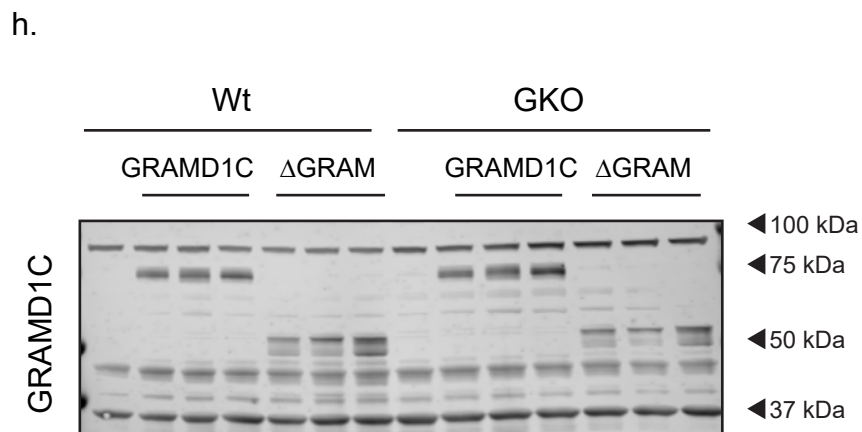
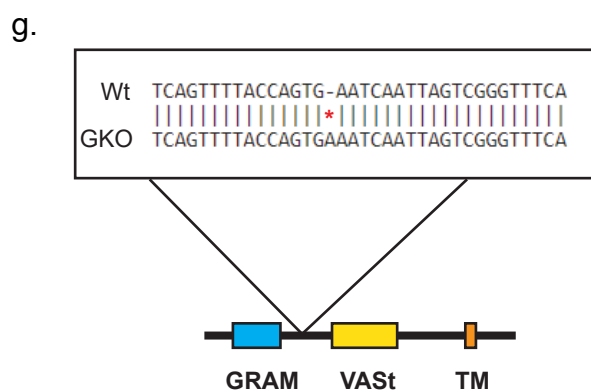
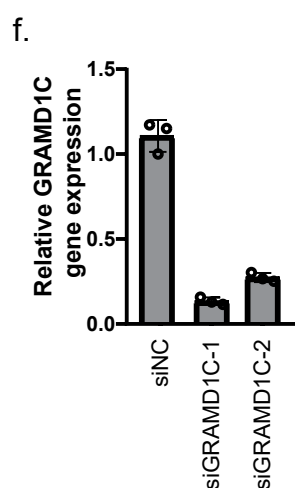
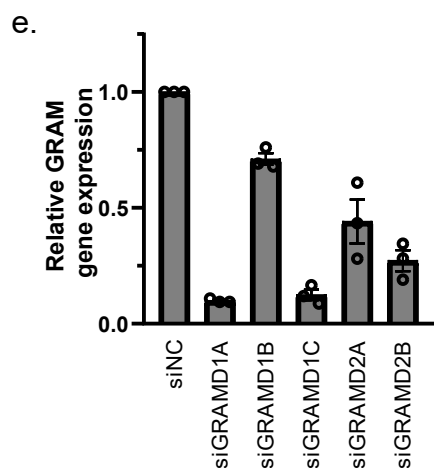
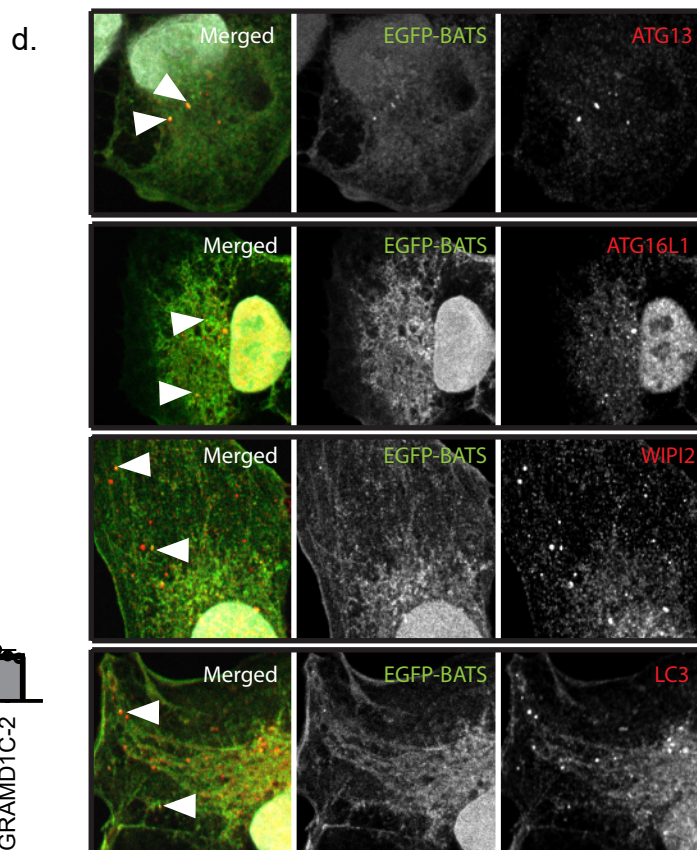
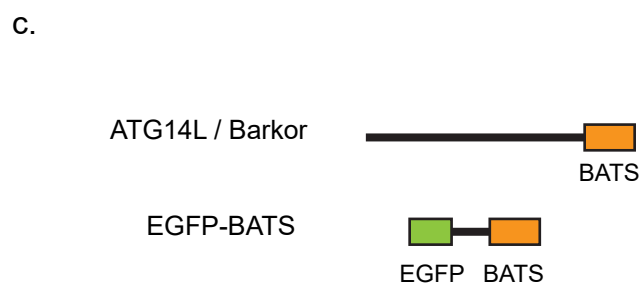
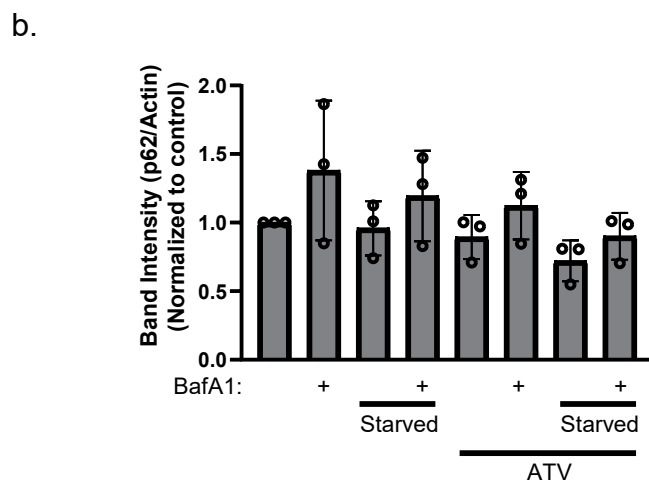
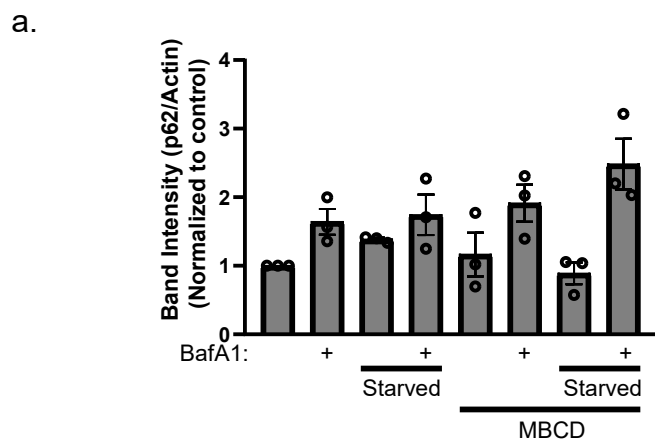
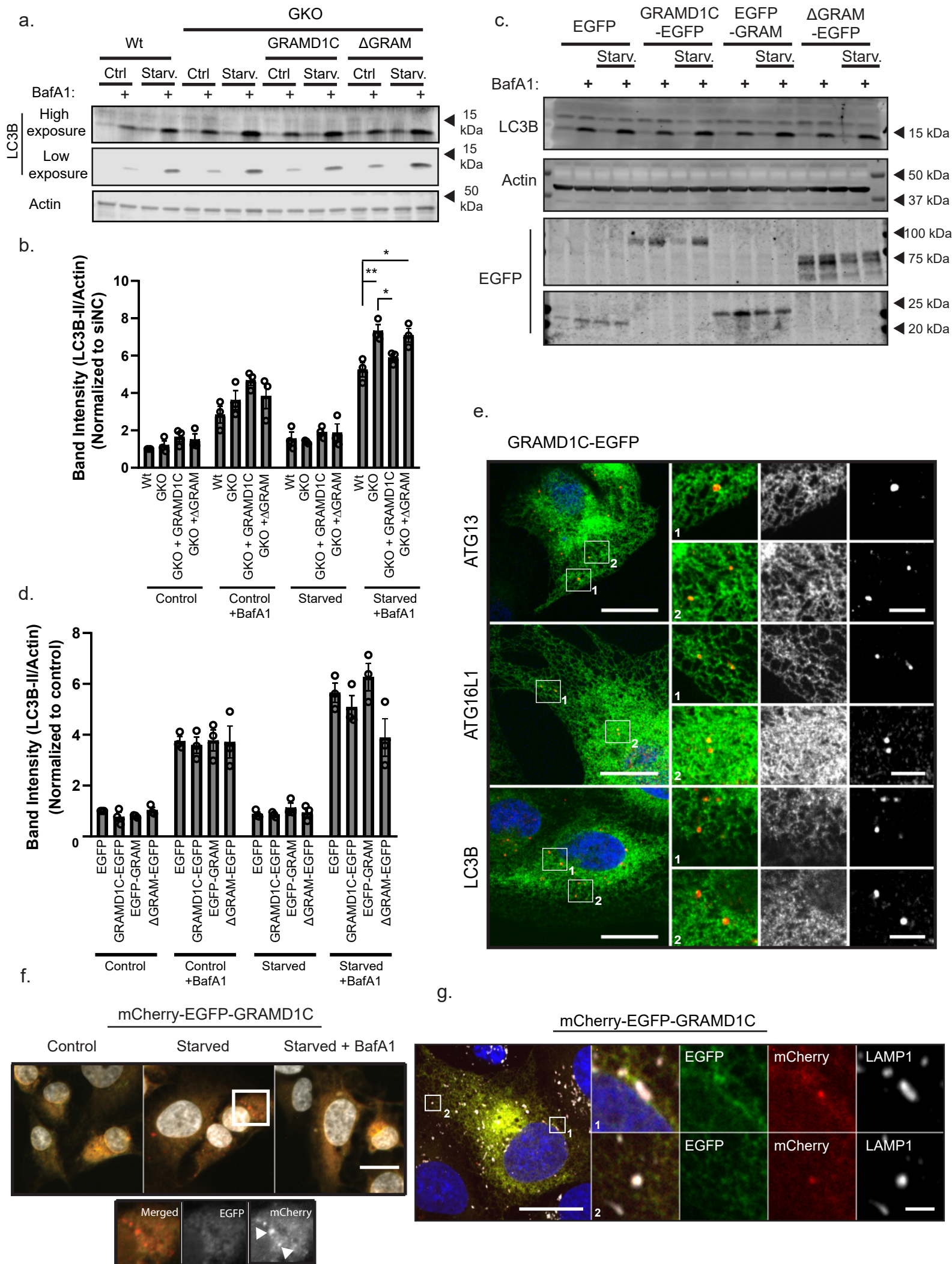




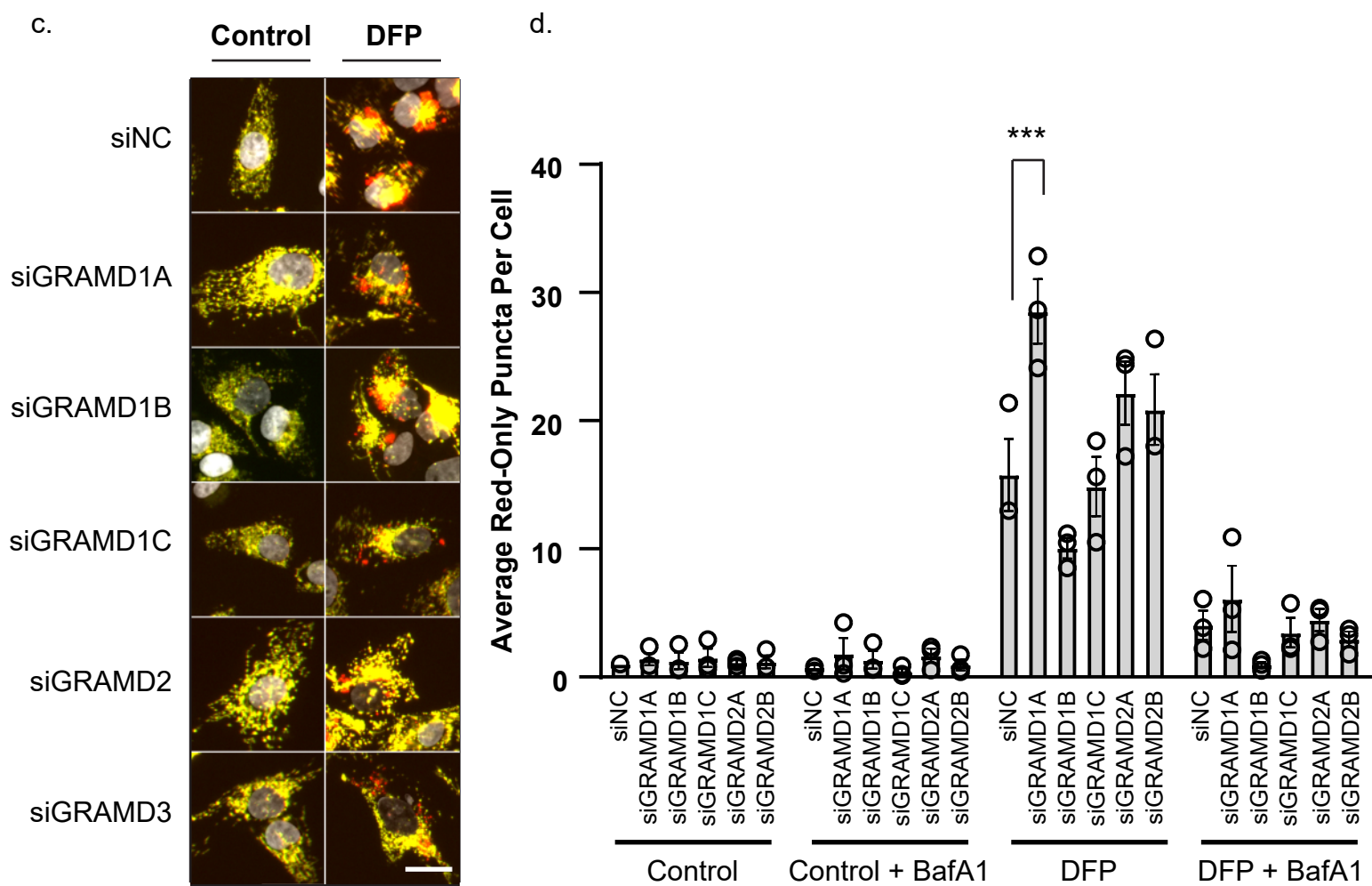
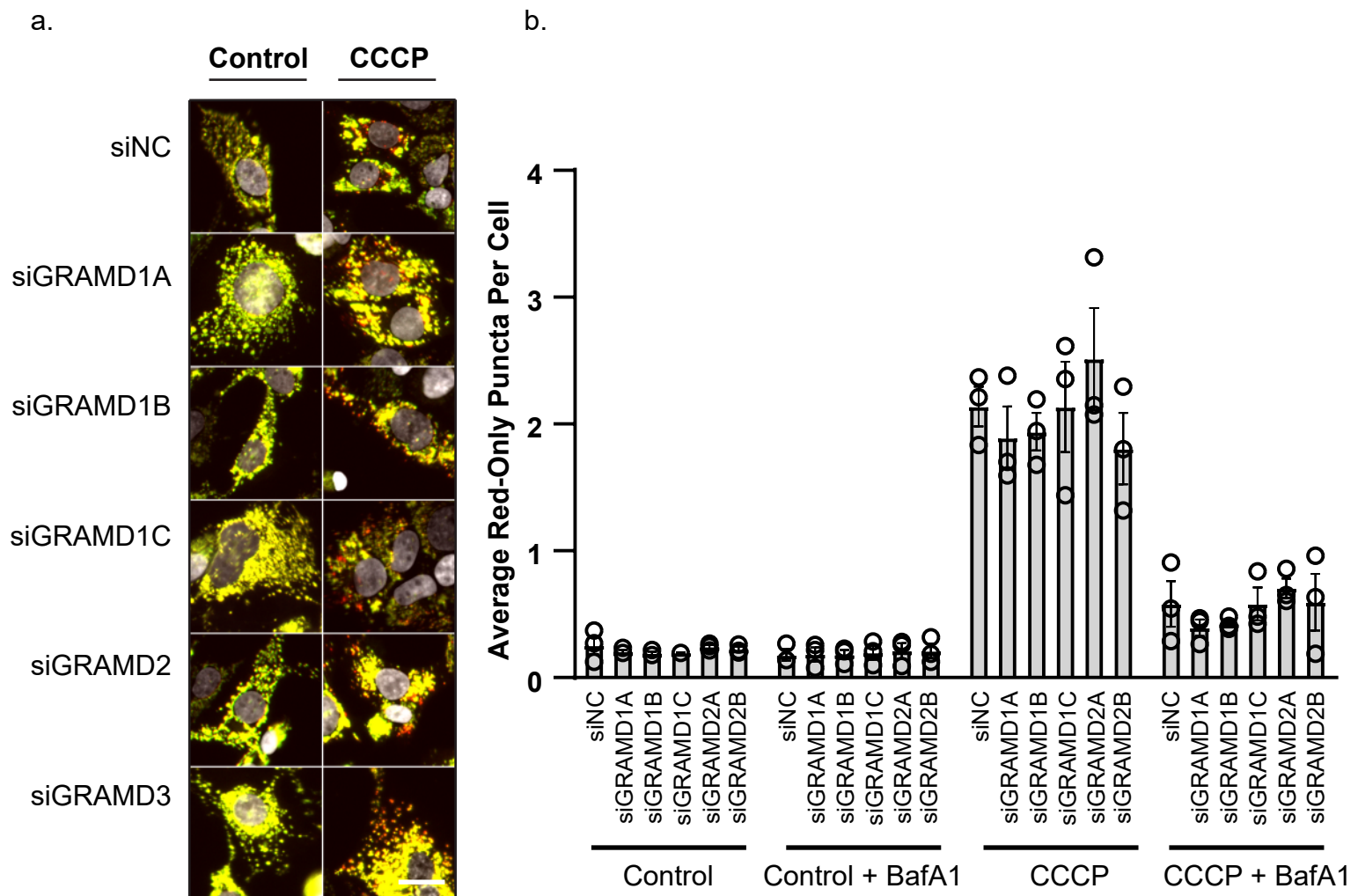
Supplementary figure 1 – EGFP-BATS are recruited to early autophagy structures

a-b The graphs represents the band intensities of p62 relative to actin and normalized to siNC from **a**. Figure 1a and **b**. Figure 1e from n = 3 biologically independent replicates. Error bars = SEM. **c** Graphical representation of ATG14L and its BATS domain, and the EGFP-BATS construct used. **d** U2OS cells stably expressing EGFP-BATS were starved in EBSS for 1 hr prior to fixation and immunostaining with the indicated antibodies prior to imaging with a confocal microscope. Scale bar = 10 μ m. n = 3 independent experiments. **e** U2OS cells were treated with siRNA against members of the GRAM family for 72 hrs before mRNA extraction, cDNA synthesis and qPCR using primers against the gene targeted by the siRNA. n = 3 biologically independent replicates. Error bar = SEM **f** U2OS cells were treated with two different siRNAs against GRAMD1C for 72 hrs before mRNA extraction, cDNA synthesis and qPCR using primers against GRAMD1C. n = 3 biologically independent replicates. Error bar = SD. **g** GRAMD1C Knockout Cells (GKO) KO were generated by CRISPR/Cas9, resulting in a single amino acid insertion in exon 7 of *GRAMD1C* leading to a premature stop codon (E245S*fs20). **h** Wt and GKO cells stably expressing untagged GRAMD1C and Δ GRAM were lysed and subjected to western blot analysis for GRAMD1C from 3 experiments. Endogenous GRAMD1C was neither detected in Wt nor GKO cells.



Supplementary figure 2 – GRAMD1C knockdown validation and degradation

a Wt cells, GKO cells and GKO cells stably expressing GRAMD1C or GRAMD1C(Δ GRAM) were starved or not in EBSS ($\pm$ BafA1) for 2 hrs prior to protein isolation and western blot analysis for the indicated proteins. **b** Quantification of LC3-II/Actin band intensity from a. from 3 independent experiments. Significance was determined using 2-way ANOVA followed by Dunnett's multiple comparison test from $n = 3$ experiments. Error bar = SEM. p value = 0.0012, 0.0044, and 0.0358. **c** U2OS cells expressing EGFP, GRAMD1C-EGFP, EGFP-GRAM, or Δ GRAM-EGFP were incubated in DMEM or EBSS $\pm$ 100 nM BafA1 for 4 hrs prior to protein isolation and western blot analysis for the indicated proteins. **d** Quantification of LC3B-II/Actin band intensity from c. from $n = 3$ replicates. Error bar = SEM **e** U2OS cells stably expressing GRAMD1C-EGFP were starved for 1 hr before fixation and immunostaining for ATG13, ATG16L1 and LC3B. Scale bar = 20 μ m, Scale bar inset = 5 μ m. An average of 10 cells were imaged per condition. **f** U2OS cells stably expressing mCherry-EGFP-GRAMD1C were starved or not in EBSS ($\pm$ BafA1) for 4 hrs, prior to fixation and widefield microscopy. Scale bar = 20 μ m. Arrows point towards red-only puncta. **g** U2OS cells stably expressing mCherry-EGFP-GRAMD1C were starved for 2 hrs prior to fixation and immunostaining for LAMP1. Scale bar = 20 μ m, Scale bar inset = 2 μ m.



Supplementary figure 3 - GRAMD1C depletion on PARKIN-dependent/independent mitophagy

a U2OS cells stably expressing MLS (mitochondria localization signal)-mCherry-EGFP were treated with the indicated siRNA for 48 hrs before treatment with 1 mM DFP (+/- 100 nM BafA1) for 24 hrs. The cells were then fixed, and immediately imaged on a high throughput widefield microscope. Scale bar = 20 μ M. **b** Quantification of images as shown in (a) from n = 3 experiments. The bars represent the average number of red only puncta per cell. Significance was determined using 2-way ANOVA followed by Tukey's comparison test. Error bar = SEM. **c** U2OS cells stably expressing MLS-mCherry-EGFP and untagged Parkin were treated with the indicated siRNA for 72 hrs before treatment with 10 μ M CCCP (+/- 100 nM BafA1) for 8 hrs. The cells were then fixed, and immediately imaged on a high throughput widefield microscope. Scale bar = 20 μ M. **d** Quantification of images as shown in c from n = 3 experiments. The bars represent the average number of red only puncta per cell. Significance was determined using 2-way ANOVA followed by Tukey's comparison test. Error bar = SEM. p value < 0.0001.

Sequence Identity

26.15%
25.76%
40.98%
40.91%
37.88%

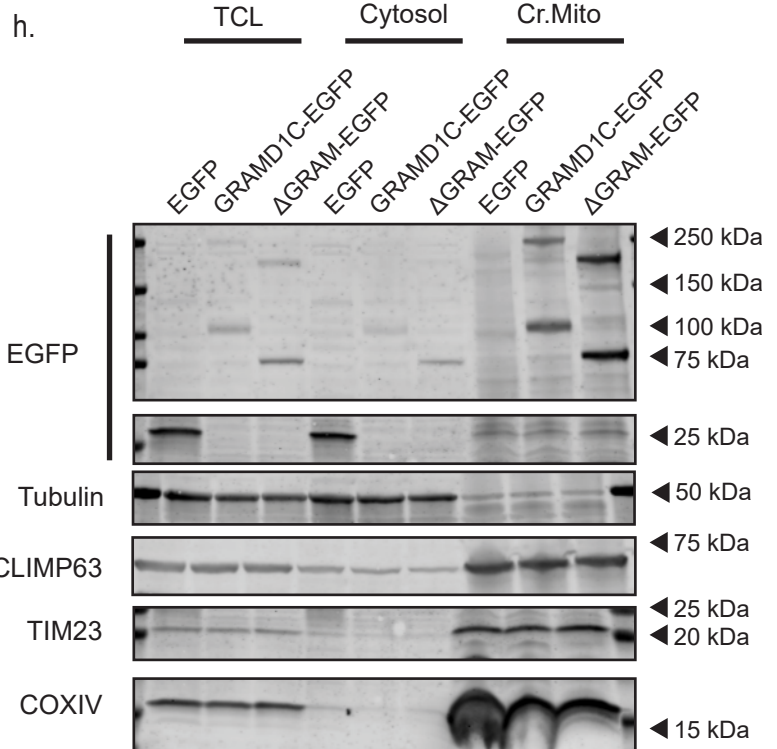
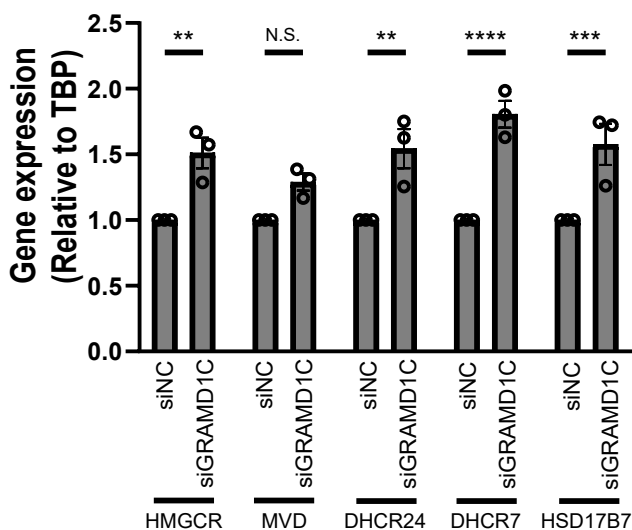
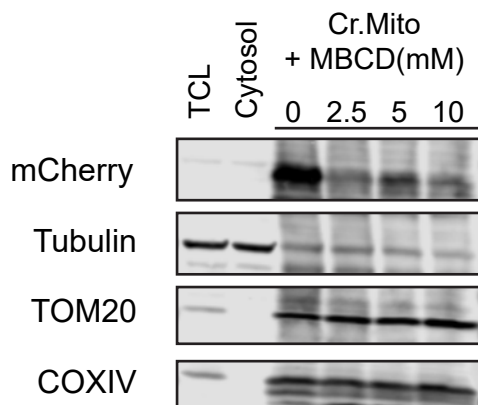
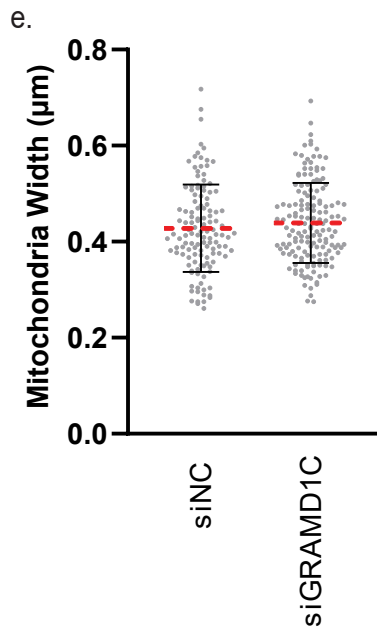
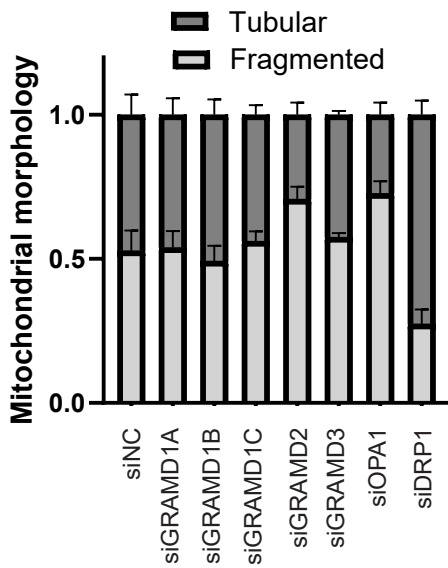
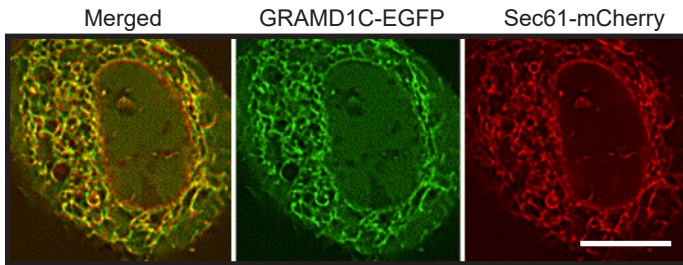
GRAM domain

LAM6 164
GRAMD1C 69
GRAMD1A 91
GRAMD1B 96
GRAMD2 72
GRAMD3 110

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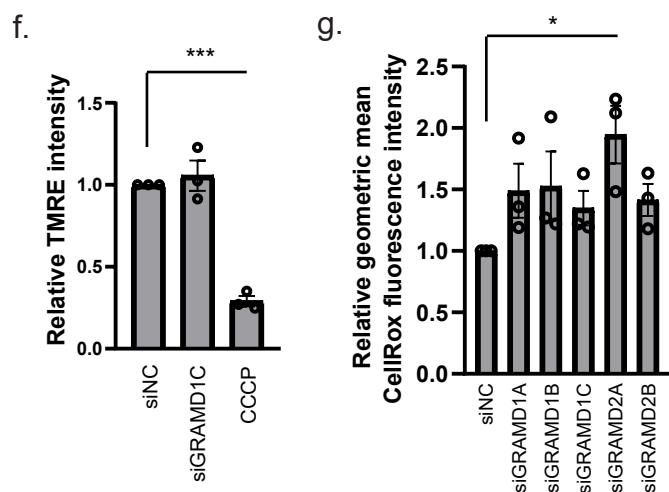
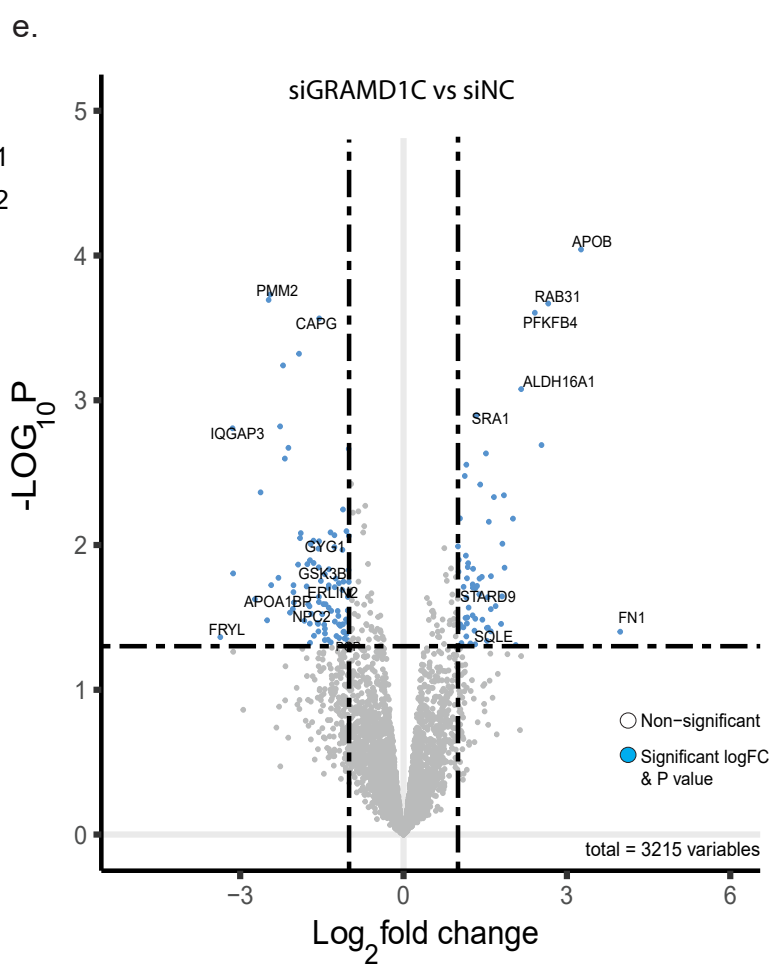
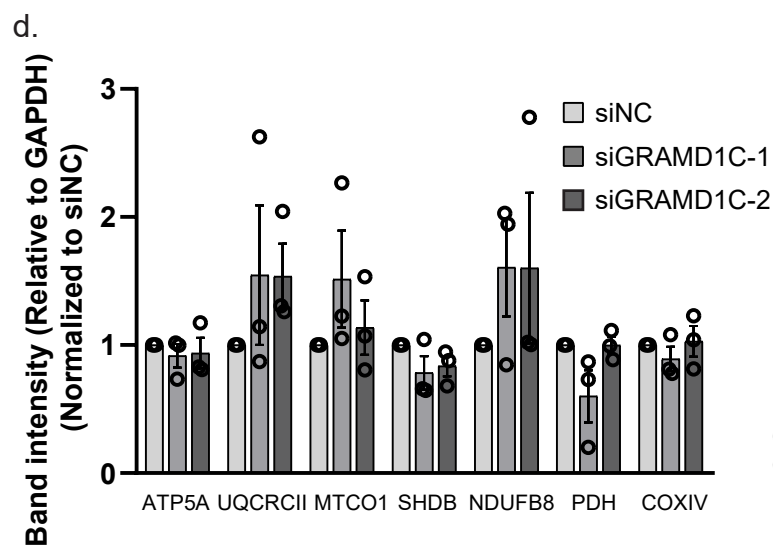
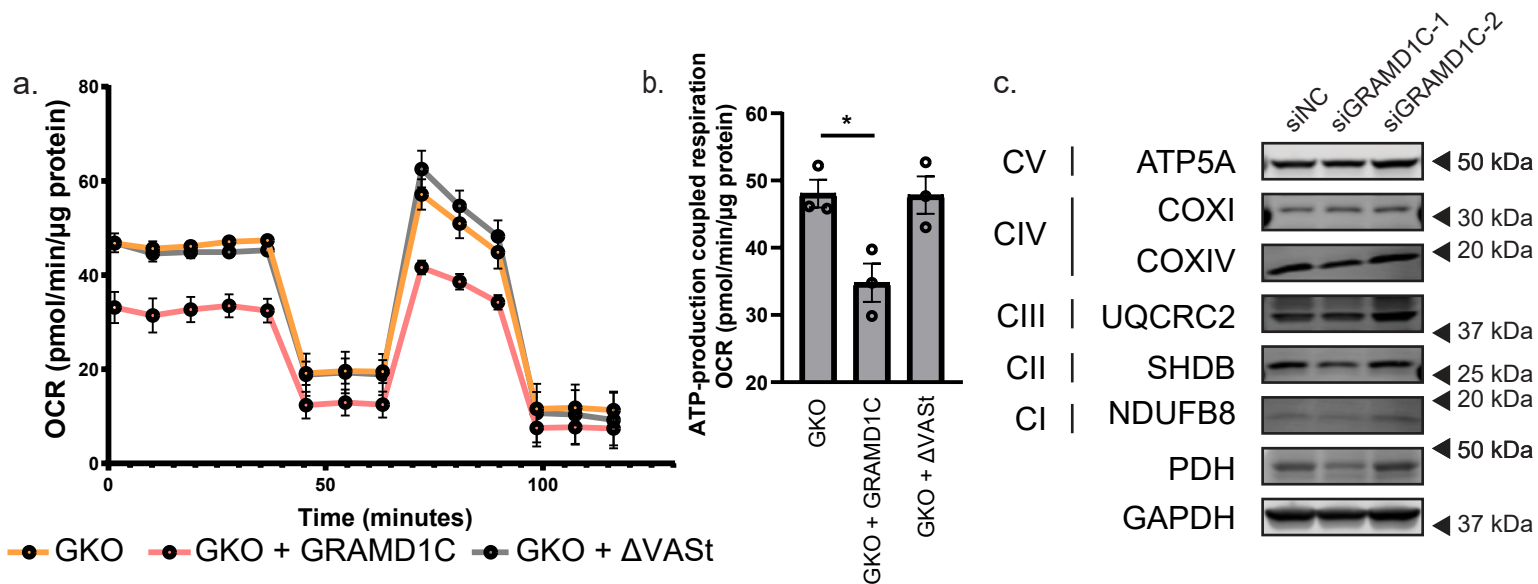
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Supplementary figure 4 - GRAMD1C depletion leads to increased SREBP target gene expression

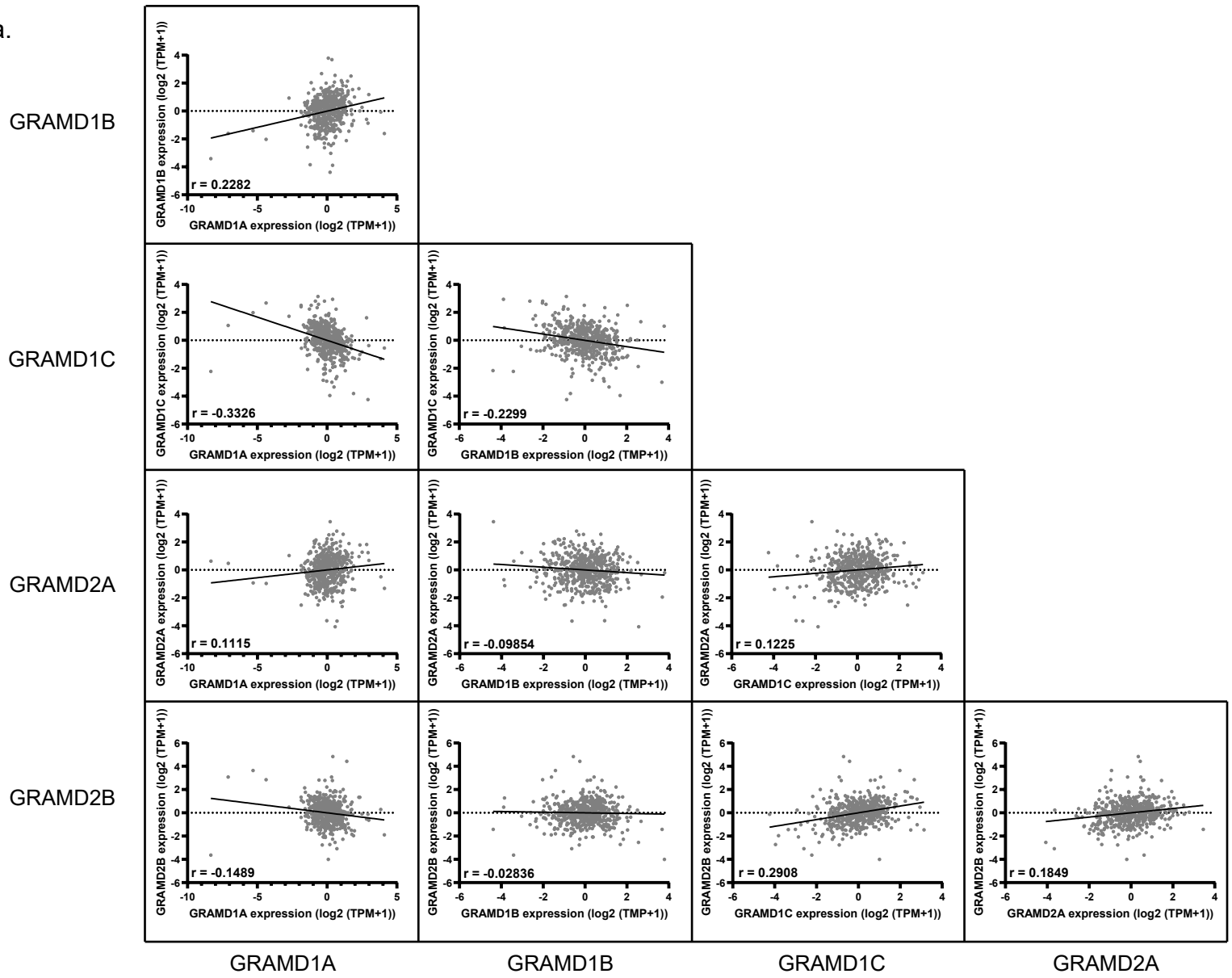
a GRAM domain sequence alignment of human *GRAMD1A*, *GRAMD1B*, *GRAMD1C*, *GRAMD2*, *GRAMD3* versus yeast *LAM6* using Clustal Omega. Sequence similarity values were obtained using BlastP. “.” represents a conservation of weakly similar properties, “:” represents a conservation of strongly similar properties, “*” represents conserved amino acids. **b** U2OS cells expressing GRAMD1C-EGFP and SEC61-mCherry were fixed and subjected to confocal microscopy analysis. Scale bar = 20 μ m. An average of 10 cells were imaged. **c-d** Mitochondrial morphology was characterized in cells expressing MLS-EGFP-mCherry. Mitochondrial EGFP signal was analyzed with CellProfiler Analyst based on the mitochondrial area, texture, intensity distribution, intensity, and shape. Mitochondria from siDRP1 and siOPA1 treated cells were positive controls for tubular and fragmented mitochondria respectively. 3 independent experiments were carried out. Error bar = SD. Scale bar = 20 μ m. **e** Mitochondria width measured from electron microscopy images (Figure 6d) from 25 siNC treated cells and 31 siGRAMD1C treated cells. The width of 5 mitochondria were measured per cell. **f** Isolated crude mitochondria were incubated with recombinant mCherry-D4 in the presence of MBCD at 37 °C for 45 mins prior to western blot analysis. **g** U2OS cells were treated with siRNA against control (siNC) or GRAMD1C before RNA extraction and cDNA synthesis. The graph represents the gene expression of the indicated SREBP target genes relative to the expression of TBP and normalized to siNC from n = 3 independent experiments. Significance was determined using One way ANOVA followed by Sidak’s multiple comparison test. Error bar = SEM. p value = 0.0023, 0.0012, <0.0001 and 0.0007. **h** U2OS cells expressing EGFP, GRAMD1C-EGFP or Δ GRAM-EGFP were subjected to crude mitochondrial fractionation (Cr.Mito) followed by western blot analysis for the indicated proteins. TLC: total cell lysate.



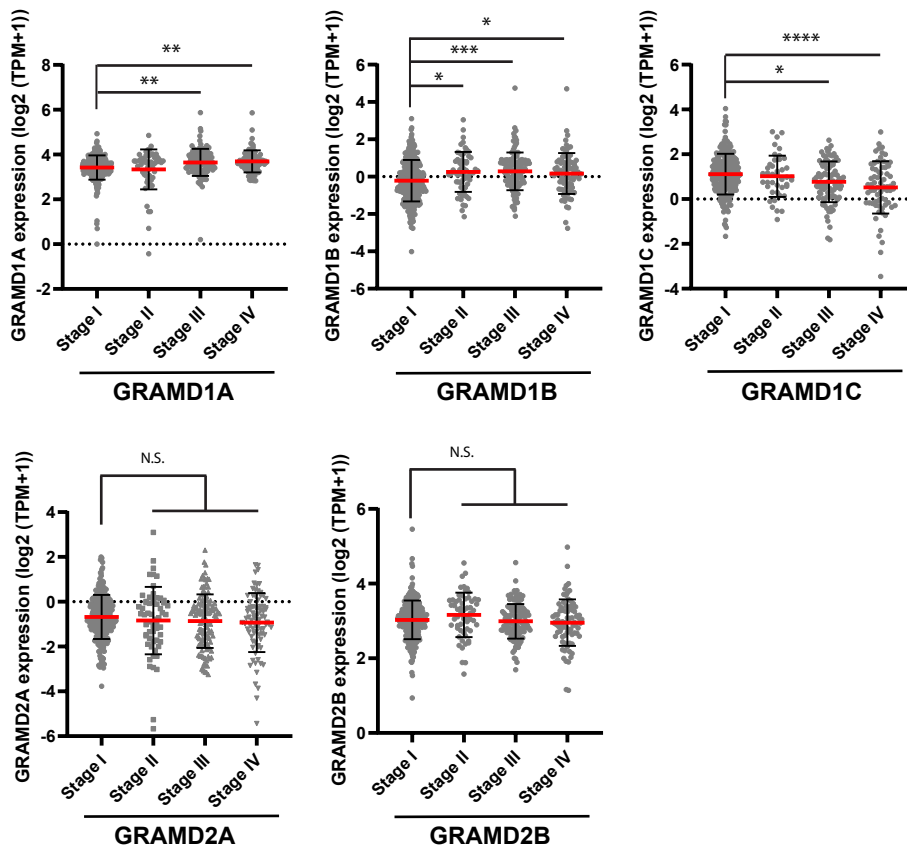
Supplementary figure 5 – GRAMD1C depletion does not alter mitochondrial morphology and proteins

a Mitochondrial oxygen consumption rate (OCR) was analyzed in GKO, GKO + GRAMD1C and GKO + Δ VAS_t cells using the Seahorse analyzer. OCR was measured after gradual addition of Oligomycin, CCCP and Rotenone/Antimycin A. $n = 3$ independent experiments were carried out. Error bar = SEM. p value = 0.0199. **b** ATP-linked respiration is calculated from the difference between the maximal respiratory capacity and the proton leak. Significance was determined using 1-way ANOVA followed by Bonferroni's comparison test from $n = 3$ experiments. Error bar = SEM. **c** Cell lysates from siNC and siGRAMD1C treated cells were subjected to western blot analysis with antibodies against the indicated OXPHOS components. **d** The graph represents the quantification of band intensities of data in a. relative to GAPDH and normalized to siNC. Significance was determined with 1-way ANOVA followed by Tukey's comparison test from $n = 3$ experiments. Error bar = SEM. **e** Lysates from U2OS cells transfected control (siNC) or GRAMD1C siRNA were subjected to mass spectrometry analysis. The graph represents the difference in protein abundance in siGRAMD1C treated cells compared to siNC cells. Blue spots represent proteins that have $p < 0.05$ and a log fold change of > 1.3 or < -1.3 . **f** U2OS cells treated with siRNA against control or GRAMD1C for 72 hrs were stained with Tetramethylrhodamine (TMRE) and dye fluorescence intensity was measured by flow cytometry. CCCP treated cells were used as positive control. Significance was determined using 1-way ANOVA followed by Tukey's comparison test. $n = 3$ experiments. Error bar = SEM. p value = 0.0003 **g** U2OS cells were treated with the indicated siRNA for 72 hrs before staining with the ROS fluorescent dye CellRox. The cells were then imaged using flow cytometry. Significance was determined using 1-way ANOVA followed by Tukey's comparison test from $n = 3$ experiments. Error bar = SEM. p value = 0.0174.

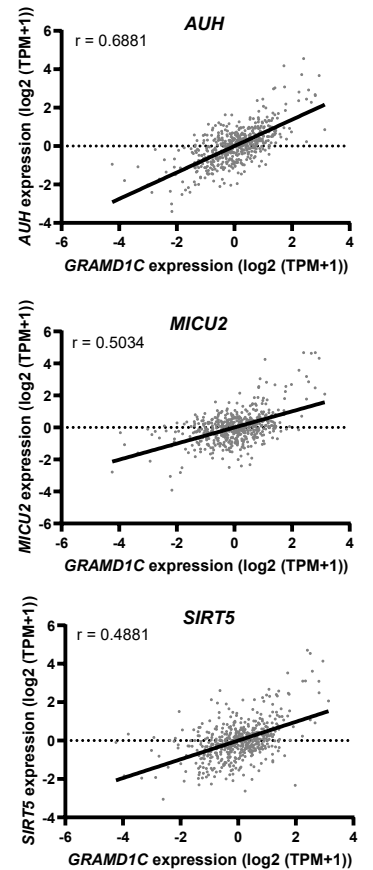
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b.

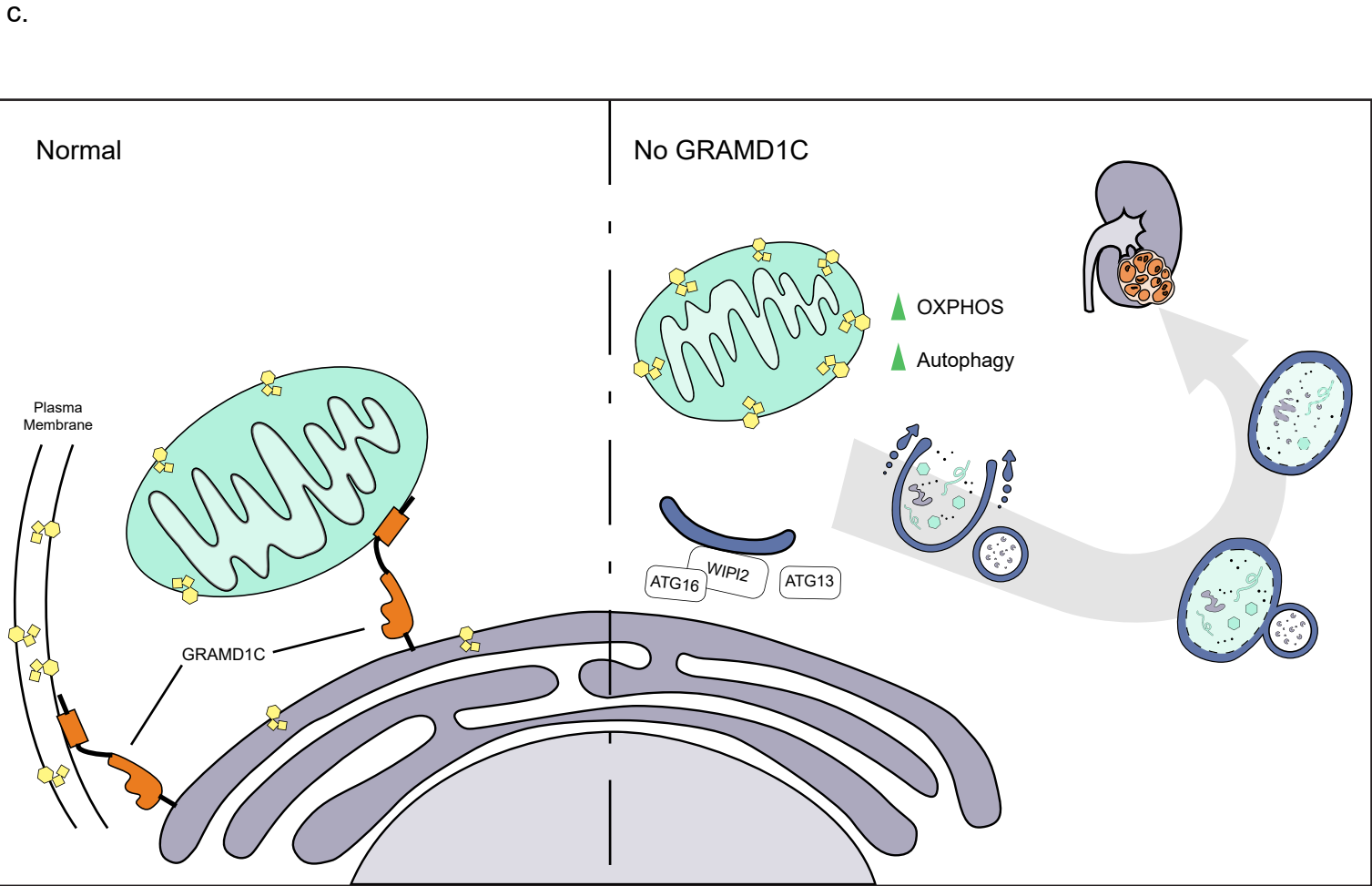
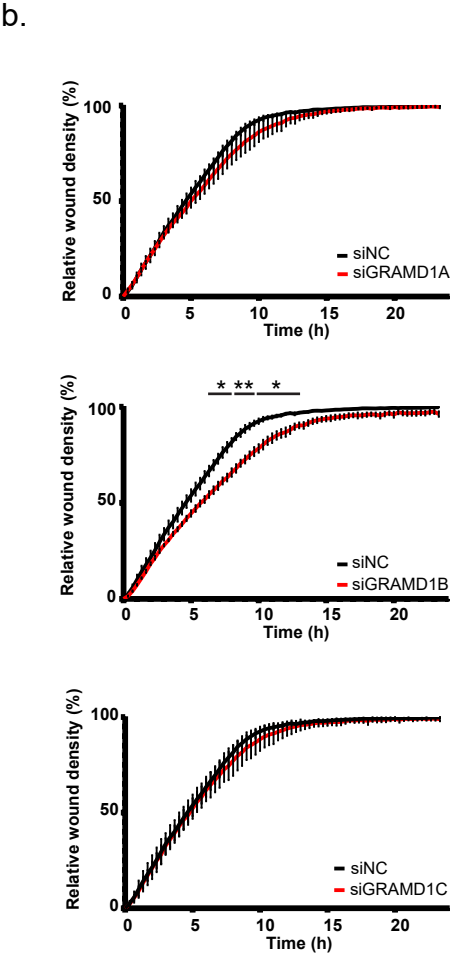
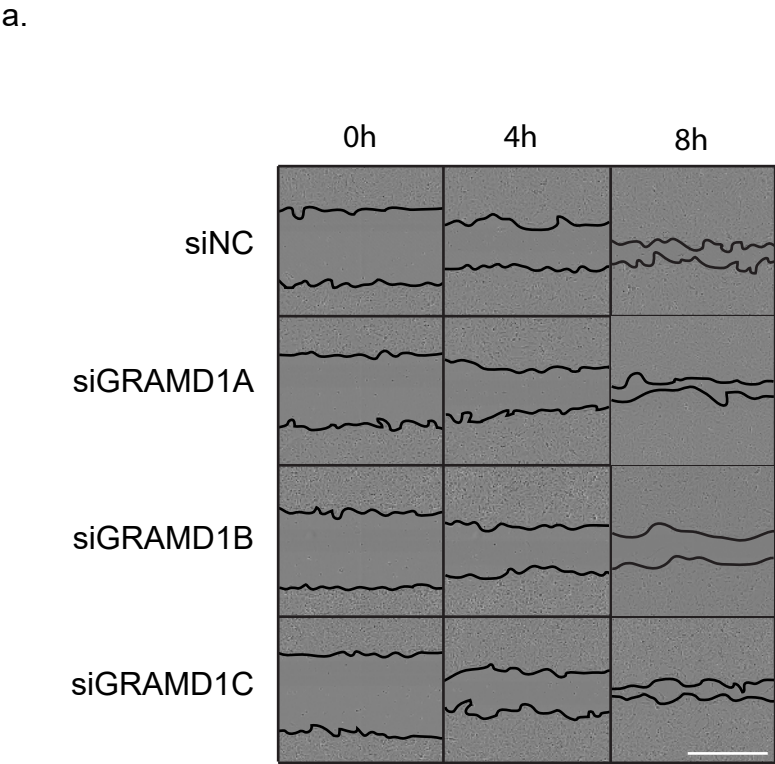


c.



Supplementary figure 6 – The expression of GRAMs in ccRCC

a The expression of individual GRAM members from the TCGA-KIRC study were plotted against each other. The Pearson's correlation values are displayed in each graph. **b** The expression of individual GRAMs were plotted against cancer stage in the TCGA KIRC cohort. Significance was determined using 1-way ANOVA followed by Tukey's comparison test. Error bar = SEM. Stage 1 n = 272, Stage 2 n = 59, Stage 3 n = 123 and Stage 4 n = 82. p value (GRAMD1A) = 0.0026 and 0.0015, (GRAMD1B) = 0.0132, 0.0001 and 0.0248, (GRAMD1C) = 0.0191 and < 0.0001. **c** Top three co-expressed mitochondrial genes with *GRAMD1C* in the KIRC TCGA cohort plotted against the expression of GRAMD1C. Top co-expressed genes of *GRAMD1C* in the KIRC TCGA dataset were downloaded from GEPIA2.cancer-pku.cn and subjected to GO Cellular Compartment enrichment using Enrichr^{89,90,91}.



Supplementary figure 7 – GRAMD1B regulates cell migration in ccRCC cells

a-b 786-O cells treated with siRNA against GRAMD1A-C were grown to confluency. A rubber stopper was then used to generate a wound, and the cells were immediately imaged at 1 hr intervals using the Incucyte. **a** shows representative images taken at 0, 4 and 8 hrs. **b** The graph represents the relative wound density over time. Significance was determined using 2-way ANOVA from n = 3 individual experiments. Scale bar = 700 μ m. Error bar = SD. p value = 0.0414, 0.0075 and 0.0202 **c** Graphical summary of the role of GRAMD1C as a negative regulator of autophagy. GRAMD1C localizes to the ER and interacts with mitochondria and the plasma membrane via its GRAM domain to facilitate cholesterol transport from these cellular structures to the ER. Such property of GRAMD1C may contribute to the suppression of autophagosome biogenesis by modulating cholesterol levels at ER membranes that are associated with autophagosome initiation sites. Upon GRAMD1C depletion membrane recruitment of early autophagic markers and autophagic flux are increased, as well as mitochondrial respiration and cholesterol abundance, potentially contributing to the decreased survival of ccRCC patients with low GRAMD1C levels.

Primers used in this study			
Target	Application	Forward (5'→3')	Reverse (5'→3')
<i>TBP</i>	qPCR	CAGAAAGTTCATCCTCTGGGCT	TATATTCGGCGTTTCGGGCA
<i>GRAMD1C</i>	qPCR	GTCCTTTTACCGTCTCCGCC	AGTCTCGGAGCACTCCCTTTA
<i>DHCR24</i>	qPCR	CGATGCACTCCGTCCGAAAA	GATGATGCGGATCTCAGCGG
<i>DHCR7</i>	qPCR	ATCGCTGACATCATCCGGGG	TAAAGTCCTGCGCCACCTTC
<i>HSD17B7</i>	qPCR	CTTCCAGCACAGCAAAGGCA	CCACATTGGAATAGAGACCCTGC
<i>HMGCR</i>	qPCR	GCCCTCAGTTCCAACCTACA	CAAGCTGACGTACCCCTGAC
<i>MVD</i>	qPCR	CGTAAGTGGCTGTGGAGCTG	CGTAAGTGGCTGTGGAGCTG
<i>GRAMD1A</i>	qPCR	AGCGAGAAGCATTTCTTCACTT	CAGTGCATTCTGCCAGAGG
<i>GRAMD1B</i>	qPCR	AGAAGGGCTCAGATCACTCCT	GTGGGGCTTAACACATTATACCA
<i>GRAMD2A</i>	qPCR	TGGCCGAGAAGGGATAACAC	GCCAGTTGGGGGAGATGTAG
<i>GRAMD2B</i>	qPCR	GCCCTTCATCTCTGCCTCTG	GCCCTTCATCTCTGCCTCTG
<i>GRAMD1C</i>	Crispr	CACCGAACCCGACTAATTGATTAC	AAACGTGAATCAATTAGTCGGGTTT
<i>EGFP-BATS</i>	Gibson	TTTAACTTAAGCTTGGTACGCCACC ATGGTGAGCAAG	TCCTGAACCTGATCCTGAACCGCTCTTG TACAGCTCGTCCATG
<i>EGFP-BATS</i>	Gibson	AGCGGTTCAAGGATCAGGTTCAAGAG CTGGAGAATCAGATGAG	TTTAAACGGGCCCTCTAGACTTAACGGT GTCCAGTGTAAG
<i>GRAMD1C</i>	Gibson	GATCTCGAGCTCAAGCTTCGGCCACC ATGGAGGGCGCT	TCCTGAACCTGATCCTGAACCGCTGCTT TCAACAGCCATGCCAGTCTTATTC
<i>GRAMD1C</i>	Gibson	AGCGGTTCAAGGATCAGGTTCAAGAG TGAGCAAGGGCGAGGAG	AGAATTATCTAGAGTCGCGGTTACTTGT ACAGCTCGTCCATG
<i>GRAMD1C-V1-EGFP</i>	Cloning	AGCGGTTCAAGGATCAGGTTCAAGAG AGGGCGCTCCGACTGTC	TTTAAACGGGCCCTCTAGACTTACTTGT ACAGCTCGTCCATGC
<i>3xHA-EGFP-OMP25</i>	Cloning	CAAAAAAGTTGGCACCATGTGCCACC ATGTATCCCTATG	TGTACAAGAAAGTTGATTACTCAGAGCT GCTTCGGTATC
<i>GRAMD1C (ΔGRAM)</i>	Cloning	TTGAGTACTTACGTAGGTACATGGAA AACTTGTCAGTGTCTG	CTGATCCTGAACCGCTGCTTTCAACAGC CATGCC
<i>GRAMD1C (ΔVAsT)</i>	Gibson	GATCTCGAGCTCAAGCTTCGGCCACC ATGGAGGGCGCT	CAATGGCCTGATGAAGATCTTTCTCAGG AACATTTTCTTCATCAACAGAATCTG
<i>GRAMD1C (ΔVAsT)</i>	Gibson	AGATCTTCATCAGGCCATTGAAGACC CTG	AGAATTATCTAGAGTCGCGGCTAGCTTT CAACAGCCATGC

Supplementary Table 1. Description of the primers used in this study

Plasmids used in this study		
Vector	Description	Source
pENTR3C	Gateway Entry vector	Invitrogen
pENTR3C-GRAMD1C	Human GRAMD1C PCR product (from U2OS cDNA) was cloned into pENTR3C.	This study
pENTR3C-GRAMD1C (Δ GRAM)	Cloning human GRAMD1C lacking the GRAM domain into a pENTR3C backbone.	This study
pLenti-PGK-GRAMD1C-EGFP	A lentiviral vector expressing C-terminal EGFP tagged GRAMD1C was generated using Gibson Assembly.	This study
pLenti-PGK-GRAMD1C (Δ GRAM)-EGFP	A lentiviral vector expressing C-terminal EGFP tagged GRAMD1C (Δ GRAM) was generated using Gibson Assembly.	This study
pCMV-VSV-G	Envelope protein vector for lentivirus production.	Gift from Bob Weinberg (Addgene # 8454)
psPAX2	Lentiviral packaging vector for lentivirus production.	Gift from Didier Trono (Addgene # 12260)
pLenti-PGK-EGFP-GRAM domain	EGFP-GRAM domain was cloned from an entry vector into a pLenti-PGK vector using Gibson Assembly.	This study
pcDNA5-MLS-EGFP-mCherry	Vector for inducible mitophagy reporter using the FlpIn system.	³⁷ Reference
pLenti-PGK-3xHA-EGFP-OMP25	3xHA-EGFP-OMP25 vector used for affinity purification of mitochondria. The insert was cloned from ⁸¹ .	This study
pLenti-PGK-EGFP-BATS	EGFP-BATS domain cloned from ATG14L plasmid into a pLenti-PGK vector using Gibson Assembly.	This study
pDest-FlpIn-mCherry-EGFP-GRAMD1C	Doxycycline inducible mCherry-EGFP-GRAMD1C construct generated using gateway cloning.	This study
pLVX-CMV-Puro-GRAMD1C	Lentiviral vector expressing GRAMD1C was generated using Gibson Assembly.	This study
pLVX-CMV-Puro- GRAMD1C (Δ GRAM)	Lentiviral vector expressing GRAMD1C (Δ GRAM) was generated using Gibson Assembly.	This study
pLVX-CMV-Puro-GRAMD1C (Δ VAS _t)	Lentiviral vector expressing GRAMD1C (Δ VAS _t) was generated using Gibson Assembly.	This study

Supplementary Table 2. Description of the plasmids used in this study.