Supporting Information

Substoichiometric hydroxynonenylation of a single protein recapitulates whole-cell-stimulated antioxidant response

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**General Materials and Methods.** BL21-CodonPlus(DE3)-RIL competent cells were from Stratagene. All primers were from IDT. Fusion Hot Start II polymerase was from Thermo. Orthovanadate, DNase I and all the restriction enzymes were from NEB. Total TRIzol® Plus RNA Purification Kit and SuperScript III Reverse Transcriptase were from Life Technologies. iQ™ SYBR® Green Supermix was from Bio-Rad. Complete EDTA-free protease inhibitor tablets were from Roche. TALON metal affinity resin was from Clontech. Sephadex G-25 resin, PD-10 Sephadex G-25M mini desalting columns and the gel filtration molecular weight standards were from GE Healthcare. Ultrafiltration Membrane Amicon Ultra centrifugal devices were from Millipore. HtPHA and HNE-alkyne were synthesized using previously published protocols.¹ Cy5-azide was from Lumiprobe (B3030). Dithiothreitol (DTT), streptomycin sulphate and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from Gold Biotechnology. Bortezomib is from LC laboratories. All other chemicals were from Sigma. pRK793 TEV [cysteine protease from tobacco etch virus (TEV)] protease (S219V mutant) bacterial expression plasmid (8827) and mammalian expression vectors for hrGFP-Keap1 (28025), GFP-Nrf2 (21549) and myc-Nrf2 (21555) were from Addgene. HEK-293 cells were from the American Type Culture Collection (ATCC). 1X PBS (Dulbecco’s phosphate-buffered saline), 1X TrypLE™ Express (stable trypsin-like enzyme with phenol red), 1X DMEM, 100X penicillin-streptomycin and 10mg/mL puromycin were from Invitrogen. Fetal bovine serum (FBS) (100 nm-triple filtered, SH30071.03) was from Hyclone. Serum-compatible broad-spectrum transfection reagent TransIT-2020 was from Mirus Bio LLC. Protein concentrations were determined using ε₂₈₀nm/M⁻¹cm⁻¹: 78,730, 138,660 and 68,555 for recombinant human His₆-Keap1, His₆-Halo-Keap1 and His₆-Nrf2, respectively. The
extinction coefficients of HtPHA and HNE-alkyne were $\epsilon_{366\text{nm}}/\text{M}^{-1}\text{cm}^{-1}$ 3953 and $\epsilon_{225\text{nm}}/\text{M}^{-1}\text{cm}^{-1}$ 16,900 respectively. All experiments with the fluorescently labeled proteins were performed in dark at the indicated temperature. Olympus CKX31 and Zeiss 510 meta confocal microscope systems were used for light and confocal fluorescence microscopy, respectively. Fluorescence-activated cell sorting (FACS) was performed on a Becton Dickinson FACSCalibur flow cytometer and FACS data analysis was carried out using FlowJo [(version v.10 (X)]. In-gel fluorescence analysis and imaging of the Coomassie-stained gels and PVDF membrane were performed on Bio-Rad ChemiDoc-MP imaging system. Densitometric quantitation was made by Bio-Rad Image Lab software (v 4.1). Cy5 excitation source was red epi illumination and emission filter used was 695/55 filter. Light exposure experiments were performed with the use of a hand-held UV lamp (Fisher, S45157, 365 nm at 0.6 mW/cm²). The lamp was positioned such that the lamp cover screen was at a distance of 1–2 cm directly above either the monolayer cell culture or solutions containing HNE precursors. His6-TEV-S219V protease was recombinantly expressed and purified from E. coli using TALON affinity chromatography (Clontech). The quantitative real-time PCR (qPCR) was performed with MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad 170-9740).

**Constructions of plasmids.** Ligase-free PCR cloning was used to construct the plasmids listed in Table S1. Briefly, the human gene of interest was cloned out from the commercially available plasmids, phrGFP-Keap1 (Addgene 28025) and eGFP-Nrf2 (Addgene 21549) using the indicated fwd-1 and rev-1 primers. The resultant PCR products were extended using the fwd-2 and rev-2 primers. These PCR products served
as mega-primers for the subsequent PCR-cloning into the linearized destination vectors indicated. The identity of each resultant plasmid was verified by sequencing of the entire gene at the genomics facility of the Cornell University Institute of Biotechnology. Plasmids were purified by Maxi- and Miniprep kits (Qiagen).

**T-REX method. (a) Targeted HNEylation in vitro** (Figure S2c). All steps were handled under dim light. Recombinant His<sub>6</sub>-Halo-Keap1 (3.0 μM) with and without His<sub>6</sub>-Nrf2 (1.5 μM) was incubated with HtPHA (1.1 equiv) (3.3 μM) in a final volume of 25 μL in 0.3 mM of TCEP and 50 mM Hepes (pH 7.6) at 37° C for 20 min, at which point the samples were directly exposed to 365 nm, 4 W lamp for 20 min at 37 °C. The light source was placed at ~1.0 inch distance from the surface of the reaction mixture. The lamp was then removed and the samples were further incubated for 20 min at 37 °C. The samples were treated with TEV protease (cysteine protease from Tobacco Etch Virus, His<sub>6</sub>-TEV-S219V) (10 μM or 0.3 mg/mL) in a final reaction volume of 27 μL at 37 °C, and subsequently exposed to Click reaction mixture in a final reaction volume of 33 μL containing in final concentrations 1.6 mM CuSO<sub>4</sub>, 0.16 mM TBTA, 1.6 mM TCEP, 4.6% v/v tBuOH, 0.9% SDS, 9.2 μM Cy-5 azide. (The freshly prepared 5X stock solution of TCEP, tBuOH, and SDS was first added, followed by the freshly prepared 5X stock solution of Cy-5 azide, CuSO<sub>4</sub>, and TBTA). Subsequent to incubation at 37 °C for 30 min, 5.0 μL 4X Laemeli dye containing 6% BME was added and the samples were further incubated for 5 min at 37 °C. 30 μL was directly loaded into each well of 10% polyacrylamide gel. Upon completion of gel-electrophoresis, the gel was rinsed with ddH<sub>2</sub>O (x2, 5 min) and analysed for Cy5 signal on a Bio-Rad ChemiDoc-MP and
subsequently coomassie-stained. Negative control samples were not exposed to light and/or TEV protease, but were treated under otherwise identical conditions.

(b) **Targeted HNEylation in live cells** *(Figure 2c, 3, 4, and S4-S8).* In experiments that use stably transfected cells, growth media were changed to complete media that do not contain puromycin 24 h prior to treatment with HtPHA. All steps hereafter were handled in the dark. Cells were treated with small molecules at a concentration of 25 µM for 2.5 h in serum-free media. Rinsing (x3) was performed every 30 min over 1.5 h with the serum-free media. For the samples designated for light exposure, plate covers were removed and mono-layered adherent cultures were exposed to 365 nm, 0.6 mW/cm² lamp for 20 min at room temperature (at ~1 inch distance from the light source) and re-incubated at 37 ºC for a further 5 min prior to harvest. Subsequent to centrifugation (500 x g, 8 min) and washing with 1X PBS (x2), the resultant cell pellets were flash-frozen in liq N₂ and subjected to 3 cycles of freeze-thaw in 15 µL lysis buffer that contained in final concentrations, 50 mM Heps (pH 7.6), 0.3 mM TCEP and 1% Nonidet. All steps hereafter were performed at 4 ºC. Debris was removed by centrifugation (18,000 x g, 8 min). A portion of the clarified lysate was made up to final volume of 25 µL containing, in final concentrations, 50 mM Heps (pH 7.6), 1.0 mg/mL lysate protein (measured by Bradford assay), and 0.2 mg/mL His₆-TEV-S219V. The mixture was incubated at 37 ºC for 45 min, and subsequently subjected to Click reaction. Briefly, in a final volume of 30 µL, the reaction mixture contained, in final concentrations, 42 mM Heps (pH 7.6), 1.7 mM TCEP, 5% v/v t-BuOH, 1% wt/v SDS, 1 mM CuSO₄, 0.1 mM TBTA, 10 µM Cy5azide and TEV-protease-treated lysate above. Subsequent to 30-min incubation at 37 ºC, the reaction was quenched with 5 µL of 4X laemmli buffer that contained 6%
BME and further incubated for 5 min at 37 °C. 20 μL was directly loaded into each well of 10% polyacrylamide gel. Upon completion of the gel-electrophoresis, the gel was rinsed with ddH₂O (x2, 5 min) and analysed for Cy5 signal on a Bio-Rad ChemiDoc-MP and, where applicable, the gel was subsequently transferred to a PVDF membrane for western blot analysis.

**Cell Culture and Transfection.** HEK-293 cells were cultured in DMEM-Glutamax (Invitrogen 41090-036) supplemented with 10% v/v FBS (100 nm-triple filtered, HyClone SH30071.03), 5% NEAA (Invitrogen A11140-050) and 5% pyruvate (Invitrogen 11360-070) in the presence of 1X penicillin-streptomycin antibiotics (Invitrogen 15140-122). HEK-293 cells stably expressing His₆-Halo-Keap1 were cultured as in HEK-293 cells except that 1.5 μg/mL puromycin (Invitrogen A11138-03) was included. Cells were cultivated in adherent culture plates (Corning) in a humidified atmosphere of 5% CO₂ at 37 °C and harvested by trypsinization (Invitrogen 25300-054). Transient transfection was performed at 50–60% confluency using Mirus TransIT-2020 (Mirus MIR5400) according to the manufacturer’s protocol.

**Generation of stably transfected cells** (Figure S1 and Figure 2a). HEK-293 cells were transfected with pMIR_DsRed-IRES-His₆-Halo-Keap1 plasmid¹ according to the Mirus protocol. TEV-protease cleavage site was encoded in the linker region between the Halo and Keap1 domains. Upon reaching full confluence, the cells were incubated with fresh media containing puromycin at 2 μg/mL, and growth was continued changing media every 4th day. Upon regaining full confluence (over ~1–2 wk period), the cells were
transferred into a small flask (e.g., 25 cm²) and cultivation was continued at the same concentration of puromycin. Western blot and FACS analysis were performed subsequently.

**Cell lysis and western blotting** (Figure 3, 4c, S3-S5, S7-S8, S11-S13). Whole cell lysates was prepared by three cycles of rapid freeze thaw in a chilled freshly prepared lysis buffer containing 50 mM Hepes buffer (pH 7.6), 1% Nonidet P-40 and 0.3 mM TCEP. Cell extract was clarified by centrifugation at 16,000 x g for 8 min at 4 °C. Total protein concentration in lysate was determined using Bradford Assay. For Nrf2 stabilization analysis, cell lysis was performed in 1X RIPA buffer: 50 mM Tris (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 1 mM orthovanadate and Roche complete mini cocktail protease inhibitor.

**Immunofluorescence** (Figure S6). Cells were grown to 70% confluence in 6-well plates with coverslips coated in 0.1% gelatin. The media was aspirated and cells were washed once with 1X PBS buffer. To fix the cells, freshly prepared solution of paraformaldehyde in PBS was added to each well (2% wt./v) and incubated for 35 min at 4 °C. The fixative was aspirated and washed with PBS (x 3) with 5-min incubation at 4 °C between each wash. Blocking and permeabilization was performed in one-step over 20 min at 4 °C by incubation in PBS containing 3% BSA and 0.2% Triton X-100. Cells were subsequently incubated with anti-c-myc antibody (1:100) in incubation buffer (1% BSA, 0.02% Triton X-100 in PBS) over 45 min at room temperature, followed by a further 35-min incubation with FITC-conjugated goat anti-mouse antibody (1:500). Cells were rinsed 3 times with
PBS after each incubation period. DAPI (Sigma) was freshly prepared in PBS from 0.5% wt./v stock solution in DMSO and added to the wells at the final concentration of 3.3×10^{-6} % wt./v. The samples were incubated in the dark for 1.5 min. Coverslips were washed once with PBS, placed on the slides with mounting medium, and stored in the dark till images were taken using a Zeiss 510 meta confocal fluorescence microscope. Image analysis was performed using Image-J (NIH).

**Confocal Microscopy** (Figure 2a, S6). Cells were cultivated in glass-bottom dishes (D35-20-1.5N, Invitro scientific) and imaged using a Zeiss 510 meta confocal fluorescence microscope 24–30 h post transfection. See also immunofluorescence protocol elsewhere.

**Flow cytometry analysis** (Figure S1). Samples were prepared by suspending the freshly harvested cells in 1% FBS in 1X PBS. Data were collected using a BD LSRII flow cytometer at the Biomedical Sciences Flow Cytometry Core Laboratory, Cornell College of Veterinary Medicine. Data analysis was carried out using FlowJo [v.10 (X)]. >10,000 cells were counted per sample.

**ARE-luciferase assays** (Figure 4a). Cignal Antioxidant Response Reporter (luc) Kit (CCS-5020L) was from Qiagen and used according to the manufacturer’s protocol. Cell lysis and measurements were performed subsequent to 18 hr incubation either post 20-min light exposure or post global HNE (25 µM) treatment.
**Nuclear/cytosol fractionation** (Figure S5). Cell fractionation protocol was adapted from Rosner et al.\textsuperscript{2} Cells from 8cm\textsuperscript{2} plate were suspended in 50 uL Buffer A containing 20 mM Tris (pH 7.6), 0.1 mM EDTA, 2 mM MgCl\textsubscript{2}, 0.5 mM NaF and 0.5 mM sodium orthovanadate and Roche complete mini cocktail protease inhibitor. The cell suspension was incubated for 2 min at room temperature followed by 10 min on ice. Nonidet P-40 was added to the cell suspension at 1% final concentration and mixed gently and thoroughly. The cell lysate was centrifuged at 500 x g for 3 min at 4 °C and 80% of the supernatant solution was extracted. The remaining white nuclear pellet was washed three times with 200 µL Buffer A containing 1% Nonidet P-40. The nuclear pellet was resuspended in 20 µL Buffer B containing 20 mM Hepes (pH 7.9), 400 mM NaCl, 25% (v/v) glycerol, 1 mM EDTA, 0.5 mM NaF, 0.5 mM sodium orthovanadate, 0.5 mM DTT and Roche. The pellet was lysed by rapid freeze thaw (x 2) followed by 20-min incubation on ice. The supernatant was collected after centrifugation at 20,000 x g for 20 min. Protein concentration was determined using Bradford assays.

**Proteasome inhibition analysis** (Figure S4). HEK cells stably expressing Halo-Keap1 were transfected with pcDNA3-myc3-Nrf2 plasmid (Addgene 21555). At 30–36 h post transfection, the cells were treated with 20 nM Bortezomib (LC laboratories). At the indicated time points after treatment, cells were harvested to probe for protein levels of RRM2 or myc-Nrf2. The results were compared with the expression levels of these two proteins obtained subsequent to T-REX-assisted HNEylation of Halo-Keap1 under otherwise identical conditions (Figure 3 and S4).
Quantitative real-time polymerase chain reaction (qPCR) analysis (Figure 4b). Total RNA was extracted and purified using TRIzol® Plus RNA Purification Kit (Life Technologies 1218355) and reverse transcribed to cDNA using SuperScript III Reverse Transcriptase (Life Technologies 18080093). The quantitative real-time PCR (qPCR) was performed with iQ™ SYBR® Green Supermix (Bio-Rad 170-8880) on the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad 170-9740). Briefly, the reaction mixture contained in a final volume of 20 µL (in final concentrations) 0.35 µM each of the indicated forward and reverse primers, and 15 ng of cDNA template in 1X SYBR® green supermix. The forward and reverse primers were first mixed with PCR master mix and water, and this mixture was loaded onto each well of the PCR multi-well plate. Corresponding cDNA template was subsequently added to the designated wells. The qPCR program was set for 3 min at 95 °C followed by 45-repeat cycles comprising heating at 95 °C for 10 s and at 55 °C for 30 s. The expected products were of ~100–200 bp in size. The primers for the detection of GAPDH, HO-1, and GCLM-1 were described previously. The sequences for all primers were shown on Table S3. Tₘ of all the primers were ~60 °C. The following criteria were adopted in determining the suitability of the primers for the qPCR analysis: (1) the threshold cycle or Ct value is no more than 30 at the concentration of the cDNA evaluated. (This requirement is met by optimizing the amount of template cDNA in the reaction); (2) no non-specific misprimed product or primer-dimer formation is observed in the melting curve analysis; (3) R² ≥ 0.95 for the linear fit of the standard curves; and (4) the primer amplification efficiency falls in the range of 80%–110%. The standard curves were generated by plotting the Ct value against log₂([cDNA]) in evaluating linearity, hence primer efficiencies. cDNA template from 20,
4, 0.8, 0.16 and 0.032 ng of total isolated purified RNA was used in generating the standard curves. In the analysis of experimental samples, cDNA template from 15 ng of total isolated purified RNA was used. Three biological replicates for each of the light-exposed and non-exposed samples were conducted. Relative quantitation was made according to Pfaffl method\(^4\) to analyze changes in gene expression levels upon T-REX-targeted HNEylation of Keap1 (i.e., compare the expression level of a specific gene of interest in light-exposed samples with respect to that in non-exposed samples). The expression levels were normalized to those of GAPDH as housekeeping gene. The abundance of each gene of interest was calculated as shown in Eq (1) as described in the SI reference no. 4. The abundance of the gene of interest from samples not exposed to light was normalized to 1.

\[
\text{Abundance} = \frac{(2^{\text{efficiency of interal control}})^{Ct_{\text{of internal control}}}}{(2^{\text{efficiency of internal gene of interest}})^{Ct_{\text{of gene of interest}}}}
\]  

(Eq. 1)

The number of independent replicates (N) for the data shown in Figure 4b (main manuscript) were: NQO1: N=12 (+), N=10 (−); HO-1: N=12 (+), N=12 (−); Trx: N=9(+), N=9 (−); and GCLM1: N=12(+), N=12 (-), where + and – indicate samples exposed (+) and not exposed (−) to light, respectively.

[NQO1, NAD(P)H quinone oxidoreductase 1. HO-1, heme oxygenase 1. Trx, thioredoxin. GCLM1, gamma-glutamylcysteine synthetase/glutamate-cysteine ligase 1].
LC-MS/MS determination of modified sites *in vitro* and *in live cells* (Figure S2c, S9, and S10, Table S4–S9). All steps were handled under dim light.

*Preparative procedure for samples from *in vitro* studies (Table S8 and S9):*

Recombinant His₆-Halo-Keap1 (3.0 µM) with His₆-Nrf2 (1.5 µM) was incubated with HtPHA or HNE alkyne (1.1 equiv) (3.3 µM) in a final volume of 50 µL in 50 mM Hepes (pH 7.6) at 37 °C for 20 min, at which point the samples were directly exposed to 365 nm, 0.6 mW/cm² hand-held lamp for 20 min at 37 °C. (The light source was placed at ~0.8 inch distance from the surface of the solution). The lamp was then removed and the samples were further incubated for 20 min at 37 °C. The samples were treated with TEV protease (10 µM or 0.3 mg/mL) in a final reaction volume of 54 µL at 37 °C. Half (27 µL) of the volume in each sample was removed and analysed by in-gel fluorescence assays as described above. The remaining half (~27 µL) in each sample was further incubated for 60 min at 37 °C prior to mixing with 5.0 µL 4X Laemmli dye (6% BME) and further incubation for 5 min at 37 °C. 30 µL was directly loaded into each well of 10% polyacrylamide gel.

*Preparative procedure for samples from *live cells* (Table S4–S7):* T-REX-targeted or global HNEylation was performed in HEK-293 cells according to the protocol described elsewhere in the SI.

*For enrichment under denatured conditions (Table S7):* The confluent monolayer cultures of HEK-293 cells were harvested from 4 x 21 cm² cultured plates and lysed in 200 µL lysis buffer A (6 M guanidine hydrochloride, 10 mM Tris, 0.1 M Na₂HPO₄, 0.1 % Triton X-100, 5 mM imidazole and 10 mM N-ethylmaleimide, pH = 8.0), vortexed, sonicated and added to 20 µL bed volume of TALON resin (Clontech®). The
suspension was incubated in the dark at 4°C for 1.5 hr with end-over-end rotation. The resin was then washed with 240 µL wash buffer A (6 M guanidine hydrochloride, 10 mM Tris, 0.1 M Na₂HPO₄, 0.1 % Triton X-100, 5 mM imidazole, pH = 8.0), 240 µL wash buffer B (8 M Urea, 10 mM Tris, 0.1 M Na₂HPO₄, 0.1% Triton X-100, pH = 8.0) and finally 240 µL wash buffer C (8 M Urea, 10 mM Tris, 0.1 M Na₂HPO₄, 0.1% Triton X-100, pH = 6.3). The proteins that remained bound to the resin were eluted in 25 µL of 50 mM Hepes, 100 mM NaCl, 300 mM Imidazole, pH=7.0.

For enrichment under native conditions (Table S4–S6): The confluent monolayer cultures of HEK-293 cells were harvested from 4 x 21 cm² cultured plates, flash-frozen in liq. N₂ and subjected to 3 cycles of freeze-thaw in 100 µL lysis buffer B (50 mM Hepes, 5 mM Imidazole, pH = 7.6). Debris was removed by centrifugation (18,000 x g, 8 min). The supernatant was recovered and added to 20 µL bed volume of TALON resin. The resin was then washed with 240 µL wash buffer D (50 mM Hepes, 100 mM NaCl, 10 mM Imidazole, pH = 7.6) and 240 µL wash buffer E (50 mM Hepes, 100 mM NaCl, 20 mM Imidazole, pH = 7.6). The proteins that remained bound to the resin were eluted in 25 µL elution buffer (50 mM Hepes, 100 mM NaCl, 200 mM Imidazole, pH = 7.6).

The eluate obtained under either denatured condition or native condition was then mixed with 4X reducing laemmlsi dye and electrophorezed through a 10% SDS-PAGE gel. The protein was visualized with Commassie blue R-250. The band representing His₆-Halo-Keap1 was excised for mass spectrometry analysis.

Sample preparation for proteomics analysis (Table S4–S9):
Upon completion of gel-electrophoresis through a Tris-Cl gel, the protein bands were visualized with Coomassie Brilliant Blue R-250 subsequent to de-staining over 24 hours.
The gel was rinsed with ddH$_2$O for 30 min and the bands representing TEV-cleaved-Keap1 were excised. After washing and dehydrating the gel pieces, the proteins were reduced with either 10 mM DTT (for data shown in Table S7–S9) or 10 mM TCEP (for data shown in Table S4–S6) in 100 mM NH$_4$HCO$_3$ solution for 45 min at 56 °C and alkylated with 55 mM iodoacetamide in 100 mM NH$_4$HCO$_3$ in dark for 45 min. After washing and drying, the gel pieces were rehydrated in 60 μL (6 μg/mL solution) of trypsin in 50 mM NH$_4$HCO$_3$ (pH 8.0) on ice for 30 min and at 30 °C overnight. Peptides were extracted sequentially with 1% formic acid, 50% acetonitrile containing 5% formic acid and 90% acetonitrile containing 5% formic acid. All supernatants were combined and dried under vacuum. LC-MS/MS analysis and data processing were performed at the Proteomics and Mass Spectrometry, Cornell University Institute of Biotechnology (Figure S2c, and Table S2 and S3).

Briefly, peptides were resuspended in 0.5% vol/vol formic acid and separated on an Ultimate 3000 (Thermo/Dionex, Inc.) nano-liquid-chromatography (LC) system coupled to a 4000 QTRAP (Applied Biosystems). Peptides were desalted onto a PepMax C18 trap column (300 μm × 5mm, Dionex, Inc.) with 98 : 2 H$_2$O : ACN (containing 0.1% vol/vol formic acid) at 20 μL/min. After a 3-min wash, peptides were separated on a PepMax nano-column (75 μm ×150 mm, Dionex, Inc.) using a 90-min linear gradient of 8% to 40% ACN in 0.1% formic acid at 300 nL/min. MS data acquisition was performed using Analyst 1.4.2 software (ABSciex) in positive ion mode for information dependent acquisition (IDA) analysis. The nanospray voltage was 1.85 kV used for all experiments in positive ion mode. Nitrogen was used as the curtain (value of 10) and collision gas (set to high) with heated interface at 150°C. The declustering potential was set at 50 eV and
Gas1 was 20 (arbitrary unit). In IDA analysis, after each survey scan for m/z 375 to m/z 1550 and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for tandem MS (MS/MS) with rolling collision energy applied for detected ions based on different charge states and m/z values. The MS and MS/MS data were then used by the Mascot search engine to search the human RefSeq downloaded from NCBInr for identification of peptides and their HNE modifications with two missed cleavage sites by trypsin allowed. The peptide tolerance was set to 1.5 Da and MS/MS tolerance was set to 0.6 Da. Several variable modifications were applied including methionine oxidation, cysteine carboxamidomethylation along with HNE (156.12), HNE alkyne (152.08), HNE alkyne reduced form (154.10), HNE alkyne dehydrated form (134.07), and reduced HNE alkyne dehydrated form (136.09) on cysteine (Figure S10). Only significant scores for the peptides defined by Mascot probability analysis (www.matrixscience.com/help/scoring_help.html#PBM) greater than “identity” with 95% confidence were considered for the peptide identification and modification site determinations. All MS/MS spectra for the identified peptides with HNE-type modifications were manually inspected and validated.

Expression and purification of recombinant human His$_{6}$-Keap1, His$_{6}$-Halo-Keap1 and His$_{6}$-Nrf2 (Figure S2). Recombinant mouse Keap1 was previously characterized to be Zinc-containing metallocprotein. Expression of Keap1 was thus carried out in M9 media supplemented with Zinc (12.8 g/L of Na$_2$HPO$_4$·7H$_2$O, 3 g/L of KH$_2$PO$_4$, 0.5 g/L of NaCl, 1.0 g/L NH$_4$Cl, 2mM MgSO$_4$, 100 µM CaCl$_2$ 50 µM ZnCl$_2$, and 0.4% of Glucose). Overnight cultures were inoculated with 50 µg/mL of CM and 50 µg/mL KAN.
Subsequent to dilution into media containing 50 µg/mL KAN, expression was induced with 250 µM IPTG at OD₆₀₀ of 0.6–0.8 over 14 h at 19 °C. For Nrf2, LB media was used and expression was induced with 250 µM IPTG at 21 °C over 13 h. Each protein was isolated by using TALON chromatography (Clontech) followed by gel filtration. For Keap1 proteins, the buffer conditions were 50 mM NaH₂PO₄ (pH 8.0), 10 mM Imidazole, 5 mM BME, 0.5 mM PMSF and 0.01% Triton X-100 for cell lysis, 50 mM NaH₂PO₄ (pH 8.0), 50 mM Imidazole, 500 mM NaCl, 5 mM BME and 0.01% Triton X-100 for washing x 3.5 bed volume, and 50 mM NaH₂PO₄ (pH 8.0), 125 mM Imidazole, 150 mM NaCl and 5 mM BME for elution, and 50 mM Tris (pH 8.0), 10 mM DTT, 100 mM NaCl and 5% glycerol for desalting by gel filtration (ÄKTA purification system, GE Healthcare HiloLoad™ 26/60 Superdex™ 200 prep grade, column ID No. 0823027) and for storage. For Nrf2 protein, lysis buffer contained 50 mM NaH₂PO₄ (pH 7.5), 10 mM imidazole, 0.05% Triton X-100, and 0.5 mM PMSF. Wash buffer contained 50 mM NaH₂PO₄ (pH 7.5), 35 mM Imidazole 500 mM NaCl, and 0.05% Triton X-100 (3 cycles of washes). His₆-Nrf2 was eluted with 50 mM NaH₂PO₄ (pH 7.5), 150 mM NaCl and 125 mM imidazole and further purified by gel filtration chromatography as above and stored in 50 mM Tris (pH 7.5) and 5% glycerol. One-shot aliquots of the purified proteins were stored in –80°C. Typical yields were 0.20, 0.24 and 0.06 mg per g of cell pellet for His₆-Keap1, His₆-Halo-Keap1 and His₆-Nrf2.

**Analytical gel filtration** (Figure S2b). Gel filtration analysis was performed using a Superdex 200 10/300 column (GE Healthcare) that had been pre-equilibrated with the running buffer (20 mM Tris, 150 mM NaCl, pH 8.0) at room temperature. Recombinant
His$_{6}$-Halo-Keap1 (3.0 μM) with His$_{6}$-Nrf2 (1.5 μM) was incubated in a final volume of 175 μL in 50 mM Hepes (pH 7.6) at 37 °C for 2 min. 150 μL of the mixture was filtered through 0.22 μm syringe filters (Millex low protein binding PVDF membrane, Millipore). 110 μL was injected onto the preequilibrated column. The flow rate was 0.5 mL/min. Absorbance was monitored at 260, and 280. Molecular mass standards (GE Healthcare): ovalbumin, 44 kDa; conalbumin, 75 kDa; aldolase, 158 kDa; ferritin, 440 kDa; thyroglobulin, 669 kDa, were run under identical conditions.
**SUPPORTING TABLES**

**Table S1.** Primers used for the construction of plasmids encoding human His$_6$-Halo-Keap1, His$_6$-Keap1, His$_6$-Nrf2 in pET28a *E. coli* expression vector, and for the construction of bicistronic mammalian expression plasmids encoding DsRed-(IRES$^\text{S}$)-His$_6$-Halo-C226S-, -C368S-, -C151S-, -C288S-, -C151S-C288S-, -C513S-, -C518S-, -C513S-C518S-Keap1, and DsRed-(IRES$^\text{S}$)-GFP-Halo. IRES, internal ribosomal entry site. Keap1 and Nrf2 genes are the longest isoforms (isoform 1) of human genes. All Halo-Keap1-fusion gene-bearing plasmids carry a TEV (cysteine protease from Tobacco Etch Virus) cleavage site in the linker region between the Halo and the Keap1 domains. In entry (4) and (5), and from (7) to (12), the sites of point mutations are underlined.

| Entry | Plasmid | Primer sequence |
|-------|---------|-----------------|
| 1)    | pET28a-His$_6$-Keap1 | Human Keap1 gene from the commercially available phrGFP-Keap1 plasmid (Addgene 28025) was cloned into an empty pET28a vector.  
**Fwd-1:**
5’-TGGTGCCTCGTGTTAGCCATATGACAGCCAGATCCCAGGC-3’  
**Rev-1:**
5’-CTCAGCCTTCCTTTCCGGCTTTTATTAACAGGTCAGTTCTGCTGTC-3’  
**Fwd-2:**
5’-ATGGGCCAGCCATCAGGACATCATCATCATCACAGCCAGCCAGCAACTCA-3’  
**Rev-2:**
5’-TATGCTAGTTATAGCTCAGGCGTGGCGACAGCCAACTCA-3’ |
| 2)    | pET28a-His$_6$-Nrf2 | Human Nrf2 gene from the commercially available pcDNA3.eGFP-cy-Nrf2 plasmid (Addgene 21549) was cloned into pET28a vector.  
**Fwd-1:**
5’-TGGTGCCCTCGTGTTAGCCATATGACAGCAGCAGCTGCTGCAATTAAC-3’  
**Rev-1:**
5’-CTCAGCCTTCCTTTCCGGCTTTTATTAACAGGTCAGTTCTGCTGTC-3’  
**Fwd-2:**
5’-ATGGGCCAGCCATCAGGACATCATCATCATCACAGCCAGCCAGCCAGCCAGCAACTCA-3’  
**Rev-2:**
5’-TATGCTAGTTATAGCTCAGGCGTGGCGACAGCCAACTCA-3’ |
| 3)    | pET28a-His$_6$-Halo- | Human Keap1 gene from phrGFP-Keap1 plasmid (Addgene 28025) was cloned into pET28a-Halo vector$^\text{S}$. |
| Keap1          | Fwd-1: 5’-TCGAGATTTCGGCTCCGGAGAAAACTTGTATTCCAGGGCTCAGGATGCGAGCCAGATC-3’  
|               | Rev-1: 5’-CTCAGCTTCCTTTCGGGCTTTTGGTTATCAACAGGTACAGTTCTGCTGGTC-3’  
|               | Fwd-2: 5’-GGACCTGATCGGACGCAGATCGCGCTGGCTGCGACGCTGAGATTTCCGGCTCCGG-3’  
|               | Rev-2: 5’-TATGCTAGTTATTTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTCCGGGCTTTTGTA-3’  
| 4) pMIR-      | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-Keap1$^1$ was used as a template for the site-directed mutagenesis.  
| DsRed-       | Fwd: 5’-TCACACTGTCCTCAGCGCACTGTTGACC-3’  
| (IRES)-      | Rev: 5’-GGTCACCAGTTGGCTGGGACAGTTGA-3’  
| His$_x$-      |  
| C226S-Keap1  |  
| 5) pMIR-      | pMIR vector encoding DsRed-(IRES)-His$_x$-Halo-Keap1$^1$ was used as a template for the site-directed mutagenesis.  
| DsRed-       | Fwd: 5’-GCCTGGCCGGCAAGCGTGGTGGG-3’  
| (IRES)-      | Rev: 5’-GCCCACCACGCTGCCGGCCAGGC-3’  
| His$_x$-      |  
| C368S-Keap1  |  
| 6) pMIR-      | pMIR vector encoding DsRed-(IRES)-GFP-RNR$\alpha^6$ was used as a template.  
| DsRed-       | Fwd-1: 5’-CACTCTCGGAGATGTCCTCCACGTC-3’  
| (IRES)-      | Rev-1: 5’-TTTAGTACTTTGAGCTGGAAGTTTCTGAGA-3’  
| GFP-Halo     | Fwd-2: 5’-GGTCCTGCGAAGTTCTGAGGACGCTGGAACGTC-3’  
|             | Rev-2: 5’-AGTTTTAAGGAAAATCCATTATTATTAAAAGTTTAGTACTTTTGAGTC-3’  
| 7) pMIR-      | pMIR vector encoding DsRed-(IRES)-His$_x$-Halo-Keap1$^1$ was used as a template for the site-directed mutagenesis.  
| DsRed-       | Fwd: 5’-CCATGGGGCGAGAAGAGTGTCCCTCCAGTC-3’  
| (IRES)-      | Rev:  
| His$_x$-      |  
|  

$^1$ pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-Keap1$^1$ was used as a template for the site-directed mutagenesis.
|   |   |   |
|---|---|---|
| C151S-Keap1 | 5'- GACGTGGAGGACACCTTTCTGCCCATGG -3' | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-Keap1$^1$ was used as a template for the site-directed mutagenesis.  
Fwd: 5'- TGCAGCTGCAAGAAGCAGGACATCATGCAG -3'  
Rev: 5'- CTGCAGATCGCTTCTGCACTGCA -3' |
| 8) | pMIR-DsRed-(IRES)-His$_6$-Halo-C288S-Keap1 | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-C151S-Keap1$^1$ was used as a template for generating the double mutant.  
Fwd: 5'- TGCAGCTGCAAGAAGCAGGACATCATGCAG -3'  
Rev: 5'- CTGCAGATCGCTTCTGCACTGCA -3' |
| 9) | pMIR-DsRed-(IRES)-His$_6$-Halo-C151S-C288S-Keap1 | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-Keap1$^1$ was used as a template for ligase-free cloning method.  
Fwd: 5'- ACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTGCAAT -3'  
AAAACACGATGATAATA-3'  
Rev: 5'- TAGCCCCCAGCAGCATAGATACAGTGTGCAGGACGCA  
GACGCTGCCCCCGCTTCGGATG-3' |
| 10) | pMIR-DsRed-(IRES)-His$_6$-Halo-C513S-Keap1 | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-C518S-Keap1$^1$ was used as a template for ligase-free cloning method.  
Fwd: 5'- ACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTGCAAT -3'  
AAAACACGATGATAATA-3'  
Rev: 5'- TAGCCCCCAGCAGCATAGATACAGTGTGCAGGACGCA  
GACGCTGCCCCCGCTTCGGATG-3' |
| 11) | pMIR-DsRed-(IRES)-His$_6$-Halo-C518S-Keap1 | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-Keap1$^1$ was used as a template for ligase-free cloning method.  
Fwd: 5'- ACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTGCAAT -3'  
AAAACACGATGATAATA-3'  
Rev: 5'- TAGCCCCCAGCAGCATAGATACAGTGTGCAGGACGCA  
GACGCTGCCCCCGCTTCGGATG-3' |
| 12) | pMIR-DsRed-(IRES)-His$_6$-Halo-C513S-C518S-Keap1 | pMIR vector encoding DsRed-(IRES)-His$_6$-Halo-Keap1$^1$ was used as a template for ligase-free cloning method.  
Fwd: 5'- ACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTGCAAT -3'  
AAAACACGATGATAATA-3'  
Rev: 5'- TAGCCCCCAGCAGCATAGATACAGTGTGCAGGACGCA  
GACGCTGCCCCCGCTTCGGATG-3' |
Table S2. Summary of antibodies. WB, western blot. IF, immunofluorescence.

| Antibody                              | Application | Catalog No.; Supplier               | Dilution |
|---------------------------------------|-------------|-------------------------------------|----------|
| Mouse monoclonal anti-Keap1 primary   | WB          | Ab119403 (clone 1B4); Abcam         | 1:1000   |
| Mouse monoclonal anti-c-myc primary   | WB, IF      | MMS-150P (clone 9E10); Covance      | 1:2000   |
| Goat polyclonal anti-lamin B primary   | WB          | sc6217 (clone M-20); Santa Cruz     | 1:1000   |
| Mouse monoclonal anti-NQO1 primary    | WB          | Sc32793 (clone A180); Santa Cruz    | 1:200    |
| Monoclonal anti-gapdh-peroxidase       | WB          | G9295; Sigma                         | 1:30,000 |
| Secondary antibody to mouse           | WB          | Ab6789; Abcam                        | 1:5000   |
| Secondary antibody to goat            | WB          | Ab97100; Abcam                       | 1:5000   |
| Secondary antibody to rabbit          | WB          | Ab97051; Abcam                       | 1:7000   |
| FITC-conjugated goat anti-mouse       | IF          | 1010-02; Southern Biotech            | 1:500    |
| secondary antibody                    |             |                                     |          |
| Name     | Sequence                                      |
|----------|-----------------------------------------------|
| NQO1 fwd | 5'-TCCTGGAAGGATGGAAGAA-3'                    |
| NQO1 rev | 5'-TCCTGCCTGGAAGTTTAGG-3'                   |
| GCLM fwd | 5'-GACAAACACAGTTGGGAACAGC-3'                 |
| GCLM rev | 5'-CAGTCAAATCTGGTGCGATC-3'                  |
| HO1 fwd  | 5'-AACTTTCAAGGGGCCAGGT-3                    |
| HO1 rev  | 5'-CTGGGCTCTCTTTGTGCTG-3'                   |
| Trx fwd  | 5'-GTCAAATGCATGCCAACAT-3'                   |
| Trx rev  | 5'-TGGCTTTCAAGCTTTTCTT-3'                   |
| GAPDH fwd| 5'-CTGACTTCAACAGCGACACC-3'                  |
| GAPDH rev| 5'-TGCTGTAGCCAAATTCGTTGT-3'                 |
Table S4. LC-MS/MS identification of HNE modifications on Keap1 pulled down (under native conditions) from HEK-293 cells treated with HNE (100 µM, 20 min).

| Human Keap1 (100%), 70.0 kDa, Mascot Score 2019, 99 unique peptides with different modifications, 4 distinct tryptic peptides with added mass of 134Da, 152Da or 154Da for possible modifications of dehydrated HNE-alkyne, HNE-alkyne or reduced HNE-alkyne, respectively (Figure S10). All the modified peptides were found present in corresponding unmodified forms of the native peptides with Cys being alkylated by carbamidomethylation. 521/624 amino acids (83% coverage) Matched peptide with HNE related modifications shown in green, other matched peptide shown in red |
|----------------|-----------------|
| MQPDPGRPSGAGACCRFLPLQSQCPEGAGDAVMYASTECKAEVTPSQHG    |
| TFSYTLEDHTKQAFIGMNELRSQOQLCDVTLQVKYQDAPAAQFMAHKVVLAS    |
| SSVPVKAMFTNGLREQMVEVSVIEGJHPKVMERLIEFAYTASISMG    |
| EKCVLHVMNGAVMYQIDSVVRACSDFLVQQLDPSNAIGIANFEAQIGCVELH |
| QRARYIYMHFGEVAKQEEEFLNSHCQLVTLISRDDLNRCESEVFHACI    |
| NWVKYDCEQRFYVQALLRARVCHSLTPNFLQMQLKCEILQDSRCKDY    |
| LVKIFEELTLHKPTQMPCRAPKVGRLIYTAGGYFRQSLSYEAYNPSD       |
| TWLRLADLQVPRSGLAGCVVGGLLYAVGGRNNSPDGNTDSSALDCYNPATM  |
| NQWSPCAPEMVSVPNIRIGVVIDGHIYAVGGSHGCIIHNSVERYEPERDEW |
| HLVAPMLTRRIGVGAVALNRLLYAVGGFDGTNRLNSAECYYPERNEWRMI    |
| TAMNTIRSGAGVCVHLHNCIYAGGGYDQDQLNSVERYDGETETWTFVAPM    |
| KHRSAALGITVHQGRIYVLG YGDHFTLDSVCEYDPDFTDWTSEVTRMTSG    |
| RSGVGVAVTM EPCRKQIDQQNCTC |
| Unique peptide with HNE-alkyne modification (asterisk suggests modification site) | MS spectra |
| C23 | |
| FLPLQSQC*PEGAGDAVMYASTECK | |
| p-value: 0.00000005 Mascot Ion Score: 74 | |
| Modification: dehydrated HNE-alkyne | |
| Protein | Peptide Sequence | Mascot Ion Score | p-value | Modification |
|---------|------------------|------------------|---------|--------------|
| C226    | QEEFFNLSC*QLVTLISR | 66               | 0.0000002 | HNE-alkyne   |
| C273    | C*HSLTPNFLQMQLQK  | 47               | 0.00002  | dehydrated HNE-alkyne |
| C368    | LADLQVPRSGLAGC*VVGGLLYAVGGR | 32               | 0.0007  | reduced HNE-alkyne |
Table S5. LC-MS/MS identification of HNE modifications on Keap1 pulled down (under native conditions) from HEK-293 cells treated with HNE (25 µM, 20 min).

Human Keap1 (100%), 70.0 kDa, Mascot Score 1688, 76 unique peptides with different modifications, 2 distinct tryptic peptides with added mass of 134 Da or 154 Da for possible modifications of dehydrated HNE-alkyne or reduced HNE-alkyne, respectively (Figure S10). Both modified peptides were found present in corresponding unmodified forms of the native peptides with Cys being alkylated by carbamidomethylation.

497/624 amino acids (79% coverage)
Matched peptide with HNE related modifications shown in **green**, other matched peptide shown in **red**

MQPDPRPSGAGACCRLFPLQŚQCPEGAGDAVMYASTECKAEVTPSQHGNRTFSYTLLEDHTKQAFIGIMNELRLSQQLCDVTLQVKYQDAPAAQFMAMHKVVLASSSPVFKAMFTNLREQQMEVVSVIEGHIPKVMERLIEFAYTASISMEGECVLMHVNGAVMYQIDSVRRACSDLVQQLDPSNAIGIANEFAEAIGCVELHQRAREYIYMHFGEVAKQEEFHNLSHQQLVTLSRDDLNVRECSEVFHACINWVKYDCEQRRFYVQALLRAVRCHSLTPNFLQMQLQCEILQSDSLCKDYLVKIFEEITLHKPTQVMPCRAPKVGRLIYTAGGFQSLSYLEAYNPSDGTWLRŁALĐQVPRSLAGCVVGGLLYAVGGRRNNSPDGNTDSSALDCYNPMTNQQWSPCAPMSVPNINKVGLVMGHIAVGGSHGCIHHNSVEREYEPDEWHLVAPMLTTRRGFLYAVGGFDGTNRLLNSAECYYPERNEWRATAMNTIRSGAVGCVLHNCIYAAAGYDGQDQLNSVERYDVESETETWTFVAPMKHRRSAŁGTVHQQRIYVLGBPMDLFDSVECDPDPTDTWSEVTRMTSGRSGVGAVMTMPCRKQIDQQNCTC

| Unique peptide with (dehydrated) HNE-alkynemodification (asterisk suggests modification site) | MS spectra |
|---|---|
| C23 | ![MS spectra image](image_url) |
| FLPLQŚQC*PEGAGDAVMYASTECK | p-value: 0.000009 Mascot Ion Score: 50 |
| Modification: dehydrated HNE-alkyne | |
**C368**

LADLQVPRSG
LAGC*VVGGL
LYAVGGGR

p-value: 0.00006
Mascot Ion Score: 42

**Modification:**
reduced HNE-alkyne
**Table S6.** LC-MS/MS identification of HNE modifications on Keap1 pulled down (under native conditions) from HEK-293 cells subsequent to T-REX-assisted Keap1-targeted HNEylation in cells.

Human Keap1 (100%), 70.0 kDa, Mascot Score 1614, 50 unique peptides with different modifications, 1 tryptic peptide with added mass of 134 Da for possible modifications of dehydrated HNE-alkyne (Figure S10).

The modified peptide was found present in corresponding unmodified form of the native peptides with Cys being alkylated by carbamidomethylation.

461/624 amino acids (73% coverage)

Matched peptide with HNE related modifications shown in **green**, other matched peptide shown in **red**

MQPDPRPSGAGACCRFLPLQSCPEGAGDAVMYASTECKAEVTSPQHGNRTFSYTLLEDHTKQAFGIMNELRLSQQLCDVTLQVKYQDAPAAQFMHVKVLASSSPVFKAMFTGLERQGMEVVSIEGHIHKVMERLIEFAYTASISMGEKCVLHVMNGAVMYQIDSVVRACSDLVFQQLDPSNAIGIANFAEQIGCVELHQRAREYIYMHFGEVAKQEEFNLSHCQLVTLSRDDLNVRCSEVFHACINWVKDYCERQRFYVQALLRARCHSLTPNFLQMQLQCKELQDSRSCKDYLVKIFEEITLHKPTQVMPCRAPKVGRILYTAGGYFRQSLSYLEAYNPDSGTWLRLADLQVPRSGLAGCVCVGLLYAVGGRNNPDGNTDSALLDCYNPMTNQWSPCAMPVPRNRIGVGVIDGHIAVGISHGIIHNSVERYEPERDEWHLVAPMLTRRIGVGVAVLRLLYAVGFGDGTNRILNSAEICYPERNEWRMITAMNTIRSGAVGCVLHNCIYAAGGYDGDQLNSVERYVETETWTYAPMKhRSLGTVHQQGRYIVLGYGDHTFLDSVECYDPDWTSEVTRMTSGRSVGAVAETMPDCKQIDQCNCTC

| Unique peptide with (dehydrated) HNE-alkynemodification (asterisk suggests modification site) | MS spectra |
|---|---|
| C513 or C518 MITAMNTIRSG AGVC*VLHNCI YAAGGYDGDQD QLNSVER or MITAMNTIRSG AGVCVLHNC*I YAAGGYDGDQD QLNSVER | |

p-value: 0.0001

Mascot Ion Score:
| 39 |
|----|
| **Modification:** dehydrated HNE-alkyne |
**Table S7.** LC-MS/MS identification of HNE modifications on Keap1 pulled down (under denatured conditions) from HEK-293 cells subsequent to T-REX-assisted Keap1-targeted HNEylation in cells.

Human Keap1 (100%), 70.0 kDa, Mascot Score 1456, 63 unique peptides with different modifications, 1 tryptic peptide with added mass of 134 Da for possible modifications of dehydrated HNE-alkyne (Figure S10). The modified peptide was found present in corresponding unmodified form of the native peptides with Cys being alkylated by 4-vinyl pyridine or N-ethylmaleimide. 443/624 amino acids (71% coverage) Matched peptide with HNE related modifications shown in **green**, other matched peptide shown in **red**

```
MQPDPGRPSGAGACCFPLPLSQQCPEGAGDAVMYASTECKAEVTPSQHGNR
TSYTLLEDHT KQAFIGMNELRSLQQLCDVT LQVKYQDAPAAQFMHVKVVL
ASSSPVFKAM FTNLREQGMEVVSIEGIHPKVMERLIEFAYTASISMGEK
CVLHMNGAVMYQIDSVVRACSDLHVQQLDPSNAIGIANF AEQIGCVELH
QRAREYIYMH FGEVAKQEEF FNLSHCOQLVTLSRDDLNVRCESEVFHACI
NWVKYDCEQR RFYVQALLRAVRCHSLTPNFLQMLQKCEILQDSRCKDY
LVKIFEEFLHLKPTQVMPCRAPKVGRLIYTAGGYFRQSLSYLEAYNPSDG
TWLRDLQVPRSLAGCVVGGLLYAVGGRNPSDGNTDSSALDCYPMNT
NWSPCAPMSVPRNIRIVGVIDGHIYAVGG SHGCIIHNSVERSEPERDEW
HLVAPMLTRIGVGAVLNLRLLYAVGGFDGTNRNLNSAEICYPERNEWRMI
TAMNTIRSGAGVCLHNCIYAGGYDQQDLNSVERYDVETETTWFPAPM
KHRASALGIVHVQGRYIVLGYGDHTFLDSVECYPDTDTWSEVTRMTSG
RSGVGVAVTM EPCRKQIDQQNCTC
```

| Unique peptide with (dehydrated) HNE-alkynemodification (asterisk suggests modification site) | MS spectra |
|---|---|
| **C513 or C518** SGAGVC*VLHNCIYAAGGYDGQDQLNSVER Or SGAGVCLHNCIYAGGYDGQDQLNSVER | |

p-value: 0.00000000002
Mascot Ion Score:
| 106 |
|-----|
| **Modification:** dehydrated HNE-alkyne |
Table S8. LC-MS/MS identification of HNE modifications on recombinant human Keap1 from global HNE treatment in vitro. McMahon et al. has previously reported that from global treatment of the cells with HNE, C151 of mouse Keap1 is modified by reduced HNE (i.e., adduction by 4-hydroxy-2-nonenol, instead of 4-hydroxy-2-nonenal). In the case of C273 modification, modification of H274 (with dehydrated HNE-alkyne), i.e., CH*SLTPNFLQMQLQK, is possible.

| Human Keap1 (100%), 70.0 kDa, Mascot Score 2610, 116 unique peptides with different modifications, 7 distinct tryptic peptides with added mass of 134 Da or 152 Da for possible modifications of dehydrated HNE-alkyne or HNE-alkyne, respectively (Figure S10). All 7 modified peptides were found present in corresponding unmodified forms of the native peptides with Cys being alkylated by carboxamidomethylation. 531/624 amino acids (85% coverage) Matched peptide with HNE related modifications shown in green, other matched peptide shown in red |
|---|
| MQPDPRPSPGAGACCLFLPLQSQCPEGADAVMYASTECKAEVTSPQHGNR TFSYTLEDHTKQAAGIMNELRLSQQLCVDTLQVKYQDAPAAQFMAHKVVL ASSSPVFKAMFTNLREQGMEVVSIEGHPKVMERLIEFAYTASIMGEK CVLHMNGAVMYQIDSVVRACSDLVLQQLDPSNAIGIANFAEQGCVELH QRAEYIYMHFGEVAKQEEFNLHSCQLVTLISRDDLNVCESEVFHACINWVKYDCEQRRFYVQALLRAVRCHSLTPNFLQMQLQKCEILQSDSRCKDYLVKIEEELTHKPTQVMPCRAPKVGRILIYTAGGYFQRLSYLEAYNPSDG TWLRLADLQVPRSGLAGCVGGGLYAVGGRNNSPDGNVTSSALDCYNPMT NQWSPCAVVPNRIGGVIDGHYAYVGGSHGCIIHNSVEREYPERDEWHLVAPMLTRRIGVGAVLNRLLYAVGGFDGTMNLNSAECYYPERNEWRMI TAMNTIRSGAGVCVLHNCSIAYAGGYDQGDQLNSVERYDVEOTWTFVAPM KHRSAALGITVHQQRIYVGLGYDGHFTFLDSVCEYDPDTDTWSEVTRMTSGRSGVGVAVTM EPCRKQIDQQNCTC |
| Unique peptide with (dehydrated) HNE-alkyne modification (asterisk suggests modification site) | MS spectra |
C77
LSQQLC*DVT
LQVK
p-value:
0.000001
Mascot Ion Score:
60
Modification:
derhydrated HNE-alkyne

C151
C*VLHVMNGA
VMYQIDSVVR
p-value:
0.00000004
Mascot Ion Score:
74
Modification:
derhydrated HNE-alkyne

C226
QEEFFNLSHC*
QLVTLISR
p-value:
0.000004
Mascot Ion Score:
54
Modification:
derhydrated HNE-alkyne
| C273 | C273 | C319 |
|------|------|------|
| **C*HSLTPNFLQ MQLQK** | **C*HSLTPNFLQ MQLQK** | **IFEELTLHKPT QVMPC*R** |
| p-value: 0.000008 | p-value: 0.000005 | p-value: 0.0001 |
| Mascot Ion Score: 61 | Mascot Ion Score: 43 | Mascot Ion Score: 40 |
| **Modification:** dehydrated HNE-alkyne | **Modification:** HNE-alkyne | **Modification:** dehydrated HNE-alkyne |

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**C273**

C*HSLTPNFLQ MQLQK

p-value: 0.000008
Mascot Ion Score: 61

**Modification:** dehydrated HNE-alkyne

**C273**

C*HSLTPNFLQ MQLQK

p-value: 0.000005
Mascot Ion Score: 43

**Modification:** HNE-alkyne

**C319**

IFEELTLHKPT QVMPC*R

p-value: 0.0001
Mascot Ion Score: 40

**Modification:** dehydrated HNE-alkyne

---

S-35
| C368 |
|------|
| SGLAGC*VVG GLLYAVGGR |
| p-value: 0.0000000003 |
| Mascot Ion Score: 95 |
| **Modification:** dehydrated HNE-alkyne |

![Graph](image)
Table S9. LC-MS/MS identification of HNE modifications on recombinant human Keap1 from T-REX-assisted targeted HNEylation in vitro. Hourihan et al.\textsuperscript{8} has previously reported the functional relevance of C226 in disulfide bond formation. C151, the residue with the 2\textsuperscript{nd}-highest ion score in the globally HNEylated sample in vitro (Table S8) was not found to be modified under T-REX conditions.

| C226 | MS spectra |
|------|------------|
| QEEFFNLSH C*QLVTISR | ![Diagram of MS spectra] |

Human Keap1 (100%), 70.0 kDa, Mascot Score 1920, 105 unique peptides with different modifications, 2 distinct tryptic peptides with added mass of 134 Da for possible modifications of dehydrated HNE-alkyne (Figure S10). Both of the two modified peptides were found present in corresponding unmodified forms of the native peptides with Cys being alkylated by carbamidomethylation. 530/624 amino acids (85% coverage) Matched peptide with HNE related modifications shown in green, other matched peptide shown in red

MQPDPRPSGAGACCRFLPLQSQCPEGADAVMYASTECKAEVTSPHQHNR TFSYTLLEDHTKQAIGMNEKLQLCDVTLQVYQDAPAAQFMHAKVVL ASSSPVFKAMFTNLREQGMEVVSIEGHIKP VMERLIEFA YTASISMGEK CVLHMNGAVMYQIDSVVRACSDFLVQQLDPSNAIGIANFAEQIGCVELQQR AREEIYMHFGEVAKQEEFFNLSHCQLVTLISRDDLNVRCSEVFHACINWK YDCEQRRFYVQALLRAVRCHELTPNFLQMLQKCEILQSDRCBKDVKIFE ETLHKPTQVMPCRAPKVGRLYTAGGYFRQSLSYLEAYNPSDGTLRLALD LPVPRSGLACVGGGLLYAVGGRNNSPDGNTDSSALDCCYNPMTNQWSPCAM SVPRNRIGVVIDGHIAVGGSHGCHHSVEYPERDEWHLVAPMLTRRIG VGAVLNRLLYAVGGFDGTNRLNSAECYPERNEWRMITAMNTSAGVCC VLHNCIYAEAGYGDQQDLNVSERYDVELETWTFVAPMKHRRSALGTVHQQ RIVLGGYDGHTFLDSVECYPDPTDTTWSEVTRMTSGRSGVVAVTMEPCRK QIDQCNCTC

### Unique peptide with HNE-alkyne modification (asterisk suggests modification site)

| QEEFFNLSH C*QLVTISR | p-value: 0.000008 | Mascot Ion Score: 51 |
|----------------------|-----------------|----------------------|
| **Modification:**    | dehydrated HNE-alkyne | |
C368

SGLAGC*VVGG
LLYAVGGR

p-value: 0.0000008
Mascot Ion Score: 61

Modification: dehydrated HNE-alkyne
Supporting Figures

Figure S1.

(a) HEK-293 cells were stably transfected with bicistronic expression plasmid pCMV_DsRed-IRES-Halo-Keap1 (Table S1) and the amount of DsRed fluorescence in live cells was quantitated by flow cytometry (left) against non-transfected controls (right). (b) Comparison of transfection efficiency between stable and transient expression of Halo-Keap1 in HEK-293 cells using the same plasmid in (a). Error bars designate S.D. (n = 3).
Figure S2.
**Figure S2.** (a) SDS-PAGE analysis of recombinant human proteins. Lane 1, MW ladder. Lane 2, His$_6$-Keap1 (72 kDa). Lane 3, His$_6$-Halo-Keap1 (107 kDa). Lane 4, His$_6$-Nrf2 (70 kDa). Nrf2 has been previously reported to migrate at an apparent MW of ~95–110 kDa.$^9$ (b) Gel filtration analyses show that Halo-Keap1 behaves similarly as Keap1, in dimerization and binding to Nrf2, and that treatment of either Keap1–Nrf2 complex with 1.1 equiv of HNE, or (Halo-Keap1)–Nrf2 complex under T-REX-mediated HNEylation conditions, did not lead to Nrf2 dissociation. The 2:1 binding stoichiometry was validated by SDS-PAGE analysis of eluted fractions of the complex in each case. Representative data sets from three independent gel filtration experiments are shown. In both panels, solid blue (—) traces designate corresponding Keap1–Nrf2 (*left*) and (Halo-Keap1)–Nrf2 (*right*) complexes (post HNE treatment). Dotted green (…) designates Nrf2 as a standard. Dashed red (---) traces respectively designate Keap1 (*left*) and Halo-Keap1 (*right*) as standards. In the left panel, ★: 19.9 min, 541 kDa (2:1 Keap1: Nrf2); ●: 22.5 min, 298 kDa (Nrf2 alone); and ✬: 24.0 min, 210 kDa (Keap1 dimer alone). Note: retention time of Keap1 dimer is known to deviate from the theoretical value predicted based on MW.$^{10}$ In the right panel, ★: 18.3 min, 782 kDa (2:1 Halo-Keap1: Nrf2); ●: 22.5 min, 298 kDa (Nrf2 alone); and ✬: 21.7 min, 357 kDa (Halo-Keap1 dimer alone). MWs were extracted from GE Healthcare MW standard curve. (c) T-REX with recombinant proteins. Arrow 1, 2, 3, 4 and 5 designate: Nrf2, Halo-Keap1, Keap1 after TEV-cleavage, Halo after TEV-cleavage, and TEV, respectively. A representative data set from six independent experiments is shown. All recombinant proteins have N-terminal His$_6$ tag. TEV is a cysteine protease from Tobacco Etch Virus.
Figure S3. Whole-cell HNE treatment inhibits Nrf2 degradation. HEK-293 cells transiently expressing myc-Nrf2 and stably expressing Halo-Keap1 were directly treated with 25 μM HNE for indicated time period. A representative data set from three independent experiments is shown. The data are compared with the results from T-REX-assisted HNEylation (Figure 3). GAPDH is used as a loading control.
Figure S4. Proteasomal inhibition stabilizes both RRM2 and Nrf2. HEK-293 cells transiently expressing myc-Nrf2 and stably expressing Halo-Keap1 were directly treated with 20 nM bortezomib. The data are compared with the results from T-REX-assisted HNEylation (Figure 3).

| Time (h) | 0 | 1 | 4 | 18 |
|---------|---|---|---|----|
| α-RRM2  |   |   |   |    |
| α-gapdh |   |   |   |    |
| α-myc   |   |   |   |    |
| α-gapdh |   |   |   |    |

20 nM Bortezomib

RRM2 stabilization

Nrf2 stabilization

Figure S4.
Figure S5. Cell fractionation analysis shows Nrf2 does not selectively accumulate in the nucleus subsequent to Keap1-alone HNEylation. Nuclear/cytosol fractionation analysis\(^5\) subsequent to T-REX-assisted Keap1-specific HNEylation. Lamin B and GAPDH respectively serve as nuclear and cytosol markers (and loading controls). A representative data set from four independent experiments is shown. The outcomes were independently validated by immunofluorescence analysis (Figure S6).
Figure S6. Immunofluorescence analysis shows Nrf2 does not selectively accumulate in the nucleus subsequent to Keap1-alone HNEylation. (a) HEK-293 cells transiently expressing myc-Nrf2 and stably expressing Halo-Keap1 were subjected to the indicated conditions against controls and subsequently fixed and immunostained using anti-myc primary and FITC-conjugated secondary antibodies. Representative images are shown with scale bars, 5 µm. (b) Results from Image-J (NIH) quantitation of immunofluorescence data. Error bars designate S.D. (n = 30). Four independent samples were analyzed per condition. The outcomes were independently validated by nuclear/cytosol fractionation analysis (Figure S5).
**Figure S7.**

HNE-alkyne signal remains on Keap1 over the time course of the western blot and ARE-luciferase assays shown in Figure 3 and 4. Results from three independent experiments are shown. Time post light indicates incubation time after 20-min light exposure. The “Cy5” signal (on dark gel) corresponds to the molecular weight expected for Keap1 after TEV-protease-mediated separation of the HaloTag from Keap1. Also see Figure 2b for a representative view of the entire fluorescence gel against the coomassie-stained PVDF membrane. GAPDH is used as a loading control.
Figure S8. T-REX-assisted Keap1-specific HNEylation at substoichiometric levels is sufficient to upregulate the protein expression level of the well-known ARE-driven gene NQO1. Western blot quantitation of the results from 5 independent biological replicates. 3 are shown in Figure 4c. Error bars designate S.D (n = 5). 25 μM HtPHA (Figure 1c) was used where applicable.
Figure S9. Domain structure of 624-amino acid-long human Keap1 and specific Cys’s modified under indicated conditions (Table S4–S9). N and C respectively designate N- (1–60) and C-terminal regions (599–624). BTB, IVR, and Kelch domains designate broad complex, Tramtrack, Bric-á-brac (61–179), intervening region (180–314), and Kelch domain (315–598), respectively. The Kelch domain houses the Nrf2 binding site and BTB is the domain via which Keap1 homodimerizes. All 9 residues characterized to be modified in this study have been previously implicated to be sensitive to HNE and other electrophiles.\(^7\)\(^{11}\) To the best of our knowledge, C151 is the only residue of Keap1 for which direct evidence of Keap1 HNEylation (specifically modified by “reduced HNE”) has been obtained in intact cells subsequent to whole-cell HNE treatment.\(^7\) All the other available MS data on Keap1 HNEylation report on modifying the recombinant protein from human or mouse in isolated systems.\(^{11}\) Differences in residue specificity between \textit{in vitro} and in cell systems (Table S6 and S7 vs. Table S9) are likely due to different conformational preference of Halo-Keap1. See also discussion in main manuscript.
Figure S10. Proposed structures accounting for Cys modifications in Table S4–S9. Shown here are possible chemical structures for HNE modifications of Keap1 Cys residues in vitro and in cells subsequent to either whole-cell or T-REX treatment approaches. Also see Table S4–S9 and Figure S9. To our best knowledge, “reduced HNE” is the only characterized modification of Keap1 cysteine subsequent to whole-cell flooding with HNE. Our own identification of “reduced HNE alkyne” under global treatment (Table S4 and S5, modification specifically on residue C368) mirrors this existing report. Identity of the reducing agent that gives rise to reduced HNE modification is presently unclear.
Figure S11. C226S and C368S (Table S9) point mutations maintain the capability of Keap1 to be HNEylated under T-REX targeting conditions, and the mutant proteins are able to stabilize Nrf2. For each mutant/wild type (viewed down vertically from top), conditions with no light exposure, or 20-min light exposure with incubation time of either 1, 4 or 18 hr, were performed. For the “Cy5” fluorescence gel (dark background), the top band is Keap1 (70 kDa) and the lower band is Halo (33 kDa) resulting from TEV-cleavage. Representative Cy5 gels and the corresponding western blots from three independent experiments are shown. Lowered intensity of Cy5 band on Keap1 for the C368S mutant is related to lower ectopic expression level achieved for this mutant as validated by western blot analysis. GAPDH is used as a loading control.
Figure S12. Consequences of Cys to Ser mutations on C513 and C518 (Table S6 and S7) in relation to T-REX-assisted Keap1-targeted HNEylation in live cells. WT and DM respectively designate wild type Keap1 and the double mutant in which both C513 and C518 have been mutated to Ser. Data sets from two independent replicates are shown. Each data set is analyzed by in-gel fluorescence, probing for efficiency of targeted HNEylation on WT or mutant Keap1 (Top band: signal from HtPHA located on Halo domain of Halo-Keap1; lower band, signal from adducted HNE-alkyne on Keap1 post TEV cleavage), and by western blot, probing for Nrf2 stabilization. Lysates are also probed with anti-Keap1 for WT vs. mutant Keap1 expression levels. Anti-GAPDH probe is used as a loading control. Although LC-MS/MS analysis identifies C513 and C518 as residues HNEylated under T-REX conditions in live cells (See Table S6 and S7), the data in this Figure suggest that the mutant Keap1 proteins are still able to sense HNE and able to block Nrf2 stabilization. Also see discussion in main manuscript.
Figure S13. ARE upregulation resulting from T-REX-assisted targeted HNEylation of WT-, C513S-, C518S-, and C513S/C518S-Keap1 in cells (see also Figure S12). Error bars designate S.D. (n ≥ 8 for WT, and n ≥ 5 for mutants).
Figure S14. Consequences of Cys to Ser mutations on C151 and C288 in relation to T-REX-assisted Keap1-targeted HNEylation in live cells. WT and DM respectively designate wild type Keap1 and the double mutant in which both C151 and C288 have been mutated to Ser. Data sets from two independent replicates are shown. Each data set is analyzed by in-gel fluorescence, probing for efficiency of targeted HNEylation on WT or mutant Keap1 (Top band: signal from HtPHA located on Halo domain of Halo-Keap1; lower band, signal from adducted HNE-alkyne on Keap1 post TEV cleavage), and by western blot, probing for Nrf2 stabilization. Lysates are also probed with anti-Keap1 for WT vs. mutant Keap1 expression levels. Anti-GAPDH probe is used as a loading control. Previous data suggest that even under non-induced conditions, C151 and C288 mutations result in hyperactivity and loss-of-function phenotype of Keap1 repressor activity, respectively\textsuperscript{11,12}. The Nrf2 levels from samples unexposed to light in this Figure are consistent with the previous reports. DM resulted in an intermediate level of Nrf2 in between the effects of each single mutant. Targeted HNEylation of C151S was able to relieve constitutive repressor function and stabilize Nrf2 to modest levels. The Nrf2 stability was not visibly affected by HNEylation of the double mutant. The varied HNEylation efficiencies based on Cy 5 gels was likely due to different mutant vs. WT protein expression levels, and/or changes in conformation of Halo-Keap1 upon mutations. Also see discussion in main manuscript.
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