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The nuclear pore complex protein ALADIN is anchored via NDC1 but not via POM121 and GP210 in the nuclear envelope

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ABSTRACT

The nuclear pore complex (NPC) consists of ~30 different proteins and provides the only sites for macromolecular transport between cytoplasm and nucleus. ALADIN was discovered as a new member of the NPC. Mutations in ALADIN are known to cause triple A syndrome, a rare autosomal recessive disorder characterized by adrenal insufficiency, alacrima and achalasia.

The function and exact location of the nucleoporin ALADIN within the NPC multiprotein complex is still unclear. Using a siRNA-based approach we downregulated the three known membrane integrated nucleoporins NDC1, GP210, and POM121 in stably expressing GFP-ALADIN HeLa cells. We identified NDC1 but not GP210 and POM121 as the main anchor of ALADIN within the NPC. Solely the depletion of NDC1 caused mislocalization of ALADIN. Vice versa, the depletion of ALADIN led also to disappearance of NDC1 at the NPC. However, the downregulation of two further membrane-integral nucleoporins GP210 and POM121 had no effect on ALADIN localization. Furthermore, we could show a direct association of NDC1 and ALADIN in NPCs by fluorescence resonance energy transfer (FRET) measurements. Based on our findings we conclude that ALADIN is anchored in the nuclear envelope via NDC1 and that this interaction gets lost, if ALADIN is mutated. The loss of integration of ALADIN in the NPC is a main pathogenetic aspect for the development of the triple A syndrome and suggests that the interaction between ALADIN and NDC1 may be involved in the pathogenesis of the disease.

Introduction

Nuclear pore complexes (NPCs) are very large protein complexes that in mammalian cells are composed of multiple copies of about 30 proteins, termed nucleoporins [1]. Embedded in the two membranes of the nuclear envelope, NPCs mediate all traffic between the cytoplasm and the nucleoplasm. Nucleoporins perform various functions within the NPCs. They can be involved in anchorage in the nuclear membrane or in the structure, or can be dynamically associated and responsible for connection, transport, recycling, and release of molecules and transport complexes [2]. In 2002 Cronshaw et al. identified ALADIN (*AL*acrima *A*chalasia a*D*renal *I*nsufficiency *N*eurologic disorder) as a new member of the nucleoporin family at the NPC [1]. ALADIN is the first nucleoporin which was found to be linked to a human inheritable disease. Mutations in the *AAAS* gene, which encodes the 60 kDa WD repeat-containing protein ALADIN causes triple A syndrome (MIM#231550) [3,4]. Triple A syndrome is a rare autosomal recessive disorder characterized by adrenocorticotropic hormone (ACTH) resistant adrenal failure, achalasia and alacrima [5]. It is associated with neurological disturbances such as dysautonomia, motor-sensory neuropathy, ataxia, parkinsonism, opticus atrophy, seizures, and mental retardation [6,7].

The localization and function of ALADIN in the NPC has not been clearly defined. From immunocytochemical investigations it was suggested that it localizes to the cytoplasmatic side of the nuclear pore [8,9]. In studies of Rabut et al. on the dynamic organization of the NPCs in single living cells, it was shown that ALADIN belongs to the group of barely exchangeable nucleoporins with an average residence time of 57 h. These nucleoporins build the central part of the NPC, which consists of a stable structural scaffold. The WD repeat domains probably play a crucial role for protein–protein interactions [10]. Until now the function and interplay of ALADIN was largely unknown. It was hypothesized that it is involved in protein transport between the cytoplasm and the nucleus [11]. Up to date, all identified mutations in ALADIN, with one exception, cause a defect in nuclear targeting, leading to the assumption that

Abbreviations: FRET, fluorescence resonance energy transfer; FLIM, fluorescence lifetime imaging microscopy; NPC, nuclear pore complex; NUP, nucleoporin; GFP, green fluorescent protein; CFP, cyan fluorescent protein.

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the mislocalization of ALADIN and therefore the lack of ALADIN at NPCs is the major mechanism leading to triple A syndrome [8,12,13]. In this study we analyzed the specific anchoring of ALADIN in the NPC and identified interaction partners of ALADIN by siRNA and fluorescence resonance energy transfer (FRET) experiments *in vivo*. A better understanding of ALADIN's localization and its role in the NPCs is important to provide insight into the relationship between its mislocalization and the pathogenesis of the triple A syndrome.

Materials and methods

Cloning of fluorescence tagged proteins. Full-length *NDC1* cDNA was amplified from HeLa derived cDNA and cloned into XhoI and KpnI sites of pEYFP-C1 and -N3 (BD Biosciences Clontech, Palo Alto, CA, USA) in frame at the carboxy- or aminoterminus of the enhanced yellow fluorescence protein (EYFP). The vector-insert boundaries and the insert were verified by sequencing.

Construction and transduction of the retroviral vectors. For the generation of pzc-CFG5.1-EGFP-AAAS the full-length coding region of ALADIN from pEGFP-AAAS was excised with KpnI/XmaI restriction and ligated into the pcz-CFG5.1-MCS. The vector-insert boundaries and insert were verified by sequencing.

Retroviral particles were generated as described previously [14]. Single HeLa cell colonies expressing GFP-ALADIN were obtained by seeding of a dilution of one half cell per well in a 96-well plate. After 10 days the colonies were analyzed by fluorescence microscopy. One clone was selected for subcultivation because of the fluorescent intensity of GFP-ALADIN at the NPC and its low overexpression in cytoplasm.

For the generation of cells expressing CFP-ALADIN or ALADIN-CFP the gammaretroviral vector SERS11.SF.gp91s.iGM.W was used as backbone for cloning [15]. Using XbaI and SalI the cassette containing gp91phox-IRES-ΔMGMT-GFP was exchanged by the full-length coding region of wild type ALADIN fused to CFP (Allele Biotechnology, San Diego, CA) at its carboxy- or amino terminus resulting in an expression of CFP-ALADIN or ALADIN-CFP in the subsequent transduced HeLa-cells. Cell-free viral supernatants were generated by transient co-transfection on HEK293T cells as described elsewhere [16]. HeLa target cells were plated in 1.5 cm dishes and were transduced with retroviral titers. Forty-eight hours after transduction the cells were subcultured. Single cell colonies were obtained by single cell sorting into 96-well plates with the flow cytometer FacsARIA (Becton Dickinson GmbH, Heidelberg, Germany) cultured and analyzed by fluorescence microscopy.

RNAi and immunofluorescence. For RNAi experiments, the following siRNAs were used: AAAS: GAUCUGUCUGAGACAACAA (sense sequence) (Qiagen, Hilden, Germany) and NDC1-1, POM121, GP210, and control non-silencing siRNA (Eurofins MWG Operon, Ebersberg, Germany) as described above [17]. Transfection of HeLa-cells stably expressing GFP-ALADIN with a final concentration of 10 nM siRNAs using HiPerFect (Qiagen, Hilden, Germany) was performed in 24-well plates four times over a distance of each 72 h. Immediately before second, third, and fourth transfection were performed, the cells were split. At the last transfection the cells were spread out into four wells and 48 h later were fixed with ice-cold Aceton for 2 min for anti-POM121 staining, or 2% PFA for 20 min for anti-NDC1 and anti-GP210 staining. PFA fixed cells were semipermeabilized with 0.5% digitonin in PBS for 20 min. Blocking was performed 30 min with 1% BSA and 10% fetal calf serum (FCS) in PBS. The anti-NDC1, anti-POM121, and anti-GP210 antibodies (kindly provided by D. Görlich, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany) were diluted 1:1000 in blocking solution and incubated with the fixed cells for 1 h at room temperature. After washing with PBS the cells were incubated with the 1:200 diluted secondary antibody Alexa Fluor® 568 goat anti-rabbit IgG (Invitrogen, Karlsruhe, Germany) for 1 h. The cover slips with the immunostained cells were transferred on object slides with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired using the confocal laser microscope TCS SP2 (Leica, Mannheim, Germany). To avoid crosstalk between fluorophors we used the sequential scan modus. The 8 bit pictures of the green and red fluorescence were pair-wise densitometrically evaluated using the program Fiji ImageJA 1.42I. The mean fluorescence intensity was calculated from approximately 50 cells using the selection brush tool to selectively check the fluorescence at the nuclear rim. The fluorescence intensity ranged from 0 to 255. We calculated the statistical significance of the several groups using a one-way ANOVA followed by Bonferroni's post hoc test.

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker™ CAMBREX, Apen, Germany) supplemented with 10% fetal bovine serum, L-glutamine and antibiotics at 37 °C and 5% CO_2. The day before transfection, HeLa cells were plated in 24-well plates on cover slips at 2×10^4 cells per well in DMEM without antibiotics. At a confluence of ca. 30% cells were transiently transfected with 0.1 μg plasmid DNA using Fugene HD (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Seventy-two hours after transfection, the cells were washed with PBS and fixed with 2% paraformaldehyde in PBS. The cover slips with the cells were transferred on object slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and analyzed by FLIM.

Fluorescence resonance energy transfer (FRET) analysis. All FLIM-FRET experiments were done in HeLa cells, which stably expressed N-terminal (CFP-ALADIN) or C-terminal (ALADIN-CFP) fluorophor-tagged ALADIN. Cells were transfected with C- or N-terminal YFP-tagged NDC1. The transfection efficiency was examined to be 90–95% prior performing the FLIM-FRET measurements.

Fluorescence lifetime measurements were performed by wide-field time-domain FLIM as described previously with minor changes [18]. Briefly, an excitation wavelength of 440 nm was used. Images were recorded on a Zeiss Axiovert S100TV with a 63× 1.4 NA oil immersion objective through a 455LP dichroic mirror and a D480/40 m emission filter (Chroma filter set 31044v2). The fluorescence decay curves were recorded by 18 images with increasing delay times (step 0.5 ns) after the excitation laser pulse with a camera binning of 4×4 and an exposure time of 200–400 ms for each image. The laser power was reduced by ND filters to avoid photo bleaching during the acquisition. Images were background-corrected by subtracting the mean value of a region without any fluorescence signal. The fluorescence lifetime of CFP was calculated by tail-fitting of the decay curve for each pixel according to a single exponential decay function $I(t) = Ae^{-t/\tau}$ with I being the measured intensity in each pixel at a time point t, A the pre-exponential factor, and τ the fluorescence lifetime. A minimum of 20 pictures of three independent experiments were analysed. Significance of the fluorescence lifetime shifts was analyzed using the two-tailed Mann–Whitney U test.

Results

Depletion of ALADIN and NDC1 causes bilateral interference

We examined the cellular localization of ALADIN after knockdown of the three transmembrane NPC anchoring nucleoporins NDC1, POM121, and GP210 in HeLa-cells stably expressing GFP-ALADIN. In parallel, we depleted ALADIN in these cells to investigate the effect on the NPC anchoring proteins. ALADIN was detected by GFP fluorescence and the transmembrane nucleoporins were detected via immunofluorescence (Fig. 1) and subsequently quantified (Fig. 2 and Table 1). When HeLa cells were treated with non-silencing siRNA (mock) we revealed a mean fluo-

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rescence intensity of 51 ± 9 for NDC1 (red) and 49 ± 9 for GFP-ALADIN (green) (Fig. 2A, black points). The knock-down of NDC1 leads to a decrease GFP-ALADIN fluorescence at the NPCs to about 50% (Fig. 1, arrows and Fig. 2A, gray rhombs). Furthermore, after ALADIN depletion the localization of NDC1 at the NPC decreased (Figs. 1 and 2A, gray triangles).

In contrast, after RNAi-mediated depletion of POM121 and GP210 no effects on ALADIN were observed (Figs. 1 and 2B, gray squares and Fig. 2C, gray points). However when ALADIN was downregulated, POM121 immunostaining increased (Figs. 1 and 2B, gray triangles) while the GP210 level remained unchanged (Figs. 1 and 2C, gray triangles).

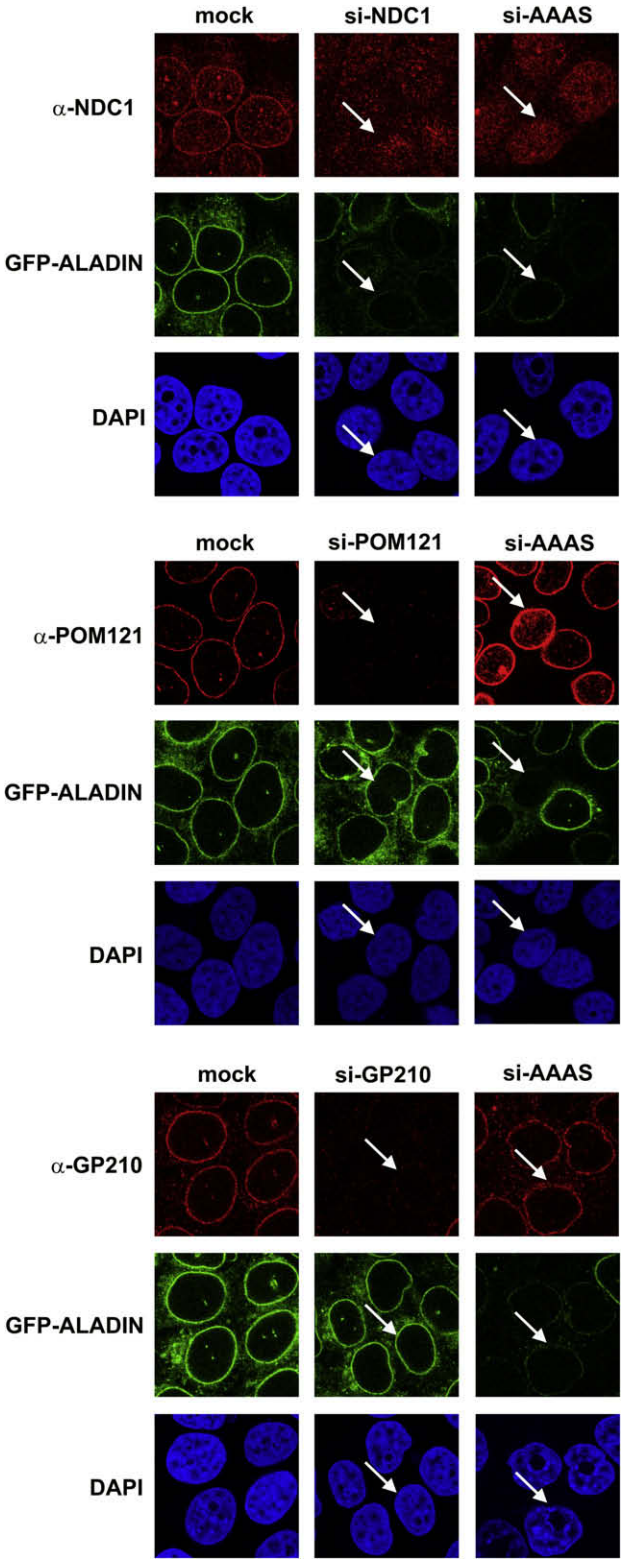

Fig. 1. Depletion of NDC1, POM121, GP210, and ALADIN from HeLa-cells stably expressing GFP-ALADIN by siRNA. HeLa-cells stably expressing GFP-ALADIN (green) were either mock treated or transfected with siRNAs to NDC1, POM121, GP210 or ALADIN. Immunofluorescence of NDC1, POM121, and GP210 under the above mentioned conditions (red). Nuclei were stained with DAPI. Arrows indicate an example of a siRNA-depleted cell. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

Identification of an association of ALADIN and NDC1

To test a potential association between ALADIN and NDC1 we performed FLIM-FRET analyses as a powerful tool to measure protein–protein association *in vivo*. FRET is a photophysical effect in which energy is transferred from an exited donor fluorophore to an acceptor fluorophore in close proximity (within 2–10 nm) [19]. FRET can be measured by FLIM. The thereby measured fluorescent lifetime is the average time which the fluorophore remains in the excited state after excitation, and is a function of the close environment of the fluorophore. If an acceptor is in close proximity, the lifetime of the donor decreases. All FLIM-FRET experiments were done using HeLa cells, which stably expressed N- or C-terminal CFP coupled ALADIN (CFP-ALADIN or ALADIN-CFP). These cells were transiently transfected with YFP-tagged NDC1 (YFP-NDC1 or NDC1-YFP). In the absence of YFP-tagged NDC1 the lifetimes of CFP-ALADIN and of ALADIN-CFP were 2.63 ± 0.02 and 2.63 ± 0.03 ns, respectively. In the presence of YFP-coupled NDC1, the fluorescence lifetime of CFP-ALADIN and ALADIN-CFP was significantly decreased indicating an energy transfer (Fig. 3). We investigated the four possible combinations of C- and N-terminal coupled protein-fluorophore variants. In the presence of YFP-NDC1 the lifetimes of CFP-ALADIN and ALADIN-CFP were reduced to 2.46 ± 0.06 and 2.50 ± 0.06 ns, respectively. In the presence of NDC1-YFP the lifetimes of CFP-ALADIN and ALADIN-CFP were also decreased to 2.54 ± 0.04 ns and 2.54 ± 0.05 ns, respectively. These results indicate an association of NDC1 and ALADIN.

Discussion

ALADIN is a four WD repeats containing protein which enable it for protein–protein interactions. In addition ALADIN lacks any motif for anchoring itself within the NPC [3,4]. Therefore we predict that the integration of ALADIN within the nuclear pore complex is permitted by other associated proteins. To proof this hypothesis, we performed RNAi experiments to knock-down the three known membrane integrated nucleoporins NDC1, GP210, and POM121. In these experiments we used a HeLa cell line stably expressing GFP-ALADIN for the following reasons: (I) in contrast to transiently transfected cells, this cell line avoids overexpression artefects leading to a mislocalisation of ALADIN in the cytoplasm; (II) all cells are expressing GFP-ALADIN at the same expression level and (III) therefore a quantification of the fluorescence intensity at the nuclear rim after RNAi treatment was possible. We could show that after depletion of NDC1 the ALADIN localization at the NPC decreases whereas the depletion of POM121 or GP210 had non effect on ALADIN distribution, suggesting that ALADIN is anchored in the NPCs via NDC1 and not POM121 or GP210. To proof this association with NDC1 *in vivo* we performed FLIM-FRET experiments. Here we could observe an energy transfer from CFP-tagged ALADIN to the C- as well as N-terminus of YFP-tagged NDC1 indicating that both sites are located at the same site of the membrane. Former studies also predicted the N- and C-terminus as well as loop 2 and 4 of the six transmembrane segments bearing NDC1 at the cytoplasmatic side [17,20].

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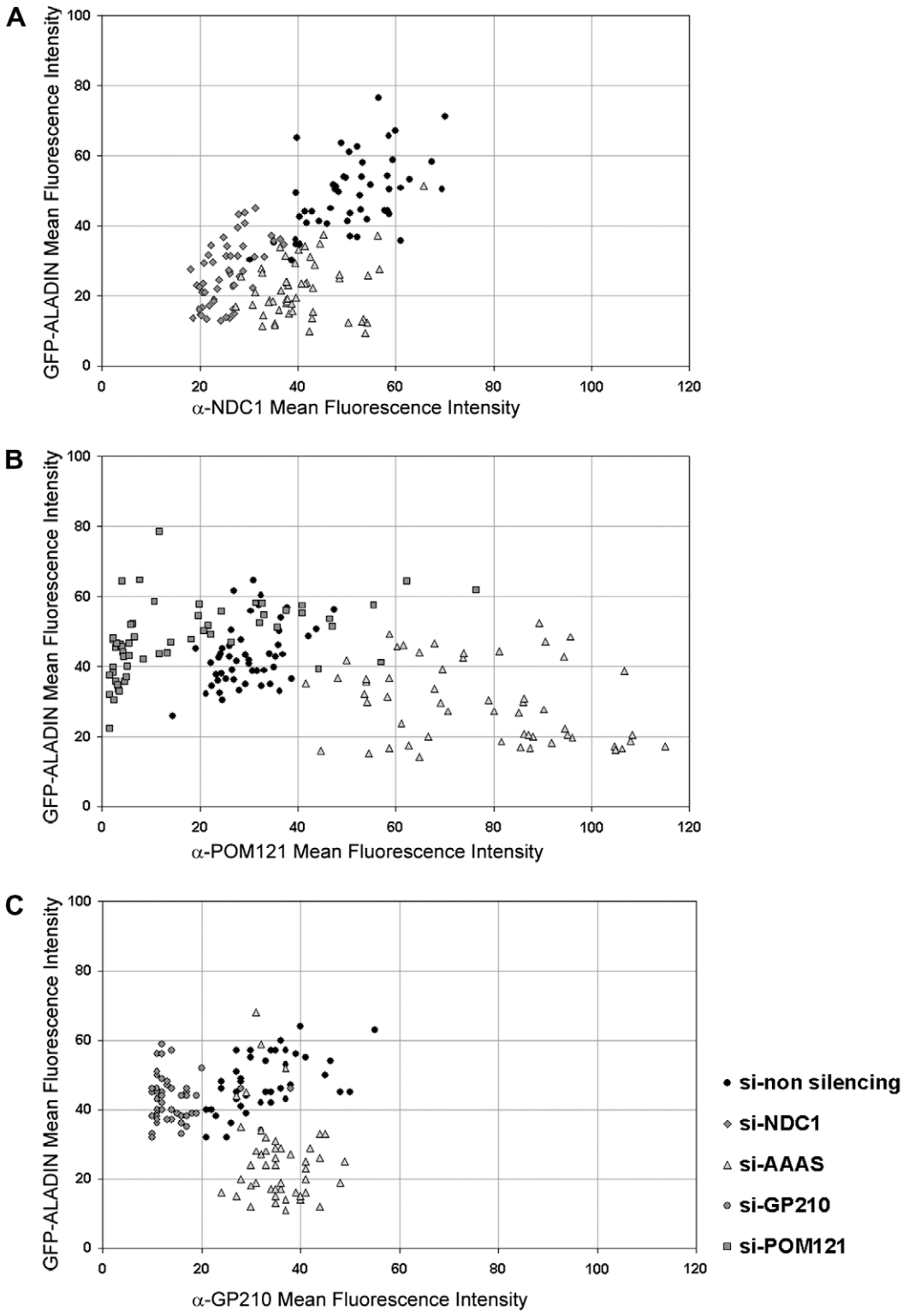

Fig. 2. Quantification of the fluorescence at the nuclear rim from the siRNA experiments. Pair-wise quantitative densitometric evaluation of the green (GFP-ALADIN) and red (-NDC1, -POM121 or -GP210) fluorescence of the nuclear rim from the siRNA experiments. Quantification of (A) NDC1 antibody stain, (B) POM121 antibody stain, and (C) GP210 antibody stain. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

These findings are further supported by GST-pull-down and immunoprecipitation data [21].

Moreover in our knock-down experiments we could show that depletion of ALADIN led to an enhanced POM121 immunostaining. A possible explanation may be that ALADIN and POM121 use same docking sites at NPCs, and when GFP-ALADIN is knocked down these sites become more accessible for POM121.

In contrast, the NDC1 localization at the NPC decreases after depletion of ALADIN. One reason may be that ALADIN stabilizes NDC1 at the NPC or influences the correct membrane integration

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Table 1
Mean fluorescence intensities of *x*- (antibody stain, red) and *y*-values (GFP-ALADIN, green) of the quantitative analysis in Fig. 2. Values are between 0 and 255 (8 bit pictures). Significance of the fluorescence intensity values was analyzed using a one-way ANOVA followed by Bonferroni's post hoc test. *P*-values <0.01 were considered as significant and were marked with two asterisks.

		Mock	Si-transmembrane NUP	Si-AAAS
α-NDC1	Mean red fluorescence	51 ± 9	26 ± 5**	41 ± 8**
	Mean green fluorescence	49 ± 11	26 ± 9**	22 ± 8**
α-POM121	Mean red fluorescence	30 ± 6	18 ± 18**	78 ± 19**
	Mean green fluorescence	43 ± 9	48 ± 10	30 ± 11**
α-GP210	Mean red fluorescence	33 ± 8	14 ± 5**	36 ± 5
	Mean green fluorescence	48 ± 8	43 ± 6	25 ± 12**

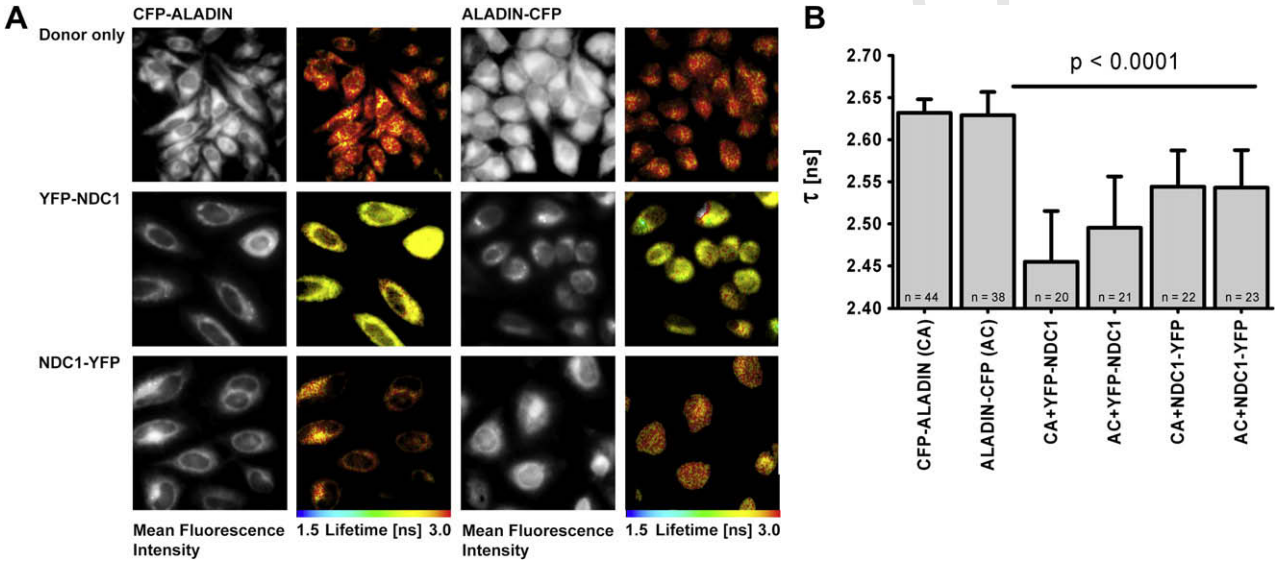

Fig. 3. FLIM-FRET. (A) HeLa-cells stably expressing CFP-ALADIN only or ALADIN-CFP only and transiently transfected with NDC1-YFP or YFP-NDC1. On the left site the fluorescence intensity of these cells is shown. On the right site the calculated lifetime for each pixel is added and shown as a false color code. (B) Statistical analysis. Average lifetime of CFP-ALADIN or ALADIN-CFP alone (FRET-donor only) and in combination with NDC1-YFP or YFP-NDC1 (FRET-donor and FRET-acceptor). The fluorescence lifetime was significantly lower if YFP-coupled NDC1 was present. Error bars are SEM. (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.)

of NDC1. NDC1 is initially integrated into the membranes of the rough endoplasmic reticulum before its assembly into NPCs [20]. We suppose that ALADIN is involved in transport processes of new build NDC1 from the rough endoplasmic reticulum to the NPC. Based on our results we assume that ALADIN and NDC1 are interaction partners. We conclude that ALADIN is anchored by NDC1 to the nuclear envelope and that this interaction is abolished, if ALADIN is mutated.

The lack of ALADIN at NPCs is the major mechanism leading to triple A syndrome and indeed all (except one) identified mutations in ALADIN cause a defect in nuclear targeting [8,12,13]. We conclude that the loss of association with NDC1 leads to the observed mislocalization of ALADIN. With our investigations we contribute to the knowledge about the nucleoporin ALADIN and its integration into the complex composition of the NPC.

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