

## SUPPLEMENTARY FIGURES AND INFORMATION

### Title

Ergothioneine exhibits longevity-extension effect in *Drosophila Melanogaster* via regulating of cholinergic neurotransmission, tyrosine metabolism, and fatty acid oxidation

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Table S1 Statistical analysis of lifespan experiments for Canton S and yw females and males

Figure	Genotype	Sex	EGT concentration (uM)	Median	Number of flies	% Increase over flies on control food	Log-rank test comparison to control
Figure 1A	Canton S	Females	0	85	129		
	Canton S	Females	10000	86	138	1%	P<0.0001
	Canton S	Females	1000	94.5	114	11%	P<0.0001
	Canton S	Females	100	97.5	124	15%	P<0.0001
	Canton S	Females	10	94	138	11%	P<0.0001
	Canton S	Females	1	85	133	0%	P<0.0001
	Canton S	Females	0.1	87	116	2%	P<0.0001
Figure 1B	Canton S	Males	0	69	142		
	Canton S	Males	10000	66	147	-4%	p=0.311
	Canton S	Males	1000	77	147	12%	p=0.0016
	Canton S	Males	100	75	152	9%	p=0.0118
	Canton S	Males	10	72	143	4%	p=0.5998
	Canton S	Males	1	65	146	-6%	p=0.0001
	Canton S	Males	0.1	66	140	-4%	p=0.0364
Figure 1C	YW	Females	0	73	142		
	YW	Females	10000	69	129	-5%	p=0.0425
	YW	Females	1000	81	140	11%	p<0.0001
	YW	Females	100	85	123	16%	p<0.0001
	YW	Females	10	70	136	-4%	p=0.1002
	YW	Females	1	74	146	1%	p=0.1067
	YW	Females	0.1	68	133	-7%	p=0.0009
Figure 1D	YW	Males	0	70	113		
	YW	Males	10000	69	137	-1%	p=0.0135
	YW	Males	1000	72	140	3%	p=0.6320
	YW	Males	100	75	145	7%	p<0.0001
	YW	Males	10	74	120	6%	p<0.0001
	YW	Males	1	60	143	-14%	p=0.9676
	YW	Males	0.1	60	137	-14%	p=0.2175

Table S2 Statistical analysis of lifespan experiments for dietary restriction

Figure	Number of	EGT	DR	Median	% Increase over	Log-rank test
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	flies	concentration (uM)	medium (% yeast)		flies on control food	comparison to control food
Fig 1G	139	0	1%	68		
	148	100	1%	72	6%	p=0.0001
Fig 1G	139	0	5%	76		
	143	100	5%	79	4%	p=0.0009
Fig 1G	141	0	10%	80		
	136	100	10%	86	8%	p=0.0086
Fig 1G	141	0	15%	72		
	149	100	15%	78	8%	p=0.0007
Fig 1G	138	0	20%	66		
	136	100	20%	75	14%	p<0.0001

Table S3 Statistical analysis of stress survival experiments for yw females

Figure	Sex	Number of flies	EGT concentration (uM)	Stresses	Median	Log-rank test comparison to control food
Fig 2A	Females	117	0	5% H <sub>2</sub> O <sub>2</sub>	32h	
	Females	118	100	5% H <sub>2</sub> O <sub>2</sub>	48h	p=0.0008
Fig 2B	Females	119	0	10mM Paraquat	36h	
	Females	117	100	10mM Paraquat	48h	p=0.0003
Fig 2C	Females	120	0	Heat (37°C)	6h	
	Females	120	100	Heat (37 °C)	12h	p<0.0001
Fig 2D	Females	120	0	Desiccation	8h	
	Females	117	100	Desiccation	8h	p=0.0042
Fig 2E	Females	118	0	Starvation	3d	
	Females	120	100	Starvation	7d	p<0.0001
Fig 2A	Male	119	0	5% H <sub>2</sub> O <sub>2</sub>		
	Male	127	100	5% H <sub>2</sub> O <sub>2</sub>		p=0.0299
Fig 2B	Male	117	0	10mM Paraquat	83h	
	Male	119	100	10mM Paraquat	83h	p=0.1418
Fig 2C	Male	120	0	Heat (37°C)	53h	
	Male	119	100	Heat (37 °C)	53h	p<0.0001
Fig 2D	Male	118	0	Desiccation	6h	
	Male	121	100	Desiccation	9h	p=0.1306
Fig 2E	Male	120	0	Starvation	4h	
	Male	120	100	Starvation	4h	p<0.0001

Table S4 Effects of EGT on the antioxidant status in brain tissues of young and aged *D.melanogaster* females

Treatment	No.	GSSG (umol/g weight)	GSH (umol/g weight)	GSH/GSSG
Ck-D10	1	2.170680617	334.284815	154
	2	2.477321814	328.3154433	132.5283786
	3	1.727861771	334.103925	193.3626466
EGT-D10	1	4.168466523	682.5209379	163.7342975
	2	5.399568035	685.2401448	126.9064748
	3	4.384449244	679.801731	155.0483751
CK-D30	1	12.31346252	258.8121562	21.01863354
	2	13.56193	263.248062	19.41081114
	3	11.1394	265.2365714	23.81066947
EGT-D30	1	6.26349892	421.2849785	67.26032588
	2	5.788336933	414.9100386	71.68035369
	3	6.652267819	418.5254699	62.91470538

Abbreviations: GSH = reduced glutathione; GSSG = oxidized glutathione; Values are represented as means  $\pm$  SD, n = 30 for the test groups)

Table S5 Quality statistics of clean sequencing data

Sample	Clean reads	Bases (bp)	AF-Q20%	AF-Q30%	AF-GC%
Ck1	40650058	6234426000	98.49%	95.42%	50.34%
Ck2	33128244	5074689300	98.52%	95.45%	49.77%
Ck3	36334318	5541768600	98.37%	95.06%	49.44%
EGT1	38655782	5970233700	98.44%	95.23%	48.45%
EGT2	39237312	6032070300	98.48%	95.31%	48.67%
EGT3	36784508	5630997900	98.44%	95.22%	48.73%

Q20, Q30 refer to the proportion of base calls with Phred scores  $> 20$  or  $30$  in the total raw reads. AF-GC% refers to the after filter; Higher scores ensured the base calling accuracy and data quality. Each group has three biological replicates.

Table S6 The KEGG pathway enrichment analysis

The column of “log<sub>2</sub> Fold Change” represents the base-two logarithm value of the fold change of the gene expression; “+” means up-regulated genes; “-” means down-regulated genes. Hypergeometric test was used for statistical analysis. The FDR < 0.05 plus the absolute log<sub>2</sub> Fold Change value > 1 were considered statistically significant.

Table S7 Primer sequences for RT-qPCR analysis of RNA-samples of *D.melanogaster* yw

ID	Targeted gene	Primer sequence	Tm (°C)
ID (Symbol)	Description	Log2(FC)	p value
<b>Metabolic pathways</b>			
FBgn0030596(CG12398)	FI24332p1, partial	1.841213726	5.99E-13
FBgn0040211(hgo)	homogentisate 1,2-dioxygenase	-1.323214493	1.63E-11
FBgn0036992(Hpd)	4-hydroxyphenylpyruvate dioxygenase	-1.504948315	2.58E-34
FBgn0283437(PPO1))	TYR; tyrosinase	-1.47312386	1.26E-12
FBgn0026755(Ugt37B1)	UDP-glycosyltransferase family 37 member B1	-1.243150982	0.000752176
FBgn0030558(CG1461)	uncharacterized protein Dmel_CG1461, isoform B	-1.184267321	6.56E-13
FBgn0033296(Mal-A7)	maltase A7, isoform B	-1.032463772	0.001440658
FBgn0040069(vanin-like)	vanin-like	1.250631126	1.84E-23
FBgn0037697(GstZ2)	GH17960p, partial	-1.086316602	9.73E-06
FBgn0036381(CG8745)	uncharacterized protein Dmel_CG8745	1.047484649	3.87E-36
FBgn0286723(hll)	heimdall	-1.80668778	7.51E-11
<b>Tyrosine metabolism</b>			
FBgn0040211(hgo)	homogentisate 1,2-dioxygenase	-1.323214493	1.63E-11
FBgn0036992(Hpd)	4-hydroxyphenylpyruvate dioxygenase, isoform A	-1.504948315	2.58E-34
FBgn0283437(PPO1)	prophenoloxidase 1	-1.47312386	1.26E-12
FBgn0030558(CG1461)	uncharacterized protein Dmel_CG1461, isoform B	-1.184267321	6.56E-13
FBgn0037697(GstZ2)	GH17960p, partial	-1.086316602	9.73E-06
<b>Protein digestion and absorption</b>			
FBgn0031406(Send1)	spermathecal endopeptidase 1	1.752841557	1.86E-24
FBgn0019928(Ser8)	IP02555p, partial	1.035536157	0.00057038
FBgn0033760(CG8785)	uncharacterized protein Dmel_CG8785, isoform A	1.153947743	1.04E-29
FBgn0000042	<i>Act 5C-F</i>	TTGTCTGGGCAAGAGGATCAG	58°C
	<i>Act 5C-R</i>	ACCACTCGCACTTGCACCTTC	
FBgn0051661	<i>CG31661-F</i>	ACCGTCACCACAAGATTCCT	58°C
	<i>CG31661-R</i>	GGTCACATTCACAGCATTAC	
FBgn0030558	<i>CG1461-F</i>	AGAGTCAGTTCCGATAACCAGAG	58°C
	<i>CG1461-R</i>	TTGCGTATGTCCTGCGATTG	
FBgn0037697	<i>GstZ2-F</i>	GATGTAACCTTGACCGTGTGAG	58°C
	<i>GstZ2-R</i>	TGACTATTGGCGTTCGTTCTTC	
FBgn0040211	<i>hgo-F</i>	CGTGGCTCTACAGGAAGTTG	58°C

	<i>hgo-R</i>	GATTCGGTGGCTGCTCATC	
FBgn0036992	<i>Hpd-F</i>	CCGTGGATGACTCGCAGAT	58°C
	<i>Hpd-R</i>	CTTCACCGTCTCCTCGTAGTT	
FBgn0032055	<i>Sgp-F</i>	TGGCATCCGATTGTTAGGGG	58°C
	<i>Sgp-F</i>	TGGCATCCGATTGTTAGGGG	
FBgn0286723	<i>Hll-F</i>	CCAATGATACTGCCGCTCTG	58°C
	<i>Hll-R</i>	CATACTTATTCGCTCGCTTGATG	
FBgn0038641	<i>ChT-F</i>	ACGACGAGGAGACCAAGGA	58°C
	<i>ChT-R</i>	CACCAGGAGACCGAGATGAG	

Abbreviations: Sgp = Sgropino; Hll = Heimdal; GstZ2 = maleylacetoacetate isomerase; hgo = homogentisate 1,2-dioxygenase; Hpd = 4-hydroxyphenylpyruvate dioxygenase; ChT = choline transporter

Table S8 ELISA assay kit measures the CTSD level in brain tissues of aged *D.melanogaster* females

CTSD level (ng/g weight)	Ck-D30	EGT-D30
1	40.56	55.76
2	35.52	69.8
3	35.62	78.12

Table S9 Feeding intake assay

1h feeding (ug/fly/1h)	Ck		EGT	
	D10	D30	D10	D30
#1	8.162881	16.901305	8.693393	17.3570515
#2	8.328666	17.0532205	8.826021	17.0194615
#3	8.295509	17.036341	8.8094425	16.9594

48 hrs feeding (ug/fly/1h)	Ck		EGT	
	D10	D30	D10	D30
#1	15.04456	16.05733	24.328285	25.678645
#2	17.914075	18.31749505	22.977925	28.379365
#3	15.38215	17.31485275	20.0915305	22.741612

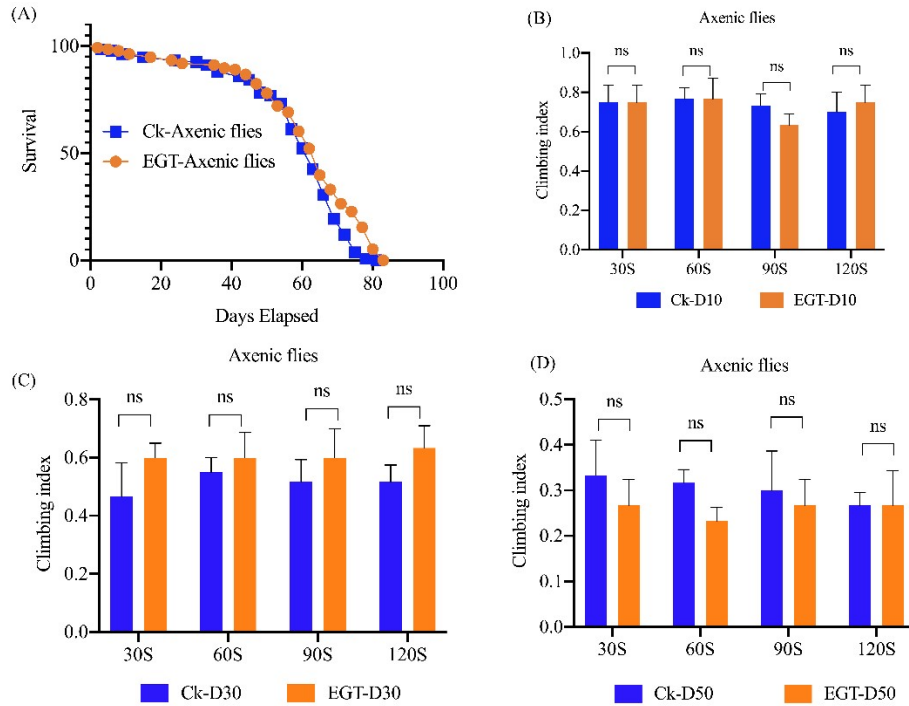


Figure S1 lifespan extension effect by EGT is abolished in axenic flies (A), and axenic flies (under conventional reared and EGT supplementation conditions) showed compromised climbing activity at different stages of life (B-D); Independently repeated three times with 20 flies per group.

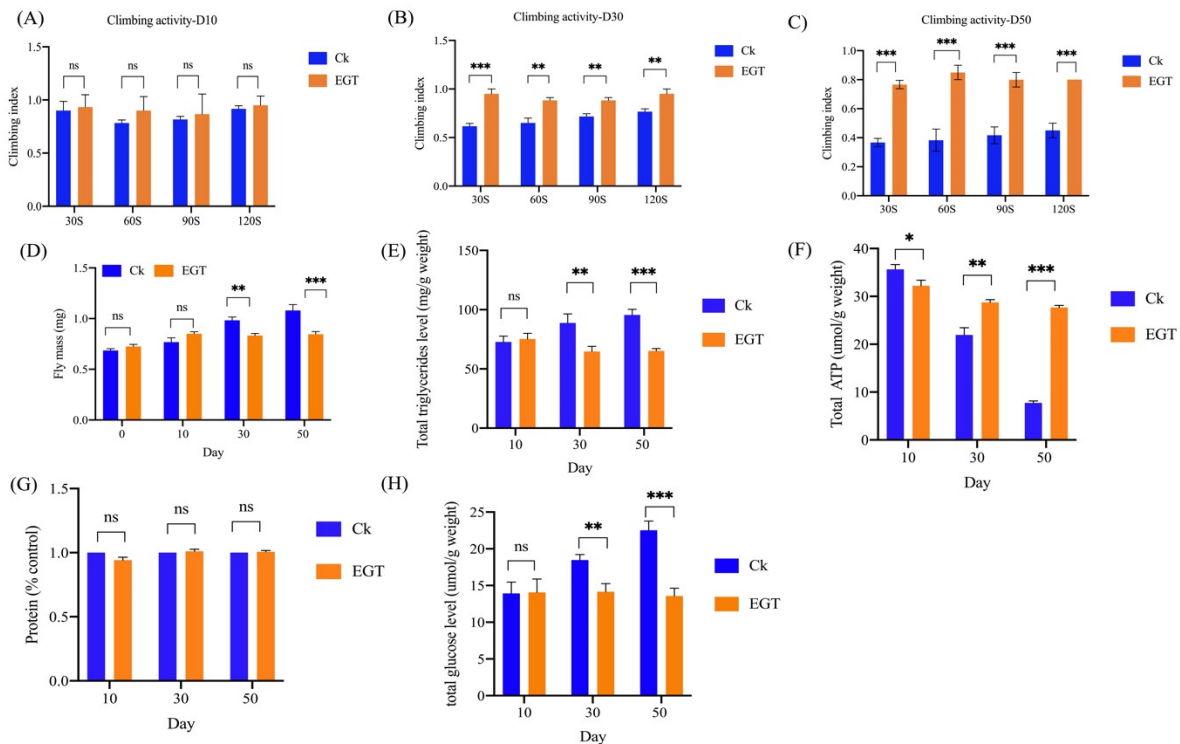
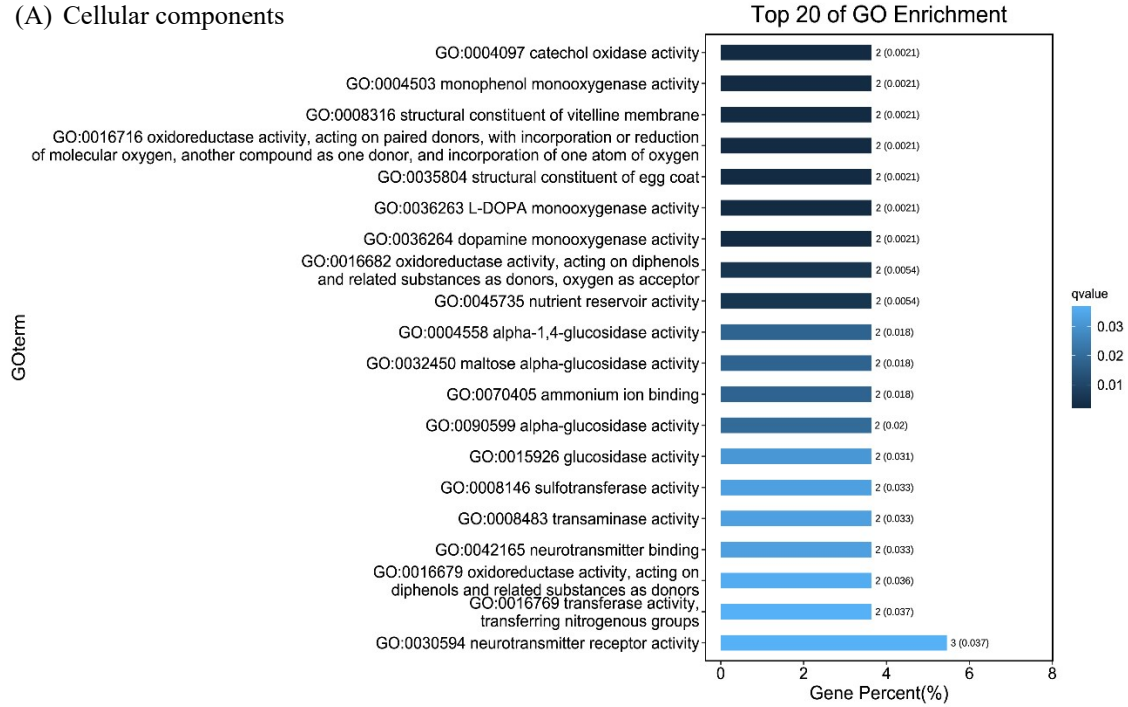


Figure S2 Supplementation with EGT improves fitness and metabolic parameters in aging *Drosophila melanogaster*. An increased climbing ability in aging flies with EGT supplementation was observed at different stages of life (A-C); Metabolic parameters, including (A) weight, (B) total triglycerides (TAG), (C) total ATP, (D) total glucose, and (E) total proteins, were measured in yw females after 10, 30, 50 days of EGT treatment from whole flies. The climbing test was independently repeated three times with 20 flies per group. Data are shown as mean  $\pm$  SEM. Each group

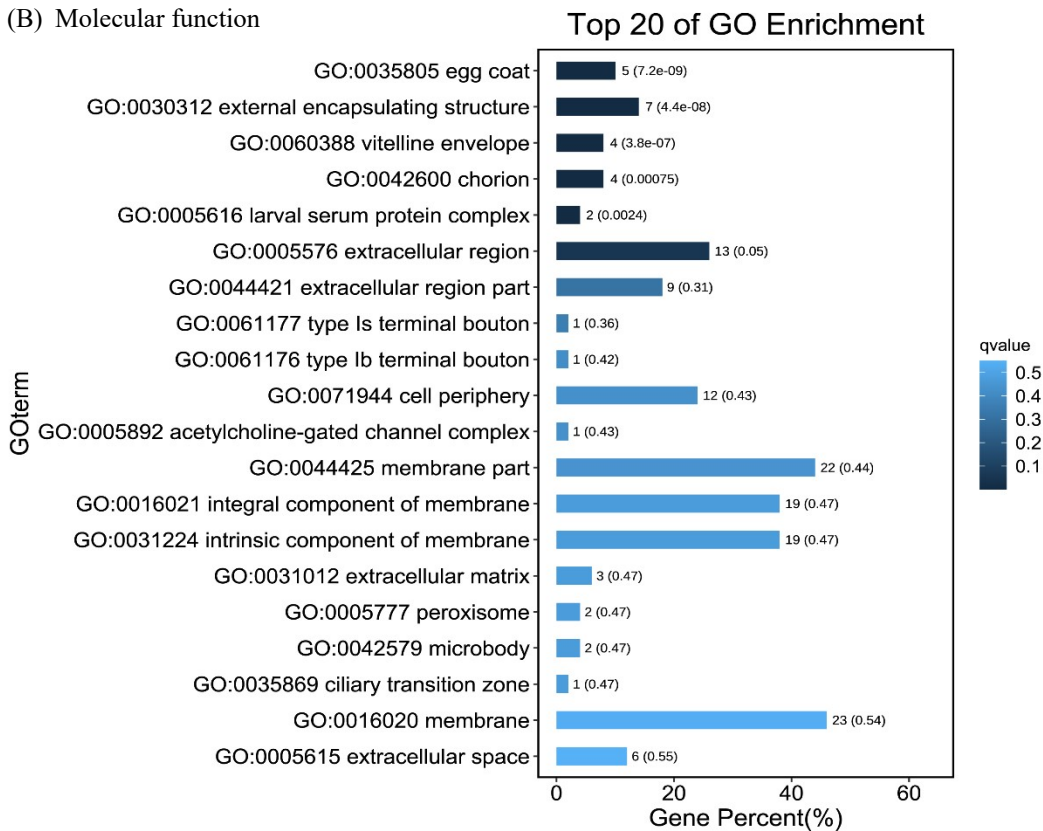


contained n = 3 independent groups and significance is indicated as stars (\*) relative to the control group with ns: p > 0.05, \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.

(A) Cellular components



(B) Molecular function



(C) Biological process

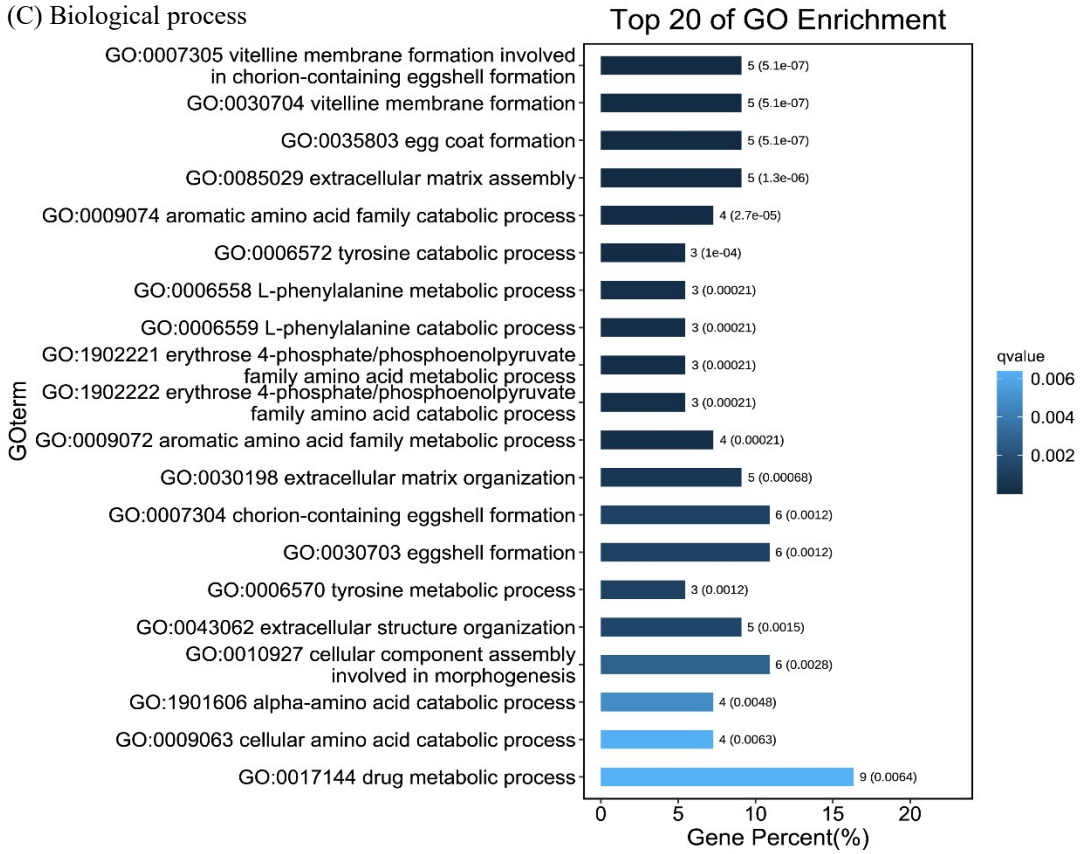


Figure S3 Bar plot of GO enrichment analysis of DEGs between Ck group VS EGT group, the top 20 GO term entries with the smallest Q value were displayed with molecular function (A), cellular components (B), and biological process (C); x-axis represents gene percentage (%). y-axis represents different functional groups (also named as different GO terms).

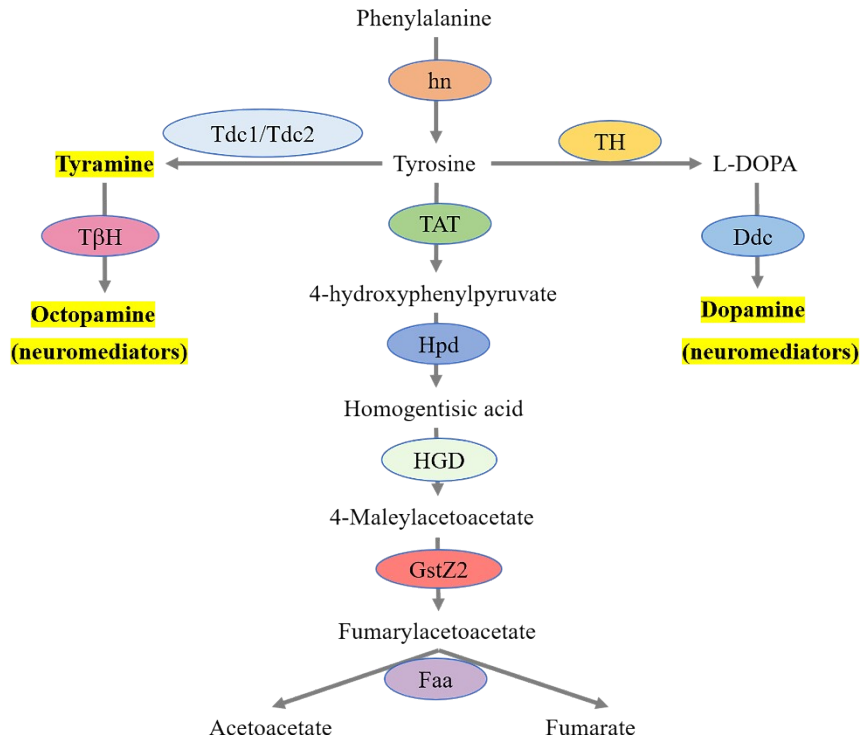


Figure S4 Schematic representation of tyrosine metabolism and the constituent enzymes.