

# Genome sequencing reveals mitochondrial thiol systems are essential for antioxidant defence in human adrenal glands

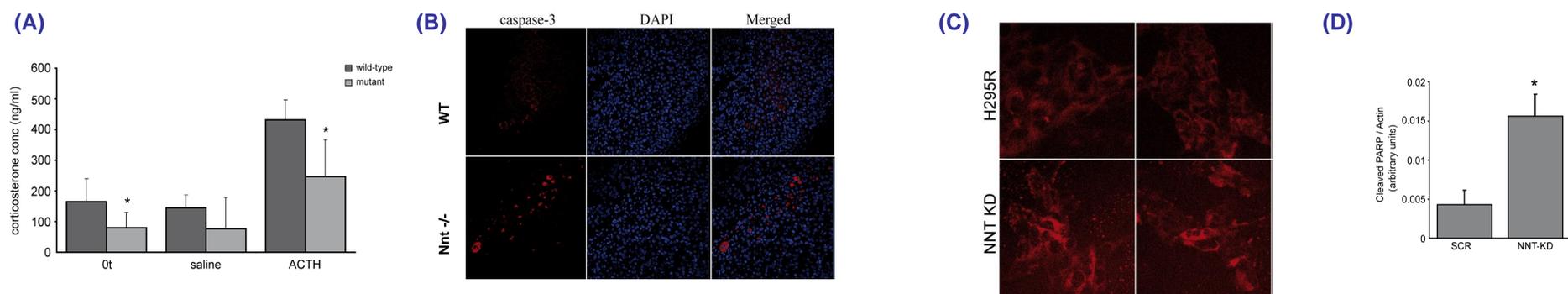
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## INTRODUCTION

Familial Glucocorticoid deficiency (FGD) is a disease in which the cells of the zona fasciculata within the adrenal cortex fail to respond appropriately to stimulation by ACTH to produce cortisol. The disease is characterised by isolated glucocorticoid deficiency and patients therefore exhibit low or often undetectable serum cortisol with high plasma ACTH levels. We have previously demonstrated that oxidative stress is implicated in the pathogenesis of the disease. Specifically we identified the first human mutations in the antioxidant gene encoding the mitochondrial anti-oxidant nicotinamide nucleotide transhydrogenase (NNT) in patients with FGD. Further whole genome sequencing of FGD patients with unknown aetiology revealed a novel homozygous mutation, p.Y447X, in the mitochondrial selenoprotein, thioredoxin reductase 2 (TXNRD2) in one large kindred and two further homozygous mutations in glutathione peroxidase 1 (GPX1) and peroxiredoxin 3 (PRDX3) in a single affected individual. In this study we aim to investigate the effect of these antioxidant genes in adrenal tissue.

## RESULTS 1



**Figure 1 Absence of NNT affects steroidogenesis in mice and induces oxidative stress in human adrenocortical H295R cells.** (A) Corticosterone levels in wt and mutant mouse serum stimulated with ACTH. Three-month old male wt and *Nnt*<sup>-/-</sup> mice were injected ip with saline or 125µg ACTH. Blood serum was collected and radioimmunoassay results revealed both basal and stimulated corticosterone levels were lower in mutant mice (\*p<0.05).

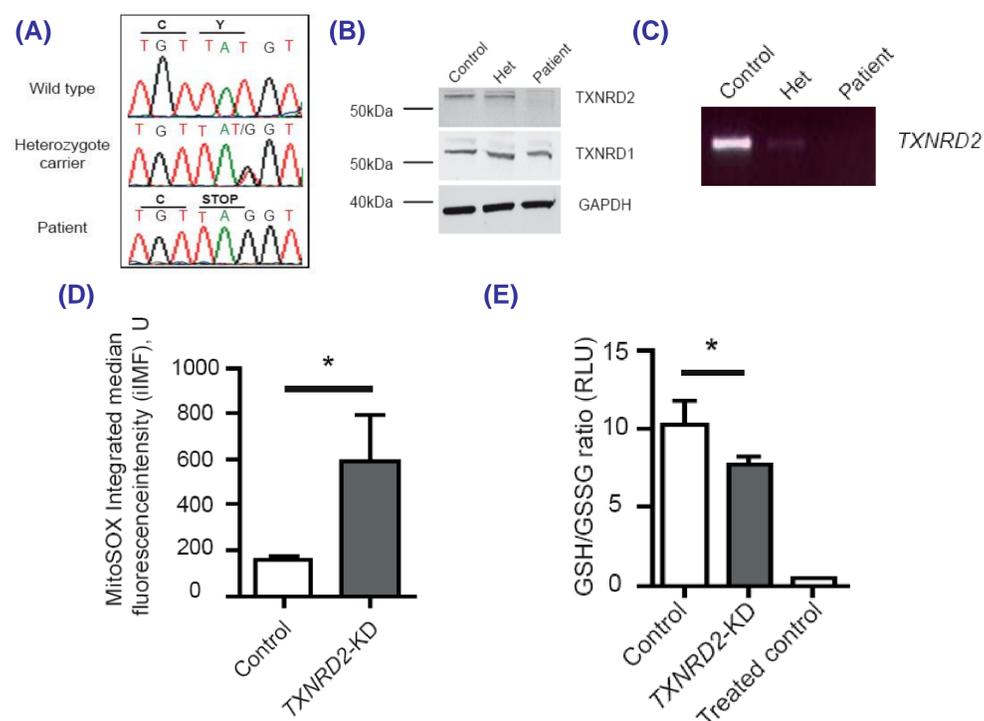
(B) Increased apoptosis in *Nnt*<sup>-/-</sup> mutant mice. Whole adrenals were immunostained with cleaved caspase-3 (c-cleaved) antibody, a marker of apoptosis. Adrenals from mutant mice showed a higher number of caspase 3 positive cells (red) present in zona fasciculata than the wt.

(C) ROS production in knockdown of *NNT* in the human adrenocortical H295R cell line by shRNA Mitosox. Quantitative analysis showed a significant increase (p<0.005) in superoxide production in NNT-KD relative to SCR cells.

(D) Densitometric analysis showed a significant (p< 0.001) increase in cleaved PARP between SCR and NNT-KD cells. Values are intensities of cleaved PARP relative to actin, n = 12.

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## RESULTS 2

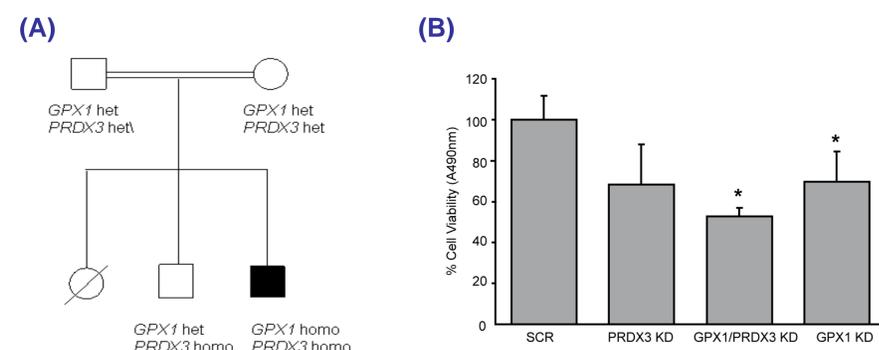


**Figure 2 p.Y447X TXNRD2 mutation leads to apparent loss of TXNRD2 protein and impairs redox homeostasis in human adrenocortical cells.**

(A) Partial sequence chromatograms of genomic cDNA from wild-type, heterozygote carrier and patient, showing a T>G base change, resulting in a premature stop codon. (B) Lysates from a homozygous patient, heterozygote carrier and control human lymphocytes were immunoblotted with an anti-TXNRD2 antibody. While control and the heterozygote carriers expressed the 56 kDa protein, this is absent in the homozygote patient with no evidence of a truncated protein. All individuals express cytoplasmic TXNRD1 normally. (C) RT-PCR of cDNA from patient, heterozygote carrier and control suggested nonsense mediated decay of mRNA since direct sequencing of the amplicon from a heterozygote carrier revealed amplification of the wild-type sequence alone. (D) Quantitative analysis of superoxide production by MitoSOX, shows a significant increase in superoxide production in knockdown cells relative to controls; (n=3). Error bars represent standard deviation (\* p< 0.05). (E) Increased pressure on the glutathione system is observed with a significant decrease in the reduced: oxidised glutathione (GSH: GSSG) ratio. Oxidative stress induced by 25 µM menadione in the control cells reduced this ratio to 0.4±0.1 (n=4).

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## RESULTS 3



**Figure 3 Double Knockout of GPX1 and PRDX3 decreases viability of adrenocortical cells.** (A) Pedigree of family showing homozygous mutations p.130-133delREAL in *GPX1* and p.Q67X in *PRDX3*. The *PRDX3* mutation was heterozygous in the parents but homozygous in the unaffected brother. Stable knockdown of both *PRDX3* and *GPX1* in human adrenocortical cells was achieved by lentiviral transduction. (B) An MTS assay was used to determine cell viability measuring absorbance at 490nm. Only disruption to both *GPX1* and *PRDX3* significantly decreased viability of adrenocortical cells. The data are expressed as % of cell viability ± SD, n=3 \*p<0.05.

## Conclusions

NNT is highly expressed in the adrenal and its knockdown/out leads to increased levels of ROS and reduced glucocorticoid production. Furthermore mutation in TXNRD2 is associated with a predominantly adrenal phenotype in humans, indicating the importance of the thioredoxinsystem in maintaining redox homeostasis in the adrenocortical environment. Loss of *PRDX3* alone is insufficient to cause FGD however the mutation in *GPX1*, either alone or in combination with *PRDX3*, may tip the redox balance to cause FGD. These findings suggest that a delicate balance of mitochondrial redox regulation controls steroidogenesis within the adrenal gland and that perturbation of one or more components of the regulatory system (Figure 4) can lead to FGD.

**Figure 4 Schematic representation of mitochondrial electron transport chain.**

