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CORRECTION

Correction: A Golgi-associated protein 4.1B variant is required for assimilation of proteins in the membrane (doi:10.1242/jcs.039644)

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This Correction updates the Expression of Concern (doi:10.1242/jcs.233080) relating to *J. Cell Sci.* (2009) **122**, 1091-1099 (doi:10.1242/jcs.039644).

The wrong western blot was used for the anti-4.1G U1 panel in Fig. 1B. The original full blots for this figure are no longer available so the corresponding author, Xiuli An, repeated the experiment with the original antibody. The new results confirm the specificity of the anti-4.1G U1 antibody, as shown here.
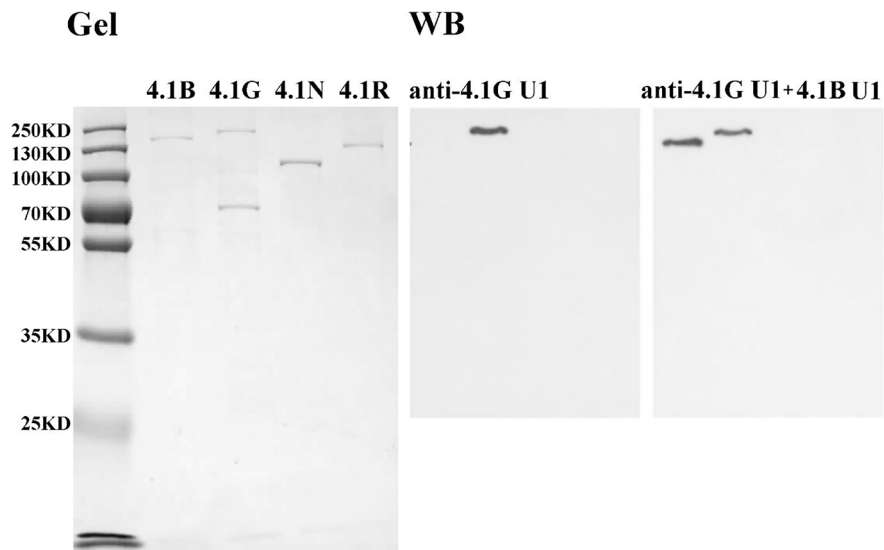
Repeat of Fig. 1B. SDS-PAGE of purified recombinant protein 4.1 family members: 4.1B, 4.1G, 4.1N and 4.1R (left). Western blotting using anti-4.1G U1 antibody (middle) followed by re-probe with anti-4.1B U1 antibody (right).

During our investigation into this matter, a reader also noticed that the cell images acquired using the anti-4.1B U2 antibody in Fig. 3 were duplicated for HBE and MDCK cells. This was an error that occurred during figure preparation. The authors provided the original images and the corrected figure is shown here. The authors apologise to readers for any inconvenience caused by these errors, which do not affect the conclusions of the paper.
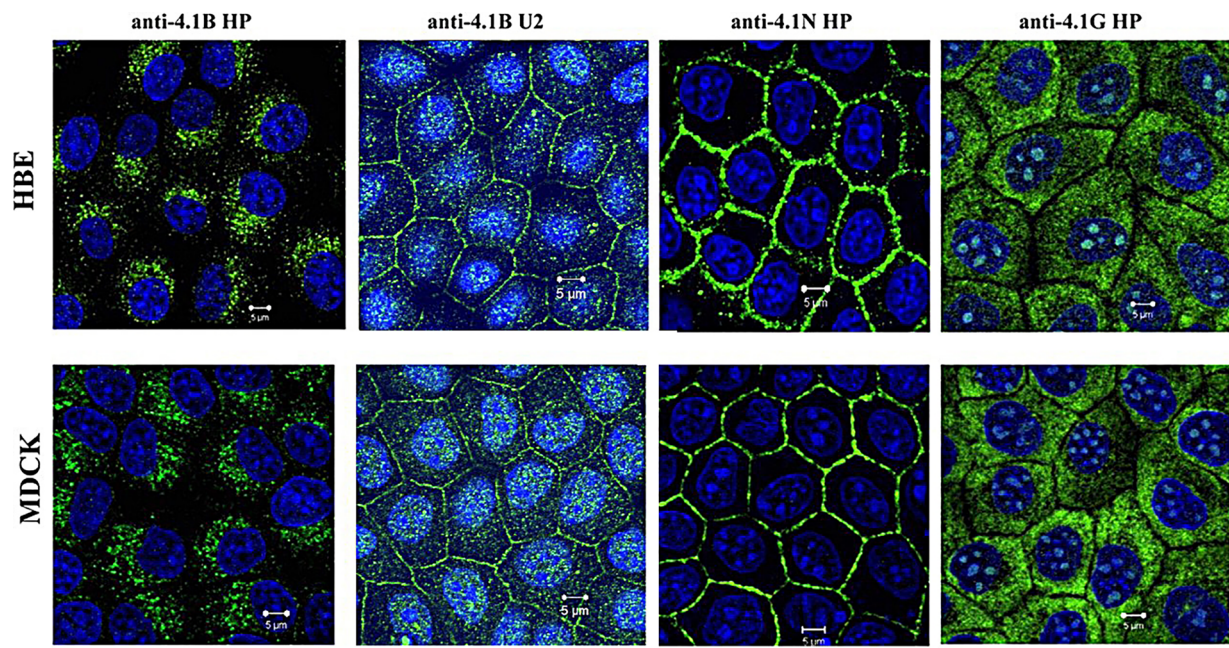
Fig. 3. Localization of 4.1 proteins in MDCK and HBE cells. MDCK and HBE cells were stained with rabbit polyclonal anti-4.1B-HP, anti-4.1B-U2, anti-4.1N-HP and anti-4.1G-HP, followed by anti-rabbit Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with Tropo 3 (blue). The images were analyzed by confocal microscopy. Note the distinct localization of different epitopes. Scale bars: 5 μm.

PUBLISHER'S NOTE

Expression of Concern: A Golgi-associated protein 4.1B variant is required for assimilation of proteins in the membrane (doi:10.1242/jcs.039644)

Qiaozhen Kang, Ting Wang, Huizheng Zhang, Narla Mohandas and Xiuli An

This Expression of Concern relates to *J. Cell Sci.* (2009) **122**, 1091-1099 (doi:10.1242/jcs.039644).

Journal of Cell Science was informed that part of the 4.1G U2 blot in Fig. 1B is duplicated in the 4.1G U1 panel above. As the corresponding author, Xiuli An, was unable to locate the original data, the journal is unable to determine whether the results and conclusions reported in the paper are compromised. Therefore, the journal is publishing this Expression of Concern to make readers aware of the issues with the western blots shown in Fig. 1B.

The authors apologise to readers for any inconvenience caused.

A Golgi-associated protein 4.1B variant is required for assimilation of proteins in the membrane

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Summary

The archetypal membrane skeleton is that of the erythrocyte, consisting predominantly of spectrin, actin, ankyrin R and protein 4.1R. The presence in the Golgi of a membrane skeleton with a similar structure has been inferred, based on the identification of Golgi-associated spectrin and ankyrin. It has long been assumed that a Golgi-specific protein 4.1 must also exist, but it has not previously been found. We demonstrate here that a hitherto unknown form of protein 4.1, a 200 kDa 4.1B, is associated with the Golgi of Madin-Darby canine kidney (MDCK) and human bronchial epithelial (HBE) cells. This 4.1B variant behaves like a Golgi marker after treatment with Brefeldin A and during mitosis. Depletion of the protein in HBE cells by siRNA resulted in disruption of the Golgi structure and failure of Na^+/K^+-ATPase, ZO-1 and ZO-2 to migrate to the membrane. Thus, this newly identified Golgi-specific protein 4.1 appears to have an essential role in maintaining the structure of the Golgi and in assembly of a subset of membrane proteins.

Key words: Golgi, Cytoskeleton, Protein 4.1B, Spectrin

Introduction

The Golgi complex is the highly organized and dynamic organelle of eukaryotic cells in which newly synthesized proteins are processed and sorted. It forms an interconnected network, consisting of cisternal stacks positioned around the perinuclear region. Earlier studies have focused on the role of golgin family proteins (Barr and Short, 2003) and microtubules (Cole et al., 1996; Ho et al., 1989; Thyberg and Moskalewski, 1985) in the structural organization and function of the Golgi. The discovery that spectrin and ankyrin (two major components of the red blood cell membrane skeleton) are present in the Golgi has led to the conjecture that the organelle might contain a spectrin-based network, resembling that of the erythrocyte (Beck, 2005; De Matteis and Morrow, 2000). Two isoforms of Golgi-associated spectrin have been identified (Beck et al., 1994; Stankewich et al., 1998), as have the ankyrin isoforms, Ank_{G119} and Ank_{195} (Beck et al., 1997; Devarajan et al., 1996). It has further been shown that disruption of the endogenous Golgi-spectrin complex by overexpression of the actin-binding and membrane-association domains of βI spectrin blocks transport of α- and β-Na^+/K^+-ATPase and of vesicular stomatitis virus G protein, testifying the participation of a spectrin-ankyrin network in vesicle trafficking (Devarajan et al., 1997). Another indispensable component of the erythrocyte spectrin-based membrane skeleton is protein 4.1R (Tchernia et al., 1981), the prototypic member of the protein 4.1 family. It might therefore be expected that such a protein would occur in conjunction with spectrin and ankyrin in the Golgi. However, the evidence for a Golgi-specific protein 4.1 is still lacking.

There are four known protein 4.1 paralogs, 4.1R (Conboy, 1987), 4.1G (Parra et al., 1998), 4.1N (Walensky et al., 1999) and 4.1B (Parra et al., 2000), encoded by separate, but homologous genes. Each of these forms undergoes extensive alternative splicing, resulting in the generation of multiple splice forms (Conboy et al., 1988; Conboy et al., 1991). Despite the enormous variety of possible splice variants, all proteins of the 4.1 family have a domain structure in common, which comprises an N-terminal membrane-binding domain (MBD), an internal spectrin-actin-binding domain (SABD), and a C-terminal domain (CTD), all with highly conserved sequences. These domains are interspersed with non-conserved domains, unique to one or other isoform, and with unknown functions.

It is generally accepted that 4.1 proteins act as linkers between the plasma membrane and the actin cytoskeleton (Sun et al., 2002), as observed in the erythrocyte (Salomao et al., 2008). Nevertheless, in nonerythroid cells, 4.1 isoforms are found in a variety of subcellular compartments, including the nucleus (Correas, 1991), the centrosome (Krauss et al., 1997), and tight junctions (Mattagajasingh et al., 2000). The implication is that some 4.1 proteins might have functions in nonerythroid cells other than simply acting as inert linkers.

In the present study, we have identified a Golgi-specific 200 kDa protein 4.1B and have shown that it is required for both structural integrity of the Golgi complex and for targeting of a subset of membrane proteins.

Results

Characterization of anti-protein-4.1 antibodies

Detection and identification of Golgi-specific 4.1 protein required a panel of antibodies specific for the different members of the family. Since the conserved regions share very high sequence homologies, the antibodies were directed against the sparse non-conserved regions. For each protein 4.1, we generated two antibodies, one against the unique upstream non-conserved region, U1 (also called head-piece, hereafter referred to as HP), the other against the non-conserved U2 region. The specificity of each antibody was confirmed by western blotting on recombinant full-length protein 4.1 isoforms, and on brain tissue (for anti-4.1B antibodies), on kidney tissue (for anti-4.1N and anti-4.1R antibodies) and lung tissue

(for anti-4.1G antibodies). Tissue samples were also taken from the cognate knockout mice as negative controls. Fig. 1A displays the schematic structure of 4.1 and the regions targeted as antigens. Fig. 1B shows that none of the antibodies crossreacts with other protein 4.1 components. Fig. 1C confirms that 4.1 isoforms seen in wild-type tissues are not detected in the corresponding knockouts, with the exception of anti-4.1B-U2 antibody, which recognizes a zone with apparent size of ~60 kDa in both $4.1B^{+/+}$ and $4.1B^{-/-}$ brain tissue. We believe this band to be 4.1B because it appears in many cell lines and can be knocked down by 4.1B-specific siRNA (data not shown). It also should be noted that some antibodies recognize multiple bands in the tissues, which are splice variants of the protein. Thus, the antibodies we have generated are highly specific.

All members of protein 4.1 family are expressed in MDCK and HBE cells

Epithelial, and especially MDCK cells, are most commonly used for Golgi studies because of their highly polarized morphology and the abundance of their Golgi complexes. As a first step towards identifying a Golgi-specific 4.1, we assayed expression of 4.1 proteins in MDCK cells and in another type of epithelial cell, human bronchia epithelial cells (HBE) by western blotting. Fig. 2 shows that all 4.1 paralogs are expressed in both cell lines, and with very similar patterns. Anti-4.1B-HP antibody stained a ~200 kDa band, and anti-4.1B-U2 antibody a ~60 kDa band. Both anti-4.1G-HP and anti-4.1G-U2 antibodies recognized a component of ~70 kDa. Similarly, both anti-4.1NHP and anti-4.1N-U2 detected a major band of ~105 kDa. Finally antibodies against both anti-4.1R exon-16 and anti-4.1R exon 19 recognized two bands, one of ~80 kDa the other of ~110 kDa, which represent the two splice variants of 4.1R in both MDCK and HBE cells. These observations suggest that such

widespread expression of 4.1 proteins may be a general phenomenon in epithelial cells. They also point to a variety of functions for 4.1 proteins in such cells and tissues.

Members of protein 4.1 family are located in a distinct subcellular compartment in MDCK and HBE cells

We used confocal immunofluorescence microscopy to locate the various antigens in MDCK and HBE cells, using antibodies that each recognized only a single band. As shown in Fig. 3, staining of completely confluent interphase MDCK and HBE cells with anti-4.1B-HP revealed a perinuclear distribution, morphologically reminiscent of the appearance of the Golgi complex. Staining with anti-4.1B-U2 indicated both nuclear and membrane (bilateral, no colocalization with E-cadherin; our unpublished data) localization. Furthermore, although the 4.1N-HP epitope occurred at the membrane (bilateral, colocalized with E-cadherin; our unpublished data), that of 4.1G-HP was discerned in both the cytosol and nucleolus. Antibodies against both anti-4.1R exon 16 and anti-4.1R exon 19 revealed no clear staining pattern. Preimmune antibodies gave no staining (data not shown). Each member of the 4.1 group of proteins thus has its own distinctive distribution and probably therefore also has a distinct function.

The 200 kDa 4.1B is associated with the Golgi in HBE cells

To gain further evidence that the 200 kDa 4.1B protein $(4.1B_{200})$ is indeed an integral part of the Golgi complex, we investigated its association with Golgi proteins by immunostaining of HBE cells. Fig. 4A shows that $4.1B_{200}$ colocalizes with four Golgi proteins in the perinuclear region: βI-spectrin, β-COPI, Golgi97 and TGN46. By contrast, $4.1B_{200}$ dose not colocalize with endosomal marker Rab5 (Fig. 4B). We also examined the association of the $4.1B_{200}$
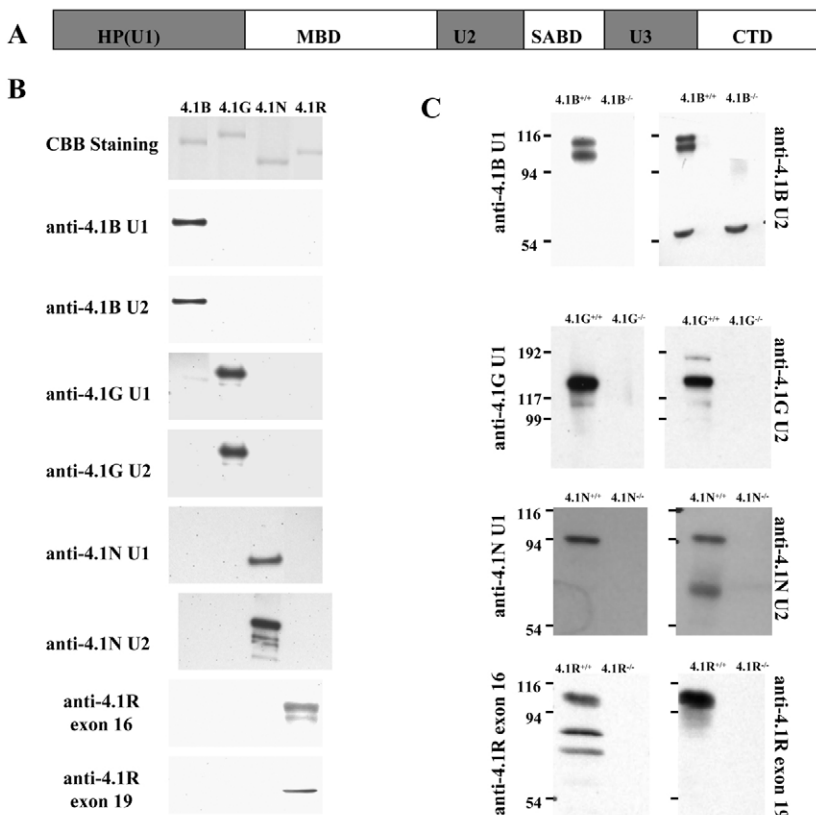

Fig. 1. Specificity of anti-4.1 antibodies. (A) Schematic representation of 4.1 domain structure. The boxes represent the structural domains of 4.1. The conserved domains are shown in empty boxes and the non-conserved unique domains (U1, U2 and U3) are shaded. Targeted regions are shown in dark gray. CTD, C-terminal domain; MBD, membrane-binding domain; SABD, spectrin-actin-binding domain. (B) Reaction of anti-4.1 antibodies with recombinant 4.1 proteins. Recombinant full-length 4.1 proteins (2 μg/lane) were subjected to immunoblot analysis with various anti-4.1 antibodies. Top panel, CBB staining of the proteins. No crossreaction of any antibody with other 4.1 proteins was seen. (C) Reaction of anti-4.1 antibodies with tissues. Cell lysates (20 μg of total protein) from wild-type and knockout mice were subjected to immunoblot analysis with various anti-4.1 antibodies. Note the absence of the relevant band in the corresponding knockout sample with the exception of anti-4.1B-U2 antibody.
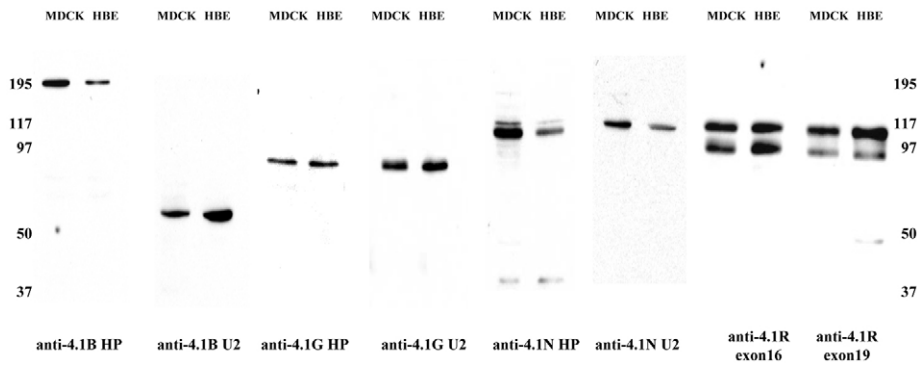
Fig. 2. Expression of 4.1 proteins in MDCK and HBE cells. Total cell lysate of MDCK and HBE cells was subjected to immunoblot analysis with the indicated polyclonal rabbit anti-4.1 antibodies. Note that the same proteins are expressed in both MDCK and HBE cells. Molecular size markers are shown in kDa on the left.

with βI-spectrin by coimmunoprecipitation, in view of the well-known interaction between spectrin and 4.1. As shown in Fig. 4C, spectrin was precipitated with $4.1B_{200}$ by anti-4.1B-HP antibody, but not by preimmune antiserum. It thus appears that the $4.1B_{200}$ is the Golgi-specific form of protein 4.1.

Dynamics of Golgi $4.1B_{200}$

One of the important characteristics of the Golgi is its dynamic nature, which is needed to satisfy the demands of membrane trafficking. Thus, extensive Golgi fragmentation occurs during mitosis, and also following treatment with Brefeldin A (BFA). It has been reported that BFA perturbs the assembly dynamics of a variety of Golgi proteins, including the membrane skeleton components, β-spectrin (Beck et al., 1994) and ankyrin (Beck et al., 1997). To ascertain whether $4.1B_{200}$ association with the Golgi membrane is likewise dynamic, we examined its distribution during mitosis. The $4.1B_{200}$ staining pattern was found to differ markedly between interphase and mitotic cells. As shown in Fig. 5A, although $4.1B_{200}$ was confined to the perinuclear region in interphase HBE and MDCK cells, it was dispersed in all stages of mitotic cells. We also examined the distribution of $4.1B_{200}$ in BFA-treated HBE cells. Fig. 5B shows that following BFA treatment, the distribution of the $4.1B_{200}$ became dispersed, and that its perinuclear concentration was restored after washing out the BFA. It should be mentioned that the spots seen in mitotic and BFA-treated cells contain Golgi markers, indicating they are Golgi, rather than other structures. We conclude that the association of $4.1B_{200}$ with the Golgi membrane, like that of β-spectrin and ankyrin, is labile.

Altered Golgi structure in $4.1B_{200}$-knockdown HBE cells

In the erythrocyte, 4.1R interacts with both spectrin and actin, and thereby preserves the structure of the membrane skeleton (Salomao et al., 2008; Yawata et al., 1997). To determine whether $4.1B_{200}$ has an analogous role in the Golgi architecture, we used RNAi to knock down the protein in HBE cells. Fig. 6A shows that although expression of $4.1B_{200}$ was substantially reduced, that of 60 kDa 4.1B remained unchanged, as did the expression of other 4.1 members (4.1R, 4.1G and 4.1), and actin. Knockdown of $4.1B_{200}$ was also revealed by significantly reduced staining of 4.1B in 4.1B-depleted cells (Fig. 6B, top panels). The morphology of the Golgi was exposed by staining with antibodies against three Golgi-specific markers, Golgi97, TGN46 and ManII. As shown in Fig. 6B, in contrast to their typical perinuclear staining pattern in control cells, all three markers were diffusely distributed throughout the cytoplasm in the $4.1B_{200}$-depleted cells. These results demonstrate that $4.1B_{200}$ has an important role in the maintenance of Golgi structure.

Impaired assembly of Na^+/K^+-ATPase, ZO-1 and ZO-2 in $4.1B_{200}$-knockdown HBE cells

The Golgi complex oversees the transport and assembly of proteins. In polarized epithelial cells, differential targeting of proteins to apical and basolateral domains is especially prominent. To study the role of $4.1B_{200}$ in protein trafficking, we systematically examined the locations of apical proteins, tight junction proteins and adherens junction proteins in control and $4.1B_{200}$-knockdown HBE cells. Fig. 7A shows that in confluent control cells, Na^+/K^+-ATPase was restricted to the bilateral membrane, whereas ZO-1 and ZO-2 were located at the apical extreme of the lateral side. By contrast, in the $4.1B_{200}$-knockdown cells, all were diffusely distributed in the cytosol. The correct distributions of Na^+/K^+-ATPase, ZO-1, ZO-2 as well as the structure of Golgi were recovered in knockdown cells cotransfected with mouse 4.1B. Under normal conditions, β-Na^+/K^+-ATPase is synthesized and glycosylated in the ER (core glycosylation), it then undergoes further glycosylation in the Golgi and eventually incorporates as an α/β heterodimer into a detergent-insoluble spectrin and ankyrin cortical skeletal lattice (Mays et al., 1995; Morrow et al., 1989; Nelson and Hammerton, 1989; Nelson and Veshnock, 1986). Thus, to further explore the mechanism by which $4.1B_{200}$ affects the localization of Na^+/K^+-ATPase, we compared the glycosylation status of β-Na^+/K^+-ATPase as well as the incorporation of α-Na^+/K^+-ATPase into the spectrin-based membrane skeleton in control and $4.1B_{200}$-depleted cells. Fig. 7B reveals increased ER-dependent core glycosylation of β-Na^+/K^+-ATPase in $4.1B_{200}$-depleted cells, indicating a block at the ER-to-Golgi transition upon $4.1B_{200}$ depletion. The transition of α-Na^+/K^+-ATPase to the detergent-insoluble fraction was also blocked, as demonstrated by the increased proportion of α-Na^+/K^+-ATPase in the soluble fraction (Fig. 7C). Absence of $4.1B_{200}$ had no effect on localization of the adherens junction proteins E-cadherin and β-catenin, nor the two apical markers, syntaxin-3 and EBP50 (Fig. 8). The expression levels of all the proteins examined were indistinguishable from those in the $4.1B_{200}$-knockdown HBE cells (Fig. 9). It thus appears that $4.1B_{200}$ selectively controls the traffic of Na^+/K^+-ATPase, ZO-1 and ZO-2.

ZO-1 and ZO-2 associate with $4.1B_{200}$ in non-confluent cells

It has been reported that disruption of the spectrin membrane skeleton of the Golgi complex results in impaired assembly of Na^+/K^+-ATPase (Devarajan et al., 1997); therefore, the altered distribution of the enzyme in the $4.1B_{200}$-knockdown cells is not unexpected. However, it is not clear why the assembly of ZO-1 and ZO-2 is also affected. To explore the mechanism of the breakdown of ZO-1 and ZO-2 assembly, we tested the possibility that ZO-1 and/or ZO-2 might transiently interact with the $4.1B_{200}$. To this end,
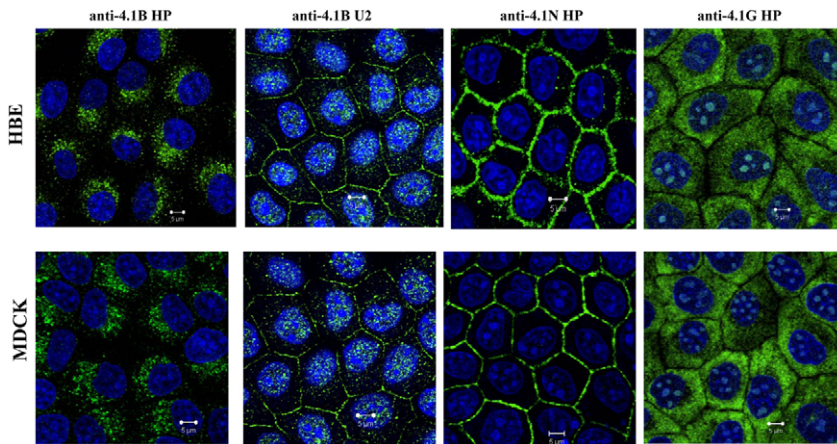
Fig. 3. Localization of 4.1 proteins in MDCK and HBE cells. MDCK and HBE cells were stained with rabbit polyclonal anti-4.1B-HP, anti-4.1B-U2, anti-4.1N-HP and anti-4.1G-HP, followed by anti-rabbit Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with Tropo 3 (blue). The images were analyzed by confocal microscopy. Note the distinct localization of different epitopes. Scale bars: 5 μm.

we examined the association of ZO-1 and ZO-2 with $4.1B_{200}$ in non-confluent HBE cells by co-staining, and in solution by co-immunoprecipitation. Fig. 10A shows that in fully confluent HBE cells, ZO-1 and ZO-2 are exclusively located at the plasma membrane and $4.1B_{200}$ remains in the Golgi. In the subconfluent cells, by contrast, ZO-1and ZO-2 appeared not only in the plasma membrane but also in some vesicles in the perinuclear region and colocalized with $4.1B_{200}$ (Fig. 10B). To confirm this association, we performed co-immunoprecipitation with anti-4.1B-HP antibody. We did indeed observe that, although ZO-1 and ZO-2 were not immunoprecipitated with $4.1B_{200}$ from complete confluent cells (Fig. 10C), they were brought down by the same antibody from non-confluent cells (Fig. 10D).

Discussion

There is evidence that a spectrin-based membrane skeleton might be implicated in the structure and function of the Golgi complex. However, in contrast to the well-characterized erythrocyte membrane skeleton, even the identities of the components of this surmised network in the Golgi-associated membrane have been elusive. Specific isoforms of spectrin and ankyrin have been recognized in the Golgi, and a recent report has suggested that the AE2 anion exchanger, a member of the red blood cell band 3 (AE1) family, is necessary for the structural integrity of the Golgi complex (Holappa et al., 2004). In the present study, we have identified a previously unknown protein 4.1 variant, $4.1B_{200}$, as a Golgi-specific component. Several lines of evidence have led us to this conclusion, namely: (1) the typical perinuclear distribution of the protein; (2) its colocalization and co-immunoprecipitation with Golgi markers; (3) dynamic changes in its distribution during the cell cycle and following BFA treatment; (4) fragmentation of the Golgi structure upon deletion of $4.1B_{200}$; and (5) impaired assembly of Na^+/K^+-ATPase, ZO-1 and ZO-2 in $4.1B_{200}$-depleted cells.

Although the precise structure of the $4.1B_{200}$ variant has yet to be defined, we feel confident that this Golgi-associated constituent is a protein 4.1B because of the high specificity of our anti-4.1B-HP antibody, as evidenced by: (1) its failure to recognize other members of protein 4.1 family; (2) its failure to recognize any band in 4.1B-knockout tissues; and (3) the reduction of $4.1B_{200}$ protein in siRNA-treated cells with a 4.1B-specific sequence. It is also interesting to note that a monoclonal antibody against canine liver Golgi membrane showed that a 200 kDa, BFA-sensitive protein was associated with the Golgi (Narula et al., 1992), although its identity and function are still uncertain.
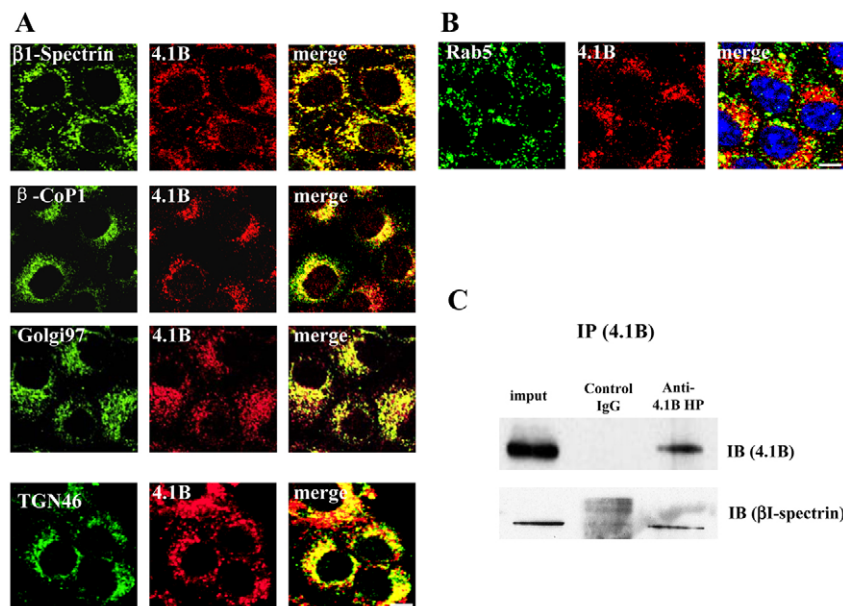

Fig. 4. Association of $4.1B_{200}$ with Golgi markers. (A) Colocalization of $4.1B_{200}$ with Golgi markers. HBE cells were co-stained with anti-4.1B-HP antibody (directly labeled with Alexa Fluor 594) and anti-βI-spectrin, anti-β-COPI, anti-Golgi97 or anti-TGN46 (directly labeled with Alexa Fluor 488). Merged images show that $4.1B_{200}$ colocalizes with all these Golgi proteins in the perinuclear region. Scale bar: 10 μm. (B) HBE cells were co-stained with anti-4.1B-HP antibody (directly labeled with Alexa Fluor 594) and anti-Rab5. Merged images show that $4.1B_{200}$ dose not colocalize Rab5. Scale bar: 10 μm. (C) Immunoprecipitation (IP) was performed using anti-4.1B-HP antibody. Proteins in the immunoprecipitate were detected with the antibodies as indicated. Note that βI spectrin was brought down with $4.1B_{200}$. Pre-immune IgG was used as negative control.
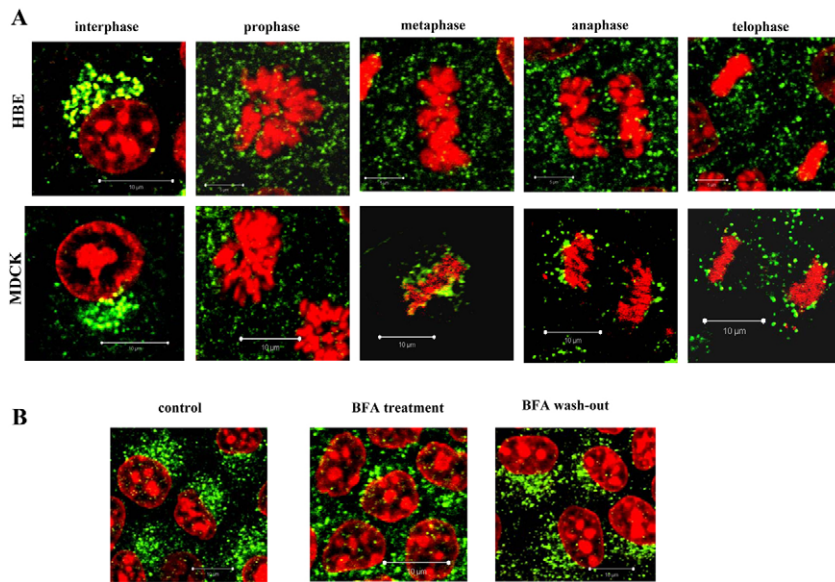
Fig. 5. Dynamics of 4.1B$_{200}$ protein. (A) Distribution of 4.1B$_{200}$ in mitotic cells. Subconfluent HEB and MDCK cells were stained with anti-4.1B-HP (green) and Tropo 3 (red). Note that the staining of 4.1B$_{200}$ is concentrated around perinuclear region in the interphase cells, but dispersed in all stages of mitotic cells. (B) Distribution of 4.1B$_{200}$ in BFA-treated cells. Subconfluent HBE cells were either treated with 1% ethanol, 6 μg/ml BFA for 60 minutes at 37°C, or by washout after BFA treatment. The cells were fixed and stained as above. Note the perinuclear staining of 4.1B$_{200}$ in control cells (left panel), dispersed staining in BFA-treated cells (middle panel) and the recovery of the perinuclear staining after BFA washout (right panel). Scale bars: 10 μm.

It has been shown that Na$^+$/K$^+$-ATPase transport from endoplasmic reticulum to Golgi depends on the Golgi spectrin-ankyrin$_{G119}$ skeleton (Devarajan et al., 1997). That the transport of Na$^+$/K$^+$-ATPase is also impaired in 4.1B$_{200}$-depleted cells strongly suggests that the 4.1B$_{200}$ is an integral part of a spectrin-based network in the Golgi.

ZO-1 and ZO-2 are well-known tight junction markers, located at the apical extreme of the lateral side of confluent epithelial cells (Denker and Nigam, 1998). The mechanism by which they are assembled in the tight junction is unclear. Our finding that correct assembly of ZO-1 and ZO-2 fails in 4.1B$_{200}$-depleted HBE cells demonstrates that 4.1B$_{200}$ is required for proper targeting of ZO-1 and ZO-2 to the tight junction. So far, no other molecule has been reported to have such a role. This finding is somewhat unexpected, because the Golgi and other transport systems are known to have important roles in proper assembly of transmembrane and secretory proteins, whereas ZO-1 and ZO-2 fall into neither class. Although we do not know the manner in which depletion of the 4.1B$_{200}$ leads
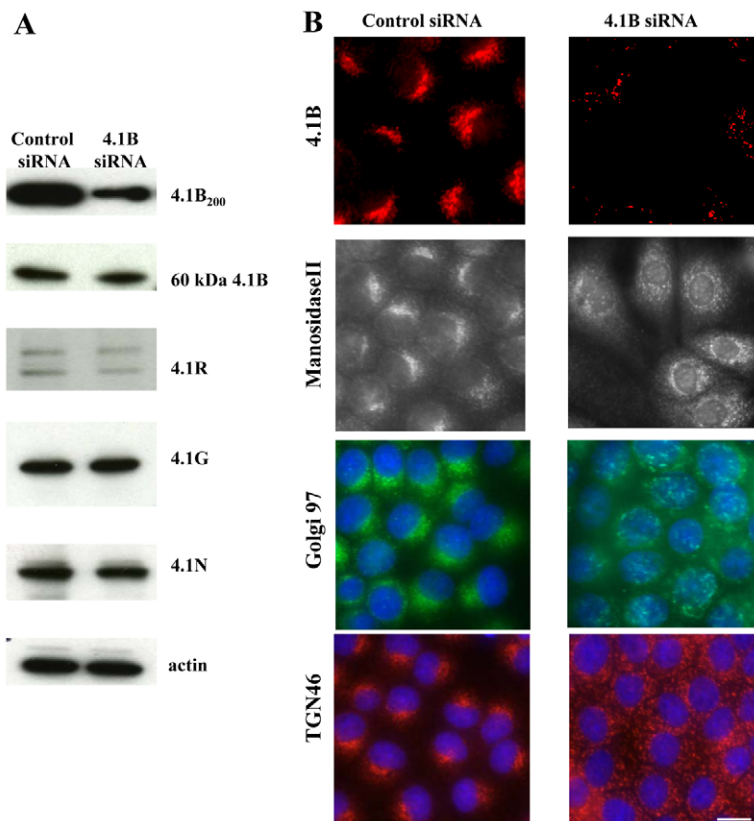

Fig. 6. Depletion of 4.1B$_{200}$ affects Golgi structure. (A) Specific knockdown of 4.1B$_{200}$ in HBE cells. Total cell lysate from HBE cells transfected with control siRNA or 4.1B siRNA were subjected to immunoblot analysis using antibodies against 4.1B-HP, 4.1B-U2, 4.1R exon16, 4.1G-HP, 4.1N-HP and actin. Note the significant reduction of only 4.1B$_{200}$. (B) Fragmentation of Golgi in 4.1B$_{200}$-depleted HBE cells. HBE cells transfected with control siRNA or 4.1B siRNA were stained with anti-4.1B-HP or Golgi markers Golgi 97, TGN46 and mannosidase II. Note the perinuclear staining of all the marker proteins in control cells (left panels) but dispersed staining in 4.1B$_{200}$-depleted cells (right panels). Scale bar: 10 μm.

to impaired assembly of ZO-1 and ZO-2, it appears that a direct interaction between them must be involved.

Spectrin has been identified on many intracellular membranes besides that of the Golgi, including those of chromaffin granules (Aunis and Bader, 1988; Perrin and Aunis, 1985), synaptic vesicles (Sikorski et al., 1991; Zagon et al., 1986), and the endoplasmic reticulum (Zagon et al., 1986). Similarly, 4.1 proteins have been found in synaptic vesicles (Baines and Bennett, 1985; Sikorski et al., 1991) and chromaffin granules (our unpublished data). Thus,

there seems a high likelihood that a membrane skeleton, containing spectrin, ankyrin, 4.1 and probably other analogs of erythrocyte components, exists in many, if not all, intracellular organelle membranes. It will be interesting in future studies to clarify the functions of spectrin-based membrane skeletons in these compartments.

It is also striking that all members of the 4.1 protein family are expressed in HBE and MDCK cells, reflecting, we suppose, the wide participation of these proteins in epithelial cell biology.
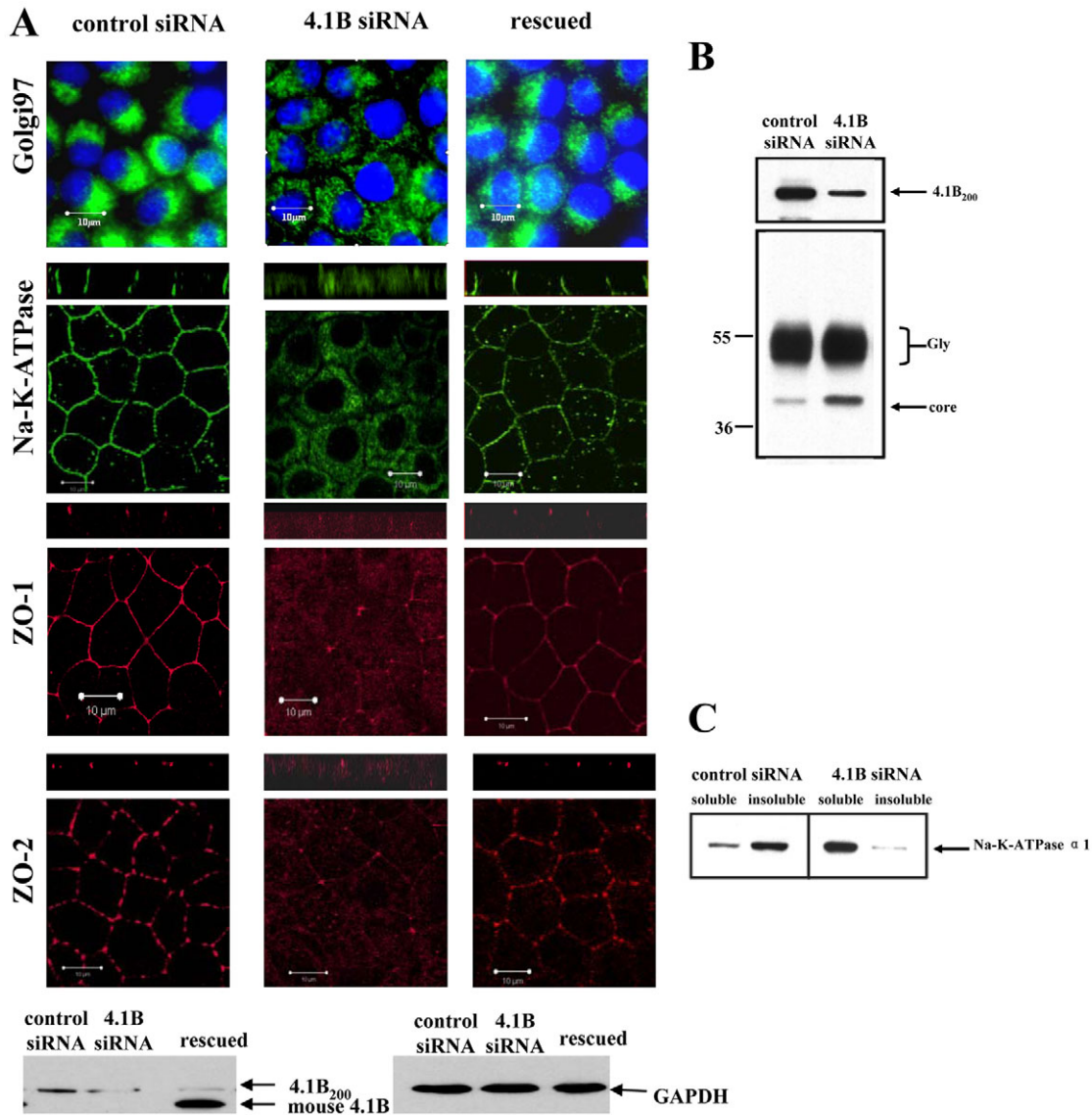

Fig. 7. Depletion of $4.1B_{200}$ results in impaired traffic of Na^+/K^+-ATPase, ZO-1 and ZO-2. (A) Mislocalization of Na^+/K^+-ATPase, ZO-1 and ZO-2 in $4.1B_{200}$-depleted HBE Cells. HBE cells were transfected with control siRNA (left panels), 4.1B siRNA (middle panels) or 4.1B siRNA co-transfected with mouse 4.1B (right panels). Transfected cells were stained with Golgi97, α- Na^+/K^+-ATPase, ZO-1 and ZO-2 antibodies. Note the diffuse distribution of these proteins in $4.1B_{200}$-depleted cells compared with their confinement to perinuclear region (for Golgi97) or the membrane (for α- Na^+/K^+-ATPase, ZO-1 and ZO-2) in control cells. Co-transfection of mouse 4.1B preserved Golgi structure and prevented the failed assembly of Na^+/K^+-ATPase, ZO-1 and ZO-2 in the $4.1B_{200}$-depleted cells. Z-sections are shown at top of each panel. Scale bars: 10 µm. Knockdown of human $4.1B_{200}$ and overexpression of mouse 4.1B was confirmed by western blot analysis (bottom panels). GAPDH was detected as loading control. (B) Altered glycosylation of β- Na^+/K^+-ATPase in $4.1B_{200}$-depleted HBE Cells. Total cell lysates from control or 4.1B-depleted cells were analyzed by western blotting using anti-β- Na^+/K^+-ATPase antibody. The broad band around 55 kDa (Gly) represents the mature glycosylated β-Na^+/K^+-ATPase and the sharp band around 40 kDa represents the ER-dependent core β-Na^+/K^+-ATPase glycosylation. Note a significantly increased core β-Na^+/K^+-ATPase glycosylation in $4.1B_{200}$-depleted cells. (C) Increased detergent-soluble fraction of α-Na^+/K^+-ATPase in $4.1B_{200}$-depleted HBE Cells. Detergent-soluble and detergent-insoluble fractions were analyzed by western blotting using anti-α-Na^+/K^+-ATPase antibody. Note that in control cells, most α-Na^+/K^+-ATPase is in the detergent-insoluble fraction, in marked contrast, in 4.1B-depleted cells, most is in the detergent-soluble fraction.

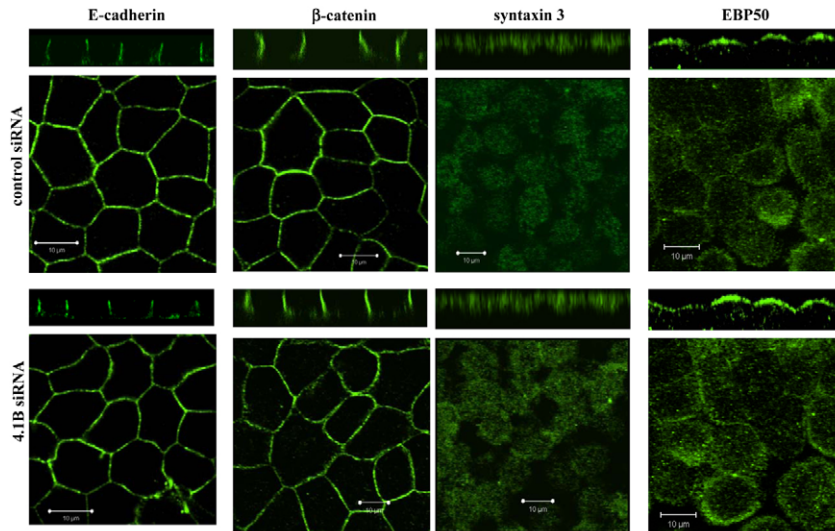**Fig. 8.** Depletion of 4.1B$_{200}$ has no effect on the assembly of adherens junction proteins and apical proteins. HBE cells were transfected with control siRNA or 4.1B siRNA. Transfected cells were stained with E-cadherin, β-catenin, syntaxin 3 and EBP50. In both control (upper panels) and 4.1B$_{200}$-depleted cells (lower panels), E-cadherin and β-catenin are located on the bilateral membrane, and syntaxin 3 and EBP50 on the apical membrane. Z-sections are shown at top of each panel. Scale bars: 10 μm.

Furthermore, the observation that different 4.1 isoforms and splice variants occur in distinct subcellular compartments also suggests that they have diverse functions in epithelial cells. It will be of interest to disentangle the activities of these proteins in epithelial structure and function in future studies.

Materials and Methods
Antibodies
All anti-4.1 antibodies were raised in rabbits at Genemed Synthesis. The antigens used for generation of antibodies against 4.1B, 4.1G and 4.1N were His-tagged recombinant HP or recombinant U2 region. Anti-βI spectrin antibody was also raised

Fig. 9. Depletion of 4.1B$_{200}$ has no effect on protein expression levels. Total cell lysate from HBE cells transfected with control siRNA or 4.1B siRNA was subjected to immunoblot analysis with the indicated antibodies. Note significant knockdown of 4.1B$_{200}$ but not of other proteins.

in rabbit and the antigen used was His-tagged repeat 12 of β-spectrin. The antibodies were affinity-purified using Affigel 10 resin (Bio-Rad) coupled to the corresponding region fused with GST. The antigens used for generation of anti-4.1R antibody were synthetic peptides from exon 16 and exon 19. The peptide antibodies were affinity-purified by using specific peptides immobilized on Sulfolink Coupling Gel (Pierce). The specificity of each antibody was confirmed by western blotting on recombinant full-length protein 4.1 isoforms, on brain tissue (for anti-4.1B antibodies), kidney tissue (for anti-4.1N and anti-4.1R antibodies) and lung tissue (for anti-4.1G antibodies). Tissue samples were also taken from the cognate knockout mice as negative controls. Rabbit polyclonal antibody to TGN46 was from Sigma. Mouse monoclonal anti- ZO-1, rat monoclonal anti-E-cadherin, rabbit polyclonal anti-ZO-2 and anti-Golgi97 were from Invitrogen. Mouse monoclonal antibody to mannosidase II, anti-β-Na$^+$/K$^+$-ATPase, rabbit polyclonal antibodies to EBP50, syntaxin3 and β-catenin were from Abcam. Mouse monoclonal anti-α-Na$^+$/K$^+$-ATPase (clone C464.6) was from Upstate. Mouse monoclonal antibody to GAPDH was from IMGENEX. Alexa Fluor 488-conjugated donkey anti-mouse IgG and anti-rat IgG, Alexa Fluor 594-conjugated donkey anti-rabbit IgG, zenon tricolor mouse IgG, as well as rabbit IgG labeling kit, were from Invitrogen. Anti-Rab5 was kindly provided by Jon S. Morrow (Yale University, New Haven, CT).

Cell culture
MDCK cells were purchased from ATCC. HBE cells were a gift from Vann Bennett (Duke University, Durham, NC). HBE cells and MDCK were routinely grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), penicillin-streptomycin (ATCC) at 37°C in 5% CO$_2$.

4.1B$_{200}$ knockdown by siRNA
To specifically knock down 4.1B$_{200}$, which contains upstream headpiece region and is detected by anti-4.1B-HP antibody, we transfected HBE cells with siRNA sequences which target exon 3 of human EPB41L3 (NM_012307). These pre-designed and validated siRNA sequences were from Ambion and targeted to human 4.1B exon 2. Their sequences are: sense, GCUCGAAUAUCAGCAAUUAtt; antisense, UAAUUGCUGAUAUUCGAGCtg. For transfection, HBE cells were trypsinized, and 2.4×10^5 cells were plated on six-well cell culture plates, 24 hours before transfection. Cells were then transfected with 100 nM of the siRNA. The non-targeting Silencer Control siRNA #1 (Ambion) was used as a negative control. NeoFX™ Transfection Agent was from Ambion and siRNA transfection was performed according to the manufacturer's instructions. 48 hours after transfection, the cells were processed either for immunofluorescence staining or Immunoblot analysis.

Immunofluorescence analysis
For confocal immunofluorescence microscopy assay, cells were grown on MatTek uncoated glass-bottom microwell cell culture dishes (MatTek). Cells were fixed with 1% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 in 0.25% paraformaldehyde-PBS. Cells were then incubated in 10% horse serum, 0.1% Triton X-100 in PBS for 30 minutes to minimize nonspecific antibody binding. The cells were incubated with primary antibodies at 4°C overnight or at room temperature for 2 hours then washed with PBS four times and incubated with the appropriate second antibody at room temperature for 30 minutes. The following primary antibodies were used: rabbit polyclonal antibodies to 4.1B-HP, 4.1B-U2, 4.1N-HP, 4.1G-HP, TGN46, β1-spectrin, β-catenin, ZO-2, EBP50 and syntaxin3; rat monoclonal antibody to E-cadherin; mouse monoclonal antibody to ZO-1, Na$^+$/K$^+$-ATPase,
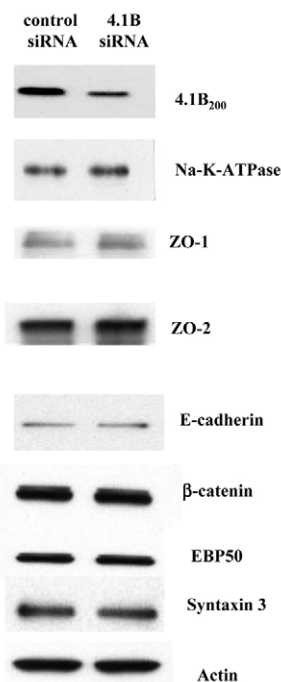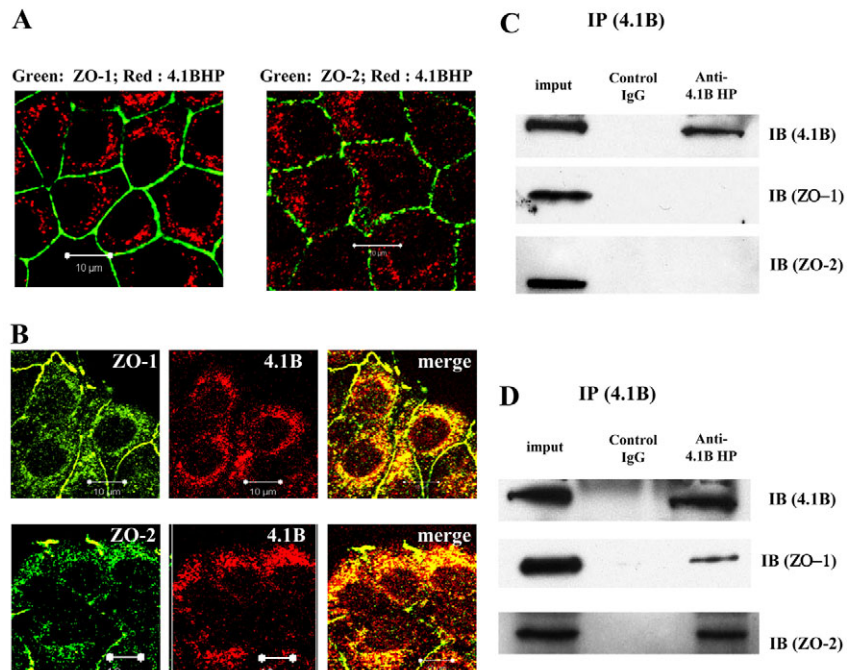
Fig. 10. Association of ZO-1 and ZO-2 with 4.1B$_{200}$ in non-confluent but not confluent HBE cells. (A) Confluent HBE cells were co-stained with rabbit polyclonal anti-4.1B-HP antibody and mouse monoclonal anti-ZO-1 by indirect fluorescence. For co-staining of 4.1B and ZO-2, rabbit anti-4.1B-HP was directly labeled with Alexa Fluor 594 and rabbit anti-ZO-2 was directly labeled with Alexa Fluor 488. No colocalization was observed. Scale bars: 10 μm. (B) Subconfluent HBE cells were co-stained with anti-4.1B-HP and anti-ZO-1 or ZO-2 as described in A. Merged images show some colocalization of 4.1B$_{200}$ with ZO-1 and ZO-2 in the perinuclear region and on the membrane. Scale bars: 10 μm. (C) Immunoprecipitation of confluent HBE cell lysates was performed using anti-4.1B-HP antibody. Proteins in the immunoprecipitate were detected with the antibodies as indicated. Note that ZO-1 and ZO-2 were not brought down with 4.1B$_{200}$. (D) Immunoprecipitation of subconfluent HBE cell lysates and immunoblot were performed as described in C. Note that ZO-1 and ZO-2 were brought down with 4.1B$_{200}$. Pre-immune IgG was used as negative control.

Golgi97 and mannosidase II. The secondary antibodies were donkey anti-rabbit, goat anti-rat and donkey anti-mouse IgG labeled with Alexa Fluor 488 or Alexa Fluor 594. Tropo3 was used to stain the nuclear. For the direct immunofluorescence staining, Alexa Fluor 488, Alexa Fluor 594 zenon rabbit IgG labeling kit (Invitrogen) were used to label the anti-bodies according to the manufacturer's instructions. Images were collected on a Zeiss LSM510 META confocal microscope using either a ×63 or a ×100 oil-immersion objective. Z-stacks were collected at Z increments of 0.2 μm. X-Z images were obtained from Z stacks using the orthogonal function of the LSM510 software.

Co-immunoprecipitation
HBE cells were lysed with ice-cold lysis buffer (50 mM HEPES, pH 8.3, 420 mM KCl, 0.1% NP-40, 1 mM EDTA) for 15 minutes on ice. Supernatant was collected after centrifugation at 16,000 g at 4°C for 10 minutes and the concentration of protein in the supernatant was determined by the Bradford method using BSA as standard (Bio-Rad). 500 μg extract was incubated with 5 μg anti-4.1B-HP antibody or preimmune IgG in 500 μl of Co-IP buffer (Active motif) at 4°C overnight with rotation. The immunoprecipitates were isolated on Protein-G beads and separated by 10% SDS-PAGE followed by transfer to nitrocellulose membrane. The membrane was probed with antibodies against 4.1B-HP, β1-spectrin, ZO-1, ZO-2 and GAPDH.

Immunoblot analysis
Cells were trypsinized, washed with PBS, then lysed with ice-cold lysis buffer (50 mM HEPES, pH 8.3, 420 mM KCl, 0.1% NP-40, 1 mM EDTA) for 15 minutes on ice in the presence of proteinase inhibitor cocktail (Sigma). After centrifugation at 16,000 g at 4°C for 10 minutes, the supernatant was collected. Detergent-soluble and detergent-insoluble fractions were prepared as follows. HBE cells were washed with ice-cold PBS, then sequential extracts were performed in the following buffers: buffer 1, 10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl$_2$, 0.5% Triton X-100 and 1.2 mM PMSF; buffer 2, identical to buffer 1 except that 250 mM ammonium sulfate was substituted for 100 mM NaCl. The first extraction was for 5 minutes on ice with buffer 1. The supernatant was collected and this yielded a detergent-soluble fraction. The pellets were further extracted on ice for 10 minutes with buffer 2. This high-salt buffer solubilized the 'cytoskeletal' fraction of proteins (detergent-insoluble fraction). Protein concentration was measured by the Bradford method using BSA as standard.10 mg protein was separated in 10% SDS-PAGE and transferred to nitrocellulose membrane. After blocking for 1 hour in blocking buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween-20, 5% non-fat dried milk powder), the blot was probed for 1 hour with the desired primary antibodies. After several washes, the blot was incubated with anti-rabbit or anti-mouse IgG coupled to HRP and developed with the SuperSignal West Pico chemiluminescence detection kit (Molecular Probes). All steps were performed at room temperature.

Brefedin A treatment
HBE cells grown on MatTek uncoated glass-bottomed microwell cell culture dishes were treated with 6 μg/ml of Brefedin A (Ebioscience) at 37°C for 60 minutes; 1%

ethanol was used as a negative control. For BFA-washout experiments, BFA-treated cells were washed with PBS and further incubated in BFA-free medium for 24 hours at 37°C.

References
Aunis, D. and Bader, M. F. (1988). The cytoskeleton as a barrier to exocytosis in secretory cells. *J. Exp. Biol.* **139**, 253-266.

Baines, A. J. and Bennett, V. (1985). Synapsin I is a spectrin-binding protein immunologically related to erythrocyte protein 4.1. *Nature* **315**, 410-413.

Barr, F. A. and Short, B. (2003). Golgins in the structure and dynamics of the Golgi apparatus. *Curr. Opin. Cell Biol.* **15**, 405-413.

Beck, K. A. (2005). Spectrins and the Golgi. *Biochim. Biophys. Acta* **1744**, 374-382.

Beck, K. A., Buchanan, J. A., Malhotra, V. and Nelson, W. J. (1994). Golgi spectrin: identification of an erythroid beta-spectrin homolog associated with the Golgi complex. *J. Cell Biol.* **127**, 707-723.

Beck, K. A., Buchanan, J. A. and Nelson, W. J. (1997). Golgi membrane skeleton: identification, localization and oligomerization of a 195 kDa ankyrin isoform associated with the Golgi complex. *J. Cell Sci.* **110**, 1239-1249.

Cole, N. B., Sciaky, N., Marotta, A., Song, J. and Lippincott-Schwartz, J. (1996). Golgi dispersal during microtubule disruption: regeneration of Golgi stacks at peripheral endoplasmic reticulum exit sites. *Mol. Biol. Cell* **7**, 631-650.

Conboy, J. (1987). Molecular cloning and characterization of the gene coding for red cell membrane skeletal protein 4.1. *Biorheology* **24**, 673-687.

Conboy, J. G., Chan, J., Mohandas, N. and Kan, Y. W. (1988). Multiple protein 4.1 isoforms produced by alternative splicing in human erythroid cells. *Proc. Natl. Acad. Sci. USA* **85**, 9062-9065.

Conboy, J. G., Chan, J. Y., Chasis, J. A., Kan, Y. W. and Mohandas, N. (1991). Tissue- and development-specific alternative RNA splicing regulates expression of multiple isoforms of erythroid membrane protein 4.1. *J. Biol. Chem.* **266**, 8273-8280.

Correas, I. (1991). Characterization of isoforms of protein 4.1 present in the nucleus. *Biochem. J.* **279**, 581-585.

De Matteis, M. A. and Morrow, J. S. (2000). Spectrin tethers and mesh in the biosynthetic pathway. *J. Cell Sci.* **113**, 2331-2343.

Denker, B. M. and Nigam, S. K. (1998). Molecular structure and assembly of the tight junction. *Am. J. Physiol.* **274**, F1-F9.

Devarajan, P., Stabach, P. R., Mann, A. S., Ardito, T., Kashgarian, M. and Morrow, J. S. (1996). Identification of a small cytoplasmic ankyrin (AnkG119) in the kidney and

muscle that binds beta I sigma spectrin and associates with the Golgi apparatus. *J. Cell Biol.* **133**, 819-830.

Devarajan, P., Stabach, P. R., De Matteis, M. A. and Morrow, J. S. (1997). Na,K-ATPase transport from endoplasmic reticulum to Golgi requires the Golgi spectrin-ankyrin G119 skeleton in Madin Darby canine kidney cells. *Proc. Natl. Acad. Sci. USA* **94**, 10711-10716.

Ho, W. C., Allan, V. J., van Meer, G., Berger, E. G. and Kreis, T. E. (1989). Reclustering of scattered Golgi elements occurs along microtubules. *Eur. J. Cell Biol.* **48**, 250-263.

Holappa, K., Munoz, M. T., Egea, G. and Kellokumpu, S. (2004). The AE2 anion exchanger is necessary for the structural integrity of the Golgi apparatus in mammalian cells. *FEBS Lett.* **564**, 97-103.

Krauss, S. W., Chasis, J. A., Rogers, C., Mohandas, N., Krockmalnic, G. and Penman, S. (1997). Structural protein 4.1 is located in mammalian centrosomes. *Proc. Natl. Acad. Sci. USA* **94**, 7297-7302.

Mattagajasingh, S. N., Huang, S. C., Hartenstein, J. S. and Benz, E. J., Jr (2000). Characterization of the interaction between protein 4.1R and ZO-2. A possible link between the tight junction and the actin cytoskeleton. *J. Biol. Chem.* **275**, 30573-30585.

Mays, R. W., Siemers, K. A., Fritz, B. A., Lowe, A. W., van Meer, G. and Nelson, W. J. (1995). Hierarchy of mechanisms involved in generating Na/K-ATPase polarity in MDCK epithelial cells. *J. Cell Biol.* **130**, 1105-1115.

Morrow, J. S., Cianci, C. D., Ardito, T., Mann, A. S. and Kashgarian, M. (1989). Ankyrin links fodrin to the alpha subunit of Na,K-ATPase in Madin-Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* **108**, 455-465.

Narula, N., McMorrow, I., Plopper, G., Doherty, J., Matlin, K. S., Burke, B. and Stow, J. L. (1992). Identification of a 200-kD, brefeldin-sensitive protein on Golgi membranes. *J. Cell Biol.* **117**, 27-38.

Nelson, W. J. and Veshnock, P. J. (1986). Dynamics of membrane-skeleton (fodrin) organization during development of polarity in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* **103**, 1751-1765.

Nelson, W. J. and Hammerton, R. W. (1989). A membrane-cytoskeletal complex containing Na+,K+-ATPase, ankyrin, and fodrin in Madin-Darby canine kidney (MDCK) cells: implications for the biogenesis of epithelial cell polarity. *J. Cell Biol.* **108**, 893-902.

Parra, M., Gascard, P., Walensky, L. D., Snyder, S. H., Mohandas, N. and Conboy, J. G. (1998). Cloning and characterization of 4.1G (EPB41L2), a new member of the skeletal protein 4.1 (EPB41) gene family. *Genomics* **49**, 298-306.

Parra, M., Gascard, P., Walensky, L. D., Gimm, J. A., Blackshaw, S., Chan, N., Takakuwa, Y., Berger, T., Lee, G., Chasis, J. A. et al. (2000). Molecular and functional characterization of protein 4.1B, a novel member of the protein 4.1 family with high level, focal expression in brain. *J. Biol. Chem.* **275**, 3247-3255.

Perrin, D. and Aunis, D. (1985). Reorganization of alpha-fodrin induced by stimulation in secretory cells. *Nature* **315**, 589-592.

Salomao, M., Zhang, X., Yang, Y., Lee, S., Hartwig, J. H., Chasis, J. A., Mohandas, N. and An, X. (2008). Protein 4.1R-dependent multiprotein complex: new insights into the structural organization of the red blood cell membrane. *Proc. Natl. Acad. Sci. USA* **105**, 8026-8031.

Sikorski, A. F., Terlecki, G., Zagon, I. S. and Goodman, S. R. (1991). Synapsin I-mediated interaction of brain spectrin with synaptic vesicles. *J. Cell Biol.* **114**, 313-318.

Stankewich, M. C., Tse, W. T., Peters, L. L., Ch'ng, Y., John, K. M., Stabach, P. R., Devarajan, P., Morrow, J. S. and Lux, S. E. (1998). A widely expressed betaIII spectrin associated with Golgi and cytoplasmic vesicles. *Proc. Natl. Acad. Sci. USA* **95**, 14158-14163.

Sun, C. X., Robb, V. A. and Gutmann, D. H. (2002). Protein 4.1 tumor suppressors: getting a FERM grip on growth regulation. *J. Cell Sci.* **115**, 3991-4000.

Tchernia, G., Mohandas, N. and Shohet, S. (1981). Deficiency of skeletal membrane protein band 4.1 in homozygous hereditary elliptocytosis: implications for erythrocyte membrane stability. *J. Clin. Invest.* **68**, 454-460.

Thyberg, J. and Moskalewski, S. (1985). Microtubules and the organization of the Golgi complex. *Exp. Cell Res.* **159**, 1-16.

Walensky, L. D., Blackshaw, S., Liao, D., Watkins, C. C., Weier, H. U., Parra, M., Huganir, R. L., Conboy, J. G., Mohandas, N. and Snyder, S. H. (1999). A novel neuron-enriched homolog of the erythrocyte membrane cytoskeletal protein 4.1. *J. Neurosci.* **19**, 6457-6467.

Yawata, A., Kanzaki, A., Gilsanz, F., Delaunay, J. and Yawata, Y. (1997). A markedly disrupted skeletal network with abnormally distributed intramembrane particles in complete protein 4.1-deficient red blood cells (allele 4.1 Madrid): implications regarding a critical role of protein 4.1 in maintenance of the integrity of the red blood cell membrane. *Blood* **90**, 2471-2481.

Zagon, I. S., Higbee, R., Riederer, B. M. and Goodman, S. R. (1986). Spectrin subtypes in mammalian brain: an immunoelectron microscopic study. *J. Neurosci.* **6**, 2977-2986.