

Supplementary Information

Supplementary tables

Peptide list obtained from the redox proteomics analysis

Peptide containing cysteine	UniProt, accession number	Master protein name	Fold change	p-value
[K].CLDAFPNLR.[D]	P10649	Glutathione S-transferase Mu 1	9.15	0.027
[K].IIFVVGPGSGKGTQC EK.[I]	Q9R0Y5	Adenylate kinase isoenzyme 1	6.63	0.002
[K].LGRICLDILK.[D]	P61089	Ubiquitin-conjugating enzyme E2 N	5.60	0.000
[R].EKDFIILSCVR.[A]	Q9EPU0	Regulator of nonsense transcripts 1	5.32	0.000
[K].NTEICDELMTR.[H]	Q8VC19	5-aminolevulinate synthase, nonspecific, mitochondrial	4.81	0.008
[R].RVNQAIWLLCTGAR.[E]	P97461	40S ribosomal protein S5	3.98	0.010
[K].EGGPNPENSSLANIL ELCR.[S]	Q8K0Z7	Translational activator of cytochrome c oxidase 1	3.33	0.037
[R].VTWDSSEFCVAVNPR.[F]	Q9WUM4	Coronin-1C	3.32	0.026
[R].VCGAVMETVK.[Q]	Q8VC19	5-aminolevulinate synthase, nonspecific, mitochondrial	3.26	0.050
[K].SLEKVCADLIR.[G]	P60867	40S ribosomal protein S20	3.20	0.038
[K].VTWDSAFCAVNPK.[F]	Q920M5	Coronin-6	3.03	0.026
[R].ITYRELLETCR.[L]	Q99NB1	Acetyl-coenzyme A synthetase 2-like, mitochondrial	2.80	0.032
[K].VVAACAMPVMKGW NILTNSEK.[S]	Q91VD9	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	2.74	0.039
[R].EGVVDIYNCVR.[E]	P28828	Receptor-type tyrosine-protein phosphatase mu	2.73	0.023
[K].VGVGPGSVCTTR.[T]	Q9DCZ1	GMP reductase 1	2.68	0.004
[R].TNGFSLESCR.[S]	O35350	Calpain-1 catalytic subunit	2.65	0.008
[R].VNQAIWLLCTGAR.[E]	P97461	40S ribosomal protein S5	2.62	0.034
[R].VGSVLQEGCEKISQLY GDLKHLK.[T]	Q8K2B3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	2.60	0.028
[K].GELLRPSPTCEIK.[A]	A2ASS6	Titin	2.52	0.023
[K].LASLTPGFSGADVAVN CNEAALIAAR.[H]	Q8JZQ2	AFG3-like protein 2	2.51	0.029
[K].LTDFGFCAQITPEQSK.[R]	O88643	Serine/threonine-protein kinase PAK 1	2.48	0.033

[R].TTANAIYCPK.[L]	Q8BG32	26S proteasome non-ATPase regulatory subunit 11	2.43	0.037
[K].ADLSNCLYK.[D]	Q0II04	Nebulette	2.38	0.039
[K].DGVVTIGCIGFPNVGK SSLINGLVGR.[K]	P36916	Guanine nucleotide-binding protein-like 1	2.35	0.029
[K].TPPYQIACGISQGLAD NTVVAK.[V]	Q9D0R2	Threonine--tRNA ligase 1, cytoplasmic	2.30	0.033
[R].ASDSSLVCSR.[Y]	Q8BYW9	EGF domain-specific O-linked N-acetylglucosamine transferase	2.24	0.035
[R].VNLVAILCTHK.[H]	Q69ZP3	Probable hydrolase PNKD	2.22	0.005
[R].VAIYMPVSPLAVAAM LACAR.[I]	Q99NB1	Acetyl-coenzyme A synthetase 2-like, mitochondrial	2.18	0.005
[K].ALNVEPDGTGLTCSLA PNILSQL.[-]	P99029	Peroxisome oxidoreductase, mitochondrial	2.11	0.006
[R].VTNDNTFCR.[L]	P29268	CCN family member 2	2.09	0.046
[K].VPTTLAEYCIK.[T]	Q6ZWZ2	Ubiquitin-conjugating enzyme E2 R2	2.09	0.048
[K].TQAIVCQQLDLTHLK.[E]	P61222	ATP-binding cassette sub-family E member 1	2.08	0.014
[R].TIQFVDWCPTGFK.[V]	Q9JJZ2; P68369; P68373	Tubulin alpha-8 chain	2.08	0.049
[R].LIIGQNGILSTPAVSCII R.[K]	Q8BZF8	Phosphoglucosyltransferase-like protein 5	2.08	0.039
[K].RPNKPLFTGLVTQCQK .[M]	Q8K4Z3	NAD(P)H-hydrate epimerase	2.07	0.020
[K].DGSASGTTLEALDCIL PPTRPTDKPLR.[L]	P10126	Elongation factor 1-alpha 1	2.04	0.017
[R].GSLLGCSINISDIR.[D]	O35350	Calpain-1 catalytic subunit	2.02	0.009
[K].EDVKSCAEFVSGSQLR .[I]	Q9DCX2	ATP synthase subunit d, mitochondrial	2.02	0.017
[K].KLPVGFTFSFPCR.[Q]	P17710	Hexokinase-1	1.98	0.014
[R].LEALELKECLAHTPK.[L]	O09131	Glutathione S-transferase omega-1	1.97	0.001
[R].SAVQYAECQSK.[A]	Q8K2I4	Beta-mannosidase	1.95	0.036
[K].ELETQLLEQCTVDTGA AK.[G]	Q9QZB7	Actin-related protein 10	1.95	0.050
[R].LDGALCSYTEK.[D]	P52624	Uridine phosphorylase 1	1.94	0.021
[K].FTCEIQGAPNVR.[F]	A2ASS6	Titin	1.94	0.018
[R].ILALCMGNHELYMR.[R]	P26041	Moesin	1.92	0.029
[K].NMMAACDPR.[H]	Q9ERD7; P68372	Tubulin beta-3 chain	1.91	0.002
[R].YVEPIEDVPCGNIVGL VGVDQFLVK.[T]	P58252	Elongation factor 2	1.89	0.045
[R].LVATDGAFSMDGDIA PLQDICR.[L]	O88986	2-amino-3-ketobutyrate coenzyme A ligase, mitochondrial	1.87	0.041
[R].GDSGLYLCK.[A]	A2ASS6	Titin	1.87	0.014
[K].LQAGIDLCETR.[T]	Q8C0M9	Isoaspartyl peptidase/L-asparaginase	1.86	0.049

[R].VFSANSTAACTELAK.[R]	Q9D0M1	Phosphoribosyl pyrophosphate synthase-associated protein 1	1.85	0.002
[R].KNANCSIEESFQR.[F]	P38060	Hydroxymethylglutaryl-CoA lyase, mitochondrial	1.84	0.003
[R].TFVSGACDASIK.[L]	P62880	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	1.84	0.015
[R].SLVANLAAANCYK.[K]	P55264	Adenosine kinase	1.81	0.016
[K].LVEEAIQCAEK.[I]	Q8BH95	Enoyl-CoA hydratase, mitochondrial	1.80	0.002
[K].VVNEINIEDLCLTK.[A]	Q9CQB5	CDGSH iron-sulfur domain-containing protein 2	1.80	0.029
[R].IVVHMAHALKPGEFG LASICNGGGGASALLIEK.[L]	Q8QZT1	Acetyl-CoA acetyltransferase, mitochondrial	1.79	0.037
[R].GTLLSLSEQELLDCDK VDK.[A]	Q9R013	Cathepsin F	1.78	0.041
[K].AVIFCLSADKK.[C]	Q9R0P5	Destrin	1.76	0.026
[R].VGAFVVCKDAEEAK.[R]	P05202	Aspartate aminotransferase, mitochondrial	1.75	0.002
[K].IYQLQVLANC.[A]	P50431	Serine hydroxymethyltransferase, cytosolic	1.74	0.016
[R].NLADCLR.[S]	Q9R1P3	Proteasome subunit beta type-2	1.74	0.041
[K].GSEDLKKHGCTVLTAL GTILK.[K]	P04247	Myoglobin	1.73	0.030
[R].AAQLCGAGMAAVVE K.[I]	P17710	Hexokinase-1	1.73	0.046
[K].SSFATPGVNVGLFCST PAVALGR.[A]	Q9D7J9	Enoyl-CoA hydratase domain-containing protein 3, mitochondrial	1.73	0.013
[R].VVDGIFAVCGVPESK.[L]	Q99KK9	Histidine--tRNA ligase, mitochondrial	1.71	0.012
[R].ILDLTECSAVQFDYSQ ER.[V]	Q9QXT1	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide	1.71	0.036
[R].MPLGFTFSFPCR.[Q]	P17710	Hexokinase-1	1.70	0.025
[K].TGVGYQLSAVIECAD SAHGLK.[G]	Q9DCZ1	GMP reductase 1	1.70	0.025
[K].LVEEAIQCAEKIASNSK.[I]	Q8BH95	Enoyl-CoA hydratase, mitochondrial	1.69	0.023
[R].DLTTAGAVTQCYR.[D]	P62717	60S ribosomal protein L18a	1.68	0.033
[R].VTNRDIICQIAYAR.[I]	P47962	60S ribosomal protein L5	1.68	0.008
[K].NLDCVVTGLQQGK.[T]	A2ASS6	Titin	1.67	0.002
[K].LPVGFTFSFPCR.[Q]	P17710	Hexokinase-1	1.67	0.036
[R].VGAFVVCKDAEEAK R.[V]	P05202	Aspartate aminotransferase, mitochondrial	1.67	0.012

[K].ASADLMSYCEEHAR.[S]	Q9DAS9	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	1.67	0.009
[R].YFSLGLPTGSTPLGCK.[K]	Q9CRC9	Glucosamine-6-phosphate isomerase 2	1.66	0.023
[K].AADLVSENCETIEAHMR.[D]	Q9JLI6	Selenocysteine lyase	1.66	0.016
[K].ISHTEDCLTFK.[V]	P01027	Complement C3	1.66	0.018
[K].IAYPLGLATLGATVCYP AQSIIAK.[I]	Q78IK4	MICOS complex subunit Mic27	1.65	0.007
[R].AHGSSILACAPLYSWR.[T]	P11688	Integrin alpha-5	1.65	0.001
[K].AVAVEEFCK.[S]	Q6PE15	Palmitoyl-protein thioesterase ABHD10, mitochondrial	1.65	0.001
[K].LAEEESCR.[E]	Q61543	Golgi apparatus protein 1	1.64	0.045
[K].IKNVDCVLLAR.[H]	Q9CQ65	S-methyl-5'-thioadenosine phosphorylase	1.63	0.035
[K].MVAAVACAK.[V]	Q3ULD5	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	1.62	0.006
[K].STLIDALCR.[K]	Q64514	Tripeptidyl-peptidase 2	1.62	0.023
[R].VIATFACSGEK.[E]	Q9JLJ2	4-trimethylaminobutyraldehyde dehydrogenase	1.61	0.008
[R].LSMEQICR.[H]	Q6P4S6	Serine/threonine-protein kinase SIK3	1.61	0.021
[R].SLGLICDECPGYTGPR.[C]	Q60675	Laminin subunit alpha-2	1.59	0.005
[R].LFVSGACDASAK.[L]	P62874	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	1.59	0.007
[R].EGITFHFTPLVCK.[D]	Q8VDL4	ADP-dependent glucokinase	1.59	0.039
[R].KGLIAAICAGPTALLA HEVGFCK.[V]	Q99LX0	Parkinson disease protein 7 homolog	1.59	0.042
[K].LLASCSADMTIK.[L]	P63005	Platelet-activating factor acetylhydrolase IB subunit beta	1.58	0.044
[R].TLSGMESYCVR.[A]	P80315	T-complex protein 1 subunit delta	1.58	0.017
[K].EILGTAQSVGCNVDG R.[H]	P35979	60S ribosomal protein L12	1.56	0.013
[K].LLTQLHYCEK.[A]	Q99KK9	Histidine--tRNA ligase, mitochondrial	1.56	0.027
[K].SAGGIGVAVSCIR.[A]	P07742	Ribonucleoside-diphosphate reductase large subunit	1.56	0.026
[K].VTYCPTEPGTYIINIK.[F]	Q8VHX6	Filamin-C	1.56	0.010
[R].GTFCSFDTPDEAIR.[N]	P61922	4-aminobutyrate aminotransferase, mitochondrial	1.55	0.037
[R].SIQFVDWCPTGFK.[V]	P68368	Tubulin alpha-4A chain	1.55	0.026

[KR].DLVRVCENIPIVLCG NK.[V]	P62827	GTP-binding nuclear protein Ran	1.55	0.043
[K].KGAFDAYVAVGGGSTM	Q8R0N6	Hydroxyacid-oxoacid transhydro- mitochondrial	1.55	0.016
[K].FGANAILGVSLAVCKA GAVEK.[G]	P17182	Alpha-enolase	1.55	0.043
[K].IREIADGLCLEVEGK.[M]	P63028	Translationally-controlled tumor protein	1.55	0.019
[R].AALEALGSCLNNK.[Y]	Q9CZN7	Serine hydroxymethyltransferase, mitochondrial	1.54	0.014
[R].ATNLNCSVIADVR.[H]	Q9D1H8	39S ribosomal protein L53, mitochondrial	1.54	0.030
[K].VFCIGPVFR.[A]	Q922B2	Aspartate--tRNA ligase, cytoplasmic	1.54	0.048
[K].GAVHQLCQSLAGK.[N]	Q8BVI4	Dihydropteridine reductase	1.52	0.010
[R].TSLDLIANVIHCK.[S]	P70404	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	1.52	0.016
[K].IEEACEIYAR.[A]	Q9DB05	Alpha-soluble NSF attachment protein	1.52	0.031
[R].TLIQNCGASTIR.[L]	P80318	T-complex protein 1 subunit gamma	1.52	0.039
[K].VFADYEEYIKCQDK.[V]	Q9WUB3	Glycogen phosphorylase, muscle form	1.52	0.006
[K].TFESLVDFCK.[T]	Q61425	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	1.51	0.010
[K].VIYDKDQFMCGETVP APSTNK.[E]	Q8K0W9	DPH3 homolog	1.50	0.046
[R].LISLNMNFCSR.[E]	Q04519	Sphingomyelin phosphodiesterase	1.50	0.049

Supplementary Table 1: List of cysteine containing peptides that were identified in the redox proteomic screen to show an increased total reversible oxidation demonstrated by a fold change of ≥ 1.5 and a p-value of ≤ 0.05 in the left ventricles of HyPer-DAO mice compared to wild type mice after 7 days of D-ala treatment. P values were calculated by two-tailed unpaired t-test.

Peptide list obtained from the redox proteomics analysis

Peptide containing cysteine	UniProt, accession number	Master protein name	Fold change	p-value
[K].TCVADESAANCDKSL HTLFGDK.[L]	P07724	Albumin	0.50	0.027
[R].MAHAMNEYPDSCAV LVR.[R]	Q9WVQ5	Methylthioribulose-1-phosphate dehydratase	0.49	0.010
[R].EVFGSGTACQVCPVH QILYEGK.[Q]	O35855	Branched-chain-amino-acid aminotransferase, mitochondrial	0.49	0.002
[K].QFSYTHICAGASAFGK .[N]	Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial	0.49	0.013
[K].TLTPGGHAEHDGQPY CHKPCYGILFGPK.[G]	Q9DCT8	Cysteine-rich protein 2	0.49	0.003
[K].YGHAACFGLQPGCLR.[Q]	Q8C1A5	Thimet oligopeptidase	0.48	0.003
[R].QCLSFPCAGVPIR.[D]	P0CW02	Lymphocyte antigen 6C1	0.48	0.043
[R].EGICGSCAMNINGGN TLACTR.[R]	Q9CQA3	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	0.48	0.006
[R].GHIEDCGHWTQIEKP TEVNQILIK.[W]	P34914	Bifunctional epoxide hydrolase 2	0.47	0.001
[R].VDLEISPDFLAVPVGG HENSHCICGNER.[K]	Q61838	Pregnancy zone protein	0.47	0.019
[R].LNQATVTSNRPLGFY GQCSEICGSNHSFMPIVLE MVPLK.[Y]	P00405	Cytochrome c oxidase subunit 2	0.47	0.016
[R].VLCVALGQLDRPLDLA DDGR.[I]	Q6P3E7	Polyamine deacetylase HDAC10	0.47	0.049
[K].TFHETLNCCGSNALTT LTTILR.[N]	P35762	CD81 antigen	0.46	0.047
[R].GDGQTCYDIDECSEQ PSR.[C]	P10493	Nidogen-1	0.46	0.003
[K].QCEAHLGHVFPDGP KPTGQR.[F]	Q78J03	Methionine-R-sulfoxide reductase B2, mitochondrial	0.46	0.031
[K].EKLCYVALDFENEMA TAASSSLEK.[S]	P68033	Actin, alpha cardiac muscle 1	0.46	0.045
[R].ELACDDPEAEQVALLA VDYLNNHLLQGFK.[Q]	P29699	Alpha-2-HS-glycoprotein	0.45	0.048
[R].LDPQLQLHCSDEIANL CAEEAAAQEQTGVVEECL K.[V]	Q61543	Golgi apparatus protein 1	0.45	0.033
[K].MVTMVEIDQMVIDG CK.[K]	P97355	Spermine synthase	0.45	0.036
[R].HYCIVANPVSR.[D]	Q9EQK5	Major vault protein	0.44	0.000
[K].ECCHGDLLECADDDRA ELAK.[Y]	P07724	Albumin	0.44	0.015
[K].LCMCADGTLFK.[V]	P82349	Beta-sarcoglycan	0.44	0.014

[R].TGQCECQPGITGQHCE R.[C]	P02468	Laminin subunit gamma-1	0.44	0.006
[R].FRCPETLFQPSFIGMESAGIHETTYNSIMK.[C]	P68033	Actin, alpha cardiac muscle 1	0.43	0.049
[R].IQPFQNLTLHPACSLGHYSLQLFEGLK.[A]	O35855	Branched-chain-amino-acid aminotransferase, mitochondrial	0.43	0.010
[K].AVLTSQETLFGGSDCTGNFCLFK.[S]	Q921I1	Serotransferrin	0.42	0.050
[R].LAQGICLELSEASSCEEFKK.[E]	Q64314	Hematopoietic progenitor cell antigen CD34	0.42	0.029
[K].SACGNCYLGDAFR.[C]	Q8WTY4	Anamorsin	0.41	0.039
[R].ACLLCSLVK.[T]	P63271	Transcription elongation factor SPT4-A	0.41	0.045
[R].LCGSGFQSIIVSGCQEI CSK.[D]	Q8BWT1	3-ketoacyl-CoA thiolase, mitochondrial	0.41	0.047
[R].RCESCAPGYEGNPIQPGGK.[C]	Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	0.40	0.023
[K].SLCPVSWVSAWDDR.[I]	P56391	Cytochrome c oxidase subunit 6B1	0.40	0.017
[R].VGTKCCTLPEDQRLPCVEDYLSAILNR.[V]	P07724	Albumin	0.40	0.007
[R].NECFLOQHKDDNPSLP PFERPEAEAMCTSFK.[E]	P07724	Albumin	0.40	0.006
[K].LCYVALDFENEMATAASSSLEK.[S]	P68033	Actin, alpha cardiac muscle 1	0.38	0.027
[R].VALMGSGSHGGFLSCHLIGQYPETYSACIAR.[N]	Q8R146	Acylamino-acid-releasing enzyme	0.38	0.045
[K].NCVILPHIGSATYK.[T]	Q91Z53	Glyoxylate reductase/hydroxypyruvate reductase	0.37	0.001
[K].GDCVECMACSDNTVR.[A]	Q924M7	Mannose-6-phosphate isomerase	0.36	0.001
[K].LYDYCDIPLCASASSFECGKPQVEPK.[K]	P20918	Plasminogen	0.36	0.009
[R].SCAPGSDPDSPLCALCVGGNNPAHMCANNAEGYHGSSGALR.[C]	Q9DBD0	Inhibitor of carbonic anhydrase	0.36	0.047
[K].QDADFPTSLSFQCVNGK.[H]	Q61129	Complement factor I	0.35	0.046
[R].RGTDECAIESIAVAaip IPK.[L]	P97821	Dipeptidyl peptidase 1	0.35	0.046
[R].KPVVPGHVLVCPLRPVER.[F]	O89106	Bis(5'-adenosyl)-triphosphatase	0.35	0.035
[R].LPCVEDYLSAILNRVCLLHEK.[T]	P07724	Albumin	0.34	0.001
[R].MLHSCTSEGSAYR.[K]	Q9R098	Hepatocyte growth factor activator	0.34	0.028
[R].DFNLDGAPYGYTPFCDSR.[R]	Q6P5E4	UDP-glucose:glycoprotein glucosyltransferase 1	0.33	0.029

[K].TPVSEHVTKCCSGSLV ERRPCFSALTVEITYVPK.[E]	P07724	Albumin	0.33	0.028
[K].TCVADESAANCDKSL HTLFGDKLCAIPNLR.[E]	P07724	Albumin	0.33	0.014
[K].VDIQTEDLEDGTCK.[V]	Q80X90	Filamin-B	0.32	0.043
[K].RDCGGAAAVLGAFR.[A]	Q6NSR8	Probable aminopeptidase NPEPL1	0.32	0.030
[R].LTASSTCGLHSPQPYCI VSHLQDEK.[K]	Q61292	Laminin subunit beta-2	0.32	0.005
[R].VTVQAACGNSVLQDS R.[L]	Q9EPR5	VPS10 domain-containing receptor SorCS2	0.32	0.024
[K].LCYHVLGTDQSEDILC AEFPDEPK.[W]	Q9QUR6	Prolyl endopeptidase	0.31	0.012
[R].LCRPCQCNDNIDPNA VGNCNR.[L]	P02468	Laminin subunit gamma-1	0.31	0.044
[R].VSVCAETFPDEEEED NDPR.[V]	P12367	cAMP-dependent protein kinase type II-alpha regulatory subunit	0.30	0.006
[K].SDQGEYTCVASSGR.[M]	P35918	Vascular endothelial growth factor receptor 2	0.30	0.018
[K].GEADAMSLDGGFAY VAGHCGLVPVLAENYLST HSSGR.[L]	Q9DBD0	Inhibitor of carbonic anhydrase	0.30	0.031
[K].TNGDQASCENELLK.[F]	P15208	Insulin receptor	0.30	0.045
[K].IDFDLHDLIPSCER.[T]	Q99P30	Peroxisomal coenzyme A diphosphatase NUDT7	0.30	0.023
[K].DWSFYILAHTFTPTTE TDTYACR.[V]	P01887	Beta-2-microglobulin	0.29	0.014
[R].GTVPGSSPCDSSSGTC FCK.[R]	Q61292	Laminin subunit beta-2	0.29	0.049
[R].VFIGKDCIGGCSDLIS MQQTGELMTR.[L]	Q9QUH0	Glutaredoxin-1	0.29	0.016
[R].LQIAACSNQDPLQGT TGLIPLLIDVWEHAYYLQ YK.[N]	P09671	Superoxide dismutase [Mn], mitochondrial	0.28	0.019
[R].FQQLVHQMTCLCWE K.[C]	Q9WVA2	Mitochondrial import inner membrane translocase subunit Tim8 A	0.28	0.021
[R].DKLCDLLVANNYFTHF FAPK.[N]	Q61702	Inter-alpha-trypsin inhibitor heavy chain H1	0.27	0.024
[R].AVDVLSELSYAPMTP DHFPTLFCK.[E]	Q7TSQ8	Pyruvate dehydrogenase phosphatase regulatory subunit, mitochondrial	0.27	0.008
[R].LCQTCYPLFQQVAIK.[M]	Q8BGT0	Osteopetrosis-associated transmembrane protein 1	0.27	0.037
[R].SICTTVLELLDK.[Y]	P68254	14-3-3 protein theta	0.26	0.027
[K].FQGPTCETCQTCLGV CAEHK.[E]	P09055	Integrin beta-1	0.25	0.032

[R].FRCPSRHEVVLDL.[H]	Q9ERP3	Tripartite motif-containing protein 54	0.25	0.025
[K].HVTEDCVFIYCQVGD KPYWK.[D]	Q9CQM5	Thioredoxin domain-containing protein 17	0.25	0.016
[R].VGAGGLDGYSVEYCQ EGCSEWTPALQGLTER.[R]	O70468	Myosin-binding protein C, cardiac-type	0.25	0.033
[R].QGTHITVVAHSRPVG HCLEAAVLSK.[E]	Q9D051	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	0.24	0.010
[K].YHIQVCTTTPCMLRDS DSILETLQR.[K]	Q9D6J6	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	0.24	0.009
[R].SQTEEDCTEELFDLH ARDHCVAHKLFK.[N]	P99028	Cytochrome b-c1 complex subunit 6, mitochondrial	0.24	0.027
[K].LCMAALSHQPQEFT YVEPTNDEICEAFR.[R]	P21614	Vitamin D-binding protein	0.23	0.042
[R].QAVDQISSGFFSPKDP DCFKDVVNMLMYHDR.[F]	Q8CI94	Glycogen phosphorylase, brain form	0.23	0.005
[K].ECCHGDLLECADDR.[A]	P07724	Albumin	0.23	0.013
[K].QYPCTLVGTWNTWY GEQDQAVHLWR.[Y]	O55126	Protein NipSnap homolog 2	0.23	0.022
[K].ISCSFHVTK.[Q]	Q8VE38	Oxidoreductase NAD-binding domain-containing protein 1	0.23	0.019
[R].FSDTCFLDTDGQATC DACAPGYTGR.[R]	Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	0.22	0.004
[K].TLTPGGHAEHDGKPF CHKPCYATLFGPK.[G]	Q9DCT8	Cysteine-rich protein 2	0.22	0.004
[K].TVQGAFFGVPVYKDH ENCISGEDITHNGIVYTPK.[H]	Q61838	Pregnancy zone protein	0.22	0.035
[R].FCLEGMEESGSEGLDE LIFAQK.[D]	Q9D1A2	Cytosolic non-specific dipeptidase	0.21	0.044
[K].DLFQCVSFIIPR.[L]	P28665	Murinoglobulin-1	0.20	0.001
[K].LVSEAIAAGIFNDLGS GSNIDLCVISK.[S]	P70195	Proteasome subunit beta type-7	0.20	0.047
[R].EEYQEELEFCEK.[L]	Q99P31	Hsp70-binding protein 1	0.19	0.000
[K].SAGYPGTLIPYRCDLS NEEDILSMFSAVR.[S]	Q3U0B3	Dehydrogenase/reductase SDR family member 11	0.19	0.000
[R].AQCIYMGGSSTYCSC L PGFSGDGR.[A]	P10493	Nidogen-1	0.19	0.000
[K].ILLCTGAIMEEQAAQL LGVK.[M]	Q9CY45	EEF1A lysine methyltransferase 1	0.19	0.000
[R].VELCAQGSPDLAHL DGPYEAGGEKEQDPR.[L]	Q61738	Integrin alpha-7	0.19	0.000
[R].ACLISMGYDLGEAEFA R.[I]	Q9JI91	Alpha-actinin-2	0.19	0.001
[K].LVQYLRECEDVMDWI NDK.[E]	P16546	Spectrin alpha chain, non-erythrocytic 1	0.18	0.000

[R].KICALDDNVCMAG LTADAR.[I]	Q9Z2U0	Proteasome subunit alpha type-7	0.18	0.001
[R].QWVLTAAHCFDIPY PDVWR.[I]	P26262	Plasma kallikrein	0.18	0.000
[K].CGPMVLDALIK.[I]	Q9CQA3	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	0.18	0.000
[K].KQEGQYYCTASNR.[A]	Q08481	Platelet endothelial cell adhesion molecule	0.18	0.001
[R].VVQCSDLGLDKVPW DFPPDTLLDLQNNK.[I]	P28654	Decorin	0.18	0.000
[K].QNCFDDFQCAAELYIK .[E]	Q9QUR6	Prolyl endopeptidase	0.17	0.003
[R].QCLSELDACTFHK.[Y]	Q9DBD0	Inhibitor of carbonic anhydrase	0.17	0.023
[R].IEDDMDGGDWSFCD GR.[L]	Q61739	Integrin alpha-6	0.17	0.001
[R].GVQDIVVGEGTHFLIP WVQKPIIFDCR.[S]	P67778	Prohibitin	0.17	0.049
[R].HYCVILDPMPDGGK.[N]	Q9EQK5	Major vault protein	0.17	0.001
[K].HQHDRVCGDTVFLQ QENVK.[D]	Q61738	Integrin alpha-7	0.17	0.001
[R].VAHMEFCYQELCQLA AER.[R]	Q62261	Spectrin beta chain, non- erythrocytic 1	0.17	0.001
[R].VTGPPIFNVPANEVYL NFESSTHCLADR.[Y]	Q07113	Cation-independent mannose- 6-phosphate receptor	0.17	0.003
[K].LGCGECCGACTVMI SK.[Y]	Q00519	Xanthine dehydrogenase/oxidase	0.17	0.002
[R].QAQLQDAGIYECESK. [T]	P29533	Vascular cell adhesion protein 1	0.17	0.001
[R].TEHSPFHEHVLGALCS LVTDFPQGVR.[E]	Q99P31	Hsp70-binding protein 1	0.17	0.005
[R].DKETPSGFTLDDVIQT GVDNPGHPFIMTVGCVA GDEESYTVFK.[D]	P07310	Creatine kinase M-type	0.17	0.001
[K].DGQIFMSACQDQTIR. [L]	Q91VU6	DDB1- and CUL4-associated factor 11	0.17	0.001
[K].TVCTIAAHEGTLAAITF NSSGSK.[L]	Q8R3E3	WD repeat domain phosphoinositide-interacting protein 1	0.16	0.001
[K].AMLFMCSGSIHSLAD EQDIR.[K]	P03921	NADH-ubiquinone oxidoreductase chain 5	0.16	0.039
[K].NCTCGLAEELEER.[E]	Q8WTY4	Anamorsin	0.16	0.001
[K].LQDPFSVYRCHTIMN CTQTCPK.[G]	Q9CQA3	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	0.16	0.004
[R].YNFYLYGCTNQGYQL LR.[S]	P42703	Leukemia inhibitory factor receptor	0.16	0.001
[R].GASIAHCPNSNLSLSS GLLNVLEVVK.[H]	Q9R111	Guanine deaminase	0.16	0.003

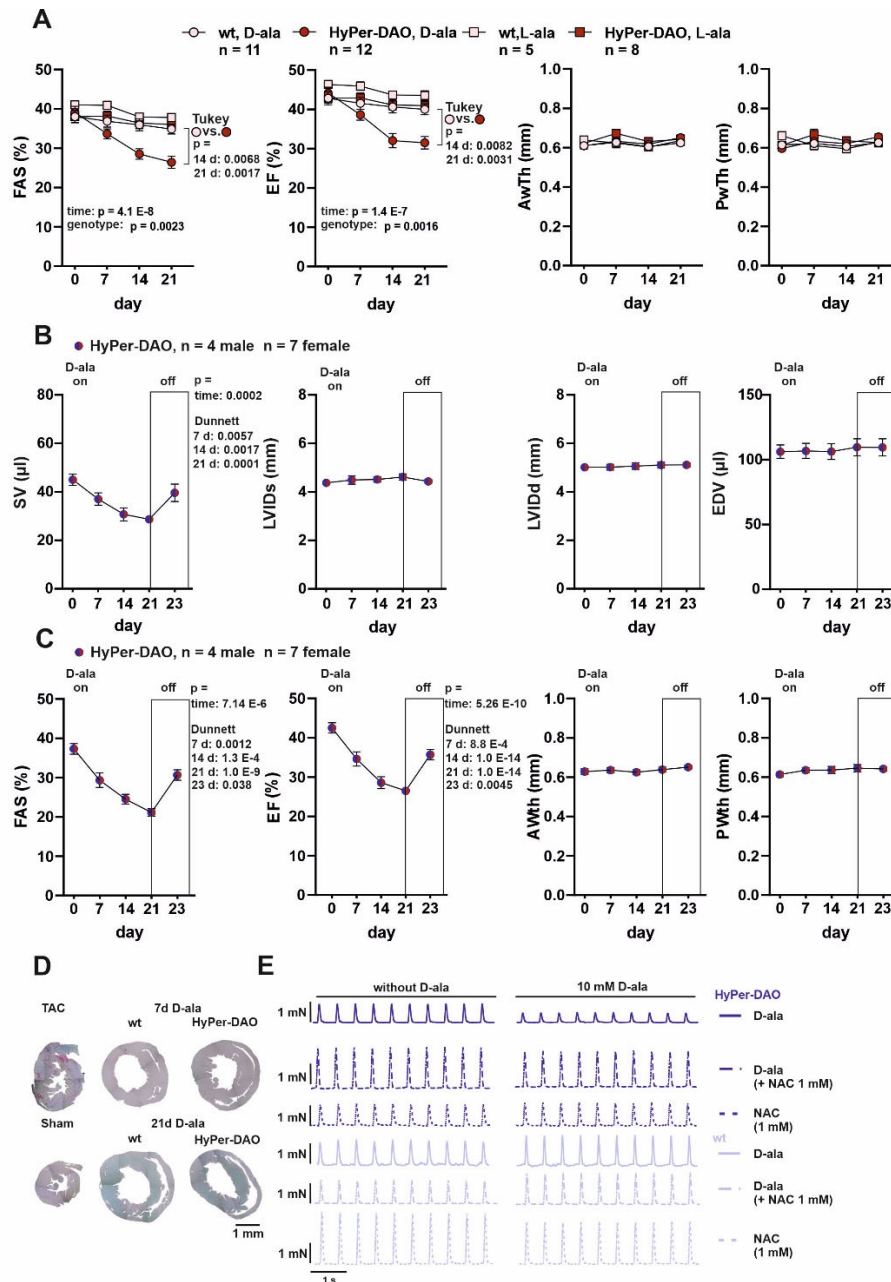
[R].LFGDKTCDLLASFK.[D]	Q60854	Serpin B6	0.16	0.007
[K].KPGVFIPSCDEDEGYR.[K]	Q9ER58	Testican-2	0.16	0.004
[K].FAPVCSICENPIIPR.[D]	Q71FD7	Filamin-binding LIM protein 1	0.16	0.004
[K].NILSHDPDVGTDTMEKEETHHACQEMELK.[V]	Q68FE8	Zinc finger protein 280D	0.16	0.001
[K].ASAFNSWFENAEEDLTDPVRCNSLEEIK.[A]	P16546	Spectrin alpha chain, non-erythrocytic 1	0.16	0.002
[K].AAFDIFVLGAEDGCISTKELGK.[V]	P19123	Troponin C, slow skeletal and cardiac muscles	0.16	0.003
[K].LCYGLNMDFVDPAQITMK.[V]	P07742	Ribonucleoside-diphosphate reductase large subunit	0.16	0.004
[K].ALGFPESLVIQAYFAC EK.[N]	P54726	UV excision repair protein RAD23 homolog A	0.16	0.003
[K].RLDLTYSFLGSQGVGQCYDSSPCER.[Q]	Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	0.16	0.004
[K].QCIVAHVPNPPYYVPLVELVPHPETAPATMDR.[T]	Q99KP3	Lambda-crystallin homolog	0.15	0.002
[R].VFIWTCDDASGNMWSPK.[L]	Q9D1M0	Protein SEC13 homolog	0.15	0.002
[K].GASSSSCSETYCGLYPESPEVK.[A]	Q9JHH6	Carboxypeptidase B2	0.15	0.002
[K].LGRPSLSSEVGVIICDISNPASLDEMAK.[Q]	Q8R127	Saccharopine dehydrogenase-like oxidoreductase	0.15	0.019
[R].VAGEPAADGTVACPCQAPTRPQALSTNLQLSR.[L]	Q1XH17	Tripartite motif-containing protein 72	0.15	0.002
[R].SSNLLGECSPAQR.[E]	Q9QY81	Nuclear pore membrane glycoprotein 210	0.15	0.018
[R].NLHMEQAPVDLDQPTSSFHVGTCTFANAESGTYFDGTGFAK.[A]	Q60675	Laminin subunit alpha-2	0.15	0.002
[R].QLEPGLQGILITCNMNER.[K]	Q99J36	THUMP domain-containing protein 1	0.15	0.015
[K].TCEWIHDSSLSASCK.[E]	Q61207	Prosaposin	0.15	0.006
[K].FFGEFTGFVDMCVQH IPSPK.[V]	O08810	116 kDa U5 small nuclear ribonucleoprotein component	0.15	0.003
[R].WCQSMQDPSASLLER.[Q]	Q9DC50	Peroxisomal carnitine O-octanoyltransferase	0.15	0.003
[K].GVCTEAGMYALR.[E]	P62196	26S proteasome regulatory subunit 8	0.14	0.022
[K].GSFPLDHFGECK.[S]	Q8K0C8	Cytochrome c oxidase assembly protein COX19	0.14	0.019
[R].AFWRELVECFQKISK.[D]	O35459	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	0.14	0.005
[R].LDSHSCLEVTAATLR.[R]	Q8VCT3	Aminopeptidase B	0.14	0.003

[K].TCNCETEDYGEK.[F]	Q8VEK3	Heterogeneous nuclear ribonucleoprotein U	0.14	0.011
[K].TYAACPQNWIGVENK.[C]	Q91V08	C-type lectin domain family 2 member D	0.14	0.012
[R].EAGTWMDDTCDISK.[Q]	Q61830	Macrophage mannose receptor 1	0.14	0.006
[K].DMFNVNVLALSICTR.[E]	Q3U0B3	Dehydrogenase/reductase SDR family member 11	0.14	0.016
[R].LPLTLGASYEGVAVNDCIESGR.[Q]	P51175	Protoporphyrinogen oxidase	0.14	0.010
[K].VCQCDSNGDCTDVD R.[I]	P15116	Cadherin-2	0.14	0.034
[R].TKCDEWSIIEGKIECE SAETTEDCIEK.[I]	Q92111	Serotransferrin	0.14	0.043
[R].ECSLQVAEDELVSTLK.[H]	P21126	Ubiquitin-like protein 4A	0.13	0.011
[K].FCASGPYGGEDCPQ WMVPITISTEDPNQAK.[L]	Q11011	Puromycin-sensitive aminopeptidase	0.13	0.020
[R].IMRPTDVPDQGLLCD LLWSDPDKDVQGWGEN DR.[G]	P62137	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	0.13	0.001
[R].DSSTCPGDYVLSVSEN SR.[V]	P47941	Crk-like protein	0.13	0.006
[K].DCSIYGEDTVTDEEGK.[F]	Q6GQT9	Nodal modulator 1	0.13	0.013
[R].GADCCVLVFDVTAPN TFK.[T]	P51150	Ras-related protein Rab-7a	0.13	0.007
[R].VGQILHPEECMYAVG QGALAVEVR.[A]	P22907	Porphobilinogen deaminase	0.13	0.013
[K].GEDFYCVTCHETK.[F]	P97447	Four and a half LIM domains protein 1	0.13	0.008
[R].FIAHGDAVMVAGG TDSCISPLSLAGFSR.[A]	Q9D404	3-oxoacyl-[acyl-carrier-protein] synthase, mitochondrial	0.13	0.041
[K].LVDGCYSFWQAGLLP LLHR.[A]	Q8K2I1	Protein farnesyltransferase subunit beta	0.13	0.009
[K].LQSLDLSSAVAQMTCTG TPPGADCSESECGPNCR.[T]	P02469	Laminin subunit beta-1	0.12	0.020
[K].DVDYVCISDNYWLKG.[N]	Q9D1A2	Cytosolic non-specific dipeptidase	0.12	0.022
[R].VCMVYDLYPTLTPLAV AYAR.[A]	Q9CWJ9	Bifunctional purine biosynthesis proteinATIC	0.12	0.008
[R].LSVSPLPTLTEDDELLC LFGDSPPHPAR.[V]	B2RXS4	Plexin-B2	0.12	0.011
[R].EGQDATAACEVNPLAC LSQTATCAPEIPAFSHGGF AYR.[D]	O70423	Membrane primary amine oxidase	0.12	0.032
[K].ECCHGDLLECADDRA ELAKYMCENQATISSK.[L]	P07724	Albumin	0.12	0.023

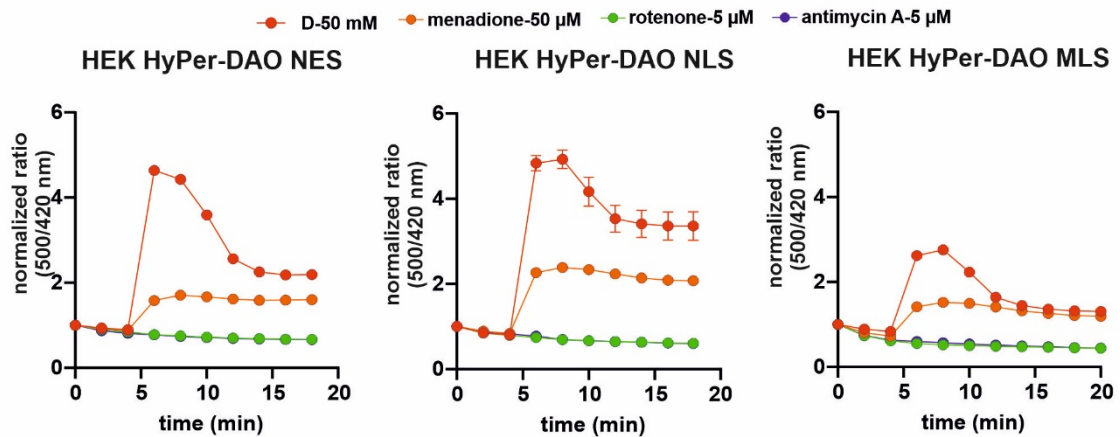
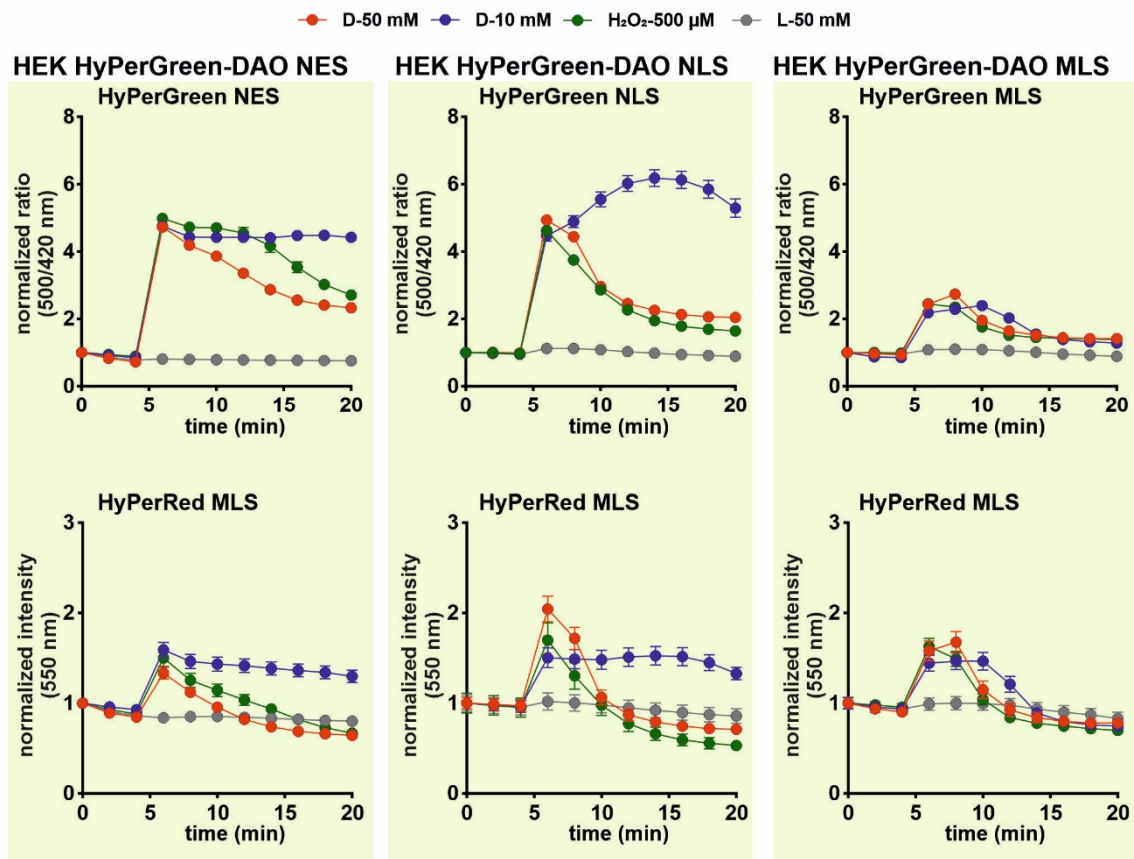
[R].FSFCFSPEPEAEQAAGP GPCER.[L]	Q64337	Sequestosome-1	0.12	0.024
[K].FPVFNMSYNPAENAV LLCTR.[A]	Q8CIE6	Coatomer subunit alpha	0.12	0.028
[K].QFLECAQNQSDVKLC EGFNEVLR.[Q]	Q9D1L0	Coiled-coil-helix-coiled-coil- helix domain-containing protein 2	0.11	0.005
[R].IIPLEQCQSYFDMK.[T]	Q9Z319	Atrial natriuretic peptide- converting enzyme	0.11	0.029
[K].DFAPGKPLKCVIKHPN GTQETILLNHTFNETQIE WFR.[A]	Q99KI0	Aconitate hydratase, mitochondrial	0.11	0.017
[K].ILVEGIGEVQEYVDVC DYAAGLSR.[M]	Q9DBF1	Alpha-aminoadipic semialdehyde dehydrogenase	0.11	0.033
[R].DDILCPDCGKDI.[-]	O70433	Four and a half LIM domains protein 2	0.10	0.007
[K].TLTSGGHAHEGKPY CNHPCYSAMFGPK.[G]	P63254	Cysteine-rich protein 1	0.10	0.030
[K].LCLSEIHK.[M]	P15208	Insulin receptor	0.10	0.031
[R].WLLLCNPGLAEIIVER. [I]	Q8CI94	Glycogen phosphorylase, brain form	0.09	0.040
[K].FVTAVATGGPDNQV HFEGYQVSNQCMALVR.[D]	P60670	Nuclear protein localization protein 4 homolog	0.09	0.048
[K].GCEECFCSGVSNR.[C]	Q60675	Laminin subunit alpha-2	0.09	0.000
[-].MEESEYESVLCVKPEVH VYR.[I]	Q9D1J1	Adaptin ear-binding coat- associated protein 2	0.09	0.033
[R].NSDVMDCVILDDGGF LLMANHDDYTNIQGR.[F]	O08532	Voltage-dependent calcium channel subunit alpha-2/delta- 1	0.09	0.040
[K].SHCAEPFTEYWTCLDY SNMQLFR.[H]	Q9DCJ5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	0.08	0.009
[R].KNGLMDHEDFRACLI SMGYDLGEAEFAR.[I]	Q9JI91	Alpha-actinin-2	0.08	0.040
[R].SQSVRPGADVTFICTA K.[S]	Q05793	Basement membrane-specific heparan sulfate proteoglycan core protein	0.07	0.039
[K].SEPDKADPFSFEGPEI VDCDGCTIDWK.[K]	Q78ZA7	Nucleosome assembly protein 1-like 4	0.07	0.040
[R].VVYLQYPSLAPHHQC SQLFLYDWYTK.[V]	Q8R146	Acylamino-acid-releasing enzyme	0.07	0.008
[K].TCGFDFSGALEDISKIP EQSVLLHACAHNPTGVD PRPEQWK.[E]	P05202	Aspartate aminotransferase, mitochondrial	0.06	0.009
[K].LFQEEFPGIPYPPDAA VECHRGECQSEGVLFQGG NR.[K]	Q91X72	Hemopexin	0.06	0.003

Supplementary Table 2: List of cysteine containing peptides that were identified in the redox proteomic screen to show a decreased total reversible oxidation demonstrated by a fold change of $\leq$

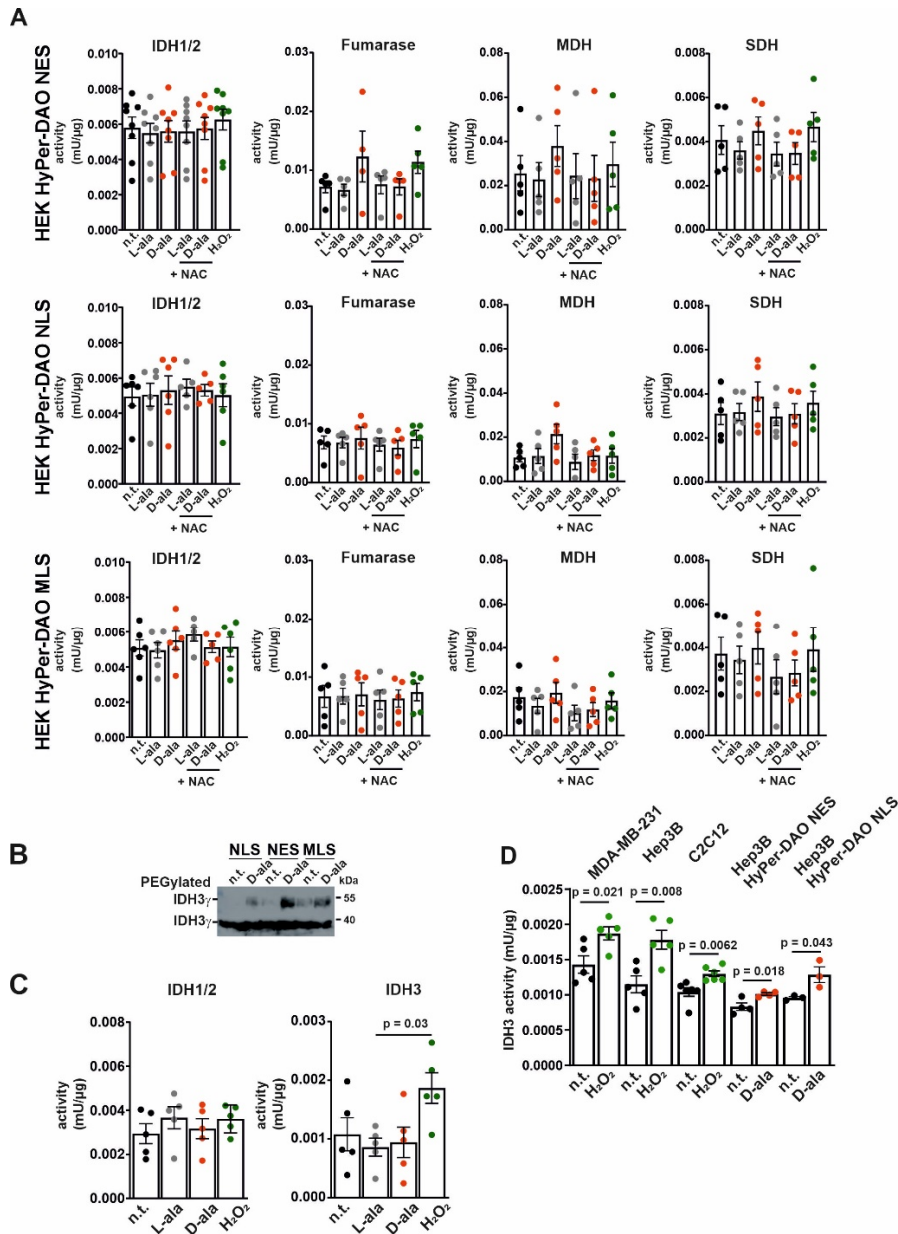
0.5 and a p-value of ≤ 0.05 in the left ventricles of HyPer-DAO mice compared to wild type mice after 7 days of D-ala treatment. P values were calculated by two-tailed unpaired t-test.



Supplementary Figure 1: Echocardiographic analysis and cardiac fibrosis in HyPer-DAO mice, related to Figure 2 (A, B and C) Echocardiographic analysis of fractional area shortening (FAS), ejection fraction (EF), anterior wall thickness (AwTh) and posterior wall thickness (PwTh), stroke volume (SV), left ventricular inner diameter in systole (LVIDs), left ventricular inner diameter in diastole (LVIDd), and enddiastolic volume (EDV) in female (in A) and a mixed group of female and male (in B and C) HyPer-DAO or wild type (wt) mice after treatment with D-ala or L-ala in the drinking water as indicated. **(D)** Representative figures of Sirius red/Fast green stained cardiac slices of HyPer-DAO mice after 7 or 21 days of D-ala treatment and mice after TAC or sham surgery. For each mouse the staining was performed once. **(E)** Original tracings of force development measured in heart slices from HyPer-DAO and wt mice after treatment 10 mM D-ala +/- 1 mM NAC. mean $\pm$ SEM, one-way ANOVA (B and C) or two-way ANOVA (A). Source data are provided as a Source Data file.

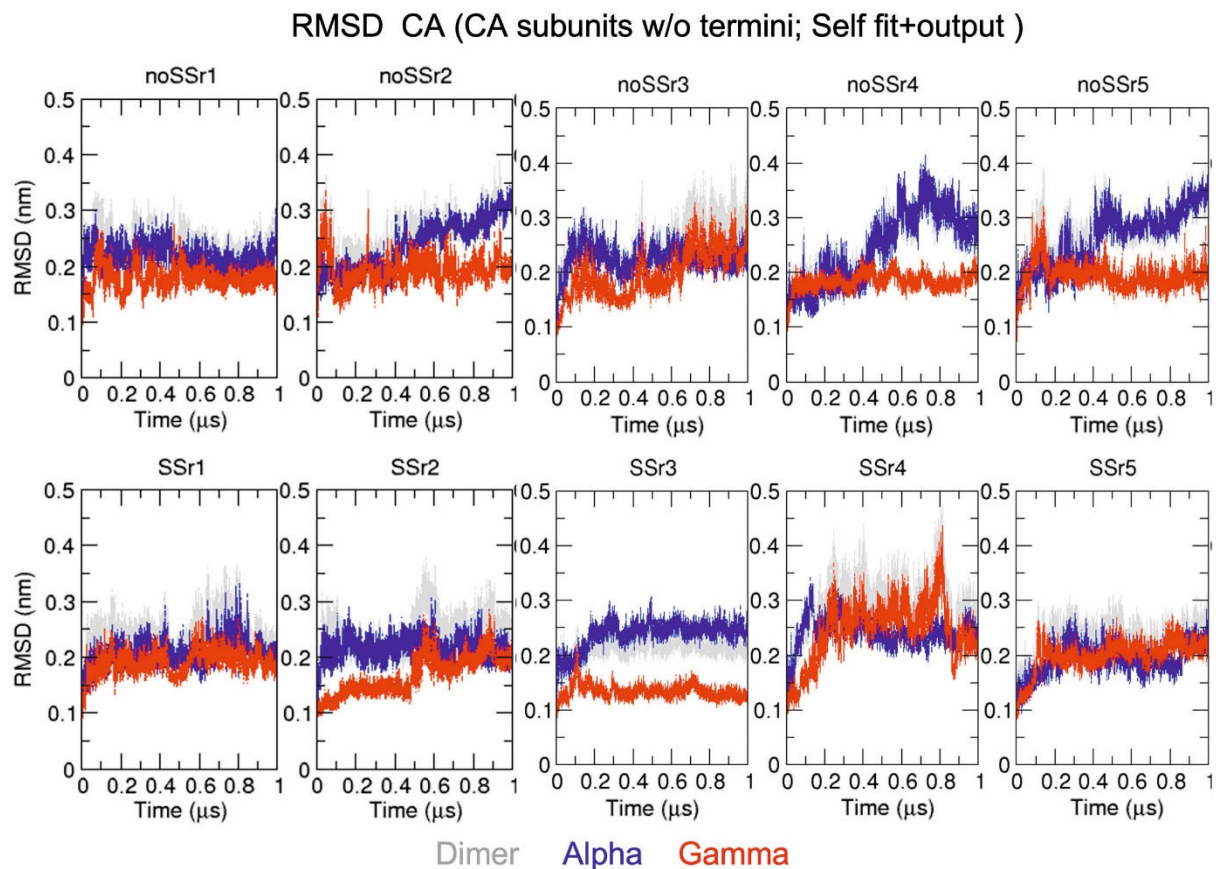
A**B****Supplementary Figure 2: Intracellular distribution of H₂O₂ in HEK cells, related to Figure 4A-C**

(A) HyPer-Green fluorescence responses were recorded after treating HyPer-DAO-NLS cells with D-ala (n = 42 independent cells), menadione (n = 44 independent cells), rotenone (n = 42 independent cells) or antimycin A (n = 40 independent cells), which were added at time point 4 min. **(B)** HEK cells overexpressing HyPer-DAO fusion protein in the cytoplasm (HyPer-DAO-NES), the nucleus (HyPer-DAO-NLS) and the mitochondrial matrix (HyPer-DAO-MLS) were transiently transfected to overexpress the H₂O₂ sensor HyPerRed localized to the mitochondrial matrix (HPerRed-MLS). The HyPer Green and HyPer Red fluorescence responses were recorded after stimulation with D-ala, L-ala or H₂O₂, which were added at time point 4 min. Ratios are normalized to the HyPer ratio prior treatment, n = 25 independent cells per condition were analyzed. Data are presented as mean values ± SEM. Source data are provided as a Source Data file.



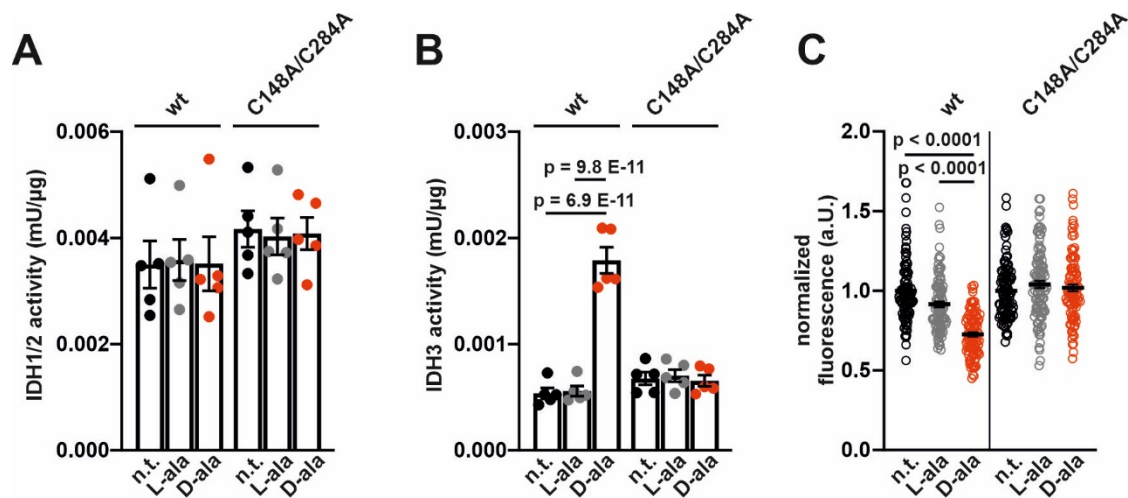
Supplementary Figure 3: TCA cycle enzyme activities in HyPer-DAO overexpressing HEK cells, related to Figure 4 D-G

(A) IDH1/2, fumarase, malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) activities in cell extracts obtained from HEK HyPer-DAO-NES, HEK HyPer-DAO-NLS and HEK HyPer-DAO-MLS cells either non-treated (n.t.) or after treatment with 50 mM D-ala, 50 mM L-ala $\pm$ 8 mM NAC or 500 μ M H₂O₂ for 20 min. IDH1/2 activity: n = 8 independent experiments for HyPer-DAO NES, n = 6 for HyPer-DAO NLS and MLS except for n = 5 for D-ala and L-ala + NAC, n = 5 independent experiments for all other conditions. **(B)** Representative immunoblot for IDH3 γ oxidation analyzed by PEG switch assay in HEK HyPer-DAO-NLS, HEK HyPer-DAO-NES and HyPer-DAO-MLS cells n.t. and after treatment with 50 mM D-ala for 20 min. The experiment has been performed three times. **(C)** IDH1/2 and IDH3 activity in cell extracts obtained from HEK wt cells either non-treated (n.t.) or after treatment with 50 mM D-ala, 50 mM L-ala or 500 μ M H₂O₂ μ M for 20 min, n = 5 independent experiments. **(D)** IDH3 activity in cell extracts obtained from MDA-MB-231, Hep3B, C2C12, Hep3B HyPer-DAO-NES and Hep3B HyPer-DAO-NLS cells either non-treated (n.t.) or 20 min treatment with 500 μ M H₂O₂ for MDA-MB-231, 250 μ M H₂O₂ for Hep3B, 5 μ M H₂O₂ for C2C12 and 5 mM D-ala for Hep3B HyPer-DAO-NES and -NLS, respectively. 5, 5, 6, 4 and 3 independent experiments for MDA-MB-231, Hep3B, C2C12, Hep3B HyPer-DAO-NES and HyPer-DAO-NLS cells, respectively. Data are presented as mean values $\pm$ SEM, one-way ANOVA (A and C) or two tailed unpaired t-test (D). Source data are provided as a Source Data file.



Supplementary Figure 4: Root mean square deviation (RMSD) computed over the C α -atoms for all MD simulations, related to Figure 6

Curves show results separately for the whole dimer (without disordered termini, grey), the α subunit (blue), and the γ subunit (red).



Supplementary Figure 5: Point mutation of IDH3γ Cys148 and Cys284 prevents H₂O₂ induced IDH3 activity and impaired ATP production in the mitochondria, related to Figure 7.

IDH1/2 (**A**) and IDH3 (**B**) activity in cell extracts obtained from HEK HyPer-DAO-NLS wild type (wt) or C148A/C284A cells non-treated (n.t.) and after treatment with 50 mM D-ala or L-ala for 20 min, $n = 5$ independent experiments per condition. (**C**) Mitochondrial ATP levels determined with the fluorescence sensor ATP-red in HEK HyPer-DAO-NLS wt and C148A/284A cells n.t. and after treatment with 50 mM D-ala or L-ala for 20 min. $n = 100$ cells per condition were analyzed. Data are presented as mean values $\pm$ SEM, one-way ANOVA (B and C). Source data are provided as a Source Data file.

Non-cropped blots

Figure 1B

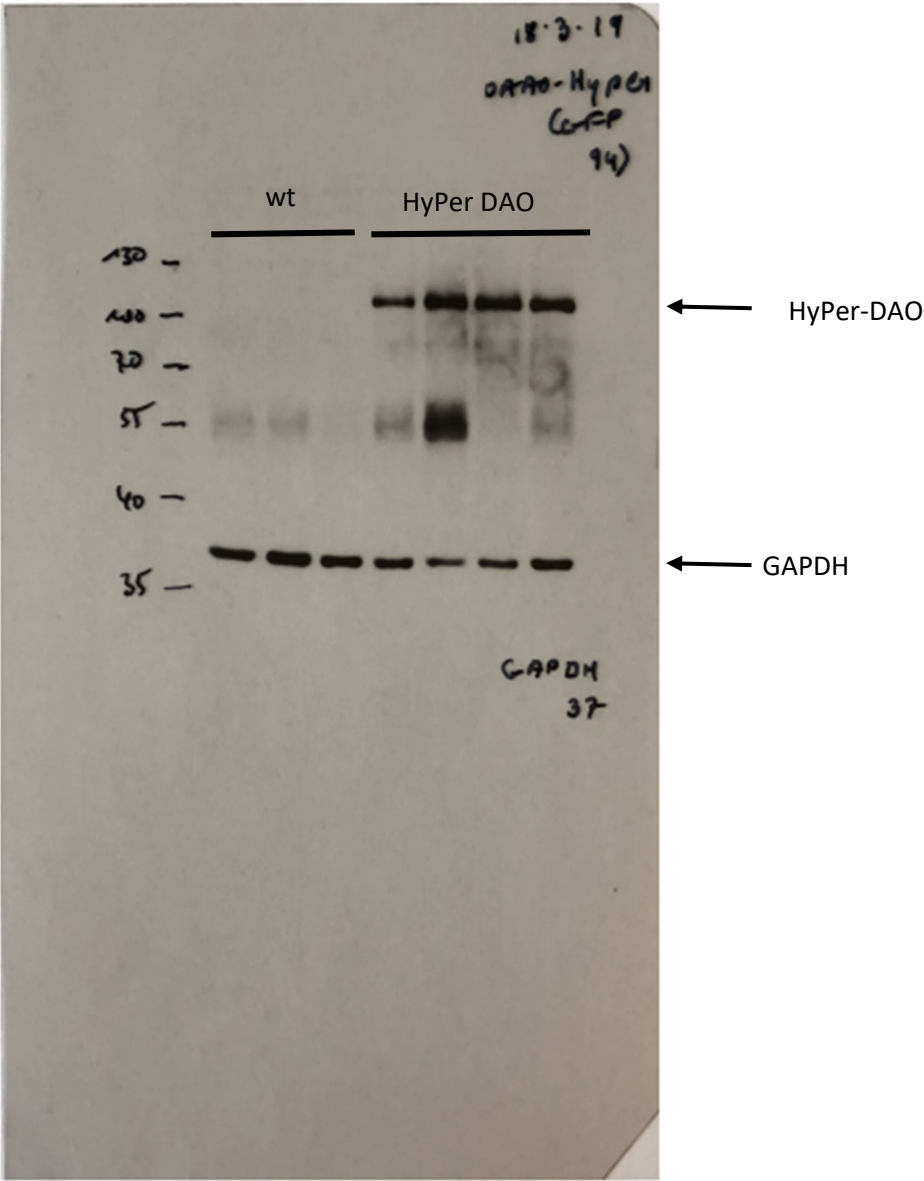


Figure 1C

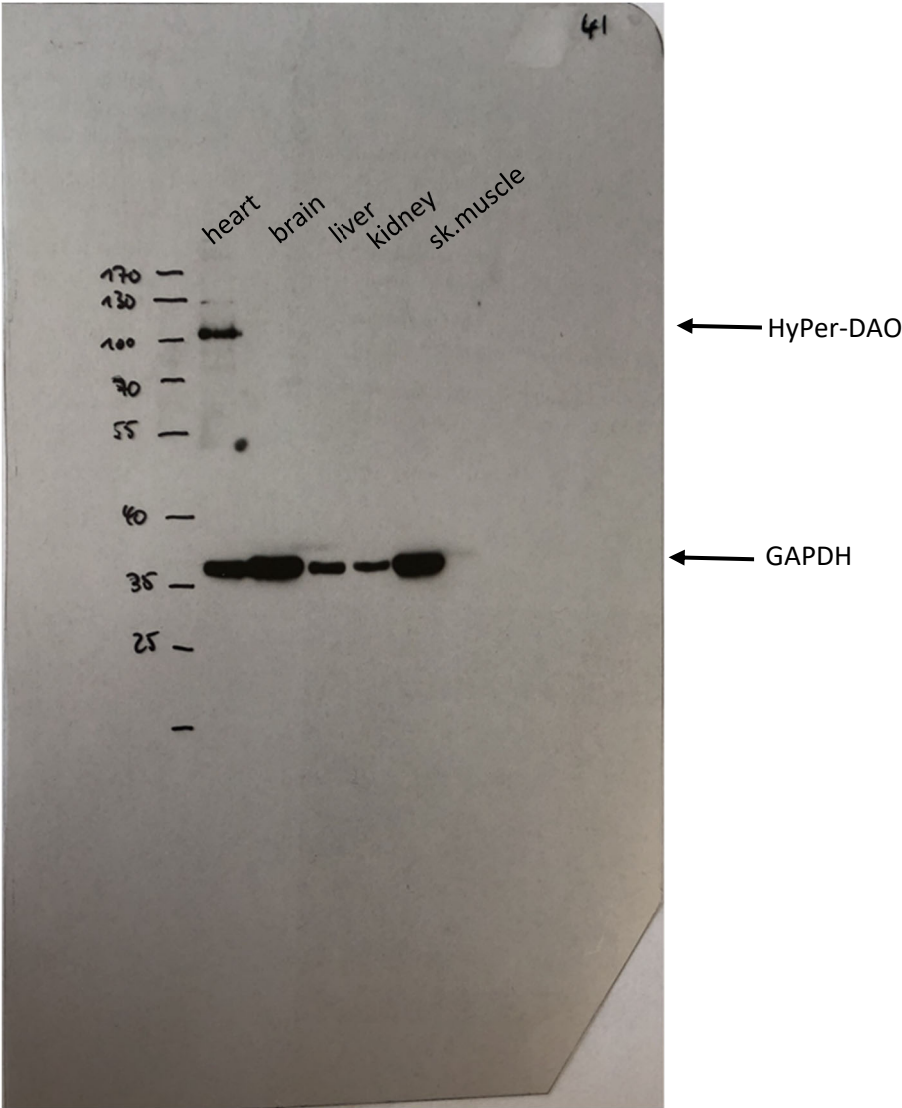


Figure 3G

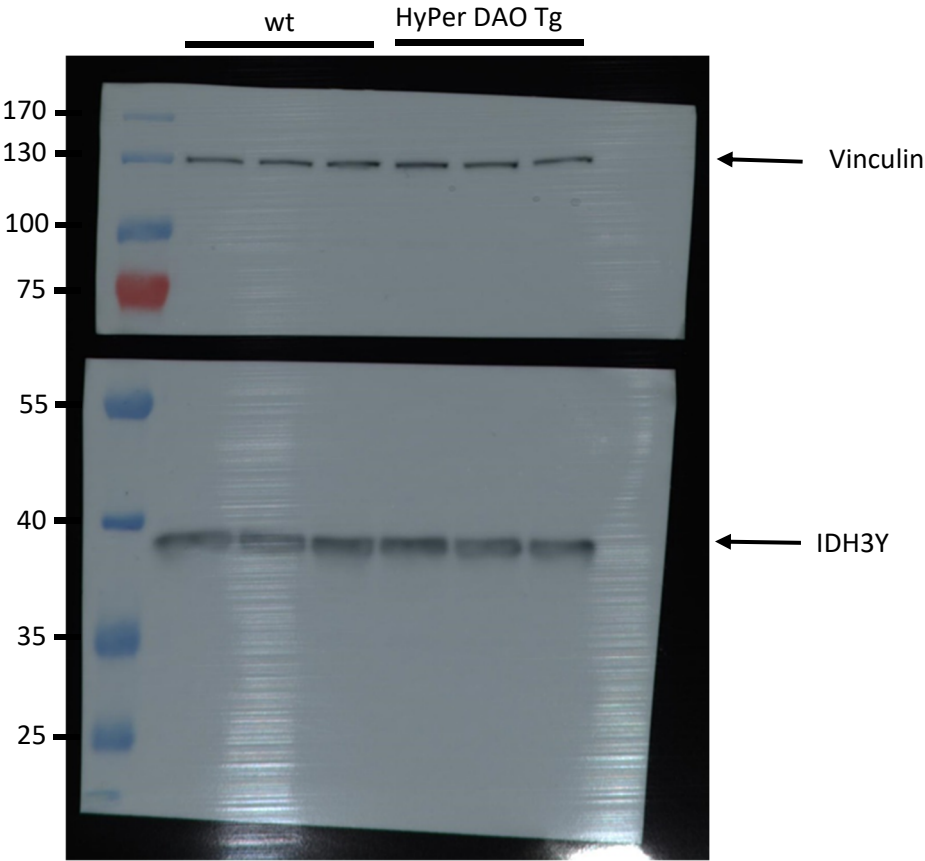


Figure 3K

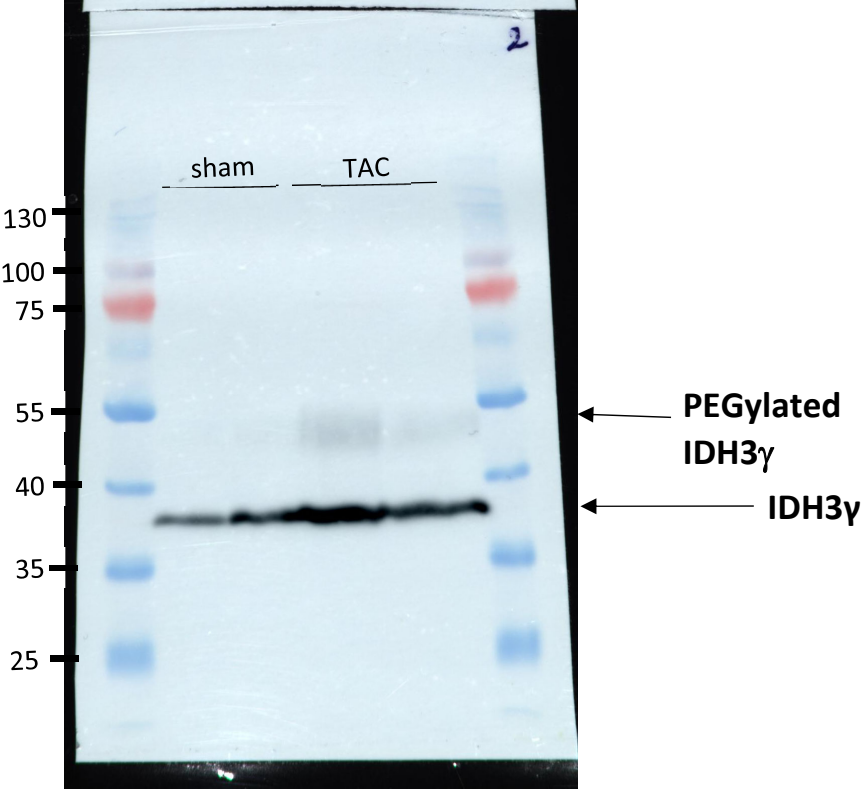


Figure 4E

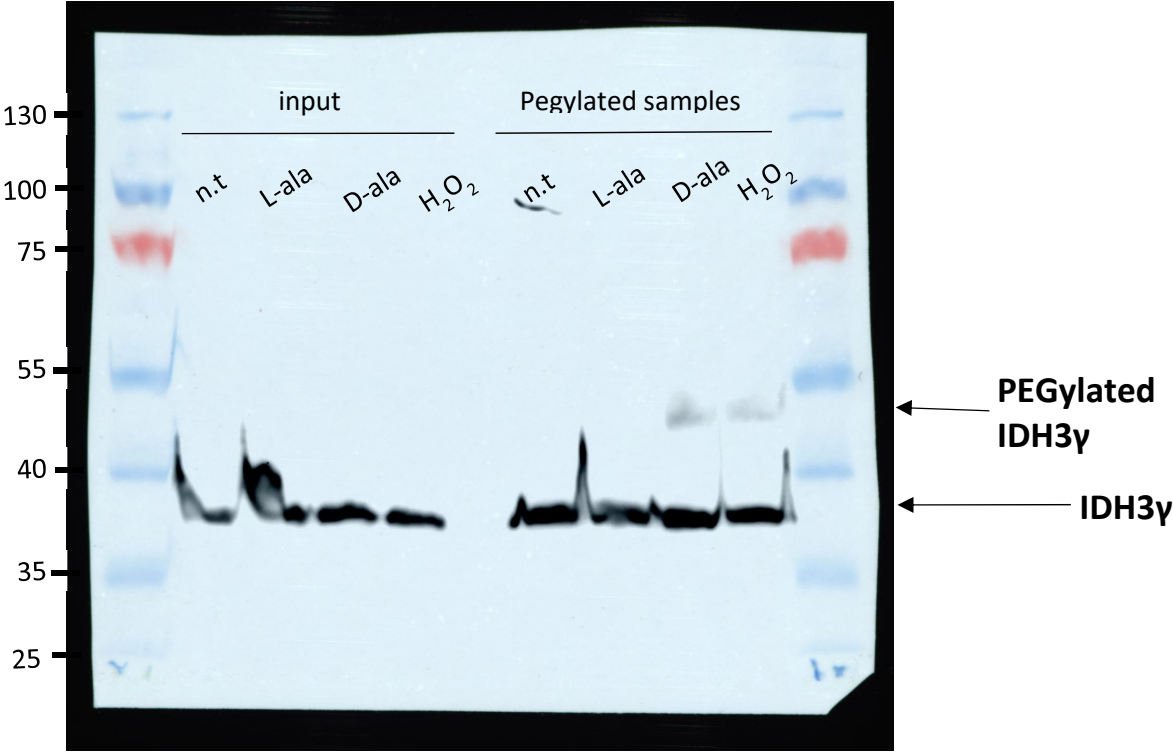


Figure 4G

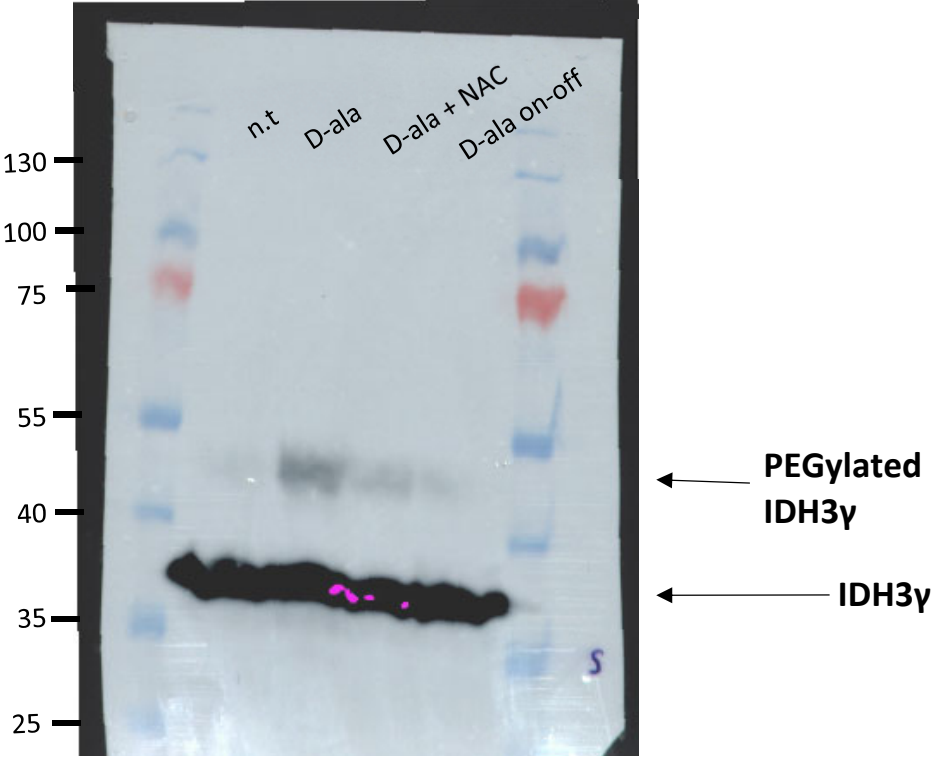
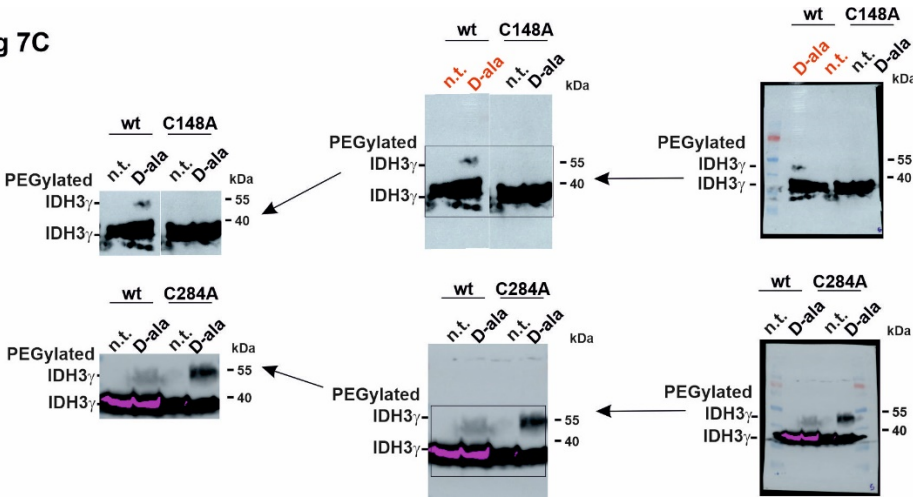
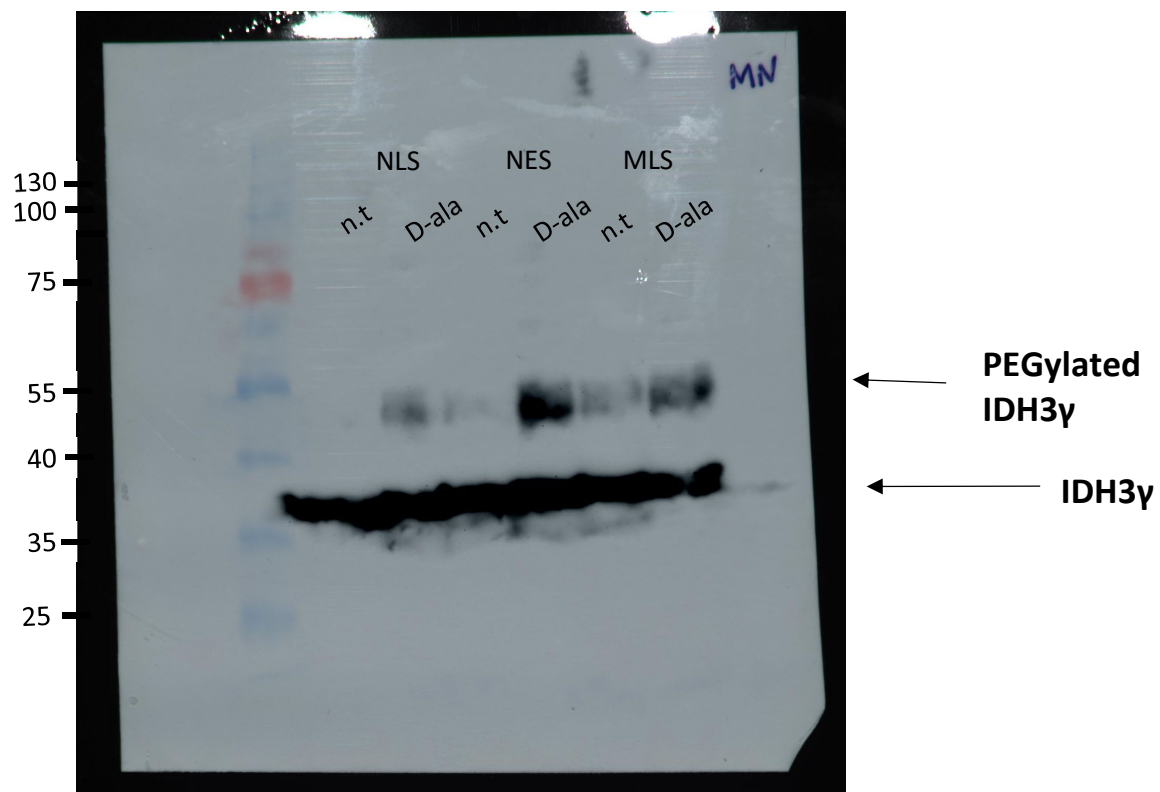


Figure 7C

Fig 7C



Supplementary Figure 3B



IDH3 γ functions as a redox switch regulating mitochondrial energy metabolism and contractility in the heart



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In their study Nanadikar and colleagues apply a genetically encoded tool for H₂O₂ production recently developed by the Belousov group (also part of this study) to study H₂O₂ linked redox metabolism in heart. By overproducing H₂O₂ in mice heart they identify specific cysteine redox switch in IDH3 that allows cells to directly sense cellular redox state. Presented work is innovative as it is one of the first examples when both a genetically encoded sensor and tool were expressed as one construct. This allows authors to both generate H₂O₂ and at the same time to directly monitor live changes in H₂O₂ levels. Overall, this study represents an important advancement in our understanding how cells sense changes in redox environment and how they respond to them via a Cys-based redox switch. This study also illustrates usefulness of the Oximouse project recently introduced by the Chouchani group. Although this study presents an advancement towards our understanding of redox regulation in live cells, the presented work needs to be improved to be considered for a publication. Below are my specific comments:

- 1). It looks like the H₂O₂ levels produced by HyPerDAO is rather modest both based on a very high K_M for D-Ala as well as on physiological effects presented. This mild H₂O₂ production perhaps represents an advantage compared to other methods of H₂O₂ generation in cellular or organismal systems. It is important to see where H₂O₂ produced by HyPerDAO lies compared to other methods of H₂O₂ production (i.e. addition of menadione or other redox cyclers that via NQO1 mediate H₂O₂ production; H₂O₂ levels due to inhibited ETC, bolus of H₂O₂ directly added to cells etc).
- 2). How the HyPerDAO construct was developed in first place? Are there any linker regions between HyPer and DAO proteins? Was HyPerDAO protein previously expressed in E.coli and purified to show that the enzymatic activity of HyPerDAO is very similar to DAO alone?
- 3). The schematic of the DAO reaction in Fig. 1A is not correct: D-ala conversion leads to imino acid formation and FAD reduction (FADH₂), then FADH₂ reacts with O₂ to produce H₂O₂. What happens next to the imino acid product? Will imino acid react with H₂O with a concomitant NH₃ production in cells? Were ammonia levels evaluated in DAO expressing cells/organs?
- 4). Not clear the reasoning why nuclear targeted HyPerDAO construct was used in mice. It seems logical to use a cyto construct (it looks like mitochondria are very good at detoxifying H₂O₂ (especially low levels produced by DAO?) and not usage of a mito construct in mice was well justified).
- 5) In Fig. 1 B-C which antibody was used to detect HyPerDAO? Loading GAPDH control was not equal in samples presented.
- 6). Fig. 2: Blue colors are very confusing, especially half blue bars with a gradient, therefore representations need to be improved to help with the clarity.

7). Why only IDH3 was explored as an enzyme under redox control? It seems like SHMT1 enzyme is also worth exploring given its role in NAD(P)H and 1C metabolism.

8). It is very difficult to gauge enzymatic activities results as in Methods only a kit from Sigma was mentioned. How exactly these assays were performed, which substrates, under Vmax conditions etc? Were cell homogenates desalted to remove endogenous substrates? This study will greatly benefit from tracer studies (at least in a cell line model presented). This will allow to directly evaluate glycolysis/TCA cycle activities and fluxes. Also, a summary schematic figure is needed to guide the reader on the state of redox Cys and downstream effects linked with the IDH3 activity. Obviously even mild H₂O₂ production by DAO leads to downstream effects in cells (including decreased ATP levels) and it's not expected that mutated redox Cys will rescue ATP production to a full extent. At the same time a connection between an increased TCA cycle activity (via IDH3) and GSH biosynthesis is not very convincing. At least in HEK293T cells NADH/NAD⁺ and NADPH/NADP⁺ levels need to be evaluated; what happens if cells are treated with buthionine sulfoximine (to inhibit GSH biosynthesis).

9). It is certainly will be more convincing to see the effect observed not only in HEK293T cells. Have authors tried another cell line? For example, C2C12 myoblasts? It will be very interesting if in a panel of cell lines IDH3 activity will be increased upon DAO-mediated H₂O₂ production.

10). As mentioned above a schematic figure is needed to guide the reader through results: the state of redox cysteine switch vs TCA activity and other downstream effects;

Reviewer #2 (Remarks to the Author):

Nandikar, et. al, propose that IDH3 γ is a redox switch responsible for losses of cardiac function under significant ROS-induced stress. The research design is of notable robustness, and the authors present a fairly convincing justification for this mechanism as a possible source of myocardial "stunning" after infarction. While the overall manuscript is strong, some parts of the results and discussion could be refined to make the message clearer.

p.6. lines 187-189. The authors state cytosolic H₂O₂ diffuses into the mitochondria, but not vice-versa. This by definition is not diffusion. Should it be termed "facilitated transport by an unknown mechanism?"

p.9 line 301. The oxygen consumption rate (OCR) is the essential element indicative of increased TCA cycle turnover. But that data is not presented (see (2) below). Instead, the authors convolve increased metabolite pool sizes with increased activity, which is plausible, but does not establish a true link. See reference:" Ragavan, Mukundan, et al. "A comprehensive analysis of myocardial substrate preference emphasizes the need for a synchronized fluxomic/metabolomic research design." *American Journal of Physiology-Heart and Circulatory Physiology* 312.6 (2017): H1215-H1223."

With the interplay between energy metabolism at IDH3 and glutathione availability, it is quite possible that changes in pool sizes are related to anaplerotic flux into the TCA cycle. "Brunengraber, Henri, and Charles R. Roe. "Anaplerotic molecules: current and future." *Journal of inherited metabolic disease* 29.2 (2006): 327-331."

A secondary question is that of IDH3 reversibility. While IDH1 and IDH2 are known to be reversible, IDH3 is not generally acknowledged to have the same properties. Did the authors find any evidence of reversibility in this context? That would add to the originality of this manuscript.

Finally, the connection simply was not clear to me between increased IDH3 function and loss of ATP production (lines 290-292). If IDH is rate limiting for TCA cycle flux as stated in the document, an increase should mean more NADH, and more ATP production, not less. Maybe I am missing the logic, but I believe the discussion could be made clearer. Lines 380-384 draw a connection between increased TCA cycle flux and glutamine cataplerosis, but simultaneously invokes glutamine anaplerosis. Please clarify.

Data to be presented:

- 1) The weights of the control versus HyPer hearts would be nice to include along with the histology of Figure 1.
- 2) The raw SeaHorse data should be included in the main figures, with a full description of the data. The actual oxygen consumption rates need to be included in a clear manner. While Figure 7E ostensibly refers to the OCR, the caption for this figure is abbreviated. Is the fluorescence on y-axis associated with OCR?

Reviewer #3 (Remarks to the Author):

This is an intriguing work identifying isocitrate dehydrogenase 3 (IDH3) as a metabolic enzyme that can act as a redox switch through reversible oxidation, alongside important insights on the metabolic consequences of elevated H₂O₂ in the heart. IDH3 oxidation was identified through the use of an aMHC-HyPer-DAO mouse model, which is an innovative in vivo system that affords the cardiomyocyte overexpression model of H₂O₂ as well as an H₂O₂ reporter in the nucleus. The overall findings that IDH3 can be reversibly redox modified and the metabolic consequences of this modification are significant and the approach was innovative. However, the physiological significance of these findings are less clear. Mitochondria are a major source of ROS/H₂O₂, whereas the nucleus is not often described as such. Accordingly, it is unclear why the authors chose to model in vivo elevated H₂O₂ signaling in the nuclear compartment when they had access to the mitochondrial construct as evidenced by use in vitro studies. Moreover, while the aMHC-HyPer-DAO mice do show that IDH3 modification can occur in the heart, does this happen physiologically, or in the context of cardiac disease? While the authors do note in the discussion that oxidation may play a particularly important role in acute myocardial

infarction/myocardial stunning, ROS signaling may also be important in other disease modalities such as cardiac pressure overload. Showing IDH3 redox modification in a physiologically relevant cardiac disease model would strengthen the study and expand the disease relevance of this work.

Concerns:

1. Using the aMHC-HyPer-DAO-NLS mice to generate a gradient of H₂O₂ is intriguing, however, the rationale underlying the choice to use the NLS HyPer-DAO to make the mouse model is not clear. Mitochondria are a major source of ROS in cardiomyocytes and in cardiac disease. The explanation for this choice, especially in light of the availability of the mitochondrially targeted construct to the authors, needs to be further expanded upon. Is the concentration of H₂O₂ produced in vivo with the NLS-targeted construct indeed higher in the nucleus than in the mitochondria? Also how far does H₂O₂ travel within the cell? Does this system reflect the levels of H₂O₂ that would be produced in a cardiac disease setting? Please include in the discussion/rationale for NLS HyPer-DAO choice.
2. In Figure 1E, the authors show serial H&E stained sections of 2 hearts HyPer-DAO vs WT to show that the hearts are unaffected with HyPer-DAO expression at baseline. These images are not informative—please include echocardiography of baseline cardiac function and morphometry to accompany these images in the supplemental data.
3. In Figure 2C, the authors show that over the course of 21 days, activation of the cardiomyocyte HyPer-DAO construct can result in reduced fractional shortening and ejection fraction) without adverse cardiac wall remodeling. Moreover, the authors show in 2D that this contractile dysfunction can be reversible. These findings are striking, and should be accompanied by the echocardiography measurements of left ventricular interior dimensions at diastole and systole, as well as the stroke volume and end diastolic volumes.
4. Are the gene expression changes observed with HyPer-DAO activation (Fig. 2A) also reversible/inducible with the removal of D-alanine (in line with the changes in cardiac function shown in the dosing regimen used in 2D)?
5. Are the mitochondrial proteins identified by mass spec to be reversibly modified in Fig3B also modified with HyPer-DAO constructs targeted to other cellular compartments?
6. The aMHC-HyPer-DAO-NLS animal is a really great tool to highlight what can be redox modified in the heart, but there is a disconnect between what is identified with this nuclear H₂O₂ source model, versus what might occur physiologically in the heart (where the mitochondria might be the major source of ROS). This study would be very much strengthened if the authors would be able to show that some of the redox modified targets identified in the HyPer-DAO mice to be similarly modified in cardiac disease settings (would be particularly of interest to know if IDH3 Cys148 plays a role). While the authors note in the discussion that this may play a role in myocardial stunning, it would also be worthwhile to look in the context of TAC, where ROS may also play a role (also because the authors already have that surgical model in use for this study).

Reviewer #4 (Remarks to the Author):

With the HyPer-DAO mouse model and redox proteomics, Nanadikar et al. identified IDH3 γ that can serve as a redox switch, revealing that the Cys148 and Cys284 are the two critical sites in regulating IDH3 activity. The experiment design and the results are generally sound. The finding on the redox regulatory role of IDH3 γ is interesting. The authors should address the following questions before it becomes acceptable.

1. The in vivo H₂O₂ abundance in mouse heart with and without D-ala treatment was not determined. How much was the basal H₂O₂ in mouse heart? and how much H₂O₂ was produced in response to D-ala?
2. Do ATP production and other energy-related metabolites differ between HyPer-DAO and WT with the D-ala treatment in mice?
3. Fig 4EG. The PEG switch experiment shows that the oxidative level of IDH3 is low. Can this minor redox level change result in sufficient regulation on total enzyme activity?
4. Fig 7C. Why was PEGylated IDH3 much higher in C-284A mutant? Should PEGylation level be correlated to its enzymatic activity? And why? It looks like the lane image of the C284A treating with D-ala was cropped from elsewhere. Please confirm this is the original image.
5. Since this is a cys-site specific analysis, the redox proteomics data should be analyzed and illustrated in a site-centric manner. The current data (e.g. fig3b and SI tables) analyses were all performed on protein level. An average value of all cys sites surely dilutes the importance of those proteins in which only a part of the cys sites show changes. I would strongly suggest the authors to re-analyze their proteomic data in a site-centric manner.
6. The MS2 and fold-changes of the important cys sites, such as cys 148 and 284 of IDH3 γ , should be added in the manuscript. Their identification with high-confidence is the foundation of follow-up studies.
7. The STN is a very weird term in describing fold-change in proteomics. Signal-to-noise has an exact definition in mass spectrometry, but rarely represents fold-change. The authors should revise this word, and related figures and tables.

8. The overall quality of the redox proteomics should be assessed. In this reviewer's opinion, there are several flaws.

a. Why was the enrichment done on proteins, not on peptides? A peptide-based enrichment would deepen the analysis coverage.

b. How much was the enrichment efficiency (cys-containing peptides vs. total peptides)? Based on several example raw data the authors provided, this reviewer found that the enrichment efficiency was bad. Roughly, only less than 10% of identified peptides contain cys. This is a sign that the experiments were not performed properly. Moreover, in the several data files I searched, the cys 148 and 284 of IDH3 γ were not identified.

c. Total numbers of cys sites and changed cys sites should be added. Their quantitative results should be included.

d. Blocking free cys with MSTP was done in a RIPA buffer, which was a mild buffer thus couldn't denature all proteins' structures. Therefore, some free cys sites might not be blocked in the first step, affecting the subsequent enrichment. The authors should evaluate the completeness of the reaction between free cys and MSTP.

9. Line 107. How does Nrf2 itself change?

10. Line 169 and fig 3e. The RNA levels of IDH3 were increased, though the protein level was not. Is the difference statistically significant?

11. Line 171 and fig 3G. Does IDH3 activity really go back to normal after removing D-ala for 2days? Is the difference between the two bars on the right statistically significant?

12. Fig 7B. the IDH3 basal activity of C236A is lower than wt IDH3 and other mutants. Are there any explanations?

Reviewer comments - Point to Point response

We would like to thank all four reviewers for their input. In response to their comments we have performed the following new experiments. A more detailed point to point response can be seen below:

1. Echocardiography analysis of cardiac output, stroke volume, left ventricular inner diameter in systole, left ventricular inner diameter in diastole, enddiastolic volume at basal level and after D-ala or L-ala treatment (Fig. 1E, Suppl. Fig. 1B).
2. Quantification of Nrf2 target RNAs in left ventricular samples obtained from wt and HyPer-DAO mice after 7 days of treatment with D-ala plus 2 additional treatment free days (Fig. 2A).
3. Analysis of heart weight to body weight of wt and HyPer-DAO mice before and after D-ala treatment (Fig. 2E).
4. Analysis of ATP/ADP levels in left ventricular samples of wt and HyPer-DAO mice after a 7 days long treatment with D-ala (Fig. 2F).
5. Repetition of the redox proteomics screen with left ventricular samples from wt and HyPer-DAO mice after 7 days of treatment with D-ala as well as a peptide centric analysis (Fig. 3B and C, Suppl. Table 1 and 2)
6. IDH3 activity and IDH3 redox modification analyzed in heart samples obtained from mice after transverse aortic constriction or sham treatment (Fig. 3J and K).
7. IDH3 redox modification analyzed in HEK HyPer-DAO NLS, HyPer-DAO MLS and HyPer-DAO NES cells by Peg switch assays (Suppl. Fig. 3B).
8. IDH3 activity in a panel of wt cells after H₂O₂ bolus treatment as well as in HyPer-DAO NLS or HyPer-DAO NES stably transfected Hep3B cells after D-ala treatment (Suppl. Fig. 3D).
9. Targeted stable isotope tracer studies using uniformly labeled ¹³C glucose (Fig. 7F and G).
10. NADH/NAD⁺ and NADPH/NADP⁺ levels in HEK HyPer-DAO NLS cells after L- or D-ala treatment (Fig. 7H).
11. ATP levels after treatment of HEK HyPer-DAO cells with buthionine sulfoximine (see rebuttal letter, reviewer #1, comment #8.3).
12. HyPer fluorescence measurements in HEK HyPer-DAO NLS, MLS and NES cells after treatment with menandione, rotenone or antimycin A (see rebuttal letter, reviewer #1, comment #1)
13. Nrf2 Western blot with protein extracts of wt and HyPer-DAO mice after a 7 days long treatment with D-ala (see rebuttal letter, reviewer #4, comment #9).
14. IDH1/2 activities, IDH3 activity and ATP levels in HEK HyPer-DAO cells harboring Cys148A/Cys284A double point mutations (Suppl Fig. 5A-C).

Reviewer #1 (Remarks to the Author):

Changes made in response to reviewer #1 are marked in **turquoise** (changes made in response to >1 reviewer are marked in **grey**) in the revised manuscript.

In their study Nanadikar and colleagues apply a genetically encoded tool for H₂O₂ production recently developed by the Belousov group (also part of this study) to study H₂O₂ linked redox metabolism in heart. By overproducing H₂O₂ in mice heart they identify specific cysteine redox switch in IDH3 that allows cells to directly sense cellular redox state. Presented work is innovative as it is one of the first examples when both a genetically encoded sensor and tool were expressed as one construct. This allows authors to both generate H₂O₂ and at the same time to directly monitor live changes in H₂O₂ levels. Overall, this study represents an important advancement in our understanding how cells sense changes in redox environment and how they respond to them via a Cys-based redox switch. This study also illustrates usefulness of the Oximouse project recently introduced by the Chouchani group. Although this study presents an advancement towards our understanding of redox regulation in live cells, the presented work needs to be improved to be considered for a publication. Below are my specific comments:

Answer: We would like to thank reviewer #1 for the in general positive evaluation of our manuscript. Please find the answers to the specific comments below.

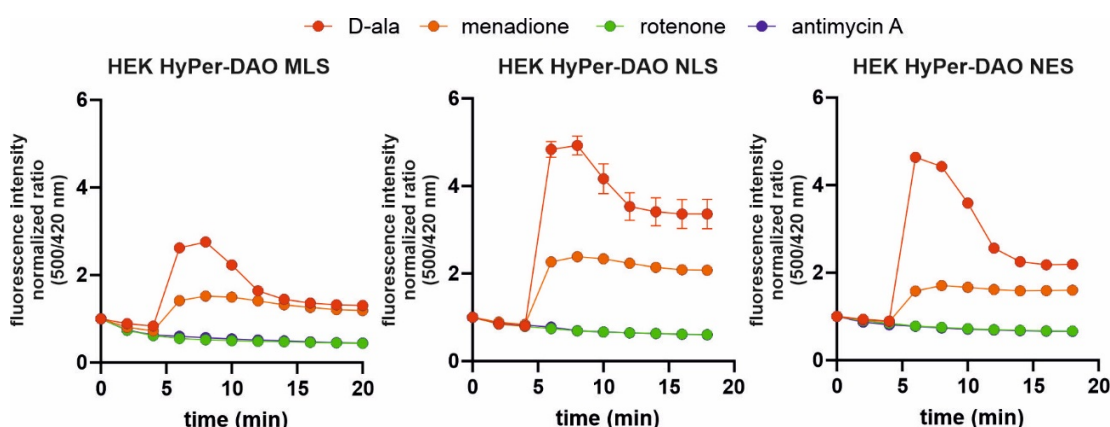
1). It looks like the H₂O₂ levels produced by HyPerDAO is rather modest both based on a very high KM for D-Ala as well as on physiological effects presented. This mild H₂O₂ production perhaps represents an advantage compared to other methods of H₂O₂ generation in cellular or organismal systems. It is important to see where H₂O₂ produced by HyPerDAO lies compared to other methods of H₂O₂ production (i.e. addition of menadione or other redox cyclers that via NQO1 mediate H₂O₂ production; H₂O₂ levels due to inhibited ETC, bolus of H₂O₂ directly added to cells etc).

Answer: Monitoring H₂O₂ production using HyPer provides some clues regarding the concentrations of H₂O₂ produced under different concentrations of D-ala. In the original paper, in which the biosensor HyPer was described, *in vitro* calibrations of purified HyPer towards different concentrations of H₂O₂ were reported (Belousov et al, Nat Meth 2006, doi: 10.1038/nmeth866). The minimal probe response was elicited to be around 25 nM, and complete oxidation to be around 200 nM H₂O₂. The basal concentration of H₂O₂ in the cytosol is in the low nanomolar range (Belousov, Oxidative Stress 2020, Chapter 6, doi.org/10.1016/B978-0-12-818606-0.00006-7). Therefore, HyPer indeed allows to detect a range of concentrations from basal level up to 1-2 orders of magnitude higher compared to the basal state, which can be interpreted by the cell as a signal or a stress, depending on the duration.

There are examples how DAO-driven H₂O₂ production can be compared to other ways of oxidative challenge. First, we frequently use bolus H₂O₂ addition to the cells at the end of a DAO experiment to determine saturation of the probe (example in Pak et al, 2020, Cell Metab,

doi: 10.1016/j.cmet.2020.02.003). Bolus addition of H_2O_2 was also applied in the current manuscript (see Fig. 4B). Typically, for bolus addition saturating concentrations of H_2O_2 are used. The main differences of H_2O_2 bolus treatment compared to DAO are the following. First, bolus addition of moderate amount gives a short transient of H_2O_2 because of the rapid degradation of the oxidant, compared to the steady production of H_2O_2 by DAO. Second, DAO can be targeted to a particular organelle or cell type, which allows to create various scenarios of endogenous H_2O_2 production to evaluate the consequences for the cell or tissue. In contrast, bolus H_2O_2 , redox cyclers, or mitochondria poisons affect equally all cells in a tissue, or, in case of bolus H_2O_2 , all compartments in the cell, which represents a less physiological scenario. In previous experiments from our group we used rotenone which resulted in much lower levels of H_2O_2 production compared to DAO, and only in the mitochondrial matrix, but not in the cytosol (see Fig 3F and G in Pak et al, 2020, Cell Metab, doi: 10.1016/j.cmet.2020.02.003). Moreover, the detection was possible with the highly sensitive HyPer7 and not with the earlier version HyPer3. In our current study we employed HyPer2, which is a similar version as HyPer3. Conceptually, giving the cell mitochondrial poisons is a disadvantaged way to induce oxidative stress, because together with poorly controlled and quantifiable H_2O_2 production this also shuts down ATP synthesis and, likely, affects many other functions of mitochondria. Instead, DAO can mimic a more or less "pure" oxidative stress or signaling depending on the enzyme location, substrate concentration, and exposure timing.

To further answer the question experimentally, we strictly followed the advice of reviewer #1, despite the above arguments, and compared the D-ala induced HyPer response with the response of cells to menadione and inhibition of the electron transport chain via treating the cells with antimycin A or rotenone.



The redox cyclers menadione but not inhibitors of the ETC induce a ROS response similar to D-ala detected by HyPer2. HEK HyPer-DAO-MLS, -NLS and -NES cells were treated either with 50 mM D-ala, 50 μM menadione, 5 μM rotenone or 5 μM antimycin A. The substances were added at time point 4 min. Subsequently, HyPer2 fluorescence responses were recorded. Ratios are normalized to the HyPer ratio prior treatment, 40 cells per condition were analyzed. Mean $\pm$ SEM.

As can be seen in these new experiments, the D-ala induced H_2O_2 responses in the nucleus, mitochondria and the cytoplasm induce a HyPer response as well as the treatment of the cells with 50 μM menandione. Inhibition of the electron transport chain with rotenone (complex I) or antimycin A (complex III), on the other hand, did not result in a response, which we were able to detect with the HyPer2 sensor, which is in line with the above described previous data from the Belousov laboratory (Pak et al, 2020, Cell Metab, doi: 10.1016/j.cmet.2020.02.003). Based on the above-mentioned arguments, we decided not including this data set in the manuscript, however, if advised by the reviewer to do so, we easily can show these data in the supplementary section.

2). How the HyPerDAO construct was developed in first place? Are there any linker regions between HyPer and DAO proteins? Was HyPerDAO protein previously expressed in E.coli and purified to show that the enzymatic activity of HyPerDAO is very similar to DAO alone?

Answer: HyPer-DAO fusion was the same as used before (Matlashov et al, 2014 Antioxid Redox Signal, doi: 10.1089/ars.2013.5618) and contained the Gly-Gly-Ser-Gly linker between HyPer and DAO. This information has been added in the Material and Method section (lines 402-403 of the revised manuscript).

Our earlier attempts to purify DAO failed, likely because E. coli have a substantial amount of some D-amino acids that induce oxidative toxicity (mutant inactive DAO version can be efficiently expressed). Instead, in the previous paper from the Belousov laboratory that describes HyPerDAO fusion (Matlashov et al, 2014, Antioxid Redox Signal, doi: 10.1089/ars.2013.5618), the authors compared HyPerDAO with DAO co-expressed with HyPer, and found that the enzyme retains its full functionality in both fused and free forms. HyPer fused to DAO appeared to be an even better detector of H_2O_2 produced by DAO compared to co-expressed version, obviously because in the fusion the generator and detector are placed in proximity to each other.

3). The schematic of the DAO reaction in Fig. 1A is not correct: D-ala conversion leads to imino acid formation and FAD reduction (FADH₂), then FADH₂ reacts with O₂ to produce H₂O₂. What happens next to the imino acid product? Will imino acid react with H₂O with a concomitant NH₃ production in cells? Were ammonia levels evaluated in DAO expressing cells/organs?

Answer: We apologize for the mistake made in Fig. 1A, which has been corrected in the revised manuscript (see Fig 1A of the revised manuscript). We also would like to point out that the presented graph is a simplified scheme of DAO reaction rather than the complete reaction scheme. Indeed, D-amino acids are dehydrogenated by DAO into imino acids that spontaneously hydrolyze to the corresponding α -keto acids and ammonia. In case of D-ala used as a substrate in our study, the resulting α -keto acid is pyruvate.

However, as the reaction is slow, and H_2O_2 is produced in nanomolar to low micromolar amounts, both ammonia and pyruvate are produced in equimolar amounts, several orders of magnitude lower than high-micromolar endogenous ammonia or millimolar pyruvate concentrations. Therefore, we assume that neither ammonia nor pyruvate produced by DAO are able to perturb

cellular metabolism. In addition, extensive ammonia generation would lead to at least a transient alcalinization of the cellular compartment of interest. Our numerous control experiments in many cell types expressing DAO with SypHer family pH probes did not show any alterations of cellular pH upon D-ala addition confirming that ammonia is produced at very low rate.

4). Not clear the reasoning why nuclear targeted HyPerDAO construct was used in mice. It seems logical to use a cyto construct (it looks like mitochondria are very good at detoxifying H₂O₂ (especially low levels produced by DAO?) and not usage of a mito construct in mice was well justified).

Answer: We indeed originally intended to generate aside from the nuclear targeted HyPer-DAO mice also transgenic mice that express HyPer-DAO in the mitochondrial matrix and the cytosol. We performed blastocyst injections twice. In the first round we obtained in total 5 HyPer-DAO NLS and 5 HyPer-DAO NES founder mice carrying the correct genotype. Functional analysis, however revealed that the cardiomyocytes of just one of the HyPer-DAO NLS mice showed a positive expression and D-ala response. In the other mice the HyPer-DAO fusion protein expression was either negligible, dislocated or completely degraded. In the second round of blastocyst injections we obtained 3 HyPer-DAO NES and 2 HyPer-DAO MLS mice genotyped positive by PCR. Cardiomyocytes of neither of them demonstrated a functional DAO-HyPer response due to the same reason as for the previous founder mice. We added this information to the revised manuscript in the result (see lines 91-93 of the revised manuscript) and in the discussion (lines 324-334 of the revised manuscript).

Although the generation of HyPer-DAO NES and HyPer-DAO MLS transgenic mice was not successful, we still believe that the data obtained with the HyPer-DAO NLS mice are of physiological relevance since (i) we verified the H₂O₂ effect on IDH3 γ redox modification (see new data set presented in Fig. Suppl. Fig. 3B) and IDH3 activity in Hek HyPer-DAO NLS, HyPer-DAO NES and HyPer-DAO MLS cells (see Fig. 4C) and more importantly (ii) we have in the meantime gained evidence that IDH3 activity is altered in a relevant cardiac disease setting (see Fig. 3J and K), i.e. cardiac hypertrophy/heart failure in consequence of an increased afterload. We have chosen this cardiac disease model based on comment #6 of reviewer #3. We discuss the importance of subcellular H₂O₂ production and especially the H₂O₂ buffering capacity of mitochondria as mentioned by the reviewer (see lines 324-330 of the revised manuscript).

5) In Fig. 1 B-C which antibody was used to detect HyPerDAO? Loading GAPDH control was not equal in samples presented.

Answer: HyPer-DAO was recognized using the anti-GFP antibody (11814460001, Sigma-Aldrich, line 620 of the revised manuscript). The antibody binds to the HyPer part of the fusion protein.

For each organ sample we indeed loaded the same amount of total protein. It is well described in literature that GAPDH expression varies by 15-fold between different tissue types, which is also reflected in Fig. 1C (Barber et al, 2005, *Physiol Genomics*, doi.org/10.1152/physiolgenomics.00025.2005). In the experiments shown, we aimed for detecting if HyPer-DAO is present in the transgenic mice and heart only and not in wt or other

organs, which can be seen in Fig. 1B and C. However, we agree with the reviewer that an absolute quantification of HyPer-DAO protein levels standardized by GAPDH protein levels would be precluded but was also not intended in the presented manuscript.

6). Fig. 2: Blue colors are very confusing, especially half blue bars with a gradient, therefore representations need to be improved to help with the clarity.

Answer: The blue color in Fig. 2 was removed as suggested by the reviewer (see Fig. 2).

7). Why only IDH3 was explored as an enzyme under redox control? It seems like SHMT1 enzyme is also worth exploring given its role in NAD(P)H and 1C metabolism.

Answer: We definitively agree with reviewer #1 that IDH3 γ is not the only interesting candidate that was identified in the redox proteomics screen. We indeed followed up possible changes in the activity of the succinate dehydrogenase, which we could exclude in the mouse and the cell model (Fig. 3E and Suppl. Fig. 3)

SHMT1, which is involved in NADPH and 1C metabolism as well as Trifunctional protein (TFP) β , which is involved in β -oxidation might link redox signaling and alterations in metabolism. Since, however, the respective necessarily follow up experiments are of different nature depending on the protein candidate to be studied, we had to make a choice for the current project. We will follow up on the other candidates, which to our opinion is out of the scope of the current manuscript. We discuss in the revised manuscript that other redox-modified target proteins might add to the phenotype observed in the Hyper-DAO mice (see lines 351-354 and lines 358-363 of the revised manuscript).

8). It is very difficult to gauge enzymatic activities results as in Methods only a kit from Sigma was mentioned. How exactly these assays were performed, which substrates, under Vmax conditions etc? Were cell homogenates desalted to remove endogenous substrates?

Answer: More detailed information on the IDH activity measurements are given in the revised manuscript (see lines 727-751 of the revised manuscript).

This study will greatly benefit from tracer studies (at least in a cell line model presented). This will allow to directly evaluate glycolysis/TCA cycle activities and fluxes.

Answer: We have performed targeted stable isotope tracer studies using uniformly ^{13}C glucose and GC-MS with the HEK HyPer-DAO NLS wt and HEK HyPer-DAO NLS C148A cells. The new data are presented in Fig. 7F and G.

Also, a summary schematic figure is needed to guide the reader on the state of redox Cys and downstream effects linked with the IDH3 activity.

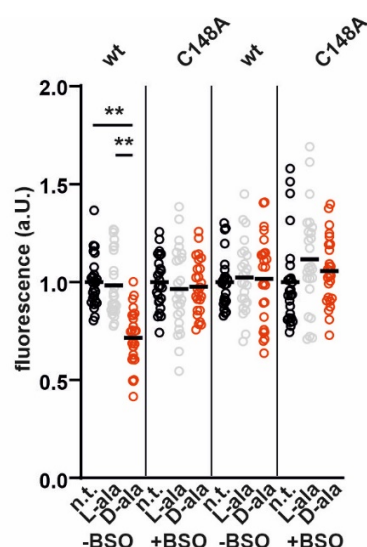
Answer: A summary schematic figure has been added in Fig. 7 (see Fig. 7I).

Obviously even mild H_2O_2 production by DAO leads to downstream effects in cells (including decreased ATP levels) and it's not expected that mutated redox Cys will rescue ATP production to a full extent. At the same time a connection between an increased TCA cycle activity (via IDH3) and GSH biosynthesis is not very convincing. At least in HEK293T cells NADH/NAD⁺ and NADPH/NADP⁺ levels need to be evaluated.

Answer: A new data set describing NADH/NAD⁺ and NADPH/NADP⁺ levels in the HEK HyPer-DAO wild type and C148A gene edited cells are presented in Fig. 7H.

What happens if cells are treated with buthionine sulfoximine (to inhibit GSH biosynthesis).

Answer: We would like to thank the reviewer for suggesting this experiment. We inhibited glutathione (GSH) biosynthesis by buthionine sulfoximine (BSO) treatment as recommended. Whereas inhibition of GSH biosynthesis rescues the decrease of ATP after D-ala stimulation in the HyPer-DAO-NLS HEK cells, this effect is not seen in the HyPer-DAO-NLS Cys148A HEK cells. These data support the notion that TCA metabolites, which are used for GSH biosynthesis after D-ala stimulation stay in the TCA cycle and are metabolized for energy production. However, since this is a very indirect measure, we decided to soften the conclusion regarding the glutathione synthesis in the manuscript.



Inhibition of glutathione synthesis rescues ATP levels. HEK HyPer-DAO-NLS wild type (wt) and HEK HyPer-DAO C148A cells were treated or not treated with 500 μ M buthionine sulfoximine (BSO) for 18 hrs. Subsequently ATP levels were measured with the ATP-red fluorescence sensor at three different conditions, i.e. non treated (n.t.), L-ala (50 mM, 20 min), and D-ala (50 mM, 20 min). 25 cells were analyzed per condition. mean, ** $p < 0.01$ by one-way ANOVA.

9). It is certainly will be more convincing to see the effect observed not only in HEK293T cells. Have authors tried another cell line? For example, C2C12 myoblasts? It will be very interesting if in a panel of cell lines IDH3 activity will be increased upon DAO-mediated H_2O_2 production.

Answer: We have analyzed IDH3 activity in a panel of cell lines after treating the cells with a bolus of H_2O_2 . We indeed find an increased activity of IDH3 in a panel of different cells (MADA-MB-231, Hep3B and C2C12). Additionally, we have included Hep3B cells that are stably expressing HyPer-DAO in the cytoplasm or the nucleus (Hep3B HyPer-DAO NES and Hep3B HyPer-DAO NLS) in our

analysis. Comparable to the effect seen in the HyPer-DAO mice and the HEK HyPer-DAO cells, IDH3 activity is increased after treating the cells with D-ala. The new data is shown in Suppl. Fig. 3D and described in lines 207-211 and lines 336-337 of the revised manuscript.

10). As mentioned above a schematic figure is needed to guide the reader through results: the state of redox cysteine switch vs TCA activity and other downstream effects;

Answer: A summary schematic figure has been added in Fig. 7 (see Fig. 7I).

Reviewer #2 (Remarks to the Author):

Changes made in response to reviewer #2 are marked in green (changes made in response to >1 reviewer are marked in grey) in the revised manuscript.

Nandikar, et. al, propose that IDH3 γ is a redox switch responsible for losses of cardiac function under significant ROS-induced stress. The research design is of notable robustness, and the authors present a fairly convincing justification for this mechanism as a possible source of myocardial “stunning” after infarction. While the overall manuscript is strong, some parts of the results and discussion could be refined to make the message clearer.

We would like to thank reviewer #2 for the in general positive evaluation of our manuscript. Please find the answers to the specific comments below.

p.6. lines 187-189. The authors state cytosolic H₂O₂ diffuses into the mitochondria, but not vice-versa. This by definition is not diffusion. Should it be termed “facilitated transport by an unknown mechanism?”

Answer: The underlying mechanisms are indeed not clear. Therefore, we believe that neither describing a pure diffusion or transport is supported by a sufficient amount of data in the literature. Therefore, we have changed the wording as suggested by the reviewer (see page 6, lines 188-190 of the revised manuscript).

p.9 line 301. The oxygen consumption rate (OCR) is the essential element indicative of increased TCA cycle turnover. But that data is not presented (see (2) below). Instead, the authors convolve increased metabolite pool sizes with increased activity, which is plausible, but does not establish a true link. See reference:” Ragavan, Mukundan, et al. "A comprehensive analysis of myocardial substrate preference emphasizes the need for a synchronized fluxomic/metabolomic research design." American Journal of Physiology-Heart and Circulatory Physiology 312.6 (2017): H1215-H1223.” With the interplay between energy metabolism at IDH3 and glutathione availability, it is quite possible that changes in pool sizes are related to anaplerotic flux into the TCA cycle. “Brunengraber, Henri, and Charles R. Roe. "Anaplerotic molecules: current and future." Journal of inherited metabolic disease 29.2 (2006): 327-331.”

A secondary question is that of IDH3 reversibility. While IDH1 and IDH2 are known to be reversible, IDH3 is not generally acknowledged to have the same properties. Did the authors find any evidence of reversibility in this context? That would add to the originality of this manuscript.

Answer: We agree with reviewer #2 that metabolic flux assays would give a better insight into the H₂O₂ induced metabolic changes. As also described above (see reviewer #1, comment #8) we have performed targeted stable isotope tracer studies using uniformly ¹³C glucose and GC-MS to obtain experimental insight into the alterations in metabolism aside from oxygen consumption rate measurements (see new data set presented in Fig. 7F and G). IDH3 is described to catalyze a non-reversible reaction. The data obtained in our experiments do not indicate that the enzymatic reaction *per se* changes upon redox-modification. However, we would like to point out that the redox-modification-induced increased enzyme activity of IDH3 is reversible upon release of the H₂O₂ stimulation (see Fig. 4F and G).

Finally, the connection simply was not clear to me between increased IDH3 function and loss of ATP production (lines 290-292). If IDH is rate limiting for TCA cycle flux as stated in the document, an increase should mean more NADH, and more ATP production, not less. Maybe I am missing the logic, but I believe the discussion could be made clearer. Lines 380-384 draw a connection between increased TCA cycle flux and glutamine cataplerosis, but simultaneously invokes glutamine anaplerosis. Please clarify.

Answer: In line with the new data set obtained with the tracer studies we have refined the discussion of the alterations in the TCA cycle flux (see page 10, lines 374-381 of the revised manuscript). As also discussed above we have softened the conclusion regarding the glutamine cataplerosis.

Data to be presented:

- 1) *The weights of the control versus HyPer hearts would be nice to include along with the histology of Figure 1.*

Answer: We have analyzed the heart weights of the wt and HyPer-DAO mice, which clearly show no change neither before nor after D-ala treatment. These data are included in Fig. 2E of the revised manuscript. Based on the comment made by reviewer #3, comment #2 we have removed the H&E sections as they were regarded as *non-informative*.

- 2) *The raw SeaHorse data should be included in the main figures, with a full description of the data. The actual oxygen consumption rates need to be included in a clear manner. While Figure 7E ostensibly refers to the OCR, the caption for this figure is abbreviated. Is the fluorescence on y-axis associated with OCR?*

Answer: We would like to politely disagree with the reviewer. Fig. 7E is correctly labelled as fluorescence for y-axis as it reflects the ATP measurements performed with the ATP-Red dye (Merck, SCT045) and not the seahorse measurements.

Reviewer #3 (Remarks to the Author):

Changes made in response to reviewer #3 are marked in **blue/green** (changes made in response to >1 reviewer are marked in **grey**) in the revised manuscript.

This is an intriguing work identifying isocitrate dehydrogenase 3 (IDH3) as a metabolic enzyme that can act as a redox switch through reversible oxidation, alongside important insights on the metabolic consequences of elevated H_2O_2 in the heart. IDH3 oxidation was identified through the use of an α MHC-HyPer-DAO mouse model, which is an innovative in vivo system that affords the cardiomyocyte overexpression model of H_2O_2 as well as an H_2O_2 reporter in the nucleus. The overall findings that IDH3 can be reversibly redox modified and the metabolic consequences of this modification are significant and the approach was innovative. However, the physiological significance of these findings are less clear. Mitochondria are a major source of ROS/ H_2O_2 , whereas the nucleus is not often described as such. Accordingly, it is unclear why the authors chose to model in vivo elevated H_2O_2 signaling in the nuclear compartment when they had access to the mitochondrial construct as evidenced by use in vitro studies. Moreover, while the α MHC-HyPer-DAO mice do show that IDH3 modification can occur in the heart, does this happen physiologically, or in the context of cardiac disease? While the authors do note in the discussion that oxidation may play a particularly important role in acute myocardial infarction/myocardial stunning, ROS signaling may also be important in other disease modalities such as cardiac pressure overload. Showing IDH3 redox modification in a physiologically relevant cardiac disease model would strengthen the study and expand the disease relevance of this work.

We would like to thank reviewer #3 for the thoughtful comments. As you can also see from the specific responses below, we have especially concentrated on answering the questions about the general conclusions made with the HyPer-DAO localized to the nuclear compartment and to prove that the changes observed are of relevance in the context of cardiac diseases.

Concerns:

1. *Using the α MHC-HyPer-DAO-NLS mice to generate a gradient of H_2O_2 is intriguing, however, the rationale underlying the choice to use the NLS Hyper-DAO to make the mouse model is not clear. Mitochondria are a major source of ROS in cardiomyocytes and in cardiac disease. The explanation for this choice, especially in light of the availability of the mitochondrially targeted construct to the authors, needs to be further expanded upon.*

Answer: As described above (please see answer to reviewer #1, comment #4) we indeed intended to generate HyPer-DAO transgenic mice with the expression of the fusion protein in either the mitochondrial matrix, the cytosol or the nucleus, from which despite several blastocyst injections only the nuclear targeted version resulted in a successful mouse line. We have in the meantime obtained a data set that indicates that modification of IDH3 γ plays a role in afterload-induced cardiac hypertrophy/failure (see comment #6).

Is the concentration of H₂O₂ produced in vivo with the NLS-targeted construct indeed higher in the nucleus than in the mitochondria? Also how far does H₂O₂ travel within the cell?

Answer: We would like to refer the reviewer to the experiments shown in Suppl. Figure 2 of the manuscript. In these experiments, we transfected the Hek HyPer (Green) DAO-NES, -NLS and -MLS cells with HyPer-Red, which was localized to the mitochondrial matrix. These experiments indeed imply that the H₂O₂ levels in the nucleus are higher than in the mitochondria in the HyPer (Green) DAO-NLS cells indicating a cellular H₂O₂ gradient. Even more, when HyPer (Green) DAO was localized to the mitochondria (right panel of the figure), the same D-ala concentration resulted in less H₂O₂ levels compared to the nuclear localization, which indicates a high H₂O₂ buffering capacity of the mitochondria. Although we cannot formally answer the question how far H₂O₂ “travels” within the cells, overall the data clearly indicate intracellular H₂O₂ gradients. This is in line with the previous characterization of the HyPer-DAO system (Mishina et al, 2019, Antioxid Redox Signal, doi: 10.1089/ars.2018.7697, 2011, Mishina et al, 2011, Antioxid Redox Signal, doi: 10.1089/ars.2010.3539).

Does this system reflect the levels of H₂O₂ that would be produced in a cardiac disease setting? Please include in the discussion/rationale for NLS HyPer-DAO choice.

Answer: As suggested in comment #6 we have by now analyzed IDH3 activity in a well described mouse heart failure model, i.e. cardiac hypertrophy and failure induced by transverse aortic constriction.

A discussion/rationale for analyzing HyPer-DAO NLS is included in the revised manuscript (see page 9, lines 324-334 of the revised manuscript).

2. *In Figure 1E, the authors show serial H&E stained sections of 2 hearts HyPer-DAO vs WT to show that the hearts are unaffected with HyPer-DAO expression at baseline. These images are not informative-please include echocardiography of baseline cardiac function and morphometry to accompany these images in the supplemental data.*

Answer: We have performed echocardiography to analyze cardiac output in non-treated male and female wild type (wt) and HyPer-DAO mice. This new data set is presented in Fig. 1E. There are no significant differences between the wt and respective HyPer-DAO mice at baseline. We would additionally like to point out that other baseline characterization (including fractional area shortening/FAS, ejection fraction/EF, anterior wall thickness/AwTh and posterior wall thickness/PwTh) is already shown in the day 0 time points presented in Fig. 2C, 2D and Suppl. Fig. 1A of the manuscript. All animals were analyzed by echocardiography before L-ala or D-ala treatment. FAS, EF, AwTh and PwTh were unaltered in the transgenic mice compared to the age-matched wt littermates. As suggested we additionally analyzed stroke volume (SV), left ventricular inner diameter in systole (LVIDs), left ventricular inner diameter in diastole (LVIDd)

and enddiastolic volume (EDV). Neither SV, LVIDs, LVIDd nor EDV differ between wt and HyPer-DAO mice before treatment at day 0 (Suppl. Fig. 1B).

3. *In Figure 2C, the authors show that over the course of 21 days, activation of the cardiomyocyte HyPer-DAO construct can result in reduced fractional shortening and ejection fraction) without adverse cardiac wall remodeling. Moreover, the authors show in 2D that this contractile dysfunction can be reversible. These findings are striking, and should be accompanied by the echocardiography measurements of left ventricular interior dimensions at diastole and systole, as well as the stroke volume and end diastolic volumes.*

Answer: As suggested, we have analyzed left ventricular interior dimensions at diastole and systole, stroke volume and end diastolic volume. Data are presented in Suppl. Fig. 1B in the revised manuscript.

4. *Are the gene expression changes observed with HyPer-DAO activation (Fig. 2A) also reversible/inducible with the removal of D-alanine (in line with the changes in cardiac function shown in the dosing regimen used in 2D)?*

Answer: According to the reviewer comment we have analyzed the Nrf2 target genes shown in Fig. 2A in left ventricular samples of HyPer-DAO mice either treated with D-ala for 7 days in the drinking water (on) or 2 additional days without D-ala (off). Wild type (wt) littermates treated for 7 days with D-ala in the drinking water served as controls. We observed a mild increase of Nrf2 target genes in the HyPer-DAO mice after D-ala treatment, the RNA levels were no longer increased after removal of D-ala. The new data set is presented in Fig. 2A of the revised manuscript and described on page 4, lines 108-110 and page 10, line 365 of the revised manuscript.

5. *Are the mitochondrial proteins identified by mass spec to be reversibly modified in Fig3B also modified with HyPer-DAO constructs targeted to other cellular compartments?*

Answer: We would like to point out that we checked in HyPer-DAO-NLS, HyPer-DAO-NES as well as HyPer DAO-MLS cells for redox-induced changes in IDH3 activity (Fig. 4C). The changes observed were independent of the cellular compartment, in which H₂O₂ was produced. In response to the reviewer comment we also analyzed IDH3 γ redox modification in the cells with the HyPer-DAO construct in the nucleus, cytosol and mitochondria by performing Peg switch assays. In line with the IDH3 activity, IDH3 γ modification was altered in response to D-ala in all three cell lines (see new data set in Suppl. Fig. 3B).

Although it would be of course very interesting to repeat the redox proteomics screen aside from the hearts obtained from HyPer-DAO NLS mice (as demonstrated in the manuscript) in comparison with all three different cell lines, we believe that this would be an independent project and beyond the scope of the current manuscript. We hope for the understanding of the

reviewer that these kinds of experiments, although already planned, will not be included in the current project.

6. The α HMC-HyPer-DAO-NLS animal is a really great tool to highlight what can be redox modified in the heart, but there is a disconnect between what is identified with this nuclear H_2O_2 source model, versus what might occur physiologically in the heart (where the mitochondria might be the major source of ROS). This study would be very much strengthened if the authors would be able to show that some of the redox modified targets identified in the HyPer-DAO mice to be similarly modified in cardiac disease settings (would be particularly of interest to know if IDH3 Cys148 plays a role). While the authors note in the discussion that this may play a role in myocardial stunning, it would also be worthwhile to look in the context of TAC, where ROS may as play a role (also because the authors already have that surgical model in use for this study).

Answer: We would like to thank the reviewer for the advice. We analyzed left ventricular samples obtained from mice that underwent transverse aortic constriction (TAC) or sham surgery for IDH3 activity and IDH3 γ redox modification. As shown in Fig. 3J and K IDH3 activity and IDH3 γ modification are both increased in TAC challenged compared to sham treated mice. The data are described on page 6, lines 172-176 and discussed on page 11, lines 395-398.

We understand that it would be of particular interest to know if IDH3 γ Cys148 plays a role. To answer this question formally correct, the generation of a Cys148 point mutated mouse model would be necessary, which clearly is beyond the scope of the first description of IDH3 γ as a redox switch protein target.

Reviewer #4 (Remarks to the Author):

Changes made in response to reviewer #4 are marked in pink (changes made in response to >1 reviewer are marked in grey) in the revised manuscript.

With the HyPer-DAO mouse model and redox proteomics, Nanadikar et al. identified IDH3 γ that can serve as a redox switch, revealing that the Cys148 and Cys284 are the two critical sites in regulating IDH3 activity. The experiment design and the results are generally sound. The finding on the redox regulatory role of IDH3 γ is interesting. The authors should address the following questions before it becomes acceptable.

We would like to thank reviewer #4 for the in general positive evaluation of our manuscript. Please find the answers to the specific comments below.

- 1. The in vivo H_2O_2 abundance in mouse heart with and without D-ala treatment was not determined. How much was the basal H_2O_2 in mouse heart? and how much H_2O_2 was produced in response to D-ala?*

Answer: These are highly relevant but likewise difficult questions to be answered due to technical limitations. To quantify basal and D-ala induced H_2O_2 one would need to analyze the *in vivo* abundance of H_2O_2 in the mouse heart in the living animal optimally with unopened chest over days. Since the HyPer biosensor relies on its fluorescence characteristics, this setting would preclude detecting the HyPer signal in living mice. A real-time fiber-optic recording of fluorescence biosensor signals has been developed in the context of acute-ischemic-stroke in the brain recently (Pochechuev et al, 2022, J Biophotonics, doi: 10.1002/jbio.202200050). A similar system would be needed to be developed for the heart. Considering that the heart contains spontaneously beating musculature, developing a suitable fiber optics set up would need to account in addition these movements. Currently a suitable system does not exist in either the authors groups nor – to the best of our knowledge - somewhere else. Establishing a suitable setup is beyond the scope of the current project. Therefore, we hope for the understanding of the reviewer that solving these technical problems would be an independent project *per se*.

2. *Do ATP production and other energy-related metabolites differ between HyPer-DAO and WT with the D-ala treatment in mice?*

Answer: We agree with the reviewer that analyzing ATP in the hearts of the HyPer-DAO mice are of interest. To this end we determined the ATP/ADP ratio in wild type and HyPer-DAO mice after a 7day long treatment with D-ala (Fig. 2F). There is a trend of a decreased ATP-ADP ratio ($p = 0.08$) in the transgenic mice. This mild effect might be caused by the fact that aside from the affected HyPer-DAO expressing cardiomyocytes, the measurement will also include the ATP-ADP ratio of the non-cardiomyocytes. This conclusion is in part supported by the fact that we saw a significant decrease in ATP levels after D-ala treatment of cardiomyocytes isolated from HyPer-DAO mice (Fig. 5C).

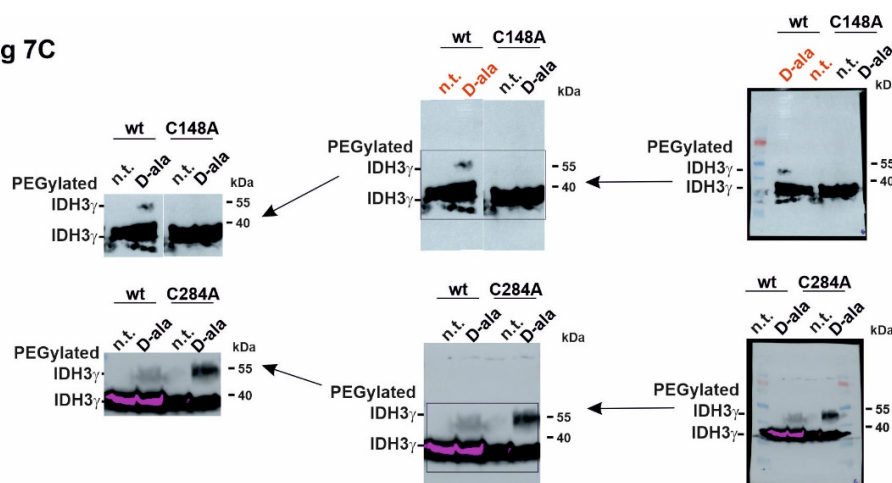
3. *Fig 4EG. The PEG switch experiment shows that the oxidative level of IDH3 is low. Can this minor redox level change result in sufficient regulation on total enzyme activity?*

Answer: We certainly agree that a minor fraction of IDH3 γ was modified according to the PEG switch assay. This still resulted in a significantly altered and robust IDH3 activity. IDH3 is a rate limiting enzyme of the TCA cycle, with IDH3 γ as its regulatory component. Therefore, one might argue that an alteration of the entire IDH3 γ pool might result in drastic metabolic changes that would harm the cells. The IDH3 γ redox modification (PEG switch, Fig. 4G and G) and IDH3 activity (enzyme activity measurements, Fig. 4C and F) that we observed after D-ala stimulation were rapidly reversible, which might result in the correct titration that is needed for the on-off characteristic of the biological consequences. We have included a respective discussion on page 9, lines 335-336 of the revised manuscript.

4. *Fig 7C. Why was PEGylated IDH3 much higher in C-284A mutant? Should PEGylation level be correlated to its enzymatic activity? And why? It looks like the lane image of the C284A treating with D-ala was cropped from elsewhere. Please confirm this is the original image.*

Answer: Please find the uncropped image below. The wt and cysteine mutated cells were indeed analyzed side by side in the same experiment and immunoblot. Since the loading of the experiment performed with the C148A and the C284A mutant and the respective wild type (wt) cells were different (see original blots on the right side), the image was cut and rearranged (for the C148A mutant cells). We left a white space in the figure 7C, where the blot was cut and will make this space even clearer in the revised manuscript.

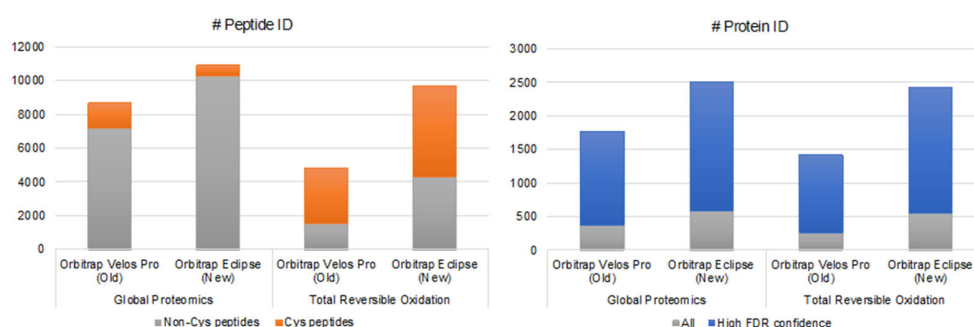
Fig 7C



In addition, it is absolutely correct that the PEGylation levels in the C284A mutant seem to be higher compared to the wt. We speculate that the reason why this band is more intense could be that the oxidation of cysteine 148 is more stable in the absence of cysteine 284. This could be explained if the disulfide is more solvent accessible than a cysteine 148 sulfenic acid.

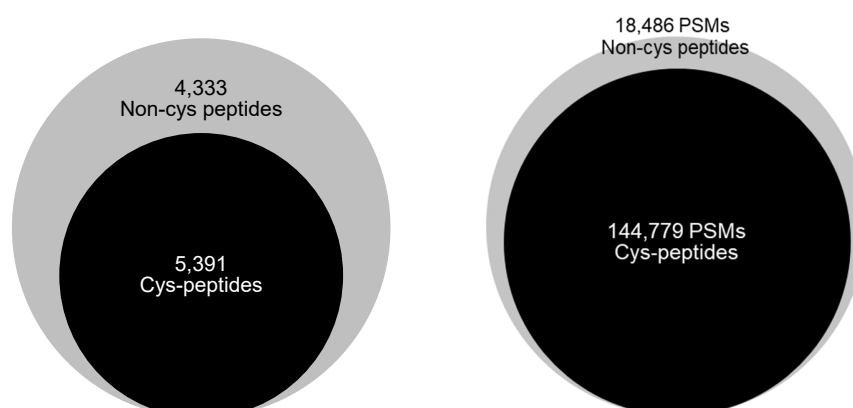
5. *Since this is a cys-site specific analysis, the redox proteomics data should be analyzed and illustrated in a site-centric manner. The current data (e.g. fig3b and SI tables) analyses were all performed on protein level. An average value of all cys sites surely dilutes the importance of those proteins in which only a part of the cys sites show changes. I would strongly suggest the authors to re-analyzed their proteomic data in a site-centric manner.*

Answer: As a part of our major effort in this revision, we repeated the LC-MS/MS analysis of the samples employing a recently acquired Orbitrap Eclipse Tribrid mass spectrometer (Thermo Scientific, Waltham, MA), an instrument superior to the previously used Orbitrap Velos Pro mass spectrometer. The new proteomics and redox proteomics data (.raw files and database search results) were uploaded onto the ProteomeXchange via PRIDE server. Improvements in both overall protein and peptide numbers were noted as shown in the bar graphs below.



The analysis shows significant enrichment in cysteine-containing peptides from ~5% in the global proteomics data to >60% in the redox proteomics data. This demonstrates that the enrichment at the protein level in the redox proteomics workflow was effective to allow monitoring of redox changes. The new analysis shows a similar trend in differential protein abundance. Most importantly, the target protein, IDH3 γ was identified with the same changes in the Cys148 peptides abundance levels upon D-ala treatment. Peptide identification quality was acceptable judging from manual inspection of their MS2 spectra and XCorr scores (Sequest scores).

Proteomics data were summarized in peptides centered manner rather than protein comparison in the revised manuscript (see Supplement table 1 and 2). In the table, cysteine containing peptides in the 7 days treatment group was compared by the ratio of peak abundance calculated by label-free quantitation. A panel (as shown below) showing the number of unique non-cys and cys containing peptides and number of peptide spectrum matches (PSM) was added to Figure 3B to summarize the new data.



Venn-diagrams demonstrate elevated yield of cysteine-containing unique peptides and total number of identified peptide spectra matches (PTM) by redox enrichment.

The new redox proteomics data (.raw files and database search results) were uploaded onto the ProteomeXchange via PRIDE server (PXD037987). Below is the reviewer's credential for data access.

- Project DOI: 10.6019/PXD037987
- Username: reviewer_pxd037987@ebi.ac.uk
- Password: nloP9oLa.

6. *The MS2 and fold-changes of the important cys sites, such as cys 148 and 284 of IDH3 γ , should be added in the manuscript. Their identification with high-confidence is the foundation of follow-up studies.*

Answer: Though the cys-284 peptide was not identified in this study, cys-148 peptide was successfully measured with high confidence (XCorr values ranging from 2.59-3.63). The fold change for Cys148 was found to be 1.52, p value 0.016. This information is provided in Supplement Table 1 and on page 5, lines 158-160 of the revised manuscript.

7. *The STN is a very weird term in describing fold-change in proteomics. Signal-to-noise has an exact definition in mass spectrometry, but rarely represents fold-change. The authors should revise this word, and related figures and tables.*

Answer: A power law global error model (PLGEM) is a widely accepted method in identifying differentially expressed genes (Pavelka et al., BMC Bioinformatics, 2004 Dec 17;5:203), but indeed is not as frequently utilized in proteomics. We have replaced STN with fold-change to enable easier evaluation by readers.

8. *The overall quality of the redox proteomics should be assessed. In this reviewer's opinion, there are several flaws.*

- a. *Why was the enrichment done on proteins, not on peptides? A peptide-based enrichment would deepen the analysis coverage.*

Answer: The experiment was performed according to the method described in Nat Protoc. 2014 Jan; 9(1): 64–75. The analysis shown by the bar graph above demonstrates significant enrichment and capture of Cys-containing peptides.

- b. *How much was the enrichment efficiency (cys-containing peptides vs. total peptides)? Based on several example raw data the authors provided, this reviewer found that the enrichment efficiency was bad. Roughly, only less than 10% of identified peptides contain cys. This is a sign that the experiments were not performed properly. Moreover, in the several data files I searched, the cys 148 and 284 of IDH3 γ were not identified.*

Answer: To clarify, 3,316 Cys-peptides out of 4,829 total peptides (68%) were found in the original redox proteomics data set submitted with the first version of the manuscript (performed with the Orbitrap Velos Pro). This is higher than the proportion of Cys-peptides found in the global proteomics data (17%). Cys148 peptide was identified in both

the proteomics and redox proteomics data. We indeed did not identify Cys284 in the redox proteomics data. However, the peptide containing Cys284 might not be as susceptible to tryptic cleavage as the Cys148 peptide. The new data set submitted with the revised manuscript (performed with the Orbitrap Eclipse) includes 2,000 more Cys-peptides, i.e. 5391 Cys-containing peptides (see Fig. 3B).

- c. *Total numbers of cys sites and changed cys sites should be added. Their quantitative results should be included.*

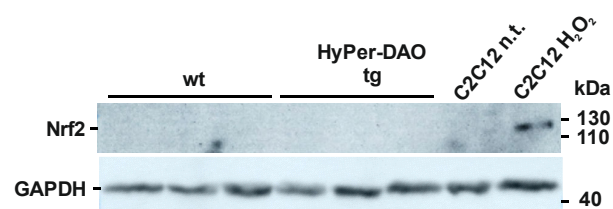
Answer: In total we identified 6374 cysteine sites in 5391 peptides. Out of these we found that 185 peptides demonstrated a significant decreased modification and 115 a significant increased modification in the samples obtained from the 7 days D-ala treated HyPer-DAO mice compared to the wt mice. These numbers are now described on page 5, lines 141-148 and Fig. 3B.

- d. *Blocking free cys with MSTP was done in a RIPA buffer, which was a mild buffer thus couldn't denature all proteins' structures. Therefore, some free cys sites might not be blocked in the first step, affecting the subsequent enrichment. The authors should evaluate the completeness of the reaction between free cys and MSTP.*

Answer: We have used RIPA with 2% SDS and higher amount of denaturing agent and also 2% of SDS was used in reconstitution of protein pellet.

9. Line 107. How does Nrf2 itself change?

Answer: We checked for NRF2 protein levels using the anti-Nrf2 antibody (Bioworld Technologies, L593). Detecting NRF2 on protein level is challenging (Lau et al, Antioxid Redox Signal. 2013, doi: 10.1089/ars.2012.4754) since some commercially available antibodies detect non-specific bands. Therefore, we used cell extracts of non-treated and H₂O₂ treated C2C12 cells as positive control. In line with the mild induction of Nrf2 target RNA expression, Nrf2 protein levels did not reach the detection level in the protein extracts obtained from left ventricles of wt and HyPer DAO mice compared to the positive control, i.e. cell extract of H₂O₂ treated C2C12 cells.



Nrf2 protein levels in hearts of wild type (wt) and HyPer-DAO transgenic (tg) after treatment with D-ala. Mice were treated with D-ala for 7 days in the drinking water. On day 7 animals were sacrificed and left ventricles were isolated. Protein extracts from the left ventricles were analyzed for Nrf2 and GAPDH protein levels by Western blot. As a positive control for Nrf2, protein extracts of non-treated (n.t.) or H₂O₂ treated C2C12 cells were loaded.

10. Line 169 and fig 3e. The RNA levels of IDH3 were increased, though the protein level was not. Is the difference statistically significant?

Answer: The differences between left ventricles from 7d or 21d wt and HyPer-DAO mice in regard to IDH3 α , β and γ are not statistically significant. Significance was tested by one-sample t-test.

11. Line 171 and fig 3G. Does IDH3 activity really go back to normal after removing D-ala for 2days? Is the difference between the two bars on the right statistically significant?

Answer: The difference between the three groups, i.e. wt-on, HyPer-DAO on and HyPer-DAO on-off was analyzed by Welch`s ANOVA test. The following comparisons were done: wt-on vs. HyPer-DAO on, p-value: 0.0307; wt-on vs. HyPer-DAO off, p-value: 0.8809, HyPer-DAO on vs. HyPer-DAO off, p-value: 0.2652.

12. Fig 7B. the IDH3 basal activity of C236A is lower than wt IDH3 and other mutants. Are there any explanations?

Answer: The basal activity of C236A was not significantly different compared to basal activity of the wt. Still, there seems to be at least a trend towards lower activity as mentioned by the reviewer. This might be explained by inducing changes through mutating a cysteine, that might have no regulatory function in redox modulation but a structural function. A short description and discussion is included on page 8, lines 275-277 of the revised manuscript.

Aside from the experiments performed in response to the reviewer comments, we have in the meantime generated HEK HyPer-DAO-NLS cells that are gene edited on both critical cysteines, i.e. Cys148 and Cys284. The respective C148A/C284A cells were analyzed regarding IDH3 activity and ATP production after D-ala stimulation. In line with our hypothesis, C148/C284A cells did not increase IDH3 activity and did show a decrease in ATP levels in response to the stimulus. The new data set is presented in Suppl. Fig. 5A-C.

We shortened the text throughout the manuscript to meet the required word count (no more than 5000 words in the main text). The word count of the revised abstract is n = 141 and of the revised main text n = 4997.

We would like to thank all reviewers for their comments and hope that with the additional experiments performed and information added, we were able to address all concerns.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Authors addressed all the major concerns that were raised. I have a few more comments/suggestions:

1) I take my words back that H₂O₂ levels generated by DAO are modest (Fig. 7H). The DAO-HyPer fusion is indeed a powerful genetically encoded tool for compartment-specific generation of H₂O₂. I anticipate that DAO-HyPer tool will be used in various studies to explore metabolic remodeling due to a pro-oxidative shift. I suggest that authors do INCLUDE as a supplement figure: “The redox cyler menadione but not inhibitors of the ETC induce a ROS response similar to D-ala detected by HyPer2”.

2) I think it needs to be stated somewhere (it will address concerns of Reviewer 2) that because DAO consumes O₂ to make H₂O₂ Seahorse measurements are not straight forward (and ATP production was used as a proxy for the ETC activity instead [both heart and HEK cells]). This of course has its caveats as in cancer cell lines (HEK and many others) majority of the ATP produced is via glycolysis.

3) I still not quite understand the connection between an increased IDH3 activity as a response to elevated H₂O₂ levels and at the same time a decrease in the TCA cycle activity (authors suggest an INCREASE of flow towards AKG only). It also looks like that succinate levels go up with H₂O₂ regardless of the state of the redox switch in IDH3 (as stated by authors). I think that in Discussion the discovery of the switch should be presented as a major finding as we still do not quite understand the role of this switch and how it is integrated in cellular metabolism. (especially since now authors dialed down on GSH metabolism in the Discussion).

4) I think that data presented in Fig. 7H is truly remarkable. In C148A IDH3 cells there is a drastic increase in NADH/NAD⁺ (a pro-reductive shift). At the same time as expected H₂O₂ production by DAO exhausted NADPH levels (very low NADPH/NADP⁺ and GSH/GSSG ratios). However, in C148A IDH3 there is a sizable increase in NADPH/NADP⁺ ratio with D-Ala (wt vs C148A). This suggests a rebalancing of reducing of NADH and NADPH equivalents (a truly remarkable observation). Also, it is very interesting that inhibition of GSH biosynthesis rescued ATP production in C148A IDH3 cells. On a similar note: it will be interesting to see the GSH/GSSG ratio in C148A IDH3 cells treated with D-Ala.

5) Lines:375-380 The entire paragraph is confusing. How an increase in IDH3 activity (NADH producing enzyme) can lead to NADH decrease? There is an interesting crosstalk between IDH3 as in C148A IDH3 cells the NADH/NAD⁺ ratio was increased substantially (see above)! Again, finding the exact mechanism of this “redox rebalancing” is outside the scope of this work.

6) Minor:

Fig. 5: panels B and C are labeled identically.

Fig. 7H: typo in “NADPH/NADP⁺”

Fig. 7I: change the schematic, it appears that TCA cycle activity is going up with H₂O₂ and wt IDH3.

Reviewer #2 (Remarks to the Author):

The authors have significantly enhanced the manuscript by adding tracer metabolism approaches, and they have properly qualified or removed statements that were not completely supported by the data.

Reviewer #4 (Remarks to the Author):

The authors have addressed all my questions or given reasonable explanations thoughtfully. Thanks.

Reviewer #5 (Remarks to the Author):

All the modifications have been read. Most concerns have been solved, but there is still a problem that I'm concerned about. The clinical relevance of this study remains unclear. Authors have demonstrated the changes of IDH3 γ induced by H₂O₂ also existed in TAC model. However, authors failed to demonstrate that if inhibiting IDH3 γ Cys148 modification could suppress the progression of cardiac dysfunction and fibrosis in TAC model in vivo, which is closely connected with the clinical significance of this study.

Reviewer comments - Point to Point response

We would like to thank all reviewers for their input.

A more detailed point to point response can be seen below:

Reviewer #1

Authors addressed all the major concerns that were raised.

We would like to thank reviewer #1 for this statement.

I have a few more comments/suggestions:

1) *I take my words back that H₂O₂ levels generated by DAO are modest (Fig. 7H). The DAO-HyPer fusion is indeed a powerful genetically encoded tool for compartment-specific generation of H₂O₂. I anticipate that DAO-HyPer tool will be used in various studies to explore metabolic remodeling due to a pro-oxidative shift. I suggest that authors do INCLUDE as a supplement figure: "The redox cyclers menadione but not inhibitors of the ETC induce a ROS response similar to D-ala detected by HyPer2".*

As suggested, we have now included the data set obtained from the stimulation experiments using menadione and the inhibitors of the ETC to see the ROS response in comparison to the D-ala stimulation (see Supplementary Figure 2A and lines 177-180).

2) *I think it needs to be stated somewhere (it will address concerns of Reviewer 2) that because DAO consumes O₂ to make H₂O₂ seahorse measurements are not straight forward (and ATP production was used as a proxy for the ETC activity instead [both heart and HEK cells]). This of course has its caveats as in cancer cell lines (HEK and many others) majority of the ATP produced is via glycolysis.*

A respective statement has been included in the revised manuscript (see lines 218-219).

3) *I still not quite understand the connection between an increased IDH3 activity as a response to elevated H₂O₂ levels and at the same time a decrease in the TCA cycle activity (authors suggest an INCREASE of flow towards AKG only). It also looks like that succinate levels go up with H₂O₂ regardless of the state of the redox switch in IDH3 (as stated by authors). I think that in Discussion the discovery of the switch should be presented as a major finding as we still do not quite understand the role of this switch and how it is integrated in cellular metabolism. (especially since now authors dialed down on GSH metabolism in the Discussion).*

We certainly agree with this statement. We have pointed out in the revised manuscript that the discovery of IDH3 γ as a redox switch is the major finding in the abstract (see line 42) as well as in the discussion (see lines 394-396).

4) *I think that data presented in Fig. 7H is truly remarkable. In C148A IDH3 cells there is a drastic increase in NADH/NAD⁺ (a pro-reductive shift). At the same time as expected H₂O₂ production by DAO exhausted NADPH levels (very low NADPH/NADP⁺ and GSH/GSSG ratios). However, in C148A IDH3 there is a sizable increase in NADPH/NADP⁺ ratio with D-Ala (wt vs C148A). This suggests a rebalancing of reducing of NADH and NADPH equivalents (a truly remarkable observation). Also, it is very interesting that inhibition of GSH biosynthesis rescued ATP production in C148A IDH3 cells. On a similar note: it will be interesting to see the GSH/GSSG ratio in C148A IDH3 cells treated with D-Ala.*

We would like to thank the reviewer for this positive statement. As suggested we have included GSH and GSSG measurements obtained from wt versus C148A IDH3 cells (see Fig. 7I and lines 378-380).

5) *Lines:375-380 The entire paragraph is confusing. How an increase in IDH3 activity (NADH producing enzyme) can lead to NADH decrease? There is an interesting crosstalk between IDH3 as in*

C148A IDH3 cells the NADH/NAD⁺ ratio was increased substantially (see above)! Again, finding the exact mechanism of this “redox rebalancing” is outside the scope of this work.

We apologize for this confusion. The reviewer is right by mentioning that the NADH/NAD⁺ ratio was significantly increasing in the sense of a pro-reductive shift. We have changed the wording of the mentioned paragraph accordingly (see lines 378-380).

6) *Minor:*

Fig. 5: panels B and C are labeled identically.

We have changed the labeling to clearly indicate that Fig. 5 panel B and C show ATP measurements in HEK cells and isolated cardiomyocytes, respectively.

Fig. 7H: typo in “NADPH/NADP⁺”

The typo has been corrected.

Fig. 7I: change the schematic, it appears that TCA cycle activity is going up with H₂O₂ and wt IDH3.

We have changed the schematic to make this point clearer.

Reviewer #2 *The authors have significantly enhanced the manuscript by adding tracer metabolism approaches, and they have properly qualified or removed statements that were not completely supported by the data.*

We would like to thank reviewer #2 for this positive statement.

Reviewer #4

The authors have addressed all my questions or given reasonable explanations thoughtfully. Thanks.

We would like to thank reviewer #4 for this positive statement.

Reviewer #5

All the modifications have been read. Most concerns have been solved, but there is still a problem that I'm concerned about. The clinical relevance of this study remains unclear. Authors have demonstrated the changes of IDH3 γ induced by H₂O₂ also existed in TAC model. However, authors failed to demonstrate that if inhibiting IDH3 γ Cys148 modification could suppress the progression of cardiac dysfunction and fibrosis in TAC model in vivo, which is closely connected with the clinical significance of this study.

In order to prove the importance of IDH3 γ Cys148 modification fully, a Cys148 gene edited mouse model would be necessarily needed to be established. It is uncertain, if a respective mouse model would be viable. As suggested by the editor we have therefore addressed this point by clearly stating this limitation in the text (see lines 369-371).

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all my questions and reasonable explanations were given. Thank you.

Very minor: Fig. 7I , not clear statistics of which columns is shown (shifted label maybe?)

Reviewer comments - Point to Point response

We would like to thank all reviewers for their input.

A more detailed point to point response can be seen below:

Reviewer #1 (Remarks to the Author):

The authors have addressed all my questions and reasonable explanations were given. Thank you.

We would like to thank reviewer #1 for this positive feedback.

Very minor: Fig. 7I, not clear statistics of which columns is shown (shifted label maybe?)

We have checked Fig. 7I and have aligned the connecting lines between the columns to show statistically significant differences in a clear manner.

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection All the commercially available softwares used are mentioned in the Methods section.

Data analysis Statistical data are shown as Mean $\pm$ standard error of mean and the analysis was performed using the Graph Pad Prism software 9.0.0. In this study, following softwares were used for the data analysis: CellSens (v1.18) from Olympus, CGenFF via ParamChem web server (v2.4.0), GROMACS (v2019.3), GROMACS_FDA (v2020), Image J win64 (v1.48), LabChart (For recording: V7.2, For analysis: V8) from ADInstruments, Proteome Discoverer (v2.5) from Thermo-Fischer Scientific, Scipy (stats module, v1.5.4), Skyline (V21.2.0.369) from MacCoss Lab software, Vevo lab (v5.5) from Fujifilm VisualSonics, MassHunter - Quantitative Analysis (vB.07.01), IsoCorrectoR (v0.1.13), CRISPOR (v5.01).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The mouse line generated in this study will be deposited to the Knockout Mouse Project (KOMP). The proteomics data generated in this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository under accession code PXD037987 and 10.6019/PXD037987 [https://www.ebi.ac.uk/pride/archive/projects/PXD037987]. All other data generated in this study are provided in the Supplementary Information/Source Data file.

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Samples sizes were not been able to be decided before starting the experiments as the results were not predictable prior to each experiment. To observe consistency in the data, it was made sure that a higher n number per experiment is utilized or a high statistically significant p value is provided whenever possible. All the n and p values for each experiment are mentioned in the figure legends section.
Data exclusions	No data were excluded.
Replication	The number of replications for each experiment and precise n-number of independent samples or mice are given in the figure legends.
Randomization	Due to the obvious fluorescence expression of the used cells, randomization was not noticeably possible while working with cells. However whenever possible it was applied. The mice used in the experiments were of mixed gender. The control group and the test group of mice were equally treated with the treatment at the same time. The echocardiography of the mice before or after the treatment was done randomly by two different investigators to avoid predisposed approach.
Blinding	Due to the obvious fluorescence expression of the used cells, blinding was not noticeably possible while working with cells. However whenever possible it was applied. The echocardiography was performed by two different investigators who were not informed about the genotype of the mice or whether the group is a control group or the test group. The samples sent for redox proteomics analysis, ADP/ATP measurements or ¹³ C6 glucose tracing analysis were kept blind folded about the control or test groups prior to the analysis.

Behavioural & social sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Briefly describe the study type including whether data are quantitative, qualitative, or mixed-methods (e.g. qualitative cross-sectional, quantitative experimental, mixed-methods case study).
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Research sample	State the research sample (e.g. Harvard university undergraduates, villagers in rural India) and provide relevant demographic information (e.g. age, sex) and indicate whether the sample is representative. Provide a rationale for the study sample chosen. For studies involving existing datasets, please describe the dataset and source.
Sampling strategy	Describe the sampling procedure (e.g. random, snowball, stratified, convenience). Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient. For qualitative data, please indicate whether data saturation was considered, and what criteria were used to decide that no further sampling was needed.
Data collection	Provide details about the data collection procedure, including the instruments or devices used to record the data (e.g. pen and paper, computer, eye tracker, video or audio equipment) whether anyone was present besides the participant(s) and the researcher, and whether the researcher was blind to experimental condition and/or the study hypothesis during data collection.
Timing	Indicate the start and stop dates of data collection. If there is a gap between collection periods, state the dates for each sample cohort.
Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, provide the exact number of exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Non-participation	State how many participants dropped out/declined participation and the reason(s) given OR provide response rate OR state that no participants dropped out/declined participation.
Randomization	If participants were not allocated into experimental groups, state so OR describe how participants were allocated to groups, and if allocation was not random, describe how covariates were controlled.

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	Briefly describe the study. For quantitative data include treatment factors and interactions, design structure (e.g. factorial, nested, hierarchical), nature and number of experimental units and replicates.
Research sample	Describe the research sample (e.g. a group of tagged <i>Passer domesticus</i> , all <i>Stenocereus thurberi</i> within Organ Pipe Cactus National Monument), and provide a rationale for the sample choice. When relevant, describe the organism taxa, source, sex, age range and any manipulations. State what population the sample is meant to represent when applicable. For studies involving existing datasets, describe the data and its source.
Sampling strategy	Note the sampling procedure. Describe the statistical methods that were used to predetermine sample size OR if no sample-size calculation was performed, describe how sample sizes were chosen and provide a rationale for why these sample sizes are sufficient.
Data collection	Describe the data collection procedure, including who recorded the data and how.
Timing and spatial scale	Indicate the start and stop dates of data collection, noting the frequency and periodicity of sampling and providing a rationale for these choices. If there is a gap between collection periods, state the dates for each sample cohort. Specify the spatial scale from which the data are taken
Data exclusions	If no data were excluded from the analyses, state so OR if data were excluded, describe the exclusions and the rationale behind them, indicating whether exclusion criteria were pre-established.
Reproducibility	Describe the measures taken to verify the reproducibility of experimental findings. For each experiment, note whether any attempts to repeat the experiment failed OR state that all attempts to repeat the experiment were successful.
Randomization	Describe how samples/organisms/participants were allocated into groups. If allocation was not random, describe how covariates were controlled. If this is not relevant to your study, explain why.
Blinding	Describe the extent of blinding used during data acquisition and analysis. If blinding was not possible, describe why OR explain why blinding was not relevant to your study.

Did the study involve field work? ☐ Yes ☐ No

Field work, collection and transport

Field conditions	Describe the study conditions for field work, providing relevant parameters (e.g. temperature, rainfall).
Location	State the location of the sampling or experiment, providing relevant parameters (e.g. latitude and longitude, elevation, water depth).
Access & import/export	Describe the efforts you have made to access habitats and to collect and import/export your samples in a responsible manner and in

Access & import/export	<i>compliance with local, national and international laws, noting any permits that were obtained (give the name of the issuing authority, the date of issue, and any identifying information).</i>
Disturbance	<i>Describe any disturbance caused by the study and how it was minimized.</i>

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Details of all the antibodies used are clearly mentioned in the Methods section anti-GAPDH Cell Signaling Cat# 2118L, RRID: AB_561053 anti-Vinculin Sigma-Aldrich Cat# V9264, RRID: AB_10603627 anti-GFP Sigma Aldrich Cat# 11814460001, RRID: AB_390913 anti-IDH3γ Sigma-Aldrich Cat# HPA002017, RRID: AB_1079096 goat anti-mouse Sigma Aldrich Cat# 12-349, RRID: AB_390192 goat anti-rabbit Santa Cruz Cat# sc-2004, RRID: AB_631746
Validation	All the primary antibodies used for this study have been validated and reported before by other users. Anti-GAPDH, anti-vinculin and anti-GFP antibodies are cited in more than 400, 250 and 200 research papers respectively. Anti-IDH3γ has been validated by the Human Protein Atlas (HPA) project and thus has been supported for extensive characterization in the industry.

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	All the information about the cell lines generated by us for the study are clearly mentioned in the methods section. The source of the commercially available cell lines used for the study are: Human: HEK293 cells ATCC Cat# CRL-1573, RRID: CVCL_0045 Human: HEK293T cells ATCC Cat# CRL-3216, RRID: CVCL_0063 Human: MDA-MB 231 cells DSMZ Cat#ACC 732, RRID: CVCL_0062 Human: Hep3B cells ATCC Cat#HB-8064, RRID: CVCL_0326 Mouse: C2C12 cells, Sigma Aldrich Cat#91031101, RRID: CVCL_0188
Authentication	The HEK293, HEK293T, MDA-MB 231, Hep3B and C2C12 were purchased from the sources indicated above. Authentication was not performed afterwards in our lab. Since the stably transduced cell lines used for the study were generated from the the parent commercially available HEK293 cells and Hep3B cells respectively, the same applies for these cells.
Mycoplasma contamination	All the cells were regularly tested for Mycoplasma. The results were consistently negative.
Commonly misidentified lines (See ICLAC register)	HEK293 cells were used in the study since they are easily transfectable. The resulting stably transfected cells were analyzed for the response of the overexpressed HyPer-DAO fusion protein. HEK293 cell responses were compared within the same cell line and not with other cell lines.

Palaeontology and Archaeology

Specimen provenance	<i>Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information). Permits should encompass collection and, where applicable, export.</i>
Specimen deposition	<i>Indicate where the specimens have been deposited to permit free access by other researchers.</i>

Dating methods

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Ethics oversight

Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

Mice: C57Bl6N purchased from Jackson laboratories were used in this study. Transgenic mice were generated by pronuclear blastocyst injection in C57Bl6N mice. The resulting transgenic mice were named HyPer-DAO mice. The detailed description of generation of the transgenic mice is clearly mentioned in the methods section. Both adult male and female mice at the age of 8-14 weeks were used in the study. The gender of the mice used is clearly indicated in the figures and figure legends. The information about the exact number of mice used for each experiment is provided in the figure legends. Mouse housing conditions: light cycle, 12 hrs light/12 hrs dark cycle. Temperature and humidity, 18-23°C with 40-60% humidity.

Wild animals

No wild animals were used in this study

Reporting on sex

Both adult male and female mice at the age of 8-14 weeks were used in the study. The information about the exact number of mice and their gender used for each experiment is provided in the figure legends.

Field-collected samples

No field collected samples were used in this study.

Ethics oversight

The study complied with all the ethical regulations for work with experimental animals. Animal experiments were carried out according to the guidelines for the care and use of laboratory animals, following directive 2010/63/EU of the European Parliament. The animal work with the HyPer-DAO transgenic mice and the animal protocol regarding transverse aortic constriction were confirmed by the institutional guidelines and were approved by the Niedersächsische Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg Germany (33.9-42502-04-16/2352 and 33.9-42502-04-16/2102).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

Clinical trial registration

Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

Study protocol

Note where the full trial protocol can be accessed OR if not available, explain why.

Data collection

Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

Outcomes

Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

Dual use research of concern

Policy information about [dual use research of concern](#)

Hazards

Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

No Yes

- | | | |
|--------------------------|--------------------------|----------------------------|
| ☐ | ☐ | Public health |
| ☐ | ☐ | National security |
| ☐ | ☐ | Crops and/or livestock |
| ☐ | ☐ | Ecosystems |
| ☐ | ☐ | Any other significant area |

Experiments of concern

Does the work involve any of these experiments of concern:

No Yes

- | | | |
|--------------------------|--------------------------|---|
| ☐ | ☐ | Demonstrate how to render a vaccine ineffective |
| ☐ | ☐ | Confer resistance to therapeutically useful antibiotics or antiviral agents |
| ☐ | ☐ | Enhance the virulence of a pathogen or render a nonpathogen virulent |
| ☐ | ☐ | Increase transmissibility of a pathogen |
| ☐ | ☐ | Alter the host range of a pathogen |
| ☐ | ☐ | Enable evasion of diagnostic/detection modalities |
| ☐ | ☐ | Enable the weaponization of a biological agent or toxin |
| ☐ | ☐ | Any other potentially harmful combination of experiments and agents |

ChIP-seq

Data deposition

- ☐ Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- ☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

May remain private before publication.

For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session

(e.g. [UCSC](#))

Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

Replicates

Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots

Confirm that:

- ☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☐ All plots are contour plots with outliers or pseudocolor plots.
- ☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument

Identify the instrument used for data collection, specifying make and model number.

Software	<i>Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.</i>
Cell population abundance	<i>Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.</i>
Gating strategy	<i>Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.</i>

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type	<i>Indicate task or resting state; event-related or block design.</i>
Design specifications	<i>Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.</i>
Behavioral performance measures	<i>State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).</i>

Acquisition

Imaging type(s)	<i>Specify: functional, structural, diffusion, perfusion.</i>
Field strength	<i>Specify in Tesla</i>
Sequence & imaging parameters	<i>Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.</i>
Area of acquisition	<i>State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.</i>
Diffusion MRI	☐ Used ☐ Not used

Preprocessing

Preprocessing software	<i>Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).</i>
Normalization	<i>If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.</i>
Normalization template	<i>Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.</i>
Noise and artifact removal	<i>Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).</i>
Volume censoring	<i>Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.</i>

Statistical modeling & inference

Model type and settings	<i>Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).</i>
Effect(s) tested	<i>Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.</i>
Specify type of analysis:	☐ Whole brain ☐ ROI-based ☐ Both
Statistic type for inference (See Eklund et al. 2016)	<i>Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.</i>
Correction	<i>Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).</i>

Models & analysis

n/a	Involvement in the study
☐	☐ Functional and/or effective connectivity
☐	☐ Graph analysis
☐	☐ Multivariate modeling or predictive analysis

Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.