

***O*-GlcNAcylation of orphan nuclear receptor ERR γ promotes hepatic
Gluconeogenesis**

Running title: *O*-GlcNAcylation of ERR γ

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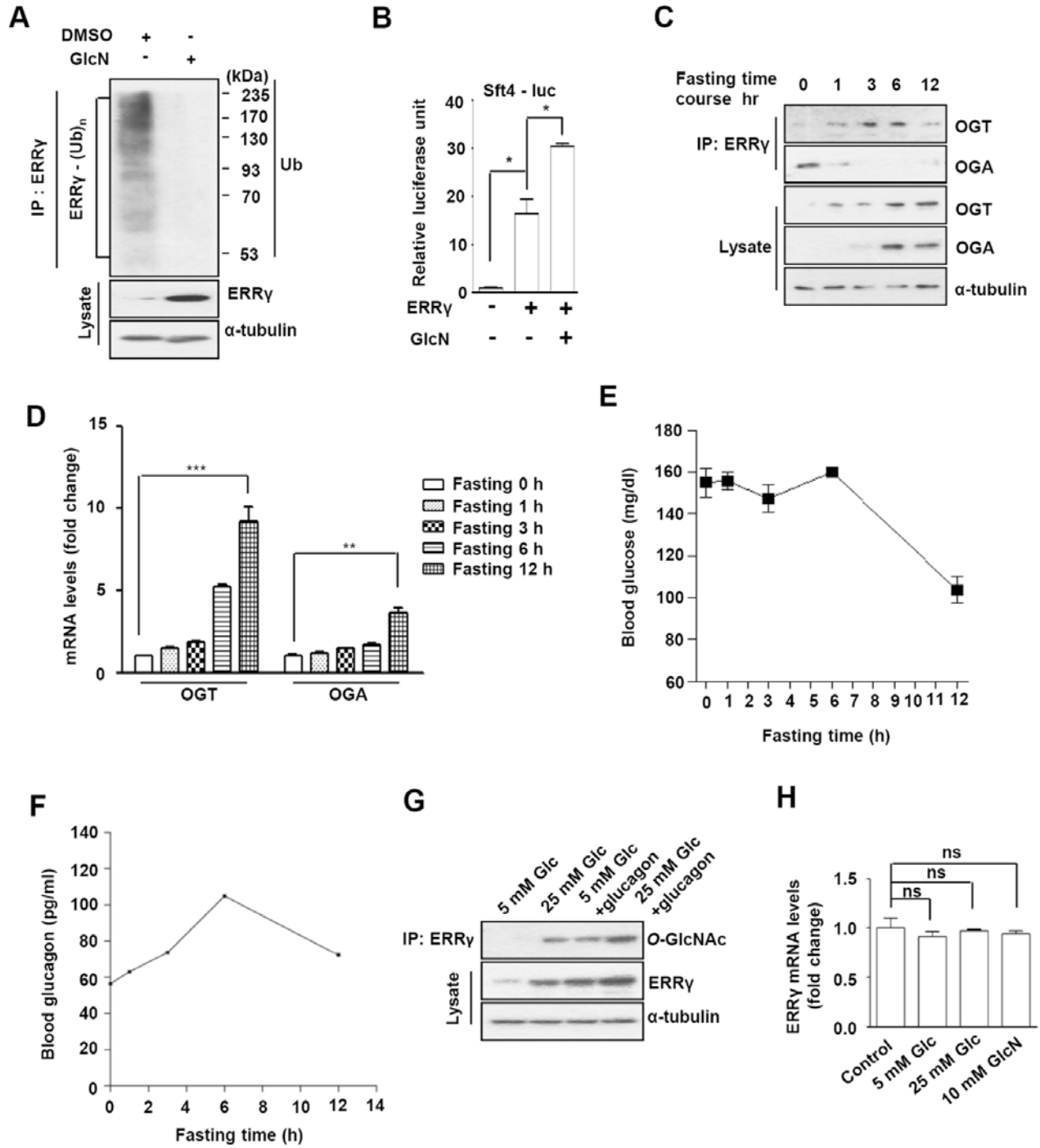
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Supplementary Figure 4. *O*-GlcNAcylation affects ERR γ protein stability and activity.

SUPPLEMENTARY DATA

Supplementary Figure 1. *O*-GlcNAcylation is affected by both glucosamine and high glucose levels. (A) AML 12 cells were treated with DMSO or GlcN (10 mM) for 6 h as indicated. Immunoprecipitation with ERR γ antibody and western blot analyses with Ub, ERR γ and α -tubulin antibodies were performed. (B) HEK 293T cells were transfected with Sft4-luc along with the indicated plasmids followed by GlcN (10 mM) treatment for 6 h. (C) Liver tissue from C57BL/6 mice (n=5) fasted for different time points as indicated, was homogenized for immunoprecipitation with ERR γ antibody and immunoblot with OGT, OGA and α -tubulin antibodies. All of the samples were combined for western blot analyses. (D) Liver tissue from C57BL/6 mice (n=5) fasted for different time points as indicated, was homogenized for RNA isolation and qPCR analyses of total RNA. (E) Fasting blood glucose levels in C57BL/6 mice (n=5). (F) Fasting blood glucagon levels in C57BL/6 mice (n=5). (G) AML12 cells were incubated in 5 mM or 25 mM glucose media overnight followed by glucagon (100 nM) treatment for 6 h. Immunoprecipitation with ERR γ antibody and western blot analyses with *O*-GlcNAc, ERR γ and α -tubulin antibodies were performed as indicated. (H) AML 12 cell were treated with GlcN or different doses of glucose for 6 h followed by ERR γ mRNA measurement by qPCR. NS, not significant. * $p < 0.05$, ** $P < 0.005$ and *** < 0.0005 using Student's t-test. Error bars show \pm s.e.m.

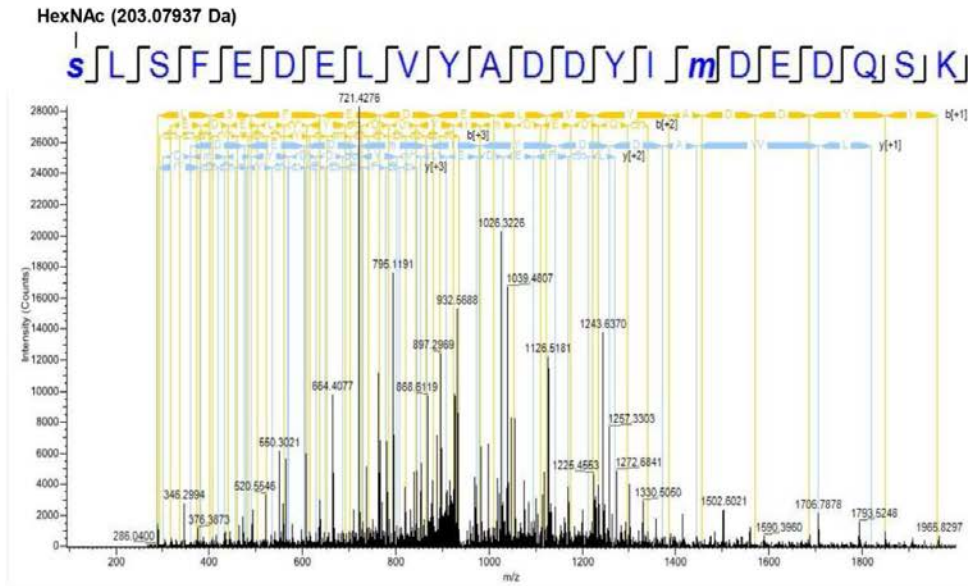
SUPPLEMENTARY DATA



SUPPLEMENTARY DATA

Supplementary Figure 2. LC-MS/MS CID site mapping of the ERR γ O-GlcNAc modification site, S317. Sequence: SLSFEDELVYAD γ DYI [m]DE γ Q[S]K. SI-HexNAc (203.07937 Da), M16-Oxidation (15.99492 Da) MS/MS: CID, Charge: +3, Monoisotopic m/z: 944.40265 Da (-1.41 mmu/-1.5 ppm), MH+: 2831.19339 Da, RT: 46.62 min, Identified with: SEQUEST (v1.13); XCorr: 2.61, Probability:3.23, Ions matched by search engine: 43/252
 Fragment match tolerance used for search: 1.2 Da Fragments used for search: a-H₂O; a-NH₃; b; b-H₂O; b-NH₃; c; y; y-H₂O; y-NH₃; z+1

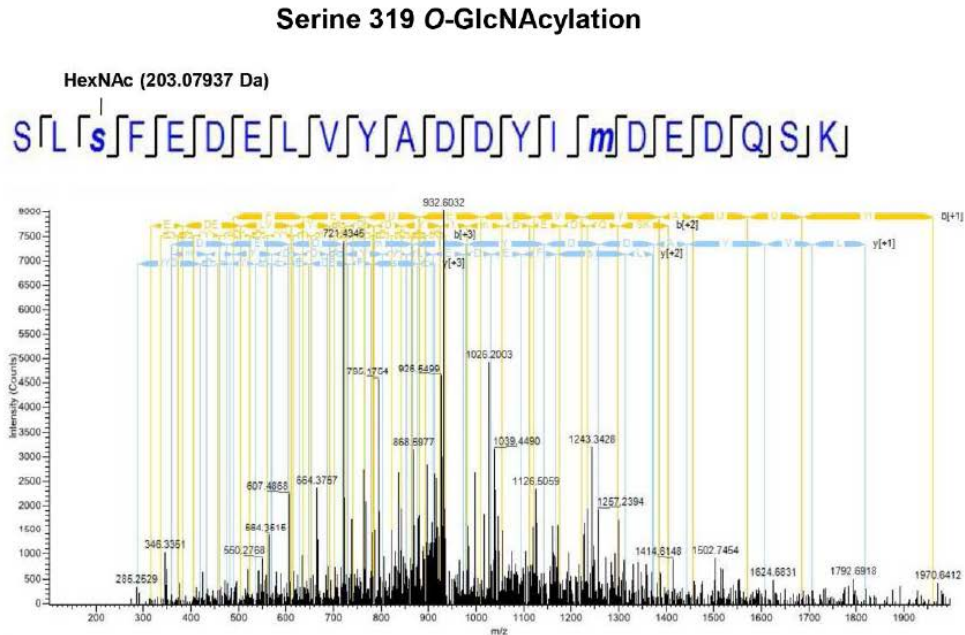
Serine 317 O-GlcNAcylation



#1	b*	b**	b**	c*	c**	c**	Seq.	y*	y**	y**	z+1*	z+1**	z+1**	#2
1	291.11868	146.06298	97.71108	308.14523	154.57625	103.38659	S-HexNAc							22
2	404.20275	202.60501	135.40577	421.22930	211.11829	141.08128	L	2541.08623	1271.04675	847.70026	2526.07533	1263.54130	842.69663	21
3	491.23478	246.12103	164.41644	508.26133	254.63430	170.09196	S	2428.00216	1214.50472	810.00557	2412.99126	1206.99927	805.00194	20
4	638.30320	319.65524	213.43925	655.32975	328.16851	219.11477	F	2340.97013	1170.98870	780.99489	2325.95923	1163.48325	775.99126	19
5	767.34580	384.17654	256.45345	784.37235	392.68981	282.12897	E	2193.90171	1097.45449	731.97209	2178.89081	1089.94904	726.96845	18
6	882.37275	441.69001	294.79577	899.39930	450.20329	300.47128	D	2064.85911	1032.93319	688.95789	2049.84821	1025.42774	683.95425	17
7	1011.41535	506.21131	337.80997	1028.44190	514.72459	343.48548	E	1949.83216	975.41972	650.61557	1934.82126	967.91427	645.61194	16
8	1124.49942	562.75335	375.50466	1141.52597	571.26662	381.18017	L	1820.78956	910.89842	607.60137	1805.77866	903.39297	602.59774	15
9	1223.56784	612.28756	408.52746	1240.59439	620.80083	414.20298	V	1707.70549	854.35638	569.90668	1692.69459	846.85093	564.90305	14
10	1386.63116	693.81922	462.88190	1403.65771	702.33249	468.55742	Y	1608.63707	804.82217	536.88387	1593.62617	797.31672	531.88024	13
11	1457.66828	729.33778	486.56094	1474.69483	737.85105	492.23646	A	1445.57375	723.29051	482.52943	1430.56285	715.78506	477.52580	12
12	1572.69523	786.85125	524.90326	1589.72178	795.36453	530.57878	D	1374.53863	687.77195	458.85039	1359.52573	680.26650	453.84676	11
13	1687.72218	844.36473	563.24558	1704.74873	852.87800	568.92109	D	1259.50068	630.25848	420.50808	1244.49878	622.75303	415.50444	10
14	1850.78550	925.89639	617.60002	1867.81205	934.40966	623.27553	Y	1144.48273	572.74500	382.16576	1129.47183	565.23955	377.16213	9
15	1963.86957	982.43842	655.29471	1980.89612	990.95170	660.97022	I	981.41941	491.21334	327.81132	966.40851	483.70789	322.80769	8
16	2110.90498	1055.95613	704.30651	2127.93153	1064.46941	709.98203	M-Oxidation	868.33534	434.67131	290.11663	853.32444	427.16586	285.11300	7
17	2225.93193	1113.46961	742.64883	2242.95848	1121.98288	748.32435	D	721.29992	361.15360	241.10482	706.28902	353.64815	236.10119	6
18	2354.97453	1177.99091	785.86303	2372.00108	1186.50418	791.33855	E	606.27297	303.64012	202.76251	591.26207	296.13467	197.75888	5
19	2470.00148	1235.50438	824.00535	2487.02803	1244.01786	829.68086	D	477.23037	239.11882	159.74831	462.21947	231.61337	154.74468	4
20	2598.06006	1299.53367	866.89154	2615.08661	1308.04895	872.36706	Q	362.20342	181.60535	121.40599	347.19252	174.09990	116.40236	3
21	2685.09209	1343.04969	895.70222	2702.11864	1351.56296	901.37773	S	234.14484	117.57606	78.71980	219.13394	110.07061	73.71617	2
22							K	147.11281	74.06004	49.70912	132.10191	66.55459	44.70549	1

SUPPLEMENTARY DATA

Supplementary Figure 3. LC-MS/MS CID site mapping of the ERRγ O-GlcNAc modification site, S319. Sequence: SLSFEDELVYAD^YDDYI [m]DE[^YD]Q[S]K], M16- Oxidation (15.99492 Da), MS/MS: CID, Charge: +3, Monoisotopic m/z: 944.40356 Da (-0.5 mmu/-0.53 ppm), MH+: 2831.19614 Da, RT: 46.73 min, Identified with: SEQUEST (v1.13); XCorr:2.56, Probability:3.11, Ions matched by search engine: 39/208
 Fragment match tolerance used for search: 1.2 Da ERRG #



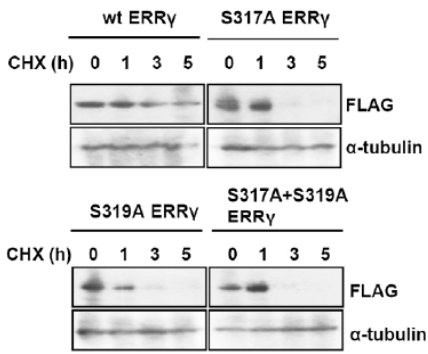
#1	b ⁻	b ⁺	b ⁺	c ⁻	c ⁺	c ⁺	Seq.	y ⁻	y ⁺	y ⁺	z+1 ⁻	z+1 ⁺	z+1 ⁺	#2
1	86.03931	44.52329	30.01795	105.06586	53.03657	35.69347	S							22
2	201.12338	101.06533	67.71264	218.14993	109.57860	73.38616	L	2744.16560	1372.58644	915.39338	2729.15470	1365.08099	910.38975	21
3	491.23478	246.12103	164.41644	508.26133	254.63430	170.09196	S-HexNAc	2631.08153	1316.04440	877.89869	2616.07063	1308.53895	872.69506	20
4	638.30320	319.65524	213.43925	655.32975	328.16851	219.11477	F	2340.97013	1170.98870	780.99489	2325.95923	1163.48325	775.99126	19
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SUPPLEMENTARY DATA

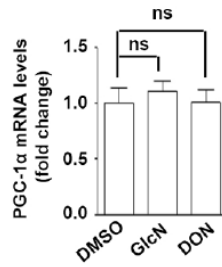
Supplementary Figure 4. *O*-GlcN Acylation affects ERR γ protein stability and activity. (A) HEK 293T cells were transfected with plasmids encoding FLAG-wt ERR γ , FLAG-S317A ERR γ , FLAG-S319A ERR γ and FLAG-S317A+S319A ERR γ . At 24 h after transfection cells were treated with cycloheximide (CHX) (10 mg/mL) for the indicated times and FLAG-ERR γ levels were detected by immunoblot using FLAG and α -tubulin antibodies. (B) AML 12 cell were treated with DMSO or GlcN or DON for 6 h followed by PGC-1 α mRNA measurement by qPCR. NS, not significant. (C) HEK 293T cells were transfected with plasmids encoding FLAG-wt ERR γ , FLAG-S317A ERR γ , FLAG-S319A ERR γ , FLAG-S317A+S319A ERR γ and HA-PGC-1 α . At 24 h post transfection cells were treated with MG-132 (10 μ M) for 6 h followed by immunoprecipitation with HA antibody and immunoblot with FLAG, HA and α -tubulin antibodies. (D) HEK 293T cells were transfected with Gal4-tk-luc along with the indicated plasmids followed by GlcN (10 mM) treatment for 6 h. (E) Effectiveness of OGT overexpression was confirmed by western blot analyses. Mouse Primary hepatocytes were first infected with Ad-US or Ad-shERR γ followed by Ad-GFP or Ad-OGT infection as indicated. Total proteins were analyzed for the FLAG expression by immunoblot. (F) Liver tissue of C57BL/6 mice (n=3) fed with NCD or HFD was homogenized for RNA isolation and qPCR analyses of total RNA. (G) Effectiveness of ERR γ overexpression was confirmed by western blot analyses. Ad-GFP, Ad-wt ERR γ or Ad-S317A+S319A ERR γ was administered via tail- vein injection into C57BL/6 mice (n=5). Total proteins were isolated at day 7 after injection and analyzed for the FLAG expression by immunoblot. (H) Effect of OGA over expression on ERR γ was examined by western blot analyses. Ad-GFP, Ad-wt ERR γ or Ad-wt ERR γ +Ad-OGA was administered via tail-vein injection into C57BL/6 mice (n=5). Total proteins were isolated at day 7 after injection and analyzed for ERR γ expression by immunoblot. *p <0.05, **P <0.005 and ***<0.0005 using Student's t-test. Error bars show \pm s.e.m.

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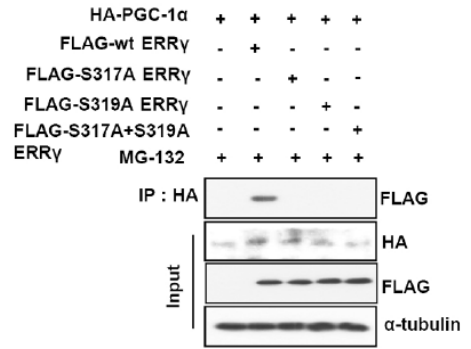
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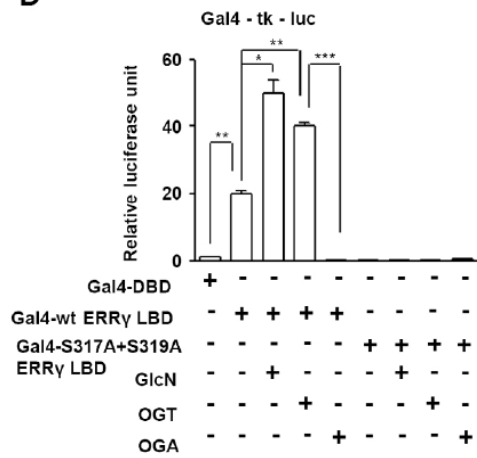
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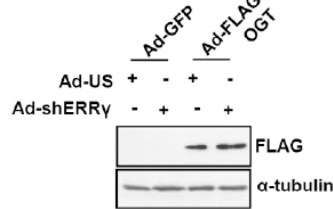
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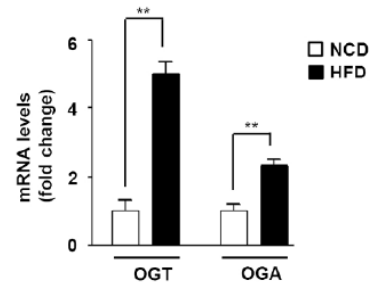
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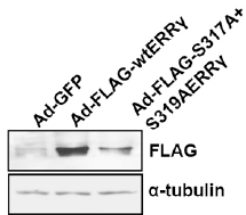
E



F



G



H

