# Chapter 21

# Detection and Characterization of Receptor Interactions with PDZ Domains

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## Abstract

Many transmembrane receptors are regulated by associations with scaffold proteins containing PDZ domains, which interact with receptor carboxyl-termini to control receptor signaling, trafficking, and localization. We describe here approaches for detecting and characterizing interactions between receptors and PDZ scaffolds. These approaches include the construction and screening of proteomic arrays, blot overlays using fusion proteins, and co-immunoprecipitation studies to assess interactions in cells.

**Key words:** G protein-coupled receptor, PDZ domain, Scaffold, Affinity, Growth factor, Tyrosine kinase

## 1. Introduction

Cell-surface receptors mediate intercellular signaling and are common therapeutic targets for drug development (1). Receptor function is often regulated by receptor-interacting proteins, which can profoundly influence receptor signaling, trafficking, and/or pharmacology (2). One of the most well-studied classes of receptor-interacting partners is the family of PDZ domain-containing scaffold proteins. PDZ domains are specialized protein–protein interaction modules, which derive their name from the first three proteins in which they were identified: the postsynaptic density protein of 95 kDa (*P*SD95), the *Drosophila* protein disc large tumor suppressor A (*D*lgA), and the tight junction protein zona-occludens 1(*z*o-1). PDZ domains are approximately 90 amino acids in length and typically recognize target motifs at the extreme C terminus of interacting proteins (3), although some PDZ

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proteins can bind to an internal PDZ ligand not found at the extreme C terminus (4). Most PDZ-interacting proteins possess a C-terminal motif consisting of a hydrophobic amino acid at the terminal position and either a hydrophobic amino acid, a hydroxyl-bearing amino acid (S or T), or an acidic amino acid (D or E) at the –2 position (5).

PDZ domain-mediated interactions are often of very high affinity and therefore amenable to detection by a number of different screening approaches. For example, many receptor/PDZ interactions have been first detected in yeast two-hybrid screens (6). Other screening approaches include phage display (7), fluorescence polarization (8), and pull-down studies from tissue samples followed by mass spectrometry (9). When novel PDZ interactions are detected via these types of unbiased screening approaches, it is natural to wonder about the specificity of the association: Was a particular PDZ scaffold detected as an interacting partner for a receptor of interest simply because the PDZ protein was very abundant in the yeast two-hybrid library or tissue sample that was chosen for the initial screen? Might there be other PDZ proteins that actually have much higher affinities for interacting with the receptor of interest? To address such questions, over the past few years a number of groups have developed screening approaches involving the creation of proteomic arrays of PDZ domains, which can be screened in a rapid and comprehensive manner for their binding to any target C terminus of interest. Commercially available PDZ domain arrays have been developed by Panomics, and several academic laboratories have also developed their own PDZ domain arrays (5, 10, 11). Here we describe a standard approach for screening a PDZ domain array with a receptor C terminus of interest to detect novel interactions, then confirming these interactions via reverse overlay and co-immunoprecipitation.

## 2. Materials

### *2.1. Screening of a PDZ Proteomic Array*

1. Purified S- and hexahistidine-tagged fusion proteins of candidate PDZ domains (see Note 1).
2. Purified receptor C-terminal (CT) GST fusion proteins (see Note 2).
3. 96-well plates (plastic).
4. Parafilm.
5. Multiblot replicator – "spotter" (V&P Scientific, Inc.).
6. Absolute (200 proof) Ethanol.
7. Aluminum foil.

8. Nytran SuperCharge 96-grid nylon membranes, 0.45 μM (Whatman).
9. Blot Buffer: 2% (w/v) nonfat powdered milk, 0.1% (v/v) Tween-20, 50 mM NaCl, 10 mM Hepes, in $dH_2O$, pH 7.4.
10. Blot trays.
11. Rocking platform.
12. Anti-GST HRP Conjugate (GE Healthcare Life Sciences).
13. Enhanced chemiluminescence (ECL) kit (ThermoScientific).
14. Autoradiography cassette.
15. X-ray film for ECL detection.

### 2.2. Reverse Blot Overlays

1. SDS-PAGE mini-gel apparatus (Invitrogen).
2. Western blot transfer apparatus (Invitrogen).
3. Power supply (BioRad).
4. SDS-PAGE 4–20% tris-glycine mini gels (Invitrogen).
5. SDS-PAGE buffer: 30.5 g Tris, 144.8 g glycine, 10 g SDS, diluted up to 10 L of $dH_2O$.
6. Transfer buffer: 5.8 g Tris, 28.8 g glycine, 800 ml methanol, diluted up to 4 L of $dH_2O$.
7. 6× Sample Buffer: 12% (v/v) β-mercaptoethanol, 12% (w/v) SDS, 30% (v/v) glycerol, 100 mM TRIS, 5 mg of bromophenol blue, $dH_2O$ up to 30 ml. Six times stock may be diluted with $dH_2O$ to generate 2× and 1× stock as needed. Store out of light.
8. Anti-S-protein HRP Conjugate (Novagen).
9. Anti-Hexahistidine HRP Conjugate (Miltenyi Biotec Inc).
10. ImageJ or similar image analysis software.

### 2.3. Confirmation of Receptor/PDZ Interaction by Co-precipitation

1. HEK-293T cells (American Type Culture Collection).
2. Complete DMEM: 10% qualified fetal bovine serum (Invitrogen), 1% penicillin and streptomycin (Invitrogen), DMEM (1×) high glucose (Invitrogen).
3. 100 mm tissue culture dishes.
4. Lipofectamine™ 2000 (Invitrogen).
5. cDNAs expression plasmids encoding the full-length receptor and candidate PDZ interactor (FLAG-tagged receptor and HA-tagged PDZ scaffold).
6. PBS/$Ca^{2+}$: phosphate-buffered saline (Invitrogen) supplemented with 0.9 mM calcium chloride.
7. Harvest Buffer: 50 mM NaCl, 20 mM Hepes, 5 mM EDTA, 1 protease inhibitor cocktail tablet (Roche Applied Science), 1% (v/v) Triton-X-100, diluted with $dH_2O$ up to 50 ml, pH 7.4.

8. 1.5 mL capped microcentrifuge tubes.
9. High-speed microcentrifuge in a cold room (4°C).
10. M2 anti-FLAG agarose beads (Sigma).
11. Table-top microcentrifuge.
12. Heat block.
13. Anti-HA (12CA5) mouse monoclonal antibody (Roche Diagnostics).
14. Anti-mouse IgG HRP Conjugate (GE Healthcare Life Sciences).

## 3. Methods

### 3.1. Preparation and Screening of the PDZ Proteomic Array

To construct the proteomic array, recombinant PDZ domains are spotted onto a nitrocellulose grid and, similar to a far Western blot, purified receptor CT fusion proteins are subsequently overlaid onto the membranes. For the generation of the PDZ domains mentioned in the below protocol, the bacterial expression vector pET30A was used to yield purified recombinant PDZ domains containing an S-tag and a hexahistidine tag. Screening multiple PDZ domain candidates simultaneously can increase the odds of detecting a candidate interaction. If using a commercially available PDZ domain array, skip to step 10.

1. Pipette 50 µl of each purified S-tagged PDZ protein (1 µg/µl) into its respective well of a 96-well plate. Keep the 96-well plate on ice while distributing PDZ proteins, in order to minimize protein degradation.
2. Cover the 96-well plate with parafilm and store at –80°C until required for experimental use. Because multiple freeze/thaw cycles can enhance the degradation or precipitation of the purified PDZ domains, limit the number of freeze–thaws.
3. To construct the proteomic array, remove the 96-well plate from the –80°C freezer and thaw on ice. It is important that all proteins are completely thawed before spotting.
4. Soak the spotter in fresh absolute ethanol.
5. Spread aluminum foil onto the bench-top and place the unused 96-grid nylon membranes on top of the foil, keeping the blue backing as a separation between the membranes and the foil. Remove the top blue covers to expose the membranes. Set the blue covers aside. It is helpful to construct multiple arrays at one time, as they may be stored for up to 1 year at 4°C.

6. Use a multichannel pipetter to thoroughly mix the thawed PDZ proteins. If any wells are lacking in volume, replenish the well(s) with purified PDZ protein (1 μg/μl).
7. Remove the spotter from the ethanol, shake off excess, and allow it to dry completely before use.
8. Dip the spotter into the 96-well plate and swish gently. The fusion proteins will be drawn up into the spotter. Then, lay the spotter onto one of the nitrocellulose membranes, beginning on one side and rolling to the other side to equally distribute the PDZ proteins. If some of the proteins do not transfer, manually pipette 1 μl of the PDZ domain stock (1 μg/μl) onto the appropriate grid block (Fig. 1).
9. Allow membranes to dry for at least 30 min at room temperature (RT), or overnight, until the spots are no longer visible.

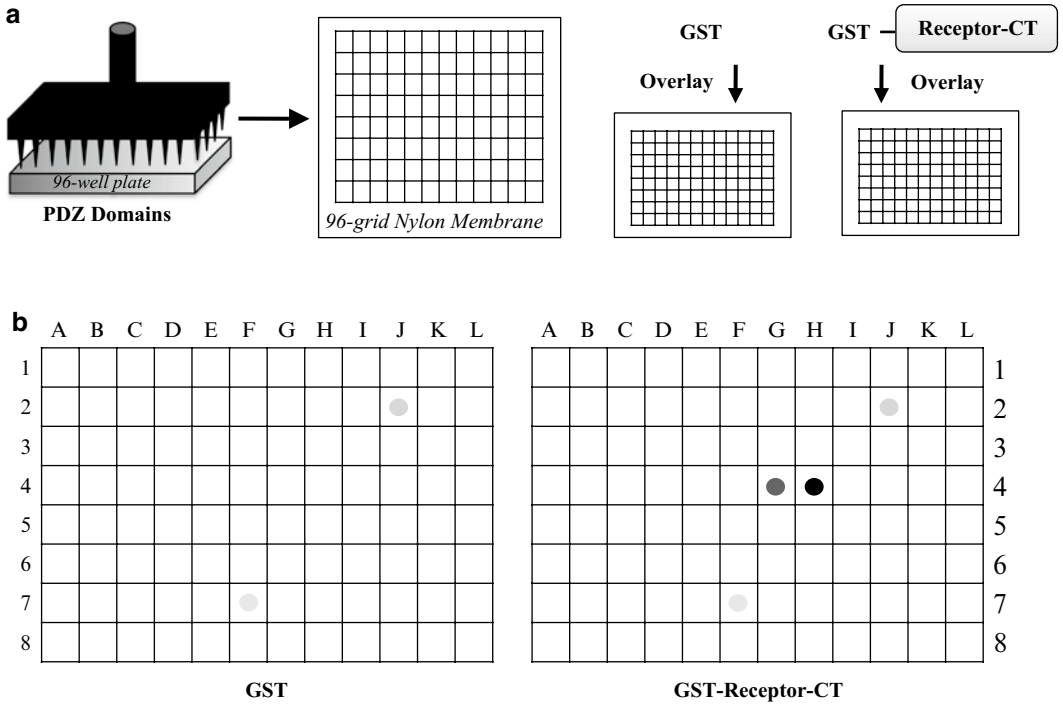


Fig. 1. Screening of the PDZ proteomic array. (**a**) A schematic is shown depicting the construction and screening of the PDZ proteomic array. A multireplicator "spotter" is used to equally distribute the PDZ domain fusion proteins onto gridded nitrocellulose. After drying overnight, purified recombinant GST alone or GST–receptor–CT fusion proteins are overlaid onto the membranes and incubated to detect PDZ interactions. (**b**) Representative data for screening GST alone (*left*) and GST–receptor–CT fusion proteins (*right*) are shown. On the GST alone blot, bins J2 and F7 depict the nonspecific binding of the GST fusion protein to the array. However, when 100 nM of GST–receptor–CT is overlaid onto the membrane, two additional positive hits are seen in bins G4 and H4, indicating that the corresponding PDZ domains most likely interact with the receptor–CT. Conversely, the intensity of the signal in bin J2 remains the same, which is representative of a "false positive" on the array. Therefore, only the PDZ proteins identified in bins G4 and H4 should be pursued further and validated using a reverse overlay and co-immunoprecipitation approaches.

Replace the blue membrane top cover and stack arrays together. Return arrays to the packaging envelope and store at 4°C until needed for use. Return the PDZ proteins in the 96-well plate for storage at −80°C.

10. To screen the array, remove membranes from the refrigerator and block them in blot buffer for 1 h at RT on a gently shaking platform.
11. Overlay 100 nM of the purified GST–receptor–CT fusion protein and 100 nM of the purified GST protein alone, diluted in 10 ml of blot buffer, onto duplicate blocked PDZ domain membranes (see Note 3).
12. Incubate membranes and GST fusion proteins for 1 h, at RT, or overnight at 4°C.
13. Wash membranes five times using 10 ml of blot buffer per 5-min wash.
14. Incubate membranes with an HRP-conjugated anti-GST monoclonal antibody (1:4,000) for 1 h at RT, with gentle shaking (see Note 4).
15. Wash membranes five times using 10 ml of blot buffer per 5-min wash.
16. Use an ECL kit to visualize the HRP.
    (a) Incubate membranes with freshly prepared ECL solution for 1–5 min.
    (b) Drain excess ECL solution and gently pat membranes dry.
    (c) Transfer membranes to a clear plastic sheet protector and tape into an autoradiography cassette.
    (d) Expose film for various time points and develop using a film developer.
    (e) Compare GST alone and GST–receptor–CT fusion proteins to determine specific binding (Fig. 1).

### 3.2. Reverse Blot Overlays and Receptor/PDZ Affinity Calculations

After screening for candidate receptor/PDZ interactions using a PDZ domain proteomic array or other screening technique, a common next step is to confirm any putative interactions and estimate their affinity. Candidate interactions should be confirmed using a different technique than was used for screening. For example, an overlay assay should be done in reverse, or a pull-down assay (9) should be performed instead. The following protocol will describe a reverse overlay approach for confirming a novel receptor/PDZ interaction.

1. Load 2 μg of purified receptor GST fusion proteins into individual wells of a SDS-PAGE gel and subject purified proteins to gel electrophoresis at 120 V for approximately 100 min.

2. Transfer separated GST fusion proteins to nitrocellulose paper using 25 V for 120 min.
3. Cut blot into vertical strips to separate groups of GST alone (negative control) and GST–receptor–CT.
4. Overlay increasing concentrations of candidate PDZ domain (S- and hexahistidine-tagged) in order to assess specificity of binding (i.e., 1, 3, 10, 30, 100, 300, 1,000, 3,000 nM).
5. Wash blots three times using 10 ml of blot buffer per 5-min wash.
6. Incubate blots with HRP-conjugated anti-S-protein antibody diluted in 10 ml of blot buffer (1:4,000) or HRP-conjugated anti-hexahistidine protein antibody diluted in 10 ml of blot buffer (1:4,000) for 1 h at RT, with gentle shaking.
7. Wash membranes three times using 10 ml of blot buffer per 5-min wash.
8. Use ECL kit to visualize HRP (see Subheading 3.1; step 16).
9. Scan blots into ImageJ (freeware from NIH.gov) or another suitable program, and calculate the optical densitometry (OD value) of each immunoreactive band.

In order to generate a binding curve, OD values must be converted into percentages of maximal binding and plotted on a graph. "Maximal binding" is defined when the amount of PDZ domain binding (OD value) does not change between two increasing concentrations (OD values) of the amount of PDZ domain overlaid. As shown in Fig. 2, the curve may then be used to estimate the affinity constant ($K_D$) of the interaction (see Note 5).

### 3.3. Confirmation of Receptor/PDZ Interaction in a Cellular Context

The first two sections describe approaches for screening a receptor C terminus for potential interactions with a large number of candidate PDZ domains and then confirming and estimating the affinities of any detected interactions. An important next step is to assess whether a given interaction actually occurs when the full-length PDZ scaffold and full-length receptor are expressed in a cellular context. This can be done using BRET or FRET approaches (12), or, as described below, by co-immunoprecipitation.

1. Maintain HEK-293T cells in Complete DMEM in a humidified incubator at 37°C, 5% $CO_2$/95% air mixture. For transfection and immunoprecipitation experiments, culture HEK-293T cells on 100 mm tissue culture-treated sterile plates.
2. Use Lipofectamine™ 2000 to transfect HEK-293T cells with 1 μg each of a FLAG-tagged receptor cDNA and an HA-tagged PDZ scaffold cDNA. It is also necessary to have a condition in which just the HA-tagged PDZ scaffold is expressed, as well as 1 μg of pcDNA3.1 (mock cDNA) to control

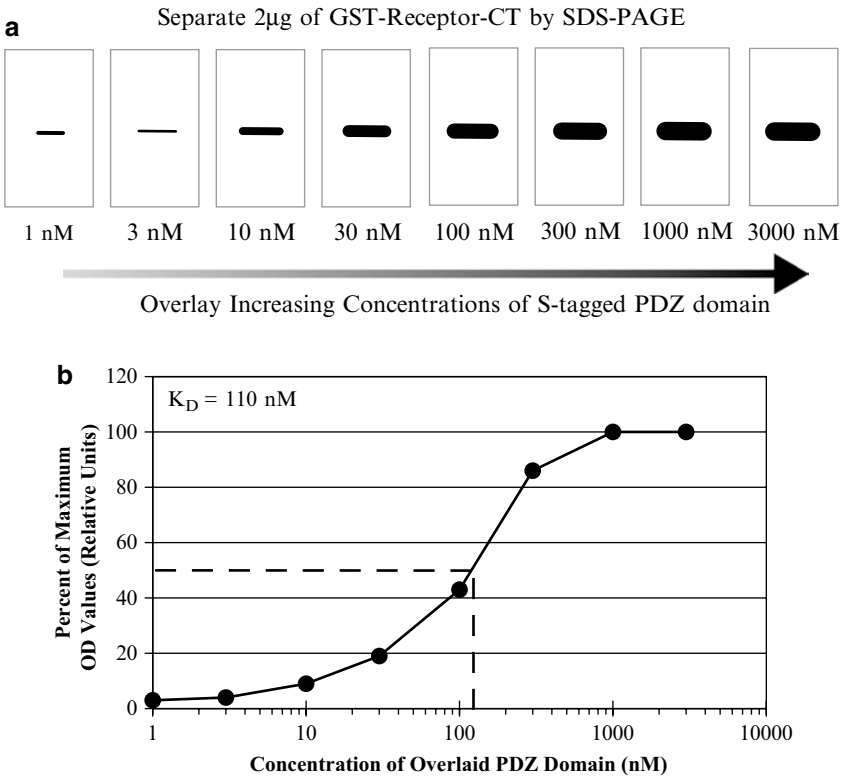


Fig. 2. Reverse blot overlays and receptor–PDZ affinity estimations. (**a**) Representative data shown for reverse blot overlay experiments. Briefly, 2 µg of GST–receptor–CT are separated by SDS-PAGE, transferred to nitrocellulose and then cut into strips. Membranes are then overlaid with increasing concentrations of an S-tagged PDZ domain that was identified as a positive hit in the original screen of the PDZ array. Importantly, no binding is seen for overlay of GST alone (*data not shown*). (**b**) After converting the immunoreactive bands in the overlay experiments into OD values, the maximum OD value can be identified as that value does not change between two increasing concentrations of S-tagged PDZ domain overlay. The remaining OD values are converted into a percentage of the maximal OD value and plotted onto a dose–response graph, in which the concentration of the PDZ domain is on a logarithmic scale. The curve can then be used to estimate the $K_D$ of the receptor/PDZ domain interaction, or the concentration of PDZ domain required for 50% of maximum binding (*dashed line*).

for any HA-tagged PDZ scaffold that may be nonspecifically pulled down by the FLAG beads used in the immunoprecipitation (see Note 6).

3. After 24–48 h, transfer cells to ice in order to slow protein degradation, aspirate old media, and wash cells two times with 5 ml of ice-cold PBS/$Ca^{2+}$ per 5-min wash.
4. Add 500 µl of ice-cold Harvest Buffer to cells and scrape cells into an Eppendorf tube.
5. Solubilize proteins for 30 min at 4°C, with end-over-end rotation.
6. Microcentrifuge the samples for 20 min at 18,000 *g* to separate insoluble membrane fraction (pellet) from the soluble lysate (supernatant).

7. Remove 50 µl of the soluble lysate to check the efficiency of the transfection and receptor solubilization. Add 10 µl 6× sample buffer to achieve a 1× sample buffer final concentration, denature proteins, and facilitate sample storage.
8. Incubate the remaining soluble lysate with 30 µl of M2 FLAG agarose beads for 2 h at 4°C, with end-over-end rotation.
9. Spin down beads using a table-top centrifuge (20 s) and carefully aspirate the supernatant. Wash beads three times with 1 ml of ice-cold lysis buffer, spinning down beads in-between washes.
10. Resuspend beads in 60 µl of 2× Sample Buffer.
11. Boil samples at 100°C for 10 min to elute proteins from beads.
12. Load 20 µl of each IP supernatant into an SDS-PAGE gel and separate proteins by gel electrophoresis at 120 V for 100 min. Load two separate gels in order to probe with an antibody for the receptor, as well as an antibody for the PDZ domain scaffold.
13. Transfer proteins to nitrocellulose at 25 V for 120 min.
14. Perform a Western blot.
    (a) Block membranes with 10 ml blot buffer for 30 min at RT.
    (b) Probe one membrane with an antibody directed against the receptor to confirm that the FLAG-tagged receptor was immunoprecipitated by the FLAG beads. Use the anti-HA 12CA5 antibody (1:4,000) to probe the other blot for the HA-tagged PDZ scaffold. Dilute primary antibodies in 10 ml of blot buffer and incubate membranes for 1 h at RT, with gentle shaking.
    (c) Wash membranes three times using 10 ml of blot buffer per 5-min wash.
    (d) Incubate membranes with the appropriate secondary HRP-conjugated antibody, directed against the host species of the primary antibody. Use the anti-mouse HRP-conjugated secondary antibody (1:4,000) to detect if the HA-tagged PDZ scaffold was co-immunoprecipitated.
    (e) Wash membranes three times using 10 ml of blot buffer per 5-min wash.
    (f) Use an ECL kit to visualize the HRP (see Subheading 3.1; step 16).

An example of a successful immunoprecipitation between a receptor and a PDZ domain-containing scaffold using a heterologous expression system is shown in Fig. 3 (see Note 7).

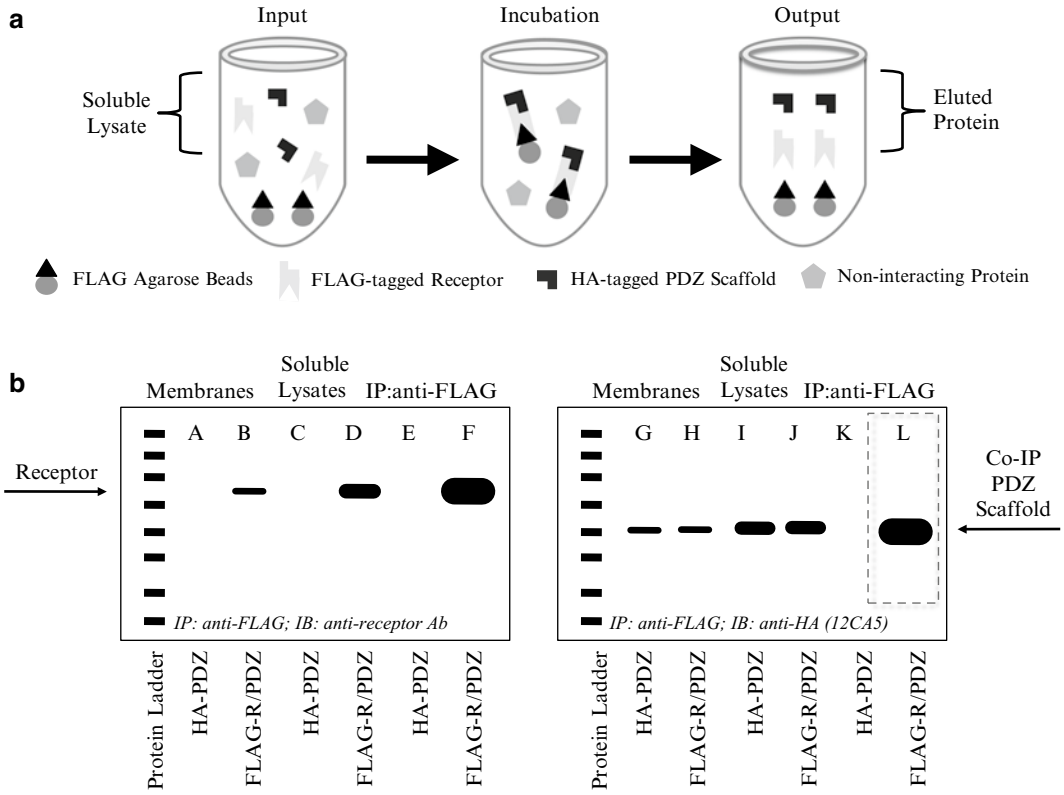


Fig. 3. Immunoprecipitation experiments to validate receptor/PDZ interactions in a cellular context. (**a**) Schematic diagram illustrating the immunoprecipitation (IP) of a FLAG-tagged receptor using FLAG agarose beads. Solublized cell lysates containing FLAG-tagged receptor and HA-tagged PDZ scaffold are incubated with FLAG-conjugated agarose beads. The FLAG antibody (*triangle*) that is covalently attached to the beads (*circle*) immunoprecipitates the FLAG-tagged receptor (*gray shape*), while the noninteracting protein (*pentagon*) does not associate. The HA-tagged PDZ scaffold (*L-shape*) also binds to the FLAG-tagged receptor and is co-immunoprecipitated by the beads. After the incubation, the beads are incubated with 2× Sample Buffer and the receptor and the PDZ scaffolds are eluted. (**b**) Representative data showing a successful immunoprecipitation (IP) of a FLAG-tagged receptor and the specific co-immunoprecipitation (co-IP) of a HA-tagged PDZ scaffold. Samples from the membrane, soluble lysate, anti-FLAG IP fractions are run on an SDS-PAGE gel, transferred to nitrocellulose, and blotted with an antibody corresponding to the receptor (*left blot*) and the HA-tag on the PDZ scaffold (*right blot*). An efficient solubilization of the receptor from the membrane is shown (*lane D* vs. *lane B*), and incubation of this soluble lysate with FLAG beads results in a robust immunoprecipitation of the FLAG-tagged receptor (*lane F*). Likewise, the HA-PDZ scaffold is solubilized efficiently (*right blot, lanes I and J* vs. *G and H, respectively*) and a band corresponding to the predicted molecular weight of the HA-PDZ scaffold is only seen in the lane in which the receptor was immunoprecipitated (*right blot, lane L*). As a negative control, the FLAG beads do not pull-down the HA-PDZ scaffold when the receptor is not co-transfected (*right blot, lane K*).

## 4. Notes

1. We typically prepare PDZ domain fusion proteins using the pET30A bacterial expression vector, which creates proteins containing a hexahistidine tag and an S-tag, separated by a thrombin cleavage site. The modular nature of the PDZ

domain makes it quite amenable to recombinant protein expression. The domain itself contains six anti-parallel β sheets sandwiched between two α helices, that together form a hydrophobic pocket in which the last amino acid of the PDZ consensus sequence can interact (3). PDZ domain fusion proteins should be generated using this entire domain.

2. Target protein C-termini are typically prepared as GST fusion proteins. The length of the CT fragment is important. It should be no less than 25 amino acids in length, in order to allow proper spacing between the PDZ-interacting motif and the GST moiety. Also, there must not be any sort of tag on the C-terminal end of the CT fragment. The GST moiety must be on the N-terminal side and the CT fragment must have a free C terminus. Please see Vikis and Guan for a detailed protocol describing the generation of GST fusion proteins (13).
3. When purifying GST proteins used in screening the proteomic array, it is important to prepare a GST alone control, alongside of the GST–receptor–CT, in order to assess non-specific background in the overlay. This will help to determine which hits, if any, are "false positives" in the screening of the PDZ proteomic array.
4. For initial experiments involving antibodies, it can be helpful to perform a pilot experiment to titrate the amount of the antibody required for optimal detection of the bands of interest. Concentrations of commercially available antibodies can vary across lots; therefore, a range of different dilutions can be employed in pilot studies to determine an optimal dilution.
5. The estimated affinities of PDZ domain-mediated interactions can vary greatly depending on the method used to assess the affinity of the interaction (3). In addition to the saturation-binding approach described here, other approaches that can be used include fluorescence polarization, ELISA, and surface plasmon resonance.
6. For the described immunoprecipitation experiment, the receptor has an N-terminal FLAG-tag, while the PDZ protein has an N-terminal HA-tag. The addition of two distinct tags will allow for the specific pull-down of the receptor with M2 FLAG agarose and subsequent detection of the PDZ domain for the co-immunoprecipitation experiment. Because most receptor/PDZ interactions occur at the extreme C terminus of the receptor, it is important to avoid having any tag on the C terminus of the receptor of interest. A tag on the C terminus of the receptor will almost certainly interfere with the binding of PDZ domain-containing partners.
7. The receptor can also be immunoprecipitated with an antibody raised against the receptor, as opposed to using tagged receptor constructs. This type of experimental approach is especially

useful when determining if the receptor/PDZ interaction occurs in native cells and/or tissues. However, it is important to avoid using an antibody that recognizes an epitope at the extreme C terminus of the receptor, as such an antibody may compete for C-terminal binding with the PDZ protein that is being assessed for potential co-immunoprecipitation.

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