

## ***Drosophila engrailed* can substitute for mouse *Engrailed1* function in mid-hindbrain, but not limb development**

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### SUMMARY

The *Engrailed-1* gene, *En1*, a murine homologue of the *Drosophila* homeobox gene *engrailed* (*en*), is required for midbrain and cerebellum development and dorsal/ventral patterning of the limbs. In *Drosophila*, *en* is involved in regulating a number of key patterning processes including segmentation of the epidermis. An important question is whether, during evolution, the biochemical properties of En proteins have been conserved, revealing a common underlying molecular mechanism to their diverse developmental activities. To address this question, we have replaced the coding sequences of *En1* with *Drosophila en*. Mice expressing *Drosophila en* in place of *En1* have a near complete rescue of the lethal *En1* mutant brain defect and most skeletal abnormalities. In contrast, expression of *Drosophila en* in the embryonic limbs of *En1*

mutants does not lead to repression of *Wnt7a* in the embryonic ventral ectoderm or full rescue of the embryonic dorsal/ventral patterning defects. Furthermore, neither *En2* nor *en* rescue the postnatal limb abnormalities that develop in rare *En1* null mutants that survive. These studies demonstrate that the biochemical activity utilized in mouse to mediate brain development has been retained by Engrailed proteins across the phyla, and indicate that during evolution vertebrate En proteins have acquired two unique functions during embryonic and postnatal limb development and that only *En1* can function postnatally.

Key words: *engrailed*, *En1*, Limb, Mid/hindbrain, Development, Transcription factor, Mouse, *Drosophila*

### INTRODUCTION

There is ample evidence to demonstrate that developmentally crucial genes have been conserved across evolution. Indeed, since the demonstration that the homeobox HOM-C and vertebrate HOX gene clusters were conserved across the phyla, isolating homologues from different species has been an effective strategy in isolating key developmental genes (Rossant and Joyner, 1989). Furthermore, components of genetically defined pathways have, in many instances, also been conserved and some striking molecular parallels exist between diverse developmental processes from very different organisms. A challenge now is to determine what aspects of the biochemical functions of genes have diverged, allowing for the evolution of new species.

One of the best-studied genetic pathways controlling *Drosophila* segment development involves the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*). These genes are essential for establishing the anterior/posterior polarity of the segments (Ingham and Martinez-Arias, 1992). *En*- and *wg*-

expressing cells lie adjacent to each other in the cellular blastoderm, spanning the parasegment boundary, and expression of these two genes is mutually exclusive, such that cells expressing *en* do not express *wg* and vice versa. The *paired* gene is involved in initiating expression of both genes. However, after their initial induction, for a period of time, expression of *en* is required for continued *wg* expression in adjacent cells as well as the converse (Perrimon, 1994). Many of the genetic components involved in this pathway have been identified and placed in a regulatory hierarchy. Components of the *wg* signaling pathway include *frizzled*, *disheveled*, *armadillo* and *APC* (Moon et al., 1997), whereas *en* represses *ci* expression in the posterior cell, allowing for hedgehog signaling to the adjacent *wg*-expressing cell (Eaton and Kornberg, 1990; Schwartz et al., 1995).

All of the genes known to be components of the *en-wg* pathway are conserved in vertebrates, although the situation is considerably more complex, as many of the ancestral genes have undergone duplications to generate larger gene families (Joyner, 1996). Evidence has emerged that many genes of this conserved

en-wg pathway operate in controlling the development of the vertebrate midbrain and cerebellum as well as the limb, although it is not clear to what extent the epistatic relationships have been retained from fly to mouse (Joyner, 1996; Wassef and Joyner, 1997). There are some notable differences between the pathway in the vertebrate midbrain/cerebellum when compared to segmentation in the *Drosophila* cellular blastoderm. For example, although expression of *En* and *Wnt-1* in the anterior neural folds appear to be dependent on each other (McMahon et al., 1992; W. Wurst and A. L. Joyner, unpublished), in this case both genes are expressed by the same cells rather than adjacent cells. In addition, a *Hedgehog* pathway does not seem to be required for anterior-posterior patterning of this region of the brain. In the developing limb, on the other hand, *En1* and *Wnt7a* are expressed in the ventral and dorsal ectoderm, respectively, and one role of *En1* appears to be to repress *Wnt7a* expression (Loomis et al., 1996, 1998; Logan et al., 1997; Cygan et al., 1997). These observations present something of a paradox, since components of the en-wg pathway are conserved across species, but the rules governing the operation of the pathway do not hold true, even within the same organism. Thus it is likely that there are numerous different pathways involving the *En* genes, and the diverse pathways probably utilize different protein-protein and/or protein-DNA interactions.

A key question in understanding the evolution of the *En* genetic pathways to allow participation in diverse developmental processes, is whether the biochemical activities of En and other protein components have been conserved. Previously, we showed that all en proteins contain five conserved regions (EH1-EH5), including the homeodomain, and their existence implies a degree of functional conservation (Logan et al., 1992). Some degree of functional activity has been shown for four of these domains: the homeodomain (EH4) mediates DNA binding (Desplan et al., 1988), and EH2 interacts with *exd* and *Pbx* homeodomain proteins to increase DNA-binding affinity and specificity (Peltenburg and Murre, 1996; Pfeifer and Wieschaus, 1990; van Dijk et al., 1995; van Dijk and Murre, 1994). The EH-1 and EH5 regions have been shown to have transcriptional repression activity (Han and Manley, 1993; Smith and Jaynes, 1996). EH-1 can bind to the corepressor *groucho* in vitro, and en repressor function in vivo has been shown to be *groucho*-dependent (Jimenez et al., 1997; Tolkunova et al., 1998). The non-conserved region C-terminal to EH-1 also appears to have a repressor activity, although this is more apparent in an in vitro assay than in vivo (Han and Manley, 1993; Smith and Jaynes, 1996). Interestingly, in an in vivo assay in flies, the activity of EH-1 from en can be replaced by EH-1 from mouse En1 (Smith and Jaynes, 1996).

It is not clear if all En proteins are capable of performing the same repertoire of activities in vivo. *En1* and *En2* are both expressed in the developing midbrain and anterior hindbrain from early embryonic stages, except that *En1* is expressed half a day earlier than *En2* (Davis et al., 1988, 1991). Despite their similar expression patterns, *En1* null mutants die at birth and lack most midbrain and cerebellar structures, whereas *En2* mutants are viable and have only mild cerebellar defects (Joyner et al., 1991; Millen et al., 1994; Wurst et al., 1994). *En1* mutants also have defects in tissues that do not express *En2*, such as the axial skeleton and presence of double dorsal paws (Loomis et al., 1996). Previously, we showed that En2 can substitute for En1 in mouse brain and axial skeleton development, as well as

embryonic patterning of the limb by gene replacement in vivo (Hanks et al., 1995). The study indicated, however, that *En2* could not rescue the requirement for *En1* in development of paw skin structures (Loomis et al., 1996). These results demonstrated that the differences in function between *En1* and *En2* in mouse, which are revealed by analysis of null mutants, are in the main part due to divergent embryonic expression patterns, rather than to differing biochemical capabilities of the two mouse En proteins. However, cell type-dependent differences in their biochemical activities could not be excluded. To extend this approach and to determine to what extent biochemical function of en has been retained across the phyla, we have compared the activities of the *Drosophila* en protein with those of En1 and En2 in transfection assays. These experiments suggest that in this limited cell system all en proteins mediate transcriptional repression. In a much more exacting test of functional conservation, we replaced mouse *En1* coding sequences with *Drosophila* en sequences by a gene-targeting knock-in strategy (Hanks et al., 1995). These studies demonstrate that *Drosophila* en can substitute for En1 during murine brain development, leading to a near complete rescue of the lethal *En1* null mutant brain phenotype. *Drosophila* en can also, for the most part, rescue the skeletal defects in *En1* mutants. Interestingly, en expression in the ventral ectoderm of the developing embryonic limb buds in place of *En1* does not fully rescue the embryonic dorsal-ventral limb defects since en can neither repress *Wnt7a* expression nor subsequent development of dorsal structures such as hairs and nails on the ventral digits. In addition, neither en nor *En2* can rescue the postnatal limb defects caused by loss of *En1* function.

## MATERIALS AND METHODS

### Generation of *Drosophila* en knock-in construct

The pKSloxPNT.enKI targeting vector was constructed as follows. The PGKNeo cassette in pKSNT was first replaced by a loxP-flanked PGKNeo cassette taken from pCCM7a.TK (a gift from Cecelia Moens) to generate pKSloxPNT (Hanks et al., 1995). Firstly, pCCM7a.TK was digested with *HindIII* and *SalI*, Klenow-blunted and self-ligated to remove unwanted polylinker sequences, to generate pLoxNeo. pKSNT was digested with *XbaI* and *HindIII*, blunted with Klenow and the 5 kb vector fragment was ligated to a 2 kb *XhoI*, Klenow-blunted fragment from pLoxNeo, which encoded the loxP-flanked PGKNeo cassette. This generated pKSloxPNT. Next, the 3' *En1* sequence was inserted into pKSloxPNT: a 4 kb *ClaI*, *SacI* *En1* fragment (the 3' arm) was cloned into *SalI*-digested, Klenow-blunted and *ClaI*-digested pKSloxPNT, to generate pKSloxPNT.En1.3'.

The entire *Drosophila* en coding sequence contained within a 2 kb *EcoRI* cDNA fragment (Poole et al., 1985) was then isolated and cloned into the *EcoRI* site of pBluescript KS+ to generate pKSen. A 4.2 kb *XbaI*, *ApaI* partial fragment of the *En1* 5' region was then ligated into *HindIII*-digested, Klenow-blunted, *XbaI*-digested pKSen to generate pKSen.En1.5'. This fused the en coding sequence to a 4.2 kb *En1* 5' fragment (the 5' homology arm) at a position approximately 90 bp upstream of the endogenous *En1* ATG.

Finally, the en coding sequence fused to the 5' *En1* arm was isolated on a 6.2 kb *XbaI*-, *ClaI*-digested, Klenow-blunted fragment, which was ligated to *EcoRI*-digested Klenow-blunted pKSloxPNT.En1.3', to generate the final knock-in vector pKSloxPNT.enKI. The en cDNA was kindly provided by Thomas Kornberg.

### ES cell culture and production of mice with knock-in mutant alleles

ES cell culture and Southern blot analysis were as described (Hanks et

al., 1995; Wurst et al., 1994). Germ line transmitting chimeric mice were generated from correctly targeted R1 ES cell lines (Nagy et al., 1993) by aggregation with CD1 morulae as described (Wood et al., 1993).

**Southwestern blotting**

GST fusion proteins with En1 and En2 were constructed as follows. A 1.1 kb *Clal*-digested, Klenow-blunted, *EcoRI*-digested En1 cDNA fragment was ligated to *BamHI*-digested, Klenow-blunted, *EcoRI*-digested pGEX-2T (Pharmacia) to produce pGST:En1. The *Clal* site is at amino acid 112 in the En1 protein. pGST:En2 was constructed by ligating a 750 bp *Clal*, *BglII*-digested, Klenow-blunted En2 cDNA fragment to *BamHI*-digested, Klenow-blunted pGEX-2T. The *Clal* site is at amino acid 68 in the En2 protein. Oligos used for southwestern DNA binding assays were: (TCAATTAATGA)<sub>4</sub>, derived from a defined *en* binding sequence (Desplan et al., 1988), and the mutated derivative, (TCAAGGAGGTGA)<sub>4</sub>, in which the essential A residues have been mutated to G (underlined).

Southwestern blots were performed as described (Staudt et al., 1988).

**Transfections, CAT and fl-Gal assays**

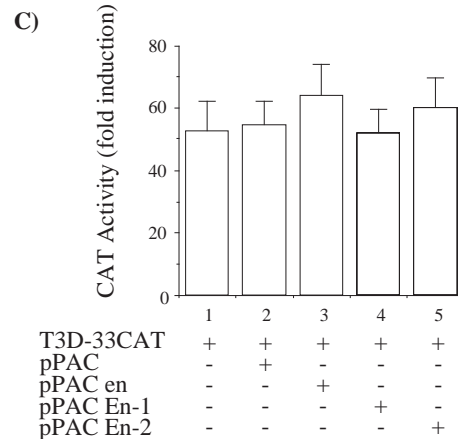
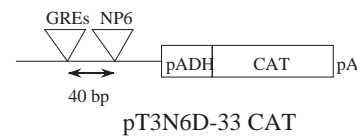
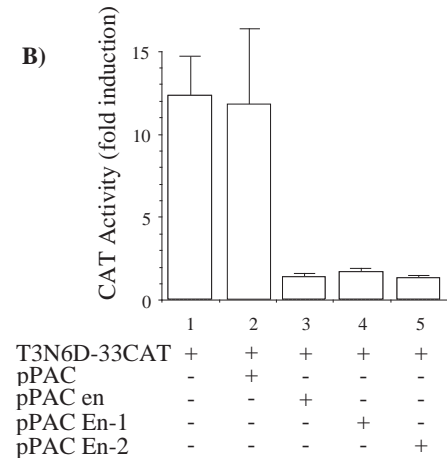
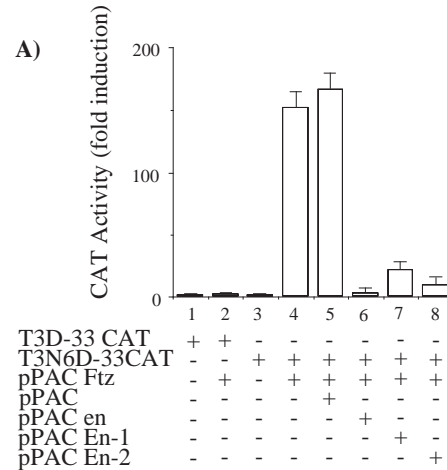
Transfections into SL2 cells (Schneider, 1972) were performed at least in triplicate and standardized using HSPLacZ cotransfection as an internal reference; CAT and fl-Gal assays were also performed as described (Jaynes and O'Farrell, 1988, 1991) pT3N6-D33CAT, pT3D-33CAT, pAC-*en* and pPAC (Krasnow et al., 1989) were kindly provided by Jim Jaynes. HSPLacZ was a gift from Vincent Figuere. pPACEn1 and pPACEn2 were constructed as follows: pPAC was digested with *BamHI* and blunted with Klenow. A 1.3 kb *EcoRI*-, *BglII*-digested, Klenow-blunted En2 cDNA fragment, containing the complete En2 coding sequence, was cloned into the blunted pPAC to generate pPACEn2. A 1.3 kb *Apal*-digested, T4 polymerase-blunted En1 cDNA fragment, containing the entire En1 coding sequence, was cloned into the blunted pPAC to generate pPACEn1. We noted that the levels of dexamethasone induction of GR-mediated CAT activity from T3D-33CAT and from T3N6D-33 CAT were consistently 10-fold less and 100-fold less, respectively, than those reported by Jaynes and O'Farrell (1991). To address this, we altered the amounts of CAT reporter constructs and GR producer plasmid in the transfection mixes, as well as the concentration of dexamethasone in the cell cultures (data not shown), but failed to achieve greater inductions than shown in Fig. 1. Jaynes and O'Farrell (1991) showed that *en* repressed GR-mediated transcriptional activation by a factor of between 4- and 10-fold in their

experiments and we observed a similar 10-fold repression activity in our assays. This suggests that, although we were unable to achieve the high levels of GR-mediated transcriptional activation of the CAT reporters used by Jaynes and O'Farrell (1991), our assays nevertheless recapitulated *en*-mediated transcriptional repression.

**In situ hybridizations and skeletal preparations**

<sup>35</sup>S- and digoxigenin-labeled RNA in situ hybridizations were

**Fig. 1.** En1, En2 and *en* are capable of active and passive transcriptional repression in SL2 cells. (A) En1, En2 and *en* passively repress Ftz-mediated transcription. Cotransfection of T3D-33CAT and pPAC Ftz resulted in an approximately 170-fold induction of the CAT reporter (lane 4) over basal levels (lanes 1 and 3). This was dependent on the presence of the NP6 homeodomain binding sequence, as Ftz had no effect on T3D-33CAT, which lacks NP6 (lane 2). Coexpression of *en* (lane 6), En1 (lane 7) or En2 (lane 8) repressed *ftz*-mediated transcriptional activation approximately 100-fold, 9-fold and 18-fold, respectively. However, equivalent amounts of empty pPAC vector had no effect on transcription (lane 5). (B) Active repression of GR-mediated transcriptional activation by En1, En2 and *en*. All samples were transfected with pPAC GR in addition to the vector pT3N6D-33CAT depicted below. Induction with dexamethasone consistently resulted in a 12-fold induction of T3N6-D33CAT (lane 1). Coexpression with pPAC *en*, pPAC En1 or pPAC En2 (lanes 3, 4 and 5), but not empty pPAC (lane 2) resulted in approximately 10-fold repression of GR-mediated transcription. (C) Repression was dependent on the presence of NP6, as the same transfection mixes resulted in a reproducible 50- to 60-fold induction of T3-D33CAT, but no repression was observed by coexpressing any of the En proteins.



performed on tissue sections and whole-mount mouse embryos as described (Hanks et al., 1995; Loomis et al., 1996). The probe to detect *Drosophila en* transcripts in situ was transcribed from a 2.1 kb *EcoRI* fragment from the *en* cDNA (Poole et al., 1985), subcloned into pBluescript KS. Mouse newborn skeletal preparations were prepared as described (Lufkin et al., 1992).

## RESULTS

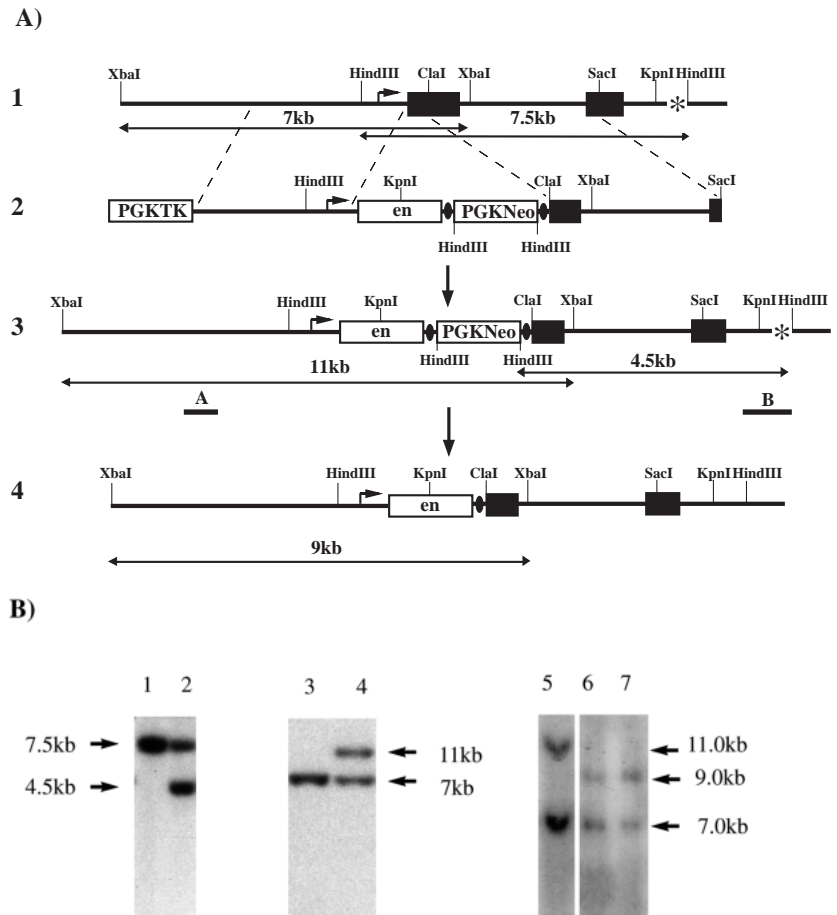
### Transcriptional repression by mouse En proteins in cultured cells

As a first approach to determine the degree of functional conservation between the *Drosophila en* and mouse En proteins, the DNA binding and transcriptional activities of the proteins were compared in vitro. Southwestern blotting was used to show that mouse En1 and 2-GST fusion proteins were capable of binding to a previously defined DNA target sequence for *en* (NP6), which consists of six tandem copies of the sequence TCAATTAATGA (Desplan et al., 1985, 1988; Hoey and Levine, 1988) (data not shown). The assays established by Jaynes and O'Farrell (1988, 1991) were modified to enable comparison of the En1 and En2 proteins with that of *en* in *Drosophila* SL2 cells. These authors had previously demonstrated, using transient transfection assays in SL2 cells, that *en* could passively repress ftz-mediated transcriptional activation of a CAT reporter construct carrying NP6. In addition, *en* was capable of repressing glucocorticoid receptor (GR)-mediated transcriptional activation from reporter constructs carrying NP6 located adjacent to binding sites for GR (GRE); this was termed active repression.

We performed similar experiments using the mouse En proteins. Cotransfection of the reporter construct T3N6D-33CAT and pPAC-Ftz resulted in an approximately 170-fold induction of the CAT reporter over basal levels (Fig. 1A). This was dependent on the presence of the NP6 homeodomain binding sequence, as Ftz had no effect on T3D-33CAT, which lacks NP6. Co-expression of *en*, En1 or En2 with ftz and T3N6D-33CAT repressed the ftz-mediated transcriptional activation approximately 100-fold, 9-fold and 18-fold, respectively (Fig. 1A). However, equivalent amounts of empty pPAC expression vector had no effect on transcription. Semi-quantitative western blots showed that similar amounts of ftz protein were present in lysates transfected with pPACftz (data not shown).

These data demonstrated that, at least qualitatively, mouse and *Drosophila* En proteins share the ability to passively repress ftz-mediated transcriptional activation.

*Drosophila en* protein was the most effective repressor, followed by mouse En2 and then En1. However, we have no accurate means of comparing the relative levels of these En proteins in the transfection lysates, so it is possible that in these assays, where repression is presumed to be a result of competition between ftz and competitor (En proteins) for their common binding site, these quantitative differences in ability to repress may reflect differing protein levels in transfected



**Fig. 2.** Generation of *En1<sup>dki</sup>* targeted ES cell lines. (A) Knock-in targeting strategy. Line 1: depiction of the *En1* genomic locus. Coding sequences are shown by solid boxes, the arrow shows the transcriptional start and the poly-adenylation signal is represented by the asterisk. Line 2: targeting vector. The selectable neomycin resistance (PGKneo), thymidine kinase (PGKTK) cassettes and the *en* coding sequence are shown. Elipses depict *LoxP* sites. The regions of homology between the genomic sequence and the targeting vector are shown by the dashed lines. Line 3: structure of the *En1<sup>dkin</sup>* targeted locus. *en* coding sequence has been integrated downstream of the *En1* promoter concomitant with a deletion of the sequences encoding the first 111 amino acids of *En1*. Line 4: structure of the *En1<sup>dki</sup>* locus after Cre-mediated removal of the PGKneo sequences from the *En1<sup>dkin</sup>* targeted locus. The 5' and 3' probes (A and B) used to identify the targeted cell lines are shown. (B) Southern blots of genomic DNA isolated from targeted ES cell lines (lanes 1-4) and from mouse tails (lanes 5-7). Lanes 1-2: DNA digested with *HindIII* and probed with B, giving a wild-type 7.5-kb fragment and *En1<sup>dkin</sup>*-targeted 4.5-kb fragment. Lanes 3-4: DNA digested with *XbaI* and probed with A, giving an endogenous 7-kb and targeted 11 kb bands for *En1<sup>dkin</sup>*-targeted cell lines. Lanes 1 and 3: wild-type cells. Lanes 2 and 4: *En1<sup>dkin</sup>*-targeted cell line. Lanes 5-7: tail DNA digested with *XbaI* and probed with A giving the endogenous 7-kb and targeted 11-kb bands as well as a 9-kb fragment, reflecting the excision of the PGKneo sequences by cre-recombinase from the targeted *En1<sup>dkin</sup>* allele to produce the *En1<sup>dki</sup>* allele. Lane 5: *En1<sup>dki/+</sup>* mice with the PGKneo sequences present; lanes 6-7: *En1<sup>dki/+</sup>* mice after excision of the PGKneo sequences.

cells. In support of this, semi-quantitative western blots, using a polyclonal antibody that recognizes both En1 and En2 (Davis et al., 1991), showed that En1 protein was always present in transfection lysates at reduced levels when compared to En2 (data not shown). However, this could reflect, at least in part, the specificity of the polyclonal antibody, which was raised against En2.

Jaynes and O'Farrell (1988) first showed that *en* was able to repress transcriptional activation mediated by the glucocorticoid receptor (GR), when bound to a site adjacent to the glucocorticoid response element (active repression). In similar assays, we found that En1 and En2 were also capable of actively repressing GR mediated transcription as effectively as *en* (Fig. 1B). Again, this effect was dependent on the presence of NP6, as no repression was observed in lysates using T3D-33CAT as the reporter (Fig. 1C).

### Replacement of mouse *En1* with *Drosophila en* by gene targeting

The most stringent test of conservation of protein function would be to determine whether substitution of a mouse En protein with *Drosophila en* in vivo can rescue the developmental effects of a loss-of-function mutation in a mouse *En* gene. We chose to rescue the *En1<sup>hd</sup>* loss of function mutation because *En1* is the first *En* gene expressed in the presumptive embryonic midbrain-cerebellum and the *En1* homozygous mutant brain phenotype is much more severe than that of the *En2<sup>hd</sup>* mutant phenotype (Millen et al., 1994; Wurst et al., 1994). In addition, *En1<sup>hd/hd</sup>* mutants show defects in limb and skeleton development, whereas the *En2<sup>hd/hd</sup>* phenotype is mild and restricted to the brain (Loomis et al., 1996; Wurst et al., 1994). Thus, rescuing the *En1<sup>hd</sup>*, rather than *En2<sup>hd</sup>*, loss-of-function mutation represents a broader test of functional conservation.

To test the ability of *Drosophila en* to substitute for *En1* in vivo, we used a gene targeting knock-in strategy (Hanks et al., 1995) in mouse embryonic stem (ES) cells to replace the coding sequences of mouse *En1* with those of *Drosophila en* (Fig. 2A). The first *En1* allele generated was designated *En1<sup>dki</sup>*, for En1 *Drosophila en* knock-in containing *neo*. One correctly targeted ES cell line (Fig. 2B) was used to generate chimeric mice giving germline transmission of the *En1<sup>dki</sup>* allele (see Materials and methods). Heterozygous *En1<sup>dki/+</sup>* animals were viable, fertile and in all respects appeared phenotypically normal. It has been found that in certain cases insertion of *neo* near a transcription unit can interfere with transcription of a neighboring gene (Olson et al., 1996). To avoid this, *neo* was removed from the *En1<sup>dki</sup>* allele by site specific recombination. Since *neo* was flanked by loxP sites, by breeding *En1<sup>dki/+</sup>* mice with transgenic mice expressing CRE from the CMV promoter (White et al., 1997), *neo* was deleted from the *En1<sup>dki</sup>* allele to produce the allele, *En1<sup>dki</sup>* (see Fig. 2).

### Removal of *neo* from the *En1<sup>dki</sup>* allele induces *En1*-like expression of *en*

Expression of the *Drosophila en* RNA transcripts from the *En1<sup>dki</sup>* and *En1<sup>dki</sup>* alleles was analyzed to determine whether it was similar to that of *En1*. Whole-mount RNA in situ hybridization using an antisense *Drosophila en* probe showed the presence of *en* transcripts in 8.0- to 10.5-day post coitus

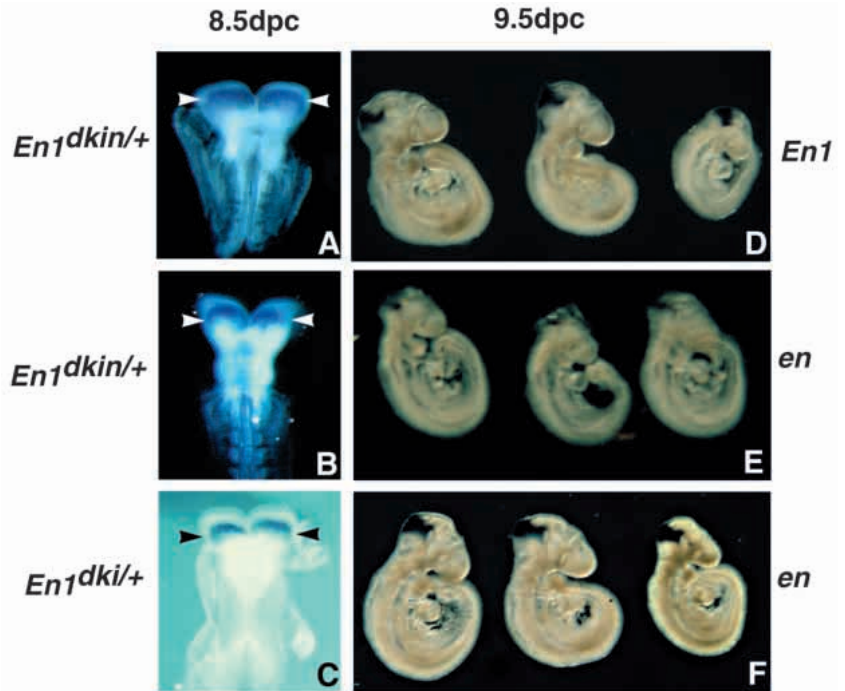
(dpc) *En1<sup>dki</sup>* and *En1<sup>dki</sup>* embryos. In *En1<sup>dki</sup>* embryos, *en* was expressed at high levels and in all tissues that normally express *En1*, including the brain, spinal cord, somites and limbs (Figs 3C,F and 4J,P). However, *en* expression in *En1<sup>dki</sup>* embryos, although restricted to the normal *En1* expression domains, was variable (Figs 3B,E and 4B,H); in the brains of *En1<sup>dki</sup>* embryos the width of the *en* expression domain was generally much narrower than that of the wild-type *En1* domain or that of the *en* domain in *En1<sup>dki</sup>* brains (Fig. 3E). Furthermore, *en* expression from the *En1<sup>dki</sup>* allele was often reduced in limb buds (Fig. 4B) and undetectable in the spinal cord and somites (Fig. 4H). Thus, the presence of a *neo* cassette in the first exon of the *En1<sup>dki</sup>* allele interferes with expression of the inserted *en* sequences, and removal of *neo* results in normal *En1*-like expression from the *En1<sup>dki</sup>* allele.

### *Drosophila en* can rescue *En1* mutant mid-hindbrain development

In contrast to *En1<sup>hd/hd</sup>* newborns, which generally never feed and die within 24 hours of birth due to deletion of midbrain and hindbrain structures, 90% of *En1<sup>dki/dki</sup>* homozygous mice (27/30) survived to weaning, although four of these died during the next 4 weeks. The animals that survived to adulthood did not show behavioral defects and many males and females were fertile. These results suggest that, if expressed in place of *En1*, *Drosophila en* can substantially rescue the brain defects of *En1* mutants. Given the incomplete expression of *en* from the *En1<sup>dki</sup>* allele, it is not surprising that survival of *En1<sup>dki/dki</sup>* animals was lower, with approximately 67% (10/15) of *En1<sup>dki/dki</sup>* pups surviving to 3-4 weeks of age, and 50% (7/15) to 8 weeks. Some *En1<sup>dki/dki</sup>* females were fertile (4/6), but no males (*n*=6) appeared fertile.

To examine the degree of morphological rescue of the *En1* mutant brain phenotype, whole-mount preparations and sagittal sections through newborn and adult brains from *En1<sup>dki/dki</sup>* and *En1<sup>dki/dki</sup>* animals were prepared (Fig. 5). All the adult (*n*=4) and newborn (*n*=5) *En1<sup>dki/dki</sup>* whole brains examined showed a near complete rescue of the *En1<sup>hd/hd</sup>* mutant phenotype, with a well-developed midbrain and cerebellum and all the cerebellar folds apparently present in adults (Fig. 5E,G). Sagittal sections through the brains of *En1<sup>dki/dki</sup>* newborns revealed that the midbrain and cerebellum appeared grossly normal (Fig. 5F), although in some animals the midbrain was slightly truncated and cerebellar folding slightly delayed. However, sagittal sections through adult *En1<sup>dki/dki</sup>* brains showed that there were subtle cerebellar foliation defects in some animals. One of four animals had a relatively normal foliation pattern (Fig. 5H), whereas another animal was missing most of the first anterior fold (Fig. 5O). This animal also had an alteration of the eighth fold that was similar to that seen in *En2* mutants (Joyner et al., 1991). The other two animals had a partial fusion of the anteriormost two folds and a partial posterior shift in the eighth fold (Fig. 5P).

As expected, *En1<sup>dki/dki</sup>* brains showed a variable rescue that correlated with the ability of *En1<sup>dki/dki</sup>* pups to feed and older animals to survive. In general, the brain rescue was less complete than that observed in *En1<sup>dki/dki</sup>* animals (Fig. 5I,K), and newborn brains exhibiting the weakest rescue were only marginally less affected than *En1<sup>hd/hd</sup>* mutants (not shown). Examination of sagittal sections through the

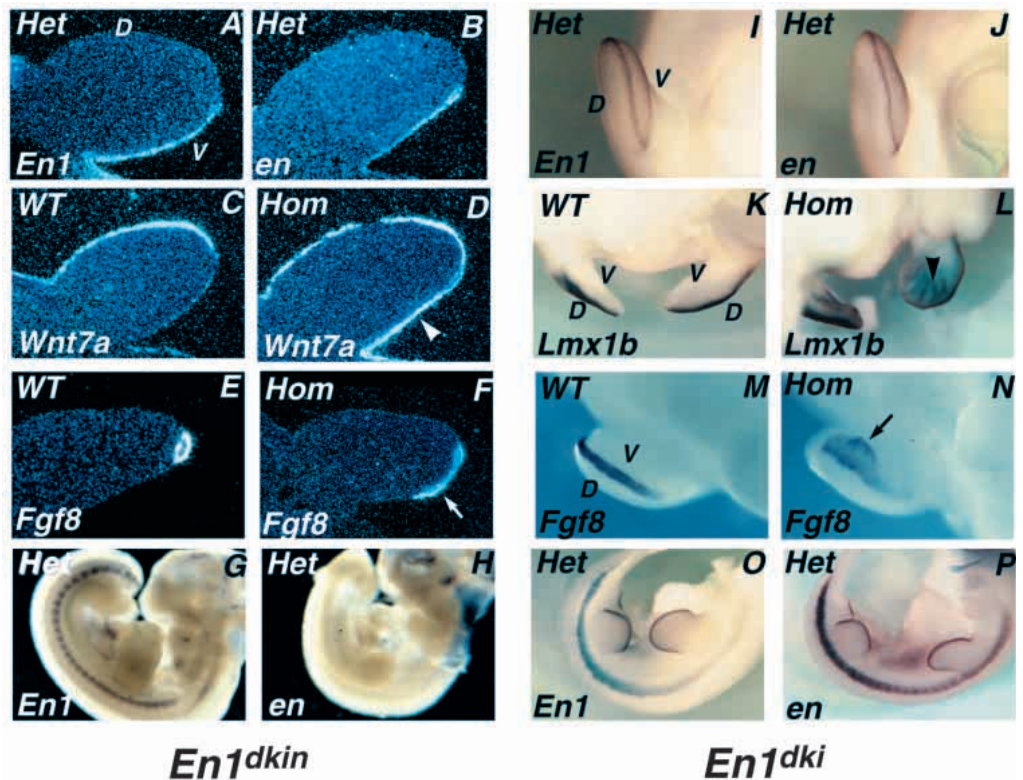


**Fig. 3.** Whole-mount RNA in situ analysis of mouse embryos analyzed with Drosophila *en* or mouse *En1* probes. 8.5 dpc *En1<sup>dkin/+</sup>* (A,B) or *En1<sup>dki/+</sup>* (C) mouse embryos probed with *En1* (A) or *en* (B,C). Expression in the anterior neural folds is indicated by arrowheads. 9.5 dpc *En1<sup>dki/+</sup>* (D,E) and *En1<sup>dki/+</sup>* (F) embryos probed with *En1* (D) and *en* (E,F) sequences, using long proteinase K treatments appropriate for detecting transcripts in the neural tube. Expression of *en* from the *En1<sup>dkin</sup>* allele is seen at the mid/hindbrain border (E), but is more variable than that seen in either *En1<sup>dkin/+</sup>* embryos probed with *En1* (D) or *En1<sup>dki</sup>* embryos probed with *en* (F).

cerebellums of adult and newborn animals confirmed that collicular and cerebellar cytoarchitecture was largely normal, though in some *En1<sup>dkin/dkin</sup>* animals that survived, regions of the posterior colliculi and medial and anterior

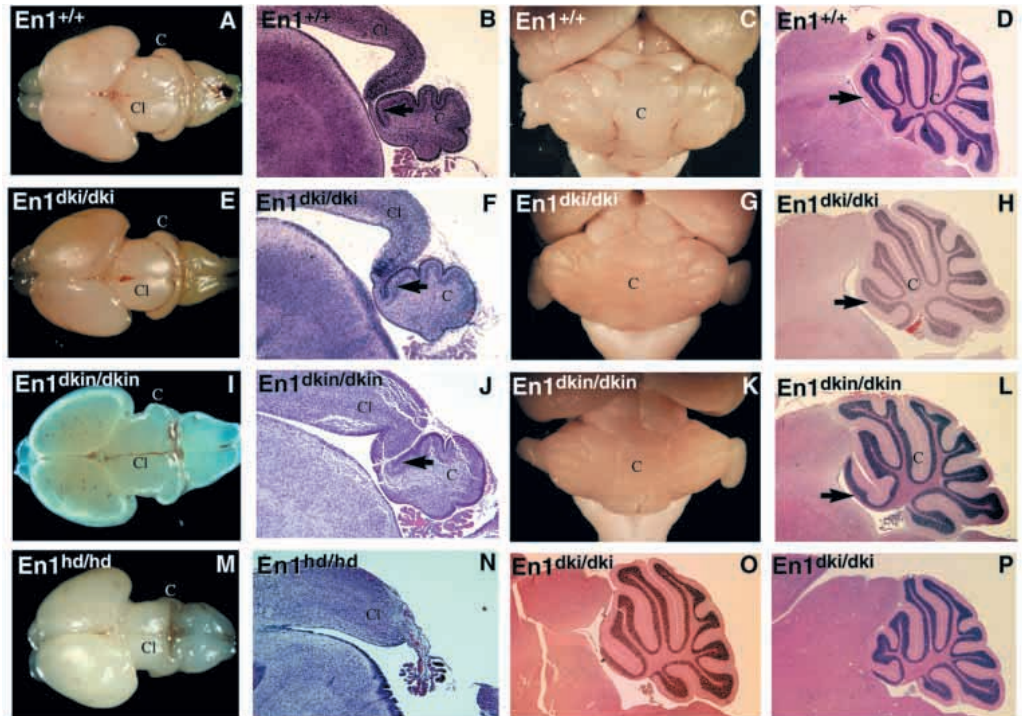
cerebellum were missing (Fig. 5L). These results suggested that the *En1<sup>dkin/dkin</sup>* brains exhibit a partial loss-of-*En1* phenotype, whereas the *En1<sup>dki/dki</sup>* brains more closely resemble the wild-type phenotype.

**Fig. 4.** Whole-mount and section RNA in situ analysis of limbs and trunk from embryos carrying the *En1<sup>dkin</sup>* or *En1<sup>dki</sup>* alleles. 11.0 dpc limb sections (A-F) and 11.5 dpc whole mounts (G-P) of *En1<sup>dkin/+</sup>* (A,B,G,H), *En1<sup>dki/+</sup>* (I,J,O,P), *En1<sup>dkin/dkin</sup>* (D,F), *En1<sup>dki/dki</sup>* (L,N) or *En1<sup>+/+</sup>* (C,E,K,M) embryos probed with *En1* (A,G,I,O), *en* (B,H,J,P), *Wnt7a* (C,D), *Lmx1b* (K,L) or *Fgf8* (E,F,M,N). Expression patterns of *En1* and *en* are similar in *En1<sup>dkin/+</sup>* and *En1<sup>dki/+</sup>* limbs (A,B,I,J), but the level of *en* expression in *En1<sup>dkin</sup>* embryos was variable and often reduced compared to *en* in *En1<sup>dki/+</sup>* limbs. *En1<sup>dkin/dkin</sup>* and *En1<sup>dki/dki</sup>* mutant limbs display ectopic ventral expression of the dorsalizing genes *Wnt7a* (D, white arrowhead) and *Lmx1b* (L, black arrowhead) as well as aberrant proximoventral expansion of the *Fgf8* expression domain at early stages of AER development (F, white arrow) and an ectopic *Fgf8*-positive rim at later stages (N, black arrow). Somites and spinal cord of *En1<sup>dkin/+</sup>* embryos did not express detectable levels of *en* (H), whereas those of *En1<sup>dki/+</sup>* embryos expressed *en* at levels comparable to endogenous *En1* (O versus P). D, dorsal; V, ventral.



Somites and spinal cord of *En1<sup>dkin/+</sup>* embryos did not express detectable levels of *en* (H), whereas those of *En1<sup>dki/+</sup>* embryos expressed *en* at levels comparable to endogenous *En1* (O versus P). D, dorsal; V, ventral.

**Fig. 5.** *En1<sup>dkin/dkin</sup>* show partial rescue, whereas *En1<sup>dki/dki</sup>* homozygous mice show more complete rescue, of the *En1<sup>hd/hd</sup>* mutant brain phenotype. Intact newborn (A,E,I,M) and adult (C,G,K) brains and matched parasagittal sections (B,D,F,H,J,L,N,O,P) from wild-type (A-D), *En1<sup>dki/dki</sup>* (E-H,O,P), *En1<sup>dkin/dkin</sup>* (I-L) and *En1<sup>hd/hd</sup>* (M,N) mice. The *En1<sup>dkin/dkin</sup>* animals depicted represent examples of the well-rescued group of mice. The deletion of colliculi and cerebellum is clearly seen in newborn brains (M,N). These structures are largely rescued in *En1<sup>dki/dki</sup>* (E-H,O,P) and in *En1<sup>dkin/dkin</sup>* (I-L) mice, although they are smaller and less well-developed in *En1<sup>dkin/dkin</sup>* mutants. Arrows depict the area of anterior cerebellum deleted in *En1<sup>dkin/dkin</sup>* homozygous brains. *En1<sup>dki/dki</sup>* brains can appear relatively normal (H) or they can display loss (O) or partial fusion (P) of the anterior folds. C, cerebellum, Cl, colliculi.

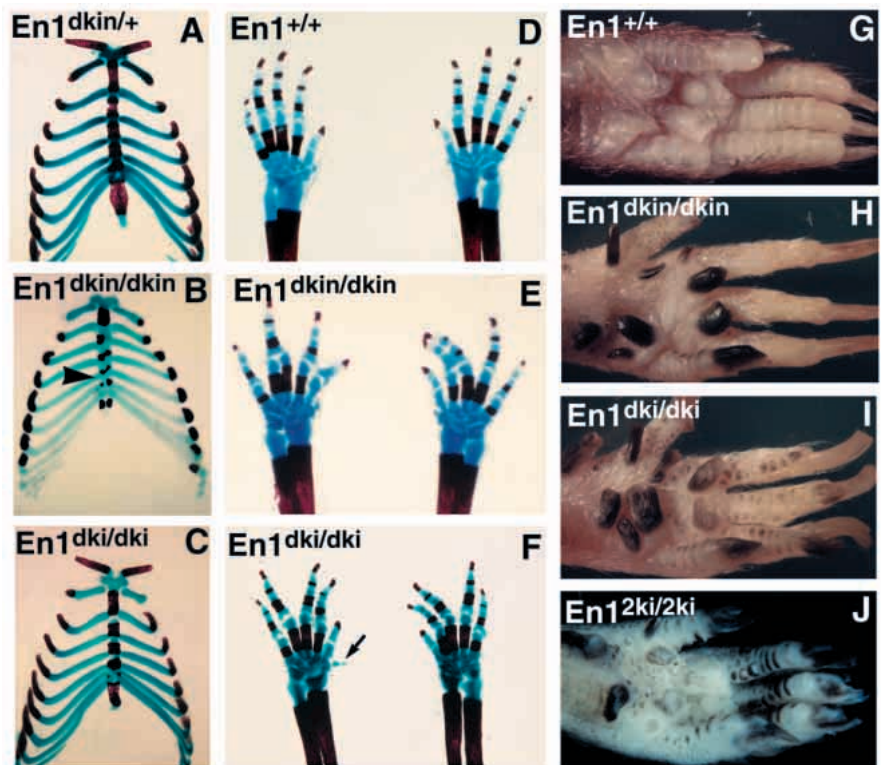


### Drosophila *en* cannot rescue *En1* mutant embryonic or postnatal limb patterning

Whole-mount and section in situ hybridizations showed that *en* expression in the embryonic limbs of *En1<sup>dkin/+</sup>* and *En1<sup>dkin/dkin</sup>* embryos was detectable though weak in most limbs (Fig. 4B and data not shown), whereas *en* was consistently expressed at appropriate levels in the limbs of *En1<sup>dki/+</sup>* and *En1<sup>dki/dki</sup>*

embryos (Fig. 4J and data not shown). Nevertheless, the limbs of both *En1<sup>dki/dki</sup>* and *En1<sup>dkin/dkin</sup>* animals displayed the double dorsal phenotype characteristic of *En1<sup>hd/hd</sup>* mutants, with ventral nails and hair, and loss of palmer eccrine glands (Fig. 6H,I and data not shown) (Loomis et al., 1996; Wurst et al., 1994). Like *En1* null mutants, the posterior paw (4th and 5th digits) displayed less severe dorsalization than the anterior

**Fig. 6.** The *En1<sup>dkin</sup>* and *En1<sup>dki</sup>* alleles fail to rescue the *En1<sup>hd/hd</sup>* limb defects. Alcian Blue (cartilage) and Alizarin Red (bone) staining (A-F) of the sternae and limbs from newborn *En1<sup>dkin/+</sup>* (A), *En1<sup>dkin/dkin</sup>* (B,E), *En1<sup>+/+</sup>* (D), and *En1<sup>dki/dki</sup>* mice (C,F). Note the incomplete and asymmetric ossification of the sternum (B, arrowhead) and skeletal digit fusion in *En1<sup>dkin/dkin</sup>* mutants as compared to wild type and *En1<sup>dki/dki</sup>* mutants. Whole mounts of adult *En1<sup>+/+</sup>* (G), *En1<sup>dkin/dkin</sup>* (H), *En1<sup>dki/dki</sup>* (I) and *En1<sup>2ki/2ki</sup>* (J) limbs. Both the *En1<sup>dkin/dkin</sup>* and *En1<sup>dki/dki</sup>* limbs (H,I) develop ectopic ventral hairs, nails and occasional ventral digits (F, arrow). The *En1<sup>2ki/2ki</sup>* mutants (J), which lack the *neo* sequences, are indistinguishable from the *En1<sup>2kin/2kin</sup>* mice, which display no limb abnormalities at birth (Hanks et al., 1995), but subsequently develop hyperpigmented, nail-like structures over the more proximal paw pads (Loomis et al., 1996).



paw. However, in the *En1<sup>dki/dki</sup>* mutants the middle digits were slightly less dorsalized than in the *En1<sup>hd/hd</sup>* and *En1<sup>dkin/dkin</sup>* mutants. *En1<sup>dkin/dkin</sup>* and *En1<sup>dki/dki</sup>* limbs also displayed the forelimb handplate defects, syndactyly and post-axial polydactyly, seen in *En1<sup>hd/hd</sup>* forelimbs, but the syndactyly was much less prominent in the *En1<sup>dki/dki</sup>* mutants (Fig. 6E,F). Occasional ectopic ventral digits similar to *En1<sup>hd/hd</sup>* homozygotes were also observed on fore- and hindpaws of both knock-in mutants (Fig. 6F). Finally, postnatal hyperpigmentation and nail-like differentiation on the ventral epidermis developed at 3-4 weeks of age in both *En1<sup>dkin/dkin</sup>* and *En1<sup>dki/dki</sup>* mice, as seen in the *En1<sup>hd/hd</sup>* animals that survive past birth (Loomis et al., 1996; Fig. 6H-I).

Our previous studies showed that the dorsalization of *En1<sup>hd</sup>* mutant paws at birth reflected changes in gene expression at early limb bud stages. In *En1<sup>hd/hd</sup>* 10.5 dpc mutant embryos, the dorsally restricted limb gene *Wnt7a* is upregulated in the ventral ectoderm of *En1<sup>hd</sup>* early limb buds and this leads to the ectopic up-regulation of *Lmx1b* in the ventral mesenchyme of 11.5 dpc limbs (Loomis et al. 1996, 1998; Cygan et al., 1997). In addition, the AER markers *Fgf8*, *Bmp2* and *Dlx2* are expanded ventroproximally at slightly later developmental stages and delineate a second rim of thickened ectoderm (Loomis et al., 1998; Fig. 4) along the proximoventral border of the expanded domain. RNA in situ analysis of *En1<sup>dki/dki</sup>* and *En1<sup>dkin/dkin</sup>* limbs showed that *Fgf8*, *Wnt7a* and *Lmx1b* were abnormally expressed, as in *En1* null mutants, despite high levels of *en* expression in the ventral ectoderm of *En1<sup>dki/dki</sup>* embryos (Fig. 4D,F,L,N and data not shown).

### En2 can rescue embryonic, but not postnatal limb patterning

In our studies of *En1<sup>2kin</sup>* mice (previously designated *En1<sup>2ki</sup>* mice), in which *En1* was replaced with *En2* (Hanks et al., 1995; Loomis et al., 1996), we had found that *En2* can rescue the embryonic patterning defects of *En1<sup>hd</sup>* mutant limbs, but not the postnatal defects. Since the *En1<sup>2kin</sup>* allele contained the *neo* gene it was possible that the lack of postnatal rescue was due to an effect of *neo* on *En2* expression in the limb. To address this, *neo* was removed by site-specific recombination to produce *En1<sup>2ki</sup>* heterozygous mice, which were interbred to produce homozygotes. *En2<sup>2ki/2ki</sup>* mice were, however, found to develop the hyperpigmentation and ventral nail-like structures of *En1<sup>hd/hd</sup>* mutants a few weeks after birth (Fig. 6J).

### en can substitute for En1 in sternum development

In contrast to the limb defects, the severity of the sternum and rib skeletal phenotypes differed between *En1<sup>dki/dki</sup>* and *En1<sup>dkin/dkin</sup>* homozygotes. In *En1<sup>dki/dki</sup>* animals, where *en* showed appropriate *En1*-specific expression, the chest wall skeletal defects appeared largely rescued (Fig. 6C). However, since *en* was poorly expressed in the developing body mesoderm of *En1<sup>dkin/dkin</sup>* embryos, as expected, these animals exhibited the disorganized sternum described for *En1<sup>hd/hd</sup>* mutants (Fig. 6B), with delayed irregular ossification, partial fusion of the two halves of the sternal primordium and asymmetric articulation of ribs 2-5. These results provide compelling evidence that *Drosophila en* can functionally replace mouse *En1* in skeletal as well as brain development, but not during embryonic or postnatal limb development.

## DISCUSSION

In summary, we have utilized in vitro transient transfection assays and an in vivo knock-in gene targeting approach to demonstrate that mouse *En1* and *Drosophila en* share common biochemical activities, despite having roles in diverse developmental processes. *En1*, *En2* and *en* have qualitatively similar biochemical activities in vitro: all can bind a common DNA sequence and can act as active and passive repressors of transcription. Additionally, *Drosophila en* can substitute for *En1* in the developing murine midbrain and cerebellum, leading to a near complete rescue of the *En1<sup>hd/hd</sup>* mutant phenotype. We further show that *en* can for the most part replace *En1* in sternal development, but cannot functionally replace *En1* in limb dorsal/ventral patterning during embryonic or postnatal stages.

A number of studies have shown that mammalian homologues of *Drosophila* genes can partially rescue *Drosophila* mutant phenotypes, or elicit similar phenotypes to their endogenous fly homologues when transiently expressed from a heat shock promoter (Bachiller et al., 1994; Dauwalder and Davis, 1995; Malicki et al., 1990, 1993; McGinnis et al., 1990; Zhao et al., 1993). Our studies are among the first to demonstrate that a gene from an invertebrate can substitute for a mammalian gene throughout development of a particular organ and contribute to development of a highly evolved structure, the brain (see also Acampora et al., 1998). Our mutant analysis also provides the first evidence for specific functional diversity of *En* proteins during evolution, based on the degree to which *en* and *En2* can rescue three different aspects of the *En1* null mutant phenotype. Furthermore, the two knock-in mutants show interesting and different intermediate phenotypes in all tissues and therefore, together with *En1<sup>hd/hd</sup>* mutants, represent an informative *En1* allelic series.

The incomplete brain and skeletal rescue of *En1<sup>dkin/dkin</sup>* mutants probably reflects the variable and weak expression of *en* from the *En1<sup>dkin</sup>* allele in the neural tube and presumptive thoracic mesenchyme. In support of this idea, removal of *neo* from the *En1<sup>dkin</sup>* allele, to produce the *En1<sup>dki</sup>* allele, results in normal *En1*-like expression of *en* in these tissues and consequent rescue of the relevant structures. The reduction of *en* expression from the *En1<sup>dkin</sup>* allele as compared with the *En1<sup>dki</sup>* allele or wild-type *En1* locus is consistent with other reports that *neo* can interfere with the proper regulation of a nearby gene (Olson, 1996). In addition, since *neo* was inserted within the *En1* transcription unit in exon 1, it could also produce an *en* mRNA that is less stable than the *En1* transcript. The expression studies suggest that in either case, there are tissue-specific effects on production of *en* mRNA from the *En1<sup>dkin</sup>* allele. Furthermore, it is interesting to note that insertion of the mouse *En2* or bacterial *lacZ* cDNAs into *En1*, instead of *en*, resulted in more faithful *En1*-like expression of these cDNAs in the presence of *neo* (Hanks et al., 1995).

The finding that some *En1<sup>dki/dki</sup>* animals died after weaning might suggest that expression from the *En1<sup>dki</sup>* allele was also variable, although based on our expression studies this was to a much smaller degree. An explanation for the post-weaning fatalities in *En1<sup>dki/dki</sup>* or *En1<sup>dkin/dkin</sup>* animals could be that variation in levels of *en* expression from the *En1<sup>dki</sup>* and *En1<sup>dkin</sup>* alleles, combined with a reduced functional capacity of *en*



compared to *En1*, sometimes results in *en* failing to operate above a critical threshold level. Consistent with this idea, our studies of *En1* and *En2* double mutants have shown a graded deletion of mid-hindbrain structures that correlates with a graded loss of total *En* expression (W. Wurst and A. L. Joyner, unpublished). Furthermore, the intermediate phenotypes seen in *En1<sup>+/-</sup>En2<sup>-/-</sup>* double mutants, loss of posterior colliculi and anterior cerebellum, are similar to those seen in some *En1<sup>dkin/dkin</sup>* mutants. One other interesting alternative in the case of *En1<sup>dki/dki</sup>* animals is that *En1* plays an essential role postnatally in some tissue where *en* is expressed, but where *en* can not completely replace *En1* function. Taken together, our studies demonstrate that, given an appropriate expression profile, *en* can functionally replace *En1* throughout brain development to a great degree.

The finding that *Drosophila en* expression in the developing limbs of *En1<sup>dki/dki</sup>* embryos is unable to repress ventral expression of *Wnt7a* raises the interesting possibility that *En1* protein has different functions in the embryonic brain and limb, with *en* capable of performing the functions required for brain-specific, but not limb-specific development. The lack of limb rescue is not likely to be due to insufficient *en* expression in the limb ectoderm, since *en* appears to be expressed at normal levels from the *En1<sup>dki</sup>* allele. Furthermore, *En1<sup>hd/+</sup>* heterozygotes have normal limbs.

Our recent analysis of targeted *En1<sup>2kin</sup>* mutants (Hanks et al., 1995; Loomis et al., 1996) and present analysis of *En1<sup>2ki</sup>* mutants, in which *En2* replaces *En1*, have shown that *En2* can rescue the *En1<sup>hd</sup>* brain defects and embryonic limb patterning defects, but not an apparent postnatal role of *En1* in the limb epidermis. The limbs of *En1<sup>2ki/2ki</sup>* animals appear normal at birth and *En2* can repress embryonic expression of *Wnt7a* and *Lmx1b* in the ventral limb (R. Kimmel and C. Loomis, unpublished data). However, by about 3-4 weeks post partum in *En1<sup>2ki/2ki</sup>* and *En1<sup>dki/dki</sup>* mutants, and in rare *En1<sup>hd/hd</sup>* animals that survive, the ventral epidermis overlying the palm pads becomes pigmented and begins to form nail-like structures. These postnatal defects correlate with postnatal expression of *En1* in epidermal structures (C. Loomis, unpublished results). Although it is possible that the postnatal limb defects in *En1* mutants result from a lack of *En1* during early embryonic limb patterning, it seems more likely that they are due to a requirement for *En1* later in the epidermis. The fact that *En2* can rescue the early dorsal/ventral patterning function of *En1* in the limb but not a later function in the epidermis, and that *en* cannot fully rescue either limb function of *En1*, suggests that the functions of *En1* protein are different in early and late limb development. Furthermore, since both *En2* and *en* can rescue the function of *En1* in the brain, the region(s) of *En1* required for this function must be conserved in both *en* and *En2*, unlike the region(s) required for limb development.

We speculate that the functional differences between the three engrailed proteins may reflect the inability of *en* and *En2* to interact with the full repertoire of *En1* accessory proteins, possibly resulting in altered DNA binding affinities for selective targets. As already mentioned, comparison of the sequences of all *En* proteins has identified five conserved domains, the largest being the homeodomain (Logan et al., 1992). Outside these domains *En1* and *En2*, or *en*, diverge to a similar degree. Thus, in a non-conserved *En* region, the coding sequence of the first vertebrate *En* gene could have

evolved a domain required for specific protein-protein or protein-DNA interactions in the limbs, which became further specialized in *En1* after the second *En* gene (now designated *En2*) was formed by duplication. In this regard it is interesting to note that *En1* proteins share a number of conserved regions that are not found in other *En* proteins (Logan et al., 1992). It is also possible that some of the conserved domains do not have the same repertoire of functions. It has been noted that EH-1, which is present in a number of transcription factors, can be divided into subfamilies based on sequence conservation (Smith and Jaynes, 1996). Furthermore, EH-1 is quite divergent between mouse and *Drosophila*. Although it was shown in a fly in vivo assay that EH-1 from mouse *En1* can functionally replace EH-1 from *en*, it is possible that during mouse development *en* EH-1 can functionally replace *En1* in the brain and skeleton, but not the limb. Thus, protein sequence and experimental data are consistent with the hypothesis that *En1* protein has evolved one or more functional domains required for vertebrate limb development.

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