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Supporting Online Material
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Ubistatins Inhibit Proteasome-Dependent Degradation by Binding the Ubiquitin Chain

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To identify previously unknown small molecules that inhibit cell cycle machinery, we performed a chemical genetic screen in *Xenopus* extracts. One class of inhibitors, termed ubistatins, blocked cell cycle progression by inhibiting cyclin B proteolysis and inhibited degradation of ubiquitinated Sic1 by purified proteasomes. Ubistatins blocked the binding of ubiquitinated substrates to the proteasome by targeting the ubiquitin-ubiquitin interface of Lys⁴⁸-linked chains. The same interface is recognized by ubiquitin-chain receptors of the proteasome, indicating that ubistatins act by disrupting a critical protein-protein interaction in the ubiquitin-proteasome system.

Unbiased chemical genetic screens can identify small molecules that target unknown proteins or act through unexpected mechanisms (1). To identify previously unknown components or potential drug targets required for cell division, we screened for small mole-

cules that stabilize cyclin B in *Xenopus* cell cycle extracts. Cyclin B degradation regulates exit from mitosis and requires activation of an E3 ubiquitin ligase called the anaphase-promoting complex/cyclosome (APC/C) (2). Because APC/C activation re-

quires mitotic entry, we anticipated that this screen would identify compounds that stabilized cyclin B indirectly by blocking mitotic entry as well as compounds that directly inhibited the cyclin proteolysis machinery. To monitor APC/C activation, we fused the destruction-box domain of *Xenopus* cyclin B1 to luciferase (3) and found that the reporter protein was degraded in mitotic but not interphase extracts (fig. S1). Proteolysis was sensitive to inhibitors of cyclin-dependent kinases and the ubiquitin-proteasome system but not affected by inhibitors of DNA replication or spindle assembly, as expected in egg extracts lacking exogenous nuclei (4, 5) (fig. S2).

We developed a miniaturized assay system (6) and screened 109,113 compounds to identify 22 inhibitors (Table 1). To distinguish compounds that blocked mitotic entry from direct inhibitors of proteolysis, we arrested extracts in mitosis before addition of the compound and the reporter protein. Sixteen compounds lost inhibitory activity under these conditions (class I, fig. S3), whereas six compounds (class II, fig. S4) remained inhibitory. We next activated proteolysis directly in interphase extracts by adding the APC/C activator Cdh1 (Cdc20 homolog 1) (7). Again we found that only class II compounds re-

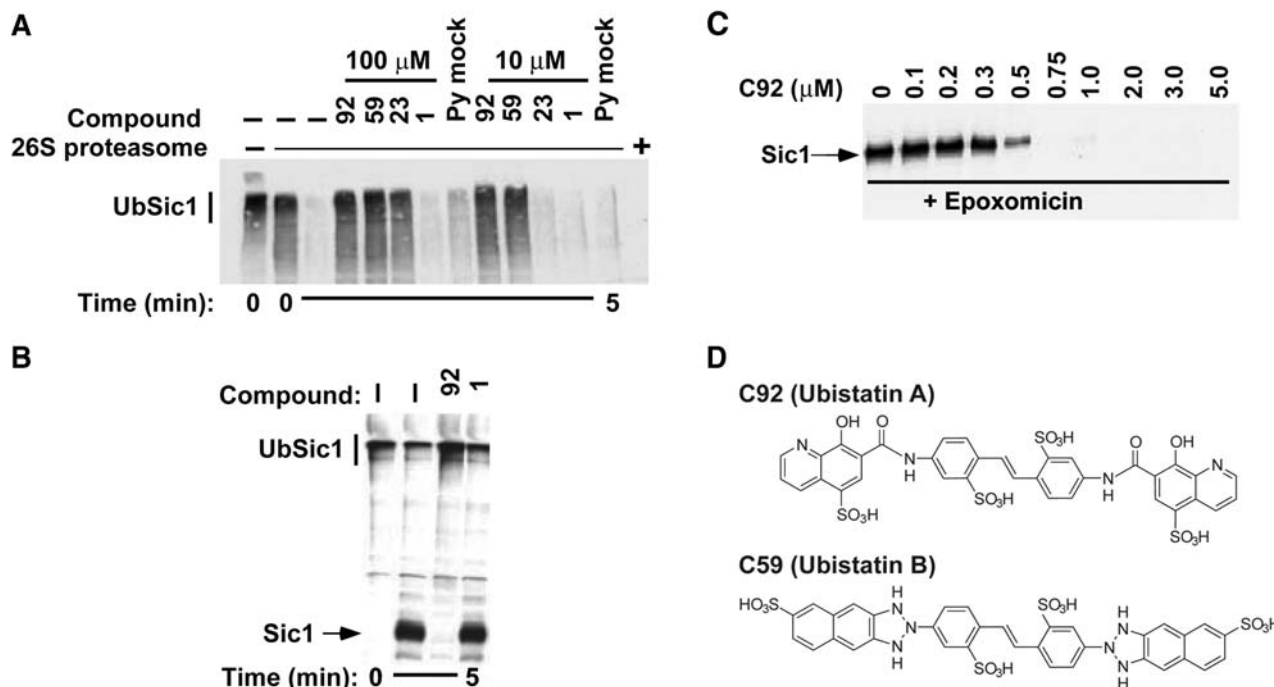


Fig. 1. Class IIB compounds inhibit degradation and deubiquitination of UbSic1 by purified 26S proteasomes. (A) Purified 26S proteasomes were preincubated in the presence or absence of test compounds. UbSic1 was then added and assayed for degradation by immunoblotting for Sic1 (3). Py mock refers to pyridine in which C23 was dissolved. (B) Purified 26S

proteasomes were preincubated with 100 μM epoxomicin in the presence or absence of 100 μM test compound. UbSic1 was then added and deubiquitination monitored by immunoblotting for Sic1 (3). (C) Titration of C92 in deubiquitination assay. (D) Structures of C92 and C59 (ubistatins A and B).

tained inhibitory activity. We concluded that class I compounds blocked entry into mitosis or APC/C activation, whereas class II compounds directly blocked components of the cyclin degradation machinery. We next examined whether the inhibitors could block turnover of a β -catenin reporter protein (8), a substrate of the SKP1/cullin/F-box protein (SCF $^{\beta}$ -TRCP, where β -TRCP is β -transduction repeat-containing protein) ubiquitin ligase (Table 1). Three class II compounds (class IIB) were inhibitory, suggesting these compounds inhibited a protein required for the degradation of both APC/C and SCF $^{\beta}$ -TRCP substrates. Class IIB compounds did not block cyclin B ubiquitination or 20S peptidase activity (9), indicating they did not inhibit E1 or act as conventional proteasome inhibitors.

To understand how class IIB compounds inhibited proteolysis, we turned to a reconstituted system using purified 26S proteasomes and ubiquitinated Sic1 (UbSic1) (10). Degradation of Sic1 requires its ubiquitination by the ligase SCF $^{\text{Cdc4}}$ (11, 12), after which UbSic1 is

docked to the 19S regulatory particle by a multi-Ub chain receptor (13). Proteolysis of UbSic1 requires removal of the multi-Ub chain, catalyzed by the metalloisopeptidase Rpn11 (14, 15). The deubiquitinated substrate

is concomitantly translocated into the 20S core particle, where it is degraded. Two class IIB molecules, C92 and C59 (Fig. 1D), strongly inhibited UbSic1 turnover in the reconstituted system (Fig. 1A). To address whether these

Table 1. Characterization of compounds in *Xenopus* extract assays. Results are reported as percent inhibition (percent stimulation). Compounds (200 μ M, except C10 and C92, tested at 100 μ M) and cyclin-luciferase (cyc-luc) were added to interphase extracts and then induced to enter mitosis by addition of nondegradable cyclin B, or extracts were pretreated with nondegradable cyclin B to allow entry into mitosis before addition of test compound and cyc-luc. Cdh1 was added to interphase extracts before addition of compound and cyc-luc. Interphase extracts were treated with recombinant axin to induce turnover of β -catenin-luciferase. Parentheses indicate those values where stimulation, rather than inhibition, was observed by addition of compound to the reaction.

Compound	Addition before mitotic entry	Addition after mitotic entry	Cdh1-activated interphase extract	β -catenin reporter protein
<i>Class IA</i>				
C77	100	4	(12)	0
C58	100	5	(8)	2
C82	100	0	0	0
C34	100	0	(8)	6
C62	84	0	(8)	0
C61	77	8	(8)	2
C13	75	0	(9)	0
C18	73	4	(7)	0
C25	66	3	(6)	0
C54	54	3	(6)	0
C67	53	3	(8)	3
C40	42	0	(6)	3
<i>Class IB</i>				
C39	100	9	(7)	67
C57	100	4	0	60
C51	100	0	0	30
C10	33	0	(4)	21
<i>Class IIA</i>				
C1	100	100	35	6
C2	80	50	100	0
C8	70	63	20	0
<i>Class IIB</i>				
C23	100	100	100	27
C59	97	100	100	70
C92	60	22	65	21

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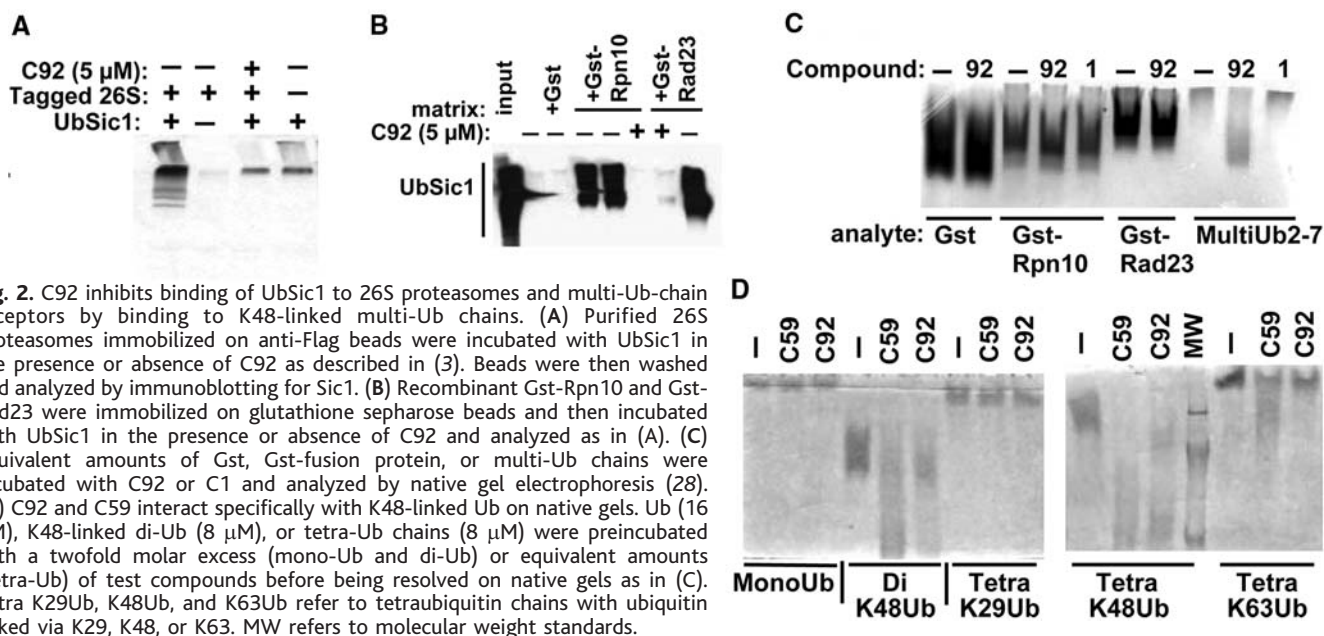


Fig. 2. C92 inhibits binding of UbSic1 to 26S proteasomes and multi-Ub-chain receptors by binding to K48-linked multi-Ub chains. (A) Purified 26S proteasomes immobilized on anti-Flag beads were incubated with UbSic1 in the presence or absence of C92 as described in (3). Beads were then washed and analyzed by immunoblotting for Sic1. (B) Recombinant Gst-Rpn10 and Gst-Rad23 were immobilized on glutathione sepharose beads and then incubated with UbSic1 in the presence or absence of C92 and analyzed as in (A). (C) Equivalent amounts of Gst, Gst-fusion protein, or multi-Ub chains were incubated with C92 or C1 and analyzed by native gel electrophoresis (28). (D) C92 and C59 interact specifically with K48-linked Ub on native gels. Ub (16 μ M), K48-linked di-Ub (8 μ M), or tetra-Ub chains (8 μ M) were preincubated with a twofold molar excess (mono-Ub and di-Ub) or equivalent amounts (tetra-Ub) of test compounds before being resolved on native gels as in (C). Tetra K29Ub, K48Ub, and K63Ub refer to tetraubiquitin chains with ubiquitin linked via K29, K48, or K63. MW refers to molecular weight standards.

compounds acted upstream or downstream of Rpn11 isopeptidase, we treated proteasomes with the 20S inhibitor epoxomicin, which results in Rpn11-dependent substrate deubiquitination (14, 16) and accumulation of deubiquitinated Sic1 within the 20S chamber (13). This reaction was completely blocked by C92 (Fig. 1B), with a median inhibitory concentration (IC_{50}) of about 400 nM (Fig. 1C). C59, which is structurally related to C92, also inhibited deubiquitination of UbSic1 (IC_{50} = 1 μ M), whereas C23 inhibited marginally (fig. S5). Thus C92 and C59 potently blocked proteolysis at or upstream of the essential isopeptidase-dependent step.

Selective recognition of the multi-Ub chain by the 26S proteasome is the first step in UbSic1 degradation (13). C92 strongly inhibited binding of UbSic1 to purified 26S proteasomes (Fig. 2A), suggesting that it inhibited UbSic1 turnover by blocking the first step in the degradation process. The multi-Ub chain receptors Rad23 and Rpn10 serve a redundant role in targeting UbSic1 to the proteasome and

sustaining its degradation (13). In the absence of the Ub-binding activities of Rpn10 and Rad23, UbSic1 is not recruited, deubiquitinated, or degraded by purified 26S proteasomes. We thus tested whether C92 could interfere with binding of UbSic1 to recombinant Rpn10 and Rad23. C92 abolished binding of UbSic1 to both proteins (Fig. 2B), even though these receptors use distinct domains [the Ub-interaction motif (UIM) and the Ub-associated (UBA) domain, respectively] to bind ubiquitin chains (17). C59 also abrogated binding of UbSic1 to Rpn10, whereas other compounds were without effect (fig. S5).

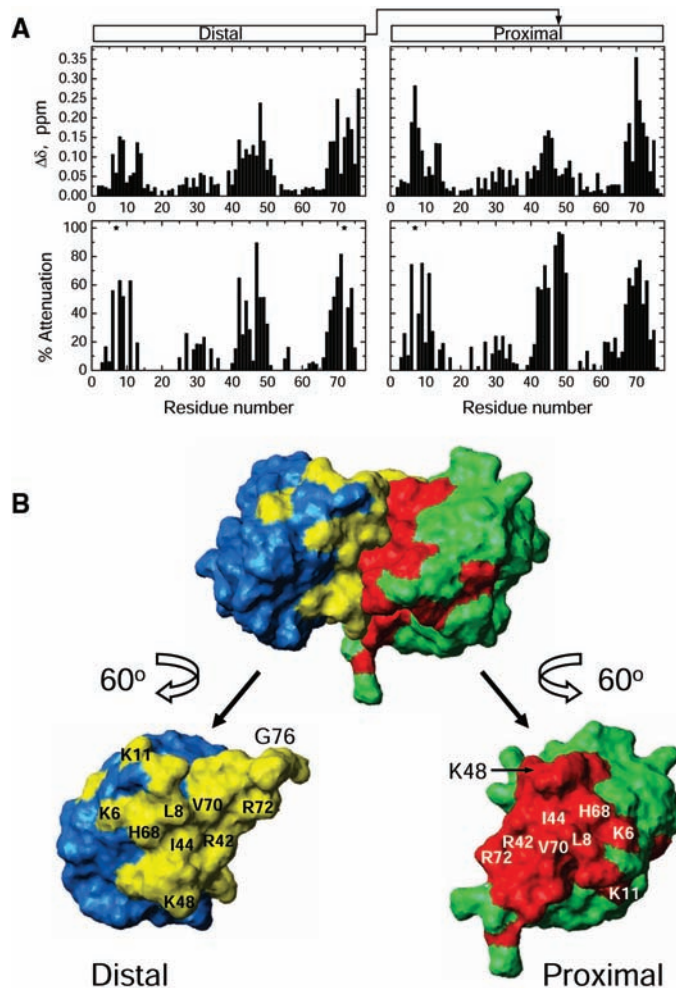
To distinguish whether C92 inhibited proteolysis by binding to proteasome receptor proteins or to the Ub chain on Sic1, we exploited the negative charge of C92 to determine whether compound binding induced a mobility shift of the target proteins upon fractionation on a native polyacrylamide gel. C92 was preincubated with recombinant Rpn10, Rad23, or a mixture of Ub chains containing two to seven Ub molecules. The

mobility of the multi-Ub chains, but not Gst-Rpn10 or Gst-Rad23, was altered by incubation with C92, suggesting that C92 bound Ub chains (Fig. 2C). Ubiquitin molecules can be linked to each other in vivo through different internal lysines, including K29, K48, and K63 (18). The K48-linked chain is the principal targeting signal in proteolysis, whereas K63-linked chains are implicated in enzyme regulation (19). Whereas C92 and C59 efficiently shifted the native gel mobility of K48-linked ubiquitin chains, they had little or no effect on K29- or K63-linked chains (Fig. 2D). Because C92 and C59 bind to ubiquitin chains and block interactions with proteasome-associated receptors without affecting 26S assembly or peptidase activity (fig. S6), we refer to these compounds as ubistatin A and B, respectively.

We next tested the ability of ubistatins to block proteolysis of ornithine decarboxylase (ODC), whose degradation does not require ubiquitin (20). Whereas a 30-fold molar excess of ubistatin A over the substrate strongly inhibited UbSic1 degradation by purified yeast proteasomes (Fig. 1A), a 100-fold molar excess of ubistatin A over the substrate had no effect on degradation of radiolabeled ODC by purified rat proteasomes (fig. S7). Ubistatin B marginally inhibited ODC turnover at this concentration (12%). In contrast, a 20-fold molar excess of cold ODC inhibited degradation of labeled ODC by 43% under the same conditions. These data indicate that ubistatins at low concentrations preferentially inhibit the degradation of ubiquitin-dependent substrates. Inhibition of ODC turnover by high concentrations of ubistatins, especially ubistatin B (fig. S7), may reflect either nonspecific activity or specific inhibition of a targeting mechanism shared by ubiquitin-dependent and ubiquitin-independent substrates of the proteasome (20).

On the basis of the selectivity of ubistatin A for binding K48-linked chains and inhibiting the ubiquitin-dependent turnover of Sic1 but not the ubiquitin-independent turnover of ODC, we tested the effect of ubistatin A on protein degradation within intact mammalian cells. Because the negative charge on ubistatin A precluded efficient membrane permeation, we introduced the compound into cells by microinjection and monitored degradation of an androgen receptor–green fluorescent protein (AR-GFP) fusion protein by fluorescence microscopy. Microinjection of a synthetic compound (protac, proteolysis-targeting chimeric molecule), which recruits AR-GFP to SCF $^{\beta}$ -TRCP, induces rapid proteasome-dependent turnover of AR-GFP (21). Microinjection of 100 nM ubistatin A into mammalian cells inhibited the Protac-induced degradation of AR-GFP as efficiently as 100 nM epoxomicin (fig. S8), demonstrating that ubistatin A is an effective

Fig. 3. Ubistatin A binding to K48-linked di-Ub induces site-specific perturbations in NMR spectra for both Ub domains. (A) Backbone NH chemical shift perturbation, $\Delta\delta$, and percent signal attenuation caused by ubistatin A binding as a function of residue number for the distal (left) and the proximal (right) domains. Ub units are called "distal" and "proximal" to reflect their location in the chain relative to the free C terminus. The diagram (top) depicts the location of the G76-K48 isopeptide bond between the two Ub domains. Asterisks indicate residues that showed significant signal attenuation that could not be accurately quantified because of signal overlap. (B) Mapping of the perturbed sites on the surface of di-Ub. The distal and proximal domains are shown in surface representation and colored blue and green, respectively; the perturbed sites on these domains are colored yellow and red and correspond to residues with $\Delta\delta > 0.075$ parts per million and/or signal attenuation greater than 50%. Numbers indicate surface location of the hydrophobic patch and some basic residues along with G76 (distal) and the side chain of K48 (proximal).



inhibitor of ubiquitin-dependent degradation in multiple experimental settings.

The specificity of ubistatin A for K48-linked ubiquitin chains suggested that it might bind at the Ub-Ub interface, which is well defined in K48-linked chains but is not present in K63-linked di-ubiquitin (Ub₂) (22). We performed nuclear magnetic resonance (NMR) titration studies of K48-linked Ub₂ by using a segmental labeling strategy (23). Well-defined site-specific perturbations were observed in the resonances of the backbone amides of both Ub units in Ub₂ (Fig. 3), indicating that the hydrophobic patch residues L8, I44, V70 (24), and neighboring sites (including basic residues K6, K11, R42, H68, and R72) experienced alterations in their molecular environment upon binding of ubistatin A. The same hydrophobic patch is involved in the formation of the interdomain interface in Ub₂ (23, 25) and mediates the binding of ubiquitin to multiple proteins containing CUE (coupling of ubiquitin conjugation to ER degradation), UBA, and UIM domains (17). At the high concentrations of compound used in the NMR titration experiments, ubistatin A induced a similar pattern of chemical shift perturbations in monomeric ubiquitin, suggesting that the effect of ubistatin A on Ub₂ arises from its direct binding to the hydrophobic patch and the basic residues around it. The same sites are perturbed when ubistatin A binds tetra-Ub chains (26).

Although there is intense interest in developing drugs for defined molecular targets, it is often difficult to know a priori which proteins can be most effectively targeted with small molecules. Our study demonstrates that chemical genetic screens in complex biochemical systems such as *Xenopus* extracts can identify small-molecule inhibitors that act through unexpected mechanisms. Although target identification remains challenging, our work highlights the value of reconstituted biochemical systems to illuminate the mechanism of action of inhibitors discovered in unbiased screens. The recent approval of the 20S proteasome inhibitor Velcade (Millenium Pharmaceuticals, Cambridge, MA) for treatment of relapsed multiple myeloma (27) has suggested that the ubiquitin-proteasome system is an attractive target for cancer drug development. The identification of ubistatins indicates that the ubiquitin chain itself provides another potential opportunity for pharmacological intervention in this important pathway.

References and Notes

1. T. U. Mayer, *Trends Cell Biol.* **13**, 270 (2003).
2. J. M. Peters, *Mol. Cell* **9**, 931 (2002).
3. Materials and methods are available as supporting material on Science Online.
4. M. Dasso, J. W. Newport, *Cell* **61**, 811 (1990).
5. J. Minshull, H. Sun, N. K. Tonks, A. W. Murray, *Cell* **79**, 475 (1994).
6. L. A. Walling, N. R. Peters, E. J. Horn, R. W. King, *J. Cell. Biochem.* **537**, 7 (2001).

7. C. M. Pfeleger, M. W. Kirschner, *Genes Dev.* **14**, 655 (2000).
8. A. Salic, E. Lee, L. Mayer, M. W. Kirschner, *Mol. Cell* **5**, 523 (2000).
9. N. Peters, R. W. King, unpublished data.
10. R. Verma, H. McDonald, J. R. Yates 3rd, R. J. Deshaies, *Mol. Cell* **8**, 439 (2001).
11. D. Skowyra *et al.*, *Science* **284**, 662 (1999).
12. J. H. Seol *et al.*, *Genes Dev.* **13**, 1614 (1999).
13. R. Verma, R. Oania, J. Graumann, R. J. Deshaies, *Cell* **118**, 99 (2004).
14. R. Verma *et al.*, *Science* **298**, 611 (2002); published online 15 August 2002; 10.1126/science.1075898.
15. T. Yao, R. E. Cohen, *Nature* **419**, 403 (2002).
16. L. Meng *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 10403 (1999).
17. R. Hartmann-Petersen, M. Seeger, C. Gordon, *Trends Biochem. Sci.* **28**, 26 (2003).
18. J. Peng *et al.*, *Nat. Biotechnol.* **21**, 921 (2003).
19. C. M. Pickart, *Cell* **116**, 181 (2004).
20. M. Zhang, C. M. Pickart, P. Coffino, *EMBO J.* **22**, 1488 (2003).
21. K. M. Sakamoto *et al.*, *Mol. Cell. Proteomics* **2**, 1350 (2003).
22. R. Varadan *et al.*, *J. Biol. Chem.* **279**, 7055 (2004).
23. R. Varadan, O. Walker, C. Pickart, D. Fushman, *J. Mol. Biol.* **324**, 637 (2002).
24. Single-letter abbreviations for the amino acid residues are as follows: H, His; I, Ile; K, Lys; L, Leu; R, Arg; and V, Val.
25. W. J. Cook, L. C. Jeffrey, M. Carson, Z. Chen, C. M. Pickart, *J. Biol. Chem.* **267**, 16467 (1992).
26. D. Fushman, unpublished data.
27. J. Adams, *Nat. Rev. Cancer* **4**, 349 (2004).
28. R. Verma *et al.*, *Mol. Biol. Cell* **11**, 3425 (2000).

29. We thank the Developmental Therapeutics Program, National Cancer Institute, for providing access to compound collections, C. Pickart for tetraubiquitin chains of defined linkages, A. Salic for recombinant Axin and β-catenin-luciferase, and C. Sawyers for AR-GFP. G.T. is supported by NIH National Research Service Award GM068276. K.M.S. is supported by a UCLA Specialized Programs of Research Excellence in Prostate Cancer Development Research Seed Grant (P50 CA92131), U.S. Department of Defense (DAMD17-03-1-0220), and NIH (R21CA108545). P.C. is supported by NIH R01 GM-45335. D.F. is supported by NIH grant GM65334. R.J.D. is supported by HHMI and the Susan G. Komen Breast Cancer Foundation (DISS0201703). R.W.K. is supported by the NIH (CA78048 and GM66492), the McKenzie Family Foundation, and the Harvard-Armenise Foundation and is a Damon Runyon Scholar. Screening facilities at the Harvard Institute of Chemistry and Cell Biology were supported by grants from the Keck Foundation, Merck and Company, and Merck KGaA. R.J.D. is a founder and paid consultant of Proteolix, which is negotiating with Caltech and Harvard to license a patent related to ubistatin. Molecular interaction data have been deposited in the Biomolecular Interaction Network Database with accession codes 151787 to 151791.

Supporting Online Material

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Materials and Methods

Figs. S1 to S8

Table S1

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Regulation of Cytokine Receptors by Golgi N-Glycan Processing and Endocytosis

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The Golgi enzyme β1,6 N-acetylglucosaminyltransferase V (Mgat5) is up-regulated in carcinomas and promotes the substitution of N-glycan with poly N-acetyllactosamine, the preferred ligand for galectin-3 (Gal-3). Here, we report that expression of Mgat5 sensitized mouse cells to multiple cytokines. Gal-3 cross-linked Mgat5-modified N-glycans on epidermal growth factor and transforming growth factor-β receptors at the cell surface and delayed their removal by constitutive endocytosis. Mgat5 expression in mammary carcinoma was rate limiting for cytokine signaling and consequently for epithelial-mesenchymal transition, cell motility, and tumor metastasis. Mgat5 also promoted cytokine-mediated leukocyte signaling, phagocytosis, and extravasation *in vivo*. Thus, conditional regulation of N-glycan processing drives synchronous modification of cytokine receptors, which balances their surface retention against loss via endocytosis.

Co-translational modification of proteins in the endoplasmic reticulum by N-glycosylation facilitates their folding and is essential in single-cell eukaryotes. Metazoans have additional Golgi enzymes that trim and remodel the N-glycans, producing complex-type N-glycans on glycoproteins destined for the cell surface. Mammalian development requires complex-type N-glycans containing N-acetyllactosamine antennae, because their complete absence in Mgat1-deficient em-

bryos is lethal (1, 2). Deficiencies in N-acetylglucosaminyltransferase II and V (Mgat2 and Mgat5) acting downstream of Mgat1 reduce the content of N-acetyllactosamine, and mutations in these loci result in viable mice with a number of tissue defects (3, 4). N-glycan processing generates ligands for various mammalian lectins, but the consequences of these interactions are poorly understood. The galectin family of N-acetyllactosamine-binding lectins has been implicated in cell