

Investigating D-Amino Acid Oxidase Expression and Interaction Network Analyses in Pathways Associated With Cellular Stress: Implications in the Biology of Aging

Bioinformatics and Biology Insights
Volume 18: 1–11
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DOI: 10.1177/11779322241234772



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ABSTRACT: D-amino acid oxidase (DAO) is a flavoenzyme that metabolizes D-amino acids by oxidative deamination, producing hydrogen peroxide (H₂O₂) as a by-product. The generation of intracellular H₂O₂ may alter the redox-homeostasis mechanism of cells and increase the oxidative stress levels in tissues, associated with the pathogenesis of age-related diseases and organ decline. This study investigates the effect of DAO knockdown using clustered regularly interspaced short palindromic repeats (CRISPR) through an *in silico* approach on its protein-protein interactions (PPIs) and their potential roles in the process of aging. The target sequence and guide RNA of DAO were designed using the CCTop database, PPI analysis using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, Reactome biological pathway, protein docking using GalaxyTongDock database, and structure analysis. The translated target sequence of DAO lies between amino acids 43 to 50. The 10 proteins that were predicted to interact with DAO are involved in peroxisome pathways such as acyl-coenzyme A oxidase 1 (ACOX1), alanine-glyoxylate and serine-pyruvate aminotransferase (AGXT), catalase (CAT), carnitine O-acetyltransferase (CRAT), glyceronephosphate O-acyltransferase (GNPAT), hydroxyacid oxidase 1 (HAO1), hydroxyacid oxidase 2 (HAO2), trans-L-3-hydroxyproline dehydratase (L3HYPDH), polyamine oxidase (PAOX), and pipercolic acid and sarcosine oxidase (PIPOX). In summary, DAO mutation would most likely reduce activity with its interacting proteins that generate H₂O₂. However, DAO mutation may result in peroxisomal disorders, and thus, alternative techniques should be considered for an *in vivo* approach.

KEYWORDS: D-amino acid oxidase, hydrogen peroxide, reactive oxygen species, aging, protein-protein interaction

RECEIVED: May 2, 2023. **ACCEPTED:** February 7, 2024.

TYPE: Original Research Article

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by KT's grants – Higher Education Malaysia for Fundamental Research Grant Scheme with Project Code: FRGS/1/2020/SKK0/USM/02/9 and Universiti Sains Malaysia for Research University Grant and RR grants—Ministry of Higher Education Malaysia through the Fundamental Research Grant Scheme (FP007-2019A).

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

The World Health Organization estimates that the world's population of those more than 60 years will double from 12% to 22% between 2015 and 2050, as the pace of population aging is faster now compared with the past. According to the National Institute on Aging (NIA), US Department of Health and Human Services, aging involves dynamic changes in a living organism's biological, physiological, environmental, psychological, behavioral, and social processes.¹ In trying to identify the biological characteristics and factors that contribute to aging, researchers have included 9 hallmarks of aging² and the 7 pillars of aging³ that were aligned to outline the following aging mechanisms: (1) genomic instability/abnormal gene expression, (2) DNA damage, (3) telomere attrition, (4) epigenetic alterations, (5) loss of proteostasis, (6) deregulated nutrient sensing/metabolism, (7) mitochondrial dysfunction, (8) oxidative stress, (9)

cellular senescence, (10) stem cell exhaustion, (11) altered inter-cellular communication, and (12) inflammation.

From the growing body of evidence, there is a large focus on oxidative stress theory that results in the irreversible accumulation of oxidative damage, due to reactive oxygen species (ROS), such as superoxide radical (O₂⁻), hydroxyl radical (HO•), and hydrogen peroxide (H₂O₂).⁴ Reactive oxygen species contribution to aging is also governed by the cellular metabolism, protective systems, and genotype, where unintended cellular activities result in damage in the form of by-products, errors, and imbalance in cellular components.⁵ The overproduction of ROS can disturb the delicate redox balance within cells, resulting in the impairment of essential cellular components to the damage of primary cellular components including DNA, lipids, and proteins.⁶ Reactive oxygen species can cause oxidative damage to these cellular structures, leading to various adverse effects



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on cell function, which eventually linked to aging and the progression of age-related diseases, such as cardiovascular disease, neurodegenerative disease, and diabetes.⁷ While mitochondria are the leading player in ROS production, peroxisomes also produce significant amounts of ROS, and their dysfunction may contribute to cell death.^{8,9} Peroxisomes are highly dynamic organelles consisting of H₂O₂-producing oxidases and the anti-oxidant enzyme, namely catalase (CAT), to detoxify the ROS.

Notably, accumulation of D-amino acids has been associated with age-related diseases, including long-term kidney disease, Alzheimer disease, and Parkinson disease.¹⁰ An enzyme known as D-amino acid oxidases (DAO/DAAO, from now on addressed as DAO) catalyzes the oxidative deamination of D-amino acids to produce α -keto acids, ammonia, and H₂O₂.¹¹ In mammals, DAO is primarily expressed in the kidney, liver, brain, and to a lesser extent, in the small intestine, and neutrophilic leukocytes.¹² In humans, DAO (1) eliminates D-amino acids in the liver and kidney, (2) modulates D-serine which is required for the activation of N-methyl-D-aspartate (NMDA) receptor in the brain, and (3) expresses in granule fraction of mature human granulocytes in counteracting foreign microorganisms.¹³

Recently, a study identified DAO upregulation in cellular senescence, persistent DNA damage, and oxidative stress.¹⁴ D-amino acid oxidase produces ROS by-products of substrate oxidation, and ROS accumulation mediates senescence. By knocking out DAO in mice, previous study has found that there was an enhancement of D-serine exposure in the brain, which improves short-term memory, and suppression of hydrogen peroxide generation in the kidney.¹⁵ However, the long-term effect of DAO knockout and knockdown has been unclear. D-amino acid oxidase is a peroxisomal enzyme, which may interact with other proteins and impact on cellular homeostasis. Considering these, this study aimed to assess the effect of DAO knockdown using CRISPR through an *in silico* approach and predict its protein-protein interactions (PPIs), as mutations may affect their binding ability and the functional interactions between the target and interacting proteins.

Materials and Methods

Sequence retrieval from National Center for Biotechnology Information

The consensus coding sequences (CCDS) of DAO were retrieved from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>). Both nucleotide (accession No. NM_001917.5) and protein (accession No. NP_001908.3) sequences of DAO were retrieved for further analysis.

Designing guide RNA and target sequence prediction

The nucleotide sequence of DAO was used to predict the guide RNA (gRNA) sequence and the target sequence (5' end of the gRNA) using the CCTop (<http://crispr.cos.uni-heidelberg.de/>).

¹⁶ The gRNA of DAO was predicted and chosen according to its efficacy score.

Mapping of target sequence onto DAO protein sequence

The best-predicted target sequence based on the *in silico* analysis was mapped onto the DAO nucleotide sequence to identify the location. The target sequence was mapped onto the DAO amino acids sequence to obtain the amino acid position of the target sequence in the DAO protein sequence.

Analysis of protein-protein interaction

D-amino acid oxidase protein interaction analysis was carried out using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database version 11.5 (<https://string-db.org/>).¹⁷ D-amino acid oxidase protein was submitted as the query protein to identify the predicted interacting proteins. The analysis was conducted with the highest confidence level settings (0.9) to avoid false positive or less significant interactions. The interaction data were then exported in tab-separated values (TSV) format, and the network illustration was exported in jpg format.

Analysis of gene ontology and Kyoto Encyclopedia of Genes and Genomes enrichment

The gene list was submitted to Database for Annotation, Visualization and Integrated Discovery (DAVID) database version 6.8 (<https://david.ncifcrf.gov/>) as a query in the search box to predict the gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways involved.¹⁸ Gene Ontology analysis was carried out to specify the cellular locations, functions, and biological processes over-represented in the gene set.¹⁹ Kyoto Encyclopedia of Genes and Genomes analysis was conducted to identify the metabolic pathways involved in the gene set.²⁰

Analysis of biological pathway using Reactome

Pathway analysis of proteins interacting with DAO was carried out using the Reactome Knowledgebase database version 79 (<https://reactome.org/>).²¹ Reactome analysis was conducted to specify cellular compartments and protein localization participating in reactions, forming a network of biological interactions grouped into pathways involved in the gene set.

Preparation of protein structures

Three-dimensional (3D) protein structures were retrieved from RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) (www.rcsb.org/)²² for the following human DAO (PDB ID: 3G3E), AGXT (PDB ID: 5F9S), CAT (PDB ID: 1DGF), CRAT (PDB ID: 1NDF), and HAO1 (PDB ID: 2NZL). For protein without experimental

structures such ACOX1, HAO2, GNPAT, PIPOX, PAOX, and L3HYPDH, homology models were built using SWISS-MODEL (<https://swissmodel.expasy.org/>).²³ The protein structures were prepared using the The University of California, San Francisco (UCSF) Chimera (<https://www.cgl.ucsf.edu/chimera/>).²⁴ The protein structures were dock prepped by removing bound ligands and solvent molecules from the protein structures. The prepared protein structures were then saved in protein data bank (pdb) format for docking analysis.

Analysis of protein docking

Docking analysis of the target protein (DAO) with the interacting proteins was conducted using GalaxyTongDock (<http://galaxy.seoklab.org/tongdock>).²⁵ D-amino acid oxidase protein structure was submitted as chain A and each interacting protein structure as chain B in GalaxyTongDock. Each pair of docking structures were downloaded in pdb format for structure analysis.

Analysis of the docked structure

Each docking pairs were analyzed using Solvent accessibility based Protein-Protein Interface Identification and Recognition (SPPIDER) database to predict the binding residues between the proteins (<http://sppider.cchmc.org/>).²⁶ The interface residues for each docking pair were saved for further analysis. The interface residues were compared with the CRISPR target sequence of DAO (gRNA) to predict the effect on the binding of the interacting proteins.

Results

D-amino acid oxidase consensus coding sequences

The CCD represents the protein-coding region of a gene. Nucleotide and protein sequences of DAO were obtained from NCBI (CCDS ID: CCDS9122.1). Nucleotide sequences followed by the protospacer adjacent motif (PAM) site NGG (5'-20N-NGG-3') were identified using CCTop. The top 10 gRNAs identified for DAO had an efficacy score of 0.74 to 0.83, as shown in Table 1 and Supplemental Table 1.

While the efficacy score for all gRNAs was high, in our past studies, we have shown that the gRNA with the best *in silico* score also performed the best in subsequent downstream assays. Therefore, for the purpose of this study, the gRNA target sequence (5'-GCCGGCAGCCACGTCGGTGGTGG-3'), with the highest efficacy score (0.83), PAM sequence (TGG), was selected for this study. The predicted nucleotide target sequence lies between positions 108885134 to 108885156 at chromosome number 12.

Target sequence mapping onto DAO protein sequence

The DNA target sequence was mapped onto the DAO nucleotide sequence. Then, the target sequence was mapped onto the DAO protein sequence. The translated target sequence

Table 1. Top 10 gRNA sequences retrieved from CCTop.

RANK	GRNA TARGET SEQUENCE	EFFICACY SCORE
1	GCCGGCAGCCACGTCGGTGGTGG	0.83
2	GAAGTTCTTCCAGCGGAAAGTGG	0.82
3	CCTGGGCCTGTTCTAATCTCGG	0.82
4	GAACATATCCAGCTCTCTGGGGG	0.80
5	CTCATGGATGCAGAGGGCGGTGG	0.80
6	TTCCTGGAAGGACACAGTTCTGG	0.79
7	CGTGTGGTGGTATTGGAGCAGG	0.79
8	GAGGCCGGCAGCCACGTCGGTGG	0.78
9	CCACACAAGCCTAATTCTGGAGG	0.76
10	GTTCCACACAAGCCTAATTCTGG	0.74

position found through this mapping method lies from amino acid positions 43 to 50 (tttdvaag), indicated in bold lowercase in the amino acid sequence, as shown in Figure 1.

PPI analysis

Network analyses were conducted to identify the interacting partners of DAO predicted by the STRING database. D-amino acid oxidase is found to have direct or indirect interaction with 10 proteins (ACOX1, AGXT, CAT, CRAT, GNPAT, HAO1, HAO2, L3HYPDH, PAOX, and PIPOX) which were gathered from various sources and evidence, most of which came from curated databases, text mining, and co-expression data as illustrated in Figure 2.

All the predicted proteins have an interaction score between 0.944 and 0.975, which is a very high confidence score, suggesting that the interaction of DAO with the predicted protein partners, either physically or functionally, is highly plausible, as shown in Table 2. Since STRING provides information on physical and functional interactions, functional enrichment analysis was conducted to determine each protein's GO and the significant pathways involved among the predicted proteins and DAO.

GO and KEGG enrichment

Based on KEGG analysis, 5 pathways are significantly enriched by the gene set, as shown in Table 3. Ten of the interacting genes and DAO are most associated considerably with the metabolic processes in the peroxisome. Five significant processes with a *P* value less than .05 were involved in the gene set. The glyoxylate metabolic process is the most significantly enriched process by the gene set ($P < .05$) as the value is the closest to 0 compared with the rest of the results. The most significant molecular function obtained is receptor binding, which is shown by 10 genes. D-amino acid oxidase, ACOX1, AGXT, CAT, CRAT, GNPAT, HAO1, HAO2,

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1   MRVVVIGAGV IGLSTALCIH ERYHSVLPQL DIKUYADRFT PLtttdvaag LWQPYLSDPN
61  NPQEADWSQQ TFDYLLSHVH SPNAENLGLF LISGYNLFHE AIPDPSWKDT VLGFRKLTTPR
121 ELDMPFDYGY GWFHTSLILE GKNYLQWLTE RLTERGVKFF QRKVESFEEV AREGADVIVN
181 CTGVWAGALQ RDPLLQPRG QIMKVDAPWM KHFILTHDPE RGIYNSPYII PGTQTVTLGG
241 IFQLGNWSEL NNIQDHNTIW EGCCRLEPTL KNARIIGERT GFRPVRPQIR LEREQLRTGP
301 SNTEVIHNYG HGGYGLTIHW GCALEAAKLF GRILEEKKLS RMPPSHL

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Figure 1. Amino acid sequences of D-amino acid oxidase.

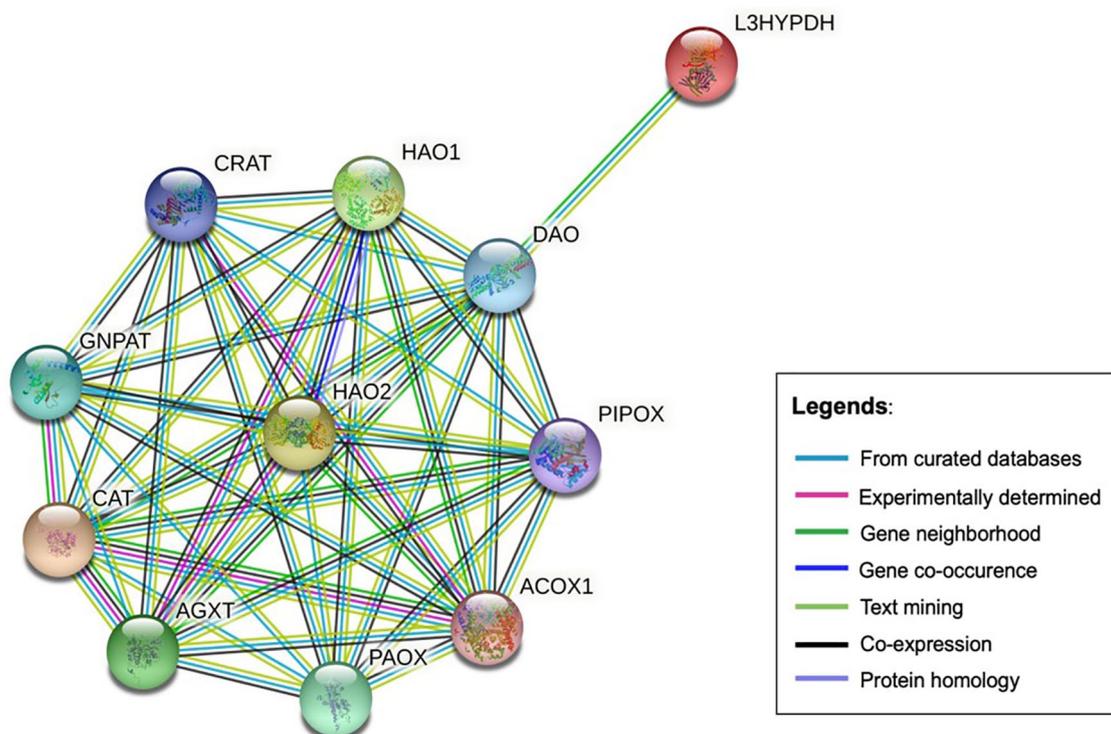


Figure 2. D-amino acid oxidase interaction network extracted from STRING.

PAOX, and PIPOX have receptor binding functions where the proteins can bind with other proteins in functional or physical interactions. The target gene and all interacting genes, except for L3HYPDH, encode peroxisome-localized proteins.

Reactome pathway analysis

Based on Reactome analysis, 10 proteins (ACOX1, AGXT, CAT, CRAT, DAO, GNPAT, HAO1, HAO2, PAOX, and PIPOX) were found as participants (members of) cargo proteins containing peroxisomal targeting signal 1 (PTS1) (ID: R-HSA-9033111 and R-HSA-9033148). This protein plays a role in the peroxisomal protein import pathway (ID: R-HSA-9033241), located in the cytosol, peroxisomal matrix, and peroxisomal membrane.

Protein-protein docking

After obtaining docked protein structures from GalaxyTongDock, the structures were analyzed through SPPIDER database to

predict the interacting residues of the protein structures. Referring to the gRNA map translated to amino acid sequence, the mutation site should be at T43 T44 T45 D46 V47 A48 A49 G50. As indel mutations in the target gene area would most likely alter the downstream amino acid sequence, the binding of DAO to its interaction partners may be disrupted. This is demonstrated in Table 4, which shows that most DAO binding residues are downstream of T43 and bolded.

Discussion

DAO catalyzes D-amino acids via three mechanisms.²⁷⁻²⁹ : (i) dehydrogenation of α -D-amino acids to their imino acid counterparts 270 with concurrent reduction of FAD to FADH₂, (ii) the FADH₂ is reoxidised by molecular oxygen 271 to produce H₂O₂, (iii) hydrolysis of the imino acid to give the corresponding α -keto acid and 272 ammonium. Excess H₂O₂ increases oxidative stress and is widely recognized as a critical initiator 273 of senescence, and their accumulation is widely observed in senescent cells.²⁹

Table 2. Predicted functional partners of DAO from STRING.

PREDICTED FUNCTIONAL GENE	PROTEIN (GENECARDS)	FUNCTION (GENECARDS)	INTERACTION SCORE	ACCESSION NUMBER
L3HYPDH	Trans-L-3-hydroxyproline dehydratase	Catalyzes the dehydration of trans-3-hydroxy-L-proline to Delta(1)-pyrroline-2-carboxylate (Pyr2C)	0.975	ENSP00000247194
CAT	Catalase	Converts the reactive oxygen species hydrogen peroxide to water and oxygen and thereby mitigates the toxic effects of hydrogen peroxide	0.974	ENSP00000241052
HAO1	Hydroxyacid oxidase 1	Has 2-hydroxyacid oxidase activity. Most active on the 2-carbon substrate glycolate, but is also active on 2-hydroxy fatty acids, with high activity toward 2-hydroxy palmitate and 2-hydroxy octanoate	0.961	ENSP00000368066
AGXT	Alanine-glyoxylate and serine-pyruvate aminotransferase	Involved in glyoxylate detoxification	0.957	ENSP00000302620
PAOX	Polyamine oxidase	Flavoenzyme that catalyzes the oxidation of N(1)-acetylspermine to spermidine and is thus involved in the polyamine back-conversion	0.955	ENSP00000278060
CRAT	Carnitine O-acetyltransferase	Catalyzes the reversible transfer of acyl groups from an acyl-CoA thioester to carnitine and regulates the ratio of acyl-CoA/CoA	0.955	ENSP00000315013
PIPOX	Pipecolic acid and sarcosine oxidase	Metabolizes sarcosine, L-pipecolic acid and L-proline	0.953	ENSP00000317721
GNPAT	Glyceronephosphate O-acyltransferase	Involved in plasmalogen biosynthesis	0.953	ENSP00000355607
HAO2	Hydroxyacid oxidase 2	Catalyzes the oxidation of L-alpha-hydroxy acids as well as, more slowly, that of L-alpha-amino acids	0.952	ENSP00000483507
ACOX1	Acyl-coenzyme A oxidase 1	Catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. First enzyme of the fatty acid beta-oxidation pathway	0.944	ENSP00000293217

We hypothesized that knocking down DAO using CRISPR/Cas9 would inhibit DNA damage-induced senescence. In this study, the best sgRNA target site of DAO was mapped onto the CCDS region to identify the translated target amino acid sequence. The target sequence lies at chromosome 12, between 108 885 134 and 108 885 156 bp, from positions 43 to 50. Mutations in this region might affect its PPI as the interface residues between the proteins might be affected due to the DAO gene knockdown.

Further STRING analysis predicted 10 interacting proteins with DAO based on evidence. GO analysis was carried out to identify the functional interaction between these proteins to better understand the mechanism at cellular levels and their localization. Nine out of 10 of the interacting proteins were found to be peroxisomal proteins (Table 3). Peroxisomes synthesize and degrade various critical cellular compounds.³⁰ Particularly, H₂O₂ that is produced is immediately processed by CAT by the peroxisome. However, in certain conditions where the balance of H₂O₂ production is altered, there is an accumulation of potentially

toxic metabolites, putting the cells on a pro-aging perturbation. The following peroxisomal proteins were found to have functional interactions with DAO: (1) AGXT, (2) CRAT, (3) HAO1, (4) HAO2, (5) ACOX1, (6) PIPOX, (7) PAOX, and (8) GNPAT.

AGXT encodes serine-pyruvate aminotransferase, which catalyzes the transamination of glyoxylate to glycine, and deficiency leads to overproduction of oxalate and glycolate in primary hyperoxaluria type 1 (PH1).³¹ Upstream of the AGXT reaction, the flavin mononucleotide (FMN)-dependent enzymes hydroxyacid oxidases (HAO1 and HAO2) catalyze the oxidation of glycolate to glyoxylate.³² Therefore, AGXT and HAO are involved in glyoxylate detoxification in the human hepatocytes. In particular, HAO action releases H₂O₂ in the peroxisomes.³³

Besides AGXT and HAO, other H₂O₂-producing oxidases include PIPOX and PAOX, which are involved in amino acid and polyamine catabolism, respectively. PIPOX converts L-pipecolic acid to 1-piperidine-6-carboxylic acid, and deficiency in the liver is associated with patients with

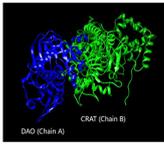
Table 3. List of KEGG pathways, biological processes, molecular functions, and cellular components involved in the gene set.

PATHWAYS/PROCESSES	GENES	P
KEGG pathway		
Peroxisome	DAO, ACOX1, AGXT, CRAT, CAT, GNPAT, HAO1, HAO2, PIPOX, PAOX	3.40×10^{-17}
Glyoxylate and dicarboxylate metabolism	AGXT, CAT, HAO1, HAO	6.40×10^{-6}
Biosynthesis of antibiotics	DAO, AGXT, CAT, HAO1, HAO2	1.60×10^{-4}
Carbon metabolism	AGXT, CAT, HAO1, HAO2	4.80×10^{-4}
Glycine, serine, and threonine metabolism	DAO, AGXT, PIPOX	1.40×10^{-3}
Biological process		
Glyoxylate metabolic process	DAO, AGXT, HAO1	1.00×10^{-4}
Fatty acid beta-oxidation using acyl-CoA oxidase	ACOX1, CRAT	7.70×10^{-3}
Fatty acid oxidation	ACOX1, HAO2	8.30×10^{-3}
Response to fatty acid	CAT, GNPAT	1.00×10^{-2}
Cellular lipid metabolic process	GNPAT, HAO2	2.40×10^{-2}
Oxidation-reduction process	DAO, PIPOX, PAOX	4.60×10^{-2}
Molecular function		
Receptor binding	DAO, ACOX1, AGXT, CRAT, CAT, GNPAT, HAO1, HAO2, PIPOX, PAOX	6.80×10^{-15}
(S)-2-hydroxy-acid oxidase activity	HAO1, HAO2	1.20×10^{-3}
Oxidoreductase activity	HAO2, PIPOX, PAOX	5.90×10^{-3}
FMN binding	HAO1, HAO2	8.90×10^{-3}
FAD binding	DAO, ACOX1	1.40×10^{-2}
Cellular components		
Peroxisomal matrix	DAO, ACOX1, AGXT, CRAT, CAT, GNPAT, HAO1, HAO2, PIPOX, PAOX	1.80×10^{-24}
Peroxisome	DAO, ACOX1, AGXT, CRAT, CAT, GNPAT, HAO1, HAO2, PIPOX	7.70×10^{-18}
Peroxisomal membrane	DAO, ACOX1, CAT, GNPAT	2.00×10^{-6}
Mitochondrion	ACOX1, CRAT, CAT, GNPAT, HAO2	2.60×10^{-3}

human peroxisome biogenesis disorders (PBDs) like Zellweger syndrome.³⁴ On the contrary, PAOX preferentially oxidizes polyamines such as N¹-acetyl-spermine or N¹-acetyl-spermidine to produce spermidine, and elevated polyamines level results in tumor progression.³⁵ Carnitine o-acetyltransferase encodes carnitine acyltransferases that catalyze acyl exchange between carnitine and coenzyme A (CoA), and reduced activity causes serious neurological and heart problems, similar to that seen in patients with Alzheimer disease.³⁶

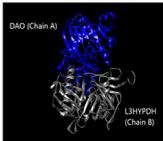
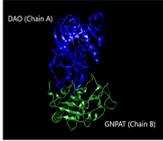
In ether lipid biosynthesis, *GNPAT* encodes the enzyme GNPAT or dihydroxyacetone phosphate acyltransferase (DHAPAT) and is responsible for the production of plasmalogens. Mutations in *GNPAT* have been found to cause rhizomelic chondrodysplasia punctata type 2 (RCDP2), marked by shortened long bones and intellectual disability.³⁷ Meanwhile, in the fatty acid beta-oxidation pathway, *ACOX1* encodes the first and rate-limiting enzyme of the very-long-chain fatty acid (VLCFA), and deficiency causes a rapid and severe loss of nervous system function.³⁸

Table 4. Protein docking structures with the respective interacting residues.

PROTEIN PARTNERS	DOCKING SCORE	PROTEIN DOCKING STRUCTURES	CHAIN A (DAO)	CHAIN B (DAO INTERACTION PARTNER)
DAO and AGXT	1054.734		D31 K33 D37 F39 T40 P41 L42 D73 L76 S77 H78 S81 P82 K142 N143 Q146 T149 T153 G156 K158 F159 F160 Q161 R162	L6 L7 V8 P10 P11 L14 M45 H83 C84 E87 R118 I119 K236 P237 F238 S239 F240 Y241 D243 W246 Y260 H261 H262 T263
DAO and CAT	1067.298		S57 D58 L91 I92 S93 Y95 H99 E100 A101 I102 P103 D104 S106 K108 D109 L112 G113 F114 R115 K116 T118 P119 R120 E121 L122 D123 M124 P126 Y128 G129 Y130 F133 T135 W209 K211 H212 P219 E220	S337 N338 E344 A345 Q352 G353 L355 F356 P359 D360 R363 P368 N369 L371 H372 V383 N385 Y386 Q387 R388 N403 Y404 Y405 P406 Q414 P416 A418 L419 E420 H421 S422 I423 Q424 Y425 V429 R431
DAO and HAO1	885.399		E21 R22 Y23 H24 S25 V26 Q28 D66 Q69 Q70 D73 Y74 L76 S77 H78 V79 H80 S81 P82 N83 D109 T110 L112 W147 R151 R155 L324 K328 R332 S340	N32 D33 E35 D39 A42 R46 V159 P162 Y163 N166 P215 S216 I217 S218 W219 E220 R240 D242 D243 E246 L265 D266 G267 V268 P269 D273 V274 E277 K362
DAO and CRAT	962.219		E21 R22 Y23 H24 S25 V26 Q63 D66 Q69 Q70 D73 Y74 L76 S77 D109 L112 W320 L324 E325 K328 G331 R332 E335 E336 S340	V109 D110 L111 Q112 R116 D206 F209 V210 E213 K214 N217 S218 L220 Q221 T222 N223 K224 R348 M356 K359 R361
DAO and ACOX1	854.546		T40 P41 L42 P82 K142 Q146 Q161 K204 D206 P208 W209 T233 Q234 T235 E249 L250 N251 N252 I253 Q254 H256 N257 T258 W260 E261 P268 T269 K271 N272 R274 I275 I276 G277 E278 R279	T139 M141 G142 H143 G144 T145 H146 T171 W175 W176 I226 P228 K229 F230 E233 Q309 S310 W383 N386 A387 E390 E391 R393 M394 G397 G398 Y401 Y411 T415 P416 C418 T419 F420 E423 T425 V426 L429 N517 S520 V521 V524 R525 E528 Q569 K570 S649 H653
DAO and HAO2	1060.998		E85 F90 L91 I92 S93 Y95 R120 E121 M124 F133 D206 P208 W209 M210 K211 H212 P231 G232 T233 Q234 T235 E267 T269 N272	L137 Q172 R174 N176 T178 Q183 P185 K186 K187 G188 N189 A190 I191 P192 Y193 F194 Q195 M196 T197 S200
DAO and PIPOX	1128.328		M1 R2 H20 H24 Q28 P29 L30 D31 K33 F39 T40 P41 L42 P82 E85 N86 K142 N143 Q146 T149 E150 R151 T153 E154 R155 G156 K158 F159 F160 Q161 R162 E173 T233 Q234 I276	F39 F40 P42 H43 S44 R45 H49 Q51 T84 Y152 K153 R156 D160 R163 Q164 I168 R170 D171 G172 S190 R191 R230 E231 P234 E263 Y264 P265 L267 R283 D284 P286 T287 R289 E312 P313 A314 V315 I316 E317
DAO and PAOX	897.664		E21 R22 Y23 H24 S25 V26 D66 Q69 Q70 D73 Y74 L76 S77 S106 K108 D109 T110 V111 L112 G113 W320 L324 E325 K328 G331 R332 E335 S340	R177 K178 L181 D345 T346 S347 L349 E350 D351 A352 A353 P354 E355 L356 Q357 D358 A359 W360 F361 R362 F390 T393 L394 S395 E397 E398 L401 C402 Q405 R408 R409

(Continued)

Table 4. (Continued)

PROTEIN PARTNERS	DOCKING SCORE	PROTEIN DOCKING STRUCTURES	CHAIN A (DAO)	CHAIN B (DAO INTERACTION PARTNER)
DAO and L3HYPDH	790.3		K163 V164 E165 S166 E168 W185 G187 A188 L189 R191 D192 P193 L194 L195 Q196 G245 N246 W247 S248 L250 N252 Q254	H26 G28 G29 C205 S206 A207 R210 K249 D250 A251 Y252 T253 K254 E255 S301 A302 T303 G304 S305 V306 T308 S328 G329 Q330 H332
DAO and GNPAT	1073.847		N61 Q63 K163 E165 S166 E168 E169 G187 A188 L189 Q190 R191 D192 P193 L194 L195 W247 R286 Q288 R290 L291 E292 R293 E294 L296 R297 E325 L329	M209 G215 N216 K217 Y219 W220 V222 F223 S224 K228 T229 R232 F240 G244 K256 F257 G258 L259 N261 M264 E265 F268 K269 L337 A338

The residues downstream of T43, the start of the mutation site are bolded.

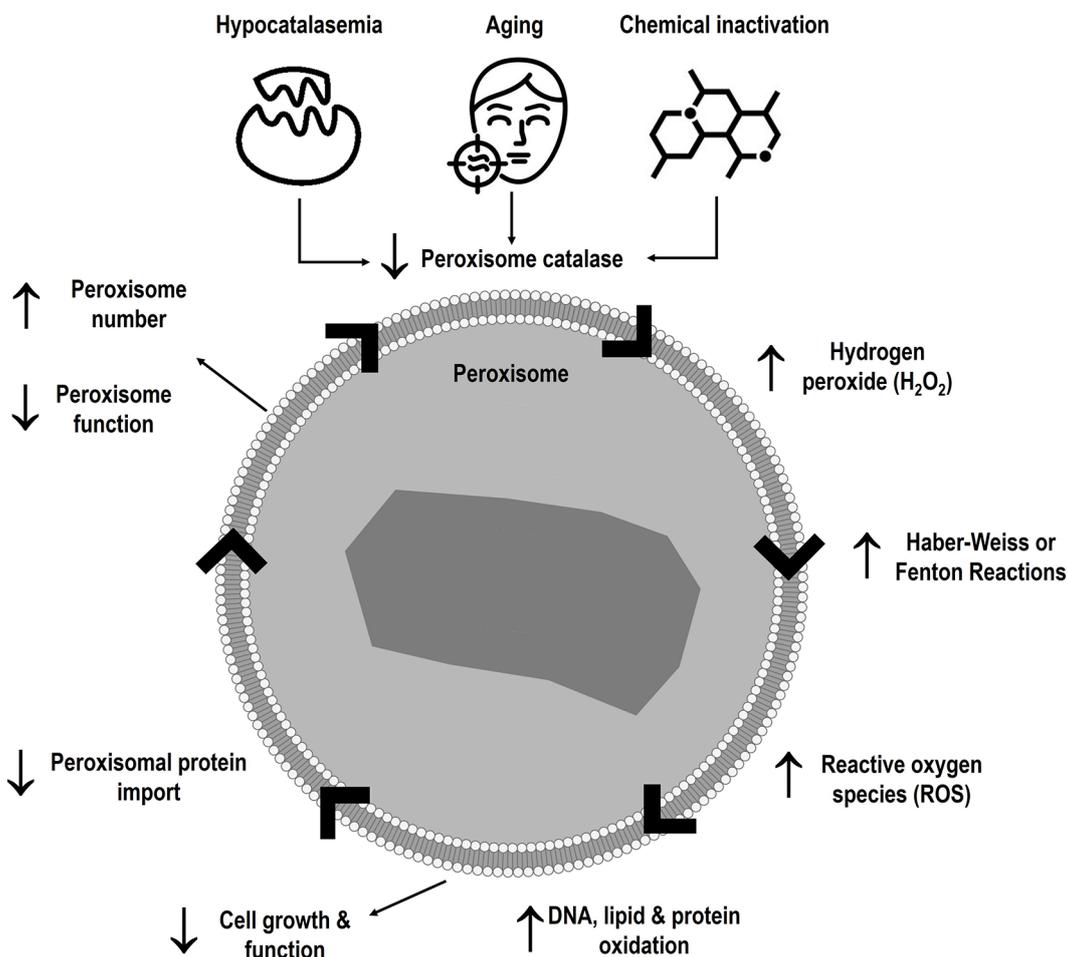


Figure 3. Peroxisome deterioration spiral.

The Reactome biological pathway analysis reported cargo proteins containing PTS1. Generally, the matrix proteins in the cytosol are imported into the peroxisome via PTS1, a carboxyl-terminal tripeptide motif and cytosolic protein, Pex5p, which is the receptor for PTS1.³⁹ A study found that aging compromises PTS1 protein import, affecting the antioxidant

activity of CAT.^{40,41} Furthermore, alterations in the number and appearance of peroxisomes were observed, and the organelles accumulated the Pex5p on their membranes. The decline in Pex5p-dependent peroxisomal import of CAT resulted in increased oxidative damage to cellular proteins and lipids, thereby initiating the "deterioration spiral," which eventually

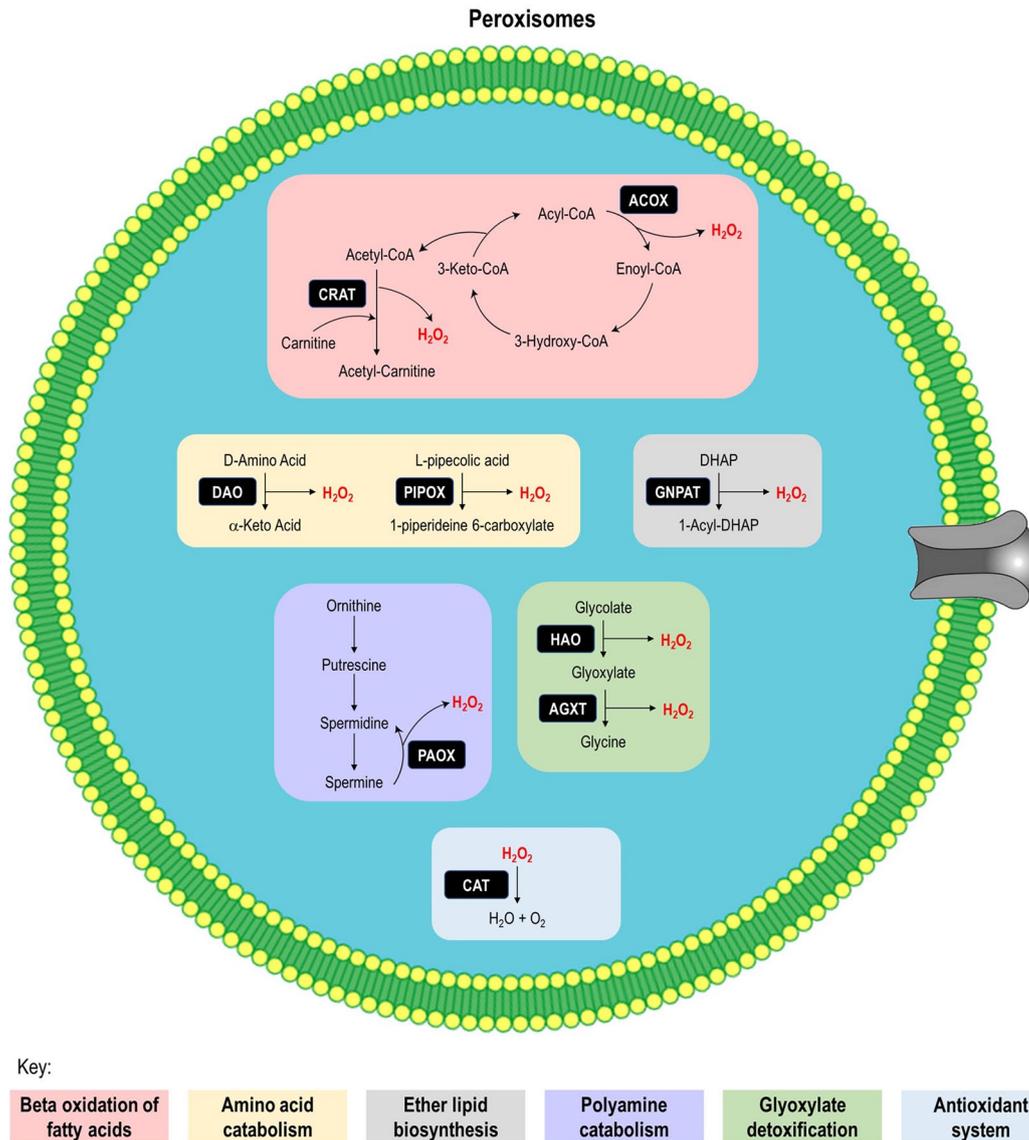


Figure 4. The major producer of peroxisomal ROS and their enzymes. ACOX and CRAT in the beta-oxidation of fatty acids, DAO and PIPOX in the amino acid catabolism, GNPAT in the ether lipid biosynthesis, PAOX in the polyamine catabolism, and HAO and AGXT in the glyoxylate detoxification. Meanwhile, CAT is recognized to be involved in the antioxidant system.

reduces peroxisomal protein import and exacerbates the effects of aging.⁴² As illustrated in Figure 3, the model described 3 potential factors: hypocatalasemia, aging, or chemical inactivation, resulting in reduced cellular CAT expression.⁴¹

Our further protein-protein docking analysis showed that all interacting proteins would most likely be unable to bind to DAO if the target sequence is mutated since all of the interacting or binding residues are located upstream and downstream of the DAO target residues. Changes in the DNA sequence generate inactivating mutations at the target site, creating frameshifts.⁴³ This might cause changes in the downstream amino acid residues and possibly influence how the protein folds into a 3D structure. Indels adjacent to the PPI interfaces may alter the protein-protein bindings, resulting in the loss or gain of new interactions.⁴⁴ Thus, this modification will randomly affect

DAO general structure and, more crucially, its binding interface, resulting in a loss of capacity to interact with its partners.

Comparative genome-wide expression analysis revealed novel genes specifically upregulated in senescence, with DAO directly regulated by p53.⁴⁵ D-amino acid oxidase cellular senescence activation was observed when DNA damage was induced through anticancer drugs, cancerous genes, or telomere shortening.¹⁴ In response, transcription is induced in DAO and p53-regulated genes, including solute carrier family 52 member 1 (SLC52A1) located at the cell membrane. Increased levels of SLC52A1 allow transport of vitamin B2, which converts to flavin adenine dinucleotide (FAD) abundantly in the cell. Increased FAD concentration increases DAO activity and damages cellular DNA, proteins, and lipids, thereby promoting cellular senescence.

Although most of the peroxisomal proteins (AGXT, ACOX1, HAO1, HAO2, PIPOX, PAOX, GNPAT, and CRAT) have a negative effect on aging due to their production of H₂O₂, as illustrated in Figure 4, deficiencies or mutations in any of these enzymes result in peroxisomal disorders.³³ In particular, the hypothetical mutation in DAO was related to amyotrophic lateral sclerosis (ALS), a fatal human disease characterized by progressive muscle weakness, atrophy, paralysis, and death from respiratory failure. When DAO activity in the spinal cord was reduced in a mice model, the D-serine levels were consequently increased.⁴⁶ The excessive D-serine released from neurons and glia leads to synaptic loss and stimulation of extra-synaptic N-methyl-D-aspartate receptor (NMDAR) currents.⁴⁷

In conclusion, the *in silico* knockdown of DAO at the predicted target site would affect its PPI network, which is involved in H₂O₂ production. D-amino acid oxidase may serve as a promoter of DNA damage-induced cellular senescence. Overproduction of ROS has a negative effect, and ROS released under stress is thought to cause illness and aging. However, *in vivo* edits using CRISPR can be done with caution, using alternatives like targeted base edits to avoid unwanted or detrimental effects in future research.

Limitations

This article focuses on the knockdown of DAO using the best gRNA predicted through an *in silico* analysis, resulting in a slight bias in selection of gRNA. An in-depth study of the top 10 gRNA may produce different results, enabling a broader option for selection of gRNA for subsequent experiments. In addition, the insights gained from the GalaxyTongDock analysis are confined to binding modes and energies, and a more in-depth atomic-level investigation could provide a more profound and complete understanding of the interactions involving DAO and its partner. Furthermore, it should be noted that the proteins and interactions chosen in this study represent only a fraction of very intricate biological landscape, potentially neglecting the involvement of other crucial proteins and pathways. Thus, while the study's findings offer insightful glimpses, they should be interpreted within the expansive framework of the multifaceted mechanisms that underlie these aging-related phenomena.

Author Contributions

DS and YSH carried out the data analysis and wrote the draft article. VK reanalyzed the data and wrote the final article with support from NZ, LKH, RR, and KTD. All authors reviewed the article.

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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