Supplemental Information

Intracellular H2S production is an autophagy dependent adaptive response to DNA damage

A high-content screen identifies genotoxic agents as regulators of intracellular H_2S levels in an autophagy dependent response to DNA damage

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Table S1. Small molecule screening data. Related to Figure 1

Category	Parameter	Description
Assay	Type of assay	A high content cell based phenotypic screen for small molecule compounds that modulate intracellular H_2S level
	Target	Biochemical pathways that control cellular H_2S level
	Primary measurement	Fluorescence intensity of the reaction product of H_2S and probe P3 (H_2S/P3), and the fluorescence intensity of DNA probe DRAQ5 in images
	Key reagents	H_2S probe P3
	Assay protocol	Described in method section "High-content compound screening"
	Additional comments	
Library	Library size	12,000
	Library composition	Small molecule Known Bioactives collection
	Source	Harvard ICCB-Longwood Screening Facility
	Additional comments	Library described in https://iccb.med.harvard.edu/known-bioactives-collection
Screen	Format	384-well
	Concentration(s) tested	0.1-30 μM
	Plate controls	DMSO as negative control, 1.3 mM AOAA as positive control
	Reagent/ compound dispensing system	ICCB in-house robot and automated pipettor (Seiko compound transfer robot and Agilent Vprep)
	Detection instrument and software	Image Xpress Micro (Molecular Devices) and MetaXpress software
	Assay validation/QC	Z-factor was 0.44 for 1.3 mM AOAA
	Correction factors	No
	Normalization	Described in method section "High-content compound screening"
	Additional comments	P3 was dissolved in DMSO and added to assay plates using automated pipettor (Agilent Vprep).
Post-HTS analysis	Hit criteria	Activators (cell survival >50%, relative intensity of H_2S/P3 > 1.4)
	Hit rate	1 %
	Additional assay(s)	Described in the method section
	Confirmation of hit purity and structure	4 representative compounds for validation were well characterized and purchased from reliable commercial sources.
	Additional comments	

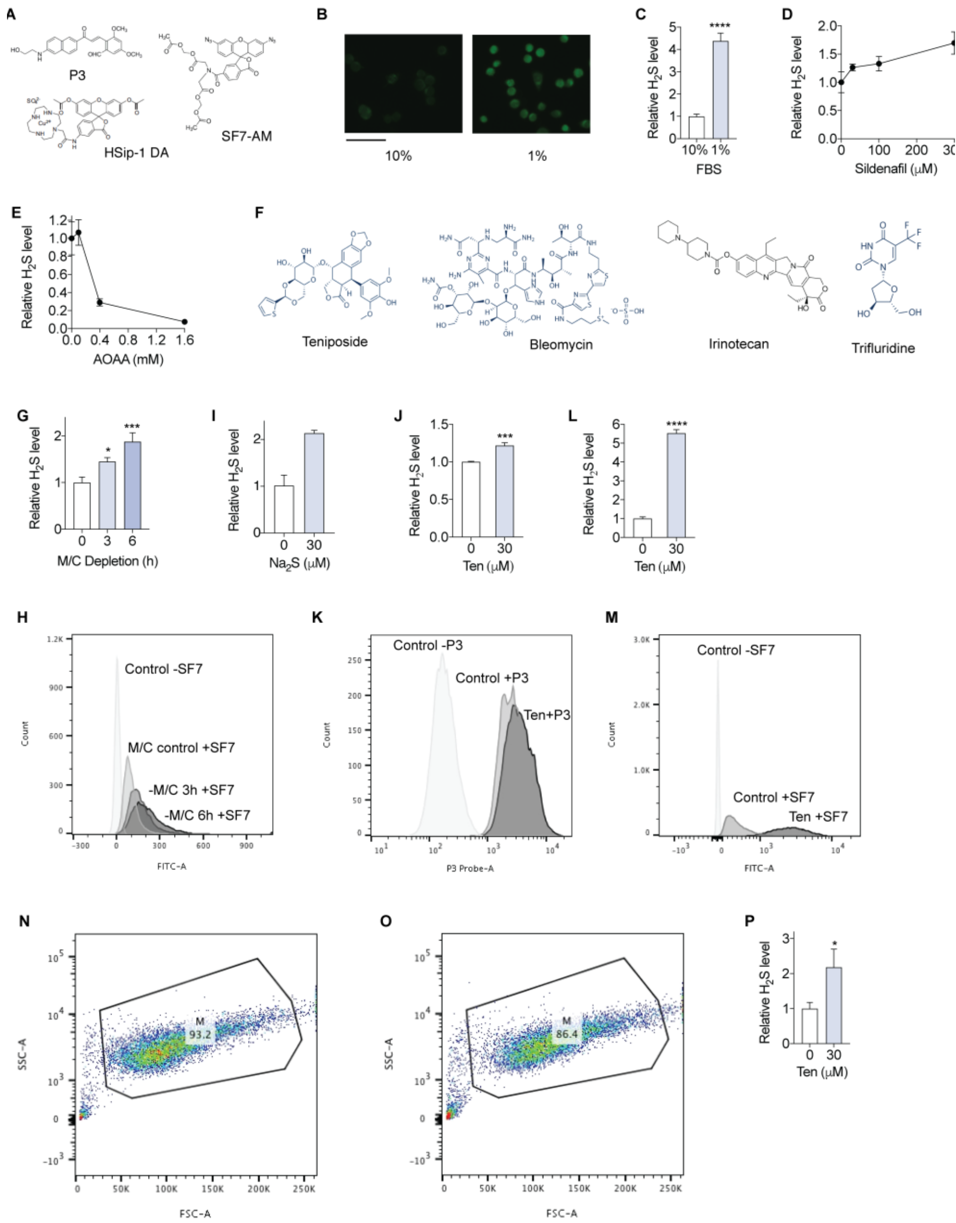
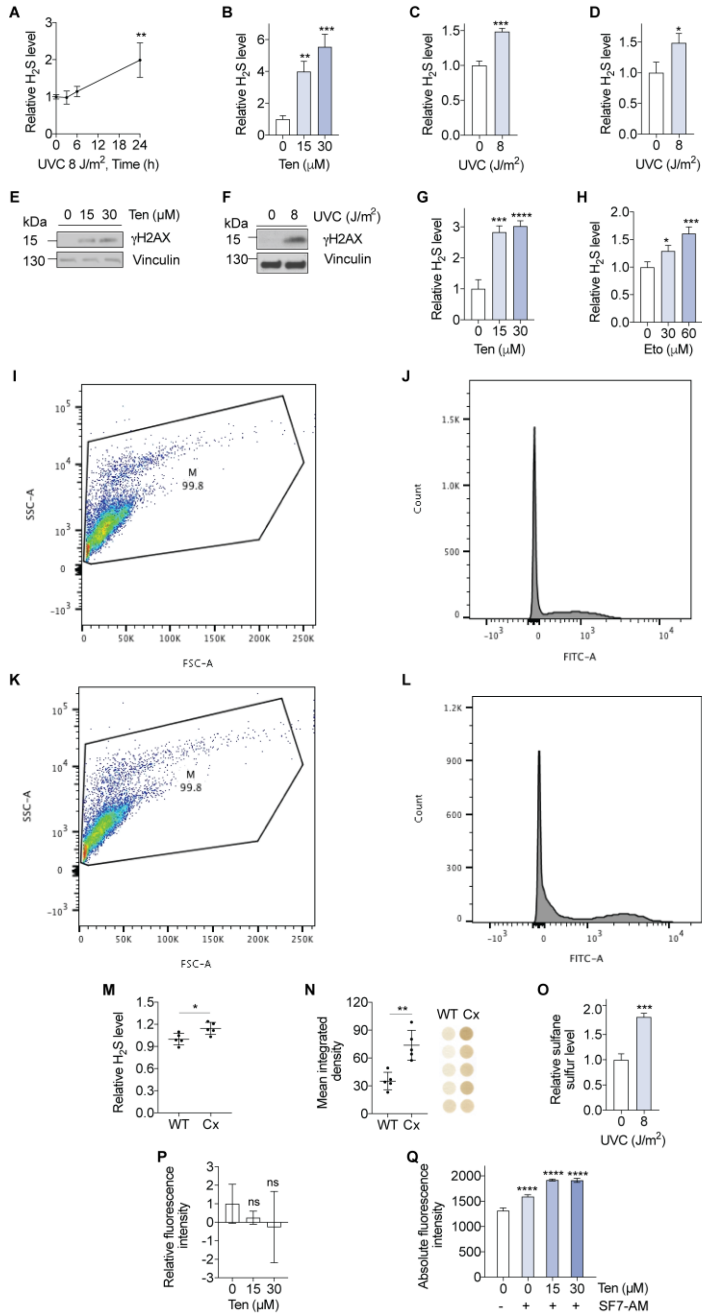


Figure S1. A high-content screen for small molecule modulators of intracellular H_2S levels. Related to Figure 1

(A) Chemical structures of H_2S probes. (B, C) Validation of P3 imaging assay. Representative P3 fluorescence images of HeLa cells grown in 10% FBS or 1% FBS media for 16 h prior to staining with P3 (B) and quantification of images (n=3 fields) (C). Scale bar, 60 μm. (D, E) Validation of the small molecule screening assay using P3. Intracellular H_2S levels were measured in HeLa cells treated with sildenafil (D) or AOAA (E) for 20 h (n=4). (F) Chemical structures of 4 representative genotoxic compound hits. (G, H) Validation of SF7-AM flow cytometry assay using M/C depletion. Quantitation of mean SF7-AM fluorescence intensity (G; n=3) and representative histogram (H) from primary MEFs grown in media lacking sulfur amino acids methionine and cysteine (-M/C) for the indicated time, stained with 0.25 μM SF7-AM for 30 min and analyzed by flow cytometry. The cells not stained with SF7 (Control -SF7) were used for the blank control. The control cells (M/C control +SF7) and cells grown in media lacking M/C for 3h (-M/C 3h +SF7) or 6h (-M/C 6h +SF7) were stained with SF7-AM. (I) Validation of SF7-AM flow cytometry assay using H_2S donor Na_2S. 0.25 μM SF7-AM was added to the medium of immortalized MEFs, and then fresh Na_2S PBS solution was added and incubated for 30 min. Intracellular H_2S levels were measured by flow cytometry (n=2). (J-M) SF7-AM is a more sensitive H_2S probe than P3 in flow cytometry assays. Quantitation of mean fluorescence intensity (J, L; n=3) and representative histograms (K, M) from immortalized MEFs treated with 30 μM Ten for 4 h, stained with 10 μM P3 (J, K) or 0.25 μM SF7-AM (L, M) for 30 min, and analyzed by flow cytometry. The cells not stained with probe were used for the blank control. The cells treated with 30 μM Ten or vehicle control (DMSO) were stained with probe. (N, O) The gating strategy for SF7-AM stained control (N) and Ten treated MEFs (O). (P) Quantification of P3 fluorescence microscopy images (n=3 fields) as shown in Fig. 1D from HeLa cells treated with Ten. Error bars indicate SD. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. Student's t-test (C, J, L, P); one-way ANOVA (G).



A
Relative H_2S level
UVC 8 J/m^2, Time (h)
B
Relative H_2S level
Ten (μM)
C
Relative H_2S level
UVC (J/m^2)
D
Relative H_2S level
UVC (J/m^2)
E
kDa
Ten (μM)
γH2AX
Vinculin
F
kDa
UVC (J/m^2)
γH2AX
Vinculin
G
Relative H_2S level
Ten (μM)
H
Relative H_2S level
Eto (μM)
I
SSC-A
FSC-A
M
99.8
J
Count
FITC-A
K
SSC-A
FSC-A
M
99.8
L
Count
FITC-A
M
Relative H_2S level
WT
Cx
N
Mean integrated density
WT
Cx
O
Relative sulfane sulfur level
UVC (J/m^2)
P
Relative fluorescence intensity
Ten (μM)
Q
Absolute fluorescence intensity
Ten (μM)
SF7-AM

Figure S2. DNA damage increase sulfide levels *in vitro* and *in vivo*. Relative to Figure 2
(A) Intracellular H_2S in immortalized MEFs up to 24 h UVC irradiation as indicated (n=3). (B) Intracellular H_2S measured using the fluorescent probe HSip-1 DA in primary MEFs treated with Ten for 6 h (n=3). (C, D) Intracellular H_2S measured using SF7-AM (C) or HSip-1 DA (D) in primary MEFs 24 h after irradiation with 8 J/m^2 UVC (n=3). (E, F) Immunoblots of γH2AX from primary MEFs treated with Ten for 6 h (E) or 24 h after UVC irradiation (F). (G) Intracellular H_2S in mouse circulating leukocytes treated *ex vivo* with Ten for 3 h (n=3). (H) Intracellular H_2S in immortalized MEFs treated with Eto for 6 h (n=3). (I-L) Flow cytometry gating strategy (I, K) and representative histograms (J, L) for circulating leukocytes from WT (I, J) and Cx (K, L) mice shown in Fig. 2H. (M) Intracellular H_2S (P3 fluorescence) in lung endothelial cells of WT vs. Cx mice (n=5 mice/group). (N) Lead sulfide darkening (right panel) and quantitation (left panel) indicative of H_2S production capacity of liver extracts from WT vs Cx mice (n=5 mice/group). (O) Intracellular sulfane sulfur in primary MEFs 24 h after UVC irradiation (n=3). (P) Teniposide does not release H_2S. Relative SF7-AM fluorescence intensity in culture media co-incubated with SF7-AM and Ten for 6 h at 37 ºC in a CO_2 incubator (n=3) is shown. (Q) Absolute fluorescence intensity data from media of primary MEFs treated with Ten for 6 h from Fig. 2J (n=3). Error bars indicate SD. ns, not significant; *P < 0.05; **P < 0.01; *** P < 0.001; **** P < 0.0001. One-way ANOVA (A, B, G, H, P, Q); Student's t-test (C, D, M-O).

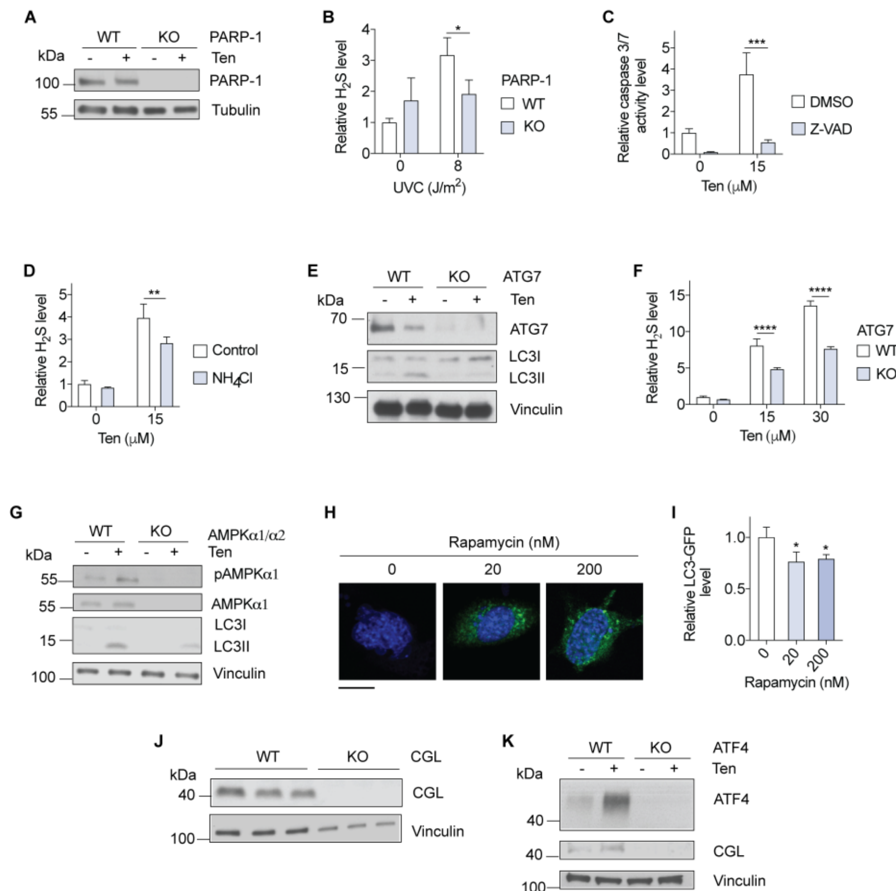


Figure S3. Autophagy and CGL mediate H_2S generation upon DNA damage. Relative to Figure 2 and 3

(A) Immunoblot of PARP-1 in immortalized WT and PARP-1 knockout (KO) MEFs treated with 30 μM Ten for 6 h. (B) Intracellular H_2S in immortalized WT and PARP-1 KO MEFs 24 h after UVC irradiation (n=3). (C) Caspase 3/7 activity in immortalized MEFs treated with 5 μM pan-caspase inhibitor Z-VAD-FMK (Z-VAD) and Ten as indicated for 24 h (n=3). (D) Intracellular H_2S in immortalized MEFs treated with 15 μM Ten and 15 mM NH_4Cl for 6 h (n=3). (E) Immunoblots of the indicated proteins in immortalized WT and ATG7 KO MEFs treated with 30 μM Ten for 6 h. (F) Intracellular H_2S in WT and ATG7 KO immortalized MEFs treated with Ten for 6 h (n=3). (G) Immunoblots of the indicated proteins in immortalized WT and AMPK$\alpha1^{-/-}/\alpha2^{-/-}$ MEFs treated with Ten for 6 h. (H, I) Autophagic puncta (green) formation (H) and quantitation of GFP-LC3 levels (I; n=3) in immortalized MEFs expressing GFP-LC3 treated with rapamycin for 24 h. Scale bar, 20 μm. (J) Immunoblot of CGL in primary WT and CGL KO MDFs. (K) Immunoblots of ATF4 and CGL in immortalized WT and ATF4 KO MEFs treated with 30 μM Ten for 6 h. Error bars indicate SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; **** $P < 0.0001$. One-way ANOVA (I); Two-way ANOVA (B-D, F).

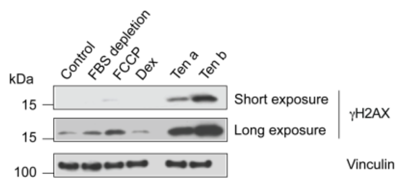


Figure S4. Effects of serum depletion, FCCP and Dex on DNA damage marker γH2AX levels. Relative to Figure 4

Immunoblots of γH2AX in immortalized MEF cells treated with serum (FBS) depletion, 20 μM FCCP, 150 μM Dex, or 3.75 μM (Ten a), 7.5 μM (Ten b) teniposide for 6h.