Intrinsic disorder and amino acid specificity modulate binding of the WW2 domain in kidney and brain protein (KIBRA) to synaptopdin

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ABSTRACT

The second WW domain (WW2) of the kidney and brain scaffolding protein, KIBRA, has an isoleucine (Ile-81) rather than a second conserved tryptophan and is primarily unstructured. However, it adopts the canonical triple-stranded antiparallel β-sheet structure of WW domains when bound to a two-PPxY-motif peptide of the synaptic protein Dendrin. Here, using a series of biophysical experiments, we demonstrate that the WW2 domain remains largely disordered when bound to a 69-residue two-PPxY-motif polypeptide of the synaptic and podocyte protein synaptopdin (SYNPO). Isothermal titration calorimetry and CD experiments revealed that the interactions of the disordered WW2 domain with SYNPO are significantly weaker than SYNPO’s interactions with the well-folded WW1 domain and that an I81W substitution in the WW2 domain neither enhances binding affinity nor induces substantial WW2 domain folding. In the tandem polypeptide, the two WW domains synergized, enhancing the overall binding affinity with the I81W variant tandem polypeptide 2-fold compared with the wildtype polypeptide. Solution NMR results showed that SYNPO binding induces small but definite chemical shift perturbations in the WW2 domain, confirming the disordered state of the WW2 domain in this complex. These analyses also disclosed that SYNPO binds the tandem WW domain polypeptide in an antiparallel manner, that is, the WW1 domain binds the second PPxY motif of SYNPO. We propose a binding model consisting of a bipartite interaction mode in which the largely disordered WW2 forms a “fuzzy” complex with SYNPO. This binding mode may be important for specific cellular functions.

KIBRA, also known as WWC1 (for WW and C2 domain containing), is a 125 kDa cytoplasmic scaffolding protein which is expressed mainly in the kidney and brain (1). It is a multifunctional protein implicated in the directional migration of podocytes (2-4), endosomal vesicle sorting (5), memory performance (6-8) cell growth and tumor suppression (9-11). While these functional roles are well-documented, much less is known about how KIBRA engages partner proteins to facilitate these diverse functions.

The KIBRA polypeptide is organized into multiple protein interacting modules. Of
particular interest are two N-terminal WW domains, WW1 and WW2. The WW domain is a ~35 residue globular domain which folds into a twisted, three-stranded anti-parallel beta sheet and binds proline-rich motifs (12-14). Two conserved tryptophans (W), from which the WW name is derived, lie on opposite sides of the beta sheet. The first tryptophan is part of a hydrophobic core essential for domain stability, and the second tryptophan, located in a solvent exposed region is a hydrogen bond donor important in ligand binding (15).

Human KIBRA WW1 and WW2 domains, are separated by a 14-residue segment and recognize proline-rich motifs of the type PPxY (where P is proline, x is any amino acid and Y is tyrosine). The domains share 61 % sequence identity with one major difference; the WW2 domain has an isoleucine (I81) at the position normally occupied by the second conserved tryptophan in most WW domain proteins. WW domain proteins with a substitution of the second conserved tryptophan are not uncommon; and a couple of these “atypical” WW domain proteins that have tyrosine or phenylalanine substitutions show reduced or complete loss of binding to specific targets (16,17). Both human and mouse KIBRA WW2 domain display weak binding to PPxY-containing peptides (1,18). Furthermore, recent extensive work by Zhang and coworkers (18) show that the mouse KIBRA WW2 domain is unstructured but adopts the canonical WW domain structure when bound to two of the three PPxY sites of the synaptic protein Dendrin. The KIBRA WW domains bind the two Dendrin PPxY sites, which are separated by two residues, with an unusually high nanomolar affinity. Given that the dissociation constants of WW domain-PPxY interactions are generally in the low micromolar range (17,19), it is plausible that this high affinity binding induces folding of the WW2 domain.

Synaptopodin (SYNPO) is a 929-residue proline-rich actin cytoskeleton-associated protein which similar to Dendrin, is found in kidney podocytes and the dendrites of neurons (1,20-22). In kidney podocytes, it is essential for the integrity of the podocyte actin cytoskeleton (23,24) and forms a WW domain-PPxY mediated complex with KIBRA implicated in cell migration (4). In dendrites, it plays an important role in regulating spine dynamics and synaptic plasticity (25). SYNPO has two PPxY motifs, PPTY (residues 562-565) and PPSY (residues 581-584) separated by fifteen amino acids and located in a region with striking sequence similarity to the region harboring the Dendrin PPxY motifs (4). Thus SYNPO is a good candidate to provide additional insight into the structural heterogeneity of the KIBRA WW2 domain.

In this work, we characterize the interaction between a 69-residue recombinant SYNPO polypeptide and variants of the human KIBRA WW domain polypeptide by a series of biophysical techniques. We show that recombinant human KIBRA WW2 domain is primarily disordered, binds SYNPO with relatively weak affinity and remains largely disordered in the complex. The two proteins interact in an antiparallel manner with the well-folded WW1 domain making substantial contacts with the SYNPO polypeptide while the WW2 domain forms a “fuzzy” complex. This structural heterogeneity of the WW2 domain is discussed in the context of specific functions of the KIBRA WW domains.

Results
SYNPO545-613 is primarily unstructured.

For this study, we used a 69-residue Synpo polypeptide (Synpo545-613) which includes both PPxY motifs, located at residues 562-565 and 581-584 and ~ 28 flanking residues. The primary sequence of the SYNPO545-613 polypeptide was analyzed with PONDR, a computational tool for predicting unstructured regions in proteins (26,27). The PONDR-generated disorder profile
of SYNPO<sub>545-613</sub> (Fig. 1) shows values greater than 0.5 indicative of a largely disordered polypeptide. To determine if SYNPO<sub>545-613</sub> is disordered as predicted, we used far UV CD and NMR spectroscopy to investigate the solution structure of the polypeptide. A strong negative signal at 200 nm in the far UV CD spectrum (Fig. 1B) and limited dispersion in the amide proton region of the <sup>1</sup>H<sup>-</sup><sup>15</sup>N HSQC spectrum (Fig. 1C) are consistent with a primarily disordered protein. To determine local structural propensities, backbone residue assignments were obtained for 57 of the 59 (S596 and H597 were not visible in the spectrum) non-proline residues. A plot of the deviations of the CA and CB chemical shift values (ΔCα-ΔCβ) from standard random coil values (28) in Fig. 1D, shows no strong propensity for this segment of SYNPO to form ordered structure. These results agree with the significant disorder reported for other members of the SYNPO family (29).

**Design of KIBRA constructs.**

Earlier studies assigned the KIBRA WW domain boundaries to residues 7-39 and 54-86, and identified P37A and P84A as mutations in the WW1 and WW2 domain respectively which abrogate binding to PPxY motifs (1). Guided by these findings and sequence-based secondary structure prediction, we designed the KWW<sub>TD</sub>, KWW<sub>1</sub> and KWW<sub>2</sub> constructs to include both, the first and the second WW domains respectively, the P37ATD and P84ATD to probe binding to each WW domain in the context of the tandem WW domain construct, and the I81W mutants, I81WT<sub>D</sub> and KWW<sub>2</sub>_I81W, to restore the binding site tryptophan in the tandem and isolated WW domain constructs respectively. A schematic of all the KIBRA constructs is shown in Fig. 2A.

The KIBRA WW domains adopt different secondary structures.

PSIPRED (30), a structure prediction algorithm which gives estimates of secondary structure based on a protein’s primary sequence predicts beta strands for residues 22-26 and 32-35 in the WW1 domain, 70-72 in the WW2 domain (designated by arrows in Fig. 2A) and disorder for the rest of the KWW<sub>TD</sub> sequence. The CD spectrum of KWW<sub>1</sub> shows a positive peak at 230 nm and a negative peak at 220 nm (black spectrum in Fig. 2B) which are signature WW domain peaks attributed to packing of the two tryptophans and beta strands respectively. Thus, the CD spectrum of KWW<sub>1</sub> is consistent with a well-folded WW domain protein. In contrast, the spectra of KWW<sub>2</sub> and KWW<sub>2</sub>_I81W lack the positive peak and show strong negative signals at 200 nm indicative of predominantly unstructured proteins (orange and brown spectra in Fig. 2B).

CD spectra for the tandem WW domain constructs, KWW<sub>TD</sub>, I81WT<sub>D</sub>, P84ATD and P37ATD are shown in Fig. 2C. KWW<sub>TD</sub>, I81WT<sub>D</sub>, and P84ATD all show positive signals at 230 nm, albeit more reduced than the corresponding signal in the spectrum of the isolated WW1 domain in Fig 2A, and negative signals at 220 nm and 200 nm. The similarities in the CD spectra suggest that the P84A and I81W point mutations do not significantly alter the secondary structure of the tandem WW domain polypeptide. The spectrum of the KWW<sub>TD</sub> polypeptide is qualitatively identical to the sum of the spectra for each WW domain (data not shown), which strongly implies that the domains retain their folded and unfolded structures in the tandem construct. In contrast, the CD spectrum of P37ATD (black spectrum in Fig. 2C) does not show a positive peak at 230 nm but only the negative signals at 220 nm and 200 nm which clearly demonstrates that this polypeptide is predominantly unfolded. To summarize, the CD data demonstrate that the WW1 domain is folded, the WW2 domain is unfolded, the I81W and P84A mutations in the WW2 domain do not result in substantial folding of the WW2 domain, and a P37A mutation in the WW1 domain is enough to disrupt the folded
Solution NMR studies of the KIBRA WW domains.

The $^1$H-$^{15}$N HSQC spectrum of KWW$_{TD}$ with backbone assignments is shown in Fig. 3A. Heterogeneous peak intensities and substantial cross-peak overlap made it challenging to assign all the backbone resonances. Nonetheless we were able to unambiguously assign the 69 peaks (82 non-proline residues expected) visible in the spectrum. Missing peaks (E13, E14, R16, D17, Y24, H27, N29, T31 – W34, E60, H74 and N75), mostly in the WW1 domain map to the 1st beta strand and loop region (E13, E14, R16, 17), ligand-binding loop (H27, N29, W34) and 3rd beta strand (T31, T32, S33) of the recent crystallographic structure of a ligand-bound mouse KIBRA WW domain (18). Chemical exchange with the solvent likely renders these signals undetectable. Two distinct peaks were assigned to N-terminal residues E5 and L8 which are adjacent to proline residues, inter-domain linker segment residues D49, C50, S52, and E54, and C-terminal residues I72, E82, and R89 (circled in red, Fig. 3A). Five Trp indole NH signals rather than the four expected are observed in the indole region ($^1$H 10.2 ppm; $^{15}$N 130 ppm) of the spectrum. Multiple peaks for the same NH are a strong indicator of multiple conformers in slow exchange. Peaks for the more folded KWW$_1$ domain, example G11 at 8.8 ppm and R40 at 7.4 ppm, are more dispersed while most peaks in the linker segment and the KWW$_2$ domain are clustered in the middle of the spectrum.

The $^1$H-$^{15}$N HSQC spectrum of P84A$_{TD}$ is shown in Fig. 3B. The spectrum is less heterogeneous than the KWW$_{TD}$ but is still missing the same peaks that were not visible in the spectrum of the WT protein. Several peaks (labeled in red, Fig. 3B) are significantly more intense than equivalent peaks in the WT spectrum. Peaks for residues D49, C50, S52, E54, E82 and R89 peaks are denoted by asterisks. These peaks displayed multiple conformations in the WT spectrum but coalesce into single peaks in the P84A$_{TD}$ spectrum. The more homogeneous P84A$_{TD}$ spectrum may be due to reduced cis-trans isomerization. The $^1$H-$^{15}$N HSQC spectrum of I81W$_{TD}$ was comparable to the KWW$_{TD}$ spectrum (see Fig. 6). However, the $^1$H-$^{15}$N HSQC spectrum of P37A$_{TD}$ was less dispersed in the amide proton region, indicative of unfolding or aggregation of the polypeptide and consistent with the CD data (data not shown).

Backbone dynamics of the KIBRA tandem WW domain polypeptides.

To gain insight into backbone dynamics of KWW$_{TD}$, P84A$_{TD}$ and I81W$_{TD}$, we acquired steady-state heteronuclear NOE (HetNOE) data which are sensitive to conformational dynamics in the picosecond to nanosecond timescale (31,32). Generally, high positive (> 0.6) HetNOE values suggest restricted internal motions, smaller positive values indicate substantial internal motion and negative values are indicative of fully disordered regions. The HetNOE plots in Fig. 3C compare the WT KWW$_{TD}$ (grey bar plot) to P84A$_{TD}$ (blue area plot) and I81W$_{TD}$ (orange area plot). All three polypeptides show HetNOEs in the range of 0.5 – 0.9 for most residues in the WW1 domain. The relatively high values are indicative of restricted internal motions which support the conclusion that the WW1 domain is folded. For the KWW$_{TD}$ polypeptide, computed HetNOEs are close to zero for most residues immediately preceding the WW2 domain and within the WW2 domain. Equivalent residues in P84A$_{TD}$ (blue plot) and to a lesser extent I81W$_{TD}$ (orange plot) display relatively higher HetNOE values. These results are a clear indication of substantial internal motions in the WW2 domain of KWW$_{TD}$.

Binding studies monitored by ITC

Binding energetics for SYNPO$_{545-613}$-WW domain complexes were determined from
isothermal titration calorimetry (ITC) experiments. Representative binding isotherms are shown in Fig. S1 and thermodynamic parameters are summarized in Table 1. All the interactions are enthalpically driven. Binding affinities are reported as dissociation constants ($K_d$), and range between 1.7 $\mu$M to 27 $\mu$M (Table 1). Binding of SYNPO$_{545-613}$ to KWW$_{TD}$ is two-fold weaker than I81W$_{TD}$ ($K_d$ values of 2.2 $\mu$M and 0.9 $\mu$M respectively) and ~1.7-fold higher than interactions with P84A$_{TD}$ (compare 2.2 $\mu$M to 3.7 $\mu$M). The slight increase in the binding affinity of I81W$_{TD}$ relative to the WT KWW$_{TD}$ polypeptide suggests that the isoleucine (I81) substitution in the WW2 domain reduces its affinity for SYNPO$_{545-613}$. A stoichiometry of 1 is recorded for all interactions with the tandem WW domain polypeptides. As SYNPO$_{545-613}$ is not expected to bind mutant domains in P37A$_{TD}$ and P84A$_{TD}$ a stoichiometry of 1 is interpreted as binding of a single WW domain to a single PPxY motif (1:1). For KWW$_{TD}$ and I81W$_{TD}$, simultaneous interactions with both WW domains (2:2) could also result in a stoichiometry of 1.

Thermodynamic parameters for the interaction of SYNPO$_{545-613}$ with the isolated KWW$_1$, KWW$_2$ and the WW2 domain mutant, KWW$_2$-I81W are shown in Table 1. The $K_a$ for the KWW$_1$ domain-SYNPO$_{545-613}$ interaction is (5.2 $\mu$M) approximately 1.5 - 2-fold weaker than the P84A$_{TD}$ mutant (binding to the WW2 domain is abolished) or the KWW$_{TD}$ construct. This suggests that residues in the WW1 domain alone are not sufficient for optimum stability of the complex. Isotherms for the SYNPO$_{545-613}$-KWW$_2$ (Fig. S1F) and SYNPO$_{545-613}$-KWW$_2$-I81W (Fig. S1G) show low heat changes upon binding. The small magnitude of the heat changes make it difficult to fit the data and derive accurate thermodynamic parameters. Fixing the stoichiometry N, in the data analysis process to 1 (assuming binding to a single PPxY motif), rather than allowing it to be optimized as an additional fitting parameter, gave a minimum $K_a$ value of 60 $\mu$M.

**Solution NMR studies to map the KIBRA-SYNPO binding interface.**

To map the binding interface of the SYNPO-KIBRA complex, we monitored chemical shift changes in the $^1$H-$^15$N HSQC spectrum of SYNPO$_{545-613}$, KWW$_{TD}$ or mutant tandem polypeptides without or with the unlabeled binding partner. A change is defined as a shift and/or a decrease in peak intensity. Data for $^{15}$N-labeled SYNPO$_{545-613}$ with unlabeled KWW$_{TD}$ added at molar ratios of 1: 0.5 and 1:1 (SYNPO:KWW$_{TD}$) are shown in Fig. 4. A plot of the normalized peak intensities of the 1:0.5 complex (Fig. 4A, grey plot) show a 43 % decrease in peak intensities for residues 564 -570, and 578-591. Increasing the molar ratio to 1:1 (orange plot in Fig. 4A and red spectrum in Fig. 4B) show complete loss of peaks corresponding to residues 558 - 571, and 575 - 588. Missing resonances are attributed to exchange broadening of peaks at the binding interface, as well as to slower tumbling of the complex. N-terminal residues 545-556, inter-PPxY motif linker segment residues 572-574 and C-terminal residues 592-613 are not perturbed (labeled peaks in Fig. 4B). A 1:2 complex did not show additional changes in chemical shift and intensities (data not shown) indicating that binding is saturated in the 1:1 complex. These observations are consistent with binding of both WW domains to both PPxY motifs.

To define the orientation of SYNPO$_{545-613}$ in the complex, unlabeled KWW$_1$ was titrated into $^{15}$N-labeled SYNPO$_{545-613}$. Normalized peak intensities for the 1:1 (grey area plot) and 1:2 (blue area plot) complexes are shown in Fig 4C. The 1:1 complex shows complete disappearance of peaks corresponding to residues 579-588 which include the second PPxY motif, and a few peaks corresponding to residues (564 -566) in the vicinity of the first PPxY motif. More peaks in the
KIBRA-Synaptopodin interaction

The 560-571 segment disappear when a higher concentration of unlabeled KWW_1 is added (blue plot). The pattern of peak disappearance clearly show that KWW_1 first binds the second (PPSY) motif before it binds the first motif.

In a reciprocal NMR titration experiment, we monitored chemical shift changes in the ^1H-^15N HSQC spectrum of KWW_TD or I81W_TD when bound to saturating concentrations of unlabeled SYNPO_545-613. Spectra of unbound (black) and SYNPO_545-613-bound KWW_TD (red) or I81W_TD (green) at molar ratios of 1:1 are shown in Fig. 5A and B. Peaks that are significantly attenuated or shifted in the SYNPO_545-613-bound spectra are labeled. We reasoned that attenuated peaks are directly or indirectly involved in binding, and attribute the broadened resonances to exchange between unbound and bound conformations as well as to slower tumbling of the complex. Increasing the concentration of added SYNPO_545-613 to a molar ratio of 1:2 did not result in additional changes in chemical shift or intensities (Fig. S3), a clear indication that binding is saturated in the 1:1 complex. Fewer WW2 domain peaks (labeled in magenta) are attenuated in the SYNPO_545-613-bound KWW_TD compared to the SYNPO_545-613-bound I81W_TD. This is consistent with the higher affinity of SYNPO_545-613 for the I81W mutant. Figure 5C show plots of the chemical shift changes in the SYNPO-bound WW2 domain of KWW_TD (red) and I81W_TD (green). Small but distinct changes in chemical shift are observed for most residues except T79 in the SYNPO-bound I81W_TD spectrum, which is significantly shifted. To summarize, SYNPO_545-613 binding is accompanied by changes in chemical shift for select residues in both WW domains. This is clear indication that SYNPO binds both WW domains. The lack of chemical shift dispersion in the SYNPO-bound spectra KWW_TD and I81W_TD is clear indication that the WW2 domain remains globally disordered in the SYNPO-bound complex.

**Binding-induced folding of the KIBRA WW2 domain**

Zhang and coworkers (18) reported that the mouse KIBRA WW2 domain adopts the canonical WW domain structure when bound to a two-PPxY motif-containing peptide of the neural and renal protein, Dendrin. The two PPxY sites in Dendrin are separated by only two residues while the SYNPO_545-613 PPxY sites are separated by a 15-residue linker. Thus we asked the question whether a SYNPO polypeptide with a two-residue linker would induce folding of the WW2 domain. To address this question, we engineered the SYNPO variant SYNPO-2A. The two PPxY sites in this variant are separated by two non-native alanine residues (Fig. 6). The ITC-measured dissociation constant of the SYNPO-2A- KWW_TD interaction was 0.1 µM (Fig 6B), which represents a 20-fold increase in binding affinity compared to the WT SYNPO_545-613 (K_d of 2.2 µM in Table 1). Next, we monitored binding between ^15N-labeled KWW_TD and SYNPO-2A by NMR spectroscopy. The ^1H-^15N HSQC spectrum of SYNPO-2A-bound KWW_TD is shown in Fig. 6C. The spectrum is well-dispersed, a clear indication that both WW domains are well-folded in the complex.

**Discussion**

Despite decades of well-documented studies on the functional roles of the KIBRA WW domains, how the domains, particularly the WW2 domain, bind partner PPxY proteins is unclear. A recent structural study of a mouse KIBRA tandem WW domain polypeptide bound to a peptide of the synaptic protein Dendrin, shows that the isolated WW2 domain is unfolded but adopts a well-folded structure in the complex (18). In contrast, the biophysical studies reported here clearly demonstrate that the WW2 domain remains largely disordered when bound to the actin-binding synaptic and podocyte protein Synaptopodin. The different folds of the WW2 domain in these independent studies clearly
demonstrate that different PPxY partners induce varying degrees of folding of the WW2 domain. The extent of folding may be important for the diverse functions of the KIBRA.

The isolated KIBRA WW2 domain binds PPxY peptides with weak affinity

In this study and previously reported studies (1,18), the isolated WW2 domain displays weak binding to PPxY-containing peptides. Two unusual features of the KIBRA WW2 domain explain why it has a weak affinity for PPxY containing peptides; intrinsic disorder and the I81 substitution of the conserved tryptophan. Disorder coupled to weak binding is inferred from observations that when the relatively well-folded WW1 domain is unfolded by introducing the P37A mutation, its affinity for the SYNPO polypeptide is also considerably weakened (Table 1). P37 is a conserved WW domain residue and part of a hydrophobic core formed by the side chains of W12 and Y24. The hydrophobic core stabilizes the anti-parallel beta sheet of the KIBRA WW1 domain (18) therefore it is not surprising that disrupting this core unfolds the WW1 domain. A mutation of the conserved proline in the WW domains of Yes-associated protein (YAP) and Formin-binding protein, FBP28 also unfold the domains (33,34). Unexpectedly, alanine substitution in the equivalent position in the WW2 domain, P84, leads to a substantial reduction in internal motions of the WW2 domain. This observation is intriguing because a stable hydrophobic core comprising W59, F71 and P84 (equivalent to W12, Y24 and P37 in the WW1 domain) is not expected to form in the primarily disordered WW2 domain. It is possible that P84 is involved in cis-trans isomerization which is abolished by the alanine mutation.

The second underlying cause of the weak affinity for PPxY peptides is the I81 substitution in the WW2 domain. We find that as reported for the WW domains of Smad ubiquitination regulatory factor 2 and WW domain-containing oxidoreductase (16,17), the loss of the binding site tryptophan reduces the affinity of the tandem WW domain polypeptide for SYNPO545-613. Isoleucine or leucine is the preferred amino acid in this position for KIBRA isoforms from Drosophila to humans, which suggests that this substitution is optimized for the functions of the KIBRA WW domains.

Similarities and differences between the KIBRA-SYNPO and KIBRA-Dendrin complexes.

The solution studies reported here provide valuable molecular-level insights into how the KIBRA WW domains, particularly, the WW2 domain, bind SYNPO. The pattern of peak disappearance in the NMR titration experiments (Fig. 4) strongly suggest that the second PPxY motif binds the N-terminal WW1 domain in the same anti-parallel orientation previously reported for the Dendrin polypeptide (18). The PPxY polypeptides may bind in a similar anti-parallel manner, but clearly there are distinct differences in how they influence the structure of the WW2 domain. The 1H-15N HSQC spectrum of the Dendrin-bound mouse KIBRA tandem WW domains shows significant peak dispersion consistent with global folding of the WW2 domain (Fig S2E in reference (18)). In contrast, the limited dispersion in the 1H-15N HSQC spectrum of the 1:1 (Fig. 5) and 1:2 (Fig. S3) KIBRA:SYNPO complexes strongly argues against global folding of the WW2 domain. We do not rule out the possibility that in this dynamic complex, a population of the WW2 domain becomes folded upon binding to SYNPO545-613. However, this population is minimal compared to the population which remains disordered in the complex. Thus, while binding of Dendrin to the tandem WW domain of mouse KIBRA induces substantial folding of the WW2 domain, the data presented here clearly show that binding of SYNPO545-613 to the tandem WW domains of
human KIBRA does not induce substantial folding of the WW2 domain.

A second major difference between the two binding partners is that while the dissociation constant of the SYNPO545-613-KIBRA complex is in the low micro-molar range, a typical range for most WW domain-PPxY interactions (17,35,36), the interaction with Dendrin occurs with an unusually high nano-molar dissociation constant. The two PPxY sites in the Dendrin peptide are separated by only two amino acids; and increasing the number of amino acids between the two PPxY sites substantially reduced the binding affinity for the KIBRA WW domains (18). On the contrary, the two PPxY sites in the SYNPO545-613 polypeptide are separated by fifteen residues. We demonstrate that a mutant SYNPO545-613 polypeptide, SYNPO-2A, with a two-residue linker binds the KWWTD polypeptide with a 20-fold higher affinity compared to the WT SYNPO545-613 polypeptide. Furthermore, initial NMR studies show that binding of SYNPO-2A to the KIBRA tandem WW domains induces substantial chemical shift changes in the 1H-15N HSQC spectrum (Fig. 6), similar to the KIBRA-Dendrin interaction (Fig S2E in reference (18)). Clearly, while the WW2 domain remains largely disordered when bound to WT SYNPO, reducing the SYNPO PPxY linker to two residues induces substantial folding of the domain.

**Model for the SYNPO-KIBRA interaction.** Figure 7 depicts the proposed binding model for the KIBRA-SYNPO dynamic complex. In this bipartite mode of interaction, SYNPO first binds the WW1 domain via the second PPxY motif, inducing folding of a small percentage of WW2 domain (panel 2) while a substantial population remains largely disordered. A small fraction of the disordered WW2 domain remains unbound to SYNPO (panel 3) while a large proportion binds the first PPxY motif (panel 1). The bound WW2 domain is best described as a “fuzzy” complex, a term used to describe IDP complexes which defy the classic stable and well-folded binding interface (37-39) and retain conformational heterogeneity in the bound state. “Fuzziness” is reduced when the SYNPO PPxY sites are separated by two residues (panel 4). We speculate that partners in which the PPxY sites are separated by two residues will induce substantial folding of the WW2 domain while partners with a longer linker will not. The structural variability of the WW2 domain may be critical for the diverse cellular functions of KIBRA.

**Experimental procedures**

**Cloning of constructs**

All constructs were prepared using the PCR-based Gibson assembly cloning protocol (New England Biolabs, MA). Briefly, a 69-residue Synaptopodin (Uniprot ID: Q8N3V7) construct, (hereafter referred to as SYNPO545-613), and KIBRA (Uniprot ID: Q8IX03) WW domain constructs KWWTD (residues 1-91), KWW1 (residues 1-48), and KWW2 (residues 45-91) were generated by PCR. We also generated a SYNPO545-613 variant, SYNPO-2A, with a two-alanine-residue linker between the two PPxY sites. PCR products were cloned into a modified pET24TM (Novagen) expression vector engineered with a tobacco etch virus (TEV) protease recognition sequence to allow cleavage of the N-terminal poly-histidine tag. A Q5TM PCR-based mutagenesis protocol (New England Biolabs, MA) was used to generate single sites mutations of the tandem WW domain construct, P37ATD and P84ATD, which abrogate binding of PPxY motifs to the WW1 and WW2 domain respectively (1), and I81W (I81W TD) and the isolated WW2 domain (KWW2_I81W). Primers for the PCR and mutagenesis reactions were purchased from Eurofins Genomics (https://www.eurofinsgenomics.com).

**Recombinant protein production**

Recombinant plasmids were transformed
into *Escherichia coli* BL21 (DE3) cells. Cells were cultured 37 °C in Lysogeny broth, or 15N-enriched MJ9 media supplemented with 12C or 13C glucose to mid-log phase (optical density of 0.6 - 0.7). The temperature was reduced to 20 °C, and 0.1 mM isopropyl 1-thio-D-galactopyranoside (IPTG) was added to the culture to induce protein expression. Cultured cells were harvested after 12 hours, lysed by sonication, and centrifuged to remove cell debris. The soluble His6-tagged proteins were first purified on a Ni-NTA-affinity (Qiagen) column, followed by treatment with TEV protease to remove the His6-tag, and a finally purified on a Superdex75 (GE life sciences) size exclusion chromatography column. Purity of recombinant proteins were assessed by SDS-PAGE. Protein concentrations were determined from the absorbance at 280 nm and computed molar extinction coefficients [http://web.expasy.org/protparam/](http://web.expasy.org/protparam/).

**Circular dichroism spectroscopy**

Far UV CD data were recorded at 25 °C, on a JASCO 720 spectropolarimeter using a bandwidth of 1.0 nm and a pathlength of 1nm. The experiments were collected on two independent samples prepared from two different protein preparations. Protein samples at concentrations of 10 – 20 µM were in buffer composed of 10 mM sodium phosphate, pH 6.8 or pH 8.0. Three scans were recorded on each sample. Reported data are the average of the experimental repeats.

**Isothermal titration calorimetry**

ITC data were collected on a VP-ITC instrument (Malvern instruments Inc, MA) set to 25 °C. 27 - 10 µl injections of 130 – 200 µM of the KIBRA constructs (KWWTD, KWW1, KWW2, P37ATD, P84ATD and I81WTD) in buffer composed of 50 mM sodium phosphate, 50 mM NaCl, 5 mM β-ME, 1 mM sodium azide, pH 8, was titrated into 12 – 24 µM of SYNPO545-613 in the same buffer. ITC data were analyzed with the Origin 7.0 software provided with the instrument. Data were fitted to a one-site model. Reported data are the average of two independent experiments performed under similar conditions using proteins from two different preparations. Three replicate titrations were carried out for each experiment. Standard deviations are computed from the average of the two experiments.

**NMR data collection and analysis.**

NMR experiments were performed at 25 °C on a Bruker Avance III, 800 MHz spectrometer (Bruker BioSpin) equipped with a triple resonance cryogenic probe. NMR samples were prepared in 10 mM sodium phosphate buffer, pH 6.8, 10 mM sodium chloride, 5 mM TCEP and 10% D2O. A series of backbone assignments experiments (1H-15N HSQC, HNCACB, CBCA(CO)NH and HBHA(CO)NH) were recorded on 13C15N isotopically labeled KIBRA constructs (WWTD, P84ATD, P37ATD, I81WTD) or SYNPO545-613 using BEST (band selective excitation short transient) (40) and/or TROSY (41) pulse sequences, and non-uniform sampling (NUS) in the indirect dimensions. NUS data were reconstructed in NMRpipe (42). 1H chemical shifts were referenced to an internal 2, 2-dimethylsilapentene-5-sulfonic acid (DSS). NMR data were visualized with the graphical program Sparky (Goddard T.D, and Kneller D.G, 2005, Sparky 3, University of California, San Francisco).

Titration data were collected on 15N-labeled SYNPO545-613 or 15N-labeled KIBRA constructs bound to the unlabeled partner. Unlabeled proteins were added at molar ratios of 1:0.5, 1:1, and 1:2 (labeled: unlabeled protein). The peak corresponding to the last residue (91 in isotopically labeled KWWTD/I81WTD and 613 in isotopically labeled SYNPO545-613) was used as an internal reference to correct for small changes in peak intensities across spectra. The relative
intensity of each peak (measured as peak heights) was calculated as the ratio of the intensity of the peak in the spectrum of the bound protein to the intensity of the same peak in the spectrum of the unbound protein.

Steady-state $^1$H-$^{15}$N Heteronuclear NOEs data were collected as two two-dimensional interleaved spectra with and without proton saturation delay of 8 seconds (43). NOE values were determined from the ratio of the peak intensities with and without proton saturation. Errors associated with the data ($\sigma_{\text{noe}}$) were estimated from the root mean square variation of the noise using the equation.

$$\sigma_{\text{noe}}/\text{NOE} = \left\{\left(\sigma_{\text{sat}}/I_{\text{sat}}\right)^2 + \left(\sigma_{\text{unsat}}/I_{\text{unsat}}\right)^2\right\}^{1/2}$$  (32).

Where $\sigma_{\text{sat}}$ and $\sigma_{\text{unsat}}$ are the standard deviation values of peak intensities with and without saturation, respectively. HetNOE experiments for KWW$_{TD}$ were collected on two independent samples prepared from two different protein preparations.

**Secondary structure propensity from NMR chemical shifts.**

Random coil values for NMR-based chemical shift propensities were compiled from the web-based algorithm of Poulsen et al (44-46). Local structural propensities were calculated from the deviations of the experimental C$\alpha$ and C$\beta$ chemical shifts from expected random coil values (28). A negative difference between the C$\alpha$ and the C$\beta$ secondary chemical shifts ($\Delta$C$\alpha$–$\Delta$C$\beta$) is suggestive of beta strand propensity; and a positive difference is attributed to helical propensity.

**Chemical shift changes.**

Chemical shift for assigned residues in the C-terminus (residue 45 -91) of KWW$_{TD}$ and I81W$_{TD}$ were computed from the 1:1 titration point. The combined $^1$H and $^{15}$N chemical shift changes were calculated as follows:

$$\Delta_{\text{ppm}} = \sqrt{(\Delta\delta\text{HN})^2 + (\Delta\delta\text{N} \times 0.17)^2}$$

where HN and N are the $^1$H and $^{15}$N chemical shifts.

**BMRB Accession code**

The chemical shifts for KIBRA KWW$_{TD}$ have been deposited in the Biological Magnetic Resonance Data Bank under accession code 27930.
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FOOTNOTES

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The abbreviations used are: NMR, nuclear magnetic resonance spectroscopy; HSQC, heteronuclear single quantum coherence; WT, wild type.

Table 1: Thermodynamics parameters for the SYNPO545-613 – KIBRA WW domain interaction

| Titrant       | N  | Kd (μM) | ΔH° (kcal/mol) | −TΔS° (kcal/mol) | ΔG° (kcal/mol) |
|---------------|----|---------|----------------|-----------------|---------------|
| KWW_{TD}      | 1.0| 2.2 ± 0.1| −38.3 ± 1.9    | 30.6 ± 1.9      | −7.71 ± 0.03  |
| P37A_{TD}     | 0.9| 27 ± 6  | −17.5 ± 4.2    | 11.2 ± 4.3      | −6.23 ± 0.13  |
| P84A_{TD}     | 0.9| 3.7 ± 0.1| −29.3 ± 0.5    | 21.9 ± 0.5      | −7.41 ± 0.01  |
| I81W_{TD}     | 1.0| 0.9 ± 0.1| −49.8 ± 0.3    | 41.7 ± 0.3      | −8.24 ± 0.02  |
| KWW_{1}       | 1.0| 5.2 ± 0.4| −23.4 ± 3.6    | 14.1 ± 3.6      | −7.21 ± 0.05  |
| KWW_{2}       | 1.0*| > 65  | N.D            | N.D             | N.D           |
| KWW_{2} (I81W)| 1.0*| > 60  | N.D            | N.D             | N.D           |

Changes in enthalpy (ΔH°), entropy (−TΔS°) and free energy of binding (ΔG°) are shown for interactions between KIBRA WW domains variants and SYNPO545-613. Values were determined at 25 °C from the average of three experiments using three different protein preparations. The error estimation is from experimental repeats. * Number of binding sites fixed to 1 in fitting procedure.
Figure 1: SYNPO545-613 is primarily unstructured. (A) Schematic representation of the SYNPO545-613 construct showing the positions of the two PPxY (PPTY and PPSY) motifs. POND- generated disorder profile of SYNPO545-613. Values > 0.5 indicate significant disorder in this segment of the protein (B) Far-UV CD spectrum of SYNPO545-613 collected in 10 mM sodium phosphate buffer, pH 8. SYNPO545-613 is predominantly unstructured as indicated by a strong ellipticity at 200 nm (C) $^1$H-$^1$N HSQC spectrum of SYNPO545-613 showing backbone assignments for 57 of the 59 non-proline residues. The spectrum was recorded at 10 °C (D) A plot of the chemical shift differences ($\Delta$13CA - $\Delta$13CB) as a function of residue number. $\Delta$CA and $\Delta$CB values were calculated by subtracting the random coil chemical shifts from experimentally determined chemical shift values.
Figure 2: Schematic representation and far UV CD profiles of KIBRA WW domain constructs. The constructs used in this study - tandem WW domain (KWW_{TD}), KWW_{TD} variants with single point mutations in the WW1 (P37A_{TD}) and WW2 (P84A_{TD} and I81W_{TD}) domains, individual WW domains (KWW_{1} or KWW_{2}) and a KWW_{2} mutant (KWW_{2-I81W}). Sequence-based secondary structure predictions are shown for the KWW_{TD} construct. Two segments in the WW1 domain and a segment in the WW2 domain have beta strand propensities (arrows). The rest of the protein is predicted to be disordered (dotted lines). (B) Far UV CD spectra of isolated WW domain constructs KWW_{1} (black), KWW_{2} (red) and KWW_{2-I81W} (brown). (C) Far UV CD spectra of KWW_{TD} (orange), P84A_{TD} (green), I81W_{TD} (magenta) and P37A_{TD} (black). The P37A mutation decreases the signal at 230 nm. CD data were collected in 10 mM sodium phosphate, pH 6.8 or pH 8.0.
Figure 3: Resonance assignments and HetNOE plots of the KIBRA tandem WW domain constructs. (A) $^1$H–$^{15}$N HSQC spectrum of KWW$_{TD}$ showing backbone assignments for 69 of the 84 non-proline residues. Residues which display multiple conformations are circled in red. (B) $^1$H–$^{15}$N HSQC spectrum of P84A$_{TD}$ showing assignments for select peaks. Peaks designated with asterisks display multiple conformations in the WT KWW$_{TD}$ spectrum in Fig 3A, but coalesce into single peaks in the P84A$_{TD}$ spectrum. Peaks labeled in red are more intense compared to equivalent peaks in Fig 3A (C) Plots of steady-state heteronuclear NOE ($I_{sat}/I_{unsat}$) of individual residues in KWW$_{TD}$ (grey bar plot), P84A$_{TD}$ (blue area plot) and I81W$_{TD}$ (orange area plot). Residues for which assignments are not available are shown in red in the KWW$_{TD}$ sequence above each plot. Most assigned residues in the WW2 domain of KWW$_{TD}$, have HetNOEs close to zero, consistent with the disordered nature of the domain. Positive HetNOEs in the WW2 domain of P84A$_{TD}$ indicate transient structural rigidity. Error bars, ± 0.01 – 0.06 are not shown for easier comparison.
Figure 4: Residue-specific mapping of the KIBRA binding site on SYNPO<sub>545-613</sub>. (A) Plots of relative peak intensity versus residue numbers of KIBRA-bound SYNPO<sub>545-613</sub> at molar ratios of 0.5:1 (grey) and 1:1 (orange) (B) Overlay of <sup>1</sup>H-<sup>15</sup>N HSQC spectra of unbound (black) and KWW<sub>TD</sub>-bound (red) <sup>15</sup>N-labeled SYNPO<sub>545-613</sub> at a 1:1 molar ratio. Peaks visible in the KIBRA-bound spectrum are labeled (C) Plots of the relative intensity versus residue numbers of KWW<sub>1</sub>-bound SYNPO<sub>545-613</sub> at molar ratios of 1:1 (grey) and 1:2 (blue). Binding is accompanied by complete loss of peaks in the vicinity of the PP<sub>x</sub>Y motifs. Peak intensities are relative to the intensity of the same peak in unbound SYNPO<sub>545-613</sub>, which is taken as one.
KIBRA-Synaptopodin interaction

A

Unbound KWW_{TD} SYNPO-bound

B

Unbound I81W SYNPO-bound

C

$\Delta$ ppm

| Residue | T79 |
|---------|-----|
| R40     |     |
| K43     |     |
| T46     |     |
| D49     |     |
| S52     |     |
| L55     |     |
| G58     |     |
| E61     |     |
| D64     |     |
| V67     |     |
| Y70     |     |
| T76     |     |
| R79     |     |
| E82     |     |
| R85     |     |
| W88     |     |
| E91     |     |

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Figure 5: Residue-specific mapping of the SYNPO545-613 binding site on the KIBRA WW domains. Overlay of 2D $^1$H-$^{15}$N HSQC spectra of $^{15}$N-labeled unbound (black) and SYNPO545-613-bound (A) KWW$_{TD}$ (red) and (B) I81W$_{TD}$ (green). Select peaks in the WW1 (black labels) and the WW2 (magenta labels) domain that completely disappear or are significantly shifted in the SYNPO545-613-bound spectra are labeled with the residue number. These peaks are directly or indirectly involved in binding. Peaks designated with asterisks are part of the expression vector. The complexes were formed by mixing equal concentrations of unlabeled and $^{15}$N-labeled proteins. (C) Plots of chemical shift perturbation in the WW2 domain of SYNPO545-613-bound KWW$_{TD}$ (red) and SYNPO545-613-bound I81W$_{TD}$ (green). Binding induced small but definite changes in chemical shift for most residues. T79 is significantly shifted in the SYNPO545-613-bound I81W$_{TD}$ spectrum.
Figure 6: SYNPO-2A binding induces changes in the KWW_{TD} spectrum. (A) Schematic representation of the SYNPO-2A construct. The residues between the two PPxY motifs were replaced with two non-native alanine residues (underlined) (B) ITC measurements for the KWW_{TD}-SYNPO-2A interaction shows a binding affinity (Kd) of 0.1 µM (C) Overlay of $^1$H-$^1$N HSQC spectra of unbound (black) and SYNPO-2A-bound KWW_{TD} (red) at KWW_{TD}:SYNPO-2A molar ratio of 1:1. Significant chemical shift dispersion in the SYNPO-2A-bound spectrum is indicative of binding-induced folding of the WW2 domain.
Figure 7. A model summarizing the interaction between the KIBRA WW domains and Synaptopodin. The KIBRA WW domains (teal) comprise a well-folded WW1 domain and a disordered WW2 domain. SYNPO (red) has two PPxY motifs. The two proteins interact in an antiparallel manner, that is, the WW1 domain binds the second PPxY motif. The model depicts the populated conformations of the WW2 domain in the complex: (1) the WW2 domain remains largely unfolded forming a "fuzzy" complex with the first PPxY motif (most populated) and minor populations in which the WW2 domain (2) undergoes a disorder-to-order transition or (3) remains disordered and unbound to SYNPO. (4) The SYNPO variant, SYNO-2A, binds the KIBRA WW domains with a higher affinity and induces substantial folding of the WW2 domain.
Intrinsic disorder and amino acid specificity modulate binding of the WW2 domain in kidney and brain protein (KIBRA) to synaptopodin
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