

## Supplemental Data

### Evaluation of a Diffusion-Driven Mechanism for Substrate Ubiquitination by the SCF-Cdc34 Ubiquitin Ligase Complex

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#### Data Quantification

For all data measurements, experiments were performed in triplicate and band intensities were measured by phosphor screen analysis using ImageJ software. Data points represent the mean from measured experiments with standard deviation from the mean error bars. The rate of ubiquitination or K0 ubiquitin incorporation was measured as the ratio of modified Sic1 to the total amount of Sic1 with respect to time (Figure 2). The 0 and 60 min time points for reactions employing F72V were used to estimate background for modified and unmodified Sic1, respectively. These values were subtracted from the values for other time points. Rates were determined by measuring the slope of the linear portion of the curve. Due to the rapid reaction conditions for WT in which some Sic1 modification occurs during the time of initiating the reaction to removing the initial time point (approximately 5 seconds), the rate measurements reported for WT may be an underestimate.

To measure fold inhibition for reactions containing increasing amounts of either C95S, L99S or F72V (Figure 3B), high molecular weight species (>98 kDa) for each reaction was measured and expressed as a percentage of the total amount of Sic1 measured per data point. The measurements were normalized with respect to the maximum amount of high molecular weight Sic1 formed (no competitor added, 5 minutes). Reaction rates were determined by curve fitting algorithms using GraphPad Prism (Figure S2). Fold inhibition was determined as the ratio of the determined rates without competitor to those rates with a particular concentration of competitor.

The rate of Cdc34~Ub loss and diubiquitin formation (Figure 5B, C) was measured for the respective species as a percentage of the measured total amount of ubiquitin per reaction.

### **Structural Modeling**

The x-ray structures for a Skp1- $\beta$ TrCP complex bound to  $\beta$ -catenin phosphopeptide (pdb 1P22) and an F-box-Skp1-Cul1-Rbx1 complex (pdb 1LDK) were structurally superimposed to generate a model of the SCF enzyme complex (Shindyalov and Bourne, 1998). The x-ray structure of yeast Ubc7 was used to generate a homology model of yeast Cdc34 using the program Modeller (Sali and Blundell, 1993). We then used the structure of the c-Cbl ubiquitin ligase bound to UbcH7 (Zheng et al., 2000) to dock Cdc34 to the SCF RING domain. Here Cdc34 was structurally aligned to UbcH7 and Rbx1 was structurally aligned to the RING domain from c-Cbl. This procedure was expected to yield reasonable results, given that the E2 and RING folds are highly conserved. No steric conflicts were observed when Cdc34 and Rbx1 were docked together. Finally, ubiquitin molecules (derived from pdb 2BGF) were covalently attached to the terminal lysine residue on the  $\beta$ -catenin phosphopeptide using the molecular modeling program 'O' (Jones et al., 1991). The K48 side-chain dihedrals and/or the C-terminal glycyl main-chain dihedrals were manually changed to change the geometry of the polyubiquitin chain. This procedure was repeated until the terminal K48 amino group was placed near the Cdc34 active site cysteine residue. Ideal bond lengths and angles were maintained by using the program CNS (Brunger et al., 1998), with the x-ray term turned off during the refinement.

### **Supplemental References**

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<b>Common Name</b>	<b>RDB Number</b>	<b>Description</b>	<b>Reference</b>
Cdc34 $\Delta$ C	RDB1529	His6-tagged Cdc34 for bacterial expression, lacks 25 C-terminal amino acids	(Seol et al., 1999)
Cdc34 $\Delta$ C (F72V)	RDB1962	Identical to RDB1529 except contains F72V mutation	This study
Cdc34	RDB1963	Full-length, untagged Cdc34 for bacterial expression	This study
Cdc34 (F72V)	RDB1964	Identical to RDB1963 except contains F72V mutation	This study
Sic1	RDB1706	Sic1 open reading frame encoding an N-terminal T7 tag and a C-terminal His6-tag, contains only the 6 lysines within the N-terminal 180 amino acids necessary for in vivo degradation	(Petroski and Deshaies, 2003)
Cdc34 $\Delta$ C (C95S L99S)	RDB1291	Identical to RDB1529 except contains C95S L99S mutations	This study
pRS313	RDB45	Plasmid for yeast containing HIS3 marker	(Sikorski and Hieter, 1989)
Cdc34-Myc13/pRS313	RDB1965	Yeast expression plasmid with HIS3 marker for Cdc34 expressed from Cdc34 promoter, contains C-terminal Myc13 tag,	This study
Cdc34-Myc13 (F72V)/pRS313	RDB1966	Identical to RDB1965 except contains F72V mutation	This study
Cdc34/pRS316	RDB1967	Yeast expression plasmid with URA3 marker for Cdc34 expression from Cdc34 promoter, no epitope tag	This study

Table S1. Plasmids Used during This Study

<b>RJD number</b>	<b>Description</b>	<b>Genotype</b>	<b>Source</b>
RJD3253	Heterozygous diploid, single genomic copy of CDC34	<i>MATa/MATα cdc34Δ::kan<sup>R</sup>/CDC34 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0</i>	Open Biosystems, YSC1021-669597
RJD3254	Haploid, sic1Δ	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems, YSC 1021-551353
RJD3255	MATa cdc34Δ expressing URA3 marked Cdc34	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1 leu2Δ0 ura3Δ0::[RDB1967-pMP351]</i>	This study
RJD3256	MATa cdc34Δ expressing URA3 marked Cdc34 with pRS313	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[pRS313] leu2Δ0 ura3Δ0::[RDB1967-pMP351]</i>	This study
RJD3257	MATa cdc34Δ expressing URA3 marked Cdc34 and HIS3 marked Cdc34-Myc13	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[RDB1965-pMP374] leu2Δ0 ura3Δ0::[RDB1967-pMP351]</i>	This study
RJD3258	MATa cdc34Δ expressing URA3 marked Cdc34 and HIS3 marked Cdc34-Myc13 (F72V)	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[RDB1966-pMP376] leu2Δ0 ura3Δ0::[RDB1967-pMP351]</i>	This study
RJD3259	MATa cdc34Δ expressing HIS3 marked Cdc34-Myc13	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[RDB1965-pMP374] leu2Δ0 ura3Δ0</i>	This study
RJD3260	MATa cdc34Δ expressing HIS3 marked Cdc34-Myc13 (F72V)	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[RDB1966-pMP376] leu2Δ0 ura3Δ0</i>	This study
RJD3261	MATa cdc34Δ rub1Δ expressing URA3 marked Cdc34 and HIS3 marked Cdc34-Myc13	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[RDB1965-pMP374] leu2Δ0 ura3Δ0::[RDB1967-pMP351] rub1Δ::LEU2</i>	This study
RJD3262	MATa cdc34Δ rub1Δ expressing URA3 marked Cdc34 and HIS3 marked Cdc34-Myc13 (F72V)	<i>MATa cdc34Δ::kan<sup>R</sup> his3Δ1::[RDB1966-pMP376] leu2Δ0 ura3Δ0::[RDB1967-pMP351] rub1Δ::LEU2</i>	This study

Table S2. Yeast Strains Used during This Study

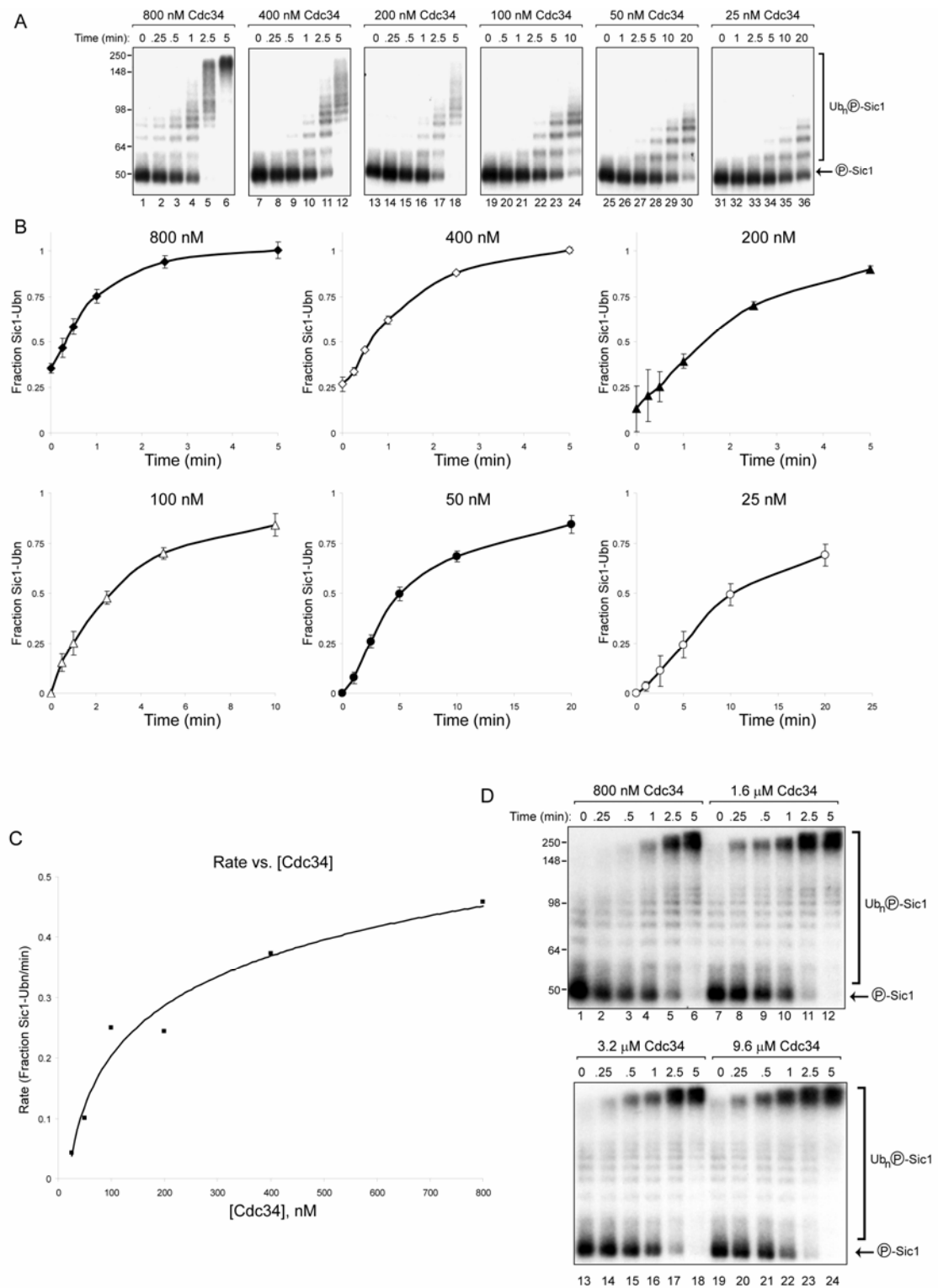


Figure S1. The Rate and Extent of Sic1 Ubiquitination by SCF-Cdc34 Depends on the Concentration of Cdc34

(A) The effect of Cdc34 concentration on the rate of Sic1 ubiquitination was measured in reactions containing the indicated amounts of Cdc34, 100 nM SCF, 100 nM E1, 77.5  $\mu$ M ubiquitin, and 0.8  $\mu$ M phosphorylated Sic1 in a total volume of 20  $\mu$ l. Reaction aliquots were removed at the indicated times and added to sample buffer prior to analysis by SDS-PAGE and autoradiography. The rate of Sic1 ubiquitination (B) was measured at each Cdc34 concentration for experiments as shown in (A). Error bars represent the standard deviation from the mean (n=3). (C) Initial rates were determined by measuring the slope of the curves in (B) for the first 3 time points measured and plotted against the corresponding Cdc34 concentration. Nonlinear regression analysis of the initial rate data by GraphPad Prism indicates that the overall rate of Sic1 conversion to conjugated species is half-maximal at 195 nM Cdc34. (D) To determine if increasing the concentration of Cdc34 greater than 800 nM increases the rate of Sic1 ubiquitination, further titration experiments as in (A) were performed. Whereas the extent of Sic1 ubiquitination increased, the overall rate of Sic1 conversion to ubiquitinated species did not significantly increase at concentrations greater than 800 nM.

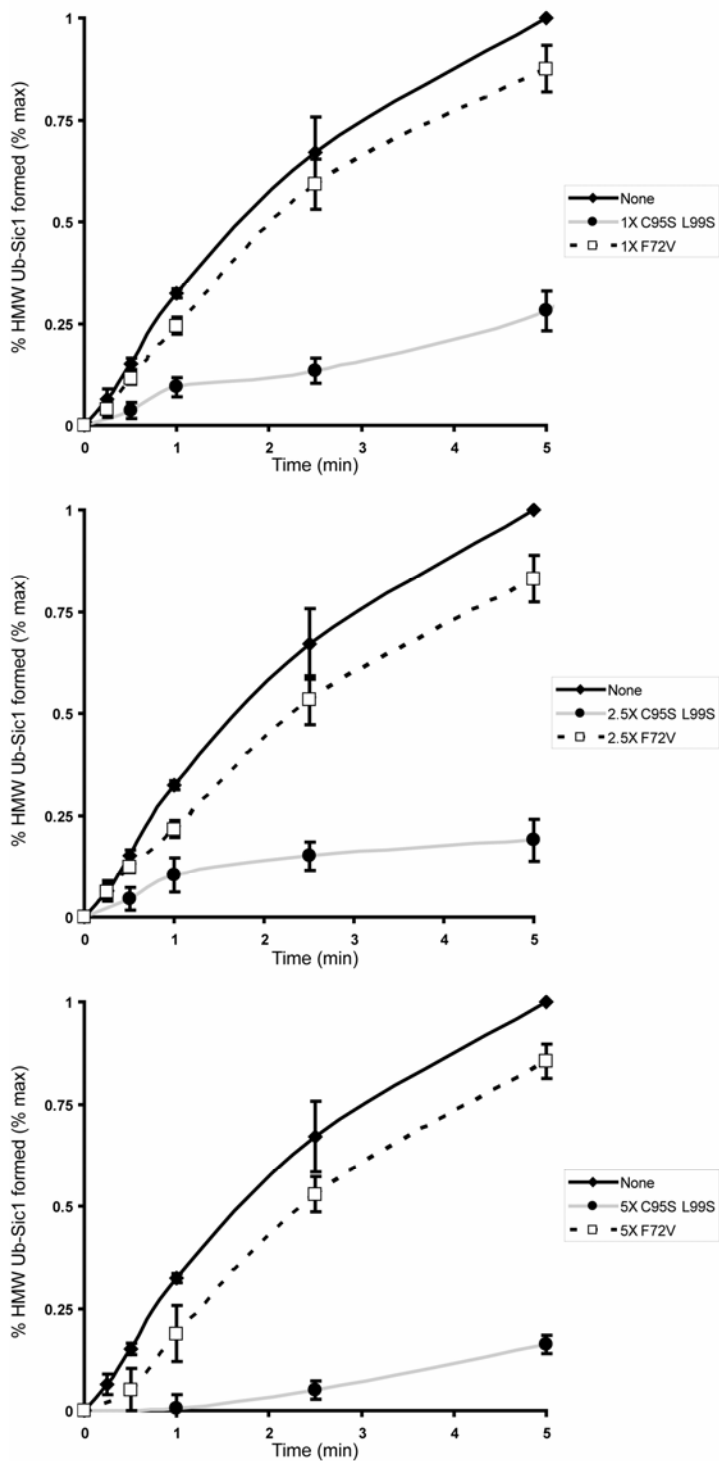


Figure S2. The Rate of Formation of High-Molecular-Weight Ubiquitinated Sic1 Is Not Affected by the Presence of Increasing Amounts of Cdc34 F72V

The rate of formation of high molecular weight ubiquitinated Sic1 was measured for experiments as shown in Figure 2A by measuring the ratio of Sic1 at greater than 98 kDa to the total amount of Sic1 as a function of time. Subsequent measurements were normalized to the maximum total of high molecular weight ubiquitinated Sic1 formed at 5 minutes with no competitor (see Figure 2A, lane 6). Each data point represents the mean (n=3) with error bars representing the standard deviation.

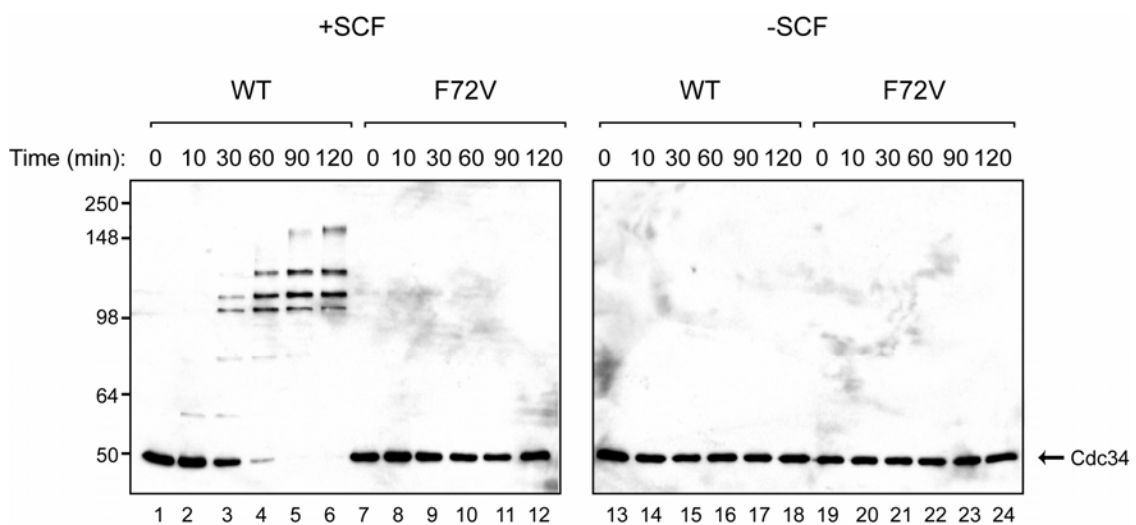


Figure S3. The “Auto Ubiquitination” of Cdc34 $\Delta$ C Depends on F72 and the Presence of SCF

To test the SCF-dependency of Cdc34 ubiquitination of either WT or F72V Cdc34 in the Cdc34 $\Delta$ C context, in vitro ubiquitination reactions were performed containing E1 (100 nM), Cdc34 $\Delta$ C (either WT or F72V, 500 nM), ubiquitin (77.5  $\mu$ M), and ATP (2 mM). Aliquots were removed at the indicated times post E2 addition and analyzed by SDS-PAGE and western blotting with anti-Cdc34 antisera.

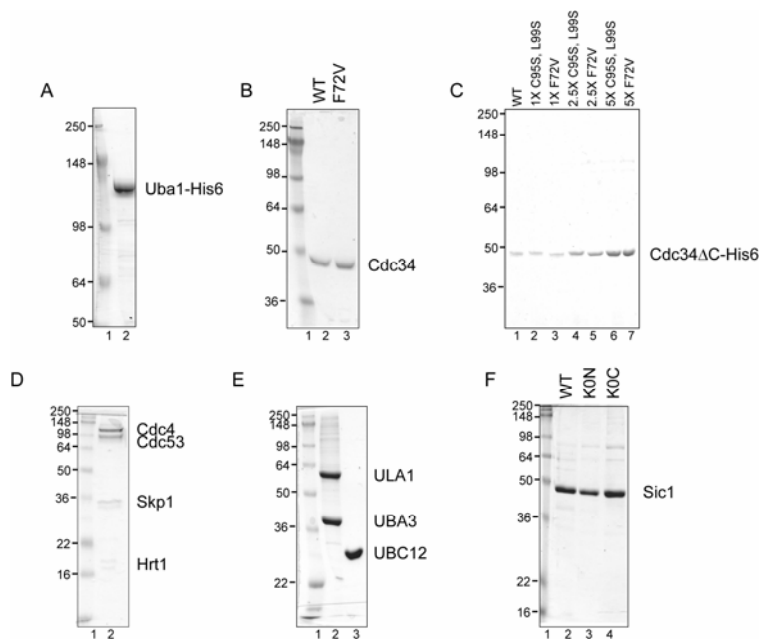


Figure S4. Purified Proteins Used in Biochemical Assays

Protein concentration was estimated by performing a BioRad protein concentration assay as per the manufacturer's instructions, using IgG to generate a standard curve. (A) Yeast E1 (2  $\mu$ g) was purified from a yeast strain (RJD979) expressing His6-tagged Uba1 under the control of a copper-inducible promoter. (B) Full-length, untagged Cdc34 (WT and F72V, 1  $\mu$ g each) were purified from *E. coli* by DEAE and monoQ chromatography. (C) Various Cdc34 $\Delta$ C derivatives used in Figure 3A were analyzed by Coomassie staining. The amount of protein loaded was: 100 ng for WT, 1X C95S, L99S, and 1X F72V, 250 ng for 2.5X C95S, L99S and 2.5X F72V, and 500 ng for 5X C95S, L99S and 5X F72V. (D) Recombinant SCF complex purified by anti-Py epitope tag affinity chromatography from baculovirus infected insect cells. Approximately 1  $\mu$ g was analyzed by Coomassie staining. (E) Recombinant neddylation components were purified by NiNTA chromatography. ULA1/UBA3 complexes were purified from baculovirus infected insect cells and UBC12 was purified from *E. coli*. Approximately 2  $\mu$ g of each was analyzed by Coomassie staining. (F) Sic1 (1  $\mu$ g of K0C, containing 6 lysine residues localized within the N-terminal region of Sic1) was purified from *E. coli* via its His6-tag by NiNTA chromatography. WT and K0N (containing the 14 C-terminal lysine residues intact) are also shown.