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Aha1 Is an Autonomous Chaperone for SULT1A1

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Abstract

The cochaperone Aha1 activates HSP90 ATPase to promote the folding of its client proteins; however, very few client proteins of Aha1 are known. With the use of an ascorbate peroxidase (APEX)-based proximity labeling method, we identified SULT1A1 as a proximity protein of HSP90 that is modulated by genetic depletion of Aha1. Immunoprecipitation followed by Western blot analysis showed the interaction of SULT1A1 with Aha1, but not HSP90. We also observed a reduced level of SULT1A1 protein upon genetic depletion of Aha1 but not upon pharmacological inhibition of HSP90, suggesting that the SULT1A1 protein level is regulated by Aha1 alone. Maturation-dependent interaction assay results showed that Aha1, but not HSP90, binds preferentially to newly synthesized SULT1A1. Reconstitution of Aha1-depleted cells with wild-type Aha1 and its E67K mutant, which is deficient in interacting with HSP90, restored SULT1A1 protein to the same level. Nonetheless, complementation of Aha1-depleted cells with an Aha1 mutant lacking the first 20 amino acids, which disrupts its autonomous chaperone function, was unable to rescue the SULT1A1 protein level. Together, our study revealed, for the first time, Aha1 as an autonomous chaperone in regulating SULT1A1. SULT1A1 is a phase-II metabolic enzyme, where it adds sulfate groups to hydroxyl functionalities in endogenous hormones and xenobiotic chemicals to improve their solubilities and promote their excretion. Thus, our work suggests the role of Aha1 cochaperone in modulating the detoxification of endogenous and environmental chemicals.

Graphical Abstract

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Supporting Information

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Western blot images and quantification results (PDF)

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.chemrestox.2c00167

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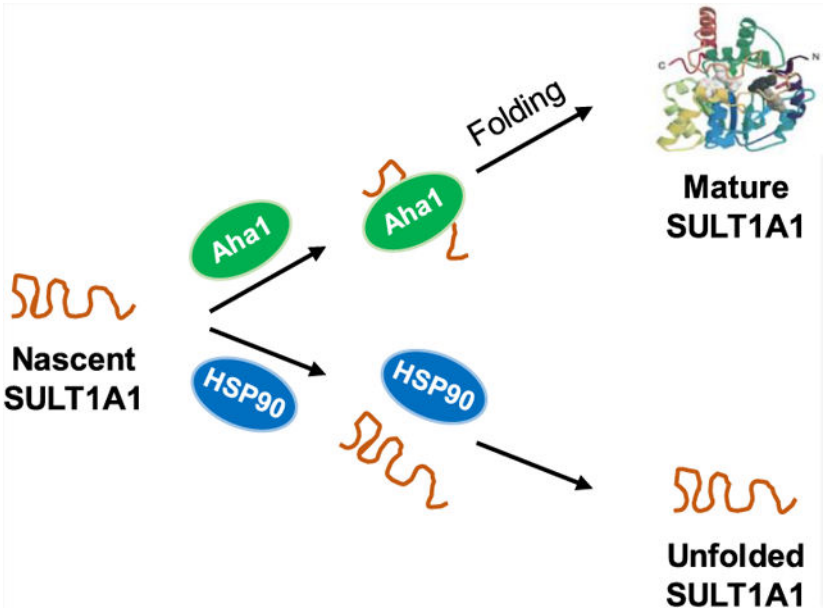

INTRODUCTION

Heat shock protein 90 (HSP90) is a central regulator of proteostasis in cells under both physiological and stress conditions, where several hundred client proteins have been documented for HSP90. Thus, HSP90 is a key modulator of many important processes encompassing protein folding, immune response, DNA repair, development, and neuro-degenerative diseases.[1–5]

Cochaperones constitute important modulators of HSP90, and they regulate the ATPase-associated conformational changes of the HSP90 dimer while processing its client proteins. The interactions of the major known cochaperones with HSP90 and their functional mechanisms have been elucidated in recent years.[6,7] Among the HSP90 cochaperones, CDC37 exhibits interactions with a subset of client proteins in HSP90 complexes and assumes a specialized role in the maturation of protein kinases.[8–10]

Aha1 (activator of HSP90 ATPase) is a potent activator of the ATPase activity of HSP90.[11] Aha1 supports the activity of steroid hormone receptors and kinases such as Raf,[12] indicating that Aha1-mediated stimulation of HSP90 ATPase activity assumes a crucial role in the folding processes of these client proteins. On the other hand, Aha1 promotes the pathological aggregation of tau protein,[13] and it exerts adverse effects on the folding of an HSP90 client, a cystic fibrosis transmembrane conductance regulator (CFTR), and its disease-causing ΔF508 variant, which ultimately promotes CFTR degradation.[14]

Identification of client proteins of the HSP90-Aha1 network will offer new knowledge about the roles of Aha1 in human diseases and shed important light on Aha1's role in the biological functions regulated by HSP90. It, however, remains unclear whether there are other client proteins of Aha1 and whether Aha1-modulated folding and maturation of these client proteins are HSP90-dependent.

Proximity labeling (PL) together with proteomics has been used to identify proteins that display weak and transient interactions with a target protein of interest in the native environment of live cells.[15–18] In PL methods, an enzyme catalyzing the biotinylation of endogenous proteins is fused with a target protein of interest, which allows proximal and/or interacting proteins to be conjugated with biotin for subsequent affinity enrichment and mass spectrometric analysis.[15–19] Engineered ascorbate peroxidase 2 (APEX2) is one of the most commonly used enzymes in PL.[17,20] After the addition of hydrogen peroxide to cells preincubated with biotin-phenol, APEX catalyzes the production of biotin-phenoxyl radicals that covalently label endogenous proteins in close proximity.[17] With a small labeling radius (<20 nm), the biotin-phenoxyl radical is highly reactive and short-lived and can conjugate with electron-rich amino acids, including Tyr, Trp, His, and Cys.[17,20–24]

By employing APEX2-labeling in combination with label-free quantitative proteomics, we recently identified a number of proximity proteins of HSP90 that are lost upon short hairpin RNA (shRNA)-mediated depletion of Aha1, including Dicer1.[25] Here, we report our characterizations of SULT1A1, another protein displaying a diminished presence in the proximity proteome of HSP90 upon Aha1 depletion, and we demonstrated that Aha1 is an autonomous, HSP90-independent chaperone for the maturation of SULT1A1.

EXPERIMENTAL PROCEDURES

Materials.

All chemicals, except described specifically, were obtained from Sigma-Aldrich. Cycloheximide (CHX), 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), ganetespib, and AT13387 were purchased from Fisher Scientific.

Cell Culture.

HEK293T and GM00637 cells were purchased from ATCC. HEK293T *AHSA1*$^{-/-}$ cells were generated previously by using CRISPR-Cas9.[25] The cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (10,000 U/mL penicillin and 10,000 U/mL streptomycin, Thermo Fisher). The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

shRNA and Plasmids.

All oligodeoxyribonucleotides (sequences listed in Table S1) were purchased from Integrated DNA Technologies. Control shRNA with a hairpin sequence of 5′-CCT AAG GTT AAG TCG CCC TCG CTC TAG CGA GGG CGA CTT AAC CTT AGG-3′ (Addgene, Cambridge, MA, USA) was used as a negative control, as described previously.[26] The shRNAs for Aha1 depletion, the HSP90-APEX plasmid, and the pRK7 3× Flag plasmids for expressing wild-type Aha1, Aha1-Δ20 (with the first 20 amino acids of Aha1 being deleted), and Aha1-E67K were described recently.[25] The coding sequence of SULT1A1 was cloned into the *Bam*HI and *Eco*RI sites of the pRK7 3× Flag vector (Addgene, plasmid no. 8996), and the sequence of the resulting plasmid was confirmed by Sanger sequencing.

Lentivirus Production and Stable Cell Line Generation.

HEK293T cells were transfected with pLKO.1/puro-shRNA plasmids along with an envelope plasmid (pLTR-G, Addgene plasmid no. 17532) and a package plasmid (pCMV-dR8.2 dvpr, plasmid no. 8455) with the use of a PolyFect transfection reagent (Qiagen). Viral particles were collected at 48 h following transfection and filtered with a 0.45 μm sterile filter. Cells were infected for 48 h with a mixture of viral particles and DMEM (5:1, v/v). The cells were subsequently screened with puromycin (1 μg/mL) for a week and cultured in complete DMEM containing the same concentration of puromycin.

Western Blotting.

Cell lysis was conducted by using a CelLytic M cell lysis reagent (Sigma-Aldrich), and the supernatant was collected for Western blot experiments. The following primary antibodies were used: human Aha1 (Santa Cruz Biotechnology, sc-166610), HSP90 (Santa Cruz Biotechnology, sc-13119), SULT1A1 (Abclonal, A1599), V5 (Proteintech, no. 14440-1-AP), Flag (Cell Signaling Technology, no. 14973S), and streptavidin (Thermo Scientific, no. S911). The secondary antibodies were the goat anti-rabbit IgG (whole molecule)-peroxidase antibody (Sigma, no. A0545) and m-IgGκ BP-HRP (Santa Cruz Biotechnology, sc-516102). Membranes were also probed with anti-tubulin (Santa Cruz Biotechnology, sc-166729) or anti-GAPDH (Santa Cruz Biotechnology, sc-32233) to verify equal protein loading.

Flag Immunoprecipitation.

The cells were lysed in a CelLytic M cell lysis reagent supplemented with a complete protease inhibitor cocktail (Sigma-Aldrich). After lysis on ice for 30 min, the samples were centrifuged at 13,000 rpm for 10 min, and the supernatant was collected. One milligram of total protein was incubated with prewashed Flag beads at 4 °C overnight. The resulting immune complexes were washed with ice-cold lysis buffer five times, and the beads were boiled in 2× loading buffer (Bio-Rad) to elute the captured proteins, which were subjected to Western blot analysis.

RESULTS

APEX Labeling-Based Proteomic Analysis Revealed SULT1A1 as a Candidate Client Protein of the Aha1 Cochaperone.

HSP90 functions together with cochaperones to achieve spatiotemporal modulation of client proteins. However, not many client proteins of HSP90 regulated by Aha1 are known. To fill in the knowledge gap, we recently conducted an APEX2-based proximity labeling of HSP90 in HEK293T cells treated with the control nontargeting shRNA or shRNA for stable knockdown of Aha1.[25] The analysis led to the quantification of more than 30 proteins displaying significant alterations (at least 1.5-fold) in abundance in the proxomity proteome of HSP90, including Dicer1 and SULT1A1 (Figure 1).[25]

SULT1A1, an important phase-II xenobiotic metabolizing enzyme, is highly expressed in the liver and mediates the sulfonation of steroids, drugs, and carcinogens.[27] SULT1A1 assumes a major role in the chemical and functional homeostasis of substrate chemicals and hormones.[27–29] Owing to its functions in the metabolism of xenobiotics and endogenous

hormones, SULT1A1 and its regulation are implicated in the pathophysiology of liver disease[30,31] and cancer.[32,33] Hence, we decided to choose this protein for further investigation.

SULT1A1 Is a Client Protein of Aha1.

Our Western blot analysis showed that shRNA-mediated knockdown of Aha1 led to diminished expression of SULT1A1 protein (Figure 2), suggesting that SULT1A1 is a potential client of Aha1.

Previous studies showed that Aha1 stimulates the ATPase activity of HSP90, thereby promoting effective folding of HSP90 client proteins, including steroid hormone receptors and kinases.[4] We, therefore, hypothesized that diminished expression of SULT1A1 in the Aha1-deficient background may involve HSP90. We previously generated CRISPR-engineered HEK293T cells, where a 3× Flag tag was fused with the C terminus of endogenous HSP90.[34] Strikingly, Flag pull-down with the lysate of these cells showed a lack of interaction between SULT1A1 and HSP90 (Figure 2d). On the other hand, Flag immunoprecipitation results showed that ectopically expressed Flag-Aha1 interacts with endogenous SULT1A1 in HEK293T cells (Figure 2e), suggesting SULT1A1 as a candidate client protein of Aha1.

Sun et al.[35] showed that molybdate stabilized the interaction between Aha1 with HSP90 in a rabbit reticulocyte lysate. Thus, we also hypothesized if molybdate treatment affects the interaction of Aha1 with HSP90 and SULT1A1, and Anti-Flag pull-down results showed that the interaction between ectopically expressed Flag-Aha1 and endogenous SULT1A1 or HSP90 in the lysate of HEK293T cells was not influenced by molybdate treatment (Figure S1). The different results obtained for the effect of molybdate on HSP90-Aha1 interactions in the lysates of rabbit reticulocytes and HEK293T cells may emanate from biological heterogeneities.

Given that Aha1 stimulates the ATPase activity of HSP90 to promote client protein folding, we next investigated if HSP90 and Aha1 interact preferentially with nascent SULT1A1 protein. The maturation-dependent interaction assay[36] revealed a progressive attenuation in binding of freshly synthesized SULT1A1 with Aha1, but not HSP90, in HEK293T cells (Figure 3a). These data suggest that Aha1 functions as an autonomous chaperone in regulating SULT1A1.

Aha1 Acts as an Autonomous Chaperone to Regulate the Expression of SULT1A1.

To further substantiate the above findings, we ectopically overexpressed wild-type Aha1 and two mutants of Aha1 that are either defective in binding with HSP90 (Aha1-E67K)[37] or lack inherent chaperone activity (i.e., Aha1-Δ20, with the deletion of the N-terminal 20 amino acids),[38,39] in HEK293T cells with Aha1 being genetically depleted with shRNAs targeting its 3′-untranslated region (3′-UTR). Our results showed that SULT1A1 levels can be restored to the same levels after overexpression of wild-type (WT) Aha1 or the E67K mutant; Aha1-Δ20, however, was unable to rescue the SULT1A1 protein level (Figure 3b and 3e). Together, these results substantiate that SULT1A1 is a client protein of Aha1 but not HSP90. In this vein, the band below SULT1A1 is attributed to a nonspecific band, and

we confirmed this by using Western blot analysis of the lysate of HEK293T cells with the *SULT1A1* gene being stably knocked down with shRNA. In particular, we found that the intensity of the SULT1A1 band was substantially reduced, whereas no change was observed for the nonspecific band upon genetic depletion of SULT1A1 (Figure S2). In addition, the nonspecificband was only observed in stable SULT1A1 or control shRNA knockdown cells, and it likely arises from a protein induced from the puromycin screening process.

Next, we examined if Aha1 regulates SULT1A1 expression in different cell lines. Our results showed that the expression of SULT1A1 protein was attenuated in GM00637 cells upon genetic depletion of Aha1, though the extents of the decrease of SULT1A1 were lower than what we found for HEK293T cells (Figure S3). Since Aha1 is a cochaperone of HSP90, we also assessed the changes in the expression level of SULT1A1 upon treatment with several small-molecule inhibitors of HSP90, namely, ganetespib, AT13387, and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, a.k.a. alvespimycin).[40] Our results showed that the HSP90 inhibitor treatments did not alter the expression level of SULT1A1 in HEK293T or GM00637 cells (Figure S4). The SULT1A1 protein did not exhibit any change in the expression level upon further inhibition of HSP90 in shAha1-treated HEK293T cells (Figure 4a,b). Furthermore, genetic ablation of Aha1 also results in a decrease in the SULT1A1 protein level, and HSP90 inhibitor treatments did not further attenuate the SULT1A1 protein level in HEK293T cells with Aha1 being genetically ablated (Figure 4c,d and Figures S5 and S6). These results suggest that Aha1 itself acts as a chaperone to regulate the expression of SULT1A1 protein.

DISCUSSION

Aha1 is a potent activator of the ATPase activity of HSP90 and promotes proper folding and maturation of client proteins.[11,12,41–43] Aha1 was found to be associated with many diseases, including Alzheimer's disease[13] and cystic fibrosis.[14] Our recently reported APEX-labeling and label-free quantitative proteomic analysis revealed a number of candidate client proteins of Aha1 in HEK293T cells.[25] Among these proteins, SULT1A1 protein was markedly attenuated in the proximity proteome of HSP90 upon shRNA-mediated knockdown of Aha1. We further validated these results by using streptavidin affinity pull-down followed by Western blot analysis (Figures 1 and 2).

Streptavidin immunoprecipitation results showed that genetic depletion of Aha1 led to decreased expression of SULT1A1 protein (Figure 2). Anti-Flag affinity pull-down data also revealed interactions of SULT1A1 with Aha1 but not with endogenous HSP90 (Figure 2). Furthermore, we observed that newly synthesized SULT1A1 binds specifically to Aha1 but not HSP90. Additionally, genetic depletion of Aha1 attenuates the expression level of SULT1A1, whereas HSP90 inhibition failed to do so (Figure 4 and Figure S4). Results from the rescue assay showed that overexpression of WT Aha1 or the E67K mutant in the Aha1-deficient background can restore the SULT1A1 protein to the same level; however, Aha1-Δ20, which abrogates its chaperone activity,[38,39] failed to rescue it (Figure 3). A previous study showed that the attenuated HSP90/Aha1 interaction accompanied with diminished stimulation of HSP90 ATPase activity confers little effect on the activation of some client proteins,[44] underscoring an additional function of Aha1 beyond its role in stimulating the

ATPase activity of HSP90. In agreement with these previous observations, we found that Aha1 itself can function as a chaperone to regulate the expression of SULT1A1. Our results are also in keeping with the previous observations that Aha1 can serve as an autonomous chaperone to prevent aggregation of stressed proteins[39] and to promote the maturation of Dicer1.[25]

SULT1A1 is a client of Aha1 but not HSP90. The biotin-phenoxyl radical produced by APEX2 is highly reactive and short-lived, has a small labeling radius, and can conjugate biotin with proximity proteins.[17,20] Being a cochaperone of HSP90, Aha1 functions in part as a complex with HSP90, which may explain why SULT1A1 is present in the proximity proteome of HSP90.

Overexpression of Aha1 destabilizes wild-type CFTR protein and the folding-deficient mutant CFTR-ΔF508, which promotes their ER-associated degradation.[14] Thus, Aha1 as a chaperone can recognize directly and engage with clients that are not folded properly under stress conditions, which challenge the cell by overloading it with unfolded proteins. In light of our observation that Aha1 serves as an autonomous chaperone to stabilize SULT1A1 protein, we conclude that the chaperone activity of Aha1 renders it functions as an alternative chaperone to help fold nascent client proteins like SULT1A1 or promotes disposal of denatured or misfolded proteins like CFTR and its mutant to protect the cellular protein folding machineries from being overloaded, thereby maintaining proteostasis.

Together, we unveiled, for the first time, SULT1A1 as a client protein of Aha1. As a phase-II metabolic enzyme, SULT1A1 assumes a major role in chemical and functional homeostasis by adding sulfate groups to hydroxyl functionalities in substrate chemicals and hormones, thereby improving their solubilities and promoting their excretion.[27–29] Due to its function in the metabolism of xenobiotics, SULT1A1 and its aberrant regulation are implicated in the pathophysiology of liver disease[30,31] and cancer.[32,33] Therefore, the regulatory role of Aha1 in the expression level of SULT1A1 protein that we uncovered herein may provide an important basis for developing approaches toward therapeutic interventions of these diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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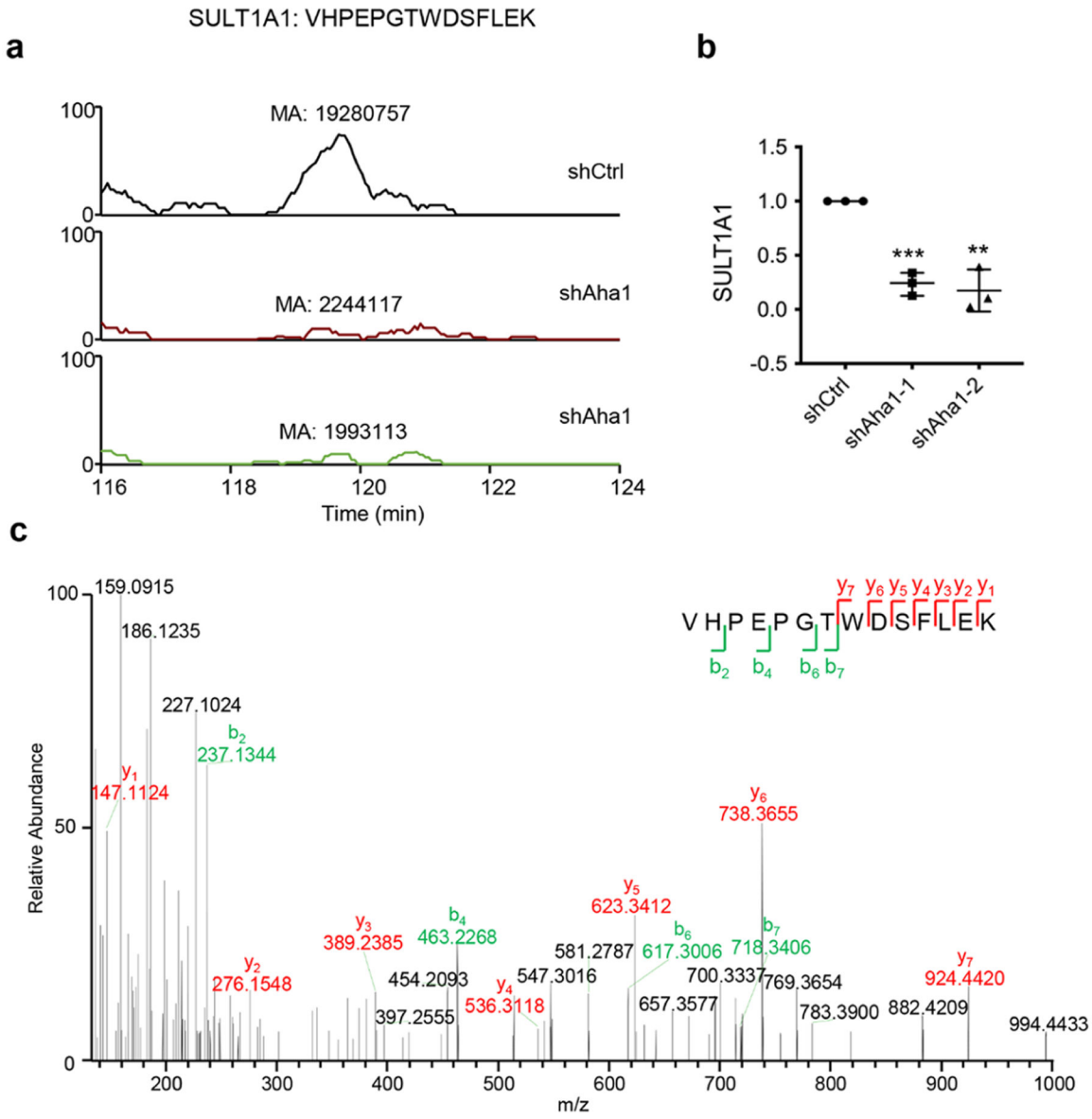

Figure 1.

APEX2-based proximity proteomics revealed Aha1-mediated interactions between HSP90 and SULT1A1. (a) Extracted-ion chromatograms (XIC) and (b) corresponding quantification results for a unique peptide of SULT1A1 identified in the proximity proteome of HSP90. Proximity labeling was performed in the control shRNA (shCtrl)-treated or Aha1 knockdown (shAha1) cells. The peak area of the SULT1A1 peptide from Aha1 knockdown cells was normalized to that of the control. Plotted are the means ± S.D. of results obtained from three biological replicates. The *p* values were calculated using two-tailed, unpaired *t*-tests (**, $0.01 < p < 0.05$; ***, $0.001 < p < 0.01$). (c) Representative MS/MS spectrum for a unique peptide of SULT1A1 identified in control shRNA-treated cells.

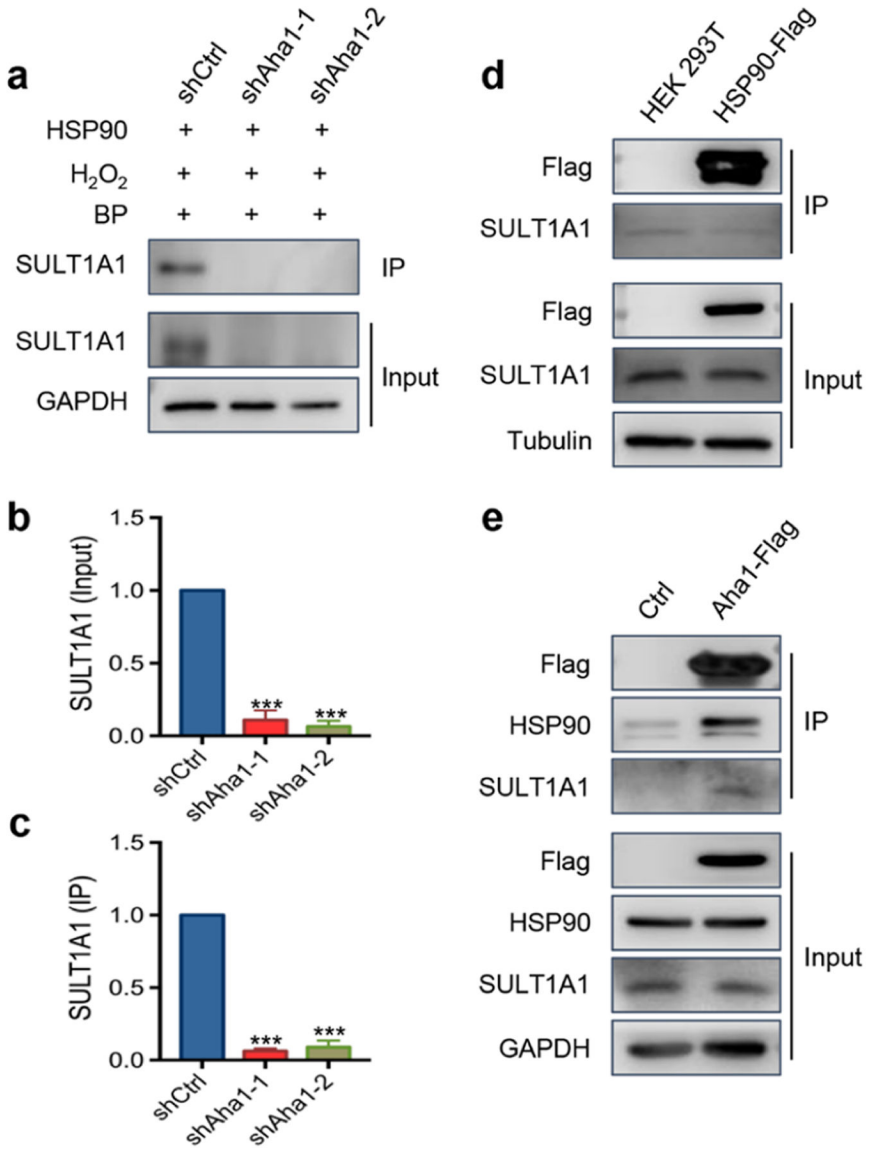

Figure 2.

SULT1A1 interacts with Aha1. (a) HEK293T cells with genetic depletion of Aha1 (shAha1) and shCtrl cells were transfected with the HSP90-APEX plasmid for 24 h and then incubated with biotin phenol for 30 min and H_2O_2 for 1 min followed by cell lysis and streptavidin affinity pull-down; the whole-cell lysate and pull-down lysate were employed for Western blot analysis. (b) Change in the expression level of SULT1A1 in the input panel of (a). (c) Alteration in the level of SULT1A1 in the proximity proteome of HSP90 based on the IP panel of (a). (d) The lysates of HEK293T cells with a 3× Flag tag on the C terminus of endogenous HSP90 were employed for immunoprecipitation using an anti-Flag antibody, and the immunoprecipitates were used to monitor the levels of HSP90-Flag and SULT1A1 proteins by Western blotting. (e) HEK293T cells were transfected with the Flag-tagged Aha1 plasmid for 24 h followed by cell lysis and affinity purification using anti-Flag agarose. The whole-cell lysate and pull-down lysate were employed for Western blotting.

SULT1A1 protein levels in (b) were quantified from band intensities using ImageJ and normalized to GAPDH first and then displayed relative to the level in HEK293T shCtrl cells. SULT1A1 levels in (c) were quantified from band intensities using ImageJ and were displayed relative to the level in HEK293T shCtrl cells. The data represent the mean ± S.D. ($n = 3$). The p values were calculated using unpaired, two-tailed Student's t-test: **, $0.001 \leq p < 0.01$; ***, $p < 0.001$.

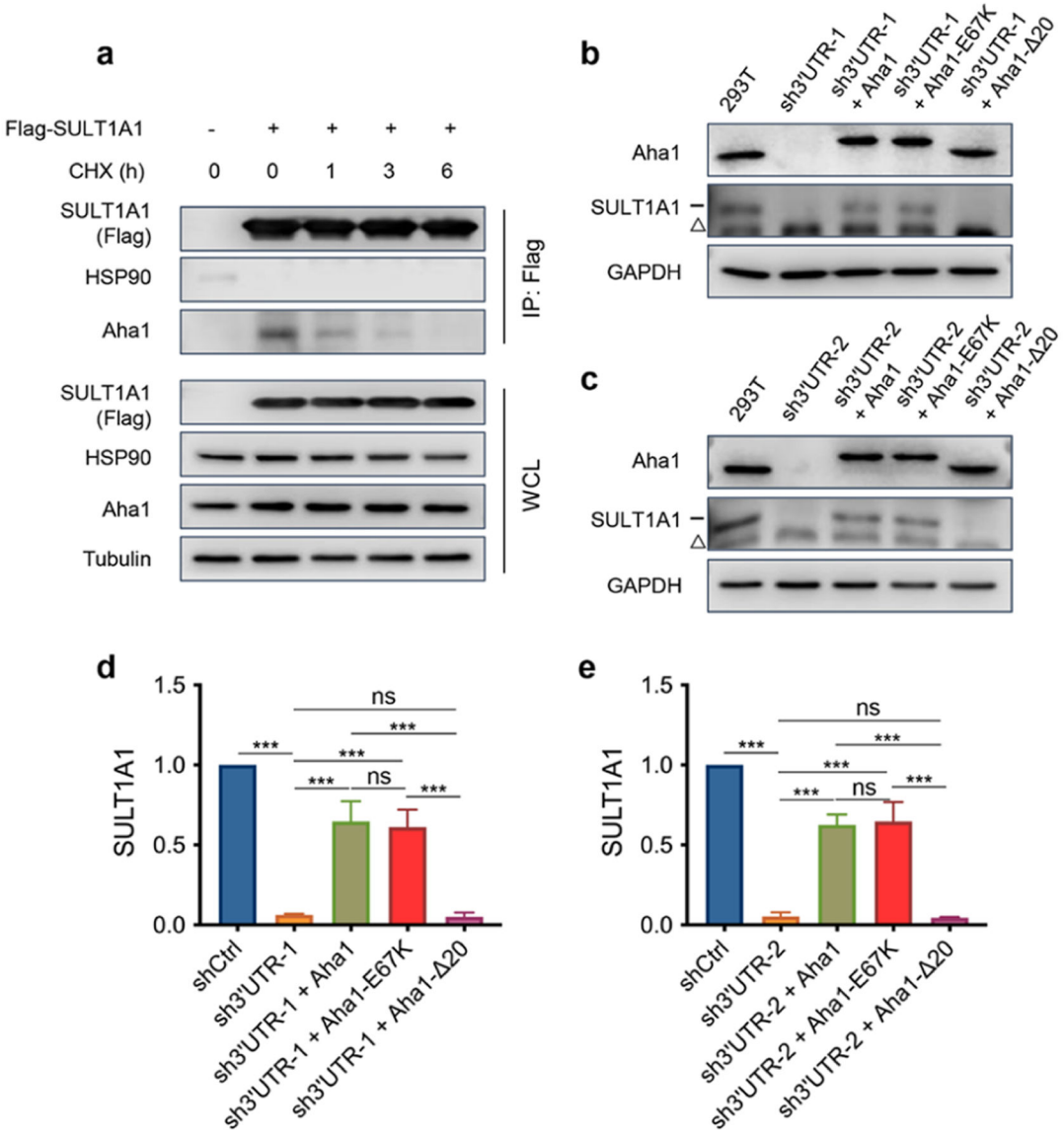

Figure 3.

SULT1A1 is a client protein of Aha1. (a) HEK293T cells were transfected with Flag-SULT1A1 for 24 h, treated with cycloheximide (CHX) to block *de novo* protein synthesis, and harvested at different time points. The cells were then lysed, and the lysates were subjected to Western blot analysis for monitoring the time-dependent alterations of SULT1A1 (Flag), HSP90, and Aha1 proteins. (b,c) HEK293T cells with Aha1 being knocked down with shRNAs targeting its 3′-UTR were reconstituted with wild-type Aha1, E67K mutant, and Aha1-Δ20 for 24 h. The cells were then harvested, and lysates were used to monitor the alterations in the expression level of SULT1A1 protein; "Δ" indicates a nonspecific band. (d,e) The data in (b) and (c) were quantified with ImageJ based on band intensities and normalized against that of GAPDH, where the values are displayed relative to those measured for shCtrl cells. The data represent the mean ± S.D. ($n = 3$). The p values

were calculated using unpaired, two-tailed Student's *t*-test: ns, $p \geq 0.05$; *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$; ***, $p < 0.001$.

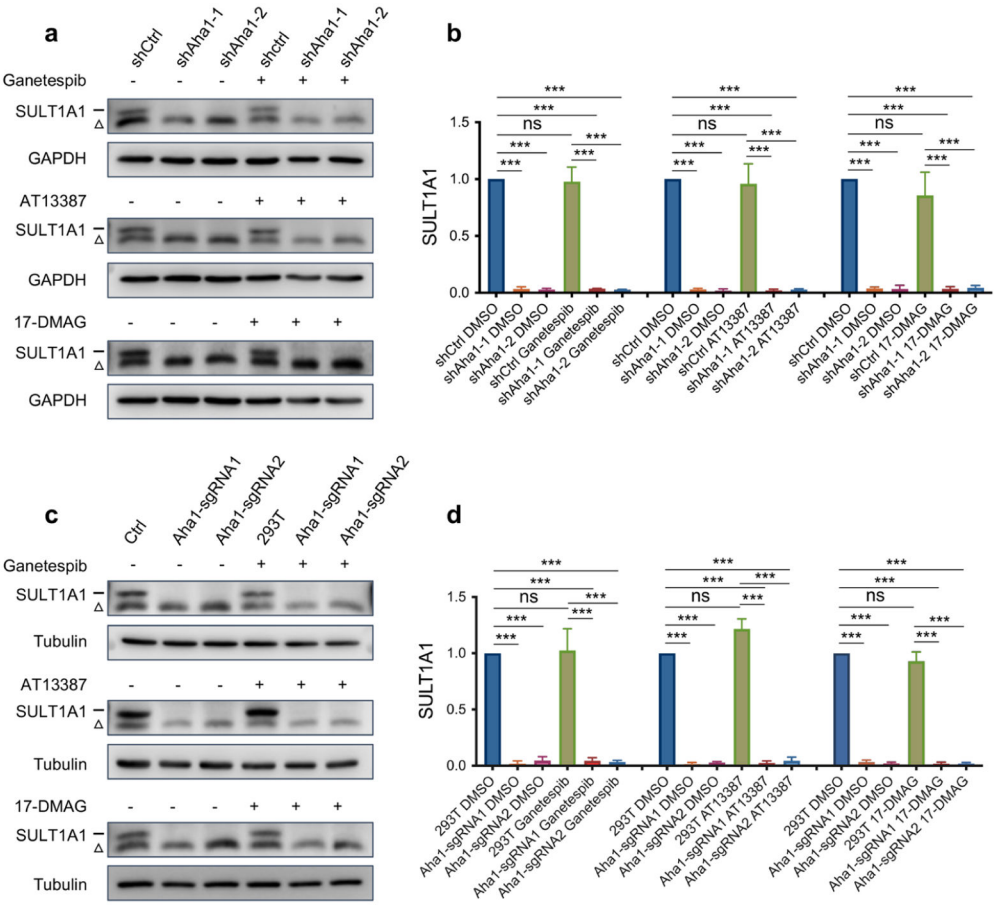

Figure 4.

Aha1 acts as an autonomous chaperone in regulating SULT1A1. (a) HEK293T cells with genetic depletion of Aha1 were incubated with HSP90 inhibitors, and cell lysates were used to monitor the alterations in the levels of SULT1A1 protein. (b) Changes in SULT1A1 protein levels in (a). (c) HEK293T cells with Aha1 being genetically ablated using CRISPR with two separate sgRNAs were incubated with HSP90 inhibitors, and cell lysates were subjected to Western blot analysis for monitoring the changes in the SULT1A1 protein level; "Δ" indicates a nonspecific band. (d) Alterations in SULT1A1 protein levels in (c). The changes in SULT1A1 protein levels were quantified from their band intensities using ImageJ and normalized against that of GAPDH or tubulin, where the values are displayed relative to the level in shCtrl cells. The data are presented as the mean ± S.D. ($n = 3$). The p values were calculated using unpaired, two-tailed Student's t-test: ns, $p \geq 0.05$; *, $0.01 \leq p < 0.05$; **, $0.001 \leq p < 0.01$; ***, $p < 0.001$.