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Developmental neurogenesis in mouse and *Xenopus* is impaired in the absence of Nosip

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ABSTRACT

Background: Genetic deletion of Nosip in mice causes holoprosencephaly, however, the function of Nosip in neurogenesis is currently unknown.

Results: We combined two vertebrate model organisms, the mouse and the South African clawed frog, *Xenopus laevis,* to study the function of Nosip in neurogenesis. We found, that size and cortical thickness of the developing brain of Nosip knockout mice were reduced. Accordingly, the formation of postmitotic neurons was greatly diminished, concomitant with a reduced number of apical and basal neural progenitor cells *in vivo*. Neurospheres derived from Nosip knockout embryos exhibited reduced growth and the differentiation capability into neurons *in vitro* was almost completely abolished. Mass spectrometry analysis of the neurospheres proteome revealed a reduced expression of Rbp1, a regulator of retinoic acid synthesis, when Nosip was absent.

We identified the homologous *nosip* gene to be expressed in differentiated neurons in the developing brain of *Xenopus* embryos. Knockdown of Nosip in *Xenopus* resulted in a reduction of brain size that could be rescued by reintroducing human *NOSIP* mRNA. Furthermore, the expression of pro-neurogenic transcription factors was reduced and the differentiation of neuronal cells was impaired upon Nosip knockdown. In *Xenopus* as well as in mouse we identified reduced proliferation and increased apoptosis as underlying cause of microcephaly upon Nosip depletion. In *Xenopus* Nosip and Rbp1 are similarly expressed and knockdown of Nosip resulted in down regulation of Rbp1. Knockdown of Rbp1 caused a similar microcephaly phenotype as the depletion of Nosip and synergy experiments indicated that both proteins act in the same signalling pathway.

Conclusions: Nosip is a novel factor critical for neural stem cell/progenitor self-renewal and neurogenesis during mouse and *Xenopus* development and functions upstream of Rbp1 during early neurogenesis.

1. Introduction

Nosip originally identified as nitric oxide synthase interacting protein (Dedio et al., 2001; Dreyer et al., 2004; Schleicher et al., 2005) is a 34 kDa protein expressed in a variety of tissues and organs, including the central and peripheral nervous system (Dreyer et al., 2003, 2004; Hoffmeister et al., 2014), trachea and lung (Konig et al., 2005) as well as liver, pancreas and gastrointestinal tract (Konig et al., 2002). Nosip belongs to the family of E3-ubiquitin ligases and harbours a split U-box domain (Friedman et al., 2003). We have shown recently that Nosip regulates the activity of protein phosphatase 2A through monoubiquitination of the catalytic subunit (Hoffmeister et al., 2014), indicating a function of Nosip in the specific modulation of signal transduction.

Genetic deletion of Nosip in mice results in holoprosencephaly (HPE) (Hoffmeister et al., 2014). HPE (OMIM 236100) is the most common developmental disorder in humans involving the forebrain and is characterised by absent or inadequate hemisphere separation and associated with craniofacial malformations (Geng and Oliver, 2009; Roessler and Muenke, 2010). The loss of Nosip in mice causes a significant increase in phosphatase 2A activity, that is most pronounced in the affected craniofacial tissues (Hoffmeister et al., 2014). Given this involvement of Nosip in brain development, in this current study we aimed to address the hitherto unknown function of Nosip in neurogenesis as the fundamental cellular process in the development of the brain.

Neurogenesis describes the generation of neurons from neural stem

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and progenitor cells (NSPCs) (Gotz and Huttner, 2005; Noctor et al., 2007; Sun and Hevner, 2014). NSPCs show the typical characteristics of stem cells such as self-renewal capacity and multipotency as they can develop into all neurons of the central nervous system. In addition, NSPCs give rise to astrocytes and oligodendrocytes in a process referred to as gliogenesis (Gotz and Huttner, 2005; Taverna et al., 2014). Tight control of the switch between proliferation and differentiation into neurons and also of the switch from neurogenesis to gliogenesis is important for proper brain development since it directly determines the number of neurons that are produced as well as the distribution of the three main cell types (Florio and Huttner, 2014; Gotz and Huttner, 2005; Taverna et al., 2014).

An important signalling molecule involved in the control of the proliferation/differentiation switch of NSPCs is retinoic acid (RA) (Janesick et al., 2015). RA is required for neuronal differentiation and promotes expression of proneural and neurogenic genes, including Pax6 and N-tubulin (Gajovic et al., 1997; Janesick et al., 2013; Miyata et al., 2010; Siegenthaler et al., 2009). The importance of RA for neurogenesis and brain development is also reflected in the fact that genetic alterations of RA signalling components or prenatal exposure to RA have been associated with the development of HPE (Bendavid et al., 2009; Cohen and Shiota, 2002; Muenke and Beachy, 2000; Petryk et al., 2015; Roessler and Muenke, 2010).

RA signalling requires a number of factors involved in RA transport and metabolism (Napoli, 2012, 2017). Amongst these cellular retinol binding protein 1, Rbp1 (Crbp1), emerged as a master regulator of retinol homeostasis due to its role as cell intrinsic regulator of vitamin A metabolism and intracellular transport of retinoids (Napoli, 2012). Interestingly, Rbp1 has been found to be silenced in a variety of human cancers (Doldo et al., 2015). This highlights the relevance of Rbp1 for RA signalling and its importance for maintaining the differentiated, non-proliferating state that is frequently lost during tumorigenesis.

Since Nosip has been shown to be highly evolutionary conserved (Dreyer et al., 2003, 2004; Hoffmeister et al., 2014; Konig et al., 2002, 2005) we combined in the present study two model systems, the mouse and the frog *Xenopus laevis*, to study the role of Nosip in neurogenesis.

2. Material and methods

2.1. Generation and histological characterisation of Nosip knockout mice

Nosip knockout mice were described previously (Hoffmeister et al., 2014). Tissue preparation, processing, sectioning and histological HE staining and microscopy were carried out as described (Hoffmeister et al., 2014; Kovacevic et al., 2015). Mice were maintained at the Goethe University Frankfurt Medical School animal facility, as approved by the responsible Veterinary Officer of the City of Frankfurt. Animal welfare was approved by the Institutional Animal Welfare Officer (Tierschutzbeauftragter) in accordance with the Animal protection act (Tierschutzgesetz, TierSchG BGBI. IS. 1206, 1313 17). For embryo collection, pregnant dams were euthanised humanely by isoflurane sedation followed by cervical dislocation. Embryos were collected between E12.5 and E15.5 and sacrificed by decapitation.

2.2. Xenopus laevis embryos

Xenopus embryos were generated, cultured and staged corresponding to standard protocols (Nieuwkoop, 1956; Sive, 2000). All treatments conformed to the Animal protection act and were approved by the German state administration Baden-Württemberg (*Regierungspräsidium Tübingen*).

2.3. Morpholino oligonucleotides and microinjections

Nosip antisense morpholino oligonucleotides (MO), Rbp1 MO and a standard Control MO were ordered from Gene Tools, Philomath, OR, USA. Nosip MO sequence: 5′- TAC AGT TTT TCC CAT GCC TTG TCA T-3′; Rbp1 MO sequence: 5′- CAT GTC TGC TGG GCG AAT GAG T-3′; Control MO: 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′. The MOs were diluted in diethyl pyrocarbonate-treated water and injected unilaterally into one animal-dorsal blastomere at eight-cell stage to target the neural tissue with an amount of 23 ng (Nosip MO) or 60 ng (Rbp1 MO), if not indicated otherwise (Rothe et al., 2017). Correct injections were controlled by co-injection of 0.5 ng *GFP* RNA and evaluated using a fluorescence microscope (Olympus, M-VX, U-RFL-T, Japan). The non-injected side served as internal, the Control MO injection as injection control. Nosip MO-injected embryos were compared to Control MO-injected ones for phenotypic analysis. For rescue experiments, the open reading frame (ORF) of human NOSIP was subcloned into the pCS2^{+} vector (Rupp and Weintraub, 1991) using *Eco*RI (NEB) and following primers: nosip_l: 5′-ATG ACA AGA CAC GGG AAA AAT TGT ACA GG-3′; nosip_r: 5′- TCA GGC CTG CAT CAC CGG CCG TGA-3′. The left primer contains three silent point mutations (here underlined) to have in total nine point mutations in the Nosip MO binding site compared to the corresponding *Xenopus* sequence. For rescue experiments, 23 ng Nosip MO, 0.5 ng full-length human NOSIP RNA and 0.5 ng *GFP* mRNA were co-injected. For rescue control experiments, 23 ng Nosip MO and 1 ng *GFP* mRNA were co-injected. For synergy experiments, 10 ng Nosip MO and 30 ng Rbp1 MO were unilaterally injected either alone or in combination.

2.4. Whole mount in situ hybridization (WMISH) and histology sections in Xenopus

For expression analysis the ORF of *Xenopus laevis nosip* and *rbp1* with the lengths of 896 bp (nosip) or 542 bp (rbp1) were cloned into the pSC-B vector (Stratagene). For opa1 and syne2, partial ORFs were cloned with the lengths of 1.044 bp (opa1) and 763 bp (syne2) into the pSC-B vector (Stratagene). Cloning primers were: xenopus_nosip_l: 5′-ATG ACA AGA CAC GGG AAA AAT TGT ACA GG-3′; xenopus_nosip_r: 5′-TCA CGC TTG CAT TAC TGG CCG-3′; rbp1l_l: 5′-GCC AGA GCT CAC ACA CAC ATA CA-3′; rbp1l_r: 5′-CTT CTA CTT GGG ATG GTG GTT TAT T-3′; opa_l: 5′-TCA GTT CAA TAC GTG CCC TTG C; opa1_r: 5′-CTT ATC ATC CTT TTG CAG CAC CAG-3′; syne2_l: 5′-CTA CAA GCT GAG CAA GAG GGA AC-3′; syne2_r: 5′-ACT GCG CCA CAT ATG TCA TAA TGG-3′. WMISH approaches were performed according to standard protocols (Hemmati-Brivanlou et al., 1990). Digoxigenin-labelled antisense RNA probes were generated by *in vitro* transcription utilizing T3 or T7 RNA polymerase (Roche). Embryos at indicated stages were embedded into gelatine and glutaraldehyde and sections were performed with a thickness of 25 µm using a vibratome (Vibratome 1500 Classic, The Vibratome Company) (Gessert et al., 2007).

2.5. TdT-mediated dUTP-biotin nick end labeling (TUNEL) and phospho histone (pH3) staining

TUNEL and pH3 staining was performed according to standard protocols (Gessert et al., 2007; Kiem et al., 2017; Rothe et al., 2017). TUNEL- and pH3-positive cells were counted in defined areas at both sides of individual embryos.

2.6. Measurement of the brain area in Xenopus

To determine the brain area, embryos at stage 42 were fixed in formaldehyde and brains were dissected using fine forceps. Brain areas of the non-injected and injected sides were labelled and calculated using the ImageJ64 software (Wayne Rasband) (Kiem et al., 2017).