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Hox genes, evo-devo, and the case of the *ftz* gene

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Abstract

The discovery of the broad conservation of embryonic regulatory genes across animal phyla, launched by the cloning of homeotic genes in the 1980s, was a founding event in the field of evolutionary developmental biology (evo-devo). While it had long been known that fundamental cellular processes, commonly referred to as housekeeping functions, are shared by animals and plants across the planet—processes such as the storage of information in genomic DNA, transcription, translation and the machinery for these processes, universal codon usage, and metabolic enzymes—*Hox* genes were different: mutations in these genes caused “bizarre” homeotic transformations of insect body parts that were certainly interesting but were expected to be idiosyncratic. The isolation of the genes responsible for these bizarre phenotypes turned out to be highly conserved *Hox* genes that play roles in embryonic patterning throughout Metazoa. How *Hox* genes have changed to promote the development of diverse body plans remains a central issue of the field of evo-devo today. For this Memorial article series, I review events around the discovery of the broad evolutionary conservation of *Hox* genes and the impact of this discovery on the field of developmental biology. I highlight studies carried out in Walter Gehring's lab and by former lab members that have continued to push the field forward, raising new questions and forging new approaches to understand the evolution of developmental mechanisms.

Keywords

Hox gene; Homeotic transformation; Molecular evolution; *ftz*; Arthropods

Introduction

Homeotic genes were first characterized in the fruit fly, *Drosophila melanogaster*, on the basis of bizarre mutant phenotypes in which one body part is transformed to an alternate, fully formed body part. These types of mutations, termed homeotic transformations by W. Bateson in the 1800s (Bateson 1894), were pursued by basic researchers such as E.B. Lewis, T. Kaufman, W. J. Gehring, and others because of their striking phenotypic effects (Gehring 1966; Lewis 1978; Kaufman et al. 1980; Lewis et al. 1980; reviewed in Lewis 1994). For

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This article is dedicated to the memory of Walter Gehring.

Compliance with ethical standards

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example, an *Antennapedia* (*Antp*) mutation *Nasombemia*, studied by Gehring when a student with Professor Ernst Hadorn at the University of Zurich, results in a classic *Antp* phenotype with antennae transformed to fully formed legs (Fig. 1).

Clever analysis of homeotic mutant phenotypes led to the realization that the wild-type function of homeotic genes is to specify the unique identity(ies) of segments along the anterior-posterior body axis (e.g., (Garcia-Bellido 1975; Lewis 1978; Lawrence and Morata 1983; Lawrence and Morata 1994). Individual homeotic genes were shown to function in different body regions, and through detailed genetic mapping, Lewis discovered the “colinearity” of homeotic genes: the activity of homeotic genes along the anterior-posterior axis of the embryo mimics the order in which genes are located in chromosomal complexes of *Drosophila* (HOM-C, Fig. 2). Later, molecular studies confirmed these genetic analyses by showing that homeotic genes are expressed in colinear patterns along the anterior-posterior axis of early embryos, each expressed in the primordia of the segment(s) transformed in the corresponding mutant. Homeotic mutations visible in adults were often found to result from gain-of-function homeotic mutations. For example, the wild-type function of *Antp* is to differentiate the second thoracic segment (T2), where *Antp* is normally expressed and active, from other body segments. In gain-of-function *Antp* mutants, *Antp* is mis-expressed in the head. Regulating genes normally active in the T2 segment, this ectopic Antp promotes the formation of a T2 leg, now in an ectopic location (Frischer et al. 1986; Schneuwly et al. 1987b; Talbert and Garber 1994).

Early on, homeotic genes were classified as “selector genes” that act to select or choose specific developmental trajectories for groups of cells, providing instructions for unique pathways of growth and differentiation that are controlled directly by downstream “cyto-differentiation genes” or “realizator genes” that encode products directly involved in morphogenesis (Garcia-Bellido 1975) (reviewed in Lawrence 1992). Unlike the housekeeping genes that were known to be shared among different species of plants and animals, these homeotic genes were expected to be wholly specific to the animal in whose genome they resided: if homeotic genes are responsible for the development of things like fly legs and wings, what role could they possibly play in taxa lacking such features (especially those without an exoskeleton)? Given this mindset, it was deemed a crazy experiment—possibly a total waste of time—to search for orthologs of *Drosophila* homeotic genes in other species.

Hox genes are the key to animal development

The story began with work of Bill McGinnis, a postdoc in the Gehring lab, who identified the “homeobox”—a sequence that cross hybridized among different *Drosophila* homeotic genes on Southern blots (McGinnis et al. 1984b; Scott and Weiner 1984). This finding was a spark for thought about mechanisms controlling animal development, and McGinnis et al. took this to a new level in their 1984 Cell paper (McGinnis et al. 1984a) proposing that “The notable conservation of the homeobox sequence in pattern-formation genes of *Drosophila* suggested to us that it might also be conserved in other organisms.” To most, the latter part of the sentence would not have followed logically from the first—rather, the notable conservation of homeobox sequences in genes determining segmental identity in a fly would

have suggested relatedness among a group of genes doing similar things *specific to* a highly specialized organism, not organisms that are obviously very different from flies. Nevertheless, whether these authors were maniacs, geniuses, or both, the story continued: Bill had found homeobox sequences, not only in *Drosophila melanogaster* but also in other species of *Drosophila*. Fellow postdoc Mike Levine was chatting with John Postlethwait at a meeting and told him about these amazing results. John suggested that maybe earthworms—also segmented animals—would have homeoboxes. In one of many late night sessions in the lab, Mike and Bill discussed this idea, and Bill decided it was worth pursuing. The next day, he went out and collected up some different kinds of animals—earthworms, houseflies, mealworms, and crickets—to test whether their genomes had sequences that hybridize with the homeobox probes from *Drosophila*. The resulting “zoo blots” (Fig. 3) that identified cross hybridization with the *Drosophila* homeoboxes in these different animals arguably launched the field of evo-devo. Not only were homeoboxes found in other insects, homology to *Drosophila* homeoboxes was found in chick, mouse, and human genomes. Although the function of the homeobox in these species would not be determined for some time, the authors speculated that “Whether the homologous genes in frogs and mice function in pattern formation or as developmental switches is still unknown, but if this is true, some elements of pattern formation in metazoans (at least metameric metazoans) would seem to be mechanistically related in a very basic way, raising the possibility of universal control mechanisms of development at the molecular level” (McGinnis et al. 1984a). This speculation, which proved to be correct, changed the way we think about animal development.

Around the same time, studies down the hall from Gehring's lab, in Eddy DeRobertis' lab identified the first homeobox-containing gene outside of *Drosophila*, in the vertebrate *Xenopus laevis* (Carrasco et al. 1984; Muller et al. 1984). Importantly, these studies showed that this homeobox-containing gene was expressed during embryonic development in *Xenopus*, supporting the notion that homeobox-containing genes might have roles in patterning in distant species. Not only were homeobox-containing genes found in flies and frogs, the sequence homology found on the McGinnis zoo blots in the human genome (Fig. 3) was shown to correspond to bona fide human genes (Levine et al. 1984). These findings and other early studies (reviewed in Ruddle et al. 1985) led to flurry of activity from labs around the world identifying homeobox-containing genes in different species. A driving force behind this explosion of interest was the difficulty in identifying developmental regulatory genes in species for which experimental systems enabling large-scale genetic screens were not well-established or were prohibitively expensive. This includes an enormous range of species from nonmodel invertebrates to mammals. The conservation of homeobox-containing genes (for further discussion, see Burglin and Affolter 2015) opened up the possibility of gaining inroads into developmental processes using what is now commonly known as a “candidate gene approach” via identification of genes containing homeoboxes that could play roles in any number of processes in invertebrate and vertebrate development. As summarized in review articles published around that time, “homeo-madness” or “homeo box fever” overtook the field of developmental biology during this period, with scientists (frantically) isolating genes containing homeoboxes, mapping them and determining their expression patterns (Gehring 1985; Marx 1986; Wilkins 1986). Even

now, more than 30 years after the discovery of the homeobox, *Hox* genes are a standard “go to” genes for analysis of the development of new species (e.g., Albertin et al. 2015). Whether and how many *Hox* genes a given species possesses is a routine, basic question for genomic and developmental analysis of emerging model systems. Given the ubiquity of *Hox* genes across Metazoa, it has become the *absence* of *Hox* genes that draws attention. For example, it was striking that sponges were found to lack *Hox* genes (Fortunato et al. 2014).

As homeobox-containing genes were isolated from more and more species in the 1980s, several interesting generalities became evident: First, the clustering of homeobox-containing genes and the colinearity observed by Lewis for *Drosophila* HOM-C also occur in other animals, including mammals (Fig. 2). The group of homeobox-containing genes present in these evolutionarily conserved complexes became referred to as “*Hox* genes” because of the homeobox they share. An early study of human homeobox-containing genes revealed four *Hox* complexes, with homeodomain sequence similarities across each complex suggestive of chromosomal duplication (Boncinelli et al. 1988). Similar clusters were also found in mice (Do and Lonai 1988; reviewed in Schughart et al. 1988; Akam 1989). *Hox* complexes are thought to have arisen by duplication and divergence from an ancestral *Hox* gene in Urbilateria (reviewed in Finnerty and Martindale 1998; Cook et al. 2001; Erwin and Davidson 2002; Gehring 2012).

Many *Hox* genes were found to be expressed during early embryogenesis, suggestive of roles in development (examples of the many studies are Krumlauf et al. 1987; Utset et al. 1987; Dolecki et al. 1988; and Mavilio et al. 1988; reviewed in Holland and Hogan 1988). A major breakthrough came with the finding that murine homeobox-containing genes are expressed in a segmental fashion in the rhombomeres of the developing hindbrain, in staggered patterns reminiscent of *Drosophila* homeotic genes (Dressler and Gruss 1989; Gaunt et al. 1989; Murphy et al. 1989; Wilkinson et al. 1989). These findings eventually led to the understanding that *Hox* genes are expressed in a colinear fashion in many species, including mammals, although differing from the situation in *Drosophila* in that it is the anterior boundaries of expression of mammalian *Hox* genes, rather than the full expression domains, that correspond to their chromosomal order (reviewed in McGinnis and Krumlauf 1992).

Second, the homeobox encodes a DNA-binding homeodomain. This was first suggested on the basis of sequence similarity to yeast transcription factors (TFs) and later verified experimentally in a number of labs (Shepherd et al. 1984; Desplan et al. 1985; Muller et al. 1988) (reviewed in Gehring et al. 1994a; Gehring et al. 1994b). Homeodomain-containing proteins function as sequence-specific DNA-binding transcription factors that regulate development by activating or repressing the expression of downstream target genes. Hox proteins function as “master regulators” that control whole developmental pathways by activating or repressing groups of downstream target genes that include other TFs and genes involved directly in growth and morphogenesis, the realizator genes proposed on the basis of genetic analysis by Garcia-Bellido years earlier. The many other homeobox-containing genes that are not members of *Hox* complexes encode a large family of evolutionarily conserved TFs that function in many different tissue types in both animals and plants (reviewed in Holland 2013).

Third, functional experiments began to reveal roles for *Hox* genes in evolutionary distant species. Indeed, *Hox* genes were not only clustered and expressed like their *Drosophila* homologs, but they also actually did function to control embryonic development in distant species, including mammals. Ectopic expression of murine *Hox* genes was shown to impact mouse development, in some cases, causing abnormalities of vertebrae that could be interpreted as homeotic transformations (Kessel et al. 1990). Further, as tools were developed for targeted mutagenesis in the mouse, the *Hox* genes were among the first to be probed. Results confirmed a role for *Hox* genes in embryonic development and in particular, in axial patterning, where homeotic-like transformations were observed (Chisaka et al. 1992; Lufkin et al. 1992; Jeannotte et al. 1993). These studies led to the completely unexpected hypothesis that *Hox* genes were functionally, not only structurally, conserved during evolution.

Functional conservation of *Hox* genes

The alignment of *Hox* genes within clusters in many different animals and the growing evidence that *Hox* genes control anterior-posterior body patterning in diverse species suggested that these genes were conserved regulators of embryonic development. Just how conserved are *Hox* genes? Once again, Bill McGinnis led the field in devising an experimental test of this question. Experiments by Stephan Schneuwly in the Gehring lab had previously shown that ubiquitous expression of *Drosophila Antp* during larval development led to *Antp*-like antennal to leg homeotic transformations (Fig. 4a, b). Using this approach, the McGinnis lab reasoned that if *Hox* genes were functionally conserved, then expression in *Drosophila* of a cognate *Hox* gene from a different species would result in a corresponding homeotic transformation. In two Cell papers published in 1990, they showed that candidate orthologs of *Antp* and of *Deformed (Dfd)* from mammals functioned as did their fly cognate genes when mis-expressed in *Drosophila* (see Fig. 4c). Specifically, a mouse ortholog of *Antp* generated antennal to leg transformations when expressed in *Drosophila* larvae and thoracic transformations when expressed in embryos (Malicki et al. 1990). Even a human *Hox* gene, an ortholog of *Dfd*, mimicked *Drosophila Dfd* effects when expressed in flies (McGinnis et al. 1990). These remarkable results suggested that the function of *Hox* genes has been retained during the >500 million years since the divergence of protostomes and deuterostomes.

Later, Jack Zhao in my own lab used this same approach to assess conservation of *Sex combs reduced (Scr)* genes (Fig. 4d). He found that expression of a mouse ortholog of *Scr* resulted in transformation of antennae to first leg, the leg specified by fly *Scr*. Further, the mouse gene correctly regulated expression of a *Drosophila Scr* target gene (Zhao et al. 1993). Follow-up experiments using this same approach with the mouse ortholog effectively mapped *Scr*-family protein domains on the basis of their functional potential when expressed in flies (Zhao et al. 1996).

Hox genes are shared among metazoans but body plans differ

If expression of mammalian *Hox* genes can promote development of fly legs, why do mice and humans not have fly-like legs? We believe that this can be explained, in part, by the

DNA-binding properties of Hox proteins. Hox proteins share very similar homeodomains and, in vitro, bind to very similar DNA sequences and with low specificity (Laughon 1991). This allows for tremendous flexibility in their DNA sequence binding-site selection in the genome—too much flexibility in fact, since Hox proteins alone would bind to millions of sites in the genome and different proteins would bind the same sites (the “Hox paradox” reviewed in Mann 1995; Mann and Carroll 2002; Mann et al. 2009). The biological specificity, which is unique to each Hox protein, results from interaction with protein cofactors such as extradenticle (Exd; mammalian Pbx). Exd, a TALE class homeodomain protein, was found to interact with many Hox proteins, increasing their DNA-binding specificity in vitro and in vivo (Chan et al. 1994; Johnson et al. 1995; Chan and Mann 1996; Zhao et al. 1996; Crocker et al. 2015). It is this partner or cofactor interaction and thus sequence-specific DNA binding that has been retained during animal radiations. Accordingly, cognate Hox proteins share DNA-binding specificity and thus the ability to bind the same DNA target sites. When mammalian Hox proteins are expressed in *Drosophila* cells, they recognize and bind to these DNA sequences, the same sequences that the cognate *Drosophila* Hox protein would bind. This explains the ability of mammalian proteins to “correctly” regulate the target genes of fly Hox proteins and thus generate ortholog-specific phenotypes.

What is not explained by this is how the same set of *Hox* genes has changed to control the development of animals with diverse morphologies. This is a question that continues to drive the field of evo-devo today. Invoking many such studies not only of *Hox* genes but also of additional gene families that are shared widely across animal taxa, the concept of the “genetic toolkit” emerged (Carroll 2000; Carroll et al. 2005; Prud'homme et al. 2007). This genetic toolkit is comprised of a set of regulatory genes—encoding TFs such as the Hox proteins, as well as signaling proteins—that are shared by diverse species but are utilized in different ways in different species such that different body plans and morphologies arise. One mechanism underlying species variation is change in *cis*-regulatory elements (CREs) that control toolkit genes. As discussed extensively by others, the modular nature of CREs gives them enhanced flexibility since change in one CRE can result in loss or gain of an expression domain without impacting other expression domains for the same gene (Carroll 2008; Stern and Orgogozo 2008). Thus, *Hox* genes can be expressed in new domains without detrimental effects on embryonic development since the embryonic expression patterns will not be affected by acquisition of new, separate CREs (Fig. 5, upper left). These changes can have large impacts on body patterning: for example, variation in expression of *Hox* genes has been associated with the evolution of the limbless snake body plan from tetrapod ancestors (reviewed in Mansfield 2013).

Perhaps even more frequent than changes in the expression of *Hox* genes themselves is the gain and loss of *Hox* targets due to changes in CREs of realizator or downstream genes (Fig. 5, upper right). Examples of this include elaborate modifications of the insect thorax, such as horns in *Onthophagus* beetles (Wasik et al. 2010) and helmets in treehoppers (Prud'homme et al. 2011), the acquisition of sexually dimorphic features such as male sex combs in *Drosophila* species resulting from the sex determination gene *doublesex* coming under control of Scr (Tanaka et al. 2011), and variations in abdominal pigmentation in drosophilids (Jeong et al. 2006). It is likely that the loose DNA-binding specificity of Hox proteins makes

them particularly amenable to this type of change, as many Hox binding sites may be preexisting in genomic regions, requiring only changes in chromatin structure or modifications of cofactor binding sites to bring a new gene under *Hox* control. Thus, the low specificity of Hox DNA binding may in part explain the extreme conservation of *Hox* genes in diverse species.

A third, more recently noted way in which changes in *Hox* genes can impact change in developmental strategies occurs at the posttranscriptional level (Fig. 5, lower right). For example, Bill McGinnis' lab found that the *Hox* gene *abd-A* is transcribed in a typical *Hox*-like pattern in *Artemia* but fails to function because it is not translated (Hsia et al. 2010). In contrast, *abd-A* is transcribed, translated, and functional in insects. Changes in noncoding regions allow for gain and loss of translational control elements that can be transcript-specific, again allowing for both the maintenance of conserved *Hox* functions and for variation in other functions, as would changes in splice sites or similar nontranscriptional regulatory elements.

Finally, protein sequence change in Hox proteins can alter their function during evolution, allowing for novel regulatory activities that impact development (Fig. 5, lower left). This mode of change is considered to be an infrequent occurrence, since functional changes in embryonic TFs would be expected to negatively impact development. However, we argue that this type of change is not nearly as infrequent as thought and it is just an understudied mode of change. For example, the conservation of function of Hox proteins across species cited above (and including work from this author's lab) demonstrated that Hox proteins from mammals can perform fly-like functions when ectopically expressed in *Drosophila*. However, these papers also revealed differences in functional properties. For example, in addition to the Scr-like effects, ectopic expression of mouse *Hox 1.3* in *Drosophila* caused Dfd-like transformations in the head of some adults (Zhao et al. 1993). Further, in all of these trans-species experiments, genes from distant species were less toxic than the *Drosophila* orthologs. This sort of finding is usually dismissed because of divergence in general factors that are less compatible between distantly related species. This is probably true, but what it reflects is change in protein function of TFs during evolution. Because these changes may be subtle and likely occur very slowly over evolutionary time, they are easily ignored. At the time that these trans-species experiments were done, the finding that functions were conserved at all over such large evolutionary spans was totally unexpected. Subtle differences in function were nothing compared to the major differences that were anticipated, and this is why they were mentioned in those publications but not emphasized. For an excellent discussion of this issue, see Lynch (2009). As discussed in detail below, extensive change in Hox protein function has been well documented for the *Hox* gene *fushi tarazu* (*ftz*) and is explained, at least in part, by the modular nature of proteins (reviewed in Lynch and Wagner 2008; Wagner and Lynch 2008).

***ftz*: one of the original homeobox-containing genes**

The last time this author met Walter Gehring was at the Molecular Mechanisms in Development and Evolution Symposium held in Basel, Switzerland, in March 2014 to celebrate his 75th birthday and the 30-year anniversary of the discovery of the homeobox. At

that meeting, I gave a talk about our progress toward understanding the functional evolution of *ftz*. Walter's comment after my talk was that I should "write the *ftz* book." In keeping with this, and since I never will write a book on this topic, I use the "*ftz* story" as an illustration of the findings and surprises in *Hox* evo-devo research.

The *ftz* gene was first identified by Wakimoto and coworkers on the basis of a mutant phenotype for this gene found as part of a screen for mutations in the *Drosophila* Antennapedia Complex (ANT-C) (Wakimoto and Kaufman 1981; Wakimoto et al. 1984). The description of this phenotype, given in detail in this original paper, made more sense after the massive screen carried out by Nusslein-Volhard and coworkers described mutations resulting in loss of alternate body segments, identifying a class of genes they named the pair-rule genes (Nusslein-Volhard and Wieschaus 1980; Jurgens et al. 1984). *ftz* is one of nine *Drosophila* pair-rule genes that serve to establish the basic body segments of the fly. Cloning of the *ftz* gene, followed by in situ hybridization and immunolocalization, revealed that *ftz* is expressed in stripes in blastoderm stage embryos in the primordia of the alternate parasegments missing in *ftz* mutants (Hafen et al. 1984; Weiner et al. 1984; Carroll and Scott 1985) (Fig. 6). These findings for *ftz*, and most other pair-rule genes, suggested that restriction of their expression to specific cells (sets of stripes) in early embryos is a key to the establishment of the segmental body pattern. Rather quickly, it was observed that *ftz* and a second homeobox-containing pair-rule gene, *even-skipped* (*eve*), are expressed in roughly complementary sets of stripes and that the anterior border of these stripes overlaps exactly with alternate stripes of *engrailed* (*en*), a segment polarity gene thought to be responsible for establishment of segmental borders (Lawrence and Johnston 1989). It has since been shown that Ftz directly regulates alternate *en* stripes through an intronic *en* enhancer (see below). Unlike the other eight pair-rule genes, *ftz* is present in the ANT-C, between homeotic genes *Scr* and *Antp*, and its localization in that chromosomal position is broadly conserved (see Fig. 2). In fact, *ftz* was one of the three genes, *Ubx* and *Antp* being the other two, used to identify the first homeobox (McGinnis et al. 1984b; Scott and Weiner 1984). Ftz encodes a DNA-binding TF, and its DNA-binding is so similar to that of its neighbors, *Scr* and *Antp*, that Markus Affolter predicted the Ftz binding sites he found in the *ftz* upstream element (Pick et al. 1990) based on published binding sites for mouse Hox 1.3, an *Scr* ortholog (Odenwald et al. 1989). Yet, the *ftz* pair-rule phenotype is distinctly nonhomeotic (Fig. 6b).

What is a pair-rule gene doing in the *Hox* complex?

As mentioned above, *ftz* mutants display a typical pair-rule phenotype, with alternate body segments missing. In contrast, mutations in homeotic *Hox* genes result in transformation of one body part into an alternate body part, as their wild-type function is to specify the identity of segments along the anterior-posterior body axis. However, the location of *ftz* in HOM-C, along with its *Hox*-like homeobox and DNA-binding, suggested that *ftz* arose as a duplication of an ancestral *Hox* gene. Unlike homeotic *Hox* genes, *ftz* is expressed in stripes in early embryos (Fig. 6c, yellow), while homeotic *Hox* genes are expressed in broad bands in the primordia of the segments whose identity they specify. This suggests that *ftz* acquired CRE(s) to generate the seven-stripe pair-rule pattern while also losing CREs for an ancestral *Hox*-like pattern of expression. Was the functional change in *ftz* from an ancestral homeotic gene to a pair-rule gene in *Drosophila* solely due to this change in expression pattern? Or,

did changes in the protein sequence occur that gave *Drosophila* Ftz a new function? To address this question, Ulrike Lohr, a graduate student in my lab, used the “Schneuwly method” to ectopically express *ftz* in a manner that reveals homeotic function for Hox proteins such as Antp, Scr, and their mammalian orthologs (Fig. 4). Strikingly, expression of *Dm-ftz*, either ubiquitously by heat shock or more persistently in imaginal discs with the *UAS-GAL4* system, did not result in an antennal to leg transformation (Lohr et al. 2001) (Fig. 7a, b). In contrast, the *ftz* ortholog from the beetle, *Tribolium castaneum* (*Tc-ftz*), generated a perfect antennal leg when expressed under the same conditions (Fig. 7c). Further, *Tc-ftz* but not *Dm-ftz* repressed expression of Hth when expressed in imaginal discs, in a fashion expected for a homeotic *Hox* gene (Lohr et al. 2001). These experiments indicated that *Dm-Ftz* has lost the potential to function as a homeotic protein, even when force-expressed in a homeotic fashion. In contrast, *Tc-ftz*, as well as the grasshopper *ftz* (*Sg-ftz*, Fig. 7d), retains homeotic potential. These findings provided strong support for the notion that *ftz* arose as a homeotic gene and that its function changed during evolution in lineages leading to *Drosophila melanogaster*. Further, based on both morphology and gene regulation, Ftz proteins from basally branching species behaved most similarly to Antp, suggesting that modern day *ftz* and *Antp* arose from an ancient duplication (see also (Telford 2000) who reached the same conclusion based on protein sequence).

Changes in Ftz protein sequence switched cofactor interactions

The findings summarized above demonstrated homeotic potential for *ftz* genes from species branching basally to *Drosophila*. At the same time, experiments in which the *ftz* genes were expressed ubiquitously in blastoderm embryos showed that both *Dm-Ftz* and *Tc-Ftz* efficiently generated anti-*ftz* phenotypes (Struhl 1985) indicative of segmentation function, while *Sg-Ftz* did not (Lohr et al. 2001). Examination of protein motifs present in these three proteins revealed the shared N-terminal arm of the homeodomain, indicative of all Ftz proteins, but differences in known protein interaction motifs. Specifically, those proteins capable of generating homeotic transformations contain the YPWM motif upstream of the homeodomain required for interaction with Exd (Johnson et al. 1995; Chan and Mann 1996), while those with segmentation potential contain an LRALL sequence.

To explain the significance of the LRALL sequence, it is necessary to back up in time a few years and discuss experiments done to probe how *Dm-Ftz* regulates target genes in *Drosophila* embryos, target genes different from those regulated by the homeotic Hox proteins. Yan Yu in my lab undertook a modified yeast interaction screen to identify partners for Ftz, using a Ftz-responsive CRE, the *ftz* proximal enhancer (Pick et al. 1990; Han et al. 1998; Yu et al. 1999; Pick et al. 2000) (also named *ftz* autoregulatory element (Schier and Gehring 1992; Schier and Gehring 1993). Our logic was that partners of Ftz might coordinately bind DNA with Ftz and that their isolation would be facilitated by screening with a native CRE that would also include binding sites for a partner protein. The orphan nuclear receptor Ftz-F1 was isolated in this screen and shown to form a stable, immunoprecipitable complex with Ftz in wild-type embryos (Yu et al. 1997). Ftz and Ftz-F1 bind DNA, activate transcription synergistically, and are each required for expression of alternate *en* stripes, a gene long known to be regulated by *ftz*. The interaction between Ftz and Ftz-F1 was found more or less at the same time by us and another former Gehring lab

member, Henry Krause (Guichet et al. 1997; Yu et al. 1997). Ftz-F1 is expressed in all somatic nuclei of early *Drosophila* embryos (Fig. 6c, green), which was at first disappointing to us as we thought that it might be a general cofactor enhancing Ftz activity rather than conferring specificity. However, when we presented this work at a *Drosophila* Research Conference, students Yan Yu and Miyuki Yussa met Willis Li from Norbert Perrimon's lab. Perrimon's lab had carried out a screen for maternal effects of zygotic lethal alleles and had a P-element insertion in the *ftz-f1* gene that produces a pair-rule phenotype in germline clone embryos (Perrimon et al. 1996; Yu et al. 1997). Working together, we confirmed that *ftz* and *ftz-f1* mutants display indistinguishable pair-rule phenotypes and that the same set of alternate *en* stripes are lost in either *ftz* or *ftz-f1* mutants (Yu et al. 1997). Similarly, Anne Ephrussi's lab isolated a maternal effect allele in *ftz-f1* that generated pair-rule mutant embryos, and a Ftz/Ftz-F1-dependent enhancer was identified in an *en* intron that binds both Ftz and Ftz-F1 and directs expression of alternate *en* stripes (Florence et al. 1997; Guichet et al. 1997). Together, these studies demonstrated that Ftz works together with Ftz-F1 to promote the formation of alternate body segments and regulate target genes, such as *ftz* itself and *en*. Later studies from our group have identified additional targets of Ftz and Ftz-F1, all of which require both partner proteins for expression (Bowler et al. 2006; Hou et al. 2009; Field et al. 2015, Targets of *Drosophila* Ftz-F1 are coordinately regulated by Ftz, submitted). The reliance of Ftz on a DNA-binding cofactor for target site selection explained its ability to regulate a discrete, nonhomeotic set of target genes in *Drosophila* embryos and also explained the ability of Ftz proteins to regulate at least some native targets in the absence of its homeodomain (Fitzpatrick et al. 1992; Copeland et al. 1996).

Once the Ftz/Ftz-F1 interaction was documented, experiments began to define the protein regions required for this interaction. The LRALL sequence was shown by the Krause lab and by our lab to mediate interactions with Ftz-F1. This LRALL sequence conforms to a typical LXXLL motif present in nuclear receptor coactivators, a motif that binds directly to nuclear receptor AF-2 domain, releasing transcriptional activity. As documented with several different techniques, the Ftz LRALL sequence directly binds the AF-2 domain of Ftz-F1 and likely functions as a coactivator, releasing Ftz-F1's transcriptional potential. Accordingly, the LRALL motif is necessary for Ftz segmentation function in vivo (Schwartz et al. 2001; Yussa et al. 2001; Suzuki et al. 2003; Yoo et al. 2011; Lu et al. 2013).

Returning to the experiments done by Ulrike Lohr: The presence of a YPWM motif in Ftz proteins that were able to perform homeotic functions when expressed in *Drosophila*, and the LXXLL sequence in those able to generate an anti-*ftz* segmentation phenotype, suggested that loss of the YPWM motif during evolution led to loss of homeotic potential for *Dm*-Ftz. To test this, the degenerate FNWS sequence in *Dm*-Ftz, present in the position of homeotic YPWM motifs just upstream of the homeodomain, was replaced with a standard YPWM motif (Lohr and Pick 2005). This modified protein generated an antennal to leg transformation when expressed in *Drosophila* (Fig. 7e). Further, inactivating mutations in the LRALL sequence combined with addition of a YPWM motif resulted in stronger homeotic transformation (Fig. 7f). Together, these experiments suggested that changes in the function of Ftz protein occurred during radiation of insect lineages, with *Dm*-Ftz losing the YPWM motif and the potential to function as a homeotic protein and gaining the ability to interact with a new partner, Ftz-F1, and participate in new regulatory processes, segment formation.

When did *ftz* expression and function change during evolution?

As summarized above, multiple changes occurred in *ftz* to switch its function from an ancestral homeotic gene to a pair-rule gene in *Drosophila*: at least two changes in its protein sequence and a switch in expression pattern from homeotic-like to pair-rule stripes. When and in what order did these changes occur? This question was bravely taken on by a graduate student in my lab, Alison Heffer, in collaboration with an arthropod systematist, Dr. Jeff Shultz (Heffer et al. 2010). Simply thinking about this problem did not provide a clear hypothesis for the order of expression versus protein change: if *ftz* took on segmentation function before loss of homeotic expression, a segmentation protein would have been expressed in a homeotic pattern; conversely, if *ftz* expression changed before it took on segmentation potential, a homeotic gene would have been expressed in stripes. We decided to sample *ftz* genes across a broad range of species, obtaining full-length sequence to identify protein interaction motifs that might be predictive of function. Results showed that the LXXLL motif was stably acquired at the base of the holometabolous insects, with all *ftz* genes sampled in this large and diverse clade retaining this sequence (Fig. 8, green). In contrast, the YPWM motif degenerated at least six times independently during arthropod radiations (Fig. 8, degen).

With respect to expression pattern and despite initial predictions, *ftz* expression changed multiple times in arthropods. In the most basally branching species sampled, *ftz* expression is homeotic-like (e.g., (Telford 2000; Hughes and Kaufman 2002; Janssen and Damen 2006; Papillon and Telford 2007; Green and Akam 2013). In the brine shrimp, *Artemia*, *ftz* expression was only marginally detectable, and this very weak expression was reminiscent of homeotic expression. In several species, neither homeotic nor striped expression was observed. *ftz* stripes were observed most basally in the firebrat *Thermobia domestica* and were observed in all holometabolous insects examined (Fig. 8). However, they are absent in the grasshopper, *Schistocera gregaria*, which expresses *ftz* in the growth zone and developing nervous system (Dawes et al. 1994). It remains to be determined whether striped expression was gained independently in two insect lineages (that leading to *Thermobia* and separately, in a branch leading to holometabolous insects) or was acquired in basal insects and lost in lineages leading to *Schistocerca*. Finally, honeybee *ftz* is maternally expressed—another new mode of expression gained within holometabolous insects (Wilson and Dearden 2012). Several conclusions can be drawn from this analysis: (1) *ftz* homeotic expression was lost before striped expression arose; (2) loss of homeotic function was relatively common and likely had no phenotypic consequence; (3) Ftz proteins found in extant species can have either a YPWM motif, an LXXLL motif, both motifs or neither; and (4) both the LXXXX motif and pair-rule stripes were acquired at the base of the holometabolous insects, suggesting that they are retained due to necessary functions. What the ancestral or modern day function(s) are and what the consequences of these acquisitions were remain, to be determined.

Why is *ftz* present in all arthropod genomes?

The loss of *ftz* homeotic expression and function is relatively easy to understand and would be typical of a gene duplication event in which one duplicate is free to lose function retained

by the other duplicate (Ohno 1970; Force et al. 1999; Lynch and Force 2000). Why was *ftz* not lost completely from at least some arthropod genomes? As noted first for *Drosophila* and *Schistocerca*, *ftz* is expressed in a discrete set of cells in the developing central nervous system (CNS) of each segment of the embryo (Dawes et al. 1994). This expression is highly conserved among arthropods (Fig. 8, orange) and even in Lophotrochozoa, where the *ftz* ortholog *Lox5* is expressed in a *Hox*-like fashion exclusively in the CNS (Kourakis et al. 1997; Telford 2000). Thus, expression in the CNS appeared to be the only stable feature of *ftz*, which may be the feature that provides selective pressure against its total loss. We reasoned that if *ftz* CNS function is the broadly conserved essential function of this gene, then neither motif that varies in different Ftz proteins would be required for its CNS role (Heffer et al. 2013b).

Making use of a fly stock established long ago in the Gehring lab, graduate student Alison Heffer was able to test this hypothesis in *Drosophila*. Yash Hiromi, in one of the first papers to use reporter genes in *Drosophila* to analyze CREs controlling expression of an embryonic regulatory gene, identified three major control elements for *ftz*: the zebra element, the neurogenic element, and the upstream element (Hiromi et al. 1985; Hiromi and Gehring 1987). In collaborative work with Chris Doe, Yash showed that expression of *ftz* under control of the zebra and upstream elements rescued the segmentation defects associated with *ftz* mutant embryos, allowing them to examine direct roles of *ftz* in the developing CNS. They found that Ftz is necessary for proper development of RP2 neurons and that Eve expression is lost specifically in these cells in *ftz* mutants (Doe et al. 1988). Luckily for us, Urs Kloter, long-time Gehring-lab member, had saved this “*ftzK*” rescue line, and we obtained it to examine the role of Ftz domains in CNS function. As Yash had shown previously, the *ftz* neurogenic element directs expression exclusively in the developing CNS (Hiromi et al. 1985), in the cells in which endogenous *ftz* is expressed. Alison used the neurogenic element to drive expression of altered Ftz proteins in the CNS in animals carrying the *ftzK* rescue construct, allowing her to assess the role of these protein domains specifically in the CNS function of *ftz*. Expression of wild-type Ftz fully rescued expression of Eve in RP2 neurons (Fig. 9), as expected. Neither protein domain that has varied during evolution was necessary for this rescue: proteins with altered LRALL or FNWS sequences both efficiently rescued Eve expression (note: rescue with the LRALL mutant was slightly less effective than wild type), while Ftz lacking its homeodomain was not able to rescue CNS function. Interestingly, the N-terminal arm of Antp substituted for the N-terminal arm of Ftz in the CNS, suggesting that the functional specificity harbored in this region of the homeodomain does not contribute to Ftz specificity in this tissue. However, the full-length Antp protein did not support rescue, suggesting that other differences between Ftz and Antp have arisen since an initial gene duplication event, which had functional consequences.

In sum, these experiments suggest that Ftz proteins lacking either the LXXLL motif or the YPWM motif function effectively in the CNS. As a number of Ftz proteins from extant species lack one, the other, or both of these motifs, a role for them in the CNS may provide selective pressure against *ftz* nonfunctionalization, even if these genes are not expressed or do not function in any other tissue. Consistent with this hypothesis is the one possible exception: thus far, no CNS expression of *ftz* has been detected in *Oncopeltus*, and the unusual structure of this gene suggests that it may be a pseudogene (Y. Lu and L.P.,

unpublished). These results further suggest that it was an early acquisition or subfunctionalization of a neurogenic CRE that differentiated *ftz* from *Antp*, giving *ftz* a unique, nonredundant role in CNS development.

Gain of function changes in an embryonic regulatory gene

The loss of *ftz* homeotic expression and function are relatively easy to understand because *Antp* retained these activities. Overlap between the ancestral homeotic expression pattern of *ftz* with that of *Antp* or even *Scr* ensured the retention of homeotic function by the “traditional” homeotic gene, allowing *ftz* to diverge. But how can a regulatory gene gain new functions without perturbing a regulatory system? How are regulatory interactions re-wired to bring new target genes under control of a TF expressed in a new set of cells? What are the impacts of such changes? These questions are being addressed in our laboratory, and while we do not have hard answers, current data allow us some speculation.

Loss of *ftz* homeotic-like expression appears to have predated the expansion of insect lineages, as no hexapod studied to date expresses *ftz* in a homeotic-like pattern (Fig. 8). This loss of expression may have resulted from accumulated small changes in positively acting homeotic-CRE(s), which could have resulted in gradual decreases in expression domains or expression levels. For example, expression of *ftz* in *Artemia* was homeotic-like but so weak as to be almost undetectable (Heffer et al. 2010). Alternatively, acquisition of silencers may have led to loss of expression, as seen recently for color patterning genes (Johnson et al. 2015). We previously proposed that it was loss- or low-level expression of *ftz* in embryos that was permissive for gain-of-function changes in its protein function. That is, if a protein with a new function is expressed at high levels in embryos, it is likely to be highly deleterious to development. However, at low levels, this protein may have little effect on gene regulation, thus avoiding negative selection. Lacking expression in a homeotic domain, and with *Antp* sufficient to carry out homeotic function, little pressure remained for Ftz to interact with Exd and perform homeotic functions. Accordingly, retention of the YPWM motif is “flickering” across the tree, likely retained in some species (Fig. 8, blue) by drift rather than selection.

Ftz gained new expression patterns more than once as expression has been detected in stripes (*Thermobia* and all holometabolous insects), in the growth zone (*Schistocerca*, (Dawes et al. 1994), and ubiquitously in embryos due to maternal deposition (honeybee, (Wilson and Dearden 2012). Separately, it gained the LXXLL motif that enables interaction with Ftz-F1 (Fig. 8, green). The latter acquisition of a new protein motif appears to have occurred at the base of holometabolous insects and has been maintained in every holometabolous insect examined. This is highly suggestive of function but, despite our original expectations, this function may not always be in Ftz-F1-dependent pair-rule patterning. Several lines of evidence suggest that *Tribolium ftz* does not have pair-rule function even though it has an LXXLL motif and is expressed in pair-rule stripes (Brown et al. 1994). A large genomic deletion removing several *Hox* genes including *ftz* did not cause a pair-rule phenotype (Stuart et al. 1991). Further, knockdown with parental RNAi (Choe et al. 2006) or embryonic RNAi (A. Heffer and L.P., unpublished) did not cause segmentation defects. Thus, either *Tribolium* Ftz lost a role in pair-rule patterning and an interaction with

Ftz-F1 present in ancestral holometabolous insects, or the Ftz/Ftz-F1 interaction was acquired in a different lineage during the radiation of holometabolous insects. This conundrum remains to be investigated, but preliminary data suggest that the role is ancestral to holometabolous insects as *ftz* from other beetles has pair-rule function (J. Xiang and L.P., unpublished). In addition to the changes in “variable” Ftz protein motifs (LXXLL and YPWM), the Ftz and/or Antp proteins diverged such that Antp did not substitute for Ftz in the CNS (see above). The functional differences in protein sequence and whether it is extant Ftz or Antp that represents more closely the basal condition remain to be investigated.

The gain of the LXXLL motif allowed Ftz to interact with Ftz-F1 and possibly other nuclear receptors. This interaction results in a completely different DNA-binding specificity from that of homeotic Hox proteins that interact with Exd: Ftz-F1 has a zinc finger DNA-binding domain and stringent consensus binding site found in all Ftz/Ftz-F1 CREs, while Exd is a homeodomain protein. Thus, Ftz/Exd complexes will bind to different genomic sequences and regulate different target genes from Ftz/Ftz-F1 complexes. It seems unlikely that an animal could survive if Ftz activated a whole new set of target genes in the embryo that were not previously expressed. The more likely explanation may lie in an ancestral role for Ftz-F1 in pair-rule patterning. In the beetle *Tribolium*, *Tc-ftz-f1* is expressed in pair-rule stripes, and embryonic RNAi knockdown revealed that it has pair-rule function (Fig. 10). In *Tribolium*, *ftz* and *ftz-f1* stripes overlap (unpublished), while *Dm*-Ftz-F1 is maternally deposited and ubiquitously expressed (Fig. 6c, green). *ftz-f1* is also expressed in stripes in other beetles and more basally branching species (unpublished). Thus, we propose that Ftz was incorporated into a preexisting network of pair-rule segmentation genes—that included *ftz-f1*—by virtue of acquisition of a physical interaction with this nuclear receptor. Since Ftz-F1 is the main determinant of DNA-binding specificity for the Ftz/Ftz-F1 heterodimer, this change would not result in sudden activation of new sets of regulatory genes but might have impacted the relative affinity of DNA binding to or levels of transcription activation of an ancestral set of target genes. At some point, perhaps gradually, Ftz came to be required for Ftz-F1 pair-rule function, taking on a required role in regulating *en* and other target genes. In turn, this dependence on Ftz weakened constraints on *ftz-f1* expression such that it is expressed ubiquitously in *Drosophila* embryos even though it only functions in the cells that also express Ftz (Fig. 6c, green vs. yellow). Current studies in our lab are investigating the steps involved in rewiring of regulatory interactions such that Ftz, added into a preexisting network, became essential for its function.

Changes in *Hox* genes and morphological evolution

ftz is not the only *Hox* gene to have changed biological function, expression pattern, or protein interaction during evolution, but it is one of the genes that has changed most dramatically. Similar dramatic changes occurred for *Hox 3* genes with this typical *Hox* gene taking on roles in extraembryonic development due to changes in both protein sequence and expression pattern (Panfilio and Akam 2007). Further, a *Hox 3* duplication in higher Diptera generated *bicoid* (*bcd*), a head-determining gene in *Drosophila* (reviewed in McGregor 2005). Similar to *ftz*, *bcd* has taken on a novel biological role and its expression pattern is distinctly nonhomeotic, with maternal expression that results in a morphogen gradient in early embryos. While it is possible that these examples are in some ways unique and

extreme, they may also represent the tip of an iceberg, with much still to be learned about molecular evolution of regulatory genes.

Other *Hox* genes have changed expression or protein interactions in ways that have been correlated with morphological variation (reviewed in Carroll et al. 2005; Prud'homme et al. 2006; Prud'homme et al. 2007; Carroll 2008; Stern and Orgogozo 2008) (see Fig. 5). For example, shifts in expression of *Hox* genes have been correlated with the loss of limbs in snakes (Cohn and Tickle 1999; Mansfield 2013). Changes in Scr-regulation of target gene expression have been implicated in the development of elaborate horns on the thorax of some types of beetles (Wasik et al. 2010) and of “helmets” on the treehopper thorax (Prud'homme et al. 2011). Increased levels of expression of *Ubx* appear to explain the lengthened T2 leg in water striders (Khila et al. 2009; Khila et al. 2014). Most of these changes are likely due to changes in *cis*-regulatory elements, either for the *Hox* gene(s) or downstream targets, although this remains to be stringently demonstrated in most cases. Finally, there is at least one example of a change in Hox protein function that has been correlated with morphological change: acquisition of a repressor domain in *Ubx* correlates with the loss of abdominal appendages in insects (Galant and Carroll 2002; Ronshaugen et al. 2002). However, of all the *Hox* gene variations that have been examined to date, it is hard to dispute the conclusion that *bcd* and *ftz* have changed the most dramatically. Given this, it is striking that no change in morphology can be correlated with changes in their expression or function. Both genes took on new roles—*bcd* in head determination and *ftz* in segment formation—that existed before these genes arose, and there is no evidence that modes of development or morphological features changed upon their integration into a preexisting regulatory network. Both regulate processes that occur in all insects (formation of a head, formation of body segments) whether or not the corresponding gene does or does not participate in the process. This contrasts with other examples given above in which smaller changes in expression or function of other *Hox* genes can be correlated with, and likely caused, changes in morphology. Notably, these other examples reflect cases in which external morphology is affected (change in appendages, bristles, or body armor) that are not vital to organismal survival per se, although changes likely impact fitness in other ways, while the *Hox* genes that changed more dramatically (*ftz*, *bcd*) control essential steps in the formation of embryos. There are too few examples to know if this is a coincidence or if there is some fundamental “rule” reflected here that applies to *Hox* genes specifically or regulatory genes overall. In any case, one thing is certain: evolutionary change in *Hox* genes, and other conserved families of regulatory genes, does not occur in one way only. Anything that can happen likely has happened during the hundreds of millions of years of evolution of mechanisms regulating embryonic development in animals.

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Fig. 1.

The classic *Antp* phenotype. The wild-type function of Antp assigns segmental identity to the second thoracic segment (T2). **a** Wild-type antenna. **b** When Antp is expressed in the head, it directs formation of a T2-leg in place of an antenna. Photo courtesy of Gehring (1998)

Fig. 2.

Chromosomal complexes of *Hox* genes are found throughout the animal kingdom. Paralog groups are indicated by the *numbers* and *color coded*. Genes are expressed along the body axis colinearly with their order in the complex, anterior to posterior. *Hox* genes have been found in most animal phyla including protostomes and deuterostomes, as indicated by the cladogram (*left*). In chordates, genome duplications generated multiple clusters (*top*). The *Drosophila Hox* complex, HOM-C, is highlighted. Genes that have taken on non-*Hox*-like functions are shown in brown and discussed in the text. Note that a large number of other genes contain homeoboxes but are not present in evolutionarily conserved chromosomal complexes. These comprise a separate category of non-*Hox*, homeobox-containing gene (reviewed in Holland 2013; Burglin and Affolter 2015). Figure from Heffer and Pick (2013).

Fig. 3.

Homeobox genes in other species: the McGinnis Zoo blot. DNA probes from Antp (A) or Ubx (U) were hybridized to Southern blots with genomic DNA from the indicated species. This is, to our knowledge, the first evidence for evolutionary conservation of the homeobox. Figure adapted from McGinnis et al. (1984a).

Fig. 4.

Ectopic expression of *Antp* or mammalian *Hox* genes generated an antennal to leg transformations. **a** Wild-type antenna. **b** Ubiquitous expression of *Antp* induced transformation of antennae to T2 legs. Specific leg structures are indicated. **c** Ectopic expression of mouse *Hox 2.2* or **d** *Hox 1.3/a5* in *Drosophila* resulted in homeotic transformations expected for the *Drosophila* orthologs, *Antp*, and *Scr*, respectively. Figure adapted from Schneuwly et al. (1987a), Malicki et al. (1990), and Zhao et al. (1993)

Fig. 5.

Variation in *Hox* gene expression and function during evolution. Changes in *Hox* gene expression and function have contributed to morphological evolution and to variation in gene regulatory networks in animals. Four key modes of change that have been reported are illustrated. From Pick and Heffer (2012)

Fig. 6.

Ftz and Ftz-F1 cooperate to promote the formation of alternate body segments. **a** Cuticle preparation of wild-type larva. **b** *fitz* mutant embryo lack alternate parasegments. **c** Ftz-F1 is present in all somatic nuclei of *Drosophila* blastoderm stage embryos (green), while Ftz is expressed in stripes (yellow due to overlap with Ftz-F1) in the primordia of the parasegments missing in *fitz* or *fitz-f1* mutants

Fig. 7.

Tribolium ftz retains homeotic potential, while *Drosophila ftz* does not. **a** Wild-type antenna. **b** Expression of *Dm-ftz* caused cell death in antennae. **c** Expression of *Tc-ftz* transformed antennae into complete T2 legs. **d** Expression of *Sg-ftz* produced weaker antennal to leg transformations. **e** Addition of YPWM to *Dm-Ftz* increased homeotic potential. **f** *Dm-Ftz* protein with an added YPWM motif and inactivated LXXLL motif transformed antennae to T2 legs. Note that the ectopic experiments in *Drosophila* reveal what these proteins *can* do, when expressed in specific conditions and in specific cell types; we refer to this as protein “potential” to emphasize the fact that these types of trans-species assays may not reveal endogenous functions (reviewed in Heffer et al. 2011). From Lohr et al. (2001) and Lohr and Pick (2005)

Fig. 8. Variation in *ftz* expression and protein domains in arthropods (*left*). Cladogram indicating major arthropod phyla and selected species from which full-length *ftz* genes have been isolated (*right*). Presence/absence of functional Ftz motifs and expression patterns during embryogenesis. The LXXLL motif (*green*) is required for pair-rule function in *Drosophila* and mediates interaction with the Ftz cofactor Ftz-F1. LXXLL was stably acquired at the base of Holometabola. The YPWM mediates interaction with the homeotic cofactor Exd. This motif is present in Ftz in some arthropods (*blue*) but has degenerated in many lineages (*red*). The early embryonic expression pattern of *ftz* has been reported as *Hox*-like (Crustacea, Myriapoda, Chelicerata), in the growth zone (Orthoptera), and in stripes (Thysanura, Hymenoptera, Coleoptera, Diptera). *ftz* CNS expression has been reported in many arthropods (*orange*). From Heffer et al. (2013b)

Fig. 9.

Motifs that vary in Ftz from extant arthropods are not required for CNS function. The neurogenic element (NE) was used to express Ftz proteins in the CNS of *ftz* mutants rescued for segmentation defects. Rescue of Eve expression in RP2 neurons is shown (percent rescue) for different transgenes, as indicated. From Heffer et al. (2013b).

Fig. 10. Ftz-F1 has pair-rule function in *Tribolium*. **a** Expression of *en* in control embryo. **b** Expression of alternate *en* stripes was decreased after knockdown of *Tc-ftz-f1* with embryonic RNAi, indicative of pair-rule function. From Heffer et al. (2013a)