Identification of a novel putative interaction partner of the nucleoporin ALADIN

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ABSTRACT

It has been shown that the nucleoporin ALADIN plays a significant role in the redox homeostasis of the cell, but its function in steroidogenesis contributing to adrenal atrophy in triple A syndrome remains largely unknown. In an attempt to identify new interaction partners of ALADIN, co-immunoprecipitation followed by proteome analysis was conducted in different expression models using the human adrenocortical tumour cell line NCI-H295R. Our results suggest an interaction of ALADIN with the microsomal protein PGRMC2. PGRMC2 is shown to be activity regulator of CYP P450 enzymes and, therefore, to be a possible target for adrenal dysregulation in triple A syndrome. We show that there is a sexual dimorphism regarding the expression of Pgrmc2 in adrenals and gonads of wild-type (WT) and Aaas knock-out (KO) mice. Female Aaas KO mice are sterile due to delayed oocyte maturation and meiotic spindle assembly. A participation in meiotic spindle assembly confirms the recently investigated involvement of PGRMC2 in mitosis and emphasises an interaction with PGRMC2 which is a regulator of the cell cycle. By identification of a novel interaction partner of ALADIN, we provide novel aspects for future research of the function of ALADIN during cell cycle and for new insights into the pathogenesis of triple A syndrome.

KEY WORDS: ALADIN, Cell division, Nuclear pore complex, PGRMC2, Triple A syndrome

INTRODUCTION

Triple A syndrome (MIM#231550) is an autosomal recessive disorder characterised by three distinct symptoms: ACTH-resistant adrenal insufficiency, oesophageal achalasia and absent tear production (alacrima) in combination with progressive neurological impairment (Allgrove et al., 1978). The disorder is caused by mutations in AAAS (achalasia-adrenal insufficiency alacrima syndrome) encoding the protein ALADIN (alacrima-achalasia-adrenal insufficiency neurologic disorder) (Handschug et al., 2001; Tullio-Pelet et al., 2000). ALADIN presents enhanced protein levels in adrenal atrophy in triple A syndrome. Our observations give the basis for further research on the association between ALADIN and steroidogenesis.

RESULTS

PGRMC2 precipitates with ALADIN in an exogenous and endogenous ALADIN adrenal cell expression model

Co-IP was conducted in NCI-H295R cells either expressing endogenous ALADIN or additionally exogenous GFP-ALADIN. In the GFP-ALADIN expression model adequate peptides of ALADIN could be detected (Fig. 1). The analysis of ALADIN co-IP resulted in less detected peptides (one exclusive unique peptide), but had a 100% probability of correct protein identification. Despite several methodological optimisations using different protocols and antibodies, ALADIN co-IP had a low yield and analysis of bound proteins identified in mass spectrometry in GFP-ALADIN co-IP and ALADIN co-IP, but which were not found in the specific control pulldown assays, are presented in Tables S1 and S2.

PGRMC2 was simultaneously identified by mass spectrometry analysis in co-IP of GFP-ALADIN using GFP-Trap_A agarose.
beads and in co-IP of ALADIN using anti-ALADIN coupled to Protein G UltraLink resin sepharose beads. Exclusive unique peptides of ALADIN and PGRMC2 detected in mass spectrometry after GFP-ALADIN are shown in Fig. 1A. Fewer peptides of PGRMC2 were detected after ALADIN pulldown (one exclusive unique peptide), with 100% protein identification probability.

We additionally confirmed the identification of PGRMC2 in GFP-ALADIN and ALADIN co-IP by western blot. Successful GFP-ALADIN pulldown in lysates of NCI-H295R cells stably expressing GFP-ALADIN (86 kDa) is presented in Fig. 2A. In accordance with our mass spectrometry results, PGRMC2 (24 kDa) could also be detected after GFP-ALADIN pulldown (Fig. 2A, 24 kDa, arrow). The negative control remained empty.

Successful endogenous ALADIN pulldown is shown in Fig. 2B. ALADIN (59 kDa) was found in the bound fraction of the ALADIN co-IP, but the negative control using normal mouse IgG was shown to be empty. In accordance with our result after GFP-ALADIN pulldown, PGRMC2 precipitated in endogenous ALADIN pulldown with no unspecific interaction in the negative control (Fig. 2B, arrow).

ALADIN precipitates with PGRMC2 in an exogenous and endogenous PGRMC2 adrenal cell expression model

In order to further evaluate a possible interaction between the nucleoporin ALADIN and microsomal PGRMC2 we conducted reciprocal co-IP assays. Reciprocal pulldowns were done in NCI-H295R cells transiently expressing exogenous PGRMC2-GFP and endogenous PGRMC2. Efficient pulldown of (PGRMC2-)GFP in lysates of NCI-H295R cells transiently expressing PGRMC2-GFP (51 kDa) is presented in Fig. 2A. Confirming our previous results identifying a possible interaction between ALADIN and PGRMC2, ALADIN could be detected after PGRMC2-GFP pulldown (Fig. 2A, 59 kDa, arrow). Additionally, PGRMC2 could be identified after PGRMC2-GFP pulldown (Fig. 2A). The negative control was empty for both.

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**Fig. 1.** PGRMC2 was identified in mass spectrometry after GFP-(ALADIN) pulldown. Identified exclusive unique peptides (yellow) (number of different amino acid sequences, regardless of any modification, that are associated only with this protein) of ALADIN and progesterone receptor membrane component 2 (PGRMC2) are highlighted. Also for PGRMC2, relative intensities of annotated spectra are shown after GFP(-ALADIN) co-IP of whole cell lysates of GFP-ALADIN expressing NCI-H295R cells.
PGRMC2 pulldown is shown in Fig. 2B. PGRMC2 was successfully detected in the bound fraction of the PGRMC2 co-IP, and the negative control using rabbit IgG remained empty. According to our results after PGRMC2-GFP pulldown, ALADIN slightly but visibly precipitated after PGRMC2 pulldown with no unspecific interaction in the negative control (Fig. 2B, arrow).

**Localisation of ALADIN and PGRMC2 in adrenal cells using different expression models**

Further evidence of possible co-localisation of ALADIN and PGRMC2 is given after immunofluorescent staining. Cells expressing GFP-ALADIN and PGRMC2-GFP were used to verify the specificity of anti-ALADIN and anti-PGRMC2 staining in NCI-H295R cells. Staining was done using anti-ALADIN, anti-PGRMC2 and anti-NPC proteins (mAb414).

Immunostaining with mAb414 in all adrenal cell expression models gave a thin circle around the nucleus indicating punctate localisations of NPCs (Fig. 3).

Immunofluorescent staining of the nucleoporin ALADIN appeared at the nuclear envelope at the proximity of NPCs in all adrenal cell expression models. In the exogenous GFP-ALADIN cell model, the fusion protein was correctly targeted to the nuclear envelope and did not accumulate to a greater extent in the cytoplasm. We showed that ALADIN almost completely co-localises with anti-NPC proteins (mAb414) immunostaining at the nuclear envelope, substantially verifying the localisation of ALADIN at the nuclear pore (Fig. 3).

In immunofluorescent staining the microsomal protein PGRMC2 localised to the central ER, but also revealed a patchy and punctate staining pattern around the nucleus to the perinuclear space in all adrenal cell expression models (Fig. 3). The same PGRMC2 immunostaining pattern was observed in human cervical carcinoma (HeLa) and human fibroblasts (Fig. S1A,B, respectively). In the exogenous adrenal cell model the PGRMC2-GFP fusion protein was still correctly targeted to the central ER and perinuclear space. In the staining with the anti-PGRMC2 in NCI-H295R cells we could also observe nuclear staining in some adrenal cells (Fig. 3, arrow). Nuclear localisation of PGRMC2 was absent in the PGRMC2-GFP adrenal cell expression model. Co-localisation between mAb414 and PGRMC2 or ALADIN and PGRMC2 was not complete but showed positivity in the perinuclear space and in the nuclear membrane in all cell expression models (Fig. 3).
Depletion of ALADIN affects localisation of PGRMC2 in human skin fibroblasts

In order to address whether depletion of ALADIN affects localisation of PGRMC2 at the perinuclear ER we immunostained PGRMC2 and ALADIN in the inducible adrenocortical ALADIN knock-down cell line (NCI-H295R1-TR/AAAS knock-down), and in the specific control cell lines (NCI-H295R1-TR/Scrambled shRNA and NCI-H295R1-TR), recently described by our group (Jühlen et al., 2015). To verify these results we also used skin fibroblasts of a triple A patient (homozygous mutation in Exon 9, c.884G>A, p.Trp295X) and human skin fibroblasts of an anonymised in-house control (Fig. 4).

In adrenocortical cells we could not detect an alteration of PGRMC2 localisation after ALADIN knock-down. However, immunostaining of PGRMC2 in NCI-H295R1-TR/Scrambled shRNA and NCI-H295R1-TR compared to NCI-H295R1-TR/AAAS knock-down was more cytoplasmic and nuclear (Fig. 4).

In human skin fibroblasts, ALADIN depletion in the triple A patient lead to an increased staining of PGRMC2 at the perinuclear ER compared to human control skin fibroblasts (Fig. 4).

The expression of PGRMC2 is not affected after AAAS knock-down in human adrenal cells

To test if PGRMC2 expression is affected when ALADIN is downregulated we used the inducible NCI-H295R1-TR cells with AAAS knock-down shRNA or scrambled shRNA as negative control (Jühlen et al., 2015).

We could not find an alteration on PGRMC2 mRNA level after induction of ALADIN depletion by doxycycline in NCI-H295R1-TR cells in at least ten triplicate experiments (Fig. S2).

Pgrmc2 exhibits a sexual dimorphism in adrenals and gonads of WT and Aaas KO mice

In order to examine the expression of Pgrmc2 in WT and Aaas KO mice we looked at the adrenals and gonads using TaqMan analysis and western blot (Fig. 5A).

Indeed, we found that Pgrmc2 displayed a sexual dimorphism in female and male WT and Aaas KO mice: the expression in testes was significantly higher independent of genotype compared to female ovaries, in female KO adrenals the expression was significantly higher compared to male KO adrenals (Fig. 5A).
Interestingly, in female adrenals the depletion of ALADIN leads to a significant increase in Pgrmc2 expression, whereas in female ovaries a decrease compared to WT ovaries was observed (Fig. 5A). The expression of Pgrmc2 was not altered in male Aaas KO adrenals or testes compared to male WT organs in at least four triplicate experiments (Fig. 5A).

To examine our findings in female WT and Aaas KO mice on PGRMC2 RNA level, we conducted western blot of several female murine WT and Aaas KO tissues, i.e. adrenals, brain, ovaries and spleen (Fig. 5B). We could confirm our results on PGRMC2 RNA level and show an increase in PGRMC2 protein in female adrenals of Aaas KO mice compared to female adrenals of WT mice (Fig. 5B). Furthermore, in ovaries of Aaas KO mice PGRMC2 protein was diminished compared to ovaries of WT mice.

**DISCUSSION**

The exact role of the nucleoporin ALADIN at the NPC and its involvement in steroidogenesis leading to the characteristic adrenal atrophy in triple A syndrome remains largely unknown. We and others have provided evidence of the involvement of ALADIN in the oxidative stress response of the cell (Jühlen et al., 2015; Kind et al., 2010; Koehler et al., 2013; Prasad et al., 2013; Storr et al., 2009). Recently, we have shown that a depletion of ALADIN in adrenocortical carcinoma cells leads to an alteration in glucocorticoid and androgenic steroidogenesis (Jühlen et al., 2015).

Despite the reported interaction between ALADIN and ferritin heavy chain 1, no other interaction partner which would lead to the identification of a plausible function and signal transduction of ALADIN in the cell is known so far (Storr et al., 2009).

In an attempt to identify new interaction partners of ALADIN, co-IP analyses showed that PGRMC2 precipitated with ALADIN. To verify the identified association between ALADIN and PGRMC2, reciprocal IP was conducted. Our results showed co-IP of ALADIN with PGRMC2. Different co-IP approaches using exogenous and endogenous expression systems in human adrenal cells have shown...
for the first time that the nucleoporin ALADIN associates in a complex with the microsomal protein PGRMC2.

PGRMC2 belongs to the MAPR family. MAPRs are proteins found at the membrane of the ER and are thought to be regulators of CYP P450 enzymes. The first identified MAPR, PGRMC1, gained widespread attention and is extensively investigated compared to its homologue PGRMC2 (Falkenstein et al., 1996). PGRMC1 is a cytochrome-related protein with several implications in cancer (Clark et al., 2016; Falkenstein et al., 1996; Kabe et al., 2016).

In this work, PGRMC2 was found to interact with the nucleoporin ALADIN. We could additionally show that PGRMC2 is detected after PGRMC2-GFP pulldown on western blot suggesting a homodimeric role of PGRMC2. It has been reported that PGRMC2 interacts with PGRMC1 as a heterodimer in an exogenous expression system in human ovarian carcinoma cells (Peluso et al., 2014), but homodimerisation of PGRMC2 has not been shown yet.

Visualising PGRMC2 in the cell using immunofluorescence and confocal microscopy has shown the presence of PGRMC2 at the central ER, and, interestingly, at the nuclear envelope and the perinuclear ER. We detected that PGRMC2 co-localises with ALADIN and with different FG-repeat NUPs [stained with anti-NPC proteins (mAb414)] to the nuclear envelope and the perinuclear ER.

Microsomal CYP P450 systems are haemoproteins and are localised to the membrane of the ER (Pandey and Flück, 2013). PGRMC2 is also restricted to the ER and is thought to be an electron donor for some CYP P450 enzymes, most likely through its cytochrome b5-similar haem-binding domain (Albrecht et al., 2012; Wendler and Wehling, 2013). Most recently, both PGRMC1 and PGRMC2 were identified as putative interacting partners of ferrochelatase, an enzyme catalysing the terminal step in the haem biosynthetic pathway, thereby possibly controlling haem release as a chaperone or sensor (Piel et al., 2016). The mixed-function oxidase system of microsomal CYP P450 enzymes requires a donor transferring electrons from NADPH to reduce the prosthetic haem group (Pandey and Flück, 2013). Interaction of ALADIN with PGRMC2 at the perinuclear ER could influence CYP P450 enzyme activity through electron transfer from NADPH and/or control haem synthesis. In triple A syndrome, altered CYP P450 enzyme activity would consecutively contribute to adrenal atrophy.

In order to address if ALADIN anchors PGRMC2 at the perinuclear ER, we conducted immunostaining in adrenocortical cells after inducible knock-down of ALADIN and in skin fibroblasts of a triple A patient. The knock-down model has been used in our lab as an in vitro model for triple A syndrome (Jühlen et al., 2015). In immunostaining we could not detect an alteration of PGRMC2 protein intensity and localisation after ALADIN knock-down; however, PGRMC2 immunostaining was more nuclear and cytoplasmic in control cell lines compared to ALADIN knock-down cells. Additionally, we showed that there is no alteration in PGRMC2 expression after ALADIN knock-down in adrenocortical cells. Localisation of PGRMC2 in triple A patient skin fibroblasts was not changed compared to human control skin fibroblasts in immunostaining analyses. Astonishingly, patient cells presented an increased staining of PGRMC2 at the perinuclear ER and nuclear envelope compared to control cells. Cytoplasmic and nuclear staining was not altered compared to control cells.

Taken together, our results in immunofluorescence microscopy using different ALADIN and PGRMC2 adrenal cell expression systems provide a basis for future research of how ALADIN and PGRMC2 possibly associate in a complex close to the nuclear envelope, and what the effects on steroidogenesis of this association would be. Additionally, ALADIN depletion affects PGRMC2...
localisation at the perinuclear ER in triple A patient cells, suggesting a regulatory role of ALADIN for PGRMC2 protein localisation. Probably because ALADIN is not fully depleted in the adrenocortical knock-down model compared to patient fibroblasts we could not detect such effect in the in vitro model.

In summary, our work identifies microsomal PGRMC2 as novel interactor for ALADIN and provides new insights into the molecular function of the nucleoporin in the pathogenesis of triple A syndrome. We found that Pgrmc2 has a sexual dimorphic role in adrenals and gonads of WT and AAas KO mice, and, of note, ALADIN depletion leads to an alteration in PGRMC2 RNA and protein level in adrenals and ovaries of female AAas KO mice. Our group reported that female mice homozygous deficient for AAas are infertile (Huebner et al., 2006). Carvalhal et al. recently presented that ALADIN is involved in mitotic and meiotic spindle assembly, chromosome segregation and production of fertile mouse oocytes (Carvalhal et al., 2015, 2016 preprint). Interestingly, both PGRMC1 and PGRMC2 seem to be involved in regulation of ovarian follicle development and therefore seem to have neuroendocrine functions (Wendler and Wehling, 2013). PGRMC1 and PGRMC2 locate to the mitotic spindle and are shown to exploit a specific role during metaphase of mitosis (Griffin et al., 2014; Peluso et al., 2014; Sueldo et al., 2015). Additionally, ALADIN and PGRMC2 have been identified to interact with the human centrosome-cilium interface (Gupta et al., 2015; Hanson et al., 2014; Yan et al., 2014). The centrosome is a fundamental organelle which participates in cell cycle progression and mitotic spindle assembly.

Future work needs to address how ALADIN associates with PGRMC2 at the perinuclear ER and influences PGRMC2 localisation. It needs to be explored which molecular mechanisms are altered by the interaction between ALADIN and PGRMC2, allowing yet another important task of ALADIN to be elucidated.

MATERIALS AND METHODS

Cell culture

NCI-H295R cells stably expressing GFP-ALADIN fusion protein or GFP were generated as described previously using the gamma-retroviral transfer vectors pcz-CFG5.1-GFP-AAAS and pcz-CFG5.1-GFP (Kind et al., 2009).

NCI-H295R cells transiently expressing PGRMC2-GFP fusion protein were generated as follows. Cells were transfected with pCMV6-AC-PGRMC2-GFP vector (RG204682) (OrGene Technologies, Rockville MD, USA) using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s protocols. Cells were harvested or fixed after 48 h.

In all exogenous expression models clones were selected by moderate expression of the desired fusion protein and true cellular localisation in order to exclude the possibility of false positive protein interactions.

NCI-H295R cells were cultured in DMEM/F12 medium (Lonza, Cologne, Germany) supplemented with 1 mM L-glutamine (Lonza, Cologne, Germany), 5% Nu-serum (BD Bioscience, Heidelberg, Germany), 1% insulin-transferrin-selenium (Gibco, Life Technologies, Darmstadt, Germany) and 1% antibiotic-antimycotic solution (PAA, GE Healthcare GmbH, Little Chalfont, UK).

NCI-H295R I-TR cells with AAas knock-down or scrambled shRNA were generated, selected and cultured as described previously (Jühlen et al., 2015).

Triple A patient skin fibroblasts and human anonymised control skin fibroblasts were obtained and cultured as described earlier by our group (Kind et al., 2010). Informed consent was obtained from all subjects and experiments were approved by the local ethics review board (Medical Faculty, Technische Universität Dresden, EK820897). HeLa cells were cultured as described previously (Kind et al., 2009).

Animals

All procedures were approved by the Regional Board for Veterinary Affairs, Dresden, Germany (AZ 24-9168.21-1-2002-1) in accordance with the institutional guidelines for the care and use of laboratory animals. C57BL/6J mice were obtained from Janvier Labs (Le Genest-Saint-Ile, France), AAas KO mice were generated as described previously (Huebner et al., 2006).

RNA extraction, cDNA synthesis and quantitative real-time PCR using TaqMan

Total RNA from cultured cells (n=10) and from frozen murine organs (at least four animals per genotype and sex) was isolated using the NucleoSpin RNA (Macherey-Nagel, Düren, Germany) according to the protocol from the manufacturer. Purity of the RNA was assessed using Nanodrop Spectrophotometer (ND-1000) (Nanodrop Technologies, Wilmington DE, USA). 300 ng of total RNA was reverse transcribed using the GoScript Reverse Transcription System (Promega, Mannheim, Germany) following the protocols from the manufacturer. Primers for the amplification of the target sequence were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies, Darmstadt, Germany) and compared to the human or murine genome database for unique binding using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer sequences are listed in Table S3.

The qPCR amplifications were performed in triplicates using the GoTaq Probe qPCR Master Mix (Promega) according to the manufacturer’s reaction parameter on an ABI 7300 Fast Real-Time PCR System (Applied Biosystems). In all results repeatability was assessed by standard deviation of triplicate Ct s and reproducibility was verified by normalizing all real-time RT-PCR experiments by the Ct of each positive control per run.

Immunoblots

After SDS-PAGE separation onto 4-12% PAGE (150 V for 1.5 h) and electroblotting (30 V for 1.5 h) (Invitrogen, Life Technologies, Darmstadt, Germany) onto Amersham hybond-ECL nitrocellulose membrane (0.45 µm) (GE Healthcare GmbH, Little Chalfont, UK) non-specific binding of proteins to the membrane was blocked by incubation in PBS containing 3% BSA (Sigma-Aldrich, Munich, Germany) at room-temperature.

The membrane was then probed with primary antibodies either anti-ALADIN (mouse, B-11: sc-374073; 1:100 in 3% PBS/BSA) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-PGRMC2 (rabbit, HPA041172; 1:200 in 5% PBS/milk powder) (Sigma-Aldrich, Munich, Germany) or anti-PGRMC2 (mouse, F-3: sc-374624; 1:100 in 3% PBS/BSA) (Santa Cruz Biotechnology, Inc.) overnight at 4°C. Secondary antibodies goat anti-mouse IgG conjugated to horseradish peroxidase (1:2000 in 3% PBS/BSA) (Invitrogen, Life Technologies, Darmstadt, Germany) or goat anti-rabbit IgG conjugated to horseradish peroxidase (1:3000 in 5% PBS/milk powder) (Cell Signalling Technology Europe B.V., Leiden, Netherlands) were incubated for 1 h at room-temperature.

Co-immunoprecipitation

For GFP co-IP lysates from NCI-H295R expressing GFP-ALADIN or PGRMC2-GFP were used. Lysates from cells expressing GFP were used as negative control. Cell lysates (500 µg protein) were added to the pre-equilibrated GFP-Trap A agarose beads (ChromoTek GmbH, Planegg-Martinsried, Germany), gently re-suspended by flipping the tube and bound overnight at constant mixing at 4°C. After washing steps, the beads were gently re-suspended in 60 µl NUPAGE 2X LDS sample buffer and in order to dissociate the captured immunocomplexes from the beads, boiled at 95°C for 10 min and western blot analysis was conducted with 20 µl of the eluate. The leftover 40 µl of the eluate using the lysates of NCI-H295R expressing GFP-ALADIN or GFP was further processed for proteomic profiling using mass spectrometry. These experiments following mass spectrometry analysis were repeated three times.

For co-IP of ALADIN or PGRMC2 lysates from NCI-H295R cells and Protein G UltraLink resin sepharose beads (Pierce, Thermo Scientific, Fischer Scientific, Schwerte, Germany) were used. Beads were gently re-
suspended in anti-ALADIN (2 µg/ml) or anti-PGRMC2 (HPA041172) (2 µg/ml) and as negative controls normal mouse or rabbit IgG (2 µg/ml) (Invitrogen, Life Technologies, Darmstadt, Germany). All antibodies were bound to the beads over-night at 4°C in a rotation chamber. After washing cell lysates (500 µg protein) were added to the beads, gently re-suspended by flipping the tube and bound overnight as described before. After washing the beads were gently re-suspended in 60 µl sample buffer containing dilution buffer, NU PAGE 1X LDS Sample Buffer and 1 x reducing agent. The captured immunocomplexes were dissociated and the eluates were collected and processed by western blot as described previously. These experiments were repeated three times. The left 40 µl of the eluate after ALADIN co-IP and negative control was further processed for proteomic profiling using mass spectrometry. Mass spectrometry analysis was conducted once.

Proteomic profiling using tandem mass spectrometry

Entire gel lanes were cut into 40 slabs, each of which was in-gel digested with trypsin (Shevchenko et al., 2006). Gel analyses were performed at the Mass Spectrometry Facility at the Max Planck Institute for Molecular Cell Biology and Genetics (Dresden) on a nano high-performance liquid chromatograph (UltiMate) interfaced on-line to a LTQ Orbitrap Velos hybrid tandem mass spectrometer as described previously (Vasilj et al., 2012).

A database search was performed against the IPI human database (Kersey et al., 2004) (downloaded in July 2010) and NCBI protein collection without species restriction (https://www.ncbi.nlm.nih.gov/protein, updated in June 2014) using MASCOT software v.2.2. Scaffold software v.4.3.2 was used to validate MS/MS-based protein identifications. Protein probabilities were assigned by the Protein Prophet algorithm (Nevesižskii et al., 2003).

Immunofluorescence microscopy

Cells grown onto glass cover slips were fixed for 5 min with 4% PFA (SAV LP, Flinschbach, Germany) in PBS, permeabilised for 5 min with 0.5% Triton-X-100 in PBS and fixed again for 5 min. Blocking was performed for 30 min with 2% BSA/0.1% Triton-X-100 in PBS at room temperature.

All antibodies used for immunofluorescence were diluted in blocking solution. Primary antibodies anti-ALADIN (1:25), or anti-PGRMC2 (HPA041172, 1:50) or anti-PGRMC2 (F-3: sc-374624, 1:25) and anti-NPC proteins (mAb414; 1:800; Covance, Berkley CA, USA) were incubated at 4°C overnight in a humidified chamber. Secondary antibodies goat anti-mouse IgG Cy3 (1:500; Amersham Biosciences, Freiburg, Germany), Alexa Fluor 488 and 555 goat anti-rabbit IgG (1:500; Molecular Probes, Life Technologies) were incubated one hour at room temperature in the dark.

Fluorescence was visualised using the confocal laser microscope TCS SP2 (Leica Microsystems, Mannheim, Germany). The experiments were repeated at least three times.

Statistics of TaqMan analyses

Statistical analyses were made using the open-source software R version 3.3.0 and R Studio version 0.99.902 (R Core Team, 2015). Unpaired Wilcoxon–Mann–Whitney U-test was performed. During evaluation of the results a confidence interval alpha of 95% and P-values lower than 0.05 were considered as statistically significant. Results are shown as box plots which give a fast and efficient overview of median, first and third quartile (25th and 75th percentile, respectively), interquartile range (IQR), minimal and maximal values and outliers.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.J., A.H. and K.K. conceived and designed the experiments. R.J. performed all experiments. D.L. assisted with immunofluorescence staining and K.K. with confocal microscopy. R.J. analysed the data and wrote the paper. A.H. and K.K. helped improving the manuscript. All authors read the final version of the manuscript and gave their permission for publication.

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Supplementary information

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