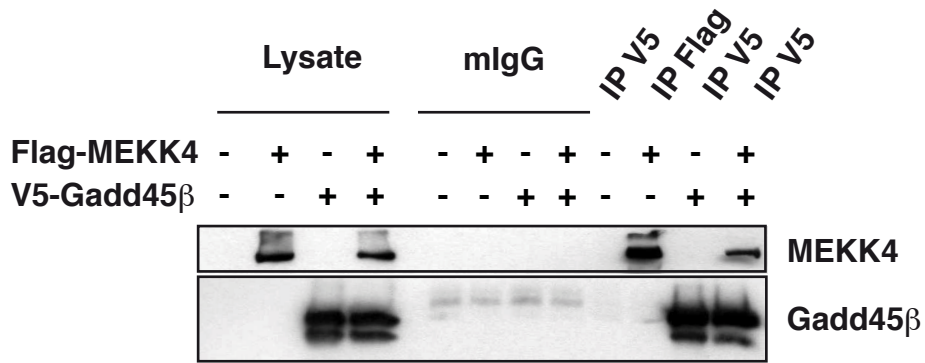
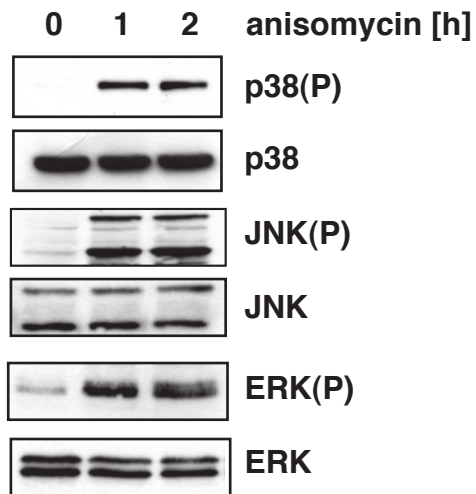
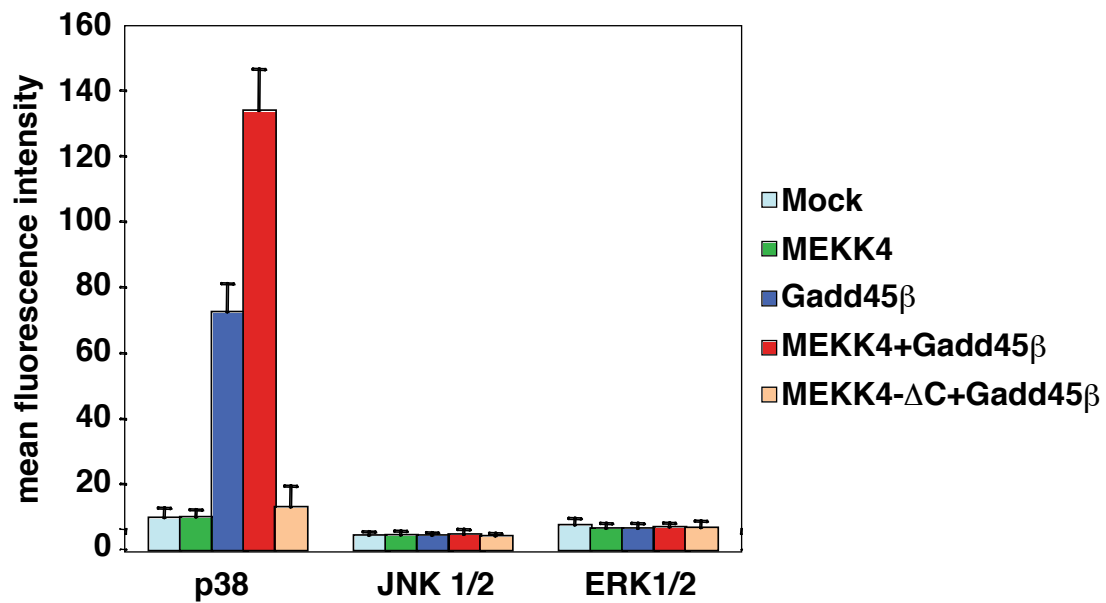


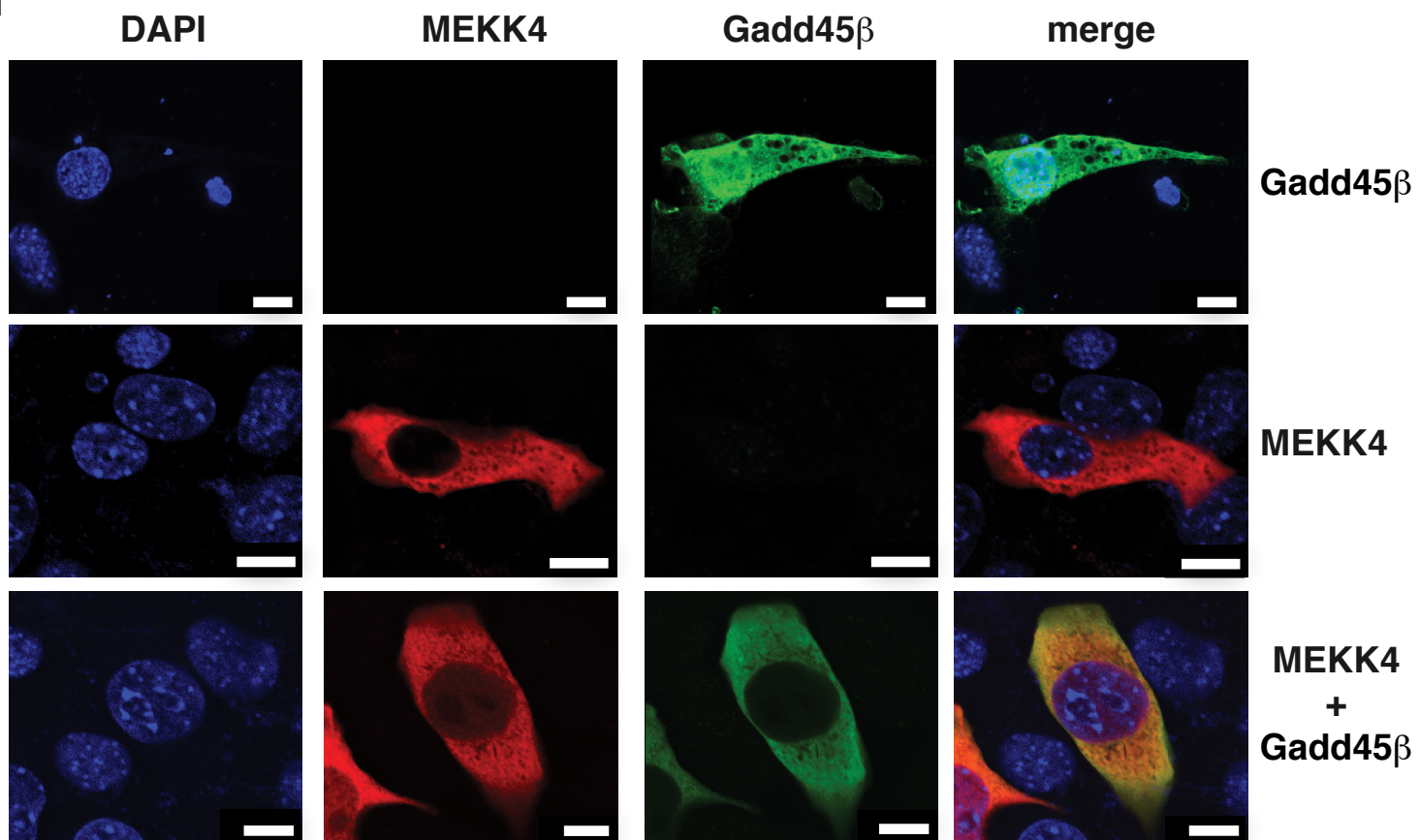
Supplemental information

Keil et al. “Phosphorylation of Atg5 by the Gadd45 β -MEKK4-p38 pathway inhibits autophagy”

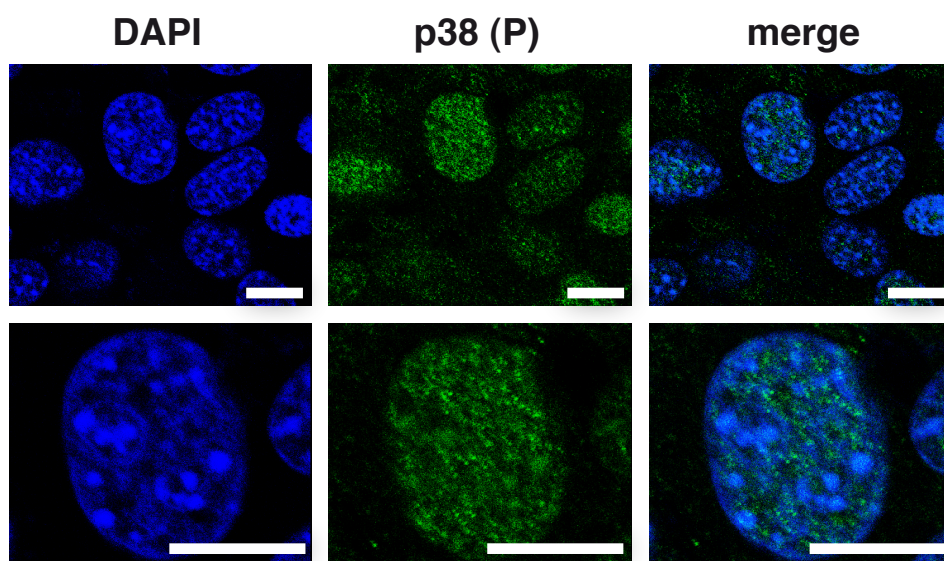
Supplementary Figure 1. (a) NIH/3T3 cells were transfected with V5-tagged Gadd45 β , Flag-tagged MEKK4 or with both constructs. Untransfected cells were used as negative control. Cellular lysates were prepared and used for immunoprecipitation with the indicated tag-specific antibodies or control mouse immunoglobulins (mIgG). Precipitates and lysates were analyzed by immunoblotting for MEKK4 or the V5 tag (Gadd45 β). (b) As a positive control for MAPK activity, NIH/3T3 cells were treated for 1 and 2 hours with 10 μ g/ml anisomycin. NIH/3T3 cellular lysates were prepared and analyzed by immunoblotting using the indicated antibodies. (c) NIH/3T3 cells were transfected as in (a). 24 hours post-transfection, a multiplex analysis of MAPK activation was performed with cytometric bead arrays (CBA) and quantified by FACS. (d) NIH/3T3 cells were transiently transfected with either V5-tagged Gadd45 β or HA-tagged MEKK4 alone or with both constructs. 24 h post-transfection, the cells were fixed and stained with anti-HA (red), anti-V5 antibody (green) and DAPI (blue). Subsequently, samples were analyzed by confocal laser scanning microscopy. Note, that Gadd45 β is localized to the cytosolic and the nuclear compartment when expressed alone but solely to the cytoplasm when co-expressed with MEKK4. (e) NIH/3T3 cells were irradiated with UV light for 1 hour. Then, cells were fixed and stained with anti-phospho-p38 (green) and DAPI (blue). Confocal microscopy demonstrates an exclusively nuclear localization of phosphorylated p38 in UV-irradiated cells. (f) NIH/3T3 cells were transiently transfected with either V5-tagged Gadd45 β and HA-tagged MEKK4, or with V5-tagged Gadd45 β and Myc-tagged MEKK4- Δ C. 24 h post-transfection, the cells were fixed and stained with anti-V5 (red), anti-HA (blue) or anti-Myc antibodies (blue) and anti-phospho-p38 (green). Subsequently, samples were analyzed by confocal microscopy.

a**b****c**

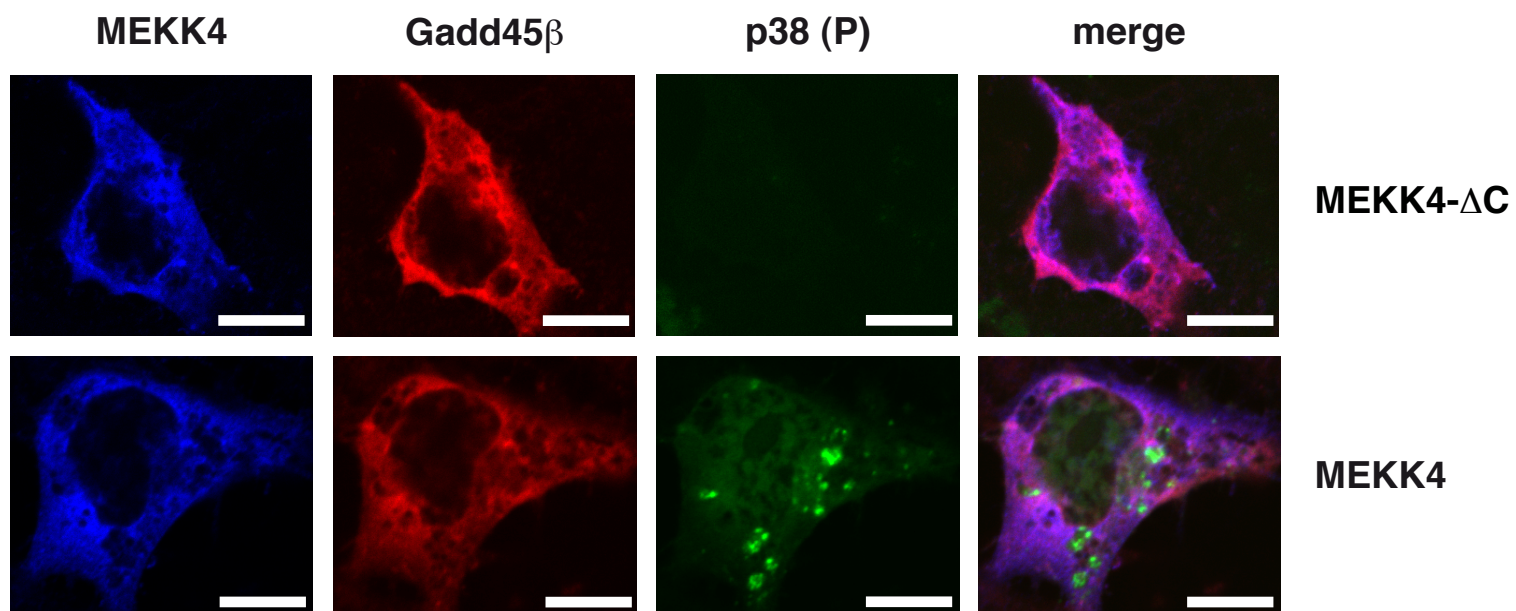
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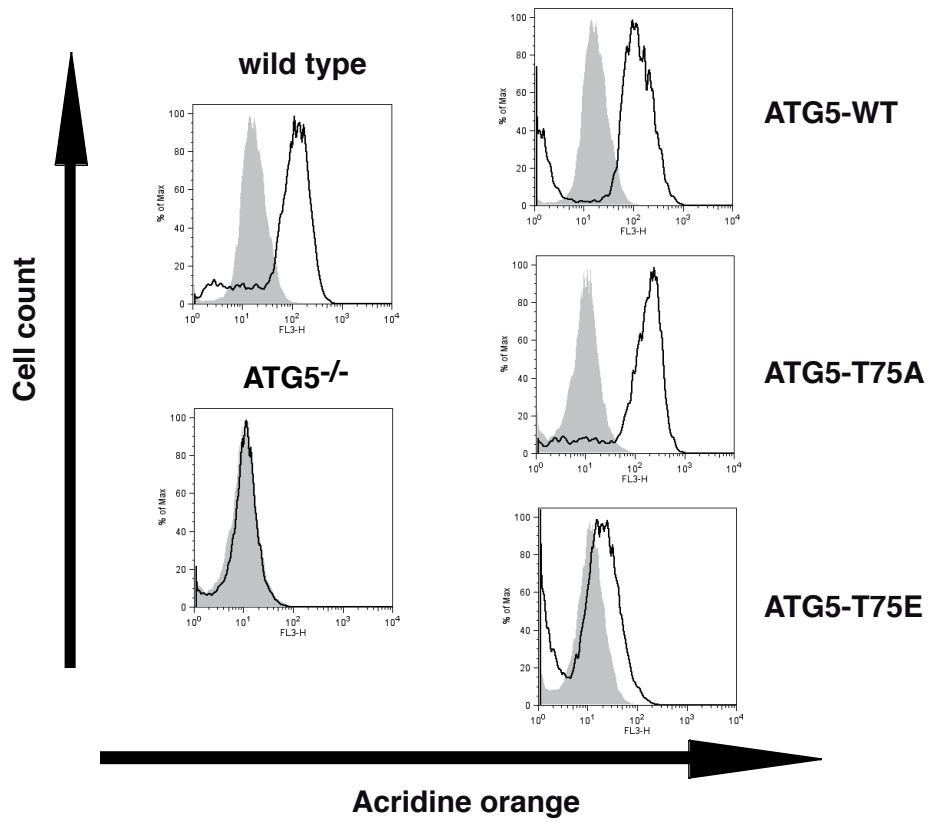
e



f



Supplementary Figure 2. (a) MEFs of the indicated genotypes were cultured for 4 h in HBSS medium to induce autophagy without the use of E64 and pepstatin A (solid line) or left in full culture medium (filled curves). Subsequently, cells were stained with acridine orange and analyzed by FACS to detect the development of acidic vesicular organelles. The acridine orange assay was done as follows: To visualize the acidic vesicular compartment in living cells the lysomotropic fluorescent dye acridine orange was used. Cells were stained with 1 $\mu\text{g/ml}$ acridine orange (Sigma) for 15 min at 37°C in culture medium. Cells were washed in PBS and red fluorescence intensity was quantified on a FACScalibur using CellQuest software (BD Bioscience). (b) Quantification of acridine orange staining as described in (c). Data are represented as mean of three independent experiments \pm s.e.m. While Atg5-deficient MEFs showed basically no autophagic activity, WT MEFs and MEFs reconstituted with WT Atg5 showed the same autophagic activity. The autophagic flux was increased in cells expressing the T75A mutant and reduced in cells expressing Atg5 T75E.

a**b**