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Prolonged *Nrf1* Overexpression Triggers Adipocyte Inflammation and Insulin Resistance

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ABSTRACT

Adipose tissue is currently being recognized as an important endocrine organ, carrying defects in a number of metabolic diseases. Mitochondria play a key role in normal adipose tissue function and mitochondrial alterations can result in pathology, like lipodystrophy or type 2 diabetes. Although *Pgc1α* is regarded as the main regulator of mitochondrial function, downstream *Nrf1* is the key regulator of mitochondrial biogenesis. *Nrf1* is also involved in a wide range of other processes, including proliferation, innate immune response, and apoptosis. To determine transcriptional targets of *Nrf1*, 3T3-L1 preadipocytes were transfected with either pNrf1 or a control vector. Two days post-confluence, 3T3-L1 preadipocytes were allowed to differentiate. At day 8 of differentiation, *Nrf1* overexpressing cells had an increased mtDNA copy number and reduced lipid content. This was not associated with an increased ATP production rate per cell. Using global gene expression analysis, we observed that *Nrf1* overexpression stimulated cell proliferation, apoptosis, and cytokine expression. In addition, prolonged *Nrf1* induced an adipokine expression profile of insulin resistant adipocytes. *Nrf1* has a wide range of transcriptional targets, stimulators as well as inhibitors of adipose tissue functioning. Therefore, post-transcriptional regulation of *Nrf1*, or stimulating specific *Nrf1* targets may be a more suitable approach for stimulating mitochondrial biogenesis and treating adipose tissue defects, instead of directly stimulating *Nrf1* expression. In addition, our results show that short-term effects can drastically differ from long-term effects. J. Cell. Biochem. 111: 1575–1585, 2010.

KEY WORDS: ADIPOCYTES; GENE EXPRESSION; MITOCHONDRIA; NRF1

The incidence of obesity and obesity-associated diseases, like cardiovascular diseases, hypertension, dyslipidemia, and Type 2 diabetes mellitus (T2DM) is rapidly increasing throughout the world [Wild et al., 2004]. Obesity and T2DM are currently being considered world-wide epidemics [Smyth and Heron, 2006]. Adipose tissue is together with skeletal muscle, a key organ in development of both obesity and T2DM. Obesity is characterized by excessive caloric intake that requires increased adipocyte proliferation and adipocyte enlargement. Prolonged excessive adipocyte overload can lead to adipocyte dysfunction, which is characterized by increased macrophage content, inflammation, lipolysis, altered adipokine secretion profile, insulin resistance, and decreased adipocyte differentiation capacity [Guilherme et al., 2008].

Central in normal lipid metabolism are the mitochondria. Mitochondria are responsible for the majority of ATP production in a cell and accomplish a number of important cellular processes, like regulating the balance between fatty acid synthesis, β-oxidation, and lipolysis. These processes are impaired in both skeletal muscle and adipose tissue from T2DM subjects [Mootha et al., 2003; Lowell and Shulman, 2005; Dahlman et al., 2006]. The

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relation between mitochondrial aberrations and abnormal lipid metabolism is also exemplified by observations that m.8344A>G, m.8363G>A, and m.3271T>C mutation carriers, and persons having a mtDNA depletion due to anti-retroviral therapy, may develop lipodystrophy and T2DM as part of the clinical spectrum [Munoz-Malaga et al., 2000; Mallon et al., 2005]. In addition, decreased mitochondrial density and activity, as well as decreased expression of key regulators of mitochondrial biogenesis have been observed in muscle and adipose tissue of T2DM patients [Mootha et al., 2003; Patti et al., 2003; Lowell and Shulman, 2005; Choo et al., 2006]. One of those regulators is peroxisome-proliferator activated receptor γ co-activator 1α (*Pgc1α*). *Pgc1α* is a transcriptional co-activator that is involved in regulation of a number of transcription factors involved in energy metabolism, for example, *PPARγ*, *Nrf1*, *ERRα*, and *CREB*. As a result, *Pgc1α* provides a direct link between external physiological stimuli and the regulation of mitochondrial biogenesis [Arany, 2008]. Thiazolidinediones (TZDs), for example, pioglitazone and rosiglitazone, are anti-diabetic agents that activate the adipogenic transcription factor *PPARγ*, resulting in improved adipose tissue mitochondrial function, insulin sensitivity, and adipokine expression [Bogacka et al., 2005; Choo et al., 2006; Lv et al., 2009; Sugii et al., 2009]. Like pioglitazone, 2 days of *Nrf1* overexpression results in increased adiponectin secretion, which regulates energy homeostasis and glucose and lipid metabolism in adipocytes [Koh et al., 2007].

Although *Nrf1* is mainly regarded as a *Pgc1α* target, which controls *Tfam* expression and mitochondrial biogenesis, altered *Pgc1α* expression does not necessarily influence *Nrf1*. For example, inactivity resulted in decreased *Pgc1α* expression whereas *Nrf1* expression was increased [Timmons et al., 2006], and *Nrf1* is not influenced by *Sirt1*, which is an upstream activator of *Pgc1α* [Gerhart-Hines et al., 2007]. *Nrf1* encodes a phosphorylated nuclear protein with a bZIP binding domain and plays, besides stimulating mitochondrial biogenesis, a crucial role in multiple other processes. Immunoprecipitation assays have shown that *Nrf1* targets ~700 promoters of genes involved in a number of functions, for example, DNA replication and repair, cell proliferation, migration, and apoptosis [Cam et al., 2004]. Taken together, current data indicates that *Nrf1* is more than a *Pgc1α* target and mitochondrial dysfunction in adipose tissue and reduced *Nrf1* are key aspects of T2DM development. In this study we aim to identify the genes that are directly regulated by *Nrf1* in adipocytes, which may help identifying new more specific downstream therapeutic targets for T2DM, or other diseases associated with mitochondrial dysfunction.

MATERIALS AND METHODS

CELL CULTURE

Mouse 3T3-L1 preadipocytes (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% FCS (Gibco) and penicillin/streptavidin (Gibco). As shown in Figure 1, 3T3-L1 preadipocytes were transfected at 70% confluence with pSPORT-Nrf1 (Openbiosystems) or pEGFP control vector with Fugene HD transfection reagents (Roche) according to the manufacturers' protocol. Transfection was repeated at days 0 and 4 of differentiation. Differentiation was induced 2 days post-confluence by changing medium with DMEM containing 0.5 mM 3-isobutyl-1-methylxantine (IBMX) (Sigma), 10 μg/ml insulin (Sigma) and 1 μM dexamethasone (Sigma). After 2 days, medium was replaced with DMEM containing 10% FCS and 10 μg/ml insulin, and changed every 2 days till day 8.

MTDNA COPY NUMBER DETERMINATION

DNA was isolated using the Qiagen DNA isolation kit according to the manufacturers' protocol (Qiagen, Hilden, Germany). The mtDNA content was determined by comparing the ratio of mtDNA (ND1) to nDNA (18S rRNA) in triplo by real-time quantitative PCR. DNA was amplified in a 12.5 μl reaction containing 5 ng DNA, 1.25 pmol of forward and reverse primer, and ABI SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA). The cycling conditions were: an initial step for 2′ at 50°C, activation of the Hot Goldstar enzyme at 95°C for 10′, 40 cycles of 15″ at 95°C followed by 1′ at 60°C (denaturation, annealing, and elongation).

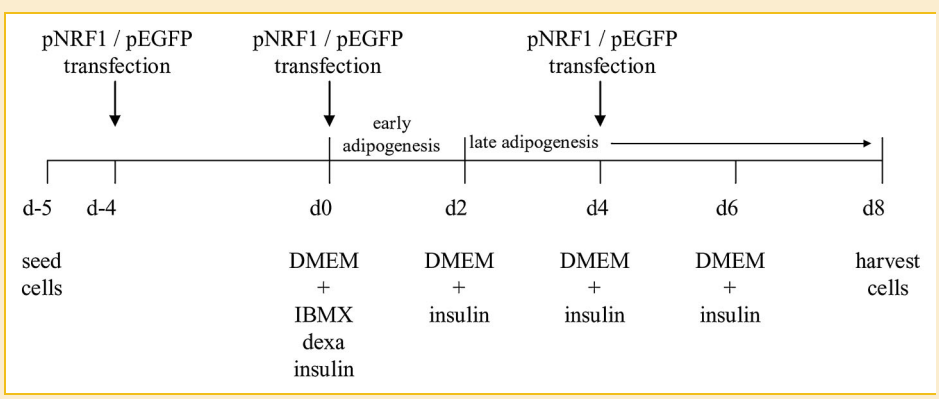

Fig. 1. Experimental setup 3T3-L1 cells were seeded at day-5 and 70% confluent at day-4 when 3T3-L1 cells were transfected with either pSPORT-Nrf1 overexpression construct or control pEGFP vector. Two days postconfluence, at day 0, transfection was repeated and differentiation mix was added. Cells were differentiated for 8 days with refreshing medium + insulin every other day. IBMX, 3-isobutyl-1-methylxanthine; dexa, dexamethasone.

GENE EXPRESSION ANALYSIS

RNA isolation and DNAse treatment were performed with the high pure RNA isolation kit (Roche) according to the manufacturers' protocol. RNA quantity and purity were determined spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE) and RNA integrity was assessed by determining the RNA 28S/18S ratio using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Mouse adipocyte RNA spiked with four bacterial RNA transcripts was reverse transcribed into cDNA and amplified in a two-round amplification reaction according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). A mixture of cDNA and added hybridization controls was hybridized on Affymetrix Mouse Genome 430 2.0 arrays, followed by staining and washing steps in the GeneChip fluidics station 400 (Affymetrix) according to the manufacturer's procedures. To assess the raw probe signal intensities, chips were scanned using the GeneChip scanner 3000 (Affymetrix).

DETECTION OF OVER-REPRESENTED TRANSCRIPTION FACTOR BINDING SITES

The online tool oPOSSUM V2.0 was used to identify over-represented transcription factor binding sites in the promoters of differentially expressed genes [Ho Sui et al., 2005]. All genes with >20% up- and down-regulated expression in NRF1-overexpressing 3T3-L1 cells at day 8 of differentiation were uploaded in oPossum. Settings were as followed: Taxonomic supergroup: vertebrates, top 10 level of conserved regions (min. conservation >70%), matrix match threshold 80%, 2,000 bp up- and downstream sequence, *z*-score ≥ 10.

STATISTICAL ANALYSIS

Statistical analysis was performed using the freely available program R [Ihaka and Gentleman, 1996] and the publicly available library "growth" [Lindsey, 1999]. The chip description file (CDF) used for the analysis was an update [Dai et al., 2005; http://brainarray.mbni.med.umich.edu] based on Ensembl (version 10). This resulted in the analysis of 15768 gene-transcripts. All genes were analyzed using a Gaussian linear regression $N(\mu, \sigma^2)$, where μ is the mean and σ^2 is the variance, including the chip's log mean intensity and the best hybridization spike. The inference criterion used for comparing the models is their ability to predict the observed data, that is, models are compared directly through their minimized minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (AIC) [Akaike, 1973]. The transcript under consideration was found to be differentially expressed, if the AIC decreased compared to the model not containing the group effect, the confidence interval did not include a fold change of 1, there was at least a 20% fold change increase or decrease, and each group had a mean signal intensity >200 or >300 in one group. Visualization and analysis of microarray data on biological pathways was performed using the Gene Map Annotator and Pathway Profiler (GenMAPP, version 2.0) [Salomonis et al., 2007]. Gene database Mm-std_20070817 and MAPPs version Mm_contributed_20080619 were used. Significantly changed pathways were identified using the MAPPFinder program, which expresses a "*z*-score" for each pathway using Fisher's exact test. *z*-scores >1.96 and permute $P \leq 0.05$ were considered to be significant [Doniger et al., 2003]. In addition, gene ontology (GO) based analysis of biological processes of level 5 were analyzed in DAVID [Dennis et al., 2003].

QUANTITATIVE REAL-TIME PCR

cDNA was generated from 1 μg RNA in a standard reverse transcriptase reaction using M-MulV reverse transcriptase (Finnzymes, Espoo, Finland). Primers were designed using Primer Express® software version 3.0 (Applied Biosystems; for primer sequences, see Electronic Supplementary Material, ESM 4). Quantification of transcripts was carried out using the ABI 7900 HT Real-Time PCR detection system using Eurogentec qPCR Mastermix Plus for SYBR Green® I (Eurogentec, Seraing, Belgium). The cycling conditions were: an initial step for 2′ at 50°C, activation of the Hot Goldstar enzyme at 95°C for 10′, 40 cycles of 15″ at 95°C followed by 1′ at 60°C. The mRNA levels of each gene were normalized to those of the housekeeping gene encoding the Cyclophilin. Primer sequences for the analyzed genes can be found in the Electronic Supplementary Material (ESM 4).

ATP PRODUCTION AND CASPASE 3/7 ASSAYS

ATP production in 3T3-L1 cells transfected with pSPORT-Nrf1 or pEGFP (control) was measured at day 8 using the CellTiter-Glo luminescent Cell viability assay (Promega, Madison, WI) according manufacturer's protocol. ATP production was corrected for cell density by quantification of dsDNA determined with the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA). Caspase 3/7 activity was measured at day 8, after trypsinization, cell were counted and an equal amount of cells per well were used for quantification of caspase 3/7 activity using the Caspase-Glo®3/7 assay (Promega) according to manufacturer's protocol.

CRYSTAL VIOLET STAINING

Cell numbers were assessed by the crystal violet assay. Cells were fixed for 1 h in 3.7% paraformaldehyde in PBS. They were stained for 30 min with a 0.1% solution of crystal violet, and destained five times in demineralized water. The dye was released with 10% acetic acid at RT for 30 min, and the A_{590} signal was then read using the Multiskan Spectrum microplate spectrophotometer (Thermo Fisher Scientific).

ELECTRON MICROSCOPY

Cells were fixed overnight in 2.5% glutaraldehyde (Merck, Darmstadt, Germany), post-fixed in 1% osmium tetroxide solution, dehydrated, and embedded in epoxy resin. Semi-thin (1 μm) serial sections were stained with toluidine blue. Ultra-thin sections (70–90 nm) were mounted on Formvar-coated 75 mesh copper grids (1595 E, Merck, Amsterdam, The Netherlands), and counterstained with uranyl acetate and lead citrate before analysis on a Philips CM100 transmission electron microscope (Philips, Eindhoven, The Netherlands). Quantitative analysis of mitochondrial size was carried out using freely available Image J software (version 1.42q).

RESULTS

Mouse 3T3-L1 preadipocytes were transfected with either a vector containing *Nrf1* or a control vector without Nrf1 (pEGFP), and were allowed to differentiate for 8 days (Fig. 1). Mitochondrial DNA copy number and gene expression of *Nrf1* and adipogenic regulators were determined at several time points after transfection and during differentiation to test if the transfection showed the expected result. On day 8 of differentiation, analysis of mitochondrial function, density, and morphology were analyzed together with global gene expression levels to determine transcriptional targets of *Nrf1*.

EFFECT OF NRF1 OVEREXPRESSION ON MITOCHONDRIAL PARAMETERS IN DIFFERENTIATING PREADIPOCYTES

MtDNA copy number was quantified by the ratio between mitochondrial encoded *ND1* and nuclear encoded *18S rRNA*. The mitochondrial DNA copy number in 3T3-L1 adipocytes was significantly increased ($P = 0.01$; $n = 4$) in pNrf1 overexpressing cells (1.53 ± 0.3-fold) compared with control pEGFP transfected cells. To identify if the mtDNA was continuously increased during differentiation, we analyzed the mtDNA content at days 0, 3, 6, and 8, and observed only a significantly higher mtDNA content in pNRF1-transfected cells at days 6 and 8, but not earlier (data not shown). To evaluate if *Nrf1* overexpression resulted in increased ATP production, we measured ATP production at day 8 of differentiation in *Nrf1* overexpressing and control pEGFP-transfected 3T3-L1 adipocytes. As shown in Figure 2, increased ATP production was observed in *Nrf1*-overexpressing cells. However, after correcting for number of cells, no difference was observed in ATP production. Since cells were seeded at same density, increased cell number in *Nrf1*-overexpressing cells suggests increased proliferation due to *Nrf1* overexpression. Crystal violet staining at days 0 and 8 showed indeed a 13% ($P = 0.07$) and 36% ($P < 0.01$) increased cell number during differentiation in *Nrf1* transfected wells. Furthermore, electron microscopic analysis of mitochondrial morphology showed that *Nrf1*-overexpressing cells had enlarged (Figs. 5 and 6) and more electron dense mitochondria than pEGFP-transfected cells at day 8 of differentiation (Fig. 5).

DIFFERENTIALLY EXPRESSED GENES DUE TO NRF1 OVEREXPRESSION IN PREADIPOCYTES

The effect of *Nrf1* overexpression on gene expression was assessed by comparing four replicates of 3T3-L1 cells transfected with pNrf1 overexpression construct with four replicates transfected with pEGFP as controls on day 8 of differentiation. Gene expression analysis was performed using Affymetrix Mouse Genome 430 2.0 arrays, and after correction for probe set annotation, 15,768 transcripts were analyzed. Using normal regression modeling, a total of 1,926 transcripts were found differentially expressed between control cells and *Nrf1* overexpressing cells with a fold-change of minimal 20%. From these 1,926 transcripts, 920 transcripts were increased, and 1,006 transcripts were decreased in *Nrf1* overexpressing 3T3-L1 cells. The majority of the differentially expressed genes had a fold change of less than twofold up or down. From the 1,006 transcripts with decreased expression, 51 (5.1%) had a fold change <0.5, and the most extreme down-regulated probe had a fold change of 0.22. Of the 920 up-regulated transcripts, 76 transcripts (8.3%) were more than two times higher. A few of the increased genes had an extreme fold change with a maximum of 57-fold. The distribution of the fold changes is depicted in electronic supplementary material (ESM) 1, and a list of the 10 most severely up- and down-regulated genes is presented in Table I.

We used a process-based approach to identify differentially expressed pathways in adipocytes due to overexpression of *Nrf1*. The 1,926 differently expressed genes that could be annotated were used

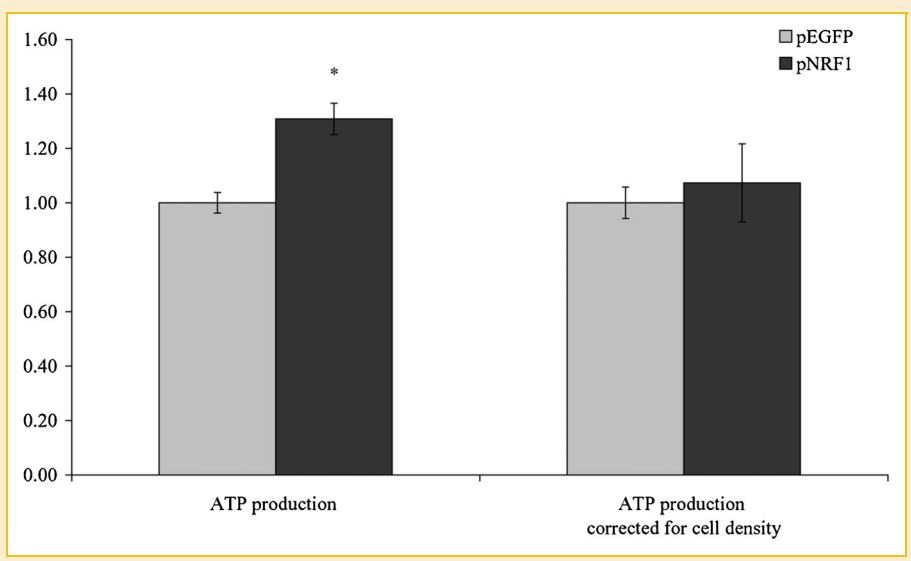

Fig. 2. ATP production is similar in pNrf1-transfected and empty vector transfected adipocytes 3T3-L1 cells were seeded at the same density, transfected at 70% confluence with pNrf1 or pEGFP and differentiation was induced 2 days post-confluence. ATP measurements were performed at day 8 of differentiation. $n = 4$, $^{*}P < 0.05$.

TABLE I. Top 10 Most Up- and Down-regulated Transcripts in *Nrf1*-overexpresing Cells

Name	Symbol	Fold change *Nrf1*-overexpressing cells
Down-regulated in NRF1 overexpressing cells		
Adiponectin	*Adipoq*	0.22 (0.11–0.43)
Na+/K+ transporting ATPase subunit beta-1	*Atp1b1*	0.22 (0.21–0.24)
Leucine-rich repeat-containing protein 17 precursor	*Lrrc17*	0.24 (0.21–0.28)
Type-2 angiotensin II receptor	*Agtr2*	0.25 (0.24–0.27)
Matrix Gla protein	*Mgp*	0.26 (0.25–0.27)
Fatty acid-binding protein 4	*Fabp4*	0.27 (0.24–0.32)
ATP-binding cassette sub-family A member 8-A	*Abca8a*	0.28 (0.27–0.29)
Dermatopontin	*Dpt*	0.31 (0.29–0.33)
DNA-binding protein inhibitor ID-2	*Id2*	0.31 (0.29–0.33)
Small proline-rich protein 1A	*Sprr1a*	0.31 (0.30–0.32)
Up-regulated in NRF1 overexpressing cells		
Cd74 antigen	*Cd74*	5.06 (4.22–6.08)
Chitinase 3-like 1	*Chi3l1*	5.17 (4.96–5.39)
Chemokine (C-X-C motif) ligand 11	*Cxcl11*	5.34 (5.15–5.53)
Complement factor B	*Cfb*	6.99 (6.67–7.33)
Prolactin family 2, subfamily c, member 4	*Prl2c4*	7.20 (6.13–8.47)
Lipopolysaccharide binding protein	*Lbp*	10.4 (10.2–10.7)
Chemokine (C-X-C motif) ligand 5	*Cxcl5*	10.5 (9.22–12.0)
Haptoglobin	*Hp*	21.8 (20.8–22.8)
Serum amyloid A3 protein	*Saa3*	35.4 (33.0–37.8)
Lipocalin 2	*Lcn2*	56.8 (53.0–60.8)

TABLE II. qPCR Analysis Supported the Gene Expression Changes Identified by Microarray Analysis

Gene	Symbol	Fold change microarray (±CI)	Fold change qPCR (±CI)
Fatty acid-binding protein 4	*Fabp4*	0.27 (0.24–0.32)	0.31 (0.26–0.39)
Superoxide dismutase 2	*Sod2*	3.35 (3.19–3.52)	1.82 (1.65–2.00)
Ceruloplasmin	*Cp*	4.22 (4.12–4.34)	4.00 (3.36–4.75)
Haptoglobin	*Hp*	21.8 (20.8–22.8)	215 (179–257)
Serum amyloid A-3 protein	*Saa3*	35.4 (33.0–37.8)	369 (312–437)
Adiponectin	*Adipoq*	0.22 (0.11–0.43)	0.22 (0.18–0.26)
Matrix Gla protein	*Mgp*	0.26 (0.25–0.27)	0.31 (0.27–0.35)

for gene ontology based analysis using the GenMAPP/MAPPFinder program, which performs global analysis of expression data in the context of hundreds of pathway MAPPs and thousands of Gene Ontology (GO) terms, calculates the cumulative total of genes changed for a local MAPP or term, and provides a statistical *z*-score to assess significance (Table II, ESM 2). Eight pathways (Table IV) were found significantly changed with MAPPFinder analysis with a *z*-score >1.96 and permute $P \leq 0.05$. In addition, 10 GO biological processes at level 5 were found significantly changed ($P \leq 0.05$) in pNrf1 transfected cells, and comprised cell-cycle regulation, inflammation and apoptosis related genes, as seen in the Mappfinder analysis (ESM 3).

DETECTION OF POTENTIAL NRF1 OVERREPRESENTED TRANSCRIPTION FACTOR BINDING SITES

Nrf1 contains a bZIP domain and oPOSSUM analysis of the up-regulated genes in *Nrf1* overexpressed cells at day 8 of adipogenesis showed that the hepatic leukemia factor (HLF), which belongs to the bZIP class of transcription factors, was solely enriched (Table III). Subsequent GO biological process analysis of all 203 up-regulated genes containing the bZIP domain showed they were involved in proliferation and cell-cycle regulation, apoptosis and chemokine/cytokine signaling (ESM 5). Within the group of down-regulated genes, a more heterogeneous group of five transcription factor binding sites were overrepresented (Table III).

TABLE III. Overrepresented Transcription-Factor Binding Sites in *Nrf1*-induced Genes

Transcription factor (TF)	TF class	No. target gene hits	*z*-score
Up-regulated genes			
Hepatic leukemia factor (HLF)	bZIP	203	12.3
Down-regulated genes			
Serum response factor (SRF)	MADS	57	20.1
Sex-determining region Y (SRY)	HMG	579	12.1
Forkhead box q1 (Foxq1)	FORKHEAD	273	11.6
Aryl hydrocarbon receptor nuclear translocator/receptor (Arnt-Ahr)	bHLH	625	11.3
SRY-related HMG-box (Sox5)	HMG	586	11.1

TABLE IV. Identification of Significantly Changed Pathways by MAPPFinder Analysis

MAPP name	% Changed	% Present	z-Score	PermuteP
Mm_Adipogenesis	29	92	4.28	0.00
Mm_Apoptosis	28	96	3.09	0.00
Mm_Id_NetPath_5	30	94	2.89	0.01
Mm_Delta-Notch_NetPath_3	28	87	2.88	0.01
Mm_Hypertrophy_model	38	80	2.48	0.03
Mm_Matrix_Metalloproteinases	33	96	2.48	0.02
Mm_TGF_Beta_Signaling_Pathway	27	94	2.30	0.03
Mm_Fas_Pathway_and_Stress_Induction_of_HSP_Regulation	29	92	2.21	0.04

z-score values >1.96 and Permute $P \leq 0.05$ were considered to be significant.

EXPRESSION ANALYSIS DURING ADIPOGENESIS

A decreased adipogenic capacity of *Nrf1*-transfected cells could also explain our results, to test this, we transfected 3T3-L1 cells with either pNrf1 or control pEGFP, and harvested at different time-points during adipogenesis. In this way we could determine if differential expression at day 8 of adipogenesis was directly caused by *Nrf1* overexpression or was a secondary result. At days 0, 3, 6, and 8, qPCR analysis was performed of the key regulators of adipogenesis, *Pparγ* and *C/EBPα*. As shown in Figure 3, expression of these two key regulators was not significantly different during adipogenesis. Moreover, *Pparγ* expression was slightly increased in *Nrf1* overexpressing cells at all time-points, but this was only significant at day 3 of differentiation. In addition, expression of superoxide dismutase 2 (*Sod2*), Ceruloplasmin (*Cp*) and Serum amyloid A-3 (*Saa3*) were higher in *Nrf1*-overexpressing cells at minimal three of the four time-point, while adiponectin and Haptoglobin (*Hp*) were only altered at day 6 and/or 8 of differentiation (Fig. 4).

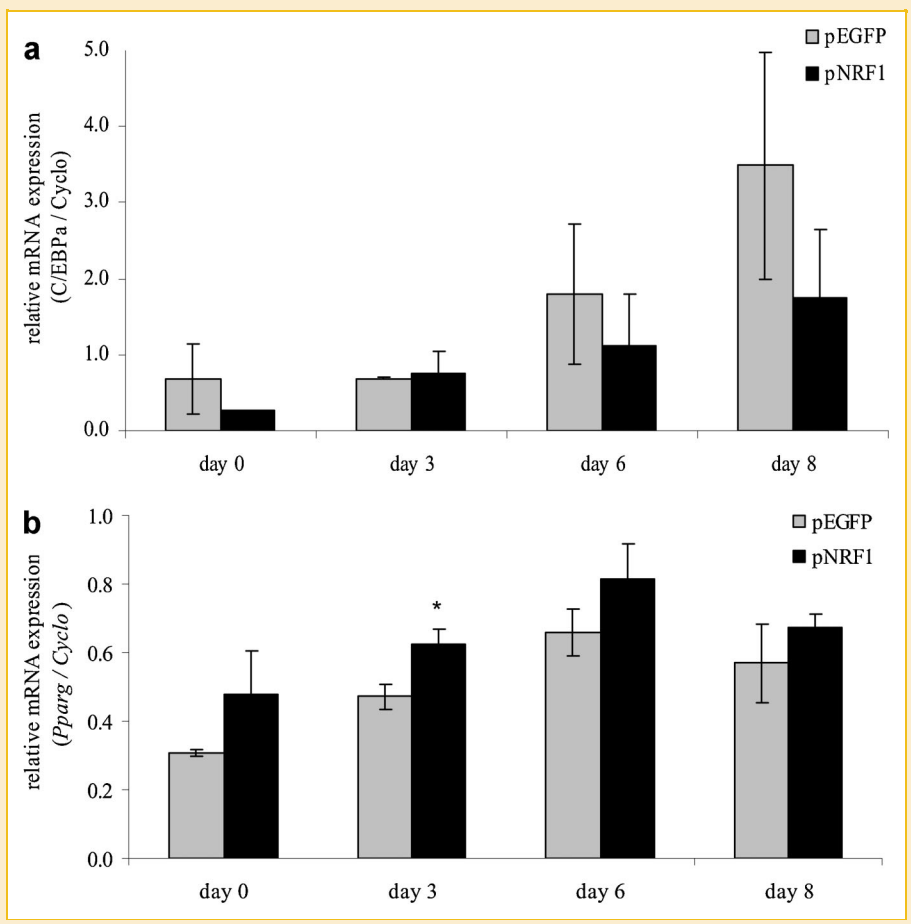

Fig. 3. Adipogenic regulators were not altered in pNrf1 transfected cells during 3T3-L1 differentiation 3T3-L1 cells were transfected with pSPORT-NRF1 or pEGFP empty vector and differentiated. Total RNA was isolated at indicated time-points, subjected to qPCR and expression was normalized to the expression of Cyclophilin A (CypA). a: C/EBPα mRNA expression of Nrf1 overexpressed 3T3-L1 cells and cells transfected with empty vector. b: Pparγ mRNA expression of Nrf1 overexpressed 3T3-L1 cells and cells transfected with empty vector Values are mean ± SD of three determinations, $^{*}P < 0.05$ when compared to pEGFP transfected cells at the same time-point.

APOPTOSIS

Gene expression analysis indicated increased apoptosis in *Nrf1* overexpressing cells. The activity of caspase 3/7 was measured, which are the main executors of apoptosis, to functionally test this at day 8 of differentiation. Caspase 3/7 activity was found significantly increased ($P < 0.05$; $n = 3$) in pNrf1 overexpressing cells (1.40 ± 0.13-fold) compared with pEGFP transfected cells (1.00 ± 0.05).

DISCUSSION

In this study we identified the gene repertoire directly regulated by Nrf1 and showed that continuous overexpression of *Nrf1* during adipogenesis mainly stimulates cell proliferation, chemokine activation, and apoptosis (Fig. 7). With regard to metabolism-related processes, besides increased mitochondrial density, no effects on energy metabolism were seen. We demonstrated that long-term changes can drastically differ from short-term effects. Long-term *Nrf1* overexpression mimics insulin-resistant adipocytes in contrast to the short-term insulin-sensitizing effect.

MITOCHONDRIAL DENSITY, ATP PRODUCTION, AND LIPID HANDLING CAPACITY IN NRF1-OVEREXPRESSING 3T3-L1 ADIPOCYTES

Adipogenesis is characterized by increased lipid deposition and triglyceride synthesis, but, *Nrf1*-overexpressing 3T3-L1 cells displayed less and smaller lipid droplets than control pEGFP-transfected cells at day 8 of differentiation. Reduced mitochondrial function results in increased lipid droplet size [Vankoningsloo et al., 2005], whereas, in contrast, *Nrf1* overexpression has been implicated to enhance mitochondrial function [Koh et al., 2007]. Therefore, we hypothesized that increased lipid oxidation might be responsible for the reduced lipid deposition in pNrf1 overexpressing 3T3-L1 cells. Our results also indicated that increased mtDNA content was not accompanied by higher ATP production. The expression of mitochondrial assembly factors was not increased, indicating that *Nrf1*-overexpression was not sufficient to assemble functional mitochondria and stimulate mitochondrial function, which was also observed by Baar et al. [2003]. The number of intact mitochondria remains the same and, therefore, ROS production capacity per mitochondrion will not change. In line with this, no changes on gene expression level were observed in pathways related to energy metabolism, indicating that the reduced lipid content was not a result of increased lipid oxidation. Expression of a number of genes involved in triglyceride synthesis, lipid transport and packaging were decreased in *Nrf1* overexpressing cells, for example, fat specific protein 27 (*Fsp27*), stearoyl-Coenzyme A desaturase 1 (*Scd1*), Fatty acid binding protein 4 (*Fabp4*) and cluster of differentiation 36 (*Cd36*). Therefore, reduced lipid deposition is most likely the result of reduced fatty acid synthesis [Rossmeisl et al., 2000], and/or increased lipolysis, as seen with knock-out of genes involved in lipid packaging, for example, perilipin (*Plin*) or *Fsp27* [Nishino et al., 2008; Toh et al., 2008].

Since lipid accumulation is a key feature of late adipogenesis and a frequently used parameter to analyze adipogenic capacity, we wanted to test if the reduced lipid content seen in *Nrf1*-overexpressing cells could be the result of reduced differentiation capacity. Gene expression analysis of two key adipogenic transcription factors, *PPARγ* and *C/EBPα*, during differentiation (Fig. 3) indicated that *Nrf1* overexpression had no significant effect on early adipogenesis. In addition, expression of Adipophilin (*Adfp*), which is a constituent of the globule surface and a characteristic of late differentiation and was increased as well in *Nrf1*-overexpressing cells, and inhibitors of adipogenesis, like Delta like kinase (*Dlk*) and GATA binding protein 2 (*Gata2*), were decreased in *Nrf1*-overexpressing cells. This excludes reduced differentiation as a possible explanation.

NRF1 INDUCED CELL PROLIFERATION

A number of genes up-regulated by *Nrf1* contained a bZIP transcription factor binding site and were involved in a variety of processes, including proliferation and cell-cycle regulation, apoptosis and chemokine/cytokine signaling. These results are supported by increased expression of a number of genes involved in cell-cycle regulation, including *cMyc*, Cyclin D1 (*Ccnd1*), and *Ccnd3*, which are important regulators of cell proliferation and adipogenesis [Morrish et al., 2003; Fox et al., 2008]. In addition, *C/EBPβ* and *C/EBPδ*, two bZIP containing transcription factors involved in clonal expansion [Tang et al., 2003; Narayanan et al., 2004], were also increased at day 8 by *Nrf1* overexpression. As indicate above, the ability to proliferate is unlikely the consequence of "slowing of differentiation," which would result in a relative increase of preadipocytes capable of proliferation. We demonstrated that expression of the early adipogenic regulators, adipocyte markers and preadipocyte specific genes was not increased in Nrf1-overexpressing cells. This data demonstrates that the pronounced increase in cell proliferation can not be explained by the presence of an increased number of preadipocytes in Nrf1-overexpressing wells. Mitochondria induced cell-cycle regulation has previously been shown in mouse embryonic fibroblast treated with mitochondrial inhibitors, which resulted in either cell-cycle arrest, repair or cell-death [Kulawiec et al., 2009]. Furthermore, the role of *Nrf1* in cell-cycle progression has previously been established by Cam et al. [2004], and may explain the embryonic lethality in $Nrf1^{-/-}$ mice [Huo and Scarpulla, 2001]. Overexpression of *Nrf1* may cause inappropriate proliferation, which is also a hallmark of cancer cells. In line with this, *Nrf1* has been previously associated as one of the transcription factors involved in breast cancer malignant progression [Niida et al., 2008].

NRF1 INDUCES APOPTOSIS

Gene expression profiling showed that a number of pathways involved in apoptosis were significantly changed in *Nrf1*-overexpressing adipocytes. This was confirmed by an increased caspase 3/7 activity. Apoptosis in *Nrf1*-overexpressing adipocytes appeared to be a result of *Nrf1* induced expression of *Fas*, which has a bZIP binding domain, and was possibly further stimulated by chemokine activation and altered adipokine expression after prolonged *Nrf1* overexpression. The observed increase in apoptosis does not contradict an increase in cell number. Increased apoptosis is likely only apparent in the final stages of differentiation, whereas there is

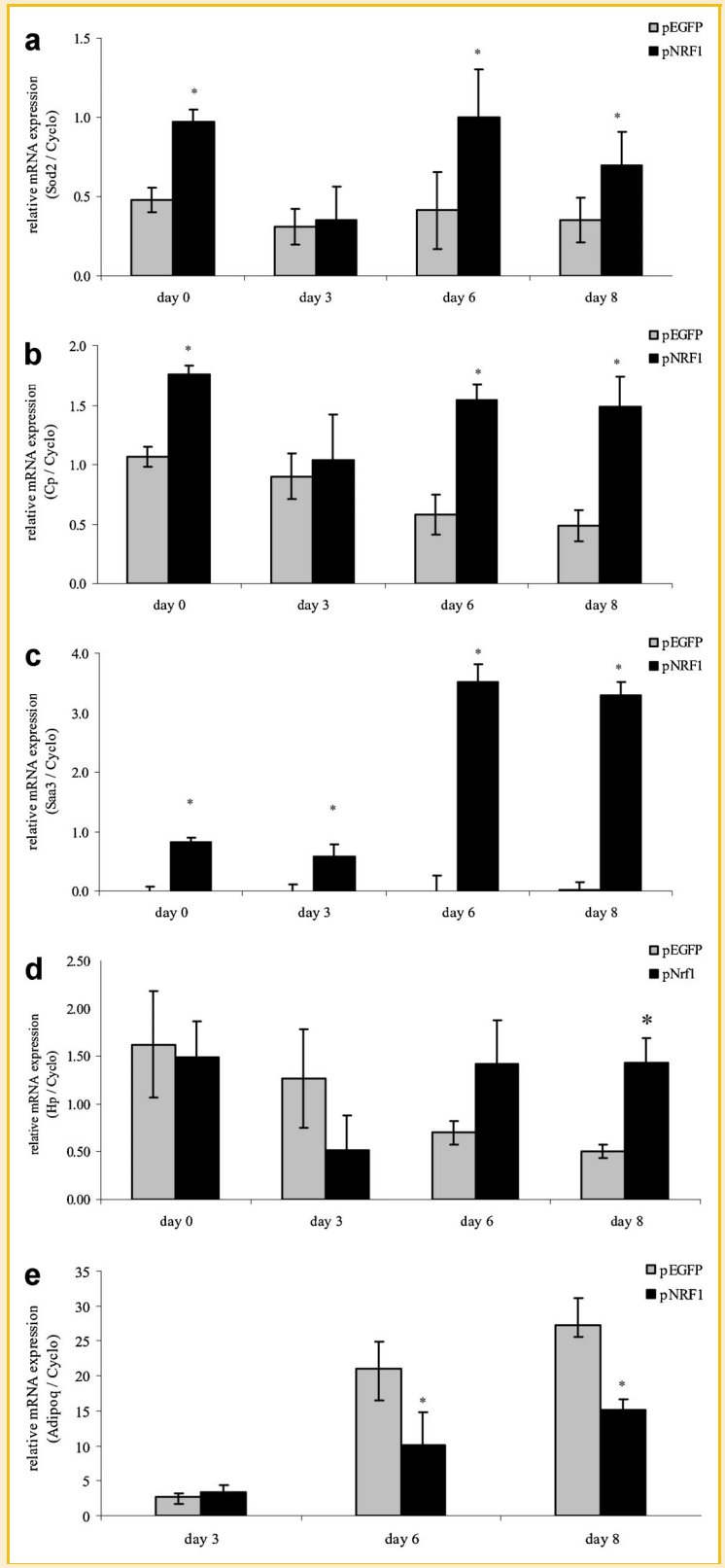

Fig. 4. Identification of direct and indirect Nrf1 targets during 3T3-L1 differentiation 3T3-L1 cells were transfected with pSPORT-Nrf1 or pEGFP empty vector and differentiated. Total RNA was isolated at indicated time-points, subjected to qPCR and expression was normalized to the expression of Cyclophilin A (CypA). Sod2 (a), Cp (b) and Saa3 (c) were considered as direct targets of Nrf1 (sign. altered $\geq$3 time-points). Hp (d) and Adipoq (e) were considered indirect targets of Nrf1. Error bars indicate SD (n = 3), $^{*}P \leq 0.05$.

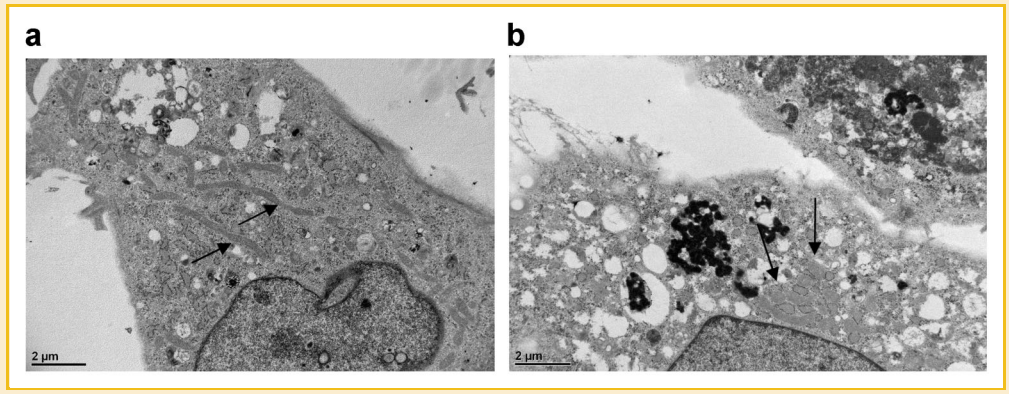

Fig. 5. Representative electron microscopy pictures of pNrf1 transfected (a) and control pEGFP transfected (b) 3T3-L1 cells at day 8 of differentiation. Arrows indicate mitochondria. Nrf1-transfected cells (a) have more electron dense and larger mitochondria than pEGFP transfected cells (b).

already a trend for increased proliferation from day 0 of differentiation. Furthermore, *Nrf1* also induced a number of genes involved in anti-oxidant defense, for example, superoxide dismutase 2 (*Sod2*). Although this feature was not clearly represented in the pathway analysis, some key anti-oxidant genes were strongly increased in *Nrf1* overexpressing cells, for example, *Sod2*, Ceruloplasmin (*Cp*), and Nuclear factor erythroid 2-related factor 1 (*Nfe2l1*). Excessive *Sod2* expression could result in oxidative stress hypersensitivity and stimulate apoptosis [Kowald et al., 2006], but the relation between *Nrf1* and oxidative stress response requires further studying. Despite increased *cMyc* expression, *Bcl2* expression was unchanged and mitochondria were not irregularly shaped in *Nrf1*-overexpressing cells, therefore we do not expect mitochondria dependent induction of apoptosis, as seen by Morrish et al. [2003]. Although we can only speculate, since we did not measure cytochrome *c* or mitochondrial membrane potential. Possibly, we did not observe a similar effect in our *Nrf1*-overexpressing adipocytes because our cells were not serum-depleted. In line with their results, *Nrf1*-overexpression in 3T3-L1 cells showed a trend of enlarged and more electron dense mitochondria (Fig. 5), likely due to increased import of mitochondrial proteins.

LONG-TERM NRF1 OVEREXPRESSION INDUCES INFLAMMATION AND INSULIN-RESISTANT ADIPOKINE EXPRESSION PROFILE

Our data indicate that inflammation in *Nrf1*-overexpressing cells is the result of both primary *Nrf1* targets, as well as triggers of inflammation induced secondary to *Nrf1* overexpression. *Nrf1* increased a number of cytokines, for example, cluster of differentiation 47 and 74 (*Cd47* and *Cd74*), which attract macrophages [Leng et al., 2003], and chemokines like C-X-C motif chemokine (*Cxcl*) 2 and 5, chemokine ligand (*Ccl*) 2 and 7. In addition, a number of adipokines, were amongst the strongest *Nrf1* induced genes at day 8, but some were also increased at earlier timepoints (Fig. 4). Strong and immediate induction of, for example, *Saa3* and *Cp* indicate that they are direct targets of *Nrf1*, and that their induction is not a consequence of increased cell density and/or

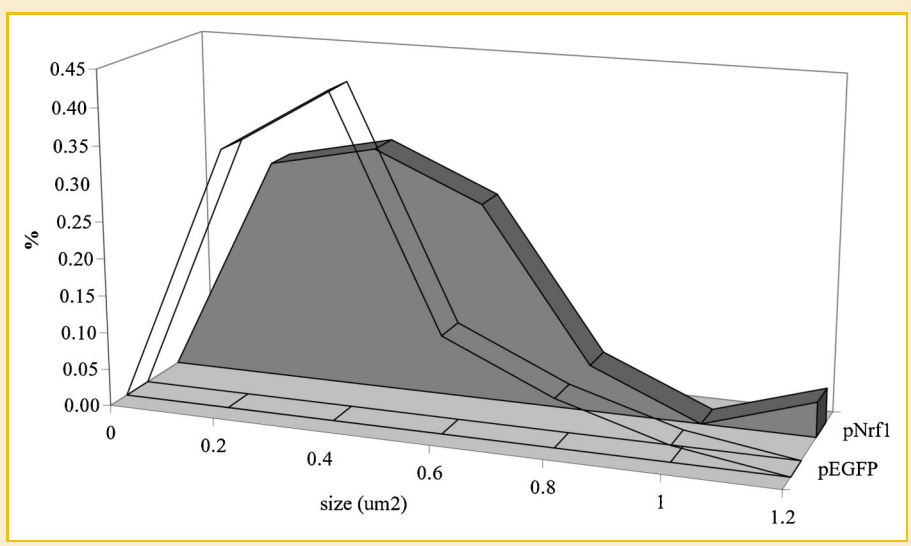

Fig. 6. Size distribution mitochondria of pNrf1 and pEGFP transfected 3T3-L1 adipocytes. Mitochondrial size was quantified using Image J analysis of electron microscopy images. pNrf1-transfected cells contained larger mitochondria than pEGFP-transfected cells.

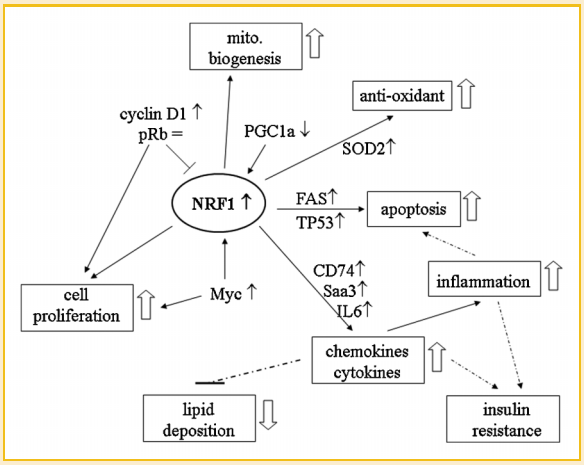

Fig. 7. Processes altered as a result of Nrf1 overexpression in 3T3-L1 adipocytes. Solid lines indicate directly stimulated processes, and dashed lines indicate secondary effects of NRF1 Gene symbols represent genes involved in the implicated processes. The change in gene expression induced by Nrf1-overexpressing is indicated with arrows; increased expression (↑), decreased expression (↓), or no change (=).

altered adipokine expression pattern later in differentiation, as seen with, for example, *Hp* and *Adipoq*. In our study, adiponectin was strongly reduced at days 6 and 8 of differentiation, despite increased mtDNA copy number and normal ATP production in *Nrf1*-overexpressing cells. These results oppose an earlier study which showed that 2 days overexpression of *Nrf1* resulted in increased mtDNA content and increased adiponectin expression in 3T3-L1 adipocytes [Koh et al., 2007]. Based on these findings we hypothesize that reduced adiponectin expression is probably not solely a result of reduced mitochondrial content and/or functioning, but from altered adipokine signaling, for example, increased TNFα and IL6 expression. Taken together, our results indicate that *Nrf1* can induce inflammation in adipocytes directly, which likely affects lipid handling in adipocytes [Gustafson and Smith, 2006]. *Nrf1*-overexpression probably triggers a pro-survival mechanism, which is also seen after exposure of cells to the endotoxin lipopolysaccharide (LPS) [Suliman et al., 2003]. We do not expect that our results are an artifact from transfection induced LPS-contamination, since inflammation is predominantly present in late adipogenesis and key adipogenic transcription factors are not decreased, and early adipogenesis is not inhibited [Chung et al., 2006]. Insulin resistance is reflected by altered adipokine expression profile, inflammation and increased lipolysis. All these parameters were observed in Nrf1-overexpressing cells at day 8 of differentiation. Therefore, we concluded that insulin resistance is a (secondary) result of Nrf1-overexpression. Exploring the exact mechanism by which Nrf1 triggers insulin resistance falls outside the scope of this study.

In summary, our results demonstrate that *Nrf1* is a key regulator of a broad variety of processes (Fig. 7). Some properties of *Nrf1* are beneficial to combat T2DM and lipodystrophy, like stimulation mitochondrial biogenesis, anti-oxidant, and cell proliferation, while others, for example, stimulation of inflammation and apoptosis, worsen insulin resistance. These data indicate that direct *Nrf1* overexpression is not recommended, but specific post-transcriptional regulation, or specific activation of *Nrf1* downstream targets could be beneficial for increasing adipocyte tissue mass thereby reducing lipid deposition in peripheral tissues [Gugneja and Scarpulla, 1997; Wang et al., 2006]. In addition, our results show that despite promising short-term *Nrf1* overexpression, long-term secondary effects can drastically differ.

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