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Cell autonomous roles of Nedd4 in craniofacial bone formation

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ABSTRACT

Nedd4 is an E3 ubiquitin ligase that has an essential role in craniofacial development. However, how and when Nedd4 controls skull formation is ill defined. Here we have used a collection of complementary genetic mouse models to dissect the cell-autonomous roles of Nedd4 in the formation of neural crest cell derived cranial bone. Removal of Nedd4 specifically from neural crest cells leads to profound craniofacial defects with marked reduction of cranial bone that was preceded by hypoplasia of bone forming os-teoblasts. Removal of Nedd4 after differentiation of neural crest cells into progenitors of chondrocytes and osteoblasts also led to profound deficiency of craniofacial bone in the absence of cartilage defects. Notably, these skull malformations were conserved when Nedd4 was specifically removed from the osteoblast lineage after specification of osteoblast precursors from mesenchymal skeletal progenitors. We further show that absence of Nedd4 in pre-osteoblasts results in decreased cell proliferation and altered osteogenic differentiation. Taken together our data demonstrate a novel cell-autonomous role for Nedd4 in promoting expansion of the osteoblast progenitor pool to control craniofacial development. Nedd4 mutant mice therefore represent a unique mouse model of craniofacial anomalies that provide an ideal resource to explore the cell-intrinsic mechanisms of neural crest cells in craniofacial morphogen-esis.

1. Introduction

Craniofacial malformations represent a significant clinical issue, with many cases requiring highly invasive and recurring surgical interventions throughout life. Unfortunately, the vast majority of these disorders have unknown genetic or pathological origin. These highly prevalent disorders manifest as a result of the com-plex array of cellular interactions and morphogenetic events re-quired for correct development of the craniofacial skeleton. In contrast to the mesodermal origin of the axial and appendicular skeleton, the mammalian skull requires the unique contribution of cells from the cranial neural crest (Trainor, 2013). In addition, the calvaria and the bones of the face form primarily through the process of intramembranous ossification in which mesenchymal progenitors differentiate directly into bone-forming osteoblasts without a cartilage precursor (Percival and Richtsmeier, 2013). Identifying the molecular mechanisms by which neural crest cells differentiate into osteoblasts and bone is critical to our under-standing of craniofacial development and the origins and potential treatments for craniofacial birth defects.

Cranial neural crest cells are multipotent cells that arise from the dorsal neural folds during early embryonic development

(Trainor, 2013). Following delamination from the neural tube, cranial neural crest cells migrate vast distances into the facial primordia where they receive instructive signals to initiate a transcriptional programme that ultimately leads to their differ-entiation into bone and cartilage (Jeong et al., 2004). In the first of a series of differentiation steps, neural crest cells entering the fa-cial primordia form mesenchymal progenitors of bone and carti-lage. Cell-type specificity is later initiated through the expression of the HMG box transcription factor *Sox9* in cartilage precursors (Bi et al., 2001) or the sequential activity of the runt family member *Runx2* (Komori et al., 1997) and the zinc finger transcription factor *Osterix* (*Osx*) (Nakashima et al., 2002) in osteoblast precursors. The outcome of these transcriptional programmes is the progression of precursor cell differentiation into bonafide chondroctyes or os-teoblasts that in turn secrete extracellular matrix components and mineral specific to cartilage or bone.

While our knowledge of neural crest cell and cranial bone formation is biased toward the roles of transcription factor net-works acting downstream of morphogenic gradients, intrinsic roles for post-translational modifications in neural crest cells are also coming to light (Wiszniak et al., 2013; Vermillion et al., 2014). Ubiquitination is a post-translational modification that instructs functional changes in target proteins by controlling degradation, localisation or biochemical properties (Rotin and Kumar, 2009). We recently identified an essential role for ubiquitination in neural crest cell development by demonstrating that the E3 ubiquitin

ligase Nedd4 plays an important role in cranial neural crest cells (Wiszniak et al., 2013). Nedd4 is expressed in neural crest cells as they delaminate from the neural tube and $Nedd4^{-/-}$ mice have profound deficiency of cranial ganglia and neural crest cell-derived cranial bones, as well as milder reductions in bone in the axial skeleton. As Nedd4 is essential for maintaining neural crest cell stem-cell identity and survival, our results suggested that the craniofacial defects may arise from a lack of neural crest cells at earlier developmental time points. However, whether Nedd4 is cell-autonomously required by neural crest cells or whether early neural crest cell deficiency underpins craniofacial defects remained unresolved. Here we have removed Nedd4 at various stages of cranial neural crest cell and bone development using Cre/LoxP technologies to answer these questions.

Consistent with our analyses in $Nedd4^{-/-}$ embryos we found that mice lacking Nedd4 specifically in neural crest cells had pronounced bone deficiency in the absence of notable cartilage defects. Our analyses at earlier embryonic stages further suggest
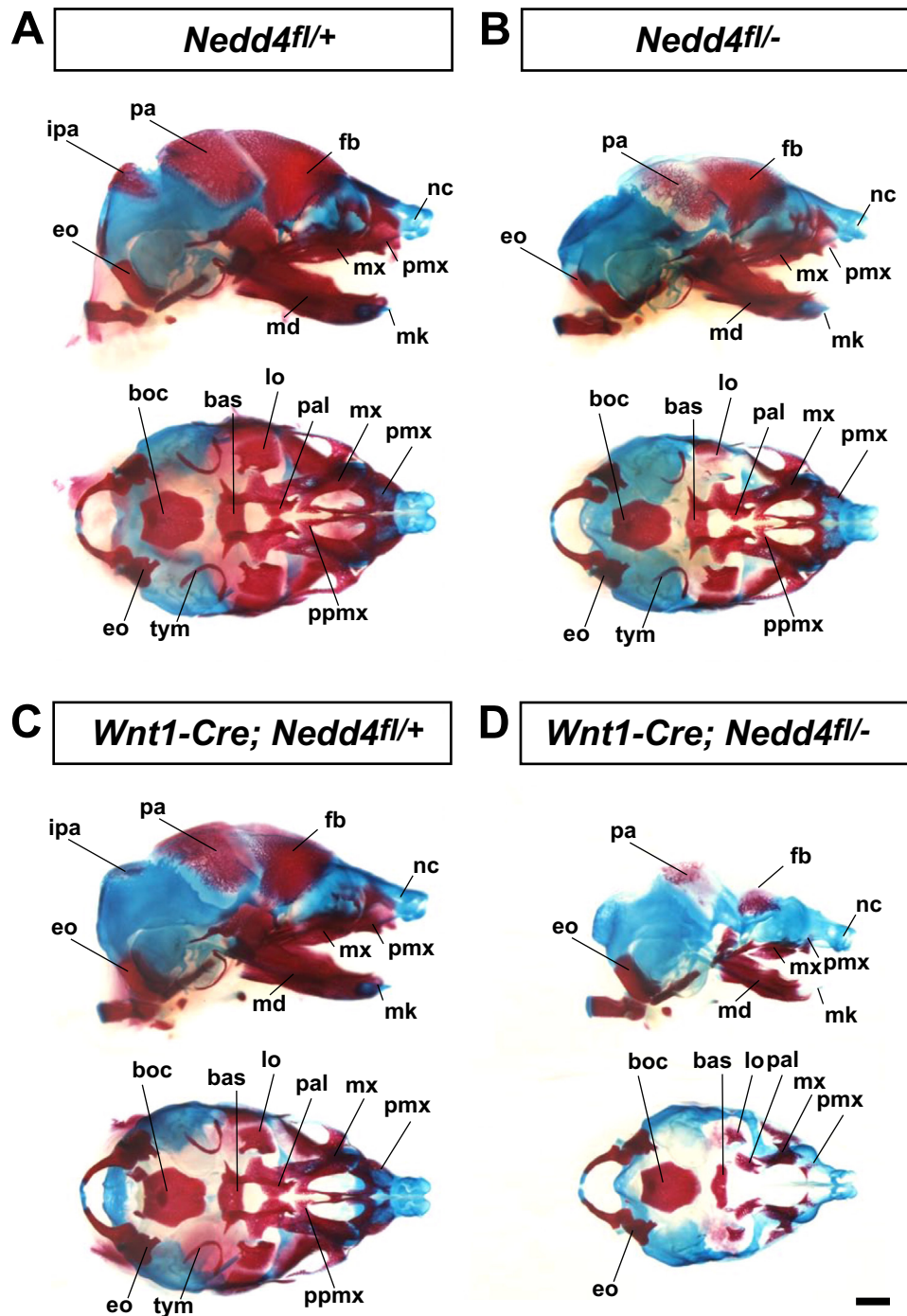

Fig. 1. Loss of *Nedd4* in neural crest cells and derivatives causes craniofacial defects. E17.5 littermate skulls stained with Alizarin red (bone) and Alcian blue (cartilage). A: *Nedd4*$^{fl/+}$ wildtype embryo. B: Loss of a single allele of *Nedd4* in *Nedd4*$^{fl/-}$ embryos causes a mild reduction in bone. C: Loss of a single allele of *Nedd4* specifically in neural crest-derived tissue in *Wnt1-Cre; Nedd4*$^{fl/+}$ embryos similarly causes a mild reduction in neural crest derived bone. D: Complete lack of *Nedd4* in neural crest-derived tissue in *Wnt1-Cre; Nedd4*$^{fl/-}$ embryos causes severe hypoplasia of craniofacial bone. bas, basisphenoid; boc, basioccipital; eo, exoccipital; fb, frontal bone; ipa, interparietal; lo, lamina obturans; md, mandible; mk, Meckel's cartilage; mx, maxillary; nc, nasal capsule; pa, parietal; pal, palatal; pmx, premaxillary; ppmx, palatal process maxillary; tym, tympanic. Scale bar=1 mm.

that the deficiency in bone deposition likely arises from a reduction in osteoblast progenitors. Removal of Nedd4 after the initial specification of neural crest cells toward mesenchymal progenitors of bone and cartilage, or after differentiation into osteoblast precursors also lead to pronounced deficiency of craniofacial bone, akin to our report in $Nedd4^{-/-}$ embryos. Analysis of osteoblast growth dynamics demonstrated significant reductions in cell proliferation and altered osteogenic differentiation. In addition to a role in maintaining the progenitor pool of neural crest cells at early stages of development, our results therefore identify a novel cell-autonomous role for Nedd4 in promoting proliferation of osteoblast precursors and their terminal differentiation into definitive osteoblasts and osteocytes. These findings further demonstrate that bone hypoplasia associated with Nedd4 deficiency arises from aberrant osteoblast formation rather than a reduction in neural crest cell numbers at earlier stages of development.

2. Results

2.1. Loss of Nedd4 in neural crest-derived tissue causes craniofacial defects

We have previously reported that complete loss of Nedd4 in $Nedd4^{-/-}$ mice causes craniofacial defects, with severe hypoplasia of neural crest-derived intramembranous bone (Wiszniak et al., 2013). This raised the hypothesis that given the developmental origin of these cranial bones, Nedd4 may be required cell-

autonomously in neural crest cells for correct craniofacial bone development. To address this, we removed Nedd4 specifically in the neural crest cell lineage by crossing a floxed Nedd4 allele to the Wnt1-Cre driver which is widely used for neural crest cell-specific gene deletion (Jiang et al., 2000). Consistent with our previous study, we found that deletion of a single copy of Nedd4, either in the whole embryo ($Nedd4^{fl/-}$, Fig. 1B) or specifically in the neural crest (Wnt1-Cre; $Nedd4^{fl/+}$, Fig. 1C) caused a mild general hypoplasia of bones in the skull at embryonic day (E) 17.5. Mild bone hypoplasia also persisted into adulthood in $Nedd4^{+/-}$ animals compared to wildtype littermates (Supp. Fig. 1). However, complete removal of Nedd4 in all neural crest-derived tissue (Wnt1-Cre; $Nedd4^{fl/-}$, Fig. 1D or Wnt1-Cre; $Nedd4^{fl/fl}$, Supp. Fig. 2) caused severe hypoplasia of craniofacial bone at E17.5, as well as clefting of the palate (Supp. Fig. 3A). Notably, the frontal bone, premaxilla, maxilla, tympanic, basisphenoid and palatine bone were markedly reduced. In contrast to $Nedd4^{fl/-}$ mice, the mesodermal derived bones, such as the parietal, were less affected in Wnt1-Cre; $Nedd4^{fl/+}$ embryos, which is expected given Wnt1-Cre does not drive expression and hence Nedd4 knockout in these tissues. The mild hypoplasia of mesodermal-derived intramembranous bones observed in Wnt1-Cre; $Nedd4^{fl/+}$ animals likely reflects a slight developmental delay in these embryos compared to the wildtype controls, given that the parietal and interparietal bones are only just beginning to ossify at E17.5, and variability of ossification in these bones is evident between wildtype littermates. Taken together, these results are consistent with our previous study, and as such the craniofacial defects observed in $Nedd4^{-/-}$ mice are
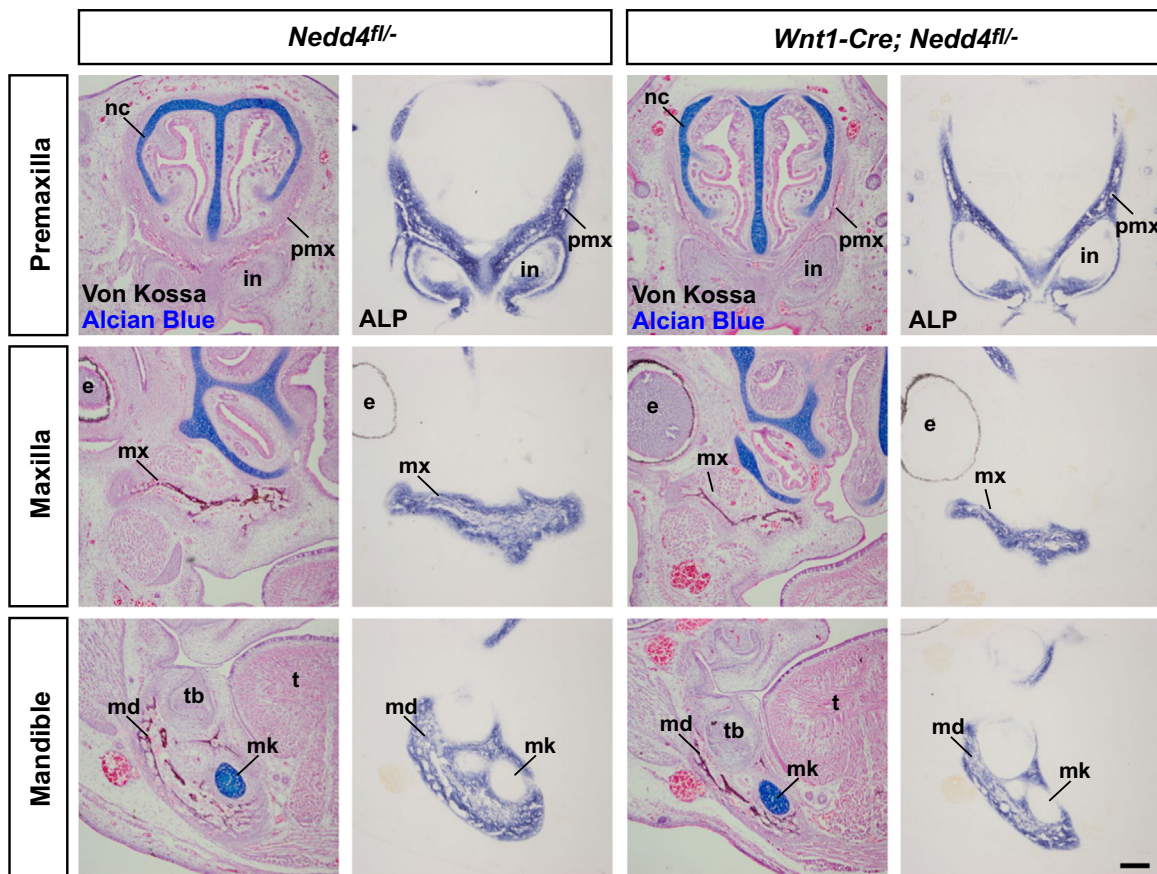

Fig. 2. Bone formation is reduced at E15.5 in Wnt1-Cre; $Nedd4^{fl/-}$ mutants. Serial frontal sections of E15.5 $Nedd4^{fl/-}$ control and Wnt1-Cre; $Nedd4^{fl/-}$ mutant embryos stained with Von Kossa and Alcian Blue (left panels) to mark mineralisation and cartilage respectively, and alkaline phosphatase (ALP) (right panels) to label osteoblast activity. Equivalent sections of control and mutant embryos are shown of the premaxilla (top), maxilla (middle) and mandible (bottom). Compared to controls, Wnt1-Cre; $Nedd4^{fl/-}$ mutants show less mineralised bone and ALP staining, whereas cartilage development is unaffected. nc, nasal cartilage; in, incisor; pmx, premaxilla; mx, maxilla; md, mandible; mk, Meckel's cartilage; tb, toothbud; t, tongue. Scale bar=200 μm.

recapitulated in the *Wnt1-Cre; Nedd4*$^{fl/-}$ model.

Bone formation was further examined earlier in development at E15.5 by histological analyses. Sections of *Nedd4*$^{fl/-}$ control and *Wnt1-Cre; Nedd4*$^{fl/-}$ mutant embryos stained with Von Kossa to mark mineralised bone and Alcian blue to mark cartilage revealed a dramatic reduction in bone formation throughout the skull, including the premaxilla, maxilla and mandible, with negligible effects on cartilage development (Fig. 2). Staining of serial sections with alkaline phosphatase (ALP), a marker of osteoblast activity, also demonstrated a marked reduction of osteoblasts in *Wnt1-Cre; Nedd4*$^{fl/-}$ mutant embryos (Fig. 2), which likely underlies the reduced bone deposition in these mutants.

2.2. Nedd4 is required in the osteoblast lineage for craniofacial bone formation

Given *Wnt1-Cre* is expressed in the earliest precursors of the craniofacial bones, the neural crest, beginning around E8.5, we sought to define the critical time at which Nedd4 plays a role in cranial bone development. Neural crest cells delaminate from the neural tube and migrate into the facial primordia where they form mesenchymal condensations at sites of the future facial bones. As development progresses, these mesechymal cells differentiate into pre-osteoblasts, and then osteoblasts, which function to deposit bone matrix that acts as a substrate for subsequent mineralisation (Komori, 2006).
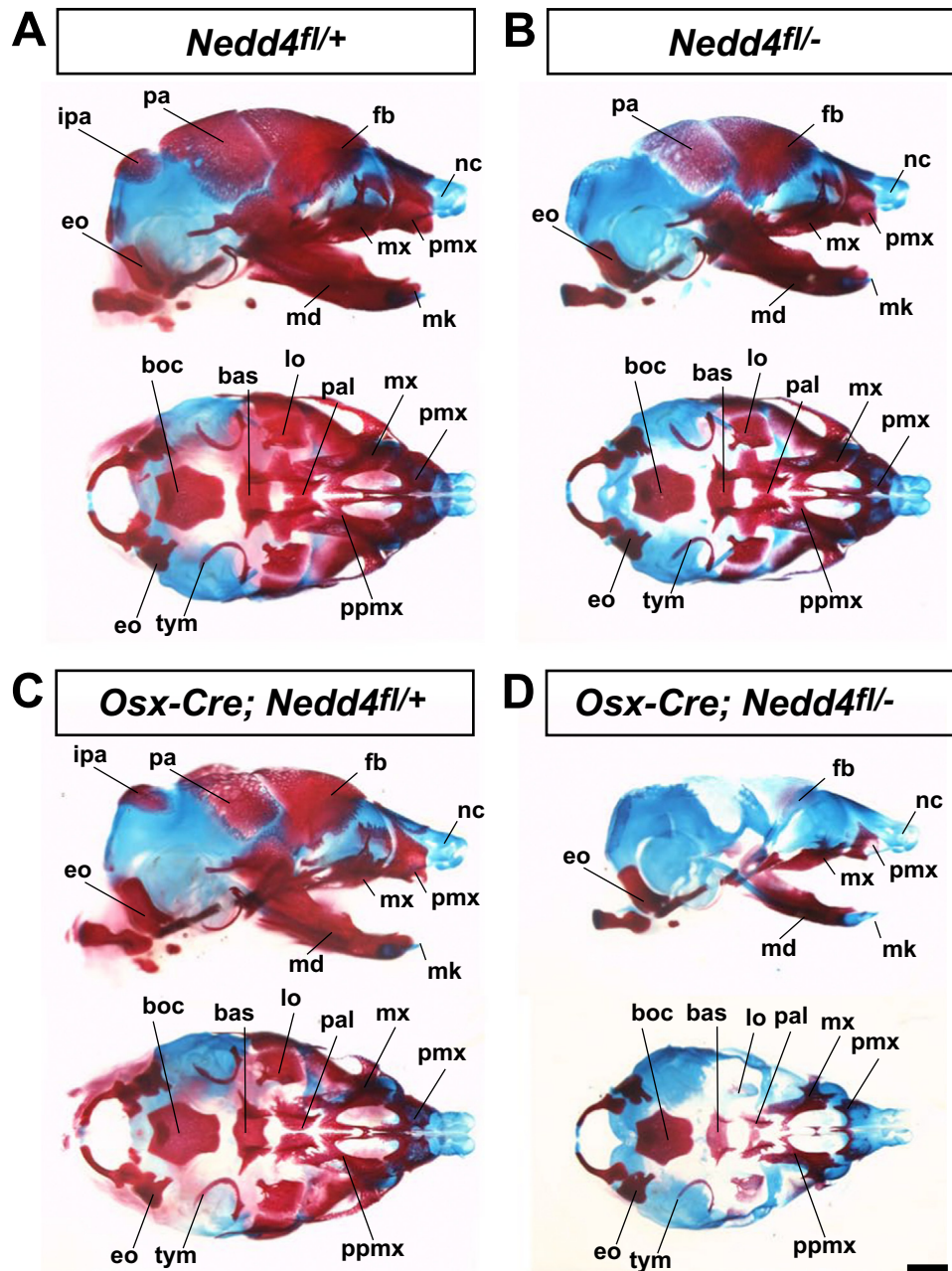

Fig. 3. *Nedd4* is required in the osteoblast lineage for craniofacial bone formation. E17.5 littermate skulls stained with Alizarin red (bone) and Alcian blue (cartilage). A: *Nedd4*$^{fl/+}$ wildtype embryo. B: Consistent with previous analyses, loss of a single allele of *Nedd4* in *Nedd4*$^{fl/-}$ embryos causes a mild reduction in bone. C: Loss of a single allele of *Nedd4* specifically in osteoblasts in *Osx-Cre; Nedd4*$^{fl/+}$ embryos similarly causes a mild reduction in bone. D: Complete lack of *Nedd4* in osteoblasts in *Osx-Cre; Nedd4*$^{fl/-}$ embryos causes severe hypoplasia of craniofacial bone. bas, basisphenoid; boc, basioccipital; eo, exoccipital; fb, frontal bone; ipa, interparietal; lo, lamina obturans; md, mandible; mk, Meckel's cartilage; mx, maxillary; nc, nasal capsule; pa, parietal; pal, palatal; pmx, premaxillary; ppmx, palatal process maxillary; tym, tympanic. Scale bar=1 mm.

To determine whether Nedd4 plays a role in bone development after neural crest cell formation and migration into the facial primordia, we removed Nedd4 in the facial mesenchyme using the *Prx1-Cre* driver, which directs Cre expression in mesenchymal lineages destined to form bone and cartilage (Logan et al., 2002). Similar to the phenotype observed in *Nedd4*$^{-/-}$ and *Wnt1-Cre; Nedd4*$^{fl/-}$ mutants (Supp. Fig. 4), we observed a dramatic reduction in cranial neural crest-derived and intramembranous bone in *Prx1-Cre; Nedd4*$^{fl/-}$ mutants (Supp. Fig. 5). Specifically, there was a marked reduction of the frontal bone, premaxilla, maxilla, tympanic, basisphenoid and palatine bone in these mice. This suggests that the cranial bone defects observed in *Nedd4*$^{-/-}$ mice are unlikely due to a defect in early neural crest cell development, and likely lie later in the bone formation pathway.

To analyse the role of Nedd4 in later stages of bone development, we removed Nedd4 specifically in the osteoblast lineage using the *Osx-Cre* driver (Rodda and McMahon, 2006) which directs expression of Cre in pre-osteoblasts beginning around E12.5 (Supp. Fig. 6). We first analysed craniofacial bone formation at E17.5. Consistent with aforementioned results, we observed a mild general hypoplasia of bones with loss of a single allele of Nedd4 (*Nedd4*$^{fl/-}$ and *Osx-Cre; Nedd4*$^{fl/+}$, Fig. 3B and C), but lack of both Nedd4 alleles specifically in pre-osteoblasts (*Osx-Cre; Nedd4*$^{fl/-}$, Fig. 3D or *Osx-Cre; Nedd4*$^{fl/fl}$ Supp. Fig. 2) caused severe hypoplasia of cranial bone. There were dramatic reductions in neural crest derived structures including the frontal bone, premaxilla, maxilla, tympanic, basisphenoid, lamina obturans, palatine bone and mandible in these mice. However, in contrast to *Wnt1-Cre;*

Nedd4$^{fl/-}$ embryos there was no palatal clefting in *Osx-Cre; Nedd4*$^{fl/-}$ or *Prx1-Cre; Nedd4*$^{fl/-}$ mutants (Supp. Fig. 3). In addition to these neural crest derived bone defects, there was also deficiency in the mesodermal derived parietal bones (Fig. 3D). Bone formation was further examined earlier in development at E15.5 by Von Kossa, Alcian blue and ALP staining. Consistent with the analyses performed on *Wnt1-Cre; Nedd4*$^{fl/-}$ mutants, *Osx-Cre; Nedd4*$^{fl/-}$ mutants demonstrated a reduction in formation of mineralised bone and osteoblast staining compared to *Nedd4*$^{fl/-}$ controls (Fig. 4 and Supp. Fig. 4). This identifies an important role for Nedd4 in craniofacial development after the onset of bone progenitor specification.

In our previous publication, we reported aberrant cranial neural crest cell death in cells of the rhombomere 2 (r2) migrating stream in *Nedd4*$^{-/-}$ embryos, and hypothesised that neural crest cell deficiency due to increased cell death may underlie the reduced cranial bone formation in these mice (Wiszniak et al., 2013). To explore whether the increased neural crest cell death observed in *Nedd4*$^{-/-}$ embryos was cell-autonomous, we analysed cleaved-Caspase 3 expression in *Wnt1-Cre; Nedd4*$^{fl/-}$ mutants at E9.5. Consistent with our previous results, neural crest cell death was similarly observed in *Wnt1-Cre; Nedd4*$^{fl/-}$ mutants (Supp. Fig. 7A). Concomitantly, at E11.5, the trigeminal ganglia, which is in part derived from neural crest cells of the r2 stream, was significantly smaller in size in *Wnt1-Cre; Nedd4*$^{fl/-}$ embryos compared to controls (Supp. Fig. 7B). In contrast, formation of the trigeminal ganglia was unaffected in *Osx-Cre; Nedd4*$^{fl/-}$ embryos (Supp. Fig. 7C). Since the craniofacial bone defects of *Nedd4*$^{-/-}$ embryos
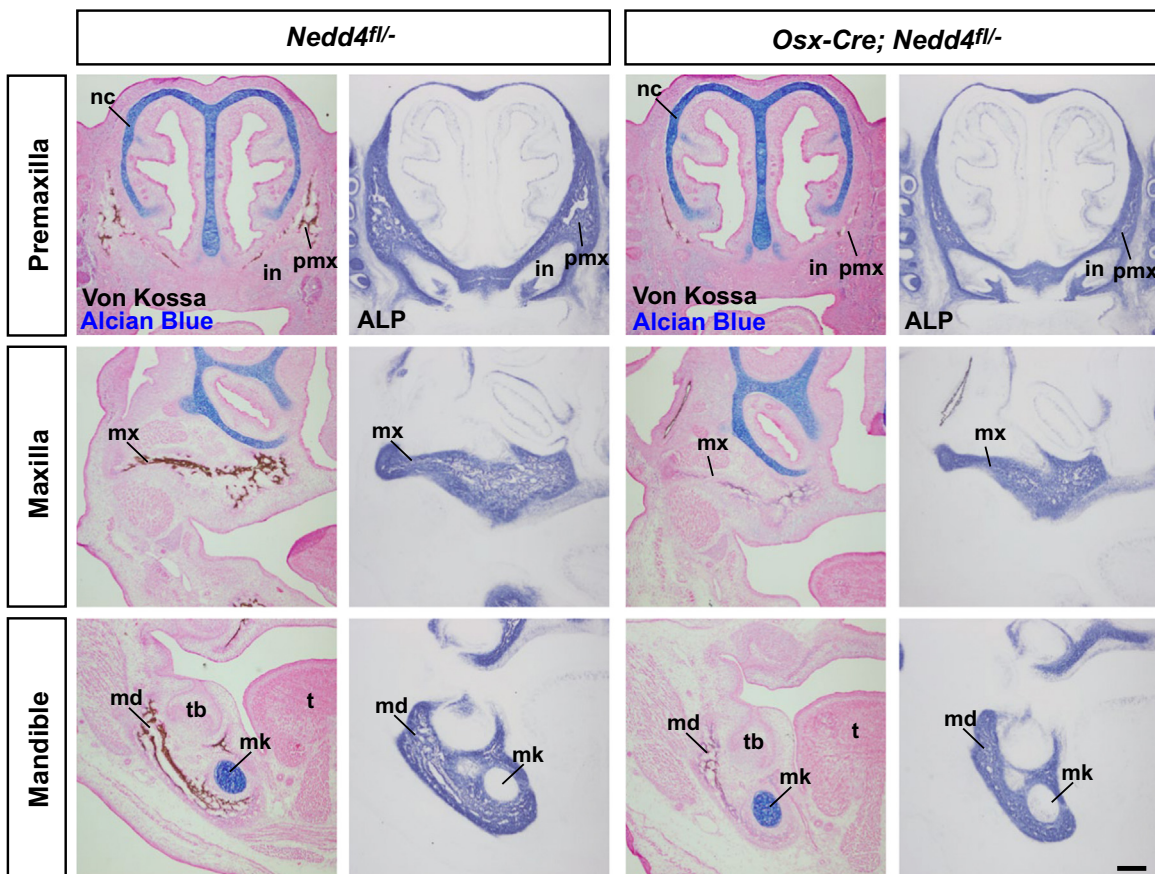

Fig. 4. Bone formation is reduced at E15.5 in *Osx-Cre; Nedd4*$^{fl/-}$ mutants Serial frontal sections of E15.5 *Nedd4*$^{fl/-}$ control and *Osx-Cre; Nedd4*$^{fl/-}$ mutant embryos stained with Von Kossa and Alcian Blue (left panels) to mark mineralisation and cartilage respectively, and alkaline phosphatase (ALP) (right panels) to label osteoblast activity. Equivalent sections of control and mutant embryos are shown of the premaxilla (top), maxilla (middle) and mandible (bottom). Compared to controls, *Osx-Cre; Nedd4*$^{fl/-}$ mutants show less mineralised bone and ALP staining, whereas cartilage development is unaffected. nc, nasal cartilage; in, incisor; pmx, premaxilla; mx, maxilla; md, mandible; mk, Meckel's cartilage; tb, toothbud; t, tongue. Scale bar=200 μm.

are replicated in mice lacking *Nedd4* at later stages in the bone development cascade, we conclude that the primary role of Nedd4 in craniofacial development lies in the formation of bone from pre-osteoblasts rather than maintaining neural crest cell survival at earlier stages of development. Taken together, our previous and current results therefore support a dual role for *Nedd4* in neural crest and craniofacial development; in promoting neural crest cell survival in cells destined to form the trigeminal ganglia, and later in the proliferation of pre-osteoblast bone progenitors.

2.3. Osteoblast progenitors are reduced in Osx-Cre; Nedd4$^{fl/-}$ embryos

Considering the marked reduction in bone formation and osteoblasts at E15.5, we sought to determine the onset of defective bone development in *Osx-Cre; Nedd4$^{fl/-}$* mutants. The *Osx-Cre* driver produces a Cre-GFP fusion protein (Rodda and McMahon, 2006) which allows for visualisation of the *Osx-Cre* expression domain by examining GFP native fluorescence or immunostaining with anti-GFP antibodies. The earliest developmental time point at which we could detect Cre-GFP protein was E12.0. We compared Cre-GFP expression in sections of *Osx-Cre; Nedd4$^{fl/+}$* control and *Osx-Cre; Nedd4$^{fl/-}$* mutant embryos at E12.5, E13.5 and E15.5 (Fig. 5). At E12.5 there was comparable expression of Cre-GFP between control and mutant embryos (Fig. 5A and quantified in

D), suggesting the initial specification of pre-osteoblasts in mutant embryos occurs normally. However, from E13.5 onwards, the area of Cre-GFP positive cells was markedly reduced in *Osx-Cre; Nedd4$^{fl/-}$* mutants compared to controls (Fig. 5B, C and quantified in E, F). This suggests that following specification, there is loss of osteoblasts in *Osx-Cre; Nedd4$^{fl/-}$* mutants, which likely underpins the reduction in mineralised bone seen later in development.

We further examined osteoblast progenitor development in *Osx-Cre; Nedd4$^{fl/-}$* mutants by *in situ* hybridisation for the pre-osteoblast markers *Runx2* and *Osx*, and the committed osteoblast markers *Col1a1* and *BSP*. At E13.5, expression of all markers was dramatically reduced in *Osx-Cre; Nedd4$^{fl/-}$* mutants compared to *Nedd4$^{fl/-}$* controls (Fig. 6A). Consistently, at E15.5, *Col1a1* expression was severely reduced in mutants (Fig. 6B), as well as *BSP* expression at E15.5 (Fig. 6C) and E18.5 (Fig. 6D).

2.4. Reduced pre-osteoblast proliferation underlies bone hypoplasia in Osx-Cre; Nedd4$^{fl/-}$ mutants

Given the reduction in Cre-GFP-positive osteoblast progenitors observed in *Osx-Cre; Nedd4$^{fl/-}$* mutants at E13.5 and E15.5 (Fig. 5), we hypothesised that reduced cell proliferation or aberrant cell death may underlie this observed reduction in Cre-GFP cells, and hence contribute to the bone hypoplasia observed in later development. We analysed cell proliferation and death by staining
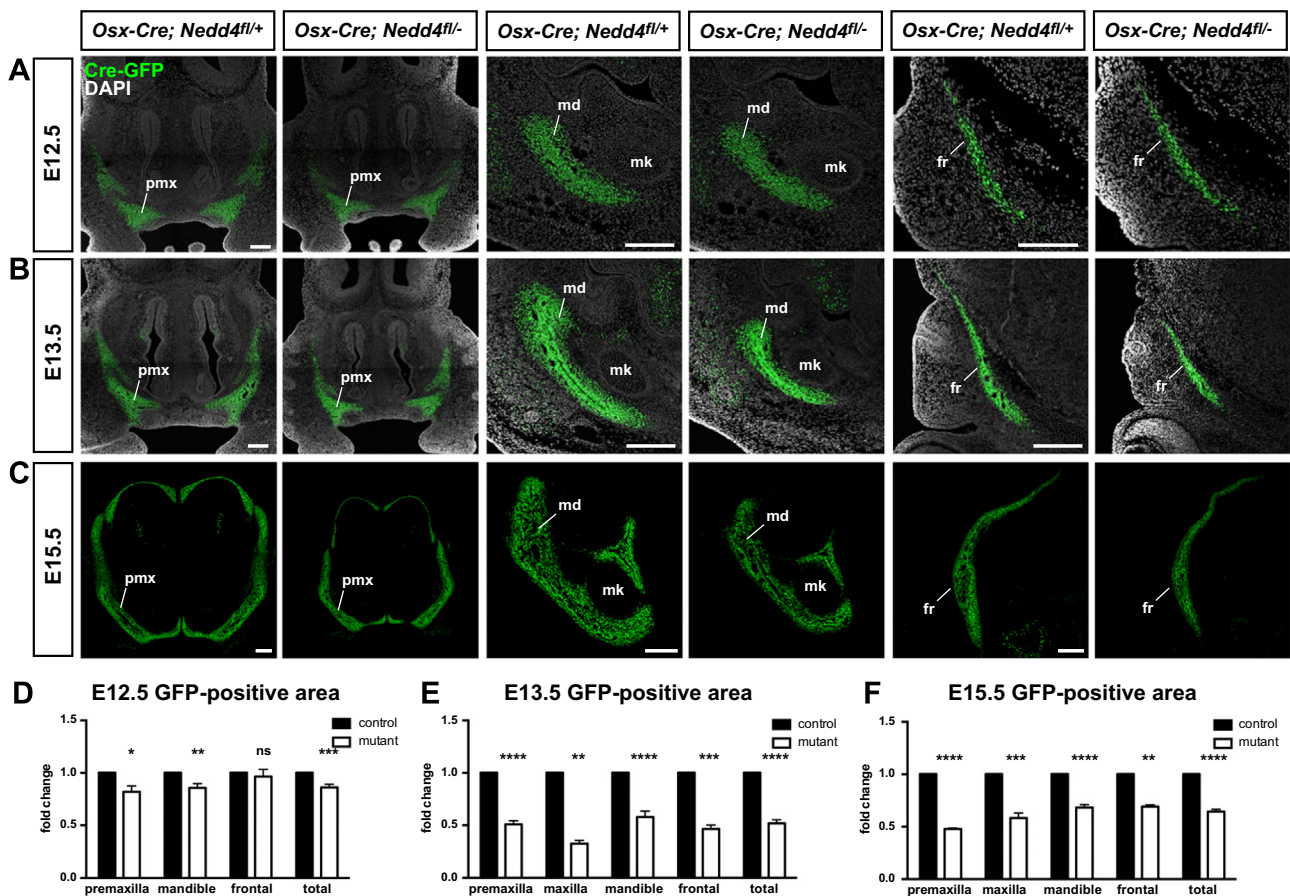

Fig. 5. Osteoblast progenitors are reduced in *Osx-Cre; Nedd4$^{fl/-}$* mutants from E13.5. The Cre recombinase driven by the *Osx* promoter is fused to GFP, which allows for visualisation of *Osx* expressing osteoblast progenitor cells. A–C: Frontal sections of *Osx-Cre; Nedd4$^{fl/+}$* control and *Osx-Cre; Nedd4$^{fl/-}$* mutant embryos were immunostained for GFP. Sections through the premaxilla are shown in left panels, sections through the mandible are shown in middle panels, and sections through the frontal bone are shown in right panels. A: At E12.5, the area of GFP expression is mildly reduced in mutant embryos. B: At E13.5 the area of GFP expressing cells, and hence osteoblast progenitors is greatly reduced. C: Similarly at E15.5 the area of GFP expressing cells is greatly reduced. pmx, premaxillary; md, mandible; mk, Meckel's cartilage. Scale bars=200 μm. D–F: Quantitation of the area of GFP expressing cells in control and mutant embryos, expressed as a fold change relative to the control. Equivalent sections in control and mutant embryos were compared, with at least 5 sections per bone per animal quantitated. Statistical significance was determined by Student's *t*-test. **** $p < 0.0001$, *** $p < 0.001$ ** $p < 0.01$, * $p < 0.05$, ns=not significant. Error bars represent SEM.
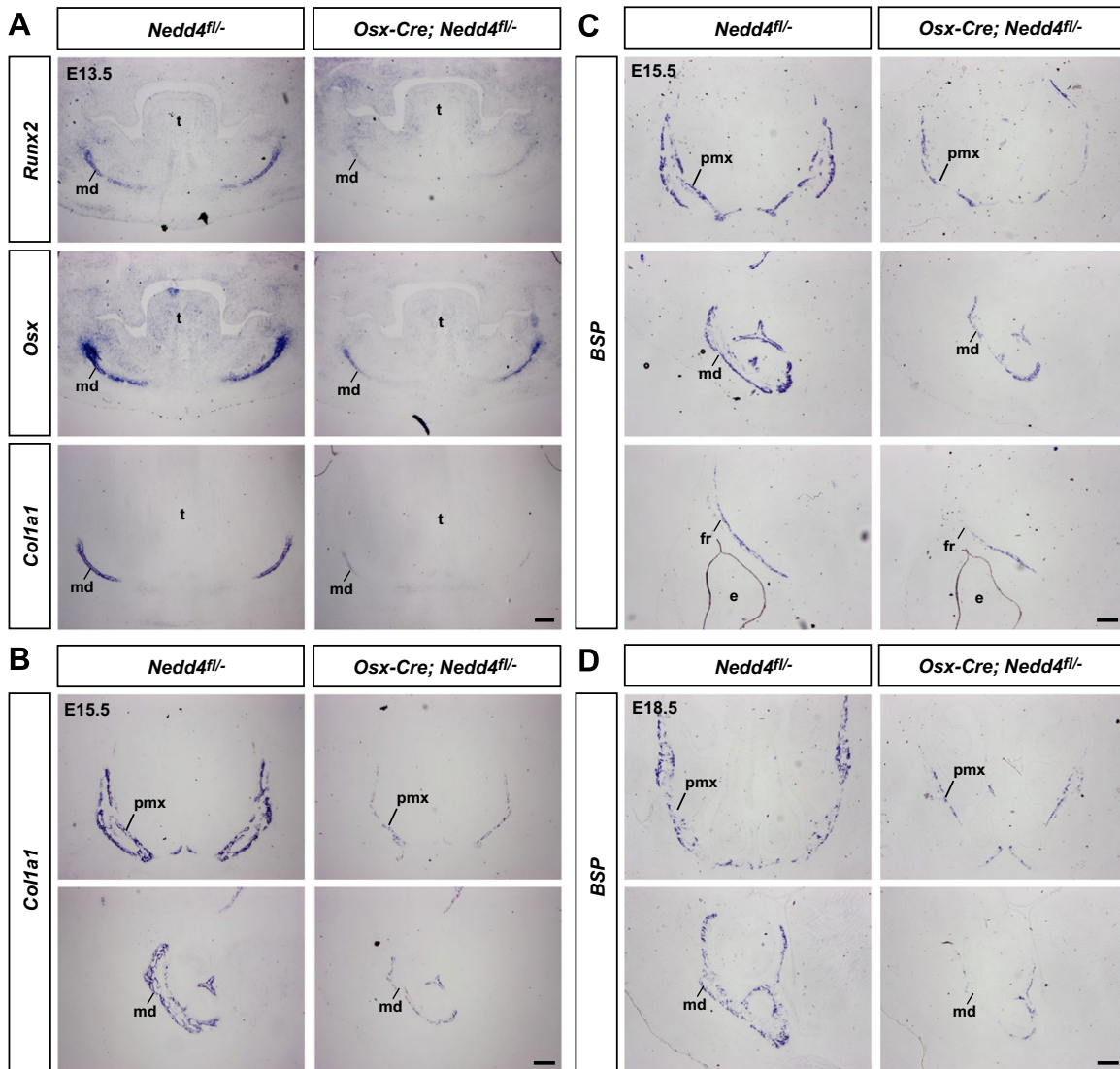
Fig. 6. Expression of osteoblast specification and differentiation markers are reduced in *Osx-Cre; Nedd4$^{fl/-}$* mutants. *In situ* hybridisation of frontal sections for the osteoblast specification markers *Runx2*, *Osx*, *Col1a1* and *BSP* in *Nedd4$^{fl/-}$* control and *Osx-Cre; Nedd4$^{fl/-}$* mutant embryos. A: At E13.5 there is a reduction in expression of all markers examined in the developing mandible of mutant embryos. B: At E15.5 there is a marked reduction of *Col1a1* expression in mutants in the premaxilla (top panels) and mandible (bottom panels). C: At E15.5 there is a marked reduction in expression of the definitive osteoblast marker *BSP* in mutants in the premaxilla, mandible and frontal bones. D: At E18.5 there was a severe reduction in *BSP* expression in the premaxilla and mandible. md, mandible; t, tongue; pmx, premaxillary; fr, frontal; e, eye. Scale bars=200 μm.

tissue sections from *Osx-Cre; Nedd4$^{fl/+}$* control and *Osx-Cre; Nedd4$^{fl/-}$* mutant embryos for EdU incorporation, phospho-histone H3 (PHH3), TUNEL and cleaved-Caspase 3. Analysis of EdU incorporation at E12.5 revealed a decrease in cell proliferation of ∼25% in mutants compared to controls (Fig. 7A and Supp. Fig. 8A). Similarly, analysis of cell proliferation by immunostaining for PHH3 (which marks cells in G2/M phase) revealed a dramatic decrease in proliferation of ∼50% at E12.5, followed by consistent but more modest decreases in proliferation at E13.5 and E15.5 (Fig. 7B and Supp. Fig. 8B). For all analyses, proliferation measurements were made for multiple cranial bones including the premaxilla, maxilla, mandible, and frontal bones, and combined into an average of total bones (Fig. 7). For measurements divided into individual bones, see Supp. Fig. 8. At all stages examined, there were no significant differences in cell death between control and mutant embryos by either TUNEL or Caspase 3 staining (Fig. 7C, D, Supp. Figs. 8C,D and 9 for individual bones). This suggests that the lack of bone observed in later development, and the reduction in osteoblasts observed from E13.5 onwards is caused by

a failure of correct proliferation of osteoblast progenitors at a critical time window between E12.5 and E13.5 in *Osx-Cre; Nedd4$^{fl/-}$* embryos. Therefore, Nedd4 likely plays an important role in positively regulating pre-osteoblast proliferation between E12.5 to E13.5. This is consistent with the expression profile of *Nedd4*, which is broadly expressed throughout development, but is highly expressed in the pre-osteoblast mesenchyme at E13.5, with reduced expression later in development at E15.5 (Supp. Fig. 10).

Bone morphogenic proteins (BMPs) play important roles in bone formation by inducing osteoblast differentiation (reviewed in (Chen et al., 2012)). BMPs signal via activation of the intracellular signalling molecules known as SMADs, in particular by phosphorylating and activating SMAD1/5/8. Nedd4 has previously been shown to negatively regulate BMP signalling via interacting directly with SMAD1 to promote its ubiquitination and proteosomal degradation (Kim et al., 2011). Consequently, siRNA knockdown of Nedd4 in human vascular smooth muscle cells increased phospho-SMAD1 and ALP activity, and induced calcification of these cells (Kim et al., 2011). Given this, and although in a different cellular
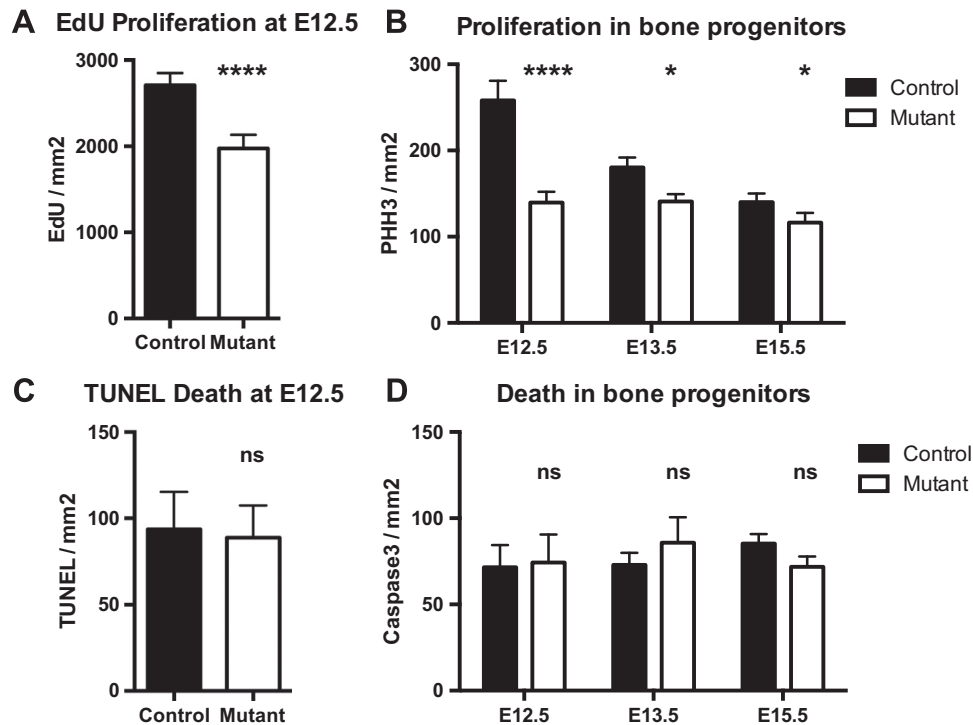
Fig. 7. Reduced pre-osteoblast proliferation, without aberrant cell death, underlies bone hypoplasia in *Osx-Cre; Nedd4*$^{fl/-}$ mutants. Cell proliferation and death was examined in *Osx-Cre; Nedd4*$^{fl/+}$ (control) and *Osx-Cre; Nedd4*$^{fl/-}$ (mutant) embryos by quantitating the number of positive cells per mm^2 of GFP-positive cells in tissue sections. A: pregnant dams were pulsed with EdU 2 hours prior to embryo harvest at E12.5 to assess cell proliferation. There is a significant reduction in EdU-positive cells in pre-osteoblasts in mutant embryos. B: Control and mutant embryos at E12.5, E13.5 and E15.5 were immunostained for phospho-histone H3 (PHH3) to assess cell proliferation. There is a marked reduction in proliferation in mutant embryos at E12.5, with significant reductions in proliferation also observed at E13.5 and E15.5. C: E12.5 sections were stained with the TUNEL method to assess cell death, with no significant differences observed between control and mutant embryos. D: Cell death was examined by cleaved caspase 3 immunostaining at E12.5, E13.5 and E15.5. No significant differences were observed between control and mutant embryos at all stages examined. Statistical significance was determined by Student's *t*-test. **** $p < 0.0001$, * $p < 0.05$, ns = not significant. Error bars represent SEM.

context, it is possible that loss of Nedd4 in preosteoblasts may induce premature differentiation via upregulation of phospho-SMAD1, at the expense of proliferation and expansion of the progenitor pool. Immunostaining of E12.5, E13.5 and E15.5 embryos for phospho-SMAD1/5/8 revealed no upregulation of phospho-SMAD in Cre-GFP-positive osteoblast progenitors in *Osx-Cre; Nedd4*$^{fl/-}$ mutants compared to controls (Supp. Fig. 11). Therefore it is unlikely that the reduced proliferation of preosteblasts in *Osx-Cre; Nedd4*$^{fl/-}$ embryos may be explained by premature differentiation of these cells.

3. Discussion

Here we have used genetic approaches to dissect the roles of the ubiquitin ligase Nedd4 in craniofacial development. Our previous analysis of *Nedd4*$^{-/-}$ mice demonstrated that Nedd4 is essential for intramembranous bone formation, however the timing at which it contributed to this process remained unknown. Indeed, based on our findings of reduced cranial neural crest cell viability in the absence of Nedd4, our previous data suggested a model in which the craniofacial defects may arise from an insufficient population of cranial neural crest cells at earlier developmental time points. Consistent with this hypothesis, we found that mice lacking Nedd4 in neural crest cells (*Wnt1-Cre; Nedd4*$^{fl/-}$) had aberrant neural crest cell death and replicated the full repertoire of craniofacial defects observed in *Nedd4*$^{-/-}$ mice.

However, since the craniofacial bone is of neural crest cell origin, the *Wnt1-Cre; Nedd4*$^{fl/-}$ genetic cross does not distinguish between early roles for Nedd4 in neural crest cell viability or later roles for Nedd4 in osteoblast formation or bone deposition. To

address this, we next generated mice in which Nedd4 was genetically deleted at later stages of bone development. Removal of Nedd4 with *Prx1-Cre* also replicated the full craniofacial phenotype of *Nedd4*$^{-/-}$ embryos. This result therefore demonstrates a primary role for Nedd4 in intramembranous ossification after the onset of neural crest cell specification toward chondrocyte or osteoblast identity. The *Wnt1-Cre* and *Prx1-Cre* crosses both remove *Nedd4* from mesenchyme with both osteoblast and chondrocyte potential, however, neither line had notable cartilage defects. This further suggests Nedd4 plays a critical role in bone development after the specification of mesenchyme toward an osteoblast or chondrocyte lineage. Consistent with this notion, removal of *Nedd4* with *Osx-Cre* also replicated the craniofacial bone hypoplasia of the other *Nedd4* deficient lines. Since *Osx-Cre* only becomes expressed after the onset of mesenchymal differentiation into osteoblast precursors, this result further demonstrates that Nedd4 has an essential role in osteoblast formation and bone deposition.

Our previous analysis of *Nedd4*$^{-/-}$ embryos identified significantly increased neural crest cell death in the craniofacial region soon after delamination, and this was confirmed to be a cell-autonomous effect in *Wnt1-Cre; Nedd4*$^{fl/-}$ embryos. However, since the craniofacial bone deficiency was also observed in *Prx1-Cre* and *Osx-Cre; Nedd4*$^{fl/-}$ mutant embryos, which do not induce Nedd4 knockout until after neural crest cell migration in to the facial primordia, our new data suggest that this early neural crest cell death does not underlie craniofacial bone defects. Rather, our analysis of the trigeminal ganglia may suggest that the cranial neural crest cell death identified at E9.5 is restricted to cells of the neuronal and glial lineage. Whether aberrant cell death in *Nedd4*$^{-/-}$ mice leads to deficiencies of other neural crest cell

derivatives such as the peripheral nervous system, thymus or cardiac outflow tract remains to be explored.

Our results suggest that reduced proliferation of osteoblast precursors at a critical time window of E12.5–15.5 represents part of the cellular mechanism underlying bone deficiency in mice lacking Nedd4. Consistent with a reduction in EdU incorporation and PHH3-positve proliferative cells at each age examined, we also observed increasing severity in the hypoplasia of pre-osteoblasts (Runx2, Osx and Cre-GFP positive cells), osteoblasts (Col1a1, BSP and ALP positive cells) and bone deposition (von kossa and Alizarin red staining) as development progressed. While this highlights an essential role for Nedd4 in maintaining or promoting the expansion of the pre-osteoblast progenitor pool, our results may also suggest that progression of osteoblast development is arrested in the absence of Nedd4. Thus, while we observed a mild reduction in pre-osteoblast proliferation and Cre-GFP positive cells, there was a profound lack of bone deposition and Col1a1 expression at later stages of development.

Progression of immature mesenchymal cells along the osteoblast lineage is controlled by expression of the master regulator Runx2. Runx2, in turn, promotes expression of Osx, and together these transcription factors are essential for osteoblast differentiation (Komori, 2006). The importance of these transcription factors in bone formation is clearly evident from the analysis of Runx2 and Osx knockout mice. In the absence of Runx2, Osx fails to be expressed and bone development is arrested at the pre-osteoblast stage (Komori et al., 1997; Otto et al., 1997). In the absence of Osx, Runx2 is expressed in pre-osteoblasts but ossification is completely blocked (Nakashima et al., 2002). Notably in Runx2 and Osx knockout mice, the progenitor pool of pre-osteoblasts is comparable between knockout and control embryos, which is in contrast to the reduction in pre-osteoblast progenitors observed in our Nedd4 knockout models. Interestingly, in our analyses we observed reduced expression of Runx2 after deletion of Nedd4 in Osx expressing cells. Given the reduction in the number of Osx-positive cells observed with Cre-GFP staining, the reduced expression of Runx2 in Osx-Cre; Nedd4$^{fl/-}$ embryos likely represents hypoplasia of the pre-osteoblast progenitor pool rather than a role for Nedd4 in feed-back regulation of Runx2 expression.

Nedd4 is the prototypic member of the C-terminal HECT domain family of E3 ubiquitin ligases that comprise N-terminal C2 domains and WW domains controlling substrate recognition (Rotin and Kumar, 2009; Kumar et al., 1997). Identifying molecular targets of Nedd4 in pre-osteoblasts now poses the next challenge in defining the mechanisms by which Nedd4 controls osteoblast proliferation and/or differentiation. As Nedd4 predominantly targets proteins for non-degradative modifications (Fukushima et al., 2015; Fujii et al., 2013) this presents a major challenge in identifying Nedd4 targets in vivo. Several established targets of Nedd4 are known to play roles in bone development. One such target is phospho-SMAD1, which signals downstream of BMP to activate osteoblast differentiation programmes (Chen et al., 2012). Nedd4 has previously been shown to negatively regulate phospho-SMAD1 in in vitro cell culture models (Kim et al., 2011), suggesting that loss of Nedd4 may induce precocious activation of phospho-SMADs and premature osteoblast differentiation. However, this was not observed in vivo in our present study. Therefore it is likely that Nedd4 regulates target proteins differentially in a cell type-specific manner. Of the other established targets of Nedd4, a strong candidate for controlling pre-osteoblast proliferation may be the IGF signalling pathway. Nedd4 positively regulates IGF signalling through controlling the abundance and activity of IGF1R (Cao et al., 2008) which has an essential role in bone growth and development (Guntur and Rosen, 2013). However, whether this or other targets of Nedd4 play important roles in osteoblast growth dynamics remain to be explored.

In conclusion, our data identify a previously unknown role for Nedd4 in osteoblast formation and define a critical time window downstream of osteoblast specification at which Nedd4 is required for bone growth and craniofacial development. Nedd4$^{-/-}$ and conditional knockout mice therefore represent novel models to decipher the mechanisms controlling craniofacial development and the origins of craniofacial birth defects.

4. Materials and methods

4.1. Mouse strains

All experiments were carried out in accordance with ethical guidelines of the SA Pathology Animal Ethics Committee. To remove Nedd4 specifically in NCCs we crossed Wnt1-Cre;Nedd4$^{+/-}$ males to Nedd4$^{fl/fl}$ females. To remove Nedd4 specifically in mesenchymal precursors of bone and cartilage we crossed Prx1-Cre; Nedd4$^{+/-}$ males to Nedd4$^{fl/fl}$ females (Logan et al., 2002). To remove Nedd4 specifically in osteoblast precursors we crossed Osx-Cre/EGFP;Nedd4$^{+/-}$ males to Nedd4$^{fl/fl}$ females (Rodda and McMahon, 2006). Nedd4$^{-/-}$ mice and Nedd4$^{fl/fl}$ (generously provided by Dr Hiroshi Kawabe, Max-Planck Institute of Medical Research) have been described previously (Cao et al., 2008; Kawabe et al., 2010). To lineage trace Osx positive osteoblasts we crossed Osx-Cre/EGFP mice to R26RLacZ reporter mice. Genotyping for Cre and the Nedd4 alleles has been described previously (Cao et al., 2008; Kawabe et al., 2010). To obtain mouse embryos of defined gestational ages, mice were mated in the evening and the morning of vaginal plug formation was counted as E0.5.

4.2. Skeletal preparations

Staining of bone and cartilage was carried out on E17.5 embryos fixed overnight in 95% ethanol then skinned and eviscerated. Embryos were placed in acetone for 2 days before being placed into a solution containing 1 volume of 0.3% Alcian Blue 8GS in 70% ethanol, 1 volume of 0.1% Alizarin Red S in 95% ethanol, 1 volume acetic acid and 17 volumes of 70% ethanol for 3 days. Embryos were then washed in water and macerated in 1% KOH for 1 day and then decolorised in 20% glycerol in 1% KOH for 1–2 days and prepared in increasing concentrations of glycerol to a final concentration of 100% glycerol.

4.3. Histology and Immunolabelling

Embryos were fixed in 4% paraformaldehyde in PBS and cryosectioned at 12 µm thickness (Leica). Von Kossa (1% silver nitrate) staining was used to mark areas of mineralisation, alkaline phosphatase staining (0.5 mg/ml NBT/BCIP) was used to mark osteoblast activity and Alcian blue (0.1% Alcian blue in 0.1 M HCl) was used to mark cartilage. Histological markers were counterstained with eosin. High resolution images were recorded on an IX81 inverted microscope (Olympus) equipped with an OCRA-ER digital CCD camera (Hamamatsu) and processed with CellR software (Olympus).

For immunolabelling, cryosections were blocked in 10% DAKO blocking solution, 0.2% Triton X-100 in PBS, and stained with the indicated primary antibodies. Antibodies used: chicken anti-GFP (Abcam) 1:1000; rabbit anti-cleaved caspase 3 (Cell Signalling Technology) 1:500; rabbit anti-phospho-Histone H3 (Millipore) 1:500; goat anti-Sox10 (Santa Cruz); rabbit anti-phospho-SMAD1/5/8 (Cell Signalling Technology). Cyrosections were mounted in Prolong Gold antifade reagent with DAPI (Molecular Probes). Confocal images were acquired on a LSM 700 (Zeiss) system. All figures were constructed in Adobe Photoshop CS4 (Adobe Systems, Inc.).

4.4. In situ hybridisation

Section *in situ* hybridisation was performed as described (Schwarz et al., 2004). Riboprobes were transcribed from plasmids containing cDNA sequences for *Isl1, Col1a1, Runx2, Osx,* and *BSP*. For each probe, control and mutant embryos were developed for the same amount of time. High resolution images were recorded on an IX81 inverted microscope (Olympus) equipped with an OCRA-ER digital CCD camera (Hamamatsu) and processed with CellR software (Olympus).

4.5. β-Galactosidase staining

Cryosections were incubated in staining solution: 19 mM Sodium dihydrogen phosphate, 81 mM Disodium hydrogen phosphate, 2 mM $MgCl_2$, 5 mM EGTA, 0.01% Sodium deoxycholate, 0.02% NP-40, 5 mM Potassium ferricyanide, 5 mM Potassium ferrocyanide and 1 mg/ml X-gal substrate, at 37 °C until blue staining was sufficient.

4.6. EdU administration

EdU (5-ethynyl-2′-deoxyuridine) (Life technologies) was prepared in 0.9% NaCl solution at 10 mg/ml and stored at -20 °C until use. To label proliferating cells EdU was delivered by intraperitoneal injections to pregnant dams at E12.5 at 100 mg/kg of body weight. Embryos were collected from pregnant dams 2 h after injection. Cryosections of EdU labelled embryos were incubated with Click-iT EdU Kit Alexa-594 conjugated (Life Technologies) following staining with primary antibodies.

4.7. TUNEL staining

TUNEL staining was performed using the TMR Red In Situ Cell Death Detection Kit (Roche), following the manufacturers recommendations.

4.8. MicroCT analysis

Adult skulls were collected and fixed in 95% EtOH. *Ex vivo* micro-CT was performed using a micro-CT system (Skyscan 1076, Brussels, Belgium) at a resolution of 26 µm/pixel. Reconstructed X-ray images were realigned to generate 3D projections that were rotated to form dorsal and lateral views.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.12.001.

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