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The transmembrane nucleoporin NDC1 is required for targeting of ALADIN to nuclear pore complexes

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ABSTRACT

NDC1 is a transmembrane nucleoporin that is required for NPC assembly and nucleocytoplasmic transport. We show here that NDC1 directly interacts with the nucleoporin ALADIN, mutations of which are responsible for triple-A syndrome, and that this interaction is required for targeting of ALADIN to nuclear pore complexes (NPCs). Furthermore, we show that NDC1 is required for selective nuclear import. Our findings suggest that NDC1-mediated localization of ALADIN to NPCs is essential for selective nuclear protein import, and that abrogation of the interaction between ALADIN and NDC1 may be important for the development of triple-A syndrome.

Introduction

Nuclear pore complexes (NPCs) are large, multiprotein complexes that span the nuclear envelope, forming a selective channel between the cytoplasm and nucleus. NDC1 is a transmembrane nucleoporin and is required for NPC assembly in vertebrate cells [1,2]. Loss of NDC1 impairs the localization of FG (Phe-Gly) repeat-containing nucleoporins that constitute a significant proportion of NPCs and disrupts nuclear assembly *in vitro*. In addition to its function as a structural basis for NPCs, NDC1 is involved in nucleocytoplasmic transport [3]. In the present study, we show that NDC1 interacts with ALADIN. ALADIN is a component of NPCs and is assumed to be involved in the selective nuclear import [4]. Moreover, mutations of ALADIN are known to be responsible for triple-A syndrome (MIM 231550; also known as Allgrove syndrome), an autosomal recessive disorder characterized by the clinical triad of achalasia of the cardia (increased tone of the lower esophagus sphincter with consecutive dilation of the esophagus), alacrima (deficiency of tear production), and adrenocorticotropic hormone (ACTH)-resistant adrenal insufficiency [5]. Furthermore, we show that NDC1 is required for anchoring ALADIN to NPCs and that NDC1 plays a critical role in selective nuclear import.

Materials and methods

Cell culture and transfection. HeLa cells were cultured in MEM supplemented with 10% fetal bovine serum (FBS). 293T cells were cultured in DMEM supplemented with 10% FBS. Plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or FuGENE HD (Roche, Mannheim, Germany).

Plasmid construction. FLAG-tagged *NDC1*, *ALADIN*, Dronpa-tagged NLS, *XRCC1* were generated by PCR and cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). For preparation of GST-fusion proteins, cDNA fragments were subcloned into pGEX-5X-1 (GE Healthcare). GST-fusion proteins were synthesized in *Escherichia coli* and isolated by adsorption to glutathione-conjugated Sepharose (GSH-Sepharose; Pharmacia, Buckinghamshire, UK). *GFP-ALADIN* was a gift from Michael J. Matunis (Rockefeller University).

Generation of anti-NDC1 and anti-ALADIN polyclonal antibodies. Rabbit polyclonal antibody to NDC1 and ALADIN were generated by immunizing rabbits with a GST-fusion protein containing amino acids 452–501 of NDC1 and a synthetic peptide containing amino acids 533–546 of ALADIN, respectively. Antibodies were purified by affinity chromatography using columns to which the antigens used for immunization had been linked.

Immunoprecipitation, GST-pull-down assay and immunoblotting. Immunoprecipitation, GST-pull-down assays and immunoblotting were performed as described previously [6]. The blot was analyzed by immunoblotting using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG

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(Promega, Madison, WI) as a secondary antibody. Primary antibodies were diluted as follows: anti-NDC1 antibody, 1:500; anti-ALADIN antibody, 1:2000; anti-FLAGM2 antibody (Sigma–Aldrich, Seelze, Germany), 1:500; anti-α-tubulin antibody (Calbiochem Novabiochem GmbH, Bad Soden/Ts., Germany), 1:1000.

Immunostaining. Cells were fixed with 10% formalin/PBS for 15 min. Fixed cells were stained with each antibody for 2 h at room temperature. Staining patterns obtained with antibodies were visualized with Alexa 488-labelled antibody or Alexa 594-labelled antibody (Molecular Probes, Eugene, OR). Primary antibodies were diluted as follows: anti-NDC1 antibody, 1:500; anti-ALADIN antibody, 1:500. Cells were photographed with a Carl Zeiss LSM510 laser scanning microscope.

shRNAs. DNA oligonucleotides encoding shRNAs were cloned into the H1-RNA gene promoter vector, pSuper-retro (OligoEngine, Seattle, WA [7]). The sequences of the region in human *NDC1-1*, *NDC1-2* and *ALADIN* targeted for shRNA were 5′-GCAGGTCGCGGGACATAC T-3′, 5′-CTTACCCAGTGGAACCTAA-3′ and 5′-GACTCAGTCCGTGT GTATA-3′, respectively. The sequence of mut-shRNA-NDC1-1 was 5′-GCAGGTCGCGGGACAGGCG-3′.

Results

Interaction between NDC1 and ALADIN

We attempted to identify NDC1-associated proteins using liquid chromatography (LC)-based electrospray tandem mass spectrometry (MS/MS) [8]. 293T cells were transfected with FLAG-tagged NDC1 and lysates from these cells were subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitated proteins were subjected to proteolysis followed by LC-based MS/MS. We found that the nucleoporin ALADIN is coprecipitated with NDC1.

To confirm that NDC1 interacts with ALADIN, we examined the ability of the *in vitro*-translated ALADIN to interact with a fusion protein consisting of a fragment of NDC1 containing amino acids 287–386 (GST-NDC1) and glutathione-*S*-transferase (GST). We found that the *in vitro*-translated ALADIN interacts with GST-NDC1, but not with GST alone (Fig. 1A).

We next examined whether endogenous NDC1 is associated with ALADIN *in vivo*. Lysates from HeLa cells were subjected to immunoprecipitation with anti-NDC1 antibody followed by

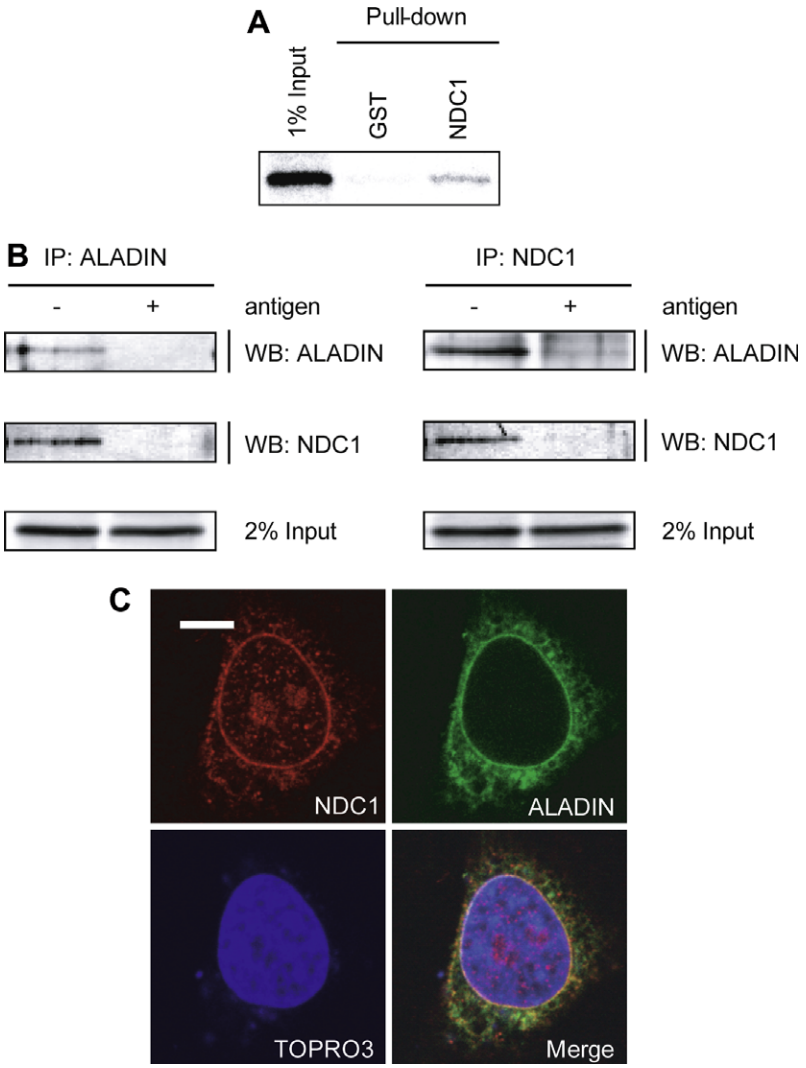

Fig. 1. Interaction between NDC1 and ALADIN *in vitro* and *in vivo*. (A) Interaction of NDC1 with ALADIN *in vitro*. ALADIN was generated by *in vitro*-translation and incubated with GST-NDC1 (amino acids 287–386) or GST bound to Sepharose. Bound proteins were analyzed by SDS–PAGE followed by autoradiography. (B) Interaction of NDC1 with ALADIN *in vivo*. Lysates prepared from HeLa cells were immunoprecipitated with the indicated antibodies, separated by SDS–PAGE and analyzed by immunoblotting with the indicated antibodies. (+) Antibodies were pre-incubated with antigen before immunoprecipitation. (C) NDC1 and ALADIN are colocalized at the nuclear envelope in HeLa cells. Cells were cotransfected with *FLAG-NDC1* and *GFP-ALADIN* and stained with anti-NDC1 antibody. Cell nuclei were counterstained with ToPro3. Scale bar, 5 μm.

immunoblotting with anti-ALADIN antibody. NDC1 was identified as a 70 kDa protein and was found to coimmunoprecipitate with ALADIN (Fig. 1B). Likewise, immunoprecipitation of the lysates with anti-ALADIN antibody followed by immunoblotting with anti-NDC1 antibody revealed an association between NDC1 and ALADIN. Coprecipitation of NDC1 and ALADIN was inhibited by preincubation of the antibodies with the antigens used for immunization.

To further confirm the above results, we examined the subcellular localization of NDC1 and ALADIN ectopically expressed in HeLa cells. When cells were transfected with FLAG-NDC1 along with GFP-ALADIN, both proteins were found to be colocalized at the nuclear rim (Fig. 1C). Together, these results suggest that NDC1 is associated with ALADIN at the nuclear envelope in living cells.

NDC1 is required for targeting of ALADIN to NPCs

It is known that NPC targeting is critical for the function of ALADIN [4,9]. To investigate the role of NDC1 in targeting ALADIN to NPCs, we performed RNAi experiments using two distinct short hairpin RNAs (shRNAs), shRNA-NDC1-1 and -2, designed to suppress the expression of NDC1 and a point mutant shRNA, mut-shRNA-NDC1-1. Immunoblotting analysis revealed that expression

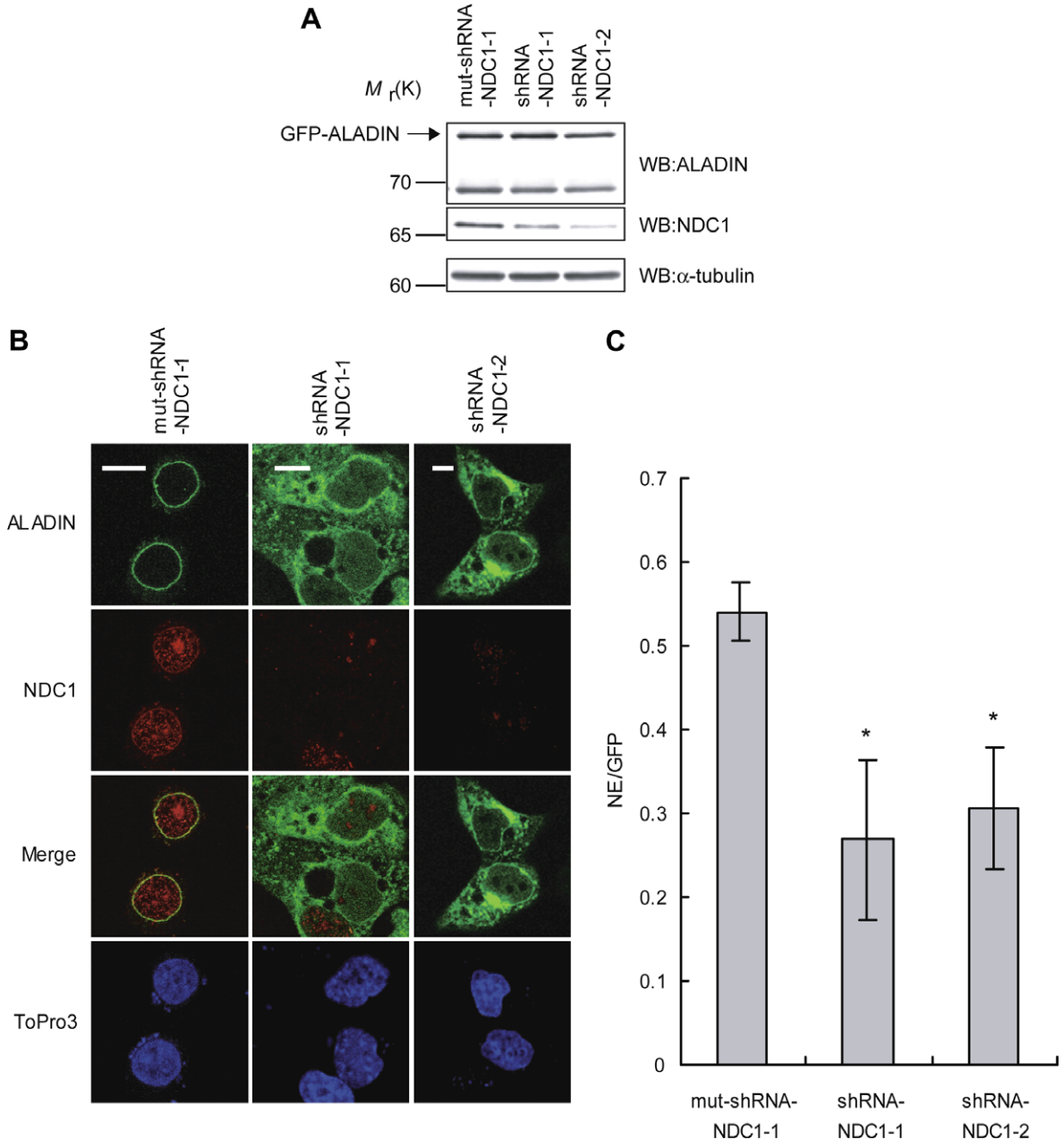

Fig. 2. NDC1 is required for targeting of ALADIN to NPCs. (A) Suppression of NDC1 expression by shRNAs. HeLa cells were transfected with GFP-ALADIN along with shRNA-NDC1-1, mut-shRNA-NDC1-1 or shRNA-NDC1-2. Lysates prepared from transfected cells were analyzed by immunoblotting with anti-NDC1 or anti-ALADIN antibody. Anti-α-tubulin antibody was used as a control. (B) Suppression of NDC1 expression by shRNA changes ALADIN localization. HeLa cells were transfected with GFP-ALADIN along with shRNA-NDC1-1, mut-shRNA-NDC1-1 or shRNA-NDC1-2. After 48 h of transfection, cells were stained with anti-NDC1 antibody. Cell nuclei were counterstained with ToPro3. Scale bar, 10 μm. (C) Quantification of cells expressing ALADIN at the nuclear envelope. The number of cells expressing ALADIN (GFP) was counted. The number of cells in which ALADIN is localized at the nuclear envelope was also counted (NE). Results are shown as means ± S.D. ($n = 3$). * denotes $p < 0.05$.

of NDC1 was almost completely inhibited in either shRNA-NDC1-1 or -2-transfected cells but not in mut-shRNA-NDC1-transfected cells (Fig. 2A). When cells were transfected with shRNAs along with GFP- ALADIN, GFP- ALADIN was localized in the cytoplasm but not at the nuclear envelope in either shRNA-NDC1-1 or -2-transfected cells (Fig. 2B and C). On the other hand, ALADIN was

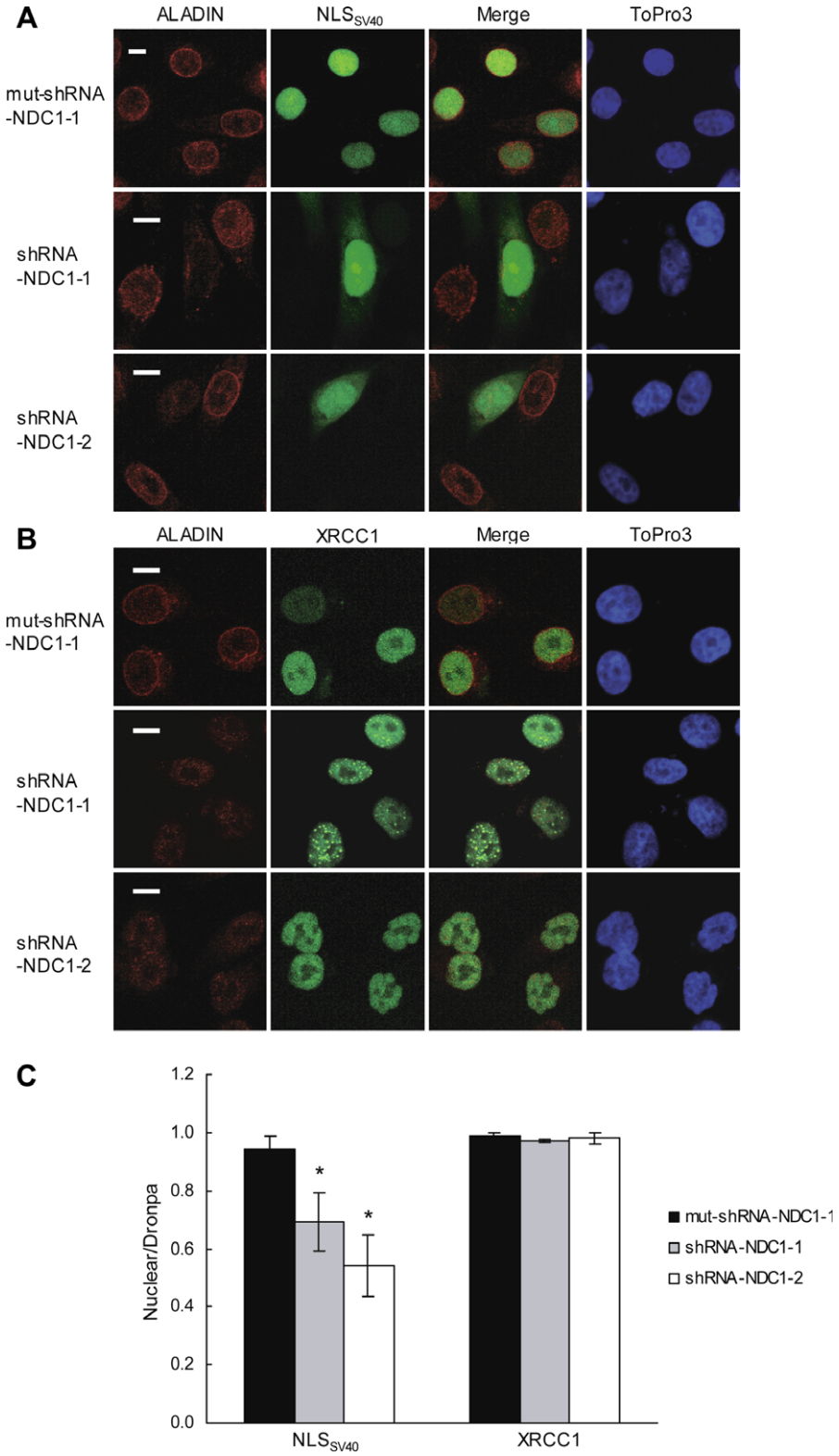

Fig. 3. NDC1 is required for ALADIN-mediated nuclear import. (A, B) Suppression of NDC1 expression by shRNA impairs ALADIN function. HeLa cells were transfected with Dronpa-NLS$_{SV40}$ (A) or Dronpa-XRCCI (B) along with shRNA-NDC1-1, mut-shRNA-NDC1-1 or shRNA-NDC1-2. After 48 h of transfection, cells were stained with anti-ALADIN antibody. Cell nuclei were counterstained with ToPro3. Scale bar, 10 μm. (C) Quantification of cells expressing Dronpa-tagged proteins at NPCs. The number of cells expressing Dronpa-tagged proteins was counted. The number of cells expressing Dronpa-tagged proteins exclusively in nucleus was also counted. Results are shown as means ± S.D. (n = 3). * denotes $p < 0.05$.

localized at the nuclear envelope in mut-shRNA-NDC1-1-transfected cells. These results suggest that NDC1 plays a critical role in the localization of ALADIN at NPCs.

NDC1 is required for selective nuclear import

ALADIN was previously shown to be involved in selective nuclear import [4]. For example, ALADIN was shown to be involved in nuclear import of proteins containing NLS of the simian virus 40 large T antigen (NLS_{SV40}) but not XRCC1 (X-ray repair cross-complementing group 1). We therefore investigated whether NDC1 is required for selective nuclear import of these proteins: we examined the subcellular localization of Dronpa-tagged NLS_{SV40} and XRCC1 in HeLa cells transfected with shRNA-NDC1-1 or mut-shRNA-NDC1-1. Dronpa-NLS_{SV40} was localized in the nucleus in cells transfected with mut-shRNA-NDC1-1, whereas it was mislocalized in the cytoplasm in cells transfected with shRNA-NDC1-1 (Fig. 3A and B). On the other hand, Dronpa-XRCC1 was localized in the nucleus in cells transfected with either shRNA-NDC1-1 or mut-shRNA-NDC1-1. These results suggest that NDC1 may play a critical role in selective nuclear import.

Discussion

NDC1 is a transmembrane nucleoporin and its carboxy-terminal region is exposed to the cytoplasm [1,2]. We have shown that NDC1 interacts with ALADIN. This result is consistent with the fact that ALADIN is localized to the cytoplasmic face of NPCs [9]. Furthermore, we found that depletion of NDC1 dramatically changes ALADIN localization. These results suggest that NDC1 functions as an anchor that links ALADIN to NPCs. Interestingly, most mutant ALADINs identified in triple-A syndrome patients are unable to localize to NPCs and predominantly reside in the cytoplasm [4,9]. Thus, NDC1-mediated localization of ALADIN to NPCs appears to be critical for its function. Moreover, we found that depletion of NDC1 results in an impairment of nuclear import of NLS_{SV40} but not XRCC1. Consistent with this, it has recently been reported that ALADIN is required for nuclear import of NLS_{SV40} but not XRCC1 [4]. These findings raise the possibility that NDC1-mediated anchoring of ALADIN to NPCs is essential for selective nuclear protein import.

It has been reported that there are some patients who are clinically diagnosed as triple-A syndrome but do not have a mutation in both alleles of *ALADIN* [10]. Our findings raise the possibility that the *NDC1* gene might be mutated in triple-A syndrome patients without mutations in *ALADIN*. Detailed analysis of NDC1 function would give new insights into the role of ALADIN at NPCs and the development of therapeutic strategies against triple-A syndrome.

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