The Nuclear Scaffold Protein NIPP1 Is Essential for Early Embryonic Development and Cell Proliferation

Aleyde Van Eynde,1†‡ Mieke Nuytten,1† Mieke Dewerchin,2 Luc Schoonjans,3 Stefaan Keppens,1 Monique Beullens,1 Lieve Moons,2 Peter Carmeliet,2 Willy Stalmans,1 and Mathieu Bollen1

Division of Biochemistry1 and Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology,2 Faculty of Medicine, Catholic University of Leuven, and Thromb-X NV,3 B-3000 Leuven, Belgium.

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NIPP1 (nuclear inhibitor of protein phosphatase 1) is a ubiquitously expressed nuclear scaffold protein that has been implicated in both transcription and RNA processing. Among its protein ligands are a protein kinase, a protein phosphatase, two splicing factors, and a transcriptional regulator, and the binding of these proteins to NIPP1 is tightly regulated by phosphorylation. To study the function of NIPP1 in vivo, we have used homologous recombination to generate mice that are deficient in NIPP1. NIPP1−/− mice developed normally. However, NIPP1−/− embryos showed severely retarded growth at embryonic day 6.5 (E6.5) and were resorbed by E8.5. This early embryonic lethality was not associated with increased apoptosis but correlated with impaired cell proliferation. Blastocyst outgrowth experiments and the RNA interference-mediated knockdown of NIPP1 in cultured cells also revealed an essential role for NIPP1 in cell proliferation. In further agreement with this function, no viable NIPP1+/− cell lines were obtained by derivation of embryonic stem (ES) cells from blastocysts of NIPP1−/− intercrosses or by forced homogenization of heterozygous ES cells at high concentrations of Geneticin. We conclude that NIPP1 is indispensable for early embryonic development and cell proliferation.

NIPP1 is a nuclear protein of 39 kDa that is expressed in both plants and animals (11, 17, 29). It was originally purified as a potent and specific inhibitor of protein phosphatase 1 (PP1); hence its name, nuclear inhibitor of PP1 (3). NIPP1 contains at least two binding sites for PP1 and is complexed to about one-third of the nuclear pool of PP1 (2, 5, 19). The NIPP1–PP1 complex is inactive but can be activated by phosphorylation of NIPP1 with protein kinase A (1, 31), protein kinase CK2 (28, 31), and protein tyrosine kinases of the Src family (5). Overexpression of NIPP1 in Drosophila melanogaster is lethal in a range of tissues and developmental stages, probably as a result of the inhibition of PP1 (25).

NIPP1 also binds to the cell-cycle-regulated maternal-embryonic leucine zipper kinase (MELK) (30). During mitosis, MELK interacts via a phosphorylated threonine with the Forkhead-associated (FHA) domain of NIPP1. Interestingly, MELK is a potent inhibitor of spliceosome assembly, and this inhibition requires a functional NIPP1-binding site. Thus, the MELK-NIPP1 interaction may contribute to splice arrest during mitosis. Additional evidence for a key role of NIPP1 in pre-mRNA splicing comes from competition experiments with NIPP1 fragments (4) and from observations that NIPP1 is enriched both in the splicing factor storage sites, or “speckles,” and in spliceosomes (4, 17, 26). The targeting of NIPP1 to these subnuclear compartments is mediated by its FHA domain (4, 17) and is likely to be accounted for by the interaction of the FHA domain with phosphorylated forms of the splicing factors CDC5L (8) and SAP155 (7).

Further insights into the functional complexity of NIPP1 have come from recent observations that NIPP1 also interacts with the Polycomb protein EED (embryonic ectoderm development), a component of the Polycomb repressive complex 2 (PRC2) that is implicated in the maintenance of genes in their repressed state (20). Both EED and NIPP1 function as transcriptional repressors of targeted genes in transient transfection experiments. Moreover, a macromolecular complex that contains NIPP1, EED, PP1, and the histone deacetylase HDAC2 has been identified, suggesting a role for histone deacetylation in transcriptional repression by NIPP1.

The human NIPP1-encoding gene, PPPIR8, can give rise to multiple splice variants (27). The NIPP1 isoform that is ubiquitously expressed is referred to as NIPP1α (or just NIPP1).

<table>
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<td>29 (56)</td>
<td>11 (21)</td>
<td>52</td>
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* Corresponding author. Mailing address: Afdeling Biochemie, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. Phone: 32-16-34 57 01, Fax: 32-16-34 59 95. E-mail: Aleyde.VanEynde@med.kuleuven.ac.be.
† A.V.E. and M.N. contributed equally to this work.

a ND, not determined. The total number of wild-type and heterozygote embryos at E6.5 was 121 (78%).
A. Targeting strategy

\[ Ppp1r8 \]

Phage 20.2

Phage 4.1

Probes A and C

ATG

Probe B

\[ \text{wt Locus} \]

\[ \text{Targeting vector} \]

\[ \text{Targeted locus} \]

\[ 9 \text{ kb (wt)} \]

\[ 5.8 \text{ kb (mutant)} \]

B. Southern on neonates

<table>
<thead>
<tr>
<th>wt</th>
<th>+/-</th>
<th>-/-</th>
<th>+/-</th>
<th>+/-</th>
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</table>

\[ 9 \text{ kb (wt)} \]

\[ 5.8 \text{ kb (mutant)} \]

C. PCR on embryos (E7.5)

\[ 500 \text{ bp} \]

\[ 402 \text{ bp (wt)} \]

\[ 696 \text{ bp (mutant)} \]

D. RT-PCR on blastocysts (E3.5)

\[ 500 \text{ bp} \]

\[ 350 \text{ bp (wt)} \]

\[ 696 \text{ bp (mutant)} \]

E. Immunostaining (E6.5)

\[ \text{NIP1} \]

\[ \text{HDAC2} \]
Another isoform is NIPP1/H9253/Ard1 (12, 27), which corresponds to the C-terminal one-third of NIPP1/H9251. Interestingly, NIPP1/H9253 displays an endoribonuclease activity with a specificity similar to that of bacterial RNase E, a key regulator of the decay and processing of various RNAs (21, 32). However, the widely expressed NIPP1/H9251 isoform shows no endoribonuclease activity (18), suggesting that the expression of this enzymatic activity is under tight control.

In conclusion, NIPP1 emerges from the available data as a multifunctional protein and as a candidate “integrator” of transcription, RNA processing, and cell cycle progression. To further study the complex function of NIPP1, we have disrupted the gene encoding NIPP1 (Ppp1r8) in mice by homologous recombination. NIPP1/H11002/H11001 mice did not display an overt phenotype, while NIPP1/H11002/H11002 embryos died at around 6.5 days postconception. This early embryonic lethality was associated with a generally decreased ability of the cells to proliferate. Blastocyst outgrowth experiments and the RNA interference

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**FIG. 1.** Targeted disruption of the NIPP1 gene. (A) Partial restriction maps of the Ppp1r8 locus (mouse NIPP1 gene), the targeting vector, and the targeted locus. Two 12.5-kb phage genomic clones, isolated from the 129SvJ mouse lambda genomic library, are shown on top. In the wild-type (wt) locus, exons 1 to 5 (E1 to E5) are shown as solid boxes and “ATG” represents the first coding exon. Restriction sites: B, BamHI (not all indicated); E, EcoRI (all indicated); P, PstI (not all indicated). Dashed lines between the wt locus and the targeting vector delineate the regions that are common to the genomic locus and the replacement vector. The neomycin resistance cassette (Neo) in the replacement vector is flanked by loxP (not indicated), and a thymidine kinase cassette (TK) lies downstream of flanking 2 in the replacement vector. The targeted locus represents the predicted structure of the NIPP1 locus after homologous recombination with the replacement vector. A 6.6-kb fragment comprising part of the promoter region, as well as exons 1 to 2, has been replaced by the Neo cassette. (B) Southern blot genotyping of neonates derived from intercrosses of NIPP1 heterozygotes. Genomic DNA was digested with EcoRI and hybridized with probe A, yielding a 9-kb band for the wt allele and a 5.8-kb band for the mutant allele. (C) PCR genotyping of E7.5 embryos obtained from crosses of NIPP1 heterozygotes. A wt-specific nested PCR, as explained in Materials and Methods, amplifies a band of 402 bp, and a mutant-specific PCR results in a band of 696 bp. M, marker; C, control. (D) RT-PCR genotyping of blastocysts (E3.5) isolated from NIPP1 heterozygous intercrosses. The presence of the wt NIPP1 allele results in amplification of a 350-bp product, while the mutated NIPP1 allele leads to amplification of a 696-bp product. (E) Detection of NIPP1 by immunostaining in E6.5 embryos from intercrosses of NIPP1 heterozygotes. Serial transverse sections of paraflin-embedded embryos were made from E6.5 deciduae. Sections were stained with anti-NIPP1 antibodies (a and c) or anti-HDAC2 antibodies (b and d). The figure shows an embryo that expresses NIPP1 (a and b) and an embryo that lacks NIPP1 (c and d). The control condition in panels c and d did not contain DNA template. Bar in panel Ea, 50 μm.
A. Transverse sections

NIPP1wt

NIPP1−/−

B. Sagittal sections

NIPP1wt

NIPP1−/−

FIG. 3. Cell lineages in NIPP1wt and NIPP1−/− embryos. Transverse (A) and sagittal (B) sections of paraffin-embedded NIPP1wt and NIPP1−/− embryos (E6.5) were immunostained for NIPP1, Gata4, Cdx2, and Oct4 as indicated. Gata4 stains specifically the visceral and parietal endoderm, Cdx2 is a marker of the extraembryonic ectoderm, and Oct4 is specifically expressed in the embryonic ectoderm. All three cell lineages can be distinguished in the NIPP1−/− embryos, but the extraembryonic and the embryonic ectoderms are not organized into a clear epithelial layer. Abbreviations are as explained in the legend to Fig. 2. VE, visceral endoderm. Bars in panels Aa and Ba, 50 μm.
A. Apoptosis

FIG. 4. Apoptosis and proliferation rates in NIPP1<sup>wt</sup> and NIPP1<sup>−/−</sup> embryos. (A) Paraffin-embedded transverse sections of NIPP1<sup>wt</sup> (a and b) and NIPP1<sup>−/−</sup> (c and d) E6.5 embryos were stained with hematoxylin and eosin (a and c) and by the TUNEL assay (b and d) for the visualization of apoptosis. No difference in apoptosis between wild-type and knockout embryos was detected. Bar in panel Aa, 50 μm. (B) Pregnant mice were injected with BrdU 1 h before sacrifice. Sections of NIPP1<sup>wt</sup> and NIPP1<sup>−/−</sup> embryos (E6.5) were analyzed for BrdU incorporation by immunostaining with anti-BrdU antibodies. Stained nuclei of five NIPP1<sup>wt</sup> and six NIPP1<sup>−/−</sup> embryos were counted and expressed as a percentage of the total number of nuclei in the section. Nearly 20% lower BrdU incorporation was found in the mutant embryos, indicating a decreased rate of cell proliferation. Results are means ± standard errors (P = 0.0074 by an unpaired t test).

B. BrdU staining

% positive nuclei

NIPP1<sup>wt</sup>  NIPP1<sup>−/−</sup>
A. Blastocyst outgrowth

Day 1

Day 3

Day 5

NIPP1\(^{wt}\)

NIPP1\(^{-/-}\)

B. NIPP1/BrdU/Hoechst staining

Ab NIPP1

Ab BrdU

Hoechst

FIG. 5. Retarded outgrowth of NIPP1-deficient blastocysts. Blastocysts from intercrosses of heterozygotes were isolated, cultured, and genotyped as described in Materials and Methods. (A) Photographs of outgrowths of NIPP1\(^{wt}\) (a to c) and NIPP1\(^{-/-}\) (d to f) blastocysts following
Rat NIP1, a new member of the PDZ protein family, is essential for cell proliferation. NIP1 contains a PDZ domain that binds to the C-terminal tails of tyrosine kinases. The mRNA of NIP1 was found to be expressed specifically in the proliferating cells of the mouse embryonic stem cells. NIP1 was then identified as a novel target of the E3 ubiquitin protein ligase Cbl, which is known to be involved in the regulation of cell proliferation. The role of NIP1 in cell proliferation was further studied by generating NIP1 knockout mice. The knockout mice showed severe defects in cell proliferation, indicating that NIP1 is essential for cell proliferation.

The authors then tested the ability of NIP1 to inhibit cell proliferation by using a ectopic expression assay. The results showed that NIP1 was indeed able to inhibit cell proliferation in a dose-dependent manner. The authors also found that NIP1 was able to inhibit cell proliferation in a time-dependent manner. These results suggest that NIP1 plays a role in the regulation of cell proliferation.

The authors then investigated the mechanism by which NIP1 inhibits cell proliferation. They found that NIP1 inhibits cell proliferation by downregulating the expression of the cell cycle regulatory protein CDK6. The authors also found that NIP1 inhibits cell proliferation by downregulating the expression of the cell cycle regulatory protein CDK4. These results suggest that NIP1 inhibits cell proliferation by downregulating the expression of the cell cycle regulatory proteins CDK6 and CDK4.

The authors then investigated the role of NIP1 in the regulation of cell proliferation in vivo. They found that NIP1 was essential for the proper development of the mouse embryo. The knockout mice showed severe defects in cell proliferation, indicating that NIP1 is essential for cell proliferation.

In conclusion, the authors showed that NIP1 is essential for cell proliferation. NIP1 inhibits cell proliferation by downregulating the expression of the cell cycle regulatory proteins CDK6 and CDK4. The authors also showed that NIP1 is essential for the proper development of the mouse embryo.

Materials and methods

Construction of a targeting vector for homologous recombination. The 5’-end primer 5’-GGCGATGCGCTGCGAATCG-3’ and the 3’-end primer 5’-GAAGAGGCC-3’ were used to amplify a 475-bp fragment from the wild-type allele. The sense primer 5’-GGCAGTGTTGAACTCTGTCAGG-3’ and the antisense primer 5’-GGCAGTGTTGAACTCTGTCAGG-3’ were used to amplify a 475-bp fragment. This amplification of the wild-type allele results in a 402-bp band, while amplification of the mutant allele yields a product of 623 bp. PCR genotyping was performed by Southern blot analysis of genomic DNA obtained from tail biopsies. Heterozygous mice were intercrossed to obtain NIP1-/- mice with an overall 50:50 R1:129:Swiss genetic background.

Genotype analysis. DNA for genotype analysis by Southern blotting or by PCR blotting was obtained from ES clones, mouse tail tips, or embryos. The correct homologous recombination of NIP1-/- clones at both flanks was confirmed by Southern blot analysis using a 0.8-kb EcoRI-PstI fragment (probe A) located immediately upstream of flank 1 and a 1.7-kb HindIII-BamHI fragment (probe B) located downstream of flank 2 (Fig. 1A). To exclude additional random integration of the targeting vector, an internal 1.1-kb XbaI fragment (probe C) located in flank 2 was used to verify the absence of additional bands in Spé digest DNA. Hybridization of EcoRI-digested DNA with probe A detects a 9-kb band for the wild-type allele and a 5.8-kb band for the targeted allele (Fig. 1B). Hybridization of Spel-digested DNA with probe B identifies a 13-kb band for the wild-type allele and an 8-kb band for the targeted allele (data not shown).

PCR genotyping of mouse tail tips and embryonic day 8.5 (E8.5) or E10.5 embryos (Table 1) was performed with a three-primer strategy: a common sense primer located in flank 1 (5’-CCTCAGCAGATAGCCCACGG-3’), and an antisense primer 5’-GCTCTGGAATTTCTTTGGA-3’ that is specific for the targeted allele. The PCR products were used as templates in a secondary PCR with the same sense primer and the nested antisense primer 5’-AGATGCTGGACACCAGGC-3’ and the antisense primer 5’-GAATCTTTGATAGCCCACGG-3’. This amplification of the wild-type allele results in a 402-bp band, while amplification of the mutant allele yields a product of 623 bp. PCR genotyping of E3.5 blastocysts showed delayed outgrowth and severely reduced incorporation of BrdU. Ab, antibody. Bars, 20 μm.

In vitro culture of blastocysts. Heterozygous NIP1 mice were intercrossed with superovulating heterozygous females. Blastocysts were flushed out of the uterus at day 3.5 of pregnancy in M2 medium (Sigma, St. Louis, Mo.) and cultured at 37°C and 5% CO2 on gelatin-coated chambered cover glasses (Nunc, Denmark) in TSX medium (Thyroid-X and Nucleobasal medium). The maximum number of blastocysts in each well was limited to 200 cells. After 2 days of culture, the blastocysts were fixed, embedded in paraffin, and sectioned as described above. The sections were stained with hematoxylin and eosin, and the nuclei were counterstained with DAPI. The sections were then analyzed by confocal microscopy.

Immunohistochemistry. The TSA Biotin system of Perkin-Elmer (Wellesley, Mass.) was used for immunohistochemical staining. Paraffin sections were rehydrated (twice, for 5 min each time, in xylene; twice, for 3 min each time, in 100% ethanol; 3 min in 70% ethanol; 3 min in 50% ethanol; and 5 min in water), boiled in citric acid buffer (1.5 mM citric acid, 8.5 mM sodium citrate, 0.05% Dett detergent) on a hot plate for 20 min, and cooled to room temperature. Sections were washed in TBS (20 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 0.1% Triton X-100) for 5 min. Endogenous peroxidase was blocked with 0.3% H2O2 in methanol for 20 min. The sections were then washed in TBS three times, for 5 min each time, and then blocked in TNB blocking buffer (Perkin Elmer) with either 20% goat serum or 20% rabbit serum (DAKO, Glostrup, Denmark) for 30 min. The primary antibodies were diluted 1:200 in TBS, and the sections were incubated at 4°C overnight with the primary antibody, rinsed three times with TBS, and incubated with a biotinylated secondary antibody (1:100 in TBS). The sections were then washed three times with TBS, and incubated with streptavidin-HRP (1:100 in TBS) for 30 min, rinsed three times with TBS, and incubated with the chromogenic substrate diaminobenzidine (1 mg/ml in 50 mM Tris-HCl [pH 7.5]). After a 5 min wash, the sections were counterstained with nuclear fast red.

Results

NIP1-/- blastocysts showed delayed and reduced incorporation of BrdU. Ab, antibody. Bars, 20 μm.
glass for 4 days. Sixteen hours later, the cells were fixed in 2% paraformaldehyde-PBS, and immunofluorescence was performed.

**RNAi.** Small interfering RNA molecules (siRNAs) for the knockdown of NIPP1 were made with the Dicer siRNA generation kit from Gene Therapy Systems, Inc. (San Diego, Calif.). To target double-stranded RNA (dsRNA) of NIPP1, the following PCR primers, with the first 20 bp derived from the T7 promoter, were used: 5'-GCGTATACCTCTATATTGGAAGGGTTCT ATGTATTGCTGA-3' and 5'-GCGTATACCTCTATATTGGAAGGGTTCT CATGCTGCTGCTTGCTACCC-3'. The PCR product was transcribed in vitro with T7 RNA polymerase to form dsRNA by using the MEGAscript T7 kit (Ambion, Huntinton, Cambridgeshire, United Kingdom). For transfection of one 24-well culture of HEK293 or U2OS cells, 1 μg of this dsRNA was cut in vitro with T1 of recombinant Dicer enzyme. The lamin A/C siRNA duplex (5'-AACUGGACUUCCGAGAACA-3') was bought from Dharmacon, Inc. (Lafayette, Colo.). The lamin dsRNA or the diced and purified NIPP1 dsRNA was transfected with the GeneSilencer siRNA transfection reagent according to the manufacturer's protocol (Gene Therapy Systems). For Western blot analysis, all cells were collected, washed twice with PBS, and subsequently lysed in a solution containing 50 mM Tris (pH 7.5), 0.5 M NaCl, 0.5% Triton X-100, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, and 5 μM leupeptin. After sonication, the lysates were cleared by centrifugation (for 10 min at 16,000 x g), and the protein concentration was measured to ensure equal loading during sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Cell culture and immunofluorescence.** HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μg of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM l-glutamine. U2OS cells were grown in McCoy's 5A medium with L-glutamine, supplemented with 10% fetal calf serum, 100 μg of penicillin/ml, and 100 μg of streptomycin/ml. For immunofluorescence experiments, cells and blastocyst outgrowths were washed in PBS for 5 min, fixed in PBS-2% formaldehyde for 10 min, permeabilized in PBS-0.5% Triton X-100 for 10 min, and finally blocked in PBS containing 3% bovine serum albumin for 20 min. For BrdU staining, blastocyst outgrowths were permeabilized in PBS-0.5% Triton–0.1 M HCl. Primary and secondary antibodies (tetramethyl rhodamine isothiocyanate [TRITC]-conjugated anti-mouse immunoglobulin G [DAKO]) were used at a final dilution of 1/200 and were left on the cells for 1 h. Each step was followed by three washes with PBS for 5 min. Microscopy was performed with a confocal microscope (Zeiss LSM 510).

**TUNEL assay.** Terminal deoxynucleotidyltransferase (TdT)-mediated incorporation of dUTP into DNA ends was carried out on sectioned embryos by using the DeadEnd colorimetric apoptosis detection system (Promega, Leiden, The Netherlands). Briefly, deparaffinized and rehydrated sections adhering to glass slides were digested with proteinase K and postfixed with 4% paraformaldehyde in PBS. Incorporation of fluorescently labeled nucleosides by the TdT enzyme was carried out on slides at 37°C for 60 min. In each staining experiment known TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive samples (DNase treated) were used as a positive control. As a negative control, the reaction was performed without TDT.

**RESULTS**

**Targeted disruption of Ppp1r8.** To generate NIPP1+-/- ES cells by homologous recombination, we constructed a replacement-type targeting vector aimed at the swapping of a 6.6-kb fragment of Ppp1r8 for a neomycin cassette (Fig. 1A). The deleted Ppp1r8 fragment included a 3.2-kb fragment of the promoter region as well as exons 1 and 2. Following electroporation of the linearized targeting vector into R1 ES cells, eight NIPP1+-/- ES cell clones were obtained. The NIPP1+-/- ES cells were aggregated into Swiss mouse morulae, which were implanted into foster mothers. The resulting chimeric males were crossed with Swiss females, and the heterozygous offspring was identified by Southern blot analysis.

NIPP1+-/- mice did not show an overt phenotype for as long as 1 year (data not shown). Thus, the heterozygotes showed normal fertility and did not develop tumors in this period. Interestingly, immunoblot analysis did not show differences between NIPP1 expression levels in organs from wild-type versus NIPP1+-/- mice (data not shown), indicating that the loss of one NIPP1 allele does not affect the cellular NIPP1 concentration.

**A deficiency of NIPP1 results in early embryonic lethality.** To examine the phenotype of NIPP1-null mice, NIPP1+-/- mice were intercrossed and their progeny was genotyped. PCR and Southern blot analysis of the neonates yielded no evidence of viable NIPP1+-/- mice, while wild-type and heterozygous animals were obtained at the expected 1:2 ratio (Table 1; Fig. 1B). To examine at which stage during embryonic development the NIPP1+-/- embryos died, we performed RT-PCR and immunohistochemical analyses of embryos from timed matings. Only 1 out of the 59 PCR-genotyped E7.5 embryos was homozygous, and no viable NIPP1+-/- embryos were detected beyond 7.5 days (Table 1; Fig. 1C). However, we noted that 20 to 30% of the E7.5 to E10.5 embryos were resorbed (Table 1), which corresponds roughly to the number of expected NIPP1+-/- embryos (25%). E3.5 blastocysts still showed a normal Mendelian distribution among NIPP1+-/+(23%), NIPP1+-/- (56%), and NIPP1+/- (21%) genotypes, as determined by RT-PCR analysis (Table 1; Fig. 1D). Also, immunostaining of sections from paraffin-embedded E6.5 embryos revealed that 19% of the embryos from the NIPP1+-/- intercrosses did not express NIPP1, while they showed a normal level of the unrelated nuclear histone deacetylase HDAC2 (Table 1; Fig. 1E). It should be noted that genotyping by immunofluorescence does not distinguish between NIPP1+-/- and NIPP1+/- genotypes; therefore, embryos genotyped in this manner will be collectively referred to as wild type (NIPP1+/+).

The data reported above indicated that NIPP1+-/- embryos die between days 6.5 and 7.5 of development. Hematoxylin and eosin staining showed that E6.5 NIPP1+-/- embryos were only about half the size of their wild-type E6.5 littermates (Fig. 2). The differences were apparent on both transverse and sagittal sections. In the wild-type embryos at E6.5, an embryonic axis could be observed (Fig. 2A), and the extraembryonic and embryonic ectodermus were organized into epithelia surrounding the proamniotic cavity (Fig. 2A to C). In the NIPP1-null embryos, however, the embryonic axis was hardly visible and the cells from the embryonic and extraembryonic ectodermus did not appear to be clearly organized into epithelial layers, accounting for the disorganized appearance of the NIPP1+-/- embryos. In addition, the proamniotic cavity in the NIPP1-null embryos was reduced in size and sometimes was not even

**FIG. 6.** Effects of the RNAi-mediated knockdown of NIPP1 on the proliferation of mammalian cells. (A) At 72 h after transfection of HEK293 cells with NIPP1-specific or lamin A/C-specific siRNAs or mock transfection (without siRNAs), the cells were lysed and processed for immunoblotting with anti-NIP1 antibodies (upper panel) and with antibodies against SIPP1, a structurally unrelated nuclear scaffold protein (lower panel). (B) Levels of NIPP1 protein in mock-transfected cells (a) and in cells transfected with NIPP1-specific siRNAs (b), as determined by immunofluorescence analysis. Bar, 10 μm. (C) Number and morphology of HEK293 cells 72 h after mock transfection (a) or after transfection with NIPP1-specific siRNAs (b). The RNAi-induced knockdown of NIPP1 results in a lower number of cells, indicating slower growth. Bar, 30 μm.
A. Western blot

B. Immunofluorescence

C. Cell morphology
The data reported above demonstrated that NIPP1α disorganized ectoderm. NIPP1α cylinder, which, however, was severely reduced in size and had these data could indicate that NIPP1 is required for the G1/S by repeated replication without intervening cell divisions (16), those in outgrowths from NIPP1wt blastocysts (Fig. 5Bc and Be). Compared to NIPP1wt blastocyst outgrowths, very few cells were labeled in NIPP1−/− blastocyst outgrowths, pointing to a considerably prolonged cell cycle for the latter condition.

**Cell lines that lack NIPP1 are not viable.** To examine whether NIPP1 is also required for the proliferation of cultured cells, we first attempted to generate NIPP1−/− ES cells. A single clone was subjected to selection with an increased Genetin concentration (2 mg/ml [23]), but among the 169 surviving ES subclones, not a single viable NIPP1−/− line was identified. As an alternative, we tried to derive ES cell lines from blastocysts of NIPP1−/+ intercrosses. Out of the 102 blastocysts that were processed for ES cell derivation, 42 cell lines were established. Genotyping by Southern blot analysis yielded 16 NIPP1−/+ (38%) and 26 NIPP1−/+ (62%) cell lines, but not a single cell line with a NIPP1−/− genotype (data not shown). Our failure to obtain NIPP1−/− ES clones suggests that NIPP1 is also essential for the viability of ES cells.

To further explore the requirement of NIPP1 for the proliferation of cultured cells, we examined the effect of the RNAi-mediated knockout of NIPP1 in HEK293 cells (Fig. 6) and U2OS cells (data not shown). For that purpose, a 700-kb fragment of dsRNA derived from the NIPP1 transcript was fragmented in vitro with the Dicer enzyme and transfected into the cells. This resulted in the nearly complete disappearance of NIPP1 within 72 h, as detected by both immunoblotting (Fig. 6A) and immunofluorescence analysis (Fig. 6B). In contrast, this transfection did not affect the level of SIPP1 (Fig. 6A), an unrelated nuclear scaffold protein (22). Also, transfection of the cells with siRNAs for the knockdown of lamin A/C did not affect the level of NIPP1 (Fig. 6A). The RNAi-induced knockdown of NIPP1 was associated with a complete failure of the cells to proliferate, as evidenced by the low number of surviving cells (Fig. 6C).

**DISCUSSION**

NIPP1 is required for early embryonic development in the mouse. To study the functional diversity of NIPP1 in vivo, we have targeted the NIPP1-encoding genes by homologous recombination. Surprisingly, the disruption of only a single NIPP1 allele did not affect the cellular NIPP1 concentration, which explains the lack of an overt phenotype in NIPP1−/+ mice. These findings fit in nicely with our previous observations that the cellular NIPP1 concentration is strictly controlled and may be subject to negative-feedback regulation. For example, transfection of mammalian cells with an expression vector for NIPP1 resulted in a huge accumulation of NIPP1 transcripts without a corresponding change in the concentration of NIPP1 protein; this could be accounted for by a translational repression mechanism involving the coding region of the NIPP1 transcript (17, 33). It is therefore possible that the concentrations of NIPP1 protein in heterozygote tissues are normal because of a lesser translational repression due to a lower basal level of NIPP1 transcripts. However, additional control mechanisms, e.g., at the level of transcription, cannot be excluded.

Disruption of both NIPP1 alleles was associated with early embryonic lethality. At E6.5, NIPP1−/− embryos were only
about half the normal size (Fig. 2), and they showed a generally lower proliferation rate, which was not, however, associated with apoptosis (Fig. 4). Also, NIPP1−/− embryos failed to form an ectoplacental cone as well as a mesoderm, and their ectoderm cells did not organize into an epithelial layer (Fig. 2 and 3). By E7.5, nearly all NIPP1−/− embryos were resorbed (Table 1). Collectively, these data strongly indicate that NIPP1 is required for the rapid growth and differentiation phase of embryos around gastrulation. An intriguing question is why NIPP1−/− embryos survive until E6.5 (Table 1) while the knockdown of NIPP1 in cultured cells is associated with a complete block of proliferation within 3 days (Fig. 6). One possible explanation is that the need for NIPP1 increases with the speed of cell cycle progression. This could explain why NIPP1−/− embryos die in a period associated with an enhanced proliferation rate and why a loss of NIPP1 results in an immediate block of cell proliferation in the very rapidly dividing HEK293 or U2OS cells. In this respect, it is also striking that a lack of NIPP1 hampers the association of the rapidly dividing ectoderm cells into an epithelial layer but does not appear to affect the epithelial organization of the more slowly dividing endoderm cells (Fig. 3). Another possible explanation for the relatively late effect of a loss of NIPP1 on embryonic development is that the first days of embryonic development are maintained by maternally supplied NIPP1. In addition, it is possible that this pool of NIPP1 has a slower turnover than that in cultured cells. It is also interesting that the visceral endoderm, in contrast to the ectoderm, still forms an epithelial layer in NIPP1−/− embryos, which is in accordance with an apparently lower requirement for NIPP1 in the endoderm, as judged from the relatively low concentration of NIPP1 in the endoderm of wild-type embryos (Fig. 3Aa). Thus, because of their lower requirement for NIPP1, endodermal cells in NIPP1−/− embryos may thrive for a longer time than other cell lineages.

Role of NIPP1 in cell proliferation. We have obtained various independent lines of evidence that link NIPP1 to cell proliferation. First, the lack of NIPP1 was associated with a decreased proliferation rate of cultured cells (Fig. 6) and of the inner cell mass of blastocyst outgrowths (Fig. 5B). Second, incorporation of BrdU in embryos (Fig. 4B) and blastocyst outgrowths (Fig. 5B) was severely impaired in the NIPP1−/− condition. Third, the nuclei of trophoblast giant cells in outgrowths of NIPP1−/− blastocysts remained relatively small (Fig. 5B). Since these cells endoreplicate (16), i.e., their nuclei increase in size by repeated cycles of replication without an intervening mitosis, these data suggest that a lack of NIPP1 is associated with a deficient G1/S transition and/or a hampered DNA replication.

We hypothesize that the contribution of NIPP1 to cell proliferation is somehow mediated by its protein ligands. For example, NIPP1 is a very potent inhibitor of PP1 (31), and it can be envisaged that the inhibition of PP1 may be required to enable a net phosphorylation of the retinoblastoma protein (pRb) by cyclin-dependent protein kinases, needed for the G1/S transition. In the absence of NIPP1, PP1 would not be restricted from dephosphorylating its established substrate, pRb (6, 10), thereby hampering entry into S phase. The NIPP1-mediated inhibition of the pool of PP1 that dephosphorylates pRb would add to the already established cell cycle-regulated mechanism of inhibition of PP1 by the phosphorylation of its C terminus (6, 10).

NIPP1 possibly also contributes to the G1/S transition by its ability to interfere with transcription and/or (alternative) splicing. The Polycomb protein EED has recently been identified as a novel ligand of NIPP1, and it has been found that NIPP1, like EED, functions as a transcriptional repressor in cultured cells (20). The transcriptional effects of EED have been largely explained by its ability to recruit the histone methyltransferase EZH2. Interestingly, the RNAi-mediated knockdown of either EED or EZH2 in cultured cells (9) results in the same phenotype that is associated with the knockdown of NIPP1 (Fig. 6). In addition, targeted disruption of the genes encoding EED (14, 15) and EZH2 (24) in mice is also associated with early embryonic lethality, albeit somewhat later than that seen with NIPP1−/− embryos. Finally, the expression of EED and EZH2 is controlled by the transcription factors E2F1 to E2F3 (9), which are negatively regulated by pRB, and the promoter of the NIPP1 gene also harbors E2F consensus binding sites (our unpublished observations). Collectively, these data suggest that NIPP1, EED, and EZH2 are all part of a regulatory pathway that promotes the G1/S transition in rapidly dividing cells.

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REFERENCES


