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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Evolution of *Hox* Gene Expression and Function
and the Effect on Limb Specification in Arthropods

A Dissertation submitted in partial satisfaction of
the requirement for the degree Doctor of Philosophy

in

Biology

by

Cheryl Chih-Jui Hsia

Committee in charge:

Professor William McGinnis, Chair
Professor Hopi Hoekstra
Professor James Kadonaga
Professor Alexandra Newton
Professor Steven Wasserman

2007

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Chair

University of California, San Diego

2007

To Kay and mizuko,

*I still think of you often
and wish you could have shared this journey with me.*

The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!' (I found it!)
but 'That's funny ...'

Isaac Asimov

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ABSTRACT OF THE DISSERTATION

Evolution of *Hox* Gene Expression and Function and the Effect on Limb Specification in Arthropods

by

Cheryl Chih-Jui Hsia

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor William McGinnis, Chair

Many common sets of genes are used to generate diverse animal body plans. One set of these genes are the *Hox* genes, transcription factors that specify segmental identity along the anterior-posterior axis of animals in early development. Many studies have been carried out to uncover how the evolution of *Hox* genes and *Hox* gene function may have precipitated the evolution of diverse body plans. I carried out functional assays in *Drosophila melanogaster* embryos to explore whether changes in protein sequence may have facilitated the divergence of six-legged insects from multi-legged crustaceans. I developed fluorescent immunohistochemistry and double *in situ*

hybridization methods in the crustacean, *Artemia franciscana*, to further clarify the HOX expression patterns in the trunk. From these studies, I found an example of a *Hox* gene capable of homeotic function, but inhibited from expression and presumably, inhibited from conferring segmental identity. This loss of segment identity function may contribute to the overall morphological body plan of *Artemia* to ensure the development of limbs throughout the trunk.

Chapter 1

Introduction

From a “simple” single cell, an animal extracts the necessary information and components to develop more cells and shape those cells into structures critical to its existence. An assortment of body plans has evolved to enable this survival. Yet, the basic sets of genetic instructions responsible for promoting these diverse body plans have been found to be relatively consistent in all animals (Carroll et al., 2004). One set of these genetic instructions are the *Hox* genes, a group of transcription factors which, in the early stages of development, act to confer segmental identity along the anterior-posterior axis.

Hox genes were first discovered in the fruit fly, *Drosophila melanogaster* as a cluster of eight genes that, when mutated, exhibited transformations of segments from one identity to another (Lewis, 1978). Later, *Hox* genes were found to be conserved between invertebrates and vertebrates and have been found in almost all metazoans (McGinnis and Krumlauf, 1992). The question of how the varied animal body plans evolved, then, would seem to be intimately tied to the evolution of *Hox* genes and *Hox* gene function. Attempts to answer this question have led to multitudes of studies on *Hox* gene expression, function and evolution in an effort to understand how the use of a common set of genes instructs the formation of diverse animal body plans.

Arthropods have been the focus of many of these studies on *Hox* gene evolution due to the immense morphological diversity found within the phylum and their relative amenability to experimentation. The ancestral arthropod *Hox* cluster is believed to have been made up of about 10 genes (Hughes and Kaufman, 2002a), although the exact makeup is the subject of ongoing deliberation. In extant

arthropods, few, if any, have maintained the complete set of ancestral *Hox* genes either in copy or function. For example, in the insect lineage, one homeotic gene evolved into a segmentation gene when the homeotic function was lost and a segmentation function acquired (Löhr et al., 2001).

One main focus of morphological variation in arthropods lies within the trunk. This variation includes differences in segment number and segment type i.e. segments bearing appendages of different kinds (antennae, feeding appendages, locomotory appendages, etc.) or none at all. *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*) and *abdominal-a* (*abd-A*) are the three *Hox* genes responsible for conferring segmental identity in the epidermis of the arthropod trunk in early development. In arthropods, they are sometimes expressed in discrete expression boundaries, other times in overlapping expression domains. In the insect, *Drosophila melanogaster*, all three trunk *Hox* genes are expressed in the epidermis of the pre-genital trunk in overlapping expression domains. *Antp* and *Ubx* are expressed in the thoracic segments that bear appendages including legs, wings and halteres. *Ubx* and *abd-A* are expressed in the abdominal segments that bear no appendages. *Artemia franciscana* are crustaceans, the sister subphyla to insects. They possess a pre-genital trunk with 11 pairs of appendages, followed by two genital segments and a post-genital trunk with no appendages. *Antp* transcript expression is expressed in the pre-genital region starting in the more posterior of two maxillary segments and extending posteriorly throughout the pre-genital trunk segments (Averof and Akam, 1995). However, *Antp* protein expression does not fully correlate with transcript expression and is only detected in

the posterior maxillary segment (Shiga et al., 2006). UBX protein expression in *Artemia*, starts at the first trunk segment extending posteriorly throughout the trunk (Shiga et al., 2006). Expression of ABD-A protein has been most clearly detected in the neuromeres that lie in the mesodermal layer (Averof and Akam, 1995) but has not been confirmed to be expressed in the pre-genital trunk epidermis in early development.

This work is an attempt to gain a deeper understanding of the evolution of transcription factors, in general, and more specifically how the *Ubx* and *abd-A* genes have evolved in arthropods to facilitate the disparate limb outcomes in the trunks of six-legged insects and multi-limbed crustaceans.

Chapter 2

Evolution of Transcription Factor Function

ABSTRACT

Functional assays in *Drosophila melanogaster* with orthologous transcription factors from other species suggest that changes in the protein-coding sequence may play a larger role in the evolution of transcription factor pathways than was previously believed. Interestingly, recent studies provide evidence that changes in transcription factor protein sequence can affect the regulation of only a subset of target genes, even in the same cells of a developing animal.

INTRODUCTION

Transcription factors regulate the spatio-temporal expression of thousands of genes to ensure the proper development and function of the organism. Until recently, studies on the evolution of transcription factor pathways have largely focused on the apparent functional changes in cis-regulatory elements (Tautz, 2000; Carroll, 2000). This focus is sensible, if one accepts the common belief that the functional evolution of transcription factors would result in alterations in the expression of many genes, and therefore likely to be disastrous for the organism. But with the knowledge that transcription factors have modular structures, sequence comparisons alone have permitted informed speculation on how their functions could be altered during evolution (Frigerio et al., 1986; Morgenstern and Atchley, 1999).

In the recent past, a few experimental studies, almost all on homeodomain proteins, have tested how sequence changes affect transcription factor functions in different animal lineages. The data suggest that changes in transcription factor coding sequence can alter the expression of a subset of downstream target genes without wholesale disruption of the entire downstream gene hierarchy. These changes can result in the acquisition of new functions by transcription factors while retaining their overall role (Ronshaugen, et al., 2002; Galant and Carroll, 2002), or in acquiring entirely new functions, which in some cases is correlated with the gain or loss of known cofactor interaction motifs (Stauber et al., 1999; Brown et al., 2001; Löhr et al., 2001; Alonso et al., 2001). This review will focus on the evolution of new functions by orthologous transcription factors in different lineages.

Evolutionarily conserved roles of transcription factors, just how conserved are they?

Despite their variations in shape and complexity, most bilateral animals possess a core set of transcription factors, inherited from a common ancestor at least 500 million years ago, with largely similar functions in controlling embryonic development. For example, in both vertebrates and many invertebrates, HOX transcription factors specify where different morphological features will develop on the head-tail axis of embryos, MEF-2 transcription factors specify skeletal muscle, Csx/Nkx2-5/Tinman transcription factors specify visceral mesoderm/heart, and Pax-6 transcription factors specify eye organ and anterior nervous system development (Veraksa et al., 2000). The amount of detailed functional variation within orthologous factors that conserve broadly similar roles has been carefully studied in only a few instances.

The most convincing experiments evaluating the extent of functional conservation versus functional variation of distantly related transcription factors would come from studies in which orthologous coding sequences from, for example a fly, precisely replaced endogenous coding sequences in a distantly related animal, for example a mouse. This would nearly guarantee that the distantly related ortholog would be expressed in the same patterns and levels as the endogenous gene. This experiment has not yet been accomplished in the precise manner described above, but it has been closely approached. For example, the *Drosophila engrailed* gene was introduced by homologous recombination into the locus of one of its mouse orthologs,

En1 (Hanks et al., 1998). In this instance, part of the endogenous *En1* gene remained at the recombined locus, and some *En1* regulatory sequences were not at the same positions relative to the promoter for *Drosophila engrailed* when compared to the endogenous *En1* promoter. Nevertheless, the *Drosophila engrailed* gene was expressed in mouse embryos in a pattern that was very similar to its *En1* ortholog. In homozygous mice with the *Drosophila engrailed* gene allele, the *En1* mutant phenotypes in the midbrain and cerebellum were largely rescued, and most mice survived to adulthood, compared to the early post-natal lethality observed in *En1* mutants. However, in the distal limbs, where EN1 function is required for normal dorsal-ventral polarity, the *Drosophila engrailed* allele did not rescue the *En1* mutant phenotype, even though expressed in the normal pattern at approximately normal levels. There have been other similar rescue experiments, for example, the chicken *Hoxb1* gene partially rescues the function of its *Drosophila* ortholog, *labial* (Lutz et al., 1996); and mouse *Pax3* partially rescues the function of a *Drosophila paired* mutation (Xue and Noll, 1996).

In such gene swapping experiments, evidence for near perfect rescue exists in only one case. Greer et al. (2000) tested the mouse *Hoxa3* and *Hoxd3* paralogs, which duplicated and began diverging 400 -500 million years ago, for their ability to rescue each other's function. The coding regions of these two *Hox* paralogs were precisely substituted for each other using homologous recombination. Even in their normal chromosomal locations, the two genes are expressed in nearly identical patterns, so that the differences in the expression patterns from the swapped genes apparently

amount to different transcript levels. The mice in which HOXA3 protein was expressed in the amounts characteristic of HOXD3 protein, and vice versa, had no apparent mutant phenotypes. However in this case, the two distantly related paralogs are likely to have been expressed in nearly identical patterns in the same animal for the past few hundred million years, and are required in tandem for the proper development of many structures. This may have imposed strong selection on the functions of HOXA3 relative to HOXD3 when compared to paralogs expressed in different patterns, or compared to orthologs in different phylogenetic lineages. In sum, the current gene replacement evidence for distant orthologs shows a great deal of functional conservation in some tissues, but in no case is the functions identical, and in some cases an orthologous gene provides no detectable rescue.

Depending on the phenotype(s) being scored, another complication exists in the interpretation of ortholog replacement experiments, or of experiments where a phylogenetically distant ortholog mimics a gain of function phenotype produced by an endogenous gene. If only one or two endogenous downstream genes need to be regulated to achieve a specific phenotype, inducing that phenotype with an orthologous factor does not provide strong support for extensive functional conservation. In at least two cases, there is evidence for such a scenario. It was shown that brief ectopic expression of mouse HOXB6 protein in a developing fly will partially transform the antennae into legs, quite similar to the phenotype seen after brief ectopic expression of its fly ortholog Antennapedia (Malicki et al., 1990). However, loss of function in the antenna primordia for any of three genes, *Distal-less*,

homothorax, or *spineless-aristapedia*, provides a similar phenotype, and it has been shown that the *homothorax* gene is indeed transcriptionally repressed in the antenna primordia by ectopic Antennapedia (Yao et al., 1999), as is *Distal-less* (C. Gross and W. McGinnis, unpublished data). So it seems likely that the ability of the HOXB6 ortholog to mimic ectopic Antennapedia function resides in its ability to repress transcription of one or more of these three genes. *Distal-less*, *homothorax*, and *spineless-aristapedia* all encode transcription factors, and their loss might result in a stable alteration of downstream gene cascades. In this context, it is relevant that recent studies have shown that, at least in some cell types during *Drosophila* development, a *Hox* gene need regulate only one target gene to accomplish its normal morphogenetic function in those cells (Lohmann et al., 2002; Brodu et al., 2002).

There is at least one more case where the conservation of orthologous transcription factor function may have been overestimated. When expressed in the imaginal disc primordia for adult *Drosophila* structures, mouse PAX6 protein can induce the development of ectopic *Drosophila* eyes, similar to its *Drosophila* ortholog Eyeless (Halder et al., 1995). Later studies have shown that *Drosophila* Eyeless activates the expression of other "eye" transcription factor genes *sine oculis*, *eyes absent*, and *dachshund*, which in certain combinations can induce ectopic eye development themselves (Chen et al., 1997; Pignoni et al., 1997). So the ability of PAX6 to induce ectopic eyes may not indicate extensive conservation of ability to regulate entire batteries of downstream genes, since in this assay it would need to activate only two downstream eye-promoting genes to mimic the function of its

ortholog Eyeless. Therefore, the apparent similarity of distantly related transcription factor functions in some ectopic expression assays (as well as in some gene swap assays) may not always indicate amazing functional conservation, since complex phenotypes such as a leg or an eye may require very few endogenous downstream genes to be activated or repressed.

Modest divergence in transcription factor functions in different lineages

We know from the fossil record that proto-hexapods with similar morphologies to modern silverfish appeared about 400 million years ago. Molecular evidence indicates that these early insect-like creatures branched from a crustacean lineage (Friedrich and Tautz, 1995). Two recent studies implicate mutations in HOX transcription factor sequence as contributing to the difference in limb number between multi-limbed crustaceans and hexapod insects. In *Drosophila*, the HOX proteins Ultrabithorax and Abdominal-A are required to repress limb development in the abdomen, whereas in the crustacean *Artemia*, their orthologs apparently do not repress limbs (Averof and Akam, 1995). One study found that the loss of serines and threonines from the C-terminal region of Ultrabithorax proteins during the transition from *Artemia* to insects could explain how Ultrabithorax evolved a limb repression function, and suggested that the loss of serine/threonine phosphorylation sites (which are not found in any insect Ultrabithorax orthologs, but in many multi-limbed arthropod Ultrabithorax orthologs) contributed to the macroevolutionary change in limb number between these two arthropod lineages (Ronshaugen et al., 2002).

Interestingly, this study suggested that a modulatory domain for an existing repression domain was evolving, not the repression domain itself. The *Artemia* Ultrabithorax protein, although unable to repress the limb promoting gene *Distal-less* in *Drosophila* embryos, still retained a transcription repressive function on another target gene, *Antennapedia*, also normally expressed in the epidermal limb primordia.

A complementary study involved swaps of protein domains between an onychophoran (a proto-arthropod) version of Ultrabithorax, and its *Drosophila* ortholog, followed by tests of limb repression in *Drosophila* embryos. The authors found evidence that a C-terminal region from *Drosophila* Ultrabithorax, which contained a glutamine/alanine rich motif present in all insect Ultrabithorax proteins, provides a transcriptional repression function that is missing from its onychophoran ancestor (Galant and Carroll, 2002). Some multi-legged arthropods conserve a portion of the glutamine/alanine rich motif, but none are as extensive as the motif found in insect Ultrabithorax orthologs. This study suggested that a new repression domain evolved in the insects, which was not present in ancestral versions of Ultrabithorax. So it seems that the evolution of amino acids involved in transcriptional repression functions (by mutations that abolished a repression modulatory domain and in the same region apparently generated an additional repression domain) may explain one step in the evolution of the hexapod body plan from multi-limbed arthropod ancestors.

Evolutionary variation in HOX protein repression function may have also occurred within the crustacean lineage. Studying the development of the crustacean *Daphnia*, Shiga et al (2002) found that diversification of anterior appendage

morphology and *Distal-less* expression patterns was associated with evolutionary variation in the expression pattern of the *Daphnia* Antennapedia HOX protein. When the *Daphnia* Antennapedia protein was tested in *Drosophila* embryos, it possessed a much stronger limb suppressing activity than its *Drosophila* cognate.

Another recently reported case of apparent transcription factor evolution was in the forkhead class protein FOXP2. Mutations in humans that reduce the dose of FOXP2 are correlated with defects in verbal articulation. Enard et al. (2002) analyzed the sequence of FOXP2 orthologs from gorillas, chimpanzees, orangutans, rhesus monkeys and mice, and found at some point after the divergence of the gorilla and human lineages, that a two amino acid sequence variation in FOXP2 was fixed and maintained only in the human lineage. The authors speculate that the two amino acid differences generated in the evolutionary branch leading to humans may have contributed to their acquisition of speech and language compared to other primates (Enard et al., 2002). This speculation seems highly unlikely given the current minimal evidence, but it isn't completely impossible.

Complete divergence of transcription factor function

Studies of Zerknullt (ZEN) and Bicoid (BCD) orthologs in different insect lineages indicate that they are homeodomain proteins that have undergone rapid evolution. In fact, the evolution of the *bcd* gene has been so rapid in cyclorrhaphan Dipterans that its derivation from *Hox* genes was unrecognized until recently. In *Drosophila*, *bcd* is maternally expressed and functions as an anterior determinant,

while *zen* is expressed in extra-embryonic tissues and functions in dorsal-ventral patterning. Although the *bcd* and *zen* genes of *Drosophila* are highly diverged in function, analysis of their counterparts in non-cyclorraphan Dipterans indicate that they are paralogs (Stauber et al., 1999) and that the complete divergence of *bcd* from *zen* in expression and function occurred within the Dipteran lineage (Brown et al., 2001; Stauber et al., 2002; Brown et al., 2002). The *zen* gene, in turn, has been found to be a derivative of a HOX3 ortholog. Phylogenetic analysis of the homeodomain sequences of Zen from *Tribolium* and *Schistocerca* groups them with *Hox3* genes, but they also share motifs outside the homeodomain with *Drosophila* ZEN (Falciani et al., 1996). Evidence for *zen*'s ancestral homeotic function lies in expression studies of the *Hox3/zen* ortholog in three chelicerates and a myriapod (Telford and Thomas, 1998; Damen and Tautz, 1998; Abzhanov et al., 1999; Hughes and Kaufman, 2002b). These studies show a HOX-like expression pattern for *Hox3/zen* along the anterior-posterior axis, suggestive of a role in segment identity. And like the non-Dipteran insect orthologs, chelicerate *Hox3/zen* genes group more closely, in phylogenetic analyses, to chordate HOX3 even though chelicerates are more closely related to *Drosophila* than to chordates (Telford and Thomas, 1998; Damen and Tautz, 1998; Abzhanov et al., 1999). Whether the *Hox3/zen* orthologs from these other arthropods also have extra-embryonic expression is not known. Functional assays to test for potential homeotic functions of the basal arthropod *Hox3/zen* orthologs are still needed, but it seems likely that *Hox3/zen* had a HOX-like function in the early arthropods, still retained in some lineages, but lost in the insect lineage (Figure 1-1). Taken together these

findings provide indirect evidence that cis-regulatory changes were involved in *Hox3/zen* functional evolution.

However, there is also evidence suggesting that protein sequence changes played a role in the transition of *Hox3* to *zen*, and from *zen* to *bcd*. The homeodomain of the HOX3 ortholog from *Cupiennius salei* (a spider) is located towards the C-terminus of the protein, a position more similar to the chordate HOX3 (and other HOX) proteins than to ZEN proteins where the DNA binding domain is located towards the N-terminus (Damen and Tautz, 1998). Additionally, the basal insect HOX3/ZEN protein acquired motifs outside the homeodomain similar to motifs in the *Drosophila* ZEN protein, and lost the YPWM motif conserved only in HOX proteins and their close relatives (Falciani et al, 1996). So it appears that there was a dramatic change in expression pattern that is correlated with an apparent loss of HOX-like protein function, as evidenced by loss and gain of protein motifs (Figure 1-1).

The divergence between ZEN and BCD protein coding sequences was also apparently accompanied by a divergence of cis-regulatory sequences during the period when cyclorraphan Dipterans evolved, although in this and the other cases described in this review it is unknown whether the novel expression pattern, or the protein sequence changes, evolved first. Rapid changes in BCD protein sequence, including a change within the homeodomain to acquire RNA binding ability (Niessing et al, 2000), resulted in a BCD that was highly divergent from ZEN, and to BCD eventually acquiring an anterior embryonic polarity function. Interestingly, BCD is a clear example of a protein acquiring a strikingly different DNA binding specificity from its

HOX ancestors, due to a substitution of lysine for glutamine at residue 50 of the homeodomain. This change does not result in a novel homeodomain binding specificity, as it converts BCD to the binding specificity of Orthodenticle/OTX family members, an ancient pre-existing anterior transcription factor in the homeodomain class (Laughon, 1991). This conversion of DNA binding specificity would have altered the expression of all BCD targets. It is possible that this dramatic change may not have been strongly selected against if BCD was only transiently present in early embryos, as it is in present day *Drosophila* embryos, and if it could bind new targets but initially had little regulatory effect on them. Then as other domains in BCD evolved, it gradually evolved the ability to regulate downstream enhancers that could bind lysine-50 homeodomains.

Evolution of transcription factor function linked to cofactor interactions

At present, the best evidence for a direct relationship between protein sequence changes and changes in cofactor interactions can be found in the evolution of the pair-rule gene, *fushi tarazu* (*ftz*). Like *zen* and *bcd*, *ftz* maps in the *Hox* cluster in insects, encodes a homeodomain protein, and is thought to have duplicated from a *Hox* gene and undergone a complete divergence in function (Telford, 2000).

Functional assays of FTZ protein orthologs in *Drosophila* (Löhr et al, 2001; Alonso et al., 2001) and expression studies of *ftz* genes in different arthropods (Hughes and Kaufman, 2002b; Telford, 2000; Mouchel-Vielh et al., 2002) indicate that *ftz* genes in different lineages has one, two or three different functions. CNS or

CNS-like expression is found for *ftz* orthologs in a myriapod (Hughes and Kaufman, 2002b), a crustacean (Mouchel-Vielh et al., 2002), and several insects (Brown et al, 1994; Dawes et al, 1994), suggesting that one ancient function of *ftz* might be in CNS development. Functional evidence for this conservation lies in the ability of *Schistocerca* FTZ to rescue *Drosophila ftz* CNS function via activation of *even-skipped* in neuronal precursors (Alonso et al., 2001). It is not yet known, though, which sequences are required for CNS function and whether they are conserved in the arthropods.

Löhr et al. (2001) tested the homeotic and segmentation abilities of FTZ proteins from *Drosophila*, *Tribolium* and *Schistocerca*. Their findings suggest that *Tribolium* FTZ and *Schistocerca* FTZ possess some HOX-like functions while *Drosophila* FTZ does not. Among the insects, with the exception of Abdominal-B orthologs, homeotic function correlates with the presence of a YPWM motif. The YPWM motif is part of an interaction domain for the HOX cofactor Extradenticle/PBX and is conserved in HOX proteins (Mann and Chan, 1996). FTZ protein sequences from crustaceans (Mouchel-Vielh et al., 2002) and chelicerates (Telford, 2000) reveal the presence of a slightly altered YPWM motif, FPWM (Figure 1-2), while sequence data from centipede FTZ, which exhibits HOX-like expression, is inconclusive as to the presence of a YPWM domain. Whether these FTZ proteins from non-insect arthropods also have HOX-like functions is unknown, although it seems very likely.

Assays for segmentation function in *Drosophila* embryos indicate that *Tribolium* FTZ possesses similar functions to *Drosophila* FTZ, but *Schistocerca* FTZ shows little or no segmentation function. Again, Löhr et al. (2001) found that segmentation ability is correlated with a specific protein sequence, an LXXLL motif. The LXXLL motif has been implicated as a domain for cofactors to facilitate interactions with nuclear hormone receptors (Heery et al., 1997), and FTZ protein has been shown to interact with the nuclear hormone receptor, FTZ -F1 through an LXXLL motif in order to carry out its segmentation function in flies (Yu et al., 1997; Guichet et al., 1997; Schwartz et al., 2001; Yussa et al., 2001). Löhr et al. (2001) further propose that competition of cofactors resulted in an exchange of homeotic function for segmentation function via the loss of the YPWM motif allowing *Drosophila* FTZ to act solely in segmentation during early embryogenesis.

The evolution of the FTZ protein in arthropods appears to be an example of a transcription factor that has altered the set of target genes it regulates from genes involved in segmental identity to genes involved in segmentation. Although not as well studied, this transition from segment identity gene to segmentation function also has occurred in the evolution of *even-skipped/Evx* orthologs. In many vertebrates, *Evx* genes map adjacent to the most posterior genes in HOX complexes, are expressed in the posterior termini of developing embryos, and are essentially *Hox* cluster genes required for posterior identities. *even-skipped* orthologs are also expressed at the posterior termini of some arthropod embryos (Hughes and Kaufman, 2002c). Thus, it seems likely that, like *ftz*, the *even-skipped*-like *Hox* genes diverged and acquired a

pair rule segmentation function sometime during insect evolution, while it retains an ancestral HOX function in many other taxa (Ferrier et al., 2001). To what extent the batteries of downstream segmentation and segment identity genes overlap is not yet known, but recent results suggest that some HOX proteins within the canonical complexes have roles in maintaining segment boundaries (Lohmann, et al., 2002; Gavalas et al., 1997), in addition to their well-known segment identity roles.

CONCLUSION

Cis-regulatory sequence mutations have been thought to be pre-eminent in the evolution of transcription factor pathways. In part, this is because cis-regulatory evolution was the only variation that was widely assayed until recently and in part because the conservation of transcription factor functions, although real, has been overemphasized. The advent of detailed assays for the role of homeodomain protein sequence variations, accompanied by comparison of ortholog expression patterns in different taxa has shown that both protein expression pattern changes and protein sequence mutations in these proteins have contributed to their functional evolution in developmental pathways. It will be fascinating to determine whether changes in expression pattern typically have occurred prior to protein functional divergence, or vice versa.

UPDATE

Evolutionary variation in HOX repression may have also occurred within the crustacean lineage. Studying the development of the crustacean, *Daphnia*, Shiga et al. (2002) found that the diversification of anterior appendage morphology and Distal-less expression patterns was associated with evolutionary variation in the expression pattern of the *Daphnia* Antennapedia HOX protein. When the *Daphnia* Antennapedia protein was tested in *Drosophila* embryos, it possessed a much stronger limb-suppressing activity than its *Drosophila* cognate.

ACKNOWLEDGEMENTS

The text of Chapter 2, in full, is a reprint of the material as it appears in Hsia C.C. and McGinnis, W. (2003). Evolution of Transcription Factor Function. *Current Opinion in Genetics and Development*, volume 13(2):199-206. I was the primary researcher and the co-author listed in this publication directed and supervised the research which forms the basis for this chapter.

FIGURES

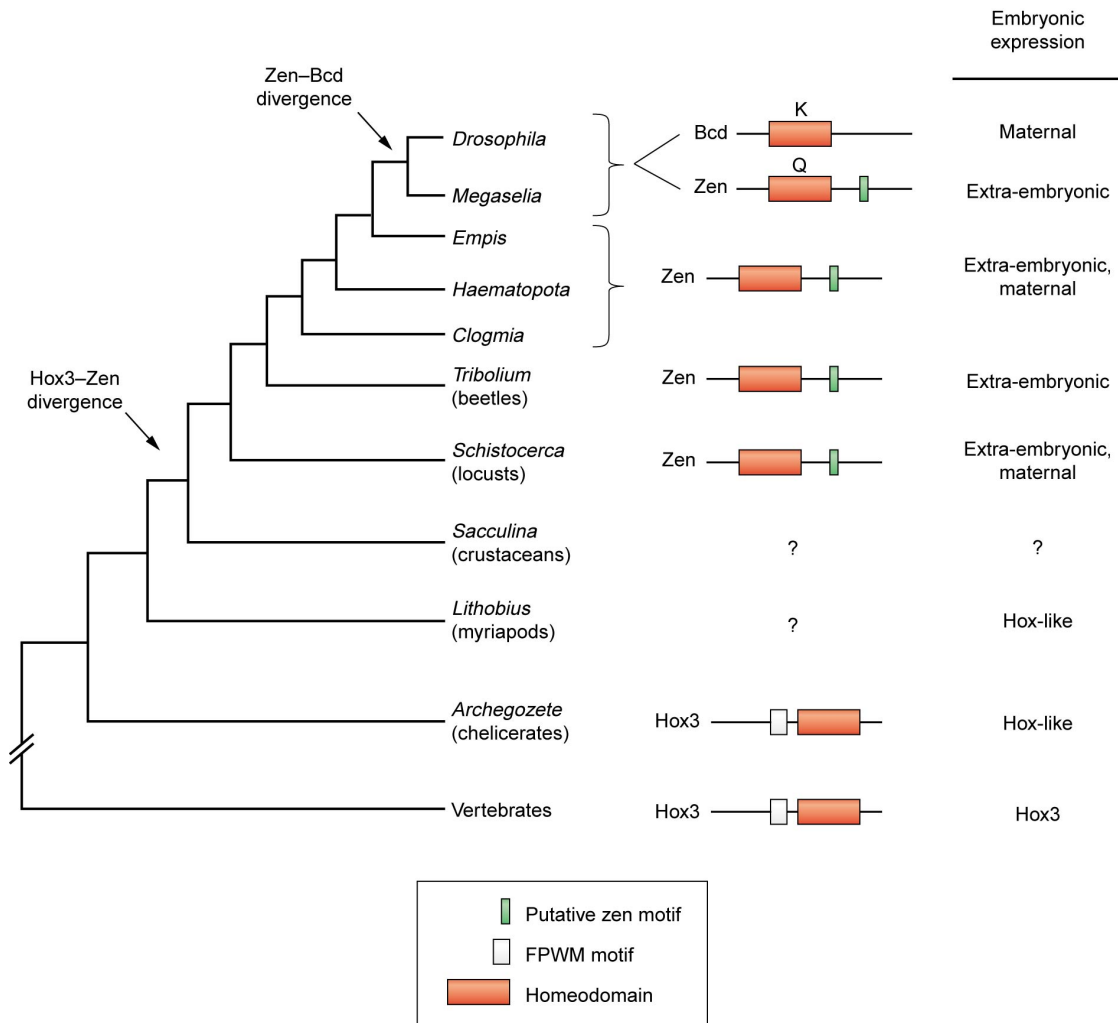


Figure 2-1: Evolution of Hox3/Zen/Bcd protein motifs and function. The embryonic expression patterns and protein diagrams denoting sequence motifs in Hox3/Zen orthologs are depicted on a phylogenetic tree. At some point before or during the early insect divergence, Hox3 lost its HOX-like expression and acquired extra-embryonic and maternal expression. During this period, the FPWM motif was lost from the Hox3 precursor, the homeodomain acquired a more N-terminal position and Zen-like motifs were acquired. The arrow marking the Hox3-Zen divergence indicates the most recent possible divergence of Zen from Hox3 based on known data, but this could have occurred prior to this time and after divergence of the clade which include myriapods, crustaceans, and insects. Then, in the insect lineage, maternal expression of *zen* was lost and the *bcd* gene was acquired through duplication and divergence of *zen* in the Drosophilids. The phylogenetic relationship of the animals is simplified and the length of lines is not indicative of relative divergence times

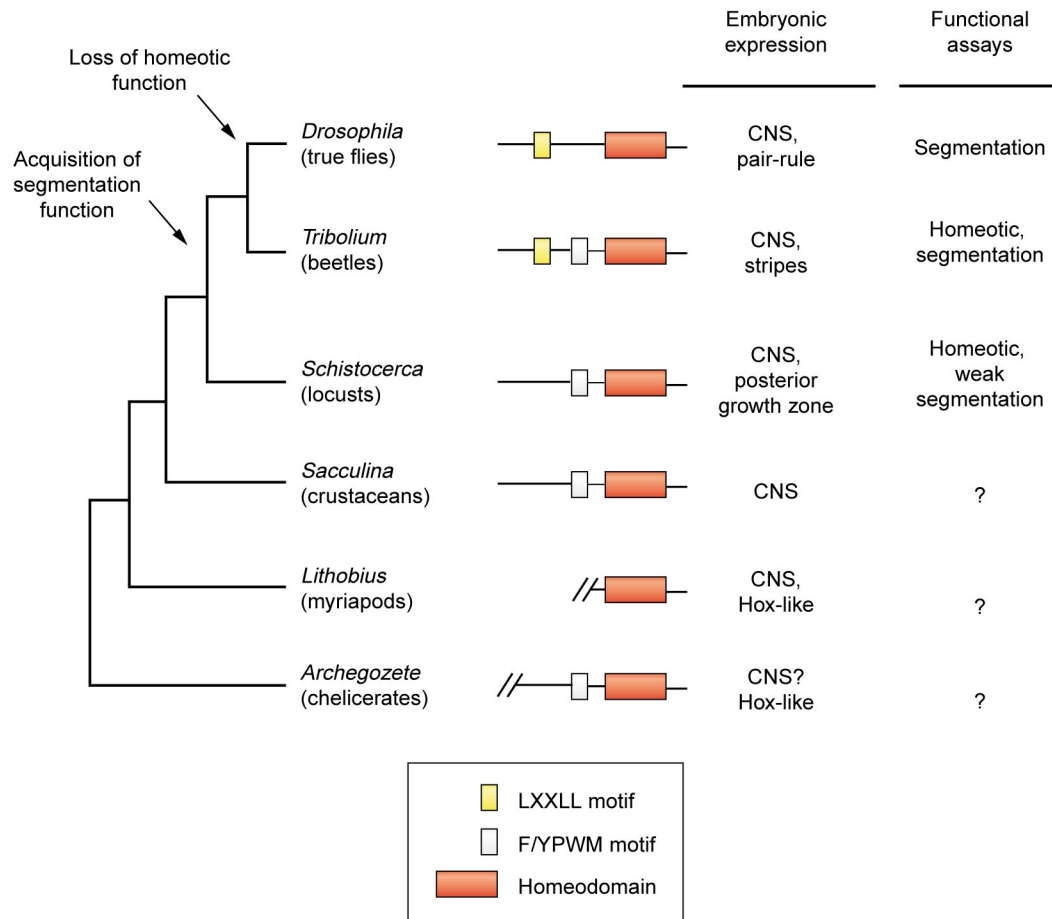


Figure 2-2: Evolution of Ftz protein function in arthropods. Expression studies of *ftz* in various arthropods suggest that HOX-like and CNS functions are ancestral functions of FTZ protein that are still retained in extant arthropods. Functional assays in *Drosophila* embryos show a correlation between the acquisition of segmentation function and the loss of homeotic function with the acquisition of an LXXLL motif and the loss of a YPWM motif, respectively, in the insect lineage.

Chapter 3

Modulating HOX protein function

INTRODUCTION

Cis-regulatory changes as the mechanism for the functional evolution of transcription factors has been the focus of many studies (Tautz, 2000; Carroll, 2000). The attention on cis-regulatory sequences is based on the long standing belief that alterations in protein sequence will likely incur pleiotropic effects to a degree that prevents viability. Certainly, some domains within transcription factors (e.g. DNA binding domains) can result in the widespread loss of target gene regulation, causing disastrous effects. But a recent functional study (Hittinger et al., 2005) on the repression function of the transcription factor Ultrabithorax (UBX) found that when a motif outside the DNA binding domain is deleted, pleiotropic phenotypes are incurred but the overall animal is viable. Although transcription factors are the regulators of a multitude of target genes, different parts of the protein may modulate different subsets of target genes to varying degrees. Functional studies in *Drosophila* embryos of orthologous HOX proteins suggest that UBX can evolve to lose the ability to regulate certain target genes without changing its ability to regulate other sets of target genes (Grenier and Carroll, 2000). Moreover, ectopic expression studies of UBX showed that a specific function carried out by a transcription factor may involve multiple motifs acting additively within the protein (Tour et al., 2005) indicating that a change or loss of one motif does not necessarily result in drastic alterations in function.

Evidence is starting to emerge that changes within protein sequences may play a larger role in the evolution of transcription factor function than previously believed. The segmentation gene *fushi tarazu* has long been suspected to have evolved from a

tandem duplication of a *Hox* gene and is still found within the *Hox* cluster. Insect studies by Löhr, et al (2001) suggest that protein sequence changes within the lineage facilitated the functional evolution of *fushi tarazu* from a protein with a HOX-like function to one with a segmentation function. Specifically, the presence and absence of specific motifs implicated in cofactor interactions can be associated with the presence and absence of HOX or segmentation functions.

A similar finding, but affecting a less extreme morphological alteration, can be found in an earlier study of the *Hox* gene *Ultrabithorax (Ubx)* in arthropods. Changes within the protein sequence of UBX may have modified its function from a transcription factor specifying trunk segments with limbs to one specifying trunk segments without limbs (Ronshaugen et. al. 2002). In the model proposed by Ronshaugen et al., the insect UBX protein lost residues capable of being phosphorylated within the C-terminus, including a serine within a consensus CK2 site. This loss allowed UBX to repress limbs in the abdominal trunk segments, contributing to the overall hexapod body plan. In the crustacean lineage, these residues were retained and act to inhibit the repression of limb-promoting genes during embryonic development.

That the loss of phosphorylation sites, specifically serines within CK2 sites, results in a modified HOX function has also been seen in studies of the *Antennapedia (Antp)* gene in *Drosophila melanogaster*. Mutations within CK2 phosphorylation sites allowed the ANTP protein to acquire the ability to repress larval limb structures known as Keilin's organs (Jaffe and Mann, 1997). *Antp*, along with *Ubx* and

abdominal-a (*abd-A*), is a central class *Hox* gene. These three genes are usually expressed in arthropod trunk segments and are believed to have arisen from two tandem duplications. The first duplication produced *Antp* and a central *Hox* gene that underwent another duplication to produce *Ubx* and *abd-A*. The six-legged insects subsequently diverged from their crustacean-like multi-limbed ancestors about 400 million years ago. It has been proposed that this divergence is due, in part, to the evolution of the three trunk *Hox* genes (Averof and Akam, 1995).

Additionally, Ronshaugen et al. (2002) found a correlation between the presence of serine/threonine residues and UBX proteins known or thought to lack the ability to repress limbs. It is possible that the ancestral trunk *Hox* gene possessed inhibitory phosphorylation sites that were lost within both the UBX and ABD-A proteins in the insect lineage. These losses permit the proteins to repress limb-promoting targets and prevent limb formation in the insect abdomen.

To further explore the role of protein sequence changes in transcription factor evolution, I set out to test two predictions from these studies of *Ubx* and *Antp* and the effect of CK2 phosphorylation on limb repression function. First, that an inverse correlation exists between the ability of the *Ubx* orthologs to be phosphorylated at serine/threonine residues within the C-terminus and the functional ability of trunk *Hox* genes to repress limbs. Second, that as a paralog to *Ubx* and *Antp*, *abd-A* orthologs of insects and crustaceans may also exhibit disparate abilities in limb function and work in concert with *Ubx* to specify differences in trunk morphology that ultimately distinguish the insect body plan from that of crustaceans.

Assays scoring the presence or absence of Keilin's organs, the embryonic structures formed from the field of cells marked by the expression of *Distal-less*, a limb promoting gene, were used to determine limb repression ability in a similar manner as described in Ronshaugen et al. (2002). In the course of carrying out these experiments, limitations of Keilin's organ repression assays became apparent, particularly in assaying the functional ability of mutant exogenous proteins. However, when there were large differences in Keilin's organ ability, conclusions about limb repression ability could still be made.

RESULTS

Ectopically expressed *Artemia* UBX is phosphorylated *in vivo*

If modulation of *Artemia* UBX function on limb promoting genes in *Drosophila* embryos involves phosphorylation of *Artemia* UBX, then *Artemia* UBX would be expected to be phosphorylated *in vivo*. To test if *Artemia* UBX is phosphorylated in *Drosophila* embryos, Western blot analysis was carried out. Whole embryo extracts of *Drosophila* embryos ectopically expressing *Artemia* UBX-HA were treated without calf intestinal phosphatase (CIP), with CIP and with CIP in the presence of an inhibitor (NaVO₃). As a control, proteins from w¹¹¹⁸ embryos, the parental line, were also extracted. The blot was then probed with α -HA to detect only the induced protein. The presence of a second, faster-migrating band is visible after treatment with CIP but only one band is detected when no CIP was added or when CIP was added in the presence of an inhibitor (Figure 3-1).

***in vitro* phosphorylation of *Artemia* UBX occurs mainly within a C-terminal CK2 consensus site**

Though Western Blots indicate that *Artemia* UBX is phosphorylated *in vivo*, the exact sites of phosphorylation are unknown. Previous studies (Ronshaugen et al., 2002) point to a consensus CK2 site within the C-terminus as one likely site for phosphorylation. *in vitro* CK2 kinase assays were performed to determine if CK2 is capable of phosphorylating *Artemia* UBX within this C-terminus consensus CK2 site and to determine if *in vitro* CK2 phosphorylation is inversely correlated with the

ability to repress limbs. Eight GST-fusion proteins of selected *Ubx* wildtype, chimeras, truncations and mutations were constructed. Six were proteins tested by Ronshaugen et al. in *in vivo* ectopic expression studies (Figure 3-2). Additionally, a GST-*Artemia* UBX fusion protein with all serine/threonine residues in the C-terminus mutated to Ala (*Art* UBX1-7) and a GST-*Artemia* UBX fusion protein with only the Ser within the CK2 consensus site mutated to Ala (*Art* UBX7) were expressed.

The amount of CK2 phosphorylation was determined relative to *Artemia* UBX, taking into account the amount of protein loaded and taking the average from two separate experiments. The *in vitro* CK2 assays showed that *Artemia* UBX but not *Drosophila* UBX was strongly phosphorylated by CK2 (Figure 3-2). GST alone was not phosphorylated by CK2 (data not shown). A truncated *Artemia* UBX missing the C-terminus (*Art* UBX CΔ) showed a 10% phosphorylation relative to *Artemia* UBX. There was very little difference in phosphorylation between the GST fusion protein with all C-terminal serines/threonines mutated to alanine (*Art* UBX 1-7) and that of the GST fusion protein with only the serine within the CK2 consensus site mutated to alanine (*Art* UBX 7). Both were phosphorylated at ~1% or less relative to wildtype protein. As was the fusion protein in which all but the threonine at position 6 were mutated to alanine (*Art* UBX 1-5,7). When the C-terminus of the *Drosophila* UBX protein was replaced with the *Artemia* C-terminus, the protein was heavily phosphorylated by CK2 at a level equivalent to *Artemia* UBX.

Keilin's organ repression assays have limitations

To try to deduce the relationship between limb repression ability and *in vitro* phosphorylation by CK2, the limb repression ability of wildtype *Artemia* UBX ectopically expressed in *Drosophila* embryos was compared to those of *Artemia* UBX mutated in either only the serine within the CK2 consensus site or all the serine/threonine residues within the C-terminus. These tests were initially performed using one line per construct and scoring for the presence of Keilin's organs, the larval equivalent of limbs. Analysis of the limb repression data from these lines and the *in vitro* CK2 data showed an inverse correlation between limb repression ability and phosphorylation at the C-terminus by CK2 (data not shown). However, when additional lines of these constructs, including wildtype *Artemia Ubx*, were tested to confirm these results, different transgenic lines expressing the same ectopic protein at similar protein levels displayed different abilities of Keilin's organ repression (Figure 3-3 A). Some of the differences in Keilin's organ repression varied 50% or more even though different lines carrying the same construct showed a difference of protein expression of around 15% with overlapping error bars.

These disparate results are, in part, explained by work in our lab using ectopic expression assays with *Drosophila* UBX using a range of protein expression. In these assays, the relationship between the level of protein expression and Keilin's organ repression ability was sigmoidal in shape, suggestive of a cooperative dose response (Tour et al., 2005). In the steep part of this curve for *Drosophila* UBX, the output of Keilin's organ repression varied between 20% and 80% within a difference of 20% in

protein expression levels. The calculated error of protein expression levels for *Artemia* UBX wildtype and mutant lines fell between 12% to 18%. If the differences in limb repression of *Artemia* UBX mutants are subtle or moderate, it may not be detectable. Additionally, Tour et al. (2005) determined that scoring the number of sensory hairs instead of Keilin's organs served as a more precise phenotypic marker for expression of *Distal-less (Dll)*, a gene required for limb formation (Cohen et al., 1989) and a target of *Drosophila Ubx* for inhibiting limb formation (Vachon et al., 1992).

***Drosophila* ABD-A has strong limb repression ability, *Artemia* ABD-A shows a dose dependent response**

Keeping in mind the limitations of the Keilin's organ repression assays and the need to survey a range of protein expression levels, Keilin's organ repression ability of *Artemia* ABD-A was tested in *Drosophila* embryos to determine if the *Artemia* ortholog exhibited a significantly different ability to repress Keilin's organs compared to *Drosophila* ABD-A. Many insect studies have shown the ability of ABD-A to act as a limb repressor. *Drosophila* embryos mutant in *Ubx* and *abd-A* show ectopic expression of *Dll* (Vachon et al., 1992) and ectopic formation of Keilin's organs throughout the limb-less abdominal segments (Lewis, 1978). Heat shock treatments to drive ubiquitous expression of *abd-A* in *Drosophila* results in the loss of Keilin's organs in the thoracic segments (Sánchez-Herrero et al., 1994). In *Tribolium*, *abd-A* mutant embryos ectopically express DLL in the abdominal segments (Lewis et al.,

2000). And in *Oncopeltus*, RNAi experiments show that both UBX and ABD-A repress limbs in their respective expression domains (Angelini et al., 2005).

Clues to the limb repression ability of ABD-A in crustaceans are less clear and are based mostly on expression data. In *Artemia*, the *abd-A* expression domain lies within the pre-genital trunk segments that bear limbs, although it is detected only from the mid-larval stage forward when limb formation has already initiated and only in the neuromeres (Averof and Akam, 1995). Many expression studies in arthropods utilize the pan-species antibody, FP6.87 which detects a conserved epitope shared by both UBX and ABD-A (e.g. Averof and Patel, 1997; Zheng et al., 1999; Abzhanov and Kaufman, 2000a; Abzhanov and Kaufman, 2000b; Blin et al., 2003). Though this conservation allows for the use of this antibody in a wide array of arthropods, it cannot distinguish between the two HOX proteins to be certain if one or both are present. In *Artemia*, FP6.87 staining shows trunk expression, although neither transcript nor protein expression of *abd-A*, specifically, in the epidermis of early stage *Artemia* development before *Dll* initiation (Panganiban et al., 1995) in the pre-genital trunk has been verified. In studies of *abd-A* transcript expression, myriapod *abd-A* is found to be co-expressed with *Ubx* (Hughes and Kaufman, 2002b; Brena et al., 2006). And in barnacles (cirripede crustaceans), it is believed that the *abd-A* gene has been lost, which is correlated with the absence of an abdomen in these animals (Mouchel-Vielh et al., 1998; Blin et al., 2003).

Drosophila ABD-A does not seem to display the concentration dependency for limb repression ability observed for *Drosophila* UBX. In ectopic expression assays,

Keilin's organs are fully repressed even when protein is expressed at ~40% of endogenous levels (Figure 3-4A). Ectopic expression of full-length, wildtype *Artemia* ABD-A could not be detected by antibody staining with an *Artemia* ABD-A specific antibody. However, a truncated protein (*Artemia* ABD-A CΔ) missing the last 54 amino acids could be ubiquitously expressed in *Drosophila* embryos and this truncated protein was used to assess limb repression ability. *Art* ABD-A CΔ was also capable of fully repressing Keilin's organs, but only when expressed at levels near endogenous ABD-A levels. When *Art* ABD-A CΔ protein expression levels fell below 100% of endogenous levels, however, limb repression ability was reduced. Although not enough protein concentrations below 100% endogenous levels were tested to generate a complete profile, *Art* ABD-A CΔ displayed a protein concentration-Keilin's organ repression ability relationship reminiscent to that of *Drosophila* UBX.

Truncated *Artemia* ABD-A shows homeotic function in *Drosophila* embryos.

Cuticle analysis of *Drosophila* embryos ectopically expressing *Art* ABD-ACA showed many phenotypes similar to embryos ectopically expressing *Drosophila* ABD-A. Embryos from the different lines ectopically expressing *Art* ABD-ACA and ranging in protein expression from 30% to 90% of endogenous levels were analyzed in parallel with the standard line ectopically expressing *Drosophila* ABD-A at 98% of endogenous levels. In all *Artemia* ABD-ACA lines, cuticles displayed a transformation of thoracic denticle belts toward abdominal A1 identity (Figure 3-4B), head involution defects, formation of denticle belts in the head and repression of the

T1 beard (data not shown), all hallmarks of ectopic *Drosophila* ABD-A expression (Sánchez-Herrero et al., 1994) and homeotic ability. The degree of the phenotypes, however, varied but many showed a correlation with the amount of protein expression (data not shown).

DISCUSSION

Keilin's organ repression ability is not a sufficient measure of limb repression function, unless a large degree of limb repression function is present

The original assessment by Ronshaugen et al. (2002) that limb repression abilities of *Drosophila* UBX and *Artemia* UBX were different was based on comparisons of Keilin's organ repression at a single level of protein expression and on the presence/absence of Keilin's organs. When multiple lines of each construct over a range of protein expression levels are compared, a more complex relationship between protein expression levels and Keilin's organ formation emerged. For *Drosophila* UBX, the protein concentration-Keilin's organ repression relationship was sigmoidal, with Keilin's organs almost completely repressed when the protein levels are above 70% of endogenous and very weakly repressed when protein levels are below 40% (Tour et al., 2005). The switching point between Keilin's organ formation and Keilin's organ repression occurs over a range of protein expression levels between 30% and 60% of endogenous expression (Tour et al., 2005).

Artemia UBX, on the other hand, seems to display a narrower region for this switching point, perhaps over as little as a 15% protein concentration change (Figure 3-3). But, the exact nature of the relationship between protein expression levels within this region requires further evaluation. And, while expression of *Artemia* UBX at levels above endogenous *Drosophila* UBX results in an increased ability to repress Keilin's organs, full repression of Keilin's organs was not observed.

The Keilin's organ repression dependence on protein expression levels for mutated *Artemia Ubx* lines is ambiguous and is further obscured by the finding that different *Art* UBX 1-7 lines can exhibit similar protein expression levels but large differences in Keilin's organ repression ability (Figure 3-3). When one *Art* UBX 1-7 line was assayed a second time for Keilin's organ repression to test the reproducibility of Keilin's organ repression ability, a difference of 20% in Keilin's organ repression was found although protein concentration levels were calculated to be similar (data not shown). Whether this disparity in Keilin's organ repression is due to variability in protein expression calculations or scoring Keilin's organs or other factors is not clear. These data, however, call into question the utility of scoring the presence/absence of Keilin's organs as an indicator of limb repression function, when the differences in repression strength may be small. The best conclusion to be made from the limb repression data of the *Artemia Ubx* lines carrying serine to alanine mutations within the C terminus is that simply scoring the presence or absence of Keilin's organs in *Drosophila* embryos is not a sufficiently precise measure of limb repression ability for these exogenous proteins.

An attempt to quantify UBX protein expression and *Dll* transcript expression on a cell by cell basis at stage 11 when *Dll* expression is initiating has proven to be more informative. In these experiments by Paré and McGinnis (unpublished), *Dll* expression is almost completely repressed by *Drosophila* UBX but *Artemia* UBX had an intermediate ability to repress *Dll* expression. Compared to wildtype *Dll* transcript levels, *Dll* was expressed at ~40% of wildtype in embryos ectopically expressing

Artemia UBX indicating a ~60% repression of *Dll* transcripts by *Artemia* UBX.

Based on Tour et al.'s (2005) characterization of *Drosophila* UBX and the effect of protein concentration on *Dll* expression, this is comparable to the effect of *Drosophila* UBX expressed at just over 30% of endogenous levels. At this level of expression, *Drosophila* UBX was capable of repressing about 10% of Keilin's organs, which is similar to the Keilin's organ repression reported for *Artemia* UBX (Ronshaugen et al., 2002). This data indicates that, when expressed at similar levels, the limb repression function of *Artemia* UBX is weaker than that of its *Drosophila* ortholog.

Phosphorylation in the C-terminus, but not CK2 phosphorylation alone, has some inhibitory effect on *Artemia* UBX limb repression function

The question still remains whether CK2 phosphorylation plays a role in modulating UBX limb repression function. Western Blot analysis on embryos ectopically expressing *Artemia* UBX indicates that the *Artemia* UBX protein is phosphorylated in *Drosophila* embryos due to the appearance of a second, faster-migrating band after treatment with phosphatase (Figure 3-1). *in vitro* CK2 kinase assays showed that the GST-fusion constructs carrying intact C-terminal CK2 sites were always phosphorylated to the same degree as wildtype *Artemia* UBX. A truncated *Artemia* UBX showed a small degree of *in vitro* phosphorylation by CK2, but *in vitro* phosphorylation by CK2 was almost non-existent in constructs carrying a serine to alanine mutation within the C-terminal CK2 consensus site. These results suggest that, although CK2 phosphorylation may occur outside the C-terminus, the

majority of *in vitro* CK2 phosphorylation within *Artemia* UBX occurs at the CK2 consensus site within the C-terminus.

However, the relationship between CK2 phosphorylation and Keilin's organ repression function in *Artemia* UBX proteins carrying serine to alanine mutations could not be determined due to the inability of the assays to detect small differences in the ability to repress Keilin's organs. Paré and McGinnis (unpublished) also tested the mutant lines *Art* UBX 1-7 and *Art* UBX 7, both shown to have ~1% of *in vitro* CK2 phosphorylation relative to wildtype *Artemia* UBX (Figure 3-2). *Dll* repression by *Artemia* UBX 7, with only the serine within the CK2 consensus site mutated to alanine, was comparable to *Artemia* UBX. But *Artemia* UBX 1-7, with all serines and threonines with the C-terminus mutated to alanine, showed a stronger ability to repress *Dll* (only ~10% of *Dll* transcript detected compared to wildtype). By this measure of *Dll* repression, the serine within the C-terminal consensus CK2 site of *Artemia* UBX does not have a detectable function in inhibiting *Dll* repression, but other serines and threonines within the C-terminus do. If phosphorylation within the C-terminus of *Artemia* UBX is responsible for inhibiting *Dll* repression, then it is due to phosphorylation of serines and threonines outside the CK2 consensus or the sum effect of removing all the serines and threonines in the C-terminus.

***Artemia* ABD-A is capable of Keilin's organ repression and homeotic function**

Although scoring Keilin's organs is a low resolution method for determining limb repression ability, this method for measuring limb repression function was

sufficient to uncover the difference in functional abilities between wildtype *Drosophila* ABD-A and truncated *Artemia* ABD-A. *Drosophila* ABD-A is a potent repressor of Keilin's organ formation, with no dependence on protein concentration down to ~40% of endogenous ABD-A levels. The truncated *Artemia* ortholog, however, showed a reduced ability to repress limbs at ~65% of endogenous levels even though at near endogenous levels, Keilin's organs were fully repressed. The protein concentration dependence of *Artemia* abdA's ability to repress limbs is not unlike that of *Drosophila* UBX (Tour et al., 2005). And, while *Artemia* ABD-A is capable of repressing 100% of Keilin's organs, *Artemia* UBX has not been found to be able to fully suppress Keilin's organ formation even at levels well above endogenous UBX levels (Figure 3-3).

The ability of *Artemia* ABD-AC Δ protein to transform thoracic structures toward abdominal identity and to repress Keilin's organs suggest that *Artemia* ABD-A protein is capable of promoting homeotic function in *Drosophila*. The presence of a limb repression function in the truncated *Artemia* ABD-A indicates that a limb repression domain is present within the *Artemia* ABD-A protein. However, the inability to induce full-length ABD-A protein in *Drosophila* embryos unless the C-terminus is deleted suggests that the C-terminus in the *Artemia* coding region may prevent translation of and/or promote rapid degradation of full-length protein. It is possible that a post-transcriptional or post-translational regulation is utilized in *Artemia* to inhibit the ABD-A limb repression function in the pre-genital trunk and allow limb formation. This possibility will be explored in the next chapter.

MATERIALS AND METHODS

CIP Assays.

0-18 hour w¹¹¹⁸ or *arm-GAL4:UAS-Art Ubx*-HA embryos were collected, flash frozen in liquid Nitrogen and stored at -80°C. Embryos were homogenized and proteins extracted in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH 8.0) plus 1µg/ml leupeptin, 2.5 µg/ml aprotinin and 1µg/ml pepstatin. Extracts were treated with 0U or 30U Calf Intestinal Phosphatase (CIP) or 30U CIP plus 2mM NaVO₃ at 37°C for 2 hours. Samples were separated using 10% SDS-PAGE and transferred to PDVF membrane. The membrane was probed with α-HA, then incubated with rabbit α-rat-biotin and detected using the Vectastain ABC Kit (Vector Labs, Cat #PK-4000).

in vitro CK2 assays.

N-terminal GST-fusion proteins of interest were expressed in *E. coli* and purified. *in vitro* CK2 kinase reactions were performed using 65ng/µl protein, 3U/µl CK2 (NEB, Cat. P6010S), 3nmols ATP and 5 µCi γ-³²P ATP. For each experiment, the percent phosphorylation was calculated relative to *Artemia* UBX phosphorylation, taking into account the amount of protein loaded. The percentages are an average of two separate experiments.

Determination of Keilin's organ repression ability.

Coding sequences were cloned into the pUAST vector with the addition of a Kozak sequence encompassing the start codon to ensure translation initiation and a hemagglutinin (HA) tag at the C-terminus to allow for detection of the transgene product. Mutations were introduced using PCR. These constructs were injected into *w¹¹¹⁸* embryos and stably integrated via P element transformation. Multiple transgenic lines of each construct were established and homozygous lines established. Ectopic expression was induced using the GAL4/UAS system (Brand and Perrimon., 1993) by crossing the UAS-lines to flies carrying *arm*-GAL4 on the third chromosome. Use of this driver allowed for proper timing of expression starting at stage 10, prior to expression of endogenous *Dll*, to assay effects on limb formation (Sánchez-Herrero et al., 1994). To determine the levels of protein expression, 4-8 hour embryos collected at 25°C were fixed for 20 min at RT. For *Ubx* constructs, the standard was a line ectopically expressing *Drosophila* UBX known to produce protein at 83%±6% of endogenous levels (Tour et al., 2005).

To establish a standard line for the *abd-A* transgenes, embryos from lines ectopically expressing *Drosophila* ABD-A and wildtype embryos (progeny of *w¹¹¹⁸* crossed to *arm*-GAL4) were stained with an α -*Drosophila* ABD-A mouse monoclonal antibody, mAbDMabd-A subclone 6A8.12 (Kellerman, et al., 1990) at 1:500. Regions corresponding to the ventrolateral position of *Dll* expression of stage 11 embryos (determined by morphological markers) were measured for average luminosity in abdominal segments 2 and 3 for wildtype and in all three thoracic segments for ectopic

abd-A lines. Three *Drosophila abd-A* lines with an average luminosity close to endogenous levels were identified.

For quantification of all other ectopically expressed *abd-A* lines, one *Drosophila abd-A* line was used as the standard ($98\% \pm 11\%$). Ectopic proteins were subsequently detected using an α -HA antibody (rat monoclonal, clone 3F10, Roche, Cat. #1 867 423). The average luminosity in regions of the thoracic segments corresponding to the ventrolateral position of *Dll* expression in stage 11 embryos was calculated and reported as % endogenous protein expression.

To determine Keilin's organ repression ability, cuticles were collected and cleared for phenotypic analysis (Wieschaus and Nüsslein-Volhard, 1986). For each line, all three thoracic segments of 35 cuticles (210 possible Keilin's organs) were scored for presence or absence of Keilin's organs.

ACKNOWLEDGEMENTS

Portions of Chapter 3 appear in Hsia, C.C., Paré, A., Ronshaugen, M., Hannon, M. and McGinnis, W. “Evolution of *Hox* Gene Expression and Function and the Effect on Limb Specification in Arthropods” (in preparation). I was the primary author and responsible for the research included in this dissertation.

FIGURES

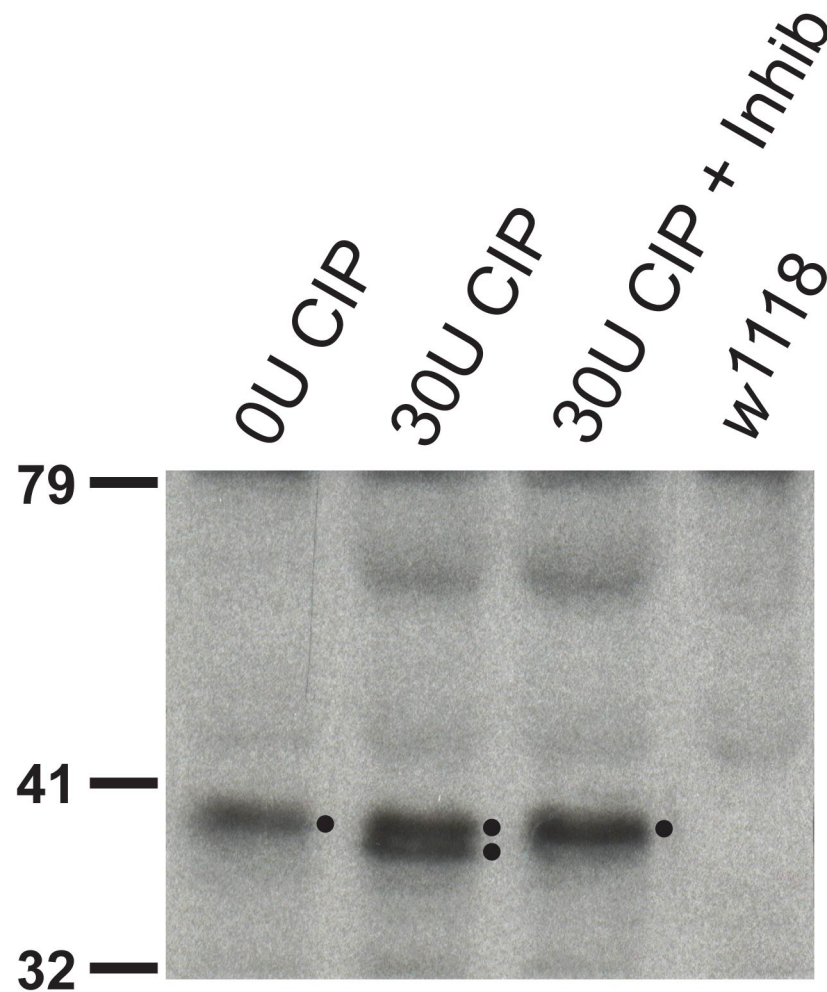


Figure 3-1: Ectopically expressed *Artemia* UBX is phosphorylated *in vivo*.

Western Blot of whole embryo extracts from 0-18 hour *Drosophila* embryos ectopically expressing *Art* UBX and treated with 0U CIP, 30U CIP, or 30U CIP plus 2mM NaVO₃ (a CIP inhibitor) at 37°C for 2 hours and the parental line, w¹¹¹⁸. A second, faster-migrating band is visible after treatment with CIP that is not visible in either the samples with no CIP added or where CIP activity was inhibited.

Figure 3-2: *in vitro* CK2 phosphorylation occurs mainly at the consensus CK2 site within the C-terminus of *Artemia* UBX. Sequence of the C-terminus of *Artemia* UBX is shown at the top with the serines/threonines in red and numbered. The CK2 consensus site is boxed. GST-fusion protein constructs were purified and *in vitro* phosphorylation reactions carried out. GST-*Art* UBX was highly phosphorylated by CK2. The amount of phosphorylation on GST-*Art* UBX by CK2, taking into account the amount of protein loaded, was set as the standard at 100%. CK2 phosphorylation for all other proteins were calculated relative to GST-*Art* UBX. There was no detectable phosphorylation of GST-*Droso* UBX by CK2. Mutant proteins of GST-*Art* UBX carrying a serine to alanine mutation at the serine within the CK2 consensus site (serine 7) showed reduced phosphorylation regardless of whether other serines/threonines (see *Art* UBX1-5, 7 and *Art* UBX 1-7) were also mutated to alanine. When serine 7 was left intact, the level of CK2 phosphorylation was equal to wildtype *Art* UBX protein. GST-*Art* UBX Δ , which is missing the C-terminus, was weakly phosphorylated, suggesting that CK2 also phosphorylates sites outside the C-terminus.

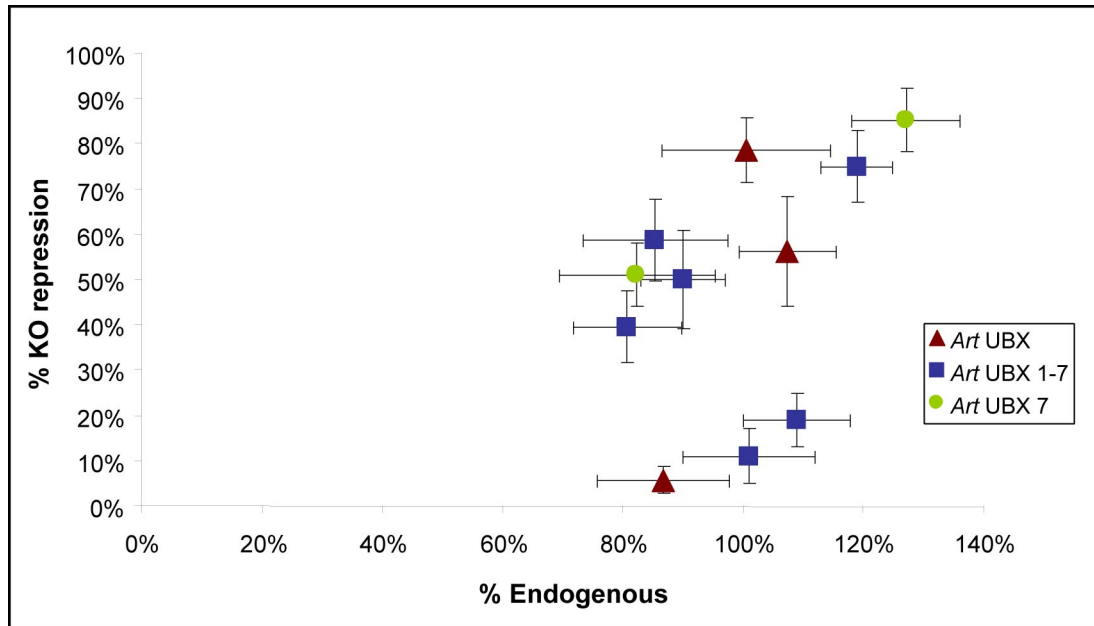


Figure 3-3: Keilin's organ repression abilities of wildtype and mutated *Artemia Ubx* lines. Two wildtype *Artemia Ubx* lines in addition to the original *Artemia Ubx* tested by Ronshaugen et al. (2002) (red triangles) and a total of two *Artemia* UBX 7 (green circles) and six *Artemia* UBX 1-7 (blue squares) lines carrying serine to alanine mutations (see Figure 3-2 for sites of mutations) were tested for limb repression ability by scoring for the presence/absence of Keilin's organs. Many lines with overlapping protein expression levels displayed different abilities to repress Keilin's organs.

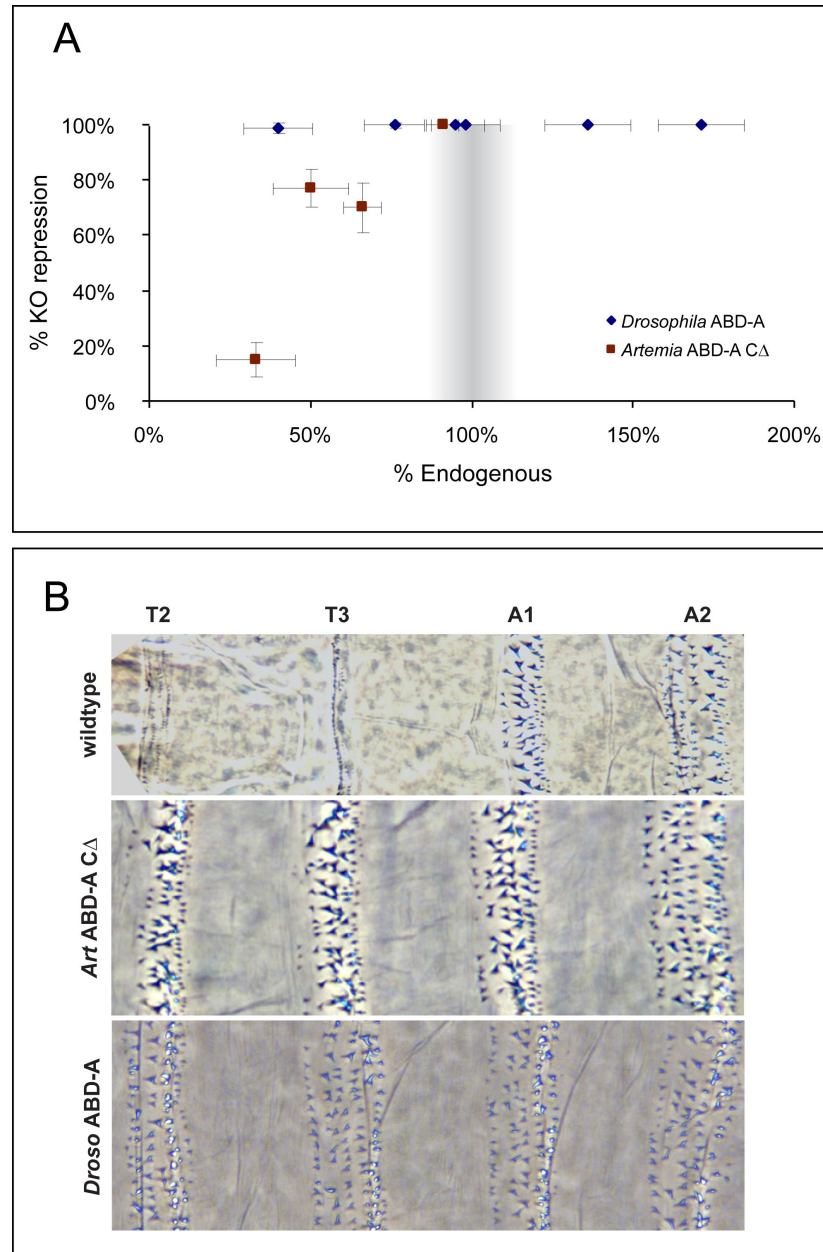


Figure 3-4: Truncated *Artemia* ABD-A is capable of repressing Keilin's organs in *Drosophila* embryos. (A) *Drosophila* ABD-A can completely repress Keilin's organs (KO) even when ectopically expressed at ~40% of endogenous levels. *Artemia* ABD-A C Δ can repress 100% of Keilin's organs when ectopic expression is near endogenous levels, but shows reduced ability to repress Keilin's organs at lower protein concentrations. (B) When ubiquitously expressed in *Drosophila* embryos, *Artemia* ABD-A C Δ transforms thoracic denticle belts towards an abdominal A1-like identity and represses formation of Keilin's organs while *Drosophila* ABD-A transforms denticle belts towards an abdominal A2-like identity

APPENDIX

The central class *Hox* genes, *Antp*, *Ubx* and *abd-A*, are believed to have come about through two tandem duplications. The *Drosophila* ANTP protein has been shown to acquire a limb repression function upon loss of CK2 phosphorylation sites (Jaffe and Mann, 1997). In the course of determining whether phosphorylation by CK2 was inversely correlated with the functional ability of trunk *Hox* genes to repress limbs in UBX orthologs, the protein sequence of *Drosophila* and *Artemia* ABD-A were also analyzed for potential CK2 sites.

Determining potential CK2 sites in ABD-A orthologs

Analysis of the amino acid sequences of the ABD-A orthologs of *Drosophila* and *Artemia* for consensus CK2 sites was carried out to determine potential sites of CK2 phosphorylation. Studies on CK2 specificity indicate there are three major considerations to efficient phosphorylation by CK2: 1) a minimal requirement of the consensus site S/T X X D/E with serine preferred over threonine 2) acidic residues clustered near the +3 position or residues promoting a beta-turn enhances phosphorylation at the serine/threonine 3) basic residues or a proline residue C-terminal to the serine/threonine usually results in weak or abolished phosphorylation by CK2 (Pinna, 1990).

Potential CK2 sites were initially identified based on the minimal requirement for a consensus CK2 site. Five putative sites each in *Drosophila* and *Artemia* ABD-A were identified. The residues within and surrounding these sites were analyzed for the

likelihood of forming a beta-turn (Kaur and Raghava, 2002) and the number of basic residues and proline residues C-terminal to serine/threonine. Of the identified consensus sites in *Drosophila* ABD-A, four out of five had a threonine instead of a serine, a basic residue within the consensus or a proline at the +1 position. The fifth consensus site in *Drosophila* ABD-A had neither favorable nor unfavorable indicators of phosphorylation. None of the five sites showed high probability for forming a beta-turn.

Analysis of the *Artemia* ABD-A CK2 consensus sites indicated two nested sites within the C-terminus with a high probability of forming beta-turns and an increasing efficiency of phosphorylation upon phosphorylation of one serine (thereby creating a basic residue). These two sites, out of all consensus sites in both ABD-A orthologs, seem to have the highest likelihood of being phosphorylated by CK2. Moreover, there is an additional serine immediately C-terminal to the serines of the nested CK2 sites followed by two aspartate residues (SDD), a motif which bears some resemblance to reported non-canonical CK2 sites (Lin et al., 1992; Manak and Prywes, 1991; Yin et al., 2000; Walton and Gill, 1983; Hill et al., 1988). Phosphorylation, if it occurs, at the non-canonical CK2 site produces another possible CK2 site creating a potential series of 4 nested CK2 sites. The other three consensus sites within *Artemia* ABD-A had no or mediocre probability of forming a beta-turn and/or had a proline residue at the +2 position.

Artemia* ABD-A but not *Drosophila* ABD-A is phosphorylated by CK2 *in vitro

To test if the ABD-A orthologs from *Artemia* and *Drosophila* can be phosphorylated by CK2 *in vitro*, GST-fusion proteins were cloned, expressed and purified from *E. coli*. CK2 assays indicated that *Artemia* ABD-A was strongly phosphorylated by CK2 but *Drosophila* ABD-A was not (Figure A-1).

To determine if CK2 phosphorylation of *Artemia* ABD-A occurred at the Ser residues within the nested series of consensus CK2 sites within the C-terminus, GST-fusions of *Artemia* ABD-A were constructed with different combinations of serines mutated to alanines. A truncated protein missing the last 54 amino acids (*Artemia* ABD-AC Δ) was also cloned. *in vitro* CK2 kinase reactions were carried out in the same manner as CK2 assays on *Drosophila* and *Artemia* UBX that were carried out previously, except that the standard for phosphorylation was *Artemia* ABD-A. *Artemia* ABD-AC Δ showed no detectable phosphorylation, indicating that all *in vitro* phosphorylation by CK2 occurs within the C-terminus of *Artemia* ABD-A.

in vitro phosphorylation is completely abolished only when serines 2-3 (Figure A-1) are mutated to alanine. These are the serines in the overlapping consensus CK2 sites and the serine immediately following it, in a non-canonical CK2 consensus site. The results of these assays suggest that *Drosophila* ABD-A is not capable of being phosphorylated by CK2 *in vitro* but *Artemia* ABD-A is phosphorylated by CK2 in its C-terminus, at consecutive serines found within canonical and non-canonical CK2 sites.

MATERIALS AND METHODS

in vitro CK2 assays.

N-terminal GST-fusion proteins of interest were expressed in *E. coli* and purified. *in vitro* CK2 kinase reactions were performed using 65ng/μl protein, 3U/μl CK2 (NEB, Cat. P6010S), 3nmols ATP and 5 μCi γ-32P ATP. For each experiment, the percent phosphorylation was calculated relative to *Artemia* ABD-A phosphorylation, taking into account the amount of protein loaded. The percentages are an average of two separate experiments.

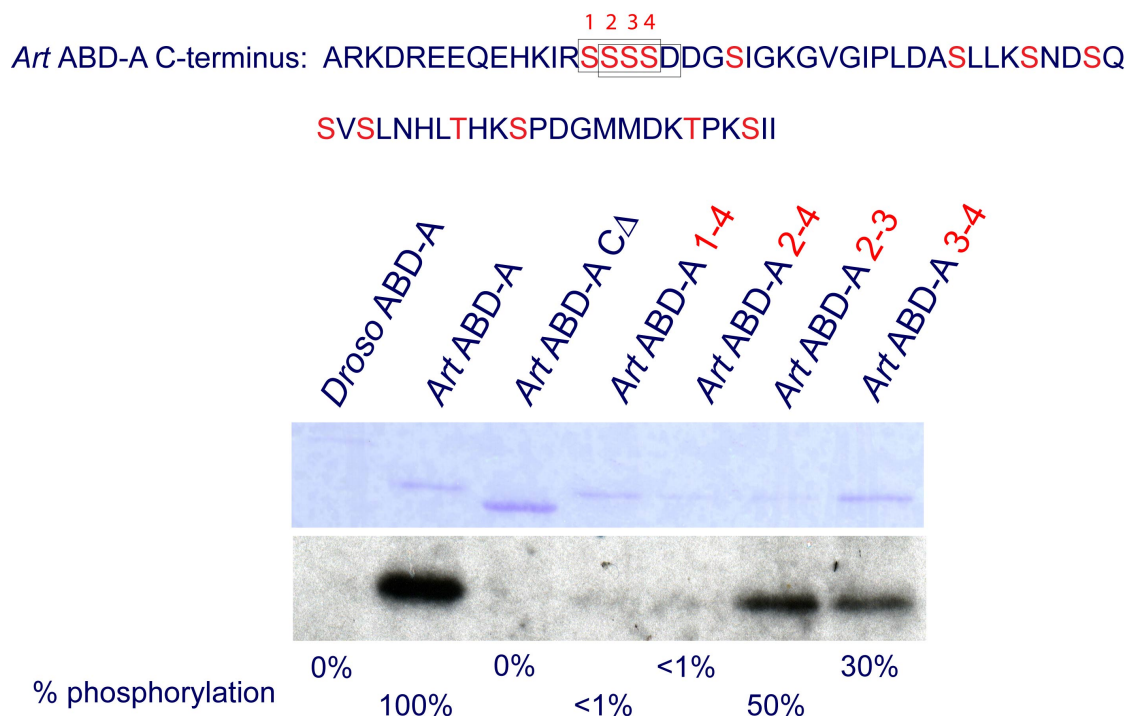
FIGURE

Figure A-1: *Artemia* ABD-A is phosphorylated at consecutive Serines within the C-terminus. The amino acid sequence of the *Artemia* ABD-A C-terminus is shown with serine and threonine residues highlighted in red and the overlapping consensus CK2 sites boxed. The serines that were mutated to alanines are numbered. Serine 4 is the suspected phosphorylation site of a non-canonical CK2 site. Both the Coomassie stained protein gel (top) and the autoradiogram (bottom) are shown. The amount of CK2 phosphorylation relative to *Artemia* ABD-A is indicated below each lane.

Chapter 4

Inhibition of ABD-A homeotic function via suppression of protein expression in *Artemia franciscana*

INTRODUCTION

Hox genes are transcription factors that specify segmental identity along the anterior-posterior axis in both invertebrates and vertebrates. In many metazoans, *Hox* genes are found within one or multiple clusters, each cluster usually consisting of eight to ten genes. Due to this clustering and the conservation of the DNA binding domains or homeodomains among *Hox* genes, it has been proposed that the *Hox* cluster first arose via tandem duplication (Lewis, 1978). It is believed that the complement of *Hox* genes continued to evolve through further duplication of individual genes, as in the case of many invertebrates, or from duplication of the complete cluster, as in the case of vertebrates. Another conserved aspect of *Hox* genes is their colinearity. The order the *Hox* genes lie on the chromosome typically correlates with the order of the anterior expression boundary along the anterior-posterior axis of the animal (McGinnis and Krumlauf, 1992). The order of gene expression, for example, in the trunk of *Drosophila* is a good, though not an exact, predictor for the order of expression of those orthologs in other arthropods.

Due to their role in conferring segmental identity, studies of how the expression and function of *Hox* genes have evolved may shed light on the evolution of morphological diversity. How does essentially the same set of genes result in the myriad of animal body plans we see today? Studies have already shown that changes in *Hox* gene expression or regulation of downstream target genes are correlated with morphological changes (Gellon and McGinnis, 1998). Arthropods are often the subjects of these kinds of studies, not only because *Hox* genes were initially

discovered in the insect, *Drosophila melanogaster*, but also due to the enormous amount of diversity in body plans that are found within the phylum.

Expression studies using an antibody that detects both Ultrabithorax (UBX) and abdominal-a (ABD-A) HOX proteins found that the anterior expression boundary correlated with the transition between segments that bear head appendages and those that bear trunk appendages (Averof and Patel, 1997). This cross-reacting antibody, FP6.87 (Kelsh et al., 1994), is widely used because its epitope is to a conserved region within both UBX and ABD-A, allowing for the detection of UBX/ABD-A proteins in many arthropods. That UBX/ABD-A expression occurs in the pre-genital trunks of various arthropods has been borne out over many species (Averof and Patel, 1997; Zheng et al., 1999; Abzhanov and Kaufman, 2000a; Abzhanov and Kaufman, 2000b; Blin et al., 2003). In *Artemia*, studies using FP6.87 showed that the UBX/ABD-A expression domain extends from the first thoracic segment towards the posterior end of the animal (Averof and Patel, 1997). An UBX specific antibody has found that UBX protein expression correlates with the expression boundaries detected by FP6.87 (Shiga et al., 2006). Additionally, abdominal-B (abd-B) expression, a *Hox* gene typically expressed in the most posterior segments (which also tend to be the genital segments in most arthropods) and therefore typically expressed more posteriorly than *abd-A*, was detected in the genital segments of *Artemia* (Averof and Akam, 1995; Copf et al., 2003).

From the expression studies accumulated thus far, colinearity would predict that *abd-A* is expressed in the pre-genital trunk segments. In published studies of *abd-*

A expression in arthropods, *abd-A* is always found to be expressed in trunk segments that do not form limbs (Tear et al., 1990; Shippy et al., 1998; Zheng et al., 1999; Abzhanov and Kaufman, 2000a; Abzhanov and Kaufman, 2000b; Zhang et al., 2005). These segments where *abd-A* is detected are also normally anterior to the genital segment(s). Although ABD-A expression has been detected in the neuromeres of the pre-genital trunk (Averof and Akam, 1995) and the domains of the nervous system expression of *Hox* genes follow closely (albeit not exactly) the domains of epidermal expression, the boundaries of expression of ABD-A protein in the pre-genital trunk epidermis needs verification.

In order to gain a deeper understanding of the molecular underpinnings responsible for the morphological differences between the *Drosophila* and *Artemia* pre-genital segments, *abd-A* expression and function within *Artemia* must be clarified. It is known that in *Drosophila*, both *Ubx* and *abd-A* are expressed in the pre-genital abdomen that bear no appendages and both proteins can function to repress limbs. In the *Artemia* pre-genital segments, all 11 segments bear limbs where UBX expression has been confirmed (Shiga et al., 2006). Several pieces of evidence suggest that in order to fully allow the formation of limbs in the *Artemia* pre-genital trunk, expression of UBX may be slightly downregulated and expression of ABD-A greatly downregulated. First, functional studies of *Artemia* UBX indicate that, when expressed at levels just below the level of expression in *Drosophila* embryos, it still has a very weak ability to repress limbs (Ronshaugen et al., 2002) and the limb-promoting gene, *Distal-less* (*Dll*) (Paré and McGinnis, unpublished). The degree of its

ability to repress limbs may be protein concentration dependent, like *Drosophila* UBX (Tour et al., 2005), as it has been observed that when the protein is ectopically expressed at higher protein concentrations in *Drosophila* embryos, *Artemia* UBX's ability to repress Keilin's organs increases (Chapter 3). Truncated *Artemia* ABD-A shows a strong ability to repress Keilin's organs in *Drosophila* embryos but also exhibits a dose dependent relationship for this ability (Chapter 3). And lastly, in *Drosophila* embryos, full-length *Artemia abd-A* ORF produced abundant cytoplasmic transcripts in ectopic expression assays but protein was not detected unless C-terminal coding sequences were deleted, suggesting a post-transcriptional mechanism for downregulation or abolition of protein expression.

To determine the specific expression pattern of *abd-A* transcript and protein in the pre-genital trunk of *Artemia*, fluorescent immunohistochemistry and *in situ* hybridization protocols were developed. There was no detectable ABD-A protein expression in the epidermal pre-genital trunk, including the limb primordia in the early stages of development just prior to initiation of the limb-promoting gene, *Dll*. This finding in *Artemia* may be the first in which a functional *Hox* gene capable of segmental identity function is silenced in early stages and therefore does not function to confer segmental identity. This loss of segment identity function may contribute to the overall morphological body plan of *Artemia* in allowing the development of limbs on all pre-genital trunk segments.

RESULTS

Trunk development and gene expression in *Artemia franciscana*

Artemia embryogenesis begins within the reproductive female. Under permissive conditions, embryogenesis progresses and results in the live birth of first instar larvae, known as L1 nauplii, into the surrounding sea water. Under stressful conditions, the embryo ceases development after initiation of gastrulation and a hard shell is deposited around the embryo, forming a cyst. These cysts are released and *Artemia* stay dormant until conditions are more favorable. Upon resumption of development, L1 nauplii hatch from the cysts.

In the L1 stage, the animal's head structures have already begun differentiation except for two gnathal segments (Schredhardt, 1987a). The larvae will undergo 14 molts before reaching adulthood. During these 14 molts, the trunk will undergo segmentation in an anterior-posterior gradient of development with anterior segments at more advanced stages of development than posterior segments, similar to the mode of development seen in short-germ band insects. Segments emanate from a growth zone that resides at the posterior end of the trunk.

Besides the two gnathal segments, 11 thoracic segments each bearing a pair of limbs will also develop. Trunk segmentation begins with the formation of segmentation furrows followed by limb bud formation (Figure 4-1). Thoracomers refer to the limbs once limb bud formation has begun. The limbs then undergo articulation, at which point the limbs are known as thoracopods. After articulation, the thoracopods differentiate so that within each limb several podites develop and setae

form and elongate. *Artemia* are staged by molts, by the number of thoracomers and thoracopods present and by the degree of differentiation the limbs have undergone. Several segments can develop between each molt (Schredhardt, 1987a).

Some expression studies have been carried out in *Artemia*. Engrailed is a conserved homeodomain protein expressed in stripes in the posterior segments of various arthropods (e.g. Fjose et al., 1985; Patel et al., 1989; Hughes and Kaufman, 2002c; Damen, 2002) including *Artemia* (Manzanares et al., 1996). In *Drosophila*, it is a segment polarity gene and involved in establishing segmental boundaries during the early stages. In later stages, it is expressed in subsets of neuroblasts. This neural expression is conserved across several species of arthropods (e.g. Harzsch, 2003; Scholtz, 1994; Thomas, 1984).

During the early stages of *Artemia* development, the most anterior Engrailed protein stripes mark the posterior compartments of the two as yet undifferentiated gnathal maxillary and maxillary segments (Manzanares et al., 1996). As the trunk develops with each subsequent molt, Engrailed protein expression within a segment precedes any noticeable morphological segmentation or thoracomere formation in the trunk (Manzanares et al., 1996). Later, as the thoracopods develop further, Engrailed protein can be detected in a subset of cells in the neuromeres of the trunk (Figure 4-2K).

Published studies using the 4F11 anti-Engrailed antibody and the FP6.87 antibody show that the anterior boundary of FP6.87 signal abuts the Engrailed stripe of the maxillary segment (the more posterior of the gnathal segments) (Panganiban et

al., 1995; Averof and Patel, 1997). Expression of UBX/ABD-A protein is detected within the trunk starting at the L1 stage (Figure 4-3F) until at least the L5 stage when the most developed trunk segments have started to articulate (Averof and Akam, 1995). As already mentioned, ABD-A protein expression was studied using an antibody made to an epitope to a region conserved in insect and *Artemia* ABD-A (Averof and Akam, 1995). Expression studies using this antibody of ABD-A within the epidermal trunk during the early stages was inconclusive, however there was strong expression within the thoracic neuromeres.

ABD-A protein is not detected in the epidermis of early stage *Artemia*

To test if ABD-A protein is present in early *Artemia* nauplii prior to trunk differentiation, polyclonal rabbit antibodies made to the N-terminal region up to and including the YPWM domain of *Artemia* ABD-A were affinity purified. The specificity of this antibody was tested on *Drosophila* embryos ectopically expressing various HA-tagged proteins. They were found to be specific to *Artemia* ABD-A and did not cross-react with *Artemia* UBX, *Drosophila* ABD-A or *Drosophila* UBX proteins (data not shown).

Double stains using anti-Engrailed antibody plus α -*Artemia* ABD-A and using FP6.87 plus α -*Artemia* ABD-A were carried out in parallel on various stages of *Artemia*. Early stage (L1 to L4) *Artemia* and *Drosophila* embryos ubiquitously expressing truncated *Artemia* ABD-A (*Art* ABD-AC Δ) were processed separately prior to incubation in primary antibodies. The animals were subsequently pooled so

that experimental and control tubes each contained a mixture of *Artemia* nauplii and *Drosophila* embryos. Since ABD-A protein expression has been detected in the neuromeres of late stage *Artemia*, L9 to L12 stage *Artemia* were also stained as a positive control for the α -*Art* ABD-A antibody (Figure 4-2 L). As a negative control, *Artemia* were stained with an α -*Drosophila* ABD-A antibody (Figure 4-2 B).

Engrailed and *Art* ABD-ACA proteins were detected in *Drosophila* embryos (Figure 4-2 C, D). In *Artemia*, Engrailed protein expression were visible in about 70% of the L1 to L4 stage animals. However, no detectable signal for ABD-A could be found within this group of animals (Figure 4-2 F, H, J). In late stage *Artemia*, ABD-A expression was detected within the neuromeres as reported previously (Averof and Akam, 1995; Figure 4-2 L). In a stage L9 animal, the ABD-A neuromere expression initiates in the trunk segments bearing thoracopods (T8, Figure 4-2 L). However, Engrailed protein expression in the neuromeres seems to have initiated in a more posterior and less developed trunk segment bearing thoracomers (T9, Figure 4-2 K).

In double antibody stains with FP6.87 plus α -*Artemia* ABD-A, *Drosophila* embryos ubiquitously expressing *Art* ABD-ACA show a low and ubiquitous signal from the FP6.87 antibody while the α -*Artemia* ABD-A gives a strong nuclear staining (compare panels C and D in Figure 4-3) suggesting that the α -*Artemia* ABD-A antibody may be a more sensitive antibody than FP6.87 in fixed embryos. In early stage *Artemia*, fewer animals showed signal with the FP6.87 antibody than with the α -Engrailed antibody. However, no detectable signal for ABD-A protein could be seen in any L1 to L4 stage animals, regardless of whether FP6.87 signal was detected in

those animals (Figure 4-3 G, K, I). In a stage L12 *Artemia*, when all of the limbs in the pre-genital trunk segments have finished differentiation, both FP6.87 and ABD-A antibodies clearly detect protein in the same cells within the neuromeres (Figure 4-3 L, M and inset).

***abd-A* transcript is not detectable in *Artemia* L4 animals**

To test for *abd-A* transcript expression in the trunk of early stage *Artemia*, *in situ* hybridizations for the simultaneous detection of two probes were developed. Different haptens were found to exhibit different sensitivities in *Artemia*. Probes labeled with DIG or FITC produced strong signals, with DIG labeled probes exhibiting better sensitivity than FITC labeled probes (data not shown). Pairwise *in situ* hybridizations of *engrailed*, *Ubx*, and *abd-A* were carried out on L1 to L4 stage *Artemia*. Sense probe controls were also performed on a subset of these animals.

Signal was detected in about three quarters of the animals in *in situ* hybridizations using *en*-DIG plus *Ubx*-FITC probes. In all early stages, the domain of *Ubx* transcript extends from just posterior of the maxillary *en* stripe to just anterior of what is believed to be the growth zone (arrow, Figure 4-4). The anterior boundary of *Ubx* transcript expression correlates with the reported anterior boundary of FP6.87 (Averof and Patel, 1997). The *Ubx* transcript expression domain, in full (Figure 4-4 B, E, H), coincides with the observed domain of expression for FP6.87 (Averof and Akam, 1995; Averof and Patel, 1997; Figure 4-4 F, J, H) and the reported UBX specific protein expression domain (Shiga et al., 2006).

Ubx transcripts were detected in at least three quarters of the animals hybridized with *Ubx*-DIG plus *abd-A*-FITC probes and *en* transcripts detected in about half of the animals hybridized with *en*-FITC plus *abd-A*-DIG probes. However, no *abd-A* transcript was detected in any stage L2 through L4 animals (Figure 4-5 B, C, K, L). In the majority of L1 nauplii, no *abd-A* transcript were detected. However, in a small percentage of L1 animals probed with *abd-A*-DIG (Figure 4-5 A), and less commonly in L1 animals probed with *abd-A* FITC, a small region in the posterior portion of the trunk showed a slightly denser pattern of signal just anterior of the growth zone. No signal was detected within the growth zone. It is not certain if the observed pattern is actual transcript and more work is necessary to determine whether this is an indication of transient expression of *abd-A* transcript or is due to experimental artifacts.

Northern blot analysis was performed to confirm the absence of *abd-A* transcript in L4 *Artemia* nauplii, the stage when *Dll* expression first initiates (Panganiban et al., 1995). PolyA RNA from L4 stage *Artemia* was purified and probed for the presence of endogenous *abd-A* and *Ubx* transcripts. Sense RNA of the respective genes were *in vitro* transcribed and loaded at 300 pg and 30 pg concentrations in order to determine the lower limit of sensitivity of the probes (Figure 4-5 N, O). 10 µg of polyA RNA was loaded for *Ubx* transcript detection and 25 µg polyA RNA loaded for *abd-A* detection. *Ubx* transcripts were detected in polyA RNA of L4 stage *Artemia*, but no *abd-A* transcripts were detectable even though more than twice the amount of polyA RNA was loaded.

In *Drosophila* embryos, *Artemia abd-A* transcript expression can be induced

Previous work testing the functional ability of *Artemia* ABD-A in *Drosophila* embryos uncovered the peculiar finding that *Artemia* ABD-A protein expression could not be induced unless the C-terminus was truncated (Chapter 3). Sequencing of the transgenes from all ten available lines carrying the UAS-*Artemia abd-A* construct was carried out. None were found to carry nonsense mutations (data not shown). When *in situ* hybridizations were carried out on three lines of *Drosophila* embryos induced to express full-length *Artemia abd-A* and two lines induced to express *Art abd-ACΔ*, transcripts were detected for both the full-length and truncated versions of *Artemia abd-A* (Figure 4-6A). Protein expression, however, was detected only for embryos induced to express the truncated *Artemia* ABD-A protein. These findings in *Drosophila* embryos coupled with the possible, transient expression of *abd-A* in *Artemia* L1 nauplii led to the question of whether the lack of ABD-A expression in the epidermal trunk in early stage *Artemia* may involve a redundant post-transcriptional/translational mechanism to inhibit ABD-A expression and ultimately, function in the trunk.

Analysis of the C-terminal region uncovered several putative motifs for various post-translational modifications including consensus sites for other kinases and for sumoylation sites (Figure 4-6B). Based on the arrangement of these motifs, additional transgenic lines carrying longer truncations of *Artemia abd-A* were generated to test their ability to express protein in *Drosophila* embryos to try to identify a region responsible for the lack of ABD-A protein expression. Multiple lines

of each truncation were tested, again using hatch test assays, along with full-length *Artemia abd-A* and w^{1118} as a control. The percentage of hatching was adjusted relative to w^{1118} . As expected, full-length *Artemia abd-A* showed 100% hatching while ubiquitous expression of the *Art ABD-A CΔ* truncation missing the last 54 residues was embryonic lethal and no larvae were observed. Addition of 17 residues to *Art ABD-A CΔ* (*Art ABD-A CΔ2*) displayed embryonic lethality indicating the presence of functional HOX protein. An addition of 18 more residues (*Art ABD-A CΔ3*) resulted in the loss of protein expression and all lines of this transgene tested showed viability. However *Art ABD-A CΔ4*, which is only nine residues longer than *Art ABD-A CΔ3* and ten residues short of full-length, again displayed embryonic lethality when ubiquitously expressed. And, as already observed, full-length *Artemia abd-A* does not produce functional HOX protein and all lines tested were 100% viable. If sequences within the *Artemia ABD-A* C-terminus acting to inhibit *Artemia ABD-A* protein expression exists, these results suggest there may be two regions responsible.

DISCUSSION

***Hox* gene expression in the *Artemia* pre-genital trunk**

Studies of the limb promoting gene, *Distal-less* (*Dll*), in *Artemia* indicate that DLL protein expression begins when the first trunk segments have developed limb buds (Panganiban et al., 1995). The earliest stage in which this development occurs in *Artemia* is stage L4 (Schredhardt, 1987a). Given that neither *abd-A* transcript nor protein is detectable in the epidermal trunk during stages L2 to L4, that *Ubx* transcript is expressed in stages L1 to L4 and that UBX protein expression has been detected throughout the trunk at stage L4 (Shiga et al., 2006), *Ubx* is the only *Hox* gene expressed in the *Artemia* pre-genital trunk when *Dll* expression begins.

It has been observed in our lab that *Dll* transcription is initiated in the A1 abdomen of *Drosophila* embryos but not maintained as it is in the thoracic segments (Kosman and McGinnis, unpublished). The transient expression of *abd-A* in the trunk epidermis of L1 nauplii, if real, is reminiscent of this *Dll* initiation. It is possible that the *abd-A* transcript is expressed at low levels in the first larval stage, but is not translated into protein nor is the expression maintained in the *Artemia* trunk and allowed to persist into the later stages. By the L2 stage neither *abd-A* transcript nor protein are observed. The lack of *abd-A* transcript in L4 animals is supported by Northern analysis, but further confirmation is needed to determine if *abd-A* transcript is actually transiently expressed in L1 nauplii.

Artemia abd-A expression in the L5 to L8 stages is not known. However, neuromere expression of ABD-A protein is seen in a thoracopod trunk segment that

has started undergoing articulation in an L9 stage *Artemia* (T8, Figure 4-2L). Engrailed protein expression in this animal can be weakly detected in the less developed trunk segment just posterior to this segment (T9, Figure 4-2K). These expression patterns suggest that initiation of Engrailed protein expression begins prior to initiation of ABD-A expression in the pre-genital neuromeres. By stage L5, neural precursor cells have begun to move towards the midline as marked by Engrailed expressing cells (Manzanares et al., 1996) and the most advanced trunk segments have articulated limbs (Schredhardt, 1987a). It can be inferred that ABD-A neuromere expression may begin as early as stage L5.

Inhibition of *Artemia* ABD-A homeotic function

Functional studies of truncated *Artemia* ABD-A protein in *Drosophila* embryos show that it is capable of homeotic function (Chapter 3). However, the finding that the *abd-A* gene in *Artemia* may not be expressed in the pre-genital trunk epidermis is unexpected. In all published studies of *abd-A* specific expression in arthropods, it is expressed in a HOX-like pattern in the trunk during the stages when limb development is underway (Tear et al., 1990; Nagy et al., 1991; Shippy et al., 1998; Peterson et al., 1999; Abzhanov and Kaufman, 2000a; Abzhanov and Kaufman 2000b; Hughes and Kaufman, 2002b; Zhang et al., 2005). That *abd-A* in *Artemia* is not expressed in the epidermis during trunk development but is expressed in neuromeres suggests it is not functioning to confer segmental identity. Expression and function in the CNS is another hallmark of *Hox* genes and of genes believed to have

evolved from *Hox* genes but is no longer capable of homeotic function, such as *fushi tarazu* (Hsia and McGinnis, 2003). The presence of protein expression in neuromeres indicates that *Artemia abd-A* may have retained this characteristic of *Hox* genes. The question still remains, however, whether *Artemia abd-A* is a bona fide *Hox* gene or whether it has evolved/is evolving other functions like *fushi tarazu* and other homeodomain genes that began as *Hox* genes. ABD-A protein expression has been detected in the female ovisacs (data not shown), although whether this expression is present in the juvenile/adult female or in early embryos is not clear.

No published studies thus far have found a lack of *abd-A* expression in the trunk epidermis when the gene is present, though there is some evidence that Collembolans may exhibit a similar peculiarity to *abd-A* expression as that seen in *Artemia*. In these hexapods, the *Ubx* transcript domain fully coincides with FP6.87 expression and *abd-A* transcripts are detected later in development but not in the developing trunk (M. Terry, personal communications). This lack of segmental identity function in ABD-A may not be unique to *Artemia* and may be a characteristic of other arthropods as well. As the ability to carry out expression and functional studies in more arthropods increases, it will be interesting to see if more examples like these are uncovered.

Although the *Hox* cluster was originally discovered in insects and thought to be made up of eight *Hox* genes, studies of invertebrate and vertebrate *Hox* clusters suggest that the original *Hox* cluster was composed of at least 10 *Hox* genes and at least two have evolved into genes without homeotic function in some lineages

(Hughes and Kaufman, 2000a). In these cases, the genes have evolved away from a homeotic function and their sequences have diverged to the extent that the lost homeotic function is not obvious. What is novel in the case of *Artemia abd-A* is the presence of an identifiable “*Hox*” gene without segmental identity function. The *Artemia* ABD-A homeodomain sufficiently resembles that of other ABD-A orthologs and a lack of segmental identity function is not immediately apparent. Additionally, functional studies in *Drosophila* embryos of a truncated version of *Artemia* ABD-A show that the protein is capable of conferring segmental ability. This ability, then, is present in *Artemia* ABD-A, but the lack of expression prevents this function from being carried out.

Suppression of *Artemia* ABD-A expression may also employ post-transcriptional mechanisms

The main mechanism to prevent *abd-A* expression in the epidermis of the *Artemia* trunk would seem to be at the cis-regulatory level. But no extensive cis-regulatory sequences have been identified for any of the *Hox* genes in *Artemia*. The genomic structure of the *Hox* genes in *Artemia* is unknown and although it is tempting to assume that *Artemia Hox* genes are clustered in a similar manner as seen in other arthropods, genome sequencing is challenging the idea that *Hox* genes are typically found in clusters. More and more examples of animals with *Hox* genes in disrupted clusters or clusters that have been dispersed are coming to light (Lemons and McGinnis, 2006). Whether these disruptions and dispersements may cause changes in

the cis-regulation of *Hox* genes and whether this is a reason why *abd-A* is not expressed in the epidermal trunk in *Artemia* requires further exploration.

Dissection of the C-terminal region of *Artemia* ABD-A using *Drosophila* ectopic expression assays implicated two separate regions that may be involved in conferring protein instability. Whether the lack of ABD-A expression in *Artemia* also employs post-transcriptional/translational regulation remains uncertain as it is not known if the protein instability seen in the *Drosophila* ectopic expression assays reflects the endogenous situation in *Artemia*. If transient *abd-A* expression in L1 stage *Artemia* can be verified, this evidence would bolster support for the use of two levels of regulation to inhibit *abd-A* expression in the *Artemia* trunk.

Mounting evidence for the use of post-transcriptional mechanisms to regulate HOX protein expression presents the possibility that the use of post-transcriptional regulation may be a conserved aspect of *Hox* genes. There are other examples that point to the possible use of post-transcriptional mechanisms to regulate the timing of HOX protein expression. In the crustacean, *Porcelio scaber*, SCR protein is detected in the early stages of development only in the posterior maxillary segment while the expression domain of *Scr* transcript is much wider and encompasses segments both anterior and posterior to the protein expression domain (Abzhanov and Kaufman, 1999). Later, as development progresses, the protein expression domains expand posteriorly (but not anteriorly) to coincide with *Scr* transcript expression. Recent work also provides evidence that the posterior boundary of SCR is maintained via miRNA regulation (Lemons and McGinnis, unpublished results).

Additionally, in *Drosophila*, a temporal gap between the time when *Hox* gene transcript is detected and the time when HOX protein expression is detected has been reported for *Ubx*, *Antennapedia* (Carroll et al., 1986) and *Scr* (Riley et al., 1987; Mahaffey and Kaufman, 1987). One explanation has been that this temporal gap is due to transcript length. However in the case of *Scr*, a 3' probe was utilized (Riley, et al., 1987) suggesting otherwise. Another example where transcript expression domains do not correspond with protein expression domains is in *Artemia* and involves the Antennapedia protein. Antennapedia protein expression has been found to be restricted to the posterior maxillary segment (Shiga et al., 2006) even though *Antennapedia* transcript is detected continuously from the posterior maxillary segment, throughout the post-gnathal trunk (Averof and Akam, 1995). In all reported cases, any post-transcriptional regulation, if it exists, could involve either sequence outside the coding region or within the coding region. However, in the case of *Artemia abd-A*, sequences responsible for any post-transcriptional/translational mechanism for inhibiting protein expression would be utilizing coding sequences only, as no UTR sequence were included in the transgenes introduced into *Drosophila* embryos.

Implications of the loss of ABD-A expression on *Artemia* morphology

The combination of an absence of *abd-A* expression in the epidermis and the ability of *Artemia* UBX to permit some *Dll* expression (Chapter 3) sheds some light into how limbs are allowed to develop in the pre-genital trunk of *Artemia*, using HOX

proteins that traditionally function to repress limbs. *Artemia* UBX was found to repress *Dll* expression relatively strongly, though not completely, when protein expression levels were induced in *Drosophila* embryos to a level almost equal to endogenous *Drosophila* UBX (Tour et al., 2005). Since *Drosophila* UBX has been shown to exhibit a reduced ability to repress *Dll* at lower protein concentrations, it's possible that if endogenous *Artemia* UBX expression levels are actually lower than what was tested in *Drosophila* embryos, *Dll* expression in *Artemia* may be high enough to explain the prevalence of limbs along the pre-genital trunk. Use of the FP6.87 antibody may be advantageous in this case for comparisons of protein expression levels of endogenous UBX in *Drosophila* embryos versus endogenous UBX in *Artemia*. It is also possible that *Drosophila Dll* is more sensitive to UBX repression than *Artemia Dll*, either because the *Drosophila* ortholog has evolved to be more sensitive to form a limb-less abdomen or the *Artemia* ortholog has evolved to be less sensitive in order to allow the limbs to form.

With a lack of *abd-A* expression in the pre-genital trunk epidermis and taking into account that all segments within the pre-genital trunk bear limbs but no limbs develop within the post-genital trunk, could *abd-A* be expressed non-colinearly in the epidermis of the post-genital trunk? There are seven post-genital segments that form in the late stages of *Artemia* larval development. Attempts to determine what, if any, *Hox* gene is expressed there have not been successful (Copf et al., 2003). However, it cannot be ruled out that *abd-A* is expressed out of colinear order and during these later developmental stages, although, in the limited number of late stage *Artemia* studied in

this work (e.g. the L9 stage *Artemia* in Figure 4-2 K, L and the L12 stage *Artemia* in Figure 4-3 L, M), no ABD-A protein was detected in the post-genital trunk segments either early in their formation or after their formation was completed (data not shown).

The argument can also be made that since there is no *abd-A* expressed in the *Artemia* trunk epidermis, there is no real abdomen in *Artemia*. In the crustacean, *Sacculina carcini*, attempts to clone the *abd-A* gene have failed, although related *Hox* genes were cloned. It has been proposed that the *abd-A* gene has been lost (Blin et al., 2003) and this loss is correlated with an absence of an abdomen. Another example can be found in comparisons of *abd-A* transcript from two ant species that have different abdominal morphology. The authors of this study proposed a correlation between the “stripey” *abd-A* transcript expression they observed in one ant species with the reduced abdominal segment in that species (Nicolita, 2005).

MATERIALS AND METHODS

Artemia husbandry.

Artemia franciscana cysts (San Francisco Bay Brand) were hatched (day 1) in 1 L 15% Instant Ocean (Aquarium Systems, Cat. # SS3-50) with continuous light and aeration overnight. Temperature of the culture was maintained between 28°C to 32°C and early development progressed at about one molt per day under these conditions until stage L4. For L1 to L4 mixed stage collections, 0.3g dehydrated cysts were hatched on day 1, an additional 0.2g cysts added on days 2 and 3, 0.1g cysts added on day 4. Live animals collected on day 5. To grow *Artemia* to late larval stages, 250µl of diluted Tahitian Blend algal paste (Brine Shrimp Direct) were added daily starting on day 3 when most animals have reached stage L2. Animals were collected and fixed once they developed to the desired stage.

Antibodies.

An α -*Artemia* ABD-A antibody was made against a GST-fusion of the N-terminal domain, up to and including the YPWM motif of *Artemia* ABD-A but missing the homeodomain and C-terminus (Pocono Rabbit Farm and Laboratory Inc.). The rabbit polyclonal sera were affinity purified through a Quickpure column (Sterogene, Cat. # QP01-01) and tested for specificity on *Drosophila* embryos at 1:200 dilution. The 4F11 α -EN antibody (Patel et al., 1989) was used at 1:30 dilution. The FP6.87 antibody (Kelsh et al, 1994) was concentrated 4-5 fold using Centricon filter

units (Millipore), then used at 1:2 dilution. Rat α -HA was purchased from Roche Applied Science (Cat. # 11867423001) and used at 1:500 dilution.

***Artemia* immunohistochemistry.**

Artemia were fixed based on protocols supplied by Nipam Patel (personal communications) with modifications. 0.2g of live L1-L4 *Artemia* or 400-500 μ l late-stage animals were fixed in 33ml 0.1M PIPES, 2mM EGTA, 1 mM MgSO₄, 3.4% Formaldehyde (ULTRAPURE ampules, Polysciences, Cat. #18814) for 5 minutes. Tween-20 was added to 0.02% and fixation continued for another 3 minutes. The *Artemia* were subsequently dehydrated using a stepwise transfer into methanol and stored at -20oC until ready to proceed with the immunodetection. Antibody staining was carried out as described for *Drosophila* embryos in Kosman, et al. (2004) with the addition of a sonication step prior to blocking. Sonications were carried out using a Branson Sonifier 150 at a maximum output of 5W in 40 ml PBT. The total sonication time was stage dependent. 100 μ l of L1 to L4 stage *Artemia* were sonicated for a total of 16 seconds, seven to eight L9-L10 stage animals for 50 seconds and seven to eight L11 to L12 stage animals for 62 seconds. Two second bursts of sonication were followed by inversion of the sample several times to mix the animals thoroughly between bursts until the total sonication time was achieved. The animals to PBT volume ratio as well as the stage specific sonication times were critical for achieving optimal permeability of reagents. For detection of nuclei to aid in staging, DAPI was added to the mounting media. Animals were mounted ventral side up. Images were

obtained using a Leica TCS SP2 AOBS confocal microscope. Though dilutions worked out for staining *Drosophila* embryos using the 4F11 antibody and FP6.87 antibody also worked well in *Artemia*, for *Artemia* stains, the rabbit α -Art ABD-A was used at a higher concentration of 1:100 to ensure that the inability to detect protein during early stages was not due to insufficient antibody.

***Artemia in situ* hybridization.**

Probes to the first 731 bp of coding sequence for *Artemia* Engrailed and to the complete coding sequence of *Artemia Ubx* and *abd-A* were prepared as described in Kosman et al. (2004). *Artemia* were fixed in the same manner as for immunohistochemistry, then hydrated using a MeOH:PBT series and sonicated as previously described. *in situ* hybridizations were carried out based on *Drosophila* protocols above with the following modifications: *Artemia* were not treated with xylenes, Protease K treatment was carried out for 2 minutes at a final concentration of 5 μ g/ml, the transfer to hybridization solution prior to the prehybridization step was carried out with reagents pre-heated to 55°C and included an extra five minute wash with hybridization solution at 55°C. The hybridization solution was modified with an addition of SDS to a final concentration of 1% as suggested by N. Patel (personal communications). Probes were hybridized for 19-19.5 hours at 55°C. After hybridization, the animals were transferred to PBT using a graded hybridization solution/PBT series pre-heated to 55°C. Subsequent washes and antibody incubations were carried out at room temperature or 4°C if carried out overnight. Detection of

probes required tyramide amplification (TSA Plus systems, Perkin Elmer, Cat. # NEL744001KT, NEL741001KT) at 1:75 for 15 minutes at RT with occasional mixing. Tyramides were resuspended in water instead of DMSO. DIG probes were detected with Cy3 tyramide and FITC probes with FITC tyramide. When detecting multiple transcripts within the same animal, sequential tyramide reactions were carried out (Kosman et al. 2004) and a post-hybridization fixation was added prior to antibody detection. The post-hybridization fixation involved incubating the *Artemia* in 1% formaldehyde for 5 minutes followed by two rinses and three 5 minute washes with PBT. Mounting was done as described above for immunohistochemistry. Images were obtained using a Leica TCS SP2 AOBS confocal microscope and deconvolved using the AutoDeblur software (MediaCybernetics).

Northern Blots.

L4 stage *Artemia* were collected by hatching 0.5g cysts as described previously. On day 2, hatched L1 nauplii were collected and transferred to fresh 15% Instant Ocean and light and aeration were continued until day 5, when L4 stage animals were collected. In this manner, ~98% of the animals were at stage L4. *Artemia* were flash frozen in liquid Nitrogen and stored until RNA was extracted using 1ml Trizol reagent (Invitrogen, Cat. # 15596-018) per 50 mg L4 *Artemia* using the manufacturer's instructions. PolyA RNA was purified using the Oligotex mRNA kit (Qiagen, Cat. #70042). Sense RNA were *in vitro* transcribed in the same manner as described above for making RNA probes except the 10X hapten-U NTP mix was

substituted with a 10X NTP mix and the RNA was not fragmented. RNA was transferred to a positively charged nylon membrane (Brightstar Plus, Ambion, Cat. #AM10102) using the NorthernMax system (Ambion, Cat. #AM1940). DIG probes made for *in situ* hybridizations were used at 1:2000 and hybridized to the membrane overnight at 68°C. High stringency washes were carried out and the membrane was blocked for 30 minutes in 1.5X Western Blocking Reagent (Roche, Cat. # 11921673001). Next, the membrane was incubated in 1:10,000 Sheep α -DIG HRP (Roche, Cat. #1 207 733) in TBS-T for 1 hour at room temperature, rinsed twice in TBS-T, followed by washes in TBS-T (15 min, 2 X 5 min). For detection, the SuperSignal West Pico Chemiluminescent Substrate detection system (Pierce, Cat. # 34080) was used following the manufacturer's instructions.

***Drosophila* embryo *in situ* hybridizations and immunohistochemistry.**

in situ hybridizations and immunochemistry were carried out as described in Kosman et al., 2004.

Hatch assays.

Ten to 20 males of the UAS-transgenic line were crossed to 20-25 virgin females carrying the *arm*-GAL4 driver on the third chromosome. Progeny from the crosses were collected for 24 hours and counted. After aging for 24 hours, unhatched embryos were counted to determine the number of embryos hatched. This number

was normalized relative to a cross of w^{1118} to *arm*-GAL4 that was carried out in parallel.

ACKNOWLEDGEMENTS

Portions of Chapter 4 appear in Hsia, C.C., Paré, A., Ronshaugen, M., Hannon, M. and McGinnis, W. “Evolution of *Hox* Gene Expression and Function and the Effect on Limb Specification in Arthropods” (in preparation). I was the primary author and responsible for the research included in this dissertation.

FIGURES

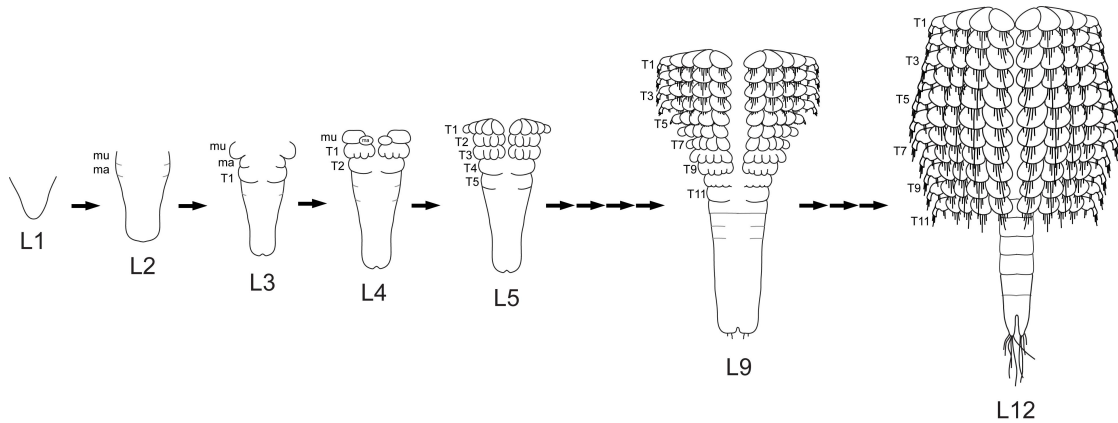


Figure 4-1: Trunk development in *Artemia*. Schematic of the trunk segments at various stages during larval development. The gnathal segments are shown in the L2 and L3 stages, otherwise head structures are not represented in these schematics. After the first molt, segmentation begins in the two gnathal segments (mu and ma). Additional thoracic segments emanate from the posterior growth zone, so that there is an anterior-posterior gradient of development with anterior segments at a further stage of differentiation than the posterior segments. Thoracomers, trunk segments with limb buds, mark the start of limb formation (e.g. T1 of L3 stage *Artemia* or T4 of L5 stage *Artemia*). As limb development proceeds, articulation begins and the trunk segments are now known as thoracopods (e.g. T2 and T3 of L5 stage). The thoracopods undergo elongation and further differentiation so that several podites form for each limb and setae develop and elongate. mu: maxillulary segment, ma: maxillary segment

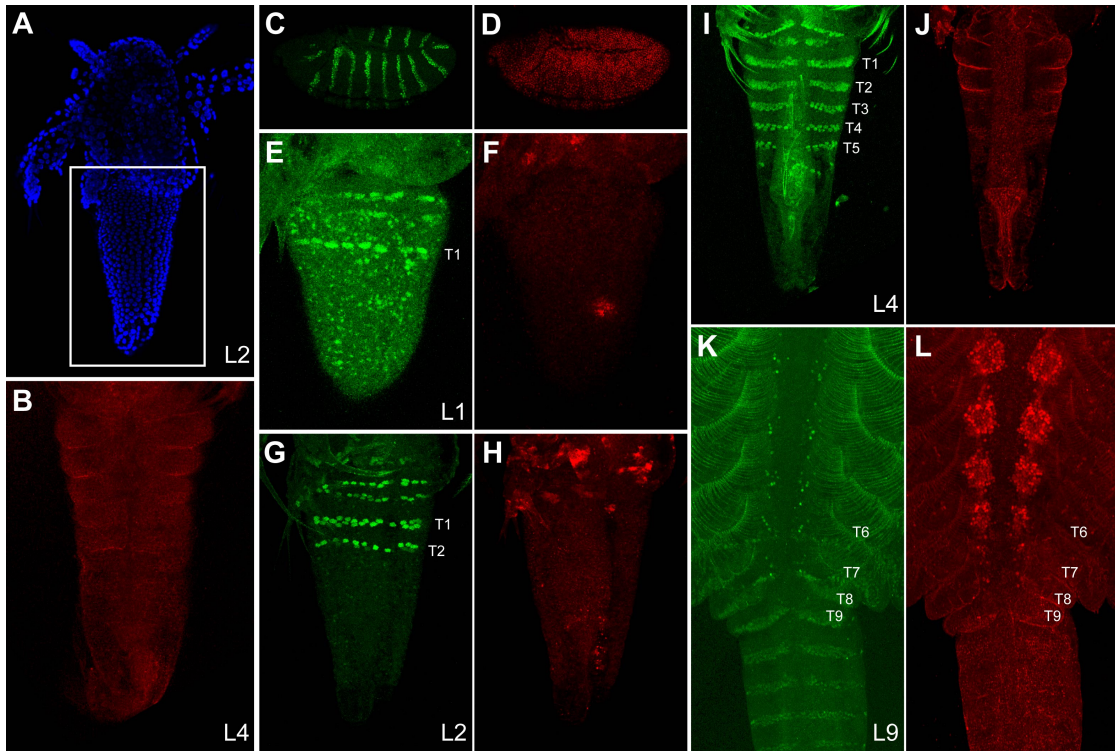


Figure 4-2: Double antibody stains with α -en and α -Art ABD-A. Engrailed is in green, ABD-A is in red and DAPI in blue. Anterior is left for *Drosophila*, up for *Artemia* for this figure and all subsequent figures. Panel A shows the DAPI stain of an L2 stage *Artemia* with a box denoting the trunk region shown for the rest of the panels in this figure and all subsequent figures of L1 to L4 stage *Artemia*. L1 to L4 mixed stages of *Artemia* were stained with *Drosophila* embryos ectopically expressing Art ABD-AC Δ . Detection of EN protein and *Artemia* ABD-AC Δ protein in *Drosophila* are shown (C, D). In *Artemia*, Engrailed stripes in the posterior of each segment are evident in early stages (E, G, I). Panels K and L show the magnification of the midline of a stage L9 *Artemia*. By the L9 stage (K), Engrailed is also detected in a few cells in the neuromeres of each segment. ABD-A protein is not expressed to detectable levels in early stages (F, H, J) but is clearly expressed in neuromeres at stage L9 (L). In the L9 stage (K, L), limbs of trunk segments anterior to T6 are fully differentiated. T6 thoracopods have almost finished differentiation. T8 thoracopods have started articulation (vertical furrows visible in panel L) and T9 thoracomeres are just about to initiate articulation. ABD-A protein expression in neuromeres have initiated in the T8 trunk segment. Engrailed protein is expressed in stripes in the undifferentiated trunk segments posterior to T9 and this striped expression starts to decrease as limb differentiation is underway (visible in T8 and faintly visible in T7). Weak Engrailed protein expression is observed in the neuromeres of trunk segment T9.

Figure 4-3: Double antibody stains with FP6.87 and α -*Artemia* ABD-A. FP6.87 is in green, ABD-A is in red. As with the previous double stains, L1 to L4 mixed stages of *Artemia* and *Drosophila* embryos ectopically expressing Art ABD-ACA Δ were pooled. Panels A and B are the negative controls for *Drosophila* embryos and show the background levels. Panel C and D are of a *Drosophila* embryo ectopically expressing truncated *Artemia* ABD-A protein. The FP6.87 antibody may have a lower sensitivity than the α -*Artemia* ABD-A antibody. In *Artemia*, FP6.87 signal is detected in early stages (F, J, H) throughout the trunk starting at the anterior border of the first thoracic segment (arrowhead). However, no ABD-A signal is detected in these animals (G, K, I). In a stage L12 *Artemia*, protein is detected by both the FP6.87 (L) and α -*Artemia* ABD-A antibodies (M) in the neuromeres. These signals are colocalized (inset in M).

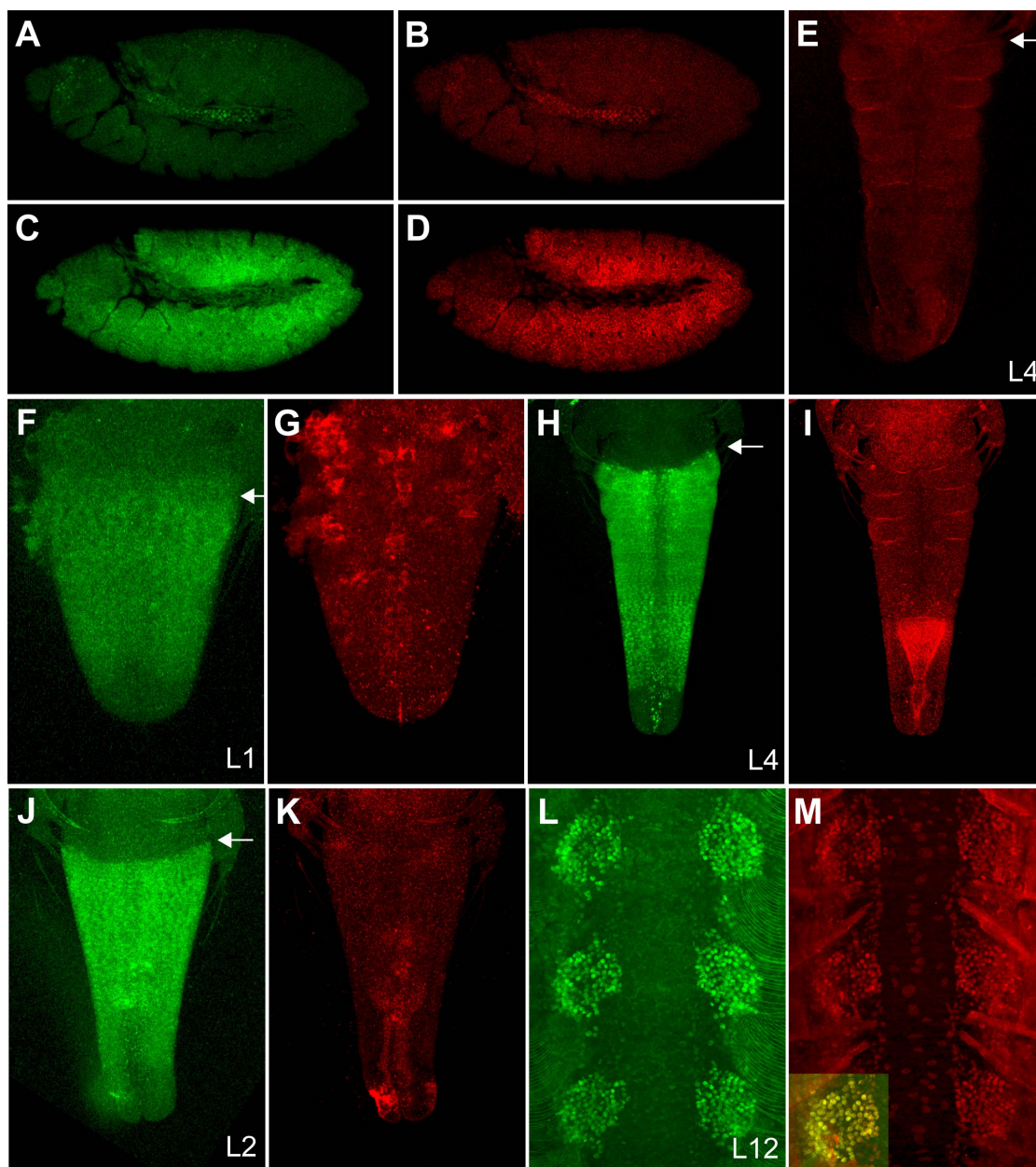
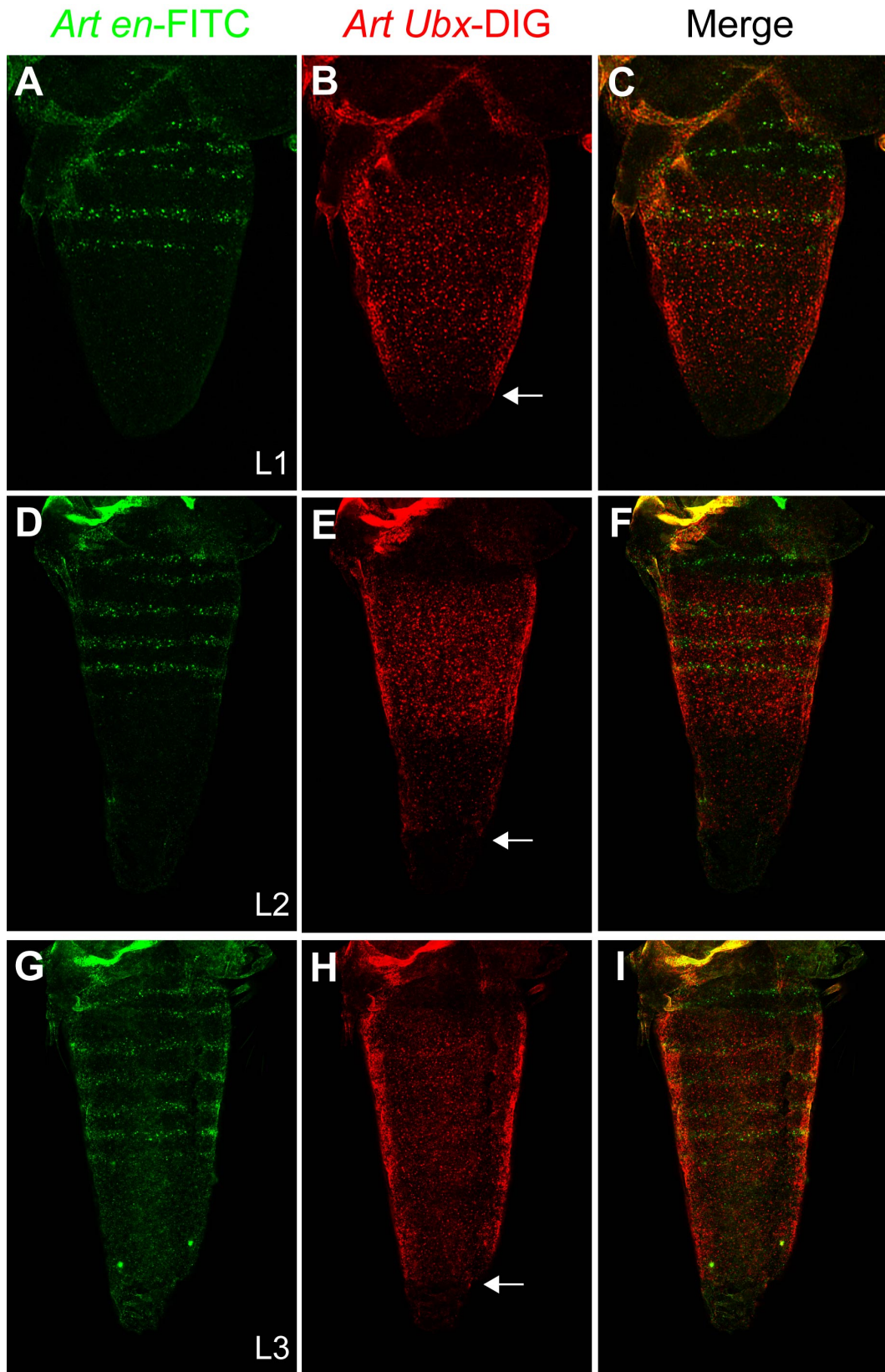


Figure 4-4: Double *in situ* hybridizations with *en* plus *Ubx* probe. *en*-FITC is in green (A, D and G) and *Ubx*-DIG is in red (B, E and H). The last column is the merge of these two channels (C, F and I). The anterior boundary of *Ubx* transcript is at the maxillary/thoracic border which coincides with the anterior boundary of FP6.87 staining. *Ubx* transcript expression extends posteriorly past the last visible *en* stripe in all stages shown and the posterior boundary (arrow) abuts what is surmised to be the growth zone.



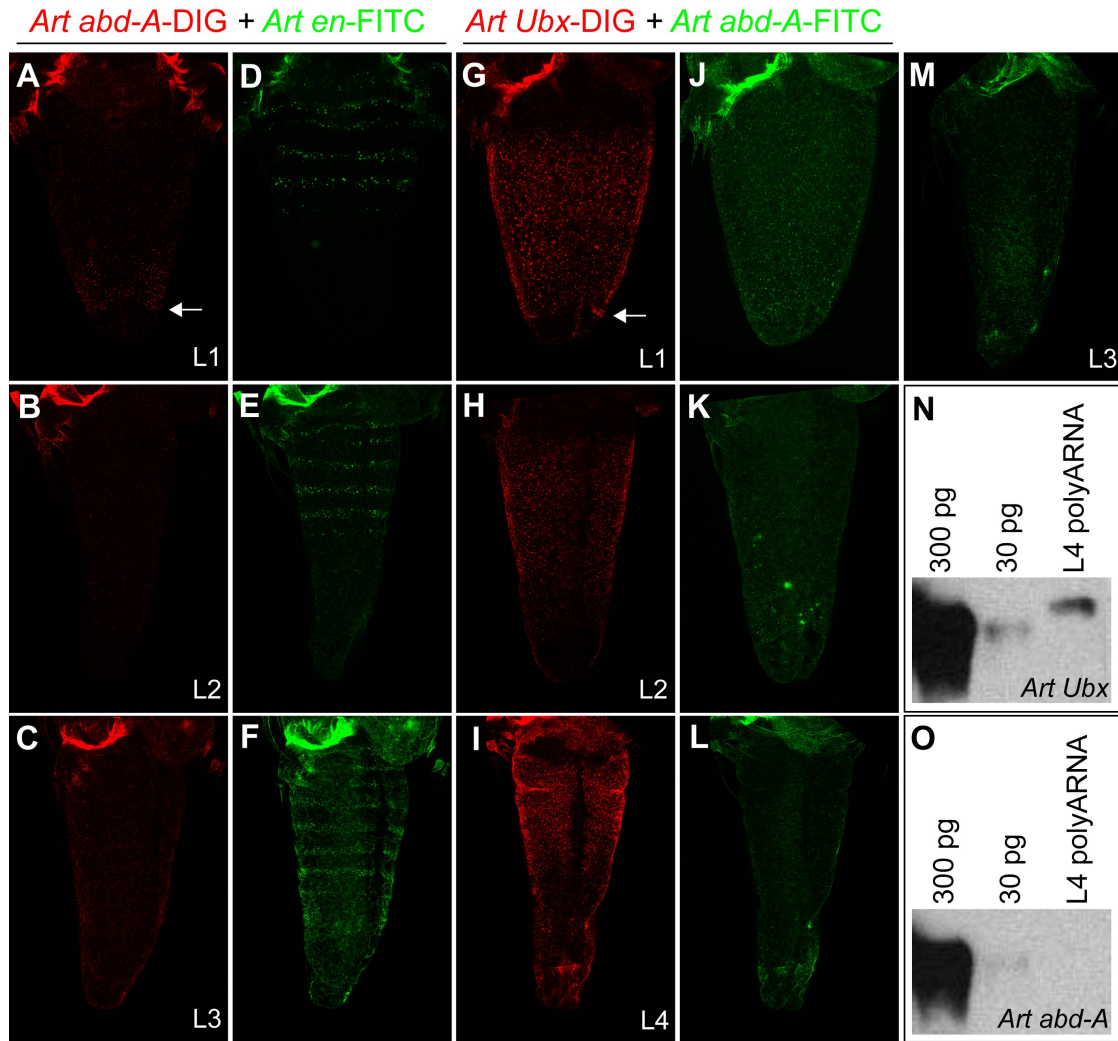


Figure 4-5: *abd-A* transcript is not detected in L4 stage nauplii. Double *in situ* hybridizations with *abd-A*-DIG (red; A, B and C) plus *en*-FITC (green; D, E and F) probes or *Ubx*-DIG (red; G, H and I) plus *abd-A*-FITC (green; J, K and L) probes. Panel M is a ~L3 stage animal stained for *Ubx*-DIG plus sense *abd-A*-FITC, with only the sense *abd-A*-FITC channel shown. The anterior boundary of the surmised growth zone is indicated by an arrow in panels A and G. Panels N and O are Northern blots hybridized with either *Artemia Ubx* (N) or *Artemia abd-A* (O) DIG probes. In Panel N, the lanes from left to right are 300 pg *in vitro* transcribed sense *Artemia Ubx*, 30 pg *in vitro* transcribed sense *Artemia Ubx* and 10 µg of polyA RNA. In Panel O, the lanes from left to right are 300 pg *in vitro* transcribed sense *Artemia abd-A*, 30 pg *in vitro* transcribed sense *Artemia abd-A* and 25 µg of polyA RNA.

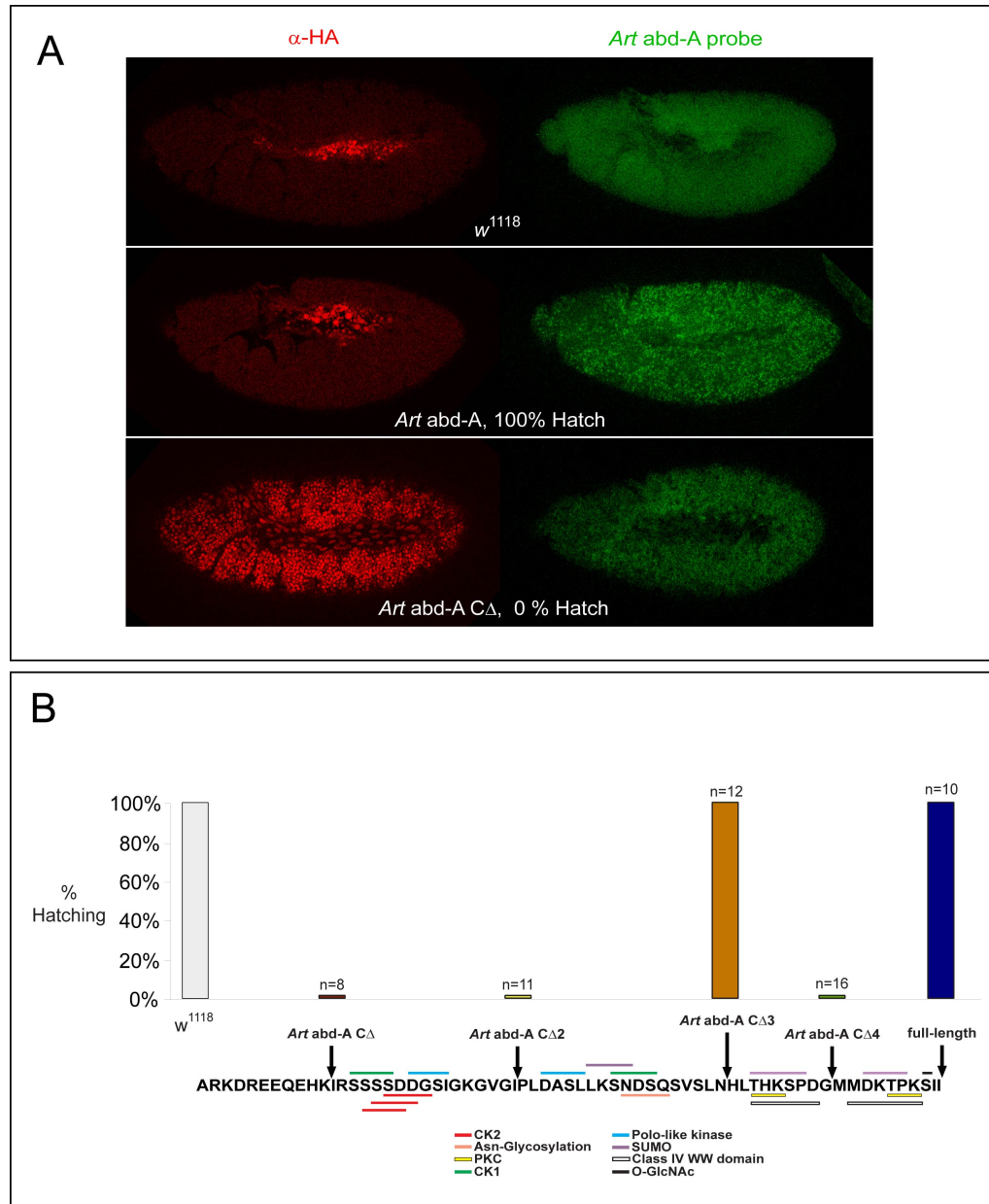


Figure 4-6: Protein expression of *Artemia* ABD-A cannot be induced in *Drosophila* embryos unless C-terminal sequences are deleted. (A) *Drosophila* embryos induced to ubiquitously express *Artemia abd-A* transgenes were tested for the presence of transcript and protein. The top panel shows the negative control of embryos from arm-GAL4 crossed to w^{1118} . Transcripts were detected in embryos expressing both full-length and truncated ABD-A, but protein was only detected for embryos expressing the truncated *abd-A* transgene. (B) Hatch assays of various *Artemia* ABD-A truncations. The truncation point for each transgene is shown below the graph. Hatch percentages were calculated relative to w^{1118} . Putative sites of post-translational modifications are marked.

Chapter 5

Concluding Remarks

On a general level, these studies suggest that evolution of transcription factor function occurs on different levels. Though cis-regulatory changes are a strong and no doubt important force in the evolution of transcription factors such as *Hox* genes, changes within protein sequences also play a part. The reluctance to assign protein sequence changes a larger role in transcription factor evolution lies largely on the correctly placed assessment that a change in protein sequence results in pleiotropic effects. However, to assume that all cases of pleiotropic phenotypes are deleterious may be an overestimation. Complete loss of specific peptide motifs within transcription factors that regulate numerous target genes has resulted in less than dramatic effects (Hittinger et al., 2005; Tour et al., 2005). A change in one motif may affect one subset of target genes minimally or drastically while leaving another subset relatively unchanged (Figure 5-1).

There are domains that are integral to transcription factor function regardless of the subset of target genes upon which it acts. These domains, such as DNA binding domains, are critical to all functions carried out by the transcription factor and, if changed, results in the deleterious, pleiotropic effects believed to be the result of protein sequence changes. However, alterations in other motifs and functional domains found within the transcription factor may have less severe ramifications. Add to that the possibility that modulation of transcription factor function may require several motifs across the protein (e.g. the repression ability of *Drosophila* UBX), and the effect of many protein sequence changes are minimal and may even go unnoticed. If transcription factors carry out their numerous functions via multiple motifs and

alterations in regulation of one subset of target genes can leave regulation of other target genes more or less unaffected, protein sequence changes need not be lethal to the organism. In some ways, altering the cis-regulation, the when and where a transcription factor is expressed, can cause far more widespread effects since it causes a change in the regulation of the complete set of target genes acted upon by that transcription factor as well as the added possibility of regulating genes not normally under its control due to target gene expression patterns.

Finally, these studies bring up the question, what is the reason for inhibiting *abd-A* expression in the pre-genital trunk epidermis of *Artemia*? A possible answer lies in the fact that *Artemia* limbs on the pre-genital trunk serve more than a locomotory function. *Artemia* are filter feeders and subsist on bacteria and unicellular algae. Starting from stage L2, the developing limbs start participating in the filter feeding of the animal which was previously carried out by the appendages in the head (Schredhardt, 1987b). Movement through the water places the *Artemia* in contact with food sources and the action of the limbs direct water flow over the food groove, found in the ventral midline, and sweeps the food toward the mouth. Additionally, the numerous setae or the hairlike projections from the limbs act to trap and concentrate the food. Later, in the adult stages the limbs also act in osmoregulation and respiration (Schredhardt, 1987b). A loss of appendages, then, could be greatly detrimental to *Artemia* as it would affect multiple aspects required for survival.

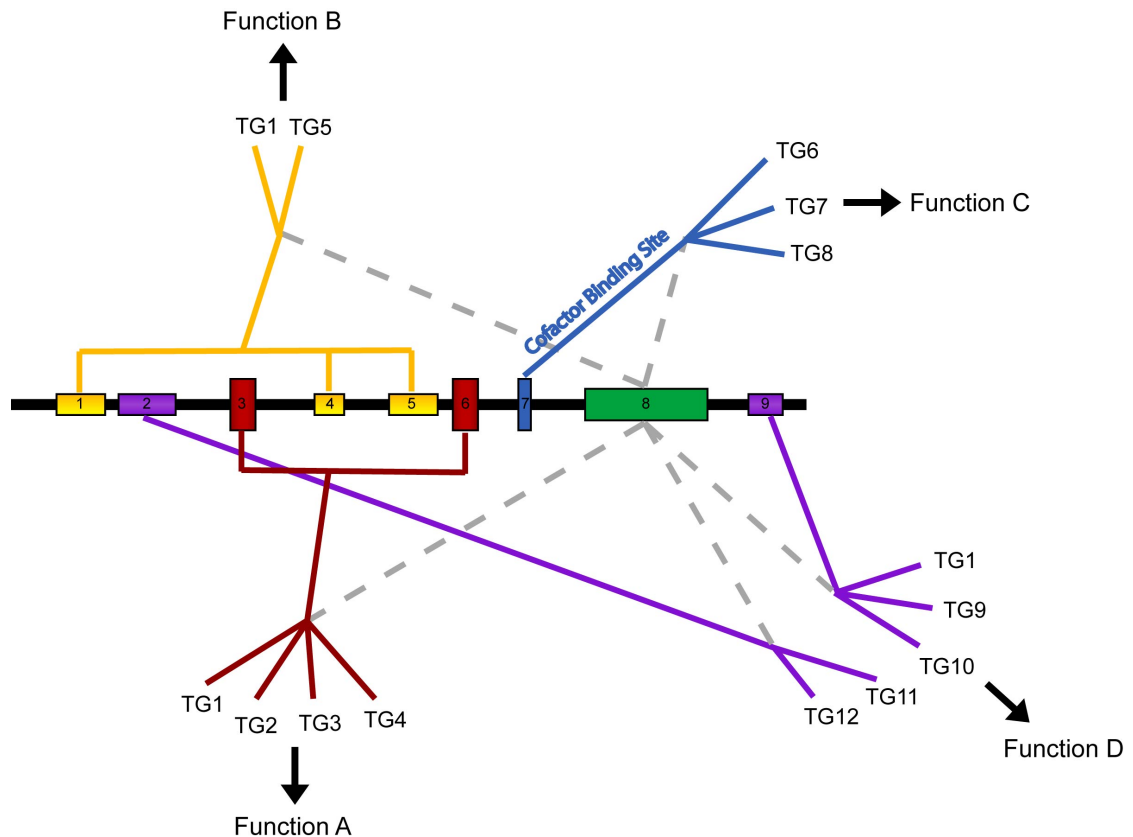
FIGURE

Figure 5-1: Model of the modulation of target gene regulation and protein function via different domains/motifs. Schematic of a transcription factor and its various domains and motifs. Same color motifs/domains act together to modulate the same function, although not necessarily the same subset of target genes (TG). Different motifs may modulate the regulation of the same target gene (e.g. TG1). Domain 8 represents a critical domain required for transcription factor function that acts on all subsets of target genes. Mutations in this domain would likely result in deleterious effects. Protein sequence changes in some motifs, for example, motif 2, may only result in mild phenotypes and slightly altered function (Function "D") with relatively no effect on other subsets of target genes. Sequence changes in motif 3 or 6 could result in pleiotropic effects to varying degrees as changes there may cause mis-regulation of TG1 which is required in several functions.

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