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The ubiquitin ligase Nedd4 regulates craniofacial development by promoting cranial neural crest cell survival and stem-cell like properties

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ABSTRACT

The integration of multiple morphogenic signalling pathways and transcription factor networks is essential to mediate neural crest (NC) cell induction, delamination, survival, stem-cell properties, fate choice and differentiation. Although the transcriptional control of NC development is well documented in mammals, the role of post-transcriptional modifications, and in particular ubiquitination, has not been explored. Here we report an essential role for the ubiquitin ligase Nedd4 in cranial NC cell development. Our analysis of *Nedd4*$^{-/-}$ embryos identified profound deficiency of cranial NC cells in the absence of structural defects in the neural tube. Nedd4 is expressed in migrating cranial NC cells and was found to positively regulate expression of the NC transcription factors Sox9, Sox10 and *FoxD3*. We found that in the absence of these factors, a subset of cranial NC cells undergo apoptosis. In accordance with a lack of cranial NC cells, *Nedd4*$^{-/-}$ embryos have deficiency of the trigeminal ganglia, NC derived bone and malformation of the craniofacial skeleton. Our analyses therefore uncover an essential role for Nedd4 in a subset of cranial NC cells and highlight E3 ubiquitin ligases as a likely point of convergence for multiple NC signalling pathways and transcription factor networks.

Introduction

Neural crest (NC) cells are a transient population of stem-cells that arise from neuroepithelial precursors during early embryonic development to generate a wide spectrum of derivatives. Cranial NC cells arise between the posterior forebrain and posterior hindbrain and migrate into the facial primordia to generate the vast majority of the vertebrate head including the cranial ganglia, connective tissue, tendons, osteoblasts, chondrocytes and melanocytes (Le Douarin et al., 2004; Osumi-Yamashita et al., 1994). Underscoring the critical importance of this cell type is the high prevalence of congenital craniofacial birth defects such as cleft lip and/or palate and Digeorge Syndrome that arise from cranial NC cell deficiencies (Cordero et al., 2010).

The understanding of NC cell development at the molecular level is heavily biased toward the role of transcription factor networks acting downstream of morphogen signalling (Steventon et al., 2005). NC induction is initiated at the interface of the neural plate and non-neural ectoderm through the combined activity of several morphogens such as BMPs, FGFs and Wnts that induce expression of NC cell specifying transcription factors including Snail, Sox10, Sox9 and FoxD3. These transcription factors not only promote an epithelial-to-mesenchymal transition by altering expression of cell adhesion and morphology molecules, they also play essential roles in the early maintenance of NC cell identity, NC cell survival and NC stem-cell properties (Steventon et al., 2005). Notably, many of these transcription factors are down-regulated after the initial stages of NC cell delamination and their early migration away from the neural tube, but are later up-regulated to play essential roles in NC-derived tissue morphogenesis.

The HMG box transcription factors Sox9 and Sox10 are initially expressed in most NC cells to promote delamination from the neural tube, however, both play disparate roles in later stages of NC development. Sox9 is down-regulated in cranial NC cells as they migrate into the facial primordia at E9.5 and later is up-regulated (from E12.5 onwards) to act as a master regulator in mesenchymal condensations that will form cartilage (Akiyama et al., 2002; Bi et al., 2001; Steventon et al., 2005). In its absence NC cells apoptose either during or shortly after delamination

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resulting in a reduced number of NC cells that will form the craniofacial skeleton. As a consequence of NC hypoplasia and aberrant cartilage differentiation, Sox9 deficient embryos have profound craniofacial defects (Cheung et al., 2005). Sox10 expression is initially maintained in NC cells of a neuroglial fate and is required for NC stem-cell self-renewal and multipotentiality (John et al., 2011; Kim et al., 2003). Sox10 deficient mice have been shown to have widespread defects in many NC derivatives that result from precocious NC cell death prior to differentiation (Kapur, 1999; Sonnenberg-Riethmacher et al., 2001; Southard-Smith et al., 1998). The forkhead transcription factor FoxD3 begins to be expressed prior to delamination, is maintained in migrating NC cells, but is later down-regulated during differentiation into most derivatives (Labosky and Kaestner, 1998). An essential role for FoxD3 in early NC cell maintenance and survival has been defined through loss-of-function studies in mice (Mundell and Labosky, 2011; Teng et al., 2008). Consequently, mouse embryos lacking FoxD3 in NC cells have profound craniofacial defects (Teng et al., 2008). *Ex vivo* studies have also shown that FoxD3 is required to direct fate choices of NC cells and to regulate NC stem-cell self-renewal and multipotentiality (Mundell and Labosky, 2011). The signalling pathways that regulate these transcription factors and how these transcriptional networks are coordinated is ill defined.

The ubiquitin pathway plays critical roles in most cellular processes by regulating proteosomal- and lysosomal-mediated protein degradation, and also by modulating the function of target substrates by altering their subcellular localisation or biochemical properties (reviewed in (Rotin and Kumar, 2009)). The ubiquitination process requires three enzymes: the E1 enzyme initially activates ubiquitin, E2 enzymes facilitate the transfer of activated ubiquitin to substrates recognised by one of many E3 ubiquitin protein ligases (for a detailed review we refer readers to (Kerscher et al., 2006)). In this manner, E3 ubiquitin ligases provide substrate specificity and are essential in identifying which proteins are targeted for ubiquitination. Their ability to recognise multiple targets provides an ideal mechanism through which several signalling pathways can converge. To date, the role of E3 ubiquitin ligases and ubiquitination in mammalian NC cell development has not been explored.

Here we uncover a hitherto unknown role for the founding member of the HECT family of E3 ubiquitin ligases, Nedd4 (neural precursor cell expressed developmentally down-regulated) (Kumar et al., 1997), in mouse NC cell development. Nedd4 has a number of putative substrates with known roles in NC cell development identified through *in vitro* studies, including Ptch1 (Lu et al., 2006), FGFR1 (Persaud et al., 2009), receptor-regulated SMAD1 (Kim et al., 2011) and ErbB4 (Zeng et al., 2009). In contrast, there is limited knowledge of the roles and substrates of Nedd4 *in vivo*: in mice it regulates Rap2A (Kawabe et al., 2010) and PTEN (Christie et al., 2011; Drinjakovic et al., 2010) to control neurite development and axonal branching, regulates Thrombospondin-1 to control cardiovascular development (Fouladkou et al., 2010), and positively regulates IGF-1R signalling to control embryonic growth (Cao et al., 2008).

Our functional analyses of *Nedd4*$^{-/-}$ embryos and Nedd4-deficient NC cells now highlights an essential role for Nedd4 in the maintenance of a subset of cranial NC cells and the promotion of stem-cell like properties. Nedd4 is expressed in cranial NC cells during their migration from the neural tube toward their final destinations in the anterior regions of the head. In the absence of Nedd4, there is a striking hypoplasia of cranial NC cells emigrating from the posterior forebrain, midbrain and rostral hindbrain. Our *in vivo* and *ex vivo* analyses suggest that Nedd4-deficient cranial NC cells form normally, but undergo aberrant apoptosis during their migration away from the neural tube. Accordingly, the trigeminal ganglia and craniofacial skeleton of *Nedd4*$^{-/-}$ embryos is dramatically affected. To understand the origin of this defect we analysed the expression of the core NC cell transcription factors. Our analyses show that *Snail*, Sox9, Sox10 and *FoxD3* are rapidly downregulated following Nedd4 knockdown, providing insight to the molecular defects underpinning NC cell death, loss of stem-cell like self-renewal capacities and cranial NC hypoplasia. Taken together, our findings identify Nedd4 as a key E3 ubiquitin ligase in modulating transcription factor networks in a subset of cranial NC cells and support a role for Nedd4 in the development of the ectomesenchyme and neuroglial cranial NC cell lineages.

Results

Nedd4$^{-/-}$ *mice have craniofacial defects*

Our analysis of *Nedd4* deficient embryos revealed that these mice have severe craniofacial defects with full penetrance (8/8 *Nedd4*$^{-/-}$ *vs.* 0/8 wildtype). Embryonic day (E) 15.5 *Nedd4*$^{-/-}$ embryos exhibit a pronounced forehead and displacement of the tongue from the oral cavity (Fig. 1A). Staining of bone and cartilage at E15.5 with Alizaran red and Alcian blue revealed that the majority of NC and paraxial mesoderm derived cartilage was present in *Nedd4*$^{-/-}$ embryos, indicating that the early stages of cartilage condensation are normal in mutant embryos (Fig. 1C). At E17.5, wholemount staining revealed that many cranial bones were either absent or severely hypoplastic in *Nedd4*$^{-/-}$ embryos (Fig. 1C). While the bulk of the cranial skeleton arises from NC cell origin, the caudal regions of the skull, including the otic capsule, the occipital and part of the sphenoid bones arise from paraxial mesoderm.

Our analysis at E17.5 found that cranial bones of NC cell origin were affected more than bone of paraxial mesoderm origin with gross defects of the mandible and maxilla, and absence of all or part of the premaxillary, frontal and tympanic bones (Fig. 1C). *Nedd4*$^{-/-}$ embryos also display a cleft palate, which results as a failure of the palatal shelves to fuse (Fig. 1B), and subsequently the palatal bone is absent at E17.5 (Fig. 1C). Analysis of bone formation by microCT scans produced skeletal models consistent with the bone stains showing that bone density in the mandible, maxilla, premaxilla, frontal, tympanic and palatal bones were reduced in *Nedd4*$^{-/-}$ embryos ($n=3$; Supplemental Fig. S1A).

While *Nedd4*$^{-/-}$ embryos are significantly smaller than their wildtype littermates due to an IGF-dependent growth defect (Cao et al., 2008), the absence or deficiency of the cranial bones is unlikely to be the sole consequence of a developmental delay. Strong evidence for this comes from normal formation of cartilage at E15.5, including the nasal capsule, Meckel's cartilage, otic capsule and frontal cartilage (Fig. 1C). At E17.5 non-NC derived bone, albeit reduced in size, was affected less than the NC-derived bone with only mild reductions in ossification of the exoccipital (eo) and basioccipital (boc) bones in the head (Fig. 1C), and the vertebrae, ribs and limbs in the body (Supplemental Fig. S1A and B). To quantify the deficiency of the craniofacial skeleton we completed bone mass calculations of microCT 3D models and normalised this to formation of the humerus. Our calculations identified developmental defects in both NC and mesoderm derived bone. However, the defects in NC derived bone (reduction of $65\pm2\%$, $n=3$) far outweighs the defects in mesoderm derived bone (reduction of $25\%\pm8\%$, $n=3$) in E17.5 *Nedd4*$^{-/-}$ embryos (see Supplemental Fig. S1A). Our data therefore suggests that Nedd4 is required for craniofacial development, and in particular, for NC cell bone formation.

To define the origin of these cranial defects, bone and cartilage were analysed at E12.5 in *Nedd4*$^{-/-}$ embryos, a time when these