Suramin inhibits cullin-RING E3 ubiquitin ligases

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Contributed by Jerard Hurwitz, January 26, 2016 (sent for review December 4, 2015; reviewed by Serge Y. Fuchs and Pengbo Zhou)

Cullin-RING E3 ubiquitin ligases (CRL) control a myriad of biological processes by directing numerous protein substrates for proteasomal degradation. Key to CRL activity is the recruitment of the E2 ubiquitin-conjugating enzyme Cdc34 through electrostatic interactions between E3’s cullin conserved basic canyon and the acidic C terminus of the E2 enzyme. This report demonstrates that a small-molecule compound, suramin, can inhibit CRL activity by disrupting its ability to recruit Cdc34. Suramin, an antitrypansomal drug that also possesses antitumor activity, was identified here through a fluorescence-based high-throughput screen as an inhibitor of ubiquitination. Suramin was shown to target cullin 1’s conserved basic canyon and to block its binding to Cdc34. Suramin inhibits the activity of a variety of CRL complexes containing cullin 2, 3, and 4A. When introduced into cells, suramin induced accumulation of CRL substrates. These observations help develop a strategy of regulating ubiquitination by targeting an E2–E3 interface through small-molecule modulators.

In eukaryotes selective protein degradation requires a cellular program known as the “ubiquitin (Ub)-proteasome system.” The Ub-proteasome system achieves target selection by tagging protein substrates with lysine-48 (K48)-linked polyubiquitin chains that drive the modified substrates for destruction by the 26S proteasome (1). K48-polyubiquitination is driven by the concerted action of three enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) such as Cdc34, and a Ub ligase (E3) family that comprises ~300 members, about half of all E3s identified in humans (2).

Typically, ubiquitination with the SCF E3 begins with the activation of a substrate degradation signal (degron) by post-translational modification such as phosphorylation that often is involved in triggering a diversified array of cellular signaling pathways (4, 5). The resulting active degron drives affinity interactions with the F-box substrate receptor protein within the E3 complex. A well-studied example is the complex formed between the β-TrCP F-box protein and the DpSgoXp5 degron motif present on IxBo and β-catenin (6). Through a mechanism yet to be defined, the E3-substrate association triggers covalent modification of Nedd8 to the E3’s cullin 1 (CUL1) scaffold subunit at its conserved lysine residue in the winged-helix B domain (WHB) (also known as “ECTD”), converting the E3 from a restrained state into an active conformation (7–9). Last, SCF, bound substrate, and Ub-charged E2-conjugating enzyme (s) are engaged in multifaceted interactions that produce K48-linked polyubiquitin chains. In this context, emerging studies have revealed the actions of two E2 enzymes: the E2 UbcH5 acts as an initiator E2 and attaches a single Ub molecule to a substrate, after which the E2 Cdc34 builds the K48-linked polyubiquitin chain (10, 11).

In an effort to target CRL-mediated K48 polyubiquitination, we developed a FRET-based in vitro reporter assay system. On the basis of this assay format, a high-throughput screen (HTS) was performed, resulting in the identification of a potent inhibitor of CRL activity, suramin. Interestingly, suramin is a century-old antitrypansomal drug that also possesses antitumor activity (12).

Results

Tracking CRL-Mediated K48 Ubiquitination by FRET. To target CRL-mediated K48 ubiquitination, we developed a FRET K48 di-Ub assay that measures the linkage-specific Ub–Ub transfer reaction by fluorescence. The assay has five components (Fig. 1A): (i) E1; (ii) E2 Cdc34 specific for K48 linkage; (iii) the E3 subcomplex ROC1–CUL1 (411–776) [called “ROC1–CUL1 C-terminal domain (CTD)”] required for E2 activation; (iv) donor Ub carrying a K48R substitution and fluorescent dye iFluor 555 at position E64C (Fig. S1A); and (v) receptor Ub bearing the G75G76 deletion and fluorescent dye iFluor 647 at position E64C (Fig. S1A). These five proteins are of >95% purity (Fig. S1B).

Interactions between E2 and E3 enzymes are key for ubiquitination, but whether such a dynamic association is susceptible to perturbation by small-molecule modulators remains elusive. By demonstrating that suramin can inhibit cullin-RING E3 ubiquitin ligase by disrupting its ability to recruit E2 Cdc34, this work suggests that the E2–E3 interface may be druggable. In addition, suramin is an antitrypansomal drug that also possesses antitumor activity. Our findings have linked the ubiquitin-proteasome pathway to suramin and suggest additional biochemical mode of action for this century-old drug.

Significance

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The FRET K48 di-ubiquitin (di-Ub) assay formed di-Ub of expected size only in the complete reaction (Fig. 1 B, lane 1). The reaction was abolished by the removal of any of the five components (Fig. 1 B, lanes 2–7), demonstrating that the formation of di-Ub is absolutely dependent on the presence of the E1/E2/E3 enzymes. For FRET, the reaction aliquot was subjected to spectroscopic analysis that revealed a robust FRET signal only in the complete reaction (Fig. 1 C). Thus, the results from the gel and spectroscopic assays are in complete agreement, showing that the observed FRET truly reflects the formation of the K48-linked di-Ub chain as a result of enzymatic synthesis.

An HTS assay is judged by Z' factor, which measures statistical effect size based on the means and SDs of both the positive and negative control samples, with a value in the range of 0.5–1 indicating an excellent assay for screening (17). The calculated Z' factor of the FRET K48 di-Ub assay was 0.86 (Fig. 1D), demonstrating a sufficient window for HTS. Providing further proof-of-principle evidence of the utility of this FRET assay for inhibitor screening (17), we determined whether suramin inhibited ubiquitination of IκBα–Ub by E2 Cdc34, which required the holo-E3 complex SCFβTrCP and Nedd8 (Fig. S4). Suramin inhibited the ubiquitination of IκBα–Ub in a dose-dependent fashion (Fig. 2 C). Together, these results establish the inhibitory activity by suramin on ubiquitination catalyzed by E2 Cdc34 and E3 ROC1–CUL1.

Suramin Targets CUL1’s Conserved Basic Canyon. We explored the mechanistic action of suramin and found that it did not inhibit the formation of Ub thiol ester with either E1 (Fig. S5 A) or Cdc34 (Fig. S5 B). However, titration experiments revealed that the inhibitory effect by suramin is strictly dependent on the concentration of ROC1–CUL1 CTD, because higher levels of the RING complex, but not of E1 or E2 Cdc34, reversed the inhibition (Fig. S6). Together these results suggested that ROC1–CUL1 CTD mediates suramin’s inhibitory effect.

Indeed isothermal titration calorimetry (ITC) experiments showed direct binding of suramin to ROC1–CUL1 CTD with a $K_d$ of 2.80 ± 0.42 μM (Fig. 3 A). Suramin inhibited the formation of Ub thiol ester with either E1 (Fig. S5 A) or Cdc34 (Fig. S5 B). However, titration experiments revealed that the inhibitory effect by suramin is strictly dependent on the concentration of ROC1–CUL1 CTD, because higher levels of the RING complex, but not of E1 or E2 Cdc34, reversed the inhibition (Fig. S6). Together these results suggested that ROC1–CUL1 CTD mediates suramin’s inhibitory effect.

**Fig. 1.** FRET K48 di-Ub assay. (A) Reaction scheme. (B) Detection by gel-based analysis. The FRET K48 di-Ub assay was carried out in a test tube as described in detail in Materials and Methods. Shown is an image of fluorescent reaction products detected by a Typhoon 9500 scanner. Only the complete reaction in which all components of the assay were present supported di-Ub formation. (C) FRET. Aliquots of the above reaction mixture were spotted onto a 384-well plate, which was subjected to analysis with the Synergy-H1 reader. The fluorescence signals ranging from 545 to 700 nm are shown. Only the complete reaction yielded FRET. (D) Z’ factor. Three independent FRET experiments were carried out. The graph represents an average of three independent experiments; error bars indicate SD. The Z’ factor at each time point was calculated and is shown. “Control” refers to reactions lacking ATP.
CTD were used (Fig. 3B). Such CUL1 mutants, containing charge-swapping substitutions K431E/K432E/K435E or K678E/K679E/R681E, disrupted the CUL1 basic canyon (15), which is a conserved region that functions to bind the acidic tail of E2 Cdc34. The inhibitory effects of the K431E/K432E/K435E or K678E/K679E/R681E mutants were confirmed by di-Ub synthesis experiments (Fig. S7). Note that neither ROC1 nor Cdc34 alone exhibited significant binding for suramin (Fig. 3B). These results strongly suggest that suramin targets the basic canyon of CUL1.

Consistent with these findings, immunoprecipitation experiments revealed that suramin inhibited the binding of Cdc34 to ROC1–CUL1 CTD by 90% (Fig. 3C, lanes 3–6). Of note, immunoprecipitation did not detect interactions between ROC1–CUL1 CTD and the Cdc34 C-terminal tail alone. Together, these results suggest that suramin’s mechanism of action involves its binding to the CUL1 conserved basic canyon, thereby competitively blocking the recruitment of the E2 Cdc34 to the E3 complex (Fig. 4). A demonstration of how suramin occupies the CUL1 basic pocket awaits future high-resolution structural work.

In addition to Cdc34, the E3 ROC1–CUL1 core complex can work with the E2 enzymes UbcH5 and Ubc12, producing mono-ubiquitinated species and neddylated CUL1 (11), respectively. Fig. S8 examined the ubiquitination of β-catenin by SCFβTrCP and E2 UbcH5c in an assay similar to that shown in Fig. 2C. It was evident that at least threefold higher levels of suramin were required for inhibition of UbcH5c activity than in the Cdc34 reaction (compare Fig. 2C and Fig. S8). Moreover, suramin levels as high as 10 μM inhibited the transfer of Nedd8 to ROC1–CUL1 CTD by <50% (Fig. S9). Together, these data suggest that Cdc34-mediated ubiquitination is more susceptible to suramin than is UbcH5 or Ubc12.

Our ITC experiments demonstrate that suramin binds ROC1–CUL1 CTD with a Kd of 2.8 μM (Fig. 3A), an affinity that appears to be weaker than expected considering that this compound inhibits the FRET K48 di-Ub synthesis with an IC50 of 0.46 μM (Fig. 2A). However, we should note that the Kd measurement by ITC was carried out with PBS, which has a significantly higher ionic strength than used in the FRET K48 di-Ub synthesis assay (Materials and Methods). This difference in ionic strength may account for the higher-than-expected Kd, because the suramin–CUL1 CTD interactions are charge-based and therefore are likely to be sensitive to salt. Moreover, it is noteworthy that a similar discrepancy between IC50 and affinities has been reported for the Cdc34 inhibitor CC0651. In the previous study, CC0651 had an IC50 of 2.5 μM in the ubiquitination assay, but an EC50 of 51 μM was observed in the binding assay to Cdc34-Ub (18).

**Suramin Is an Inhibitor of CRL.** Cullins 2–5 (CUL2–5) share a conserved, C-terminally located basic canyon originally identified in CUL1 (15). In support, a more recent work revealed direct binding of the CUL2 basic canyon to the Cdc34 C-terminal tail (16). Given the evidence that suramin targets CUL1’s basic pocket (Fig. 3), we reasoned that suramin inhibits other CRL complexes as well. To test this hypothesis, we performed di-Ub synthesis reactions catalyzed by Cdc34 in the presence of ROC1–CUL2, ROC1–CUL3, or ROC1–CUL4A. Suramin inhibited all reactions tested (Fig. 5A). ROC1–CUL2 and ROC1–CUL4A appeared to be less sensitive to suramin than the ROC1–CUL1 CTD and ROC1–CUL3 complexes (Figs. 2A and 5A). The basis for these differences is presently unclear. However, note that in contrast to SCF/CRL1, whose E3 ligase activity can be measured in many reconstituted assays, well-developed in vitro systems for quantifying other CRL complexes are lacking in general. Such limitation precludes in-depth comparison of various CRL complexes in their responses to suramin.

We next attempted to examine the impact of suramin on the stability of CRL substrates. Because of its predominantly anionic structure, suramin was proposed to be membrane impermeant, and limited cellular uptake of suramin may occur by the process...
of receptor-mediated fluid phase endocytosis (19). Subsequently, Baghdiguian et al. (20) performed quantitative autoradiographic analysis revealing that in the absence of serum albumin labeled suramin was found to enter the human colon adenocarcinoma cells and distribute over the nucleus, the Golgi apparatus, and the mitochondria. In addition, using an indirect acridine orange reporter assay, Huang et al. (21) reported that suramin enters and accumulates in low-pH intracellular compartments (endosomes, lysosomes, and trans-Golgi complex) of normal and v-sis-transformed NIH 3T3 cells. To facilitate the cellular analysis of suramin, we used a serum-starvation protocol developed by Baghdiguian et al. (20) to increase the uptake of suramin and treated U2OS cells with this compound in concentrations ranging from 62.5 to 500 μM. The selection of this range of compound concentration was in line with previous studies showing that suramin at high concentrations (200–400 μM) inhibits the growth of a variety of cancer cell lines (22).

We used MLN4924, a well-characterized inhibitor of neddylation that inactivates CRL (23), as a positive control for the immunoblots analysis (Fig. 5B). In agreement with previous findings (23), MLN4924 caused stabilization of SCF(CRL1)Skp2 substrate p27, CRL3Keap1 substrate Nrf2, and CRL4Cdt2 substrate CDT1 (Fig. 5B), albeit with varying degrees of effect. As shown, suramin at 62.5 μM accumulated p27 and CDT1 (Fig. 5B). Accumulation of Nrf2 was observed in cells treated with higher concentrations of suramin (250–500 μM). It thus appeared difficult to correlate these cellular effects of suramin and the in vitro response shown by various CRL complexes (Figs. 2 and 5A). On the other hand, cellular effects are complex and likely are influenced by many factors, including subcellular localization. In this context, note that although both CDT1 and p27 are nuclear proteins (24, 25), CRL3Keap1 appears to function in the cytoplasm (26). Given the previous observations that suramin is preferentially enriched in

Fig. 3. Suramin binds to CUL1’s conserved basic canyon. (A) ITC was carried out as described in Materials and Methods. (B) Mutations in the CUL1 basic canyon impair binding to suramin. ITC was carried out with suramin (or its analog NF449) and ROC1–CUL1 CTD with wild-type or substituted CUL1 to disrupt the CUL1 basic canyon, as specified. ROC1 alone or Cdc34 showed no significant binding to suramin. It appears that two molecules of suramin bind to one CUL1 molecule. In support of this binding mode, NF449, an analog that resembles two suramin molecules, bound to ROC1–CUL1 CTD in a 1:1 fashion. However, conclusive proof for this claim requires future high-resolution structural studies. The ROC1/Rbx1–CUL1 structure and the zoomed in basic cleft of CUL1 were derived from Protein Data Bank (PDB) ID code 1LDJ (43) and were visualized using PyMOL. ROC1/Rbx1 is shown in magenta, and the basic residues in the basic cleft of CUL1 (K431, K432, K435, K678, K679, R681) that were replaced by glutamates are highlighted in yellow. The CDC34 catalytic core (residues 7–182) was derived from PDB ID code 2O84 and was visualized using PyMOL. The RING domain of ROC1/Rbx1 was derived from PDB ID code 2LGV (14) and was visualized using PyMOL. (C) Suramin blocks the binding of ROC1–CUL1 CTD to Cdc34. Immunoprecipitation experiments were carried out with HA-Cdc34 and ROC1–CUL1 CTD in the presence of the indicated compounds, in amounts specified and as described in SI Materials and Methods. A darker exposure of the gel is shown to visualize ROC1 better, because this smaller protein stained poorly.
properties. When tested in the FRET K48 di-Ub assay with at least one aromatic spacer on either side of the central urea. anionic substituent, preferably sulfonates. It also must contain a macophore appears to be a structure with two assemblies of highly polysulfonated naphthylurea. A panel of commercially available ROC1–CUL1 CTD and ROC1–CUL3 (Fig. 6). Previous studies have shown that NF340 inhibits the ligand binding to the P2Y11 receptor (27) and angiogenesis (28). Although NF023 and NF340 are structurally similar overall, they differ in the number of sulfonate groups (six in NF023 and four in NF340) and in the position of sulfonates on the naphthyl ring (4, 6, and 8 in NF023; 3 and 7 in NF340). On the other hand, it should be noted that NF110, which contains four sulfonate groups, inhibits ROC1–CUL1 CTD with an IC50 of 1.03 μM (Fig. 6). Together, these results suggest that the position of sulfonates on the naphthyl ring impacts selectivity. It is possible that NF340 binds to the P2Y11 receptor but binds poorly to CUL1 because of slight differences in the basic pocket in P2Y11 and CUL1.

Overall, ROC1–CUL3 appeared more sensitive than ROC1–CUL1 CTD in response to suramin and its analogs (Fig. 6). Although cullins share a conserved basic canyon, subtle differences may result in the differential abilities of suramin and analogs to interact with the cullins. These results suggest that compounds that selectively target distinct classes of CRL complexes might be developed in the future.

Discussion

The FRET K48 di-Ub Assay: A Strategy for Targeting CRL-Mediated K48 Ubiquitination. The Food and Drug Administration (FDA)-approved antimalaria and anti-CRL4cycloprodrug lenalidomide/thalidomide (29, 30) provides proof-of-concept evidence in favor of promoting CRL-based therapies. This study describes the development of an in vitro ubiquitination reporter system, the FRET K48 di-Ub assay, that can be used effectively for an HTS campaign to discover small-molecule chemical probes that target the CRL subcomplex containing ROC1 and its cullin partner as well as E1 and E2 Cdc34. Our FRET strategy is innovative, and easily adaptable for other K48-dependent ubiquitination HTS assays. First, by allowing only one nucleophilic attack, it produces a single Ub–Ub isopeptide bond (Fig. 1A). In contrast, previous assays have used the wild-type fluorescent Ub that yields polyubiquitination (31, 32). The restriction to a single Ub–Ub linkage eliminates the complexity associated with polyubiquitin chains and allows straightforward quantification of each assay. This innovation ensures a high degree of reproducibility that is critical for HTS success, as validated in our present screen (Fig. 1D and Fig. S2A). Second, FRET is produced by two Ub-linked fluorophores that become juxtaposed upon Ub–Ub conjugation (Fig. S1A). Each fluorophore is uniquely engineered to either donor or receptor Ub at a specific site (Fig. S1A). Note that optimal positioning of the fluorophore in Ub is critical, because N-terminally labeled Ub, commonly used in previous publications (31, 33), was inactive in our system. Commercial fluorescent Ub proteins do not specify the location of the fluorescent dye in Ub, and it is unclear if such reagents can be generally optimal for HTS FRET assays. On the other hand, our FRET strategy should be applicable to other HTS campaigns that depend on K48 ubiquitination but require different E3/E2 enzyme combinations.

Cullin Conserved Basic Canyon: An Emerging Target of Regulation by Chemical Probes. The CRL superfamily, which contains ~300 members and accounts for nearly half of the E3s identified in humans, affects a myriad of biological processes by directing numerous protein substrates for proteasomal degradation (2, 3). Key to CRL activity is the recruitment of the E2 Cdc34 through electrostatic interactions between E3’s cullin conserved basic canyon and the acidic C terminus of the E2 enzyme (15, 16). Cdc34 is the only E2 required for cell viability in yeast (34). Using a chimera strategy, yeast genetic studies have shown that fusion of the Cdc34 acidic C-terminal tail to another E2 (such as Ub2/Rad6) can transform the chimeric enzyme into a Cdc34-like protein essential for supporting cell growth, underscoring an indispensable role for the E2’s negatively charged C terminus (35, 36). Subsequent biochemical experiments have demonstrated the interactions between Cdc34 and E3 SCF (CRL1) (37), mapped the binding activity to the E2 acidic C terminus and E3’s ROC1–CUL1 CTD subcomplex (38), and finally localized the contacts to CUL1 CTD’s basic canyon and the E2 negatively charged C-terminal tail (15, 16). Canonical cullins 1–5 share a conserved, C-terminally located basic canyon (15). Indeed, recent work has demonstrated direct binding of the CUL2 basic canyon to the Cdc34 C-terminal tail (16). In further support, the biochemical reconstitution assays have revealed that a variety of CRL complexes tested, including ROC1–CUL2, ROC1–CUL3, and ROC1–CUL4A, can support Cdc34 for di-Ub synthesis (Fig. S4).

In the present work we demonstrate that the cullin conserved basic canyon is a target of regulation by chemical probes. Suramin, a small-molecule inhibitor identified through an HTS using the FRET K48 di-Ub assay format (Fig. 2), is shown to bind to the ROC1–CUL1 CTD complex directly (Fig. 3A), most likely at the CUL1’s conserved basic canyon (Fig. 3B). Consequently, suramin is found to block the binding of Cdc34 to the ROC1–CUL1 CTD complex in vitro (Fig. 3C) and to accumulate CRL substrates in vivo (Fig. 5B).

Fig. 4. A model of suramin competing with Cdc34 for binding to the conserved basic canyon of CUL1. The ROC1/Rbx1–CUL1 structure was derived from PDB ID code 1LDJ (43) and was visualized using PyMOL.

Published online March 21, 2016 | E2015

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BIOCHEMISTRY

E2 Cdc34 acidic C-terminal tail

Suramin

Basic Cleft

Culin-1

E2 Cdc34 acidic C-terminal tail

Suramin

Basic Cleft

Culin-1
One caveat is that suramin has many cellular targets (12), presumably because of its ability to bind highly positively charged regions that are analogous to the cullin conserved basic canyon. However, analyses of suramin analogs (Fig. 6) may have shed some light on the prospect of improving selectivity. The differential inhibition observed among suramin target protein–protein complexes suggests that subtle differences in basic pockets of the cullins might result in measurable differences in their affinity to suramin and analogs. Such postulated differences, if validated, might be exploited for developing selective suramin analogs with differential abilities to target distinct classes of CRL complexes.

We have reproducibly observed that suramin exhibits differential inhibitory potency toward ubiquitination reactions mediated by SCF or its subcomplexes. Suramin inhibited the ROC1–CUL1 CTD-dependent FRET K48 di-Ub synthesis or discharge reaction with similar potency, exhibiting an IC50 of 0.46 μM or 1 μM, respectively (compare Fig. 2 A and B). However, the ubiquitination of IκBα–Ub by SCFβTrCP appeared less sensitive to suramin (Fig. 2C). A possible explanation of this paradox is the existence of suramin-insensitive mechanisms for the recruitment of Cdc34 by the holo-SCFβTrCP E3 complex. Ubiquitination of IκBα–Ub requires the assembly of the SCFβTrCP-IκBα-Ub complex. It is possible that, in addition to using electrostatic interactions mediated by CUL1’s basic canyon and E2’s acidic C terminus, which is sensitive to suramin, the IκBα-linked Ub may bind and help recruit Cdc34. Furthermore, in the context of the SCFβTrCP-IκBα-Ub complex, the ROC1 RING domain may exhibit affinity to

![Diagram](image-url)

**Fig. 5.** Suramin is an inhibitor of CRL. (A) Suramin inhibits CRL complexes containing CUL2-4. The FRET K48 di-Ub assay was used to test suramin inhibition with ROC1–CUL3. The 32P-di-Ub synthesis assay was used with ROC1–CUL2 and ROC1–CUL4A. Both assays are described in SI Materials and Methods. In all cases, Cdc34 was used. Detected di-Ub products in the presence or absence of suramin were quantified and are shown graphically. (B) Suramin accumulates CRL substrates. Extracts from U2OS cells treated or not treated with compounds were subject to immunoblot analyses using the indicated antibodies, as described in SI Materials and Methods. The Nedd8 inhibitor MLN4924 is known to accumulate CRL substrates (23) and was used as a positive control. Detected CRL substrate signals were quantified by Odyssey infrared imaging (LI-COR); the relative values are presented under each blot.
the Cdc34 core domain at levels significantly higher than those displayed by a subcomplex (ROC1–CUL1 CTD). Both E2–Ub and RING-E2 interactions are driven by hydrophobic interactions (39) and thus are unlikely to be sensitive to suramin. However, despite subtle differences, suramin effectively inhibits SCF/CRL1 and other CRL complexes (Figs. 2 and 5A).

Recent studies have revealed that Cdc34 activity can be regulated by a protein inhibitor or small-molecule compound. Gloxinin, a HEAT-repeat–containing protein, was shown to bind the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Like- wise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41). Likewise, the small-molecule inhibitor of Cdc34a, CC0651, stabilizes a noncovalent complex between the E2 and the donor Ub, thereby blocking Ub discharge (18). To our knowledge, disrupting the E2-interacting surface of the ROC1/RBX1 RING domain and to inhibit CRL-mediated chain formation by Cdc34 (40, 41).

Materials and Methods

Detailed procedures for the HTS experiment, protein preparation, assays, and cell-based experiments are provided in SI Materials and Methods.

Preparation of Fluorescently Labeled Ub. Site-directed mutagenesis was used to create Ub–Q31C/K48R (donor Ub) and Ub–E64C/GG (acceptor Ub), each containing a single cysteine residue for labeling with maleimide-based fluorescent dyes. To prepare fluorescent Ub, purified Ub–Q31C/K48R and Ub–E64C/GG, 150 mg each, were treated with 10 mM Tris(2-carboxyethyl) phosphine (TCEP) (Sigma) in degassed PBS for 15 min at room temperature and then were bound to Ni-NTA agarose (Qiagen) for 1 h at 4 °C. After binding, the beads were washed with degassed PBS to remove the TCEP and any unbound protein. The resulting beads were incubated with 15 mg of iFluor 555 maleimide (UbQ31C/K48R) or iFluor 647 maleimide (UbE64C/GG) (AAT Bioquest), dissolved in dimethylformamide (Sigma), for 1 h at room temperature and then overnight at 4 °C in the dark. Note that the above handling was performed inside an anaerobic glove box (Captair Pyramid; Erlab). The subsequent steps were performed in normal air. Following incubation, beads were washed with PBS to remove unreacted dye. Then the labeled proteins were eluted with PBS plus 250 mM imidazole. The eluted proteins were dialyzed at 4 °C in the dark, against buffer containing 25 mM Tris·HCl (pH 7.4), 1 mM EDTA, 10% glycerol, 150 mM NaCl, 0.01% Nonidet P-40, and 1 mM DTT. The final yields were ∼108 mg labeled Ub–Q31C/K48R and ∼129 mg for labeled Ub–E64C/GG.

FRET K48 di-Ub Assay. The reaction mixture (15 μL) was assembled onto a 384-well microtiter plate (or in a test tube). Each well contained 33 mM Tris·HCl (pH 7.4), 1.7 mM MgCl2, 0.33 mM DTT, 0.07 mg/mL BSA, 14 nM Ub E1, 124 nM Ub E2 Cdc34, 1 μM ROC1–CUL1 CTD, 0.93 μM Ub C31-iFluor 555 (donor), and 1.62 μM Ub C64-iFluor 647 (receptor) in the absence or presence of a compound such as suramin, in amounts as indicated. ATP (0.66 mM) was added to the mixture followed by a brief centrifugation to settle the mixture. The resulting plate was incubated at 30 °C in a Synergy-H1 reader (BioTek), and the fluorescence signal was monitored. Ubiquitination was quantified based on the ratio of acceptor:donor fluorescence (excitation 515 nm; donor emission 570 nm, acceptor emission 670 nm).

IC50 Determination. Compound stock solutions in DMSO were titrated into 384-well plates using an HP D300 digital dispenser. Assays were performed in a 384-well microtiter plate as described above using the Synergy-H1 reader. Results were analyzed and graphed using SigmaPlot software (SyStat). The IC50 curve was determined using four-parameter logistic standard curve analysis.

ITC. ITC was performed on a MicroCal iTC200 system (GE) at 25 °C. Samples were prepared in degassed PBS. Typically 0.15–0.3 mM suramin was titrated in 2.5-μL injections, administered at 3-min intervals, into the sample cell containing 10–30 μM protein (or protein complex). The data were analyzed in Origin software (OriginLab).
**Supporting Information**

**Wu et al. 10.1073/pnas.1601089113**

**SI Materials and Methods**

**Protein Preparations.**

Plasmids and protein preparations for fluorescently labeled Ub. Site-directed mutagenesis was used to create Ub-Q31C/K48R (donor Ub) and Ub-E64C/ΔGG (acceptor Ub), each containing a single cysteine residue for labeling with maleimide-based fluorescent dyes. For both, yeast Ub in pET3a with an N-terminal tobacco etch virus (TEV) cleavable His6-tag (pHisTEVUb) was used as the template plasmid. For Ub-Q31C/K48R, the QuikChange Multi Site Directed Mutagenesis Kit (Agilent) was used, using two primers that contain the Q41C and K48R mutations, respectively. For Ub-E64C/ΔGG, the QuikChange II XL Site Directed Mutagenesis Kit (Agilent) was used, using a primer pair that contains both the E64C and G75X mutations on the primer. The plasmids were transformed and expressed in *Escherichia coli* Rosetta 2 (DE3)pLysS cells (EMD Millipore). The proteins were purified on Ni-NTA agarose (Qiagen) and dialyzed against 25 mM Tris-HCl (pH 7.4), 10% (vol/vol) glycerol, 50 mM NaCl, 0.01% Nonidet P-40, and 1 mM DTT.

**Preparation of IκBα (EE)-Ub.** To construct IκBα (EE)-Ub containing the phosphor-mimetic degron, pGEX-2TK-IκBα (1–20)-Ub-IκBα (23–54) (10) was used as a template. The standard PCR-based mutagenesis strategy was applied to convert the IκBα degron motif DS<sup>13</sup>GLDS<sup>36</sup> to DE<sup>19</sup>GLDE<sup>36</sup> (32 and 36 denote the amino acid positions in human IκBα). Mutagenesis was confirmed by DNA sequencing. To express IκBα (EE)-Ub, pGEX-2TK-IκBα (1–20)-Ub-IκBα (23–54)-EE was transformed into *E. coli* Rosetta2 (DE3) pLysS cells (EMD Millipore). After standard IPTG induction at 37 °C and purification through glutathione matrix, GST was cleaved by thrombin digestion yielding pure IκBα (EE)-Ub. The yield was about 6 mg/L induced culture.

**Other reagents.** The following reagents were prepared using established protocols. Baculovirally expressed SCF<sup>α</sup>TrCP and bacterially expressed PK-Ub, HA-Cdc34, and Ubc12 were prepared using the methods described by Chong et al. (13). For large-scale preparation of human Cdc34 and E1, His<sub>6</sub>-TEV Cdc34 C191S/C223S in pET28a (14) and Ubc1 (E1)-His<sub>6</sub> in pET3a (Addgene plasmid #3571) were used. ROC1 alone (ROC1 12–108) and ROC1–CUL4A were prepared following the protocols by Spratt et al. (14) and Angers et al. (44), respectively.

ROC1–CUL2 and ROC1–CUL3 were purchased from Ubiquigent. Nedd8, Neddl E1, human Ub, and UbK0 were purchased from Boston Biochem. Suramin, NF023, sodium 1-naphthalenesulfonate, and 1,3 (6, 7)-naphthalene-trisulfonic acid were purchased from Sigma. NF-340, NF-449, and NF-546 were purchased from Tocris. Cdc34 inhibitor CC0651 was a generous gift of Thimo Kurz (University of Dundee, Dundee, Scotland).

**Assays.**

IκBα di-ubiquitination assay.

**Preneddylation of ROC1–CUL1.** The preneddylation mixture contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 2 mM ATP, 0.5 mM DTT, 0.1 mg/mL BSA, ROC1–CUL1 (0.45 μM), Neddl8 (20 μM), Neddl E1 (83 mM) and Ubc12 (15 μM). The reaction was incubated at room temperature for 10 min.

**Assembly of SCF<sup>α</sup>TrCP-IκBα (EE)-Ub complex.** Skp1-βTrCP (0.45 μM) was added to the above preneddylation mix. Incubation continued at room temperature for 10 min. Then 3.7 μM IκBα (EE)-Ub was added, and the reaction volume was adjusted to 5 μL. The reaction was incubated for additional 10 min at room temperature.

**Assembly of Cdc34–Ub K0.** The E2 charging reaction was assembled in a 5-μL mixture that contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 2 mM ATP, 0.5 mM DTT, 0.1 mg/mL BSA, 40 μM Ub K0, 0.05 μM E1, and human 10 μM Cdc34. The reaction was incubated for 5 min at 37 °C.

IκBα di-ubiquitination. The above mixture containing SCF<sup>α</sup>TrCP, IκBα (EE)-Ub or Cdc34–Ub K0 was mixed (final volume, 10 μL). The reaction was incubated at 37 °C for 30 min. The products were separated by 4–20% SDS/PAGE, visualized by Coomassie stain, and quantified on a Typhoon FLA 9500 laser scanner (GE).
The **32P-di-Ub synthesis**. The **32P-di-Ub synthesis reaction was carried out by preassembling the donor Ub mix. The 5-μL mixture contained 50 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, 0.5 mM DTT, 0.1 mg/mL BSA, 2 mM NaF, 10 nM okadaic acid, 1.7 μM **32P-PK Ub**, 0.05 μM E1, and 1 μM human Cdc34. The reaction was incubated for 5 min at 37 °C. Suramin, in the amounts indicated, was added followed by the addition of 0.5 μM ROC1–CUL1 CTD and 43 μM human Ub. The final volume was adjusted to 10 μL. The reaction was incubated for 30 min at 37 °C. The products were separated by 4–20% SDS/PAGE and were visualized and quantified on a Typhoon FLA 9500 laser scanner (GE).

**Immunoprecipitation.** The reaction mixture (20 μL) contained buffer A [25 mM Tris-HCl (pH 7.5), 1 mM DTT, 10% glycerol, 0.01% Nonidet P-40], 50 mM NaCl, HA-tagged human Cdc34 (in amounts as specified), ROC1–CUL1 CTD (in amounts as specified), and compounds (where indicated). The mixture was incubated at room temperature for 10 min. The resulting mixture was adsorbed to the matrix (20 μL) containing anti-HA antibodies (Sigma) for 2 h at 4 °C on a mixer (1,300 rpm). The unbound protein was removed by washing the beads three times with 0.5 mL of buffer A plus 50 mM NaCl. The bound proteins were eluted by SDS, separated by 4–20% SDS/PAGE, visualized by Coomassie stain, and quantified on a Typhoon FLA 9500 laser scanner (GE).

**Suramin treatment and Western blot analysis.** Human U2OS cells were maintained on a 35 × 100 mm dish in DMEM (Life Technologies) supplemented with 10% heat-inactivated FBS and penicillin (100 U/mL)-streptomycin (100 μg/mL). Cells (6 × 10⁵) were seeded 24 h before drug treatment. To increase suramin uptake (20), complete medium was replaced by serum-free medium containing increasing amounts of suramin. Cells were cultured with drug in the absence of serum for 6 h. Serum then was added to a final concentration of 10%. Cells were cultured for an additional 18 h before harvest. Whole-cell lysates were prepared from cells as described previously (10). Extracts were run on 4–20% Tris-glycine gels, and were transferred to membranes using standard Western blotting procedures. For the analysis of p27 or Nrf2, 30 μg of extract proteins were used. For CDT1, 1 μg of extract proteins was used. The following primary antibodies were used: CDT1 and Nrf2 (Santa Cruz Biotechnology), p27, (BD Biosciences), and tubulin, (Sigma). Signals were visualized and quantified using the LI-COR Odyssey IR Imaging System.

**HTS.**

**Library compounds.** HTS was carried out at the Integrated Screening Core of the Icahn School of Medicine at Mount Sinai, New York using the FDA library, which is composed of ~2,000 compounds (MicroSource Discovery Systems) approved for use in humans or animals.

**Assay optimization.** The FRET K48 di-Ub assay described in Materials and Methods was optimized for an HTS in a 384-well assay format. Because the assay system includes three enzyme components, the assay was formatted using two reagent-addition steps to maximize the possibility of identifying inhibitors for any of the critical reaction steps. The FDA library compounds, stored in 10 mM stock plates (DMSO; 320 compounds per plate), were transferred by pin tool (V&P Scientific) into assay plates containing a 15-μL reaction mixture containing 14 nM Ub E1, 124 nM Cdc34 E2, 1 μM ROC1–CUL1 CTD, 0.93 μM Ub C31-I555 (donor), and 1.62 μM Ub C64-I647 (receptor) in the presence of 33 mM Tris-HCl (pH 7.4), 1.7 mM MgCl₂, 0.33 mM DTT, and 0.07 mg/mL BSA. ATP (0.66 mM) was added to the protein/small-molecule mixture. Following incubation at room temperature, ubiquitination was quantified based on the ratio of acceptor:donor fluorescence (excitation 515 nm; donor emission 570 nm, acceptor emission 670 nm) quantified using an EnVision high-throughput plate reader (Perkin-Elmer). Fig. S2A shows the results of an automated assay demonstrating that the reaction progressed in an almost linear fashion up to 20 min and was significantly inhibited by the Cdc34 inhibitor CC0651.

**Primary screen.** The screen was carried out by placing the FDA library compounds onto 2 × 8 of 384-well microtiter plates. On each plate, 320 small molecules were distributed in 22 columns; the first and last two columns were left empty to allow the addition of the no-ATP negative control and the complete-reaction-without-compound positive control. At the 15- and 30-min reaction time points, plates in duplicate (sets A and B) were read at 570 nm and 670 nm. Fig. S2B shows the readings of plates 6A and 6B recorded at both the 15- and 30-min time points, revealing that both wells 6A E4 and 6B E4 (containing compound suramin) possessed a 670/570 value similar to that of the no-ATP negative control.
Fig. S1. FRET K48 di-Ub assay and inhibition by Cdc34 inhibitor CC0651. (A) Placement of fluorescent dye on a pair of residues on the donor and receptor Ub. The model of the K48 di-Ub chain (PDB ID code 1F9J) (45) is shown. The positions of residues Q31 and E64 are marked, and these residues are replaced by cysteine and labeled with the indicated fluorescent dye as described in Materials and Methods. The calculated distance between Ub C31-linked iFluor 555 and Ub C64-linked iFluor 647 in the K48-linked di-Ub chain is 38 Å. The Förster radius (Ro) for iFluor 555/iFluor 647 is about 51 Å (at this distance the resonance transfer efficiency is 50%). (B) Purity of the five assay components used for FRET. Shown are the five reaction components resolved on SDS/PAGE. Fluorescently labeled donor and receptor Ub were imaged by a Typhoon FLA 9500 scanner. E1, Cdc34, and ROC1–CUL1 CTD were visualized by staining with Coomassie. (C and D) Inhibition of FRET K48 di-Ub synthesis by Cdc34 inhibitor CC0651. (C) Gel analysis of FRET K48 di-Ub synthesis reaction without or with CC0651 (2, 10, and 100 μM). (D) FRET assay was performed with increasing amounts of CC0651 and IC50 was determined as in Fig. 2A.
Fig. S2. HTS identification of the inhibitor suramin. (A) FRET automation assay. A FRET automation time-course experiment is shown. Proteins in buffer lacking ATP were mixed with the Cdc34 inhibitor CC0651 (10 μM), followed by the addition of ATP to initiate the reaction. Blank represents the no-ATP negative control. Each data point is the average of 32 independent reactions. Deviation is shown for each data point. (B) The FDA library screen format. Screening was carried out in a 384-well format as described in SI Materials and Methods. Shown are assay plates 6A and 6B with 320 small molecules distributed in 20 columns; the first and last two columns were left empty to allow the addition of assay-specific controls (no ATP and complete, no compound). The numbers show the ratio of 670/570 nm. Ratio values >0.75 are colored red, indicating no or very poor inhibition. Values <0.25 are colored cyan, indicating severe inhibition. Values between 0.25 and 0.75 are colored green, indicating modest inhibition. This screen shows the readings of duplicated plates recorded at both the 15- and 30-min time points, revealing that both wells 6A E4 and 6B E4 (suramin) possessed a 670/570 nm value similar to that of the no-ATP negative control. (C) Follow-up assays confirmed the inhibitory activity by suramin. Gel analysis of a panel of reaction mixtures retrieved from the indicated wells on the marked assay plates that showed reduced FRET signals. When tested in gel assays, reaction mixtures retrieved from either well 6A E4 or 6B E4 (suramin) showed no di-Ub product (lanes 13 and 14). (D) Confirmation by reassay. A di-Ub reaction was carried out as in Fig. 1; the reactions were treated with suramin obtained from the Mount Sinai Screening Core.
Fig. S3. Suramin inhibits discharge by ROC1–CUL1 CTD with or without neddylation. The $^{32}\text{P-Di-Ub}$ synthesis reaction was carried out as described in SI Materials and Methods. Where indicated, ROC1–CUL1 CTD was neddylated as described by Kovacev et al. (11). The di-Ub product was quantified, and the percentage of inhibition by suramin is shown. Nearly identical results were obtained with or without neddylation.
**Fig. S4.** The IκBα-Ub fusion. (A) The IκBα phosphor-degron. IκBα contains a phosphor-degron and K21/K22 to accept Ub. IκBα(1–54) EE-Ub contains a phosphor-mimetic degron with S32 and S36 replaced by glutamate (E), and an Ub in place of K21/K22. The N-terminal GST tag can be removed by thrombin cleavage. (B) IκBα (EE)-Ub supports ubiquitination. The reaction was carried out as described in SI Materials and Methods. It contains eight components: IκBα (EE)-Ub; the donor Ub (K0; terminating chain elongation); E1; Cdc34; SCFβTrCP (E3); and neddylation agents (Nedd8, Nedd8 E1, and Ubc12) that activate the E3. IκBα (EE)-Ub contains an Ub moiety that is fused upstream of IκBα’s phosphor-mimetic degron (DEGLDE). This reaction yielded IκBα di-ubiquitin species (lane 1) in a manner that requires intact SCFβTrCP (lanes 3 and 4) and neddylation (lane 2). In this system, IκBα (EE)-Ub is bifunctional so that the IκBα (EE) degron anchors SCFβTrCP, whereas the fusion Ub provides K48 that attacks the E3-bound Cdc34–Ub K0 (10).

**Fig. S5.** Suramin does not target E1 or Cdc34. (A) Suramin did not inhibit the formation of the E1–S-Ub thiol ester complex. Increasing amounts of suramin were incubated with Ub E1 and iFluor 555-labeled donor Ub (Fig. S1A). The reaction was terminated by SDS under nonreducing conditions. The levels of E1–S-Ub were quantified using a GE FLA 9500 scanner and are presented graphically. (B) Suramin did not inhibit the formation of the Cdc34–S-Ub thiol ester complex. Suramin, in the amounts indicated, was incubated with Ub E1, Cdc34 E2, and iFluor 555-labeled donor Ub. The reaction was terminated by SDS under nonreducing conditions. Where indicated, DTT (0.1 M) was treated to reduce Cdc34–Ub thiol ester. The levels of Cdc34–S-Ub were quantified by a GE FLA 9500 scanner and are presented graphically. Note that species migrating between 20 and 25 KDa represent small amounts of the dimeric forms of Ub.
Suramin targets ROC1–CUL1 CTD: Titration experiments. The FRET K48 di-Ub assay was carried out with various concentrations of suramin and E3 ROC1–CUL1 CTD (A) and E1 or E2 Cdc34 (B) as indicated. A 90-min time course is shown in which the FRET signal was measured at 5-min intervals. On the graph in A and B, Left, red denotes a complete reaction without compound, and green represents the negative control without ATP. Blue indicates the reaction with compound suramin at the indicated concentrations. On the graph in B, Right, blue denotes a complete reaction, and green represents the negative control. Red indicates the reaction with suramin. FRET activity was quantified and expressed against the enzyme/suramin concentrations. The inhibitory effect of suramin is strictly dependent on the concentrations of ROC1–CUL1 CTD (but not E1/E2), so that the highest levels of the RING complex completely reversed the inhibition.
Fig. S7. CUL1 basic canyon disruptive mutations inhibit ubiquitination. The $^{32}$P-di-Ub synthesis assay, carried out as described in SI Materials and Methods, was used to determine the effects of ROC1–CUL1 CTD that contains the CUL1 mutations K431E/K432E/K435E (A) or K678E/ K679E/R681E (B).
Fig. S8. Effects of suramin on the ubiquitination of β-catenin by SCFβTrCP and UbcH5c. The reaction was initiated by combining two preformed mixtures that contained UbcH5c–S–Ub and SCFβTrCP–β-catenin, respectively. The E2 charging reaction was assembled in a 5-μL mixture that contained 50 mM Tris·HCl (pH 7.4), 5 mM MgCl₂, 2 mM NaF, 10 nM okadaic acid, 2 mM ATP, 0.5 mM DTT, 0.1 mg/mL BSA, 40 μM Ub-K0, 0.2 μM E1, and 2 μM UbcH5c. The reaction was incubated for 5 min at 37 °C. To assemble the E3-substrate complex, a 5-μL mixture containing 0.3 μM Nedd8-SCFβTrCP (prepared as in Fig. 2C) and 0.4 μM 32P-β-catenin peptide [prepared as described by Kovacev et al. (11)] was incubated in the presence of 0.1 M Na-glutamate for 10 min at room temperature. Suramin, at the indicated concentrations, was added to Nedd8-SCFβTrCP–β-catenin. This mixture then was added to UbcH5c-Ub-K0 (in a final volume of 10 μL), and the combination was incubated at 37 °C for 15 min. The reaction products were visualized by autoradiography after separation by 4–12% SDS/PAGE, and the levels of input substrate and products were quantified by phosphoimaging.

Fig. S9. Effects of suramin on CUL1 neddylation in vitro. CUL1 neddylation was initiated by combining two preformed mixtures that contained Ubc12–S–Nedd8 and ROC1–CUL1 CTD, respectively. The Ubc12 charging reaction was assembled in a 5-μL mixture that contained 50 mM Tris·HCl (pH 7.4), 5 mM MgCl₂, 2 mM ATP, 0.5 mM DTT, 0.1 mg/mL BSA, 10 μM Nedd8, 20 mM Nedd8 E1, and 100 nM Ubc12. The reaction was incubated for 5 min at 37 °C. The second mixture contained 1 μM ROC1–CUL1 CTD and 10 μM of suramin or analogs as indicated. The two mixtures were combined and incubated at room temperature for 10 min. The reaction products were visualized by Coomassie stain after separation by 4–12% SDS/PAGE, and the levels of CUL1 CTD were quantified by imaging using a Typhoon 9500 scanner.