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Comparative Study in Various Model Organisms Regarding the Effect of the Loss of Glyoxalase 1

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Content

Abbreviations

4-HNE	4-hydroxynonenal
AC	Acrolein
AGE	Advanced glycation endproduct
AKR	Aldo-keto reductase
ALDH	Aldehyde dehydrogenase
AmpB	Amphotericin B
ARE	Antioxidant response element
DHAP	Dihydroxyacetone phosphate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
FA	Formaldehyde
FCS	Fetal calf serum
GAD3P	Glyceraldehyde-3-phophate
GLO1	Glyoxalase 1
$GLO1^{-/-}$	Glyoxalase 1 knock-out
GLO2	Glyoxalase 2
GO	Glyoxal
GPX	Glutathione peroxidase
GSH	Glutathione, reduced form
GSR	Glutathione reductase
GSSG	Glutathione disulphide, oxidized form
H_2O_2	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMOX1	Heme oxygenase (decycling) 1
HOG1-MAPK	high osmolarity glycerol 1/mitogen-activated protein kinase
hpf	Hours post fertilization
HTA	Hemithioacetal
KO	Knock-out
LD 50	Median lethal dose 50%
MDA	Malondialdehyde
MG	Methylglyoxal
MG-H1	Methylglyoxal-derived Hydroimidazolone
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NGM	Nematode growth media
NQO1	NADH dehydrogenase (quinone) 1
NRF2	Nuclear factor E2-related factor 2
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween 20
Pen	Penicillin
PRDX	Peroxiredoxin
RAGE	Receptor for AGE
RCS	Reactive carbonyl species
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SRXN	Sulfiredoxin
Strep	Streptomycin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TXNDR	Thioredoxin reductase
WT	Wildtype

1. Introduction

Diabetes is a major metabolic disease and a leading cause for several pathological conditions affecting more than 400 million people worldwide [IDF Diabetes Atlas 2017]. Globally, the numbers of patients suffering from diabetes and associated late complications, such as retinopathy, neuropathy and nephropathy are rapidly increasing. The molecular mechanisms of diabetic complications are still not clearly understood, leading to an inadequate treatment and therapeutic outcome for patients [Forbes, J.M. and Cooper, M.E. 2013].

T1D is characterized by the impairment of insulin signalling due to the destruction of pancreatic β cells [Alberti, K.G. and Zimmet, P.Z. 1998]. The pathology of T2D is associated with biochemical aspects such as insulin resistance, hyperglycaemia and obesity [Han, T.S. and Lean, M.E. 2016]. The standard therapy for diabetes is the glycaemic control. However, the DCCT has shown that only 11% of late complications in T1D [Lind *et al.* 2010] are explained by increased HbA1c levels and similar findings are observed in T2D patients [Gerstein *et al.* 2008]. Therefore, it is not surprising that patients despite well controlled HbA1c still develop late complications such as neuropathy [Ismail-Beigi *et al.* 2010]. These findings show that additional mechanisms are involved in the onset of diabetes and the development of late complications and that these mechanisms need to be further investigated.

One of the commonly accepted theories for the development of late complications is the unifying theory [Brownlee, M. 2001]. The theory is based on the assumption that increased glucose uptake by insulin-independent cells leads to elevated intracellular glucose concentrations and subsequently to the accumulation of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO•). Under normal physiological conditions, antioxidative defence systems in form of enzymes such as NADH dehydrogenase (quinone) 1 (NQO1) [Ross, D. and Siegel, D. 2004], superoxide dismutase (SOD) [Pias *et al.* 2003], catalase [Chelikani *et al.* 2004], glutathione peroxidase [Mills, G.C. 1957] or ROS scavengers such as glutathione (GSH) [Pompella *et al.* 2003] or vitamins [Deponte, M. 2013] are responsible for the detoxification of ROS to achieve low levels of oxidative stress within the cell [Finkel, T. and Holbrook, N.J. 2000]. An inefficient detoxification leads to deleterious effects on various cell compartments due to oxidative stress [Davis, K.J.A. 1995]. Under conditions of high glucose flux, the polyol, hexosamine and protein kinase C pathways are activated and furthermore, the increase in glucose metabolism leads to the accumulation of advanced glycation end products (AGEs). The unifying theory presupposes an important role of high glucose flux on cellular dysfunction and subsequently on the development of late complications [Brownlee, M. 2005; Giacco, F. and Brownlee, M. 2010].

1.1 The reactive metabolite methylglyoxal and advanced glycation end products

It has been shown that AGEs were involved in the development and progression of diseases including diabetes [Baynes, J.W. 2003; Yamagishi *et al.* 2012; Yap *et al.* 2012]. During glycolysis, AGEs are derived from reactive carbonyl species (RCSs). RCSs such as glyoxal (GO), 4-hydroxynonenal (4-HNE), acrolein (AC) or malondialdehyde (MDA) are formed endogenously during glycolysis, by lipid peroxidation and during the carbohydrate metabolism [O'Brien *et al.* 2005]. The major precursor of AGEs during glycolysis is methylglyoxal (MG), an α-oxoaldehyde. MG is formed non-enzymatically as a by-product of the glycolysis from the spontaneous degradation of glyceraldehyde 3-phosphate (GAD3P) and dihydroxyacetone phosphate (DHAP) [Phillips, S.A. and Thornalley, P.J. 1993; Thornalley, P.J. 2008]. It is estimated that the concentration of MG in human blood plasma are in the range of 100-120 nM, while cellular concentrations are in the range of 1-5 µM [Thornalley, P.J. 2008]. As a glycation agent, MG reacts with free amino groups on proteins to form AGEs, especially with arginine residues to form methylglyoxal-derived hydroimidazolones (MG-H1), the most physiologically relevant protein modification (›90%) regarding AGEs ubiquitously found in all body tissues [Rabbani, N. and Thornalley, P.J. 2012].

Within the cell, the formation of MG and its capability to promote posttranslational modifications leads to damaging effects such as changes in gene transcription, cell growth arrest and apoptosis, modification of extracellular proteins and pro-inflammatory response through an increased binding of glycated plasma proteins to the receptor for AGEs (RAGE) [Bierhaus *et al.* 2005; Brownlee, M. 2005; Lukic *et al.* 2008; Allaman *et al.* 2015]. Subsequently, the link between increased glycolytic flux and increased MG formation has been shown in patients suffering from diabetes, resulting in an association with late complications due to an increased AGE accumulation [Thornalley, P.J. 1988; Phillips, S.A. and Thornalley, P.J. 1993; Beisswenger *et al.* 2003; reviewed in Fleming *et al.* 2011]. For instance, in patients suffering from diabetic neuropathy, it has been shown that MG-derived AGEs in peripheral neurons triggered pain caused by a change in the firing pattern of the sodium-gated voltage channels [Bierhaus *et al.* 2012; Eberhardt *et al.* 2012].

1.2 The detoxification of methylglyoxal via the glyoxalase system

The glyoxalase system, present in the cytosol, is considered to be the main enzyme system for the detoxification of MG. The system consists of two enzymes using a catalytic amount of GSH to catalyse the conversion of MG to D-lactate [Rabbani, N. and Thornalley, P.J. 2012]. First, MG and GSH form, non-enzymatically and spontaneously, a hemithioacetal (HTA). Glyoxalase 1 (GLO1) catalyses the isomerization of HTA to S-2-hydroxylacylglutathione. In the second step, glyoxalase 2 (GLO2) converts the S-2-hydroxylacylglutathione to D-lactate [Thornalley, P.J. 2003]. In the past, scientific research has focused mainly on the initial and rate-limiting enzyme GLO1. Its activity prevents the accumulation of MG and therefore the formation of MG-derived AGEs. Increased concentrations of MG are associated with decreased activity of GLO1 in diabetes and is linked to the development of late complications [Vander Jagt, D.L. 2008; Rabbani, N. and Thornalley, P.J. 2011]. The observation that two enzymes are essential for the detoxification of MG and the role as an anti-glycation defence, were already made in 1951 [Racker, E. 1951]. Therefore, GLO1 and its role in detoxification has been characterized in many different organisms, from *Saccharomyces cerevisiae* [Aronsson *et al.* 1978], to plants [Deswal R. and Sopory S. K. 1991] up to humans [Cameron *et al.* 1997].

The role of glyoxalase 1 in *Saccharomyces cerevisiae*

S. cerevisiae represents a simple eukaryote in which glucose metabolism, the effect of MG and the capacity of GLO1 has been investigated. In fact, the activity of GLO1 is one of the most important parameters for controlling the intracellular MG concentration in yeast [Ponces *et al.* 2014]. It has been shown that osmotic stress, such as high glucose concentrations, leads to an increased MG formation resulting in elevated AGE formation such as argpyrimidine [Gomes *et al.* 2005]. Elevated MG levels, either via exogenous MG stimuli or due to high glucose flux, leads to an increase of GLO1 expression via the high osmolarity glycerol 1/mitogen-activated protein kinase (HOG1-MAPK) pathway [Maeta *et al.* 2005]. Interestingly, yeast cells lacking GLO1 are viable and even further, GLO1 knock-out (Δglo1) yeast cells show a hormetic effect when cultured with low doses of MG and this enables yeast cells to deal with toxic metabolites [Zemva *et al.* 2017].

The role of glyoxalase 1 in *Caenorhabditis elegans*

In *C. elegans*, it has been shown that the activity of GLO1 is reduced with age and a decrease of GLO1 activity lead to a drastic accumulation of MG which can be reduced by overexpressing GLO1. Furthermore, the mean lifespan in nematodes with a GLO1 overexpression is increased from 13.3 days to 17.2 days, while RNAi-mediated knock-down of GLO1 decreased mean lifespan from 18 days to 8.6 days.

The overexpression of GLO1 also leads to decreased MG-derived AGEs (50%) and this accumulation is associated with an elevated formation of superoxide in the mitochondria [Morcos *et al.* 2008; Schlotterer *et al.* 2009].

The role of glyoxalase 1 in *Drosophila melanogaster*

D. melanogaster has been emerged as a useful model to study human diseases including neurodegenerative disease, cancer and metabolic dysfunction [Bellen, H.J. and Yamamoto, S. 2015]. To obtain consistent phenotypes, the group of Aurelio Teleman (German Cancer Research Center, Heidelberg, Germany) generated a GLO1 knock-out fly [Moraru *et al.* 2018]. They have reported that GLO1 knock-out flies are viable and normal in appearance. Endogenous MG was mildly elevated and complications characteristic for T2D were detected in these animals. Young GLO1 knock-out flies were found to be insulin resistant; they became obese and hyperglycemic during aging. This data would suggest that the loss of GLO1 lead to elevated MG levels and thereby to the onset of a diabetes-like phenotype [Moraru *et al.* 2018].

The role of glyoxalase 1 in *Danio rerio*

D. rerio (zebrafish) has been proven as a model organism to study vascular development, function and mechanisms with shared functions in mammals. Indeed, besides *S. cerevisiae* [Tyedmers, J. 2012], *C. elegans* [Mendler *et al.* 2012] and *D. melanogaster* [Teleman *et al.* 2012], zebrafish has evolved as a model organism for investigations of metabolic disorders such as hyperglycemia-induced pathologies [Gleeson *et al.* 2007; Olsen *et al.* 2010]. To target the role of GLO1 in zebrafish, the group of Jens Kroll (University Heidelberg, Medical Faculty Mannheim, Germany) generated a GLO1 knock-out zebrafish. The first evidence for elevated MG levels when GLO1 is impaired has been shown in GLO1 transient morpholino-induced knock-down animals resulting in vascular changes [Jörgens *et al.* 2015]. The GLO1 knock-out animals also showed mildly increased MG levels but no altered vasculature structures caused by endogenously applied MG [Lodd *et al.* 2018, under revision].

The role of glyoxalase 1 in mammals

In 1998, it has been shown on bovine endothelial cells that the overexpression of GLO1 leads to the prevention of hyperglycemia-induced elevated MG and AGE [Shinohara *et al.* 1998]. Furthermore, it was shown that the overexpression of GLO1 prevented the impairment of angiogenesis under hyperglycemic conditions in human endothelial cells [Ahmed *et al.* 2008]. The downregulation of GLO1 via RNA interference results in a loss of viability in neurons [Bélanger *et al*. 2011] and the knock-down of GLO1 via small interfering RNA (45-65% reduction of GLO1 expression) leads to

increased MG-H1 formation in murine glomeruli and tubular cells [Giacco *et al*. 2014]. *In vitro* studies from 2016 in human aortic endothelial cells have shown that the knock-down of GLO1 leads to increased MG, enhanced inflammation markers and apoptosis which might contribute to vascular damage [Stratmann *et al.* 2016]. In mice and rats, overexpression of GLO1 in rats leads to reduced levels of AGEs under diabetic conditions [Brouwers *et al.* 2011] and to the prevention of impaired vascular dilatation in diabetic rats [Brouwers *et al.* 2010; reviewed Rabbani, N. and Thornalley, P.J. 2012]. The impairment of GLO1 activity, by a partial reduction, leads to hyperalgesia and nephropathy in mice and in patients suffering from diabetes [Bierhaus *et al.* 2012; Giacco *et al.* 2014]. Furthermore, it has been hypothesized that the activity of GLO1 correlates with diabetic neuropathy because it has been shown that in human blood samples from T1D and T2D patients with neuropathy the activity of GLO1 is reduced [Skapare *et al.* 2013]. Based on these findings, it has been hypothesized that increasing expression of GLO1 may improve metabolic health in humans. Therefore, a GLO1 inducer was developed which can activate GLO1 activity via a functional regulatory antioxidant response element (ARE), induced by the transcriptional factor nuclear factor E2-related factor 2 (NRF2) [Xue *et al.* 2015; Xue *et al*. 2015; Xue *et al*. 2016]. It was shown that the GLO1 inducer corrected insulin resistance, improved glycaemic control and vascular inflammation in healthy overweight and obese subjects [Rabbani, N. and Thornalley, P.J. 2018].

In contrast, in 2017 has been shown that the total loss of GLO1 in murine Schwann cells did not lead to elevated MG and MG-H1 levels and furthermore, the total knock-out of GLO1 and its function to detoxify MG, was compensated by other enzymes such as aldo-keto-reductases (AKR) and aldehyde dehydrogenases (ALDH) [Morgenstern *et al.* 2017]. Additionally, a viable GLO1 knock-out mouse was created and showed a reduced anxiety-like and an increased depression-like behavior [Jang *et al.* 2017]. A viable GLO1 knock-out mouse was generated using the CRISPR/Cas9 technique. No elevated MG or MG-H1 levels were found even under hyperglycemic conditions, no aggravation in nephropathic changes were detected and subsequently, AKR subtypes as compensatory enzymes were identified in kidney and liver [Schuhmacher *et al.* 2018].

1.2.1 Alternative detoxification of methylglyoxal

Even though GLO1 is the main enzyme to detoxify MG, various alternative enzymes has been described which are able to detoxify MG. Studies have focused on the NADP/NADPH-dependent subtypes of the AKR and ALDH families, which were found to convert MG into non-toxic end products [Vander Jagt, D.L. and Hunsaker, L.A. 2003].

The family of AKRs contains more than 190 subtypes and have a broad range of substrates such as MDA, 4-HNE, GO and MG. The AKR family consists of about 60 members which are also able to detoxify MG *in vivo* [Baba *et al.* 2009]. The subtypes AKR1a1, 1b1 and 1b3 are able to convert MG into hydroxyacetone and lactaldehyde [Vander Jagt *et al.* 1992; Vander Jagt, D.L. and Hunsaker, L.A. 2003]. *In vivo*, AKR1b3 null mice showed an increased AGE formation in the heart and an increased atherosclerotic lesion formation [Baba *et al.* 2009]. In murine Schwann cells, AKR1b3 has been shown to play an essential role in the detoxification of MG [Morgenstern *et al.* 2017]. Both findings, *in vitro* and *in vivo*, confirmed the important role of AKRs as an alternative detoxification system for MG. Additionally, the family of ALDHs has been studied regarding the detoxification of reactive metabolites, such as MG. The subtypes ALDH1, 2, 3 and 9 are able to convert MG into pyruvate *in vitro* [Izaguirre *et al.* 1998]. When GLO1 is knocked-out, the activity of AKR1b3 is increased, particularly in the kidney. Additionally, there is evidence that the activity of ALDHs in the liver is also increased which are also known to detoxify MG *in vivo* [Schuhmacher *et al.* 2018].

In addition, other organisms also have alternative detoxification mechanisms. It has been shown that the transcription factor Yap 1 is up-regulated and constitutively active in the nucleus in Δglo1 yeast cells and activates the expression of target genes responsible for the GSH metabolism and detoxification such as GSH synthesis (GSH1, GSH2) [Grant *et al*. 1996] and GSH-dependent antioxidant enzymes (GPX2) [Tsuzi *et al.* 2004; Maeta *et al.* 2004; reviewed in Inoue *et al.* 2011]. It has been shown that MG activates Gcn2 followed by the activation of Gcn4, a master regulator of gene expression. In Δgcn4 yeast cells, the response to MG stress was impaired indicating that Gcn4 may play an important role, but the mechanism behind the adaptive response is still under investigation [Nomura *et al.* 2008]. Another concept of regulating MG stress is the activation of the yeast aldose reductase (Gre3) via the high osmolarity glycerol mitogen-activated protein kinase (HOG-MAPK) pathway [Aguilera, J. and Prieto, J.A. 2001]. It has been shown that the overexpression of Gre3 increases MG tolerance and furthermore compensates the loss of GLO1 in Δglo1 cells [Aguilera *et al.* 2005]. In *C. elegans*, 11 ALDH genes have been identified [Sophos *et al*. 2001**]**. Most of the ALDH genes were predicted to have oxidoreductase activity, but only alh-1 and alh-10 has been shown to moderately contribute to the oxidation of 4-HNE [Singh *et al*. 2008]. The substrates of the other enzymes are still not clear. *D. melanogaster* expresses various AKR genes [Davidson *et al.* 1978], but the mechanisms and the physiological role of these proteins are still under investigation. ALDH2 is capable of the detoxification of aldehydes [Rothacker, B. and Ilg, T. 2008] with a broad substrate specificity, probably necessary to protect from various toxic aldehydes generated by lipid peroxidation or acetaldehyde accumulation [Esterbauer *et al.* 1991; Fry, J.D. and Saweikis, M. 2006; Chakraborty, M. and Fry, .JD. 2011].

In zebrafish, it is known that they express the AKRs 1a1a, 1a1b (members of AKR1a1) and 1b1 [Thisse *et al.* 2004] but it is still not fully understood whether these genes could compensate the function of GLO1 to detoxify MG.

1.3 Aim of the study

Over the last two decades, the role of GLO1 has been studied extensively. In *S. cerevisiae* and *C. elegans*, it has been shown that GLO1 is one of the most important detoxification enzymes for MG [Ponces *et al.* 2014; Zemva *et al.* 2017; [Morcos *et al.* 2008; Schlotterer *et al.* 2009]. In 2014, it has been shown that the GLO1 knock-down leads to increased MG-H1 formation in murine glomeruli and tubular cells [Giacco *et al.* 2014]. However, conflicting data regarding the detoxification of MG have recently been published. It has been reported that the knock-out of GLO1 does not influence the MG metabolism in murine Schwann cells [Morgenstern *et al.* 2017] and in GLO1$^{-/-}$ mice [Jang *et al.* 2017; Schuhmacher *et al.* 2018] due to alternative detoxification mechanisms.

The contradictory findings lead to the question whether the loss of GLO1 can cause different effects across species. Thereby raises the question whether findings from simple organisms are transferable to mammals. This study aims to define possible effects of the loss of GLO1 in simple organisms such as *S. cerevisiae* and *C. elegans* up to zebrafish and mammals. The comparison of various model organisms can help to better understand the contribution of GLO1 in simple and complex organisms. To address this question, the MG content, the proliferation/fertilization rate and the response towards toxins will be analysed.

From the previous findings in mice [Schuhmacher *et al.* 2018], it is known that alternative mechanisms could compensate the loss of GLO1 in mice with regard to the detoxification of MG. As these compensatory enzymes are also involved in the detoxification of other toxic substrates, the contribution of general protective mechanisms *in vitro* and *in vivo* will be determined.

2. Material and Methods

2.1 Material

Chemicals and reagents: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (MTT, Sigma, Steinheim, Germany); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma, Steinheim, Germany); 4-hydroxynonenal (4-HNE, Sigma, Steinheim, Germany); Acetaldehyde (AA, Sigma, Steinheim, Germany); Acrolein (AC, Sigma, Steinheim, Germany); Agar (Sigma, Steinheim, Germany); Amphotericin B (AmpB, Gibco, Thermo Fisher Scientific, Karlsruhe, Germany); Dimethyl sulfoxide (DMSO, Honeywell, New Jersey, United States); Dithiothreitol (DTT, Sigma, Steinheim, Germany); Dulbecco's modified eagle's medium (DMEM, Gibco, Thermo Fisher Scientific, Karlsruhe, Germany); Ethanol (Roth, Karlsruhe, Germany); Ethylenediaminetetra acetic acid (EDTA, Roth, Karlsruhe, Germany); Fetal calf serum (FCS, Gibco, Thermo Fisher Scientific, Karlsruhe, Germany); Formaldehyde (FA, Sigma, Steinheim, Germany); Glucose (Merck, Darmstadt, Germany); Glutathione (GSH, Sigma, Steinheim, Germany); Glycerol (Applichem, Darmstadt, Germany); Glycin (Applichem, Darmstadt, Germany); Hydrogenperoxide (H_2O_2, 37%, Sigma, Steinheim, Germany); Malondialdehyde (MDA, Sigma, Steinheim, Germany); Methanol (Sigma, Steinheim, Germany); Methylglyoxal (MG, 40%, Sigma, Steinheim, Germany); Monopotassium phosphate (Sigma, Steinheim, Germany); Nicotinamide adenine dinucleotide phosphate (NADPH, Sigma, Steinheim, Germany); NP 40 (Applichem, Darmstadt, Germany); Penicillin-streptomycin (Pen-Strep, Gibco, Thermo Fisher Scientific, Karlsruhe, Germany) ; Phosphate buffered saline (PBS, Sigma, Steinheim, Germany); Pierce protein-free blocking solution (Thermo Fisher Scientific, Karlsruhe, Germany); Protease inhibitor cocktail (PIC, Sigma, Steinheim, Germany); Sodium dodecyl sulfate (SDS, Roth, Karlsruhe, Germany); Sodium chloride (Sigma, Steinheim, Germany); Sodium hypochlorit (Roth, Karlsruhe, Germany); Sodium phosphate di-sodium salt (Applichem, Darmstadt, Germany); Tris (hydroxymethyl) amino methane (TRIS, Roth, Karlsruhe, Germany); Triton-X 100 (Applichem, Darmstadt, Germany); Trypsin-EDTA (0.05% Gibco, Thermo Fisher Scientific, Karlsruhe, Germany); Tween 20 (Roth, Karlsruhe, Germany); β-Mercaptoethanol (Merck, Darmstadt, Germany).

Consumables and hardware: Bacto peptone (BD Bioscience, Heidelberg, Germany); Bacto yeast extract (BD Bioscience, Heidelberg, Germany); Biophotometer (Eppendorf, Hamburg, Germany); Bioruptor (Diagenode, New Jersey, United States); Cell culture clean bench (Thermo Fisher Scientific, Karlsruhe, Germany); Cell culture dishes (Cellstar, Greiner Bio-One, Nürtingen, Germany); Cell culture flasks (Cellstar, Greiner Bio-One, Nürtingen, Germany); Enhanced Chemiluminescence (ECL) Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, United Kingdom); FluoStar Omega

Multiplate Reader (BMG Labtech, Ortenberg, Germany); High Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher Scientific, Karlsruhe, Germany); Incubator (New Brunswick, Eppendorf, Hamburg, Germany); Light Cycler 480 Instrument II (Roche, Mannheim, Germany); Loading dye concentrated (Fermentas, St. Leon-Rot, Germany); Milk powder (Roth, Karlsruhe, Germany); Mini-Protean TGX precast gels (4-20% acrylamide, Biorad, München, Germany); Nitrocellulose blotting membrane (GE Healthcare, Buckinghamshire, United Kingdom); Page-ruler pre-stained protein ladder (Fermentas, St. Leon-Rot, Germany); PeqGOLD Total RNA Kit (Peqlab, Erlangen, Germany); Primers (Eurofins Genomics, Ebersberg, Germany); Protein extraction beads (Diagenode, New Jersey, United States); Quanti Nova Sybr Green Kit (Qiagen, Hilden, Germany); Tandem mass spectrometry (LC-MS/MS, TQS, TQXS, Waters Corporation, Massachusetts, United States); Trans Blot Turbo Transfer System (Biorad, München, Germany); Western Blotting chamber (Biorad, München, Germany); X-ray film processor (Cawo Solutions, Schrobenhausen, Germany); X-ray films (GE Healthcare, Buckinghamshire, United Kingdom).

2.1 *In Vitro* Methods

2.2.1 Cell culture

Cells were cultured in a humidified incubator with 5% CO_2 at 37 °C. Cells were grown to 80% confluence for experiments and passaged at 90% confluence using 0.05% Trypsin-EDTA (Gibco). Cell culture experiments were performed under sterile conditions. Immortalized murine endothelial cardiac (MCEC), renal tubular epithelial (M1), kidney mesangial (MES13) and Schwann cells (SW10) were obtained from ATCC® (Virginia, United States). For all cell types, GLO1-deficient cells ($GLO1^{-/-}$) were generated via the CRISPR/Cas9 technique and kindly provided by Dr. Thomas Fleming and Dr. Jakob Morgenstern (University Hospital Heidelberg, Germany). Cells were grown in DMEM (Gibco) containing 1 g/mL glucose (5.5 mM) or 4.5 g/mL glucose (25 mM), 10% FCS, 1% HEPES buffer (Sigma), 1% Pen-Strep (10,000 units/mL, 10 mg/mL, Gibco) and 1% AmpB (250 µg/mL, Gibco).

2.2.2 Colorimetric MTT assay

The colorimetric MTT assay was used for the determination of viability as described previously [Mosmann, T. 1983]. Cells were seeded in a 96-well-plate and allowed to adhere overnight at 37 °C. At 80% confluency, cells were treated with the specific compound and incubated in 0.1% FCS containing DMEM media for further 48 h at 37 °C. For cell viability determination, 50 µL of MTT solution (2 mg/mL in PBS) was added to the media and incubated at 37 °C for 2-3 h.

Reduced MTT was solubilized by adding 200 µL DMSO. The absorbance was measured at 590 nm using a FLUOstar OMEGA multiplate reader (BMG Labtech). Data were given in percentage and plotted as cell viability (%) vs. concentration. To calculate the concentration at which 50% cell viability (LD 50) is achieved, data were fitted by nonlinear regression using the GraphPad PRISM 7 software (GraphPad Software Inc.).

2.2.3 Real-time quantitative PCR

Cells were grown in a T175 cell culture flask (Thermo Fisher Scientific) and harvested and collected as described previously at 90% confluency (2.2.1). For RNA extraction, the PeqGOLD Total RNA Kit (Peqlab) was used as described in the manufacturer protocol. 1 µg RNA was converted into cDNA with the High Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher Scientific) according to the manufacturer protocol. The quantitative real-time PCR was performed with 10 µL cDNA using the Quanti Nova Sybr Green PCR Kit (Qiagen, 10 µl of reaction buffer contains 1 x SYBR Green Mastermix, 200 nM forward primer, 200 nM reverse primer). The analysis was performed using a Roche Lightcycler 480 applying the following protocol (tbl. 1).

Table 1. Thermocycler program for real-time quantitative PCR.

Step	Temperature [°C]	Time	Number of cycles
Pre-incubation	95	7 min	1
Amplification	95	10 sec	45
	60	20 sec	
	72	1 sec	
Melting curve	95	5 sec	1
	65	1 min	
	97	5 acquisitions per °C	
Cooling	4	∞	1

Signals of amplified products were verified using melting curve analysis. The mRNA levels were normalized to the geometric mean of housekeeper genes. Following primer sequences were used (tbl. 2).

Table 2. Aldo-keto-reductase and aldehyde-dehydrogenase primer.

Gene	PrimerBank	Forward primer	Reverse primer
AKR1a1	10946870a1	5'-AGCCTGGTCAGGTGAAAGC-3'	5'-GGCCTCCCCAATCTCAGTT-3'
AKR1b3	31981909a1	5'-AGGCCGTGAAAGTTGCTATTG-3'	5'-ATGCTCTTGTCATGGAACGTG-3'
AKR1b8	6679791a1	5'-GACCAAGGCAGAATCCTCACC-3'	5'-AGATGCCCTTCGAGTGACAGT-3'
AKR1b10	31982057a1	5'-CTAGTGCCAAACCAGAGGACC-3'	5'-TCCTGTATTCGAGAAGGTGTCA-3'
AKR1c14	19527294a1	5'-GTGTGGTACTAAACGATGGTCAC-3'	5'-CAAATAAGCGGAGTCAAAATGGC-3'
AKR1c18	19527284a1	5'-TCGTCCAGAGTTGGTCAGAC-3'	5'-CTTTAGGCAAAAGCTCATTCCCT-3'
AKR1c20	16905111a1	5'-ATCGGGGTGTGCAACTTTAAC-3'	5'-AGCACTATGAGCAACCAAAACA-3'
AKR1cl	13386240a1	5'-TTGGAACAATCCCTGAGAAAGC-3'	5'-TGGCTAACCCTGAATCCTTACA-3'
AKR7a5	7659728a1	5'-CGGCCAGTCCGAGAACATC-3'	5'-TTCAGTGACTTCCCTTCCCAG-3'
ALDH1a1	7304881a1	5'-ATACTTGTCGGATTTAGGAGGCT-3'	5'-GGGCCTATCTTCCAAATGAACA-3'
ALDH1a2	6677665a1	5'-CAGAGAGTGGGAGAGTGTTCC-3'	5'-CACACAGAACCAAGAGAGAAGG-3'
ALDH1a3	31542123a1	5'-GGGTCACACTGGAGCTAGGA-3'	5'-CTGGCCTCTTCTTGGCGAA-3'
ALDH1b1	21312260a1	5'-CTCCAGGGCAGGACTACCTC-3'	5'-CATGCCACTCGTTGTTGATGA-3'
ALDH2	6753036a1	5'-GACGCCGTCAGCAGGAAAA-3'	5'-CGCCAATCGGTACAACAGC-3'
ALDH3a1	6680676a1	5'-AATATCAGTAGCATCGTGAACCG-3'	5'-GGAGAGCCCCTTAATCGTGAAA-3'
ALDH3a2	6680678a1	5'-TCTCTGCCCTTTGGAGGTGT-3'	5'-AGCTGATCCTTGACAATCACAG-3'
ALDH3b1	34328288a1	5'-ATGGACTCGTTTGAAGACAAGC-3'	5'-GATGGCAATCTCAGACACCTC-3'
ALDH9a1	9910128a1	5'-GGCCGAGTGATTGCCACTT-3'	5'-AGGCCACTTTTCTTACTCCAGA-3'

2.2.4 Preparation of total protein extracts

$3x10^6$ cells were harvested and collected as described previously (2.2.1). 500 µL of ice-cold radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% TritonX-100; 0.5% Na-deoxycholate; 0.1% SDS; fresh: 0.5 DTT; 1% PIC (Sigma) were added to each sample. After 10 min incubation on ice, cells were sonicated at 60% power for 30 sec (3 cycles). Cells were centrifuged at 14,000 rpm for 10 min at 4 °C and afterwards, the supernatant was transferred into a new tube. Protein concentration was determined using Bradford reagent as described previously [Bradford, M. 1976].

2.2.5 Quantification of proteins via western blotting

For the quantification of proteins via Western Blotting, samples with a final concentration of 1 µg/µL were incubated in 1x LaemmLi buffer (Sigma) for 10 min at 95 °C. Proteins were separated by a Mini-PROTEAN TGX pre-casted gel (4–20% acrylamide, Bio-Rad) for 45 min at 180 volt. Afterwards, proteins were transferred to a nitrocellulose membrane for 30 min at 25 volt.

Membranes were blocked with 5% milk in PBS containing 0.05% Tween (PBS-T) or with Pierce protein-free blocking buffer for MG-H1 (Thermo Fisher Scientific) at room temperature for 1 h. Primary antibodies were incubated overnight at 4 °C. After washing the membranes with PBS-T, secondary antibodies were incubated for 1 h at room temperature. Primary and secondary antibodies were listed in table 2. After final washing steps with PBS-T, proteins were visualized on X-ray films using ECL Western Blotting Detection Reagent (GE Healthcare). Exposure time depended on protein of interests (approx. 30 sec – 2 min). Protein expression was determined via GS-800 calibrated densitometer and the analyzing software QuantityOne (Bio-Rad). Protein quantification was normalized to β-actin expression.

Table 3. Antibodies.

Antibody	Isotope	Dilution	Company
Primary			
anti-GLO1	rat	1: 1000	Abcam (81461)
anti-MG-H1	rat	1:450	Provided by Dr. T. Fleming and Dr. J. Morgenstern (University Hospital Heidelberg, Germany) in collaboration with the monoclonal antibody core facility at Helmholtz Centre Munich, Germany [Morgenstern *et al.* 2017]
anti-ß-actin	rabbit	1:1000	Cell Signalling Technology (4967)
Secondary (HRP-linked)			
anti-Rat		2x dilution of	Cell Signalling Technology (7077)
anti-Rabbit		primary antibody	Cell Signalling Technology (7074)

2.2.6 Enzyme activity assays

The activity of GLO1 was determined by measuring the increase of absorbance at 235 nm due to the formation of S-D-lactoylglutathione generated from HTA by pre-incubation of MG and GSH as described previously [Thornalley, P. 1988]. Cells were harvested (2.2.1) and proteins were lysed as described previously (2.2.4). For the assay, 10µg/10 µL protein were pipetted into a 96-well UV microplate and incubated with 190 µL reaction buffer (100 mM sodium phophaste buffer pH 6.6; 20 mM GSH; 20 mM MG) at 37°C for 10 min. The absorbance at 235 nm was recorded every 30 sec for a period of 10 min using a FLUOStar Omega Microplate Reader (BMG Labtech).

The activity of AKR, ALDH and GSH reductase was determined as described previously [Srivastava *et al.* 1999; Skapare *et al.* 2013; Carlberg, I. & Mannervik, B. 1985] with minor changes, supported by Dr. Jakob Morgenstern (University Hospital Heidelberg, Germany). Briefly, AKR activity was determined by following the loss of NADPH (0.1 M sodium phosphate pH 7.2, 0.1–2 mM MG, 0.1 mM NADPH); ALDH activity was measured by the rate of NADPH formation (75 mM Tris-HCl pH 9.5, 10 mM DL-2-amino-1-propanol, 0.5 mM NADP, 0.1–2 mM MG) both at 340 nm.

2.2.7 Quantification of methylglyoxal and MG-derived proteins

$1x10^6$ cells were collected as described previously (2.2.1). The determination of MG and MG-H1 via liquid chromatography with tandem mass spectrometry (LC-MS/MS) (TQS, TQXS, Waters®) was performed by the CRC1118 LC-MS/MS Core Facility (S01) by Dr. Thomas Fleming (University Hospital Heidelberg, Germany) as described previously [Rabbani, N. & Thornalley, P.J. 2014; Thornalley, P.J. & Rabbani, N. 2014]. The content of MG-H1 was determined via a stable isotope-dilution with LC-MS/MS after an enzymatic hydrolysis as described [Thornalley *et al.* 2003].

2.3 *In vivo* Methods

2.3.1 *Saccharomyces cerevisiae* strains and standard growth conditions

The *S. cerevisiae* strain BY4741 (Euroscarf) was used either as a control (wildtype; WT) and further to create a GLO1 knock-out strain (Δglo1). Both strains were kindly provided and introduced by Dr. Jens Tyedmers (University Hospital Heidelberg, Germany). Yeast cells were grown in Yeast Extract/Peptone/Dextrose (YPD) media containing 100 mM glucose (10 g Bacto Yeast Extract (BD), 20 g Bacto Peptone (BD), 100 mM glucose for 1 L YPD) at 30 °C on a shaker (120 - 150 rpm) and spotted on YPD plates at 30 °C.

2.3.2 Glyoxalase 1 activity assay in *Saccharomyces cerevisiae*

To determine the activity of GLO1, 1 mL of a 50 mL cell suspension was spectrophotometrically measured at the wavelength of 600 nm to determine the optical density of the sample (OD_{600}) (BioPhotometer, Eppendorf). When the cells reached an OD_{600} value of 0.3 (logarithmic growth phase), cells were lysed in 150 µL lysis buffer (10 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.1% NP40; 0.5 M EDTA; fresh: 10 µL/mL protease inhibitor cocktail (P9599, Sigma)). 50 mg of protein extract beads (Diagenode) were added to the sample and sonicated in a Bioruptor Pico Waterbath (Diagenode, sonication cycle 30 sec., total sonication for 30 cycles, at 4 °C).

The sonication was repeated in total three times. Afterwards, cells were centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was transferred into a new 1.5 mL Eppendorf tube. The protein concentration was determined as described in 2.2.4 and the further GLO1 activity assay was performed as previously described in 2.2.6.

2.3.3 Quantification of methylglyoxal and MG-derived proteins in *Saccharomyces cerevisiae*

For the quantification of MG and MG-H1, 50 mL of cells were grown under standard growth conditions (see 2.3.1). At OD_{600} 0.3, cells were harvested, lysed and sonicated (see 2.3.2). The protein concentration was determined by the CRC1118 LC-MS/MS Core Facility (S01) by Dr. Thomas Fleming (University Hospital Heidelberg, Germany) as described in 2.2.4. The quantification of MG and MG-H1 via LC-MS/MS was performed as described in 2.2.7.

2.3.4 Determination of proliferation rate in *Saccharomyces cerevisiae*

For determination of the proliferation rate, cells were grown in YPD media (see 2.3.1) at 30 °C. 1 mL of the cell suspension was measured spectrophotometrically. At OD_{600} 0.1, the OD_{600} was measured every 30 minutes until cells reached their plateau growth phase (OD_{600} 0.8). The different time points were normalized to the starting OD_{600} value.

2.3.5 Toxicity assay in *Saccharomyces cerevisiae*

Cells were grown under standard growth conditions (see 2.3.1) to the logarithmic growth phase. At OD_{600} 0.3, cells were treated with increasing concentrations either with MG, hydrogen peroxide (H_2O_2) or formaldehyde (FA). Non-treated cells served as control. Cells were incubated for 60 min at 30 °C. Afterwards, OD_{600} was measured and cells were re-suspended in a new 1.5 mL Eppendorf tube to reach the same OD_{600} (reflecting the cell number) in each sample. Subsequently, cells were spotted onto YPD plates in a serial dilution (1:5). Plates were incubated for 48 h at 30 °C to determine cell survival.

2.3.6 *Caenorhabditis elegans* strains and standard growth conditions

The WT strain N2 Bristol and the GLO1 knock-out ($GLO1^{-/-}$) strain VC343 [glod-4(gk189)III] were purchased from the Caenorhabditis Genetics Centre (University of Minnesota, USA). Both strains were cultured on nematode growth media (NGM) plates (for 1 L: 17 g agar, 3 g NaCl, 2.5 g peptone; after autoclaving 25 mL 1 M KPO_4, 1 mL 1 M $MgSO_4$, 1 mL cholesterol (50 mg/mL), 1 mL 1 M $CaCl_2$) at 20 °C or 25 °C. Nematodes were fed with the concentrated *Escherichia coli* (*E.coli*) strain OP50 (provided by the Caenorhabditis Genetics Centre) in LB-media (4 g Bacto peptone, 2 g Bacto yeast

extract, 4 g NaCl in 400 ml water). For synchronization of same-aged nematodes, plates (60 mm diameter petri dishes, Greiner) containing eggs were washed in M9 buffer (for 1 L: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl; after autoclaving 400 µL 2.5 M $MgSO_4$). After collecting the pellet in a 15 mL falcon, nematodes were bleached for 5 min at room temperature in bleaching solution (3.3 mL sodium hypochlorit solution (Sigma), 12.5 mL 2 M NaOH (Sigma), 9.5 mL water). The pellet was washed three times in M9 buffer and centrifuged for 3 min at 1700 rpm. Afterwards, the eggs were transferred onto new NGM plates covered with OP50. After 12-14 h nematodes in the same age were hatched (L1 larvae). After additional 40-48 h, the nematodes were grown up to L4 larval/young adult stage at 20 °C.

2.3.7 Glyoxalase 1 activity assay in *Caenorhabditis elegans*

For the determination of GLO1 activity, 10 plates (60 mm diameter, Greiner) completely covered with a heterogeneous population of nematodes were collected in M9 buffer (2.3.6). Nematodes were lysed in 150 µL lysis buffer (20 mM Tris-HCl pH 8.0; 137 mM NaCl; 1% Triton-X 100; 2 mM EDTA; fresh: 10 µL/mL protease inhibitor cocktail (P9599, Sigma)). After adding the lysate buffer, the procedure for sonication, protein concentration and GLO1 activity measurement was followed as described in 2.3.2.

2.3.8 Quantification of methylglyoxal and MG-derived proteins in *Caenorhabditis elegans*

The quantification of MG and MG-H1 via LC-MS/MS was performed as described in 2.2.7 by the CRC1118 LC-MS/MS Cora Facility (S01) by Dr. Thomas Fleming (University Hospital Heidelberg, Germany). For the determination, 20,000 nematodes were collected in M9 buffer as described in 2.3.7.

2.3.9 Determination of egg laying rate in *Caenorhabditis elegans*

The determination of the egg laying rate was performed with nematodes in the age of young adults. Five adult nematodes were transferred onto new NGM plates with OP50 and incubated for 24 h at 20 °C. After the incubation time, the eggs were counted under a microscope.

2.3.10 Toxicity assay in *Caenorhabditis elegans*

The toxicity assay was performed with L4 larvae's in a sterile 96-well plate (Greiner). Eight nematodes were transferred into each well containing 50 µL M9 buffer. The 96-well plate was incubated for 30 min at 20 °C. Dead nematodes were replaced before starting the experiment for a total number of eight nematodes per well. Nematodes in each well were counted (time point before toxin treatment) and afterwards treated with increasing concentrations of MG, H_2O_2 or FA.

Non-treated cells served as control. Cells were incubated at 20 °C for 2 h (MG), 20 min (H_2O_2) or 1 h (FA). After incubation, living nematodes were counted and percentage of survival was calculated.

2.3.11 Toxicity assay in *Danio rerio*

WT and GLO1 knock-out ($GLO1^{-/-}$) zebrafish embryos were kindly provided at the age of 48 hours post fertilization (hpf) by Prof. Dr. Jens Kroll (CRC1118, B01, University Heidelberg, Medical Faculty Mannheim, Germany). 20 embryos per condition were transferred into new plates (60 mm diameter, Greiner) and treated with 10 mL of increasing concentrations of MG, H_2O_2 or FA (toxins were diluted in cleared egg water). Non-treated embryos served as control. Embryos were incubated at 28.5 °C for 1 h (MG), 2 h (H_2O_2) or 1 h (FA). After incubation, living embryos were counted and percentage of survival was calculated.

2.3.12 Mouse strains and standard conditions

WT (C57BL/6) mice were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA). GLO1 knock-out ($GLO1^{-/-}$) mice were generated via CRISPR/Cas9 technique and kindly provided by Prof. Marc Freichel and Dr. Dagmar Schuhmacher (CRC1118, S03N, University Heidelberg, Institute of Pharmacology, Germany) [Schuhmacher *et al.* 2018]. Mice were maintained under standard laboratory conditions: constant temperature (22 ± 2 °C) and humidity (55 ± 5%), 12 h light/12 h dark cycle and free access to food and water. Animal studies were approved by the Animal Care and Use Committee by the regional authority in Karlsruhe, Germany (G295/15). Mice were sacrificed by CO_2 inhalation and directly perfused through the heart with ice cold PBS. For analysis, organs (heart, liver, kidney, muscle and brain) were dissected and flash-frozen in liquid nitrogen for direct analysis or storage at -80 °C.

2.3.13 Real-time quantitative PCR in tissue

The flash-frozen tissue was pulverized using an ice-cold mortar and 10 mg were used for further RNA extraction, cDNA synthesis and Real-Time PCR as described in 2.2.3. Primer sequences were used as followed (tbl. 4).

Table 4. Primer sequences for antioxidant genes.

Gene	PrimerBank	Forward primer	Reverse primer
Catalase	157951740c1	5'-GGAGGCGGGAACCCAATAG-3'	5'-GTGTGCCATCTCGTCAGTGAA-3'
GPX1	6680075a1	5'-AGTCCACCGTGTATGCCTTCT-3'	5'-AGTCCACCGTGTATGCCTTCT-3'
GPX4	22022299a1	5'-GATGGAGCCCATTCCTGAACC-3'	5'-GATGGAGCCCATTCCTGAACC-3'
GSR	34328489a1	5'-GACACCTCTTCCTTCGACTACC-3'	5'-GACACCTCTTCCTTCGACTACC-3'
HMOX1	6754212a1	5'-AAGCCGAGAATGCTGAGTTCA-3'	5'-AAGCCGAGAATGCTGAGTTCA-3'
NQO1	6679080a1	5'-AGGATGGGAGGTACTCGAATC-3'	5'-AGGCGTCCTTCCTTATATGCTA-3'
PRDX1	6754976a1	5'-AATGCAAAAATTGGGTATCCTG-3'	5'-AATGCAAAAATTGGGTATCCTG-3'
PRDX6	6671549a1	5'-CGCCAGAGTTTGCCAAGAG-3'	5'-CGCCAGAGTTTGCCAAGAG-3'
SOD1	12805215a1	5'-AACCAGTTGTGTTGTCAGGAC-3'	5'-AACCAGTTGTGTTGTCAGGAC-3'
SOD2	31980762a1	5'-CAGACCTGCCTTACGACTATGG-3'	5'-CAGACCTGCCTTACGACTATGG-3'
SRXN1	21313268a1	5'-CCCAGGGTGGCGACTACTA-3'	5'-CCCAGGGTGGCGACTACTA-3'
TXNDR1	12658437a1	5'-CCCACTTGCCCCAACTGTT-3'	5'-CCCACTTGCCCCAACTGTT-3'

2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc.) and are expressed as mean values ± S.D. Statistical significance was analysed using two-tailed unpaired *t* test with Welch's correction or one- or two-way ANOVA (comparison of more than one group). Differences were considered significant at: $p > 0.05$ n.s.; $p < 0.0001$***, $p < 0.001$**, $p < 0.05$*.

3. Results

3.1 The total loss of glyoxalase 1 leads to an increased sensitivity towards toxins in *Saccharomyces cerevisiae*

GLO1-deficient (Δglo1) and wildtype (WT) yeast cells used in this work were kindly provided by Dr. Jens Tyedmers (University Hospital Heidelberg, Germany). To characterize Δglo1 yeast cells, the activity of GLO1 and the proliferation rate under basal conditions were determined. The activity of GLO1 was significantly reduced in Δglo1 cells as compared to WT (fig. 1 A).

Furthermore, the proliferation rate of Δglo1 cells was significantly decreased (fig. 1 B). Regarding the detoxification capacities, MG and MG-H1 were determined under basal conditions. The measurement of MG and MG-H1 content via LC-MS/MS was performed by Dr. Thomas Fleming (LC-MS-MS Core Facility, CRC1118, S01, University Hospital Heidelberg, Germany). The KO of GLO1 led to increased MG (fig. 1 C) and MG-H1 (fig. 1 D) formation. Additionally, cross-toxicity studies were performed. Cells were treated either with MG, H_2O_2 or FA and spotted in serial dilutions (1:5). For all three toxins, Δglo1 cells were more sensitive as compared to WT cells. The LD 50 for MG in Δglo1 cells was 20 mM (fig. 1 E, left), for H_2O_2 3 mM (fig. 1 E, middle) and for FA 17.5-20 mM (fig. 1 E, left).

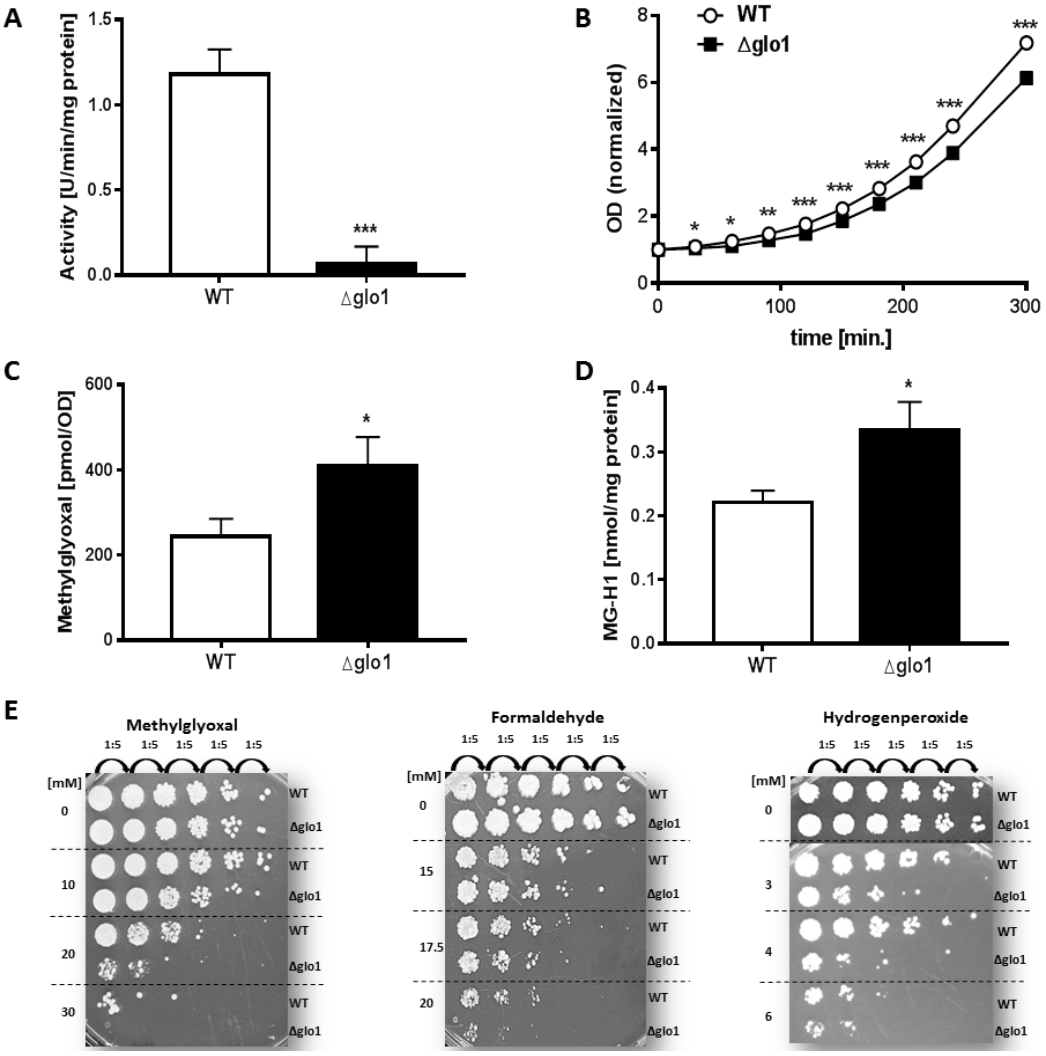

Figure 1. Effect of the total loss of glyoxalase 1 in *Saccharomyces cerevisiae*. White bars/dots representing wildtype (WT) and black bars/squares GLO1-deficient (Δglo1) yeast cells. **A:** GLO1 activity under basal conditions (100 mM glucose). **B:** Proliferation rate was determined via the optical density (OD_{600}). **C** and **D:** Intracellular methylglyoxal (C) and MG-modified arginine residues (MG-H1) (D) levels under basal conditions (100 mM glucose) measured via LC-MS/MS. **E:** Effect of methylglyoxal (0-30 mM), hydrogen peroxide (0-6 mM) and formaldehyde (0-20 mM). Cells were treated for 1 h. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

3.2 Increased toxin sensitivity in glyoxalase 1 deficient *Caenorhabditis elegans*

WT (N2 Bristol) and GLO1$^{-/-}$ (VC343 [glod-4(gk189)III] nematodes used in this work were provided by the Caenorhabditis Genetics Centre (University of Minnesota, USA). Reduced GLO activity in GLO1$^{-/-}$ nematodes was validated (fig. 2 A). Furthermore, adult GLO1$^{-/-}$ nematodes showed a significantly decreased egg laying rate (fig. 2 B) as compared to their WT counterparts. The loss of GLO1 in nematodes was accompanied with an increased MG (fig. 2 C) and MG-H1 (fig. 2 D) formation under basal conditions.

The measurement of MG and MG-H1 content via LC-MS/MS was performed by Dr. Thomas Fleming (LC-MS-MS Core Facility, CRC1118, S01, University Hospital Heidelberg, Germany). To determine the toxic effect of MG, H_2O_2 and FA, the LD 50 was calculated (fig. 2E). GLO1$^{-/-}$ nematodes were significantly more sensitive to MG (35.7 vs. 20.7 mM), H_2O_2 (1.2 vs. 0.3 mM) and FA (26.2 vs. 7.6) compared to WT nematodes.

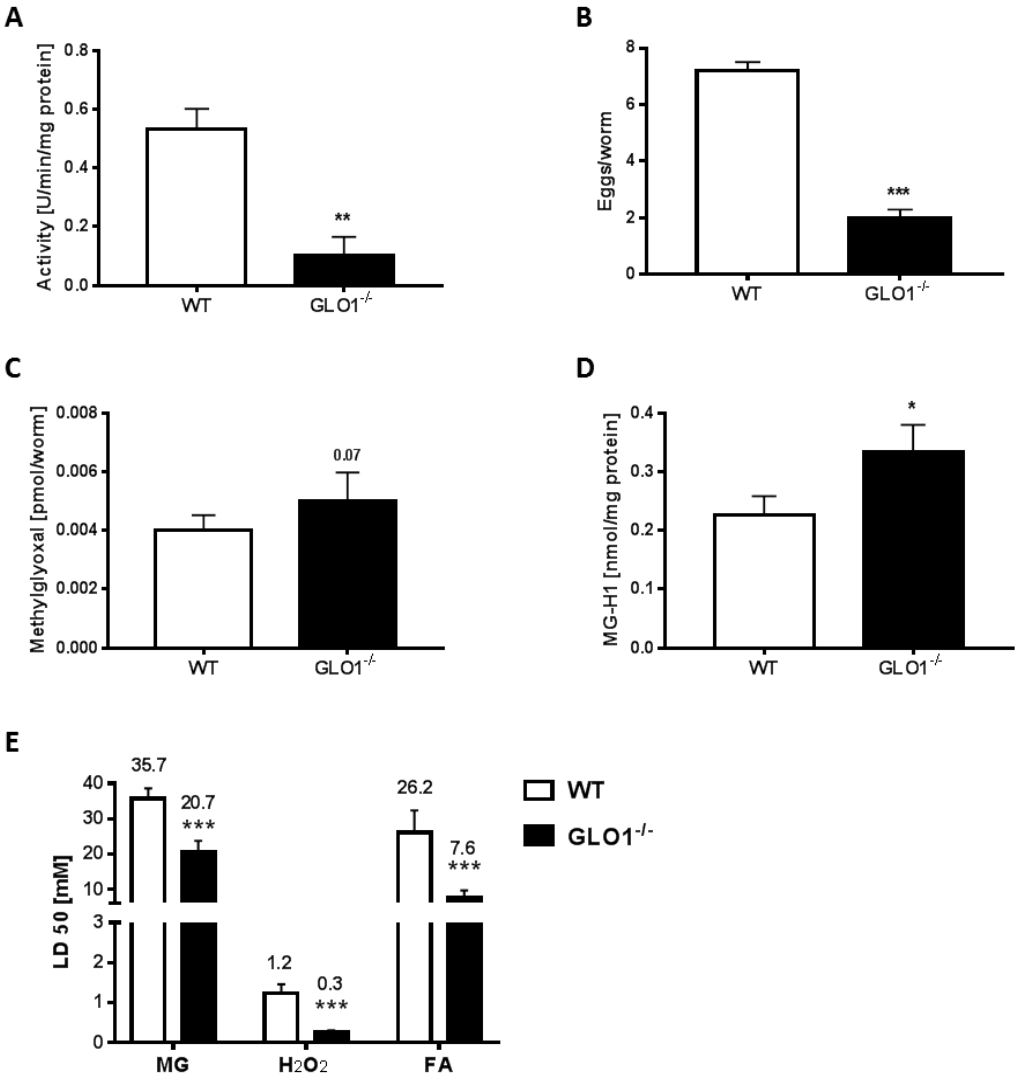

Figure 2. Effect of the total loss of glyoxalase 1 in *Caenorhabditis elegans.* White bars representing wildtype (WT); black bars GLO1-deficient (GLO1$^{-/-}$) nematodes. **A:** GLO1 activity in L4-stage nematodes. **B:** For egg laying rate, eggs from young adults were counted 24 h after seeding. **C** and **D:** Intracellular methylglyoxal (C) and MG-modified arginine residues (MG-H1) (D) levels measured via LC-MS/MS. **E:** Lethal dose 50 (LD 50) value of methylglyoxal (MG), hydrogen peroxide (H_2O_2) and formaldehyde (FA) in L4-stage nematodes. Nematodes were treated for 2 h (MG), 20 min (H_2O_2) or 1 h (FA). Values above the bars representing the lethal dose 50 (LD 50) value [mM]. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype nematodes ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

3.3 Total loss of glyoxalase 1 leads to a decreased sensitivity in zebrafish

WT and GLO1$^{-/-}$ zebrafish embryos were kindly provided at the age of 48 hours post fertilization (hpf) by Prof. Dr. Jens Kroll (CRC1118, B01, University Heidelberg, Medical Faculty Mannheim, Germany). GLO1$^{-/-}$ zebrafish showed a decreased sensitivity to MG, H_2O_2 and FA (fig. 3). The effect was strong for FA (7.7 vs. 39.6 mM), sufficient for MG (9.5 vs. 15.0 mM) and weak for H_2O_2 (63.1 vs. 74.1 mM).

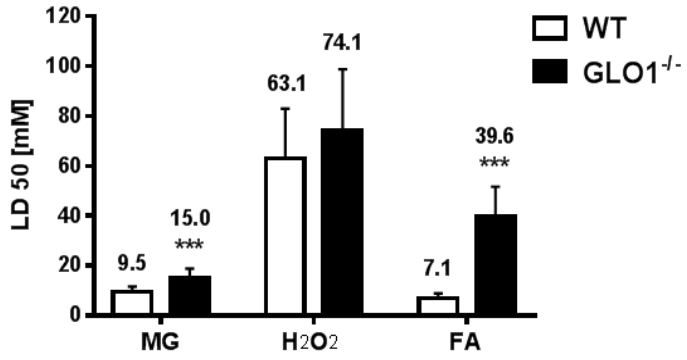

Figure 3. Total loss of glyoxalase 1 leads to decreased sensitivity in zebrafish. White bars representing wildtype (WT) and black bars GLO1-deficient (GLO1$^{-/-}$) animals. Lethal dose 50 (LD 50) value of methylglyoxal (MG), hydrogen peroxide (H_2O_2) and formaldehyde (FA) in 48 hpf old embryos. Zebrafish were treated for 1 h (MG), 2 h (H_2O_2) or 1 h (FA). Values above the bars representing the LD 50 value [mM]. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype nematodes ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

3.4 Total loss of glyoxalase 1 leads to an increased tolerance in mammal cells

To investigate the consequences of the loss of GLO1 in mammal cells, three murine cell lines with a full knock-out of GLO1 were used (GLO1$^{-/-}$). In all cell lines, the activity and protein content of GLO1, levels of MG and MG-H1 and their response to toxins and UV-C radiation were determined. MG and MG-H1 content was measured via LC-MS/MS by Dr. Thomas Fleming (LC-MS/MS Core Facility, CRC1118, S01, University Hospital Heidelberg, Germany). GLO1 activity and protein content in renal tubular GLO1$^{-/-}$ cells were significantly reduced (fig. 4 A). There was no elevation in MG (fig. 4 B) or MG-H1 (fig. 4 C) in the GLO1$^{-/-}$ renal tubular cells neither under basal (5 mM glucose) nor under hyperglycaemic conditions (25 mM glucose). When treated with other toxins or UV-C radiation, GLO1$^{-/-}$ renal tubular cells were more tolerant in a toxin-specific manner.

GLO1$^{-/-}$ cells treated with FA, UV-C, 4-hydroxynonenal (4-HNE) and H_2O_2 showed the highest increase regarding the LD 50 compared to WT cells. The effects for acetaldehyde (AA), acrolein (AC) or MG were moderate or lower to WT cells (fig. 4 D).

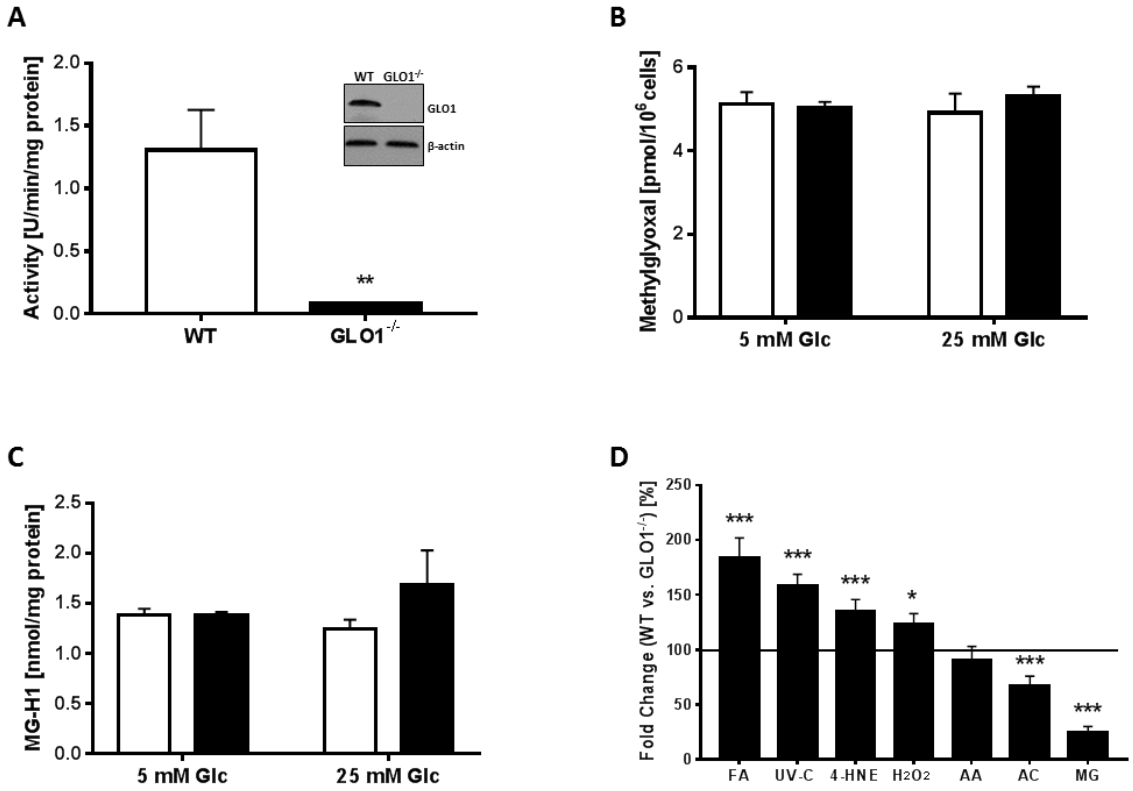

Figure 4. Loss of glyoxalase 1 leads to increased tolerance in renal tubular cells. White bars representing wildtype (WT) and black bars GLO1-deficient (GLO1$^{-/-}$) renal tubular cells. **A:** GLO1 activity and protein content (probed with anti-GLO1 and β-actin antibody). **B:** Intracellular methylglyoxal levels under basal (5 mM, right bars) and hyperglycaemic (25 mM, left bars) conditions measured via LC-MS/MS. **C:** Intracellular levels of MG-modified arginine residues (MG-H1) under basal (5mM, right bars) and hyperglycaemic (25 mM, left bars) conditions measured via LC-MS/MS. **D:** Lethal dose 50 (LD 50) [%] of GLO1$^{-/-}$ cells compared to wildtype cells (100 %, black line). Cells were treated for 48 h (formaldehyde, FA; UV-C; 4-hydroxynonenal, 4-HNE; hydrogen peroxide, H_2O_2; acetaldehyde, AA; acrolein, AC; methylglyoxal, MG). Toxicity was determined via MTT assay. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

Comparable results were detected in cardiac endothelial cells (fig. 5 A-C). GLO1$^{-/-}$ endothelial cells were significantly more tolerant to UV-C, H_2O_2 and AC (fig. 5 D). In GLO1$^{-/-}$ neuronal cells, GLO1 activity was significantly reduced (fig. 6 A). For MG and MG-H1 content, there were no differences compared to neuronal WT cells (fig. 6 B-C). Furthermore, in those cells, the tolerance towards UV-C, 4-HNE, H_2O_2 and AC was increased (fig. 6 D)

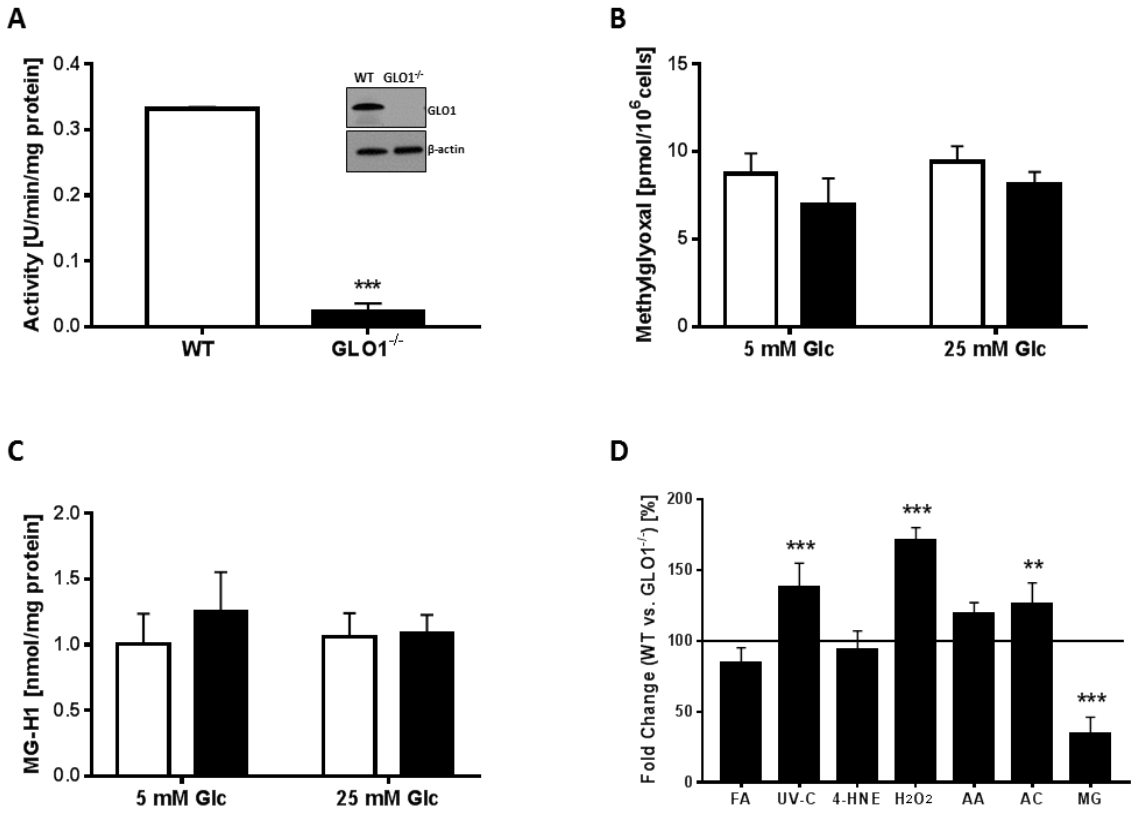

Figure 5. Loss of glyoxalase 1 leads to increased tolerance in endothelial cells. White bars representing wildtype (WT) and black bars GLO1-deficient ($GLO1^{-/-}$) cardiac endothelial cells. **A:** GLO1 activity and protein content (probed with anti-GLO1 and β-actin antibody). **B:** Intracellular methylglyoxal levels under basal (5 mM, right bars) and hyperglycaemic (25 mM, left bars) conditions measured via LC-MS/MS. **C:** Intracellular levels of MG-modified arginine residues (MG-H1) under basal (5mM, right bars) and hyperglycaemic (25 mM, left bars) conditions measured via LC-MS/MS. **D:** Lethal dose 50 (LD 50) [%] of $GLO1^{-/-}$ cells compared to wildtype cells (100 %, black line). Cells were treated for 48 h (formaldehyde, FA; UV-C; 4-hydroxynonenal, 4-HNE; hydrogen peroxide, H_2O_2; acetaldehyde, AA; acrolein, AC; methylglyoxal, MG). Toxicty was determined via MTT assay. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

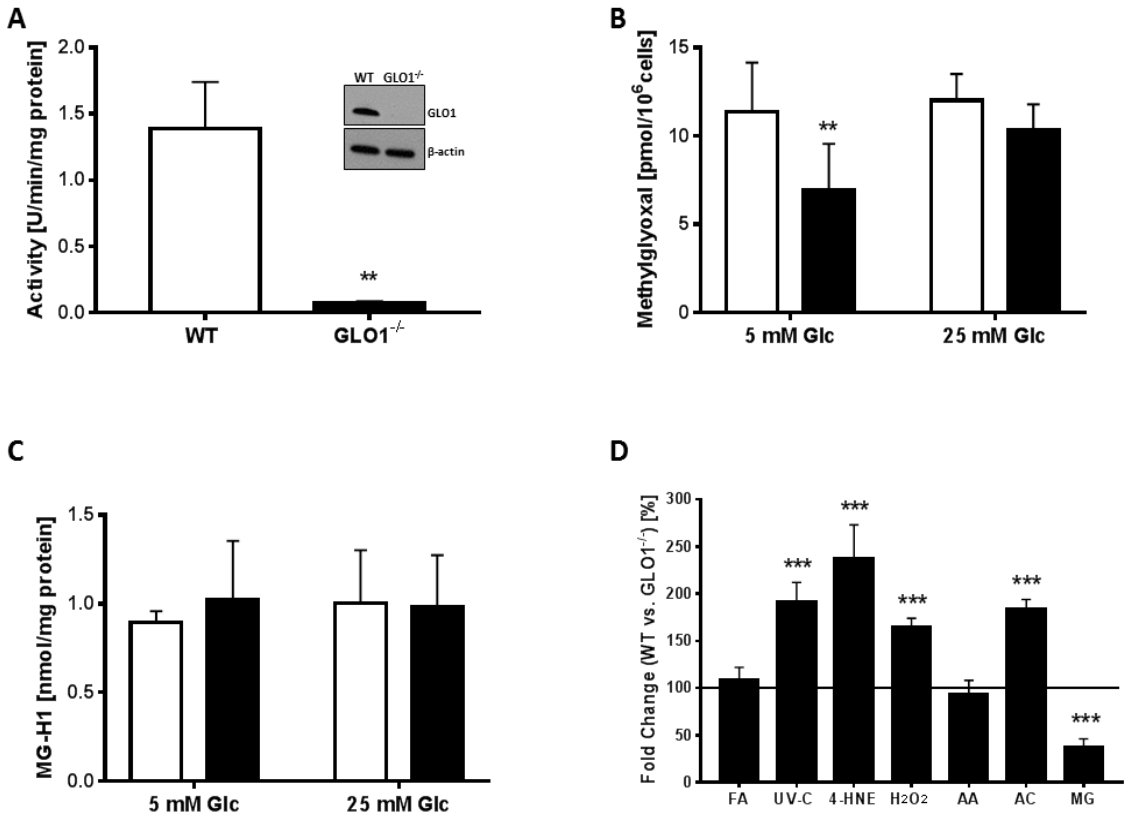

Figure 6. Loss of glyoxalase 1 leads to increased tolerance in neuronal cells. White bars representing wildtype (WT) and black bars GLO1-deficient (GLO1$^{-/-}$) neuronal Schwann cells. **A:** GLO1 activity and protein content (probed with anti-GLO1 and β-actin antibody). **B:** Intracellular methylglyoxal levels under basal (5 mM, right bars) and hyperglycaemic (25 mM, left bars) conditions measured via LC-MS/MS. **C:** Intracellular levels of MG-modified arginine residues (MG-H1) under basal (5mM, right bars) and hyperglycaemic (25 mM, left bars) conditions measured via LC-MS/MS. **D:** Lethal dose 50 (LD 50) [%] of GLO1$^{-/-}$ cells compared to wildtype cells (100 %, black line). Cells were treated for 48 h (formaldehyde, FA; UV-C; 4-hydroxynonenal, 4-HNE; hydrogen peroxide, H_2O_2; acetaldehyde, AA; acrolein, AC; methylglyoxal, MG). Toxicty was determined via MTT assay. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

3.5 Determination of alternative detoxification pathways in mammal cells

The mRNA content of various oxidoreductase subtypes was measured. In GLO1$^{-/-}$ renal tubular cells, the AKR subtypes 1b3, 1b8, 1c14, 1c18 and the ALDH subtypes 1a1 and 9a1 were up-regulated compared to WT cells (fig. 7 A). The loss of GLO1 in endothelial cells led to a significantly elevated expression of AKR 1a1, 1c14 and 1c20 and ALDH3a2 (fig. 7 B). In GLO1$^{-/-}$ neuronal cells AKR subtypes 1a1, 1b3, 7a5, 1cl, ALDH 1a2, 3a1, 1b1 and 9a1 were up-regulated compared to WT cells (fig. 7 C).

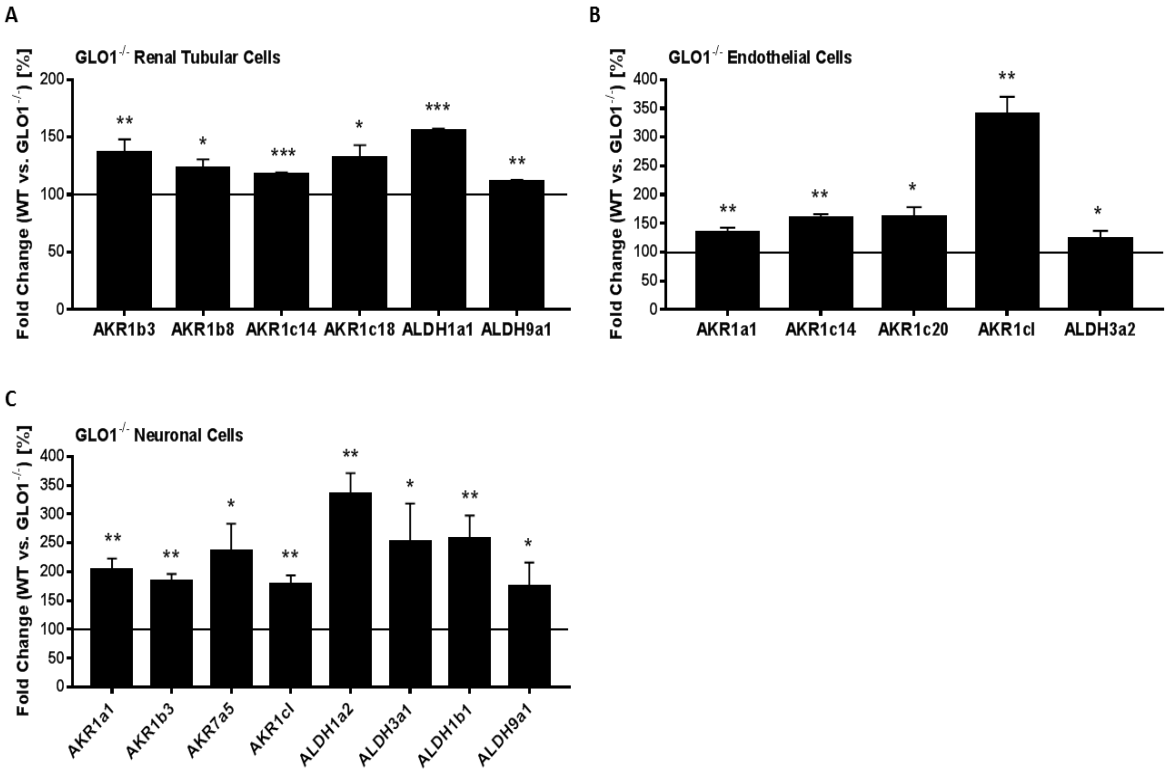

Figure 7. Loss of glyoxalase 1 leads to an up-regulation of oxidoreductases in mammal cells. Fold change (% of WT vs. GLO1$^{-/-}$) of mRNA expression of different subtypes of AKRs and ALDHs in GLO1$^{-/-}$ mammal cells (black bars). **A:** Renal tubular cells. **B:** Cardiac Endothelial cells. **C:** Neuronal cells (Schwann cells). Values for wildtype cells are standardized to 100% (black line). Expression data are normalized to ß-actin. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

In GLO1$^{-/-}$ mammal cells, an increased tolerance towards various toxic substances were found (see fig. 4, 5 and 6 D). To determine whether the up-regulated AKR and ALDH subtypes in renal tubular, endothelial and neuronal cells able to detoxify other toxic substances, activity assays with different substrates and co-factors were performed supported by Dr. Jakob Morgenstern (University Hospital Heidelberg, Germany). In GLO1$^{-/-}$ renal tubular, endothelial and neuronal cells, AKR enzymes revealed an increased activity towards FA (only moderate in renal tubular cells) and 4-HNE as substrates (fig. 8 A, black bars). The activity of ALDH enzymes were increased towards the substrates AC (only moderate in renal tubular cells), 4-HNE and MDA (fig. 8 B, black bars). Additionally, two essential enzymes in the GSH metabolism (GSH reductase, peroxidase) showed a significant increased activity in the absence of GLO1 (fig. 8 C).

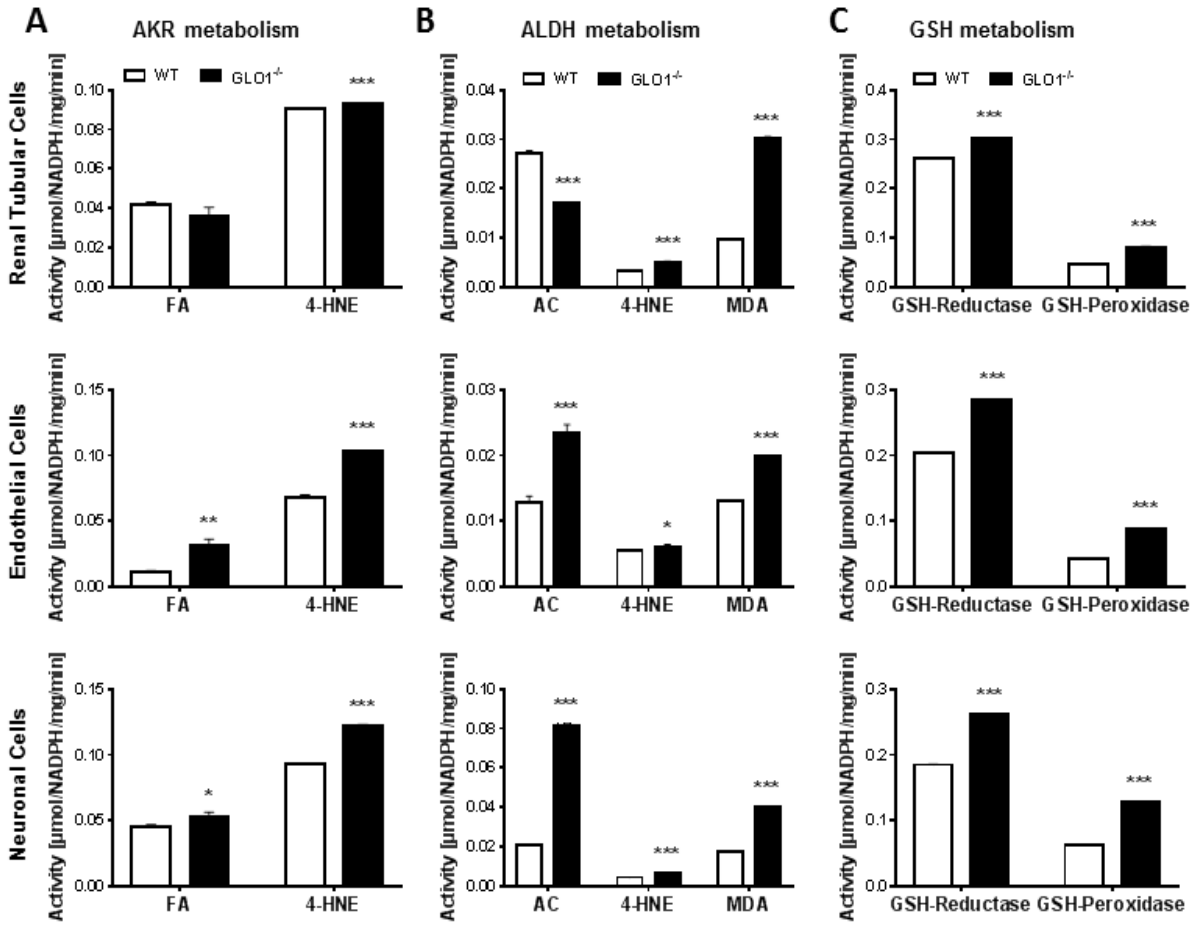

Figure 8. Increased enzyme activity in GLO1$^{-/-}$ mammal cells. Enzyme activity in wildtype (white bars) and GLO1$^{-/-}$ mammal cells (black bars) towards various substrates (formaldehyde, FA; 4-hydroxynonenal, 4-HNE; acrolein, AC; malondialdehyde, MDA; glutathione, GSH). **A:** Aldo-keto-reductase (AKR) activity. **B:** Aldehyde-dehydrogenase (ALDH) activity. **C:** Glutathione (GSH) reductase and GSH peroxidase activity. Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

3.6 Loss of glyoxalase 1 leads to up-regulation of protective enzymes corresponding to increased natural survival in mice

The loss of GLO1 lead to enhanced tolerance towards various toxins and UV-C radiation, an up-regulation of oxidoreductases correlating with an increased activity towards different substrates in mammal cells.

GLO1$^{-/-}$ mice were kindly provided by Prof. Dr Marc Freichel (University Heidelberg, Pharmacology, Germany) [Schuhmacher *et al.* 2018]. To investigate potential defence mechanism *in vivo*, the mRNA content was screened for antioxidant enzymes. Several enzymes involved in the defence against free radicals were increased in the liver of GLO1$^{-/-}$ mice: Peroxiredoxin 6 (PRDX6), Glutathione reductase (GSR), NAD(P)H dehydrogenase (quinone) 1 (NQO1), Superoxide dismutase 2 (SOD2), Catalase and Heme oxygenase (decycling) 1 (HMOX1) (fig. 9 A, black bars). These findings in mice were linked to an increased natural survival rate in GLO1$^{-/-}$ mice (fig. 9 B) when compared to their WT counterparts.

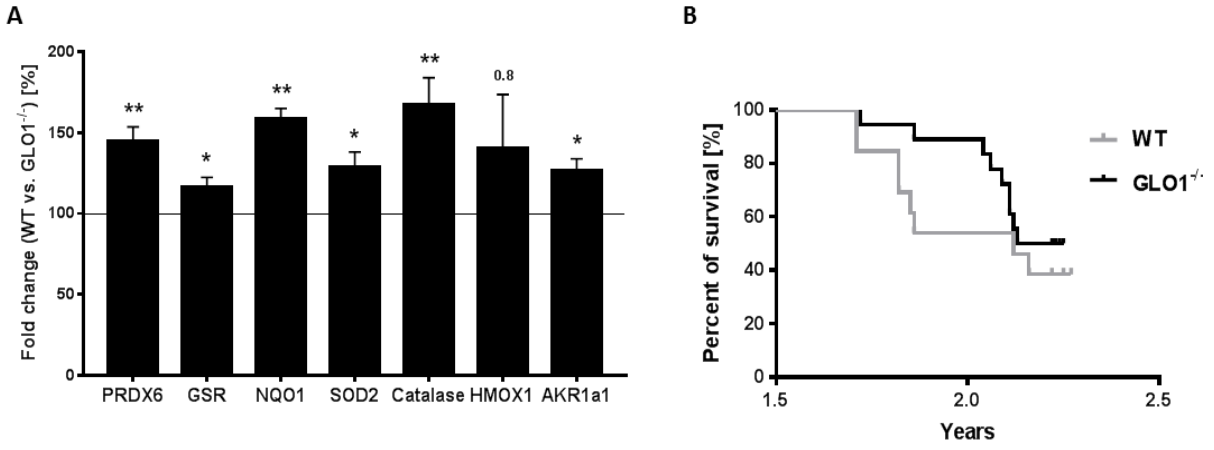

Figure 9. Loss of glyoxalase 1 correlates with increased defence and survival in mice. A: Fold change (WT vs. GLO1$^{-/-}$) of mRNA expression of different antioxidant enzymes in liver from 6 month old GLO1$^{-/-}$ mice (black bars). Values for wildtype mice are standardized to 100% (black line). Peroxiredoxin 6, PRDX6; Glutathione reductase, GSR; NAD(P)H dehydrogenase (quinone) 1, NQO1; Superoxide dismutase 2, SOD2; Catalase and Heme oxygenase (decycling) 1, HMOX1. **B:** Natural survival of wildtype and GLO1$^{-/-}$ mice (n=13-18). Data represent the mean of at least three individual experiments ± S.D. P-values indicating significant changes in comparison to wildtype cells ($p<0.0001$***, $p<0.001$**, $p<0.05$*).

4. Discussion

4.1 The contribution of glyoxalase 1 decreases in complex organisms

This study has demonstrated the decreasing dependency for GLO1 as the main detoxification system for MG from simple organisms up to mammals. In summary, in *S. cerevisiae* and *C. elegans*, the total loss of GLO1 leads to increased MG and MG-H1 levels, a decreased proliferation or fertilization rate and an increased sensitivity towards toxins. In zebrafish, the total loss of GLO1 resulted in a decreased sensitivity towards toxins. In GLO1-deficient murine cell lines, no increased MG and MG-H1 levels under basal or hyperglycaemic conditions were determined and surprisingly, an increased tolerance towards toxins was detected.

Our studies in *S. cerevisiae* and *C. elegans* showed that they cannot detoxify MG effectively when GLO1 is absent, resulting in an intracellular accumulation of MG and MG-H1. Zemva *et al.* (2017) showed that with increasing MG concentrations cell survival decreases in WT and even more in Δglo1 yeast cells. This leads to the presumption that increased MG could be the possible driver for the decreased proliferation rate shown in our study. In *C. elegans*, Morcos *et al.* (2009) showed that in GLO1 knock-down nematodes elevated MG-H1 correlated with a decreased lifespan. The decreased lifespan could result in a shorter fertile phase which could be responsible for the decreased egg production in $GLO1^{-/-}$ nematodes shown in our study. Further studies are required to determine whether the fertile phase is impaired in $GLO1^{-/-}$ nematodes compared to WT. Another explanation for the decreased fertilization shown in this study could be the rescue mechanism of *C. elegans*. It is known that *C. elegans* enters a stage of arrested development in which the development and the reproduction stops due to unfavourable conditions [Golden, J.W. and Riddle, D.L. 1984; reviewed in Androwski *et al*. 2017]. It is not known yet whether MG is a possible stressor to induce a dauer stage. Therefore, a definite conclusion is not possible. Further studies are required to better understand whether the impaired fertilization is a consequence of the shorter lifespan or the dauer stage. Both events could possibly triggered by an elevated MG content in the $GLO1^{-/-}$ nematodes. Furthermore, a direct damaging effect of MG or an unknown consequence of the loss of GLO1 cannot be ruled out. The determination of the egg laying rate when treated with MG could help to address this question.

Besides of the accumulation of MG, this study shows that the loss of GLO1 leads to an increased sensitivity in *S. cerevisiae* and *C. elegans* towards exogenously added MG as compared to WT. Alternative detoxification of MG in *S. cerevisiae* could be substituted by Yap 1 [Grant *et al.* 1996; Maeta *et al.* 2004; Tsuzi *et al.* 2004; reviewed in Inoue *et al.* 2011], Gcn2 and Gcn4 [Nomura *et al.*

2008] or the yeast aldose reductase (Gre3) via the HOG1-MAPK [Aguilera, J. and Prieto, J.A. 2001; Aguilera *et al.* 2005]. For *C. elegans*, less is known about alternative detoxification of MG. It is known that a few ALDH genes have been identified and that most of them are predicted to have oxidoreductase activity but whether the ALDH genes could alternatively detoxify MG is still investigated. Nevertheless, our data indicates that the alternative detoxification mechanisms are not effective as compared to GLO1 in the detoxification of MG. Furthermore, both organisms showed an increased sensitivity towards endogenously added H_2O_2 or FA, implicating that in the absence of GLO1 other detoxification systems are also impaired. More investigations are required to address the mechanisms behind the sensitive response towards other toxins. So far, it seems that simple organisms are more dependent on GLO1 as a defence system, not only for MG but also for other toxins.

This study also showed a decreased sensitivity towards toxins in the GLO1$^{-/-}$ zebrafish as compared to WT. Lodd *et al.* (2018, under revision) showed only moderately elevated MG levels in GLO1$^{-/-}$ zebrafish. One explanation for the only mildly elevated MG and the decreased sensitivity could be possibly the up-regulation of AKR1a1a, 1a1b or 1b1 which can alternatively detoxify MG even the detoxification is not effective as through GLO1. Furthermore, the up-regulation of AKRs/ALDHs could have a positive side effect on the detoxification of H_2O_2 or FA resulting in a tolerance towards these toxins. To investigate which compensatory enzymes are activated and leading to an alternative defence mechanism towards stress, further studies regarding the double knock-out of AKR/ALDH and GLO1 in zebrafish would be helpful. In addition, Moraru *et al*. (2018) showed that MG and MG-H1 were mildly elevated in GLO1$^{-/-}$ flies and they tolerate H_2O_2-induced oxidative stress better than WT flies similar to the findings in zebrafish. One would expect that GLO1$^{-/-}$ flies with elevated MG levels show a reduced lifespan as observed in *C. elegans*. Surprisingly, GLO1$^{-/-}$ flies show a prolonged lifespan. An explanation for this could be the up-regulation of alternative detoxification mechanisms in GLO1$^{-/-}$ flies. At that time, only ALDH2 is known for its capacity to detoxify aldehydes [Rothacker, B. and Ilg, T. 2008] but nothing is known about the specific capacity to detoxify MG in the absence of GLO1. Further analyses are required to determine possible alternative detoxification enzymes in GLO1$^{-/-}$ flies.

In summary, our toxicity data in GLO1$^{-/-}$ zebrafish and the findings shown in GLO1$^{-/-}$ flies from Moraru *et al.* (2018) would suggest that there is no loss of defence in the absence of GLO1 probably due to alternative detoxification mechanisms. It must be noted, that to confirm these findings, further studies are required with zebrafish. Because of the low number of eggs, the experiment needs to be repeated with an increased number.

Furthermore, these studies were performed in 48 hpf young embryos, therefore the study should be repeated in adult zebrafish, in which the alternative detoxification capacity could be changed.

In GLO1$^{-/-}$ murine cells, this study demonstrated that MG or MG-H1 is not elevated under basal or hyperglycaemic conditions. This is consistent with the data from Morgenstern *et al.* (2017) in murine Schwann cells. Here, it is important to mention that there are discrepancies when compared with previous studies in GLO1 knock-down mice. Giacco *et al.* (2014) showed an increased MG-H1 content in non-diabetic GLO1 knock-down mice. However, these mice still had a residual GLO1 activity left while our GLO1 knock-out murine cell lines and the GLO1$^{-/-}$ mice from Schumacher *et al.* (2018) showed no GLO1 activity. This would suggest that silencing of GLO1 is not sufficient for the up-regulation of alternative detoxification mechanisms and that only a total loss of GLO1 leads to an effective compensation due to the up-regulation of alternative detoxification mechanisms. To verify this, alternative detoxification mechanisms in GLO1 knock-down mice should be investigated.

4.2 Up-regulation of protective mechanisms corresponds to increased natural survival in the absence of glyoxalase 1 in a mouse model

Our study shows that the loss of GLO1 leads to an increased tolerance towards a broad range of toxins as well as UV-C radiation in murine cell lines. The tolerance induction by the loss of GLO1 was cell- and toxin-specific as well as being more pronounced than in either *D. melanogaster* or zebrafish. This leads to the assumption that the loss of GLO1 induces a tolerance towards toxins. One explanation for the tolerance induction is the up-regulation of various AKRs and ALDHs in the GLO1$^{-/-}$ murine cell lines as shown in this study. And that these enzymes are able to detoxify a broad range of toxins.

Additionally to the up-regulation of alternative detoxification enzymes resulting from the loss of GLO1, this study shows that the GSH reductase and GSH peroxidase are also up-regulated in the GLO1$^{-/-}$ murine cells. This indicates that the cells handle cellular stress not only by the up-regulation of alternative detoxification enzymes, but also by increasing their defence capacities. This is consistent with data from GLO1$^{-/-}$ flies where the GSH/GSSG ratio is increased and the animals tolerate H_2O_2 treatment [Moraru *et al*. 2018]. One could suggest that changes in the GSH/GSSG ratio were caused probably due to the loss of GLO1 and that this would be a beneficial effect for the cells to handle MG-independent stress.

Additionally, our study shows that not only GSH metabolism enzymes are up-regulated but also the activity of AKRs and ALDHs towards different reactive aldehydes is increased. This would confirm the hypothesis of an increased defence cascade in complex organisms when GLO1 is absent. The *in vitro* data are in line with our studies in GLO1$^{-/-}$ mice, where in the liver an increased expression of AKR1a1 and antioxidant enzymes (NQO1, SOD2, Catalase and GSH reductase) was observed. It is to mention that only the expression in liver was determined. As it is known from the study of Schuhmacher *et al.* (2018), differences between tissues regarding the expression of AKRs and ALDHs were observed in GLO1$^{-/-}$ mice. Therefore, a variety of tissues needs to be further analysed to establish tissue or even cell-specific responses to the loss of GLO1. It is to mention that the mice in this study were at the age of 6 month. It would be interesting to determine the antioxidative capacity during aging to establish whether there are changes. Interestingly, an increased natural survival of the GLO1$^{-/-}$ mice were observed. This phenomenon could correlate with the increased up-regulation of protective enzymes. This finding could be confirmed by the increased lifespan observed in GLO1$^{-/-}$ flies [Moraru *et al.* 2018]. In fact, further investigations need to be done to understand the molecular mechanism behind the increased longevity, but it is conceivable that the described mechanisms could be mainly responsible for the increased natural survival.

With regard to the role of GLO1 in humans, the literature provides conflicting data regarding the activity of GLO1 in diabetic patients. In 1994, it has been shown that red blood cells from diabetic patients with complications have an increased GLO1 activity [McLellan *et al.* 1994]. In contrast, 2013 it has been shown that in human blood samples from T1D and T2D patients with neuropathy the activity of GLO1 is reduced [Skapare *et al.* 2013]. Interestingly, Schumacher *et al.* (2018) revealed that there is evidence for an AKR-dependent MG detoxification in erythrocytes of T2D patients without nephropathy but not for patients with nephropathy. They suggest that diabetic patients without late complications are protected due to alternative detoxification mechanisms. Therefore, it is of high interest to investigate possible antioxidative capacities in diabetic patients with and without complications and to determine whether this antioxidative capacity could be useful in respect to develop new approaches for treating late complications.

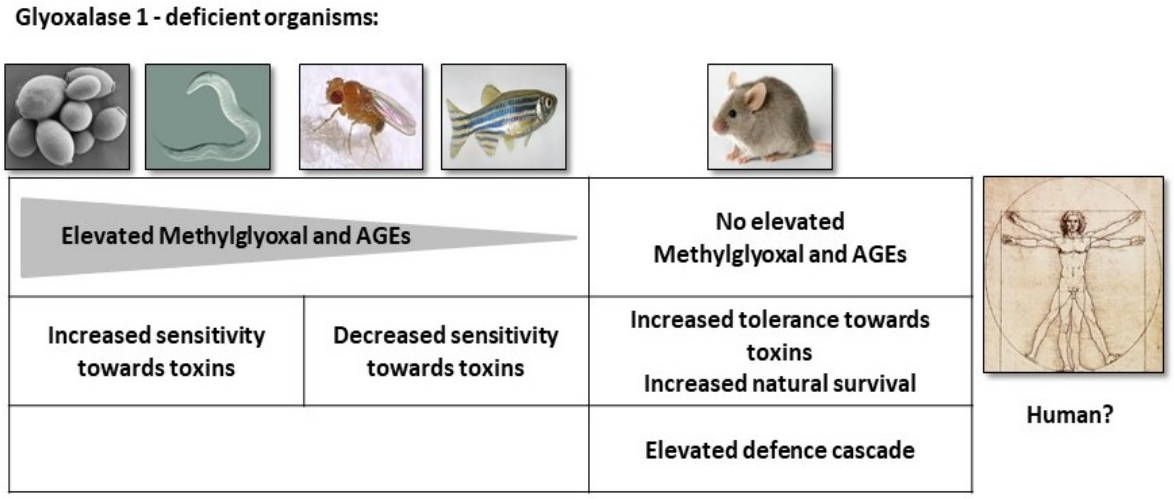

Figure 10. Regulation of toxic metabolites increases during evolution in the absence of Glyoxalase 1.

In conclusion, this study showed that in simple organisms, GLO1 is the main detoxification enzyme for MG and even though potential alternative detoxification mechanisms are present, they are not equivalent to the GLO1 system. Admittedly, it has been shown by Zemva *et al.* (2017) that low levels of MG have beneficial effects in *S. cerevisiae*, but apparently the accumulation of MG due to the loss of GLO1, exceeds this level. Furthermore, the loss of GLO1 does also affect the detoxification of other toxins. In zebrafish and *D. melanogaster* alternative detoxification mechanisms are able to detoxify most of the MG and these organisms showed a tolerance towards selected toxins. This might be a beneficial side effect of the up-regulation of alternative detoxification mechanisms. This is in line with the findings in GLO1$^{-/-}$ murine cell lines and mice, showing an up-regulation of alternative detoxification mechanisms as well as additional antioxidative capacities. This suggests that the alternative detoxification mechanisms in mice are at least equal to GLO1 and because of the so far determined positive side effect even superior. Interestingly, in *C. elegans* it has been shown recently, that mildly elevated MG levels leads to an extended lifespan [Ravichandran *et al.* 2018] and in *D. melanogaster*, it has been found that in the absent of GLO1 the lifespan is increased [Moraru *et al.* 2018]. Data from our study indicating furthermore an increased natural survival in GLO1$^{-/-}$ mice possibly through an up-regulation of a defence cascade of alternative detoxification and antioxidant enzymes.

Altogether, the data of this study implies that, at least with respect to the GLO1 system, lower organisms are no suitable model organisms to address the detoxification of MG. Especially the ability to enter dauer stages is a critical point in respect to the comparison of *C. elegans* to humans. Whether the data obtained from the mouse model can be transferred to humans needs further investigations.

Why the importance of GLO1 differs massively between the different organisms has still to be investigated. A possible explanation might be a change in nutrition and environmental factors, resulting in the production of toxic metabolites such as ROS and RCS. Throughout the evolution, the increasing complexity of organisms is accompanied by a more complex energy metabolism to ensure energy production and healthiness even under unfavourable conditions. *S. cerevisiae* and *C. elegans* can handle unfavourable conditions by entering rescue stages. The energy metabolism of zebrafishes depends on the surrounding temperature. Therefore, they might have evolved other mechanisms as compared to warm-blooded organisms. For *D. melanogaster*, the eggs will only hatch if a food source is present. Therefore, changes in energy metabolism are probably limited due to this fact. For mice and subsequently also for humans, they have to maintain their energy metabolism and body temperature to ensure wellbeing. While fasting by itself will at some point be negative for the wellbeing, the food intake afterwards will cause an elevated energy flux and results possibly in the production of toxic metabolites. But subsequently, nature has evolved mechanisms, which enables mammals to handle negative consequences from elevated energy flux as seen for the alternative detoxification mechanisms and the antioxidative capacity in the absence of GLO1.

5. Summary

The generation of methylglyoxal-derived advanced glycation end products plays an important role in the development of diabetes and late complications. Detoxification of methylglyoxal by glyoxalase 1 is therefore a key element in the context of dicarbonyl-induced damage in patients suffering from diabetes. This assumption was based on findings in simple organisms such as *S. cerevisiae* and *C. elegans*. Recent findings in *D. melanogaster*, zebrafish and murine cell lines indicate that glyoxalase 1 is less significant than in higher organisms. It has been shown that the loss of glyoxalase 1 is compensated by increased aldo-keto-reductase and aldehyde dehydrogenase activity. Therefore, the major aim of this study was to address potential differences in various organisms regarding their dependency on glyoxalase 1 and the determination of physiological consequences of the loss of glyoxalase 1.

Glyoxalase 1-deficient S. *cerevisiae* and *C. elegans* accumulate more methylglyoxal, the proliferation rate and egg laying rate is decreased and they are sensitive to cellular stress induced by other toxins. In zebrafish, MG is mildly elevated, but interestingly they show a decreased sensitivity in part towards toxins compared to wild-type animals. Furthermore, in three glyoxalase 1-deficient murine cell lines, methylglyoxal is not elevated and they tolerate xenobiotics such as toxins or UV-C radiation in a cell and toxin-specific manner possibly through an up-regulation of compensatory enzymes. *In vivo* data reveal furthermore the up-regulation of antioxidant enzymes when glyoxalase is absent, subsequently correlating with an elevated natural survival in $GLO1^{-/-}$ mice.

Complex organisms are less dependent on glyoxalase 1 and less prone to damage despite the loss of glyoxalase 1 than expected. In glyoxalase 1-deficient cells and mice, the phenotype even shows a protective character. Therefore, the data from this study suggests that the loss of glyoxalase 1 has less severe effects on complex organisms than expected and might even lead to an advantage on survival through the activation of antioxidant enzymes. The clinical relevance of glyoxalase 1 and possible compensatory pathways on the development of late complications in different organs has to be addressed in future studies.

6. Zusammenfassung

Der während der Glykolyse gebildete toxische Metabolit Methylglyoxal beeinflusst die Entwicklung und Progression von diabetischen Spätfolgen. Daher kommt dem Abbau von Methylglyoxal über das Glyoxalase-System im Organismus eine wichtige Rolle zu. Die ersten Untersuchungen von Glyoxalase 1, der wichtigsten Komponente des Gyloxalase-Systems, wurden in einfachen Organismen wie *S. cerevisiae* und *C. elegans* durchgeführt. Es wurde ermittelt, dass Glyoxalase 1 in diesen Organismen das wichtigstes Entgiftungsenzym für Methylgloyxal darstellt. Neuere Daten in höher-entwickelten Organismen wie *D. melanogaster*, Zebrafisch und in murinen Zelllinien weisen darauf hin, dass Glyoxalase 1 im Laufe der Evolution an Bedeutung verliert, da ein Verlust der Glyoxalse 1 in murinen Schwann-Zellen und in Glyoxalse 1-defizienten Mäusen durch alternative Mechanismen kompensiert werden kann.

Ziel der vorliegenden Studie war es, potentielle Unterschiede in verschiedenen Modellorganismen in Bezug auf deren Glyoxalase 1-Abhängigkeit zur Entgiftung und anschließend die daraus resultierenden physiologischen Konsequenzen zu erforschen. Glyoxalase 1-defiziente Modelle von *S. cerevisiae* und *C. elegans* weisen eine erhöhte Akkumulation von Methylglyoxal auf. Zudem sind einfache Organismen in ihrer Proliferation und Fertilität eingeschränkt, sowie sensitiver gegenüber anderen toxischen Substanzen. In Glyoxalase 1-defizienten Zebrafischen konnte eine nur leicht erhöhte Akkumulation von Methylglyoxal festgestellt werden. Überraschenderweise reagierten diese Tiere teilweise toleranter auf die Behandlung mit toxischen Substanzen als Wildtyp-Tiere. Zudem konnte gezeigt werden, dass Methylglyoxal in drei murinen Glyoxalase 1-defizienten Zelllinien nicht erhöht ist. Diese Zellen wiesen darüber hinaus eine Resistenz gegenüber anderen toxischen Substanzen und UV-C-Strahlung auf. Ein Grund für die erhöhte Toleranz könnte die erhöhte Aktivität von Aldo-Keto-Reduktasen und Aldehyde-Dehydrogenasen sein, die außer Methylglyoxal weitere toxische Substanzen entgiften können. Zudem wird die Abwehr in Glyoxalase 1-defizienten Mäusen durch die Aktivierung von Antioxidantien verstärkt. Dies führt vermutlich in Glyoxalase 1-defizienten Mäusen zu einer gesteigerten Lebenserwartung.

Säugetiere scheinen demnach weniger abhängig von Glyoxalase 1 als erwartet. Der Verlust der Glyoxalase 1 hat einen protektiven Charakter, indem mehrere Schutzmechanismen gegen verschiedene Substanzen aktiviert werden. Unsere Daten lassen demnach einen Vorteil vermuten, wenn Glyoxalase 1 nicht mehr vorhanden ist. Die Natur hat im Laufe der Evolution mehrere parallele Reaktionswege entwickelt, um komplexe Organismen vor schädlichen Umwelteinflüssen zu schützen und den Verlust eines einstmaligen wichtigen Enzyms zu kompensieren.

7. Literature

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Eidesstattliche Versicherung

1. Bei der eingereichten Dissertation zu dem Thema *Comparative study in various model organisms regarding the effect of the loss of glyoxalase 1* handelt es sich um meine eigenständig erbrachte Leistung.

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