Biochimica et Biophysica Acta 1843 (2014) 2871–2877

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Impaired trafficking of the very low density lipoprotein receptor caused by missense mutations associated with dysequilibrium syndrome

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ARTICLE INFO

Article history:
Received 29 May 2014
Received in revised form 18 August 2014
Accepted 22 August 2014
Available online 27 August 2014

Keywords:
VLDLR
Dysequilibrium syndrome
ERAD
Protein misfolding
Reelin

ABSTRACT

Dysequilibrium syndrome (DES, OMIM 224050) is a genetically heterogeneous condition that combines autosomal recessive non-progressive cerebellar ataxia with mental retardation. The subclass dysequilibrium syndrome type 1 (CAMRQ1) has been attributed to mutations in the *VLDLR* gene encoding the very low density lipoprotein receptor (VLDLR). This receptor is involved in the Reelin signaling pathway that guides neuronal migration in the cerebral cortex and cerebellum. Three missense mutations (c.1459G>T; p.D487Y, c.1561G>C; p.D521H and c.2117G>T; p.C706F) have been previously identified in *VLDLR* gene in patients with DES. However, the functional implications of those mutations are not known and therefore we undertook detailed functional analysis to elucidate the cellular mechanisms underlying their pathogenicity. The mutations have been generated by site-directed mutagenesis and then expressed in cultured cell lines. Confocal microscopy and biochemical analysis have been employed to examine the subcellular localization and functional activities of the mutated proteins relative to wild type. Our results indicate that the three missense mutations lead to defective intracellular trafficking and ER retention of the mutant VLDLR protein. This trafficking impairment prevents the mutants from reaching the plasma membrane and binding exogenous Reelin, the initiating event in Reelin signaling. Collectively, our results provide evidence that ER quality control is involved in the functional inactivation and underlying pathogenicity of these DES-associated mutations in the VLDLR.

1. Introduction

Dysequilibrium syndrome (DES, OMIM 224050) is a rare autosomal recessive non-progressive cerebellar disorder characterized by ataxia, mental retardation, cerebellar hypoplasia and delayed ambulation and in some patients with strabismus, seizures and short stature [1]. DES is inherited as an autosomal recessive trait and a subgroup of this disorder has been associated with mutations in the gene encoding the very low density lipoprotein receptor (VLDLR) and therefore recognized as VLDLR-associated DES [2–8]. VLDLR is a multi-ligand apolipoprotein E (ApoE) receptor and an integral part of the Reelin signaling pathway that directs the proper positioning of migrating neuroblasts in the cerebral cortex and cerebellum during embryonic brain development [9]. Reelin is a glycoprotein secreted by the Cajal–Retzius cells and cerebellar granule cells during cortical plate development, which directly binds to the extracellular domains of the VLDLR and the related low density lipoprotein (LDL) receptor ApoER2 (LRP8) on the cell surface, triggering a signaling cascade involving the intracellular adaptor protein Dab1, that ultimately results in the cortical layer formation in the developing brain [10,11]. VLDLR and ApoER2 mediate canonical Reelin signaling and deletion of the genes encoding both these receptors in mice results in inversion of cortical layers and absence of cerebellar foliation [12]. Consistent with the findings in animal models with *VLDLR* homologue knock-outs, a small compact cerebellum and absence of foliation have been observed in MRI images of brains of patients with VLDLR mutations associated with DES [2,4,13,14].

Structurally, VLDLR is a type I transmembrane receptor belonging to the LDL receptor family. Like the other members of this family, VLDLR has five highly conserved structural domains as shown in Fig. 1A. An extracellular N-terminal ligand binding domain with cysteine-rich repeats, six modules of amino acids containing YWTD motif which form the extracellular β-propeller domain, an epidermal growth factor (EGF) domain, an O-linked sugar domain, a single transmembrane sequence and a cytoplasmic region containing an NPxY motif [15]. VLDLR mRNA transcripts are highly abundant in the brain and fatty

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http://dx.doi.org/10.1016/j.bbamcr.2014.08.013
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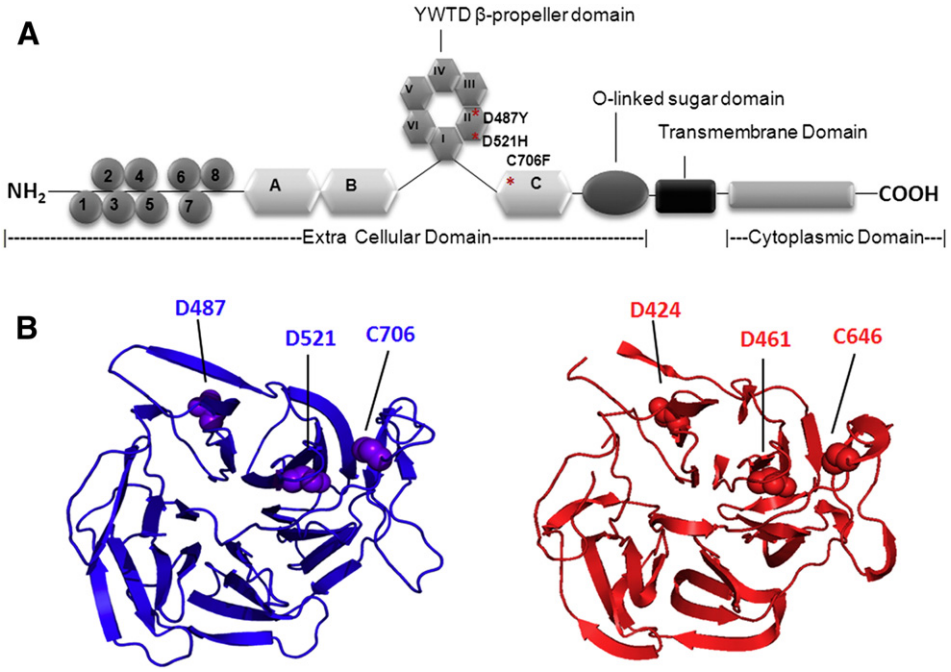

Fig. 1. Schematic domain structure of VLDL receptor: (A) The VLDLR extracellular domain contains eight cysteine rich class A repeats (1–8), three EGF-like repeats (A–C), six YWTD modules (I–VI) forming a β-propeller domain and an O-linked sugar domain. The positions of disease causing mutations are indicated by asterisks. (B) Homology model of the VLDLR as predicted by the Phyre2 Protein Fold Recognition Server [44]. VLDLR protein was modeled in intensive mode. Residues 69–752 (78% of the submitted sequence) of the VLDLR were modeled with 100% confidence using the LDLR structure (PDB identifier 1N7D) as a structural template. Structural predictions for the propeller domain (residues 439–697) and subsequent EGF domain (residues 702–750) are shown on the left with the mutated residues as spheres. Homologous conserved residues in the LDLR structure are indicated on the right. Figure was prepared using PyMOL [45].

acid-active tissues (adipose tissues, skeletal muscle and heart) supporting its roles in neuronal signaling and fatty acid metabolism [16].

Several mutations in *VLDLR* associated with DES are null mutations involving either complete deletion of the gene or loss of functional alleles resulting from premature stop codons, a frameshift or missense mutations. Regardless of the lesion type, the consequence of VLDLR loss-of-function is indistinguishable at the neuroanatomical level [3,5,6,13] suggesting that the brain abnormality resulting from VLDLR deficiency is likely to be caused by impaired Reelin signaling. We have identified the first homozygous missense founder mutation (RefSeq NM_003383.3:c.2117G>T; NP_003374.3: p.C706F) in VLDLR to cause DES in two unrelated Omani families from UAE [13]. Two other pathogenic missense mutants P.D487Y [17] and p.D521H [5], also located in the highly conserved extracellular β-propellar domain of VLDLR, have been recently described (Fig. 1A). Intriguingly, these residues are also conserved in the closely related receptor, the LDLR. Moreover, structural analysis indicates their positioning in these receptors is similar, likely reflecting a conserved function (Fig. 1B). It has been proposed that the functional loss associated with mutations at these key residues is likely a result of misfolding, ER retention and subsequent degradation by the ER associated degradation pathways [5,13,17]. However, the exact cellular mechanisms underlying these pathogenic mutations have not been established and are therefore investigated in this study.

2. Materials and methods

2.1. Antibodies

The antibodies with their dilutions and sources were as follows: antibodies for immunofluorescence: mouse anti-HA-tag monoclonal antibody (1:200; Cell Signaling Technology), rabbit anti-calnexin polyclonal antibody (1:200, Santa Cruz), mouse anti-Reelin (1:200, Millipore clone G10), Alexa Fluor 568-goat anti-mouse IgG (1:200; Molecular Probes), Alexa Fluor 568-goat anti-rabbit IgG (1:200; Molecular Probes), Alexa Fluor 488-goat anti mouse IgG (1:200, Molecular Probes). Antibodies for Western blotting: rabbit anti-HA polyclonal antibody (1:4000, Sigma), goat anti-rabbit IgG-peroxidase (1:30,000; Sigma), rabbit anti-mouse IgG-peroxidase (1:30,000; Sigma).

2.2. Generation of mutant expression constructs

All the mutations described in this study are with reference to the coding VLDLR sequence represented by Genebank accession number NM_003383.3 and protein sequence represented by NP_003374.3. The missense mutations (p.D487Y, p.D521H, p.C706F) were introduced into the C-terminally tagged VLDLR-HA expression vector [18] by site directed mutagenesis using *Pfu* Ultra HF polymerase (Stratagene). The primers used to introduce the mutations were as follows: VLDLR- D487-F: CAGA AACTATTCTGGGCC**TAC**CTAAGCCAAAAGGCTATC; VLDLR-D487Y-R: GATAGCCTTTTGGCTTAGGTAGGCCCAGAATAGTTTCTG; VLDLR-D521H-F: CTGCAGCCATTGCTGTT**CAC**TGGGTGTACAAGACCATC; VLDLR-D521H-R: GATGGTCTTGTACACCCA**GTG**AACAGCAATGGCTGCAG; VLDLR-C706F-F: CCATCAGGTAAAAATTGGT**TC**GAAGAAGACATGGAGAATG; and VLDLR-C706F-R: CATTCTCCATGTCTTCTTC**GA**ACCAATTTTTACCTGATGG. All the plasmids have been sequenced to confirm the introduction of intended changes. Sequencing was performed using the dideoxy Sanger method by fluorescent automated sequencing on an ABI 3130xl genetic analyzer (Applied Biosystems).

2.3. Cell culture and transfection

HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin at 37 °C with 5% CO2. For localization experiments, cells were grown on sterile cover slips in 24-well tissue culture plates and transient transfection was performed by FuGENE HD transfection reagent (Promega) according to the manufacturer's instructions using 0.5 μg plasmid DNA. GFP-H-Ras plasmid [19] was used as a plasma membrane marker and co-transfected with HA-tagged wild type [18]

or mutant plasmids. The cells were processed for staining and imaging after 24 h of transfection.

Human embryonic kidney cells (HEK-293T, ATCC) were cultured in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (10 U/ml) and streptomycin (100 μg/ml) at 37 °C with 5% CO2. For transfection, cells were grown in 6 well tissue culture plates and transfected with 1 μg plasmid DNA using FuGENE HD transfection reagent.

Reelin-conditioned medium was prepared as previously reported [18]. Briefly, HEK-293T cells stably expressing Reelin (a kind gift from Dr. Thomas Curran, Children's Hospital of Philadelphia) [20] were grown to ~75% confluence, washed twice with phosphate-buffered saline and cultured in Opti-MEM medium (Invitrogen) for an additional 24 h. Conditioned medium was collected, filtered, and stored at −80 °C.

2.4. Immunocytochemistry and imaging

Twenty-four hours after transfection, HeLa cells grown on cover slips were washed with phosphate-buffered saline (PBS), fixed by methanol at −20 °C for 5 min. Fixed cells were washed with PBS three times and blocking was carried out in 1% BSA (Sigma) in PBS for 30 min at room temperature. After blocking, the cells were incubated with either mouse monoclonal anti-HA antibody alone or co-stained with both mouse monoclonal anti-HA antibody and rabbit polyclonal anti-calnexin antibodies, for 45 min at room temperature. The cells were washed with PBS, and incubated with the respective secondary antibodies for 45 min at room temperature, washed several times with PBS and mounted in immunofluor medium (ICN Biomedicals). Confocal microscopy and imaging was performed with a Nikon Eclipse system (Nikon Instruments Inc) equipped with FITC and TRITC filters. Images were obtained with 100× oil immersion objective lens. All images presented are single sections in the z-plane. Images were color enhanced and merged using ImageJ software [21].

2.5. Immunoprecipitation and Western blotting analysis

Forty eight hours after transfection, HEK-293T cells were lysed in CelLytic-M lysis reagent (Sigma) containing protease inhibitors (Sigmafast protease inhibitor cocktail, Sigma) according to the manufacturer's instructions. Total protein concentration was determined by Bicinchoninic Acid protein Assay (BCA kit, Pierce). HA tagged proteins were immunoprecipitated using anti-HA agarose beads (Pierce). Briefly, equal amounts of total cell lysates were incubated with anti-HA agarose beads for 2 h at 4 °C with rotation. Immunoprecipitates were collected by centrifugation and washed thrice with lysis buffer. For Western blotting, the proteins were eluted from the beads by boiling in Laemmli sample buffer. The samples were then resolved on 7.5% SDS-PAGE gel followed by blotting onto nitrocellulose membranes (Whatman Protran) and probed with respective antibodies. Detection was performed using Enhanced Chemiluminescence Plus reagent (Pierce) and Typhoon FLA 9500 Imager (GE Healthcare Biosciences).

2.6. Endoglycosidase H sensitivity assay

For Endoglycosidase H deglycosylation assay, the immunoprecipitates were denatured in 1× glycoprotein denaturation buffer (0.5% SDS and 1% β-mercaptoethanol) for 5 min at 100 °C. The denatured proteins were then divided into two equal aliquots, which were incubated for 4 h at 37 °C in the presence or absence of 10 U of Endoglycosidase H (Sigma). The digested samples were then resolved on a 7.5% SDS-PAGE gel and analyzed by Western blotting as described above.

2.7. Reelin binding assay

Reelin binding assay was performed as described in [18]. Briefly, HEK-293T cells were transfected with the different VLDLR expression plasmids. Subsequently, 48 h post-transfection, cells were washed thrice with Opti-MEM (Invitrogen) and incubated with Reelin-containing conditioned media on ice. Binding was allowed to proceed for 30 min after which cells were vigorously washed five times with PBS. Preparation of cell lysates and immunodetection was done as detailed above.

3. Results

3.1. The three missense mutations affect the trafficking and cell surface expression of VLDLR

To investigate the impact of the mutations at the cellular level, we generated by site directed mutagenesis the three missense mutations (P.D487Y, p.D521H and p.C706F) found in DES patients. To determine the subcellular localization of the mutant receptors, the constructs were co-expressed in HeLa cells along with the plasma membrane indicator, H-Ras tagged with EGFP [19]. The wild type HA-tagged VLDLR localized largely to the plasma membrane as confirmed by its co-localization with GFP-H-Ras (Fig. 2, panels A–C). The intracellular localization patterns of the three DES-associated VLDLR mutants were distinct from that of the wild type and showed a more cytoplasmic, reticular and perinuclear distribution, which is characteristic for ER localized proteins (Fig. 2, panels D–L). To confirm the ER localization of the mutants, HeLa cells over-expressing wild type or mutants were co-stained with the ER marker, calnexin. As apparent from panels A–C in Fig. 3, the localization pattern of the wild type receptor was distinct from the staining of calnexin. A small fraction of the wild type protein was also found to co-localize with calnexin, which likely reflects nascent synthesized protein in transit. All the three mutants showed co-localization with calnexin (Fig. 3, panels D–L).

3.2. Altered glycosylation status confirms ER retention of VLDLR mutants

The mature form of type-1 VLDLR contains two types of carbohydrates, N-linked and O-linked sugars. Hence type 1 VLDLR is detected as two bands in immunoblots, a faster migrating precursor form and a slower migrating fully glycosylated mature form [22]. In total cell lysates overexpressing the wild type VLDLR, a precursor form of apparent molecular weight 130 kDa and a mature form of apparent molecular weight 150 kDa were observed (Fig. 4). In cell lysates overexpressing the mutants the mature receptor form was absent and only the precursor form was observed. To analyze the glycosylation status of the mutant and wild type VLDLR, immunoprecipitated wild type and mutant proteins were subjected to Endoglycosidase H digestion (Endo H), which specifically removes oligosaccharides of the high mannose and hybrid (pre-Golgi) forms, but not complex carbohydrate structures attained in the Golgi. Figure 4 shows that Endo H treatment reduced the molecular mass of the mutant VLDLRs, as well as of the precursor form of the wild type receptor. The higher molecular weight form observed in the wild type VLDLR was however resistant to EndoH treatment, consistent with it representing advanced glycosylation status attained in the Golgi. Taken together with the localization studies, this result establishes that the mutant receptors are retained in the ER, explaining also the absence of complex Golgi-dependent glycosylation.

3.3. Mutant forms of VLDLR fail to bind Reelin

VLDLR is one of the receptors for Reelin, an extracellular matrix protein that directs the organization of cortical structures in the developing cerebral cortex and cerebellum. Binding of Reelin to the lipoprotein receptors on the cell surface is a prerequisite for triggering the canonical Reelin signaling pathway in migrating neurons. Therefore, ER retention and subsequent degradation of VLDLR is likely to disrupt Reelin signaling mediated by VLDLR. To test this possibility we incubated HEK-293T cells expressing wild type or mutant VLDLR with Reelin-containing

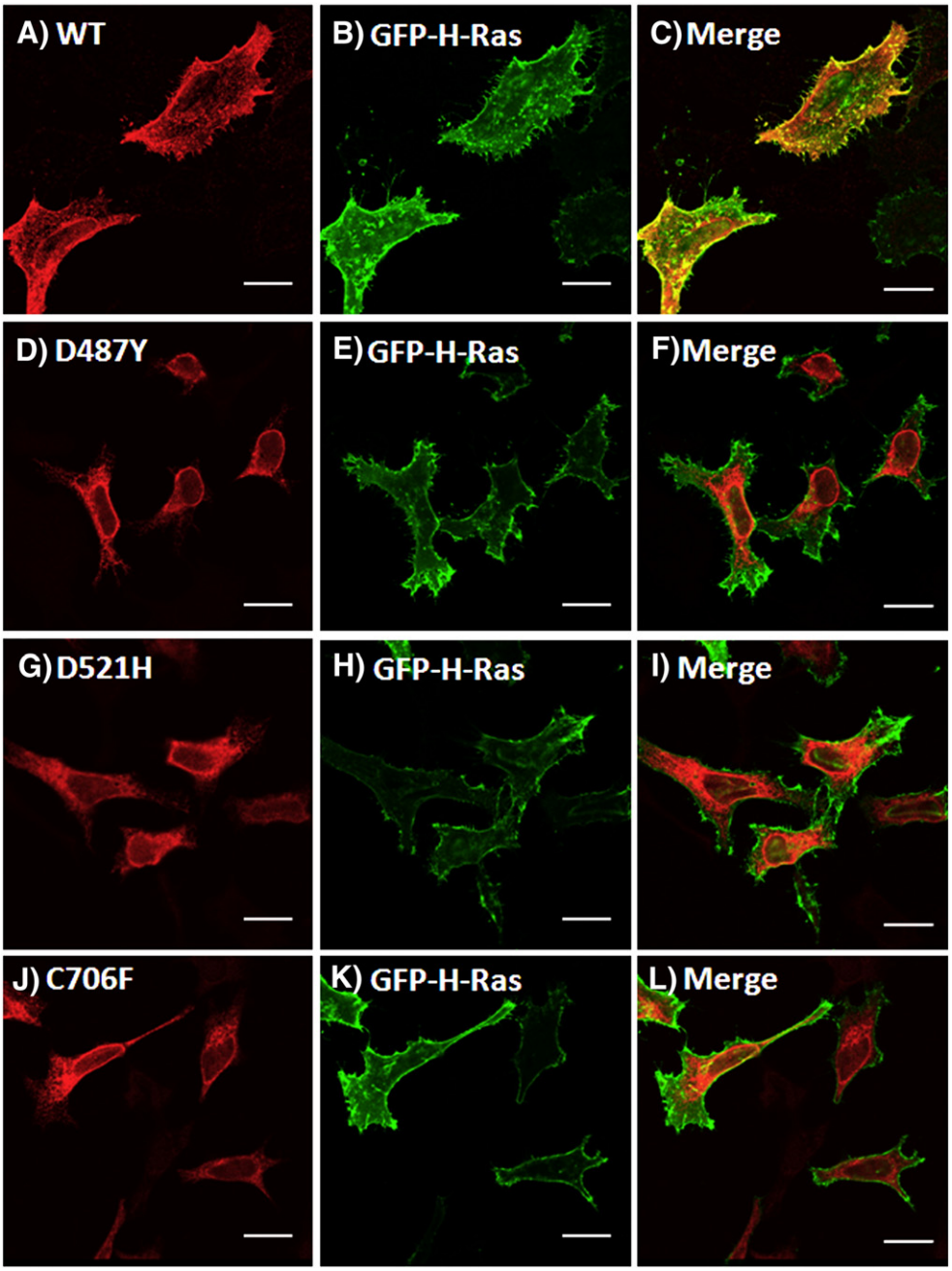

Fig. 2. Comparison of intracellular localization of VLDLR wild type and mutant variants in relation with plasma membrane marker H-Ras: HeLa cells were transiently co-transfected with the indicated HA-tagged VLDLR plasmids and EGFP tagged H-Ras and stained with anti-HA antibodies. (A), (D), (G) and (J) show the distribution of over-expressed HA-tagged VLDLR proteins. (B), (E), (H) and (K) show the localization pattern of GFP-H-Ras, which is on the plasma membrane. (C), (F), (I) and (L) are the composite images indicating the extent of co-localization of VLDLR proteins with GFP-HRas. Scale bar is 20 μM.

medium and the extent of cellular Reelin binding was analyzed by immunoblotting, as previously reported [18]. Consistent with our previous finding, Fig. 5 shows that only the Endo H resistant mature form of VLDLR found in the wild type reaches the plasma membrane and is able to bind Reelin. In agreement with their subcellular localization, the ER retained forms of VLDLR failed to bind extracellular Reelin (Fig. 5), suggesting that all the three missense mutations result in functionally inactive receptors in vivo.

4. Discussion

In this report we have analyzed the cellular consequences of three, recently identified [5,13,17], missense mutations in the *VLDLR* gene associated with DES. We provide evidence that these mutations lead to a dysfunctional receptor due to ER retention of the mutant receptors that precludes it from reaching the plasma membrane and binding Reelin.

The disease-causing mutations p.D487Y and p.D521H affect amino acid residues in the conserved β-propeller domain (Fig. 1A, B). The crystal structure of the closely related receptor, LDLR revealed that in the consensus repeat motifs (Tyr-Trp-Thr-Asp), the Asp residues serve as clasps between adjacent blades of the β-propeller, stabilizing the structure by hydrogen-bonding with the backbone and Trp side chains [23]. Since both the carboxylated oxygens of Asp residues are acceptors of structurally conserved hydrogen bonds, the pathogenic mutations will probably disrupt the structure and subsequently, trafficking of the

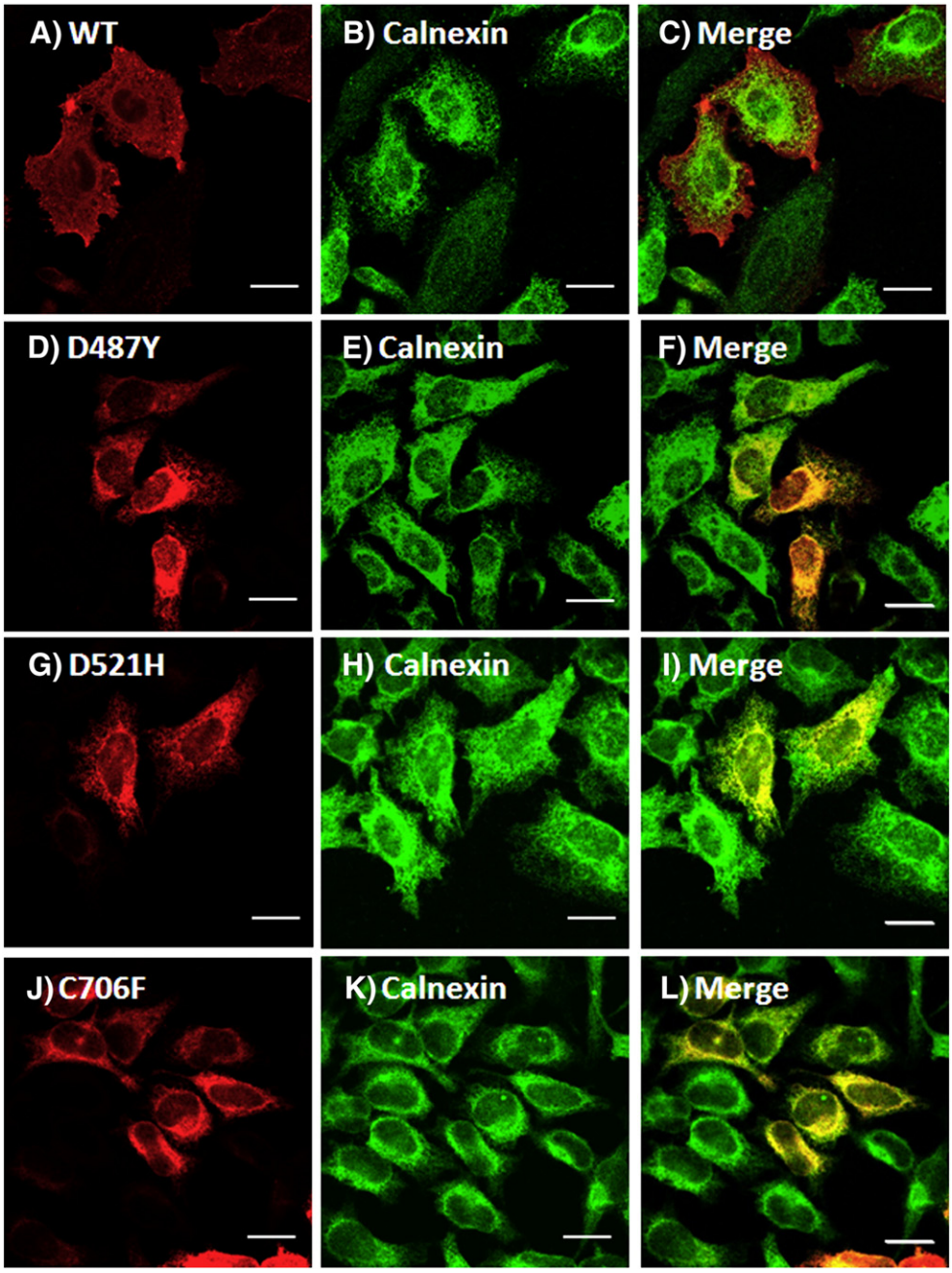

Fig. 3. Comparison of intracellular localization of VLDLR wild type and mutant variants in relation with the ER marker calnexin: HeLa cells transiently transfected with the indicated HA-tagged VLDLR constructs were fixed and co-stained with antibodies against HA-tag and calnexin. (A), (D), (G) and (J) show the intracellular distribution of over-expressed HA-tagged VLDLR proteins. (B), (E), (H) and (K) show the intracellular localization of the ER resident protein calnexin. (C), (F), (I) and (L) are the merged images showing the extent of co-localization of VLDLR protein with calnexin. For presentation purpose images A, D, G and J were pseudocolored as red and images B, E, H and K were pseudocolored as green using image J software. Scale bar is 20 μM.

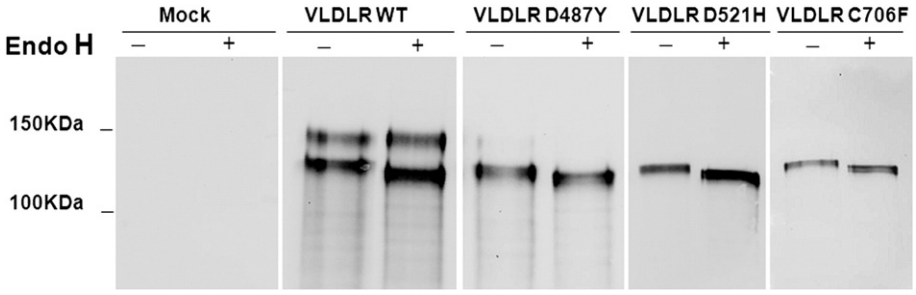

Fig. 4. Endoglycosidase H (EndoH) susceptibility of the wild-type VLDLR and its mutants: HA-tagged wild type VLDLR or mutant variants were transiently expressed in HEK-293T cells. HA-tagged proteins were immunoprecipitated, treated with Endoglycosidase H for 4 h at 37 °C (+) or left untreated for 4 h at 37 °C (−) and analyzed by immunoblotting with anti-HA antibody. The mature form of the receptor was detectible only in the immunoprecipitates from the wild type. ER forms of the wild type as well as the mutants were sensitive to EndoH treatment.

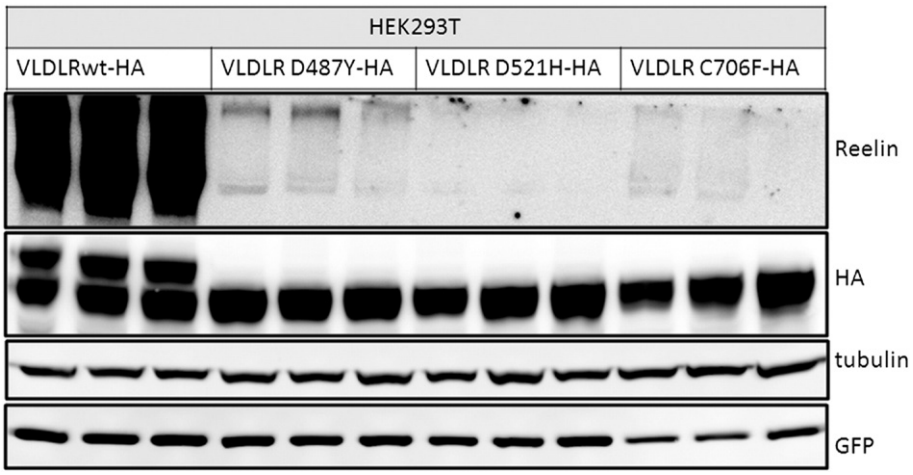

Fig. 5. Defective cellular trafficking of the VLDLR disrupts Reelin binding: HEK-293T cells were transfected with the indicated HA-tagged VLDLR expression plasmids. A GFP-encoding expression plasmid was co-transfected and served to control transfection efficiency. Subsequently, cells were incubated with Reelin-containing conditioned medium for 30 min on ice followed by extensive washing. Total cell lysates were subjected to SDS-PAGE and immunoblotted as indicated.

protein. Similarly, the p.C706F mutation is located in the extracellular EGF-like 3 domain of the VLDLR protein. The cysteine residue at position 706 is highly conserved and is predicted to be involved in a disulfide bond with cysteine 719. Therefore, disruption of this residue is likely to result in misfolding of the protein. These three residues (D487, D521, C706) are also conserved in the LDLR (D424, D461, C646), and homology modeling indicates that their positioning in the VLDLR and LDLR is highly similar (Fig. 1B). Intriguingly, mutations occurring in the corresponding positions in LDLR have been reported to cause familial hypercholesterolemia (FH) [24,25]. We show here that all the pathogenic amino acid substitutions reported in VLDLR indeed result in the retention of the mutant variants in the ER and impaired transport to the cell surface, likely due to misfolding. In contrast, the wild type protein localized predominantly to the plasma membrane, with only a smaller fraction observed in the ER. Biochemical analysis of the mutants and wild type protein confirmed their intracellular distribution. The ER has a quality control system to ensure that only correctly folded proteins reach their final destination. Incompletely folded or misfolded proteins are retained in the ER, eventually translocated to the cytoplasm and degraded by the ubiquitin proteasome pathway, a process collectively termed as ER-associated degradation (ERAD) [26]. Alternatively, prolonged retention of misfolded proteins in the ER can induce ER stress and initiate macroautophagy, via an ER-activated autophagy (ERAA) pathway [27]. Retention of misfolded proteins in ER and subsequent degradation by ERAD has been implicated in the pathogenicity of many congenital disorders [28–31]. Mutations that generate transport deficient variants of LDLR, account for ~50% of the pathogenic variants causing FH, and proteasomal degradation has been reported to be the principal pathway for the degradation of many of LDLR mutants [32].

Functionally, VLDLR deficiency at the cell surface could translate to impaired binding of the receptor to its corresponding ligands in specific cell types. VLDLR appears to play a crucial role in triglyceride metabolism [33] and is an integral part of the Reelin signaling pathway which guides neuronal migration in the cerebral cortex and cerebellum [34]. Reelin-Dab1 signaling has been found to regulate changes in Golgi morphology in neurons that drive neuronal polarization and brain lamination [35]. Reelin signaling is regulated through internalization and rapid uncoupling of the ligand from the VLDL receptor, whereupon the ligand is targeted for lysosomal degradation and the receptor is recycled to the cell membrane [36]. The neurodevelopmental phenotype in DES has been attributed to defects in Reelin signaling since mutations in the other components of this pathway have also been reported to produce identical phenotypes to VLDLR receptor deficiency [5,37]. Our results from in vivo Reelin binding assay indicated that, ER retention of the mutant variants of the VLDLR indeed lead to impaired binding to Reelin.

Recently it has been reported that Reelin also mediates signals via a non-canonical pathway in the post-natal brain which does not involve the VLDLR/ApoER2 receptors [38]. A few reports suggest that processes related to neuronal migration including maturation and secretion of Reelin are sensitive to purtubations in the ER quality control (ERQC) machinery, since mutations affecting the integral components of the ERQC has been found to result in cortical dysplasia in animals [39,40]. Chronic ER stress due to persistent accumulation of misfolded proteins has also been linked to the pathogenesis of several neurodegenerative diseases [41]. A recent study indicates that VLDLR itself may be an important component of the ER stress responsive pathway since increased expression of VLDLR transcripts has been observed in response to ER stress [42] and also conditions that promote ER stress such as hypoxia [43].

In conclusion, our results indicate that ER retention is involved in the pathogenic mechanism leading to DES by the *VLDLR* missense mutations studied here. The possibility of restoring the functionality of mutant proteins that are entrapped in the ER due to folding disabilities is being explored as a therapeutic approach for several human genetic diseases, and could be potentially implemented for treatment of DES.

Acknowledgments

We are grateful to Mr. Saeed Tariq, Confocal facility, College of Medicine and Health Sciences, UAEU for helping with confocal microscopy imaging.

Funding

This work was supported by the United Arab Emirates University grants (grant nos. 31M092 and NP/14/08) to BRA. NZ is supported by a VIDI grant (grant no.17.106.355) from the Netherlands Organisation of Scientific Research (NWO), and by an ERC Consolidator grant (grant no. 617376) from the European Research Council.

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