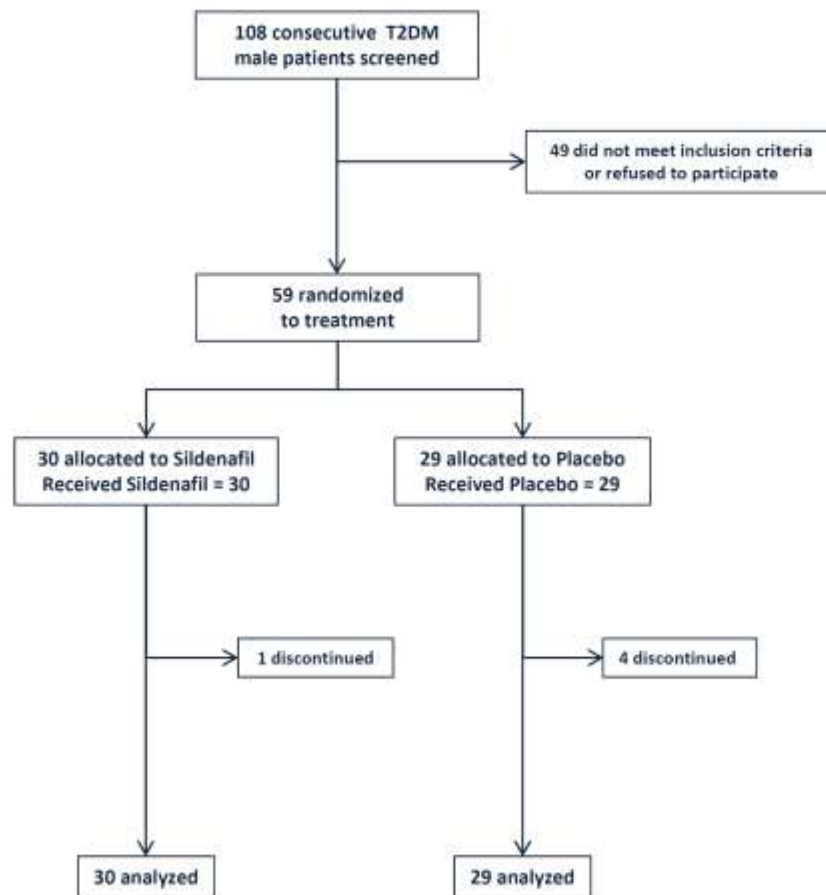
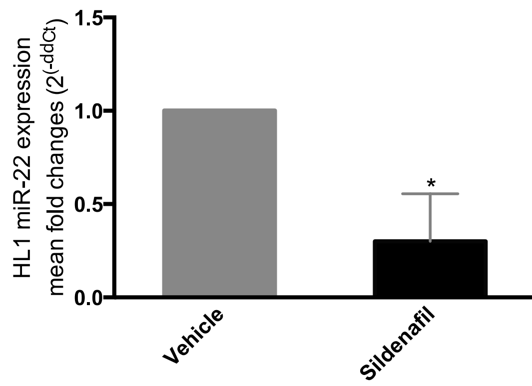
Supplemental Figure 1.

Study flow chart.



Supplemental Figure 2.

Histograms represent fold changes of mir-22 in HL-1 cells. Vehicle-treated (gray) or sildenafil-treated (black b) cells. Results are expressed as mean ± SD. * $P < .05$.



Supplemental Material and Methods.

Human subcutaneous fat biopsies.

Superficial subcutaneous AT samples of ~1–2 cm^3 (corresponding to ~1–2 g) were obtained from the periumbilical area, under local anesthesia (1% xylocaine), from a comparable site of the abdomen. An area 5 cm lateral from the navel (either to the left or right side of the abdomen) was sterilized. 2 mL of a local anesthetic agent was injected through a half circular small dermal (intracutaneous) injection. After 5 min, the skin was sterilized again. A cannula was then adapted to a 20-mL syringe and the piston compressed. Approximately one-third of the length of the cannula was inserted into the subcutaneous fat, and the piston was released and locked, creating a vacuum. Tissue resistance was established by the surgeon gripping the abdominal wall with one hand with the other hand rotating the needle through the tissue in an up-down motion. Once the tissue was aspirated, the cannula was withdrawn and the piston removed. The samples were immediately placed in ice-cold 0.9% (w/v) saline, then frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

Serum miRNA expression analysis of different human serum samples using Agilent Human microRNA Microarrays (one- color).

The miRNA data were normalized, and log2 intensities were calculated. The quantile normalized log2-transformed values were used for comparisons. Fold change expression values were calculated. In the first step of the discriminatory miRNA analysis (DGA), statistical tests were performed to identify any miRNAs differentially expressed between the treatment groups. Statistical differences in the variance between the sample groups are documented by the P-value. To correct for type I error, the Benjamini-Hochberg multiple testing correction method was applied to adjust P-values. In this analysis, a P-value < 0.05 was used to select miRNAs with significant expression differences in the test relative to the reference samples. However, the P-value indicates only a measure of the probability that the two groups are non-identical; it is not a measure of the magnitude of the change, nor of biological relevance. We therefore used a combination of both significance and fold-change criteria to identify biologically meaningful expression changes. In addition to the P-value,

differentially expressed genes were selected by filtering for an at least 1.5-fold difference between the median expression in the test and reference samples. To reduce potentially false positive results, a measure of detectability was included in the analysis. In the discriminatory analysis, the regulated miRNAs were obtained by additional filtering for miRNAs with the majority of intensity values > 5 LU. Inspection of the results of the discriminatory miRNA analysis with ‘standard’ selection methods using statistical tests and effect size appeared too stringent. An alternative DGA selection method which is more robust to highòy variable data, as is the case with individual patient data, was therefore applied. miRNAs were analyzed on individual samples and not by pooling sera from different patients. The alternative selection method was used to identify putative candidate miRNAs with a similar expression trend in the majority of samples.

The following selection criteria were applied to identify similar expression trends for upregulated miRNA: 1) at least 50% of the test samples with higher expression values than the median of the reference group; 2) at least 0.5-fold (log2) higher expression in the lower 25th percentile value of the test sample group compared to the reference samples; 3) at least 2-fold (log2) higher expression in the 75th percentile values of the test samples relative to the reference samples; 4) the majority of samples of the group with higher expression reaching at least 5 LU signal intensity.

The following selection criteria were applied to identify similar expression trends for downregulated miRNA: 1) at least 50% of the test samples with lower expression values than the median of the reference group; 2) at least -0.5-fold (log2) expression of the lower 25th percentile value of the test sample group compared to the reference samples; 3) at least -2-fold (log2) lower expression in the 75th percentile values of the test samples relative to the reference samples; 4) the majority of samples of the group with higher expression reaching at least 5 LU signal intensity.

MicroRNA quantification of circulating miR-22 by real-time quantitative PCR in human samples.

SYBR-green qRT-PCR was used for miRNA quantification in serum samples. Briefly, 30 ng of plasma RNA containing miRNA was polyadenylated by poly(A) polymerase and reverse transcribed to cDNA using miScript II Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Real-time qPCR was

performed using miScript SYBR Green PCR kit (Qiagen) with the manufacturer provided miScript Universal primer and the miRNA-specific forward primers in ABI PRISM 7500 Real-time PCR system (Applied Biosystems). The miRNeasy Serum/Plasma Spike-In Control is a C. Elegans miR-39 miRNA (Qiagen) used as spike reference; Hsa_miR-22_1 miScript Primer Assay (000398, Thermo Fischer) for miR-22 and hsa-miR-39 as candidate reference (000200, Thermo Fisher). Each reaction was performed in a final volume of 10 µL containing 1 µL of the cDNA, 0.5 mM of each primer and 1x SYBR Green PCR Master mix (Qiagen). The amplification profile was: denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 34 s, in which fluorescence was acquired. Each sample was run in triplicate for analysis. The miRNA expression levels were normalized to mir-103. The relative change in miR-22 expression was calculated using the formula: fold change=2^(ddCt)= 2dCt(treated samples)2dCt(control samples), where dCt=Ct(detected miRna)-Ct (miR-103) and Ct is the threshold number.

SIRT-1 levels by PCR in human fat samples.

RNA isolation and analysis: RNA was extracted from frozen human fat biopsies using the RNeasy Lipid Tissue kit (Qiagen UK) according to the manufacturer's instructions. Extraction was followed by a DNAase digestion step to remove any contaminating genomic DNA. Total RNA concentration was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo SCIENTIFIC) and purity was estimated by absorption at 260 nm and 280 nm in an aliquot volume of each sample RNA. 1µg of RNA from each sample was reverse transcribed using high capacity cDNA reverse transcription kit (Applied Biosystem). The total cDNA pool obtained served as the template for subsequent PCR amplification with the following primers: hSirt1 Fw 5-CTGGACAATTCCAGCCATCT and hSirt1 Rw 5-GGGTGGCAACTCTGACAAA, hGAPDH Fw 5-AGGTCGGAGTCAACGGATTT and hGAPDH Rw 5-GTGATGGCATGGACTGTGGT as the internal reaction control. The PCR program used 30 cycles (95 °C, 30 seconds; 52 °C, 30 seconds; 72 °C, 40 seconds).

Tissue Preparation for Flow Cytometry.

For all experiments, VAT was obtained from the omental depot. Tissues were minced with scissors until homogeneous. One mL CollagenaseD (1.5u/mL, Roche Biochemicals,) with Dispase II (Roche Biochemicals, 2.4 u/mL), supplemented in total with 10 mM $CaCl_2$, was applied to each gram of homogenized tissue. The preparation was incubated at 37C for 2 hours then washed and cells were collected by centrifugation at 200 relative centrifugal force for 10 minutes. The floating adipocyte layer and supernatant were removed, and the remaining stromovascular cells were passed through a 40-um cell strainer prior to staining for flow cytometry.

Antibodies and Flow Cytometry.

Stromavascular cells were incubated with primary antibodies using standard methods. We used the following monoclonal primary antibodies: anti-CD31 (BD), anti-CD45 (BD), anti-Sca1 (eBioscience), anti-F4/80- (eBioscience), anti-Mrc1 (R&D System). Cells were stained with propidium iodide (1 µg/mL) and Hoechst 33,342 (2.5 µg/mL) and resuspended at $\sim 1 \times 10^7$ cells/ml density. All samples were analyzed with CyAn™ ADP cytometer (DAKO). The biexponential analysis was performed using Summit V4.3 software and FlowJo X (Treestar) software.

MicroRNA quantification of miR-22 by real-time quantitative PCR in mice fat samples.

The reverse transcription reaction of 10 ng RNA extract was carried out with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and contained 10ng of RNA, 1 mmol/L of each deoxyribonucleotide triphosphate, 50 units of Multiscribe Reverse Transcriptase, 1 reaction buffer, 4 units RNase inhibitor, and 1 miRNA-specific primer. Nuclease-free H_2O was added to a final volume of 15 µL. For the synthesis of cDNA, the reaction mixture was incubated at 16 °C for 30 minutes, followed by an incubation step at 42 °C for 30 minutes. The enzyme was inactivated at 85 °C for 5 min. Afterward, 1.5 µL of the cDNA solution was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems), 1 miRNA-specific primers/probe (hsa-miR-22 000398, Thermo Fischer), and nuclease-free H_2O in a final volume of 10 µL. The quantitative PCR was run on an ABI PRISM 7500 Real-time PCR system (Applied Biosystems) with an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles with a denaturation step at 95 °C for 15 seconds, and an annealing/elongation step at 60 °C for 60 seconds. Each sample was run in triplicate for analysis. The miRNA expression levels were

normalized to U6 snRNA (001973).

SIRT-1 quantification by real-time quantitative PCR in mice fat samples.

RNA isolation and analysis: RNA was extracted from frozen mice fat samples using the RNeasy Lipid Tissue kit (Qiagen UK) according to the manufacturer's instructions. Extraction was followed by a DNAase digestion step to remove any contaminating genomic DNA. Total RNA concentration was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo SCIENTIFIC), and purity was estimated by absorption at 260 nm and 280 nm in an aliquot volume of each sample RNA. 1 µg of RNA from each sample was reverse transcribed using high capacity cDNA reverse transcription kit (Applied Biosystem). The total cDNA pool obtained served as the template for subsequent PCR amplification with the following primers: mSirt1 Fw 5-TCCTTCAGTGTCATGGTTCCT and mSirt1 Rw 5-GCTTCATGATGGCAAGTGGC, mGAPDH Fw 5-GTGAAGGTCGGTGTGAACG and mGAPDH Rw 5-ATTTGATGTTAGTGGGGTC as the internal reaction control. The quantitative PCR was run on an ABI PRISM 7500 Real-time PCR system (Applied Biosystems) with an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles with a denaturation step at 95 °C for 15 seconds, and an annealing/elongation step at 60 °C for 60 seconds. Each sample was run in triplicate for analysis.