Activity-dependent Neuroprotective Protein Constitutes a Novel Element in the SWI/SNF Chromatin Remodeling Complex*

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Shmuel Mandel¹ and Illana Gozes²
From the Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

Complete deficiency in activity-dependent neuroprotective protein (ADNP), a heterochromatin 1-binding protein, results in dramatic changes in gene expression, neural tube closure defects, and death at gestation day 9 in mice. To further understand the cellular roles played by ADNP, the HEK293 human embryonic kidney cell line that allows efficient transfection with recombinant DNA was used as a model for the identification of ADNP-interacting proteins. Recombinant green fluorescent protein (GFP)-ADNP was localized to cell nuclei. When nuclear extracts were subjected to immunoprecipitation with specific GFP antibodies followed by polyacrylamide gel electrophoresis, several minor protein bands were observed in addition to GFP-ADNP. In-gel protein digests followed by mass spectrometry identified BRG1, BAF250a, and BAF170, all components of the SWI/SNF (mating type switching/sucrose nonfermenting) chromatin remodeling complex, as proteins that co-immunoprecipitate with ADNP. These results were verified utilizing BRG1 antibodies. ADNP short hairpin RNA down-regulation resulted in morphological changes that are closely associated with the SWI/SNF complex multifunctionality. Taken together, the current study uncovers a molecular basis for the essential function of the ADNP gene and protein.

Activity-dependent neuroprotective protein (ADNP) was originally discovered as a gene product associated with neuroprotection/neuroglia interactions. ADNP immunoreactivity was shown to localize to the astrocyte nucleus as well as to the cytoplasm and to the extracellular space, where the protein enhances neuronal survival. Further studies suggested very high gene conservation in mammals including humans, increased expression in malignant cells, and close association with cellular survival. In the mature brain, ADNP expression was modulated by injury, suggesting a potential protective effect for the protein in vivo. Additionally, ADNP expression shows sexual dichotomy and is modulated during the estrous cycle in the arcuate nucleus of the hypothalamus, and in the vagina, suggesting a potential neurotrophic/plasticity-associated effect for the protein.

Importantly, complete deficiency in ADNP results in neural tube closure defects and death at gestation day 9 in mice. ADNP-deficient embryos exhibit dramatic increases in gene transcripts associated with lipid metabolism coupled to a reduction in organogenesis/neurogenesis related transcripts. In pluripotent P19 cells, ADNP was shown to interact with specific chromatin regions in the neuro-differentiated state, which was associated with binding to heterochromatin protein 1. To further understand ADNP function, the HEK293 human embryonic kidney cell line that allows efficient transfection with recombinant DNA was used as a model for the identification of ADNP-interacting proteins and cellular function, suggesting that ADNP interacts with several major proteins of the essential SWI/SNF (mating type switching/sucrose nonfermenting) chromatin remodeling complex.

EXPERIMENTAL PROCEDURES

HEK293 Cell Culture and Transfection with Green Fluorescent Protein (GFP)-ADNP—Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum and grown at 37 °C with 5% CO₂. HEK293 cells were transfected using the transfection reagent Lipofectamine 2000 (Invitrogen) with a pEGFP-C1 (Clontech, Palo Alto, CA) expression vector carrying the human ADNP gene transcript. The ADNP construct (named 2S) contained the full-length ADNP transcript without the first and second untranslated exons (NCBI accession code NM_015339). Selection for Hek cells, human embryonic kidney cells; Mops, 4-morpholinepropanesulfonic acid; LC, liquid chromatography; MS, mass spectrometry; IP, immunoprecipitation.
transfected cells was achieved using G418 neomycin antibiotics (Roche Applied Science) as the plasmid contained a neomycin resistance epitope.

**Monoclonal Stable Cell Line Formation**—A monoclonal stable cell line was established by dilution of transfected cells. Under G418 antibiotic selection, individual colonies grew from individual cells. The cells assimilated the GFP-ADNP construct into their genome by non-homologous recombination, and two stable cell lines were formed, a GFP-ADNP stable cell line HEK293–2S and a control stable cell line HEK293–5S containing GFP with a 20-kDa ADNP N-terminal fragment that resulted from non-homologous recombination and was identified by Western blot analysis (see below).

**Controls for Specificity**—The recombinant ADNP is a fused protein containing an additional 28-kDa protein fragment that constitutes a functional GFP tag. Thus, the discrimination between endogenous and recombinant ADNP relies on both size differences as well as on antibody specificity with GFP antibodies recognizing only the recombinant protein. Three sets of antibodies were used for immunoprecipitation experiments; 1) GFP antibodies recognizing the fused GFP-ADNP, 2) antibodies against ADNP, recognizing both endogenous and recombinant ADNP, which can be differentiated by size, and 3) nonspecific preimmune serum verifying specificity. As an additional control, a truncated recombinant GFP-ADNP including only the 20-kDa-terminal portion of ADNP (5S, described above) was used to ascertain specific binding to ADNP.

**ADNP, BRG1 Co-immunoprecipitation**—Nuclear proteins were extracted from HEK293–2S GFP-ADNP transfected stable cell line and from a control stable cell line HEK293–5S. Protein extraction was performed using Pierce NE-PER nuclear extraction buffer (Pierce) according to the manufacturer’s protocol and supplemented with protease inhibitor mixture (Sigma). 300 μg of nuclear proteins were used for ADNP/BRG1 immunoprecipitation using anti-ADNP (BD Biosciences), anti-BRG1 (Santa Cruz Biotechnology, Delaware, CA), anti-GFP beads (MLB International, Woburn, MA), or nonspecific rabbit IgG antibodies (Sigma). 2 μg of each of the antibodies or 30 μl of the anti-GFP beads were added to the nuclear extracts, and the sample was rotated overnight at 4 °C in 1 ml of radioimmune precipitation assay buffer (1% Nonidet P-40, 1 mM EDTA, 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% deoxychloric acid) with a protease inhibitor mixture (Sigma). For the ADNP precipitation pre-cleared slurry (30 min of rotation with beads at 4 °C before antibody addition) was supplemented with protein A/G Plus-agarose beads (30 μl; Santa Cruz Biotechnology), and the mixture was incubated for 1 h at 4 °C. The protein sample was then subjected to centrifugation at 2000 × g for 30 s to pellet the beads, and the supernatant was removed. The beads were then washed four times with wash buffer (radioimmune precipitation binding buffer, as above) followed by a wash with phosphate-buffered saline. SDS-PAGE sample buffer was added to the beads, and the proteins were boiled for 5 min. Proteins were separated by a gradient SDS-PAGE gel electrophoresis (NuPAGE, Invitrogen) as described below.

**Western Analysis**—SDS-PAGE for GFP-ADNP, ADNP, and BRG1 was performed essentially as described (5). In brief, proteins were separated by electrophoresis on a 4–12% (w/v) gradient NuPAGE Mops gel (Invitrogen). Molecular weights were determined using Wide Range (6–250 kDa) Multicolor Protein Markers (see Blue, Invitrogen). The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), and nonspecific antigen sites were blocked using a solution containing 5% nonfat dried milk (w/v) in 10 mM Tris, pH 8, 150 mM NaCl, and 0.05% Tween 20. Antigen detection was performed using anti-Bethyl-ADNP 1:2,000 (Bethyl Laboratories, Montgomery, TX) or anti-BRG1 (1:200). Antibody-antigen complexes were detected using horseradish peroxidase-conjugated goat anti-rabbit 1:25,000 (Jackson ImmunoResearch, West Grove, PA) and visualized by ECL Plus Western blotting detection system (Amersham Biosciences).

**Staining and Protein Sequencing**—Staining of the protein gel was performed using “SeeBand Forte” following the manufacturer’s guidelines (GeBa, Kfar Hanagid, Israel). The bands of interest were cut from the gel, digested by trypsin, analyzed by liquid chromatography/mass spectrometry (LC-MS/MS) on DECA/LCQ, and identified by Pep-Miner and Sequest software against a non-redundant data base of human, mouse, rat, bovine, and rabbit. A peptide was considered high quality if its Pep-Miner identification score was greater than 80 and the Sequest Xcore was 1.5 for single-charged peptides, 2.5 for double-charged peptides, and 3 for triple-charged peptides.

**Short Hairpin RNAs (shRNA) Constructs and Transfection**—ADNP shRNA (Sigma) clones were used for ADNP silencing in HEK293 cells. Three hairpin forming fragments were used in a plKO.1 <-puro vector: SH1, CCGGGC GCCGAGAGTAGTATTCTCGAGAATACTACTCTTCTTCTCGGCTTTTTT; SH3, CCGGGC GCCGAGAGTAGTATTCTCGAGAATACTACTCTTCTTCTGCGTTTTTT; SH5, CCGG CGCAGCGGAGGAAATGACTCGAGTTCCATTTC TCTCGTAAGTGGCCTTTT. The control shRNA vector contained a nonspecific sequence that does not have a match in the human genome (Fragment): GATCCCCGGAGGCACACATCTTCTTCTTCAAGAGAGAAGAGATGGTGCGTCCTTTTTTGAA.

Transfection was performed using Lipofectamine2000 (Invitrogen), and cells were selected for positive transfection with 2–5 μg/ml puromycin (in accordance with the manufacturer instructions (Sigma)). ADNP reduction was shown both by quantitative real time PCR analysis (described below) as well as by Western analysis as described above. To control for nonspecific effects by puromycin, GFP shRNA was used side by side with different ADNP shRNA species, and cells transfected with GFP shRNA (controls) were also treated with puromycin.

**RNA Extraction and Quantitative Real Time PCR Assay**—Total RNA was extracted from ADNP shRNA-transfected and shRNA control HEK293 cells. RNA was isolated using Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). ADNP RNA expression levels were determined using specific primers for ADNP (sense, 5′-ACTTACGAAAAACCGAGACTATC-3′; antisense-5′-GACATTGGGAAATGACT-3′). HPRT control primers were 5′-TATGGACAGGA TGAGTC-3′ (sense) and 5′-ATCCAGCAGGTACGAAA-3′ (antisense). After being treated by DNase I (Ambion, Austin, TX), 0.2 μg of RNA/sample was reverse-transcribed by SuperScript III reverse
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transcriptase (200 units, Invitrogen) using random hexamer primers (10 min at 25 °C, 50 min at 50 °C, 5 min at 85 °C). Real time reverse transcription-PCR was performed using the SYBER GREEN PCR Master mix and ABI PRISM 7900 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA).

Antibodies—Rabbit polyclonal C-terminal-ADNP (BL1034) antibodies were obtained from Bethyl Laboratories (Montgomery, TX), and mouse monoclonal tubulin antibodies (TUB2.5) were used as before (12). Rabbit BRG1 antibodies were from Santa Cruz Biotechnology. Anti-GFP covalently conjugated to agarose beads was from MBL International (Woburn, MA). Other antibodies included mouse monoclonal anti-C-terminal-ADNP (BD Biosciences) and nonspecific IgG from rabbit serum (Sigma). Secondary antibodies were goat anti-mouse-HRP (Jackson ImmunoResearch) and goat anti-rabbit-HRP (Sigma).

Immunocytochemistry and Confocal Microscopy—HEK293 cells were fixed using 4% paraformaldehyde. Immunostaining was performed as follows. Triton X-100 (0.2%) was added for 5 min at room temperature. After washing with phosphate-buffered saline followed by washing with 2% bovine serum albumin in phosphate-buffered saline, nonspecific antibody binding was blocked using 50 μg/ml goat IgG (Sigma). The cells were then incubated for 1 h at room temperature with the primary mouse monoclonal ADNP antibody (1:60), rabbit BRG1 polyclonal antibodies (1:200), or tubulin mouse monoclonal antibodies, TUB2.5 (1:20), followed by a 30-min incubation with affinity-purified Cy3-labeled secondary goat-anti-mouse IgG (1:200) or Cy2 goat anti rabbit IgG (1:300). Slides were then washed twice (5 min/wash) in phosphate-buffered saline containing 0.1% Triton X-100, stained for DNA with Hoechst 33258 at 2 mg/ml, and mounted. Fluorescently stained cells were analyzed using the Zeiss confocal laser-scanning microscope. Zeiss LSM 410 inverted microscope (Oberkochen) equipped with a 25-milliwatt krypton-argon laser (488- and 568-nm maximum lines). A 40 × 1.2-watt Apochromat water immersion lens (Axiovert 135M; Zeiss) was used for all imaging.

Metabolic Activity Measurements—Metabolic activity of shRNA-transfected HEK293 viable cells was measured by the CellTiter 96 kit in accordance with the manufacturer’s instructions (Promega, Madison, WI). The assay uses a colorimetric method employing a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, (MTS)) and electron-coupling reagent phenazine methasulfate. MTS is bio-reduced by the living cells to the formazan form that is detected at 490 nm.

RESULTS

GFP-ADNP Stable Cell Line Formation—ADNP is known to localize mostly to the nucleus and in some cases to the cell cytoplasm (3). To trace ADNP in the cell and to broaden our understanding regarding its mechanism of action, GFP was attached to ADNP to form a GFP-ADNP chimeric protein. The construct was formed and used to transfect HEK293 human kidney cell line. The cells with the attained construct resistance to G418 neomycin antibiotics were diluted and grown separately to form monoclonal GFP-ADNP overexpressing colonies (Fig. 1). This model enabled real time detection of ADNP localization in the cell as well as precipitation of the protein from its N-terminal GFP tag in addition to ADNP antibodies against the ADNP C-terminal domain. The GFP-ADNP protein showed the expected increase of 27 kDa in its molecular mass compared with endogenous ADNP due to the GFP addition (Fig. 1A) and also showed distinct localization to the nuclei (Fig. 1B).

ADNP Co-precipitates with the SWI/SNF Complex through ADNP C-terminal Interaction—Immunoprecipitation (IP) experiments of GFP-ADNP were performed using two antibodies, anti-GFP and anti-C-terminal ADNP. Anti-GFP co-immunoprecipitated GFP-ADNP with other proteins (Fig. 2A, lane 1). Control IP of a GFP 20-kDa N-terminal-truncated ADNP chimera (see the “Experimental Procedures”) was performed (Fig. 2A, lane 2) to indicate that the interaction with other proteins was neither with the GFP region nor with the N-terminal ADNP region of the chimeric GFP-ADNP.

A second IP assay was performed using C-terminal ADNP antibody that recognize a 136-amino acid region on the ADNP region of the chimeric GFP-ADNP.
FIGURE 2. ADNP co-precipitates with BAF250a, BRG1, and BAF170, all SWI/SNF chromatin remodeling complex subunits. A, nuclear proteins were extracted from the GFP-ADNP-2S stable cell line and from the control-truncated GFP-ADNP-5S cell line (20 kDa of ADNP N-terminus fused to 27 kDa of GFP). On the first two lanes precipitation was conducted using anti-GFP antibodies conjugated to agarose beads (precipitation from the N-terminal region). On the third lane anti-C-terminal ADNP precipitation is shown, and on the fourth lane only A/G beads were used as nonspecific precipitation control. As shown in both cases, ADNP immunoprecipitation results differ between the two antibodies used. B, analysis carried out by LC-MS/MS on DECA/LCQ and identified by Pep-Miner and Sequest software against a non-redundant data base of human, mouse, rat, bovine, and rabbit. The four heaviest bands were identified as BAF250a, BRG1, ADNP, and BAF170. All three proteins (other than ADNP) belong to the SWI/SNF chromatin remodeling complex. C, on the left, validation of ADNP detection using Western blot analysis of the IP results with anti-ADNP. Controls show that ADNP was not precipitated nonspecifically. On the right, detection with anti-BRG on the IP products is shown. Results showed that BRG1 can only be detected with the N-terminal ADNP IP but not with the anti-C-terminal ADNP IP. D, immunoprecipitation with anti-BRG1 shows co-immunoprecipitation with endogenous ADNP. Left membrane, validation of the LC-MS/MS protein sequencing results by co-IP with GFP antibodies (on agarose beads) in HEK293 nuclear extract and detection with BRG1 antibodies. Lane 1, BRG1 precipitates with full-length ADNP (2S). Lane 2, BRG1 does not precipitate with the truncated ADNP control (5S). Lane 3, nuclear extract (without immunoprecipitation) showing endogenous BRG1. On the right membrane we show immunoprecipitation with BRG1 antibodies and detection with ADNP antibodies. Lanes 1–3 and BRG1 antibodies to validate the above results (lanes 4 and 5). The – sign indicates precipitation control with nonspecific pre-immune rabbit serum, and the (+) sign indicates specific antibodies. Extract, nuclear extract with/without IP. Arrows indicate BRG1 (~200 kDa) and endogenous ADNP (~150 kDa). E, double-immunohistochemistry of ADNP and BRG1 in HEK293 cells (“Experimental Procedures”) shows similar patterns of nuclear staining, reinforcing co-localization of both proteins. Results in blue indicate Hoechst nuclear staining, in red indicate ADNP staining (ADNP mouse monoclonal antibody), and in green indicate BRG1 staining. Yellow indicates co-localization of BRG1 and ADNP immunostaining.
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C terminus (Fig. 2A, lane 3). The C-terminal ADNP antibody precipitated both endogenous ADNP (150 kDa) and chimeric GFP-ADNP (~180 kDa). However, no proteins co-precipitated with ADNP when using this antibody. Protein-A/G beads were used as a nonspecific control (Fig. 2A, lane 4). Four bands obtained by the anti-GFP antibody co-IP (Fig. 2A, lane 1, arrows) were analyzed using trypsin digestion and LC-MS.

Results of the protein analysis showed that of the four bands, one belonged to the GFP-ADNP chimeric protein (~180 kDa) and the other three to components of the SWI/SNF chromatin remodeling complex: BAF170, BRG1, and BAF250a (13) (Fig. 2, A and B). This result indicates interaction between ADNP and the SWI/SNF complex through the ADNP C-terminal region. When precipitated with anti-C-terminal ADNP, both endogenous ADNP and GFP-ADNP precipitated, but the interaction with the SWI/SNF proteins was abolished (Fig. 2A).

Validation of Co-immunoprecipitation Results—To validate the LC-MS results, Western analysis was performed on the immunoprecipitated proteins from nuclear extracts of GFP-ADNP HEK293 stable cells (2S, see “Experimental Procedures”) and control GFP-truncated ADNP cells (5S, see “Experimental Procedures”) using anti-GFP and anti-C-terminal ADNP (Fig. 2, C and D). Results showed that GFP-ADNP (Fig. 2C, lane 1, left membrane) precipitated with anti-GFP and that mostly endogenous ADNP as well as GFP-ADNP was precipitated with anti C-terminal ADNP (Fig. 2C, lane 3, left membrane). BRG1 was selected as a SWI/SNF representative component, and its presence was validated using Western analysis on the same IP results (Fig. 2C, lane 1, right membrane). These results also showed that co-precipitation with BRG1 was eliminated when precipitation was performed with C-terminal ADNP antibodies (Fig. 2C, lane 3, right membrane). This reinforces the fact that ADNP and SWI/SNF complex-related proteins interact through the ADNP C-terminal region.

Interaction between BRG1 and Endogenous ADNP—IP with anti-BRG1 was performed to reinforce the ADNP/BRG1 interaction results and to show that not only recombinant GFP-ADNP is involved in this interaction but also endogenous ADNP. HEK293 nuclear extract was used for anti-BRG1 IP. Nonspecific rabbit serum was used as control antibody. Results showed that endogenous ADNP (Fig. 2D, lane 2, right membrane) precipitates with BRG1 (Fig. 2D, lane 5, right membrane). In particular, BRG1 was shown here to co-precipitate with full-length GFP-ADNP (2S) (Fig. 2D, lane 1, left membrane) and not with the truncated GFP-ADNP control (5S) when GFP antibodies were used for immunoprecipitation (Fig. 2D, lane 2, left membrane). Nuclear extract (without immunoprecipitation) showed detectable levels of endogenous BRG1. On the right membrane (Fig. 2D) we show immunoprecipitation with BRG1 antibodies and detection with ADNP antibodies (lanes 1–3) and BRG1 antibodies to validate the above results (lanes 4 and 5).

To further substantiate the interaction between ADNP and BRG1 at the cellular level, we have performed triple cellular precipitation was performed with C-terminal ADNP antibodies showed that co-precipitation with BRG1 was eliminated when GFP antibodies were used for immunoprecipitation (Fig. 2, A and B). This result indicates interaction between ADNP and the SWI/SNF complex through the ADNP C-terminal region. When precipitated with anti-C-terminal ADNP, both endogenous ADNP and GFP-ADNP precipitated, but the interaction with the SWI/SNF proteins was abolished (Fig. 2A).

FIGURE 3. shRNA reduction of ADNP expression in HEK293 cells. A, two ADNP shRNA constructs in a puromycin vector were transfected into HEK293 cells and grown under puromycin selection conditions. A puromycin resistance-containing vector was used as control. The cells were grown for 4 days in each passage and assayed for ADNP RNA levels. A quantitative real time PCR (QRT-PCR) assay shows that ADNP levels in both SH1 and SH3 were significantly reduced (a ~75% and ~80%, respectively, reduction compared with control); B, a cellular protein extract was used to asses the ADNP protein levels using Western blot analysis (anti-ADNP bethyl antibody). As shown, a reduction of protein levels correlates the reduction of the RNA levels.
Cell counting results were corroborated using MTS metabolic activity measurements, indicating a significant 15% decrease in cell viability in the SH3 ADNP shRNA-treated cells in comparison to the GFP shRNA-treated cells (Fig. 4D). These results indicate that the lower cell density measured in the SH3-treated cells (Fig. 4C) was associated at least in part with decreased metabolic activity.

Changes in cell shape and the ADNP potential downstream mechanisms were addressed using monoclonal anti-tubulin antibodies and triple immunofluorescence staining as follows. Fig. 5A shows nuclear localization for ADNP (green) together with Hoechst staining (blue). Tubulin staining was dispersed in the cytoplasm (red) of the control shRNA-transfected cells. In contrast, ADNP shRNA treatment (SH3) resulted in almost complete disappearance of ADNP immunostaining (attesting to the specificity of the antibody) coupled to microtubule “shrinkage” and changes of cellular condensation and shape (Fig. 5B). Fig. 5C shows that the degree of cell shape changes may depend on the degree of the reduction in ADNP as follows. A 50% reduction in ADNP expression resulted in a similar morphology to control cells, whereas an 80% reduction in ADNP was correlated with distinct morphological changes.

**DISCUSSION**

The current study shows for the first time interaction between ADNP and three members of the SWI/SNF chromatin remodeling complex: BAF250a, BRG1, and BAF170. Furthermore, this paper shows that ADNP expression is associated with cell shape determination.

Mammalian SWI/SNF is a multiprotein chromatin-remodeling complex that is composed of at least 10 elements. Two distinct SWI/SNF complexes were described, each characterized by the presence of a unique subunit, either BAF250 or BAF180, defined as BAF and PBAF, respectively. In addition, BAF can contain either BRG1 or BRM as the core motor subunit, whereas PBAF only contains BRG1 (13). Here, ADNP precipitated with the BAF250a subunit as well as with the BRG1.
subunit, suggesting that ADNP associates with the BAF complex containing the BRG1 core motor subunit.

To ascertain specificity for the ADNP BRG1 interaction and given that the endogenous expression of ADNP in HEK293 is high, we have taken the following measures. 1) We have performed repeated immunoprecipitation experiments using antibodies against the GFP portion of the recombinant ADNP fused to GFP resulting in BRG1 co-immunoprecipitation (Fig. 2, A–D). The recombinant ADNP is a fused protein containing an additional 28-kDa protein fragment constituting a functional GFP tag. Thus, the discrimination between endogenous and recombinant ADNP relies on both size differences as well as on antibody specificity with GFP antibodies recognizing only the recombinant protein. 2) Antibodies against the endogenous ADNP C terminus, although precipitating endogenous ADNP, did not co-precipitate BRG1, suggesting that a free ADNP C terminus is required for interaction with BRG1 (Fig. 2C). 3) Precipitation with BRG1 antibodies showed co-precipitation with endogenous ADNP (Fig. 2D). 4) Nonspecific serum precipitation verified specificity (Fig. 2D, H11011 indicates control nonspecific serum). 5) We have also performed co-immunoprecipitation experiments with truncated ADNP fused to GFP, including only the 20-kDa N-terminal portion of ADNP (5S, described above) to ascertain specific binding to ADNP, showing that the full-length ADNP is required for BRG1 interaction and co-immunoprecipitation. Furthermore, we performed triple-labeling experiments with Hoechst nuclear staining, ADNP staining (green) and tubulin staining (red) indicated that extensive morphological changes were observed only with SH3, i.e. in the presence of 80% reduction in ADNP. Shcontrol, short hairpin control.

Previously, complete ADNP deficiency was shown to block neurogenesis in the mouse embryo (10) in parallel with specific inhibition of Neurogenin1 and Neurod1 (11) (genes that are associated with neuronal fate determination (14)). In addition,
ADNP was shown to be associated with neuronal maturation through differential interaction with chromatin in the pluripotent p19 cells that were differentiated into neuronal/glial phenotype (11). These previous results correlate well with the fact that BRG1 (and by inference the SWI/SNF complex) is an essential regulator of neuronal differentiation during vertebrate nervous system formation (15) with direct interaction with Neurogenin1 and Neurod (16). ADNP was also associated with repression of lipid transport and metabolism-related genes (11). This functional group of genes was significantly up-regulated in the ADNP knock-out embryos. Promoter analysis of this group of genes revealed a significant enrichment in the regulatory properties (11). Intact SWI/SNF chromatin remodeling complexes are essential for the activation of adipogenic genes as well as for the activation of peroxisome proliferator-activated receptor γ (PPARγ) transcription factor binding site, indicating ADNP/PPARγ co-regulatory properties (11). Intact SWI/SNF chromatin remodeling complexes are essential for the activation of adipogenic genes as well as for the activation of peroxisome proliferator-activated receptor γ (PPARγ) (17) reinforcing and explaining the ADNP/PPARγ effect on embryonic lipid transport and metabolism.

SWI/SNF is a multiprotein complex able to remodel chromatin toward cellular and environmental requirements. In addition to its transcriptional activator functions, it was described also as a transcription repressive element in concert with the SUV39H1/HP1 complex (18, 19). An interaction between ADNP and HP1α through the ADNP conserved PXVXL HP1α biding motif (11) may explain some of the SWI/SNF-HP1α repressive association.

Down-regulation of ADNP by shRNA resulted in morphological changes that are in line with the fact that ADNP contains a homeodomain profile (2) and with the SWI/SNF complex function that is associated with cellular differentiation. Indeed, previous studies have shown that BRG1 loss induces an altered cellular morphology and disruption in the organization of the actin cytoskeleton (20), and mutations in the SWI/SNF complex resulted in changes in cell size and attachment area (21). Our results suggest that ADNP functionality plays a role in these changes. In addition, in the current studies, changes in cell morphology as a result of decreased ADNP expression may have further led to cellular death and detachment from the cell culture plate. Cell counting as well as metabolic activity measurements indicated a 15–50% decrease in cell viability in the SH3 ADNP shRNA-treated cells in comparison to the GFP shRNA-treated cells.

Previous results have indicated that ADNP down-regulation in cancer cell lines result in cell death that was associated with an increase in the expression of the proapoptotic protein p53 (5). In line with this finding, the addition of recombinant ADNP to pheochromocytoma cells under severe oxidative stress resulted in increased cellular survival coupled to decreases in p53 (4). ADNP may be involved in the p53 pathway by mediating the recruitment of SWI/SNF to the chromatin of genes implicated in growth suppression and apoptosis in response to several cell stress-dependent pathways (22).

Other studies have suggested shuttling for ADNP between the nucleus and the cytoplasm, where ADNP-like immunoreactivity may interact with the microtubule cytoskeleton (3) that is associated with determination of cellular structure in combination with other cytoskeletal elements, including actin microfilaments and intermediate filaments (23). Here, microtubule reorganization paralleled changes in cellular morphology. Interestingly, BAF53, an actin-related protein that also shuttles between nucleus and cytoplasm, constitutes an integral component of many chromatin-modifying complexes including SWI/SNF as well as TIP60, TRRAP, and TIP48/49. Furthermore, recent studies have associated BAF53 with p53-dependent transcription (24).

shRNA constructs were used to target endogenous ADNP and as control a GFP shRNA was used. From our shRNA experiments it was apparent that the amount of ADNP in the cell is important for cell morphology and survival. Indeed, when we partially reduced ADNP mRNA by 50% (SH5, Fig. 5C), we did not see the obvious changes in cell morphology observed with 80% (SH3) ADNP mRNA reduction (Fig. 5D). Furthermore, our in vivo data suggest that complete knock-out of ADNP is lethal, whereas the ADNP heterozygous mice survive but show multiple deficits (25). Thus, partial reduction in ADNP expression in vivo in ADNP+/- mice results in multiple changes in gene expression, decreased cellular activity, and a tausopathy-like phenotype (25), in agreement with the current data that suggest ADNP-related multigene regulation through the SWI/SNF complex that affects cytoskeletal organization and cell shape determination.

Comprehensive work performed by Taylor and Knife (26) revealed BRG1 to be exploited by the herpes simplex virus as part of its mechanisms of replication and infection in the mammalian cell. In this work the authors did not detect ADNP in the viral-associated complex containing BRG1. The lack of ADNP co-interaction in the viral BRG1 complex could be trivial and related to the antibodies used or may suggest that ADNP is not...
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utilized by the herpes simplex virus like many other known BRG1 co-interacting proteins.

Taken together, the current results lead to the uncovering of the molecular basis for ADNP as a vital gene important for cellular differentiation and cellular protection (Scheme 1), paving the path for novel drug design. In this respect the ADNP-derived eight-amino acid peptide fragment, NAP (NAPVSIPQ) (27), which interacts with the neuronal microtubule network (28) and reduces p53 expression (29) without affecting cell division (10), is currently in phase II clinical development.

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