Published in final edited form as:

FEBS J. 2020 May ; 287(10): 1985–1999. doi:10.1111/febs.15132.

Direct regulation of Chk1 protein stability by E3 ubiquitin ligase HUWE1.

Katelyn B. Cassidy^{1,#}, Scott Bang^{2,#}, Manabu Kurokawa^{1,2,3,*}, Scott A. Gerber^{1,3,*}

¹Department of Molecular & Systems Biology, Geisel School of Medicine, Hanover, NH 03755

²Department of Biological Sciences, Kent State University, Kent, OH 44242

³Norris Cotton Cancer Center, Geisel School of Medicine, Lebanon, NH 03756

Abstract

The HECT E3 ubiquitin ligase HUWE1 is required for a wide array of important functions in cell biology. Although HUWE1 is known to play a role in DNA damage signaling, the mechanism(s) that underlies this function remains elusive. HUWE1 regulates effectors of DNA replication and genotoxic stress tolerance. However, the loss of HUWE1 can also result in the accrual of significant endogenous DNA damage due to insufficient remediation of replication stress induced by an overabundance of key substrates. We discovered that HUWE1 depletion leads to a significant increase in levels of the single strand break effector kinase Chk1, independent of the DNA damage response, activation of apical DNA damage repair (DDR) signaling kinases (ATM and ATR), and the tumor suppressor p53. We also identified multiple lysine residues on Chk1 that are poly-ubiquitinated by HUWE1 *in vitro*, many of which are within the kinase domain. HUWE1 knockdown also markedly prolonged the protein half-life of Chk1 in steady state conditions and resulted in greater stabilization of Chk1 protein than depletion of Cul4A, an E3 ubiquitin ligase previously described to control Chk1 abundance. Moreover, prolonged replication stress induced by hydroxyurea or camptothecin resulted in a reduction of Chk1 protein levels, which was rescued by HUWE1 knockdown. Our study indicates that HUWE1 plays a significant role in the regulation of the DDR signaling pathway by directly modulating the abundance of Chk1 protein.

Keywords

DNA damage; replication stress; ubiquitination; protein degradation

*Corresponding Authors: Manabu Kurokawa: phone : (330) 672-2979, mkurokaw@kent.edu, mailing address: 211 Cunningham Hall, Department of Biological Sciences, Kent State University, Kent OH 44242., Scott Gerber: phone: (603) 653-3679, scott.a.gerber@dartmouth.edu, mailing address: One Medical Center Dr, Rubin 7th, HB-7937, Lebanon, NH 03756.

#These authors contributed equally to the work

Author Contributions

KBC, MK, and SAG conceived the study and designed the experiments. KBC and SB performed the cell culture and Western blot experiments. KBC carried out the mass spec analysis as well as the *in cellulo* and *in vitro* ubiquitination assays. KBC, MK, and SAG analyzed the data and wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Introduction

The HECT E3 ubiquitin ligase HUWE1 (also known as ARF-BP1, LASU1 and Mule) was initially discovered as a regulator of the key cell cycle effector protein p53 [1]. Through findings reported across the past decade, HUWE1 is now understood to regulate the stability of a diverse complement of proteins, including p53, Mcl-1, and c-Myc, among others. Given the diversity of its substrates, the role of HUWE1 in cellular homeostasis is complex. Indeed, some of the HUWE1 substrates are oncoproteins, suggesting HUWE1 functions as a tumor suppressor, while other substrates are tumor suppressors, indicating that HUWE1 might also function as an oncoprotein. As such, the role of HUWE1 in cancer may be tissue specific or context-dependent.

Accumulating evidence strongly suggests that HUWE1 plays a key role in genome integrity and cellular responses to DNA damage. It has been shown that HUWE1 contributes to restarting stalled replication forks [2], targeting unassembled proteins for degradation [3] and regulating base excision repair [4]. HUWE1 may also promote apoptosis in cells that are unable to repair sustained DNA damage by promoting the degradation of anti-apoptotic Bcl-2 family protein Mcl-1 [5, 6].

A recent study that aimed to define the significance of HUWE1 to mammalian protein quality control demonstrated a putative relationship between HUWE1 and the replication stress response protein Chk1 [3]. Chk1 is a serine/threonine kinase [7] that plays an essential role in preserving genome integrity in cells experiencing impaired replication [2] or genotoxic stress [8, 9], and delays late origin firing until replication has resumed at stalled forks [10]. In addition to modulating replication stress, Chk1 also has important functions within an unperturbed cell cycle: Chk1 participates in genome surveillance [11], prevents premature mitotic entry [12] and regulates the spindle assembly checkpoint [13, 14]. In response to endogenous and acute DNA damage, Chk1 is activated by the apical kinase ATR. ATR phosphorylates and thereby activates Chk1 at two conserved sites (Ser 317 and Ser345) [15]. DNA damage-dependent Chk1 activation is facilitated by Claspin [10, 16, 17] and BRCA1 [18, 19].

Deletion of Chk1 is embryonically lethal in mice due to severe proliferation defects that result in p53-independent apoptosis [11]. Conversely, overexpression of Chk1 in fission yeast results in an accumulation of inactive p34^{cdc2} and subsequent sustained G₂ arrest [20]. In cultured gastric adenocarcinoma cells, Chk1 overexpression significantly increases resistance to radiotherapy [21]. Therefore, it is critical for cells to have mechanisms to carefully regulate Chk1 abundance.

Chk1 levels are tightly controlled by proteasomal degradation [8, 22–25]. To date, two putative SCF (Skp1-Cullin-Fbox) E3 ligase complexes have been described as regulators of Chk1 abundance: one containing Cullin (Cul1)/FbxO6 and another containing Cullin4A (Cul4A)/DDB1 [8, 22, 24, 25]. However, evidence exists that other E3 ubiquitin ligases likely also play a role in Chk1 turnover [26], as partial redundancy/multiplicity is common in the ubiquitin proteasome system (UPS) [27].

Here, we report that HUWE1 is a prominent Chk1 E3 ubiquitin ligase that directly regulates the stability of Chk1 protein in steady state conditions as well as under prolonged replication stress.

Results

HUWE1 depletion induces phosphorylation of Chk1 kinase independently of ATM and ATR

It has been shown previously that cells depleted of HUWE1 incur significant DNA damage [28]. However, in our hands we did not observe induction of DNA double strand break (DSB) marker γ -H2AX upon siRNA-mediated depletion of HUWE1 in HeLa cells using a pooled siRNA (Fig. 1A). In fact, HUWE1 depletion did not result in the activation of the apical DSB pathway kinase ATM (phospho-S1981), nor activation of the apical single strand break (SSB) pathway kinase ATR (phospho-S1989) (Fig. 1B), which is consistent with our finding that HUWE1-depleted cells do not accumulate unresolved DNA lesions (Fig. 1A). Surprisingly, we did observe modest accumulation of Chk1 kinase protein as early as 24 hours post transfection with HUWE1 siRNA (Fig. 1B). To confirm the specificity of our HUWE1 siRNA pool, we repeated the RNA interference experiment with two single siRNAs targeting HUWE1 in separate experiments, including one published previously [29] and observed a similar increase in Chk1 protein for both of them (Fig. 1C). Moreover, this increase in total Chk1 level was accompanied by a concomitant increase in activated Chk1 (phospho-S296) levels by 72 hours post-HUWE1 knockdown (Fig. 1C). These results suggest that the stability of Chk1 protein is either directly or indirectly linked to HUWE1, despite the absence of upstream DDR signaling.

HUWE1 depletion stabilizes Chk1 protein

Because HUWE1 is known to regulate the stability of substrate proteins by targeting them for proteasome-mediated degradation [5, 29–32], we hypothesized that the abundance of Chk1 protein was directly regulated by HUWE1. To assess whether Chk1 abundance in HUWE1-depleted cells was regulated post-translationally, we performed a cycloheximide chase experiment in which U2OS cells were treated with either control or HUWE1 siRNA for 72 hours, followed by addition of 100 μ g/mL cycloheximide and Western blotting at multiple timepoints across an 8-hour time window. Whereas Chk1 protein exhibited a turnover half-life of 5.9 hours in U2OS cells treated with control siRNA, its degradation was significantly slower in HUWE1-depleted cells (turnover half-life 13.3 hours; Fig. 2A). Likewise, in HeLa cells, HUWE1-depletion resulted in significant stabilization of Chk1 protein (turnover half-life: 6.1 vs. 26.0 hours; Fig. 2B). Collectively, these results indicate that the increase in abundance of Chk1 protein following HUWE1 depletion occurs post-translationally.

HUWE1 facilitates the ubiquitination and degradation of p53 [1]. In addition, p53 negatively regulates Chk1 levels via modulation of Chk1 transcription [33]. Thus, the increase in Chk1 protein observed when HUWE1 is depleted occurs in spite of a decrease in Chk1 transcription. To confirm that the p53-Chk1 transcriptional circuit is intact in this system, and to determine if Chk1 abundance is also regulated by a different E3 ubiquitin ligase that regulates p53, we treated p53 wild-type U2OS cells with siRNA targeting the well-known

p53 regulator MDM2 [34]. Depletion of MDM2 led to a greater increase in p53 as compared to that seen in HUWE1-depleted cells, as well as a significant decrease in Chk1 protein levels (Fig. 3). We therefore surmise that the increased stability of total Chk1 is not a general feature of p53 E3 ligases. Furthermore, Chk1 regulation by HUWE1 was observed in both p53 proficient (U2OS) and deficient (HeLa) cell lines (Fig. 2).

HUWE1 poly-ubiquitinates active Chk1 in living cells and in vitro

To further establish a regulatory relationship between Chk1 protein stability and HUWE1, we performed ubiquitination assays by transfecting HeLa cells with a plasmid encoding HA-ubiquitin following control or HUWE1 siRNA (Fig. 4A). We retrieved ubiquitinated proteins with anti-HA immunoprecipitation and analyzed the ubiquitination level of endogenous Chk1 by Western blot. As shown in Fig. 4B, HUWE1 depletion resulted in marked reduction in poly-ubiquitination of endogenous Chk1. Taken together, these results are consistent with our hypothesis that HUWE1 directly poly-ubiquitinates and targets Chk1 to the proteasome.

We next assessed the ability of recombinant HUWE1 to ubiquitinate Chk1 *in vitro*. To do this, we expressed and isolated GST-tagged, recombinant catalytically active as well as inactive mutant (C4341S) HUWE1 HECT domains from bacteria and full-length, wild-type HUWE1 from insect cells. We then performed *in vitro* ubiquitination reactions using the active and inactive HECT domains, ubiquitin cascade enzymes (UBE1 and UBE2), recombinant substrates, ubiquitin and ATP. After 3 hours of incubation, the reactions were quenched and analyzed by Western blotting and shotgun mass spectrometry. We confirmed that our preparation of the HUWE1 HECT domain was catalytically active using the known HUWE1 substrate p53 via observation of HUWE1-mediated ubiquitin-dependent laddering of p53 in Western blots (Fig. 5A). We also observed extensive ubiquitination of Chk1 *in vitro* in the presence of the active HUWE1 HECT domain (Figs. 5B,C and Table 1) or full-length HUWE1 (Fig. 6), but not in the presence of another p53-targeting E3 ligase (MDM2) (Fig. 6). These data strongly support the hypothesis that Chk1 is a HUWE1 substrate.

Although HUWE1 is a large protein with multiple functional domains, it interacts with some of its substrates primarily via the C-terminal HECT domain. For instance, it has been shown that poly-ubiquitination of p53 and CDC6 by HUWE1 only requires the HECT domain [1,29]. Since our results indicate that the HUWE1 HECT domain is also sufficient to polyubiquitinate Chk1 *in vitro* (Fig. 5), we next sought to determine whether the HUWE1 HECT domain interacts with Chk1 in cells. Towards this end, we incubated recombinant GST or GST-HECT protein with cell lysates prepared from HeLa cells (Fig. 7A). After incubation, we retrieved proteins bound to GST or GST-HECT by glutathione Sepharose beads and analyzed proteins bound to the HECT domain by Western blot. As shown in Fig. 7B, GST-HECT, but not GST alone, strongly bound to Chk1 protein. Interestingly, Chk1 purified with the HECT domain (Fig. 7B arrowhead) appeared at a molecular weight that was slightly higher than Chk1 in cell lysates (Fig. 7B arrow). Moreover, treatment of the cells with hydroxyurea prior to lysate preparation further enhanced the interaction between Chk1 and the HUWE1 HECT domain (Fig. 7B). The nonspecific protein Vinculin did not bind to GST or GST-HECT, indicating the binding specificity between Chk1 and the HUWE1 HECT domain (Fig. 7B). Taken together, these results demonstrate that the

HUWE1 HECT domain is sufficient to bind and polyubiquitinate Chk1. This data also suggests that the interaction between Chk1 and HUWE1 might be regulated by DNA damage signaling.

Next, we mapped ubiquitination sites on recombinant Chk1 protein via LC-MS/MS analysis. Chk1 was ubiquitinated by HUWE1 at multiple lysine residues and many of these modification sites are located in regions that are known to be occluded by the auto-inhibitory domain within the C-terminus of Chk1 [35, 36], including the kinase domain (Fig. 5C, Table 1). These data suggest that at least some recombinant Chk1 was in an open or active conformation upon interaction with HUWE1. Thus, in addition to ubiquitin modifications, we searched for evidence of Chk1 activity in our *in vitro* ubiquitination reactions. We found Chk1 auto-phosphorylation sites in this assay (Table 2), including S296, a site typically used as a marker of an active Chk1 conformation in cells. We also observed phosphorylation of the HECT domain by Chk1 in these *in vitro* reactions (Table 3).

LC-MS/MS analysis of ubiquitin linkages also indicated that the HUWE1 HECT domain predominantly assembled K48-linked poly-ubiquitin *in vitro* (Table 4). Canonical K48-linked poly-ubiquitin chains permit the recognition and subsequent degradation of modified substrates by the 26S proteasome [37], which is consistent with a role for HUWE1 in regulating Chk1 abundance. Interestingly, we also identified K6 and K63 ubiquitin linkages (Table 4), indicating that HUWE1 assembles non-canonical poly-ubiquitin linkages either on itself or on Chk1. This is consistent with previous findings of HUWE1 HECT domain poly-ubiquitin chain type specificity [38].

HUWE1 is a prominent Chk1 E3 ubiquitin ligase

Multiple E3 ubiquitin ligases have been observed to target Chk1 for proteasomal degradation following DDR, including Cul4A/DDB1 [8, 24] and Cul1/FbxO6 [8, 22], although only Cul4A was reported to contribute to Chk1 stability in unperturbed cells [24]. In our hands, HUWE1 knockdown resulted in a significant increase in steady-state Chk1 protein levels versus control- as well as Cul4A siRNA-treated cells (Fig. 8). Co-depletion of Cul4A and HUWE1 did not have a detectable additive or synergistic effect on Chk1 protein levels (Fig. 8), suggestive of HUWE1 as a key housekeeping E3 ubiquitin ligase for Chk1. Curiously, we also observed a modest reduction in Cul4A levels when treating cells with HUWE1 siRNA, although not to the degree observed with Cul4A RNAi.

We further investigated the contributions of HUWE1 and Cul4A to the regulation of Chk1 protein levels in response to genotoxic agents. It is known that Chk1 is both activated and destabilized in response to prolonged replication stress [22, 24, 39], which is commonly induced by treatment with the ribonucleotide reductase inhibitor hydroxyurea (HU) or topoisomerase inhibitor camptothecin (CPT). As with steady-state turnover of Chk1, depletion of Cul4A resulted in only modest Chk1 stabilization from replication stress-elicited turnover in HeLa cells (Fig. 9). HUWE1 depletion, on the other hand, elicited a significant rescue of the destabilization of Chk1 induced by exposure to HU and CPT (Fig. 9). Thus, we conclude that HUWE1 also plays a significant role in modulating Chk1 abundance in response to replication stress.

Discussion

In the present study, we have identified HUWE1 as an additional E3 ubiquitin ligase that controls Chk1 protein stability in the unperturbed cell cycle and in response to replication stress.

Our *in vitro* ubiquitination assay data indicates that HUWE1 multiply poly-ubiquitinates regions of Chk1 that are known to be occluded in the inactive/auto-inhibited form [35, 36] (Table 1). This data, as well as our observation that the phosphorylated form of Chk1 accumulates with HUWE1 knockdown (Fig. 1B), may indicate that HUWE1 preferentially targets active Chk1 for proteasomal degradation. In this regard, it is interesting to note that endogenous Chk1 precipitated by the HUWE1 HECT domain migrated to a slightly higher molecular weight in our western blot than the prominent Chk1 pool in the input cell lysate (Fig. 7B). It remains to be determined whether this shift in molecular weight is due to phosphorylation or other post-translational modifications of Chk1 that permit interaction with the HUWE1 HECT domain, such as mono-ubiquitination. Regardless, our *in vitro* ubiquitination assay results clearly indicate that the presence of other protein effectors (besides ubiquitin cascade components) are not essential to promote the ubiquitination of Chk1 by HUWE1. This does not rule out the existence of other mechanisms that could refine or inhibit HUWE1 ubiquitination of Chk1 in cells but does indicate that additional factors are not required to initiate HUWE1-mediated Chk1 ubiquitination. For example, our finding that Chk1 hyper-phosphorylates the HUWE1 HECT domain *in vitro* (Table 3) is suggestive of a regulatory step for targeting of Chk1 by HUWE1. Phosphorylation of serine or threonine residues within the last ~60 amino acids of the HECT domain could influence ubiquitin chain assembly, as sequence determinants within this region dictate poly-ubiquitin chain linkage preferences [38]. Alternatively, the high concentrations of Chk1 required for efficient ubiquitination by HUWE1 *in vitro* may simply result in spurious phosphorylation of HUWE1 that is non-physiological. Further analysis of the HUWE1-Chk1 complex formation requirements and experimental validation would be necessary to determine if Chk1 primes its own ubiquitination via phosphorylation of HUWE1. Finally, our results demonstrated that the binding between Chk1 and the HUWE1 HECT domain increases in response to DNA damage (Fig. 7B), the mechanism of which remains to be elucidated. It is noteworthy that DNA damage may promote the translocation of HUWE1 from the cytoplasm to the nucleus [54], suggesting another mechanism that regulates the interaction between Chk1 and HUWE1 as Chk1 predominantly localizes to the nucleus.

Previous studies uncovered that two SCF complexes containing Cul1/FbxO6 and Cullin4A (Cul4A)/DDB1 regulate Chk1 protein stability [22, 24]. Our ubiquitination experiment in HeLa cells indicated that Chk1 is poly-ubiquitinated in the presence and absence of HUWE1 (Fig. 4), which is consistent with the presence of multiple active Chk1 E3 ubiquitin ligases. When comparing the contributions of Cul4A and HUWE1 to the regulation of steady-state Chk1, we observed that HUWE1 knockdown caused a slight reduction in Cul4A protein levels, albeit not to the degree that was observed by directly targeting Cul4A via RNAi (Fig. 8A). It would be interesting to investigate whether the stability of Chk1 is coordinately regulated by these multiple E3 ligases and whether crosstalk exists between HUWE1 and other Chk1 E3 ligases. Any potential relationship between HUWE1 and Cul4A appears

likely to be indirect, as we observed a decrease in Cul4A levels, rather than an increase, in response to HUWE1 depletion. Our study shows that HUWE1 knockdown results in a higher relative increase in the levels of Chk1 protein abundance than that of Cul4A (Figs. 8 and 9), suggesting that HUWE1 may be a more prominent E3 ubiquitin ligase that regulates the stability of total Chk1 protein in HeLa cells.

While preparing this manuscript for submission, a study was published that ties HUWE1 to the K6-linked ubiquitination of Chk1 [40, 41]. This is consistent with another recent publication that demonstrated that HUWE1 is the predominant mammalian K6 ubiquitin E3 ligase [3]. Our data is also in accord with previously published reports of HUWE1 assembling K63-linked poly-ubiquitin on a variety of substrates such as c-Myc [42] and Disheveled [43]. K6 and K63 linkages create distinct poly-ubiquitin topographies that can determine protein fates other than the degradation of the substrate. K63 poly-ubiquitination, for instance, can regulate the formation of signaling complexes [44] and K6 linkages can inhibit proteasomal degradation [45]. We believe that uncovering the mechanistic relevance of these modifications would be of great interest to the field.

It has been suggested that maintaining supraphysiological levels of Chk1 could be advantageous for cell proliferation and survival [46, 47]. However, others have noted that Chk1 overexpression can also trigger a Wee1 kinase-mediated G₂ arrest [20]. Accordingly, we also noticed that prolonged exogenous overexpression of GFP-Chk1 results in cell cycle arrest, which prohibited us from generating a stable cell line (unpublished observation). One could speculate that perhaps a cell type-specific sensor exists for monitoring Chk1 abundance and activity. It would be useful to study the factors responsible for this capability, as tolerance of Chk1 overexpression has been reported to desensitize cells to intra-S phase checkpoint triggers. Indeed, Chk1 is capable of remediating the increased replication stress induced by rapidly dividing cells during oncogenic transformation [46]. Interestingly, in our study, HUWE1 knockdown per se did not cause apoptosis or cell cycle arrest (unpublished observation), suggesting that endogenous Chk1 levels are coordinately regulated at multiple levels.

The role of HUWE1 in cancer remains a matter of debate in the field. We view our finding that a putative oncogene (Chk1) is destabilized by HUWE1 as another example of HUWE1 serving as a tumor suppressor, and it would be interesting to determine if this relationship is preserved across a variety of cancers and tissue types.

Methods

Cell culture and reagents

U2OS and HeLa were maintained in DMEM (Corning-CellGro) supplemented with 8% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Corning). Cycloheximide was used at a final concentration of 100 µg/mL, for up to 8 hours. Hydroxyurea (HU) was used at a final concentration of 20 mM for 8 hours. Camptothecin (CPT) was used at a final concentration of 1 µM for 8 hours. Etoposide was used at a final concentration of 10 nM. The Chk1 inhibitor (MK-8776) was used at a final concentration of 2 µM for up to 2 hours.

Immunoblotting, antibodies and plasmids

Cell pellets were harvested and then lysed in a buffer consisting of: 10mM HEPES pH 7.5, 10mM KCl, 1% NP-40, with phosphatase inhibitor (PhosSTOP from Sigma Aldrich) and a protease inhibitor cocktail (Roche cOmplete EDTA-free). Lysates were cleared of chromatin and membranous debris by centrifugation (10–15 minutes at 21,100 x *g*) and lysate concentrations were determined by Bradford (Biorad) or BCA assay (Pierce).

A total of 30–60 µg of protein was loaded per lane. Proteins were separated on a 4–12% Bis-Tris gradient gel (Biorad) and blotted onto PVDF (Millipore) or nitrocellulose (GE).

Antibodies utilized are as follows: HUWE1/Lasu1/Urb1 (Bethyl), Chk1 (SCBT), pATM S1981 (CST), ATM (CST), pATR T1989 (CST), ATR (CST), pChk1 S296 (CST), Actin-HRP (Abcam), GFP (GeneTex), MDM2 (R&D Systems), Cul4A (Bethyl), HA (SCBT), p53 (SCBT), γ-H2AX (CST) and H2AX (CST). Blots were incubated overnight in primary antibody at 4°C with the exception of HRP-conjugated Actin, which was incubated for 1 hour at room temperature.

Immunoblots were quantified using ImageJ and values were plotted in Excel.

The Chk1-eGFP expression construct was generously provided by Dr. Youwei Zhang (Case Western Reserve University, Cleveland, Ohio). The HA-Ub expression construct was described previously [49].

Transient RNA interference

Cells were plated the evening before transfection in penicillin-strep-free DMEM. Cells were transfected the following morning in Optimem (Gibco) with either RNAimax (ThermoFisher) or INTERFERin (Polyplus). We used Dharmacon siGENOME SMARTpool for Cul4A (M-012610-01) (Figs. 8 & 9), Dharmacon siGENOME SMARTpool for HUWE1 (M-007185-01) (Figs. 1A, 1B, 2–4, 8 & 9), Dharmacon siGENOME individual siRNA (D-007185-06) (Fig. 1C, “HUWE1-B”), and a custom single HUWE1 target siRNA that had been previously described [29] (“HUWE1-A” in Fig. 1C). In addition, we utilized two different control (unspecific) siRNAs: Dharmacon siGENOME Non-targeting siRNA pool #1 (D-001206-13-05) and GFP siRNA [29]. Dharmacon SMARTpool siRNA were utilized at a final concentration of 20 nM. Single siRNAs, including HUWE1 siRNA-A/B and GFP control, were used at a final concentration of 10 nM. Media was replaced on transfected cells the morning after transfection and cells were replated as necessary.

In cellulo ubiquitination assay

Chk1 *in cellulo* ubiquitination assays were performed as previously described [48]. On day 1, HeLa cells were transfected with either non-targeting control siRNA or HUWE1 siRNA. On day 2, these cells were replated into a 10cm dish and, after attaching, transfected with 2 µg of a plasmid encoding HA-tagged ubiquitin. On day 3 cells were treated with MG132 (20µM) for 8 hours then, harvested and lysed in IP buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40) and precipitated with an anti-HA beads (Roche), as previously described [49]. Eluates were immunoblotted with the aforementioned antibodies.

In vitro ubiquitination assay

Reactions were performed as previously described [49]. Recombinant ubiquitin, Ube1 (E1), UbcH7 (E2), UbcH5a (E2), MDM2 and p53 were purchased from R&D systems. Recombinant GST-Chk1 was purchased from Enzo biosciences. The mutant and WT HUWE1 HECT domains were cloned and expressed in BL21 bacterial cells. Full-length recombinant HUWE1 protein was expressed in Sf9 insect cells. The HUWE1 HECT domain and full-length HUWE1 were expressed as a GST-fusion protein and purified with glutathione sepharose beads. UbcH7 was used as the E2 in HUWE1 mediated reactions whereas UbcH5a was used in MDM2 mediated reactions. Ubiquitination sites were mapped via sequencing tryptic Chk1 peptides. Briefly: each *in vitro* ubiquitination reaction mixture was reduced and alkylated and then run approximately 1 mm into a 12% SDS-PAGE gel. Regions above 50kDa were excised and processed as previously described [50]. Eluates were analyzed on a Q Exactive Plus Quadrupole-Orbitrap mass spectrometer (Thermo Fisher). MS/MS spectral data was searched against a human proteome reference database (FASTA derived). Peptide matches were subjected to internal quality filters [51] to ensure that we were reporting hits with high confidence (~1% FDR). Phosphorylation sites were assigned a localization confidence score with phosphoRS [52]. Ubiquitinated lysines were searched as a dynamic modification and linkage sites were manually annotated.

Proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [53].

Acknowledgements

This work was funded by Norris Cotton Cancer Center Holland Awards (to MK), Prouty Cancer Center Pilot Funds (to MK), American Cancer Society grant # IRG-82-003-30 (to MK), NIH R03 CA230828 (to MK) and NIH R01s CA155260 and GM122846 (to SAG).

We would like to thank Dr. Youwei Zhang (Case Western Reserve University) for the eGFP-Chk1 plasmid and Dr. Jean Cook (University of North Carolina, Chapel Hill) for the GST-HUWE1 HECT expression plasmid. We would also like to thank Dr. Duane Compton and Dr. Yolanda Sanchez (Geisel School of Medicine at Dartmouth) for valuable discussion.

Abbreviations

ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
Cul1	Cullin 1
Cul4A	Cullin4A
Chk1	Chek1
HECT	Homologous to the E6-AP Carboxyl Terminus
HUWE1	HECT, UBA and WWE domain-containing protein 1
MDM2	Double minute 2 protein
SCF	Skp, Cullin, F-box

DDB1	DNA damage-binding protein 1
BRCA1	Breast cancer type 1 susceptibility protein
Bcl-2	B-cell lymphoma 2
Mcl-1	Induced myeloid leukemia cell differentiation protein
UPS	Ubiquitin Proteasome System
DDR	DNA Damage Response
DSB	Double Strand Break
SSB	Single Strand Break
KD	knockdown
LC-MS	Liquid chromatography mass spectrometry
geLC-MS	In-gel digest liquid chromatography mass spectrometry FASTA, FAST-all
GST	Glutathione S-transferase
GFP	Green fluorescent protein
HA	Human influenza hemagglutinin
HU	Hydroxyurea
CPT	Camptothecin
FL	Full length
DMEM	Dulbecco's Modified Eagle Media
FBS	Fetal Bovine Serum
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride
HRP	Horseradish peroxidase

References

1. Chen D, Kon N, Li M, Zhang W, Qin J & Gu W (2005) ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor, Cell. 121, 1071–83. [PubMed: 15989956]
2. Choe KN, Nicolae CM, Constantin D, Imamura K, Kawasawa Y, Delgado-Diaz MR, De S, Freire R, Smits VA & Moldovan GL (2016) HUWE1 interacts with PCNA to alleviate replication stress, EMBO reports. 17, 874–86. [PubMed: 27146073]
3. Xu Y, Anderson DE & Ye Y (2016) The HECT domain ubiquitin ligase HUWE1 targets unassembled soluble proteins for degradation, Cell Discov. 2, 16040. [PubMed: 27867533]

4. Parsons JL, Tait PS, Finch D, Dianova II, Edelmann MJ, Khoronenkova SV, Kessler BM, Sharma RA, McKenna WG & Dianov GL (2009) Ubiquitin ligase ARF-BP1/Mule modulates base excision repair, *The EMBO journal*. 28, 3207–15. [PubMed: 19713937]
5. Zhong Q, Gao W, Du F & Wang X (2005) Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis, *Cell*. 121, 1085–95. [PubMed: 15989957]
6. Pervin S, Tran A, Tran L, Urman R, Braga M, Chaudhuri G & Singh R (2011) Reduced association of anti-apoptotic protein Mcl-1 with E3 ligase Mule increases the stability of Mcl-1 in breast cancer cells, *British journal of cancer*. 105, 428–37. [PubMed: 21730980]
7. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H & Elledge SJ (1997) Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25, *Science*. 277, 1497–501. [PubMed: 9278511]
8. Zhang YW, Otterness DM, Chiang GG, Xie W, Liu YC, Mercurio F & Abraham RT (2005) Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway, *Molecular cell*. 19, 607–18. [PubMed: 16137618]
9. Vera J, Raatz Y, Wolkenhauer O, Kottek T, Bhattacharya A, Simon JC & Kunz M (2015) Chk1 and Wee1 control genotoxic-stress induced G2-M arrest in melanoma cells, *Cellular signalling*. 27, 951–60. [PubMed: 25683911]
10. Kumagai A & Dunphy WG (2000) Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts, *Molecular cell*. 6, 839–49. [PubMed: 11090622]
11. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA & Elledge SJ (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint, *Genes & development*. 14, 1448–59. [PubMed: 10859164]
12. Matsuyama M, Goto H, Kasahara K, Kawakami Y, Nakanishi M, Kiyono T, Goshima N & Inagaki M (2011) Nuclear Chk1 prevents premature mitotic entry, *Journal of cell science*. 124, 2113–9. [PubMed: 21628425]
13. Tang J, Erikson RL & Liu X (2006) Checkpoint kinase 1 (Chk1) is required for mitotic progression through negative regulation of polo-like kinase 1 (Plk1), *Proceedings of the National Academy of Sciences*. 103, 11964–11969.
14. Zachos G, Black EJ, Walker M, Scott MT, Vagnarelli P, Earnshaw WC & Gillespie DAF (2007) Chk1 Is Required for Spindle Checkpoint Function, *Developmental cell*. 12, 247–260. [PubMed: 17276342]
15. Zhao H & Piwnica-Worms H (2001) ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1, *Molecular and cellular biology*. 21, 4129–39. [PubMed: 11390642]
16. Chini CC & Chen J (2003) Human claspin is required for replication checkpoint control, *The Journal of biological chemistry*. 278, 30057–62. [PubMed: 12766152]
17. Liu S, Bekker-Jensen S, Mailand N, Lukas C, Bartek J & Lukas J (2006) Claspin Operates Downstream of TopBP1 To Direct ATR Signaling towards Chk1 Activation, *Molecular and cellular biology*. 26, 6056–6064. [PubMed: 16880517]
18. Draga M, Madgett EB, Vandenberg CJ, du Plessis D, Kaufmann A, Werler P, Chakraborty P, Lowndes NF & Hiom K (2015) BRCA1 Is Required for Maintenance of Phospho-Chk1 and G2/M Arrest during DNA Cross-Link Repair in DT40 Cells, *Molecular and cellular biology*. 35, 3829–40. [PubMed: 26324327]
19. Yarden RI, Pardo-Reoyo S, Sgagias M, Cowan KH & Brody LC (2002) BRCA1 regulates the G2/M checkpoint by activating Chk1 kinase upon DNA damage, *Nature genetics*. 30, 285. [PubMed: 11836499]
20. O'Connell MJ, Raleigh JM, Verkade HM & Nurse P (1997) Chk1 is a wee1 kinase in the G2 DNA damage checkpoint inhibiting cdc2 by Y15 phosphorylation, *The EMBO journal*. 16, 545–54. [PubMed: 9034337]
21. Bargiela-Iparraguirre J, Prado-Marchal L, Fernandez-Fuente M, Gutierrez-Gonzalez A, Moreno-Rubio J, Munoz-Fernandez M, Sereno M, Sanchez-Prieto R, Perona R & Sanchez-Perez I (2016)

- CHK1 expression in Gastric Cancer is modulated by p53 and RB1/E2F1: implications in chemo/radiotherapy response, *Scientific reports*. 6, 21519. [PubMed: 26867682]
22. Zhang YW, Brognard J, Coughlin C, You Z, Dolled-Filhart M, Aslanian A, Manning G, Abraham RT & Hunter T (2009) The F box protein Fbx6 regulates Chk1 stability and cellular sensitivity to replication stress, *Molecular cell*. 35, 442–53. [PubMed: 19716789]
 23. Park C, Suh Y & Cuervo AM (2015) Regulated degradation of Chk1 by chaperone-mediated autophagy in response to DNA damage, *Nature communications*. 6, 6823.
 24. Leung-Pineda V, Huh J & Piwnica-Worms H (2009) DDB1 Targets Chk1 to the Cul4 E3 Ligase Complex in Normal Cycling Cells and in Cells Experiencing Replication Stress, *Cancer research*. 69, 2630–2637. [PubMed: 19276361]
 25. Huh J & Piwnica-Worms H (2013) CRL4(CDT2) targets CHK1 for PCNA-independent destruction, *Molecular and cellular biology*. 33, 213–26. [PubMed: 23109433]
 26. Kim AJ, Kim H-J, Jee HJ, Song N, Kim M, Bae Y-S, Chung JH & Yun J (2011) Glucose deprivation is associated with Chk1 degradation through the ubiquitin-proteasome pathway and effective checkpoint response to replication blocks, *Biochimica et biophysica acta*. 1813, 1230–1238. [PubMed: 21440578]
 27. Ionomou M & Saunders DN (2016) Systematic approaches to identify E3 ligase substrates, *Biochemical Journal*. 473, 4083–4101. [PubMed: 27834739]
 28. Fok KL, Bose R, Sheng K, Chang CW, Katz-Egorov M, Culty M, Su S, Yang M, Ruan YC, Chan HC, Iavarone A, Lasorella A, Cencic R, Pelletier J, Nagano M, Xu W & Wing SS (2017) Huwe1 Regulates the Establishment and Maintenance of Spermatogonia by Suppressing DNA Damage Response, *Endocrinology*. 158, 4000–4016. [PubMed: 28938460]
 29. Hall JR, Kow E, Nevis KR, Lu CK, Luce KS, Zhong Q & Cook JG (2007) Cdc6 Stability Is Regulated by the Huwe1 Ubiquitin Ligase after DNA Damage, *Molecular biology of the cell*. 18, 3340–3350. [PubMed: 17567951]
 30. Kim HC & Huibregtse JM (2009) Polyubiquitination by HECT E3s and the determinants of chain type specificity, *Molecular and cellular biology*. 29, 3307–18. [PubMed: 19364824]
 31. Zhao X, Heng JI, Guardavaccaro D, Jiang R, Pagano M, Guillemot F, Iavarone A & Lasorella A (2008) The HECT-domain ubiquitin ligase Huwe1 controls neural differentiation and proliferation by destabilizing the N-Myc oncoprotein, *Nature cell biology*. 10, 643–53. [PubMed: 18488021]
 32. Inoue S, Hao Z, Elia AJ, Cescon D, Zhou L, Silvester J, Snow B, Harris IS, Sasaki M, Li WY, Itsumi M, Yamamoto K, Ueda T, Dominguez-Brauer C, Gorrini C, Chio IL, Haight J, You-Ten A, McCracken S, Wakeham A, Ghazarian D, Penn LJ, Melino G & Mak TW (2013) Mule/Huwe1/Arf-BP1 suppresses Ras-driven tumorigenesis by preventing c-Myc/Miz1-mediated down-regulation of p21 and p15, *Genes & development*. 27, 1101–14. [PubMed: 23699408]
 33. Gottifredi V, Karni-Schmidt O, Shieh SS & Prives C (2001) p53 down-regulates CHK1 through p21 and the retinoblastoma protein, *Molecular and cellular biology*. 21, 1066–76. [PubMed: 11158294]
 34. Haupt Y, Maya R, Kazaz A & Oren M (1997) Mdm2 promotes the rapid degradation of p53, *Nature*. 387, 296–9. [PubMed: 9153395]
 35. Katsuragi Y & Sagata N (2004) Regulation of Chk1 Kinase by Autoinhibition and ATR-mediated Phosphorylation, *Molecular biology of the cell*. 15, 1680–1689. [PubMed: 14767054]
 36. Emptage RP, Schoenberger MJ, Ferguson KM & Marmorstein R (2017) Intramolecular autoinhibition of checkpoint kinase 1 is mediated by conserved basic motifs of the C-terminal kinase-associated 1 domain, *The Journal of biological chemistry*. 292, 19024–19033. [PubMed: 28972186]
 37. Thrower JS, Hoffman L, Rechsteiner M & Pickart CM (2000) Recognition of the polyubiquitin proteolytic signal, *The EMBO journal*. 19, 94–102. [PubMed: 10619848]
 38. Pandya RK, Partridge JR, Love KR, Schwartz TU & Ploegh HL (2010) A structural element within the HUWE1 HECT domain modulates self-ubiquitination and substrate ubiquitination activities, *The Journal of biological chemistry*. 285, 5664–73. [PubMed: 20007713]
 39. Tu Y, Liu H, Zhu X, Shen H, Ma X, Wang F, Huang M, Gong J, Li X, Wang Y, Guo C & Tang T-S (2017) Ataxin-3 promotes genome integrity by stabilizing Chk1, *Nucleic acids research*. 45, 4532–4549. [PubMed: 28180282]

40. Heidelberger JB, Voigt A, Borisova ME, Petrosino G, Ruf S, Wagner SA & Beli P (2018) Proteomic profiling of VCP substrates links VCP to K6-linked ubiquitylation and c-Myc function, *EMBO reports*. 19.
41. Michel MA, Swatek KN, Hospenthal MK & Komander D (2017) Ubiquitin Linkage-Specific Affimers Reveal Insights into K6-Linked Ubiquitin Signaling, *Molecular cell*. 68, 233–246 e5. [PubMed: 28943312]
42. Adhikary S, Marinoni F, Hock A, Hulleman E, Popov N, Beier R, Bernard S, Quarto M, Capra M, Goettig S, Kogel U, Scheffner M, Helin K & Eilers M (2005) The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation, *Cell*. 123, 409–21. [PubMed: 16269333]
43. de Groot RE, Ganji RS, Bernatik O, Lloyd-Lewis B, Seipel K, Sedova K, Zdrahal Z, Dhople VM, Dale TC, Korswagen HC & Bryja V (2014) Huwe1-mediated ubiquitylation of dishevelled defines a negative feedback loop in the Wnt signaling pathway, *Science signaling*. 7, ra26. [PubMed: 24643799]
44. Silva GM, Finley D & Vogel C (2015) K63 polyubiquitination is a new modulator of the oxidative stress response, *Nature structural & molecular biology*. 22, 116–23.
45. Shang F, Deng G, Liu Q, Guo W, Haas AL, Crosas B, Finley D & Taylor A (2005) Lys6-modified ubiquitin inhibits ubiquitin-dependent protein degradation, *The Journal of biological chemistry*. 280, 20365–74. [PubMed: 15790562]
46. Lopez-Contreras AJ, Gutierrez-Martinez P, Specks J, Rodrigo-Perez S & Fernandez-Capetillo O (2012) An extra allele of Chk1 limits oncogene-induced replicative stress and promotes transformation, *J Exp Med*. 209, 455–61. [PubMed: 22370720]
47. Sarmiento LM, Pova V, Nascimento R, Real G, Antunes I, Martins LR, Moita C, Alves PM, Abecasis M, Moita LF, Parkhouse RM, Meijerink JP & Barata JT (2015) CHK1 overexpression in T-cell acute lymphoblastic leukemia is essential for proliferation and survival by preventing excessive replication stress, *Oncogene*. 34, 2978–90. [PubMed: 25132270]
48. Yun J & Lee W-H (2003) Degradation of Transcription Repressor ZBRK1 through the Ubiquitin-Proteasome Pathway Relieves Repression of Gadd45a upon DNA Damage, *Molecular and cellular biology*. 23, 7305–7314. [PubMed: 14517299]
49. Kurokawa M, Kim J, Geradts J, Matsuura K, Liu L, Ran X, Xia W, Ribar TJ, Henao R, Dewhirst MW, Kim W-J, Lucas JE, Wang S, Spector NL & Kornbluth S (2013) A Network of Substrates of the E3 Ubiquitin Ligases MDM2 and HUWE1 Control Apoptosis Independently of p53, *Science signaling*. 6, ra32–ra32. [PubMed: 23652204]
50. Shevchenko A, Tomas H, Havlis J, Olsen JV & Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes, *Nature protocols*. 1, 2856–60. [PubMed: 17406544]
51. Faherty BK & Gerber SA (2010) MacroSEQUEST: efficient candidate-centric searching and high-resolution correlation analysis for large-scale proteomics data sets, *Analytical chemistry*. 82, 6821–9. [PubMed: 20684545]
52. Taus T, Kocher T, Pichler P, Paschke C, Schmidt A, Henrich C & Mechtler K (2011) Universal and confident phosphorylation site localization using phosphoRS, *Journal of proteome research*. 10, 5354–62. [PubMed: 22073976]
53. Vizcaino JA, Cote RG, Csordas A, Dianes JA, Fabregat A, Foster JM, Griss J, Alpi E, Birim M, Contell J, O'Kelly G, Schoenegger A, Ovelheiro D, Perez-Riverol Y, Reisinger F, Rios D, Wang R & Hermjakob H (2013) The PRoteomics IDentifications (PRIDE) database and associated tools: status in 2013, *Nucleic acids research*. 41, D1063–9. [PubMed: 23203882]
54. Wang X, Lu G, Li L, Yi J, Yan K, Wang Y, Zhu B, Kuang J, Lin M, Zhang S, Shao G.(2014) HUWE1 interacts with BRCA1 and promotes its degradation in the ubiquitin-proteasome pathway. *Biochem Biophys Res Commun*. 444, 290–5. [PubMed: 24342616]

Figure 1. HUWE1 negatively regulates Chk1 protein abundance.

(A) HeLa cells were subjected to control, Chk1, or HUWE1 siRNA. Cells were harvested 48 hours after siRNA transfection. Immunoblot was performed for HUWE1, Chk1, γ H2AX (S139) and Actin (N=3). (B) HeLa cells were subjected to control (C) or HUWE1 (H) siRNA. Twenty-four and 48 hours later, the cells were harvested and immunoblot was performed for the indicated proteins. As a positive control, the cells were also treated with 25 μ M Etoposide (Etop) for 8 hours (N=3). (C) HeLa cells were subjected to control siRNA, HUWE1-A, or HUWE1-B siRNA. Cells were harvested 72 hours after siRNA transfection. Immunoblot was performed for HUWE1, Chk1, pChk1 (S296), and Actin (N=3).

Figure 2. HUWE1 depletion results in the stabilization of Chk1 protein.

Seventy-two hours following control or HUWE1 siRNA transfection, U2OS (**A**) and HeLa cells (**B**) were treated with 100 μ g/mL cycloheximide (CHX) for the indicated times and immunoblotted for Chk1 and actin (top). Densitometric analysis of the abundance of Chk1 from 4 independent experiments (mean $\pm$ SEM) is shown (bottom). Light grey series and trendline is HUWE1 KD, dark grey is control. * $p < 0.05$ by a paired, two tailed Student's *t*-test.

Figure 3. MDM2 does not regulate Chk1 protein abundance.

U2OS cells were subjected to scramble, HUWE1, or MDM2 siRNA. Cells were harvested 72 hours after siRNA transfection. Immunoblot was performed for the indicated proteins. “Scramble” = scramble siRNA, “HUWE1” = HUWE1 siRNA and “MDM2” = MDM2 siRNA (N=2).

Figure 4. HUWE1 depletion diminishes Chk1 ubiquitination in normally cycling cells. HeLa cells were transfected with either control or HUWE1 siRNA. Forty-eight hours after siRNA, the cells were re-plated and further transfected with HA-ubiquitin. Twenty-four hours later, ubiquitinated proteins were retrieved by anti-HA immunoprecipitation. **(A)** Confirmation of HUWE1 KD and Chk1 stabilization in immunoprecipitation input by Western blot (N=3). **(B)** Western blot of immunoprecipitation eluates with an anti-Chk1 antibody (N=3).

Figure 5. Recombinant HUWE1 HECT poly-ubiquitinates active Chk1 *in vitro*

A) In vitro ubiquitination assays were performed with the active HUWE1 HECT domain (lane 2) and the inactive HECT point mutant (C4341S: lane 1) using recombinant p53 as a substrate. The non-ubiquitinated form of p53 is indicated by the arrow. (N=2) **B)** In vitro ubiquitination assays were performed using wild type (lane 2) or the catalytically inactive mutant form (lane 1) of the HUWE1 HECT with recombinant Chk1 as a substrate. The arrow indicates the non-ubiquitinated form of Chk1. (N=3) **C)** Functional domain map of Chk1 indicating 9 ubiquitin modification sites (red dots) as determined by LC-MS/MS analysis. Each indicated ubiquitinated site was sequenced at least twice (2 TP). Stacked dots indicate the presence of multiple ubiquitinated sites found adjacent to one another (2 amino acids).

Figure 6. Full-length HUWE1 ubiquitinates Chk1 and MDM2 does not ubiquitinate Chk1 *in vitro*.

Full-length HUWE1 or MDM2 was incubated with Chk1 for 3 hours *in vitro*, with and without E2 (lanes 1, 2, & 3; N=2). MDM2 was incubated with p53 under identical conditions *in vitro* (lanes 4 & 5; N=2)

Figure 7. HUWE1 HECT domain interacts with endogenous Chk1.

(**A**) Recombinant GST (arrow) and GST-HECT proteins (arrowhead) were produced in *E. coli* and were purified. Shown is Coomassie Brilliant Blue staining. (**B**) HeLa cells were left untreated or treated with hydroxyurea (50 mM) for 30 min (N=3). Cell lysates were prepared and incubated with GST or GST-HECT protein for 1 hour. Proteins bound to the GST proteins were retrieved by glutathione Sepharose beads and subjected to immunoblot for Vinculin and Chk1. Endogenous Chk1 in the cell lysates and Chk1 bound to GST-HECT are indicated by arrow and arrowhead, respectively.

Figure 8. HUWE1 is a prominent steady-state E3 ubiquitin ligase for Chk1.

HeLa cells were transfected with the indicated siRNA pools and harvested 48 hours after transfection. **A**) Representative blot. **B**) Quantification of the average of five replicate experiments, normalized to loading (actin). * = p-value < 0.05, Student's t-test. N.S. = not significant. Error bars indicate standard deviation from the mean

Figure 9. HUWE1 also regulates Chk1 stability in cells under replication stress.

HeLa cells were transfected with the indicated siRNA pools for 48 hours followed by treatment with either 20mM hydroxyurea or 1 μ M camptothecin for 8 hours. **A)**

Representative Western blot where: “-” = mock/reagent only; “H” = siHUWE1; “C” = siCul4A. **B)** Quantification of the average of five replicate experiments, normalized to loading (actin). * = p-value < 0.05, Student’s t-test. N.S. = not significant. Error bars indicate standard deviation from the mean

Table 1.

The HUWE1 HECT domain multiply ubiquitinates Chk1.

Sequence	PTM-site In Chk1	TP (WT)	TP (C4341S)
MCGT#LPYVAPELL K !R	T170 : K180	1	0
MCGTLPYVAPELL K !R	K180	1	0
E K !KT#YLNPWK	K224 : T226	1	0
K !TYLNPWK	K225	1	0
K !IDS#APLALLHK	K233 : S236	1	0
K !IDSAPLALLHK	K233	1	0
IT#IPDI K ! K !DR	T255: K260:K261	1	0
ITIPDI K ! K !DR	K260:K261	1	0
DRWYN K !PLK	K266	1	0
DRWYN K !PLKK	K266	2	0
WYN K !PLK	K267	2	0
WYN K !PLKK	K267	2	0
WYN K !PL K !K	K267:K270	2	0
WYN K !PL K !GAK	K267:K271	1	0
WYNKPL K !K	K270	3	0
LDAD K !SYQCLK	K388	1	0
ETCE K !LGYQWK	K399	1	0
K !SCMNQVTISTTDRR	K405	1	0
RAVDCPEN K !K	K53	1	0
AVDCPEN K !K	K53	1	0
EICIN K !MLNHENVVK	K60	2	0
MLNHENVV K !FYGHR	K69	1	0

Total peptide (TP) counts for reactions containing either the WT or the catalytically dead (C4341S) HECT domain. “**K**!” indicates an ubiquitinated lysine. “#” indicates the previous residue is phosphorylated and “*” indicates an oxidized methionine. The PTM-site column indicates the position of the modified residue within the Chk1 protein. Use of a “ : ” indicates that more than one modifiable residue (K/S/T/Y) on a given peptide was determined to be modified. Sites highlighted in yellow are mapped in Fig. 5C.

Table 2.

Recombinant Chk1 is phosphorylated during the *in vitro* ubiquitination reaction.

Sequence	Phos-sites	WT TP	C4341S TP
DIKPENLLDNERDNLKIS#DFGLATVFR	S147	1	1
DNLKIS#DFGLATVFR	S147	2	1
IDS#APLALLHK	S236	5	2
KIDS#APLALLHK	S236	7	4
K!IDS#APLALLHK	S236	2	0
ILVENPS#AR	S251	1	1
ILVENPS#ARIT#IPDIKK	S251:T255	3	3
VTS#GGVSESPSGFSK	S280	2	2
VTS#GGVS#ESPSGFSK	S280:S284	1	1
HIQS#NLDFSPVNSASSEENVK	S296	0	1
HIQS#NLDFS#PVNSASSEENVK	S296:S301	1	2
HIQS#NLDFSPVNS#ASSEENVK	S296:S305	3	1
HIQS#NLDFSPVNSAS#SEENVK	S296:S307	1	0
YSS#SQPEPR	S316	2	2
YSS#SQPEPR	S317	1	1
TGLS#LWDTSPSYIDK	S326	1	0
TGLS#LWDTSPSYIDK	S326:S331	1	1
TGLSLWDTSPSYIDK	S333	1	0
LDADKS#YQCLK	S389	1	2
KS#CM*NQVTISTTDR	S407	1	0
KS#CMNQVTISTTDRR	S407	0	1
KS#CM*NQVT#ISTTDRR	S407:T413	1	0
KS#CMNQVT#ISTTDRR	S407:T413	0	1
LIDIVS#SQK	S467	3	2
ISDFGLAT#VFR	T153	1	1
MCGT#LPYVAPELLK	T170	1	1
M*CGT#LPYVAPELLK!R	T170	1	0
M*CGT#LPYVAPELLK	T170	1	0
MCGT#LPYVAPELLK!R	T170	1	0
M*CGT#LPY#VAPELLK	T170:Y173	1	0
MCGT#LPY#VAPELLK	T170:Y173	0	1
KT#YLNPKW	T226	3	1
EKKT#YLNPKW	T226	2	1
T#YLNPKW	T226	1	1
KT#Y#LNPKW	T226:Y227	1	1
IT#IPDIK!K!DR	T255	1	0
IT#IPDIKK	T255	2	0
RMT#RFFT#KLDADK	T378:T382	1	2
RM*T#RFFT#KLDADK	T378:T382	1	0

Sequence	Phos-sites	WT TP	C4341S TP
FFT#KLDADK	T382	2	1
FFT#KLDADKSYQCLK	T382	1	1
FFT#KLDADKS#YQCLK	T382:S389	0	2
SCM*NQVT#ISTTDRR	T413	1	1
SCMNQVT#ISTTDR	T413	0	1
DRWY#NKPLKK	Y265	0	1
LGY#QWK	Y402	1	0
LGY#QWKK	Y402	1	0

A comparison of total peptide counts for sequences containing the indicated phosphorylated residues from either the WT or catalytically inactive (C4341S) HECT *in vitro* ubiquitination reactions. “#” indicates the previous residue is phosphorylated and “*” indicates an oxidized methionine. The phos-site column indicates the position of the phosphorylated residue (or residues) within the Chk1 protein. Use of a “ : ” indicates that more than one phosphoacceptor (S/T/Y) on a given peptide was modified.

Table 3.

HUWE1 is phosphorylated by Chk1.

Sequence	Phos-sites	WT TP	C4341S TP
DHVFEDS#YRELHR	S4030	2	2
RDHVFEDS#YR	S4030	0	1
RDHVFEDS#YRELHR	S4030	2	2
VTYTINPS#SHCNPNHLSYFK	S4096	1	1
VTYTINPS#S#HCNPNNHLSYFK	S4096:S4097	0	1
YQS#NSIQIQWFWR	S4273	0	1
ALRS#FDQADR	S4287	2	2
DDRS#TDRLPSAHTCFNQDLDPAYESFEK	S4331	1	0
STDRLPS#AHTCFNQDLDPAYESFEK	S4337	1	0
STDRLPS#AHT#CFNQDLDPAYESFEK	S4337:T4340	1	0
VTYT#INPSSHCHNPNNHLSYFK	T4092	0	2
VTYT#INPS#SHCNPNHLSYFK	T4092:S4096	0	1
DLKPNGANILVT#EENKK	T4199	0	1
FLQFVT#GTSKVPLQGFAALEGMNGIQK	T4301	0	2
LPSAHT#CFNQDLDPAYESFEK	T4340	1	0
STDRLPSAHT#CFNQDLDPAYESFEK	T4340	1	0
TSPGDRVTY#T#INPSSHCHNPNNHLSYFK	Y4091:T4092	0	1

Total peptide (TP) counts for each phosphorylated peptide for reactions containing either the WT or the catalytically inactive (C4341S) HECT domain. “#” indicates a phosphorylated residue. The “Phos-sites” column indicates the position of the modified residue within the HUWE1 protein. Use of a “:” indicates that more than one phosphoacceptor (S/T/Y) on a given peptide was modified.

Table 4.

The HUWE1 HECT domain predominantly assembles K48-linked poly-ubiquitin in *in vitro* reactions with Chk1.

Sequence	Ub-sites	WT TP	C4341S TP
MQIFV K !TLTG K !TITLEVEPSDTIENVK	K6:K11	1	0
MQIFV K !TLTGK	K6	8	2
IQD K !EGIPPDQQR	K33	5	0
TITLEVEPSDTIENV K !AK	K27	1	0
TLTG K !TITLEVEPSDTIENVK	K11	4	1
LIFAG K !QLEDGR	K48	19	2
TLSDYNIQ K !ESTLHLVLR	K63	2	1
TLSDYNIQ K !ESTLHLVLR	K63	2	0

Total peptide (TP) counts for each ubiquitin peptide for reactions containing either the WT or the catalytically inactive (C4341S) HECT domain. “**K**!” indicates a ubiquitinated lysine. The “Ub-sites” column indicates the position of the modified residue within the ubiquitin protein. Use of a “:” indicates that more than one lysine on a given peptide was ubiquitinated.

Table 5.The HUWE1 HECT domain auto-ubiquitinates *in vitro*.

Sequence	Ub-sites	WT TP	C4341S TP
VLD F DV K !R	K3996	2	0
VLD F DV K !RK	K3996	1	0
LDEGLR K !EDM*AVHVR	K4014	2	0
LDEGLR K !EDMAVHVR	K4014	2	0
IV A K!AVYDNR	K4116	1	0
SFY K !HILGK	K4134	2	0
DL K !PNGANILVTEENKK	K4190	2	0
DLKPNGANILVTEEN K !KEYVHLVCQMR	K4203	1	0
A K !FLQFVTGTSK	K4295	1	0
FLQFVTGT S K!VPLQGFAALEGM*NGIQK	K4305	1	0
FLQFVTGT S K!VPLQGFAALEGMNGIQK	K4305	1	0
VPLQGFAALEGM*NGIQ K !FQIHR	K4322	1	0
VPLQGFAALEGMNGIQ K !FQIHR	K4322	1	0
LPSAHTCFNQLDLPAYESFE K !LR	K4355	1	0

Total peptide (TP) counts for each HUWE1 peptide for reactions containing either the WT or the catalytically inactive (C4341S) HECT domain. “**K**!” indicates a ubiquitinated lysine and “*” indicates an oxidized methionine. The “Ub-sites” column indicates the position of the modified residue within HUWE1 protein.