

# FTZ-Factor1 and Fushi tarazu interact via conserved nuclear receptor and coactivator motifs

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**To activate transcription, most nuclear receptor proteins require coactivators that bind to their ligand-binding domains (LBDs). The *Drosophila* FTZ-Factor1 (FTZ-F1) protein is a conserved member of the nuclear receptor superfamily, but was previously thought to lack an AF2 motif, a motif that is required for ligand and coactivator binding. Here we show that FTZ-F1 does have an AF2 motif and that it is required to bind a coactivator, the homeodomain-containing protein Fushi tarazu (FTZ). We also show that FTZ contains an AF2-interacting nuclear receptor box, the first to be found in a homeodomain protein. Both interaction motifs are shown to be necessary for physical interactions *in vitro* and for functional interactions in developing embryos. These unexpected findings have important implications for the conserved homologs of the two proteins.**

**Keywords:** *Drosophila*/FTZ-F1/Fushi tarazu/homeodomain/nuclear receptor

## Introduction

The *Ftz-Factor1* (*Ftz-F1*) and *fushi tarazu* (*ftz*) genes act together in the *Drosophila* embryo to define the identities of alternate segmental regions (Guichet *et al.*, 1997; Yu *et al.*, 1997). *Ftz-F1* encodes a member of the nuclear receptor gene superfamily (Lavorgna *et al.*, 1991). The gene is transcribed both maternally and zygotically from two different promoters (Lavorgna *et al.*, 1993). This differential promoter usage results in two splice variants referred to as  $\alpha$ FTZ-F1 and  $\beta$ FTZ-F1, each of which encodes a receptor with a unique N-terminus. It is the maternally provided  $\alpha$ FTZ-F1 splice form that is required for segmentation. Due to this maternal deposition,  $\alpha$ FTZ-F1 is uniformly distributed in the early embryo (Yu *et al.*, 1997).

Like *Ftz-F1*, *ftz* is also a member of an important gene superfamily, the homeobox genes. Unlike *Ftz-F1*, however, *ftz* is expressed zygotically in a seven-stripe pattern (Hafen *et al.*, 1984; Carroll and Scott, 1985). These stripes correspond in position to the future even-numbered parasegments that are missing in both *ftz* (Wakimoto

*et al.*, 1984; Martinez-Arias and Lawrence, 1985) and  $\alpha$ FTZ-F1 (Guichet *et al.*, 1997; Yu *et al.*, 1997) mutant embryos. Thus, *ftz* provides the spatial information for the combinatorial function of the two genes.

Nuclear receptors are transcription factors that bind DNA via type II zinc-finger motifs (reviewed in Schwabe and Rhodes, 1991). They also contain structurally conserved ligand-binding domains (LBDs) that generally serve as activity-regulating modules. For receptors with known ligands, this regulation occurs through the binding of ligands such as steroid hormones (reviewed in Mangelsdorf and Evans, 1995). Ligand binding causes structural changes in the LBD that switch binding specificities for competing corepressor and coactivator proteins (Wurtz *et al.*, 1996; Westin *et al.*, 1998).

FTZ-F1 is a member of the orphan class of nuclear receptors, meaning that a ligand that binds and controls its activity has not been identified. Consistent with the absence of a known ligand, the published sequences of both the *Drosophila* and silkworm (*Bombyx mori*) FTZ-F1 proteins lack an important motif required for ligand binding (Lavorgna *et al.*, 1991; Sun *et al.*, 1994). This motif, termed the activation function 2 (AF2) motif, is an  $\alpha$ -helix found at the C-terminal end of most receptors (Durand *et al.*, 1994). In solved LBD structures, the AF2 helix folds over the ligand and its pocket, placing the AF2 in an appropriate position to participate in coactivator binding (Wurtz *et al.*, 1996). Most coactivators identified thus far either help activate transcription by recruiting histone acetyltransferase complexes, or by providing histone acetyltransferase activity themselves (Horwitz *et al.*, 1996). Interestingly, although the two cloned insect *Ftz-F1* genes do not appear to encode AF2 motifs, the LBDs of vertebrate and *Caenorhabditis elegans* *Ftz-F1* homologs do (Ikeda *et al.*, 1993; Ellinger-Ziegelbauer *et al.*, 1994; Galarneau *et al.*, 1996; Gissendanner and Sluder, 2000).

FTZ-F1 was first identified as a putative regulator of *ftz* gene expression, hence its name (Lavorgna *et al.*, 1991). More recently, mutations that specifically affect maternal *Ftz-F1* expression were isolated (Guichet *et al.*, 1997; Yu *et al.*, 1997). As might have been expected for a regulator of *ftz* expression, these mutations cause a pair-rule phenotype indistinguishable from that of *ftz*. However, further examination of the mutant embryos revealed that *ftz* transcript and protein expression were normal while FTZ target gene expression was not. This suggested that FTZ-F1 might act as a cofactor for FTZ function rather than as a requisite regulator of *ftz* gene expression. Consistent with this possibility, FTZ and FTZ-F1 were shown to interact directly *in vitro* and in a mutually dependent fashion *in vivo* (Guichet *et al.*, 1997; Yu *et al.*, 1997).

FTZ contains a highly conserved homeodomain (Kuroiwa *et al.*, 1984; Laughon and Scott, 1984; Weiner *et al.*, 1984) that is required for DNA binding activity (Desplan *et al.*, 1988; Hoey and Levine, 1988; Laughon *et al.*, 1988). Homeodomain-containing proteins are found in all metazoans and are key regulators of embryonic patterning (reviewed in Veraksa *et al.*, 2000). Interestingly, FTZ can perform some of its regulatory functions in the absence of its homeodomain (Fitzpatrick *et al.*, 1992; Copeland *et al.*, 1996). This finding suggested that FTZ can be recruited to target gene promoters by interacting with specific cofactors. FTZ-F1 may be one of these cofactors since it is required for a number of homeodomain-independent FTZ activities (Guichet *et al.*, 1997).

To understand better how FTZ and FTZ-F1 cooperate to regulate target gene expression, we have carried out a deletional analysis to define domains that mediate contact between them. Surprisingly, we find that insect FTZ-F1 proteins do in fact contain a highly conserved AF2 motif. This motif is shown to be crucial for interactions with FTZ, both *in vitro* and *in vivo*. Correspondingly, we show that FTZ contains a previously unnoticed nuclear receptor box, a motif that is found in all nuclear receptor coactivators that interact with the LBD (Heery *et al.*, 1997). This motif is also required, both *in vitro* and *in vivo*, to interact with FTZ-F1. These findings have important implications for other members of both protein super-families.

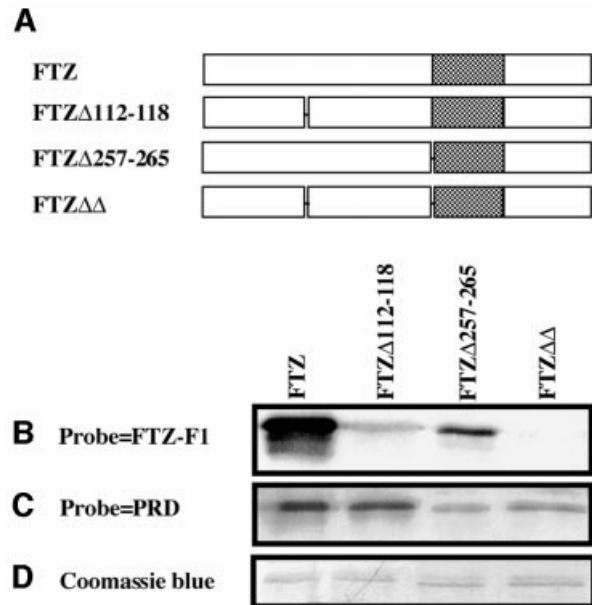
## Results

### The nuclear receptor box of FTZ is required for contact with FTZ-F1

In previous work, we showed that a 50 amino acid region in the N-terminus of FTZ is required for contact with FTZ-F1 (Guichet *et al.*, 1997). This region contains a conserved sequence, LRALLT, which is closely flanked by proline residues on each side, and further away by charged residues. We now note that the central portion of this sequence matches the LXXLL consensus motif referred to as a nuclear receptor box (Heery *et al.*, 1997). Nuclear receptor boxes are found in most coactivators of nuclear receptors. This coincidence prompted us to test whether the LRALLT sequence in FTZ is specifically required to interact with FTZ-F1, and if so, with what part of FTZ-F1.

Residues that span the FTZ nuclear receptor box were deleted to generate the construct FTZ $\Delta$ 112–118 (see Figure 1A). Previous work has shown that the FTZ–FTZ-F1 interaction can be specifically detected by a variety of techniques including far western blotting (Guichet *et al.*, 1997). Figure 1B shows a far western blot containing several FTZ deletion constructs probed with <sup>35</sup>S-labeled FTZ-F1. The full-length FTZ polypeptide is recognized well by the radiolabeled FTZ-F1 probe (Figure 1B, lane 1). In contrast, FTZ $\Delta$ 112–118 yields a much fainter signal (Figure 1B, lane 2). Thus, the nuclear receptor box in FTZ plays an important role in the FTZ-F1 interaction *in vitro*.

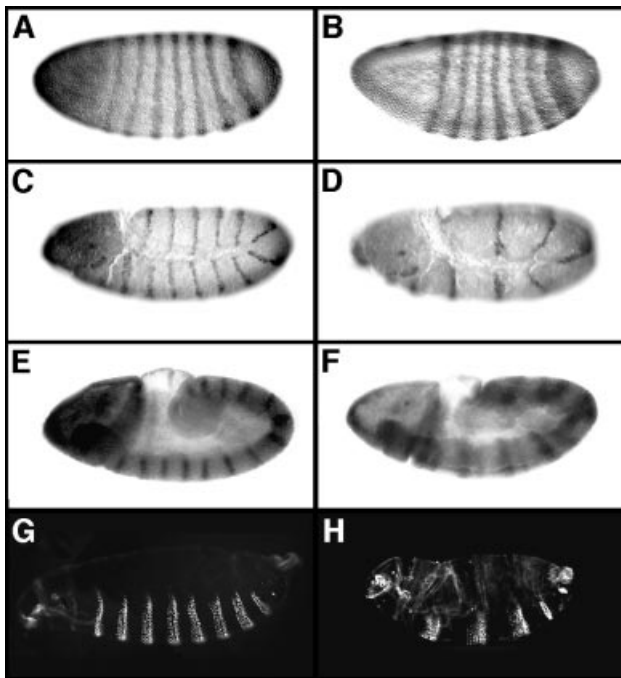
While removal of the nuclear receptor box in FTZ $\Delta$ 112–118 greatly reduced the intensity of the FTZ-F1 signal, it did not eliminate it. This suggests the



**Fig. 1.** Domains in FTZ required to bind FTZ-F1. (A) Line drawings of the FTZ deletion constructs that affect binding to FTZ-F1. The N- and C-terminal regions of FTZ are represented by open boxes and the homeodomain by a hatched box. FTZ $\Delta$ 112–118 is missing conserved amino acids that comprise a consensus nuclear receptor box. FTZ $\Delta$ 257–265 is missing the first nine residues of the homeodomain. FTZ $\Delta$  $\Delta$  contains both deletions. (B) A far western blot used to test binding of a <sup>35</sup>S-labeled FTZ-F1 probe to the immobilized FTZ polypeptides depicted in (A). The FTZ protein present in each lane is indicated above. (C) A far western blot in which radiolabeled Paired protein (PRD) was used to probe the same polypeptides as in (B). (D) A Coomassie Blue stained gel of the same proteins loaded in (B) and (C).

existence of a second FTZ-F1 interaction domain within the FTZ polypeptide. A previous study showed that a FTZ polypeptide lacking the N-terminal half of the protein (including the nuclear receptor box) could bind cooperatively with FTZ-F1 to adjacent binding sites on DNA (Florence *et al.*, 1997), suggesting a potential contact region for FTZ-F1 within the homeodomain-containing C-terminal region. A series of deletion constructs that remove different parts of the FTZ C-terminus were tested for effects on FTZ-F1 binding (J.W.R. Copeland and H.M. Krause, unpublished). A deletion that removes the first nine residues of the FTZ homeodomain (FTZ $\Delta$ 257–265) was found to have a modest effect (Figure 1B, lane 3). When these nine residues and the N-terminal nuclear receptor box are both deleted (FTZ $\Delta$  $\Delta$ ), binding to the <sup>35</sup>S-labeled FTZ-F1 probe is no longer detected (Figure 1B, lane 4). Hence, there appear to be two FTZ-F1 interaction domains in FTZ, the nuclear receptor box and a second contact point that includes the beginning of the homeodomain.

As a control for the specificity of action of the two FTZ deletions, we tested them for effects on binding to another FTZ-interacting protein, Paired (PRD). Like FTZ-F1, PRD has been shown to require contact points within both the N- and C-terminal regions of FTZ (Copeland *et al.*, 1996). Figure 1C shows a far western blot with the same FTZ polypeptides used in Figure 1B, but with <sup>35</sup>S-labeled PRD used as the probe. Unlike FTZ-F1, PRD binds equally well to both full-length FTZ and FTZ $\Delta$ 112–118 (Figure 1C,



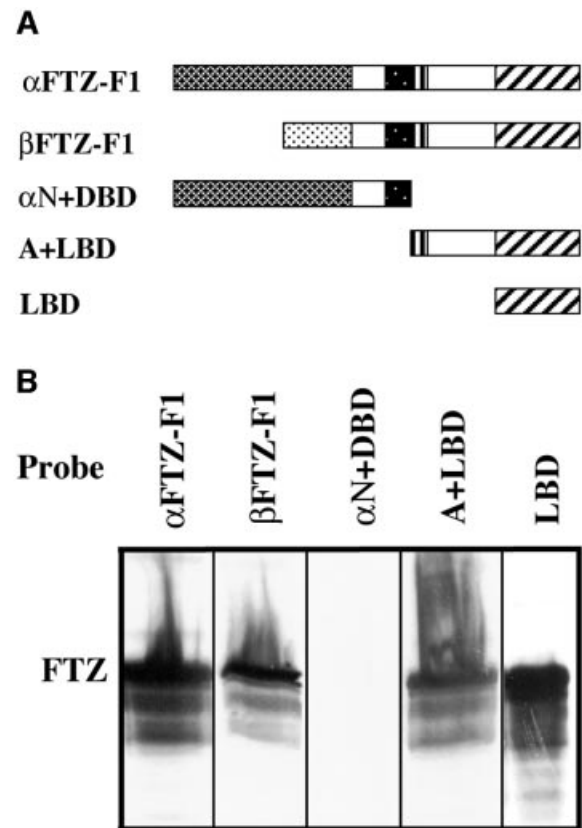
**Fig. 2.** The FTZ nuclear receptor box is required for FTZ-F1-dependent FTZ activities. Full-length FTZ and FTZ $\Delta$ 112–118 proteins were tested for rescuing activity in transgenic embryos. (A and B) FTZ expression; (C and D) EN expression; (E and F) WG expression; (G and H) cuticular phenotypes. (A)–(F) are also double-stained for  $\beta$ -galactosidase expression. (A), (C), (E) and (G) are internal *ftz*<sup>+</sup> controls from *ftz* $\Delta$ 112–118 rescue crosses. These contain a wild-type *ftz* chromosome marked with *hb-lacZ*. The embryos in (B), (D), (F) and (H) are homozygous mutant for the endogenous *ftz* gene and express only the *ftz* $\Delta$ 112–118 transgene.

lanes 1 and 2). Thus, the nuclear receptor box of FTZ is specifically required to interact with FTZ-F1. On the other hand, deletion of residues 257–265 did affect binding to PRD (Figure 1C, lane 3), suggesting that this region is either a common contact point for both PRD and FTZ-F1, or that it affects other contact points via general effects on protein conformation. Removal of both regions had no further effect on the binding of PRD (Figure 1C, lane 4).

#### **Deletion of the FTZ nuclear receptor box disrupts FTZ-F1-dependent functions in vivo**

Previous studies showed that loss of  $\alpha$ *Ftz-F1* expression in the early embryo results in the loss of most FTZ activities, one exception being the ability to autoregulate its own promoter. Thus, if the FTZ nuclear receptor box in FTZ is specifically required to contact FTZ-F1, removal of this motif should have a similar effect on FTZ activities. FTZ $\Delta$ 112–118 should be able to regulate its own expression but should not be able to regulate other target genes or to generate *ftz*-dependent cuticle. This hypothesis was tested by expressing transgenic *ftz* genes, containing or missing the nuclear receptor box, in a *ftz* mutant background. Full-length and  $\Delta$ 112–118 *ftz* sequences were subcloned back into a 10 kb genomic rescue construct previously shown to be capable of rescuing all *ftz*-dependent processes (Hiromi *et al.*, 1985), and transgenic lines containing the constructs were established.

Seventy-five percent of the embryos from the *ftz* rescue crosses possess a wild-type *ftz*-containing chromosome



**Fig. 3.** Mapping domains in FTZ-F1 required for binding to FTZ. (A) Schematic drawings of the full-length and deleted FTZ-F1 constructs initially used to map FTZ-interacting regions. The light and dark hatched regions at the N-terminal ends of the proteins represent the alternative N-termini of the  $\alpha$  and  $\beta$  FTZ-F1 isoforms. The black region is the zinc-finger DNA-binding domain. The vertically striped region immediately behind is the A-box and the diagonally hatched region at the C-terminus is the LBD. (B) A far western blot in which the FTZ-F1 deletion constructs shown in (A) were radioactively labeled and used to probe full-length FTZ immobilized on a membrane after SDS-PAGE. Lanes 1–4 and lane 5 are from separate blots.

that is marked with a *lacZ*-expressing reporter gene (*hb-lacZ*). Figure 2A shows one of these internal controls, double-stained for both FTZ and  $\beta$ -galactosidase. The normal pattern of seven FTZ stripes is present, along with the anterior  $\beta$ -galactosidase pattern. The same FTZ pattern is observed in homozygous *ftz* mutant embryos (no  $\beta$ -galactosidase expressed) when either the wild-type (not shown) or *ftz* $\Delta$ 112–118 (Figure 2B) rescue construct is present. In contrast, the *ftz* $\Delta$ 112–118 construct failed to carry out any of the other *ftz*-dependent activities tested. FTZ $\Delta$ 112–118 was unable to restore *ftz*-dependent *en-grailed* stripes (Figure 2D), to prevent the widening of odd-numbered *wingless* stripes (Figure 2F) or to rescue FTZ-dependent cuticle (Figure 2H). Thus, deletion of the FTZ nuclear receptor box phenocopies the loss of  $\alpha$ *Ftz-F1*. Similar results were obtained when transgenic FTZ and FTZ $\Delta$ 112–118 activities were tested via ectopic expression (data not shown).

#### **The LBD of FTZ-F1 is required for binding to FTZ**

We next performed a deletional analysis of the FTZ-F1 protein to identify domains required to interact with FTZ. Figure 3A shows the  $\alpha$  and  $\beta$ FTZ-F1 constructs that were

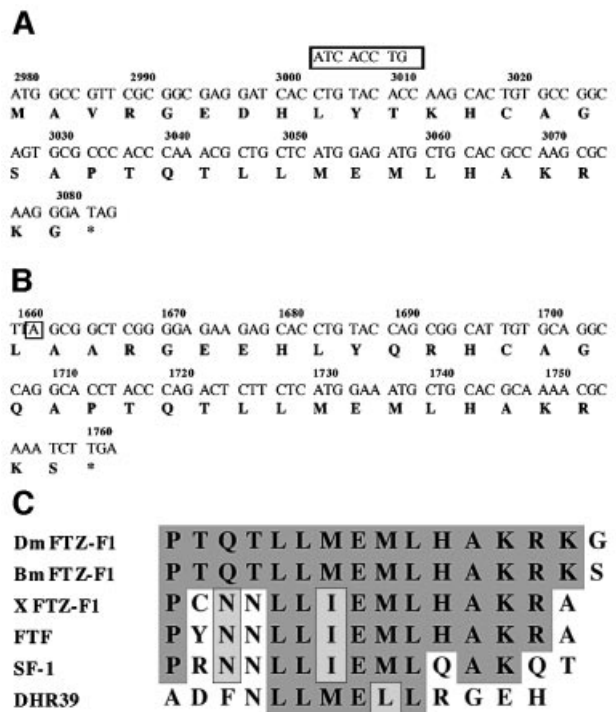
made and tested. The unique N-terminal region of the maternally expressed  $\alpha$ FTZ-F1 isoform suggests the possibility that this region may have evolved as a FTZ-specific contact point. However, both  $\alpha$  and  $\beta$  isoforms of FTZ-F1 bind to FTZ (Figure 3B, lanes 1 and 2), indicating that the  $\alpha$ -specific N-terminus is not essential for FTZ binding. This assumption is supported by the finding that a polypeptide containing the unique N-terminus of  $\alpha$ FTZ-F1 along with the DNA-binding domain ( $\alpha$ N+DBD) shows no detectable binding to FTZ (Figure 3B, lane 3). Consistent with a C-terminal contact point, a complementary polypeptide that spans from the A-box region to the C-terminal end of FTZ-F1 (A+LBD) is sufficient for FTZ binding (Figure 3B, lane 4). Further removal of residues to the beginning of the LBD does not disrupt the interaction (Figure 3B, lane 5). Thus, the FTZ-F1 LBD appears to be both necessary and sufficient for an interaction with FTZ.

### FTZ-F1 contains a conserved AF2 helix

*Ftz-F1* deletion constructs were sequenced to ensure that no errors had been introduced during the course of PCR and cloning procedures. These analyses revealed a discrepancy between our sequences and those of the published *Ftz-F1* sequence (Lavorgna *et al.*, 1991). An 8 bp repeat beginning at position 3006 of the  $\alpha$ *Ftz-F1* sequence was absent in our sequences (Figure 4A). The predicted coding region of the revised sequence diverges at amino acid 1003 and encodes an additional 24 amino acids. This revised sequence contains an AF2 consensus that is highly similar to those of vertebrate FTZ-F1 homologs (see Figure 4C for comparisons). Confirmation of this revised sequence has recently been provided by the *Drosophila* genome sequencing project (Adams *et al.*, 2000).

The sequence of the FTZ-F1 protein of *B.mori* has also been reported to lack an AF2 motif (Sun *et al.*, 1994). A brief examination of the DNA coding sequence revealed that the addition of a single nucleotide in an appropriate location would change the reading frame and yield an AF2 nearly identical to that of the *Drosophila* protein. Hence, a *Bombyx Ftz-F1* cDNA was obtained and sequenced. An additional adenosine nucleotide was found that follows the thymidine at position 1659 (Figure 4B). The shifted reading frame generates a new C-terminus with 27 of the 34 predicted amino acids identical to the *Drosophila* sequence. An additional four residues are conservative substitutions. The C-terminal AF2s of the two insect proteins are identical at 15 of 16 positions. We conclude that both insect proteins contain highly conserved AF2 motifs.

Figure 4C shows the homology between the AF2 motifs of the insect, *Xenopus* and human FTZ-F1 homologs. Humans have two receptors with high homology to FTZ-F1, suggesting the possibility of an evolutionary gene duplication. The fetoprotein transcription factor (FTF) AF2 exhibits the highest homology to the FTZ-F1 AF2 (67% identity and 80% similarity). The steroidogenic factor-1 (SF-1) AF2 has 53% identity and 67% similarity to the FTZ-F1 AF2. *Xenopus* has two FTZ-F1 homologs, both of which are more closely related to FTF than to SF-1 (Ellinger-Ziegelbauer *et al.*, 1994). Consistent with this relationship, their homologies to the FTZ-F1 AF2 are the same as that of FTF. DHR39, also sometimes referred to as



**Fig. 4.** *Drosophila melanogaster* and *B.mori* FTZ-F1 sequences encode conserved AF2 motifs. (A) The corrected DNA and protein sequences for the final 34 amino acids of the *Drosophila* FTZ-F1 coding region. The boxed sequence above position 3006 indicates the repeat that is present in the previously published sequence but not found in ours. The numbering above the DNA sequence is for the  $\alpha$  transcript. The corrected protein sequence is provided below the DNA sequence. (B) The corrected DNA and protein sequences for the final 34 amino acids of the *B.mori* FTZ-F1 coding region. The boxed adenosine at position 1660 represents a missing nucleotide in the previously published sequence. The corrected protein sequence is provided below the DNA sequence. (C) A comparison of the AF2 helices of FTZ-F1 homologs and the most closely related *Drosophila* receptor DHR39. Dark shading indicates identical residues and light shading similar residues. DmFTZ-F1 is the *Drosophila* protein, Bm FTZ-F1 the *B.mori* protein and XFTZ-F1 the *Xenopus laevis* protein. FTF is human fetoprotein transcription factor, SF-1 is steroidogenic factor 1 and DHR39 is the *Drosophila* hormone receptor at genomic position 39.

FTZ-F1 $\beta$ , is the *Drosophila* nuclear receptor that is most similar to FTZ-F1 (Ayer *et al.*, 1993; Ohno and Petkovich, 1993). DHR39 binds to the same DNA sequences as FTZ-F1, and regulates the same reporter genes in cultured cells (Ayer *et al.*, 1993). However, the only part of the DHR39 AF2 that is homologous to the FTZ-F1 AF2 is the core (five of six amino acids).

### The AF2 motif of FTZ-F1 is required for contact with FTZ

Since the AF2 helix in other nuclear receptors provides an essential contact surface for the binding of nuclear receptor box-containing coactivators (Darimont *et al.*, 1998; Nolte *et al.*, 1998; Shiau *et al.*, 1998), we wished to know whether the AF2 in FTZ-F1 was necessary for contact with FTZ. This was tested by removing the AF2 and by replacing it with homologous or non-homologous AF2 motifs. Figure 5A shows the results of a far western blot containing deleted and chimeric FTZ-F1 constructs probed with <sup>35</sup>S-labeled FTZ (Figure 5B shows a loading

control). The C-terminal half of FTZ-F1 (A+LBD) binds well to the FTZ probe (Figure 5A, lane 1), as previously shown in Figure 3B. However, when the AF2 is removed (A+LBD $\Delta$ AF2), little signal is detected (Figure 5A, lane 2). Binding is re-established by adding either of the human FTZ-F1 homolog AF2 motifs back to the AF2-deleted FTZ-F1 construct (Figure 5A, lanes 3 and 4). A weaker signal is observed when the AF2 of the more divergent DHR39 receptor is added (Figure 5A, lane 5).

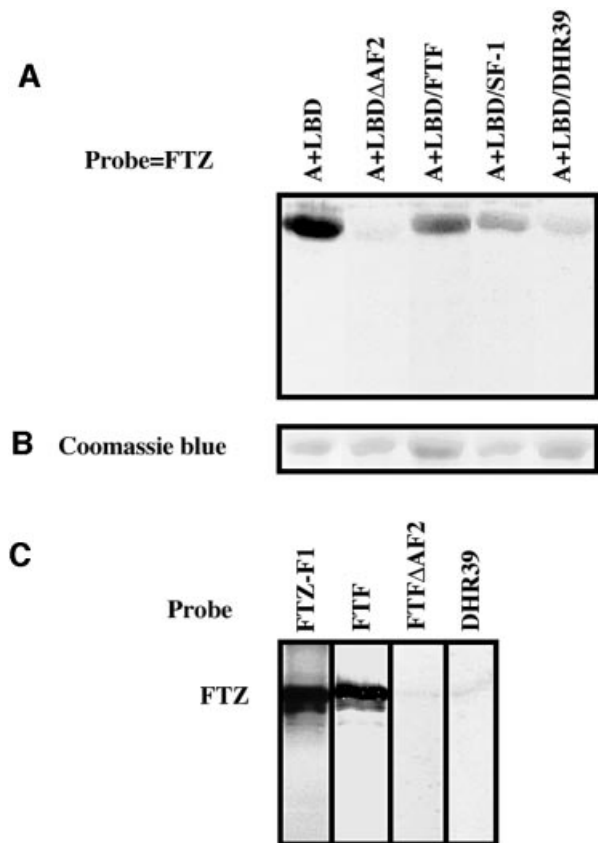
In a reciprocal blot, equal loadings of full-length FTZ were probed with labeled nuclear receptor proteins (Figure 5C). Lanes 1 and 2 show that full-length FTZ-F1 and FTF both bind to the immobilized protein. However, removal of the FTF AF2 motif results in a substantial loss of signal (Figure 5C, lane 3). The signal generated with labeled DHR39 is also very weak (Figure 5C, lane 4). Thus, a conserved AF2 motif is required for a strong interaction *in vitro*.

### A conserved AF2 motif is essential for FTZ-F1 function *in vivo*

The results of Figure 2 show that FTZ loses FTZ-F1-dependent functions *in vivo* when the nuclear receptor box is removed. We wished to know whether removal or exchange of the FTZ-F1 AF2 helix would cause a reciprocal loss of FTZ-dependent activities *in vivo*. This was tested by making transgenic fly lines that express  $\alpha$ FTZ-F1 with or without its AF2, or with the AF2s of other receptors swapped in. The constructs were expressed in both wild-type and  $\alpha$ *Ftz-F1* mutant backgrounds under the control of a GAL4-dependent promoter and an *armadillo*-GAL4 driver (see Materials and methods for details). The *armadillo*-GAL4 driver is transcribed both maternally and zygotically (Riggleman *et al.*, 1989).

The *Ftz-F1* mutant line used for rescue (*Ftz-F1*<sup>209</sup>) has previously been reported to be null for the maternally provided protein (Guichet *et al.*, 1997). The majority of embryos laid by *Ftz-F1*<sup>209</sup> mothers exhibit a pair-rule phenotype as shown in Figure 6B. However, we find that this line does produce a low level of escapers (~10% of eggs hatch as viable larvae). Since this number was consistent between lines and experiments, it was treated as background. A minimum of four transgenic lines for each construct were obtained and tested. The results described below (Figure 6; Table I) were obtained with representative lines that express equivalent levels of each protein construct (determined by western blot analysis; data not shown).

Figure 6C shows a typical *Ftz-F1*<sup>209</sup> mutant embryo rescued by a full-length *Ftz-F1* construct. Ninety-nine percent of these embryos are rescued to either wild-type or partially rescued phenotypes (Table I). However, when the AF2 helix is removed, rescuing activity is reduced dramatically (Figure 6D). Sixty-six percent of the embryos resemble the unrescued pair-rule phenotype, and another 28% show a partial pair-rule phenotype (Table I). Similar results are obtained when the DHR39 AF2 motif is substituted for that of FTZ-F1 (Figure 6E; Table I). Thus, the FTZ-F1 AF2 is required for full rescuing activity. This AF2 requirement is further supported by the results of a conservative SF-1 AF2 swap. This construct rescues the FTZ-F1 phenotype to about the same degree as full-length FTZ-F1 (Figure 6F; Table I). Thus, the SF-1 AF2



**Fig. 5.** Binding to FTZ requires the AF2 helix of FTZ-F1. (A) A far western blot in which various FTZ-F1 derivative proteins are probed with <sup>35</sup>S-labeled FTZ. Each of the FTZ-F1 polypeptides begins at the A-box and contains all (A+LBD) or most of the LBD. A+LBD $\Delta$ AF2 has the AF2 helix deleted. A+LBD/FTF contains the FTF AF2 in place of the FTZ-F1 AF2. Similarly, A+LBD/SF-1 and A+LBD/DHR39 contain the SF-1 and DHR39 AF2 helices in place of the FTZ-F1 AF2. (B) A Coomassie Blue-stained gel loaded with the same amounts of proteins loaded in (A). (C) A far western blot in which different full-length and deleted nuclear receptor proteins were <sup>35</sup>S-labeled and used to probe blots in which equivalent amounts of FTZ were loaded. The probe used is indicated above each lane. FTF $\Delta$ AF2 is FTF with its AF2 helix removed.

retains the functional specificity of its *Drosophila* counterpart.

Although embryos that express wild-type *Ftz-F1* and *Ftz-F1/SF-1* show good rescue of the *Ftz-F1* segmentation phenotype, many of these embryos exhibit a novel cuticular phenotype (similar to Figure 6G). The cuticles tend to be swollen and rounded up, the head and tail regions improperly formed and denticles incompletely developed. These defects are likely to be caused by the persistent expression of  $\alpha$ FTZ-F1 during later stages of embryogenesis due to use of the *armadillo*-GAL4 driver. Maternally provided  $\alpha$ FTZ-F1 is normally gone by early gastrulation, and  $\beta$ *Ftz-F1* expression does not begin until late embryogenesis (Lavorgna *et al.*, 1993). Hence, the expression of  $\alpha$ *Ftz-F1* during these later stages of embryogenesis may cause inappropriate expression or overexpression of FTZ-F1 target genes. Alternatively, late expression of the  $\alpha$  isoform may compete non-productively with  $\beta$ FTZ-F1 for necessary cofactors.

To determine whether these defects are due to inappropriate FTZ-F1 activities, ubiquitous  $\alpha$ *Ftz-F1* expression

was induced under *armadillo*-GAL4 control in a wild-type background. None of the eggs produced from this cross hatch as larvae, and all exhibit a phenotype similar to that shown in Figure 6G. Thus, expression of  $\alpha$ *Ftz-F1* after gastrulation is lethal.

## Discussion

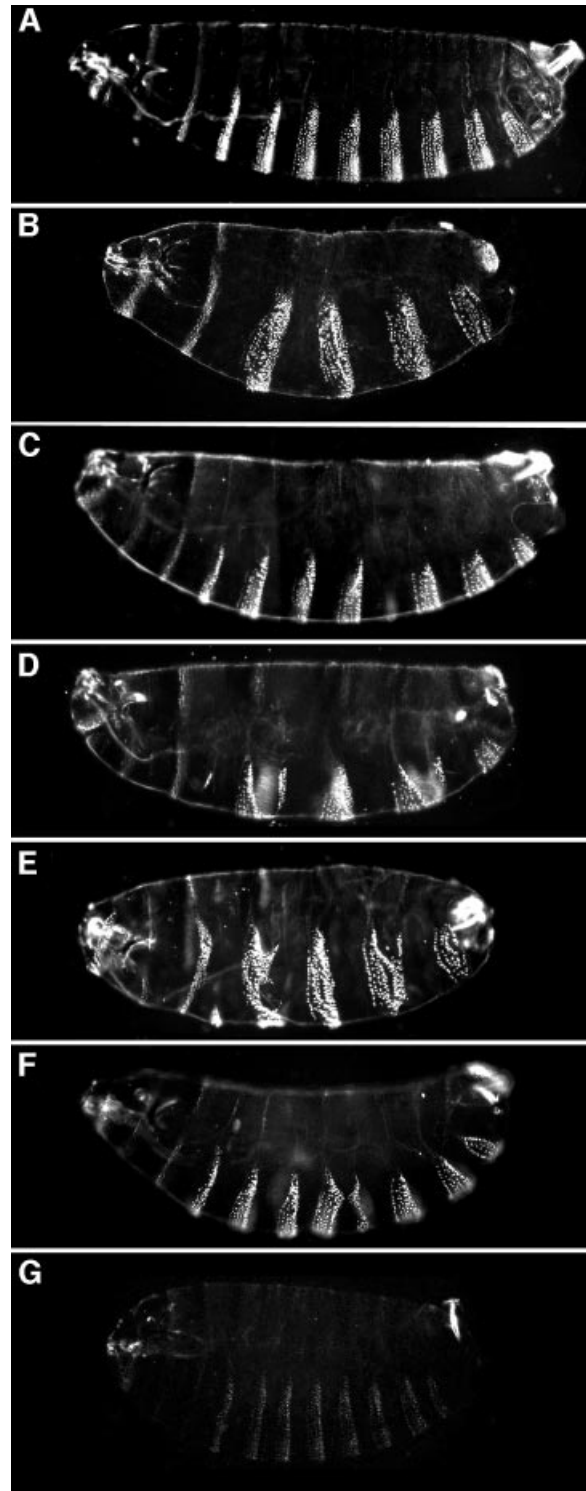
### *FTZ and FTZ-F1 interact via canonical motifs*

Recent studies have shown that a number of nuclear receptors activate transcription through the selective recruitment of histone acetyltransferase complexes (reviewed in Freedman, 1999; Xu *et al.*, 1999; Chen *et al.*, 2000). This is achieved with the aid of coactivator molecules that generally bind to counterpart nuclear receptor LBDs. The major finding of this study is that FTZ and FTZ-F1 interact via the same canonical motifs used by these other receptors and their coregulators, a nuclear receptor box in the case of FTZ and an AF2 motif in the case of FTZ-F1. FTZ is the first homeodomain protein shown to contain a functional nuclear receptor box. In the case of FTZ-F1, both of the insect *Ftz-F1* homologs previously sequenced had been found to lack coactivator-binding AF2 motifs, suggesting that they may activate transcription by other means. Our finding that FTZ-F1 does indeed have an AF2 motif, and FTZ a counterpart nuclear receptor box, has important implications for the functions of both proteins and their conserved homologs.

Although this is the first example of a nuclear receptor box in a homeodomain protein, other homeodomain proteins may also contain related motifs. Our *in vitro* binding experiments also identified a second FTZ-F1 interaction motif at the beginning of the FTZ homeodomain. The residues shown to be important are highly conserved in all Antennapedia class homeodomains (reviewed in Gehring *et al.*, 1994; Sharkey *et al.*, 1997), suggesting that other homeodomain proteins may also be capable of interacting with FTZ-F1 or with other nuclear receptors via their homeodomains. Thus, interactions between homeodomain proteins and nuclear receptors could be fairly common.

When residues of the FTZ-F1 AF2 helix are plotted on a helical wheel, the conserved residues cluster on two faces of the helix. Q1014, L1017, L1021 and K1024 are on the side that is predicted to interact with the ligand-binding pocket (Feng *et al.*, 1998; Nolte *et al.*, 1998; Shiau *et al.*, 1998; Westin *et al.*, 1998), and L1016, E1019 and A1023 are on the side predicted to contact coactivator nuclear receptor boxes. M1020 is also conserved and lies between these two clusters. Residues that differ from the DHR39 AF2, which interacts poorly with FTZ, are on either side of the consensus AF2 core, suggesting that it is these flanking regions that provide the specificity for FTZ.

Although SF-1 was initially considered to be the human FTZ-F1 homolog, its AF2 is only 53% identical to FTZ-F1. The closer homology of FTF suggests the interesting possibility that FTF may be the more ancestral form of the two proteins, and that it may serve functional roles more equivalent to those of FTZ-F1. Consistent with this suggestion, zebrafish and trout appear to have only one FTZ-F1 homolog each (Liu *et al.*, 1997; Ito *et al.*, 1998), and these most closely resemble FTF.



**Fig. 6.** Phenotypes of rescued *Ftz-F1*<sup>209</sup> embryos. FTZ-F1 constructs were used to rescue embryos lacking maternally provided  $\alpha$ FTZ-F1. The embryos shown are representative of the cuticular phenotypes obtained with each of the listed genetic backgrounds. (A) A wild-type cuticle. (B) A *Ftz-F1*<sup>209</sup> cuticle. (C) A *Ftz-F1*<sup>209</sup> embryo rescued with a full-length FTZ-F1 transgene. Embryos in (D)–(F) were rescued with a FTZ-F1 $\Delta$ AF2 transgene (D), a FTZ-F1/DHR39 transgene (E) or a FTZ-F1/SF-1 transgene (F). (G) A typical example of an embryo in which full-length FTZ-F1 was expressed in a wild-type background.

The FTZ-F1 rescue experiments suggest that, like FTZ, FTZ-F1 may also contain additional interaction motifs, as removal of the FTZ-F1 AF2 did not destroy all rescuing

**Table I.** *Ftz-F1* construct rescue efficiency

Construct	Total	% PR	% pseudo-PR	% partial-PR	% pseudo-WT
None	1227	65	16	13	6
<i>Ftz-F1</i>	215	0	1	10	89
<i>Ftz-F1</i> ΔAF2	272	13	53	28	7
<i>Ftz-F1/DHR39</i>	391	17	44	32	6
<i>Ftz-F1/SF-1</i>	422	1	0	24	75

Constructs present in the transgenic lines used are indicated on the left. Total indicates the number of cuticles counted for each of the transgenic lines (one representative line chosen/construct). Percentages shown are for the different degrees of rescue observed with each of the *Ftz-F1* rescue constructs. The cuticular phenotypes observed were divided up into four categories. PR indicates pair-rule; pseudo-PR indicates partial splitting of 1–2 fused denticle belts; partial-PR indicates most denticle belts are distinguishable but are partially fused; pseudo-WT indicates a wild-type or nearly wild-type (1–2 denticle belts partially fused or incomplete) cuticle.

activity. Although fully rescued embryos were not observed, the number of weakly rescued embryos rose significantly. Indeed, further deletional mapping of FTZ-F1 suggests that there may be a second interaction motif N-terminal to the LBD (data not shown).

#### **How do FTZ and FTZ-F1 regulate transcription?**

As stated previously, many nuclear receptors are now known to use corepressors and coactivators to recruit histone deacetylase and acetyltransferase complexes (reviewed in Xu *et al.*, 1999; Egea *et al.*, 2000). It is not yet known how FTZ and FTZ-F1 regulate their target genes. Interestingly, FTZ and FTZ-F1 are known to function as both transcriptional activators and repressors (Guichet *et al.*, 1997). Thus, FTZ may be able to act as both a coactivator and corepressor for FTZ-F1 function. Alternatively, these differential activities may depend on the actions of other cofactors, some of which may be target gene specific.

The fact that FTZ-F1 utilizes FTZ as a coregulator of target genes, and that FTZ contains a nuclear receptor box, suggests the possibility that, like other nuclear receptor cofactors, FTZ may help recruit histone acetyltransferase or deacetylase complexes. On the other hand, FTZ may act via regulatory factors or complexes that are quite different from these. For example, FTZ target genes such as *engrailed* and homeotic genes of the *Antennapedia* and *bithorax* gene clusters are regulated by chromatin-organizing Polycomb and Trithorax group protein complexes (Chinwalla *et al.*, 1995; LaJeunesse and Shearn, 1996; Strutt *et al.*, 1997; Kennison *et al.*, 1998). The FTZ–FTZ-F1 complex may play a role in recruiting these larger protein complexes.

#### **Does FTZ-F1 have a ligand?**

There are several observations that argue both for and against the existence of a FTZ-F1 ligand. First, motifs and residues required for ligand binding by other receptors are conserved in FTZ-F1. These include residues in the AF2 helix that, based on other receptor structures, are expected to contact the ligand-binding pocket (Wagner *et al.*, 1995; Wurtz *et al.*, 1996; Nolte *et al.*, 1998). FTZ-F1 also contains a conserved residue in the third helix of the LBD (N840) that has been shown in other receptors to be a key ligand-contacting residue (Williams and Sigler, 1998). Although a study with the FTZ-F1 homolog SF-1 suggested that its transcriptional activity could indeed be enhanced in the presence of certain oxysterols (Lala *et al.*,

1997), subsequent studies have been unable to validate this finding (Mellon and Bair, 1998). A more general observation in favor of a ligand is that while there are a large number of orphan nuclear receptors whose ligands have not been identified, there are currently no clear examples of receptors that are fully functional as activators in the absence of ligand.

Observations that argue for the absence of a FTZ-F1 ligand include the avid binding of FTZ to FTZ-F1 observed *in vitro* in the absence of ligand. For most nuclear receptors with known ligands, the ligand is required to position the overlying AF2 in the proper orientation for coactivator binding (Wurtz *et al.*, 1996). The ability of FTZ to bind in the absence of ligand suggests that coactivator binding to FTZ-F1 may be constitutive, and that temporal and spatial regulation of FTZ-F1 activity is controlled by the presence or absence of cofactors such as FTZ rather than by ligands. On the other hand, conditions in the cell may be more stringent, making the FTZ–FTZ-F1 interaction ligand dependent.

#### **Evolution of the FTZ–FTZ-F1 interaction**

The role of the FTZ–FTZ-F1 interaction in the metameric patterning of other insects has yet to be determined. In the relatively primitive grasshopper (*Schistocerca*), a *ftz* gene is present, but is only expressed in the central nervous system (Dawes *et al.*, 1994). In *Tribolium*, *ftz* is expressed in both the nervous system and emerging segments, but deletion of the gene has no effect on segment number (Brown *et al.*, 1994). This has led to the suggestion that *Tribolium* may represent an evolutionary intermediate in which the pair-rule function of *ftz* is just emerging. Interestingly, the *Schistocerca ftz* gene, which is not segmentally expressed, also does not contain a nuclear receptor box. Thus, the FTZ–FTZ-F1 interaction may have evolved for a role in segmentation. The expression pattern and phenotype of the *Tribolium Ftz-F1* gene should help determine how and where this interaction evolved and for what initial purpose.

FTZ-F1 homologs may play related roles in the patterning of other metazoans. Our results also suggest the possibility that these receptors may have homeo-domain protein partners. Interestingly, FTZ-F1 and FTZ regulate homeotic genes in *Drosophila* in much the same way that RAR receptors and HOX proteins regulate HOX genes in vertebrates (reviewed in Langston and Gudas, 1994; Lufkin, 1996; Eichele, 1997). This suggests the possibility that the latter may also interact directly, and

that the regulatory complexes they form may share similar components or functions.

## Materials and methods

### Plasmids

The *Ftz* deletions  $\Delta 112-118$  and  $\Delta 257-265$  were generated by PCR using the plasmid p18-FKZ (a variant of pGemF1; Krause *et al.*, 1988) as template. All reactions were performed with high fidelity *Pfu* DNA polymerase (Stratagene). The primer pair 5'-CTTCACGGGTGTGCTGGTCGCTCCTC-3' and 5'-CCCAGCACACCCGTGAAGAAGCTGAAAG-3' was used to generate the  $\Delta 112-118$  deletion and the primer pair 5'-GCTATGCGGCCGAGGAGCTAGCTATAAAC-3' and 5'-GCACTGCGGCCGCTAACTGAGCATCGCCGGAC-3' was used to generate the  $\Delta 257-265$  deletion. Full-length versions of the deleted and wild-type proteins were generated using the primer pair 5'-TGC-AAAGACCGCTACCAGACCTGGAG-3' and 5'-CTGGTAGCGGCTTTGCAATCTGATGC-3'. The resulting products contain *NotI* sites at both ends. These were used to insert the fragments into the unique *NotI* site of the heat shock-inducible vector pNMT4 (Schneuwly *et al.*, 1987) generating the constructs pI (full-length), pR27 ( $\Delta 112-118$ ) and pVIII ( $\Delta 257-265$ ).

To make a bacterial expression plasmid containing FTZ $\Delta 112-118$ , a *BamHI* site was generated at the translation start site of FTZ $\Delta 112-118$  using PCR and the oligo 5'-ACGGATCCACCGATATGGCTACCAAAAC-3' and a plasmid-specific oligo. The 1.5 kb *BamHI* fragment was then transferred into pRSET to make pFTZ $\Delta 112-118$ . To generate a bacterial expression plasmid carrying FTZ $\Delta 257-265$ , the N-terminal 0.5 kb *BamHI-SalI* fragment of pGemF1 (Krause *et al.*, 1988) was cloned into pRSET to make pFNS. Then the C-terminal 0.85 kb *SalI-NotI* fragment from pVIII was added to pFNS to generate pFTZ $\Delta 257-265$ . Similarly, to make the bacterial expression plasmid carrying both the FTZ $\Delta 112-118$  and FTZ $\Delta 257-265$  deletions (FTZ $\Delta\Delta$ ), the N-terminal 0.5 kb *BamHI-SalI* fragment from pFTZ $\Delta 112-118$  was substituted for the non-deleted fragment in pFTZ $\Delta 257-265$ .

Two *ftz* transgenes that express full-length FTZ (pCasper4Ftz) and FTZ $\Delta 112-118$  (pCasper4Ftz $\Delta$ ) were generated via PCR and the following subcloning steps. To construct pCasper4Ftz $\Delta$ , a *BstEII* fragment from pFTZ $\Delta 112-118$  was swapped with the corresponding wild-type *BstEII* fragment of pBlueftzX (a modified Bluescript vector containing a genomic *ftz XmnI* fragment; Furukubo-Tokunaga *et al.*, 1992; Dong *et al.*, 1998), to make pBlueftzX $\Delta$ . The *XmnI* fragments from pBlueftzX and pBlueftzX $\Delta$  were then excised with *Asp700* (Roche Molecular Biochemicals) and ligated into pBKM (a vector containing 8.5 kb of *ftz* genomic DNA deleted for the *XmnI* fragment; Furukubo-Tokunaga *et al.*, 1992; Dong *et al.*, 1998). The reconstructed 10 kb *ftz* genomic fragments were then excised with *KpnI* and subcloned into the P-element vector pCasper4 to generate pCasper4Ftz and pCasper4Ftz $\Delta$ .

Plasmids for the expression of FTF and FTFAAF2 were provided by L. Belanger. The SF-1 expression plasmid was provided by B. Schimmer. The plasmids TL $\alpha$ FTZ-F1, TL $\beta$ FTZ-F1 and TLFTZ-F1 $\beta$ , used for bacterial expression of  $\alpha$ FTZ-F1,  $\beta$ FTZ-F1 and DHR39, respectively, were provided by M. Petkovich.

New bacterial FTZ-F1 expressing constructs were made as follows. To make  $\alpha$ N+DBD, PCR was first used to create a *BamHI* site at the 5' end of the  $\alpha$ FTZ-F1 open reading frame using the oligo 5'-GCGGAA-TTCTTAAGTATCCCGTGTC-3' and a plasmid-specific oligo. A 1.7 kb fragment, extending from this 5' *BamHI* site to an internal *XhoI* site, was then ligated into pET15. The A+LBD construct was made by moving the 1.5 kb *XhoI-EcoRI* fragment of TL $\alpha$ FTZ-F1 into pRSET. The LBD-expressing construct was made using PCR and the oligos 5'-GGG-GATCCTTAAGAGTCTCACCG-3' and 5'-GCGGATCCTGTCACGTTCTCCCGACC-3' to generate a 0.7 kb fragment beginning at nucleotide 2371 of the  $\alpha$ FTZ-F1 reading frame. This fragment was then inserted into the *BamHI* site of pET15.

To generate the chimeric bacterial expression constructs, the final 126 bp of the FTZ-F1 open reading frame, beginning at the *HpaI* site at position 2952, was recreated with a series of three pairs of oligonucleotides. The first two pairs had the following complementary sequences: 5'-AACATCTTACCGAAATCCATGCCATGGCCGTTCCGCGGAG-3', 5'-GCGAACGGCCATGGCATGGATTTCCGGTAA-GATGTT-3', 5'-GATCACCTGTACACCAAGCACTGTGCGGCAG-TGCG-3' and 5'-GCCGGCAGTGCTTGGTGTACAGGTGATCCTCGCC-3'. When annealed and ligated, these recreated the FTZ-F1 sequence from the *HpaI* site to the beginning of the AF2 helix. The final

pairs provided the sequence for distinct AF2 motifs followed by a stop codon and an *Asp718* restriction site. The sequences for these oligos were: 5'-CCCTACAATAACCTGCTCATCGAGATGCTGCACGCCAAGC-GCGCCTAG-3' and 5'-GTACCTAGGCGCGCTTGGCGTGCAAGCATCTCGATGAGCAGGTTATTGTAGGGCGCACT-3' for FTZ-F1/FTF, 5'-CCCCTGATTTCAATTTGCTAATGGAGCTTTTGGCGGGAGA-GCATTAG-3' and 5'-GTACCTCAATGCTCTCCGCGCAAAGCTCCATTAGCAAATTGAAATCAGCGGGCGCACT-3' for FTZ-F1/DHR39, and 5'-CCCCGAATAACCTGCTCATCGAGATGCTGCAAGCCAAGCAGACCTAG-3' and 5'-GTACCTAGGTCTGCTTGGCTTGCAAGCATCTCGATGAGCAGGTTATTGCGGGCGCACT-3' for FTZ-F1/SF-1. The fully annealed and ligated sequences were then inserted into Bluescript. These plasmids were cut with *HpaI* and *Asp718*, and the 126 bp restriction fragments inserted into the corresponding sites of the A+LBD plasmid, creating A+LBD/FTF, A+LBD/DHR39 and A+LBD/SF-1.

*In vivo* rescue plasmids were generated by carrying out three-piece ligations using the newly generated 1.4 kb *XhoI-Asp718* restriction fragments removed from the chimeric A+LBD plasmids, the 1.7 kb *BamHI-XhoI* fragment from the  $\alpha$ N+DBD construct described above and the P-element vector pUAST, which had been linearized with *BglII* and *Asp718*.

To remove the AF2, the oligo 5'-GCGAATCTCACGACTGCCGGCACAGTGCT-3' was used together with a plasmid-specific oligo and PCR to remove the penultimate 48 bp that encode the AF2, generating A+LBD $\Delta$ AF2 in pRSET.

All constructs generated by PCR were sequenced to confirm integrity. Discrepancies found between our FTZ-F1 sequences and previously published sequences were confirmed by sequencing the 3' ends of available *Ftz-F1* cDNAs.

### Far western analyses

Far western assays were carried out as previously described (Guichet *et al.*, 1997) using expression constructs and probes that are described above and in the figure legends.

### Ftz rescue assays

P-elements contained within pCasper4Ftz and pCasper4Ftz $\Delta$  were inserted into the genome of *y,w;  $\Delta 2-3$ , SbTM6, Ubx* flies by P-element transformation (Rubin and Spradling, 1982). Several independent lines with insertions on the second chromosome were obtained. Flies homozygous for the transgenes, and carrying either *ftz<sup>11</sup>/hb-lacZ* TM3 or *ftz<sup>13</sup>/hb-lacZ* TM3, were generated. Reciprocal crosses were carried out and the embryos collected for cuticles. FTZ was detected using a rabbit  $\alpha$ FTZ antibody (Krause *et al.*, 1988), Engrailed using the mouse monoclonal antibody Mab 4D9 (Patel *et al.*, 1989) and Wingless using the mouse monoclonal antibody Mab 4D4 (Brook and Cohen, 1996). Homozygous *ftz* mutant embryos were identified by the absence of  $\beta$ -galactosidase, as detected using a mouse monoclonal antibody (Promega). Antibody complexes were detected using a Vectastain elite ABC kit (Vector Laboratories). Cuticles were prepared 24–28 h after egg-laying (Roberts, 1986).

### Zygotic rescue of *Ftz-F1<sup>209</sup>* and ectopic expression assays

pUAST expression constructs encoding FTZ-F1 and derivative proteins were transformed into flies and at least four transgenic lines obtained per construct. Expression was achieved by crossing the transgenic flies to *w; armadillo-GAL4; Ftz-F1<sup>209</sup>* or *w; armadillo-GAL4; +/-* females (Brand and Perrimon, 1993). Eggs were collected for 24 h and then allowed to develop for a minimum of 24 h. Eggs and larvae were counted to determine the hatching frequencies, and then cuticles from the unhatched eggs prepared and observed by dark-field microscopy.

For quantitation of expression levels, eggs were collected for ~18 h and then dechorionated with 50% bleach. Eggs were then suspended in a solution of three parts phosphate-buffered saline and one part 3 $\times$  SDS-PAGE loading buffer at a concentration of two eggs per microliter, and boiled. After electrophoresis on a 10% polyacrylamide gel, proteins were blotted to a membrane and subjected to western analysis using anti-FTZ-F1 antibodies (provided by C. Wu) and chemiluminescent substrates. Expression levels of FTZ-F1 proteins were normalized by comparison with  $\beta$ -tubulin signals.

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