Supplementary data

Supplementary materials and methods

Patients

This study was approved by the Outer North East London Research Ethics Committee, reference number 09/H0701/12. Similar to other forms of FGD caused by mutations in MC2R pathway (MC2R and MRAP) or steroid synthetic pathways (StAR) patients present with high ACTH levels, low cortisol and hyperpigmentation but with normal renin and aldosterone levels. Affected individuals were diagnosed with FGD, by their local clinicians, on the basis of clinical history and biochemical measurements. Patients were all diagnosed before 40 months (range 6-40 months) and had cortisol levels <5µg/dl (normal range 5-25), ACTH levels ranging between 624 - >2000pg/ml (normal range <50) and normal renin, aldosterone and electrolyte levels. Where an ACTH stimulation test was performed the patients all failed to mount a suitable response. We isolated genomic DNA from blood samples of affected individuals and family members after obtaining informed consent from them and/or their parents. In all cases, the sequences of MC2R, MRAP and STAR had been determined to be normal.

Mouse strains

C57BL/6NHsd from Harlan (Harlan Laboratories UK) is wild-type for Nnt, C57BL6J from Charles River (Charles River UK Ltd) has an in-frame deletion of 5 Nnt exons. Three-month old male C57BL6J mice from Charles River were used as $Nnt^{-/-}$ whereas three-month old male C57BL6NHsd mice from Harlan were used as wild-type control $Nnt^{+/+}$. Genomic DNA was extracted from the mouse tail tissue using a Qiagen DNeasy tissue kit. Mice were genotyped for Nnt status using previously published primers [Huang] (Supplementary Figure 6). Briefly PCRs were performed targeting; exon 1 as a positive PCR control which amplifies in both mouse sub-strains, exon 6 which is deleted in the Nnt mutant (6J) but will amplify in the wild-type 6NHsd mice, and the breakpoint junction of the deletion which will amplify in 6J but not in 6NHsd. The animal protocol used in this study was approved by United Kingdom Home Office.

Methods

Genotyping

For the whole-genome scan, the GeneChip mapping 10 K array *Xba142* (Affymetrix, Santa Clara, CA) was used in accordance with the manufacturer's guidelines as previously described [ref Metherell2]. We performed homozygosity mapping in ten families introducing consanguineous loops into all families. Parametric linkage analysis was performed with a new version of MERLIN (Abecasis et al, 2002 and 2005) that allows for marker clustering to compensate for linkage disequilibrium (LD) between adjacent markers. We assumed

recessive inheritance with full penetrance and 0.0001 disease allele frequency. The parametric curve under heterogeneity showed the highest peak on chromosome 5, a maximum HLOD score of 2.34 (alpha = 44%) was calculated for the critical interval.

Mutation discovery

Sequence Capture Array and sequencing

A custom 385K Sequence Capture array targeting the exons, 50bp up- and downstream, plus 1Kb upstream of the transcription start site for each REFSEQ gene within the areas of homozygosity (coordinates 5:31.5- 68.1Mb from rs1382882 to rs33296) was designed and manufactured by Roche NimbleGen (Madison, WI). A 472-fold enrichment of the targeted regions was achieved and paired-end sequencing of 2 x 36bp was performed on a single lane of the Illumina GAII analyzer. Single Nucleotide Polymorphisms, with a threshold coverage of at least 10 reads on the respective nucleotide, were called with the MAQ alignment and downstream analysis tools. The results were checked against the Ensembl SNP database, release 54. The number of variants was reduced by the following strategy; (i) considering variants within the disease-linked locus only, (ii) excluding variants that were heterozygous, (iii) removing variants annotated in SNP databases (Ensembl SNP database, release 54), (iv) evaluating non-synonymous coding variants, splice variants and indels only.

Conventional sequencing

Genomic DNA was extracted from peripheral blood leukocytes, and each exon of *NNT* including intronic boundaries was amplified by PCR using specific primers (primers available on request).

Mutation detection

We carried out PCR using primers directed to intronic sequences in a total volume of 50µl. Primer sequences are available on request. The reaction mixture contained 100 ng of DNA template, 1 X PCR buffer, 200 µM each dNTP, 200 nM each primer and 1 U *Taq* DNA polymerase (Sigma-Aldrich). After an initial denaturation step of 5 min at 95°C, PCR cycling was performed for 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min, followed by a final extension step at 72°C for 5 min. PCR products were visualized on 1% agarose gel and sequenced using the ABI Prism Big Dye sequencing kit and an ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions.

RT-PCR to determine tissue distribution.

We investigated expression of NNT and GAPDH using a panel of cDNAs derived from 20 adult tissues (adrenal, liver, heart, kidney, spleen, lung, bladder, skeletal muscle, small intestine, ovary, placenta, thymus, trachea, testes, colon, adipose tissue, prostate, cervix, oesophagus and thyroid). We carried out RT-PCR using the KAPA Sybr Fast qPCR protocol for NNT and GAPDH in each tissue in a

reaction mixture containing cDNA, 1 X KAPA SYBR Fast qPCR master mix (Anachem), 200nM each primer and 50nM ROX_{LOW} in a total volume of 10µl. PCR cycling was performed as follows 95°C for 3 min, then 40 cycles of [95°C for 3s, 60°C for 20s and 72°C for 1s], then 1 cycle of [95°C for 1 min, 60°C for 30s, 72°C for 30s].

Animal Studies

Histology and Immunofluorescence

Adrenal tissues attached to kidney were dissected and kept in 4% paraformaldehyde overnight at 4^{o}C. Next day tissues were dehydrated and embedded in paraffin and 7µm sections were cut and stained with hematoxylin and eosin using standard protocols.

For immunofluorescence, paraffin-embedded tissues were deparaffinised, boiled in 0.5M sodium citrate pH 6.0 for 30 mins, and blocked with 10% normal horse serum (Sigma Aldrich) in PBS-T. Sections were then incubated at 4^{o}C overnight with mouse anti-CYP11B1 (1:20) and rabbit anti-side chain cleavage (1:1000; Millipore) or rabbit anti-caspase 3 (1:1000 Santa Cruz). Next day slides were washed 3 times with PBS-T, and then incubated for 2 hrs with goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa fluor 568 (1:1000; Invitrogen). After further washes, slides were coverslipped and images were acquired using Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Corticosterone measurements

Six mice per group were sacrificed at t=0, 6 mice were ip injected with 125µg of synacthen and sacrificed after 1 hr, and 6 mice were injected with saline (sham injection) and sacrificed after 1 hr. Blood was collected by cardiac puncture. Plasma corticosterone was determined using a commercially available kit (DRG Instruments GmbH, Germany, ^{125}I RIA kit) and according to manufacturer's instructions.

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

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

Immunoblotting analysis
Immunoblotting with anti-NNT antibody was used to confirm protein knockdown. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (SIGMA) and then left on ice for 30mins. Samples were centrifuged for 15 mins at 13,000 rpm. Supernatant was collected and an equal volume of Laemmli buffer was added. Samples were heated at 95-100^{o}C for 5mins and then loaded on 4-12% SDS-PAGE gels. Proteins were then transferred to nitrocellulose membrane (Sigma Aldrich) using semi-dry transfer blot (Biorad) at 15V for 1 hr. Membranes were probed with mouse anti-NNT (1:1000; MitoScience). Visualisation of the proteins was performed by using Alexa-fluor 680 (1:1000; Invitrogen) secondary and the Li-CoR Odyssey system.

PARP Cleavage
SCR and NNT-KD cells were plated in 6 well plates and allowed to reach 80% confluency. Cells were lysed and analysed by Western blotting analysis using PARP polyclonal rabbit antibody at 1:500 (Cell signalling). Cleaved PARP was normalized against the signal from an ACTIN antibody at 1:10,000 (AbCam).

Detection of superoxide production
Mitosox staining was performed according to the manufacturer's instructions. Briefly, NNT-KD, and SCR stable cells were incubated with 5μM MitoSox Red (Invitrogen) for 10mins. After incubation cells were washed 3 times with HANKS buffer. Detection of superoxide was performed by imaging cells on a confocal microscope (63X).

GSH/GSSG measurements
Measurement of total glutathione (GSH + GSSG) or oxidized glutathione (GSSG) was performed by using GSH/GSSG-Glo Assay (Promega) a luminescence based system, and according to manufacturer's instructions. Briefly SCR and NNT-KD cells were plated on a 96 well plate at a density of 20,000 cells per well and duplicate samples were assayed for total glutathione or GSSG. GSH/GSSG ratios were calculated directly from Net RLU measurements using the equation GSH/GSSG ratio = [Net total glutathione RLU-Net GSSG RLU]/ [Net GSSG RLU/2].

References
Huang TT, Naeemuddin M, Elchuri S, Yamaguchi M, Kozy HM, Carlson EJ, Epstein CJ. Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase. *Hum. Mol. Genet.* ***15,***1187-94 (2006).

Abecasis, G.R., Cherny, S.S., Cookson, W.O. & Cardon, L.R. Merlin–rapid analysis
of dense genetic maps using sparse gene flow trees. *Nat. Genet.* **30**, 97–101 (2002).

Abecasis GR, Wigginton JE. Handling marker-marker linkage disequilibrium: pedigree analysis with clustered markers. *Am J Hum Genet*. **77**:754-67 (2005).

Metherell, L.A. *et al.* *J. Clin. Endocrinol. Metab.* **94,** 3865-71 (2009).

Supplementary Table 1
Variants for validation

Gene name	Variation from*	Variation to*	Reference sequence	Variant	Ensembl gene ID	Ensembl exon ID	Protein change	Forward primer**	Reverse primer**
SPEF2	35682541	35682541	A	G	ENSG00000152582	ENSE00001006048	Arg201Gly	AGTCTCTTGGCTAATTGCTG	AAGCAAATGTGAAGTTTTGG
C5orf42	37218659	37218659	G	A	ENSG00000197603	ENSE00001578843	Pro675Leu	GGAATGAGTGGATTATTTGG	ATTAAAGGAACCACTGGAAG
CARD6	40889761	40889761	C	T	ENSG00000132357	ENSE00001143108	Ser857Leu	CTAAGTGGTTCCATCCTTTG	AGAATGGAAGGGTTTAGGC
NNT	43685159	43685159	C	T	ENSG00000112992	ENSE00000742794	Ala533Val	GTGGAATTTAAAAGCATTGG	ATAAACGGCATTGATAGTCC
GPBP1	56577983	56577983	A	G	ENSG00000062194	ENSE00001126759	Asn200Ser	AGTTATTTTACCCAGGAGGC	CTAGAACTCAGCACCCCAC

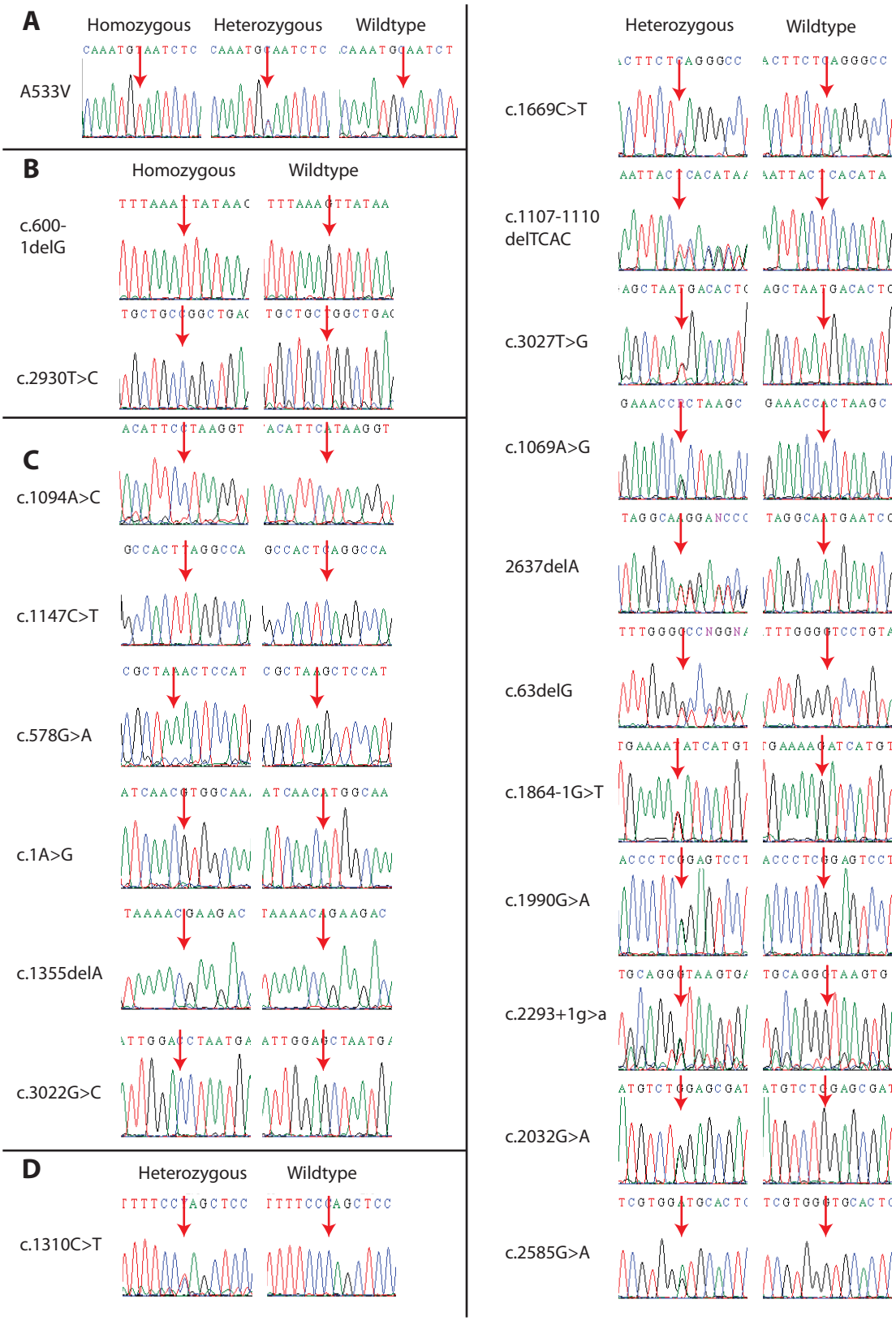
* sequence numbering based upon Feb. 2009 (NCBI37/hg19) human reference sequence assembly, ** primers utilised for both PCR and sequencing

Supplementary Table 2

Clinical data for probands from 15 kindreds with Familial Glucocorticoid Deficiency

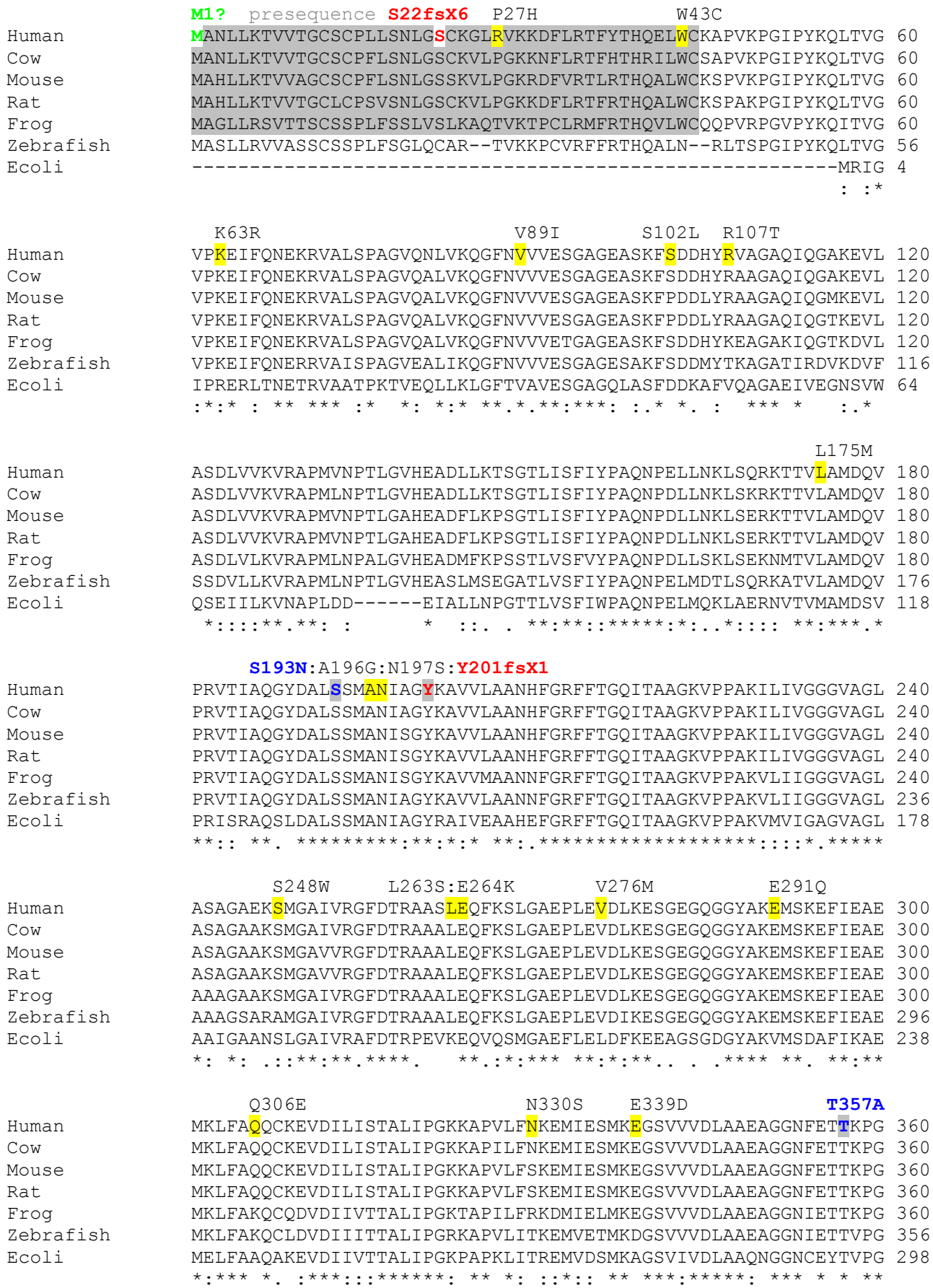
Family	Age of onset (months	presenting feature	cortisol nmol/L (nr >200)	ACTH (pg/ml) [nr 0-50]
1	9	hypoglycaemia, hyperpigmentation	54	624
2	18	hypoglycaemia, hyperpigmentation	<5	1460
3	unknown	unknown	low	high
4	15	hypoglycaemia, hyperpigmentation	low	high
5	6	collapse, hypoglycaemia	<5	660
6	12	hypotonia, febrile	4.6	654
7	12	unknown	low	high
8	27	pigmentation at 12m, febrile convulsions at 19m	undetectable	1139
9	12	exhausted not wanting to eat	310	high
10	unknown	unknown	low	high
11	29	hypoglycaemia	1.2	1022
12	14	hypoglycaemia	<11	>1000
13	7	hyperpigmentation, hypoglycaemic seizure following a cold	<0.6	3412
14	39	hypoglycaemia seizures	undetectable	>1000
15	29	ITU admission with encephalopathy following mumps	undetectable	>2000

Supplementary Figure 1



Partial chromatograms showing the NNT mutations detected in families with FGD. A left the homozygous A533V mutation in the proband, middle the mutation is heterozygous in the parents and right wild-type sequence for comparison. B c.600delG and c.2390T>C mutations seen in the two other chromosome 5-linked probands. C homozygous and D heterozygous mutations found in FGD patients, in each case the mutant sequence is on the left and on the right is a wild type sequence, arrows indicate the position of the mutations.

Supplementary Figure 2



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                  H365T
            L362V H365P H370X        Q383X                              T418M
Human           ELYIHK-GITHIGYTDLPSRMATQASTLYSNNITKLLKAISPDKDNFYFDVKDDFDFGTM 419
Cow             ELYVHK-GITHIGYTDLPSRMATQASTLYSNNITKLLKAISPDKDNFYFEVKDDFDFGTM 419
Mouse           ELYVHK-GITHIGYTDLPSRMATQASTLYSNNITKLLKAISPDKDNFHFEVKDDFDFGTM 419
Rat             ELYVHK-GITHIGYTDLPSRMATQASTLYSNNITKLLKAISPDKDNFHFEVKDDFDFGTM 419
Frog            DIYVHK-GVTHIGYTDIPSRMASQASTLYSNNITKLLKAISPDKDNFYYDIKDDFDYGTM 419
Zebrafish       ELSVHK-GVIHVGYTDIPSRLPTQASTLYSNNITKLIRAISPDKETFYFDVKNEFDFGTM 415
Ecoli           EIFTTENGVKVIGYTDLPGRLPTQSSQLYGTNLVNLLKLLCKEKD---GNITVDFD---- 351
                ::   : *:  :****:*.*:.:*:* **..*:.:*:: :. :*:    ::. :**

              V422I:V427A:G432D:P437L:P441L:G447D:Q452fsX44:K453T:E461K:T475M
Human           GHVIRGTVVMKDGKVIFPAPTPKNIPQGAPVKQKTVAELEAEKAATITPFRKTMSTASAY 479
Cow             GHVIRGTVVMKDGQVIFPAPTPKNIPQGAPVKQKTVAELEAEKAATITPFRKTMTSASVY 479
Mouse           SHVIRGTVVMKDGKVIFPAPTPKNIPEEAPVKPKTVAELEAEKAGTVSMYTKTLTTASVY 479
Rat             SHVIRGTVVMKDGNVIFPAPTPKNIPKEAPAKQKTVAELEAEKAGTVSMYTKTLRTASVY 479
Frog            DHVIRGTVVMKDGNVIFPAPPPNKIPQAAPVKQKSVAQIEAEKAASISPFRKTMNGAAAY 479
Zebrafish       DHVIRGSVVMQDGKVLFPAPQPQNVPVAAPPKQKTVQELQKEKASAVSPFRATLTTAGVY 475
Ecoli           DVVIRGVTVIRAGEITWPAP-PIQVSAQPQAAQKAAPEVKTEEKCTCSPWRKYALMALAI 410
                . **** .*:: *:: :*** * ::.  .    *:. ::: *:  : : :      * .

                                                                    A533V
Human           TAGLTGILGLGIAAPNLAFSQMVTTFGLAGIVGYHTVWGVTPALHSPLMSVTNAISGLTA 539
Cow             TAGLTGILGLGIAAPNLAFSQMVTTFGLAGIVGYHTVWGVTPALHSPLMSVTNAISGLTA 539
Mouse           SAGLTGMLGLGIVAPNVAFSQMVTTFGLAGIIGYHTVWGVTPALHSPLMSVTNAISGLTA 539
Rat             SAGLTGMLGLGIVAPNLAFSQMVTTFGLAGIIGYHTVWGVTPALHSPLMSVTNAISGLTA 539
Frog            TAGLGTLLSLGIASPHSAFTQMVTTFGLAGIVGYHTVWGVTPALHSPLMSVTNAISGLTA 539
Zebrafish       TGGLGTAIGLGLCAPNAAFTQMVTTFGLAGIVGYHTVWGVTPALHSPLMSVTNAISGLTA 535
Ecoli           IL-----FGWMASVAPKEFLGHFTVFALACVVGYYVVWNVSHALHTPLMSVTNAISGIIV 465
                       :.     .   *   .*.*.** ::**:.**.*: ***:***********: .

                                Q557X                   R587C:T589S
Human           VGGLALMGGHLYPSTTSQGLAALATFISSVNIAGGFLVTQRMLDMFKRPTDPPEYNYLYL 599
Cow             VGGLVLMGGHLYPSTTSQGLAALATFISSVNIAGGFLVTQRMLDMFKRPTDPPEYNYLYL 599
Mouse           VGGLALMGGHFYPSTTSQSLAALATFISSVNIAGGFLVTQRMLDMFKRPTDPPEYNYLYL 599
Rat             VGGLALMGGHFYPSTTSQSLAALATFISSVNIAGGFLVTQRMLDMFKRPTDPPEYNYLYL 599
Frog            VGGLALMGGGYLPTNTHELLAVLAAFVSSINIAGGFLVTQRMLDMFKRPTDPPEYNYLYL 599
Zebrafish       VGGLSLMGGGYLPSSTAETLAVLAAFISSVNIAGGFLVTQRMLDMFKRPTDPPEYNYLYL 595
Ecoli           VGALLQIGQGGWVS----FLSFIAVLIASINIFGGFTVTQRMLKMFRKNMS--------- 512
                **.*  :*     :     *: :*.:::*:** *** ******.**::  .

                      V606A    I622fsX1   S627L  G633S                 M652T
Human           LPAGTFVGGYLAALYSGYNIEQIMYLGSGLCCVGALAGLSTQGTARLGNALGMIGVAGGL 659
Cow             LPAGTFVGGYLASLYSGYNIEQIMYLGSGLCCVGALAGLSTQGTARLGNALGMIGVAGGL 659
Mouse           LPGGTFVGGYLAALYGGYNIEEIMYLGSGLCCVGALGGLSTQGTARLGNALGMIGVAGGL 659
Rat             LPGGTFVGGYLAALYGGYNIEEIMYLGSGLCCVGALGGLSTQGTARLGNALGMIGVAGGL 659
Frog            LPGGGFVGGYAAALHSGYDIEQMVYLGSGLCCVGALAGLSTQGTARLGNSLGMMGVAGGI 659
Zebrafish       LPTGVFVGGYGVALQSGYNIEQMMYLGSGLCCVGALGGLSTQSTARLGNALGMIGVAGGI 655
Ecoli           -------GG---LVTAAYIVAAILFIFS-------LAGLSKHETSRQGNNFGIAGMAIAL 555
                       **    : ..* :  :::: *       *.***.: *:* ** :*: *:* .:

               L663F:G664R    G678R  T689fsX320 R693H
Human           AATLGVLKPGPELLAQMSGAMALGGTIGLTIAKRIQISDLPQLVAASHSLVGLAAVLTCI 719
Cow             AATLGGLKPCPELLAQMSGAMALGGTIGLTIAKRIQISDLPQLVAAFHSLVGLAAVLTCI 719
Mouse           AATLGGLKPDPQLLAQMSGAMAMGGTIGLTIAKRIQISDLPQLVAAFHSLVGLAAVLTCM 719
Rat             AATLGGLKPDPQLLAQMSGAMAMGGTIGLAIAKRIQISDLPQLVAAFHSLVGLAAVLTCM 719
Frog            AATLGALKPSPELLAQMSGAMALGGTIGLTIAKRIQISDLPQLVAAFHSLVGLAAVLTCV 719
Zebrafish       AATFGVLKPSPELMAQMSAAMAVGGTAGLTIAKKIQISDLPQLVAAFHSLVGLAAVLTCV 715
Ecoli           IATI--FGPDTGNVGWILLAMVIGGAIGIRLAKKVEMTEMPELVAILHSFVGLAAVLVGF 613
                 **:  : * .  :. :  **.:**: *: :**:::::::*:***  **:*******. .

                           T731M
Human           AEYIIEYPHFATDAAANLTKIVAYLGTYIGGVTFSGSLIAYGKLQGLLKSAPLLLPGRHL 779
Cow             AEYIIEYPHFATDAAANLTKIVAYLGTYIGGVTFSGSLVAYGKLQGILKSAPLLLPGRHL 779
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Mouse          AEYIVEYPHFAMDATSNFTKIVAYLGTYIGGVTFSGSLVAYGKLQGILKSAPLLLPGRHA 779
Rat            AEYIVEYPHFAMDATSNFTKIVAYIGTYIGGVTFSGSLVAYGKLQGILKSAPLLLPGRHA 779
Frog           AEYMIEYPHFATDPAANLTKIVAYLGTYIGGVTFSGSLVAYGKLQGVLNSAPLLLPGRHM 779
Zebrafish      AEYMVEYPHFATDPAANLTKIVAYLGTYIGGVTFSGSLVAYGKLQGLLNSAPLMLPGRHA 775
Ecoli          NSYLHHDAGMAP-ILVNIHLTEVFLGIFIGAVTFTGSVVAFGKLCGKISSKPLMLPNRHK 672
                .*: . . :*:    *:    .::* :**.***:**::*:*** * :.* **:**.**

                           M796V:S800E:T802P/I        V817E           V834I
Human          LNAGLLAASVGGIIPFMVDPSFTTGITCLGSVSALSAVMGVTLTAAIGGADMPVVITVLN 839
Cow            LNAGLLAGSVGGIIPFMMDPSFTTGITCLGSVSALSAVMGVTLTAAIGGADMPVVITVLN 839
Mouse          LNAGLLAASVGGIIPFMADPSFTTGITCLGSVSGLSTLMGVTLTAAIGGADMPVVITVLN 839
Rat            LNAGLLAASVGGIIPFMADPSFTTGIMCLGSVSALSTLMGVTLTAAIGGADMPVVITVLN 839
Frog           LNAGLLTASVGGIIPYMLDPSYTTGITCLGSVSALSAVMGVTLTAAIGGADMPVVITVLN 839
Zebrafish      LNATLMAASVGGMIPYMLDPSYTTGITCLGSVSALSAVMGLTLTAAIGGADMPVVITVLN 835
Ecoli          MNLAALVVSFLLLIVFVRTDSVGLQVLALLIMTAIALVFGWHLVASIGGADMPVVVSMLN 732
               :*   :. *.  :* ::   *    : .*  ::.:: ::*  *.*:*********:::**

                             T859S  G862D      Y874C  M880X
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Cow            SYSGWALCAEGFLLNNNLLTIVGALIGSSGAILSYIMCVAMNRSLANVILGGYGTTSTAG 899
Mouse          SYSGWALCAEGFLLNNNLLTIVGALIGSSGAILSYIMCVAMNRSLANVILGGYGTTSTAG 899
Rat            SYSGWALCAEGFLLNNNLLTIVGALIGSSGAILSYIMCVAMNRSLANVILGGYGTTSTAG 899
Frog           SYSGWALCAEGFLLNNNLLTIVGALIGSSGAILSYIMCVAMNRSLTNVILGGYGTTSTAG 899
Zebrafish      SYSGWALCAEGFLLNNNLLTIVGALIGSSGAILSYIMCVAMNRSLANVILGGYGTSSTGT 895
Ecoli          SYSGWAAAAAGFMLSNDLLIVTGALVGSSGAILSYIMCKAMNRSFISVIAGGFGTDGSST 792
               ****** .* **:*.*:** :.***:***** ****** *****: .** **:** .:.

               P902S:T910M:N913S:I918V:A924V:S926N   A937V
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Cow            GKPMEISGTHTEINLDNAIDMIREANSIIITPGYGLCAAKAQYPIADLVKMLSEQGKKVR 959
Mouse          GKPMEISGTHTEINLDNAVEMIREANSIVITPGYGLCAAKAQYPIADLVKMLTEQGKKVR 959
Rat            GKPMEISGTHTEINLDNAVEMIREANSIVITPGYGLCAAKAQYPIADLVKMLTEQGKKVR 959
Frog           GKPMEITGTHTEINLDNAVEYIREANNIIITPGYGLCAAKAQYPIADLVKILKEAGKNVR 959
Zebrafish      GKPMEITGTHTEVNVDQTVDLIKEAHNIIIVPGYGLCAAKAQYPIADLVKSLTDQGKKVR 955
Ecoli          GDDQEVG-EHREITAEETAELLKNSHSVIITPGYGMAVAQAQYPVAEITEKLRARGINVR 851
               *.  *:   * *:. ::: : :::::.:::.****:..*:****:*::.: *   * :**

                                L977P            I993V    A1008P:N1009K
Human          FGIHPVAGRMPGQLNVLLAEAGVPYDIVLEMDEINHDFPDTDLVLVIGANDTVNSAAQED 1019
Cow            FGIHPVAGRMPGQLNVLLAEAGVPYDIVLEMDEINHDFPDTDLVLVIGANDTVNSAAQED 1019
Mouse          FGIHPVAGRMPGQLNVLLAEAGVPYDIVLEMDEINSDFPDTDLVLVIGANDTVNSAAQED 1019
Rat            FGIHPVAGRMPGQLNVLLAEAGVPYDIVLEMDEINSDFPDTDLVLVIGANDTVNSAAQED 1019
Frog           FGIHPVAGRMPGQLNVLLAEAGVPYDIVLEMDEINEDFPETDLVLVIGANDTVNSAAQED 1019
Zebrafish      FGIHPVAGRMPGQLNVLLAEAGVPYDVVLEMDEINEDFPETDLVLVIGANDTVNSAAQED 1015
Ecoli          FGIHPVAGRLPGHMNVLLAEAKVPYDIVLEMDEINDDFADTDTVLVIGANDTVNPAAQDD 911
               *********:**::******* ****:******** **.:** **********.***:*

          P1020A:I1023F                                          A1075V:A1078E
Human          PNSIIAGMPVLEVWKSKQVIVMKRSLGVGYAAVDNPIFYKPNTAMLLGDAKKTCDALQAK 1079
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Mouse          PNSIIAGMPVLEVWKSKQVIVMKRSLGVGYAAVDNPIFYKPNTAMLLGDAKKTCDALQAK 1079
Rat            PNSIIAGMPVLEVWKSKQVIVMKRSLGVGYAAVDNPIFYKPNTAMLLGDAKKTCDALQAK 1079
Frog           PNSIIAGMPVLEVWKSKQVIVMKRSLGVGYAAVDNPIFYKPNTSMLLGDAKKTCDSLQAK 1079
Zebrafish      PNSIIAGMPVLEVWKSKQVVVMKRSLGVGYAAVDNPIFYKPNTSMLLGDAKKTCDALSAK 1075
Ecoli          PKSPIAGMPVLEVWKAQNVIVFKRSMNTGYAGVQNPLFFKENTHMLFGDAKASVDAILKA 971
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Human          VRESYQK 1086
Cow            VRESYQK 1086
Mouse          VRESYQK 1086
Rat            VRESYQK 1086
Frog           VRESYQN 1086
Zebrafish      VREG--- 1079
Ecoli          L------ 972
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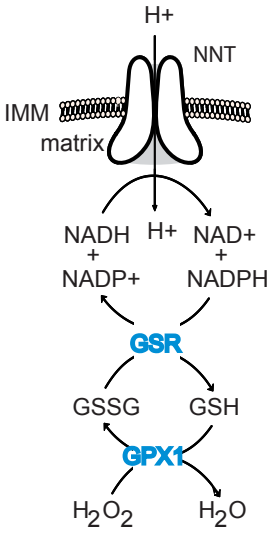
Supplementary Figure 2 (continued)

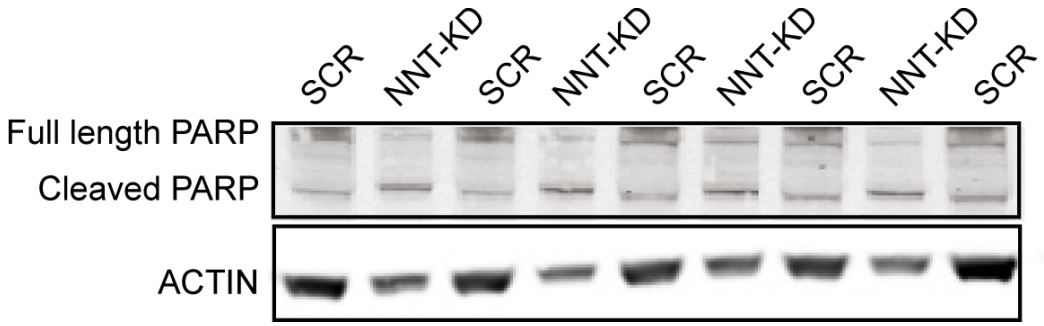
Multiple sequence alignment of NNT protein sequences. Alignment source file accession numbers: Human gi:1110520, gb:AAC51914; Cow gi:163410, gb:AAA30660; Mouse gi:11245971,gb:AAF72982; Rat gi:60552131, gb:AAH91271; Frog gi:148231869, NP_001087704; Zebrafish gi:161611474, gb:AAI55746; E. coli sequence is a composite of [Escherichia coli str. K12 substr. MG1655] pyridine nucleotide transhydrogenase, alpha subunit gi:16129561, gb:NP_416120 and pyridine nucleotide transhydrogenase, beta subunit gi: 16129560, gb:NP_416119, the end of the alpha subunit is highlighted in red and the beginning of the beta subunit is highlighted in green. Sequence conservation is beneath the alignment, * = total conservation, : = partial conservation. The positions of nonsense/frameshift (in red) and missense mutations (in blue) are indicated. The M1? change to the initiating methionine is indicated in green and the 44 residue presequence targeting the protein to mitochondria is shaded in grey. All known non-synonymous coding SNPs from The NHLBI "Grand Opportunity" Exome Sequencing Project (GO-ESP) [https://esp.gs.washington.edu/drupal/] and other SNP databases are highlighted in yellow (minor allele frequencies ranging from 0.0093 to 4.43%). The gene has now been sequenced in >10,000 alleles (consisting of approx 7000 European American and 3700 African American alleles) as part of the NHLBI GO-ESP. None of the mutations reported in the paper occur in these populations. 55 missense variants exist in this database almost all with minor allele frequencies (MAF) <0.5% and there are no homozygous individuals, although there may be compound heterozygotes but at extremely low frequencies. The exceptions to this are three SNPS rs75710404, rs35201656 and rs41271083 resulting in amino acid changes T731M, K63R and L663F; having MAF 0.83%, 4.43% and 4.3%; with 1, 4 and 4 homozygous individuals in the database respectively. These variants have been annotated on the alignment in supplementary figure 2, highlighted in yellow.

Supplementary Figure 3



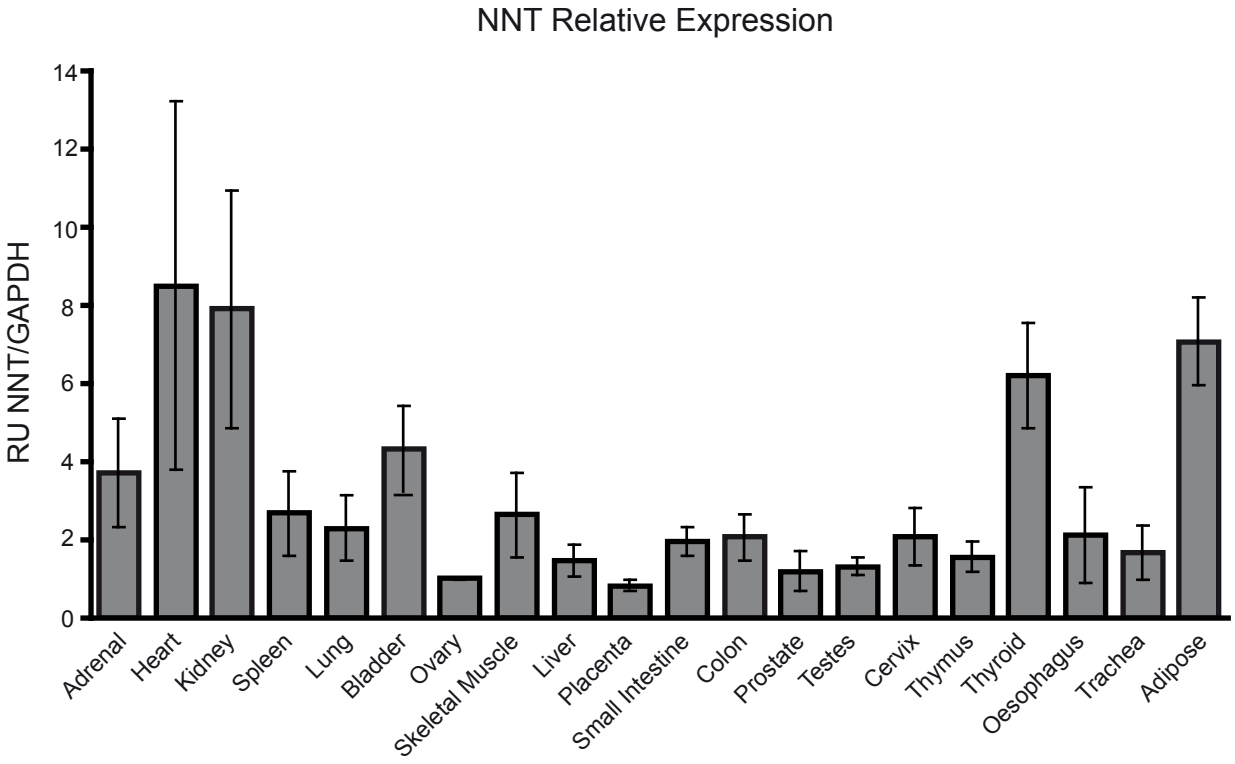
NNT pumps protons across the inner mitochondrial membrane (IMM). Detoxification of reactive oxygen species (ROS) depends upon maintenance of a high GSH/GSSG ratio.

Supplementary Figure 4



Western blot analysis showing full length and cleaved PARP from NNT-KD and SCR cells (upper panel) and actin (lower panel). NNT-KD cells show an increase in PARP cleavage relative to SCR controls $p<0.001$

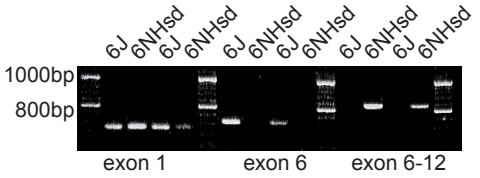
Supplementary Figure 5



Graph indicating mRNA expression of NNT relative to GAPDH in human tissues analyzed by RT-PCR. Expression was most readily detectable in adrenal gland, heart, kidney, thyroid and adipose tissue.

Supplementary Figure 6

	6J	6NHsd
exon 1	242bp	242bp
exon 6	312bp	-ve
exon 6-12	-ve	542bp



Mice were genotyped for Nnt status using previously published primers [Huang]. Briefly PCRs were performed targeting; exon 1 as a positive PCR control which amplifies in both mouse sub-strains, exon 6 which is deleted in the Nnt mutant (6J) but will amplify in the wild-type 6NHsd mice, and the breakpoint junction of the deletion which will amplify in 6J but not in 6NHsd.