Novel regulatory interactions revealed by studies of murine limb pattern in
Wnt-7a and En-1 mutants

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SUMMARY

Classical embryological experiments have demonstrated that dorsal-ventral patterning of the vertebrate limb is dependent upon ectodermal signals. One such factor is Wnt-7a, a member of the Wnt family of secreted proteins, which is expressed in the dorsal ectoderm. Loss of Wnt-7a results in the appearance of ventral characteristics in the dorsal half of the distal limb. Conversely, En-1, a homeobox transcription factor, is expressed exclusively in the ventral ectoderm, where it represses Wnt-7a. En-1 mutants have dorsal characteristics in the ventral half of the distal limb. Experiments in the chick suggest that the dorsalizing activity of Wnt-7a in the mesenchyme is mediated through the regulation of the LIM-homeodomain transcription factor Lmx-1. Here we have examined the relationship between Wnt-7a, En-1 and Lmx-1b, a mouse homolog of chick Lmx-1, in patterning the mammalian limb. We find that Wnt-7a is required for Lmx-1b expression in distal limb mesenchyme, and that Lmx-1b activation in the ventral mesenchyme of En-1 mutants requires Wnt-7a. Consistent with Lmx-1b playing a primary role in dorsalization of the limb, we find a direct correlation between regions of the anterior distal limb in which Lmx-1b is miss-regulated during limb development and the localization of dorsal-ventral patterning defects in Wnt-7a and En-1 mutant adults. Thus, ectopic Wnt-7a expression and Lmx-1b activation underlie the dorsalized En-1 phenotype, although our analysis also reveals a Wnt-7a-independent activity for En-1 in the repression of pigmentation in the ventral epidermis. Finally, we demonstrate that ectopic expression of Wnt-7a in the ventral limb ectoderm of En-1 mutants results in the formation of a second, ventral apical ectodermal ridge (AER) at the junction between Wnt-7a-expressing and nonexpressing ectoderm. Unlike the normal AER, ectopic AER formation is dependent upon Wnt-7a activity, indicating that distinct genetic mechanisms may be involved in primary and secondary AER formation.

Key words: mouse, limb bud, dorsoventral pattern, Wnt-signaling, apical ectodermal ridge

INTRODUCTION

The vertebrate limb is a model system for studying pattern formation during development. The proper elaboration of this structure requires that cells of the developing limb integrate and respond to patterning signals from each of the three limb axes: proximal-distal (P-D), anterior-posterior (A-P) and dorsal-ventral (D-V). A source of patterning signals for each axis has been identified by grafting experiments performed principally in the chick (for reviews see Johnson et al., 1994; Tickle, 1995). Signals from the apical ectodermal ridge (AER) are necessary for P-D outgrowth and patterning, since removal of the AER terminates limb outgrowth and results in the loss of distal limb elements (Saunders, 1948; Summerbell, 1974). Signals from the zone of polarizing activity (ZPA) control patterning across the A-P axis. When grafted to the anterior margin of a host limb bud, the ZPA is capable of inducing a mirror-image duplication of structures along the A-P axis (Saunders and Gasseling, 1968; Tickle et al., 1975). Manipulations of the D-V axis have revealed that more than one mechanism is involved in establishing polarity along this axis. The limb bud ectoderm controls D-V patterning, but only in the distal limb. When the limb ectoderm is rotated 180° in relation to the underlying mesenchyme, the D-V polarity of distal limb elements is reversed, while the proximal limb is unaffected (MacCabe et al., 1974; Geduspan and MacCabe, 1987). Thus, signals from the limb bud ectoderm control D-V patterning of the distal limb; however, the mechanism by which the proximal limb is patterned is not yet resolved.

The signaling molecule Wnt-7a was identified as a putative regulator of D-V patterning in the developing vertebrate limb based on its restricted expression in dorsal limb bud ectoderm (Dealy et al., 1993; Parr et al., 1993). In the mouse, Wnt-7a transcripts are present in the dorsal ectoderm prior to limb outgrowth and remain dorsally restricted throughout the patterning process (Parr and McMahon, 1995). Loss of Wnt-7a function affects only the dorsal half of the limb and results in a ventralized phenotype (Parr and McMahon, 1995). This includes the suppression of dorsal hair growth, and the appearance of ectopic footpads and striated epidermis (both ventral
characteristics) on the dorsal surface of digits. Further, tendons and bones (mesenchymally derived tissues) also adopt a ventral fate. However, these transformations are restricted to the distal limb, consistent with the limb bud ectoderm controlling D-V patterning of only distal regions.

The identification of a possible target of Wnt-7a signaling has provided further insights into the role of Wnt-7a in D-V patterning. The LIM-homeodomain transcription factor Lmx-1 is expressed in the dorsal mesenchyme of the developing limb (Riddle et al., 1995; Vogel et al., 1995). The onset of Lmx-1 expression is concurrent with the onset of Wnt-7a expression in the overlying ectoderm (Riddle et al., 1995). Lmx-1 expression in the distal limb appears to be dependent on signals from the dorsal ectoderm, since removal of the dorsal ectoderm results in a loss of Lmx-1 expression in distal limb mesenchyme (Riddle et al., 1995; Vogel et al., 1995). Cells expressing Wnt-7a are sufficient to maintain Lmx-1 expression in the distal limb mesenchyme in the absence of limb ectoderm, indicating that Wnt-7a is sufficient to activate Lmx-1 (Riddle et al., 1995). However, it is uncertain if Wnt-7a regulates Lmx-1 throughout limb development, for although Lmx-1 expression is lost only in the distal dorsal mesenchyme following ectoderm removal, it is possible that Lmx-1 is activated in proximal mesenchyme in response to Wnt-7a and, once activated, Lmx-1 expression is maintained independent of Wnt-7a signals.

Lmx-1 expression can also be induced in ventral mesenchyme in response to ectopic Wnt-7a in the ventral ectoderm (Riddle et al., 1995; Vogel et al., 1995). It has been suggested that Lmx-1 mediates the dorsalizing Wnt-7a signal, since ectopic expression of Lmx-1 in ventral mesenchyme of the chick limb is sufficient to dorsalize the distal ventral limb in the absence of Wnt-7a (Riddle et al., 1995; Vogel et al., 1995). Since Wnt-7a is required for Lmx-1 expression in dorsal mesenchyme, and can induce Lmx-1 in ventral mesenchyme, it is possible that the reversal of D-V polarity in the distal limb that results from ectoderm rotation can be explained by the Wnt-7a-induced expression of Lmx-1 in the ventral, rather than dorsal, distal limb mesenchyme.

Ventrally, the homeodomain transcription factor En-1 represses Wnt-7a since, in the absence of En-1 function, Wnt-7a is ectopically expressed in the ventral ectoderm (Loomis et al., 1996). The ectopic expression of Wnt-7a in the ventral ectoderm may account for the dorsalized phenotype of En-1 mutant limbs, which have dorsal characteristics such as hair and pigmentation on the ventral surface of the adult limb, and a dorsal pattern of tendon and bone formation (Loomis et al., 1996). Although it is also possible that En-1 positively regulates ventralizing signals, En-1 is not ectopically expressed in the dorsal ectoderm of Wnt-7a mutants, which display ventral characteristics dorsally (Parr and McMahon, 1995).

There is extensive evidence for coordination in patterning along the three limb axes (Vogel and Tickle, 1993; Lauber et al., 1994; Niswander et al., 1994; Yang and Niswander, 1995; Chiang et al., 1996). Both Wnt-7a and En-1 are required for the proper patterning of the other limb axes. In addition to regulating D-V patterning, En-1 also regulates P-D patterning by repressing the expression of AER markers in the ventral ectoderm. In the En-1 mutant limb bud, Fgf-8 and Bmp-2, which are normally expressed in the AER (Lyons et al., 1990; Crossley and Martin, 1995), expand into the ventral ectoderm (Loomis et al., 1996). This may be related to the appearance of ectopic ventral digits at a low frequency in En-1 mutants (Loomis et al., 1996). Wnt-7a is required for proper patterning along the A-P axis. In Wnt-7a mutants, there is a decrease in the number of cells expressing the ZPA signal Shh, and a frequent loss of the most posterior digit of the adult limb (Parr and McMahon, 1995). Experiments in the chick have demonstrated that Wnt-7a can rescue the loss of Shh expression that results from removal of the dorsal ectoderm, which indicates that Wnt-7a maintains Shh expression (Yang and Niswander, 1995). Interestingly, the D-V patterning aspect of the Wnt-7a phenotype shows an A-P bias as well, since ventral characteristics appear more frequently on anterior digits (Parr and McMahon, 1995). The basis for this anterior bias in the localization of D-V patterning defects is unknown.

To analyze the interrelationship between Wnt-7a, Lmx-1b and En-1 in D-V patterning of the mouse limb, we have examined Wnt-7a, En-1 and Wnt-7a/En-1 double mutant embryos. These studies map more extensively the regions affected in Wnt-7a and En-1 mutants and indicate that Lmx-1b may be the primary effector of pattern alteration in these mutants. Further, they reveal an unexpected role for Wnt-7a signaling in the formation of an ectopic AER.

**MATERIALS AND METHODS**

**Collection and genotyping of embryos**

Embryos from Wnt-7a+/−; En-1+/− intercrosses were collected at noon on the day of dissection. Each embryo was dissected into PBS, and the yolk sacs were collected for genotyping. Embryos were fixed in PBS/methanol series and stored in methanol at −20°C until use. The Wnt-7a genotype was determined by PCR-mediated amplification of yolk sac DNA in a reaction that amplified both the wild-type and targeted alleles. A 220 bp product was amplified from the wild-type allele (primers 553 and 1144), and a 350 bp product was amplified from the targeted allele (primers 553 and 1143). The amplification reaction consisted of a 5 minute denaturation at 94°C followed by 34 cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 1 minute at 72°C. The En-1 genotype was determined by Southern blot of yolk sac DNA digested with XhoI and probed with an 850 bp EcoRI-HindIII genomic fragment as described (Wurst et al., 1994).

Primer sequences: oligo 553: 5′-TCACGTTACCGCAATCCAGC-3′; oligo 1143: 5′-CTCTTCCGTGGTTACTCTGTGG-3′; oligo 1144: 5′-CCCTCAGAGACATCTGACGC-3′.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed essentially as described (Parr et al., 1993; modified by Knecht et al., 1995). A plasmid containing the entire coding region of Lmx-1b was linearized with HindIII and transcribed with T7 polymerase to generate a 1.1 kb antisense probe. A probe for Wnt-10b was synthesized from a 400 bp PCR fragment cloned as described by Gavin et al. (1990), linearized with EcoRI and transcribed with T3 polymerase. Probes for Wnt-7a (Parr et al., 1993), Fgf-8 (Crossley and Martin, 1995) and Shh (Echelard et al., 1993) were made as described. For double-label whole-mount in situ hybridization, one of the probes was labeled with digoxigenin, the other with fluorescein. Both probes were hybridized at the same time, but detected sequentially with alkaline phosphatase-conjugated antibodies. Following detection of the first probe with BM Purple (Boehringer-Mannheim), the alkaline phosphatase was inactivated by heating at 65°C in 10 mM EDTA for 30 minutes followed by dehydration through methanol. The second probe was detected with INT-BCIP (Boehringer-Mannheim).
Histological analysis
Samples that had previously been stained by whole-mount in situ hybridization were rinsed several times in PBS. Tissue was embedded in 2% agarose in PBS and vibratome-sectioned at 50-100 μm. Sections were placed on a glass slide, allowed to equilibrate overnight in 70% glycerol under a coverslip and photographed under Nomarski optics. Alternatively, unstained samples were dehydrated into methanol, rehydrated into PBS and rinsed several times. Tissue was embedded and sectioned as described above. Sections were dehydrated in methanol, then taken through the whole-mount in situ hybridization procedure. After staining and fixation, sections were mounted and photographed as above.

Generation of Wnt-7a/En-1 double mutant adults
Wnt-7a+/−; En-1+/− females were crossed to a male carrying the WEXPZ-En-1 transgene (Danielian and McMahon, 1996). A PCR assay was designed to analyze tail DNA for the presence of the WEXPZ-En-1 transgene. Amplification with primers 890 and 891 produced a 200 bp band in all animals and an additional 300 bp band in animals that were WEXPZ-En-1+. The amplification reaction consisted of 36 cycles of 30 seconds at 93°C, 30 seconds at 55°C, and 1 minute at 72°C, followed by a 5 minute termination step at 72°C. Wnt-7a+/−; En-1+/−; WEXPZ-En-1+ animals were bred to Wnt-7a+/−; En-1+/−; and the offspring were examined at 2 weeks for limb abnormalities. Those displaying a limb phenotype were genotyped using tail DNA as described above.

Primer sequences: oligo 890: 5′-TCTACTGCACCGCCTATTCTTG-3′; oligo 891: 5′-CTGTGTTGAATGTTGTAACAG-3′.

Skeletal preparations
3-week forelimbs were skinned and stained for cartilage (alcian blue) and ossified bone (alizarin red S) by the method described (Parr and McMahon, 1995).

RESULTS

Expression of Lmx-1b during limb development
Experiments in the chick (Riddle et al., 1995; Vogel et al., 1995) have suggested that Lmx-1 may play a role in dorsalization of the limb. To determine whether its mammalian counterpart, Lmx-1b (R. Johnson et al., unpublished), is expressed in a spatiotemporal pattern that is consistent with a function in D-V patterning of the mouse limb, we examined Lmx-1b expression in the developing limb. Lmx-1b transcripts are first detected in the lateral plate mesoderm of the presumptive forelimb field at the 10 somite stage, or 8.5 dpc (days post coitum) (arrows in Fig. 1A), coincident with the onset of Wnt-7a expression (Parr and McMahon, 1995). When the limb buds emerge, Lmx-1b transcripts are restricted to the dorsal mesenchyme (Fig. 1B; data not shown), within cells approximately 9-12 cell layers beneath the dorsal ectoderm at 11.5 dpc (Fig. 1C). By 13.5 dpc, when interdigital necrosis is occurring, Lmx-1b expression is downregulated in the interdigital regions, but remains high within the digits (Fig. 1D). At 14.5 dpc, when the digits become morphologically distinct, expression levels appear to have dropped considerably in the forearm, but expression in the dorsal mesenchyme of the autopod (the paw or hand) persists at higher levels (Fig. 1E). As at earlier stages, transcripts are restricted to the dorsal mesenchyme (Fig. 1F). These results are consistent with Lmx-1b playing a role in D-V patterning of the limb.

Lmx-1b is a target of Wnt-7a signaling
To assess whether Lmx-1b is a target of Wnt-7a signaling, we examined Lmx-1b expression in Wnt-7a mutants. Up until 11.5 dpc, the distribution of Lmx-1b transcripts is identical to that of wild type (data not shown). At 11.5 dpc, expression is lost from both the distal anterior mesenchyme and around the lateral margin of the limb bud (Fig. 2A-D). These results indicate that Lmx-1b expression may be dependent on Wnt-7a in distal regions of the limb bud.

To determine if Lmx-1b expression can be induced in ventral mesenchyme in response to ectopic Wnt-7a, we examined Lmx-1b expression in En-1 mutants, which ectopically express Wnt-7a in the ventral limb ectoderm at 11.5 dpc (Loomis et al., 1996; Fig. 5F). The distribution of Lmx-1b transcripts is similar to wild type in the dorsal mesenchyme (Fig. 2E), but there is a separate domain of ectopic expression in the ventral mesenchyme (arrow in Fig. 2F). Expression levels are highest at the anterior distal edge of the ectopic domain, and are patchy throughout the distal ventral mesenchyme. Interestingly, although dorsal Lmx-1b expression is only dependent upon Wnt-7a activity in distal regions, ectopic Wnt-7a expression in the ventral ectoderm leads to distal and proximal Lmx-1b activation. To determine if Wnt-7a is required for the ectopic Lmx-1b expression in En-1 mutants, we looked at Lmx-1b expression in Wnt-7a/En-1 double mutant embryos. The expression pattern in the dorsal mesenchyme is very similar to that observed in the Wnt-7a mutant, although overall expression levels appear somewhat lower (Fig. 2G). Lmx-1b transcripts are not detected in the ventral mesenchyme (Fig. 2H), indicating that Wnt-7a is required to induce Lmx-1b in the ventral mesenchyme of En-1 mutants. Together, these results indicate that Wnt-7a signaling is necessary and most likely sufficient to induce Lmx-1b expression in the distal forelimb of the mouse.

Expression of Lmx-1b correlates with the expression of the D-V patterning marker Wnt-10b at 14.5 dpc
We observed at 11.5 dpc that Lmx-1b expression is preferentially affected in the anterior mesenchyme of Wnt-7a and En-1 mutants. To determine if this pattern persists as limb development proceeds, and if it can be correlated with changes in D-V patterning, we examined the distribution of Lmx-1b transcripts in forelimbs of Wnt-7a mutant, En-1 mutant, and Wnt-7a/En-1 double mutant embryos at 14.5 dpc. In the Wnt-7a mutant, Lmx-1b transcripts are always absent from digit 2, the anterior half of digit 3, and the distalmost regions of digits 1, 2, 3, and 4, and which correspond to the lateral margin of the 11.5 dpc limb bud (Fig. 3I). In addition, Lmx-1b is sometimes absent throughout digits 1 and 3 of the Wnt-7a mutant. In the En-1 mutant, the ectopic Lmx-1b expression in the anterior ventral mesenchyme at 11.5 dpc has refined by 14.5 dpc to expression in the ventral mesenchyme of digits 1, 2, 3, and the anterior half of digit 4 (arrowheads in Fig. 3G). Surprisingly, on the dorsal surface of the En-1 mutant, Lmx-1b expression is absent from the distal tip of digits 2 and 3 (arrows in Fig. 3E). The double mutant displays a complete absence of Lmx-1b expression in digits 1, 2, 3, and the anterior half of digit 4 (Fig. 3K). The more severe loss of Lmx-1b expression in the Wnt-7a/En-1 double mutant than is observed in the Wnt-7a mutant may be due to differences in the genetic background. The Wnt-7a mutation was created on a 129/Sv background, while the Wnt-7a/En-1 double mutants...
are a mixed 129/Sv; C57Bl/6J background. In all cases, however, it appears that the pattern of *Lmx-1b* expression which is established at 11.5 dpc is maintained as the autopod develops.

Previous studies of *Wnt-7a* mutant forelimbs have demonstrated that dorsal to ventral transformations can be characterized molecularly at 15.5 dpc by the expression of a footpad marker on the dorsal side of the mutant autopod (Parr and McMahon, 1995). To determine whether this correlates with regions of the mesenchyme in which *Lmx-1b* expression is altered at 14.5 dpc, we examined the expression of a second Wnt family member, *Wnt-10b*. In the 14.5 dpc forelimb, *Wnt-10b* is expressed on the dorsal surface of the autopod in the developing hair follicles (Wang and Shackleford, 1996; Fig. 3B), and diffusely in the epithelium of the digits (Fig. 3B). *Wnt-10b* is also expressed on the ventral surface, in the epithelium and mesenchyme of the developing footpads (Fig. 3D; data not shown). We compared alterations in the normal *Wnt-10b* expression pattern with changes in *Lmx-1b* expression in mutant forelimbs. In the *Wnt-7a* mutant forelimb, the normal expression of *Wnt-10b* in the dorsal epithelium is absent from digits 2 and 3, suggestive of a lack of hair follicle development, but there is a region of high expression at the base of each of

![Image of developing limb analyzed by whole-mount in situ hybridization](image)

**Fig. 1.** *Lmx-1b* expression in the developing limb analyzed by whole-mount in situ hybridization. (A) Dorsal view of an 8.5 dpc (10 somite) embryo. *Lmx-1b* is expressed in the lateral plate mesoderm at the presumptive forelimb level (arrows). (B) Close-up of a 10.5 dpc forelimb showing that *Lmx-1b* expression is dorsally restricted. (C) Vibratome section along the P-D axis of an 11.5 dpc forelimb. *Lmx-1b* is expressed in cells approximately 9-12 cell layers beneath the dorsal ectoderm. (D) 13.5 dpc forelimb showing high levels of *Lmx-1b* expression in the digits and lower levels in the interdigital regions. Dorsal (E) and lateral posterior (F) view of a 14.5 dpc forelimb. *Lmx-1b* transcripts are detected in the autopod (E), and are still restricted to the dorsal mesenchyme (F). Anterior is up in A,B,D,E. Dorsal is up in C,F; V, ventral; sm, somitic mesoderm; psm, presomitic mesoderm.

![Image of Wnt-7a regulating Lmx-1b expression in the distal limb](image)

**Fig. 2.** Wnt-7a regulates *Lmx-1b* expression in the distal limb.

Whole-mount in situ hybridization analyzing *Lmx-1b* expression in wild-type (A,B), *Wnt-7a* mutant (C,D), *En-1* mutant (E,F) and *Wnt-7a/En-1* double mutant (G,H) forelimbs at 11.5 dpc. (A,B) *Lmx-1b* is expressed throughout the dorsal mesenchyme of the wild-type forelimb. (C,D) In the *Wnt-7a* mutant, *Lmx-1b* expression is absent in the dorsal mesenchyme around the lateral margin of the limb bud. Note that the anterior mesenchyme is more severely affected (arrowheads). (E) *Lmx-1b* expression is similar to wild type in the dorsal mesenchyme of the *En-1* mutant, and ectopic Wnt-7a in the ventral ectoderm has induced an additional ectopic domain of *Lmx-1b* expression in the ventral mesenchyme (arrow in F). (G) *Lmx-1b* expression in the dorsal mesenchyme of a *Wnt-7a/En-1* double mutant. Notice that *Lmx-1b* expression is absent in the same regions as in the *Wnt-7a* mutant (C), particularly the distal anterior mesenchyme (arrowheads). (H) In the absence of Wnt-7a activity in the ventral ectoderm, *Lmx-1b* is not expressed in the ventral mesenchyme of the double mutant. For dorsal views, anterior is on top, proximal is to the left; for lateral views, dorsal is up, proximal is to the left.
these digits, indicative of ectopic footpad development (arrows in Fig. 3J). These transformations localize to the digits in which \textit{Lmx-1b} expression is absent in \textit{Wnt-7a} mutants (compare Fig. 3I and J). On the ventral surface of the \textit{En-1} mutant, \textit{Wnt-10b} is ectopically expressed in the proximal ventral epithelium of digits 1-4 (closed arrowheads in Fig. 3H), the same digits in which \textit{Lmx-1b} is ectopically expressed in the ventral mesenchyme in these mutants (compare Fig. 3G and H). In the double mutant, the changes in \textit{Wnt-10b} expression reflect the more severe loss of \textit{Lmx-1b} expression (compare Fig. 3K and L). A large region of the dorsal epithelium, including digits 2, 3, part of 4 and a large region at the base of these digits, lacks \textit{Wnt-10b} expression, with the exception of highly expressing patches indicative of ectopic footpad development (arrows in Fig. 3L).

Two other genes were examined that have distinct D-V domains of expression at 14.5 dpc: \textit{Fz-1}, a putative Wnt receptor (Wang et al., 1996; Bhanot et al., 1996), normally expressed in the mesenchyme of the developing footpads (data not shown), and \textit{Lef-1} (Travis et al., 1991), a transcriptional target of \textit{Wnt-10b} signaling (H. Dassule and A.P.M., unpublished data), which is expressed in the epithelium of the hair follicles (Zhou et al., 1995), and the epithelium and mesenchyme of the footpads (data not shown). The expression pattern of both of these genes changed in the mutants in a manner similar to that observed for \textit{Wnt-10b} (data not shown). Thus, there is a strong correlation between changes in \textit{Lmx-1b} expression and altered expression of D-V patterning markers at 14.5 dpc.

**Adult D-V phenotypes correlate with \textit{Lmx-1b} distribution**

The \textit{Wnt-7a} mutant adult forelimb displays D-V patterning defects that primarily localize to the autopod, the region of the forelimb in which \textit{Lmx-1b} transcripts are lost. We conducted a statistical analysis to rigorously examine the localization of D-V patterning alterations in the mutant adults and to determine if there is a correlation between \textit{Lmx-1b} misexpression at 14.5 dpc and the localization of patterning defects in the adult. We scored each digit of the \textit{Wnt-7a} mutant autopod for striations in the distal epidermis and the appearance of an ectopic footpad, both of which are ventral features (Table 1). Footpads are present at the base of digits 2 and 3 with a very high frequency, while they rarely appear at the base of digits 4 or 5. Striations also appear more frequently on anterior digits 2 and 3, though they are occasionally observed on a portion of digit 4 or 5. Often the series of striations are present on only the posterior half of a digit (data not shown), which may correlate to a border between \textit{Lmx-1b}-expressing cells and cells not expressing \textit{Lmx-1b} within a digit (see digit 3 in Fig. 3I). To analyze the \textit{En-1} adult limb phenotype we were able to rescue the \textit{En-1} perinatal brain lethality by expressing \textit{En-1} in the midbrain using a \textit{WEXPZ-En-1} transgene (Danielian and McMahon, 1996). We found that ectopic ventral \textit{Lmx-1b} expression at 14.5 dpc correlates with the appearance of ventral-to-dorsal transformations. All seven of the animals examined had ectopic hair growth on the ventral side of anterior digits. Surprisingly, we observed ectopic dorsal footpads at the distal tip of digits 2 and 3 in three of the seven
Table 1. Percentage of Wnt-7a<sup>−/−</sup> adults displaying dorsal-ventral patterning defects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Digit 2</th>
<th>Digit 3</th>
<th>Digit 4</th>
<th>Digit 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectopic dorsal footpad at the base</td>
<td>100</td>
<td>94</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>of the digit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectopic dorsal footpad at the distal</td>
<td>97</td>
<td>80</td>
<td>11</td>
<td>60</td>
</tr>
<tr>
<td>tip of the digit</td>
<td></td>
<td>(entire)</td>
<td>(entire)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at the distal tip of the digit</td>
<td>14</td>
<td>14</td>
<td>46</td>
<td>(posterior)</td>
</tr>
</tbody>
</table>

A total of 35 animals were killed at 8 weeks of age and scored under a dissecting microscope.

animals. This is consistent with the loss of Lmx-1b expression that is sometimes observed at the tip of these digits at 14.5 dpc (arrows in Fig. 3E). Though there is some variability in Lmx-1b expression among mutant embryos and in the resulting adult phenotypes, there is a consistent correlation between areas of Lmx-1b misexpression and the perturbation of normal D-V patterning in the limb.

Wnt-7a/En-1 double mutant adults have a D-V phenotype similar to Wnt-7a mutants, and reveal a new role for En-1 in D-V patterning

Based on Lmx-1b expression in the developing limb of double mutant embryos, we predicted that double mutant adults would have a similar phenotype to Wnt-7a mutants, that is, distal limb elements would be ventralized due to the loss of Lmx-1b expression, but ventral structures would be unaffected. To determine if this is the case, we generated Wnt-7a/En-1 double mutants to determine if there is a correlation between the D-V phenotype and Lmx-1b expression. Four Wnt-7a<sup>−/−</sup>; En-1<sup>−/−</sup>; WEXPZ-En-1* animals were generated from a total of 207 offspring, a frequency lower than the 1/32 expected. The viability of the double mutants ranged from 3 weeks post partum to animals that are healthy at 4 months. Fig. 4 shows a double mutant forelimb (Fig. 4J,K) in comparison with wild type and single mutants of the same age. The dorsal surface displays ventral characteristics similar to those observed in Wnt-7a mutants, including ectopic footpads at the base of digits 2, 3 and 4, and striations at the distal tip of digits 2, 3 and 5 (Fig. 4J). Other double mutant animals lack digit 5, and all the remaining digits have darkly pigmented dorsal striations and ectopic dorsal footpads (data not shown).

The ventral autopod of the double mutant appears similar to wild type in the distribution of footpads and the absence of hair, but the epidermis is deeply pigmented and the footpads have hardened. This ventral pigmentation is also observed in the En-1 mutant, but not in wild type or Wnt-7a mutant forelimbs (Fig. 4B,E,H,K). Thus, it appears that En-1 is required to repress pigmentation in the ventral epithelium, and this does not involve Wnt-7a. Consistent with this, digits of the Wnt-7a and double mutant that lack dorsal Lmx-1b expression and display ventral characteristics are still pigmented normally (Fig. 4D,J).

To determine if the D-V patterning of mesenchymally derived structures in the double mutant was consistent with that of the overlying epidermis, we performed a skeletal analysis of mutant forelimbs at 3 weeks post partum. Digit 3 was examined for the appearance of sesamoid processes, ventrally located bones that serve to attach tendons to the digit (open arrows in Fig. 4C). In the Wnt-7a mutant these processes are duplicated on the dorsal side, indicating that the dorsal mesenchyme is ventralized (Parr and McMahon, 1995; arrows in Fig. 4F). En-1 mutants lack the normal ventral sesamoid processes, displaying a double-dorsal character (Loomis et al., 1996; Fig. 4I), whereas the double mutant has ectopic dorsal sesamoid processes (arrows in Fig. 4L), indicating that dorsal mesenchymally derived structures are ventralized. In all three genotypes the D-V character of the mesenchymally derived structures reflects the predicted Lmx-1b distribution. The same is true of epithelially derived structures, with the exception of the ventral pigmentation of the double mutant, which is a dorsal characteristic that appears in the ventrally epithelial independent of Wnt-7a and Lmx-1b. Thus, we conclude that En-1 has two functions in D-V patterning. The first is to regulate the localization of Wnt-7a/Lmx-1b, since the bidorsal character of the En-1 mutant autopod can be explained by the ectopic ventral activation of Wnt-7a/Lmx-1b. The second is to repress melanocyte migration, proliferation or maturation in the ventral ectoderm, to prevent pigmentation of the ventral epidermis.

The P-D patterning phenotype of En-1 mutants requires Wnt-7a function

Previous studies have indicated a role for En-1 in P-D patterning of the limb (Loomis et al., 1996; Laufer et al., 1997; Logan et al., 1997; Rodriguez-Esteban et al., 1997). However, Wnt-7a does not appear to be required for P-D patterning (Parr and McMahon, 1995). Thus, it is unclear whether changes in P-D patterning observed in En-1 mutants are dependent on Wnt-7a activity. One aspect of the En-1 mutant phenotype is the appearance, at a low frequency, of ectopic digits originating from the anterior side of the ventral autopod (Loomis et al., 1996). We did not observe these ventral outgrowths in viable Wnt-7a/En-1 double mutants; however, since we were not able to look at a large number of such mutants we cannot be certain that these outgrowths are always suppressed in the double mutant.

To address this issue more thoroughly we analyzed the expression of the AER marker Fgf-8 at 11.5 dpc. Fgf-8 expression is similar in the AER of wild-type and Wnt-7a mutant embryos, indicating that Wnt-7a is not necessary to initiate or maintain the normal AER (Parr and McMahon, 1995; Fig. 5A,B). However, in 1 of 12 Wnt-7a mutant limb buds examined, there is a patch of ectopic Fgf-8 expression in the dorsal ectoderm (arrow in Fig. 5B; Fig. 5E). Strikingly, in all of the En-1 mutant limb buds examined, there is a second distinct ridge of Fgf-8-expressing cells in the ventral ectoderm that has the morphology of an AER (Fig. 5C,F). This ectopic ridge of cells originates at the anterior end of the endogenous AER and often terminates at approximately the midline of the A-P axis. In addition, there is often an additional patch or patches of Fgf-8-expressing cells proximal to this ectopic ventral ridge (arrows in Fig. 5C). Previous analysis of Fgf-8 expression in En-1 mutants was performed at 10.5 dpc and revealed a ventral expansion of Fgf-8 expression, but not two separate ridges of Fgf-8 expressing cells (Lyons et al., 1996). To confirm that other genes which are expressed in the endogenous AER are also expressed in a distinct ridge of cells in the ventral ectoderm at 11.5 dpc in En-1 mutants, we analyzed the expression of two other AER markers, Bmp-2 (Lyons et al., 1990) and Wnt-10b (H. Dassule and A.P.M., unpublished data). At 11.5 dpc, in addition to expression in the endogenous AER, both Bmp-2 (data not shown) and Wnt-10b (Fig. 5G) are
expressed in a second distinct ridge of cells in the ventral ectoderm. In contrast, the Wnt-7a/En-1 double mutant embryos, like the Wnt-7a mutants, display a single ridge of cells expressing Fgf-8 (Fig. 5D) and Wnt-10b (data not shown). These results suggest that by 11.5 dpc, there is a distinct ectopic AER in the ventral ectoderm of En-1 mutants, and that Wnt-7a is required for the formation of this second, ventral AER.

To address whether Wnt-7a signaling is required for the dorsal character of ventral ectoderm in En-1 mutants, we performed whole mount in situ hybridization with a Wnt-7a probe, then sectioned the forelimbs of wild-type, En-1 mutant and double mutant embryos. Ectopic Wnt-7a transcripts are detected in the ventral ectoderm of both En-1 mutant and Wnt-7a/En-1 double mutant embryos (Fig. 5E-G). Thus, in both the En-1 and Wnt-7a/En-1 mutants, the ventral ectoderm is dorsalized, but in the absence of a Wnt-7a signal in the latter a secondary AER is not formed.

Lmx-1b is related to the Drosophila gene apterous (Cohen et al., 1992), which is involved in specifying the Drosophila wing margin, which may be the equivalent of the AER in the Drosophila wing (Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Irvine and Wieschaus, 1994; Williams et al., 1994; Kim et al., 1995). The wing imaginal disc is a single cell-layer epithelial sheet, in which Apterous functions in a cell-autonomous manner. A boundary of apt+lap− clones establishes a new wing margin, which is often characterized by local outgrowths (Diaz-Benjumea and Cohen, 1993). If Lmx-1b, expressed in the mesenchyme, is involved in establishing the position of the AER in the vertebrate limb bud, it would have to function indirectly, by activating a molecule capable of signaling to the overlying ectoderm. Lmx-1b transcripts are present in the ventral mesenchyme of En-1 mutant, but not in double mutant, embryos (Fig. 2F,H). To determine if an Lmx-1b expression boundary is associated with ectopic AER formation, we performed double-label whole-mount in situ hybridization with Lmx-1b (purple) and Fgf-8 (orange) probes. In the Wnt-7a/En-1 double mutant limb that do not express Lmx-1b during development are ventralized in the adult limb. Wnt-7a, presumably through the regulation of Lmx-1b during development, is required for the dorsalized phenotype in the distal limb of En-1 mutants. These results are in good agreement with Lmx-1b expression experiments in the chick, which demonstrate that Lmx-1 can dorsalize the distal ventral limb (Riddle et al., 1995; Vogel et al., 1995), and that the default state of the limb is ventral, i.e. there is an absence of Lmx-1 expression in the mesenchyme.

Lmx-1b is also expressed in the dorsal mesenchyme of the proximal limb, where Wnt-7a signal is not necessary to activate or maintain Lmx-1b expression. Furthermore, in the chick, Lmx-1 is expressed throughout the dorsal mesenchyme, and in proximal regions the maintenance of Lmx-1 expression appears to be independent of ectodermal signals (Riddle et al., 1995; Vogel et al., 1995). These results raise the question of how proximal Lmx-1b expression may be regulated. Recent evidence indicates that the somitic mesoderm may produce a
factor that dorsalisizes the early limb bud (Michaud et al., 1997). Such a signal may act directly on the limb mesenchyme, or indirectly by first dorsalisizing the ectoderm. The activation of Wnt-7a expression in presumptive limb ectoderm is concurrent with the activation of Lmx-1 expression in limb mesenchyme (Riddle et al., 1995), suggesting that one signal may be responsible for dorsalisizing both cell layers. Whatever the mechanism of early Lmx-1b activation, its expression in the proximal limb suggests that Lmx-1b may play a more substantial role in regulating dorsal fates along the entire P-D axis of the limb, which will require the generation of Lmx-1b null mutants to determine.

Although only the distal dorsal mesenchyme requires Wnt-7a to express Lmx-1b, we observe that Lmx-1b can be ectopically activated by Wnt-7a in both proximal and distal ventral mesenchyme of En-1 mutants (Fig. 2F). This would indicate that ventral mesenchyme is competent to respond to dorsalisizing signals at proximal levels where the equivalent dorsal mesenchyme does not actually require Wnt-7a activity. There is further evidence to this effect from ectoderm rotation experiments in the chick. Following ectoderm rotation, the tendon pattern is normal in the most proximal region, and reversed in the most distal region of the resulting limb. There is a medial region, however, which displays a bidorsal tendon pattern irrespective of the D-V character of the ectoderm itself. Using expression of Lmx-1b as a marker of dorsal character in the ectoderm, the dorsal ectoderm of the Wnt-7a mutant, and both the dorsal and ventral ectoderm of the En-1 and Wnt-7a/En-1 double mutants are of dorsal character. This ‘dorsal’ ectoderm can be induced by ‘ventral’ (non-Lmx-1b-expressing) mesenchyme to form ventral structures such as ectopic footpads and striated epidermis in Wnt-7a mutants, whereas hair development is suppressed. Similarly, in Wnt-7a/En-1 double mutants, the ‘dorsalized’ ventral ectoderm gives normal ventral derivatives. Thus, the identity of ectodermal structures is determined by the underlying mesenchyme, as is apparent.

Although ectopic Lmx-1b expression in the proximal ventral mesenchyme of En-1 mutants appears widespread early on, expression eventually refines to only the anterior digits. There is a similar anterior bias to the loss of Lmx-1b expression in Wnt-7a mutants. Though there are signaling molecules that are expressed specifically in the posterior mesenchyme, there is no evidence that they regulate Lmx-1b expression. Shh, for example, is expressed in the ZPA at the posterior margin of the limb bud (Echelard et al., 1993; Riddle et al., 1993). Although expression of Lmx-1b persists in posterior regions of the distal limb bud in the absence of Wnt-7a, Lmx-1b expression is absent where Shh expression levels are highest (compare Figs 2C and 6B). Possibly there are other A-P specific determinants that have not been identified that are involved in Lmx-1b regulation. Alternatively, the anterior mesenchyme may be more sensitive to Wnt-7a signals. At this time we cannot account for the biased A-P distribution of Lmx-1b expression in the mesenchyme of Wnt-7a and En-1 mutants.

Our analysis indicates that the mesenchyme can induce dorsal- or ventral-specific structures in the overlying ectoderm, irrespective of the D-V character of the ectoderm itself. Using expression of Wnt-7a RNA as a marker of dorsal character in the ectoderm, the dorsal ectoderm of the Wnt-7a mutant, and both the dorsal and ventral ectoderm of the En-1 and Wnt-7a/En-1 double mutants are of dorsal character. This ‘dorsal’ ectoderm can be induced by ‘ventral’ (non-Lmx-1b-expressing) mesenchyme to form ventral structures such as ectopic footpads and striated epidermis in Wnt-7a mutants, whereas hair development is suppressed. Similarly, in Wnt-7a/En-1 double mutants, the ‘dorsalized’ ventral ectoderm gives normal ventral derivatives. Thus, the identity of ectodermal structures is determined by the underlying mesenchyme, as is apparent.

Fig. 4. Dorsalization of the ventral En-1 mutant limb requires Wnt-7a. (A,B,D,E,G,H,J,K) Wild-type and mutant autopods at 3 weeks post partum. The dorsal surface of the En-1 mutant (G) appears similar to wild type (A); however, the Wnt-7a mutant (D) and the Wnt-7a/En-1 double mutant (J) have ectopic footpads and striations. Note that the striations are pigmented. The ventral surface of the Wnt-7a mutant (E) is similar to wild type (B); however, the En-1 mutant has ectopic hair growth (H), and both the En-1 mutant (H) and Wnt-7a/En-1 double mutant (K) have pigmented footpads. The double mutant also has pigmented striations (K), but the striations are absent in the En-1 mutant (H). Note that digits 2 and 3 are fused in the En-1 mutant (H). For dorsal views anterior is up; for ventral views posterior is up. (C,F,I,L) Skeletal analysis of digit 3 from the same posterior is up. (C,F,I,L) Skeletal analysis of digit 3 from the same posterior is up. (C,F,I,L) Skeletal analysis of digit 3 from the same posterior is up. (C,F,I,L) Skeletal analysis of digit 3 from the same posterior is up.
5029Limb patterning in Wnt-7a and En-1 mutants (Saunders et al., 1957). We would predict that D-V specific mesenchymal signals may regulate these events.

Pigmentation of the epidermis, in contrast, does not appear to be influenced by the D-V character of the underlying mesenchyme, but rather by the D-V character of the ectoderm itself. In the normal limb, pigment is associated with the dorsal autopod, and remains dorsal in Wnt-7a mutants (Parr and McMahon, 1995; Fig. 4E), but pigment is also present ventrally in both En-1 and Wnt-7a/En-1 double mutants (Loomis et al., 1996; Fig. 4H,K). There are at least two possible explanations for the observed D-V distribution of pigmentation. En-1 activity may repress melanocyte migration, proliferation or maturation in the ventral ectoderm. This repression would be alleviated in the ventral ectoderm of En-1 and Wnt-7a/En-1 double mutants, resulting in pigmentation in the ventral ectoderm (arrow).

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epidermis. Alternatively, ectoderm of dorsal character may promote melanocyte development, which would account for the pigmentation of dorsal epidermis in Wnt-7a mutants, and the presence of ventral pigmentation in both En-1 and Wnt-7a/En-1 double mutants. In either case, correct pigment development is independent of Wnt-7a. Thus, as a result of the double mutant analysis, it is possible to separate D-V characteristics in the ectoderm that are regulated by the ectoderm from those regulated by the underlying mesenchyme.

**Axial interactions in limb patterning**

Recent evidence indicates that reciprocal interactions amongst the limb axes regulate the final pattern. For example, expression of Shh, a posteriorizing signal, depends on Fgf signaling by the AER, and Shh itself is required to maintain AER activity (Laufer et al., 1994; Niswander et al., 1994). Furthermore, Wnt-7a has been shown to regulate Shh (Parr and McMahon, 1995; Yang and Niswander, 1995), and En-1 appears to repress the expression of both Wnt-7a and Fgf-8, directly or indirectly (Loomis et al., 1996; Logan et al., 1997). Interestingly, in En-1 mutants there are a number of changes in the expression of both AER and ZPA activities, and these appear to depend on Wnt-7a signaling.

One surprising observation is the appearance of a second distinct ridge of cells that express AER markers in the ventral ectoderm of En-1 mutants at 11.5 dpc. Previous analysis at 10.5 dpc revealed an expansion of the AER markers Fgf-8 and Bmp-2 into the ventral ectoderm, but not two distinct expression domains (Loomis et al., 1996). Though we observe two ridges of cells expressing AER markers, they do not possess the characteristic morphology of the AER. Both the endogenous and ectopic 'ridges' appear flattened. It is possible that the entire region between these 'ridges' is a single broadened AER, and in fact the AER marker genes are now only expressed at the dorsal and ventral boundaries of this territory.

Our analysis of En-1 mutants indicates that Wnt-7a is required for the formation of a second AER in the ventral ectoderm. Expression of the AER marker Fgf-8 is induced in the ventral ectoderm of En-1 mutants precisely at the boundary between Wnt-7a-expressing and nonexpressing cells. Further, this ectopic AER is lost in Wnt-7a/En-1 double mutants. Thus, secondary AER formation is dependent upon Wnt-7a signaling. Whether it is the boundary of Wnt-7a expression in the ectoderm, or the resulting boundary of Lmx-1b-expressing and nonexpressing cells in the mesenchyme, that is required for ectopic AER formation remains to be determined. The argument that Lmx-1b could regulate the formation of the AER is appealing, since Lmx-1b is related to Drosophila apterous, which is involved in establishing the wing margin of the Drosophila wing imaginal disc (Diaz-Benjumea and Cohen, 1993; Irvine and Wieschaus, 1994). However, the Drosophila imaginal disc is a single cell-layer epithelial sheet, while the vertebrate limb bud is composed of two distinct cell layers. Lmx-1b could not regulate AER formation in a cell-autonomous manner, as Apterous does in the Drosophila imaginal disc, but would rather have to activate a secondary signal to relay its identity to the overlying ectoderm. In addition, our observation that, in the dorsal limb of Wnt-7a mutants, a new boundary of Lmx-1b-expressing and nonexpressing mesenchyme occurs between proximal and distal regions and no AER is formed also argues against Lmx-1b being involved in AER formation. In one instance we observed a small patch of Fgf-8 expression in the dorsal ectoderm of a Wnt-7a mutant limb bud, though a complete AER was never observed. This may be an issue of timing, since this Lmx-1b expression boundary does not form until almost 11.5 dpc, when the mesenchyme may no longer be able to support an ectopic AER. However, it seems unlikely that it is the juxtaposition of these mesenchymal cells which is important.

Interestingly, expression of Lmx-1b is patchy in the ventral mesenchyme of En-1 mutants when it is first observed (Fig. 5H). This presumably reflects uneven activation of Wnt-7a in the overlying ectoderm. We observed that early in the formation of the ectopic AER, there is a similar patchy expression of Fgf-8 induced in the ventral ectoderm. Although it has not been determined, the induction of Fgf-8 may be the result of the formation of a boundary of Wnt-7a-expressing and nonexpressing cells in the ectoderm, or Lmx-1b-expressing and nonexpressing cells in the mesenchyme. By 11.5 dpc, expression of Wnt-7a is uniform in the ventral ectoderm, and both the Wnt-7a+/Wnt-7a– and Lmx-1b+/Lmx-1b– boundaries demarcate the secondary AER. Although neither a Wnt-7a nor an Lmx-1b boundary is necessary to maintain the endogenous AER, it is possible that either or both of these boundaries are requisite for the genetic program that results in ectopic AER formation in the ventral ectoderm.

Experiments in the chick are consistent with AER formation being dependent on the expression of Radial fringe (R-fng) (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). An AER forms at the boundary between R-fng-expressing and nonexpressing cells, and since R-fng is normally expressed in the dorsal ectoderm, the primary AER forms at the D-V boundary. Ectopic expression of R-fng in the ventral ectoderm results in the formation of ectopic ventral AERs, independent of Wnt-7a or Lmx-1 (Rodriguez-Esteban et al., 1997; C. Tabin, personal communication). En-1 represses R-fng expression in the ventral ectoderm, and expression of En-1 in the dorsal ectoderm leads to ectopic dorsal AER formation (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Although En-1 also represses Wnt-7a (Logan et al., 1997), the repression of Wnt-7a is not involved in dorsal AER formation, since in the eudiplopodia mutant, ectopic AERs form in the dorsal ectoderm, while Wnt-7a expression is unaffected (Laufer et al., 1997). Thus, experiments in the chick would argue that Wnt-7a is not required for secondary AER formation.

It remains to be determined if a similar mechanism is responsible for secondary AER formation in the mouse limb. If so, the loss of En-1 activity should result in ectopic R-fng in the ventral ectoderm, and the formation of an ectopic ventral AER would require a new R-fng boundary in the ventral ectoderm. Surprisingly, none of the three published fng genes that have been identified in the mouse are expressed in the limb (Johnston et al., 1997). However, even if there is a mouse Fng that performs a similar function to chick R-fng, unlike the chick, Wnt-7a activity is required to form an ectopic ventral AER in the mouse.

We also observed that expansion of Shh expression into the ventral mesenchyme of En-1 mutants requires Wnt-7a. Shh expression expands to the level of ectopic Fgf-8 expression in the ventral ectoderm, which is also a Wnt-7a+ boundary. Although Wnt-7a has been demonstrated to maintain Shh expression, it does not appear that Wnt-7a induces the
ectopic Shh expression, since the ectopic Shh expression is localized to mesenchyme underlying non-Wnt-7a-expressing ectoderm. Thus, it appears that the requirement for Wnt-7a in ectopic Shh expression is indirect, possibly through the ectopic production of Fgfs in the secondary AER. The additional levels of Shh activity in the distal limb may be a contributing factor to the syaddactyly or ectopic digits observed in En-1 mutants.

In summary, these studies have allowed the relationship between Wnt-7a, Lmx-1 and En-1 to be more precisely defined. It is now apparent that more complex mechanisms operate to regulate D-V patterning and AER formation than were previously appreciated. The anterior bias to the loss of Lmx-1b expression in the Wnt-7a mutant limb bud, the formation of a second AER in En-1 mutants and the requirement for Wnt-7a in this process are just some examples. Our results also raise a cautionary note as to whether the pathways that lead to secondary AER formation are really relevant to the normal mechanism that establishes the AER. Clearly, the identification of additional genes, and the genetic dissection of their involvement in axial patterning and AER formation, will provide a greater context for interpretation of these events.

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