**Supplemental Information:**

**Reply to Vangala et al.**

# Complete inhibition of the proteasome reduces new proteasome production by causing Nrf1 aggregation, not by blocking its processing

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## Supplemental Results

### Fig S1 Effect of different concentrations of carfilzomib (CFZ) on proteasome inhibition and the formation of processed Nrf1 in the lysate

A. HEK293A cells were treated with the indicated concentrations of CFZ for 16h to analyze the levels of glycosylated (G), deglycosylated full-length (FL), and processed (P) forms of endogenous Nrf1 by Western blotting (left). Densitometry of the levels of Processed (P-Nrf1) and Full-length (FL-Nrf1) forms of Nrf1 was performed and normalized with GAPDH as the loading control (Right Panel).
B. Flowchart for the protocol for the NMS873 chase used in Fig 1A. HEK293A cells were treated with 10 μM NMS873 for 2h. Then NMS873 was removed, but 100 μg/ml cyclohexmide were added, and the cells were cultured for 2, 6, or 20h. To avoid cell killing by cycloheximide, its concentration was reduced to 20 μg/ml after 6h. During the release phase from NMS873, cells were cultured in the presence of 0.1 or 10 μM CFZ, or a combination of 0.1 μM CFZ and 0.5 μM E1-In to inhibit the proteasome or ubiquitination. To ensure that proteasomes and ubiquitination were inhibited at the

beginning of the release phase, CFZ and E1-In were added 1h before the removal of NMS873.

C. To verify the partial inhibition of the proteasome by 0.1 μM CFZ and the complete inhibition by 10 μM CFZ, the relative amounts of chymotrypsin-like and caspase-like activities of the proteasome were measured in the lysate as in Fig 1A. The amounts of activity in untreated cells were set as 100%.

D. HEK293A cells were transfected with a plasmid expressing N-terminally-tagged HA-Nrf1 [S1], then treated as in Fig S1B and Fig 1A to assay the generation of the p75 product of HA-Nrf1 after 20h treatment with 100 nM CFZ, or the generation of the p110 product after release from the p97 inhibitor NMS873. Both p75 and p110 have the same molecular weight and lack the N-terminal HA tag, indicating that they are the same processed (P) form of HA-Nrf1.

E. Lysates of WT and Ddi2KO HAP1 cells arrested with NMS873 and then released for 2h (as in Fig S2B) were blotted with Nrf1 antibody to reveal the glycosylated, full-length (FL), or processed (P) forms of Nrf1. Alternatively, lysates of untreated WT HAP1 cells were blotted with antibodies against NF-κB (p50/p105), p97, PI3 Kinase (p110α), and PI3 Kinase (p85). The positions of these bands were compared on the same membrane with each other plus two molecular weight standards (SeeBlue, Thermo LC5925, used in our manuscript [S1] and Bluestain, Gold Biotechnology P008). It appears obvious that company-assigned molecular weights are higher for Bluestain than for Seeblue, supporting our prediction that referring to company-assigned molecular weight values of different markers may cause variations in molecular weight estimation. We estimate that glycosylated Nrf1 is 105-110kDa because its position is similar to p105 and p110. The

full-length Nrf1 is estimated to be 100kDa because it is smaller than p105 but larger than p97. The processed Nrf1 is estimated to be 90-95kDa because it is bigger than p85 but slightly smaller than p97 (p97 appears slightly bigger than Bluestain-93kD but P-Nrf1 appears slightly smaller than Bluestain-93kD). Importantly, the Nrf1 antibody only recognized these three major forms, and there is not a second cleavage product of Nrf1.

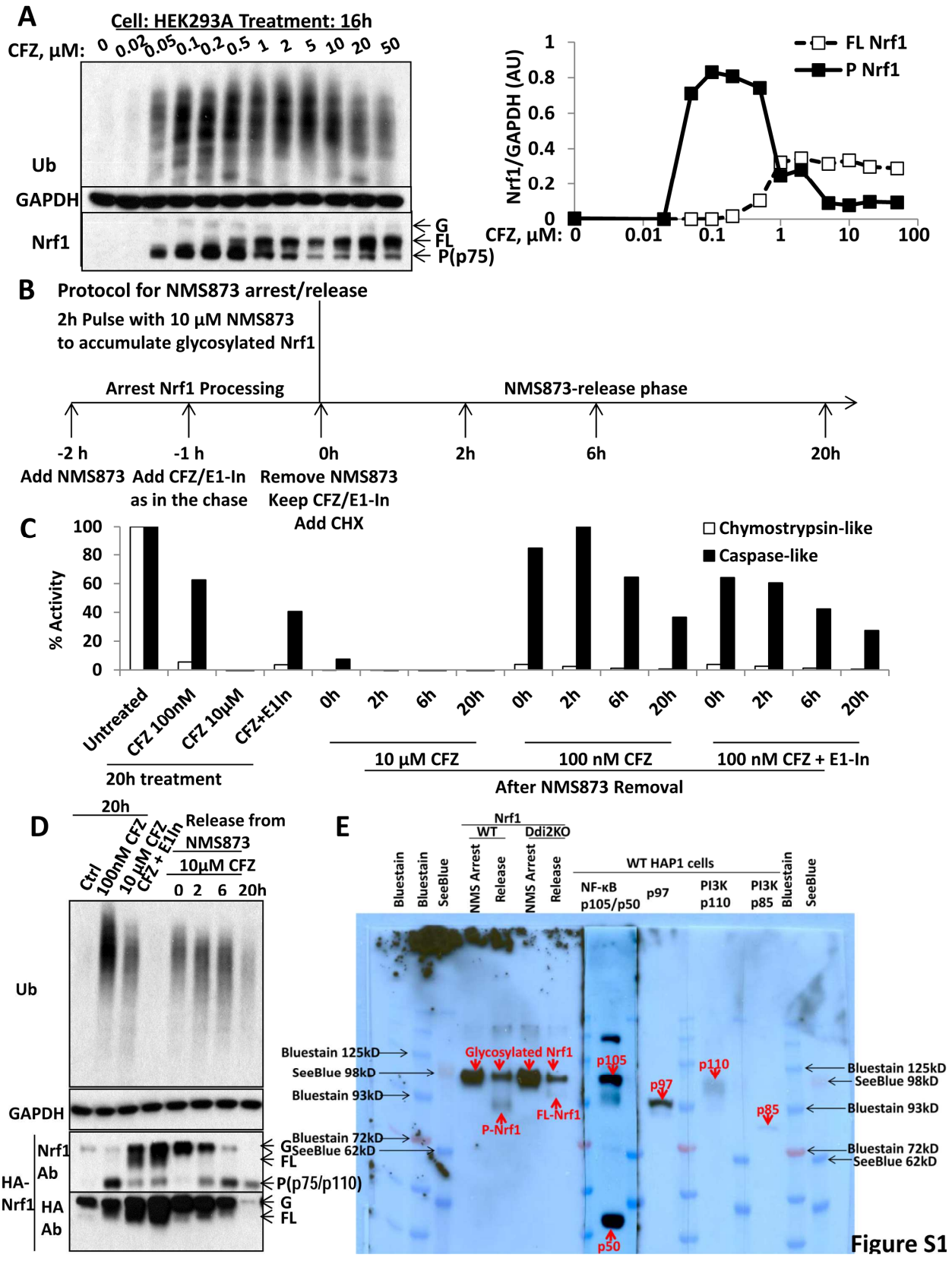
A
Cell: HEK293A Treatment: 16h
CFZ, μM: 0 0.02 0.05 0.1 0.2 0.5 1 2 5 10 20 50
Ub
GAPDH
Nrf1
G
FL
P(p75)
Nrf1/GAPDH (AU)
FL Nrf1
P Nrf1
CFZ, μM: 0 0.01 0.1 1 10 100
B
Protocol for NMS873 arrest/release
2h Pulse with 10 μM NMS873
to accumulate glycosylated Nrf1
Arrest Nrf1 Processing
NMS873-release phase
-2 h
Add NMS873
-1 h
Add CFZ/E1-In
as in the chase
0h
Remove NMS873
Keep CFZ/E1-In
Add CHX
2h
6h
20h
C
% Activity
Chymostrypsin-like
Caspase-like
Untreated
CFZ 100nM
CFZ 10μM
CFZ+E1In
20h treatment
10 μM CFZ
100 nM CFZ
100 nM CFZ + E1-In
After NMS873 Removal
D
20h
Ctrl
100nM CFZ
10 μM CFZ
CFZ + E1In
Release from NMS873
10μM CFZ
0 2 6 20h
Ub
GAPDH
Nrf1 Ab
HA-Nrf1
HA Ab
G
FL
P(p75/p110)
E
Nrf1
WT
Ddi2KO
Bluestain
SeeBlue
NMS Arrest
Release
WT HAP1 cells
NF-κB p105/p50
p97
PI3K p110
PI3K p85
Bluestain 125kD
SeeBlue 98kD
Bluestain 93kD
Bluestain 72kD
SeeBlue 62kD
Glycosylated Nrf1
P-Nrf1
FL-Nrf1
p105
p50
p97
p110
p85

Figure S1

**Fig S2 Ddi2 is required for the processing of Nrf1 induced by proteasome-inhibition**

WT or Ddi2KO HAP1 cells were treated for 16h with 0.1 or 10 μM CFZ (A), or Nrf1 processing was arrested with NMS873 and then released for 2h (B) as described in Supplemental Fig S1B. The levels of G, FL, P forms of Nrf1 in cell lysate were measured by western blotting. In Ddi2KO cells, the processing of Nrf1 is completely abolished, and there was no P-Nrf1 in the pellet fraction (data not shown). These findings confirm the conclusions of Lehrbach and Ruvkun [S2] and Murata (personal communication).

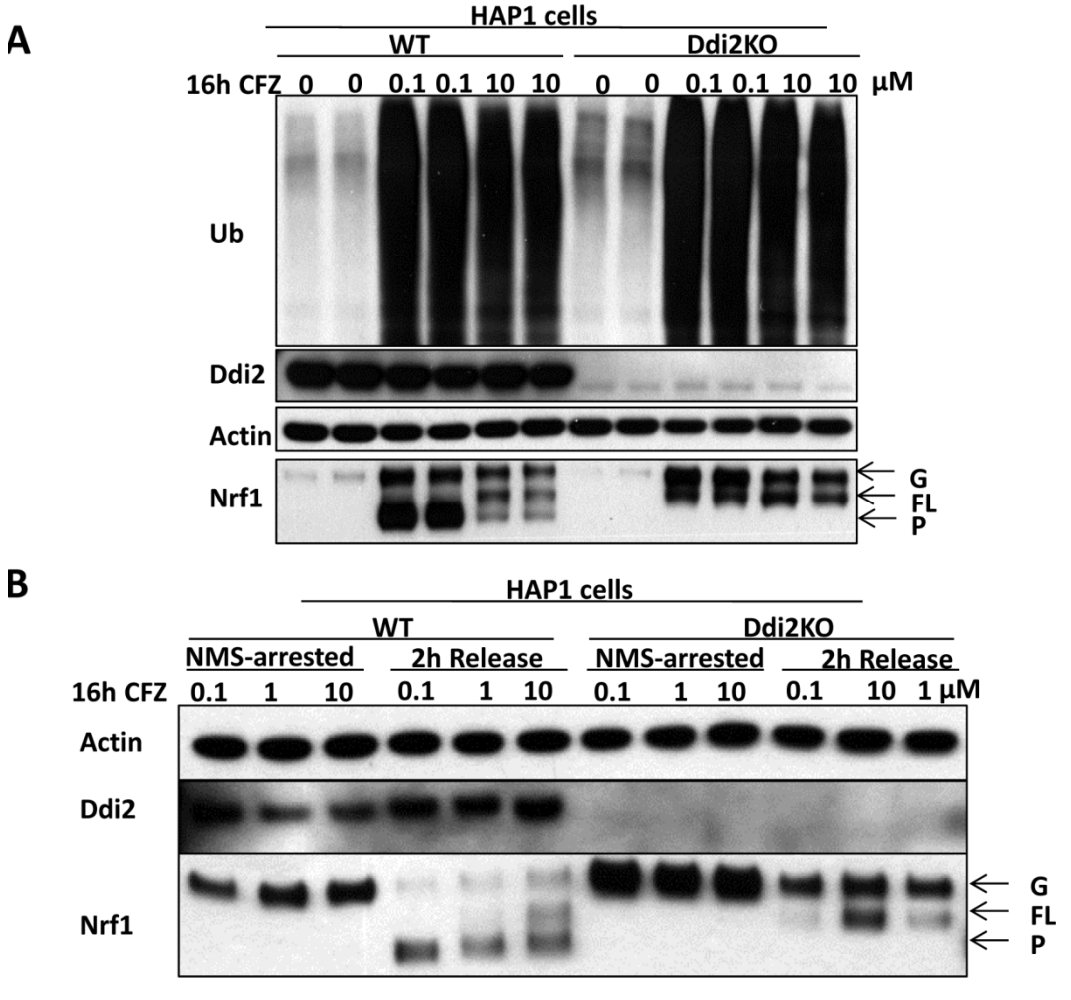


Figure S2

## Supplemental Experimental Procedures

### Cell lines and growth conditions

Ddi2 knock out cells and parental HAP1 cells were purchased from Horizon (HZGHC000182c006) and cultured in IMDM media (Thermo, 12440061). HEK293A cells and HEK293F cells were cultured in DMEM media (Westnet, 10-013-CV). All media contains 10% FBS (SIGMA, F2442-500mL) and 1% Penicillin-Streptomycin solution (Life Technologies, 15070-063). All cells were maintained in a humidified incubator at 37°C and 5% $CO_2$.

#### Overexpression of HA-Nrf1 in HEK293A cells

HEK293A cells were transfected with the plasmid expressing HA-Nrf1 (EX-F0807-M06, Genecopoeia Inc.) using Lipofectamine 2000 (Life Technologies, 11668-019) as described in [S1]. Cells were treated 48h after the transfection.

### Treatment with proteasome inhibitors or other compounds

Stock solutions were prepared Bortezomib (Millenium Pharmaceuticals, 1 µM, $H_2O$), Carfilzomib (UBPBio, 10 µM, DMSO), Cycloheximide (SIGMA, 10 mg/ml, DMSO), NMS873 (SIGMA, 10 mM, DMSO). The E1 inhibitor ML00603997 (20 mM, DMSO) was kindly provided by Dr. Laurence R. Dick at Millenium Pharmaceuticals.

### Western Blotting

Cells were lysed in 1% TX-100 lysis buffer (50mM NaCl, 30mM $Na_4P_2O_7$, 50 mM NaF, 10 mM Tris-Cl pH 7.6, 5 mM EDTA, 1 mM $Na_3VO_4$, 1% TX-100, Roche Protease Inhibitor Cocktail Tablet, 100 µl per well (24-well)) as described in [S1]. The pellet after centrifugation (10,000 ×g, 10 min) was solubilized in 2% SDS (prepared in 50 mM Tris-Cl pH 7.4). Antibodies against

Nrf1(Cell Signaling, 8052S, 1:1000), HA(SIGMA, H9658-.2ML, 1:10,000), GAPDH(SIGMA, G8795-200µl, 1:10,000), Actin (SIGMA, A3854-200UL, 1:10,000), Ubiquitin (P4D1, Santa Cruz, sc-8017, 1:5,000), Ddi2 (Bethyl, A304-629A, 1:2,000), p97 (BD, 612182, 1:1,000), NF-κB (p50/p105) (EMD Millipore, 06-886, 1:1,000), PI3 Kinase p85 subunit (Cell Signaling, 4092S, 1:1,000), PI3 Kinase p110α subunit (Cell Signaling, 4255S, 1:1,000), HA (SIGMA, H9658-.2ML, 1:10,000), were used to detect corresponding proteins.

**Proteasomal Peptidase Activity**

Peptidase activity assay was set up as described in [S1] except that the caspase-like activity was measured by 20 µM Ac-nLPnLD-AMC (Bachem, 4030084, 20 mM in DMSO).

**Supplemental References**


S1. Sha, Z., and Goldberg, A.L. (2014). Proteasome-mediated processing of Nrf1 is essential for coordinate induction of all proteasome subunits and p97. Current biology : CB *24*, 1573-1583.

S2. Lehrbach, N.J., and Ruvkun, G. (2016). Proteasome dysfunction triggers activation of SKN-1A/Nrf1 by the aspartic protease DDI-1. eLIFE *In press*.


**Author Contributions**

Conceptualization, Z.S. and A.L.G.; Methodology, Z.S. and A.L.G.; Investigation, Z.S.; Writing –Original Draft, Z.S. and A.L.G.; Writing –Review & Editing, Z.S. and A.L.G.; Funding Acquisition, A.L.G.; Resources, A.L.G.; Supervision, A.L.G.