Gong et al., https://doi.org/10.1083/jcb.201606095

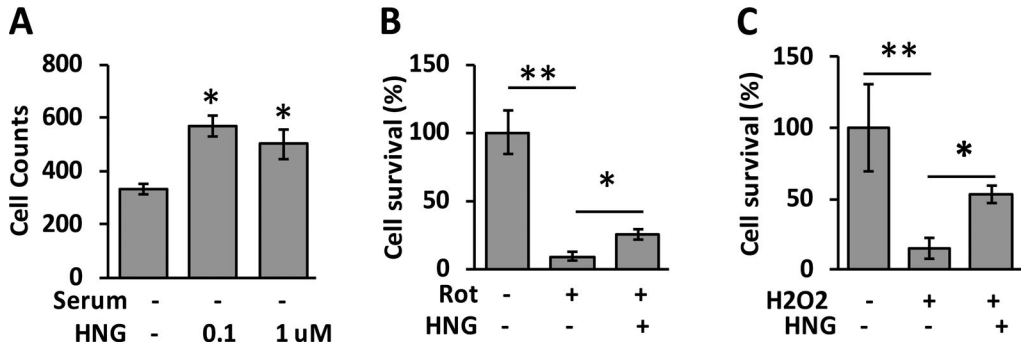

Figure S1. **HNG increases survival in MN9D cells in response to stressors. (A–C)** Protective effects of HNG on MN9D cells during starvation (A), rotenone (Rot; B), and H_2O_2 (C) treatment. *, P < 0.05; **, P < 0.01. Error bars show SEM.

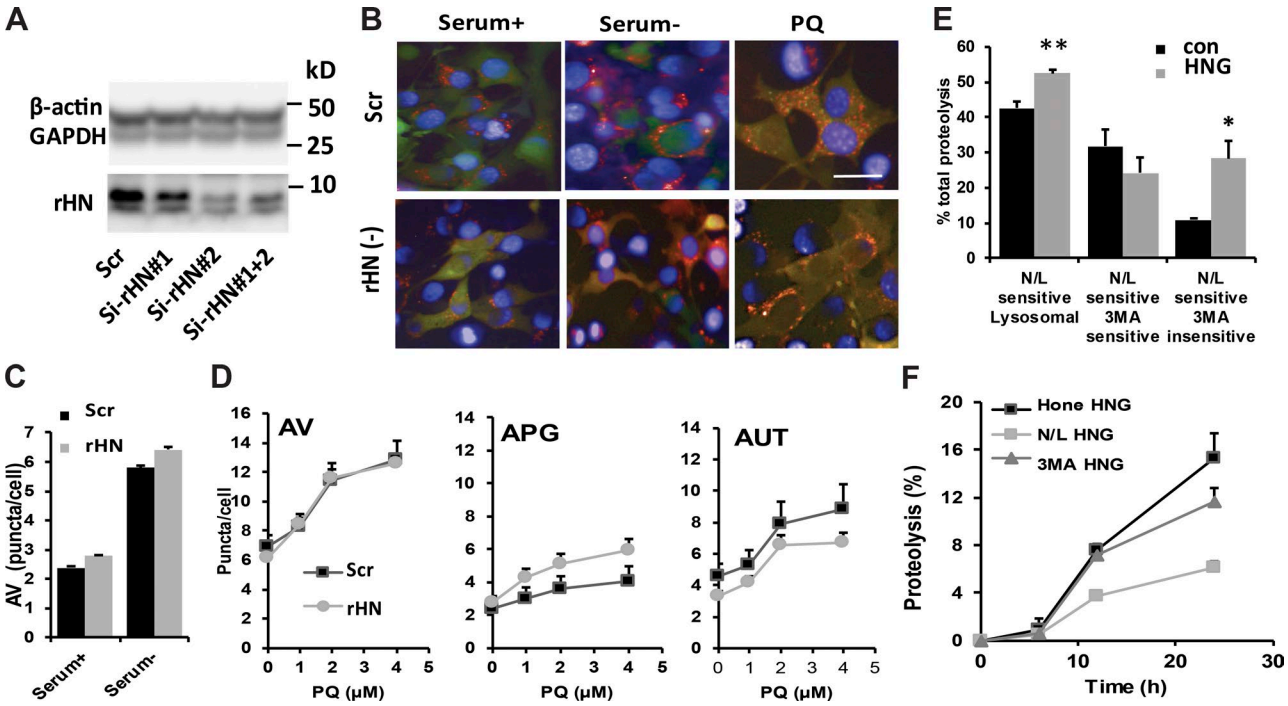

Figure S2. **Effect of endogenous rHN on macroautophagy. (A)** Knockdown of endogenous rHN in NIH3T3 cells using siRNAs. **(B–D)** Analysis of macroautophagy activity in NIH3T3 cells stably expressing the mCherry-GFP–LC3-II tandem reporter and exposed to siRNA against rHN or scrambled siRNA (Scr). Cells were maintained in serum-supplemented (+) or serum-depleted (–) media in the presence of 5 µM PQ (B) or the indicated increasing concentrations of PQ (D). **(B)** Representative images. Nuclei are stained with DAPI. Bar, 10 µm. **(C)** Quantification of the mean number of mCherry-positive puncta (autophagic vacuoles; AVs) per cell in cells maintained in presence or absence of serum. **(D)** Quantification of the number of autophagic vacuoles, autophagosomes (APGs; puncta positive for both fluorophores), and autolysosomes (AUTs; puncta positive for mCherry only) per cell in cells exposed to the indicated concentrations of PQ. All values are means + SEM and come from the quantification of nine different fields (~3,500 cells total per condition) in triplicate wells. No significant differences were detected between rHN(–) and control cells. **(E and F)** Effect of HNG on macroautophagy-dependent degradation. NIH3T3 cells were incubated with [^{3}H]leucin for 48 h, and after extensive washing, cells were placed in media supplemented with or without HNG and inhibitors of lysosomal proteolysis (NH_4Cl and leupeptin; N/L) or of 3-MA to block macroautophagy-dependent degradation. **(E)** Proteolysis rates at the indicated times in HNG-supplemented cells to show that most protein degradation of lysosomal origin is not sensitive to 3-MA. **(F)** Rates of lysosomal-dependent degradation (N/L sensitive) and degradation via macroautophagy (N/L and 3-MA sensitive) and CMA (N/L sensitive and 3-MA insensitive). All values are means + SEM of $n = 3$ with triplicate wells. Differences with untreated samples are significant for *, P < 0.01; **, P < 0.001.

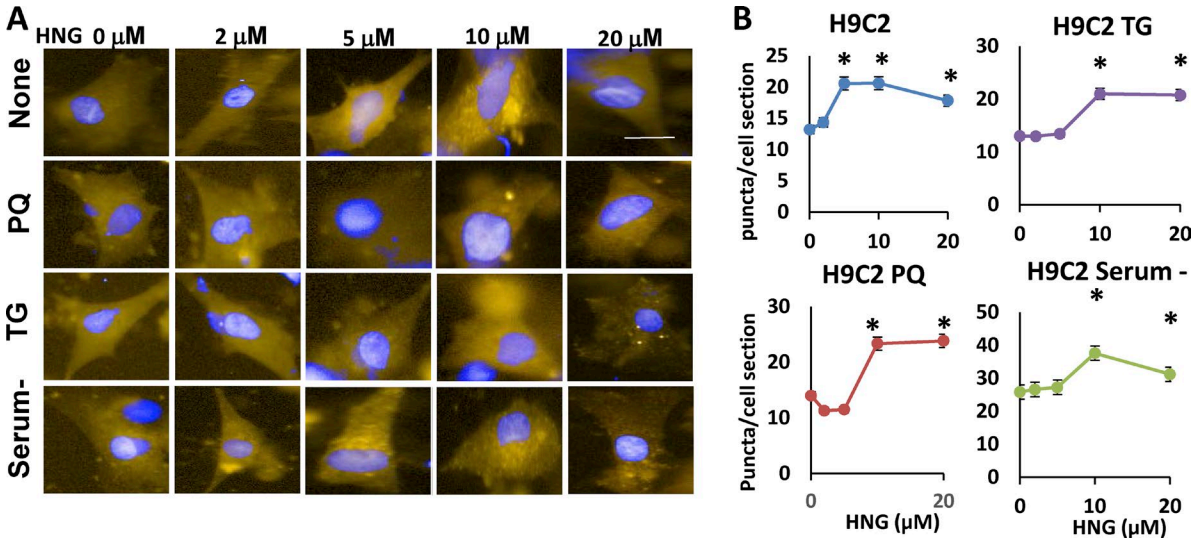

Figure S3. **HNG induces CMA in H9C2 cells. (A and B)** H9C2 cells stably transduced with lentivirus carrying a KFERQ-dendra reporter used to monitor CMA activity were incubated with indicated concentrations of HNG in the absence or presence of PQ, thapsigargin (TG), or serum deprivation (serum–). **(A)** Representative images (single cells) from confocal microscopy. Bar, 10 µm. **(B)** Quantification of changes in the mean number of puncta per cell section quantified with high-content microscopy in *n* = 1,500 cells per condition. Values are means + SEM (all SEM were <5% of the mean value). Differences are significant for *, P < 0.001.

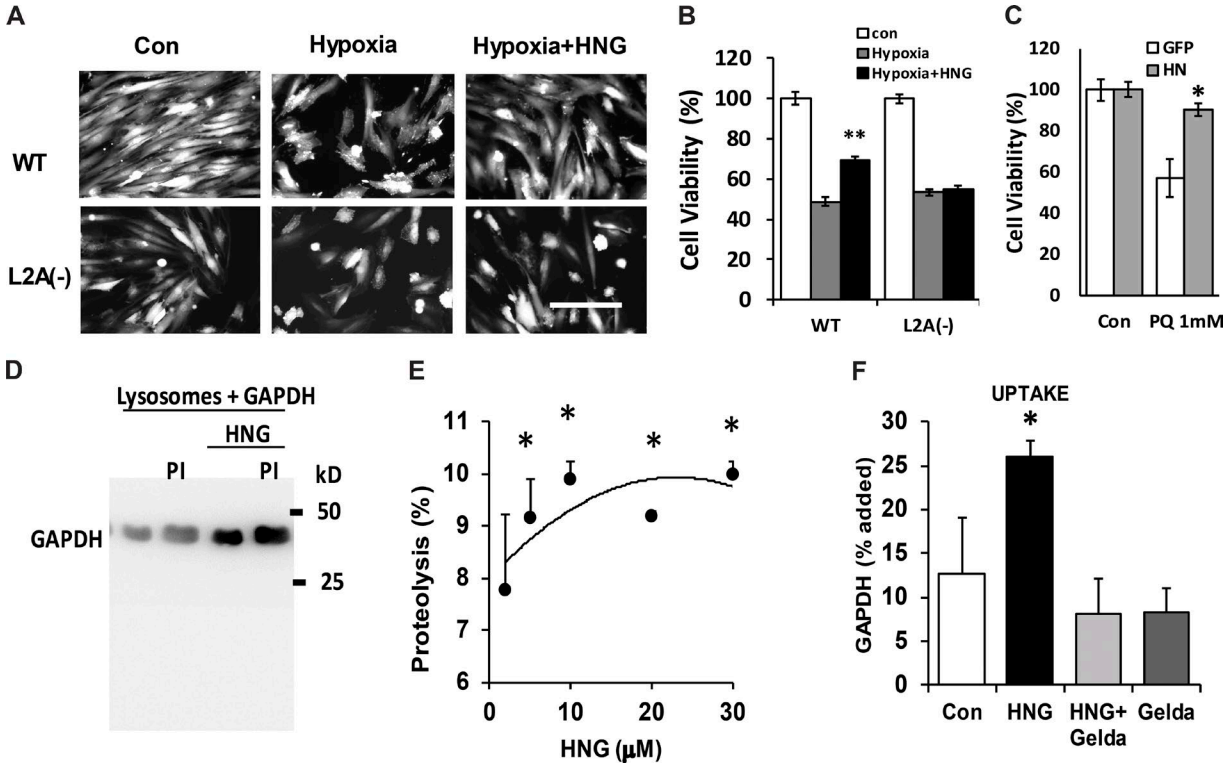

Figure S4. **Effects of HN on stress-induced cell survival and substrate uptake. (A and B)** Effects of HNG on survival under hypoxia is lost in L2A⁻ cells. Representative images (A) and quantification (B) for cell survival of WT or L2A⁻ H9C2 cells under hypoxia with or without HNG treatment. Bar, 50 µm. **(C)** Overexpression of GFP-HN protects H9C2 cell from 1 mM PQ-induced cell death. **(D and E)** Effects of HNG on uptake of CMA substrates. **(D)** Intact rat liver lysosomes untreated or preincubated with protease inhibitors (PIs) were incubated with GAPDH in the presence of 20 µM HNG. This experiment is as the one displayed in Fig. 5 D, except it was repeated to show untreated and protease inhibitor samples in the same blot. **(E)** Intact rat liver lysosomes were incubated with a cocktail of radiolabeled cytosolic proteins enriched in CMA substrate proteins in the presence of increasing concentrations of HN. Proteolysis was measured at the end of the incubation as the percentage of acid-precipitable radioactivity (protein) transformed into acid-soluble radioactivity (amino acids and peptides). *n* = 3. **(F)** Uptake of GAPDH by intact rat liver lysosomes incubated in the presence of HNG and/or geldanamycin calculated by immunoblots as the ones shown in Fig. 6 D. *n* = 5. Values are all means ± SEM. Differences with control are significant for *, P < 0.05; **, P < 0.01.

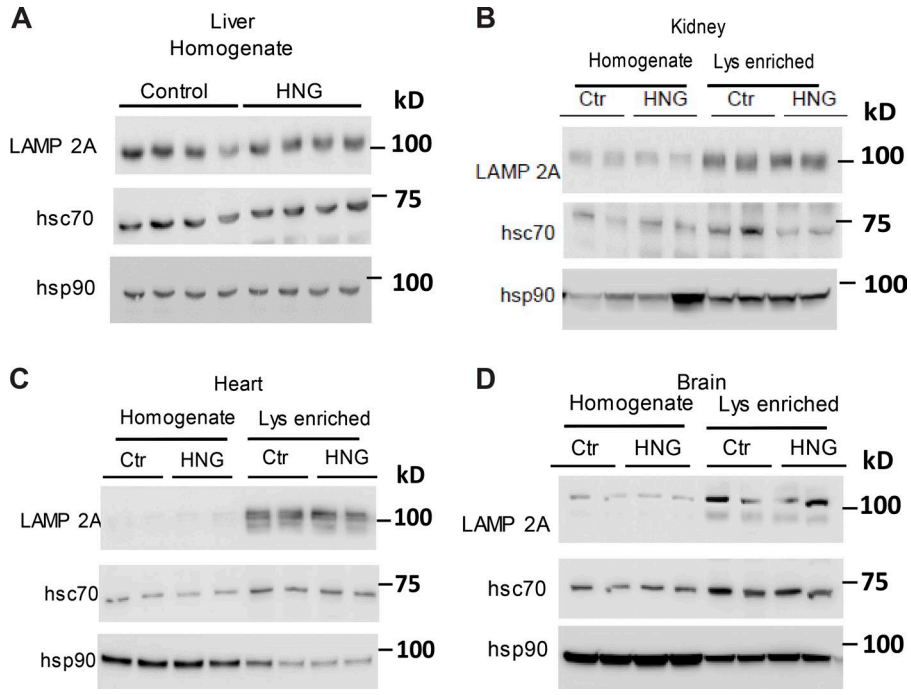

Figure S5. **Effect of in vivo treatment with HNG on CMA. (A)** Immunoblot for the indicated proteins in homogenates from livers of rats treated with 2 mg/kg of HNG immunoprecipitate for 4 h. **(B–D)** Immunoblots for the indicated proteins in homogenates and lysosome-enriched fractions isolated from kidneys (B), hearts (C), or brains (D) of animals treated as in A.