Activation of Polycystin-1 Signaling by Binding of Stalk-derived Peptide Agonists

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ABSTRACT

Polycystin-1 (PC1) is the membrane protein product of the PKD1 gene whose mutation is responsible for 85% of the cases of autosomal dominant polycystic kidney disease (ADPKD). ADPKD is primarily characterized by the formation of renal cysts and potential kidney failure. PC1 is an atypical G protein-coupled receptor (GPCR) consisting of 11 transmembrane helices and an autocatalytic GAIN domain that cleaves PC1 into extracellular N-terminal (NTF) and membrane-embedded C-terminal (CTF) fragments. Recently, signaling activation of the PC1 CTF was shown to be regulated by a stalk tethered agonist (TA), a distinct mechanism observed in the adhesion GPCR family. A novel allosteric activation pathway was elucidated for the PC1 CTF through a combination of Gaussian accelerated molecular dynamics (GaMD), mutagenesis and cellular signaling experiments. Here, we show that synthetic, soluble peptides with 7 to 21 residues derived from the stalk TA, in particular, peptides including the first 9 residues (p9), 17 residues (p17) and 21 residues (p21) exhibited the ability to re-activate signaling by a stalkless PC1 CTF mutant in cellular assays. To reveal molecular mechanisms of stalk peptide-mediated signaling activation, we have applied a novel Peptide GaMD (Pep-GaMD) algorithm to elucidate binding conformations of selected stalk peptide agonists p9, p17 and p21 to the stalkless PC1 CTF. The simulations revealed multiple specific binding regions of the stalk peptide agonists to the PC1 protein including an "intermediate" bound yet inactive state. Our Pep-GaMD simulation findings were consistent with the cellular assay experimental data. Binding of peptide agonists to the TOP domain of PC1 induced close TOP-putative pore loop interactions, a characteristic feature of the PC1 CTF signaling activation mechanism. Using sequence covariation analysis of PC1 homologs, we further showed that the peptide binding regions were consistent with covarying residue pairs identified between the TOP domain and the stalk TA. Therefore,

structural dynamic insights into the mechanisms of PC1 activation by stalk-derived peptide agonists have enabled an in-depth understanding of PC1 signaling. They will form a foundation for development of PC1 as a therapeutic target for the treatment of ADPKD.

Keywords: Polycystin-1, Peptide Gaussian accelerated Molecular Dynamics (Pep-GaMD), Peptide Agonists, Autosomal dominant polycystic kidney disease (ADPKD), Cellular Signaling, Sequence Covariation.

INTRODUCTION

Polycystin-1 (PC1) is the protein product of the PKD1 gene that is mutated in the majority of cases (~85%) of autosomal dominant polycystic kidney disease (ADPKD)[1]. ADPKD is a potentially lethal disease, affecting >0.6 million individuals in the US. It causes renal cyst formation that could consequently lead to kidney failure. Approximately one-third of PKD1 mutations are non-truncating and could encode a partially functional PC1 protein[2-4]. Mutation-specific therapies designed to increase the level of PC1 function are currently being pursued[2, 5-7]. However, this approach remains difficult due to incomplete knowledge of the physiological mechanisms and disease related functions of PC1. Currently, a small-molecule antagonist Jynarque™ is the only approved ADPKD treatment, but it is inadequate due to its limitations in only reducing disease progression and causing adverse side effects[8]. Therapeutic treatments targeting PC1 remain a promising approach for the treatment of ADPKD.

PC1 shares characteristics with the Adhesion class of GPCRs (ADGRs), including a conserved GPCR autoproteolysis inducing (GAIN) domain that directs autocatalytic cleavage at an embedded GPCR proteolysis site (GPS) motif[9]. Intramolecular cleavage at the GPS motif generates two non-covalently attached fragments - the extracellular N-terminal fragment (NTF) and the membrane-embedded C-terminal fragment (CTF)[10, 11]. The NTF consists of multiple adhesive domains that interact between cells and with the extracellular matrix[12-16], while the CTF harbors G protein-coupled and additional signaling activity[17]. PC1 is referred to as an atypical GPCR as it is composed of 11 transmembrane (TM) helices and a short C-terminal tail (C-tail) that has been shown to interact with G proteins for signal activation or regulation[1, 14, 18]. Previous studies demonstrated critical importance of cleavage at the PC1 GPS site to prevent renal cystogenesis in mouse models[5, 19, 20]. For the ADGRs, a tethered agonist (TA) model has been

proposed for activation of G protein signaling. After dissociation of the NTF, the N-terminal stalk of the CTF interacts with its membrane-embedded TM domains to induce conformational rearrangements that mediate activation of G protein signaling[21-24]. Exogenous peptides consisting of various lengths of the N-terminal sequence of the stalk have been shown to function as soluble agonists in activation of signaling by full-length and CTF mutants for numerous ADGRs[17, 25].

In previous studies of the PC1 CTF and ADPKD-associated variants within the stalk, we revealed a stalk TA-mediated molecular mechanism underlying PC1 signaling to a pGL3-NFAT promoter luciferase reporter through complementary biochemical experiments and all-atom Gaussian accelerated Molecular Dynamics (GaMD) simulations. In this study, we have tested the ability of synthetic peptides of various lengths derived from the N-terminal portion of the PC1 CTF stalk sequence to rescue signaling of a stalkless CTF expression construct ($CTF^{\Delta st}$) to the NFAT reporter in transiently transfected cells. Notably, peptides including the first 7 residues (p7), 9 residues (p9), and 17 residues (p17) could activate signaling to the reporter in $CTF^{\Delta st}$ -transfected cells, while p19 and p21 showed agonistic activity irrespective of ectopic $CTF^{\Delta st}$ expression. These experimental results suggest that stalk TA-derived peptides may act as soluble peptide agonists and exhibit an ability to restore signaling function of mutant PC1. We hypothesized that the soluble, activating peptides bind to the TOP domain of PC1 in a manner mimicking the tethered stalk in order to reactivate signaling of the stalkless $CTF^{\Delta st}$ mutant.

Biomolecules can be simulated over time at an atomistic level using Molecular dynamics (MD)[26]. Protein-peptide interactions have been modeled over microsecond timescale using conventional MD (cMD)[27-30]. However, biological processes over millisecond timescales and beyond are often challenging to simulate using cMD simulations and would require enhanced sampling techniques. Gaussian accelerated MD (GaMD) is an unconstrained enhanced sampling

method that works by adding a harmonic boost potential to reduce large biomolecular energy barriers. GaMD is able to recover the original free energy landscape of biomolecules through cumulant expansion to the second order[31]. Complex biological processes including ligand binding[32-39], protein-protein/membrane/nucleic acid interactions[40-46], protein folding[32, 47] and GPCR activation[33] have been successfully captured in GaMD simulations. Based on GaMD, the Peptide GaMD (Pep-GaMD) algorithm was recently developed to characterize peptide-protein binding processes more efficiently[48]. Repetitive binding and dissociation of highly flexible peptides to target proteins were captured in microsecond Pep-GaMD simulations[48].

Another avenue for identifying functional interactions within and between proteins is by analysis of homologous protein sequence covariation. Potts analysis is one of the pioneering covariation methods, in which a protein fitness model is inferred based on observed mutational covariation patterns in multiple sequence alignments (MSAs) of homologous proteins[49]. The statistical interactions found by this method are well established to be strong predictors of contacting residues in the 3D structures of proteins and their assemblies in the functional conformations visited during the protein lifecycle[50-52], including interactions corresponding to experimentally unobserved conformational states[52], protein-protein interfaces[53, 54] and more. Covariation patterns arise due to various functional constraints on a protein, including structural constraints that may be difficult to uncover experimentally because the functional state is transient or only appears in specific conditions. For this reason, covariation analysis complements our structure-based analysis, providing an alternate basis of evidence for important residue interactions between stalk-TA derived peptides and other domains of PC1 CTF.

Here, we have combined *in vitro* and *in silico* studies to identify peptide agonists targeting PC1 and investigate their activation mechanisms of PC1 signaling. Cellular assays were performed

to identify stalk sequence-derived synthetic peptides that demonstrated signal re-activation of the stalkless PC1 CTF construct. In addition, we combined peptide docking and Pep-GaMD simulations of selected peptide agonists p9, p17 and p21 to gain insight into their binding mechanism to the stalkless PC1 CTF. Pep-GaMD was able to successfully refine the docking conformations of the peptides bound to the extracellular TOP domain of PC1. In our previous study[55], we revealed a novel allosteric transduction pathway for the PC1 CTF that involves signal activation initiated by the Stalk interacting with the TOP domain followed by close interactions between the TOP and pore loop (PL) domains. GaMD simulations of the wildtype PC1 CTF sampled the "Closed/Active" low-energy state relative to the large number of Stalk-TOP contacts and the R3848-E4078 ionic interaction[55]. In the new Pep-GaMD simulations, the key salt bridge interaction between R3848 and E4078 from the TOP domain and PL, respectively, was observed upon binding of the peptides to stalkless PC1 CTF. Using Potts covariation analysis, we identified residues in the PC1 stalk with direct mutational covariation with residues in the TOP domain, which were strikingly consistent with the binding interfaces identified in docking and simulation studies. Overall, our analyses yielded mechanistic insights underlying the stalk peptide agonist mediated signal re-activation of PC1 CTF. Such insights provide significant contributions toward the future design and development of peptide modulators targeting PC1 for an effective ADPKD therapeutic treatment.

RESULTS

Synthetic, stalk-derived peptides re-activate NFAT reporter by CTF$^{\Delta st}$ *in trans*

Our previous study utilized expression constructs of human PC1 CTF, however, in order to prepare for eventual *in vivo* experiments in mouse models, we generated expression constructs of mouse

(m) PC1 consisting of the CD5 signal peptide sequence fused in frame with the stalk sequence of mCTF beginning with residue T3041, or with a 'stalkless' CTF lacking the first 21 residues of the stalk (mCTF$^{\Delta st}$) beginning with residue S3062 (**Fig. 1A**). Transient transfection of HEK293T cells with either empty expression vector (ev), CTF or CTF$^{\Delta st}$ showed that the CTF$^{\Delta st}$ mutant exhibited a dramatic loss of NFAT reporter activation that was essentially reduced to ev control levels (**Fig. 1B**). Both total (**Fig. 1 C-D**) and cell surface (**Fig. S1A-B**) expression levels of CTF$^{\Delta st}$ were comparable to CTF, which suggests that neither protein stability nor membrane trafficking was responsible for the inability of CTF$^{\Delta st}$ to activate the NFAT reporter. These results are consistent with those obtained using expression constructs of human PC1 that demonstrated the stalk region of PC1 CTF acts as a tethered peptide agonist[55].

To further investigate the agonistic property of the CTF stalk, we synthesized peptides (p) consisting of the N-terminal 7, 9, 11, 13, 15, 17, 19 or 21 residues from the stalk sequence of mPC1. All peptides were appended with a C-terminal, 7-residue hydrophilic sequence (GGKKKKK) to increase solubility. HEK293T cells were transiently transfected with empty expression vector or mCTF$^{\Delta st}$ along with the NFAT luciferase reporter and then treated with stalk peptides p7 through p21 or with addition of culture medium only ('no peptide' control). The NFAT reporter was significantly activated in CTF$^{\Delta st}$-transfected cells by treatment with p7, p9 or p17 as compared to their corresponding ev + peptide treatment controls. These stalk peptides also significantly increased reporter activity in comparison to the CTF$^{\Delta st}$ with no peptide treatment control (**Fig. 1E**). Treatment of CTF$^{\Delta st}$-transfected cells with p19 or p21 also significantly increased reporter activation in comparison to the CTF$^{\Delta st}$ + no peptide control; however reporter activation occurred in both ev- and CTF$^{\Delta st}$-transfected cells treated with either p19 or p21, suggesting that p19- and p21-mediated activation was not dependent on exogenous expression of

the mouse CTF$^{\Delta st}$ and could be activating the endogenous human PC1 protein. Such results were consistent with soluble stalk-derived peptides acting as PC1 CTF agonists *in trans*, and provided additional support for the PC1 CTF stalk region harboring tethered agonist (TA) activity[55]. From among the active stalk-derived peptides, we selected p9, p17 and p21 that exhibited the highest agonist activity in activating CTF$^{\Delta st}$ and the NFAT reporter (**Fig. 1E**) for further computational simulation studies.

Docking and Pep-GaMD simulations of peptide agonist binding to stalkless PC1 CTF

Using a computational model of the ΔStalk PC1 CTF developed previously[55], we successfully docked the p9, p17 and p21 stalk peptides with HPEPDOCK[56] (**See SI**). The peptides all bound to the TOP domain and the interface between the TOP domain and extracellular loop 1 (ECL1) of CTF (**Fig. S2A-B**). In particular, peptide p21 occupied a closely similar binding region as the stalk in wildtype CTF as observed in the previous study[55]. We then performed five independent 500 ns Pep-GaMD simulations on each of the three stalk peptide agonists p9, p17 and p21 bound to ΔStalk CTF to refine their HPEPDOCK docking conformations (**See SI**).

With the Pep-GaMD simulation frames, we performed structural clustering of each peptide using the hierarchical agglomerative algorithm in CPPTRAJ[57]. The top-ranked conformations of each peptide bound to ΔStalk CTF were compared to their initial docking conformations. Next, we calculated 2D free energy profiles of the peptides-bound ΔStalk CTF by reweighting the Pep-GaMD simulations. The R3848-E4078 residue distance and the number of contacts between the peptides (p9, p17 and p21) and TOP domains were selected as the reaction coordinates. In the subsequent analyses, stalk and peptide residues are numbered relative to the N terminus of the

stalk as starting from 1, while residues of the Δstalk CTF are numbered according to the human PC1 protein sequence.

Active conformation of peptide p9-bound PC1 CTF

From the free energy profile of the p9-bound ΔStalk CTF, we identified "Unbound" and "Bound" low-energy states (**Fig. 2A**). In the docking conformation, peptide p9 bound to the interface between the TOP and ECL1 of ΔStalk CTF (**Fig. S2**). In Pep-GaMD simulations, the p9 peptide dissociated from the TOP-ECL1 binding pocket and rebound to the TOP domain in a slightly different region (**Fig. 2B**). The p9 peptide sequence is mostly composed of hydrophobic residues. Polar interactions between the main chain atoms of peptide-protein residues were observed in the top-ranked representative conformation of the p9-bound ΔStalk CTF. Protein residues R3892 and H3864 formed hydrogen bonds with p9 residues A2 and A5, respectively (**Fig. 2C**). The distance between the TOP domain residue R3848 and PL residue E4078 was 3.9 Å (**Fig. 2D**), suggesting that the top-ranked representative conformation of the p9-bound ΔStalk CTF was in the "Closed/Active" low-energy state.

Active and Intermediate conformational states of peptide p17-bound PC1 CTF

The free energy profile of the p17-bound ΔStalk system allowed us to identify three low-energy states - "Unbound", "Intermediate", and "Bound" (**Fig. 3A**). In the docking conformation, peptide p17 bound to the interface between the TOP and ECL1 of ΔStalk CTF (**Fig. S2**). In the Pep-GaMD refined "Bound" state, a folded antiparallel ß-strand conformation was observed for the peptide p17 at the interface of ECL1 and the TOP domain (**Fig. 3B**). Peptide residues T1, F3, A5, F8, F16

and V17 formed hydrophobic interactions with the protein residues H3311, R3314 and Y3307 from ECL1, and E3708, S3711, Q3707, A3704, R3700 and L3701 from the TOP domain (**Fig. 3C**). The distance between the TOP domain residue R3848 and PL residue E4078 was 4.1 Å (**Fig. 3D**), suggesting that the top-ranked representative conformation of the p17 bound ΔStalk CTF was in the "Closed/Active" low-energy state.

In the "Intermediate" state, p17 with a short helical turn was also observed to bind the TOP domain of ΔStalk CTF (**Fig. 3B**). Hydrophobic residue interactions were also formed between the peptide and protein. In particular, peptide residues T1, F3, P10, P11, H13, R15, F16 and V17 formed hydrophobic interactions with the protein residues P3859, A3704, S3741, Q3739, Y3734, P3733, H3729, W3726, R3712 and R3856 from the TOP domain (**Fig. 3E**). The distance between the TOP domain residue R3848 and PL residue E4078 was 14.6 Å (**Fig. 3F**), suggesting that this representative conformation (ranked the second among the Pep-GaMD structural clusters) of the p17-bound ΔStalk CTF was in the "Intermediate" low-energy state.

Active conformational state of peptide p21-bound PC1 CTF

Finally, the free energy profile of the p21-bound ΔStalk CTF allowed us to identify only a broad low-energy well corresponding to the "Bound" state (**Fig. 4A**). The docking conformation of p21-bound ΔStalk CTF was refined through Pep-GaMD simulations, where folding of the peptide was observed on the protein surface of the TOP domain (**Fig. 4B**). The p21 peptide occupied a similar binding region as the stalk in wildtype CTF as observed in the previous study[55]. Hydrophobic contacts were observed between peptide residues L7, F8, P10, S12, H13, V14, V17, P19, E20 and P21 and protein residues L3863, L3701, I3705, L3709, E3708, R3712, F3714, H3729, W3726,

V3730, L3732, P3733, N3738, R3856 and S3741 (**Fig. 4C**). The distance between the TOP domain residue R3848 and PL residue E4078 was 3.8 Å (**Fig. 4D**), suggesting that the top-ranked representative conformation of the p21-bound ΔStalk CTF was in the "Closed/Active" low-energy state.

Peptide binding regions correlated with covarying residue pairs identified between the TOP domain and stalk TA

To provide an independent basis of evidence supporting the observation of "Bound" and "Intermediate" states of agonist peptide binding, we constructed a multiple sequence alignment (MSA) with an effective count of 1022 evolutionarily diverged PC1 homologs (illustrated in **Fig. S3)** from which we inferred a Potts statistical model (**Fig. 5**). Columns of the MSA with "direct" statistical interactions, as detected using the Potts inference method, reflect compensatory mutation pairs maintained through evolution supporting a conserved function. We limited our MSA to 394 residues on the extracellular side of PC1 because of the computational challenge of fitting the entire PC1 sequence (**Fig. 5B** and **Fig. S3**).

Fig. 5A shows the pairs of positions with strong Potts interaction scores where one position is either in the stalk, the nearby GAIN domain, or the TM1 helix. Some predicted interaction pairs recapitulated beta-sheet contacts within the GAIN domain observed in the homolog rat latrophilin-1[9] as well as predicted by Alphafold[58] (**Fig. S4**) or between the extracellular ends of the TM2/4 and TM5/6 alpha helices known from cryo-EM structures[59] or predicted by Alphafold, validating that our model detected biologically functional interactions.

We identified strong interactions between the stalk and other residues from the Potts model. They were not observed in the cryo-EM structure, in which the flexible stalk is missing. For interactions with the TOP domain, out of the 4875 possible pairs (25 stalk residues by 195 TOP domain residues in our Potts model), this analysis detected a stringent set of 6 strongly interacting pairs. Remarkably, multiple positions in this small set were among those relevant to the "Intermediate" binding conformation of p17 and "Bound" conformation of p21 as identified from the Pep-GaMD simulations. These were W3726 and S3741 in the TOP domain, both interacting with T1 of the stalk, and P3859 interacting with N25 at the end of the stalk (Fig. 5A). Additionally, we identified E3743 to be strongly interacting with F16 in the stalk, and it was also near the observed binding region in the TOP domain for the peptide p17 in the "Intermediate" state near S3741 (**Fig. 3E**). The remaining two strong interactions between the stalk and TOP domain involved Q3821 with T1 and L3893 with G4. L3893 is adjacent to R3892 that was identified to interact with the peptide p9 in the "Bound" state (**Fig. 2C**) and mutating it may affect its neighbor's positioning. Besides the interactions between the TOP domain and the stalk TA, we also found a set of interactions between the stalk and the extracellular ends of TM2-TM3 helices and TM4-TM5 helices, in which stalk residues G4, P10, F16 and E20 interact with W3298, A3296, and S3579, respectively, as well as a strong interaction between V3077, three residues past the end of the stalk, and T3856 in the TOP domain. TOP domain residue T3856 was also identified as relevant to the binding region of peptide p17 in the "Intermediate" state (**Fig. 3E)** and peptide p21 in the "Bound" state (**Fig. 4C)**. These interactions could additionally play a role in stalk-TA activation or could be related to other functionality such as cleavage in the GPS motif.

To gain further insight and to validate that these detected "direct" interactions reflect biologically meaningful functional interactions and are not artifacts of the data, we examined the

residue-specific covariation observed in the MSA (**Fig. 5C**), which measures the difference between the observed pairwise residue frequency and its null expectation under assumption of independent variation. Values greater than ~1% are commonly found to be indications of a statistically reliable mutational covariation (see **SI Computational Methods**), and many of the covarying pairs discovered between the stalk and TOP domain were significantly above this value. We validated that the covarying residue pairings were consistent with biophysical interaction. For example, for the position-pair 20-3579, here annotated such that the first index is the stalk residue numbered relative to the N terminus of the stalk and the second index is the TOP domain residue numbered according to the human PC1 protein sequence, there were excess residue-pair counts in the MSA consistent with opposite-charge or polar pairing such as K20-E3579, N20-Q3579, and others, and a dearth of repulsive like-charge pairs such as E20-E3579. Similarly, position-pair 1-3741 favored certain combinations of polar residues such as T1-S3741. Other position-pairs appeared consistent with hydrophobic packing interactions, such as F16-A3296, G4-W3298, and T1-W3726. A large residue F or W at position 3298 in the TOP domain was commonly paired with a G at stalk position 4, while a smaller I or L residue at position 3298 was more commonly paired with T at stalk position 4.

DISCUSSION

In *in vitro*, cell-based signaling assays, PC1 CTF-mediated activation of the NFAT reporter is dependent on its N-terminal, extracellular stalk, as shown by the loss of reporter activity with the CTF stalk-deletion expression construct, $CTF^{\Delta st\ 55}$ (**Fig. 1B**) and by the ability of synthetic, stalk sequence-derived peptides to reactivate signaling by $CTF^{\Delta st}$ *in trans* (**Fig. 1E**). A series of synthetic peptides derived from the N-terminal sequence of the mouse PC1 CTF stalk were used to

determine their agonistic activity in PC1 CTF$^{\Delta st}$-transfected cells. Notably, treatment with stalk peptides p7, p9, p17, p19 and p21 resulted in significant NFAT reporter activity over CTF$^{\Delta st}$ control (no peptide treatment), wherein the effects of p7, p9 and p17 were specific to mouse CTF$^{\Delta st}$-expressing cells. These data are consistent with the stalk peptides acting as soluble TA peptide agonists for PC1, and provide further evidence for the activation of PC1 signaling via an ADGR-like TA mechanism.

To reveal the molecular mechanisms of the soluble stalk-derived peptides, we chose to perform HPEPDOCK docking and novel Pep-GaMD simulations to sample the peptide interactions with the ΔStalk PC1 CTF. Pep-GaMD simulations were able to refine the docking conformations of peptide agonists bound to the ΔStalk PC1 CTF. Pep-GaMD simulations sampled an antiparallel ß-strand and a short helical secondary structure of peptide p17 bound to the ΔStalk CTF. Furthermore, peptides p9 and p21 adopted a more folded structure as compared to their disordered loop conformations in the docking poses. We also observed TOP-PL interactions, particularly the salt bridge between residues R3848-E4078 that is a key feature of the stalk TA-mediated activation of signaling for PC1 CTF[55]. Signal transduction was initiated upon binding of the stalk (TA) to the TOP domain, which was transmitted to the PL via a salt bridge formation between residue R3848 in the TOP domain and residue E4078 in the PL. The bound peptide agonists p9, p17 and p21 maintained the ΔStalk CTF in its "Closed/Active" conformation as observed in the wildtype PC1 CTF simulations[55].

The interacting pairs identified using sequence-based covariation analysis matched the pairs identified by Pep-GaMD simulations, providing complementary evidence of the importance of these interactions and of the existence of the "Bound" and "Intermediate" binding states of the stalk TA and stalk-derived peptide agonist. This suggests that such stalk TA binding states are

evolutionarily conserved across PC1 orthologs.. Covariation analysis identifies interactions important during any part of the protein lifecycle, and alone cannot be used to distinguish which conformational state an interaction arises in. By comparison to the conformations found in the Pep-GaMD simulations, we found that most of the identified interactions between the stalk TA and TOP domain were consistent with either the "Intermediate" or "Bound" binding states of the stalk-derived peptides, which are related to CTF inactive and active signaling states, however, it remained possible that other interactions, such as between the start of the stalk-TA and TM2/TM3, may be related to conformational states necessary for cleavage of the GAIN/GPS domain. Additionally, structural contacts may be incompletely detected at some positions when the statistical signal of covariation is masked by high conservation, subfamily specialization, or misalignment.. This can explain why some interactions identified in the binding interface through docking are not detected using covariation analysis. Despite this, the specific subset of interactions detected using covariation analysis suggest broader peptide binding interfaces, and we found these to be consistent with those observed in the Pep-GaMD simulations, and the covarying residue pairings were consistent with functional biophysical interactions.

The proposed binding interactions of the PC1 stalk peptides shares some similarity with those observed for the ADGRs. Specifically, Xiao et al. resolved cryo-EM structures of active ADGRG2 and ADGRG4 in complex with tethered Stachel sequences[25]. The structures showed that the 15 residue Stachel sequence inserts into the TM bundle to form intense hydrophobic interactions[25]. A hydrophobic F/Y/LXφφφXφ motif identified in the ADGR tethered sequences formed five finger-like projections in the hydrophobic pits of the TM bundle[25]. In our study, we observed a similar pattern of intense hydrophobic interactions between the peptide agonists p9, p17 and p21 and the hydrophobic pockets in the TOP domain of PC1 CTF. Notably, a closely

similar TOP binding pocket was identified for interaction of the tethered agonist (Stalk) in our previous study[55] and for binding of peptide agonist p21 in this study. The TOP domain hydrophobic pocket may serve as a significant candidate binding site for designing new synthetic peptides or small molecules to aid in rescue of PC1 function levels. Moreover, the shorter peptide agonists' (p9 and p17) binding sites also serve as novel pockets for design and development of therapeutic approaches for treating ADPKD.

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Author Contributions

R.M. and Y.M. designed research; S.P., B. S. M., E. N. M. and A.H. performed research; S.P., B. S. M., E. N. M., A.H., R.M. and Y.M. analyzed data; and S.P., A.H., R.M. and Y.M. wrote the paper.

Competing Interest Statement

No competing interests.

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Figure Captions

Figure 1. Synthetic peptides derived from the stalk sequence of PC1 can stimulate signaling of stalkless PC1 CTF. (**A**) Alignment of CTF stalk sequences from human (h) and mouse (m) PC1. $CTF^{\Delta st}$ has a 21-residue deletion from the N-terminal end of the stalk region. Arrow, GPS cleavage site. Non-identical residues shown in bolded blue. **(B)** Activation of the NFAT-luc reporter by transfected mCTF or $mCTF^{\Delta st}$ expression constructs shown relative to empty expression vector (ev) as means (+ standard deviation, SD) of 3 wells/construct from each of 7 independent experiments. (**C**) Representative Western blot of total cell lysates from one of the experiments in (B), probed with antisera A19 against mouse PC1 C-tail. ns, non-specific. (**D**) Summary of the total expression levels (means +SD) of $CTF^{\Delta st}$ relative to CTF from the experiments in (B). (**E**) Stalk peptide treatment of ev- or $mCTF^{\Delta st}$-transfected cells. Sequences of stalk-derived peptides P7-P21 are shown. Graph represents the fold NFAT-luc activation for both eV- (gray bars) and $CTF^{\Delta st}$- (blue bars) transfected cells relative to the $CTF^{\Delta st}$ control after 24 hr treatment with or without peptide. Results are the means (+SD) of 3 separate experiments, each with 3 wells/condition. *, $p < 0.05$; ***, $p = 0.0001$; ****, $p < 0.0001$. Analysis by 1-way ANOVA with Tukey-Kramer post-test.

Figure 2. (**A**) Free energy profile of the p9-bound ΔStalk CTF regarding the number of atom contacts between p9 and extracellular domains of CTF and the distance between the CZ atom of R3848 and the CD atom of R4078 in CTF calculated from Pep-GaMD simulations. (**B**) Comparison of HPEPDOCK docking (cyan) and Pep-GaMD refined (magenta) conformations of peptide p9. (**C**) Polar interactions between peptide-protein residues observed in the top-ranked representative conformations of p9. Peptide residues are numbered relative to the N terminus of the stalk with the peptide starting from 1, while residues within Δstalk CTF are numbered

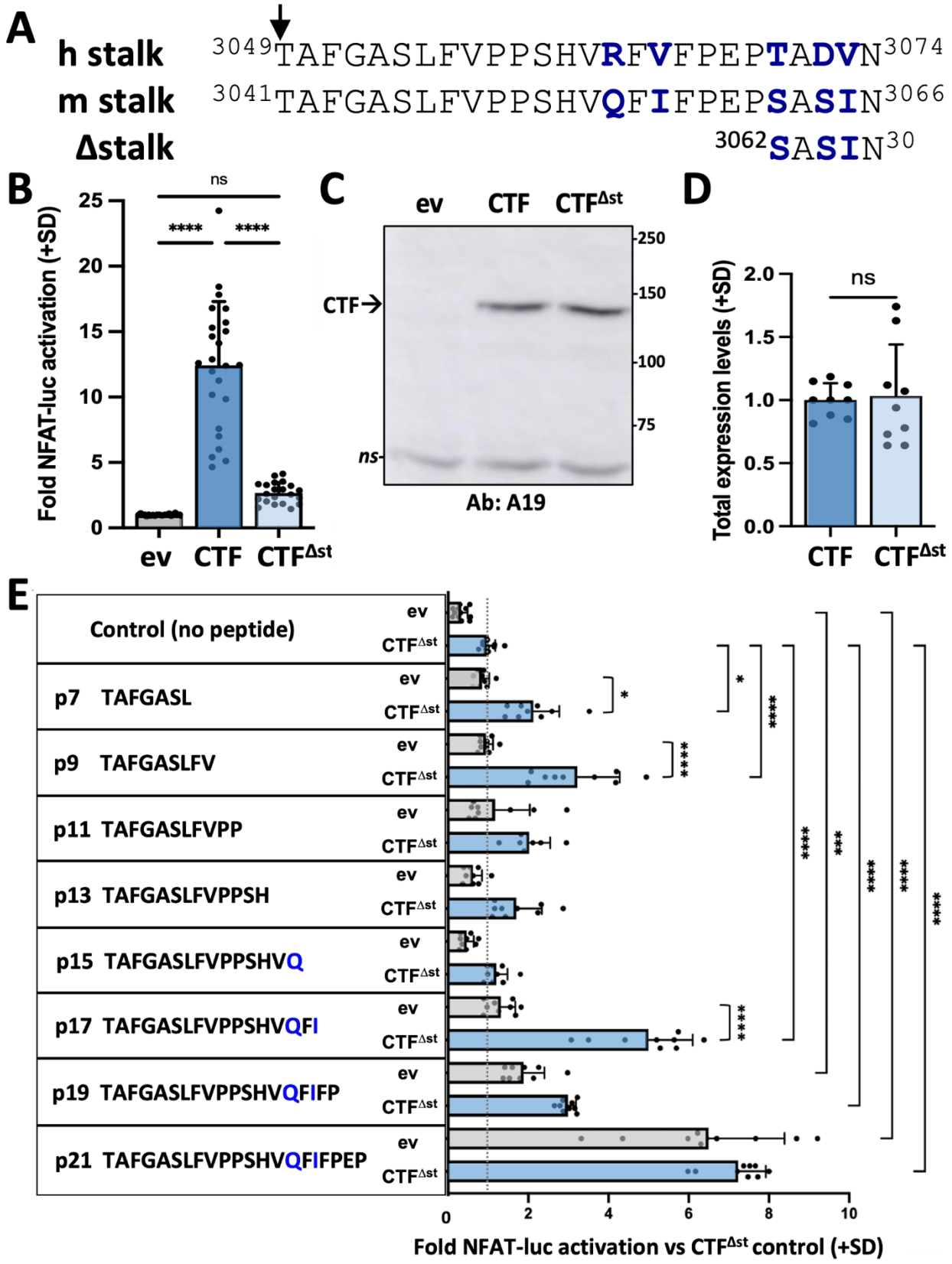
according to the standard PC1 residue number. (**D**) Distance between TOP domain residue R3848 and PL residue E4078 observed in p9-bound ΔStalk CTF.

Figure 3. (**A**) Free energy profile of the p17-bound ΔStalk CTF regarding the number of atom contacts between p17 and extracellular domains of CTF and the distance between the CZ atom of R3848 and the CD atom of R4078 in CTF calculated from Pep-GaMD simulations. (**B**) Comparison of HPEPDOCK docking (cyan) and Pep-GaMD refined (magenta) conformations of peptide p17. Hydrophobic interactions (red dashed lines) between peptide-protein residues observed in the (**C**) "Bound" and (**E**) "Intermediate" low-energy conformations of p17-bound ΔStalk CTF. Distance between TOP domain residue R3848 and PL residue E4078 observed in the (E) "Bound" and (F) "Intermediate" low-energy conformations of p17-bound ΔStalk CTF.

Figure 4. (**A**) Free energy profile of the p21-bound ΔStalk CTF regarding the number of atom contacts between p21 and extracellular domains of CTF and the distance between the CZ atom of R3848 and the CD atom of R4078 in CTF calculated from Pep-GaMD simulations. (**B**) Comparison of HPEPDOCK docking (cyan) and Pep-GaMD refined (magenta) conformations of peptide p21. (**C**) Polar interactions between peptide-protein residues observed in the top-ranked representative conformations of p21. (**D**) Distance between TOP domain residue R3848 and PL residue E4078 observed in p21-bound ΔStalk CTF.

Figure 5. (**A**) Potts interaction map based on the PKD1 multiple-sequence-alignment illustrated in **Figure S3**, showing interactions with the stalk. Gray dots are shown for residue position-pairs with Potts covariation scores above a threshold, colored darker for higher scores, and selected interacting pairs are annotated with the stalk residue (horizontal, numbered from the stalk N-terminus) and other residue (vertical, standard PKD1 numbering) with the PKD1 residue at each position. The secondary structure as a function of position is annotated along the axes. (**B**) Cartoon

showing the subset of PC1 included in the Potts covariation analysis colored as in the secondary structure in panel A, using a structure predicted by AlphaFold. Gray regions were excluded from the Potts model. (**C**) Residue Covariation scores for selected position-pairs. The scores reflect the percentage excess frequency of the residue-pair relative to the null expected frequency if the MSA columns were uncorrelated, with blue values reflecting excess and red dearth . Only the most common residue types are shown.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Δstalk 3062SASIN30
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Fold NFAT-luc activation (+SD)
ns
****
****
ev
CTF
CTFΔst
C
ev
CTF
CTFΔst
CTF
ns
250
150
100
75
Ab: A19
D
Total expression levels (+SD)
ns
CTF
CTFΔst
E
Control (no peptide)
p7 TAFGASL
p9 TAFGASLFV
p11 TAFGASLFVPP
p13 TAFGASLFVPPSH
p15 TAFGASLFVPPSHVQ
p17 TAFGASLFVPPSHVQFI
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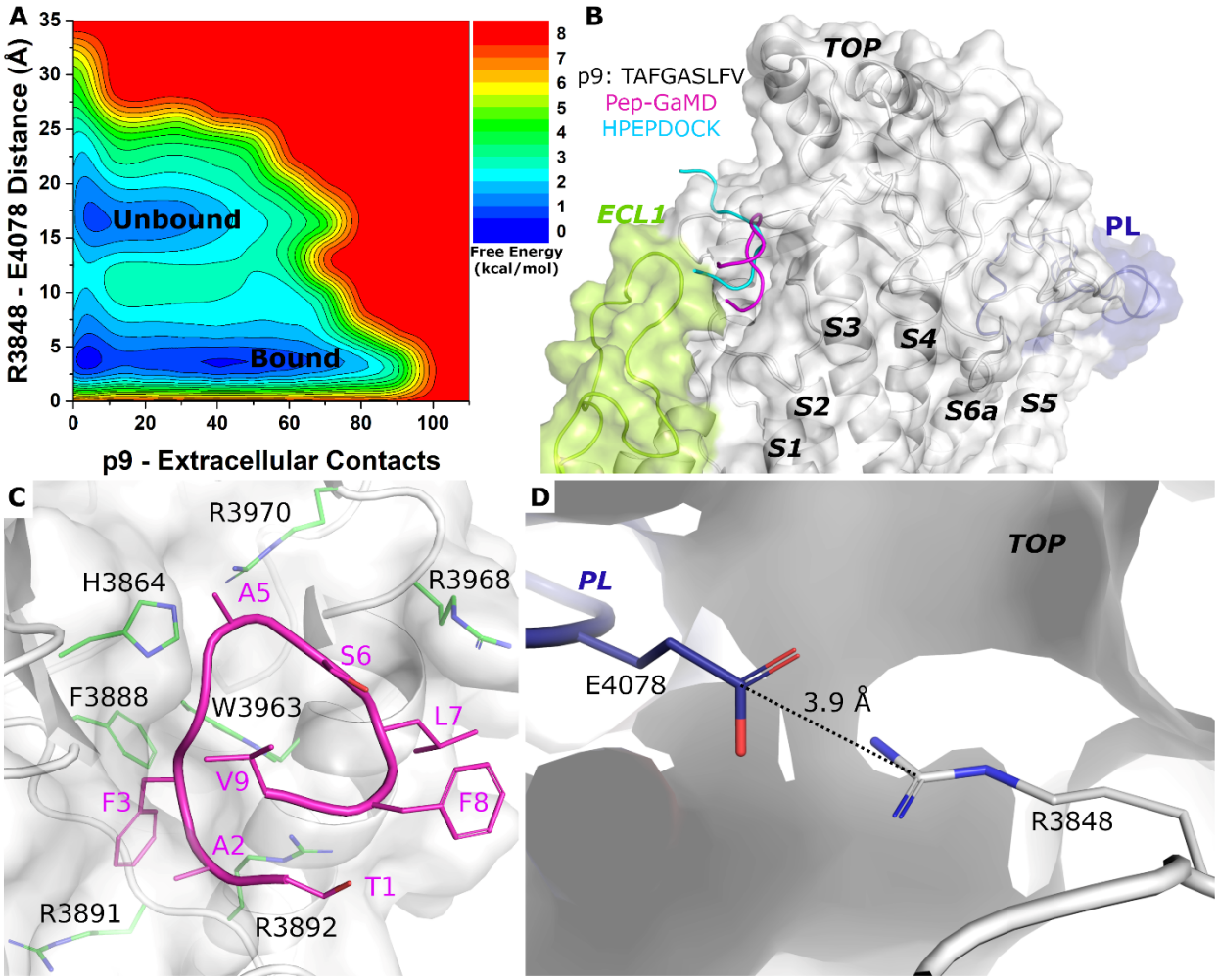
ev
CTFΔst
Fold NFAT-luc activation vs CTFΔst control (+SD)


A
R3848 - E4078 Distance (Å)
p9 - Extracellular Contacts
Unbound
Bound
Free Energy (kcal/mol)
B
p9: TAFGASLFV
Pep-GaMD
HPEPDOCK
TOP
ECL1
PL
S1
S2
S3
S4
S5
S6a
C
R3970
H3864
A5
R3968
S6
F3888
W3963
L7
V9
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F8
A2
T1
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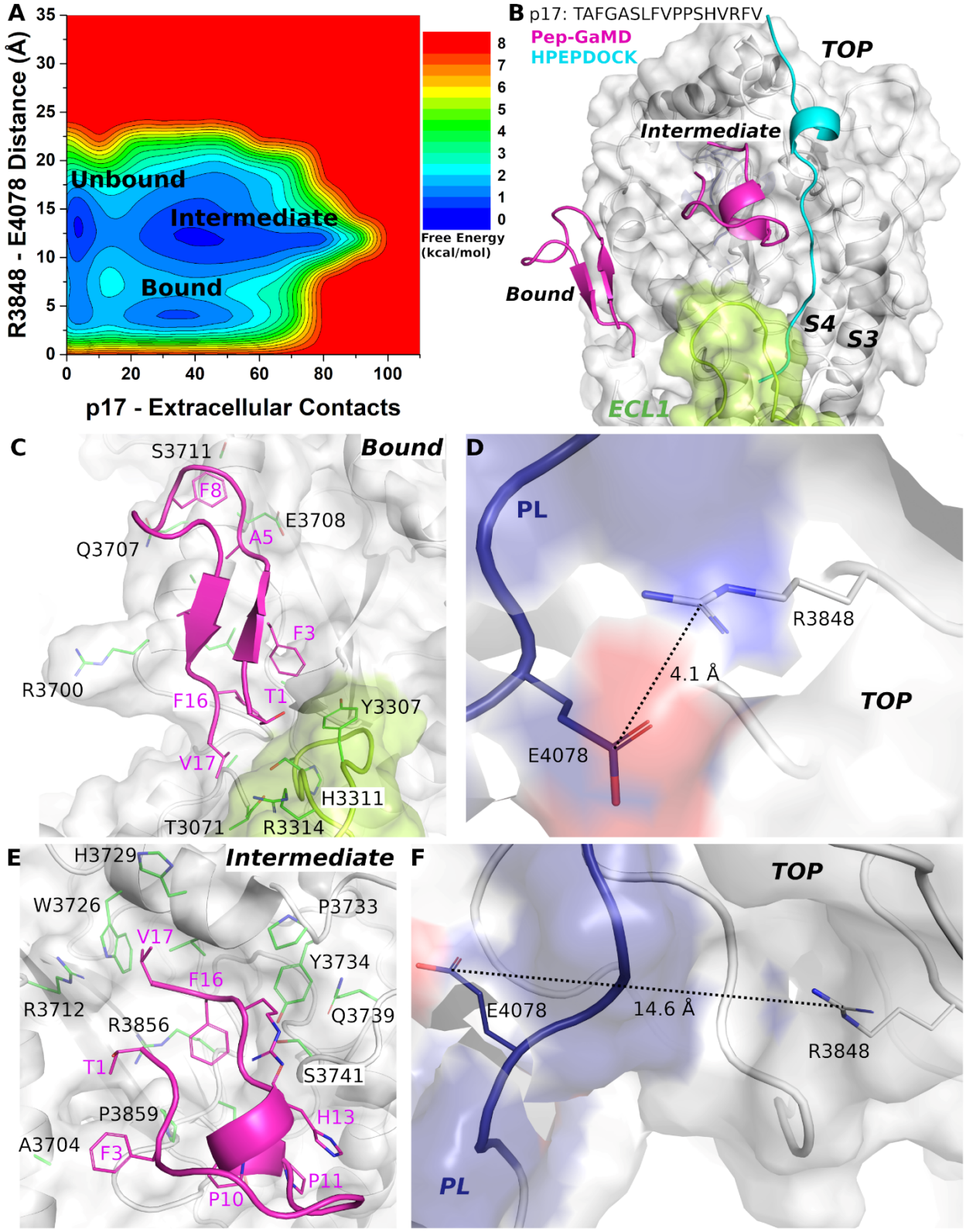
R3892
D
TOP
PL
E4078
3.9 Å
R3848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R3848 - E4078 Distance (Å)
p17 - Extracellular Contacts
Unbound
Intermediate
Bound
Free Energy (kcal/mol)
B
p17: TAFGASLFVPPSHVRFV
Pep-GaMD
HPEPDOCK
TOP
Intermediate
Bound
S4
S3
ECL1
C
Bound
S3711
F8
E3708
Q3707
A5
F3
R3700
F16
T1
Y3307
V17
H3311
T3071
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D
PL
R3848
4.1 Å
TOP
E4078
E
Intermediate
H3729
W3726
P3733
V17
Y3734
F16
R3712
R3856
Q3739
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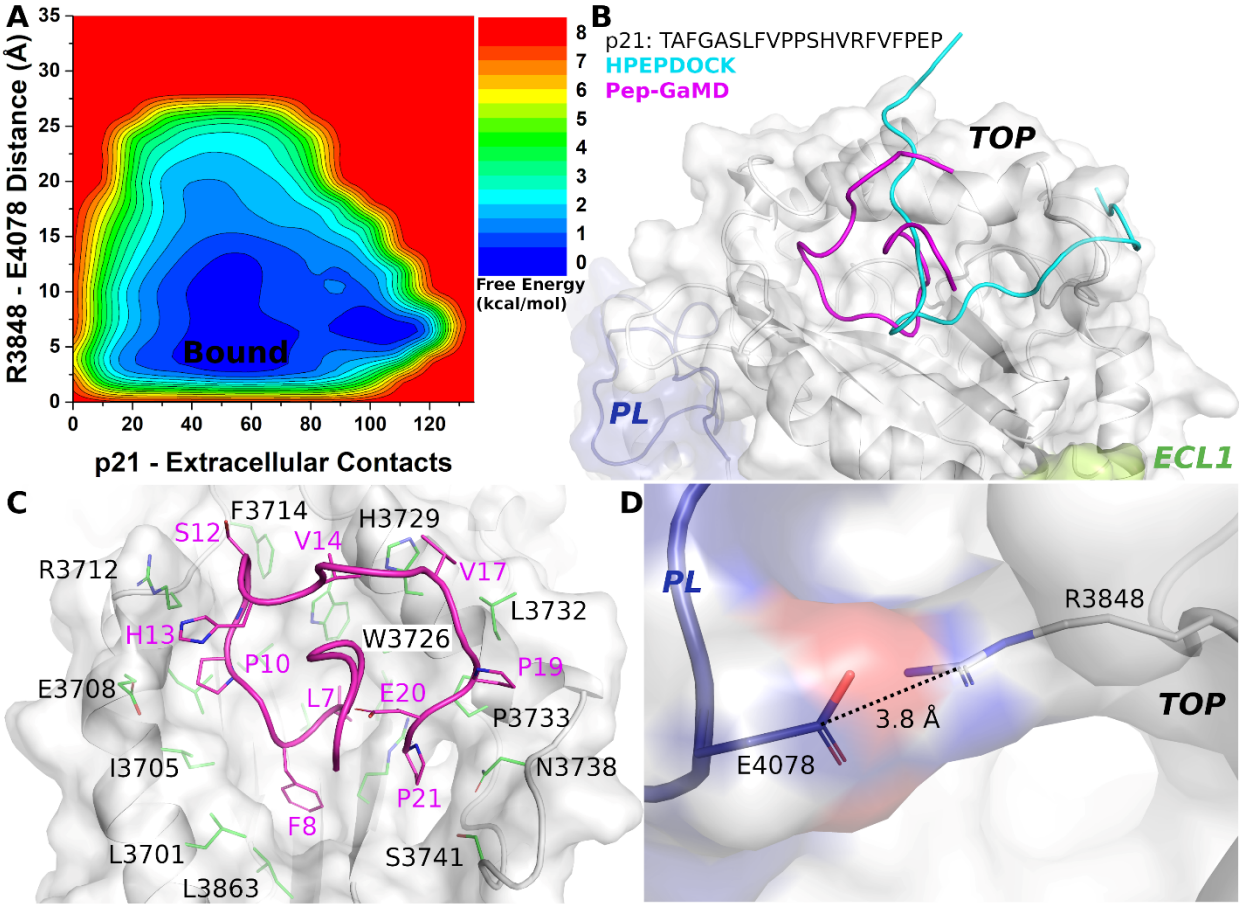
A
R3848 - E4078 Distance (Å)
p21 - Extracellular Contacts
Bound
Free Energy (kcal/mol)
B
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HPEPDOCK
Pep-GaMD
TOP
PL
ECL1
C
F3714
H3729
S12
V14
V17
R3712
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H13
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L7
E20
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TOP
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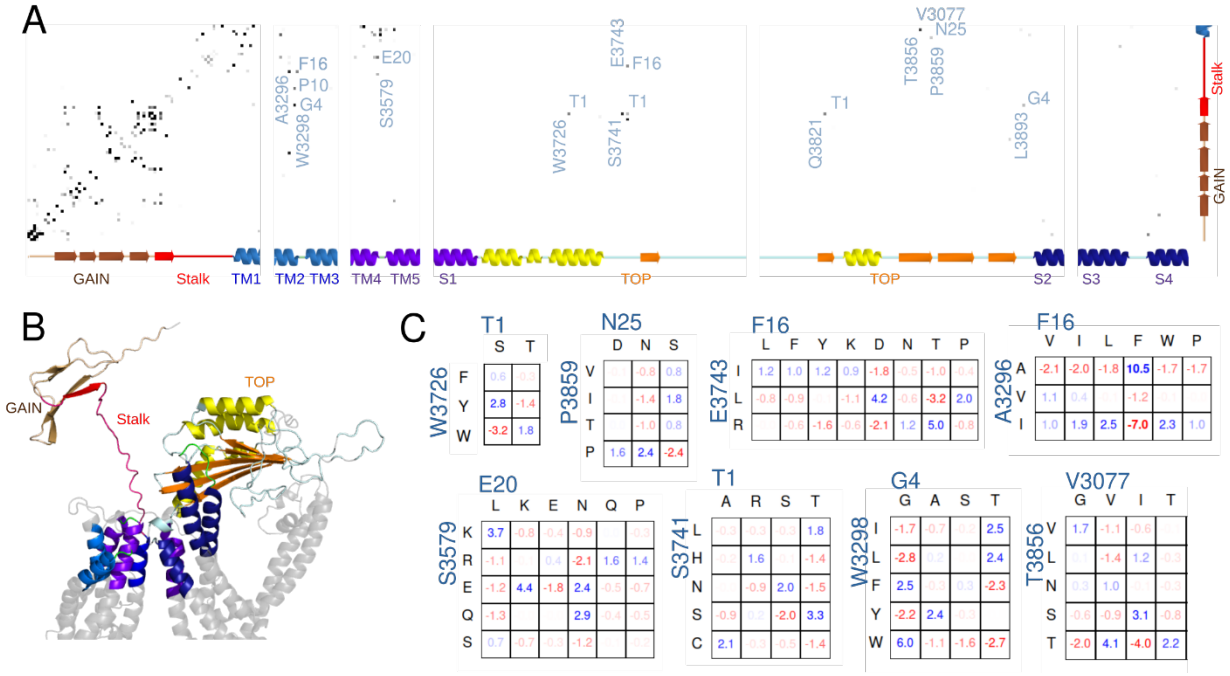
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GAIN
GAIN
Stalk
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TM2
TM3
TM4
TM5
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TOP
TOP
S2
S3
S4
B
GAIN
Stalk
TOP
C
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S T
F 0.6 -0.3
Y 2.8 -1.4
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V I L F W P
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I 1.0 1.9 2.5 -7.0 2.3 1.0
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L K E N Q P
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E -1.2 4.4 -1.8 2.4
Q -1.3 2.9
S 0.7 -0.7 -1.2
T1
S3741
A R S T
L 1.8
H 1.6 -1.4
N -0.9 2.0 -1.5
S -0.9 -2.0 3.3
C 2.1 -1.4
G4
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G A S T
I -1.7 -0.7 2.5
L -2.8 2.4
F 2.5 -2.3
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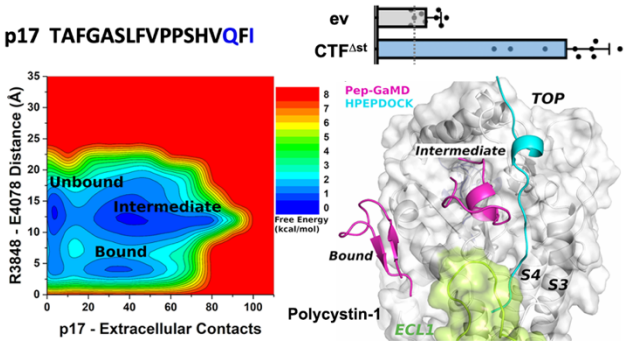
T3856
G V I T
V 1.7 -1.1 -0.6
L -1.4 1.2
N 1.0
S -0.9 3.1 -0.8
T -2.0 4.1 -4.0 2.2

TOC Graphic



Structural dynamic models are presented for binding of novel synthetic, soluble peptide agonists and associated activation of Polycystin-1 through a combination of complementary cellular signaling assays, accelerated molecular simulations and sequence coevolutionary analysis. The p17 peptide derived from the first 17 residues of the protein stalk tethered agonist is shown here.