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Initial Characterization of a Conserved Active Site Residue for the Cdc34 Ubiquitin Conjugating Enzyme

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INITIAL CHARACTERIZATION OF A CONSERVED ACTIVE SITE RESIDUE FOR THE CDC34 UBIQUITIN CONJUGATING ENZYME

By

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Abstract

Ubiquitin-conjugating enzymes (E2s) covalently modify protein substrates with ubiquitins. The active site cysteine residues on E2s are essential for catalyzing the transfer of ubiquitin from the E2 active site onto the protein substrate, however there is a limited amount of information available concerning additional active site residues for E2s that may also participate in catalysis. Cdc34 is an essential E2 that has merited the lion's share of attention for biochemical analysis of the E2 family. Previous phylogenetic analysis of Cdc34 amino acid sequences has identified an invariably conserved histidine residue close to the active site cysteine in the primary structure, however whether this residue actually participates in Cdc34 function is unknown. Here we demonstrate that histidine 98 on the human Cdc34 ubiquitin conjugating enzyme is vital to the enzymatic activity of the E2. Recombinant His98Ala mutant Cdc34 was isolated from bacterial cells engineered to express the protein and compared to wild-type Cdc34 through two complementary ubiquitination assays. Substitution of the histidine residue with alanine resulted in a nearly complete loss of function. These results uncover the possible roles that histidine 98 may play during Cdc34 function.

Introduction

The ubiquitin proteasome system (UPS) is the single largest pathway in eukaryotic cells and is responsible for the regulated degradation of proteins.[1] There are three primary reasons why a protein is targeted for degradation. First, proteins become old and/or damaged over time and must be efficiently disposed of. Secondly, some proteins do not fold correctly after their translation from ribosomes, and they must be degraded before they aggregate and perturb normal cellular function. Lastly, some proteins control various biological processes and when their functions are completed, their presence may disrupt the cell and thus they also must be removed at this point.

There is great medical relevance to studying the molecular processes of the UPS. For instance, we now appreciate that when several genes that function in the UPS become mutated, various forms of cancer can arise.[2] Furthermore, a link between malfunction of the UPS and neurodegenerative disorders has also been made.[3] Thus, a greater appreciation for how proteins in the UPS function may eventually lead to human therapies. For example, a proteasome inhibitor (note that the proteasome is a macromolecular protease that binds to and degrades ubiquitinated proteins), Bortezomib, has been approved by the FDA for the treatment of myeloma, a form of cancer.[4]

Proteins are targeted for degradation by the covalent attachment of ubiquitin, a conserved 76 amino acid protein.[1] Once a protein is modified by an ubiquitin molecule, this serves as the site for the addition of more ubiquitins and the eventual formation of a poly-ubiquitin chain. This is referred to as ubiquitination. This is relevant to proteolysis because the 26S proteasome (see above) recognizes poly-ubiquitinated proteins that contain an ubiquitin chain of at least 4 or more protomers.[1]

The ubiquitination of a protein substrate of the UPS involves three enzymes: E1, E2 and E3. The E1, also known as ubiquitin activating enzyme, uses the energy in ATP to form a high energy covalent intermediate between the E1 active site and the C-terminus of ubiquitin (Fig. 1a). This enzyme then passes this activated ubiquitin to the E2 active site, also known as ubiquitin conjugating enzyme (Fig. 1b). The E2~ubiquitin then binds to the E3 ubiquitin ligase that recognizes and binds to the protein substrate. The E3 then promotes the transfer of ubiquitin from the E2 to the E3 (Fig. 1c).

Cdc34 is an ubiquitin conjugating enzyme or E2 that has great biological significance.[5] Cdc34 functions with the cullin-RING ligase family of E3s, the most numerous family in human cells. The mechanism of action of Cdc34 has been under investigation since the discovery of ubiquitin conjugating enzymes, and thus it is reasonable to consider Cdc34 the archetypal E2.[6]

Even though a considerable amount of time has been spent on determining the molecular mechanism of action of Cdc34, large gaps in our understanding of the details for how this enzyme works exist. For instance, Cdc34 contains several highly conserved residues in the active site.[7] However, the molecular roles for these residues are, in some cases, unknown. For instance, human Cdc34 (huCdc34) contains an invariably conserved histidine residue at position 98 (His 98). Histidine residues are commonly involved in enzyme mechanisms such as general acid-base catalysis or in the binding of substrate, however it is unknown whether either or both of these roles pertain to His 98 in huCdc34.[8] The aim of this thesis is to begin a preliminary investigation into the molecular function of His 98. Here we determine that this residue greatly contributes to Cdc34 catalysis and our results pave the way for future more detailed mechanistic work.

Methodology

Constructs and Plasmids: A previously described plasmid, which is designed to express huCdc34 containing an N-terminal 6-histidine tag for purification in bacteria, was used as a starting point.[10] DNA primers were generated using online software that introduced a mutation in the huCdc34 DNA sequence that corresponds to the replacement of histidine at position 98 with alanine.[11] A polymerase chain reaction was assembled from a commercially available kit followed by 22 rounds of thermal cycling to produce the DNA product.[12] This was then digested with 1 μL of Dpn1 restriction enzyme at 37 degrees C for 1 hour, followed by the transformation of DNA into chemically competent E. *coli* cells. The plasmid expressing huCdc34 is also marked by a gene that confers ampicillin resistance, and therefore bacteria that took up the plasmid were selected for on LB-ampicillin plates. Single colonies were isolated and grown in selective liquid media overnight, followed by the purification of plasmid DNA with a commercially available mini-prep kit.[13] The presence of the His98Ala mutation in the huCdc34 amino acid sequence was verified by DNA sequencing.

Protein expression: Expression of a human recombinant protein in bacteria requires using a special strain of E. *coli* named Rosetta. This is an engineered strain of bacteria that has been genetically altered for the efficient expression of recombinant protein. Plasmids corresponding to either wild-type or His98Ala huCdc34 were transformed into Rosetta competent bacterial cells and transformants were selected for on solid LB media with selective antibiotics overnight. Around twenty colonies were selected and transferred to a liquid LB medium with the

proper selective antibiotics. The media was incubated in a Thermo MaxQ4000 shaker incubator for approximately twelve hours at 37 degrees C.[14] Twenty mL of the inoculated media was then transferred to a fresh liter of LB plus antibiotics, which was then incubated in a Thermo MaxQ8000 shaker/incubator at 37 degrees C.[14]

The OD_{600} of the culture was periodically checked to estimate the growth of the bacteria until it reached approximately 0.8. The bacterial suspension was then introduced to the presence of 0.4 mM IPTG, which signals the bacteria for the production of recombinant protein. After approximately 3 hours, the bacteria were separated from the growth media by centrifuging the suspensions at 5000xG for ten minutes. The supernatants were discarded, and then 25 mL of phosphate-buffered saline (PBS) was used to wash and re-suspend the pellets.[20] These suspensions were centrifuged again, the supernatants discarded, and their remaining pellets (containing the bacterial cells) flash frozen using liquid nitrogen. The frozen cells were then stored in a minus 80 °C freezer for later use.

Protein purification: The bacterial pellets were thawed and suspended in 30 mL of lysis buffer.[14] This suspension was then sonicated at least three times to disrupt the bacterial cell walls and cellular membranes. The lysates were transferred to labeled Oakridge tubes and centrifuged at approximately 23,000xG for forty minutes. The supernatants, which contain both recombinant huCdc34 and endogenous bacterial protein, were then transferred to 50 mL Falcon™ tubes containing Nickel-agarose resin that had been pre-washed in lysis buffer. Note that Nickel-agarose has high affinity for proteins containing the 6-histidine tag

that was introduced into the recombinant huCdc34 amino acid sequence.[21] These solutions were incubated with agitation for 2 hours at 4 degrees C. The solutions were then centrifuged at 1000xG to collect the resins, and washed multiple times with lysis buffer. The washed resins were then transferred to disposable columns and 2.7 mL of nickel elution buffer was added, which allows for the dissociation and collection of a solution containing the Cdc34 proteins. This protein solution was then dialyzed 3X against a storage buffer.[15]

Enzyme assays: Cdc34 is an ubiquitin conjugating enzyme that joins ubiquitin protomers into a poly-ubiquitin chain. In living cells, Cdc34 collaborates with the SCF ubiquitin ligase to build poly-ubiquitin chains onto protein substrates that bind to SCF. Cdc34 has its own intrinsic activity, which can be measured in the absence of SCF. Cdc34 activity can also be assessed in the presence of SCF and SCF-bound substrate. Both of these assays, named the di-ubiquitin synthesis assay (for the SCF-independent Cdc34 activity) and the ß-catenin ubiquitination assay (for SCF-dependent activity) were used to track the rates of ubiquitination for both wild-type and mutant Cdc34.

Description of the di-ubiquitin synthesis assay (SCF-independent): In this assay, Cdc34 is pre-incubated with ^{32}P-labeled ubiquitin and purified E1 enzyme (generous gift from A. Ziemba).[18] This forms a thioester bond between Cdc34 and radio-labeled ubiquitin (shown as Cdc34~ubiquitin hereafter). The reaction was then initiated by the addition of unlabeled ubiquitin, which resulted in the formation of a covalent di-ubiquitin product over time. Time-points were taken at various intervals. The reaction was quenched by the addition of 2X SDS-PAGE (poly-

acrylamide gel electrophoresis) running buffer, which serves to denature the native state of the proteins. The products and reactants were separated by SDS-PAGE followed by auto-radiography and the quantitation of products and reactants using a Typhoon image scanner and ImageQuant software.[16] The amount of product is calculated as a percentage of total products and reactants followed by the normalization of signal to the enzyme concentration. These values were then plotted in at least duplicate versus time and followed by linear regression.[17] The slope of the line represents the rate of Cdc34 reaction. Thus, wild-type and His98Ala Cdc34 intrinsic enzyme activity can be quantitatively compared.

Description of ß-catenin ubiquitination assay (SCF-dependent): In this assay, huCdc34 is pre-incubated with unlabeled ubiquitin and E1 as before, followed by the addition of the SCF ubiquitin ligase, and finally the reaction was initiated by adding 5 μM ^{32}P labeled ß-catenin peptide (kind gift from G. Kleiger).[19] Time-points were taken and products and reactants were separated as above. Note that a product is considered as a ß-catenin peptide containing one or more ubiquitins. The rate of ß-catenin ubiquitination was calculated by linear regression as above.

Results

The basis for understanding the role of His 98 relies on its innate charge and polarity. Specifically, histidine residues contain an imidazole side-chain that can act as both a hydrogen bond donor and acceptor. This can both promote the formation of the enzyme-substrate complex and/or promote catalysis by forming hydrogen bonds that stabilize the transition state complex. Additionally, histidine residues can act in general acid-base chemistry. In summary, all of these potential mechanisms may promote Cdc34 activity. As such, if any of these mechanisms are relevant to Cdc34 activity, our hypothesis is that the His98Ala mutation would have a negative effect on product formation during Cdc34 catalysis. Since alanine is an uncharged nonpolar amino acid, it should disrupt all the potential roles for the histidine residue mentioned above. To test our hypothesis, we generated a His98Ala mutant huCdc34 protein and compared its activity to wild-type huCdc34 using two specific ubiquitination assays (see methods).

The rate of ubiquitination for His98Ala mutant huCdc34 enzyme was significantly slower than its wild-type counterpart in both ubiquitination assays. For instance, in the di-ubiquitin synthesis assay, the rate of product formation for His98Ala huCdc34 was approximately eleven times slower than for wild-type (0.00165 ± 0.00037 min^{-1} versus 0.01941 ± 0.004108 min^{-1}, respectively, Fig. 2). Additionally, in the ß-catenin ubiquitination assay, His98Ala huCdc34 was more than 110 times slower (0.0024 ± 0.0003 min^{-1} versus 0.28 ± 0.04 min^{-1}, Fig. 3). In fact, His98Ala huCdc34 activity was so defective in this assay that product formation was barely apparent, and we had to apply special techniques to detect the signal for true product over background (see discussion).

Therefore, these results leave no doubt that His 98 plays a prominent role during the catalysis of ubiquitination by the huCdc34 ubiquitin conjugating enzyme.

Note that the rate of product formation was estimated by linear regression (methods). The data fit extremely well to a linear model as evidenced by the statistical significance of the R^2 values for the goodness of fit being close to 1. Furthermore, the standard error of measurement (SEM) was estimated during the fitting procedure and was based on at least duplicate data points for all results. These SEM values are provided next to the rates of product formation (shown in the inset next to the graphs in Figs. 2 and 3). Notice that the errors are substantially lower than the estimates for the rates of the reactions. Some concern might be drawn over the high SEM values associated with the rates for His98Ala huCdc34 in both ubiquitination assays. This would initially create a large uncertainty as to the statistical significance of the mutant Cdc34 ubiquitination rates. However, note that the rate of product formation is negligible compared to its wild-type counterpart and easily identifiable from the autoradiographs, such that the difference between the wild-type and His98Ala rates far outweighs the large SEM values for mutant huCdc34.

Discussion

We determined that the His 98 residue in huCdc34 is crucial for the mechanism of catalysis during the ubiquitination reaction. There are several potential explanations for why this could be so. First, His 98 could be forming a hydrogen bond with the ubiquitin substrate. This could serve to orient the lysine on the ubiquitin (note that ubiquitin has seven potential lysine acceptors on its surface).[9] In fact, Cdc34 is known to catalyze ubiquitin chains where Lys 48 is the preferred acceptor during chain formation.[6] Perhaps His 98 forms a hydrogen bond with ubiquitin during this reaction to thus orient Lys 48 in the active site to promote its iso-peptide bond formation with Cdc34~ubiquitin. Second, His 98 could be forming a hydrogen bond with the transition state conformation of the substrate. A tetrahedral intermediate during iso-peptide bond formation is known to occur during the E2 catalytic cycle. This could involve an H-bond between His 98 and the oxyanion that forms in the transition state complex, which would serve to lower the transition state energy and therefore the activation energy barrier. Finally, we can also envision a role for H98 in acid-base catalysis. For instance, the acceptor lysine on either SCF-bound substrate or on ubiquitin must be deprotonated before it can participate in a nucleophilic reaction. The basic imidazole side-chain of histidine could theoretically deprotonate lysine.

Several complications arose during the quantitation of His98Ala huCdc34 for the ß-catenin ubiquitination assay, and novel methods had to be developed to overcome this temporary hurdle. One of the earliest obstacles encountered involved the radioactivity of the ^{32}P-labeled ß-catenin. Due to this isotope's relatively short half-life (approximately 14 days), the signal for the images during autoradiography was very weak. Therefore,

autoradiography with a phosphor screen, which normally needs only one hour of exposure, under these conditions involved timing the experiments so that the gels could be exposed overnight. This new technique, although it introduced more background interference and the potential oversaturation of signals from substrate, was essential in quantitating the faint product bands for the ß-catenin ubiquitination assay and His98Ala mutant Cdc34.

Another problem with His98Ala mutant huCdc34 quantitation arose from its inherent deficiency in ubiquitin conjugation. Even with a longer exposure time on the phosphor screen, the faintness of the product bands made it such that the background noise significantly interfered with the detection of product. This initially resulted in an extremely low R^2 (<0.3) using a typical procedure for background correction. We circumvented the problem by performing individual background corrections for each product band. These two techniques allowed for the quantitation, albeit with a significant amount of error, of the results for His98Ala Cdc34 from the β-catenin ubiquitination assay.

Conclusion

Here we conclusively demonstrate that His 98 is vital to huCdc34's enzymatic activity. Since this histidine is conserved among all eukaryotic Cdc34 orthologs, it is reasonable to assume that the residue contributes to the functions of all Cdc34 proteins from all eukaryotic organisms. Although the actual mechanistic role is still unknown, we note that there are several interesting possibilities. Our results pave the way for more sophisticated biochemical approaches that can uncover the exact mechanism of action for how this histidine residue functions in the Cdc34 enzyme.

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14. Lysis buffer: 30mM Tris pH 7.5, 250mM NaCl, 20mM imidazole, 0.1% IgePal, 5% Glycerol, 1mM β-mercaptoethanol

15. Storage buffer: (-) DTT, 30 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% Glycerol

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18. Di-ub synthesis assay conditions: ATP buffer, 250 nM E1, 5 μM K48R Ub, 2 μM (His98Ala or WT) huCdc34, 50 μM D77 Ub

19. Beta-catenin ubiquitination assay conditions: ATP buffer, 1 μM E1, 10 nM (His98Ala or WT) huCdc34, 30 μM WT Ub, 100 nM SCF-Skp-βTrcp, 5 μM β-catenin

20. Phosphor Buffered Saline Solution: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot 2 H_2O$, 2.0 mM KH_2PO_4 pH 7.4

21. *Ni-NTA agarose*. (2013, May). Retrieved from http://www.qiagen.com/products/catalog/sample-technologies/protein-sample-technologies/purification-kits-and-resins/ni-nta-agarose

Figures and Tables

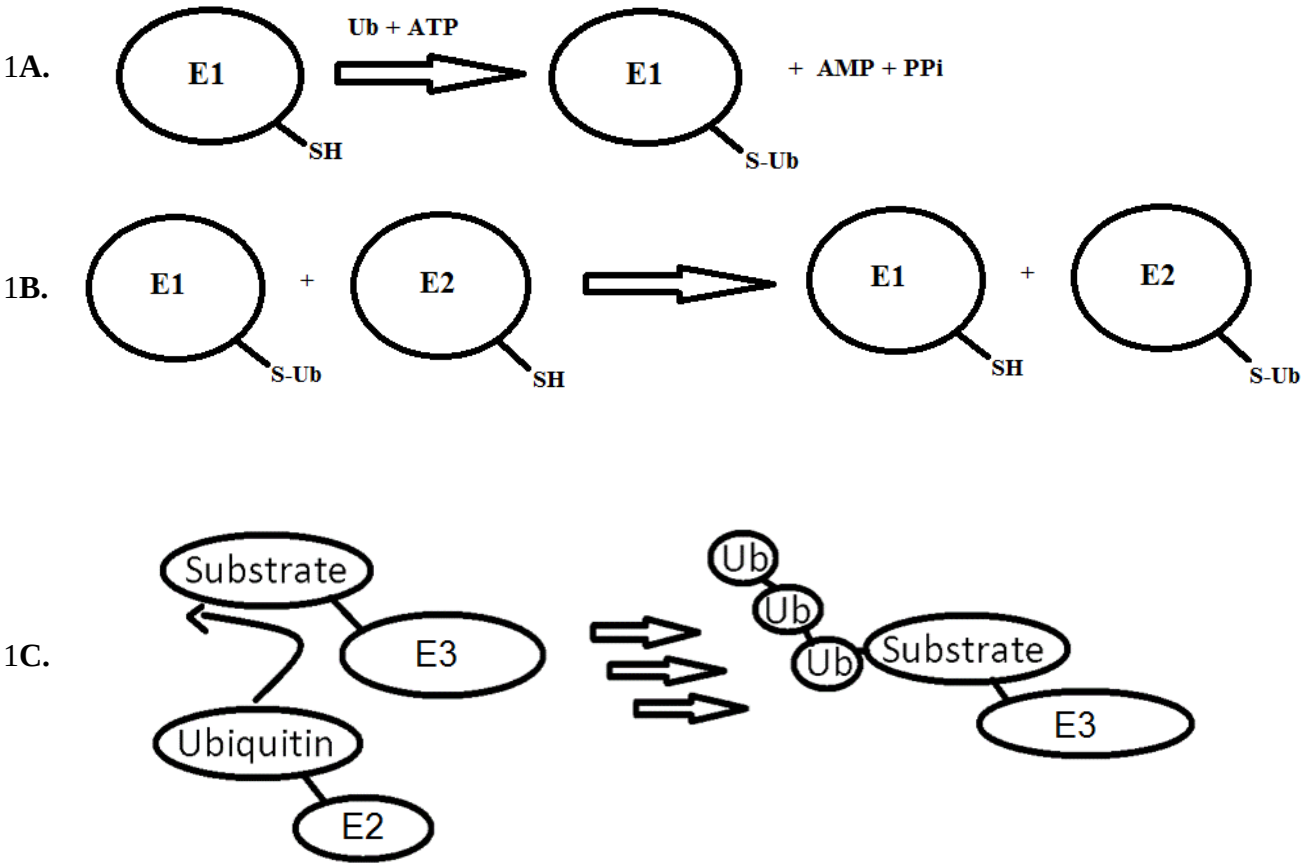
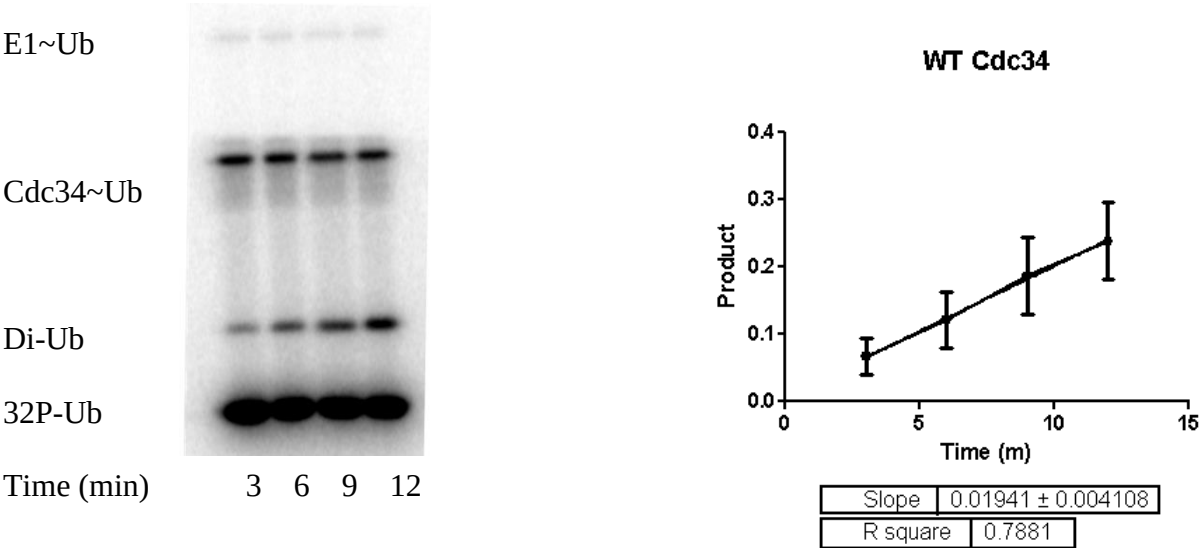


Figure One: The ubiquitination of a protein substrate involves the sequential action of three classes of enzymes: E1, E2 and E3. (A) The E1 enzyme binds to ubiquitin and ATP and promotes the formation of a high energy thioester bond intermediate between the C-terminal end of ubiquitin and the E1 active site cysteine residue. (B) The E2 enzyme binds to the E1~ubiquitin and the ubiquitin is transferred to the E2 active site cysteine. (C) The E3 binds to both the protein substrate that is to be modified and to the E2~ubiquitin, which promotes the transfer of ubiquitin from the E2 to the protein substrate.

2**A:** Di-ubiquitin synthesis assay for wild-type huCdc34. 2**B:** Plot of 2**A**.



2**C:** Di-ubiquitin synthesis assay for His98Ala mutant huCdc34. 2**D:** Plot of 2**C**.

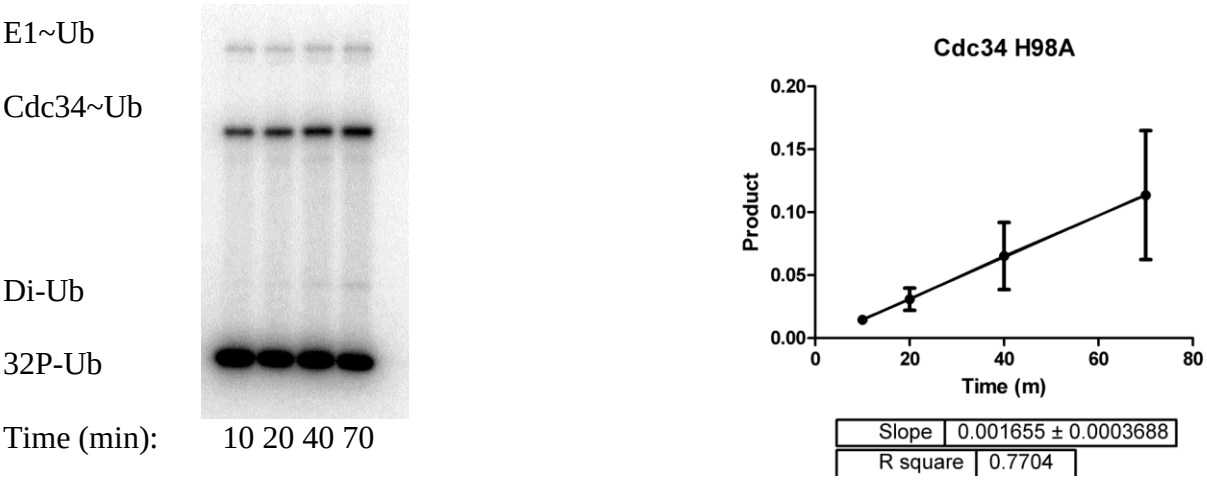
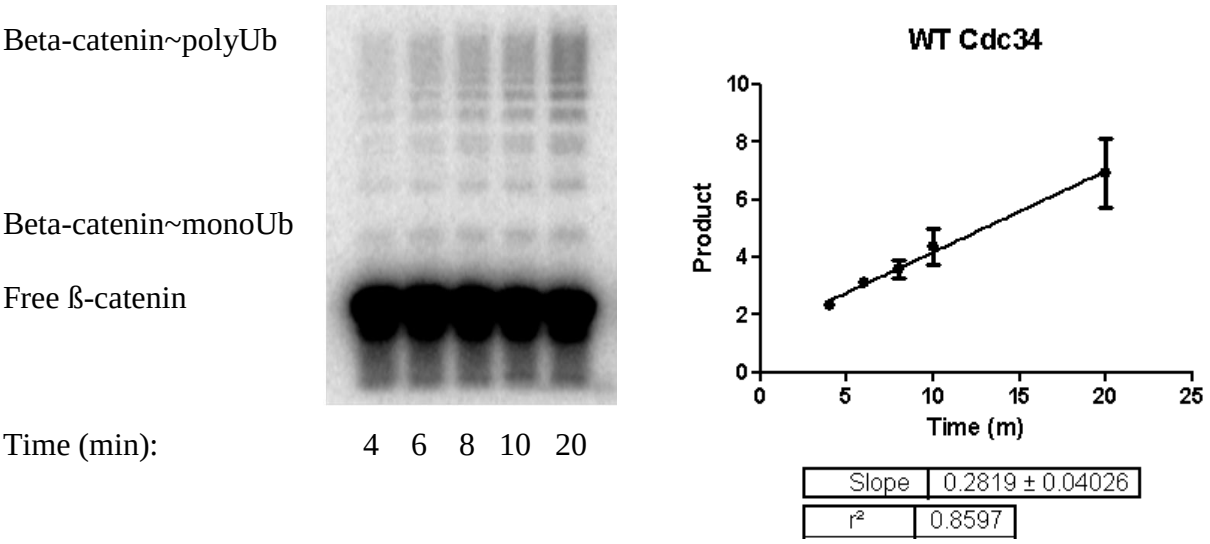


Figure Two: Results for the comparison of wild-type and His98Ala Cdc34 using the di-ubiquitin synthesis assay. (A) Autoradiograph showing the formation of product for wild-type Cdc34 over time. Note the increasing amount of di-ubiquitin (product) shown over time. (B) Graph showing the amount of di-ubiquitin normalized for Cdc34 concentration over time. Linear regression was used to determine the line of best fit to the

data. (C) Same as in (A) except using His98Ala Cdc34. (D) Same as in (B) except using His98Ala Cdc34 (Ub is ubiquitin).

3**A:** Beta-catenin ubiquitination assay for wild-type huCdc34. 3**B:** Plot of 3**A**.



3**C:** Beta-catenin ubiquitination assay for His98Ala huCdc34. 3**D:** Plot of 3**C**.

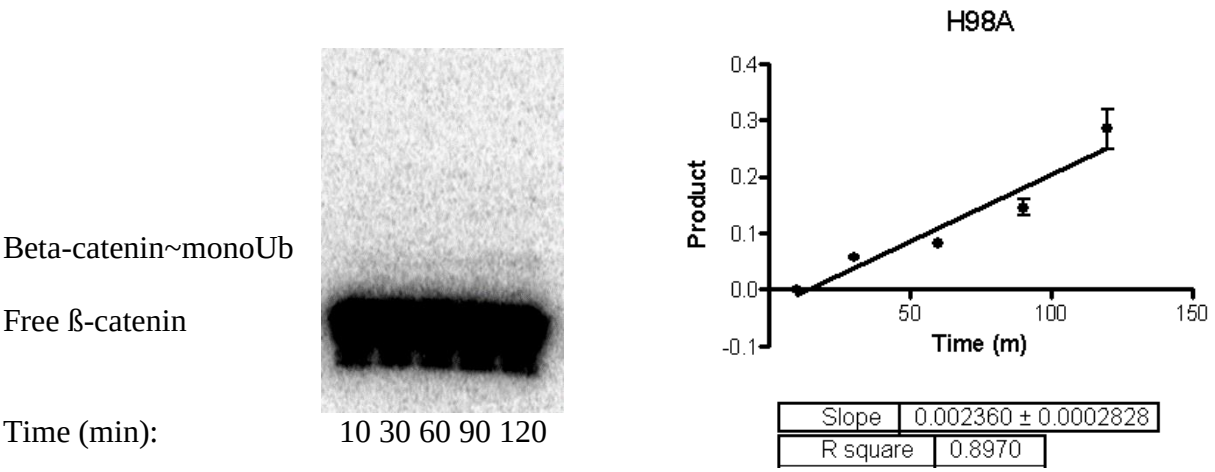


Figure Three: Results for the comparison of wild-type and His98Ala Cdc34 using the β-catenin ubiquitination assay. (C) While it is difficult to detect the formation of product by eye from the autoradiogram, notice that the increase in product is easily perceptible through computational quantitation (D).

Table One: Wild-type huCdc34 in duplicate for the di-ubiquitin reaction.

Time (m)	WT huCdc34	WT huCdc34
3.	0.04781488	0.08477971
6.	0.09041639	0.149125
9.	0.1460612	0.2271989
12.	0.1972842	0.2788746

Table Two: His98Ala Mutant huCdc34 in duplicate for the di-ubiquitin reaction.

Time (m)	His98Ala huCdc34	His98Ala huCdc34
10.	0.01682636	0.01230089
20.	0.0371301	0.0246475
40.	0.08405849	0.04633586
70.	0.1498405	0.07737828

Table Three: Wild-type huCdc34 in duplicate for the ß-catenin reaction.

Time (m)	WT huCdc34	WT huCdc34
4.	2.240333	2.466434
6.	2.944260	3.263175
8.	3.254951	3.916962
10.	3.764723	4.987077
20.	5.737291	8.126178

Table Four: His98Ala mutant huCdc34 in duplicate for the ß-catenin reaction. Note the nullification of the first time-point to achieve a linear result.

Time (m)	His98Ala huCdc34	His98Ala huCdc34
10.	0.000000	0.000000
30.	0.05646447	0.05801594
60.	0.07365134	0.09258923
90.	0.1599235	0.1302997
120.	0.3201147	0.2499201

All table data were generated using the following equation:

$$\frac{Product}{Product + Substrate} \times \frac{[Substrate]}{[Enzyme]}$$

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