Supporting Information

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SI Materials and Methods

All primer sequences are listed in Table S1.

Gene Targeting. To produce a targeting construct for protein O-mannosyltransferase 2 (*Pomt2*), 1.9-kb (primers a and b) and 6.4-kb (primers c and d) DNA fragments were amplified from genomic DNA (129/OLA mouse strain) and cloned into pGEM-T easy (Promega), resulting in vectors ko2 and ko3, respectively. The 6.4-kb XhoI fragment comprising the 3′ end of *Pomt2* exon 3 to *Pomt2* exon 6 was isolated from ko3 and subcloned into pKO SelectNeo V800 (Lexicon Pharmaceuticals), producing the vector ko6-2. A 759-bp repetitive sequence was removed from the *Pomt2* genomic sequence contained in ko2 (1,856 bp of *Pomt2* intron 2 and 50 bp of *Pomt2* exon 2) by HindIII digestion and religation. A 1.2-kb XhoI/SacII fragment was excised from the resulting vector and was cloned into ko6-2 to produce vector ko7. This vector then was linearized, and the DNA was transferred into embryonic day 14.1 (E14.1) ES cells by electroporation. Clones with the desired homologous recombination events were identified by PCR using primers 1 and 2 and were confirmed by Southern blotting using a ^{32}P-labeled DNA probe (base pairs 5,731–6,263; GenBank accession no. AY090489). Two independent targeted ES-cell clones (6B7 and 7B7) were injected into BALB/c blastocysts to create chimeric mice. Heterozygous *Pomt2*$^{+/-}$ progeny were mated, and the offspring were genotyped by PCR using primers 1–4.

Genotype Analysis of Preimplantation Embryos. DNA from preimplantation embryos was prepared as described elsewhere (1). DNA was precipitated in the presence of linear polyacrylamide (15 μg/mL; Ambion). Complete samples were used in the first round of a nested-PCR strategy. Primers 14 and 15 were used to amplify the WT and mutant *Pomt2* loci. Then 5 μL of first-round products were amplified further using primer pair 16/18 or primer pair 17/18, which are specific for the WT and transgenic alleles, respectively.

Zygosity Assay. Genomic DNA was prepared from ES cells using the DNeasy Blood and Tissue kit (Qiagen). Fifty nanograms of genomic DNA served as template for quantitative PCR using an iCycler iQ real-time PCR detection system (Bio-Rad) and iQ Sybr-Green Supermix (Bio-Rad). PCR reactions were performed to amplify the WT allele (primers 10 and 11), the null allele (primers 12 and 13), or both *Pomt2* alleles (primers 8 and 9) (Fig. S1). The zygosity status of the two *Pomt2* alleles was calculated relative to the total *Pomt2* content, using the $\Delta\Delta C_T$-method (2).

In Vitro Assays for POMT and Dolichol Monophosphate-Activated Mannose Synthase. Microsomal membranes were used to determine enzymatic activities as described previously (3, 4). Reactions were supplemented with DMSO or rhodanine-3-acetic acid derivative compound 5a (R3A-5a) as indicated.

Cytotoxicity and Cell Proliferation Assays. MDCK cells (American Type Culture Collection CRL-2936) were cultured under standard conditions (5). The culture medium was supplemented with 0.5% DMSO or R3A-5a dissolved in DMSO for 3 d. Cytotoxicity of R3A-5a and cell proliferation were measured using the CytoTox-One homogeneous membrane integrity assay kit (Promega).

Protein Extraction and Enrichment. α-Dystroglycan (α-DG)–enriched fractions from MDCK cells were prepared as described elsewhere (6). For protein extracts containing epithelial cadherin (E-cad), cells were solubilized in lysis buffer [50 mM Tris·HCl (pH7.4), 150 mM NaCl, 1% Triton X-100, supplemented with protease inhibitors]. For affinity purification of E-cad, rat monoclonal E-cad antibody (DECMA-1) was cross-linked to protein G agarose (Pierce) using dimethylpimelimidate (Sigma) as described elsewhere (7). E-cad–containing protein extracts were incubated with the affinity matrix overnight and were washed extensively with ice-cold lysis buffer. α-DG from porcine skeletal muscle was partially purified by diethylaminoethyl cellulose- and wheat germ agglutinin-chromatography as recently described (8). Recombinant 6×HIS-tagged sGas1^{523} was expressed in *Pichia pastoris* (9), whereas hNfasc186, hDGdel2, and hDG5 constructs were expressed in HEK293T cells (10, 11). Recombinant proteins were purified from the culture supernatant by nickel-nitrilotriacetic acid affinity chromatography as described elsewhere (9–11).

Western Blot Analysis. Protein samples were separated on 6% or 10% SDS-polyacrylamide gels and were transferred to nitrocellulose. Immunoblots were decorated with mouse monoclonal VIA4-1 (1:500, Millipore), rat monoclonal DECMA-1 (1:5,000; anti–E-cad; Sigma), mouse monoclonal anti-tubulin (1:1,000; Sigma), mouse monoclonal anti-pentaHis (1:2,500; Qiagen), goat polyclonal anti–core-αDG (1:100, Santa Cruz), goat polyclonal anti–K-cadherin (1:1,000; anti–K-cad; Santa Cruz), rabbit polyclonal anti–β-DG (1:1,000; Santa Cruz), and rabbit polyclonal anti–threonine O-mannosyl–conjugated epitope (T[α1-Man]) (1:25, this study) antibodies. Proteins were visualized by enhanced chemiluminescence using the Amersham ECL system.

Tryptic Digestion and LC-MS Analysis. Purified human extracellular domain E-cad (10204-H08H; Sino Biological) was digested by trypsin using the Filter-Aided Sample Prep (FASP) method with minor modifications (12). Five micrograms of E-cad were denatured in 8 M urea. The urea then was replaced with ammonium bicarbonate, and 1 μg trypsin (Promega) was added. After incubation overnight at 37 °C, the peptides were separated from undigested protein by ultrafiltration (Microcon 30; Millipore). For LC-MS analysis, a nanoAcquity Ultra Performance Liquid Chromatography system (Waters) was coupled to an Orbitrap XL (Thermo Scientific). Peptides were trapped (5 μm Symmetry 180 μm × 20 mm, Waters) at a flow rate of 15 μL/min in 100% buffer A (0.1% formic acid in HPLC-grade water). After 5 min of loading and washing, peptides were eluted on an analytical column [75 μm (inner diameter) × 250 mm (length) 1.7 μm (particle size) BEH130 C18 column; Waters] and were separated using a 40-min gradient from 3% to 40% of buffer B [0.1% formic acid in 90% (vol/vol) acetonitrile] at a flow rate of 200 nL/min. Full-scan mass spectra were acquired in the Orbitrap at 60,000 resolution. In a first analysis, the six most intense precursors were selected for collision-induced dissociation (CID) fragmentation. CID spectra were acquired using the ion-trap detector. Candidates for glycosylated peptides were selected based on a dominant neutral loss of hexose, *N*-acetylhexosamine, and *N*-acetylneuraminic acid, using the neutral fragment option of the Qualbrower software (ThermoScientific). Candidates were analyzed in MS3 experiments in a data-independent mode, using the first fragmentation event to remove the glycan, followed by a second fragmentation of the deglycosylated peptide.

Treatment of Peptides with α(1-2,3,6)-Mannosidase. To remove specific α-mannose residues from the synthetic peptide [LSDAGT (α1-Man)VVSGQIR, 33 nM] or the tryptic E-cad peptides (FASP purification), samples were treated with or without 3 U/mL

α(1-2,3,6)-mannosidase (Jack Bean; Prozyme) (13). After 18 h at 37 °C in 100 mM sodium acetate and 2 mM ZnCl (pH 5.0), the enzyme was removed by ultrafiltration (Microcon 30; Millipore). Samples were acidified by adding a one-third volume of 0.1% trifluoroacetic acid (in HPLC-grade water) and were analyzed by LC-MS. Extracted ion chromatograms were done using the QualBrowser software (ThermoScientific) with the calculated peptide mass tolerance of 10 ppm.

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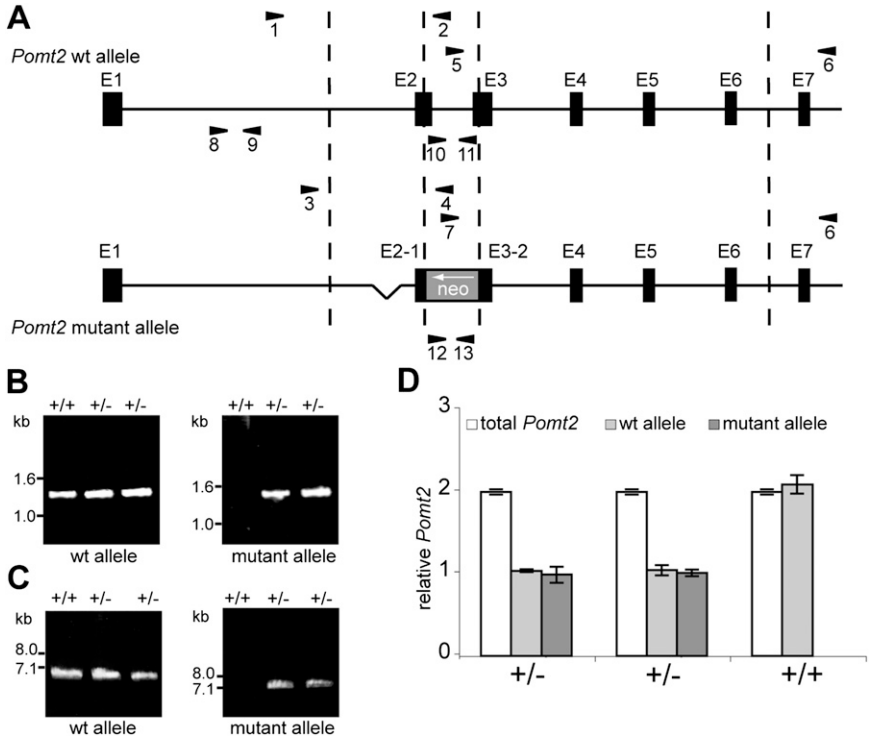

Fig. S1. Targeted disruption of the *Pomt2* gene in ES cells. (*A*) Schematic representation of the *Pomt2* genomic locus and the mutant *Pomt2* allele produced by homologous recombination. Arrowheads indicate PCR primers. (*B* and *C*) PCR analysis to confirm correct homologous recombination in targeted ES cells. (*B*) Correct homologous recombination of the 5′ arm was confirmed by PCR analysis using primers 1 and 2, which identify the WT allele (1.3-kb fragment; *Left*), and primers 3 and 4, which are specific for the targeted allele (1.4-kb fragment; *Right*). (*C*) Correct homologous recombination at the 3′ arm was confirmed by PCR amplification of either the WT allele (primers 5 and 6; 7.1-kb fragment; *Left*) or the mutant allele (primers 6 and 7; 7.2 kb; *Right*). A mutant allele-specific PCR fragment is not detectable in WT ES cells. (*D*) Quantification of *Pomt2* alleles in ES cells. Amounts of the WT (primers 10 and 11) and mutant (primers 12 and 13) alleles relative to the amount of both *Pomt2* alleles (primers 8 and 9), as determined by quantitative PCR. The total amount of *Pomt2* alleles was arbitrarily set to 2.

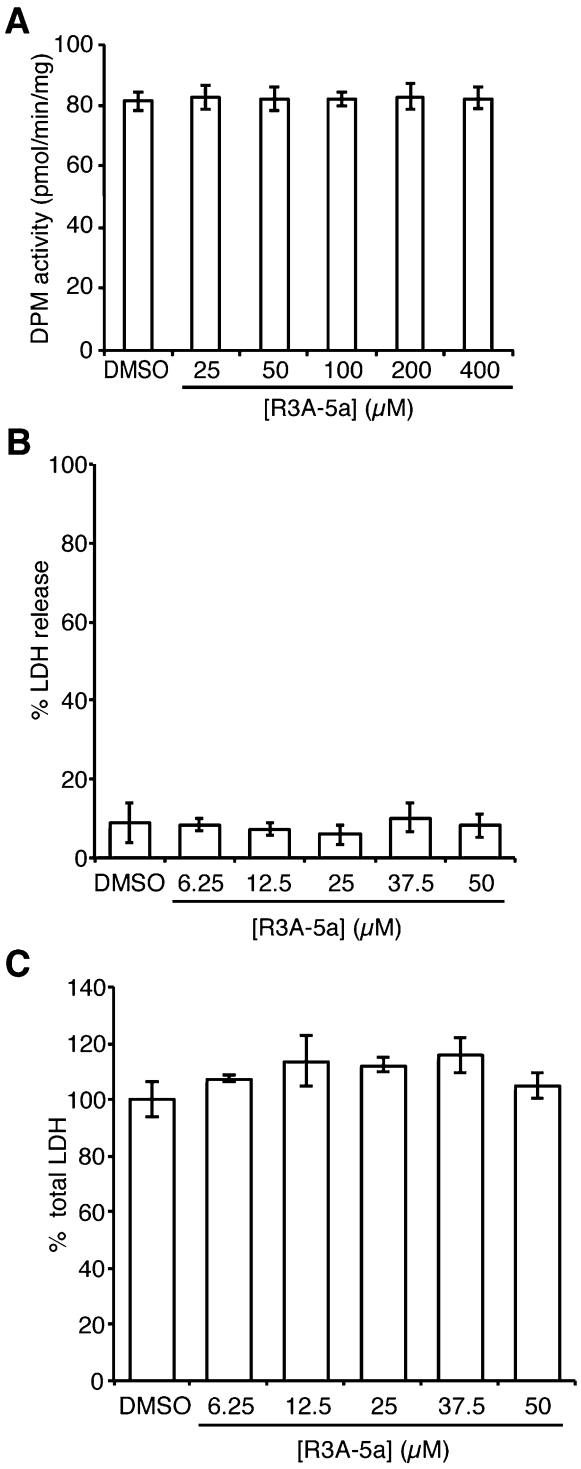

Fig. S2. Characterization of POMT inhibitor R3A-5a. (*A*) Effect of R3A-5a on in vitro activity of dolichol monophosphate-activated mannose synthase in mouse-liver membranes. No inhibitory effect is observed. (*B* and *C*) Cytotoxicity and proliferation of MDCK cells in the presence of R3A-5a. Cells were cultured in the presence of DMSO or R3A-5a at the concentrations indicated. (*B*) Quantitation of lactate dehydrogenase (LDH) membrane leakage as a readout of R3A-5a cytotoxicity. (*C*) Quantitation of LDH content as a readout of cell proliferation in the presence of R3A-5a.

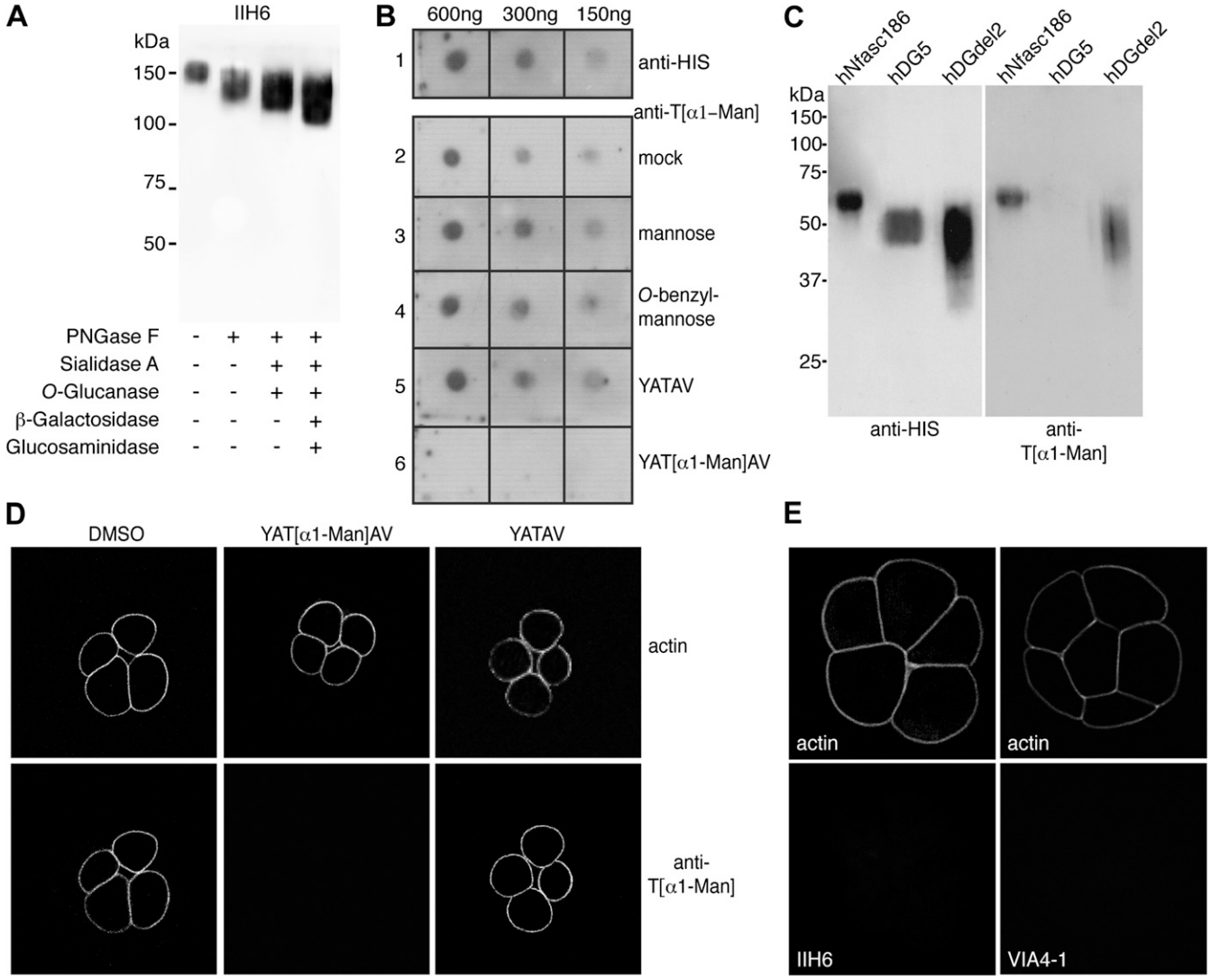

Fig. S3. Characterization of O-mannosyl glycans in preimplantation embryos. (*A–D*) Characterization of T[α1-Man] antibodies. (*A*) α-DG–enriched glycoprotein fractions were treated with glycosidases as indicated following the GLYCOPRO regime described by Combs and Ervasti (8) and were analyzed by Western blotting using IIH6 antibodies. N-linked glycans were removed using peptide-*N*-glycosidase F (PNGase F), and sialylated core 1 and core 3 O-linked oligosaccharides were removed by combined treatment with sialidase A and O-glucanase. Further trimming of the O-mannosyl glycan core structure Galβ1–4GlcNAcβ1–2Man-Ser/Thr was achieved by treatment with β-galactosidase and *N*-acetyl-β-glucosaminidase. Although glycosidase treatment resulted in a clear decrease in the molecular weight of the protein, high-molecular-weight species of α-DG still were detectable. In addition, glycosidase treatment led to an increase in signal intensity. Our data closely resemble the previous results of Combs and Ervasti (8), which demonstrated that the applied GLYCOPRO regime did not remove all of the protein-bound carbohydrates. Their study further suggested that increased signal intensities might be a consequence of a more efficient transfer of the deglycosylated protein (8). We obtained highly similar results when our T[α1-Man]–specific antibody was used for Western blotting (Fig. 2*A*). Alternatively, one could conclude that epitopes are more accessible after glycosidase treatment or even that new epitopes are generated. However, whether the polyclonal T[α1-Man]–specific antibody recognizes only unextended O-linked mannoses is not known. (*B* and *C*) Characterization of affinity-purified antibodies. (*B*) The specificity of crude serum, as assessed by immunoreactivity following preadsorption. The indicated amounts of the highly O-mannosylated yeast protein Gas1 [sGas1^{523} (9)] were spotted on nitrocellulose and probed with anti-His antibodies (lane 1) or preadsorbed anti–T[α1-Man] serum (lanes 2–6). Immunoreactivity toward Gas1 of the serum was not affected when preadsorbed with mannose (lane 3), O-benzylmannose (lane 4), or peptide YATAV (lane 5) but was lost in the presence of the O-mannosylated peptide (lane 6). (*C*) Fifty nanograms of the O-mannosylated recombinant proteins neurofascin (hNfasc186) (10), a truncated version of α-DG (hDGdel2) (11), and a nonmannosylated truncated form of α-DG (hDG5) (11) were analyzed in immunoblots probed with anti-His (*Left*) and anti–T[α1-Man] antibodies. Only the mannosylated proteins hNfasc186 and hDGdel2 were detected by the anti–T[α1-Man] antibody. (*D*) Immunofluorescence analysis of four-cell–stage mouse embryos stained with the anti–T[α1-Man] antibody preadsorbed to DMSO, O-mannosylated YATAV, or the nonmannosylated peptide. Immunoreactivity was inhibited only upon preadsorption with the mannosylated peptide. In combination with the results shown in Fig. 2, these data demonstrate the specificity of the newly established anti–T[α1-Man] antibody. (*E*) Whole-mount immunofluorescence analysis of O-mannosylated α-DG in WT embryos. Morula-stage embryos were stained with IIH6 and VIA4-1 monoclonal antibodies as indicated. O-glycosylated α-DG was not detectable at this developmental stage. In *D* and *E,* cortical actin was stained with phalloidin.

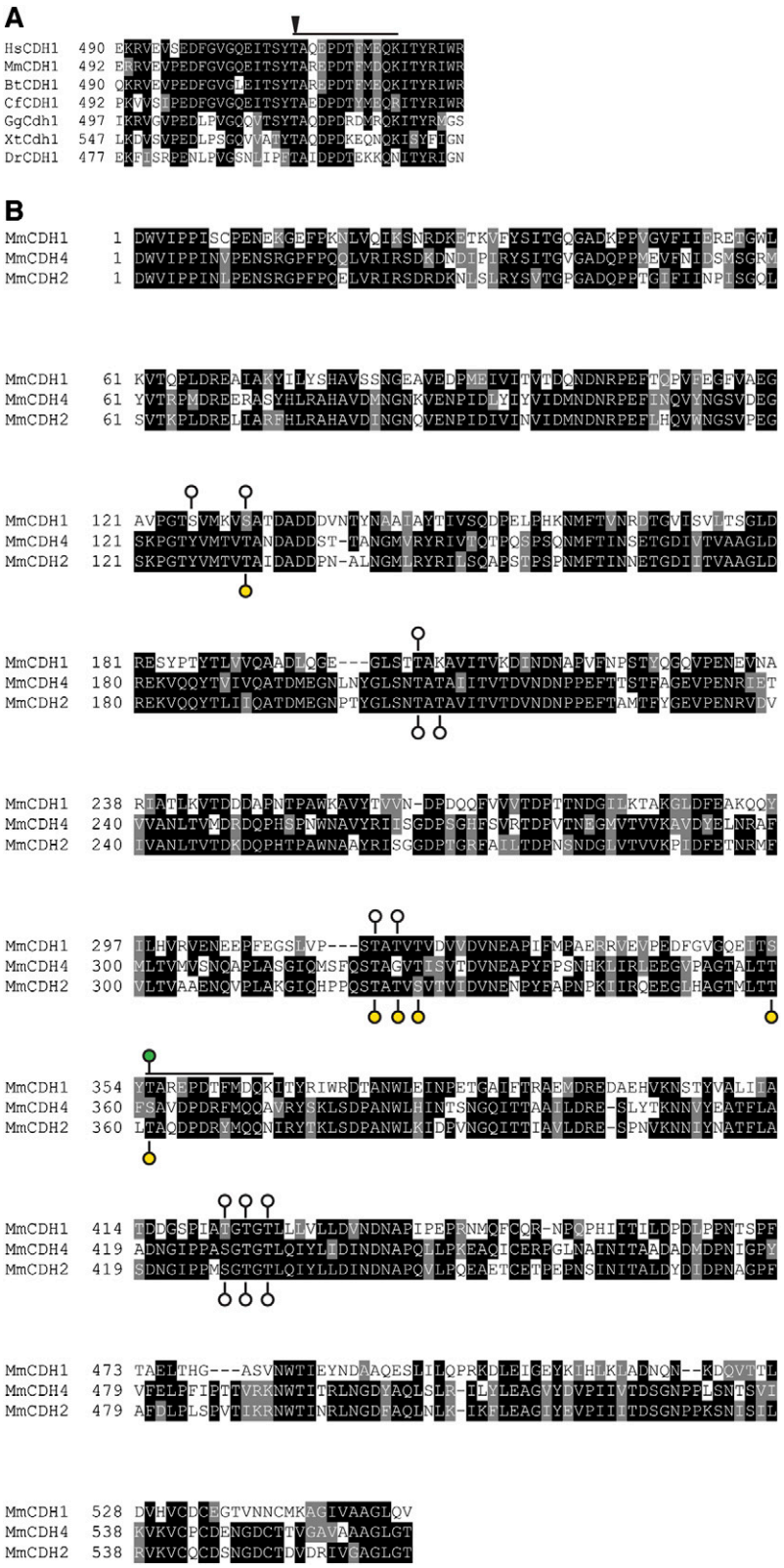

Fig. S4. Conservation of O-mannosylation sites in type I classical cadherins. (*A*) Conservation of the identified O-mannosylation site of E-cad–derived TAQEPDTFMEQK, located in EC4. Alignment [ClustalW (1)] of multiple sequences from vertebrates [*Homo sapiens* (Hs), *Mus musculus* (Mm), *Bos taurus* (Bt), *Canis familiaris* (Cf), *Gallus gallus* (Gg), and *Danio rerio* (Dr)]. The peptide TAQEPDTFMEQK is indicated by the horizontal line. In relation to the crystal structure of mouse E-cad (2), the O-mannosylated threonine is depicted by an arrow. (*B*) Alignment of the sequences of the extracellular domains of *Mus musculus* E-cad (MmCDH1), retinal cadherin (R-cadherin, MmCDH4), and neuronal cadherin (N-cadherin, MmCDH2). Putative O-mannosylation sites, according to the crystal structures of E- and N-cadherin, are depicted as open circles (2). O-mannosylation sites that recently were confirmed on N-cadherin are shown in yellow (3). The

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majority of O-mannosyl glycosites are highly conserved among these cadherins. The O-mannosylated peptide TAQEPDTFMEQK we identified is indicated. The conserved O-glycosylation site is shown in green.

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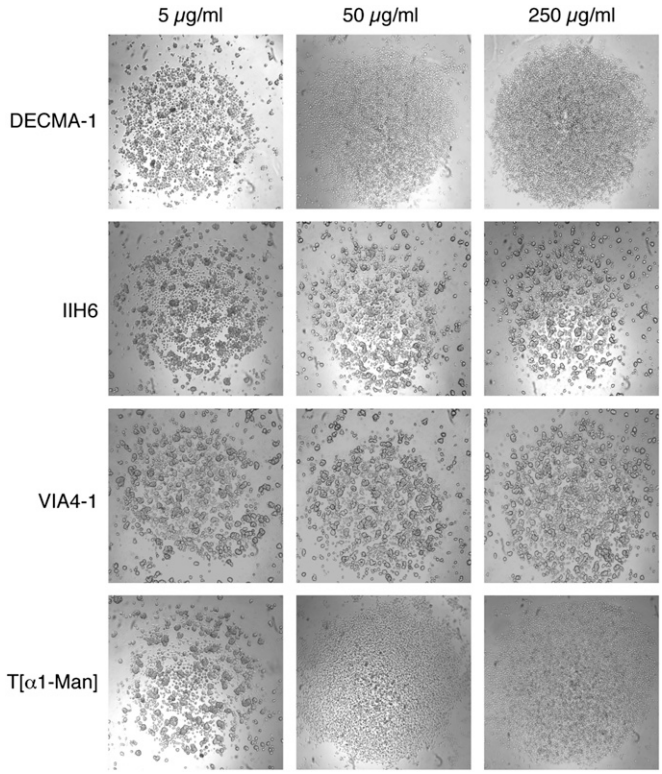

Fig. S5. Slow-aggregation assays of MDCK cells. MDCK cells were aggregated overnight in the presence of E-cad–specific (DECMA-1) and T[α1-Man]–specific antibodies and antibodies directed against O-mannosidically linked carbohydrate epitopes present on α-DG (IIH6 and VIA4-1). Antibodies were used at concentrations of 5 μg/mL, 50 μg/mL, and 250 μg/mL as indicated. Aggregation was inhibited to a similar extent by the E-cad–specific (DECMA-1) and T[α1-Man]–specific antibodies but not by antibodies directed against O-mannosylated α-DG (IIH6 and VIA4-1).

Table S1. PCR Primer sequences

Primer a*	5′-ccg*ctcgag*cttcaaggctgacagaaaatattgg-3′
Primer b†	5′-tcc*ccgcgg*aa*gtcgac*gtgcggttaatatagtaacttcc-3′
Primer c*	5′-ccg*ctcgag*gctacatgggaatgagaggag-3′
Primer d*	5′-ccg*ctcgag*gagaggctaatggtaagcagc-3′
Primer 1	5′-agctgtggcacaagaaagggtttg-3′
Primer 2	5′-ggatgcagttcagatcgtgaactag-3′
Primer 3	5′-gggctgtaagagcctgagagag-3′
Primer 4	5′-cttctatcgccttcttgacggttc-3′
Primer 5	5′-gattcctctctcctcagatgctga-3′
Primer 6	5′-tacctgtaggaccttgggaaag-3′
Primer 7	5′-tacctgtaggaccttgggaaag-3′
Primer 8	5′-cacagtggtgacaaccaggaa-3′
Primer 9	5′-cctatgcactgctcttccaca-3′
Primer 10	5′-catccaccactgggaaaggta-3′
Primer 11	5′-gactgcatttgtgtcttcatcca-3′
Primer 12	5′-ccttcccgcttcagtgaca-3′
Primer 13	5′-ggctatgactgggcacaaca-3′
Primer 14	5′-aaccaatagcctggcagtatagca-3′
Primer 15	5′- aagtccctccatctccactctga-3′
Primer 16	5′-gggactggggagtcttttgatctaa-3′
Primer 17	5′-gtccatctgcacgagactagtgaga-3′
Primer 18	5′-gccttactcctctcattcccatgta-3′
Primer 19‡	5′-tga*actagt*ggagctggagcctgagtcctgc-3′
Primer 20§	5′-cttga*gtcgac*gtcgtcctaccaccgcc-3′
Pomt1 forward	5′-ctacatcccaggaccagtgctcaga-3′
Pomt1 reverse	5′-agcgggaccaggcatcctca-3′
Pomt2 forward	5′-tccagcatgttgacaggtatcctatgg-3′
Pomt2 reverse	5′-cataagccagagggtggaagaggtaga-3′
Ppia forward	5′-cgcgtctccttcgagctgtttg-3′
Ppia reverse	5′-gtaaagtcaccaccctggcacatg-3′

*XhoI site in italics.
†SacII and SalI sites in italics.
‡SpeI site in italics.
§SalI site in italics.