Structural basis of interaction between dimeric cyclophilin 1 and Myb1 transcription factor in *Trichomonas vaginalis*

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Cyclophilin 1 (TvCyP1), a cyclophilin type peptidyl-prolyl isomerase present in the human parasite *Trichomonas vaginalis*, interacts with Myb1 and assists in its nuclear translocation. Myb1 regulates the expression of ap65-1 gene that encodes for a disease causing cytoadherence enzyme. Here, we determined the crystal structures of TvCyP1 and its complex with the minimum binding sequence of Myb1 (Myb1104–111), where TvCyP1 formed a homodimer, unlike other single domain cyclophilins. In the complex structure, one Myb1 104–111 peptide was bound to each TvCyP1 protomer, with G106-P107 and Y105 fitting well into the active site and auxiliary S2 pocket, respectively. NMR data further showed that TvCyP1 can catalyze the cis/trans isomerization of P107 in Myb1104–111. Interestingly, in the well-folded Myb1 protein (Myb1 35–141), the minimum binding sequence adopted a different conformation from that of unstructured Myb1 104–111 peptide, that could make P107 binding to the active site of TvCyP1 difficult. However, NMR studies showed that similar to Myb1 104–111 peptide, Myb1 35–141 also interacted with the active site of TvCyP1 and the dynamics of the Myb1 135–141 residues near P107 was reduced upon interaction. Together, the structure of TvCyP1 and detailed structural insights on TvCyP1-Myb1 interaction provided here could pave the way for newer drugs to treat drug-resistant strains.
Parasitic cyclophilins influence different stages of a parasite's development, and CsA or its analogues have been found with anti-parasitic activity. Most of the parasitic cyclophilins reported to date possess high sequence similarity and identity to HcypA. In most cases, the biological substrates of these cyclophilins are unknown and extensive structural studies on cyclophilin-substrate interactions have not been reported.

TvCyP1 is a cyclophilin present in the parasite Trichomonas vaginalis (T. vaginalis) showing high sequence identity to HcypA. T. vaginalis is the most common, sexually transmitted, non-viral pathogen infecting humans, causing Trichomoniasis. The infection causes several adverse reproductive outcomes and even cervical and prostate cancers. T. vaginalis also maintains a symbiotic relationship with various other parasitic organisms, which can eventually lead to an inflammatory response or resistance to metronidazole (MTZ). The availability of only a single class of drugs to treat the disease is a major concern for public health, with the emergence of drug-resistant strains, especially due to the higher transmission of HIV and HPV in infected individuals.

Cytoadherence is one of the most important contact-dependent mechanisms by which T. vaginalis begins and continues chronic infection. Among the several hydrogenosomal enzymes that also function as adhesins, AP65, encoded by ap65-1 gene, plays an important role in cytoadherence of the parasite to human vaginal epithelial cells. AP65 is a 65 kDa hydrogenosomal malic enzyme primarily involved in carbohydrate metabolism, but also act as adhesin at the surface of the parasite. The temporal and iron-inducible expression of AP65 is regulated by the coordinated actions of three Myb-like transcription factors, Myb1, Myb2 and Myb3, by their differential and competitive selection of the entry sites in the promoter of ap65-1 gene. Nuclear import of these Myb proteins is a crucial checkpoint that regulates transcription, which in Myb2 and Myb3 occurs via their own DNA binding domains (DBDs). However, for Myb1, the nuclear translocation is regulated by TvCyP1. The interaction of TvCyP1 with Myb1 leads to the cytoplasmic release of Myb1 from membrane-bound vesicles, an important step preceding translocation to nucleus. We also identified a minimal region TvCyP1-binding sequence in Myb1, in which YGP was found critical in TvCyP1 interaction. Mutations of G106 and P107 in Myb1 changed the cytoplasmic retention and nuclear translocation of overexpressed Myb1 protein.

In this study, we determined the crystal structures of TvCyP1 in the absence and presence of Myb1. Unlike other dimeric or multimeric single domain cyclophilins, TvCyP1 formed a homodimer without hindering the substrate binding site and existed as a dimer even in solution. In the complex structure, P107 of one Myb1 104–111 molecule was bound to the active site of each TvCyP1 protomer. Presence of tyrosine preceding the Gly-Pro bond in Myb1 104–111 allowed optimal interaction with the S2 pocket. Using NMR, we have also confirmed the catalytic behavior of TvCyP1 and inhibition of catalysis by CsA. Although our attempts to crystallize the Myb1-TvCyP1 complex were not successful, with NMR and site-directed mutagenesis, we showed that P107 of Myb1 104–111 interacts with the active site of TvCyP1 similar to Myb1 104–111 peptide fragment. NMR Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion experiments suggested that many Myb1 104–111 residues exhibit milliseconds time-scale dynamics and these conformational exchanges were notably reduced for the residues surrounding P107 upon addition of TvCyP1. By and large, our study reports for the first time the structure of a catalytically active dimeric cyclophilin possessing only a single cyclophilin domain and provides structural details of its interaction with an intriguing biological substrate. The data shown here could provide further insights into the design of newer drugs to treat Trichomoniasis.

Results
TvCyP1 is a divergent, single domain cyclophilin and forms a stable dimer in solution. TvCyP1 is a single domain cyclophilin that shows high sequence identity to human and parasitic cyclophilins, all of which are monomers. Like Caenorhabditis elegans Cyp3 (Ccy3; PDB: 1DYW) and Brugia malayi CypB (BcyB; PDB: 4JCP), TvCyP1 is a divergent loop cyclophilin and possesses an additional loop in the residues 49–55 (KSGMPLS). Although TvCyP1 contains the conserved E84, which helps to lock and hitch the loop into a certain conformation, the divergent loop shows variation from the consensus loop sequence (**GK**LH), observed in all other divergent cyclophilins. Because of the presence of serine instead of a highly conserved H54 seen in Cyp3, TvCyP1 lacks the metal ion coordination site formed by the imidazole sidechain of H54 and sulfhydryl groups of C168 and C40 (numbering based on 1DYW). To evaluate whether TvCyP1 exists as a monomer or dimer in solution, we performed size-exclusion chromatography with multi-angle light scattering (SEC-MALS) and analytical ultracentrifugation–sedimentation velocity (AUC-SV). The results from both the experiments confirmed that the protein exists as a dimer in solution (Fig. 1a,b). This is in contrast to the behavior of all other single domain cyclophilins in solution. In addition, the sensitivity of 3D triple resonance experiments was not sufficient to assign any of the side chain carbons and protons, possibly because of shorter transverse relaxation rate (T₂), typical of a larger-sized protein. Presence of only a single set of peaks in [1H, 15N]-transverse relaxation optimized spectroscopy–heteronuclear single quantum coherence (TROSY-HSQC) spectrum further confirmed the symmetric structure of TvCyP1 in solution (Supplementary Fig. S2).

Crystal structure of TvCyP1. To elucidate the structure of TvCyP1, we crystallized it and obtained hexagonal crystals that gave optimal quality X-ray diffraction and belonged to the space group P6₁22 with the following unit cell dimensions: a = b = 38.51 Å, c = 365.70 Å, and α = β = 90°, γ = 120°. The structure of TvCyP1 was solved by molecular replacement by using the structure of Ccy3 (PDB: 1DYW) as a search model, which shares 70% and 82% sequence identity and similarity, respectively, with TvCyP1. The final crystal structure was refined to 2.27 Å resolution with R_work and R_free values of 21.20% and 27.80%, respectively (Table 1).

The crystal structure of TvCyP1 displays the canonical cyclophilin fold with a β-barrel structure composed of eight anti-parallel β-strands capped by two α-helices at the top and bottom (Fig. 2a). With one of the crystallographic symmetry mates, TvCyP1 seems to form an anti-parallel side-to-side dimer, which was earlier confirmed in solution. An interesting structural feature of TvCyP1 is the presence of an extra small beta sheet between α1 and β4.
The prototypical cyclophilin, HcypA, shows high sequence identity (62%) to TvCyP1. Superposition of HcypA (PDB: 1OCA) with TvCyP1 revealed high structural identity (root mean square deviation [RMSD] = 0.910 Å) between HcypA and the protomer of TvCyP1 (Fig. 2b). The active site pocket is far from the dimer interface so that substrate binding to the active site of each protomer is not hindered (Fig. 2c). The dimer interface forms a pocket and is stabilized by hydrogen bonds, salt bridges and hydrophobic interactions between non-conserved residues residing in the loops between β1 and β2, α1 and β3 and α3 and β8. Hydrogen bond interactions between conserved G51 of the divergent loop and R161, R14 and conserved K57 and a salt bridge interaction between D16 and K57 are seen at the dimer interface (Fig. 2d). Hydrophobic interactions at the interface are formed by the residues M60, F78, M163 and M165 (Fig. 2e). Like Ccyp3, TvCyP1 does not form a disulfide bridge between conserved residues C169 and C41, and these cysteines could play a role in a signaling mechanism during oxidative stress conditions30.

Structure of TvCyP1–Myb1104–111 complex. We reported earlier that Myb1 has a minimum TvCyP1 binding sequence, Myb1104–111, with 105YGPK108 critical for TvCyP1 interaction27. To further confirm this observation, we designed four different fluorescein isothiocyanate (FITC)-labeled peptides starting from Myb1104–108 (104EYGPK) to Myb1104–111 (104EYGPKWNK) for fluorescence polarization (FP) experiments. Binding isotherms from FP experiments confirmed the eight-residue Myb1 peptide, Myb1104–111, having the strongest binding to TvCyP1, with Kd value of 16.81 ± 0.58 μM (Fig. 3a).

To obtain a greater clarity on the interaction between TvCyP1 and Myb1104–111, we co-crystallized them. The orthorhombic crystals gave optimal-quality X-ray diffraction and belonged to the space group P212121, with the following unit cell dimensions: a = 37.79 Å, b = 78.07 Å, c = 117.71 Å, and α = β = γ = 90°. The structure was solved and refined to 2.06 Å resolution with Rwork and Rfree values of 16.79% and 21.29%, respectively (Table 1). The asymmetric unit contained two TvCyP1 molecules forming an anti-parallel side-to-side dimer with non-crystallographic symmetry.

In the initial refinement cycle, continuous electron densities (at 1σ cutoff) located in the active site of TvCyP1 were observed and identified as parts of the Myb1104–111 peptide: 105YGPKWN110 in one protein molecule and 104EYGPK108 in the other (Fig. 3b). The 106GP105 atoms were inserted deeply into the hydrophobic active site.
Peptide chain Y105 preceding G106 in Myb1 104–111 enabled optimal interaction with the S2 pocket in CyP1. However, the two Myb1 104–111 peptides bound to each protomer adopted a similar conformation because of Y105 preceding G106 in Myb1 104–111. Presence of catalytic amounts of CyP1 apo and peptide-bound structures. RMSD, root mean square deviation. aValues in parentheses are for the highest resolution shell. bCompleted the 1H resonance assignments of Myb1 104–111 peptide. Separate resonance signals were observed for different side-chain orientations of K108. In protomer I, 105YGPKWN 110 was in an open conformation, while in protomer II, 105YGPKWN 110 was in a closed conformation due to an intramolecular salt bridge interaction between E104 and K108 (Fig. 3b). However, the two Myb1 104–111 peptides bound to each protomer adopted a similar conformation of the critical interacting residues Y105 preceding G106 in Myb1 104–111. Peptidyl-prolyl isomerase activity of CyP1. Since CyP1 is the only known dimeric cyclophilin with the active sites exposed, we tried to obtain direct evidence for catalysis activity by using NMR rotating-frame Overhauser effect spectroscopy (ROESY). In a ROESY spectrum, the sign of the cross-peaks from chemical exchange is the same as the diagonal peaks, while the cross-peaks between neighboring hydrogens display an opposite sign. In order to monitor the catalysis of cis/trans isomerization of P107 in Myb1 104–111 by CyP1, we completed the 1H resonance assignments of Myb1 104–111 peptide. Separate resonance signals were observed for the cis and trans conformations of residues from Y103 to K111 (Supplementary Fig. S4), indicating that each conformation lies in a distinct chemical environment and the cis/trans isomerization is slow on NMR time scale (exchange rate <0.1 s⁻¹). Because of the slow exchange rate of the un-catalyzed isomerization, only the cross-peaks between neighboring hydrogens (with opposite sign relative to the diagonal peaks) are observed in the ROESY spectrum (Fig. 4a). Presence of catalytic amounts of CyP1 enhanced the cis/trans isomerization of P107, as revealed by the appearance of exchange cross-peaks connecting cis and trans Hα peaks of G106, P107.

### Table 1. Data collection and refinement statistics of TvCyP1 apo and peptide-bound structures.

| Crystal parameters | Apo form | Peptide-bound form |
|--------------------|----------|---------------------|
| Space group | P6₁,22 | P2₁,2,2₁ |
| Unit cell parameters | | |
| a, b, c (Å) | 38.51; 38.51; 365.70 | 37.79; 78.07; 117.71 |
| α, β, γ (°) | 90, 90, 120 | 90, 90, 90 |
| Monomers per asymmetric unit cell | 1 | 2 |

| Data collection | | |
| Wavelength (Å) | 0.9998 | 0.9762 |
| Resolution range (Å) | 29.25−2.28 (2.35−2.28) | 29.45−2.06 (2.14−2.06) |
| Unique no. of reflections | 8106 | 21875 |
| Total no. of reflections | 29747 | 115767 |
| Completeness (%) | 90.0 (86.0) | 91.0 (90.0) |
| Redundancy | 3.7 (2.7) | 5.3 (3.9) |
| CC₁/₂ | 0.966 (0.880) | 0.996 (0.769) |

| Refinement statistics | | |
| Resolution (Å) | 2.27 | 2.06 |
| Rwork (%) | 21.20/27.80 | 16.79/21.29 |
| MRMSD (Å) | 0.009 | 0.007 |
| Peptide | 27.35 | 24.99 |
| Solvent | 31.90 | 32.07 |
| Ramachandran plot (%) | 82.2/17.1/0.7/0 | 85.7/14.0/0.3/0 |

TvCyP1 is the only known dimeric cyclophilin with the active sites exposed, and we tried to obtain direct evidence for catalysis activity by using NMR rotating-frame Overhauser effect spectroscopy (ROESY). In a ROESY spectrum, the sign of the cross-peaks from chemical exchange is the same as the diagonal peaks, while the cross-peaks between neighboring hydrogens display an opposite sign. In order to monitor the catalysis of cis/trans isomerization of P107 in Myb1 104–111 by CyP1, we completed the 1H resonance assignments of Myb1 104–111 peptide. Separate resonance signals were observed for the cis and trans conformations of residues from Y103 to K111 (Supplementary Fig. S4), indicating that each conformation lies in a distinct chemical environment and the cis/trans isomerization is slow on NMR time scale (exchange rate <0.1 s⁻¹). Because of the slow exchange rate of the un-catalyzed isomerization, only the cross-peaks between neighboring hydrogens (with opposite sign relative to the diagonal peaks) are observed in the ROESY spectrum (Fig. 4a). Presence of catalytic amounts of CyP1 enhanced the cis/trans isomerization of P107, as revealed by the appearance of exchange cross-peaks connecting cis and trans Hα peaks of G106, P107.
Figure 2. Dimeric structure of TvCyP1 by x-ray crystallography. (a) Structure of TvCyP1 dimer showing secondary structural elements (PDB: 5YB9). The divergent loop of TvCyP1 is highlighted in red. (b) Structural alignment of TvCyP1 protomer (blue) with HcypA (orange). Both structures show the typical cyclophilin fold and exhibit high similarity. (c) Surface representation of TvCyP1 highlighting the active site pocket and S2 pocket. The active site residues and “gatekeeper” residues that guard the S2 pocket are shown in sticks. The structure shows the antiparallel nature of TvCyP1 homodimer. (d) and (e) Close-up views of polar/salt bridge (d) and hydrophobic interactions (e) at TvCyP1 dimer interface. The residues that form a dimer interface from either of the protomers are shown in different colored sticks. Dotted lines indicate hydrogen bond/salt bridge interactions. Oxygen, nitrogen and sulfur atoms are shown in red, blue and yellow, respectively.
and K108 (Fig. 4b). The addition of 1.0 molar equivalent of CsA, a strong binding HcypA inhibitor, hindered catalysis, as observed by the disappearance of exchange cross-peaks (Fig. 4c). This finding suggests that CsA or CsA analogues can bind to the active site of \( \text{TvCyP1} \) with greater affinity than \( \text{Myb1}^{104-111} \) (shown as green and magenta sticks; PDB: 5YBA). Each protomer binds to one molecule of Myb1 peptide. Although the peptide used was \( \text{Myb1}^{104-111} \), electron densities of only \( \text{105YGP}^{110} \) (protomer I) and \( \text{104EYGPK}^{108} \) (protomer II) were obtained in the complex structure. Intramolecular salt bridge interaction between K108 and E104 is shown as a green dashed line (in \( \text{104EYGPK}^{108} \) bound to protomer II). (c and d) Close-up views of interactions between \( \text{TvCyP1} \) and Myb1 peptide. Residues of \( \text{TvCyP1} \) interacting with Myb1 peptide are shown as orange sticks. Fragments of Myb1 peptide, \( \text{105YGP}^{110} \) and \( \text{104EYGPK}^{108} \), bound to either protomer, are represented as green and magenta sticks in (c) and (d), respectively. The hydrogen bond between the \( \text{C}=\text{O} \) of P107 and the side chain NH of the catalytic residue R63 is shown as a black dashed line. See Supplementary Fig. S2 for a close-up view of the conformation of the Myb1 peptide bound to either protomer and the conformation of the same sequence in \( \text{Myb1}^{35-141} \). See Supplementary Fig. S3 also for a 2D diagram by Ligplot showing detailed interactions between Myb1 peptide and \( \text{TvCyP1} \).

**Figure 3.** The minimum binding sequence in Myb1 and the complex structure of \( \text{TvCyP1} \) with the same. (a) Binding curves from fluorescence polarization experiments of Myb1 fragments with \( \text{TvCyP1} \). Error bars are standard deviation (SD). Binding affinities were derived from a one-site binding model by using GraphPad Prism 6. \( \text{104EYGPKWN}^{111} \) (Myb1\textsuperscript{104-111}) showed the highest binding affinity to \( \text{TvCyP1} \) (16.81 ± 0.58μM). (b) Electrostatic surface of \( \text{TvCyP1} \) dimer in complex with the minimum binding sequence of Myb1, \( \text{Myb1}^{104-111} \) (shown as green and magenta sticks; PDB: 5YBA). Each protomer binds to one molecule of Myb1 peptide. Although the peptide used was \( \text{Myb1}^{104-111} \), electron densities of only \( \text{105YGP}^{110} \) (protomer I) and \( \text{104EYGPK}^{108} \) (protomer II) were obtained in the complex structure. Intramolecular salt bridge interaction between K108 and E104 is shown as a green dashed line (in \( \text{104EYGPK}^{108} \) bound to protomer II). (c and d) Close-up views of interactions between \( \text{TvCyP1} \) and Myb1 peptide. Residues of \( \text{TvCyP1} \) interacting with Myb1 peptide are shown as orange sticks. Fragments of Myb1 peptide, \( \text{105YGP}^{110} \) and \( \text{104EYGPK}^{108} \), bound to either protomer, are represented as green and magenta sticks in (c) and (d), respectively. The hydrogen bond between the \( \text{C}=\text{O} \) of P107 and the side chain NH of the catalytic residue R63 is shown as a black dashed line. See Supplementary Fig. S2 for a close-up view of the conformation of the Myb1 peptide bound to either protomer and the conformation of the same sequence in \( \text{Myb1}^{35-141} \). See Supplementary Fig. S3 also for a 2D diagram by Ligplot showing detailed interactions between Myb1 peptide and \( \text{TvCyP1} \).

**Identification of \( \text{TvCyP1} \)-binding site on \( \text{Myb1}^{35-141} \) protein.** Unlike the typical substrates for cyclophilins that exhibit flexible structures to fit into the active site pocket, Myb1 is a well-folded protein with \( \text{TvCyP1} \)-binding key residues, \( \text{105YGP}^{107} \), lying in a short loop structurally restrained by two stable helices. Hence, the conformation of the minimum binding sequence, \( \text{104EYGPKWN}^{111} \), in Myb1 protein is very different from those observed in the complex structure (Fig. 5a). Our previous NMR structural study on Myb1\textsuperscript{35-141} indicated that all residues from E104 to K111 adopt a \( \text{trans} \) conformation and there was no NMR signal from the \( \text{cis} \) conformer\textsuperscript{35}. As a result, it was not possible to study the catalysis of \( \text{cis/trans} \) isomerization of \( \text{105GP}^{107} \) bond in \( \text{Myb1}^{35-141} \) protein by \( \text{TvCyP1} \). Also, we tried to co-crystallize the \( \text{TvCyP1} \)–\( \text{Myb1}^{35-141} \) protein complex but could not obtain any crystal.
To identify the TvCyP1-binding site on Myb135–141 protein, we used NMR. The titration of unlabeled TvCyP1 to 15N-labeled Myb135–141, which includes the R2-R3 DNA binding domains of Myb1, resulted in significant line-width broadening of all residues but very little chemical shifts perturbation (Supplementary Fig. S5). To identify the TvCyP1 binding site on Myb135–141 protein, we used cross-saturation transfer NMR that helps to identify the residues involved in direct intermolecular contacts33. We prepared uniformly 15N, 2H (>95%)-labeled Myb135–141 to avoid excitation of its aliphatic protons by the radio-frequency pulses. The labeled Myb135–141 protein mixed with unlabeled TvCyP1 was dissolved in 50% 2H2O to reduce saturation transfer from spatially crowded amide protons by 1H2O. The plot of intensity ratios of amide peaks with and without saturation versus residue number showed a significant decrease in amide peak intensity for the residues near P107, with the minimum at G106 and

Figure 4. Catalysis of the cis/trans isomerization of Myb1104–111 peptide by TvCyP1. (a) Selected region of the 300-ms mixing-time ROESY spectrum of Myb1104–111 peptide (4.4 mM). The positive peaks are colored in blue and negative peaks in red. The 1H resonances of G106, P107 and K108 are marked, and the resonances from cis and trans conformers are labeled as C and T respectively. The negative cross-peaks between neighboring hydrogens are shown in red and connected by red dashed lines. (b) Exchange between cis and trans conformations was accelerated in the presence of 22μM TvCyP1, as evidenced by the appearance of positive exchange cross-peaks between cis and trans resonances shown in blue and connected by black dashed lines. (c) Inhibition of TvCyP1 isomerase activity by CsA (22μM) resulted in a loss of the exchange cross-peaks between cis and trans resonances.
K108 (Fig. 5b). Although there are 5 proline residues in Myb1$_{35-141}$, P70, P83, P89, P92 and P107, cross-saturation transfer NMR clearly showed that TvCyP1 interacts with the P107-containing loop in Myb1$_{35-141}$ protein (Fig. 5c).

**Mapping the Myb1$_{35-141}$ protein interaction region on TvCyP1.** Further, we tried to map the Myb1$_{35-141}$ protein binding site on TvCyP1 by NMR. To achieve this goal, we firstly completed the $^1$H, $^{13}$C and $^{15}$N backbone chemical shift assignment of TvCyP1 using standard triple-resonance NMR spectra acquired from a $^2$H, $^{13}$C, $^{15}$N (>95%)-labeled protein sample. The $^1$H, $^{13}$N TROSY-HSQC of TvCyP1 features an extremely well dispersed set of resonances (Supplementary Fig. S2), agreeing with the well-folded structure of this protein. To map the

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**Figure 5.** TvCyP1 interacts with the P107-containing loop in Myb1$_{35-141}$ protein. (a) Conformations of the minimum binding sequence in protomer I (left), protomer II (middle) and Myb1$_{35-141}$ protein (right), showing that the structure of this sequence in 3 states is different from each other. (b) The intensity ratio of the amide proton resonances, with and without irradiation of the aliphatic protons, plotted against the Myb1$_{35-141}$ residue number. The dashed line signifies one SD from the average intensity ratio (~0.8). (c) Mapping the cross-saturation transfer NMR results on Myb1$_{35-141}$ protein structure. Proline residues are shown as black spheres. The residues exhibiting intensity ratios lower that 0.8 are shown as red spheres and others as cyan spheres. Since TvCyP1 is a PPlase, significant reduction in peak intensities of residues near P107 indicates that TvCyP1 binds to the loop containing P107.
Myb\textsuperscript{35–141} protein interaction site on \textit{TvCyP1}, we titrated 2 folds of unlabeled Myb\textsuperscript{35–141} protein to \textit{15N}-labeled \textit{TvCyP1}, resulting in severe line-width broadening of the peaks of the active site and “gatekeeper” residues of \textit{TvCyP1} (Fig. 6a,b and Supplementary Fig. S6). Apart from the active site and “gatekeeper” residues, certain residues in faraway loops were also perturbed or line-width–broadened during titration owing to conformational exchange during substrate turnover\textsuperscript{34}. To confirm the specificity of interaction, we produced a Myb\textsuperscript{35–141} mutant protein with P107 mutated to alanine (P107A-Myb\textsuperscript{35–141}) and titrated unlabeled P107A-Myb\textsuperscript{35–141} to \textit{15N}-labeled \textit{TvCyP1} (Supplementary Fig. S7). The nearly identical NMR spectra from \textit{TvCyP1} with and without P107A-Myb\textsuperscript{35–141} suggested that \textit{TvCyP1} recognizes P107A-Myb\textsuperscript{35–141} weakly. Of note, despite 5 proline residues in Myb\textsuperscript{35–141}, only P107 can bind to \textit{TvCyP1} active site pocket specifically.

Although we tried to measure the binding affinity of \textit{TvCyP1–Myb\textsuperscript{104–111}} interaction using several biophysical techniques, we were not successful. Hence, to compare \textit{TvCyP1–Myb\textsuperscript{104–111}} and \textit{TvCyP1–Myb\textsuperscript{35–141}} interactions, we also titrated Myb\textsuperscript{104–111} to \textit{15N}-labeled \textit{TvCyP1}. The titration resulted in a notable chemical shift perturbation (CSP) of several \textit{TvCyP1} residues (Fig. 6c and Supplementary Fig. S6). Structural mapping of these residues showed that most of the \textit{TvCyP1} residues that underwent significant chemical shift perturbation on Myb\textsuperscript{104–111} peptide titration also exhibited severe line-width broadening on Myb\textsuperscript{35–141} protein titration (Fig. 6d). Although, in the well-folded Myb\textsuperscript{35–141} protein, the minimum binding sequence adopts a very different conformation from that observed in the complex structure, our NMR studies suggest that the interaction between \textit{TvCyP1} and Myb\textsuperscript{35–141} protein is similar to the highly specific recognition between \textit{TvCyP1} and Myb\textsuperscript{104–111} peptide.

**Interaction with \textit{TvCyP1} reduces the slow dynamics in Myb\textsuperscript{35–141} around P107.** We carried out NMR Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion experiments at 1H frequency of 600 and 850 MHz on Myb\textsuperscript{35–141} in the free form and in complex with \textit{TvCyP1} to probe the millisecond time scale conformational exchange of Myb\textsuperscript{35–141} in 2 different states. The \textit{15N} relaxation dispersion profiles of individual residues

![Figure 6. Interaction of \textit{TvCyP1} with Myb\textsuperscript{35–141} protein is similar to that of its binding to Myb\textsuperscript{104–111} peptide.](image-url)
of Myb135–141 were fitted to a two-site exchange process using CR72 or TSMFK01 model by the software relax37 (Fig. 7a) and the extracted dynamic parameters are listed in Supplementary Tables S1 and S2 for the CR72 and TSMFK01 models, respectively. Plenty of residues in free Myb135–141 displayed conformational exchange (Fig. 7b), suggesting that Myb1 is a highly dynamic protein. However, the exchange rates for the residues around P107 are too different to be fitted globally to a single model. The residues with very slow exchange rates were fitted to the TSMFK01 model and those with faster exchange rates were fitted to the CR72 model, shown as yellow and red spheres respectively in Fig. 7b,c. In free Myb135–141, E104 and several residues in α5 are restricted to become very slow dynamics and can be fitted with the TSMFK model.

Discussion
Dimerization via the interaction of hydrophobic residue pairs in a mirror-image–like fashion as seen in TvCyP1 has been observed in various dimeric bacterial FKBP-type PPIases38,39, but not in any other cyclophilins. Most of the cyclophilins known to date are monomeric in nature, with certain exceptions such as Aspergillus fumigatus.
cyclophilin (Asp f11; PDB: 2C3B), cyclophilin from Mimivirus (Mimicyc; PDB: 2OSE) and cyclophilin from *Hirschia baltica* (AquaCyp300; PDB: 5EX1). However, AquaCyp300 is not a single domain cyclophilin because N- and C-terminal extensions together with the large insertion in the cyclophilin domain form a contiguous structural entity termed the NIC domain40. The dimer interface in AquaCyp300 is formed by residues of both the cyclophilin domain and NIC domain. Although Asp f11 and Mimicyc are single-domain cyclophilins with swapped dimeric and trimeric structures, respectively, they were found to be monomeric in solution28,29. Moreover, multimerization in these two proteins resulted in obstruction of the active site, thereby hindering substrate interaction. In contrast, TvCyP1 exhibits a natural tendency to form a dimer even in solution, as observed from a single dimeric peak in SEC-MALS and AUC-SV analyses, and has the active site of both protomers exposed for interaction with the substrate. Hence, TvCyP1 is the only single-domain dimeric cyclophilin reported so far that can still bind to its substrates.

The complex structures of cyclophilin with its biologically relevant substrate are available for only HcypA. The complex structures of HcypA with HIV capsid protein and CrkII protein show high structural similarity because both contain a Gly-Pro motif binding to the active site with proline fitting deep inside the hydrophobic pocket of the active site41,42. However, neither of these biological substrates show strong interaction with the S2 pocket because of the absence of residues that exhibit surface complementarity to the S2 pocket of HcypA.

Myb1 is an ideal cyclophilin substrate due to the presence of Tyr-Gly sequence preceding the interacting proline that enables a strong interaction with the active site and S2 pocket of TvCyP117. As a result, in the TvCyP1–Myb1104–111 complex structure, we observed that Y105 was inserted well inside the S2 pocket. However, Myb1105–141 is a well-folded protein with interacting proline, lying in a short loop surrounded by two stable helices. Hence, in the well-folded Myb1 protein, the interaction of Y105 with the S2 pocket is structurally hindered and therefore could occur only if some conformational changes take place. This was found to be true from CPMG relaxation dispersion experiments which showed that Myb1105–141 underwent slow conformational exchange. The slow dynamics near the region containing P107 was greatly reduced upon interaction with TvCyP1 further suggesting that the interaction is facilitated by dynamics.

In the complex structure, we observed a difference in the conformation of Myb1104–111 peptide bound to both protomers of TvCyP1 because of different side-chain orientations of K108. Structural comparison showed that the conformation of 104EYGPK108 in protomer II is more similar to the conformation of the loop in Myb1 containing P107 (Fig. 2a). Hence, with some conformational changes at Y105, the minimum binding sequence in Myb1105–141 upon interaction with TvCyP1 could probably adopt a similar structure as that of 104EYGPK108 bound to protomer II.

Nuclear translocation of Myb1 is an important step regulating the expression of disease causing *ap65–1* gene. Hence, preventing the nuclear import by blocking the interaction between Myb1 and TvCyP1 can be a good strategy to treat the disease. From ROESY experiments, we observed that CsA can inhibit Myb1104–111 binding to the active site. Although CsA has long been proposed to be an inhibitor for cyclophilin 4, the deleterious effect of its immunosuppressive activity44 has always been a major hindrance in using it as a drug to treat diseases. Moreover, CsA is not specific to any of the isoforms of cyclophilins45. Many attempts to design and synthesize an isoform-specific drug for cyclophilins have not been successful and the S2 region has been proposed as an ideal site for isoform-specific drug development. However, because HcypA and TvCyP1 possess the same set of “gate-keeper” residues, a drug targeting the S2 region of TvCyP1 may also be able to interact with HcypA in humans. However, the harmful effect of this unwanted interaction would probably not be as detrimental as immunosuppression and hence is worth studying. An alternative and probably better drug target site would be the dimer interface. As shown in Fig. 2c, there is a pocket formed by the dimer interface in TvCyP1. A CsA analogue that can recognize both the active site and the dimer interface pockets may specifically recognize TvCyP1 but not the monomeric HcypA.

Collectively, our study has helped in understanding the unique dimeric structure of TvCyP1 and obtaining detailed information about its interaction with the biological substrate Myb1. TvCyP1 has also been found to interact with Myb327, and future work will elucidate the details of the Myb3–TvCyP1 interaction. It is also possible that TvCyP1 dimerization may help in the simultaneous binding of Myb1 and Myb3 at the active sites of either protomer. Indeed, much needs to be studied at the cellular level to further understand the role of CyP1 and the significance of its dimerization on the parasite's development and pathogenicity. However, our current data shows that TvCyP1 is a good drug target and provides a rationale to design drugs specific for the protein or to disrupt its interaction with Myb1.

**Methods**

**Expression and purification of TvCyP1 and TvCyP1 mutants.** TvCyP1 gene was cloned into pET-28a vector (Novagen) and was expressed with an N-terminal His-tag followed by a TEV digestion recognition site in the *Escherichia coli* strain BL21 (DE3). After TEV protease digestion, TvCyP1 protein had an additional Glycine and Serine at its N-terminus. For unlabelled or uniformly labeled samples, *E. coli* cells were cultivated in hysogeny broth medium or M9 minimal medium at 37 °C, respectively. The recombinant TvCyP1 was expressed at 16 °C by the addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) with the culture medium reaching optical density at 600 nm of 0.6. Cells were harvested 16 hr after induction and resuspended in Tris-HCl (pH 8.0) and lysed by using a M-110S microfluidizer (Microfluidics). The insoluble fraction was removed by centrifugation at 12,000 rpm for 30 min. The supernatant was passed through Q ion-exchange resin (Q sepharose fastflow, GE healthcare) and the flow-through containing TvCyP1 was further purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity resin (Qiagen, Hilden, Germany) equilibrated with 20 mM Tris-HCl, 100 mM NaCl, and 10 mM Imidazole (pH 8.0). TvCyP1 was eluted from the Ni-NTA resin in 500 mM imidazole, 100 mM NaCl and 20 mM Tris solution at pH 8.0. The eluate was treated with TEV protease overnight at room temperature and then diluted to the final imidazole concentration of 10 mM and passed through the Ni-NTA resin again. The
flow-through containing TvCyP1 was buffer-exchanged in 20 mM NaH2PO4, 50 mM NaCl and 0.5 mM NaN3 (pH 6.0) by centrifugation with 10,000 Da MWC membrane ultrafiltration (Millipore). The 3H/35/35N-labeled TvCyP1 was prepared in the same way, but the host cells were grown in M9 minimal medium containing D2O supplemented with 15N-NH4Cl (1 g/L) and 13C-glucose (2 g/L). The protein purity was verified by SDS-PAGE, and the concentration of TvCyP1 monomers was assessed by using a molar absorption coefficient of $E_{280} = 8,480 \text{ M}^{-1}\text{cm}^{-1}$. TvCyP1 mutants were expressed and purified using the same protocol.

**Preparation of Myb1, Myb1 mutants and Myb1 peptides.** The gene encoding Myb135–141 was cloned into the pET-29b (Novagen) vector and expressed in *E. coli* BL21(DE3) cultured in lysogeny medium or minimal medium for 1H/15N-labeled samples. The expressed Myb135–141 has two extra residues in the C-terminus, leucine and glutamic acid, followed by a His-tag. Myb135–141 was expressed and purified as described32. 1H,13C,15N-labeled Myb1 was prepared in the same way, but the host cells were grown in M9 minimal medium containing D2O supplemented with 15N-NH4Cl (1 g/L) and 13C,1H glucose (2 g/L). The purity of Myb135–141 was confirmed by SDS-PAGE and the concentration was calculated by using a molar absorption coefficient of $E_{208} = 33,460 \text{ M}^{-1}\text{cm}^{-1}$. The final protein sample used for experiments was in a buffer containing 20 mM NaH2PO4, 50 mM NaCl and 0.5 mM NaN3 (pH 6.0). Myb1 mutants were expressed and purified using the same protocol. Myb1 peptides were purchased from Yao-Hong Biotechnology Inc. (Taiwan) and FITC-labeled Myb1 peptides were synthesized by the peptide synthesis facility at the Institute of Biological Chemistry, Academia Sinica.

**Size-exclusion chromatography coupled to multi-angle static light scattering (SEC-MALS).** A total of 300 μg of TvCyP1 was injected into a size-exclusion chromatography column (Enrich™ SEC. 70 10 × 300 Column, Bio-Rad Laboratories, Inc.) and analyzed by static and dynamic light scattering45 at a flow rate of 0.5 mL min$^{-1}$ in 20 mM monosodium phosphate, 50 mM NaCl, 0.5 mM NaN3 (pH 6.0) at 25 °C. The column is in line with four detectors: a static light-scattering detector (miniDAWN TREOS, Wyatt Technology), a quasi-elastic light-scattering detector (QELS, Wyatt Technology), a refractive index detector (Optilab T-rEX, Wyatt Technology) and an ultraviolet-visible (UV) detector (Agilent, USA). Bovine serum albumin (Sigma, A1900) was used for system calibration. Molecular weight was calculated by using ASTRA 6 (Wyatt Technology) with the dn/dc value set to 0.185 mL g$^{-1}$.

**Analytical ultracentrifugation.** AUC-SV experiments were conducted on a Beckman Coulter ProteomeLab XL-I ultracentrifuge with an AN 50Ti rotor. The sample was collected from a Superdex 75 gel filtration column (GE Healthcare Life Sciences) after equilibrating the column extensively with 20 mM monosodium phosphate, 50 mM NaCl, 0.5 mM NaN3 (pH 6.0). We loaded 400 μL of 29 μM TvCyP1 and collected data at 20 °C at 50,000 rpm at 280 nm. The scans were collected every minute, with a total 150 scans obtained per sample. Buffer density, viscosity and partial specific volume were calculated by using SednTerp. The data were edited and processed by using SedFit software (https://sedfitsedphat.nibib.nih.gov).

**Fluorescence polarization measurements.** Myb1 peptides for fluorescence polarization experiments were labeled with FITC at the N-terminus. The indicated amounts of TvCyP1 were added to wells containing 0.5 μM FITC-labeled Myb1 peptide in 20 mM monosodium phosphate, 50 mM NaCl, 0.5 mM NaN3 (pH 6.0) at 298 K. Triplicate measurements of the reactions were acquired by using the SpectraMax Paradigm microplate reader (Molecular Devices, CA, USA) with excitation wavelength 485 nm and emission wavelength 535 nm. Data were analyzed and fitted to a one-site binding model by using GraphPad Prism 6 (San Diego, CA, USA).

**Protein crystallization and data collection.** For crystallography screening experiments, TvCyP1 sample was further purified on a Superdex 75-gel filtration column (GE Healthcare Life Sciences) after equilibrating the column extensively with 20 mM Bis-Tris, 50 mM NaCl and 0.5 mM NaN3 (pH 6.0). Initial protein crystallization trials were performed at 283 K by the sitting-drop vapour-diffusion method with commercial crystallization screen kits, 96-well Intelli-plates (Art Robbins Instruments) and a Phoenix robot (Art Robbins Instruments). For co-crystallizing with Myb1104–111 peptide, purified TvCyP1 protein was mixed with the synthetic Myb1104–111 peptide (Glu-Tyr-Gly-Pro-Lys-Trp-Asn-Lys) in a molar ratio of 1:4 before crystallization trials. Each crystallization drop was prepared by mixing 0.3 μL TvCyP1 or TvCyP1/peptide at 9 mg/mL with an equal volume of mother liquor, and the mixture was equilibrated against 100 μL reservoir solution. The crystals of TvCyP1 in apo and Myb1 peptide-bound forms were grown at 283 K within 7–14 days with the optimal conditions of 100 mM HEPES, pH 7.0, 30% (v/v) Jefamine M-600 and 100 mM Tris-HCl pH 8.0, 30% (v/v) polyethylene glycol 400, respectively. For diffraction data collection, the crystal was cryoprotected in mother liquor supplemented with 20% glycerol, and flash-frozen in liquid nitrogen at 100 K. The diffraction images of TvCyP1 in apo and Myb1 peptide-bound forms were recorded at the National Synchrotron Radiation Research Center (Taiwan) on a MX300HS detector in TPS 0SA and a Q315r detector in TLS BL13C1 beamlines, respectively. Diffraction data were processed and scaled by using HKL2000 software46.

**Structure determination and refinement.** The crystal structures of TvCyP1 and TvCyP1 in complex with Myb1104–111 peptide were determined by molecular replacement with the Phaser-MR program47, by using the Ccyp3 structure from *C. elegans* (PDB: 1DYW) as a search model. Crystallographic refinement involved repeated cycles of conjugate-gradient energy minimization and temperature-factor refinement with the program phenix.refine in the PHENIX package48. Amino-acid side chains and water molecules were fitted into 2Fo-Fc and Fo-Fc electron-density maps by using COOT49. The model was evaluated by using PROCHECK50 and MOLPROBITY51. The data collection and structure refinement statistics are in Table 1. Final coordinates and structure factors of TvCyP1 and the TvCyP1–Myb1 peptide complex have been deposited in the Protein Data Bank (PDB: 5YB9 and 5YBA, respectively).
NMR experiments. TvcyP1 and Myb115-111 samples for NMR experiments were prepared in 20 mM monosodium phosphate, 50 mM NaCl, 0.5 mM NaN3 (pH 6.0). To improve the spectral quality of TvcyP1, the sample used for backbone assignment was 1H, 15N, 13C-labeled. The NMR spectra were acquired on a Bruker AVANCE 600-, 800- and 850-MHz spectrometers equipped with a z-gradien TCI cryoprobe (Bruker, Karlsruhe, Germany) at 310 K. Backbone assignment of TvcyP1 was based on TROSY-HNCA/CB and -HNCACB spectra32. Backbone assignment of FM-TvcyP1 was based on the HNCACB spectrum with a 15N, 13C-labeled sample. Backbone assignment of Myb115-111 was reported previously32. Observation of chemical-shift changes as well as reduction in peak intensity in 1H, 15N TROSY-HSQC of 15N-enriched TvcyP1 upon titrating unlabeled Myb1 peptide or Myb115-111 was used to confirm interactions and to determine the binding site of TvcyP1. The weighted CSPs for backbone 15N and 1H resonances were calculated with the equation 
\[
\Delta \delta = \frac{1}{2}(\Delta \delta_{15N}^0 + (\Delta \delta_{1H}^0)^2)^{1/2}.
\]
All experiments with Myb1 were performed at 298 K. The sample for cross-saturation transfer experiments contained a 1H, 15N-labeled Myb115-111 sample (333 μM) and unlabeled TvcyP1 (1000 μM) in 20 mM monosodium phosphate, 50 mM NaCl, 0.5 mM NaN3 (pH 6.0). To avoid spin diffusion, the sample was prepared in 50% D2O. The deuteration percentage of Myb135–141 is more than 95%. The saturation pulses were centered at 516.8 Hz (0.608 ppm) at which we observed large proton peaks from TvcyP1 and no peak from Myb115-114. The measurement time was 5.5 h, with relaxation delay and saturation time 5 s.

1H-TOCSY, 1H-COSTY and 1H-NOESY were performed at 300 K with a mixing time of 75 ms (TOCSY) and 300 ms (NOESY) on the Myb1104–111 peptide to complete the assignment of proton resonances. The peptide was dissolved in 20 mM monosodium phosphate, 50 mM NaCl, 0.5 mM NaN3 (pH 6.0) to obtain a concentration of 2 mM. All ROESY experiments were performed at 300 K with 300 ms mixing time. For catalysis experiments, Myb1104–111 peptide (2000 μM) was mixed with TvcyP1/FM-TvcyP1 (13.3 μM) at a ratio of 250:1.

The 15N, 13C-labeled Myb115-111 (0.3 mM) in the free form and in complex with unlabeled TvcyP1 (0.36 mM) or unlabeled FM-TvcyP1 (0.36 mM) were prepared for CPMG measurement. The constant-time 15N relaxation dispersion profiles of individual residues at both 600 and 850 MHz were acquired at 298 K on both 600 MHz and 850 MHz NMR spectrometers with νCPMG of 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 Hz and a total CPMG delay of 40 ms. The spectra with νCPMG of 100 and 700 Hz were collected twice to estimate experimental errors. The peak intensities in each spectrum were extracted by using an automated routine in NMRView. The R2eff was calculated as R2eff(νCPMG) = (1/T) ln[I(νCPMG)]/I0, where I(νCPMG) and I0 are the intensities of peaks recorded with and without the CPMG intervals. The 15N relaxation dispersion profiles of individual residues at both 600 and 850 MHz were fitted to a two-site exchange process by the software relax37. Two models, the Carver and Richards equation (CR72) that describes 2-site exchange for most time scales and TSMFK0136, which is appropriate for 2-site very slow exchange within range of microsecond to second time scale, were fitted. The Akaike’s model selection38 was performed to judge statistical significance of the models. Successful fit to CR72 model yields the Δωex, the exchange rate (kex), the population of the major state (pA) and the chemical shift difference between 2 states (Δω0). The fit to TSMFK01 model yields R2A, kex and Δω0. The extracted dynamic parameters are listed in Supplementary Tables S1 and S2 for the CR72 and TSMFK01 models, respectively.

All NMR spectra, except the spectra for cross-saturation transfer and CPMG relaxation dispersion experiments, were processed by using Topspin 3.1 (Bruker) and analyzed by using NMRView34. For cross-saturation transfer experiments, the spectra were processed by using Topspin 3.1 (Bruker) and analyzed by using Sparky (T.D. Goddard and D.G. Kneller, Sparky 3, University of California, San Francisco). For CPMG-based relaxation dispersion experiments, the spectra were processed by using NMRpipe35 and analyzed by using NMRviewJ.

Data availability. The datasets generated during the current study are available in the RCSB PDB repository, [https://www.rcsb.org/pdb/home/home.do]. The backbone chemical shift assignment of TvcyP1 generated during the current study are available in BMRB under accession code 12014.

Accession Numbers. The atomic coordinates and structure factors for TvcyP1 and TvcyP1-Myb1 peptide complex are deposited under RCSB PDB accession codes PDB: 5YB9 and 5YBA respectively. The backbone NMR chemical shift of TvcyP1 has been deposited in the BMRB under accession code 12014.

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Author Contributions
T.M. and C.C. designed all the experiments; S.Y.W. cloned the protein constructs; T.M. expressed and purified the proteins; T.M. and S.S. performed mutagenesis experiments; C.C. Chou, C.C. Cho and M.H.L. crystallized the protein and protein-peptide complex, collected diffraction data; C.H.H. determined the crystal structures; S.Y.W. cloned the protein constructs; T.M. expressed and purified the proteins; T.M. performed NMR experiments and fluorescence polarization experiments and analyzed the data; Y.C.L. performed CPMG relaxation dispersion NMR experiments and analyzed the data; T.M. analyzed AUC and SEC-MALS data; C.C. supervised the research; T.M., Y.C.L., C.H.H., J.H.T. and C.C. wrote the manuscript.

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