Coordination between metabolic transitions and gene expression by NAD+ availability during adipogenic differentiation in human cells.

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21 **ABSTRACT**

22 Adipocytes are the main cell type in adipose tissue, a critical regulator of metabolism,

23 highly specialized in storing energy as fat. Adipocytes differentiate from multipotent

24 mesenchymal stromal cells through adipogenesis, a tightly controlled differentiation

25 process involving closely interplay between metabolic transitions and sequential

26 programs of gene expression. However, the specific gears driving this interplay remain

27 largely obscure. Additionally, the metabolite nicotinamide adenine dinucleotide (NAD$^+$)

28 is becoming increasingly recognized as a regulator of lipid metabolism, being postulated

29 as promising therapeutic target for dyslipidemia and obesity. Here, we explored the

30 effect of manipulating NAD$^+$ bioavailability during adipogenic differentiation from human

31 mesenchymal stem cells. We found a previously unappreciated strong repressive role

32 for NAD$^+$ on adipocyte commitment, while a functional NAD$^+$-dependent deacetylase

33 SIRT1 appeared crucial for terminal differentiation of pre-adipocytes. Remarkably,

34 repressing the NAD$^+$ biosynthetic salvage pathway during adipogenesis promoted the

35 adipogenic transcriptional program, suggesting that SIRT1 activity during adipogenesis

36 is independent from the NAD$^+$ salvage pathway, while two photon microscopy and

37 extracellular flux analyses suggest that its activation relies on the metabolic switch.

38 Interestingly, SIRT1-directed control of subcellular compartmentalization of redox

39 metabolism during adipogenesis was evidenced by two-photon fluorescence lifetime

40 microscopy.

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43 **Significance Statement**

44 Adipocyte differentiation occurs from mesenchymal stem cells through the adipogenic

45 process, involving sequential activation of both transcriptional and metabolic programs

46 in a tightly coordinated manner. However, how transcriptional and metabolic transitions

47 reciprocally interact during adipogenic differentiation remains largely obscure. Here we

48 describe that the metabolite NAD^+ is suppresses adipogenesis trough rewiring

49 transcription, while a functional NAD^+-dependent deacetylase SIRT1 is essential for

50 terminal differentiation of pre-adipocytes. Using two-photon fluorescence lifetime

51 microscopy, we created a metabolic map of NADH and lipid content simultaneously in

52 live cells and described a new role for SIRT1 in the control of compartmentalization of

53 redox metabolism during adipogenesis. These findings advance our understanding to

54 improve therapeutical approaches targeting the NAD^+-SIRT1 axis as treatment for

55 obesity and dyslipemia.

56

57 **INTRODUCTION**

58 Adipose tissue is a crucial regulator of body metabolism through storing calories as

59 lipids in response to excessive nutritional intake and serving as a source of energy by

60 mobilizing these lipids during starvation, amongst others. Notably, the adipose tissue is

61 a relevant endocrine organ, producing several adipokines such as leptin or

62 adiponectin(1). It is primarily composed of adipocytes, and a fraction of a

63 heterogeneous collection of cell types which include mesenchymal stem cells (MSC),

64 endothelial precursors, immune cells, smooth muscle cells, pericytes and

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65   preadipocytes(2). Disfunction of the adipose compartment is common to metabolic

66   diseases including obesity or type 2 diabetes. Indeed, increased white adipose tissue

67   (WAT) mass observed in obesity is due to both adipocyte hypertrophy and increased

68   proliferation and differentiation of adipocyte progenitors(3, 4), which originate from

69   MSCs though the adipogenic process (5, 6). Hence, understanding the mechanisms

70   underlaying adipogenesis in humans is crucial to design therapeutic strategies for

71   prevalent metabolic dysfunctions.

72   MSCs are multipotent progenitor cells able to differentiate to osteoblasts, myocytes,

73   chondrocytes, and adipocytes. Fate decision is determined by specific signaling

74   pathways such as transforming growth factor-beta (TGFβ)/bone morphogenic protein

75   (BMP) signaling, wingless-type MMTV integration site (Wnt) signaling or fibroblast

76   growth factors (FGFs)(7, 8). In particular, the adipogenic process occurs in two major

77   phases: commitment to progenitors and terminal differentiation; both of which are tightly

78   regulated by intertwined transcriptional, epigenomic and metabolic transitions(8). At the

79   transcriptional level, the master regulators of adipogenesis are the key transcription

80   factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer

81   binding protein α (C/EBPα)(9, 10), which promote growth arrest and the progressive

82   expression of a lipogenic transcriptional program including the hormones adiponectin

83   and leptin, and the lipases adipose triglyceride lipase (ATGL) and lipoprotein lipase

84   (LPL)(8). Concomitantly, a switch from highly glycolytic to oxidative metabolism with

85   increased mitochondrial reactive oxygen species (ROS) is essential for adipocyte

86   differentiation(11, 12). However, how transcriptional and metabolic transitions

87   reciprocally interact during adipogenic differentiation remains an open question.

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88   During the past few years, energy metabolism is becoming increasingly recognized as

89   an effective therapeutic target for obesity. Specifically, therapies aiming to increase

90   endogenous nicotinamide adenine dinucleotide (NAD^+) levels have been proven

91   effective to reduce adiposity in both mouse and human[13-16]. Indeed, NAD^+ levels

92   decline in metabolic tissues of obese mice and humans[14, 15, 17-20], which may

93   contribute to metabolic disfunction by, for example, reducing the activity of SIRT1, a

94   deacetylase using NAD^+ as cofactor and known to regulate mitochondrial function and

95   metabolism[21, 22]. These evidences suggest that NAD^+ metabolism might be a central

96   player on adipose tissue homeostasis probably by regulating mitochondrial function and

97   consequently, adipocyte differentiation. Along these lines, in mouse preadipocytes,

98   NAD^+ synthesis through the salvage pathway and SIRT1 activity appear essential for

99   adipogenesis[23]; however, the interplay between NAD^+ bioavailability and SIRT1

100   function during adipogenesis in humans remains poorly understood.

101   In this study, we explored the coordinated dynamics of the transcriptional and energy

102   metabolism reprogramming during adipogenic differentiation of human MSC (hMSC).

103   Using two-photon fluorescence lifetime microscopy (2P-FLIM) on live hMSC, we created

104   a non-invasive metabolic map of NADH compartmentalization at a submicron resolution

105   to define the dynamics of redox metabolism during adipogenesis, which appeared

106   tightly synchronized with mitochondrial function and transcriptional reprogramming.

107   Moreover, we described a previously unappreciated robust inhibitory role for NAD^+ on

108   adipocyte commitment, while SIRT1 activity appeared essential for terminal

109   differentiation. Surprisingly, suppressing the NAD^+ salvage pathway during

110   adipogenesis led to increased expression of adipocyte markers, indicating that SIRT1

111   activation during adipogenesis doesn´t depend on NAD^+ biosynthesis through the

112   salvage pathway. Remarkably, SIRT1-directed control of compartmentalization of redox

113   metabolism during adipogenesis was evidenced by 2P-FLIM.

114

RESULTS

NAD^+ obstructs adipogenic differentiation and lipid accumulation in hMSC.

117   To approach the question whether variations in NAD^+ bioavailability impact

118   adipogenesis, we induced adipogenic differentiation in hMSCs in the presence or

119   absence of NAD^+ (Figure 1A). Interestingly, NAD^+ treatment obstructed neutral lipid

120   accumulation, as visualized by significantly less accumulation of Oil-red-O (ORO) stain

121   at terminal differentiation than in non-treated (NT) cells (Figure 1A, day 16, $P<0,01$,

122   Two-way ANOVA with Tukey's post-test). Concomitantly, a treatment with FK866, a

123   potent and highly selective inhibitor of NAMPT(24, 25), the rate limiting enzyme in the

124   NAD^+ salvage biosynthetic pathway, led to significantly more neutral lipids at day 16

125   (Figure 1A, day 16, $P<0,01$, Two-way ANOVA with Tukey's post-test), further reinforcing

126   the idea that NAD^+ bioavailability might oppose lipogenesis. As it has been largely

127   shown that SIRT1 is a mayor effector of NAD^+ signaling, we reasoned that it might be

128   dispensable for adipogenesis. Surprisingly, selective inhibition of SIRT1 during

129   adipogenic differentiation strongly hindered neutral lipid accumulation (Figure 1A, day

130   16, $P<0,01$, Two-way ANOVA with Tukey's post-test). Interestingly, distinct dynamic

131   changes in lipid accumulation were observed at days 4, 8 and 12 of adipogenic

132   inductions with the different treatments (Figure 1A).

133 To further confirm that lipid accumulation is changing with the treatments in living cells

134 while avoiding potential artifacts from fixation or ORO stain, we performed label-free

135 Fluorescence lifetime microscopy of intrinsic lipid-associated fluorophores in living cells

136 (see Methods section). Third harmonic generation (THG) images were used as

137 reference to identify lipid droplets (26, 27) (Figure 1B, S1A, S1B), and they showed

138 bright round dots for lipid droplets smaller than 1μm and hollow round structures at the

139 interface between cytoplasm and lipid droplets larger than 1μm. FLIM images (Figure

140 1B) indicated that lipid droplets associated fluorophores have a longer lifetime (τ_m) with

141 respect to the rest of the cell cytoplasm, hence we determined a threshold to

142 automatically and systematically identify and segment lipid droplets (Figure 1B, S1B,

143 Methods section). This method allowed us to quantitively assess lipid accumulation in

144 live hMSCs during adipogenic induction (Figure 1C). We imagined cells at days 4, 6, 8

145 and 12, corresponding to those when more dynamic changes were previously observed,

146 and found that NAD^+ treatment significatively impaired lipid accumulation after day 8

147 (Figure 1C, D; day 8 and 12, $P<0.001$, Two way ANOVA with Tukey's post-test). With

148 this approach, we didn´t find significant differences in lipid accumulation in live cells

149 when comparing FK866 and EX527 treatments to non-treated cells.

150 PPARγ is considered a master transcription factor for adipogenesis(28, 29). It´s

151 expression is induced in early stages(30), and sustains the specific transcriptional

152 program for adipocyte differentiation(31). For these reasons, we explored how NAD^+

153 levels impact PPARγ protein expression during adipogenic induction of hMSCs (Figure

154 1E, 1F). We found that PPARγ1 isoform is strongly expressed since day 4 of

155 differentiation for all tested conditions (Figure 1E, F). Yet, while its expression was

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156   overall sustained along the adipogenic process in control cells, NAD⁺ triggers a very

157   significant reduction in PPARγ1 protein expression while favoring expression of the

158   PPARγ2 isoform at differentiation day 8, which is lost at later stages (Figure 1E, F;

159   *P*<0.01, Two-way ANOVA with Tukey's post-test). Interestingly, EX527 treatment led to

160   significantly decreased expression of PPARγ1 at the end of differentiation. Conversely,

161   FK866 significantly increased PPARγ2 isoform with respect to the control without

162   affecting PPARγ1 protein expression. As expected, a 16-days treatment of hMSC with

163   any of the compounds led to detectable changes in PPARγ expression (Figure S1C).

164   Together, these differential dynamics on PPARγ protein expression point to a mayor

165   role for transcriptional control in the adipogenic potential shown after each treatment,

166   which is also in line with the distinct outcomes in lipid accumulation.

167   **Extensive and specific reprogramming of the transcriptome during adipogenic**

168   **differentiation by NAD⁺**

169   To decipher the dynamic changes occurring in the transcriptome of hMSC during

170   differentiation, we performed RNA-seq analyses from undifferentiated hMSC, and

171   differentiated cells at two different time points: at 8 days of adipogenic differentiation

172   (middle of differentiation protocol) and at 16 days (end of differentiation), in untreated

173   cells and in cells treated with NAD⁺, FK866 or EX527. We first performed an unbiased

174   principal component analysis (PCA) revealing that, as expected, the largest variation

175   was due to the differentiation process (PC1, 24,92%; Figure 2A and S2A). Interestingly,

176   the second component retaining 21.03% of the original variance between samples was

177   mostly related to NAD⁺ treatment (Figure 2A, S2A). Finally, a third component could be

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178   due to the progress of the differentiation process itself (PC3, 8.98%; Figure 2A, S2A).

179   We then performed differential gene expression analyses to compare between samples

180   using DESeq2 (32), (see methods for details and Table S1 for normalized counts).

181   These analyses further reinforced that NAD$^+$ treatment had a large impact in the

182   transcriptome, comparable to the differentiation process itself, as more than 3000 genes

183   were differentially expressed (DE) in differentiating cells treated with NAD$^+$ when

184   compared with their untreated controls (adjusted P <0.05) (Figure S2B). Concomitantly,

185   inhibition of NAD$^+$ biosynthesis by FK866 lead to just 171 differentially expressed genes

186   when compared with the untreated controls at the end of the adipogenic process (Figure

187   S2B, AD16_FKvsAD16). Hence, we sought to determine the molecular signatures of

188   NAD$^+$ treatment during adipogenic differentiation of hMSC. To do so, we first selected

189   the transcripts specifically dysregulated by NAD$^+$ treatment and found 660 common DE

190   genes in NAD$^+$ treated cells when compared with any other treatment or hMSC, which

191   conform a unique NAD$^+$ molecular signature (Figure 2B). Out of these, 157 genes were

192   consistently upregulated, while 407 were always silenced by the treatment (Figure 2B,

193   Table S2). Next, we sought to explore the molecular routes responsible for the impaired

194   adipogenic capacity of NAD$^+$ treated cells. Hence, we selected the genes which

195   appeared consistently dysregulated in NAD$^+$ treated cells whAmong these, en

196   compared with the rest of differentiating cells (AD, AD_EX, AD_FK). We identified 2,057

197   of these genes at day 8 and 1,969 at day 16 after adipogenic induction. 993 genes were

198   shared, out of which 70% (703) were consistently downregulated, while 28% (279) were

199   always overexpressed (Figure 2C, 2D, Table S2). Gene ontology (GO) analyses

200   revealed that most of the upregulated genes are implicated in apoptotic and response to

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201   stress processes, while downregulated genes relate to mRNA metabolism, cellular

202   motility, and differentiation processes (Figure 2E, 2F, Table S2). A subsequent pathway

203   mapping in KEGG of the same transcripts revealed that many genes involved in steroid

204   biosynthesis (*SQLE, DHCR7, FDFT1, SC5D, MSMO1*) were upregulated by NAD$^+$

205   treatment during adipogenic differentiation of hMSCs. Also, apoptotic signaling

206   pathways appeared active, as suggested by the overexpression of several death

207   receptors (*TNFRSF -10A*, *-10B*, *-10D*) , probably triggered by activated ER stress and

208   unfolded protein response, evidenced by increased transcription of *IRE1α* (*ERN1*), BiP

209   (*HSPA5*) and *XBP1* (Fig. 1G, Table S2). Interestingly, increased cholesterol

210   biosynthesis induces ER stress in macrophages(33). In contrast, a unique set of genes

211   downregulated by an excess of NAD$^+$ during adipogenic differentiation pertained to the

212   ribosome pathway, involving many transcripts for ribosomal proteins (Figure 2H, S3A),

213   indicating that their expression is strongly suppressed by NAD$^+$ in an adipogenic

214   context. Additionally, many transcripts involved in cell adhesion and motility, which

215   become expressed during differentiation, were downregulated in NAD$^+$ treated cells,

216   indicating that the adipogenesis process is arrested. Furthermore, we found that the

217   JAK-STAT pathway was impaired in NAD$^+$ treated cells, with downregulated transcripts

218   including *STAT5A and STAT5B,* known positive regulators of the master adipogenic TF

219   PPARγ (34)*,* or the leptin encoding gene *LEP* (Figure 2H, Table S2). Taken together,

220   these data points towards NAD$^+$ promoting a proapoptotic and anti-adipogenic

221   environment, therefore inhibiting differentiation and maturation of adipocytes.

222   Accordingly, a promoter screening for transcription factor binding motifs revealed

223   significant enrichment for SMAD motifs within promoters of upregulated genes (Figure

224   2I, $P = 10^{-7}$), and for CEBP:AP1 motifs amongst downregulated genes´ promoters

225   (Figure 2J, $P = 10^{-8}$). Indeed, SMADS are well known apoptotic regulators, while CEBP

226   is a master adipogenic TF(29, 35).

227   SIRT1 regulates terminal differentiation of pre-adipocytes but is dispensable for

228   adipogenic commitment.

229   If an excess of NAD$^+$ hinders the adipogenic transcriptional reprogramming, while

230   inhibiting NAD$^+$ biosynthesis has only a mild transcriptional effect at the end of the

231   adipogenic process, it is conceptually apparent that critical NAD$^+$ consuming enzymes

232   such as SIRT1 would not be essential for adipocyte differentiation. Accordingly, SIRT1

233   inhibition by EX527 had only a mild effect on the transcriptome at day 8, with just 57 DE

234   genes compared with the untreated cells (Figure S2B, AD8_EX vs AD8, Table S3).

235   Strikingly, in terminally differentiated adipocytes we found 2040 differentially expressed

236   genes, with 1095 upregulated and 945 downregulated transcripts (Figure S2B,

237   AD16_EX vs AD16, Figure 3A, Table S3). This is in line with the absence of lipid

238   accumulation in these cells, and indicates that probably, SIRT1 is dispensable for

239   lineage commitment, but essential for terminal differentiation of adipocytes.

240   Interestingly, at day 8 of differentiation, the anti-adipogenic genes *EGR1* and *NR4A1*

241   (36, 37) were overexpressed in EX527 treated cells (Table S3, *P*<0,0001, Fold change

242   > 2), and these have previously been shown to be upregulated in the early mitotic clonal

243   expansion phase during preadipocyte differentiation(37), further reinforcing that SIRT1

244   inhibition compromises maturation, but not commitment of preadipocytes. Accordingly,

245   at day 16 many genes pertaining to pathways such as fatty acid metabolism,

246   degradation or lipolysis were downregulated in cells treated with EX527, most of them

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247 implicated in PPAR signaling (Figure 3B, S4A, Table S3). Amongst these, the adipokine

248 genes leptin (*LEP*), leptin receptor (*LEPR*), and adiponectin (*ADIPOQ*) showed

249 significatively lower levels in EX527 treated cells (Table S3). Interestingly, SIRT1

250 inhibition upregulated transcripts enriched for adhesion and locomotion, critical

251 processes during the onset of the adipogenesis, including the fibronectin gene *FN1*,

252 which inhibits adipocyte maturation(38) (Figure 3C, S4B. Table S3). Accordingly, gene

253 set enrichment analysis (GSEA) identified adipogenesis and apical junction as the top-

254 ranked hallmarks enriched in untreated and EX527 treated cells respectively (Figure

255 3D, E). Together, these data indicate that SIRT1 activity is essential for adipogenic

256 differentiation, specifically by tightly controlling the transition between adipocyte

257 commitment and maturation, and the maturation process itself. Remarkably, even

258 though SIRT1 mRNA expression increased at the beginning of adipogenic induction

259 (Figure 3F), the protein significantly increased after 8 days of adipogenic induction

260 (Figure 3G, $P<0,0001$, Kluskal-Wallis with Dunn´s post-test), probably due to

261 posttranscriptional regulation, and further reinforcing the notion that SIRT1 is essential

262 for adipocyte maturation. Strikingly, a motif analysis revealed that pharmacological

263 inhibition of SIRT1 downregulated preferred targets genes for distinct members of the

264 FOX family of transcription factors (Figure 3H, $P=1E-11$), while overexpressed genes

265 were enriched for motifs binding homeobox (NKX3-1,-2) or b-Helix- loop-helix (b-HLH)

266 transcription factors such as CLOCK:BMAL (Figure 3I, $P=1E-8$). Indeed, most of these

267 are well-known targets for SIRT1 deacetylation.

268 SIRT1 activity heavily relies on NAD^+ availability, hence the treatment with the NAMPT

269 inhibitor FK866, known to dampen intracellular NAD^+ levels, would prevent SIRT1

270 activation and the subsequent adipocytic gene expression program. Surprisingly, FK866

271 treatment during adipogenesis led to enhanced adipocytic differentiation, through

272 overexpression of adipogenic hallmarks and increased adipocytokine and PPAR

273 signaling over the control, non-treated adipocytes, at terminal differentiation (Figure

274 4A,B, Table S4). Indeed, 39 genes involved in lipid metabolism, including *CD36*,

275 *CEBPA*, *ADIPOQ*, *LPL*, *ACLY*, *FASN*, *ACACA*, *ACACB*, *PLIN1* and *RETSAT*, were

276 highly expressed in terminal adipocytes treated with FK866 (Figure 4C). Moreover,

277 genes related to ossification or cartilage development were downregulated in these cells

278 (Figure 4D), indicating that low NAD^+ levels induced by FK866 treatment potentiate the

279 adipogenic over the osteogenic lineage in hMSCs(7). This is in line with our previous

280 observation that NAD^+ treatment hinders differentiation of hMSC to adipocytes; yet, it is

281 in contrast with the need of SIRT1 activity, which relays on its cofactor NAD^+, for

282 adipocyte maturation. Furthermore, out of 57 differentially expressed genes between

283 the control and EX527 treated cells at day 8 of differentiation, 33 (58%) were also

284 dysregulated by FK866 treatment, suggesting that SIRT1 is not active in FK866 treated

285 cells at day 8 (Figure 4E). Accordingly, SIRT1 expression both at the mRNA and protein

286 levels was dampened by EX527 and FK866 to similar levels during adipogenic induction

287 (Figure 4F, Two-way ANOVA with Turkey´s post-test; Figure 4G, Kluskal-Wallis with

288 Dunn´s post-test; Figure S5A). These data suggest that SIRT1 function might be

289 dispensable for adipocyte commitment, but necessary for differentiation, and the source

290 of NAD^+ essential for SIRT1 activity does not require the salvage pathway.

291 **Increased NAD^+ bioavailability during the adipogenic process impairs the rise of**

292 **mitochondrial respiration capacity in hMSCs.**

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293   A major shift in metabolic phenotype is a hallmark of adipogenic differentiation. Hence,

294   we investigated the functional effect of altering NAD^+ balance and SIRT1 activity during

295   hMSC adipogenesis on energy metabolism by performing extracellular flux analysis for

296   measuring cellular bioenergetics. As expected, non-treated hMSCs progressively

297   increased their respiratory capacity during the adipogenic differentiation when

298   compared to undifferentiated hMSCs, which retained low oxygen consumption rates

299   across all tested days (Figure 5A-D). We observed that at day 4, all tested conditions

300   retained low respiratory capacity, comparable to undifferentiated cells (Figure 5A), while

301   the most prominent increase in respiration capacity occurs between days 8 to 16

302   (Figure 5B-D). Indeed, FK866 treatment overall allowed the metabolic reprogramming

303   during adipogenesis of hMSCs; however, NAD^+ treatment obstructed the progressive

304   increase in mitochondrial respiration (Figure 5A-D). Interestingly, pharmacological

305   inhibition of SIRT1 with EX527 showed major effect after day 12, consisting of markedly

306   reduced respiratory capacity compared to the untreated cells. These results confirm that

307   metabolic reprogramming during adipogenesis is compromised by SIRT1 inhibition

308   specifically at late differentiation stages and reinforce the notion that SIRT1 is essential

309   for adipocyte maturation. We observed major differences between treatments in

310   maximal respiration and spare respiratory capacity at days 12 and 16, when induced

311   cells either untreated or treated with FK866 showed a very significant increase

312   compared to the rest of the conditions (Figure 5E-H, $P < 0.0001$, Two-way ANOVA with

313   Tukey's post-test), indicating a high rate of oxidative phosphorylation in these cells.

314   Non-mitochondrial respiration and proton leak did not show significant differences at

315   any of the studied conditions (Figure S5). Notably, extracellular acidification rate

316 (ECAR) measurements revealed that undifferentiated hMSC exhibit a glycolytic

317 phenotype, while treatment with NAD$^+$ during adipogenic induction also diminished the

318 glycolytic flux (Figure 5I-L), indicating that these cells are metabolically less active.

319 Next, we performed label-free two-photon fluorescence lifetime microscopy (2P-FLIM)

320 of the intrinsic metabolic biomarker NADH in live cells (see Methods section). We

321 examined the fraction of bound NADH (fB_NADH) during adipogenic differentiation with

322 all treatments with a micrometer pixel resolution (Figure 6A). The results were in

323 agreement with the extracellular flux analyses, and we saw that the fB_NADH

324 progressively increased during adipogenic differentiation, as a result of the metabolic

325 shift from a glycolytic to an OXPHOS phenotype (Figure 6A, S6A-E). Also, NAD$^+$

326 treatment induced consistently low fB_NADH across all tested days (Figure 6A),

327 reinforcing the notion that increased NAD$^+$ bioavailability during adipogenic

328 differentiation opposes the metabolic shift towards OXPHOS. With this approach, we

329 only found significant reduction of fB_NADH in cells treated with EX572 at the end of

330 the differentiation process (Figure 6A), which is in line with the extracellular flux

331 analyses indicating that SIRT1 inhibition hinders adipocytic maturation.

332 **Subcellular compartmentalization of NADH metabolism during adipogenesis**

333 **depends on SIRT1 and is impaired by abnormally high NAD$^+$ levels.**

334 Our FLIM and extracellular flux analyses showed important disparities specifically for

335 the cells treated with EX527 during adipogenic differentiation. Interestingly, at days 8

336 and 12, two photon-FLIM didn´t show significant differences between EX527 and

337 untreated cells, while extracellular flux analyses indicated an important reduction in

338 respiratory capacity of EX527-treated cells after day 8. For this reason, we ought to

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339 determine the metabolic signatures at distinct subcellular compartments, in order to

340 dissect[i] the subcellular location of the metabolic changes in these cells (see Methods

341 section and Figure S1). This technique allowed us to capture intensity images and maps

342 of fB_ NADH in the entire cell and in different cellular compartments such as

343 mitochondria and nucleus/cytoplasm, as shown in Figure 6B for a hMSC, and at day 8

344 after adipogenic induction in untreated (AD) or NAD^+ treated cells (AD_NAD^+). Our

345 single cell analysis at day 8 showed a wide distribution of cellular metabolic states in

346 every culture condition (UD, AD, AD-NAD+, AD-FK866 or AD-EX527) (Figure 6D).

347 Considering the metabolic fingerprint of the entire cell, NAD^+ treated cells showed lower

348 fB_NADH than untreated cultures, while FK866 and EX527 treatments didn´t show

349 differences with untreated cells (Figure 6C, Two-way ANOVA with Tukey's post-test).

350 Interestingly, we observed distinct subcellular compartmentalization of NADH

351 metabolism depending on the treatment (Figure 6D, E). Consistently, fB_NADH at both

352 mitochondria and nucleus/cytoplasm were lower in NAD^+ treated cells (Figure 6D-E).

353 Moreover, the fB_NADH in the cytoplasm/nucleus was higher than in mitochondria in

354 differentiating cells, while remained similar in hMSC; yet NAD^+ treatment showed an

355 opposite ratio, where the mitochondrial fraction showed significantly higher levels of

356 fB_NADH (Figure S6F). Interestingly, the fB_NADH of mitochondria appeared

357 significantly decrease in cells treated with the SIRT1 inhibitor, while the fB_NADH

358 increased within the nucleus/cytoplasm compartment in these cells when compared with

359 the non-treated or with the FK866 treated cells (Figure 6D,E; P<0,05, Two-way ANOVA

360 with Tukey's post-test). Together, out data reveal that energy metabolism progress

361 during adipogenic differentiation is impeded by both SIRT1 inhibition and high NAD^+

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362   levels, through different mechanisms at distinct subcellular compartments. These

363   observations have further implications for disease, as provide previously unappreciated

364   sub-cellular insights into the previously reported efficacy of NAD^+ as a treatment for

365   diet-induced obesity and metabolic dysfunction(39, 40).

366

367   **DISCUSSION**

368   In this study, we have demonstrated a previously unappreciated role for NAD+-SIRT1

369   interplay in adipogenesis from hMSC which is dependent on the differentiation stage.

370   We have shown that SIRT1 activity is essential for terminal adipocyte differentiation,

371   and unexpectedly, NAD^+ availability fine tunes the adipogenic process. Mounting

372   research demonstrates that SIRT1 inhibits adipogenesis in mesenchymal stem cells(41-

373   47), however the dynamics of SIRT1 activity during adipogenesis remains poorly

374   understood. Our gene expression data reveals that SIRT1 activity is dispensable for

375   adipocyte commitment, as out of ~5,000 DE genes at day 8 of differentiation, less than

376   60 were significantly altered between normally differentiating cells and those with

377   inhibited SIRT1 activity. Unexpectedly, transition to mature adipocytes was strongly

378   dampened by SIRT1 inhibition, demonstrating SIRT1 is essential. These results are in

379   line with the notion that reducing SIRT1 activity specifically in fat could lead to improved

380   metabolic function in obesity(48) . Moreover, in mice lacking SIRT1 specifically in MSC,

381   their adipogenic capacity appears compromised leading to significant reduction in

382   subcutaneous fat(49), further reinforcing this notion.

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383   A number of studies report that the stem cell redox status is tightly regulated during

384   differentiation, and thus activation of oxidative pathways constitute a metabolic

385   signature of stem-cell differentiation(50, 51). Along the same lines, stem cells appear to

386   contain lower levels of reactive oxygen species (ROS) than their mature progeny, and

387   that ROS accumulation triggers intracellular signaling required for differentiation(52-55).

388   Hence, it appears that metabolic reprogramming activates specific signaling cascades

389   promoting either stem cell maintenance and self-renewal (reduced state) or stem cell

390   proliferation and differentiation (oxidized state). Here, we have shown that fueling

391   energy metabolism with NAD^+ markedly obstructs adipogenic differentiation. Imposing

392   an oxidative redox state during early differentiation triggers a transcriptional program

393   leading to translational arrest and induction of proapoptotic pathways, and these cells

394   acquire a quiescent metabolic phenotype (Figure 2, 4). NAD^+ levels rise at late stages

395   of differentiation(23), probably as a result of the increased oxidative metabolism which

396   in our hMSC adipogenic differentiation model rapidly emerges between days 8-12

397   (Figure 5, 6A). This is coincident with increased levels of SIRT1 transcription and

398   protein expression (Figure 4F, 4G), reinforcing the idea of a SIRT1-dependent late

399   stage of adipogenic differentiation. At this regard, obstructing the NAD^+ salvage

400   pathway through constant inhibition of the rate-limiting enzyme NAMPT during

401   adipogenesis didn´t hinder the adipogenic capacity, demonstrating that the metabolic

402   switch triggered by the transcriptional rewiring might control intracellular variations on

403   NAD^+ levels and activity of NAD^+- consuming enzymes. Indeed, we observed that

404   NAMPT inhibition upregulated transcription of key lipid metabolism genes, and

405   adipocytic identity at terminal differentiation (Figure 4A-C), thus favoring lipid

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406 accumulation (Figure 1A). This suggests that the NAD$^+$ salvage pathway fine tunes

407 adipogenesis in its late stages, and is in line with its protective role in diet-induced

408 obesity(56, 57).

409 To gain insights into our observations in live, single cells at a submicrometric resolution,

410 we developed a new method based on two-photon fluorescence lifetime microscopy

411 (2P-FLIM) of intrinsic fluorophores that provides directly quantitative metrics of

412 adipogenic stem cell differentiation, metabolic state of subcellular compartments. We

413 measured a long lifetime of lipid droplets associated fluorophores (Figure 1B, 1C), as

414 previously shown(58). We used the lifetime contrast to select autofluorescence from

415 lipids and from NADH within cells (Figure S1A, S1B). Third harmonic generation

416 microscopy was performed to visualize lipid droplets (26, 59) . Based on NADH intensity

417 contrast, we also implemented an automated segmentation of mitochondria and

418 cytoplasm/nuclear compartments that relies on their different concentration of NADH

419 (Figure S1B). With this method, we observed lipid accumulation and increased fraction

420 of bound NADH during adipogenic differentiation in live single cells, reflecting the

421 metabolic shift from a glycolysis to OXPHOS metabolism during differentiation(60, 61).

422 We provided here the first description of intracellular NADH metabolic signature in

423 different subcellular compartments and at distinct stages of adipogenic differentiation in

424 human cells, since subcellular characterisation of NAD$^+$/NADH metabolism was

425 previously demonstrated during few hours after adipogenic induction from 3T3-L1

426 murine preadipocytes(62, 63). Our results show that spatial subcellular

427 compartmentalisation of NADH metabolism is highly dependent on the differentiation

428 stage and is intimately linked with the transcriptional reprograming allowing progressive

19

429 lipid accumulation. Hereby, our observations are in agreement with the emerging view

430 that temporal and spatial subcellular metabolic compartmentalisation contributes to

431 numerous biological roles and regulation of intracellular signalling and transcription (24,

432 64) and that NAD$^+$ compartmentalisation regulates adipogenesis(63).

433 Finally, the quantitative metrics based on fluorescence lifetime microscopy developed in

434 this study serves as a label-free biomarker to simultaneously measure lipogenesis and

435 metabolic shifts in single cell. Quantitative characterization of subcellular states from

436 adipose tissues in health and disease using our two-photon microscopy-based method

437 could provide means to uncover new roles of hMSC in obesity, thus paving the way for

438 the development of MSC-based treatments.

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455

AUTHOR´S CONTRIBUTION:

457 LA-A and CS conceived and designed the study. ES-R, TPLU, XdT-R, GF-O conducted

458 experiments. ES-R, TPLU, XdT-R, RO-S, CS and LA-A analyzed and interpreted the

459 data. LN and AT assisted with Seahorse extracellular flux analyses. JJM provided

460 hMSC and validated the model. Two-photon fluorescence lifetime microscopy was

461 performed in CS lab and molecular biology and RNA-seq were done in LA-A lab. LA-A,

462 CS, TPLU, ES-R and GF-O wrote the manuscript. All authors reviewed the manuscript.

463

DECLARATION OF INTEREST

465 The authors declare that the research was conducted in the absence of any commercial

466 or financial relationships that could be construed as a potential conflict of interest.

467

DATA AVAILABILITY

469 All relevant data to this manuscript are available from the authors.

470

471 **METHODS**

472 **Isolation and characterization of BM-MSCs.** Bone marrow derived MSCs were

473 obtained from healthy donors and have been previously described(65). Briefly,

474 mononuclear cells (MNCs) from BM were obtained by density gradient centrifugation,

475 and $2x10^5$ MNCs/cm^2 were seeded in low glucose Dulbecco's modified Eagle's medium

476 (LG-DMEM, Gibco) with 10% fetal bovine serum (FBS; Gibco), 4mM L-glutamine and

477 antibiotics. Cells were incubated at 37°C and 5% of CO_2. At 80% of confluence,

478 adherent cells were trypsinized and reseeded at a density of $0.2x10^4$ cells/cm^2.

479 Experiments were done at 3 - 5 passages. Cell surface markers expression of MSCs

480 were determined by flow cytometry following our previously implemented and described

481 protocols (65, 66), where MSCs were selected to express CD73, CD90 and CD105

482 markers, while being negative to the hematopoietic markers CD45, CD34 and CD14

483 (Figure S7A). Differentiation capacity was assessed using the StemPro™ Adipogenic

484 and Osteogenic differentiation kits (Gibco A1007001, A1007201), and the Chondrogenic

485 Differentiation Medium (Cambrex Bio Science) supplemented with 10 ng/ml of TGFβ

486 (Peprotech 100-21C), following manufacturer´s instructions. Adipogenic differentiation

487 was evaluated by Oil Red O (Sigma O0625) staining after 16 days of induction,

488 osteogenic differentiation was revealed by detecting alkaline phosphatase activity

489 (Sigma B5655) after 14 days of induction, and chondrogenic differentiation was

490 indicated by the presence of mucopolysaccharides positive to alcian blue (Sigma, ca.

491 no. A5268), in micromasses cross-sections, after 28 days of induction(65, 66).

492 Representative images are shown in Figure S7B.

22

493   All protocols were compliant with the Declaration of Helsinki and approved by the Ethics

494   Committee of Villacoapa Hospital, Mexican Institute for Social Security (IMSS).

495   Informed consent was given by the participants. Additionally, a BM-MSC line was

496   purchased from ATCC .

497   **Cell culture and maintenance:** hMSCs were maintained in LG-DMEM (Gibco, cat. no.

498   31600-034) supplemented with 10% FBS, 4mM L-glutamine, 100 μg/ml of penicillin and

499   streptomycin (Gibco), and incubated at 37°C and 5% of CO_2. Adipogenic differentiation

500   was induced in MSCs in growing phase (70%-80% confluency), and drugs were present

501   in the medium as indicated in the text and figures, following our previously standardized

502   protocols(24). The medium and drugs were replaced every four days during the

503   differentiation process. For live cell imaging, BM-hMSCs (ATCC) were cultured on 3.5-

504   cm glass bottom petri dishes (MatTek, Ashland, MA, USA) in 2.5 ml LG-DMEM per well

505   without phenol red and 10% FBS and 100 UI/mL penicillin and 100 µg/ml streptomycin.

506   Cells were plated at the initial density of $1,1x10^4$ cells/cm^2 and allowed to attach

507   overnight in a humidified cell culture incubator at 37 °C in 5% CO_2 before proceeding

508   with treatments. During the imaging experiments, we replaced the adipogenic medium

509   by basal medium without the phenol red before imaging. To determine the metabolic

510   trajectory of NADH lifetime, MSCs were treated with rotenone 50 µM in DMSO (R8875;

511   Sigma-Aldrich, St. Louis, MO, USA) and hydrogen peroxide (H_2O_2) 4mM in DMSO

512   (216763; Sigma-Aldrich, St. Louis, MO, USA) to block the respiratory chain via complex

513   I and to increase NAD^+:NADH ratio via oxidative stress respectively. All cultures tested

514   negative for mycoplasma contamination.

515 **Antibodies and reagents:** The antibodies used in this study are as follows: anti-SIRT1,

516 Millipore cat. no. 07-131; anti-PPARγ, Cell Signaling, cat. no. 81B8; anti-GAPDH-HRP

517 conjugated, GeneTex, cat. no. GTX627408-01; Goat anti-Rabbit Alexa Fluor® 594

518 conjugated, Invitrogen cat. no. R37117; anti rabbit IgG-HRP conjugated, Invitrogen cat.

519 no. 65-6120. All purchased antibodies were validated for mammalian studies (as shown

520 on the manufacturers' websites). EX527 (E7034), FK866 (F8557) and NAD^+ (N8535)

521 were purchased from Sigma.

522 **Oil-Red-O staining:** Cells grown on slides were briefly washed with PBS and fixed for

523 45 min with 4% fresh paraformaldehyde. Preparation of Oil Red O (SIGMA, cat.

524 no.O1391) working solution and staining of slides were performed as described(67).

525 ORO was applied on the slides for 5 min at RT. Slides were washed twice during 10

526 min. in water, and mounted in vectashield mounting media (Vector Labs, cat. no. H-

527 1000). The images were captured with the camera Axiocam EEc 5s coupled to a ZEISS

528 Primovert microscope, using a 20X magnification. Lipid droplets were quantified using

529 the ImageJ software, by converting RGB to 8-bit grayscale images, and then using the

530 "analyze particles" plug-in as described(68). Four frames per slide were used for image

531 analyses and quantification (n=3 biological replicates with 4 technical replicates).

532 **Quantitative real-time PCR:** Total RNA was extracted from MSCs using TRIzol™

533 Reagent (Invitrogen cat. no. 15596018) following the manufacturer´s instructions. cDNA

534 was obtained by retrotranscription of 1 µg of total mRNA with iScript cDNA synthesis kit

535 (Bio-Rad) according to the manufacturer's instructions. Real-time RT-PCR was done

536 with the real-time CFX96 detection system (Bio-Rad). For a 10 µl PCR reaction, 25 ng

537 of cDNA template was mixed with the primers to final concentrations of 200 nM and

24

538   mixed with 5 µl of iTaqTM Universal SYBR® Green Supermix kit (Bio-Rad). The

539   reactions were done in triplicates with the following conditions: 30 sec at 95 °C, followed

540   by 45 cycles of 30 s at 95 °C and 30 s at 60 °C. Expression levels were calculated using

541   the ddCt method. The PCR primers were as follows: SIRT1: Fw, 5´-

542   GCTGGAACAGGTTGCGGGAA-3´; Rv, 5´-GGGCACCTAGGACATCGAGGA-3´. β-

543   actin: Fw, 5´-CTTGTACGCCAACACAGTGC-3´; Rv, 5´-ATACTCCTGCTTGCTGATCC-

544   3´

545   **Immunofluorescences:** MSCs were seeded on Lab-Tek Chamber slides (Thermo

546   Fisher) at $9x10^3$ cells/cm^2. Cells were washed with phosphate buffered saline (PBS;

547   Gibco), fixed with 1% of paraformaldehyde at 37°C for 10 min, washed twice with PBS

548   and permeabilized with PBS 0.1% triton during 15 min. The slides were then washed

549   with PBS, and incubated with blocking buffer (PBS, 0.1% Tween® 20, 2% BSA) for 1

550   hour. Incubation with anti-SIRT1 (1:500) was performed over night at 4°C, and the

551   secondary antibody (1:2,000) was incubated during 1 hour at RT. 1:5,000 dilution of

552   Hoechst 33342 was used for nuclear counterstain (Invitrogen H1399) by incubating

553   during 10 min at RT. Coverslips were mounted using VECTASHIELD® antifade

554   mounting medium (Vector labs H1000) and sealed with nail polish. Fluorescence

555   images were acquired by an Olympus DP70 Digital Camera in an Olympus BX51

556   fluorescence microscope. Hoescht stain was acquired at a 1/300 s exposure, while

557   SIRT1 intensity was acquired at 1/200 s. Densitometry was performed using ImageJ on

558   3 cells at 4 different fields from 2 biological replicates.

559   **Western blotting.** MSCs were harvested from confluent 6-well dishes, washed with

560   PBS and lysed with RIPA buffer supplemented with HDAC inhibitors (50mM Tris pH 8,

25

561   150mM NaCl, 1% 5mM EDTA pH8, 1% NP-40, 0.5% Na deoxycholate, and 0.1% SDS;

562   all from Sigma). Cells where left on ice during 20 min. and centrifuged at 14,000 RPM

563   during 15 min at 4ºC. The protein extracts in the supernatants were snap frozen and

564   stored at -80°C. Protein quantification was done using the Bradford colorimetric assay

565   (Sigma B6916). 20µg of proteins were separated run on a 10% SDS-PAGE gel, at 100

566   V for 2 hours using a mini-PROTEAN system (BIO-RAD) and transferred to

567   nitrocellulose membrane (Millipore) at 40 mV overnight at 4°C. A 1:1,500 dilution of anti-

568   PPARγ in blocking buffer (Tris-buffered saline plus Tween-20 -TBST- and 5% nonfat

569   milk) was used to detect the protein. Antibodies to GAPDH protein at 1:20,000 dilution

570   in PBST were used as a loading control. Proteins were revealed using

571   chemiluminescent detection (Immobilon Western, Millipore WBKLS0100) and visualized

572   using a Kodak GEL Logic 1500 Imaging System with Transilluminator.

573   **Extracellular flux analysis.** OCR and ECAR were measured using the Seahorse

574   XFe96 Analyzer (Agilent), using The Cell Mito Stress Test Kit (Agilent, cat. no. 103015-

575   100). MSCs were seeded at a density of 10,000 cells/well in a XF96 cell culture 96-well

576   microplate (Agilent 101085-004) precoated with 10 µg/ml of fibronectin (Sigma F1141).

577   Adipogenic induction and treatments were initiated after two days of seeding. One hour

578   prior the Seahorse analysis, MSCs culture were washed with 200 µl/well of XF assay

579   media supplemented with 10 mM glucose, 2mM glutamine and 1 mM pyruvate. Then,

580   180 µl/well of this medium was added and the plate was equilibrated for 30 min at 37°C

581   in a CO_2-free incubator before being transferred to the Seahorse XFe96 analyzer.

582   Measurement of OCR and ECAR was done at baseline and following sequential

583   injections of (i) 2 µM oligomycin, an ATP synthase inhibitor, (ii) 0.5 µM carbonyl

26

584 cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), a protonophoric uncoupler, and

585 (iii) 0,5 µM of rotenone, an inhibitor of complex I of the electron transport chain. Briefly,

586 oligomycin inhibits mitochondrial ATP synthase, and the resulting drop in OCR and rise

587 in ECAR are attributed to ATP-linked OCR and the compensation of glycolysis for the

588 loss of mitochondrial ATP production. FCCP uncouples the mitochondrial proton

589 gradient and oxygen consumption from ATP synthase, hereby driving maximal OCR.

590 Rotenone inhibits complex I of the electron transport chain, hence it hinders

591 mitochondrial oxygen consumption. Therefore, the residual OCR is regarded as

592 nonmitochondrial.

593 **Expression profiling (RNA-seq) and analysis**. Total RNA from MSCs was extracted

594 using Quick RNA MiniPrep Kit (Zymo Research, USA) following the manufacturer´s

595 instructions. RNA samples with RNA integrity number (RINs) > 7.0 were sent for library

596 preparation and sequencing to Novogene Corporation Inc., California, USA. Briefly,

597 mRNA was isolated using oligo(dT) beads and randomly fragmented by adding

598 fragmentation buffer, followed by cDNA synthesis primed with random hexamers. Next,

599 a second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I

600 were added for second-strand synthesis. After end repair, barcode ligation and

601 sequencing adaptor ligation, the double-stranded cDNA library was completed with size

602 selection to 250-300 bp, and PCR enrichment. Sequencing was performed on an

603 Illumina NovaSeq 6000 Sequencing System with paired-end 150 bp reads, at 9 G raw

604 data/sample. Total and mapped reads per sample are shown in Supplementary Table

605 S1.

606   **RNA-seq data processing.** Human Reference genome and gene model annotation

607   files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly.

608   Indexes of the reference genome was built using STAR and paired-end clean reads

609   were aligned to the *Homo sapiens* assembly GRCh38/hg38, with the STAR aligner v2.5

610   (69). STAR uses the method of Maximal Mappable Prefix (MMP) which can generate a

611   precise mapping result for junction reads. HTSeq v0.6.1 was used to count the read

612   numbers mapped of each gene. Afterwards, FPKM of each gene was calculated based

613   on the length of the gene and reads count mapped to it (70). Differential expression

614   analysis between conditions (three biological replicates per condition) was performed

615   with the DESeq2 R package (2_1.6.3), which uses a model based on the negative

616   binomial distribution (32). The resulting *P*-values were adjusted using the Benjamini and

617   Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an

618   adjusted *P*-value <0.05 were assigned as differentially expressed. The Venn diagrams

619   were prepared using the function vennDiagram in R based on the gene list for different

620   groups, or with Venny V 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). Differentially

621   expressed genes were subjected to functional analyses using the "Compute Overlaps"

622   tool to explore overlap with the CP (Canonical Pathways) and the GO:BP (GO biological

623   process) gene sets at the MSigDB (molecular signature database). The tool is available

624   at: https://www.gsea-msigdb.org/gsea/msigdb/annotate.jsp, and estimates statistical

625   significance by calculating the FDR q-value. This is the FDR analog of the

626   hypergeometric P-value after correction for multiple hypothesis testing according to

627   Benjamini and Hochberg. Gene set enrichment analysis (GSEA) was performed using

628   GSEA v. 4.0.3. (71) to determine the enrichment score within the Hallmark gene set

629   collection in MSigDB v7.0(72), selecting the Signal2Noise as the metric for ranking

630   genes. The findMotifs.pl program in the HOMER software [73] was used for motif

631   discovery and enrichment, searching within the genomic regions encompassing 300 Kb

632   upstream and 50 Kb downstream the TSS, and selecting 6-8 bp for motif length. Motif

633   enrichment is calculated by findMotifs.pl using the cumulative hypergeometric

634   distribution.

635   All RNA-seq raw and processed data will be made publicly available at Gene

636   Expression Omnibus (GEO) under the accession number GSE178615

637   **Two-photon excited fluorescence lifetime imaging (FLIM) and third harmonic**

638   **generation (THG).** Imaging was performed on a laser scanning microscope

639   (TriMScope, Lavision Biotec, Germany). A simplified scheme of the multiphoton

640   microscope is shown in Figure S7C. The Excitation is provided by a dual-output

641   femtosecond laser (Insight DS++, Spectra-Physics, Santa Clara, CA, USA) with a first

642   beam turnable from 680 nm to 1300 nm (120 fs pulses, 80 MHz) and a second, fixed

643   wavelength beam at 1040 nm (200 fs pulses). A water Immersion objective (25X,

644   NA=1.05, XLPLN-MP, Olympus, Japan) is used to focus the laser on the sample and

645   collect fluoresce signal. Fluorescence signal is epi-detected by a hybrid photomultiplier

646   tube (R10467U, Hamamatsu, Japan -), whereas and third-harmonic generation (THG)

647   signal is forward detected by a photomultiplier (H6780-01, Hamamatsu, Japan). To

648   perform Fluorescence lifetime microscopy of NADH, 760 nm wavelength excitation was

649   used with a typical power of 12 mW (Figure S7D). A band-pass filter was used in front

650   of the detector to collect NADH autofluorescence (Semrock FF01–460/80).Time-

651   correlated single photon counting (TCSPC) electronics (Lavision Biotec, Germany) with

652 5,5 ns dead time, and 27 ps time bins was used to measure the arrival time of the

653 fluorescence photons with respect to the laser pulse and perform FLIM imaging. The

654 laser trigger reference was taken from the fixed wavelength beam using a photodiode

655 (PDA10CF-EC, Thorlab). Calibration of the FLIM system was performed by measuring

656 the lifetime of fluorescein at pH=9 with a single exponential of 4 ns (Figure S7E). We

657 measured the lifetime of free NADH in solution (Sigma Aldrich n. N8129, St. Louis, MO,

658 USA) to calculate the fraction of bound NADH (Figure S6B). We typically collected 500

659 photons for FLIM images of live cells with a pixel dwell time of 240 μs/pixel and a total

660 acquisition time on the order of one minute. Third harmonic generation was performed

661 using a wavelength of 1100nm with a typical power of 12mW and the signal was

662 collected with a band-pass filter (Semrock FF01–377/50) (Figure S7D). We typically

663 collected 800 photons for THG images with a a pixel dwell time of 53 μs/pixel and a total

664 acquisition time of the order of one minute.

665 **Analysis of the Fluorescence Lifetime microscopy images.** Intensity images were

666 analysed with Fiji-ImageJ (NIH, Bethesda, MD, USA). All FLIM data was processed and

667 analysed with SimFCS (developed by the laboratory for Fluorescence Dynamics

668 https://www.lfd.uci.edu/globals/) and with a Matlab (Mathworks, Natick, MA, USA)

669 custom written software. FLIM data were transformed by using FFT and plotted in the

670 phasor plot as previously described (74) (75) (see Supplementary material). Briefly, the

671 coordinates g and s in the phasor plot were calculated from the fluorescence intensity

672 decay of each pixel of the image (Figure S7F) by using the transformations defined in

673 the Supplementary material (equation 1 and 2). We applied an intensity threshold to

674 eliminate the background of the cellular medium and a median filter on the g and s

30

675 images to reduce the variance of the phasor location without decreasing the spatial

676 resolution of the image. For every pixel of the image, we calculated the value of τ_φ

677 (equation (5) in Supplementary material) and τ_m (equation (6) in Supplementary

678 material) starting from the g and s images (Figure S1A). Fraction of bound NADH

679 (equation (7) in Supplementary material) was graphically calculated as the distance

680 from the experimental point to the location of free NADH (Figure S1A and Figure S6B).

681 **Subcellular segmentation of lipid droplets, cell NADH, mitochondria and nucleus**

682 **and cytoplasm.** Image processing and segmentation was performed by a Matlab

683 custom written software. The principles of the segmentation are illustrated in Figure

684 S1B. A threshold (2.87 ns) was applied in the τ_m lifetime image to automatically

685 separate lipid droplets and NADH of the cell. Pixels with longer lifetime were assigned

686 to a lipid mask while pixels with shorter lifetime were highlighted to the NADH cell mask.

687 The τ_m threshold was determined empirically to match the lipid mask border with the

688 THG signal of the lipid droplet (Figure 1B). Then the mask of NADH cells was used to

689 calculate fB_NADH of the same ROI or cell. For quantification, we used the average

690 values of fB_NADH and lipid ratio. To quantify the lipid droplets in ROIs or in single

691 cells, we calculated the ratio between the number of pixels of the lipid mask and the

692 total number pixels of the cell: Single cell analysis was performed manually. We

693 performed subcellular segmentation of mitochondria and nucleus plus cytoplasm

694 applying a threshold (150 photons) to the intensity image multiplied with the NADH

695 mask (Figure S1B). The threshold was determined based on the different NAD^+/NADH

696 ratios in mitochondria (~ 10) and cytoplasm and nucleus (~ 50-1000)[76]. Pixels with

697 higher number of photons were assigned to mitochondria while pixels with lower

31

698 number of photons are assigned to nucleus and cytoplasm. The masks of mitochondria

699 and nucleus and cytoplasm were applied to the map of fraction of bound NADH, and the

700 average fraction of bound NADH was calculated in different cellular compartments for

701 statistical analysis.

702 **Statistics and images analyses.** Data are shown as mean with SEM. Statistical

703 analyses were performed using GraphPad Prism 8.2. The statistical tests were

704 performed as indicated in the figure legends, mostly consisting of Two way ANOVA

705 followed by Turkey´s multiple comparisons test, or Kruskal–Wallis H test . Statistical

706 significance was considered when the P value was <0.05. When possible, experimental

707 evaluation was performed blind to the experimental conditions (i.e., specifically for

708 western blot quantification, image processing and subsequent quantifications).Western

709 blot analyses and image processing for overlay in different channels from

710 immunofluorescences were performed with ImageJ software. Figures were arranged

711 using Adobe Illustrator.

712

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891

892 **FIGURE LEGENDS:**

893 **Figure 1: NAD$^+$ hinders adipogenic differentiation and lipid accumulation in**

894 **hMSC.**

895 Adipogenic differentiation was induced in hMSC in the absence or presence of the

896 indicated drugs: NAD$^+$ (5 mM), FK866 (1 nM) or the SIRT1-specific inhibitor EX527 (50

897 µM). **A)** Neutral lipids were stained with Oil-Red-O (ORO) at the indicated days after

898 adipogenic induction, and representative images from are shown. Quantification was

899 performed by densitometry from n=4 technical and 3 biological replicates. **B)**

900 Representative images from Label free quantification of lipid droplets by Fluorescence

901 Lifetime Microscopy of an adipocyte at terminal differentiation. Intensity , modulation

902 lifetime tauM (τ_m) lipid mask determined by a τ_m threshold, and Third harmonic

903 generation (THG) intensity are shown as indicated. The graph on the right shows a

904 cross sectional profile of lipid mask created by a τ_m threshold and THG signal of one

905 lipid droplet of 5 µm diameter. **C)** Representative images of intensity (left), modulation

906 lifetime τ_m (middle) and lipid mask (right) of hMSCs during adipogenic differentiation at

907 the indicated days of culture under selected treatments **D)** Quantification from lipid ratio

908 at indicated days and treatments during the differentiation process of hMSC. n=14

909 single cells; experiments were conducted in triplicate. **E)** PPARγ1 and PPARγ2 protein

910 expression levels were measured by western blot in whole cell extracts at the indicated

911 days after adipogenic induction. p84 was used as loading control. **F)** quantification of

912 PPARγ1 and PPARγ2 protein expression, normalized to p84 loading control. n=3

913 biological replicates. For all graphs, data is presented by mean ± SEM (*p< 0,05, **p<

914 0,01, ***p< 0,001, Two-way ANOVA with Tukey's post-test)

37

915 **Figure 2: NAD$^+$ treatment during adipogenesis from hMSC induces profound and**

916 **specific changes in the transcriptome.**

917 RNA-seq was performed per triplicate from multipotent hMSC (MSC, UD0), or at days 8

918 (AD8) and 16 (AD16) after adipogenic induction in the absence (AD8, AD16) or

919 presence of 5 mM NAD+ (AD8_NAD, AD16_NAD), 1 nM FK866 (AD8_FK866,

920 AD16_FK866) or 50 μM EX527 (AD8_EX527, AD16_EX527) **A)** Principal component

921 analysis (PCA) was computed for the whole data. **B)** Heatmap comparing expression

922 from 660 genes DE exclusively in NAD$^+$ treated cells (FDR-adjusted *P*-value <0.05) **C)**

923 Overlap of DE transcripts between NAD+ treated cells and the rest of the tested

924 conditions at day 8 and day 16 after adipogenic induction **D)** Heatmap comparing

925 expression from 994 genes DE exclusively in NAD$^+$ treated cells at both days 8 and 16

926 after adipogenic induction when compared with the rest of the samples. **E-H)** Functional

927 annotation of the 994 DE genes constituting the NAD$^+$ transcriptional signature:

928 biological processes (E, F) or KEGG pthaways (G-H) for consistently upregulated (E, G)

929 or downregulated (F, H) transcripts. **I, J)** Homer *de novo* motif discovery analyses from

930 promoters of genes specifically upregulated (I) or downregulated (J) after NAD$^+$

931 treatment during adipogenic induction.

932 **Figure 3: SIRT1 activity is essential for terminal differentiation of pre-adipocytes.**

933 **A)** Heatmap comparing expression from 2040 genes DE between EX527-treated cells

934 (50 μM) and untreated cell at day 16 after adipogenic induction on hMSC. (FDR-

935 adjusted *P*-value <0.05) **B, C)** KEGG pathway enrichment analyses from genes

936 downregulated (B) or upregulated (C) by EX527 treatment during adipogenic

38

937   differentiation, at day 16 after induction, compared with untreated, terminally

938   differentiated adipocytes. **D, E)** Gene set enrichment analysis (GSEA) investigated

939   within the molecular signature database (MSigDB) "Hallmark" gene set collection.

940   Genes were rank-ordered by differential expression between terminally differentiated

941   adipocytes untreated (AD16) or treated with EX527 (AD16_EX). **F)** *SIRT1* gene

942   expression levels were assessed by RT-qPCR at the indicated days after adipogenic

943   induction on hMSC. n= 3 biological and 2 technical replicates. One-way ANOVA

944   followed by Tukey´s post test. * p <0.05, ** p <0.01, *** p <0.001. Symbol key for

945   multiple comparisons: *: day 0 vs days 3, 6; $: day 0 vs days 9-16; #: days 3, 6 vs days

946   9-16. **G)** SIRT1 protein expression and subcellular location was explored by

947   immunofluorescence at the indicated days after adipogenic induction on hMSC. Boxplot

948   shows densitometric analyses from n= 2 biological and 7 technical replicates. Kluskal-

949   Wallis test followed by Dunn's multiple comparisons test was applied. * p <0.05, ** p

950   <0.01, *** p <0.001. **H, I)** Homer *de novo* motif discovery analyses from promoters of

951   genes downregulated (H) or upregulated (I) by EX527 treatment during adipogenic

952   induction, at terminal differentiation (day 16).

953   **Figure 4: The NAD$^+$ salvage pathway is dispensable for adipogenesis**

954   **A)** Gene set enrichment analysis (GSEA) was investigated within the molecular

955   signature database (MSigDB) "Hallmark" gene set collection. Genes were rank-ordered

956   by differential expression between terminally differentiated adipocytes untreated (AD16)

957   or treated with 1 nM FK866 (AD16_FK). **B)** Functional annotation (Biological processes

958   and KEGG pathways) for 144 genes upregulated by FK866 treatment during

959   adipogenesis, at terminal adipogenic differentiation (day 16). **C)** Heatmap comparing

39

960 expression levels between the indicated samples at day 16 form 39 genes involved in

961 lipid metabolism. **D)** Biological processes enriched in 44 genes downregulated by

962 FK866 treatment during adipogenesis, at terminal differentiation (day 16). **E)** Overlap of

963 DE genes (up- or downregulated) comparing EX527 and FK866 treated cells during

964 adipogenic induction, at day 8 **F)** *SIRT1* gene expression levels were assessed by RT-

965 qPCR at the indicated days after adipogenic induction on hMSC either untreated (AD)

966 or treated with the indicated drugs. n= 3 biological and 2 technical replicates. One-way

967 ANOVA followed by Tukey´s post test. ** p <0.01, *** p <0.001. **G)** Boxplot showing

968 SIRT1 protein levels analyzed by immunofluorescence at days 8 and 16 after

969 adipogenic induction on hMSC. Cells were either untreated (AD), or treated with the

970 indicated compounds. Densitometric analyses are from n= 2 biological and 7 technical

971 replicates. Kluskal-Wallis test followed by Dunn's multiple comparisons test was applied.

972 n.s.: non-significant * p <0.05, ** p <0.01, *** p <0.001.

973 **Figure 5: NAD+ impairs mitochondrial bioenergetics during adipogenic induction**

974 **in hMSC.**

975 Analysis of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

976 was performed using Seahorse XF analyzer to assess mitochondrial respiration and

977 lactate production from n = 3 biological replicates with 6-10 technical replicates each. **A-**

978 **D)** OCR was measured at days 4 (A), 8 (B), 12 (C) or 16 (D) after adipogenic induction

979 in hMSC, in the absence or presence of the indicated treatments. with sequential

980 addition of oligomycin (Oligo, complex V inhibitor), FCCP (a protonophore), and

981 Rotenone/antimycin A (Rot/AA, complex III inhibitor), **E-F**) Mitochondrial bioenergetic

982 parameters calculated from extracellular flux analyses: basal respiration, maximal

40

983   respiratory capacity, spare respiratory capacity, and ATP production. Two-way ANOVA

984   followed by Tukey´s post test. * p <0.05, ** p <0.01, *** p <0.001. **I-L)** ECAR was

985   measured after serial addition of oligomycin and FCCP. Data is presented by mean
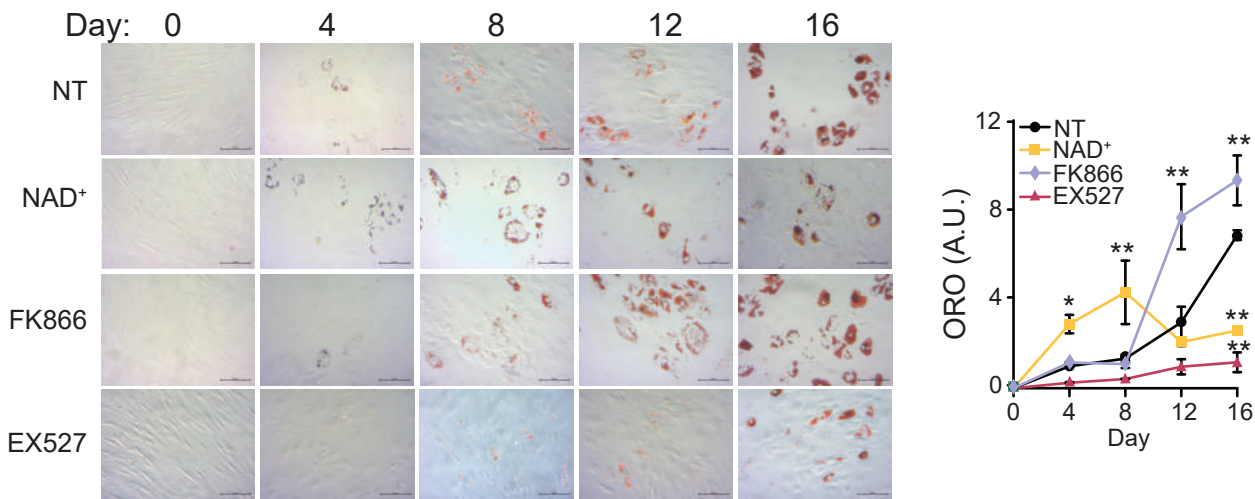
986   ±SEM.

987   AD: adipogenic induced cells; NAD+ adipogenic induced cells treated with 5 mM NAD$^+$;

988   FK866: adipogenic induced cells treated with 1 nM FK866; EX527: adipogenic induced

989   cells treated with 50 μM EX527. MSC: untreated, undifferentiated hMSC.

990   **Figure 6: Subcellular compartmentalization of NADH metabolism during**

991   **adipogenesis depends on SIRT1 activity.**

992   **A)** Representative images of fraction of bound NADH (fB_NADH) of hMSCs during

993   adipogenic differentiation at days 4, 6, 8, 12 and 16 of induction in the absence (NT) or

994   presence of the indicated treatments: 5 mM NAD$^+$, 1 nM FK866 or 50 μM EX527. Low

995   fB_NADH (blue colors) corresponds to a cellular glycolytic phenotype, while high

996   fB_NADH (red colors) corresponds to an OXPHOS phenotype. Quantification of fraction

997   of bound NADH in each culture condition was performed from n= 14 single cells.

998   Experiments were conducted per triplicate. Data is presented by mean ±SEM. **B)**

999   Representative images of intensity and fB_NADH of hMSCs, pre-adipocytes (AD) and

1000   cells treated with NAD+ during adipogenic induction (AD_NAD$^+$) imaged at day 8 of

1001   differentiation, show different spatial distributions of fraction of bound NADH in different

1002   cell compartments such as mitochondria, nucleus and cytoplasm. **C-E)** Quantification of

1003   fB_NADH in single cells at day 8 from hMSC (MSC), pre-adipcyte (AD) and cells treated

1004   with the indicated compounds during adipogenic differentiation. Quantification from n=

1005   63-125 cells was performed in the whole cell (C), or the mitochondrial (D) and

1006   nuclear/cytoplasmic (E) subcellular compartment. Two-way ANOVA followed by

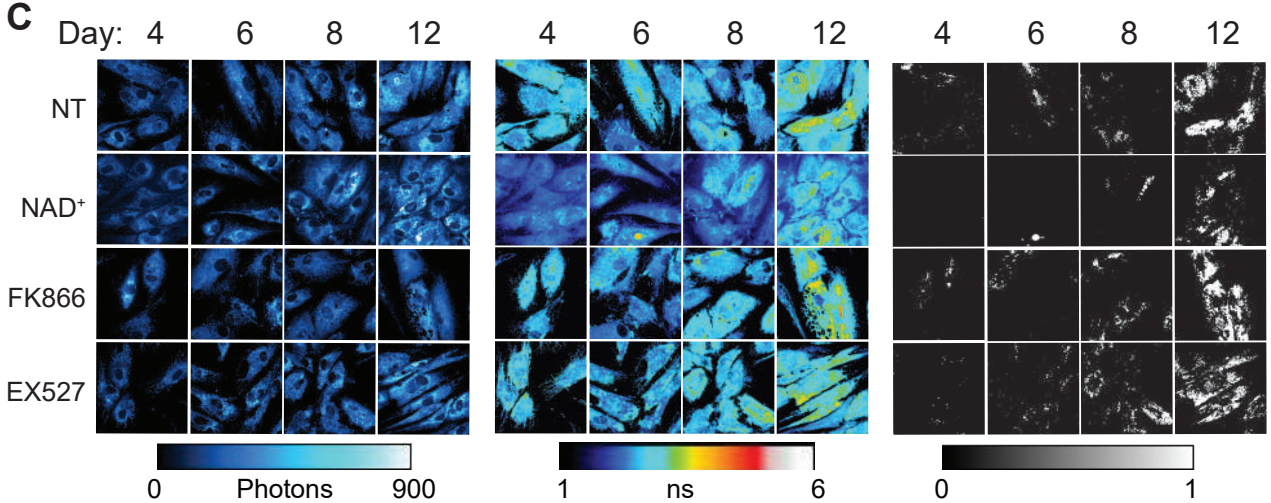1007   Tukey´s post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s.: non-significant.
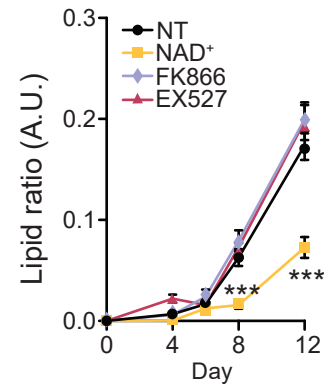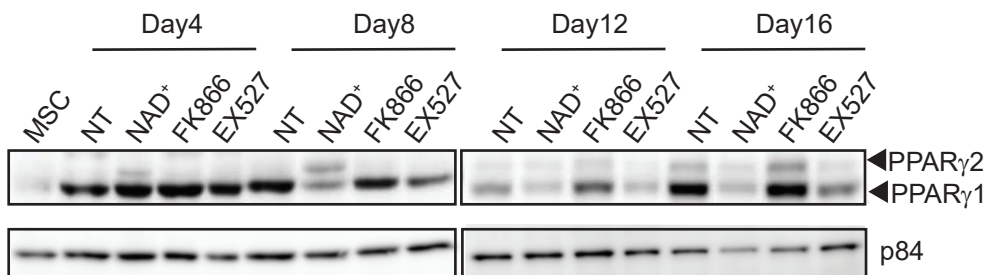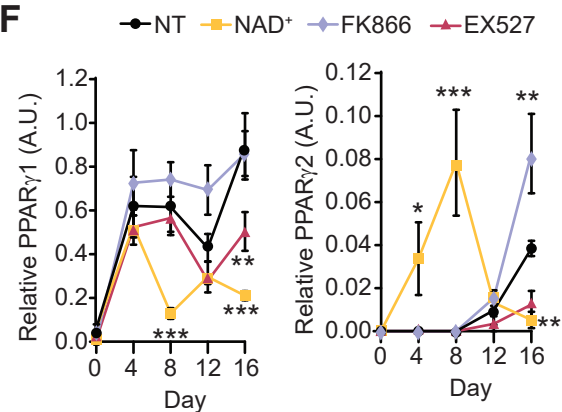
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Edgar Sanchez-Ramirez, Thu-Phuong Lien Ung *et al.*, **Figure 2**

A

B

C NAD+ molecular signatures

Day 8 | Day 16

1064 | 993 | 976

D

E NAD+ Upregulated genes

Biological Processes — -Log10 p value; FDR<0.001

- Apoptotic Process
- Regulation of Response to Stress
- Regulation of Cell Death
- Response to ER Stress
- Ion Transport
- Response to Cytokine
- ER Unfolded Protein Response
- Immune Effector Process
- Regulation of Macroautophagy
- Carbohydrate Metabolic Process
- Lipid Biosynthetic Process
- Neuron Development
- Neuron Differentiation
- Response to Oxygen Levels
- Apoptotic Signaling by p53 Class Mediator

F NAD+ Downregulated genes

Biological Processes — -Log10 p value; FDR<0.0001

- Cotranslational Protein Targeting to Membrane
- Nucl. Transcribed mRNA Catabolic Process NMD
- Translational Initiation
- Biological Adhesion
- Nuclear Transcribed mRNA Catabolic Process
- Locomotion
- RNA Catabolic Process
- Cellular Macromolecule Localization
- Cellular Component Morphogenesis
- Regulation of Cell Differentiation
- Cardiovascular System Development
- Neuron Differentiation
- Ossification
- Response to Growth Factor
- Skeletal System Development

G NAD+ Upregulated genes

KEGG Pathway — -Log10 p value; FDR<0.01

- Antigen Processing and Presentation
- Steroid Biosynthesis
- Protein Processing in ER
- Lysosome
- Apoptosis
- Glycosaminoglycan Biosynthesis
- p53 Signaling Pathway
- PI3-Akt Signaling Pathway

H NAD+ Downregulated genes

KEGG Pathway — -Log10 p value; FDR<0.001

- Ribosome
- Focal Adhesion
- Hipertrophic cardiomyopathy
- Regulation of actin cytoskeleton
- PI3-Akt Signaling Pathway
- Biosynthesis of Unsaturated Fatty Acids
- Rap1 Signaling Pathway
- Jak-STAT Signaling Pathway

I

NAD+ Upregulated genes *de novo* motif	P-value % of targets	Similar known motifs
1 CGACTATA	1 e-7 13,81% (5,07%)	TBP SOX17
2 CTCGTCTG	1 e-7 8,21% (2,03%)	SMAD4 SMAD2
3 GAGTCGAC	1 e-7 16,24% (6,84%)	ZPF691

Known motif enrichment	P-value/q-value % of targets	Motif name/ID
TATGCAAATTA	1 e-4 / 0,0326 6,34% (2,12%)	BRN1 / NPC-Brn1-ChIP-seq (GSE35496)

J

NAD+ Downregulated genes *de novo* motif	P-value % of targets	Similar known motifs
1 ATGTAAGC	1 e-8 12,29% (6,22%)	TBP SIX6
2 TTTGCAGC	1 e-8 19,19% (11,49%)	POU5 CEBP:AP1
3 GTCGCCTT	1 e-7 20,99% (13,17%)	NR5A1 NR4A2

Known motif enrichment	P-value/q-value % of targets	Motif name/ID
CCATATATGG	1 e-5 / 0,0009 7,05% (3,32%)	SRF / PUER-Srf-ChIP-seq (*Sullivan Et. Al.*)

Edgar Sánchez-Ramírez, Thi Phuong Lien Ung *et al.*; **Figure 4**

Edgar Sanchez-Ramirez, The Phuong-Lien Ung et al., **Figure 5**
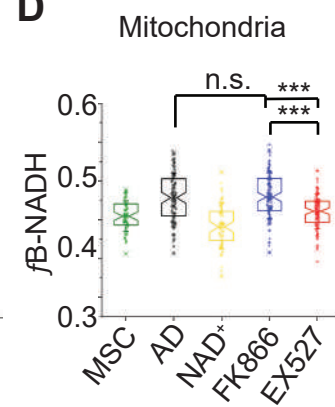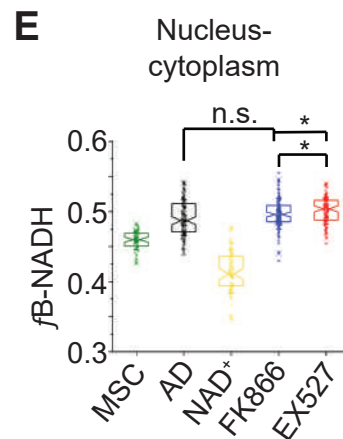
Supplementary Information for

Coordination between metabolic transitions and gene expression by NAD$^+$ availability during adipogenic differentiation in human cells.

Edgar Sanchez-Ramírez, Thi Phuong Lien Ung, Ximena del Toro-Rios, Guadalupe R. Fajardo-Orduña, Lilia G. Noriega, Armando R. Tovar, Juan José Montesinos, Ricardo Orozco-Solís, Chiara Stringari and Lorena Aguilar-Arnal.

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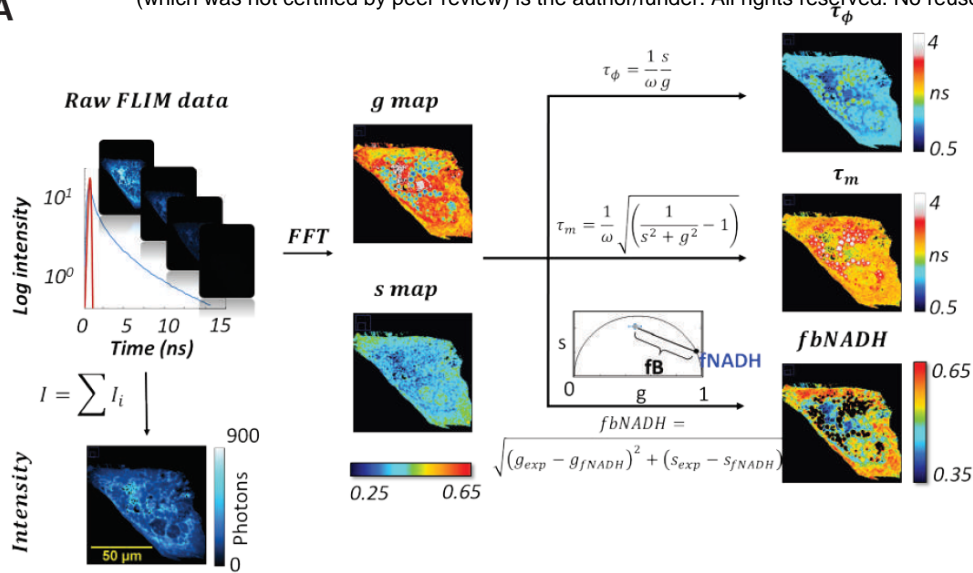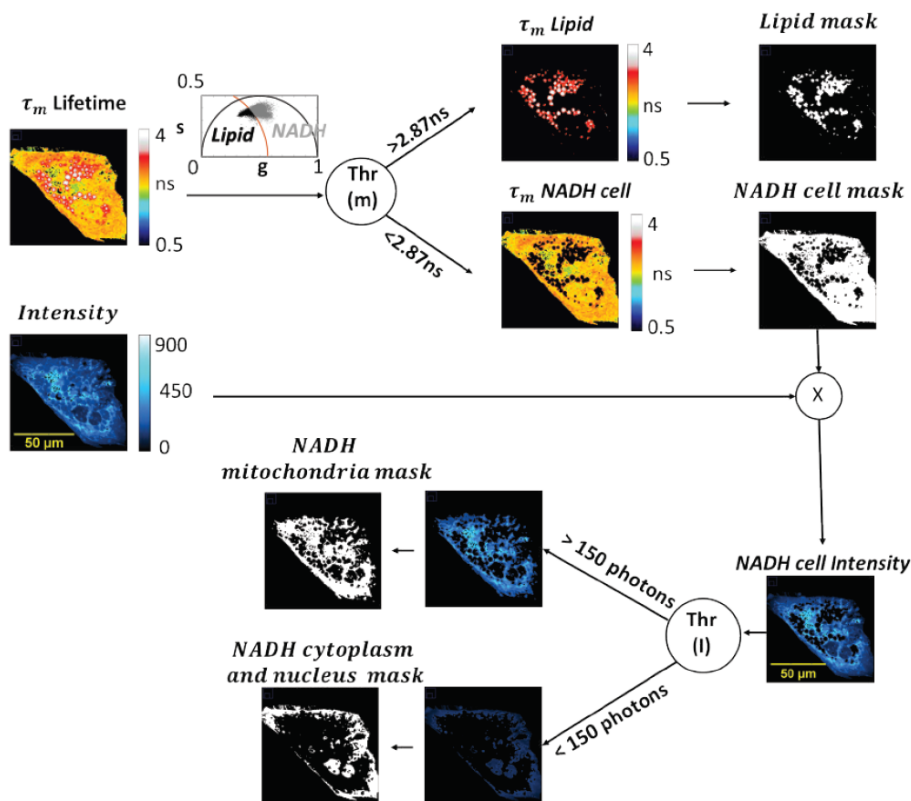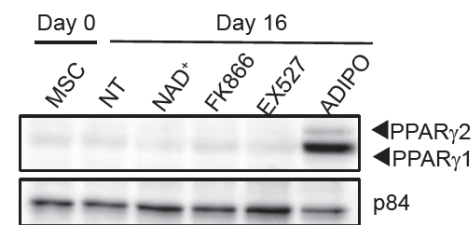
Fig. S1. Image processing workflow for FLIM and Lipid and NADH segmentation A) Workflow of FFT based Phasor analysis of Fluorescence Lifetime Microscopy images. B) Workflow of sub-cellular segmentation based on lifetime and intensity thresholds. A threshold on modulation lifetime (THR (m) = 2.87ns) is applied to separate lipid droplets and NADH in entire cell. The pixels of the FLIM image with τm > THR (m) (black points in the phasor plot) are assigned to lipid droplets while the pixels with τm < THR (m) (grey points in the phasor plot) are assigned to the NADH signal in the rest of the cell. A threshold (150 photons) on intensity of cell NADH is applied to segment mitochondria and nucleus plus cytoplasm. The pixels with intensity> THR (I) are assigned to mitochondria while pixels with Intensity< THR (I) are assigned to the cytoplasm and nucleus. C) PPARγ1 and PPARγ2 protein expression levels were measured by western blot in whole cell extracts from hMSC untreated (NT) or treated with the indicated compounds for 16 days. Terminally differentiated adipocytes (ADIPO) were also included. p84 was used as loading control.

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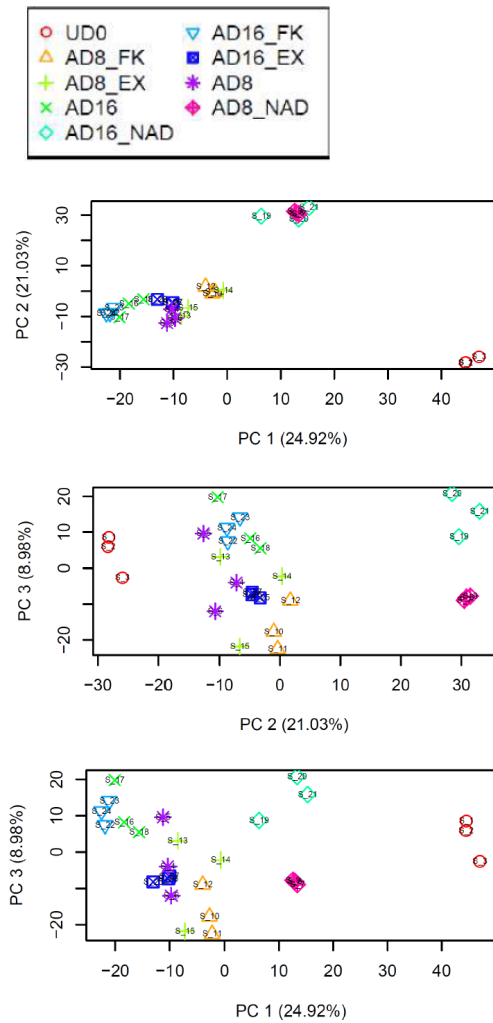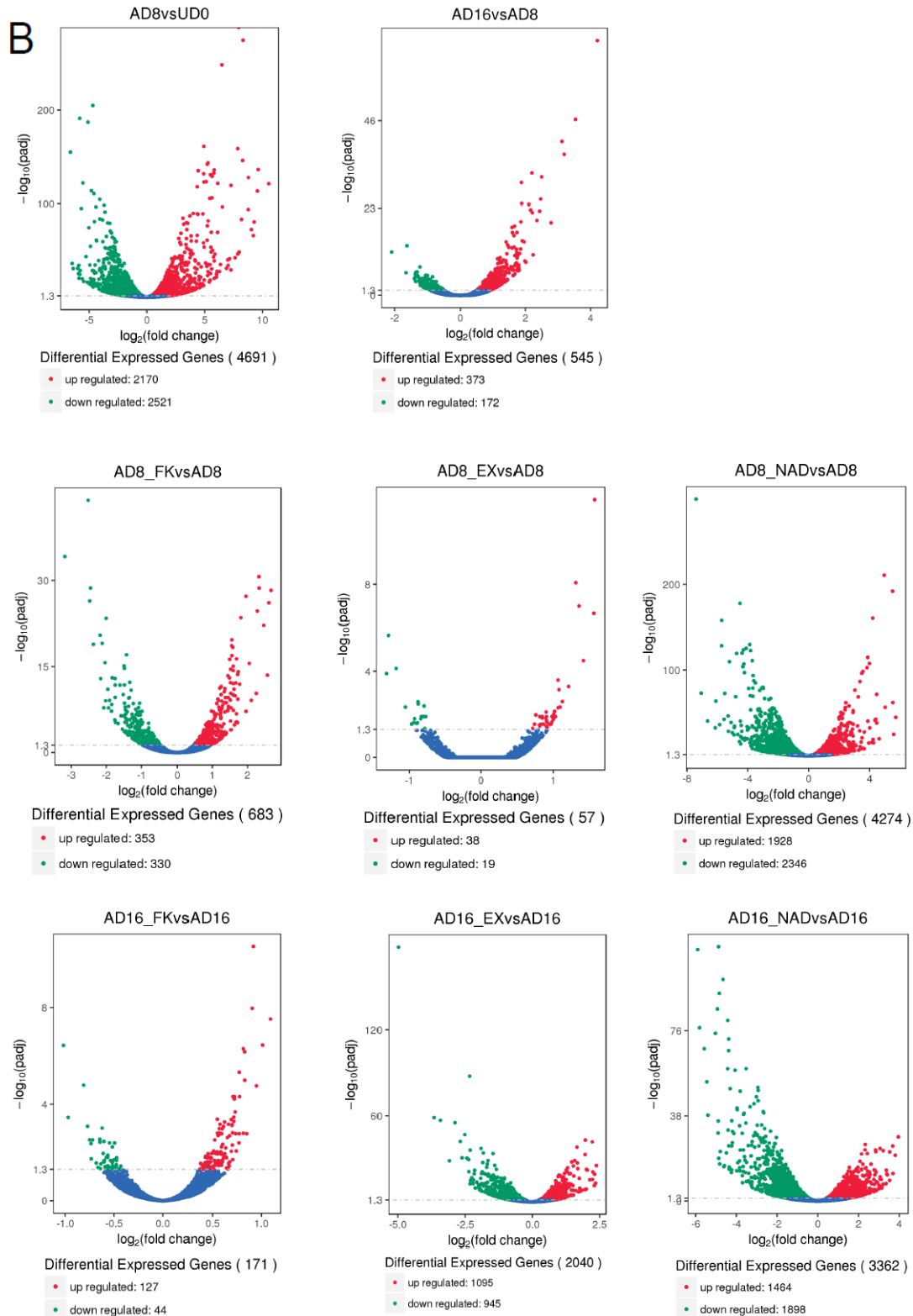
Fig. S2. RNA-seq data analyses. A) Principal component analyses (PCA) was computed and plotted in two dimensional PCA score plots, showing clustering of undifferentiated hMSC (UD) vs differentiated cells (top), NAD+-treated (AD8_NAD, AD16_NAD) vs untreated cells (middle) and day 8 (adipocyte commitment) vs day 16 (terminally differentiated adipocytes) (bottom). **B)** Volcano plots show DE genes from the indicated comparisons (FDR-adjusted P-value <0.05).
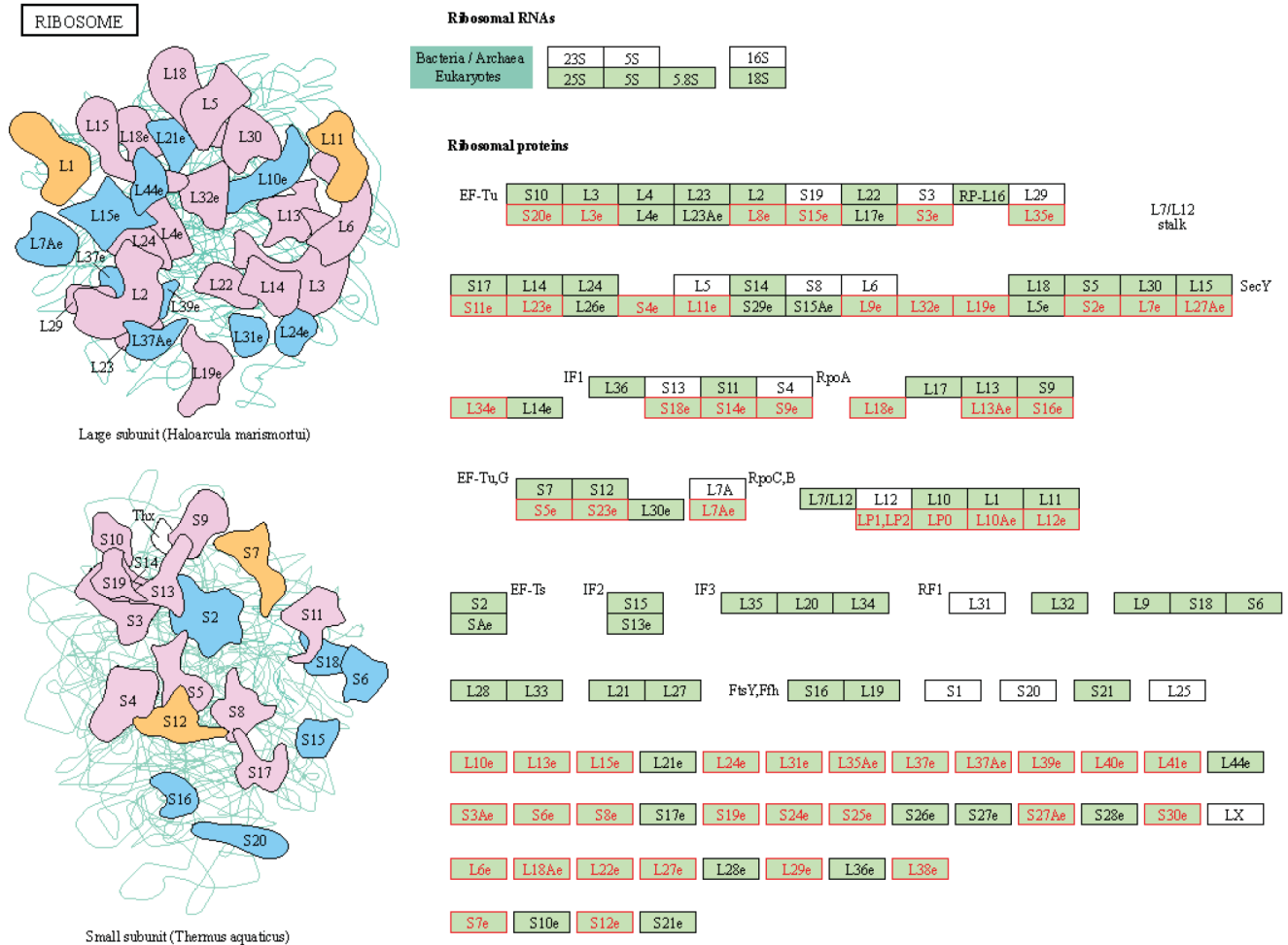
Fig. S3. Expression of the ribosomal pathway is impaired by excess of NAD+ during adipogenesis.
Illustration of the Ribosomal Pathway according to the KEGG. Ribosomal components are illustrated (left) and listed (right). Ribosomal proteins whose mRNA expression is consistently downregulated by NAD+ treatment during adipogenesis are highlighted in red color..
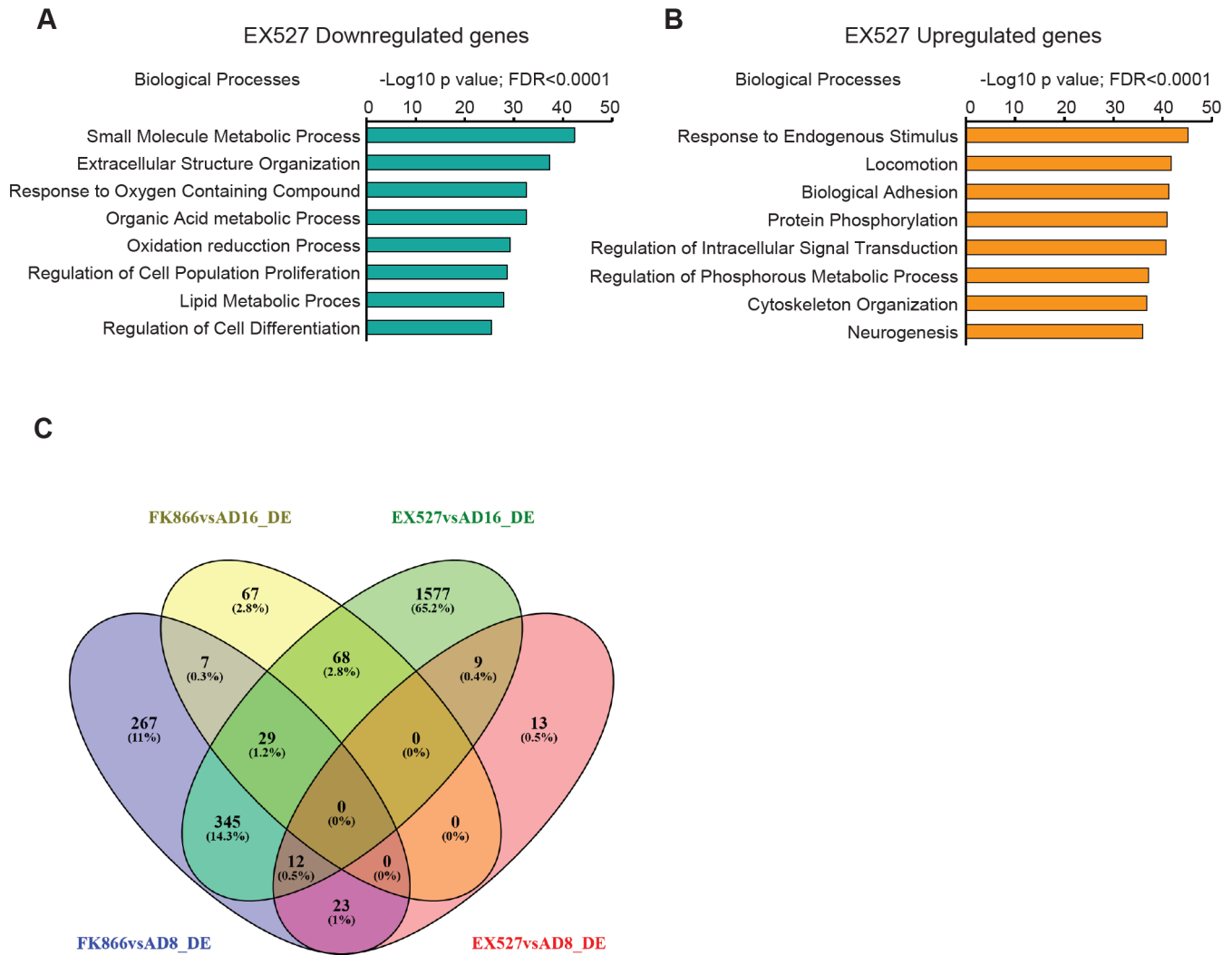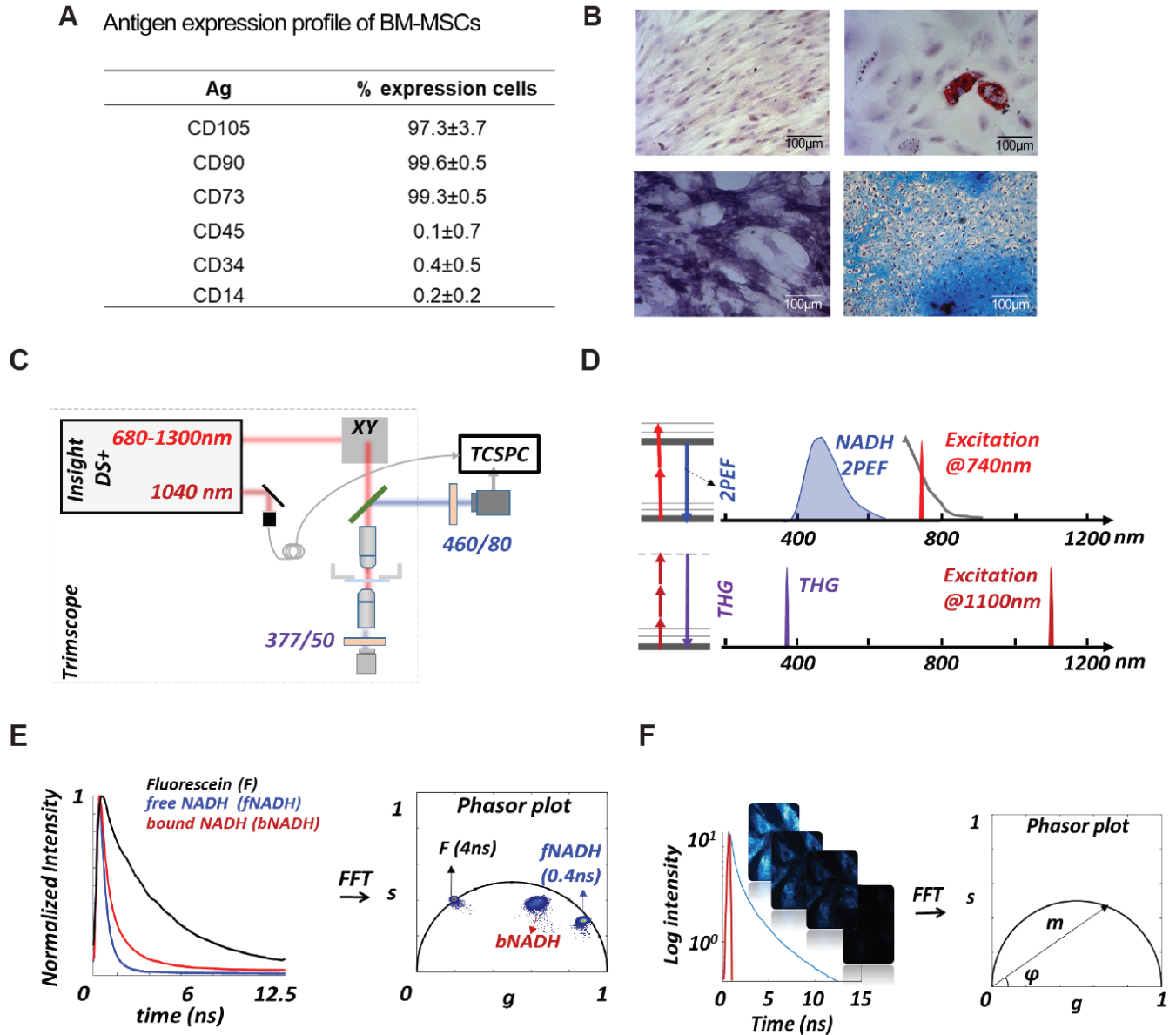
Fig. S4. Transcriptional rewiring during adipogenic differentiation triggered by SIRT1 or NAPT inhibition.
A, **B**) Biological processes enrichment analyses from genes downregulated (**A**) or upregulated (**B**) by EX527 treatment during adipogenic differentiation, at day 16 after induction, compared with untreated, terminally differentiated adipocytes. C) Venn diagram shows overlapping DE genes between indicated comparisons: FK866vsAD8_DE and FK866vsAD16_DE: mRNA was analyzed from cells during adipogenic differentiation (day 8 or day 16) from untreated (AD) or treated with 1nm FK866 during differentiation. EX527vsAD8_DE and EX527vsAD16_DE: mRNA was analyzed from cells during adipogenic differentiation (day 8 or day 16) from untreated (AD) or treated with 50μM EX527 during differentiation.
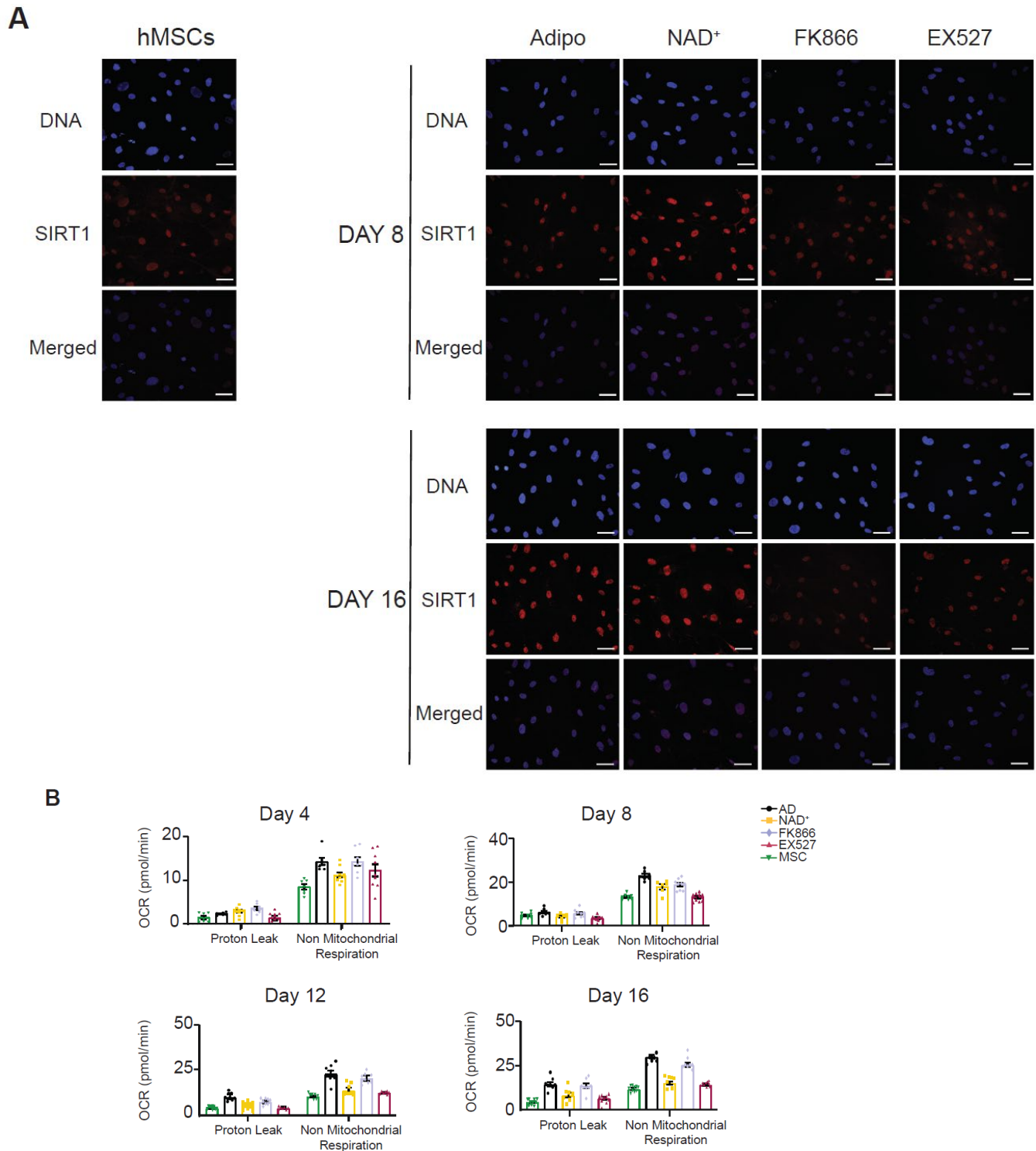
Fig. S5. SIRT1 levels and mitochondrial bioenergetics during adipogenic differentiation. A) SIRT1 protein levels and subcellular localization were analyzed by immunofluorescence at days 8 and 16 after adipogenic induction on hMSC. Cells were either untreated (Adipo), or treated with the indicated compounds. n= 2 biological and 7 technical replicates. **B)** Mitochondrial bioenergetic parameters calculated from extracellular flux analyses: Proton leak and non-mitochondrial respiration. AD: adipogenic induced cells; NAD$^+$ adipogenic induced cells treated with 5 mM NAD+; FK866: adipogenic induced cells treated with 1 nM FK866; EX527: adipogenic induced cells treated with 50 μM EX527. MSC: untreated, undifferentiated hMSC.
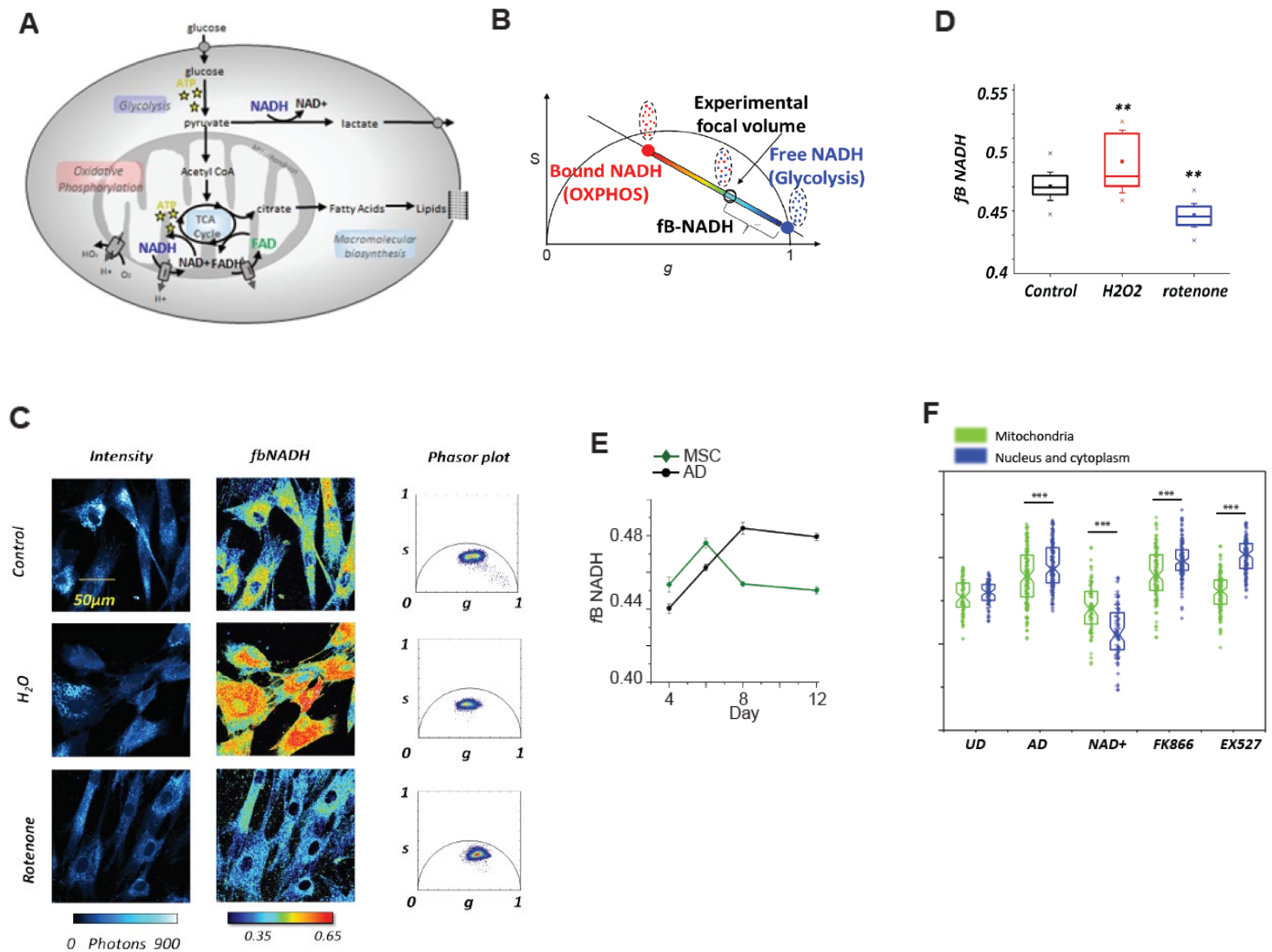
Fig. S6. Metabolic trajectories assessed by 2P-FLIM on NADH. A) Schematic representation of cellular metabolism. Glucose breakdown through glycolysis and the TCA cycle generates reduced NADH and FADH2. Quiescent cells have a basal rate of glycolysis, converting glucose to pyruvate, which is then oxidized in the TCA cycle. As a result, the majority of ATP is generated by oxidative phosphorylation (OXPHOS). Non proliferating, differentiated cells are characterized by a low NADH/NAD$^+$ ratio. During proliferation, the large increase in glycolytic flux rapidly generates ATP in the cytoplasm. Most of the resulting pyruvate is converted into lactate by lactate dehydrogenase A, which regenerates NAD$^+$ from NADH. Proliferating cells are characterized by a high NADH/NAD$^+$ ratio. Rotenone blocks the respiratory chain via complex I while H$_2$O$_2$ increase the NAD$^+$:NADH ratio. **B)** Metabolic trajectory between free NADH and bound NADH indicates a shift from a glycolytic to a OXPHOS cellular phenotype as free/bound NADH ratio corresponds to NAD$^+$:NADH ratio. The fraction of bound NADH (fB_NADH) of the experimental point is graphically calculated from the location of free NADH. **C)** Representative images of intensity, fB_NADH and phasor plot of hMSCs with different treatments: control, rotenone (respiratory chain inhibitor) and H2O2 (induces oxidative stress). Accumulation of reduced NADH by blocking the respiration chain shifts the cellular metabolic signature toward free NADH, while oxidative stress shifts the cellular metabolic signature towards bound NADH. **D)** Quantification of fraction of bound NADH in a ROI with different metabolic treatments. One-way ANOVA followed by Tukey´s post test. * $p < 0.05$, ** $p < 0.01$. **E)** Quantification of fraction of bound NADH during adipogenic differentiation at with (black) or without adipogenic culture medium (dark green). Data is presented as mean ±SEM F) Quantification of fB_NADH in mitochondria (green) and in nucleus/cytoplasm (blue) in single cells at day 8 of adipogenic differentiation in the absence (AD) or in the presence of the indicated treatments. hMSC (UD) were also assessed. n= 63-125 cells; ***$p < 0,001$, Student's t-test.

A Antigen expression profile of BM-MSCs

Ag	% expression cells
CD105	97.3±3.7
CD90	99.6±0.5
CD73	99.3±0.5
CD45	0.1±0.7
CD34	0.4±0.5
CD14	0.2±0.2

B

C

D

E

F

Fig. S6. hMSC characterization and experimental setup. A) Expression cell markers in MSCs was determinated by Flow Cytometry, data correspond to mean percentage of cells positive to each marker ± SD, n=3 biological replicates. **B)** hMSCs stained with toluidine blue (top left), adipogenic differentiation was determined by the presence of lipid vacuoles positive to ORO (top right), osteogenic differentiation was determined by Alkaline Phosphatase assay (bottom left) and chondrogenic differentiation was assessed by matrix positive to Alcian blue in cryosections of micromasses (bottom right). n=3 biological replicates. **C)** Scheme of the experimental setup used for this work. **D)** Principles of 2 photon excitation fluorescence and THG signal generation: Two-photon excitation of NADH occurs at 740 nm with emission collected with band-pass filters centered at 460nm, which resulted primarily from NADH. The excitation of THG occurred at 1100nm, with emission collected with a band-pass filter centered at 377nm. **E)** Example of fluorescence intensity decay of fluorescein and free and bound NADH in solution and their locations in the phasor plot. **F)** The multi-exponential fluorescence intensity decay in every pixel of the image is transformed with a Fourier transform; the real (g) and imaginary (s) parts are plotted in the graphical phasor plot.

Dataset S1 (separate file). Summary of mapping results. Sample name: sample identification; Total reads: total clean reads suitable for analysis; Total mapped: numbers of reads being mapped on the genome; Uniquely mapped reads: numbers of reads being mapped to a single position of the genome; Multiple mapped reads: numbers of reads being mapped to more that one genomic sites; Total mapping rate: (mapped reads)/(total reads)*100; Uniquely mapping rate: (uniquely mapped reads)/(total reads)*100; Multiple mapping rate: (multiple mapped reads)/(total reads)*100.

Dataset S2 (separate file). List of differentially expressed genes from NAD+-treated cells genes and their functional analyses.

Dataset S3 (separate file): List of DE genes in from EX527-treated cells at terminal differentiation (day 16) and their functional analyses.

Dataset S4 (separate file): List of DE genes in from FK866-treated cells at terminal differentiation (day 16) and their functional analyses