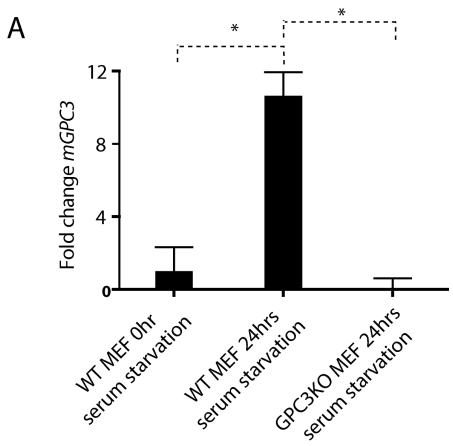
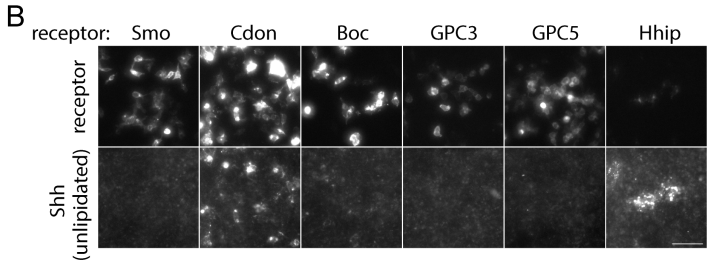
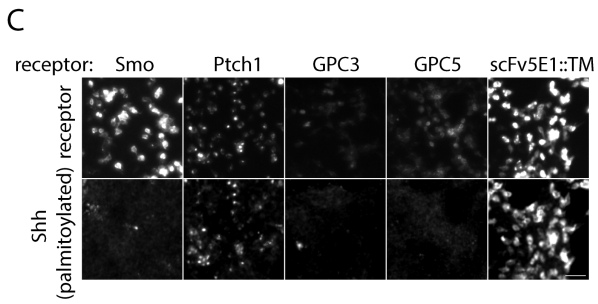
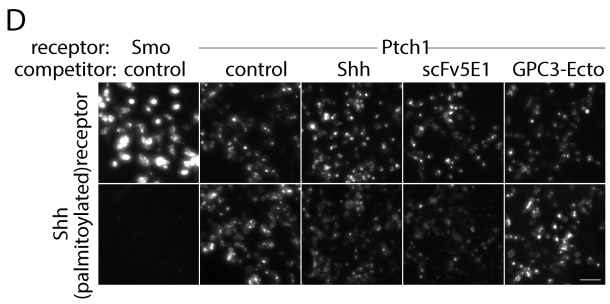
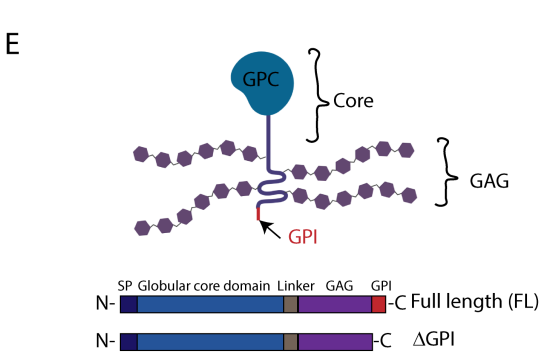
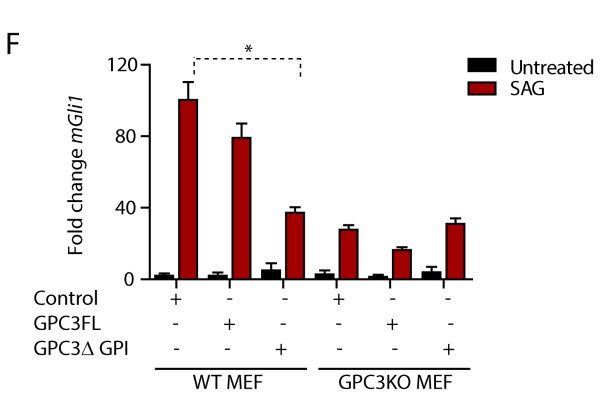

A
*
*
12
8
4
0
Fold change mGPC3
WT MEF 0hr serum starvation
WT MEF 24hrs serum starvation
GPC3KO MEF 24hrs serum starvation


B
receptor:
Smo
Cdon
Boc
GPC3
GPC5
Hhip
receptor
Shh (unlipidated)


C
receptor:
Smo
Ptch1
GPC3
GPC5
scFv5E1::TM
receptor
Shh (palmitoylated)


D
receptor:
Smo
Ptch1
competitor:
control
control
Shh
scFv5E1
GPC3-Ecto
Shh (palmitoylated)
receptor


E
GPC
Core
GAG
GPI
SP
Globular core domain
Linker
GAG
GPI
N-
-C Full length (FL)
N-
-C
ΔGPI


F
*
120
80
40
0
Fold change mGli1
Untreated
SAG
Control
GPC3FL
GPC3Δ GPI
WT MEF
GPC3KO MEF

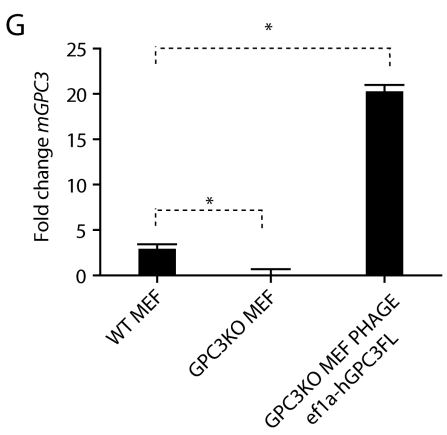

G
*
*
25
20
15
10
5
0
Fold change mGPC3
WT MEF
GPC3KO MEF
GPC3KO MEF PHAGE ef1a-hGPC3FL

Fig. S1. Related to Figure 1

(A) WT or *Gpc3*^{KO} MEFs were serum-starved for the indicated time, and levels of *mGPC3* transcript were measured by qRT-PCR. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$. *mGPC3* transcripts are present in WT, but not *Gpc3*^{KO}, MEFs. Serum starvation increases *mGPC3* transcript levels.

(B) Representative fluorescence micrographs for the experiment quantified in Figure 1B, showing expression of eGFP-tagged receptors (top) and corresponding binding of AlexaFluor 594–labeled unlipidated Shh (bottom). Scale bar = 50μm.

(C) As in (B), but for the experiment quantified in Figure 1C, showing binding of TMR-labeled palmitoylated Shh (bottom). Scale bar = 50μm.

(D) As in (C), but for the experiment quantified in Figure 1D. Scale bar = 50μm.

(E) Cartoon representation of full length (FL) and ΔGPI GPC3 constructs.

(F) WT or *Gpc3*^{KO} MEFs, transduced with constructs expressing GPC3 FL or GPC3 ΔGPI, were incubated with SAG (1μM) or control media for 24 hours, and Hh signaling was measured by qRT-PCR for *Gli1*. GPC3ΔGPI expression inhibits Hh signaling in WT cells. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$.

(G) *mGPC3* transcript levels were measured by qRT-PCR in WT MEFs, *Gpc3*^{KO} MEFs, and *Gpc3*^{KO} MEFs transduced with a lentivirus expressing GPC3 FL. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$.

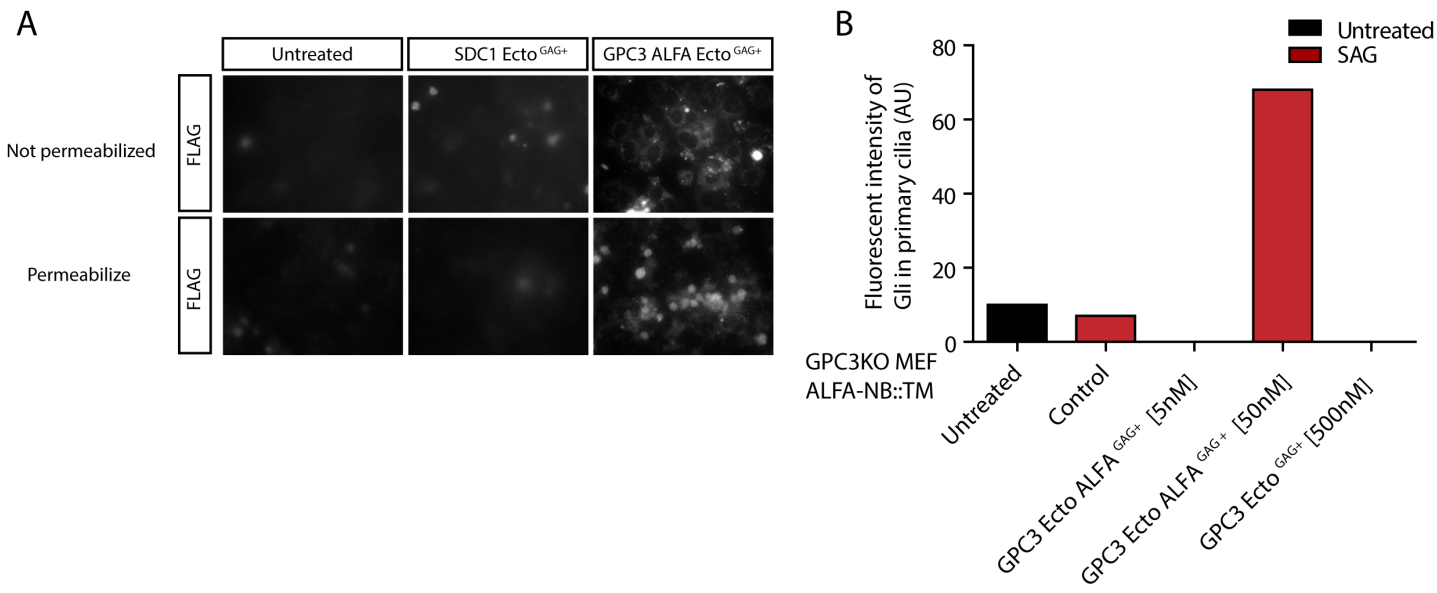
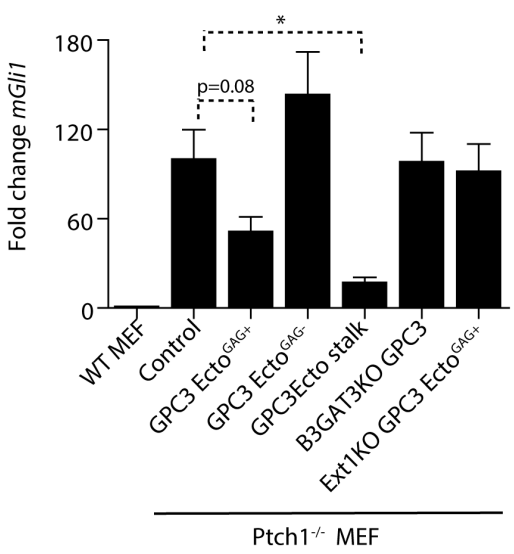


Fig. S2. Related to Figure 2.

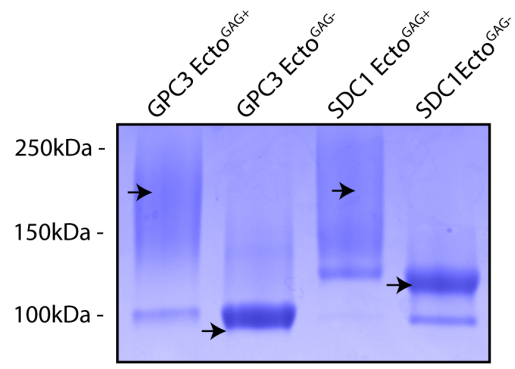
(A) *Gpc3*^{KO} MEFs expressing ALFA-NB::TM were incubated for 24 hours with 50 nM FLAG-tagged GPC3-ALFA-Ecto^{GAG+} or SDC1-Ecto^{GAG+} (negative control), followed by anti-FLAG immunofluorescence microscopy, with or without permeabilization with 1% Triton X-100. GPC3-ALFA-Ecto^{GAG+} is recruited to cells expressing ALFA-NB::TM.

(B) *Gpc3*^{KO} MEFs expressing ALFA-NB::TM were incubated with SAG (1μM), in the presence of the indicated concentrations of purified GPC3-Ecto-ALFA^{GAG+} for 6hrs. Ciliary intensity of endogenous Gli protein was measured by immunofluorescence microscopy. 100-150 cilia were measured per condition. While 50 nM GPC3-Ecto-ALFA^{GAG+} rescues Gli recruitment to cilia, 500 nM GPC3-Ecto-ALFA^{GAG+} is inhibitory.

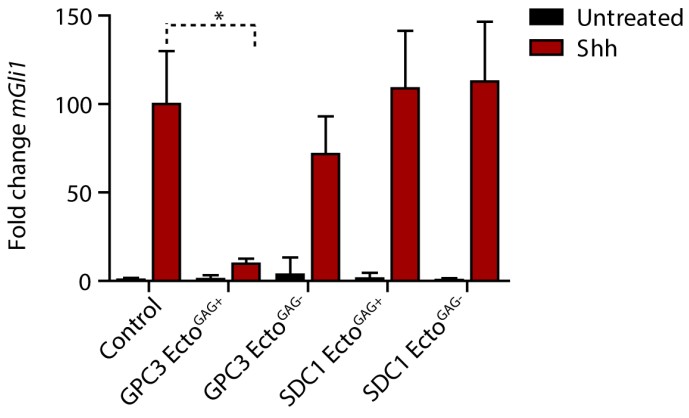
A


Fold change mGli1
180
120
60
0
*
p=0.08
WT MEF
Control
GPC3 Ecto GAG+
GPC3 Ecto GAG-
GPC3Ecto stalk
B3GAT3KO GPC3
Ext1KO GPC3 Ecto GAG+
Ptch1-/- MEF

B


GPC3 Ecto GAG+
GPC3 Ecto GAG-
SDC1 Ecto GAG+
SDC1Ecto GAG-
250kDa -
150kDa -
100kDa -

C


Fold change mGli1
150
100
50
0
*
Untreated
Shh
Control
GPC3 Ecto GAG+
GPC3 Ecto GAG-
SDC1 Ecto GAG+
SDC1 Ecto GAG-

D

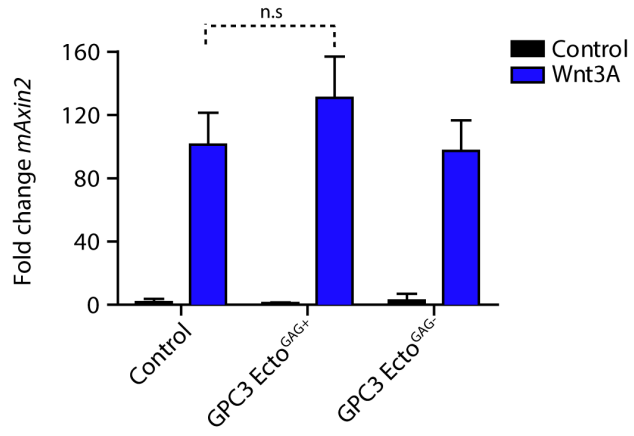

Fold change mAxin2
160
120
80
40
0
n.s
Control
Wnt3A
Control
GPC3 Ecto GAG+
GPC3 Ecto GAG-

Fig. S3. Related to Figure 3

(A) *Ptch1*$^{-/-}$ MEF cells were incubated with the indicated purified proteins (1μM) for 24 hours, and Hh pathway activity was measured by qRT-PCR for *Gli1*. HS-modified GPC3-Ecto and stalk domain antagonize constitutive Hh pathway activation. GPC3-Ecto proteins purified from *EXT1*^{KO} or from *B3GAT3*^{KO} cells (CS-modified and unmodified, respectively) are inactive. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$.

(B) GPC3-Ecto and SDC1-Ecto fused to HaloTag were expressed in HEK293T cells and were affinity purified from conditioned media. The proteins were then separated by size-exclusion chromatography, to isolate GAG-modified and GAG-unmodified fractions, which were analyzed by SDS-PAGE and Coomassie staining. Arrows indicate relevant protein species in each gel lane.

(C) WT MEFs were incubated with Shh or control media for 24 hours, in the absence or presence of the indicated purified proteins (1μM). Hh signaling was measured by qRT-PCR for *Gli1*. SDC1-Ecto has no effect on Hh signaling, while GPC3-Ecto inhibits signaling in a GAG-dependent manner. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$.

(D) As in (C), but with Wnt3A treatment and assaying Wnt pathway activation by qRT-PCR for *Axin2.* GAG-modified and unmodified GPC3-Ecto has no effect on Wnt signaling. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$.

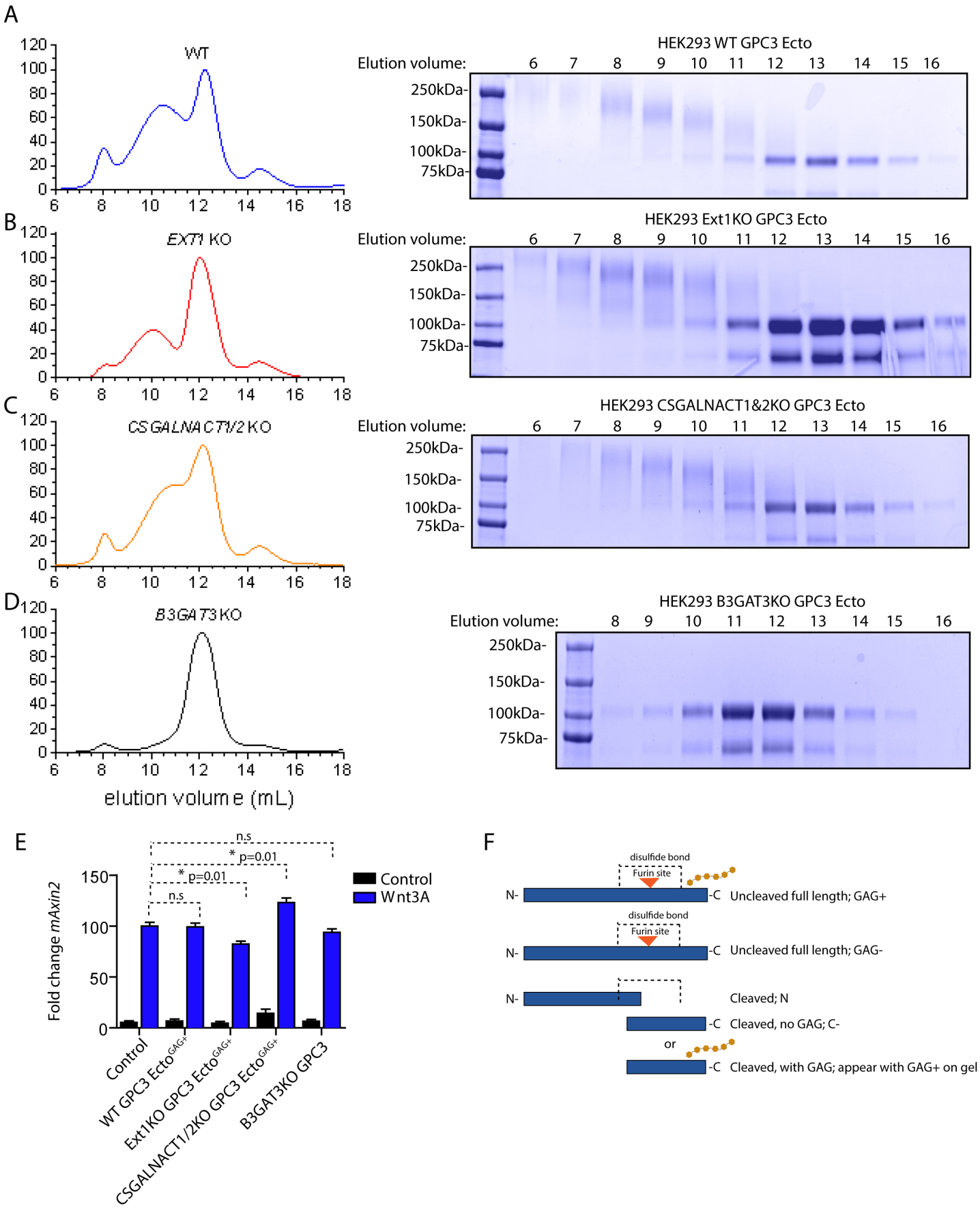


Fig. S4. Related to Figure 4

(A) UV trace for size exclusion chromatography of GPC3-Ecto, affinity-purified from media conditioned by WT HEK293T cells (left). The indicated fractions were analyzed by SDS-PAGE and Coomassie staining (right).

(B) As in (A), but with GPC3-Ecto secreted by *Ext1*^{KO} cells.

(C) As in (A), but with GPC3-Ecto secreted by *CSGALNACT1,2*^{KO} cells.

(D) As in (A), but with GPC3-Ecto secreted by *B3GAT3*^{KO} cells.

(E) WT MEFs were incubated with Wnt3A or control media for 24 hours, in the absence or presence of GPC3-Ecto proteins (1μM), expressed and purified from the indicated cells. Wnt signaling was measured by qRT-PCR for *Axin2*. Bars show average fold-change for three replicates, and error bars show SEM. * denotes statistical significance, $p<0.05$. n.s =$p>0.05$.

(F) Cartoon representation of GPC3 cleavage by Furin, related to Figure 4B.

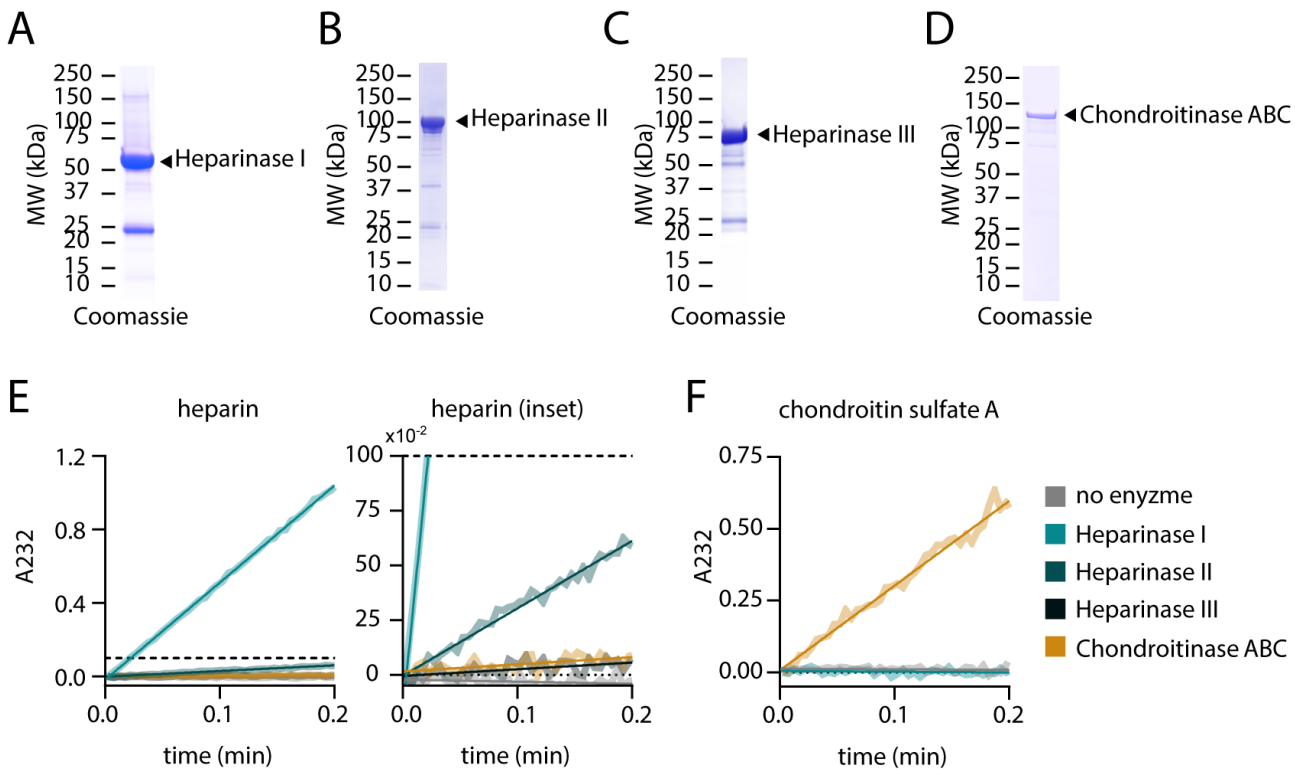


Fig. S5. Related to Materials and Methods

(A) GST-tagged *B. thetaiotaomicron* heparinase I (*bt4675*) was purified by glutathione affinity chromatography followed by gel filtration on a Superdex 200 26/60 column. Protein species well separated from the void volume were pooled, concentrated, and analyzed by SDS-PAGE and Coomassie staining.

(B) As in (A), but for *B. thetaiotaomicron* heparinase II (*bt4652*).

(C) As in (A), but for *B. thetaiotaomicron* heparinase III (*bt4657*).

(D) As in (A), but for *B. thetaiotaomicron* chondroitinase ABC (*bt3350*).

(E) Purified recombinant enzymes (20 μg) from (A)-(D) were incubated with 1 mg/mL porcine intestinal heparin in a total volume of 1 mL, and liberation of unsaturated non-reducing ends was quantified over time by continuous measurement at A232. Data (thick, light line) are fit with a linear regression (thin, dark line), used to calculate the specific activities reported in Table S4. Heparinase I (left) and heparinase II (see inset, right) act on heparin. Heparinase III, which acts on less highly sulfated HS substrates, and chondroitinase ABC are inactive against the heparin substrate.

(F) As in (E), except assaying enzyme activity on chondroitin sulfate A. Only chondroitinase ABC exhibits activity in this assay.

Table S1. Generation of null cell lines by CRISPR-Cas9

Target Gene	gRNA #	NCBI Reference Sequence	Target Site Description	gRNA Sequence	gRNA Orientation	Associated Plasmid	Associated Cell Line
B3GAT3	1	NM_012200	Exon 3	TTCCGCTGCTCGACACCACG	antisense	pBMW673	BMW170.41
EXT1	1	NM_000127	Exon 1	GCCAGAAATGATCCGGACTG	antisense	pBMW606	BMW171.17
CSGALNACT1	1	NM_001130518	Exon 4	GGGTGCAGGCCAACATGTAC	antisense	pBMW703	BMW301.16
CSGALNACT2	1	NM_018590	Exon 2	GCCAAACTACCCAGTGAGTA	sense	pBMW704	BMW301.16
Gpc3	1	NM_016697	Exon 2	TGAGTTCCATACTCGCAGAC	antisense	pYCL1	YCL1.24
Gpc3	2	NM_016697	Exon 3	TGCGGTGGTTATTGCAATGT	sense	pYCL2	YCL1.24

Target Gene	gRNA #	Direction	Barcode Sequence	Target Recognition Sequence
B3GAT3	1	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CCTCCTCTTCACACACCTGG
B3GAT3	1	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CAAAGTAGACGACTCCTTGGGT
EXT1	1	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TTGTCTCGCCCTTTTGTTTTAT
EXT1	1	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	AAATGTGCACGCTGGAATC
CSGALNACT1	1	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TTCCTGAATGATGATGGTTCG
CSGALNACT1	1	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TGGTACCCCTCCTTCCCC
CSGALNACT2	1	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACAAAGAGCAAGCACCTAGTGA
CSGALNACT2	1	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	CTTTTCTTCAGGATGGCGAGT
Gpc3	1	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CAACATGCTGCTCAAGAAAGAT
Gpc3	1	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GCCATTGAACAGTACATCGAAA
Gpc3	2	Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACACTACCGACCACCTCAAGTT
Gpc3	2	Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TACTTGTCGATCTCCACCACAC

gRNA #	Allele 1 Sequence	Allele 1 Type	Allele 1 % Aligned Reads	Allele 2 Sequence	Allele 2 Type	Allele 2 % Aligned Reads	Allele 3 Sequence	Allele 3 Type	Allele 3 % Aligned Reads
1	AGCCTGGCTG//TGGGTGGGGA	68-nt deletion	51%	CGTG-TGTCGAGCAGCGGAA	1-nt deletion	26%	CGTGG(G)TGTCGAGCAGCGGAA	1-nt insertion	23%
1	GCACCACCCC//CCGCTTCCCG	20-nt deletion	92%	GGCTTGCACC//[CCC]CCCCGCTTCC	20-nt deletion / 3-nt mutation	8%	--	--	--
1	CTGTGCTATC//ACTGCCCAGG	53-nt deletion	74%	GTA--TGTTGGCCTGCACCC	2-nt deletion	26%	--	--	--
1	GCCAAACTACCCAGTGA(A)GTA	1-nt insertion	100%	--	--	--	--	--	--
1	GTCTGCGAGTATGGACTCA	wild-type	72%	GT----------GGAACTCA	10-nt deletion	28%	--	--	--
2	TGCG-TGGTTATTGCAATGT	1-nt deletion	35%	TGCGG(G)TGGTTATTGCAATGT	1-nt insertion	30%	TGCGGT---TATTGCAATGT	3-nt deletion	35%

Table S2. List of expression constructs

pBMW Identifier	Vector	Promoter	Short Name	Full Transgene	Eukaryotic Resistance Marker	Bacterial Resistance Marker	Category	Subcategory	Reference
pBMW673	pX459	U6	*B3GAT3*^{KO}	hB3GAT3_CRISPR_KO_gRNA1	Puro	Amp	CRISPR knockout	GAG biosynthesis	this paper
pBMW606	pX459	U6	*EXT1*^{KO}	hEXT1_CRISPR_KO_gRNA1	Puro	Amp	CRISPR knockout	GAG biosynthesis	this paper
pBMW703	pX459	U6	*CSGALNACT1*^{KO}	hCSGALNACT1_CRISPR_KO_gRNA1	Puro	Amp	CRISPR knockout	GAG biosynthesis	this paper
pBMW704	pX459	U6	*CSGALNACT2*^{KO}	hCSGALNACT2_CRISPR_KO_gRNA1	Puro	Amp	CRISPR knockout	GAG biosynthesis	this paper
pYCL1	pX459	U6	*Gpc3*^{KO}	mGpc3_CRISPR_KO_gRNA1	Puro	Amp	CRISPR knockout	*Gpc3*^{KO}	this paper
pYCL2	pX459	U6	*Gpc3*^{KO}	mGpc3_CRISPR_KO_gRNA2	Puro	Amp	CRISPR knockout	*Gpc3*^{KO}	this paper
pAS63	pCS2	IE94-CMV	Smo	mSMO-FL::fTEV-EGFP	--	Amp	binding experiments	negative control	Wierbowski et al., 2020
pAS58	pHAGE2	CAG-CMV	Ptch1	mPTCH1-Del(C)Tail::fEGFP	Blast	Amp	binding experiments	positive control	Wierbowski et al., 2020
pBMW177	pHAGE2	CAG-CMV	Cdon	hCDON-Del(C)TM1::hCDON-Del(N)FNs::fTEV-EGFP	Blast	Amp	binding experiments	positive control	Wierbowski et al., 2020
pBMW281	pCS2	IE94-CMV	Boc	hBOC-Del(C)TM1::hBOC-Del(N)FNs::fTEV-EGFP	--	Amp	binding experiments	positive control	Wierbowski et al., 2020
pBMW241	pCS2	IE94-CMV	Hhip	EGFPf::hHHIP-FL	--	Amp	binding experiments	positive control	this paper
pBMW304	pHAGE2	CAG-CMV	scFv5E1::TM	scFv5E1-LH::hCDON-Del(N)FNs::fTEV-EGFP	Blast	Amp	binding experiments	positive control	Wierbowski et al., 2020; Maun et al., 2010
pBMW243	pCS2	IE94-CMV	GPC3	EGFPf::mGPC3-FL	--	Amp	binding experiments	experimental sample	this paper
pBMW244	pCS2	IE94-CMV	GPC5	EGFPf::hGPC5-FL	--	Amp	binding experiments	experimental sample	this paper
pAS295	pHAGE2	CAG-CMV	ALFA-NB::TM	HPC-NbALFA::hCDON-TM-CTD	Blast	Amp	stable mammalian expression	ALFA-NB recruitment system	Wierbowski et al., 2020; Gotzke et al., 2019
pAS283	pHAGE2	CAG-CMV	GPC3	FLAG-mGPC3-FL	Blast	Amp	stable mammalian expression	*Gpc3*^{KO} rescue	this paper
pAS173	pHAGE2	CAG-CMV	GPC3-Ecto	FLAG-HT7-PreSci::mGPC3-Del(C)GPI	Blast	Amp	stable mammali	*Gpc3*^{KO} rescue	this paper

							an expression		
pAS272	pTWIN	T7lac	unlipidated Shh	SHH(C24A)-N	--	Amp	bacterial protein production	Shh ligand for binding experiments; competitor for Ptch1 interaction	Wierbowski et al., 2020
pBMW643	pGEX-2TK	Ptac	Heparinase I	GST-Thromb::B.theta.HeparinaseI	--	Amp	bacterial protein production	heparinases	this paper
pBMW644	pGEX-2TK	Ptac	Heparinase II	GST-Thromb::B.theta.HeparinaseII	--	Amp	bacterial protein production	heparinases	this paper
pBMW645	pGEX-2TK	Ptac	Heparinase III	GST-Thromb::B.theta.HeparinaseIII	--	Amp	bacterial protein production	heparinases	this paper
pBMW678	pGEX-2TK	Ptac	Chondroitinase ABC	GST-Thromb::B.theta.ChondroitinaseABC	--	Amp	bacterial protein production	chondroitinase	this paper
pAS48	pCS2	IE94-CMV	Shh	hSHH-N	--	Amp	transient mammalian protein production	Shh conditioned medium production	Wierbowski et al., 2020
pAS75	pCS2	IE94-CMV	palmitoylated Shh	hSHH-N::fHT7-PreSci-HPC	--	Amp	transient mammalian protein production	Shh ligand for binding experiments	Wierbowski et al., 2020
pBMW814	pHAGE2	CAG-CMV	scFv5E1	scFv5E1::PreSci-fHT7-HPC	Blast	Amp	stable mammalian protein production	competitor for Ptch1 interaction	Wierbowski et al., 2020
pAS290	pHAGE2	CAG-CMV	GPC3-Ecto-ALFA	FLAG-mGPC3-Ecto-ALFA	Blast	Amp	stable mammalian protein production	ALFA-NB recruitment system	this paper
pBMW637	pHAGE2	CAG-CMV	SDC1-Ecto	hSDC1-Ecto::PreSci-fHT7-HPC	Blast	Amp	stable mammalian protein production	negative control	this paper
pAS174	pHAGE2	CAG-CMV	GPC3-Ecto	mGPC3-Del(C)GPI::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	experimental sample	this paper
pBMW697	pHAGE2	CAG-CMV	GPC3-Ecto^{PreScission}	FLAG-mGPC3-Core::PreSci::mGPC3-Stalk::fHT7-HPC	Blast	Amp	stable mammalian protein production	cleavable GPC3-Ecto	this paper
pBMW675	pHAGE2	CAG-CMV	GPC3-Stalk	HPC-HT7-PreSci::mGPC3-Stalk	Blast	Amp	stable mammalian protein production	stalk sufficiency test	this paper

pBMW554	pHAGE2	CAG-CMV	GPC1-Ecto	hGPC1-Del(C)GPI::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	core necessity test	this paper
pBMW555	pHAGE2	CAG-CMV	GPC2-Ecto	hGPC2-Del(C)GPI::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	core necessity test	this paper
pBMW833	pHAGE2	CAG-CMV	$GPC1^{core}$-$GPC3^{stalk}$-Ecto	hGPC1-Core::mGPC3-Stalk::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	core necessity test	this paper
pBMW834	pHAGE2	CAG-CMV	$GPC3^{core}$-$GPC1^{stalk}$-Ecto	mGPC3-Core::hGPC1-Stalk::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	core necessity test	this paper
pBMW817	pHAGE2	CAG-CMV	$GPC2^{core}$-$GPC3^{stalk}$-Ecto	hGPC2-Core::mGPC3-Stalk::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	core necessity test	this paper
pBMW818	pHAGE2	CAG-CMV	$GPC3^{core}$-$GPC2^{stalk}$-Ecto	mGPC3-Core::hGPC2-Stalk::fHT7-PreSci-HPC	Blast	Amp	stable mammalian protein production	core necessity test	this paper

Table S3. List of qRT-PCR primers

Target	Forward qPCR Primer	Reverse qPCR Primer
mGli1	TACCATGAGCCCTTCTTTAGGA	GCATCATTGAACCCCGAGTAG
mCyclo	GGAGATGGCACAGGAGGAA	GCCCGTAGTGCTTCAGCTT
mAxin2	GCTCCAGAAGATCACAAAGAGC	AGCTTTGAGCCTTCAGCATC

Table S4. Specific activities of recombinant heparinases and chondroitinase

Gene	Protein	Substrate	Specific Activity (pmol/min/µg)
bt3350	*B. theta* Chondroitinase ABC	chondroitin sulfate A	38802.63
		heparin	*N.D.*
bt4675	*B. theta* Heparinase I	chondroitin sulfate A	*N.D.*
		heparin	69500.00
bt4652	*B. theta* Heparinase II	chondroitin sulfate A	*N.D.*
		heparin	4057.89
bt4657	*B. theta* Heparinase III	chondroitin sulfate A	*N.D.*
		heparin	*N.D.*