Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 306 (2007) 208–221
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

The *Hectd1* ubiquitin ligase is required for development of the head mesenchyme and neural tube closure

Irene E. Zohn [a,1], Kathryn V. Anderson [b], Lee Niswander [a,*]

[a] *Howard Hughes Medical Institute, Department of Pediatrics, Section of Developmental Biology, University of Colorado at Denver and Health Sciences Center-Fitzsimmons Campus, PO Box 6511, MS 8322, 12800 E. 19th Ave., Aurora, CO 80045, USA*
[b] *Developmental Biology Program, Sloan-Kettering Institute, New York, NY, 10021, USA*

Received for publication 3 January 2007; revised 7 March 2007; accepted 12 March 2007
Available online 20 March 2007

Abstract

Closure of the cranial neural tube depends on normal development of the head mesenchyme. Homozygous-mutant embryos for the ENU-induced *open mind* (*opm*) mutation exhibit exencephaly associated with defects in head mesenchyme development and dorsal-lateral hinge point formation. The head mesenchyme in *opm* mutant embryos is denser than in wildtype embryos and displays an abnormal cellular organization. Since cells that originate from both the cephalic paraxial mesoderm and the neural crest populate the head mesenchyme, we explored the origin of the abnormal head mesenchyme. *opm* mutant embryos show apparently normal development of neural crest-derived structures. Furthermore, the abnormal head mesenchyme in *opm* mutant embryos is not derived from the neural crest, but instead expresses molecular markers of cephalic mesoderm. We also report the identification of the *opm* mutation in the ubiquitously expressed *Hectd1* E3 ubiquitin ligase. Two different Hectd1 alleles cause incompletely penetrant neural tube defects in heterozygous animals, indicating that Hectd1 function is required at a critical threshold for neural tube closure. This low penetrance of neural tube defects in embryos heterozygous for Hectd1 mutations suggests that Hectd1 should be considered as candidate susceptibility gene in human neural tube defects.

Keywords: Neural tube closure; Neural tube defect; Head mesenchyme; Ubiquitin ligase; ENU mutagenesis

Introduction

The central nervous system of the vertebrate embryo originates during gastrulation with the formation of the neural plate. During subsequent development, the neural plate undergoes extensive morphogenic movements resulting in formation of the neural tube. When the neural tube fails to close completely during its morphogenesis, neural tube defects result. Neural tube defects are one of the most common human congenital malformations occurring in approximately one out of every one thousand live births (Copp et al., 2003; Zohn et al., 2005). Common forms of neural tube defects include spina bifida and exencephaly where the neural tube remains open in the most caudal and rostral aspects of the neural axis, respectively. In humans, neural tube defects represent a complex disease with multiple environmental and genetic contributing factors. Because of the multifaceted etiology of human neural tube defects, identification of causative mutations has been problematic.

Vertebrate model systems have been indispensable for the discovery of the processes required for neural tube closure. The mouse has been particularly useful for identification of genes required for proper morphogenesis of the neural tube and the generation of numerous mouse models for neural tube defects has implicated a long list of candidate genes for human neural tube defects (Copp et al., 2003; Zohn et al., 2005). These genes regulate cell movement, apoptosis, proliferation, patterning and differentiation of not only the neural tissue, but also the surrounding mesenchyme and non-neural ectoderm. Moreover,

* Corresponding author.
E-mail address: Lee.Niswander@UCHSC.edu (L. Niswander).
[1] Present address: Center for Neuroscience Research, Children's Research Institute, Children's National Medical Center, Washington, DC 20010, USA.

0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.03.018

in some cases, identification of key regulators of neural tube closure in mice has helped to uncover the genetic basis of neural tube defects in humans (Gelineau-van Waes and Finnell, 2001).

Neural tube closure is a complex morphogenic process where the neural plate rolls into a tube forming the central nervous system (Copp et al., 2003; Zohn et al., 2005). The neural folds form at the edges of the neural plate and rise towards the dorsal midline due to forces from both the neural tissue and the surrounding epithelium and mesenchyme. Apical constriction of cells in the midline and in more lateral regions results in the formation of medial and dorsal-lateral hinge points respectively. In the cranial neural tube, neural fold elevation is accompanied by an expansion of the head mesenchyme (Morriss and Solursh, 1978) and reviewed in (Copp, 2005). This expansion is mediated by both increased cell proliferation and an increase in the extracellular space between the mesenchymal cells and is thought to be critical to allow the elevation of the neural folds. The molecular signals regulating these cellular behaviors of the head mesenchyme remain unknown.

Cells that originate from both the cephalic paraxial mesoderm and the neural crest populate the head mesenchyme (Noden and Trainor, 2005). The cephalic mesoderm is derived from the cells in the primitive streak immediately caudal to the node. As gastrulation progresses, cells from the paraxial mesoderm spread medio-laterally from the primitive streak to a position beneath the developing neural plate. In contrast, the cranial neural crest is derived from cells that are located at the junction of the neural and non-neural ectoderm. Once specified, neural crest cells migrate ventral-laterally between the surface ectoderm and the paraxial mesoderm. During later stages of development, the paraxially-derived cephalic mesoderm contributes to multiple structures such as the smooth and skeletal muscles and some of the cartilaginous and bony elements of the skull. The neural crest contributes to cranial nerves, blood vessels and many of the bony elements of the head and face.

Cranial neural tube closure is critically dependent on the proliferation and cellular rearrangement of the head mesenchyme. Mouse models with deletions in the *Twist1*, *Cart1* or *Tcfap2a* genes exhibit cranial neural tube closure defects linked to defects in development of the head mesenchyme (Chen and Behringer, 1995; Schorle et al., 1996; Zhang et al., 1996; Zhao et al., 1996). *Twist1* function is required in the head mesenchyme where it is expressed in both the paraxial mesoderm and the neural crest lineages (Chen and Behringer, 1995). Yet it is unknown if either *Twist1* or *Cart1*are required in the mesoderm or neural crest or both lineages. Deletion of *Tcfap2a* specifically in the neural crest results in exencephaly, indicating that *AP-2α* function in the neural crest is essential for cranial neural tube closure (Brewer et al., 2004).

Here we describe the further characterization of the neural tube defects in the ENU-induced *open mind* (*opm*) mutant mouse line. Homozygous *opm* mutant embryos exhibit severe defects in cranial neural tube closure (Kasarskis et al., 1998). We demonstrate that the neural tube closure defect in *opm* mutant embryos is associated with a failure of dorsal-lateral hinge point formation and an abnormal organization of the head mesenchyme surrounding the neural tube during closure. In spite of the severe defects in cranial neural tube closure and the abnormal head mesenchyme, *opm* mutant embryos exhibit apparently normal development of neural crest-derived structures such as facial bones and palate. Neural crest development also appears normal in *opm* mutant embryos as assayed by both molecular marker and lineage-tracing analysis. In contrast, analyses of the expression pattern of molecular markers that mark the cephalic mesoderm suggest that this tissue is abnormally dense in *opm* mutants. By positional cloning we identify the *opm* mutation in an uncharacterized ubiquitin ligase (*Hectd1*) that is ubiquitously expressed throughout early development of the mouse embryo. Our data also indicate that a critical threshold of Hectd1 function is required for neural tube closure as up to 20% of mutant heterozygotes exhibit neural tube defects depending on the mutant allele. The low penetrance of neural tube defects in heterozygous embryos suggests that Hectd1 should be considered as a candidate susceptibility gene in human neural tube defects.

Materials and methods

Analysis of mutant phenotype

Whole-mount and section RNA in situs were performed as described (Holmes and Niswander, 2001; Liu et al., 1998) using the following probes: *Fgf8* (Crossley and Martin, 1995), *BMP4* (Jones et al., 1991), *Sox10* (Pusch et al., 1998), *AP-2α* (Mitchell et al., 1991), *Tbx1* (Chapman et al., 1996), *Snail* (Nieto et al., 1992), *Twist* (Chen and Behringer, 1995), *PDGFRα* (Schatteman et al., 1992). The expression pattern of *Hectd1* was determined using an anti-sense RNA probe synthesized from IMAGE clone: 3672615 or determining LacZ activity in $Hectd1^{XC/+}$ embryos. For immunofluorescence experiments, embryos were dissected from the decidua and fixed for 1 hour in 4% paraformaldehyde in PBST (Phosphate-buffered saline (PBS) plus 0.1% Tween-20), washed 3 times in PBST, cryopreserved in 30% sucrose in PBS, embedded in OCT compound (Tissue-Tek) and sectioned at 10 μM. Fixed frozen sections were processed as described (Timmer et al., 2002) using the anti-phospho-Histone H3 Mitosis Marker (1:250; Upstate Biotechnology) for proliferation assays or the cleaved Caspase-3 antibody (1:250; Cell Signaling #9661) for apoptosis assays. Sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories; H-1200) or stained with Hoechst (10 μg/ml; Sigma) to stain nuclei and allow counts of the total number of cells. For Hematoxylin and Eosin staining, embryos were fixed overnight in 4% paraformaldehyde in PBST and processed for frozen sectioning as described (Timmer et al., 2002). Skeletal and β-galactosidase staining were performed as described (Hogan et al., 1994).

Mouse strains and genotyping

The *opm* mouse line was identified in a screen for recessive ENU-induced mutations that cause defects in neural tube closure at E9.5 (Garcia-Garcia et al., 2005; Kasarskis et al., 1998; Zohn et al., 2005). The *opm* mutation was generated on a C57BL/6J genetic background and backcrossed to C3H/HeJ for at least 10 generations to obtain a congenic line. The mapping of the *opm* mutation was done during the creation of the congenic line, while the high resolution mapping data was generated once the congenic line (e.g. >N10) was established. Approximately 5% of *opm*/+ embryos exhibit defects in cranial neural tube closure (see Table 1). However, the neural tube defect in these heterozygous embryos is less severe (only mid-and hindbrain exencephaly) and can be easily distinguished at E9.5 and E10.5 from *opm/opm* embryos (exencephaly from the forebrain to the hindbrain). For this reason, mapping of the *opm* mutation was done using only E9.5 and E10.5 embryos from crosses of *opm*/+ females mated to *opm*/+ males. In a mapping cross of 590 opportunities for recombination, *opm* was mapped between the Massachusetts Institute of