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The homeoprotein engrailed 1 has pleiotropic functions in calvarial intramembranous bone formation and remodeling

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The membranous bones of the mammalian skull vault arise from discrete condensations of neural crest- and mesodermally-derived cells. Recently, a number of homeodomain transcription factors have been identified as critical regulators of this process. Here, we show that the homeoprotein engrailed 1 (EN1) is expressed during embryonic and perinatal craniofacial bone development, where it localizes to the skeletogenic mesenchyme, and, subsequently, to calvarial osteoblasts and osteoprogenitors. Mice lacking *En1* exhibit generalized calvarial bone hypoplasia and persistent widening of the sutural joints. A reduction in calvarial membranous bone deposition and mineralization (osteopenia) is coupled to enhanced osteolytic resorption in *En1* mutants. Consistent with these observations, expression of established osteoblast differentiation markers reveals that *En1* function is required for both early and late phases of calvarial osteogenesis. Further analysis shows that EN1 regulates FGF signaling in calvarial osteoblasts. Moreover, EN1 indirectly influences calvarial osteoclast recruitment and bone resorption by regulating the expression of receptor activator of NFκB ligand (RANKL) in osteoblasts. Thus, during intramembranous bone formation, EN1 acts both cell autonomously and non-cell autonomously. In summary, this study identifies EN1 as a novel modulator of calvarial osteoblast differentiation and proliferation, processes that must be exquisitely balanced to ensure proper skull vault formation.

KEY WORDS: Calvarial bone, *En1*, *Osterix*, Osteoblasts, Osteoclasts

INTRODUCTION

The mammalian skull vault (roof) comprises an amalgamation of skeletal elements that specifically evolved to encase and protect the brain and sensory organs. Consisting of the frontal, parietal, interparietal and occipital bones (in mouse), the skull vault is developmentally complex and receives lineage contributions from both the cranial neural crest (CNC) and the paraxial mesoderm (Jiang et al., 2002). These populations migrate into defined locations overlying the cerebral hemispheres, and subsequently differentiate into condensing osteogenic or chondrogenic mesenchyme (between E7.5-E11.5 in mouse). The osteogenic mesenchyme is characterized by the expression of *Runx2* and *Osterix* (*Osx*; *Sp7* – Mouse Genome Informatics), the earliest molecular determinants of bone formation (Komori et al., 1997; Nakashima et al., 2002; Otto et al., 1997). In contrast to the endochondral bone formation that ensues from a cartilaginous template, most elements of the skull vault develop by intramembranous ossification, characterized by the direct differentiation of osteogenic mesenchyme into osteoblasts. Osteoblasts are specialized cells that produce bone extracellular matrix or osteoid, and that subsequently regulate its mineralization (Aubin, 2002).

During the morphogenetic phases of skull development, a pool of highly proliferative osteoprogenitors populates the margins (osteogenic fronts) of each enlarging bone anlagen, thereby maintaining calvarial bone expansion through osteoblast replenishment (Iseki et al., 1999; Opperman, 2000). After each bone has acquired its basic form (E15.5), the individual skeletal elements remain separated by fibrous joints, or sutures, composed of skeletogenic mesenchyme and fibroblasts (Opperman, 2000). Continued production of osteoprogenitors in the sutures ensures that calvarial expansion is coordinated with growth of the underlying brain. Thus, sutures serve as major centers for calvarial osteoblast differentiation and new bone formation postnatally. Finally, in addition to osteoblast-mediated bone formation, calvarial bones undergo dynamic modeling and remodeling of their three-dimensional microarchitecture, through the coordinated resorptive activity of haematopoietically derived osteoclasts (Takahashi, 2002).

Genetic and molecular evidence has implicated a number of growth and transcription factors as being important regulators of skull formation. The fibroblast growth factors (FGFs) are a family of secreted polypeptides that act through four related tyrosine kinase receptors (FGFR1-FGFR4) to regulate a plethora of developmental processes, and they are of central significance to intramembranous ossification (Ornitz and Marie, 2002). Human diseases that manifest the precocious osseous obliteration of sutures, known as craniosynostosis, often result from gain-of-function mutations in FGF receptors 1-3 (*FGFR1/2/3*) (Webster and Donoghue, 1996; Wilkie, 1997). Mouse models of loss- or gain-of-function mutations in *Fgfr1* and *Fgfr2* have provided further evidence that FGF signaling regulates the proliferation and differentiation of calvarial osteoblasts and osteoprogenitors (Eswarakumar et al., 2004; Eswarakumar et al., 2002; Yu et al., 2003; Zhou et al., 2000). Elucidating the precise mechanisms of osteoblastic FGF signaling, however, has been complicated by the fact that at least four potential ligands (*Fgf2*, *Fgf4*, *Fgf9* and *Fgf18*) for *Fgfr1-Fgfr3* are expressed in the developing mouse calvarium (Kim et al., 1998; Ohbayashi et al., 2002; Rice et al., 2000). In addition, although a growing number of intracellular antagonists for receptor tyrosine kinases (RTKs) have been identified as being important modifiers of FGF-responsiveness in many developmental contexts, information on how these may regulate calvarial bone formation is currently lacking (Furthauer et al., 2002; Kawakami et al., 2003; Wakioka et al., 2001).

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At the transcriptional level, a number of homeodomain proteins have been shown to participate in regulating calvarial bone development. Perturbed calvarial ossification is observed in humans and mice harboring loss-of-function mutations in the homeoproteins *Msx2*, *Dlx5* and *Alx4* (Robledo et al., 2002; Satokata et al., 2000; Wilkie et al., 2000; Wuyts et al., 2000). Interestingly, regulation of the osteocalcin gene promoter by Runx2 has been shown to be influenced by FGF signaling and the modifying activities of Dlx5 and Msx2 (Newberry et al., 1998). Furthermore, FGF signaling has been shown to directly induce *Msx2* gene expression in calvarial sutures (Ignelzi et al., 2003). These findings are indicative of crucial interactions between growth factors and transciptional regulators of calvarial osteogenesis, and raise the possibility that other such modifiers remain to be identified.

Engrailed 1 (*En1*), the homolog of *Drosophila en*, is a homeodomain-containing transcription factor that participates in the regulation of multiple mammalian developmental processes, such as dorsoventral patterning of the distal limb and mid-hindbrain specification (Loomis et al., 1996; Wurst et al., 1994). Interestingly, several ossification and growth abnormalities observed in the sternae and phalanges of *En1*-null mice, as well as the observed expression of *En1* in developing vertebrae, have implicated its involvement in skeletal development (Davidson et al., 1988; Wurst et al., 1994). We show that *En1* is expressed during the early and late phases of calvarial osteogenesis. Through the characterization of novel phenotypic features of *En1*-ablated mice, we demonstrate that EN1 plays a crucial role in regulating intramembranous ossification during craniofacial bone development. In addition, evidence is provided to suggest that EN1 regulates calvarial ossification by influencing FGF responsiveness in osteoblasts. We further show that EN1 has a non cell-autonomous function in regulating osteoclast recruitment and activation, thereby affecting calvarial bone resorption and remodeling.

MATERIALS AND METHODS

Mouse mutants and genotyping

En1 knockout mice (Hanks et al., 1995; Matise and Joyner, 1997; Wurst et al., 1994) were maintained on a wild-type Swiss Webster background. Mice harboring either the $En1^{hd}$ (homeobox deleted) or $En1^{lki}$ (*lacZ* 'knock in') null alleles were intercrossed to generate homozygous mutants lacking *En1* function.

Whole-mount skeletal preparations and β-galactosidase staining

Visualization of the cartilaginous and mineralized skeleton was facilitated by Alcian Blue and Alizarin Red staining, as previously described (Loomis et al., 1996). For detection of β-galactosidase activity in tissues, specimens were treated for several hours with a gluteraldehyde fixative, washed in phosphate-buffered saline (PBS) and stained with standard X-gal solution for 16 hours at 4°C (Matise and Joyner, 1997).

Quantitative micro-computed tomography (microCT)

Micro-computed tomography (microCT) was performed at the Centre for Bone and Periodontal Research located on McGill campus (Montreal, Quebec). Data were acquired on a SkyScan T-1072 microtomograph (Skyscan, Aartselaar, Belgium). Cross-sections along the specimen axis were reconstructed and images quantified using the Cone-Beam Reconstruction Software supplied by SkyScan.

Histological and immunohistochemical analysis of calcified and decalcified tissues

Dissected newborn skulls were fixed overnight at 4°C in 4% paraformaldehyde (PFA), and decalcified for 4 days in 40 mM EDTA (pH 7.3), before dehydrating and embedding in paraffin. X-gal-stained tissues were dehydrated in isopropanol.

Immunolocalization of phosphorylated extracellular signal-regulated kinase (pERK) was performed on 6-μm deparaffinized sections using a rabbit monoclonal antibody specific to phosphorylated ERK1/2, according to the manufacturer's instructions (20G11, Cell Signaling). Signal detection was performed using the ABC-AP Kit (Vector Laboratories) coupled to the BM-Purple substrate (Roche).

In situ hybridization (ISH) and probes

Probes for osteopontin (*Opn*), osteocalcin (*Ocn*), bone sialoprotein (*Bsp*) and osterix (*Osx*), were amplified by reverse transcriptase (RT)-PCR (iScript cDNA Synthesis Kit, BioRad), employing specific primers to each mRNA and total RNA extracted from primary calvarial osteoblasts as a template. Amplified cDNA fragments were cloned into the pGEMT-Easy cloning vector (Promega). Probes for murine *Fgfr1* and *Fgfr2* were obtained from Dr G. Morriss-Kay (Oxford). The probe for *Fgf18* was obtained from Dr B. Hogan (Duke). The probes for *Spry1* and *Spry2* were obtained from Dr G. Martin (UCSF). Digoxigenin-UTP labeling of RNA riboprobes was performed with the MEGAscript transcription kit (Ambion). In situ hybridization was performed on paraffin-embedded sections or whole-mount calvariae, essentially as described by Wilkinson (Xu, 1999).

In vivo proliferation analysis

Bromodeoxyuridine (BrdU, Sigma; 100 μg/g body weight) was administered intraperitoneally into pregnant mice at the indicated gestational stages; then, 1.5 hours later, mice were sacrificed and embryos collected and fixed overnight in 4% PFA. Immunodetection of BrdU was performed as previously described (Ishii et al., 2003). A comparative analysis of the osteogenic fronts of *En1*-null and wild-type littermates was performed, and statistical significance analyzed by ANOVA one-way assessment of variance (Graph Prism).

Assessment of alkaline phosphatase (ALP) activity, tartrate-resistant acid phosphatase (TRAP) and mineral content

ALP activity was quantitated as previously described (Deckelbaum et al., 2002). The histological detection of $TRAP^+$ osteoclasts in paraffin-embedded sections was performed as previously described (Miao et al., 2004). Whole-mount detection of $TRAP^+$ osteoclasts was performed as described (Holt et al., 1994). For the detection of mineralized matrix, cultured cells were formalin fixed, ethanol dehydrated, stained with 2% $AgNO_3$ under ultraviolet light, and then treated with 5% sodium thiosulfate. Detection of mineralized bone matrix in non-decalcified tissue sections was performed as described previously (Valverde-Franco et al., 2004).

Culture of murine primary calvarial osteoblasts

Dissected calvarial bones were collected in Hank's balanced salt solution (HBSS, Gibco). To extract osteoblasts, bone fragments were treated with collagenase type IA (1 mg/ml in Hank's balanced salt solution; Sigma) for 30 minutes at 37°C, and then with EDTA (4 mM in PBS), and released cells were plated at a density of 10^6 cells/35 mm in complete culture media [cCM: 10% FCS (Gemini), α-MEM (Gibco), 100 U/ml penicillin, 50 μg/ml streptomycin]. At confluence, cCM was supplemented with 100 μg/ml ascorbate and 5 mM β-glycerophosphate to promote osteogenic differentiation and mineralization (Bakker, 2003).

Northern blot analysis

Northern blot analysis was performed as previously described using total RNA (20 μg) extracted from primary osteoblasts, separated on a denaturing formaldehyde gel, and blotted onto supported nitrocellulose (Deckelbaum et al., 2002). Quantitative assessment of autoradiograms was performed using ScionImage software.

RESULTS

Engrailed 1 is expressed during calvarial bone development

In addition to its well-described activation in the embryonic mid-hindbrain, we noted *En1* expression in the developing murine skull. To address a possible role for *En1* in craniofacial development, we first analyzed its expression at various embryonic stages using a

mouse line that harbors a functional β-galactosidase reporter cassette inserted into the first exon of the *En1* locus (Hanks et al., 1995; Matise and Joyner, 1997). The *En1-lacZ* allele (*En1*^{lki}), from which the expression of functional EN1 protein is abrogated, precisely recapitulates the endogenous *En1* expression. Importantly, *En1*$^{lki/+}$ heterozygotes display no discernable phenotype, and are used interchangeably with wild-type mice in this study. Furthermore, the *En1* homolog *En2* is not expressed by any calvarial tissues (data not shown), excluding the possibility that it compensates for *En1* function in these tissues.

Calvarial expression of *En1*, as detected by whole-mount X-gal staining, initiates at embryonic day (E) 11.5 within lateral aspects of the head (Fig. 1A, part a; arrow), overlying the diencephalic-telencephalic border in the forebrain. Between E12.5 and E13.5, *En1* expression expands rostrocaudally and anteriorly, encompassing the frontonasal and mandibular prominences (Fig. 1A, parts b,c; arrows). During phases of overt intramembranous ossification [E14.5 to postnatal day (P) 1], *En1* is detected in all developing calvarial bones and sutures (Fig. 1A, parts d-f; arrows, asterisk). Coronal sections of E11.5 heads revealed *En1* expression within the presumptive calvarial bone mesenchyme, as confirmed by its co-localization with the early osteogenic marker ALP (Fig. 1B, part a; data not shown). By E13.5, this domain had expanded further toward the base and apex of the skull (Fig. 1B, part b). Histological analysis of the neonatal skull showed that *En1* is predominantly expressed by ectoperiosteal osteoblasts lining the bone surfaces (Fig. 1B, parts c,d; arrow), as well as by osteoblasts and osteocytes populating the endosteal surfaces and the matrix of the cranial bone trabeculae (Fig. 1B, part d; arrowheads). Curiously, we found *En1* expression to differ between the types of calvarial sutures. Within the abutting interfrontal suture, *En1* expression remains restricted to the sutural mesenchyme and is excluded from the osteoprogenitors populating the bone margins (Fig. 1B, parts c,e). By comparison, the osteoprogenitors outlining the frontal and parietal bones exhibit *En1* expression (Fig. 1B, part f). Taken together, our expression analyses suggest potential roles for *En1* in regulating both the primordial and later stages of calvarial ossification.

Calvarial bone hypoplasia and osteopenia in *En1*$^{-/-}$ mice

To determine whether *En1* function is required for calvarial bone formation, we first compared the skulls of *En1*$^{-/-}$ and wild-type mice at P1 by staining with Alcian Blue for cartilage and Alizarin Red for mineralized bone (Fig. 2A). Although displaying no overt patterning defects, the cranial membranous bones of *En1* mutants exhibit profound hypoplasia (reduced size). Notably, the expansion of unossified areas result in pronounced frontal foramina, and gaping coronal and lambdoid sutures (Fig. 2A, parts b,c). Although most *En1*$^{-/-}$ mice die during early postnatal life, secondary to mid-hindbrain defects, analysis of the calvariae of rare surviving mutants revealed persistent coronal and lambdoid suture widening. Moreover, the interfrontal suture, which is normally obliterated by P35 through osseous fusion of the abutting frontal bones, undergoes incomplete closure in *En1*$^{-/-}$ mice (Fig. 2B, parts a,b).

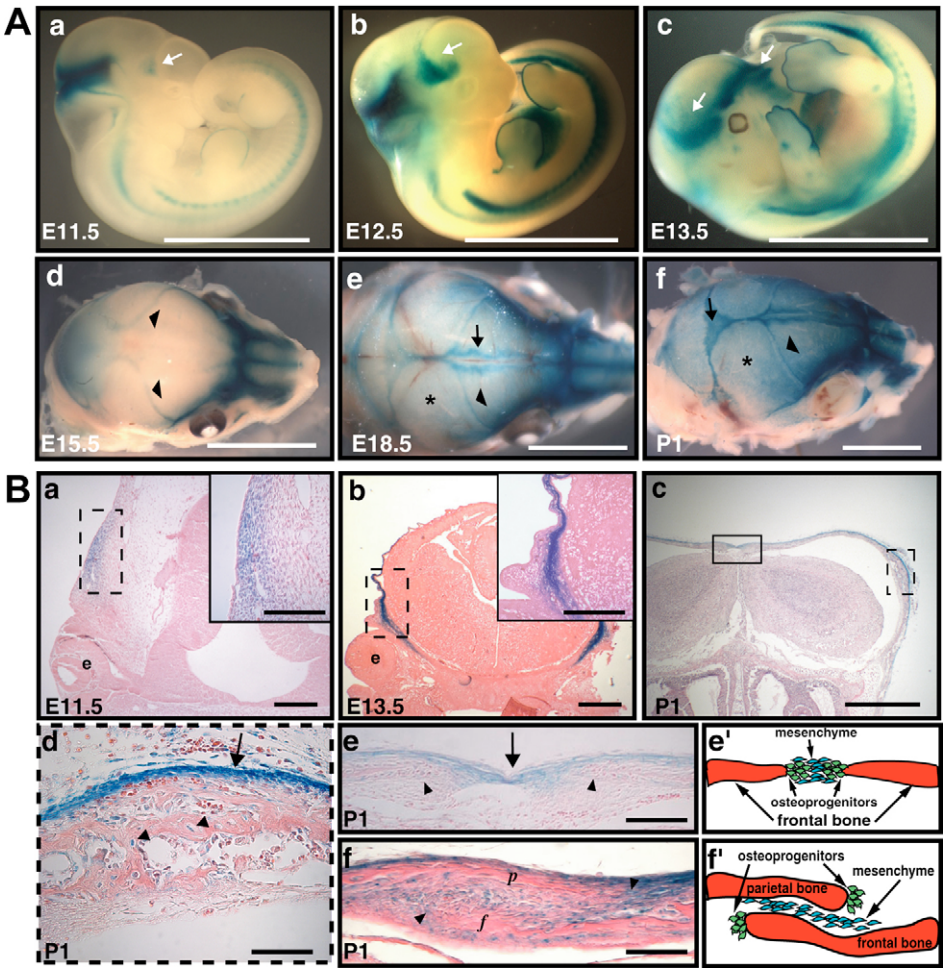

Fig. 1. Analysis *of En1* expression during calvarial bone development between E11.5 and P1, as recapitulated by X-gal staining of *En1*$^{lki/+}$ mice. (**A**,**B**) Calvarial *En1* expression within the craniofacial mesenchyme (Aa-Ac, white arrows) at E11.5 (Aa,Ba), E12.5 (Ab) and E13.5 (Ac,Bb). Expression of *En1* during overt calvarial bone formation (E15.5-P1) is prominent along the osteogenic fronts (arrowhead) and developing sutures (black arrows) (Ad-Af); it is expressed by ectocranial periosteal osteoblasts (Ac, Af, asterisks; Bc,Bd, arrow) as well as by the endosteal osteoblasts and osteocytes of the frontal bone trabeculae (Bc,Bd, arrowheads). In the interfrontal suture, *En1* is expressed in the mesenchyme (Bc,Be, arrow; Be'), but not in osteoprogenitors populating the frontal bone margins (Be, arrowheads). By contrast, *En1* is expressed by osteoprogenitors within the coronal suture (Bf, arrowheads; Bf'). *p*, parietal bone; *f*, frontal bone; e, eye. Scale bars: 2 mm in A; 1 mm in Ba-Bc; 0.1 mm in insets in Ba,Bb, and in Bd-Bf.

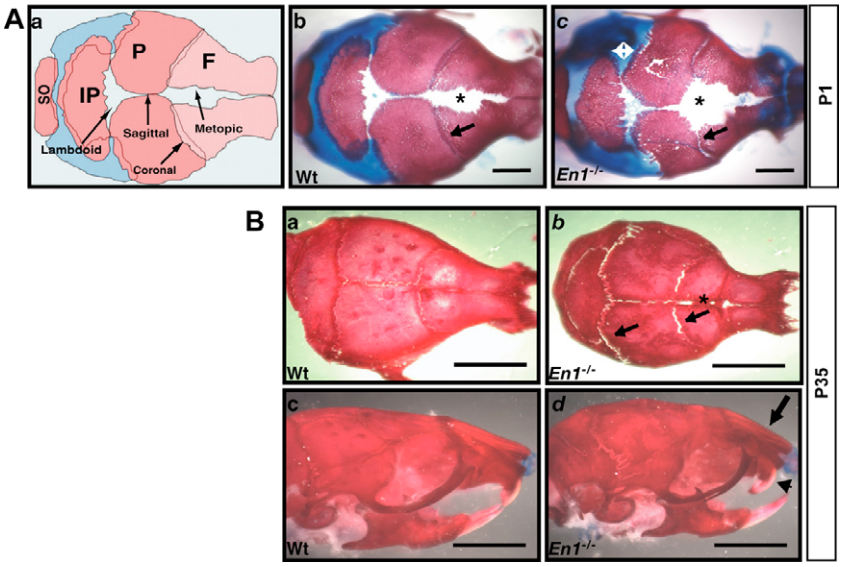

Fig. 2. Gross calvarial malformations in *En1*$^{-/-}$ mice. (**A**) Schematic diagram and staining at P1. (Aa) Schematic depicting the organization of the murine skull vault consisting of: the paired frontal bones (F), the paired parietal bones (P) and the interparietal bone (IP). These are intervened by the lambdoid, coronal, sagittal and interfrontal (metopic) sutures. (Ab,Ac) Alizarin Red and Alcian Blue staining of mineralized bone and cartilage, demonstrating a generalized calvarial hypoplasia, coronal and lambdoid suture gaping (arrow, double-headed arrow), and frontal foramina (asterisk) in the skull vault of *En1*$^{-/-}$ mice at P1. (**B**) At P35, *En1* mutants (Bb,Bd) exhibit abnormal coronal, lambdoid and interfrontal suture patency (Bb, arrows, asterisk), and severe malocclusion due to maxillary and nasal bone hypoplasia (Bd, arrow, arrowhead) compared with wild type (Ba,Bc). Scale bars: 2 mm in A; 5 mm in B.

In addition, adult *En1* mutants display a severe hypoplasia of the frontonasal and maxillary prominences that results in malocclusion and misalignment of the upper and lower incisors (Fig. 2B, parts c,d). Although hypoplasia of the skull base is often the primary cause for malocclusion in other animal models (Chen et al., 2003), we found no differences between wild-type and *En1*$^{-/-}$ mice in this regard.

To gain a more precise three-dimensional perspective of the skull, we performed quantitative microCT on P1 and P5 calvariae of wild-type and *En1*$^{-/-}$ mice (Fig. 3A). Our analysis confirmed the calvarial ossification defects in *En1*$^{-/-}$ mice and indicated that these do not resolve by P5 (Fig. 3A, part b). Quantitative morphometric analyses reveal a significant reduction in bone volume (BV) compared with tissue volume (TV) in *En1* mutants (BV/TV: wild type, 3.460±0.4033, n=8; *En1*$^{-/-}$, 2.205±0.3031, n=6; P=0.0301). Although trabecular thickness is unaltered, a significant increase in the distance separating the trabecular spicules was observed in the mutants (mean distance: wild type, 0.7007±0.05129, n=8; *En1*$^{-/-}$, 0.8362±0.02945, n=6; P=0.0448), which is suggestive of marrow space expansion (Fig. 3B). Histological analysis of non-decalcified calvariae revealed that, by comparison with the wild type, the membranous bones of *En1* mutants are composed of reduced and under-mineralized osteoid that is heavily perforated by fibrous tissue (Fig. 3C). These observations suggest that the hypoplasia observed in *En1*$^{-/-}$ calvariae results from decreased bone matrix deposition (osteopenia) and mineralization.

To evaluate the cellular basis for the defects in calvarial bone mineralization, we evaluated the capacity of *En1* mutant osteoblasts to induce mineralization in culture. Osteoblasts released from newborn wild-type and *En1*$^{-/-}$ calvariae were cultured for 21 days under conditions that promote extracellular matrix mineralization. We observed that, although wild-type osteoblasts formed extensive mineralized three-dimensional nodules, *En1*$^{-/-}$ osteoblasts exhibited a poor capacity to induce mineralization over the culture period (Fig. 3D). Moreover, ALP activity, the osteoblastic expression of which is indispensable for bone mineralization (Fedde et al., 1999; Murshed et al., 2005), was significantly reduced in *En1*$^{-/-}$ osteoblasts in comparison to the wild-type population (Fig. 3E). Collectively, these results imply that *En1* is required for calvarial osteoblast differentiation and bone mineralization.

Impaired osteogenesis and osteoblast function in calvarial bones of *En1*$^{-/-}$ mice

We therefore investigated the possibility that the calvarial defects in *En1* mutants result from impaired osteoblast differentiation in vivo. The expression of osteopontin (*Opn*), a secreted phosphoglycoprotein that is normally activated in differentiating calvarial osteoblasts (Iseki et al., 1997), was examined by whole-mount ISH. We first detected calvarial expression of *Opn* in wild-type mice at E14.5, within the ossification centers of the presumptive parietal bones (Fig. 4A). By comparison, *Opn* was nearly absent in *En1*$^{-/-}$ skulls. By E16.5, *Opn* expression extended into all calvarial membranous bones of wild-type and *En1*$^{-/-}$ skulls but its domain was considerably reduced in the mutants. This deficiency in *Opn* expression persisted at E18.5 in *En1*$^{-/-}$ rudiments, indicating that *En1* plays a role in promoting osteoblast differentiation during the prenatal stages of calvarial bone development.

To evaluate whether the delay in *Opn* initiation in *En1* mutants arises from impaired commitment and early differentiation of the cranial skeletogenic mesenchyme, we examined the expression of the osteogenic determinant *Osx* in E13.5 skulls. Interestingly, *Osx* and *En1* expression overlapped in the calvarial skeletogenic mesenchyme of wild-type embryos (Fig. 4B; compare with Fig. 1B, part b). By contrast, virtually no expression of *Osx* was observed in the calvarial mesenchyme of *En1*$^{-/-}$ littermates. As comparable levels of *Osx* occurred within the mutant and wild-type mandibular mesenchyme (not shown), this result suggests that *En1* has a selective role in enhancing the osteogenic potential of the skull vault mesenchyme. Furthermore, *Osx* expression remained reduced in *En1*$^{-/-}$ calvarial osteoblasts both in vivo and in vitro (Fig. 4B,D). These findings suggest that *En1* function is specific to and indispensable for inducing early phases of calvarial osteogenesis, possibly through the activation of *Osx*.

To gain a more detailed perspective on calvarial osteogenesis, we examined the coronal suture morphology and the expression of osteoblastic genes on sagittal sections of wild-type and *En1*$^{-/-}$ skulls at P1. We made three unanticipated observations. First, the thin layer of mesenchyme, which typically separates the overlapping parietal and frontal bone fronts in the wild-type coronal suture, was considerably thickened in *En1* mutants (Fig. 4C, arrow). Moreover, the margins of the mutant frontal and parietal bones failed to overlap within the suture proper. Second, although *En1* mutants at this

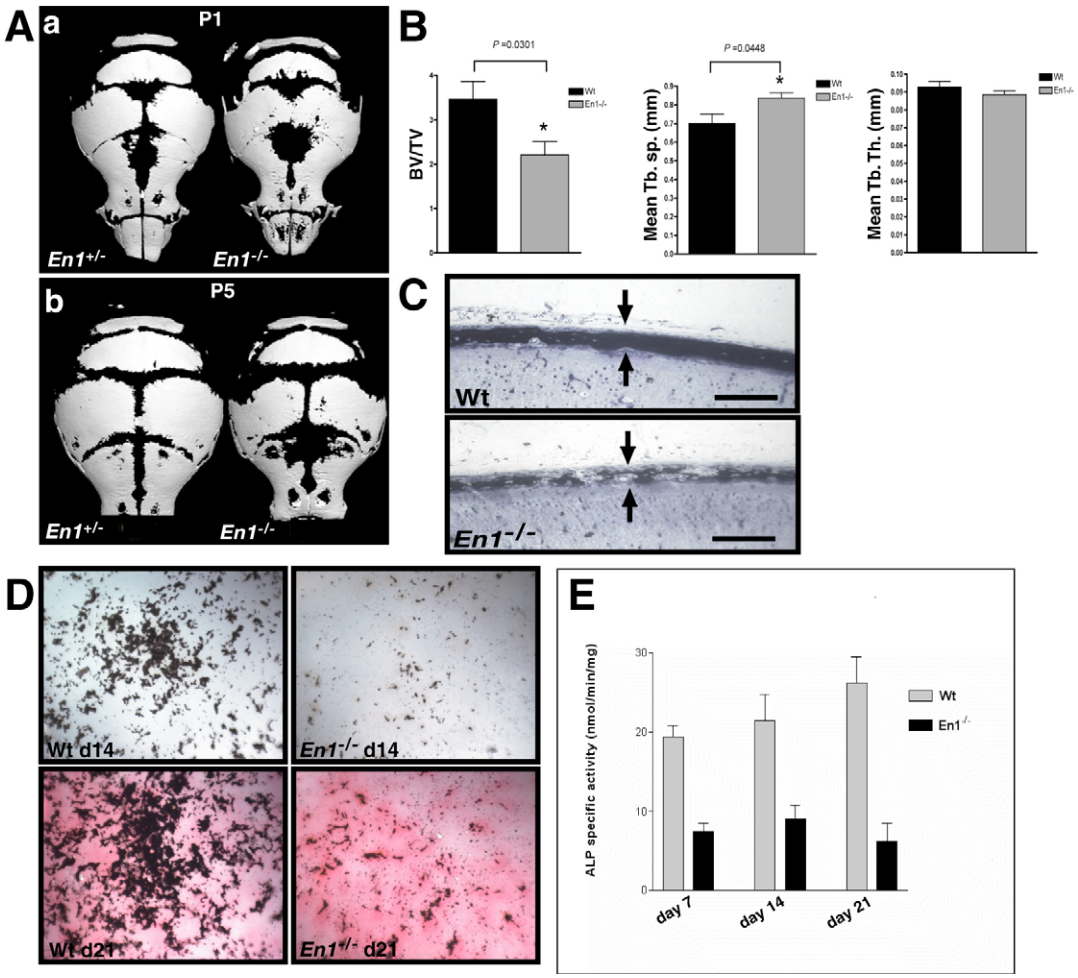

Fig. 3. Osteopenia and impaired calvarial bone mineralization in *En1*$^{-/-}$ mice. (**Aa**,**Ab**) Micro-CT analysis of heterozygous and *En1*$^{-/-}$ calvariae at P1 and P5, demonstrating reduced ossification in the mutants. (**B**) Quantitative morphometric measurements of calvariae ultrastructure obtained by micro-CT analysis, demonstrating reduced bone volume and increased trabecular separation in *En1*$^{-/-}$ mice. (**C**) Histological assessment of tissue mineralization by Von Kossa staining. The parietal bone matrix of *En1*$^{+/-}$ mice is mostly mineralized at birth, whereas that of *En1* mutants is heavily perforated and undermineralized (arrows). (**D**) *En1*-null calvarial osteoblasts exhibit a poor capacity to mediate extracellular marix mineralization over the course of a 21-day differentiation period. (**E**) ALP activity, assessed spectrometrically between 7-21 days of culture, is 2- to 3-fold higher in wild-type osteoblasts than in *En1*$^{-/-}$ cells. Scale bar: 0.1 mm in C.

developmental stage exhibited normal *Opn* expression in ectoperiosteal and endosteal osteoblasts, terminally differentiated osteocytes continued to express this gene inappropriately (4C, insets, arrowheads). Third, the calvarial expression of *osteocalcin* (*Ocn*), a specific marker of late osteoblast differentiation, was almost abolished in *En1* mutants. In corroboration, the expression of *Ocn* and *bone sialoprotein* (*Bsp*), an additional marker of the mature osteoblast, was impaired in cultures of differentiating *En1*$^{-/-}$ calvarial osteoblasts (Fig. 4D). These findings suggest that *En1* functions beyond early osteogenesis to regulate the expression of the late osteoblastic genes commonly associated with matrix mineralization. In addition, the morphological changes within the suture suggest that EN1 may regulate osteoprogenitor proliferation and/or differentiation.

En1 differentially regulates osteoprogenitor proliferation in distinct sutures

In addition to impaired osteoblast differentiation, another contributing factor to the observed frontal foramina of *En1*$^{-/-}$ calvariae could be a reduction in the proliferative capacity of cells populating the osteogenic fronts of the frontal bones. We therefore examined the proliferation of cells within the rudimentary frontal bones and osteogenic fronts of the interfrontal suture by BrdU labeling wild-type and *En1*$^{-/-}$ embryos between E12.5 and E18.5. Although no significant difference in proliferation was observed between E12.5 and E16.5 (Fig. 5A,B; data not shown), a significant decrease in BrdU incorporation was observed in the interfrontal sutures of *En1*$^{-/-}$ calvariae at E18.5. These results suggest that *En1* function is important for osteoprogenitor proliferation following, but not prior to, interfrontal suture formation. The lack of *En1* expression in the interfrontal osteoprogenitors, however, implies that *En1* affects their proliferation through indirect mechanisms.

We next evaluated BrdU incorporation within the coronal sutures of wild-type and *En1*$^{-/-}$ calvariae at E18.5. The wild-type coronal suture characteristically comprised a thin layer of mesenchyme containing relatively few proliferating cells at the osteogenic fronts of the overlapping parietal and frontal bones (Fig. 5C). By contrast, the *En1*$^{-/-}$ suture was considerably thickened and hypercellular, and

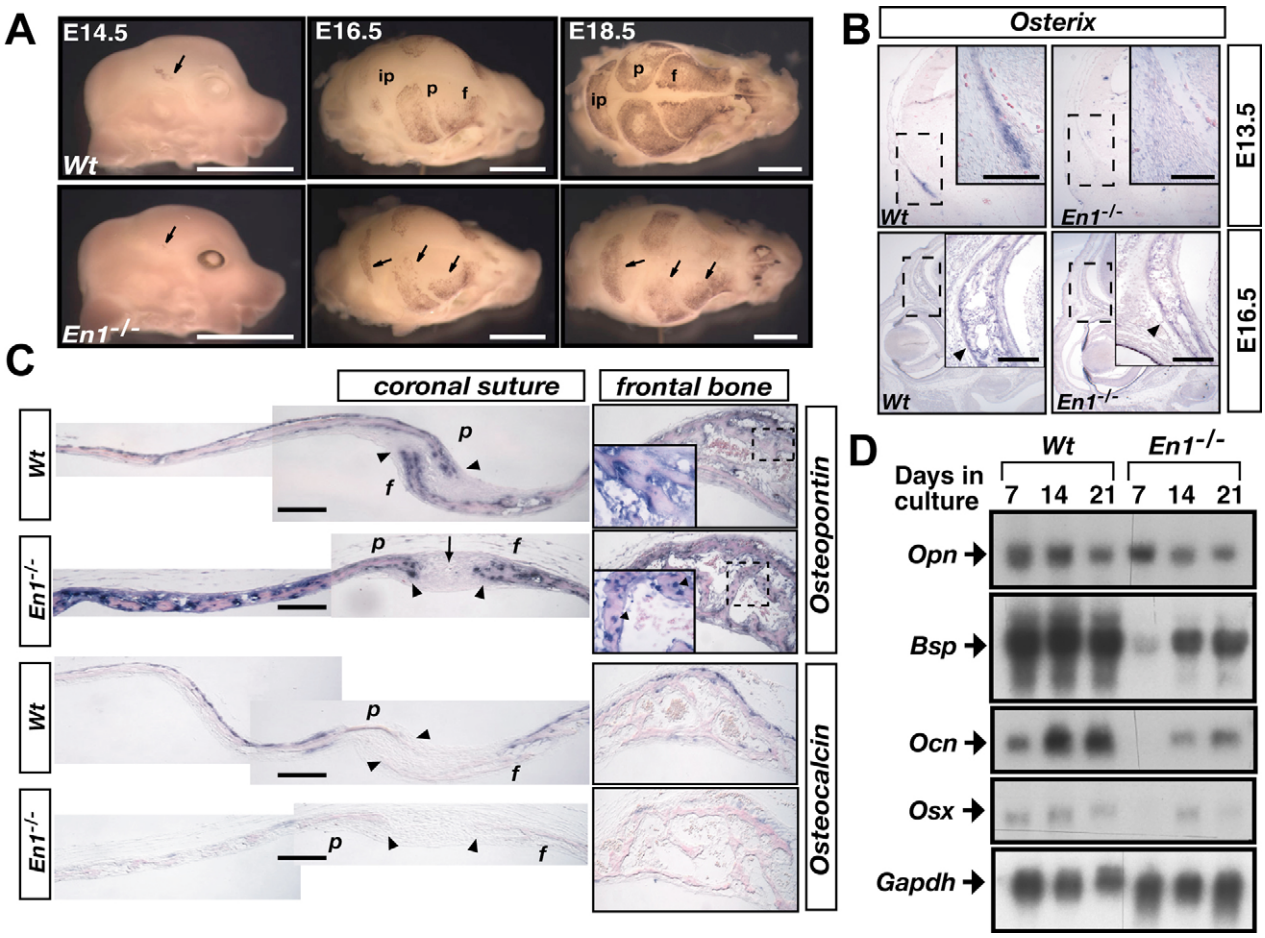

Fig. 4. Impaired calvarial osteogenesis in *En1* mutant mice. (**A**) Whole-mount in situ hybridization analysis of calvarial *Opn* expression, showing that *En1*$^{-/-}$ embryos exhibit a delay in the commencement of osteogenesis (E14.5), followed by deficient osteoblast differentiation (arrows, E16.5-E18.5). (**B**) Section ISH analysis demonstrating the absence of *Osx* expression in the mesenchyme of *En1*$^{-/-}$ calvariae at E13.5. At E16.5, *Osx* is expressed by wild-type osteoblasts of the frontal bone, but is reduced in *En1*$^{-/-}$ osteoblasts (arrowheads). (**C**) Analysis of osteopontin (*Opn*) and osteocalcin (*Ocn*) on sagittal sections of wild-type and *En1*$^{-/-}$ calvariae at P1. Although *Opn* normalizes to wild-type levels in *En1*$^{-/-}$ calvariae postnatally, it is aberrantly expressed by terminally differentiated osteocytes of the mutant frontal bones (inset, arrowheads). By comparison, normal *Ocn* expression in ectoperiosteal osteoblasts is nearly abolished in *En1*$^{-/-}$ calvariae. (**D**) Northern blot analysis of *Opn*, *Bsp*, *Ocn* and *Osx* expression by calvarial osteoblasts over a 21-day culture period. *p*, parietal bone; *f*, frontal bone. Scale bars: 1.5 mm in A; 0.1 mm in B; 0.2 mm in C.

contained increased numbers of BrdU-positive cells. These findings indicate that *En1* plays differential roles in regulating osteoprogenitor proliferation within distinct suture types.

En1 is required for mediating FGF signaling during calvarial bone formation

In order to assess *En1* function within the context of known regulators of cranial osteogenesis, we analyzed components of the fibroblast growth factor (FGF) signaling pathway in wild-type and *En1*-null calvariae. Like EN1, this pathway impacts on both cellular proliferation and differentiation during calvarial bone formation. We first compared the expression pattern of *Fgfr1* and *Fgfr2* between wild-type and *En1* mutants on sagittal sections of newborn calvariae. In the wild type, *Fgfr1* expression was observed in ectoperiosteal osteoblasts lining the calvarial bones and the endosteal osteoblasts of the frontal bone trabeculae, but was excluded from the osteogenic fronts and mesenchyme of the coronal suture (Fig. 6A). By comparison, *Fgfr2* was co-expressed with *Fgfr1* in wild-type osteoblasts in most locations; however, it was also detected within sutural osteoprogenitors. *En1* mutants displayed a similar pattern of receptor expression in bone-lining osteoblasts with one exception: *Fgfr1* and, more significantly, *Fgfr2* were strongly upregulated within the coronal sutural mesenchyme. Examination of *Fgfr1* and *Fgfr2* in wild-type and *En1*$^{-/-}$ cultured calvarial osteoblasts showed that both receptors were upregulated over the course of a 21-day differentiation period (Fig. 6B); however, no differences in relative expression between the two populations were observed by semi-quantitative assessment (data not shown). Sharing phenotypic similarities with *En1* mutants, *Fgf18*$^{-/-}$ mice display cranial ossification defects as a result of impaired osteoprogenitor proliferation and osteoblast differentiation (Ohbayashi et al., 2002). We found comparable expression of *Fgf18* in sutural osteoprogenitors and mature calvarial periosteal osteoblasts between wild-type and *En1*$^{-/-}$ mice. These findings suggest that *En1* is crucial for attenuating *Fgfr1* and *Fgfr2* expression within the coronal suture mesenchyme, and raises the possibility that *En1* regulates osteoprogenitor proliferation through FGFR signaling.

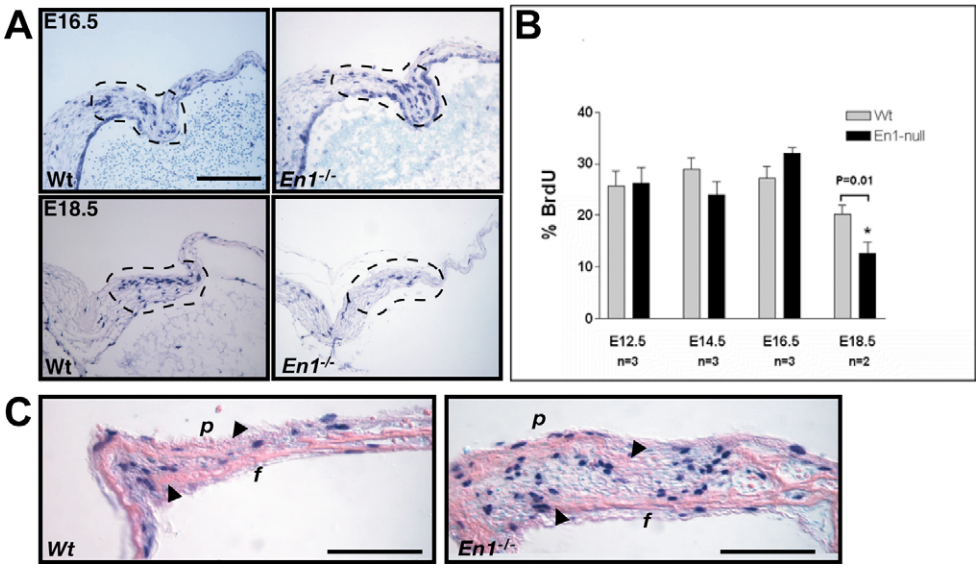

Fig. 5. Osteoprogenitor proliferation at the interfrontal and coronal sutures of wild-type and *En1*$^{-/-}$ embryos between E12.5-E18.5.
(**A**) Immunohistochemical detection of BrdU-labeled cells within the interfrontal sutures (dashed outline). (**B**) Proliferation was numerically assessed as the fraction of labeled to non-labeled cells. A specific reduction in osteoprogenitor proliferation was observed in *En1*$^{-/-}$ calvariae at E18.5. (**C**) Enhanced osteoprogenitor proliferation within the coronal suture (arrowheads) of *En1* mutants at E18.5. *p*, parietal bone; *f*, frontal bone. Scale bar: 0.2 mm.

In addition, we examined the possibility that the impact of *En1* on osteoblast differentiation may be mediated through regulating events downstream of FGFR signaling. Members of the *Sprouty* (*Spry1-Spry4*) gene family encode intracellular antagonists of receptor tyrosine kinases (RTKs) and are also transcriptional targets of FGF signaling in vertebrates (Minowada et al., 1999). As *Spry1* and *Spry2* are commonly expressed in FGF-responsive tissues (Minowada et al., 1999; Zhang et al., 2001), we examined their expression in the calvarial bones of wild-type and *En1*$^{-/-}$ mice. Although *Spry1* was not detected in wild-type or *En1*$^{-/-}$ calvariae (data not shown), *Spry2* was expressed by wild-type ectoperiosteal osteoblasts, osteoprogenitors of the coronal suture, and, to a lesser extent, endosteal osteoblasts of the frontal bone trabeculae (Fig. 6C). In contrast to the expression in wild-type calvariae, we found almost no expression of *Spry2* in parallel sections of *En1* mutants. This finding, together with the FGF ligand/receptor expression data, suggests that EN1 is required for regulating the signal transduction events downstream of FGFR that are necessary for activating *Spry2* transcription (see Fig. 6E for proposed model).

The induction of *Spry2* expression is mediated in part by the ERK-signaling cascade downstream of FGFR (Ozaki et al., 2001). More importantly, ERK phosphorylation is crucial for promoting FGFR-mediated osteogenesis in calvarial cell and organ cultures (Kim et al., 2003; Xiao et al., 2002). To evaluate the role of this signal transduction pathway in vivo, we used an antibody specific to the phosphorylated form of ERK (pERK) to probe sections of calvarial bone. As shown in Fig. 6D, we observed weak pERK expression in the osteogenic fronts of the parietal and frontal bones of wild-type and *En1*-null calvariae, whereas no activity was detected in periosteal osteoblasts of either genotype. By contrast, strong pERK activity was observed in the endosteal osteoblasts of wild-type frontal bone trabeculae at E16.5 (not shown) and P1. Strikingly, the number of endosteal osteoblasts exhibiting pERK activity in *En1*$^{-/-}$ calvariae was significantly reduced. These in vivo findings show that *Spry2* and pERK lie within distinct spatial domains of calvarial bone, which strongly suggests that signal transduction pathways other than the ERK/MAPK cascade mediate osteoblastic induction of *Spry2* downstream of FGFs. In addition, ERK activity correlates with advanced osteoblast maturation, and its deficiency in *En1*$^{-/-}$ calvariae suggests that impairment in FGF signaling might be a contributing factor to the perturbed osteogenic differentiation that characterizes *En1* mutants.

Increased resorption and aberrant osteoclast activation in *En1*$^{-/-}$ calvarial bone

In addition to impaired osteogenesis, increases in osteoclast number or activity could potentially contribute to the calvarial osteopenia in *En1* mutants. Our observations of multiple perforations within the mature frontal and parietal bones of *En1*$^{-/-}$ skulls suggested an osteolytic process (Fig. 6A). This prompted us to evaluate osteoclast activity in wild-type and *En1*$^{-/-}$ calvariae by using whole-mount staining for TRAP activity. TRAPs are produced by both mono- and multi-nuclear activated osteoclasts, and are localized primarily to the osteoclast ruffled border and the extracellular resorptive space (Minkin, 1982). We observed that TRAP-staining in the parietal bones of wild-type mice at P5 was stronger near the bone margins, whereas areas distant from the sutures stained less intensely (Fig. 6B). In contrast to wild-type bones, the analogous bones of *En1*$^{-/-}$ mice showed uniform TRAP activity throughout. Histological analysis of coronal sections of wild-type calvariae at P1 confirmed that osteoclasts were restricted to areas of mature trabecularized bone, and predominantly occupied the endocranial surfaces (Fig. 6C, arrows). By comparison, *En1*$^{-/-}$ cranial bones displayed significantly more TRAP^{+}-osteoclasts, which were often larger and located along trabeculae distant from the endocranial surface (Fig. 6C, arrowheads). Substantiating these observations, quantitative histomorphometry performed on E18.5 and P1 calvariae showed increased osteoclast numbers in *En1* mutants, consistent with the hypothesis that dysregulated osteoclastogenesis underlies the osteolytic phenotype of *En1* null calvariae (E18.5: wild type, 17.11±1.559, *n*=9; *En1* null, 47.11±2.796, *n*=9; P1: wild type, 46.83±5.974, *n*=6; *En1* null, 76.50±2.766, *n*=6). These results suggest that increased osteoclast numbers may be a contributing factor to the osteopenic phenotype of *En1* mutants.

To determine whether increased osteoclast recruitment in *En1* mutants results from alterations in known osteoclastogenic regulators, we examined the expression of receptor activator of NFκB ligand (RANKL; TNFSF11 – Mouse Genome Informatics), a TNF-related cytokine that promotes osteoclast differentiation

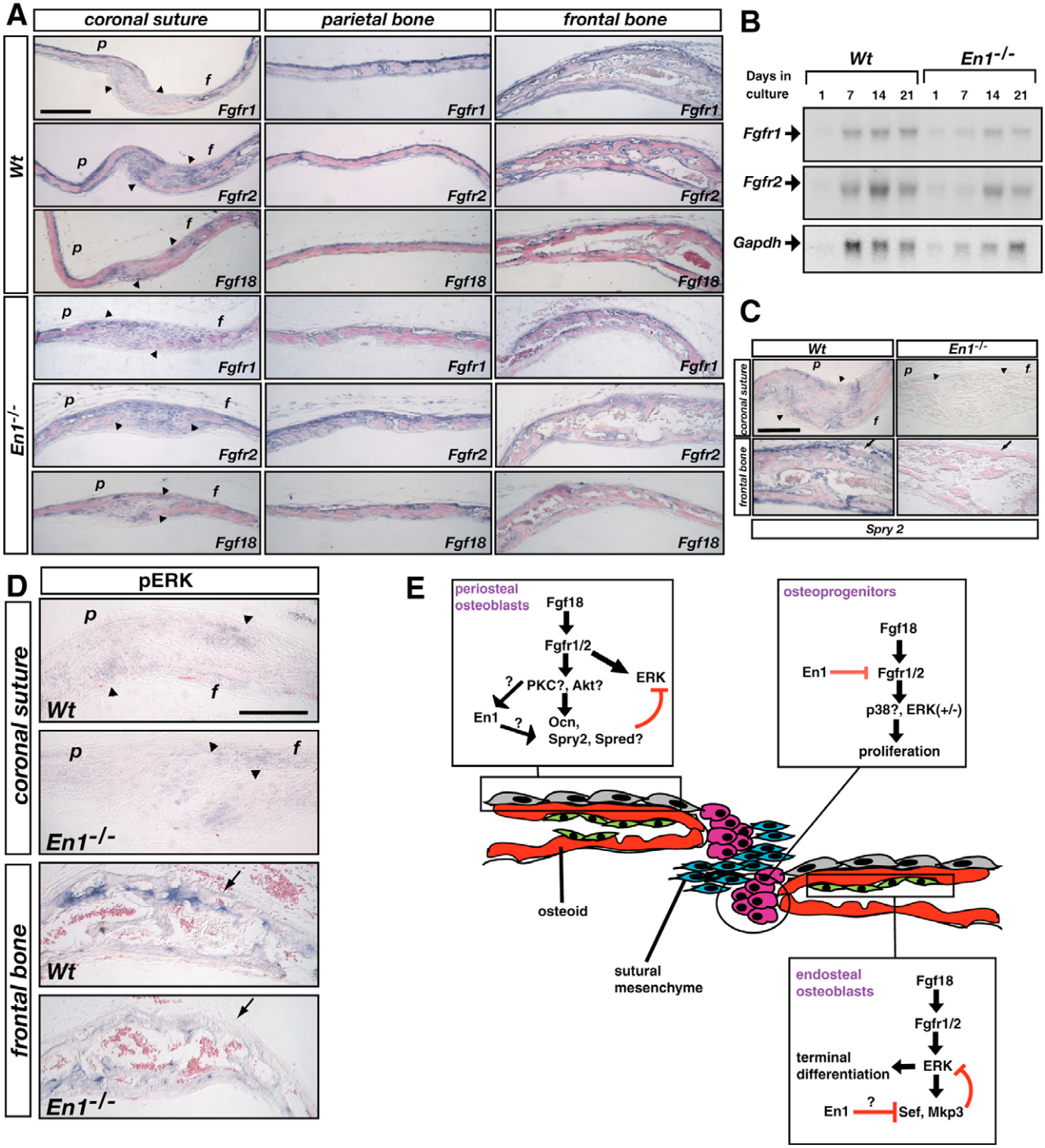

Fig. 6. Reduced FGF signaling in *En1* mutant calvariae. (**A**) Expression of *Fgfr1*, *Fgfr2* and *Fgf18* in the coronal suture, and the parietal and frontal bones, at P1. *Fgfr1*, *Fgfr2* and *Fgf18* are co-expressed by ectocranial periosteal osteoblasts in the parietal and frontal bones. *Fgfr1* is absent from wild-type sutural osteprogenitors, whereas *Fgfr2* and *Fgf18* are expressed at these locations. In *En1* mutants, *Fgfr1* and *Fgfr2* are upregulated in the sutural mesenchyme (arrowheads indicate the parietal and frontal bone margins). (**B**) Northern blot analysis of *Fgfr1* and *Fgfr2* expression during the differentiation of cultured primary calvarial osteoblasts. Relative to *Gapdh*, no significant differences in *Fgfr1* or *Fgfr2* levels were observed in wild-type and *En1*$^{-/-}$ osteoblasts. (**C**) Loss of *Spry2* expression in the calvarial osteoblasts (arrow) and osteoprogenitors (arrowheads) of *En1* mutants. (**D**) Expression of pERK in calvarial bones of wild-type and *En1*$^{-/-}$ mice at P1. pERK displays strong activity in wild-type endosteal osteoblasts lining the frontal bone trabeculae, but is only weakly present at the osteogenic fronts (arrowheads), and is absent from ectoperiosteal osteoblasts (arrow). *En1* mutants display severely reduced pERK in endosteal osteoblasts. *p*, parietal bone; *f*, frontal bone. (**E**) Proposed model for the interactions between EN1 and FGF signaling events during calvarial osteogenesis. The calvarial bone is postulated to divide into spatial subdomains (sutural osteoprogenitors, ectoperiosteal osteoblasts, endosteal osteoblasts) that respond differentially to FGF signaling. Scale bars: 0.2 mm in A; 0.1 mm in C; 0.1 mm in D.

(Takahashi, 2002). Primary osteoblasts were released from wild-type and *En1* mutant calvariae at P2 and cultured for 7 days under conditions promoting mineralization. Under these conditions, *Rankl* was strongly upregulated in $En1^{-/-}$ cells (Fig. 6E,F). By comparison, expression of osteoprotegerin (*Opg*), a decoy receptor and inhibitor of *Rankl*, was comparable between wild-type and $En1^{-/-}$ cells. These findings point toward an additional and non cell-autonomous role for *En1* in regulating osteoclast differentiation and/or recruitment.

DISCUSSION

En1 regulates osteogenic differentiation during calvarial bone formation

The present study demonstrates a novel and important requirement for *En1* in regulating calvarial osteogenesis. Although not exhibiting gross patterning defects of the skull vault, $En1^{-/-}$ mice develop calvarial bone hypoplasia and osteopenia, increased suture patency, and postnatal malocclusion. As both CNC- and mesodermally-derived bones are similarly affected in *En1* mutants, it is unlikely that the phenotypes observed resulted from an impairment in CNC cell migration or determination (Jiang et al., 2002). Furthermore, *En1* expression initiates in the calvarial skeletogenic mesenchyme at E11.5, several days following the cessation of CNC migration (Jiang et al., 2002). Interestingly, *En1* expression temporally precedes that of the osteogenic determinant *Osx*, and, in the absence of *En1*, the onset of *Osx* expression is delayed. As *Osx* is necessary for potentiating the osteogenic fate of the skeletogenic mesenchyme (Nakashima et al., 2002), its perturbed expression provides a mechanistic basis for the delayed calvarial ossification in $En1^{-/-}$ mice. Furthermore, that *Osx* expression remains impaired in *En1*-null osteoblasts, suggests that *En1* also lies upstream of *Osx* during later phases calvarial osteogenesis, and thus mediates distinct functions in osteoblast differentiation.

Consistent with a later role for *En1* in osteoblast differentiation and function, our quantitative morphometric analysis showing reduced bone volume in *En1* mutants is indicative of generalized calvarial osteopenia. In correlation with this, $En1^{-/-}$ osteoblasts were deficient in mediating osteoid mineralization and exhibited reduced ALP activity, an enzyme that is essential for this process (Fedde et al., 1999; Murshed et al., 2005). Corroborating its role in osteoblast function, *En1* is expressed postnatally by ectoperiosteal and endosteal osteoblasts, as well as by terminally differentiated osteocytes. Moreover, ablation of *En1* results in impaired *Ocn* and *Bsp* expression, genes that are normally associated with advanced osteoblast differentiation (Aubin, 2002). *Ocn* expression has also been shown to be dependent on *Osx* (Nakashima et al., 2002). However, the fact that *Opn* expression in $En1^{-/-}$ calvariae is restored to wild-type levels postnatally, suggests that early phases of osteoblast differentiation can eventually occur in the absence of *En1* (Fig. 4C,D). Taken together, our results strongly indicate that, in addition to its role in early osteogenic commitment, *En1* is directly required for mediating late calvarial osteoblast differentiation and bone matrix mineralization.

EN1 interacts with FGF signaling to regulate osteoblast differentiation and proliferation

Our study demonstrates that the $En1^{-/-}$ calvarial phenotype arises in part from alterations in FGF signaling. The FGF signaling pathway is intimately involved in regulating calvarial osteogenesis, where it plays a major function in promoting osteoblast differentiation (Ornitz and Marie, 2002). Activating mutations in *FGFR1* or *FGFR2* that cause craniosynostosis in humans are recapitulated by knock-in mouse models that exhibit enhanced osteoblast differentiation (Eswarakumar et al., 2004; Zhou et al., 2000). Conversely, selective ablation of *Fgfr2IIIc*, the mesenchymal splice variant of the *Fgfr2*, results in impaired calvarial ossification and osteoblast differentiation (Eswarakumar et al., 2002). Mice lacking FGF18 ligand share phenotypic similarities with *En1* mutants: they display defective calvarial ossification and delayed terminal osteoblast differentiation (Ohbayashi et al., 2002). Thus, the associated alterations in osteoblastic differentiation affecting $En1^{-/-}$ calvariae are consistent with an underlying perturbation in FGF signaling. Importantly, our data demonstrate that these effects are not due to changes in the osteoblastic expression of *Fgfr1*, *Fgfr2* and *Fgf18*, but are rather attributed to hampered events downstream of FGF receptor activation.

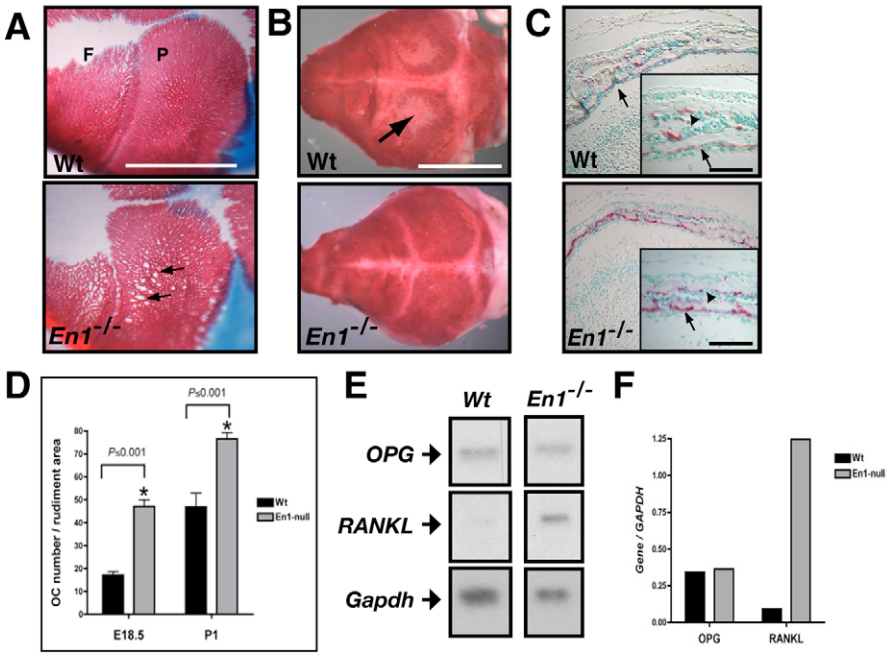

Fig. 7. Increased remodeling and osteoclast recruitment in $En1^{-/-}$ calvariae. (**A**) Alizarin Red stained wild-type and $En1^{-/-}$ calvariae at P1 showing multiple perforations in the mutants (arrows). (**B**) Whole-mount detection of osteoclast-specific TRAP activity in calvariae at P5, depicting increased staining in *En1* mutants (compare with wild type, arrow). (**C**) Histological detection of $TRAP^{+}$ osteoclasts located along the endocranial (arrows) and trabecular (arrowheads) bone surfaces of the frontal bones at P1. (**D**) Quantitative histomorphometric assessment of osteoclast number in sections of wild-type and $En1^{-/-}$ frontal bones. (**E,F**) Northern blot and semi-quantitative analysis of *OPG* and *Rankl* expression in cultured calvarial osteoblasts. At day 7 of postconfluent growth, wild-type and $En1^{-/-}$ cells express similar levels of *OPG*, whereas expression of *Rankl* is strongly upregulated in mutant osteoblasts. F, frontal bone; P, parietal bone. Scale bars: 2 mm in A,B; 0.1 mm in C.

Two lines of evidence indicate that *En1* regulates signaling mediated by FGFRs. First, the activation ERK, normally restricted to the mature endosteal osteoblasts of wild-type calvarial bone, is severely impeded in *En1* mutants (Fig. 7D,E). Second, *En1* ablation results in loss of the FGF target gene *Spry2* in ectoperiosteal osteoblasts. The significance of limiting pERK to the most mature osteoblasts in bone is not entirely clear; however, it correlates with previous studies ascribing inductive functions for pERK during calvarial bone formation and advanced osteoblast differentiation (Kim et al., 2003; Xiao et al., 2002). Therefore, *En1* might mediate the terminal differentiation of endosteal osteoblasts by potentiating ERK activity (Fig. 6E). The ERK/MAPK cascade has been demonstrated as an important intracellular mediator of FGF-signaling in multiple developmental contexts. Interestingly, recent studies have shown that ERK activity is frequently limited to the sub-domains of FGF-responsive regions (Corson et al., 2003). Reciprocally, a number of FGFR inhibitors (*Sprouty*, *Sef*, *Spred*, *Mkp3*) are induced by FGF signaling, and are expressed in patterns consistent with their role in restricting ERK activity (Kawakami et al., 2003; Lin et al., 2002; Minowada et al., 1999; Wakioka et al., 2001; Zhang et al., 2001). Here, we present novel evidence demonstrating the existence of select domains for ERK activation in calvarial bone. Accordingly, we found that *Spry2*, a biochemical antagonist of the ERK/MAPK pathway (Hanafusa et al., 2002), is preferentially expressed by ectocranial osteoblasts and sutural osteoprogenitors, indicating that it may play a role in spatially modulating FGF responsiveness. However, the fact that loss of *Spry2* expression in $En1^{-/-}$ calvariae did not result in enhanced ERK activity in ectoperiosteal osteoblasts suggests that other antagonists may also modulate ERK. Indeed, in the developing limb bud, *Fgf8* signal responsiveness is attenuated in the mesenchyme by the cooperative activities of *Spry1* and *Mkp3*, limiting ERK activation to the overlying ectoderm (Corson et al., 2003; Kawakami et al., 2003; Minowada et al., 1999). We postulate that EN1 regulates the establishment of a negative-feedback loop within the calvarial skeletal rudiments by inducing the expression of *Spry2* in ectocranial osteoblasts, while potentially repressing the expression of other FGFR-signaling attenuators (e.g. *Mkp3*, *Sef*) in endosteal osteoblasts (Fig. 6E). Consequently, loss of *En1* function would result in the observed reduction in endosteal pERK (Fig. 6E). Furthermore, *En1* may regulate alternative FGF-signaling effectors known to affect osteoblast differentiation, such as p38 MAPK or PKC (Kozawa et al., 1999; Lemonnier et al., 2000; Lomri et al., 2001). A precise temporal and spatial delineation of these intracellular pathways will enable a better understanding of how osteoblastic differentiation is coordinated by EN1 and FGFs.

Differential effects of *En1*-ablation on osteoprogenitor proliferation

In addition to defects in calvarial osteoblast differentiation, altered osteoprogenitor proliferation is likely to contribute to the frontal foramina and gaping of the coronal sutures in *En1* mutants. The interfrontal suture forms late in development (E18.5-P1) through the gradual approximation of the frontal bone margins. Prior to suture closure at the cranial apex, the bone margins are interposed by extensive mesenchyme that would preclude the effectiveness of regulatory signals between the opposing osteogenic fronts. By contrast, the coronal suture is established early (E12-E14) along the CNC-mesodermal lineage boundary, where a thin layer of mesenchyme maintains a consistent separation between the closely juxtaposed, but non-fusing frontal and parietal bones (Jiang et al., 2002). Together with recent studies showing distinct FGF-responsiveness between the sutures, it is reasonable to infer that the interfrontal and coronal sutures represent unique sites for intramembranous bone formation (Ignelzi et al., 2003).

Interestingly, *En1* is selectively expressed by the interfrontal sutural mesenchyme, but is excluded from the osteoprogenitors (Fig. 1). We postulate that the proliferative defect in the interfrontal suture, which becomes apparent only by E18.5, stems from deficient commitment of the mesenchyme rather than from a direct requirement for *En1* in promoting osteoprogenitor mitosis. By comparison, in the coronal suture *En1* is expressed by osteoprogenitors along the opposing bone margins, and its absence results in increased proliferation and mesenchymal thickening. This suggests that EN1 is a direct negative regulator of osteoprogenitor proliferation at this location. Previous studies indicated that *Fgfr1* and *Fgfr2* elicit a differential mitogenic response to FGFs in coronal suture osteoprogenitors (Ignelzi et al., 2003; Iseki et al., 1997; Iseki et al., 1999). Moreover, specific activating mutations in *Fgfr2* are known to enhance proliferation within this population (Eswarakumar et al., 2004). It is therefore possible that upregulation of *Fgfr2* and *Fgfr1* in the coronal sutures of $En1^{-/-}$ calvariae results in the enhancement of an FGF-mediated mitotic response.

EN1 affects calvarial bone remodeling by regulating osteoclastogenesis

Following the completion of calvarial morphogenesis, prenatal and postnatal cranial bone expansion is modulated by the resorptive activity of osteoclasts. Calvarial osteoclast activity has been shown to initiate as early as E16.5 in the mouse and is important for the modeling and remodeling of the skull vault during brain growth (Rice et al., 1997). Here, we show that loss of *En1* function results in focal calvarial osteolytic lesions that correlate with a significant increase in osteoclast number and activation. Consistent with resorptive bone loss, *En1*-ablated mice exhibit reduced calvarial bone volume and increased marrow space. In agreement with this, osteoclast numbers within the calvarial bone rudiments of $En1^{-/-}$ mice are significantly increased.

Osteoclast differentiation and activation occurs in response to specific cytokines and growth factors secreted by osteoblasts and their progenitors within the bone marrow microenvironment (Takahashi, 2002). By binding to its cellular receptor RANK, RANKL mediates signal transduction pathways that result in overt osteoclast differentiation. Osteoclastogenesis, in turn, is balanced by the osteoblast-specific expression and secretion of osteoprotegrin (OPG), a decoy receptor capable of binding and inhibiting RANKL. Curiously, osteoprogenitors and less differentiated osteoblasts have been shown to express higher levels of RANKL and to support osteoclastogenesis to a greater extent than differentiated osteoblasts (Atkins et al., 2003; Gori et al., 2000). Accordingly, we have demonstrated that *En1*-null osteoblasts, arrested in an early stage of differentiation, display a specific increase in RANKL expression. Furthermore, the aberrant expression of *Opn*, a known osteoclastic chemoattractant, by terminally differentiated osteocytes has been associated with increased bone remodeling and osteoclast recruitment (Terai et al., 1999; Yamazaki et al., 1999). Interestingly, we observed abnormal *Opn* expression in $En1^{-/-}$ calvarial osteocytes, suggesting an additional mechanism for increased osteoclast recruitment in these animals. In addition to its direct role in regulating calvarial osteogenesis, these findings demonstrate a novel role for *En1* in inhibiting osteoclastogenesis by osteoblasts. The *En1*-null mouse thus provides a valuable tool for studying the interactions between osteogenesis and osteoclastogenesis during intramembranous ossification.

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