Drosophila fushi tarazu: a gene on the border of homeotic function

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Background: *Hox* genes specify cell fate and regional identity during animal development. These genes are present in evolutionarily conserved clusters thought to have arisen by gene duplication and divergence. Most members of the *Drosophila Hox* complex (HOM-C) have homeotic functions. However, a small number of HOM-C genes, such as the segmentation gene *fushi tarazu* (*ftz*), have nonhomeotic functions. If these genes arose from a homeotic ancestor, their functional properties must have changed significantly during the evolution of modern *Drosophila*.

Results: Here, we have asked how *Drosophila ftz* evolved from an ancestral homeotic gene to obtain a novel function in segmentation. We expressed Ftz proteins at various developmental stages to assess their potential to regulate segmentation and to generate homeotic transformations. *Drosophila* Ftz protein has lost the inherent ability to mediate homeosis and functions exclusively in segmentation pathways. In contrast, Ftz from the primitive insect *Tribolium* (Tc-Ftz) has retained homeotic potential, generating homeotic transformations in larvae and adults and retaining the ability to repress *homothorax*, a hallmark of homeotic genes. Similarly, *Schistocerca* Ftz (Sg-Ftz) caused homeotic transformations of antenna toward leg. Primitive Ftz orthologs have moderate segmentation potential, reflected by weak interactions with the segmentation-specific cofactor Ftz-F1. Thus, Ftz orthologs represent evolutionary intermediates that have weak segmentation potential but retain the ability to act as homeotic genes.

Conclusions: *ftz* evolved from an ancestral homeotic gene as a result of changes in both regulation of expression and specific alterations in the protein-coding region. Studies of *ftz* orthologs from primitive insects have provided a "snap-shot" view of the progressive evolution of a Hox protein as it took on segmentation function and lost homeotic potential. We propose that the specialization of *Drosophila* Ftz for segmentation resulted from loss and gain of specific domains that mediate interactions with distinct cofactors.

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Background

Hox genes control the body plans of a diverse array of organisms throughout the animal kingdom. These genes are clustered in complexes, which have been highly conserved through evolution. *Hox* genes with equivalent function are located in equivalent positions within *Hox* complexes of animals as diverse as fly and human [1]. This suggested that modern-day *Hox* complexes arose from a basal *Hox* complex in a common ancestor of protostomes and deuterostomes. This complex may have contained primordial *Hox* genes whose function was to distinguish the identity of head, tail, and trunk segments [2]. The ancestral *Hox* cluster was then elaborated by duplication and divergence within the complex, as well as by duplication of entire clusters in the vertebrate lineage [1, 3, 4].

The *Drosophila Hox* complex, HOM-C, is composed primarily of homeotic genes, genes that, by definition, have the ability to transform the characteristics of one body part into those of another body part [5]. However, several genes within HOM-C, such as the segmentation gene *fushi tarazu* (*ftz*), have no known homeotic function [6]. *ftz* is located in HOM-C between the homeotic genes *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*). Ftz contains an Antp-class homeodomain (HD) [7], and its in vitro DNA binding specificity is indistinguishable from that of other Hox proteins [8, 9]. The Ftz HD is most closely related to those of its neighbors, Scr and Antp, with only two amino acid differences from Antp and four from Scr in the variable N-terminal arm shown to be important for Hox specificity in vivo [10–12]. Initially found only in *Drosophilids*, putative *ftz* orthologs have been found in *Hox* complexes of primitive insects [13, 14], arthropod outgroups such as mites and tardigrades [15], and onychophorans [16]. The best-studied orthologs are *Tc-ftz* from the beetle *Tribolium castaneum* [14] and Sg-Ftz from

the grasshopper *Schistocerca gregargia* [13]. These Ftz orthologs contain a YPWM motif found in and required for function of homeotic Hox proteins [17, 18], which is not present in Dm-Ftz. This suggests that primitive *ftz* orthologs may be more closely related to an ancestral *Hox* gene than is *Dm-ftz* [6, 15].

Dm-ftz behaves as a pair-rule segmentation gene necessary for the development of even-numbered parasegments [19, 20]. *Dm-ftz* is expressed in seven stripes in blastoderm embryos in the primordia of the cells missing in *ftz* mutants [21]. Because of the correlation between expression pattern and function, most efforts to understand the role of *Dm-ftz* in segmentation focused on the complex regulation of its striped expression pattern [9, 22, 23]. *Tc- ftz* is also expressed in a striped pattern in beetle embryos [14], although its role in segmentation is unclear, as embryos deficient for a genomic region containing *Tc-ftz* do not show segmentation defects [24]. *Sg-ftz*, on the other hand, is expressed in a single broad posterior domain of grasshopper embryos [13]. The striped expression pattern of *Dm-ftz* in the blastoderm differs dramatically from that of homeotic genes that are expressed in more-restricted domains and at slightly later developmental stages than *Dm-ftz*.

If *ftz* genes evolved from ancestral homeotic genes, their function in segmentation might have changed as a result of the acquisition of a striped expression pattern. That is, Ftz proteins might be homeotic proteins whose function was redirected by virtue of a change in spatial and temporal expression. Alternatively, Ftz proteins themselves may have acquired intrinsic protein properties that are involved in directing segmentation rather than homeosis. To address this, we expressed Ftz proteins in *Drosophila* in a fashion previously used to reveal the homeotic function of *Drosophila* and vertebrate Hox proteins [25–28]. We compared the intrinsic properties of Dm-Ftz with those of Ftz orthologs from more primitive insects; Tc-Ftz from beetles and Sg-Ftz from grasshoppers. Our results suggest that Dm-ftz has become highly specialized for segmentation, while primitive Ftz proteins retain homeotic potential.

Results

Tc-Ftz functions in an Antp-like manner

Antp gain-of-function mutations produce dramatic homeotic transformations of antenna-to-leg identity [29, 30]. These transformations can be mimicked by misexpressing *Antp* [25] or, more weakly, by other leg-determining genes such as *Sex combs reduced* (*Scr*) [10] or *Ultrabithorax* (*Ubx*) [31] during larval development. Based upon sequence and position in HOM-C, *ftz* appears to be most closely related to the leg-determining homeotic genes. To test the homeotic potential of Ftz proteins, they were transiently expressed during larval development using a heat-inducible promoter. Expression of Dm-Ftz had no effect on antennal development (Figure 1b). Surprisingly, Tc-Ftz caused antenna toward leg transformations (Figure 1c), in which the arista took on characteristics of the tarsus. These transformations resemble weak transformations induced by ectopic expression of Scr (Figure 1d) or Antp [32]. Antenna-to-leg transformations were not observed when Sg-Ftz was expressed with the heat shock promoter (data not shown).

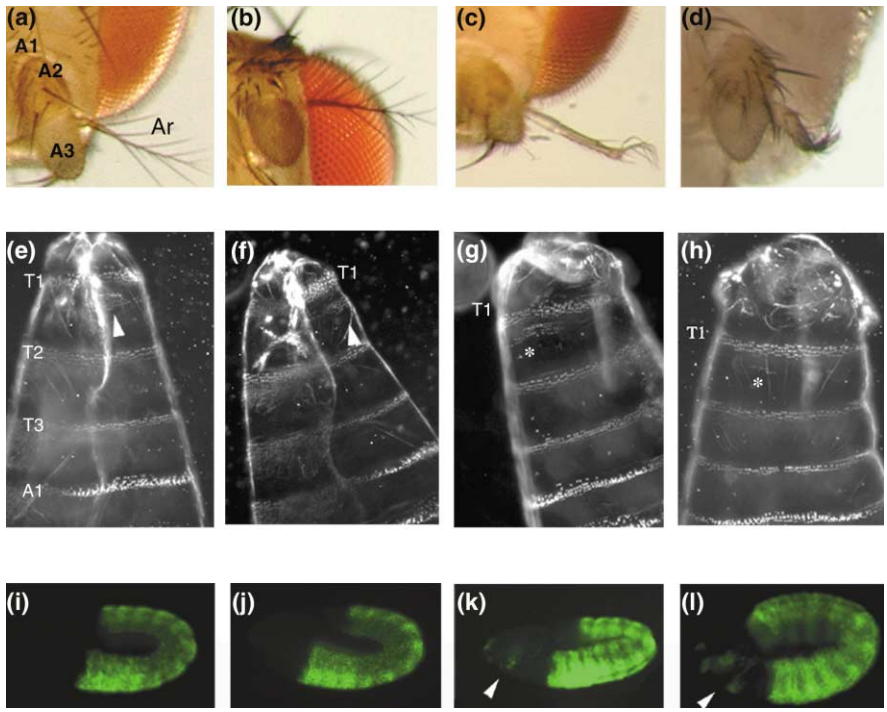
To assess the ability of Ftz proteins to generate homeotic transformations during embryogenesis, expression was induced by heat shock at 5.5 hr after egg laying (AEL), the time when misexpression of Scr and Antp cause the strongest homeotic transformations of the larval cuticle [10, 32]. Ectopic expression of Dm-Ftz at this stage had no effect on cuticle development (Figure 1f). However, *Tc-ftz* expression at this stage caused head involution defects, transformation of the T1 denticle toward T2 identity, and a reduction of the T1 ventral beard (Figure 1g). This phenotype strongly resembles an embryonic *Antp* gain-of-function phenotype (Figure 1h) [10].

Given the similarity of phenotypes induced by Tc-Ftz and Antp in the larval cuticle, the ability of Ftz proteins to mediate homeotic effects in the same pathway as Antp was examined at the molecular level. Ectopic expression of Antp at ~5 hr AEL activates *teashirt* (*tsh*) expression in the head of the embryo (Figure 1l) [33]. Expression of Dm-Ftz at this time in development had no effect on *tsh* expression (Figure 1j). In contrast, ectopic expression of Tc-Ftz (Figure 1k) activated *tsh* in much the same manner as ubiquitous *Antp* (Figure 1l). The effects of Tc-Ftz on the larval cuticle and on *tsh* expression were not mediated via induction of Dm-Antp by Tc-Ftz, since ectopic Dm-Antp expression was not observed in these embryos (data not shown). These results suggest that Dm-Ftz has lost the potential to act as a homeotic gene, whereas Tc-Ftz has retained such functions.

Persistent expression of Tc-Ftz and Sg-Ftz causes antenna-to-leg transformations

To ask if homeotic properties of Dm-Ftz and also of Sg-Ftz are too weak to be revealed when the protein was induced by brief heat pulses, the UAS-Gal4 system [34] was used to induce more sustained expression. Ftz proteins were expressed in the *Distal-less* (*Dll*) domain, with a driver that maintains constant gene expression in the distal region of imaginal discs throughout development [35]. This persistent ectopic expression of Dm-Ftz resulted in truncation of the arista and malformation of the third antennal segment (AIII) (Figure 2b). No transformation to an alternate body part was discernable. In contrast to this, Tc-Ftz caused the complete transformation of the arista and AIII into tarsus (Figure 2c). All five tarsal segments including claw (arrow) were formed. The ec-

Figure 1



The *Tribolium ftz* gene has homeotic potential. The ability of *ftz* genes from *Drosophila* and *Tribolium* to cause homeotic transformations during larval and embryonic development was assessed. **(a–d)** Adult phenotypes induced by ectopic expression of *ftz* genes. Antennal segments A1, A2, A3, and arista (Ar) are indicated. (a) Heat treatment of ry^{-} larvae had no effect on antennal development. (b) Dm-Ftz had no effect on antennal morphology. (c) Tc-Ftz caused the transformation of arista to tarsus, similar to (d) Scr. **(e–h)** Homeotic transformations of the larval cuticle. (e) Heat treatment of *yw* control embryos had no effect on larval cuticles. T1, T2, T3, and A1 denticle belts are indicated. The arrowhead indicates the T1 ventral beard, which marks this segment. (f) Dm-Ftz caused no alteration in larval cuticles. (g) Tc-Ftz caused head involution defects and transformation of T1 toward T2, marked by reduction in the T1 ventral beard (asterisk). This phenotype resembles that caused by (h) Antp. All heat treatments were performed in parallel to ensure that results could be compared across lines carrying different expression constructs. Similar results were obtained when *ftz* genes were expressed with Gal4-UAS using an *armadillo* driver (data not shown). **(i–l)** Tc-Ftz activates the homeotic target gene *tsh*. Expression of Tsh is shown at stage 11. The anterior is directed toward the left. (i) Heat treatment of *yw* embryos had no effect on Tsh localization, which is restricted to the trunk region of the embryo. (j) Dm-Ftz did not alter Tsh expression. (k) Ectopic Tsh was induced in the head by Tc-Ftz (k; arrowhead) and similarly by Antp (l; arrowhead).

topic tarsus did not have sex combs on the first segment, a characteristic of the *Scr*-dependent T1 leg, nor were transverse bristles observed, a characteristic of the *Ubx*-dependent T3 leg. These observations suggest that Tc-Ftz mimics Antp in inducing transformations of the antenna toward T2 leg identity. Expression of Sg-Ftz in the *Dll* domain transformed the antenna toward the leg (Figure 2d). Only the fourth and fifth tarsal segments, including claw (arrow) were formed. The tarsi that were formed showed no distinct bristle pattern. The homeotic effects of Tc-Ftz and Sg-Ftz were not caused by the induction of endogenous Dm-Antp, as ectopic expression of Dm-Antp was not observed in these discs (data not shown). These results demonstrate that both Tc-Ftz and Sg-Ftz have retained homeotic potential, although Sg-Ftz has weaker activity than Tc-Ftz when expressed in *Drosophila*. Dm-Ftz, on the other hand, cannot mediate homeotic functions during embryogenesis or larval development.

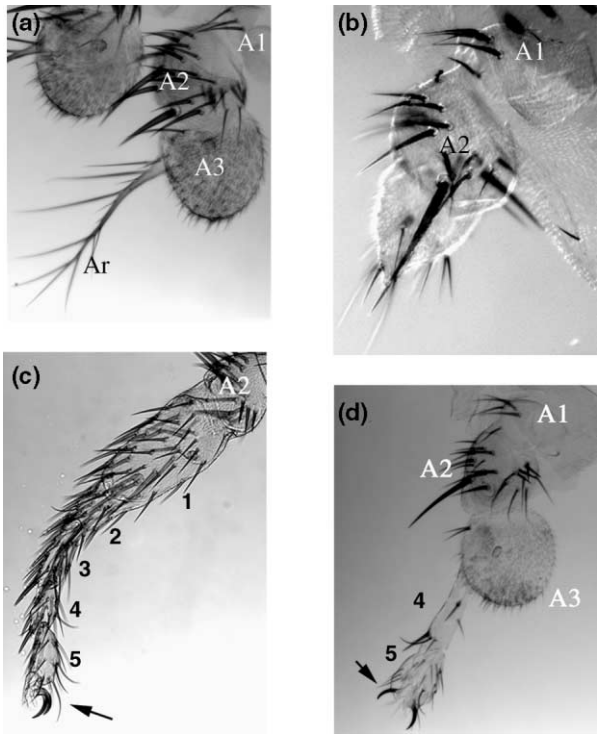
Dm-Ftz influences cell survival when ectopically expressed in the antennal discs

Sustained expression of Dm-Ftz in the *Dll* domain caused appendage truncations, without any apparent transformation of segment identity. To test if these truncations were due to ectopic cell death, imaginal discs expressing Ftz proteins in the *Dll* domain were stained with acridine orange. As shown in Figure 3b, Dm-Ftz triggered massive cell death in the distal region of the antennal disc. In discs in which Tc-Ftz or Sg-Ftz had been ectopically expressed, minimal cell death was observed scattered throughout the disc (Figure 3c,d). This confirms that Dm-Ftz does not act to transform the antennae into legs, but rather influences cell survival in the distal region of the disc. We suggest that the loss of distal cells may in turn lead to the malformation of AIII.

Tc-Ftz represses *homothorax* (*hth*) expression in a homeotic fashion

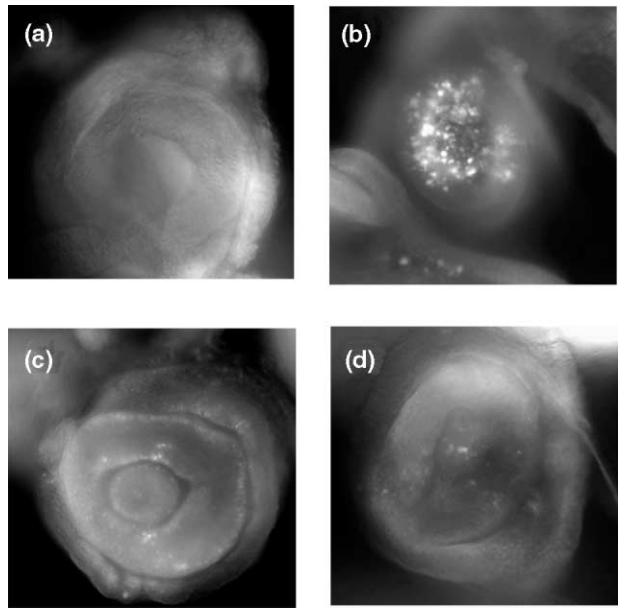
Homeotic genes are thought to cause antenna-to-leg transformations by repressing the expression of *homothorax* (*hth*) [36]. If Ftz orthologs were functioning in a true homeotic manner, they should thus repress *hth* in the antennal discs. The effects of ectopic expression of Ftz proteins on *hth* expression was tested using a disc-specific *dpp*-Gal4 driver, which induces gene expression in a proximodistal wedge of cells (Figure 4; green). Hth is uniformly expressed in wild-type antennal discs, excluding only the most distal region, which gives rise to the arista ([37]; Figure 4a,b; red). Dm-Ftz had no effect on *hth* (Figure 4c,d; Hth and GFP overlap), confirming that Dm-Ftz cannot interact in homeotic pathways at the molecular

Figure 2



Primitive *ftz* genes cause antenna-to-leg transformations. Ftz proteins were expressed in imaginal discs using the *Dll*-GAl4 driver. **(a)** *lacZ* had no effect on antennal formation (A1, A2, A3, and arista [Ar] are indicated). **(b)** Dm-Ftz caused deletion of the arista and malformation of A3; A2 and A1 developed normally. **(c)** Tc-Ftz caused the transformation of arista to complete tarsus; all five tarsal segments are indicated, including claw (arrow). **(d)** Sg-Ftz caused the transformation of the arista into two tarsal segments, including claw (arrow).

Figure 3



Dm-ftz influences cell survival, not segmental identity. Ftz proteins were expressed in the same manner as for Figure 2. Acridine orange was used to determine cell viability after persistent expression of *ftz* genes. **(a)** *lacZ* had no effect on cell viability. **(b)** Dm-Ftz caused massive cell death. **(c)** Tc-Ftz or **(d)** Sg-Ftz induced minimal cell death.

level. In keeping with its ability to generate strong homeotic transformations, Tc-Ftz repressed Hth expression (Figure 4e,f). As in previous experiments, no ectopic Antp expression was observed as a result of ectopic Tc-Ftz expression (data not shown). Sg-Ftz had no effect on Hth expression (data not shown), consistent with the observation that Sg-Ftz only caused homeotic transformations in the distal-most region of the disc, where Hth is never expressed. Thus, the strong antenna-to-leg transformations caused by Tc-Ftz were generated in a homeotic manner via repression of Hth.

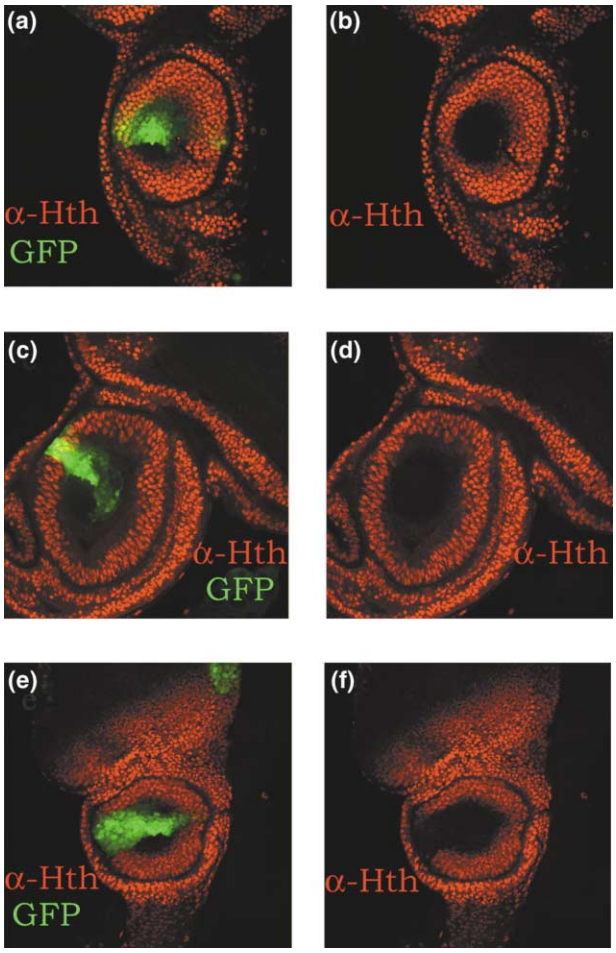
Sg-Ftz represses *spineless-aristapedia* (*ss*) in antennal discs

The antenna-to-leg transformations caused by expression of Sg-Ftz in the *Dll* domain were reminiscent of weak *spineless-aristapedia* (*ss*) loss-of-function phenotypes. *ss* is necessary to establish AIII and arista identity in the antenna and from the second to fourth tarsal segments in the leg [38]. Accordingly, *ss* is expressed uniformly in the distal region of the antennal disc, which gives rise to the arista ([39]; Figure 5a). In leg discs, *ss* is expressed in a transient distal ring corresponding to the tarsal region, during late second and early third larval instar stages ([39], Figure 5b). Ectopic expression of leg-determining homeotic genes in antennal discs represses expression of *ss* in the center of the discs and causes its pattern to resemble that found in the leg discs [39]. To test if Sg-Ftz mediates arista to tarsus transformations in a homeotic fashion, Ftz proteins were expressed in the *Dll* domain with the UAS-Gal4 system, and effects on *ss* expression were determined by in situ hybridization to *ss* RNA. As shown in Figure 5c,d, both Tc-Ftz and Sg-Ftz repressed *ss* expression in the center of the disc. The expression of *ss* in these antennal discs strongly resembled the pattern in which *ss* is normally expressed in leg discs (Figure 5b). These results indicate that Sg-Ftz and Tc-Ftz transformed the arista toward tarsus identity in a homeotic manner.

Both Tc-Ftz and Sg-Ftz have segmentation potential

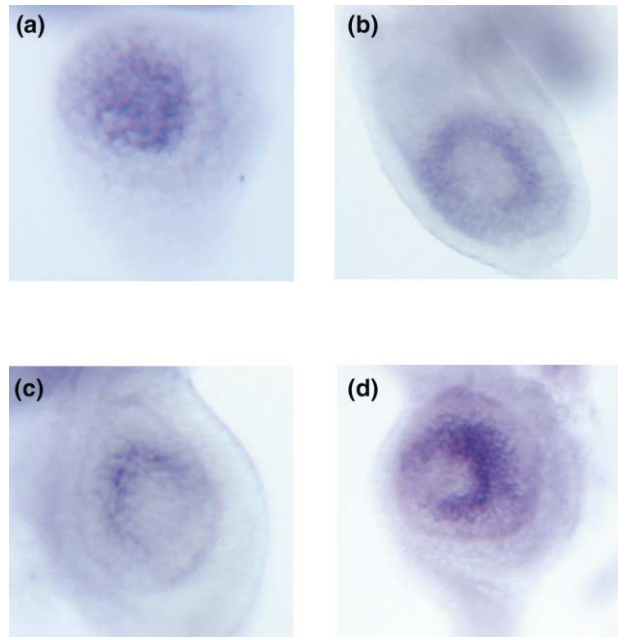
Taken together, the results reported above indicate that Dm-Ftz has lost homeotic potential that has been retained in Ftz orthologs from more primitive insects. To what extent can this be correlated with a specialization for segmentation-specific functions? Ectopic expression of Dm-Ftz during the blastoderm stage causes deletion of *ftz*-independent segments, a phenotype nearly reciprocal to that caused by *ftz* loss-of-function mutations ("anti-*ftz* phenotype") [40]. To examine the potential of Ftz

Figure 4



Tc-ftz functions in a homeotic manner to repress *hth*. *Dm-ftz* or *Tc-ftz* were expressed using a *dpp*-Gal4 driver linked to a UAS-GFP transgene. **(a,b)** *lacZ* had no effect on Hth. Where *lacZ* expression, indicated by GFP (green), overlaps Hth (red), Hth expression remained uniform. **(c,d)** Dm-Ftz had no effect on Hth. Note the uniform expression of Hth and the overlap of Dm-Ftz and Hth (yellow). **(e,f)** Tc-Ftz repressed Hth. Hth immunoreactivity was not observed in regions of the antennal disc expressing Tc-Ftz. Sg-Ftz had no effect on Hth (data not shown).

Figure 5

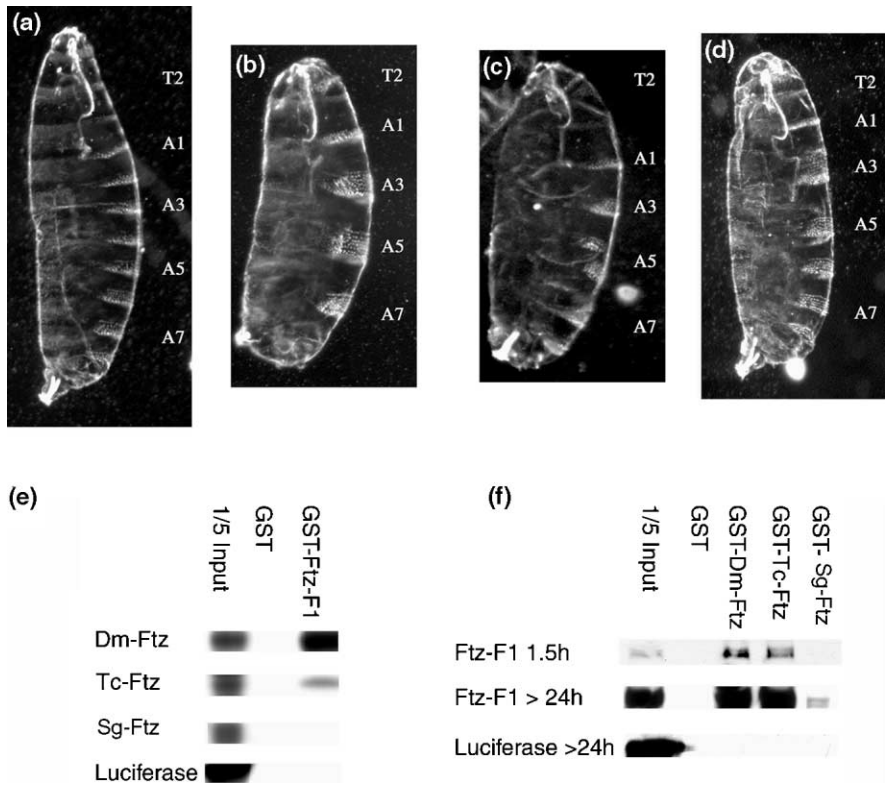


Ftz orthologs repress *ss* in the antennal disc. Ftz proteins were expressed in antennal discs using the *Dll*-Gal4 expression system. *ss* expression was observed with in situ hybridization. **(a)** *lacZ* expression had no effect on *ss* expression in the antennal disc. *ss* is expressed in the distal domain from late second instar larva onward. **(b)** In the leg discs, *ss* is expressed transiently from late second to early third larval instar in a ring. *ss* expression in the leg disc was not altered by *lacZ* expression. **(c)** The expression of *ss* was altered by Tc-Ftz to resemble *ss* expression in the leg disc (compare to [b]). This expression is transient, as it is in the leg disc. **(d)** Sg-Ftz repressed expression of *ss* in the center of the antennal disc. Uniform *ss* expression in the antennal disc was transformed to a transient ring similar to that seen in leg discs (compare to [b]).

orthologs to mediate segmentation functions in *Drosophila*, Tc-Ftz and Sg-Ftz were ectopically expressed at the blastoderm stage. Ectopic expression of Dm-Ftz produced an anti-*ftz* phenotype in almost all cuticles (Figure 6b). Tc-Ftz also caused an anti-*ftz* phenotype, but the effect was weaker, causing complete loss of *ftz*-independent segments in only a small percentage of cuticles (Figure 6c). Sg-Ftz rarely induced a complete anti-*ftz* phenotype, but rather caused partial pairing defects. In the cuticle shown, anterior denticle belts were fused, while posterior denticles resembled wild-type cuticle (Figure 6d). The parasegments affected by ectopic expression of Sg-Ftz varied from embryo to embryo. Expression of Scr or Antp under the same conditions did not cause anti-*ftz* or any other pair-rule-like phenotypes (data not shown), confirming that homeotic genes do not have segmentation potential.

The segmentation function of Dm-Ftz is strictly dependent on its cofactor, the orphan nuclear hormone receptor Ftz-F1. Ftz and Ftz-F1 physically interact to regulate target gene expression [41–43]. The ability of Tc-Ftz and Sg-Ftz to interact directly with Ftz-F1 was tested in GST pull-down assays (Figure 6e,f). Consistent with the in vivo results, GST-Ftz-F1 interacted strongly with ^{35}S-labeled Dm-Ftz and, to a lesser extent, with Tc-Ftz (Figure 6e). No interaction was detected between GST-Ftz-F1 and labeled Sg-Ftz in this experiment. In the reciprocal experiment, Ftz proteins fused to GST interacted with ^{35}S-labeled Ftz-F1 protein (Figure 6f). The interaction between Ftz-F1 and GST-Dm-Ftz was stronger than that of Ftz-F1 with GST-Tc-Ftz (see short exposure time of 1.5 hr). An interaction between GST-Sg-Ftz and Ftz-F1 was also observed; however, this was detectable only after

Figure 6



Ftz proteins from *Schistocerca* and *Tribolium* have weak segmentation properties. **(a–d)** *ftz* genes induce an anti-*ftz* phenotype. The ability of primitive *ftz* genes to mediate segmentation functions was assessed by ectopic expression at the blastoderm stage. (a) *yw* embryos developed normally. Denticle belts of all thoracic (T) and abdominal (A) segments were formed. Denticles of the *ftz*-dependent parasegments are marked. (b) Dm-Ftz caused an anti-*ftz* phenotype in >80% of embryos: odd-numbered parasegments were deleted. The remaining denticle belts are indicated. (c) Tc-Ftz caused anti-*ftz* phenotypes. Most of the *ftz*-independent parasegments are deleted. In this cuticle, a small portion of A4 and A8 remain. Approximately 30%–40% of embryos were affected by ectopic Tc-Ftz; complete anti-*ftz* phenotypes were rarely observed. (d) Sg-Ftz caused weak pair-rule-like defects. A cuticle in which only anterior *ftz*-independent parasegments were deleted is shown. Only 5%–9% of embryos were affected; complete anti-*ftz* phenotypes were never observed. All heat treatments were performed in parallel to ensure that results could be compared across lines carrying different expression constructs. Results were confirmed in at least three separate experiments. **(e–g)** Tc-Ftz and Sg-Ftz interact with the segmentation-specific cofactor Ftz-F1. (e,f) GST pull-down assays. GST-fusion proteins were expressed in pGex5X-1. Approximately equivalent amounts of ^{35}S-labeled protein were incubated with 10 μg GST-fusion proteins bound to beads. Reactions were analyzed by SDS-PAGE and autoradiography. (e) ^{35}S-labeled Dm-Ftz interacted strongly with GST-Ftz-F1. The interaction between ^{35}S-labeled Tc-Ftz and Ftz-F1 was weaker, while an interaction between ^{35}S-labeled Sg-Ftz was not observed. No interaction between ^{35}S-labeled proteins and GST alone was observed. GST-Ftz-F1 did not interact with ^{35}S-labeled luciferase. (f) ^{35}S-labeled Ftz-F1 interacted with GST-Dm-Ftz and GST-Tc-Ftz (1.5 hr exposure time). The weak interaction between ^{35}S-labeled Ftz-F1 and GST-Sg-Ftz was detectable after a longer exposure (>24 hr).

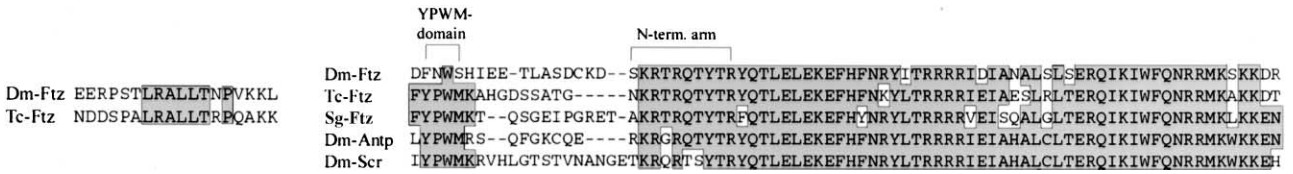
a prolonged exposure (>24 hr). These results indicate that Sg-Ftz interacts with Ftz-F1 in a significantly weaker fashion than do Ftz and Tc-Ftz proteins. This observation is consistent with the in vivo results (Figure 6d) that showed that Sg-Ftz caused only very mild segmentation phenotypes. No interaction was detected between GST-Ftz, Tc-Ftz, Sg-Ftz, or Ftz-F1 and in vitro-translated luciferase or between in vitro-translated Ftz-F1 or Ftz proteins and GST alone (Figure 6e,f), demonstrating the specificity of these interactions.

Since the levels of ectopically expressed proteins in *Drosophila* could not be directly compared, the ability of each individual Ftz protein to mediate segmentation phenotypes cannot be precisely quantified. However, taken together with the in vitro results, these experiments reveal a trend in the ability of the different Ftz proteins to mediate pair-rule functions: Dm-Ftz generated strong segmentation phenotypes and interacted strongly with Ftz-F1, Tc-Ftz caused moderate phenotypes and interacted with Ftz-F1 to a lesser extent than Dm-Ftz, while Sg-Ftz generated extremly weak phenotypes and only interacted marginally with Ftz-F1. The weak interaction between Sg-Ftz and Ftz-F1 in the GST pull-down assays is consistent with the fact that Sg-Ftz contains only one of the two known Ftz-F1 interaction domains: Dm-Ftz and Tc-Ftz contain the LXXLL motif and the homeodomain [44, 45], while Sg-Ftz contains only the homeodomain (Figure 7, and see below). These results suggest that the potential to interact with a segmentation-specific cofactor was already present in ancestral forms of Ftz. However, Dm-Ftz has become highly specialized for segmentation, loosing the ability to act as a homeotic gene.

Discussion and conclusions

ftz is a rapidly evolving member of the *Hox* gene family [6] and thus provides an opportunity to study the mechanisms underlying the functional evolution of Hox proteins. Based on the sequence similarity between Ftz and Antp in the N-terminal arm of the HD (Figure 7), Telford [15] suggested that *ftz* arose as a duplication of an ancestral *Antp* gene (reviewed in [46]). Our experimental data supports this hypothesis, considering the similarity of phenotypes induced by ancestral *ftz* genes and *Dm-Antp*. We

Figure 7



Distinct protein domains mediate the interaction between Ftz proteins and cofactors. Amino acid sequences of cofactor interaction domains of Ftz and homeotic proteins are shown. N-terminal regions of Dm-Ftz and Tc-Ftz share an LRALL sequence that is not found anywhere in Sg-Ftz. This sequence conforms to an "LXXLL" motif [47] and mediates protein-protein interaction between Dm-Ftz and the Ftz-F1 [44, 45]. Tc-Ftz and Sg-Ftz retain the YPWM motif found in homeotic Hox proteins, which interacts with Exd [17, 49, 50, 51]. This motif has degenerated in Dm-Ftz. The homeodomains of Dm-Ftz, Tc-Ftz, and Sg-Ftz are virtually identical, including the N-terminal arm of the HD, which confers specificity in vivo [10–12]. This portion of the Ftz-HD distinguishes this gene family from neighboring homeotic genes, Scr and Antp.

further suggest that redundancy between *ftz* and *Antp* relieved constraints on the primordial *ftz* gene, resulting in changes within both its regulatory and protein-coding regions.

The expression of *ftz* has evolved from that which is typical of *Hox* genes found in mites [15] to a *Hox*-like pattern in grasshoppers [13] and a striped expression in beetles [14], which is typical for segmentation genes. In addition, two major changes occurred in the protein-coding region of *ftz* genes to facilitate the functional evolution from a homeotic to a segmentation protein. First, Ftz proteins acquired segmentation functions not present in the homeotic Hox proteins. This change appears to have happened gradually, as Sg-Ftz displays very weak segmentation potential, Tc-Ftz has moderate segmentation potential in our assays, and a major function of Dm-Ftz is to promote segmentation. This change correlates with the ability to interact with the cofactor Ftz-F1 (Figure 6). Sg-Ftz was able to interact weakly with Ftz-F1, and this interaction was enhanced by the addition of an LXXLL motif in the Tc-Ftz and Dm-Ftz proteins [44, 45]. This motif has been shown to strengthen the interactions of coactivators with nuclear hormone receptors [47], to which family Ftz-F1 belongs. The LXXLL motif was presumably acquired after the divergence of grasshoppers and beetles, although we cannot rule out the possible loss of a more ancient LXXLL motif in the grasshopper lineage. Second, Ftz proteins lost their ancestral homeotic properties. Homeotic functions of Hox proteins are dependent on the presence of a YPWM domain [17, 18] upstream of the HD (Figure 7), which mediates the interactions with a cofactor, the HD protein Extradenticle (Exd) [48, 49] in vivo and in vitro [17, 50, 51]. The loss of homeotic potential of Ftz correlates with the loss of the YPWM motif: Sg-Ftz and Tc-Ftz retain a YPWM motif and homeotic potential, whereas Dm-Ftz has lost the YPWM motif and the ability to mediate homeotic transformations. Kellerman et al. [52] showed that *Dm-ftz* gain-of-function mutants in which Ftz protein is abnormally stable display unique properties suggested to be homeotic. Whether this mutation has revealed residual homeotic activity of Dm-Ftz remains to determined.

Mayr suggested that "if, in the course of evolution, some of the proteins in an organism undergo evolutionary changes . . . this might create a selection pressure in favor of remodeling other proteins in order to improve interaction" [53]. We suggest that the capability of ancestral Ftz to interact with Ftz-F1 was selected for, leading to the acquisition of a protein domain that stabilizes this interaction and the loss of a domain that could cause competition with another cofactor. This competition was observed in our experiments with Tc-Ftz, which contains interaction motifs for both Exd and Ftz-F1. Ectopic expression of *Dm-ftz* produced anti-*ftz* phenotypes when induced over a broad time range (2.25 and 3.25 hr AEL). Ectopic expression of *Tc-ftz* caused anti-*ftz* phenotypes in less than half of the embryos scored when induced early (2.25 and 2.75 hr AEL). Slightly later, when Exd has become nuclear, Tc-Ftz induced only homeotic transformations and head involution defects. Since Ftz-F1 is nuclear throughout embryogenesis [41], but Exd is only nuclear at approximately 3 hr AEL [54], these observations suggest that Tc-Ftz preferentially interacts with Exd when it is present, and, thus, its segmentation functions are inhibited. In *Drosophilids*, loss of the YPWM motif abolished this competition for cofactors, allowing for exclusive interaction of Ftz with Ftz-F1 such that the function of Dm-Ftz was devoted entirely to segmentation. Both Hox cofactors, Exd and Ftz-F1, influence Hox protein function by cooperative binding to composite binding sites that contain DNA recognition sequences for both the Hox protein and the partner [17, 41, 43, 51]. The different partner pairs recognize qualitatively different sets of DNA sequences in the genome and thus regulate different sets of target genes. Homeotic genes function to determine regional identity [3], while Dm-Ftz, in conjunction with Ftz-F1, has evolved to regulate a unique set of target genes, pre-

sumably involved in promoting cell survival and morphogenesis during segmentation.

The ability of Ftz proteins to regulate segmentation in *Drosophila* correlates with the germ band length of its native organism: *Sg-ftz*, from an extreme short germ band insect in which only the anterior-most segments are established during blastoderm and all other segments are established during gastrulation, shows the weakest ability to function as a segmentation gene. In the case of Tc-Ftz, which is native to an intermediate germ band insect, in which head and trunk segments are established during blastoderm and remaining segments during gastrulation, segmentation properties were more pronounced. Dm-Ftz is entirely devoted to segmentation in the long germ band insect *Drosophila*, in which all segments are established at the blastoderm stage. It is possible that the segmentation functions of Ftz evolved to fit the needs of more complex forms of embryogenesis, such as that found in *Drosophila*. It remains to be seen if *ftz* orthologs from ancestral insect species with a long germ band mode of embryogenesis have segmentation functions.

Elegant studies in recent years in other labs have highlighted the importance of changes in *cis*-regulatory regions that drive the functional evolution of homeobox genes [4, 6, 55–57]. The results presented here suggest that the functional evolution of the *Hox* gene *ftz* was dependent not only upon such regulatory changes, but also upon changes in protein sequence that modified interactions with specific protein partners. Studies of these molecular modulations in rapidly evolving control genes, such as *ftz*, provide insight into the developmental and evolutionary mechanisms that promote changes in the body plan and ultimately the diversity of species throughout the animal kingdom.

Materials and methods

Plasmid construction

Plasmid construction: *ftz* cDNA was cloned into pKS, pCaSpeR-hs, pUAST, and pGEX5X-1 as an EcoRI fragment. *Tc-Ftz* was cloned into the SmaI/NotI sites of pCaSpeR-hs and filled in BglII and NotI sites of pUAST. For in-frame cloning, an EcoRI site was added just 5′ of the endogenous ATG by PCR, and *Tc-ftz* was inserted as an EcoRI/NotI fragment into pKS and pGEX5X-1. *Sg-ftz* cDNA was subcloned into pUC19 as a KpnI/BstUI fragment, released as an EcoRI/XbaI fragment, and inserted directly into pCaSpeR-hs or pUAST. For in-frame cloning, an EcoRI site was added just 5′ of the endogenous ATG by PCR; *Sg-ftz* was inserted into pKS and pGEX5X-1 as an EcoRI/HindIII fragment. Full-length *ftz-f1* cDNA was inserted into pGEX5X-1 and pKS as a HindIII/BamHI fragment. All PCR products were verified by sequencing.

GST pull-down assays

To express proteins fused to a Gluthathione S-transferase (GST) moiety, pGEX5X-1 (Pharmacia) vectors were used. Overnight cultures of BL21 cells containing the appropriate plasmids were diluted 1:100 with LB containing 50 μg/ml ampicillin and were grown to $OD_{600} \approx 0.8$ at 37°C. Protein expression was induced with 0.2 M IPTG. Cells containing the Ftz-F1 plasmid were incubated at 37°C for 1.5 hr, while cells expressing Ftz proteins were incubated at 30°C for 2 hr. Cells were harvested and resuspended in 1.75 ml lysis buffer (1× PBS containing 1% Triton X-100 and 1 mM DTT) per 100 ml culture. Cells were lysed by sonication and incubated on ice for 30 min. Insoluble debris was removed by centrifugation at 11,000 × g for 10 min. Cell lysate was incubated with 500 μl 50% slurry of equilibrated GST beads (Pharmacia) for 30 min at room temperature. After washing, beads were stored at 4°C in 1× PBS with a protease inhibitor mixture (50 μg/ml trypsin inhibitor, 1 mM benzamidine, 2 U/ml aprotinin, 1 μg/ml antipain and 1 μg/ml bacitricin). The amount of GST-protein bound to beads was estimated by analyzing aliquots on a 10% polyacrylamide SDS gel. ^{35}S-labeled methionine proteins were produced by in vitro translation (TnT-Coupled Wheat Germ Extract System or TnT-Coupled Reticulocyte Lysate System, Promega) and were incubated with 10 μg fusion protein in 200 μl binding buffer (50 mM Tris-HCl [pH 8.0], 100 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 0.3 mM DTT and protease inhibitor mixture) for 2 hr at 4°C. After washing, beads were resuspended in 20 μl SDS loading buffer, boiled, and separated through 10% polyacrylamide SDS gels. After electrophoresis, gels were fixed in 25% methanol and 12% acetic acid. The EN^{3}HANCE kit (NEN) was used to enhance the ^{35}S-labeled methionine signal. Gels were dried and exposed to autoradiography film for the appropriate length of time.

Phenotypic analysis

P element transformation of *Drosophila* used standard methods. Fly lines containing UAS-*ftz* constructs were crossed to Gal4 lines. *Dll*-Gal4 [35] and *arm*-Gal4 were obtained from the Bloomington stock center. Flies carrying *dpp*-Gal4, UAS-GFP were a kind gift of J. Wu and M. Strigini. The expression of transgenes was confirmed at different developmental stages by conducting in situ hybridizations on embryos and discs carrying transgenes under the control of UAS and various Gal4 drivers. Significant differences in levels of mRNA expression were not observed (data not shown). For cuticle preparations, embryos from the appropriate crosses were collected for 6 hr, aged for 22 hr at 25°C, and treated as described [28]. Adult fly parts were dissected in isopropanol, directly mounted in Hoyers, and incubated at 45°C. Results were confirmed with two independent transformants for each transgene.

Embryos and imaginal discs were prepared and fixed using standard methods [58]. For discs expressing GFP, fixation time was reduced to 20 min. Immunohistochemistry was preformed using standard methods. Rat anti-Tsh [59] and rat anti-Hth (J. Wu, unpublished data) antibodies were detected using the TSA-Direct Kit (NEN) or with anti-rat antibody coupled with Texas red (Vector Labs).

The *ss* probe was made using the Dig RNA-labeling kit (Roche/Boehringer). In situ hybridization was carried out using standard methods. The dig-labeled probe was detected using an alkaline phosphatase-labeled anti-Dig Fab fragment (Roche) and visualized using the 1 Step NBT/CIP (Pierce).

Embryos, discs, and fly parts were photographed on a Zeiss Axiophot microscope, using a digital camera (Diagnostic Instruments) with dark field or fluorescent optics. Confocal imaging was carried out on a Leica TCS-SP (UV) confocal laser scanning microscope.

Embryonic and larval heat shocks

To synchronize embryonic development, embryos were precollected for 30 min at 25°C for all heat shock experiments. For ectopic expression of genes at blastoderm, embryos were collected for 30 min and aged for 2 hr and 15 min at 25°C. Embryos were washed off apple juice plates and submerged in a 37°C water bath for 8–10 min. We found that the optimization of conditions for the early heat shocks is crucial. Accordingly, the conditions for these early heat shocks were extensively optimized. For heat shocks during later stages of embryogenesis, embryos were collected for 1 hr and aged 5 hr at 25°C. Embryos were heat shocked for 45 min in a 37°C water bath. All embryos were allowed to develop into larvae at 25°C, and cuticle preparations were carried out as described above. All heat treatments were preformed in parallel to ensure that results could be compared across lines carrying different

expression constructs. Results were confirmed in at least three independent experiments. Heat shocks to detect ectopic *tsh* expression were carried out as described in [12]. For larval heat shocks, embryos were collected for 1 hr at 25° and were allowed to hatch. We selected 20 larvae of the same genotype and placed them in a food vial. Vials were placed in a 37°C water bath for 45 min at 4-hr intervals from 68 hr to 98 hr AEL and were than kept at 25°C. Flies were allowed to hatch or pharates were dissected from pupal cases.

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