Molecular Cell, Volume *27*

Supplemental Data

The MUC1 and Galectin-3 Oncoproteins Function in a MicroRNA-Dependent Regulatory Loop

Selvi Ramasamy, Sekhar Duraisamy, Sergei Barbashov, Takeshi Kawano, Surender Kharbanda, and Donald Kufe

Supplemental Experimental Procedures

Plasmid constructions. The pIRESpuro2-MUC1 and pIRESpuro2-MUC1-C vectors have been described (Huang et al., 2003; Li et al., 2001b). pRNA-U6.1/Neo-MUC1siRNA plasmids were constructed by ligation of MUC1siRNA#2 (AAGGTACCATCAATGTCCACG), MUC1siRNA#4 (AAGTTCAGTGCCAGCTCTAC) or random control (CsiRNA; CGCTTACCGATTCAGAATGG) sequences into pRNA-U6.1 (GenScript). pCR3.1-hFc-MUC1-C extracellular domain (MUC1-C/ED) was constructed by PCR amplification of MUC1-C/ED from pIRESpuro2-MUC1-C and cloned into the mammalian pCR3.1 vector. The human Fc fragment (hFc) was amplified from the CD5-IgG1 plasmid (Aruffo et al., 1990). The N36A mutation in pIRESpuro2-MUC1-C was introduced using the QuickChange kit (Stratagene). For vectors encoding galectin-3 and galectin-3 fragments, cDNAs were generated from ZR-75-1 mRNA using specific primers and cloned into the PET22b+ vector (Novagene). Plasmids expressing GST-galectin-3 or GST-galectin-3 fragments were generated by PCR from PET22b+-galectin-3 and cloned into the pGEX4T1 vector (Amersham Pharma Biotech). The galectin-3 promoter regions spanning −3000 to +141 and −836

to +141 bp from the transcription start site were amplified using the failsafe PCR kit (Epicentre) and cloned into the pGL3 basic vector NheI and HindIII sites. The galectin-3 3'-UTR was cloned into the pMIR reporter plasmid (Ambion).

Cell transfections. pIRESpuro2, pIRESpuro2-MUC1, pIRESpuro2-MUC1-C and pIRESpuro2-MUC1-C(N36A) were transfected into BT549 cells with Fugene6 (Roche) and stable clones were selected in the presence of 300 ng puromycin (Calbiochem-Novabiochem). The pCR3.1-hFc-MUC1-C/ED plasmid was transfected into CHO-K1 cells with Fugene6 and selected in the presence of 1 mg/ml G418 (Invitrogen). ZR-75-1 cells were stably transfected to express an empty vector, a control scrambled siRNA (CsiRNA) or a MUC1siRNA#2 as described (Ren et al., 2004). Transient transfections with a MUC1 siRNA pool, galectin-3 siRNA pool or a non-specific control duplex IX RNA (Dharmacon) were performed in the presence of Lipofectamine 2000 (Invitrogen). DU145 cells were transfected with pRNA-U6.1/Neo-CsiRNA, pRNA-U6.1/Neo-MUC1siRNA#2 or pRNA-U6.1/Neo-MUC1siRNA#4 and selected in 200 μg/ml neomycin. DU145/MUC1siRNA#4 cells were transfected with pIRES-puro2 or pIRES-puro2-MUC1-C and selected in the presence of 400 ng/ml puromycin.

Quantitative real-time PCR. Total RNA was extracted from cells with TRIzol (Invitrogen) and reverse transcribed with oligo(dT) priming and Superscript III reverse transcriptase (Invitrogen). Quantification of galectin-3 transcripts was

performed with primer pairs (CAACCAGTACTTGTATTTTGAATG and CAATGAGAACAACAGGAGAGTCA) using Power SYBR Green QPCR Mastermix and ABI Prism 7000 sequence detections system (Applied Biosystems). Relative gene expression was determined using the comparative Ct method with GAPDH as the internal control.

Nuclear run-on assays. Nuclei were isolated from $5x10^6$ cells and assayed as described (Patrone et al., 2000). In brief, run-on transcription was performed in the presence of biotin-16-UTP (Roche Applied Science) for 30 min at 30°C. RNA was purified using TRIzol reagent. The biotinylated RNA was isolated with Dynabeads M-280 (Invitrogen). Galectin-3 and, as an endogenous control, β-actin nascent transcripts were analyzed by quantitative RT-PCR. The difference in transcript level was calculated by the comparative C_T method.

mRNA stability assays. Cells were treated with 1.3 μg/ml actinomycin D and harvested at different intervals. Total RNA was isolated and analyzed for galectin-3 and GAPDH mRNA levels by quantitative RT-PCR. The half life of galectin-3 mRNA was calculated using linear regression analysis.

Protein purification. CHO-K1 cells expressing hFc-MUC1-C/ED were adapted to grow as a suspension culture in mAb production medium (BD Bioscience) using the Cell-line 1000 chamber flask. The secreted hFc-MUC1-C/ED protein was purified using a protein-A column (Pierce). The galectin-3 proteins were purified from *E.*

coli using an Asialofetulin column as described (Pelletier and Sato, 2002).

Identification of MUC1-C/ED cell surface binding proteins. Purified hFc-MUC1-C/ED and hFc (US Biologicals) were cross-linked to agarose beads. ZR-75-1 cells were grown in 0.1% FBS for 48 h and washed with salt solution (10 mM MES, pH 6.2, 750 mM NaCl, 2 mM EDTA). The salt extract was passed through a 0.45 um filter, diluted 3x in water, adjusted to 1 mM $CaCl_2$/3 mM $MgCl_2$ (pH 7.4) and passed through a tandem control Fc and hFc-MUC1-C/ED column. The column was washed with 10 mM Tris-HCl, pH 7.4, 250 mM NaCl, 1 mM $CaCl_2$ and 3 mM $MgCl_2$, and eluted with 2 M NaCl and 5 mM EDTA. The eluate was dialyzed against PBS, concentrated and analyzed by SDS-PAGE and silver staining.

Biacore analysis. Surface plasmon resonance measurements were determined using a BIACORE 3000 instrument at 25°C. Galectin-3 and galectin-3(63-250) were covalently coupled to CM5 Biacore sensor chips (500-1000 RU) at 30 μg/ml using the Biacore Amine Coupling kit. hFc-MUC1-C/ED and hFc diluted in running buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.005% NP-40) were injected across galectin-3, galectin-3(63-250) or control flow cells at a rate of 30 μl/min. Regeneration of the sensor surface was performed with a 30 sec pulse of 100 mM lactose. Association and dissociation kinetic constants were calculated by BIAevaluation software using 1:1 binding with a drifting base model.

In vitro deglycosylation. MUC1-C was immunoprecipitated from cell lysates, eluted from the beads with 50 mM glycine-HCl, pH 2.6, dialyzed against 50 mM sodium phosphate buffer, pH 7.0, and deglycosylated under denaturing conditions using the Glycokit (ProZyme).

Immunofluorescence microscopy. Cells were fixed in 3.7% formaldehyde, permeabilized in 0.2% Triton X-100 and post-fixed in 3.7% formaldehyde. The cells were blocked with 10% normal goat serum and stained with anti-MUC1-N (MAb DF3) and anti-EGFR (Santa Cruz Biotechnology), followed by fluorescein-conjugated anti-rabbit IgG or Texas Red-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories). The cells were then mounted onto coverslips using slow-fade mounting reagent (Molecular Probes). Images were captured with a Zeiss LSM510 confocal microscope under x63 magnification and 1024 x 1024 resolution.

Supplemental References

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B., and Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell *61*, 1303-1313.

Patrone, G., Puppo, F., Cusano, R., Scaranari, M., Ceccherini, I., Puliti, A., and Ravazzolo, R. (2000). Nuclear run-on assay using biotin labeling, magnetic bead capture and analysis by fluorescence-based RT-PCR. Biotechniques *29*, 1012-1014, 1016-1017.

Pelletier, I., and Sato, S. (2002). Specific recognition and cleavage of galectin-3 by Leishmania major through species-specific polygalactose epitope. J Biol Chem *277*, 17663-17670.

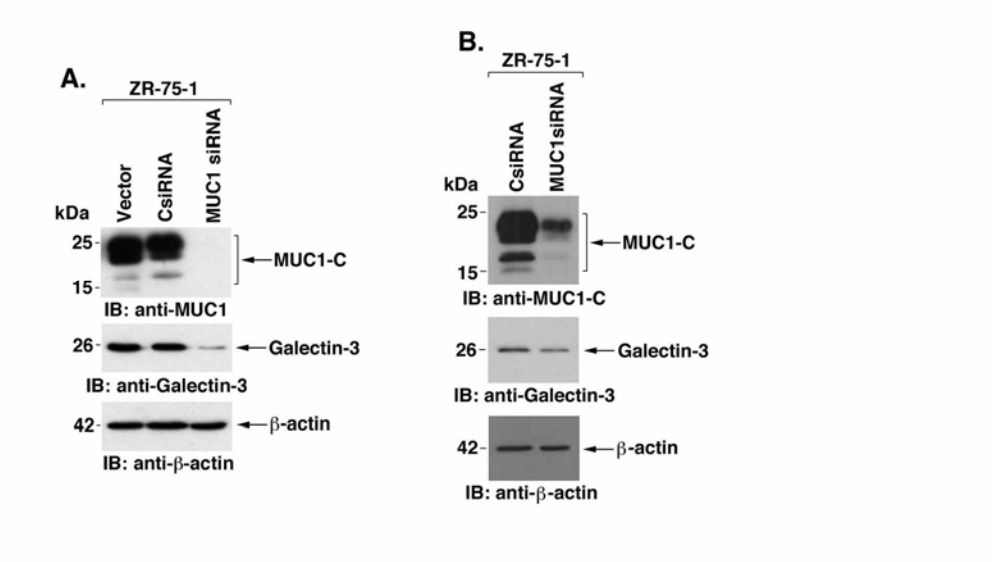

Figure S1. A. Lysates from ZR-75-1 cells stably expressing the empty vector (ZR-75-1/vector-B), a control scrambled siRNA (ZR-75-1/CsiRNA) or MUC1siRNA#2 (ZR-75-1/MUC1siRNA#2-B) were immunoblotted with the indicated antibodies. **B.** ZR-75-1 cells were transiently transfected with a control siRNA (CsiRNA) or a pool of MUC1 siRNAs. At 72 h after transfection, lysates were immunoblotted with the indicated antibodies.

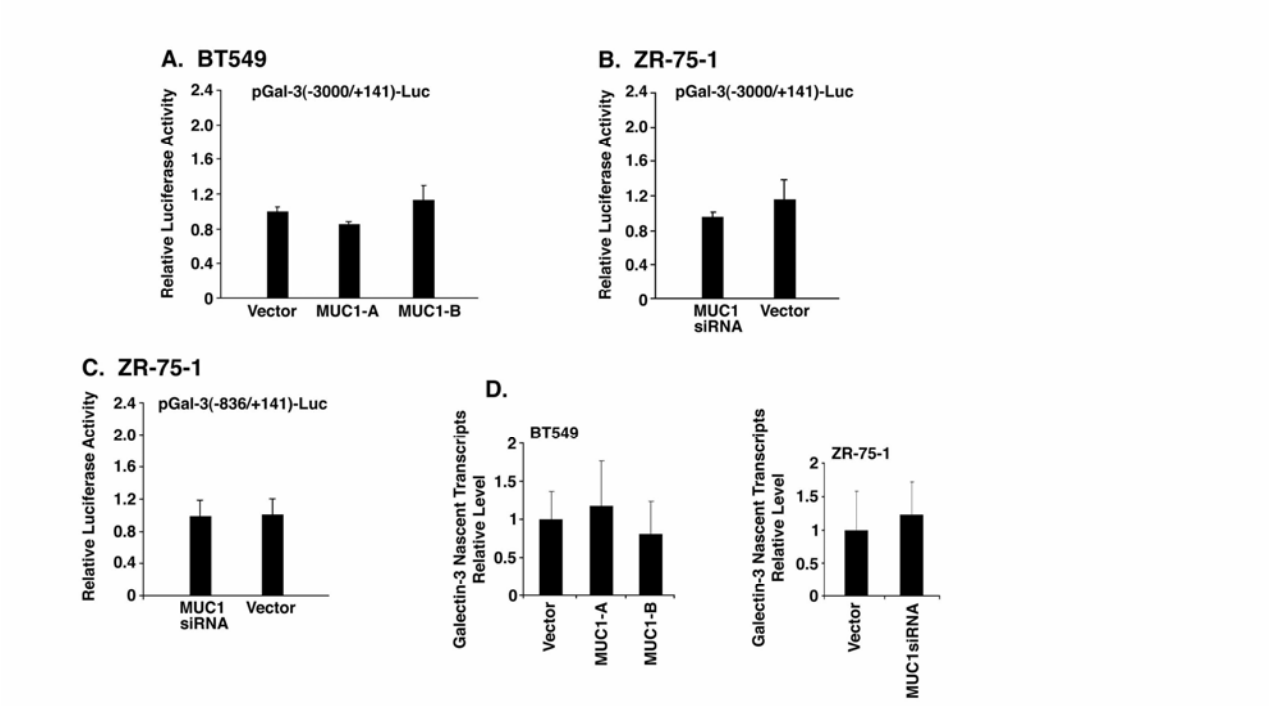

Figure S2. A. The indicated BT549 cells were transfected with pGal-3(-3000/+141)-Luc and pcDNA-LacZ plasmids. **B and C.** ZR-75-1/MUC1siRNA and ZR-75-1/vector cells were transfected with the indicated pGal-Luc constructs and pcDNA-LacZ. The cells were assayed for luciferase and β-gal activities at 24 h after transfection. The results (mean±SD from three experiments) are expressed as relative galectin-3 promoter activity compared to that obtained with the BT549/vector (A) or ZR-75-1/MUC1siRNA (B and C) cells (assigned a value of 1). **D.** *Galectin-3* gene transcription was assayed for the indicated BT549 (left) and ZR-75-1 (right) cells in run-on assays using the *β-actin* gene as an internal control. The results (mean±SD from three replicates) are expressed as the relative change in the abundance of newly transcribed galectin-3 mRNA.

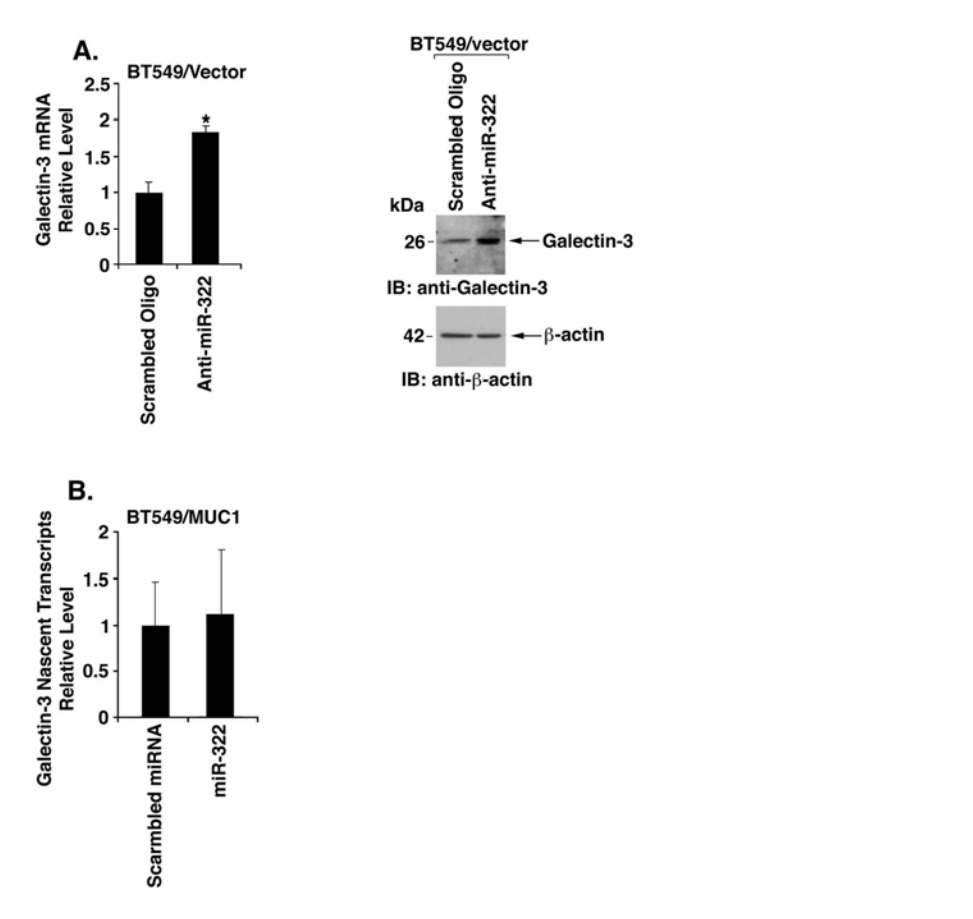

Figure S3. A. BT549/vector cells were transfected with an antisense 2′-O-methyl oligoribonucleotide targeted against miR-322 or a scrambled 2′-O-methyl oligoribonucleotide as a control for 72 h. The cells were analyzed for galectin-3 and GAPDH mRNA levels by quantitative RT-PCR (left) or galectin-3 and β-actin protein levels by immunoblotting (right). The RT-PCR results (mean±SD from three replicates) are expressed as the relative galectin-3 mRNA levels (normalized to GAPDH) compared to that obtained with the scrambled miRNA (assigned a value of 1). The asterisk (*) denotes a significant difference at $p<0.01$ as compared to normal. **B.** BT549/MUC1 cells were transfected with a pre-miR-322 or a scrambled miRNA and selected in the presence of

blasticidin. Nuclei were assayed for *galectin-3* and *β-actin* gene transcription in run-on assays. The results (mean±SD from three replicates) are expressed as the relative change in the abundance of newly transcribed galectin-3 mRNA.

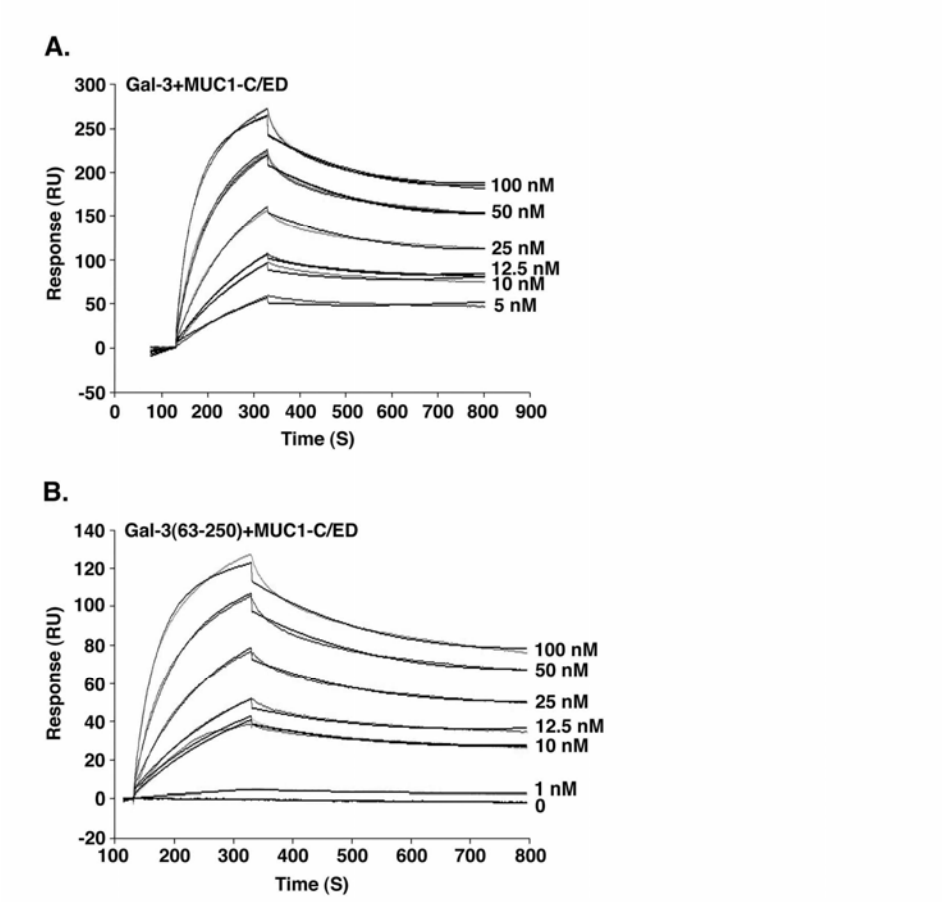

Figure S4. A and B. hFc-MUC1-C/ED was injected at concentrations ranging from 0 nM to 100 nM at a constant flow rate of 30 μl/min over a galectin-3 immobilized chip (A), over a galectin-3(63-250) immobilized chip (B) and over a control dextran surface. The association was monitored for 200 seconds and the dissociation observed for 500 seconds. The dark curve represents the fit of data to 1:1 binding with a drifting base model.

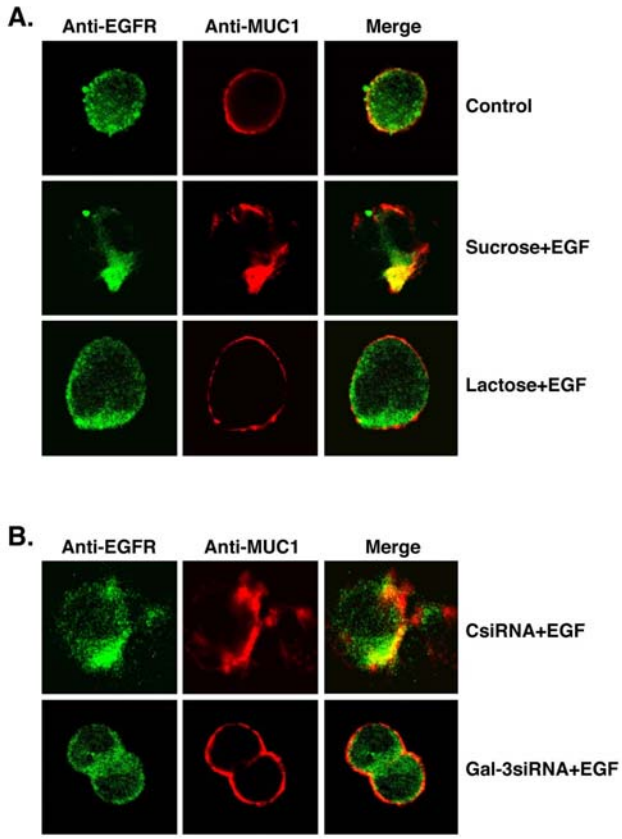

Figure S5. A. ZR-75-1 cells were incubated with sucrose or lactose in complete medium for 24 h and then with sucrose and lactose for an additional 24 h in 0.1% serum. The cells were then stimulated with EGF for 5 min. **B.** ZR-75-1 cells were transfected with a control siRNA or a galectin-3 siRNA pool for 48 h and then grown in the presence of 0.1% serum for 24 h. The cells were then stimulated with EGF for 5 min. Cells were analyzed by confocal microscopy after fixation and staining with anti-EGFR and anti-MUC1-N.