Supplementary Information

Supplementary Materials and Methods

RNA isolation and quantitative RT-PCR analysis

Total RNA was prepared using RNA-lysis reagent (PRIME) as described previously [1]. The primers used are shown in Supplementary Table S1. Quantitative RT-PCR was performed using SYBR Green Master Mix (Takara) on an ABI instrument (Applied Biosystems, Inc., USA).

Preparation of galectin-3-expressing vectors

The construction of a plasmid expressing full-length human galectin-3, pcDNA3.1-NT-GFP-gal3; the pcDNA3.1-NT-GFP vector; pcDNA3.0-FLAG-galectin-3(1–250); pcDNA3.0-FLAG-galectin-3(Δ44–58); and the lentiviral vectors that overexpress LacZ, galectin-3(1–250), and galectin-3(Δ44–58) in pLECE3 have been previously described [2].

Immunoprecipitation and western blot analysis

Immunoblotting was done with primary antibodies against β-catenin, galectin-3, STAT3, HA, GSK-3β, GSK-3β(ser9) (Santa Cruz Biotechnology, USA), pSTAT3(Tyr705), STAT3, survivin, cyclin D1, lamin A/C (Cell Signaling, USA), and FLAG (Merck, USA). β-Actin and GAPDH were purchased from Santa-Cruz Biotechnology. Quantitative analysis of western blot data from more than three experiments was performed with ImageJ and was expressed as mean ± SD. Immunoprecipitation was carried out with protein A/G agarose beads coated with galectin-3 and STAT3, and proteins were detected with antibodies against galectin-3, STAT3, β-catenin, and HA using western blotting. Mouse/rabbit IgG was used as the negative control.

Immunocytochemical (ICC) analysis

Cells were cultured in a chamber slide and fixed with 3.7% formaldehyde and then permeabilized with 0.5% Triton X-100. The cells were blocked with 5% BSA in PBS, incubated with primary antibodies against galectin-3, pSTAT3 (Tyr705), and STAT3 (diluted 1:200 in PBS containing 1% BSA), and then incubated with secondary antibodies labeled with FITC and Cy5 (Zymed) (diluted 1:200 in PBS containing 1% BSA). Finally, the samples were mounted with mounting medium containing DAPI, covered with a cover glass, and analyzed on a confocal microscope (Carl Zeiss, Germany).

Luciferase assays

STAT3 transcriptional activity was measured as described previously [3]. The STAT3 luciferase vector was kindly provided by Dr. Sungpil Yoon (National Cancer Center) [3, 4]. TOP flash and FOP flash luciferase vectors were used to examine TCF4-mediated transcription induced by Wnt signaling, as previously reported [5]. STAT3, TOP flash, and FOP flash luciferase vectors, and the drug treatments are described in the figure legends. The cells were harvested at 48 h post transfection, and luciferase activity was measured using a luciferase assay system (Promega, USA), according to the manufacturer's instructions.

Mouse embryo fibroblasts (MEFs)

Female and male *lgals3*$^{+/-}$ mice were crossed to produce wild-type and *lgals3*$^{-/-}$ MEFs. To obtain MEFs from 13.5-day-old embryos, the embryos were washed with PBS and minced. Next, MEFs were incubated with 3 mL of trypsin/EDTA (Gibco, USA) at 37 °C for 15 min. The trypsinized MEFs were transferred to 150 mm culture dishes containing 20 mL of DMEM supplemented with 10% FBS and incubated at 37 °C for 4–8 h. After incubation, the medium was exchanged. The MEFs were maintained in the same medium and subcultured 1:3 upon reaching confluence.

Structural modeling

Modeling was performed using Biovia Discovery Studio 2017 (DS). CDocker was used as the main docking tool. The STAT3 protein structure (PDB: 4E68) was used as the receptor, and two peptides

from the SH2 binding motif in galectin-3 (Supplementary Fig. S10), wild-type (WT; GAYPGQ) and Y45P (GApYPGQ), were used as ligands. The peptides were manually placed in the binding site (Radius = 20 Å, XYZ = -188.373, -5.108, 20.363552) at pH 7.5, correcting for missing side chains and deletion of all water molecules. The algorithm was adopted from the CHARMm protocol, and CDocker was run to identify the top 10 hits for each peptide. The CDocker interaction energies were analyzed for each hit. The best score was selected [WT(GAYPGQ): -CDOCKER ENERGY = 70.1911, -CDOCKER INTERACTION ENERGY = 63.2348; Y45P(GApYPGQ): -CDOCKER ENERGY = 101.004, -CDOCKER INTERACTION ENERGY = 81.2132].

Analysis of the gene expression signatures of patients with GC in a public dataset

The gene-expression relationships were analyzed in various GC types using the Gene Expression Omnibus (GEO) profile GSE27342 (www.ncbi.nlm.nih.gov/geoprofiles) [6]. The GSE27342 dataset is composed of 80 paired gastric tumors and adjacent normal tissues. The dataset was normalized using GEO2R, and a scatter plot was generated for the expression pattern analysis.

References

1. Kim SJ, Hwang JA, Ro JY, Lee YS, Chun KH. Galectin-7 is epigenetically-regulated tumor suppressor in gastric cancer. Oncotarget. 2013;4(9):1461-71.
2. Kang HG, Kim DH, Kim SJ, Cho Y, Jung J, Jang W, et al. Galectin-3 supports stemness in ovarian cancer stem cells by activation of the Notch1 intracellular domain. Oncotarget. 2016;7(42):68229-41.
3. Ahn YH, Yi H, Shin JY, Lee KD, Shin SP, Lee SJ, et al. STAT3 silencing enhances the efficacy of the HSV.tk suicide gene in gastrointestinal cancer therapy. Clin Exp Metastasis. 2012;29(4):359-69.
4. Kim JH, Lee SC, Ro J, Kang HS, Kim HS, Yoon S. Jnk signaling pathway-mediated regulation of Stat3 activation is linked to the development of doxorubicin resistance in cancer cell lines. Biochem Pharmacol. 2010;79(3):373-80.
5. Choi SW, Song JK, Yim YS, Yun HG, Chun KH. Glucose deprivation triggers protein kinase C-dependent beta-catenin proteasomal degradation. J Biol Chem. 2015;290(15):9863-73.
6. Cui J, Chen Y, Chou WC, Sun L, Chen L, Suo J, et al. An integrated transcriptomic and computational analysis for biomarker identification in gastric cancer. Nucleic Acids Res. 2011;39(4):1197-207.

Supplementary Table

Supplementary Table S1. The primer sequences used in this study

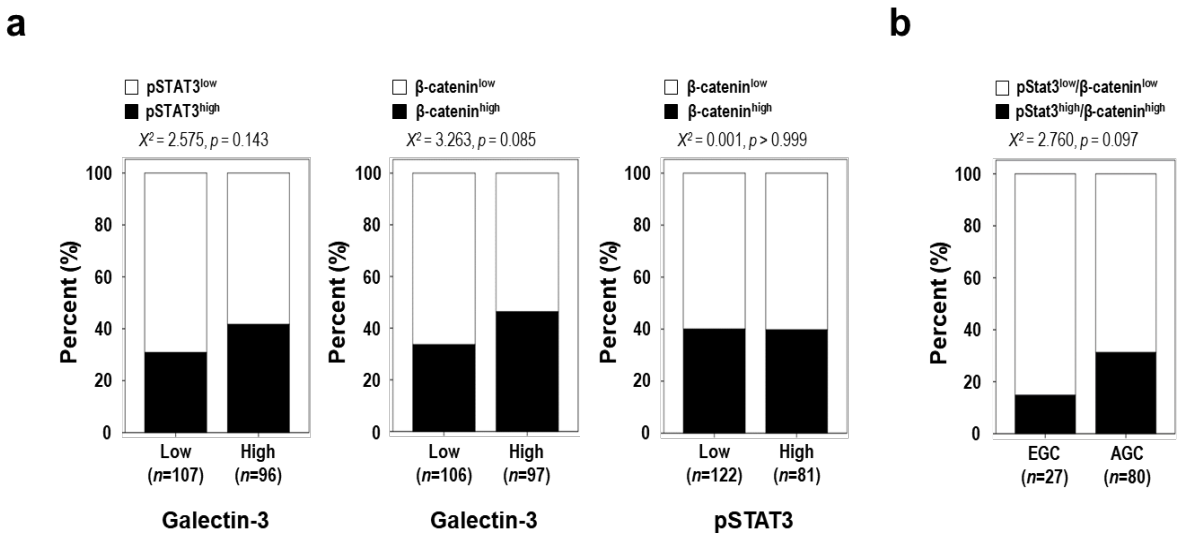
Gene name		sequence
mIL-1β	Sense	5′- CAACCAACAAGTGATATTCTCCATG-3′
	Anti-sense	5′- GATCCACACTCTCCAGCTGCA-3′
mIL-6	Sense	5′- GAGGATACCACTCCCAACAGACC-3′
	Anti-sense	5′- AAGTGCATCATCGTTGTTGTTCATACA-3′
mIL-11	Sense	5’- GATGTCCTACCTCCGGCAT -3’
	Anti-sense	5’- TCAAGAGCTGTAAACGGCG -3’
mIL-27	Sense	5’- GTGACAGGAGACCTTGGCTG -3’
	Anti-sense	5’- CTGACTGTGAATTCCCTGCG -3’
mLIF	Sense	5’- CCCCATTTGAGCATGAACTT -3’
	Anti-sense	5’- AGCAGCAGTAAGGGCACAAT -3’
mIL-10	Sense	5′- GGTTGCCAAGCCTTATCGGA -3′
	Anti-sense	5′- ACCTGCTCCACTGCCTTGCT -3′
mIL-24	Sense	5’- AGGCCTTCTGGACTGTGAAG -3’
	Anti-sense	5’- TGGGCAAGGTAACAGCTCTC-3’
mIL-28	Sense	5’- AAAGGATTGCCACATTGCTC -3’
	Anti-sense	5’- AGATGAGGTGGGAACTGCAC -3’
mIFN-γ	Sense	5′- TCAAGTGGCATAGATGTGGAAGAA -3′
	Anti-sense	5′- TGGCTCTGCAGGATTTTTCATG -3′
mTNFα	Sense	5′- CGTCAGCCGATTTGCTATCT -3′
	Anti-sense	5′- CGGACTCCGCAAAGTCTAAG -3′
mβ-actin	Sense	5′- GGCTGTATTCCCCTCCATCG -3′
	Anti-sense	5′- CCAGTTGGTTGGTAACAATGCCATGT -3′

Supplementary Table S2. Clinicopathological characteristics

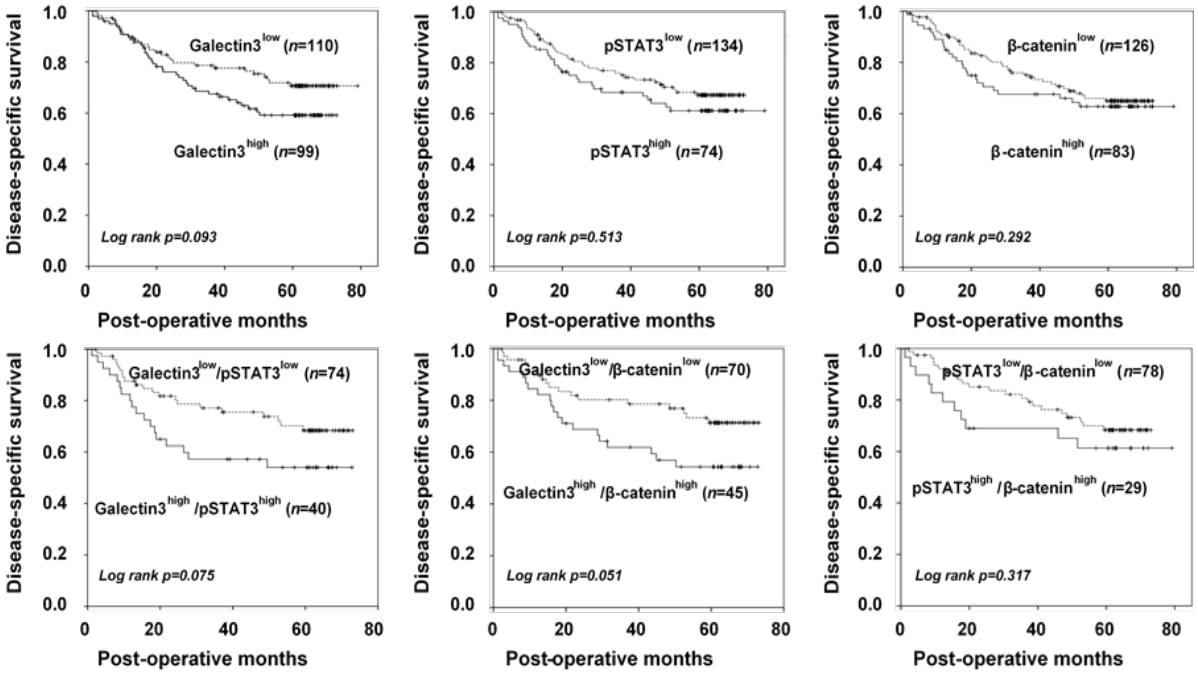
Group	Frequency	(%)
Age	56.9 ± 12.0*	
Sex		
Male	65	31.1
Female	144	68.9
Differentiation		
Well differentiated	18	8.6
Moderately differentiated	65	31.1
Poorly differentiated	96	45.9
Signet ring cell carcinoma	25	12.0
Mucinous adenocarcinoma	5	2.4
T stage		
1	49	23.4
2	39	18.7
3	73	34.9
4	48	23.0
Lymph node metastasis		
Absent	87	41.6
Present	122	58.4
Lymphovascular invasion		
Absent	116	55.5
Present	93	44.5

* mean value

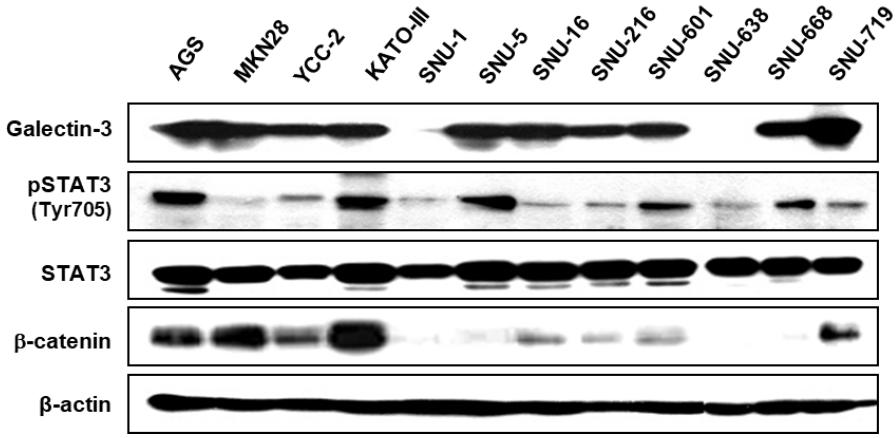
Supplementary Figures



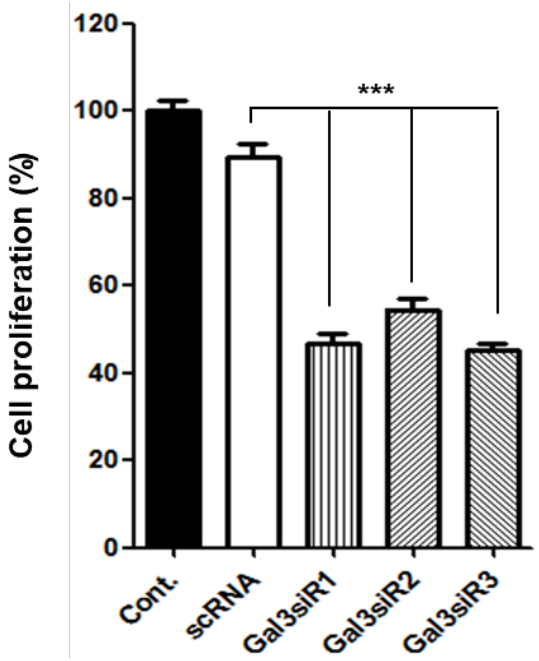
Supplementary Figure S1. Galectin-3, pSTAT3, and β-catenin expression in gastric cancer specimens. (a) Galectin-3 expression shows the tendency of positive correlation with pSTAT3 and β-catenin in gastric cancer specimens, while there is no correlation between pSTAT3 and β-catenin. **(b)** The cases of combined pSTAT3^{high}/β-catenin^{high} expression was observed more frequently in advanced gastric cancer cases compared to those of early gastric cancers, but this did not reach statistically meaningful values.



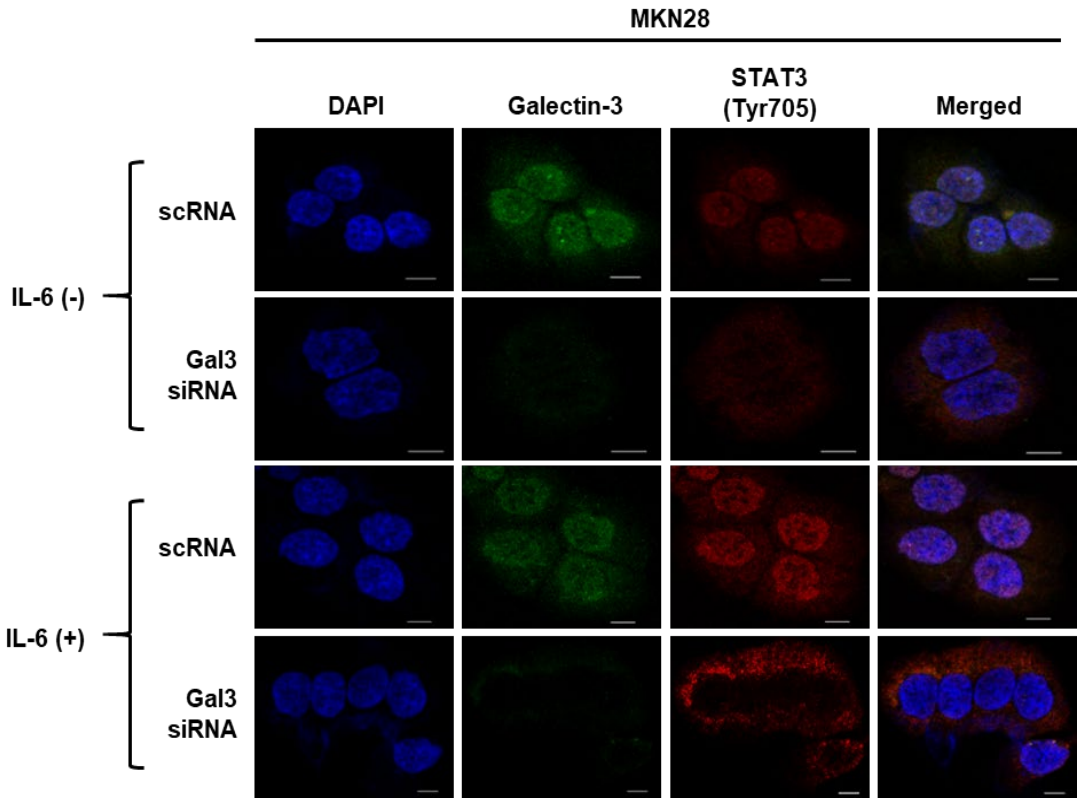
Supplementary Figure S2. Disease-free survival was analyzed by Kaplan-Meier plot. Patients with combined galectin-3^{high}/pSTAT3^{high} (mean survival time; 38.6 months, $p = 0.075$) and galectin-3^{high}/β-catenin^{high} (mean survival time; 41.5 months, $p = 0.051$) expressions exhibited a tendency of worse disease-specific survival, than patients with galectin-3^{low}/pSTAT3^{low} (mean 45.0 months) and galectin-3^{low}/β-catenin^{low} (mean 46.8 months) expressions.



Supplementary Figure S3. Detection of expression levels of β-catenin, STAT3, phosphorylated STAT3(Tyr 705) and galectin-3 in 12 gastric cancer cell lines. Protein expression was analyzed by western blot analysis. β-actin was used as a loading control.



Supplementary Figure S4. Depletion of galectin-3 reduces cell proliferation. Detection of cell viability after 3 type of galectin-3 siRNA transfection by WST assay.

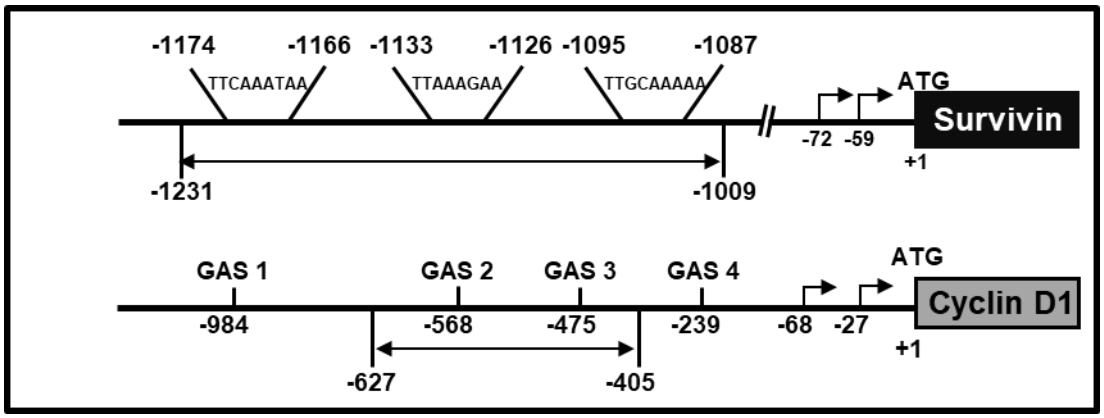


Supplementary Figure S5. IL-6 induces phosphorylation of STAT3 with galectin-3 dependent manner in MKN28 gastric cancer cells. After galectin-3 specific siRNA treatment, IL-6 cytokine were subjected for 6 hrs. Cells were fixed and immunocytochemical analysis was performed using confocal microscope. DAPI was used to figure out the nucleus (Blue). The bars indicate 10 μm.

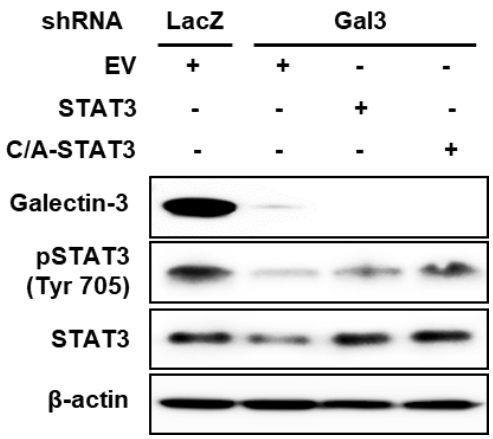
Galectin-3 protein sequence (250 Amino acid)
MADNFSLHDA LSGSGNPNPQ GWPGAWGNQP AGAGGYPGAS YPGA**YPGQ**AP
PGA**YPGQ**APP GAYPGAPGAY PGAPAPGVYP GPPSGPGAYP SSGQPSATGA
YPATGPYGAP AGPLIVPYNL PLPGGVVPRM LITILGTVKP NANRIALDFQ
RGNDVAFHFN PRFNENNRRV IVCNTKLDNN WGREERQSVF PFESGKPFKI
QVLVEPDHFK VAVNDAHLLQ YNHRVKKLNE ISKLGISGDI DLTSASYTMI

YPGQ : STAT3 SH2 domain binding motif

Supplementary Figure S6. Amino acid sequence of Galectin-3. Galectin-3 has two prediction sites for STAT3 SH2 domain binding motif (AA number 45-48, 54-57). Also, galectin-3 has a nuclear localization signal (NLS)-like sequence (222-230).



Supplementary Figure S7. Schematic models of STAT3 binding site regions in the promoter of survivin and Cyclin D1. Survivin promoter has a 3 site of STAT3 binding motif and Cyclin D1 promoter has a STAT3 binding site. ChIP primers was performed as described in “Materials and Methods” to detect STAT3 binding on the promoter of survivin (-1231~ -1009) and Cyclin D1 (-627 ~ -405).



Supplementary Figure S8. Preparation of cells with galectin-3 depletion and STAT3 overexpression for experiments of xenograft in nude mouse. Stabled cells after co-transfection with galectin-3 shRNA lenti-virus vector and STAT3 overexpression vector or galectin-3 shRNA lenti-virus vector and constitutive activated (C/A) STAT3 overexpression vector. LacZshRNA lenti-virus vector was used as a transfection control. Cell lysates were prepared and expression level of galectin-3, STAT3 and phosphorylated STAT3 were detected by western blotting. β-actin was used as a loading control.