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Unglycosylation at Asn-633 made extracellular domain of E-cadherin folded incorrectly and arrested in endoplasmic reticulum, then sequentially degraded by ERAD

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Abstract The human E-cadherin is a single transmembrane domain protein involved in Ca^{2+}-dependent cell–cell adhesion. In a previous study, we demonstrated that all of four potential N-glycosylation sites in E-cadherin are occupied by N-glycans in human breast carcinoma cells *in vivo* and the elimination of N-glycan at Asn-633 dramatically affected E-cadherin expression and made it degraded. In this study we investigated the molecular mechanism of E-cadherin, which lacks N-glycosylation at Asn-633 (M4), degradation and the role of the N-glycan at Asn-633 in E-cadherin folding. We treated cells stably expressed M4 E-cadherin with MG123, DMM, respectively. Either MG132 or DMM could efficiently block degradation of M4 E-cadherin. M4 E-cadherin was recognized as the substrate of ERAD and was retro-translocated from ER lumen to cytoplasm by p97. It was observed that the ration of M4 E-cadherin binding to calnexin was significantly increased compared with that of other variants, suggesting that it was a misfolded protein, though cytoplasmic domain of M4 E-cadherin could associate with β-catenin. Furthermore, we found that N-glycans of M4 E-cadherin were modified in immature high mannose type, suggesting that it could not depart to Golgi apparatus. In conclusion, this study revealed that N-glycosylation at Asn-633 is essential for E-cadherin expression, folding and trafficking.

Keywords E-cadherin · N-glycosylation · ERAD · ER–Golgi trafficking

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Abbreviation

EC	extracellular domain
AJs	adherence junction complexes
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
WT	wild type E-cadherin
M1	E-cadherin lacking N-glycan at Asn-554 site
M2	E-cadherin lacking N-glycan at Asn-566 site
M3	E-cadherin lacking N-glycan at Asn-618 site
M4	E-cadherin lacking N-glycan at Asn-633 site
M123	E-cadherin has a single N-glycan at Asn-633 site
Endo H	endoglycosidase
PNGase F	peptide N-glycosidase F
PBS	phosphate-buffered saline

Introduction

The human E-cadherin belongs to a classic cadherin family (*e.g*. E-, P-, and N-cadherin), which is involved in Ca^{2+}-dependent cell–cell adhesion, morphogenetic processes, differentiation and establishing and maintaining cell polarity [1, 2]. E-cadherin has an extracellular domain composed of five tandemly repeated subdomains of about 110 amino acids each, a single transmembrane region and a cytoplasmic domain. The five extracellular subdomains of classic cadherins together coordinate several Ca^{2+} ions to maintain the rod-like conformation of the entire extracellular region [3] and allow interaction with other cadherins, resulting in their adhesive activity [4].

The cytoplasmic domain of E-cadherin is the most conserved region. The interactions of the cytoplasmic parts with the actin cytoskeleton are mediated by the catenins, such as β-catenin, P120 (plakoglobin), and α-catenin. β-Catenin and P120 (plakoglobin) bind directly to the distal regions of the cadherin cytoplasmic tail in a mutually exclusive manner, whereas α-catenin was recruited by β-catenin and P120 (plakoglobin) to link E-cadherin, either directly or indirectly, and mediate association of E-cadherin–catenin complex and actin cytoskeleton [5–9]. These interactions seem to be involved in regulation of E-cadherin adhesive activity [10, 11].

Human E-cadherin has four potential N-glycosylation sites, two in the extracellular subdomain 4 (EC4) at Asn residues 554, 566, two in the extracellular subdomain 5 (EC5) at Asn residues 618, 633. Experiments with cadherin mutants and prediction of the cadherin three-dimensional structure show that EC1 and EC2 subdomains play a main role in the homophilic binding specificity of the cadherins [12]. Analysis of fractional E-cadherin crystals suggests that EC1 and EC2 participate in forming lateral homodimers in Ca^{2+}-dependent manner [13]. These homogenous lateral dimers were fundamental units for E-cadherin mediated cell–cell adhesion, but not the sole critical step in regulating E-cadherin adhesion activity [14]. Evidences also showed that N-glycosylation has an important role in regulation of E-cadherin adhesion activity. Increasing a specific structure, the bisecting GlcNAc, in E-cadherin could delay E-cadherin turnover and decreased release from the cell surface. Furthermore, the changes of N-glycan types could enhance the cell–cell adhesion and down-regulate the intracellular signal pathway [15, 16]. The variation in E-cadherin N-glycosylation from high mannose N-glycans to complex N-glycans could affect the molecular organization of adherence junction complexes (AJs) by influencing their composition and association with the actin cytoskeleton. These changes could regulate the assemble or disassemble of cell–cell adhesion during different environmental conditions [17]. The basic function of the proteins N-glycosylation in eukaryotic cells have been known for several years, but only recently has the full significance of this covalent modification in glycoprotein folding become evident. N-glycans provide bulky, highly hydrophilic groups that help to maintain glycoproteins in solution during the folding process or modulate protein conformation by forcing amino acids close to the linking Asn units to be in the proximity of the water–glycoprotein interphase [18].

If folding correctly, glycoproteins destined for distal compartments exit the ER via vesicular or tubular structures [19–25].

Because aberrant proteins may expose hydrophobic regions and free cysteines and have a tendency to aggregation, and these misfolded or aggregated proteins are potentially cytotoxic, cells develop a complex quality control system including many chaperones to monitor proteins folding and remove aberrant proteins. UGGT (UDP-Glc:glycoprotein glucosyltransferase) was one of members of the quality control system. UGGT can recognize the misfolded proteins released from calnexin/calreticulin, and bind to the monoglucosylates N-glycans in regions with exposed hydrophobic patches, then these glycoproteins reglycosylated and reassociate with calnexin/calreticulin until these glycoproteins achieve proper folding. This process was known as calnexin/calreticulin cycles [26, 27]. α-Mannosidase I was another important monitor in quality control system. If after a certain period of time the glycoproteins are not properly folded, a specific mannose residue (mannose-b) would be removed by α-mannosidase I to generate a $Man_8GlcNAc_2$ structure. This mannose trimming is known as the beginning signal of degradation. Glycoproteins with $Man_8GlcNAc_2$ can still be reglucosylated, and enter the calnexin/calreticulin cycles. Finally, progressive mannose trimming yield Man_{6}-$GlcNAc_2$ structure and effectively removes the glycoproteins from the calnexin/calreticulin cycles [28–30]. These misfolded glycoproteins with $Man_{8\text{-}6}GlcNAc_2$ sequentially associated with a putative lectin EDEM, a homologous to ER α-mannosidase I, but lacks enzymatic activity [31, 32]. The EDEM-misfolded complex recruits other chaperons to ubiquitinate substrates and form retro-translocation complexes. A growing number of E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases, ubiquitin-binding proteins, shuttles or delivery proteins, and deubiquitinating enzymes have been identified in the retro-translocation complexes [33, 34]. A transmembrane protein on the ER membrane, VIMP associates with retro-translocation complexes, and recruits a cytosolic AAA ATPase p97. p97 retro-translocates the substrates from ER lumen into cytoplasm through Sec61β proteins channel and deliver them to proteasomes for degradation, this pathway is also called ER-association degradation (ERAD) [35–37].

Our previous study had showed that all of four potential N-glycosylation consensus sites were utilized by human E-

cadherin *in vivo*. But once eliminated the N-glycan at Asn-633, the expression of E-cadherin could not be detected, suggesting that N-glycosylation at Asn-633 is crucial for E-cadherin stability [38]. To investigate the degradation of E-cadherin lacking N-glycan at Asn-633 in detail, we generated N-glycosylation-deficient mutants by substituting glutamine for asparagine in each N-glycosylation consensus sequence of human E-cadherin, either individually or in combination, by site-directed mutagenesis using PCR. Wild type and mutated cDNAs were expressed in human breast carcinoma cells line MDA-MB-435. We found that N-glycosylation at Asn-633 is essential for E-cadherin expression, folding and trafficking. Unglycosylation at Asn-633 site made E-cadherin recognized as a misfolded protein and finally degraded via ERAD pathway.

Materials and methods

Cell lines, antibodies and reagents

The E-cadherin-negative human breast carcinoma cell lines MDA-MB-435 were maintained in culture medium (DMEM, 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin) in an atmosphere containing 5% CO_2 at 37°C. MG132, the inhibitor of proteasomes, DMM (1,5-Dideoxy-1,5-imino-D-mannitol hydrochloride), the inhibitor of α-mannosidases I, and chloroquine, the inhibitor of lysosomes were purchased from Sigma (Sigma, St. Louis, MO). PNGase F and Endo H were purchased from Roche (Roche Diagnostics). Monoclonal mouse antibodies against α-catenin, β-catenin, calnexin, and α-tubulin were obtained from Santa Cruz (Santa Cruz, CA, USA). Monoclonal mouse antibody against p97 was obtained from Biodesign (Biodesign, Saco, USA). Monoclonal mouse antibody against E-cadherin was obtained from BD Transduction Laboratories (Becton, Dickinson and Company, USA). Monoclonal mouse antibody against ubiquitin was obtained from Biomol. (Biomol international Lp, USA). Monoclonal mouse antibody against GAPDH and secondary antibodies conjugated with HRP were from Kang-Chen Biotech (Shanghai, China). The protein A/G plus-agarose was purchased from Santa Cruz (Santa Cruz, CA, USA). The production of horseradish peroxidase-linked lectins *Galanthus nivalis agglutinin* (GNA) and *wheat germ agglutinin* (WGA) were performed as described previously [39].

Plasmids construction, site-directed mutagenesis and transfections

The plasmid pcDNA3.0-E-cad contains human full-length *E-cadherin* cDNA (Gene Bank Accession No. L08599) was kindly supplied by Dr. Cara J. Gottardi (Memorial Sloan-Kettering Cancer Center, New York, USA). Human E-cadherin contains four N-glycosylation sites (Asn-Xaa-Ser/Thr); Asn-554, Asn-566, Asn-618, Asn-633, as shown in Fig. 1, which are located in the extracellular segment and are well conserved in the human, mouse and rat. The E-cadherin variants were constructed with unglycosylation of a selected single N-glycan or different combinations as shown in Fig. 1: four single mutants (M1, M2, M3, M4); combined mutants (M123).

To create either individual or combined mutants of N-glycosylation sites of E-cadherin, a PCR-based site-directed mutagenesis was carried out using a three-round method. In the first-round PCR, the forward primer was 5′-AGT GAC GAA TGT GGT ACC TTT TGA-3′ (for N554, 566, 618 and 633), and the reverse primers were 5′-TTA GGG CTG TGT ACG TGC TTT GCT TCA-3′ (for N554), 5′-AGC AAC TGG AGA ACC TTG GTC TGT AGC TAT-3′ (for N566), 5′-TGA AGG GAG ATG TTT GGG GAG GAA GGT C-3′ (for N618), and 5′-TAC TGA ATG GTC CATTGG GGC ACT CGC C-3′ (for N633). In the second round, the forward primers were 5′-TGA AGC AAA GCA CGT ACA CAG CCC TAA-3′ (for N554), 5′-ATA GCT ACA GAC CAA GGT TCT CCA GTT GCT-3′ (for N566), 5′-GAC CTT CCT CCC CAA ACA TCT CCC TTC A-3′ (for N618), 5′-GGC GAG TGC CCC AAT GGA CCA TTC AGT A-3′ (for N633), and the reverse primer was 5′-GC TCT AGA TCT CGA GTC CCC TAG TGG TCC-3′ (for N554, 566, 618, and 633). In the last round, PCR products from the first two steps were purified, ligated and used to replace the similar fragment of human E-cadherin cDNA plasmid. Mutants were confirmed by automatic DNA sequencing. Recombinant expression plasmids carrying the wild type or mutated E-cadherin cDNAs were purified and transfected into 3×10^5 cells by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Cell lines stably expressed E-cadherin variants were selected by G418 (800 μg/ml) and screened by RT-PCR and Western blotting analysis.

PNGase F and Endo H sensitivity assay

Total cell lysates were digested with 1 U of PNGase F or Endo H for 1 h at 37°C, loaded onto a 10% SDS-PAGE, and examined by Western blotting. For controls, samples were incubated without the enzymes.

Western blotting

Triton-soluble cells extracts were prepared by directly lysing cells in a buffer containing 10 mM Tris–HCl, pH 6.8, 1 mM EDTA, 150 mM NaCl, 0.25% Nonidet P-40, 1% Triton X-100, and 1 mM PMSF, 1 mM Na_3VO_4 followed

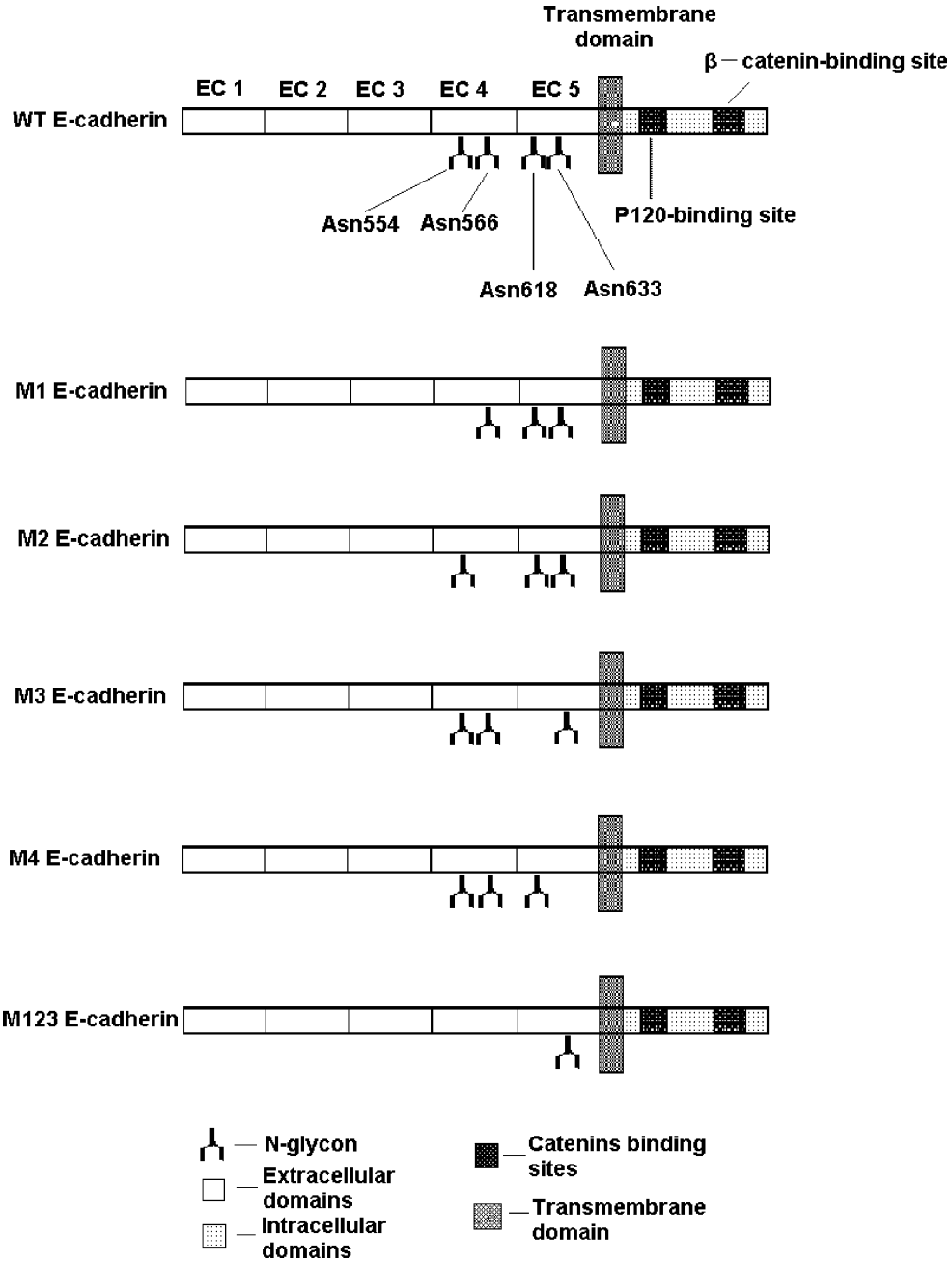

Fig. 1 Schematic illustration of potential N-glycosylation sites of the human E-cadherin. The sites corresponding to putative N-glycosylation sites (Asn-554, Asn-566, Asn-618, Asn-633) are shown by cartoon icon. A wild type E-cadherin cDNA (Genebank, L08599) was used as template to generate mutants in potential N-glycan addition sites singly and in combinations, by substituting asparagine (Asn) within the N-glycan addition consensus sequences with glutamine (Gln), at indicated sites in EC4 and EC5 domain. M1 E-cadherin, lacking Asn-554; M2 E-cadherin, lacking Asn-566; M3 E-cadherin, lacking Asn-618; M4 E-cadherin, lacking Asn-633; M123 E-cadherin, lacking Asn-554, Asn-566, and Asn-618

by a spin at 16,000×*g*. For Triton-insoluble extracts, the remaining pellet was re-extracted twice with lysis buffer to ensure that all detergent-soluble material was removed. The final pellet after a spin at 16,000×*g* was extracted with an SDS-containing buffer (10 mM Tris–HCl, pH 6.8, 2 mM EDTA, 150 mM NaCl, 1% SDS). The samples were quantitated using the BCA protein assay. For total proteins, cells were lysed in 1× SDS lysis buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM PMSF, and 1 mM Na_3VO_4). Equal amount of cells lysates were fractionated on either 7.5–10% SDS-PAGE and blotted onto PVDF membranes (Millipore, Corp.). For comparison of expression levels of the wild type E-cadherin and its N-glycosylation deficient variants in MDA-MB-435 cells, typically 50 μg of total cellular proteins were used. For immunoblot analysis of Triton-soluble fractions, samples were loaded at equal amounts of protein (30 μg). Their respective insoluble fractions were loaded at volumes equal to the soluble fractions so that direct comparisons could be made. The samples were blocked in PBS–Tween (PBST) (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.05% Tween-20.) or TBS–Tween (TBST) (0.1 M Tris–HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween-20) with 5% nonfat dry milk, and membranes were incubated with primary antibodies at

appropriate dilutions in PBST/TBST with 5% nonfat milk overnight at 4°C. Next, membranes were washed three times with PBST/TBST solution, followed by incubation with horseradish peroxidase-linked secondary antibody (1:3,000) in PBST/TBST with 5% nonfat milk. The results were visualized by fluorography using an enhanced chemiluminescence system (Perfect Biotech, Shanghai, China).

Immunoprecipitation

Cells were washed with ice-cold PBS, and lysed in lysis buffer (containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 15 mM EGTA, 0.5% (*w*/*v*) Nonidet P-40, 1 mM PMSF, 1 mM DTT, 1 mM Na_2VO_3, 100 mM NaF, 5 μg/ml leupeptin, 5 μg/ml aprotinin). The cells lysates were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were collected, and protein concentration was determined by means of Lowry protein assay. Equal amounts of protein samples (1 mg) were incubated with 1 μg of each antibody for 1.5 h and then 20 μl of protein A/G plus-agarose for incubation at 4°C overnight. The immunoprecipitates were washed four times with lysis buffer. Samples were resuspended in 15 μl of 2× SDS sample buffer and boiled 3 min at 95°C prior to analyses by Western and lectin blotting.

Lectin blotting

PVDF membranes were incubated overnight with horseradish peroxidase-linked lectins *Galanthus nivalis agglutinin* (GNA), *wheat germ agglutinin* (WGA) at 4°C. Next the blots were washed three times with PBST solution. The results were visualized with ECL Plus Detection Reagents.

Immunofluorescence

For immunofluorescence analysis, cells stably expressed E-cadherin variants were cultured on glass coverslip for 8 h. Before fix, cells stably expressed M4 E-cadherin were treated with MG132 (50 μM) for 2 h. When cells grown to 70% confluence, wild type and mutated E-cadherin transfected MDA-MB-435 cells were fixed in 4% paraformaldehyde for 0.5 h at 4°C, permeabilized with 0.1% Triton X-100 for 0.5 h at 4°C, and washed three times with PBS. The samples were blocked with PBS containing 3% BSA, and incubated with primary antibodies against to E-cadherin for 4 h at 37°C. Cells were wash three times with PBS containing 3% BSA, and then incubated with FITC-linked secondary antibodies (Sigma, St. Louis, MO.) for 1 h at 37°C. Finally, cells were washed three times with PBS. The immunostained samples were analyzed with a Nikon Eclipse TE300 epifluorescence microscope.

Conformation predication

The prediction of three-dimensional structure of E-cadherin was performed by SWISS-MODEL WORKSPACE online sever (available at http://swissmodel.expasy.org/workspace/), and the display of modeling projection file using program DeepView, available at: http://www.expasy.org/spdbv/. The evaluation of hydrophobicity of amid acid residues was performed by ExPASy Proteomics tools online sever: Protscale (available at http://www.expasy.ch/tools/protscale.html).

Results

E-cadherin lacking N-glycosylation at Asn-633 was degraded by proteasomes in ubiquitin-dependent manner

Analysis of sequences of E-cadherin showed that there are four potential N-glycosylation sites in the EC4 and EC5 subdomain, our previous study had demonstrated that all of four N-glycosylation sites were utilized by human E-cadherin *in vivo*. Compared with other sites, N-glycans at Asn-554 and Asn-566 were more important in E-cadherin-mediated calcium-dependent cell–cell adhesion. Surprisingly, N-glycan at Asn-633 site affected the stability of E-cadherin, compared with other variants, M4 E-cadherin could not be expressed, even though its mRNA could be detected [38]. To investigate the degradation of M4 E-cadherin in detail, we treated cells stably expressing M4 E-cadherin with inhibitor of proteasomes, MG132 and inhibitor of lysosomes, chloroquine, respectively. As expected, MG132 blocked the degradation of M4 E-cadherin effectively as observed in the 4 h (Fig. 2a). In contrast, chloroquine had no effect on M4 E-cadherin degradation (Fig. 2b). However, according to Liwosz *et al.* [17], E-cadherin lacking N-glycan at Asn-633 still could be stably expressed in CHO and MDCK cells. To reconcile the discrepancy, we transfected other three cell lines with M4 E-cadherin to research the degradation of M4 E-cadherin in different cell lines. Results showed that E-cadherin lacking N-glycan at Asn-633 had different destiny in different cell lines. After transfection, 7721 cells could not express M4 E-cadherin, and in Hela cells, a trace of E-cadherin could be observed. However, the expression of E-cadherin significantly increased in 293T cells, which likely ascribe to the expression of M4 E-cadherin plasmid (Fig. 2c).

Next we examined whether the E-cadherin was ubiquitinated while it was degraded by proteasomes, a co-immunoprecipitation assay was carried out. The ubiquitinated E-cadherin ladder bands were detected, and as control, no ubiquitinated E-cadherin bands were

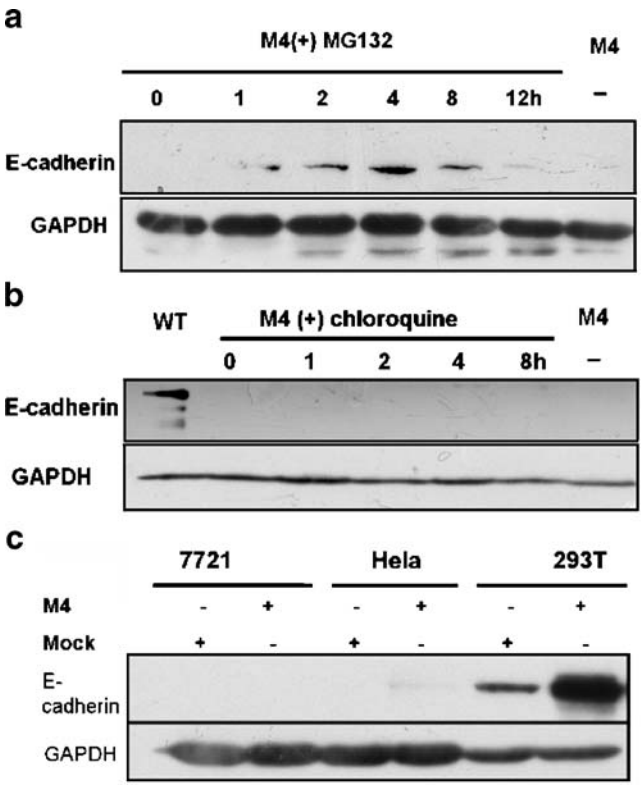

Fig. 2 M4 E-cadherin was degraded by proteasomes. **a** M4 E-cadherin was degraded by proteasomes. Cells stably expressing M4 E-cadherin were treated with M132 (50 μM) for different time. Thirty μg of total protein was fractionated on 10% SDS gel and the presence of M4 E-cadherin was detected by using antibodies against to E-cadherin. Results showed that MG132 could successful suppress the degradation of M4 E-cadherin at 1 h, and the expression level of M4 E-cadherin reach peak at 4 h. **b** Lysosomes did not participate in the degradation of M4 E-cadherin. Cells stably expressing M4 E-cadherin were treated with chloroquine (40 μM) for different times. Thirty μg of total protein was fractionated on 10% SDS gel and the presence of M4 E-cadherin was detected by using antibodies against to E-cadherin. Chloroquine had no effect on the expression of M4 E-cadherin. **c** The degradation of M4 E-cadherin in three cell lines. The 7721 cells, Hela cells, and 293T cells were transiently transfected with M4 E-cadherin. After cultured 48 h, cells were lysed used 1× SDS lysis, then 30 μg of total proteins were fractionated on 10% SDS gel. The presence of M4 E-cadherin was detected use antibody against to E-cadherin

detected in the mock cell stably expressing the vector (Fig. 3). These results clearly demonstrated that N-glycosylation at the Asn-633 is essential for E-cadherin expression. The elimination of this N-glycan made E-cadherin degraded by proteasomes in ubiquitin-dependent manner. Because of the importance of N-glycosylation at Asn-633 of E-cadherin, we focus our interest on M4 E-cadherin in the following works.

M4 E-cadherin was substrate of ERAD

Correctly transmembrane proteins, after folded are transported through secretory pathway, while misfolded or unfolded proteins would retain in ER. These unfolded proteins expose hydrophobic amino-acid residues and tend to form protein aggregation. Protein aggregations are so toxic that they induce apoptotic cell death. To deal with these toxic proteins accumulation in ER, cells developed an effective mechanism which called ER associated degradation (ERAD) to retro-translocated unfolded proteins to the cytoplasm and make them degraded by the proteasomes [40]. α-mannosidase I is an important sensor in ERAD. It can recognize misfolded glycoprotein and trims a specific mannose residue to generate $Man_8GlcNAc_2$ type N-glycans which are known as the signal of ERAD [28–30]. 1-Deoxymannojirimycin (DMM), an inhibitor of α-mannosidase I, could specifically suppressed ERAD in Chinese hamster ovary cells [19]. To investigate the effect of DMM on the degradation of M4 E-cadherin, we treated cells stably expressing M4 E-cadherin with DMM (0.5 μM), as expected, DMM effectively made M4 E-cadherin re-expressed as observed in 0.5 h (Fig. 4a). $p97/VCP^{Ufd1–Npl4}$ is a hexameric type II AAA ATPases which mediates disparate cellular functions including ERAD. A general model for the role of $p97/VCP^{Ufd1–Npl4}$ in ERAD involves binding of polyubiquitinated ERAD substrates at the cytoplasmic face of the ER membrane both before and after substrate ubiquitination, followed by a complete substrate extraction/dislocation and its transfer to the 26S proteasomes [41]. To examine the interaction between p97 and M4 E-cadherin, a co-immunoprecipitation assay was carried out by using antibodies against to p97 and E-cadherin, respectively. As shown in Fig. 4b, association of M4 E-cadherin

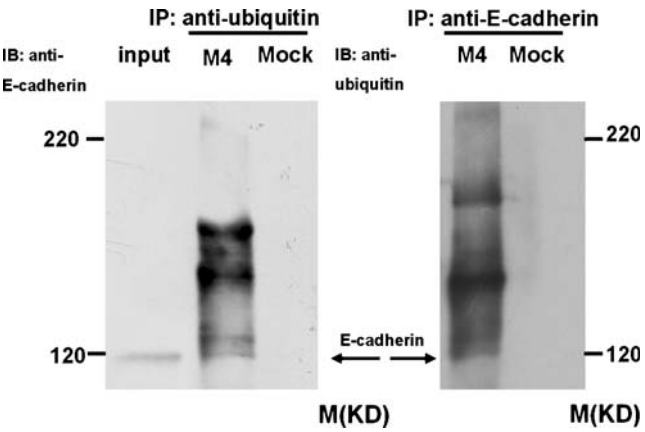

Fig. 3 Ubiquitination of the E-cadherin in MDA-MB-435 cells. Cells stably expressing M4 E-cadherin were incubated with MG132 for 2 h and were lysed in 0.5% NP-40 lysis buffer, followed by immunoprecipitation and Western blotting by using antibodies against to E-cadherin and Ubiquitin, respectively. Results showed that M4 E-cadherin binds to ubiquitin, and forms polyubiquitin–E-cadherin complex ladder. (After cells were lysed, 20 μg of total protein was conserved, then these cell lysates were fractionated on 7.5% SDS-gel with immunoprecipitates together to mark the location of E-cadherin bands. The portion of proteins was "input")

and p97 was observed, suggesting that M4 E-cadherin underwent a retro-translocation.

Calnexin and calreticulin is a pair of chaperone-like lectin proteins residing in ER, which work as chaperons for helping nascent peptides folding. Calnexin localized near the translocon, can interact with nascent chains of N-glycosylated proteins. A prerequisite for both calnexin and calreticulin binding to newly synthesized glycoproteins is a sequential, initially co-translational, action by a-glucosidases I and II that trim two glucose residues, creating a monoglucosylated glycan. Calnexin mainly associates with transmembrane glycoproteins, and calreticulin is the chaperon for soluble glycoproteins [28–30]. To determine the possible involvement of calnexin in the folding of E-cadherin variants, we examined the association of calnexin with these mutants. As shown in Fig. 5a,b, a significant increase in calnexin binding were observed in M4, M2, and M123 E-cadherin, compared with that in wild type E-cadherin. In addition, the highest ratio of calnexin binding was found in M4 E-cadherin, suggesting that this mutant is a misfolded protein and can not escape from calnexin/calreticulin cycling. Furthermore, the ratio of calnexin binding in M2 and M123 was also considerably higher than that in wild type E-cadherin, but lower than that in M4 E-cadherin. This may reflect the important role of N-glycan at Asn-566 in E-cadherin conformation folding. Moreover, the ratio of calnexin binding in M1 and M3 was slight higher than that of wild type E-cadherin, suggesting that being unglycosylated at these two N-glycosylation sites did not affect E-cadherin folding. Then we examined the association of E-cadherin with calnexin in M4 cells treated with or without inhibitors. Whether M4 cells treated with or without inhibitor, the capture of M4 E-cadherin with calnexin were all observed (Fig. 5c). And the results showed that MG132 do not affect calnexin binding to M4 E-cadherin. The relative rations of calnexin binding to E-cadherin had no significant difference between cells treated with or without MG132. But DMM could increase the ration of calnexin binding to E-cadherin moderately (Fig. 5d).

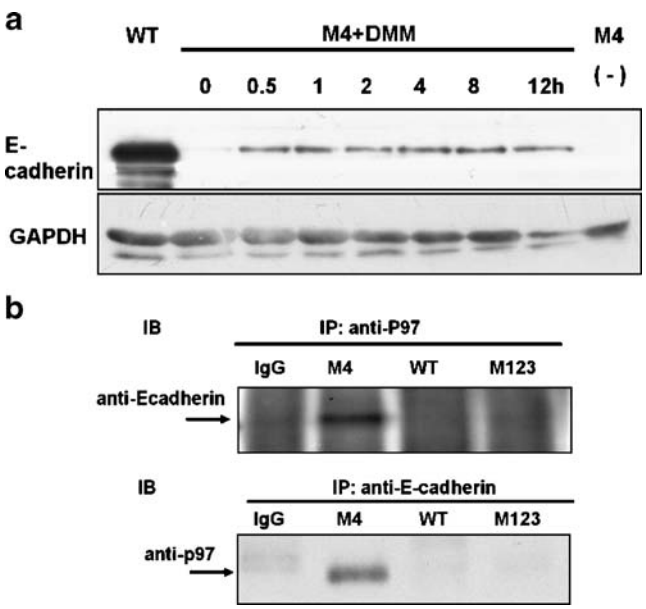

Fig. 4 M4 E-cadherin was degraded via ERAD. **a** Immunoblot analysis of the effect of DMM on the expression of M4 E-cadherin in the MDA-MB-435 cells. 0.5 μM DMM was added to culture medium, the time of treatment was indicated in the (**a**). And 30 μg of total protein was fractionated on 10% SDS-PAGE. Expression of M4 E-cadherin was analyzed by using antibodies against E-cadherin. Results showed that DMM could successful suppress the degradation of M4 E-cadherin at 0.5 h. **b** Interactions between M4 E-cadherin and p97. Interaction between M4 E-cadherin and p97 was detected by immunoprecipitation and immunoblot analysis using anti-E-cadherin and anti-p97, respectively. Before extraction, cells stably expressing M4 E-cadherin were treated with MG132 (50 μM) for 2 h, and total cell lysates were immunoprecipitated with an antibody against to E-cadherin or p97, respectively. Immune complexes were analyzed for the presence of E-cadherin or p97

Taking together, N-glycosylation at Asn-633 site was essential for E-cadherin folding correctly. It was difficult for E-cadherin to form the mature conformation without N-glycan at Asn-633 site. As misfolded proteins, M4 E-cadherin was arrested in calnexin/calreticulin cycling, then marketed as substrate of ERAD by α-mannosidase I.

Unglycosylation at Asn-633 site destroyed the conformation of E-cadherin luminal domains, but not cytoplasmic fragments

It was demonstrated above that M4 E-cadherin was misfolded. However, the role of N-glycan at Asn-633 in E-cadherin conformation folding was poorly understood. Did the lesion made by the unglycosylation limit in extracellular domains or was the whole structure of E-cadherin collapsed. To explore the role of the N-glycan at Asn-633 site in E-cadherin conformation structure, we first examined the association of M4 E-cadherin, with catenins.

E-cadherin has a conserved fragment which is called catenin-binding site in its cytoplasmic domain (Fig. 1). When newly synthesized E-cadherin enters into ER for its folding, β-catenin binds to E-cadherin coinstantaneously and sequentially transported with E-cadherin as complexes to Golgi apparatus together [42]. After E-cadherin–β-catenin complexes arrived at cells surface, α-catenin was recruited by β-catenin and worked as a ‘bridge’ to mediate the interaction between E-cadherin–catenin complexes and cytoskeletons [5–9]. As shown in Fig. 6a, even though recognized as misfolded proteins, M4 E-cadherin could associate with β-catenin, but interaction between M4 E-cadherin and α-catenin was not detected. These results suggested that cytoplasmic domain of M4 E-cadherin was still functional. At least, it could form complexes with β-

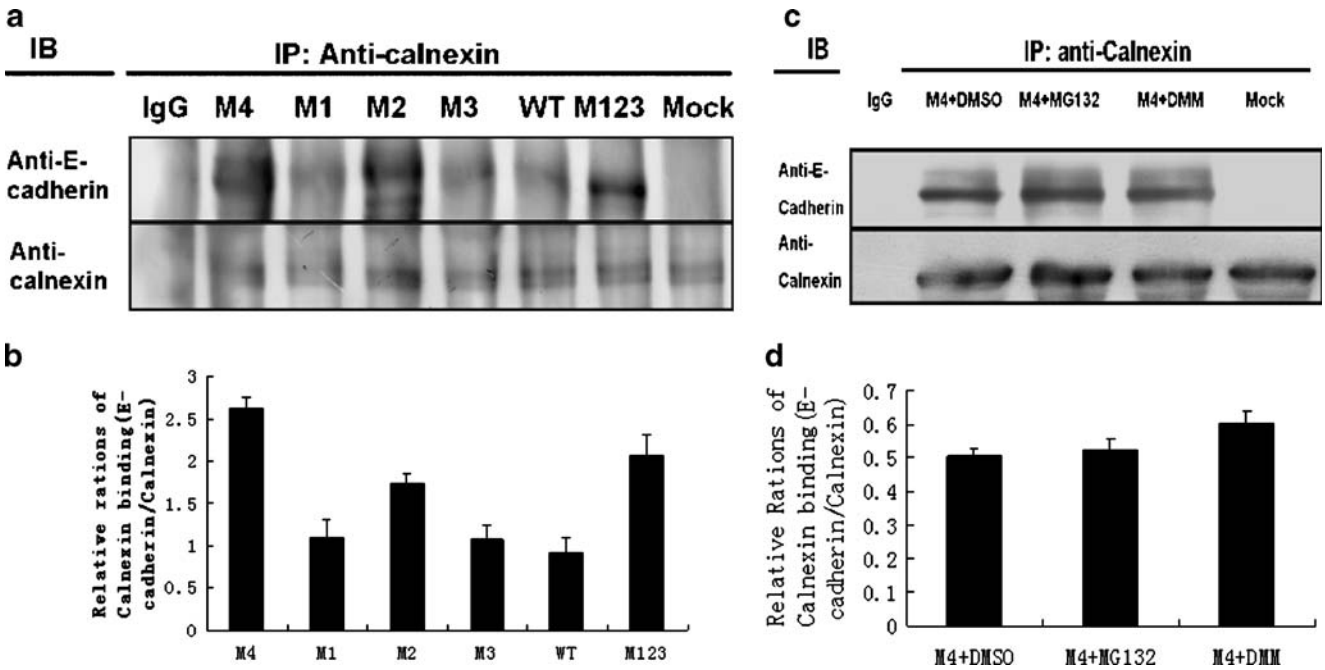

Fig. 5 Association of the E-cadherin variants with calnexin. **a** Association of E-cadherin variants with calnexin. Cells stably expressing wild type and E-cadherin unglycosylation mutants were lysed and immunoprecipitated with antibodies against to calnexin. Before lysed, cells stably expressing M4 E-cadherin were treated with MG132 (50 μM) for 2 h. The samples were then subjected to 10% SDS-PAGE and immune complexes were detected with antibodies against to E-cadherin or calnexin as described under "Materials and methods", respectively. **b** Relative ratios of calnexin binding to E-cadherin were determined by dividing the mean intensity of E-cadherin to that of calnexin. Results represent one of three independent experiments. **c** Association of M4 E-cadherin with calnexin in M4 cells. Cells stably expressing M4 E-cadherin were treated with DMSO, MG132, DMM for 2 h, respectively. Then lysates were immunoprecipitated with antibody against to calnexin. The samples were then subjected to 10% SDS-PAGE and immune complexes were detected with antibody against to E-cadherin or calnexin, respectively. **d** Relative ratios of calnexin binding to E-cadherin were determined by dividing the mean intensity of E-cadherin to that of calnexin. Results represent one of three independent experiments

catenin. Because M4 E-cadherin was a misfolded protein, it likely could not be transported to the cell surface. This may explain why the association of M4 E-cadherin and α-catenin could not be detected.

Analysis of the crystal structure of E-cadherin was considered a perfect way to know the role of N-glycans in E-cadherin dimensional structure. However, it was still a challenge. A prediction of the E-cadherin three-dimensional structure was performed by homology modeling method. And evaluation of hydropathicity of E-cadherin amid acid residues was also carried out using arithmetic from Kyte J. and Doolittle R.F. [43]. Alignment of E-cadherin sequences with templates saved in Swiss Model Template Library (ExPDB) revealed a crystal structure (C-cadherin Ectodomain, PDB No: 1l3wA) shared 57% sequence identity with E-cadherin extracellular domain, and the humorous amid acid residues range from 151 to 692. So we selected it as homology template for E-cadherin modeling. The images of three-dimensional structure are displayed in Fig. 7a,b. The whole extracellular domain of E-cadherin was folded into five subdomains (EC1–EC5), and secondary structure of E-cadherin extracellular domain only has β-sheet (Fig. 7a). Analysis of crystal structure of C-cadherin revealed that extracellular region of C-cadherin contains five subdomains, and each subdomain consists of a barrel-like core region, which had rigid 3D structure and a relaxed loop fragment, which connect to adjoining core regions. From Fig. 7b, β-sheets in EC4 and EC5 coiled and formed a hollow barrel-like structure, this region bears analogy with

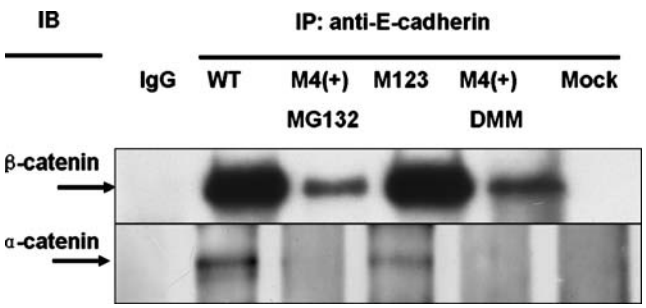

Fig. 6 Association of the unglycosylation mutants with catenins. Cells stably expressed wild type, M4, M123 E-cadherin were lysed and immunoprecipitated with antibody against to E-cadherin. Before lysed, cells stably expressed M4 E-cadherin were treated with MG132 (50 μM) and DMM (0.5 mM) for 2 h, respectively. The samples were then subjected to 10% SDS-PAGE and immune complexes were detected with antibody against to α-catenin and b-catenin as described under "Materials and methods"

the core region of C-cadherin. Based on the structure of C-cadherin, we speculate that these barrel-like structures were the core regions of E-cadherin. Because these core regions were high ordered, disturbed the structures would break down the three dimensional structure of E-cadherin. Asn-566 and Asn-633 both localized at these core regions, suggesting that N-glycans at these two sites were important in maintain stability of core regions. In contrast, Asn-554 and Asn-618 localized at relaxed loop region, which could be postulated as intrinsically disordered regions, lacks rigid 3D structure, existing instead as the dynamic ensembles of interconverting structures. And changes of N-glycans at these flexible regions could alter the function of E-cadherin.

N-glycosylation at Asn-633 site was essential for E-cadherin trafficking to Golgi apparatus

There was no association of M4 E-cadherin and α-catenin, hinting that after blocking the degradation, M4 E-cadherin likely could not be transported to the cell surface and anchored to adherence junction complexes (AJs). To investigate the subcellular distribution of E-cadherin variants, we examined the expression of E-cadherin in Triton-X100 soluble (membrane) and insoluble (cytoskeleton) fractions from cells stably expressing E-cadherin variants. In stable cell–cell adhesion, the E-cadherin–β-catenin complex of the AJs is anchored to the actin cytoskeleton via α-catenin, and this association is manifested by the insolubility of the complex components in TritonX-100 [44, 45]. As shown in Fig. 8a, wild type and M123 E-cadherin were both detected in TritonX-100 insoluble and soluble fractions, and the quantity of wild type and M123 E-cadherin in TritonX-100 insoluble fraction was much larger than that in TritonX-100 soluble fraction. After treated with MG132, degradation of M4 E-cadherin was blocked efficiently, but the distribution of M4 E-cadherin was different from that of wild type and M123 E-cadherin, the bands of M4 E-cadherin only could be detected in the TritonX-100 soluble fraction (Fig. 8a).

These results suggested that N-glycosylation at Asn-633 was required for E-cadherin trafficking. Moreover, other N-glycosylation sites seemed to have no effect on E-cadherin trafficking, because M123 E-cadherin with a single N-glycan conserved at Asn-633 still could be transported to the cell surface efficiently and anchored to AJs complexes to form

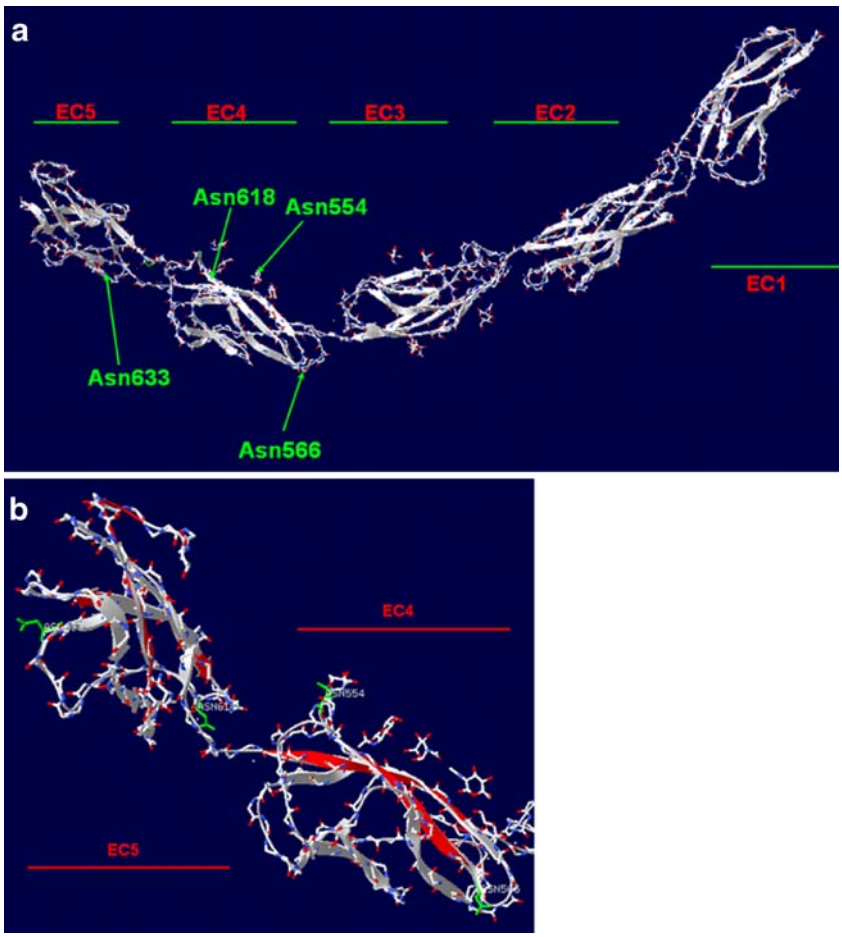

Fig. 7 Three-dimensional structure of extracellular domain of E-cadherin. **a** The full conformation of extracellular domain. β-Sheet is shown in white strip representation, and location Asn-554, Asn-566, Asn-618, and Asn-633 are indicated used *green arrow*. **b** Amplificatory structure of EC4 and EC5 domain, β-sheet is shown in *white strip representation*, hydrophilic residues are represented with red strip, N-glycosylation sites were indicated in *green* and number, and oxygen atoms, nitrogen atoms and carbon atoms are represented with *red*, *blue*, *white*, respectively

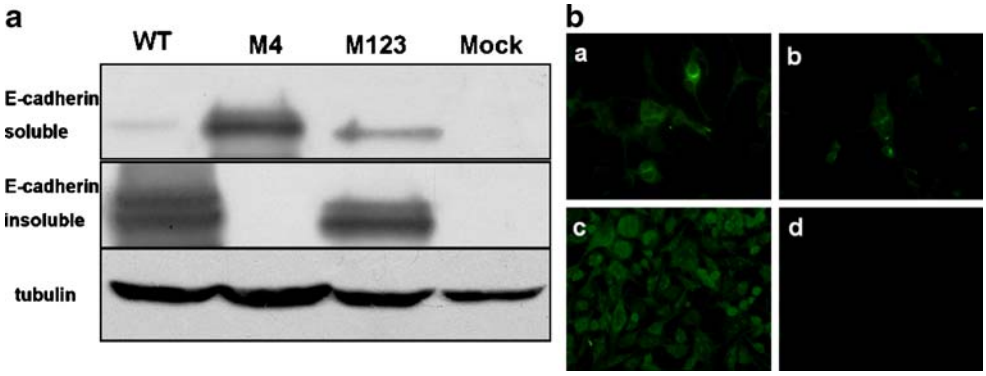

Fig. 8 Glycosylation on Asn-633 is required for the transportation of E-cadherin to cell surface. **a** Triton insolubility of E-cadherin N-glycosylation variants. MBA-MD-435 cells stably expressing wild type, M123, M4 E-cadherin and empty vector, were extracted with the TritonX-100 extraction buffer, before extraction, cells stably express M4 E-cadherin were treated with MG132 (50 μM) for 2 h. TritonX-100-soluble and -insoluble fractions were analyzed for E-cadherin, α-tubulin (control) expression by Western blotting, as described in "Materials and methods". Wild type and M123 E-cadherins migrated as two bands, where the upper species most likely represented unprocessed E-cadherins (pro-peptides), the down one, mature E-cadherin. In TritonX-100 soluble and insoluble fractions, wild type and M123 E-cadherin were detected, but M4 E-cadherin was only detected in the TritonX-100 soluble fraction. **b** Immunofluorescence analysis of MBA-MD-435 cells stably expressing wild type and M4 E-cadherin, immunostained with antibodies against to E-cadherin (*green* fluorescence of secondary antibodies labeled with FITC). *a* Cells stably expressed wild type E-cadherin; *b* cells stably expressed M123 E-cadherin; *c* cells stably expressed M4 E-cadherin, which were treated with MG132 (50 μM) for 2 h prior to analysis; *d* cells stably expressing empty vector (control). Original magnification, ×400

stable adherence complex. Immunofluorescence analysis also showed that wild type and M123 E-cadherin mainly located at the cell surface membrane, but the distribution of M4 E-cadherin was different. From Fig. 8b—c, M4 E-cadherin mainly distributed in cytoplasm and formed visible granules.

E-cadherins are synthesized as inactive precursor proteins containing a prosequence followed by five Ca^{2+}-dependent repeated subdomains, the length of E-cadherin prosequence is 152 amid acid residues. The N-terminal prosequence is proteolytically cleaved off in the later Golgi at the conserved consensus site R-X1-K-R-X2-W, and the mature E-cadherin is then transported to the cell surface [46]. In our previous experiments, prolonged the exposure times afforded, two bands in wild type E-cadherin (Fig. 8a), the upper band is the pro-peptide, the lower one is mature E-cadherin. But in these results, the M4 E-cadherin has only a band, and its mobility was similar to the pro-peptide of wild type E-cadherin. We speculated that M4 E-cadherin might not enter into Golgi apparatus, and retained in ER. Analysis of N-glycan types of E-cadherin might be a valid method to study whether the proteins enter into Golgi apparatus. Because after nascent glycopeptides were synthesized and entered into the ER lumen, a high mannose N-glycan composed of two *N*-acetylglucosamine, nine mannose and three glucose residues, will attached to the asparagines in consensus Asn-x-Ser/Thr motifs. If folded properly, the nascent glycoproteins would be trafficked to Golgi apparatus I, in the compartment, the immature high mannose type of N-glycans were modified to complex type [21]. Therefore, whether the M4 E-cadherin was transported to Golgi apparatus could be evaluated by the types of N-glycans. Endo H is an endoglycosidase that hydrolyzes only immature N-glycans at the chitobiose core, whereas PNGase F is an amidase that cleaves most N-glycans, including high mannose, hybrid, and complex structures, at the asparagines residues. As expected, wild type E-cadherin is sensitive to PNGase F but not to Endo H. After treated with PNGase F, the mobility of wild type becomes faster on SDS-PAGE gel, whereas the Endo H could not affect the mobility of wild type E-cadherin (Fig. 9a). These results suggested that wild type E-cadherin was mainly modified with complex N-glycans. In contrast, based on the mobility shifts after PNGase F and EndoH treatment, M4 E-cadherin was primarily PNGase F and Endo H sensitive, and this provided evidence that M4 E-cadherin was modified with immatural high mannose N-glycan type. This finding was extensively confirmed by the lectins stain of E-cadherins with WGA and GNA lectins. WGA only binds to complex N-glycans, whereas GNA specifically binds to high mannose N-glycans. As shown in Fig. 9b, wild type E-cadherin was stained by WGA, and slightly stained by GNA. We proposed that slight stain may from the newly synthesized wild type E-cadherin, which retained in ER for its folding. Notably, M4 E-cadherin had a different stain pattern, it only could be stained by GNA, suggesting that the N-glycans of M4 E-cadherin were modified with high mannose type. Taking these results together, the difference of N-glycan types between wild type E-cadherin and M4 E-cadherin reflects the importance of N-glycosylation at Asn-633 in E-cadherin trafficking from ER to Golgi apparatus, unglycosylation at this site blocked the mutant to be transported to Golgi apparatus.

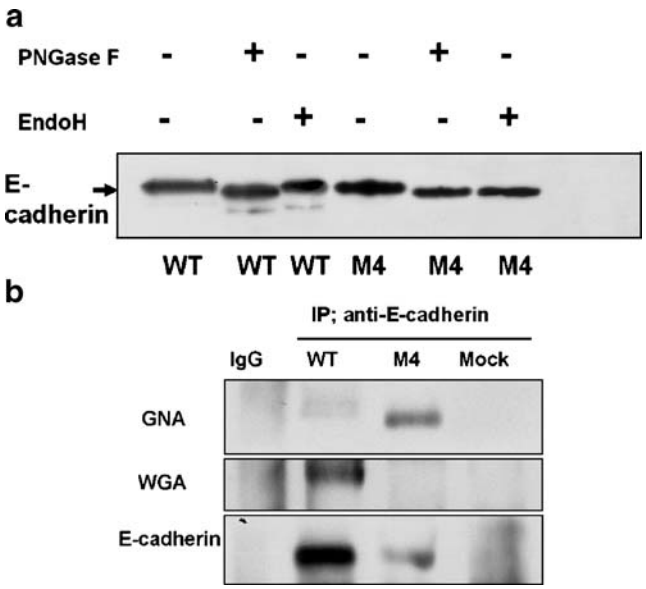

Fig. 9 Difference of glycan types between wild type and M4 E-cadherin. **a** Different sensitivity of wild type and M4 E-cadherin to PNGase F and Endo H. Total cell lysates from cells stably expressing wild type E-cadherin and M4 E-cadherin were treated with either Endo H or PNGase F prior to analysis on Western blots. Before extraction, cells stably expressing M4 E-cadherin were treated with MG132 (50 μM) for 2 h. M4 E-cadherin underwent a significant shift in mobility following PNGase F and Endo H treatment, indicating that it was modified with high mannose N-glycans. Wild type E-cadherin exhibited a significant shift in mobility following PNGase F treatment, but no shift in mobility after Endo H treatment, indicating that wild type E-cadherin was modified with complex N-glycans. **b** Analysis of N-glycans of wild type and M4 E-cadherin using lectins blotting. Before extraction, cells stably expressed M4 E-cadherin were treated with MG132 (50 μM) for 2 h. Cell extracts (1 mg of total protein) from cells stably expressing wild type and M4 E-cadherin were immunoprecipitated with anti-E-cadherin antibodies (1 μg), immunoprecipitates were fractionated on 10% SDS-PAGE, blotted onto PVDF membranes, and probed with lectins WGA and GNA. Wild type showed significant WGA stains, and slightly GNA stains, whereas M4 E-cadherin only could be stained by GNA, which recognizes high mannose N-glycan form specifically

Discussion

Our previous study had showed that human E-cadherin *in vivo* utilizes all of four potential N-glycosylation sites, and in this study we reported that the elimination of a single N-glycan at Asn-633 profoundly affected the E-cadherin stability, whereas obliteration of any other N-glycan had no effect on molecular stability. Lysosomes and proteasomes are two main compartments, which are responsible for protein degradation in mammal cells. For wild type E-cadherin, after being synthesized, it was delivered to the cell surface to participate in adherence junction, but pool of E-cadherins underwent endocytosis, when cells are in unstable cell–cell contact phase such as proliferation [47]. Some of them could be recycled to the cell surface, others would ubiquitinated by a tyrosine phosphorylation kinase, Hakai which works as a E3 ubiquitin-ligase and sequentially degraded by 26S proteasomes [48]. To distinguish which were responsible for M4 E-cadherin degradation, we treated cells stably expressed M4 E-cadherin with MG132, chloroquine and DMM, respectively. MG132 and DMM, but not chloroquine could result in the retardation of M4 E-cadherin degradation. Association of M4 E-cadherin with ubiquitin revealed that degradation of M4 E-cadherin was ubiquitin-dependent. Our results also demonstrated that p97 participated in degradation of M4 E-cadherin. These findings clearly showed that unglycosylation at Asn-633 made E-cadherin degraded via ERAD. According to Liwosz's results, unglycosylation at Asn-633 (V3) did not affect the expression of E-cadherin in CHO and MDCK cells [17]. These results contradict with our observations. To reconcile the discrepancy, we checked degradation of M4 E-cadherin in other cell lines. Interestingly, the destiny of M4 E-cadherin was different in different cell lines (Fig. 2c). These results suggested that although unglycosylation at Asn-633 site made E-cadherin a misfolded protein, but in some cell lines, such as CHO cells and 293T cells, it could escape from quality control system in ER. Liwosz's results also showed that though V3 E-cadherin could be stably expressed, compared with other variants, it mostly distributed in cytoplasm, suggesting the deficiency of V3 E-cadherin in function.

N-glycosylation is necessary for glycoprotein folding has been observed in some glycoproteins. For example, elimination of only one N-glycan would affect folding of human α-galactosidase A [49], elimination of five N-glycans on β-propeller domain of integrin α5 subunit result in its misfolding [51]. However, in some glycoproteins, the role of N-glycosylation in protein folding is not required. Such as Mouse procathepsin L, even after removal of its unique functional N-glycosylation, it still can fold correctly [51]. Calnexin is a ER membrane-bond chaperone-like protein, which works as a chaperon to help nascent glycoproteins folding [28–30]. Our results showed that calnexin was involved in E-cadherin folding, as well as a highest ratio of calnexin binding was observed in M4 E-cadherin, suggesting that it is a misfolded protein. However, the ratio of calnexin binding also increased significantly in M2 and M123 E-cadherin, suggesting that N-glycan at Asn-566 also plays a complementary role in helping proteins folding. Compared with N-glycans at Asn-633 site, unglycosylation at Asn-566 did not affect the expression of E-cadherin, but made M2 E-cadherin retained in calnexin/calreticulin cycling in more time. These likely reflected the different role of N-glycosylation at Asn-566 and Asn-633 sites in E-cadherin conformation structure. Elimination of N-glycan at Asn-566 site may lead the conformation of E-cadherin changed, but the lesion may be tolerant for 'quality control systems' in ER, so M2 E-cadherin, but not M4 E-cadherin could pass the check points. To investigate the 3D structure of E-cadherin in detail, we simulated the 3D structure of E-cadherin by

using the homology modeling method. Protein structural comparison has shown that if more than 45% of the amino acid positions are identical, the amino acids should be quite superimposable in the 3D structure of the proteins. Thus, if the structure of one of the aligned proteins is known, the structure of the second protein and positions of the identical amino acids in this structure may be reliably predicted. This method to predict three-dimensional structure is called Homology Modeling [52]. Fortunately, a resolved crystal structure saved in Swiss Model Template Library which share 57% identity in protein sequences with E-cadherin had been found. Based on the template, we predicted a dimensional structure of E-cadherin with high reliability. At first we presumed that degradation of M4 E-cadherin was due to exposure of hydrophilic residues surrounding the Asn-633 site, but analysis of the image revealed that none of N-glycosylation sites surrounded by high hydrophilic residues (red strip in Fig. 7b). In contrast, we found that the sites of Asn-566 and Asn-633 were localized at the core regions of EC4 and EC5 domains, suggesting the important role of N-glycans at these two sites in E-cadherin dimensional structure. In contrast, Asn-554 and Asn-566 localized in flexible regions, which are likely involved in biological function. Our previously study had demonstrated that unglycosylation of E-cadherin at Asn-554 and Asn-566 would result in increasing the tyrosine phosphorylation levels of β-catenin and affect cell–cell adhesion which is mediated by E-cadherin [38].

Our findings also demonstrated that N-glycosylation at Asn-633 was required for E-cadherin trafficking from ER to Golgi apparatus. Analysis of M4 E-cadherin subcellular distribution and N-glycan types showed that M4 E-cadherin could not arrive in Golgi apparatus. Furthermore, removal of other three N-glycans synchronously, but a single N-glycan preserved at Asn-633 had no effect on E-cadherin trafficking, the M123 E-cadherin still could be secreted to the cell surface normally.

Correctly folded protein destined for distal compartments exit ER via vesicular or tubular structures. Vesicles transport of correctly folded protein involves concentration of proteins in COPII components or with ER export cargo receptors. Such ER export cargo receptor typically span the membrane with the luminal domain binding soluble cargo in the ER lumen, while the cytoplasmic domains of the receptor is responsible for the packaging of soluble cargo protein. Many membrane-spanning proteins contain specific cytosolic exit signals, including the di-acidic (D/E-x-D/E) motif that interacts directly with components of the COPII coat [53]. Misfolded proteins were retro-translocated to cytoplasm for degradation by ERAD. But not all misfolded proteins are retained in ER, a number of misfolded proteins exit the ER and traffick to the Golgi apparatus, such as mutant Z form of A1PiZ and misfolded carboxypeptidase Y (CRY). Efficiently degraded these protein dependent on ER–Golgi trafficking [54, 55]. Vashist and Ng [56] proposed a ERAD-L/C model to explain how are ERAD substrates have sorted to each pathway. They suggested that proteins are sorted based on the sites of lesion. At the ERAD-C pathway, monitors in cytoplasm check the folding state of cytosolic domains of membrane proteins and rapidly clean misfolded proteins from the ER. This occurs without regard to the state of the luminal domain. Should the conformation of cytosolic domains pass the ERAD-C checkpoint, the ERAD-L pathway will monitor the state of luminal domains. If a lesion is detected, the protein is sorted for ER–Golgi transport. Naturally, all soluble proteins bypass the ERAD-C pathway, as they are entirely luminal. However, Copper and Kincaid [57] argued that ER exit signals play a role in the ER exit of misfolded proteins, and these ER exit signals can localized on either side of membrane in different proteins. Misfolded protein utilized the same machinery, which is responsible for exporting correctly folded protein to depart from ER, and ERAD and ER exit machinery can compete for binding of misfolded proteins. Loss or deletion of the ER export signals, or disruption of signal presentation contributes to misfolded proteins remaining in ER. Our results are consistent with Copper's hypothesis. Because the elimination of N-glycan at Asn-633 did not affect association of E-cadherin with β-catenin, but made M4 E-cadherin retained in calnexin/careticulin cycles. These suggested that the lesion of M4 E-cadherin was on the luminal domain. Furthermore, according to the Ng's theory it should be sorted as the substrates of ERAD-L pathway. But our findings showed that even the ERAD pathway was blocked, M4 E-cadherin could not traffick to the Golgi apparatus. So we proposed that unglycosylation at Asn-633 likely sterically interferes with incorporation into COPII vesicles or exit signal presentation and finally account for failure to exit ER.

In conclusion, this study reports that glycosylation at Asn-633 is essential for E-cadherin expression, folding, and trafficking.

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