Engineering selective competitors for the discrimination of highly conserved protein-protein interaction modules

Charlotte Rimbault1,2, Kashyap Maruthi3,4, Christelle Breillat1,2, Camille Genuer1,2, Sara Crespiño1,2, Virginia Puente-Muñoz1,2, Ingrid Chamma1,2, Isabel Gauthereau1,2, Ségalène Antoine1,2, Coraline Thibault1,2, Fabienne Wong Jun Tai5, Benjamin Dartigues5, Dolors Grillo-Bosch1,2, Stéphane Claverol6, Christel Poujol7, Daniel Choquet1,2,7, Cameron D. Mackereth3,4* & Matthieu Sainlos1,2*

Designing highly specific modulators of protein-protein interactions (PPIs) is especially challenging in the context of multiple paralogs and conserved interaction surfaces. In this case, direct generation of selective and competitive inhibitors is hindered by high similarity within the evolutionary-related protein interfaces. We report here a strategy that uses a semi-rational approach to separate the modulator design into two functional parts. We first achieve specificity toward a region outside of the interface by using phage display selection coupled with molecular and cellular validation. Highly selective competition is then generated by appending the more degenerate interaction peptide to contact the target interface. We apply this approach to specifically bind a single PDZ domain within the postsynaptic protein PSD-95 over highly similar PDZ domains in PSD-93, SAP-97 and SAP-102. Our work provides a paralog-selective and domain specific inhibitor of PSD-95, and describes a method to efficiently target other conserved PPI modules.
Protein–protein interactions (PPIs) are involved in the complex and intricate cellular networks that dynamically govern processes such as transport, localization and signal transduction. Preventing specific interactions can provide insight into physiological role of each protein partner or reduce the deleterious effects of abnormal protein function. It is in the latter context that PPI inhibitors have seen increasing interest as potential drug targets. Despite their promise, the study and targeting of PPI modules still represent a challenge, due in part by stronger evolutionary conservation of residues at the PPI interface compared to the rest of the protein domain. Processes such as domain recombination and gene duplication have led to paralogs, as well as distantly related proteins, that can share highly conserved interfaces with similar specificity. This tendency is exemplified by protein domains that bind short peptide sequences, such as the PDZ, SH3, SH2 and WW domains. Large-scale interactome studies on PDZ and SH3 domains highlight shared binding preferences for protein family clusters. The development of a selective inhibitor for a specific PPI must, therefore, avoid interaction with similar coexisting PPI interfaces, or risk adverse off-target effects.

The postsynaptic density protein 95 (PSD-95; also known as SAP90 or DLG4) is highly studied and one of the main postsynaptic scaffold proteins. PSD-95 plays an important role in the organization of the postsynaptic density, with the anchoring synaptic scaffold proteins. PSD-95 plays an important role in the context that PPI inhibitors have seen increasing interest as deleterious effects of abnormal protein function. It is in the latter into physiological role of each protein partner or reduce the interactome studies on PDZ and SH3 domains highlight ces, such as the PDZ, SH3, SH2 and WW domains. Large-scale SAP90 or PSD-95 binders. In contrast, less-conserved patches can be found on the opposite sides of the binding grooves (Fig. 1b, c). We reasoned that by first targeting these patches, it will be possible to generate the higher selectivity required for family member-specific binding, which could in turn be exploited to engineer the final selective competitors by fusion of an element that directly interacts with the PDZ domain binding groove (for specificity vs selectivity precisions, see Supplementary Note 1).

We used a phage display strategy with a library of diversified 10FN3 domains as a robust scaffold to yield convex surface binders. The 10FN3 domain has been previously used to obtain high-affinity binders of numerous protein domains with good stability, ease of production and the absence of disulphide bridges as found in VH or variable regions of antibodies. We used the sequence improved by the group of Koide and inserted the 10FN3 scaffold as a fusion to the g3p minor phage coat protein (Supplementary Fig. 2a). We next performed diversification of the 10FN3 BC and FG loops using NNK degenerate codons by both varying all residues as well as the length of the two loops by the Pfunkel method (Supplementary Fig. 2b). This provided us with a library of about 10^10 unique clones as estimated by the sequence analysis of 96 randomly picked colonies (Supplementary Fig. 2c). In parallel, we produced the biotinylated tandem PDZ domains of PSD-95, as well as the tandems of the other DLG family members by introducing a biotin acceptor peptide tag on their N-terminus and co-expressing the resulting modified gene with a plasmid encoding for the biotin ligase BirA in Escherichia coli. The targets were purified to homogeneity and biotinylation levels were above 75% as judged by gel assay.

Three rounds of selection were performed on streptavidin-coated magnetic beads functionalized with the biotinylated PDZ domains, using our phage library produced with the M13KO7 phage helper to favour the display of a single diversified domain per phage (Supplementary Fig. 2d). The target concentration was decreased between each round to increase the stringency (from 100, 50 to 25 nM). After the third round, 96 isolated colonies were randomly picked and analysed by phage-ELISA and sequencing. We identified 11 different clones that showed a strong ELISA response for PSD-95 with various levels of enrichment (Fig. 2b). No consensus sequence emerged from the sequence analysis,
Fig. 1 Analysis of PSD-95 tandem PDZ domains as a target for specific binders. a Sequence alignment of the first two PDZ domains of the PSD-95 paralog family (Rattus norvegicus). The red asterisks indicate residues directly involved in the binding of partner proteins. b Surface representations of PSD-95 tandem PDZ domains (PDB ID 3GSL, domain 1 on the left and domain 2 on the right) with ligand modelled in (RTTPV aligned from PDB ID 3JXT, black sticks) and with non-identical residues coloured in shades of blue according to the other tandem it is compared to (SAP97, PSD-93 or SAP102). c Scheme summarizing the location of highly conserved and more diverging regions on the surface of PSD-95 tandem PDZ domains with respect to other family members.

Fig. 2 Isolation and selectivity of clones targeting PSD-95 tandem PDZ domains. a Structure of the protein scaffold used for selection (PDB ID 3K2M). The diversified loops (BC and FG) are represented in blue. b Library design and loop sequences of the isolated clones (Xph#) against PSD-95. c Library design and loop sequences of the isolated clones (Xph#) against PSD-95. d Phage-ELISA of isolated clones against tandem PDZ domains from PSD-95 family. e FRET systems used for measurement of the donor lifetime (FLIM). f Lifetime of eGFP inserted in PSD-95, SAP97 and PSD-95 (C3S, C5S) in presence of the respective partners. g Unprocessed original gels are shown in Supplementary Fig. 4.
which can be explained by the much higher theoretical diversity of the designed library as compared to the actual diversity that can be handled with the phage display method.

**Clones characterization.** Specificity of the isolated clones was first evaluated by phage-ELISA response towards the tandem PDZ domains of PSD-95, as compared to the other DLG family members (Fig. 2c). Most clones showed specificity for PSD-95, which was remarkable given the absence of negative selection. Performing the same analysis with the isolated second PDZ domain of PSD-95 failed to show a strong response, suggesting that the isolated clones were either binding to the first PDZ domain or had epitopes on both domains (Supplementary Fig. 3). We excluded clones that contained multiple cysteines from further study, and confirmed the binding properties of the remaining clones with a pull-down approach on recombinant proteins (Fig. 2d and Supplementary Fig. 4). The evolved 10FN3 domain clones were produced in E. coli with a deca-His-tag, directly isolated from the lysates with Ni-NTA magnetic beads, and then incubated with purified tandem PDZ domains. The material left on the beads following the wash was eluted with imidazole and analysed by densitometric analysis of the colloidal blue-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) band intensity. The results were similar to measurements by phage-ELISA, indicating that recognition of PSD-95 tandem PDZ domains is indeed mediated by the evolved 10FN3 domains. To ensure that the binding capacities of the clones were preserved in a cellular environment, the seven best binders were further evaluated by a cell-based FRET/FLIM (Fürster resonance energy transfer/fluorescence-lifetime imaging microscopy) assay. The FRET system was based on one previously developed to investigate divalent ligands25 (Fig. 2e). The donor fluorescent protein, EGFP, was inserted after the second PDZ domain in PSD-95 or SAP97. The acceptor, mCherry, replaces the C-terminal PDZ domain-binding motif (PBM) of the transmembrane protein Stargazin, and is followed by a 20-amino-acid linker and the 10FN3 clone. All clones showed strong binding to full-length membrane-bound PSD-95 as indicated by reduction of the mean lifetime of the donor fluorescent protein to around 2.2 ns as compared to the lifetimes above 2.4 ns obtained with the donor alone or in presence of a naïve clone (Xph0; Fig. 2f). In contrast, only weak binding could be observed with SAP97 with mean lifetimes around 2.4 ns for all the clones we tested (Fig. 2f and similar results were obtained for PSD-93, Supplementary Fig. 5). Strong binding was also observed with a soluble mutant of PSD-95 (ref. 34) that can be more directly compared to the cytosolic SAP97. Together these results indicate that the evolved 10FN3 domains we have selected are robust and specific binders of epitopes on the PSD-95 tandem PDZ domains.

**Epitope mapping.** Following the specificity evaluation, five final clones (Xph15, Xph17, Xph18, Xph20 and Xph25) stood out based on their relative binding strength and specificity. We selected three representative clones (Xph15, Xph18 and Xph20) to further investigate binding properties with a series of in vitro assays. To maximize the solubility and stability, we used two strategies: the first consisted of a fusion to the SUMO protein tag on the C-terminus of the clone (an N-terminal tag resulted in loss of binding), the second approach involved mutation of serine 65 into a lysine as previously reported by the group of Koide35. Both strategies improved our capacity to concentrate and freeze-store the proteins while maintaining homogeneity of the samples, as judged by analytical size exclusion chromatography (Supplementary Fig. 6). Thermal stability evaluation of the three S63K mutant clones (Supplementary Fig. 7) showed inflection in their thermal unfolding curves around 69 ºC for Xph18 and Xph20, and at 77 ºC for Xph15, suggesting only partial loss of stability to that of wild-type 10FN3 (82.5–88 ºC)36,37. Finally, nuclear magnetic resonance (NMR) spectroscopy was used to test the binding specificity of the recombinant clones towards 15N-labelled tandem PDZ domains of all four DLG family members (Supplementary Fig. 8). Binding was detected by the change in 15N-HSQC peak positions, and in keeping with the previous results, all three clones resulted only in significant changes to the PSD-95-12 spectra, with the exception of Xph20 and SAP97 for which partial binding could be observed in these conditions (>80 μM). Overall, these results indicate that the several recombinant forms of the selected clones are specific for PSD-95-12 and constitute solid candidates for further investigation and engineering.

To allow for precise engineering of subsequent tools based on Xph15, Xph18 and Xph20, detailed information on the mode of interaction and the precise epitopes of each clone are required. NMR spectroscopy was chosen to access residue-specific information, and therefore we first obtained chemical shift assignment of the PSD-95 tandem PDZ domain (Supplementary Fig. 9). A comparison of spectra for PSD-95-12 in the unbound form and bound to each clone reveals residue-specific changes in NMR crosspeak positions or signal disappearance due to broadening (Fig. 3; Supplementary Figs. 10–12). Quantification of the clone-dependent changes in the crosspeak positions shows that Xph15 and Xph20 interact with similar residues on PSD-95 situated on the opposite side of the binding groove of PDZ domain 1. In contrast, the Xph18 epitope involves both PDZ domains 1 and 2, as well as the connecting linker, but similarly encompasses a region distant from the binding grooves.

The epitope mapping experiments from Xph15 and Xph20 reveal a binding epitope nearly exclusive to the first PDZ domain. To probe this interaction in more detail, we repeated the NMR binding experiments with isolated PDZ1 and PDZ2 from PSD-95 (annotated reference spectra in Supplementary Fig. 13). Addition of Xph15 and Xph20 to 15N-labelled PDZ1 resulted in nearly identical chemical shift perturbation as for the tandem construct (Supplementary Figs. 14 and 15). Also similar to the previous binding studies, there were no significant changes, and thus no apparent interaction, for the isolated PDZ2 domain. Using similar protein concentrations for Xph18, neither isolated PDZ domain from PSD-95 displayed evidence of interaction (Supplementary Fig. 16). At higher protein concentrations, a specific interaction between Xph18 and PDZ1 could, however, be detected. Thus, for Xph18, it appears that a primary interaction still involves PDZ1, although the epitope extends across both PDZ1 and PDZ2 and the presence of both domains is a requisite for binding.

Based on the specific complexes formed between each of the clones and PSD-95, we decided to generate putative atomic models in order to better define key structural elements of the epitopes. To this end, we used the NMR-based interaction data to generate docking models for all three clones supplemented with additional information. For Xph15 and Xph20, we first collected reciprocal binding data from isotopically labelled clones in the unbound and PDZ1-bound forms (Supplementary Figs. 17 and 18). During the analysis, we also noted that there were two populations in the NMR spectra, but only for the unbound state. Assignment of backbone chemical shifts for the two equal populations of free Xph15, as well as the major and minor populations of free Xph20, localized the differences to the BC and FG loops, and the C-terminal β-strand (Supplementary Figs. 19 and 20). Further investigation revealed that these two populations were unaffected by temperature (283–308 K) and remained in equilibrium during the titration with Xph15 or Xph20, with a population exchange rate >1 s⁻¹ (Supplementary Fig. 21).
Using the identified binding surfaces on Xph15 and Xph20, as well as the corresponding interaction regions on PDZ1, we generated a series of docking models (Fig. 4a and Supplementary Fig. 22). For both clones, the model reveals that the bound Xph15 or Xph20 likely extends away from the ligand-binding groove, and is not expected to sterically hinder interaction to the C-termini of PSD-95 interaction partners. In addition, the bound clones are remote from the interdomain contacts to PDZ2, and we can reasonably speculate that the interaction has limited impact on the orientation of the two domains. This is further supported by the strong similarity of chemical shifts when comparing Xph15 and Xph20 binding to PDZ domain 1 alone and to the tandem. There should also be minimal impact on the dynamic interconversion of two major domain arrangements of the PDZ1 and PDZ2 domains that has been recently described.38

For Xph18, we first created a docking model of Xph18 with PDZ1 (Supplementary Fig. 22c). Since we knew that PDZ2 also contributes to the interaction with Xph18, we collected residual dipolar coupling data on the Xph18:PSD-95-12 complex in order to fix the orientation of the PDZ2 domain relative to PDZ1, and to confirm that the two domains are relatively fixed in position relative to each other (Supplementary Fig. 22d, e). A final model of the complex illustrates that this PDZ domain arrangement explains the previously observed extended interaction surface with Xph18 (Fig. 3b). Despite the fixed arrangement, the binding grooves on both PDZ1 and PDZ2 are still accessible to ligand binding.

Overall, these results demonstrate a primary requirement of PDZ1 and a specificity that is achieved by binding to regions remote from the binding groove. For all three clones, the origin of the specificity could be attributed principally to a single phenylalanine residue (F119) that is replaced by an arginine in all three other proteins from the DLG family (Supplementary Fig. 22g).

Affinity determination. Binding affinities of Xph15, Xph18 and Xph20 were evaluated by single-cycle kinetics surface plasmon resonance (SPR) and by isothermal titration calorimetry (ITC) (Fig. 5 and Supplementary Figs. 23 and 24). With both techniques, the affinity of the three clones were similarly ranked with Xph15 being the weakest binder and Xph20 the strongest. Xph15 presented a dissociation constant in the micromolar range with fast kinetics ($k_{on}$ 9.5 x 10^4 M$^{-1}$ s$^{-1}$ and $k_{off}$ 0.4 s$^{-1}$). In comparison, Xph20 presented a faster $k_{on}$ and a slower $k_{off}$.

Fig. 3. Epitope mapping. a-c 15N-HSQC NMR spectrum of unbound 80 μM [15N]PSD-95-12 (black) with spectra following addition of 100 μM natural abundance Xph15 (blue) (a), Xph18 (orange) (b) or Xph20 (teal) (c). Histograms show the combined 1H, 15N chemical shift perturbation $\Delta\delta_{(H,N)}$ of the backbone amide crosspeaks for residues in PSD-95-12 that result from addition of the clones. Residues with $\Delta\delta_{(H,N)}$ values greater than 0.3 ppm (dark coloured bars) or 0.15 ppm (light coloured bars) are also highlighted on surface representations of PSD-95-12 (same orientations as in Fig. 1). Dark grey shading indicates residues with ambiguous or missing NMR data. Yellow bars in b indicate crosspeaks that are broadened upon addition of Xph18. In all three cases, addition of 1.2 molar equivalents of Xph15, Xph18 or Xph20 was sufficient to fully shift the [15N]PSD-95-12 crosspeaks to the bound population, consistent with 1:1 stoichiometry for the complexes. Source data are provided as Source Data file.
leading to submicromolar dissociation constants of 330 nM and 67 nM by SPR and ITC, respectively. Finally, Xph18 showed a different kinetic profile with slower $k_{on}$ and $k_{off}$ yielding a micromolar to submicromolar dissociation constant. The slower association rate constant observed for this clone can be justified by the fact that its epitope is spread over both domains 1 and 2, which are to some extent mobile with respect to one another and the two domains must adopt the appropriate orientation so that the epitope can be fully recognized. Compared to antibodies, our binder affinities were at the lower limit ($K_D$ ranging from 10 μM to 100 nM) and display relatively fast dissociation rate constants (half-lives <1 min). Nevertheless, they are in the range of natural binders such as PSD-95 partners with PBMs that bind in the low micromolar range with fast rate constants. Of note, further investigation of the sole unspecific binding observed between Xph20 and SAP97, that was partially observed by NMR titration, resulted in a $K_D$ estimation over 150 μM (Supplementary Fig. 23).
These results were further confirmed by using a different experimental configuration for the SPR (i.e., immobilization of the tandem PDZ domain instead of the clones); however, the biphasic behaviour of the clones prevented a quantitative analysis (Supplementary Fig. 23). Interestingly, single point mutations on PSD-95 and SAP97 tandems (F119R and R278F, respectively, in order to swap what appears as a key residue for Xph15, Xph18 and Xph20 specificity) significantly impaired binding of Xph20 to the PSD-95 mutant by SPR and NMR, while it allowed recognition and binding to the SAP97 mutant (Supplementary Fig. 25). This highlights the critical role of F119 in the generation of specific binders and further validates our structural studies.

Selective competitor engineering and characterization. In order to convert our specific binders (Xph15, Xph18 and Xph20) to selective inhibitors, it is necessary to add a peptide motif that interacts with the PDZ domain binding groove despite the fact that available PBMs are relatively non-selective for specific PDZ domains from PSD-95, SAP97, SAP102 or PSD-93 (Fig. 6a). One of the chosen peptides was derived from the AMPAR auxiliary subunit stargazin with higher affinity for the second PDZ domain19, and the second peptide is a consensus sequence isolated from a peptide phage display investigation that binds both domains9. To preserve the terminal carboxylate group, which is key to PDZ domain binding, the inhibiting peptides must be inserted C-terminal to the Xph clones with sufficiently long connecting linkers.

The engineered constructs were first evaluated by SPR (Fig. 6b, Supplementary Fig. 26 and Supplementary Table 1). Fusion of the binding motif can in principle bind to each domain (overlay of the two situations). The reported concentrations represent the highest concentration used on the injections are a series of twofold dilutions. The coloured curves represent measured data points and black lines represent the global response in the sensorgrams were only obtained with over 100-fold higher analyte concentrations and resulting dissociation constants more than a 1000-fold weaker than for PSD-95 (Supplementary Table 1). Overall, all constructs that we generated showed dramatically improved affinities while maintaining a strong selectivity.

The competing capacity of one of the most potent clones was next evaluated by a pull-down assay (Fig. 6c, Supplementary Fig. 27 and Supplementary Table 2). PDZ domain tandems for all four family members were first bound to streptavidin-coated magnetic beads functionalized via a biotinylated divalent peptide-based ligand25 and the resulting PDZ domain complexes were then incubated in presence of increasing amounts of the competitors. The biomimetic divalent ligand was used here as a model for complex multivalent interaction systems such as those that can be found in cells. The affinities of the divalent ligand were first determined by fluorescence polarization for each tandem PDZ domains (Supplementary Fig. 28). The similarity of the $K_s$ that were measured illustrates the sequence identity of the binding grooves and its consequences even on complex ligands. Quantification of the competition was performed by loading on SDS-PAGE the material left on the beads and densitometric analysis of the colloidal blue-stained band intensity. The titration results were consistent with the SPR experiments, with lower concentrations of Xph20-ETWV required to compete off the divalent ligand from PSD-95 PDZ domains as compared to SAP97, SAP102 or PSD-93. The residual competition capacity of Xph0-ETWV towards all DLG family members is equal to that observed for Xph20-ETWV binding to SAP97, SAP102 or PSD-93. The lower competition activity appears to be due solely to the ETWV motif, as further supported by measured $K_i$ values in the range expected for the isolated ETWV peptide (Supplementary Fig. 28). Efficient competition of Xph20-ETWV against PSD-95 therefore involves an essential combination of the targeting and competing elements.

Compared to the SPR measurements, we observe here a reduction of the $K_i$ difference between PSD-95 tandem PDZ domains and the other tandems (10-fold vs 1000-fold). We attribute this discrepancy to several factors. First, the binding modes are different as the divalent ligand can bind both PDZ domains, whereas our competitor only binds a single binding groove. Second, the divalent ligand presents a much weaker affinity compared to Xph20-ETWV, which prevents a robust
quantitative determination of the inhibition constant. Finally, despite our efforts, some detectable amounts of proteolyzed ligand missing the PBM (Supplementary Fig. 29 and Supplementary Table 3) were present together with the competitor. These by-products reduce the effective concentration of the competing ligand and also act as a blocker of the binding site of Xph20 in the Xph20-ETWV construct.

In order to confirm that the binding properties of the engineered competitors are maintained with the full-length endogenous protein, we next evaluated the Xph20-ETWV capacity to recognize PSD-95 from rat brain lysates and primary hippocampal neuron culture. Using a biotinylated competitor and magnetic streptavidin-coated beads, we performed pull-downs on adult rat brain lysates. In comparison to a control protein (mScarlet-I), Xph20-ETWV was clearly able to efficiently bind endogenous PSD-95 as observed by western blot analysis (Fig. 7a). To further confirm binding to PSD-95 in its native environment, we appended a protein transduction domain from the HIV-1 TAT sequence on the competitor, and attempted co-immunoprecipitation (co-IP) in neuron primary cultures. We first evaluated several PSD-95 antibodies for co-IP to avoid potential epitope overlap with Xph20 (Supplementary Fig. 30). Out of four antibodies, three presented an epitope on the first two PDZ domains. Unfortunately, the remaining antibody performed poorly as a PSD-95 immunoprecipitant and only a faint signal could be observed for Xph20-ETWV by western blot after incubation of the cell culture with the TAT-derived competitor. Nevertheless, co-IP with an anti-His tag antibody allowed us to clearly detect PSD-95 (Fig. 7b) thereby demonstrating that Xph20-ETWV can bind endogenous PSD-95 in its native environment.

Finally, we investigated whether our strategy could preserve the selectivity of Xph20-ETWV for PSD-95 in a complex cellular environment where a large number of PDZ domain-containing proteins can in principle interact with class I PBMs such as ETWV. To this end, we performed a proteomic analysis of the pull-down material from a rat brain lysate using the biotinylated competitor by comparison to a neutral control protein (mScarlet-I). The results show a clear enrichment for PSD-95 (Fig. 7c) with only two other PDZ domain-containing proteins detected (PSD-93 and SAP97) albeit at much lower abundance. While the presence of these proteins could be the consequence of the ETWV motif, they could also be co-precipitates of PSD-95-associated proteins as previously observed. The enrichment of other PSD-95-associated proteins not containing PDZ domain together with the recent report of supercomplexes composed of PSD-95, PSD-93 and NMDA receptors are consistent with the latter possibility. Comparison of the results obtained in the same conditions with Xph0-ETWV (Fig. 7d), in which numerous PDZ-domain containing proteins are detected in addition to PSD-95, confirms that high selectivity for PSD-95 in complex cellular extracts is achieved by combining Xph20 and the ETWV PBM.

Precise target identification. As the PBMs we used can in principle interact with either of the two PDZ domains within the complex, we sought to determine if the first or second PDZ domain was principally targeted by our tools. For this purpose, we used a photocrosslinking approach (Fig. 8a). The unnatural amino acid p-azidophenylalanine (pAzF) photocrosslinker was introduced in the construct sequence by the amber suppression method. We hence placed an amber stop codon at the -5 position of the PBMs by site-directed mutagenesis. This position was chosen as it is not highly conserved in the consensus recognition motif of both domain 1 and 2, and we have previously shown that incorporation of aromatic residues at this site did not significantly modify the binding properties of partner sequences. The pAzF-containing mutants of both Xph20-Stg and
adducts (as seen in Supplementary Fig. 31), strongly supports the groove. The absence of observed fragments from PDZ1, together with robustness of the pAzF-PBM to generate photocrosslink adducts (as seen in Supplementary Fig. 31), strongly supports the pAzF residue within the second PDZ domain-binding region of interaction with the motif.

Xph20-ETWV were produced in E. coli in the presence of an additional plasmid encoding an engineered pair of tRNA and aminoacyl-tRNA synthetase to direct the incorporation of the unnatural amino acid. Crosslinking was verified at 365 nm in the presence of the tandem PDZ domains, and generated a single additional band with a molecular weight consistent with a covalent bond with the target PDZ domain in the region of interaction with the motif.

Supplementary Fig. 31c) and supplementary Table 6). Next we attempted to selectively target the PDZ1 by stargazin in the presence of Xph20-ETWV and controls (**P < 0.0001, ANOVA test followed by a Tukey’s Multiple Comparison Test 2 by 2.). Each data point represents a unique cell. Co-immunoprecipitation of PSD-95 by stargazin in the presence of Xph20-ETWV and controls (n = 2 independent experiments). The bars represent the mean ± s.d. Schematics of LRRTM2/PSD-95 interactions and bar graph of the percentage of LRRTM2 clusters containing PSD-95. The bars represent the mean ± s.e.m. and numbers in the columns indicate the number of cells (**P < 0.01, non-parametric ANOVA test followed by a Dunn’s Multiple Comparison Test 2 by 2.). Source data are provided as Source Data file.

Competing properties in cellular environment. Finally, the capacity of the engineered competitor to disrupt specific interactions was assessed in a cellular environment using three approaches (Fig. 8d–f and Supplementary Fig. 35). We first used a FRET/FLIM approach. As initial attempts with the stargazin/PSD-95 system did not yield conclusive results, we designed an intramolecular FRET system that only reports binding to the second PDZ domain. The system consists of the tandem PDZ domains 1 and 2 structure (PDB ID 3GSL) with ligands modelled in (RTTPV, black sticks). The red spheres represent pAzF position in the binding motifs. Bottom: zoom in the identified region on PSD-95. The salmon and red sticks represent the identified fragment with the red highlighting the most likely candidate residues. Competition in cellular environment measured by FRET/FLIM. The box plots show median, first and third quartile, with whiskers extending to the minimum and maximum of all individual data points (****P < 0.0001).

**Fig. 8** Selective blocking of PSD-95 PDZ domain 2 with engineered ligands. a Schematics of the photocrosslinking assay. Amber codon suppression method is used to insert p-azidophenylalanine (pAzF) into the competitor in the vicinity of PDZ domain-binding motif to create a covalent bond with the target PDZ domain in the region of interaction with the motif. b Photocrosslinking of Xph20 derivatives with PSD-95 tandem PDZ domain analysed by anti-His tag western blot. The red arrow indicates the photocrosslink products. For uncropped blots, see Supplementary Fig. 28. c Position of PSD-95 photocrosslinked fragment with Xph20-ETWV* and -Stg* identified by LC-MS/MS. Top: PSD-95 domains 1 and 2 structure (PDB ID 3GSL) with ligands modelled in (RTTPV, black sticks). The red spheres represent pAzF position in the binding motifs. Bottom: zoom in the identified region on PSD-95. The salmon and red sticks represent the identified fragment with the red highlighting the most likely candidate residues. d Competition in cellular environment measured by FRET/FLIM. The box plots show median, first and third quartile, with whiskers extending to the minimum and maximum of all individual data points (****P < 0.0001, ANOVA test followed by a Tukey’s Multiple Comparison Test 2 by 2.). Each data point represents a unique cell. e Co-immunoprecipitation of PSD-95 by stargazin in the presence of Xph20-ETWV and controls (n = 2 independent experiments). The bars represent the mean ± s.d. f Schematics of LRRTM2/PSD-95 interactions and bar graph of the percentage of LRRTM2 clusters containing PSD-95. The bars represent the mean ± s.e.m. and numbers in the columns indicate the number of cells (**P < 0.01, non-parametric ANOVA test followed by a Dunn’s Multiple Comparison Test 2 by 2.). Source data are provided as Source Data file.
domains in which the donor protein (eGFP) was inserted on the N-terminus of PSD-95 PDZ1. The acceptor protein (mCherry) was inserted on the C-terminus of PDZ2 with a long linker to separate mCherry from the tandem and the donor. The C-terminus of mCherry was functionalized with the last 14 amino acids of stargazin to allow monitoring of PDZ domain competing binders. Additionally, to be able to exclusively focus on interactions mediated by the second PDZ domain, the PDZ1 was mutated (H130V) to impair its binding properties55. The reporter showed a shorter lifetime than soluble eGFP indicating proximity of the two fluorescent proteins and the lifetime was increased upon expression of a reporter deletion mutant lacking the stargazin PBM. A similar increase in lifetime was observed when the reporter was co-expressed with the Xph20-ETWV competitor, but not with the Xph0-ETWV construct, demonstrating that efficient competition can be achieved in cells and requires both Xph20 and the PBM. This result was confirmed by a co-IP assay (Fig. 8e and Supplementary Fig. 35d, e) between stargazin and a mutated PSD-95 in which only PDZ2 was left fully functional (for the reasons mentioned above). Stargazin was then immunoprecipitated from 293T cell lysates transfected with stargazin, the PSD-95 mutant and either Xph0-ETWV, Xph0-ETWV or Xph20. In these conditions, and in agreement with the FRET assay, only Xph0-ETWV was able to reduce the amount of co-precipitated PSD-95. Finally, the competing property of Xph20-ETWV was evaluated with a fully functional PSD-95 against a synaptic adhesion protein, LRRTM2, reported to be a PSD-95 partner that binds PDZ domains 1 and 2 (ref. 46) (Fig. 8f and Supplementary Fig. 35f, g). In COS-7 transfected cells, recruitment of PSD-95 at LRRTM2 clusters was not detectably affected by co-expression of Xph0 or Xph0-ETWV. In contrast, expression of Xph20-ETWV led to more than a 50% decrease of recruited PSD-95 as a direct result of competition for PDZ2. These experiments together demonstrate the capacity of Xph20-ETWV to selectively and efficiently bind the PSD-95 second PDZ domain and to consequently block PDZ domain 2-mediated interactions in a cellular context.

Discussion

We report here a semi-rational strategy used to design selective binders of a single PPI domain within a family of highly sequence-conserved PPI domains. By combining a non-selective minimal binding motif to a protein scaffold evolved to specifically recognize a target, we have dramatically increased the selectivity for the PSD-95 second PDZ domain. PDZ domains constitute a representative class of PPI module that is characterized by fast kinetic interactions in the micromolar range that depend on a minimal set of consensus residues (at 0 and −2 positions from the C-terminus). Designing and validating a methodology to generate molecular tools that selectively target and modulate the function of a single PDZ domain of PSD-95 addresses challenges that pertain both to the general conception of PPI modulators and to the investigation of one of the main synaptic scaffold proteins.

The selection that we used to evolve PDZ domain binders was not directed at any specific sites and in particular was not performed by blocking the PDZ domain binding grooves. It is interesting to note that, based on the phage-ELISA and pull-down results, most of the clones that we isolated with such an unbiased selection scheme showed strong specificity for PSD-95. Although epitopes were only mapped for three of the clones, we can reasonably speculate that given their specificity most bind to regions outside of the conserved binding grooves. This observation contrasts with the reported tendency of 10FN3-derived binders to principally target functional sites (peptide-binding grooves as would be expected for PDZ domains)30. Our results suggest that parts of the first PDZ domain of PSD-95 constitute hot-spots for PPI as they appear to be involved in the binding of all the isolated clones. This finding validates the benefit of choosing to keep intact the tandem PDZ domain supramodule for our selection. Specificity of the characterized clones is principally driven by a single residue, which indicates that even minimal surface variations can be exploited to generate specific protein- and more specifically 10FN3-based binders. It is of interest that epitopes for the selected clones are conserved within numerous species (Supplementary Fig. 36) and are not involved in any reported post-translational modifications47 (Supplementary Fig. 37). This property thus extends the scope of application beyond the species we have used for selection (Rattus norvegicus) and guarantees recognition of PSD-95 regardless of its cellular state.

An unexpected finding during structural characterization of the unbound clones was that they exist in two conformations, and this may constitute an inevitable trade-off for installing diversity on two of the main loops. Molecular dynamics studies also predict strand swapping at high temperature for these 10FN3 terminal strands48. This structural plasticity of the clones may have consequences on their interaction with PSD-95 and result in a complex interaction mode. In our analyses, quantitation of the binding properties were performed using a simple 1:1 Langmuir binding model as we only observe a unique 1:1 complex for all the clones as investigated by NMR.

If we compare the 10FN3 domain part of our modulators to some of the commercially available PSD-95-specific antibodies, epitopes are also found in fragments that encompass the same surface region targeted by Xph15, Xph18 and Xph20 (Supplementary Fig. 30b), suggesting shared epitopes that may rely on the same residues to achieve specificity. In comparison to antibodies, the small size and synthetic nature of our binders constitute an advantage by allowing extensive biophysical and biochemical characterization of the tools, although the monovalence and reduced binding surface of unappended Xph compared to antibodies can lead to reduced affinities. Only two other protein-based binders of PSD-95 have been developed by other groups for imaging purposes. One is derived from a single chain variable fragment (PF11)59 that was obtained from a selection against the full-length palmitoylated form of PSD-95. The other molecule is an evolved 10FN3 domain (PSD95.FingR)30 that was selected against the SH3-GuK domains of PSD-95. While PF11 is described as recognizing only the palmitoylated form of PSD-95 (and not the one of PSD-93), PSD95.FingR also binds to SAP97 and SAP102. In both cases, the epitopes were not clearly established and the affinities not determined. In contrast, Xph15, Xph18 and Xph20 represent specific and synthetic small domains that bind to PSD-95 with precisely identified epitopes. Their high specificity and apparent non-hinderance of peptide ligand binding to PSD-95 also constitute a base for further engineering to develop imaging tools to monitor endogenous PSD-95 with minimal perturbation.

The combination of synthetic binders has already been described as a strategy to improve the affinity for a target; examples include fluorescent proteins51, anti-EGFR VHHs52, a PDZ domain binder53,54, or even kinases with 10FN3-derived binders35. Our aim here was different, although the final effect also relies on affinity improvement: we use the 10FN3 moieties to increase the selectivity of a competing motif towards a protein of interest. The fusion of moderate to weak binders (100 nM–10 μM) created strong binders with affinities in the picomolar range, arising from slower k_off Determination of the effective concentrations (C_{eff})35 resulting from the tethering of two binders via a linker, as a quantitative indicator related to the affinity enhancement, provided us with values mostly in the 10–50 mM range (Supplementary Table 8). These values are
overall higher than those reported for a bivalent system involving PDZ domain-mediated interactions. While the high $c_{\text{eff}}$ values are a direct consequence of the strong affinity of the fusions, they may also reflect our particular case where the tethered PBM can bind to two different and also tethered PDZ domains. In this context, the deliberate use of weaker PBMs is a possible approach to improve the competitor selectivity by reducing the off-target effects. This increased selectivity would result from a loss of interaction with cellular concentrations of SAP97, SAP102 or PSD-93 at the expense of an acceptable loss of the competitor affinity. Other directions for further engineering are prompted by the characterization of the PDZ domain binding groove targeted by the two competitors (Xph20-ETWW and Xph20-Stg). In a first step, the linker length could be adjusted to closely match the distance between the epitope and peptide-binding groove. Secondly, in order to generate PDZ1 blockers, the nature of the peptide source could be varied to include PBMs more selective for this domain (such as attempted with GluK2 PBMM) together with an adaptation of the linker length.

As an important validation step, we have shown that the core properties and the selectivity of the clone Xph20-ETWW were preserved in cellular environments. Given the lack of chemical tools to selectively inhibit single PDZ domains within conserved protein families, our competitors constitute a unique opportunity to investigate the role of endogenous PSD-95 without impacting other paralog family members. Natural partners of the second PDZ domain of PSD-95 include essential synaptic proteins such as TARPs, AMPAR receptors, NMDA receptors, LRRTMs and nNOS. Even though some of these partners form multimeric protein complexes that rely on multivalent interactions to efficiently anchor to synaptic PSD-95, we have nevertheless demonstrated the capacity of Xph20-ETWW to compete against a divalent ligand. An additional advantage of the relatively small size of the protein-based competitors (~16 kDa) is that they can either be genetically encoded to control levels of expression and cell-type targeting, or exogenously produced as a cell-permeant version for acute effect, such as done for V$_H^\text{Hi}$. Finally, the pAzF-containing ligands could also constitute selective irreversible inhibitors of the second PDZ domain of PSD-95 after light-triggered crosslinking.

In summary, our two-step strategy allowed us to circumvent the challenges associated with direct generation of selective or specific competitive binders targeting a highly conserved binding groove within members of a same protein family. With the recent emergence of directed evolution techniques and development of small size synthetic binding domains, we foresee that our strategy will constitute an attractive and powerful method to address the design of selective inhibitors to target other conserved PPI surfaces.

**Methods**

**Phagemid construction.** The plasmids generated and the primers used in this study are listed in Supplementary Tables 9 and 10, respectively. The phagemid (referred to as pSEX84) was derived from the commercial phagemid (pSEX81 Surface Expression Phagemid Vector (PROGEN)). The pSEX81 vector EcoRI restriction site was modified to a HindIII site and the LacI$_\text{cr}$ transcriptional repressor gene (containing a single T→C mutation at the -35 position of the promoter region of LacI) was inserted between Xhol restriction site followed by a strong terminator gene sequence (TTP upstream of the lac promoter). The sequence of the tenth subunit of human bronectin type III repeat (10FN3) was designed as in Karatan et al. and the gene was synthesized by GenScript. The synthesized gene, Xph8, was flanked by HindIII and BamHI restriction sites in the first and third position, respectively. The BC, DE and FG loops were replaced by serines in place of the wild-type residues to generate an inert template. A ribosome binding site, 5′-agga-3′, was added after the HindIII restriction site and before the methionine initiation codon (ATG). The DsbA signal sequence (MKKWLALAGLVLAFSASA-) was also included N-terminally to Xph8 for the secretion of the fusion protein into the periplasmic space thus replacing the pelB signal peptide sequence present in the pSEX81 vector, to ensure a greater surface display of 10FN3. Additional restriction sites (NotI and KpnI) were added into Xph8 sequence as well as His$_\text{tag}$, a FLAG tag and the Amber stop codon at the end of the sequence, after a short GGGGS linker. The sequence was optimized for expression in E. coli. The resulting construct was subcloned into the pSEX84 vector between HindIII and BamHI restriction sites, in frame with the phage particle minor coat protein pII(bgp), under the lac operon gene regulatory system.

**Vector construction.** To produce PDZ domains and Xph clones under various forms, the commercial vector pET-24a(+) (Novagen) was modified to generate pET-IG, pET-Gcc, pET-SUMoC, pET-IG-TAT, pBG and pIGC. In order to express proteins with a N-terminal His$_\text{tag}$-tag, pET-24a(+) was modified between Ndel/Xhol sites to generate pET-IG that presents from 5′ to 3′, after the start codon and in frame, a His$_\text{tag}$, the Tobacco Etch Virus (TEV) cleavage site (-ENLYFQG-) and SUMO acceptor site (BamHI-Xhol). In order to express proteins with a C-terminal His$_\text{tag}$-tag, pET-24a(+) was modified between Ndel and HindIII sites to generate pET-Gcc that presents from 5′ to 3′, after the start codon and in frame, a multiple cloning site (NdeI-XhoI) and a His$_\text{tag}$-tag immediately followed by a stop codon. In order to express proteins with an N-terminal TAT sequence, pET-24a(+) was modified between Ndel/Xhol sites to generate pET-Gcc that presents from 5′ to 3′, after the start codon and in frame, a HIV-1 TAT sequence (YGRKKRRQRRR), a His$_\text{tag}$-tag, the TEV cleavage site and a multiple cloning site (BamHI-Xhol). In order to express proteins with a C-terminal biotinylation tag and a His$_\text{tag}$-tag, pET-24a(+) was modified between Ndel/Xhol sites to generate pET-Gcc that presents from 5′ to 3′, after the start codon and in frame, a Biotin acceptor peptide (AP-tag or AviTag, -GLNDIFEAQKIEWHE-), a His$_\text{tag}$-tag, the TEV cleavage site and a multiple cloning site (BamHI-Xhol). In order to express proteins with a C-terminal biotinylation tag and a His$_\text{tag}$-tag, pET-24a(+) was modified between Ndel/Xhol sites to generate pET-Gcc that presents from 5′ to 3′, after the start codon and in frame, a His$_\text{tag}$-tag immediately followed by a stop codon. The pET-NO vector was previously described and contains after the initial methionine a His$_\text{tag}$-tag, the TEV cleavage site (-ENLYFQG-) and a multiple cloning site (BamHI-Xhol). To improve biotinylation yields, the biotin ligase BirA from the pDisplay-BirA-ER, gift from Alice Ting, Stanford University, Addgene plasmid #20856, was cloned into a PCYC-dsK-1 vector as a fusion to mCherry to generate pACYC-mCherry-BirA.

**Plasmid construction.** The amino acid sequences of the main constructs are listed in Supplementary Table 11.

The cDNAs of PSD-95, SAP97, SAP102 and PSD-93 (gift from Nathalie Sans, University of Bordeaux) were all from the rat species. Boundaries used for the first two PDZ domains (bandem) are the following residues 61–249 for PSD-95 (PSD-95-12), 220–408 for SAP97 (SAP97-12), 220–408 for SAP102 (SAP102-12), 220–408 for SAP102 (SAP102-12), 152–249 for PSD-93 (PSD-93-12), 152–249 for PSD-93 (PSD-93-12). Boundaries for the isolated PDZ domains 1 and 2 of PSD-95 were, respectively, residues 61–152 and 152–249. Xph8 was synthesized as described above and Xph clones were directly PCR-amplified from the isolated colonies obtained at the end of the selection after sequencing. The plasmid cloning in X ph clones was performed by site-directed mutagenesis (for the wild-type 10FN3, PDB ID 1FNA), F119R in PSD-95 and R278F in SAP97 were introduced by site-directed mutagenesis. PSD-95-eGFP was obtained by cloning in two steps the N-terminal and C-terminal parts of PSD-95 into the psDNA3.1 vector. A control construct was produced similarly by omitting the strong terminator gene sequence (tHP) upstream of the lac promoter. The BC, DE and FG loops were replaced by serines in place of the wild-type residues to generate an inert template. A ribosome binding site, 5′-agga-3′, was added after the HindIII restriction site and before the methionine initiation codon (ATG). The DsbA signal sequence (MKKWLALAGLVLAFSASA-) was also included N-terminally to Xph8 for the secretion of the fusion protein into the periplasmic space thus replacing the pelB signal peptide sequence present in the pSEX81 vector, to ensure a greater surface display of 10FN3 (refs. 31,63). Additional restriction sites (NotI and KpnI) were added into Xph8 sequence as well as His$_\text{tag}$, a FLAG tag and the Amber stop codon at the end of the sequence, after a short GGGGS linker. The sequence was optimized for expression in E. coli. The resulting construct was subcloned into the pSEX84 vector between HindIII and BamHI restriction sites, in frame with the phage particle minor coat protein pII(bgp), under the lac operon gene regulatory system.
The gene corresponding to Xph20 followed by a linker composed of 12 repeats between the BamHI and XhoI sites. The constructs were then PCR amplified inserted into pIG-Xph15 after Xph15 sequence with a classical ligation using the linker followed by Stargazin 13 last amino acids was synthesized (Genscript) and inserted into pET-IG and pbIG vectors between BamHI and XhoI sites. All other Xph20 [S63K], obtained by PCR amplification and directed mutagenesis at -11 and -12 positions leading to Xph20-ETWV*[S63K] constructs, respectively, in order to facilitate proteomics experiments.

**Isotopically labelled proteins production.** For minimal media expression and purification of isotopically labelled proteins, isotopically labelled constructs were expressed in BL21 plysY (New England Biolabs, C30101) from LB plates containing 30 μg mL⁻¹ kanamycin. Cells pellets were obtained overnight cultures in 10 mL of LB medium containing 30 μg mL⁻¹ kanamycin. Cells were used for protein expression with 0.5 mM IPTG, and incubated at 37 °C, 200 rpm for 12–16 h. The cells were collected by centrifugation for 15 min at 4500 × g and resuspended in sonication buffer (50 mM NaCl, 20 mM imidazole, 50 mM Na2SO4, 50 mM phos- phate buffer, pH 7.5). The elite containing protein was pooled and the buffer exchanged to PBS by using a PD-10 column (GE Healthcare). Constructs with a cleavable N-terminal His-tag were incubated overnight with TEV protease at room temperature for 12–16 h, then the TEV protease, unreacted protein and the manufacturer’s protocol (Thermo Fisher Scientific) or by an SDS-PAGE assay.

**Proteins were recovered, characterized by SDS-PAGE and concentrated using Amicon Ultra-15 Centrifugal Filter Devices (Merck Millipore) with a 10 kDa cut-off. Protein concentration was determined by absorbance at 280 nm and molar extinction coefficient predicted by ProtParam (web.expasy.org). The proteins were finally aliquoted and flash-frozen with liquid nitrogen for conservation at −80 °C.**

**Unnatural amino-acid-containing proteins production.** The plasmids containing the amber codon were co-transformed in BL21(DE3) (Thermo Fisher Scientific, C600003) with the pEVOL-pAxF (a gift from Peter Schultz, Scripps Research Institute).
The five phosphorylated oligonucleotides were combined in equimolar amounts (1.5 pmol total; Eurogentec) and the seven FG-phosphorylated were annealed to uracilated ssDNA template, at a molar ratio of 4 (oligonucleotides/ssDNA) in a 0.5-ml Eppendorf tube containing 80 mM Tris-HCl pH 7.5, 25 mM MgCl2, 20 mM dithiothreitol (DTT), 0.5 mM NAD+ (New England BioLabs), 0.2 mM dNTPs, and 4% DMSO in a total volume of 94 μL. The annealing was performed by heating to 95 °C for 3 min, then at 60 °C for 3 min. After the annealing step, 200 units of cohesin end Taq ligase (New England BioLabs) and 2.5 units of PfuTurbo CX hotstart DNA polymerase were added. Ten units of UDG (uracil-DNase, New England BioLabs) and 30 units of ExonIII (Exonuclease III, New England BioLabs) were added and the mixture was incubated at 37 °C for 1 h followed by an inactivation step for 20 min at 70 °C. Before purification, 1 μL of the PfuKlen solution was transformed into 10 μL of E. coli TG1 chemically competent cells (Lucigen, 60106-2) and were plated onto 2x YT medium agar-plate with 50 μg·mL⁻¹ carbenicillin 1 for kanamycin and chloramphenicol 10 μg·mL⁻¹. The typical volume obtained was 20 μL. The purified DNA was immediately used for electrotransformation into TG1 electrocompetent cells (Lucigen).

In four pre-chilled 0.1 cm cuvettes (BioRad), the DNA library was electrotransformed into 250 μL of TG1 cells at 1800 V with an electroporator (BioRad LabSquare 20) for 60 min at 37 °C and resuspended in fresh 2x YT medium for two times. Bacteria were then flash-frozen with liquid nitrogen after the addition of 20% sterile glycerol and were kept at −80 °C for later use.

**Sequencing analysis software.** SynDiVa is a tool implemented in Python for analyzing multiple sequencing results in the context of directed evolution projects using diversified gene libraries. SynDiVa works as follows. First, SynDiVa pre-processing the sequencing input data using BioPython 68. It determines the orientation of the sequences and their reading frame, translates the nucleotide sequences into protein sequences and then filters the sequences by removing the ones that do not have pre-defined restrictions or sites or contain stop codons in variable regions. Pairwise alignment is performed on the valid sequences using NW-align (http://zhmali.csdb.med.unich.edu/nwalign). Sequence clustering is accomplished using MCL-algorithm 69 and multiple sequence alignment is done using Clustal Omega 70. SynDiVa outputs all the results in an HTML report.

**Biopanning procedure.** For the phage library preparation, an aliquot of frozen bacterial library was resuspended in 500 μL of 2x YT in the presence of 50 μg·mL⁻¹ carbenicillin. During the exponential phase of growth OD₆₀₀ = 0.6-0.8, bacteria were infected with M13K07 helper phage particles (multiplicity of infection or MOI = 20) for 60 min at 37 °C. A total of 2.5 μM IPTG and 30 μg·mL⁻¹ kana-mycin were added and bacteria were grown overnight at 37 °C at 200 rpm. The culture was centrifuged at 16,000g for 10 min at 4 °C and resuspended in fresh 2x YT medium for two times. Bacteria were then flash-frozen with liquid nitrogen after the addition of 20% sterile glycerol and were kept at −80 °C for later use.

**Biopanning procedure.** For the phage library preparation, an aliquot of frozen bacterial library was resuspended in 500 μL of 2x YT in the presence of 50 μg·mL⁻¹ carbenicillin. During the exponential phase of growth OD₆₀₀ = 0.6-0.8, bacteria were infected with M13K07 helper phage particles (multiplicity of infection or MOI = 20) for 60 min at 37 °C. A total of 2.5 μM IPTG and 30 μg·mL⁻¹ kanamycin were added and bacteria were grown overnight at 37 °C at 200 rpm. The culture was centrifuged at 16,000g for 10 min at 4 °C, the supernatant was transferred to a new tube, mixed with 1/5 volume of PEG8000 solution and incubated for 5 min at 37 °C. The number of colonies obtained generally depends on pfunkel efficiency and the sequence diversity of the DNA library was estimated by performing Sanger sequencing of 96 randomly picked colonies from the obtained transforms. The library was then purified using Amicon Ultra-Centrifugal Filter Devices (Merck Millipore) with a 30-kDa cut-off according to the manufacturer’s instructions. The typical volume obtained was 20 μL. The purified DNA was immediately used for electrotransformation into TG1 electrocompetent cells (Lucigen).
plates resulting from the last round of panning. The individual colonies were
picked from the agar. For binding determination, 96 colonies were randomly
picked from the agar plate with a pipette. Phages were isolated by precipitation
with PEG/NaCl solution (as in the phage library preparation step) and
resuspended in 1.3 mL of PBT2. Their concentration was determined by an
inverted stand (Leica Microsystems, Mannheim, Germany), using objectives HC
Plan Apo CS 63X oil NA 1.32 and HCX Plan Apo 100X oil NA 1.40. The lasers
were obtained using this method.

The FLIM measurements were done with the LIFA fluorescence lifetime
attachment (Lambert Instrument, Roden, Netherlands), which allows the
generation of lifetime images by using the frequency domain method. This
system consisted of a modulated intensified CCD camera L2 CAM MD, a modulated
light source, a monochromator (Princeton Instruments, Trenton, New Jersey),
and an inverted stand (Leica Microsystems, Mannheim, Germany), using objectives HC
Plan Apo CS 63X oil NA 1.32 and HCX Plan Apo 100X oil NA 1.4 and an
Evolve EMCCD camera (Photometrics, Tucson, USA) or a HQ2 CCD
camera (Photometrics, Tucson, USA). The diode lasers used were at 408, 491, 561
and 638 nm. The 37 °C atmosphere was created with an incubator box and an air
heating system (Life Imaging Services, Basel, Switzerland). This system was
compared to an MetaMorph software (Becker and Hickl). Experiments corresponding to Fig. 2f
were obtained using this method.

The FLIM measurements were done with the LIFA fluorescence lifetime
test as measured by mIRFP670

Comparison Test 2 by 2.

NMR spectroscopy. NMR spectra were recorded at 298 K using Bruker Avance III
700 MHz or 800 MHz spectrometers equipped with a triple resonance gradient
NMR spectroscopy. NMR spectra were recorded at 298 K using Bruker Avance III
700 MHz or 800 MHz spectrometers equipped with a triple resonance gradient
donor

landscape and modulation for each pixel of the image. Consequently, we could
classically detect the

and FLIM images were obtained using this method. For competition
experiments, only cells presenting a high level of expression of the competitor or
control as measured by mIRFP670 fluorescence were taken into consideration.
Datasets were analyzed by an ANOVA test followed by a Tukey’s Multiple
Comparison Test 2 by 2.

NMR spectroscopy. NMR spectra were recorded at 298 K using Bruker Avance III
700 MHz or 800 MHz spectrometers equipped with a triple resonance gradient
standard probe or cryoprobe, respectively. Topspin versions 2.1, 3.2 and 3.5
(Bruker BioSpin) were used for data collection. Spectra processing used
NMRPipe

by following analysis with Sparky 3 (T.D. Goddard and D.G. Kneller,
University of California) or CARA (RL. Keller, ETH Zurich).
Backbone chemical shift assignment of PSD-95. Spectra for the assignment of backbone H^1, 13C, 15N, 1H and 13C nuclei of the first PDZ domain of rat PSD-95-1 were measured by 2D 1H-NMR in a 1:2 mixture of 15N-labeled sample in PBS with 10% D_2O added for the lock. NMR assignment used 2D 15N-HSQC, 3D HNCO, 3D HNCA, 3D CBCA(CO)NH and 3D HNCA(CB) spectra. The backbone assignment is nearly complete, except for missing assignments for the initial Ser-Gly-Ser- remaining from the His-tag, the backbone carbonyl for Asp91, and the amide H^1 and 15N chemical shifts for Asn72, Ser73, Leu75 and Glu122. Also assigned are sidechain 15N^ε, 1H^ε and 13C^ε nuclei from Asn72, Asn85, Asn114 and Asn121, as well as sidechain 15N^β, 1H^β and 13C^β nuclei from Gln107. Chemical shift assignments for PSD-95 PDZ1 were deposited in the Biological Magnetic Resonance Data Bank (BMRB) as entry 27330. An annotated 15N-HSQC is included in Supplementary Fig. 13a.

Spectra for the assignment of backbone H^1, 13C^α, 13C^β, 13C^γ and 15N chemical shifts of the second PDZ domain of rat PSD-95-1 (residues 135–249 with an N-terminal Ser-Gly-Ser- remaining after cleavage by TEV protease) were collected on an 80 μM 1H,13C,15N-labeled sample in PBS with 10% D_2O added for the lock. NMR assignment used 2D 15N-HSQC, 3D TROSY-HNCO, 3D TROSY-HNCA, 3D TROSY-HNCA(CB), 3D TROSY-BCA(CN)CO, 3D TROSY-HC(CO)NH, 3D HNCA(CB), and 3D HNCA(CB) spectra. The backbone assignment is nearly complete, except for missing assignments for the initial Ser-Gly-Ser- remaining from the His-tag, all nuclei for Pro161, the amide H^1 and 15N chemical shifts for Lys168, Leu170 and Ser171. Also assigned are sidechain 15N^β, 1H^β and 13C^β nuclei from Lys118, Lys119, Asn180, Asn187, Asn216, Asn234 and Asn248, as well as sidechain 15N^δ, 1H^δ and 13C^δ nuclei from Glu181 and Gln207. Chemical shift assignments for PSD-95 PDZ2 were deposited in the Biological Magnetic Resonance Data Bank (BMRB) as entry 27310. An annotated 15N-HSQC is included in Supplementary Fig. 13b.

Spectra for the assignment of backbone H^1, 13C^α, 13C^β, 13C^γ and 15N chemical shifts of the tandem PDZ1-PDZ2 domains of rat PSD-95 (residues 61–249 with an N-terminal Ser-Gly-Ser- remaining after cleavage by TEV protease) were collected on a 190 μM 1H,13C,15N-labeled sample in PBS with 10% D_2O added for the lock. NMR assignment used 2D 15N-HSQC, 3D TROSY-HNCO, 3D TROSY-HNCA, 3D TROSY-HC(CO)NH, 3D HNCA(CB), and 3D HNCA(CB) spectra. Assignments are missing for all nuclei in Pro153, Pro154, Lys157, Pro167 and the initial Ser-Gly-Ser- remaining from the His-tag. Amide H^1 and 15N chemical shifts are missing for Asn72, Ser73, Leu75, Ser171, Asn121, Glu122, Ala155, Lys168 and Leu170, and the backbone carboxyl is missing for Asn72, Asp91, Asn121, Ile183, His225 and Glu226. Residues His225, Glu226, Lys157, Pro167 and the initial Ser-Gly- remaining from the His-tag, the backbone carbonyl for Asp91, and the amide 1HN and 15NH chemical shifts for Asn72, Ser73, Leu75 and Glu122. Also assigned are sidechain 15N^ε, 1H^ε and 13C^ε nuclei from Gln107 and Gln207. Chemical shift assignments for PSD-95 PDZ2 were deposited in the Biological Magnetic Resonance Data Bank as entry 27310. An annotated 15N-HSQC is included in Supplementary Fig. 9.

Chemical shift assignment for PSD-95 bound to Xph15 or Xph20. Titration of [15N]PSD-95-12 with 1.2 molar equivalents of natural abundance Xph15 or Xph20 resulted in large changes for numerous crosspeaks in the slow exchange regime, such that it was not possible to unambiguously assign the bound forms due to peak broadening and are indicated in yellow in Fig. 3b.

Combined chemical shift perturbations (Δδ(H,N)) were calculated from the unbound and bound 2D 15N-HSQC spectra using the following equation:

\[
\Delta \delta(H, N) = \left( \Delta \delta_{14N} \right)^2 + \left( \Delta \delta_{15N} \right)^2
\]

where Δδ_{14N} and Δδ_{15N} are the changes in backbone amide chemical shifts for H^1N and 15N, respectively, between the free and bound form of the protein.

Residual dipolar coupling (RDC). The sample contained 160 μM [70%-2H,15N] with 180 μM natural abundance Xph18 in PBS. Reference spectra to measure isotropic 1H,15N used interleaved spin-state-selective TROSY experiments at 700 MHz and 298 K, averaged from two separate measurements. Anisotropy was introduced by using the gel-stretch method by using the 6 mm to 4.2 mm gel-stretch kit from New Era Enterprises. The gel was prepared by mixing 125 μL of 40% 19:1 acrylamide/bisacrylamide with 860 μL of 5% TBE and 0.1% (w/v) ammonium persulfate, and initiating polymerization with 0.05% (w/v) TEMED. After 1 h in the gel chamber, the gel was dialyzed for 48 h at room temperature in distilled water. The dialyzed gel was then cut into two pieces of equal length, and dried overnight on parafilm at room temperature. One of the dried gel pieces was placed in the gel chamber to which was added 500 μL of the same [70%-15N,1H]PSD-95-12:Xph20 complex used for the isotropic NMR spectra. After the sample had entered the gel over 24 h at ~4°C, the excess liquid was removed and the gel was squeezed into an NMR tube open at both ends by using the assembled gel press. The top and bottom plugs were inserted and the 1H,15N + 1D,15N Values were measured by again using interleaved spin-state-selective TROSY experiments, averaged from two separate measurements. Subtraction of average 1J(H,N) from average 1J(H,N) + 1D(N) yielded 1D(N) values for 60 residues (24 from PDZ1, 36 from PDZ2).

Docking-based models. Models were generated by using HADDOCK2.2 as implemented in the HADDOCK2.2 webserver made available by WeNMR. For Xph15, an homology model was first generated by using SWISS-MODEL with the template PDB entry 3RWZ, and the PDZ1 domain of PSD-95 was taken from the PDB entry 3GL. The HADDOCK protocol used the WeNMR GRID-enabled docking server with the Easy interface and 1000 calculated structures. Active
residues were identified by chemical shift perturbation analysis (Supplementary Figs. 14 and 17) and predicted solvent accessibility, with passive residues automatically identified. Active residues included residues 18, 31, 33, 35, 55, 56, 58, 60, 81, 82, 83, 84, 95–97, 118, 119, 121, 124, 143, 145, 147, and 149 from PSD-95–1. From the 1000 calculated structures, 200 structures were further refined in explicit water, and 186 of these final structures (93.0%) were clustered into six clusters, from which the top cluster based on Z-score (−1.8) was taken as the predicted ensemble. For Xph20, the protocol was similar and used an homology model for Xph20 based on PDB entry 3K2M. Active residues were again selected based on chemical shift perturbation (Supplementary Figs. 15 and 18) and predicted solvent accessibility. Active residues on Xph20 included residues 29–31, 33, 57, 58, 60, 61–63, 65, 66, 67, 68, 72, 119, 121, 124, 126, 143, 145, 147, 149 and 150. From the 200 water-refined structures, 174 (87.0%) were clustered into 11 clusters, with the top cluster selected as the predicted ensemble (Z-score of −1.7). For Xph18, the homology model was based on PDB entry 3RWZ but the model calculation strategy was carried out in two steps. Unlike Xph15 and Xph20, initial chemical shift perturbation studies using Xph18 with isolated PSD-95–1 and PSD-95–2 did not reveal binding to either domain. Upon repeating the study with doubled protein concentrations, numerous 1H, 15N crosspeaks for PSD-95–1 displayed perturbation or peak broadening, with still no observed binding to PDZ2 (see Supplementary Fig. 16). Therefore, an initial HADDOCK docking used Xph18 and PDZ2, with active residues for PSD-95–1 including residues 61, 63, 65, 67, 90, 91, 119, 124, 127, 145, 147, 149 and 151. Active residues for Xph18 comprised solvent accessible residues from the BC, DE and FG loops (residues 28, 29, 30, 31, 32, 33, 34, 35, 57, 58, 59, 80, 81, 82, 48, 85, 86, 87, 88 and 89). The main cluster represented 123 of the 200 calculated models, with a HADDOCK score of −115.9 and a Z-score of −2.5. The lowest energy model was then used to generate a structure calculation with ARIA2.3/CNS1.2 for the complete PSD-95–12:Xph18 complex, by maintaining the structure of Xph18:PDZ1 complex with synthetic NOE distances derived from the HADDOCK model. Additional NOE distances and dihedrals were obtained for all non-hydrogen atoms in PDB entry 3GSL, while leaving linker residues 151 to 155 free of constraint. The NET-MD protocol was included for 700,000 steps between PDZ2 and Xph18. Active residues for PDZ2 included 155, 156, 157, 159, 211, 213, 214, 216, 218, 220, 240, 242 and 244, with no specification for a particular region on Xph18. Finally, 60 RDC values for the Xph18-bound conformation of PSD-95–12 were included, with rhombicity of 0.4 and magnitude −16. The lower energy structures were formed in a cluster which was taken as the final ensemble of the Xph18:PSD-95–12 complex. HADDOCK docking statistics also appear in Supplementary Fig. 22.

Surface plasmon resonance. SPR measurements were performed on a BiACore X100 or a BiACore T200 instrument with analysis temperature set to 25°C. CAP Sensor chips (GE Healthcare) that allow the reversible capture of biotinylated ligands as an immobilization system were used. During the experiments, reagents were kept at room temperature or at 6–10°C for the X100 and T200, respectively. Two experimental configurations were used for the study of Xph clones, either immobilized biotinylated Xph clones to monitor the interaction with tandem PDZ domains or the opposite. Immobilization levels were optimized to reflect a compromise between minimal surface density of the ligand to avoid rebinding effects and generation of interpretable sensorgrams when possible. Sensorsgrams were collected using single cycle kinetics as Xph clones or tandem PDZ domains were injected at various concentrations (using two-fold dilutions unless otherwise stated) in PBS containing 500 mM NaCl (pH 7.4), supplemented with 1% BSA. For each concentration, the initial unbound and the saturating states were monitored twice. To determine the corresponding affinities (apparent $K_d$), curves were fitted using a non-linear regression function $78$ with GraphPad Prism v7.04 after normalizing the values of each protein series between the initial unbound and the saturating states.

For competitive titrations, experiments were designed such that the starting polarization value represent 75% of the maximal shift of the direct titrations. For the divalent stargazin ligand, PSD-95–12, SAP97–12, PSD-93–12 and SAP102–12 were used at respective concentrations of 206, 800, 118 and 982 nM. Tandem PDZ domains, bound to the fluorescein-labelled stargazin divalent peptide (50 nM), were titrated against a range of increasing concentrations of the different recombinant PDZ domains in a 100-μl final volume. Fluorescence polarization was measured in millipolarization units ($\mu$P$m$) at an excitation wavelength of 485 ± 5 nm and an emission wavelength of 520 ± 5 nm using a POLARStar Omega (BMG Labtech) microplate reader. Titrations were conducted at least in duplicate and measured twice. To determine the corresponding affinities (apparent $K_d$), curves were fitted using a non-linear regression function $78$ with GraphPad Prism v7.04 after normalizing the values of each protein series between the initial unbound and the saturating states.

Competitive pull-down assays. The competitive pull-down assays and control experiments were performed using a KingFisher Duo system in 96 deep-well plates (see Supplementary Note 3 for a detailed protocol). Streptavidin-coated magnetic beads (Dynabeads M-280; Invitrogen) were incubated in PT buffer with 0.5% BSA (1 × PBS with 500 mM NaCl, pH 7.4 ± 0.1 °C 20 ± 1 °C) and incubated with 120 pmol of biotinylated [Stg 15]$_2$ or biotin in PT buffer. The beads were washed with PT buffer with 0.5% BSA and incubated next with 180 pmol of purified tandem PDZ domains. The tandem PDZ domains–ligands complexes were then incubated with the beads next to the magnetic bead purification column. Unbound peptides were then washed using the ImageLab software (lanes and bands detection) and the intensity of the band corresponding to the tandem PDZ domains without competitor was taken as the 100% intensity. The relative intensities were reported in GraphPad Prism 7.02 software in function of the logarithm of the peptide concentration. A typical example is shown on equation “One site – Fit $K_d$” with constrained values for the concentrations of labelled ligand (tandem PDZ domains, in nanomolar) and for the equilibrium corresponding heats of dilution. The experimental thermograms were baseline corrected, blank subtracted and the peaks were integrated to determine the binding heats. To determine the binding hindrance, the binding enthalpies were corrected by subtraction of the heat of dilution using corrected, blank subtracted and the peaks were integrated to determine the binding...
Dissociation constant of the labelled ligand (Kd) for [85S]cGMP determined by fluorescence polarization in nanomolar).

**Pull-down on brain lysates.** Adult rat brain lysates were produced as previously described. Brains were obtained from adult (2-3 months old) Sprague-Dawley rats raised in the animal facility of Bordeaux University B 33 063 917. Animals were killed by decapitation after isoflurane anesthesia (5%, 3 min), in accordance with the European 2010/63/EU directive and approved by the Bordeaux University Ethics Committee (CE50). Briefly, frozen brains (2 x 1.5 g) were thawed in 20 mL of ice-cold dissecting buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% CHAPS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA) containing a protease inhibitor mixture (1:1000; Protease Inhibitor Cocktail set III; Calbiochem) for about 5 min and cut into small pieces. The tissues were homogenized using a glass/teflon homogenizer. Homogenates were centrifuged at 7500 × g for 25 min at 4°C to remove cell debris. The supernatant was aliquoted and stored at −80°C until the affinity-based isolation (pull-down) experiments were performed.

For Western blot analysis, the pull-down assay was performed using a KingFisher Duo system in 96-deep-well plates. Streptavidin-coated magnetic beads (50 μL of Dynabeads M-280 Streptavidin per well) were equilibrated in RIPA buffer with 0.5% BSA. Beads were then incubated with the Xph20-ETWV–lysate pre-mix and were washed twice with 1 mL of RIPA–BSA and twice with 1 mL of RIPA without BSA and finally transferred to 2 × sample loading buffer. The samples were heated for 5 min at 75°C. Western blot analyses were performed as described for the anti-His Co-IP.

For mass-spectrometry analysis, the pull-down assay was performed using a KingFisher Duo system in 96 deep-well plates. As a pre-clearing step, brain lysate (200 μL per well) was first incubated with 50 μL of streptavidin-coated magnetic beads and 1.5 mL of sample loading buffer. After 10 min of incubation, the magnetic beads were captured and the cleared brain lysate supernatant was recovered and placed in new low binding tubes into which biotinylated proteins were added at a concentration of 60 nM (12 pmol of biotinylated Xph20–ETWV). The samples were heated for 5 min at 75°C. The samples were equilibrated in RIPA buffer with 0.5% BSA. Beads were then incubated with the proteins–lysat pre-mix and were washed three times with 1 mL of RIPA–BSA and nine times with 1 mL of RIPA without BSA and finally transferred to 2 × sample loading buffer. The samples were heated for 5 min at 75°C.

**Immunoprecipitation on hippocampal neuron culture.** For IP experiments, hippocampal neurons (E18) were plated at a density of 300,000 cells/well in 6-well culture plates in Neurobasal medium supplemented with SM1 supplement (Stemcell). 2 mM Glutamine and 10% horse serum. At 3 DIV, the horse serum was exchanged every 3 days by replacing half of the supplemented medium by BrainPhys neuronal medium (Stemcell), 2 mM Glutamine and 10% horse serum. At 3 DIV, the horse serum was exchanged every 3 days by replacing half of the supplemented medium by BrainPhys neuronal medium (Stemcell), 2 mM Glutamine and 10% horse serum. At 3 DIV, the horse serum was exchanged every 3 days by replacing half of the supplemented medium by BrainPhys neuronal medium (Stemcell), 2 mM Glutamine and 10% horse serum. At 3 DIV, the horse serum was exchanged every 3 days by replacing half of the supplemented medium by BrainPhys neuronal medium (Stemcell), 2 mM Glutamine and 10% horse serum.

**Photo-crosslinking experiments.** For determining the selectivity, photo-crosslinking reactions were performed in PBS buffer at room temperature. Xph20-ETWV* (15 μM) was mixed with one, two or three tandem PDZ domains protein (s) in a large excess (45 μM each) in a final volume of 20 μL in 0.2 mL clear polypropylene PCR tubes (Bio-Rad). Long-wave UV irradiation (λ = 365 nm) was performed in a Vilber Lourmat® Biolink® BLX UV Crosslinker for 10 min (0.120 J cm−2). Samples were analysed by SDS-PAGE using 4–20% gradient Miniprotein TGX Precast gels (Bio-Rad) and colloidal blue staining. The gel image was obtained using a ChemiDoc XRS + imager (Bio-Rad).

For proteomic analysis of the region of interaction of the binding motif, photo-crosslinking reactions were performed in PBS buffer at room temperature. Xph20-ETWV* at a final concentration of 10 μM was mixed with the four DLG tandems (20 μM each) in a final volume of 20 μL in 0.2 mL clear polypropylene PCR tubes (Bio-Rad). Samples UV irradiation and SDS-PAGE analysis were conducted as described above.

For proteomic analysis of the selectivity, photo-crosslinking reactions were performed in PBS buffer at room temperature. Xph20-ETWV* at a final concentration of 10 μM was mixed with the four DLG tandems (20 μM each) in a final volume of 20 μL in 0.2 mL clear polypropylene PCR tubes (Bio-Rad). Samples UV irradiation and SDS-PAGE analysis were conducted as described above.

**Proteomic analyses.** Protein samples were solubilized in Laemli buffer and were deposed onto SDS-PAGE. After colloidal blue staining, bands were cut out from the gel subsequently cut in 1 mm × 1 mm pieces. Gel pieces were destained in 25 mM ammonium bicarbonate 50% acetonitrile (ACN), rinsed twice in ultrapure water and shrunk in ACN for 10 min. After ACN removal, gel pieces were dried at room temperature, covered with the trypsin solution (10 ng μL−1 in 50 mM NH4HCO3), rehydrated at 4°C for 10 min, and finally incubated overnight at 37°C. Spots were then incubated in 100 mM NH4HCO3, 4% (v/v) acetonitrile, and 1% (v/v) formic acid at room temperature with rotary shaking. The supernatant was collected, and an H2O/ACN/HCOOH mixture ((47:5:2:5) extraction solution) was added onto gel slices for 15 min. The extraction step was repeated twice. Supernatants were pooled and concentrated in a vacuum centrifuge to a final volume of 30 μL. Digests were finally acidified by addition of 1.4 μL of formic acid and stored at −20°C.

Pep-tide mixture was analysed on an Ultimate 3000 nanoLC system ( Dionex, Amsterdam, The Netherlands) coupled to an Electrospray mass spectrometer.

For the analysis of pull-down material, 10 μL of peptide digestions were loaded onto a 300-μm-inner diameter × 5-mm C18 PepMapTM trap column (LC Packings). For 1.4 pmol, the peptide ions were enriched by photo-irradiation for 50 ms using a 300-nm monochromator. The peptides were eluted with a linear peptide sample loading buffer (1.6 μM) and were added at a concentration of 60 nM (12 pmol of biotinylated Xph20–ETWV). The samples were heated for 5 min in RIPA–BSA and nine times with 1 mL of RIPA without BSA and finally transferred to 2 × sample loading buffer. The samples were heated for 5 min at 75°C. The samples were equilibrated in RIPA buffer with 0.5% BSA. Beads were then incubated with the proteins–lysat pre-mix and were washed three times with 1 mL of RIPA–BSA and nine times with 1 mL of RIPA without BSA and finally transferred to 2 × sample loading buffer. The samples were heated for 5 min at 75°C.
Clustering assay of Stargazin on the same lane was performed using the average intensity of each of the IP fraction of PSD-95 co-immunoprecipitated normalized to the IP fraction of an anti-rabbit IgG, light chain specific immunoprecipitations were washed three times with lysis buffer and eluted in SDS-(Invitrogen, 10004D) pre-coated with 2 μg of rat anti-HA antibody (Sigma-Aldrich, Biochemistry & Biotechnology, General Biochemistry) 79 was used for the detection of protein amount. Protein ratios were calculated based on unique peptides intensities. Normalization was performed based on total protein amount. Protein ratios were calculated based protein abundancies. A statistical test (ANOVA) was calculated based protein individual values. Quantitative data were considered for proteins quantified by a minimum of two peptides, fold changes above 2 and a statistical p-value < 0.05.

Immunoprecipitation assay. 293T cells (ECACC-12020101) were plated at a density of 300,000 cells per well in 6-well culture plates in DMEM medium supplemented with 2 mM glutamax and 10% FBS. The day after plating, co-transfection of PSD-95 H130V, H372V eGFP and either Xph20-ETWV or Xph20-ETWV was performed using Xtreme GENE HP DNA transfection reagent (Roche) as per the manufacturer’s instructions. A 1:5 molar ratio between PSD-95, Stargazin and the ligands was used.

After 18–20 h of expression, cells were lysed on ice with 200 μL per well of lysis buffer (125 mM NaCl, 25 mM HEPES, 1% NP40, 1 × protease inhibitor cocktail (Calbiochem) and 2 × Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific)). Cell lysates were collected, homogenated with a micropestle and spun down at 15,000 g for 10 min. Proteins concentration of each lysate was quantified using BCA reagent (Thermo Fisher Scientific). For co-IP, 50 μg of protein per condition were incubated overnight at 4 °C with 20 μL of Dynabeads protein-G (Invitrogen, 10004D) pre-coated with 2 μg of rat anti-HA antibody (Sigma-Aldrich, 1 867 020-A) and Arg with 3 and 2 missed cleavages, respective minimum peptide length; 5, static modification: acetylation of N-term by IAA, variable modification: oxidation of C, cross-linker: AzidoPhe (-N3), precursor and fragment mass accuracy: 10 ppm, signal-to-noise ratio: 2, precursor mass correction activated, prescore cut-off at 10 intensity, FDR cut-off: 5%.

Clustering assay. COS-7 cells were cultured in DMEM (GIBCO/BRL) supplemented with 10% FBS, 100 units/mL 1,000 U/mg penicillin and 100 μg/mL 3 streptomycin. Cells were electroporated with PSD-95-eGFP [23], AP-LRRTM2, Brr3D9 (gift from Alice Ting, Stanford University, Addgene plasmid #20856) and either Xph20-ETWV or Xph20-ETWV with the membrane was incubated overnight with rabbit anti-GFP (1:5000, Abcam, ab290) and anti-mCherry (1 1000, Abcam, ab167453). Following incubation with an anti-rabbit IgG, light chain specific IRDye-800W secondary antibody (1 15 000, Jackson Immunoresearch, 211-652-171), blots were imaged using an Odyssey Imaging System. Analysis was done using the Odyssey software and quantification of the IP fraction of PSD-95 co-immunoprecipitated normalized to the IP fraction of Stargazin on the same lane was performed using the average intensity of each single band on line range values.

Cells expressing AP-LRRTM2 were detected using mSA-STAR635P and kept on the setup in Tyrode’s medium at 37 °C for <1 h. The nuclear miRFP670 was used to identify co-transfected cells and constructs expression level. Images of LRRTM2 and PSD-95 were acquired using the MetaMorph software (Molecular Devices) under the same acquisition parameters. Analysis was performed using a custom-made macro on MetaMorph. Briefly, images of AP-LRRTM2 and PSD-95-eGFP were segmented using a wavelet-based segmentation method to identify clusters. Segmented images from the AP-LRRTM2 signal were used to draw regions of LRRTM2 clusters. These regions were transferred onto the segmented images of PSD-95 to determine the percentage of LRRTM2 clusters containing PSD-95 (apposed and colocalized clusters). Datasets were analysed by a non-parametric ANOVA test followed by a Dunn’s Multiple Comparison Test 2 by 2.

Molecular graphics. All figures depicting the protein structures were generated with PyMOL (Version 2.0.7 Schrödinger, LLC).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Backbone H, Cα and N chemical shift assignments for PSD-95-12, PSD-95-1 and PSD-95-2 were deposited in the Biological Magnetic Resonance Data Bank (BMRB) as entries 27309, 27309 and 27310, respectively. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD015313 (cellular target identification) and PXD015366 (photocrosslinking). Key plasmids constructed in this study are available directly from Addgene at https://www.addgene.org/Matthieu_Samuelsen/. The authors declare that the data supporting the findings of this study are available within the paper, its Supplementary Information file and Source Data file. Additional raw data and other materials are available from the corresponding authors upon reasonable request.

Received: 12 August 2018; Accepted: 14 September 2019;
Published online: 04 October 2019

References
1. Arkin, M. R., Tang, Y. & Wells, J. A. Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. Chem. Biol. 21, 1102–1114 (2014).
2. Bakail, M. & Ochsenbein, F. Targeting protein–protein interactions, a wide open field for drug design. C. R. Chim. 19, 19–27 (2016).
3. Milroy, L. G., Grossmann, T. N., Hennig, S., Brunsved, L. & Ottmann, C. Modulators of protein-protein interactions. Chem. Rev. 114, 4695–4748 (2014).
4. Laraia, L., McKenzie, G., Spring, D. R., Venkitaraman, A. R. & Huggins, D. J. Overcoming chemical, biological, and computational challenges in the development of inhibitors targeting protein-protein interactions. Chem. Rev. 22, 689–703 (2015).
5. Corbi-Verge, C. & kim, P. M. Motif mediated protein-protein interactions as drug targets. Cell Commun. Signal. 14, 8 (2016).
6. Andreani, J. & Guerri, R. Evolution of protein interactions: from intermolecular to interfaces. Arch. Biochem. Biophys. 554, 65–75 (2014).
7. Teppa, E., Zea, D. J. & Marino-Budjde, C. Protein-protein interactions leave evolutionary footprints: high molecular coevolution at the core of interfaces. Protein Sci. 26, 2438–2444 (2017).
8. Jin, J. et al. Eukaryotic protein domains as functional units of cellular networks. Sci. Signal. 7, ra46 (2014).
9. Tonikian, R. et al. A specificity map for the PDZ domain family. PLoS Biol. 6, e239 (2008).
10. Xin, X. et al. SH3 interactome conserves general function over specific form. Mol. Syst. Biol. 9, 652 (2013).
11. Geifler, D. Uncovering new aspects of protein interactions through analysis of specificity landscapes in peptide recognition domains. FEBS Lett. 586, 2764–2772 (2012).
12. Teyra, J., Sidhu, S. S. & Kim, P. M. Elucidation of the binding preferences of peptide recognition modules: SH3 and PDZ domains. FEBS Lett. 586, 2631–2637 (2012).
13. Won, S., Levy, J., Nicoll, R. A. & Roche, K. W. MAGUKs multifaceted synaptic organizers. Curr. Opin. Neurobiol. 43, 94–101 (2017).
14. Zhu, J., Shang, Y. & Zhang, M. Mechanistic basis of MAGUK-organized synaptic organizers. PLoS Biol. 15, e2002114 (2017).
15. Gardoni, F., Marcello, E. & Di Luca, M. Postsynaptic density-membrane associated guanylate kinase proteins (PSD-MAGUKs) and their role in CNS disorders. Nat. Rev. Neurosci. 16, 234–333 (2015).
16. Coley, A. A. & Gao, W. J. PSD95: a synaptic protein implicated in schizophrenia or autism? Prog. Neuropharmacol. Biol. Psychiatry 82, 187–194 (2018).
17. Cook, D. J., Teves, L. & Tymianski, M. Treatment of stroke with a PSD-95 inhibitor in the gyrencephalic primate brain. Neuron 48, 213–217 (2012).
18. Emes, R. D. & Grant, S. G. Evolution of synapse complexity and diversity. Annu. Rev. Neurosci. 35, 111–131 (2012).
19. Grant, S. G. The molecular evolution of the vertebrate behavioural repertoire. Philos. Trans. R. Soc. Lond. B Biol. Sci. 370, 20150051 (2016).
20. Nithanathanarajah, J. et al. Synaptic scaffold evolution generated components of a shared cognitive complexity. Nat. Neurosci. 16, 16–24 (2013).
21. Sun, Q. & Turrigiano, G. G. PSD-95 and PSD-93 play critical but distinct roles in synaptic scaling up and down. J. Neurosci. 31, 6800–6808 (2011).
22. Howard, M. A., Elias, G. M., Elias, L. A., Swat, W. & Nicoll, R. A. The role of SAP97 in synaptic glutamate receptor dynamics. Proc. Natl Acad. Sci. USA 107, 3805–3810 (2010).
23. Levy, L. J., Chen, X., Reece, T. S. & Nicoll, R. A. Synaptic consolidation normalizes AMPAR quantal size following MAGUK Loss. Neuron 87, 534–548 (2015).
24. Xu, W. PSD-95-like membrane associated guanylate kinases (PSD-MAGUKs) and synaptic plasticity. Curr. Opin. Neurobiol. 21, 306–312 (2011).
25. Sainlos, M. et al. Dynamic and specific interaction between synaptic NR2-NMDA receptor and PDZ proteins. Proc. Natl Acad. Sci. USA 107, 19561–19566 (2010).
26. Nissen, K. B. et al. Targeting protein-protein interactions with trimeric ligands: high affinity inhibitors of the MAGUK protein family. PLoS ONE 10, e0136685 (2015).
27. Grillo-Bosch, D., Choquet, D. & Sainlos, M. Inhibition of PDZ domain-mediated interactions. Drug Discov. Today Technol. 10, e531–e540 (2014).
28. Koide, A. & Koide, S. Monobodies: antibody mimics based on the scaffold of the fibronectin type III domain. Methods Mol. Biol. 352, 95–109 (2007).
29. Sha, F., Salzman, G., Gupta, A. & Koide, S. Monobodies and other synthetic binding proteins for expanding protein science. Nat. Rev. Drug Discov. 16, 910–924 (2017).
30. Wójcik, J. et al. A potent and highly specific FN3 monobody inhibitor of the Ab1 SH2 domain. Nat. Struct. Mol. Biol. 17, 519–527 (2010).
31. Schierle, C. F. et al. The DnBa signal sequence directs efficient, cotranslational export of passenger proteins to the Escherichia coli periplasm via the signal recognition particle pathway. J. Bacteriol. 185, 5706–5713 (2003).
32. Firnberg, E. & Ostermeier, M. PFunkel: efficient, expandable, user-defined mutagenesis. PLoS ONE 7, e52031 (2012).
33. Topinka, J. R. & Bredt, D. S. N-terminal palmitoylation of PSD-95 regulates cellular cognitive complexity. Int. J. Cancer 129, 2013–2024 (2011).
34. Firnberg, E. & Ostermeier, M. PFunkel: efficient, expandable, user-defined mutagenesis. PLoS ONE 7, e52031 (2012).
35. Jacobs, S. A. et al. Design of novel FN3 domains with high stability by a consensus sequence approach. Protein Eng. Des. Sel. 25, 107–112 (2012).
36. Coen, P. J., Karantan, E. et al. Molecular recognition properties of FN3 monobodies that bind the Src SH3 domain. J. Am. Chem. Soc. 130, 112–120 (2008).
37. van Zundert, G. C. P. et al. The HADDOCK2.2 web server: user-friendly biomolecular NMR. J. Struct. Biol. 213, 178–185 (2018).
38. Doss, B., Casamayor, F. et al. Inclusion of an upstream transcriptional terminator in phage display vectors abolishes background expression of toxic fusions with coat protein g3p. Gene 178, 71–74 (1996).
39. Karatan, E. et al. Molecular recognition properties of FN3 monobodies that bind the Src SH3 domain. J. Biol. Chem. 281, 38799–38807 (2006).
40. Steiner, D., Forrer, P., Stumpf, M. T. & Pluckthun, A. Signal sequences directing cotranslational translocation expand the range of proteins amenable to phage display. Nat. Biotechnol. 24, 823–831 (2006).
41. Howarth, M. et al. Monovalent, reduced-size quantum dots for imaging receptors on living cells. Nat. Methods 5, 397–399 (2008).
42. Studier, F. W. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. 41, 207–234 (2005).
43. Sorrentino, A. E., Askin, S. P. & Schaffer, P. M. In-gel detection of bivalent protein conjugates with a green fluorescent streptavidin probe. Anal. Methods 7, 2087–2092 (2015).
44. Tonikian, R., Zhang, Y., Boone, C. & Siddhu, S. S. Identifying specificity profiles for peptide recognition modules from phage-displayed peptide libraries. Nat. Protoc. 2, 1368–1388 (2007).
45. Cock, P. J. et al. Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics 25, 1422–1423 (2009).
46. Enright, A. J., Van Dongen, S. & Ouzounis, C. A. An efficient algorithm for large-scale detection of protein families. Nucleic Acids Res. 30, 1575–1584 (2002).
47. Sievers, F. et al. Fast, scalable generation of high quality protein multiple sequence alignments using Clustal Omega. Mol. Biol. Evol. 31, 158 (2014).
48. Delaglio, F. et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293 (1995).
49. Tycko, R., Blanco, F. J. & Ishii, Y. Alignment of biopolymers in strained gels: a new way to create detectable dipole–dipole couplings in high-resolution biomolecular NMR. J. Am. Chem. Soc. 122, 9340–9341 (2000).
50. Dominguez, C., Boelens, R. & Bonvin, A. M. HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. J. Am. Chem. Soc. 125, 1731–1737 (2003).
51. van Zundert, G. C. P. et al. The HADDOCK2.2 web server: user-friendly integrative modeling of biomolecular complexes. J. Mol. Biol. 428, 720–726 (2017).
52. Wassenaar, T. A. et al. WeNMR: structural biology on the grid. J. Grid Comput. 10, 743–767 (2012).
53. Chung Bryan, H. et al. A systematic family-wide investigation reveals that >30% of mammalian PDZ domains engage in PDZ-PDZ interactions. Chem. Biol. 20, 1433–1444 (2013).
54. Pazos, E. et al. Rational design of a cyclin A fluorescent peptide sensor. Org. Biomol. Chem. 9, 7629–7632 (2011).
Acknowledgements
This research was financially supported by grants from the Centre National de la Recherche Scientifique, the Conseil Régional de la Nouvelle Aquitaine, the National Infrastructure France Bioimaging (grant ANR-10INBS-04-01), the Agence Nationale de la Recherche (CheMePPI, ANR-13-B807-0019-01) to C.M., C.P. and M.S., the European Research Council advanced grants nano-dyn-syn (232942) and ADOS (339541) to D.C., the Labex BRAIN (ANR-10-LABX-43) to C.R. and a Marie-Curie postdoctoral training grant to D.G. B. (neuroCHEMbiotools, FP7-PEOPLE-2010-IEF project #273817). We also thank the Biochemistry and Biophysics Core Facility of the Bordeaux Neurocampus funded by the Labex BRAIN (ANR-10-LABX-43) and J.M. Blanc and Y. Ruffin for technical assistance as well as the Structural Biophysics-Chemistry plateform (UMS3033/US001) of the Institut Européen de Chimie et Biologie (Pessac, France) for access to the T200 Biacore instrument and Laetitia Minder for technical assistance as well as the Structural Biophysics-Chemistry plateform (UMS3033/US001) of the Institut Européen de Chimie et Biologie (Pessac, France) for access to the T200 Biacore instrument and Laetitia Minder for technical assistance and the IINS cell culture facility and Emeline Verdier for technical assistance. Financial support from the IR-RMN-THC Fr3050 CNRS for conducting the research is gratefully acknowledged. The FP7 WeNMR (project #261572), H2020 West-Life (project #675858) and the EOSC-hub (project #777536) European e-Infrastructure projects are acknowledged for the use of their web portals, which make use of the EGI infrastructure with the dedicated support of CESNET-MetaCloud, INFN-PADOVA, NCG-INGRID-PT, TW-NCHC, SURFsara and NIKHEF, and the additional support of the national GRID initiatives of Belgium, France, Italy, Germany, the Netherlands, Poland, Portugal, Spain, UK, Taiwan and the US Open Science Grid.

Authors contribution
C.R., C.D.M. and M.S. designed the research and wrote the article. C.R. generated the library and performed phage display selections and biophysical experiments with the help of C.B., C.G., I.G., S.Cr., S.A., C.T. and D.G.B. C.D.M. and K.M. designed the NMR experiments, performed all NMR experiments and analysed the data. C.B., V.P. and C.P. performed the FRET/FLIM experiments. I.C. developed and performed the cellular LRRTM2 binding assay. C.B. performed the immunoprecipitation experiments. F.W.J.T. and B.D. developed the sequencing analysis tool. D.G.B. synthesized the divalent ligands. S.C.l. performed the proteomic analysis. D.C. provided intellectual input, material and financial support. C.D.M. and M.S. coordinated and oversaw the research project. All authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-12528-4.

Correspondence and requests for materials should be addressed to C.D.M. or M.S.

Reprints and permission information is available at http://www.nature.com/reprints

Peer Review Information Nature Communications thanks Yves Nominé, and other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019