Supplemental Information

Chondroitin Sulfate Is Indispensable for Pluripotency and Differentiation of Mouse Embryonic Stem Cells

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Supplemental Methods

Embryoid body (EB) formation

ES cells were maintained on feeder cells and transferred to gelatin for three passages prior to EB generation. For differentiation into EBs, ES cells were trypsinized and transferred into bacterial dishes at 5000 cells/ml in 20% KnockOut™ Serum Replacement without LIF, and medium was changed every second day. CSase (10 mIU/ml), HSase (5 mIU/ml)/Hepase (5 mIU/ml), Heparin (10 μg/ml), HS (10 μg/ml), CS-A (10 μg/ml), CS-C (10 μg/ml), or CS-E (10 μg/ml) was added.

Immunofluorescence

For immunofluorescence, cells were fixed in 4% paraformaldehyde for 10 min and blocked in 3% BSA for 60 min, with PBS washes between all steps. Antibodies were diluted in blocking solution. The primary antibody was E-cadherin (ECDD-2, 1:100; Takara). An appropriate secondary antibody (Alexa488-conjugated 1:200) was obtained from Invitrogen. To visualize nuclei, cells were incubated in DAPI (Dojindo) for 10 min at room temperature. Fluorescent images were obtained using a laser-scanning confocal microscope LSM710 (Carl Zeiss).

Quantitative real-time RT-PCR

Total RNA was extracted from ES cells using TRIzolR reagent (Invitrogen). cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega) and a random nonamer primer (TaKaRa Bio Inc., Shiga, Japan). The primer sequences used were: Gata6, forward primer 5′-AGGAATTCAAACCAGGAAACG-3′ and reverse primer 5′-TGGTCGCTTGTGTAGAAGG-3′; Tbx6, forward primer 5′-ATGTACCATCCACGAGAGTTGT-3′ and reverse primer 5′-GGTAGCGGTAACCCTCTGTC-3′; E-cadherin, forward primer 5′-GGACAGCAACATCAGCGA-3′ and reverse primer 5′-GGCTTCAGGAATACATGGACAAA-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward primer 5′-CATCTGAGGGCCCACTG-3′ and reverse primer 5′-GAGGCCATGTAGGCCATGA-3′. Quantitative real-time RT-PCR was performed using FastStart DNA Master plus SYBR Green I in a LightCycler ST300 (Roche). The expression level of each gene was normalized to that of the GAPDH transcript.

Protein extraction and western blot analysis

Cells were lysed with immunoprecipitation buffer containing 0.5 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture (Roche), 1% Triton X-100, and 1 mM sodium orthovanadate. Cell lysates were centrifuged at 12,000 ×*g* for 20 min at 4°C, and the supernatants were stored at −80°C. Protein quantification was performed using the bicinchoninic acid protein assay reagent (Pierce). Protein aliquots (10 μg) were denatured in SDS sample buffer and electrophoresed on 10% polyacrylamide-SDS gels. The membranes were immunoblotted using a primary antibody against E-cadherin (ECDD-2, 1:100; Takara) and β-actin (1:1000; Sigma). The antigen-antibody complexes were visualized using appropriate secondary antibodies (Sigma) and the enhanced chemiluminescence detection system, as recommended by the manufacturer (GE Healthcare).

ES cell culture with CSase or HSase

Before beginning differentiation experiments, the ES cell cultures were depleted of feeder cells by incubating trypsinized cells in ES cell medium on culture dishes for 30 min, during which only the feeder cells attached to the dish. Subsequently, the remnant ES cells were plated on gelatin-coated dishes, cultured overnight, and feeder-depleted again in ES cell medium with the addition of CSase (10 mIU) or HSase (Heparinase and heparitinase, 5 mIU each) on culture dishes for 60 min before they were counted. ESCs were transferred into bacterial dishes at 5000 cells/ml in 20% KnockOut™ Serum Replacement without LIF and with the addition of CSase (10 mIU) or HSase (Heparinase and heparitinase, 5 mIU each). The medium with CSase or HSase was changed every second day.

Adhesion assay

To prepare the E-cad-Fc-coated surface, E-cad-Fc (R&D Systems) was directly added to untreated polystyrene 96-well plates. After incubation for 2 h at 37°C, plates were washed with PBS once, and cells were seeded at a density of 3.0×10^4 cells/well into 96-well plates precoated with 0.1% gelatin or E-cad-Fc. After 4 h of culture, medium and non-adherent cells were removed, and cells were washed with culture medium. Adherent cells were stained with Cell counting lit-8 (Dojindo), and absorbance at 570 nm was measured using a microplate reader.

Supplemental Figure Legends

Figure S1. Quantitative analysis of differentiation marker genes.

A, Real-time PCR results of *Gata6* (emdoderm), *Nestin* (ectoderm), and *Tbx6* (mesoderm) from EBs cultured with GAGs at day 6. GAPDH transcripts were used as an internal control. Statistical analysis was carried out by Student's *t* test. *P < 0.01 and **P < 0.005. B, Real-time PCR results of *Gata6* (emdoderm), *Nestin* (ectoderm), and *Tbx6* (mesoderm) from EBs cultured with CSase or HSase at day 6. GAPDH transcripts were used as an internal control. Statistical analysis was carried out by Student's *t* test. *P < 0.01 and **P < 0.005.

Figure S2. CS-A and CS-E rescued differentiation of *GlcAT-I*$^{-/-}$ ESCs and anti-E-cadherin antibody inhibited EB formation.

A, Phase-contrast microscopy of day 0 to day 4 EBs. B, Phase-contrast microscopy of day 4 EBs. C, Proliferation of EBs. Final cell number per ml at EB day 6; the starting cell density was 5000 cells per ml. *P < 0.01, Wt versus Wt ES cells treated with CSase, **P < 0.005, Wt versus *GlcAT-I*$^{-/-}$ ES cells, *GlcAT-I*$^{-/-}$ ES cells treated with CS-E versus *GlcAT-I*$^{-/-}$ ES cells treated with anti-E-cadherin antibody and CS-E, and ***P < 0.001, *GlcAT-I*$^{-/-}$ ES cells versus *GlcAT-I*$^{-/-}$ ES cells treated with CS-A, *GlcAT-I*$^{-/-}$ ES cells treated with CS-A versus *GlcAT-I*$^{-/-}$ ES cells treated with anti-E-cadherin antibody and CS-A, and *GlcAT-I*$^{-/-}$ ES cells versus *GlcAT-I*$^{-/-}$ ES cells treated with CS-E.

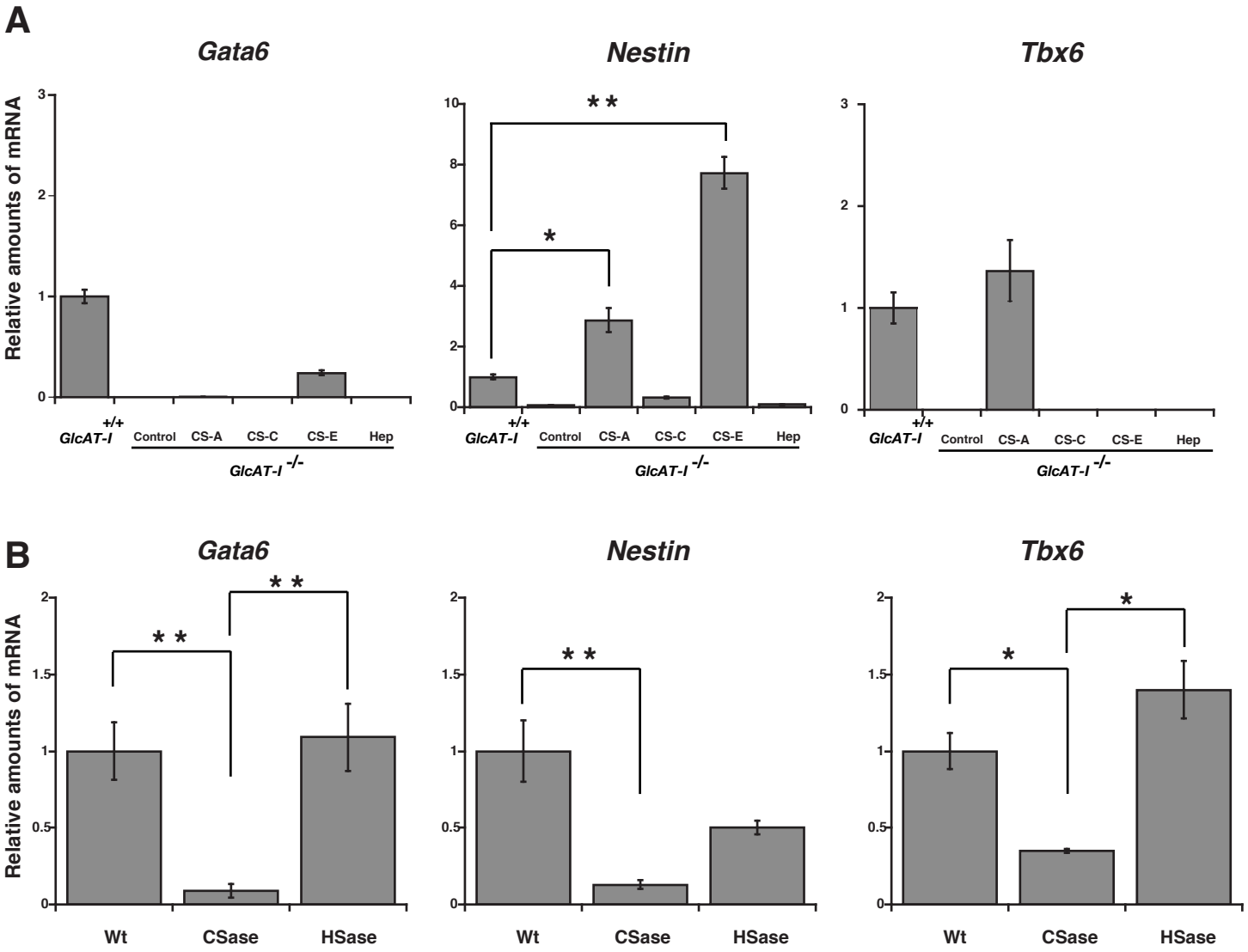
Figure S3. E-cadherin expression was increased in *GlcAT-I*$^{-/-}$ ES cells

A, Wild-type (left) or *GlcAT-I*$^{-/-}$ ES cells (right) were stained with E-cadherin (green). Nuclei were visualized with DAPI staining (blue). B, Real-time PCR and C, western blot results of *E-cadherin* in Wt or *GlcAT-I*$^{-/-}$ ES cells. The gels have been run under the same experimental condition. The blot image was cropped around the region of interest. The results are representative of at least three independent experiments. Statistical analysis was carried out by Student's *t* test. **P < 0.005.

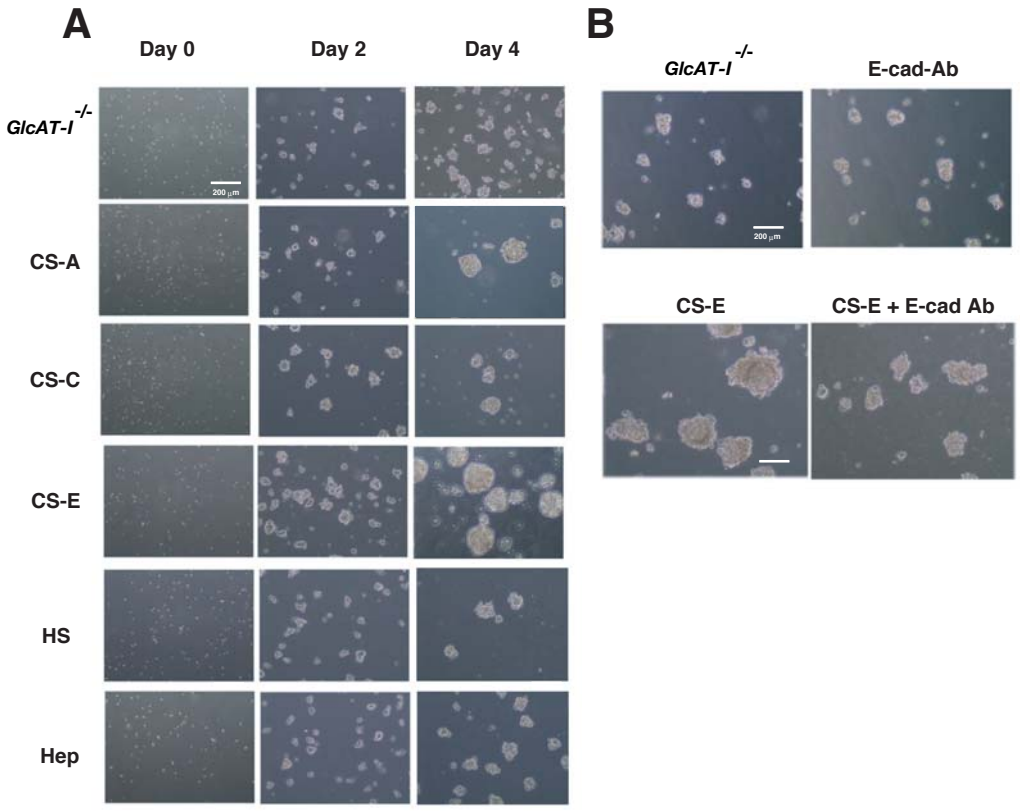
Figure S4. E-cadherin homophilic interactions were unaffected in *GlcAT-I*$^{-/-}$ ES cells

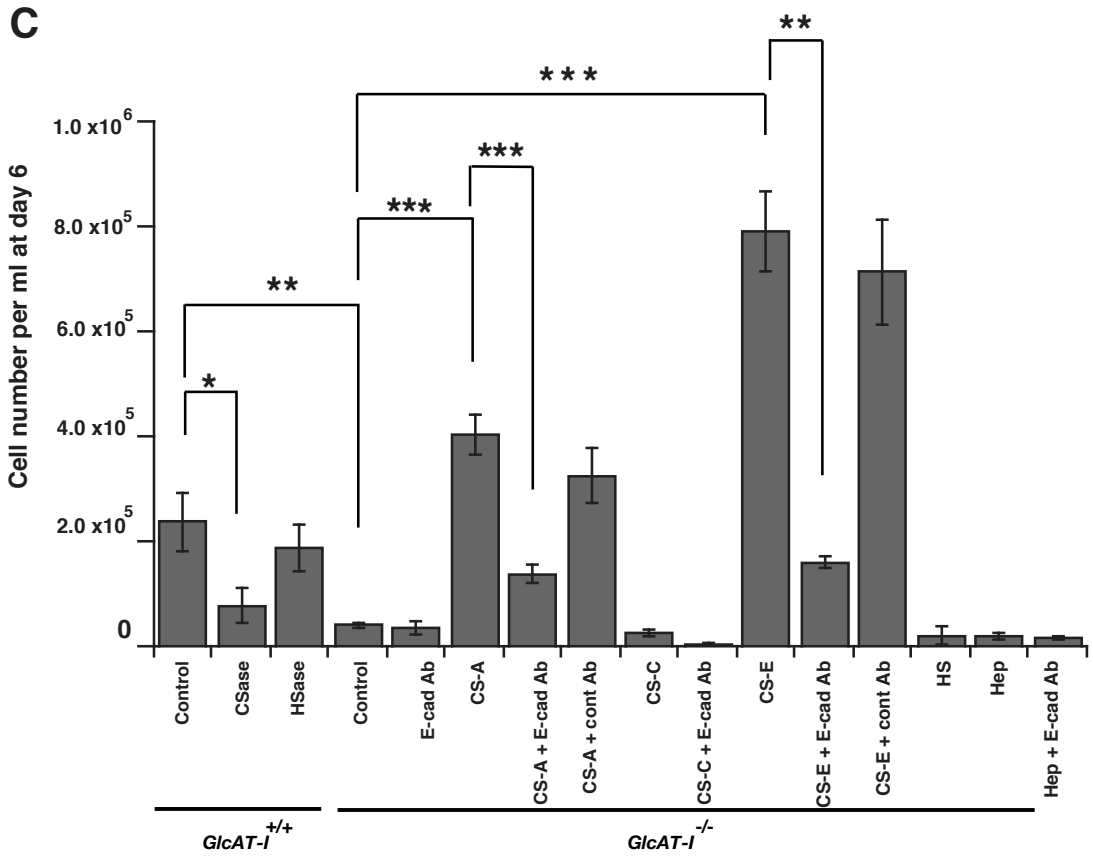
Adhesion of mouse ES cells onto the surface of a 96-well plate coated with E-cadherin-Fc was examined after 4 h of incubation. Wt ES cells adhered to the E-cadherin-Fc-coated surface with equivalent efficiency as to a 0.1% gelatin-coated surface. The data indicate means ± S.D. of three separate experiments. **P < 0.005, Wt versus Wt ES cells treated with EGTA, *GlcAT-I*$^{-/-}$

ES cells versus *GlcAT-I*$^{-/-}$ ES cells treated with EGTA, and Wt versus *GlcAT-I*$^{-/-}$ ES cells.

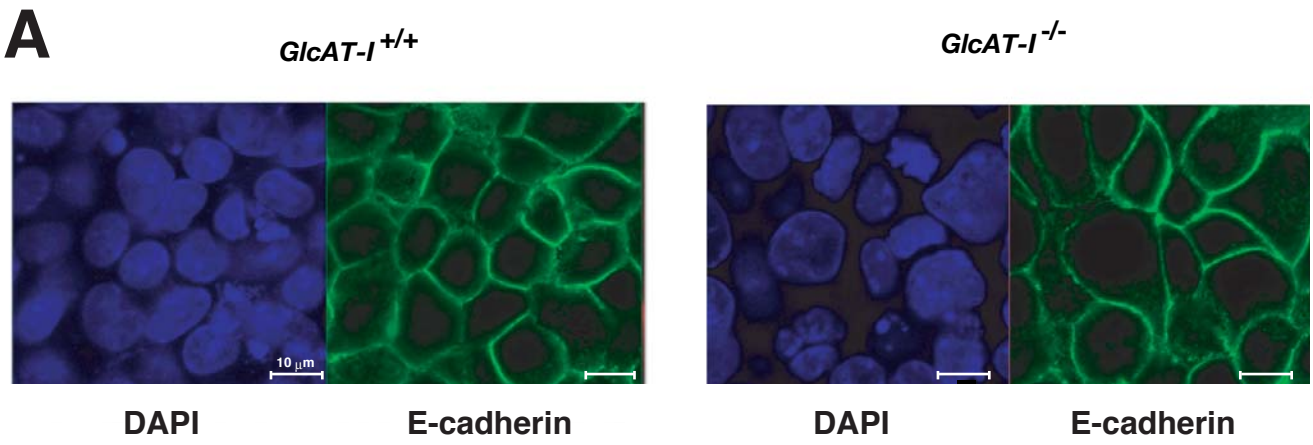


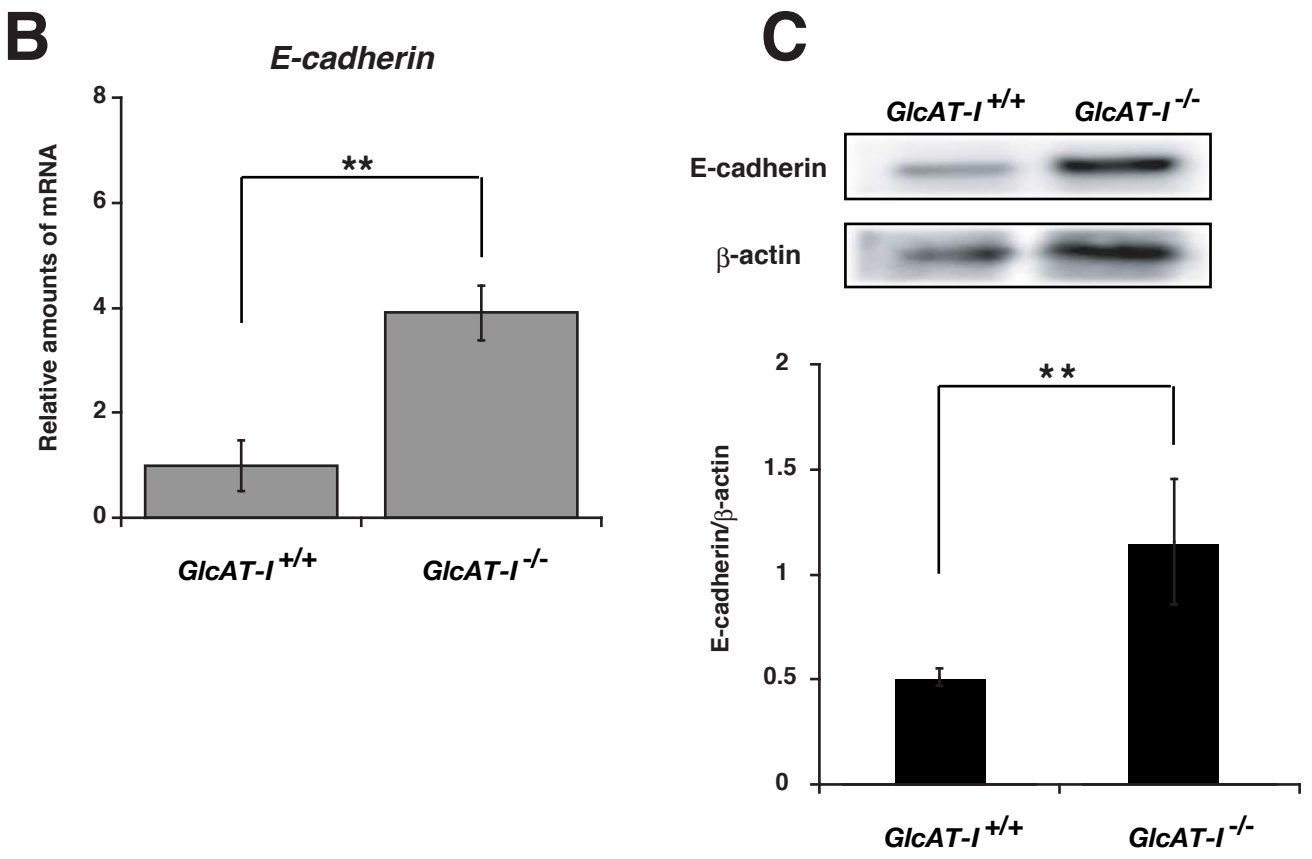
Supplementary Fig. S1



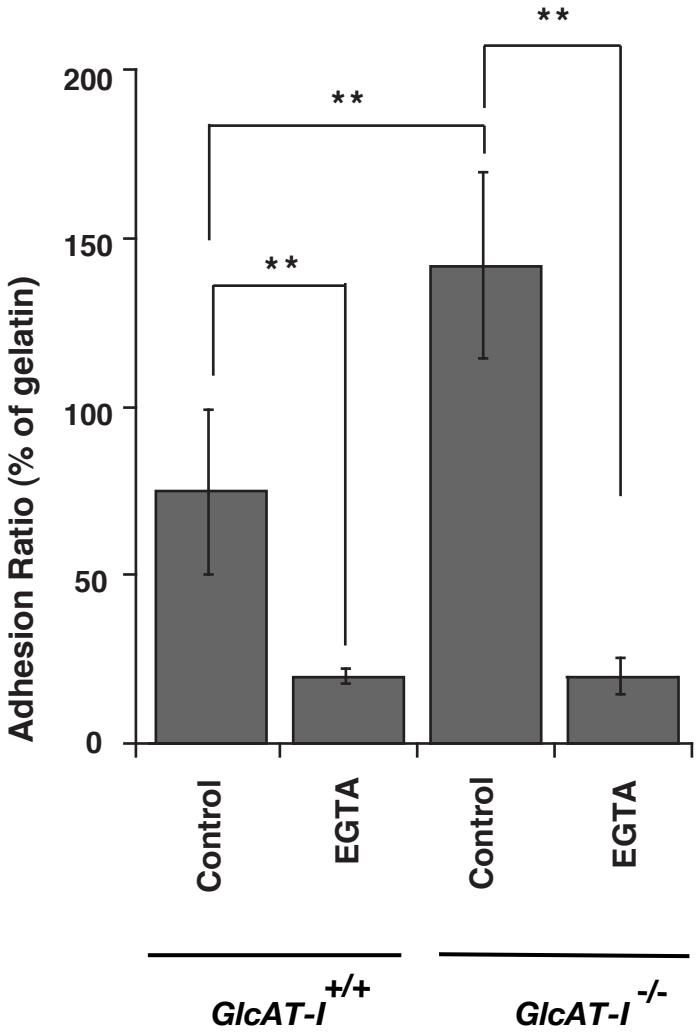


Supplementary Fig. S2





Suppplementary Fig. S3



Supplementary Fig. S4

Table S1

Disaccharide composition of HS from *GlcAT-I*$^{+/+}$, *GlcAT-I*$^{+/-}$, and *GlcAT-I*$^{-/-}$ embryonic stem cells

The values are expressed as pmol of disaccharide per mg of dried homogenate of these cells.

Disaccharides[a]	pmol/mg (mol%)[b]		
	GlcAT-I$^{+/+}$	*GlcAT-I*$^{+/-}$	*GlcAT-I*$^{-/-}$
ΔDiHS-0S	281.9 ± 11.8 (76)	185.5 ± 8.0 (72)	N.D.[c]
ΔDiHS-6S	2.0 ± 0.7 (1)	1.5 ± 0.2 (1)	N.D.
ΔDi-NS	34.9 ± 1.7 (9)	36.8 ± 3.2 (14)	N.D.
ΔDi-diS$_1$	13.1 ± 3.2 (4)	7.1 ± 2.8 (3)	N.D.
ΔDi-diS$_2$	29.7 ± 5.2 (8)	25.0 ± 5.9 (9)	N.D.
ΔDi-triS	7.3 ± 2.6 (2)	1.9 ± 0.7 (1)	N.D.
Total (pmol/mg)	368.9 ± 20.8	257.8 ± 21.9	N.D.

[a]Abbreviations: ΔDiHS-0S, ΔHexUAα1-4GlcNAc; ΔDiHS-6S, ΔHexUAα1-4GlcNAc(6-*O*-sulfate); ΔDi-NS, ΔHexUAα1-4GlcN(2-*N*-sulfate); ΔDi-diS$_1$, ΔHexUAα1-4GlcN(2-*N*-sulfate,6-*O*-sulfate); ΔDi-diS$_2$, ΔHexUA(2-*O*-sulfate)α1-4GlcN(2-*N*-sulfate); ΔDi-triS, ΔHexUA(2-*O*-sulfate)α1-4GlcN(2-*N*-sulfate,6-*O*-sulfate).

[b] The values are the means from three independent experiments.

[c] N.D., not detected.

Table S2

Disaccharide composition of CS from Wt EB

Disaccharides	pmol/mg (mol%)	
	Day4 [a]	Day6 [a]
ΔDi-0S	32 ± 5 (17)	45 ± 9 (13)
ΔDi-6S	12 ± 3 (5)	8 ± 2 (2)
ΔDi-4S	148 ± 10 (75)	285 ± 4 (79)
ΔDi-diS$_D$	2 ± 1 (1)	5 ± 1 (1)
ΔDi-diS$_E$	3 ± 1 (2)	17± 2 (5)
Total (pmol/mg)	197 ± 15	360 ± 21

[a] The values are the means from two independent experiments.

Table S3
Kinetic parameters for the interaction of various CS with immobilized E-cadherin

GAG	*ka*	*kd*	*Kd*
	$M^{-1}s^{-1}$	s^{-1}	*nM*
CS-A	1.91×10^{4}	2.84×10^{-3}	1.49×10^{2}
CS-C	ND	ND	ND
CS-E	9.59×10^{4}	8.34×10^{-4}	9.21

The apparent association (k_a) and dissociation (k_d) rate constants and equilibrium dissociation constants (K_d) for the interaction of various GAGs with immobilized E-cadherin were determined using a BIAcore system.