Supplementary Information

Ube2V2 is a Rosetta Stone bridging redox and ubiquitin codes, coordinating DDR responses

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Supplemental Figures S1–S9 and Supplemental Figure Legends.

General Materials and Methods.

Statistical Analysis.

Supplemental Tables S1–S6.

Supplemental References.
Supplement figures and figure legends

Figure S1

A. T-REX: interrogation into on-target functional redox response of low-occupancy modifications

B. Hi-PreHNE or DMSO

C. His-Halo Titration (μg)

D. Hi-PreHNE treatment

E. Streptavidin pulldown of HNE(alkyne)ylated proteins

F. Streptavidin pulldown of HNE(alkyne)ylated proteins

G. Streptavidin-HRP

H.孑 LEN-pulldown LMW privileged sensors identified by G-REX

Table:

| Condition | Protein | Fold Change | p-value |
|-----------|---------|-------------|---------|
| Control   | Halo    | 1           | 0.01    |
| Light     | Halo    | 2           | 0.002   |

Cell/fish lysis

TEV-azide Click

Streptavidin pulldown of RES-modified PFR

Western

Coomassie

Validation of target specificity and quantitation of the extent of PFR-specific modification

Cy5-azide Click

In-gel fluorescence
**Figure S1.** G-REX identifies privileged first-responding redox-sensor cysteines genome-wide. A. Workflow for executing T-REX on-demand RES-targeting in cells/in vivo to a specific privileged first responder (PFR), one of the hits from G-REX screen in this study (see Fig. 1A for G-REX set-up). PFR fused to HaloTag is ectopically expressed in live cells or fish. The bioinert photocaged precursor targetable to Halo [Ht-PreHNE(alkyne)] (see fig. S1B) binds to Halo domain. After excess probe has been rinsed out, low-energy light exposure liberates of HNE(alkyne) (t_{1/2} < 1–2 min) in substoichiometric amounts and ‘Class II proximity enhancement’ (1) enables (i) targeted HNEylation of PFR in cells/fish and (ii) direct readout of functional redox responses downstream in an otherwise unperturbed proteome. Light source: 365 nm, 0.3 mW/cm² hand-held UV-lamp placed 1 inch above samples [3–10 min in cells (G-REX/T-REX) or fish embryos (T-REX)]. B. Schematic illustration of T-REX/G-REX experimental setup and chemical structures of Ht-PreHNE(alkyne) and Ht-PreHNE(no alkyn). C. Estimation of the amount of Halo ectopically expressed in live HEK293T cells (and thus, the amount of HNE released in situ during G-REX based on 60% photocaging efficiency of Ht-PreHNE bound to Halo). standard curve (right panel) was obtained using recombinant His₆-Halo over a range of concentrations across which a linear relationship between the amount of recombinant Halo protein loaded and the intensity of the anti-Halo western blot signal was maintained. Calculations: amount of Halo ~ ~55 ng in 1.24 × 10⁵ cells. Taking 1.44 × 10⁻¹² L as the volume of each individual cell (assuming perfect spherical shape) (2), average concentration of total Halo expressed per cell is ~9 µM. See SI for detailed calculations. Using <photo-uncaging efficiency> ~60%, <[HNE]> ~5 µM is released intracellularly per cell under G-REX conditions. D. Time course experiment comparing extent of global proteome HNEylation following G-REX in-situ HNE delivery and exogenous HNE-alkyne exposure (5 µM) of HEK293T cells ectopically expressing Halo construct. After respective treatment, the cells were harvested at the indicated time post light illumination (in G-REX) or at the indicated time of HNE-alkyne treatment period (in bolus), lysed, and lysates were subjected to Click coupling with Cy5-azide followed by in-gel fluorescence analysis. Inset shows quantitation normalized against Coomassie for loading in each gel-lane and where each non-zero time-point data are further normalized against the values from photocaged-probe-alone treatment (i.e., no light exposure) in G-REX or no HNE-alkyne treatment in Bolus (set to 1 on the Y-axis). E. Control experiment for G-REX proof-of-concept. Samples from G-REX-treated cells analyzed by Coomassie. See Fig. 1A for G-REX workflow except that proteins eluted from streptavidin beads were analysed by SDS-PAGE in place of proteomics analysis. ‘Alkyn’ vs. ‘no-alkyne’ respectively describes whether the small-molecule photocaged precursor, upon photouncaging, liberates alkyn functionalized-HNE (capable of biotinylation via Click coupling with biotin-azide) or native HNE (incapable of biotinylation). As expected, the ‘Elute’ fraction in the case of ‘no-alkyne’ probe showed no detectable proteins, validating specificity of Click biotinylation and streptavidin pulldown. [Unless otherwise stated, “Ht-PreHNE” in this manuscript designates the version with alkyn functionalization. See fig. S1B for chemical structure of Ht-PreHNE (alkyne vs. no-alkyne)]. Note: the Cy5 signal in the ‘+ alkyn’ sample” at ~39 kDa corresponds to the TEV-cleaved Halo domain carrying the remaining non-photouncaged Ht-PreHNE that is also recognized by Click. F. Control experiment for specificity of Biotin-Streptavidin enrichment. either HNE alkyn or HNE No alkyn (15 µM) was incubated with native HEK293T lysates and subjected to Click coupling with Biotin-azide followed by streptavidin enrichment. Samples analyzed by Coomassie (top) and streptavidin-HRP blot (bottom). The eluted samples from streptavidin beads were analyzed as indicated. ‘No-alkyne’ version of HNE (designated as “–” for corresponding gel lanes) gave no detectable biotinylated proteins in ‘Elute’ fraction, confirming specific binding. G. Similar experiment to workflow in fig. 1A and fig. S1B. Sample eluted from streptavidin beads was resolved by SDS PAGE. A band between 15–25 kDa (shown by a red box) was excised and analyzed by digest LC-MS/MS. H. Top hits from LC-MS/MS analysis of excised band within the gel from fig. S1F. The hits in red are novel HNE sensors discovered by G-REX in this study; the hits in blue are known HNE-sensors. (A slightly higher MW observed for the Halo band in fig. S1E compared to fig. S1G, was due to the extra genetically-encoded tag present in the construct used for fig. S1E). “M” designates molecular weight marker lane in all gels/blots in this and all supplemental figures elsewhere.
Figure S2

A Human Ube2V1 and Ube2V2

| Protein       | Sequence                                                                 | Length |
|---------------|---------------------------------------------------------------------------|--------|
| Ube2V1        | MVQEYASVLYSGLSGKLDGRELLEPETFH>GKDKSFQPSQFRLLEELGKEQGKVQGIGTYSGVGL      | 60     |
| Ube2V2        | MVSTGKVPRFRLLEELGKEQGKVQGIGTYSGVGL                                    | 35     |

B Ube2V1

| Species        | Sequence                                                                 | Length |
|----------------|---------------------------------------------------------------------------|--------|
| D. rerio      | MRRLFTYVYSLFPLRKPSAASQFRILLEQGKGKGVGQIGCYSGVGL                         | 53     |
| H. sapiens    | MCVQVLYSGLSGKLDGRELLEPETFH>GKDKSFQPSQFRLLEELGKEQGKVQGIGTYSGVGL      | 60     |
| P. troglodytes| MCVQVLYSGLSGKLDGRELLEPETFH>GKDKSFQPSQFRLLEELGKEQGKVQGIGTYSGVGL      | 60     |
| X. laevis     | MTRRTFYVYSLFPLRKPSAASQFRILLEQGKGKGVGQIGCYSGVGL                         | 37     |

C Ube2V2

| Species        | Sequence                                                                 | Length |
|----------------|---------------------------------------------------------------------------|--------|
| X. laevis      | MWSQGKVKPRFRLLEELGKEQGKVQGIGTYSGVGL                                     | 60     |
| D. rerio      | MWAQYKVKPRFRLLEELGKEQGKVQGIGTYSGVGL                                     | 60     |
| H. sapiens    | MVSTGKVPRFRLLEELGKEQGKVQGIGTYSGVGL                                    | 35     |
| P. troglodytes| MVSTGKVPRFRLLEELGKEQGKVQGIGTYSGVGL                                    | 35     |

D Ube2V1 and Ube2V2
**Figure S2.** Inter-isofrom and inter-species conservation of Ube2V1 and Ube2V2. A. Clustal-Ω alignment of human Ube2V1 (isoform 2, the longest isoform) and Ube2V2. Cysteines are highlighted in yellow. C94 and C69 are conserved between the two proteins. C69 of Ube2V2 was identified as an HNE-sensitive residue (see ribbon structure in Fig. 1C). B–C. Clustal-Ω alignment of zebrafish, human, chimpanzee, mouse, and African-clawed frog Ube2V1 (B) and Ube2V2 (C). D. Sequence logo highlighting amino-acid conservation within the linker region of either Ube2V1 (top) and Ube2V2 (bottom), across 16 and 12 species, respectively (vide infra). Relative sizes of residues reflect their frequency and the Y-axis indicates the information content of the position in bits. The arrows indicate the respective conserved cysteines. The sequence logo was generated using WebLogo (invented by Steven E. Brenner et al., Computational Genomics Research Group, University of California, Berkeley). For Ube2V1, the human protein sequence has been aligned with: *Bos taurus* (100%), *Canis lupus familiaris* (100%), *Sus scrofa* (100%), *Heterocephalus glaber* (99%), *Taeniopygia guttata* (99%), *Castor Canadensis* (99%), *Gallus gallus* (98%), *Xenopus laevis* (93%), *Danio rerio* (88%), *Salmo salar* (87%), *Xenopus tropicalis* (90%), *Trichinella britovi* (64%), *Trichinella T8* (64%), *Mus musculus* (91%), *Trichinella native* (51%). For Ube2V2: *Sus scrofa* (99%), *Orcinus orca* (99%), *Mus musculus* (98%), *Gallus gallus* (97%), *Danio Rerio* (94%), *Xenopus laevis* (100%), *Schizosaccharomyces pombe* (94%), *Saccharomyces cerevisiae* (93%), *Kluyveromyces marxianus* (91%), *Scheffersomyces stipites* (93%). The number in parenthesis indicates the extent of sequence identity (in percentage) when aligned with the full-length sequences (see fig. S2A) of Homo Sapiens Ube2V1 and Ube2V2, respectively.
Figure S3

A

Ube2V2

wt

C69S

Time (min) 0 0.5 1 2 0 0.5 1 2

C69S

Cy5

Coomassie

Cy5

Coomassie

Independent replicates

B

Ube2V2

wt

C69S

M

wt Ube2V2 without HNE treatment

wt Ube2V2 with HNE treatment

C

Wavelength (nm)

θ (mdeg)

D

Ube2V2 C69S wt buffer

Ube2V2 C69S wt buffer

Anti-K63-Linked Ub

Independent Replicates

E

Wavelength (nm)

θ (mdeg)

F

CHX (0.1 mg/ml, 8h)

+ + – –

wt C69S wt C69S

Anti-HaloUbe2V2 (FLAG)

Anti-βActin

G

HNEylated Ube2V2 (%) 0 50 75 100

Non-HNEylated Ube2V2 (%) 100 50 25 0

HNEylated Ube2V2 (%) 0 50 75 100

Non-HNEylated Ube2V2 (%) 100 50 25 0

K63 linked Ub5

K63 linked Ub6

K63 linked Ub3

K63 linked Ub4

K63 linked Ub2

Anti-K63 linked Ub

Independent Replicates

Non-HNEylated Ube2V2 HNEylated Ube2V2

ns

p = 0.0001

p = 0.0018

p = 0.0096
Figure S3. Recombinant Ube2V2 is a more efficient HNE-sensor (over Ube2N) in vitro; its sensing defective mutant is functional, stable, and folded similarly to wt; and this site-specific HNEylation promotes Ube2N’s ability to catalyze K63 linked ubiquitin chains in vitro. A. In vitro HNE labeling of Ube2V2 wt and C69S mutant: either recombinant wt or mutant (22 µM) (see fig. S3B) was incubated with 22 µM HNE(alkyne) at 37 °C. At each indicated time point, an aliquot of the reaction was 20-fold diluted into chilled HEPES buffer (pH 7.6) and immediately subjected to Click coupling assay with cy5-azide. The mixture was further incubated at 37 °C for 30 min before running on a SDS-PAGE gel for in-gel fluorescence analysis. See supporting methods for detailed conditions. (n = 2 independent biological replicates). Also see Fig. 1B. B. Coomassie-stained SDS-PAGE gel for recombinantly-purified Ube2V2 wt and C69S mutant (15 µg) (MW: 18 kDa). C. Circular dichroism (CD) spectra of Ube2V2 wt and C69S. Each data set represents a background-subtracted average of three independent biological replicates. See supplemental methods for experimental details. D. In vitro ubiquitination assay comparing Ube2V2 wt and C69S mutant in facilitating Ube2N-catalyzed construction of K63-linked ubiquitin chains. See supporting methods for detailed conditions. The chain formation was probed by anti-K63 antibody. Inset: quantitation of fig. S3D. (n = 3 independent biological replicates). E. Wt-Ube2V2 was treated with equimolar amount of HNE or DMSO for 20 min at 37 °C, and subjected to circular dichroism (CD) analysis at 25 °C. Each trace represents a background-subtracted average of 3 independent biological replicates. See supplemental methods for experimental details. F. Non-synchronized HEK293T cells at logarithmic phase ectopically expressing Halo-Ube2V2 (either wt or C69S) were treated with cycloheximide (0.1 mg/ml) or DMSO for 8 h, and subjected to standard western blot analysis. Right: quantitation. RNR2 protein with known cellular half-life of ~3 h is used as positive control (inset). See supplemental method for details. G. Experiments were set up as in fig. S3D except that the indicated ratios of non-HNE modified wt-Ube2V2 to HNEylated variant replaced the Ube2V2 protein. Inset: quantitation (n = 3 independent biological replicates). See supplemental method for details.
Figure S4

A. Halo-TEV-FLAG-POI Ube2N Ube2V1 Ube2V2

Light TEV

+ + + + +

C5

WB

+ + + + +

Halo-TEV-FLAG-POI Halo (Post TEV-cleavage) TEV protease Ube2V1/ Ube2V2 Ube2N

C5

WB

+ + + + +

Halo-TEV-FLAG-POI (FLAG)

Anti-βActin

C. Halo-TEV-FLAG-Ube2V2 HA-Ube2N

HA-Ube2N

+ + + + +

Halo-TEV-FLAG-POI Halo (Post TEV-cleavage) TEV protease Ube2N (all FLAG tagged)

C5

WB

+ + + + +

Halo-Ube2V2 Ube2V2

Anti-Ube2v2 (FLAG)

Anti-Ube2N (HA)

D. Halo-TEV-FLAG-Ube2V1 HA-Ube2N

HA-Ube2N

+ + + + +

Halo-TEV-FLAG-POI Halo (Post TEV-cleavage) TEV protease Ube2N (both FLAG-tagged)

C5

WB

+ + + + +

Halo-Ube2V2 Ube2V1

Anti-HaloUbe2V1 (FLAG)

Anti-Ube2N (HA)

E. Halo-TEV-FLAG-Ube2V2

HA-Ube2N

+ + + + +

Halo-TEV-FLAG-POI Halo (Post TEV-cleavage) TEV protease Ube2N

C5

WB

+ + + + +

Halo-Ube2V2 Ube2V2

Anti-Ube2V2 (FLAG)

F. Input Biotin-Click pulldown

Hi-ProHNE Light No alkyne

+ + + + +

M

Anti-Ube2N (HA)

M

Anti-HaloUbe2V2 (FLAG)

M

Anti-GAPDH

M

HaloUbe2V2

Anti-HaloUbe2V2 (FLAG)
Figure S4. *Ube2V2 is the most efficient electrophile-sensor among Ube2V1, Ube2V2, and Ube2N, and its sensing function is specific to C69.* A. Whole Cy5 gel and corresponding western blots for Fig. 1E. B. Whole Cy5 gel and corresponding western blots for Fig. 1F. C. Whole Cy5 gel and corresponding western blots for Fig. 1G. D. Identical experiment to C except Halo-(FLAG)-Ube2V1-expression plasmid replaces that for Ube2V2. E. Identical experiment to C except no co-transfection with Ube2N or empty vector. F. Cells were transfected with Halo-(FLAG)-Ube2V2 and HA-Ube2N. Cells were then treated either with Ht-PreHNE, or its no-alkyne variant (incapable of Click coupling) (see chemical structures in fig. S1B). These samples were either exposed to light or not as indicated. Cells were lysed, biotin was attached to HNE(alkyne)-modified proteins via Click coupling with biotin-azole, and samples were enriched by streptavidin pulldown. Inputs (left panel) were analyzed by western blot using indicated antibodies for loading control and for validation of similar protein expression across different conditions. Eluate (right panel) was analysed by anti-HA(Ube2N) blot to examine the extent of Ube2N-HNEylation under these various conditions (see workflow in fig. S1A: ‘biotin azide Click’ panel on the lower right of the flow chart; however, note: there is no TEV treatment in this experiment; thus, the band intensity on the Halo-POI in “no-light” lane is greater than “post T-REX (light exposed sample)”.)
Figure S5. Ube2V2(C69)-specific HNEylation enhances its (K63-linked)-poly-ubiquitylation. A. HEK293T cells ectopically expressing either [Halo-(FLAG)-Ube2V2 (either wt or C69S-mutant) and ‘HA-Ube2N] or [empty plasmid (EV) and HA-Ube2N] were treated with either Ht-PreHNE or DMSO, and subsequently exposed to light or not, as indicated. Cells were lysed, and expressed protein was immunoprecipitated using FLAG resin (Input: top panel; IP: lower panel). Eluates and inputs were analyzed by western blot using indicated antibodies. B. Similar experiment to A, except Input and IP (FLAG) samples were loaded on the same gel. C. HEK293T cells co-expressing Halo-(FLAG)-Ube2V2 and HA-Ube2N were subjected to T-REX [against various T-REX-controls (from right to left): untreated, Ht-PreHNE probe alone, and light alone]. At 1.5 h prior to T-REX execution, half of the set was treated with MG132 (5 µM, 1.5 h), and this concentration of MG132 was maintained for an additional 3 h post light shining until cell harvest. Cells were lysed by sonicating cell pellets, and Halo-(FLAG)-Ube2V2 was immunoprecipitated using FLAG resin. Changes in the extent of mono-Ubiquitnation on Ube2N and the extent of endogenous ubiquitination as a consequence of T-REX with or without MG132 treatment, were analyzed by western blot using indicated antibodies (IP, lower panels; Input, top panels). D. HEK293T cells transfected with the same plasmids as in C were subjected to T-REX conditions against ‘no-Ht-PreHNE’-controls. Cells were treated with or without bortezomib (200 nM, 2 h prior to T-REX, maintained over 3 h post light-shining period, until cell harvest). Post lysis, Ube2V2 was enriched using HA IP (for ubiquitinated proteins), then both input and IP were analyzed on the same blot. E. Quantitation of the relative amount of poly-ubiquitinated Halo-Ube2V2 (wt or C69S) enriched from cells subjected to T-REX against controls. See Fig. 2C, E, and fig. S5B for representative blots. (mean +/- s.e.m., at least four independent sets of biological replicates at different passages were performed, n = 9 for wt-Halo-(FLAG)-Ube2V2, including T-REX and controls; n = 4 for C69S-mutant, including T-REX and controls).
Figure S6. Ube2V2(C69)-specific HNEylation does not elicit K48-linked poly-ubiquitylation A. HEK293T cells co-expressing HA-Ube2N and either Halo-(FLAG)-Ube2V2 (either wt or C69S mutant) or empty vector (EV) were subjected to T-REX against indicated controls. The extent of poly-ubiquitination on the wt and mutant protein was analyzed subsequent to FLAG-immunoprecipitation. B. Quantitation of the relative amount of K63-linked poly-ubiquitinated Halo-Ube2V2 (wt or C69S) enriched from cells subjected to T-REX against controls. See Fig. 2C for a representative blot. [mean +/- s.e.m., at least three independent sets of biological replicates at different passages were performed, for wt-Halo-(FLAG)-Ube2V2, n = 5 (T-REX), n = 6 (light alone), n = 6 (Ht-PreHNE alone), n = 6 (DMSO); for C69S-mutant, n = 3 (T-REX), n = 3 (light alone), n = 3 (Ht-PreHNE alone), n = 2 (DMSO)]. C. HEK293T cells transfected with wt-Halo-(FLAG)-Ube2V2 and HA-Ube2N were subjected to T-REX conditions against all possible negative controls of T-REX. Cells were lysed, and expressed protein was pulled down using biotin K63 tandem ubiquitin binding entity (TUBE) (procedures described in the SI) (pulldown: lower panel; Input: top panel) Changes in K63-linked Ube2V2-polyubiquitination were assessed by western blot. D. HEK293T cells co-expressing HA-Ubiquitin (either wt or K63R mutant) and Halo-(FLAG)-Ube2V2 were subjected to T-REX. Following FLAG-immunoprecipitation (Input: left panel; IP: right panel), the role of K63R-mutation on Ub, in regulating the Halo-(FLAG)-Ube2V2 poly-ubiquintination was analyzed by western blot. E. HEK293T cells co-expressing Halo-(FLAG)-Ube2V2, T7-Ube2N, and either empty (EV) or HA-Ubiquitin were subjected to T-REX against all controls. 3-h Post light shining, cells were lysed, immunoprecipitated with FLAG resin (Input: top panels; IP: lower panels), and analyzed for the extent of reduced mono-ubiquitination on Ube2N by western blot using anti-HA(Ub) as well as anti-T7(Ube2N).
Figure S7

A  Input

B  Whole cell lysate after sonication

C  Relative Ub2N knockdown efficiency (normalized band intensity of Ub2N)

D  Input

E  Input
**Figure S7.** *Ube2V2(C69)*-specific HNEylation and K63-linked-poly-ubiquitination is accompanied by reduced mono-ubiquitinated Ube2N: this redox–Ub signaling exchange process requires Ube2N. A. HEK293T cells co-expressing Halo-(FLAG)-Ube2V2 and HA-Ube2N were subjected to T-REX and treated with DMSO or the proteasome inhibitor bortezomib (Bz, 200 nM) for a further 3 h before harvest. HA-immunoprecipitated samples (IP: lower panels; Input: top panels) were analyzed by western blot using indicated antibodies in order to evaluate the extent to which the proteasome degradation pathway is influenced by the newly-discovered HNE-initiated signalling response. (i). There is no further ubiquitination of Ube2N upon Bz treatment (conditions that stop proteasome degradation; validated by increase in endogenous HMW-Ubiquitinated proteins in an anti-Ub blot). (ii). Sonication of pellet (insoluble fraction) (Right Panel) shows there is no formation of insoluble HMW-Ubiquitinated-Ube2N complex, further consistent with mono-ubiquitination of Ube2N. B. Knockdown lines expressing different shRNAs targeting Ube2N (line #’s: 16; 17; and 18) were generated using lentiviral transduction. Levels of Ube2N in these lines were assessed relative to wt and lines expressing two different control shRNAs (namely, shLacZ-D11 and shLacZ-B12). C. Quantitation of Ube2N knockdown efficiencies. (mean +/- s.d., Two independent replicates were performed. In total, n = 3 for shUbe2N-#16, n = 2 for shUbe2N-#17, n = 4 for shUbe2N-#18, n = 2 for sh-LacZ-D11 control). D. HEK293T cells expressing either shRNA targeting Ube2N (lines #16 and #18 that express different shRNA’s) or a control shRNA, were transfected with Halo-(FLAG)-Ube2V2, and subjected to T-REX against ‘light-alone’-T-REX-control. Halo-(FLAG)-Ube2V2 was immunoprecipitated (IP: lower panels) and input and eluates were analyzed by western blot using anti-FLAG antibody. See fig S7B–C for knockdown efficiencies. E. Same as the set-up in D except that the cells were co-transfected with HA-Ubiquitin, and HA-immunoprecipitation (instead of FLAG) was performed (IP: lower panels) and input and eluted samples were analyzed by western blot using indicated antibodies to evaluate the Ube2N-dose-dependent changes in the extent of polyUb of HaloUbe2V2.
Figure S8

A

B

C

D

E

F

HaloUbe

D

HaloUbe

E

HaloUbe

F

HaloUbe

G

HaloUbe

H

HaloUbe

I

HaloUbe

J

HaloUbe

K

HaloUbe

L

HaloUbe

M

HaloUbe

N

HaloUbe

O

HaloUbe

P

HaloUbe

Q

HaloUbe

R

HaloUbe

S

HaloUbe

T

HaloUbe

U

HaloUbe

V

HaloUbe

W

HaloUbe

X

HaloUbe

Y

HaloUbe

Z
Figure S8. Both Ube2V2(C69S) and Ube2N-knockdown cells are hypomorphic for γ-H2AX upregulation and DNA-synthesis suppression. **A.** Schematic illustration for dual-pulse experiment. **B.** Representative images for Fig. 4F. HEK293T cells that had been transfected with Halo-(FLAG)-Ube2V2 (wt or C69S mutant), and subsequently exposed to the indicated conditions; followed by immunofluorescence analyses (detailed in SI methods). Scale bars, 5 µm. **C.** HEK293T cells expressing control shRNA (shLacZ-D11) or shRNA targeting Ube2N were transfected with Halo-(FLAG)-Ube2V2 (wt); and subjected to T-REX conditions against various T-REX-controls. 3-h post light exposure (or equivalent time in respective controls), cells were sequentially treated with EdU then BrdU. Cells where then fixed, immunostained and analyzed (detailed in SI methods). [mean +/- s.e.m., for shUbe2N-#16, n = 66 (T-REX), n = 186 (Light alone), n = 270 (Ht-PreHNE alone), n = 240 (DMSO); for shLacZ-D11, n = 185 (T-REX), n = 247 (Light alone), n = 233 (Ht-PreHNE alone), n = 234 (DMSO)]. **D.** HEK293T cells were transfected with Halo-(FLAG)-Ube2V2 (wt or C69S mutant) and exposed to T-REX conditions against various T-REX-controls, or treated with mitomycin C (10 µg/ml, 3 h). 3-h post light exposure (or equivalent time in respective controls), cells were fixed, immunostained for γ-H2AX and analyzed by immunofluorescence (detailed in methods). [mean +/- s.e.m., for wt-Halo-(FLAG)-Ube2V2, n = 50 (T-REX), n = 50 (Light alone), n = 50 (Ht-PreHNE alone), n = 50 (DMSO), n = 180 (Mitomycin C); for C69S-Halo-(FLAG)-Ube2V2, n = 95 (T-REX), n = 59 (Light alone), n = 55 (Ht-PreHNE alone), n = 55 (DMSO), n = 55 (Mitomycin C)]. **E.** HEK293T cells were transfected with Halo-(FLAG)-Ube2V2 (wt or C69S mutant). Cells were exposed to T-REX conditions, then γ-H2AX levels were assessed as a function of time post light exposure by immunofluorescence microscopy. [mean +/- s.e.m., for wt-Halo-(FLAG)-Ube2V2, n = 143 (0 h), n = 137 (3 h), n = 172 (24 h); for C69S-Halo-(FLAG)-Ube2V2, n = 164 (0 h), n = 138 (3 h), n = 156 (24 h)]. **F.** Representative images for Fig. S8D. HEK293T cells that had been transfected with Halo-(FLAG)-Ube2V2 (wt or C69S mutant), and subsequently exposed to the indicated conditions; followed by immunofluorescence analyses (detailed in SI methods). Scale bars, 5 µm.
**Figure S9.** HNEylation of Ube2V2 in zebrafish upregulates γ-H2AX. **A.** Casper zebrafish were either not injected or injected with mRNA encoding Halo-(FLAG)-Ube2V2 and subjected to T-REX against ‘light-alone’ control. Fish were then dechorionated, deyolked, lysed and HNEylated proteins were captured by Click coupling with biotin-azide and subsequent streptavidin pulldown (see workflow in fig. S1A, Clicking with biotin-azide). HNEylation of Ube2V2 in fish also led to polyubiquitination of Ube2V2, indicating mechanistic conservation between fish and humans, and functional relevance of this electrophile regulatory pathway in vivo. Also see Fig. 5C. **B.** Casper embryos were either non-injected or injected with mRNA coding for Halo-Ube2V2 and treated with the stated conditions. After 36-h fish were dechorionated and imaged using a stereomicroscope. Scale bars, 545 µm.
General Materials and Methods.

All procedures related to zebrafish studies conform to the National Institutes of Health guidelines regarding animal experimentation and were approved by Cornell University's Institutional Animal Care and Use committees. All primers were from IDT. Phusion HotStart II polymerase was from Thermo Scientific. All restriction enzymes were from NEB. Complete EDTA free protease inhibitor was from Roche. 1X RIPA buffer was from Santa Cruz biotech. 1X Bradford dye was from BioRad. Pre-HNE and HaloTag-targetable photocaged precursor HNE alkyne (Ht-PreHNE) were synthesized as described previously(3, 4). Cyanine5 (Cy5)-azide and Cu(TBTA) were from Lumiprobe. Dithiothreitol (DTT), streptomycin sulfate, isopropyl β-D-1-thio-galactopyranoside (IPTG), TCEP-HCl, Coelenterazine, and D-Luciferin were from Goldbio Biotechnology. Conenzyme A was from Avanti Polar lipids. trans-1,2-Diaminocyclohexane-N,N,N′,N′-tetraacetic acid monohydrate (CDTA) was from Alfa Aesar. EdU (5-ethynyl-2’-deoxyuridine) and BrdU (5-Bromo-2’-deoxyuridine) were from Chem-Impex. Adenosine triphosphate disodium salt hydrate (ATP) was from Fisher. Biotin-dPEG®\textsubscript{11}-azide was from Quanta Biodesign. Streptavidin sepharose beads were from GE Healthcare. ANTI-FLAG® M2 affinity gel (A2220) and monoclonal anti-HA-agarose, clone HA-7 (A2095) were from Sigma-Aldrich. Bovine Serum Albumin (BSA) powder was from Thermo Scientific. All other chemicals were from Sigma. BL21 (DE3)-RIL codon plus cells were from Stratagene. The plasmid for recombinant expression of TEV protease (pRK793, Addgene #8827), ubiquitin-conjugating enzyme E2 variant 1 (Addgene #31429), ubiquitin-conjugating enzyme E2 variant 2 (Addgene #31430), ubiquitin-conjugating enzyme E2N (Addgene #12461), histone H2A (Addgene #63560) and the empty pCS2+8 vector (Addgene #34931) were from Addgene. HA-Ubiquitin plasmid was a gift from Prof. Ling Qi (Cornell...
University). Myc-MCM6 was a gift from Prof. John Schimenti (Cornell University). 3X FLAG peptide was from APEXBIO (A6001). HEK293T cells were from American Type Culture Collection (ATCC). 1X DPBS, 1X Trypsin (TrypLe), 100X NEAA, 100X sodium pyruvate, 100X Penicillin-Streptomycin and 1X MEM+Glutamax media were from Life Technologies. Fetal Bovine Serum (FBS) was from Sigma (F2442). TransIT 2020 transfection reagent was from Mirus Bio LLC. Polyethylenimine, linear, MW 25,000 (PEI, 23966-1) was from Polysciences, Inc. 365 nm UV lights were from Spectroline (for handheld size, ENF240C and if larger surface area is needed, XX15N). For T-REX™ experiments, the lamps were positioned above confluent monolayer of cells or zebrafish embryos in 6-well plates such that the power of UV irradiation was ~ 5 mW/ cm² (as measured by a hand-held power sensor (Spectroline, XDS-1000). For all confocal imaging experiments, a Zeiss LSM710 confocal microscope was used. Quantitation of fluorescence intensity was performed using Image-J software (NIH, version 1.50g). In-gel fluorescence analysis and imaging of western blots and Coomassie stained gel were performed using BioRad Chemi-Doc MP Imaging system. Densitometric quantitation was performed using BioRad Image Lab software (version 4.1). Cy5 excitation source was epi illumination and 695/55 emission filter was used. Cell counting was done by Countess II FL (A25750). His₆-TEV S219V protease was recombinantly expressed and purified from BL21(DE3)-RIL cells using TALON resin. Dual luciferase assay was performed using a BioTek Cytation™ 3 Cell Imaging Multi-mode reader with dual reagent injectors. Proteomics data from G-REX and SILAC−T-REX experiments are provided as separate excel files (corresponding to Supplementary Table 1 and 3, respectively). MS analysis of the site of modification is provided as Supplementary Table 2 (also see Fig. 1D). Information on cloning primers, shRNA sequences, and antibodies used are listed in Supplementary Table
4, 5, and 6, respectively. All sterile cell culture plastic-ware was from CellTreat, except for glass-bottomed dishes used for imaging that were from In Vitro Scientific. Enzymes for in vitro ubiquitin assay, including Ube1 (E-306), Ube2N (E2-660) and Ubiquitin (U-100H) were purchased from Boston BioChem. The TEV protease (V6101) used for recombinant purification of Ube2V2 protein was purchased from Promega. Recombinant His6-Halo protein used for Halo protein level in vivo was obtained from purified pET28a-His-Halo-HuR followed by TEV cleavage. The circular dichroism (CD) analysis was performed using AVIV Biomedical Circular Dichroism Spectrometer Model 201-01, AVIV Biomedical Inc., Lakewood NJ. Mycoplasma testing for all the cell lines in this work was performed using Sigma LookOut® PCR detection kit (MP0035) every 3-month.

**Safety comments:** All experiments are performed according to standards of operational practice in compliance with the expected SOP for chemical safety and biosafety. All experiments involving zebrafish conform to the NIH guidelines and are approved by Cornell University's IACUC.

**Validation of antibodies:** Many of the antibodies were themselves used to show knockdown of proteins using multiple shRNAs. The antibody specificities of the transgenes of interest were independently verified using targeted siRNA knockdowns. In addition, for many experiments, results were replicated by detecting ectopic expression of an epitope-tagged version (where the gene of interest from both non-transfected and transfected cells can be viewed in the same frame, validating the specificity of the antibody). By this metric, we confirmed that the data for ectopically-overexpressed proteins are consistent with the immunofluorescence (IF) data for the endogenous protein. Wherever possible, anti-
FLAG/myc/HA were used to detect the ectopic proteins, thereby eliminating doubts about antibody specificity. Halo protein was confirmed to be expressed ubiquitously throughout the transfected cells and that the photocaged small-molecule probe (Ht-PreHNE) co-localized with Halo; for instance, when Halo was fused to nuclear localization signal sequence.

**G-REX in mammalian cells:** HEK 293T cells were maintained in 1X MEM+ Glutamax™ media supplemented with 10% FBS, 1X NEAA, 1X sodium pyruvate and 1X Pen-Strep. Cells were grown in a humidified, 5% CO₂ incubator at 37 °C. 24 h later, cells were transfected with pfN21a-Halo plasmid using TransIT-2020 transfection reagent per the manufacturer’s recommendation. Subsequent steps were performed under dim light. 24–36 h post transfection, monolayer of cells were treated with 25 μM Ht-PreHNE in serum-free media and incubated for 2.5 h. Cells were gently rinsed with serum-free media three times every 30 min over the next 1.5 h. UV lamps were turned on 10 min prior to use. (Light source: 365 nm, 0.3 mW/cm² hand-held UV-lamp placed 1 inch above samples). For samples designated as “samples exposed to light”, lids were removed from the culture dishes and cells were placed under 365 nm UV light for 5–8 min. The cells were harvested, washed two times with ice-cold PBS and frozen in liquid nitrogen. After cell lysis (lysis procedure varies depending on assay methods; please see elsewhere in the supplementary methods), click with Biotin-azide (as described in Biotin/streptavidin pulldown procedure elsewhere in the supplementary methods), and pulldown enrichment by Streptavidin, the protein enriched would be eluted by 2 X Laemmeli dye containing 6% βME at 98 °C for 10 min. The sample was subjected to SDS-PAGE followed by Coomassie stain and the gel band(s) corresponding to specific region(s) of interest is excised (in this scenario, a gel band between 15–25-kDa
region) and sent for MS identification (see detailed methods regarding In-gel trypsin digestion of SDS gel bands, Protein Identification by nano LC/MS/MS Analysis, and LC-MS/MS data analysis reported elsewhere in the methods section).

**Construction of plasmids:**

Ligase-free cloning method was used to clone various plasmids (Supplementary Table 4) for expression in mammalian cells and in zebrafish. In order to clone any desired fusion genes in any vector of choice, the gene of interest (GOI) was PCR-amplified out from the original plasmid using the indicated forward (fwd-1) and reverse primers (rev-1) in Supplementary Table 4a and 4b. The resultant PCR product was extended using the indicated fwd-2, and rev-2 primers. The resultant “megaprimer” was inserted into the destination vector of interest that had been linearized with an appropriate restriction enzyme (NEB) using PCR. The plasmid was verified by sequencing the entire gene at the genomics facility of Cornell Institute of Biotechnology. Plasmids were purified using EZ-10 spin column plasmid DNA miniprep kits (Bio Basic, BS614).

**Cell growth and culture maintenance:**

HEK 293T cells were maintained in 1X MEM+ Glutamax™ media supplemented with 10% FBS, 1X NEAA, 1X sodium pyruvate and 1X Pen-Strep. Cells were grown in humidified, 5% CO₂ incubator at 37 °C. All cell lines were tested negative for mycoplasma [testing was performed every 3 months using LookOut® Mycoplasma PCR Detection Kit (Sigma)]. For SILAC–T-REX protocol, SILAC HEK293T cells were cultured and passaged at least five times (more than two weeks) using, in final concentrations, SILAC drop-off media 1X DMEM (ThermoFisher # 89985) supplemented with 10% dialyzed FBS (Sigma Aldrich # F0392),
the corresponding light/heavy amino acids, light amino acids: 146 μg/mL of L-lysine (Sigma Aldrich # L8662) and 84 μg/mL of L-arginine (Sigma Aldrich # A8094) or the same concentration of heavy amino acids: L-lysine-13C6, 15N2 hydrochloride (Sigma Aldrich # 608041) and L-arginine-13C6, 15N4 hydrochloride (Sigma Aldrich # 608033) respectively, 1X sodium pyruvate and 1X Pen-Strep. Cells were maintained in a humidified, 5% CO2 incubator at 37 °C.

**Generation of lentiviral-based knockdown lines:**

HEK293T packaging cells were seeded and grown overnight in antibiotic free media in 6 well plates. At 80% confluence, each well was transfected with packaging plasmid (pCMV-R8.74psPAX2, 500 ng), envelope plasmid (pCMV-VSV-G, 50 ng) and pLKO vector (500 ng) using TransIT.LT1 as per the manufacturer’s protocol. After 18 h media were removed and replaced with 20% serum containing media. After 24 h, media containing viruses were collected, spun down and passed through a 0.6 micron filter and stored at −80 °C or used directly.

Cells in log phase were treated with 0.6 ml of virus supernatant (from above) in 8 μg/ml polybrene in a total of 6 ml of media in a 6-well plate. After 24 h, media were removed and replaced with media containing 2 μg/ml puromycin (which was completely toxic to all lines used in this study). Cells were cultured till plate was confluent, then cells were split and moved to a 10 cm dish in 2 μg/ml puromycin containing media and grown again until reaching confluence. At this point the line was considered to be “selected”, and target gene
expression was analyzed by western blot and compared to shRNA controls. Cells up to passage 5 were used for assays and they were typically grown in 1.5 μg/ml puromycin.

**T-REX in mammalian cells:**

HEK 293T cells were maintained in 1X MEM+ Glutamax™ media supplemented with 10% FBS, 1X NEAA, 1X sodium pyruvate and 1X Pen-Strep. Cells were grown in a humidified, 5% CO₂ incubator at 37 °C. For in-gel fluorescence analysis and western blot, ~0.7–0.8 ×10^6 HEK 293T cells were seeded in 8 cm² tissue culture dishes. 24 h later, cells were transfected using TransIT-2020 transfection reagent per the manufacturer's recommendation. Subsequent steps were performed under dim light. 24–36 h post transfection, monolayer of cells were treated with 25 μM Ht-PreHNE in serum-free media and incubated for 2.5 h. Cells were gently rinsed with serum-free media three times every 30 min over the next 1.5 h. Meanwhile, UV lamps were turned on 10 min prior to UV irradiation time. For samples designated for light exposure, lids were removed from the dishes and cells were placed under 365 nm UV light for 5–8 min. The cells were harvested, washed two times with ice-cold PBS and frozen in liquid nitrogen.

**Western blotting:**

Cells were lysed in 1X RIPA buffer containing in final concentrations 1X Protease inhibitor, 1 mM sodium orthovanadate and 1 mM PMSF, by rapid freeze-thaw (x3). Cell debris was removed and the supernatant was collected after centrifugation at 18,000x g for 20 min at 4 °C. Protein concentration was determined using Bradford assay. 30–50 μg of total lysates were subjected to SDS-PAGE and the gel was transferred onto a PVDF membrane at 100 V
for 1 h at 4 °C or at 40 V overnight at 4 °C. Membrane was blocked with 10% milk and probed with various antibodies at the indicated dilutions (Supplementary Table 6).

**In-gel fluorescence assay:**

All steps were performed in dark or under dim light. Cells from 8cm² plates were lysed in 30 µL buffer containing 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail, and 0.3 mM TCEP by rapid freeze-thaw (x3). Cells debris was removed by centrifugation at 18,000xg for 20 min at 4 °C. Protein concentration of the clarified lysate was determined using Bradford assay. A portion of the lysate protein was made up to 22 µL final volume containing, in final concentrations, 50 mM HEPES (pH 7.6), 150mM NaCl, 1.0 mg/mL lysate protein, 0.3 mM TCEP, and 0.2 mg/mL TEV protease. The sample was incubated at 37 °C for 45 min, and subsequently subjected to Click reaction. In a final volume of 27 µL, the click reaction mix consisted of 1.7 mM TCEP, 5% t-BuOH, 1% SDS, 1 mM CuSO₄, 0.1 mM Cu(TBTA), 10 µM Cy5 azide and the lysate from above. The samples were incubated at 37 °C for 30 min and subsequently quenched with 5 µL 4X Laemmeli dye containing 6% βME. After additional 5-min incubation at 37 °C, 25 µL of the lysate was subjected to SDS-PAGE. After electrophoresis, the gel was rinsed 3X with ddH₂O with 5-min each rinse on a shaker and imaged on a Biorad Chemi-doc-MP Imager. Where applicable, the gel was transferred to a PVDF membrane for western blot analysis.

**Biotin azide pull down for mammalian lysate:**

HEK 293T cells were seeded in 60 cm² plate. After the cells reached 60% confluence (~18–24 h), the old media were replaced with fresh 8 mL complete media. Cells were transfected with 7.5 µg of the designated plasmids encoding the HaloTag fusion gene and 30 µL PEI (1
mg/mL) in 600 µL in Opti-MEM media for 24–36 h after which the cells were treated with 25 µM Ht-PreHNE or without alkyne (control) for 2.5 h. Rinsing and light shining protocol were as described above. Cells were harvested, washed twice with chilled 1X DPBS and flash frozen.

Mammalian cell lysis was performed in 200 µL of lysis buffer containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 and 1X Roche complete, mini, EDTA-free protease inhibitor cocktail by rapid freeze-thaw (x3). Lysate was clarified by centrifugation at 18,000 x g for 30 min at 4 °C.

Zebrafish cell lysis was performed with similar procedure as mammalian cells: 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.2 mg/ml soybean protease inhibitor (Sigma), and 3X Roche complete, mini, EDTA-free protease inhibitor cocktail by rapid freeze-thaw and vortexing with zirconia beads (Bio spec 0.7 mm beads; 11079107zx).

Total protein concentration from either mammalian cell lysate or zebrafish lysate was determined using Bradford assay with BSA as standard. The lysate was subsequently diluted to 2 mg/mL with a buffer made up of 50 mM HEPES (pH 7.6) and 0.3 mM TCEP, and subjected to Click reaction with biotin azide for 30 min at 37 °C. The final concentrations of each components were: 1% SDS, 5% t-BuOH, 200 µM Biotin azide, 2 mM TCEP, 0.9 mM CuSO₄ and 0.1 mM Cu(TBTA). The lysate proteins were precipitated by adding 4 volumes of EtOH pre-chilled at −20 °C (EtOH final concentration is 80%). The sample was vortexed and incubated at −80 °C overnight (or at least 4 h) to facilitate precipitation. The precipitant was collected by centrifugation at 21,000 x g for 120 min at 4 °C and washed twice with pre-chilled MeOH, once with pre-chilled acetone. The pellet was air-dried, then redissolved in 20–50 µL 50 mM HEPES (pH 7.6), 4% LDS and 0.5 µM EDTA and dissolved by vortexing and heating at 42 °C.
for 5 min. LDS was diluted to a final concentration of 0.5% with 350 μL of 50 mM HEPES (pH 7.6) and added to 50 μL bed volume of Strepavidin sepharose beads pre-equilibrated with 50 mM HEPES (pH 7.6) and 0.5% LDS. The sample was incubated with beads for 2–3 h at room temperature by end-over-end rotation after which time the supernatant was removed by centrifugation at 500 x g for 3 min. The beads were washed three times with 500 μL of 50 mM HEPES (pH 7.6) with 0.5% LDS with end-over-end rotation at room temperature for 30 min during each wash. The bound protein was eluted by boiling the beads at 98 °C for 10 min with 30 μL of 2 X Laemmeli dye containing 6% βME. The sample was subjected to SDS-PAGE followed by Coomassie stain or transferred to a PVDF membrane for western blot analysis.

**Anti-FLAG and anti-HA pull down from mammalian lysate:**

HEK 293T cells were seeded in 2 X 60 cm² plates. After the cells reached 60% confluence (~18–24 h), the old media was replaced with 8 mL of fresh complete media. Cells were transfected with 7.5 μg of the designated Halo clone and 30 μL PEI (1 mg/mL) in 600 μL in Opti-MEM media for 24–36 h after which the cells were treated with 25 μM Ht-PreHNE for 2.5 h. Rinsing and light shining protocol were as described above. Cells were harvested, pooled, washed twice with chilled 1X DPBS and flash frozen. Cell lysis was performed in 100-200 μL per 1.5×10⁶ cells of either: lysis buffer [containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 and 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail] for Flag pull down; or RIPA buffer [containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, and 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail]
for HA pull down, followed by rapid freeze-thaw cycles (x3). Lysate was clarified by centrifugation at 18,000 xg for 10 min at 4 °C. Total protein concentration was determined using Bradford assay with BSA as standard. The lysate was subsequently diluted to 2 mg/mL with binding buffer containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail, and 0.1% Tween-20. This diluted lysate was subjected to either 50–100 µL bed volume of ANTI-FLAG® M2 affinity gel (A2220, Sigma) or monoclonal anti-HA-agarose (clone HA-7, A2095, Sigma) that had been pre-equilibrated with the binding buffer above. The sample was incubated with beads for 2–3 h at 4 °C by end-over-end rotation after which time the supernatant was removed post-centrifugation at 1000 x g (anti-FLAG pull down) or 5000 x g (anti-HA pull down) for 3 min. The beads were washed three times at 4 °C with 500 µL wash buffer containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail, and 0.1% Tween-20, using end-over-end rotation over 10 min during each wash. The bound protein was eluted by either incubating with 0.15 mg/mL 3 X flag peptide for 2 h at 4 °C (anti-Flag pull down) or by boiling the beads at 98 °C for 10 min with 30 µL of 3 X Laemmeli dye containing 6% βME (anti-HA pull down). The sample was subjected to SDS-PAGE and transferred to a PVDF membrane for western blot analysis described above.

For hydroxylamine treated FLAG eluent assay, after eluting with 3 X FLAG-peptide, the eluent was boiled with Laemmeli dye containing 6% βME and 100 mM freshly prepared hydroxylamine for 10 min followed by SDS-PAGE and Western Blot analysis.
Isolation procedure for ubiquitylated proteins from mammalian cell lysates:

K63-linked with K63-TUBE (Biotin)

Cells were grown and transfected as described above. After T-REX, cells were harvested and lysed in 100–200 µL (~1.5×10^6 cells) of lysis buffer [containing in final concentrations 50 mM HEPES (pH 7.6), 300nM Biotin K63-TUBE, 150 mM NaCl, 5mM EDTA, 3 mM 1,10-phenanthroline, 5 mM NEM, 20 µM PR-619, 1% Nonidet P-40 and 1X Roche Complete, mini, EDTA-free protease inhibitor cocktail] according to manufactures’ manual (Biotin K63 TUBE, Lifesensors). Lysate was clarified by centrifugation at 18,000 xg for 10 min at 4 °C. Total protein concentration was determined using Bradford assay using BSA as standard. The lysate was subsequently diluted to 5–10 fold with binding buffer containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1X Roche Complete, mini, EDTA-free protease inhibitor cocktail, and 0.05% Triton-X 100 while adjusting the concentration of Biotin K63-TUBE and all inhibitors accordingly. This diluted lysate was treated with 100 µL bed volume streptavidin sepharose beads that had been pre-equilibrated with the binding buffer (vide supra). Upon incubation by end-over-end rotation at 4 °C for 3–5 h, supernatant was removed post-centrifugation at 500 x g for 1 min. The beads were washed three times with 500 µL of 50 mM HEPES (pH 7.6), 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 × Roche Complete, mini, EDTA-free protease inhibitor cocktail, and 0.05% Triton-X 100 with end-over-end rotation at 4 °C for 20 min during each wash. Bound protein was eluted by boiling the beads at 98 °C for 10 min with 30 µL of 2 X Laemmeli dye containing 6% βME. The sample was subjected to SDS-PAGE and transferred to a PVDF membrane for western blot analysis.
Luciferase assay (for NF-κB-reporter assay):

1.5–1.6 X10^5 HEK293T cells were seeded in each well of a 48-well plate. 24 h later, the cells were transfected with 120 ng of the designated HaloTag fusion gene plasmid and 120 ng of NF-κB-Firefly luciferase: pCMV-Renilla luciferase (40:1) mix, using TransIT–2020 transfection agent. 24 h post-transfection, cells were treated with 25 µM Ht-PreHNE or vehicle (corresponding volume of DMSO alone) for 2.5 h, rinsed three times and irradiated with 365 nm UV light for 10 min. The cells were incubated for a further 8 h. For dual luciferase assay, cells in each well were gently washed with 1X DPBS and lysed in 65 µL of 1X passive lysis buffer containing in final concentrations 25 mM Tris (pH 7.8), 2 mM 1,2-CDTA, 2 mM DTT, 1 mg/mL BSA, 1 % Triton X-100, and 10% Glycerol. 20 µL of the lysate was transferred to a white opaque 96-well plate (Corning). Firefly luciferase was read after adding 50 µL Firefly substrate (75 mM HEPES pH 8.0, 4 mM MgSO₄, 20 mM DTT, 0.1 mM EDTA, 0.53 mM ATP, 0.27 mM Coenzyme-A, 0.47 mM D-Luciferin Firefly). Subsequently, 50 µL of Stop and Glow substrate (7.5 mM sodium acetate pH 5.0, 400 mM sodium sulfate, 10 mM CDTA, 15 mM sodium pyrophosphate, 0.025 mM APMBT, 5.5 µM Coelenterazine) was added and Renilla luciferase activity was measured.

In-gel trypsin digestion of SDS gel bands:

The enriched Halo-Ube2V2 protein band from the SDS-PAGE gel above from anti-Flag pull down was cut and subjected to in-gel digestion with reconstituted Arg-C (Promega) followed by extraction of the peptides as previously reported(5). The excised gel pieces were washed consecutively in 200 µL distilled water, 100 mM ammonium bicarbonate (Ambic, pH 7.8)/acetonitrile (1:1) and acetonitrile (ACN). The gel pieces were reduced with 70 µL 5 mM
TCEP in 50 mM Ambic solution (pH 7.8) for 45 min at room temperature and alkylated with 100 μL of 55 mM Iodoacetamide in 100 mM Ambic at room temperature in dark for 60 min. After wash steps as described above, the gel slices were dried and rehydrated with 50 μL Arg-C in 50 mM Ambic, 10% ACN (20 ng/μL) at 37 °C for 16 hrs. The digested peptides were extracted twice with 70 μL of 50% ACN, 5% formic acid (FA) and once with 70 μL of 90% ACN, 5% FA. Extracts from each sample were combined and lyophilized.

**Protein identification by nano LC/MS/MS analysis:**

The in-gel tryptic digests were reconstituted in 20 μL of 0.5% FA for nanoLC-ESI-MS/MS analysis, which was carried out by an Orbitrap Fusion mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with a “CorConneX” nano ion source device (CorSolutions LLC, Ithaca, NY). The Orbitrap was interfaced with a Dionex UltiMate3000RSLCnano system (Thermo, Sunnyvale, CA). The gel extracted peptide samples (5 μL) were injected onto a PepMap C18 trap column-nano Viper (5 μm, 100 μm x 2 cm, Thermo) at 20 μL/min flow rate for on-line desalting and then separated on a PepMap C18 RP nano column (3 μm, 75 μm x 25 cm, Thermo) which was installed in the nano device with a 10-μm spray emitter (NewObjective, Woburn, MA). The Orbitrap calibration and nanoLC-MS/MS operation were as described previously(6). Peptides were eluted with a 90-min gradient of 5% to 38% ACN in 0.1% FA at a flow rate of 300 nL/min, followed by a 5-min ramping to 95% ACN-0.1% FA and a 7-min hold at 95% ACN-0.1% FA. The Orbitrap Elite was operated in positive ion mode with nano spray voltage set at 1.5 kV and source temperature at 250 °C.

The instrument was operated in parallel data-dependent acquisition (DDA) under FT-IT mode using FT mass analyzer for one MS survey scan from m/z 375 to 1800 with a
resolving power of 120,000 (fwhm at m/z 400) followed by MS/MS scans on top 15 most intense peaks with multiple charged ions above a threshold ion count of 10,000 in FT mass analyzer. External calibration using Ultramark 1621 for both FT mass analyzer and IT mass analyzer is performed. Dynamic exclusion parameters and normalized collisional energy were set same as previously(6, 7). All data were acquired under Xcalibur 2.2 operation software (Thermo-Fisher Scientific).

**LC-MS/MS data analysis:**

The DDA raw files for CID MS/MS only were subjected to database searches using Proteome Discoverer (PD) 1.4 software (Thermo Fisher Scientific, Bremen, Germany) with the Sequest HT algorithm. The database search was conducted against a human UniProt database containing 160,672 entries with two-missed Arg-C cleavage sites allowed. The peptide precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 Da. Variable modification of cysteine carboxymethylation, methionine oxidation, N-terminal acetylation and deamidation of asparagine/glutamine were set along with HNE alkyne (152.08 Da) and reduced HNE alkyne (154.10 Da) as well as their dehydrated HNE alkyne (134.07 Da) and dehydrated and reduced HNE alkyne (136.09 Da) on cysteine and all of these modifications also on histidine and lysine residues. Only high confidence peptides defined by Sequest HT with a 1% FDR by Percolator were considered for the peptide identification. All MS/MS spectra for identified Cam and HNE Cys modified peptides from initial database searching were manually inspected and validated using Xcalibur 2.2. Results are shown in [Supplementary Table 2](#) and Main Text Fig. 1d.
Immunofluorescence (IF):

Cells were grown to 70% confluence in 35 mm glass-bottomed dishes and transfected with HaloTagged plasmids. T-REX™ was performed as mentioned above. 3 h post irradiation by UV light, cells were fixed by adding -20 °C pre-chilled MeOH and incubating at 4 °C for 20 min. The fixative was aspirated and the cells washed three times with 1X DPBS with 5-min incubation at room temperature. Blocking and permeabilization was performed in one-step by incubation at 37 °C for 1 h in 1X DPBS containing 3% BSA and 0.2% Triton X-100. Cells were subsequently incubated with primary antibody (1:300, Supplementary Table 6) in incubation buffer (1% BSA, 0.02% Triton X-100 in 1X DPBS) for 2 h at room temperature. Cells were rinsed 3 times with 1X DPBS with 5 min incubation for each wash and subsequently incubated in dark for 1 h at room temperature with corresponding second antibody (1:1000, Supplementary Table 6) in incubation buffer. Cells were rinsed 3 times with 1X DPBS with 5 min incubation for each rinse. DAPI (Sigma) was freshly prepared in 1X DPBS from 2 mg/mL wt/v stock solution in water and added to the wells at the final concentration of 0.2 µg/mL. The samples were incubated in dark for 1 min and washed once with 1X DPBS and stored at 4 °C in dark till images were taken using a Zeiss LSM 710 meta confocal fluorescence microscope. Image analysis was performed using Image-J (NIH).

Estimation of intracellular Halo concentration in G-REX

HEK293T cells were cultured and transfected by either pFN21a-Halo or empty vector (control) using the method described above. After 36 hours of transfection, cells were harvested, pooled, washed twice with chilled 1X DPBS and the number of cells were counted and normalized before being flash frozen (in this case 1.24 × 10^5 cells on average were
collected across all the transfected cells). Cell lysis was performed in 50 µL of lysis buffer [containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40 and 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail]. The overall lysate protein concentration was determined by Bradford assay described above, followed by normalization across all samples with appropriate amounts of lysis buffer. The recombinant His₆-Halo was obtained by cleavage of purified His₆-Halo-Tev-HuR with TEV protease for 20 min, at 37 °C. Varied amounts of the resultant His₆-Halo protein were loaded on the same SDS-PAGE gel along with the cell lysate. The Halo protein amount in the lysate was determined (~5.5 ng from ~10 % lysate, see Fig. S1C) using the standard curve for His₆-Halo with the linear fit, Y (Halo protein signal intensity) = 640.6 × (Halo protein amount). Thus ~55 ng of Halo protein is expressed in 1.24 × 10⁵ cells (i.e., ~44 × 10⁻⁵ ng, per cell. In other words, taking Halo MW as 34 kDa, ~1.3 × 10⁻⁸ nmol of Halo per cell). According to Bionumbers (http://bionumbers.hms.harvard.edu/bionumber.aspx?id=108893), the diameter of an HEK cell is 13 µm, thus assuming the cell is spherical, the cell’s approximate radius is ~7 µm, and the approximate volume of each cell (\( \frac{4}{3} \pi r^3 \)) is ~ 1.44 × 10⁻¹² L. The average amount of Halo protein expressed in each cell is thus ~9 µM. Since the average photo-uncaging efficiency following 5-8 min exposure to 5 mW/cm², 360 nm lamp, is ~60%, HNE released in cells under G-REX conditions is ~5 µM.

**Isolation and purification of recombinant Ube2V2 (wt and C69S)**

The protein preparation procedure for both wt and C69S mutant of Ube2V2 was modelled on that previously reported(8). Human Ube2V2 genes (wt and C69S) were first cloned into pMAL vector to obtain plasmid DNA encoding MBP-His₆-Tev-Ube2V2 fusion protein. The
plasmids were transformed into BL21 DE3 E. coli cells and at OD$_{600}$ 0.5-0.6 induced by IPTG (0.3 mM) at 16 °C and further grown overnight. E. coli cells lysis, protein binding to nickel resin, and elution steps of the protein purification were performed according to the general procedure previously reported(9). The eluted protein was dialyzed over 5 h and cleaved with Pro TEV (Promega) overnight at 4 °C in 25 mM HEPES (pH 7.6) 50 mM NaCl, 5% glycerol, and 1 mM DTT. Subsequent incubation with Ni resin for 1 h removed the His tagged protein. After collecting the flow through, the mixture was run on G75 size exclusion column with the same dialysis buffer above except 3 mM of TCEP was used in place of DTT. Both wt and C69S mutant were eluted at approximately 180 min (corresponding to monomeric molecular weight). The eluted protein was concentrated and stored in -80 °C.

**In vitro HNEylation assay for Ube2V2 (wt/C69S) and Ube2N**

Recombinant purified proteins were normalized to the same concentration (12 µM for wt Ube2V2 and Ube2N comparison experiments, or 22 µM for wt and C69S mutant for mutant vs. wt comparison) in the storage buffer stated above containing TCEP as non-thiol-based reducing agent. Each sample was then treated with HNE-alkyne (equal concentration as protein under study, either 12 or 22 µM). The reaction mixture was incubated for the indicated time at 37 °C followed by a rapid dilution (20-fold) into chilled (50 mM HEPES (pH 7.6), 1% NP-40) buffer. The diluted reaction mixture was subjected to Click mixture containing Cy5-azide according to the procedure described above. Samples were analyzed by SDS-PAGE and in-gel fluorescence.
Circular dichroism (CD) analysis

Proteins were diluted 3–5-fold to a final concentration of 0.2–0.3 mg/ml in 4.5 mM HEPES (pH 7.6), 20 mM NaCl, 0.6–1% glycerol (v/v), and 0.5 mM TECP and the samples were placed in 0.1 cm quartz cuvettes in final volume of 300 µL. The AVIV Biomedical Circular Dichroism Spectrometer Model 201-01 spectrometer with a 150 W Suprasil Xenon lamp was used for all of the analyses. Except for the melting temperature testing, all other CD experiments were done at 25 °C, scanning from 260 nm down to 193 nm, while keeping the dynode threshold under 700 volts. 300 µL of diluted blanked buffers were performed before each sample analysis. For the HNE-labelled protein, the purified recombinant wt Ube2V2 protein 0.6 mg/ml (22 µM) was incubated with equal concentration of HNE-alkyne or buffer for 20 min at 37 °C before dilution to CD buffer indicated above.
In vitro K63-linked ubiquitin chain formation assay

| In vitro Ubiquitination assay | Final concentrations |
|-------------------------------|----------------------|
| Ubiquitin                    | 100 µM               |
| UBE1 (E1)                    | 0.1 µM               |
| Ube2N                        | 2.5 µM               |
| TCEP                          | 0.5 mM               |
| Ube2V2 (either wt/C69S)      | 2 µM                 |
| ATP                           | 3 mM                 |
| HEPES                        | 50 mM                |
| NaCl                          | 100 mM               |
| MgSO₄                         | 10 mM                |

The assay was performed using the conditions shown above at 37 °C, 30 min - 1h.

- For the functional comparison between wt and C69S Ube2V2, the corresponding assay components (E1, Ube2N, Ubiquitin, and TCEP) listed in the table above were mixed into one master stock that was then divided into different conditions with either wt Ube2V2, C69S mutant, or equal amount of blank buffer. The ubiquitination reaction was initiated by addition of ATP and quenched by Laemmli dye without reducing reagent. The entire reaction mixture was loaded on a SDS-PAGE gel followed by electrophoresis and transfer to PVDF membrane. The formation of K63-linked ubiquitin chains was quantified using Image-J (NIH) by measuring intensity of individual K63-linked ubiquitin bands from 15 kDa (dimer) till 150 kDa (19-mer). Data were plotted, and analysed by the t test (with another two independent replicates) using Prism.

- For the functional comparison of the HNEylated Ube2V2 in facilitating construction of ubiquitin chain, Recombinant Ube2V2 wt protein was made into one master stock and then split into two, one treated with equal concentration of HNE 20 min at 37 °C and the other treated with equal volume of vehicle. After the modification period, corresponding amounts of HNEylated or non-HNEylated Ube2V2 were mixed to obtain indicated different ratios. This
mixture was added into each ubiquitination reaction mixture and the reaction was initiated by addition of ATP and quenched by Laemmlı dye without reducing reagent. The entire reaction mixture was loaded on a SDS-PAGE gel followed by electrophoresis and transfer to PVDF membrane. The membranes were blotted by anti-K63. The ladders of K63-linked ubiquitin were assigned based on antibody's manual ([Merck Millipore anti K63 linked antibody, clone Apu3]) [http://www.emdmillipore.com/US/en/product/Anti-Ubiquitin-Antibody-Lys63-Specific-clone-Apu3-rabbit-moniclonal,MM_NF-05-1308] and quantified, plotted as described above.

**Cycloheximide (CHX) treatment assay for protein stability test**

HEK293T cells were cultured as described elsewhere, transfected with plasmids encoding either Halo-Tev-FLAG-(wt)Ube2V2 or the Halo-Tev-FLAG-(wt)Ube2V2(C69S) mutant for 36 h as described above before treating with either 0.1 mg/ml CHX or equal volume of vehicle (DMSO) for 8 h. Cells were harvested, pooled, washed twice with chilled 1X DPBS and flash frozen before lysing by RIPA buffer [containing in final concentrations 50 mM HEPES (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, and 1X Roche cOmplete, mini, EDTA-free protease inhibitor cocktail]. The normalized lysates were run on a SDS-PAGE gel and transferred onto a PVDF membrane before blotting with the indicated antibody. RNR2 protein with short cellular half-life (~3 h) ([10]) was used as a positive control.
**Measurement of DNA-replication efficiency by BrdU/EdU staining (in fixed cells):**

4.8 ×10⁵ cells were plated in glass-bottomed dishes (In Vitro Scientific) and allow to grow for 24 h. Cells were transfected with the indicated plasmid and allowed to grow for another 24 h. Cells were then treated with 3 μM Ht-PreHNE no alkyne for 2.5 h followed by two rinses with serum-free medium. After this time, 20 μM EdU (final concentration) was added into cell culture media for 30 min before the 3rd rinse. The cells were rinsed another two times before light shining. 2.5 h post light irradiation, cells were treated with 50 μM BrdU for 30 min at 37 °C. Then the cells were fixed with −20 °C pre-chilled MeOH for 20 min and then carried on to immunofluorescence assay as described elsewhere.

**ELISA:** Antibody was bound to the plate at the stated concentration (1–3 μg/mL) in sodium bicarbonate buffer pH 9.6 for at least 24 h in a 96 well white plate (80 μL per plate) at 4 °C. Maximum incubation time was 2 days. Next, incubation buffer was removed, washed once with TBS-Tween (100 mM Tris (pH 7.6), 150 mM NaCl, 0.03% Tween-20) and then blocked in 5% BSA in TBS-Tween (280 μL per plate) for 3-5 h at rt. Then BSA was washed away twice using TBS-Tween, and wells were filled with 150 μL blocking buffer (1.1 % BSA, 5 mM sodium orthovanadate, 20 mM NaF). Cells were lysed in 50 mM HEPES pH 7.6, 1 % NP-40, 5 mM sodium orthovanadate, 20 mM NaF and 2X ROCHE complete minus EDTA protease inhibitors. 30 μg each lysate (quantified by Bradford relative to BSA) was added to each well
(approximately 30 μL lysate, but equal amounts of total lysate buffer were always added for each set). For selected samples (usually those yielding the most protein), the amount of lysate loaded was doubled in separate wells and the value recorded was ultimately compared to the value obtained for 1X lysate. This gave equal signal (proving saturation conditions, meaning that the amount of ubiquitinated protein detected reflects the ratio of ubiquitinated to non-ubiquitinated protein in the lysate). This was incubated at 4 °C overnight. Next, wells were washed with TBS-Tween 3 times, then primary antibody was added in 1.1 % BSA in TBS-Tween overnight at 4 °C. Then, wells were washed and HRP-conjugated secondary antibody was added in 1% milk in TBS-Tween. After 1 h at r.t., wells were washed 3 times with TBS-Tween, for 15 min, then one time with TBS for 20 min after which time 50 μL TBS was added to each well. HRP was detected using a Biotek cytation 3 plate reader. Femto ELISA substrate was used, injecting 50 μL Femto ELISA substrates 1 and 2 per well. Signals were calculated relative to well coated in antibody and treated with untransfected lysate.

**In vitro transcription:** All genes were cloned into pCS2+8 vector using the primers in **Supplementary Table 4.** Prior to in-vitro transcription, the genes were PCR amplified using RNA fwd and rev primers. In vitro transcription was performed using the mMessage mMachin SP6 transcription kit according to manufacturer’s suggestion.

**Fish injection and T-REX.** All procedures conform to the National Institutes of Health guidelines regarding animal experimentation and were approved by Cornell University’s Institutional Animal Care and Use committees.
Fertilized eggs at the 1–2 cell stage from casper zebrafish were injected with mRNA (1.6 mg/ml) into the yolk sack. Immediately after injection, embryos were pooled, and separated into two petri dishes (10 cm) filled with 30 mL 10% Hank’s salt solution with methylene blue and penicillin (100 U/mL) / streptomycin (100 µg/ml). To one set was added the HaloTag-targetable photocaged precursor to HNE (Ht-PreHNE, hereafter) (<15 µM) and the other DMSO in the dark. Embryos were maintained at 28 °C in the dark for 28 h then they were washed in 10% Hank’s solution with no methylene blue/antibiotic (3 times for 30 min each). Embryos were moved to 6-well plates. Half of the embryos (Ht-preHNE-treated or -untreated) were irradiated with UV light (5 min), and the other half of each set was not irradiated. Embryos were left for 8 h at which point they were euthanized, washed with cold 1X DPBS and dechorionated (and deyolked if protein analysis was to be undertaken).
Statistical analysis:

n for imaging experiments represents the number of single cells or zebrafish cells quantified from at least ten fields of view on at least three independent plates.

n for western blot represents the number of lanes on western blots under identical experimental conditions and each lane is derived from a separate culture plate.

Fig 2A, at least two biological replicates for each condition. N ≥ 3 independent sets of biological replicates, except n =2 for compound alone control of Halo-(FLAG)-Ube2V1(C94S).

Fig. 3C, n = 3 independent sets of biological replicates at different passages.

Fig. 4B, n = 3 independent sets of biological replicates except n = 1 for shUbe2N-17.

Fig. 4E, two independent replicates were performed. For each set of cells transfected with wt- Halo-(FLAG)-Ube2V2, n = 3 (T-REX), n = 3 (DMSO); for cells transfected with C69S- Halo-(FLAG)-Ube2V2, n = 3 (T-REX), n = 3 (DMSO).

Fig. 4F, for wt-Halo-(FLAG)-Ube2V2, n = 339 (T-REX), n = 375 (Light alone), n = 300 (Ht-PreHNE alone), n = 465 (DMSO), n = 280 (Mitomycin-C), for C69S- Halo-(FLAG)-Ube2V2, n = 266 (T-REX), n = 212 (Light alone), n = 283 (Ht-PreHNE alone), n = 312 (DMSO), n = 305 (Mitomycin-C).

Fig. 4G, for wt-Halo-(FLAG)-Ube2V2, n = 648 (0h), n = 624 (1h), n = 634 (3h), n = 571 (6h), n = 542 (18h); for C69S- Halo-(FLAG)-Ube2V2, n = 585 (0h), n = 615 (1h), n = 644 (3h), n = 649 (6h), n = 646 (18h).

Fig. 4H, for shLacZ-D11, n = 434 (T-REX), n = 390 (Light alone), n = 483 (Ht-PreHNE alone), n = 434 (DMSO), n = 445 (Mitomycin-C); for shUbe2N-#16, n = 379 (T-REX), n = 297 (Light alone), n = 342 (Ht-PreHNE alone), n = 402 (DMSO), n = 241 (Mitomycin-C).
Fig. 5C, n = 69 (T-REX), n = 74 (Light alone), n = 64 (Ht-PreHNE alone), n = 69 (DMSO).

Fig. S3C and S3E (CD measurements), n = 3 independent sets of replicates;

Fig. S3D, n = 3 independent sets of biological replicates;

Fig. S3F, n = 4 independent sets of biological replicates;

Fig. S3G, n = 3 independent sets of biological replicates;

Fig. S5E, at least four independent sets of biological replicates at different passages were performed, n = 9 for wt-Halo-(FLAG)-Ube2V2, including T-REX and controls; n = 4 for C69S-mutant, including T-REX and controls.

Fig. S6B, at least three independent sets of biological replicates at different passages were performed, for wt-Halo-(FLAG)-Ube2V2, n = 5 (T-REX), n = 6 (light alone), n = 6 (Ht-PreHNE alone), n = 6 (DMSO); for C69S-mutant, n = 3 (T-REX), n = 3 (light alone), n = 3 (Ht-PreHNE alone), n = 2 (DMSO).

Fig. S7C, two independent replicates were performed. In total, n = 3 for shUbe2N-#16, n = 2 for shUbe2N-#17, n = 4 for shUbe2N-#18, n = 2 for sh-LacZ-D11 control.

Fig. S8C, for shUbe2N-#16, n = 66 (T-REX), n = 186 (Light alone), n = 270 (Ht-PreHNE alone), n = 240 (DMSO); for shLacZ-D11, n = 185 (T-REX), n = 247 (Light alone), n = 233 (Ht-PreHNE alone), n = 234 (DMSO).

Fig S8D, for wt-Halo-(FLAG)-Ube2V2, n = 50 (T-REX), n = 50 (Light alone), n = 50 (Ht-PreHNE alone), n = 50 (DMSO), n = 180 (Mitomycin-C); for C69S-Halo-(FLAG)-Ube2V2, n = 95 (T-REX), n = 59 (Light alone), n = 55 (Ht-PreHNE alone), n = 55 (DMSO), n = 55 (Mitomycin-C).

Fig. S8E, for wt-Halo-(FLAG)-Ube2V2, n = 143 (0h), n = 137 (3h), n = 172 (24h); for C69S-Halo-(FLAG)-Ube2V2, n = 164 (0h), n = 138 (3h), n = 156 (24h).
Supplementary Tables

Supplementary Table S1:

Proteomics data for protein targets identified from G-REX obtained under the experimental setup and conditions described in the manuscript.

Data in excel spreadsheet attached.
Supplementary Table S2:
(a) LC-MS/MS-based identification of the site of modification on Ube2V2 post T-REX-targeted HNEylation in HEK293T cells.

Human UBE2V2 (100%), 16.3 kDa, Mascot Score 1240.38, 3 unique peptides with different modifications, 1 distinct tryptic peptides with added mass of 154.1 Da for possible modifications of HNE(alkyne) Michael adduct (See Supplementary Table S2b). 92/145 amino acids (63.5%) coverage. Matched peptide with HNE related modifications shown in green, other matched peptide shown in red.

MAVSTGKVPR RNFRLLHS EGGKGYGDGT VSWGLEDDE MTLRWTGMI
IGPPRTNYEN R/LSLKVECG PKYPEAPPsv RFVTKINMNG INNNSGMVDA
RSIPVLAKWQ NSYSIKVVLQ ELRRLMMSKE NMKLQPQPEG QTYNN

Unique peptide with modification (asterisk suggests modification site)

MS/MS mass of Cys69 (HNE Alkyne) of human Ube2V2 (M+3H)

![MS/MS spectrum of m/z 795.7565* identifying an HNE modified peptide: IYSLKVECGPKYPEAPPsv in which Cys69 is modified by HNE alkyne](image)
(b) Chemical structures of HNE- and HNE-derived-adducts on cysteine residue on peptides.

Data in Supplemental Table S2a and Figure 1c (main manuscript) suggest Structure A is the most likely adduct among the three commonly observed HNEylated cysteines in cellular context (3, 4, 11).

| Entry | Chemical structures |
|-------|---------------------|
| A     | ![Michael adduct](image) | MW + 152.1 Da<sup>+</sup> |
| B     | ![Reduced Michael adduct](image) | MW + 154.1 Da<sup>+</sup> |
| C     | ![Dehydrated Michael adduct](image) | MW + 134.1 Da<sup>+</sup> |
**Supplementary Table S3:**

Proteomics data for protein targets identified from SILAC–T-REX obtained under the experimental setup and conditions described in the manuscript.

Data in excel spreadsheet attached.
**Supplementary Table S4:** Primers used for the construction of human Halo-TEV-Flag-Ube2V2-wt, Halo-TEV-Flag-Ube2V2-C69S, Flag2-Ube2V2-wt, Flag2-Ube2V2-C69S, Halo-TEV-Flag-Ube2V1-wt, T7-Ube2N, NF-κB-Luciferase, Myc-H2A, T7-H2A, and HA-H2A in either pFN21a, pMAL, or or pCS2+8 vector.

(a) List of primers for cloning of the gene of interest into pFN21a or pMAL vector

| Entry | Plasmid                  | Primers                                                                 |
|-------|--------------------------|-------------------------------------------------------------------------|
| (1)   | Halo-TEV-Flag-Ube2V2-wt  | Fwd-1 AGCGATAACGCGATCGCCGACTACAAGGATGACGACGATAAG                       |
|       |                          | Fwd-2 GATTTCCGCGAGCCAACCACTGGAGGATCTGTACTTTCAGGC                       |
|       |                          | GATAACGCGATCGCC Rev-1 TAGAGGATCCCCCGGTACCAGCAGCGCGACTTTATAATGTGA       |
|       |                          | TATGTTTGTCTTCTGG Rev-2 TGTTAGCCAGCAGCAGCGCGACTTTCTACTTTCAGGC          |
|       |                          | GAGGATCCCCCGGTACC                                                     |
| (2)   | Halo-TEV-Flag-Ube2V2-C69S| Fwd-1 GAAAAACAGAATATAGCTGAAAGTAGAAAGTGACGGACCTAAAT                   |
|       |                          | ACCCAGAAGCTCCCTCC Rev-1 GGAGGAGCTTCTGGGTATTTAGGTCACCTTTACCTTTCAGGC   |
|       |                          | TATATATTGCTTTTC                                                       |
| (3)   | Flag2-Ube2V2-wt          | Fwd-1 TGACGACGATAAGGACTACAAGGATGACGACTACAAGGATGACGTCCTCCACAGGA        |
|       |                          | GGTCTCCACAGGA Fwd-2 ATAGGGCTAGCAAGCCACATGGGATTACAAGGATGACGACGTCCTCG   |
|       |                          | ATAGGGACTACAAGGAT Rev-1 TAGAGGATCCCCCGGTACC                           |
|       |                          | GAGCCCGAAATTTGTTATGTGTTGTTATGTGTTTGTCTTCTGG Rev-2                    |
|   |   | Forward Primers | Reverse Primers |
|---|---|----------------|-----------------|
| 4 | Flag-2Ube2V2-C69S | TGGTAGCAGCCGGATCGCTTGAGCATGCCCTGCAGGTCGACTCTAG | GAGGATCCCCGGGTACC |
| 5 | Halo-TEV-Flag-Ube2V1-wt | AGCGATAACCGGACTGCCGACTCAAAGGATGACGACGATAAG | ATGCCAGGAGGTTCAG |
|   |   |   | GATTTCCGAGGCAAACCTACTGAGATCTGTACTTTCAAGGC | GATAACCGGATCGCC |
|   |   |   | Rev-1 | TAGAGGATCCCGGGGTACC |
|   |   |   | Rev-2 | TGTAGCGACGGATCGCTTGATCGCTGCAGGTCGACTCTA |
| 6 | T7-Ube2N | GCAAAGCCACCATGGCCAGCATGCCAGGACGCAGCATGGCCGGCCTGCCGCCC | GAGCTCGCTAGCGGAATTCCGGGGACTTTCCGGGAAATTTCG |
|   |   |   | Fwd-2 | AGCTCTTAAGGGCTAGAGTATTATACGACTCAGCTATAGGGCTAA |
|   |   |   | Rev-1 | TAGAGGATCCCGGGGTACC |
|   |   |   | Rev-2 | TGTAGCGACGGATCGCTTGATCGCTGCAGGTCGACTCTA |
| 7 | NF-kB-Luciferase | GAGCTCGCTAGCGGAATTTCGGGACTTTCCGGGGAATTTCGGG |
|   | Sequence                        |
|---|--------------------------------|
| Fwd-2 | TGGCCGGTACCTGAGCTCGTAGCGGGAA  |
| Rev-1 | CGAGGCCAGATCTGGAATTCGGAAAGTCCCGGAAATTCGCCGAAAG  |
| Rev-2 | TGGCCGGCCGAGGCGATCTGGAAATT |
| Rev-3 | AGATCTGGCCTCGCGCGCCAAGCTTAGACACTAGAGGTTAT  |
| (8) | HA-H2AX                        |
| Fwd-1 | CTACCCATACGACGTCGCCAGACTACGCTGATACTAGGATCCAGTCGGGAC  |
| Rev-1 | TCGACTCTAGAGGATCCCCGGGTACCGAGCCCGAATTCGTCTACTTGCCCTTGGCCTTGT  |
| Rev-2 | GGGCTTTGTTAGCAGCCGGATCAGCTTTGCTAGCTGCAGGTCGACTCTAGAGGATCCCCG  |
| (9) | Myc-H2AX                       |
| Fwd-1 | ACAAAAACTCATCTCAGAAGGATCTGCTAGCTAGGATCCAGTCGGGAC  |
| Rev-1 | TCGACTCTAGAGGATCCCCGGGTACCGAGCCCGAATTCGTCTACTTGCCCTTGGCCTTGT  |
| Rev-2 | GGGCTTTGTTAGCAGCCGGATCAGCTTTGCTAGCTGCAGGTCGACTCTAGAGGATCCCCG  |
|   |   |   |
|---|---|---|
| (10) | T7-H2AX | Fwd-1  
|   |   | CCAGCATGACCGGCGGCCAGCAGATGGGCGTACCTAGGATCC  
|   |   | AGTCGGGAC  
|   |   | Fwd-2  
|   |   | AGAGTATTAATACGACTCACTATAGGGCTAGCAAGCCACCAT  
|   |   | GGCCAGCATGACCGGCG  
|   |   | Rev-1  
|   |   | TCGACTCTAGAGGATCCCCGGGTACCGAGCCCGAATTCGTCT  
|   |   | ACTTGCCCTTGCCCTGT  
|   |   | Rev-2  
|   |   | GGGCTTTGTTAGCAGGCCAGATCAGCTTGCATGCCTGCAGGTC  
|   |   | GACTCTAGAGGATCCCCG |
| (11) | pMAL-MBP-His-  
|   |   | Tev-Ube2V2 (both  
|   |   | wt and C69S  
|   |   | mutant)  
|   | Fwd-1 | CTACGACATCCCCCTCTCCCGAGAAGCTTGACTTCAGGGATCC  
|   |   | ATGGCGGTCTCCACAGG  
|   | Fwd-2 | GGAAGGATCAGCGGCCACCACCACCACCACCACCACCACCACGAC  
|   |   | TACGACATCCCCCTCCTCC  
|   | Rev-1 | ACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTTTAATTGTG  
|   |   | GTATGTTTTGTCTCTTCTG  
|   | Rev-2 | CAAGGCGATTAAGTTGGGAACAGCCAGGACGCTCAGTGCAGCAG  
|   |   | ACGTTGAAAAACGACGGG |
| (12) | Halo-TEV-Flag-  
|   |   | Ube2V1-C94S  
|   | Fwd-1 | TATGAAAACCGAATATATAGCCTAAATAGAAAAAGTGACCTAA  
|   |   | ATACCCAGAAGCACC  
|   | Rev-1 | ACGACGTTGTAAAACGACGGG |
|   | GGGTGCTTCTGGGATTTTAGGTCCACTCTCTATATTTAAGGCTAT  
|   |   | ATATCGGGTTTTCATA |
### (b) List of primers for cloning of the gene of interest into PCS2+8 vector

| Entry | Plasmid           | Primers                                                                 |
|-------|-------------------|-------------------------------------------------------------------------|
| (1)   | Halo-TEV-Flag-    | Fwd-1                     | TAATTAAGGCCGCCAGCGATCGCGACATGGCAGAAATC                                  |
|       | Ube2V2-wt         |                           | GGTACTGG                                                             |
|       |                   | Fwd-2                     | GCTACTTGTTTGTGAGCCACTAGTGGCACGAGGCAATA                                  |
|       |                   |                           | ATTAAGGCCGCAGGGGCA                                                   |
|       |                   | Rev-1                     | TTCTAGAGGCTGAGAGCTTCCTCGAAGCG                                       |
|       |                   |                           | CTTATCATGTGATTACGACTCATACTAGTTCTA                                    |
|       |                   |                           | GAGGCTAGAGGCTGCTGG                                                  |
| (2)   | Halo-TEV-Flag-    | The same as entry 1       | The same as entry 1                                                   |
|       | Ube2V2-C69S       |                           | The same as entry 1                                                   |
| (3)   | HA-Ubiquitin      | Fwd-1                     | TAATTAAGGCCGCCAGCGATCGCGACATCCCATAGTTCTA                              |
|       |                   |                           | GCTCCAG                                                             |
|       |                   | Fwd-2                     | GCTACTTGTTTGTGAGCCACTAGTGGCACGAGGCAATA                                  |
|       |                   |                           | ATTAAGGCCGCAGGGGCA                                                   |
|       |                   | Rev-1                     | TTCTAGAGGCTGAGAGCTTCCTCGAAGCG                                       |
|       |                   |                           | CTTATCATGTGATTACGACTCATACTAGTTCTA                                    |
|       |                   |                           | GAGGCTAGAGGCTGCTGG                                                  |
| (4)   | HA-UBE2N          | The same as entry 3       | The same as entry 3                                                   |
**Supplementary Table S5: shRNA sequences**

The following shRNA sequences were used in pLKO1 vector. For details, see lentivirus production and infection method sections.

| Entry | Plasmid           | Sequences                                                                 |
|-------|-------------------|---------------------------------------------------------------------------|
| (1)   | shUbe2N-#16       | CCGGCCATAGAAACAGCTAGAGCATCTCGAGATGCTCTAGCTGTTTCTATGGTTTTTG               |
| (2)   | shUbe2N-#17       | CCGGAGACAAGTTGGGAAGAATATGCTCGAGCATATTTCTTCCC AACTTGTTTTTTTG             |
| (3)   | shUbe2N-#18       | CCGGGCTGAGGCTTGTGAGTCTTCTCGAGAAGACTCACAAATGCCTCAGCTTTTT                  |
| (4)   | shLac Z (shRNA control)-D11 | CGCGATCGTAATACCCGAGT                                                      |
### Supplementary Table S6: Summary of antibodies

| Antibody                                             | Application | Catalog number; Supplier                  | Dilution |
|------------------------------------------------------|-------------|-------------------------------------------|----------|
| Mouse monoclonal anti-β-Actin-HRP                   | WB          | A4700; Sigma Aldrich                      | 1:30000  |
| Rabbit polyclonal anti-Halo                          | WB          | G9281; Promega                            | 1:1000   |
| Rabbit anti-Ubiquitin                                | WB          | Ab7780, Abcam                             | 1:500    |
| Mouse monoclonal anti-monoo- and polyubiquitylated conjugates FK2 | WB          | BML-PW8810-0100, Enzo Life Sciences       | 1:500    |
| Monoclonal anti-gapdh-peroxidase                     | WB          | G92296; Sigma                             | 1:30000  |
| Anti-ubiquitin, Lys63-Specific, clone Apu3, rabbit monoclonal | WB          | 05-1308, Merck Millipore                  | 1:500    |
| Anti-ubiquitin, Lys-48-specific, clone Apu2, rabbit monoclonal | WB          | 05-1307, Merck Millipore                  | 1:7000   |
| Rabbit polyclonal anti-FLAG                          | IF          | PA1-984B, Fisher Pierce                    | 1:3000   1:300 |
| Rabbit polyclonal anti-Ube2N                         | WB          | Ab25885, Abcam                            | 1:2000   |
| Rabbit polyclonal anti-Ube2V2                        | WB          | 10689-1-AP, ProteinTech                    | 1:3000   |
| Rabbit polyclonal anti-53bp1                         | IF          | Sc-22760, Santa Cruz                      | 1:300    |
| Goat polyclonal anti-FLAG                            | IF          | Ab1257, Abcam                             | 1:300    |
| Rat monoclonal anti-HA                               | IF          | 11867423001, Sigma Aldrich                | 1:3000   1:300 |
| Rabbit polyclonal anti-PCNA                          | ELISA       | Sc-7907, Santa Cruz                       | 1:1000   |
| Goat polyclonal anti-myc tag                         | WB          | Ab 9132, Abcam                            | 1:2000   |
| Mouse monoclonal anti BrdU, MoBU-1                   | IF          | B35128, ThermoFisher                      | 1:500    |
| Rat monoclonal anti BrdU, BU1/75 (ICR1)              | IF          | Ab6326, Abcam                             | 1:1000   |
| Anti-phospho-Histone H2A.X (Ser139) Antibody, mouse monoclonal | IF          | 05-636, Merck Millipore                   | 1:300    |
| Secondary antibody to rabbit IgG, HRP linked         | WB          | 7074; Cell Signaling Technology           | 1:5000   |
| Secondary antibody to mouse, HRP linked               | WB          | Ab6789; Abcam                             | 1:5000   |
| Donkey Anti-rabbit IgG AlexaFluor® 647               | IF          | Ab150075; Abcam                           | 1:1000   |
| Goat Anti-Rat IgG H&L AlexaFluor® 568                | IF          | Ab175710; Abcam                           | 1:1000   |
| Goat Anti-rabbit AlexaFluor® 488                     | IF          | A11008; Invitrogen                        | 1:1000   |
| Goat Anti-Mouse Ig, Human ads-FITC                   | IF          | 1010-02, Southern Biotech                 | 1:1000   |
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