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An allosteric inhibitor of substrate recognition by the SCF^{Cdc4} ubiquitin ligase

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Abstract

The SCF ubiquitin ligases target proteins for degradation by recruitment factors called F-box proteins^{1,2}. We identified a bi-planar dicarboxylic acid compound, called SCF-I2, as an inhibitor of substrate phosphodegron recognition by the yeast F-box protein Cdc4. SCF-I2 inhibits the binding and ubiquitination of full length phosphorylated substrates by SCF^{Cdc4}. A crystal co-structure reveals that SCF-I2 inserts between the β -strands of blades 5 and 6 of the WD40 propeller domain of Cdc4 at a site that is 25 Å remote from the substrate binding site. Long-range transmission of SCF-I2 interactions distorts the substrate binding pocket and impedes recognition of key determinants in the Cdc4 phosphodegron. Mutation of the SCF-I2 binding site abrogates its inhibitory effect and explains specificity in the allosteric inhibition mechanism. Mammalian WD40 domain proteins may exhibit similar allosteric responsiveness and hence represent an extensive new class of druggable target.

The ubiquitin-proteasome system (UPS) mediates the intracellular degradation of many proteins through a cascade of enzyme activities, termed E1, E2 and E3, which serially activate and then transfer ubiquitin to substrate proteins³. E3 enzymes, also referred to as ubiquitin ligases, specifically recognize discrete sequence motifs in substrates termed degrons. The human genome encodes at least 600 E3 enzymes, each of which has the potential to recognize multiple substrates⁴. The largest class of E3 enzymes, the cullin-RING ligases (CRLs), were discovered through identification of the multi-subunit Skp1–Cdc53/Cullin–F-box protein (SCF) complexes^{1,2}. A large family of F-box proteins recruit substrates to the core SCF complex via protein interaction domains, typically leucine rich repeats (LRRs) or WD40 repeats, often in a phosphorylation dependent manner^{1,2,5-7}. The SCF enzymes likely target hundreds of different substrates^{4,8-10} and thus hold untapped potential for drug discovery⁴.

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The WD40 repeat is an ancient conserved motif that functions in many different cellular processes^{11, 12}. Tandem arrays of five to eight WD40 repeats form a circularly permuted β -propeller domain structure¹³. In yeast, recognition of the cyclin-dependent kinase (CDK) inhibitor Sic1 by the WD40 domain of the F-box protein Cdc4 depends on phosphorylation of multiple Cdc4 phospho-degron (CPD) motifs in Sic1^{6, 14}. SCF^{Cdc4} also targets other substrates including Far1, Cdc6 and Gcn4¹. Human Cdc4, also known as Fbw7, recruits a number of important regulatory factors for ubiquitination including cyclin E, Myc, Jun, Notch, SREBP and presenilin⁹. Cdc4 is a haploinsufficient tumor suppressor that is mutated in many cancer types^{9, 15}, and also likely influences stem cell renewal by virtue of its effects on Myc and other factors¹⁶. Given the central role of Cdc4/Fbw7 in growth and division, we sought to identify small molecules that inhibit substrate recognition by Cdc4.

We adapted a previously established fluorescence polarization (FP) assay to monitor the displacement of a fluorescein-labeled CPD peptide ($K_d \approx 0.2 \mu\text{M}$) from yeast Cdc4 (Supplementary Fig. 1a)¹⁴. The FP assay achieved a Z-factor of 0.8, based on negative (DMSO solvent only) and positive (unlabelled CPD peptide) controls. A screen against a 50,000 compound library enriched for drug-like molecules¹⁷ yielded 44 hits that inhibited the CPD-Cdc4 interaction by at least 50% (Fig. 1a). Two of these compounds, denoted SCF-I2 and SCF-I6, strongly inhibited the interaction of full length phospho-Sic1 with Cdc4 and prevented Sic1 ubiquitination by SCF^{Cdc4} (Fig. 1b). We pursued only SCF-I2 because SCF-I6 appeared to cause non-specific loss of Skp1-Cdc4 complex from the capture resin (Fig. 1b). SCF-I2 corresponds to 1-(2-carboxynaphth-1-yl)-2-naphthoic acid, which is a derivative of 1,1'-binaphthyl-2,2'-diol, also known as BINOL, a bi-planar axially chiral atropisomer that is widely used as a scaffold in chiral synthesis¹⁸. The two hydroxyl groups of BINOL are substituted by carboxylic acid groups in SCF-I2 (Fig. 1c). The form of 1-(2-carboxynaphth-1-yl)-2-naphthoic acid used in all of our assays was an undefined racemic mixture of the R- and S- enantiomers, which are non-interconvertible at even high temperature¹⁸. SCF-I2 was 10-fold less potent than unlabeled CPD peptide in the FP assay, with an $\text{IC}_{50} = 6.2 \mu\text{M}$ versus $0.5 \mu\text{M}$, respectively (Fig. 1c). SCF-I2 inhibited binding and/or ubiquitination of both full length Sic1 and Far1 with an IC_{50} of $\sim 60 \mu\text{M}$ (Supplementary Fig. 1b,c); the weaker apparent affinity of SCF-I2 in these assays may reflect differences in the interaction of peptides and full length substrates with Cdc4. SCF-I2 did not affect the activity of the closely related E3 enzyme SCF^{Met30}, which recruits its substrate Met4 via the WD40 domain of the F-box protein Met30 (Supplementary Fig. 1d)¹⁹.

We determined the crystal structure of SCF-I2 bound to a Skp1-Cdc4 complex²⁰ to $2.6 \text{ \AA}$ resolution (see Supplementary Table 1 for data collection and refinement statistics). Unbiased difference electron density maps revealed that SCF-I2 binds to the WD40 repeat domain of Cdc4 at a site that is $25 \text{ \AA}$ distant from the CPD binding pocket (Fig. 2a). The eight WD40 repeat motifs of Cdc4 form a canonical propeller structure in which each propeller blade consists of four anti-parallel β -strands and intervening loop regions (Supplementary Fig. 2)²⁰. SCF-I2 embeds in a deep pocket on the lateral surface of the β -propeller between blades 5 and 6 (Fig. 2a,b; Supplementary Fig. 2). Cdc4 engages only one of two enantiomers of SCF-I2, the (R)-(+)-equivalent of BINOL. The top naphthalene ring system of SCF-I2 inserts deeply between blades 5 and 6 forming extensive hydrophobic contacts with Leu628, Ile594, Leu634, Trp657, and Ala649 (Fig. 2b). In addition, the

carboxyl group of the top ring system hydrogen bonds to the NH group of the Trp657 side chain and forms a salt bridge with the side chain of Arg664. The bottom naphthalene ring system is more solvent exposed and forms a stabilizing co-planar stacking interaction with the side chain of Arg664 and van der Waals interactions with the side chains of Ser667 and Arg655. The carboxyl group of the bottom ring system also forms ionic interactions with the side chains of Arg655 and His631.

In the apo-Skp1-Cdc4 structure, there is no obvious pre-existing pocket that might anticipate the binding mode of SCF-I2 (Fig. 2c). Rather, the SCF-I2 binding pocket is induced by separation of blades 5 and 6 and a drastic shift of the β 21- β 22 linker that connects the two blades (Fig. 2d). The reorientation of the β 21- β 22 linker entails a 5 Å shift of the main chain and a massive 13 Å shift of the side chain of His631 from a buried to solvent-exposed position (Fig. 2d,e). These large conformational alterations create an inter-blade void that is filled by the rearrangement of residues proximal to the CPD binding pocket (Fig. 2d,e). The void is filled in part by a swap of side chain positions between Val635, which is normally buried and adjacent to His631, and the normally solvent-exposed Leu634 side chain; as a consequence of this rearrangement, the side chains of Val635 and Leu634 traverse 6 and 8 Å, respectively. The position vacated by Leu634 in turn is filled by rotation of the side chain of Tyr574. Critically, both Tyr574 and Leu634 comprise part of the highly conserved CPD binding infrastructure. In the CPD peptide-Skp1-Cdc4 complex²⁰, Tyr574 and Leu634 line the hydrophobic P-2 binding pocket within the central pore (Fig. 2e) and thereby dictate the preference for hydrophobic residues at the P-2 position of the CPD consensus motif^{14, 20}. The P-2 pocket is thus severely distorted by the reoriented side chains of Tyr574 and Leu634 in the SCF-I2 bound structure. In addition, the hydroxyl group of Tyr574 participates in stabilizing H-bond interactions with the side chain of Arg572, one of the four invariant essential Arg residues found in all Cdc4 orthologs²⁰. Arg572 stabilizes the orientation of Tyr548, which in turn directly hydrogen bonds to the CPD phosphate group in the P0 position. Thus, SCF-I2 critically compromises the main binding pockets for the P-2 and P0 positions of the CPD consensus sequence^{14, 20}. As predicted by this structural model, the effects of SCF-I2 are mimicked by a Tyr574Ala mutation, which results in a 20-fold reduction in affinity of Cdc4 for the CPD peptide (Fig. 2f).

We explored the determinants of the SCF-I2-Cdc4 interface. The two carboxylic acid groups of SCF-I2 exhibit marked charge complementarity with the guanidinium side chains of Arg655 and Arg664. Mutation of each Arg residue individually to Ala attenuated the inhibition of Cdc4 by SCF-I2 by at least 50-fold (Fig. 2g). Alleles bearing either mutation fully complemented Cdc4 function in vivo, indicating that this region of Cdc4 does not normally play a critical role in substrate recognition or SCF catalytic activity (Supplementary Fig. 3a). To investigate the structural features of SCF-I2 required for Cdc4 inhibition, we tested a panel of available BINOL analogs for activity in the FP assay (Supplementary Fig. 3b,c). This series demonstrated the importance of the naphthalene ring systems that participate in numerous hydrophobic interactions and the carboxylate groups that form electrostatic interactions with the two Arg residues on Cdc4. These mutational and structure-activity results validate the binding mode for SCF-I2 observed in the crystal structure.

We next assessed the activity SCF-I2 towards human Fbw7. The key Cdc4 residues Arg655 and Arg664 are replaced in Fbw7 by Lys and Cys respectively, suggesting that Fbw7 might be resistant to inhibition by SCF-I2. This proved to be the case as SCF-I2 inhibited the CPD-Fbw7 interaction only at high concentrations (Fig. 3a). The residual inhibitory activity of SCF-I2 towards Fbw7 might be due to the conservative Arg-to-Lys substitution and the conservation of most other residues that form the induced SCF-I2 binding pocket (Fig 3b; Supplementary Fig. 2). Alignment of all human WD40 domains revealed that, aside from the two surface Arg residues, the pattern of SCF-I2 contact residues is often conserved (Supplementary Fig. 4). We are currently exploring whether the BINOL scaffold can be modified to more potently interact with Fbw7 and other human WD40 domain proteins.

The β subunits of heterotrimeric G proteins, which transduce signals from a host of G protein coupled receptors (GPCRs), are the most thoroughly studied WD40 domain proteins²¹. Notably, the interaction of the regulatory protein phosducin with the $G_T\beta$ subunit of the heterotrimeric G-protein transducin also causes significant structural rearrangements between adjacent WD propeller blades²². These rearrangements induce a binding pocket for the C-terminal farnesyl moiety of the partner $G_T\gamma$ subunit, which may serve to regulate membrane association of the $G_T\beta\gamma$ complex²². Comparison of our SCF-I2-Cdc4 structure and the phosducin- $G\beta\gamma$ structure reveals three highly similar features. First, the ligand-bound forms of both structures exhibit an analogous buried-to-exposed transition of the conserved His residue at the apex of the connector between the affected blades (Fig. 3c). Second, the Cdc4 and $G\beta$ structures show a remarkably close juxtaposition of induced binding pockets for the SCF-I2 and farnesyl ligands, respectively (Fig. 3c). Third, these rearrangements occur between blades 5 and 6 for both WD40 structures. That two functionally unrelated and evolutionarily distant proteins undergo similar induced conformational changes hints that allosteric responsiveness may be an intrinsic and conserved feature of the WD40 domain.

In contrast to conventional protein interaction inhibitors that directly block the substrate binding site, such as the p53-Mdm2 inhibitor nutlin²³, SCF-I2 elicits its effect by an allosteric mechanism. A structural feature of WD40 domains, and other β -propeller structures such as the Kelch domain, is the variability in blade number, which in known WD40 structures ranges from five to eight blades per domain¹³. The circular β -propeller structure can exhibit inter-blade separation²⁴ and structural tolerance to artificial insertion of an additional repeat²⁵. WD40 domains may thus be inherently susceptible to disruption by insertion of appropriately configured small molecules between adjacent blades. Although it remains to be determined whether all WD40 domains exhibit allosteric responsiveness, in other protein families ultra-conserved residues can transmit long range allosteric effects²⁶.

To our knowledge, SCF-I2 represents the first example of a WD40 domain inhibitor. As our data with Cdc4 and Fbw7 shows, allosteric inhibition can discriminate between even highly related domains that recognize identical substrate motifs; it may be thus feasible to design other inhibitors that are selective for particular WD40 domain proteins. Moreover, allosteric inhibitors may be combined with conventional binding pocket inhibitors to increase potency²⁷. The yeast genome encodes at least 113 proteins with WD40 or WD40-like domains that function in signaling, transcription, chromatin remodeling, mRNA splicing,

DNA replication and repair, protein synthesis, the ubiquitin system, autophagy, vesicle trafficking, the cytoskeleton and organelle biogenesis (Supplementary Table 2). In humans, WD40 domains occur in at least 256 different proteins and perform similarly diverse functions (Supplementary Table 3). Biomedically important WD40 domain proteins include the F-box proteins Fbw7 and β -TrCP⁸, target of rapamycin (TOR) kinase complex subunits²⁸ and G β -subunits of heterotrimeric G proteins^{21, 27}. Our findings suggest that the WD40 domain may be generally accessible to allosteric modulation by small molecules.

Methods

Chemicals and reagents

An N-terminally labeled fluorescein phosphopeptide derived from cyclin E (GLLP^{TPPQSG}, called CPD) was synthesized by the W.M. Keck Biotechnology Resource Center (New Haven, CT). Non-fluorescently labeled peptide Ac-GLLP^{TPPQSG} was synthesized by Dalton Chemical (Toronto, Canada). Small molecules were purchased from Maybridge plc (Cornwall, England). Skp1-Cdc4²⁶³⁻⁷⁴⁴, Skp1-Cdc4²⁶³⁻⁷⁴⁴ R655A, Skp1-Cdc4²⁶³⁻⁷⁴⁴ R664A and Skp1-Fbw7 were purified as previously described²⁰. Purified complexes were passed over a Superdex S75 gel filtration column (GE Healthcare) equilibrated in 10 mM HEPES (pH 7.5), 250 mM NaCl, and 1 mM DTT and concentrated to 20 mg/mL.

Fluorescence Polarization Assays

A 50,000 compound Maybridge Screening Collection library (www.maybridge.com) was screened in a 384 well format on a Beckman-Coulter Integrated Robotic System at the McMaster University HTS Laboratory (Hamilton, Canada). Assays contained 0.21 μ M Skp1-Cdc4 complex and 10 nM fluorescently labeled cyclin E-derived phosphopeptide in 10 mM HEPES (pH 7.5), 40 mM NaCl, 0.1 mg/mL BSA and 1 mM DTT in a final volume of 23.5 μ L per well. 1.5 μ L of each library compound from a 1 mM stock in DMSO was added to a final concentration of 60 μ M, mixed and incubated at room temperature for 30 min. Samples were excited at 485 nm and emission was read at 535 nm on an Analyst HT plate reader (Molecular Devices Corp., Sunnyvale CA). High controls contained 1.5 μ L of DMSO only and low controls contained 1.5 μ L of unlabelled cyclin E peptide in DMSO at a final concentration of 10 μ M. Binding activity was calculated as the average sample value minus the mean of low controls divided by the mean of high controls minus the mean of low controls. Compounds were classified as initial hits if the binding value was below 50% of control. Dose response curves were carried out using the same conditions as above and EC₅₀ values calculated as previously described¹⁷. Determination of IC₅₀ was performed using a non-linear regression analysis using a sigmoidal dose response equation (variable slope) with no weighting or restraints (Graphpad Software, San Diego CA). mP units were normalized to no compound controls, i.e., 100% binding, for graphical representation.

Interaction and ubiquitination assays

For full length phospho-Sic1 substrate interaction assays, compounds (50 μ M final) were added to 500 ng of GstSkp1-HisCdc4²⁶³⁻⁷⁴⁴ immobilized to glutathione sepharose resin in PBS, incubated for 30 min at 4°C, followed by incubation with 0.1 μ M of phosphorylated Sic1 for 1 h at 4°C. Resin was washed 3 times with PBS and captured Sic1 protein

visualized by anti-Sic1 immunoblot³⁰. For ubiquitination assays, compounds were pre-incubated with recombinant E1 (0.4 μ M), Cdc34 (2 μ M), SCF^{Cdc4} (0.2 μ M) for 15 min at room temperature in 12.5 μ L reaction buffer containing 50 mM Tris (pH 7.5) 10 mM MgCl₂, 2 mM ATP, 50 μ M DTT and 24 μ M ubiquitin. Reactions were initiated by addition of recombinant HisSic1 (0.2 μ M) that had been previously phosphorylated with Cln2-Cdc28 kinase; reactions were incubated at 30°C for indicated times and products detected by anti-Sic1 immunoblot³⁰. Ubiquitination of Met4 by SCF^{Met30} was carried out as described¹⁹.

X-ray structure determination

Crystals of Skp1-Cdc4²⁶³⁻⁷⁴⁴ were derived as described previously²⁰. Crystals were transferred to buffer containing 100 mM Tris (pH 8.5), 1.5 M ammonium sulfate, 15% glycerol and 1mM 1-(2-carboxynaphth-1-yl)-2-naphthoic acid (SCF-I2) and incubated at room temperature for 30 min in order to incorporate SCF-I2 into the crystal lattice and to cryoprotect the crystal. Soaked crystals retained the same space group of P3₂ (a=108.281, b=108.281, c=165.594, $\alpha=\beta=90^\circ$, $\gamma=120^\circ$). The structure was refined to 2.6 Å to a working R_{value} of 21.1% and R_{free} of 26.3% (Supplementary Table 1). Details of data processing and refinement are provided in the Supplementary Information.

Supplementary Material

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Figure 1.

Small molecule inhibitors of the Cdc4-substrate interaction. **a**, Distribution of hits from 50,000 compound Maybridge library screen. Interaction between a fluorescein-labeled high affinity cyclin E-derived phosphopeptide (GLLpTPPQSG) and recombinant Cdc4 was monitored by fluorescence polarization. 44 compounds fell below the 50% inhibition cutoff (red line). Yellow dashed lines indicate three standard deviations above and below the mean. Z and Z' factor scores were 0.8 and 0.66 respectively. At one standard deviation (σ), high controls were 4.6%, low controls 6.8%, and sample data 7.0%. **b**, Inhibition of interaction between full length phospho-Sic1 and Cdc4 (top panel). Phosphorylated Sic1 (0.1 μ M) was incubated in the presence of recombinant Skp1-Cdc4 resin (500 ng) and the indicated compounds (50 μ M). Bound protein was visualized by anti-Sic1 immunoblot. Total protein on resin after capture and wash was determined by Ponceau S stain (middle panel). DMSO solvent alone and 10 μ M Gcn4 phosphopeptide (FLPpTPVLED) served as negative and positive controls. Inhibition of Sic1 ubiquitination in vitro (bottom panel). Phosphorylated Sic1 (0.2 μ M) was incubated with recombinant SCF^{Cdc4} (0.2 μ M), E1 (0.4 μ M), E2 (2 μ M), ubiquitin (24 μ M), and ATP (1 mM) in the presence of 80 μ M indicated compound or control. Reaction products were visualized by anti-Sic1 immunoblot. **c**, Inhibition curves for SCF-I2 (red) and unlabeled CPD peptide (black) in the FP assay. (R) and (S) enantiomers of 1-(2-carboxynaphth-1yl)-2-naphthoic acid (SCF-I2) are shown.

Figure 2.

Structure analysis of the SCF-I2–Skp1–Cdc4 complex. **a**, SCF-I2 intercalates between β -propeller blades 5 and 6 of the Cdc4 WD40 repeat domain, approximately 25 Å from the CPD phosphopeptide binding site. SCF-I2 is shown in yellow. Red dot indicates modeled position of the P0 phosphate position. PB indicates propeller blade. **b**, Stereo diagram of SCF-I2 bound between PB5 and PB6 of the WD40 domain of Cdc4. SCF-I2 is shown in yellow; critical contact residues in Cdc4 are shown in blue stick representations. **c**, Surface representation of SCF-I2 binding region on Cdc4 in the absence (top) and presence (bottom) of bound SCF-I2. **d**, Stereo diagram of main chain conformational shifts induced by SCF-I2. The structure of Cdc4 in the absence of SCF-I2 but in the presence of a CPD phosphopeptide substrate (yellow) is shown in purple; the structure of Cdc4 in the presence of SCF-I2 (yellow) is shown in blue. **e**, Schematic of allosteric alterations caused by binding of SCF-I2. Positions of SCF-I2 bound conformations are shown in red; X indicates abrogation of an H-bond caused by rotation of Tyr574. **f**, Binding curves for WT Skp1–Cdc4 (black) and Skp1–Cdc4^{Y574A} (red) interactions with cyclin E-derived phosphopeptide by fluorescence polarization. **g**, SCF-I2 inhibition curves for WT Skp1–Cdc4 (black), Skp1–Cdc4^{R655A} (green) and Skp1–Cdc4^{R664A} (blue) binding to cyclin E phosphopeptide by FP.

Inset shows binding inhibition by unlabeled cyclin E phosphopeptide for the same three proteins.

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Figure 3.

Inhibition and allosteric modulation of human WD40 domains. **a**, Fluorescence polarization competition binding curves for *S. cerevisiae* Cdc4 (black) and human Cdc4/Fbw7 (red) with SCF-I2. Inset shows inhibition by unlabeled cyclin E phosphopeptide for yeast Cdc4 (black) and human Fbw7 (red). **b**, Stereo view overlay of the inhibitor binding site region of *S. cerevisiae* Cdc4 (PDB 1NEX) in the absence of SCF-I2 (blue) with the corresponding region of human Cdc4/Fbw7 (green) (PDB 2OVR)^{20, 29}. Only residues which differ between the human and *S. cerevisiae* proteins are labeled. **c**, Stereo view comparison of induced pockets in the WD40 repeat domain of Cdc4 and the bovine transducin Gβ subunit. Top displays a superposition of Cdc4 (blue) bound to SCF-I2 (yellow) with the G_Tβ subunit (dark green) bound to bovine retinal phosducin (pink) and a farnesyl ligand (magenta) from an associated G_Tγ subunit (PDB 1A0R). Bottom displays a superposition of unliganded forms of Cdc4 (grey) and the G_Tβ subunit (light green) (PDB 1TBG). For illustrative purposes, SCF-I2 and farnesyl ligands from the top image have been modeled into the lower image.