Figure S1.

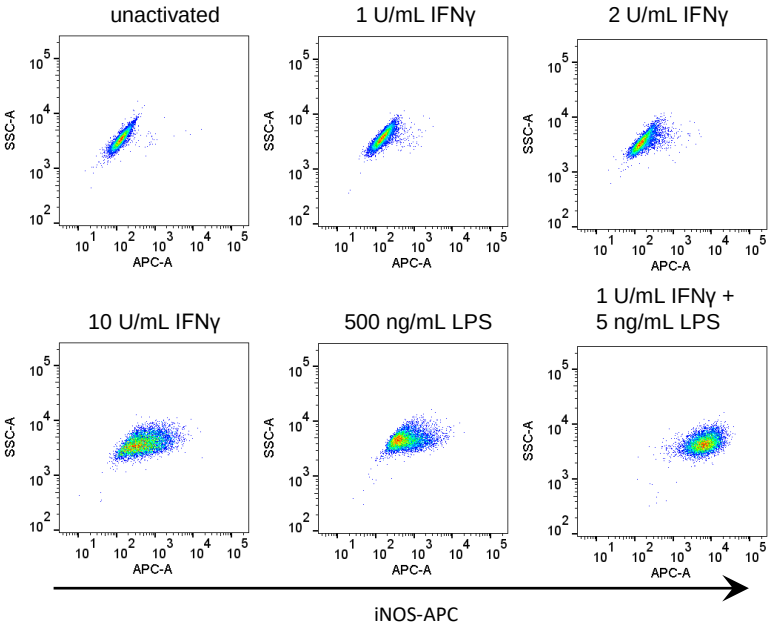

unactivated
1 U/mL IFNγ
2 U/mL IFNγ
10 U/mL IFNγ
500 ng/mL LPS
1 U/mL IFNγ +
5 ng/mL LPS
SSC-A
APC-A
iNOS-APC

Figure S2.

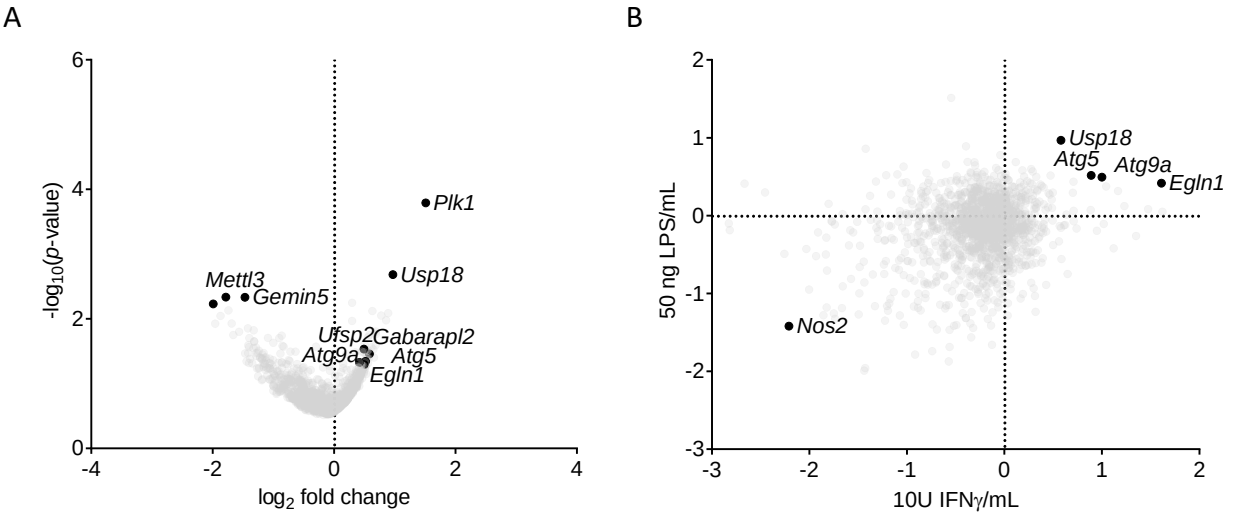

A
B
-log10(p-value)
log2 fold change
Plk1
Usp18
Mettl3
Gemin5
Ufsp2
Gabarapl2
Atg9a
Atg5
Egln1
50 ng LPS/mL
10U IFNγ/mL
Usp18
Atg5
Atg9a
Egln1
Nos2

Figure S3.

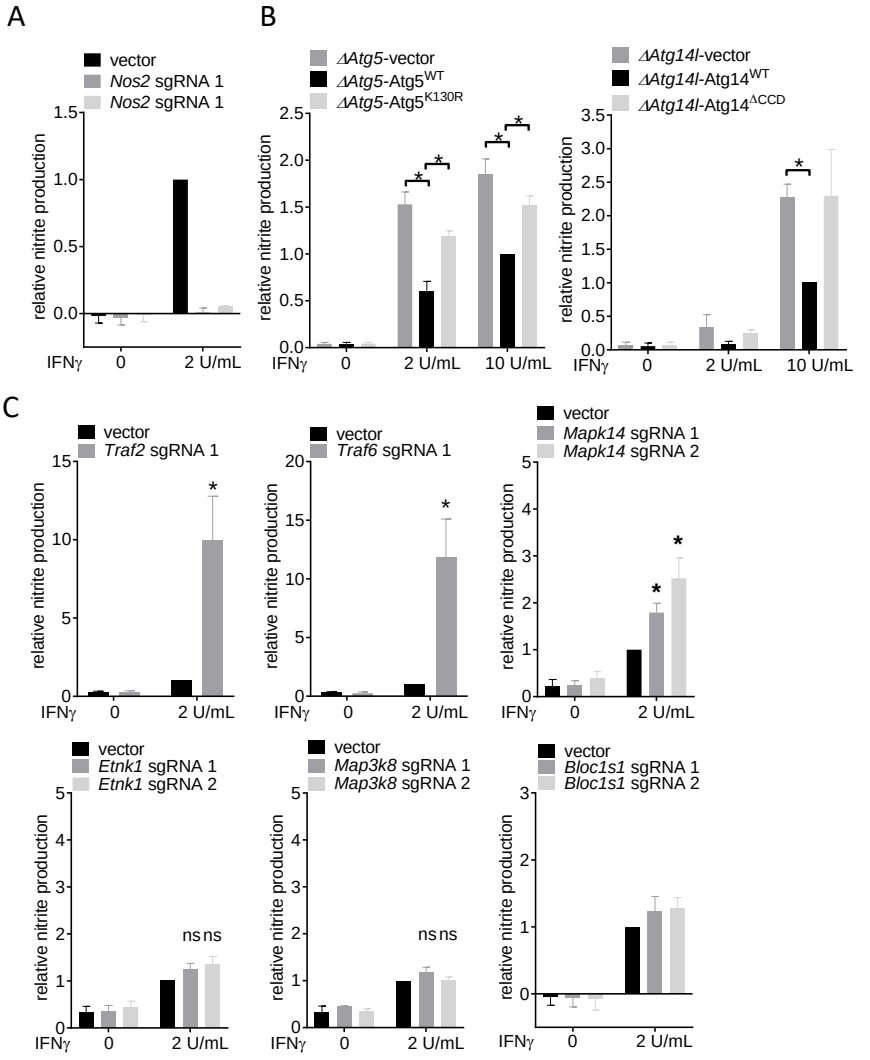
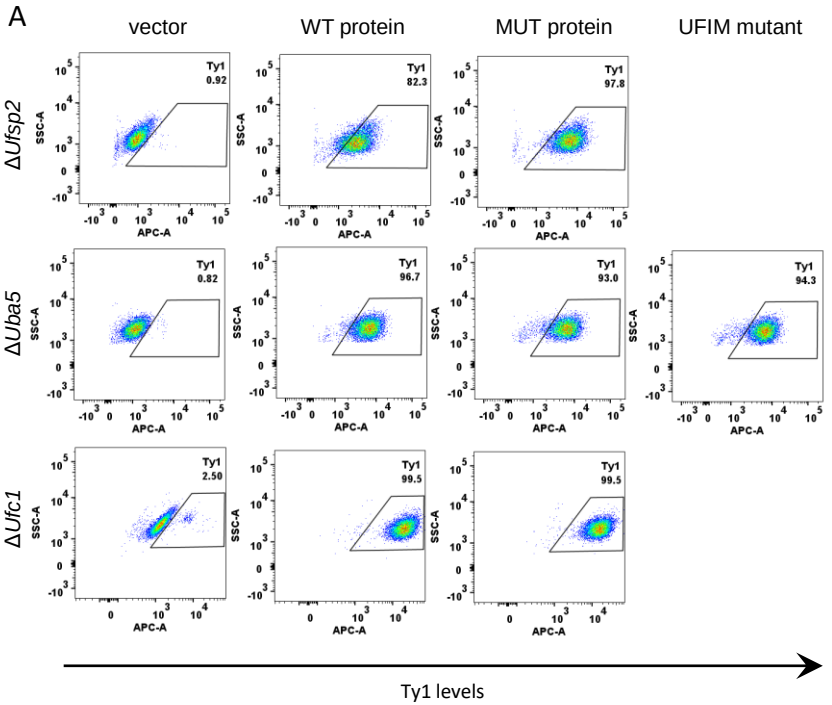

A
vector
Nos2 sgRNA 1
Nos2 sgRNA 1
relative nitrite production
IFNγ 0 2 U/mL
B
ΔAtg5-vector
ΔAtg5-Atg5WT
ΔAtg5-Atg5K130R
ΔAtg14l-vector
ΔAtg14l-Atg14WT
ΔAtg14l-Atg14ΔCCD
IFNγ 0 2 U/mL 10 U/mL
C
vector
Traf2 sgRNA 1
Traf6 sgRNA 1
Mapk14 sgRNA 1
Mapk14 sgRNA 2
Etnk1 sgRNA 1
Etnk1 sgRNA 2
Map3k8 sgRNA 1
Map3k8 sgRNA 2
Bloc1s1 sgRNA 1
Bloc1s1 sgRNA 2
ns ns
IFNγ 0 2 U/mL

Figure S4.


A
vector
WT protein
MUT protein
UFIM mutant
ΔUfsp2
ΔUba5
ΔUfc1
Ty1
0.92
82.3
97.8
0.82
96.7
93.0
94.3
2.50
99.5
99.5
SSC-A
APC-A
Ty1 levels

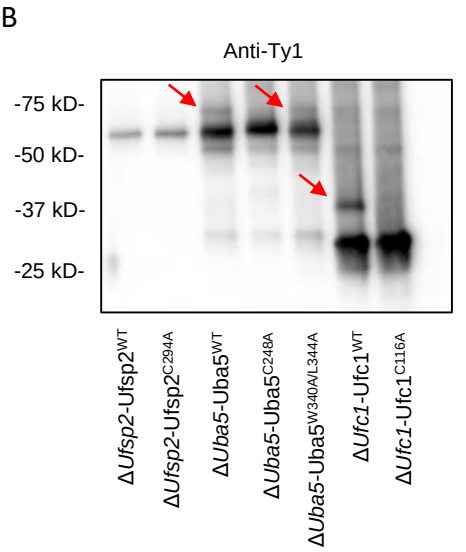

B
Anti-Ty1
-75 kD-
-50 kD-
-37 kD-
-25 kD-
ΔUfsp2-Ufsp2WT
ΔUfsp2-Ufsp2C294A
ΔUba5-Uba5WT
ΔUba5-Uba5C248A
ΔUba5-Uba5W340A/L344A
ΔUfc1-Ufc1WT
ΔUfc1-Ufc1C116A

Figure S5

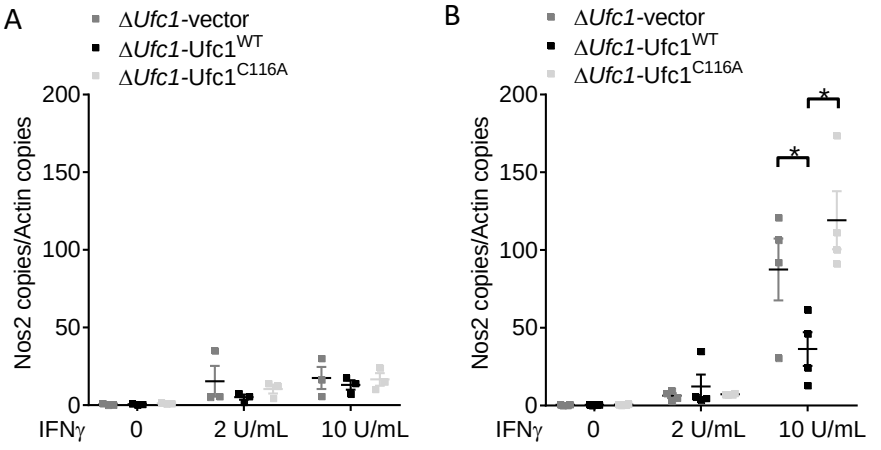
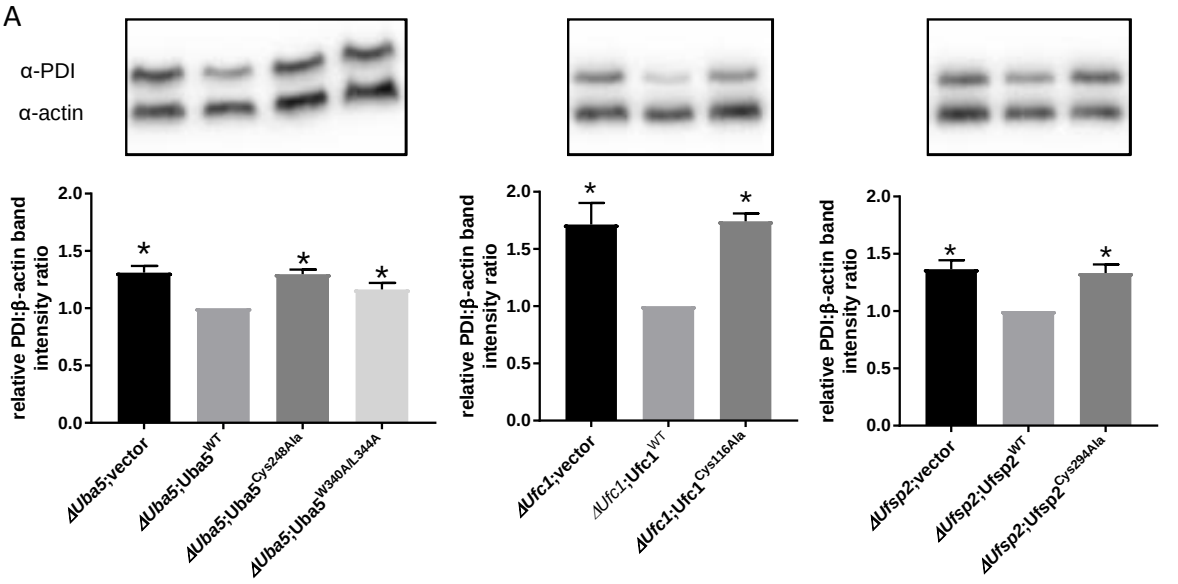
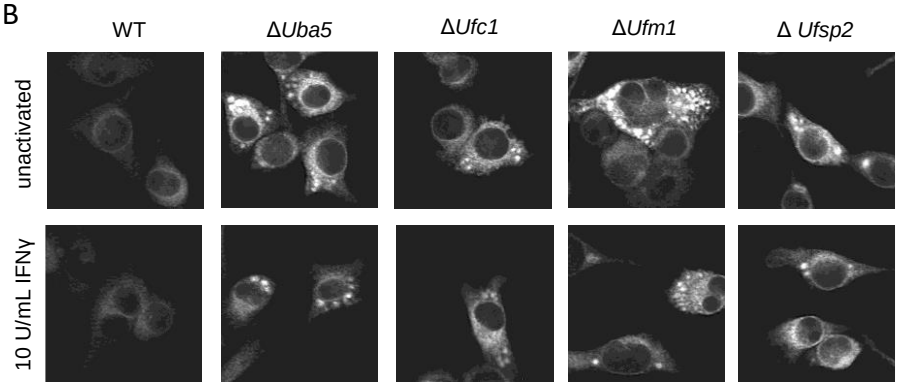

A
ΔUfc1-vector
ΔUfc1-Ufc1WT
ΔUfc1-Ufc1C116A
Nos2 copies/Actin copies
200
150
100
50
0
IFNγ
0
2 U/mL
10 U/mL
B
ΔUfc1-vector
ΔUfc1-Ufc1WT
ΔUfc1-Ufc1C116A
Nos2 copies/Actin copies
200
150
100
50
0
*
*
IFNγ
0
2 U/mL
10 U/mL

Figure S6.


A
α-PDI
α-actin
relative PDI:β-actin band intensity ratio
2.0
1.5
1.0
0.5
0.0
*
ΔUba5;vector
ΔUba5;Uba5WT
ΔUba5;Uba5Cys248Ala
ΔUba5;Uba5W340A/L344A
ΔUfc1;vector
ΔUfc1;Ufc1WT
ΔUfc1;Ufc1Cys116Ala
ΔUfsp2;vector
ΔUfsp2;Ufsp2WT
ΔUfsp2;Ufsp2Cys294Ala


B
WT
ΔUba5
ΔUfc1
ΔUfm1
Δ Ufsp2
unactivated
10 U/mL IFNγ

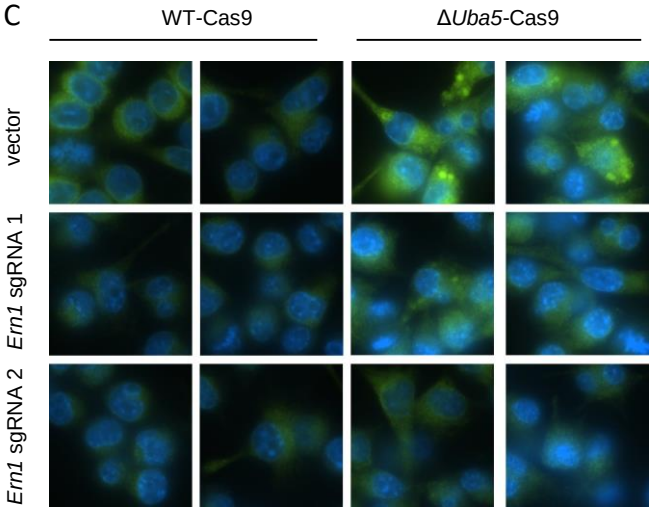
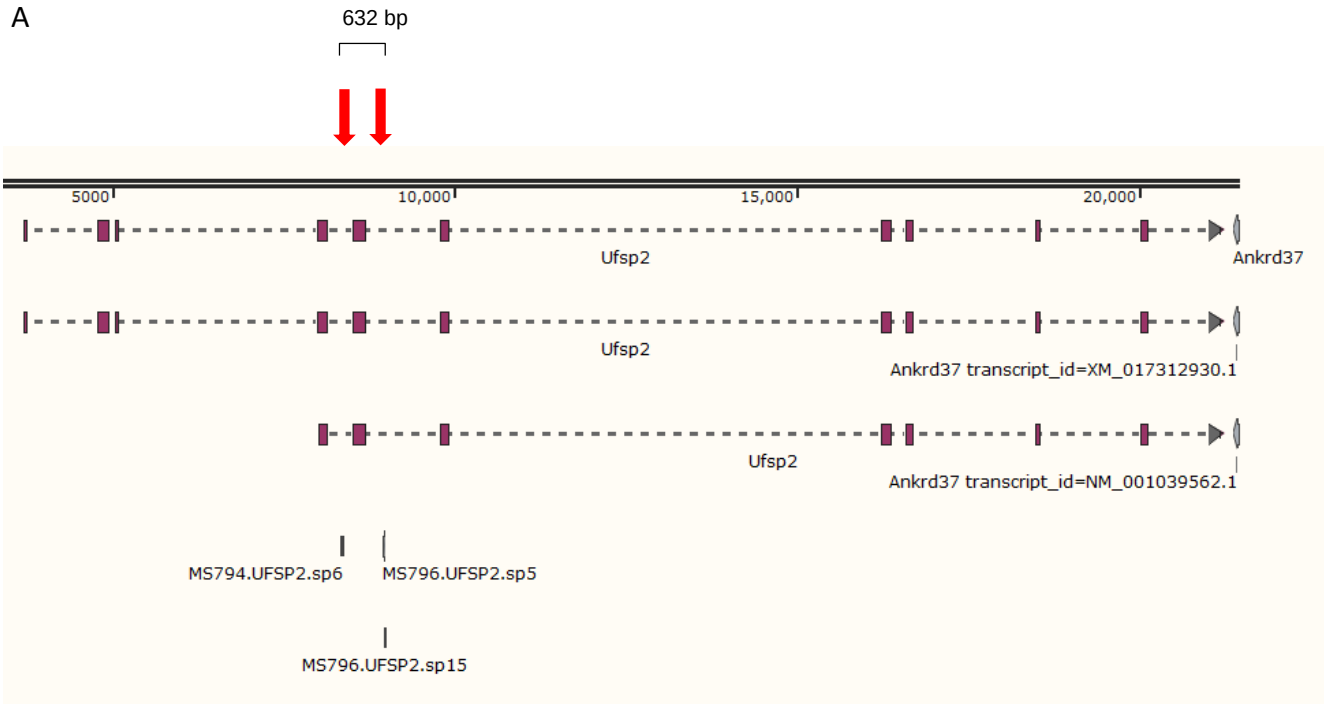

C
WT-Cas9
ΔUba5-Cas9
vector
Ern1 sgRNA 1
Ern1 sgRNA 2

Figure S7.


A
632 bp
5000
10,000
15,000
20,000
Ufsp2
Ankrd37
Ufsp2
Ankrd37 transcript_id=XM_017312930.1
Ufsp2
Ankrd37 transcript_id=NM_001039562.1
MS794.UFSP2.sp6
MS796.UFSP2.sp5
MS796.UFSP2.sp15

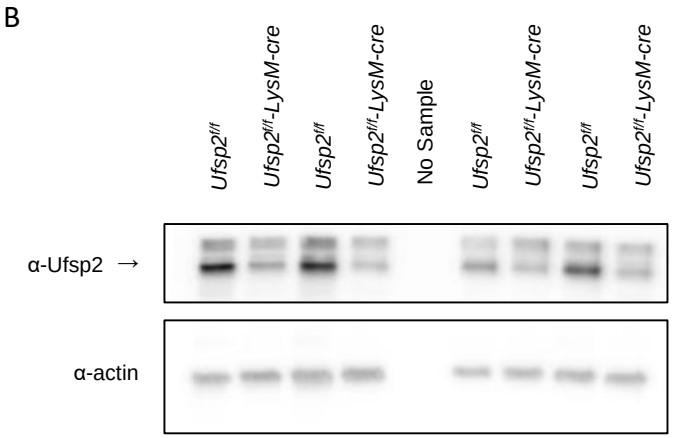

B
Ufsp2f/f
Ufsp2f/f-LysM-cre
Ufsp2f/f
Ufsp2f/f-LysM-cre
No Sample
Ufsp2f/f
Ufsp2f/f-LysM-cre
Ufsp2f/f
Ufsp2f/f-LysM-cre
α-Ufsp2 →
α-actin

Figure S1. IFNγ- and LPS-dependent iNOS expression in BV2 cells. Related to Figure 1. (A) Representative flow cytometry plots of two independent experiments showing iNOS expression in WT BV2 cells stimulated for 16 hrs with increasing concentrations of IFNγ and/or LPS.

Figure S2. Results of subpool library cells treated with LPS. Related to Figure 1. (A) Volcano plot of genes enriched in the top 10 % of iNOS2-high library BV2-Cas9 cells treated with 50 ng LPS/mL. The average log2 fold change (LFC) of all sgRNAs for each gene is plotted against the –log10(p-value) for each gene. Select genes of interest are labelled. (B) Comparison of gene-level average LFC from the LPS and IFNγ (10 U/mL) subpool screen results.

Figure S3. Validation of additional CRISPR screen hits. Related to Figure 2. (A) Average NO production in cells expressing sgRNAs targeting *Nos2* (2 independent experiments). (B) Average NO production in Δ*Atg5* and Δ*Atg14l* cells expressing empty vector, WT protein or known functional mutants. Data are represented as mean ± SEM of 3 independent experiments relative to Δ*Atg5*/Δ*Atg14l* expressing WT protein stimulated with 10 U IFNγ/mL. (C) Average NO production in cells expressing sgRNAs targeting additional CRISPR screen hits. Data are represented as mean ± SEM of 4-6 independent experiments normalized to IFNγ-activated WT BV2:Cas9 cells transduced with lentivirus carrying empty vector. (2 independent experiments for *Bloc1s1*) * $P < 0.05$ determined by ANOVA with Tukey's multiple comparison test. ns = not statistically significant.

Figure S4. Expression of complemented proteins in Δ*Ufsp2*/Δ*Uba5*/Δ*Ufc1* cells. Related to Figure 3. (A) Flow cytometry plots showing Ty1 levels (detected using anti-Ty1 and AF647-conjugated secondary) in Δ*Ufsp2*/Δ*Uba5*/Δ*Ufc1* cells and cells reconstituted with WT protein or functionally inactive mutant protein or the UFIM mutant for *Uba5*. (B) Western immunoblot for Ty1-tagged WT protein or functionally inactive mutant protein or the UFIM mutant for *Uba5* in Δ*Ufsp2*/Δ*Uba5*/Δ*Ufc1* cells. Red arrows indicate presence of an additional band which may represent the intermediate thioester between Ty1-tagged Uba5/Ufc1 and Ufm1.

Figure S5. Ufc1 decreases the steady state levels of mRNAs for *Nos2* in response to IFNγ activation. Related to Figure 4. (A-B) RT–qPCR measurements of *Nos2* transcripts normalized to Actin transcripts in cells activated with IFNγ for (A) 8 hr and (B) 24 hr. Data are represented as mean ± SEM. * $P < 0.05$ determined by ANOVA with Tukey's multiple comparison test.

Figure S6. UFMylation regulates ER membrane expansion. Related to Figure 5 and Figure 6. (A) Western immunoblot representative images and measurements of the PDI to Actin band intensity ratio in Δ*Ufsp2*/Δ*Uba5*/Δ*Ufc1* cells and cells reconstituted with WT protein or functionally inactive mutant protein or the UFIM mutant for *Uba5*. Average PDI:actin band intensity ratio pooled from 4-5 independent experiments are shown as mean ± SEM relative to knockout cells expressing WT protein. Data are represented as mean ± SEM. * $P < 0.05$ determined by ANOVA with Tukey's multiple comparison test. (B) Immunofluorescence of the ER protein PDI in WT and Δ*Uba5*/Δ*Ufc1*/Δ*Ufm1*/Δ*Ufsp2* cells. (C) Representative immunofluorescence images of PDI in WT-Cas9 and Δ*Uba5*-Cas9 expressing control vector or sgRNAs targeting *Ern1*.

Figure S7. Generation of *Ufsp2$^{f/f}$-LysM-cre* mice. Related to Figure 7. (A) *Ufsp2* floxed allele strategy. (B) Western immunoblot for Ufsp2 and beta-actin in the adherent fraction of peritoneal exudate cells from the indicated mice (4 independent mice from each genotype).