

Supplemental Information

Defective Regulation of Autophagy upon Leucine Deprivation Reveals a Targetable Liability of Human Melanoma Cells In Vitro and In Vivo

Joon-Ho Sheen, Roberto Zoncu, Dohoon Kim, and David M. Sabatini

INVENTORY OF SUPPLEMENTAL INFORMATION

- 1) Figure S1, related to Figure 1.
- 2) Figure S2, related to Figure 3.
- 3) Figure S3, related to Figure 4.
- 4) Figure S4, related to Figure 5.
- 5) Figure S5, related to Figure 7.

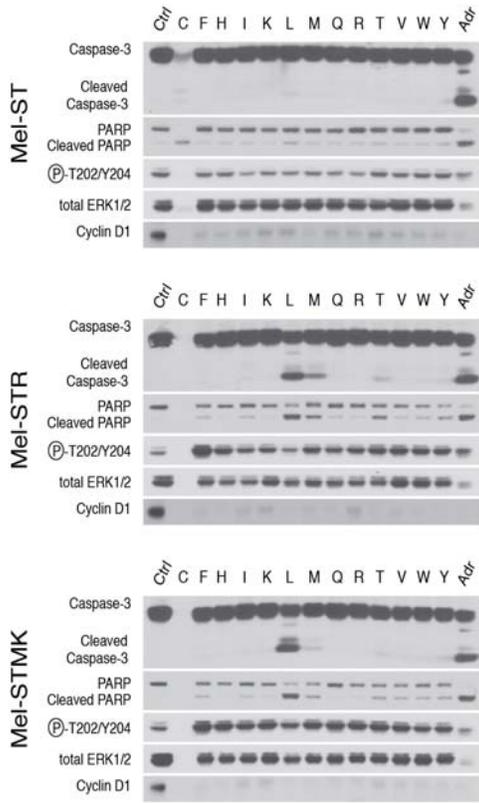
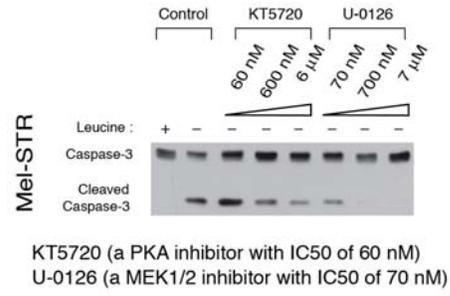
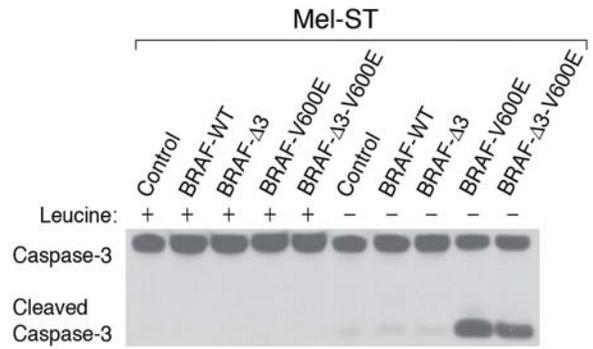
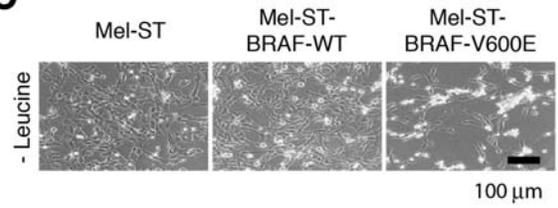
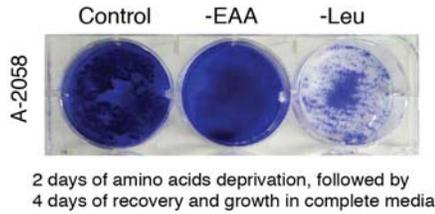
A**B****C****D**

Figure S1, related to Figure 1. Hyperactivation of the RAS-MEK pathway renders melanocytes sensitive to leucine deprivation. (A) Immunoblot analyses showing hyperactivated MAPK signaling in Mel-STR and Mel-STMK cells using dual phosphorylation of Thr-202 and Tyr-204 of ERK1/2 as a measure of MAPK pathway activity. Cyclin D1 levels were used to detect cell cycle arrest upon deprivation of any single essential amino acid. Ctrl, control RPMI-1640 media; C-F-H-I-K-L-M-Q-R-T-V-W-Y, deprivation of the indicated single amino acid (single-letter code for amino acid); Adr, adriamycin at 2 $\mu\text{g/ml}$. (B) Inhibition of MAPK signaling with a MEK1/2 inhibitor (U-0126) suppresses apoptosis upon leucine deprivation. Immunoblots show the effects of an increasing amount of a MEK1/2 inhibitor or a PKA inhibitor (KT5720) on the leucine deprivation-induced apoptosis in Mel-STR cells. (C) Representative micrographs show morphological changes in Mel-ST cells expressing oncogenic BRAF-V600E following deprivation of leucine for 2 days. BRAF- $\Delta 3$ is an allele of BRAF that interacts poorly with CRAF. Scale bar = 100 μm . Immunoblot analyses show activation and cleavage of caspase-3 upon leucine deprivation in the same cells. (D) Clonogenic survival assay showing effects on viability of A-2058 melanoma cells following deprivation for all essential amino acids or just leucine. After indicated deprivations, cells were re-seeded into complete media and allowed to grow and proliferate for 4 days after which cells were visualized by staining them with crystal violet.

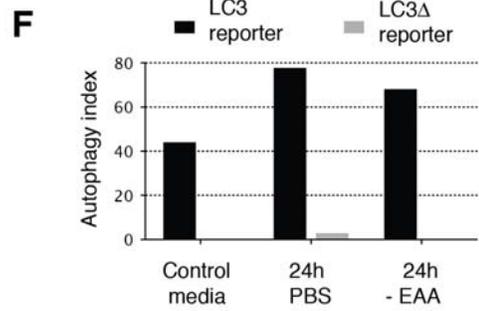
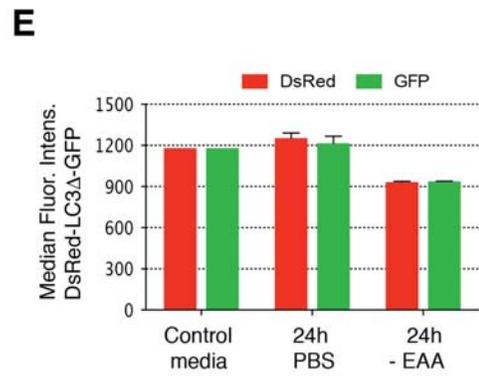
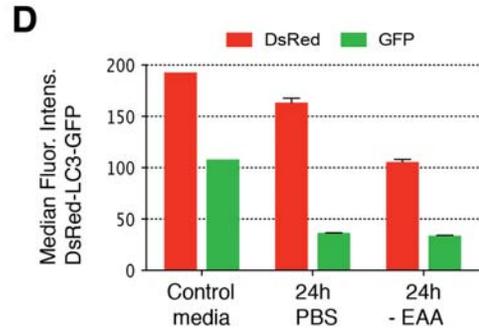
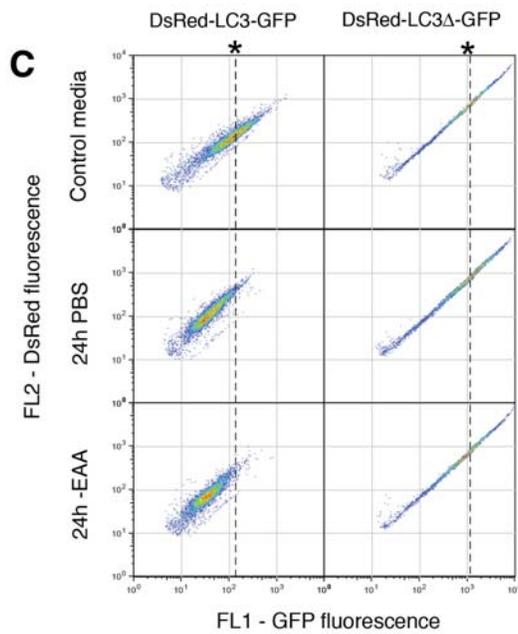
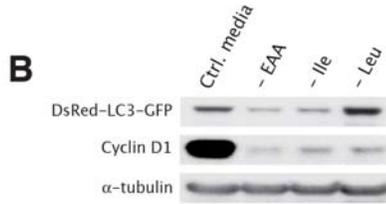
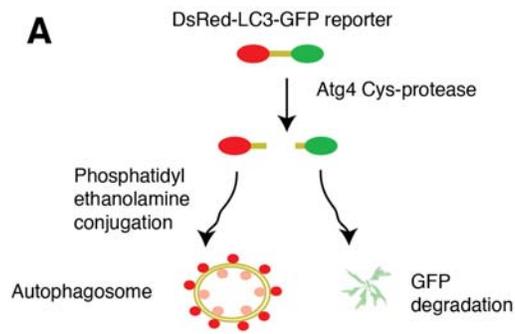


Figure S2, related to Figure 3. Development and validation of a dual color reporter for measuring autophagy activity. (A) Schematic diagram showing the concept behind the DsRed-LC3-GFP autophagy reporter. (B) Immunoblot analyses of the levels of full-length DsRed-LC3-GFP reporter following deprivations of amino acids, isoleucine, or leucine. (C) Flow cytometric data showing changes in levels of fluorescence of GFP upon nutrient deprivation. *, dotted line indicates the median fluorescence intensity of FL1 (Fluorescence 1, green fluorescence from GFP). DsRed-LC3-GFP, the dual-color reporter wild type LC3; DsRed-LC3 Δ -GFP, a control dual-color reporter with mutant LC3 that lacks the TALAV sequence (see Experimental Procedures for additional details). (D, E) Bar graphs indicate the median fluorescence intensities of the DsRed-LC3-GFP and DsRed-LC3 Δ -GFP reporters upon nutrient starvation. (F) The bar graph indicates the autophagy index upon nutrient starvation, which was assessed by measuring relative changes in the median fluorescence intensity of FL1 following normalization with the median fluorescence intensity of FL2 (Fluorescence 2, DsRed fluorescence).

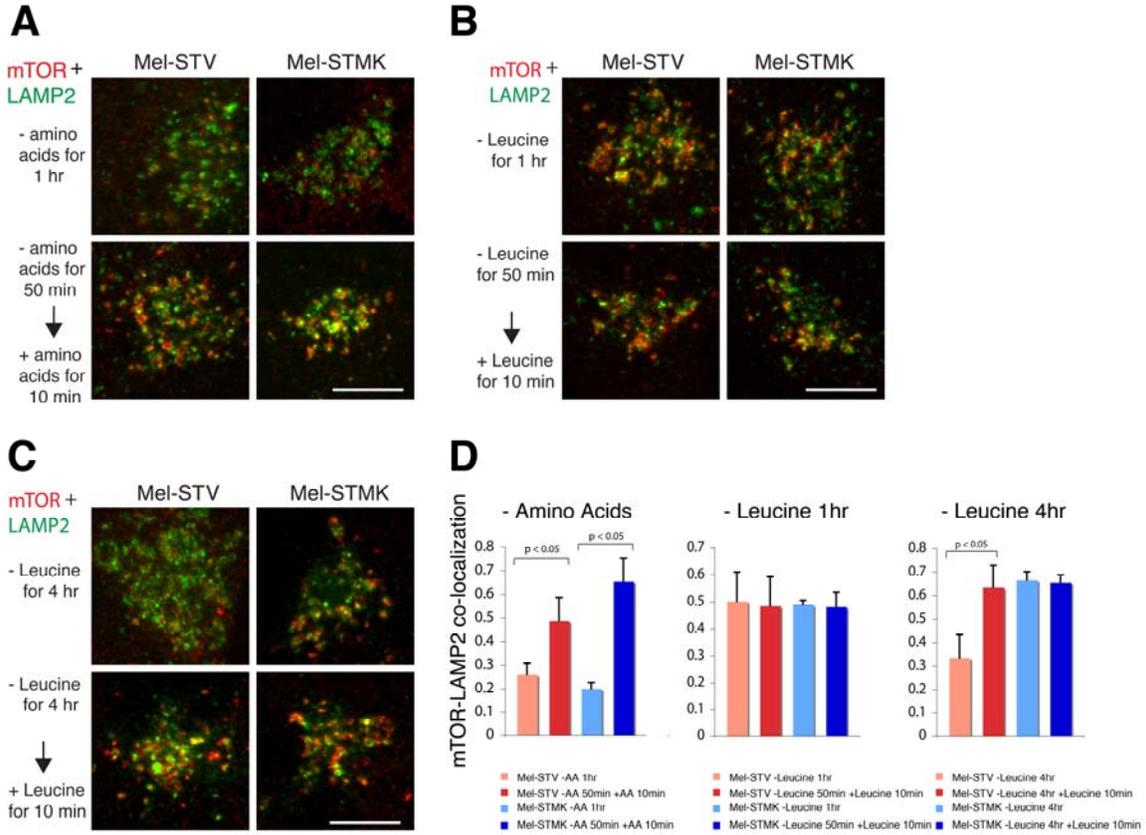


Figure S3, related to Figure 4. Quantitative analyses of the mTOR-LAMP2 co-localization upon deprivations of all amino acids or leucine. (A - C) Representative images of the co-localized mTOR and LAMP2 in Mel-STV cells (left) and Mel-STMK cells (right) co-stained for mTOR (red) and LAMP2 (green). Scale bar = 10 μ m. (D) Bar graphs exhibit fractions of the co-localized mTOR and LAMP2 following deprivations of all amino acids or just leucine. Each value represents the average of 15 cells from 3 independent fields.

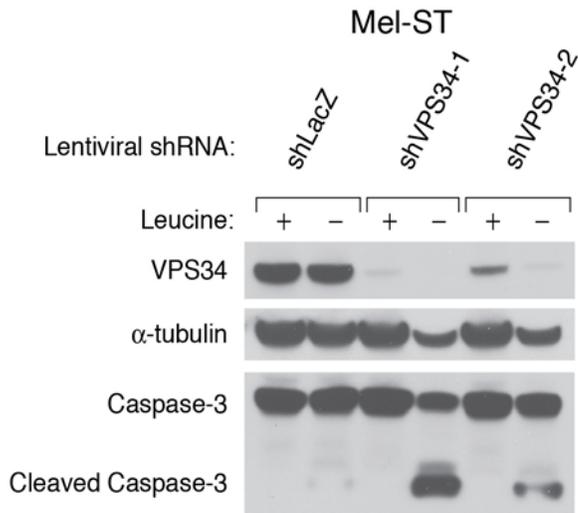
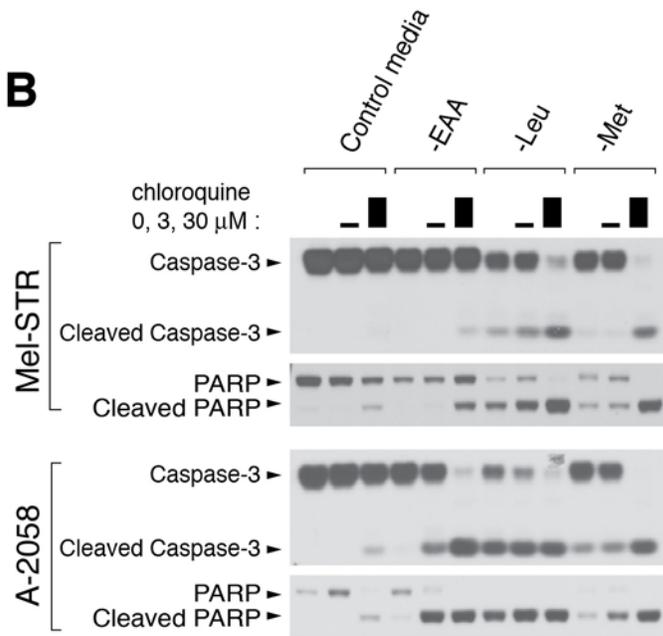
A**B**

Figure S4, related to Figure 5. Autophagy inhibition sensitizes melanoma cells to apoptosis upon deprivation of essential amino acids. (A) Knockdown of *VPS34* sensitizes Mel-ST cells to leucine deprivation. Immunoblots show the decreased level of endogenous VPS34 following shRNA-mediated RNA interference as well as cleavage of caspase-3. (B) Chloroquine synergizes with leucine deprivation (-Leu) in inducing apoptosis of melanoma cells, as well as renders the melanoma cells sensitive to apoptosis upon deprivation of all essential amino acids (-EAA) or just methionine (-Met). Immunoblots demonstrate cleavage and activation of caspase-3 and cleavage of PARP.

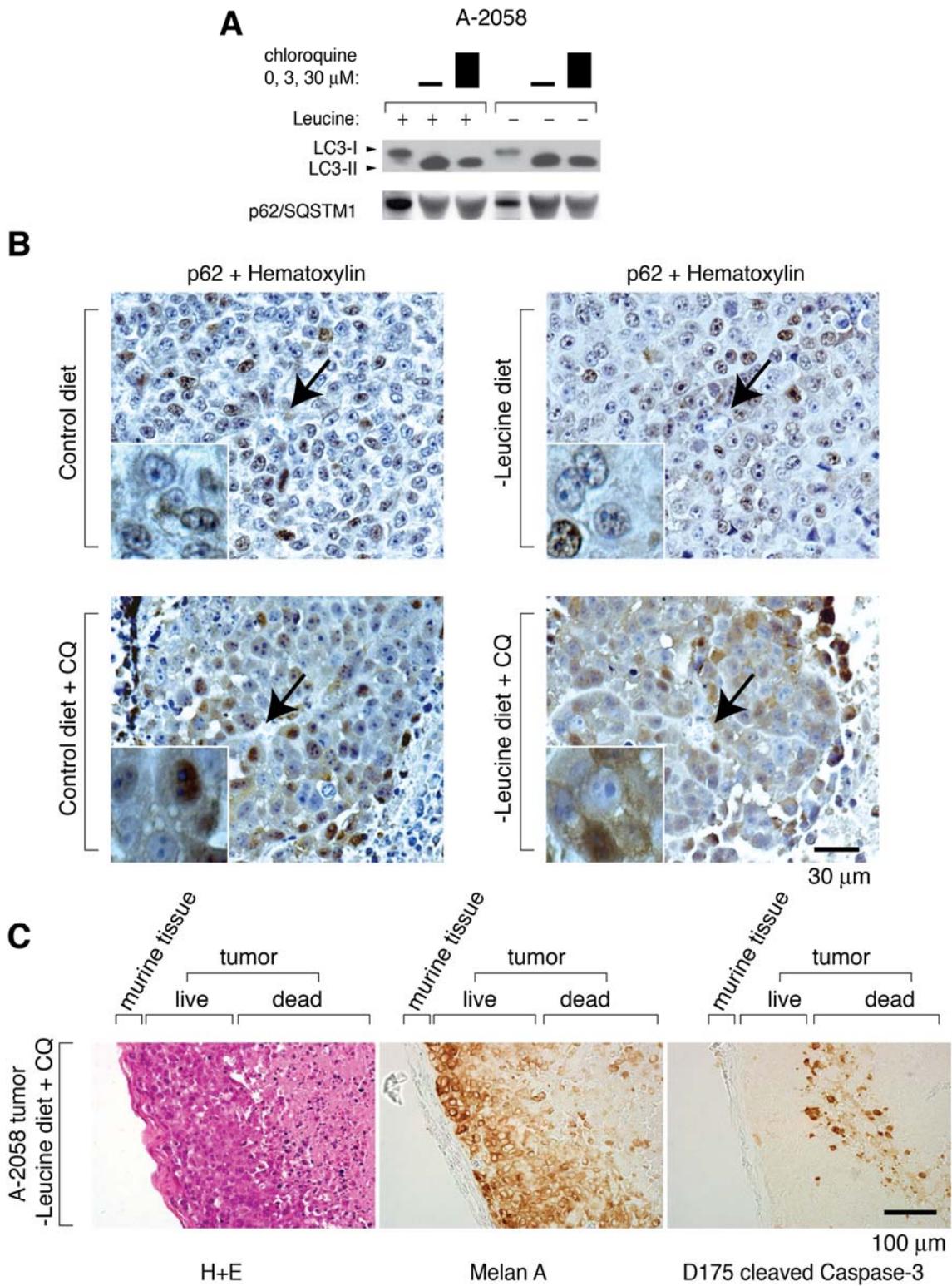


Figure S5, related to Figure 7. Effect of chloroquine on autophagy activity *in vitro* and *in vivo*, and on the activation of caspase-3 *in vivo*. (A) Immunoblot analyses showing changes in autophagy related markers (LC3, p62/SQSTM1) upon leucine deprivation in the presence or absence of chloroquine. (B) Immunohistochemical analyses for p62 in sections of tumor xenografts from animals fed indicated diets and treated with or without chloroquine (CQ). Images were taken at a 400X magnification and insets show the features of 2-3 cells. Capillaries in the tumor section are denoted with arrows. Scale bar = 30 μm . (C) Immunohistochemical analyses for Melan A, a melanocyte specific IHC marker, and D175 caspase site specific cleaved caspase-3 in sections of A-2058 tumor xenografts from animals fed leucine free diet and treated with chloroquine (CQ). Micrographs show the border area between tumors and murine tissues surrounding the xenografts. Scale bar = 100 μm .