**Structure**

**Structural Basis for Substrate Selectivity of the E3 Ligase COP1**

**Graphical Abstract**

Conserved top surface of COP1 β-Propeller

Tribbles peptide bound to top surface

ΔΔG for substitutions of DQIVPEY

**Highlights**

- X-Ray structures reported for human and plant COP1 β-propeller-peptide complexes
- Trib1 peptides bind to a conserved surface on the top face of the propeller
- Structures reveal a universal mechanism for motif recognition by COP1
- Competition binding assays confirm importance of consensus VP sequence

**Authors**

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**In Brief**

COP1 proteins are E3 ubiquitin ligases that regulate phototropism in plants and target transcription factors for degradation in mammals. The substrate-binding region of COP1 resides within a WD40-repeat domain that also binds to Trib proteins, the adaptors for C/EBPα degradation. Uljon et al. identify the structural basis and key interactions for motif recognition by COP1, and hint at how Trib1 autoinhibition is overcome to target C/EBPα for degradation.

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Structural Basis for Substrate Selectivity of the E3 Ligase COP1

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SUMMARY

COP1 proteins are E3 ubiquitin ligases that regulate phototropism in plants and target transcription factors for degradation in mammals. The substrate-binding region of COP1 resides within a WD40-repeat domain that also binds to Trib proteins, which are adaptors for C/EBPα degradation. Here we report structures of the human COP1 WD40 domain in isolation, and complexes of the human and Arabidopsis thaliana COP1 WD40 domains with the binding motif of Trib1. The human and Arabidopsis WD40 domains are seven-bladed β propellers with an inserted loop on the bottom face of the first blade. The Trib1 peptide binds in an extended conformation to a highly conserved surface on the top face of the β propeller, indicating a general mode for recognition of peptide motifs by COP1. Together, these studies identify the structural basis and key interactions for motif recognition by COP1, and hint at how Trib1 autoinhibition is overcome to target C/EBPα for degradation.

INTRODUCTION

COP1 proteins are E3 ubiquitin ligases that regulate phototropism in plants and target transcription factors for degradation in mammals. In Arabidopsis thaliana, where COP1 was first described, it represses the light response by promoting the ubiquitination and degradation of HY5/HYH family transcription factors, which contain a targeting motif with the consensus amino acid sequence ESDEExxxVP[DE] (Ang et al., 1998; Deng et al., 1991, 1992; Holm and Deng, 1999; Holm et al., 2002). A. thaliana COP1 can also promote degradation of transcription factors that do not contain canonical COP1 recognition sequences, such as PIF1 and LAF1 (Maier et al., 2013; Seo et al., 2003). Emerging evidence also suggests that COP1 is regulated both by light-dependent sequestration (via the protein UVXR8) and by its participation in multisubunit cullin-based E3 ligase complexes that promote degradation of specific transcription factors (Huang et al., 2013).

In mammals, COP1 (gene name RFWD2) is essential, and has been implicated as both a tumor suppressor and a cancer promoter. Supporting a tumor-suppressor function, COP1 hypomorphic mice develop tumors at much higher rates than wild-type mice (Migliorini et al., 2011). In addition, Jun and ETS transcription factors, which contain COP1-binding motifs, are direct targets of COP1-mediated degradation, and levels of COP1 inversely correlate with amounts of Jun and ETS in human and murine prostate cancers (Bianchi et al., 2003; Migliorini et al., 2011; Vitari et al., 2011). In contrast, COP1 is highly expressed in several other human cancers (Dorman et al., 2004a). The tumor suppressors p53 and p27 are proposed to be degradation targets of COP1 and, in the case of p27, there is evidence of a direct interaction requiring a VPA motif in p27. These data suggest that COP1 promotes cancer in these contexts (Choi et al., 2015; Dorman et al., 2004b).

Another context in which COP1 promotes tumorigenesis is in a mouse model of acute myelogenous leukemia (AML). In this model, forced expression of either Trib1 or Trib2, each of which is a homolog of Drosophila tribbles, gives rise to AML with high penetrance. Tumor formation in this context relies on the integrity of a COP1-binding motif near the Trib C terminus, recently shown to be held tethered to the N lobe of Trib1 in the absence of COP1 (Murphy et al., 2015). Additional studies probing the molecular mechanism of leukemogenesis show that COP1 does not efficiently degrade Trib1 or Trib2. Instead, it appears that Trib1 and Trib2 serve as adaptors that bind COP1, and direct it to degrade the differentiation-promoting transcription factor C/EBPα, which lacks a canonical COP1-binding motif (Keeshan et al., 2006, 2010).

Despite the fundamental importance of the COP1 E3 ligase in both the plant and animal kingdoms, it is not known how COP1 recognizes substrates, or how it binds Trib1 to alter its target substrate selectivity. We report here the structure of the human COP1 WD40 domain without bound peptide, and of the human and A. thaliana COP1 WD40 domains bound to a COP1 consensus motif from Trib1, revealing a mode of substrate recognition that relies on interfacial residues that are 100% conserved. These structures, together with biochemical and cell-based assays of COP1-Trib1 complexes, identify the structural basis for motif recognition by COP1, clarify the basis for substrate selectivity among various WD40-containing E3 ligases, and hint at how Trib1 autoinhibition is overcome to target C/EBPα for degradation.
RESULTS

The COP1 WD40 Domain Is Necessary and Sufficient for Trib1 Binding

In order to uncover binding partners of Trib proteins using an unbiased approach, we used tandemly tagged full-length Trib1 and Trib2 proteins as bait in a proteome-wide search for Trib interactors in HeLa cells. COP1 peptides were consistently retrieved in all experiments (Figure S1 and Table S1), confirming several previous reports that Trib proteins are in complexes with COP1 (Du et al., 2003; Vitari et al., 2011). We then performed anti-Flag immunoprecipitations (IPs) on cells expressing HA-COP1 and Flag-Trib1 or HA-COP1 and a Flag-Trib1 protein lacking the last 21 residues which encompass the putative COP1-binding motif (Flag-Trib1-ΔC). Flag-Trib1 recovered COP1 but the Trib1-ΔC construct did not, supporting the inference that this motif is required for COP1 binding (Figures 1 and S1).

In order to determine the region of human COP1 responsible for Trib1 binding, we created a series of COP1 molecules with serial truncations from the N terminus, and tested whether they co-immunoprecipitated with Flag-tagged Trib1 (Figure 1). Full-length COP1, as well as truncated COP1 molecules starting at residue 177 (immediately after the RING domain) or 386 (immediately preceding the predicted start of the WD40 domain) co-immunoprecipitate with Flag-tagged full-length Trib1 (Figure 1B, lanes 6–8), but an attempt to trim the N-terminal end of COP1 further (beyond residue 386) failed to support co-precipitation with Trib1 (Figure 1B, lane 9). The COP1-Trib1 interaction was also dependent on the presence of the 21 C-terminal residues of Trib1 in the western blot assay, as predicted from the proteomic data (Figure 1B, lane 10). These data show that the WD40 domain (residues 386–731) of COP1 is both necessary and sufficient for Trib1 interaction, and also confirms the requirement for the presence of the C-terminal part of Trib1 containing the proposed COP1-interacting motif.

Structural Basis for COP1 Recognition of Trib1

To determine how COP1 recognizes Trib1 and other proteins, we determined X-ray structures of the WD40 domain of human COP1 alone to 2.0-Å resolution, a complex of human COP1 with a Trib1 peptide spanning the COP1-recognition motif to 3.9-Å resolution, and a complex of the Arabidopsis COP1 WD40 domain with a Trib1 peptide to 1.6-Å resolution (Table 1). The WD40 domain of COP1 is a seven-bladed β-propeller (Figure 2A), in which the N-terminal β-strand (residues 403–412) forms the last strand of the seventh blade (7D), completing the fold and stabilizing the propeller on its bottom face (Figure 2A). In the human COP1 structures, this face is stabilized by an N-terminal helix (residues 390–401), which contacts blade six of the propeller by a salt bridge from K398 to D653, located between strands 1A and 1B.

The bottom face also contains an atypical 15-residue loop connecting strands 1C and 1D (residues 449–463) enriched in acidic residues. The WD40 domain of Arabidopsis COP1 structure is very similar, with an all-atom root-mean-square deviation of 1.6 Å, and has the same connectivity (Figure S2). One notable difference between the two molecules is that the Arabidopsis structure lacks the N-terminal buttressing helix seen in the human structure, as that region was not included in the cDNA used to produce the plant protein (the region immediately N-terminal to the expressed WD40 domain is predicted to be disordered and was cleaved in limited proteolysis studies; data not shown). Second, a loop enriched in acidic residues connecting β-strand 6D to 7A (A. thaliana residues 630–646) is visible on the top face of the plant structure (Figure 2A, solid arrow), but is partly disordered in the human structure. This loop does not make contact with bound peptide (see below).

The top face of the propeller shows an extraordinarily high degree of conservation across many sequences ranging from plants to humans (Figure 2B). As residues within 4 Å of the bound peptide are identical between plants and humans (Figure S2), interactions between bound peptide and the WD40 domain in the complexes will be described in terms of both the human and plant residue numbers, with the Arabidopsis COP1 number first and the corresponding human COP1 residue in parentheses. A key feature of the motif recognition surface is the shallow binding pocket for the consensus V-P sequence of the bound peptide, residues V358–P359 of Trib1. This hydrophobic pocket is bounded by F595 (F645), T568 (T618), and C509 (C559), and the aromatic residues W467 (W517) and F595 (F645) form a ridge between the smaller V-P pocket and the larger central donut hole (Figures 2C, 2D, and S3). The non-consensus Trib1 residue I357 points out into solvent, while Q356 sits in the central cavity formed by COP1 residues S375 (S425), F595 (F645), and W467 (W517) (Figures 2C and 2D). This role for W467 is consistent with published data showing that mutation of this residue abrogates Hy5 binding in A. thaliana (Holm et al., 2001). Positively
charged residues on the top face of COP1 are also important for binding. A negatively charged residue toward the N terminus of the peptide (D/E) is part of the consensus for the COP1-binding motif. In our structure, this Trib1 residue is D355, which forms a salt bridge with K422 (K472). Surprisingly, Trib1 E360 does not form a salt bridge with the conserved K550 (K600) residue in the structure of either complex, despite the proposal that the analogous residue in HY5 (E45) is indispensable for COP1 interaction in plants (Holm et al., 2001).

We also compared the mode of peptide binding in the COP1 complexes to substrate recognition modes for other WD40 b-propeller domains that recognize substrates and target them for ubiquitination (Figures 3 and S3) (Hao et al., 2007; Orlicky et al., 2003; Tian et al., 2012; Wu et al., 2003). In each of these examples, the top face engages the target motif and the bottom face and/or sides of the b-propeller are available to bind other components of the respective E3 ligase complexes. However, the peptide-binding groove and its chemical characteristics vary among the different ligases, imparting distinct specificities among the ligases for their various target motifs (Figure 3B). It also appears that binding selectivity relies on residues unique to each propeller but often conserved in orthologs of the same protein, as illustrated for the top face of COP1 (Figure 2B). DDB2, which recognizes DNA molecules that are not direct degradation targets (loosely analogous to binding of the Trib adaptors by COP1), also uses the top face for binding (Fischer et al., 2011; Scrima et al., 2008).

Relative Binding Contributions from Trib1 Residues across the Binding Motif
We developed a fluorescence polarization assay to measure the binding affinity of the human COP1 b-propeller for a labeled Trib1 peptide spanning the COP1-binding region. The Trib1 peptide bound with high affinity to COP1 with a K_d of 250 ± 40 nM (Figure 4A). Remarkably, the isolated Trib1 peptide has a stronger half maximal inhibitory concentration (IC50) in the competition assay than a Trib1 molecule containing residues 83–372 (Figure 4B). This result is consistent with a recent structure of Trib1 in which the COP1-interacting motif engaged in intramolecular interactions with the adjacent pseudokinase domain (Murphy et al., 2015).

In order to determine which residues from Trib1 contribute most to the affinity for human COP1, we performed alanine and arginine scans across the binding motif using the fluorescence competition

### Table 1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Human COP1</th>
<th>Human COP1 with Trib1 Peptide</th>
<th>Arabidopsis COP1 with Trib1 Peptide</th>
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*aValues in parentheses are for highest-resolution shell.

*bA single crystal was used for each structure determination.
The data show that the central valine and proline residues of the motif do indeed provide a large energetic contribution to binding, as anticipated. The glutamine, which is inserted into the donut hole in our structure, is more permissive to alanine substitution than arginine. However, the acidic residue that follows the V-P consensus is surprisingly permissive to charge reversal when replaced by arginine (Figure 4D), suggesting that the pool of COP1-binding proteins is likely to be larger than expected based on the existing consensus.

**Trib1 Competes with Substrates for COP1 Binding**

The Trib1 COP1-binding motif shares consensus residues with known ubiquitination targets of COP1 such as the Jun and ETS family transcription factors, suggesting that the Trib1 and Trib2 substrate adaptors likely compete with Jun and ETS for binding to the top face of COP1 (Figure S4). To test this idea directly, we established a fluorescence competition assay with unlabeled Trib1, and assessed whether COP1 substrates do indeed compete with Trib1 for binding to human COP1 (Figure 5). Trib1 competes with all tested substrates for COP1 binding, and the COP1 affinity for Trib1 is several-fold tighter than for Jun and ETS family ubiquitination substrates. These results show that the Trib1-binding site is shared by other protein substrates for ubiquitination by COP1.

Using the competition assay, we also addressed the effect of phosphorylation on the binding of an ETS family member lacking the consensus COP1-binding motif but reported to be targeted for degradation by COP1 by changes in phosphorylation (Lu et al., 2014). Indeed, a doubly phosphorylated ETS1 peptide (ETS1P; Figure 5A) competes with Trib1 for COP1 binding almost two orders of magnitude more effectively than the unphosphorylated ETS1 peptide (Figure 5B), and more effectively than a consensus-bearing c-Jun peptide. A peptide from the *A. thaliana* HY5, which shares the consensus motif with the mammalian sequences (Figure S4), also binds to human COP1, which is consistent with the similarities seen in our two structures.

**DISCUSSION**

COP1 family E3 ubiquitin ligases play central roles in the biology of higher eukaryotes from both plant and animal kingdoms (Ang...
The function of COP1 has been extensively studied in Arabidopsis, where it is a central hub of light response regulated by as-sembly into larger E3 ligase complexes (Lau and Deng, 2012). A primary activity of COP1 in these diverse species is to regulate transcription factor abundance by degrading molecules such as HY5 in Arabidopsis, or ETS, c-Jun, or C/EBPβ in mammals. Substrate recognition requires recognition of a binding motif directly, as for HY5, ETS, and c-Jun, or indirectly through an adaptor such as a protein of the Trib family, as for C/EBPβ.

Arabidopsis COP1 is incorporated into several multiprotein complexes, including a Cul4-DDB1 E3 ligase where it appears to function in substrate recognition (Chen et al., 2010a). DDB1 serves to connect substrate recognition elements, called DDB1- and Cul4-associated factors (DCAFS) to Cul4. The crystal structure of the human Cul4-DDB1 in complex with the DCAF DDB2 shows the interaction to be mediated by two WD40 domains of DDB1 and a short helix-loop-helix (HLH) at the N terminus of DDB2 (Fischer et al., 2011). The structures of DDB1 in complex with HLH motifs from other known DCACS confirm this mode of interaction (Li et al., 2010). In human cells, COP1 has been found associated with DDB1 (Qi et al., 2006), raising the possibility that COP1 could function as a DCAF in humans. Structural alignment between DDB2 and the WD40 domain of human COP1 reveals that both propellers are ori-ented with the N-terminal helix on the opposite side from the substrate-binding site, which would be permissive for concur-rent binding to DDB1 and substrate. The idea that COP1 might function as a DCAF in Arabidopsis has also been proposed, along with the suggestion that a WDxR motif in Arabidopsis COP1 is involved in the interaction (Chen et al., 2010a). However, our structure shows that residues of this WDxR motif are buried and cannot be accessed without destroying the propeller. Further study will be required to determine if COP1 is a DCAF and, if so, what the function of the COP1 RING domain is in these complexes.

The work presented here reveals that COP1 binds its recognition motif on the Trib1 protein using the top face of its WD40 β propeller domain. Comparison of the recognition interfaces for several multicomponent E3 ligases with β propeller domains also shows that the architectural similarity among the various propellers is striking, with substrate specificity dictated by a small number of critical differences on the propeller surface. Among the various propellers, COP1 and βTrCP1 are the most alike. Both are seven-bladed propellers that bind the peptide on the top surface with a similar peptide alignment with respect to the propeller face. The charge profile of the two propellers is also similar (Figure 3B), with both molecules containing positively charged residues between blades 4 and 5, K550 (K600) in COP1 and R431 of βTrCP1. These residues are positioned to exert favorable electrostatic interactions with consensus motifs in their substrates (a conserved pSer, pSer37 in β-catenin for βTrCP, and the consensus D/E of the COP1-binding motif, E360 of human Trib1), even if direct contacts are not present. Trib1 and β-catenin share a second negatively charged residue (D355 in human Trib1 and pSer37 in β-catenin), which pair with analogous residues K422 (K472) and R285, respectively. Indeed, the affinity of the phosphory-lated ETS1 peptide for COP1 (Figure 5) suggests that pSer res-idues readily substitute for D/E residues in COP1 binding (Lu et al., 2014). However, the shape of the binding pockets of COP1 and βTrCP1 are distinct (Figure S3). In addition to its donut hole, COP1 has a separate shallow pocket accommodating the consensus residues valine and proline. Even the addi-tion of a single methyl group in a Val to Ile mutation is not toler-ated by the COP1 surface. This constraint is particularly instructive because superimposition of the COP1 and βTrCP1
structures places the lie of the β-catenin peptide closest to the Val of the COP1 consensus site, clashing with COP1 residues S553 (S603) and T568 (T618). Thus, selectivity is achieved by both the shape of the hydrophobic pocket and the charge of the exposed peptide residues. In comparison with COP1 and βTrCP1, Cdc20 has a very shallow binding pocket where the base is formed by an insert between blades 1 and 7 (Figure S3). The insert of the propeller itself (rather than the substrate) occupies the central cavity, leaving only a shallow dimple for substrate binding. The substrate KEN box forms a helix with few contacts to the propeller, and this minimal interface allows for specificity to be determined by the combined engagement of substrates with separate binding sites on the top and the side of the propeller. By contrast, COP1 has a single known site of substrate recognition (Tian et al., 2012). Lastly, the two eight-bladed E3 ligase propeller structures available, Fbw7 and cdc4, bind substrate in an orientation perpendicular to the alignment of the peptides with the seven-bladed propellers described above (Figure S3) (Hao et al., 2007; Orlicky et al., 2003).

The Trib1 peptide binds to human COP1 at the substrate recognition site with nanomolar affinity (Figure 4A), but there is no evidence that Trib1 is itself a target for COP1-mediated ubiquitination. Instead, current evidence suggests that tribbles serves to alter COP1 substrate specificity by directing the activation of COP1 toward C/EBPα (Keeshan et al., 2006). A structure of the human Trib1 pseudokinase domain containing the C-terminal COP1-binding peptide, recently published by Murphy et al. (2015), shows the C-terminal COP1-binding site tethered to the N lobe of the Trib1 pseudokinase domain, suggesting that the molecule normally adopts a conformation in which the COP1-binding motif is masked by autoinhibitory interactions with the kinase-homology domain. We thus propose the following model for the molecular mechanism of degradation of and other targets by COP1 in the presence of a substrate such as C/EBPα. The recruited C/EBPα substrate is then ubiquitinated and degraded by the proteasome-dependent pathway. This model is consistent with the requirement for the formation of a Trib1-COP1 complex for C/EBPα ubiquitination and with our observation that the isolated Trib1 peptide has higher affinity for COP1 than does the larger Trib1 protein (Figure 4B).

Regulation of COP1 substrate specificity by Trib family members also provides a potential explanation for how COP1 can act as a tumor suppressor or promoter depending on context. Although Trib autoinhibition may regulate the creation of Trib-COP1 complexes and subsequent targeting of C/EBPα, the high affinity of Trib1 for the COP1 WD40 domain also has the potential to displace Jun and ETS from COP1, raising the possibility that the formation of the Trib1-COP1 complex may concurrently regulate the abundance of other transcription factors. In plants, which lack Trib proteins, COP1 functions as a repressor of photomorphogenesis, but under UV-B light COP1 promotes...
photomorphogenesis by assembling with different protein partners (Huang et al., 2013). The extensive literature regarding COP1 in plants can guide future studies of whether and in what context Trib binding regulates the formation of cullin-based COP1 complexes in humans.

### EXPERIMENTAL PROCEDURES

#### Protemic Studies: Tandem-IP Experiments

HelA cell lines were transduced with retrovirus expressing the desired Trib proteins, and stable cell lines were generated by sorting for co-expressed interleukin-2 receptor (IL2R). For proteomic studies, the resulting cell lines were grown in DMEM with 10% bovine growth serum in spinner flasks to a density of 2.5 × 10^6 cells/ml and harvested. After Downe homogenization and separation of the nuclear and cytoplasmic fractions by centrifugation, the tandem affinity purification (TAP) samples were directly processed in solution. Cysteine residues were reduced with 10 mM DTT for 30 min at 56°C in the presence of 0.1% RapiGest SF (Waters). Cysteines were then alkylated with 22.5 mM iodoacetamide for 20 min at room temperature in the dark. Proteins were digested overnight at 37°C using 5 μg of trypsin after adjusting the pH to 8.0 with 1 M Tris. The digests were acidified by adding trifluoroacetic acid (TFA) to a final concentration of 1% and desalted by C18 solid-phase extraction followed by strong cation exchange, both performed in batch-mode format. Eluted peptides were concentrated in a vacuum concentrator and reconstituted with 20 μl of 0.1% TFA. Purified peptides were loaded via autosampler injection (NanoAcquity Sample Manager; Waters) onto a pre-column (4 cm POROS 10R2; Applied Biosystems) and eluted with a high-pressure liquid chromatography (HPLC) gradient (NanoAcquity Binary Sample Manager; Waters; 0%–35% B in 45 min; A = 0.1 M acetic acid in water, B = 0.1 M acetic acid in acetonitrile). Peptides were resolved on a self-packed analytical column (12 cm Monitor C18; Column Engineering) and introduced into the mass spectrometer (Orbitrap XL; Thermo Scientific) equipped with a Digital PicoView (New Objective) electrospray source platform (electrospray ionization voltage = 2.2 kV) (Ficarro et al., 2009). The mass spectrometer was operated in data-dependent mode where the top eight most-abundant ions in each mass spectrometry (MS) scan were subjected to collisionally activated dissociation (35% normalized collision energy, isolation width = 2.8 Da, threshold = 20,000). Dynamic exclusion was enabled with a repeat count of one and exclusion duration of 30 s. MS spectra were recalibrated using the background ion (Si(CH3)3O2 at m/z 445.12 ± 0.03 and converted into a Mascot generic file format (.mgf) using multiplier scripts (Askenazi et al., 2009; Parkh et al., 2009). Spectra were searched using Mascot (version 2.4) against three appended databases consisting of: (1) human protein sequences (downloaded from RefSeq on 07/11/2011); (2) common laboratory contaminants; and (3) a decoy database generated by reversing the sequences from these two databases. For Mascot searches, precursor tolerance was set to 20 ppm and product ion tolerance to 0.6 Da. Search parameters included trypsin specificity, up to two missed cleavages, fixed carboxamidomethylation (C, +57 Da) and variable oxidation (M, +16 Da). Peptides matching to peptides from the reverse database were used to calculate a global false discovery rate (FDR) and were discarded. Data were further processed to remove peptide spectral matches to the forward database with an FDR greater than 1.0%. For each protein identified we counted only peptides that matched to a single gene. We annotated the final results to indicate the frequency with which a protein was detected on a large compendium of negative TAP controls (Rozenblatt-Rosen et al., 2012).

#### 293T Flag IPs

293T cells were grown in standard medium in 100 × 15 mm dishes. Cells were transfected at 60% confluence with a pcDNA3.0 vector expressing the Flag-Trib1 proteins (Invitrogen) and a pTriEx1.1 vector for the COP1 proteins (Novagen). The cells were lysed after 3 days. An anti-Flag IP was performed as above and the resulting lysates and eluates were run on a 4%–20% gradient gel blotted with anti-Flag (Sigma #A8592) or anti-COP1 (Abcam 56400) antibodies (Figure 1).

#### Structural Studies

Crystalization (human constructs): his-tagged COP1 constructs (376–731) and (386–731) were purified from Hi5 cells by metal affinity chromatography using an imidazole gradient followed by ion-exchange chromatography on a Mono Q column (GE Life Sciences). The proteins were buffer-exchanged into crystallization buffer (20 mM Tris 8.0, 5% glycerol, 250 mM NaCl, and (386–731) were purified from Hi5 cells by metal affinity chromatography using an imidazole gradient followed by ion-exchange chromatography on a Mono Q column (GE Life Sciences). The proteins were buffer-exchanged into crystallization buffer (20 mM Tris 8.0, 5% glycerol, 250 mM NaCl, and (386–731) were purified from Hi5 cells by metal affinity chromatography using an imidazole gradient followed by ion-exchange chromatography on a Mono Q column (GE Life Sciences). The proteins were buffer-exchanged into crystallization buffer (20 mM Tris 8.0, 5% glycerol, 250 mM NaCl, and

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**Table A**

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<th>Name</th>
<th>Sequence</th>
<th>IC50 (μM)</th>
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<tr>
<td>Trib1</td>
<td>Ac-SKICTSDQVFPYQEDSDI-NH2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Trib2</td>
<td>Ac-GAKXLYQDSQYNMEENL-NH2</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>ETS1P</td>
<td>Ac-QSSFPNpSLQKPSYDSFPEI-NH2</td>
<td>4.6 ± 1.2</td>
</tr>
<tr>
<td>ETS1</td>
<td>Ac-QSSFNLSQVRPSYDSDSf-NH2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Hy5</td>
<td>Ac-ISSLSEIRVPFGGEAVG-NH2</td>
<td>5.1 ± 1.4</td>
</tr>
<tr>
<td>C-Jun</td>
<td>Ac-QALRSEPTQVPMGETFP-NH2</td>
<td>10 ± 1.4</td>
</tr>
<tr>
<td>JUNB</td>
<td>Ac-SPTRETSPQVTPAEARDAT-NH2</td>
<td>43 ± 12</td>
</tr>
</tbody>
</table>

**Figure 5. Trib1 Peptide Binds Human COP1 and Competes with COP1 Substrates**

(A) Peptide sequences used in a competition experiment to compare binding affinities of reported COP1 substrates with binding of the Trib1 peptide. Red text indicates the COP1 consensus binding residues. IC50 values were calculated from the data in (B). Phosphorylated serine residues are shown in blue in the ETS1P peptide. (B) Competition experiment in which Trib2, ETS, and Jun family peptides displace FITC-Trib1 at a fixed human COP1 concentration.

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Adelmann et al., 2012) with minor modifications. For protein digestion, proteins from the tandem affinity purification (TAP) samples were directly processed in solution. Cysteine residues were reduced with 10 mM DTT for 30 min at 56°C in the presence of 0.1% RapiGest SF (Waters). Cysteines were then alkylated with 22.5 mM iodoacetamide for 20 min at room temperature in the dark. Proteins were digested overnight at 37°C using 5 μg of trypsin after adjusting the pH to 8.0 with 1 M Tris. The digests were acidified by adding trifluoroacetic acid (TFA) to a final concentration of 1% and desalted by C18 solid-phase extraction followed by strong cation exchange, both performed in batch-mode format. Eluted peptides were concentrated in a vacuum concentrator and reconstituted with 20 μl of 0.1% TFA. Purified peptides were loaded via autosampler injection (NanoAcquity Sample Manager; Waters) onto a pre-column (4 cm POROS 10R2; Applied Biosystems) and eluted with a high-pressure liquid chromatography (HPLC) gradient (NanoAcquity Binary Sample Manager; Waters; 0%–35% B in 45 min; A = 0.1 M acetic acid in water, B = 0.1 M acetic acid in acetonitrile). Peptides were resolved on a self-packed analytical column (12 cm Monitor C18; Column Engineering) and introduced into the mass spectrometer (Orbitrap XL; Thermo Scientific) equipped with a Digital PicoView (New Objective) electrospray source platform (electrospray ionization voltage = 2.2 kV) (Ficarro et al., 2009). The mass spectrometer was operated in data-dependent mode where the top eight most-abundant ions in each mass spectrometry (MS) scan were subjected to collisionally activated dissociation (35% normalized collision energy, isolation width = 2.8 Da, threshold = 20,000). Dynamic exclusion was enabled with a repeat count of one and exclusion duration of 30 s. MS spectra were recalibrated using the background ion (Si(CH3)3O2 at m/z 445.12 ± 0.03 and converted into a Mascot generic file format (.mgf) using multiplier scripts (Askenazi et al., 2009; Parkh et al., 2009). Spectra were searched using Mascot (version 2.4) against three appended databases consisting of: (1) human protein sequences (downloaded from RefSeq on 07/11/2011); (2) common laboratory contaminants; and (3) a decoy database generated by reversing the sequences from these two databases. For Mascot searches, precursor tolerance was set to 20 ppm and product ion tolerance to 0.6 Da. Search parameters included trypsin specificity, up to two missed cleavages, fixed carboxamidomethylation (C, +57 Da) and variable oxidation (M, +16 Da). Spectra matching to peptides from the reverse database were used to calculate a global false discovery rate (FDR) and were discarded. Data were further processed to remove peptide spectral matches to the forward database with an FDR greater than 1.0%. For each protein identified we counted only peptides that matched to a single gene. We annotated the final results to indicate the frequency with which a protein was detected on a large compendium of negative TAP controls (Rozenblatt-Rosen et al., 2012).
For Arabidopsis COP1-peptide co-crystals, a fragment encoding residues (349–675) was subcloned with a hexahistidine tag, expressed in insect Hi5 cells, and purified to homogeneity on SDS-PAGE as above. The complex with peptide (Ac-SDQIVPEY-NH₂ obtained from LifeTein Technologies) was crystallized in sitting drops using a 96-well plate format using a 1.2:1 ratio of peptide to protein. The crystallization well solution was 0.1 M Tris 8.5, 16% PEG 3350, 2% Tacsimate 8.0. Crystals were cryoprotected by quickly dipping in a mixture of 50% well solution, 10% sucrose, 2% glucose, 9% glycerol, and 9% ethylene glycol, then plunged into liquid nitrogen.

Data Collection and Refinement

For human COP1 apoprotein, X-ray diffraction data were collected at NE-CAT (Beam ID 24E) at cryogenic temperatures (100 K). The data were indexed with the XDS package and scaled with Aimless as part of the CCP4 package (Collaborative Computational Project, 1994; Kabsch, 2010). An initial molecular replacement solution was found using the unliganded human COP1 structure as a search model using Phaser (McCoy et al., 2007). In order to further improve the value of Rfree and refine the side-chain orientation of the ligand, a second model was constructed by homology modeling using the structural information from the Arabidopsis COP1/peptide crystal. Because all residues at the binding interface are identical, the residues in the binding pocket of the working model of the human complex were manually adjusted and aligned to the Arabidopsis COP1/peptide structure. The resulting model was further refined using Phenix with secondary structure restraints and reference model restraints as well as TLS refinement. The final structure was analyzed and validated by MolProbity (Chen et al., 2010b). Statistics, structure validation, and stereochemical quality are reported in Table 1. Graphics figures were rendered in PyMOL (Schroedinger software). Conservation analysis was performed using the program Consurf (Ashkenazy et al., 2010).

Fluorescence Polarization Assay

Fluorescence polarization assays were performed with the purified COP1 propeller (residues 376–731) in buffer containing 0.01 M HEPES (pH 7.4), 0.15 M NaCl, and 0.2% (v/v) Surfactant P20, supplemented with 2 mM TCEP. Fluorescein isothiocyanates (FITC)-labeled and unlabeled, competing peptides were obtained from LifeTein at a purity of >95%, as assessed by reverse-phase HPLC, and used without further purification. Experiments were conducted at 30 μl well volume in 384-well plates and read at 538 nM on a Spectramax M5 plate reader (Molecular Devices). Plots present data from at least three independent experiments. Error bars represent SEM (Figures 4 and 9).

ACCESSION NUMBERS

Coordinates of the isolated COP1 β propeller and of the Arabidopsis and human COP1-Trib1 complexes have been deposited in the PDB (PDB: 5HQG, 5IGO, and 5IGQ).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2016.03.002.
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