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Protein 4.1N is required for the formation of the lateral membrane domain in human bronchial epithelial cells

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Abstract

The membrane skeleton forms a scaffold on the cytoplasmic side of the plasma membrane. The erythrocyte membrane represents an archetype of such structural organization. It has been documented that a similar membrane skeleton also exits in the Golgi complex. It has been previously shown that βII spectrin and ankyrin G are localized at the lateral membrane of human bronchial epithelial cells. Here we show that protein 4.1N is also located at the lateral membrane where it associates E-cadherin, β-catenin and βII spectrin. Importantly, depletion of 4.1N by RNAi in human bronchial epithelial cells resulted in decreased height of lateral membrane, which was reversed following re-expression of mouse 4.1N. Furthermore, although the initial phase of lateral membrane biogenesis proceeded normally in 4.1N-depleted cells, the final height of the lateral membrane of 4.1N-depleted cells was shorter compared to that of control cells. Our findings together with previous findings imply that 4.1N, βII spectrin and ankyrin G are structural components of the lateral membrane skeleton and that this skeleton plays an essential role in the assembly of a fully functional lateral membrane.

Graphical Abstract

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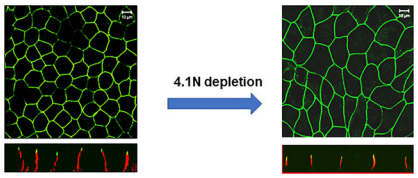
Author Contributions

Y.W, H.Z, Q.K, J.L, H.W, W.L performed research and analyzed the data. N.M. analyzed the data and edited the paper. L.C designed experiments and analyzed the data. X.A designed experiments, analyzed the data and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.



Keywords

Protein 4.1N; human bronchial epithelial cells; lateral membrane

INTRODUCTION

Epithelial cells are polarized cells with three structurally and functionally distinct membrane domains: apical, basal and lateral domains. The formation and maintenance of specialized plasma membrane domains are essential for the physiological function of epithelial cells. Many studies have focused on the role of intracellular sorting in the establishment of epithelial cell polarity. More recent studies have demonstrated that isoforms of cytoskeletal proteins, spectrin and ankyrin are localized at the lateral membrane of epithelial cells and they collaborate in the biogenesis of lateral membrane of human bronchial epithelial cells[1,2]. These findings have led to the conjecture that lateral membranes of epithelial cells have a spectrin-based membrane skeleton similar to that of erythrocytes. In addition to spectrin and ankyrin, another major component of the spectrin-based membrane skeleton in erythrocyte is protein 4.1R[3], the prototypic member of protein 4.1 family. Thus it is reasonable to expect that such a protein would also be present in conjunction with spectrin and ankyrin within the lateral membrane of epithelial cells. However, to date the association of protein 4.1 with lateral membrane has not been documented.

Protein 4.1 family consists of four paralogs: 4.1R[4], 4.1G[5], 4.1N[6], and 4.1B[7]. They are encoded by distinct genes. Due to extensive alternative splicing[8], an enormous variety of possible splice variants of 4.1 family of proteins can be generated. In erythrocytes, an 80 kDa 4.1R serves as a core for the assembly of both transmembrane proteins and spectrin-based membrane skeleton into a macromolecular complex[9]. In non-erythroid cells, 4.1 isoforms are found in a variety of subcellular compartments, including the nucleus[10], the centrosome[11], tight junctions[12], adherens junctions[13] and immunological synapse[14], implying a diverse function for members of protein 4.1 family.

Members of protein 4.1 have been shown to be expressed in the epithelial cells of mouse kidney[15]. We have previously shown that all protein 4.1 members are expressed in the cultured epithelial cell lines MDCK and HBE (human bronchial epithelial cells)[16]. Interestingly, they exhibited distinct sub-cellular localization. For example, a 200 kDa 4.1B was found in Golgi and is required for both structural integrity of the Golgi apparatus and for targeting of a subset of membrane proteins[16]. In contrast, 4.1N was found at the plasma membrane of HBE cells but the function of which has yet to be defined. Using non-small cell cancer cell lines, our group has recently documented the role of 4.1N as a potential tumor suppressor by mediating signal transduction pathways [17,18]. In the present study, using HBE cell line in conjunction with RNAi, we show for the first time that 4.1N is

a component of spectrin-based membrane skeleton within the lateral membrane and it is required for the assembly of a fully functional lateral membrane of HBE cells. Together, our findings imply the diverse functions for 4.1N in various cell types.

MATERIALS AND METHODS

Antibodies.

Mouse monoclonal antibodies against ZO-1 and E-cadherin were purchased from Invitrogen. Rabbit polyclonal antibodies to EBP50, syntaxin3 and beta-catenin were from ABCAM. Mouse monoclonal antibodies against β-tubulin and goat polyclonal antibody against beta-catenin were from Santa Cruz Biotechnology. Mouse monoclonal antibody to GAPDH was from IMGENEX. Rat anti-E-cadherin was from Zymed. Alexa 488 conjugated donkey anti-mouse IgG, Alexa 488 conjugated donkey anti-rabbit IgG and Alexa488 conjugated donkey anti rat IgG; Alexa 594 conjugated donkey anti-rabbit IgG, Alexa 594 conjugated donkey anti-rat IgG, Alexa 594 conjugated donkey anti-mouse IgG and zenon tricolor mouse IgG and Rabbit IgG Labeling kit were from Invitrogen. The affinity-purified anti-βII spectrin antibody was a gift of Dr. Vann Bennett (Duke University). All anti-protein 4.1 antibodies were generated in our laboratory and described in our previously published studies[16].

Cell culture.

Human bronchial epithelial cell line (HBE) was a gift of Dr. Vann Bennett. These HBE cells have been previously used to study the membrane biogenesis of epithelial cells[19–23]. The cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco) at 37°C in 5% CO_2. HBE cells for confocal immunofluorescence microscopy assay were grown on MatTek uncoated glass bottom micro-well cell culture dishes (MatTek).

Construction of shRNA for 4.1N knockdown.

The pENTR™/H1/TO plasmid was from Invitrogen. 19-ntRNA target regions chosen in human 4.1N and mouse 4.1N were 5'-GCAACATCACTCGAAATAA-3'(NM_012156) and 5'-GCAACATCACTCTAGATAT-3' (NM_001006664.2) respectively. The targeting sequence was cloned into the pENTR™/H1/TO plasmid according to the manufacturer's instructions. The constructs were designated as pENTR-h4.1N and pENTR-m4.1N for human and mouse sequences respectively.

Generation of stable cell lines.

One day before transfection, 5×10^5 HBE cells in 2000 μl of growth medium were plated into a six-well plate. 24 hours later, the cells were transfected with pENTR-h4.1N or pENTR-m4.1N with Lipofectamine 2000 (Invitrogen). Four to six hours after transfection, the medium was removed and replaced with fresh growth medium and cultures maintained overnight at 37°C. The cells were trypsinized and re-plated into six-well plate in fresh complete medium containing 800 μg/ml of Zeocin™ (Invitrogen) for selection. The selection was carried out for 10–14 days. Ten Zeocin™-resistant colonies were picked and expanded as stable cell lines. Stable cell lines were kept in medium containing 200μg/ml of Zeocin™.

Immunofluorescence microscopy.

Cells were fixed using 1% paraformaldehyde for 15 min. The cells were then permeabilized with 0.1% TritonX-100 in 0.25% paraformaldehyde/PBS. Cells were subsequently incubated in 10% horse serum, 0.1% Triton X-100/PBS for 30 min to minimize nonspecific antibody binding. The cells were incubated overnight with primary antibodies at 4 °C or room temperature for 2 hr, then washed four times with PBS and incubated with the appropriate second antibody at room temperature for 30 min. The titer of primary antibodies were as follows: anti-4.1NHP (0.5 μg/ml), anti-EBP50 (0.5 μg/ml), anti-syntaxin3 (0.5 μg/ml), anti-βII spectrin (0.5 μg/ml); mouse anti-E-cadherin (2 μg/ml), anti-β-tubulin (1 μg/ml), and ZO-1 (2 μg/ml); anti-β-catenin (2 μg/ml); rat anti-E-cadherin (2 μg/ml). The titer of secondary antibodies was 1/700. Images were collected on a ZeissLSM510 META confocal microscope using either a 63x oil objective or a 100x oil objective. Z stacks were collected at Z increments of 0.2 μm. XZ images were obtained from Z stacks using the orthogonal function of the LSM510 software. The cross-section area (surface area) of apical membrane was derived from XY images and both the cross-section area and the heights of lateral membrane were measured using LSM510 ZEN2009 software. The data were analyzed using Student's t tests.

Immunoblot Analysis.

Cultured cells were trypsinized and washed with PBS. The cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl pH 8.3, 420 mM KCl, 0.1% NP-40, 1 mM EDTA). After incubation for 15 min on ice, the samples were centrifuged at 16,000 × g at 4°C for 10 min and the supernatants were collected. Protein concentration was measured with Bradford method using BSA as standard. 20 μg of protein was loaded to 10% SDS-PAGE gels and the proteins were subsequently transferred onto nitrocellulose membrane. The membrane was blocked in TBS with 1 % BSA/5 % milk powder. Membranes were probed overnight with primary antibodies, and detected with the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) followed by exposure to SuperSignal WestPico Chemiluminescent substrate (Pierce).

Co-Immunoprecipitation.

Confluent monolayers of HBE cells (100-mm plates) were washed once in phosphate-buffered saline and lysed at 4°C in RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1%Triton×100, 1% Na deoxycholate, 0.1% SDS, 1mM PMSF,0.1μg/ml DTT, 0.5μg/ml protease inhibitor cocktail) for 15 min on ice. The lysate was spun for 10 min at 16,000 × g at 4°C, and the supernatant was cleared by treatment with normal rabbit serum (50 μl/ml lysate) and Protein A Sepharose beads for 30 min on ice. Supernatant was collected after centrifugation at 16,000g at 4°C for 10 min. The concentration of protein in the supernatant was determined by the Bradford method using BSA as standard (BioRad). 500μg of extract was incubated with 5 μg of anti-4.1N-HP antibody or pre-immune IgG in 500 μl of Co-IP buffer (Active motif) at 4°C overnight with rotation. The immunoprecipitates were isolated on Protein A beads and separated by 10% SDS-PAGE followed by transfer to nitrocellulose membrane. The membrane was probed with antibodies against 4.1N or βII-spectrin.

Expression and purification of recombinant proteins.

The His-tagged recombinant mouse full length 4.1N cDNA was kindly provided by Dr. Phillipe Gascard (University of California at San Francisco). The GST-tagged βIIN-terminus (amino acids 1–313), βII repeats 1–4 (amino acids 284–744) cDNAs were described previously[24]. The cDNA encoding the above polypeptides was transformed into the BL21 bacterial strain. The expression of recombinant proteins was induced by 0.1mM IPTG at 16°C overnight. The GST-tagged polypeptides were purified on a glutathione-Sepharose 4B affinity column, and His-tagged proteins on a nickel column. Protein concentrations were determined by Bradford method, using BSA as standard. All peptides were clarified by ultracentrifugation at 230,000xg for 30 min at 4°C before use.

In vitro GST pull down assay.

To test whether 4.1N directly binds to GST, GST-tagged βIIR1–4 or βIIN, these proteins was coupled to glutathione-Sepharose 4B beads at room temperature for 30 min, followed by pelleting and washing with buffer. His-tagged 4.1N was added to the coupled beads in a final volume of 100 μl at a protein concentration of 1 μM, then incubated for 1 hr at room temperature, pelleted, washed and eluted with 10% SDS. The supernatant was analyzed by SDS-PAGE and transferred to nitrocellulose membrane. The binding of His-tagged 4.1N to spectrin fragments was detected by anti-His antibody.

Construction and transient transfection of GFP or GFP-m4.1N into 4.1N depleted-HBE cells.

The GFP-tagged m4.1N was generated by sub-cloning the full length 4.1N into pEGFP-C3 vector (Clontech) using restriction enzymes EcoRI and SalI upstream and downstream respectively. The fidelity of the construct was confirmed by sequencing. 4.1N depleted-HBE cells were trypsinized, and 2×10^5 cells were plated into six-well cell culture plates and cultured for 24 hrs. Cells were then transfected with GFP or GFP-m4.1N using lipofactamine-2000. 48 hrs after transfection, the cells were processed for staining and immunofluorescence microscopy as described above.

RESULTS

4.1N is associated with the lateral membrane of human bronchial epithelial cells.

We have previously shown that six variants of protein 4.1 are expressed in cultured MDCK and HBE cells. These include ~80kDa 4.1R, ~110 kDa 4.1R, ~70 kDa 4.1G, ~200 kDa 4.1B, ~60 kDa 4.1B, and ~105 kDa 4.1N. Of these six 4.1 variants, only the ~60 kDa 4.1B, and the ~105 kDa 4.1N were shown to be associated with the plasma membrane[16]. To identify the lateral membrane associated protein 4.1N, we performed immunofluorescence image analysis. Fig 1A showed the representative image of double staining of 4.1N with ZO-1, a tight junction marker. XY images revealed that both 4.1N and ZO-1 were localized at the membrane but they did not co-localize. XZ images further revealed that while ZO-1 was located at the tight junction, 4.1N was located on lateral membrane. The lateral membrane localization of 4.1N was further confirmed by its co-localization with E-cadherin and β-catenin (Fig 1B and 1C), both of which are hallmarks of the lateral membrane. In contrast,

although the ~60 kDa 4.1B recognized by anti 4.1B U2 antibody also appeared on the plasma membrane, it did not co-localize with E-cadherin (supplementary Fig1). These findings have enabled us to identify 4.1N as a component of lateral membrane in HBE cells.

Association of 4.1N with βII-spectrin at the lateral membrane of HBE cells.

It has been previously shown that in HBE cells, βII-spectrin is located at the lateral membrane and it collaborates with ankyrin G in biogenesis of the lateral membrane[2]. To confirm 4.1N is a component of spectrin-based membrane skeleton within the lateral membrane, we examined the association of 4.1N with βII-spectrin in HBE cells. For this, we first performed double staining of 4.1N and βII-spectrin. As shown in Fig 2A, 4.1N was localized at the lateral membrane where it co-localized with βII-spectrin. We also performed co-immunoprecipitation experiments with anti-4.1N antibody. As shown in Fig 2B, the anti-4.1N antibody pulled down both βII-spectrin and 4.1N. To further confirm the direct binding between 4.1N and βII-spectrin, we performed an in vitro GST-pull down assay. In red cells, 4.1R binds to the N-terminal region of βI-spectrin containing the two calponin homology domains [25]. Fig 2C showed that similar to the binding between 4.1R and βI-spectrin, 4.1N bound to the N-terminal domain (amino acids 1–313) of βII specrtin but not to the adjacent repeats 1–4 of βII-spectrin(amino acids 284–744). These results imply that 4.1N participates in the formation of spectrin-based membrane skeleton within the lateral membrane of HBE cells through its direct binding to a specfici domian of βII-spectrin.

Specific depletion of 4.1N in HBE cells.

We utilized siRNA methodology to explore the function of 4.1N. pENTR™/H1/TO vector with a specific small interfering RNA was used to drive down the expression of human 4.1N. The 19-nucleotide target sequences from human and mouse are shown in Fig 3A and they differed at three positions which rendered the mouse sequence ineffective in knocking down human 4.1N[26]. The mouse sequence was used as an experimental control. The plasmids directing the expression of siRNA of human and mouse 4.1N were designated pENTR-h4.1N and pENTR-m4.1N respectively. As shown in Fig 3B while pENTR-h4.1N significantly decreased the expression of 4.1N in HBE cells, pENTR-m4.1N was ineffective. Importantly, as shown in Fig 3C while the expression of 4.1N was substantially reduced following transfection with pENTR-h4.1N, the expression levels of other 4 members of protein 4.1 family (4.1B, 4.1R and 4.1G) remained unchanged.

Decreased height of lateral membrane in 4.1N-depleted HBE cells.

It has been previously shown that depletion of either ankyrin G or βII-spectrin resulted in loss of lateral membrane[1,2]. These findings led to the notion that spectrin-based membrane skeleton play critical roles in the biogenesis or stability of lateral membrane. Having documented that 4.1N is a component of spectrin-based membrane skeleton within the lateral membrane of HBE cells, we hypothesized that 4.1N might play a role similar to that of ankyrin G and βII-spectrin. To test this thesis, we examined the lateral membrane of HBE cells by staining with three specific lateral membrane markers: βII-spectrin, β-catenin and E-cadherin. Representative images of XY or XZ sections of control HBE cells and cells transfected with siRNAs and stained with βII-spectrin or ZO-1 are shown in Fig 4A and 4B respectively. In contrast to control or pENTR-m4.1N transfected cells that express normal

levels of 4.1N, cells transfected with pENTR-h4.1N with markedly reduced expression of 4.1N exhibited significantly shortened lateral membrane height. Quantitative analyses showed that in control and pENTR-m4.1N transfected cells the average height of lateral membrane (from the tight junction marked by ZO-1 to the base of the cell in the XZ section) were 8.4±1.37μm and 8.5±1.42 μm, respectively (Fig 4C). In marked contrast, in pENTR-h4.1N transfected cells the average height was only 5.6±0.79 μm ($p<0.05$). The decreased lateral membrane height was also confirmed by staining with another lateral membrane marker E-cadherin (supplementary Fig 2). The decrease of lateral membrane height of pENTR-h4.1N transfected cells was accompanied by an increase in cross-sectional area. Quantitative analysis showed that the average cross-sectional area of control and pENTR-m4.1N transfected cells were 650.0±150.0 μm^2 and 666.7±152.8 μm^2, respectively while that of pENTR-h4.1N transfected cells increased to 1045.3±145.0 μm^2 ($p<0.05$) (Fig 4D).

Restoration of lateral membrane height by expressing mouse 4.1N in human 4.1N-depleted HBE cells.

To further confirm that the decreased lateral membrane height is specifically due to depletion of 4.1N, we performed rescue experiments by transfecting either GFP or GFP-m4.1N into pENTR-h4.1N transfected cells. As shown in Fig 5, while GFP was diffusively distributed in the cell, GFP-m4.1N was localized at the lateral membrane. Importantly, while the lateral membrane height of GFP-expressed cell was still significantly shorter than that of control cells, expression of GFP-m4.1N increased the lateral membrane height.

Depletion of 4.1N affects the growth of lateral membrane.

It has been shown that while 190 kDa ankyrin G and βII-spectrin are required for the *de novo* biogenesis of lateral membrane[1,2], α adducin may play a role in defining the set point of the lateral membrane height in dividing cells[27]. To examine the role played by 4.1N in this process, we monitored the biogenesis of lateral membrane in both control and 4.1N-depleted HBE cells during cytokinesis using the method established by Kizhatil K and Bennett V[1]. β-tubulin staining (green) was used to identify late anaphase and telophase cells, while β-catenin staining (red) was used as a lateral membrane marker to monitor the formation of the lateral membrane. The upper left panels show that while the lateral membrane can be clearly seen in anaphase control cells (white arrow), it is significantly reduced in 4.1N-depleted cell. Similarly, as shown in the lower panels there are also clear differences in the height of the lateral membrane of telophase normal and 4.1N-depleted cells as indicated by the white arrows. These results demonstrate that while the biogenesis of lateral membrane proceeds in both control and 4.1N-depleted HBE cells, the height of the lateral membrane is signficantly shorter in the 4.1N-depleted HBE cells compated control cells. These findings suggest that although lack of 4.1N does not affect the initial phase of lateral membrane biogenesis, it does play a role in defining the set point for the extent of lateral membrane height.

Depletion of 4.1N has no effect on apical markers.

To examine whether depletion of 4.1N has effects on apical membrane, we examined the location of two apical markers, syntaxin-3 and EBP50. As shown in Fig 7 both these two proteins localized at the apical part of both control and pENTR-h4.1N transfected cells. We

would like to note that because the height of lateral membrane was significantly decreased following 4.1N depletion, the images give the impression that these markers are located at basal membranes of human 4.1N-depleted cells. Additionally, the expression levels of all the proteins examined were indistinguishable between control and pENTR-h4.1N transfected cells (Fig 8). Together these findings strongly suggest that 4.1N selectively affect the lateral membrane in HBE cells.

DISCUSSION

Extensive studies during the last three decades on red cell membrane have enabled the development of a detailed understanding of the molecular components and the structural organization of red cell membrane skeleton. The first well characterized, as well as the most abundant structural component of the red cell membrane is spectrin[28]. Subsequently, a number of other protein constituents of red cell membrane skeleton have been identified. These include actin, ankyrin R, protein 4.1R, adducin, protein 4.2, dematin (protein 4.9), tropomyosin, tropomodulin and p55[29,30]. Interestingly, homologues of spectrin (αI and αII-spectrin, βI-IV-spectrin), ankyrin (ankyrin R, ankyrin G and ankyrin B), and protein 4.1 (4.1R, 4.1G, 4.1B and 4.1N) are found in a variety of non-erythroid cells where they play diverse functions[31,32].

There is increasing evidence that the above described spectrin-based membrane skeleton of red cell is a feature of many if not all plasma membranes as well as membranes of intracellular organelles. However, much less is known about the function of this surmised network in other systems. We and others have demonstrated that a network consisting of βI-spectrin, ankyrin G_{119} and $4.1B_{200}$ exits in the Golgi complex[16]. The existence of a spectrin-based membrane skeleton in the lateral membrane of epithelial cells has been documented by the identification of 190 kDa ankyrin-G, βII-spectrin, adducin and tropomodulin 3 in the lateral membrane[1,2]. In the present study, we identified 4.1N as a component of spectrin-based membrane skeleton within the lateral membrane of HBE cells and showed it plays an essential role in the assembly of lateral membrane of human bronchial epithelial cells. Our conclusion is based on several lines of evidence: 1) the typical localization of the protein on the lateral membrane; 2) its co-localization and co-immunoprecipitation with lateral membrane protein markers; 3) specific depletion of 4.1N by pENTR-h4.1N but not pENTR-m4.1N; 4) decreased lateral membrane height in 4.1N-depleted cells and 5) restoration of the lateral membrane height by re-expression of mouse 4.1N in human 4.1N-depleted cells.

It has been previously shown that depletion of 190-kDa ankyrin-G, or βII-spectrin or α-adducin lead to defects in lateral membrane domain formation[1,2]. The finding that the height of lateral membrane is also decreased in 4.1N-depleted cells strongly suggests that the 4.1N is an integral part of a spectrin-based network within the lateral membrane. However, it should be noted that depletion of 190-kDa ankyrin-G or βII-spectrin resulted in almost complete loss of lateral membrane due to the effect of 190-kDa ankyrin-G or βII-spectrin on the *de novo* biogenesis of lateral membrane at the initial stage of its formation following cell division. In contrast, depletion of 4.1N only resulted in decrease in the height of lateral membrane without affecting the *de novo* biogenesis of lateral membrane, a finding

also noted following depletion of α-adducin. These findings imply that 4.1N and adducin affect the lateral membrane formation and integrity in a similar manner and differently from that of 190-kDa ankyrin-G or βII-spectrin. Thus, it appears that both 190-kDa ankyrin-G and βII-spectrin are essential for the initial biogenesis of the lateral membrane while 4.1N and adducin (both of which promote spectrin network formation) are required for the growth or maintenance of the lateral membrane. The mechanisms by which various components of the spectrin-based membrane skeleton affect the formation and maintenance of lateral membrane are yet to be fully defined.

In addition to Golgi complex and lateral membrane, ankyrins and spectrins are also shown to co-localize in nodes of Ranvier and initial segments of neurons[33,34], costameres[35] as well as neuromuscular junctions in striated muscle[36]. It will be interesting to examine the expression and function of members of protein 4.1 family as well as other components (such as adducin, dematin, tropomodulin, tropomyosin and p55) of spectrin-based membrane skeleton in these specialized cells. Findings from these studies may help to identify a conserved function of spectin-based membrane skeleton for the formation of diverse membrane domains and in regulation of cell function.

In the context of epithelial cells, previous studies have demonstrated the expression of homologues of spectrin, ankyrin and protein 4.1 in a variety of epithelial cells, suggesting important roles for spectrin-based membrane skeleton in epithelial cell biology. We now show that 4.1N together with 190-kDa ankyrin-G and βII-spectrin contributes the spectrin-based membrane skeleton within the lateral membrane. It will be of interest to explore the function of the spectrin-based membrane skeleton in other aspects of epithelial structure and function in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Protein 4.1N is a structural component of lateral membrane skeleton of human bronchial epithelial cells.
- Protein 4.1N is required for biogenesis of the lateral membrane.
- 4.1N-βII spectrin-ankyrin G based lateral membrane skeleton plays an essential role in the assembly of a fully functional lateral membrane of epithelial cells.

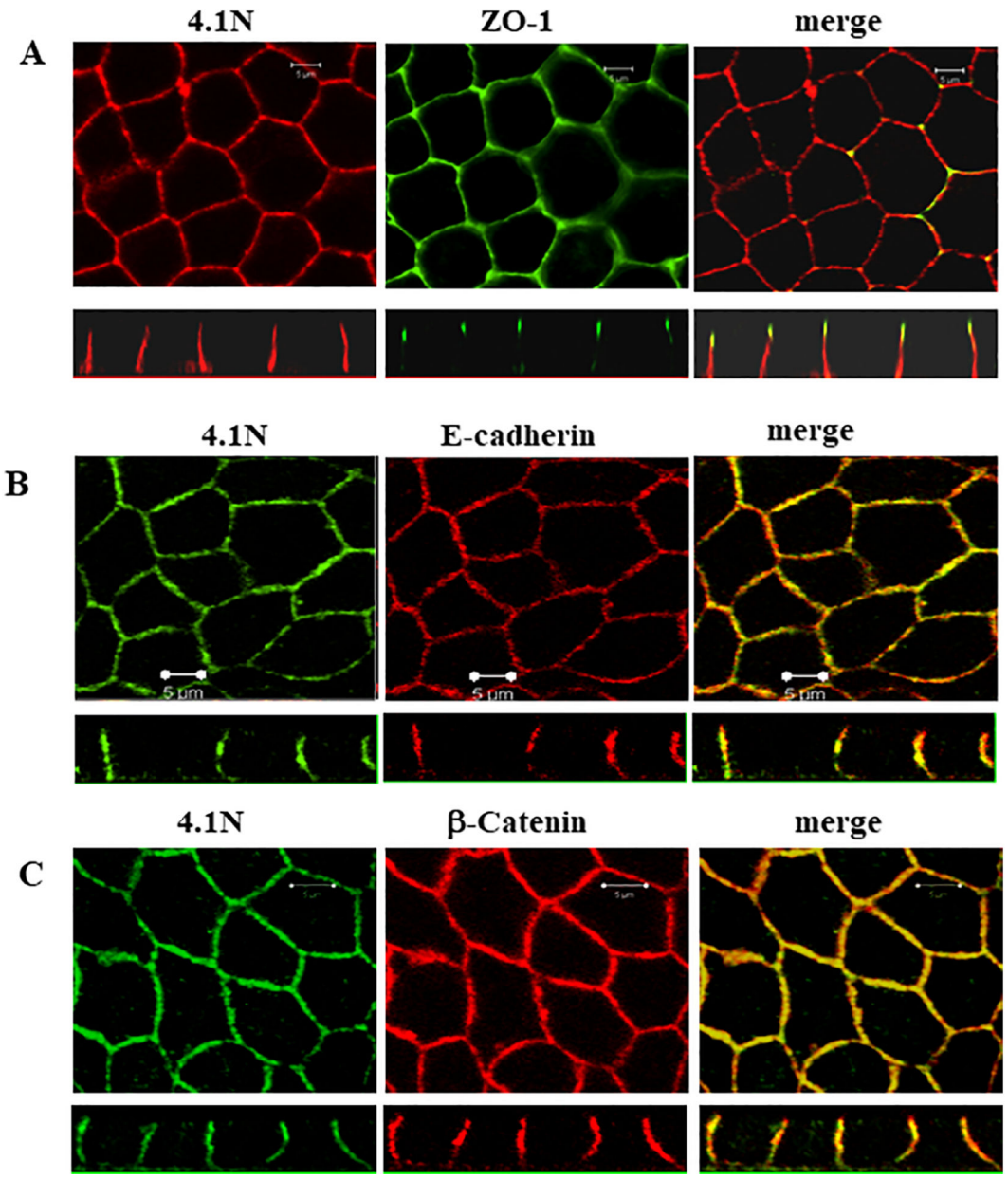

Fig 1. 4.1N is localized at the lateral membrane of human bronchial epithelial cells.

HBE cells were fixed and double stained with **(A)** 4.1N (red) and ZO-1 (green); **(B)** 4.1N (green) and E-cadherin (red); **(C)** 4.1N (green) and β-catenin (red). XY images reveal that 4.1N is located on the plasma membrane and ZY images further reveal that it co-localizes with lateral membrane markers E-cadherin and β-catenin but not with the tight junction marker ZO-1. Scale bar, 5 μm.

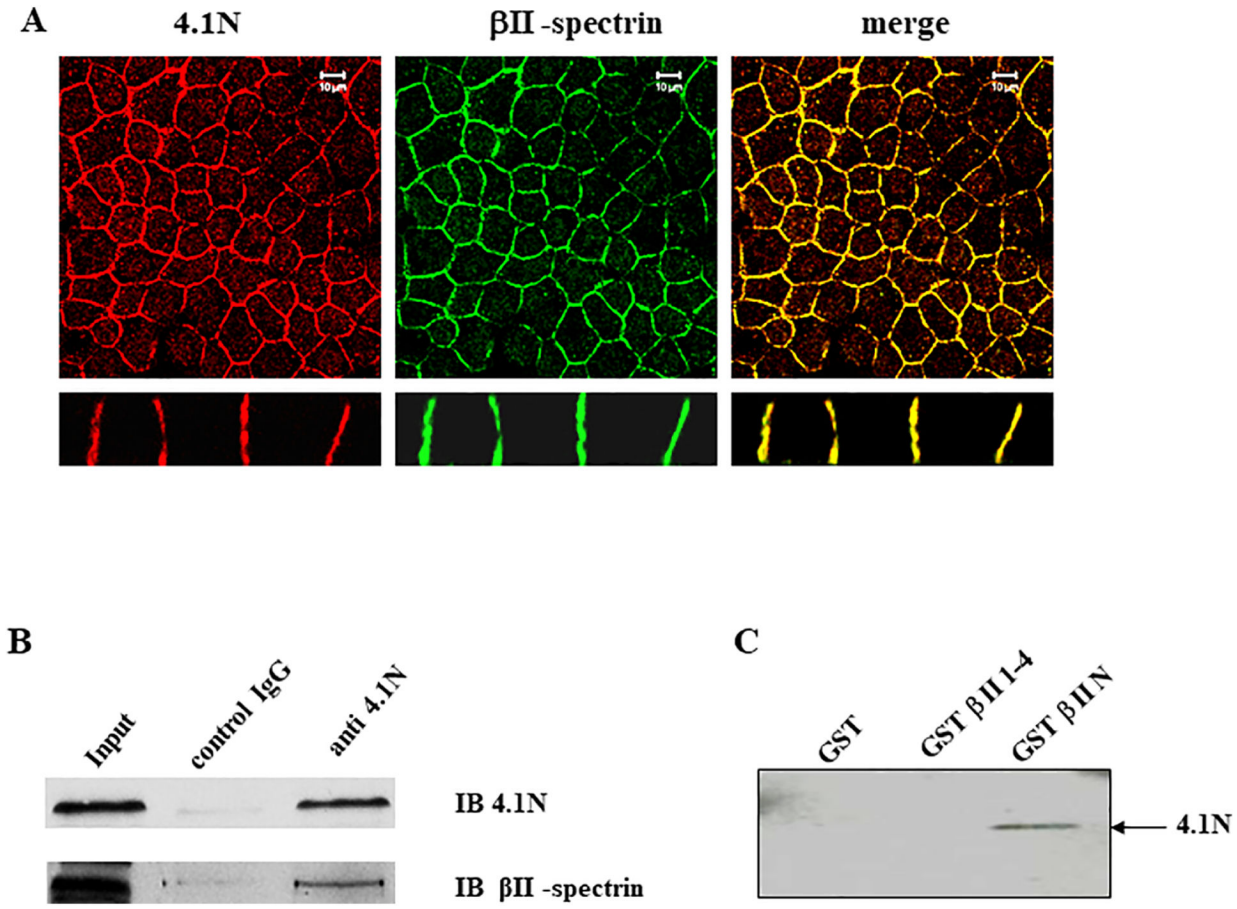

Fig 2. 4.1N is associated with βII spectrin at the lateral membrane.

(A) Co-localization of 4.1N with βII spectrin. HBE cells were co-stained with goat anti-4.1N headpiece antibody (green) and rabbit anti-βII-spectrin antibody (red). Merged image shows that 4.1N co-localizes with βII-spectrin on the lateral membrane. **(B) Co-immunoprecipitation of βII-spectrin with 4.1N.** 4.1N was immunoprecipitated from HBE cell lysate using anti 4.1N antibody or pre-immune control IgG. 4.1N or βII-spectrin in the immunoprecipitate was detected using anti 4.1N antibody (upper panel) or anti-βII-spectrin antibody (lower panel) respectively. Note that βII spectrin was brought down with 4.1N. **(C) Binding of 4.1N and βII-spectrin.** Recombinant His-tagged 4.1N was incubated with GST, GST-tagged βII-repeat 1–4, or GST-tagged βII-N Binding was assayed by pull down assay, using anti-His antibody for detection. Note that 4.1N only bound to βII-N region.

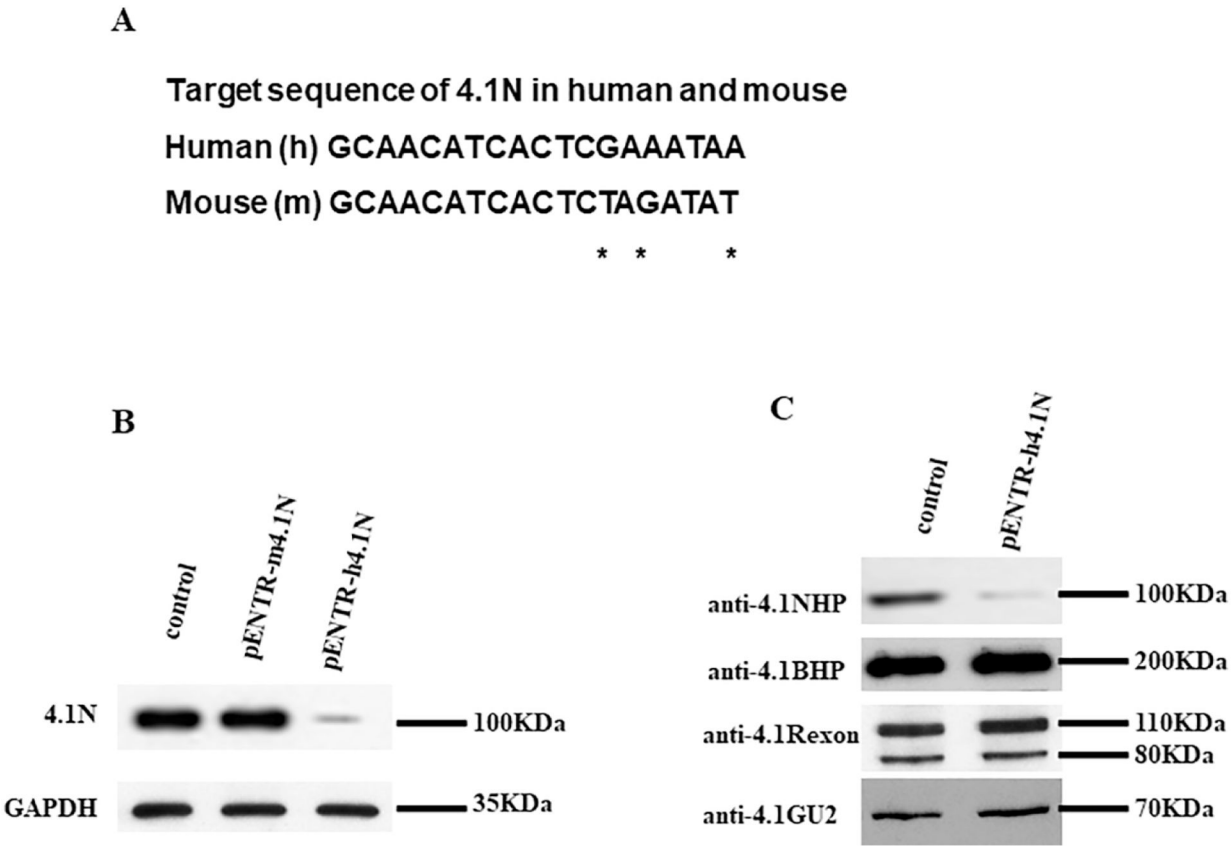

Fig 3. Specific knockdown of 4.1N in HBE cells is achieved by pENTR-h4.1N.

(A) 19-nt shRNA target regions in human 4.1N (h, top) and the mouse 4.1N (m, bottom). *Indicates the bases that are different between human and mouse nucleotide sequences. **(B)** Total cell lysate from control HBE cells or HBE cells that were stably transfected with pENTR-m4.1N or pENTR-h4.1N were subjected to immunoblot analysis using antibodies against 4.1N and GAPDH. Note the significant reduction of 4.1N in pENTR-h4.1N transfected HBE cells but not in pENTR-m4.1N transfected cells. **(C)** Total cell lysate from control and pENTR-h4.1N was subjected to immunoblot analysis with the indicated antibodies. Note significant knockdown of 4.1N but not of other 4.1 family of proteins.

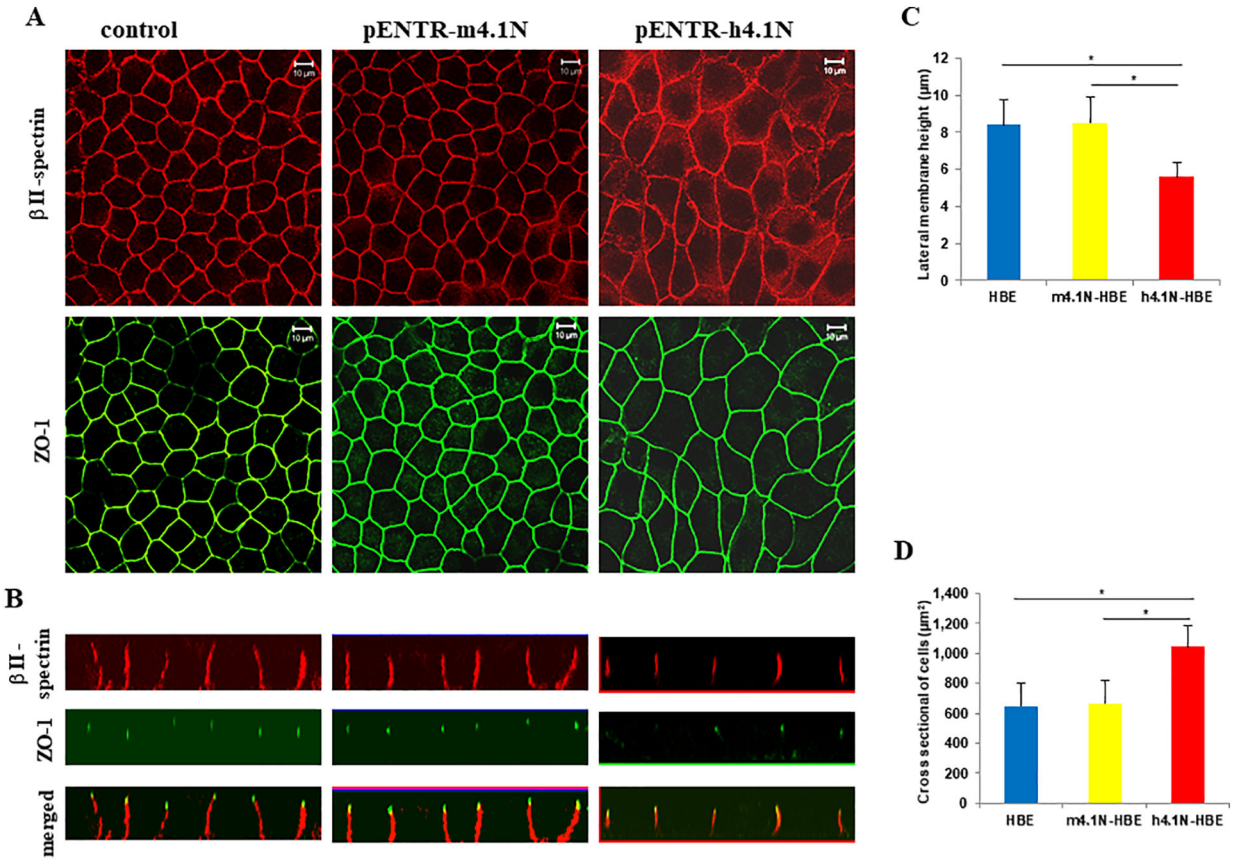

Fig 4. Knockdown of 4.1N results in shortened lateral membrane and expansion of apical membrane.

Cells were double stained with anti-βII-spectrin (red) and ZO-1 (green). XY confocal sections of cells are shown in **(A)** and XZ sections are shown in **(B)**. Note the increased cross-sectional area and decreased height of lateral membrane of pENTR-h4.1N transfected cells. Scale bar, 10 μm. Quantitative analyses of cross-section area and lateral membrane height are shown in panels **(C)** and **(D)** respectively.

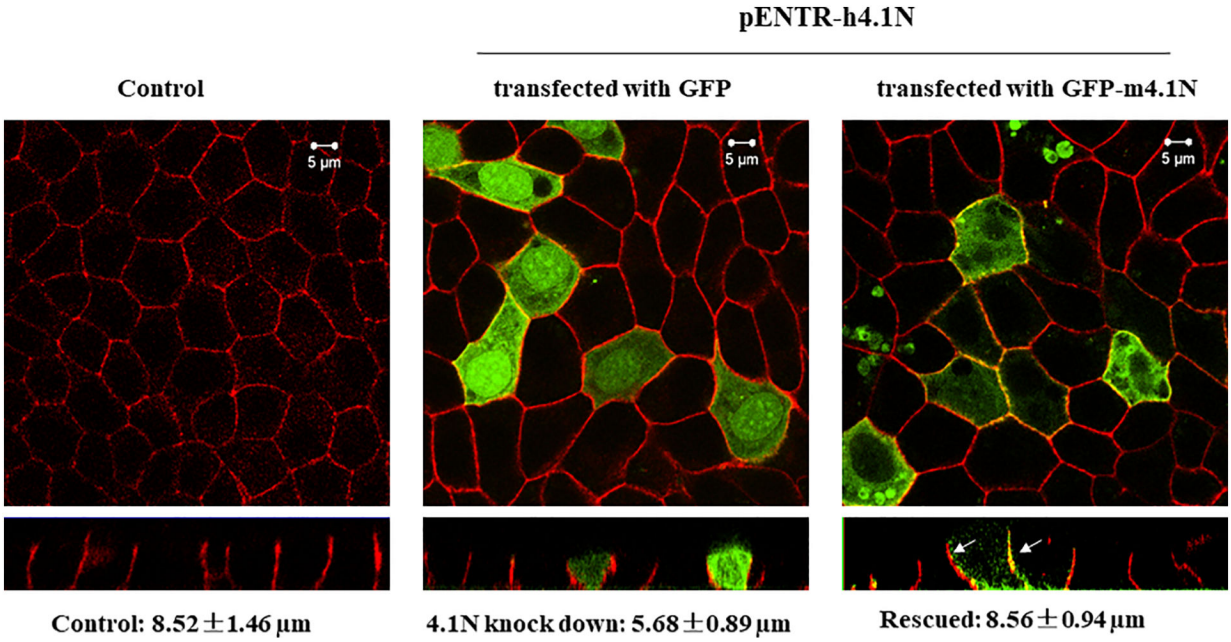

Fig 5. The height of lateral membrane is restored following expression of mouse 4.1N.

Human 4.1N-depleted HBE cells were transfected with GFP or GFP-m4.1N, and stained with E-cadherin (red). Note that the height of lateral membrane is restored (white arrows) by GFP-m4.1N but not by GFP alone. Scale bar, 5 μm.

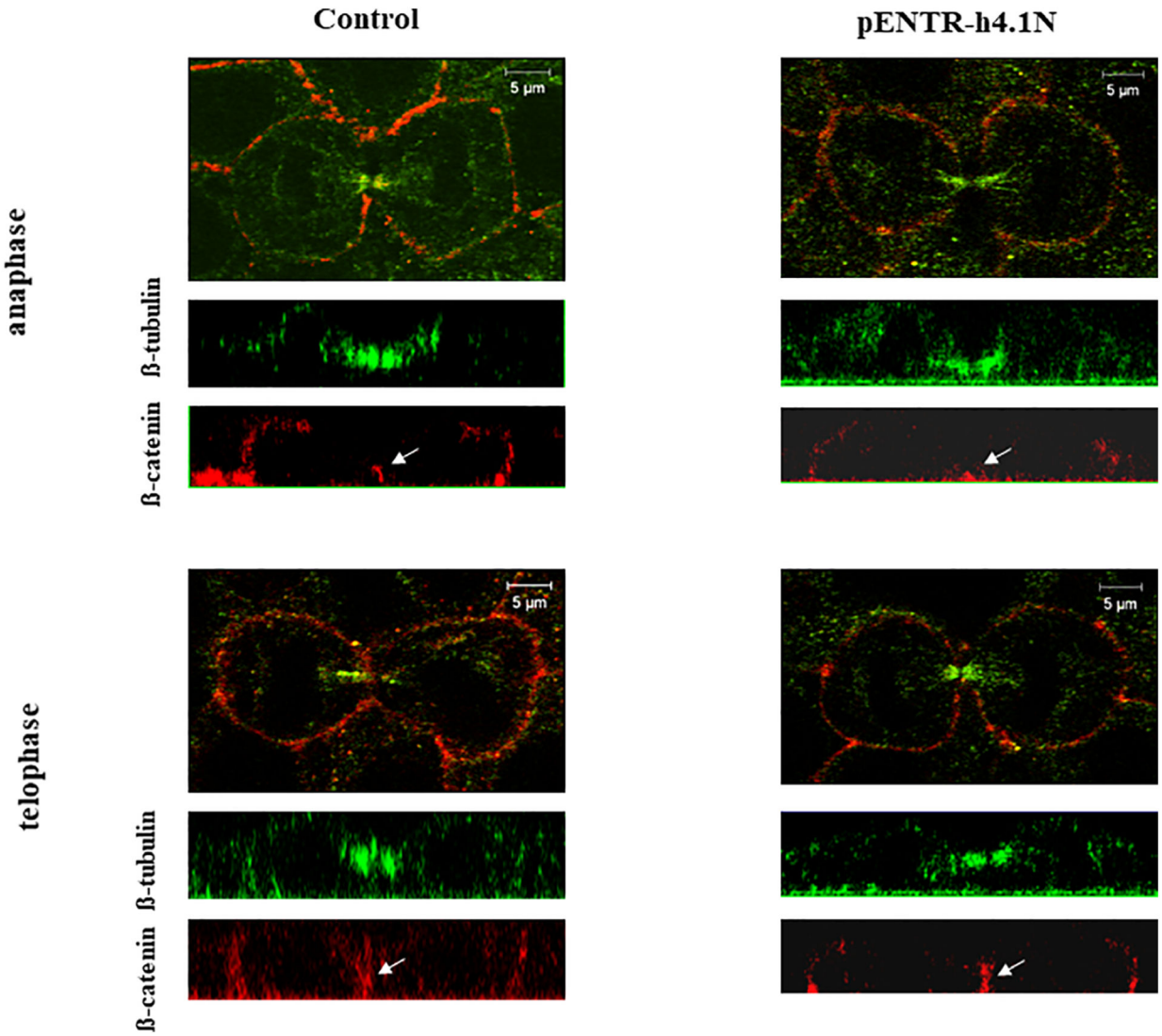

Fig 6. Post mitotic biogenesis of the lateral membrane proceeds normally in 4.1N-depleted HBE cells.

Control or human 4.1N-depleted HBE cells were fixed and co-stained with β-tubulin (green) and β-catenin (red). Each set of panels consists of an XY confocal image of a dividing cell and an XZ image of the same cell. β-tubulin staining reveals inter zonal microtubules in the late anaphase and the midbody structure in the late telophase. The arrows indicate the lateral membrane between the dividing cells. Note that although the lateral membrane was formed in the late telophase of both cell types but the height of lateral membrane was decreased in the 4.1N-depleted cells. Scale bar, 5 μm.

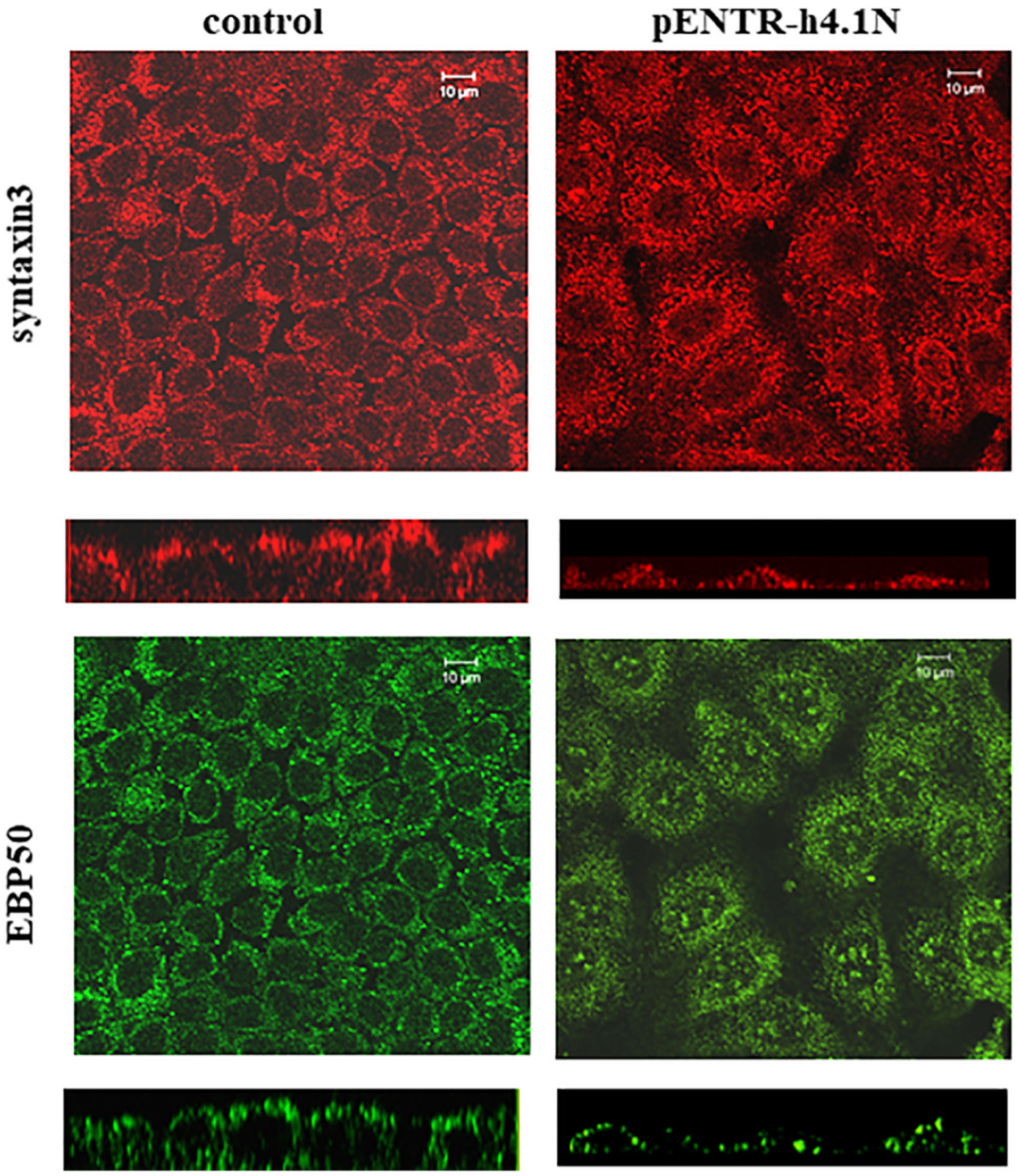

Fig 7. Knockdown of 4.1N does not affect the localization of apical proteins syntaxin3 and EBP50.

HBE cells or HBE cells transfected with pENTR-h4.1N were fixed and stained with two apical markers syntaxin 3 (red) or EBP50 (green). Note that syntaxin 3 and EBP50 are located at the apical membrane in both control and pENTR-h4.1N transfected cells. Scale bar: 10 μm.

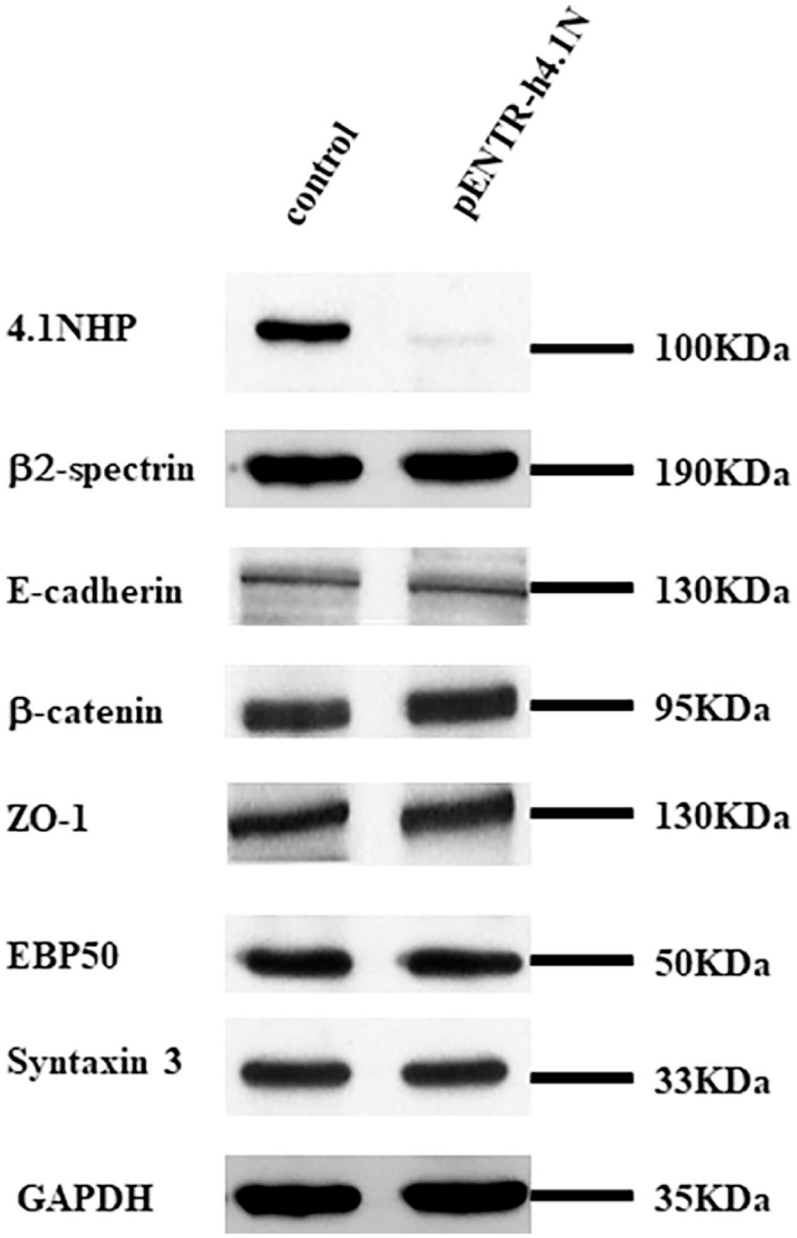

Fig 8. Effect of knockdown of 4.1N in HBE cells on the expression levels of various proteins.
Total cell lysate from control HBE cells or HBE cells transfected with pENTR-h4.1N was subjected to immunoblot analysis with the indicated antibodies. Note significant knockdown of 4.1N but not of other proteins.