Ndrg1 promotes adipocyte differentiation and sustains their function

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Adipocytes play a central role in maintaining metabolic homeostasis in the body. Differentiation of adipocyte precursor cells requires the transcriptional activity of peroxisome proliferator-activated receptor-γ (Pparγ) and CCAAT/enhancer binding proteins (C/Ebps). Transcriptional activity is regulated by signaling modules activated by a plethora of hormones and nutrients. Mechanistic target of rapamycin complexes (mTORC) 1 and 2 are central for the coordination of hormonal and nutritional inputs in cells and are essential for adipogenesis. Serum glucocorticoid kinase 1 (Sgk1)-dependent phosphorylation of N-Myc downstream-regulated gene 1 (Ndrg1) is a hallmark of mTORC2 activation in cells. Moreover, Pparγ activation promotes Ndrg1 expression. However, the impact of Ndrg1 on adipocyte differentiation and function has not yet been defined. Here, we show that Ndrg1 expression and its Sgk1-dependent phosphorylation are induced during adipogenesis. Consistently, we demonstrate that Ndrg1 promotes adipocyte differentiation and function by inducing Pparγ expression. Additionally, our results indicate that Ndrg1 is required for C/Ebpα phosphorylation. Moreover, we found that Ndrg1 phosphorylation by Sgk1 promotes adipocyte formation. Taken together, we show that induction of Ndrg1 expression by Pparγ and its phosphorylation by Sgk1 kinase are required for the acquisition of adipocyte characteristics by precursor cells.

White adipocytes are central for the regulation of lipid and metabolic homeostasis. Importantly, both deficits in adipose tissue development (lipodystrophy) and the excessive accumulation of adipose tissue result in metabolic disorders, including type 2 diabetes. Differentiation of mesenchymal precursor cells into adipocytes is crucial for adipose tissue acquisition. The terminal differentiation of adipocytes requires the coordinated expression of genes regulating their specific functions. Peroxisome proliferator-activated receptor-γ (Pparγ) and CCAAT/enhancer binding proteins (C/Ebps) are major transcription factors promoting the acquisition of molecular adipocyte characteristics by precursor cells. A plethora of extracellular signals, including hormones and nutrients, activate intracellular signaling cascades that modulate Pparγ, C/Ebps and other transcription factors to regulate adipogenesis.

Mechanistic target of rapamycin (mTOR) activity is dependent on both hormones (insulin) and nutrients levels. In cells, mTOR kinase is present in two large multi-component signaling complexes; mTORC1 and mTORC2. mTORC1 is defined primarily by raptor protein, while mTORC2 by the presence of the rictor subunit. Depletion of raptor specifically abrogates the activity of mTORC1, while rictor is specifically required for mTORC2 function. Genetic and pharmacological studies revealed that both mTORC1 and mTORC2 are required for adipogenesis and promote adipocyte function. Yet, the function of distal mTOR effectors in the regulation of adipocyte formation is not completely understood.

mTORC2 activates Sgk1, which is required for adipocyte formation. Ndrg1 is a major phosphorylation target of Sgk1. Moreover, expression of Ndrg1 in adipocytes seems to be promoted by Pparγ. Ndrg1 was previously implicated in regulation of progression of multiple tumors, peripheral neuropathy, T cell energy, as well as bone remodeling and macrophage differentiation. Additionally, a number of studies suggest that Ndrg1 might regulate other signaling cascades that influence adipocyte differentiation. This includes the Wnt/β-Catenin cascade, glycogen synthase kinase 3 (Gsk3), as well as extracellular regulated kinase 1/2 (Erk1/2). However, the impact of Ndrg1 on the regulation of adipogenesis and adipocyte function has not been elucidated. Here, we show that Ndrg1 promotes adipogenesis and sustains adipocyte function by promoting...
Pparγ expression and possibly C/Ebpa activity. Moreover, our results suggest that Sgk1-dependent phosphorylation of threonine 346 on Ndrg1 promotes adipocyte differentiation.

Results

Ndrg1 is induced during adipocyte differentiation. To test whether Ndrg1 could regulate adipocyte differentiation, we first measured its expression in the 3t3l1 pre-adipocyte cell line, which was subjected to an adipocyte-differentiation cocktail for different time points. Expression of Ndrg1 mRNA increased progressively during differentiation, reaching more than 10-fold elevation in fully differentiated cells compared to undifferentiated 3t3l1 cells (Fig. 1A). Previous studies indicated that Ndrg1 is a target of the master transcription factor promoting adipogenesis – Pparγ16, 17. In fact, 3t3l1 cells differentiated in the presence of the Pparγ agonist rosiglitazone presented markedly higher expression of Ndrg1 after 8, 12 and 14 days of differentiation (Fig. 1A). Consistently, protein levels for Ndrg1 also increased during 3t3l1 differentiation (Fig. 1B).

Upon mTORC2-dependent activation of Sgk1 kinase, Sgk1 phosphorylates Ndrg1 at five residues (S300, T328, T346, T346 and T366)13–15. Importantly, using a phospho-specific antibody, we found that the levels of Ndrg1 phosphorylated by Sgk1 on T346 also increased during adipocyte differentiation (Fig. 1B). These results suggest that Ndrg1 could play an important role in the regulation of adipocyte differentiation, function, or both.

Ndrg1 is required for adipocyte differentiation. To assess the role of Ndrg1 in adipocyte differentiation, we generated stable 3t3l1 cell lines expressing shRNA against Ndrg1. Expression of shRNA sequence 3 and 4 against Ndrg1 (Ndrg1 sh3 and sh4) resulted in efficient knockdown of Ndrg1 protein (Fig. 1C). Using AdipoRed-mediated triglyceride (TG) quantification and neutral lipid OilRedO staining, we demonstrated that knockdown of Ndrg1 in 3t3l1 cells subjected to 14 days of adipogenic differentiation resulted in roughly 50% reduced lipid accumulation compared to control cells (Fig. 1D and E). Consistently, de novo lipid production (lipogenesis) was also markedly reduced in the absence of Ndrg1 (Fig. 1F). Next, we tested whether Ndrg1 deficiency also impacts other functions of adipocytes. To assess the release of TG stored in lipid droplets during
the process of lipolysis, we measured the levels of glycerol and free fatty acids (FFAs) released to the cell culture medium from differentiated 3t3l1 cells. Lipolysis was markedly reduced in the absence of Ndrg1 (Fig. 1G and H). Indicating that also adipocytes function is affected by depletion of Ndrg1.

Ndrg1 promotes the expression of Pparγ and phosphorylation of C/Ebpα. Next, we tested the expression levels of key transcription factors regulating adipocyte differentiation. mRNA levels of Pparγ were markedly reduced in cells expressing shRNA against Ndrg1 (Fig. 2A). Induction of C/Ebpα transcriptional activity is also required for proper adipocyte differentiation, however depletion of Ndrg1 did not influence C/Ebpα mRNA expression. Moreover, the levels of transcriptional targets of Pparγ were reduced in cells depleted

Figure 2. Ndrg1 promotes Pparγ expression and stability as well as C/Ebpα phosphorylation. (A) Relative mRNA levels for indicated genes in control and Ndrg1-deficient differentiated 3t3l1 cells. (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 according to t-test). (B) Western blot for Pparγ and Gapdh in control and Ndrg1-depleted differentiated 3t3l1 cells stimulated with cycloheximide for indicated time points. Representative picture was chosen from three biological replicates. (C) Quantification of relative amount of Pparγ in cells treated with cycloheximide in relation to the initial (without treatment) levels of Pparγ in respective control and Ndrg1-depleted cells (n = 3, *P < 0.05, **P < 0.01 according to t-test). (D) Relative TG content in control and Ndrg1 sh 3t3l1 cells subjected to adipocyte differentiation cocktail with rosiglitazone (Rosi) or troglitazone (Tro). Each data point represents a biological replicate. Stars indicate significance for given parameters between control and Ndrg1-depleted cells. (*P < 0.05, **P < 0.01, ***P < 0.001 according to ANOVA followed by the Post hoc Tukey test). (E) Western blot analysis using indicated antibodies in control and Ndrg1-deficient cells stimulated with insulin (Ins.) for the indicated time.
from Ndrg1 (Fig. 2A), including diacylglycerol acyltransferase (Dgat) as well as two crucial lipases promoting lipolysis, adipose triglyceride lipase (Atgl) and hormone sensitive lipase (Hsl)30–32. Consistently, Pparγ protein levels were markedly reduced in the absence of Ndrg1 (Fig. 2B – without cycloheximide and Supplementary Fig. 1). To test if Ndrg1 also regulates the stability of Pparγ protein, we subjected Ndrg1-deficient 3t3l1 cells to a cycloheximide chase experiment. Cycloheximide treatment resulted in a higher rate of disappearance of Pparγ, in Ndrg1-deficient cells, indicating that Ndrg1 promotes Pparγ stability (Fig. 2B and C). Next, we differentiated Ndrg1-depleted and control 3t3l1 cells in the adipocyte differentiation cocktail supplemented with different concentrations of Pparγ agonists (rosiglitazone, troglitazone). Both agonists markedly induced TG accumulation in 3t3l1 control and Ndrg1-deficient cells. However, treatment with Pparγ agonists did not fully abrogate the difference in TG content between control and Ndrg1-deficient cells (Fig. 2D), suggesting that Ndrg1 could additionally promote the differentiation of adipocytes by targeting other factors than Pparγ.

Previous studies indicated that Ndrg1 might regulate Gsk3β and Erk1/2 activity, as well as the Wnt/β-Catenin pathway27. Gsk3β and Erk1/2 have been shown to promote adipogenesis33, 34, while expression of β-Catenin suppresses pre-adipocyte differentiation35. We tested expression and activation of these proteins in cells depleted of Ndrg1. Western blot analysis revealed that Ndrg1 depletion does not influence the activation or levels of Erk1/2 (Fig. 2E and Supplementary Fig. 1). Similarly, levels of phosphorylated (inactive) and total Gsk3β, as well as β-Catenin were not altered by Ndrg1 silencing (Fig. 2E and Supplementary Fig. 1). Interestingly, Gsk3β promotes phosphorylation of C/Ebpα on threonine 222/22633. Therefore, we tested whether Ndrg1 is required for C/Ebpα phosphorylation. We observed that depletion of Ndrg1 suppresses basal and insulin stimulated phosphorylation of C/Ebpα on threonine 222/226. Therefore, we tested whether Ndrg1 is required for C/Ebpα phosphorylation. We observed that depletion of Ndrg1 suppresses basal and insulin stimulated phosphorylation of C/Ebpα on threonine 222/226, but does not influence total C/Ebpα protein levels (Fig. 2E).

These results suggest that Ndrg1 promotes adipogenesis by enhancing Pparγ expression. In addition, Ndrg1 might also influence C/Ebpα activity through phosphorylation. However, the precise mechanisms of Ndrg1 action needs to be determined.

**Overexpression of Ndrg1 promotes adipocyte differentiation.** To determine if overexpression of Ndrg1 is sufficient to enhance adipogenesis, we generated 3t3l1 cells ectopically expressing Flag-tagged Ndrg1 (FlagNdrg1). Stable expression of FlagNdrg1 was confirmed by Western blotting (Fig. 3A). Overexpression of

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**Figure 3.** Overexpression of Ndrg1 promotes adipogenesis. (A) Western blot using indicated antibodies on extracts isolated from 3t3l1 control and FlagNdrg1-expressing cells. (B) Relative TG content and (C) neutral lipid staining (OilRedO) of differentiated 3t3l1 control and FlagNdrg1-expressing cells. (D) Relative mRNA levels for indicated genes in differentiated control and Ndrg1-overexpressing 3t3l1 cells. (F) Relative glycerol and (G) FFA levels in medium from differentiated 3t3l1 control cells or Ndrg1-overexpressing cells stimulated with control medium or Iso. Each data point represents a biological replicate (for bar plots n = 3). Stars indicate significance for given parameters between control and Ndrg1-overexpressing cells. (*) P < 0.05, (**) P < 0.01, (***P < 0.001 according to ANOVA followed by the Post hoc Tukey test).
Ndrg1 resulted in an over two-fold elevation in the levels of TG in differentiated 3t3l1 cells compared to control cells (Fig. 3B and C). On the molecular level, overexpression of Ndrg1 lead to elevated levels of markers defining adipocyte function. Specifically, we observed increased expression of Pparγ, Dgat, stearoyl-CoA-desaturase (Scd1), acetyl-CoA carboxylase (Acc1), Atgl, Hsl, monoglyceride lipase (Mgl), adiponectin and leptin (Fig. 3D). Moreover, Ndrg1 overexpression enhanced adipocyte function as indicated by increased lipolytic activity (Fig. 3E and F). Altogether, these data suggest that Ndrg1 overexpression promotes adipogenesis and adipocyte function.

**Sgk1-dependent phosphorylation of Ndrg1 on T346 is required for adipogenesis.** As a downstream effector of mTORC2, Sgk1 phosphorylates Ndrg1 on multiple sites (S300, T328, T346, T356 and T366)13–15. Previous studies indicated that Sgk1 is required for adipocyte differentiation11. To test if Sgk1-dependent phosphorylation of Ndrg1 is required for its function, we generated Ndrg1 alanine mutants that cannot be phosphorylated at the respective residues and expressed them in 3t3l1 cells. Western blot analysis confirmed that all mutants were equally overexpressed as the control wild type form of Ndrg1 (Fig. 4A). Next, we subjected all the cell lines overexpressing the indicated phospho-mutants of Ndrg1 to an adipogenic differentiation protocol. As expected, overexpression of the wild type form of Ndrg1 resulted in markedly elevated TG accumulation compared to cells expressing empty vector (Fig. 4B). Overexpression of S300A, T328A, T346A and T366A mutants also resulted in a marked increase in TG accumulation in cells, comparable to wild-type overexpression (Fig. 4B). However, overexpression of the T346A mutant led to markedly lower TG accumulation than cells overexpressing the wild-type form of Ndrg1 (Fig. 4B). These results indicate that Sgk1-dependent phosphorylation of Ndrg1 on T346 promotes adipogenesis.

**Ndrg1 sustains adipocyte function after differentiation.** To test if Ndrg1 plays a role in the regulation of mature adipocyte function, we knocked down Ndrg1 using transient siRNA transfection in fully differentiated 3t3l1 cells. Effective knockdown of Ndrg1 was confirmed by Western blot (Fig. 5A). Consistent with our previous results, siRNA-mediated silencing of Ndrg1 resulted in a reduced rate of lipogenesis (Fig. 5B). Moreover, knockdown of Ndrg1 decreased the rate of isoproterenol-induced lipolysis in 3t3l1 cells, as indicated by a more than two-fold reduction in the concentration of FFAs and a more than three-times reduced levels of glycerol in the medium compared to control cells (Fig. 5C and D). To confirm these results, we knocked down Ndrg1 in adipocytes differentiated from primary stromal vascular cells isolated from wild type mice. Ndrg1 knockdown in primary cells resulted in a reduced rate of lipogenesis (Fig. 5E), demonstrating that the observed phenotype is not restricted to 3t3l1 cells.

**Discussion**

Our results indicate that Ndrg1 is both activated during and required for adipogenesis. Moreover, in fully differentiated adipocytes Ndrg1 stimulates lipolysis. Ndrg1 promotes adipocyte formation partially by enhancing expression of the crucial pro-adipogenic transcription factor Pparγ. However, Ndrg1 might also regulate C/EBPα adipogenic activity (Fig. 5F).

Ndrg1 is a distal effector of mTORC2 activation in cells9. Disruption of mTORC signaling by silencing its component rictor results in reduced adipocyte differentiation16. Sgk1 kinase is directly activated by mTORC2; consistently, its depletion results in attenuated adipogenesis10–12. In agreement with these data, we showed that Sgk1-dependent phosphorylation of Ndrg1 is required for its pro-adipogenic function. However, previous results indicate that the mTORC2 signaling module suppresses lipolysis9,16. As our results indicate that Ndrg1 promotes lipolysis, mTORC2 must therefore suppress lipolysis by utilizing an Ndrg1-independent mechanism. Of note, expression of Atgl and Hsl, which are targets of Pparγ9,16 were reduced in the absence of Ndrg1. These results explain the attenuated lipolysis rate in the absence of Ndrg1. Moreover, our results might indicate that defective lipolysis in cells deficient for Ndrg1 may be a direct consequence of reduced differentiation of these cells. We showed that Pparγ activation increases Ndrg1 expression. On the other hand, our results indicate that Ndrg1 promotes expression and stability of Pparγ. Therefore we postulate that Ndrg1 acts as a component of a positive feedback loop promoting Pparγ action.
However, agonist-mediated activation of \( \text{Ppar}\gamma \) did not completely rescue the adipogenesis defect in \( \text{Ndrg1}\)-deficient cells. This might be caused by the lower protein levels of \( \text{Ppar}\gamma \) in cells depleted of \( \text{Ndrg1} \).

Alternatively, this might indicate that \( \text{Ndrg1} \) also regulates other components of the molecular machinery defining adipocyte differentiation. In fact, we observed that phosphorylation of \( \text{C/Ebp}\alpha \) on threonines 222/226 is markedly reduced in the absence of \( \text{Ndrg1} \). Threonines 222 and 226 on \( \text{C/Ebp}\alpha \) were originally identified as targets for \( \text{Gsk3}\beta \)-dependent phosphorylation 33. However, a recent study indicated that \( \text{C/Ebp}\alpha \) is a poor substrate for \( \text{Gsk3}\beta \)37. In fact, our results suggest that \( \text{Ndrg1} \) does not influence the total levels of \( \text{Gsk3}\beta \) or its phosphorylated (inactive) form. This suggests that \( \text{Ndrg1} \) modulates \( \text{C/Ebp}\alpha \) phosphorylation by influencing \( \text{Gsk3}\beta \) activity in another manner or by utilizing a \( \text{Gsk3}\beta \)-independent mechanism.

Also, the impact of \( \text{C/Ebp}\alpha \)T222/T226 phosphorylation on adipogenesis has not been directly assessed. Phosphorylation on these sites was originally proposed to enhance adipogenesis 33, but a recent study indicates that it might reduce \( \text{C/Ebp}\alpha \) activity 37.

Previous studies implicated \( \text{Ndrg1} \) in the regulation of \( \text{Wnt}/\beta\)-\text{Catenin} as well as \( \text{Erk1/2} \) action 27. Our results indicate that \( \text{Ndrg1} \) does not influence any of these pathways in adipocytes. Therefore, we postulate that \( \text{Ndrg1} \) primarily promotes adipogenesis by enhancing expression of \( \text{Ppar}\gamma \). However, \( \text{Ndrg1} \) might also influence other adipogenic factors and the exact mechanism of its action needs to be defined further.

**Methods**

**Pre-adipocyte culture and differentiation.** \( \text{3t3l1} \) preadipocytes were cultured in DMEM supplemented with 10% fetal calf serum (FCS), and 40 \( \mu \)g/ml Gentamicin at 37 °C in a humidified atmosphere. For differentiation, \( \text{3t3l1} \) were grown to confluency. Two days post-confluency, the cells were switched to medium containing 10% fetal bovine serum, 1.5 \( \mu \)g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 \( \mu \)M Dexamethasone (Dex) for 48 hours. This step was repeated for an additional 48 hours and then the induced cells were incubated with “Differentiation Medium” (DMEM supplemented with 10% FBS and 1.5 \( \mu \)g/ml insulin) for up to 10 days to achieve differentiation to adipocytes. In the indicated experiments, \( \text{Ppar}\gamma \) agonists (rosiglitazone or troglitazone) were supplemented to the differentiation medium at indicated concentrations throughout the differentiation process. Fresh agonists were added to the medium every 48 h. All compounds were purchased from Sigma-Aldrich if not specified.

Stromal vascular cells (SVCs) containing pre-adipocytes were isolated from adipose tissue Briefly, subcutaneous white adipose tissue were collected from wild type BL6 mice and cut into small fragments with a scalp. The sliced SVC fractions were treated with 2 \( \mu \)g/ml Collagenase D (Roche) in PBS containing 5 mM CaCl\(_2\), 1% BSA
and P/S with shaking (300 rpm) at 37 °C for 40 minutes. After digestion of adipose tissue, the solution was filtered through a cell strainer with 45 μm pore size and centrifuged at 1250 rpm for 10 minutes to collect SVCs. SVCs were cultured in complete medium (DMEM/F12 containing 10% FBS, 1% sodium pyruvate (SP), 1% non-essential amino acids (NEAA) and P/S) until two days post-confluent and transferred into induction medium (DMEM/ F12 containing 10% FBS, 1% SP, 1% NEAA and P/S, 0.2 μM Indomethacin, 0.5 μM Rosiglitazone, 0.5 mM IBMX and 1 μM Dexamethasone) for two times 48 hours. Then the SVCs were cultured into complete medium with 1.5 μg/mL insulin for up to 6 additional days.

**siRNA transfection.** Fully differentiated 3T3L1 cells were transfected using Dharmafect Duo transfection reagent with siRNA against Ndrg1 or NonTarget control (Dharmacon) according to the manufacturer's protocol. Cells were used for experiments 48 hours post transfection.

**Generation of stable knockdown and overexpression of wild type and mutant Ndrg1.** shRNA against Ndrg1 was introduced into 3T3L1 cells using lentiviral particles. Specific shRNA sequences (available in Supplementary Table 1) were cloned in pGIPZ vector was described previously. The infected 3T3L1 cells were then selected by puromycin treatment (5 μg/mL). The pBabe-Puro vector was used for retrovirus-driven Flag-Ndrg1 expression. A full length coding sequence of Ndrg1 was introduced between EcoRI and BamHI restriction sites of this vector. Platinum-E (Plat-E) cell were utilized to produce retroviral particles. Infected 3T3L1 cells were selected with puromycin (5 μg/mL). Ndrg1 mutants were generated using the site-directed mutagenesis kit from New England Biolabs according to the manufacturer's protocol.

**Analyses of mRNA expression and protein levels.** Quantitative polymerase chain reaction (qPCR) was performed using SYBR green Universal PCR master mix (Roche). mRNA was isolated using trizol reagent from Sigma. Lipogenesis assay. For determination of de novo lipogenesis, 3T3L1 cells were incubated with 3H-glucose (1 μCi/mL, Perkin Elmer) in the presence or absence of insulin for 3 h. Cells were washed twice with PBS and lysed in 0.1 N HCl. Lipids were then extracted with chloroform/methanol (2:1, v/v). The lipid-containing chloroform phase was used for liquid scintillation counting.

**Statistical analysis.** For multiple comparisons, one way analysis of variance (ANOVA) followed by the Post hoc Tukey test was used. For determination of significance between two experimental groups T-Test was used. Significance was accepted at the level of 0.05. Exact statistics are indicated for each figure.

**References**

1. Rosen, E. D. & Spiegelman, B. M. What we talk about when we talk about fat. *Cell* **156**, 20–44, doi:10.1016/j.cell.2013.12.012 (2014).
2. Cristancho, A. G. & Lazar, M. A. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol* **12**, 722–734, doi:10.1038/nrm3198 (2011).
3. Lefterova, M. I. & Lazar, M. A. New developments in adipogenesis. *Trends Endocrinol Metab* **20**, 107–114, doi:10.1016/j.ten.2008.11.005 (2009).
4. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. *Cell* **149**, 274–293, doi:10.1016/j.cell.2012.03.017 (2012).
5. Zhang, H. H. et al. Insulin stimulates adipogenesis through the Akt-TSC2-mTORC1 pathway. *PLoS One* **4**, e6189, doi:10.1371/journal.pone.0006189 (2009).
6. Tang, Y. et al. Adipose tissue mTORC2 regulates ChREBP-driven de novo lipogenesis and hepatic glucose metabolism. *Nat Commun* **7**, 11365, doi:10.1038/ncomms11365 (2016).
7. Yoon, M. S., Zhang, C., Sun, Y., Schoenherr, C. J. & Chen, J. Mechanistic target of rapamycin controls homeostasis of adipogenesis. *J Lipid Res* **54**, 2166–2173, doi:10.1194/jlr.M037705 (2013).
8. Polak, P. et al. Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. *Cell Metab* **8**, 399–410, doi:10.1016/j.cmet.2008.09.003 (2008).
9. Cybulski, N. & Hall, M. N. TOR complex 2: a signaling pathway of its own. *Trends Biochem Sci* **34**, 620–627, doi:10.1016/j.tibs.2009.09.004 (2009).
10. García-Martínez, I. M. & Alessi, D. R. mTORC2 complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J* **416**, 375–385, doi:10.1042/Bj20081668 (2008).
11. Di Pietro, N. et al. Serum- and glucocorticoid-inducible kinase 1 (SGK1) regulates adipocyte differentiation via forkhead box O1. *Mol Endocrinol* **24**, 370–380, doi:10.1210/me.2009-0265 (2010).
12. Pearce, L. R., Sommer, E. M., Sakamoto, K., Wullschleger, S. & Alessi, D. R. Protor-1 is required for efficient mTORC2-mediated activation of SGK1 in the kidney. *Biochem J* **436**, 169–179, doi:10.1042/Bj20102103 (2011).
13. Murakami, Y. et al. Identification of sites subjected to serine/threonine phosphorylation by SKG1 affecting N-myf downstream-regulated gene 1 (NDRG1)/Cap43-dependent suppression of angiogenic CXCR chemokine expression in human pancreatic cancer cells. *Biochem Biophys Res Commun* **396**, 376–381, doi:10.1016/j.bbrc.2010.04.100 (2010).

14. McGaig, C., Potter, L., Abramczyk, O. & Murray, J. T. Phosphorylation of NDRG1 is temporally and spatially controlled during the cell cycle. *Biochem Biophys Res Commun* **411**, 227–234, doi:10.1016/j.bbrc.2011.06.092 (2011).

15. Inglish, S. K. et al. SKG1 activity in Na+ absorbing airway epithelial cells monitored by assaying NDRG1-Thr346/356/366 phosphorylation. *Pfugers Arch* **457**, 1287–1301, doi:10.1007/s00424-008-0587-1 (2009).

16. Wang, H., Liu, L., Lin, J. Z., Abrahamian, T. R. & Farmer, S. R. Browning of White Adipose Tissue with Roscovite Induces a Distinct Population of UCP1+ Adipocytes. *Cell Metab* **24**, 835–847, doi:10.1016/j.cmet.2016.10.005 (2016).

17. Wang, H., Qiang, L. & Farmer, S. R. Identification of a domain within peroxisome proliferator-activated receptor gamma regulating expression of a group of genes containing fibroblast growth factor 21 that are selectively repressed by SIRT1 in adipocytes. *Mol Cell Biol* **28**, 188–206, doi:10.1128/MCB.00992-07 (2008).

18. Kovacevic, Z. et al. The Metastasis Suppressor, N-MYC Downstream-regulated Gene-1 (NDRG1), Down-regulates the ErbB Family of Receptors to Inhibit Downstream Oncogenic Signaling Pathways. *J Biol Chem* **291**, 1029–1052, doi:10.1074/jbc.M111.889653 (2016).

19. Sahni, S. et al. The metastasis suppressor, N-myf downstream-regulated gene 1 (NDRG1), inhibits stress-induced autophagy in cancer cells. *J Biol Chem* **289**, 9692–9709, doi:10.1074/jbc.M111.329511 (2014).

20. Fang, B. A. et al. Molecular functions of the iron-regulated metastasis suppressor, NDRG1, and its potential as a molecular target for cancer therapy. *Biochim Biophys Acta* **1845**, 1–19, doi:10.1016/j.bbadis.2013.11.002 (2014).

21. Stein, S. et al. NDRG1 is a p53-dependent apoptosis. *J Biol Chem* **279**, 48390–48400, doi:10.1074/jbc.M400386200 (2004).

22. Murakami, Y. et al. N-myf downstream-regulated gene 1 promotes tumor inflammatory angiogenesis through JNK activation and autocrine loop of interleukin-1alpha by human gastric cancer cells. *J Biol Chem* **288**, 25025–25037, doi:10.1074/jbc.M113.472068 (2013).

23. Okuda, T. et al. Ndr1-deficient mice exhibit a progressive demyelinating disorder of peripheral nerves. *Mol Cell Biol** **24**, 3949–3956 (2004).

24. Echaniz-Laguna, A. et al. NDRG1-linked Charcot-Marie-Tooth disease (CMT4D) with central nervous system involvement. *Neuromuscular Disord* **17**, 163–168, doi:10.1016/j.nmd.2006.10.002 (2007).

25. Oh, Y. M. et al. Ndr1 is a T-cell clonal anomaly factor negatively regulated by CD28 costimulation and interleukin-2. *Nat Commun* **6**, 8698, doi:10.1038/ncomms9698 (2015).

26. Watari, K. et al. Impaired differentiation of macrophage lineage cells attenuates bone remodeling and inflammatory angiogenesis in Ndr1 deficient mice. *Sci Rep* **6**, 19470, doi:10.1038/srep19470 (2016).

27. Sun, J. et al. Metastasis suppressor, NDRG1, mediates its activity through signaling pathways and molecular motors. *Carcinogenesis* **34**, 1943–1954, doi:10.1093/carcin/bgt163 (2013).

28. Wanggu, X. et al. The metastasis suppressor, NDRG1, inhibits “stemness” of colorectal cancer via down-regulation of nuclear beta-catenin and CD44. *Oncotarget** **6**, 33893–33911, doi:10.18632/oncotarget.5294 (2015).

29. Lu, W. J., Chua, M. S., Wei, W. & So, S. K. NDRG1 promotes growth of hepatocellular carcinoma cells by directly interacting with GSK-3beta and Nur77 to prevent beta-catenin degradation. *Oncotarget** **6**, 29847–29859, doi:10.18632/oncotarget.4913 (2015).

30. Sugii, S. et al. PPARgamma activation in adipocytes is sufficient for systemic insulin sensitization. *Proc Natl Acad Sci USA* **106**, 22504–22509, doi:10.1073/pnas.0912478106 (2009).

31. Kershaw, E. E. et al. PPARgamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. *Am J Physiol Endocrinol Metab* **293**, E1736–1745, doi:10.1152/ajpendo.00122.2007 (2007).

32. Deng, T. et al. Peroxisome proliferator-activated receptor-gamma transcriptionally up-regulates hormone-sensitive lipase via the involvement of specificity protein-1. *Endocrinology* **147**, 875–884, doi:10.1210/en.2005-0623 (2006).

33. Ross, S. E., Erickson, R. L., Hemati, N. & MacDougall, O. A. Glycogen synthase kinase 3 is an insulin-regulated C/EBPalpha kinase. *Mol Cell Biol** **19**, 8433–8441 (1999).

34. Prusty, D., Park, B. H., Davis, K. E. & Farmer, S. R. Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. *J Biol Chem** **277**, 46226–46322, doi:10.1074/jbc.M207776200 (2002).

35. Liu, J. & Farmer, S. R. Regulating the balance between peroxisome proliferator-activated receptor gamma and beta-catenin signaling during adipogenesis. A glycogen synthase kinase 3beta phosphorylation-defective mutant of beta-catenin inhibits expression of a subset of adipogenic genes. *J Biol Chem** **279**, 45020–45027, doi:10.1074/jbc.M100386200 (2004).

36. Lamming, D. W. & Sabatini, D. M. A Central role for mTOR in lipid homeostasis. *Cell Metab** **18**, 465–469, doi:10.1016/j.cmet.2013.08.002 (2013).

37. Liu, H. K. et al. Functional characterisation of the regulation of CAAT enhancer binding protein alpha by GSK-3 phosphorylation of Threonines 222/226. *BMC Mol Biol** 7**, 14, doi:10.1186/1471-2199-7-14 (2006).

38. Fellmann, C. et al. An optimized microRNA backbone for effective single-copy RNAi. *Cell Rep** 5**, 1704–1713, doi:10.1016/j.celrep.2013.11.020 (2013).

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Author Contributions

Kai Cai performed most of the experiments and contributed to experimental design. Rabih El-Merahi and Alexander E. Mayer performed some experiments and contributed to experimental design. Mona Loeffler performed some experiments, contributed to experimental design and writing of the manuscript. Grzegorz Sumara was responsible for experimental design and wrote the manuscript.

Additional Information

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