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Stripy Ftz target genes are coordinately regulated by Ftz-F1

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Abstract

During development, cascades of regulatory genes act in a hierarchical fashion to subdivide the embryo into increasingly specified body regions. This has been best characterized in *Drosophila*, where genes encoding regulatory transcription factors form a network to direct the development of the basic segmented body plan. The pair-rule genes are pivotal in this process as they are responsible for the first subdivision of the embryo into repeated metameric units. The *Drosophila* pair-rule gene *fushi tarazu* (*ftz*) is a derived *Hox* gene expressed in and required for the development of alternate parasegments. Previous studies suggested that Ftz achieves its distinct regulatory specificity as a segmentation protein by interacting with a ubiquitously expressed cofactor, the nuclear receptor Ftz-F1. However, the downstream target genes regulated by Ftz and other pair-rule genes to direct segment formation are not known. In this study, we selected candidate Ftz targets by virtue of their early expression in Ftz-like stripes. This identified two new Ftz target genes, *drumstick* (*drm*) and *no ocelli* (*noc*), and confirmed that Ftz regulates a serotonin receptor (*5-HT2*). These are the earliest Ftz targets identified to date and all are coordinately regulated by Ftz-F1. Engrailed (En), the best-characterized Ftz/Ftz-F1 downstream target, is not an intermediate in regulation. The *drm* genomic region harbors two separate 7-stripe enhancers, identified by virtue of predicted Ftz-F1 binding sites and these sites are necessary for stripe expression in vivo. We propose that pair-rule genes, exemplified by Ftz/Ftz-F1, promote segmentation by acting at different hierarchical levels, regulating first, other segmentation genes; second, other regulatory genes that in turn control specific cellular processes such as tissue differentiation; and, third, 'segmentation realizator genes' that are directly involved in morphogenesis.

Keywords

Segmentation; *Hox* genes; pair-rule genes; Ftz; Ftz-F1

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INTRODUCTION

The development of multicellular organisms from single fertilized egg cells is a hierarchical process in which cell fates are increasingly specified as development proceeds. Nowhere have we learned more about this process than from genetic screens carried out in *Drosophila melanogaster* (Nusslein-Volhard et al., 1985). These screens identified sets of regulatory genes that sequentially subdivide the *Drosophila* embryo into increasingly specified body parts along the anterior-posterior axis of the egg, culminating in the formation of the body segments that are the basis of the insect body plan. The segmentation genes identified in these screens were found to participate in a complex regulatory cascade: the overlapping and staggered expression patterns of maternal and gap genes are required for striped expression of pair-rule genes, which in turn regulate segmental expression of segment polarity genes and region-specific expression of homeotic genes. Together, these genes form an integrated network of regulatory genes that control early embryonic development (reviewed in Nasiadka et al., 2002; Schroeder et al., 2004).

The pair-rule genes are the first genes to be expressed in repeated patterns in the early embryo in sets of transverse stripes in the primordia of alternating segmental units. Each pair-rule gene is expressed in the primordia of the alternating metameric region missing in embryos carrying mutations in that pair-rule gene. For example, the pair-rule gene *fushi tarazu* (*ftz*) is expressed in the primordia of the even numbered parasegments which are missing in *ftz* embryos (Carroll and Scott, 1985; Hafen et al., 1984). Elegant studies have explained how pair-rule stripes can be generated by the combinatorial action of activating and repressing maternal and gap transcription factors which act on stripe-specific cis-regulatory elements (reviewed in Arnosti et al., 1996; Small et al., 1996). However, less is known about how the pair-rule genes, once expressed in striped patterns, impact segment morphogenesis. We have begun to address this by identifying target genes of the pair-rule segmentation protein Ftz. Ftz contains a DNA-binding homeodomain and activates transcription (Florence et al., 1991; Han et al., 1989; Nelson and Laughon, 1990; Pick et al., 1990; Winslow et al., 1989; Yu et al., 1999). However, like other Hox proteins, Ftz binding to DNA is weak and promiscuous, with >14 million predicted binding sites in the *Drosophila* genome (Bowler et al., 2006). The specificity of Ftz target site selection is achieved by Ftz interaction with a specific cofactor, the orphan nuclear receptor Ftz-F1, whose DNA binding specificity is much more stringent than that of Ftz (Florence et al., 1997; Guichet et al., 1997; Yu et al., 1997); reviewed in (Pick et al., 2006). Ftz and Ftz-F1 form a stable complex in vivo and bind cooperatively to DNA (Yu et al., 1997; Yussa et al., 2001). Because Ftz-F1 DNA binding specificity is greater than Ftz DNA binding specificity, the predominant determinant of Ftz/Ftz-F1 target genes is the Ftz-F1 binding site. In fact, when Ftz is overexpressed, interaction with Ftz-F1 obviates the need for Ftz DNA binding, and the *anti-ftz* phenotype can be generated by a Ftz protein lacking its DNA binding homeodomain (Copeland et al., 1996; Fitzpatrick et al., 1992; Guichet et al., 1997).

The best-characterized Ftz/Ftz-F1 target genes are *ftz* itself (autoregulation) and the downstream target *engrailed* (*en*). The Ftz/Ftz-F1-dependent enhancers that regulate *ftz* and *en* each contain composite Ftz/Ftz-F1 binding sites that are necessary for gene expression in vivo (Florence et al., 1997; Han et al., 1998; Pick et al., 1990; Schier and Gehring, 1993a). The composite binding sites in the *ftz* and *en* enhancers differ in spacing and orientation; in the *ftz* enhancer, the Ftz binding site is 5' to the Ftz-F1 site, with 7 nucleotides between them while in the *en* enhancer, the Ftz-F1 site is 5' to the Ftz site and the sites are separated by 11 nucleotides (see Bowler et al., 2006). A previous study utilized these *ftz*-like and *en*-like binding site configurations, along with a compilation of all experimentally verified Ftz and Ftz-F1 binding sequences, to identify additional Ftz/Ftz-F1 targets in the *Drosophila* genome. This study identified *apontic* (*apt*) and *Sulfated* (*Sulf1*) as downstream targets of Ftz/Ftz-F1 but most of the genes harboring multiple *ftz*-like or *en*-like Ftz/Ftz-F1 binding sites were not in fact

regulated by Ftz/Ftz-F1 (Bowler et al., 2006). This suggested that other approaches would be required to identify Ftz/Ftz-F1 targets genes.

Here, we have made use of the availability of an in situ database from the Berkeley *Drosophila* Genome Project (Tomancak et al., 2002) to identify candidate Ftz target genes that are expressed in striped patterns — 'stripy genes.' We characterized three genes that are expressed in stripes that overlap the Ftz stripes. Each genomic region contains multiple potential Ftz-F1 binding sites and each gene requires Ftz as well as Ftz-F1 for striped expression. These genes were *5-HT2*, previously shown to require Ftz for expression in stripes (Colas et al., 1995); *noc*, a zinc finger transcription factor; and *drm*, which is expressed in an *en*-like pattern of 14 stripes, 7 of which require Ftz and Ftz-F1. We identified two regulatory elements for *drm* that direct expression in Ftz-like stripes, each containing predicted Ftz-F1 binding sites that were shown to be required for expression of *drm-lacZ* fusion genes in vivo. Together, these results suggest that Ftz and Ftz-F1 coordinately regulate downstream target genes that function at different levels of a gene hierarchy, with some target genes encoding regulatory proteins and others encoding products directly involved in morphogenesis.

MATERIALS AND METHODS

Fly Stocks

Flies were maintained at 25°C on a standard diet. The *ftz* mutant was ftz^{9H34} balanced over TM3, *hb-lacZ* to identify mutant embryos. Embryos derived *from ftz-f1* germline clones (referred to as *ftz-f1* mutants) were generated with the autosomal FLP-DFS technique (Chou and Perrimon, 1992; Chou and Perrimon, 1996; Chou et al., 1993) using $ftz\text{-}f1^{19}$(Broadus et al., 1999; Fortier et al., 2003; Pick et al., 2006). Ftz was ectopically expressed throughout blastoderm embryos by mating *UAS-myc-ftz* males (Lohr and Pick, 2005) with females homozygous for an *NGT40/GAL4* driver (Tracey et al., 2000). Expression was examined for two *en* alleles, en^1, *Df(2R)42* and en^E, each balanced over *CyO, hb-lacZ* to identify mutants. Transgenic fly lines were generated by Rainbow Transgenic Flies, CA. Multiple independent lines were established for each construct, maintained over balancer chromosomes or as homozygotes.

Analysis of embryonic expression patterns

Standard protocols were followed for in situ hybridization (Kosman and Small, 1997; Tautz and Pfeifle, 1989) and antibody staining (Gutjahr et al., 1994). Digoxigenin-labeled RNA probes were made with cDNA clones LD26791 (*drm*), LD28078 (*noc*), and RH04788 (*5-HT2*) from the *Drosophila* Genome Resource Center. Protein/RNA double staining followed standard in situ hybridization protocols, including the Proteinase K treatment followed by addition of primary antibodies, rat anti-Ftz (1:200) (Kosman et al., 1998) and sheep anti-digoxigenin (1:1000, Roche). Secondary antibodies, anti-rat Alexa Fluor 488 and anti-sheep Alexa Fluor 555 (Invitrogen), were used at 1:600. After washes, including a final wash in PBST overnight at 4ºC, and rinses with PBS, embryos were mounted in 90% glycerol, 0. 1M Tris-HCl, pH 7.9. For double RNA in situs, embryos were incubated simultaneously with digoxigenin-labeled *lacZ* or target RNA and biotinylated *ftz* RNA probes, followed by detection with mouse anti-biotin (Roche) and anti-digoxigenin antibodies as described above. For *drm* reporter constructs, embryos were stained with anti-β-galactosidase antibody (Cappel, 1:1000) (Gutjahr et al., 1993). Expression patterns shown here were observed in at least 3–5 independent lines for each construct. For *drm5-lacZ*, 5 independent lines were analyzed and in only one of these was expression detected. Expression was early in 7 stripes and developed into 14-stripes during germ band extension. Although the initial 7 stripes appeared to overlap with Ftz, the fact that this expression was detected in only one *drm5-lacZ* line, which was PCR-verified, suggests that it was due to a position effect and not enhancer sequences in the *drm5*

fragment. In situ hybridizations were visualized using a LeicaDMRB microscope. Fluorescent staining was captured using the Zeiss LSM 510 confocal microscope with a 16X Zeiss objective with oil immersion. Multiple fluorescent images were captured with multitrack switching. Alexa 488 antibodies were excited with a 488nm laser and detected at 505–530nm. Alexa 555 antibodies were excited with a 543nm laser and detected above 560nm.

Identification of *drm* transcription start site and enhancer construct design

RNA was extracted from 0–9 hr. *D. melanogaster* w^{1118} embryos with TRIzol (Invitrogen, CA) and a Qiagen RNA Extraction Kit (Qiagen, CA). The TSS was mapped using 5' RLM-RACE (Ambion, TX). Briefly, RNA was treated with CIP and TAP to select full-length mRNAs. The RACE adapter was ligated to the 5' end, and cDNA was made using reverse transcriptase. Two rounds of nested PCR resulted in a PCR product with the 5' transcription start site immediately downstream of the adapter. The *drm* gene contains a perfect match to an INR, an almost perfect Downstream Promoter Elemet (DPE), and lacks an apparent TATA box. To construct *drm* enhancer-reporter transgenes, genomic fragments were isolated by PCR (primer sequences available upon request). Genomic location of fragments is: *drm1*, −606–2314; *drm2*, +513–1511; *drm 34*, +3198–4469; *drm5*, +11,900-12,714. Site-specific mutagenesis was carried out as described (Lohr and Pick, 2005). For expression in *Drosophila*, fragments were inserted directionally into the P-element vector pX28, upstream of a basal *hsp70* promoter and *lacZ* reporter gene (Bowler et al., 2006; Segalat et al., 1994).

RESULTS

Identification of stripy genes as candidate Ftz targets

To identify candidate targets of Ftz in promoting segmentation, we searched the BDGP in situ expression database (Release 2, http://fruitfly.org:9005/cgi-bin/ex/insitu.pl; (Tomancak et al., 2002) for genes expressed early enough to be possible direct targets of pair-rule genes and with expression patterns that are modulated along the A-P axis. This identified 95 genes, corresponding to 6.7% (95/1403) of the genes in the database. Many of these are expressed in seven or fourteen stripes ('stripy genes'). Some stripy genes show additional modulation, such as restriction of stripes to the ventral side, restriction to the posterior abdominal regions of the embryo, or additional expression in the head (data not shown). These stripy genes are likely to be regulated by one or more of the seven pair-rule transcription factors expressed in different registers along the anterior-posterior axis. To narrow down potential Ftz targets among these genes, we searched computationally for genes harboring Ftz/Ftz-F1 binding sites with spacing matching those of the Ftz/Ftz-F1 binding sites in either the *ftz* or *en* enhancers (Introduction). Thirty of the 95 genes contain composite Ftz/Ftz-F1 sites within 20 kb of their annotated TSS (Bowler, 2004). Analysis of the expression of these 30 genes in wild type and *ftz-f1* mutants identified 10 genes in addition to the previously characterized direct targets *en*, *apt* and *Sulf1*, that appeared to be responsive to Ftz-F1. These were: *5-HT2*, *noc*, *drm*, *CG12094*, *ImpL2*, *Sema-5c*, *Ama*, *Cyt-b5*, *danr*, and *RhoGAP71E*. The remaining genes in the list of 95 stripy genes may be targets of other segmentation genes that control gene expression in different registers.

ftz-f1, as well as ftz, is required for *5-HT2* expression

Among the earliest candidate target genes to be expressed during development is the *5-HT2* gene. As shown previously by Colas et al. (1995), *5-HT2* is expressed in stripes in the early embryo. Expression was first observed in seven stripes in blastoderm embryos. The first two and last two stripes were stronger than the middle three stripes (Fig. 1Ai). *5-HT2* RNA continued to be detectable in seven stripes during gastrulation, with the 4^{th} and 5^{th} stripe remaining weaker than the others (Fig. 1Aii). Stripes began to fade during germ band extension (Fig. 1Aiii). The *5-HT2* stripes overlap Ftz stripes in the ectodermal primordia (Fig. 1B, *5-*

HT2 red, Ftz green; and see (Colas et al., 1995). The overlap was apparent as early as the cellular blastoderm stage and during gastrulation. This is earlier than the overlap between Ftz and its other characterized downstream target genes, *en*, *apt* and *Dsulf1* (Bowler et al., 2006; Lawrence et al., 1987). In *ftz* mutant embryos, *5-HT2* expression levels were reduced and stripes were no longer distinct (Fig. 1Aiv). Similarly, at later stages, low levels of *5-HT2* mRNA were detectable but distinct stripes were lost. These patterns are similar to those previously reported (Colas et al., 1995). As was the case for *ftz* mutants, in *ftz-f1* mutants, low-level diffuse expression was seen at blastoderm and during germ band extension (Fig. 1Av). When Ftz was ectopically expressed throughout blastoderm embryos using the *UAS/GAL4* system with an *NGT40* driver (Tracey et al., 2000), changes in *5-HT2* expression were evident as early as cellular blastoderm, when the first two stripes were fused and the five posterior stripes were expanded (Fig. 1Avi). The fusing continued to be apparent during gastrulation and germ band extension when the first two stripes appeared as one broad stripe and the remaining stripes were expanded beyond their normal registers. The genomic region surrounding the *5HT-2* coding region contains 6 Ftz-F1 binding sites predicted computationally using experimentally verified Ftz-F1 binding sites to search the *Drosophila* genome, as done previously (Bowler et al., 2006). Each of these predicted Ftz-F1 sites is surrounded by multiple potential Ftz binding sites that could mediate cooperative interactions with Ftz-F1. In addition, four matches to a 7mer sequence, which is thought to act as a general enhancer for zygotic transcription via the zinc-finger transcription factor Zelda (De Renzis et al., 2007; Liang et al., 2008) are located in the *5HT-2* genomic region. Together, these results demonstrate that Ftz and its partner Ftz-F1 are necessary for the spatial patterning of *5-HT2*. Future studies will determine whether the predicted Ftz/Ftz-F1 binding sites directly regulate *5HT-2* gene expression.

noc is responsive to *ftz* and *ftz-f1*

noc is expressed in a striped pattern in early embryos. *noc* transcripts were first detected at the cellular blastoderm stage, in the procephalic ectoderm, and one stripe (*noc* stripe 1). Subsequently, 7 additional stripes arose, with *noc* stripe 2 stronger than the other stripes (Fig. 2Ai). Stripes appeared stronger ventrally. Stripe 8 and stripes 3–7 became stronger as development progressed, with expression in the head remaining strong (Fig. 2Aii). Near the end of germ band extension, stripes in the central body region developed into doublets (Fig. 2Aiii), reminiscent of segment polarity gene expression. In *ftz* mutant embryos, *noc* stripes 2–7 were not detectable (Fig. 2Aiv). Expression in the head, and in *noc* stripe 1, which do not overlap with *ftz* (Fig. 2B and data not shown), were unaffected. In addition, *noc* stripe 8, which only partially overlaps *ftz* stripe 7 (Fig. 2B) was detected in *ftz* mutant embryos. During late germband extension, some stripes appeared in weak doublet configurations in the central region of the embryo suggesting that, at later stages, additional factors regulate *noc*. However, these stripes were diffuse and lacked the clear pattern seen in the wild type (data not shown). Similar expression patterns were seen in *ftz-f1* mutants (Fig. 2Av), although it appeared that loss of *ftz-f1* function also affected *noc* stripes 1 and 8. As for the *ftz* mutants, a diffuse, partial stripy pattern was evident at late germband extension. When Ftz was ectopically expressed throughout blastoderm embryos, an extra stripe just prior to the terminal stripe appeared at blastoderm, and soon after this stripes became diffuse, covering much of the central region of the embryo (Fig. 2Avi). Stripes continued to appear fused or expanded during germband extension. Together, these results demonstrate that Ftz and Ftz-F1 are required to establish the striped pattern of *noc* in the central region of the embryo and that ectopic expression of Ftz results in rapid ectopic activation of *noc*. In keeping with potential direct regulation, 7 potential Ftz-F1 binding sites were found within a region of 10 kb upstream of the annotated *noc* TSS (data not shown). Each of these Ftz-F1 sites is surrounded by multiple Ftz binding sites that could potentially mediate cooperative interaction. However, since Ftz and Ftz-F1 regulate *en* and En is itself a transcriptional regulator thought to mediate effects of Ftz and other pair-rule genes (DiNardo and O'Farrell, 1987;Howard and Ingham, 1986;Lawrence et al., 1987), we

asked whether Ftz/Ftz-F1 effects on *noc* could be indirect via *en*. In fact, expression patterns in *en* mutants (Fig. 2B) were indistinguishable from wild type animals: Expression of *noc* stripes 2–7 overlapped precisely with the seven stripes of Ftz at cellular blastoderm and this overlap in register persisted through later stages (Fig. 2B and data not shown, *noc* red, Ftz green). *noc* stripe 8 overlapped with, but extended slightly posterior of *ftz* stripe 7. No overlap with *ftz* was seen in the head or with *noc* stripe 1, as expected. In sum, the early expression of *noc* in stripes, the dependence of stripes on both Ftz and Ftz-F1, the rapid responsiveness to ectopic Ftz expression, and the presence of potential Ftz/Ftz-F1 binding sites, together suggest that Ftz and Ftz-F1 regulate *noc* expression and that En is not an intermediate in this regulation.

drm is regulated by Ftz and Ftz-F1

drm encodes a zinc finger transcription factor involved in differentiation, morphogenesis and cell movement during gut morphogenesis (reviewed in (Lengyel and Iwaki, 2002). We found that *drm* is expressed in a seven-stripe pattern at the cellular blastoderm stage (*drm* primary stripes). A weaker set of seven secondary stripes appeared during gastrulation (*drm* secondary stripes) resulting in a fourteen-stripe pattern, reminiscent of segment polarity genes (Fig. 3A). These stripes persisted through gastrulation and early germ band extension, with the primary stripes thicker and stronger than the secondary stripes (Fig. 3B). Additional expression in the head in the proventricular and hindgut primordia was evident at early stages and became stronger as development proceeded. During late germ band extension, the stripes became more equal in intensity and the expression in the hindgut primordium increased (Fig. 3C). In *ftz* mutant embryos, half of the stripes were missing while hindgut expression was unaffected, as expected. The loss of alternate stripes was apparent as early as stage 6, and persisted through late germ band extension (Fig. 3D–F). This pattern was also observed in *ftz-f1* mutants (Fig. 3G–I). When Ftz was ectopically expressed, the spacing of *drm* stripes became irregular (Fig. 3J, K) and at late germ band extension the stripes adopted a doublet configuration (Fig. 3L) reminiscent of the *en* response to ectopic Ftz expression (Ish-Horowicz et al., 1989).

Ftz protein and *drm* co-localized in the primary *drm* stripes (white arrows, Fig. 4A,*drm* RNA red; Ftz green). Expression in the head region, hindgut primordium, and the secondary stripes did not overlap with Ftz. Quantitation of expression based upon fluorescence confirmed that the primary *drm* stripes that overlap Ftz are stronger than the secondary stripes (data not shown). To determine which set of *drm* stripes was lost in *ftz* embryos, we made use of the strong *ftz* allele, ftz^{9H34} that expresses *ftz* RNA but no protein (Furukubo-Tokunaga et al., 1992); Fig. S1). As shown in Fig. 4B, the set of seven *drm* stripes remaining in *ftz* mutants was out of register with *ftz* (*drm* RNA red, *ftz* RNA green), demonstrating that the missing set of stripes in these mutants are the primary *drm* stripes that overlap with *ftz* in wild type (Ftz-dependent *drm* stripes). These Ftz-dependent stripes require both Ftz and Ftz-F1 for expression. However, as for *noc* (see above), it was possible that regulation of *drm* was mediated via the activation of En by Ftz/Ftz-F1. To test this, *drm* expression was examined in *en* mutants (Fig. 4C). The Ftz-dependent *drm* stripes were present and still overlapped with Ftz stripes in *en* mutant embryos. Thus, En is not a necessary intermediate in the regulation of the primary *drm* stripes by Ftz/Ftz-F1. It appeared that the secondary *drm* stripes were weaker in *en* mutants, suggesting that En may contribute to the regulation of the Ftz-independent *drm* stripes (data not shown). Additionally, it was reported that *drm* expression levels were lower in *hh* mutants at later stages (Hatini et al., 2005).

In sum, *drm* is expressed in a pattern of 7 primary and 7 secondary stripes, in addition to expression in the head and hindgut primordia. Ftz overlaps with *drm* in the primary *drm* stripes. *ftz* and *ftz-f1* are each required for expression of these alternate stripes and ectopic Ftz expression alters the pattern of *drm* expression. The Ftz-dependent *drm* stripes are not

dependent upon *en*, demonstrating that En is not an intermediate in the regulation of the primary *drm* stripes by Ftz/Ftz-F1.

drm contains independent stripe enhancers

To ask whether Ftz/Ftz-F1 directly regulate *drm* expression, the major embryonic *drm* TSS was mapped (Materials and Methods) and potential Ftz-F1 binding sites were identified computationally based upon a position list for experimentally identified Ftz-F1 binding sites, BS**AAGG**HYRHH (Bowler et al., 2006). Within a 20kb *drm* genomic region, there are five potential Ftz-F1 sites (Fig. 5A, green triangles). Each of these has multiple potential Ftz binding sites within 25 bases of the core Ftz-F1 site (not shown). To determine whether these predicted Ftz/Ftz-F1 binding sites are components of functional enhancers, fragments of ~1kb spanning them were inserted upstream of a basal promoter and a *lacZ* reporter gene in the P-element vector pX28. Multiple independent transgenic lines were generated for each construct and expression was analyzed with anti-β-galactosidase antibody (Figure 5B). *drm1* contains the only predicted Ftz-F1 binding site located upstream of the TSS, at – 1.9 kb. This site is flanked by 5 potential Ftz binding sites. Two constructs were generated: the first, *drm1-lacZ*, spans 1 kb and does not contain any 7mer sequences. The second, *drm1–7mer*, was generated because no expression was detected for *drm1*. This construct is extended by ~ 700 bp to contain five 7mer sequences located 3' of the end of *drm1* (Fig. 5A, yellow diamonds). *drm1–7mer-lacZ* expression was not detected before late germ band extension. At this time, it was expressed in fourteen lines or patches that extended only partially around the embryo (Fig. 5B). This pattern evolved into a complex segmental pattern that closely resembled expression of endogenous *drm* at stages 9–12 (Tomancak et al., 2002). These results indicate that *drm1–7mer* harbors an enhancer that directs *drm* expression at late stages of development (*drm* Late Enhancer, Fig. 5A). Although clearly not a strict test of 7mer function, the finding that *drm1-lacZ* was not expressed is consistent with a role for the 7mer sequences in facilitating expression directed by a region-specific late *drm* enhancer.

drm2 contains one potential Ftz-F1 binding site, located in the first *drm* intron. This Ftz-F1 site is flanked by 7 potential Ftz binding sites and one 7mer. *drm2-lacZ* was expressed in seven strong stripes (Fig. 5C). These stripes were evident at late gastrulation and became more prominent during early germ band elongation. Expression in stripes remained strong during mid- and late- germ band extension stages. Additional expression in the amnioserosa was detected, which may be a result of vector sequences. The *drm2-lacZ* stripes are in register with Ftz (Fig. 6A–C) and thus represent the primary *drm* stripes. This stripe pattern, directed by the *drm* 7-Stripe Enhancer, is reminiscent of *ftz-lacZ* fusion genes that are directly responsive to Ftz/Ftz-F1 (Han et al., 1998;Pick et al., 1990;Yussa et al., 2001).

drm34 contains 2 Ftz-F1 potential binding sites, located about 800 bp apart in the first *drm* intron. The predicted Ftz-F1 binding site in *drm3* is flanked by 6 potential Ftz binding sites and in *drm4* by 7 potential Ftz sites. *drm34-lacZ* was also expressed in a strong 7-stripe pattern that was detectable as early as the cellular blastoderm stage (Fig. 5E; Early 7-Stripe Enhancer, Fig. 5A). During gastrulation and germ band extension, expression in the 7 stripes increased and additional expression in the proventriculus and hindgut primordia became apparent. Expression in seven stripes, the head and hindgut persisted at least through the end of germ band extension. Surprisingly, these stripes also overlapped with Ftz, as did the *drm2-lacZ* stripes. Complete overlap between Ftz and β-galactosidase expression was evident at the cellular blastoderm stage (Fig. 6D–F) and continued through germ band elongation (Fig. 6G–I) indicating that the *drm34* Early 7-Stripe Enhancer directs expression in the primary *drm* stripes.

drm5 is located downstream of the *drm* coding region. This potential Ftz-F1 binding site is flanked by 5 potential Ftz binding sites. No reproducible pattern of expression was observed,

suggesting that it either does not harbor a *drm* enhancer element or that it also harbors repressors that mask functional enhancer sequences.

In sum, this analysis identified 3 independent enhancers of *drm* (Fig. 5A). Each directs expression in portions of the endogenous *drm* pattern. The upstream *drm1* region harbors a Late Enhancer. The first intron harbors two independent enhancers: the *drm2* 7-Stripe Enhancer directs expression in 7 stripes that overlap Ftz. The *drm34* Early 7-Stripe Enhancer directs expression in 7 stripes that also overlap Ftz but which initiate earlier than *drm2*-directed stripes. The *drm34* enhancer also directs expression in the proventriculus and hindgut. The two 7-stripe enhancers each direct expression in the primary, Ftz-dependent *drm* stripes.

Predicted Ftz-F1 binding sites are functional in vivo

To ask whether the computationally identified Ftz-F1 binding sites in the two 7-stripe enhancers are functional in vivo, point mutations were generated in the core Ftz-F1 binding sequences (AAGG to AGAT) to generate a sequence known to abolish binding of purified protein Ftz-F1 and Ftz-F1 protein in *Drosophila* nuclear extracts (Han et al., 1998). Fragments carrying Ftz-F1 site mutations were inserted upstream of a basal promoter and a *lacZ* reporter gene in the P-element vector pX28, as above, and multiple independent transformant lines were generated. Expression of *drm2M-lacZ,* containing a mutation in the single predicted Ftz-F1 binding site in this 7-stripe enhancer, was drastically decreased (Fig. 5D). In two independent transformant lines, no expression was detected; in one line, reasonably strong stripes were found; and in two independent lines, very weak striped expression was observed, as shown in the figure. We interpret this result as indicating that the predicted Ftz-F1 binding site is necessary for full expression of this enhancer, but that other factors are also able to generate weak stripes in the absence of Ftz-F1.

Expression of *drm34-lacZ* fusion genes with mutations in either the *drm3* or *drm4* predicted Ftz-F1 binding sites or simultaneous mutations in both the *drm3* and *drm4* sites was abolished (Fig. 5F, one example shown). Expression of *drm3M4-lacZ, drm34M-lacZ,* or *drm3M4M-lacZ* was undetectable in 5/5 independent transgenic lines for each of these constructs. These results demonstrate that each of the Ftz-F1 sites in this *drm 34* Early 7-Stripe Enhancer is necessary for striped expression.

DISCUSSION

The regulatory transcription factors that direct *Drosophila* development have been studied in great detail. These transcription factors interact in a largely linear hierarchy of maternal, gap, pair-rule, segment-polarity and homeotic genes, with cross-regulation occurring at each level of the hierarchy. This network of transcription factors provides a blueprint for the development and differentiation of body segments. How this blueprint of regulatory or instructive information is translated into morphology is of considerable interest. Although some progress has been made in understanding control of morphology by homeotic selector genes (reviewed in (Pearson et al., 2005), it is less clear if genes acting earlier in the hierarchy impact morphology or function solely to establish the expression patterns of segment polarity and homeotic genes, which then impact morphology. In particular, there is considerable debate as to whether the pair-rule transcription factors are purely pre-patterning genes that regulate solely other selector genes in the hierarchy, or if they are involved in regulating segment formation independent of the segment polarity and homeotic genes, by controlling genes more intimately involved with segment formation and morphogenesis. We have begun to address this issue by identifying downstream targets of the pair-rule transcription factors Ftz and Ftz-F1, regulators that direct formation of alternate parasegments in the *Drosophila* embryo. Our findings support those of others (see below) in suggesting that the pair-rule genes do not participate in a strictly linear hierarchy, regulating only other selector genes to indirectly control segmentation, but

that they control the expression of a range of different classes of genes, thereby providing branch points in a linear hierarchy that amplify the information provided by striped pair-rule expression patterns.

Ftz and Ftz-F1 coordinately regulate the expression of multiple target genes

This study identified Ftz targets based upon a search for genes expressed in striped patterns in the early *Drosophila* embryo (Fig. 1–Fig. 4). Each of these Ftz-dependent genes is also regulated by Ftz-F1, an orphan nuclear receptor previously shown to interact with Ftz in vitro and in vivo (Guichet et al., 1997;Yu et al., 1997). Unlike Ftz, which is expressed in a striped pattern in the *Drosophila* blastoderm, Ftz-F1 is expressed ubiquitously, in all somatic cells at the blastoderm stage (Yussa et al., 2001). The finding here that all three additional Ftz-dependent genes, identified by virtue of their striped expression patterns, require Ftz-F1 for expression in stripes lends support to the model that interaction with Ftz-F1 is the key to Ftz functional specificity as a segmentation protein. The three genes characterized in this study, *5-HT2*, *noc* and *drm*, are the earliest identified downstream targets of Ftz. Expression in stripes was observed at the cellular blastoderm stage when Ftz-F1 is highly expressed throughout the embryo and the 7 Ftz stripes are at their peak levels. These early target gene stripes were lost in *ftz* and also in *ftz-f1* mutants. In addition, ectopic expression was observed at early stages when Ftz was ectopically expressed throughout the embryo. En, long thought to be a major mediator of Ftz function in segmentation, is expressed later than these target genes and we verified that En is not required for the Ftz-dependent stripe expression of *noc* or *drm*. These findings suggest that Ftz and Ftz-F1 directly regulate expression of these three target genes. This new study brings to seven the targets of Ftz that appear to be directly co-regulated by Ftz and Ftz-F1: *ftz* itself (Han et al., 1998;Hiromi and Gehring, 1987;Pick et al., 1990;Schier and Gehring, 1993a;Schier and Gehring, 1993b;Yussa et al., 2001), *en* (Florence et al., 1997;Kassis, 1990), *apt*, *Dsulf1* (Bowler et al., 2006), *5HT-2*, *noc* and *drm*. For each gene, multiple potential Ftz-F1 binding sites were found within a 15–20 kb genomic region. In all cases, multiple potential Ftz binding sites surround the Ftz-F1 binding sites that could mediate cooperative interactions between Ftz and Ftz-F1. Many of these sites have been maintained during evolution and are present in distant *Drosophila* species (Bowler et al., 2006;Maier et al., 1990); data not shown). Other Ftz targets, such as *Ubx* (Muller and Bienz, 1992), *prd*, *odd* (Nasiadka and Krause, 1999) and *tsh* (Core et al., 1997) are also likely to be co-regulated by Ftz-F1.

The seven Ftz/Ftz-F1 target genes identified to date play diverse roles in segmentation and act at different levels of the embryonic hierarchy. First, Ftz acts in a cross-regulatory fashion to modulate expression of other pair-rule genes: it interacts with Ftz-F1 in autoregulation and also has been shown to regulate the pair-rule genes *prd*, *odd* and *slp* (Baumgartner and Noll, 1990; Gutjahr et al., 1994; Nasiadka and Krause, 1999). Second, Ftz/Ftz-F1 directly regulate components of the segment polarity system: first, they activate *en* expression in alternate stripes, and, second, they regulate *Dsulf1*, thought to modulate Wg activity (Lai et al., 2002). Ftz has also been shown to repress *wg* expression (Ingham et al., 1988; Nasiadka and Krause, 1999). Ftz/Ftz-F1 thus indirectly control compartment border formation, via regulation of En and Wg. Third, Ftz/Ftz-F1 regulate transcription factors that in turn control the differentiation of specific cell types: *apt*, *noc*, *drm*. *drm* encodes an odd-skipped family zinc finger transcription factor that it is required for patterning the dorsal epidermis, thus regulating the differentiation of specific cell types (Hatini et al., 2005). *noc* plays a role in trachael morphogenesis with mutants displaying defects in branch migration and expanded expression of trachael-specific genes (Dorfman et al., 2002). Similarly, *apt* is involved in this process as a regulator of the migration of trachael precursor cells (Bowler et al., 2006; Liu et al., 2003). Finally, Ftz/Ftz-F1 regulate a target gene more directly involved in morphogenesis, *5HT-2*. *5-HT2* encodes a serotonin receptor that demonstrates specific ligand binding in transfected cells and in *Drosophila* embryo extracts (Colas et al., 1995). Phenotypic analysis suggested a role

for *5-HT2* and other genes involved in serotonin biosynthesis in morphogenetic movements during gastrulation: deficiency embryos lacking *5HT-2* displayed delayed and incomplete movements during germband extension accompanied by mislocalization of Armadillo protein, suggestive of abnormalities in adherens junctions (Colas et al., 1999a; Colas et al., 1999b; Schaerlinger et al., 2007). It will be of interest in the future to determine whether other pair-rule genes direct expression of additional cell surface proteins that coordinate these processes.

drm is regulated by multiple stripe enhancers

We have identified enhancers of *drm* by combining bioinformatics with enhancer-reporter gene expression analysis in vivo (Fig. 5,Fig. 6). Fragments chosen for the in vivo analysis contained one or more matche(s) to a Ftz-F1 binding site. Three of the four fragments directed expression in *drm*-like patterns in vivo (Fig. 5). The upstream fragment, *drm1*, harbors a late stage enhancer that directs segmental expression of *drm*. *drm2* directed expression in seven strong stripes. *drm34* harbors enhancers for the region-specific expression of *drm* in the proventriculus and hindgut, expression that is important for the development of the fore- and hindgut (Johansen et al., 2003), as well as an early 7-stripe enhancer. We have not investigated whether any of these enhancers also direct expression in the leg imaginal discs (Hao et al., 2003). Two of the fragments tested here, *drm2* and *drm34*, directed expression in 7-stripe patterns. Surprisingly, for each of them, the set of seven stripes is in register with Ftz, suggesting that both regulate expression of the *drm*-primary stripes (Fig. 6). Although unexpected, this phenomenon has been observed in other cases where it was suggested that enhancers directing the same or similar expression patterns function as shadow enhancers to enhance the precision of expression patterns and facilitate the rapid evolution of cis-regulatory sequences (Hong et al., 2008). Point mutations of either or both of the predicted Ftz-F1 binding sites in the *drm34* Early 7-Stripe Enhancer abolished expression of *lacZ* fusion genes (Fig. 5F). Stripe expression was decreased but not completely abolished by mutation of the single predicted Ftz-F1 binding site in the *drm2* 7-Stripe Enhancer (Fig. 5D), suggesting additional inputs into regulation of the *drm* primary stripes by this enhancer. Together, these results suggest that Ftz-F1 activates expression in the primary *drm* stripes via the *drm34* Early 7-Stripe Enhancer. We speculate that following this initial activation, autoregulation by Drm may augment Ftz-F1 activation of stripes via the *drm2* 7-Stripe Enhancer to raise levels of transcription in *drm* primary stripes.

The role of pair-rule patterning in *Drosophila* segmentation

Drosophila ftz is a typical pair-rule gene: *ftz* mutant embryos die lacking even-numbered body segments (Wakimoto et al., 1984). How this wild type function of *ftz*, and other pair-rule genes, is executed is not yet known. As for other segmentation mutants, the pair-rule mutant phenotype results from cell death (Magrassi and Lawrence, 1988; Pazdera et al., 1998). However, this cell death appears to be an indirect effect (Hughes and Krause, 2001). Similarly, pair-rule genes regulate segment border formation indirectly, via activation of the segment polarity genes such as *en* and *wg* (Carroll, 1990; Carroll et al., 1988; DiNardo and O'Farrell, 1987; Howard and Ingham, 1986; Ingham et al., 1988; Jaynes and Fujioka, 2004; Lawrence and Johnston, 1989; Lawrence et al., 1987; Lawrence and Pick, 1998). In addition to this, segment-polarity-independent roles for the pair-rule genes in morphogenesis have been revealed by careful studies from the Wieschaus lab (reviewed in (Dawes-Hoang and Wieschaus, 2001; Wieschaus et al., 1991). For example, it was found that cell intercalation and germ band extension are regulated by the pair-rule genes, independent of segment polarity genes (Irvine and Wieschaus, 1994). Similarly, cellular studies defined two subtle morphogenetic processes that occur before gastrulation – one, controlled by the pair-rule gene *paired* (Blankenship and Wieschaus, 2001). More recently, studies have shown that the planar polarity and organization of intercalating cells during germ band extension are controlled by the striped expression patterns of *eve* and *runt* (Blankenship et al., 2006; Zallen and Wieschaus, 2004) and that the longitudinal division of cells during germ band extension is controlled by *eve* (da Silva and Vincent,

2007). These studies are suggestive of direct roles for the pair-rule system in cell shape changes and rearrangements during germ band extension (reviewed in Pilot and Lecuit, 2005; Zallen and Blankenship, 2008). Together, these studies support the notion that combinatorial expression of early patterning genes assigns unique identities in the blastoderm at a single cell level, as originally proposed by Gergen et al., 1986; Scott and O'Farrell, 1986). Here we have shown that the pair-rule gene *ftz* regulates target genes prior to and independent of En. These findings support the model that the stripes of pair-rule genes play active roles in patterning the embryo rather than serving solely as intermediary patterns whose function is to produce the segmental stripes of segment polarity genes. One role for these pair-rule stripes may be to establish differential adhesiveness to groups of cells in the blastoderm embryo (Irvine and Wieschaus, 1994) and see above). Future work identifying additional pair-rule targets will be required to explain the fundamental biological roles of pair-rule patterning and to understand how the assignment of positional identities by pair-rule genes, prior to morphogenesis, translates into the development and differentiation of body segments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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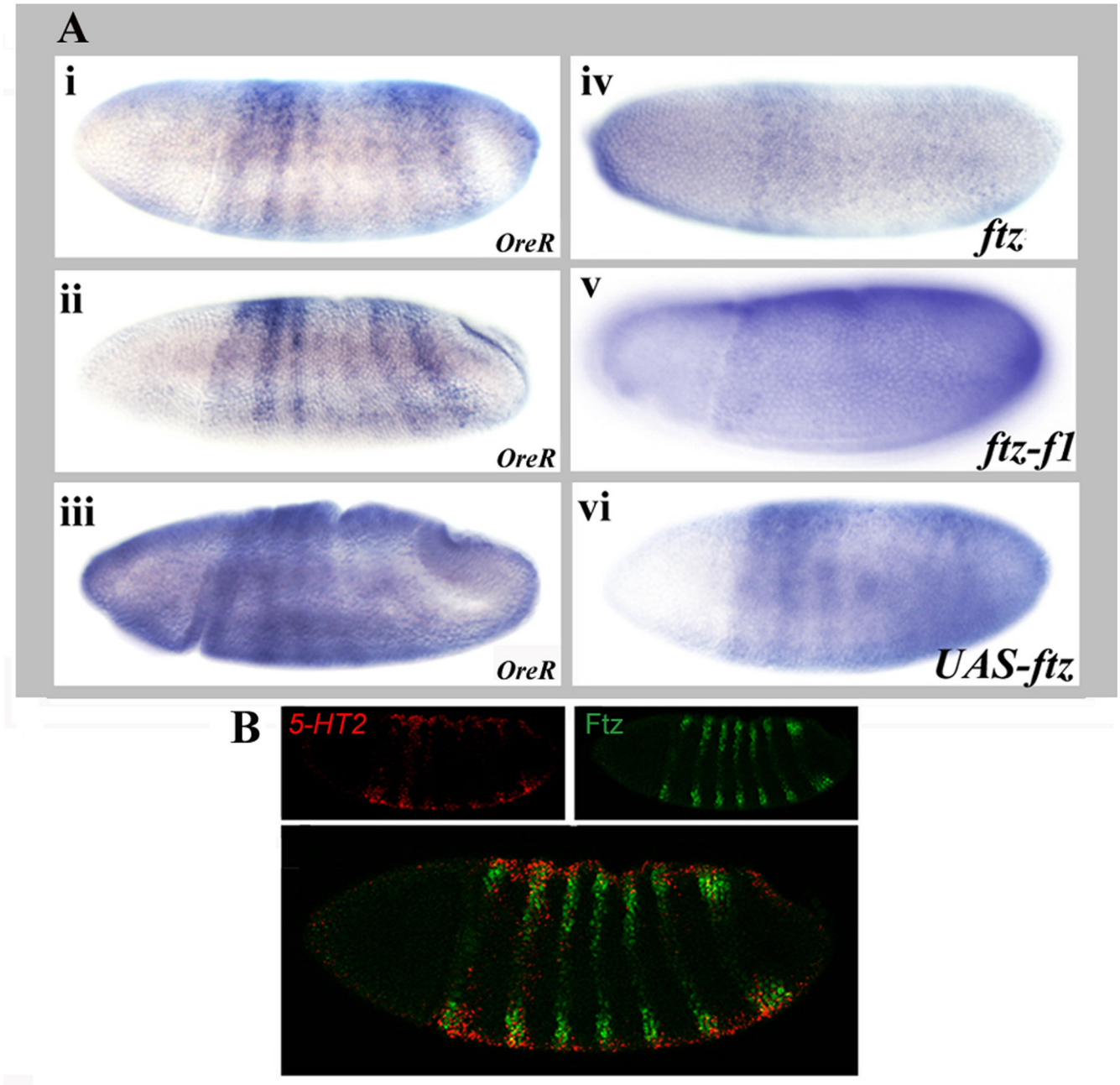

Figure 1. Ftz and its partner Ftz-F1 are necessary for 5-HT2 stripe expression

(A)5-*HT2* expression is dependent upon *ftz* and *ftz-f1*. In situ hybridization of *5-HT2* mRNA: (i,ii,iii) *OreR*, at cellular blastoderm, gastrulation and early germ band extension; (iv) ftz^{9H34} mutant embryo; (v) $ftz\text{-}f1^{19}$ mutant embryo; and (vi) *UAS-myc-ftz/NGT40* embryo. In either *ftz* or *ftz-f1* mutants, *5HT-2* stripes were replaced by diffuse, low level expression. Ectopic *ftz* expression induced ectopic *5HT-2* expression. **(B)***5-HT2 and Ftz are coexpressed*. Confocal images of embryos stained for *5-HT2* mRNA and Ftz protein. Stage 7 embryo: *5-HT2* RNA (red), Ftz protein (green), *5-HT2* RNA and Ftz protein merged. Note that overlapping expression in stripes does not produce a yellow color because Ftz protein is nuclear and *5-HT2* RNA is cytoplasmic.

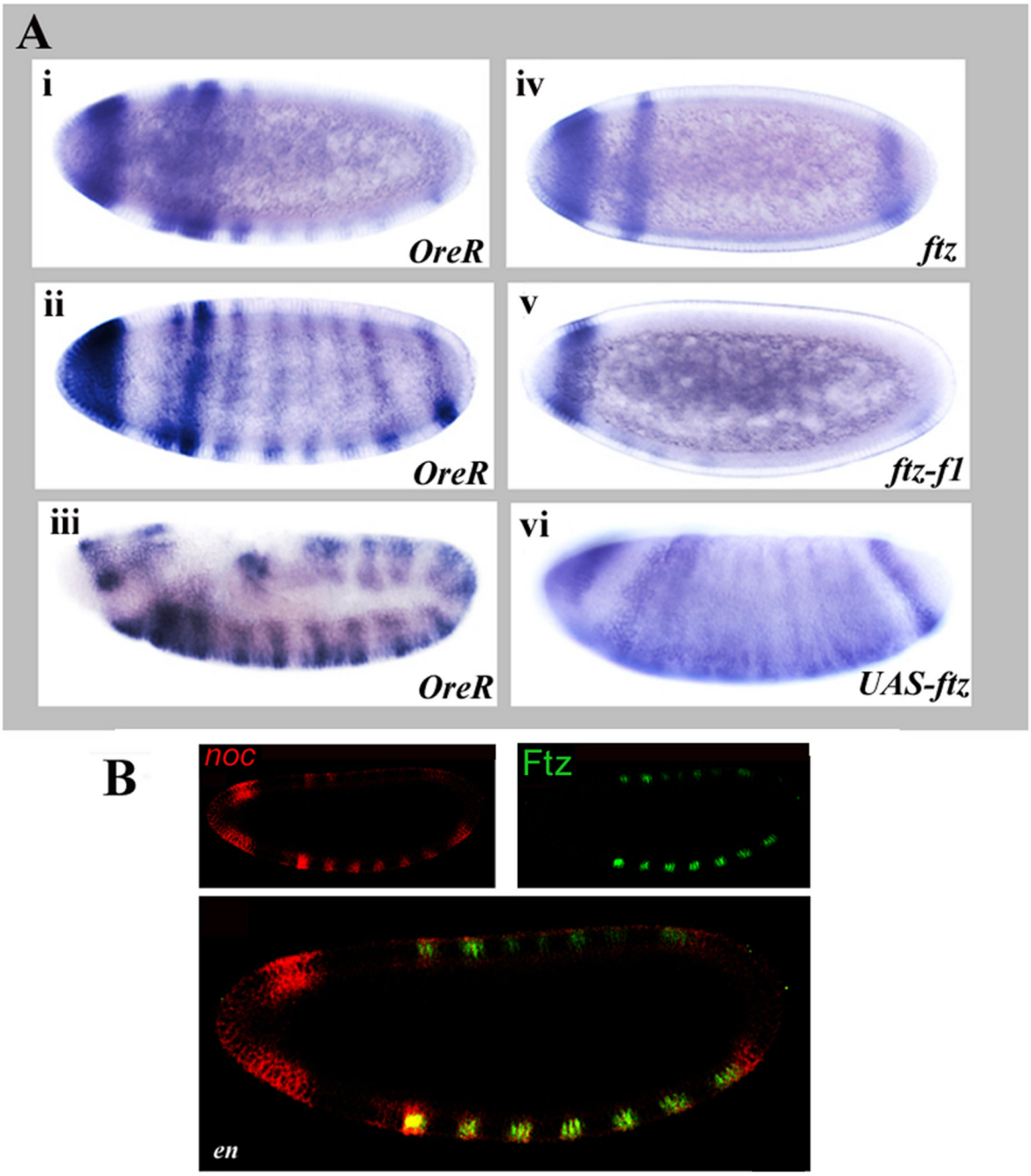

Figure 2. ftz and ftz-f1 regulate noc expression

(A) *noc* expression is dependent upon *ftz* and *ftz-f1*. In situ hybridization of *noc* mRNA: (i,ii,iii) *OreR*, at cellular blastoderm and late germ band extension stages show early expression in the head and in eight stripes, developing into a 14 stripe pattern; (iv) ftz^{9H34} mutant embryo; (v) $ftz\text{-}f1^{ex19}$ mutant embryo; and (vi) *UAS-myc-ftz/NGT40* embryo. In *ftz* mutant embryos, the six central *noc* stripes are missing while ectopic Ftz induced ectopic *noc* expression. **(B)** Ftz regulates *noc* expression independent of *en*. Confocal images of embryos stained for *noc* mRNA (red) or Ftz protein, as indicated, in an en^{1} mutant embryo: *noc* RNA (red), Ftz protein (green). Note that Ftz protein is nuclear and *noc* RNA is cytoplasmic, so cellular colocalization

does not produce a yellow color. No change was observed in the *noc* expression pattern in *en* mutants, as compared to wild type controls.

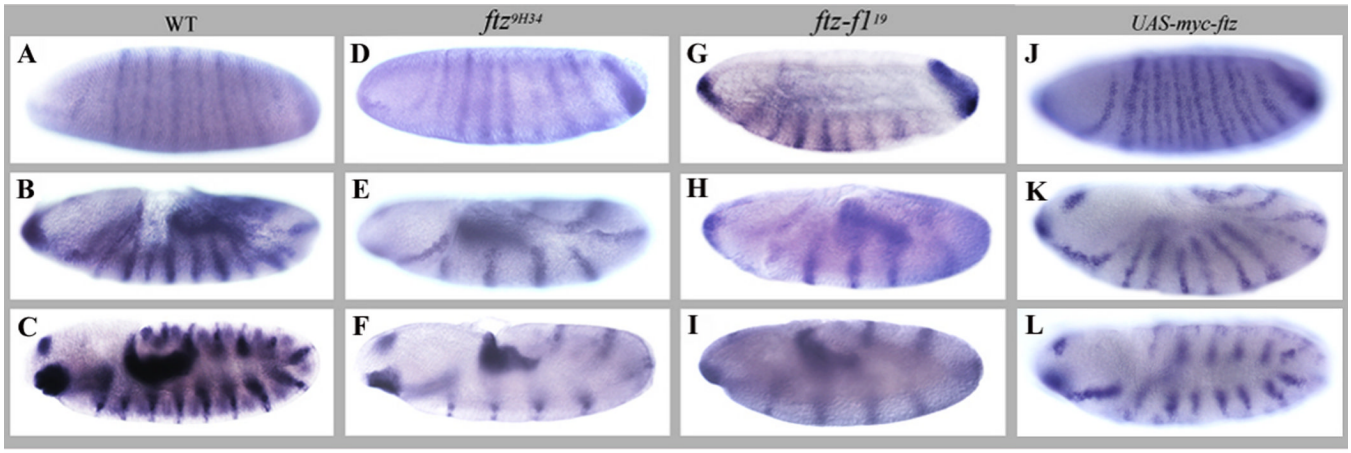

Figure 3. Alternate drm stripes require Ftz and Ftz-F1

In situ hybridization of *drm* mRNA to embryos at late cellular blastoderm, early germ band extension and full germ band extended stages: (A, B, C) *OreR*; (D, E, F) ftz^{9H34} mutant; (G, H, I) $ftz\text{-}f1^{19}$ mutant; and (J, K, L) *UAS-myc-ftz/ NGT40* embryos. *drm* is expressed in seven strong primary stripes, with a set of weaker secondary stripes becoming stronger as development proceeds. In *ftz* or in *ftz-f1* mutant embryos, half of the stripes seen in the wild type are missing, while ectopic Ftz expression alters the pattern of *drm* stripes.

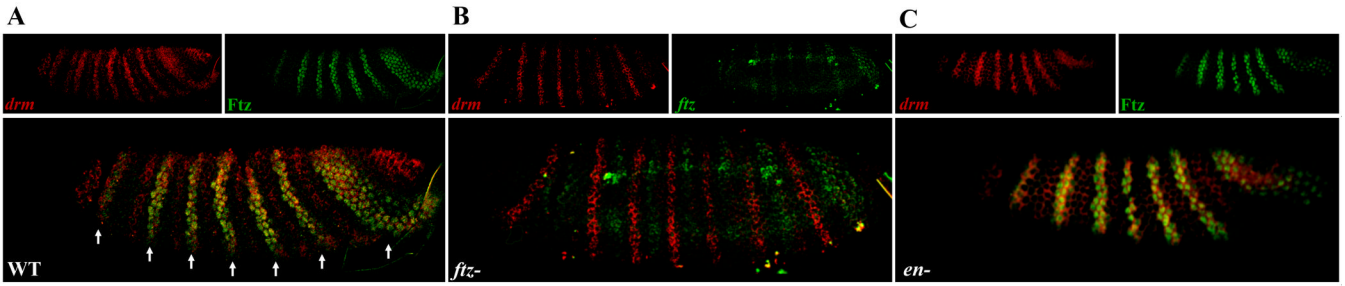

Figure 4. *ftz* is required for expression of the primary *drm* stripes independent of *en*

Confocal images of embryos double stained for *drm* mRNA and Ftz protein (A,C) or *ftz* RNA (B). **(A)***Ftz and drm overlap in the primary drm stripes.* *OreR*, stage 6 embryo: *drm* RNA (red), Ftz protein (green). **(B)***Primary drm stripes are lost in ftz mutants*. ftz^{9H34} stage 5 embryo: *drm* RNA (red), *ftz* RNA, (green). The remaining *drm* stripes in the *ftz* mutant embryo are out of register with the *ftz* stripes. **(C)***Primary drm stripes do not require en.* Stage 6 *en* embryo: *drm* RNA (red), Ftz protein (green). No change in the Ftz-dependent *drm* stripes was observed in *en* mutants.

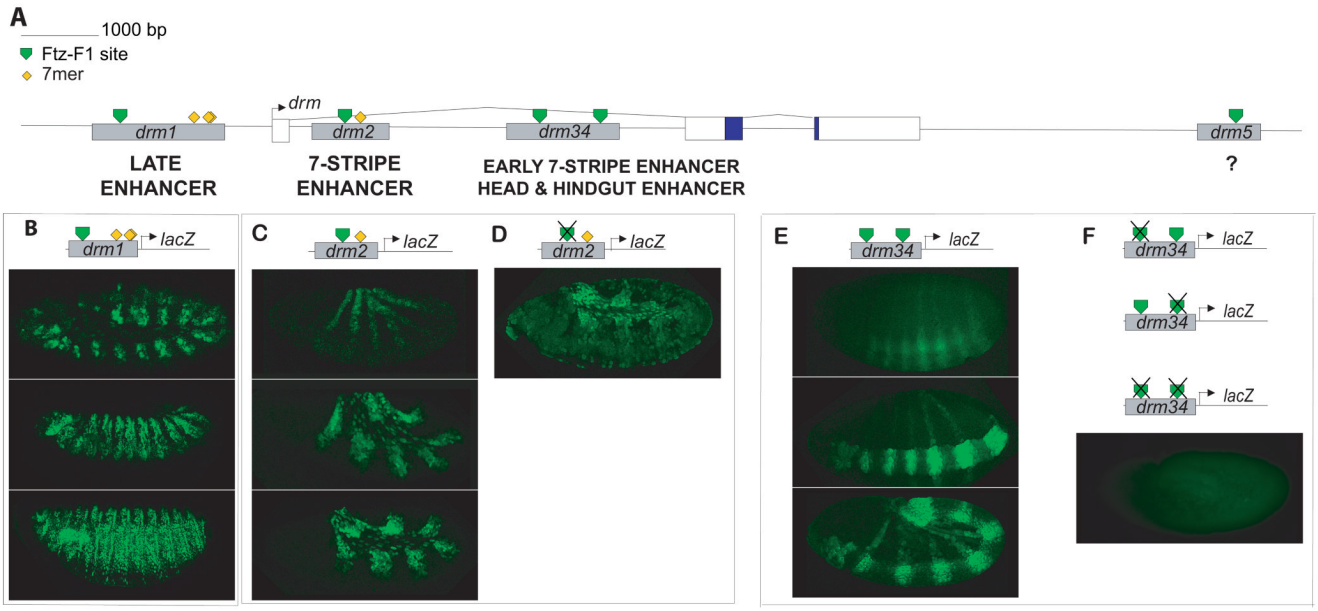

Figure 5. Multiple stripe enhancers control *drm* expression

(A) Schematic of the structure of the *drm* gene. The *drm* gene includes three exons, and two introns, with the coding region initiating in exon 2. Five potential Ftz-F1 binding sequences (green triangles) and multiple 7mers (of a total of eighteen) are indicated. Putative cis-regulatory elements for *drm* were identified by virtue of the presence of potential Ftz-F1 binding sites. Fragments containing these sites (gray rectangles, *drm1-7mer*, *drm2*, *drm34*, *drm5*, as indicated, were inserted upstream of a basal promoter and *lacZ* reporter gene in the P-element vector pX28. Enhancers identified in this analysis are indicated below the line. No reproducible expression pattern was obtained for *drm5-lacZ* ***(B–F)****Expression of drm-lacZ transgenes.* **(B)***drm1-7mer-lacZ* expression at the end of germ band extension and in a complex segmental pattern through germ band retraction. **(C)** Expression of *drm2-lacZ* in seven stripes at early, mid and late germ band extension stages. **(D)** Expression of *drm2M-lacZ*, in which the predicted Ftz-F1 binding site was mutated, in seven weak stripes at germ band extension. Amnioserosa expression appears to be due to vector sequences. **(E)** Expression of *drm34-lacZ* in seven stripes at the cellular blastoderm stage and during gastrulation. Striped expression persisted through germ band extension. Expression in the head and hindgut was detected from gastrulation through germ band extension. **(F)** Mutation of the *drm3, drm4* or both *drm3 and 4* predicted Ftz-F1 binding sites abolished expression of *lacZ* fusion genes.

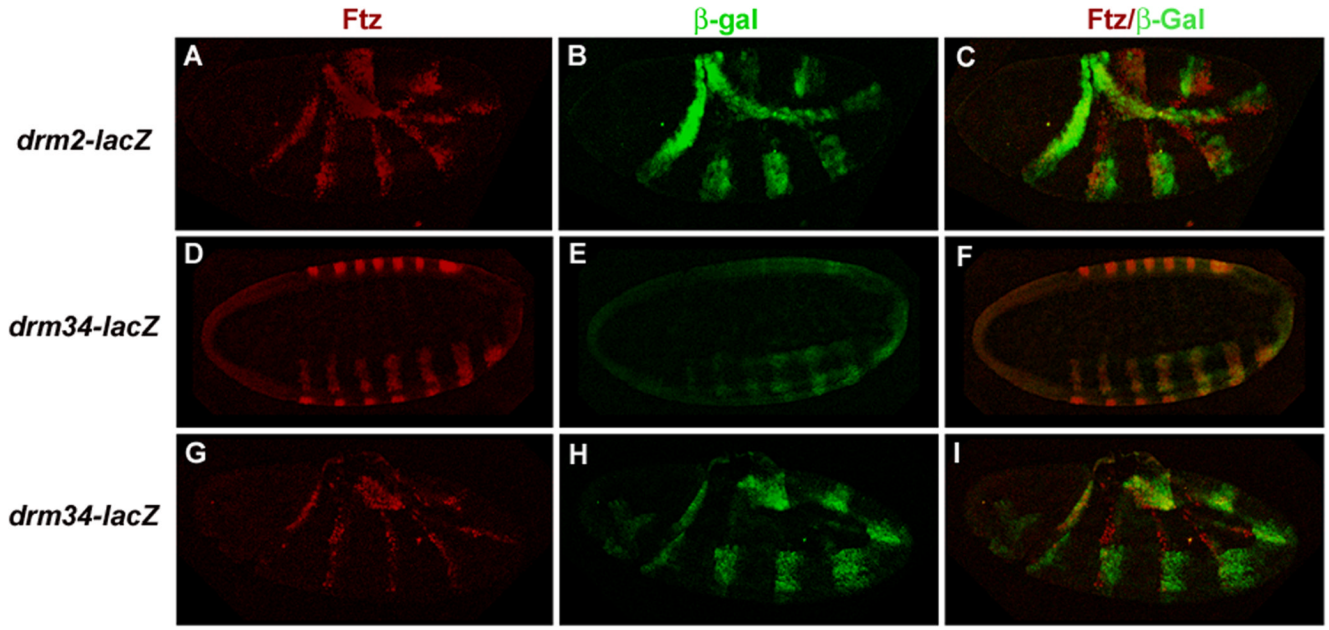

Figure 6. Two independent *drm* enhancers direct expression in Ftz-like stripes

Immunohistochemical staining of *drm-lacZ* transgenic embryos with anti-Ftz antibody (red) and anti-β-galactosidase antibody (green). (A–C) Expression of *drm2-lacZ* in seven stripes was detected during germ band elongation. Ftz and β-galactosidase overlap. Note that Ftz stripes have become thinner by this stage, but the stability of β-galactosidase results in thick stripes for the *drm-lacZ* transgene. (D–F) A confocal cross-section through a *drm34-lacZ* transgenic embryo at cellular blastoderm. Ftz and β-galactosidase expression overlap exactly. As the Ftz stripes thin during germ band elongation (G–I), Ftz and *drm34-lacZ* stripes continue to overlap but β-galactosidase expression is broader, because of the stability of β-galactosidase protein, resembling the pattern seen for *ftz* autoregulatory elements that are direct Ftz targets (Pick et al., 1990).