Regulation of Ubiquitin-like with Plant Homeodomain and RING Finger Domain 1 (UHRF1) Protein Stability by Heat Shock Protein 90 Chaperone Machinery*

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As a protein critical for DNA maintenance methylation and cell proliferation, UHRF1 is frequently highly expressed in various human cancers and is considered as a drug target for cancer therapy. In a high throughput screening for small molecules that induce UHRF1 protein degradation, we have identified the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG). We present evidence that UHRF1 interacts with HSP90 chaperone complex and is a novel HSP90 client protein. Pharmacological inhibition of HSP90 with 17-AAG or 17-demethoxygeldanamycin results in UHRF1 ubiquitination and proteasome-dependent degradation. Interestingly, this HSP90 inhibitor-induced UHRF1 degradation is independent of CHIP and CUL5, two previously identified ubiquitin E3 ligases for HSP90 client proteins. In addition, this degradation is dependent neither on the intrinsic E3 ligase of UHRF1 nor on the E3 ligase SCFβ-TRCP that has been implicated in regulation of UHRF1 stability. We also provide evidence that HSP90 inhibitors may suppress cancer cell proliferation in part through its induced UHRF1 degradation. Taken together, our results identify UHRF1 as a novel HSP90 client protein and shed light on the regulation of UHRF1 stability and function.

Ubiquitin-like with PHD§ and RING finger domain 1 (UHRF1) was originally isolated in a yeast one-hybrid screen as a protein that binds to the CCAAT box within the topoisomerase II α gene promoter (1). UHRF1 (also known as ICBP90 in humans and Np95 in mice) is now well recognized for its essential role in DNA maintenance methylation via targeting DNMT1 to DNA replication forks during the S phase of the cell cycle (2, 3). UHRF1 itself binds to DNA replication forks through a coordinated recognition of hemimethylated CpG dinucleotides by its unique SRA domain and histone H3 tails with di- or trimethylated lysine 9 by a concerted function of a tandem Tudor domain and PHD (4–9). DNMT1 is then recruited most likely through a physical interaction with both UHRF1 and ubiquitinated H3; the latter is catalyzed by the E3 ligase activity of UHRF1 (6, 10–12).

In addition to its critical role in DNA methylation, UHRF1 is also known as a key regulator of cell proliferation. Numerous studies have shown that UHRF1 is overexpressed in various cancers, and its overexpression correlates with tumor progression (13–15). Moreover, several studies have demonstrated that overexpression of UHRF1 drives cell proliferation, whereas knockdown of UHRF1 results in a strong cell cycle arrest, hypersensitivity to DNA damage and chemotherapeutic agents, and increased apoptosis (16–18). Collectively, these studies have led to the proposal that UHRF1 is not only a tumor biomarker but also a potential target for cancer therapy. However, currently there is no reported small molecule that either inhibits UHRF1 activity or induces its degradation.

HSP90 (90-kDa heat-shock protein) is one of the most abundant and conserved molecular chaperone essential in eukaryotic cells (19). Unlike HSP70, HSP90 is not required for de novo folding of most proteins but promotes the final maturation of a selected group of proteins termed HSP90 client proteins (20). The HSP90 client proteins are enriched in protein kinases, transcription factors, nuclear steroid receptors, and regulatory proteins (21, 22). Most commonly, HSP90 facilitates their stabilization, and activation and inactivation of HSP90 by small molecule inhibitors, such as the 17-allylamino-17-demethoxygeldanamycin (17-AAG), often leads to their degradation through the ubiquitin-proteasome system (20–22). Given that numerous oncproteins have been shown to be HSP90 client proteins and that HSP90 is highly expressed in tumors compared with normal tissues,
the development of HSP90 inhibitors has become a new strategy in cancer therapy (23).

In an effort to screen for small molecules that affect UHRF1 stability, we identified the HSP90 inhibitor 17-AAG as a potent inducer of UHRF1 degradation. We present evidence that UHRF1 specifically interacts with HSP90 and is a novel HSP90 client protein.

**Results**

Identification of HSP90 Inhibitor 17-AAG as a Small Molecule Potently Inducing Down-regulation of UHRF1 Proteins—Because UHRF1 is aberrantly highly expressed in multiple types of cancers and UHRF1 knockdown causes cell cycle arrest, activation of DNA damage response, and apoptosis in different types of cancer cells (16, 17), UHRF1 has been considered as a druggable target for cancer therapy (24, 25). We thus wished to screen for small molecules that could induce UHRF1 degradation in cancer cells. To this end, we generated a stable HeLa cell line that constitutively expresses a GFP-tagged UHRF1 under the control of the CMV promoter. The cells were cultured in 384-well plates and treated with various compounds for 24 h. In this study, a library of 2240 chemicals in total (244 protein kinase inhibitors (Merck), 84 cancer regulators, 480 known bioactivators (ICCB), 43 epigenetic regulators, 303 regulators in stem cells, 446 therapeutic molecules from the National Institutes of Health collection, and 640 agents from a Food and Drug Administration-approved drug library) was used. Using an automated fluorescence microscopy system, we screened for compounds that significantly diminished the intensity of GFP fluorescence (Fig. 1A). 39 compounds were positive for decreasing GFPmean to ~600 nm, compared with a GFPmean of ~1002 nm in DMSO (1%) and mock controls. Among them, 17 compounds were able to reduce GFP intensities in GFP-UHRF1-HeLa cells but not the control GFP-HeLa cells, suggesting that these compounds were probably targeting at UHRF1 rather than GFP.

**FIGURE 1.** Identification and characterization of HSP90 inhibitors as small molecules down-regulating UHRF1 post-transcriptionally. A, HeLa cells stably expressing GFP-UHRF1 were treated with a small molecule library of ~2000 compounds in a format of 384-well plates. After 24 h of treatment, the levels of GFP-UHRF1 fluorescence were acquired and analyzed through an automated microscopy system. B, immunostaining assay showing that both HSP90 inhibitors, 17-AAG and 17-DMAG, down-regulated endogenous UHRF1 in HeLa cells. The experiments were repeated three times, and representative results are shown. C, immunostaining assay showing HSP90 inhibitors 17-AAG and 17-DMAG down-regulated ectopically expressed FLAG-UHRF1 in NIH3T3 cells. For all Western blots, the experiments were repeated at least three times, and representative results were shown. D, Western blotting analysis showing that 17-AAG treatment down-regulated UHRF1 in various cancer cells. E, Western blotting analysis showing that 17-DMAG treatment down-regulated UHRF1 in various cancer cells. F, both HSP90 inhibitors 17-AAG and 17-DMAG down-regulated UHRF1 in a dose-dependent manner. HeLa cells were treated with 0.01, 0.1, and 1 μM 17-AAG or 17-DMAG for 24 h. G, HSP90 inhibitors had no effect on UHRF1 transcription. Shown is mean ± S.D. (error bars) of triplicate biological assays.
The HSP90 inhibitor 17-AAG was identified as the top hit in this screen, and for this study we focused on this molecule.

To validate the result of this screen, we treated the parental HeLa cells with 17-AAG and its analogue 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) for 24 h, and subsequent immunofluorescence staining for endogenous UHRF1 revealed that treatment with either 17-AAG or 17-DMAG led to a marked reduction of endogenous UHRF1 (Fig. 1B). Furthermore, we tested the ability for both HSP90 inhibitors to down-regulate the levels of FLAG-UHRF1 stably expressed in NIH3T3 cells. As shown in Fig. 1C, both inhibitors down-regulated the levels of FLAG-UHRF1 in these NIH3T3 cells. These data thus demonstrate that HSP90 inhibitors are able to down-regulate both endogenous and ectopically expressed UHRF1.

To test whether 17-AAG could down-regulate UHRF1 in various types of cancer cells, we treated HeLa (cervical cancer), HCT116 (colon cancer), A549 (lung cancer), and 22RV1 (prostate cancer) cells with 17-AAG for 12, 24, and 36 h, respectively. The cells were then collected and subjected to Western blotting analysis. As shown in Fig. 1D, 17-AAG treatment induced a time-dependent decrease of UHRF1 proteins in all of the cell lines tested. Note that 17-AAG treatment for 12 h was sufficient to induce the UHRF1 down-regulation (Fig. 1D). This effect on UHRF1 is specific, because 17-AAG treatment did not significantly affect the levels of DNMT1 and DNMT3A in HeLa and A549 cells (Fig. 1D) and HCT116 and 22RV1 (data not shown).

As a positive control, we confirmed by Western blotting analysis that 17-AAG treatment led to a marked increase of HSP70 proteins, in agreement with the notion that inhibition of HSP90 induces HSP70 expression. Consistent with the immunostaining results in Fig. 1B, the down-regulation of UHRF1 was also observed by Western blotting analysis when another HSP90 inhibitor, 17-DMAG, was used (Fig. 1E). As expected, down-regulation of UHRF1 by both HSP90 inhibitors is dependent on the concentration of the inhibitors (Fig. 1F). Furthermore, we found that treatment with 17-AAG or 17-DMAG had little effect on the levels of UHRF1 mRNA, although it drastically increased the levels of HSP70 mRNAs (Fig. 1G). Together these data demonstrate that both HSP90 inhibitors induce down-regulation of UHRF1 proteins via a post-transcriptional mechanism.

**HSP90 Inhibitors Induce UHRF1 Degradation via a Ubiquitin-dependent Proteasome Pathway**—Previous studies indicated that HSP90 inhibitors can induce HSP90 client degradation via the ubiquitin-proteasomal degradation pathway (20–22). We thus tested whether the HSP90 inhibitors induce UHRF1 down-regulation via proteasome degradation. We added, with or without MG132, a proteasome inhibitor together with the HSP90 inhibitors and examined the levels of UHRF1 by Western blotting analysis. As shown in Fig. 2A, the addition of MG132 blocked the 17-AAG- and 17-DMAG-induced UHRF1 decrease in HeLa, A549, and LNCaP cell lines, indicating that the reduced protein levels of UHRF1 upon
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HSP90 inhibitor treatment are indeed mediated through the proteasome pathway. To ascertain that HSP90 inhibitor treatment could induce UHRF1 degradation, we determined the effects of 17-DMAG on the stability of the UHRF1. To this end, HeLa and stable FLAG-UHRF1 NIH3T3 cells were treated with cycloheximide to block new protein synthesis and with or without 17-DMAG for 0–9 h as indicated. The cells were collected and subjected to Western blotting analysis using either anti-UHRF1 or anti-FLAG antibody. The representative results in Fig. 2B and quantitative results in Fig. 2C showed that 17-DMAG treatment shortened the half-life of both endogenous and exogenous expressed UHRF1 in HeLa and NIH3T3 cells, respectively.

To assess whether UHRF1 underwent ubiquitination upon treatment with HSP90 inhibitors, HeLa cells were cotransfected with FLAG-tagged UHRF1 and HA-Ub and treated with or without 17-AAG or 17-DMAG for 24 h. The cells were lysed under denaturing conditions, and FLAG-tagged UHRF1 protein was immunoprecipitated by anti-FLAG M2-agarose beads and then probed with anti-HA antibody to evaluate the levels of ubiquitination. As shown in Fig. 2D, the levels of UHRF1 polyubiquitination were significantly enhanced upon treatment with either 17-AAG or 17-DMAG. Collectively, these experiments indicate that HSP90 inhibitors induce UHRF1 ubiquitination and subsequently a ubiquitin-dependent proteasome degradation.

17-AAG-induced UHRF1 Degradation Is Dependent on HSP70—HSP70 is a central player in protein folding and proteostasis control (26). Earlier studies have shown that the correct folding and maturation of HSP90 client proteins is a stepwise process in which the client proteins form “early complex” with HSP70/HSP40 first and then are transferred to HSP90 (27, 28). Once HSP90 is inhibited, HSP70 is likely to target the immature client proteins for ubiquitin-dependent degradation (28, 29). Thus, to test further whether UHRF1 is a bona fide HSP90 client protein, we asked whether UHRF1 interacts with HSP70 and HSP90 and whether 17-AAG-induced UHRF1 degradation is dependent on HSP70. In this regard, both HSP70 and HSP90 were found to associate with endogenous UHRF1 in our previous proteomic study (data not shown). To confirm these proteomic data, we performed a co-immunoprecipitation assay. HEK293T cells were co-transfected with FLAG-tagged UHRF1 and HA-tagged HSP70 or HSP90 expression vectors. The whole cell extracts were then prepared and subjected to a co-immunoprecipitation assay. Subsequent Western blotting analysis showed that FLAG-UHRF1 was detected in anti-HA immunoprecipitates only when HA-HSP70 or HA-HSP90 was expressed (Fig. 3A, top). Reciprocally, HA-HSP70 and HA-HSP90 was specifically detected in anti-FLAG immunoprecipitates, indicating that HSP70 and HSP90 indeed interact with UHRF1 (Fig. 3A, bottom). We next attempted to examine the interaction between endogenous UHRF1 and HSP70 and HSP90 and also test how HSP90 inhibitor treatment affects their interaction by a co-immunoprecipitation assay. However, this attempt was unsuccessful due to high background of immunoglobulin heavy chain resulting from immunoprecipitation with anti-UHRF1 antibody. We thus resorted to a HeLa cell line stably expressing FLAG-UHRF1 that allowed us to immunoprecipitate UHRF1 with anti-FLAG M2 beads. As shown in Fig. 3B, treatment with HSP90 inhibitors for 24 h induced UHRF1 degradation and up-regulation of HSP70 and HSP90. However, IP-Western blotting analysis indicated that, despite reduced levels of UHRF1 proteins, HSP90 inhibitor treatment resulted in increased interaction between UHRF1 and HSP70 and HSP90 (Fig. 3C), suggesting that inactivation of HSP90 by 17-AAG increased the levels of immature UHRF1 proteins. This observation was further confirmed by a time course experiment in which a marked increase of interaction between HSP70 and HSP90 was observed in both control sh-Vector or sh-HSP70 as indicated and knockdown of HSP70 was confirmed by Western blot analysis. Note that 17-AAG treatment failed to induce down-regulation of UHRF1 in sh-HSP70 cells.

To address whether 17-AAG-induced UHRF1 degradation is dependent on HSP70, we constructed a HeLa cell line stably expressing an shHSP70. The efficient knockdown of HSP70 in this cell line was confirmed by Western blotting analysis. We found that HSP70 knockdown significantly mitigated 17-AAG-induced degradation of UHRF1 (Fig. 3E). Taken together, these data confirm that UHRF1 is a bona fide HSP90 client protein and provide evidence that HSP70 mediates HSP90 inhibitor-induced UHRF1 degradation.

The E3 Ligases CHIP and CIUL5 Are Not Required for HSP90 Inhibitor-induced UHRF1 Degradation—The C terminus of Hsc70-interacting protein (CHIP; also known as STUB1) is a co-chaperone that has an intrinsic E3 ubiquitin ligase activity. CHIP interacts with both HSP70 and HSP90 and has been
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The Intrinsic E3 Ligase Activity of UHRF1 Is Not Required for HSP90 Inhibitor-induced UHRF1 Degradation—UHRF1 possesses an intrinsic E3 ligase activity due to the presence of a RING domain within its C terminus. This E3 ligase activity has been shown to catalyze UHRF1 autoubiquitination as well as ubiquitination of histones and non-histone substrates, and UHRF1 autoubiquitination has been implicated in regulation of UHRF1 stability (6, 10–12, 39). Having excluded CHIP and CUL5 as the E3 ligase(s) mediating HSP90 inhibitor-induced UHRF1 degradation, we asked whether HSP90 inhibitor-induced UHRF1 degradation would be mediated through its intrinsic E3 ligase activity. To this end, we generated HeLa cell lines stably expressing FLAG-tagged wild-type UHRF1 (WT), UHRF1 with deletion of RING domain (∆RING), or UHRF1 with deletion of RING plus the polybasic region between SRA and RING (residues 1–675) (Fig. 4D). These cells were treated with or without 17-DMAG in the presence or absence of the proteasome inhibitor MG132 for 24 h, and the levels of FLAG-tagged UHRF1 and deletion mutants were determined by Western blotting analysis. The representative results in Fig. 4D showed that 17-DMAG treatment induced the degradation of all three types of FLAG-UHRF1. Furthermore, the addition of MG132 substantially elevated the levels of both wild-type and mutant UHRF1, indicating that these proteins were constantly degraded via the ubiquitin-dependent proteasome pathway. Together these data indicate that the HSP90 inhibitor-induced UHRF1 degradation is also independent of its intrinsic E3 ligase activity.

The SCFβ-TRCP E3 Ligase Complex Is Dispensable for HSP90 Inhibitor-induced UHRF1 Degradation—The SCF ubiquitin ligase complex is composed of S phase kinase-associated protein 1 (SKP1); the scaffold protein Cullin 1 (CUL1); the small RING protein RBX1, which interacts with E2 enzyme; and the variable F-box protein (40). The F-box proteins confer substrate selectivity by targeting multiple substrates, including β-catenin, CDC25A, IκB, and DEPTOR for ubiquitination-dependent degradation (41). Recently, it was reported that the F-box protein β-TRCP1 can mediate proteasomal degradation of UHRF1 via SCFβ-TRCP E3 ligase upon DNA damage (42). Hence, we asked whether the SCFβ-TRCP E3 ligase would mediate HSP90 inhibitor-induced UHRF1 degradation. We first made use of one siRNA that has been commonly used to knock down both β-TRCP1 and β-TRCP2, two highly related proteins (β-TRCP1/2) (24). By using antibody against β-TRCP1, we confirmed that treatment with this specific siRNA effectively knocked down both β-TRCP1 and β-TRCP2, two highly related proteins (β-TRCP1/2) (24). Using antibody against β-TRCP1, we confirmed that treatment with this specific siRNA effectively knocked down both β-TRCP1 and β-TRCP2, two highly related proteins (β-TRCP1/2) (24). Using antibody against β-TRCP1, we confirmed that treatment with this specific siRNA effectively knocked down both β-TRCP1 and β-TRCP2, two highly related proteins (β-TRCP1/2) (24). Using antibody against β-TRCP1, we confirmed that treatment with this specific siRNA effectively knocked down both β-TRCP1 and β-TRCP2, two highly related proteins (β-TRCP1/2) (24).
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FIGURE 5. The β-TRCP1/2 and CUL1 SCF E3 ligases are not required for HSP90 inhibitor-induced UHRF1 degradation. A, knockdown of β-TRCP1/2 had no effect on the steady-state levels of UHRF1 in HeLa cells. HeLa cells were transfected with either control scrambled siRNA (NC) or an siRNA against both β-TRCP1 and β-TRCP2 for 72 h and then harvested for immunoblotting analysis using antibodies as indicated. B, knockdown β-TRCP1/2 had no effect on steady-state levels of UHRF1 in 293T, HCT116, and A549 cells. C, stable knockdown of β-TRCP1/2 by shRNA had no effect on the steady-state levels of UHRF1 proteins in 293T, HeLa, HCT116, and A549 cell lines. D, β-TRCP1/2 knockdown had no effect on HSP90 inhibitor-induced UHRF1 degradation. The stable knockdown cell lines as in C were treated with 1 μM 17-AAG, 17-DMAG, or solvent control for 24 h and then immunoblotted with antibodies as indicated. E, knockdown of CUL1, a scaffold of the SCF complex, had no effect on the steady-state levels of UHRF1. F, CUL1 knockdown had no effect on HSP90 inhibitor-induced UHRF1 degradation. The CUL1 stable knockdown cells were treated with 1 μM 17-AAG, 17-DMAG, or solvent control for 24 h and then immunoblotted with antibodies as indicated.

also had little, if any, effect on the steady-state levels of UHRF1 proteins in all four cell lines tested (25) (Fig. 5C), indicating that the SCFβ-TRCP E3 ligase has no effect on the steady-state levels of UHRF1 under regular growth conditions. We next tested whether knockdown of β-TRCP1/2 could affect HSP90 inhibitor-induced UHRF1 degradation. As shown in Fig. 5D, we found that knockdown of β-TRCP1/2 did not affect 17-AAG- and 17-DMAG-induced UHRF1 degradation. We thus conclude that β-TRCP1/2 are also not essential for HSP90 inhibitor-induced UHRF1 degradation.

In the human genome, there are 69 F-box proteins that can be categorized into three families (40, 41), raising the possibility that the F-box proteins other than β-TRCP1/2 may mediate HSP90 inhibitor-induced UHRF1 degradation. To test this possibility, we made use of two different shRNAs to stably knock down CUL1, the common subunit of the SCFβ-TRCP E3 ligase in HEK293T and HeLa cell lines (Fig. 5E). Although knockdown of CUL1 led to accumulation of β-catenin in 293T and HeLa cells (Fig. 5E), it again did not significantly alter the levels of UHRF1 proteins. Furthermore, knockdown of CUL1 did not affect 17-AAG and 17-DMAG-induced UHRF1 degradation (Fig. 5F). Together we concluded that SCFβ-TRCP1/2 E3 ligase is not required for HSP90 inhibitor-induced UHRF1 degradation.

Down-regulation of UHRF1 May Partly Account for the Pharmacological Anti-tumor Effect of HSP90 Inhibitors—HSP90 is required for the stability and function of numerous oncogenic proteins, and its inhibitors display multiple anticancer effects, including cell cycle arrest, increased DNA damage, and apoptosis (44–46). UHRF1 is considered an oncogenic protein, because it is overexpressed in various cancers, and knockdown of UHRF1 has also been shown to cause cell cycle arrest, increased DNA damage, and apoptosis (16, 17). We thus wished to compare the effect of UHRF1 knockdown with that of HSP90 inhibitors. We confirmed that treating HeLa or A549 cells with either 17-AAG or 17-DMAG substantially reduced the numbers of viable cells, most likely as a combinatorial effect on inhibition of cell proliferation and induction of apoptosis (Fig. 6A). Knockdown of UHRF1 in either HeLa or A549 cells also reduced the numbers of viable cells, although less dramatically compared with the treatment with HSP90 inhibitors (Fig. 6B).

The knockdown of UHRF1 in these experiments was confirmed by quantitative RT-PCR (Fig. 6B, right). However, it should be pointed out the effect of UHRF1 knockdown on cell proliferation is probably underestimated in this study, possibly due to the presence of residual UHRF1, because complete UHRF1 knockout by CRISPR-cas9 in HeLa cells caused massive cellular apoptosis (data not shown). On the other hand, overexpression of UHRF1 in NIH3T3 fibroblast cells enhanced cell proliferation (Fig. 6C). To compare DNA damage and apoptosis induced by HSP90 inhibitors and UHRF1 knockdown, we examined the levels of γH2AX and the cleavage product of apoptosis effector poly(ADP-ribose) polymerase (PARP). As shown in Fig. 6D, both HSP90 inhibitor treatment and UHRF1 knockdown resulted in elevated levels of γH2AX and PARP cleavage. Again, HSP90 inhibitor is more effective than UHRF1 knockdown in inducing PARP cleavage and generation of γH2AX. Together these data indicate that UHRF1 knockdown generates a phenotype similar to but less dramatic than that of HSP90 inhibitor treatment, suggesting that down-regulation of UHRF1 by HSP90 inhibitors may partly account for the pharmacological anticancer effect of HSP90 inhibitors.

To test whether the levels of UHRF1 proteins indeed influence the anti-proliferation effect of HSP90 inhibitors, we first determined the dose effect of 17-AAG on HeLa cell proliferation (Fig. 7A). We found that 0.25 μM 17-AAG was sufficient for a nearly complete inhibition of HeLa cell proliferation (Fig. 7A). We then tested whether overexpression of UHRF1 would render HeLa cells more resistant to 17-AAG. As shown in Fig. 7B (left), a HeLa cell line with stable expression of FLAG-UHRF1 (Fig. 7B, top right) was more resistant to inhibition by 0.25 μM 17-AAG than the parental HeLa cells. Western blotting analysis confirmed that 17-AAG treatment led to a progressive reduction of UHRF1 proteins for both cell lines (Fig. 7B, bottom right). To test this further, we knocked down UHRF1 in HeLa cells by two different lentiviral UHRF1-specific shRNAs (Fig. 7C, right). Because shRNA-1 was more efficient in knocking...
down UHRF1, the stable shRNA-1-infected HeLa cells and shVector-infected HeLa cells were subjected to treatment with 0.25 μM HSP90 inhibitor 17-AAG in parallel. The representative results in Fig. 7C (left) show that knockdown of UHRF1 indeed rendered HeLa cells more sensitive to 17-AAG.

**Discussion**

In this study, we have identified 17-AAG, an HSP90 inhibitor, as a potent small molecule that induces UHRF1 degradation in various cell lines. Thus, UHRF1 joins a growing list of oncogenic HSP90 "client proteins." Because HSP90 chaperone is not required for general protein folding and its early stage but plays a critical role in protein maturation and stability, the identification of UHRF1 as an HSP90 client suggests a complex and metastable nature of the UHRF1 structure. This is actually in good agreement with the multistructural domain organization of UHRF1 proteins. In this regard, in addition to the previously recognized five structural domains, namely (in order from N to C terminus) ubiquitin-like domain, tandem Tudor domain, PHD, SRA, and RING, a recent study reported a polybasic region that is located between SRA and RING and regulates UHRF1 conformation and function through binding PI5P (47).

Furthermore, a recent structural study suggests that USP7 regulates UHRF1 chromatin association through an allosteric interaction with UHRF1 (48). Thus, the conformation of UHRF1 under physiological conditions is probably dynamic and unstable and therefore requires the action of HSP90 chaperone.

We demonstrate that HSP90 inhibitors induce UHRF1 degradation through a ubiquitin-dependent proteasome pathway (Fig. 2A). Consistent with this idea, we observed that HSP90 inhibitors promoted UHRF1 ubiquitination (Fig. 2D). We demonstrate that UHRF1 degradation induced by HSP90 inhibitors is dependent on HSP70 (Fig. 3C), suggesting that the failure of transfer of the "early HSP70-UHRF1" complex to HSP90 would lead to UHRF1 degradation. Although this result suggests that one or more HSP70-interacting E3 ligases may mediate HSP90 inhibitor-induced UHRF1 degradation, we demonstrate that the UHRF1 degradation is not dependent on the best studied HSP70-interacting E3 ligase CHIP (Fig. 4, A and B). In addition, we provide evidence that HSP90 inhibitor-induced UHRF1 degradation is dependent on neither E3 ligase CUL5 (Fig. 4C) nor SCF E3 ligase (Fig. 5). Furthermore, HSP90 inhibitor-induced UHRF1 degradation is not dependent on the intrinsic E3
ligase activity of UHRF1 (Fig. 4D). Thus, a major challenge for future study is to identify the exact E3 ligase that mediates UHRF1 ubiquitination and subsequent degradation upon inactivation of HSP90. In this regard, a recent study showed that HSP90 interacts with more than 100 E3 ubiquitin ligases (49), raising the possibility that many E3 ligases may potentially mediate HSP90 inhibitor-induced client degradation. It also remains to be determined to what extent HSP70 interacts with E3 ubiquitin ligases.

Given the broad overexpression and pro-proliferation function of UHRF1 in cancer cells, UHRF1 has become an attractive target for cancer therapy. In support of this notion, we show that knockdown of UHRF1 results in inhibition of cell proliferation, DNA damage, and apoptosis (Fig. 6). Similarly, treatment with HSP90 inhibitors also results in inhibition of cell proliferation, DNA damage, and apoptosis (Fig. 6). Furthermore, we show that overexpression of UHRF1 renders HeLa cells more resistant to HSP90 inhibitor-induced client degradation. It also remains to be determined to what extent HSP70 interacts with E3 ubiquitin ligases.

Experimental Procedures

Reagents and Antibodies—The following chemicals were used in this study. 17-AAG (catalog no. 1774-1,5) was purchased from BioVision (Milpitas, CA), 17-DMAG (S1142) and MG132 (S2619) from Selleckchem (Houston, TX), and cycloheximide (catalog no. 01810) from Sigma-Aldrich. The polyclonal UHRF1 antibody was described previously (8). The sources of commercial antibodies were as follows: HSP70 (A0284), HSP90 (A0365), and CHIP (A1169) from ABclonal (Cambridge, MA); /H9252-TRCP1 (catalog no. 4394) and PARP (catalog no. 9532) from Cell Signaling Technology (Danvers, MA); CUL5 (ab184177), CUL1 (ab75817), and /H9252-H2AX from Abcam (Cambridge, UK); FLAG (F1804) and /H9252-actin (A5441) from Sigma-Aldrich; HA (26D11) and GAPDH (3B3) from AbMART (Berkeley Heights, NJ); ubiquitin (P4D1) from Santa Cruz Biotechnology, Inc. (Dallas, TX); and /H9252-catenin (AB-37) from SAB (College Park, MD).

Small Molecule Screening—To identify inhibitors targeting UHRF1, a high-content screening platform based on automatic imaging of GFP-UHRF1-HeLa cells was established with the Operetta system (PerkinElmer Life Sciences). Cells were seeded at 1 × 10^4 cells/well on a 384-well plate, and the final concentration for chemicals used was at 1–10 μM. The inhibitory effect of small molecules on GFP-UHRF1 fusion fluorescent intensity was quantified by the Columbus analysis system (PerkinElmer Life Sciences) 24 h after chemical treatment.
**Plasmids**—The plasmids for GFP-UHRF1, FLAG-UHRF1, FLAG-UHRF1 (ΔRING), FLAG-UHRF1(1–675), and HA-Ub were constructed in our laboratory. The cDNA encoding HSP70 was amplified from HEK293T cDNAs and cloned into HA-tagged pCDNA3.0 vector. HA-tagged HSP90 plasmid was kindly provided by the laboratory of Dr. Xiaotao Li (East China Normal University, Shanghai, China).

**Cell Culture and Transient Transfection**—HeLa, HEK293T, 22Rv1, and NIH3T3 cells were routinely cultured in regular DMEM supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified incubator with 5% CO2. HT116 cells were maintained in McCoy 5A plus 10% FBS. A549 and LNCaP cells were maintained in 1640 medium containing 10% FBS. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**RNA Interference and Transfections**—The siRNA oligonucleotides were synthesized by GenePharma (Shanghai, China). siRNA sequences for human CHIP and UHRF1 were as follows: 5’-AGCGGAGAGGAGCUCAAGG-3’ (CHIP-1), 5’-GGGCCUU-GUGCUACCCUAGA-3’ (CHIP-3), 5’-GCCAUACCCUCUGACAUATT-3’ (UHRF1-1), 5’-AGGGUGGCAUCA-GACUACA-3’ (UHRF1-4).

The siRNA for human β-TRCP1/2 (5’-AAGUGGAAUUUGUGGAAAC-3’) was reported previously (51). The control (NC) siRNA sequence was 5’-UUUCUGCAACUGUCAUG-3’. The siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**shRNA Lentivirus Production and Transduction**—To generate shRNA constructs against human UHRF1, HSP70, CUL5, CUL1, and β-TRCP genes, the corresponding shRNA oligonucleotides were cloned into pLKO.1 vectors with the AgeI and EcoRI sites. The resulting constructs were sequencing-clarified by centrifugation at 12,000 rpm for 20 min at room temperature. Resulting plasmids were packaged into lentiviruses by cotransfection with pMD2.G and psPAX2 plasmids into HEK293T cells. The lentiviruses encoding the desired shRNA were then used to transduce the cell lines as indicated, and stable transduced cells were selected for 1 week in the culture medium containing 100 μM puromycin (Sigma-Aldrich). The shRNA sequences were as follows: shUHRF1-1, 5’-GCCTTTGATT-CGTTCTCTTCTT-3’; shUHRF1-3, 5’-GGCTGGCTCTC-AACTGCTTT-3’; shHSP70, 5’-GGCCAAACAGATCACCAC-ATCTT-3’; shCUL5-1, 5’-GTCTCATTCTCCTACATTG-3’; shCUL5-2, 5’-CAGACCTGAATTGAAGAT-3’; shβ-TRCP1/2, 5’-GTGGATTTTGGAAACATC-3’; shCUL1-1, 5’-GCCAGCATGATCTCAGGTTA-3’; shCUL1-2, 5’-GAGA-AAGATGATGAACTGAA-3’. The shβ-TRCP1/2 sequences were described previously (43).

**cDNA Preparation and Quantitative RT-PCR Analysis**—Total RNAs were harvested, and total RNAs were extracted with TRIzol (Invitrogen) according to the manufacturer’s instructions. cDNAs were prepared with the EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Transgen) by following the manufacturer’s protocol. The cDNAs were then used for quantitative RT-PCR analysis through CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad). Either β-actin or GAPDH was used as an internal control. The primers used were as follows: β-actin, 5’-GGCACCCCGACAAATGAGATCACA-3’ (forward) and 5’-TAAAGGATCTTGGCAGTGGAGT-3’ (reverse); GAPDH, 5’-GAAGGTGAAGTCGGAGATC-3’ (forward) and 5’-GAAGATGTTGATGGGATTTCC-3’ (reverse); HSP70, 5’-AGACGGGAGCCGCA-GAG-3’ (forward) and 5’-CACCTTGGAGTGGAC-3’ (reverse); HSP90, 5’-AGAGGTTAGACGCTGTC-3’ (forward) and 5’-AGAGTTCGATCTTGTGGTGC-3’ (reverse).

**Cycloheximide Chase Assay**—The half-life of endogenous or ectopically expressing UHRF1 was determined by a cycloheximide chase assay. In brief, cells were seeded in 12-well plates. After preincubation with 1 μM 17-DMAG or solvent control for 12 h, cells were treated with cycloheximide (100 μg/ml) and collected at the indicated time points, and the levels of UHRF1 were then subjected to Western blotting analysis.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation and Western blotting analysis were as described (8). For denaturing immunoprecipitation, the cells were lysed in denaturing lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 8% glycerol, 1% Triton X-100, 1% SDS, 1% sodium deoxycholate), and the lysates were heated at 100 °C for 15 min and clarified by centrifugation at 12,000 rpm for 20 min at room temperature. Western blotting analysis, the Odyssey infrared imaging system (LI-COR Bioscience) was used. The ImageJ software was used for densitometric analyses of Western blots, and the quantification results were normalized to the loading control.

**In Vivo Ubiquitination Assay**—For the in vivo ubiquitination assay, 36-h post-transfected cells were treated with 10 μM proteasome inhibitor MG132 for 8 h before being collected for preparation of whole cell extracts using denaturing lysis buffer. Western blotting analysis was then performed either with the whole cell extracts or UHRF1 immunoprecipitated under denaturing conditions.

**Cell Proliferation Assay**—To measure proliferation, cells were cultured in 6-well plates in medium with various concentrations of inhibitors and incubated for various times, as indicated. For cell counting, the cells were collected by treatment with 2.5% trypsin solution (Gibco), and the cells were counted with a hemocytometer. Results are the average of three duplicates.

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