Published in final edited form as:

Biochem Biophys Res Commun. 2020 April 09; 524(3): 595–607. doi:10.1016/j.bbrc.2020.01.114.

Redefining PTB domain into independently functional dual cores

Jun Zhang*, Akhil Padarti, Xiaoting Jiang, Johnathan Abou-Fadel

Department of Molecular and Translational Medicine (MTM), Texas Tech University Health Science Center El Paso, El Paso, TX, 79905, USA

Abstract

Current understanding of phosphotyrosine binding (PTB) domain is limited. Recently, we revealed a novel atypical phosphotyrosine binding (aPTB) domain in CCM2, making it a dual PTB domain-containing protein. Since aPTB domain is only 1/3 of the size of typical PTB domain, we explored the possibility to decrease the size of PTB domain and demonstrate that the typical PTB domain can be divided into two similarly structural and functional cores that can independently bind to NPXY motif. Further, we reduced each PTB core into a minimum core motif (mCore) which is the functional unit of PTB domains and structurally similar to the novel aPTB domain. Based on structural data, we developed several *cis*- and *trans*-inhibitors for NPXY binding scheme, with potential applications for therapeutic strategies in human health conditions.

Keywords

NPXY motifs; Phosphotyrosine binding domain (PTB) and atypical PTB (aPTB) domains; PTB core; Minimal PTB core motif

1. Introduction

Phosphotyrosine binding (PTB) domain was initially the second largest group of protein domains, behind SH2 domain, binding to the phosphotyrosine residue. PTB domain containing proteins play major roles in various cellular signaling cascades [1], however, current understanding of PTB domain is limited [2,3]. Considering its broad role in signal transduction, more efforts are needed to elucidate its binding mechanism with NPXY motifs. Recently, we demonstrated that some CCM2 isoforms contain two PTB domains, one typical (PTB) and one atypical (aPTB), making CCM2 a dual PTB domain-containing protein [4]. Here, we report that the typical PTB domain can be divided into halves, as two similarly functional cores (N-terminal portion PTB-core-1 and C-terminal portion PTB-core-2) that can each independently bind to NPXY motif. Through mutagenesis, we reduced each PTB -core into the minimum core motif (mCore) which is the basic essential structure of the functional component of PTB-core and structurally similar to the core of the aPTB domain identified in the recent report [4]. Based on structural data, we have developed

*Corresponding author. Department of Molecular and Translational Medicine (MTM), Texas Tech University Health Science Center El Paso, 5001 El Paso Drive, El Paso, TX, 79905, USA. jun.zhang2000@gmail.com (J. Zhang).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2020.01.114>.

several *cis*- and *trans*-inhibitors for PTB-NPXY binding scheme, which might have great potential applications for future therapeutic strategies in cancer and vascular-related human conditions.

The data gained from this study would revolutionize the current paradigm of PTB/PH domain structure and function, leading to better understanding of the molecular mechanism between PTB/PH - NPXY interactions, and provide new insights into the roles of membrane receptors in signal transduction cascades. The novel discovery of PTB-core may also lead to new therapeutic applications.

2. Materials and methods

2.1. Plasmid construction and yeast two-hybrid (Y2H) analysis

Fragments from PTB/PH domains (Suppl. Table 1), cytoplasmic domains of β integrins (ITGBs) and receptor tyrosine kinases (RTKs) (Suppl. Table 2) and NPXY-containing fragments of CCM1 (Suppl. Table 3) were amplified as described [5]. For Y2H, both GAL4 binding domain and activation domain fusion constructs were assembled, as described [5,6]. For *in vitro* protein binding assays, PTB domain and NPXY motif constructs were assembled into pcDNA3.1/V5/HIS-TOPO and pcDNA4/HisMax-TOPO vectors (Invitrogen) for mammalian expression and pGEX-4T-1 (GE), pBAD-TOPO (Invitrogen), and pCOLDII (TaKaRa) vectors for bacterial expression [7].

In Y2H analysis, binding was determined by β -galactosidase liquid assay in selective (–His/–Ade) medium and is calculated as Miller units. To minimize intergroup measurement variation, the measurement of each transformant was normalized and converted to relative β -galactosidase activity (RBGA) as described [4–7].

2.2. Protein preparation, co-immunoprecipitation (CO-IP), and modeling of tertiary structures

Proteins were expressed in BL 21 cells for GST-tagged (GE) or LMG194 cells for HIS-tagged (TaKaRa) constructs (Invitrogen) for *in-vitro* CO-IP experiments. Initially, potential target proteins were expressed with TNT® Quick Coupled Transcription/Translation and *E. coli* S30 Extract Systems (Promega) with S35-labeled methionine and confirmed by Western blots. *In vitro* CO-IP was performed as previously described [4–6].

Recombinantly expressed proteins were purified utilizing combinational chromatography methods; GST-tagged fragments from PTB/PH domains were purified with GSTrap HP column (GE), while HIS-tagged NPXY motifs were purified using HiTrap TALON column (GE), followed by size-exclusion chromatography on S100-HR column (GE) as described [4,7].

The molecular modeling of tertiary structure of PTB/PH domains was established by Iterative Threading ASSEmbly Refinement (I-TASSER) [8]. RasMol (version 2.7.5) was used for the structure visualization as described [4,7].

2.3. Label-free measurement of molecular interactions

Binding interactions between PTB/PH domains and NPXY motifs were quantified by three label-free detection platforms; Bio-Layer Interferometry (BLI), Surface Plasmon Resonance (SPR) and Isothermal Titration Calorimetry (ITC) as described [4,7].

2.4. Statistical analysis

For multiple comparisons, one-way Analysis of Variance (ANOVA) was used to detect the differences in the mean values among the treatment groups. All pairwise multiple comparison procedures were used to test differences between each group as described before [4–6].

3. Results

3.1. CCM2 isoforms with truncated PTB domain can interact with multiple NPXY motifs

NPXY motifs in the cytoplasmic side of membrane proteins (Suppl. Table 2) serve as binding targets for CCM2 to explore their interactions with CCM2 isoforms. CCM2 isoforms display a broad range of interaction with many cytoplasmic tails of β integrins through Y2H (Fig. 1A). Longer CCM2 isoforms contain *bona fide* typical PTB domain (PTB) structure (encompassing exons 2, 3, 4, 5) and atypical PTB domain (aPTB) (encompassing exons 6 and 6A) [4]. Intriguingly, several isoforms (CCM2-402, 102, 107, 117, 210, 1209) contain a partial or none of the first PTB domain (missing some of exons 2, 3, 4, 5, or all of them) but retain binding to NPXY motifs (Fig. 1A). Mutagenesis further confirmed this binding (Fig. 1B). The shortest NPXY-binding CCM2 isoform, CCM2-402, having an incomplete first PTB domain and absent aPTB domain, showed an increasing trend of binding to constructs with an increasing number of NPXY tandem motifs (0-12) (Fig. 1C). This suggests the entire PTB domain isn't needed for NPXY motif binding (Fig. 1A–C). Therefore, a partial PTB domain suffices for binding to NPXY.

3.2. PTB domain contains two functional cores that can independently interact with NPXY motifs

A region of N-terminal PTB domain was absent in some isoforms (601, 101, and 201), while the C-terminal portion of PTB domain is absent in other isoforms (102, 117, and 210), suggesting either N-terminal or C-terminal portion alone is sufficient for NPXY binding [4]. The structure of PTB domain is a palindromic β -barrel formed by two anti-parallel β -sheets (4 β -strands enclosed with one α -helix) [7]. Therefore, we hypothesized that PTB domain contains two redundant and functionally independent cores that can each bind to NPXY motifs. We divided the first PTB domain of CCM2 in the middle into N-terminal half termed PTB-core-1 (Suppl. Figs. 1A1 and 1A2) and C-terminal half termed PTB-core-2 (Suppl. Figs. 1B1 and 1B2). Each core (one α -helix and one β -sheet) was tested for potential interactions with NPXY motifs. Many PTB-cores displayed binding to protein fragments containing NPXY motifs through β -galactosidase assays (Fig. 1D), which can be further visualized through plate-based yeast two-hybrid assays (Fig. 1E). Mutagenesis confirmed this binding for CCM1 (Fig. 1F). Several PTB cores showed increasing binding affinity to constructs with increasing number of NPXY tandem motifs (0-12), validating each core's

sufficiency to bind NPXY motifs. Interestingly, these PTB-cores prefer to bind either 2 or 4 NPXY motif tandem repeats, suggesting that these NPXY motif repeats might have favorable conformation for PTB core binding (Fig. 1G).

3.3. PTB-core-NPXY interaction validated by in vitro co-immunoprecipitation (Co-IP)

Through Y2H, PTB domain is shown to contain two functional cores that can independently interact with NPXY motifs and are supported by *in vitro* Co-IP. Several CCM2 isoforms, with partial PTB domains, can pull-down cytoplasmic tails of β integrins (right panel, Fig. 2A); likewise, cytoplasmic tails of β integrins can pulldown CCM2 isoforms with partial PTB domains (left panel, Fig. 2A), supporting truncated PTB domains ability to interact with NPXY motifs (Fig. 1A, B, 1C). Similarly, the PTB-cores ability to pulldown NPXY motifs (right panel, Fig. 2B), and NPXY motifs ability to pull-down both PTB-cores (left panel, Fig. 2B), validates our findings that two cores in PTB domains can independently interact with NPXY motifs (Fig. 1D, E, 1F, 1G).

Like β integrins, cytoplasmic tails of RTKs contain multiple NPXY motifs (Suppl. Table 2). Therefore, we hypothesize that among CCM2 isoforms, each possess its own unique spectrum of interaction with NPXY motifs of RTKs. Co-IP data indicates that various CCM2 isoforms can pull-down many NPXY motifs-containing cytoplasmic tails of RTKs (Left panel, Fig. 2C), likewise, cytoplasmic tails of RTKs can also pull-down CCM2 isoforms with various number of PTB-cores (right panel, Fig. 2C). Data were validated with BLI data (Table 1), demonstrating that CCM2 isoforms can interact with many NPXY motif-containing RTKs.

3.4. PTB-C binding was supported by C-terminal split PH domains

The potential binding ability of PTB-C (PTB-core-2) to NPXY motif is understandable since it contains α 3 helix and β sheet 2 (predominantly by β 5, β 6, β 7 strands) (Suppl.Figs.1A1 and 1A2), that form a *bona fide* NPXY motif binding pocket. To date, ~80% of binding sites recorded with NPXY motifs occur in C-terminus [9]. A full β sheet 2 contains 4 β strands (β 1A, β 5, 6 and 7 strands). The loss of β 1A strand (folded from the extension of N-terminal α 2 helix) from β sheet 2 in the PTB-C (PTB-core-2) could possibly obstruct PTB-C binding to NPXY motif. However, our data show that PTB-C (PTB-core-2) can independently bind NPXY, validated by our recent report [7].

Multiple well defined C-terminal half of split PH domains (naturally occurring structures) which resemble structures identical to PTB-C (Suppl.Fig.1A2) were confirmed to have strong binding to NPXY motifs by three non-labelling binding assays (Table 2), reaffirming PTB-C NPXY motif binding (Fig. 1D, E, 1F, 1G, and 2B).

3.5. PTB-core-1 binding ability was validated by the N-terminal PTB, PH, and split PH domains

Through structure (Suppl. Fig.1B) and binding analyses (Fig. 1D–G, 2B and Table 2), we found PTB-core-1, which contains α 2 helix and β sheet 1 (Suppl. Figs.1B1), can also bind to NPXY motifs (Fig. 1D–G, 2B and Table 2). However, PTB-core-1 from primitive PTB and PH domains (Suppl. Fig. 1B2), which have primitive α 2 helices, such as PTB domains

of FRS2 and DOK4, do not bind well to NPXY motif (Fig. 1D–G, 2B and Table 2). Likewise, N-terminal half of split PH domains do not bind at all (Table 2), emphasizing the importance of a matured α -helix and β sheet for competent NPXY binding.

We found the number of β strands in PTB-core-1, four or three, showed no difference in binding affinity to NPXY motifs (Fig. 2B and Suppl.Fig.1B1). This agrees with our previous [7] and current observations on PTB-C (PTB-core-2) (Fig. 1D–G, 2B and Suppl. Figs 1A1 and 1A2).

3.6. PTB cores reduced to minimal core motifs

From isoform CCM2-402 (containing exons 2, 3, and partial exon 4), a common 90 amino acid sequence encompassing exons 3 and 4 from all CCM2 isoforms was deduced as a PTB domain essential sequence (PTBes). PTBes showed strong affinity for NPXY motif (Fig. 1H). Comparing PTB1-N (PTB-core-1) and PTBes of CCM2, demonstrated PTB1-N of CCM2 (77 amino acids) can be minimized (Fig. 1D–H, Suppl. Table 4). By deletion, we generated the fragment, PTB1-core-N-s of CCM2 (67 amino acids), PTBes-A(55 amino acids), and PTBes-B (45 amino acids) (Suppl. Table 4). PTBes-B, having α 2 helix and β sheet1 formed by two β strands (β 1 and β 2), still retains binding to NPXY motifs. Further deletion (PTBes-C, 42 amino acids) resulted in loss of binding ability (Figs. 1H and 3A, Suppl. Table 4), therefore, we define this 45 amino acid fragment, PTBes-B, as PTB domain minimal core1 (PTB-mCore1). No binding was found in other fragments of PTBes (PTBes-C, PTBes-D and PTBes-H), suggesting the importance of α 2 helix at its N-terminus (Figs. 1H and 3A, Suppl. Table 4).

Subsequently, we also reduced one β strand from PTB-C of NUMB1 to create a minimum core 2, PTB-mCore2 (α 3 helix and β sheet 2 with two β strands, β 6 and 7) of NUMB1, which also retains binding ability to NPXY motifs (Figs. 1I and 3B, Table 2). By comparing both minimum cores (PTB-mCore1, 2) binding to NPXY motif, PTB-mCore2 is superior to PTB-mCore1 (Fig. 1I), indicating PTB-mCore2 is more matured. In summary, both N-terminal core and C-terminal core (PTB-core-1, PTB-core-2) of a PTB domain can be reduced to a minimal core motif (mCore1 and mCore2). PTB-mCore1 and PTB-mCore2 are the basic functional unit for NPXY-binding. This unit is structurally identical to the aPTB domain described recently [4]. One isoform (CCM2-1209) without PTB-mCore1 or PTB-mCore2 was not a functional PTB domain despite other identical amino acid sequences, reaffirming the importance of the PTB domain basic unit, PTB-mCore (Table 2).

3.7. Novel findings suggest the potential manipulation of PTB-NPXY interaction

The competitive binding properties of cis-inhibitors of NPXY motifs, which contain tandem NPXY repeats to PTB cores, have been tested (Fig. 1C and G). Many CCM2 isoforms with various number of PTB cores (0–4) were also tested with NPXY motif cis-inhibitors (Fig. 1C, Table 1) [4]. This shows no clear correlation between the number of NPXY motifs and PTB cores in terms of binding affinity, however, an increased number of PTB cores or NPXY motifs will increase the chances of binding. This increased binding potential likely results from proper orientation of the NPXY motif and PTB core structure (Tables 1, 2, Fig. 3A and B) which can be used to manipulate PTB-NPXY interaction.

4. Discussion

4.1. Matching the number of Junctional PTB to NPXY motifs

A big puzzle for PTB-NPXY interaction paradigm is that there is usually one PTB domain and a mismatched number of NPXY motifs in cytoplasmic portions of membrane receptors (dual NPXY motifs in most cases) (Suppl. Table 3). Although there have been explanations, such as preference and specificity of paired binding, this unbalanced pairing remains unsolved. Our findings of two NPXY binding pockets within one PTB domain have successfully addressed this issue, suggesting that there may be a transient transition between two binding sites if both sides have the potential to pair with NPXY motifs. This occurs, simultaneously or sequentially, with differentiated affinities, based on flanking sequences. This is shown by each pocket of typical PTB domain having a preference for certain NPXY motifs (Table 2).

4.2. The horseshoe structure formed by one α helix and one β sheet is essential for NPXY binding

We divided the typical PTB domain into two functional cores (core-N and core-C, or core-1 and core-2). Each core has an NPXY binding pocket formed by one α helix and one β sheet (consists of 3 or 4 β strands), which can independently bind to NPXY motifs. The binding affinity for each core is comparable to that of the entire PTB domain. We created a minimal core (mCore) where the NPXY binding pocket is formed by one α helix and one β sheet (with 2 β strands) and still retains binding to NPXY motifs. This was unexpected since most binding sites were mapped to β strands 5 (β sheet 2) and 4 (β sheet 1) in reports [9,10]. The binding ability of mCore-2 was confirmed by the similar tertiary structure (horseshoe confirmation) of exon6A, in newly identified aPTB domain [4]. Our findings of a dual binding pocket of PTB domain are in line with a previous report that ligand binding pocket is similar to a curved half pipe with one end in the C-terminus, between the α 3 helix and β sheet 2, while the other end in the N-terminus, between α 2 helix and β sheet 1 [11]. Our data indicate that a mCore, in which either β strands 4 or 5 have been removed, still retain the binding ability to NPXY motif, consistent with reports [12]. Although some reports found essential electrostatic properties in β strand 5 (positively charged amino acids) determine the binding to NPXY motifs [1,13], we believe those are rare cases, since the majority of PTB-NPXY interactions are independent of tyrosine phosphorylation [3]. Many NPXY motifs are NPXF motifs, invalidating the electrostatic argument.

Interestingly, the structure of aPTB-core1 (exon 6) is a horseshoe-shape structure formed by two-helices, while the C-terminal aPTB-core2 (exon 6A) itself forms a pocket with one α helix and one β sheet, structurally comparable to PTB1-mCore2. However, the full-length aPTB domain becomes a structure containing two horseshoe-shape pockets formed by four-helix bundles, suggesting that the β sheet can be replaced with another α -helix and maintain binding. Whether this is a common or rare phenomenon, needs further investigation.

4.3. A new function for split PH domain-binding to NPXY motif

PH domains can be split into two halves (N-terminal PH_N and C-terminal PH_C) by a linker sequence. Split halves coordinate to enhance binding to inositol phospholipids of either a

full PH domain [14] or a PDZ domain [15]. Although it was reported that the binding ability to inositol phospholipids can be recovered by tethering each half of split PH domains together with coiled-coil modules [16], it is still debated whether a C-terminal half of split PH domain in PLC-g1 works with N-terminal half of split PH domain in TRPC3 to form an “intermolecular PH domain”, which can bind to inositol phospholipids. Here, we defined the C-terminal half of split PH domain binding to NPXY motifs as a C-terminal core-2 of PTB domains, which are structurally identical. Interestingly, we found an NPXY motif exposed in the pore region of TRPC3 possibly interacting with C-terminal half of split PH domain.

By creating both *cis*- and *trans*-inhibitors (Fig. 1C, G and 2C), we hope to generate biologically active molecules to modulate NPXY-PTB interaction, due to ubiquitous binding of NPXY motifs and PTB/PH domains, similar to membrane inositol phospholipids and PH domains [17]. We believe transient bindings between pairs mentioned above trump their binding strength during signal transduction. Therefore, *trans*-inhibitors (tandem PTB core repeats) can be utilized with diverse combinations for regulating RTKs (Suppl. Fig. 3). *Cis*-inhibitors, tandem NPXY repeats (Fig. 1C and G), can be used similarly for pharmaceutical and therapeutic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Yanchun Qu, Shen Sheng, Junli Zhang, Amna Siddiqui, Saafan Malik, Adam Banda, Lillian Dominguez, Joshua Kallman, Carly Levin, Jasmine Cazares and Edna Lopez, Victor Erives, Pallavi Dubey, Aileen Zhang, Zhuoran Yao, Elias Gonzalez, Venkata Muthyala, Khalid Shouakt, Mark Smith, and Ahmed Badr at Texas Tech University Health Science Center El Paso. This work was supported by the National Institute of Neurological Disorders and Stroke of the National Institutes of Health (NINDS/NIH) (1R21NS061191), Texas Tech University Health Science Center El Paso (04132015) and the Coldwell Foundation (03902231).

Abbreviations:

PTB	Phosphotyrosine binding domain
mCore	Minimal PTB core motif

References

- [1]. Padarti A, Zhang J, Recent advances in cerebral cavernous malformation research, *Ves. Plus* 2 (2018) 23, 10.20517/2574-1209.2018.34.
- [2]. Yaffe MB, Phosphotyrosine-binding domains in signal transduction, *Nat. Rev. Mol. Cell Biol.* 3 (2002) 177–186, 10.1038/nrm759. [PubMed: 11994738]
- [3]. Uhlik MT, Temple B, Bencharit S, Kimple AJ, Siderovski DP, Johnson GL, Structural and evolutionary division of phosphotyrosine binding (PTB) do-mains, *J. Mol. Biol.* 345 (2005) 1–20, 10.1016/j.jmb.2004.10.038. [PubMed: 15567406]
- [4]. Jiang X, Padarti A, Qu Y, Sheng S, Abou-Fadel J, Badr A, Zhang J, Alternately spliced isoforms reveal a novel type of PTB domain in CCM2 protein, *Sci. Rep.* 9 (2019) 15808, 10.1038/s41598-019-52386-0. [PubMed: 31676827]
- [5]. Zhang J, Rigamonti D, Dietz HC, Clatterbuck RE, Interaction between krit1 and malcavernin: implications for the pathogenesis of cerebral cavernous malformations, *Neurosurgery* 60 (2007)

- 353–359, discussion 359. [10.1227/01.NEU.0000249268.11074.83.0006123-200702000-00017](https://doi.org/10.1227/01.NEU.0000249268.11074.83.0006123-200702000-00017) [pii]. [PubMed: 17290187]
- [6]. Zhang J, Clatterbuck RE, Rigamonti D, Chang DD, Dietz HC, Interaction between krit1 and icap1alpha infers perturbation of integrin beta1-mediated angiogenesis in the pathogenesis of cerebral cavernous malformation, *Hum. Mol. Genet.* 10 (2001) 2953–2960. [PubMed: 11741838]
- [7]. Zhang J, Dubey P, Padarti A, Zhang A, Patel R, Patel V, Cistola D, Badr A, Novel functions of CCM1 delimit the relationship of PTB/PH domains, *Biochim. Biophys. Acta* 1865 (2017) 1274–1286, [10.1016/j.bbapap.2017.07.002](https://doi.org/10.1016/j.bbapap.2017.07.002).
- [8]. Kryshchuk A, Monastyrskyy B, Fidelis K, Moulton J, Schwede T, Tramontano A, Evaluation of the template-based modeling in CASP12, *Proteins* 86 (Suppl 1) (2018) 321–334, [10.1002/prot.25425](https://doi.org/10.1002/prot.25425).
- [9]. DiNitto JP, Lambright DG, Membrane and juxtamembrane targeting by PH and PTB domains, *Biochim. Biophys. Acta* 1761 (2006) 850–867, [10.1016/j.bbali.2006.04.008](https://doi.org/10.1016/j.bbali.2006.04.008). [PubMed: 16807090]
- [10]. Schlessinger J, Lemmon MA, SH2 and PTB domains in tyrosine kinase signaling, *Sci. STKE* 2003 (2003) RE12, [10.1126/stke.2003.191.re12](https://doi.org/10.1126/stke.2003.191.re12). [PubMed: 12865499]
- [11]. Siegal G, The surprisingly flexible PTB domain, *Nat. Struct. Biol.* 6 (1999) 7–10, [10.1038/4873](https://doi.org/10.1038/4873). [PubMed: 9886281]
- [12]. Uhlik MT, Temple B, Bencharit S, Kimple AJ, Siderovski DP, Johnson GL, Structural and evolutionary division of phosphotyrosine binding (PTB) domains, *J. Mol. Biol.* 345 (2005) 1–20, [10.1016/j.jmb.2004.10.038](https://doi.org/10.1016/j.jmb.2004.10.038). [PubMed: 15567406]
- [13]. Ogura K, Tsuchiya S, Terasawa H, Yuzawa S, Hatanaka H, Mandiyan V, Schlessinger J, Inagaki F, Conformation of an Shc-derived phosphotyrosine-containing peptide complexed with the Grb2 SH2 domain, *J. Biomol. NMR* 10 (1997) 273–278. [PubMed: 9390405]
- [14]. Lu Q, Yu J, Yan J, Wei Z, Zhang M, Structural basis of the myosin X PH1(N)-PH2-PH1(C) tandem as a specific and acute cellular PI(3,4,5)P(3) sensor, *Mol. Biol. Cell* 22 (2011) 4268–4278, [10.1091/mbc.E11-04-0354](https://doi.org/10.1091/mbc.E11-04-0354). [PubMed: 21965296]
- [15]. Yan J, Wen W, Xu W, Long JF, Adams ME, Froehner SC, Zhang M, Structure of the split PH domain and distinct lipid-binding properties of the PH-PDZ supramodule of alpha-syntrophin, *EMBO J.* 24 (2005) 3985–3995, [10.1038/sj.emboj.7600858](https://doi.org/10.1038/sj.emboj.7600858). [PubMed: 16252003]
- [16]. Lemmon MA, Pleckstrin homology domains: two halves make a hole? *Cell* 120 (2005) 574–576, [10.1016/j.cell.2005.02.023](https://doi.org/10.1016/j.cell.2005.02.023). [PubMed: 15766521]
- [17]. Scheffzek K, Welte S, Pleckstrin homology (PH) like domains - versatile modules in protein-protein interaction platforms, *FEBS Lett.* 586 (2012) 2662–2673, [10.1016/j.febslet.2012.06.006](https://doi.org/10.1016/j.febslet.2012.06.006). [PubMed: 22728242]

Fig. 1. Molecular interactions defined with yeast two-hybrid system.

A). Interactions between various NPXY-motif containing protein fragments and CCM2 isoforms with truncated PTB domain (CCM2-102, CCM2-117, CCM2-402, CCM2-210). CCM2-200 and CCM2-107 serves as positive control for PTB and aPTB domains, while CCM2-1209 as negative control. pGAD-T with p53 is system control. B). Interactions between wild type (W, NPXY) and mutated (M, APXA) forms of three NPXY motifs of CCM1 (K2, K5, K8) and CCM2 isoforms with truncated PTB domain (CCM2-117, CCM2-402, CCM2-210). CCM2-102 serves as positive control, while CCM2-1209 as negative control. C). Interactions between protein fragments containing various numbers of tandem NPXY repeats (TRN, 0–12) and CCM2 isoform with truncated PTB domain (CCM2-402). CCM2-206 serves as positive control, while CCM2-1209 as negative control.

D). Interactions between various NPXY-motif containing protein fragments and several half-PTB domains (N-terminal half: PTB-core-1; C-terminal half: PTB-core-2. Core number is named from N- to C-terminus) from various PTB-containing proteins (CCM2-PTB1-core-1, CCM2-PTB1-core-2; DOK4-PTB-core-1 DOK4-PTB-core-2; ICAP1-PTB-core-1, ICAP1-PTB-core-2; DAB1-PTB-core-1 DAB1-PTB-core-2; TBC1D1-PTB-core-1, TBC1D1-PTB-core-2; FRS2-PTB-core-1, FRS2-PTB-core-2; SHC1-PTB-core-1, SHC1-PTB-core-2; APBB1-PTB1-core-1, APBB1-PTB1-core-2 and APBB1-PTB2-core-1, APBB1-PTB2-core-2). CCM2-PTBes serves as positive control, while pGAD-T with p53 is a system control. E). The two-hybrid β -galactosidase activity results for PTB cores above (Fig. 1D) were further presented by plate-based yeast two-hybrid assays with the rows indicating targeted fragments fused with GAL4 activation domain constructs (GAL4AD; rows) and columns for GAL4 binding domain fusions (GAL4BD; columns). Growth of all colonies in -Trp/-Leu medium indicates that all transformations were successful, while colonies growing in selective -Trp/-Leu/-His/-Ade medium indicate high stringency interaction between the indicated molecules. The robust interaction between SV40 large T antigen (SV40-T) and p53 is shown as a positive control, and the lack of any interaction of either SV40-T or p53 with other tested fragments shown as negative controls. F). Interactions between wild type (W, NPXY) and mutated (M, APXA) forms of three NPXY motifs of CCM1 (K2, K5, K8) and various half-PTB-containing proteins (CCM2-PTB1-core-1, CCM2-PTB1-core-2; DOK4-PTB-core-1, DOK4-PTB-core-2; ICAP1-PTB-core-1, ICAP1-PTB-core-2; DAB1-PTB-core-1; DAB1-PTB-core-2; TBC1D1-PTB-core-1, TBC1D1-PTB-core-2; FRS2-PTB-core-1, FRS2-PTB-core-2; SHC1-PTB-core-1, SHC1-PTB-core-2; APBB1-PTB1-core-1, APBB1-PTB1-core-2 and APBB1-PTB2-core-1, APBB1-PTB2-core-2; N-ss-NUMB-PTB, C-ss-NUMB-PTB). pGAD-Large-T serves as negative control. G). Interactions between protein fragments containing various numbers of tandem NPXY repeats (TRN, 0–12) and representative half-PTB-containing proteins (FRS2-PTB-core-2; APBB1-PTB1-core-1, APBB1-PTB1-core-2 and APBB1-PTB2-core-1, APBB1-PTB2-core-2). pGAD-Large-T serves as positive and negative system controls. H). Interactions between various NPXY-motif containing protein fragments and several portions of CCM2-PTB domain (CCM2-PTBes-B, CCM2-PTBes-C and CCM2-PTBes-H). CCM2-PTBes serves as positive control, while pGAD as negative control. pGAD-T with p53 is a system control. I). Interactions between various NPXY-motif containing protein fragments and two PTB domain minimum core (mCore1, mCore2). CCM2-PTBes serves as positive control, while pGAD-T as negative control. For the Y2H, β -galactosidase activity of each transformant was measured, normalized, and converted to relative β -galactosidase activity (RBGA), then compared to the negative control data (red colored baseline). Lamin C (LAM) serves as a negative system control and pGAD-T with p53 is positive system control. The normalized data were represented with means and standard deviations ($M \pm SD$) generated from at least three independent assays ($n = 3$). RBGA^{***},^{**}: significantly higher than that observed in any negative controls ($P < 0.001$, 0.01 respectively). K2, K5, and K8 are fragments containing the first, second, and third NPXY motif in CCM1 respectively. Cyto-ITGB represents cytoplasmic tails of β integrins (usually containing two NPXY motifs).. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. Molecular interactions defined by co-immunoprecipitation (CO-IP).

Interactions between NPXY motifs and PTB cores were confirmed by IP with MagneGST beads pulldown. In Co-IP of GST-tagged PTB domains with HIS-tagged NPXY motifs, protein lysates were pulled down with: A) MagneGST beads for either GST-tagged cytoplasmic tails of β -Integrins (Cyto-IRGBs, left panel) or CCM2 isoforms (right panel). Interactions between cytoplasmic tails of β -Integrins and CCM2 isoforms were confirmed by IP with MagneGST beads pulldown. B) MagneGST beads for either GST-tagged NPXY motifs from cytoplasmic tails of β -Integrins and CCM1 fragment (left panel) or PTB cores

(right panel). CCM2–1209 (CCM2 C-terminal fragment, Residues 303–444, no PTB domain), CCM1 fragments, K5 (2nd NPXY motif), K8 (3rd NPXY motif), and mock control. Mock control is host cell lysate. C). MagneGST beads for either GST-tagged CCM2 isoforms containing different number of PTB-cores (left panel) or GST-tagged cytoplasmic tails of RTKs (right panel). NPXY motifs containing protein fragments are cytoplasmic tails of RTKs. CCM2 isoforms contain various number of PTB cores. CCM2-1209 (CCM2 C-terminal fragment, residues 303–444, no PTB-core), mock control is host cell lysate. MagneGST Glutathione Particles (Promega) were used for GST-tagged bait proteins and dynabeads (Invitrogen) for HIS-tagged bait proteins. All target proteins were labeled with radioactive with S³⁵.

Fig. 3. Structures of the PTB cores.

Ribbon representations of A). Mapping PTB minimum-Core-N from PTB1es and PTB1-core-1. CCM2 PTB1-core-1 (N terminal portion of PTB1) and CCM2 PTB1-core-2 (C terminal portion of PTB1) equally bind to NPXY motif. PTB1es, a common 90 amino acid sequence encompassing exons 3 and 4 from all CCM2 isoforms, also binds to NPXY motif. Through a series of mutagenesis, reduction of amino acid sequences resulted in PTB1es-B. Structures in black retained binding ability to NPXY motif while structures in blue lost ability to bind to NPXY motif. B). Mapping PTB minimum-Core-C (NUMB1-mCore2) from NUMB1-core2. Through a series of mutagenesis, reduction of amino acid sequences resulted in NUMB1-mCore2 which bears resemblance to C terminal core of atypical PTB domain (exon6A), both are capable of binding to NPXY motif. Red color: c-terminus and blue color: N-terminus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The molecular interactions between NPXY motifs and two CCM2 isoforms.

Binding affinity data were estimated from Bio-Layer Interferometry (BLI) and presented as the equilibrium dissociation constant (K_D). B in the table corresponds to (binding detected), SB (strong binding-out of range of measurement) and NB, no binding. Cyto-: cytoplasmic tails of membrane receptors, -EXT: extracellular domains, ECSCR: Endothelial Cell Surface Expressed Chemotaxis and Apoptosis Regulator. DLC: Rho GTPase Activating Protein. Full R^2 : correlation coefficient.

NPXY Domains	CCM2-100		CCM2-107	
	K_D	Full R^2	K_D	Full R^2
cyto-PTPRB	5.06×10^{-8}	0.96241	2.16×10^{-7}	0.913409
cyto-mErbb2				
cyto-ERBB3	5.14×10^{-8}	0.992922		
cyto-ERBB4				
cyto-IGFIR	1.32×10^{-7}	0.957725		
cyto-IL4R	5.92×10^{-8}	0.98762	2.32×10^{-8}	0.905622
cyto-LTK1				
cyto-mLtk1	1.91×10^{-8}	0.987901	2.84×10^{-9}	0.973516
cyto-NTRK1	1.34×10^{-8}	0.980651	2.24×10^{-8}	0.908504
cyto-NTRK2	1.7×10^{-7}	0.960898	WB	
cyto-NTRK3	1.45×10^{-7}	0.94013		
cyto-mLRP2	3.27×10^{-8}	0.985951	6.25×10^{-8}	0.987026
cyto-ALK1	2.25×10^{-7}	0.979202		
cyto-APLP1			B	
cyto-APLP2				
cyto-mAPP				
cyto-HEG1	NB		NB	NB
cyto-INSR2				
CCM1-K1	NB		NB	NB
CCM1-K2	0.93×10^{-7}	0.939259	B	
CCM1-K5				

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

NPXY Domains	CCM2-100		CCM2-107	
	K _D	Full R ²	K _D	Full R ²
CCM1-K6	NB		NB	
CCM1-K8	9.19x10 ⁻⁸	0.986794	SB	
cyto-B1A	2.65x10 ⁻⁷	0.945016		
cyto-B1D	4.11x10 ⁻⁶	0.990045	B	
cyto-B2	0.95x10 ⁻⁷	0.956119	B	
cyto-B3				
cyto-B4	3.58x10 ⁻⁸	0.994194	B	
cyto-B5	7.15x10 ⁻⁷	0.864664		
cyto-B6	2.27x10 ⁻⁷	0.987234	B	
cyto-B7	2.24x10 ⁻⁶	0.976346		
ECSCR	1.45x10 ⁻⁷	0.910896	4.98x10 ⁻⁷	0.950943
mNOTCH-EXT	2.8x10 ⁻⁸	0.972817	4.25x10 ⁻⁸	0.880229
DLC	5.39x10 ⁻⁷	0.958849	B	

Kinetic analysis of the interactions between NPXY motif of CCM1 and cores of PTB/PH domains.

Table 2

Affinity data obtained using three different platforms were presented. Binding Affinity was presented with the equilibrium dissociation constant (K_D). SB, strong binding but out of range of measurement; NB, no binding. Lysate data (Lysate BLI and SPR) are estimated from cell lysate and not purified, while Full kinetics data (SPR and ITC) are more precise measurement data from purified target proteins.

PTB/PH Core	Lysate BLI			Lysate SPR			Full-Kinetics		
	CCM1-K8	CCM1-K2	cyto-APLP1	CCM1-K8	CCM1-K2		SPR		ITC
	CCM1-K8	CCM1-K2		CCM1-K8	CCM1-K2		CCM1-K8	CCM1-K2	CCM1-K2
APBB1-PTB1-CORE1	3.09×10^8						2.04×10^{-6}		2.07×10^{-5}
APBB1-PTB1-CORE2	SB							1.07×10^{-7}	3.12×10^{-7}
APBB1-PTB2-CORE3								1.85×10^{-7}	3.05×10^{-7}
APBB1-PTB2-CORE4	SB	SB						1.15×10^{-7}	2.23×10^{-6}
DAB2-PTB-CORE1				1.31×10^{-7}	1.87×10^{-7}				2.39×10^{-6}
DAB2-PTB-CORE2	8.79×10^{-8}				1.10×10^{-6}				8.7×10^{-5}
CCM2-PTB1-CORE1								4.14×10^{-8}	5.88×10^{-5}
CCM2-PTB1-CORE2								5.1×10^{-7}	6.33×10^{-7}
FRS1-PTB-CORE1								2.58×10^{-8}	5.88×10^{-6}
FRS1-PTB-CORE2			7.09×10^{-5}						2.05×10^{-6}
TBCID4-PTB2-CORE1									1.85×10^{-6}
TBCID4-PTB2-CORE2	2.03×10^{-7}								1.12×10^{-6}
LDLRAP1-PTB-CORE1	NB							1.65×10^{-7}	1.12×10^{-6}
LDLRAP1-PTB-CORE2	SB								5.21×10^{-6}
APPL1-PTB-CORE1		SB			4.34×10^{-7}				1.9×10^{-5}
APPL1-PTB-CORE2					5.0×10^{-5}			2.31×10^{-7}	7.58×10^{-7}
DAB1-PTB-CORE1									6.06×10^{-7}
DAB1-PTB-CORE2									5.78×10^{-7}
TBCID1-PTB2-CORE1									1.07×10^{-5}
TBCID1-PTB2-CORE2	1.15×10^4							6.42×10^{-7}	6.32×10^{-7}
ICAP1-PTB-CORE1					1.31×10^{-5}				7.81×10^{-6}
ICAP1-PTB-CORE2									2.92×10^{-6}
								1.74×10^{-7}	

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

PTB/PH Core	Lysate BLI			Lysate SPR			Full-Kinetics		
	CCM1-K8			CCM1-K8			CCM1-K8		
	CCM1-K2			CCM1-K2			CCM1-K2		
PTB/PH Core	cyto-APLP1			SPR			ITC		
	CCM1-K8			CCM1-K8			CCM1-K8		
NUMB-PTB-CORE1									
NUMB-PTB-CORE2									
TBC1D4-PTB1-CORE1									
TBC1D4-PTB1-CORE2									
CCM2-1209									
MYO10-PH1A									
MYO10-PH1B									
PLCG1-PH1B									
PLCG1-PH1A									
SNTA1-PH1B									
AGAP2-PH2A									