Wnt directs the endosomal flux of LDL-derived cholesterol and lipid droplet homeostasis

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SUPPLEMENTARY INFORMATION

Analysis of the Wnt-induced transcriptional response

Principal component analysis showed that biological triplicates clustered together and that the major source of data variability originated from Wnt3A stimulation (Fig S4A). The addition of Wnt3a resulted in the perturbation of 2224 mRNAs (p<0.01) with 92 of these increasing in abundance greater than two-fold and 18 decreasing by two-fold as compared to values measured in the mock-treated control cells (Fig S5B; Table S1). The transcriptomic analysis was validated by pathway enrichment analysis, which showed that, as expected, Wnt addition significantly changed (p=0.0015) the expression of the Wnt-responsive genes. Intriguingly, pathway analysis revealed that lipid homeostasis (GO:6629, lipid metabolism process; p=5.0x10^(-8)) and cholesterol biosynthesis (GO:6695, cholesterol biosynthetic process; p=2.3x10^(-6)) were among the ontologies with the strongest statistical enrichment (Fig S4C), consistent with our observation that Wnt3a-treatment dramatically affects cellular lipid homeostasis. As discussed, Wnt3A also decreased LDLR mRNA (Fig 4C) and protein (Fig 4D), consistent with the transcriptomic analysis (Table S1; ≈ 1.27-fold reduction, p=0.005).

Wnt-dependent regulation of LD formation: CEs and TAGs

Wnt3A increased the flux of CEs through the endosomes, providing the source of cholesterol for re-esterification by SOAT and incorporation into Wnt-induced LDs (Fig 2-4). In addition, Wnt also increased the mRNA levels of the diacylglycerol O-acyltransferase DGAT2, and stimulated the accumulation of TAGs in LDs (Fig 2E,K).
Moreover, incubation in LPDS, which dramatically reduced the amount of CEs in the presence of Wnt3a, did not prevent TAG esterification (Fig 3C; right), indicating that lipid storage is not strictly dependent on cholesterol. Finally, we found that the LIPA inhibitor Orlistat and the SOAT inhibitor 58-035, which prevented the neo-synthesis of CEs (Fig 3F-G) did not affect TAG accumulation in response to Wnt (Fig. 3G; right). Altogether, these observations further support the notion that Wnt3a upregulates the lipid homeostatic machinery (see Fig S4). These observations also imply the existence of a feedback system able to compensate the disruption of intracellular cholesterol trafficking with TAG accumulation in LDs.

LEGENDS OF THE SUPPLEMENTARY FIGURES

Fig. S1. Image-based screening reveals the Wnt pathway as a regulator of endosomal lipids.

A. Principle component visualization of the screening results. Both canonical Wnt pathway annotated genes (blue; see Table S3) and the remainder of the genome (red) are shown.

B. Key hits in the Wnt pathway; single hits are green and multiple hits blue. The colour of the protein label indicates siRNA toxicity.

Fig. S2. Validation of Wnt-3a-conditioned media and Wnt signaling.

A. Western blot analysis of β-catenin in Hela-MZ and L cells treated with control- and Wnt3a-conditioned medium as indicated.

B. L cells were treated with control- or Wnt3a-conditioned media for 24h before fixation and immunostaining with specific antibodies to β-catenin. The bar is 10 µm.
C. HeLa-MZ cells cultured with control- or Wnt3a-conditioned media for 24h, were fixed, stained with filipin to reveal membrane cholesterol, and imaged by confocal microscopy. Pixel intensities are presented using the indicated lookup table (top right). Size bars = 10 µm.

D. In parallel, images in (B) were captured by automated microscopy and the mean integrated filipin signal was quantified for similarly treated cells. Statistical values are comparison (Student’s t test) with the control conditioned media at the same time of treatment.

Fig. S3. Effect of Wnt3a-treatment on LDs and lipid homeostasis.

A-C. HeLa cells were treated with control- or Wnt3a-conditioned media for 24h before quantitation of: PNPLA2 mRNA by PCR (A), number of lipid droplets per cell with antibodies to PLIN2 (N=30) (B), and incorporation of radioactive oleate into CE and TAG by TLC (C). The qPCR analysis was as in Fig 2K and the data represent ddCt values (number of PCR cycles PNPLA2 and 18S rRNA, normalized to the 0h time point). The number of LDs was quantified as in Fig 2F. The incorporation of [3H] oleate into into CE and TAG was analyzed by TLC as in Fig 2E.

D. Cells were treated with 100 µg/mL of oleic acid in the presence or absence of Wnt3a before fixation and staining with BODIPY. This is a representative image of the data presented in Fig 2I-J.

E. L Cells were treated for 24h with control- and Wnt3a-conditioned media containing lipoprotein-depleted serum (LPDS) before staining with BODIPY and the automated analysis of the number of lipid droplets in each cell performed. Data are the mean for 2 independent experiments.

Fig. S4. Pathway analysis of Wnt3a transcriptome. mRNA was extracted from HeLa-MZ cells treated as in Fig 2A, and analysis using Affymetrix microarrays.

A. A principled component analysis (PCA) of the biological replicates of the microarray experiment.
**Fig. S5. Comparison of previous transcriptomes by others.**

A. Previous gene array data sets examining the effect of Wnt3a on transcription were collected and the enrichment for Lipid Metabolic Process (GO:0006629) determined (black) and compared with that found in this study (grey). Statistical significance (p=0.05; hypergeometric test) is indicated by the dotted grey line. Details of the data sets are in the Materials and Methods.

B. HeLa-MZ cells were treated with control- or Wnt3a-conditioned media for 24h before quantitation of LDLR mRNA by PCR as in Fig 4C. N=3

C. We have analyzed the transcriptome of cells treated with U18666A, which mimics the cholesterol-storage disorder NPC (Chamoun et al, 2013). The panel shows a STRING analysis of the top hits in this U18666A experiment that are also perturbed by Wnt3a treatment. The general functional classes are indicated on the panel by colored circles.

**Fig. S6. Effect Wnt3a-treatment on the endocytic pathway.**

A. Western blotting of proteins extracted from L cells treated with control or Wnt3a conditioned media for 24h.

B. HeLa-MZ cells were treated as above with or without Wnt3a conditioned media before addition of 100 ng/mL EGF for the indicated times. Samples were then analyzed by SDS gel electrophoresis and western blotting using antibodies against EGFR to assess EGFR degradation and against p-Erk to assess EGF signaling response. Antibodies against tubulin were used as a loading control.

C. L cells were treated as above before addition of DND-99 (LysoTracker Red) to assess the presence of acidic compartments. The total cellular DND-99 signal was
quantified and the mean of a representative population ± standard deviations of at least 8500 cells per condition are shown (N=3).

**D.** L cells were treated as above before treatment with 10 mg/mL Texas-Red Dextran for 1h before imaging and cellular quantification to assess endocytosis. Data are the mean ± standard deviation of at least 5000 cells per condition.

**E.** L cells were treated as above before incubation with 2 mg/mL of horseradish peroxidase (HRP) for the indicated times. After homogenization, HRP was quantified in cell extracts using an enzymatic assay and the amounts of endocytosed HRP are normalized to the value measured after 5min in the control. Mean of three independent experiments.

**F.** The influence of the LDLR on VSV infection was studied after binding VSV to the surface of Hela-MZ cells (MOI ~1) for 1 h at 4 °C. Cells were then fixed and stained with anti VSVG antibodies to reveal bound virus. The number of particles per cell was determined by automated imaging and analysis, and the normalized mean particles per cell calculated (mean of 2 independent experiments).

**G.** Hela-MZ cells were treated with siRNAs to LDLR for two days before addition of conditioned media for 24h. Cells were then infected with VSV, fixed and analyzed by automated microscopy Data are the mean index of infection of 2 independent experiments relative to the non-targeting control.

**H.** HeLa-MZ were transfected with plasmids encoding GFP, or LDLR-GFP before 24 h treatment with conditioned media. Cells were then infected with VSV and the infection rate in transfected cells measured by automated microscopy and image analysis. Data are the mean index of infection of 2 independent experiments.
SUPPLEMENTARY TABLES

Supplementary Table 1.

List of the most perturbed genes after Wnt3a treatment.

Supplementary Table 2.

Effect of Wnt3a treatment on the top hits from the U18666A gene array.

Supplementary Table 3.

Collected list of canonical Wnt pathway genes used in this work.

SUPPLEMENTARY REFERENCE