Substrate Recognition by the Cdhl Destruction Box Receptor Is a General Requirement for APC/C\textsuperscript{Cdhl} -mediated Proteolysis*

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The anaphase-promoting complex, or cyclosome (APC/C), is a ubiquitin ligase that selectively targets proteins for degradation in mitosis and the G\textsubscript{1} phase and is an important component of the eukaryotic cell cycle control system. How the APC/C specifically recognizes its substrates is not fully understood. Although well characterized degron motifs such as the destruction box (D-box) and KEN-box are commonly found in APC/C substrates, many substrates apparently lack these motifs. A variety of alternative APC/C degrons have been reported, suggesting that multiple modes of substrate recognition are possible or that our definitions of degron structure are incomplete. We used an in vivo yeast assay to compare the G\textsubscript{1} degradation rate of 15 known substrates of the APC/C co-activator Cdhl under normal conditions and conditions that impair binding of D-box, KEN-box, and the recently identified ABBA motif degrons to Cdhl. The D-box receptor was required for efficient proteolysis of all Cdhl substrates, despite the absence of canonical D-boxes in many. In contrast, the KEN-box receptor was only required for normal proteolysis of a subset of substrates and the ABBA motif receptor for a single substrate in our system. Our results suggest that binding to the D-box receptor may be a shared requirement for recognition and processing of all Cdhl substrates.

The anaphase-promoting complex, or cyclosome (APC/C), polubiquitylates numerous cell cycle regulatory proteins to target them for proteasomal degradation. In doing so, the APC/C triggers anaphase chromosome segregation, the inactivation of cyclin-dependent kinases and other mitotic kinases, cytokinesis, and the separation of stable G\textsubscript{1} state (1–3). Proper coordination of late mitotic events by the APC/C is critical for faithful genome transmission during cell division (4–6). How the APC/C selectively targets the appropriate substrates at the correct time to orchestrate cell cycle events is an important question and an active area of investigation (3, 7–9).

The APC/C co-activators Cdc20 and Cdhl contribute to substrate recognition via docking sites on their conserved WD40 repeat domains (7, 9). Two substrate degron motifs that bind distinct sites shared by both co-activators have been characterized in detail. The destruction box (D-box), originally identified in sea urchin cyclin B and subsequently found in many other APC/C substrates, has the consensus sequence RXXLXX(I/V/L)XN but is often found with alternative amino acids at the I/V/L and N positions (10–12). The KEN-box was first identified as an APC/C degron in human Cdc20 (13) and has since been found in many other APC/C substrates (9). The location and structure of the D-box and KEN-box binding sites on Cdhl and Cdc20 were recently revealed through x-ray diffraction and high resolution cryoelectron microscopy studies, providing detailed views of how common degrons are recognized by APC/C co-activators (12, 14–16).

Despite these significant recent advances, our overall understanding of APC/C substrate recognition determinants is still incomplete. Although mutation of D- and KEN-box degrons stabilizes substrates in vivo, these motifs are often not sufficient for degradation, suggesting that other sequence elements are important as well (9, 11). Moreover, the RXXL and KEN consensus sequences are found frequently in proteins that are not APC/C substrates and thus must exist in an appropriate structural context to function as degrons. Some APC/C substrates contain both D- and KEN-boxes, whereas others appear to contain only one or the other, and there are now many examples of substrates that lack functional consensus sequences for both (7, 9). The source of binding specificity and affinity in substrates lacking one or both canonical degron motifs remains unknown in most cases. Finally, although Cdc20 and Cdhl have very similar WD40 domain structures with nearly identical D- and KEN-box binding sites (12, 14), they recognize distinct sets of substrates, suggesting again that additional factors influence specificity (17).

Many alternative APC/C degrons have been identified, often in substrates lacking consensus D- or KEN-boxes. These include GXEN in Xenopus Xkid (18), the A box or DAD motif (RXLPSN) in Xenopus Aurora A (19, 20), the O-box (PASPLTEKNAK, essential residues underlined) in Drosophila ORC1 (21), LXXXXXXN in Saccharomyces cerevisiae Spo13 (22), LLK in human Claspin (23), the CRY-box in mouse Cdc20 (24), NKSEN in budding yeast Sgo1 (25), and the ABBA motif, FX(I/V/L)(F/Y/H)X(D/E), in human cyclin A, Bub1, and budding yeast Clb5 (26, 27). Only the ABBA motif has a known binding site on the co-activator WD40 domains (12, 28). Some alternative degrons resemble D- and KEN-boxes, including RQLF in budding yeast Ase1 (29) and GXEN and NKSEN in...
Substrate Recognition by APC/C\textsuperscript{Cdh1}

**Results**

Acm1 Inhibits Proteolysis of Diverse Cdh1 Substrates—The Acm1 protein is a pseudosubstrate inhibitor of APC/C\textsuperscript{Cdh1} that acts by competitively blocking substrate binding to Cdh1 using canonical D- and KEN-box degron motifs and the recently identified ABBA motif (originally called the A-motif in Acm1) (37–39). Acm1 has only been shown to inhibit the ubiquitylation and degradation of a small number of classical APC/C\textsuperscript{Cdh1} substrates that contain well defined KEN- and/or D-box degrons (12, 37–40). It remains unclear whether Acm1 can also inhibit degradation of substrates with non-canonical degrons that lack consensus KEN- and/or D-box motifs. To answer this question, we established an *in vivo* assay in which substrate stability can be compared in the presence and absence of Acm1 (Fig. 1A). We used yeast cells harboring plasmids that conditionally express protein A-tagged substrates from the galactose-inducible GAL1 promoter and a stabilized Acm1 mutant (Acm1\textDelta52), which retains its central Cdh1 inhibitory domain (41), from the methionine-repressible MET25 promoter. The stable Acm1\textDelta52 mutant was necessary because Acm1 itself is highly unstable in the G1 phase because of an APC/C independent proteolytic mechanism (41). The cells were first arrested in the G1 phase, when APC/C\textsuperscript{Cdh1} is active, using α-factor pheromone. Substrate expression was then induced briefly with galactose either in the absence or presence of methionine to control Acm1\textDelta52 expression. Finally, substrate expression was terminated with glucose and cycloheximide, and stability was monitored over time by anti-protein A immunoblotting.

We tested a panel of 15 known Cdh1 substrates (Table 1), including proteins containing functional KEN- and D-box consensus motifs, either a KEN- or D-box but not both, a non-canonical degron motif without consensus KEN- or D-boxes, or otherwise undefined degrons. The presence of Acm1 strongly stabilized all Cdh1 substrates, regardless of degron type (Fig. 1, B and C, and Table 1).

This result could be explained either by Acm1 having a general effect on APC/C catalytic function independent of competing for substrate binding or by all substrates having one or more common or overlapping docking sites required for ubiquitylation by APC/C. Currently, there is no evidence that Acm1 directly inhibits the catalytic activity of APC/C toward substrates. Instead, current models for Acm1 action suggest that it acts as a competitive inhibitor of substrate binding (38, 40, 42, 43). However, we cannot rule out general inhibition of the catalytic function of APC/C by Acm1 in addition to its effect on substrate binding.

The ABBA Motif in Acm1 Is a Functional APC/C\textsuperscript{Cdh1} Degron—To independently test whether diverse Cdh1 substrates share a common docking site, we engineered yeast strains expressing cdh1 mutant alleles with altered degron binding sites to compare the stability of the same substrate collection with a wild-type CDH1 strain. Docking sites for the KEN-box, D-box, and ABBA motif have been identified through high resolution structural studies (12, 14–16, 28, 44), although the ABBA motif has not been demonstrated to be a functional *in vivo* degron for APC/C\textsuperscript{Cdh1} as it is for APC/C\textsuperscript{Cdc20} (26, 27). It was first identified as an element in Acm1 required for high affinity binding and inhibition of budding yeast APC/C\textsuperscript{Cdh1} (38, 40). Replacement of amino acids in all three of these Cdh1 docking sites disrupts binding of their respective ligand motifs *in vitro* (12, 14, 45). For strain validation we monitored endogenous Clb2 levels in cycling and G1- arrested cells. Clb2 remained stable in the G1 phase in the *cdh1\textDeltaabr* and *cdh1\textDeltaabr* strains containing mutations in the D-box receptor and KEN-box receptor, respectively, but was degraded in the *cdh1\textDeltaamer* strain containing mutations in the ABBA motif receptor (Fig. 2A), consistent with the presence of functional D- and KEN-boxes in Clb2 that direct its APC/C-mediated degradation (46, 47). Stabilization of endogenous Clb2 was not due to differences in expression level of the mutant proteins (Fig. 2B) or efficiency of G1 arrest (Fig. 2C). The receptor mutations also did not disrupt overall Cdh1 structure because the Cdh1\textDeltaabr and Cdh1\textDeltaabr proteins still interacted by co-immunopurification (co-IP) with Acm1 via its other interaction motifs (Fig. 2D). All three receptor mutants also interacted with the core APC/C similar to wild-type Cdh1 (Fig. 2E).
Substrate Recognition by APC/C^{CDh1}

We tested whether the Acm1 ABBA motif is a functional APC/C^{CDh1} degron using the same assay from Fig. 1, except with Acm1Δ52 expressed from the GAL1 promoter as the substrate. Paradoxically, Acm1 can be converted into an APC/C^{CDh1} substrate in vitro by mutation of its central KEN- and D-box motifs (37, 38, 40). Consistent with these in vitro results, mutation of the D- and KEN-box motifs in Acm1Δ52 (Acm1^{db/kb}) destabilized the protein in vivo, dependent on Cdh1 (Fig. 2F). Additional mutation of ABBA motif residues to create an Acm1^{db/kb/am} triple mutant stabilized the protein (Fig. 2G). In the cdh1^{amn} strain Acm1Δ52^{db/kb} was strongly stabilized compared with a wild-type CDH1 strain and was more stable than in

FIGURE 1. Acm1 blocks in vivo proteolysis of diverse Cdh1 substrates. A, flow chart for the in vivo substrate stability assay. The methionine-repressible Acm1Δ52 expression is only relevant to Fig. 1 and is omitted from the assays used in subsequent figures. Control cells lacked the Acm1Δ52 expression plasmid to establish basal instability of substrates. CHX, cycloheximide. See “Experimental Procedures” for more details. B and C, stability of the representative Cdh1 substrates that either contain (B) or lack (C) canonical D-box and/or KEN-box degrons, expressed with C-terminal protein A epitope tags, was monitored over time by anti-protein A immunoblot in the absence or presence of the Acm1Δ52-protein A fusion protein (labeled as Acm1 in all panels). Time 0 is the point where protein expression is terminated. Both substrate and Acm1 were detected with anti-protein A antibody. G6PD is a load control in all panels. When present, an asterisk indicates that two different exposures of the same blot are compared, separated by a white bar, so that the time 0 signals are of comparable intensity. This sometimes provides a better visualization of differences in substrate half-lives between the two conditions because stabilization can lead to increased steady-state protein levels. See Table 1 for results from complete substrate set. All experiments were performed three times with equivalent results.
that variations on this motif can also functionally engage the KEN-box receptor. This suggests that the ABBA motif in Acm1 is a functional D-box but does not match the RXL consensus D-box motif.

When testing the relative contributions of the three degron docking sites, we found that the stability of most was strongly dependent on the ABBA motif receptor site. However, in some cases like Spo12, KEN sequences completely independent of the KEN-box receptor in the cdh1Δ52 strain. Nonetheless, prolylolysis of a subset of substrates was strongly impaired in cdh1Δ52 cells. The most for most substrates exhibiting dependence on the KEN-box receptor contained consensus KEN-box motifs. In some cases, like Spo12, KEN sequences have not been functionally linked to APC/C-mediated protein degradation. The Spo13 and Spo11 proteins were interesting in that prolylolysis was strongly dependent on the KEN-box receptor, yet neither contains a consensus KEN-box motif. This suggests that variations on this motif can also functionally engage the KEN-box docking site on Cdh1, consistent with experiments using the GXEN motif in XKid (12). The Kip1 protein also lacks a KEN-box in the C-terminal region required for APC/C-mediated proteolysis (32) but requires the KEN-box receptor for efficient protein degradation in our assay.

We tested a subset of our Cdh1 substrate panel in the cdh1Δ52 strain, including those that showed no dependence on the KEN-box receptor, and found that the stability of most was unaffected by disruption of the ABBA motif binding site. In the cdh1Δ52 strain, several substrates exhibited a very slight increase in stability that could reflect a minor overall effect of ABBA motif receptor mutations on Cdh1 function or a weak contribution to substrate binding but did not meet our criteria for stabilization. We found a single Cdh1 substrate, Cik1, whose stability was strongly dependent on the ABBA motif receptor site (Fig. 5B), even though the Cik1 N terminus is necessary and sufficient for APC/C-mediated proteolysis (31) lacks any sequence resembling the ABBA motif consensus. Thus, in addition to the known function in promoting binding of the inhibitor Acm1 to Cdh1, the ABBA motif (or variants of it) appears to be capable of promoting substrate recognition by budding yeast APC/C/CDH1.

Novel APC/C Substrates Can Be Identified Using in Vivo Stability Assays and Cdh1 Inhibition by Acm1—The universal stabilizing effect of Acm1Δ52 on Cdh1 substrates in our assay in Fig. 1 suggested that it could be used as a tool for identifying novel substrates. To explore this idea, we tested two proteins, Ipl1 and Swe1, whose abundances during the cell cycle were previously demonstrated to be dependent on APC/C function (28), but, to our knowledge, have not been validated as direct APC/C substrates. Ipl1 is the budding yeast ortholog of Aurora B kinase, and Swe1 is the ortholog of Wee1 kinase. The stability of both proteins in G1-arrested cells increased when Acm1Δ52 was co-expressed (Fig. 6A). To verify that APC/C can target Ipl1

**TABLE 1**

Summary of APC/C/CDH1 substrates and results

| Substrate* | Functional degrons | Stability assay results |
|------------|--------------------|------------------------|
|            | D-box | KEN | ABBA | Other | Acm1 | D-box receptor | KEN receptor | ABBA receptor |
| Ase1 (29)  | ×     | ×   | ×    | RQLF  | ×    | ×             | ×             | NT*           |
| Cdc5 (21)  | ×     | ×   | ×    | Undefined | × | × | × | NT |
| Cdc20 (34, 59, 60) | × | × | × | Undefined | × | × | × | NT |
| Cik1 (31)  | ×     | ×   | ×    | Undefined | × | × | × | NT |
| Cin8 (30)  | ×     | ×   | ×    | Undefined | × | × | × | NT |
| NRM1 (52)  | ×     | ×   | ×    | Undefined | × | × | × | NT |
| Pds1 (12, 65, 66) | × | × | × | Undefined | × | × | × | NT |
| Spo11 (25) | ×     | ×   | ×    | Undefined | × | × | × | NT |

* Sources providing evidence for functional degrons or degron-containing regions are included after each substrate name. Underlining indicates substrates that lack a consensus D-box motif required for their APC/C-mediated proteolysis.

* Only degrons with experimental evidence demonstrating their requirement for substrate degradation are noted with ×.

* Stabilization (denoted with ×) is defined as a minimum of 30-min increase in the time required to reach 50% of the initial protein level compared with control. The Acm1 column reports results of assays measuring inhibition of protein degradation by the Acm1Δ52 protein. The last three columns report results of assays measuring dependence of substrate degradation on the D-box, KEN-box, and ABBA motif receptor sites of the Cdh1 WD40 domain.

* RQLF sequence in Ase1 was reported as a D-box but does not match the RXL consensus D-box motif.

* NT, not tested.

* The N-terminal 80 amino acids of Cik1 are necessary and sufficient for degradation but lack consensus degron motifs.

* The N-terminal 42 amino acids of Iqg1 are required for degradation but lack consensus degron motifs.

* The C-terminal 101 amino acids of Kip1 are necessary and sufficient for degradation but lack consensus degron motifs.

* Spo12 contains consensus D-box and KEN-box motifs, but motif binding of both did not prevent degradation.

The cdh1Δ52 and cdh1Δ48 strains (Fig. 2F). Taken together, these results demonstrate that the ABBA motif in Acm1 is a functional APC/C/CDH1 degron. With this information in hand, we proceeded to test the relative contributions of the three degron docking sites to Cdh1 substrate degradation.

The D-box Receptor on Cdh1 Is Required for Efficient Proteolysis of Most, if Not All, Cdh1 Substrates—Strikingly, mutation of the D-box receptor site on Cdh1 by alteration of just 4 conserved amino acids (40, 45) strongly stabilized all 15 Cdh1 substrates in our in vivo stability assay (Fig. 3 and Table 1), although not always to the same degree as Acm1Δ52 overexpression. This result was surprising in that roughly half of the substrates tested lack recognizable, consensus D-box motifs. The variability in the stabilizing effect across substrates could reflect different contributions of other elements to Cdh1 binding or differences in the nature of interactions with the D-box receptor region of Cdh1.

KEN-Box and ABBA Motif Receptors Are Required for Normal Proteolysis of Distinct Subsets of Cdh1 Substrates—We next explored the requirement for functional KEN-box and ABBA motif receptor sites on Cdh1 for substrate proteolysis. Unlike the universal dependence of normal proteolysis rates on the D-box receptor, the proteolysis of some substrates appeared completely independent of the KEN-box receptor in the cdh1Δ48 strain (Fig. 4A and Table 1). Nonetheless, proteolysis of a subset of substrates was strongly impaired in cdh1Δ48 cells (Fig. 4, B and C, and Table 1). For the most part, substrates exhibiting dependence on the KEN-box receptor contained consensus KEN-box motifs. In some cases, like Spo12, KEN sequences have not been functionally linked to APC/C-mediated proteolysis. The Spo13 and Spo11 proteins were interesting in that proteolysis was strongly dependent on the KEN-box receptor, yet neither contains a consensus KEN-box motif. This suggests that variations on this motif can also functionally engage the KEN-box docking site on Cdh1, consistent with experiments using the GXEN motif in XKid (12). The Kip1 protein also lacks a KEN-box in the C-terminal region required for APC/C-mediated proteolysis (32) but requires the KEN-box receptor for efficient proteolysis in our assay.

We tested a subset of our Cdh1 substrate panel in the cdh1Δ52 strain, including those that showed no dependence on the KEN-box receptor, and found that the stability of most was unaffected by disruption of the ABBA motif binding site (Fig. 5A and Table 1). Several substrates exhibited a very slight increase in stability that could reflect a minor overall effect of ABBA motif receptor mutations on Cdh1 function or a weak contribution to substrate binding but did not meet our criteria for stabilization. We found a single Cdh1 substrate, Cik1, whose stability was strongly dependent on the ABBA motif receptor site (Fig. 5B), even though the Cik1 N terminus is necessary and sufficient for APC/C-mediated proteolysis (31) lacks any sequence resembling the ABBA motif consensus. Thus, in addition to the known function in promoting binding of the inhibitor Acm1 to Cdh1, the ABBA motif (or variants of it) appears to be capable of promoting substrate recognition by budding yeast APC/C/CDH1.

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Substrate Recognition by APC/C^{Cdhi}

Figure 2. The ABBA motif is a functional APC/C^{Cdhi} degron in budding yeast. A, immunoblot analysis of endogenous Cbl2 levels in asynchronous and α-factor-treated G, cultures of the indicated strains. WT is the parental strain from which cdh1Δ was engineered. The remaining strains have 3FLAG-CDH1 or degron receptor mutant alleles (dbr, D-box receptor mutations; kbr, KEN-box receptor mutations; amr, ABBA motif receptor mutations) expressed from the CDH1 promoter integrated into cdh1Δ. B, anti-FLAG immunoblot from whole cell extracts of asynchronous cultures of cdh1Δ and the indicated derivative strains demonstrating equivalent expression from the integrated 3FLAG-Cdh1 alleles. C, the ability of the integrated 3FLAG-CDH1, 3FLAG-cdh1kk, and 3FLAG-cdh1mm strains used for substrate stability assays to stably arrest in the G1 phase in response to α-factor treatment was determined by anti-Acm1 immunoblot and flow cytometry. Acm1 is highly unstable specifically in G1 cells. Acm1 expression from GAL1p was terminated at time 0. Flow cytometry data are from 60-min point to illustrate maintenance of arrest throughout the experiment. D, the interaction of endogenous Acm1 with 3FLAG-Cdh1^{dbr} and 3FLAG-Cdh1^{kbr} mutants was assessed by anti-FLAG co-IP. WCE, whole cell extract. E, the interaction of 3FLAG-Cdh1 and the three degron docking site mutants with the core APC was assessed by anti-FLAG co-IP. NC, negative control using parental strain lacking 3FLAG-Cdh1. F, stability of Acm1Δ52-protein A and a variant with mutations in the central D- and KEN-boxes (Acm1Δ52^DK) was evaluated in CDH1 and cdh1Δ strain backgrounds. G, same as F comparing stability of Acm1Δ52^DK with a variant containing an additional mutation of the ABBA motif (Acm1Δ52^DK/ABBA) in the CDH1 strain. H, stability of Acm1Δ52-protein A with mutations in its D- and KEN-boxes was compared in strains expressing either wild-type CDH1 or one of three cdh1Δ degron receptor mutants. In this experiment Acm1 was detected with an anti-Acm1 antibody, and the load control is a nonspecific band from this blot.

and Swe1 for degradation we compared their stability in CDH1 and cdh1Δ strains. Stability of both Ipl1 and Swe1 increased in the cdh1Δ strain, confirming their APC/C^{Cdhi}-mediated turnover and demonstrating its D-box receptor dependence (Fig. 6B). Although it remains unclear whether APC/C-mediated degradation of Ipl1 and Swe1 has functional significance in yeast, this work demonstrates that, in principle, our in vivo assay can be used as a tool to determine whether a protein of interest is recognized as a substrate by APC/C^{Cdhi} in a physiological environment. Given the apparent conservation of APC/C structure and substrate recognition (16), it could potentially be used to test candidate APC/C substrates from other species as well.

Discussion

The most significant conclusion from this study is that efficient degradation of most, if not all, APC/C^{Cdhi} substrates requires engagement of the D-box receptor site on Cdhi, despite the lack of a universal D-box sequence motif common to all substrates. Thus, the apparent diversity in APC/C^{Cdhi} substrate degron motifs may not reflect complexity in the mechanisms of substrate recognition. The D-box was the first APC/C degron identified and characterized (10), and it contains elements that bind both to the WD40 domain of the co-activator proteins and to the Apc10 subunit of the core APC/C (44, 45, 49–54). The broad requirement of the D-box receptor for efficient APC/C^{Cdhi} substrate proteolysis suggests that the D-box may contribute more than just binding affinity, consistent with recent structural and mechanistic studies. D-box engagement may be important for positioning substrate lysines for ubiquitin transfer, for the co-activator-induced conformational change in the core APC/C that brings the catalytic RING module into close proximity with the substrate (16), for enhancing functional interaction with the E2 (55), or for enhancing catalysis in some other way. Despite the universal
Substrate Recognition by APC/CCdh1

FIGURE 3. The D-box receptor on Cdh1 is essential for normal proteolysis of all substrates. A, stability assays (based on the protocol from Fig. 1A) with representative protein A-tagged substrates containing consensus D-box degrons were performed in G₁-arrested CDH1 and cdh₁<sup>Δ<sub>αα</sub></sup> strains. Substrate expression from GAL1p was terminated at time 0, and the protein level was monitored over time by anti-protein A immunoblotting. G6PD is a load control. An asterisk indicates that two different exposures of the same blot are compared with comparable time 0 intensities. B, same as A with substrates that lack functional D-box consensus motifs. See Table 1 for results from complete substrate set. All experiments in A and B were performed three times with equivalent results. C, example flow cytometry results from cells at the 60-min point in the assays (Pds1 experiment shown) demonstrating maintenance of G₁ arrest throughout time course.

dependence of Cdh1 substrate degradation on an intact D-box receptor, we note that turnover of the artificial Acn<sub>1,3v/kb</sub> mutant was only slightly impaired in the cdh₁<sup>Δαα</sup> strain (Fig. 2H), presumably because of the unusual contributions of other high affinity docking motifs required for effective Cdh1 inhibition (12, 38, 40).
Substrate Recognition by APC/C<sup>Cdh1</sup>

In contrast to the D-box, the selective requirement of other motifs, e.g. the KEN-box and ABBA motif, for substrate proteolysis is consistent with these motifs providing primarily specificity and binding affinity and not a crucial contribution to the catalytic reaction. Although it is unclear what the functional implications of having a KEN box versus an ABBA motif are, the use of a variety of short linear motifs for substrate docking ensures highly selective binding while allowing for flexibility in the substrate structural requirements. It seems likely that additional docking sites on Cdh1 remain undiscovered. For example, it is unclear how a D-box alone could provide sufficient binding affinity and specificity compared with substrates that use multiple degrons motifs, and degradation of several of the substrates examined here (e.g. Nrm1, Mps1, and Cdc20) was independent of both the KEN-box and ABBA motif receptors. Nonetheless, the widespread use of D-box receptor docking argues that all Cdh1 substrates share some aspects of recognition and processing by APC/C<sup>Cdh1</sup>.

The ABBA motif was originally identified in Acm1 as a unique element required for full APC/C<sup>Cdh1</sup>-mediated proteolysis (40). Our results with Cik1 and the Acm1<sup>db/kb</sup> mutant demonstrate clearly that the ABBA motif is another functional APC/C<sup>Cdh1</sup> degron, consistent with recent reports identifying the ABBA motif as a degron for APC/C<sup>Cdc20</sup> in both yeast and humans (26, 27) and with prior in vitro work with Acm1 (40). Considering that Acm1 possesses three distinct consensus APC/C<sup>Cdh1</sup> degrons, it remains unclear how it evades ubiquitination by APC/C<sup>Cdh1</sup>, although a lack of suitably positioned lysines has been proposed (40). The ABBA motif that binds budding yeast Cdc20, identified in the S phase cyclin Cib5 (27), is distinct from the Acm1 ABBA motif that binds Cdh1. The two differ primarily at the beginning of the motif, where Clb5 orthologs contain a pair of conserved basic amino acids instead of the conserved phenylalanine of the ABBA motif consensus sequence FX(I/V/L)(F/Y/H) (27). In principle, differences in the ABBA motif receptor of Cdh1 and Cdc20 could contribute to the distinct specificities of the two APC/C co-activators, although it does not seem to contribute significantly to recognition of most Cdh1 substrates. Interestingly, the ABBA motif recognized by human APC/C<sup>Cdc20</sup> more closely resembles the motif recognized by APC/C<sup>Cdh1</sup> in budding yeast, and the residues in human Cdc20 that form the ABBA motif receptor are likewise similar to those found in budding yeast Cdh1 (28).

Our results emphasize that the current definitions of the three best characterized APC/C substrate degrons (D-box, KEN-box, and ABBA motif) are still incomplete. We identified substrates that require the D-box, KEN-box, and ABBA motif receptors yet lack sequences that match the current consensus for these degrons. Additional work is needed to identify the functional degrons in many substrates lacking the consensus motifs, for example the D-box in the kinesin Cin8, the D- and KEN-boxes in the kinesin Kip1, the D-box and ABBA motif in Cik1, and the KEN-box in Spo13. The NKS marker sequence in Sgo1 that is required for its APC/C-mediator proteolysis was reported to be part of an unconventional D-box (25) yet resembles the KEN-box consensus as well. Mutations of consensus D- and KEN-box sequences in the small Spo12 protein were pre-
Substrate Recognition by APC/C<sub>Cdh1</sub>

Experimental Procedures

Reagents, Strains, and Plasmids—All yeast strains used in this study are described in Table 2. All plasmid constructs are described in Table 3. Strain YKA412 was generated by standard PCR-mediated deletion of the entire CDH1 coding region with the KanMX4 cassette. Strains YKA627, YKA628, YKA897, YKA898, and YKA1006 were constructed by integrating plasmids expressing wild-type 3xFLAG-CDH1 or the mutant alleles 3xFLAG-cdh1<sup>dbr</sup>, 3xFLAG-cdh1<sup>kbv</sup>, 3xFLAG-cdh1<sup>omr1</sup>, and 3xFLAG-cdh1<sup>lawar2</sup> (pHIP032, pHIP042, pHIP174, pHIP175, and pHIP176) from the natural CDH1 promoter, respectively, into the his3A<sup>i</sup> locus of parental strain YKA412 following linearization with SpeI restriction enzyme.

BG1805-based plasmids expressing APC/C substrates with C-terminal protein A tags are from the Yeast ORF Collection (GE Dharmacon; YSC3868). For plasmids expressing ASE1-TAP, SPO12-TAP, and SPO13-TAP (pHLP520, pHLP521, and pHLP522), the coding regions were amplified by PCR from genomic DNA and inserted into the Gateway<sup>TM</sup> entry vector pENTR/D-TOPO (Life Technologies; K240020) as directed by the manufacturer. The genes were then transferred into destination vector pAG416GAL-ccdB-TAP (Addgene; catalog no. 14267) (57). pHLP523 was constructed by moving an XbaI and XhoI fragment containing the <i>acmanΔ52 protein A</i> fusion from pHLP400 described previously (41) into p415MET for control by the methionine-repressible MET25 promoter. pHIP032 and pHIP042 were constructed previously (42).

Cycloheximide (C1988), 3xFLAG peptide (F4799), EZview anti-FLAG resin (F2426), rabbit anti-protein A polyclonal antibody (P3775), anti-FLAG-M2 monoclonal antibody (F1804), and rabbit anti-G6PDH polyclonal antibody (A9521) were from Sigma-Aldrich. Restriction endonucleases and other cloning enzymes were from New England Biolabs. α-Factor peptide was synthesized and purified by Genscript. Rabbit anti-Cib2 polyclonal antibody (SC9071) was from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-rabbit (catalog no. 111-035-003) and anti-mouse (catalog no. 115-035-003) secondary antibodies were from Jackson ImmunoResearch. Rabbit anti-Acm1 polyclonal antibody was described previously (41). Apc1 and Cdc23 polyclonal antibodies were kindly provided by Dr. Adam Rudner (University of Ottawa). Luminata Crescendo chemiluminescent detection reagent (catalog no. WBLUR0500) was from EMD Millipore.

Site-directed Mutagenesis—All site-directed mutagenesis was performed using the QuikChange Lightning multisite-directed mutagenesis kit (Agilent Technologies; catalog no. 210513) following the provided instructions. All plasmid constructs and site-directed mutations were confirmed by DNA sequencing. In pHLP530 and pHLP538, the Acm1 D-box mutation included R119A and L122A substitutions, the KEN box mutation included K98A, E99A, and N100A substitutions, and the ABBA motif mutation included F61A, L63A, Y64A, E65Q, and E66Q substitutions. The plasmids expressing Cdh1 with altered D-box, KEN-box, and ABBA-receptor sites (pHIP042, pHIP174, pHIP175, and pHIP176) were generated using pHIP032 as a template. The D-box receptor mutant allele <i>cdh1<sup>dbr</sup></i> encodes L255V, P258A, G535A, and D536A substitutions described previously (40). The KEN-box receptor mutant <i>cdh1<sup>kbv</sup></i> encodes N405A, N407A, Q473A, and R517L substitutions described previously (14). Two ABBA motif receptor mutants were generated. One, <i>cdh1<sup>omr1</sup></i>, encodes I331S and K333T mutations and the other, <i>cdh1<sup>lawar2</sup></i>, encodes I331S, K333T, L325N, I344G, and P374K substitutions described previously (12).

Cell Growth—YP medium contained 1% yeast extract and 2% peptone and was supplemented with 2% of the appropriate carbon source (either dextrose, raffinose, or galactose). Synthetic dropout medium contained 6.7 g/liter yeast nitrogen base, the appropriate amino acid supplement mixture for maintaining plasmid selection or inducing expression from MET25p, and 2% of the appropriate carbon source. All liquid yeast cultures were grown at 30 °C with shaking at 225 rpm. For G<sub>1</sub> arrest, α-factor was added to 100 μg/L for 2–3 h until a uniform “schmoo” morphology was observed by microscopy. All cell cycle arrests were confirmed by flow cytometry on an Accuri C6 flow cytometer as described previously (43).

| Strain name | Relevant genotype | Source |
|-------------|-------------------|--------|
| YKA233      | bar1::hisG        | Ref. 43|
| YKA412      | bar1::hisG cdh1::KanMX4 | This study|
| YKA627      | bar1::hisG cdh1::KanMX4 | This study|
| YKA628      | bar1::hisG cdh1::KanMX4 his3::3xFLAG-cdh1<sup>dbr</sup>His3 | This study|
| YKA897      | bar1::hisG cdh1::KanMX4 his3::3xFLAG-cdh1<sup>kbv</sup>His3 | This study|
| YKA898      | bar1::hisG cdh1::KanMX4 his3::3xFLAG-cdh1<sup>omr1</sup>His3 | This study|
| YKA1006     | bar1::hisG cdh1::KanMX4 his3::3xFLAG-cdh1<sup>lawar2</sup>His3 | This study|

All yeast strains used in this study are derived from BY4741: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0. YKA898 and YKA1006 differ in the nature of the <i>cdh1<sup>2omr1</sup></i> mutation as described under "Experimental Procedures." YKA898 was used in the experiment in Fig. 2H and YKA1006 was used in the experiments in Figs. 2 (A and E) and 5.
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**TABLE 3**

| Plasmids | 
| --- | 
| Plasmid name | Plasmid type | Marker | Promoter | Expressed protein | Source |
| --- | --- | --- | --- | --- | --- |
| BG1805-Clb2 | 2 micron | URA3 | GAL1 | Clb2-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Cin8 | 2 micron | URA3 | GAL1 | Cin8-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Cdk1 | 2 micron | URA3 | GAL1 | Cdk1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Fin1 | 2 micron | URA3 | GAL1 | Fin1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Kip1 | 2 micron | URA3 | GAL1 | Kip1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Pds1 | 2 micron | URA3 | GAL1 | Pds1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Mps1 | 2 micron | URA3 | GAL1 | Mps1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Sgo1 | 2 micron | URA3 | GAL1 | Sgo1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Cdc5 | 2 micron | URA3 | GAL1 | Cdc5-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Ipl1 | 2 micron | URA3 | GAL1 | Ipl1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Swe1 | 2 micron | URA3 | GAL1 | Swe1-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Nrm1 | 2 micron | URA3 | GAL1 | Nrm-His<sub>6</sub>/HA/3C/ZZ | GE |
| BG1805-Cdc20 | 2 micron | URA3 | GAL1 | Cdc20-His<sub>6</sub>/HA/3C/ZZ | GE |
| pHLP520 | CEN/ARS | LEU2 | GAL1 | Ase1-TAP | This study |
| pHLP521 | CEN/ARS | LEU2 | GAL1 | Spo12-TAP | This study |
| pHLP400 | CEN/ARS | LEU2 | GAL1 | Acm1<sub>Δ52</sub>-Z2 | Ref. 41 |
| pHLP523 | CEN/ARS | LEU2 | GAL1 | Acm1<sub>Δ52</sub>-Z2 | This study |
| pHLP530 | CEN/ARS | LEU2 | GAL1 | Acm1<sub>Δ52</sub>-<sub>K</sub>-Z2 | This study |
| pHLP538 | CEN/ARS | LEU2 | GAL1 | Acm1<sub>Δ52</sub>-<sub>K</sub>-an<sub>K</sub>-Z2 | This study |
| pHIP032 | Integrating | HIS3 | CEN | 3Flag-Cdh1 | Ref. 42 |
| pHIP042 | Integrating | HIS3 | CEN | 3Flag-Cdh1<sub>amr2</sub> | Ref. 42 |
| pHIP174 | Integrating | HIS3 | CEN | 3Flag-Cdh1<sub>amr1</sub> | This study |
| pHIP175 | Integrating | HIS3 | CEN | 3Flag-Cdh1<sub>amr1</sub> | This study |
| pHIP176 | Integrating | HIS3 | CEN | 3Flag-Cdh1<sub>amr2</sub> | This study |

In Vivo Protein Stability Assay—Yeast were cultured at 30 °C overnight until saturated in the appropriate selective synthetic dropout medium for plasmid maintenance. Saturated cultures were diluted to $A_{600} \approx 0.1$ in YP with 2% raffinose. Growth was continued to $A_{600} = 0.2$ – 0.3 and then α-factor was added. For the Acm1Δ52 inhibition assay, arrested cells were pelleted by centrifugation at 5,000 × g for 3 min, washed with 10 ml of water, and pelleted again. The cell pellet was resuspended in synthetic dropout medium lacking methionine (to induce Acm1Δ52 expression) and containing 2% raffinose as carbon source and α-factor to maintain the arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expressing cdh1 degron receptor mutants, no medium change was needed following G1 arrest. After 30 min, galactose was added to 2% to induce expression of APC/C substrates. For assays in strains expr...
platform for 20 min at room temperature. The pooled eluates were then analyzed by SDS-PAGE and immunoblotting.

**Author Contributions**—L. Q. designed and conducted most of the experiments, analyzed the results, and, with M. C. H., wrote and edited the manuscript. D. S. P. F. G. conducted experiments characterizing the ABBA motif as a functional degron and analyzed the results. M. M. helped establish the assay system for studying Acm1 inhibition of Cdh1 substrate degradation. M. C. H. designed experiments, analyzed data, and wrote the manuscript.

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