A serum-free and insulin-supplemented cell culture medium ensures fatty acid synthesis gene activation in cancer cells

Su Wu[1,2*] and Anders M. Näär[1,2, #*]

[1] Massachusetts General Hospital Center for Cancer Research, Charlestown, Massachusetts, United States of America

[2] Department of Cell Biology, Harvard Medical School, Boston, Massachusetts, United States of America

[#]Current Address: Department of Nutritional Sciences & Toxicology, University of California, Berkeley, California, United States of America

[*] Corresponding authors

E-mail: swu16@mgh.harvard.edu (SW) and naar@berkeley.edu (AMN)

Abstract

While investigating the role played by *de novo* fatty acid biosynthesis (DNFA) in cancer cells, we sought a medium condition that would support cell proliferation without providing any serum lipids. Here we report that a defined serum free cell culture medium condition containing insulin, transferrin and selenium (ITS) supports controlled study of DNFA regulation in melanoma cell lines. This lipid-free ITS medium is able to support proliferation of melanoma cell lines that fulfill their lipid requirements via DNFA. We show that the ITS medium stimulates gene transcription in support of both DNFA and *de novo* cholesterol synthesis (DNCS), specifically mediated by SREBP1/2 in melanoma cells. We further found that the ITS medium promoted SREBP1 nuclear localization and occupancy on DNFA gene promoters. Our data show clear utility of this serum and lipid-free medium for melanoma cancer cell culture and lipid-related areas of investigation.

Introduction

de novo fatty acid biosynthesis (DNFA) is the metabolic pathway that converts carbohydrates into fatty acids. In healthy adults, DNFA occurs in liver and adipose tissues for energy storage or distribution to other tissues. Many malignant cancer cells also exhibit elevated DNFA as a hallmark adaptation to support proliferation and survival (1, 2). Certain cancer cells can proliferate entirely by relying on lipids generated from DNFA; others rely additionally upon lipid uptake (3). However, DNFA appears to be required for cell survival even with the presence of external lipids, suggesting that DNFA is essential for cancer cells regardless of proliferative state (3). Thus, DNFA is of particular interest as a potential therapeutic target for cancers (4, 5). DNFA is primarily regulated at the mRNA level of catalytic enzymes that drive the biosynthetic reactions (6), a transcriptional process under the control of the sterol regulatory element-binding protein 1 (SREBP1) (7). However, DNFA gene regulation is still not fully understood at the molecular level. Cell culture studies of DNFA with widely-accepted serum-containing medium conditions are often confounded by the presence of external lipids, with consequent difficulty to disentangle the respective effects of lipid synthesis and lipid update, both of which may occur even among cells that are able to survive and proliferate using DNFA alone (7, 8).

Most cell culture media are composed of serum supplements and basal mediums (BMs), each of which may contain confounding factors for DNFA studies. Typical serum often contains external lipids in two forms: non-esterified free fatty acids (FFAs) associated with albumin, and lipoproteins that carry triglycerides, cholesterol and phospholipids encapsulated by apoproteins (9, 10). Cells can take up FFAs *via* physical diffusion across the cellular membrane (11) or *via* active transport aided by membrane-associated protein CD36 or FATPs (12). Lipoprotein uptake occurs when a lipoprotein binds to a cell surface receptor (e.g. LDL receptor; LDLR) and the bound

56 lipoprotein/receptor complex undergoes endocytosis (13). After transport from serum
57 to cells, both FFAs and lipoprotein lipids are potent DNFA inhibitors (14). Intracellular
58 cholesterol interferes with retrograde trafficking of SREBP1 from the ER to the Golgi
59 apparatus – an essential step in post-translational processing and maturation of SREBP1
60 – and consequently inhibits DNFA enzyme expression (7). Polyunsaturated FFAs have
61 also been observed to inhibit both SREBP1 mRNA transcription and protein maturation,
62 resulting in decreased expression of DNFA enzyme genes (15-17). Lipid metabolism
63 studies have often employed lipoprotein-deficient serum (LPDS), in which lipoproteins
64 are removed by ultracentrifugation, but FFAs – which are confounding for DNFA
65 studies – are retained (18). An alternative is delipidated serum (8), but that is prepared
66 by organic solvent extraction and is not completely lipid-free (19). Preparation
67 protocols vary widely in organic solvent composition and extraction time, and quality
68 variation between batches is common (20, 21).

69 Basal media (BM) often contain glucose, which besides its bioenergetic role as
70 fuel for ATP synthesis also serves as a carbon source for biosynthesis of amino acids,
71 nucleotides, other carbohydrates, and lipids. Two of the most common BMs for cancer
72 cell culture are Roswell Park Memorial Institute (RPMI) 1640 medium (22), with 11.11
73 mM glucose concentration (normal glucose level); and Dulbecco's modified Eagle's
74 medium (DMEM), which contains 25 mM glucose (high glucose level). In cultured
75 hepatocytes and adipocytes, high glucose conditions stimulate lipogenesis and DNFA
76 gene expression (23, 24). Healthy livers employ glucose for DNFA principally by
77 glycolytic citrate generation and the tricarboxylic acid (TCA) cycle with oxidative
78 phosphorylation (25, 26). Glycolysis produces pyruvate that mitochondria metabolize
79 into citrate and ATP in the TCA cycle. The citrate then translocates to cytosol where
80 it is cleaved by ATP-citrate lyase (ACLY) to produce acetyl-CoA as a substrate for
81 DNFA (27-29). In contrast to healthy livers, tumors display enhanced glycolytic

82 activity but impaired oxidative phosphorylation. Pyruvate is often metabolized to
83 lactate within the cytoplasm of cancer cells preferentially to the TCA cycle (Warburg
84 effect) (30). Furthermore, elevated glucose stimulates glycolysis and inhibits aerobic
85 respiration in tumors (Crabtree effect) (31). Thus, high-glucose BMs promote
86 conversion of glucose to pyruvate and NADH generation but inhibit production of
87 substrates for DNFA via the TCA cycle (32, 33). In general, the glucose content of
88 DMEM may distort normal cellular behavior and thus render it unsuitable for molecular
89 study of DNFA transcription regulation. Both aerobic respiration and anaerobic
90 glycolysis contribute to glucose catabolism in cancer cells cultured with RPMI-1640
91 medium (34). Therefore, between the two common BMs, RPMI-1640 seems preferable
92 for lipogenesis-related investigations of cancer cells.

93 The commercially available insulin, transferrin, and selenium (ITS) supplement
94 is a serum replacement, supporting cell survival and growth but containing no lipids.
95 ITS supplement has been used for *in vitro* culture of mesenchymal stem cells isolated
96 from adipose or cartilage tissues, to maintain their differentiation and proliferation
97 capacities for tissue transplantation (35, 36). Insulin is a growth factor with a mitogenic
98 effect in cell culture that promotes the uptake of glucose and amino acids (37, 38). In
99 livers, insulin stimulates *SREBF1c* mRNA expression (23) as well as its proteolytic
100 processing to stimulate DNFA gene expression (23, 39). Transferrin is a glycoprotein
101 that transports Fe^{3+} in blood plasma and delivers Fe^{3+} to cells through binding to
102 transferrin receptor on cell surface (41). Fe^{3+} is an essential component of heme-
103 containing enzymes like cytochromes for oxidative phosphorylation process and
104 various non-heme iron enzymes, such as ribonucleotide reductase for DNA synthesis
105 (42). Transferrin provides Fe^{3+} necessary to support cell survival and proliferation in
106 culture (43). Selenium is required for proper function of antioxidant enzymes, including
107 glutathione peroxidase and thioredoxin reductase, in which selenocysteine is

108 indispensable for their catalytic activities (44). Components of ITS have been
109 individually assembled in various serum-free media to support cell growth in cancer
110 studies (45), but reports relevant to lipid metabolism are missing. Here, we introduce
111 the combination of RPMI-1640 and ITS (ITS medium) as a straightforward serum-free
112 medium condition for activating DNFA gene expression in cancer cell culture. The ITS
113 medium stimulates cell growth, exhibits consistent effect across batches, and is free of
114 confounding lipid factors. We expect that it may be adopted for investigations in lipid
115 metabolism and will facilitate *in vitro* screening for inhibitors of DNFA pathway.
116

117 Materials and Methods

118 Cell culture reagent

119 The melanoma cell line HT-144 was obtained from the MGH Center for
120 Molecular Therapeutics. Cell line was cultured in RPMI-1640 medium (21870092,
121 Thermo Fisher Scientific) with 10% fetal bovine serum (Gibco), 2 mM L-glutamine
122 (Gibco) and 50 U/ml penicillin-streptomycin (Gibco) in a 37 °C incubator with 5%
123 CO_2. 0% FBS medium contained RPMI-1640 medium, with 2 mM L-glutamine
124 (Gibco) and 50 U/ml penicillin-streptomycin (Gibco). 1% ITS medium contained the
125 RPMI-1640 medium with 1× Insulin-Transferrin-Selenium (ITS-G, Thermo Fisher
126 Scientific), 2 mM L-glutamine (Gibco) and 50 U/ml penicillin-streptomycin (Gibco).
127

128 Cell proliferation assay

129 For proliferation assay, HT-144 cells were seeded at a density of 5,000 cells per
130 well in 24-well plates (Corning) in RPMI-1640 medium with10% FBS. Sixteen hours
131 after seeding, cells were washed twice with PBS buffer and then cultured in three

132 different medium conditions for 6 days. Relative cell viability was quantified with the
133 alamarBlue cell viability reagent (DAL1025, Thermo Fisher Scientific) and
134 fluorescence signals were measured with the Envision 2103 multilabel microplate
135 reader (Perkin Elmer). Each data point was measured with four replicates. Because the
136 culture medium influences resazurin fluorescence, the background fluorescence of
137 medium-only with alamarBlue reagent was subtracted for data normalization.
138

139 Reverse transcription quantitative PCR (RT-qPCR)

140 Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen)
141 and treated with RNase-free DNase (Qiagen). RNA concentrations were quantified
142 with Qubit™ RNA BR assay kit (Thermo Fisher Scientific). 1 μg RNA was used for
143 cDNA synthesis with RNA to cDNA EcoDry™ premix (TaKaRa) containing both
144 random hexamer and oligo(dT)18 primers (Double Primed). qPCR was carried out in
145 triplicates on a LightCycler® 480 instrument (Roche) using LightCycler® 480 SYBR
146 green I master (Roche). qPCR primers were pre-designed by MGH primer bank
147 (https://pga.mgh.harvard.edu/primerbank/) and the primer sequences are listed in Table
148 1. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, normalized
149 to the 18S housekeeping gene, and the mean of negative control samples was set to 1.
150

151 **Table 1. The primers used for RT-qPCR.**

ACACA forward	5' – ATGTCTGGCTTGCACCTAGTA – 3'
ACACA reverse	5' – CCCCAAAGCGAGTAACAAATTCT – 3'
ACLY forward	5' – TCGGCCAAGGCAATTTCAGAG – 3'
ACLY reverse	5' – CGAGCATACTTGAACCGATTCT – 3'
ACSS2 forward	5' – AAAGGAGCAACTACCAACATCTG – 3'

ACSS2 reverse	5' – GCTGAACTGACACACTTGGAC – 3'
FASN forward	5' – AAGGACCTGTCTAGGTTTGATGC – 3'
FASN reverse	5' – TGGCTTCATAGGTGACTTCCA – 3'
SCD forward	5' – TCTAGCTCCTATACCACCACCA – 3'
SCD reverse	5' – TCGTCTCCAACTTATCTCCTCC – 3'
ACSL1 forward	5' – CCATGAGCTGTTCCGGTATTT – 3'
ACSL1 reverse	5' – CCGAAGCCCATAAGCGTGTT – 3'
SREBF1 forward	5' – ACAGTGACTTCCCTGGCCTAT – 3'
SREBF1 reverse	5' – GCATGGACGGGTACATCTTCAA – 3'
SREBF2 forward	5' – AACGGTCATTCACCCAGGTC – 3'
SREBF2 reverse	5' – GGCTGAAGAATAGGAGTTGCC – 3'
HMGCS1 forward	5' – GATGTGGGAATTGTTGCCCTT – 3'
HMGCS1 reverse	5' – ATTGTCTCTGTTCCAACTTCCAG – 3'
HMGCR forward	5' – TGATTGACCTTTCCAGAGCAAG – 3'
HMGCR reverse	5' – CTAAAATTGCCATTCCACGAGC – 3'
LDLR forward	5' – ACCAACGAATGCTTGGACAAC – 3'
LDLR reverse	5' – ACAGGCACTCGTAGCCGAT – 3'
18S forward	5' – GTAACCCGTTGAACCCCATT – 3'
18S reverse	5' – CCATCCAATCGGTAGTAGCG – 3'

152

153 **siRNA transfection**

154 The human-specific siRNAs targeting *SREBF1* (6720) and *SREBF2* (6721)
155 were the pre-designed ON-TARGETplus SMARTpool siRNA reagents from
156 Dharmacon. Each ON-TARGETplus SMARTpool siRNA was a mixture of four siRNA
157 duplexes. siRNAs were suspended in RNase-free 1 × siRNA Buffer (Dharmacon) to

158 yield 20 μM stock solutions. HT-144 cells were transfected with siRNAs at final
159 concentration of 50 nM using Lipofectamine RNAiMAX transfection reagent (Thermo
160 Fisher Scientific) at a density of 2 × 10^5 cells/ well in a 6-well plate. For each
161 transfection, 2.5 μl of siRNA stock solution was mixed with 4 μl of Lipofectamine
162 RNAiMAX in 200 μl of Opti-MEM I medium and then incubated for 10-20 minutes at
163 room temperature. HT-144 cells were diluted in 10% FBS/RPMI-1640 medium without
164 antibiotics so that 800 μl medium contains 2.5 × 10^5 HT-144 cells. 200 μl of
165 siRNA/Lipofectamine RNAiMAX complexes was mixed with 800 μl of the diluted
166 HT-144 cells in one well of a 6-well plate. Transfected HT-144 cells were incubated at
167 37°C for 16 hours before medium changes.
168

169 **Immunoblot assay**

170 HT-144 cells were seeded in 10 cm^2 plates (Corning) in 10% FBS/RPMI-1640
171 medium at day one. Sixteen hours after seeding, cells were washed twice with PBS
172 buffer and then cultured in different medium conditions. Total cell lysate was harvested
173 with RIPA buffer containing protease inhibitors (protease inhibitor cocktail tablets,
174 Roche) on the third day. Nuclear and cytoplasmic protein fractions were prepared with
175 NE-PER™ nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific).
176 Protein samples were separated on the 4-15% Mini-PROTEAN® TGX™ precast SDS-
177 PAGE gels (Bio-Rad) and then transferred to polyvinyl difluoride (PVDF) membranes
178 (Immobilon-P, Millipore) for immunoblot analysis. The following primary antibodies
179 were used: mouse anti-SREBP1 (IgG-2A4, BD Biosciences), rabbit anti-FASN
180 (C20G5, Cell Signaling), rabbit anti-SCD (23393-1-AP, Proteintech), rabbit anti-
181 ACSL1 (D2H5, Cell Signaling), rabbit anti-histone H3 (9715, Cell Signaling) and
182 rabbit anti-beta-actin (13E5, Cell Signaling). After being incubated with primary
183 antibodies overnight in PBST solution with 5% non-fat dry milk, immunoblot

184 membranes were probed with HRP-conjugated affinity-purified donkey anti-mouse or
185 anti-rabbit IgG (GE Healthcare) as secondary antibodies and visualized with the
186 immobilon Western Chemiluminescent HRP substrate (Millipore).
187

188 Chromatin immunoprecipitation (ChIP) quantitative PCR
189 (ChIP-qPCR)

190 For each ChIP assay, 5×10^7 HT-144 cells were used. HT-144 cells were seeded
191 in 10% FBS medium, and then medium was replaced with 10% FBS or 1% ITS medium
192 and cultured for 24 hours before ChIP-qPCR analyses. Chromatin from HT-144 cells
193 was fixed with 1% formaldehyde (Polysciences) and prepared with Magna ChIP™
194 HiSens chromatin immunoprecipitation kit (EMD Millipore). Nuclei were sonicated on
195 a sonic dismembrator 550 (Fisher Scientific) with a microtip (model 419) from Misonix
196 Inc. Lysates were sonicated on ice with 10 pulses of 20 seconds each (magnitude setting
197 of 3.5) and a 40-sec rest interval. The supernatant was used for immunoprecipitation
198 with the following antibodies: rabbit anti-SREBP1 (H-160, Santa Cruz Biotechnology),
199 rabbit anti-CBP (C-22, Santa Cruz Biotechnology), rabbit anti-RNA polymerase II
200 (8WG16, BioLegend) and rabbit anti-histone H3 (acetyl K27) (ab4729, Abcam). qPCR
201 reactions in triplicates were performed on a LightCycler® 480 instrument (Roche)
202 using LightCycler® 480 SYBR green I master (Roche). The ChIP-qPCR primers were
203 designed with software Primer 3. The primer sequences are listed in Table 2.
204

205 **Table 2. The primers used for ChIP-qPCR.**

SCD 12F (TSS)	5' – GTGGCACCAAATTCCCTTCG – 3'
SCD 12R (TSS)	5' – GACACCGACACCACACCA – 3'
SCD 245F	5' – CTTGGCAGCGGATAAAAGGG – 3'
SCD 245R	5' – GCACGCTAGCTGGTTGTC – 3'

206

207 # Results

208 ## A lipid-free and insulin-supplemented (ITS) medium
209 ## supports melanoma cell proliferation

210 We performed time-course analysis to measure cell growth rates of a melanoma
211 cell line, HT-144, under three different cell culture medium conditions: RPMI-1640
212 supplemented with either 10% FBS, 0% FBS, or 1% ITS. Viable HT-144 cells were
213 quantified with alamarBlue daily for six days. We found that HT-144 cells cultured in
214 10% FBS and 1% ITS medium conditions displayed a time-dependent increase of
215 fluorescence reads in the alamarBlue assay (Fig. 1A). We also observed increased cell
216 number, which confirms that HT-144 cells proliferate in both 10% FBS and 1% ITS
217 medium conditions. Because 1% ITS medium does not contain any external lipids, the
218 lipids for membrane synthesis during proliferation are products entirely derived from
219 DNFA. We observed a growth plateau of HT-144 cells in 0% FBS medium, which lacks
220 growth factors such as insulin, and in which HT-144 cells therefore remain quiescent.

221 The alamarBlue assay measures the fluorescence emission of resorufin
222 molecules converted from resazurin by reductants such as NADPH or NADH (46, 47).
223 The alamarBlue assay is thus an indicator of cellular NADPH/NADH concentration in
224 living cells. Because cytosolic NADPH serves as the reductant for biosynthesis
225 pathways such as lipid and nucleic acid synthesis, alamarBlue assay may provide a hint

226 of the DNFA activity. Therefore, we compared the alamarBlue reads of cells cultured
227 in different medium conditions at day one, when they had the same cell numbers in the
228 three medium conditions. HT-144 cells cultured in 1% ITS medium had the highest
229 fluorescence reads, whereas those in 10% FBS medium had the lowest reads (Fig. 1B).
230 This result suggests that even when the cell number remains the same, HT-144 cells
231 cultured in 1% ITS possibly have higher NADPH/NADH concentrations.
232

233 **Fig 1. A lipid-free and insulin-supplemented medium supports**
234 **proliferative cell state in melanoma cells.**

235 (A) HT-144 cells were seeded in 10% FBS medium at day zero. On day one, cells were
236 washed with PBS and changed to the indicated medium conditions. Cell proliferation
237 was measured with alamarBlue assay at each day. Each data point represents the mean
238 ±SD of quadruplicate samples. The results were analyzed using two-way repeated
239 measures ANOVA followed by *post hoc* Tukey's multiple comparison tests. For culture
240 time, $F = 1505$, $P < 0.0001$; for culture condition, $F = 2153$, $P < 0.0001$; for interaction
241 between culture time and condition, $F = 409.4$, $P < 0.0001$. (B) HT-144 cells were
242 seeded in 10% FBS medium at day zero. On day one, cells were washed with PBS and
243 changed to the indicated medium conditions. alamarBlue assay was performed on the
244 cells cultured in the indicated medium for one hour. Results were analyzed using one-
245 way ANOVA followed by *post hoc* Tukey's multiple comparison tests. $F = 202.7$.
246 Significant differences between medium conditions are indicated as *$P < 0.05$, **$P <$
247 0.01, ***$P < 0.001$ and ****$P < 0.0001$. ns, not significant.
248

249 **ITS medium increases DNFA and DNCS gene expression in**

250 **melanoma cells**

251 To determine whether the increase of fluorescence emission by resorufin in 1%
252 ITS condition (Fig. 1B) was due to elevated DNFA activity, we performed RT-qPCR
253 analyses of DNFA mRNA, including *ACLY*, *ACSS2*, *ACACA*, *FASN*, *SCD*, and *ACSL1*.
254 We also examined the expression of *de novo* cholesterol synthesis (DNCS) genes,
255 including *HMGCS1* and *HMGCR*. HT-144 cells were seeded in 10% FBS medium, and
256 then medium was replaced with 0% FBS or 1% ITS medium and cultured for 24 hours
257 before RT-qPCR analyses. We observed a significant increase in the expression of
258 DNFA (Fig. 2A-F), DNCS (Fig. 2G, H) and *LDLR* (Fig. 2I) genes when comparing
259 cells cultured in 0% FBS and 1% ITS medium conditions to cells in 10% FBS medium.
260 DNFA and DNCS gene expression remained higher in 0% FBS and 1% ITS three days
261 after changing the media (data not shown). These results suggest that the increase in
262 lipogenic gene expression persists in cells cultured in 0% FBS and 1% ITS media. We
263 observed lower DNFA and DNCS gene expression in cells cultured in 10% FBS
264 medium than in 0% FBS medium, consistent with repression of DNFA and DNCS by
265 lipids derived from the serum supplement.

266 Figure 1A indicates that HT-144 cells remain quiescent in 0% FBS medium
267 and proliferate in 1% ITS. Quiescent HT-144 cells have enhanced DNFA and DNCS
268 gene expression, even though there is no requirement for membrane lipid synthesis to
269 support proliferation, and the reasons for this remain unclear. DNFA and DNCS gene
270 expression is elevated in 1% ITS medium as compared with 0% FBS medium, which
271 may suggest that the insulin component of the ITS supplement contributes to
272 stimulation of DNFA gene expression.

273 We further observed significantly increased expression of *SREBF1* and
274 *SREBF2* genes in cells cultured in 0% FBS and 1% ITS medium conditions, compared

275 to 10% FBS medium (Fig. 2J, K). These results suggest that SREBP1 and SREBP2 are
276 transcriptionally activated in lipid-free and insulin-supplemented conditions. Because
277 SREBP1/2 can bind to the sterol regulatory element (SRE) at its own promoter,
278 SREBP1/2 protein possibly regulate its mRNA production through auto-activation (48)
279 and potentially contributes to elevated DNFA and DNCS gene expression.
280

281 **Figure 2. Lipid depletion combined with insulin supplement yields**
282 **increased expression of lipogenic genes.**

283 (A-K) The expression level of DNFA and DNCS genes was analyzed by RT-qPCR
284 assay. HT-144 cells were cultured in 10%, 0% FBS or 1% ITS medium for 24 hours.
285 Significant differences between medium conditions are indicated as *P < 0.05, **P <
286 0.01, ***P < 0.001 and ****P < 0.0001 using one-way ANOVA followed by *post hoc*
287 Tukey's multiple comparison tests. ns, not significant. Each data point represents the
288 mean ±SD of results from quadruplicate samples.
289

290 ITS medium increases DNFA and DNCS gene expression via
291 SREBP1 and SREBP2, respectively

292 SREBP1 and SREBP2 are the master transcription regulators of DNFA and
293 DNCS pathways, respectively (49). To verify cellular dependence on SREBP1 and
294 SREBP2 for lipid biosynthesis pathways, we transfected HT-144 cells with pooled
295 siRNAs to deplete mRNAs encoding *SREBF1* and *SREBF2*. The transfected cells were
296 cultured in 10% FBS, 0% FBS or 1% ITS medium conditions for two days and then
297 assayed with RT-qPCR for DNFA (Fig. 3) and DNCS (Fig. 4) gene expression.
298 In the group treated with scrambled siRNA (blue bars in Figs. 3 and 4), we
299 found that DNFA and DNCS gene expression was significantly elevated in 0% FBS

300 and 1% ITS medium conditions compared to 10% FBS, consistent with our
301 observations in Fig. 2. However, the DNFA gene expression is significantly less in the
302 *SREBF1* depleted group than in the scramble siRNA group, particularly in 0% FBS and
303 1% ITS medium conditions (Fig. 3A-F, statistical comparisons marked in black). The
304 DNCS gene expression is significantly less in the *SREBF2* depleted group than in the
305 scramble siRNA group, in 0% FBS and 1% ITS medium conditions (Fig. 4A, B,
306 statistical comparisons marked in black). Two-way ANOVA analysis detects a
307 significant interaction between *SREBF1/2* depletion and medium condition for DNFA
308 and DNCS gene expression. This result provides a statistical indication that SREBP1
309 and SREBP2 participated in activation of lipogenic gene expression in 0% FBS and 1%
310 ITS conditions. We further found that *SREBF1* siRNA has the greatest effect on DNFA
311 gene expression (Fig. 3A-F), and *SREBF2* siRNA has the greatest effect on DNCS gene
312 expression (Fig. 4A, B). LDLR appears to be regulated primarily through SREBP1
313 rather than SREBP2 (Fig. 4C). Our results are consistent with known roles for SREBP1
314 and SREBP2 in activation of DNFA and DNCS gene expression (49).

315 In scrambled siRNA treated groups, we observed significantly higher DNFA
316 and DNCS gene expression in 1% ITS than in 0% FBS medium, suggesting additional
317 influence of the ITS supplement on DNFA gene expression (Figs. 3 and 4, statistical
318 comparisons marked in blue). In *SREBF1*-depleted group, we observed no significant
319 increase of DNFA gene expression when comparing 1% ITS to 0% FBS condition (Fig.
320 3A-F, statistical comparisons marked in red). Similarly, in *SREBF2*-depleted group
321 there is no significant elevated expression of DNCS genes, *HMGCS1* and *HMGCR*, in
322 1% ITS medium compared to 0 % FBS (Fig. 4A, B, statistical comparisons marked in
323 green). Altogether, these results support the utility of the 1% ITS medium condition to
324 study SREBP1- and SREBP2-targeted interventions to inhibit DNFA and DNCS
325 respectively, in the absence of confounding serum lipids.

326

327 **Fig 3. DNFA gene expression of HT-144 cells increases in ITS medium,**

328 **and this increase is dependent upon SREBPs.**

329 (A-F) HT-144 cells were transfected over one day with siRNAs at 50 nM concentrations
330 for non-target control, *SREBF1* or *SREBF2*. Transfected cells were cultured in 10%,
331 0% FBS or 1% ITS for two more days before RT-qPCR analyses. RT-qPCR results are
332 presented as expression of DNFA genes relative to their expression under scramble
333 siRNA treatment (siNegative) in 10% FBS medium (set as 1 and marked with a dashed
334 line). When a significant interaction between siRNA treatment and medium condition
335 was detected by two-way ANOVA, individual gene expression was compared within
336 groups by *post hoc* Tukey's multiple comparison tests. Each data point represents the
337 mean ±SD of triplicate samples. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P <$
338 0.0001.

339

340 **Fig 4. DNCS gene expression of HT-144 cells increases in ITS medium,**

341 **and this increase is dependent upon SREBPs.**

342 (A-E) HT-144 cells were transfected over one day with siRNAs at 50 nM
343 concentrations for non-target control, SREBF1 or SREBF2. Transfected cells were
344 cultured in 10%, 0% FBS or 1% ITS for two more days before RT-qPCR analyses. RT-
345 qPCR results are presented as expression of genes relative to their expression under
346 scramble siRNA treatment (siNegative) in 10% FBS medium (set as 1 and marked with
347 a dashed line). When a significant interaction between siRNA treatment and medium
348 condition was detected by two-way ANOVA, individual gene expression was
349 compared within groups by *post hoc* Tukey's multiple comparison tests. Each data point

350 represents the mean ±SD of triplicate samples. *, P < 0.05; **, P < 0.01; ***, P < 0.001;
351 ****, P < 0.0001.
352

353 ITS medium increases the level of nuclear SREBP1

354 To confirm that lipid-free and/or insulin-supplemented medium conditions
355 influence SREBP1 protein production in general and its nuclear form in particular, we
356 examined the cytoplasmic and nuclear levels of SREBP1 in HT-144 cells cultured
357 under the three conditions (Fig. 5A). Our results revealed a dramatic increase of nuclear
358 SREBP1 in 0% FBS and 1% ITS medium conditions. However, we did not observe a
359 decrease of full-length SREBP1 in the cytoplasmic fraction from 0% FBS and 1% ITS
360 medium conditions. The overall SREBP1 protein level increased in 0% FBS and 1%
361 ITS medium conditions, which is likely due to transcriptional activation of the *SREBF1*
362 gene in Fig. 2J.
363 Consistent with the increased mRNA for DNFA genes (Fig. 2D-F), there is
364 increased production of DNFA enzymes including FASN, SCD and ACSL1 in the
365 cytoplasmic fraction from 0% FBS and 1% ITS medium conditions. The increase of
366 DNFA enzyme production correlates with the increase of nuclear SREBP1. We
367 interpret this as evidence that lipid depletion combined with insulin supplement greatly
368 enhances the abundance of the SREBP1 nuclear form and thus stimulates DNFA
369 enzyme production.
370

371 **Fig 5. Lipid depletion combined with insulin supplement yields**
372 **increased expression of nuclear SREBP1.**

373 (A) The cytoplasmic and nuclear fractions of HT-144 cells cultured in 10%, 0% FBS
374 or 1% ITS medium were isolated for Western blot analysis. HT-144 cells in 1% ITS
375 medium have increased levels of nuclear SREBP1 protein and lipogenic enzymes,

376 detected with the indicated antibodies. Histone H3 serves as the positive control for
377 nuclear fractionation during biochemical preparation of the cell samples.
378

379 Culture in ITS medium increases SREBP1 binding at the 380 *SCD* gene promoter

381 Finally, we investigated medium condition impact upon the molecular
382 mechanism by which nuclear SREBP1 controls DNFA gene expression. We used
383 chromatin immunoprecipitation (ChIP)-qPCR to examine the occupancy of SREBP1,
384 transcriptional co-activator CBP (50, 51), RBP1 (the largest subunit of RNA
385 polymerase II) and histone marker H3K27Ac (a marker for active enhancers and
386 promoters) (52) on the promoter of DNFA gene *SCD* (Fig. 6A-D). We observed a
387 significant increase of SREBP1 and CBP binding at the *SCD* transcription start site
388 (TSS) in 1% ITS relative to 10% FBS (Fig. 6A, C, statistical comparisons marked in
389 black). A corresponding slight increase of RBP1 at the *SCD* TSS site is not statistically
390 significant, however we observe a significant RBP1 increase downstream of TSS in the
391 gene body (Fig. 6B, statistical comparisons marked in black), consistent with active
392 RNA polymerase II elongation (3). 1% ITS does not significantly increase the
393 H3K27Ac signal at the *SCD* TSS nor downstream in the gene body compared to 10%
394 FBS (Fig. 6D, statistical comparisons marked in black). However, we observed
395 abundant H3K27Ac signals in the gene body for both 10% and 1% ITS (Fig. 6D,
396 statistical comparisons marked in blue and red). This result suggests that the *SCD* gene
397 body may be constitutively active, with open chromatin architecture.
398 Two-way ANOVA analysis detects significant interactions between gene locus
399 and medium condition in SREBP1 and RNA polymerase II ChIP-qPCR data. This
400 result indicates that 1% ITS significantly promotes enrichment of SREBP1 at TSS
401 region and RNA polymerase II at gene body region. We reason that, in 1% ITS medium,

402 the likely transcription regulation mechanism after SREBP1 binding is promotion of
403 the transition from RNA polymerase II pausing to active elongation (3).
404

405 **Fig 6. Lipid depletion combined with insulin supplement increased**
406 **SREBP1 binding at the *SCD* gene promoter.**

407 (A-D) ChIP-qPCR analyses for enrichment of transcriptional regulation factors on the
408 TSS and gene body region (245 bp downstream of TSS) of the *SCD* gene locus. qPCR
409 was used to quantify chromatin immunoprecipitated with the indicated antibodies from
410 HT-144 cells cultured in 10% FBS or 1% ITS medium. Quantification of enrichment
411 was determined as percentage of input chromatin before immunoprecipitation. Each
412 data point represents the mean ±SD of triplicate samples. Significant differences are
413 indicated as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 using two-way
414 ANOVA followed by *post hoc* Tukey's multiple comparison tests. ns, not significant.
415

416 # Discussion

417 We report here that a serum-free and insulin-supplemented culture medium
418 condition, 1% ITS supplemented RPMI-1640, supports DNFA and DNCS pathway
419 activation as well as proliferation and survival of the human melanoma cell line HT-
420 144. Under this condition, HT-144 cells proliferate while relying entirely on *de novo*
421 lipid synthesis to meet lipid requirements. Expression of DNFA and DNCS enzymes
422 increases significantly in cells when cultured in 1% ITS as compared with other cell
423 culture conditions. We found that 1% ITS medium activates DNFA and DNCS gene
424 expression through the transcription regulators SREBP1 and SREBP2, respectively. In
425 particular, culturing cells in 1% ITS medium promoted the transcription activation of
426 SREBP1 and accumulation of nuclear SREBP1 protein, as compared with other

427 medium conditions, and cells cultured in 1% ITS medium exhibited further increased
428 binding of SREBP1 at a DNFA gene promoter, consistent with high SREBP1-
429 dependent gene activation under this medium condition.

430 Unlike free fatty acids, free cholesterol and cholesteryl esters are only
431 minimally soluble in blood and must be transported within lipoproteins (52).
432 Lipoprotein-deficient serum (LPDS) is thus more efficient in removing cholesterol and
433 cholesteryl esters than fatty acids. LPDS has been used in lieu of full serum as an
434 activating medium for SREBPs, because it alleviates sterol inhibition (54). However,
435 LPDS contains free fatty acids that enter cells through passive membrane diffusion or
436 active transport through membrane receptors (55). LPDS has therefore also been used
437 for studies of cellular fatty acid uptake and lipid storage from extracellular fatty acids
438 (56). By contrast, ITS medium contains no external free fatty acids or lipoproteins.
439 Membrane lipids for cell proliferation in ITS medium rely entirely upon DNFA and
440 DNCS. Thus, ITS represents an attractive culture medium as compared with LPDS for
441 studying DNFA-supported proliferation and cell survival of cancer cells.

442 Our results support the idea that active DNFA is necessary for cell survival but
443 insufficient by itself for cell proliferation. Lipid depletion in 0% FBS activated DNFA
444 and DNCS gene expression, possibly due to removal of exogenous cholesterol, the
445 classic feedback inhibitor of SREBP processing and lipid synthesis (57). HT-144 cells
446 are able to persist and survive under these conditions, but become quiescent, likely due
447 to removal of growth factors in the serum necessary to support active proliferation. This
448 notion is supported by our finding that insulin present in the serum free ITS medium
449 has both metabolic and mitogenic functions in cancer cells, promoting cell survival and
450 active proliferation. It is presently unclear how DNFA is regulated to support cell
451 survival independent of insulin but may involve the AKT/GSK3 signaling pathway (58,
452 59).

453 Our data indicate that insulin promotes both SREBP1 processing and
454 transcription. Nuclear SREBP1 was elevated in cells cultured in 1% ITS and 0% FBS
455 media, compared to 10% FBS medium. However, cytoplasmic SREBP1 precursor
456 levels remained similar in both 1% ITS and 10% FBS medium conditions. This could
457 indicate that SREBP1 precursor levels are maintained regardless of whether a portion
458 of SREBP1 has been processed and migrated into the nucleus. Combining these
459 observations with the gene expression data, we believe that insulin also has a
460 stimulative effect on *SREBF1* transcription in cancer cells, in agreement with previous
461 findings from liver (23, 39).

462 DNFA is necessary for cell survival even in quiescent cancer cells that do not
463 need membrane lipid synthesis for proliferation. In those cells, fatty acid oxidation
464 (FAO) pathway hyperactivity has also been observed (59). DNFA and FAO are
465 antagonistic lipid metabolism pathways that compose a futile cycle in cancer cells, but
466 may represent a metabolic adaptation to promote cell survival under adverse (lipid-
467 depleted) conditions (60). DNFA primarily relies on cytosolic NADPH derived from
468 the pentose phosphate pathway (PPP) by glucose-6-phosphate dehydrogenase (G6PD),
469 malic enzyme (ME) and isocitrate dehydrogenase (IDH1), all of which are direct targets
470 of SREBP1 (62). Cancer cells frequently promote NAPDH synthesis to support
471 production of cellular anti-oxidants (e.g. glutathione) to counter elevated reactive
472 oxygen species (ROS). These metabolic adaptations together may then represent a pro-
473 survival mechanism, dependent on SREBP1.

474

475 # Conclusions

476 In summary, we have identified and validated a serum-free and insulin
477 supplemented (ITS) medium condition that is well suited for controlled study of

478 lipogenic gene activation and its mechanism of action through SREBP processing and
479 nuclear migration, and consequent active transcription elongation in melanomas, and
480 perhaps other cancer cell types.
481

482 # Author Contributions

483 Conceived and designed the experiments: SW and AMN. Performed the experiments:
484 SW. Analyzed the data: SW. Wrote the paper: SW and AMN.
485

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647

Figure 1.

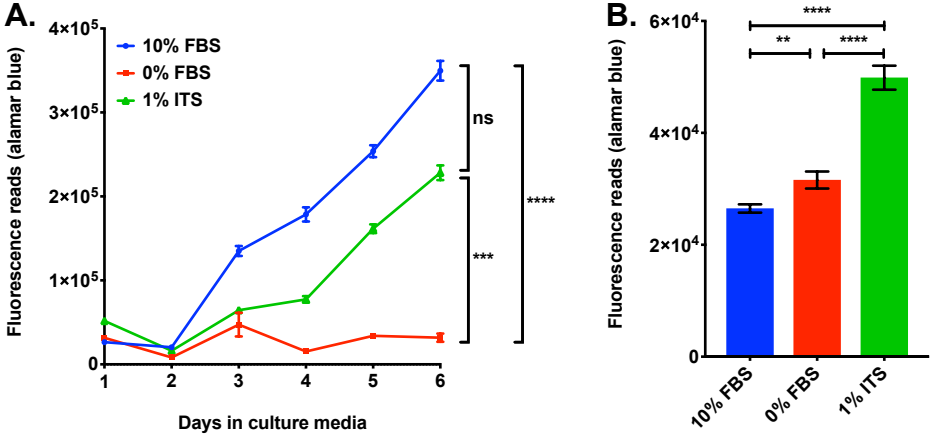

A.
Fluorescence reads (alamar blue)
4×10^5
3×10^5
2×10^5
1×10^5
0
10% FBS
0% FBS
1% ITS
ns
***
****
1
2
3
4
5
6
Days in culture media
B.
Fluorescence reads (alamar blue)
6×10^4
4×10^4
2×10^4
0
****
**
****
10% FBS
0% FBS
1% ITS

Figure 2.

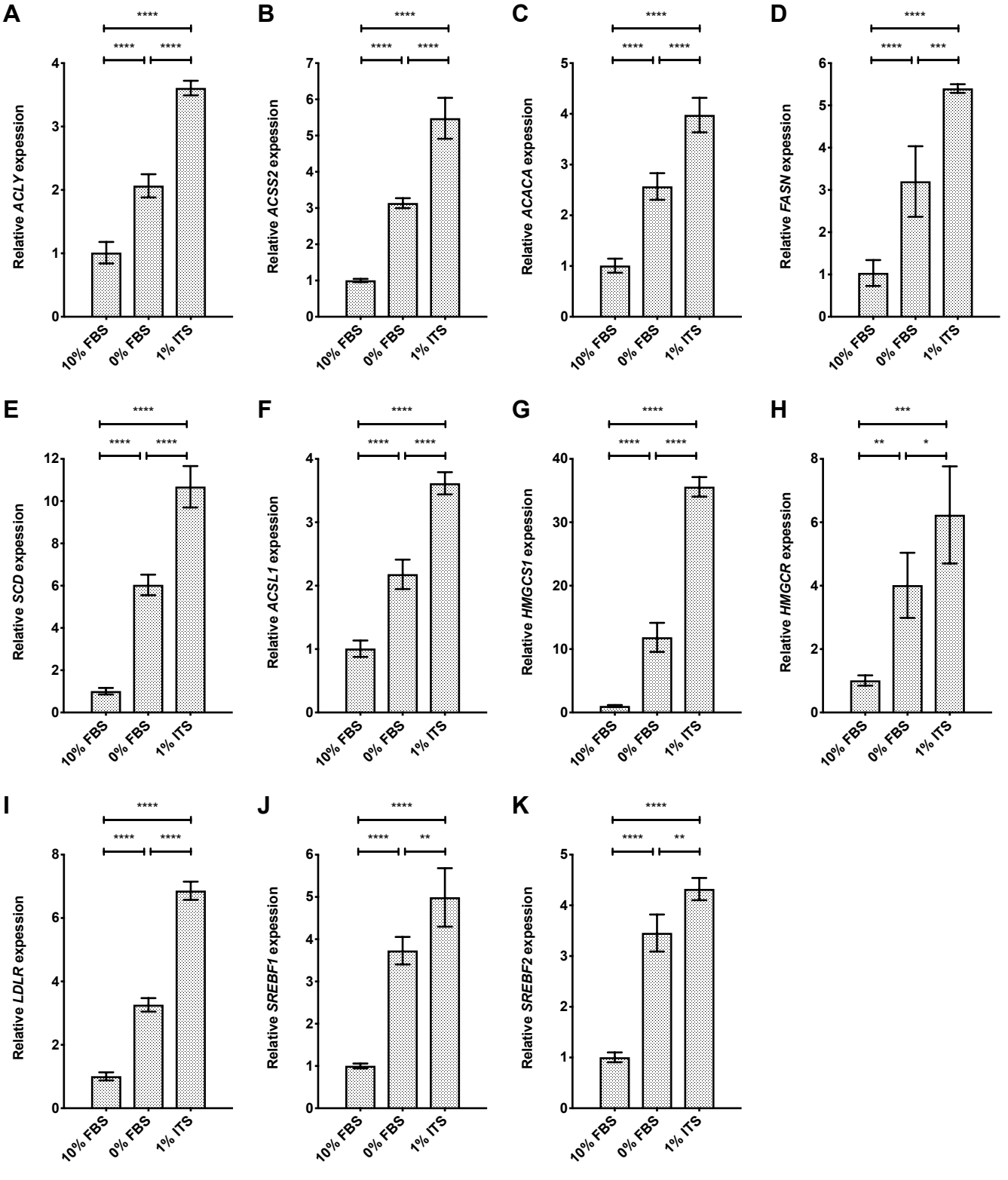

A
B
C
D
E
F
G
H
I
J
K
Relative ACLY expression
Relative ACSS2 expression
Relative ACACA expression
Relative FASN expression
Relative SCD expression
Relative ACSL1 expression
Relative HMGCS1 expression
Relative HMGCR expression
Relative LDLR expression
Relative SREBF1 expression
Relative SREBF2 expression
10% FBS
0% FBS
1% ITS
****
***
**
*

Figure 3.

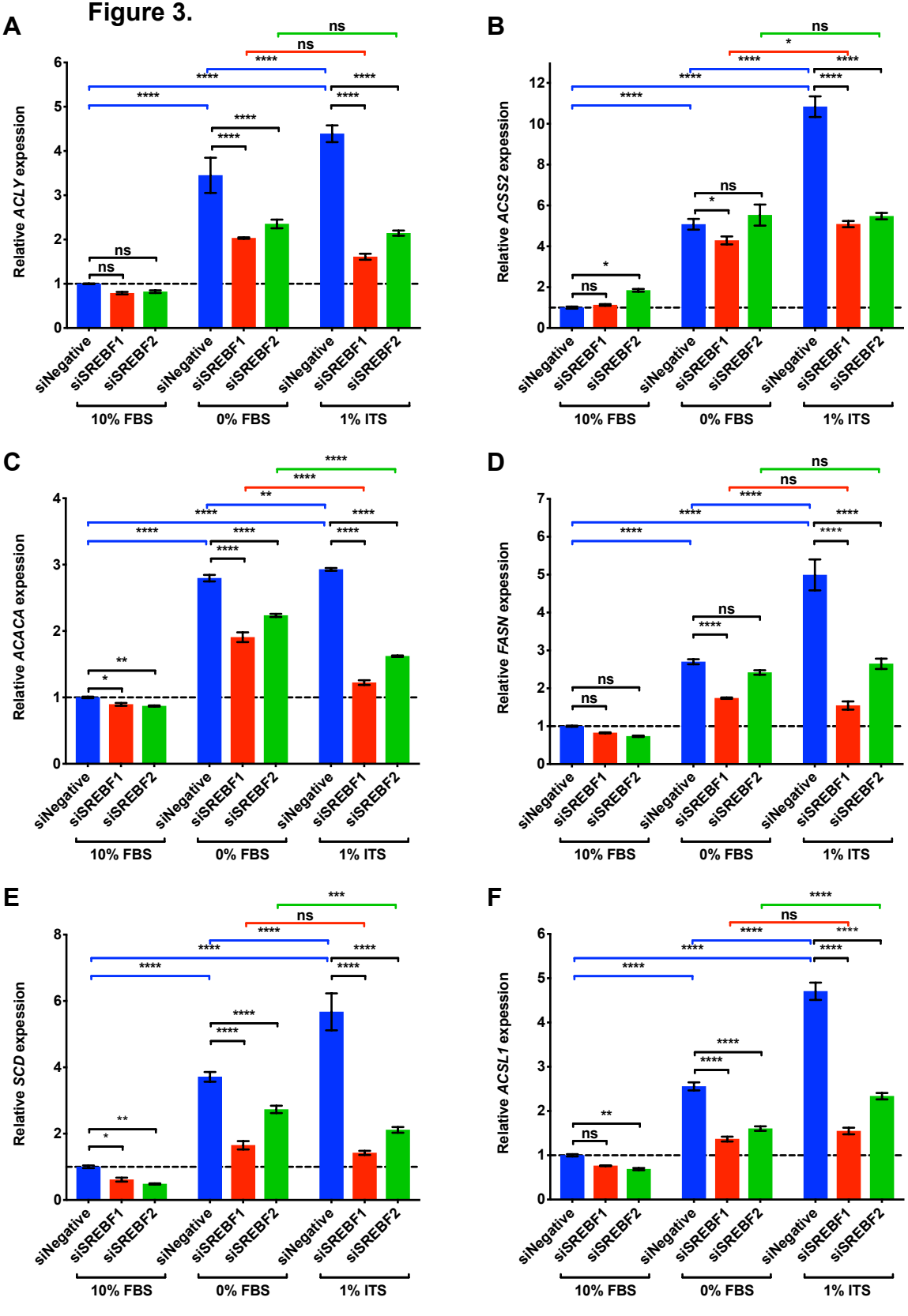

A
Relative ACLY expression
B
Relative ACSS2 expression
C
Relative ACACA expression
D
Relative FASN expression
E
Relative SCD expression
F
Relative ACSL1 expression
siNegative
siSREBF1
siSREBF2
10% FBS
0% FBS
1% ITS
ns
*
**
***
****

Figure 4.

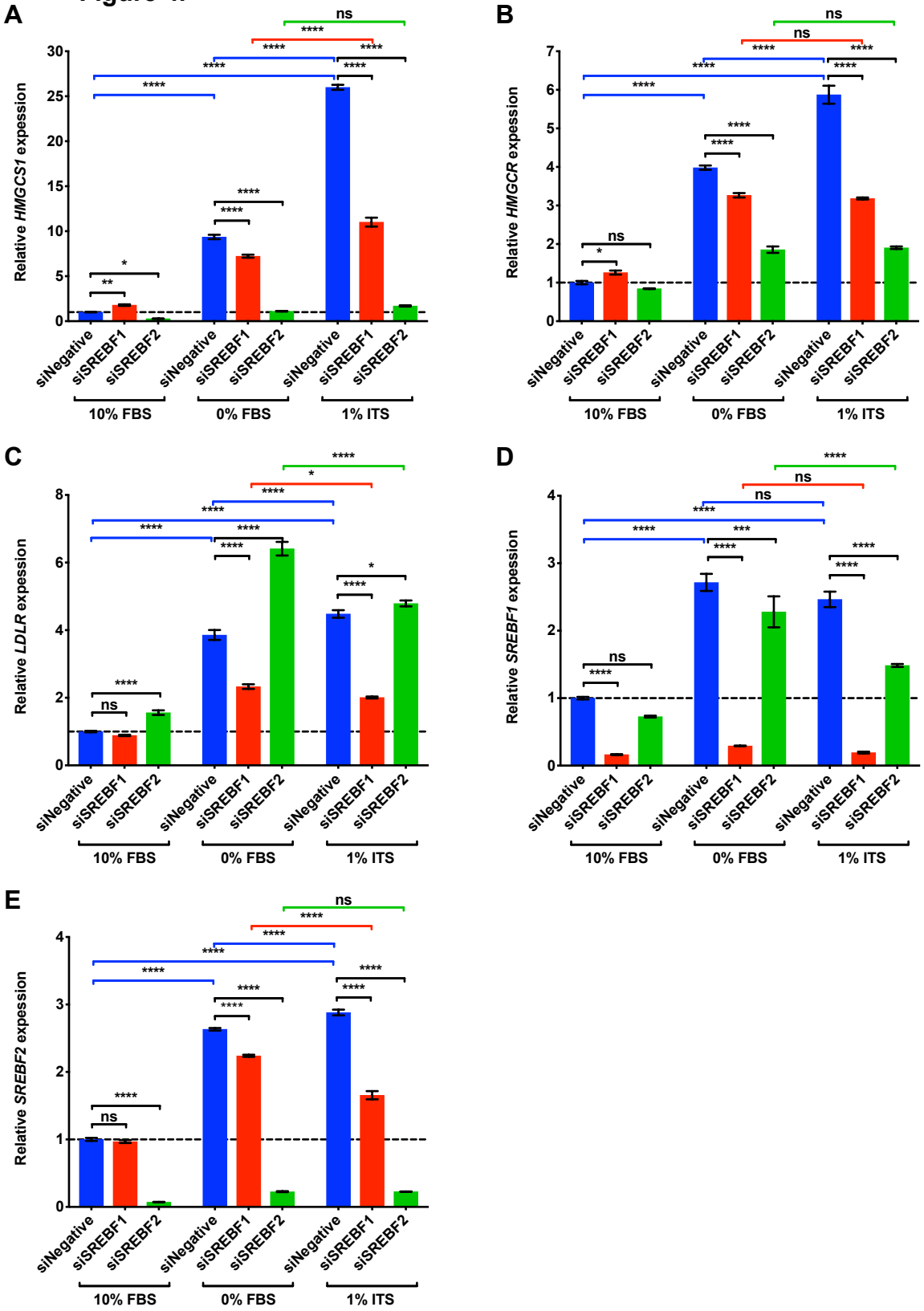

A
Relative HMGCS1 expression
B
Relative HMGCR expression
C
Relative LDLR expression
D
Relative SREBF1 expression
E
Relative SREBF2 expression
siNegative
siSREBF1
siSREBF2
10% FBS
0% FBS
1% ITS
ns
*
**
***
****

Figure 5.

A.

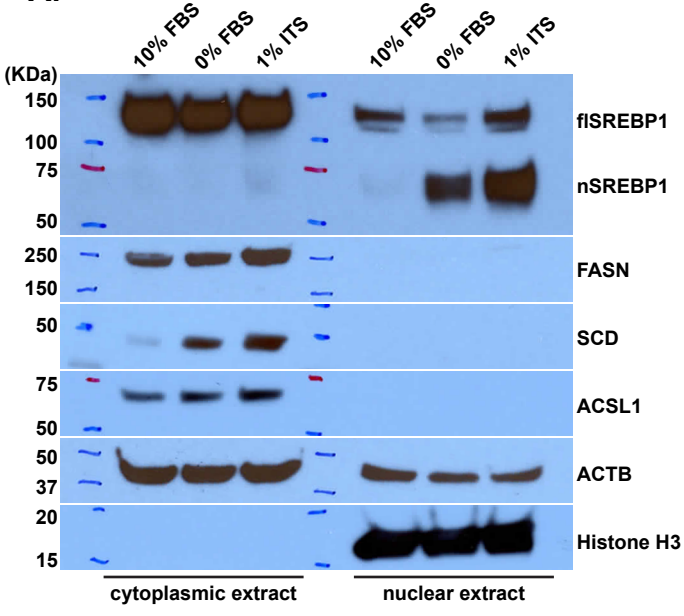
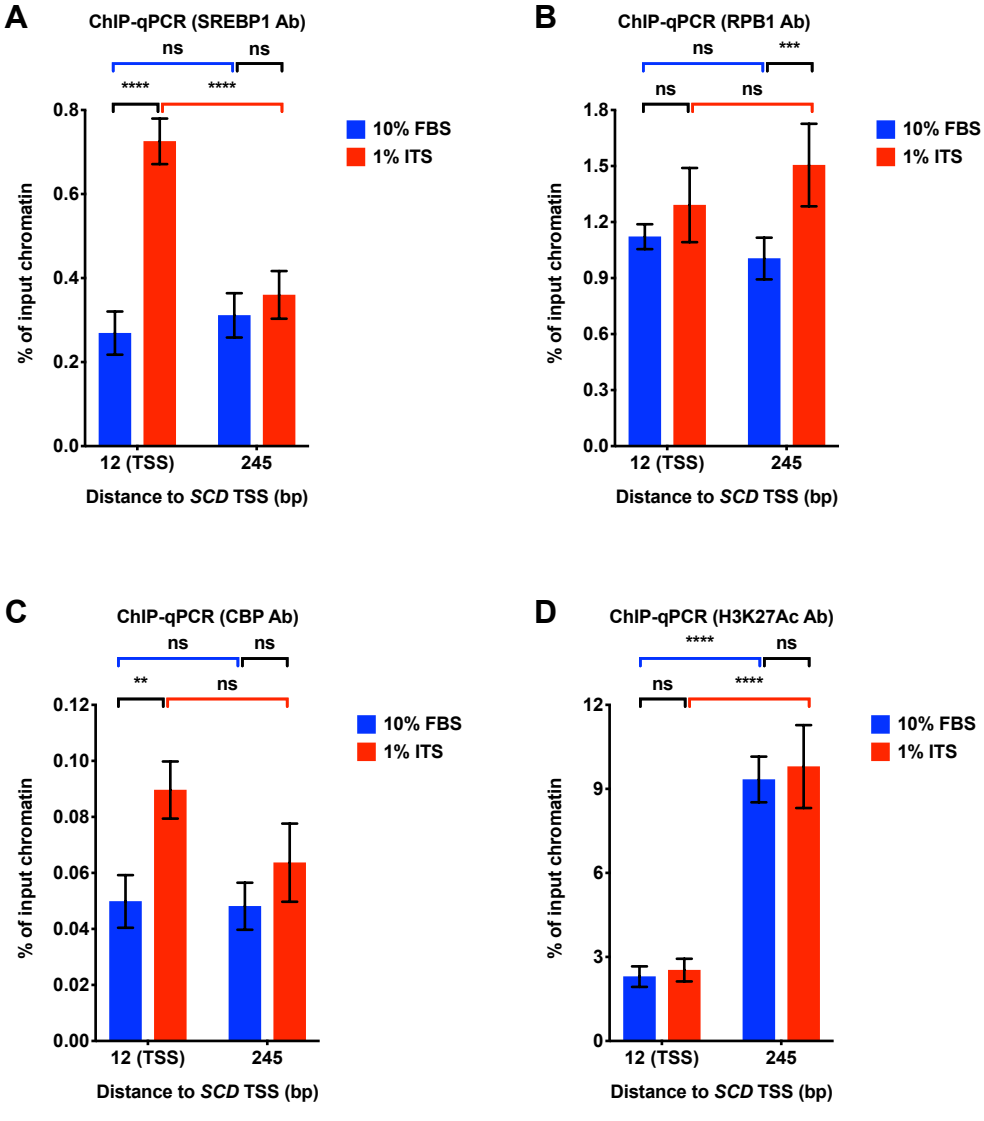

10% FBS
0% FBS
1% ITS
10% FBS
0% FBS
1% ITS
(KDa)
150
100
75
50
250
150
50
75
50
50
37
20
15
flSREBP1
nSREBP1
FASN
SCD
ACSL1
ACTB
Histone H3
cytoplasmic extract
nuclear extract

Figure 6.


A
ChIP-qPCR (SREBP1 Ab)
ns
ns
****
****
0.8
0.6
0.4
0.2
0.0
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS
B
ChIP-qPCR (RPB1 Ab)
ns
***
ns
ns
1.8
1.5
1.2
0.9
0.6
0.3
0.0
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS
C
ChIP-qPCR (CBP Ab)
ns
ns
**
ns
0.12
0.10
0.08
0.06
0.04
0.02
0.00
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS
D
ChIP-qPCR (H3K27Ac Ab)
****
ns
ns
****
12
9
6
3
0
% of input chromatin
12 (TSS)
245
Distance to SCD TSS (bp)
10% FBS
1% ITS