Supplemental material

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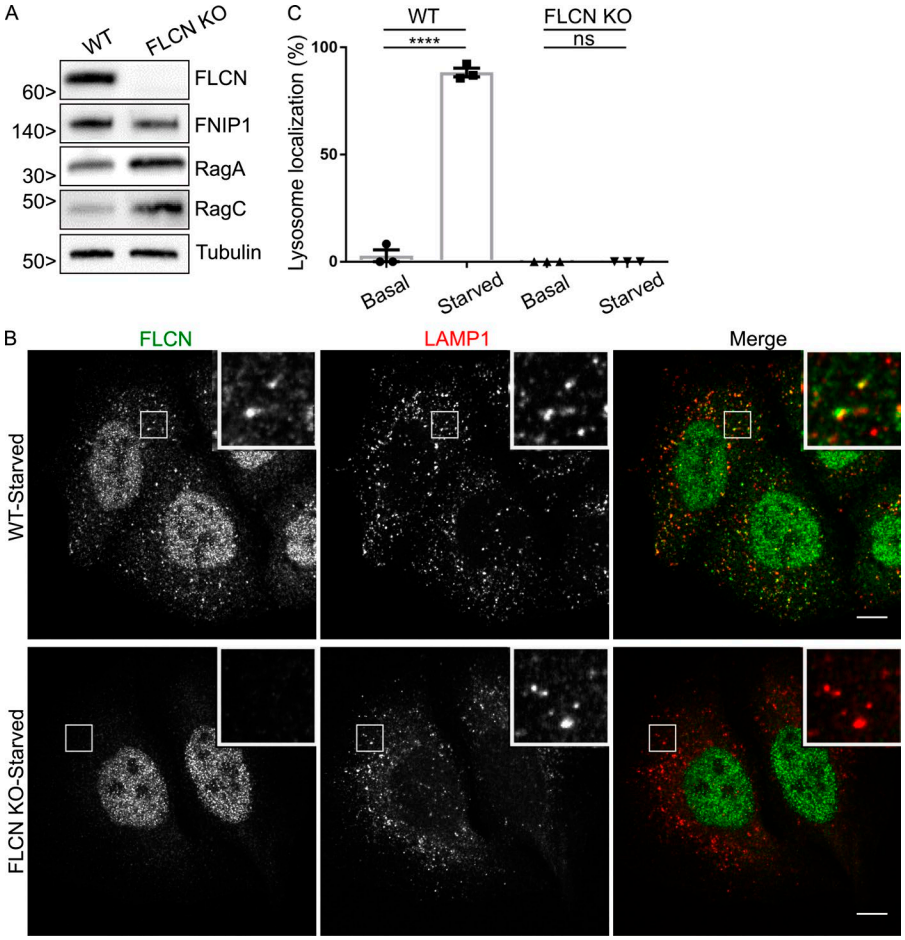

Figure S1. **Analysis of *FLCN* KO cells demonstrates anti-FLCN antibody specificity. (A)** Representative immunoblots of WT and FLCN KO HeLa cells. Molecular masses are given in kilodaltons. **(B)** Anti-FLCN immunofluorescent staining of WT versus FLCN KO cells that were starved for 2 h before fixation reveals selective loss of diffuse cytoplasmic and punctate (lysosome) signal in the *FLCN* KO cells. In contrast, a nonspecific nuclear signal was present in cells of both genotypes. **(C)** Quantification of percentage of cells with five or more FLCN spots that are positive for LAMP1 signal (mean ± SEM; $n = 3$ experiments; 57–63 cells per condition). ****, $P < 0.0001$; ANOVA with Bonferroni's post hoc test. Bars, 10 µm. Insets are 7.8 µm wide.

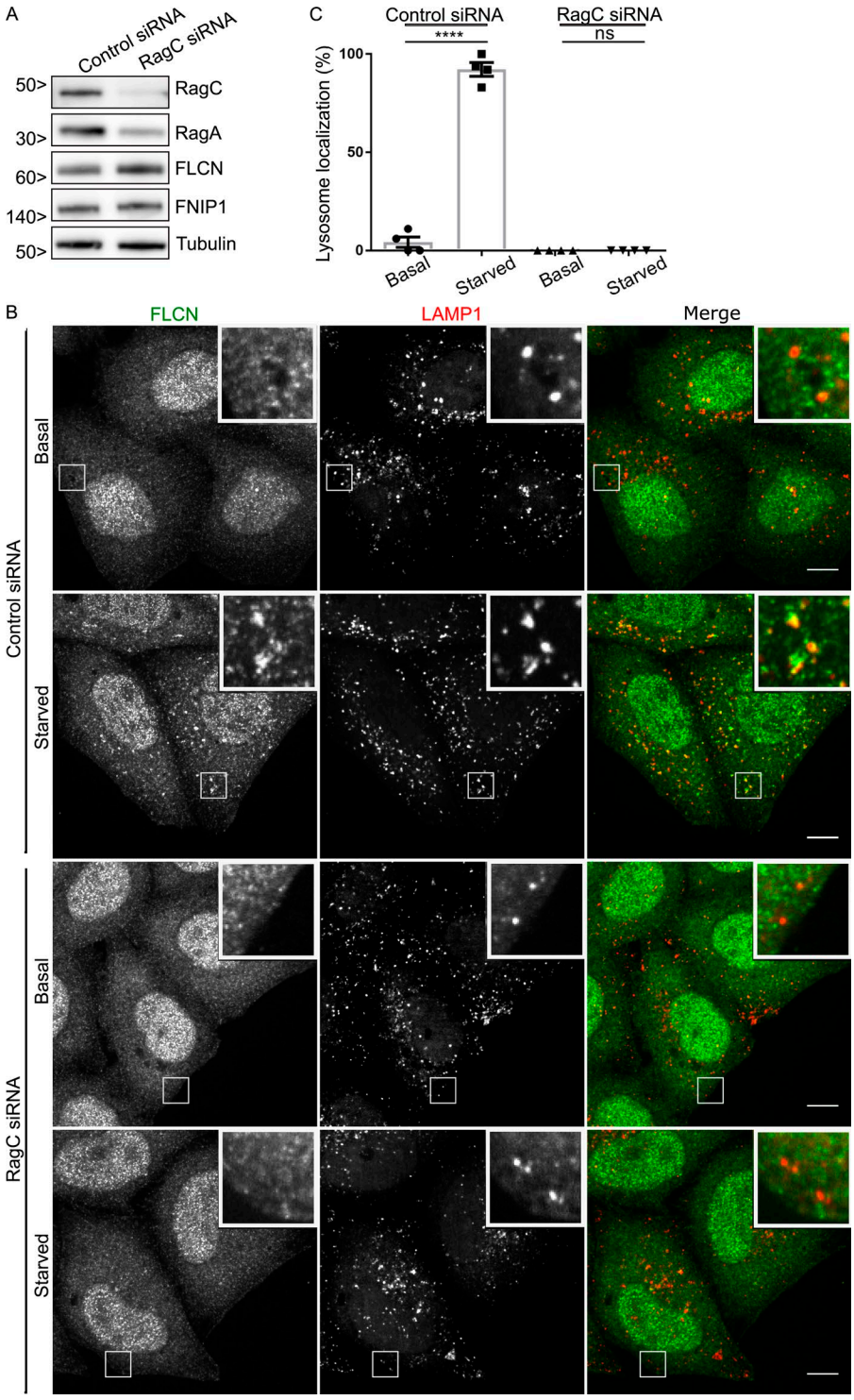

Figure S2. **Regulation of the lysosomal recruitment of FLCN by amino acid availability and Rag GTPases. (A)** Immunoblot analysis of the endogenous FLCN, FNIP1, RagA, RagC, and tubulin proteins in control conditions or after RagC knockdown. Molecular masses are given in kilodaltons. **(B)** Representative immunofluorescence experiment showing the effect of starvation in control conditions or after RagC knockdown. **(C)** Quantification of percentage of cells with five or more FLCN spots that are LAMP1-positive (mean ± SEM; *n* = 4 experiments; 61–77 cells per condition; ****, P < 0.0001; ANOVA with Bonferroni's post hoc test). Bars, 10 µm. Insets are 7.8 µm wide.

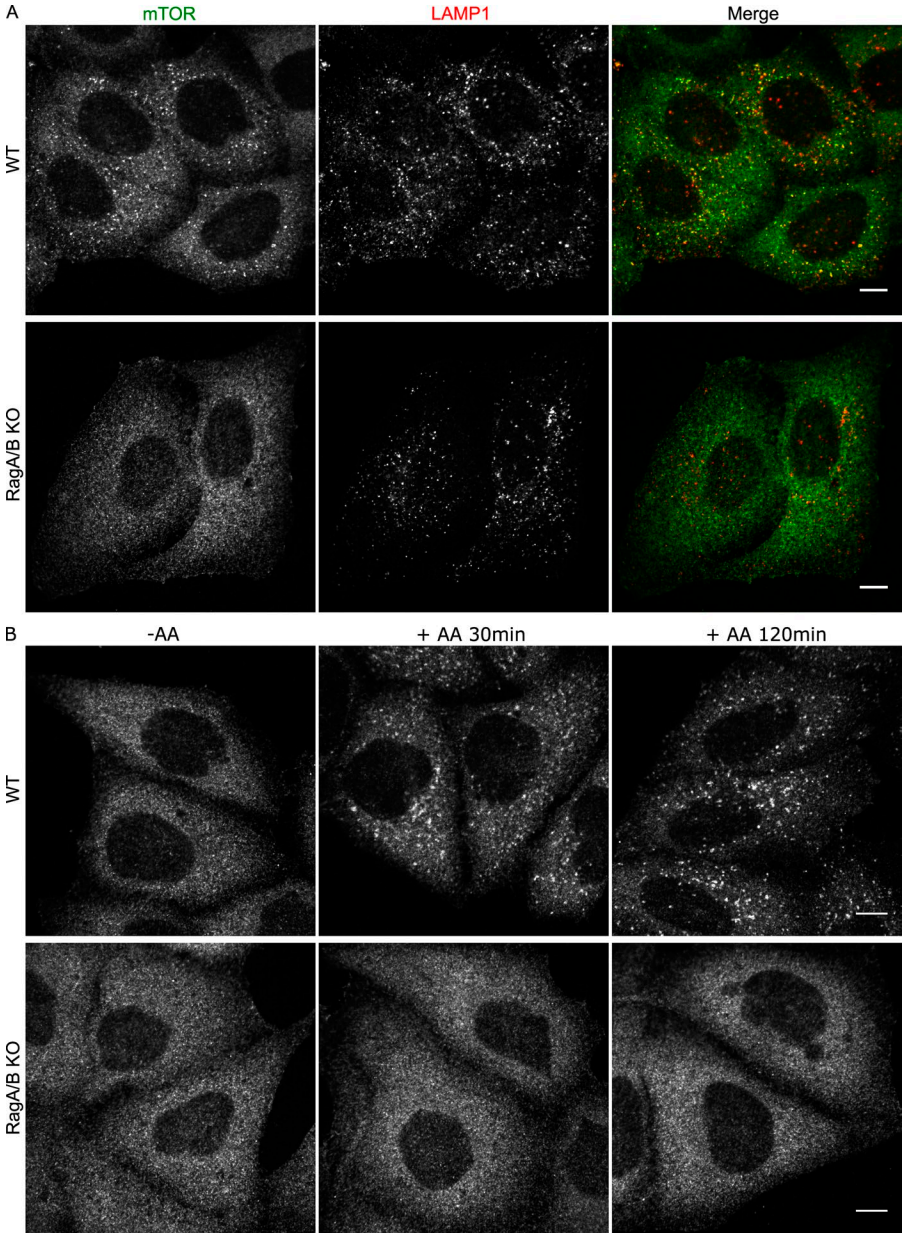

Figure S3. **mTOR lysosome localization in response to amino acids is defective in the absence of RagA and RagB. (A)** Immunofluorescence analysis showing mTOR and LAMP1 in WT and *RagA/B* KO HeLa cells under basal growth conditions. **(B)** Immunofluorescence analysis depicting mTOR and LAMP1 in WT and *RagA/B* KO cells that were starved of amino acids (AA) and then stimulated with amino acids after the indicated time. Bars, 10 µm.

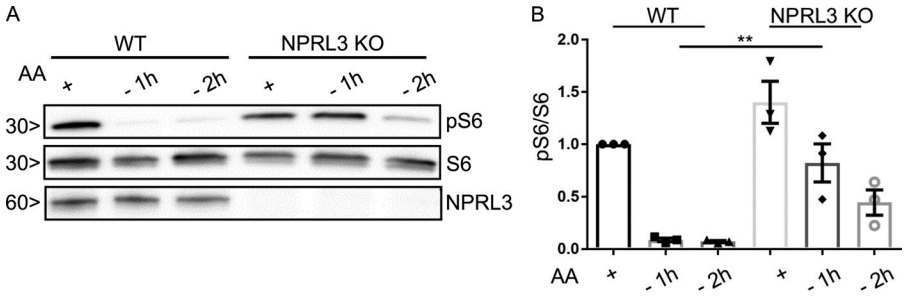

Figure S4. **mTORC1 signaling is resistant to amino acid starvation in *NPRL3* KO cells. (A and B)** Representative Western blots (A) and quantification (B) showing the phosphorylation of reporters of mTORC1 activity in WT and *NPRL3* KO cells upon starvation (1 h and 2 h). **, P < 0.01; ANOVA with Bonferroni's post hoc test. Molecular masses are given in kilodaltons. AA, amino acid.

Table S1. **Characterization of *FLCN* KO, *RagA/B* KO, *NPRL3* KO, and 2×HA-FNIP1 knock-in cell lines**

Gene	Mutation
FLCN	11 bp deletion
	2 bp deletion
	1 bp insertion
RagA	2 bp deletion
	102 bp insertion
RagB	11 bp deletion
	13 bp deletion
	1 bp deletion
NPRL3	2 bp deletion
	1 bp deletion
	4 bp deletion
FNIP1	2 bp deletion
	2×HA insertion

Table S2. **Oligonucleotide sequences for gene-editing experiments**

Gene	Use	Sense (5′–3′)	Antisense (5′–3′)
FLCN	Guide RNA for KO	CACCGTCTCAGCAAGTACGAGTTTG	AAACCAAACTCGTACTTGCTGAGAC
FNIP1	crRNA for 2×HA tag insertion	GAGCUUCUGGAACAGCGUAGGUUUUAGAG	–
FNIP1	2×HA tag template	GCGCCCATGGGGGTGGCGGGGCGGCTGTAG GAGCAGGGGCCTAGCAAGCGCCCAGCGGAG CGACCCCTGCCTGGCCGTGGCTAGCATGGC CTACCCATACGATGTTCCAGATTACGCTTA CCCATACGATGTTCCAGATTACGCTCCTAC GCTGTTCCAGAAGCTCTTCAGCAAGAGGAC CGGGC	–
NPRL3	Guide RNA for KO	CACCGGAACTACGCCCGACGTGCAC	AAACGTGCACGTCGGGCGTAGTTCC
RagA	Guide RNA for KO	CACCGATTACATTGCTCGCGACACC	AAACGGTGTCGCGAGCAATGTAATC
RagB	Guide RNA for KO	CACCGAATCCCACAGGTTCAATACC	AAACGGTATTGAACCTGTGGGATTC

Table S3. **Oligonucleotide primers for PCR amplification of genomic DNA**

Target	Sense (5′–3′)	Antisense (5′–3′)
2×HA-FNIP1	CCGTGGCCGTTTGAAGTGACTAATT	AGCGCCCTACCTGAACCCGCAATCT
FLCN	GTCTTTTCACCAGCAGCCTTCCCCA	CCACCACATCCACAGACAGGTTCTG
NPRL3	GTGATCTTAAGAGATGCTCTCCTGC	AAGGGCCCAGGAGAAACCTGGGATT
RagA	CGGGTGATGCCAAATACAGCCATGA	CAGTCCCACAGGTTCAGCACCAGGT
RagB	CTTATTTTGAATTTCACTTCACCTG	ATCTATTACAAATGATAGTTACTCT

Table S4. **Oligonucleotide primers for PCR amplification of HA-RagA cDNA**

Type	Sequence (5′–3′)
Sense	TAGTACCGGTAGGCCTGTCGACGATGCCACCATGGGATATCCGTACGATGTCCCAGACTATGCAGGTGGATCAGGAGGTTC AGGTGGAT
Antisense	AGGATCCAGCGGCCGCGGGCCCGATTCAACGCATAAGGAGACTGTGCTTG

Table S5. **Summary of antibodies used**

Target protein	Source	Catalog no.
FLCN	Cell Signaling Technology	3697
FNIP1	Epitomics	EPNCIR107
HA	Cell Signaling Technology	2367
HA-HRP	Roche	12013819001
LAMP1	DSHB	H4A3
mTOR	Cell Signaling Technology	2983
NPRL3	Sigma-Aldrich	HPA011741
Phospho-S6	Cell Signaling Technology	4858
Phospho-S6K	Cell Signaling Technology	9234
RagA	Cell Signaling Technology	4357
RagC	Cell Signaling Technology	9480
S6	Cell Signaling Technology	2217
S6K	Cell Signaling Technology	9202
Tubulin	Sigma-Aldrich	T5168