A haploid mammalian genetic screen identifies UBXD8 as a key determinant of HMGCR degradation and cholesterol biosynthesis

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Extended materials and methods

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Reagents

25-HC was purchased from Steraloids. MG132 and Simvastatin sodium salt were purchased from Calbiochem. [^{14}C]-acetate and [^{14}C]-pyruvate were obtained from American Radiolabeled Chemicals. All other reagents were purchased from Sigma.

Cell culture

Hap1, HepG2, IHH, and Hepa1-6 cells were from the ATCC. Cells were cultured at 37°C and 5% CO_2. HepG2 and Hepa1-6 cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS. Hap1 cells were cultured in Iscove's modified eagle's medium (IMDM) supplemented with 10% FBS. IHH cells were cultured in William's E medium supplemented with 2 mM Glutamine, 10% FBS, 20 mU/mL bovine insulin and 50 nM Dexamethasone, as previously reported [1]. Primary rat hepatocytes were isolated and maintained as described previously [2]. Where indicated, cells were sterol-depleted by culture in sterol-depletion medium (DMEM or IMDM supplemented with 10% lipoprotein-deficient serum (LPDS), 2.5 μg/ml simvastatin, and 100 μM mevalonate to support isoprenoid synthesis) for 24 hrs, or by culture in β-MCD-containing medium (DMEM supplemented with 3 mM β-methyl-cyclodextrin (β-MCD) and 10% LPDS).

CRISPR-Cas9-mediated genome editing

Knockout cell lines were generated by CRISPR/Cas9-mediated genome editing, as described recently [3]. Briefly, cells were co-transfected with pSC-TIA-p2A-Blast and a guide RNA cloned as a *BbsI* fragment into px330 (Addgene #42230). Subsequently, cells were selected with 5 μg/mL Blasticidin. To insert the mNeon reporter cassette into the endogenous *HMGCR* locus we used microhomology-based CRISPR/Cas9-CRIS-PITCh

methodology, as recently described by Nakade *et al* [4]. Briefly, this technique allows integration of a mNeon-2A-PURO cassette in the *HMGCR* locus and selection of puromycin resistant clones. The donor fragment containing the microhomology and mNeon-2A-PURO sequences, as well as the sgRNA guides are shown in Table S1. Independent clones were expanded and correct genome editing was confirmed by sequencing, immunoblotting, and immunofluorescence. The sequences of the guide RNA corresponding oligonucleotides are shown in Table S2.

Plasmids and expression constructs

The plasmids px330 and pENTR/pTER+ (430-1) were from Addgene (#42230 and #17453, respectively). Lentiviral expression plasmids encoding N-terminally FLAG-tagged UBXD8 (WT, ΔUBA, ΔUBX) were a kind gift from Dr. M. van der Weijer and Dr. Emmanuel Wiertz (University of Utrecht). Lentiviral particles were generated and used to obtain Zeocin-resistant target clones. All plasmids used in mammalian transfection experiments were isolated by $CsCl_2$ gradient centrifugation, and the correctness of all constructs used in this study was verified by sequencing.

Antibodies and immunoblot analysis

Total cell lysates were prepared in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 100 mM Tris-HCl, pH 7.4) supplemented with protease inhibitors (Roche). Lysates were cleared by centrifugation at 4 °C for 10 min at 10,000 x g. Samples were separated on NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. Membranes were probed with the following antibodies: ß-actin (Merck, 1:10000), FLAG (Sigma, M2, 1:1000), UBXD8 (Thermo Scientific, 1:1000), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; abcam, 1:1000), Calnexin (Sigma, 1:2000), low-density lipoprotein receptor (LDLR; Biovision, 1:1000), squalene epoxidase (SQLE; Proteintech, 1:1000), HMGCR (ATCC CRL-1811; IgG-A9, undiluted hybridoma supernatant), and ubiquitin (Sigma, U5379, 1:100). Secondary horseradish peroxidase-conjugated antibodies (Invitrogen) were used and visualized with chemiluminescence on a LAS4000 (GE Healthcare). All immunoblots shown are representative of at least three independent experiments with similar results.

Generation and amplification of adenoviral particles

To generate sh*Ubxd8*-adenoviral particles, the pAD-BLOCK-iT^{TM} system (Invitrogen) was used. Briefly, oligonucleotides targeting 3 different regions of Ubxd8 were designed and cloned into pTER+/pENTR (430-1), which had been modified by addition of a CMV-GFP cassette. The resulting pTER+/pENTR-GFP-*mUbxd8*^{shRNA} or pTER+/pENTR-GFP-*Scrambled*^{shRNA} constructs were recombined into pAD-BLOCK-iT^{TM} using Gateway cloning. Adenoviral particles were generated and evaluated as previously reported [5], and the one showing the most potent *Ubxd8* silencing was subsequently amplified, purified and titered by Viraquest.

RNA isolation and qPCR

Total RNA was isolated from cells using a Direct-zol RNA MiniPrep kit (Zymo Research). One microgram of total RNA was reverse transcribed using a cDNA synthesis kit (Biotool). SensiFAST SYBR (Bioline) was used for real-time quantitative qPCR

(qPCR) performed on a LightCycler 480 II system (Roche). Gene expression levels were normalized to the expression level of 36B4. Sequences for qPCR primers are available upon request.

FACS analysis of HMGCR-mNeon

Cells were treated as indicated in the figure legends, dissociated, washed and resuspended in FACS buffer (2 mM EDTA, 0.5% (w/v) BSA in PBS) before analysis on a CytoFLEX Flow cytometer (Beckman Coulter). Viable cells were gated and 10,000 events per condition acquired. Data were analyzed using the FlowJo software package. Relative HMGCR-mNeon intensity was calculated from GEOmean values, and presented as mean ± SD.

Cell Imaging

For confocal imaging, Hap1-HMGCR-mNeon cells were fixed with 4% paraformaldehyde for 10 minutes. Subsequently, cells were incubated with anti-calnexin antibody followed by Alexa 594-conjugated secondary antibody, counter-stained with DAPI (4', 6-diamidino-2-phenylindole), and viewed with A Leica TCS SP8 confocal microscope equipped with a 63x objective. The mNeon images were taken with identical laser power, gain, and offset in order to compare intensities between the different samples. For live cell imaging, we used a Leica IR-BE (Leica Microsystems GmbH) inverted wide field microscope equipped with a 63x objective (Leica Microsystems) and a custom-built incubator (set to 37 °C and 5% CO_2).

Determination of sterol synthesis rate

Cells were sterol-depleted by culturing in β-MCD-containing medium for 16 hrs. Subsequently, cells were pulse-labeled for 45 minutes with 0.5 μCi [^{14}C]-acetate or for 2 hrs with [^{14}C]-pyruvate in the presence of 10 μM 25-HC and 2.5 μg/ml simvastatin, as indicated. Cells were washed twice with PBS and lysed in 0.1 M NaOH. Cell lysates were adjusted for equal protein concentrations and saponified, as described [6]. The non-saponifiable lipid fraction was subjected to scintillation counting to determine radioactive content.

Haploid genetic screen for regulators of HMGCR levels

In order to identify regulators of HMGCR expression and/or degradation, we prepared a library of mutagenized Hap1-HMGCR-mNeon-2A-Puro cells using a gene-trap retrovirus expressing blue fluorescent protein (BFP), as described previously [7]. Briefly, 5x10^8 Hap1-HMGCR-mNeon cells were seeded and transduced with virus from two combined harvests on three consecutive days in the presence of 8 μg/mL protamine sulfate (Sigma). Mutagenized cells were expanded to thirty T175 flasks at a confluence of ~80%. Subsequently, cells were cultured in sterol-depletion medium for 24 hrs and with 10 μM 25-HC and 5 mM mevalonate during the last 2 hrs to stimulate degradation of HMGCR-mNeon. At the end of this treatment, the cells were washed twice with PBS, dissociated with trypsin, pelleted, and fixed with BD Fix Buffer I (BD biosciences) for 10 min at 37 °C. After washing twice with PBS containing 10% FCS, the cells were filtered through a 40 μm strainer (BD Falcon^{TM}) before sorting two populations of cells (i.e. HMGCR-mNeon^{LOW} and HMGCR-mNeon^{HIGH}) that constitute ~5% of the lowest and

highest HMGCR-mNeon expressing cells from the total cell population, respectively (as in Brockman et al.[8]). In addition, in order to reduce potential confounding effects of diploid cells, which are heterozygous for alleles carrying gene-trap integrations, the cells were sorted in parallel for DNA content (1n) by staining with 3 µM 4',6-diamidino-2-phenylindole (DAPI). Cell sorting was carried out on a Biorad S3 Cell sorter until ~10 million cells of each population were collected. Sorted cells were pelleted and genomic DNA was isolated using a DNA mini kit (Qiagen). To assist de-crosslinking of genomic DNA the cell pellets were resuspended in PBS supplemented with Proteinase K (Qiagen) followed by overnight incubation at 56°C with lysis buffer AL (Qiagen) with agitation. Viral insertion sites of each sorted cell population were amplified and mapped as described previously using a Linear AMplification polymerase chain reaction (LAM-PCR) on the total yield of isolated genomic DNA [7]. Samples were subsequently submitted for deep sequencing and gene-trap insertion sites were mapped and analysed as previously described[8]. Briefly, insertion sites were retrieved from trimmed reads (50b) that aligned unambiguously to HG19 using Bowtie allowing for one mismatch [9]. Using intersectBed, aligned reads were mapped to non-overlapping Refseq gene-coordinates. Intragenic gene-trap insertions in sense orientation with its gene were considered disruptive and kept for further analysis. For each gene, the number of unique disruptive insertions was compared between the $mNeon^{low}$ and $mNeon^{high}$ population. Genes that were significantly enriched for insertions in either of the two populations (2-sided Fisher's Exact test with Benjamini-Hochberg multiple testing correction, $p< 0.05$) were considered as regulators of HMGCR-mNeon abundance. To reflect the directionality of the effect on HMGCR-mNeon abundance a mutational index (MI)-score was calculated by counting the number of unique inserts as follows:

$$\left(\frac{\sum inserts\ in\ gene\ X\ in\ mNeon^{HIGH}}{\sum inserts\ in\ mNeon^{HIGH} - \sum inserts\ in\ gene\ X\ in\ mNeon^{HIGH}}\right) \Big/ \left(\frac{\sum inserts\ in\ gene\ X\ in\ mNeon^{LOW}}{\sum inserts\ in\ mNeon^{LOW} - \sum inserts\ in\ gene\ X\ in\ mNeon^{LOW}}\right)$$

For those genes where in only one of the two populations disruptive insertions were identified, 1 insertion was assigned to the other population to prevent these genes to be omitted from the plots.

Cell fractionation

Cell fractionation was done as previously reported [10]. Briefly, cells were cooled on ice, washed 3 times with PBS and subsequently permeabilized for 60 min on ice in permeabilization buffer (PBS supplemented with 5 mM EDTA, 5 mM EGTA, 0.05% (w/v) digitonin, 2 mM PMSF, deubiquitylase-, proteasome- and protease inhibitors). Permeabilized cells were collected and centrifuged at 20,000 g for 30 min at 4 °C to obtain supernatant and crude membrane fractions. The supernatant fraction was further centrifuged at 100,000 g for 30 min to obtain the cytosolic fraction. The 20,000 g crude membrane fraction was solubilized in lysis solution (PBS supplemented with 5 mM EDTA, 5 mM EGTA, 1% Sodium deoxycholate, 1% NP-40, 2 mM PMSF, DUB-, proteasome- and protease inhibitors) and cleared by a 30 min centrifugation at 20 000 g.

The protein content of the different fractions was determined with the BCA assay and the indicated amounts of the different fractions analyzed by immunoblotting.

Statistics

Statistical analyses were performed with the Prism software package. Results were evaluated by Student's t-test when comparing two groups, or by one-way ANOVA for grouped analysis. SD is indicated by error bars and p values are indicated by asterisks: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Materials and Methods references

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