Appendix for

MOAP-1-mediated dissociation of the p62/SQSTM1 bodies suppresses the p62-Keap1-Nrf2 signaling axis

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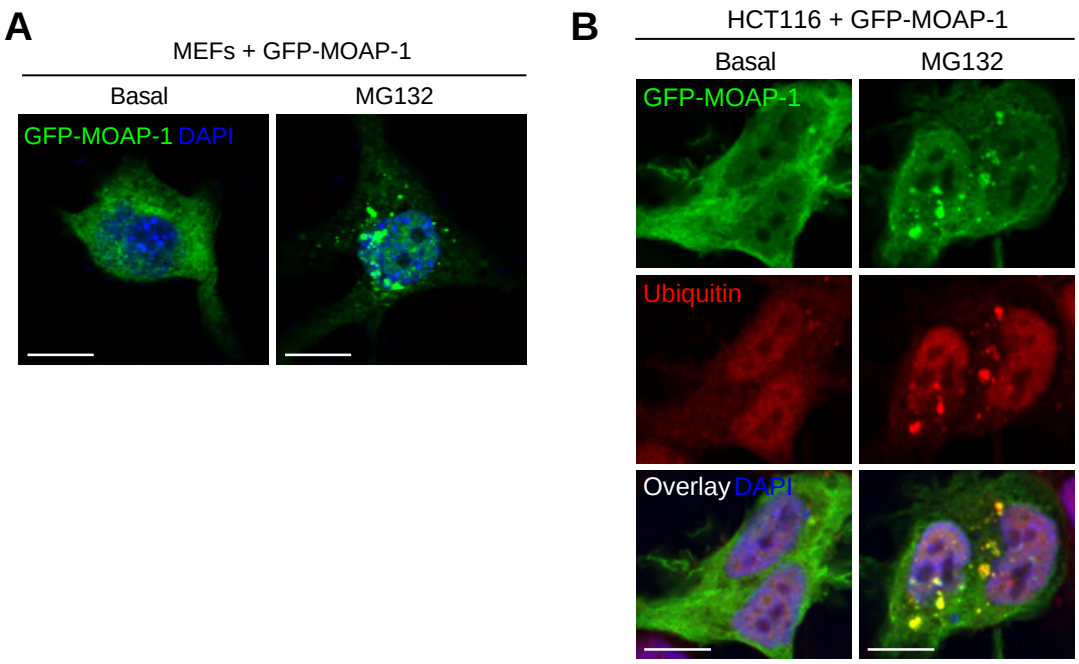
This appendix contains six supplemental figures (S1-S6)

Appendix Fig S1



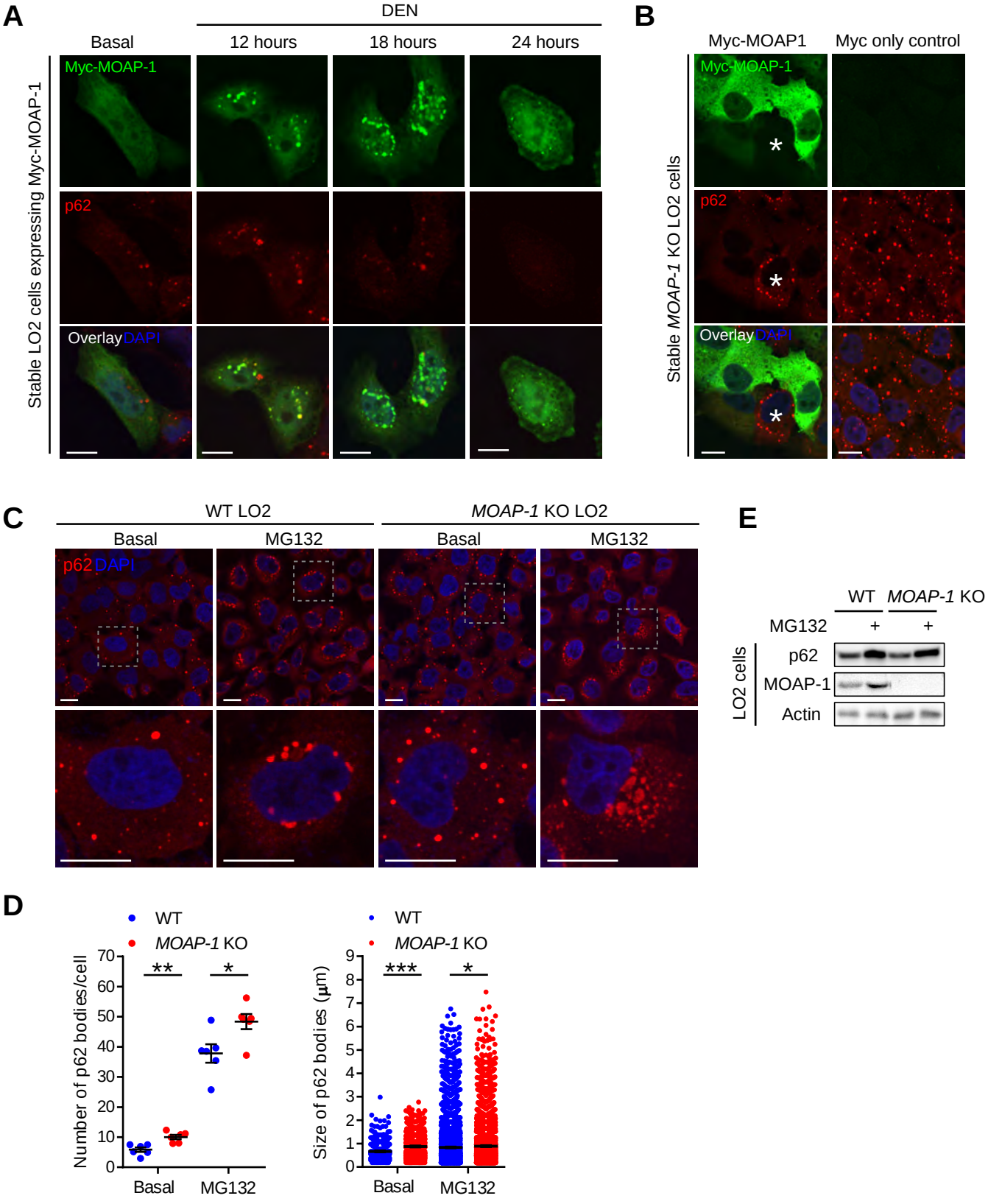
Appendix Figure S1 - MOAP-1 localizes to protein aggregates upon proteolytic stress.

A MOAP-1 localizes in aggregated patterns upon proteasome inhibition. MEFs were transfected with plasmid encoding GFP-MOAP-1. 14 hours later, the transfected cells were treated with MG132 (5 μM) for 8 hours and subjected to IF analysis with anti-GFP (in green) antibody.

B MOAP-1 co-localizes with ubiquitin in the cytoplasmic inclusion bodies upon proteasome inhibition. HCT116 cells were transfected with plasmid encoding GFP-MOAP-1. 14 hours later, cells were treated with the proteasome inhibitor MG132 (5 μM) for 8 hours and analyzed by IF with anti-GFP (in green) and anti-ubiquitin (in red) antibodies.

Data information: In (A and B) Nuclei were counterstained with DAPI (blue). Scale bar: 10 μm.

Appendix Fig S2



Appendix Figure S2 - MOAP-1 downregulates p62 bodies.

A MOAP-1 downregulates the levels of the diethylnitrosamine (DEN)-induced p62 bodies in a time-dependent manner. LO2 cells stably expressing Myc-MOAP-1 were treated with DEN (200 μM) for the indicated durations before being subjected to IF with the anti-Myc (in green) and anti-p62 (in red) antibodies.

B Re-expression of MOAP-1 in the MOAP-1 deficient LO2 cells efficiently downregulates the levels of p62 bodies. *MOAP-1* KO LO2 cells stably expressing Myc-MOAP-1 or Myc-tag only control were subjected to IF with the anti-p62 (in red) and anti-Myc (green) antibodies. The cell that does not express Myc-MOAP-1 was marked by asterisk as an internal control to document that the antibiotic selection process does not result in downregulation of the p62 bodies.

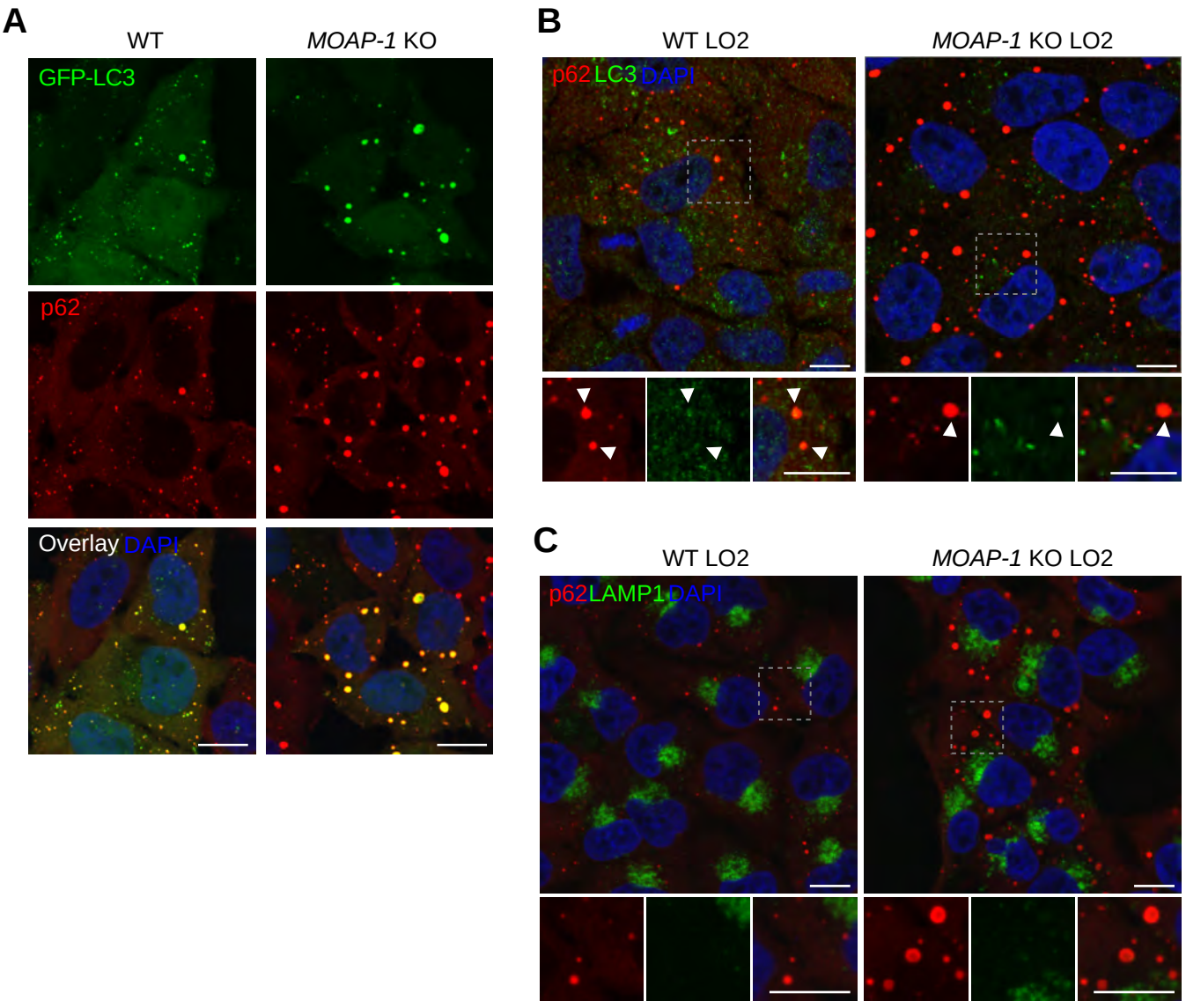
C Loss of MOAP-1 elevates levels of p62 bodies in the LO2 cells at the basal and MG132-treated states. WT and *MOAP-1* KO treated with or without MG132 (10 μM, 8 hours) were subjected to IF with anti-p62 antibody (in red). Insets in the upper panels are enlarged in the lower panels.

D Quantification of the p62 bodies in the WT and *MOAP-1* KO LO2 cells under basal and MG132-treated conditions. (Left panel) number of p62 bodies per cell. n = 6 independently plated samples. (Right panel) size of p62 bodies. n = 408 (WT, basal), 852 (*MOAP-1* KO, basal), 3409 (WT, MG132) and 3295 bodies (*MOAP-1* KO, MG132) from three independently plated samples. Error bar represents S.E.M. *P<0.05, **P<0.01, ***P<0.001, student's *t*-test.

E MOAP-1 deficiency does not affect total protein levels of p62 in the LO2 cells. Western blotting analysis of p62 and MOAP-1 protein levels in (C). Actin as loading control.

Data information: In (A, B, C) Nuclei were counterstained with DAPI (blue). Scale bar: 10 μm.

Appendix Fig S3



Appendix Figure S3 – Loss of MOAP-1 does not perturb localization of p62 bodies to auto-lysosomal structures.

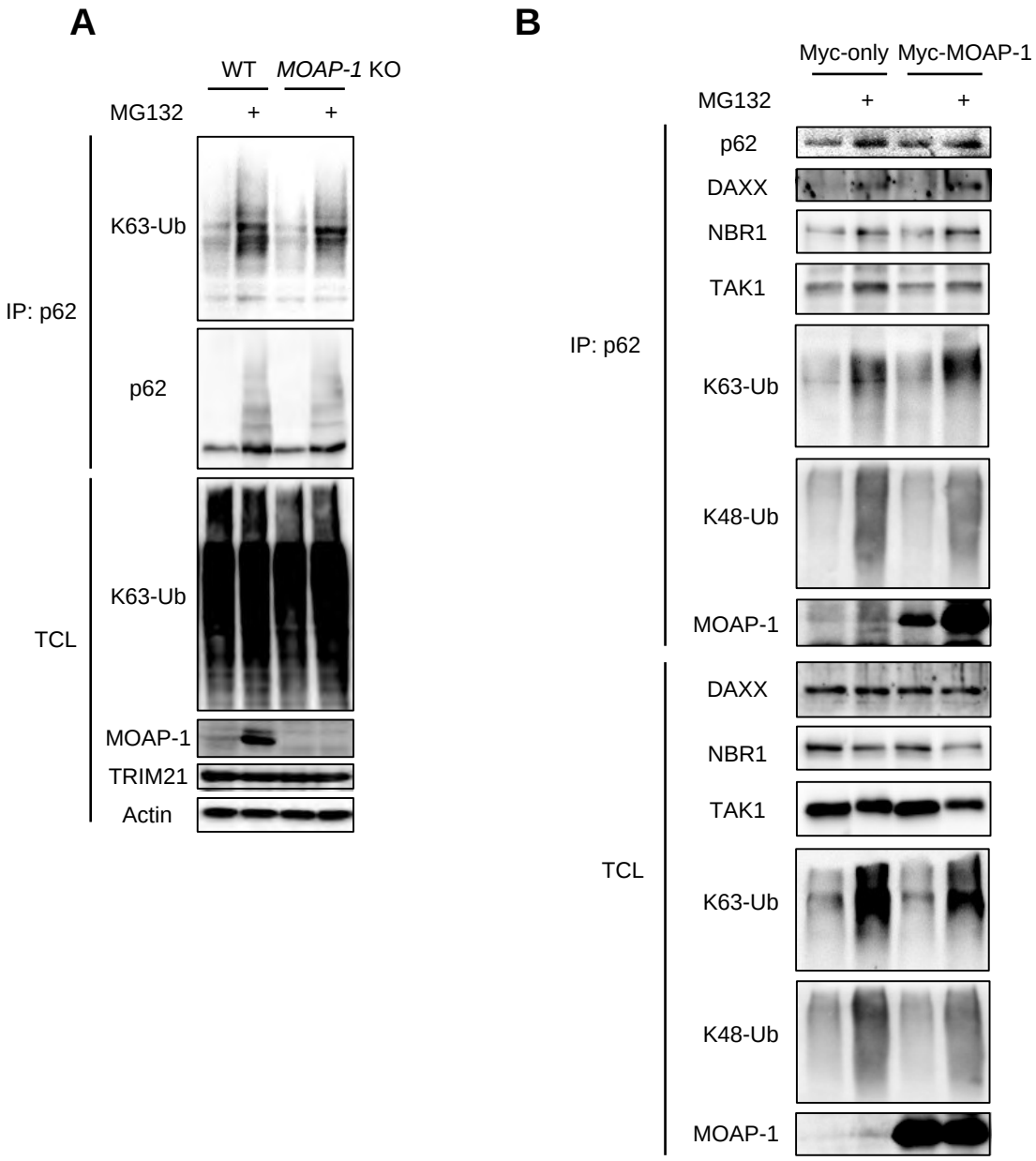
A Loss of MOAP-1 does not affect recruitment of GFP-LC3 to p62 bodies. WT and *MOAP-1* KO LO2 cells were transfected with plasmid encoding GFP-LC3. 24 hours later, the transfected cells were subjected to IF with the anti-p62 (in red) and anti-GFP (green) antibodies.

B Endogenous LC3 poorly colocalizes with p62 bodies in both WT and *MOAP-1* KO LO2 cells. WT and *MOAP-1* KO LO2 cells were subjected to IF with the anti-p62 (in red) and anti-LC3 (green) antibodies. Arrowheads indicate the p62 bodies with poor localization of endogenous LC3 protein.

C p62 bodies are not in close proximity with LAMP1, a lysosome-resident protein, in both WT and *MOAP-1* KO LO2 cells. WT and *MOAP-1* KO LO2 cells were subjected to IF with anti-p62 (in red) and anti-LAMP1 (green) antibodies.

Data information: In (A-C) Nuclei were counterstained with DAPI (blue). Scale bar: 10 μm.

Appendix Fig S4

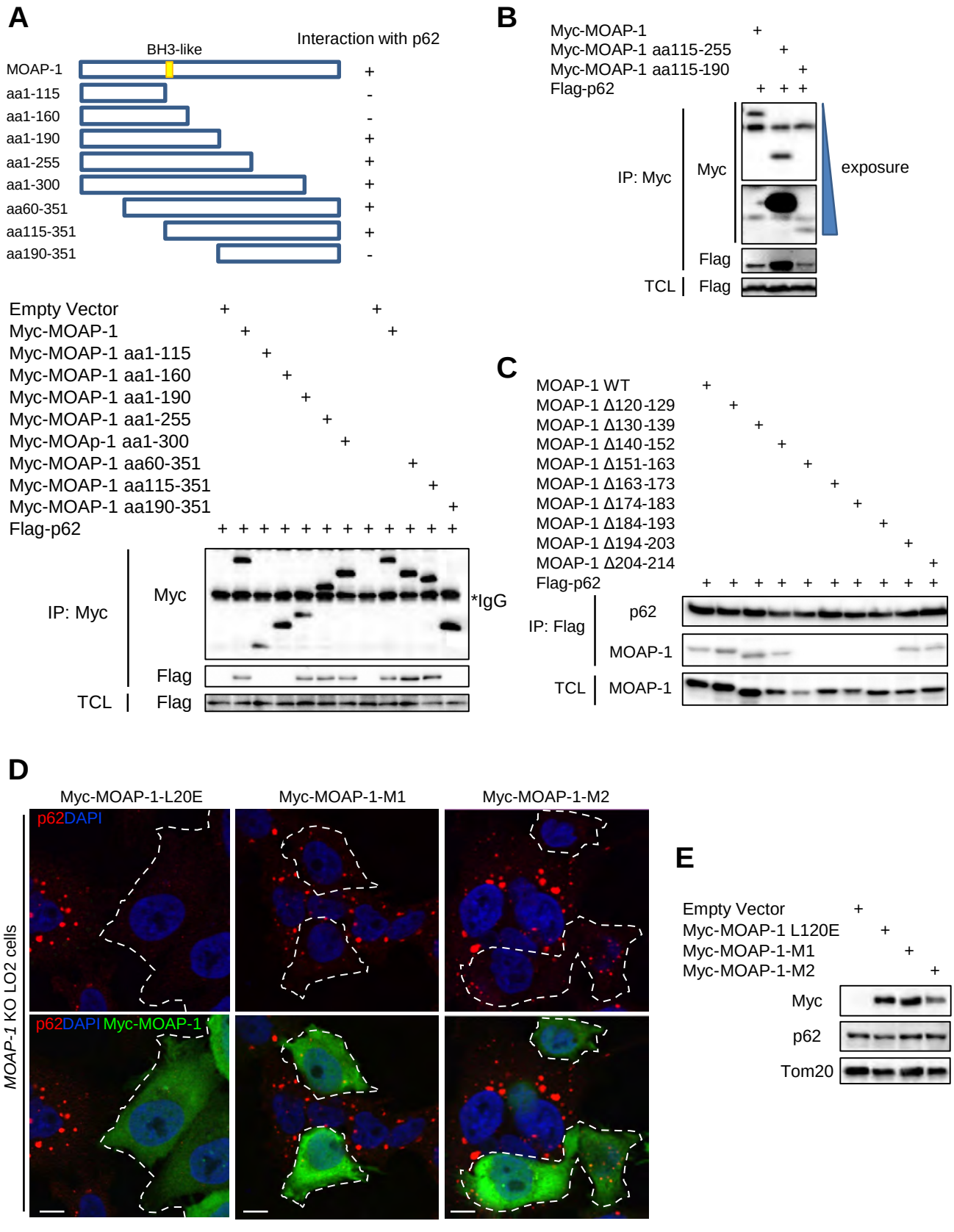


Appendix Figure S4 - MOAP-1 does not regulate the K63-linked poly-ubiquitylation of p62 nor its interaction with the known regulators of p62 bodies formation.

A MOAP-1 deficiency does not alter K63-linked poly-ubiquitylation of p62 under basal and MG132-treated conditions. WT and *MOAP-1* KO LO2 cells were treated MG132 (10 μM) for 12 hours and subjected to ubiquitylation assay under denaturing conditions. Immunoprecipitation (IP) of p62 was analyzed by Western blot analysis using anti-K63-linkage specific ubiquitin antibody.

B Overexpression of MOAP-1 does not affect the binding of p62 to the known regulators of p62 bodies formation including DAXX, NBR1, TAK1 and poly-ubiquitin chains. HepG2 cells stably overexpressing Myc-MOAP-1 or Myc-only control were treated with or without MG132 (10 μM) for 12 hours, before being subjected to co-IP analysis.

Appendix Fig S5



Appendix Figure S5 - The region spanning aa150-190 of MOAP-1 is critical for mediating its interaction with p62.

A Mapping of the region in MOAP-1 required for mediating its interaction with p62. (Upper panel) Schematic representation of a series of N- or C-terminally truncated mutants of MOAP-1 tested for their ability to interact with p62. Among the truncated mutants, aa1-190, aa1-255, aa1-300, aa60-351 and aa115-351 interacted with p62. (Lower panel) LO2 cells were transfected with the expression vectors encoding Myc-tagged MOAP-1 or its mutant and Flag-tagged p62, and cells were subjected to immunoprecipitation (IP) with anti-Myc antibody 24 hours later. TCL, total cell lysates.

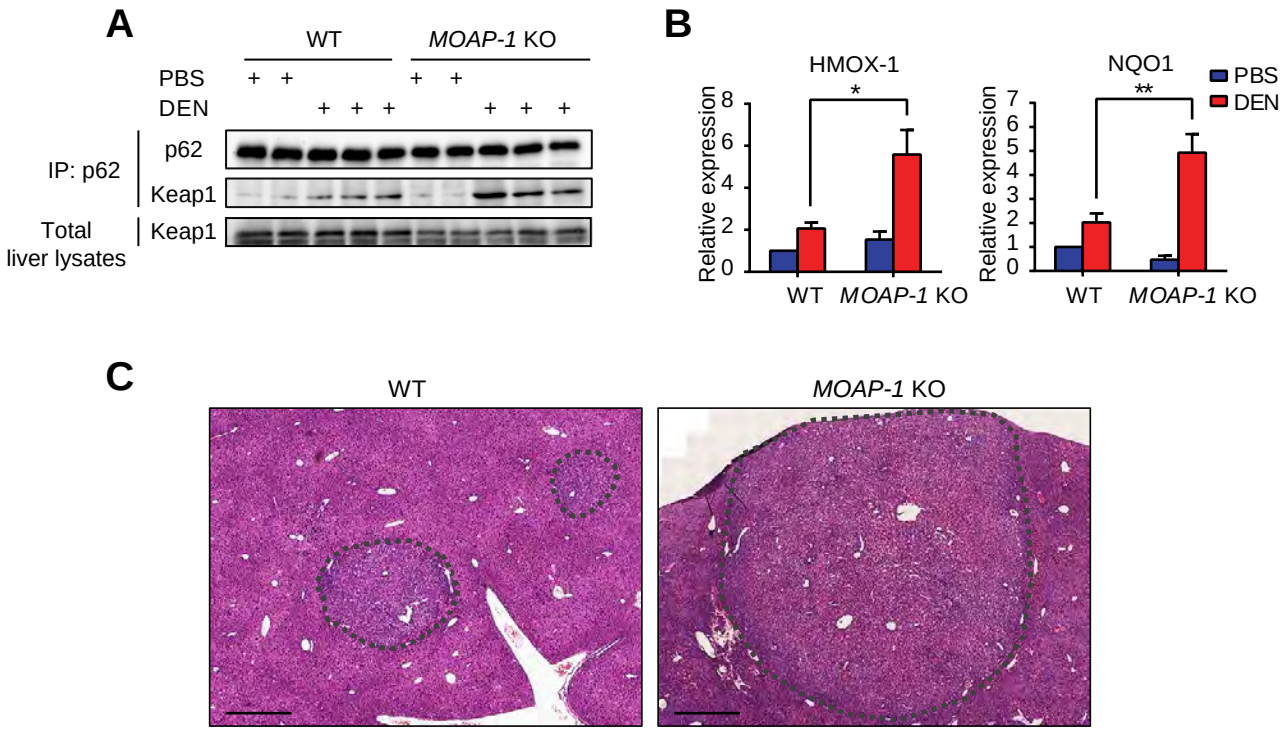
B The amino acid (aa) 115-190 region of MOAP-1 is sufficient for mediating interaction with p62. LO2 cells were transfected with Myc-MOAP-1 or fragments spanning aa115-255 or aa115-190 and Flag-p62, and then subjected to IP with anti-Myc antibody.

C The aa150-190 region of MOAP-1 is required for mediating interaction with p62. A series of deletion mutants with 10-16aa residues of MOAP-1 removed at a time in the aa120-214 region was systematically tested for their ability to associate with p62 in LO2 cells by IP analysis.

D Re-expression of MOAP-1-L120E, MOAP-1-M1 and MOAP-1-M2 downregulates levels of p62 bodies in MOAP-1 deficient cells. The MOAP-1 KO LO2 cells were transfected with plasmid encoding Myc-MOAP-1-L120E, Myc-MOAP-1-M1 or Myc-MOAP-1-M2 for 24 hours and subjected to IF with anti-p62 (in red) and anti-Myc (green) antibodies. Nuclei were stained with DAPI (blue). Scale bar: 5 μm.

E Western blotting of *MOAP-1* KO LO2 cells transfected with Myc-MOAP-1 or Myc-MOAP-1-M3 mutant as described in (D). Tom20 as loading control.

Appendix Fig S6



Appendix Figure S6 - Loss of MOAP-1 renders hyperactivation of Nrf2 and elevated tumorigenesis in the liver upon exposure to DEN.

A MOAP-1 deficiency promotes p62/Keap1 interaction in liver subjected to acute diethylnitrosamine (DEN) treatment. co-immunoprecipitation analysis of the liver lysates prepared from the WT and *MOAP-1* KO mice 48 hours post-injection with 100 μg/g body weight of DEN.

B Upregulation of mRNA levels of HMOX-1 and NQO1 in the MOAP-1 deficient liver upon acute DEN treatment. Livers dissected from the mice injected with PBS or DEN as described in (A) were harvested for RT-PCR analysis. *n* = 3 WT and 3 *MOAP-1* KO mice injected with PBS or DEN each. Error bars represent S.E.M. *P<0.05, **P<0.01, Student's *t*-test.

C Representative images of Hematoxylin and Eosin (H&E)-stained sections of liver tumors from 8.5-month-old male WT and MOAP-1 KO mice injected with a single dose of DEN (25 μg/g body weight) at 15-day-old. Scale bar: 600 μm.