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BAT3 modulates p300-dependent acetylation of p53 and autophagy-related protein 7 (ATG7) during autophagy.

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Salwa Sebti, Christine Prébois, Esther Pérez-Gracia, Chantal Bauvy, Fabienne Desmots, et al.. BAT3 modulates p300-dependent acetylation of p53 and autophagy-related protein 7 (ATG7) during autophagy.. Proceedings of the National Academy of Sciences of the United States of America, 2014, 111 (11), pp.4115-20. 10.1073/pnas.1313618111 . inserm-00966883

HAL Id: inserm-00966883

<https://inserm.hal.science/inserm-00966883>

Submitted on 27 Mar 2014

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Supplemental Materials and Methods

Autophagy measurement

Staining of endogenous LC3: 12 μm -thick liver and lung sections of paraffin-embedded WT and *Bat3*^{-/-} E18.5 mouse embryos were deparaffinized, and subjected to heat-induced epitope retrieval in citrate buffer pH 6.0 at sub-boiling temperature for 30 min. Endogenous biotin activity was blocked using the ABC Avidin-Biotin System according to the supplier's instructions (Vector Laboratories, INC., Burlingame, CA, USA). Sections were processed using the M.O.M. Basic Immunodetection kit (Vector Laboratories, INC., Burlingame, CA, USA), and hybridized at 4°C with an anti-mouse LC3B primary antibody at a final concentration of 2.5 $\mu\text{g/ml}$ (clone 5F10 from Nanotools, Teningen, Germany) overnight. Sections were then incubated with FITC-streptavidin (1:100) (Vector Laboratories, INC., Burlingame, CA, USA for 30 min). After three washes in PBS, slides were mounted with Vectashield mounting medium (Vector Laboratories, INC., Burlingame, CA, USA) and analyzed using a Leica DMIRE2 microscope equipped with an oil immersion X63/1.4 apochromatic objective and a 12-bit Coolsnap FX CCD camera (Princeton Instruments), both controlled by the MetaMorph imaging software (Universal Imaging).

Electron microscopy - Cells were immersed in a solution of 2.5% glutaraldehyde in sorenson's buffer (0.1M, pH 7.4) overnight at 4°C. After rinsing in sorenson's buffer, cells were post-fixed in a 0.5% osmic acid for 2 h at dark and room temperature. After two rinses in sorenson's buffer, the cells were dehydrated in a graded series of ethanol solutions (30-100%). The cells were embedded in EmBed 812 using an Automated Microwave Tissue Processor for Electronic Microscopy, Leica EM AMW. Thin sections (70 nm; Leica-Reichert Ultracut E) were collected at different levels of each block. These sections were

counterstained with uranyl acetate and observed using a Hitachi 7100 transmission electron microscope in the Centre de Ressources en Imagerie Cellulaire de Montpellier (France)

GFP-LC3 assay: When required, co-transfections (ratio: peGFP-LC3: plasmid = 1:3) were performed using Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA, USA). Autophagy was measured by counting the number of GFP-LC3 dots/cell (at least 50–100 cells per condition) using an Axioplan microscope (Carl Zeiss, Oberkochen, Germany). When required, Chloroquine (Sigma-Aldrich, St Louis, MO, USA) was used at 20 μ M for 6 hours.

Measurement of long-lived protein degradation - Cells were for 18h at 37C with 0.2 Ci/ml L-[14C]valine. After three rinses with PBS, cells were incubated for 2h with CM or EBSS plus 0.1% BSA and 10 mM cold valine and then precipitated in 10% (v/v) trichloroacetic acid at 4° C. The precipitated proteins were separated from soluble radioactivity by centrifugation at 600g for 10 min. The rate of protein degradation was calculated as acid-soluble radioactivity recovered from both cells and medium.

Staining of endogenous p300

Cells on coverslips were fixed in 2% paraformaldehyde for 10 min, permeabilized with 0.4% Triton X-100 for 5 min and incubated at 37°C with a primary anti-p300 antibody (1:200, N15) for 30 min and with a secondary Alexa488 goat anti-rabbit antibody (Life technologies, Carlsbad, CA, USA) for 20 min. Slides were observed under a fluorescence microscope (Zeiss Axioplan II Imaging) with a 63x oil immersion objective and an AxioCam MRm microscope (Carl Zeiss, Oberkochen, Germany), both of which were controlled by the AxioVision software (Carl Zeiss, Oberkochen, Germany). Fluorescence intensity in the cytosol was quantified with Image J.

Western blotting

The following antibodies were used for western blot analyses: anti-BAT3 1:2000 (Desmots et al., 2005), anti-ATG12 1:1000 (Novus Biologicals, Littleton, CO), anti-LC3 1:5000, anti-ATG7 1:1000 (Sigma-Aldrich, St Louis, MO, USA), anti-p53 1:1000 (1C12, Cell Signaling Technology, Danvers, MA, USA), anti-ERK2 1:2000, anti-p300 1:1000 (N15), anti-ATG5 1:1000 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), anti-acetyl-p53 Lys373 1:1000 and anti-acetyl-p53 Lys320 1:1000 (Upstate Biotechnology, Lake Placid, NY, USA), anti-TBP 1:1000, anti-LAMP2 1:1000 (Abcam, Cambridge, UK) and anti-GAPDH 1:1000 (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA). The appropriate secondary horseradish peroxidase-linked species-specific whole secondary antibodies were next used, and bound antibodies were detected using the ECL Plus Western blotting detection system (GE Healthcare, Waukesha, WI, USA).

Immunoprecipitation

For detection of acetylated ATG proteins, 2 mg of cell lysates were immunoprecipitated with a rabbit anti-acetylated-lysine antibody (1:100) in lysis buffer supplemented with 50 mM TSA and 500 mM nicotinamide (deacetylase inhibitors, Sigma-Aldrich, St Louis, MO, USA) followed by western blotting using an anti-ATG7; -ATG5, -ATG12 or -LC3 antibodies.

Immunoprecipitation

To immunoprecipitate endogenous p300, cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 100 mM NaF, 10 mM NaPPi, 1 mM EDTA, 10% glycerol, 1% Triton X-100 and protease inhibitors) (Complete, Roche Applied Science, Indianapolis, IN, USA) at 4°C for 1 hour, and overnight immunoprecipitation was performed at 4°C with a rabbit polyclonal anti-p300 antibody (1:1000; N15). Protein A Sepharose beads (Amersham Biosciences, GE Healthcare, Waukesha, WI, USA) were added at 4°C for 2 hours

and then washed three times with washing buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol and 1% Triton X-100). Immunoprecipitates were separated by SDS-PAGE gel electrophoresis, and BAT3 or ATG7 were detected by immunoblotting.

Quantitative real time PCR

Real-time PCR was performed in triplicate with the SYBR Green master mix and a Light Cycler 480 system (Roche Applied Science, Indianapolis, IN, USA) with the following cycling parameters: 95°C for 10 min; 40 cycles [95°C 15sec, 60°C 10sec, 72°C 15sec] and then a melting curve protocol. The primers used to amplify *Bat3*, *p53*, *Dram1*, *Dram2*, *Sestrin1*, *Sestrin2*, *Tigar*, *Puma* and *p21* were 5'-TGCTGACAGAGGCAGCTATG-3' and 5'-AGCCTGGAGGTACTGGTGAA-3'; 5'-GCAACTATGGCTTCCACCTG-3' and 5'-CTCCGTCATGTGCTGTGACT-3'; 5'-CCGCGCCGTTAGCTGTCTGT-3' and 5'-CAGAGAGCACCGCGACCACG-3'; 5'-ATGCCTAAAAGTGCAGCGAT-3' and 5'-ACACTCCACCATGTTGACCC-3'; 5'-GTGGACCCAGAACGAGATGACGTGGC-3' and 5'-GACACTGTGGAAGGCAGCTATGTGC-3'; 5'-TCCGAGTGCCATTCCGAGAT-3' and 5'-TCCGGGTGTAGACCCATCAC-3'; 5'-GAAACCCAGTCTCCGAAAGG-3' and 5'-CTTGACCGTTATCCGCCAT-3'; 5'-TGTCGATGCTGCTCTTCTTG-3' and 5'-GTGTGGAGGAGGAGGAGTGG-3'; 5'-ATCACCAGGATTGGACATGG-3' and 5'-CGGTGTCAGAGTCTAGGGGA-3', respectively. The mRNA expression levels were normalized versus the expression level of the two housekeeping genes *Mrs9* and *18S*.

Retroviral infection

Retroviral particles were produced in 293T cells by transfection of gag/pol and env VSV-G and the following viral vector: pSIREN shLuc or pSIREN shp300 or pQCXIH CMV/TO DEST BAT3FL or pQCXIH CMV/TO DEST BAT3ΔNLS using JET-PEI (Polyplus

transfection, Illkirch, France). 48h after transfection, cell supernatants containing viral particles were harvested and added overnight to the culture medium of MEFs with polybrene 8 µg/mL (Sigma-Aldrich, St Louis, MO, USA).

BAT3 FL and BAT3ΔNLS sequences from PCI-HA BAT3 FL and PCI-HA BAT3ΔNLS were sub-cloned into pQCXIH CMV/TO DEST using the Gateway Cloning Technology (Invitrogen, Life Technologies, Carlsbad, CA, USA). *BAT3*^{-/-} MEFs stably expressing BAT3 FL and BAT3ΔNLS were selected using hygromycin B 100µg/ml (Sigma-Aldrich, St Louis, MO, USA). ShRNA directed against mouse p300 mRNA (CCATGTTGCATTCAACTATAA) or firefly luciferase mRNA as a control were cloned into the retroviral vector RNAi Ready pSIREN (Clontech, Mountain View, CA, USA) using manufacturer's instructions. ShRNA expressing cells were selected with 2 µg/mL of puromycin (Sigma-Aldrich, St Louis, MO, USA) 48h after infection.

Supplemental Legends

Figure S1

Quantification of autophagosomes per cell (A) in WT and WT and *ATG7*^{-/-} MEF clones transfected with GFP-LC3 and representative images of GFP-LC3 staining (B). Results are the mean (SD) of the autophagosome number per cell from 3 experiments. Arrows indicate GFP-LC3 dots (i.e., autophagosomal structures).

Figure S2

Quantification of autophagosomes per cell (A) in WT and two different *BAT3*^{-/-} MEF clones transfected with GFP-LC3 and representative images of GFP-LC3 staining (B). Results are the mean (SD) of the autophagosome number per cell from 3 experiments. Arrows indicate GFP-LC3 dots (i.e., autophagosomal structures).

Figure S3

Western blot analyses of p53 acetylation at lysine 373 and lysine 320 in WT and *BAT3*^{-/-} MEFs in CM (-) or EBSS (+) for 30 min. Cell extracts were immunoblotted using anti-p53 acetylated at lysine 373, anti-p53 acetylated at lysine 320 and anti-Actin antibodies.

Figure S4

Western blot analysis of ATG5, ATG12 and LC3 acetylation after immunoprecipitation (IP) of 2 mg of protein lysates using an antibody that recognizes the acetyl-lysine residues in WT and *BAT3*^{-/-} MEFs. Cells were grown in CM (-) or EBSS (+) for 2 hours prior to immunoprecipitation. The densitometric intensity was measured for each condition and compared to WT MEF in CM.

Figure S5

Co-immunoprecipitation of ATG7 with an anti-p300 antibody in WT and two different *BAT3*^{-/-} MEF clones in CM (-) or switched to EBSS (+) for 2 hours. The control is the same as in (Figure 4A).

Figure S6

A) - Representative light microscopic images of endogenous p300 in WT and *BAT3*^{-/-} MEFs. Nuclei were visualized with DAPI stain. (B) - Quantification of the fluorescence intensity of p300 in the cytosol of WT and *BAT3*^{-/-} MEFs. Results are the mean (SD) cytosol fluorescence intensity (in arbitrary units) of 100 cells in five independent experiments.