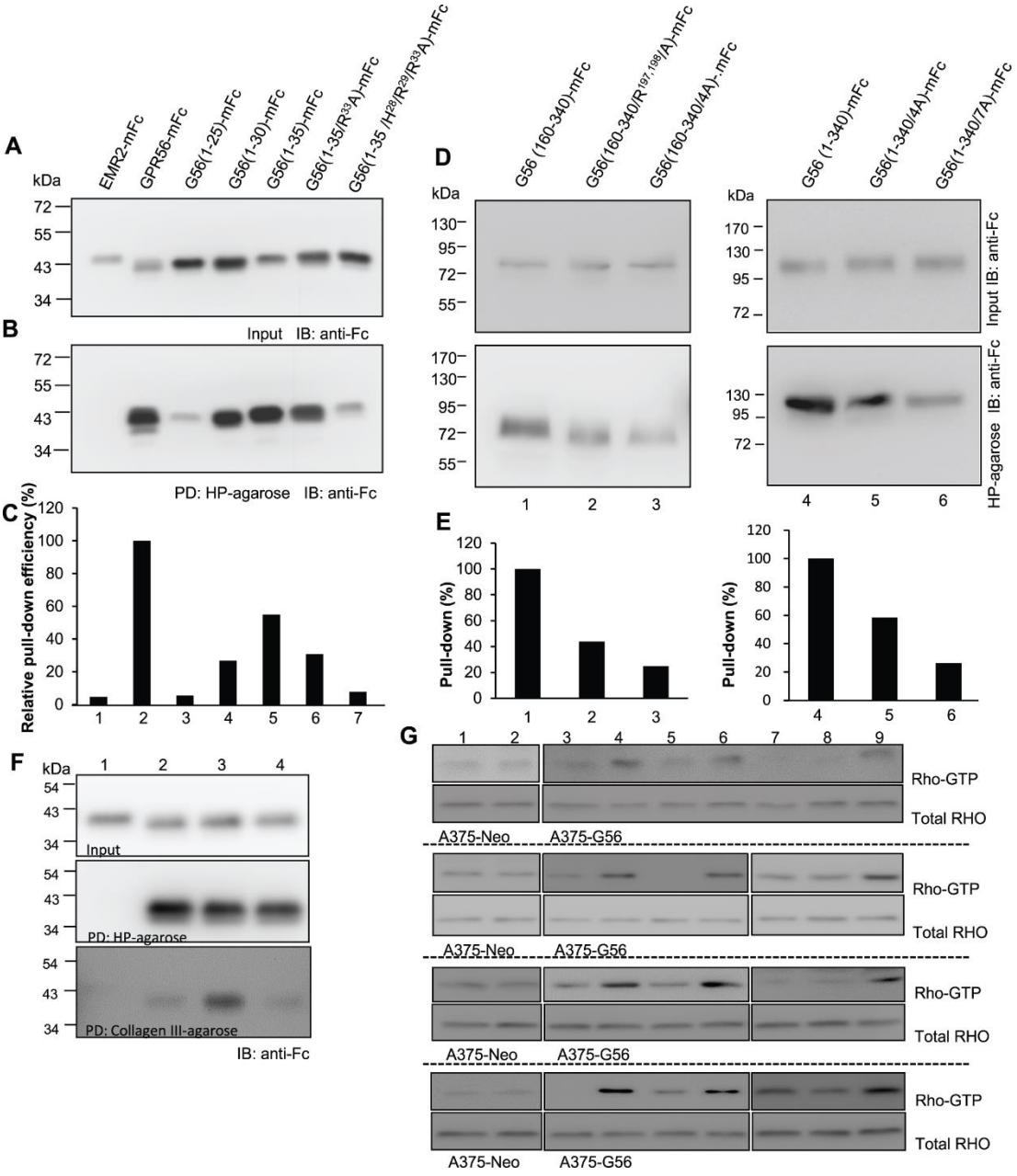
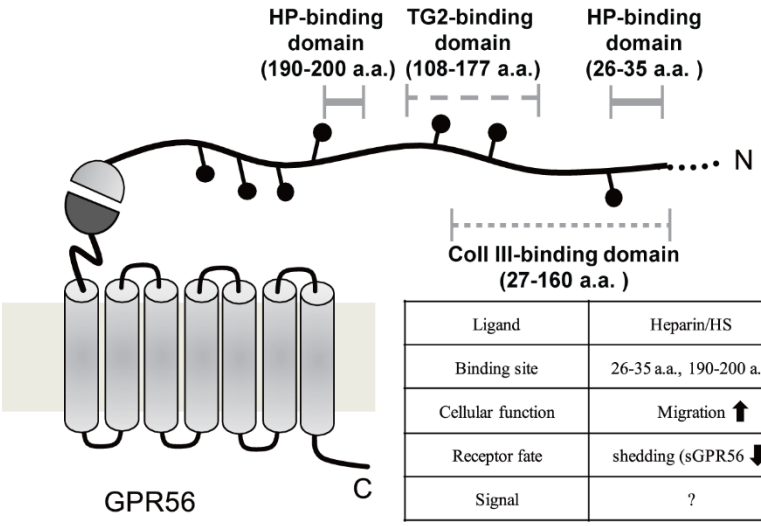


Fig. S3. Mapping of the potential heparin-binding sites at the N-terminus of GPR56 and the effect of heparin to GPR56-protein ligand interaction. (A, B) Western blotting analysis of the in vitro heparin-agarose pull-down assay. The input control (A) and heparin-agarose pull-down (B) were subjected to SDS-PAGE and detected with the anti-Fc Ab. (C) The graph shows the relative pull-down efficiency

by comparing the band intensity of the input and the pull down blots with the result of GPR56-mFc (lane 2) set as 100% (lower panel). The samples analyzed include: EMR2-mFc (lane 1), GPR56-mFc (lane 2), GPR56(1-25)-mFc (lane 3), GPR56(1-30)-mFc (lane 4), GPR56(1-35)-mFc (lane 5), GPR56(1-35/R^{33}A)-mFc (lane 6), and GPR56(1-35/H^{28}/R^{29}/R^{33}A)-mFc (lane 7). (D) Western blotting analysis of the in vitro heparin-agarose pull-down assay of recombinant GPR56-mFc fusion proteins as indicated. The input control (upper panel) and heparin-agarose pull-down (lower panel) were subjected to SDS-PAGE and detected with the anti-Fc Ab. (E) The graph shows the relative pull-down efficiency by comparing the band intensity of the input and the pull-down blots with the result of GPR56(160-340)-mFc and GPR56(1-340)-mFc set as 100% for the left and right panels, respectively. (F) Western blotting analysis of the pull-down assays using heparin-agarose (the second panel) and collagen III-agarose (the third panel). Samples included EMR2-mFc (lane 1), GPR56-mFc (lane 2), GPR56-mFc pre-incubated with collagen III (84 nM) (lane 3, second panel) or GPR56-mFc pre-incubated with heparin (25 μg/ml) (lane 3, third panel) for 1 hr. In lane 4, respective agarose beads were pre-incubated with collagen III (84 nM) (second panel) and heparin (25 μg/ml) (third panel) for 1 hr, followed by GPR56-mFc. HP: heparin; PD: pull down. Data shown are one representative of three independent experiments with similar results. (G) Heparin binding to GPR56 interfered with the RhoA activation induced by GPR56-collagen III interaction. Samples include cell lysate of stable A375-Neo cells stimulated with serum-free medium (lane 1), collagen III (84 nM, lane 2), and cell lysate of stable A375-GPR56 cells stimulated with serum-free medium (lane 3), complete medium with 10% serum (lane 4), heparin (25 μg/ml, lane 5), collagen III (84 nM, lane 6), collagen III and heparin together (lane 7), pre-incubated with heparin (25 μg/ml) for 10 min followed by collagen III (lane 8), pre-incubated with collagen III (84 nM) for 10 min followed by heparin (lane 9). Samples were subjected to the GTP-Rho pull-down assay, followed by WB analysis using the anti-RhoA mAb. The total Rho protein in cell lysate was used as a control. Results of 4 independent experiments are shown.



Ligand	Heparin/HS	Collagen III	TG2
Binding site	26-35 a.a., 190-200 a.a.	27-160 a.a.	108-177 a.a.
Cellular function	Migration ↑	Migration ↓	Tumor metastasis ↓
Receptor fate	shedding (sGPR56 ↓)	shedding (sGPR56 ↑)	Internalization ↑
Signal	?	$G_{\alpha 12/13}$, Rho A	PKC_{α}

Fig. S4. Summary of the interaction of three identified binding partners with GPR56. The ligand-binding region(s) of the three GPR56 binding partners (collagen III, TG2, and heparin) are shown. The effects of ligand binding on the cellular function, receptor fate, and signaling pathways are compared. ↑: increase; ↓: decrease.

Table S1. List of primer and restriction enzymes (RE) used in the study

Primer	Sequence(5'-3')	RE
GPR56 F	AATAAGCTTACAGGTGGTGACTTCCAAGAGT	Hind III
GPR56 R	CTAAGCGGCCGCGATGCGGCTGGACGAGGTGCT	Not I
G56-340	TTAAGCGGCCGCCTTCGGCTGTAGCTGGTGCTG	Not I
GPR56-180	AATTGCGGCCGCTTTGAGCTCGCACATGTCCAC	Not I
G56-160 F	TTCACCTTCTCCGCGGCCGCTCCTCCCCACACG	Not I
G56-160 R	CGTGTGGGGAGGAGCGGCCGCGGAGAAGGTGAA	Not I
G56-120 F	TCTAGCCTCCTCGCGGCCGCGCACCAGGAGGAG	Not I
G56-120	CTCCTCCTGGTGCGCGGCCGCGAGGAGGCTAGA	Not I
G56-90 F	CTCTACCACTTCGCGGCCGCCTGGAACCGACAT	Not I
G56-90 R	ATGTCGGTTCCAGGCGGCCGCGAAGTGGTAGAG	Not I
G56-60 F	TCCATCGAGAACGCGGCCGCGGCCCTCACAGTC	Not I
G56-60 R	GACTGTGAGGGCCGCGGCCGCGTTCTCGATGGA	Not I
G56-40	ATGCGGCCGCCTGGTTCCGCTGGCTGCAGAAGC	Not I
G56-35	TGCGGCCGCGCAGAAGCGAAAGCTTTCCCTGTG	Not I
G56-30	AATTGCGGCCGCTTCCCTGTGGCCCCTGCCGTG	Not I
G56-25	AGCGGCCGCGCCGTGGGCACCTTGGACCAGGAA	Not I
G56-35(33A)	TGCGGCCGCGCAGAATGCAAAGTCTTC	
G56-m1 F	ACGGCAGGGGCGCAGCAGAAGACTTTCGCTTC	
G56-m1 R	AAGCGAAAGTCTTCTGCTGCGCCCCTGCCGTG	
G56-3A F	GCAGCAGAAGACTTTGCATTCTGCAGCCAGCGG	
G56-3A R	CCGCTGGCTGCAGAATGCAAAGTCTTCTGCTGC	
G56-4A F	TTCCTGGCAGCTCCCCAGGCAGCCTCAGCAGC	
G56-4A R	TGCTGCTGAGGCTGCCTGGGGAGCTGCCAGG	
G56-2A F	CAGAAGGCCTCAGCAGCACCCTCGGCTGCC	
G56-2A R	GGCAGCCGAGGGTGCTGCTGAGGCCTTCTG	
G56-4A.1 F	CTCAGCCAGTTCCTGGCAGCTCCCCAGAAG	
G56-4A.1 R	CTTCTGGGGGAGCTGCCAGGAACTGGCTGAG	