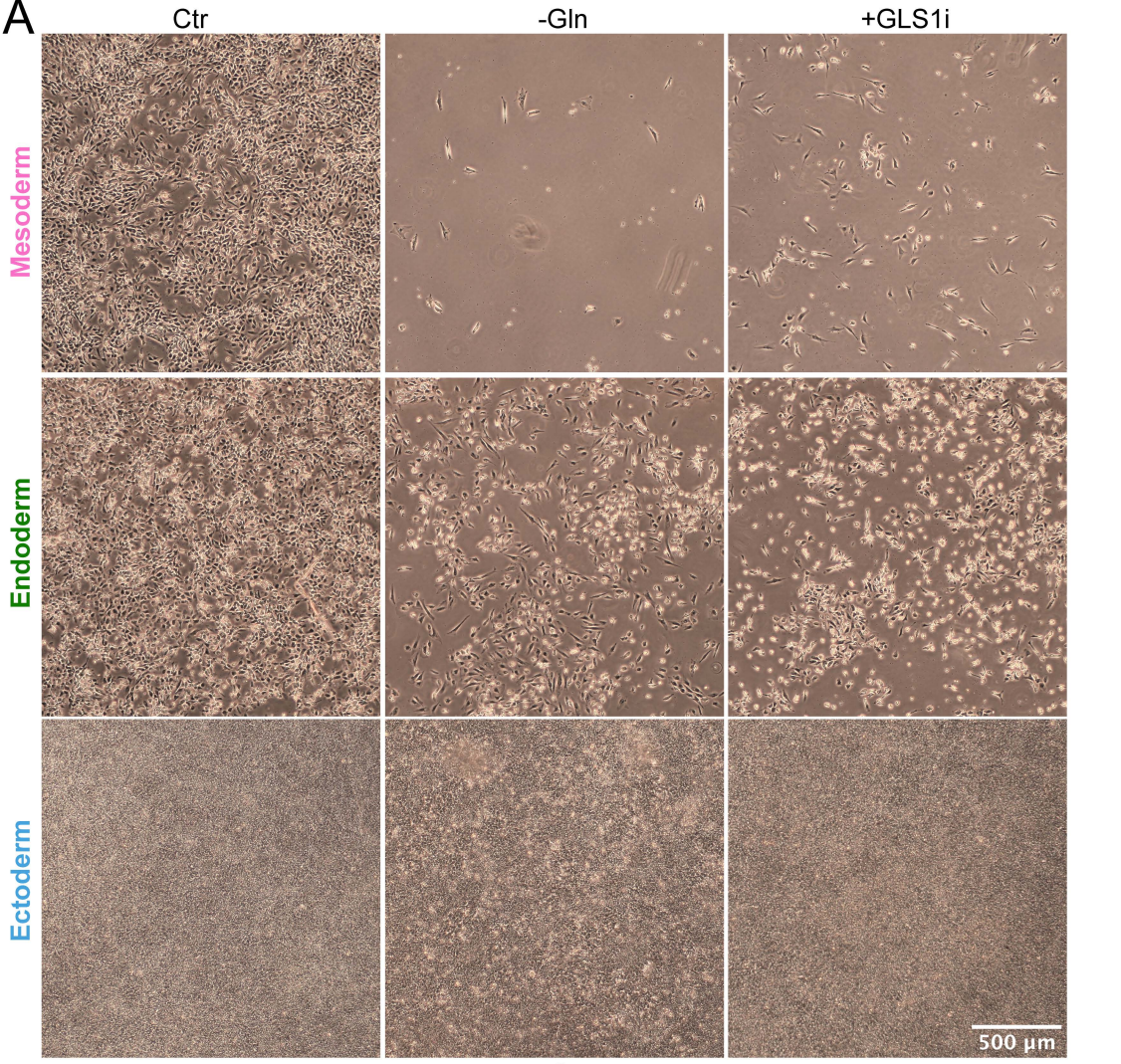
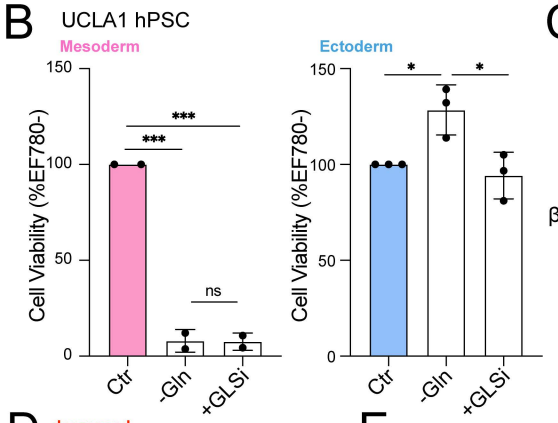
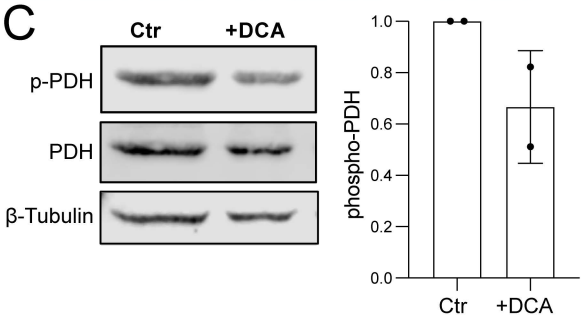
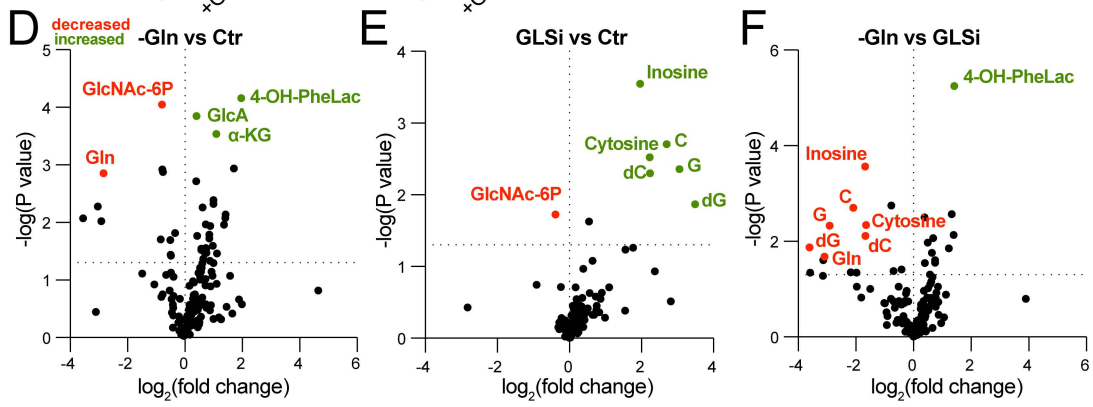
Figure S1


A
Ctr
-Gln
+GLS1i
Mesoderm
Endoderm
Ectoderm
500 µm


B
UCLA1 hPSC
Mesoderm
Ectoderm
Cell Viability (%EF780-)
***
***
ns
*
*
Ctr
-Gln
+GLSi


C
Ctr
+DCA
p-PDH
PDH
β-Tubulin
phospho-PDH
Ctr
+DCA


D
decreased
increased
-Gln vs Ctr
GlcNAc-6P
4-OH-PheLac
GlcA
α-KG
Gln
-log(P value)
log2(fold change)
E
GLSi vs Ctr
Inosine
Cytosine
C
dC
G
dG
GlcNAc-6P
F
-Gln vs GLSi
4-OH-PheLac
Inosine
C
G
Cytosine
dG
dC
Gln

Supplemental Figure S1. Cell viability images and quantification under Gln deprivation
Related to Figure 1
(A) Representative brightfield images of cell viability and confluency of D5 mesoderm, D3 endoderm, and D5 ectoderm grown in Gln-supplemented (Ctr), Gln-free (-Gln), or glutaminolysis-inhibited (+GLS1i) conditions. Scale bar indicates 500 μm.
(B) Cell viability of UCLA1-derived D5 mesoderm (n=2) and ectoderm cells relative to Ctr.
(C) Immunoblot and densitometry quantification of phosphorylated pyruvate dehydrogenase (PDH) and total PDH expression after 24h 1 mM DCA treatment (n=2).
(D-F) Volcano plots of differential metabolite abundance in (**D**) -Gln vs Ctr, (**E**) GLS1i vs Ctr, and (**F**) -Gln vs GLS1i conditions, quantified by UHPLC-MS. Differential fold change is plotted against raw p values for each metabolite comparison.

Data represent mean ± SD of n ≥ 3 biological replicates unless indicated otherwise. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$. The p values were determined by (**B**) one-way ANOVA with correction for multiple comparisons, or (**D-F**) using the Student's t test

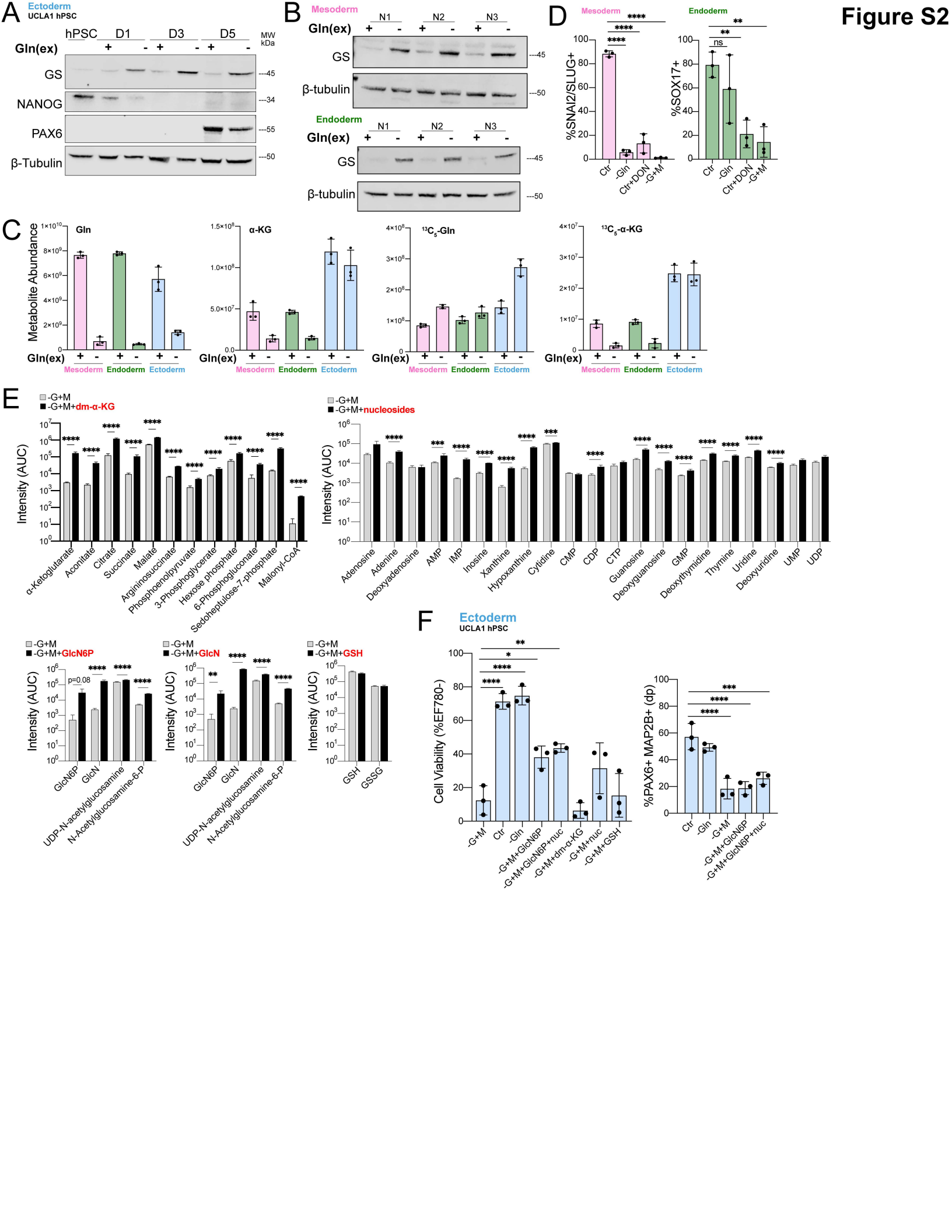

A
Ectoderm
UCLA1 hPSC
hPSC
D1
D3
D5
MW kDa
Gln(ex)
GS
NANOG
PAX6
β-Tubulin
B
Mesoderm
N1
N2
N3
Gln(ex)
GS
β-tubulin
Endoderm
D
Mesoderm
Endoderm
%SNAI2/SLUG+
%SOX17+
Ctr
-Gln
Ctr+DON
-G+M
ns
C
Metabolite Abundance
Gln
α-KG
13C5-Gln
13C5-α-KG
Gln(ex)
Mesoderm
Endoderm
Ectoderm
E
-G+M
-G+M+dm-α-KG
Intensity (AUC)
α-Ketoglutarate
Aconitate
Citrate
Succinate
Malate
Argininosuccinate
Phosphoenolpyruvate
3-Phosphoglycerate
Hexose phosphate
6-Phosphogluconate
Sedoheptulose-7-phosphate
Malonyl-CoA
-G+M+nucleosides
Adenosine
Adenine
Deoxyadenosine
AMP
IMP
Inosine
Xanthine
Hypoxanthine
Cytidine
CMP
CDP
CTP
Guanosine
Deoxyguanosine
GMP
Deoxythymidine
Thymine
Uridine
Deoxyuridine
UMP
UDP
-G+M+GlcN6P
p=0.08
GlcN6P
GlcN
UDP-N-acetylglucosamine
N-Acetylglucosamine-6-P
-G+M+GlcN
-G+M+GSH
GSH
GSSG
F
Ectoderm
UCLA1 hPSC
Cell Viability (%EF780-)
-G+M
Ctr
-Gln
-G+M+GlcN6P
-G+M+GlcN6P+nuc
-G+M+dm-α-KG
-G+M+nuc
-G+M+GSH
%PAX6+ MAP2B+ (dp)
Ctr
-Gln
-G+M
-G+M+GlcN6P
-G+M+GlcN6P+nuc

Figure S2

Supplemental Figure S2. Tri-lineage metabolomics profiles in Gln-free media

Related to Figure 2

(A-B) Immunoblot of GS expression in (**A**) UCLA1-derived ectoderm and (**B**) H9-derived mesoderm and endoderm cells differentiated in Gln-free media.

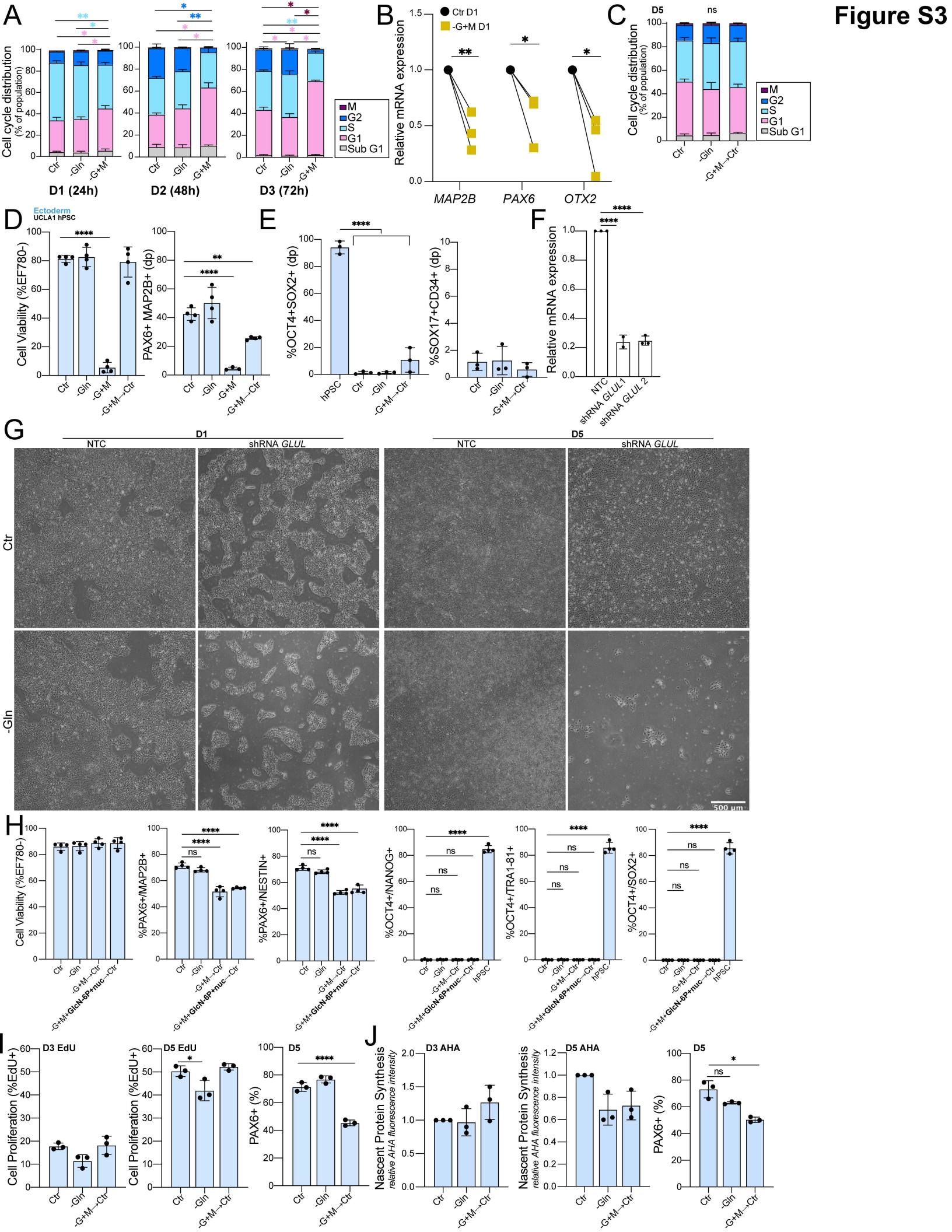
(C) Intracellular amounts of Gln, α-KG, $^{13}C_5$-Gln, and $^{13}C_5$-α-KG derived from $^{13}C_5$-glutamic acid in D3 mesoderm, endoderm, and ectoderm cells grown in Gln-supplemented or Gln-free media.

(D) Percentage of (Left) D5 mesoderm (SNAI2/SLUG$^+$) and (Right) D3 endoderm (SOX17$^+$) cells grown in Gln-supplemented or Gln-free conditions with 50 μM DON or 1 mM MSO.

(E) Verification of intracellular uptake and conversion to related metabolites upon respective metabolite supplementations (denoted in red), quantified by UHPLC-MS. Intensity values represent area under the curve (arbitrary units).

(F) (Left) Cell viability of UCLA1-derived D5 ectoderm cells differentiated in Gln-starvation and supplemented with GlcN6P, dm-α-KG, nucleosides, or GSH. (Right) Percentage of D5 ectoderm (PAX6$^+$MAP2B$^+$) cells grown in Gln-starvation with added GlcN6P or both GlcN6P and nucleosides (nuc). Data represent mean ± SD of n = 3 biological replicates. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$. The p values were determined using (**C, D, F**) one-way ANOVA with correction for multiple comparisons or (**E**) Student's t test.

Figure S3


A
Cell cycle distribution (% of population)
M
G2
S
G1
Sub G1
Ctr
-Gln
-G+M
D1 (24h)
D2 (48h)
D3 (72h)
B
Ctr D1
-G+M D1
Relative mRNA expression
MAP2B
PAX6
OTX2
C
D5
ns
Cell cycle distribution (% of population)
D
Ectoderm
UCLA1 hPSC
Cell Viability (%EF780-)
PAX6+ MAP2B+ (dp)
-G+M→Ctr
E
%OCT4+SOX2+ (dp)
%SOX17+CD34+ (dp)
hPSC
F
Relative mRNA expression
NTC
shRNA GLUL1
shRNA GLUL 2
G
D1
D5
NTC
shRNA GLUL
Ctr
-Gln
500 µm
H
Cell Viability (%EF780-)
%PAX6+/MAP2B+
%PAX6+/NESTIN+
%OCT4+/NANOG+
%OCT4+/TRA1-81+
%OCT4+/SOX2+
-G+M+GlcN-6P+nuc→Ctr
I
D3 EdU
D5 EdU
D5
Cell Proliferation (%EdU+)
PAX6+ (%)
J
D3 AHA
D5 AHA
D5
Nascent Protein Synthesis
relative AHA fluorescence intensity
PAX6+ (%)

Supplemental Figure S3. Cell cycle distribution, cell proliferation, and nascent protein synthesis during Gln repletion following 14h Gln-starvation

Related to Figure 3

(A) Flow cytometry cell cycle distribution of ectoderm cells differentiated in continuous Gln-starvation for D1 (24h), D2 (48h), and D3 (72h), colored ∗p symbols represent corresponding cell cycle stage with significant difference across indicated conditions.

(B) qRT-PCR analysis of *MAP2B*, *PAX6*, and *OTX2* expression in D1 (24h) ectoderm cells grown in Gln-starvation.

(C) Flow cytometry cell cycle distribution of D5 ectoderm cells after initial 24h Gln-starvation (-G+M→Ctr).

(D) (Left) Cell viability and (Right) percentage of UCLA1-derived D5 ectoderm ($PAX6^+MAP2B^+$) cells after initial 24h Gln-starvation (G+M→Ctr).

(E) Percentage of pluripotent ($OCT4^+SOX2^+$) and endoderm/mesoderm ($SOX17^+CD34^+$) biomarkers in D5 ectoderm cells after initial 24h Gln-starvation (-G+M→Ctr).

(F) qRT-PCR analysis of *GLUL* (encoding GS) in H9 hPSCs expressing shRNA targeting *GLUL* (shRNA *GLUL*1, clone 1; shRNA *GLUL*2, clone 2) or a non-targeting control (NTC).

(G) Representative brightfield images of H9-derived ectoderm cells expressing shRNA targeting *GLUL* (shRNA *GLUL*) or a non-targeting control (NTC) grown continuously in Gln-free media at D1 (24h) and D5 (120h). Scale bar indicates 500 μm.

(H) Cell viability and percentage of ectoderm ($PAX6^+MAP2B^+$, $PAX6^+NESTIN^+$) and pluripotent ($OCT4^+NANOG^+$, $OCT4^+TRA1\text{-}81^+$, $OCT4^+SOX2^+$) biomarkers in D5 ectoderm cells after initial 14h Gln-starvation (-G+M→Ctr) or metabolite-supplemented Gln-starvation (-G+M+GlcN-6P+nuc →Ctr).

(I) Cell proliferation (EdU+ staining) in D3 and D5 ectoderm cells after initial 14h Gln-starvation.

(J) Nascent protein synthesis (relative AHA staining) in D3 and D5 ectoderm cells after initial 14h Gln-starvation.

Data represent mean ± SD of n ≥ 3 biological replicates. ∗p ≤ 0.05; ∗∗p ≤ 0.01; ∗∗∗p ≤ 0.001; ∗∗∗∗p ≤ 0.0001. The p values were determined by (**A, C**) two-way ANOVA, (**B**) Student's t test, (**D-F, H-J**) or one-way ANOVA with correction for multiple comparisons.

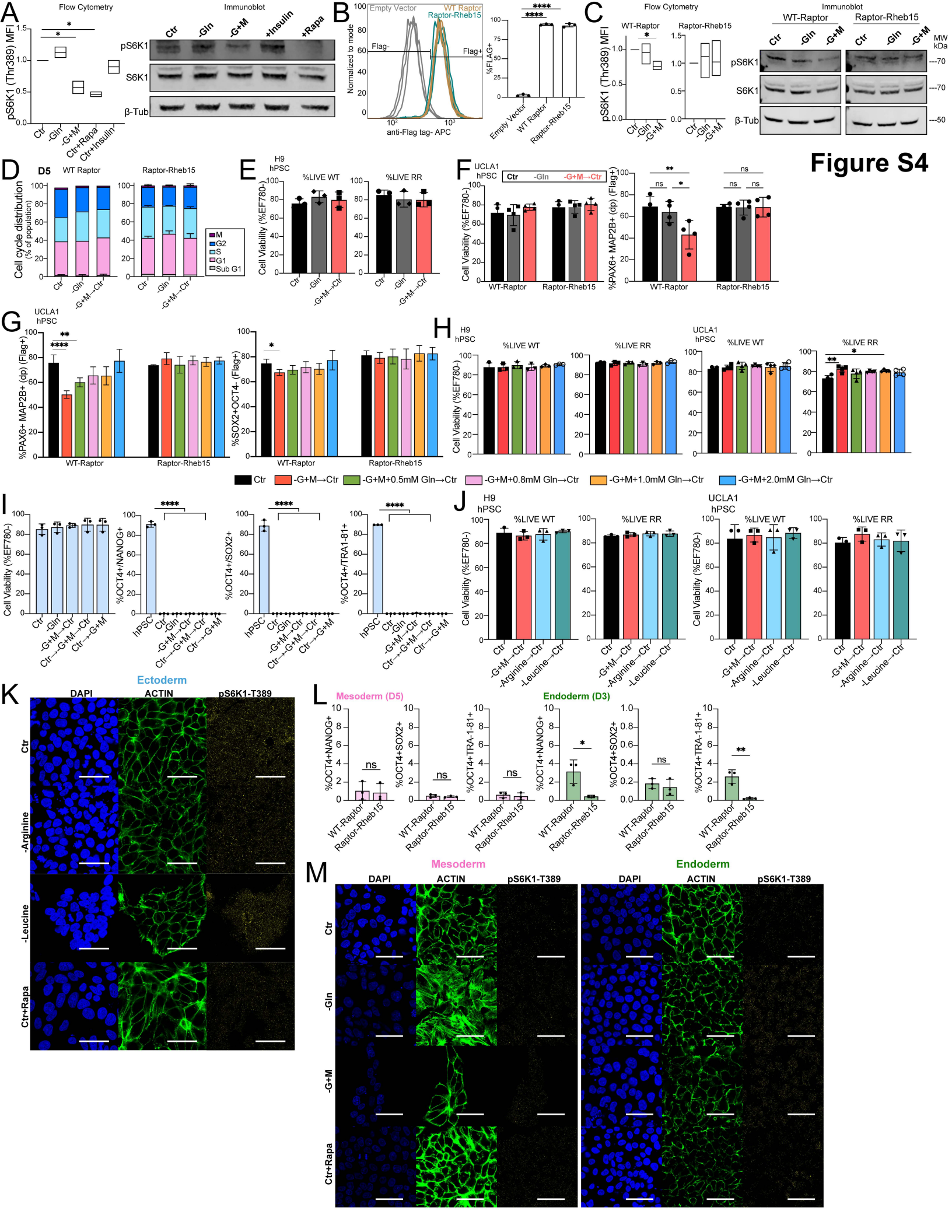


Figure S4
A
Flow Cytometry
Immunoblot
pS6K1 (Thr389) MFI
Ctr
-Gln
-G+M
+Insulin
+Rapa
pS6K1
S6K1
β-Tub
Ctr+Rapa
Ctr+Insulin
B
Empty Vector
WT Raptor
Raptor-Rheb15
Normalized to mode
Flag-
Flag+
anti-Flag tag- APC
%FLAG+
C
Flow Cytometry
WT-Raptor
Raptor-Rheb15
Immunoblot
MW kDa
D
D5
WT Raptor
Raptor-Rheb15
Cell cycle distribution (% of population)
M
G2
S
G1
Sub G1
-G+M→Ctr
E
H9 hPSC
%LIVE WT
%LIVE RR
Cell Viability (%EF780-)
F
UCLA1 hPSC
Ctr
-Gln
-G+M→Ctr
%PAX6+ MAP2B+ (dp) (Flag+)
ns
G
%SOX2+OCT4- (Flag+)
H
I
%OCT4+/NANOG+
%OCT4+/SOX2+
%OCT4+/TRA1-81+
hPSC
-G+M→Ctr
Ctr→G+M→Ctr
Ctr→G+M
J
-Arginine→Ctr
-Leucine→Ctr
K
Ectoderm
DAPI
ACTIN
pS6K1-T389
-Arginine
-Leucine
L
Mesoderm (D5)
Endoderm (D3)
M
Mesoderm
Endoderm

Supplemental Figure S4. mTORC1 inactivation under Gln-starvation

Related to Figure 4

(A) (Left) Flow cytometry quantification and (Right) representative immunoblot of mTORC activation (pS6K1-Thr389) immediately after 24h Gln-starved ectoderm differentiation.

(B) Flow cytometry traces and quantification of %$FLAG^+$ cells in H9-derived Empty Vector, WT-Raptor, and Raptor-Rheb15 hPSC lines.

(C) (Left) Flow cytometry quantification and (Right) representative immunoblot of mTORC activation (pS6K1-Thr389) immediately after 14h Gln-starved ectoderm differentiation in H9-derived WT-Raptor or Raptor-Rheb15 hPSC lines.

(D) Flow cytometry cell cycle distribution of H9-derived D5 WT-Raptor or Raptor-Rheb15 ectoderm cells after initial 14h Gln-starvation (-G+M→Ctr).

(E) Cell viability of H9-derived D5 WT-Raptor or Raptor-Rheb15 ectoderm cells after initial 14h Gln-starvation (-G+M→Ctr).

(F) (Left) Cell viability and (Right) percentage of UCLA1-derived D5 WT-Raptor or Raptor-Rheb15 ectoderm ($PAX6^+MAP2B^+FLAG^+$) cells after initial 14h Gln-starvation (-G+M→Ctr).

(G) Percentage of UCLA1-derived D5 WT-Raptor or Raptor-Rheb15 ectoderm ($PAX6^+MAP2B^+FLAG^+$, $SOX2^-OCT4^+FLAG^+$) cells after initial 14h Gln-starvation with increasing concentrations of exogenous Gln supplementation.

(H) Cell viability of (Left) H9 or (Right) UCLA1-derived D5 ectoderm cells after initial 14h Gln-starvation with increasing concentrations of exogenous Gln supplementation.

(I) (Left) Cell viability and (Right) percentage of pluripotent ($OCT4^+NANOG^+$, $OCT4^+SOX2^+$, $OCT4^+TRA1\text{-}81^+$) biomarkers in H9-derived D5 ectoderm cells after initial (-G+M→Ctr), intermediate (Ctr→-G+M→Ctr), or late (Ctr→-G+M) 14h Gln-starvation.

(J) Cell viability in (Left) H9 or (Right) UCLA1-derived D5 ectoderm differentiation after initial 14h Gln-starvation, Arg deprivation, or Leu deprivation in WT-Raptor or Raptor-Rheb15 hPSC lines.

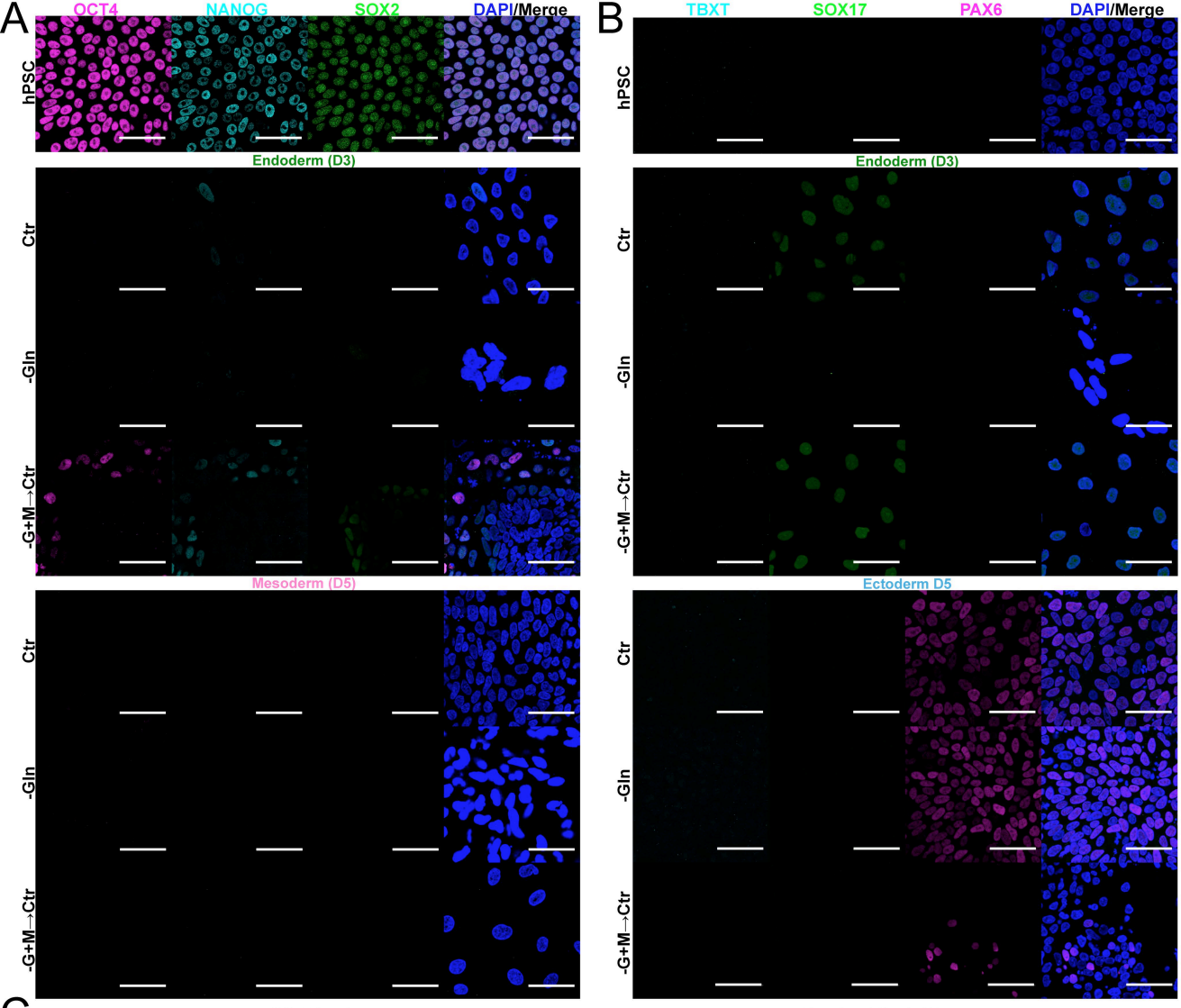
(K) Representative immunofluorescence images of mTORC activation in H9-derived ectoderm cells grown for 14h in Gln-supplemented (Ctr), Arginine-free (-Arginine), Leucine-free (-Leucine), or 200 nM rapamycin treatment (Ctr+Rapa). DAPI (blue, nucleus); ACTIN (green, cytoskeleton); pS6K1-Thr389 (yellow, mTORC1 activation). Scale bar indicates 50 μm.

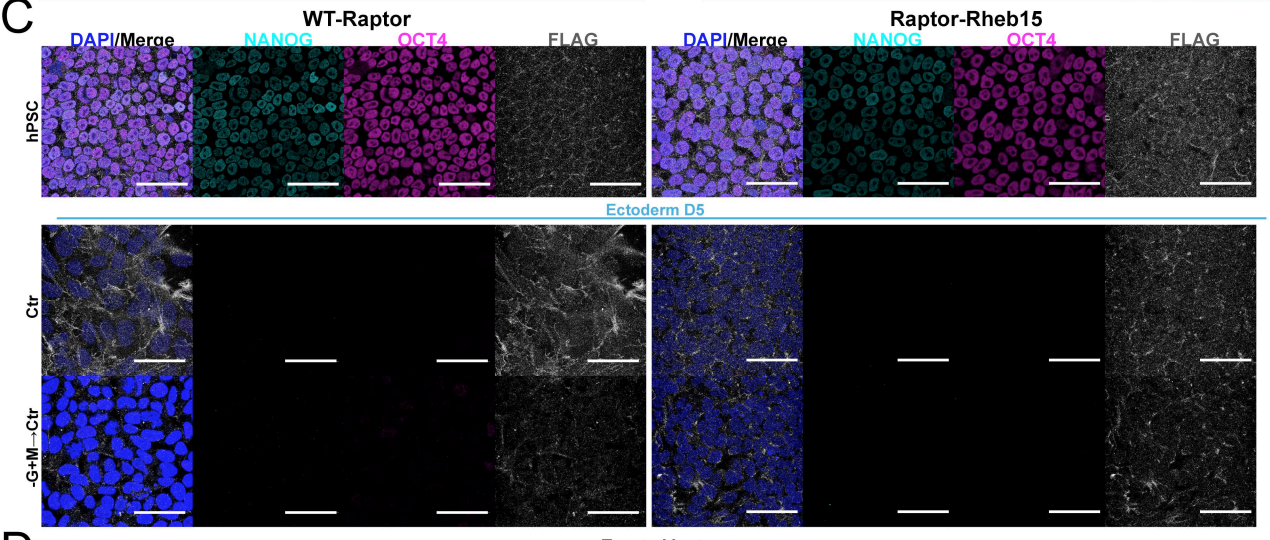
(L) Percentage of pluripotent ($OCT4^+NANOG^+$, $OCT4^+SOX2^+$, $OCT4^+TRA1\text{-}81^+$) biomarkers in H9-derived WT-Raptor or Raptor-Rheb15 D5 mesoderm and D3 endoderm cells.

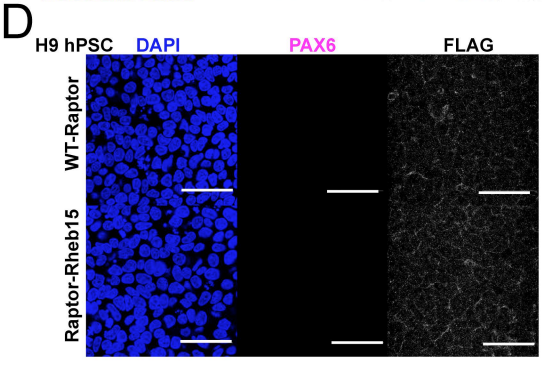
(M) Representative immunofluorescence images of mTORC activation in H9-derived (Left) mesoderm and (Right) endoderm cells grown for 24h in Gln-supplemented (Ctr), Gln-free (-Gln), Gln-starvation (-G+M), or 200 nM rapamycin treatment (Ctr+Rapa). DAPI (blue, nucleus); ACTIN (green, cytoskeleton); pS6K1-Thr389 (yellow, mTORC1 activation). Scale bar indicates 50 μm.

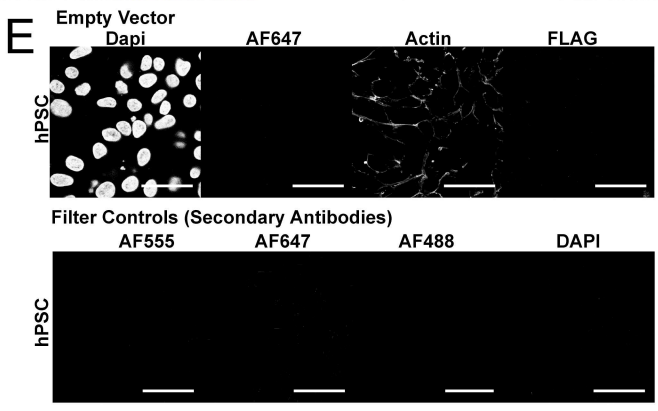
Data represent mean ± SD or (**A, C**) min and max of n ≥ 3 biological replicates. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; $****p \leq 0.0001$. The p values were determined by (**A-C, E, H-J)** one-way ANOVA, (**D, F-G**) two-way ANOVA with correction for multiple comparisons, or (**L**) Student's t test.

Figure S5


A
OCT4
NANOG
SOX2
DAPI/Merge
hPSC
Endoderm (D3)
Ctr
-Gln
-G+M→Ctr
Mesoderm (D5)
B
TBXT
SOX17
PAX6
DAPI/Merge
Endoderm (D3)
Ectoderm D5


C
WT-Raptor
Raptor-Rheb15
DAPI/Merge
NANOG
OCT4
FLAG
hPSC
Ectoderm D5
Ctr
-G+M→Ctr


D
H9 hPSC
DAPI
PAX6
FLAG
WT-Raptor
Raptor-Rheb15


E
Empty Vector
Dapi
AF647
Actin
FLAG
hPSC
Filter Controls (Secondary Antibodies)
AF555
AF647
AF488
DAPI

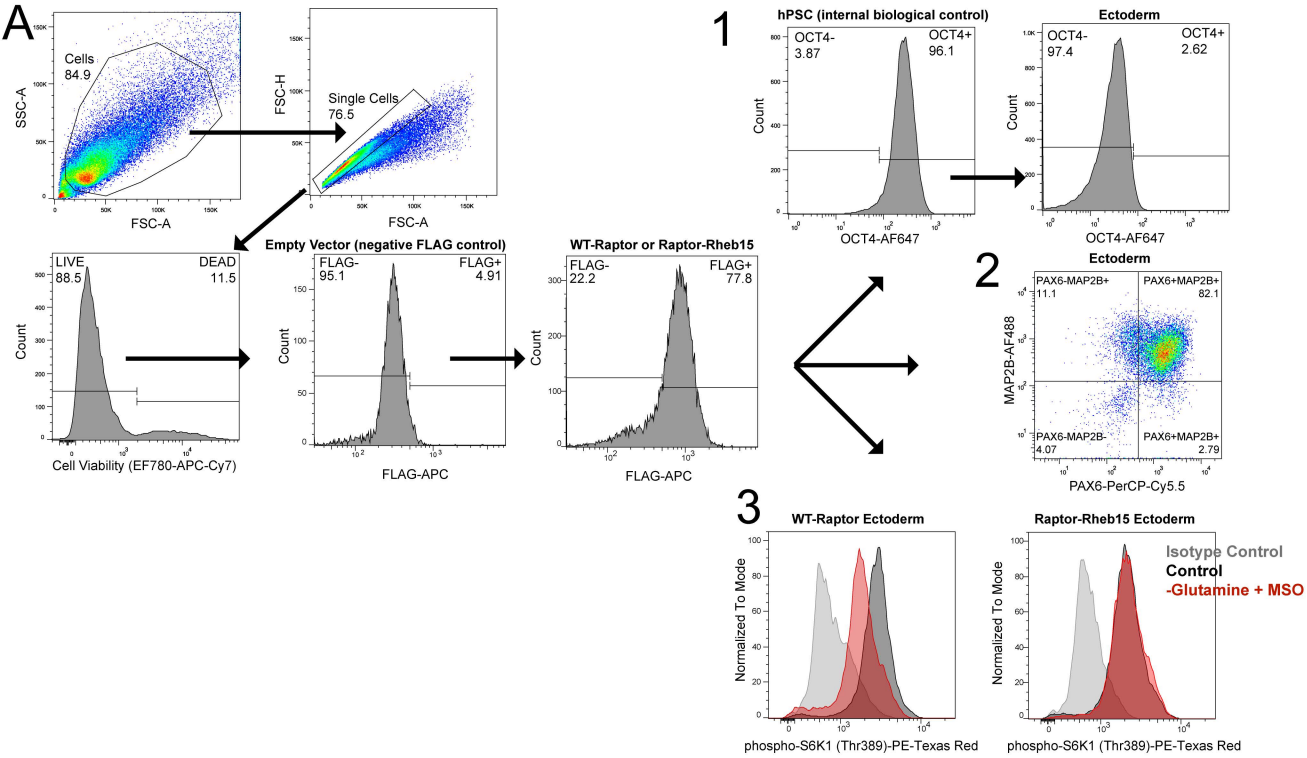
Supplemental Figure S5. Immunofluorescence images for biological and technical controls
Related to Figure 3 and 4

(A) Representative immunofluorescence images of pluripotent biomarkers in (Top) H9 hPSCs grown in mTESR, and H9-derived (Middle) D3 endoderm and (Bottom) D5 mesoderm cells grown in Gln-supplemented (Ctr), Gln-free (-Gln), or Gln-repletion follow initial 14h Gln-starvation (-G+M→Ctr). OCT4 (magenta, pluripotent); NANOG (cyan, pluripotent); SOX2 (green, pluripotent); DAPI (blue, nucleus). Scale bar indicates 50 μm.

(B) Representative immunofluorescence images of tri-lineage biomarkers in (Top) H9 hPSCs grown in mTESR, and H9-derived (Middle) D3 endoderm and (Bottom) D5 ectoderm cells grown in Gln-supplemented (Ctr), Gln-free (-Gln), or Gln-repletion follow initial 14h Gln-starvation (-G+M→Ctr). TBXT (cyan, mesoderm); SOX17 (green, endoderm); PAX6 (magenta, ectoderm); DAPI (blue, nucleus). Scale bar indicates 50 μm.

(C) Representative immunofluorescence images of pluripotent biomarkers in H9-derived D5 (Left) WT-Raptor or (Right) Raptor-Rheb15 ectoderm cells grown in Gln-supplemented (Ctr) or Gln-repletion follow initial 14h Gln-starvation (-G+M→Ctr). DAPI (blue, nucleus;) NANOG (cyan, pluripotent); OCT4 (magenta, ectoderm); FLAG (grey, transduced cell line reporter). Scale bar indicates 50 μm.

Figure S6


A
Cells
84.9
SSC-A
FSC-A
Single Cells
76.5
FSC-H
FSC-A
LIVE
88.5
DEAD
11.5
Count
Cell Viability (EF780-APC-Cy7)
Empty Vector (negative FLAG control)
FLAG-
95.1
FLAG+
4.91
Count
FLAG-APC
WT-Raptor or Raptor-Rheb15
FLAG-
22.2
FLAG+
77.8
Count
FLAG-APC
1
hPSC (internal biological control)
OCT4-
3.87
OCT4+
96.1
Count
OCT4-AF647
Ectoderm
OCT4-
97.4
OCT4+
2.62
Count
OCT4-AF647
2
Ectoderm
PAX6-MAP2B+
11.1
PAX6+MAP2B+
82.1
PAX6-MAP2B-
4.07
PAX6+MAP2B+
2.79
MAP2B-AF488
PAX6-PerCP-Cy5.5
3
WT-Raptor Ectoderm
Normalized To Mode
phospho-S6K1 (Thr389)-PE-Texas Red
Raptor-Rheb15 Ectoderm
Normalized To Mode
phospho-S6K1 (Thr389)-PE-Texas Red
Isotype Control
Control
-Glutamine + MSO

Supplemental Figure S6. Flow cytometry gating strategy

Related to Figure 3 and 4

(A) Gating strategy for flow cytometry analysis. This figure reflects gating strategy used for flow cytometry quantifications in Figures 1-4, S1-S4.