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Supplemental Information

FGF2 Induces Migration of Human Bone Marrow Stromal Cells by Increasing Core Fucosylations on N-Glycans of Integrins

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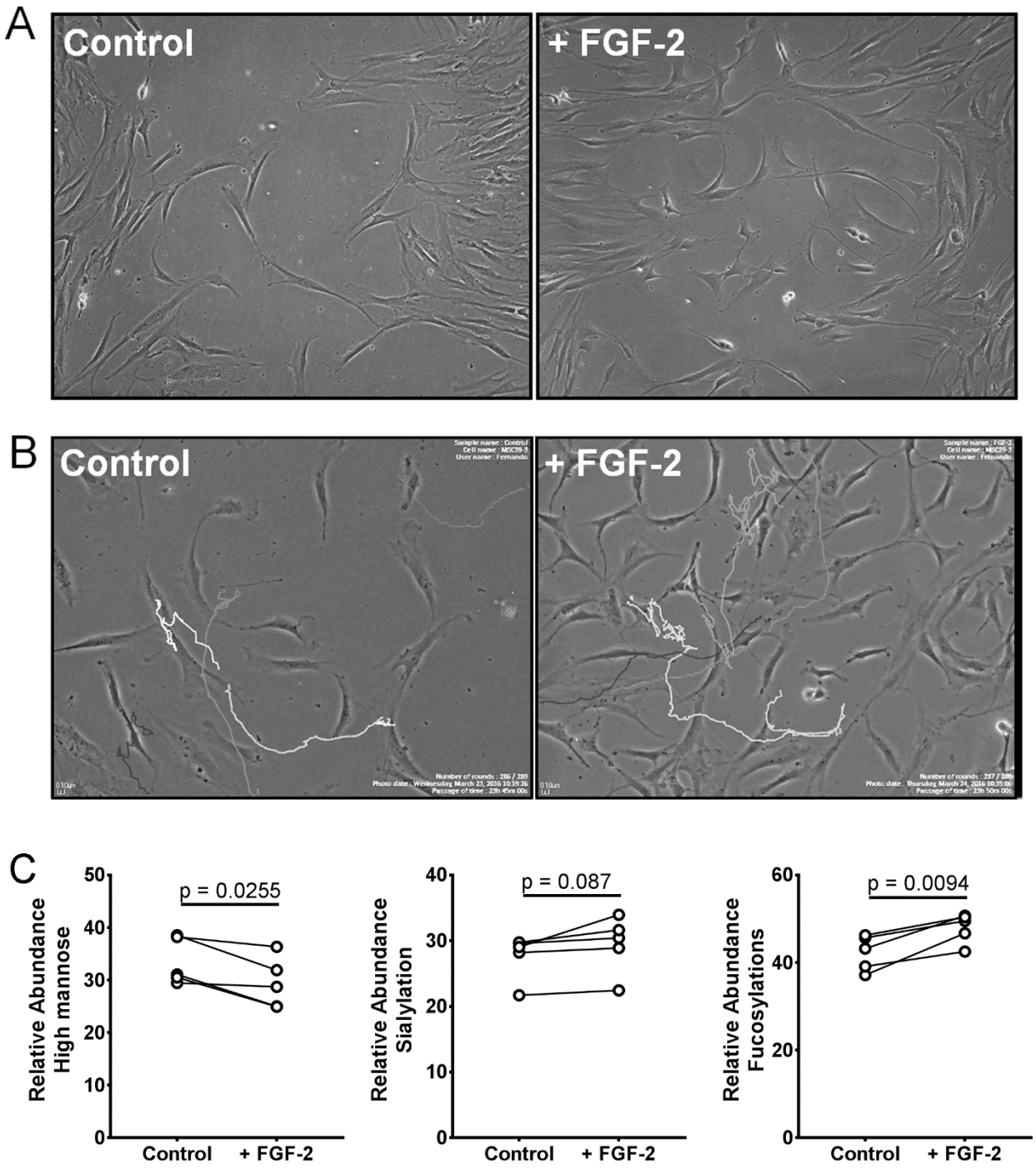


Figure S1. FGF-2 promotes migration and alters N-glycans in MSCs. (A) Wound/scratch assay. MSCs were plated into 24-well plates containing an insert that creates a uniform barrier. Representative images of wound closure. (B) Cell tracking using videomicroscopy. MSCs displacement over time (speed) was recorded and tracked using ImageJ software. Tracks of individual cells are shown as color lines on representative phase contrast images. (C) Changes in high mannose, sialylation and fucosylations in N-glycans of MSCs treated with or without FGF2. Each dot represents MSCs derived from a different donor.

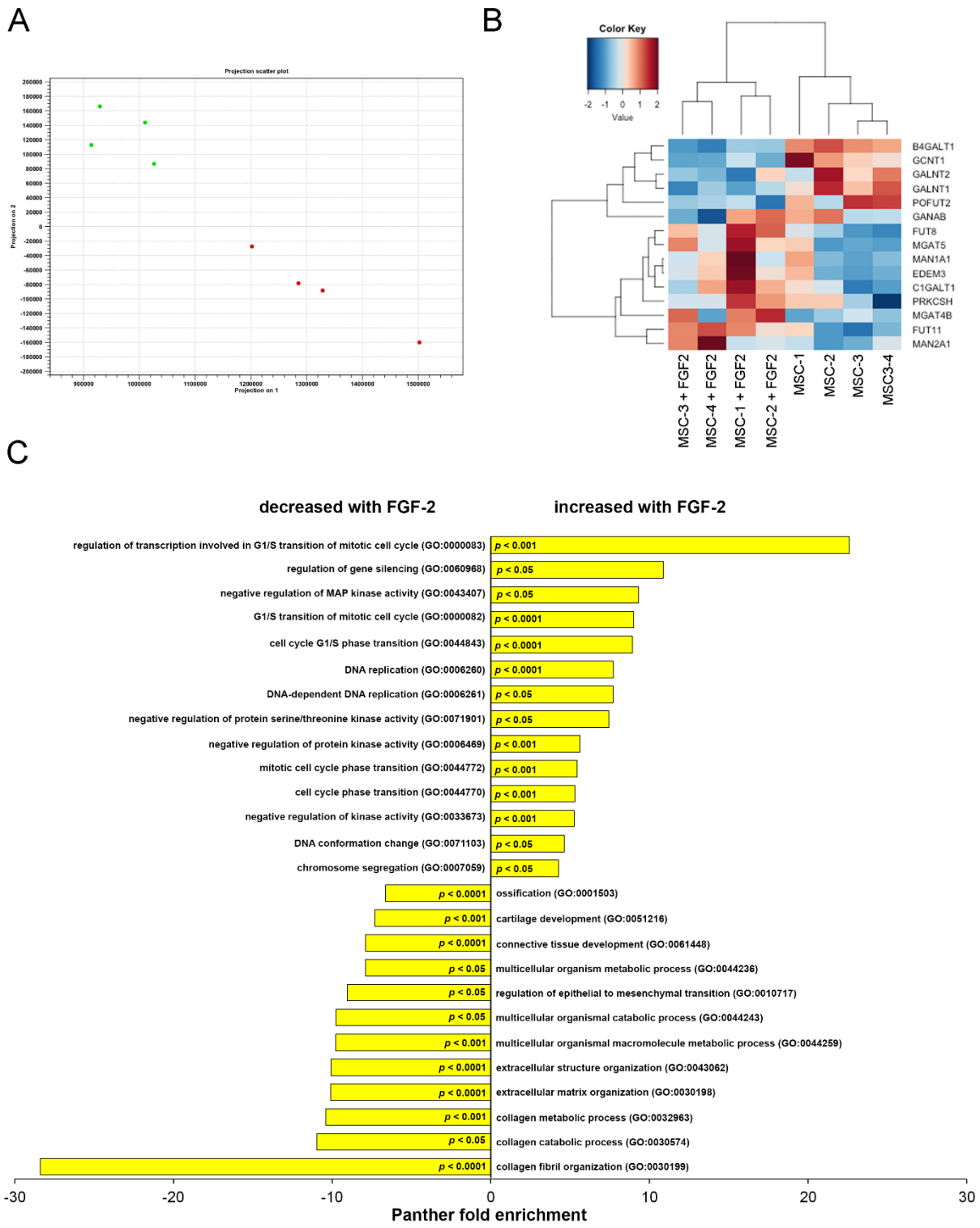


Figure S2. RNAseq of MSC treated with FGF2 (A) Cluster analysis of MSCs treated with (green) or without (red) FGF2. (B) Heat map analysis and hierarchical clustering of differentially expressed genes associated to glycan synthesis. Samples are shown in bottom, where numbers denote MSCs derived from different donors. (C) PANTHER analysis (Mi et al., 2013) of differentially expressed genes, in MSCs treated with FGF2 compared to untreated (control) cells.

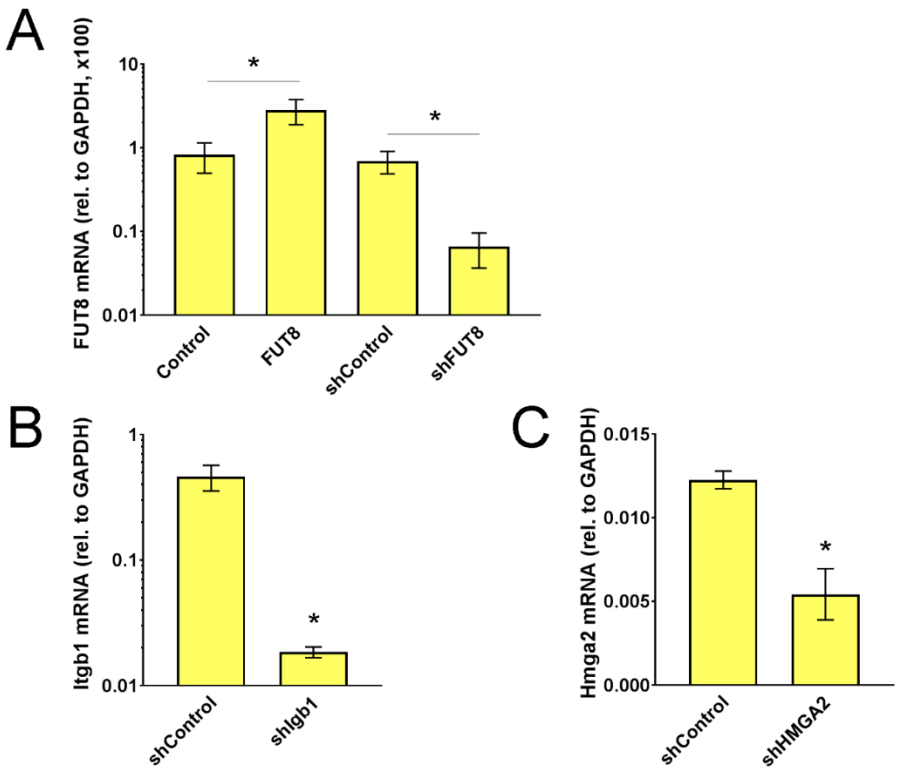


Figure S3. Silencing and over-expression efficiencies using lentivirus. MSCs were transduced with the respective lentiviral vectors. 3 days after, total RNA was extracted and mRNAs measured by real time PCR. (A) N = 6. (B) N = 4. (C) N = 3. * $p < 0.05$ analyzed using a paired Student's *t* test.

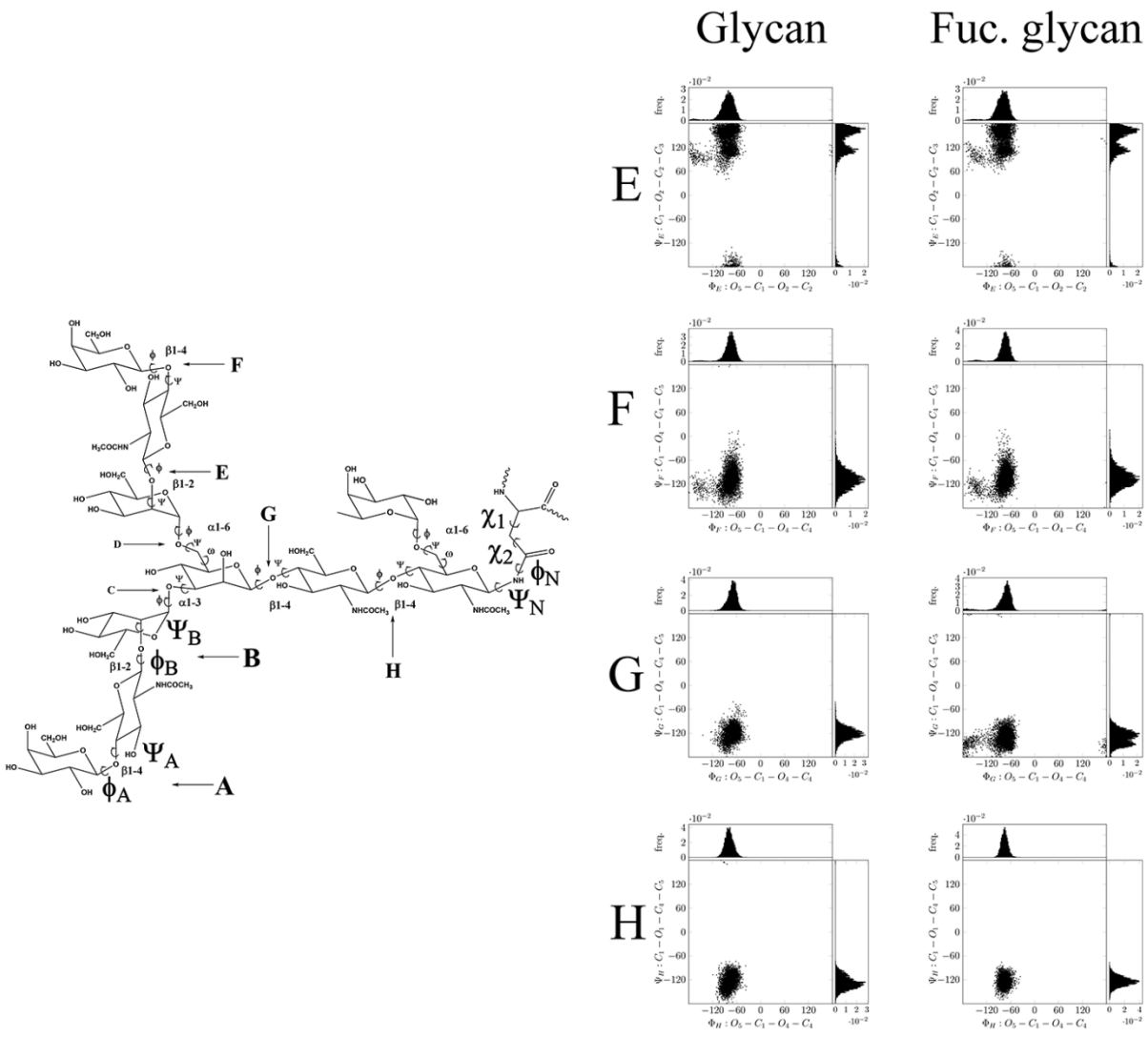
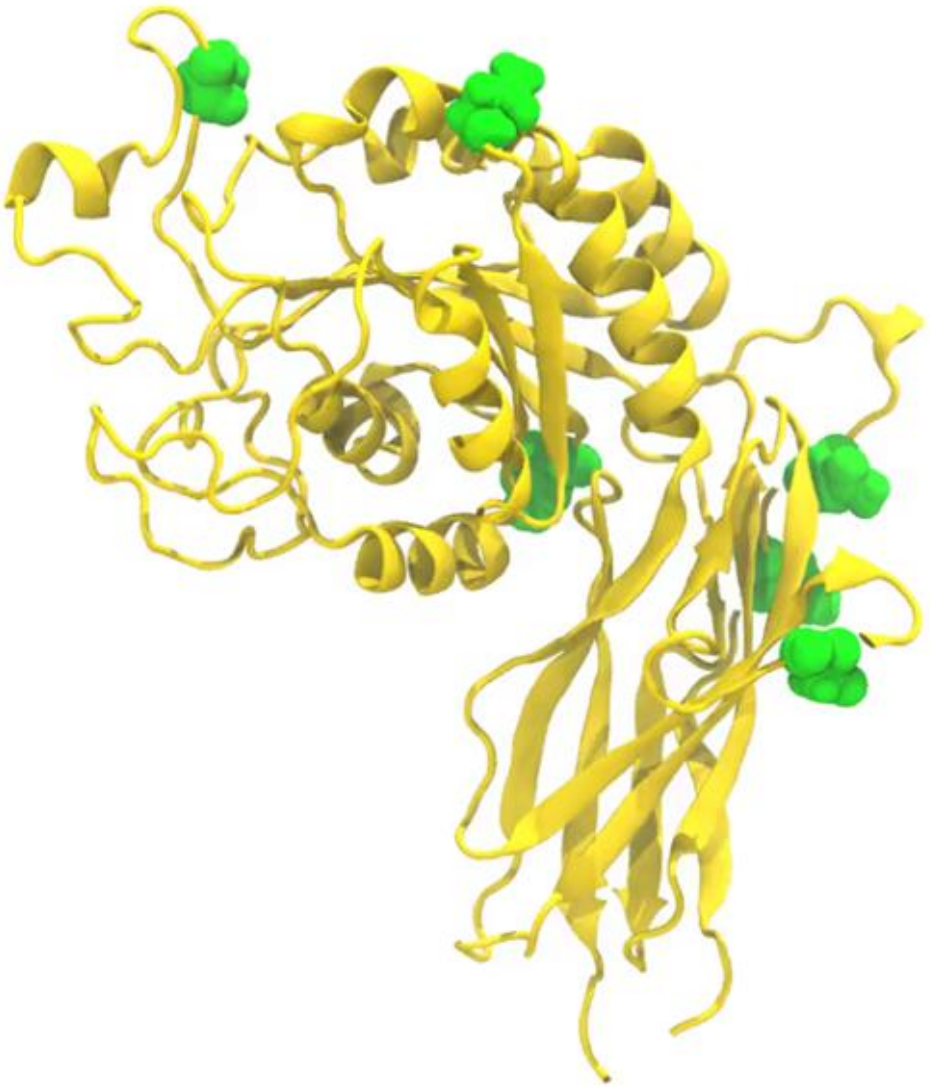
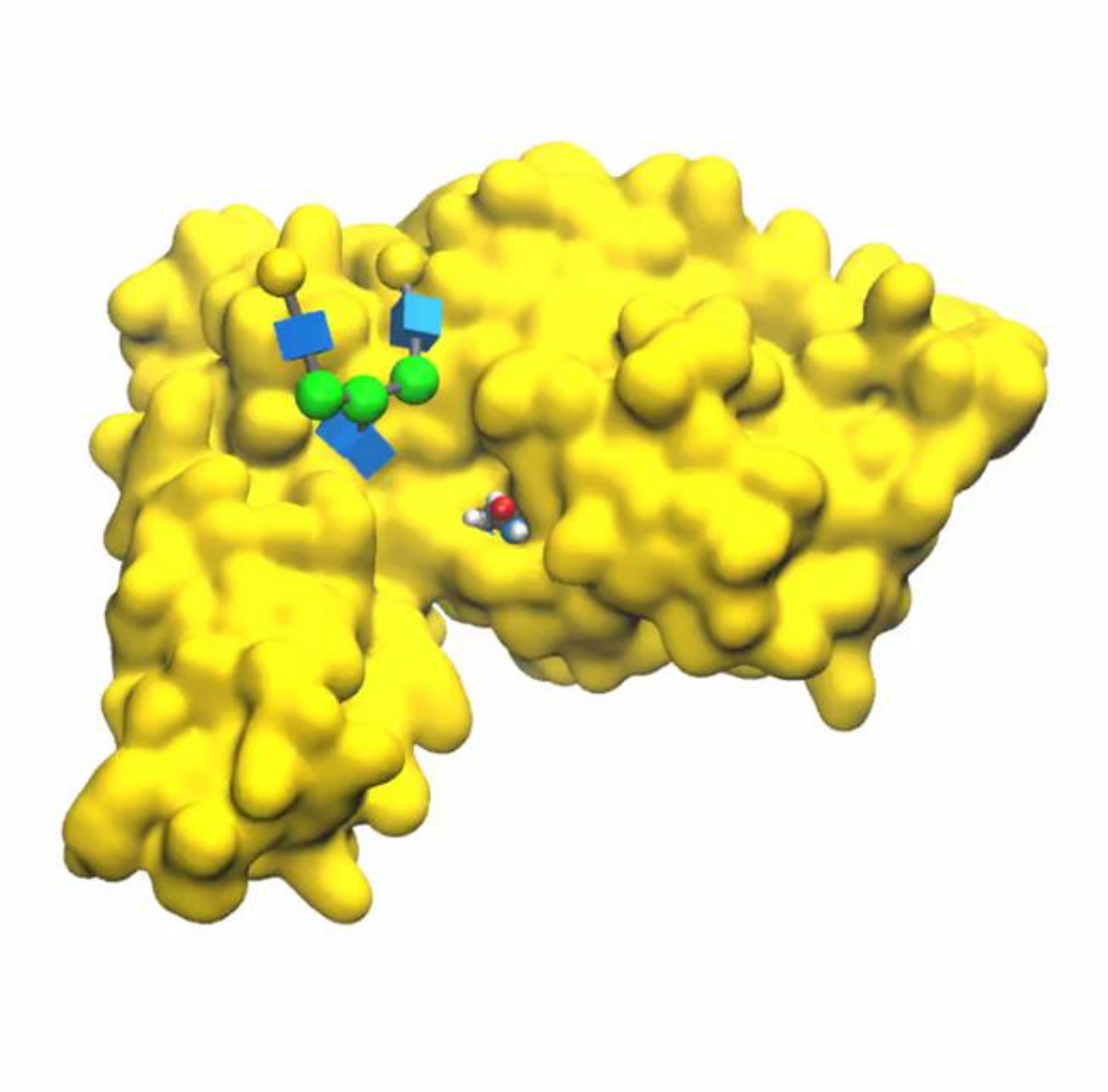


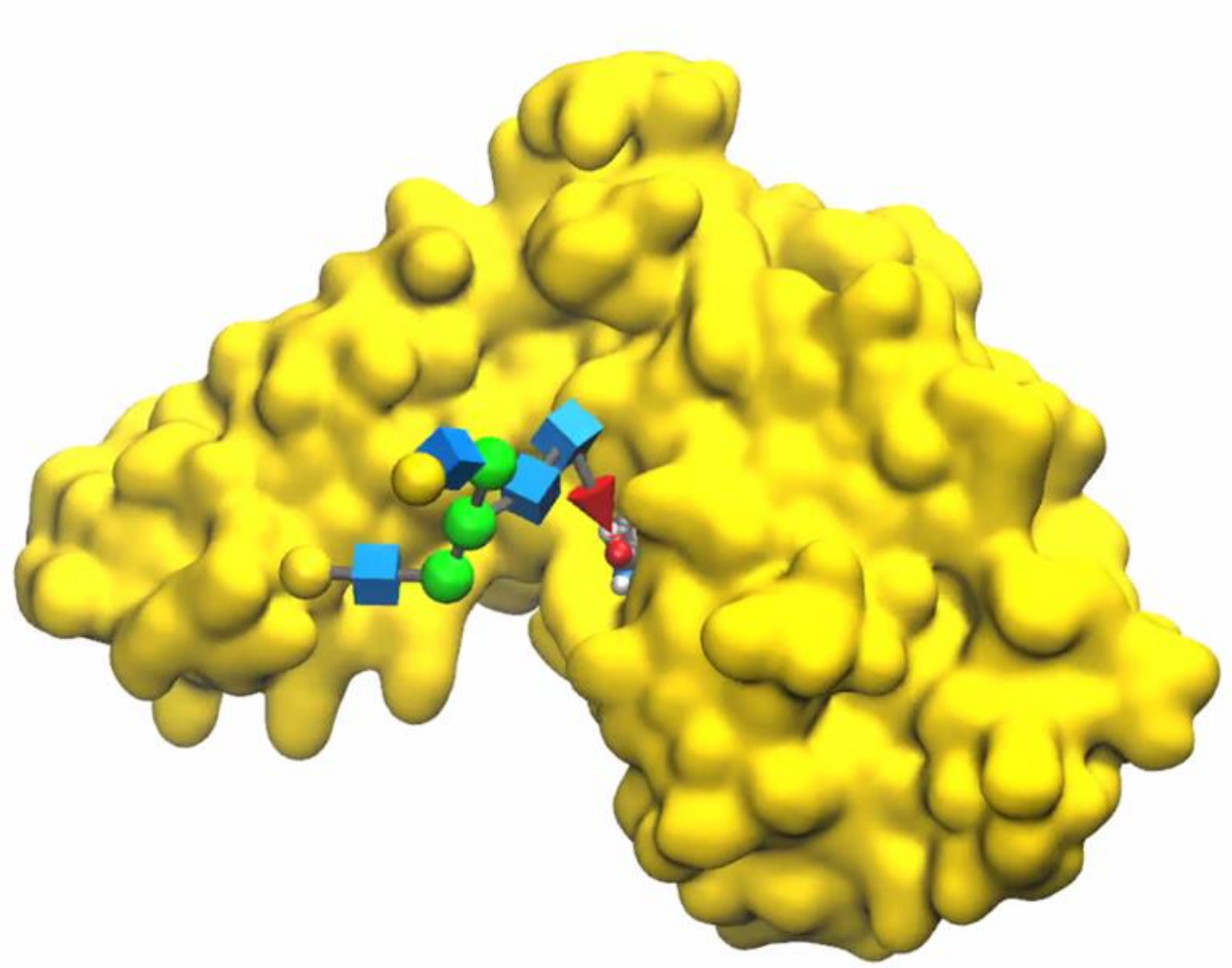
Figure S4. Torsion dihedral angles different to those shown in Figure 4.



Movie S1 (still image). 3D structure of Integrin β1, with glycosylated residues (green)



Movie S2 (still image). Simulation of Integrin β_1 with an N-glycan without core-fucosylation. A glycan (see cartoon in Figure 4) was attached to Asn329 *in silico*. This movie shows how that glycan moves freely, with no hydrogen bond with Asn265 (highlighted).



Movie S3 (still image). Simulation of Integrin β_1 with an N-glycan with core-fucosylation. A glycan with core-fucosylation (red cone) was attached to Asn329 *in silico*. This movie shows how that glycan has limited movement, at least partially due to interaction (hydrogen bond) with Asn265 (highlighted).

Supplementary Experimental Procedures

Cell Culture

Mesenchymal Stem Cells (MSCs) were isolated from bone marrow aspirates from healthy donors (Fierro et al., 2011). Fresh bone marrow aspirates (StemExpress) were mixed 1:1 with PBS, layered over Ficoll-Paque PLUS (GE Healthcare) and centrifuged for 30 min at 600 × *g*. Total mononuclear cells were then plated in tissue culture flasks using MEM-alpha (HyClone) supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% Penicillin-Streptomycin. After 2 days, non-adherent cells were washed off. Remaining cells were expanded, acquiring the characteristic morphology, immune phenotype and differentiation potential of MSCs (Horwitz et al., 2005). To isolate mouse MSCs, two months old, male 129vJ mice were humanely euthanized. Bone marrows from both femurs and tibiae, were flushed using cell culture media, and directly plated into 6-well plates. After 2 days adherent cells were washed off and for the next two passages, Trypsin treatment was limited to 4 minutes, to purify MSC from other adherent cells. All experiments were performed with MSCs in passage 2 - 6, where each passage represents 3-4 population doublings (5-7 days in culture). Each experiment repetition (N) was performed with MSCs derived from a different donor. All experiments with FGF2 use 24 hours incubation, 10ng/ml FGF2.

Wound/scratch assay

MSCs were seeded into 24-well plates with Cytoselect inserts (Cell Biolabs), at 25,000 cells per side (6 replicates per condition). The next day, inserts were removed and media was changed. Pictures were taken immediately and 24 hours after, unless otherwise described. Wound area was quantified using TScratch software (Geback et al., 2009). For experiments with engineered cells, transductions were performed 3 days prior to the assay. For experiments with FGF2, supplementation was started at the moment of seeding the cells (24 hours prior to removing the inserts). For experiments with extracellular matrix, coating was performed for 1 hour at 37C, followed by two washes with PBS, prior to seeding the cells. For collagen (type I, from rat tail, Sigma-Aldrich), Fibronectin (from human plasma, Gibco), Laminin (Sigma-Aldrich), the final concentrations were all 10μg/ml.

Videomicroscopy

MSCs were plated in 35mm petri-dishes (20,000 cells/dish) and placed in a BioStation microscope (Nikon) for videomicroscopy, while maintaining the cells at 37˚C and 5% CO_2. During recording, each dish was photographed in 6-9 fields of view over 24 hours (unless otherwise

stated). Movies were then analyzed using ImageJ software to determine individual cell displacement over time (speed). For experiments with extracellular matrix, coating was performed as described for the wound/scratch assays, but in 35mm petri-dishes.

RNA-seq

Total RNA from four different donors treated with or without FGF2 (One million cells per sample) was extracted using a Direct-zol RNA Mini-Prep kit (Zymo Research), following manufacturer's instructions. Total RNA was then submitted to Beijing Genomics Institute (BGI) at UC Davis. Here, RNA quality was determined using Agilent 2100. For all samples, RNA integrity number was above 8.0. cDNA library from total mRNA was built using an Illumina kit. Sequencing was performed in two SE50 lanes using the Hiseq2000 platform (Illumina). Average number of reads per sample was 20.7 x 10^6. Results were analyzed, normalized and semi-quantified using DESeq2 software (Love et al., 2014).

Real time PCR

RNA extraction was performed using a Direct-zol RNA Mini-Prep kit (Zymo Research), following manufacturer's instructions. Real time PCR were performed using Taqman gene expression assays (Applied Biosystems) and Taqman Universal Master Mix reagents (Applied Biosystems). The sequences are undisclosed by the manufacturer, but are identified by the following Assay ID: FUT8: Hs00189535_m1, FUCA1: Hs00609173_m1, HMGA1: Hs00852949_g1*, HMGA2: Hs04397751_m1, E2F1: Hs00153451_m1, FGFR2: Hs01552918_m1, DOCK4: Hs00206807_m1, PODXL: Hs01574644_m1, ITGA3: Hs01076879_m1, RHOB: Hs05051455_s1*, ITGA11: Hs01012939_m1, ITGAV: Hs00233808_m1, ITGB1: Hs01127536_m1, GAPDH: Hs02786624_g1. For all assays, the probe spans exons, except for those with asterisk (*), where both primers and probe map within a single exon.

NanoLC/ESI-QTOF-MS

One million MSCs treated with or without FGF2; derived from five different donors, where lifted with Trypsin, washed with PBS and resuspended in homogenization buffer (0.25 M sucrose, 20 mM HEPES-KOH (pH 7.4), and 1:100 protease inhibitor mixture (EMD Milipore)). Cells were lysed on ice using a probe sonicator (Qsonica) and lysates were pelleted by centrifugation at 2000 × *g* for 10 min to remove the nuclear fraction and unlysed cells followed by a series of ultracentrifugation steps at 200,000 × *g* for 45 min to remove other nonmembrane subcellular

fractions (Park et al., 2015). Membrane pellets were then suspended with 100 μl of 100 mM NH_4HCO_3 in 5 mM dithiothreitol and heated for 10 s at 100 °C to thermally denature the proteins. To release the glycans, 2 μl of peptide N-glycosidase F (New England Biolabs) was added to the samples, which were then incubated at 37 °C in a microwave reactor (CEM Corporation) for 10 min at 20 watts. After addition of 400 μl of ice-cold ethanol, samples were frozen for 1 h at -80 °C to precipitate deglycosylated proteins and centrifuged for 20 min at 21,130 × *g*. The supernatant containing N-glycans was collected and dried.

Released N-glycans were purified by solid-phased extraction using porous graphitized carbon packed cartridges (Grace, Deerfield, IL). Cartridges were first equilibrated with alternating washes of nanopure water and a solution of 80% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid in water. Samples were loaded onto the cartridge and washed with nanopure water at a flow rate of 1 ml/min to remove salts and buffer. N-Glycans were eluted with a solution of 40% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid in water and dried. Analysis was performed as previously described (Park et al., 2015).

Lentiviral constructs and transduction

To silence FUT8, an shRNA was designed based on a previously published target site (Sasaki et al., 2013). This was then cloned into the lentiviral construct pCCLc-U6-shRNA-PGK-dTomato, where shRNA is either a non-targeting sequence (Control) or the shRNA targeting FUT8. To silence HMGA2, the previously published lentivirus shHMGA2-2 (Kalomoiris et al., 2016) was used. Overexpression of FUT8 was performed by cloning the protein coding sequence of FUT8 (1,728bp) into the lentiviral construct pCCLc-MNDU3-X-PGK-EGFP-WPRE, where X is either no sequence (control) or FUT8. All transductions were performed using protamine sulfate (20μg/ml) and with sufficient lentivirus to generate 90–95% GFP/dTomato positive MSCs, 3 days after transduction.

Western Blot

Transduced MSCs, treated with or without FGF2 were lysed for protein extraction using RIPA Buffer (Thermo Scientific) with 1% Halt Proteinase and Phosphatase Inhibitor Cocktail (Thermo Scientific). Proteins were extracted by strong agitation for 20 min at 4˚C, then centrifuged at 12,000g for 10 min and stored at -80°C. For western blots, 30μg of proteins were loaded into 10% polyacrylamide gels and transferred into polyvinylidene fluoride membranes (BioRad). Blots were then incubated with anti-FUT8 polyclonal antibody (250ng/ml, RnD Systems) overnight. After

protein detection, both membranes were stripped and incubated with anti-actin (200ng/ml, clone AC15, Sigma-Aldrich).

LC-MS/MS analysis of glycopeptides

Membrane proteins were dissolved in 8 M urea, denatured at 55°C with 18 mM dithiothreitol, and alkylated with 27 mM iodoacetamide in dark at room temperature. The mixture was then diluted to 1 M urea with 50 mM ammonium bicarbonate, and incubated with 1 µg trypsin at 37°C overnight. Solid-phased extraction using iSPE-HILIC cartridges (Nest Group, MA) was performed to enrich the glycopeptides. Cartridges were conditioned with alternating washes of 0.1% (v/v) trifluoroacetic acid in water, and 80% (v/v) acetonitrile with 1% (v/v) trifluoroacetic acid in water. Peptides were loaded onto the column, and washed with 1% (v/v) trifluoroacetic acid and 80% (v/v) acetonitrile in water. Enriched glycopeptides were eluted with a solution of 0.1% (v/v) trifluoroacetic acid in water and dried. Analysis of the enriched glycopeptides was performed on a Q Exactive Plus mass spectrometer (Thermo Scientific, CA). Glycopeptide identification was performed using Byonic software (Protein Metrics Inc.).

Migration assay of MSCs in zebrafish

Wild type zebrafish embryos of the NHGRI-1 (LaFave et al., 2014) strain were collected shortly after fertilization and allowed to develop until the mid blastula stage (3-4 hpf). Embryos were dechorionated manually and mounted on an agarose mold for transplantations. MSCs were loaded into a borosilicate pulled Pasteur pipette and were transferred into zebrafish embryos by mild pressure using a mouth piece and latex tubing or a custom-made Hamilton syringe; 20 to 50 MSCs were introduced into each embryo. Embryos were incubated at 28.5˚C for one day before imaging and scoring the position of the transplanted cells under fluorescent illumination. Images were captured on a Leica M165 FC dissecting scope equipped with a Leica DFC7000 T camera.

Migration assay toward bone fracture in mice

Closed transverse diaphysis fractures of the right femur were generated in ten 2-month-old mice using as previously described [Zhang et al 2017]. Briefly, fractures were created at the mid-femur using a drop-weight blunt guillotine device. Immediately after, mouse MSC (transduced with either shControl or shFUT8, and pre-incubated for 24 hours with 10ng/ml FGF2) were injected intramuscularly (5 mice per group; 10^5 cells per mouse), near the site of fracture and on the opposite (left, unfractured) thigh. After 7 days, mice were euthanized and samples embedded

in optimum cutting temperature (OCT) for cryosections. Injected cells were directly visualized based on tdTomato expression and DAPI staining for total nuclei.

Modelling System Composition, Minimization, Equilibration, and Production

The crystal structure of β_1 integrin was taken from the α5β1 integrin headpiece in complex with an RGD peptide (PDB ID: 3VI4) (Nagae et al., 2012). Chain D, one of the β_1 integrins, was selected and truncated to Asn_{78} and Cys_{460}. To prevent addition of charges, the ends were capped with either an acetyl or amide group at the N-terminus and C-terminus, respectively. First, the non-glycosylated structure was used as control, while two other structures with a glycan and a core fucosylated glycan in Asn_{265}, were also built. The glycans structures (DGalb1-4DGlcpNAcb1-2DManpa1-3[DGalb1-4DGlcpNAcb1-2DManpa1-6]DManpb1-4DGlcpNAcb1-4DGlcpNAcb1-OH, and DGalb1-4DGlcpNAcb1-2DManpa1-3[DGalb1-4DGlcpNAcb1-2DManpa1-6]DManpb1-4DGlcpNAcb1-4[LFucpa1-6]DGlcpNAcb1-OH) were built using Glycam (Kirschner et al., 2008a). The primary hydroxyl groups of DGlcpNAcb1-OH were deleted for each glycan and then docked near the glycosylation site with UCSF Chimera (Pettersen et al., 2004). Each system was composed using AmberTools16 packages (Case DA, 2017), and simulated with Amber16's GPU accelerated PMEMD (Salomon-Ferrer et al., 2013).

Each structure was loaded into tLeap using the protein ff14SB (Maier et al., 2015) for the protein and GLYCAM 06j-1 for carbohydrates (Kirschner et al., 2008b). Using tLeap, disulfide bonds for β1 integrin, the covalent bond between the Asn_{265} amide group and the glycans, and monosaccharide bonds in the glycans were made. Each structure was then solvated with a rectangular box with at least 10 Å of TIP3P water layer around. Two sodium ions were then added to neutralize each system. The ending size of each system was 57,469 atoms (control), 70,528 atoms for glycosylated β_1), and 63,459 atoms for β_1 with core fucosylated glycan.

Structures were then minimized using a two-step method. In the first minimization of 100,000 cycles, a 1 kcal/mol−Å restrain was placed on the glycans, and proteins, excluding their hydrogens. During the second step, the restrain was removed and the systems were further minimized for another 200,000 steps. The systems were then equilibrated at constant volume and temperature, and gradually warmed to 300˚K over 500 ps with a 2-fs time step and with the Langevin Thermostat—this time step and thermostat with a collision frequency of 2 was used hereafter. Once at 300˚K, the systems were maintained for an additional 500ps. After equilibration, four independent simulations of 70 ns with constant temperature (300˚K), and constant pressure (1 atm)—maintained by the Berendsen Thermostat—were collected.

Modelling Analysis

All trajectory analysis was conducted with CPPTRAJ (Roe and Cheatham, 2013). The root means square deviation (RMSD) between the alpha carbons in β_1 was calculated relative to the minimized structure. During simulation, trajectories converged after 10 ns for β_1 with glycan and 20-30 ns for β_1 with core fucosylated glycan. Although, RSMD were different, both equilibrated for each structure. Trajectory analysis for the core fucosylated glycan with integrin was conducted for both times yielding the same results. To compare glycans with or without core fucose, torsion angles were collected from each simulation with a temporal resolution of 50 ps. The torsion angles were defined as Φ: O_5-C_1-O_1-C'_X, Ψ psi: C_1-O_1-C'_X-C'_{X+1}, ω: O_1-C'_6-C'_5-O'_5, χ_1: N–C_α–C_β–C_γ, χ_2: C_α–C_β–C_γ–N; Ψ_N: C_β–C_γ–N–C_1; Φ_N: C_γ–N–C_1–O_5. The torsion angles are plotted in double distribution plots to better understand the flexibility of each glyosidic linkage.

The trajectory for each simulation was visualized with Visual Molecular Dynamics (VMD) (Humphrey et al., 1996). To better represent each structure, Tachyon ambient occlusion lighting and 3D-symbol nomenclature (Varki et al., 2015) for glycans was used. The trajectory for β_1 was smoothed with a windows size of 4, while the glycan trajectory was not modified. This allowed the representation of the full motion of the glycan while reducing fluctuations in β_1.

Statistical analysis

Results were presented as mean ± SEM. Depending on the number of compared conditions, 1-way ANOVA, 2-way ANOVA or two tailed Student's t-test were conducted using Graph-Pad Prism Software. P values <0.05 were considered statistically significant.

Acknowledgements

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Supplementary References

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