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Supplemental Information

Insulin Promotes Mitochondrial Respiration and Survival through PI3K/AKT/GSK3 Pathway in Human Embryonic Stem Cells

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Figure S1

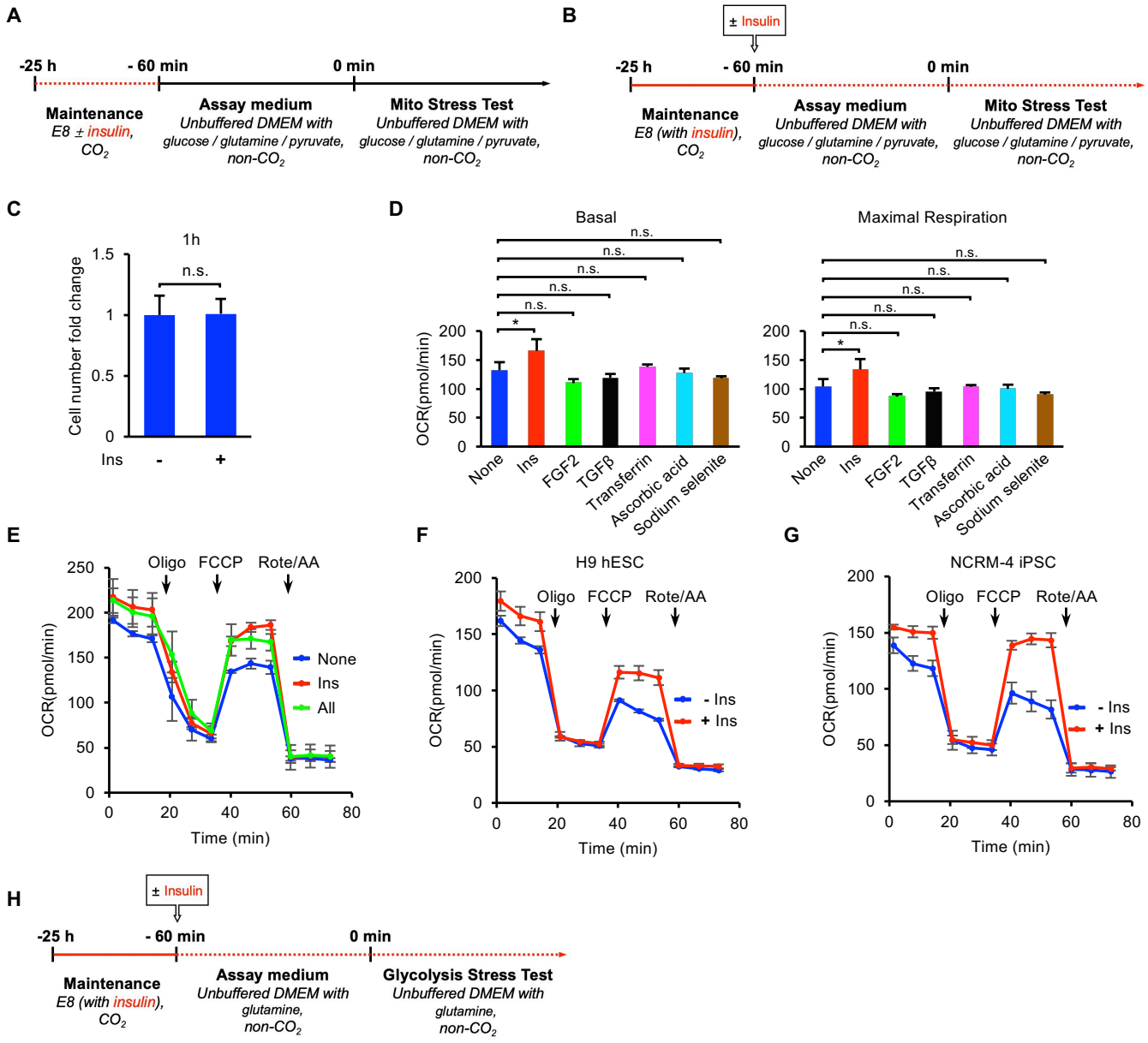

Figure S1, Related to Figure 1. Insulin is required to maintain mitochondrial respiration in hESCs.
(A) Schematic of Mito Stress Test assay workflow related to Figure 1B.
(B) Schematic of Mito Stress Test assay workflow related to Figure 1D.
(C) Survival of H1 hESCs after one hour of treatments with or without insulin (10 µg/mL). The cell numbers were determined using a BD Accuri C6 flow cytometer (n = 4 independent replicates) .
(D) Basal and Maximal Respiration calculated from Figure 1F.
(E) OCR measurement by Mito Stress Test in H1 hESCs following one hour of pre-incubation with insulin, all six essential factors (insulin, FGF2, TGFβ, transferrin, ascorbic acid, sodium selenite) or none.
(F-G) OCR measurement by Mito Stress Test in H9 hESCs or NCRM-4 iPSCs following one hour of pre-incubation with or without insulin.
(H) Workflow for Glycolysis Stress Test related to Figure 1H.
Data are presented as means ± SD.

Figure S2

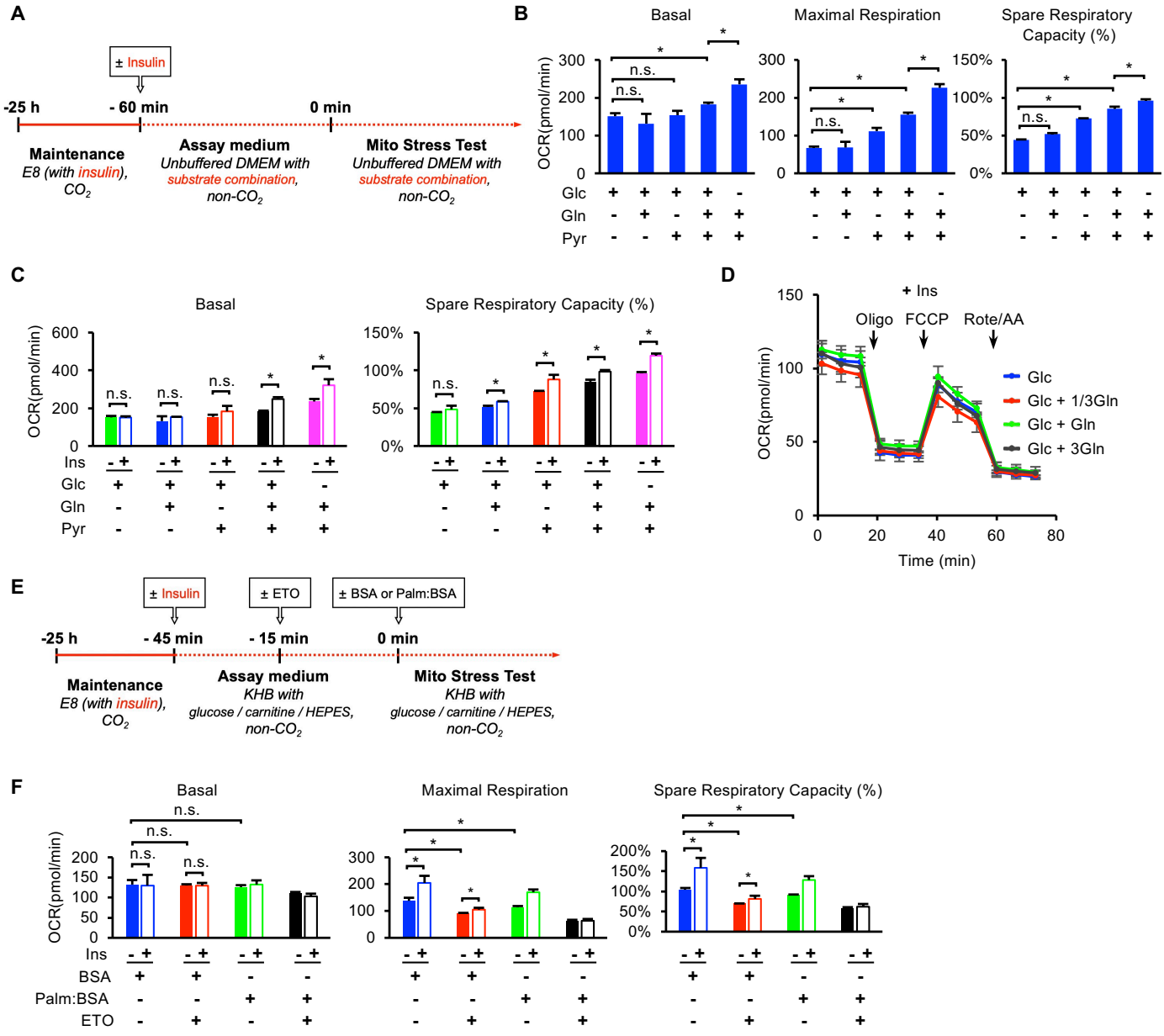

Figure S2, Related to Figure 2. Insulin promotes mitochondrial respiration through specific energy substrates.
(A) Workflow for Mito Stress Test with different substrate combinations.
(B) Basal, Maximal Respiration and Spare Respiratory Capacity calculated from Figure 2A.
(C) Basal and Spare Respiratory Capacity calculated from Figures 2A and 2B.
(D) OCR measurement by Mito Stress Test in H1 hESCs following one hour of pre-incubation with treatments of glucose (Glc) in combination with different doses of glutamine (Gln).
(E) Workflow for FAO assay.
(F) Individual respiration parameters, including Basal, Maximal Respiration and Spare Respiratory Capacity, calculated from Figures 2I and 2J.
Data are presented as means ± SD.

Figure S3

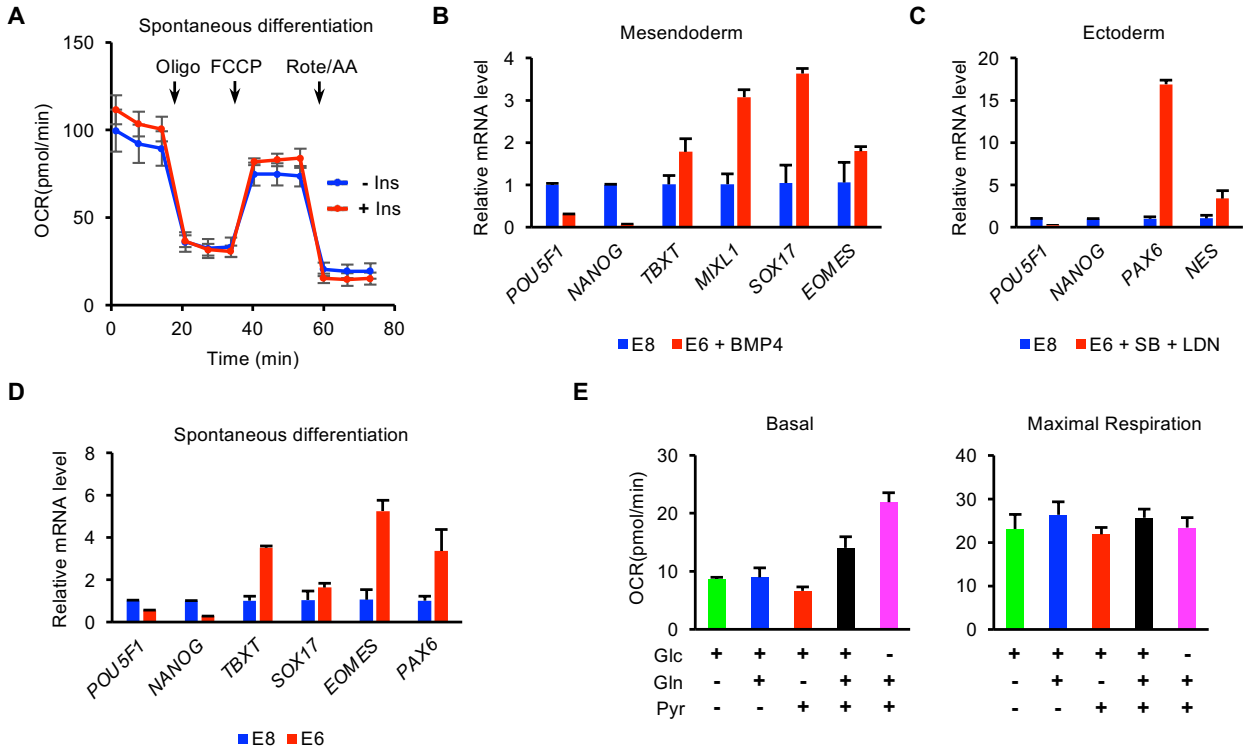

Figure S3, Related to Figure 3. Insulin-dependent mitochondrial respiration is cell type-specific.
(A) OCR was measured by Mito Stress Test in spontaneously differentiated cells from H1 hESCs after one hour of pre-incubation with or without insulin.
(B) qPCR analysis of pluripotency markers *POU5F1* and *NANOG* and mesendoderm makers *TBXT*, *MIXL1*, *SOX17* and *EOMES* of cells in E6 (E8 without FGF2 and TGFβ) supplemented BMP4 (20 ng/mL) for 2 days.
(C) qPCR analysis of pluripotency markers *POU5F1* and *NANOG* and ectoderm makers *PAX6* and *NES* of cells in E6 supplemented SB431542 (10 μM) + LDN193189 (100 nM) for 2 days.
(D) qPCR analysis of pluripotency markers *POU5F1* and *NANOG* and a mesoderm maker *TBXT*, endoderm markers *SOX17* and *EOMES* and an ectoderm marker *PAX6* of cells in E6 for 2 days.
(E) Basal and Maximal Respiration calculated from Figure 3D.
Data are presented as means ± SD.

Figure S4

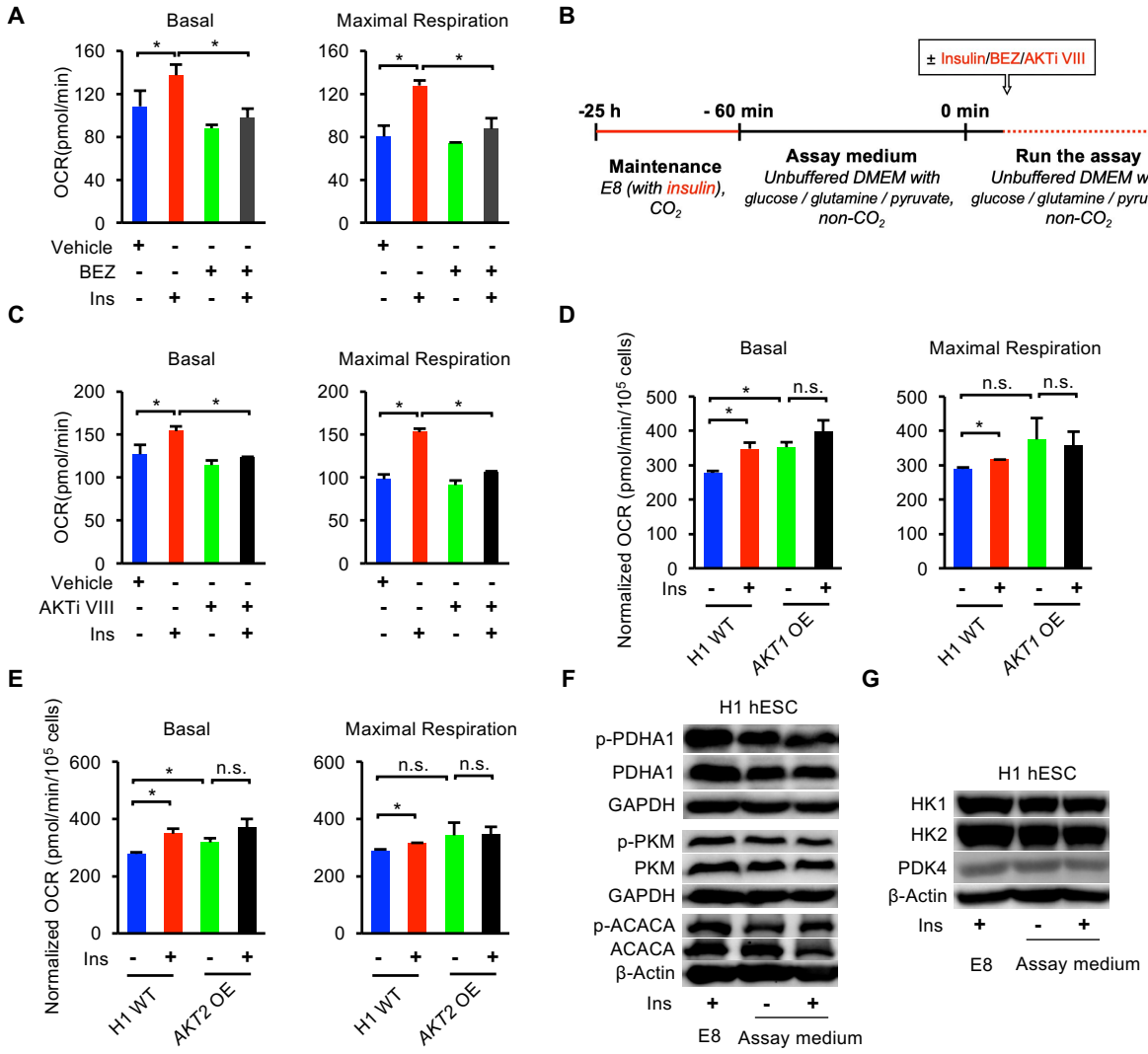

Figure S4, Related to Figure 4. Insulin regulates mitochondrial respiration via PI3K/AKT in hESCs.

(A) Basal and Maximal Respiration calculated from Figure 4B.

(B) Workflow for kinetic OCR response analysis with insulin, BEZ235 or AKTi VIII in Figures 4C and 4E.

(C) Basal and Maximal Respiration calculated from Figure 4D.

(D) Basal and Maximal Respiration calculated from Figure 4F.

(E) Basal and Maximal Respiration calculated from Figure 4G.

(F) Western blot analysis of p-PDHA1 (phospho-pyruvate dehydrogenase E1-alpha subunit), PDHA1, p-PKM (phospho-pyruvate kinase M2), PKM, p-ACACA (phospho-acetyl-CoA carboxylase), ACACA in H1 hESCs cultured in E8 or Mito Stress Test assay medium with or without insulin for one hour. β-Actin, loading control.

(G) Western blot analysis of HK1 (hexokinase 1), HK2 (hexokinase 2), PDK4 (pyruvate dehydrogenase kinase 4) in H1 hESCs cultured in E8 or Mito Stress Test assay medium with or without insulin for one hour. β-Actin, loading control.

Data are presented as means ± SD.

Figure S5

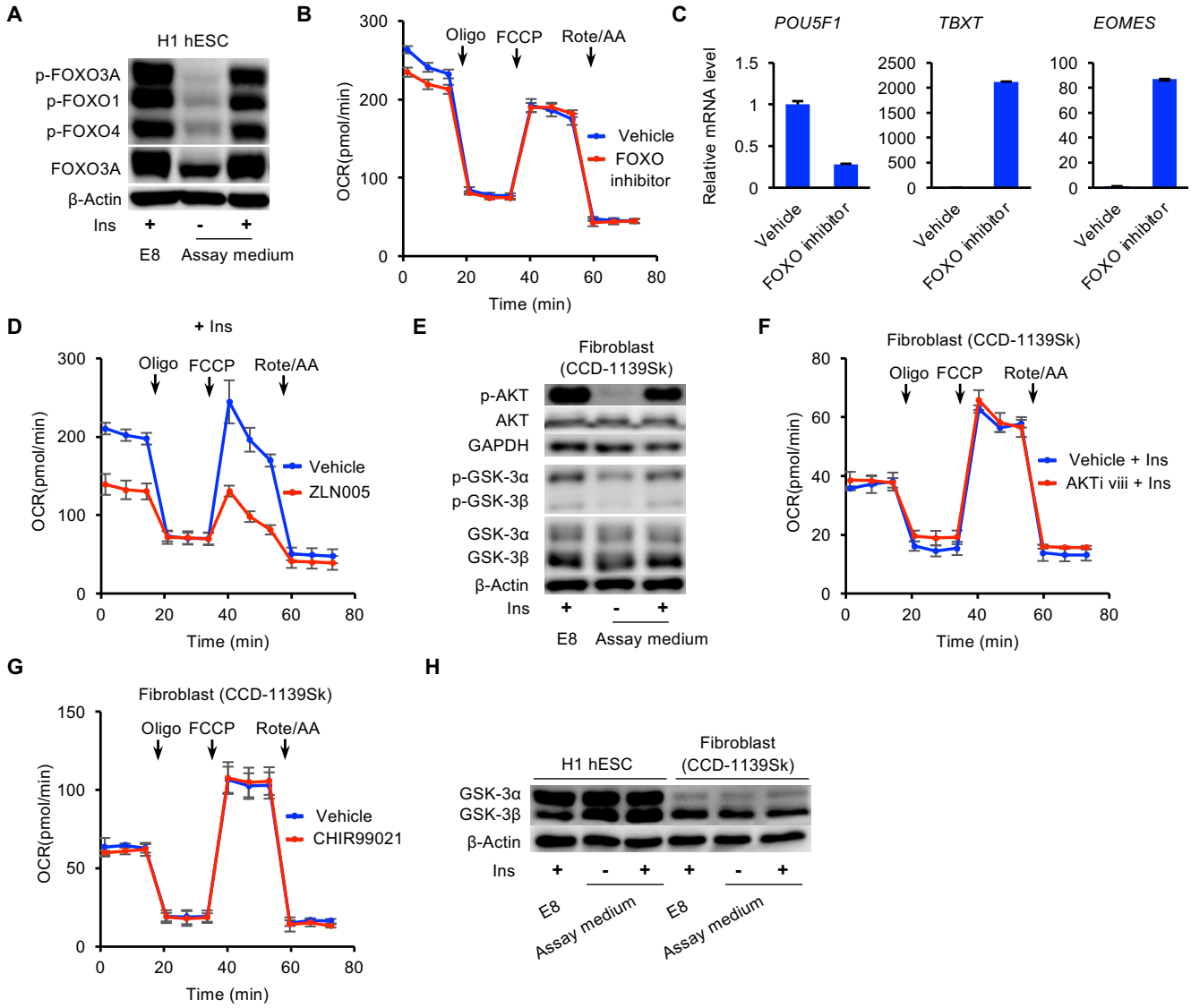

Figure S5, Related to Figure 5. GSK3 inhibition promotes mitochondrial respiration in hESCs.

(A) Western blot analysis of p-FOXO1/3A/4 and FOXO3A in H1 hESCs cultured in E8 or Mito Stress Test assay medium with or without insulin for one hour. β-Actin, loading control.

(B) OCR was measured by Mito Stress Test in H1 hESCs after one hour of pre-incubation with the FOXO inhibitor (AS1842856, 10 μM) or vehicle.

(C) qPCR analysis of a pluripotency marker *POU5F1*, and mesoderm makers *TBXT* and *EOMES* of cells in E8 or E8 supplemented with the FOXO inhibitor (AS1842856, 2 μM) for 2 days.

(D) OCR was measured by Mito Stress Test in H1 hESCs after one hour of pre-incubation with ZLN005 (a PGC1α inhibitor) or vehicle in the presence of insulin.

(E) Western blot analysis of p-AKT, AKT, p-GSK-3α/β, GSK-3α/β in fibroblasts cultured in E8 or Mito Stress Test assay medium with or without insulin for one hour. GAPDH and β-Actin, loading controls.

(F) OCR measurement in fibroblasts by Mito Stress Test following one hour of pre-incubation with vehicle + insulin or AKTi VIII + insulin.

(G) OCR measurement in fibroblasts by Mito Stress Test following one hour of pre-incubation with vehicle or the GSK3 inhibitor CHIR99021.

(H) Western blot analysis of GSK-3α/β in H1 hESCs and fibroblasts cultured in E8 or Mito Stress Test assay medium with or without insulin for one hour. β-Actin, loading control.

Data are presented as means ± SD.

Figure S6

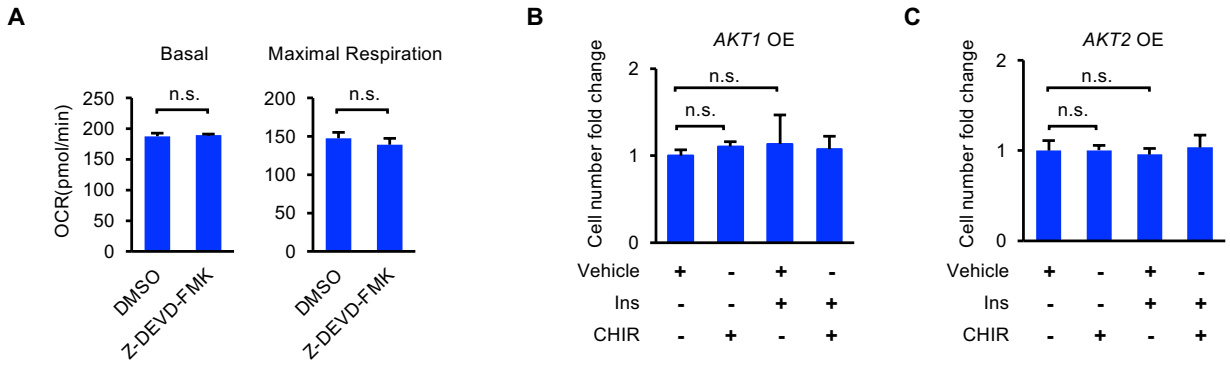

Figure S6, Related to Figure 6. GSK3 inhibition promotes the survival of hESCs.
(A) Basal and Maximal Respiration calculated from Figure 6C.
(B-C) Cell survival of dissociated *AKT1*- and *AKT2*- overexpressing H1 cells 24 hours after plating with vehicle ± insulin or CHIR99021 (1 μM) ± insulin as indicated. Vehicle, DMSO. The cell numbers were determined using a BD Accuri C6 flow cytometer (n = 3 independent replicates).
Data are presented as means ± SD.

Supplemental Experimental Procedures

Immunostaining

H1 hESCs were grown on Matrigel-coated glass coverslips in E8 medium. Then the growth medium was changed to Mito Stress Test assay medium supplemented with insulin (10 μg/mL, Sigma) for one hour. Cells were incubated with 100 nM MitoTracker Red CMXRos (Invitrogen) for 30 minutes. The cells were fixed with 4% paraformaldehyde for 10 minutes on ice, and then permeabilized with 0.1% Triton X-100 with 0.5% BSA for 15 minutes. Subsequently, the cells were incubated with primary antibodies overnight at 4 ℃. The primary antibody used was AKT-S473 (9271, Cell signaling technology). On the following day, the cells were washed with PBS and incubated with secondary antibody (111-545-046, Jackson ImmunoResearch Lab, Inc) in room temperature for one hour, followed by staining with Hoechst for 10 minutes in the dark. Confocal microscopy images were obtained with Carl Zeiss Confocal LSM710.

Mitochondrial fraction extraction

After two hours of treatments with or without insulin in Mito Stress assay medium, H1 hESCs were collected and lysed on ice with subcellular fraction buffer containing HEPES (Sigma, 20 mM), KCl (Sigma, 10 mM), $MgCl_2$ (Sigma, 2mM), EDTA (Sigma, 1 mM), EGTA (Sigma, 1mM), DTT (Promega, 1mM) and protease inhibitors (Roche). The lysate was passed through a needle (27G) 10 times. Then the lysate was centrifuged at 720 g for 5 min, resulting supernatant containing mitochondria, cytoplasm and membrane. The supernatant was further centrifuged at 10000 g for 5min. Then the pellet was washed with subcellular fraction buffer twice. The pellet was designated as the mitochondrial faction.

Western blot analysis

H1 hESCs were collected with 2x Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) with protease inhibitors (Roche). Samples were preheated at 100 ℃ for 5 minutes. The lysates were separated electrophoretically on SDS-PAGE gels and were transferred to PVDF membranes. Then 5% non-fat milk in TBST was employed to block non-specific binding. The membranes were blotted with primary antibodies overnight at 4 ℃. The primary antibodies used were AKT (4691, Cell signaling technology), AKT-S473 (9271, Cell signaling technology), GAPDH (sc-25778, Santa cruz biotechnology), GSK-3α/β (5676, Cell signaling technology), p-GSK-3α/β (9331,Cell signaling technology), β-actin (sc-47778, Santa cruz biotechnology), cleaved caspase 3 (9664, Cell signaling technology), HK1 (2024, Cell signaling technology), HK2 (2867, Cell signaling technology), PDHA1 (ab110334, Abcam), p-PDHA1 (ab92696, Abcam), PDK2 (ab172065, Abcam), p-PKM (3827, Cell signaling technology), PKM (4053, Cell signaling technology), p-ACACA (11818, Cell signaling technology), ACACA (3676, Cell signaling technology), PDK4 (sc-14495, Santa cruz biotechnology), p-FOXO1/3A/4 (2599, Cell signaling technology), FOXO3A (2497, Cell signaling technology). The secondary antibodies were horse radish peroxidase-conjugated and were purchased from Jackson ImmunoResearch Lab, Inc. After incubation with secondary antibodies conjugated to horseradish peroxidase for 2 hours at room temperature, the immune complexes were detected by enhanced chemiluminescence method (ThermoFisher).

Cell differentiation

The differentiation protocols used in this study are described below. To induce the differentiation of hESCs to different lineages, H1 hESCs maintained in E8 medium were passaged 1:6 onto Matrigel-coated plates (Corning) with DPBS/EDTA in the presence of Y-27632 (Selleck) in E6 medium (E8 without FGF2 and TGFβ). The cells were culture in E6, E6 + BMP4 (20ng/mL, R&D), or E6 + SB431542 (10μM, Selleck) + LDN193189 (100nM, Selleck) for the next two days to induce spontaneous differentiation, mesendoderm and ectoderm differentiation, respectively. The medium was changed every day.

To test the activity of the FOXO inhibitor (AS1842856), H1 hESCs were passaged 1:6 in E8 medium. Then the cells were switched to E8 medium supplemented the inhibitor (2 μM) for two more days. The medium was changed every day.

Quantitative RT-PCR (qPCR)

Total RNA from cells was extracted using RNAiso Plus reagent (Takara) according to the manufacturer's protocol. cDNA was synthesized from RNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Inc.). Takara SYBR® Premix Ex TaqTM II was applied to perform real-time PCR on

Applied Biosystems QuantStudio 7 Flex. The data were normalized by GAPDH. Primers used are listed in the table below:

mRNA Transcript	Forward primer	Reverse primer
POUR5F1	AGCGAACCAGTATCGAGAACC	CTGATCTGCTGCAGTGTGGGT
NANOG	GATGCCTCACACGGAGACTG	GCAGAAGTGGGTTGTTTGCC
TBXT	CCCTATGCTCATCGGAACAA	CAATTGTCATGGGATTGCAG
MIXL1	AGCTGCTGGAGCTCGTCTTC	TGGAAGGATTTCCCACTCTG
SOX17	CGCACGGAATTTGAACAGTA	GGATCAGGGACCTGTCACAC
EOMES	GTGCCCACGTCTACCTGTG	CCTGCCCTGTTTCGTAATGAT
PAX6	TCGAAGGGCCAAATGGAGAAGAGAAG	GGTGGGTTGTGGAATTGGTTGGTAGA
NES	CTACCAGGAGCGCGTGGC	TCCACAGCCAGCTGGAAC
GAPDH	GTGGACCTGACCTGCCGTCT	GGAGGAGTGGGTGTCGCTGT