

Expanded View Figures

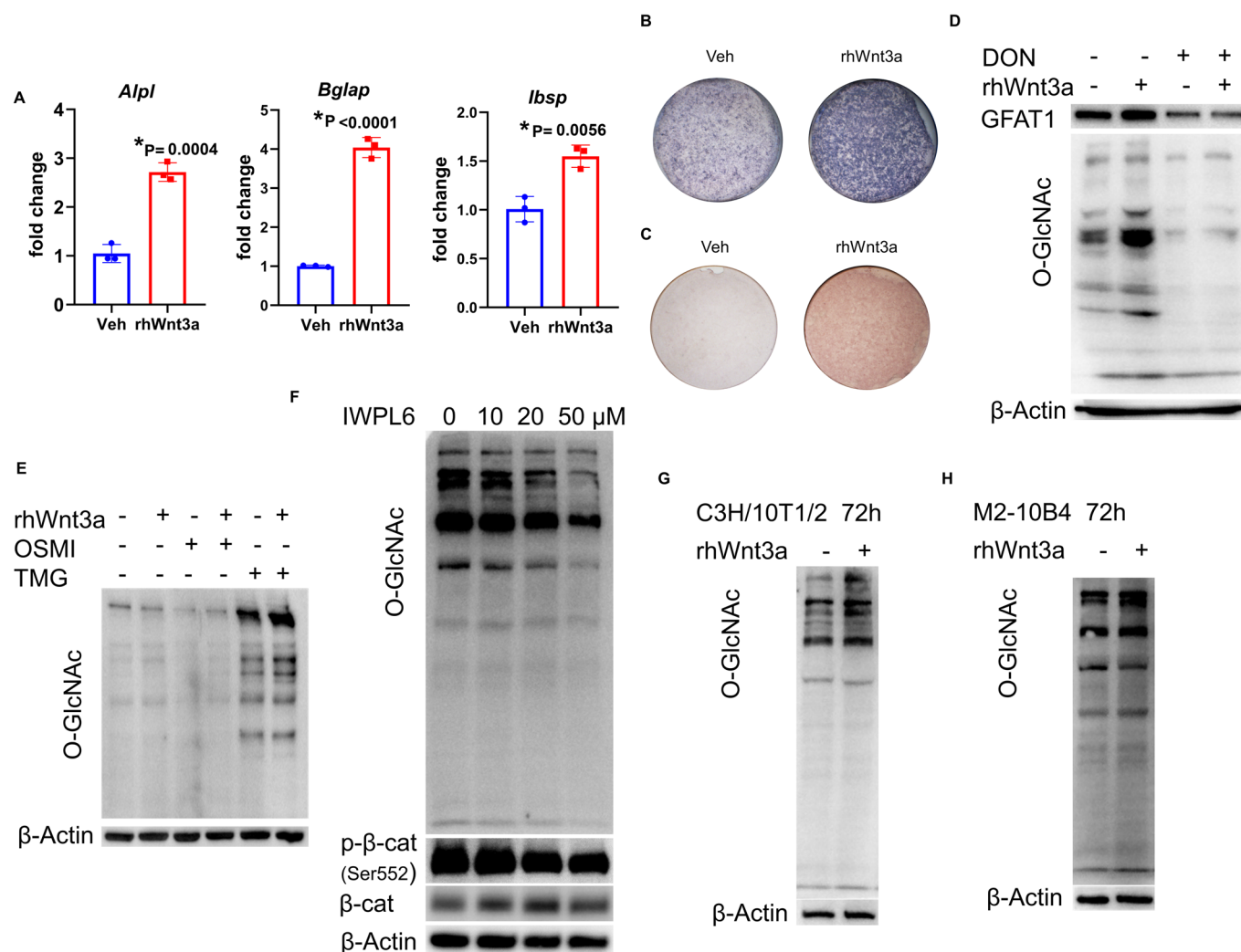


Figure EV1. Wnt3a increases protein O-GlcNAcylation that mediates Wnt-induced osteogenesis in vitro.

(related to Figs. 1 and 2) (A) ST2 cells were treated with 50 ng/ml rhWnt3a for 3 days and relative expression of osteogenic genes was tested by qPCR. Each dot represented one single experiment. Error bars: mean $\pm$ SD. * $p < 0.05$; $n = 3$, biological replicates (two-tailed Student's t-test). (B) ALP staining, and (C) Alizarin red S staining were performed after rhWnt3a treatment for 3 days and 14 days, respectively. (D) ST2 were pretreated with GFAT antagonist DON for 6 h and then administrated with rhWnt3a for 2 days. GFAT1 and O-GlcNAc levels were detected by Western blotting. (E) ST2 were pretreated with OGT and OGA inhibitors, OSMI and TMG, respectively for 6 h and then administrated with rhWnt3a for 2 days. O-GlcNAc levels are detected by Western blotting. (F) ST2 cells were pretreated with IWPL6 for 2 days, and O-GlcNAc, phospho-β-catenin(Ser⁵⁵²) and total β-catenin levels were detected by Western blotting. (G, H) O-GlcNAcylation expression levels of total proteins in C3H/10T1/2 and M2-10B4 cells after 72 h induction by rhWnt3a.

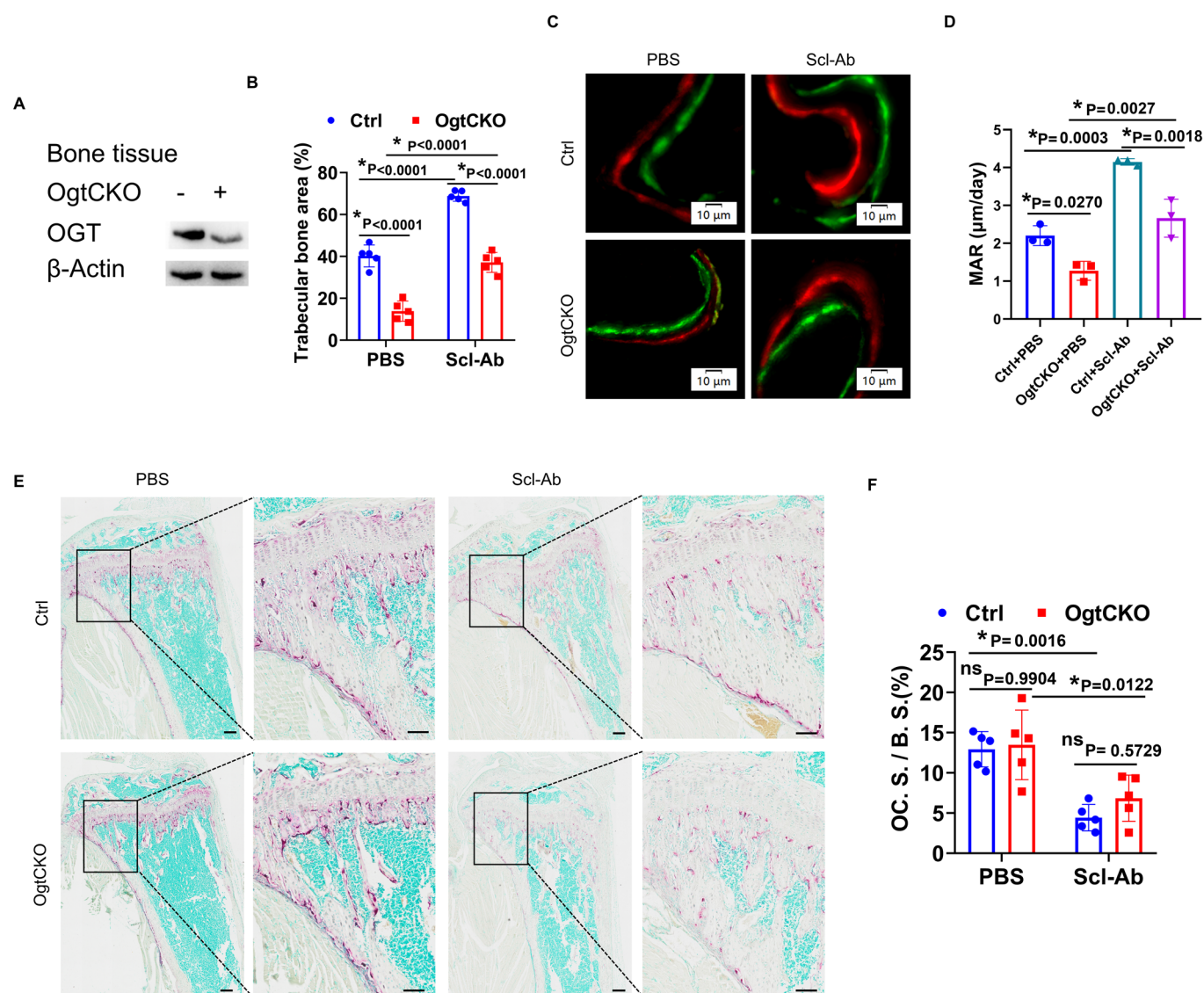


Figure EV2. Deletion of O-GlcNAcylation in osteoblast-lineage cells diminishes Wnt-induced bone formation.

(related to Fig. 3) (A) An examination of OGT protein was conducted in femoral and tibial diaphysis tissues from Ctrl mice and OgtCKO mice. (B) Quantification of proximal tibial trabeculae in Fig. 3H. (C) Representative images of calcein-alizarin red double labeling in the trabecular bone of femurs. Scale bar, 10 μm. (D) The quantification of the Mineral Apposition Rate (MAR) of trabecular bone is shown. (E) TRAP staining for the tibia from Ctrl or OgtCKO mice with or without Scl-Ab injection, scale bar, 200 μm. Boxed area is shown at a high magnification to the right, scale bar, 100 μm. (F) Osteoclast (TRAP-positive multinucleated cells) surface normalized to bone surface (OC.S./B.S.) is shown. The ROI was around 0.09 mm² region (300 × 300 μm) underneath the growth plate. Each dot represented one animal. Error bars: mean ± SD. **p* < 0.05, ANOVA followed by Tukey's multiple comparisons test.

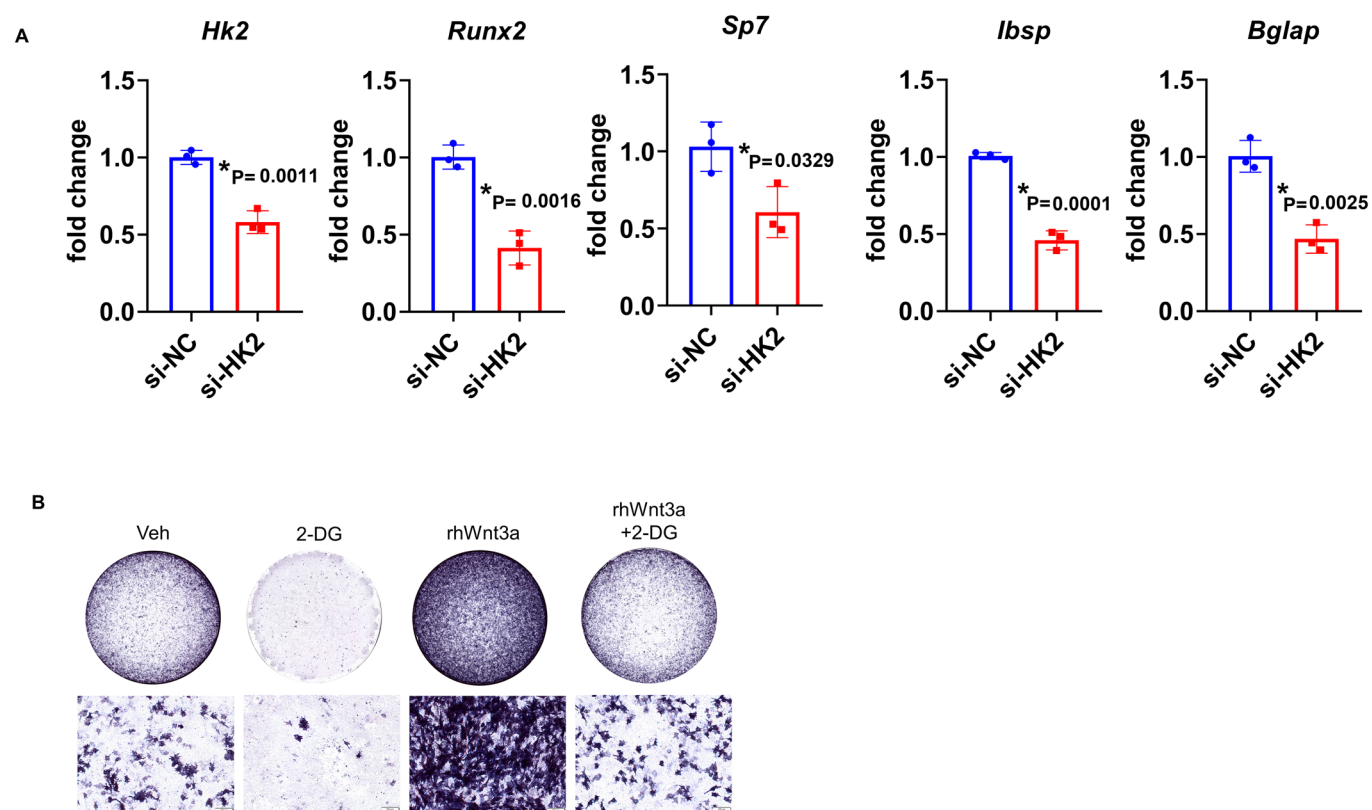


Figure EV3. O-GlcNAcylation is indispensable for Wnt3a-increased aerobic glycolysis.

(related to Figs. 5 and 8) (A) Osteoblastic gene detection by qPCR in the absence of HK2. Each dot represented one single experiment. Error bars: mean $\pm$ SD. * $p < 0.05$; $n = 3$ (two-tailed Student's *t*-test). (B) ST2 cells were pretreated with 2-DG for 1 day and then exposed to rhWnt3a for 3 additional days. Then ALP staining was performed.

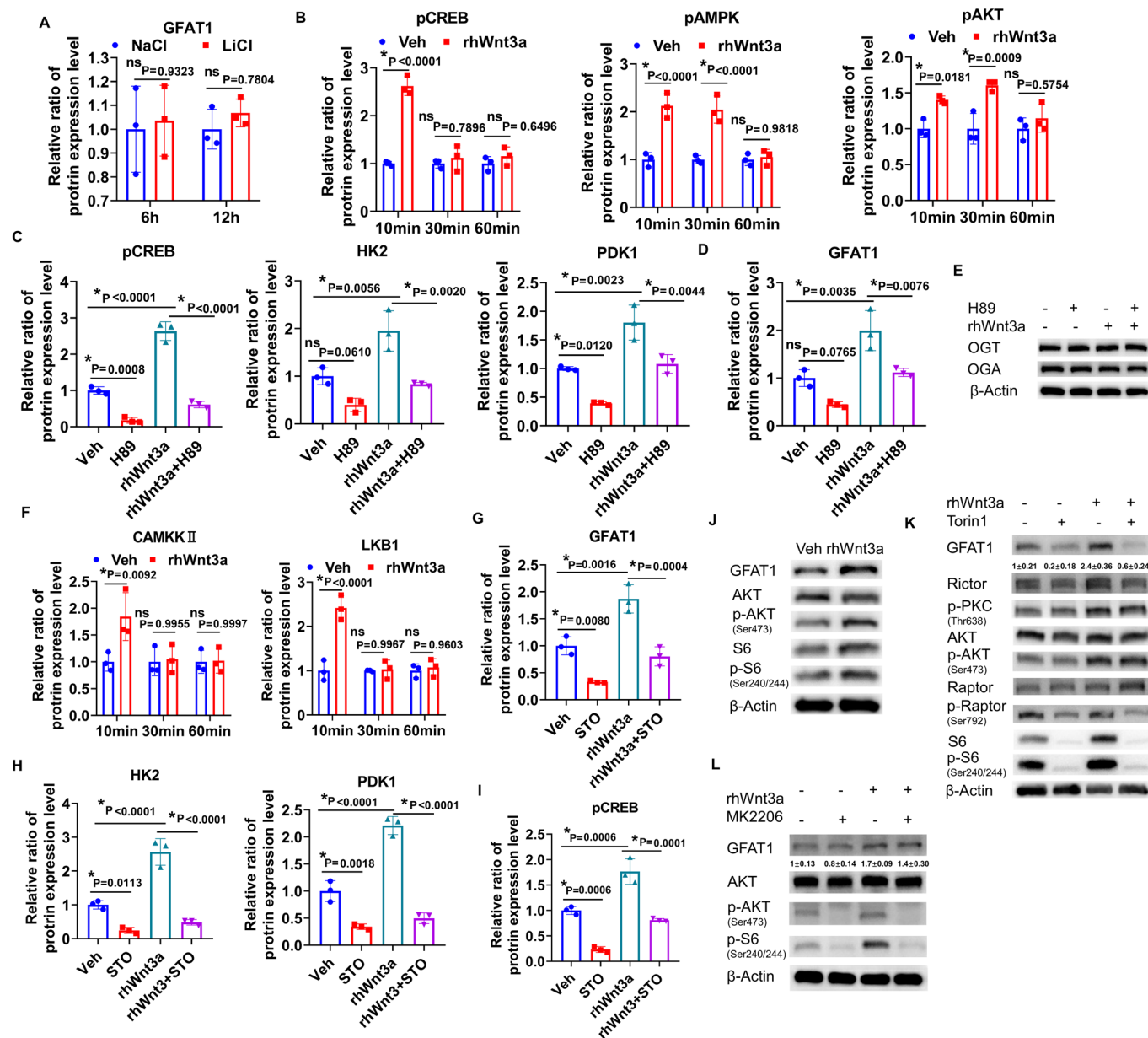


Figure EV4. Wnt rapidly increases O-GlcNAcylation via the Ca^{2+} -PKA-GFAT1 axis.

(related to Fig. 7) (A) Quantification of GFAT1 protein shown in Fig. 7A. (B) Quantification of pCREB, pAMPK, and pAKT proteins shown in Fig. 7B. (C) Quantification of pCREB, HK2, and PDK1 proteins shown in Fig. 7C. (D) Quantification of GFAT1 protein shown in Fig. 7D. (E) ST2 cells were treated with rhWnt3a, H89, or both for 6 h. OGT and OGA expression levels were detected. (F) Quantification of CAMKKII and LKB1 proteins shown in Fig. 7F. (G) Quantification of GFAT1 protein shown in Fig. 7G. (H) Quantification of HK2 and PDK1 proteins shown in Fig. 7H. (I) Quantification of pCREB protein shown in Fig. 7I. (J) The mTOR downstream targets expression in response to rhWnt3a. (K) The mTOR inhibition decreases GFAT1 protein level. (L) The GFAT1 expression detection after the mTORC2 inhibition. All data are shown as mean $\pm$ SD, $n = 3$, biological replicates. * $p < 0.05$, (A, B, F) two-way ANOVA followed by Sidak's multiple comparisons test, (C, D, G, H and I) one-way ANOVA followed by Tukey's multiple comparisons test.

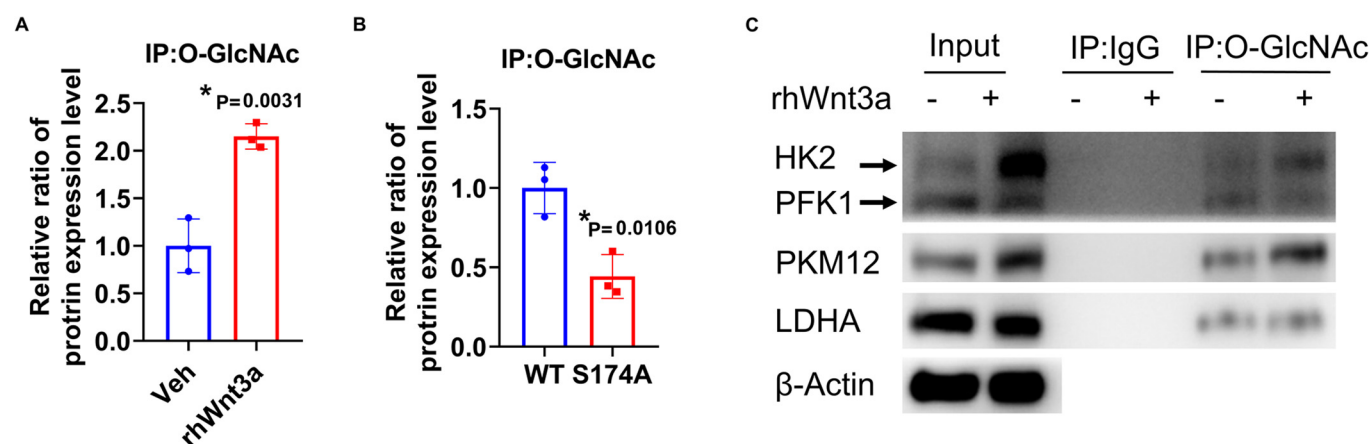


Figure EV5. O-GlcNAcylation on the Ser¹⁷⁴ site stabilizes PDK1 and facilitates Wnt3a-induced osteogenesis.

(related to Fig. 8) (A) Quantification of the IPed O-GlcNAc protein in Fig. 8B. (B) Quantification of the IPed O-GlcNAc protein in Fig. 8E. (C) Immunoprecipitation with endogenous O-GlcNAc and detection with HK2, PFK1, PKM12, and LDHA, respectively. Error bars: mean $\pm$ SD. * $p < 0.05$; $n = 3$, biological replicates (two-tailed Student's t -test).