Expression of NUDEL in manchette and its implication in spermatogenesis

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Abstract Nuclear distribution gene E-like product (NUDEL) is a mammal homologue of fungal NUDE and is involved in neuronal migration during brain development. High levels of NUDEL were expressed in murine testis as well as brain. During spermatogenesis, NUDEL was not detected until postnatal day 12 (P12), rising to significant levels at P27. NUDEL was localized predominantly along microtubules of the manchette in elongated spermatids. In maturing spermatids, NUDEL was observed only in the centrosomes, while mature testicular spermatozoa did not show any NUDEL expression. These results suggest that NUDEL plays an important role in germ cell formation, including nucleoplasmic transport and nucleus shaping by manchette microtubules.

Keywords: NUDEL; LIS1; Spermatogenesis; Manchette; Microtubule

1. Introduction

Nuclear distribution gene E-like product (NUDEL) is identified as a protein that interacts with LIS1, a product of the causative gene for type I lissencephaly. Type I lissencephaly is a malformation characterized by a smooth surface of the cortex. The smooth surface is due to a defect in neuronal migration during brain development. LIS1 is a part of a highly conserved evolutionary pathway that regulates cytoplasmic motor function [1]. This pathway was first outlined in fungi, where it participates in nucleokinesis during hyphal stalk formation [2], and is functionally conserved in mammals. NUDEL is also a member of this pathway. It not only interacts with LIS1 but also with a cytoplasmic dynein heavy chain and intermediate chain, which are microtubule minus-end-directed motor components, and thereby positively regulates the retrograde movement of dynein, mitotic spindle orientation, and mitotic progression [3]. NUDEL is phosphorylated by Cyclin-dependent kinase 5 (Cdk5)/p35, a complex known to be essential for axon outgrowth, axon guidance, and neuronal migration. Inhibition of phosphorylation by using either a NUDEL mutant or the Cdk5 inhibitor roscovitine induces neuritic swelling [4]. Thus, NUDEL appears to link the Cdk5 signaling pathway with the LIS1/dynein pathway in neurons.

LIS1 was also identified biochemically from bovine brain extracts as a non-catalytic subunit of type I platelet-activating factor acetylhydrolase (PAF-AH (I)). PAF-AH (I) is an oligomeric complex containing a dimer of two homologous catalytic subunits, α1 and α2, and a non-catalytic β subunit [5,6]. The β subunit was later found to be identical to LIS1. Although the precise function of the PAF-AH (I) catalytic subunits in the LIS1 pathway is still unclear, the catalytic subunits and NUDEL were found to bind to LIS1 in a competitive manner. We have recently shown that PAF-AH (I) catalytic subunits as well as LIS1 are expressed at high levels in both murine brain and testis, and that α1- and α2-deficient mice show a significant reduction in testis size and severe impairment in spermatogenesis [7]. These results prompted us to study the expression and localization of NUDEL, another LIS1-interacting protein, in testis.

2. Materials and methods

2.1. Source of tissues

C57BL/6J mice obtained from Japan SLC (Shizuoka, Japan) were used as source of tissues for Western blot analysis and immunohistochemical analysis.

2.2. Antibodies

Rat NUDEL protein was expressed in *Escherichia coli* as a his-tagged protein using the pET system (Novagen). WYK/Izm rats were immunized via the hind footpads with the recombinant protein using Freund's complete adjuvant. The enlarged medial iliac lymph nodes from the rats were used for cell fusion with mouse myeloma cells, PAI. The antibody-secreting hybridoma cells were selected by screening with ELISA, immunofluorescence and Western blotting. The monoclonal antibody established in this study, clone 4-9C, reacted with rat, mouse and human NUDEL, but did not cross react with NUDE. Anti-α-tubulin (monoclonal, clone DMIA) was purchased from Sigma. Anti-Cdk5 (polyclonal, C-8) was purchased from Santa Cruz Biotechnology. Anti-LIS1 (monoclonal, clone 338.40) was a kind gift from Dr. O. Reiner. Polyclonal antibody against LIS1 (N-19, Santa Cruz Biotechnology) was used for an immunohistochemical analysis.

2.3. Cell culture and transient transfection

HeLa cells were maintained in DMEM supplemented with 10% fetal calf serum. cDNA of full-length rat NUDEL and deletion mutants

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Abbreviations: NUDE, Nuclear distribution gene E product; NUDEL, NUDE-like; PAF-AH, Platelet-activating factor acetylhydrolase; Cdk5, Cyclin-dependent kinase 5

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that lack amino acids #1–#91 (Δ1-91NUDEL) or amino acids #101–#124 (Δ101-124NUDEL) were inserted into *Bam*HI/*Not*I sites of Myc-tagged pcDNA3 [8]. The resulting cDNAs were transfected into HeLa cells using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol.

2.4. Immunohistochemistry

The avidin-biotinylated-peroxidase complex (Vectastain ABC kit, Vector Laboratories) method was used for the immunohistochemistry analysis. First, paraffin-embedded sections were deparaffinized by two 10-min incubations in xylene. Tissues were then rehydrated by passage through graded ethanol and then washed in TBS and microwaved for 10 min in 0.1 M citrated buffer, pH 6.0. Endogenous peroxidase activity was inhibited by incubating sections in methanol solution containing 3% hydrogen peroxide for 20 min. Sections were blocked by incubating with 10% rabbit serum for 30 min. Sections were incubated with the primary antibody for 60 min and then incubated with biotinylated secondary antibody generated against the appropriate species (Vector Laboratories). Immunostaining was visualized using diaminobenzidine. The sections were counterstained with hematoxylin and eosin. Negative controls involved omission of the primary antibody.

2.5. Western blot analysis

Tissues and samples of brain at various developmental stages from C57BL/6J mice were homogenized in four volumes (w/v) of 10 mM Tris–HCl (pH 7.4) containing 1 mM EDTA and 250 mM sucrose, as described [9], and ultracentrifuged at $100\,000 \times g$ at 4 °C. The supernatants were used as the cytosol fractions. Protein concentrations were determined by the BCA assay (Pierce). An aliquot (50 μg) of each cytosol fractions was separated by SDS–PAGE and the proteins were transferred to nitrocellulose filters using the Bio-Rad protein transfer system. The filters were blocked with Tris-buffered saline containing 5% (w/v) skimmed milk and 0.05% (v/v) Tween 20, incubated with the required antibody in Tris-buffered saline containing 5% skimmed milk and 0.05% Tween 20, and then treated with anti-mouse or rat IgG conjugated with horseradish peroxidase. Proteins bound to the antibodies were visualized using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

2.6. Northern blot analysis

Total RNA was extracted from murine brain using Isogen (Nippon Gene). Total RNA (10 μg/lane) was separated by 1% agarose-formaldehyde gel electrophoresis and transferred to Hybond-N membrane (Amersham Biosciences) in 20 X SSC. The membranes were hybridized in Rapid-hyb buffer (Amersham Biosciences) at 65 °C and washed with 0.5 X SSC, 0.1% SDS at 65 °C. A probe for NUDEL was obtained by RT-PCR from mouse RNA and labeled by ^{32}P[dCTP] (Amersham Biosciences) using the Rediprime II DNA labeling system (Amersham Biosciences). The membrane was stripped and rehybridized with human glyceraldehydes-3-phosphate dehydrogenase probe (Clonetech) to ensure equal loading.

3. Results

3.1. Expression of NUDEL in developing murine testis

A specific monoclonal antibody against rat NUDEL was raised in mice. The specificity of the anti-NUDEL antibody was confirmed by Western blotting with lysates from HeLa cells overexpressing rat NUDE, full-length NUDEL or deletion NUDEL mutants. In control HeLa cells, no endogenous NUDEL protein was detected by the monoclonal antibody under the present conditions (Fig. 1A, lane 1), whereas a single band of about 45 kDa was detected upon transfection of myc-tagged rat NUDEL cDNA (Fig. 1A, lane 2). This band was also recognized by anti-myc antibody (Fig. 1B, lane 3), identifying it as NUDEL.

To further characterize the anti-NUDEL antibody, HeLa cells transiently transfected with rat NUDEL or rat NUDE cDNA were analyzed by Western blot (Fig. 1B). Although anti-myc antibody recognized both myc-tagged NUDEL and

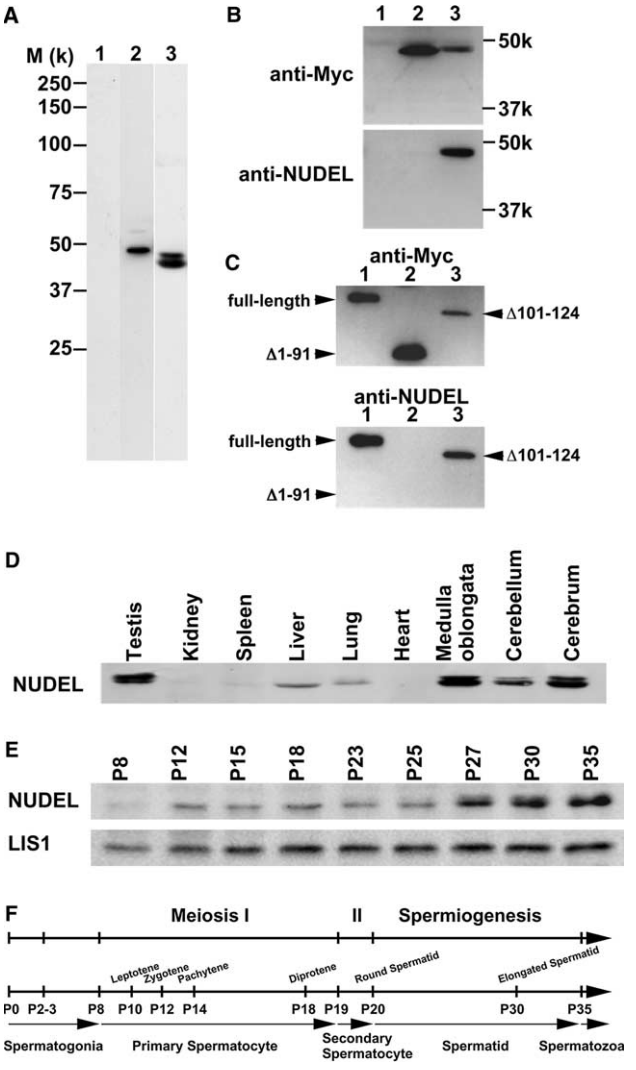

Fig. 1. Expression of NUDEL protein in murine tissues. (A) Lysates (20 μg/lane) prepared from HeLa cells, that were transfected with an empty expression vector (lane 1) or myc-tagged rat NUDEL cDNA (lane 2), and lysates (50 μg/lane) from murine brain were subjected to SDS–PAGE and immunoblotted with anti-NUDEL monoclonal antibody 4-9C. (B) Lysates (20 μg/lane) prepared from HeLa cells that were transfected with an empty expression vector (lane 1), myc-tagged rat NUDE cDNA (lane 2), or myc-tagged rat NUDEL cDNA (lane 3), were immunoblotted with a monoclonal anti-myc antibody (upper panel) or anti-NUDEL 4-9C antibody (lower panel). (C) Lysates prepared from HeLa cells expressing myc-tagged full-length NUDEL (lane 1), myc-tagged Δ91NUDEL (lane 2) or myc-tagged Δ101-124NUDEL were immunoblotted with monoclonal anti-myc (upper panel) or anti-NUDEL (lower panel) antibody. (D) Proteins were isolated from various tissues from adult mice and 50 μg of each protein was subjected to western blot analysis. (E) Western blot analysis of protein extracts from testis of postnatal stages (8–35 days) illustrates the accumulation of NUDEL in comparison to LIS1. (F) Age-dependent stages (proliferative, meiotic, spermiogenic stages) of mouse testis are illustrated.

NUDE, the anti-rat NUDEL antibody recognized only NUDEL but not NUDE. We also found that the epitope recognized by the anti-NUDEL antibody was located within the first 91 amino acids of the NUDEL protein due to its reactivity with full-length protein and Δ101-124NUDEL, but not with Δ1-91NUDEL. A comparison of the amino acid sequence of rat NUDEL in this region reveals 100% identity with the

mouse and human proteins, supporting our unpublished observation that the antibody also recognizes human NUDEL. When adult murine brain extract (50 μg protein/lane) was analyzed by Western blot using this antibody, two bands of about 43 and 44 kDa were detected (Fig. 1A, lane 3), which is consistent with previous observations [3,4]. The upper band is most probably a phosphorylated form of NUDEL, since the upper band disappeared after treatment of brain extracts with alkaline phosphatase (unpublished observations). These results indicated that the antibody raised in this study specifically recognizes NUDEL, but not NUDE.

Next, we examined the expression patterns of NUDEL in murine tissues by Western blot. NUDEL protein was expressed at high levels in brain (cerebrum, cerebellum and medulla oblongata) and testis (Fig. 1D). Only very low levels were detected in other tissues, in agreement with previous reports [3,10]. The phosphorylated form of NUDEL (upper band) was detected in both brain and testis. Because PAF-AH (I) catalytic subunits are expressed at high levels in murine testis and also play an important role in spermatogenesis [7], we further analyzed NUDEL expression in the testis.

In murine testis, the seminiferous epithelium develops postnatally and meiotic-stage germ cells can first be found around postnatal day 8 (P8) (Fig. 1F). Round spermatids are seen around P20 and elongated spermatids are seen around P30 [11,12]. As shown in Fig. 1E, NUDEL became detectable on P12, the day zygotene spermatocytes first appear, and the band density remained the same for the following 15 days, followed by a five fold increase on P27. LIS1 expression in testis also increased postnatally, but not parallel to NUDEL expression.

3.2. Immunohistochemical analysis of NUDEL in adult murine testis

The above findings suggest that NUDEL is required in testis, especially during spermatogenesis, as well as in brain. An immunohistochemical analysis of adult mouse testis showed that NUDEL was primarily localized at the bundles of microtubules surrounding the nucleus in the elongating spermatids (Fig. 2A). This microtubular array forms the manchette, a transient structure involved in nuclear shaping in elongating spermatids during spermatogenesis. A similar staining pattern for LIS1 and α-tubulin was observed in manchettes of elongating spermatids (Fig. 2B and C; arrows). LIS1 and α-tubulin were also localized in meiotic spindles of metaphase spermatocytes (Fig. 2B and C; arrowheads), while no NUDEL staining was observed in this region. Cdk5, which is known to phosphorylate NUDEL in the brain, was localized within the cytoplasm of Sertoli cells, but was not detected in relevant amounts in germ cells at any stage (Fig. 2D). These data suggest that NUDEL phosphorylation is not regulated primarily by Cdk5 in the testis, although the phosphorylated form of NUDEL was detected in the testis as well as brain (data not shown).

3.3. Stage-dependent expression and localization of NUDEL during spermatogenesis

In the mouse, progression of the seminiferous epithelium cycle can be divided into twelve stages (I–XII) according to the criteria described previously [13]. As shown in Fig. 3A, NUDEL immunoreactivity was seen in the cytoplasm of round spermatids (RS) at stage VIII. At stages II–III and X,

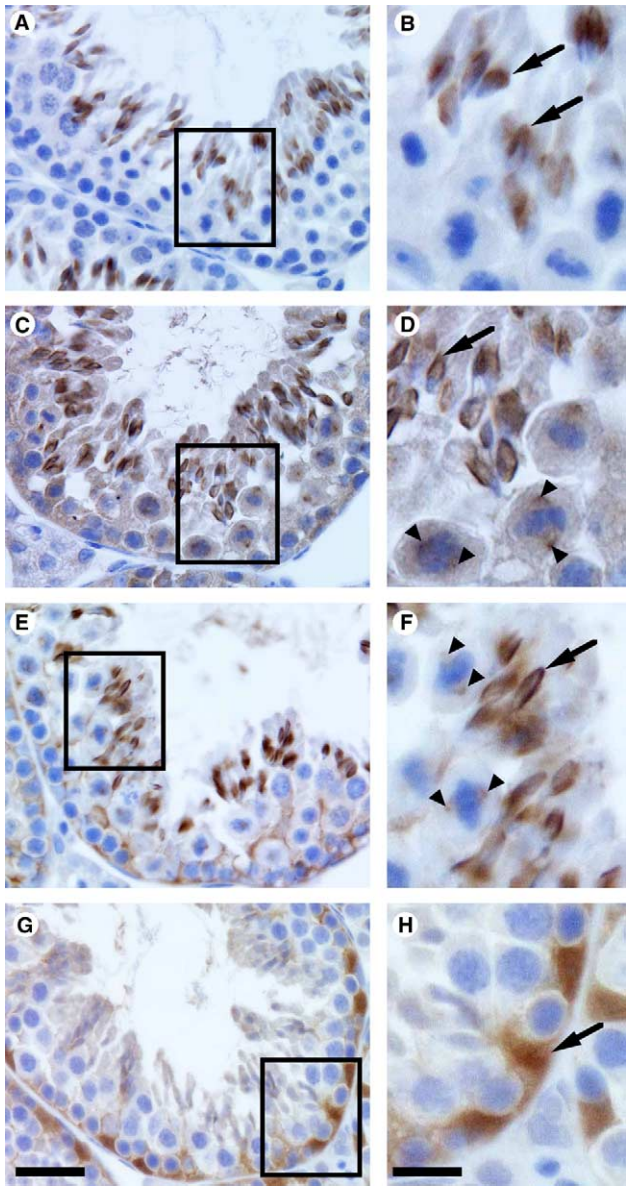

Fig. 2. Localization of NUDEL in murine testis. Immunohistochemical staining of testis cross-sections of postnatal stage (90 days) was performed using specific antibodies for NUDEL (A, B), LIS1 (C, D), α-tubulin (E, F), and Cdk5 (G, H). Hematoxylin was used for counterstaining. NUDEL was primarily localized in manchettes of elongating spermatids (B; arrow). LIS1 and α-tubulin were localized in manchettes of elongating spermatids (D, F; arrows) and meiotic spindles of metaphase spermatocytes (D, F; arrowheads), whereas Cdk5 was localized in cytoplasm of Sertoli cells (H; arrow). Scale bars: A, C, E, G, 50 μm; B, D, F, H, 10 μm.

spermatids develop manchettes extending from the posterior region of the spermatid nuclei to form elongated spermatids. Intense immunoreactivity was associated with these manchettes (Fig. 3B and C). In maturing spermatids (stage IV), when the manchette has already disassembled, spherical-shaped NUDEL immunoreactivity was observed in the caudal-ventral region of the elongated and condensed spermatid nucleus, where the centrosome is located (Fig. 3D). NUDEL immunoreactivity was very low in spermatids at stage VIII (Fig. 3A), spermatogonia (SG) and spermatocytes (SC).

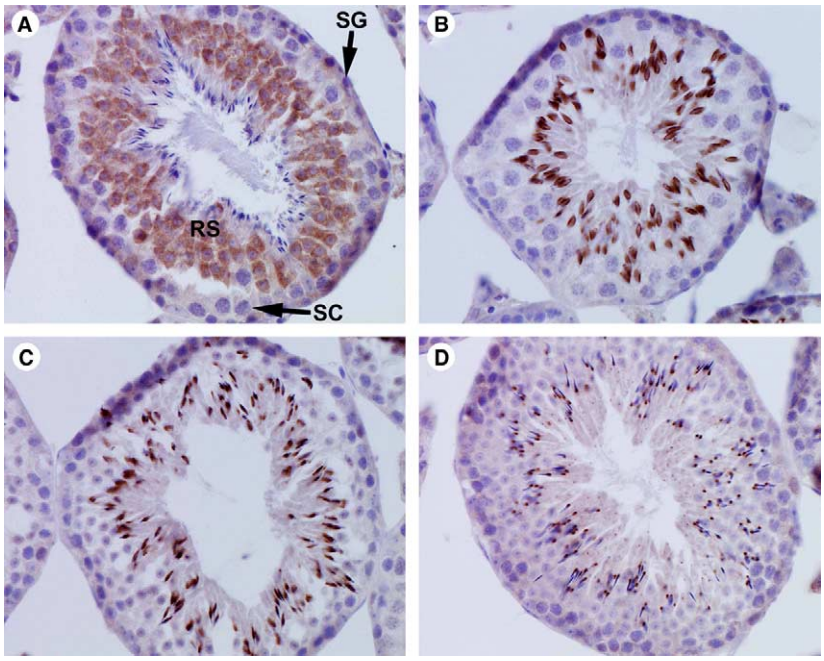

Fig. 3. Immunohistochemical localization of NUDEL in different stages of seminiferous tubules. Immunohistochemical staining of testis cross-sections of postnatal stage (90 days) was performed using specific antibodies for NUDEL. (A) Round spermatids, corresponding to stage VIII, display immunoreactivity in the cytoplasm. Elongated spermatids, corresponding to stage II–III (B) and stage X (C), display NUDEL immunoreactivity in the manchette. (D) At stage IV, when the manchette has disassembled, NUDEL round-shaped immunoreactivity is detected in the caudal-ventral region of the spermatid nuclei. RS, round spermatids; SC, spermatocytes; SG, spermatogonia.

3.4. *NUDEL protein expression in the testes of $\alpha 2^{-/-}$ and $\alpha 1^{-/-}/\alpha 2^{-/-}$ adult mice*

We recently reported the targeted disruption of PAF-AH (I) catalytic subunits in which LIS1 protein levels were significantly reduced in the testes of $\alpha 2^{-/-}$ and $\alpha 1^{-/-}/\alpha 2^{-/-}$ mice. Since NUDEL is a LIS1-associated protein, we also examined the NUDEL protein levels in knockout mice. In $\alpha 2^{-/-}$ mice and $\alpha 1^{-/-}/\alpha 2^{-/-}$ mice, testis NUDEL levels were reduced to approximately 30% and 20%, respectively (Fig. 4A). On the other hand, the levels of α-tubulin (Fig. 4A), a microtubule component, and other microtubule-associated proteins, such as p150-glued and γ-tubulin (data not shown), were not reduced significantly in these mice. *NUDEL* mRNA expression in the brain of $\alpha 1^{-/-}/\alpha 2^{-/-}$ mice was not significantly reduced compared to wild-type mice (Fig. 4B).

The testis of $\alpha 1^{-/-}/\alpha 2^{-/-}$ mouse had significantly reduced numbers of spermatocytes beyond the pachytene stage and round spermatids, and hardly any elongated spermatids (Fig. 4C). NUDEL as well as LIS1 were observed mainly in the remaining round spermatids. The reductions of NUDEL and LIS1 expression may have been due to the severe depletion of germ cells, which express these proteins at high levels. However, this did not appear to be the reason because NUDEL and LIS1 levels were also reduced significantly in the brain and isolated embryonic fibroblasts from mice deficient in the respective catalytic subunits (Fig. 4A).

4. Discussion

In this study, we demonstrated that the levels and intracellular localization of the NUDEL protein were stage-specific during spermatogenesis. Spermatogenesis is an intricately regulated morphogenetic process during which many structural and functional changes are necessary in order to produce mature spermatozoa. Spermatogenesis is divided into three phases, namely the proliferative, meiotic and differentiation phase. In the proliferative phase, the diploid SG undergo rapid successive divisions while in the meiotic phase, the genetic material of the diploid primary spermatocytes is segregated in order to give rise to the haploid secondary spermatocytes and spermatids. In the differentiation or spermiogenic phase, the spermatids undergo transformation to spermatozoa. We found by Western blot analysis that low levels of NUDEL became detectable from P12, the time zygotene primary spermatocytes are first seen (see Fig. 1F). Although immunohistochemistry did not unequivocally show NUDEL expression in meiotic-phase spermatocytes (Fig. 3A), these data suggest an involvement of NUDEL in spermatocyte differentiation. NUDEL was detected at significant levels in the round spermatid during the differentiation phases (Fig. 3A). It is homogeneously present in the cytoplasm of the round spermatid (stage VIII) and is localized at the manchette of spermatid at stages II–III. In parallel to the disappearance of the manchette, NUDEL protein was reduced to almost undetectable levels in mature spermatozoa. These observations suggest that NUDEL is critically involved in the late phase of spermatogenesis, most probably in manchette formation and function. The manchette is a unique set of parallel cytoplasmic microtubules that encircles the nucleus of elongating spermatids [14,15]. Microtubule-dependent motor proteins, such as dynein and kinesin, as well as microtubule-associated proteins, such as MAP4 [16], tau [17], E-MAP-115 [18] and MAST205 [19], are located in the manchette [20,21]. Together with these proteins, NUDEL

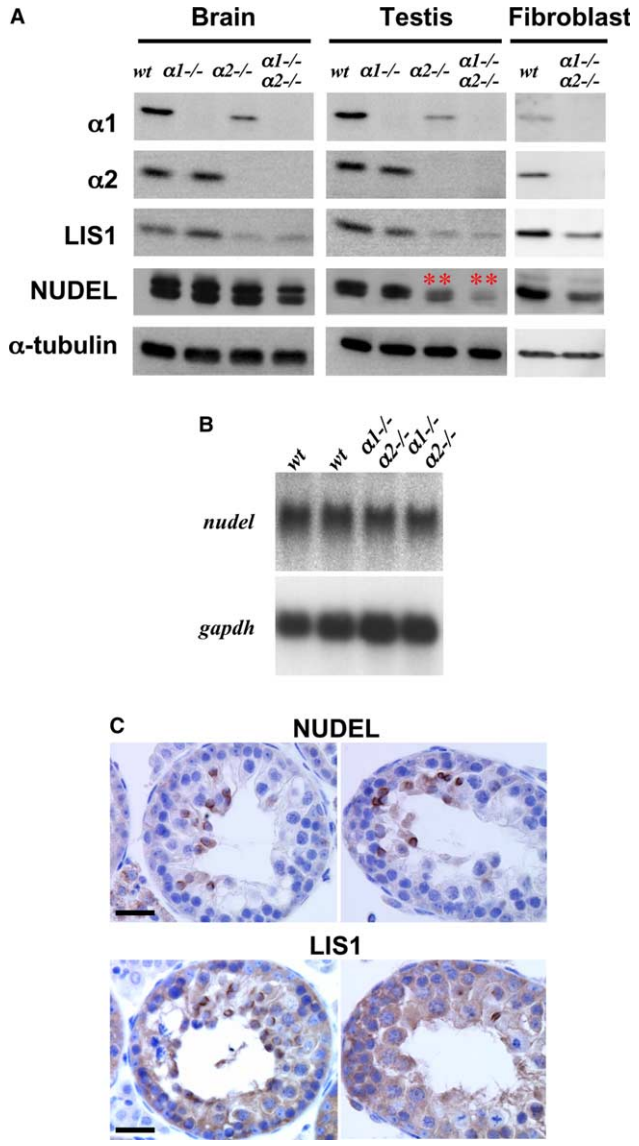

Fig. 4. NUDEL protein expression is reduced in $\alpha2^{-/-}$ and $\alpha1^{-/-}/\alpha2^{-/-}$ adult mice. (A) Western blot analysis of PAF-AH (I) catalytic subunits in adult testis of the wild-type mice and various mutant mice. Expression of NUDEL and LIS1 is reduced in $\alpha2^{-/-}$ and $\alpha1^{-/-}/\alpha2^{-/-}$ mice compared with wild-type mice (**). (B) Northern blot analysis of NUDEL mRNA expression in brains of wild type and $\alpha1^{-/-}/\alpha2^{-/-}$ mice. Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) is included as a control for loading. (C) Immunohistochemical staining of testis cross-sections of $\alpha1^{-/-}/\alpha2^{-/-}$ adult mouse was performed using specific antibodies specific for NUDEL (upper panels) and LIS1 (lower panels). Two different sections are shown, respectively. Hematoxylin was used for counterstaining. Scale bars: 20 μm.

is most likely involved in the nuclear shaping and the process of spermatid elongation.

LIS1, a NUDEL-interacting partner, is also expressed at high levels in murine testis [7]. Nayernia et al. [22] recently generated a mouse mutant by gene trap integration leading to selective loss of a LIS1 protein in testis. Mutant male mice are infertile and spermiogenesis is severely blocked. In the absence of LIS1, spermatids frequently retain large nuclei of irregular shape, a defect most probably derived from incorrect manchette formation [22]. LIS1 is a protein that associates with microtubules and modulates their dynamics [23,24]. Thus, the concentrated localization of both NUDEL and LIS1 in the microtubular manchette might be necessary to maintain its rod-like structures or generate the force exerted on the nucleus, possibly by a microtubule-dependent mechanism.

The expression pattern of LIS1 is somewhat different from that of NUDEL. LIS1 immunoreactivity is present in essentially all seminiferous tubule cell types [7]. The most intense staining of LIS1 is observed in meiotic spindles of spermatocytes and manchettes of elongating spermatids, both of which are specific microtubule structures. LIS1 protein is still present in mature sperm cells where it co-localizes with the acrosomes [22], while NUDEL protein was reduced to almost undetectable levels in mature spermatozoa. Therefore, LIS1 appears to be involved in a variety of processes during spermatogenesis in addition to meiosis and manchette formation and function, although precise functions are unclear so far.

Niethammer et al. [4] have proposed that NUDEL is phosphorylated by Cdk5/p35 in the brain. In fact, NUDEL co-localizes with Cdk5 in axonal growth cones and cell bodies of cultured embryonic hippocampal neurons [4]. In the testis, however, Cdk5 was located predominantly in the cytoplasm of Sertoli cells and mitotically dividing spermatocytes [25], neither of which express NUDEL at high levels. These data indicate that NUDEL is primarily phosphorylated by kinases other than Cdk5 in the testis. In accordance with this proposal, Niethammer et al. reported that the phosphorylated form of NUDEL was still present in $p35^{-/-}/p39^{-/-}$ brain extracts, suggesting that NUDEL can be phosphorylated by other kinases as well as Cdk5 [4]. Moreover, Yan et al. [26] showed that NUDEL was mainly phosphorylated in the M-phase, probably by Cdc2, Erk1 or Erk2 in HEK293 cells.

We have also shown that NUDEL protein levels were greatly reduced both in the testis and brain of mice with mutations of the PAF-AH (I) catalytic subunits. NUDEL may be post-transcriptionally influenced by the PAF-AH (I) catalytic subunits, since NUDEL mRNA levels are not altered in $\alpha1^{-/-}/\alpha2^{-/-}$ mice (Fig. 4B). There is no evidence that NUDEL directly interacts with these catalytic subunits. Loss of the catalytic subunits may induce destabilization of their partner LIS1 [7]. The reduced levels of LIS1 in the PAF-AH (I) catalytic subunits-deficient mice may in turn result in the destabilization of LIS1-associated protein NUDEL. Major reductions of NUDEL and LIS1 may lead to a severe impairment in spermatogenesis, especially in the formation of elongated spermatids.

In conclusion, the present observations support the idea that NUDEL plays an important role in the terminal differentiation of spermatids, and that this protein is intricately involved in manchette formation and function.

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