Compensation for chronic oxidative stress in ALADIN null mice

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Key words: ALADIN, oxidative stress, paraquat, redox homeostasis, triple A syndrome.

Summary statement: This study demonstrates that ALADIN knock-out mice present with an unexpected compensated metabolism of glutathione after application of chronic oxidative stress, whilst lacking a phenotype resembling human triple A syndrome.
ABSTRACT
Mutations in the AAAS gene coding for the nuclear pore complex protein ALADIN lead to the autosomal recessive disorder triple A syndrome. Triple A patients present with a characteristic phenotype including alacrima, achalasia and adrenal insufficiency. Patient fibroblasts show increased levels of oxidative stress, and several in vitro studies have demonstrated that the nucleoporin ALADIN is involved in both the cellular oxidative stress response and adrenal steroidogenesis. It is known that ALADIN knock-out mice lack a phenotype resembling human triple A syndrome. The objective of this study was to determine whether the application of chronic oxidative stress by ingestion of paraquat would generate a triple A-like phenotype in ALADIN null mice. Adult male mice were fed with either a paraquat (0.25 g/kg diet) or control diet for 11 days. After application of chronic oxidative stress, ALADIN knock-out mice presented with an unexpected compensated glutathione metabolism, but lacked a phenotype resembling human triple A syndrome. We did not observe increased levels of oxidative stress and alterations in adrenal steroidogenesis in mice depleted for ALADIN. This study stresses the species-specific role of the nucleoporin ALADIN, which in mice involves a novel compensatory mechanism for regulating the cellular glutathione redox response.

INTRODUCTION
The triple A syndrome (OMIM #231550), a rare autosomal recessive disorder, is caused by homozygous or compound heterozygous mutations in the AAAS (achalasia-adrenal insufficiency-alacrima syndrome) gene encoding the nucleoporin ALADIN (alacrima-achalasia-adrenal insufficiency neurologic disorder) (Handschug et al., 2001; Tullio-Pelet et al., 2000). ALADIN is anchored within the nuclear pore complex by the transmembrane nucleoporin NDC1 (nuclear division cycle 1 homologue (S. cerevisiae)) (Kind et al., 2009; Yamazumi et al., 2009). Rabut et al. suggested that ALADIN forms part of the structural backbone of the nuclear pore complex but is not required for the integrity of the complex (Rabut et al., 2004). Triple A patients present with the characteristic triad of adrenocorticotropic hormone-resistant adrenal insufficiency, achalasia of the stomach cardia and alacrima in combination with progressive neurological impairments (Allgrove et al., 1978). Phenotypic appearance of all symptoms is heterogeneous and highly variable. Adrenal atrophy may occur later in life and may develop gradually (Huebner et al., 2002; Milenkovic et al., 2010).

In contrast to other organs with high metabolic rates, the adrenal gland has high levels of enzymatic and non-enzymatic anti-oxidants (Prasad et al., 2014). Imbalances in reactive oxygen species (ROS) result in cellular oxidative stress and have been implicated in a variety of diseases (Prasad et al., 2014). Adrenocortical mitochondrial steroidogenesis increases ROS formation in the cell because the uncoupling of the cytochrome P450 enzyme (CYP) redox reaction can occur at
several steps of the reaction (Dekant, 2009; Rapoport et al., 1995). Under these circumstances, superoxide anions and hydrogen peroxide can leak and escape from the redox reaction (Dekant, 2009). Therefore, a balanced level of anti-oxidative mechanisms is crucially important in adrenocortical cells.

It has previously been reported that ALADIN is involved in the cellular oxidative stress response in adrenocortical and fibroblast cells in vitro; however, the role of ALADIN in adrenal steroidogenesis and how ALADIN might contribute to adrenal atrophy in triple A patients remains unclear (Hirano et al., 2006; Jühlen et al., 2015; Kind et al., 2010; Koehler et al., 2013; Prasad et al., 2013; Storr et al., 2009). We have shown that depletion of ALADIN in human adrenocortical carcinoma cells leads to an alteration in glucocorticoid and androgen steroidogenesis (Jühlen et al., 2015). Recently, we identified progesterone receptor membrane compartment 2 (PGRMC2) as a novel protein interactor of ALADIN (Jühlen et al., 2016). Microsomal PGRMC2 itself seems to be involved in adrenal steroidogenesis either by regulating heme synthesis, the prosthetic group of microsomal CYPs, or by acting as an electron donor for several CYPs (Piel et al., 2016; Wendler and Wehling, 2013).

Furthermore, in previous work our group has shown that female homozygous mice deficient for Aaas are infertile while otherwise ALADIN null mice present with a mild phenotype (Huebner et al., 2006). Carvalhal et al. showed that female sterility in ALADIN-deficient mice is caused by impaired chromosomal segregation and maturation of oocytes (Carvalhal et al., 2016). More recently, it was shown that conditional ablation of ALADIN interactor PGRMC2 from the female reproductive tract results in reproductive senescence (Clark et al., 2016).

Here, we attempted to verify the critical role of ALADIN in the cellular redox regulation using ALADIN null mice. We hypothesized that application of oxidative stress in mice deficient for ALADIN would generate a phenotype similar to that of triple A patients. In order to increase the sensitivity for oxidative stress we used, in addition to our Aaas knock-out (KO) mice, offspring from intercrossed heterozygous (Het) Sod2 (superoxide dismutase 2) and Aaas KO mice to obtain Aaas KO/Sod2 Het mice. Because heterozygous Sod2 null mice exhibit increased levels of ROS it seemed reasonable to hypothesize that Aaas KO/Sod2 Het mice would present with increased susceptibility to oxidative stress exposure. Unexpectedly, ALADIN null mice still lacked a phenotype related to triple A syndrome in humans and showed a compensated glutathione metabolism.
RESULTS

- Chronic oxidative stress is independent of redox-regulated \textit{Hmox1} expression

In order to increase the sensitivity for chronic oxidative stress we used double transgenic \textit{Aaas KO/Sod2 Het} mice. SOD2 is a mitochondrial antioxidant enzyme essential for transforming and detoxification of free superoxide anions leaking from the mitochondrial aerobic respiration (Li et al., 1995). Mitochondrial SOD2 catalyzes the conversion of free anion radicals to hydrogen peroxide, which in turn can be neutralized by downstream enzymes. Measuring the hepatic and adrenal \textit{Sod2} expression by quantitative real-time PCR (qPCR) we determined as expected that \textit{Sod2} expression was about two-fold diminished in \textit{Aaas KO/Sod2 Het} mice under control or paraquat diet compared to WT and \textit{Aaas KO} mice of the same diet (Fig 1A).

In the next step, the level of oxidative stress adrenal \textit{Hmox1} gene expression was measured by qPCR. \textit{Hmox1} is a widely-used redox-regulated gene whose transcriptional activation is dependent on upstream transcriptional regulators which are induced by a broad spectrum of conditions involving oxidative stress, nitrosative stress, thiol-reactive substances and cytokines (Ryter and Choi, 2005). We did not see an increased expression of \textit{Hmox1} in animals under a paraquat diet compared to a control diet (Fig 1B). However, under the control diet the expression was significantly decreased in \textit{Aaas KO/Sod2 Het} compared to \textit{Aaas KO} animals.

- Adrenal steroid output is comparable after control and paraquat diet

The investigation of the expression of different enzymes of steroidogenesis revealed that the expression of \textit{Star} was increased in \textit{Aaas KO} versus WT animals after paraquat diet (Fig 2A). Furthermore, \textit{Aaas KO/Sod2 Het} mice under paraquat diet presented with decreased expression of \textit{Star} compared to \textit{Aaas KO} mice on the same diet. However, neither expression levels of \textit{Cyp21a1}, \textit{Cyp11a1}, \textit{Cyp11b1}, \textit{Cyp11b2}, and \textit{Hsd3b2} were changed nor could we see a specific effect depending on the genotype of the mice (Fig S1A-E).

Plasma levels of pregnenolone, progesterone, deoxycorticosterone, corticosterone, aldosterone and dehydroepiandrosterone sulfate were not significantly altered by paraquat diet or among the different genotypes (Fig S2A-F). Plasma levels of 17-hydroxyprogesterone and dehydroepiandrosterone were under detection threshold. Production of androstenedione, which mice only synthesize in the gonads, increased about five-fold in WT animals after ingestion of paraquat compared to WT animals on the control diet (Fig 2B). Furthermore, androstenedione levels in \textit{Aaas KO} animals decreased about 25-fold after paraquat diet compared with WT animals also assigned a paraquat diet (Fig 2B).
• Paraquat diet and ALADIN depletion decrease body weight gain

In the mice fed the control diet, food intake over an 11 day period significantly decreased in *Aaas KO/Sod2* Het mice compared to WT mice (Fig 3A). There was also a slight decline in intake in *Aaas KO* animals. Weight gain in *Aaas KO* and *Aaas KO/Sod2* Het mice was about two-fold diminished in the control diet compared to the WT animals (Fig 3B).

WT mice on the paraquat diet consumed less compared to those on the control diet; however, food intake in *Aaas KO/Sod2* Het mice was higher versus the WT (Fig 3A). Accordingly, WT animals gained about 20-fold less weight compared to the control diet and weight gain was also about four-fold lowered in *Aaas KO* and *Aaas KO/Sod2* Het animals versus the control diet despite increased food intake (Fig 3A-B).

• Hepatic glutathione levels are balanced in ALADIN null mice

Glutathione (GSH) is an important antioxidant in cells and is converted to its oxidized form, glutathione disulfide (GSSG), to detoxify ROS. The ratio of reduced to oxidized glutathione (GSH/GSSG) decreases when cells are exposed to increased levels of oxidative stress; GSSG accumulates and GSH is consumed. Surprisingly, hepatic GSH/GSSG ratios in *Aaas KO/Sod2* Het animals on both the control and paraquat diets increased about five-fold compared to WT animals on the same diet (Fig 4A). Additionally, the paraquat diet increased the ratio significantly in *Aaas KO/Sod2* Het animals compared to the same genotype under the control diet. GSH/GSSG ratios of *Aaas KO* mice were similar to those of WT mice.

GSH concentrations were higher in *Aaas KO/Sod2* Het livers under either the control or paraquat diet versus WT and *Aaas KO* mice on the same diet (Fig 4B). Hepatic GSH content in *Aaas KO* mice were comparable to WT mice.

Similarly, hepatic GSSG concentrations were about two-fold diminished in *Aaas KO/Sod2* Het animals compared to WT and *Aaas KO* animals on the same diet (Fig 4C). Furthermore, GSSG concentrations decreased in *Aaas KO/Sod2* Het mice on the paraquat diet. Interestingly, GSSG content in *Aaas KO* mice on the control diet was significantly higher compared to WT and *Aaas KO/Sod2* Het mice. This effect was reversed under the paraquat diet: GSSG concentration in *Aaas KO* animals decreased compared to the control diet.

Glutathione peroxidase (GPX) catalyzes the detoxification of hydrogen peroxide to water by producing GSSG. Glutathione reductase (GSR) maintains GSH levels from GSSG by consuming nicotinamide adenine dinucleotide phosphate (NADPH), which in turn is made from nicotinamide adenine dinucleotide (NADH) by mitochondrial nicotinamide nucleotide transhydrogenase (NNT). To
verify our previous measurements of hepatic GSH/GSSG ratios, we assessed the hepatic expression of *Gpx1* and *Gsr* as well as the adrenal and hepatic expression of *Nnt*. We could see no alteration in the expression of *Gpx1* in control and paraquat diet animals and in the different genotypes (Fig S3A). However, *Aaas KO/Sod2 Het* mice under paraquat diet presented with decreased expression of *Gsr* compared to *Aaas KO* mice (Fig S3B). Most strikingly, hepatic and adrenal expression of *Nnt* was about two-fold increased in *Aaas KO* and *Aaas KO/Sod2* Het mice under control diet versus the WT (Fig 4D). Under paraquat diet hepatic *Nnt* expression was still higher in *Aaas KO* animals compared to WT animals and adrenal *Nnt* expression was significantly increased in *Aaas KO/Sod2* Het mice compared to WT animals under paraquat diet.

We additionally measured the end-products of hepatic lipid peroxidation (thiobarbituric acid reactive substances, TBARS) to estimate the level of oxidative stress in our animals. Relative liver and lung weights and hepatic TBARS values were not altered upon oxidative stress exposure using a paraquat diet or in the different genotypes (Figs S4 and S5). No pathological differences in histology sections of brain, duodenum, liver and lung could be found (data not shown).

**DISCUSSION**

In the present study we investigated the role of the nucleoporin ALADIN in chronic paraquat-induced oxidative stress in male mice. ALADIN-deficient mice lack a triple A syndrome-characteristic phenotype (Huebner et al., 2006). Previous studies have demonstrated that ALADIN employs a crucial role in the redox response of the cell *in vitro* (Hirano et al., 2006; Jühlen et al., 2015; Kind et al., 2010; Koehler et al., 2013; Prasad et al., 2013; Storr et al., 2009). Triple A patients as well suffer from increased cellular oxidative stress as shown by Fragoso et al. (Fragoso et al., 2017). Thus, we hypothesized that chronic oxidative stress would unmask the distinct phenotype in ALADIN null mice.

Overall, we did not see a triple A syndrome-characteristic phenotype in mice depleted for ALADIN after chronic oxidative stress exposure. Prior to this study we performed a pilot experiment inducing acute oxidative stress in mice by injection with paraquat i.p. (25 mg/kg body weight) but no involvement of ALADIN in the acute oxidative stress response was ascertained (data not shown). The results for our *in vivo* murine model do not follow those expected from various human *in vitro* cell systems in which depletion of ALADIN leads to disturbed redox homeostasis and altered adrenal steroidogenesis (Jühlen et al., 2015; 2010; Prasad et al., 2013). We assume that this discrepancy is either a result of a possible species-specific role of ALADIN or of the experimental nature of the *in vitro* versus *in vivo* models. The ALADIN gene is highly conserved between human, mouse (93.6%) and rat (92.3%). The pairwise alignment of *M.musculus* and *R.norvegicus* ALADIN sequence
revealed a 97.1% homology between these rodents.

Our data indicate that on one hand, mice depleted for ALADIN during basal conditions and after chronic oxidative stress exposure sustain balanced hepatic glutathione levels by up-regulation of Nnt resulting in a WT-like phenotype. On the other hand, Aaas KO/Sod2 Het mice under basal conditions increase hepatic glutathione levels by increasing Nnt expression. This effect was intensified after chronic oxidative stress exposure. In the cell, transmembrane nicotinamide nucleotide transhydrogenase (NNT) plays a key role in the mitochondrial defense system against reactive oxygen species (ROS) by producing NADPH (Fig 5). NADPH is in turn consumed by glutathione reductase (GSR) maintaining reduced glutathione (GSH) levels from oxidized glutathione (GSSG) (Krengel and Törnroth-Horsefield, 2015). ROS and in particular superoxide anions leaking during mitochondrial aerobic respiration or produced by exogenous stressors are converted to hydrogen peroxide by mitochondrial superoxide dismutase (SOD2). Hydrogen peroxide is then neutralized to water by consuming GSH by several peroxidases (GPX).

It has already been shown that heterozygous deficiency for Sod2 in mice activates mitochondrial uncoupling to reduce ROS production and increases aerobic glycolysis by a free radical-mediated mechanism (Xu et al., 2015). Mice heterozygously deficient for Sod2 exhibit increased levels of ROS and shift from mitochondrial oxidative phosphorylation to a cytosolic glycolytic pathway (Xu et al., 2015). During aerobic glycolysis, a high rate of energy is produced by metabolizing glucose into pyruvate which then feeds into cytosolic lactic acid fermentation rather than mitochondrial oxidation, commonly known as the Warburg effect (Vander Heiden et al., 2009; Xu et al., 2015). It can thus be assumed that the phenotypic effects observed in Aaas KO/Sod2 Het mice in the present study are caused by both the Warburg effect and increased expression of Nnt. These additive actions lead to transient increase of glutathione oxidative capacity and to an enhancement of the compensatory effect seen in Aaas KO mice, which lack typical symptoms of triple A syndrome. Thus, we suggest that ALADIN plays a crucial role in regulating NADPH levels in the cell and concommitantly enhances oxidative capacity of glutathione by altered gene expression of NNT. Interestingly, it has been demonstrated that both under- or overexpression of Nnt reduced corticosterone output in mice implying a central role for it in the control of steroidogenesis (Meimaridou et al., 2017). Furthermore, gene down-regulation of Nnt has been associated with age-related neurodegeneration in Alzheimer disease-like mouse neurons (Ghosh et al., 2014). It has been reported that NAD(P)H redox control is more critical than GSH content in promoting neurodegeneration (Ghosh et al., 2014). This result partly explains why mice depleted for ALADIN do not present with a triple A syndrome-distinct phenotype but rather behave like WT animals.
We based our study of chronic paraquat-induced oxidative stress on the work of Aoki and colleagues in which a 0.025% paraquat enriched diet was also used to induce oxidative stress in four-week-old juvenile male rats (Aoki et al., 2002). In contrast to our results, Aoki et al. found that by feeding rats the paraquat diet animals suffered from elevated hepatic lipid (TBARS) and glutathione (GSSG) oxidation, liver organ shrinkage and lung enlargement (Aoki et al., 2002). We could not reproduce these results in our mice. This may be due to the different age of the animals or to different anti-oxidant defenses in the two rodent species. Results from Aoki et al. regarding food intake and body weight gain were consistent with our study (Aoki et al., 2002). Here, we show that depletion of ALADIN in mice negatively affected body weight gain under normal control and paraquat diet. This result is underlined by increased food intake under paraquat diet in these animals.

Our in vivo study is the first to highlight a species-specific role of the nucleoporin ALADIN. The data suggest involvement of a complex cellular system that compensates for the depletion of ALADIN, which seems to have an important role in balancing cellular NADPH levels. Future research on oxidative stress pathways in mice shall address how this possible compensating mechanism is function and may further clarify the role of ALADIN in the pathogenesis of triple A syndrome.

MATERIALS AND METHODS

- Experimental animals and treatments

All mice were housed in the animal care facility (Experimental Center) of the Technical University Dresden, Dresden, Germany. All procedures were approved by the Regional Board for Veterinarian Affairs, Dresden, Germany (AZ 24-9168.11-1/2010-49) in accordance with the institutional guidelines for the care and use of laboratory animals. Animals were group housed except during actual experimental procedures, when single housing was required. Mice were kept under specific-pathogen-free conditions at a constant temperature (22 ± 1°C) and a 12 hours light-dark cycle at all times. Mice were weaned onto ssniff R/M-H (19% protein, 4.9% fibers, 3.3% fat, 12.2 MJ/kg). (ssniff GmbH, Soest, Germany) if not stated otherwise and drank water ad libitum. Aaas-deficient mice were generated as described previously (Huebner et al., 2006) and backcrossed to strain C75BL/6J for ten generations. A heterozygous Sod2 mouse strain was obtained from The Jackson Laboratory, Bar Harbor, ME USA (Strain #002973 B6.129S7-Sod2tm1Leb/J). Heterozygous Sod2 female mice were intercrossed for two generations with Aaas KO male mice to obtain Aaas KO/Sod2 Het mice.
For chronic oxidative stress exposure one-year-old adult male mice of three different genotypes [wild-type (WT) (n=16), Aaas KO (n=16) and Aaas KO/Sod2 Het (n=10)] were used and randomly divided into two groups (stress and control group). All were placed on a commercial diet (ssniff R/M-H) for 3 days to allow acclimation to these conditions. Mice were then fed with paraquat diet (0.25 g/kg diet) (ssniff GmbH) in the stress group and with control diet (ssniff GmbH) for 11 days. Body weight and diet weight were determined every day during the feeding period. At the end (day 11) of the feeding period animals were sacrificed. Lungs and liver were surgically removed, washed in ice-cold PBS and weighed. Different parts of the liver were prepared for glutathione measurement and assessment of lipid peroxidation. Adrenals and liver sections were surgically excised and quickly frozen in liquid nitrogen and stored at -80°C before RNA extraction.

- **Hepatic glutathione assay**

  Small samples (40-100 mg) of liver tissue were rapidly cut on an ice-cold petri dish to prevent oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) during preparation. Each small sample was immediately placed with a forceps in liquid nitrogen. Samples in the tubes were re-weighed and the weight of the tissue was determined. Ten volumes of ice-cold 5% sulfosalicylic acid (Carl Roth, Karlsruhe, Germany) were added to each tube, the sample was transferred to a tissue grinder and homogenized until evenly suspended. The suspension was added to the same tube and centrifuged at 4°C at 14000x g for 10 minutes. The supernatant was transferred to a new tube and equal volume of ice-cold 500 mM HEPES (pH 8) (Gibco, Thermo Fisher Scientific, Schwerte, Germany) were added.

  Each sample was diluted 60-fold in ice-cold 250 mM HEPES (pH 7.5) to be in linear detection range for measurement of total and oxidized glutathione using the GSH/GSSG-Glo assay (Promega, Mannheim, Germany). Measurements were done in duplicate as outlined in the protocol of the manufacturer and as reported elsewhere (Jühlen et al., 2015).

- **Hepatic lipid peroxidation measurement**

  End-products of hepatic lipid peroxidation, malondialdehyde precursors and other thiobarbituric acid reactive substances (TBARS), were extracted from liver sections as described before by centrifugation at 1600x g for 10 minutes (Mihara and Uchiyama, 1978). TBARS were quantified in triplicate spectrophotometrically at 535 and 520 nm as outlined previously (Mihara and Uchiyama, 1978) on a 96-well culture dish (200 μl/well) (Corning Costar, Kaiserslautern, Germany) using an Infinite 200 PRO Microplate Reader with the Magellan Data Analysis Software v6.6 (Tecan Group AG, Männedorf, Switzerland).
RNA extraction, cDNA synthesis and quantitative real-time PCR using TaqMan

Total RNA from frozen murine liver and adrenals was isolated, purity assessed, reverse transcribed and qPCR amplifications in 20 μl total volumes performed as outlined elsewhere (Jühlen et al., 2016).

As a reference gene for normalization beta-actin was evaluated and used. Positive controls contained a random mix of cDNA and negative controls contained nuclease-free water instead of cDNA. In all real-time qPCR experiments relative gene expression was calculated by the C_t method using standard and semi-log plots of amplification curves. In all results repeatability was assessed by standard deviation of triplicate C_t s and reproducibility was verified by normalizing all real-time RT-PCR experiments by the C_t of each positive control per run.

Primers for the amplification of the target sequence of beta actin (Actb), Cyp11a1, Cyp11b1, Cyp11b2, Cyp21a1, glutathione peroxidase 1 (Gpx1), glutathione reductase (Gsr), heme oxygenase 1 (Hmox1), hydroxy-delta-5-steroid dehydrogenase (Hsd3b2), nicotinamide nucleotide transhydrogenase (Nnt), superoxide dismutase 2 (Sod2) and steroidogenic acute regulatory protein (Star) were designed using Primer Express 3.0 (Applied Biosystems, Life Technologies, Darmstadt, Germany) and compared to the murine genome database for unique binding using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer sequences and gene accession numbers are listed in S1 Table.

The guidelines of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments were followed in this study to allow more reliable interpretation of real-time RT-PCR results (Bustin et al., 2013).

LC-MS/MS measurement of steroids

Blood for plasma steroid measurement by liquid chromatography tandem mass spectrometry (LC/MS-MS) was collected by cardiac puncture. Plasma steroids pregnenolone (Preg), progesterone (P), 17-hydroxyprogesterone (17OHP), deoxycorticosterone (DOC), corticosterone (B), aldosterone (ALDO), androstenedione (AE), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) were determined simultaneously by LC-MS/MS as reported previously (Peitzsch et al., 2015). Quantification of steroid levels was done by comparisons of ratios of analyte peak area obtained from plasma samples to the respective peak area of stable isotope labelled internal standard calibrators.
Histology
Sections of brain, duodenum, liver and lung were washed in PBS and fixed in 4% formaldehyde (SAV LP, Flinsbach, Germany) for 24 hours. Organs were then transferred to PBS and prepared for histology at the Histology Facility of the Joint Technology Platform (Technische Universität Dresden, Biotec, CRTD).

Tissues were embedded into paraffin with the Microm STP 420 D dehydration/infiltration unit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the EGF 1160 embedding station (Leica, Wetzlar, Germany). This included stepwise dehydration in a graded alcohol series, transfer to xylol, as well as paraffin infiltration and sample orientation. Paraffin-embedded samples were sectioned using a Microm HM 340E (Thermo Fisher Scientific) and stained with haematoxylin-eosin (Carl Roth).

Statistical analysis
Statistical analyses were made using the open-source software R version 3.3.2 and R Studio version 1.0.136 (R Core Team, 2016). Unpaired two-tailed Wilcoxon–Mann–Whitney U-test was performed. During evaluation of the results a confidence interval alpha of 95% and P-values lower than 0.05 were considered as statistically significant.

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COMPETING INTERESTS
The authors declare no competing interests.

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Figures

Fig 1. Expression analysis of redox-regulated adrenal *Hmox1* and adrenal and hepatic *Sod2*.

(A) Adrenal and hepatic *Sod2* and (B) redox-regulated adrenal *Hmox1*. Mice were fed with paraquat diet (0.25 g/kg diet) and with control diet for 11 days. P-values: * P<0.05, ** P<0.01. Significant differences were measured with unpaired Wilcoxon–Mann–Whitney U-test. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
**Fig 2. Oxidative stress affects expression of Star and testicular synthesis of androstenedione.**

(A) Adrenal expression of *Star* and (B) testicular synthesis of androstenedione. Mice were fed with paraquat diet (0.25 g/kg diet) in the stress group and with control diet for 11 days. P-values:* P<0.05. Significant differences were measured with unpaired Wilcoxon–Mann–Whitney U-test. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
Fig 3. Alteration of food intake and body weight gain by oxidative stress.

(A) Murine food intake and (B) body weight gain after oxidative stress exposure. Mice were fed with paraquat diet (0.25 g/kg diet) in the stress group and with control diet for 11 days. Body and diet weight were determined every day during the feeding period. P-values: (between different genotypes in one diet) * P<0.05, ** P<0.01 and (between different diets in one genotype) ## P<0.01, ### P<0.001. Significant differences were measured with unpaired Wilcoxon–Mann–Whitney U-test. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
Fig 4. Balance of hepatic glutathione levels in ALADIN null mice.

Mice were fed with paraquat diet (0.25 g/kg diet) in the stress group and with control diet for 11 days. GSH, reduced glutathione. GSSG, oxidized glutathione. P-values: (between different genotypes in one diet) * P<0.05, ** P<0.01 and (between different diets in one genotype) # P<0.05, ## P<0.01. Significant differences were measured with unpaired Wilcoxon–Mann–Whitney U-test. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
Fig 5. Mitochondrial redox defense system.
Transmembrane nicotinamide nucleotide transhydrogenase (NNT) contributes to the mitochondrial redox defense system by producing NADPH. NADPH is consumed by glutathione reductase (GSR) maintaining reduced glutathione (GSH) levels from oxidized glutathione (GSSG). Electrons leaking during mitochondrial aerobic respiration result in superoxide anion radicals ($O_2^\cdot$) and are converted to hydrogen peroxide ($H_2O_2$) by mitochondrial superoxide dismutase (SOD2). Hydrogen peroxide is neutralized to water ($H_2O$) consuming GSH by glutathione peroxidase (GPX).
Table 1 – Real-time qPCR primer oligonucleotides.

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**Fig. S1 Adrenal steroidogenesis gene expression after paraquat exposure.**
Mice were fed with paraquat diet (0.25 g/kg diet) and with control diet for 11 days. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
Fig. S2 Steroid output after paraquat exposure.

Mice were fed with paraquat diet (0.25 g/kg diet) and with control diet for 11 days. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
Fig. S3 Expression analysis of hepatic *Gpx1* and *Gsr*.

Mice were fed with paraquat diet (0.25 g/kg diet) and with control diet for 11 days. P-values: * P<0.05. Significant differences were measured with unpaired Wilcoxon–Mann–Whitney U-test. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
Fig. S4 Relative liver and lung weights are unchanged upon fed diets containing paraquat.
Mice were fed with paraquat diet (0.25 g/kg diet) and with control diet for 11 days. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.
**Fig. S5 The level of hepatic lipid peroxidation is not altered after the paraquat diet.** Mice were fed with paraquat diet (0.25 g/kg diet) and with control diet for 11 days. TBARS, thiobarbituric acid reactive substances. Boxplot widths are proportional to the square root of the samples sizes. Whiskers indicate the range outside 1.5 times the inter-quartile range above the upper and below the lower quartile. Outliers were plotted as dots.