. and Durston, A. J. (2008). MiR-10 represses HoxB1a and HoxB3a in zebrafish. *PLoS One* **3**, e1396.

Wu, C. I., Shen, Y. and Tang, T. (2009). Evolution under canalization and the dual roles of microRNAs: a hypothesis. *Genome Res* **19**, 734-43.

Wu, J. and Cohen, S. M. (1999). Proximodistal axis formation in the Drosophila leg: subdivision into proximal and distal domains by Homothorax and Distal-less. *Development* **126**, 109-17.

Yekta, S., Shih, I. and Bartel, D. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**, 594 - 6.

Younossi-Hartenstein, A., Nassif, C., Green, P. and Hartenstein, V. (1996). Early neurogenesis of the Drosophila brain. *J Comp Neurol* **370**, 313-29.

Zakany, J. and Duboule, D. (2007). The role of Hox genes during vertebrate limb development. *Curr Opin Genet Dev* **17**, 359-66.

Zembrzycki, A., Griesel, G., Stoykova, A. and Mansouri, A. (2007). Genetic interplay between the transcription factors Sp8 and Emx2 in the patterning of the forebrain. *Neural Dev* **2**, 8.

Zerucha, T. and Ekker, M. (2000). Distal-less-related homeobox genes of vertebrates: evolution, function, and regulation. *Biochem Cell Biol* **78**, 593-601.

Zerucha, T. and Prince, V. E. (2001). Cloning and developmental expression of a zebrafish meis2 homeobox gene. *Mech Dev* **102**, 247-50.

Zhang, X., Friedman, A., Heaney, S., Purcell, P. and Maas, R. L. (2002). Meis homeoproteins directly regulate Pax6 during vertebrate lens morphogenesis. *Genes Dev* **16**, 2097-107.

Zhu, H. and Bendall, A. J. (2006). Dlx3 is expressed in the ventral forebrain of chicken embryos: implications for the evolution of the Dlx gene family. *Int J Dev Biol* **50**, 71-5.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**, 3406-15.