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Supplementary Materials for

Ammonia Derived from Glutaminolysis Is a Diffusible Regulator of Autophagy

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The PDF file includes:

Fig. S1. Measurement of autophagic flux.
Fig. S2. Characterization of CCM.
Fig. S3. CO_2, H_2O_2, NO, and lactate do not promote autophagy.
Fig. S4. Induction of autophagy is unaffected by intracellular Ca^{2+} buffering or inhibitors of mRNA transcription and translation.
Fig. S5. Atg5 or Ulk1 knockdown enhances TNF-α sensitivity.

Figure S1

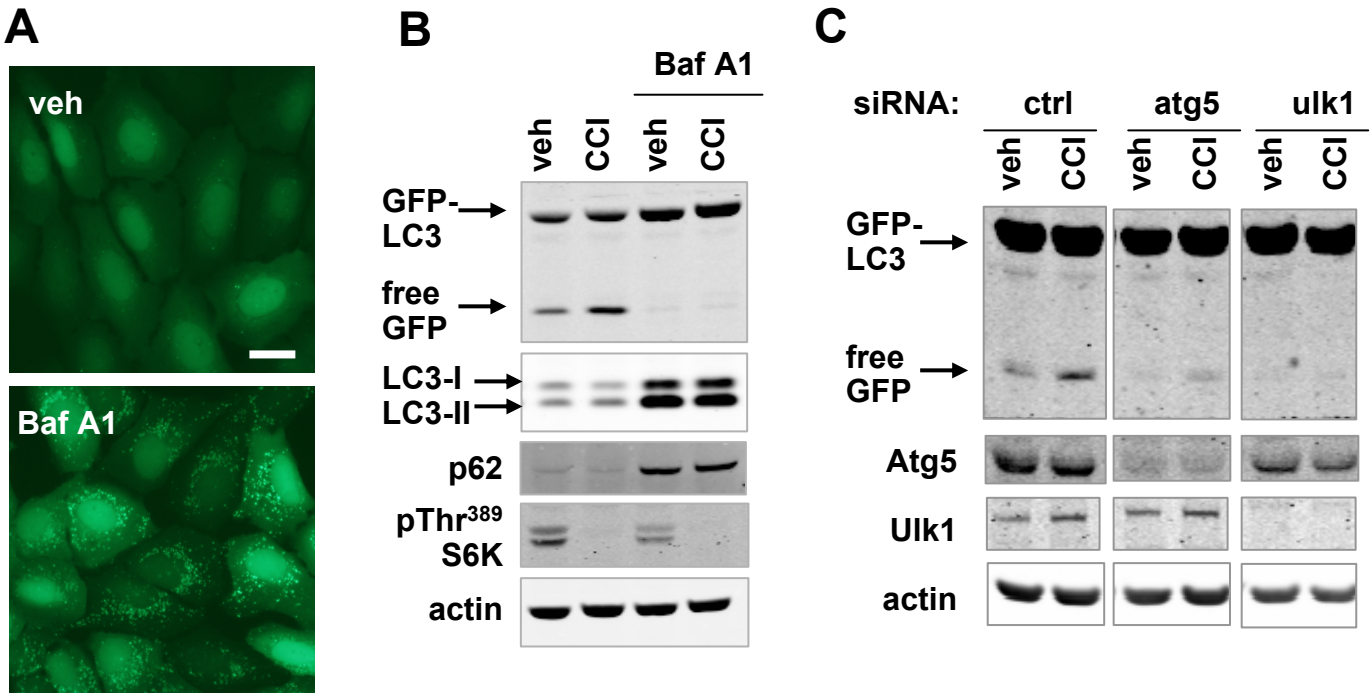

Figure S1. Measurement of autophagic flux. (**A**) U2OS GFP-LC3 cells were treated for 16 hours with 200 nM bafilomycin A1 and imaged live for GFP-LC3 puncta. Scale bar, 50 µm. (**B**) U2OS GFP-LC3 cells were treated for 24 hours with CCI-779 or vehicle alone (DMSO), in the presence or absence of 200 nM bafilomycin A1. Cells were lysed, and autophagic activity was determined by immunoblotting for free GFP, endogenous p62, and endogenous LC3 lipidation. (**C**) U2OS GFP-LC3 cells were transfected twice at 24 hour intervals with pooled siRNA duplexes targeting Atg5 or Ulk1. 72 hours after the first transfection, cells were treated for 24 hours with CCI-779, then lysed and immunoblotted for GFP. Panels depict non-adjacent lanes from the same blot.

Figure S2

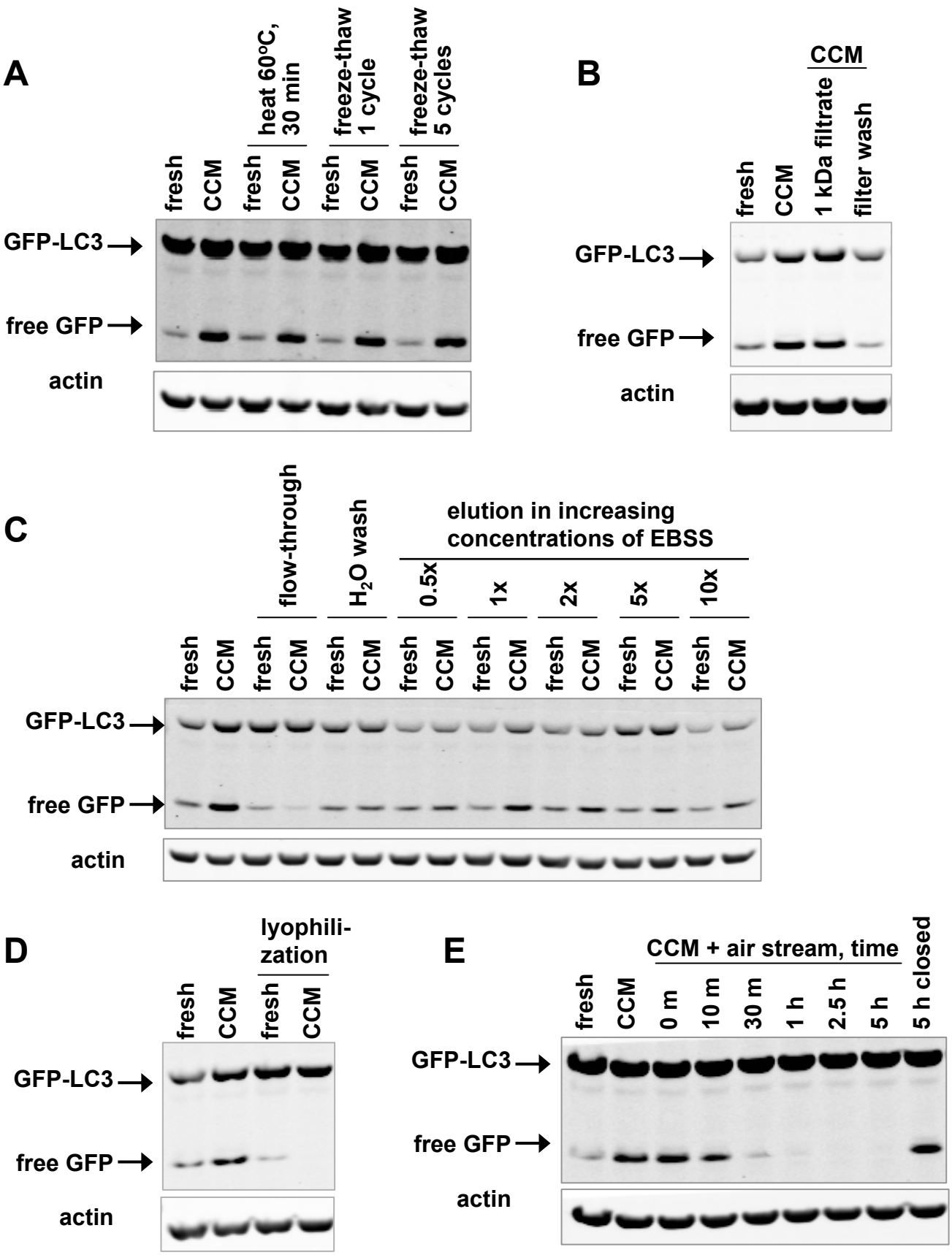
A
fresh
CCM
fresh
CCM
heat 60°C, 30 min
fresh
CCM
freeze-thaw 1 cycle
fresh
CCM
freeze-thaw 5 cycles
GFP-LC3
free GFP
actin
B
CCM
fresh
CCM
1 kDa filtrate
filter wash
GFP-LC3
free GFP
actin
C
flow-through
H2O wash
elution in increasing concentrations of EBSS
0.5x
1x
2x
5x
10x
fresh
CCM
GFP-LC3
free GFP
actin
D
lyophili-zation
fresh
CCM
GFP-LC3
free GFP
actin
E
CCM + air stream, time
fresh
CCM
0 m
10 m
30 m
1 h
2.5 h
5 h
5 h closed
GFP-LC3
free GFP
actin

Figure S2. Characterization of CCM. (**A**) Fresh or CCM was subjected to heating or multiple freeze-thaw cycles. The treated medium was applied to U2OS GFP-LC3 cells, and, after 24 hours, cells were lysed and analyzed for GFP-LC3 processing. (**B**) CCM was pressure-filtered through a regenerated cellulose membrane (molecular mass cutoff, 1 kDa) under a nitrogen atmosphere. After filtration, retained material was re-solubilized (wash), and both the filtrate and wash were applied to the U2OS GFP-LC3 reporter cell line. (**C**) Fresh medium or CCM was diluted with water to a final osmolarity of 25 mOsm, and applied to cation exchange spin columns. The columns were washed with water, and bound material was eluted with increasing concentrations of Earle's balanced salt solution (EBSS). (**D**) Fresh medium or CCM was frozen, and then lyophilized to dryness. The resulting residue was re-solubilized prior to treatment of cells. (**E**) CCM was exposed for the indicated times to an air stream at room temperature. The evaporated medium was restored to its original volume with distilled water, and was tested for activity with the U2OS GFP-LC3 reporter cell line.

Figure S3

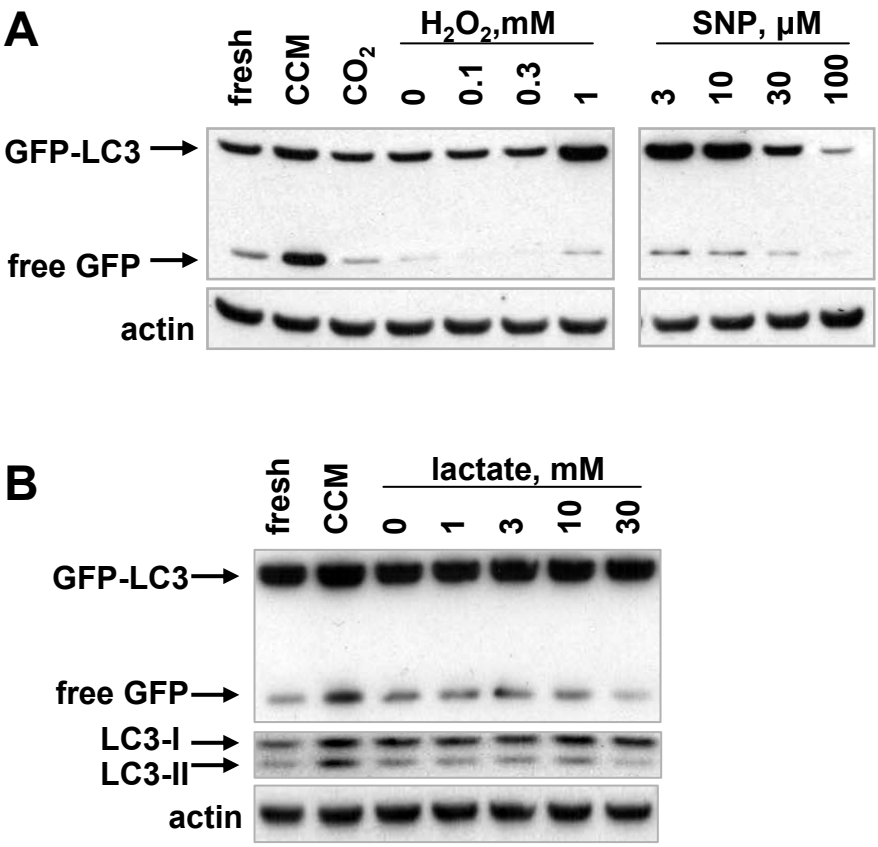

Figure S3. CO_2, H_2O_2, NO, and lactate do not promote autophagy. (**A**) U2OS GFP-LC3 cells were treated for 24 hours with serum-free CCM or fresh medium containing the indicated amounts of the nitric oxide donor sodium nitroprusside (SNP), hydrogen peroxide (H_2O_2), or fresh medium bubbled with CO_2 for 30 min. Cells were lysed and assayed for GFP-LC3 processing. Panels depict non-adjacent lanes from the same blot. (**B**) Cells were treated for 24 hours with CCM or fresh medium containing the indicated concentrations of lactic acid (after pH neutralization). Cells were lysed and assayed for GFP-LC3 processing and LC3 lipidation.

Figure S4

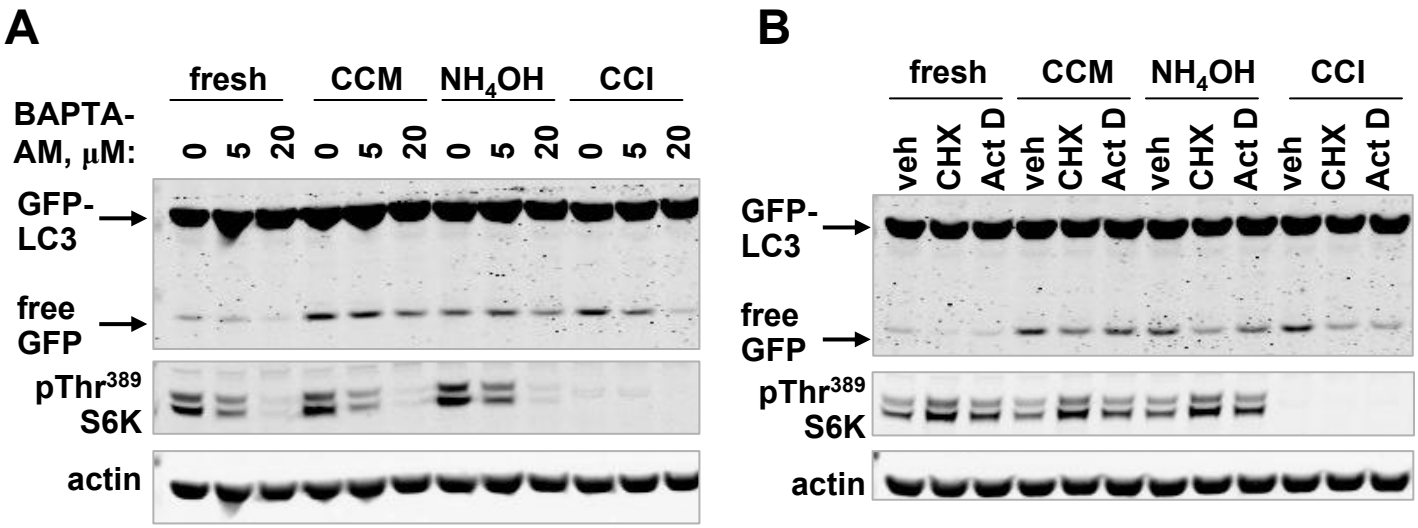

Figure S4. Induction of autophagy is unaffected by intracellular Ca^{2+} buffering or inhibitors of mRNA transcription and translation. (**A**) Cells were treated for 4 hours with CCM or fresh medium containing CCI-779 or 1 mM NH_4OH. BAPTA-AM (5 or 20 μM) was added for an additional 4 hours. Cells were lysed and assayed for GFP-LC3 processing. (**B**) Cells were treated with CCM or fresh medium containing CCI-779 or 1 mM NH_4OH. The indicated samples also contained 5 μg/ml cycloheximide (CHX) or 0.1 μg/ml actinomycin D (ActD). After 8 hours, cells were lysed and assayed for GFP-LC3 processing.

Figure S5

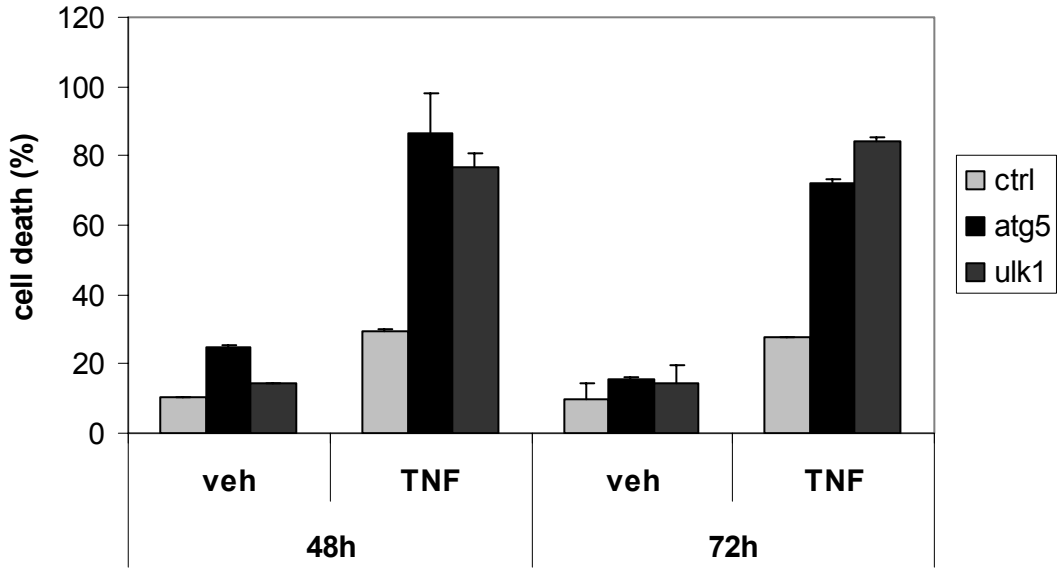

Figure S5. Atg5 or Ulk1 knockdown enhances TNF- α sensitivity. U2OS GFP-LC3 cells were transfected twice at 24 hour intervals with siRNA directed against Ulk1 or Atg5. The transfected cells were replated, and, 72 hours after the first transfection, the cells were treated for the indicated times with 100 ng/ml TNF-α. Cell viability was assayed by Guava Viacount. The data represent the mean ± CV from duplicate samples. Representative data from one of three independent experiments are shown.