

1 **NNT is a key regulator of adrenal redox homeostasis and steroidogenesis in male**
2 **mice**

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29 **ABSTRACT**

30 **Introduction:** Nicotinamide nucleotide transhydrogenase, NNT, is a ubiquitous protein of the
31 inner mitochondrial membrane with a key role in mitochondrial redox balance. NNT produces
32 high concentrations of NADPH for detoxification of reactive oxygen species by glutathione
33 and thioredoxin pathways. In humans, NNT dysfunction leads to an adrenal specific
34 disorder, glucocorticoid deficiency. Certain sub-strains of C57BL/6 mice contain a
35 spontaneously occurring inactivating *Nnt* mutation and display glucocorticoid deficiency
36 along with glucose intolerance and reduced insulin secretion. To understand the underlying
37 mechanism(s) behind the glucocorticoid deficiency we performed comprehensive RNA-seq
38 on adrenals from wild-type (C57BL/6N), mutant (C57BL/6J) and BAC transgenic mice
39 overexpressing *Nnt* (C57BL/6J^{BAC}). **Results:** Our data suggests that *Nnt* deletion (or
40 overexpression) reduces adrenal steroidogenic output by decreasing expression of crucial,
41 mitochondrial antioxidant (*Prdx3* and *Txnrd2*) and steroidogenic (*Cyp11a1*) enzymes.
42 Pathway analysis also revealed upregulation of heat shock protein machinery and
43 haemoglobins possibly in response to the oxidative stress initiated by NNT ablation.

44 **Conclusion:** Using transcriptomic profiling in adrenals from three mouse models we showed
45 that disturbances in adrenal redox homeostasis are mediated not only by under expression
46 of NNT but also by its overexpression. Further we demonstrated that both under- or
47 overexpression of NNT reduced corticosterone output implying a central role for it in the
48 control of steroidogenesis. This is likely due to a reduction in the expression of a key
49 steroidogenic enzyme, *Cyp11a1*, which mirrored the reduction in corticosterone output.

50

51 **BACKGROUND**

52 Adrenal insufficiency is a rare, potentially fatal, endocrine disorder resulting from a failure of
53 the adrenal cortex to respond to hormonal stimuli. In Familial (or isolated) Glucocorticoid
54 Deficiency adrenal hormone output is preserved apart from a specific deficit of
55 glucocorticoids. Normally, under the control of hypothalamic Corticotropin Releasing

56 Hormone (CRH) and arginine vasopressin (AVP), the pituitary releases Adrenocorticotrophic
57 hormone (ACTH) which acts on the adrenal via the ACTH receptor (otherwise known as
58 *MC2R*) to produce glucocorticoids, mainly cortisol. This in turn acts on the hypothalamus
59 and pituitary to suppress further production of ACTH in a negative feedback loop [Figure 1A]
60 [Keller-Wood & Dalman 1984]. The human adult adrenal is characterised by three distinctive
61 cortical zones surrounding the medulla; the zona glomerulosa (ZG) where mineralocorticoids
62 are produced, the zona fasciculata (ZF) which synthesises glucocorticoids (mostly cortisol; in
63 mice, the major glucocorticoid is corticosterone) and the zona reticularis (ZR) where
64 androgen synthesis occurs [Vinson 2003]. The first step of steroidogenesis occurs once
65 cholesterol is transported from the outer to the inner mitochondrial membrane by the
66 steroidogenic acute regulatory protein (STAR) and is converted to pregnenolone by the
67 cholesterol side chain cleavage enzyme (CYP11A1). To make cortisol, pregnenolone then
68 undergoes a series of intermediate reactions catalysed by microsomal enzymes (CYP17A1,
69 HSD3B2 and CYP21A2) before the final step of cortisol production, catalysed by 11beta-
70 hydroxylase (CYP11B1) or aldosterone production, catalysed by CYP11B2, both occurring in
71 mitochondria, [Figure 1B] [Miller & Auchus 2011]. The activities of steroidogenic cytochrome
72 P450 (CYP) enzymes are reliant upon electron-donating redox partners; for mitochondrial (or
73 Type 1) enzymes, electrons are transferred from the reduced form of nicotinamide adenine
74 dinucleotide phosphate (NADPH) by ferredoxin reductase (FDXR) and ferredoxin (FDX1)
75 whereas the microsomal enzymes (Type 2) use P450 oxidoreductase (POR) as their redox
76 partner. Hence the first and last steps of cortisol production occur in the mitochondria and
77 require a constant supply of reductant NADPH. This NADPH is regenerated from NADP by a
78 few pathways including the thioredoxin and glutathione pathways, which are ultimately
79 enabled by NNT [Figure 2].

80 Perturbations in this pathway cause a number of steroidogenic defects affecting adrenal and
81 gonadal steroidogenesis. *STAR* mutations give rise to lipoid congenital adrenal hyperplasia
82 (OMIM 201710) a severe syndrome of adrenal and gonadal insufficiency resulting in XY sex
83 reversal, *CYP11A1* defects give a similar clinical picture but without the lipid build up in

84 steroidogenic tissues seen with *STAR* mutations. *HSD3B2*, *CYP17A1*, *CYP21A2*, *CYP11B1*,
85 and *POR* mutations give rise to four variants of congenital adrenal hyperplasia (OMIM
86 201810, 202110, 201910, 202010, and 201750 respectively) and *CYP11B2* mutations give
87 rise to hypoadosteronism (OMIM 203400) [Figure 1B] [Miller & Auchus 2011]. No mutations
88 have yet been described in humans in *FDXR/FDX1* – perhaps due to embryonic lethality.
89 Partial loss-of-function changes in *STAR* and *CYP11A1* can present with a less severe
90 phenotype akin to our disease of interest, Familial or isolated Glucocorticoid Deficiency
91 (FGD) [Baker et al., 2006; Metherell et al., 2009; Rubtsov et al., 2009; Sahakitrungruang et
92 al., 2010; Parajes et al., 2011; Sahakitrungruang et al., 2011]. In FGD, the two most
93 common gene defects are mutations in the melanocortin 2 receptor and its accessory protein
94 (*MC2R* and *MRAP*), but recently we have described defects in NADPH supply to and/or
95 antioxidant defence in mitochondria, with defects in two genes, *NNT* and thioredoxin
96 reductase 2 (*TXNRD2*), giving disorders of adrenal insufficiency primarily compromising
97 glucocorticoid production [Meimaridou et al., 2012; Meimaridou et al., 2013; Prasad et al.,
98 201]. *MC2R* and *MRAP* are adrenal zone and ACTH pathway specific so it is unsurprising
99 that they give rise to isolated glucocorticoid deficiency whereas *NNT* and *TXNRD2* are
100 ubiquitously expressed.

101 *NNT* is the major mitochondrial enzymatic source of NADPH contributing 45% of the total
102 NADPH supply [Nickel et al., 2015]. It exists as a dimer and spans the inner mitochondrial
103 membrane modulating H^+ movement and supplying the high concentrations of NADPH
104 required for the detoxification of ROS by glutathione and thioredoxin pathways [Figure 2].
105 Even though the gene is ubiquitously expressed, the organ specific physiological roles of
106 *NNT* are only gradually being revealed by the study of a C57BL/6J mouse substrain that has
107 a spontaneous mutation in *Nnt* (an in frame 5-exon deletion) resulting in the truncation of the
108 message and absence of the protein [Nickel et al., 2015]. The first consequence of this
109 murine *Nnt* deletion, described by Toye et al in 2005, was glucose intolerance and reduced
110 insulin secretion. Subsequent to the finding of human mutations causing FGD we showed
111 that 3-month-old mice had 50% lower basal and stimulated levels of corticosterone than their

112 wild-type counterparts. Histological examination of their adrenals revealed a slightly
113 disorganized zona fasciculata with higher levels of apoptosis than the wild-type C57BL/6N
114 strain [Meimaridou et al., 2012]. More recently it was reported that liver mitochondria from
115 C57BL/6J mice have major redox impairments resulting in an inability to maintain NADP and
116 glutathione in their reduced states [Ronchi et al., 2013].

117 Previously, we have shown that H295R cells where *Nnt* has been stably knocked down
118 undergo oxidative stress as demonstrated by low glutathione levels, and increased
119 mitochondrial superoxide production [Meimaridou et al., 2012]. Similar defects in energy
120 metabolism due to *Nnt* ablation have also been demonstrated in other mouse tissues (heart,
121 liver, pancreas) emphasising the importance of NNT for cellular bioenergetics [Sauer et al.,
122 2004; Ronchi et al., 2013; Nickel et al., 2015]. However, the mechanism by which loss of *Nnt*
123 causes the adrenal specific pathology we observe is unclear.

124

125 Here we aim to investigate the effect of NNT loss and overexpression in the adrenal cortex
126 by performing RNA-seq on adrenals from mice which are wild-type (C57BL/6N, *Nnt*^{+/+}), null
127 (C57BL/6J, *Nnt*^{-/-}), or 2-fold overexpressors (BAC transgenic, *Nnt*^{BAC}) of *Nnt* [Freeman et al.,
128 2006].

129

130 **MATERIALS AND METHODS**

131 **Mouse strains**

132 All mice were bred, housed and culled at MRC Harwell and therefore the husbandry was
133 identical for all 3 substrains. The mouse strains used were C57BL/6NHsd originally from
134 Harlan (Harlan Laboratories UK) which is wild-type for *Nnt* (*Nnt*^{+/+}), C57BL/6J originally from
135 Charles River (Charles River UK Ltd) which has an in-frame deletion of 5 *Nnt* exons (*Nnt*^{-/-})
136 and C57BL/6J mice carrying a BAC transgene to restore murine *Nnt* (*Nnt*^{BAC}; [Freeman et
137 al., 2006]) which we show are 2-fold overexpressors. For RNA-seq 18-month-old male mice
138 of the three different substrains were utilised, 5 mice per group. Mice were culled with an
139 overdose of Euthatal (to allow for the collection of blood) and tissues were then removed

140 quickly and either fixed or flash frozen in liquid nitrogen. All mice were culled between 10
141 and 11.30 am and adrenals removed. The animal protocols used in this study were
142 approved by United Kingdom Home Office.

143

144 **Genotyping**

145 Genomic DNA was extracted from the mouse tail tissue using a Qiagen DNeasy tissue kit.

146 Mice were genotyped for *Nnt* status using previously published primers [Huang et al., 2006].

147

148 **Mouse histology**

149 Mouse adrenals from *Nnt*^{+/+}, *Nnt*^{-/-} and *Nnt*^{BAC} were fixed in 4% paraformaldehyde (Sigma)
150 and embedded in paraffin. Sections were obtained using a microtome (Microm HM 325,
151 Thermo Fisher) at 6-µm thickness, Hematoxylin & Eosin (H&E) staining was performed
152 using standard procedures [Guasti et al., 2011].

153 To assess changes in lipid content between the three mice strain, we performed oil red O
154 staining as described previously
155 (www.ihcworld.com/protocols/special_stains/oil_red_o.htm). Briefly sections of fresh frozen
156 adrenal tissues were obtained at 5µm thickness and fixed in ice cold 10% formalin for 5
157 mins. Sections were air dried and placed in absolute propylene glycol for 5 mins to avoid
158 carrying water into Oil Red O. Sections were then stained with pre-warmed Oil Red O
159 solution for 8-10min at 60°C and then washed twice with distilled water. Images were
160 acquired using a Leica DMR microscope (Leica), and digital images were captured using a
161 Leica DC200 camera (Leica) and DCViewer software (Leica).

162

163 **Steroid profile**

164 Serum steroids were quantified using liquid chromatography-tandem mass spectrometry
165 (LC-MS/MS) as previously described [O'Reilly et al., 2014]. Steroids were extracted from

166 200µl of serum (after addition of internal standard) using 1ml tert-butyl methyl ether (MTBE).
167 After freezing at -20 °C for 1 hour, the MTBE layer was transferred into a 96-well plate and
168 evaporated under nitrogen at 55°C. Samples were reconstituted in 125 µl of a 50:50 solution
169 of Methanol (Sigma) and H₂O (Sigma). Steroids were analysed on a Waters Xevo with
170 Acquity uPLC, steroids were eluted from a HSS T3 1.8µm, 1.2x50mm column with a
171 methanol/water 0.1% formic acid gradient system. Two mass transitions were used to
172 identify and quantify each steroid (corticosterone: 347.2 > 121.2 and 347.2 > 97;
173 deoxycorticosterone: 331 > 97 and 331 > 109.

174

175 **Generation of stable NNT knockdown (KD) and scrambled (SCR) H295R cell lines**

176 Lentiviral plasmids (RHS4430-98851990; RHS4430-98913600; RHS4430-98524425;
177 RHS4430-101033169 RHS4430-101025114) were obtained from OpenBiosystems in a
178 p.GIPZ backbone and contained shRNA specific for human NNT (NM 012343) under the
179 control of the CMV promoter, plus the puromycin resistance and green fluorescence protein
180 (GFP) genes. HEK293T cells (packaging cells) were transiently transfected with the shRNA
181 plasmids, two days after transfection virus containing media was collected, filtered using a
182 0.22µm filter and used to transduce H295R cells. Four days after infection GFP-positive cells
183 were selected in 4µg/ml puromycin. Transduction efficiency was determined by fluorescence
184 microscopy. A scrambled (control) cell line was generated in a similar fashion using a non-
185 specific shRNA.

186

187 **NADP/NADPH assay**

188 To measure total and reduced nicotinamide adenine dinucleotide phosphates (NADP⁺ and
189 NADPH respectively) stably transfected H295R cells were plated onto white-walled and
190 white bottomed 96-well culture dishes (Corning Costar). After 24hrs, NADP⁺ and NADPH
191 were measured using NADP/NADPH-Glo assay (Promega), a luminescence based system
192 and according to the manufacturer's protocol. Luminescence was recorded after 15min using
193 Omega Luminometer (BMGLabTech) and with integration time of 0.5 sec.

194

195 Oxygen Consumption rate- XF Extracellular Flux Analyser

196 Scrambled (SCR) and stable knock-down H295R cells (NNT-KD) were cultured on Seahorse
197 XF-96 microplates and allowed to grow overnight. On the day of metabolic flux analysis,
198 cells were changed to unbuffered DMEM (DMEM base medium supplemented with 10 mM
199 glucose, 1 mM sodium pyruvate, 2 mM L-Glutamine, pH 7.4) and incubated at 37°C in a
200 non-CO₂ incubator for 1 h. All medium and injection reagents were adjusted to pH 7.4 on the
201 day of assay. Baseline measurements of oxygen consumption rate (OCAR, measured by
202 oxygen concentration change) and extracellular acidification rate (ECAR, measured by pH
203 change) were taken before sequential injection of treatments / inhibitors: oligomycin (ATP
204 synthase inhibitor, 4 µM), FCCP (mitochondrial respiration uncoupler, 1 µM), and rotenone
205 (Complex I inhibitor, 1 µM).

206

207 RNA-seq

208 RNA from mouse adrenal tissues (*Nnt*^{+/+}, *Nnt*^{-/-}, *Nnt*^{BAC}) was extracted using the RNeasy Mini
209 kit (Qiagen). Once RNA quality and concentration were tested samples, were processed by
210 Oxford Gene Technology (www.ogt.co.uk). Enrichment and library preparation were
211 performed using Illumina TruSeq RNA Sample Prep Kit v2; Total RNA was captured with
212 oligo-dT coated magnetic beads. The mRNA was fragmented and then first strand cDNA
213 synthesis was initiated from random primers, followed by second strand synthesis. After end-
214 repair, phosphorylation and A-tailing, adapter ligation and PCR amplification was performed
215 to prepare the library for sequencing. Paired-end sequencing was performed over 100 cycles
216 on the Illumina HiSeq2000 platform using TruSeq v3 chemistry. Fastq files were generated
217 from the sequencing platform via the manufacturer's proprietary software. For mapping and
218 alignment reads were processed through Tuxedo suite [Trapnell et al., 2013]. Reads were
219 mapped to their location on the appropriate Illumina iGenomes build using Bowtie and splice
220 junctions were identified using Tophat. Cufflinks was used to perform transcript assembly,
221 abundance estimation and differential expression and regulation for the samples. RNA-Seq

222 alignment metrics were generated using Picard.

223 **Lipid Peroxidation Assay.**

224 Lipid peroxidation in mouse adrenal lysates was assessed by using a lipid peroxidation
225 assay kit (AbCam, UK) based on the detection of malondialdehyde (MDA) in the samples.
226 Adrenal tissues from *Nnt^{+/+}*, *Nnt^{-/-}* and *Nnt^{BAC}* mice were excised and homogenised in MDA
227 lysis buffer provided in the kit. Lysates were then centrifuged at 13,000 x g for 10mins and
228 the supernatant collected for lipid peroxidation measurements. Samples were incubated with
229 thiobarbituric Acid (TBA) which interacts with MDA present in the samples to generate MDA-
230 TBA adducts. These adducts were quantified colorimetrically at 532 nm.

231

232 **Immunoblotting analysis**

233 Immunoblotting was used to assess protein expression. Cells were lysed in RIPA buffer
234 containing protease and phosphatase inhibitors (SIGMA) and then left on ice for 30mins.
235 Samples were centrifuged for 15mins at 13,000 rpm. Supernatant was collected and an
236 equal volume of Laemmli buffer was added. Samples were heated at 95-100°C for 5mins
237 and then loaded on 4-12% SDS gels. Protein separation was performed by using the
238 Invitrogen electrophoresis system. Proteins were then transferred to nitrocellulose
239 membrane (Sigma Aldrich) using semi-dry transfer blot (Biorad) at 15V for 1hr. Membranes
240 were probed with one of; mouse anti-Nnt (1:1000; SIGMA, HPA004829), mouse anti-actin
241 (1:5000; SIGMA, A5441), rabbit anti-TXNRD2 (1:1000;SIGMA, SAB2702064), rabbit anti-
242 PRDX3 (1:500; ProteinTech, 55087-1-AP), rabbit anti-GPX1 (1:500; Abcam ab108429),
243 mouse anti-STAR (1:1000;Abcam, ab58013), rabbit anti-CYP11A1 (1:1000;Cell Signalling,
244 14217), rabbit anti-HSD3B2 (1:500; Aviva Biosystems, OAGA02009), rabbit anti-CYP21A2
245 (1:500; SIGMA HPA053371). Visualisation of the proteins was performed by using Alexa-
246 fluor 680 and 800 secondary antibodies (1:5000; Invitrogen) and the Li-CoR Odyssey
247 system.

248

249

250 Statistics

251 Statistical analyses were performed using a combination of one-way Anova using *Tukey*
252 *HSD* (honest significant difference) test and a two-tailed student's *t*-tests assuming unequal
253 variance in 5 mice per group. All values were expressed as a mean \pm standard error of the
254 mean and p values <0.05 were considered significant.

255

256 RESULTS**257 Mouse phenotyping**

258 We have previously reported that 3-month-old mice carrying a spontaneous *Nnt* 5-exon
259 deletion (C57BL/6J, *Nnt*^{-/-}) have significantly lower levels of corticosterone (50%) than their
260 wild type counterparts (C57BL/6N, *Nnt*^{+/+}) [Meimaridou et al., 2012]. To identify the
261 mechanism by which loss of NNT affects steroidogenesis we employed the same mouse
262 models but also included an additional mouse line where the *Nnt* loss had been rescued in
263 C57BL/6J mice by transgenic expression of the entire murine *Nnt* gene contained within a
264 bacterial artificial chromosome (BAC transgenic, *Nnt*^{BAC}) [Freeman et al., 2006]

265

266 Glucocorticoid levels in the three mouse strains

267 The adrenal steroid output of the three mouse strains was measured at 18 months in murine
268 serum samples employing liquid chromatography-tandem mass spectrometry (LC-MS/MS).
269 Changes in steroid production were observed predominantly for corticosterone, with
270 reduction to 14% of wild-type levels in *Nnt*^{-/-} mice [Figure 3A]. Corticosterone levels in the
271 *Nnt*^{BAC} mice restored serum corticosterone in part, but only to 40% of wild-type (*Nnt*^{+/+})
272 levels, suggesting that overexpression of *Nnt* also perturbs steroidogenesis. 11-
273 deoxycorticosterone levels were not significantly different amongst the mice and therefore
274 the ratio of 11-deoxycorticosterone/corticosterone was significantly higher in *Nnt*^{-/-} and
275 *Nnt*^{BAC} mice indicating lower enzyme activity of *Cyp11b1* [Figure 3B]. We also noted that,

276 over time, the deficit in glucocorticoid output worsened for the *Nnt*^{-/-} mice, between 3 and 18
277 months corticosterone levels in *Nnt*^{+/+} were unaltered whereas there is a 60% decrease for
278 *Nnt*^{-/-} mice, suggesting progressive loss of function [Figure 3C].

279

280 **No changes to adrenal histology**

281 In humans with FGD there is a specific loss of glucocorticoid output from the zona
282 fasciculata and relative preservation of steroid output from the other zones suggesting loss
283 of this specific zone. Consistent with these findings, *Mc2r*^{-/-} mice have smaller adrenals with
284 preservation of zona glomerulosa and medulla but atrophied zona fasciculata [Chida et al.,
285 2007]. Interestingly all *Mc2r*^{-/-} mice on a pure C57BL/6J background die within two days of
286 birth whereas those on a mixed B6/Balbc have much higher survival rates, with half making
287 it to adulthood perhaps due to the restoration of *Nnt* levels [Chida et al., 2009], making *Nnt* a
288 genetic modifier of their adrenal phenotype akin to the situation in another mouse model
289 where NNT has a protective role in superoxide dismutase deficient mice [Huang et al.,
290 2006]. To determine whether loss of ZF occurs in *Nnt*^{-/-} mice we performed H&E staining in
291 adrenal sections from the three mouse substrains. This showed no morphological
292 differences among the strains in adrenal zonation [Figure 3D]. This was supported by the
293 finding that genes differentially expressed between zones were not altered (see below). This
294 suggested no major remodelling of the adrenals due to NNT loss and gave us confidence
295 that the RNA-seq variations were not due to changes in zonation. Furthermore, on Oil Red O
296 staining, we observed no differences in lipid levels between the mouse strains suggesting
297 there is neither a dearth of cholesterol supply for steroidogenesis nor a surplus due to a
298 cholesterol transport defect as seen with *Star* mutations [Sasaki et al., 2008] [Figure 3E].

299

300 ***Nnt* deletion causes oxidative stress in mouse adrenals.**

301 We measured lipid peroxidation (LPO) by a malondialdehyde (MDA) assay as a measure of
302 oxidative stress in these mice (5 mice per group). There was a significant increase in LPO in
303 the adrenals of *Nnt*^{-/-} mice which returned towards wild-type levels in *Nnt*^{BAC} mice indicating

304 lipid damage by free radicals in adrenals upon *Nnt* deletion [Figure 4A]. We saw the same
305 trend in H₂O₂ levels (increased in *Nnt*^{-/-} and reduced again in *Nnt*^{BAC}) [Data not shown].
306 Impaired redox homeostasis caused by lack of *NNT* was further supported by *in vitro* studies
307 in human adrenocortical H295R cells. The total cellular NADP/NADPH ratio was significantly
308 higher in H295R with lentiviral knockdown of *NNT* (NNT-KD) compared to scrambled control
309 cells (SCR), suggesting that *NNT* is required to maintain the redox state of the intracellular
310 NADPH and NADP⁺ pools [Figure 4B].
311 The perturbation in NADP/NADPH balance affected mitochondrial respiration resulting in
312 significantly lower oxygen consumption rates (OCAR) in cells where *NNT* is knocked down
313 [Figure 4C].

314

315 **Transcriptome profiling by RNA-seq in adrenals from the C57BL/6 mouse substrains**

316 To investigate the cause of the reduced steroid production upon *Nnt* deletion, RNA was
317 extracted from *Nnt*^{+/+}, *Nnt*^{-/-}, and *Nnt*^{BAC} mouse adrenals and the transcriptome profiled by
318 RNA-seq. Using an Illumina HiSeq 2000 sequencer we obtained an average of 30 million
319 reads per sample, with ~98% of these reads mapped to the mouse reference genome. In
320 total, 27,622 genes were analysed in *Nnt*^{+/+}, *Nnt*^{-/-} and *Nnt*^{BAC} adrenals [Figure 5A &
321 Supplementary Table 1]. The most highly expressed categories of genes were
322 mitochondrially encoded electron transport chain genes (*mt-Co1*, *mt-Co2*, *mt-Atp6*, *mt-Co3*,
323 *mt-Cytb*, *mt-Nd4*, *mt-Nd2*, *mt-Nd1*, *mt-Atp8*, *mt-Nd6*, *mt-Nd5*, *mt-Nd4l*, *mt-Nd3*, *mt-Rnr1*),
324 followed by steroid pathway genes *Star*, *Cyp21a1* and *Hsd3b1*, which are high up in the
325 steroidogenic cascade [Supplementary Table 1] perhaps reflecting the high mitochondrial
326 content of and high demand for steroidogenesis in the adrenal.

327 Initial data analysis was performed between paired samples comparing *Nnt*^{+/+} and *Nnt*^{-/-},
328 *Nnt*^{+/+} and *Nnt*^{BAC} and *Nnt*^{-/-} and *Nnt*^{BAC} adrenals. To identify differentially expressed genes
329 from each group we used the following criteria; (1) gene expression level greater than or
330 equal to 1 read per kilobase of exon per million fragments mapped (RPKM) in all samples;
331 (2) change in expression level greater than or equal to 1.5-fold; and (3) significance p value

332 < 0.05. This revealed differential expression (fold change ≥ 1.5 ; $p < 0.05$) of 400 genes in total
333 in the pairwise comparisons [Figures 5B, 5C and Supplementary Table 2]. Only 1 gene
334 varied between all three pairwise analyses and that was *Nnt* itself (see below and Figure
335 5C). We hypothesized that genes that were up- or down-regulated in *Nnt*^{-/-} and their levels
336 restored in *Nnt*^{BAC} would be genes that were modulated by NNT.

337

338 **NNT levels in the three mouse strains**

339 *Nnt* expression levels in the three mouse strains were determined and while we observed
340 very low mRNA levels in *Nnt*^{-/-} mice (3.7-fold downregulation $p = 0.012$), there was a 2.7-fold
341 ($p = 1.8 \times 10^{-5}$) increase in *Nnt* expression in *Nnt*^{BAC} mice over that for *Nnt*^{+/+} [Figure 6A]. To
342 investigate whether mRNA levels corresponded to protein expression we performed a
343 western blot on adrenal lysates from the same mice. NNT was undetectable in the *Nnt*^{-/-}
344 mice whereas a two-fold increase in NNT was observed in the adrenals of *Nnt*^{BAC} mice, in
345 keeping with the RNA-seq data [Figure 6B]. These results suggest that the *Nnt*^{BAC}
346 represents a modest *Nnt* overexpressor.

347

348 **No differential expression of other genes with polymorphisms identified between sub-** 349 **strains**

350 Recently, comparative genomics between C57BL/6J and C57BL/6N strains has identified
351 many SNPs and structural variants that may contribute to the phenotypic differences
352 between the two strains [Simon et al., 2013]. To check whether these SNPs altered
353 expression of these genes in the adrenal we specifically looked at their mRNA expression
354 levels. Except for *Cilp*, which was up in *Nnt*^{BAC} (1.6 fold over *Nnt*^{+/+} [$p = 0.013$]) but unaltered
355 between *Nnt*^{+/+} and *Nnt*^{-/-}, we found no alterations in expression levels of the genes with
356 interstrain variations, suggesting that the effects we describe are largely due to differential
357 *Nnt* levels.

358

359

360 **No differential expression of zone specific genes between sub-strains**

361 Many genes show differential expression between the zones of the adrenal, classically for
362 example tyrosine hydroxylase (*Th*), aldosterone synthase (*Cyp11b2*), 3-beta-hydroxysteroid
363 dehydrogenase (*Hsd3b2*), cytochrome b5 (*Cyb5*) and aldo-keto reductase family 1 member
364 C3 (*Akr1c3*). More recently transcriptomic analyses have identified hundreds of other genes
365 with differential expression between ZF and ZG [Nishimoto et al., 2012; Rege et al., 2014].
366 None of these genes showed differential expression in our study suggesting no major
367 remodelling of adrenals glands in *Nnt*^{-/-} or *Nnt*^{BAC} mice.

368

369 ***Nnt* deletion does not alter the levels of ACTH receptor pathway genes or other genes**
370 **associated with adrenal insufficiency**

371 *Mc2r* expression was unaltered between *Nnt*^{+/+} vs *Nnt*^{-/-} but up in *Nnt*^{BAC} vs *Nnt*^{-/-} (1.6-fold
372 p=0.023) whereas *Mrap* levels were the same across the 3 groups (data not shown). No
373 significant expression level changes were observed at RNA level for other genes causing
374 adrenal insufficiencies in humans (*Aaas*, *Abcd1*, *Aire*, *Cdkn1c*, *Cyp11a1*, *Cyp11b1*,
375 *Cyp17a1*, *Cyp21a1*, *Mcm4*, *Nr5a1*, *Por* and *Txnrd2*).

376

377 ***Nnt* deletion alters antioxidant gene levels**

378 *Nnt* provides a constant supply of NADPH required for ROS detoxification by the thioredoxin
379 and glutathione systems. Malic enzyme 3 (*Me3*) and isocitrate dehydrogenase 2 (*Idh2*) are
380 alternate NADPH suppliers although NNT is the major contributor [14]. To investigate
381 whether *Nnt* deletion leads to perturbation of other antioxidant enzymes perhaps to
382 compensate for its loss, we looked at the mRNA and protein levels of antioxidant enzymes in
383 these pathways. There was a modest reduction in gene expression levels of the antioxidants
384 *Prdx3* and *Txnrd2* which did not reach statistical significance, however at the protein level
385 they were significantly reduced in *Nnt*^{-/-} mice vs *Nnt*^{+/+} and the levels remained significantly
386 low in *Nnt*^{BAC} when compared to *Nnt*^{+/+} [Figure 6C, 6D]. This is likely because *Nnt*^{BAC}

387 overexpress NNT which may also cause redox imbalance, implying fine tuning of NNT is
388 required for redox homeostasis. We observed no compensatory increase in the alternative
389 NADPH suppliers *Me3* or *ldh2*.

390

391 ***Nnt* deletion alters mitochondrial cytochrome P450scc levels**

392 STAR is a protein involved in the transport of cholesterol from the outer to the inner
393 mitochondrial membrane and specific partial loss-of-function mutations in *STAR* account for
394 10% of FGD cases [Meimaridou et al., 2013]. Similarly, “mild” mutations in the cholesterol
395 side chain cleavage enzyme (*CYP11A1*, the first enzyme in the steroid pathway), can give
396 rise to FGD [Rubtsov et al., 2009; Parajes et al., 2011; Sahakitrungruang et al., 2011].
397 Mutations in 11 β -hydroxylase (*CYP11B1*, the last enzyme in the glucocorticoid pathway)
398 cause congenital adrenal hyperplasia. Previous work revealed that oxidative stress, resulting
399 from the application of exogenous ROS, lead to inhibition of STAR protein expression and
400 steroidogenesis in MA-10 Leydig cells, with no effect on *CYP11A1* [Diemer et al., 2003]. We
401 hypothesized that a similar phenomenon might occur with endogenous mitochondrial
402 oxidative stress resulting from NNT deficit affecting *Star* and/or the mitochondrial CYP450
403 enzymes, *Cyp11a1* and *Cyp11b1/b2*. RNA-seq and western blot analysis showed no
404 significant changes in the expression of *Star* at mRNA or protein level, indicating that a
405 defect in cholesterol transport due to oxidative insult is an unlikely mechanism [Figure 7A,
406 7C]. However, a suggestive 25% decrease in *Cyp11a1*, *Cyp11b1* and *Cyp11b2* mRNA
407 levels in *Nnt*^{-/-} mice with incomplete recovery in *Nnt*^{BAC} suggested a possible mechanism for
408 steroid depletion. In agreement with this we observed a 65% reduction in *CYP11A1* at
409 protein level in *Nnt*^{-/-} and partial restoration (to approx. 50% of *Nnt*^{+/+} levels) in *Nnt*^{BAC} [Figure
410 7A, 7C]. We were unable to assess *CYP11B1/B2* levels since no specific murine antibody
411 exists. The failure to completely recover *CYP11A1* levels in the *Nnt*^{BAC} mice may be due to
412 redox imbalance in these overexpressing mice and, significantly, protein abundance mirrors
413 the levels of corticosterone in the three mouse substrains [Figures 3A, 7A]. This is
414 analogous to partial loss-of-function mutations in *CYP11A1*, which give rise to adrenal

415 insufficiency in humans; the proteins may retain 30-40% of wild-type activity this is
416 insufficient to maintain normal cortisol production [Parajes et al., 2011].

417 In contrast the intermediate steps of steroidogenesis occur in the ER, once pregnenolone is
418 synthesized it undergoes reactions catalysed by 3 β -hydroxysteroid dehydrogenase (*Hsd3b2*)
419 and 21-hydroxylase (*Cyp21a1*) to produce progesterone and deoxycorticosterone
420 respectively [Figure 7B]. No significant changes were observed at mRNA or protein level in
421 these enzymes between *Nnt*^{-/-} vs *Nnt*^{+/+} mice, suggesting that mitochondrial ROS does not
422 affect them and that a reduction of these enzymes is not the reason for their corticosterone
423 deficiency [Figure 7A, 7C].

424

425 **Transcriptomics- differentially expressed genes**

426 In total 400 genes were differentially expressed in pairwise analyses between the mouse
427 substrains [Supplementary Table 2]. Differentially expressed genes between *Nnt*^{+/+} vs *Nnt*^{-/-}
428 numbered 187 (89 up and 98 down-regulated), between *Nnt*^{-/-} vs *Nnt*^{BAC} numbered 157 (130
429 up- and 37 down-regulated) and between *Nnt*^{+/+} vs *Nnt*^{BAC} numbered 141 (119 up- and 22
430 down-regulated) [Supplementary Tables 3 - 8].

431 We hypothesized that genes with altered expression in *Nnt*^{-/-} that reverted to wild-type levels
432 in the *Nnt*^{BAC} would be genes that were modulated by *Nnt* levels. 40 genes including *Nnt*
433 were altered in *Nnt*^{-/-} with their expression levels rescued in *Nnt*^{BAC}. 23 of these were
434 downregulated in *Nnt*^{-/-} and back up in *Nnt*^{BAC}, whilst 17 were upregulated in *Nnt*^{-/-} and back
435 down in *Nnt*^{BAC} [Table 1]. Two groups of genes were enriched in this list; chaperones and
436 haemoglobins. Specifically, there was a 25-fold increase in *Hspa1a* and *Hspa1b* in *Nnt*^{-/-}
437 mice, and these levels were restored to *Nnt*^{+/+} levels in the *Nnt*^{BAC} mice. A smaller, but still
438 significant, increase was observed in another heat shock (*Hspb1*) and a co-chaperone
439 (*Dnajb1*) in *Nnt*^{-/-} mice. [Figure 8A].

440 The upregulation of heat shock proteins suggests that proteins are undergoing damage due
441 to increased ROS and the molecular chaperone machinery is activated to correct or degrade
442 such damaged or misfolded proteins. Interestingly alpha- and beta-haemoglobins (*Hba-a1*,

443 *Hba-a2*, *Hbb-b1* and *Hbb-b2* [aka *Hbb-bs* and *Hbb-bt*] were 4-5-fold upregulated in *Nnt*^{-/-}
444 mice (p<0.025) and their levels returned to normal in *Nnt*^{BAC} [Figure 8B]. Erythroid
445 contamination of the tissues from *Nnt*^{-/-} mice was considered but ruled out as other genes
446 highly expressed in the development of erythroid lineages were not significantly upregulated
447 (51 genes from An et al. 2014). Recently the expression of haemoglobins in tissues other
448 than erythrocytes has been reported suggesting their role in other basic cellular functions
449 apart from O₂ transport [Fordel et al., 2006; Vinogradov & Moens 2008].

450

451 **DISCUSSION**

452 We have previously shown that mutations in NNT cause adrenal dysfunction in humans
453 primarily affecting the zona fasciculata cells of the adrenal cortex responsible for cortisol
454 production, and observed a 50% reduction in corticosterone levels in 3-month-old *Nnt* null
455 mice [Meimaridou et al., 2012]. Further we showed mitochondrial perturbations and limited
456 antioxidant capacity in human adrenocortical carcinoma cells where NNT expression was
457 stably knocked-down [Meimaridou et al., 2012]. In this study, we investigated the mechanism
458 by which NNT affects steroidogenesis in older mice by utilising three models with differing
459 expression levels of *Nnt*; wild-type *Nnt*^{+/+}, null *Nnt*^{-/-} and two-fold overexpressing *Nnt*^{BAC} mice.
460 Gene expression and western blotting analysis revealed restricted levels of key
461 mitochondrial antioxidant and steroidogenic proteins in *Nnt*^{-/-} mice leading to glucocorticoid
462 deficiency which was partially rescued in the over-expressing mice. Interestingly we
463 demonstrate for the first time that overexpression of *Nnt* also negatively impacts
464 steroidogenesis, this may be due to a persistent redox imbalance initiated by the oversupply
465 of NADPH by NNT.

466

467 The mouse inbred C57BL/6 strain is widely used for genetic and functional studies. There
468 are two substrains of these mice depending on their site of origin; C57BL/6J established in
469 Jackson laboratory and C57BL/6N line from the National Institutes of Health (NIH). In 2005 a
470 spontaneous loss-of-function *Nnt* mutation in C57BL/6J was characterised which was

471 associated with impaired glucose tolerance [Ronchi et al., 2016]. Since then, these mice
472 have been used to clarify the roles of NNT in mammalian biology.

473 More recently, comparative genomics between C57BL/6J and C57BL/6N strains has
474 identified many SNPs and structural variants that may contribute to the phenotypic
475 differences between the two strains [24]. We compared expression levels of the genes noted
476 in this publication but revealed no differences in mRNA levels exception for *Nnt*. This
477 suggests that the transcriptome changes and the endocrine phenotype observed in *Nnt*^{-/-}
478 mice is largely due to differential NNT levels.

479

480 In this study, we have employed, in addition, a mouse strain with transgenic rescue of *Nnt*
481 expression (*Nnt*^{BAC}) [Freeman et al., 2006]. The *Nnt* replacement has previously been shown
482 to induce improvements in glucose tolerance and insulin secretion rescuing the phenotype
483 seen in *Nnt*^{-/-} mice. Corticosterone levels recovered somewhat in the *Nnt*^{BAC}; however, they
484 remained significantly lower than wild-type levels.

485

486 In this *in vivo* model, we also showed that the antioxidant capacity of the *Nnt*^{-/-} adrenals is
487 significantly compromised when compared to *Nnt*^{+/+} counterparts. The protein levels of key
488 mitochondrial antioxidant enzymes PRDX3 and TXNRD2 are significantly reduced in *Nnt*^{-/-}
489 mice and fail to recover in *Nnt*^{BAC}. This strongly suggests that a set level of *Nnt* expression is
490 required to maintain mitochondrial redox homeostasis. Furthermore, since mitochondrial
491 NADPH can be regenerated not only by *Nnt*, but also by isocitrate dehydrogenase 2 (*Idh2*)
492 and malic enzyme 3 (*Me3*) we excluded a possible compensatory mechanism, as
493 expression levels of these enzymes remain unchanged between *Nnt*^{+/+} and *Nnt*^{-/-} mice.
494 Studies by other groups have similarly demonstrated that liver, heart, brain, and skeletal
495 muscle mitochondria from *Nnt*^{-/-} mice have unaltered *Idh2* and *Me3* enzymatic activities
496 meaning they cannot compensate for the loss of NNT to restore NADPH levels [Nickel et al.,
497 2015, Ronchi et al., 2016]. When *NNT* is ablated in human adrenocortical cells we also see
498 a disturbance of redox balance but here this does not affect cortisol output, perhaps due to

499 adaptive alterations in sulfiredoxin and peroxiredoxin III levels which are known to occur in
500 H295R cells, and even primary adrenocortical cells, when grown in culture (Kil et al. 2013).
501 NNT dysregulation not only affects the antioxidant capacity of the adrenal but also its
502 steroidogenic capacity. We demonstrated that, in *Nnt*^{-/-} mice, adrenal steroidogenesis is
503 severely affected (86% reduction in corticosterone in 18m old mice), due to the low protein
504 levels of a crucial steroidogenic enzyme, CYP11A1. Interestingly, *Nnt*^{BAC} mice also exhibit
505 glucocorticoid deficiency as indicated by 60% reduction in levels of corticosterone
506 suggesting that *Nnt* overexpression also impacts on the steroidogenic output of these mice.
507 It is increasingly recognised that redox balance is key to physiological health. Where one
508 might assume that underexpression of antioxidants would lead to oxidative stress and
509 overexpression would give reductive stress this is not necessarily what occurs *in vivo*
510 (reviewed in [Lei et al., 2016]), with clear examples of antioxidant gene knockdown inducing
511 reductive stress [Yan et al., 2017] and antioxidant gene overexpression also causing
512 reductive stress [Zhang et al., 2010]. In addition, paradoxically, both reductive and oxidative
513 insult can lead to overproduction of ROS [Barrett et al., 2004; Arrigo et al., 2005; Filomeni et
514 al., 2005; Ali et al., 2014; Yu et al., 2014; Korge et al., 2015] which can cause protein
515 damage. We suggest that this may be the explanation for the failure to rescue glucocorticoid
516 secretion in the overexpressing mice with the demonstration of higher levels of lipid
517 peroxidation in the *Nnt*^{BAC} mice compared to *Nnt*^{+/+} mice lending support to this.

518

519 Differential gene expression studies between *Nnt*^{+/+} and *Nnt*^{-/-} revealed a significant
520 upregulation of heat shock proteins in *Nnt*^{-/-} mice. The failure of ROS detoxification may lead
521 to oxidative damage of proteins and the canonical chaperone machinery will be upregulated
522 to cope with the resultant protein misfolding and degradation. The increased ROS will also
523 render cells more susceptible to apoptosis but heat shock proteins 27 and 70 (Hsp27 and
524 Hsp70 respectively) are activated by mitochondrial ROS and are protective of cells
525 preventing apoptosis by replenishing reduced glutathione and reducing intracellular iron
526 levels [Barrett et al., 2004; Arrigo et al., 2005; Filomeni et al., 2005].

527 In addition to heat shock protein machinery, we show haemoglobins are regulated by NNT
528 levels. There is a significant increase in the mRNA levels of haemoglobins in *Nnt*^{-/-} mice
529 possibly as a compensatory mechanism to combat oxidative stress. Haemoglobins are
530 composed of alpha and beta HbA chains and their accepted main function is to transport O₂
531 to cells thorough the vascular network. However, their involvement in other fundamental
532 cellular functions and in non-erythroid cells is increasingly being recognised. Detection of
533 haemoglobin chains in macrophages, alveolar cells, kidney, brain and vaginal epithelial cells
534 has been reported and their function has been linked with antioxidant defence and the
535 regulation of mitochondrial activity [Liu et al., 1999; Arrigo et al., 2005; Newton et al., 2006;
536 Nishi et al., 2008, Saha et al. 2017]. In adrenal, overexpression of α -Hb in rat
537 pheochromocytoma (PC12) cells resulted in downregulation of *Gpx1* and *Sod1* mRNAs
538 suggesting that it may have a role in the scavenging of ROS [Biagioli et al., 2009; Maria et
539 al., 2012]. Whether a similar mechanism explains the upregulation we see in intact adrenals
540 requires further investigation.

541 In this study, we showed that the reduced steroidogenic capacity of the adrenals in *Nnt*^{-/-} and
542 *Nnt*^{BAC} mice is due to the inability of other antioxidant enzymes to compensate for redox
543 imbalance resulting from altered *Nnt* levels. This leads to limited availability of mitochondrial
544 CYP11A1 and a reduction in corticosterone output. Recently a similar mechanism was
545 demonstrated to underlie the adrenal dysfunction seen in Triple A, a disorder of adrenal
546 insufficiency, alacrima and achalasia due to mutations in *AAAS* encoding the protein
547 ALADIN. A deficiency of ALADIN results in cytosolic, as opposed to mitochondrial, oxidative
548 stress and a deficit of microsomal, rather than mitochondrial, CYP450 enzymes thereby
549 retarding adrenal steroidogenesis [Juhlen et al., 2015].

550

551

552

553

554 **CONCLUSIONS**

555 Using transcriptomic profiling in adrenals from three mouse substrains we showed that
556 disturbances in adrenal redox homeostasis are mediated not only by under expression of
557 NNT but also by its overexpression. Further we demonstrated that both under- or
558 overexpression of NNT reduces corticosterone output implying a central role for it in the
559 control of steroidogenesis. Reduced expression of CYP11A1, a key mitochondrial
560 steroidogenic enzyme, mirrored the reduction in corticosterone output. Our data also
561 suggests that oxidative stress and/or ROS damage to proteins is activating mito- and
562 cytoprotective proteins (haemoglobins and heat shock proteins respectively) that may help
563 maintain cell viability but do not rescue the steroidogenic phenotype.

564

565 **DECLARATIONS**

566 **Competing Interests**

567 The authors declare that they have no competing interests.

568

569 **Author Contributions**

570 EM, LAM designed the study and analysed the RNA-seq data. EM and FE performed the
571 immunoblotting and mouse histology. MG and RC generated and validated mouse
572 substrains. VC, PAF and WA performed the steroidogenic profiling of mice and analysed the
573 mass spectrometric data. EM, LAM prepared the draft manuscript. All authors contributed to
574 the discussion of results and edited and approved the final manuscript.

575

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580 **REFERENCES:**

- 581 Ali ZA, de Jesus Perez V, Yuan K, Orcholski M, Pan S, Qi W, Chopra G, Adams C,
582 Kojima Y, Leeper NJ, et al., 2014 Oxido-reductive regulation of vascular remodeling by
583 receptor tyrosine kinase ROS1. *J Clin Invest.* 124(12):5159-74.
- 584 An X, Schulz VP, Li J, Wu K, Liu J, Xue F, Hu J, Mohandas N, Gallagher PG. 2014
585 Global transcriptome analyses of human and murine terminal erythroid differentiation.
586 *Blood.* 123(22):3466-77.
- 587 Arrigo AP, Viot S, Chaufour S, Firdaus W, Kretz-Remy C, Diaz-Latoud C 2005 Hsp27
588 consolidates intracellular redox homeostasis by upholding glutathione in its reduced form
589 and by decreasing iron intracellular levels. *Antioxid Redox Signal.* 7(3-4):414-22.
- 590 Baker B, Lin L, Kim C, Raza J, Smith C, Miller W, Achermann J. 2006 Nonclassic
591 congenital lipid adrenal hyperplasia: A new disorder of the steroidogenic acute
592 regulatory protein with very late presentation and normal male genitalia. *J Clin*
593 *Endocrinol Metab* 91:4781-4785.
- 594 Barrett MJ, Alones V, Wang KX, Phan L, Swerdlow RH. 2004 Mitochondria-derived
595 oxidative stress induces a heat shock protein response. *J Neurosci Res.* 78(3):420-9.
- 596 Biagioli M, Pinto M, Cesselli D, Zaninello M, Lazarevic D, Roncaglia P, Simone R,
597 Vlachouli C, Plessy C, Bertin N et al., 2009 Unexpected expression of alpha- and beta-
598 globin in mesencephalic dopaminergic neurons and glial cells. *Proc Natl Acad Sci U S A*
599 106(36):15454-9
- 600 Chida D, Nakagawa S, Nagai S, Sagara H, Katsumata H, Imaki T, Suzuki H, Mitani F,
601 Ogishima T, Shimizu C et al., 2007 Melanocortin 2 receptor is required for adrenal gland
602 development, steroidogenesis, and neonatal gluconeogenesis. *Proc Natl Acad Sci U S*
603 *A.* 104:18205-10.
- 604 Chida D, Sato T, Sato Y, Kubo M, Yoda T, Suzuki H, Iwakura Y 2009 Characterization of
605 mice deficient in melanocortin 2 receptor on a B6/Balbc mix background. *Mol Cell*
606 *Endocrinol.* 300:32-6.

- 607 Diemer T, Allen JA, Hales KH, Hales DB 2003 Reactive oxygen disrupts mitochondria in
608 MA-10 tumor Leydig cells and inhibits steroidogenic acute regulatory (StAR) protein and
609 steroidogenesis. *Endocrinology* 144(7):2882-91
- 610 Filomeni G, Aquilano K, Rotilio G, Ciriolo MR 2005 Antiapoptotic response to induced
611 GSH depletion: involvement of heat shock proteins and NF-kappaB activation. *Antioxid*
612 *Redox Signal.* 7(3-4):446-55.
- 613 Fordel E, Thijs L, Martinet W, Lenjou M, Laufs T, Van Bockstaele D, Moens L, Dewilde S
614 2006 Neuroglobin and cytoglobin overexpression protects human SH-SY5Y
615 neuroblastoma cells against oxidative stress-induced cell death. *Neurosci Lett.*
616 20;410(2):146-51;
- 617 Freeman HC, Hugill A, Dear NT, Ashcroft FM, Cox RD 2006 Deletion of nicotinamide
618 nucleotide transhydrogenase: a new quantitative trait locus accounting for glucose
619 intolerance in C57BL/6J mice. *Diabetes* 55(7):2153-6.
- 620 Guasti L, Paul A, Laufer E, King P 2011 Localization of Sonic hedgehog secreting and
621 receiving cells in the developing and adult rat adrenal cortex. *Mol Cell Endocrinol.*
622 336(1-2):117-122.
- 623 Huang TT, Naeemuddin M, Elchuri S, Yamaguchi M, Kozy HM, Carlson EJ, Epstein CJ
624 2006 Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide
625 dismutase. *Hum Mol Genet.* 15:1187-94.
- 626 Jühlen R, Idkowiak J, Taylor AE, Kind B, Arlt W, Huebner A, Koehler K 2015 Role of
627 ALADIN in human adrenocortical cells for oxidative stress response and steroidogenesis.
628 *PLoS One.* Apr 13;10(4):e0124582.
- 629 Keller-Wood ME., Dallman MF 1984 Corticosteroid inhibition of ACTH secretion. *Endocr*
630 *Rev.* 5:1-24.
- 631 Kil IS, Bae SH, Rhee SG 2013 Study of the signaling function of sulfiredoxin and
632 peroxiredoxin III in isolated adrenal gland: unsuitability of clonal and primary
633 adrenocortical cells. *Methods Enzymol.* 527:169-81.

- 634 Korge P, Calmettes G, Weiss JN 2015 Increased reactive oxygen species production
635 during reductive stress: The roles of mitochondrial glutathione and thioredoxin
636 reductases. *Biochim Biophys Acta*. 1847(6-7):514-25.
- 637 Lei XG, Zhu JH, Cheng WH, Bao Y, Ho YS, Reddi AR, Holmgren A, Arnér ES 2016
638 Paradoxical Roles of Antioxidant Enzymes: Basic Mechanisms and Health Implications.
639 *Physiol Rev*. 96(1):307-64.
- 640 Liu L, Zeng M, Stamler JS 1999 Hemoglobin induction in mouse macrophages. *Proc Natl*
641 *Acad Sci U S A* 96(12):6643-7
- 642 María T. Marcos-Almaraz, José A. Rodríguez-Gómez, José López-Barneo, Alberto
643 Pascual 2012 α -Haemoglobin regulates sympathoadrenal cell metabolism to maintain a
644 catecholaminergic phenotype. *Biochemical Journal* 441 (3) 843-852.
- 645 Meimaridou E, Hughes CR, Kowalczyk J, Chan LF, Clark AJ, Metherell LA 2013 ACTH
646 resistance: genes and mechanisms. *Endocr Dev* 24:57-66.
- 647 Meimaridou E, Kowalczyk J, Guasti L, Hughes CR, Wagner F, Frommolt P, Nürnberg P,
648 Mann NP, Banerjee R, Saka HN et al., 2012 Mutations in NNT encoding nicotinamide
649 nucleotide transhydrogenase cause familial glucocorticoid deficiency. *Nat Genet*
650 44(7):740-2.
- 651 Metherell LA, Naville D, Halaby G, Begeot M, Huebner A, Nürnberg G, Nürnberg P,
652 Green J, Tomlinson JW, Krone NP et al., 2009 Nonclassic lipoid congenital adrenal
653 hyperplasia masquerading as familial glucocorticoid deficiency. *J Clin Endocrinol Metab*
654 94(10):3865-71.
- 655 Miller WL, Auchus RJ 2011 The molecular biology, biochemistry, and physiology of
656 human steroidogenesis and its disorders. *Endocr Rev*. Feb;32(1):81-151.
- 657 Newton DA, Rao KM, Dluhy RA, Baatz JE 2006 Hemoglobin is expressed by alveolar
658 epithelial cells. *J Biol Chem* 281(9):5668-76.
- 659 Nickel AG, von Hardenberg A, Hohl M, Löffler JR, Kohlhaas M, Becker J, Reil JC,
660 Kazakov A, Bonnekoh J, Stadelmaier M, et al., 2015 Reversal of Mitochondrial

661 Transhydrogenase Causes Oxidative Stress in Heart Failure. *Cell Metabolism* 22(3):472-
662 84.

663 Nishi H, Inagi R, Kato H, Tanemoto M, Kojima I, Son D, Fujita T, Nangaku M 2008
664 Hemoglobin is expressed by mesangial cells and reduces oxidant stress. *J Am Soc*
665 *Nephrol* (8):1500-8

666 Nishimoto K, Rigsby CS, Wang T, Mukai K, Gomez-Sanchez CE, Rainey WE, Seki T
667 2012 Transcriptome analysis reveals differentially expressed transcripts in rat adrenal
668 zona glomerulosa and zona fasciculata. *Endocrinology*. 153(4):1755-63.

669 O'Reilly MW, Taylor AE, Crabtree NJ, Hughes BA, Capper F, Crowley RK, Stewart PM,
670 Tomlinson JW, Arlt W 2014 Hyperandrogenemia predicts metabolic phenotype in
671 polycystic ovary syndrome: the utility of serum androstenedione. *J Clin Endocrinol*
672 *Metab*. 99(3):1027-36.

673 Parajes S, Kamrath C, Rose IT, Taylor AE, Mooij CF, Dhir V, Grötzinger J, Arlt W, Krone
674 N 2011 A novel entity of clinically isolated adrenal insufficiency caused by a partially
675 inactivating mutation of the gene encoding for P450 side chain cleavage enzyme
676 (CYP11A1): *J Clin Endocrinol Metab* 96: E1798-806.

677 Prasad R, Chan LF, Hughes CR, Kaski JP, Kowalczyk JC, Savage MO, Peters CJ,
678 Nathwani N, Clark AJ, Storr HL, Metherell LA. 2014 Thioredoxin Reductase 2 (TXNRD2)
679 mutation associated with familial glucocorticoid deficiency (FGD). *J Clin Endocrinol*
680 *Metab*. 99(8):E1556-63.

681 Rege J, Nakamura Y, Wang T, Merchen TD, Sasano H, Rainey WE 2014 Transcriptome
682 profiling reveals differentially expressed transcripts between the human adrenal zona
683 fasciculata and zona reticularis. *J Clin Endocrinol Metab*. 99(3):E518-27.

684 Ronchi JA, Figueira TR, Ravagnani FG, Oliveira HC, Vercesi AE, Castilho RF 2013 A
685 spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of
686 C57BL/6J mice results in mitochondrial redox abnormalities. *Free Radic Biol Med*.
687 63:446-56

- 688 Ronchi JA, Francisco A, Passos LA, Figueira TR, Castilho RF 2016 The Contribution of
689 Nicotinamide Nucleotide Transhydrogenase to Peroxide Detoxification Is Dependent on
690 the Respiratory State and Counterbalanced by Other Sources of NADPH in Liver
691 Mitochondria. *J Biol Chem.* 291(38):20173-87
- 692 Rubtsov P, Karmanov M, Sverdlova P, Spirin P, Tiulpakov A 2009 A novel homozygous
693 mutation in CYP11A1 gene is associated with late-onset adrenal insufficiency and
694 hypospadias in a 46,XY patient: *J Clin Endocrinol Metab* 94: 936-9.
- 695 Sahakitrungruang T, Soccio RE, Lang-Muritano M, Walker JM, Achermann JC, Miller WL
696 2010 Clinical, genetic, and functional characterization of four patients carrying partial
697 loss-of-function mutations in the steroidogenic acute regulatory protein (StAR). *J Clin*
698 *Endocrinol Metab.* 95(7):3352-9.
- 699 Saha D, Koli S, Reddy KVR 2017 Transcriptional regulation of Hb- α and Hb- β through
700 nuclear factor E2-related factor-2 (Nrf2) activation in human vaginal cells: A novel
701 mechanism of cellular adaptability to oxidative stress. [Am J Reprod Immunol.](#) 77(6).
- 702 Sahakitrungruang T, Tee MK, Blackett PR, Miller WL 2011 Partial defect in the
703 cholesterol side-chain cleavage enzyme P450_{scc} (CYP11A1) resembling nonclassic
704 congenital lipid adrenal hyperplasia: *J Clin Endocrinol Metab* 96: 792-8.
- 705 Sasaki G, Ishii T, Jeyasuria P, Jo Y, Bahat A, Orly J, Hasegawa T, Parker KL 2008
706 Complex role of the mitochondrial targeting signal in the function of steroidogenic acute
707 regulatory protein revealed by bacterial artificial chromosome transgenesis in vivo. *Mol*
708 *Endocrinol.* 22(4):951-64.
- 709 Sauer U, Canonaco F, Heri S, Perrenoud A, Fischer E 2004 The soluble and membrane-
710 bound transhydrogenases UdhA and PntAB have divergent functions in NADPH
711 metabolism of *Escherichia coli*. *Journal of Biological Chemistry*, 279: 6613–6619
- 712 Simon MM, Greenaway S, White JK, Fuchs H, Gailus-Durner V, Wells S, Sorg T, Wong
713 K, Bedu E, Cartwright EJ, et al., 2005 A genetic and physiological study of impaired
714 glucose homeostasis control in C57BL/6J mice. *Diabetologia* 48(4):675-86.

715 Roberson LA, Rozman J, Sanderson M, Santos L, Selloum M, Shannon C, Southwell A,
716 Tocchini-Valentini GP, Vancollie VE, Westerberg H et al., 2013 A comparative
717 phenotypic and genomic analysis of C57BL/6J and C57BL/6N mouse strains. *Genome*
718 *Biology* 14(7):R82.

719 Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L 2013 Differential
720 analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol.*
721 31(1):46-53.

722 Vinogradov SN, Moens L 2008. Diversity of globin function: enzymatic, transport,
723 storage, and sensing. *J Biol Chem* 283(14):8773-7

724 Vinson GP 2003 Adrenocortical zonation and ACTH. *Microsc Res Tech.*;61(3):227–239.

725 Yan J, Guo Y, Fei Y, Zhang R, Han Y, Lu S 2017 GPx1 knockdown suppresses
726 chondrogenic differentiation of ATDC5 cells through induction of reductive stress. *Acta*
727 *Biochim Biophys Sin (Shanghai)*. 49(2):110-118.

728 Yu Q, Lee CF, Wang W, Karamanlidis G, Kuroda J, Matsushima S, Sadoshima J, Tian R
729 2014 Elimination of NADPH oxidase activity promotes reductive stress and sensitizes the
730 heart to ischemic injury. *J Am Heart Assoc.* 3(1):e000555.

731 Zhang X, Min X, Li C, Benjamin IJ, Qian B, Zhang X, Ding Z, Gao X, Yao Y, Ma Y, et al.,
732 2010 Involvement of reductive stress in the cardiomyopathy in transgenic mice with
733 cardiac-specific overexpression of heat shock protein 27. *Hypertension.* 55(6):1412-7.

734

FIGURE LEGENDS

Figure 1. Hypothalamic-Pituitary-Adrenal (HPA) Axis and Enzymes responsible for cortisol production.

(A) The HPA axis is a major component for adaptation of the stress response and cortisol release and consists of a complex set of feedback interactions that connect the central nervous and endocrine systems. In response to stress the paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) that acts on the adrenal to stimulate glucocorticoid synthesis. **(B)** The conversion of cholesterol to cortisol is achieved by a series of catalytic reactions catalysed by mitochondrial (in blue) and microsomal (in pink) enzymes. Mutations in these key steroidogenic enzymes result in diseases of adrenal and gonadal insufficiency, indicated to the right with their reference numbers from online inheritance in man (OMIM). STAR, steroidogenic acute regulatory protein; CYP11A1, cytochrome P450 side chain cleavage enzyme; CYP17A1, 17-alpha hydroxylase; HSD3B2, 3-beta hydroxysteroid dehydrogenase, CYP21A2, 21-hydroxylase; LCAH, lipoid congenital adrenal hyperplasia; FGD, familial glucocorticoid deficiency; CAH, congenital adrenal hyperplasia.

Figure 2. Detoxification of free radicals in the mitochondria.

NNT encodes a protein, integral to the inner mitochondrial membrane, which under normal physiological conditions uses energy from the mitochondrial proton gradient to generate high concentrations of NADPH. This is required for many processes in the cell including the supply of reductive power to a network of antioxidant enzymes, specifically the glutathione (GSH/GSSG) and thioredoxin ($\text{Trx}(\text{SH})_2/\text{TrxS}_2$) systems, to allow the detoxification of H_2O_2 . Manganese superoxide dismutase (MnSOD) converts $\text{O}_2^{\cdot -}$ into H_2O_2 and protects ROS-sensitive proteins from oxidative damage. H_2O_2 is then removed by glutathione peroxidases (e.g. GPX1) or peroxiredoxins (e.g. PRDX3) using GSH and $\text{Trx}(\text{SH})_2$ as co-factors. GSH and $\text{Trx}(\text{SH})_2$ can be regenerated by glutathione reductase (GR) and thioredoxin reductase-2 (TXNRD2), respectively, using the reducing power from NADPH. Without *NNT*, the production of NADPH is compromised, causing the mitochondria to become more sensitive

to oxidative stress. Enzymes underlined in red are affected by one or more mutations in FGD patients.

Figure 3. Biochemical and phenotypic characterisation of *Nnt*^{+/+}, *Nnt*^{-/-} and *Nnt*^{BAC} mice.

(A) Serum corticosterone in *Nnt*^{+/+}, *Nnt*^{-/-} and *Nnt*^{BAC} mice was measured by LC-MS/MS and showed 80% and 50% reduction in *Nnt*^{-/-} and *Nnt*^{BAC} mice, respectively. **(B)** The 11-deoxycorticosterone (DOC)/corticosterone ratio (CYP11B1 inhibition) was significantly higher in *Nnt*^{-/-} and *Nnt*^{BAC} than *Nnt*^{+/+} mice. **(C)** Corticosterone synthesis deteriorates in 18 months *Nnt*^{-/-} mice whereas there is no significant difference in the levels between 3 and 18 month *Nnt*^{+/+} mice. **(D)** H&E staining of mouse adrenals showed no major histological differences in architecture or zonation (left panel) and **(E)** Oil Red O staining revealed no difference in lipid content of the adrenals among the three mouse strains (right panels). Results are means ± standard deviation (SD); n=5 mice per group, *p<0.05, **p<0.01, ***p<0.001.

Figure 4. Oxidative stress on NNT ablation. (A) Lipid peroxidation represented by MDA

levels were measured in adrenals of *Nnt*^{+/+}, *Nnt*^{-/-} and *Nnt*^{BAC} mice. MDA levels were significantly increased in the adrenals of *Nnt*^{-/-} mice with a partial rescue in *Nnt*^{BAC} mice indicating lipid damage by free radicals in adrenals upon *Nnt* deletion **(B)** The cellular levels of NADP/NADPH in H295R cells with a stable knockdown of NNT (NNT-KD) were measured by using a luciferin based assay. Total cellular NADP/NADPH ratio was significantly higher in NNT-KD compared to scramble (SCR), suggesting that NNT is required to maintain the redox state of the intracellular NADPH and NADP⁺ pools. **(C)** Mitochondria respiration was assessed by measuring oxygen consumption rates using Seahorse XF-96 metabolic Flux Analyser. NNT-KD cells had a significantly lower basal OCAR compared to scrambled cells; the addition of oligomycin (complex V inhibitor) resulted in an OCAR decline which was significantly lower in NNT-KD cells compared to controls, indicating that the ATP turnover was compromised in NNT-KD cells. Furthermore, maximal respiration capacity as measured by the addition of an uncoupling agent, FCCP, was also significantly lower in NNT-KD cells.

The addition of rotenone and antimycin (complex I and II inhibitors respectively) reflecting the spare respiratory capacity of the cells resulted in a reduction of OCAR which was significantly lowered in NNT-KD cells when compared to scrambled cells. OCAR values were normalised to total protein concentration. Results are means \pm standard deviation (SD); n=5 per group, *p<0.05, **p<0.01, ***p<0.001.

Figure 5. RNA-seq analysis flowchart and differential gene expression.

(A) Flowchart of initial RNA-seq analysis of mouse adrenals. (B) Representative heat map of RNA-seq analysis for substrain-specific differentially expressed genes (between *Nnt*^{-/-} and *Nnt*^{BAC}) within mouse adrenals. Genes were clustered by Partek hierarchical clustering based on gene expression values. Normalisation was performed by genes shifted to mean of zero and scaled to standard deviation of 1. Arbitrary signal intensity from RNA-seq data is represented by colours (red, higher expression, blue lower expression). (C) Venn diagram showing the number of differential genes in pairwise analyses between; *Nnt*^{+/+} vs *Nnt*^{-/-} (187), *Nnt*^{-/-} vs *Nnt*^{BAC} (157) and *Nnt*^{+/+} vs *Nnt*^{BAC} (141). Genes at the intersection of the pairwise analyses *Nnt*^{+/+} vs *Nnt*^{-/-} and *Nnt*^{-/-} vs *Nnt*^{BAC} represent genes that are modulated by *Nnt* levels (39 + *Nnt*) [see Table 1 and Supplementary Tables 3-8].

Figure 6. Effect of NNT loss on redox homeostasis.

(A) mRNA *Nnt* levels in the three mouse strains. (B) No NNT protein expression was observed in *Nnt*^{-/-} (western blot) however there was a two-fold upregulation in *Nnt*^{BAC} when compared to *Nnt*^{+/+}. (C) protein levels of TXNRD2, PRDX3 and GPX1 in the *Nnt*^{+/+}, *Nnt*^{-/-} and *Nnt*^{BAC} mouse adrenals were normalised to actin with representative western blots shown to the right.

Figure 7. Effect of NNT loss on steroidogenesis. (A) mRNA and protein fold change of enzymes involved in glucocorticoid synthesis, starting from cholesterol transport (STAR), to the first step of steroid synthesis (mitochondrial CYP11A1), and subsequent reactions

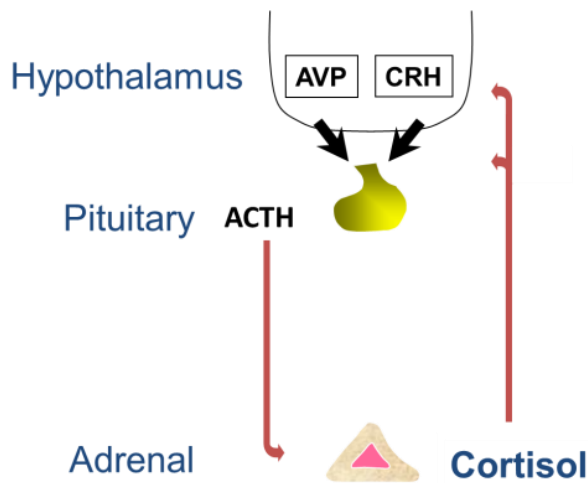
catalysed by microsomal enzymes (HSD3B2, CYP21A1) to the final synthesis of corticosterone. **(B)** The conversion of cholesterol to corticosterone in mice is achieved by a series of catalytic reactions catalysed by mitochondrial (in blue) and microsomal (in pink) enzymes (right panel). **(C)** Panel of western blots representing changes in expression of protein involved in steroidogenesis. Results are means \pm standard deviation (SD); n=5 mice per group, *p<0.05, **p<0.01, ***p<0.001.

Figure 8. Enrichment of gene pathways in response to oxidative stress.

(A) mRNA levels of heat shock proteins revealed significant upregulation (*Hspa1a* 26-fold; *Hspa1a* 25-fold; *Hsph1* 3-fold; *Dnajb1* 6-fold) in *Nnt*^{-/-} mice and restoration of the levels in *Nnt*^{BAC} mouse adrenals. **(B)** Similarly haemoglobin gene mRNA expression was significantly upregulated in *Nnt*^{-/-} mice when compared to *Nnt*^{+/+} and reversed in *Nnt*^{BAC} (*Hbb-b1* 4-fold; *Hbb-b2* 5-fold; *Hba-a1* 5-fold; *Hba-a2* 5-fold).

Figure 1

(A)



(B)

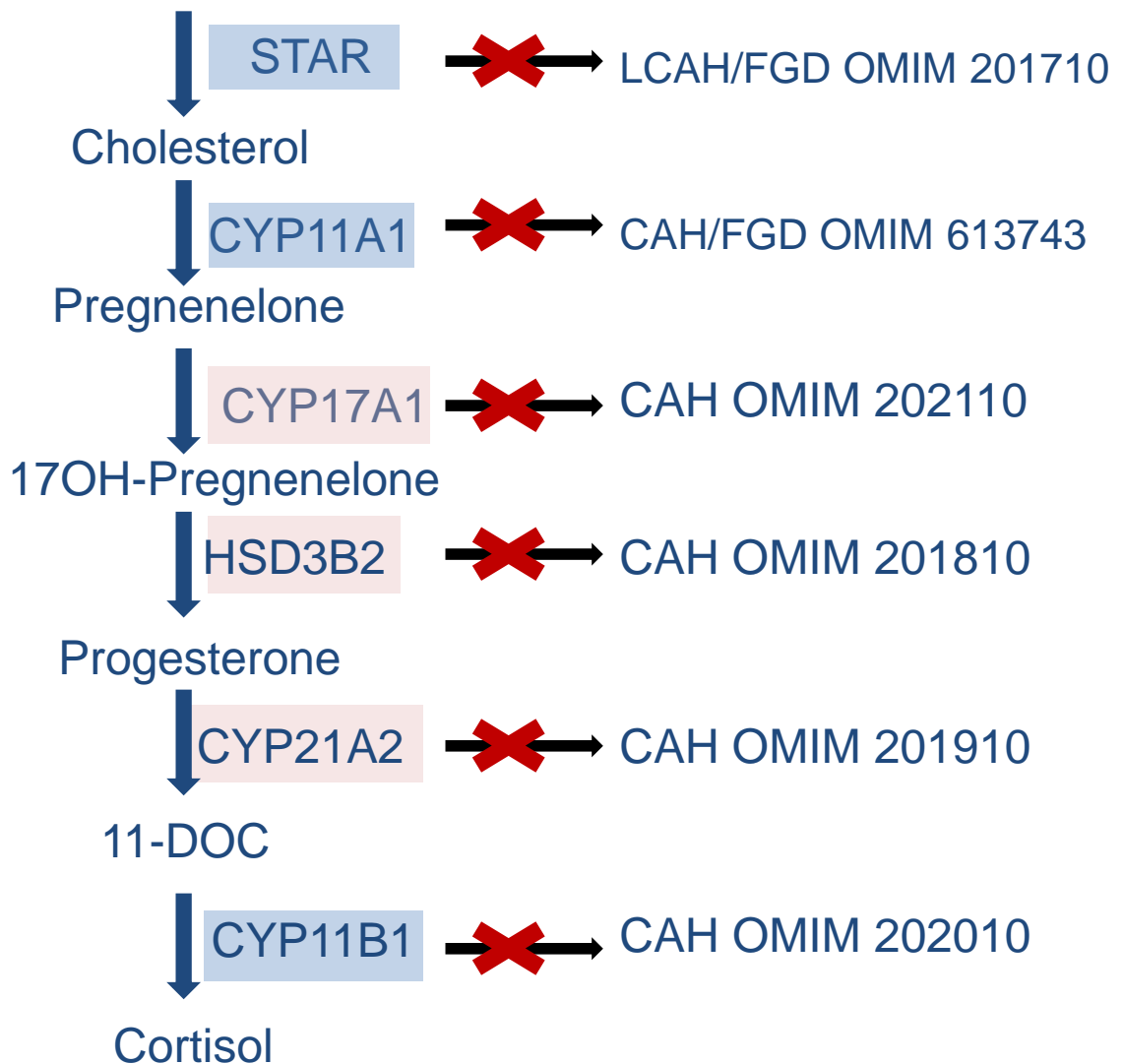


Figure 2

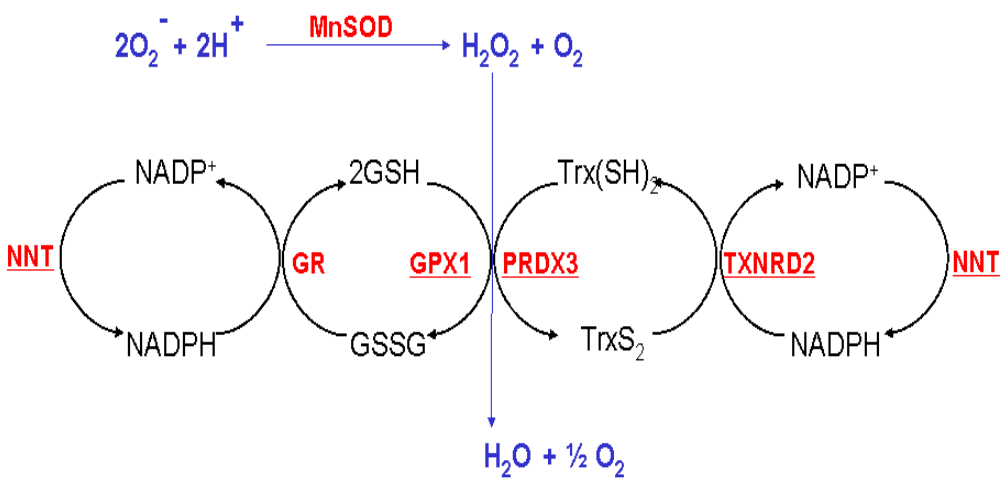


Figure 3

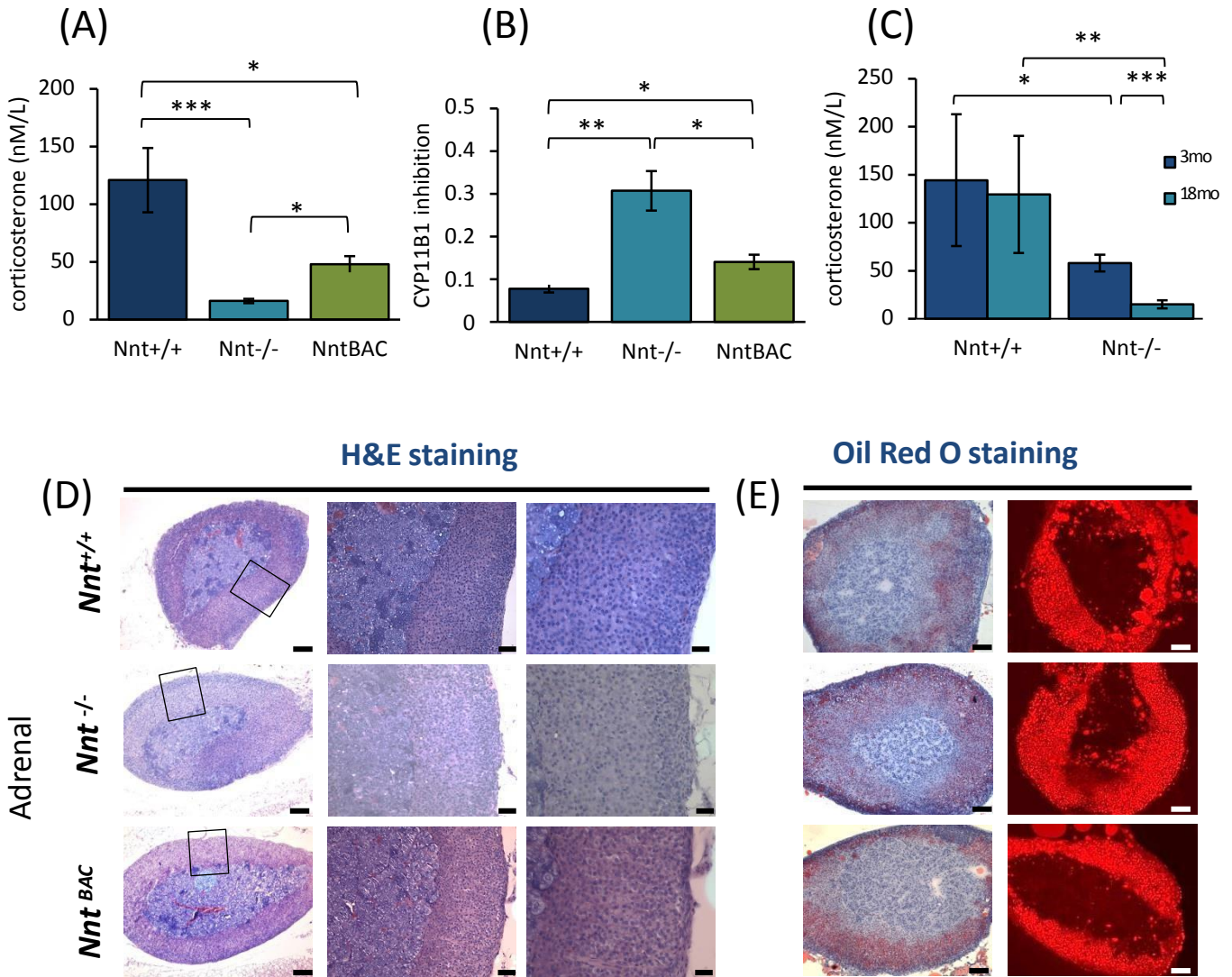
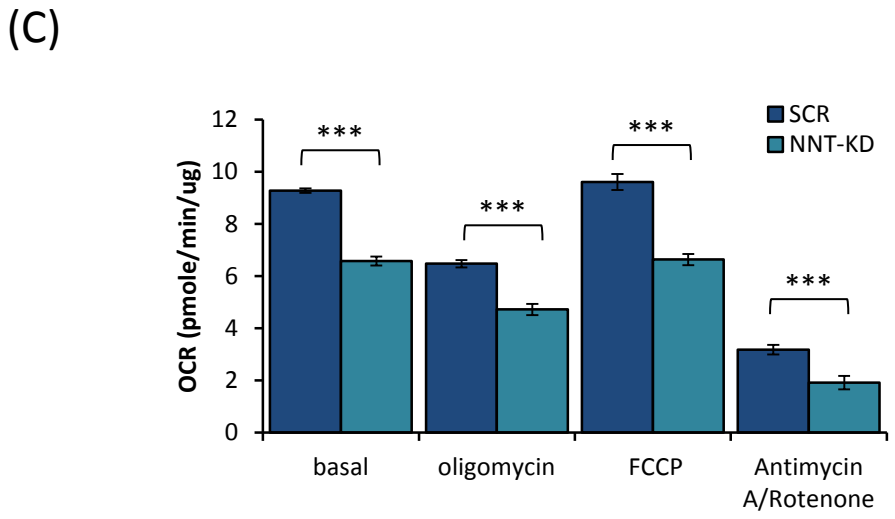
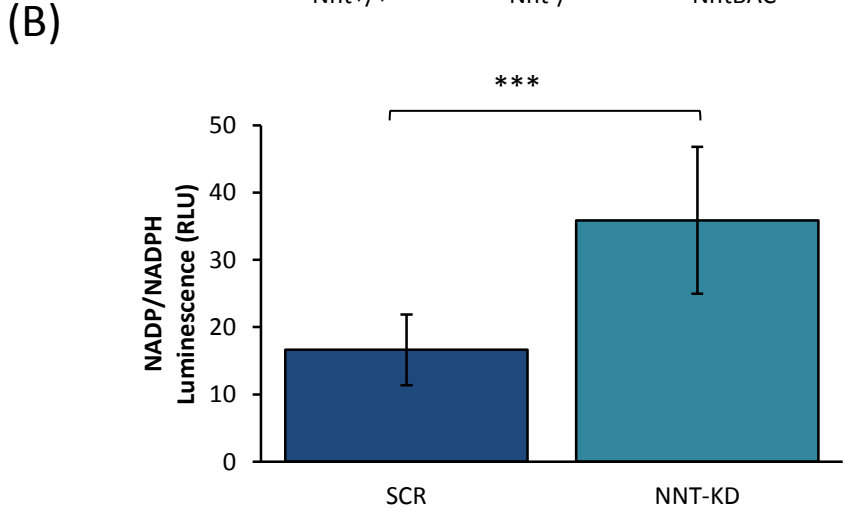
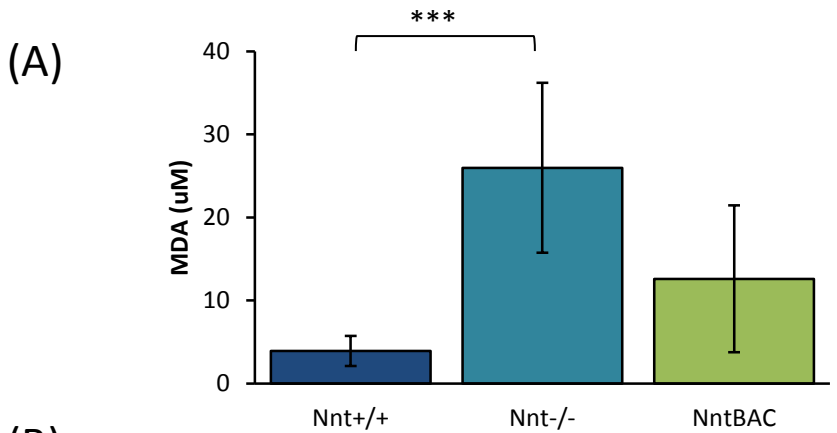


Figure 4



RNAseq on mouse adrenals n=5 per group

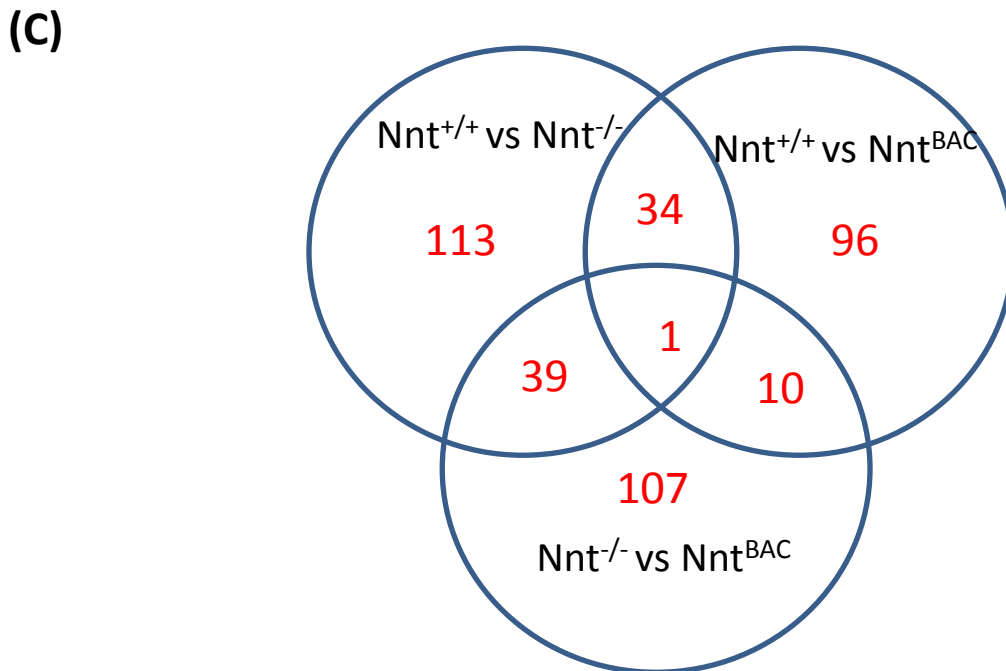
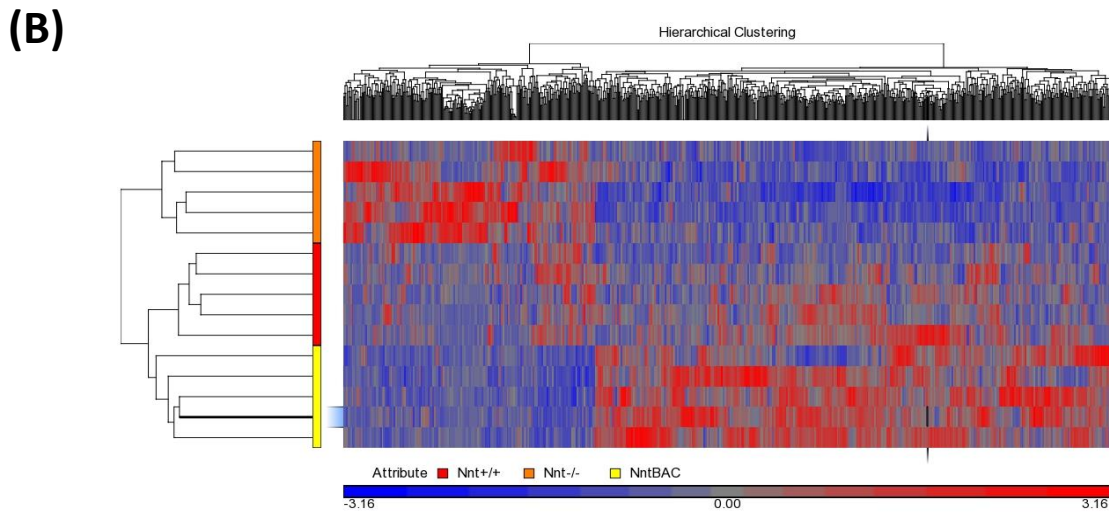
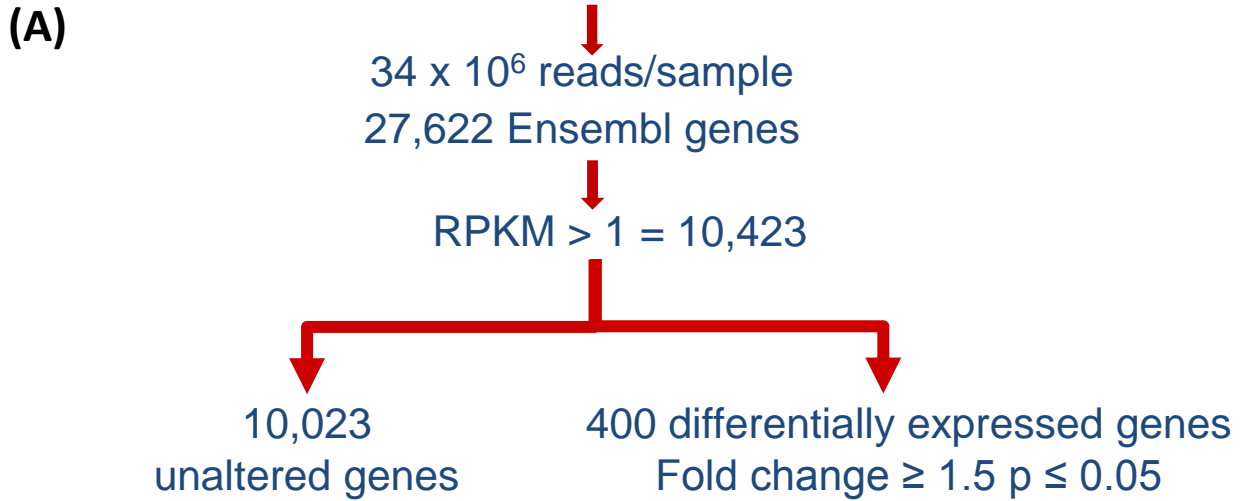


Figure 6

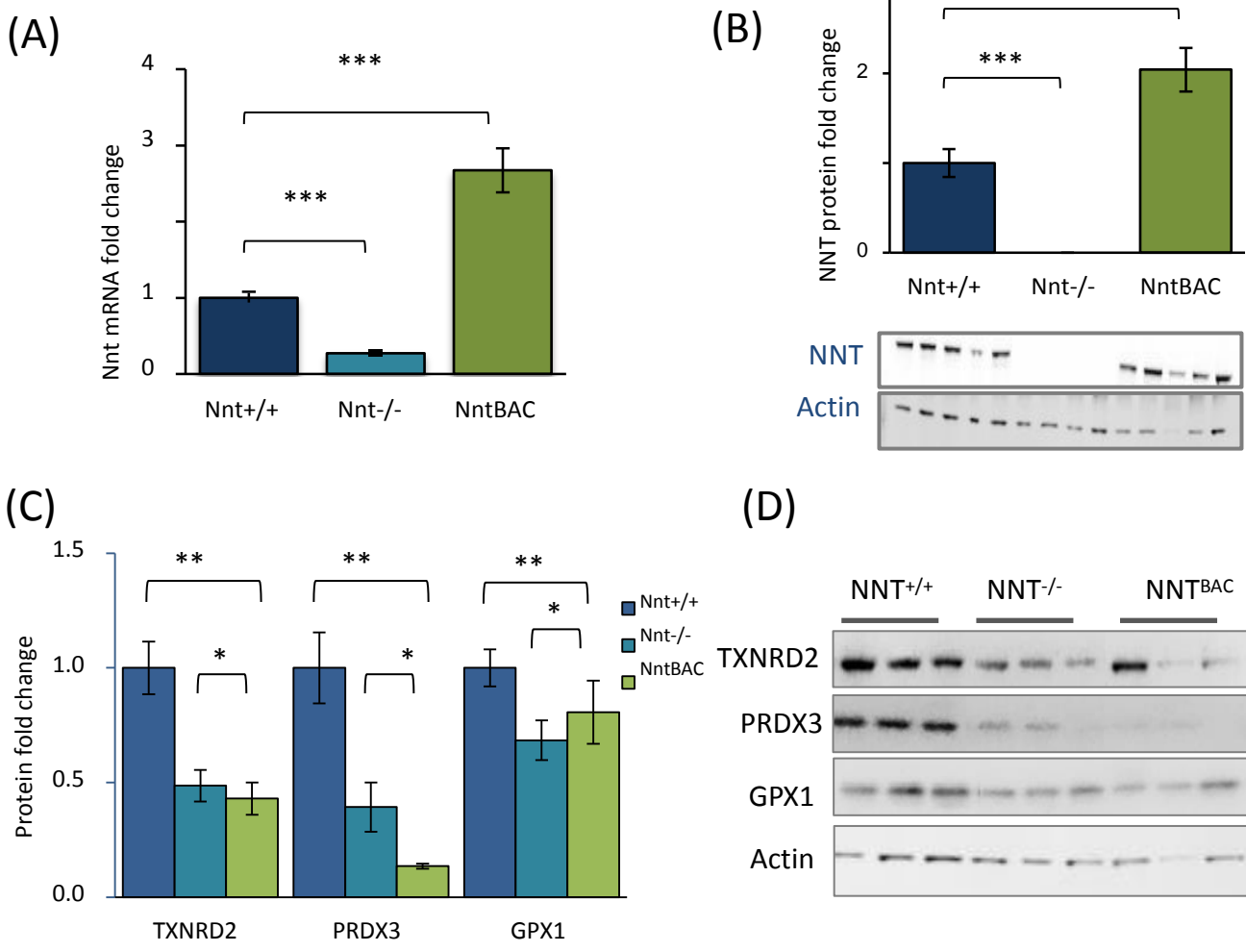
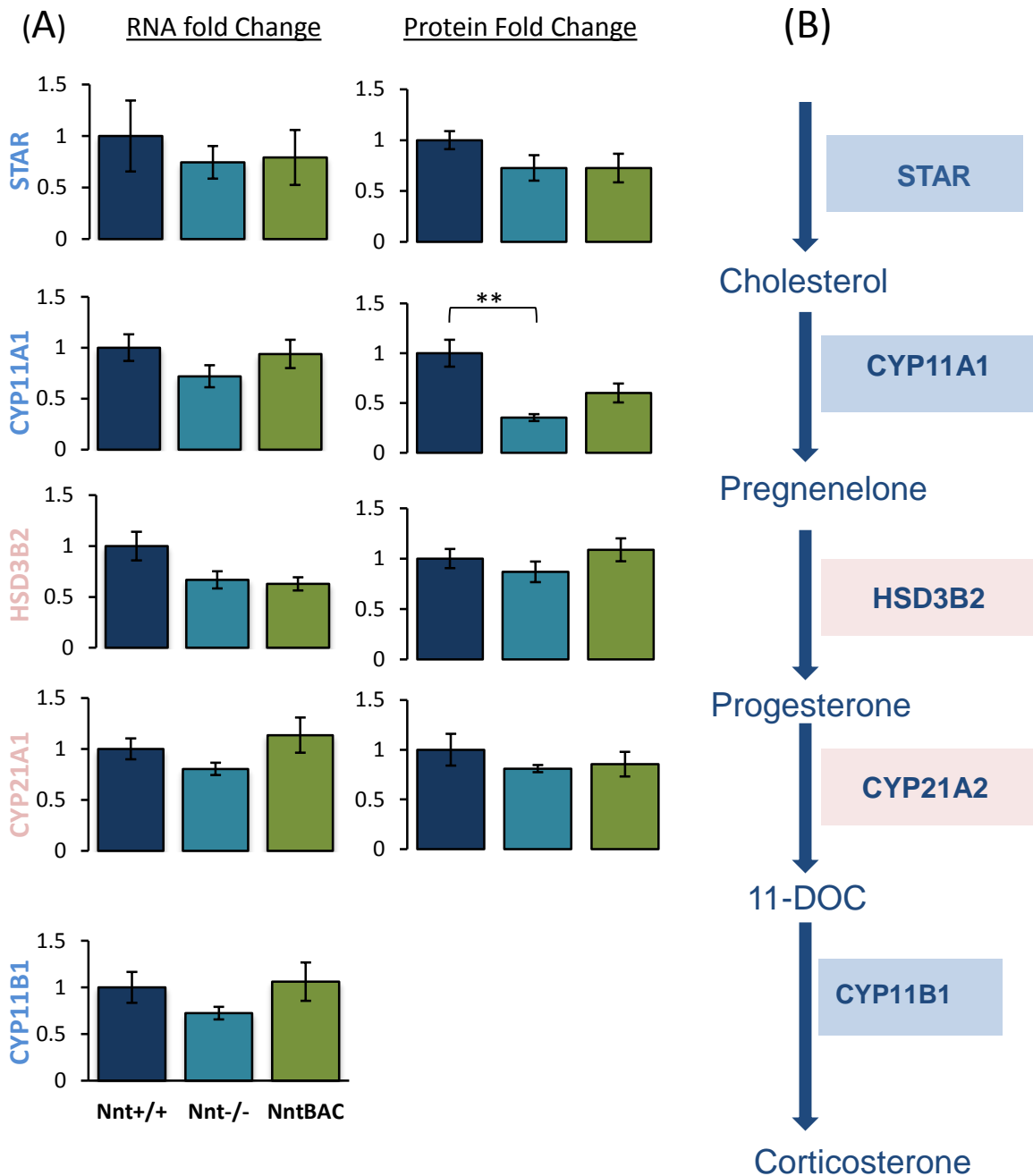
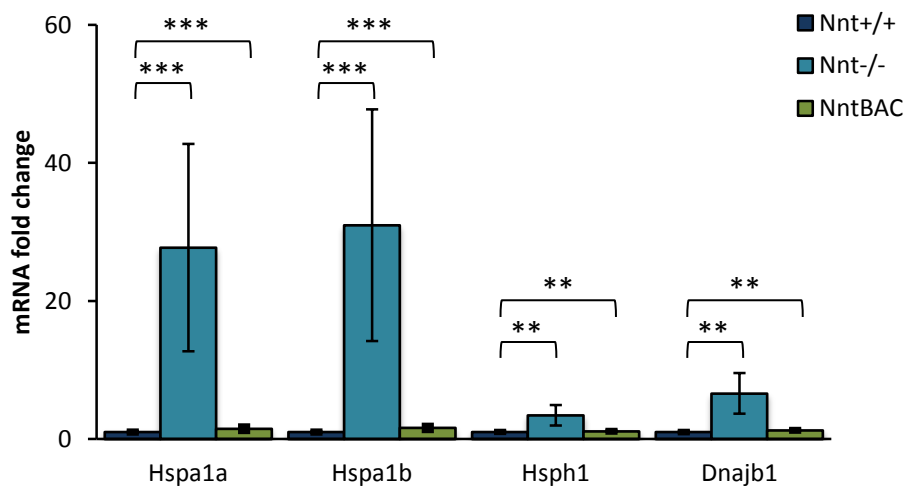


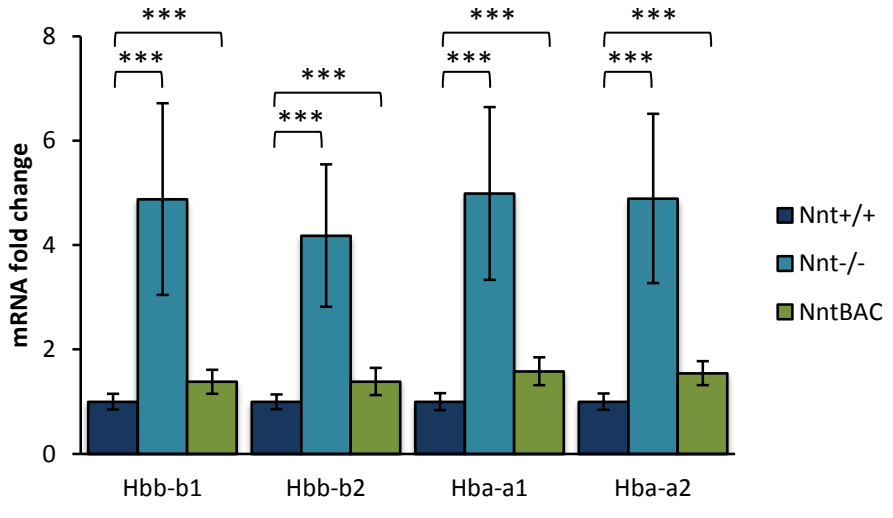
Figure 7



A



B



Genes downregulated in *Nnt*^{-/-} and restored in *Nnt*^{BAC}

Gene ID	Gene Description
1500015O10Rik	RIKEN cDNA 1500015O10 gene
9330151L19Rik	ENSMUSG00000097061 - Uncharacterized protein
A530020G20Rik	RIKEN cDNA A530020G20 gene
C1qtnf6	C1q and tumor necrosis factor related protein 6
Ccdc160	Coiled-coil domain containing 160
Ctxn3	Cortexin 3
Cyp21a2-ps	Cyp21a2 pseudogene
E330017L17Rik	RIKEN cDNA E330017L17 gene
Epb4.1l4aos	Erythrocyte membrane protein band 4.1 like 4a, opposite strand
Gnat2	Guanine nucleotide binding protein, alpha transducing 2
Ism1	Isthmin 1 homolog (zebrafish)
Kcnn2	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
Lilr4b	Leukocyte immunoglobulin-like receptor, subfamily B, member 4B
Ly96	Lymphocyte antigen 96
Nnt	Nicotinamide nucleotide transhydrogenase
Pacsin3	Protein kinase C and casein kinase substrate in neurons 3
Sox12	SRY-box containing gene 12
Steap1	Six transmembrane epithelial antigen of the prostate 1
Trim12c	Tripartite motif-containing 12C
Trim21	Tripartite motif-containing 21
Trim30d	Tripartite motif-containing 30D
Tuft1	Tuftelin 1
Vsn1	Visinin-like 1

Genes upregulated in *Nnt*^{-/-} and restored in *Nnt*^{BAC}

Gene ID	Gene Description
Arl4d	ADP-ribosylation factor-like 4D
Cwc25	CWC25 spliceosome-associated protein homolog (<i>S. cerevisiae</i>)
Cyr61	Cysteine rich protein 61
Dnajb1	Dnaj (Hsp40) homolog, subfamily B, member 1
Egr1	Early growth response 1
Fam46a	Family with sequence similarity 46, member A
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma
Hba-a1	Hemoglobin alpha, adult chain 1
Hba-a2	Hemoglobin alpha, adult chain 2
Hbb-bs	Hemoglobin, beta adult s chain
Hbb-bt	Hemoglobin, beta adult t chain
Ier2	Immediate early response 2
Ier3	Immediate early response 3
Irs2	Insulin receptor substrate 2
Klf4	Kruppel-like factor 4 (gut)

Nr4a2	Nuclear receptor subfamily 4, group A, member 2
Zfp36l2	Zinc finger protein 36, C3H type-like 2