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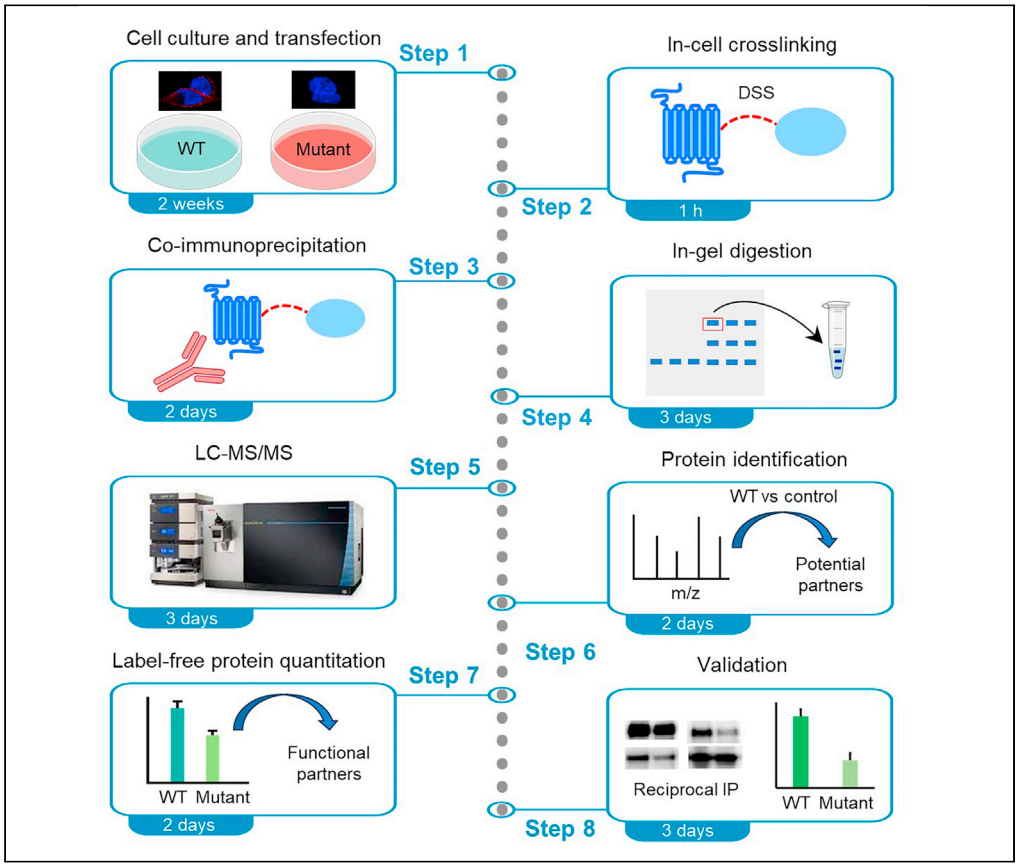
Protocol for identifying physiologically relevant binding proteins of G-protein-coupled receptors



G-protein-coupled receptors (GPCRs) are important therapeutic targets expressed on the cell surface. Here, we present a protocol for identifying physiologically relevant binding proteins of adhesion GPCR GPR110. We describe steps for in-cell chemical crosslinking, immunoprecipitation, and quantitative high-resolution mass spectrometry. Notably, we detail a label-free quantitation strategy that eliminates irrelevant interacting proteins using an inactive GPR110 mutant with impaired surface expression. Furthermore, we outline procedures for validating the identified partners.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Chemical crosslinking captures protein interactions in living cells

Affinity purification and mass spectrometry identify potential interacting proteins

Mutagenesis with quantitative mass spectrometry pinpoints true binding partners

Reciprocal immunoprecipitation validates protein-protein interactions

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Protocol for identifying physiologically relevant binding proteins of G-protein-coupled receptors

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SUMMARY

G-protein-coupled receptors (GPCRs) are important therapeutic targets expressed on the cell surface. Here, we present a protocol for identifying physiologically relevant binding proteins of adhesion GPCR GPR110. We describe steps for in-cell chemical crosslinking, immunoprecipitation, and quantitative high-resolution mass spectrometry. Notably, we detail a label-free quantitation strategy that eliminates irrelevant interacting proteins using an inactive GPR110 mutant with impaired surface expression. Furthermore, we outline procedures for validating the identified partners.

For complete details on the use and execution of this protocol, please refer to Huang et al. (2023).[1]

BEFORE YOU BEGIN

This protocol was used to identify proteins that interact with GPR110 in living cells. GPR110 is an adhesion G-protein coupled receptor (GPCR) known to play significant roles in neurodevelopment and implicated in cancer.[2,3] It is believed to participate in cellular adhesion processes, which involve interactions between cell adhesion molecules, including transmembrane proteins on the cell surface, as well as G protein-mediated signaling. Like most GPCRs, the low abundance of the receptor and limited availability of specific antibodies pose challenges for immunoprecipitation of endogenous GPR110 and its associated proteins. Therefore, this protocol employs transient expression of GPR110 in HEK cells using a *C*-terminal 3xHA-tagged human GPR110 plasmid construct containing the full-length open reading frame sequence (Figure 1). The expressed GPR110 has been shown to reside on the plasma membrane and exhibit functional activity such as increasing cAMP like the endogenous GPR110.[3,4] The HA tag and an HA antibody facilitated immunoprecipitation (IP) of GPR110-HA which was subsequently eluted with the co-immunoprecipitated products using an HA peptide for downstream analysis.[3,4]

To capture GPR110-protein interactions in living cells, a bifunctional amine-reactive crosslinker disuccinimidyl suberate (DSS) was introduced into HEK cells expressing GPR110. After IP, the co-immunoprecipitates were separated by SDS-PAGE, and selected gel bands were subjected to in-gel tryptic digestion, followed by LC-MS/MS analysis. To eliminate non-specific proteins, parallel experiments were conducted using cells overexpressing an HA-tagged empty vector control (M45). Proteins identified in this negative control were considered as background proteins. Additionally, proteins that were not reproducibly identified in three independent experiments were excluded. Physiologically relevant partners interacting with GPR110 on the cell surface were distinguished by quantitatively comparing the binding partners of the WT GPR110 with F663S, a GPR110 mutant that is minimally expressed on the cell surface, using label-free protein quantitation.

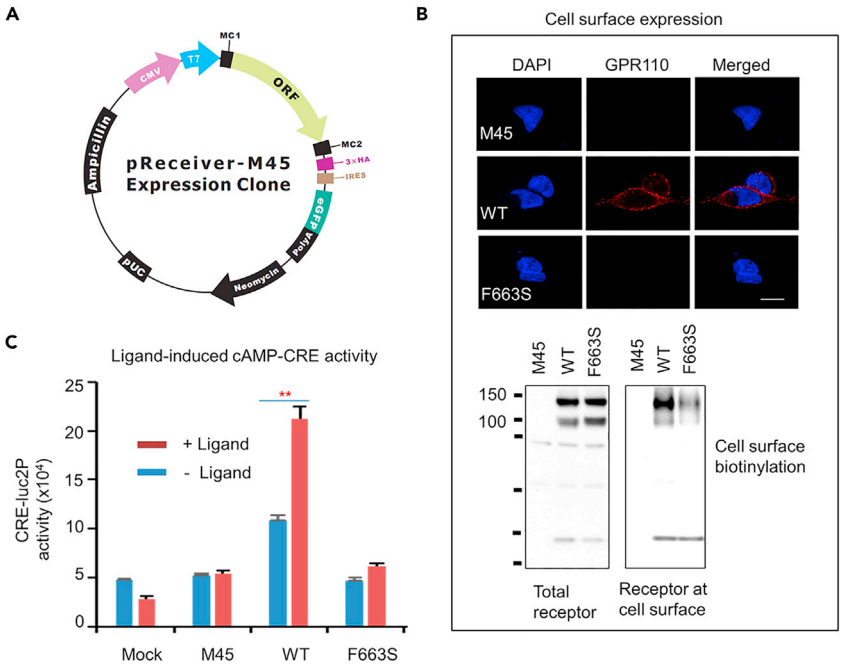

Figure 1. Expression and bioactivity profile of GPR110 and F663S mutant

(A) Plasmid map. A 3x Flag tag is incorporated in the *C*-terminal of GPR110.

(B) The F663S mutant is not properly expressed on the cell surface according to the non-permeable immunofluorescence microscopic and biotinylation detection of surface GPR110.

(C) Unlike WT GPR110, F663S mutant fails to increase cAMP production upon ligand stimulation. Statistical analysis was performed using unpaired Student's t-test (**$p < 0.01$). Data are means ± SEM of biological replicates (n = 3). Modified from Huang et al.[1]

The GPR110 F663S mutant was initially identified in a schizophrenic case from the Swedish exome sequencing study. We performed non-permeable immunostaining using a GPR110 antibody (*N*-Terminus) and cell surface biotinylation assay to evaluate the surface expression of the F663S mutant. Minimal expression of the F663S mutant in the plasma membrane was apparent compared to the WT GPR110 (Figure 1). Furthermore, CRE-luciferase assay indicated that unlike the WT GPR110, the F663S mutant failed to trigger cAMP-dependent signal transduction upon ligand stimulation, which was consistent with the impaired cell surface-expression (Figure 1). Accordingly, the F663S mutant was used as the negative control for identifying partners that interact with functional GPR110 on the cell surface.

This protocol provides a valuable approach for investigating the interacting partners of GPR110 and their functional relevance in cellular processes.[1] It should also be applicable to other GPCRs or cell surface receptors once mutants with impaired surface expression are available. In addition to the techniques mentioned above, alternative methods such as flow cytometry represent valid options for analysis of surface expression.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HA-antibody (dilution 1:1,000 WB, 1:100 IP)	Santa Cruz Biotechnology	Cat#: 7392; PRID: AB_627809
Occludin antibody (dilution 1:1,000 WB, 1:100 IP)	Proteintech	Cat#: 13409-1-AP PRID: AB_2156308
β-Actin (C4) antibody (dilution 1:1,000 WB)	Santa Cruz Biotechnology	Cat#: sc-47778 PRID: AB_626632

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
IgG from mouse serum (dilution 1:100 IP)	Millipore Sigma	Cat#: I8765 PRID: AB_1163672
Anti-mouse IgG (whole molecule) peroxidase antibody produced in goat (dilution 1:2,000)	Millipore Sigma	Cat#: A4416 PRID: AB_258167
Anti-rabbit IgG (whole molecule) peroxidase antibody produced in goat (dilution 1:2,000)	Millipore Sigma	Cat#: A6154 PRID: AB_258284
Anti-ADGRF1 (GPR110) antibody (dilution 1:100)	Millipore Sigma	Cat#: HPA038438 PRID: AB_10672645
Alexa Fluor Plus 555, anti-rabbit (dilution 1:500)	Thermo Fisher Scientific	Cat#: A32732 PRID: AB_2633281
Chemicals, peptides, and recombinant proteins		
HA peptide	Thermo Fisher Scientific	Cat#: 26184
Succinimidyl suberate (DSS)	Thermo Fisher Scientific	Cat#: A39267
Halt Protease Inhibitor Cocktail	Thermo Fisher Scientific	Cat#: 78430
Fetal bovine serum	Millipore Sigma	Cat#: F2442
Lipofectamine 2000	Thermo Fisher Scientific	Cat#: 11668-019
Opti-MEM reduced serum medium	Thermo Fisher Scientific	Cat#: 31985-070
SimplyBlue SafeStain	Thermo Fisher Scientific	Cat#: LC6060
Restore western blot stripping buffer	Thermo Fisher Scientific	Cat#: 21059
PBS (pH 7.4, without calcium and magnesium)	Thermo Fisher Scientific	Cat#: 10010-031
Acetonitrile, LC-MS grade	J.T. Baker	Cat#: UN1648
Water, LC-MS grade	J.T. Baker	Cat#: 9831-03
NuPAGE LDS sample buffer (4X)	Thermo Fisher Scientific	Cat#: NP0007
MOPS SDS running buffer	Thermo Fisher Scientific	Cat#: NP0001
Dynabeads protein G	Thermo Fisher Scientific	Cat#: 10004D
Dynabeads M-280 streptavidin	Thermo Fisher Scientific	Cat#: 11205D
Trizma hydrochloride solution (Tris-HCl, pH 7.4)	Sigma	Cat#: T2194-1L
Formic acid	Thermo Fisher Scientific	Cat#: 28905
Trifluoroacetic acid	Thermo Fisher Scientific	Cat#: 28904
Enhanced chemiluminescence (ECL) substrate	Thermo Fisher Scientific	Cat #: 34080
Sulfo-NHS biotin	Thermo Fisher Scientific	Cat#: 21217
Glycine hydrochloride	Millipore Sigma	Cat#: G2879
Synaptamide	This paper	N/A
Biotinylated-synaptamide	This paper	N/A
Trypsin/Lys-C mix	Promega	Cat#: V5073
DL-dithiothreitol	Millipore Sigma	Cat#: D9779
Iodoacetamide	Millipore Sigma	Cat#: I1149
Ammonium bicarbonate	Millipore Sigma	Cat#: A6141
Dimethyl sulfoxide	Millipore Sigma	Cat#: D2650-5X10ML
Tween 20	Fisher Scientific	Cat#: BP337-500
Tris-buffered saline	Santa Cruz	Cat#: sc-362305
Precision plus protein standards	Bio-Rad	Cat#: 161-0374
Cell lysis buffer	Cell Signaling Technology	Cat#: 9803
PMSF	Cell Signaling Technology	Cat#: 8553
BSA	Millipore Sigma	Cat#: 126609
DAPI	Millipore Sigma	Cat#: D9542
Eagle's minimum essential medium (EMEM)	ATCC	Cat#: 30-2003
Dulbecco's modified Eagle's medium (DMEM)	ATCC	Cat#: 30-2002
Critical commercial assays		
Dual-Glo Luciferase Assay	Promega	Cat#: E2920
Deposited data		
Mass spectrometry raw data	https://www.ebi.ac.uk/pride/	PXD041011
Experimental models: Cell lines		
HEK293 cells	ATCC	Cat#: CRL-1573
GloResponse CRE-luc2P HEK293	Promega	Cat#: E8500

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Mascot Distiller	Matrix Science	https://www.matrixscience.com/distiller.html
Progenesis QI for proteomics software	Waters Corporation	https://www.nonlinear.com/progenesis/qi-for-proteomics/
Kodak 1D imaging analysis software	Eastman Kodak Company	N/A
Other		
Human GPR110 plasmid	GeneCopoeia, Inc.	Cat#: EX-H0565-M45
Human GPR110 F663S plasmid	GeneCopoeia, Inc.	Cat#: CS-H0565-M45-18
Empty control vector for pReceiver-M45	GeneCopoeia, Inc.	Cat#: EX-NEG-M45
NuPAGE 4%–12%, Bis-Tris gel, 1.0 mm × 10 well	Thermo Fisher Scientific	Cat#: NP0321BOX
NuPAGE 4%–12%, Bis-Tris gel, 1.5 mm × 15 well	Thermo Fisher Scientific	Cat#: NP0336BOX
Trans-blot turbo transfer pack	Bio-Rad	Cat#: 1704156
C-18 ZipTip pipette tips	Millipore	Cat#: ZTC18S096
Acclaim PepMap100 C18 trap column	Thermo Fisher Scientific	Cat#: 164535
Acclaim RSLC column	Thermo Fisher Scientific	Cat#: 164534
Stainless steel emitter	Fisher Scientific	Cat#: ES542
2 mL clear wide opening crimp vial	Agilent	Cat#: 5181-3375
250 µL polypropylene insert	Agilent	Cat#: 5182-0549
Cap, 9 mm blue screw, PTFE/RS	Agilent	Cat#: 5182-0717
Nano HPLC system	Thermo Fisher Scientific	UltiMate 3000
Mass spectrometer	Thermo Fisher Scientific	Orbitrap Fusion Lumos Tribrid
XCell SureLock mini-cell module	Thermo Fisher Scientific	EI0001
CO_2 incubator	Thermo Fisher Scientific	HERACELL 150i
Vacuum concentrator	Labconco	7970010
Refrigerated centrifuge	Eppendorf	5427R
Refrigerated centrifuge	Eppendorf	5702R
Low-speed microcentrifuge	Tomy Tech, Inc.	PMC-880
Vortex-Genie 2	Fisher	12-812
Hybridization incubator	FINEPCR	Combi-H12
Trans-Blot Turbo transfer system	Bio-Rad	1704150
Trans-Blot Turbo mini 0.2 µm PVDF transfer packs	Bio-Rad	1704156
Rotator mixer	Benchmark Scientific, Inc.	B3D-1008
Magnetic stand (DynaMag-2)	Thermo Fisher Scientific	12321D
Rocking shaker	Reliable Scientific, Inc.	N/A
Imager	Azure Biosystems	Sapphire RGBNIR
Ultrapure water system	ResinTech, Inc.	CLS-5400-S-1

MATERIALS AND EQUIPMENT

Lysis buffer		
Reagent	Final concentration	Amount
Cell lysis buffer (10x)	1x	2.0 mL
Halt Protease Inhibitor Cocktail (100X)	1x	200 µL
Ultrapure water	N/A	17.8 mL
Total	N/A	20 mL
Add protease Halt inhibitors and keep cold at 4°C before use. Can be stored at −20°C for up to 6 months.		

Note: 1X lysis buffer contains 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin, and 1X Halt protease inhibitor cocktail.

⚠ CRITICAL: Halt protease inhibitor cocktail causes serious eye irritation and skin irritation. To prevent exposure, wear protective gloves/protective clothing/eye protection/face protection.

IP elution buffer

Reagent	Final concentration	Amount
HA peptide	1 mg/mL	1 mg
Lysis buffer	N/A	1 mL
Total	N/A	1 mL

Can be stored at −20°C for up to 6 months.

Tryptic digestion solution

Reagent	Final concentration	Amount
Mass Spectrometry Grade Trypsin/Lys-mix	12.5 ng/μL	20 μg
25 mM NH_4HCO_3	25 mM	1.6 mL
Total	N/A	1.6 mL

Prepare freshly and keep cold at 4°C before use. Do not keep.

Ammonium bicarbonate solution (25 mM)

Reagent	Final concentration	Amount
NH_4HCO_3	25 mM	2 mg
Ultrapure water	N/A	100 mL
Total	N/A	100 mL

Prepare freshly. Can be stored at room temperature for up to 1 week.

Reduction solution (10 mM dithiothreitol (DTT))

Reagent	Final concentration	Amount
DTT	10 mM	15 mg
25 mM NH_4HCO_3	25 mM	10 mL
Total	N/A	10 mL

Prepare freshly, do not keep.

⚠ CRITICAL: DTT (DL-Dithiothreitol) is harmful if swallowed and causes serious eye damage and skin irritation. To prevent exposure, wear protective gloves/protective clothing/eye protection/face protection.

Alkylation solution (110 mM iodoacetamide)

Reagent	Final concentration	Amount
Iodoacetamide	110 mM	200 mg
25 mM NH_4HCO_3	25 mM	10 mL
Total	N/A	10 mL

Prepare freshly, do not keep.

⚠ CRITICAL: Iodoacetamide is toxic if swallowed and causes severe skin burns and eye damage. It may cause allergy or asthma symptoms or breathing difficulties if inhaled. It may cause an allergic skin reaction. To prevent exposure, wear protective gloves/protective clothing/eye protection/face protection.

De-staining solution (25 mM NH_4HCO_3/acetonitrile (1:1))

Reagent	Final concentration	Amount
25 mM NH_4HCO_3	12.5 mM	25 mL
Acetonitrile	N/A	25 mL
Total	N/A	50 mL

Can be stored at room temperature for up to several weeks.

⚠ CRITICAL: Acetonitrile is a highly flammable liquid and vapor. It is harmful if swallowed, in contact with skin or if inhaled. It causes serious eye irritation. To prevent exposure, wear protective gloves/protective clothing/eye protection/face protection.

Peptide extraction solution (5% formic acid/acetonitrile (1:1))

Reagent	Final concentration	Amount
Formic acid	2.5%	0.5 mL
Ultrapure water	N/A	9.5 mL
Acetonitrile	N/A	10 mL
Total	N/A	20 mL

Can be stored at room temperature for up to 2 weeks.

⚠ CRITICAL: Formic acid is harmful if swallowed or inhaled and causes severe skin burns and eye damage. To prevent exposure, wear protective gloves/ eye protection/ face protection.

Desalting elution solution (0.1% trifluoroacetic acid/acetonitrile (1:1))

Reagent	Final concentration	Amount
Trifluoroacetic acid	0.1%	10 µL
Ultrapure water	N/A	4.99 mL
Acetonitrile	N/A	5 mL
Total	N/A	10 mL

Can be stored at room temperature for up to 2 weeks.

⚠ CRITICAL: Trifluoroacetic acid harmful if swallowed and causes severe skin burns and eye damage. To prevent exposure, wear protective gloves/protective clothing/eye protection/face protection.

Trap column LC solvent

Reagent	Final concentration	Amount
Formic acid	0.1%	1 mL
Water (LC-MS grade)	N/A	979 mL
Acetonitrile (LC-MS grade)	2%	20 mL
Total	N/A	1000 mL

Prepare freshly. Replace biweekly.

Nano-LC mobile phase A		
Reagent	Final concentration	Amount
Formic acid	0.1%	200 µL
Water (LC-MS grade)	N/A	199.8 mL
Total	N/A	200 mL
Prepare freshly. Replace biweekly.		

Nano-LC mobile phase B		
Reagent	Final concentration	Amount
Formic acid	0.1%	200 µL
Water (LC-MS grade)	2%	4 mL
Acetonitrile (LC-MS grade)	98%	195.8 mL
Total	N/A	200 mL
Prepare freshly. Replace biweekly.		

SDS electrophoresis buffer		
Reagent	Final concentration	Amount
MOPS SDS running buffer 20x	1x	50 mL
Ultrapure water	N/A	950 mL
Total	N/A	1000 mL
Can be stored at room temperature for up to 6 months.		

Western blotting washing buffer (TBS with 1% Tween 20 (TBST))		
Reagent	Final concentration	Amount
TBS (pH 7.4) 20x	1x	50 mL
Ultrapure water	N/A	949 mL
Tween-20	1%	1 mL
Total	N/A	1000 mL
Can be stored at room temperature for up to 6 months.		

4x LDS sample buffer		
Reagent	Final concentration	Amount
NuPAGE LDS sample buffer 4x	4x	0.95 mL
2-Mercaptoethanol	5%	50 µL
Total	N/A	1 mL
Can be stored at −20°C for up to 6 months.		

STEP-BY-STEP METHOD DETAILS

Cell culture and transfection

Timing: 2 weeks

In this step, WT GPR110 or F663S mutant is transiently expressed in HEK293 cells.

We recommend performing two experiments on three biological replicates. The first experiment aims to filter out the background proteins by identifying the common binding proteins of the

expressed GPR110 and M45 control. The second experiment is to identify physiologically relevant partners by comparing the data from the WT GPR110 with the F663S mutant. Six 15-cm dishes of cells are required for each experiment.

1. Cell culture.

Culture and maintain HEK293 cells in 15-cm dishes in EMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified CO2 (5%) incubator.

⚠ CRITICAL: Cells with passage numbers of 8–12 are used for transfection.

2. Cell transfection.
   a. Seed HEK293 cells.
      Seed HEK293 cells (~8×10^6 cells) in 15-cm dishes and incubate the cells at 37°C in a CO_2 incubator for 16–20 h.

   Note: Cell density should be 60%–80% confluent at the time of transfection.

   b. Transfect HEK293 cells.

   Note: The following protocol is used for transfection of a 15-cm dish of cells. The regents should be scaled up for triplicate samples.

      i. Dilute 5 μg of GPR110, or F663S, or M45 plasmid in 700 μL of Opti-MEM I. Reduced Serum Media (Opti-MEM).
      ii. Dilute 50 μL Lipofectamine 2000 in 700 μL of Opti-MEM.
      iii. Mix solutions from Steps i and ii and incubate for 15 min at room temperature (25°C).
      iv. Add the mixed solution to the 15-cm dish of cells from Step a drop by stop using a pipette. Gently rock the dish back and forth.
      v. Incubate the cells at 37°C and under 5% CO_2 for 24 h.

In-cell crosslinking

Timing: 1 h

In this step, protein-protein interactions are captured in living cells by chemical crosslinking with DSS.

3. In-cell crosslinking with DSS.
   a. Replace the medium with PBS.
      i. Remove and discard the medium by aspiration.
      ii. Wash cells with 10 mL PBS.

   Note: Gently add 10 mL PBS to the side of the dish to avoid disturbing the cell layer, and gently rock the dish back and forth 3–5 times. Also, aspiration should be performed gently.

      iii. Remove and discard the PBS by aspiration.
      iv. Add 5 mL PBS.

   ⚠ CRITICAL: Crosslinking cannot be performed in buffers containing primary amines.

   b. Crosslink proteins with DSS.
      i. Prepare freshly 200 mM DSS in DMSO.

ii. Add 25 μL of 200 mM DSS to cells in PBS (Step 3iv). Gently rock the dish back and forth 3–5 times. Incubate for 30 min at room temperature (25°C).

c. Quench crosslinking.
Add 150 μL of 1M Tris-HCl buffer (pH = 7.4) to quench the crosslinking reaction. Mix gently by rocking the dish back and forth several times. Incubate for 10 min at room temperature.

Note: It is normal that some cells become floating after crosslinking.

Immunoprecipitation (IP)

Timing: 2 days

In this step, cells are lysed and the HA-tagged GPR110 and its interacting proteins are pulled down by HA-antibody conjugated to protein G beads. The immunoprecipitated proteins are eluted from the beads by HA peptide.

4. Preparation of cell lysate.
   a. Harvest cells.
      i. Detach the cells without removing PBS using a cell lifter.
      ii. Transfer the cell suspension to a 15-mL conical tube.
      iii. Centrifuge the sample for 5 min at 4°C at 1,000 × *g*.
      iv. Discard the supernatant by aspiration.

   Note: Care should be taken not to disturb the cell pellet.

   ⚠ CRITICAL: Do not directly remove PBS from the culture dishes which will result in loss of the floating cells. Do not harvest the cells by trypsin digestion, as trypsin digestion accelerates the degradation of proteins in the lysate.

   b. Lyse cells.
      i. Add 1.5 mL cold lysis buffer to the pellet. Resuspend the pellet by pipetting up and down 3–5 times.
      ii. Transfer the cell suspension to a 1.5 Eppendorf tube.
      iii. Incubate the cell suspension on ice for 30 min. Vortex for 1–2 s every 5 min.
      iv. Centrifuge the cells for 15 min at 4°C at 15,000 × *g*.
      v. Collect and transfer the supernatant to a new Eppendorf tube. Keep samples on ice.
      vi. Determine the protein concentration using BCA Protein Assay kit. Adjust the protein concentration to the lowest sample concentration with lysis buffer if necessary.

   Note: We use 1.3 mL (~3.5 mg proteins) of the lysate for each IP. Additionally, 20 μL of each sample is used for western blotting with HA antibody to confirm the expression level of total GPR110 and F663S.

5. Immunoprecipitation with HA antibody.
   a. Incubate the lysate with HA antibody.
      i. Transfer 1.3 mL of the lysate (Step 4bv) for each sample to an Eppendorf tube.
      ii. Add 40 μL of HA antibody to the lysate. Incubate samples on a rotator overnight (16–20 h) at 4°C.
   b. Prepare protein G beads.
      i. Add 40 μL of well-resuspended magnetic Dynabeads Protein G to a 1.5-mL Eppendorf tube.
      ii. Place the tube in the rack of DynaMag-2 magnet for 1 min. Remove and discard the supernatant.

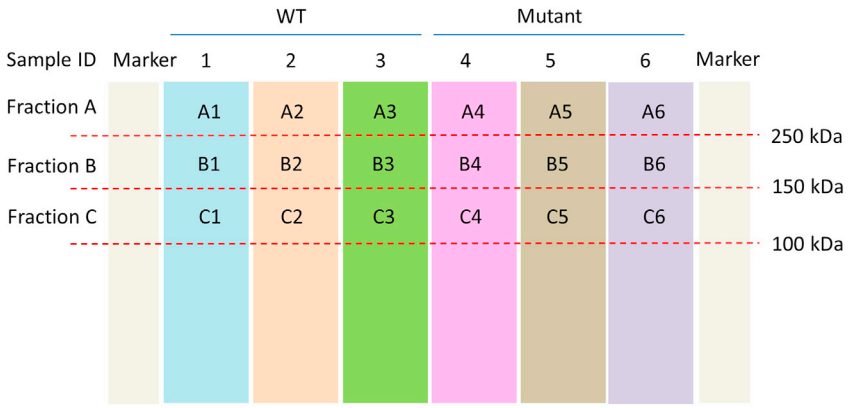

Figure 2. Gel fractionation for in-gel digestion, LC-MS/MS and label-free quantitation

Gel bands are cut out into fractions (i.e., fraction A-C) from each sample (i.e., sample number 1–6) according to the molecular weight range of interest.

Note: Remove the supernatant with a pipette while the tube is in the magnet rack. Care should be taken not to disturb the accumulated beads.

c. Conjugate antibody to protein G beads.
   i. Transfer the HA antibody-incubated lysate (from Step 5aii) to the tube containing the Dynabeads Protein G (Step 5bii).
   ii. Incubate for 4 h at 4°C on a rotator.
d. Wash non-specifically bound proteins.
   i. Place samples in the rack of DynaMag-2 magnet for 1 min. Remove and discard the supernatant.
   ii. Remove the tubes from the magnet. Wash beads by adding 800 μL of lysis buffer. Gently shake the tubes by hand. Spin down the beads briefly using a low-speed microcentrifuge.
   iii. Place the tubes on the magnet for 1 min. Remove and discard the supernatant.
   iv. Repeat Steps ii and iii two more times.

Note: Remove the supernatant with a pipette while the tubes are in the magnet rack. Care should be taken not to disturb the accumulated beads.

e. Elute co-immunoprecipitated proteins.
   i. Prepare 1 mg/mL HA peptide in lysis buffer.
   ii. Add 50 μL of 1 mg/mL HA peptide to each tube from Step 5div.
   iii. Place the samples on a rotisserie rotator for 20 min at 30°C.
   iv. Place the samples on DynaMag-2 magnet for 1 min.
   v. Collect and transfer the supernatant to a new Eppendorf tube.

Pause point: The samples containing co-immunoprecipitated proteins can be stored at −80°C until further use.

In-gel digestion

Timing: 3 days

In this step, GPR110 and its interacting protein partners are separated by sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and digested by trypsin.

6. In-gel digestion.
   a. Separate proteins with SDS-PAGE.
      i. Mix 40 μL of the protein solution eluted from Step 5ev with 13.3 μL 4x lithium dodecyl sulfate (LDS) sample buffer containing 5% β-mercaptoethanol.
      ii. Incubate the samples at 37°C for 30 min.
      iii. Assemble a XCell SureLock Mini-Cell system with NuPAGE 4%–12% Bis-Tris mini gel (1.0 mm × 10 well) and MOPS SDS running buffer.
      iv. Load 45 μL of each sample. Load 5 μL protein standards before the 1st sample and after the last sample.
      v. Start electrophoresis with a constant voltage of 200 V. Stop when the dye front is around 1 cm away from the base of the gel.

   Note: The electrophoresis time is about 60 min.

   b. Stain gel with Coomassie G-250.
      i. Remove the gel and wash the gel with 100 mL ultrapure water (18.2MΩ-cm) three times for 5 min. Discard each rinse.
      ii. Stain the gel with 20 mL of SimplyBlue SafeStain (Coomassie G-250) for 1 h at room temperature (25°C).
      iii. Remove and discard the staining solution.
      iv. Incubate the gel in 100 mL of ultrapure water for 1 h.
   c. Cut out and excise gel bands.

⚠ CRITICAL: Wear gloves, sleeve protectors, mask, and cap to avoid keratin contamination. Clean gel cutting tools (blades and forceps) subsequently with methanol and water. Clean workspace (glass plates and bench). Do not talk over samples.

      i. Place gel on a clean glass plate. Cut out gel bands using a wide stainless steel surgical prep blade.

   Note: In the case of GPR110, the monomeric receptor band appears at ~130 kDa. Bands above ~130 kDa are expected to contain interacting partners that are crosslinked to GPR110 via DSS. To identify more proteins, we divided the gel slice above 100 kDa into three fractions, 100–150 kDa, 150–250 kDa and 250 kDa to the top of the gel (Figure 2).

      ii. Dice the gel slice of each lane (i.e., each sample) into 1 × 1 mm pieces using a single edge razor blade. Collect the gel pieces into a 1.5-mL Eppendorf tube containing 200 μL water using filter forceps.

   Note: Water in the collecting tube helps separate gel slices from the forceps.

      iii. Remove and discard water from the tube using a gel loading tip.
   d. De-stain gel pieces.
      i. Add 200 μL (or enough to cover) of de-staining solution (25 mM NH_4HCO_3 in 50% ACN). Vortex and spin briefly with a low-speed microcentrifuge. Incubate samples in a shaker for 30 min at room temperature (25°C).
      ii. Remove and discard the supernatant using a gel loading tip.
      iii. Repeat Steps i-ii.
      iv. Add 50 μL 100% ACN to dehydrate the gel pieces. Vortex and spin briefly. Incubate on a shaker for 5 min. Discard the supernatant.

   Note: Gel pieces become white and semi-opaque after this step.

e. Reduce and alkylate proteins.
   i. Dry the gel pieces completely with a vacuum concentrator (typically 30 min at 25°C).
   ii. Add 100 μL (or enough to cover) of 10 mM DTT freshly made in 25 mM NH_4HCO_3 to the dried gel pieces. Vortex and spin briefly. Incubate the samples at 56°C for 1 h.
   iii. Add 100 μL 110 mM iodoacetamide freshly made in 25 mM NH_4HCO_3. Vortex and spin briefly.
   Incubate at room temperature (25°C) for 45 min in the dark (e.g., cover the tubes and the rack with aluminum foil and place them in a drawer).
   iv. Remove and discard supernatant.
   v. Add 200 μL of 25 mM NH_4HCO_3. Vortex and spin briefly. Incubated on a shaker for 10 min.vi. Remove and discard supernatant.
   vi. Add 200 μL 25 mM NH_4HCO_3 in 50% ACN. Incubated on a shaker for 10 min.
   vii. Remove and discard supernatant.
   viii. Repeat Steps vi-vii.
   ix. Add 50 μL 100% ACN to dehydrate the gel pieces. Vortex and spin briefly. Incubate on a shaker for 5 min. Discard the supernatant.

Note: After adding ACN, the gel pieces turn white and semi-opaque.

   x. Dry the gel pieces completely with a vacuum concentrator (typically 30 min at 25°C).

Pause point: The gel pieces are now ready for digestion. However, they can be stored at −80°C for up to several weeks.

f. Digest proteins in-gel.
   i. Prepare freshly 12.5 ng/μL trypsin/Lys-C in 25 mM NH_4HCO_3.

⚠ CRITICAL: Ensure that the trypsin/Lys-C pellet is at the bottom of the vial before adding 25 mM NH_4HCO_3. Gently tap down the pellet that sticks to the cap if necessary.

   ii. Add 100 μL of 12.5 ng/μL trypsin to dried gel pieces. Incubate on ice for 30 min to allow absorption of the solution into the gel.

Note: To ensure gel pieces are fully submerged, the volume of trypsin solution should be at least three times as that of the dried gel pieces. If necessary, more trypsin solution can be added to completely cover the gel pieces.

   iii. Incubate the samples at 37°C in an incubator for 16–20 h.

g. Extract peptides.
   i. Prepare peptide extraction solution (PXS). Mix 5% formic acid in water with ACN at 1:1 ratio.
   ii. Add 100 μL of PXS (or the volume of the trypsin/Lys-C solution added in Step 6 fii). Vortex and spin briefly. Incubate in a shaker for 10 min at room temperature.
   iii. Collect and transfer the supernatant to a new tube.
   iv. Add 100 μL of PXS to the gel pieces. Vortex and spin briefly. Incubate in a shaker for 10 min at room temperature.
   v. Collect and pool the supernatant to the same tube in step iii.
   vi. Repeat iv and v.
   vii. Dry the pooled supernatant containing tryptic peptides with a vacuum concentrator (typically 2–3 h at 25°C).

Pause point: The dried peptide samples can be stored at −80°C freezer until further use.

Table 1. Nano-LC gradient profile

Time	Flow (nL/min)	Percent of B	Percent of A
0	300	4	96
5	300	4	96
95	300	35	65
100	300	90	10
102.9	300	90	10
103	300	4	96
120	300	4	96

A: 99.9% H_2O, 0.1% formic acid; B: 97.9% ACN, 2% H_2O, 0.1% formic acid.

Liquid chromatography/high resolution mass-spectrometry

Timing: 3 days

In this step, peptides are purified and subjected to LC-MS/MS analysis.

7. Prepare peptides for LC-MS/MS analysis.
   a. Desalt peptides.
      i. Dissolve the dried peptides by adding 10 μL of 0.1% TFA. Vortex and spin briefly.

Note: Check the pH of the peptide solution using a pH paper. We perform this by pipetting a minimal amount of the solution (0.07 μL with a 2.5 μL pipette) into a strip of pH paper. Typically, the pH value is around 2–3.

⚠ CRITICAL: The pH should be less than 4 before the desalting step described below. Otherwise, the binding of peptides to C18 ZipTip column is compromised, and peptides are lost during washing steps.

      ii. Remove salts and contaminants using Millipore C18 ZipTip according to manufacturer's instructions. In this step, the purified peptides are eluted with 10 μL 0.1% TFA in 50% ACN.

Note: We use 10 μL Millipore ZipTip pipette tips with 0.6 μL C18 resin. The binding capacity of the ZipTip is 3 μg peptides. Adjust the amounts of peptides to be desalted if necessary.

   b. Dry the purified peptides to dryness with a vacuum concentrator (typically 30 min at 25°C).

Pause point: At this point, dried desalted peptides can be stored at −80°C until further use.

Note: Alternatively, dry the sample down to 5 μL to remove ACN. The sample is ready for LC-MS/MS analysis (Step 8b).

8. LC-MS/MS.
   This section describes LC-MS/MS procedures for analyzing peptides on an Orbitrap Fusion Lumos Tribrid Mass Spectrometer equipped with an Ultimate 3000 RSLCnano system (Thermo Scientific). Alternatively, mass spectrometers and liquid chromatography systems from other manufacturers can be used for analysis of the samples.
   a. Reconstitute peptide samples.
      i. Add 5 μL of 0.1% TFA to each dried desalted peptide sample. Vortex for 10 s. Spin down briefly with a microcentrifuge.

Table 2. Orbitrap Lumos method summary	
Global settings	
Method duration (min)	120
Ion source type	NSI
Positive Ion (V)	2200
Ion transfer tube temp (°C)	275
Default charge state	2
Master scan	
Detector type	Orbitrap
Resolution	120,000
Scan range (m/z)	350-1700
Max injection time (ms)	50
AGC target	Standard
Data type	Profile
Filters	
Monoisotopic peak detection	Peptide
Intensity threshold	1.0×10^4
Include charge state	2-6
Exclude after n times	1
Exclusion duration (s)	60
Mass tolerance (ppm)	10
ddMS[2]	
Data dependent mode	Cycle time
Time between master scans (s)	2
Isolation mode	Quadrupole
Isolation window (m/z)	1.6
Activation type	CID
Collision energy (%)	30
Detector type	Ion trap
Ion trap scan rate	Rapid
Max injection time (ms)	Dynamic
AGC target	Standard

ii. Transfer the samples into vials compatible with the autosampler of the liquid chromatography system.

Note: We use 2 mL glass vials with a 250 µL polypropylene conical insert with flexible feet and PTFE cap.

iii. Place samples in the autosampler of the liquid chromatography system.

⚠ CRITICAL: To ensure longer shelf life of the peptide samples, it is important to set the temperature of the autosampler at 4°C.

b. Set up sequence for LC-MS/MS runs.
   i. Select LC-MS/MS method, injection volume (e.g., 2 µL), vial position, etc.
   ii. Run sequence.

Note: During LC-MS/MS, peptides are injected onto a C18 peptide trap column and washed with 25 µL of trap column LC solvent at a rate of 5 µL/min for 5 min. The peptides are then eluted from the trap column into an analytical column and separated with 120-min gradients of nano-LC Mobile Phase B at a rate of 300 nL/min. The nano-LC eluent is analyzed by high resolution mass spectrometry in full scan mode and MS/MS in data-dependent acquisition

mode (DDA). A recommended mobile phase gradient and mass spectrometry method are suggested in Tables 1 and 2, respectively.

Protein identification

Timing: 2 days

In this step, proteins are identified by searching of MS/MS data against NCBInr database. Potential interacting proteins of GPR110 are identified by comparing the binding proteins of GPR110 with the M45 control.

9. Identify potential interacting proteins.
   a. Process batches of raw files for GPR110- and M45-transfected samples using Mascot (v2.3.2, Matrix Science).

   Note: Other search engines such as Proteome Discovery represent valid options for the analysis.

      i. Import the raw data files into Mascot Daemon using Mascot Distiller as the data import filter.
      ii. Search for protein identification using Mascot against the NCBInr database.

   Note: Search parameters are set as follows: enzyme, trypsin; precursor ion mass tolerance, 10 ppm; fragment ion mass tolerance, 0.3 Da; maximum missed cleavages allowed 2; carbamidomethyl of cysteine residues for fixed modification; oxidation of methionine and addition of 156. 07864 Da on lysine or N-terminal (end-capping modification) for variable modification. The criteria used to filter results included 1% false positive threshold and expect value of less than 0.05 for significant peptide matches. The expect score was calculated using the homology threshold or the significance threshold as per a standard Mascot protein family report.

   b. Export the search results to Excel files.
   c. Compare proteins identified from GPR110-transfected samples with those from M45 controls.
   d. Identify potential interacting proteins of GPR110.
      i. Exclude non-specific binding proteins that are found in M45 samples.
      ii. Exclude proteins that are not reproducible in three independent experiments.

Label-free protein quantification

Timing: 2 days

In this step, physiologically relevant partners of GPR110 are identified by quantitatively comparing the binding proteins of WT GPR110 with F663S mutant. We describe here a label-free quantitation procedure using Progenesis QI for Proteomics (version 1.05156.29278, Waters). Alternative softwares from other sources such as MaxQuant[5] may also provide valid options for quantitative analysis of proteins.

10. Identify physiologically relevant partners by label-free protein quantitation.
    a. Open Progenesis QI for Proteomics.
    b. Create a "LC-MS Experiment" for a gel fraction (e.g., Fraction A, B or C as in Figure 2).
       i. Name "Experiment" (e.g., Fraction A).
       ii. Select "Profile data" as Data type.

Figure 3. Alignment for accurate label-free protein quantitation

An example is shown for fraction C of 6 individual samples. Progenesis QI for proteomics software aligns six LC-MS/MS data to compensate for between-run variation in the LC separation technique, resulting in increased reliability and precision of peak picking and peptide abundance measurements. An alignment vector is a representation of the difference between the chromatographic retention times of an ion in one run and the equivalent ion in the reference run. The quality of the alignment is indicated by the scores.

iii. Select "High resolution mass spectrometer" as Machine type.
iv. Select Experiment folder.
v. Click on "Create experiment".

c. Import data.
Import raw mass spectrometric files of the fraction (e.g., A1, A2, A3, A4, A5, and A6 in Figure 2).

d. Start automatic processing.
i. Select "Assess all runs in the experiment for suitability" in "Select an alignment reference" box.
ii. Select "yes" in "Automatic alignment" box.
iii. Select "perform peak picking" in "Peak picking" box.
iv. Select "Relative Quantitation using non-conflicting peptides" in "Protein Quantitation" box.

e. Review alignment (Figure 3).

⚠ CRITICAL: Review the alignment result. Alignment makes it possible to accurately compare the peptides in multiple runs by correcting any drift in retention times.

f. Process "Filtering".
   Exclude features with charge of 1 and charge > 7 for the analysis.
g. Set up experiment design.
   i. Create new experiment design (e.g., WT vs. KO).
   ii. Set up conditions (e.g., group A1, A2 and A3 as "WT" and group A4, A5 and A6 as "KO").
h. Identify peptides.
   i. Select "Export as mgf file" under the "Identify peptides" window.
   ii. Search for protein identification using Mascot (v2.3.2, Matrix Science) against the NCBInr human database as described in Step 9aii.
   iii. Export Mascot results to xml files.
   iv. Import the xml result files under the "Identify peptides" window.
i. Save the processed data for the "Experiment" (e.g., Fraction A).
j. Repeat Steps 10a-10i for each fraction (e.g., Fractions B and C).
k. Combine processed data.
   i. Import processed Experiments for each fraction (e.g., Fractions A, B and C).
   ii. Recombine processed Experiments for all fractions from each sample. Combine runs from each fraction from the same sample (e.g., combine A1, B1 and C1 for sample 1 (Figure 2)).

Note: Normalization between fractions is performed during this step.

   iii. Setup experimental design (e.g., WT vs. F663S).
   iv. Export protein quantitation data to Excel.
l. Analyze data.
   i. Obtain the ratio of binding protein over GPR110 for WT GPR110- and F663S-transfected samples, for each or selected potential interacting protein identified from Step 9.
   ii. Calculate p value using Student's test.
m. Identify physiologically relevant partners.
   The proteins that show significant reduction in the F663S sample are deemed as physiologically relevant partners of GPR110.

Validation of functional binding partners

Timing: 3 days

In this section, we describe the validation of occludin, a GPR110 partner identified from the MS-based approach described above (Step 10 m), as an example. Reciprocal immunoprecipitation assay, the gold standard for the validation of protein-protein interactions, is used to confirm the interaction of occludin with GPR110. Notably, not only the interaction with WT GPR110 but also the reduced interaction with the F663S mutant are considered the validation criteria in this antibody-based technique.

11. Validate occludin as GPR110 interacting partner by reciprocal co-immunoprecipitation assay.
    a. Prepare cell lysate.
       i. Culture and transfect HEK293 cells with WT GPR110, F663S mutant, or empty vector M45 as described in Steps 1–2.
       ii. Remove and discard medium after 24 h transfection.
       iii. Add 10 mL PBS. Gently rock the culture dish back and forth. Remove and discard PBS.
       iv. Repeat Step iii.
       v. Add 1.5 mL lysis buffer to the cells. Scrape with a cell lifter. Transfer the cell suspension into an Eppendorf tube.
       vi. Prepare the lysate as described in Steps 4biii-v.
    b. Prepare pre-IP lysate samples.

i. Take 60 μL of each lysate, add 20 μL 4x lithium dodecyl sulfate (LDS) sample buffer containing 5% β-mercaptoethanol. Vortex and spin.
ii. Incubate the samples at 37°C for 30 min.

Note: These samples are to be used for normalization in reciprocal western blot analysis.

c. Perform reciprocal co-immunoprecipitation.
   i. Split 1.3 mL of each lysate into two Eppendorf tubes.
   ii. To each tube, add 20 μL HA antibody or 20 μL occlduin antibody.
   iii. Proceed immunoprecipitation procedure as described in Steps 5b-5d.
d. Elute the co-immunoprecipitated proteins.
   i. Add 40 μL 2x lithium dodecyl sulfate (LDS) sample buffer containing 2.5% β-mercaptoethanol.
   ii. Incubate the samples at 37°C for 30 min.
   iii. Place tubes in the rack of DynaMag-2 magnet for 1 min.
   iv. Collect and transfer the supernatant to a new Eppendorf tube. The samples are ready for western blotting described below.

Pause point: Samples can be stored in −80°C for up to several weeks.

e. Separate proteins using SDS-PAGE.
   We present procedures on an XCell SureLock Mini-Cell system with NuPAGE precast gels (Thermo Scientific). Alternative systems and gels from other manufacturers can achieve similar results.
   i. Assemble the XCell SureLock Mini-Cell system with 10- or 15-well NuPAGE 4%–12% Bis-Tris mini gels and MOPS SDS running buffer.
   ii. Load sequentially 5 μL protein makers, 15 μL of each pre-IP lysate samples (M45, WT, and F663S), 15 μL of each HA-IP samples (M45, WT, and F663S), or 15 μL of each occludin-IP samples (M45, WT, and F663S), and 5 μL protein makers.
   iii. Start electrophoresis with a constant voltage of 200 V. Stop when the dye front is around 1 cm away from the base of the gel.

Note: The electrophoresis time is about 60 min.

f. Transfer proteins to PVDF membrane.
   We describe a procedure on a Trans-Blot Turbo transfer system with Trans-Blot Turbo Mini 0.2 μm PVDF Transfer Pack that includes filter paper, buffer, 0.2 μm PVDF membrane (Bio-Rad). Alternative systems and reagents can achieve similar results.
   i. Carefully remove the precast gel from the XCell SureLock Mini-Cell system.
   ii. Assemble the transfer blot "sandwich" in the cassette of the Trans-Blot Turbo transfer system using a Trans-Blot Turbo Mini 0.2 μm PVDF Transfer Pack.
   iii. Start transferring proteins to PVDF membrane at a constant 25 V for 30 min.
g. Conduct western immunoblotting.
   i. Remove the PVDF membrane from the cassette.
   ii. Block the membrane with TBST containing 5% skim milk with agitation for 1 h at room temperature (25°C).
   iii. Wash the membrane in TBST 3 times with agitation for 5 min each.
   iv. Cut the membrane according to the molecular weight of target proteins. Incubate the membrane slices in 5 mL of respective primary antibody diluted in TBST containing 5% BSA overnight (16–20 h) at 4°C on a low-speed shaker.

Note: We cut the membrane at 75 kDa. The top membrane slice is incubated with HA-antibody to detect GPR110. The bottom membrane slice is incubated with occludin antibody.

v. Remove the primary antibody solution. Wash the membrane in TBST 3 times with agitation for 5 min each.
vi. Incubate the membranes in peroxidase-conjugated secondary antibody diluted in TBST containing 5% milk with agitation for 1 h at room temperature (25°C).
vii. Remove the secondary antibody solution. Wash the membrane in TBST 3 times with agitation for 5 min each.
viii. Incubate the membrane with ECL Substrate reagent for 5 min.
ix. Visualize the protein bands using an imager.

Optional: Step g is an optional step for detecting β-actin, a housekeeping protein as loading control.

h. Reprobe the bottom membrane with β-actin.
   i. Wash the membrane with TBST for 5 min.
   ii. Incubate the membrane in 20 mL of stripping buffer (Restore western blot stripping buffer, Thermo Scientific) with agitation for 15 min at room temperature (25°C).
   iii. Wash the membrane in TBST 3 times with agitation for 5 min each.
   iv. Block the membrane with TBST containing 5% skim milk with agitation for 30 min at room temperature (25°C).
   v. Wash the membrane in TBST 3 times with agitation for 5 min each.
   vi. Incubate the membrane in 5 mL of β-actin antibody diluted in TBST containing 5% BSA overnight (16–20 h) at 4°C on a low-speed shaker.
   vii. Proceed to Steps fv-ix.
i. Data analysis.
   i. Confirm occludin is co-immunoprecipitated with WT GPR110, but not with the M45 control.
   ii. Confirm WT GPR110 is co-immunoprecipitated with occludin, but not with the M45.
   iii. Quantify band intensity using appropriate software.
   iv. Calculate the ratio of co-immunoprecipitated occludin over the bait GPR110. Compare the ratio of WT GPR110 transfected-sample with that of the F663S-transfected sample.
   v. Calculate the ratio of co-immunoprecipitated GPR110 over the bait occludin. Compare the ratio of WT GPR110 transfected-sample with that of the F663S-transfected sample.

Note: The ratio of the prey protein over the bait protein is expected to decrease for the F663S-transfected sample compared to the WT sample. The data from pre-IP lysate is used for normalization of the bait protein.

EXPECTED OUTCOMES

This protocol aims to identify physiologically relevant interacting partners of GPCRs using in-cell chemical crosslinking, co-immunoprecipitation and mass spectrometry. The bait GPCR, in our case, the overexpressed GPR110, is expected to be the top-ranking protein in the database search results (Figure 4). The molecular weight of an interacting protein crosslinked with GPR110 (MW = 130 kDa) is expected to match the molecular weight range of the gel band in which it is identified (Figure 4), enabling the exclusion of non-relevant proteins. After further excluding the non-specific proteins that are found in the empty vector-transfected control and those not reproducible in biological triplicate, a couple of hundred proteins are expected to be identified as potential interacting proteins. Many of these proteins can be selected readily for further validation based on the interaction captured by DSS in living cells. However, the physiologically relevant partners are expected to be narrowed down markedly using mutants that do not properly express on the cell surface. In our experiment with WT GPR110- or F663S- transfected HEK cells, we identified less than 20 membrane proteins out of 200 candidate partners, interacting with GPR110 WT but insignificantly with F663S mutant as shown for occludin (Figure 5). These proteins are expected to be true binding partners

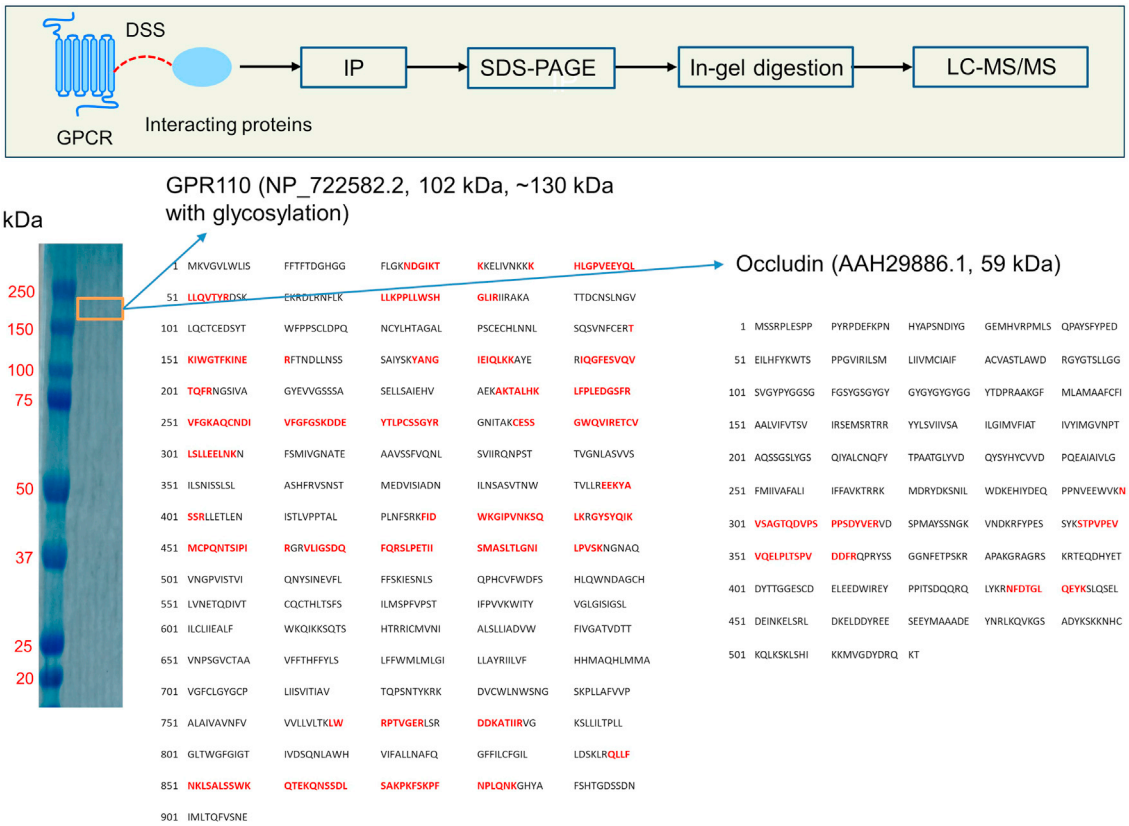

Figure 4. Identifying interacting proteins of a GRCR by chemical crosslinking, immunoprecipitation and mass spectrometry

HEK cells expressing GPR110-HA or empty vector control (M45) are subjected to DSS crosslinking in living cells followed by pull-down with HA antibody. The immunoprecipitates are separated by SDS-PAGE, digested with trypsin, and analyzed by LC-MS/MS. Occludin (59 kDa), along with GPR110 (130 kDa), is identified in the gel band (~160–230 kDa) from the GPR110-HA transfected cells but not the control cells. GPR110 is the top-ranking protein in the database search results with 30% sequence identified. Unique peptides identified by MS/MS are shown in red. Both proteins are not detected in the M45 transfected cells. Adapted from Huang et al.[1]

that interact with the receptor at the cell surface. The validation of these proteins can be readily achieved by reciprocal immunoprecipitation assay (Figure 5) or/and further functional analysis. The identified binding partners of GPCRs should provide new insights into their roles in physiological or pathological processes.[1]

QUANTIFICATION AND STATISTICAL ANALYSIS

Significance was determined by Student's t-test using Excel. P values < 0.05 were considered significant. Data are presented as mean ± SEM (standard error of the mean) of three independent experiments.

LIMITATIONS

The experimental outcome of this protocol relies on the inclusion of a negative control, which involves utilizing a mutant that impairs the expression of the GPCR of interest on the cell surface. However, it should be acknowledged that such mutants may not be readily available for certain GPCRs, and identifying or designing them may require significant efforts.

Moreover, it is worth noting that overexpressing a GPCR can lead to its binding with proteins that it does not naturally interact with, potentially leading to false-positive results. Therefore, optimizing the receptor expression is crucial to ensure that it exhibits endogenous receptor-like bioactivity and maintains similar protein-protein interactions.

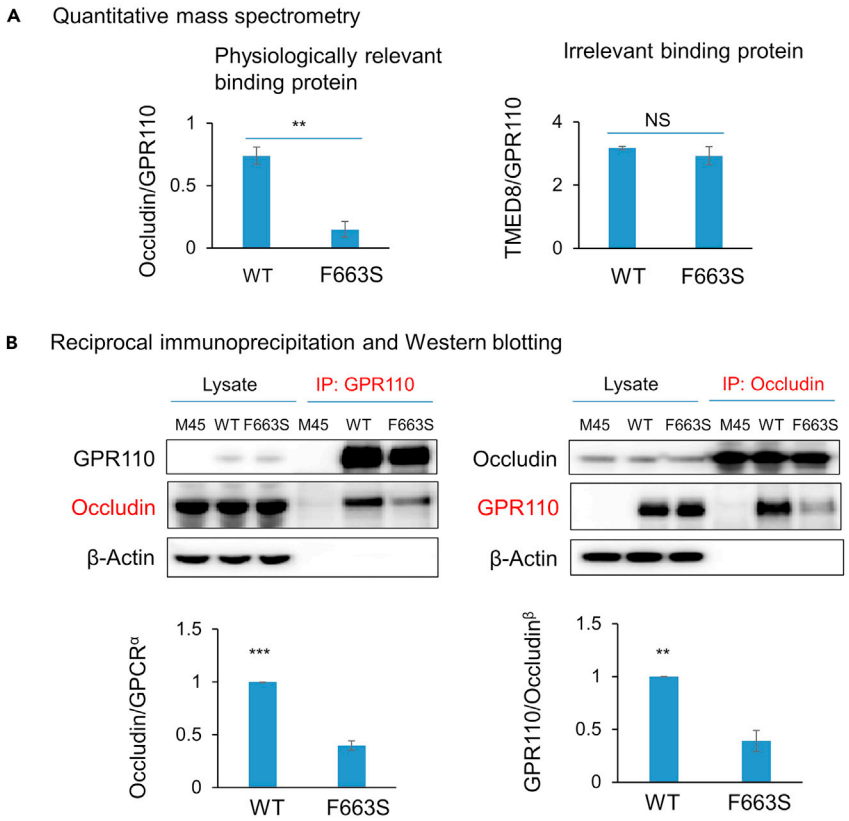

Figure 5. Representative data for identifying and validating functional interacting partners of GPR110

GPR110 F663S mutant, which is minimally expressed on the cell surface, is used to facilitate the identification of physiologically relevant binding proteins.

(A) Quantitative mass spectrometry distinguishing functional partners from non-irrelevant binding proteins.

(B) Reciprocal immunoprecipitation assay for validating the identified partner. Cells expressing F663S or WT GPR110 or M45 (empty vector) were subjected to immunoprecipitation with anti-HA antibody or anti-occludin antibody. The Co-IP partners were analyzed by western blotting. α, normalized to occludin in the lysate. β, normalized to GPR110-HA in the lysate. Statistical analysis was performed using unpaired Student's t-test (**$p < 0.01$, ***$p < 0.001$). Data are means ± SEM of biological replicates (n = 3). NS, not significant. Modified from Huang et al.[1]

In this protocol, DSS, a well-established crosslinker,[6] is used to capture interacting proteins, including weak and transient partners. Although identifying the specific sites where DSS crosslinking occurs may provide valuable insights into protein-protein interactions, it remains challenging. To improve the identification of crosslinked residues, other crosslinkers such as enrichable or MS-cleavable crosslinkers[7–9] may be integrated into the protocol. Alternatively, proximity labeling techniques may also offer a valid approach for capturing low-affinity and transient protein-protein interactions in living cells.[10,11]

TROUBLESHOOTING

Problem 1

Keratin is the most abundant protein identified in samples, much more abundant than the overexpressed GPCR (related to Step 9).

Potential solution

Keratin is produced naturally in human body. Keratin can be easily introduced to the samples from skin hair, nails, etc, particularly during the in-gel digestion procedure. Wearing a hair cover (or at least tie your hair), gloves, sleeves, and a mask, cleaning all tools and bench area used for gel cutting,

and not talking over the samples, can help minimize the keratin contamination (see comments in the CRITICAL section following Step 6c).

Problem 2

The expressed receptor is not one of the most abundant proteins (related to Step 9).

Potential solution

Special attention should be given to cell collection after in-cell crosslinking. Floating cells can be observed after crosslinking. This doesn't affect the experimental outcome because protein-protein interactions have been already captured by the crosslinker. Do not try to remove PBS by aspiration after crosslinking which will result in cell loss. Instead, scrape all cells and transfer the cell suspension to a conical tube prior to cell pelleting (see comments in the CRITICAL section following Step 4a).

It is also possible that the expression of the receptor is weak in your cell line. We use a 15-cm dish of GPR110-transfected cells for each IP. However, you can try starting with two dishes of the cells for each IP.

Problem 3

There are too few potential interacting proteins identified (related to step 9).

Potential solution

It is possible that peptides are lost during extraction and desalting steps. Make sure to confirm that the pH of the peptide solution is less than 4. Otherwise, it will affect the peptide binding to the C18 resin (see comments in the CRITICAL section following Step 7ai) and therefore the recovery of the peptides.

It is also possible that trypsin/Lys-C solution is not prepared correctly. Ensure that the trypsin/Lys-C is at the bottom of the vial by gentle tapping (see comments in the CRITICAL section following Step 6fi).

Problem 4

The alignment score is low for certain raw files (related to Step 10).

Potential solution

The alignment scores are expected to be >80% for all six samples (e.g., three WT GPR110, three F663S) from each fraction (Figure 3). If one or two files do not align well with others, check the raw MS data. Special attention should be given to the retention time and signal level. Rerun the low-score sample(s) if necessary. We recommend adding a QC sample (e.g., 1 fmol of BSA digests) between several LC-MS/MS runs, which often help identify any LC-MS/MS related issues before re-running samples.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hee-Yong Kim (hyk@nih.gov).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The study did not generate any new datasets or code.

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AUTHOR CONTRIBUTIONS

B.X.H. and H.-Y.K. designed the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Huang, B.X., Chen, H., Joo, Y., Kwon, H.S., Fu, C., Spector, A.A., and Kim, H.Y. (2023). Interaction between GPR110 (ADGRF1) and tight junction protein occludin implicated in blood-brain barrier permeability. iScience *26*, 106550. https://doi.org/10.1016/j.isci.2023.106550.

2. Abdulkareem, N.M., Bhat, R., Qin, L., Vasaikar, S., Gopinathan, A., Mitchell, T., Shea, M.J., Nanda, S., Thangavel, H., Zhang, B., et al. (2021). A novel role of ADGRF1 (GPR110) in promoting cellular quiescence and chemoresistance in human epidermal growth factor receptor 2-positive breast cancer. FASEB J *35*, e21719. https://doi.org/10.1096/fj.202100070R.

3. Lee, J.W., Huang, B.X., Kwon, H., Rashid, M.A., Kharebava, G., Desai, A., Patnaik, S., Marugan, J., and Kim, H.Y. (2016). Orphan GPR110 (ADGRF1) targeted by N-docosahexaenoylethanolamine in development of neurons and cognitive function. Nat. Commun. *7*, 13123. https://doi.org/10.1038/ncomms13123.

4. Huang, B.X., Hu, X., Kwon, H.S., Fu, C., Lee, J.W., Southall, N., Marugan, J., and Kim, H.Y. (2020). Synaptamide activates the adhesion GPCR GPR110 (ADGRF1) through GAIN domain binding. Commun. Biol. *3*, 109. https://doi.org/10.1038/s42003-020-0831-6.

5. Tyanova, S., Temu, T., and Cox, J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat. Protoc. *11*, 2301–2319. https://doi.org/10.1038/nprot.2016.136.

6. Piersimoni, L., Kastritis, P.L., Arlt, C., and Sinz, A. (2022). Cross-Linking Mass Spectrometry for Investigating Protein Conformations and Protein-Protein Interactions-A Method for All Seasons. Chem. Rev. *122*, 7500–7531. https://doi.org/10.1021/acs.chemrev.1c00786.

7. Kaake, R.M., Wang, X., Burke, A., Yu, C., Kandur, W., Yang, Y., Novtisky, E.J., Second, T., Duan, J., Kao, A., et al. (2014). A new in vivo cross-linking mass spectrometry platform to define protein-protein interactions in living cells. Mol. Cell. Proteomics *13*, 3533–3543. https://doi.org/10.1074/mcp.M114.042630.

8. Yu, C., and Huang, L. (2018). Cross-Linking Mass Spectrometry: An Emerging Technology for Interactomics and Structural Biology. Anal. Chem. *90*, 144–165. https://doi.org/10.1021/acs.analchem.7b04431.

9. Zhang, H., Tang, X., Munske, G.R., Tolic, N., Anderson, G.A., and Bruce, J.E. (2009). Identification of protein-protein interactions and topologies in living cells with chemical cross-linking and mass spectrometry. Mol. Cell. Proteomics *8*, 409–420. https://doi.org/10.1074/mcp.M800232-MCP200.

10. Kim, D.I., and Roux, K.J. (2016). Filling the Void: Proximity-Based Labeling of Proteins in Living Cells. Trends Cell Biol. *26*, 804–817. https://doi.org/10.1016/j.tcb.2016.09.004.

11. Lobingier, B.T., Hüttenhain, R., Eichel, K., Miller, K.B., Ting, A.Y., von Zastrow, M., and Krogan, N.J. (2017). An Approach to Spatiotemporally Resolve Protein Interaction Networks in Living Cells. Cell *169*, 350–360.e12. https://doi.org/10.1016/j.cell.2017.03.022.