Re-appraisal of putative glyoxalase 1 deficient mouse and dicarbonyl stress on embryonic stem cells *in vitro*

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Abstract

Glyoxalase 1 (Glo1) is a cytoplasmic enzyme with a cytoprotective function linked to metabolism of the cytotoxic side-product of glycolysis, methylglyoxal. It prevents dicarbonyl stress – the abnormal accumulation of reactive dicarbonyl metabolites increasing protein and DNA damage. Increased Glo1 expression delays ageing and suppresses carcinogenesis, insulin resistance, cardiovascular disease and vascular complications of diabetes and renal failure. Surprisingly gene trapping by the International Mouse Knockout Consortium (IMKC) to generate putative Glo1 knockout mice produced a mouse line with phenotype characterised as normal and healthy. Here we show that gene trapping mutation was successful but the presence of *Glo1* gene duplication, likely in the embryonic stem cells before gene trapping, maintained wild-type levels of Glo1 expression and activity and sustained the healthy phenotype. In further investigation of the consequences of dicarbonyl stress in embryonic stem cells (ESCs), we found prolonged exposure of mouse ESCs in culture to high concentrations of methylglyoxal and/or hypoxia led to low level increase in Glo1 copy number. In clinical translation, we found increased prevalence of low level GLO1 copy number increase in renal failure where there is severe dicarbonyl stress. In conclusion, the IMKC Glo1 mutant mouse is not deficient in Glo1 expression through duplication of the *Glo1* wild-type allele. Dicarbonyl stress and/or hypoxia induces low level copy number alternation in embryonic stem cells. Similar processes may drive rare GLO1 duplication in health and disease.

Summary statement

Unrecognised *Glo1* gene duplication led to failure of IMKC project production of a Glo1 deficient mouse. Dicarbonyl stress and/or hypoxia induced low level *Glo1* copy number increase in mouse embryonic stem cells. Copy number alternation may drive clinical GLO1 copy number increase.

Running title: Copy number alteration of glyoxalase 1.

Keywords: glyoxalase; copy number variation; methylglyoxal; embryonic stem cell; dicarbonyl stress; hypoxia.

Abbreviations: AF, attachment factor; Btbd9, BTB (POZ) Domain Containing 9; CAT, catalase; CGH, comparative genomic hybridization; CNV, copy number variation; Dnahc8, dynein, axonemal, heavy chain 8; EDTA, ethylenediaminetetra-acetic acid; EMMA, European Mutant Mouse Archive; ESC, embryonic stem cell; Glo1, *Glo1* and GLO1, protein, murine gene and human gene of glyoxalase 1, respectively; Glo2, glyoxalase 2; Glp1r, glucagon-like peptide 1 receptor; GSH, reduced glutathione; HIF1α, hypoxia-inducible factor 1-alpha; IMKC, International Mouse Knockout Consortium; KDM4A, lysine-specific demethylase 4A; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDR, multidrug resistance; MEF, mouse embryonic fibroblast; mESC, mouse embryonic stem cell; MG, methylglyoxal; MGdG, 3-(2'-deoxyribosyl)-6,7-dihydro-6,7-dihydroxy-6/7-methylimidazo-[2,3-b]purine-9(8)one; MG-H1, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine**;** Nrf2, Nuclear factor erythroid 2–related factor 2; Oct4, octamer-binding transcription factor 4; PCR, polymerase chain reaction; PRT, paralogue ratio test; PLS-DA, partial least squares-discriminant analysis; RT-PCR, reverse transcription- polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism; SOX2, sex determining region Y-box 2; TERT, Telomerase reverse transcriptase; Tfrc, transferrin receptor; Vmn2r111 and Vmn2r112**,** vomeronasal 2, receptors 111 and 112 respectively.

INTRODUCTION

Glyoxalase 1 (Glo1) is part of the glyoxalase metabolic pathway in all mammalian cells and tissues [1] (Figure 1a). The glyoxalase pathway consists of two enzymes, Glo1 and glyoxalase 2 (Glo2), and a catalytic amount of reduced glutathione (GSH). The major function of the pathway is detoxification of methylglyoxal (MG). Glo1 catalyses the conversion of the hemithioacetal formed non-enzymatically from MG and GSH to S-D-lactoylglutathione; and Glo2 catalyses the hydrolysis of S-D-lactoylglutathione to D-lactate, reforming GSH consumed in the Glo1-catalysed step. MG is formed mainly by the non-enzymatic degradation of triosephosphate intermediates of glucose metabolism – which is an unavoidable minor "leak" of *ca.* 0.1% glucose flux from anaerobic glycolysis [2, 3]. MG is a highly potent glycating agent and, although formed at relatively low flux, its high reactivity leads to formation of some of the most quantitatively important endogenous damaging modifications of protein and DNA: arginine-derived hydroimidazolone MG-H1 and deoxyguanosine-derived imidazopurinone MGdG [4, 5] (Figure 1b and 1c). Glo1 suppresses the steady-state level of MG and thereby also suppresses the level of MG-H1 and MGdG to low tolerable levels; 1 – 2% of protein and 1 in 10^5 nucleotides, respectively [4, 5]. Suppression of DNA modification by MG and related mutation likely underlies the finding that *Glo1* is a tumour suppressor gene [6]. Increased MG-H1 and related protein inactivation and dysfunction contributes to ageing [7], insulin resistance [8, 9], cardiovascular disease [10], vascular complications of diabetes [4, 11] and renal failure [12]. Increased MG concentration, a metabolic state called dicarbonyl stress, is driven by increased formation of MG and/or decreased activity of Glo1 [1]. To counter periods of increased MG formation, expression of Glo1 is under stress-responsive transcriptional control through transcription factor Nrf2 which binds to a functional regulatory antioxidant response element and increases Glo1 expression [13].

To investigate the role of Glo1 activity in maintenance of health and prevention of disease we acquired putative Glo1 knockout mice produced by the International Mouse Knockout Consortium (IMKC) [14]. Murine *Glo1* is located at 17a3.3 (30592866–30612659) and has 6 exons. *Glo1* is diallelic with each allele coding for a 21.5 kDa subunit of Glo1 dimer protein, running at 23 kDa in SDS-PAGE [15]. A rare polymorphism produces genotypes *Glo1a* (common) and *Glo1b* (rare) which, like human GLO1, gives rise to

allozymes of similar molecular mass and enzymatic activity [16, 17]. The IMKC *Glo1* mutant mouse was produced by gene trapping, with retroviral insertion of vector VICTR48 between exons-1 and -2 in strain 129SvEv^{Brd}-derived mESCs and bred in a C57BL/6J background. From the phenotypic characterisation data provided by the supplier - Lexicon Pharmaceuticals (Woodlands, Texas, USA) and included in the European Mutant Mouse Archive (EMMA) described of the Glo1 mutant mouse, it was surprising to find report of a normal, healthy phenotype in both putative homozygous and heterozygous *Glo1* mutant mice. Rare mutation of GLO1 in the human population is embryonically lethal in homozygous inheritance with increased risk of severe neurological disorders in heterozygous inheritance [18]. Glo1 knockout mice have otherwise been elusive and rather a mouse strain with Glo1 deficiency was developed by viral insertion of a vector expressing Glo1 siRNA [19]. Silencing and chemical inhibition of Glo1 in mice was linked to increased vascular disease [20-22]. If Glo1 expression had been decreased by 50% in heterozygote *Glo1* mutant mice by successful gene trapping - as expected for functional knockout, a *ca.* 2-fold increased MG and consequent impaired metabolic, vascular and neurological health in the mutant mouse model is expected [8, 20-22]. In the IMKC project, the putative mutation of the Glo1 gene was assessed by measurement of Glo1 mRNA only.

The aim of this study was to assess if the Lexicon *Glo1* mutant mice were protected against dicarbonyl stress through an alternative fate of MG metabolism, or other mechanism. We therefore developed genotyping methodology for detection of the *Glo1* mutation and evidence of putative disruption of Glo1 expression. We also characterised the glyoxalase system of mouse embryonic stem cells (mESCs) *in vitro* and modelled dicarbonyl stress by exposure to exogenous MG. Clinical translation of the findings was assessed in patients with renal failure – an example of severe dicarbonyl stress.

Herein we present conclusive evidence that mutant mice created by gene trapping of *Glo1* in the IMKC project are not deficient in Glo1 expression and activity, which explains why the healthy phenotype was preserved. We confirm that *Glo1* was mutated and reveal the compensatory mechanism for Glo1 expression – wild-type *Glo1* gene duplication, which was likely present in the mESC line before gene trapping. Additionally we show that experimental dicarbonyl stress and hypoxia induce low level Glo1 copy number increase in mESCs *in vitro* and in clinical translation we found increased prevalence of low level GLO1 copy number increase in renal failure, suggesting dicarbonyl stress and/or hypoxia-induced copy number alteration of GLO1 may occur physiologically.

EXPERIMENTAL

Glo1 mutant and wild type mice

Glo1 mutant mice produced by Lexicon Pharmaceuticals (the Woodlands, Texas, USA) were obtained as a heterozygote breeding pair from EMMA, Heidelberg, Germany. Wild-type control siblings of heterozygote C57BL/6 mice were produced in the Biological Services Unit, University of Warwick. Animal breeding was performed under Licence number 80/2556 (Breeding and maintenance of genetically altered rodents) approved by the Home Office U.K. and conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986. Control mouse liver samples of strains C57BL/6J (stock number: 664) and DBA/1J (stock number: 670) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) via UK agent Charles River (Margate, Kent, UK).

Mouse embryonic stem cells

Irradiated mouse embryonic fibroblasts (MEFs) as a feeder layer and mESCs of C57BL/6 mouse strain were purchased from Life Technologies (Paisley, U.K.). MEFs were cultured in high glucose D-MEM media supplemented with 10% fetal bovine serum (FBS; embryonic

stem cell certified). Cells were seeded in the cell culture flasks coated with 0.1% attachment factor (AF) at a density 25,000 cells/cm^2 and cultured at 37°C in 5% CO_2 and 95% air-water saturated atmosphere. After 2 - 4 days, mESCs were seeded over the feeder layer at density 50,000 cells/cm^2 and incubated under the same atmosphere. The media used for mESCs was Knockout™ D-MEM supplemented with 15% knockout serum replacement, 1% MEM non-essential amino acids solution, 1% L-glutamine, 0.001% leukaemia inhibitory factor and 0.00182% 2-mercaptoethanol. For the experiments performed under hypoxic condition, cells were incubated in hypoxia chamber gassed with 3% oxygen, 5% carbon dioxide and 92% nitrogen at 37°C. The mESC media and hypoxic atmosphere were changed daily and mESCs were passaged every 2 - 3 days when 80-90% confluent. To impose dicarbonyl stress on mESCs, we added exogenous high purity MG. High purity MG was prepared and purified as described [23]. To avoid phenotypic drift or differentiation of ESCs in culture, batches of new mESCs were revived from cryostorage every 3 months and used for experiments at ≤ passage 15. mESC viability was assessed as the percentage of cells that exclude Trypan blue and was determined after 3, 6 and 12 days of culture under 3% and 20 % oxygen atmosphere conditions.

When the mESCs were ready for passage, medium was removed and flasks washed with phosphate-buffered saline (PBS). Approximately 1 ml/25 cm^2 StemPro Accutase solution (Life Technologies) was added to each flask and cells returned to 37°C incubator for 3 - 5 min. StemPro Accutase solution was neutralised with supplemented D-MEM media. Cell pellets were diluted to required concentrations depending on cell density and added to new sterile polystyrene culture vessels coated with 0.1% AF and without a feeder layer.

Human blood samples

Whole blood samples were collected from healthy human subjects and patients with renal failure (stage 5 chronic kidney disease). Samples were collected with written informed consent using ethylenediaminetetra-acetic acid (EDTA) as anticoagulant. Clinical characteristics of the participants are given (Supplementary Table S1). Healthy human subjects and patients were recruited at the University Hospitals Coventry & Warwickshire NHS Trust, Coventry, U.K. The study was approved by National Research Ethics Service Committee West Midlands - Coventry & Warwickshire; project number 05/Q2802/26. Samples were stored at -80ºC until analysis. The experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

DNA Extraction

DNA extraction was performed using the DNeasy Blood & Tissue kit according to the manufacturer's instructions (Qiagen, Manchester, UK). Samples were: 1 x 10^6 cells, 10 - 25 mg tissue or 200 µl whole human blood. The quality and concentration of DNA was determined spectrophotometrically using a NanoDrop 1000 spectrophotometer.

PCR analysis for *Glo1* mutant mouse genotype

Primers used are given in Supplementary Table S2. The reaction mixture contained: 1 mM dNTPs, 1.5 mM Mg^{2+}, 0.2 pmol/µl forward and reverse primers, 2 µl 100 ng/µl DNA, Taq polymerase, stabilizer and buffer to the final volume of 20 µl. The protocol used for PCR was: initial denaturation 4.5 min at 94°C, 30 cycles of denaturation (0.5 min at 94°C)-annealing (0.5 min at 58°C)-elongation (0.5 min at 72°C), and final elongation (7.0 min at 72°C). PCR product (5 µl) was electrophoresed (100 V, 90 min) on 2% agarose gels stained with 0.5 µg/ml ethidium bromide. The bp marker used was Hyperladder V (Bioline, London,

U.K.). Gels were photographed using ultraviolet light by ChemiGenius^2 (Syngene, Cambridge, U.K.).

Gene copy number and gene expression analysis - TaqMan® method

Glo1 copy number assays were performed by the TaqMan® Copy Number Assays, with reference genes Tfrc and TERT for mouse and human DNA samples, respectively (Applied Biosystems, Paisley, Scotland). They were performed using the manufacturers protocol (PN 4397425) and data analysed by CopyCaller™ software. mRNA expression analysis was performed using the Taqman gene expression assay protocol (PN 4333458) according to the manufacturer's instructions. Rn 18s was internal reference gene with primers QT01036875 (Qiagen).

***Glo1* copy number analysis - Paralogue ratio test**

Accuracy of qPCR in application for copy number change analysis has been challenged and an alternative procedure developed and applied called the paralogue ratio test (PRT) [24]. A disadvantage of the PRT is greater sensitivity to template degradation than qPCR [25]. PRT primers (Supplementary Table S2) were designed, using online software PRTPrimer (www.prtprimer.org) as described [24], to amplify a sequence unique to the *Glo1* and exactly one other unlinked reference locus in a single PCR reaction with the same pair of primers. The reference primers used amplified the region of *Glo1* on chromosome 17 of chr17:30603393-30603637 which produced a PCR product of 244 bp. The primers also apply the reference locus on chromosome 3 at chr3:58404865-58405084 which produced a product of 219 bp. The PCR reaction mixture (20 µl) contained: 2 x Biomix red (10 µl; Bioline), 270 ng mouse genomic DNA, 0.375 µM forward and reverse primers and 3.75 mM $MgCl_2$. The protocol used for PCR was: initial denaturation 4.5 min at 94°C, 30 cycles of denaturation (0.5 min at 94°C)-annealing (0.5 min at 60°C)-elongation (0.5 min at 72°C), and final elongation (7.0 min at 72°C). The PCR products were then separated and quantified by capillary electrophoresis on Applied Biosystems capillary sequencer (ABI 3730) using fluorescently tagged PCR fragments and GeneScan™ LIZ® 500 Size Standard (Thermo Scientific, Lutterworth, U.K.). Total run time was 45 min and denature time was 3 min at 95 ^oC. Data were analysed by Peak Scanner™ Software 2 (Applied Biosystems, Foster City, USA). Ratios of Glo1/reference locus PCR product intensities were calibrated by PRT assay of DNA from C57BL/6J and DBA/1J mice (*Glo1* copy no = 2.0 and 4.0, respectively) – extracted from liver samples from reference strain sources.

Comparative genomic hybridization (CGH) oligonucleotide array

CGH array analysis was performed using SurePrint G3 mouse CGH Microarrays 1x1M according to the manufacturer's instructions (protocol no G4410-90010: version 7.2; Agilent Technologies LDA UK Limited, Stockport, Cheshire). Reference samples were from C57BL/6 mouse strain, gender-matched to sample DNA. Briefly, genomic DNA samples (0.5 µg in 20.2 µl acetate-EDTA buffer) were digested by Alu I/Rsa I restriction enzyme mixture using SureTaq complete DNA labelling kit, according to the manufacturer's instructions (Agilent). The master mix was added to each reaction tube containing genomic DNA to a total volume of 26 µl. Samples were loaded in a thermocycler and incubated for 2 h at 37ºC, 20 min at 65ºC and then at kept 4ºC. The digested DNA (2 µl) was loaded on to 0.8% agarose gels to analyse digestion fragments (200 and 500 bp). Labelled DNA was mixed with Tris-EDTA buffer (430 µl; pH 8.0) and purified with the mini-column provided in the SureTaq DNA labelling kit, repeating the wash step and collecting the DNA (total volume: 80.5 µl). An aliquot (1.5 µl) was used for determination of yield, degree of labelling and specific activity by Nanodrop spectrophotometry. The remaining sample and reference

DNA were mixed (158 µl), hybridisation master mix added, and heated to 95ºC for 3 min and then 37ºC for 30 min. The mixture was hybridized to the 1 M array using the Oligo aCGH/ChIP-on-chip hybridization kit: loading 490 µl to the slide gasket and covering with the active side of the microarray. The sandwich slides were loaded into an Agilent microarray hybridization chamber. This was placed in the hybridization oven heated to 60ºC and rotated at 20 rpm for 40 h. Following hybridization, the array was washed with Oligo aCGH/ChIP-on-chip wash buffer 1 and 2 and scanned using a Surescan microarray slide holder and Surescan microarray scanner. All data was processed and collected by Feature Extraction software and data analysed with Genomic Workbench Edition 7.0 (Agilent).

PCR–restriction fragment length polymorphism for *Glo1* genotyping

C419A polymorphism in exon-4 of *Glo1* was determined by PCR–restriction fragment length polymorphism. PCR mixtures contained: 200 ng human genomic DNA, Biomix red (10 µl) and C419A genotyping forward primer and reverse primer. Each reaction contained 1 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 pmol/µl forward and reverse primers, Taq polymerase, stabilizer and buffer to the final volume of 20 µl. The PCR products were digested with SfaNI restriction enzyme (New England Biolabs Ltd, Hitchen, UK) for 1 h at 37ºC. The digested product were resolved by 2% agarose gel electrophoresis and the fragments were visualized under UV light after staining with ethidium bromide to identify the single base pair change. The restriction digest reveals 453 bp and 260 bp fragments for the C419 allele (rs4746 C332) and 713 bp fragment for the A419 (rs4746 A332) allele [26].

Characterisation of the glyoxalase system and methylglyoxal metabolism

Activities of Glo1 and Glo2 were assayed spectrophotometrically, Glo1 protein and Glo1 mRNA were assayed by Western blotting and RT-PCR, and MG, glycation adduct MG-H1 and creatinine were assayed by stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described [23, 27-30]. MG reductase activity was determined spectrophotometrically as described [31] with minor modification: using 1 mM MG as substrate and 50 mM sodium phosphate buffer, pH 7.4 at 37°C, as the assay buffer. D-Lactate was measured by endpoint enzymatic assay with microplate fluorimetry [32]. Total protein was assayed by the Bradford method.

Statistical analysis

Data of copy number change, mRNA and other variables of mouse samples were analysed for normality (Kolmogorov Smirnov test), confirmed as approximating to normal distribution and sample group means analysed for significance of difference by independent sample Student's *t*-test. Similar data of mESC analysis was assumed to be normally distributed and the *t*-test applied. Clinical copy number data was similarly analysed for normality, confirmed and analysed by one sample t-test (99% CI) to identify renal failure patients with abnormally low or high *Glo1* copy number. Urinary MG-H1 data were non-parametrically distributed and sample groups were analysed for significance of difference by Mann-Whitney U test. DNA microarray data were analysed by the ADM-2 algorithm with threshold $P<1 \times 10^{-6}$, with ≥3 consecutive probes and $\log_2$ R ratio of ≥ 0.25. Factors linked to increased GLO1 copy number in the clinical study were explored by partial least squares-discriminant analysis (PLS-DA) using XLSTAT software (Addinsoft, New York, USA). For healthy control subjects and patients with chronic kidney disease combined, GLO1 gene copy number association was analysed with the following variables: age, gender, body mass index (BMI), ethanol consumption, smoking status, systolic and diastolic blood pressure, haemoglobin, plasma glucose, glycated haemoglobin HbA_{1c} and serum creatinine – Table S1. A similar PLS-DA was performed on glyoxalase-related variables (Glo1 activity, Glo1 protein, Glo1

mRNA, liver MG and urinary MG-H1) in wild-type and Glo1 (+/-) mutant mice combined to assess associations with presence of the Glo1 mutant allele – Table 1. An association was judged present when the 95% confidence interval of the variable coefficient excluded zero.

RESULTS

IMKC Mutant mice have normal Glo1 expression with two wild-type copies and one or more mutated copies of *Glo1*

We initially assessed the genotype of the Lexicon *Glo1* mutant mice by qPCR using 3 pairs of primers: one to detect wild-type *Glo1* and two others to detect each end of the inserted DNA in mutated *Glo1* (with primer sequence design for inserted DNA made in collaboration with Lexicon Pharmaceuticals) – Supplementary Table S2. Forty-four wild-type and *Glo1* mutant mice were genotyped. The product size with each primer was 237 bp, 139 bp and 242 bp respectively (Figure 1d and 1e). The genotype distribution of the mice was 9 wild-type and 35 heterozygote. No homozygotes were detected. We analysed activity of Glo1 in tissues (liver, kidney, brain, heart, skeletal muscle, brain and pancreas) of wild-type and *Glo1* mutant mice at 3 and 7 months of age and found no significant difference in any tissue at either time point (Table 1). We analysed Glo1 expression by immunoblotting of Glo1 protein (Supplementary Figure S1) and by RT-PCR of Glo1 mRNA. We found no significant difference between wild-type controls and *Glo1* mutant mice (Table 1). We also analysed other components of the glyoxalase system – activity of Glo2, concentrations of MG and D-lactate, and tissue protein content of MG-derived glycation adduct MG-H1 and found no significant change in *Glo1* mutant mice, with respect to wild-type controls (Table 1). There was also no change in whole body formation of MG-H1 glycation adducts, as judged by flux of urinary excretion of MG-H1 free adducts (median [lower – upper quartile]; nmol per mg creatinine): wild-type controls - 47.0 [24.7 – 66.7], n = 10, and Glo1(+/-) mutant - 31.9 [13.0 – 39.1], n = 10; P>0.05, *Mann-Whitney U*. Where Glo1 is functionally inactivated, an alternative fate of MG metabolism is by aldoketo reductases [33]. There was also no change in activity of MG reductase; MG reductase also had very low activity in the liver of both wild-type control and Glo1(+/-) mutant mice (Table 1). Therefore, *Glo1* mutant mice have a mutated *Glo1* gene but with compensatory Glo1 expression and MG metabolism identical to that of wild-type controls. Glo1 expression and levels of MG in *Glo1* Lexicon mutant mice found herein are typical of wild-type controls. This explains the unexpected normal, healthy phenotype of *Glo1* mutant mice reported in the IMKC project.

To explore the mechanism of compensatory Glo1 expression we quantified *Glo1* copy number by TaqMan® method, normalizing response to transferrin receptor protein-1 *Tfrc* known to be present in two copies and located on chromosome 16 - different to that of *Glo1*. For quantification of *Glo1* DNA, we chose primers targeted to the 3'-end of exon 1 and the 5'-end of exon 6 in the *Glo1* gene. DNA was extracted from the liver, kidney, brain and pancreas. DNA from wild-type siblings was used as control with two copies of *Glo1* and also DNA from DBA/1J mice known to have gene duplication and 4 copies of the *Glo1* gene. *Glo1*(+/-) mutant mice had 3 copies of *Glo1* in all tissues analysed with amplification extending from 3'-end of exon 1 to the 5'-end of exon 6 (Table 2). Compensatory expression of Glo1 occurred, therefore, from an additional wild-type copy of *Glo1*. We designed a further Taqman copy number assay with primers and probe that detected DNA across the insertion point of *Glo1* and VICTR48 to quantify also the number of mutant $Glo1^{Gt(OSTGST\_4497\text{-}D9)Lex}$ alleles (abbreviated to $Glo1^{Gt(..)Lex}$). Combination of these Taqman copy number assays enabled assessment of the number of wild-type *Glo1* and mutant $Glo1^{Gt(..)Lex}$ copies. Most mutant mice contained two copies of wild-type *Glo1* and one copy of mutant $Glo1^{Gt(..)Lex}$ - designated $Glo1(+/+)^{Gt(..)1Lex}$. In some cases, however, we found 2

copies of both *Glo1* and *Glo1*$^{Gt(..)Lex}$ – designated *Glo1(+/+)*$^{Gt(..)2Lex}$. We investigated inheritance patterns of the *Glo1*$^{Gt(..)Lex}$ alleles in litters from mating of different genotypes. The offspring genotype pattern suggested a simple Mendelian inheritance (Table 3). From mating of wild-type and *Glo1(+/+)*$^{Gt(..)2Lex}$ mice, offspring of only *Glo1(+/+)*$^{Gt(..)1Lex}$ mice indicated the presence of a *Glo1*$^{Gt(..)Lex}$ mutant allele on each homologue of chromosome 17 (Table 3). From the principles of Mendelian inheritance, mating of wild-type mouse and *Glo1(+/+)*$^{Gt(..)2Lex}$ mutant mouse producing a litter with all offspring having a single mutant *Glo1* allele, *Glo1(+/+)*$^{Gt(..)1Lex}$, indicates that each offspring has received a mutant *Glo1* allele and wild-type Glo1 allele from the *Glo1(+/+)*$^{Gt(..)2Lex}$ mutant mouse parent originating from either of the chromosome homologues inherited. Hence, *Glo1(+/+)*$^{Gt(..)2Lex}$ mice have a mutant Glo1 allele and a wild-type Glo1 allele on each chromosome homologue. Accordingly, it was not possible to produce progeny with < 2 copies of wild-type *Glo1* alleles and deficient in Glo1 expression and activity since each homologue of chromosome 17 retained a *Glo1* wild-type allele. This was indeed observed throughout all breeding of *Glo1* mutant mice.

PLS-DA analysis showed no significant association of Glo1-linked variables with presence of the mutant allele, *Glo1(+/+)*$^{Gt(..)1Lex}$, supporting the presence of wild-type glyoxalase-related expression and metabolism in Lexicon Glo1 mutant mice.

Paralogue ratio test for *Glo1* copy number

We measured copy number in this study by qPCR and later DNA microarray – see above. Accuracy of qPCR in application for copy number change analysis has been challenged and an alternative procedure developed and applied called the paralogue ratio test (PRT). We developed a PRT for murine Glo1. Application of this gave similar corroborative estimates of *Glo1* copy number by PRT were: genotype *Glo1(+/+)*, 2.00 ± 0.11; *Glo1(+/+)*$^{Gt(..)1Lex}$, 2.84 ± 0.20 ($P<0.01$ with respect to *Glo1(+/+)*); *Glo1(+/+)*$^{Gt(..)2Lex}$, 4.18 ± 0.09 ($P<0.001$ with respect to *Glo1(+/+)* and *Glo1(+/+)*$^{Gt(..)1Lex}$). Estimates of *Glo1* copy number by qPCR and PRT also corroborated well with DNA microarray analysis – see below.

Copy number increase is focussed to and selective for the *Glo1* genomic domain

Finding that Lexicon mutant mice had increased *Glo1* copy number, we sought to identify the location of copy number increase in genomic DNA. We performed high intensity genome-wide DNA microarray analysis, analysing 3 mice of each genotype: *Glo1* (+/+), *Glo1(+/+)*$^{Gt(..)1Lex}$ and *Glo1(+/+)*$^{Gt(..)2Lex}$. In wild-type controls, there was no cop number increase detected at the *Glo1* locus, confirming that wild-type mice had two copies of *Glo1*. In *Glo1(+/+)*$^{Gt(..)1Lex}$ mice, a segment of 473,479 bp DNA hybridising with 300 oligonucleotides had mainly one additional copy; mean copy number 2.76. In *Glo1(+/+)*$^{Gt(..)2Lex}$ this fragment had mainly 2 additional copies; mean copy number 3.61 (Figure 2). The copy number change region comprised *Glo1* and *Dnahc8* (dynein, axonemal, heavy chain 8) and part of *Btbd9* (BTB (POZ) Domain Containing 9) and *Glp1r* (glucagon-like peptide 1 receptor) genes. Other regions of the mouse genome showed random duplication in different areas occurring in only one donor and not linked to the *Glo1* mutant genotype. Only two genes in a different common domain were duplicated in *Glo1* mutant mice: *Vmn2r111* and *Vmn2r112* (vomeronasal 2, receptors 111 and 112), on chromosome 17 (22673192 to 22797105) and detected by 20 oligonucleotides in the *Glo1(+/+)*$^{Gt(..)1Lex}$ and 20 oligonucleotides in *Glo1(+/+)*$^{Gt(..)2Lex}$ mice with mean copy numbers of 2.70 and 3.27, respectively. Copy number change of *Btbd9*, *Dnahc8* and *Vmn2r112* did not produce concomitant increased expression, as judged by change in relative mRNA level; Vmn2r111 was not investigated. Indeed, expression of Vmn2r112 was decreased moderately in *Glo1(+/+)*$^{Gt(..)1Lex}$ mice and markedly in *Glo1(+/+)*$^{Gt(..)2Lex}$ mice (Supplementary Figure S2).

Copy number increase of Glo1 in mouse embryonic stem cells induced by dicarbonyl stress *in vitro*

We hypothesised that dicarbonyl stress in mESCs may increase *Glo1* copy number by induced copy number alteration as an adaptive genomic cytoprotective response. To explore this we studied the effect of exposure of mESCs to exogenous MG *in vitro*. Survey of expression of mESC markers, SOX2, Nanog and Oct4, confirmed maintenance of the mESC phenotype throughout. mESC growth and viability and the glyoxalase system were studied under atmospheres containing 20% and 3% oxygen - typical of most cell culture conditions and mESC oxygen exposure *in vivo*, respectively. mESC growth and expression and activity of Glo1 was decreased in 3% oxygen growth conditions (Figure 3a – 3d). Consumption of glucose and net formation of L-lactate was increased in 3% oxygen cultures, reflecting increased anaerobic glycolysis (Figure 3e and 3f). Concomitant increased flux of formation of D-lactate was found – a measure of flux of formation of MG in anaerobic glycolysis and metabolism of MG by the glyoxalase system (Figure 3g).

To impose dicarbonyl stress on mESCs, we added exogenous high purity MG. Incubation of mESCs with MG for 2 days under 20% oxygen growth conditions produced decreased growth and viability with a median growth inhibitory concentration GC_{50} = 848 ± 42 μM (N = 15) - Figure 4a. In addition, cell viability of mESCs decreased with > 50 μM MG under 20% oxygen growth conditions – Figure 4b, suggesting the inhibition of cell growth by MG is due to both growth arrest and toxicity. This was potentiated under 3% oxygen growth conditions (Figure 4a and 4b). We incubated mESCs for 12 days with 200 μM MG, changing the medium and added fresh MG every 24 h. At 3, 6 and 12 days, the copy number of *Glo1* was increased to a maximum of +16%. Copy number of other genes duplicated with *Glo1* in mutant mice, *Btbd9* and *Dnahc8*, was examined and no change was found (Table 4). The increase in *Glo1* copy number at day 12 with MG treatment was associated with an increase in Glo1 protein: Glo1 protein (normalised to β-actin) – control, 0.49 ± 0.01 versus 0.62 ± 0.04, + 27%; P<0.01 (n = 3) (Figure 4c). Similar increase in *Glo1* copy number occurred in mESCs incubated under 3% oxygen but it was not increased further with addition of exogenous MG (Figure 3h). Percentage viability of mESCs at 3, 6 and 12 days incubated under 20% oxygen was: control – 98 ± 1, 97 ± 1 and 96 ± 1, and + 200 μM MG - 96 ± 2, 97 ± 1 and 96 ± 1 (n = 3). Similar percentage viability of mESCs at 3, 6 and 12 days incubated under 20% oxygen was: control – 96 ± 8, 97 ± 3 and 97 ± 2, and + 200 μM MG - 94 ± 16, 64 ± 9 and 94 ± 10 (n = 3). Daily exposure to 200 μM MG continually slowed cell growth and thereby likely decreased toxicity to MG.

***Glo1* copy number increase in clinical dicarbonyl stress**

The relevance of *Glo1* copy number increase to human health and disease was assessed in clinical translation by study of a disease associated with severe dicarbonyl stress - renal failure (stage 5 chronic kidney disease) [34]. We also characterised genotype of subjects with respect to the common single nucleotide polymorphism (SNP) C419A (Supplementary Figure S3). We examined DNA of peripheral blood mononuclear cells of healthy subjects and patients with renal failure. Subject and patient characteristics are summarised (Supplementary Table S1). In healthy subjects, GLO1 copy number was (mean ± SD): 2.00 ± 0.13 (n = 21). In 20 renal failure patients, we found 15 patients had GLO1 copy number indistinguishable from healthy controls, one had an abnormally low GLO1 copy number (1.69, P<0.001) and 5 renal failure patients had GLO1 copy number higher than healthy controls for which GLO1 copy number (mean ± SD) was 2.18 ± 0.03 (n = 5, P<0.001). In renal failure patients, therefore, the prevalence of *Glo1* copy number increase was 25% with a mean GLO1 DNA increment of 9%. This copy number increase is of relatively high prevalence but low in magnitude

compared to GLO1 duplication in the healthy population. For the SNP C419A, in renal failure patients the genotype distribution (CC, CA, AA) was: no GLO1 copy number increase - 3:7:4, presence of GLO1 copy number increase – 0, 2, 3. The SNP for one patient could not be discerned. The change in genotype distribution was not significantly different (Fisher Exact test). There was no distinctive renal failure etiological or clinical chemistry characteristics of patients with GLO1 copy number increase.

PLS-DA analysis showed a significant association of increased GLO1 copy number with increased serum creatinine and decreased alcohol consumption. Standardised coefficients were: serum creatinine 0.363 ± 0.155, and alcohol consumption – 0.173 ± 0.082. Model cumulative $Q^2 = 0.155$.

DISCUSSION

Herein we show that mutation to *Glo1* by gene trapping in the IMKC project failed to produce a Glo1 deficient mouse because of the presence of an additional copy of the wild-type *Glo1* gene. The genetic domain duplicated is similar to that found endogenously in some strains of mice [35]. *Glo1* duplication is not present in the genetic background of C57BL/6J mice – the strain used in production of the Lexicon *Glo1* mutant mice [35]; nor was it present in the $129SvEv^{Brd}$ ESC line when assessed in 2005 [36]. However, the genome sequence of the $129SvEv^{Brd}$ ESC cell line assessed later in the Mouse Genomes Project (Sanger Institute) shows *Glo1* duplication [37]. The disparity between this and the earlier contradictory report may be due to instability of copy number increases in ESC lines. In a study of copy number variation (CNV) stability in ESC lines, about 50% of CNVs studied in multiple clonal lines were not found in their common parental ESC line, indicating that CNVs may change through CNV instability during mitosis with prolonged sub-culturing [38]. The origin of the additional *Glo1* gene copy in Lexicon mutant mouse is likely due to *Glo1* duplication in the $29SvEv^{Brd}$ ESC clone used before gene trapping. Glo1 deficiency was thereby prevented in all siblings and progeny of the Lexicon *Glo1* mutant mouse. The current description of this mouse line in the EMMA as Glo1 deficient mouse is, therefore, incorrect and requires amendment.

DNA microarray analysis of Lexicon *Glo1* mutant mice revealed focussed and selective functional *Glo1* duplication with secondary effects on genes susceptible to copy number change, *Vmn2r111* and *Vmn2r112*. There is no known link of the glyoxalase system to the pheromone signalling in mammals. Pheromone receptor genes are highly enriched in copy number changes, being present in large gene families characterized by rapid evolution with many pseudogenes and highly variable gene content between species [39]. This may account for the copy number change of *Vmn2r111* and *Vmn2r112* herein. Engineered copy number change or mutation of single genes has previously been found to induce copy number change of unrelated genes and mutations in secondary effect genes leading to impaired expression [40]. For Vmn2r112 there was moderate and markedly decrease in expression in $Glo1(+/+)^{Gt(..)1Lex}$ and $Glo1(+/+)^{Gt(..)2Lex}$ mutant mice, respectively. This decrease in expression indicates that copies of *Vmn2r112* are weakly or mostly non-functional and in *Glo1* mutant mice which is likely linked to *Vmn2r112* gene disruption.

To explore the possibility that *Glo1* may suffer dicarbonyl stress-induced copy number alteration, we studied prolonged exposure of mESCs *in vitro* to severe dicarbonyl stress imposed by high concentrations of exogenous MG [1]. For physiological relevance we studied Glo1 activity and copy number changes also under an atmosphere of 3% oxygen – equivalent to ambient oxygen concentration for embryonic stem cells *in vivo* [41]. We found low level increased copy number focussed to the *Glo1* genomic domain induced to a similar level by addition of exogenous MG, 3% oxygen, and exogenous MG and 3% oxygen together. A possible driver for this is aneuploidy which occurs spontaneously in ESCs in long

term culture. This is an unlikely explanation for Glo1 copy number increase, however, as: (i) Glo1 is not a genetic locus found increased in ESCs in genome-wide studies when cultured under 20% oxygen atmosphere [42]; (ii) genes proximate to the Glo1 genetic locus, *Btbd9* and *Dnahc8*, were not increased in copy number along with Glo1 – indicating that the increase in copy number was specific for the Glo1 locus and not the whole or substantial fragment of the chromosome; and (iii) Glo1 copy number was increased in mESCs *in vitro* under an atmosphere of 3% oxygen, or hypoxia, which are conditions where genomic instability and aneuploidy in ESCs is decreased [43, 44]. Rather, it appears that increase in Glo1 copy number in response to dicarbonyl stress and hypoxia is a programmed and not a spontaneous characteristic of mESCs *in vitro*.

Interestingly there are precedents of hypoxia-induced gene copy number increase: Black *et al*. reported genetic domain specific increase of gene copy number to levels similar to those of Glo1 herein in primary human T-lymphocytes and tumours – including genes linked to multidrug resistance (MDR) [45]. This was driven by histone demethylase KDM4A [46]. Glo1 is implicated in cancer MDR [47]. It is likely that Glo1 copy number increase herein was driven by a similar mechanism. Blank *et al*. did not investigate copy number change at the Glo1. Glo1 had increased expression in hypoxia-adapted tumour cell lines but copy number was not investigated as a possible mediating factor [48].

For the hypoxia response, we found decreased Glo1 activity in mESCs under 3% oxygen (Figure 3b). This occurred despite a concurrent small increase in Glo1 copy number (Figure 3h). This was expected because down-regulation of Glo1 expression in hypoxia is driven by hypoxia-inducible factor 1-alpha (HIF1α) and typically decrease Glo1 expression by *ca.* 30% [49], greater than the increase in Glo1 copy number in short-term culture, producing an overall decrease in Glo1 expression and activity (Figure 3b – 3d). It is likely that hypoxia-induced Glo1 copy number is increased by KDM4A or similar mechanisms – see above. This remains to be determined in future studies. We propose that high concentrations of MG either activate KDM4A or by direct glycation achieve similar functional change in histone proteins to KDM4A-driven demethylation. This would explain why hypoxia and MG treatment achieve the same level of induced Glo1 copy number increase individually and together. We propose that hypoxia-activated processes in ESCs under 3% oxygen environment *in vivo* may contribute to low GLO1 duplication in mouse and human populations [35, 50, 51].

Glo1 may be a focus of copy number increase due to the protection afforded by increased Glo1 protein and activity against dicarbonyl stress-induced cytotoxicity. Severe dicarbonyl stress also induces decreased cell growth and viability – as we and others have found previously in ESCs, other cell types, mouse oocytes, fertilization, and fetal development [52-54]. Mild dicarbonyl stress was induced by mESC culture in 3% oxygen - through down regulation of Glo1 expression and increased anaerobic glycolysis with increased flux of formation of MG. The latter was indicated by increased flux of formation of D-lactate, formed from metabolism of MG by the glyoxalase system [3]. We found increased consumption of glucose and net formation of L-lactate in 3% oxygen cultures reflecting increased anaerobic glycolysis, as found previously [55].

Cytoprotective responses in cells are usually achieved through signalling pathways activating change in gene expression through regulation of transcription and translation [56, 57]. A higher regulatory level of generational duration producing increased expression of cytoprotective genes may be achieved by inducible functional gene copy number increase or copy number alteration [58]. Inducible copy number increase of *Glo1* in dicarbonyl stress provides an example of adaptive genomics for cytoprotective response. A precedent for this is oxidative damage-induced gene duplication arising from chromosomal instability in *Saccharomyces cerevisiae* [59]. This was not focused for genes associated with alleviation of

oxidative stress. Also, there is the precedent of chronic exposure of HA1 Chinese hamster fibroblasts *in vitro* to supraphysiological concentrations of oxygen or hydrogen peroxide (95% oxygen or 800 μM hydrogen peroxide for >200 days) which increased copy number of cytoprotective catalase (CAT) [60]. Genome-wide copy number change was not investigated and hence it is uncertain if the copy number increase was focussed to and selective for CAT.

Dicarbonyl stress increases modification of both protein and DNA by MG [4, 5]. MG modification is a major cause of endogenous DNA damage: MGdG is the major quantitative adduct of endogenous DNA damage *in vivo* and is associated with mutagenesis [5]. Increased functional *Glo1* copy number provides for cytoprotective response and survival against dicarbonyl stress. This survival advantage may account for why GLO1 copy number increase is retained in some instances. Large transcription units drive locus-specific genomic instability during DNA replication. They are replicated late in the cell cycle and organize copy number duplications in their flanking regions. This is thought to occur through transcription-dependent double-fork failure in DNA replication [61]. The Glo1 gene is present in a large transcriptional domain of the genome at the flanking region of *Btbd9* in which copy number increases are enriched.

Increased gene copy number found and retained in the mammalian genome are considered to be limited to genes where copy number increase confers cell growth or survival advantage [62]. Cahan *et al*. found *Glo1* copy number increase in mice produced increased Glo1 expression in all tissue and cell types tested - hypothalamus of brain, adipose tissue and hematopoietic stem cells, with similar copy number increase at all sites [50]. A genome-wide scan of copy number variation in the human population, however, did not reveal a predominance of cytoprotective genes [51]. Many copy number increases are found at low prevalence (<5%) and may occur stochastically. Hence, unless survival pressures are experienced population-wide and continually, copy number alteration for cytoprotective responses may go undetected among a background of random copy number increases. No example of copy number increase in response to exposure to high and toxic levels of endogenous metabolites focussed to gene linked to cytoprotective response has hitherto been presented.

In clinical translation we found evidence of *Glo1* copy number increase in renal failure. We studied renal failure as an example of severe dicarbonyl stress: plasma MG concentration in healthy subjects is *ca.* 100 nM and increases 5 – 7 fold in renal failure [1, 63]. The prevalence of increased GLO1 copy number was higher than spontaneous GLO1 duplication in the human population of *ca.* 2% [51]. Chronic hypoxia is also a common characteristic of renal failure [64]. We propose GLO1 copy number increase in renal failure is due to dicarbonyl stress and/or hypoxia. Decreased GLO1 copy number in one renal failure patient was likely due to DNA damage induced by uremic toxins [65]. There was no association of increased GLO1 copy number with the common SNP C419A. This is expected as the polymorphism gives rise to dimer allozymes with identical enzymatic activity [66, 67] and therefore genotypes may be equally exposed to dicarbonyl stress and thereby similarly susceptible to GLO1 copy number increase. Increased GLO1 copy number in peripheral blood mononuclear cells of patients with renal failure may, therefore, reflect induced GLO1 copy number increase in hematopoietic stem cells and their progeny. The clinical significance of increase in GLO1 copy number in renal failure remains to be determined and could be linked to increased survival. The positive association of increased Glo1 copy number with serum creatinine concentration in PLS-DA is consistent with renal failure as a driver for GLO1 copy alteration. The negative association with alcohol consumption was surprising and interesting but requires validation in further studies.

Studies of GLO1 copy number alteration in other health impairment and disease are of interest – particularly cancer. We previously found increased GLO1 copy number in some

drug naïve human tumours associated with increased Glo1 expression. Increased GLO1 copy number was often functional giving rise to high Glo1 expression and activity permissive for tumour growth in a background of high flux of MG formation [5, 68]. Increased Glo1 expression also confers MDR in cancer chemotherapy [69]. Copy number alteration of GLO1 in the early stages of tumour development by exposure to increased MG from high glycolytic activity associated with tumour metabolic re-programming and hypoxia [70, 71] may, through clonal selection, lead to dominant functional increased functional GLO1 copy number and MDR. This is supported by observations in malignant melanoma, for example, where GLO1 copy number increase has low prevalence (2%) in early stages and high prevalence (80 - 89%) in advanced stages [68, 72, 73]. Recent studies have found very high prevalence of GLO1 copy number increase also in gastric cancer (33%) [74] and in "triple negative" breast cancer lacking expression of oestrogen and progesterone receptors and epidermal growth factor receptor-2 genes (83%) [75]. These tumour types do not respond well to currently available chemotherapy; such tumours may be sensitive to permeable Glo1 inhibitor chemotherapy when available clinically [47]. In gastric cancer increased GLO1 copy number was linked to Glo1 expression and was a negative survival factor [74]. GLO1 copy number alteration through dicarbonyl stress and/or hypoxia may contribute to the development of Glo1-linked MDR in cancer chemotherapy. Other conditions of clinical hypoxia may display low level GLO1 copy number increase in mitotic cells.

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Author contributions
A.S., M.X., G.B., P.J.T. and N.R. designed the experiments, D.Z. arranged and oversaw the clinical study, A.S. performed the experiments, N.R. and P.J.T. wrote the manuscript and all authors edited and approved the submitted manuscript.

Competing financial interests
The authors declare that they have no competing financial interests.

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Table 1 Activity and expression of Glo1 and related metabolites in *Glo1* mutant mice and wild-type controls.

Analyte	Study group	Liver	Kidney	Brain	Heart	Pancreas	Spleen
Glo1 activity	Wild-type	4.36 ± 0.77	0.77 ± 0.14	1.89 ± 0.34	1.00 ± 0.16	0.60 ± 0.05	0.33 ± 0.08
(U/mg protein)	Glo1(+/-) mutant	3.68 ± 0.70	0.78 ± 0.13	2.02 ± 0.43	0.96 ± 0.09	0.64 ± 0.05	0.45 ± 0.12
Glo1 protein	Wild-type	4.98 ± 1.46					
(normalised to β-actin)	Glo1(+/-) mutant	5.12 ± 1.93					
Glo1 mRNA	Wild-type	0.43 ± 0.02	0.55 ± 0.03				
(normalised to Rn 18s)	Glo1(+/-) mutant	0.41 ± 0.02	0.54 ± 0.04				
Glo2 activity	Wild-type	0.11 ± 0.02					
(mU/mg protein)	Glo1(+/-) mutant	0.11 ± 0.01					
Methylglyoxal	Wild-type	4.33 ± 0.65					
(pmol/mg wet weight)	Glo1(+/-) mutant	4.31 ± 1.02					
D-Lactate	Wild-type	0.34 ± 0.06					
(nmol/mg wet weight)	Glo1(+/-) mutant	0.34 ± 0.06					
MG-H1	Wild-type	0.45 (0.33 – 0.51)					
(mmol/mol arg)	Glo1(+/-) mutant	0.38 (0.32 – 0.42)					
MG Reductase activity	Wild-type	0.00278 ± 0.00089					
(U/mg protein)	Glo1(+/-) mutant	0.00284 ± 0.00091					

Data are mean ± SD or median (lower – upper quartile); n = 8, wild-type, and n = 9, Glo1(+/-) mutant mice.

Table 2 Copy number variation in *Glo1* mutant mice and wild type controls.

	Exon-1		Exon-6	
Tissue	Wild-type	Glo1 (+/-) mutant	Wild-type	Glo1 (+/-) mutant
Liver	2.02 ± 0.27	3.17 ± 0.28***	2.01 ± 0.20	3.19 ± 0.30***
Kidney	2.02 ± 0.32	2.98 ± 0.26***	2.00 ± 0.10	3.24 ± 0.29***
Brain	2.00 ± 0.06	2.97 ± 0.17***	2.00 ± 0.11	3.04 ± 0.18***
Pancreas	2.00 ± 0.11	2.95 ± 0.20***	2.01 ± 0.13	3.01 ± 0.22***

Data are mean ± SD (n = 8 wild-type and n = 9 *Glo1* mutant mice). Significance: ***, p<0.001; Student's *t*-test.

Table 3 Inheritance of the *Glo1*$^{Gt(OSTGST\_4497\text{-}D9)Lex}$ mutant allele.

Mated couple	Litter size	*Glo1(+/+)*	*Glo1(+/+)*$^{Gt(..)1Lex}$	*Glo1(+/+)*$^{Gt(..)2Lex}$
♀: *Glo1(+/+)* ♂: *Glo1(+/+)*$^{Gt(..)1Lex}$	4	2	2	0
♀: *Glo1(+/+)*$^{Gt(..)1Lex}$ ♂: *Glo1(+/+)*$^{Gt(..)1Lex}$	6	2	3	1
♀: *Glo1(+/+)* ♂: *Glo1(+/+)*$^{Gt(..)2Lex}$	7	0	5	0

Glo1 Copy number was determined by the Taqman method and allele typing by qPCR.

Table 4 Effect of exogenous methylglyoxal on copy number of Glo1 and neighbouring genes in murine embryonic stem cells *in vitro*.

	Incubation period (days)						
	0	3		6		12	
Gene	Copy number	Copy number	Change (%)	Copy number	Change (%)	Copy number	Change (%)
Glo1(Exon1)	2.00 ± 0.10	2.11 ± 0.07**	5	2.15 ± 0.11**	7	2.32 ± 0.09***	16
Glo1(Exon6)	2.00 ± 0.09	2.19 ± 0.09**	9	2.15 ± 0.11**	7	2.16 ± 0.05***	8
Btbd9	2.00 ± 0.05					1.94 ± 0.20	
Dnahc8	2.00 ± 0.05					2.04 ± 0.09	

All analyses were performed in triplicate and copy number analyses were performed using Taqman Copy number assay. Data are mean ± SD (n = 3). Significance: **: $p<0.01$, ***; $p<0.001$; Student's *t*-test.

FIGURE LEGENDS

Figure 1 Protein and nucleotide glycation and glyoxalase metabolism in dicarbonyl stress and DNA Electrophoresis for PCR analysis of *Glo1* mutant and wild-type control mice. (**a**) Metabolism of methylglyoxal by the glyoxalase system. (**b**) Formation of hydroimidazolone MG-H1 from arginine residues in protein. (**c**) Formation of imidazopurinone isomers in DNA. The common 2'-deoxyribosyl group has been omitted for clarity. (**d**) DNA Electrophoresis for PCR analysis of *Glo1* mutant and wild-type control mice. The figure shows an agarose gel stained with ethidium bromide. The first lane on the left-hand side, Hyperladder V, shows electrophoresis of DNA bp size calibrators. Then lanes are labelled by sample genotype: wild-type, Glo1(+/+)$^{Gt(..)1Lex}$ and Glo1(+/+)$^{Gt(..)2Lex}$. (**e**) Location of Primer target sequences in the *Glo1* and *Glo1* mutated gene. Solid bar, wild-type DNA; hollow bar – inserted vector; arrows, primers. LTR = long terminal repeat. *, location of VICTR48 insertion into *Glo1* (+ 706 from the start codon).

Figure 2 Comparative genomic hybridization array of *Glo1* mutant mice. An extract of chromosome 17, 894 kb from 30379127 to 31273646. Genotype: (**a**) *Glo1(+/+)*,(**b**) *Glo1(+/+)*$^{Gt(..)1Lex}$, and (**c**) *Glo1(+/+)*$^{Gt(..)2Lex}$. Response key: $\log_2 R < 0$, green; $\log_2 R > 0$, Reference DNA was from wild-type C57BL/6 mice. Data were analysed by Agilent Genomic Work bench software. R is the ratio of the probe intensity to the average of reference DNA (DNA combined from male and female wild-type mice). Normalised data were analysed with algorithm ADM-2 with threshold of $P<1 \times 10^{-6}$ and minimum of 3 consecutive probes per region.

Figure 3 Glyoxalase pathway metabolism in mouse embryonic stem cells *in vitro* and effect of low oxygen concentration. (**a**) Viable cell number per flask. (**b**) Glo1 activity**.** (**c**) and (**d**) Glo1 protein – immunoblotting images and densitometric quantitation. (**e**) Glucose consumption**.** (**f**) Net flux of L-lactate formation. (**g**) Flux of D-lactate formation**.** mESCs were incubated for 6 days under the stated conditions. (**h**) Effect on *Glo1* copy number of incubation with and without 200 μM MG for 12 days under atmosphere of 20% or 3 % oxygen. Data are mean ± SD (n = 3). Significance: * and ***, $P<0.05$ and $P<0.001$, respectively; Student's *t*-test.

Figure 4 Inhibition of growth of mouse embryonic stem cells by methylglyoxal *in vitro* – effect of oxygen concentration. (**a**) Effect on cell growth, viable cell number count as a percentage of control. (**b**) Cell viability, percentage of cells remaining that excluded Trypan blue. Key: □-□, cell incubations in 20% oxygen atmosphere; ■-■ cell incubations in 3% oxygen atmosphere. mESCs were incubated as described in the methods section with 0 – 200 μM MG for 48 h and Data are mean ± SD (n = 3). (**c**) Western blot analysis of Glo1 protein in mESCs incubated with 200 μM MG. Control and test incubation blots are given in triplicate showing internal reference protein, β-actin (42 kDa), and Glo1 (subunit molecular mass 23 kDa).

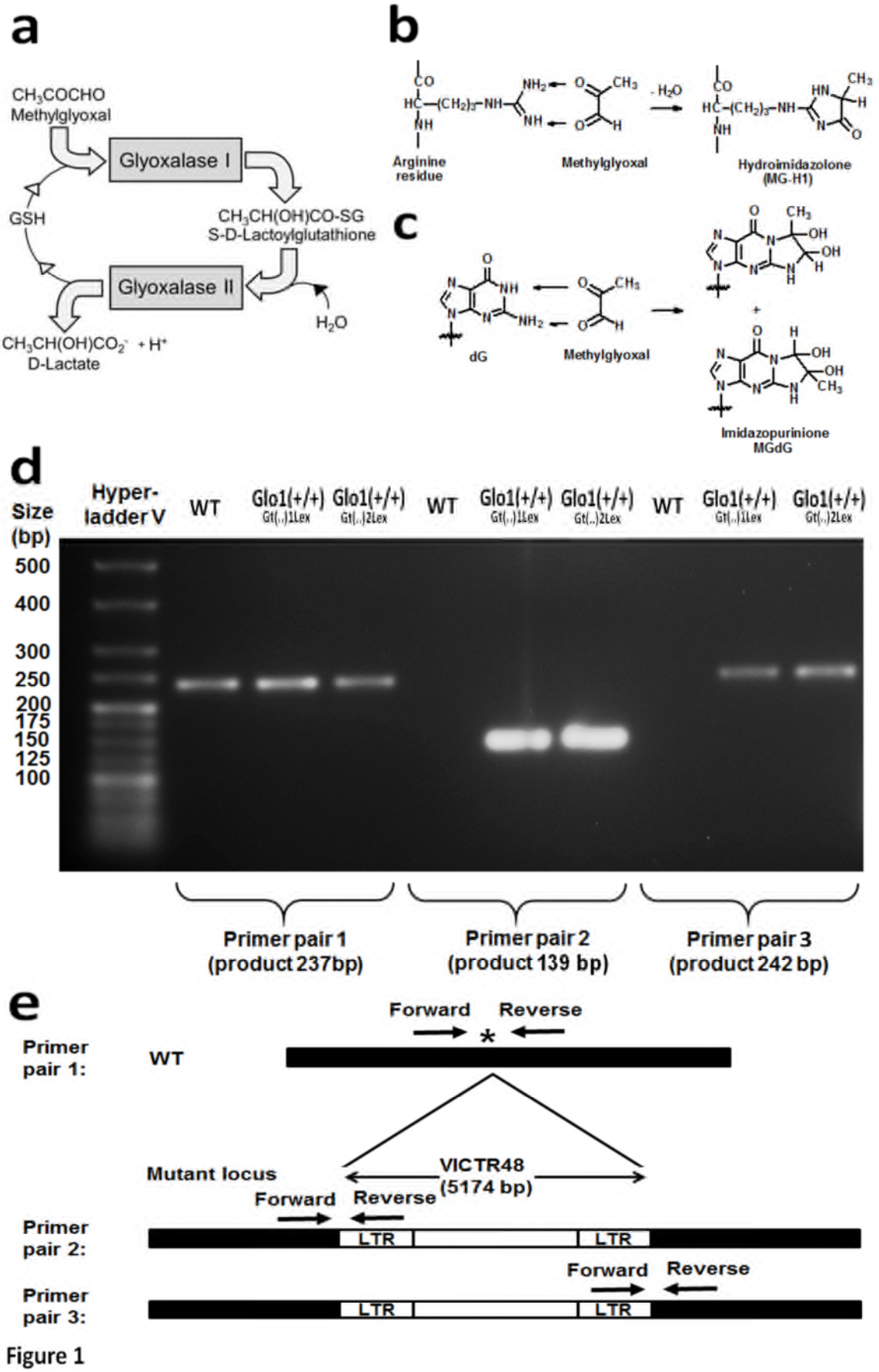
a
CH3COCHO
Methylglyoxal
Glyoxalase I
GSH
CH3CH(OH)CO-SG
S-D-Lactoylglutathione
Glyoxalase II
H2O
CH3CH(OH)CO2- + H+
D-Lactate
b
Arginine residue
Methylglyoxal
- H2O
Hydroimidazolone (MG-H1)
c
dG
Methylglyoxal
Imidazopurinione MGdG
d
Size (bp)
Hyper-ladder V
WT
Glo1(+/+) Gt(..)1Lex
Glo1(+/+) Gt(..)2Lex
500
400
300
250
200
175
150
125
100
Primer pair 1 (product 237bp)
Primer pair 2 (product 139 bp)
Primer pair 3 (product 242 bp)
e
Primer pair 1:
WT
Forward
Reverse
Mutant locus
VICTR48 (5174 bp)
Primer pair 2:
LTR
Primer pair 3:

Figure 1

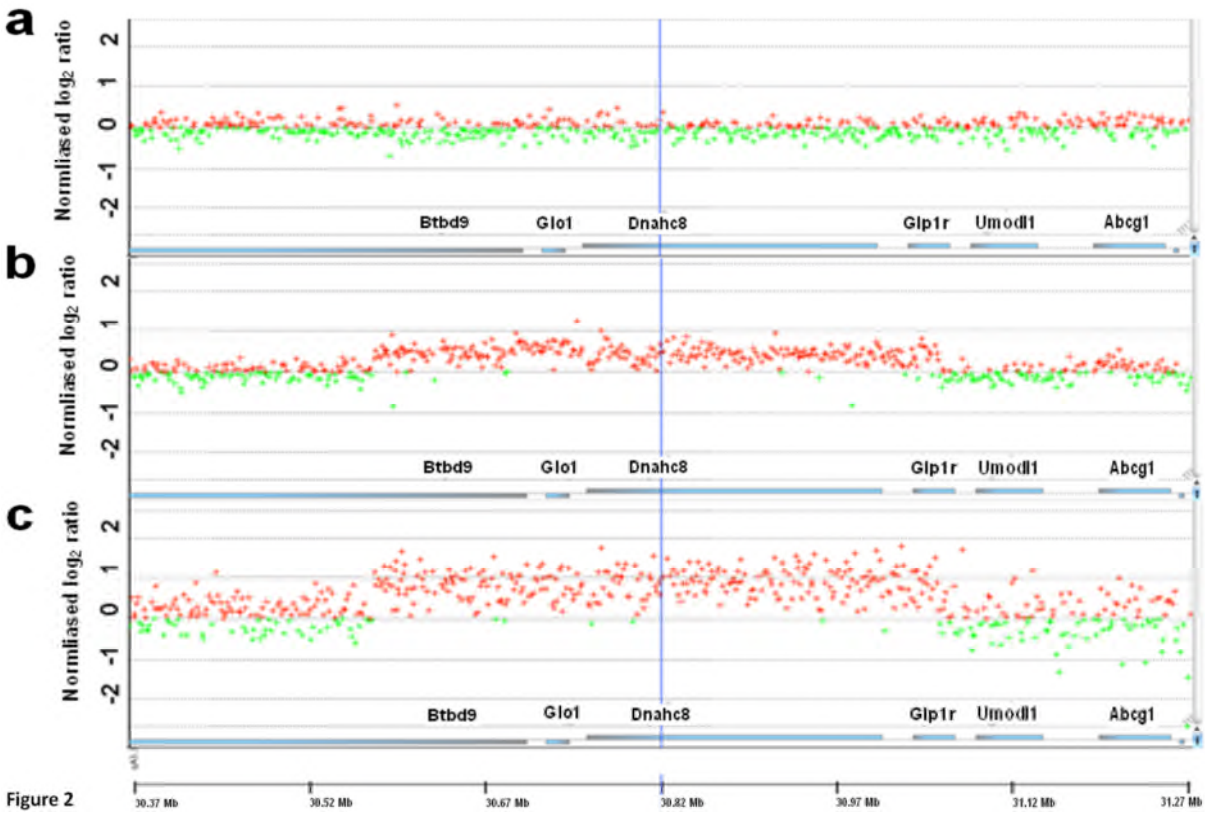

Figure 2

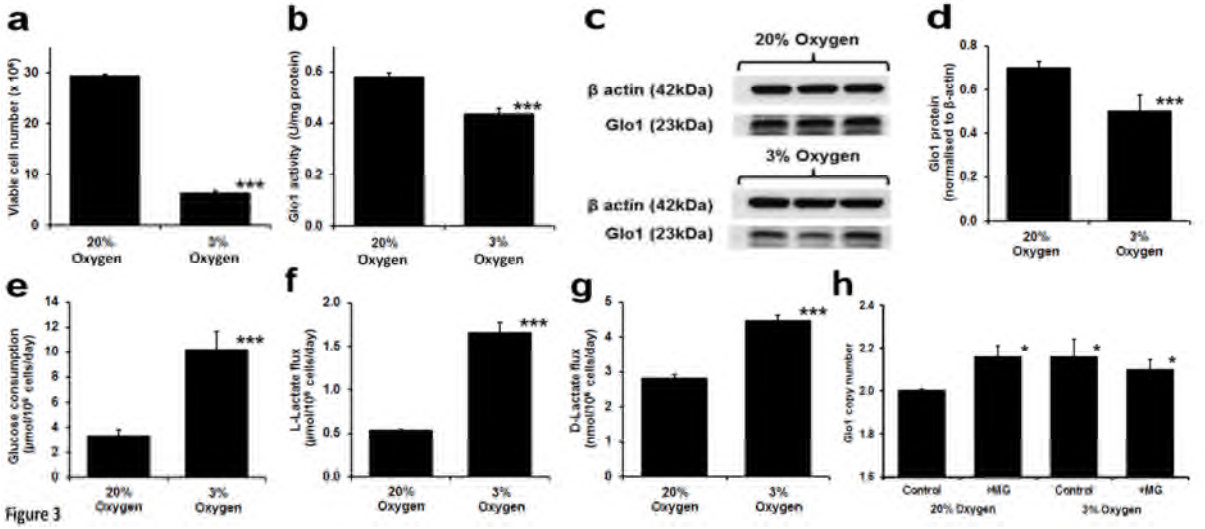

Figure 3

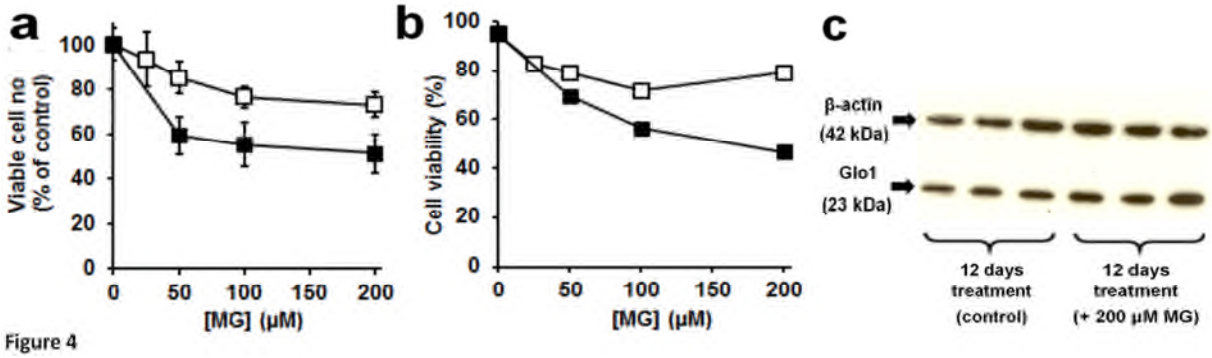

Figure 4

Supplementary information

Re-appraisal of putative glyoxalase 1 deficient mouse and dicarbonyl stress on embryonic stem cells *in vitro*

Alaa Shafie, Mingzhan Xue, Guy Barker, Daniel Zehnder, Paul J Thornalley and Naila Rabbani.

Supplementary Table S1 Characteristics of renal failure patients and healthy control subjects.

Variable	Healthy subjects	Renal failure patients
N	21	20
Gender (M/F)	11/10	10/10
Age (years)	54 ± 11	54 ± 9
Dialysis modality (pre-dialysis/peritoneal dialysis/hemodialysis)	----	6/2/12
Duration of dialysis (years)	----	3.3 (1.3 – 7.8)
Serum creatinine (μM)	90 ± 17	622 ± 242***
BMI (kg/m^2)	26.1 ± 3.3	26.2 ± 3.3
Ethnicity (Caucasian/Asian)	19/2	20/0
Alcohol consumption (Yes/No)	19/2	12/8
Smoking (Current/Ex/Never)	4/7/10	4/2/14
Systolic blood pressure (mmHg)	125 ± 14	139 ± 27*
Diastolic blood pressure (mmHg)	76 ± 9	77 ± 13
Hemoglobin (g/L)	135 ± 15	115 ± 15***
Fasting plasma glucose (mM)	5.5 ± 1.4	6.1 ± 1.5
HbA_{1c} (mM)	40 ± 7	36 ± 5
GLO1 genotype rs4746 A/C332 (AA/AC/CC)	3/9/6	3/9/7

Data are mean ± SD or median (lower – upper quartile). Significance: * and ***, $P<0.05$ and $P<0.001$ respectively; Student's t-test. The causes of renal failure were: polycystic kidney disease 4, Alport syndrome 2, interstitial nephritis 2, chronic pyelonephritis 2, reflux nephropathy 2, hypertensive nephropathy 1, vasculitis 1, obstructive nephropathy 1, IgA nephropathy, post-streptococal glomerulonephritis 1, undetermined 3. GLO1 genotype was not determined in 3 subjects. Characteristics of patients with GLO1 copy number alteration: dialysis modality – hemodialysis, 4, pre-dialysis, 1; cause of renal failure - polycystic kidney disease 2, obstructive nephropathy 1, undetermined 2; GLO1 genotype rs4746 – AA, 3 and AC, 2, CC, 0; and serum creatinine 746 ± 351 μM.

Supplementary Table S2 PCR and PRT primers used in the study.

Study	Primer no	Sequence (Sense, Antisense)
Glo1 mutant mouse genotype	1 (for wild-type Glo1)	Forward: 5'-TTGCTTGCTTGGCTTTGCCATTGC-3' Reverse: 5'-GGACCACCACCTGAATGAGTCTTGC-3'
	2 (for 5'- *Glo1*-VICTR48 link)	Forward: 5'-TTGCTTGCTTGGCTTTGCCATTGC-3' Reverse: 5'-TAAACCCTCTTGCAGTTGCATC-3'
	3 (for VICTR48-3'-*Glo1* link)	Forward: 5'-AAATGGCGTTACTTAAGCTAGCTTGC-3' Reverse: 5'-GGACCACCACCTGAATGAGTCTTGC-3'
C419A genotyping		5'-TCAGAGTGTGTGATTTCGTG-3' 5'-CATGGTGAGATGGTAAGTGT-3'
PRT (mouse)		Forward: 5'-GGCACAGGTCTGTTTTGTGA-3' Reverse: 5'-ATATTTCCAACTGGGTGGGAG-3'

Supplementary figures

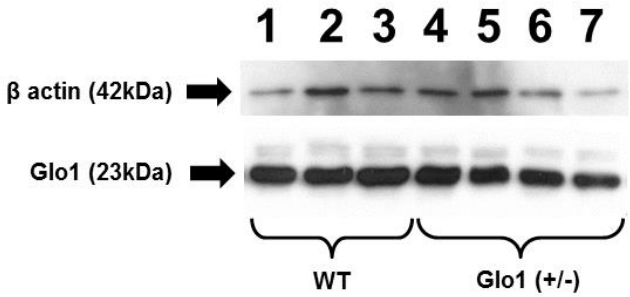

Figure S1 Glyoxalase 1 protein content of liver of wild-type and *Glo1* mutant mice. Specimen gels of immunoblotting for Glo1 protein with β-actin as house-keeping control. WT (n = 3) and Glo1(+/-) (n = 4) showing Glo1 protein (subunit molecular mass 23 kDa) and internal reference protein, β-actin (42 kDa).

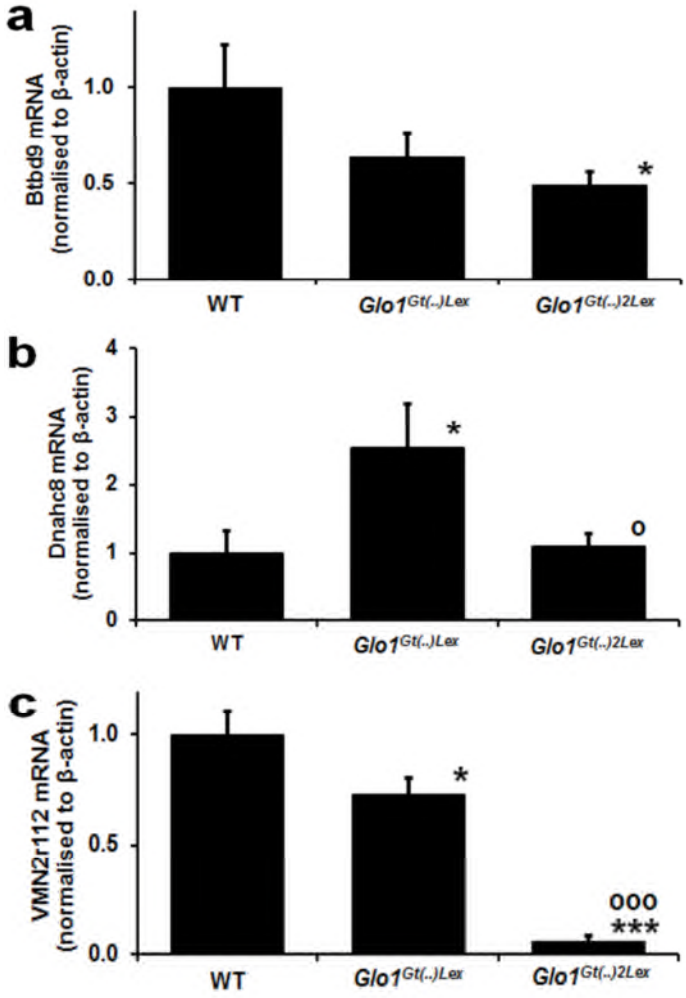

Figure S2 **mRNA expression of the duplicated genes in liver tissue of wild-type mice, *Glo1*(+/+)$^{Gt(..1)Lex}$ and *Glo1*(+/+)$^{Gt(..)2Lex}$** mice**.** Relative mRNA content: (a) Btbd9, (b) Dnahc8, and (c) VMN2r112. **Data are mean ± SD (n = 3). Significance: * and ***, P<0.05 and P<0.001 with respect to** wild-type**; and o and ooo, P<0.05 and P<0.001 with respect to *Glo1*(+/+)$^{Gt(..)1Lex}$; Student's** *t*-test.

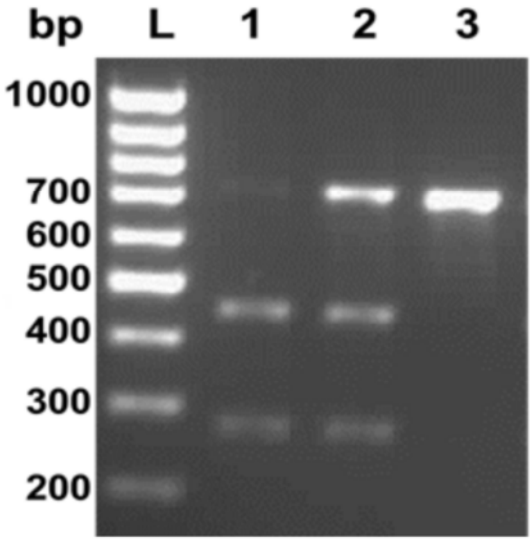

Figure S3 Specimen genotyping for C419A SNP in *GLO1* gene. DNA ladder (bp calibration standards) (Lane L). Two bands of 260 and 453 bp fragments indicative of homozygous C419 allele (rs4746 C332) (Lane 1). Three bands of 713, 453 and 260 bp indicative of heterozygous C/A419 alleles (Lane 2). A single band of 713 bp fragment indicative of homozygous A419 allele (rs4746 A332) (Lane 3). The gel image shown is cropped; all lanes were run in a single gel under the same conditions.