

1 **Nicotinamide Nucleotide Transhydrogenase as a novel treatment**
2 **target in adrenocortical carcinoma**

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45 **Abstract**

46 Adrenocortical Carcinoma (ACC) is an aggressive malignancy with poor response to
47 chemotherapy. Here we evaluated a potential new treatment target for ACC, focusing on the
48 mitochondrial NADPH generator Nicotinamide Nucleotide Transhydrogenase (NNT). NNT has a
49 central role within mitochondrial antioxidant pathways, protecting cells from oxidative stress.
50 Inactivating human NNT mutations result in congenital adrenal insufficiency. We hypothesized
51 NNT silencing in ACC cells will induce toxic levels of oxidative stress. To explore this, we
52 transiently knocked down NNT in NCI-H295R ACC cells. As predicted, this manipulation
53 increased intracellular levels of oxidative stress; this resulted in a pronounced suppression of cell
54 proliferation and higher apoptotic rates, as well as sensitization of cells to chemically-induced
55 oxidative stress. Steroidogenesis was paradoxically stimulated by NNT loss, as demonstrated by
56 mass spectrometry-based steroid profiling. Next, we generated a stable NNT knockdown model in
57 the same cell line to investigate the longer-lasting effects of NNT silencing. After long-term
58 culture, cells adapted metabolically to chronic NNT knockdown, restoring their redox balance and
59 resilience to oxidative stress, although their proliferation remained suppressed. This was associated
60 with higher rates of oxygen consumption. The molecular pathways underpinning these responses
61 were explored in detail by RNA sequencing and non-targeted metabolome analysis, revealing
62 major alterations in nucleotide synthesis, protein folding and polyamine metabolism. Our study
63 provides the first pre-clinical evidence of the therapeutic merit of antioxidant targeting in ACC as
64 well as illuminating the long-term adaptive response of cells to oxidative stress.

65 **Introduction**

66 Adrenocortical carcinoma (ACC) is a rare but aggressive malignancy. The majority of
67 patients present with, or eventually develop, metastatic disease, which shows limited or no
68 responsiveness to cytotoxic chemotherapy (1, 2). A recent randomized trial revealed a median
69 survival of <15 months for patients with disseminated disease receiving combination
70 chemotherapy (3). Glucocorticoid or androgen excess often constitutes an additional clinical
71 burden on ACC patients, undermining their quality of life (1). Unfortunately, the obvious need
72 for more effective medical treatment options in ACC patients remains unmet, despite the
73 remarkable progress in our understanding of the molecular biology of ACC in the last two decades
74 (1).

75 Recent genetic studies have provided new insights into adrenal pathophysiology, revealing
76 that inactivating mutations in the gene encoding the antioxidant enzyme Nicotinamide Nucleotide
77 Transhydrogenase (NNT) underlie a rare, hereditary form of primary adrenal insufficiency (4).
78 Affected patients present in early childhood with failure to thrive, hypotension and hypoglycemia,
79 due to inability of the adrenal glands to produce sufficient cortisol (4). Intriguingly, despite the
80 key role of NNT in preserving cellular redox balance and its ubiquitous expression, the adrenal
81 glands are the only affected organ in most patients; this observation suggests a selective sensitivity
82 of the adrenal glands to NNT loss (4, 5). Supportive of this, NNT-deficient mice harbor adrenal
83 glands with disorganized cortical architecture and high apoptotic rates in their adrenal zona
84 fasciculata, the location of glucocorticoid synthesis, but no other abnormality (4).

85 NNT is a dimeric proton pump that resides in the inner mitochondrial membrane of
86 eukaryotic cells and uses the transmembrane proton gradient to catalyze the transfer of reducing
87 equivalents from NADH to NADP⁺, according to the reaction

88 $\text{NADH} + \text{NADP}^+ + \text{H}^+_{\text{Intermembrane}} \leftrightarrow \text{NAD}^+ + \text{NADPH} + \text{H}^+_{\text{matrix}}$ (6, 7)

89 NADPH is an essential donor of reducing power to the two main mitochondrial antioxidant
90 pathways, the glutathione and the thioredoxin pathways, which protect the mitochondria from the
91 deleterious effects of oxidative stress with their capacity to detoxify reactive oxygen species (e.g.
92 hydrogen peroxide, H_2O_2). Reactive oxygen species (ROS), the molecular mediators of oxidative
93 stress, are continuously produced within the mitochondria by electron leakage along the respiratory
94 chain complexes and the tricarboxylic acid cycle (TCA); in adrenocortical mitochondria,
95 steroidogenesis represents an important additional source of ROS (8-11). Excessive levels of
96 oxidative stress lead to irreversible DNA, protein and lipid damage, which can culminate in
97 apoptotic cell death (**Fig. 1A**) (12).

98 Drawing on these data, which indicate a distinct metabolic vulnerability of the adrenal
99 cortex to oxidative stress, we explored the value of antioxidant targeting as a novel therapeutic
100 approach in ACC, focusing on NNT as a putative treatment target. Interrogating a publicly
101 available whole genome gene expression database (13), we observed that NNT is up-regulated in
102 ACCs in comparison to benign adrenocortical adenomas and healthy adrenals (**Fig. 1B**).
103 Therefore, we hypothesized NNT silencing in ACC cells will impair their antioxidant capacity and
104 lead to progressive accumulation of ROS, inducing unsustainable oxidative toxicity within the
105 mitochondria and eventually triggering cellular apoptosis. We also postulated that steroidogenesis
106 will be suppressed as a result of NADPH depletion and/ or increased oxidative stress.

107 **Materials and Methods**

108 *Cell culture protocol and cell line validation*

109 NCI-H295R (**RRID:CVCL_0458**) ACC cells (passage 10-25) were cultured under
110 standard conditions using DMEM/Ham's F-12 medium (Gibco, Thermo Fisher) supplemented
111 with 2.5% Nu serum (Corning), 1% penicillin-streptomycin (Gibco, Thermo Fisher) and 1% ITS+
112 universal cell culture premix (Corning). Cell line identity was confirmed through Short Tandem
113 Repeat (STR) genetic analysis performed by the DNA Diagnostics Company (London, UK)
114 followed by comparison to genetic profiles provided by the American Tissue Culture Collection
115 (ATCC) (<http://www.lgcstandards-atcc.org>) (**Suppl. Table 1**).

116 *Small-interfering RNA (siRNA) transfection*

117 Transient NNT gene silencing was achieved through transfection of NCI-H295R cells with
118 small interfering RNA (siRNA), using Viromer[®] Blue (Lipocalyx) molecules as transfection
119 vehicles. Three alternative siRNAs targeting different areas of the NNT gene were tried (Life
120 Technologies, HSS118900, HSS118901 and HSS118902), and the one exhibiting the most
121 consistent efficiency in knocking down NNT (HSS118902) was selected for subsequent
122 experiments. HSS118901 was used to corroborate results in proliferation and apoptosis assays,
123 whose results are open to confounding by off-target effects. A scrambled, non-sense siRNA
124 (Silencer Select 1 negative control, Life Technologies) was used as negative control (SCR siRNA).
125 Viromer-siRNA transfection was performed according to the manufacturer's instructions in 6-well
126 plates (300,000 cells/well) and 96-well plates (6-8,000 cells/well).

127 *Short-hairpin RNA (shRNA) transfection*

128 Lentiviral vectors were obtained from Dharmacon (UK) in a p.GIPZ backbone and contained

129 5 shRNAs specific for human NNT (RHS4430-98851990; RHS4430-98913600; RHS4430-
130 98524425; RHS4430-101033169; RHS4430-101025114) under the control of the CMV promoter,
131 as well as the puromycin resistance and green fluorescence protein (GFP) genes. Vectors
132 expressing non-sense, scrambled shRNA (SCR shRNA) were used as negative controls. HEK293T
133 cells (packaging cells, **RRID:CVCL_0063**) were transfected with the shRNA particles by
134 lipofectamine transfection (Thermo Fisher), according to the manufacturer's instructions. Cell
135 media containing the viral particles was collected 48-72 h post-transfection and used to transduce
136 NCI-H295R cells. Four days after transfection, GFP-positive cells were selected in 4µg/ml
137 puromycin. Transduction efficiency was determined by fluorescence microscopy and Western
138 Blotting for NNT expression.

139 *Gene expression*

140 Gene expression for NNT and steroidogenic enzymes was evaluated at a transcriptional level
141 by quantitative real-time polymerase chain reaction (qRT-PCR). RNA extraction was performed
142 using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Reverse
143 transcription to generate complementary DNA (cDNA) was carried out using the TetrocDNA
144 Synthesis Kit (Bioline), following the manufacturer's instructions (500 – 2,000 ng of RNA per
145 reaction used). cDNA concentration was determined by use of a fluorescent DNA dye (Quant-
146 iT™ PicoGreen® dsDNA reagent, Thermo Fisher), comparing sample fluorescence to the
147 fluorescence exhibited by a dilution series of samples of known concentrations (Wallac Victor
148 1420 multilabel counter). Gene expression was then quantified by qRT-PCR, using the Taqman
149 Gene Expression System (Thermo Fisher). Reactions were run in a 7500 ABI qRT-PCR analyzer
150 [50°C incubation for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds
151 (denaturation) then 60°C for 1 minute (annealing-extension)]. All reactions were normalized

152 against the housekeeping gene RPLPO (large ribosomal protein). Data are expressed as ΔCt values
153 [$\Delta\text{Ct}=(\text{Ct of the target gene}) - (\text{Ct of the housekeeping gene})$] or fold-change to control cells ($2^{-\Delta\Delta\text{Ct}}$), where $\Delta\Delta\text{Ct} = \Delta\text{Ct}$ (NNT knockdown cells) – ΔCt (control cells).

155 ***Protein expression***

156 Protein lysate generation was performed by applying RIPA buffer (Sigma-Aldrich) with
157 protease inhibitor cocktail (Sigma-Aldrich) to adherent cells grown in 6-well plates and subsequent
158 collection by scraping. Total protein concentration was estimated colorimetrically using the BCA
159 Protein Assay Kit (Thermo Fisher) as per the manufacturer's instructions, measuring absorbance
160 at 560 nm (Wallac Victor 1420 multilabel counter). NNT protein expression level was assessed by
161 Western Blotting. Samples were run in 10% SDS-PAGE (polyacrylamide) Gels (Thermo Fisher)
162 and transferred to a nitrocellulose membrane using the iBLOT™ Dry Transfer System (Thermo
163 Fisher). Membranes were subsequently probed with anti-NNT antibody produced in rabbit
164 (HPA004829, Sigma-Aldrich, [RRID:AB_1079495](#)) at a 1:500 dilution and secondary anti-rabbit
165 antibody (sc-2030, Santa-Cruz, USA, [RRID:AB_631747](#)) at a 1:2,000 dilution. β -actin was used
166 as control protein (primary antibody A5441, [RRID:AB_476744](#) from Sigma-Aldrich and
167 secondary anti-mouse antibody from Santa-Cruz (sc-2005, [RRID:AB_631736](#)), dilutions 1:10,000
168 and 1:20,000, respectively).

169 ***Reduced to oxidized glutathione ratio (GSH/GSSG)***

170 Total cell glutathione (GSH + GSSG) and oxidized glutathione (GSSG) were measured by
171 luminescence in cells growing in opaque-walled 96-well plates, using the GSH/GSSG-Glo Assay
172 (Promega) according to the manufacturer's instructions. The resulting luminescent signal was
173 measured in Wallac Victor 1420 multi-label counter, using triplicate samples per treatment group
174 and subtracting blank measurements to produce net results. GSH/GSSG ratios were calculated

175 directly from Net Relative Luminescence Units (RLU) measurements using the equation
176 $\text{GSH/GSSG ratio} = [\text{Net total glutathione RLU} - \text{Net GSSG RLU}] / [\text{Net GSSG RLU}/2]$.

177 ***Metabolic Flux Analysis (Seahorse XF)***

178 Metabolic Flux analysis in a Seahorse XF 24 Analyzer was used to assess the effect of
179 NNT knockdown on mitochondrial bioenergetics, applying the Seahorse XF Cell Mito Stress kit.
180 Cells were plated in Seahorse XF microplates the day before the experiment at a density of 100,000
181 cells/ well. Changes in oxygen concentration provide the oxygen consumption rate (OCR), which
182 is a measure of mitochondrial respiration. Changes in proton concentration (or pH) provide the
183 extracellular acidification rate (ECAR), reflective of the rate of glycolysis. Measurements were
184 taken at baseline and after successive application of compounds interfering with oxidative
185 phosphorylation: oligomycin (complex V inhibitor, 2 μM), Carbonyl cyanide-p-
186 trifluoromethoxyphenylhydrazone (FCCP, mitochondrial uncoupler, 1 μM) and Antimycin A +
187 Rotenone (Complex I and III inhibition, 1 μM). Results were normalized to protein concentration,
188 measured by the BCA Protein Assay Kit (Thermo Fisher).

189 ***Cell proliferation and apoptosis***

190 Cell proliferation was assessed in 96-well plates (loading concentration 6-8,000 cells/well),
191 using the CyQuant Proliferation Assay Kit (Thermo Fisher) and following the manufacturer's
192 instructions. Cell DNA fluorescence was measured at the end of the time course, i.e. 166 hours
193 post siRNA transfection and/ or 96 hours after treatment. The beginning of treatment was used as
194 the baseline time point ($t=0$) for each proliferation series; for siRNA knockdown experiments, 72
195 hours post transfection was taken as the baseline time point. Proliferation rates were provided by
196 the following ratio: $[(\text{end cell number} - \text{baseline cell number})] / \text{baseline cell number}$.

197 Cellular apoptosis was assessed using the Caspase-Glo 3/7 Assay kit (Promega), a
198 luminescence-based assay measuring Caspase 3 and 7 activity in cell lysates, and following the
199 manufacturer's instructions. Luminescent signals were quantified using the Wallac Victor 1420
200 multilabel counter. At the end of the assay, media and reagents were removed from all wells and
201 stored at -80°C. The next day, relative quantification of cell number was performed by use of the
202 CyQuant® Proliferation Assay Kit, as described above. Luminescence values obtained in the
203 caspase assay were normalized to the fluorescence results of the proliferation assay.

204 Paraquat and auranofin were purchased from Sigma-Aldrich (UK). Buthionine sulfoximine
205 (BSO) was purchased from Cayman Chemical (USA).

206 *In vitro steroid profiling by liquid chromatography-tandem mass spectrometry (LC-MS/MS)*

207 Steroid synthesis by NCI-H295R cells was assessed by comprehensive multi-steroid
208 profiling employing liquid chromatography-tandem mass spectrometry (LC-MS/MS), as
209 described previously (14, 15). Steroid extraction and analysis by LC-MS/MS are discussed in
210 **Supplementary Methods.**

211 *RNA sequencing*

212 RNA was prepared in triplicate from NCI-H295R KD siRNA, SCR siRNA (72 hours post-
213 transfection), KD shRNA and SCR shRNA cells using the RNeasy Mini kit (Qiagen). Libraries
214 were generated using the TruSeq Stranded mRNA Library Prep Kit (Illumina, USA). 4 nM library
215 (containing the 16 pooled libraries) was sequenced on a NextSeq500 System (Illumina). Pathway
216 analysis on sequencing data was completed using GAGE V2.22 package from Bioconductor
217 release 3.2 and referencing the KEGG pathways. Differentially expressed genes were considered
218 significant applying a false discovery rate of <5% ($q < 0.05$). Differentially regulated pathways

219 were called at a p value of <0.01. A detailed description of the methodology for RNA sequencing
220 and pathway analysis can be found in **Supplementary Methods**.

221 In addition, RNA sequencing data from recently published work on three different mouse
222 strains (*Nnt* inactivating mutation, C57BL/6J (RRID:MGI:3702942) ; wild-type, C57BL/6NHsd
223 (RRID:MGI:2161078) ; and transgenic *Nnt* overexpressor, C57BL/6J^{BAC}) (16) were re-analyzed
224 employing the same pathway analysis as for the human cell-based model; detailed information on
225 this dataset can be found in **Supplementary Methods**.

226 *Metabolome analysis*

227 Cell and media samples were prepared for non-targeted metabolome analysis through
228 quenching cell metabolism with a mix of acetonitrile, methanol and water (Sigma-Aldrich). The
229 process of sample generation and analysis is described in more detail in the **Supplementary**
230 **Methods**.

231 *Statistical analysis*

232 Statistical analysis was performed using GraphPad Prism 7 Software
233 (RRID:SCR_002798). Data are represented as mean_± SEM values, unless otherwise stated.
234 Comparisons were made using Student's paired t-test for normally distributed data or Wilcoxon's
235 signed-rank test for data not following a Gaussian distribution. Multiple comparisons (BSO and
236 auranofin treatments) were performed by one-way ANOVA followed by post-hoc multiple
237 comparison testing. Statistical methods for the RNA sequencing and untargeted metabolome
238 analysis are detailed in **Supplementary Methods**.

239 **Results**

240 *Transient and stable NNT knockdown*

241 Transient NNT silencing by siRNA knockdown was employed to explore the acute effects
242 of NNT loss on ACC cells. NNT siRNA transfection in NCI-H295R cells yielded efficient gene
243 silencing for at least 166 hours post-transfection with two different siRNAs (**Suppl. Fig. 1A-B**).
244 All subsequent experiments were performed with the siRNA that gave the best knockdown results
245 on real-time PCR and Western Blotting (referred to here as KD siRNA). The second siRNA (KD
246 siRNA2) was used to corroborate the results of proliferation and apoptosis assays, whose results
247 are most likely to be distorted by off-targets effects.

248 Stable NNT silencing by shRNA knockdown was used to delineate the long-term effects
249 of NNT loss on ACC cells. Stable NNT knockdown in NCI-H295R cells was achieved by lentiviral
250 transfection with shRNA-expressing plasmids and selection with puromycin, and resulted in
251 permanent NNT silencing (**Suppl. Fig. 1C-D**).

252 *NNT siRNA knockdown increases cellular oxidative stress*

253 Given the central role of NNT within the mitochondrial ROS scavenging network, we
254 hypothesized that NNT knockdown will increase oxidative stress in NCI-H295R cells. To test this,
255 we measured the intracellular ratio of reduced to oxidized glutathione (GSH/GSSG), an established
256 marker of oxidative stress; a decrease in the GSH/GSSG ratio indicates that the proportion of
257 oxidized intracellular glutathione is increased as a result of higher intracellular ROS levels. Indeed,
258 we observed a statistically significant ($p < 0.05$) decrease in the GSH/GSSG ratio in NNT KD
259 siRNA-transfected cells 96 hours post-transfection (**Fig. 2A**).

260 *NNT siRNA knockdown suppresses cell proliferation and induces apoptotic cell death*

261 Cell proliferation rates were assessed over the time window from 72 to 166 hours post-
262 transfection, a period with consistent NNT knockdown confirmed at protein level (**Suppl. Fig. 1**).
263 NNT knockdown by KD siRNA transfection led to a marked decrease in cellular proliferation rates
264 (**Fig. 2B**). These results were corroborated by use of a second siRNA against NNT, which
265 completely obliterated cell proliferation (**Suppl. Fig. 2**).

266 To establish whether the increased oxidative stress observed with NNT KD leads to higher
267 rates of apoptosis - as predicted by ROS physiology- we measured intracellular caspase 3 and 7
268 activity 120 hours post-transfection. We also quantified relative cell numbers by DNA
269 fluorescence at the same time-point to standardize results to cell number. NNT KD siRNA cells
270 exhibited significantly higher caspase 3/7 activity than SCR siRNA cells ($p < 0.05$), confirming our
271 hypothesis that NNT knockdown triggers cell death by apoptosis (**Fig. 2C**). The effect was even
272 more marked with the alternative siRNA against NNT (**Suppl. Fig. 2**).

273 *NNT siRNA knockdown sensitizes cells to oxidative stress*

274 Next, we evaluated changes in mitochondrial respiration by direct measurement of the
275 cellular oxygen consumption rate (OCR), using Extracellular Flux analysis. Despite the location
276 of NNT in the inner mitochondrial membrane, we observed no statistically significant difference
277 between NNT KD siRNA- and SCR siRNA-transfected cells, either at baseline or in response to
278 mitochondrial respiration disruptors (**Fig. 2D**). Baseline extracellular acidification rate (ECAR),
279 representative of the glycolytic rate, was also similar between the two groups (**Fig. 2E**).

280 Considering the integral role of NNT in mitochondrial antioxidant defense and the
281 detrimental impact of NNT inhibition on redox balance, we further hypothesized that NNT loss
282 will render NCI-H295R cells more sensitive to chemically induced oxidative stress. To assess this
283 assumption, we treated NCI-H295R cells with a sub-toxic dose of paraquat, a pesticide which

284 induces oxidative stress *in vitro* generating superoxide. Treatment with 10 μ M of paraquat for 96
285 hours led to a statistically significant decrease in cell proliferation in cells transfected with KD
286 siRNA, but not in their counterparts that had been transfected with SCR siRNA (**Fig. 2F**).

287 ***Redox adaptation develops with stable NNT knockdown***

288 To explore the long-term metabolic consequences of NNT silencing in NCI-H295R cells,
289 we employed a different model, involving stable transfection with shRNA against NNT. With
290 long-term culture under persistent NNT silencing (4-12 weeks post-transfection), cells managed
291 to restore their redox balance to the levels of their SCR shRNA-transfected counterparts (**Fig. 3A**).

292 ***ACC proliferation remains suppressed with stable NNT knockdown***

293 The distinct metabolic consequences of NNT silencing in the stable knockdown setting, in
294 comparison to acute knockdown by NNT siRNA, translated into an attenuated response with
295 respect to cellular proliferation and viability. Proliferation rates remained significantly lower in
296 KD shRNA-transfected cells compared to the SCR shRNA-transfected controls; however, this was
297 less pronounced than the decrease in proliferation we observed with siRNA-mediated knockdown
298 (**Fig. 3B**). Apoptotic rates did not differ between SCR shRNA and KD shRNA cells (**Fig. 3C**), in
299 keeping with the restoration of redox homeostasis we had ascertained based on the reduced/
300 oxidized glutathione ratio.

301 Interestingly, NNT KD shRNA cells consumed more oxygen than SCR shRNA cells at
302 baseline (**Fig. 3D**). This finding potentially reflects higher energy needs in NNT deficient cells.
303 The same trend was observed in ECAR, a surrogate marker of glycolysis, but without reaching
304 statistical significance (**Fig. 3E**). Finally, stable NNT knockdown did not enhance cell sensitivity
305 to oxidative stress induced by paraquat. (**Fig. 3F**).

306 *Transient, but not stable, NNT knockdown paradoxically stimulates steroidogenesis*

307 The effects of NNT silencing on steroidogenesis were evaluated by comprehensive multi-
308 steroid profiling in cell media by LC-MS/MS, as well as gene expression analysis by qRT-PCR.
309 We postulated that NNT silencing will disrupt steroidogenesis, either depriving mitochondrial
310 steroidogenic monooxygenases [cholesterol side-chain cleaving enzyme (CYP11A1), 11 β -
311 hydroxylase (CYP11B1), aldosterone synthase (CYP11B2)] of their essential electron donor
312 NADPH, or due to oxidative stress-induced down-regulation of key steroidogenic enzymes.
313 Surprisingly, NNT KD siRNA-transfected cells actually produced significantly more
314 glucocorticoids (cortisol) and androgens (androstenedione) than controls (**Fig. 4A-B**). Individual
315 enzyme activities were determined as product-to-substrate ratios for three key steroidogenic
316 enzymes, 11 β -hydroxylase (CYP11B1), 21-hydroxylase (CYP21A2), and CYP17A1 17/20-lyase
317 activity; all three displayed higher activity in NNT KD siRNA-transfected cells, in keeping with a
318 paradoxical generalized stimulation of steroidogenesis by acute NNT loss (**Fig. 4C-E**).

319 By contrast, in the shRNA-transfected cells with chronic NNT silencing we observed no
320 significant impact on steroidogenesis, with similar rates of cortisol or androstenedione synthesis
321 between KD shRNA and SCR shRNA cells (**Fig. 4A-E**).

322 We also explored the gene expression alterations underpinning the enhanced steroid
323 production of cells transfected with KD siRNA, comparing the expression of core steroidogenic
324 genes (StAR, CYP11A1, CYP21A2, CYP17A1, 3 β HSD2) between KD siRNA and SCR siRNA
325 cells by qRT-PCR. There was a statistically significant increase in the expression of cytochrome
326 P450 (CYP) type 2 steroidogenic enzymes located in the ER CYP21A2 (p<0.05), CYP17A1
327 (p<0.05), as well as the ER dehydrogenase HSD3B2 (p<0.01) in NNT KD siRNA cells (**Suppl.**

328 **Table 2).** CYP11B1 and CYP11B2 expression levels were too low to be quantified by qRT-PCR
329 in our cells.

330 Analysis of gene expression by RNA sequencing in an extended panel of 14 steroidogenic
331 genes indicated a significant up-regulation of CYP21A2 ($q < 0.05$) and CYP17A1 ($q < 0.01$), as well
332 as the ACTH receptor MC2R ($q < 0.01$) in the transient NNT knockdown model; no significant
333 changes were observed in the stable knockdown model (**Fig. 4F**).

334 *NCI-H295R cells are sensitive to glutathione depletion and thioredoxin reductase inhibition*

335 Given the effects of NNT inhibition on NCI-H295R cell proliferation, we went on to
336 evaluate the sensitivity of ACC cells to isolated inhibition of each of the two pillars of
337 mitochondrial antioxidant defense: the glutathione pathway and the thioredoxin pathway.

338 We used buthionine sulfoximine (BSO), a potent inhibitor of the glutathione-producing
339 enzyme c-glutamylcysteine ligase, to deplete intracellular glutathione. We observed a decline in
340 cell proliferation with a BSO dose of $\geq 100 \mu\text{M}$ after 96 hours of treatment (**Fig. 5A**).

341 Pharmacological manipulation of the alternative mitochondrial antioxidant pathway, the
342 thioredoxin pathway, was achieved by auranofin, a gold complex agent with a well-established
343 capacity to inhibit thioredoxin reductase. NCI-H295R treatment with doses of $\geq 1 \mu\text{M}$ was
344 associated with major cytotoxicity (**Fig. 5B**).

345 Finally, dual inhibition of the glutathione and thioredoxin pathways by co-administration
346 of low-dose BSO ($50 \mu\text{M}$) and auranofin ($0.2 \mu\text{M}$) resulted in marked cytotoxicity, suggesting that
347 a potent synergistic effect can be achieved by dual pathway targeting (**Fig. 5C**).

348 *Whole transcriptome and metabolome analyses reveal extensive metabolic perturbations with*
349 *transient NNT knockdown, as well as changes in protein processing and polyamine metabolism*

350 *with stable NNT knockdown*

351 In order to uncover the molecular mechanisms that underpin the effects we observed in the
352 two models and the discrepancies between them, we applied whole transcriptome analysis in RNA
353 extracted from four groups of NCI-H295R cells (NNT KD siRNA vs SCR siRNA, NNT KD
354 shRNA vs SCR shRNA). 842 genes were differentially regulated between NNT KD siRNA and
355 SCR siRNA cells; 247 genes were differentially regulated between NNT KD shRNA and SCR
356 shRNA cells ($q < 0.05$). Of note, only 17 of the genes regulated differentially between KD and SCR
357 cells were identical in the two models, NNT KD siRNA and shRNA (**Suppl. Table 3**).

358 Differentially regulated pathways are visualized in **Fig. 6A+B** ($p < 0.01$) and tabulated in
359 **Suppl. Table 4**. In NNT KD siRNA cells, significant changes were observed in crucial pathways
360 affecting cellular proliferation and viability (p53 pathway, MAPK pathway, checkpoint kinases).
361 Interestingly, in KD shRNA cells one of the borderline significantly altered pathways ($p < 0.01$,
362 $q = 0.11$) controlled protein processing in the ER, with up-regulation of genes encoding heat shock
363 proteins (predominantly in the HSP40 family), chaperone proteins that facilitate correct protein
364 folding and transfer of misfolded proteins to proteasomes for degradation (**Suppl. Table 4**) (17,
365 18). Other significantly up-regulated pathways with stable NNT KD included ribosomal genes
366 ($p < 0.01$, $q < 0.05$) and pyrimidine metabolism ($p < 0.01$, $q = 0.11$), including an up-regulation of RNA
367 II polymerases. Taken together, these findings hint at a higher protein turnover which may allow
368 cells to swiftly replace proteins that have sustained irreversible oxidative damage.

369 Of note, recent rodent-based work by Meimaridou et al. performed RNA sequencing on
370 mouse adrenal glands derived from three different mouse strains: a strain which carries an
371 inactivating mutation of *Nnt* (C57BL/6J), a strain with wild-type *Nnt* expression (C57BL/6N), and
372 transgenic mice overexpressing *Nnt* on the background of the NNT-deficient mouse strain (rescue

373 model; C57BL/6J^{BAC}) (16). We have extended this work here, carrying out additional, pathway
374 analysis on this data. Detailed information on significantly dysregulated pathways in C57BL/6J vs
375 C57BL/6N mice and C57BL/6J vs C57BL/6J^{BAC} mice are presented in **Suppl. Table 5**. Significant
376 dysregulation of the major cell signaling pathway of mitogen activated signaling kinases (MAPK)
377 was one of the salient molecular changes in both comparisons; the same pathway was also
378 dysregulated with transient NNT knockdown in NCI-H295R cells. Oxidative phosphorylation was
379 up-regulated in both comparisons, but changes were much more pronounced in the C57BL/6J vs
380 C57BL/6N comparison and likely to reflect a strain, rather than gene, effect.

381 RNA sequencing in NCI-H295R cells was complemented by whole metabolome analysis
382 performed separately in cells and corresponding cell culture supernatant. In the siRNA knockdown
383 model, NNT silencing was associated with a significant metabolic perturbation when compared to
384 the SCR siRNA cells. An increase in the presence of 44 oxidized fatty acids supports a shift to a
385 more oxidized intracellular microenvironment (**Fig. 6C** and **Suppl. Tables 6 and 7**). This was not
386 observed in the stable NNT knockdown model. Pathway enrichment analysis demonstrated that
387 six important metabolic pathways were enriched ($q < 0.05$): tricarboxylic acid (TCA) cycle,
388 arginine and proline metabolism, pyrimidine metabolism, nicotinate and nicotinamide metabolism
389 and glutathione metabolism. Additional to this, we observed statistically significant changes
390 ($q < 0.01$) for 16 acyl carnitines, 25 fatty acids, 15 acyl amino acids, 20 purine and pyrimidine
391 metabolites and 5 metabolites present in the nicotinate and nicotinamide metabolic pathway. Taken
392 together, these indicate a perturbation in mitochondrial fatty acid beta-oxidation (as shown by
393 changes in fatty acids, acyl carnitines and TCA metabolites), changes in nucleotide synthesis and
394 a potential overload of acetyl units.

395 In the stable NNT shRNA knockdown model, pathway enrichment analysis highlighted

396 changes in purine metabolism ($q < 0.05$) and NNT knockdown cells exhibited a significant ($p < 0.05$)
397 rise in several purine and pyrimidine metabolites (**Fig. 6D** and **Suppl. Tables 6 and 7**), mirroring
398 the results of the transcriptome analysis. Polyamine (spermine, spermidine) metabolism was also
399 significantly modified in both models: KD siRNA cells displayed a dramatic increase in polyamine
400 catabolism (accumulation of diacetyl-spermine, diacetyl-spermidine, spermine dialdehyde)
401 leading to depletion of spermine and spermidine, a response that has been associated with arrest
402 of cell growth (**Suppl. Tables 6 and 7**) (19). Conversely, KD shRNA cells exhibited a significant
403 rise in spermine, a polyamine that can act as a ROS scavenger (20, 21).

404 Discussion

405 With this work, we have explored the immediate and longer-term impact of NNT silencing
406 on ACC cells with respect to redox balance, mitochondrial bioenergetics, cell proliferation and
407 viability, and steroidogenesis, using two distinct *in vitro* knockdown models in the human
408 adrenocortical carcinoma cell line NCI-H295R. Our aim was to establish whether NNT inhibition
409 can have therapeutically beneficial effects with respect to control of tumor growth and steroid
410 excess. We hypothesized that NNT inhibition would compromise the ability of adrenocortical
411 mitochondria to deal with oxidative stress, leading to progressive accumulation of ROS. ROS
412 excess has multiple toxic sequelae, and can directly impair cell viability triggering apoptosis (8,
413 12). Importantly, the adrenal-specific clinical phenotype in humans and the reported increased rate
414 of adrenocortical cell apoptosis in otherwise healthy NNT mutant mice suggest that this
415 manipulation may selectively target ACC cells, sparing other organs. This susceptibility of
416 adrenocortical cells to mitochondrial antioxidant pathway disruption can be explained by the fact
417 that enzymes involved in the rapid adrenal steroid response to stress represent a major additional
418 source of ROS in the adrenals, increasing their dependence on efficient ROS scavenging (10, 11).

419 In keeping with our hypothesis, we found that in the acute setting (siRNA-mediated
420 transient knockdown), NNT loss increased intracellular oxidative stress. Redox balance
421 perturbations in response to NNT loss have been previously demonstrated in a limited number of
422 cell lines *in vitro*, as well as in lymphocytes derived from NNT mutant patients *ex vivo* (4, 22-25).
423 These findings are in line with the biological role of NNT as a major mitochondrial generator of
424 NADPH, the essential provider of reducing equivalents to the two main antioxidant pathways (26).

425 Importantly, in our study we observed that NNT silencing led to an immediate and marked
426 inhibition of cell proliferation accompanied by increased apoptotic rates. **This anti-tumor effect**

427 was even more pronounced when using a second anti-NNT siRNA (KD siRNA2). This apparent
428 difference in degree (but not in direction) of cell response raised the possibility of additional, off-
429 target effects triggered by KD siRNA2; on interrogation of the NCBI Basic Local Assignment
430 Search tool, however, neither of the two siRNAs share substantial homology with any genes that
431 would be expected to impact cell proliferation and viability. The association between excessive
432 oxidative stress and mitochondrial apoptosis has been well established in the literature (8, 12), but
433 data on the effects of NNT loss on cellular proliferation and viability are limited. Transient NNT
434 silencing was previously shown to increase rates of apoptosis in PC12 (rat pheochromocytoma)
435 cells (22); stable NNT knockdown in human melanoma cells was associated with reduced viability
436 and high apoptotic rates *in vitro*, as well as slower growth of melanoma xenografts in mice (27).
437 Meimaridou et al reported high levels of apoptosis in the zona fasciculata of the adrenal cortex
438 from NNT mutant mice, as well as NCI-H295R cells stably transfected with shRNA against NNT
439 *in vitro* (4). Although ROS have typically been associated with a stimulation of cellular
440 proliferation, a number of *in vitro* models have demonstrated the opposite effect (suppression of
441 cell division), in a complex relationship that may depend on the magnitude of ROS excess and/or
442 tissue type (28, 29). NNT inhibition may also interfere with cellular proliferation in a ROS-
443 independent way, curtailing the amount of NADPH available to fuel the pressing anabolic needs
444 of malignant cells. In keeping with the major impact on cellular viability and proliferation, we
445 observed far-reaching metabolic effects of NNT knockdown implicating several areas of cell
446 metabolism, including mitochondrial fatty acid oxidation, polyamine metabolism and nucleotide
447 synthesis. The enhanced cellular sensitivity to oxidative stress in the aftermath of NNT silencing
448 (paraquat treatment) is translationally important, as oxidative stress is induced by a number of
449 classic chemotherapy agents, contributing to their cytotoxic effect (8, 30). NNT inhibition could

450 represent a feasible strategy to sensitize ACC to such drugs

451 The longer-term effects of NNT loss on ACC cells, as delineated in the stable knockdown
452 model, were disparate from the ones encountered in the acute setting. Importantly, with long-term
453 culture under constant NNT silencing, NCI-H295R ACC cells managed to restore their redox
454 balance. This compensation abrogated the pro-apoptotic early impact of NNT loss. Interestingly,
455 a persistent proliferative handicap was demonstrated, though this was less marked than the one
456 observed in the acute setting. This may be attributable to the limited supply of NADPH in the
457 absence of NNT. Extracellular flux analysis revealed higher rates of oxygen consumption in KD
458 shRNA cells, a response that may reflect higher energy needs or be driven by the spare NADH
459 which fails to be converted to NADPH in NNT-deficient cells. Previous studies on the effect of
460 NNT silencing on oxygen consumption have shown mixed results, which may be cell type-
461 dependent (22, 25, 31).

462 Redox adaptation to oxidative stress has been previously described in tumor models *in*
463 *vitro*; this process is driven by the strong selective pressure applied by oxidative toxicity and
464 promoted by the genomic instability which characterizes the oxidized intracellular
465 microenvironment (32). We obtained insights into how this adaptation was facilitated in our model
466 by comprehensive transcriptome and metabolome analysis. In NNT KD shRNA cells, we observed
467 an up-regulation of genes that are involved in protein folding in the ER, as well as in the
468 identification and degradation of damaged proteins. Purine and pyrimidine metabolism was
469 activated in these cells, and ribosomal genes were up-regulated. Taken together, these findings
470 hint at increased protein turnover, involving degradation of damaged protein and acceleration of
471 new protein synthesis. This may represent a key compensatory mechanism against oxidative stress,
472 achieving the timely removal and replacement of irrevocably damaged (oxidized) proteins. The

473 observed increase in oxygen consumption could provide additional energy to fuel this process. Of
474 note, we recently described up-regulation of chaperone proteins in the adrenals of NNT-deficient
475 mice (16). The additional pathway analysis we performed on the same RNA sequencing data from
476 that rodent work displayed otherwise limited overlap with our *in vitro* model, likely reflecting the
477 expected biological differences between a healthy mouse adrenal and a malignant human adrenal
478 cell line.

479 Interestingly, polyamine metabolism exhibited dramatic shifts in opposite directions in the
480 two models. Polyamines (spermine, spermidine) are versatile cationic molecules involved in a
481 number of cell processes, including ROS scavenging and cell proliferation (19, 33). High
482 endogenous polyamine levels have been found in a number of cancer types (34). Acute NNT loss
483 was accompanied by a rapid accumulation of acetylated catabolic products of polyamines, leading
484 to depletion of spermine and spermidine. Polyamine catabolism can both be triggered by oxidative
485 stress and generate hydrogen peroxide (H₂O₂), creating a vicious cycle that propagates ROS
486 accumulation (33, 35). Indeed, stimulated polyamine catabolism has been associated with growth
487 arrest and cell death in various *in vitro* models (19, 36). Conversely, in the chronic setting, stable
488 NNT knockdown cells demonstrated increased spermine concentrations and no evidence of
489 accelerated polyamine catabolism. This response is likely to represent a major facilitator of the
490 successful redox adaptation in this model. Our findings underscore the importance of polyamine
491 homeostasis in adrenocortical carcinoma cells.

492 Within the same framework, we also explored alternative antioxidant targets focusing on
493 the glutathione and thioredoxin pathways. Pertinently, human mutations in thioredoxin reductase
494 2 have also been shown to result in isolated glucocorticoid deficiency (37). We used BSO to inhibit
495 glutathione synthesis. BSO has shown anti-proliferative effects against a number of cell lines *in*

496 *vitro* (38-43). We observed a significant suppression of cell growth with doses of $\geq 100 \mu\text{M}$, i.e.
497 at doses that are clinically attainable in plasma with no serious toxicity (38). Auranofin, a gold
498 complex-based agent able to inhibit thioredoxin reductase, also suppressed cell proliferation at
499 doses $\geq 1 \mu\text{M}$ and was associated with marked cytotoxicity at doses of $\geq 2 \mu\text{M}$. Auranofin has also
500 displayed anti-tumor activity against a number of cell lines *in vitro* and is currently being
501 investigated in clinical trials against leukemia (44-47). Applying combined treatment with low
502 doses of both agents, we observed a dramatic cytotoxic impact, suggesting that dual antioxidant
503 targeting can achieve potent synergistic results.

504 The observed effects of NNT silencing on NCI-H295R steroidogenesis were surprising. In
505 the acute setting, i.e. NNT siRNA knockdown, we observed a generalized stimulation of
506 steroidogenesis, leading to increased glucocorticoid and adrenal androgen output by the cells. This
507 was corroborated by a significant up-regulation of a number of steroidogenic enzymes. This
508 response is contrary to what one might have anticipated considering mitochondrial NADPH is an
509 essential cofactor to the steroidogenic cytochrome P450 enzymes CYP11A1, CYP11B1 and
510 CYP11B2. **Elucidating the mechanisms that drive this transient effect will require additional**
511 **studies.** The few studies exploring the relationship between ROS and steroidogenesis (mostly on
512 testicular Leydig cell tumor cells) have reported a down-regulation of steroidogenic enzymes with
513 oxidative stress (14, 48-50). Human patients with inactivating NNT mutations (4) and a murine
514 *Nnt* deletion model (16) has been shown to have disrupted steroidogenesis; the data from our *in*
515 *vitro* NNT knockdown models suggest that NNT loss is not limiting for adrenal steroidogenesis.
516 Interestingly, Zhao et al. demonstrated a biphasic relationship between ROS and steroidogenesis,
517 indicating that the direction of the effect is dose-dependent (51) .

518 Taken together, we show that NNT silencing can induce cytotoxicity and impede cell

519 growth in adrenocortical carcinoma cells, as well as sensitize them to chemically-induced
520 oxidative stress. Moreover, we have demonstrated how the plasticity of ACC cells can lead to the
521 development of a compensatory molecular response with time and described how changes in
522 polyamine metabolism and ER protein processing are involved in this process (**Fig. 7**). **A limitation**
523 **of our work is that it is based on a single cell line; however, NCI-H295R remains the only**
524 **established, well characterized steroidogenic human ACC cell line.** These results merit further
525 exploration with *in vivo* studies to corroborate the effectiveness of mitochondrial anti-oxidant
526 pathway targeting and explore its durability, alone or in combination with other pro-oxidant agents.
527 The unique features of adrenocortical cells, with their high-volume ROS generation due to
528 steroidogenesis, make ACC a most amenable target to this approach.

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680 **Figures**

681 **Fig. 1: A, Interaction between NNT and the mitochondrial antioxidant pathways.** ETC: electron
682 transfer chain; ATP: adenosine triphosphate; GSR: glutathione reductase; GSSG: oxidized
683 glutathione; GSH: reduced glutathione; GPX1: Glutathione peroxidase 1; TXNRD2: thioredoxin
684 reductase 2; TXN: oxidized thioredoxin; TXN-SH: reduced thioredoxin; GPX3: peroxiredoxin 3;
685 O₂⁻: superoxide; H₂O₂: hydrogen peroxide, SOD2: Superoxide dismutase 2. **B, NNT expression**
686 **in patients with ACC (n=33), compared to patients with adrenocortical adenomas (n=22) and**
687 **healthy adrenals (n=10).** Gene expression was quantile-normalized and log-transformed as
688 described (13); bars represent median and interquartile range (IQR). Gene expression across
689 groups was compared applying the Kruskal-Wallis test, followed by post-hoc Bonferroni test.
690 **p<0.01.

691

692 **Fig. 2: Effects of transient (siRNA-mediated) NNT silencing on NCI-H295R cell redox balance,**
693 **respiration, proliferation and viability.** Bars represent mean ± SEM values, unless otherwise
694 stated. **A,** GSH/GSSG ratio in NCI-H295R cells transfected with KD siRNA (96 hours post-
695 transfection), normalized to the corresponding ratio of SCR siRNA-transfected cells. Significant
696 suppression of the GSH/GSSG ratio in KD siRNA cells suggests higher intracellular oxidative
697 stress. Bars represent median ± IQR values. *p<0.05; n=8 independent experiments. **B,**
698 Proliferation rates observed in siRNA-transfected NCI-H295R cells, 72-166 hours post-
699 transfection. ***p<0.001; n=14. **C,** Caspase 3/7 activity ratio in KD siRNA cells to SCR siRNA-
700 transfected cells, after standardization to cell numbers (120 hours post-transfection). *p<0.05;
701 n=8. **D,** Seahorse XF24 analysis of cellular oxygen consumption rate (OCR) at baseline and after
702 successive application of three mitochondrial respiration inhibitors (166 hours post-transfection).
703 Results were standardized to protein concentration. Bars represent median ± IQR values. FCCP:
704 Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone. A-mycin/Rot: Antimycin A plus Rotenone.
705 p>0.05; n=4. **E,** Extracellular acidification rate (ECAR), surrogate marker of anaerobic
706 glycolysis, standardized for protein concentration. p>0.05; n=4. **F,** Proliferation under low-dose
707 chemically induced oxidative stress (paraquat 10 μM) in KD siRNA and SCR siRNA-transfected
708 cells, normalized to corresponding cell proliferation without paraquat treatment. *p<0.05; n=6.

709

710 **Fig. 3: Effects of stable (shRNA-mediated) NNT silencing on NCI-H295R cell redox balance,**
711 **respiration, proliferation and viability.** Bars represent mean ± SEM values, unless otherwise
712 stated. **A,** GSH/GSSG ratio in NCI-H295R cells transfected with KD shRNA, normalized to the
713 corresponding ratio cells transfected with SCR shRNA. Bars represent median ± IQR values.
714 p>0.05, n=10. **B,** Proliferation rates over a 96-hour period. *p<0.05; n=13. **C,** Caspase 3/7
715 activity ratio in KD shRNA cells to SCR shRNA-transfected cells, after standardization to cell

716 numbers. $p > 0.05$; $n = 4$. **D**, Seahorse XF24 analysis of cellular oxygen consumption rate (OCR) at
717 baseline and after successive application of three mitochondrial respiration inhibitors. Results
718 were standardized to protein concentration. Bars represent median \pm IQR values. FCCP:
719 Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone. A-mycin/Rot: Antimycin A plus Rotenone.
720 $*p < 0.05$; $n = 7$. **E**, Extracellular acidification rate (ECAR) standardized for protein concentration.
721 $p > 0.05$; $n = 7$. **F**, Proliferation under chemically induced oxidative stress (paraquat) in KD shRNA
722 and SCR shRNA cells, normalized to corresponding cell proliferation without paraquat treatment.
723 $p > 0.05$; $n = 13$.

724

725 **Fig. 4: Effects of NNT silencing on NCI-H295R steroidogenesis, delineated by LC-MS/MS**
726 **steroid profiling in serum-free cell media and RNA sequencing.** Cortisol production (**A**) and
727 androstenedione production (**B**) over a 48-hour period in NCI-H295R cells transfected with siRNA
728 or shRNA. A significant stimulation of cortisol and androstenedione synthesis was observed 72-
729 120 hours post-transfection with KD siRNA. $**p < 0.01$, $*p < 0.05$; $n \geq 5$. **C-E**, specific enzyme
730 activity derived from product to substrate ratios for 11 β -hydroxylase (CYP11B1) (**C**), 21-
731 hydroxylase (CYP21A2) (**D**) and 17,20-lyase (CYP17A1) (**E**) in siRNA and shRNA-transfected
732 cells. $**p < 0.01$, $***p < 0.001$; $n \geq 5$. **F**, Heat-map representation of steroidogenic gene expression
733 changes induced by transient and stable NNT knockdown, as revealed by RNA sequencing. Scale
734 represents log₂fold changes in NNT knockdown cells compared to their respective (siRNA or
735 shRNA) scrambled controls. $*q < 0.05$, $**q < 0.01$; $n = 3$.

736

737 **Fig. 5: Effect of glutathione and thioredoxin pathway inhibition on NCI-H295R cell**
738 **proliferation.** **A**, 96-hour treatment with incremental doses of BSO (0-200 μ M), inhibitor of
739 glutathione synthesis. Control cells were treated with vehicle only. $*p < 0.05$, $n = 9$. **B**, 96-hour
740 treatment with incremental doses of auranofin (0-5 μ M), a thioredoxin reductase inhibitor. Control
741 cells were treated with vehicle only. Negative proliferation rates indicate net decrease in cell
742 number after 96 hours of treatment. $**p < 0.01$, $***p < 0.0001$; $n = 9$. **C**, Combined glutathione and
743 thioredoxin inhibition by use of low-dose BSO (50 μ M) and auranofin (0.2 μ M). $*p < 0.05$; $n = 3$.

744

745 **Fig. 6: Whole transcriptome sequencing and non-targeted metabolome analysis of KD siRNA,**
746 **SCR siRNA, KD shRNA and SCR shRNA-transfected NCI-H295R cells.** **A+B**, Whole
747 transcriptome sequencing in siRNA and shRNA-transfected NCI-H295R cells. **A**, Significantly
748 altered molecular pathways ($p < 0.01$, $q < 0.05$) between KD siRNA and SCR siRNA cells and
749 number of associated genes that are up/down-regulated in KD siRNA cells. **B**, Significantly altered
750 molecular pathways ($p < 0.01$) between KD shRNA and SCR shRNA cells and number of associated

751 *genes that are up/down-regulated in KD shRNA cells. C+D, Non-targeted metabolome analysis in*
752 *siRNA and shRNA-transfected NCI-H295R cells. C, Significantly up- and down-regulated*
753 *intracellular metabolites ($q < 0.01$) in KD siRNA-transfected cells, as compared to SCR siRNA-*
754 *transfected cells. D, Significantly up- and down-regulated intracellular metabolites ($p < 0.05$) in*
755 *KD shRNA-transfected cells, as compared to SCR shRNA-transfected cells (no metabolites with*
756 *$q < 0.05$ in this comparison).*

757

758 ***Fig. 7: Response of NCI-H295R cells to NNT silencing in the acute (transient knockdown) and***
759 ***chronic (stable knockdown) setting, with proposed redox adaptation mechanisms. Acute NNT***
760 ***knockdown induces oxidative stress as predicted by NNT's role as a major NADPH generator;***
761 ***enhanced steroidogenesis and polyamine catabolism further accentuate ROS accumulation,***
762 ***triggering apoptosis and a sharp decline in cell proliferation. With time (stable knockdown), cells***
763 ***manage to adapt removing damaged proteins and enhancing spermine synthesis as an alternative,***
764 ***NADPH-independent ROS scavenger. This restores redox homeostasis and abrogates the original***
765 ***pro-apoptotic effect, but cellular proliferation remains suppressed. Horizontal arrows represent***
766 ***paucity of change.***

