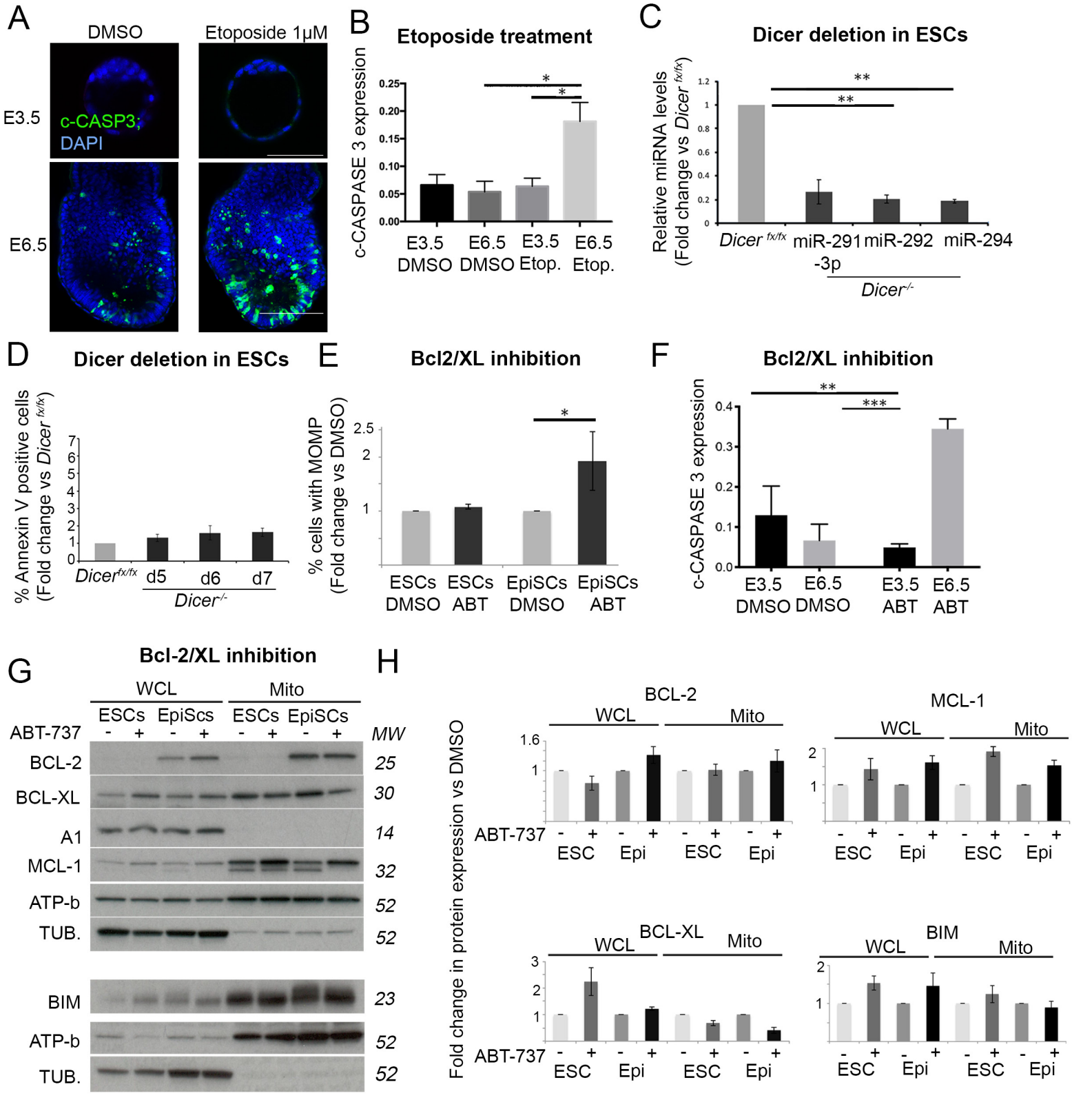
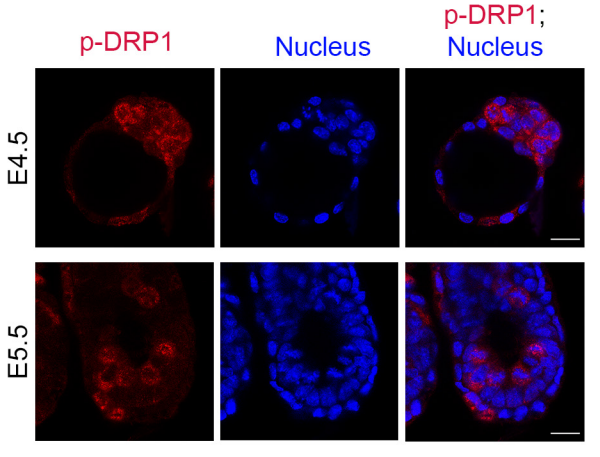
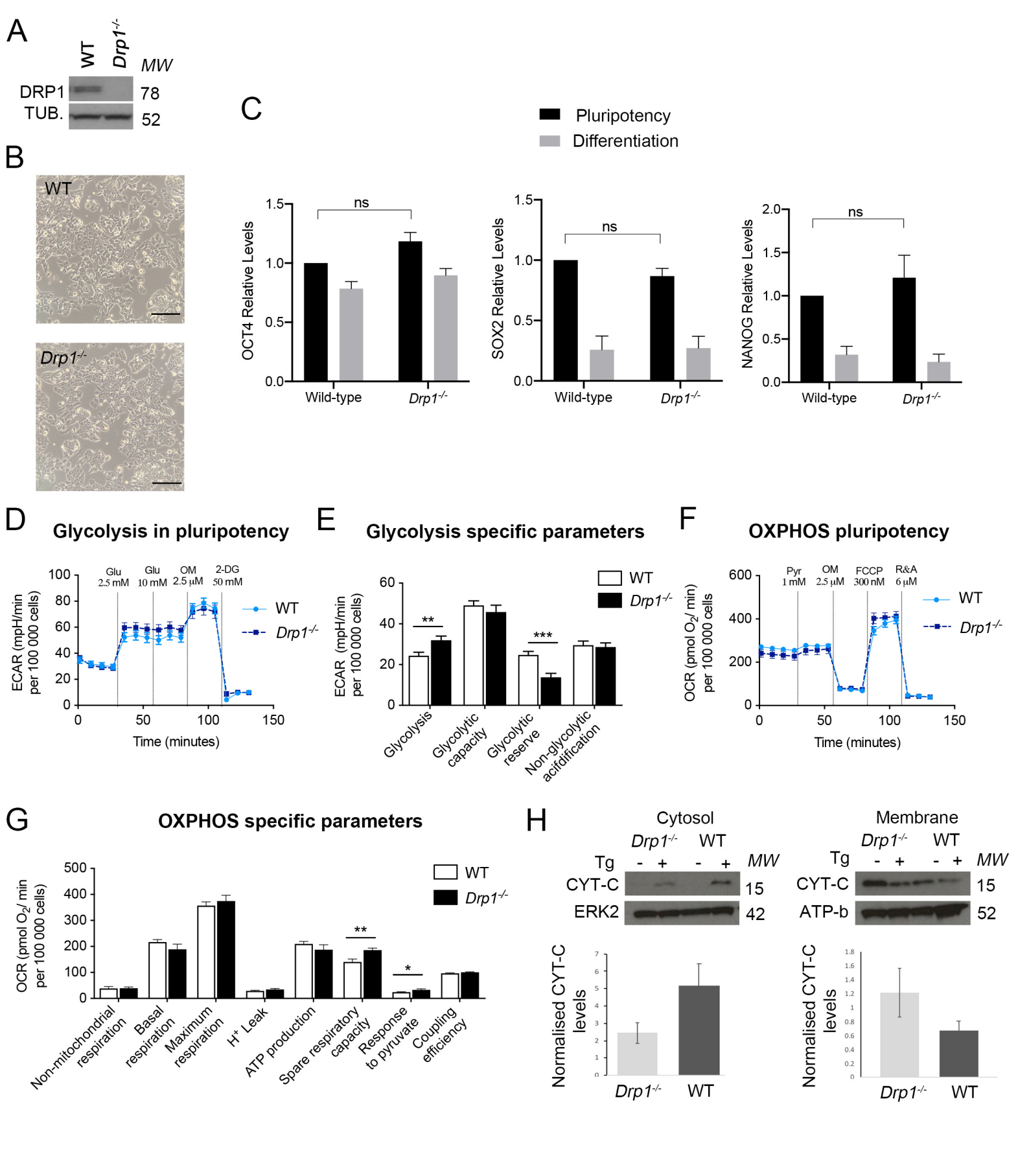

A
DMSO
Etoposide 1μM
E3.5
E6.5
c-CASP3;
DAPI
B
Etoposide treatment
c-CASPASE 3 expression
E3.5 DMSO
E6.5 DMSO
E3.5 Etop.
E6.5 Etop.
C
Dicer deletion in ESCs
Relative miRNA levels (Fold change vs Dicer fx/fx)
Dicer fx/fx
miR-291-3p
miR-292
miR-294
Dicer-/-
D
Dicer deletion in ESCs
% Annexin V positive cells (Fold change vs Dicer fx/fx)
Dicer fx/fx
d5
d6
d7
Dicer-/-
E
Bcl2/XL inhibition
% cells with MOMP (Fold change vs DMSO)
ESCs DMSO
ESCs ABT
EpiSCs DMSO
EpiSCs ABT
F
Bcl2/XL inhibition
c-CASPASE 3 expression
E3.5 DMSO
E6.5 DMSO
E3.5 ABT
E6.5 ABT
G
Bcl-2/XL inhibition
WCL
Mito
ESCs
EpiScs
EpiSCs
ABT-737
MW
BCL-2
BCL-XL
A1
MCL-1
ATP-b
TUB.
BIM
H
BCL-2
MCL-1
BCL-XL
BIM
WCL
Mito
ABT-737
ESC
Epi
Fold change in protein expression vs DMSO

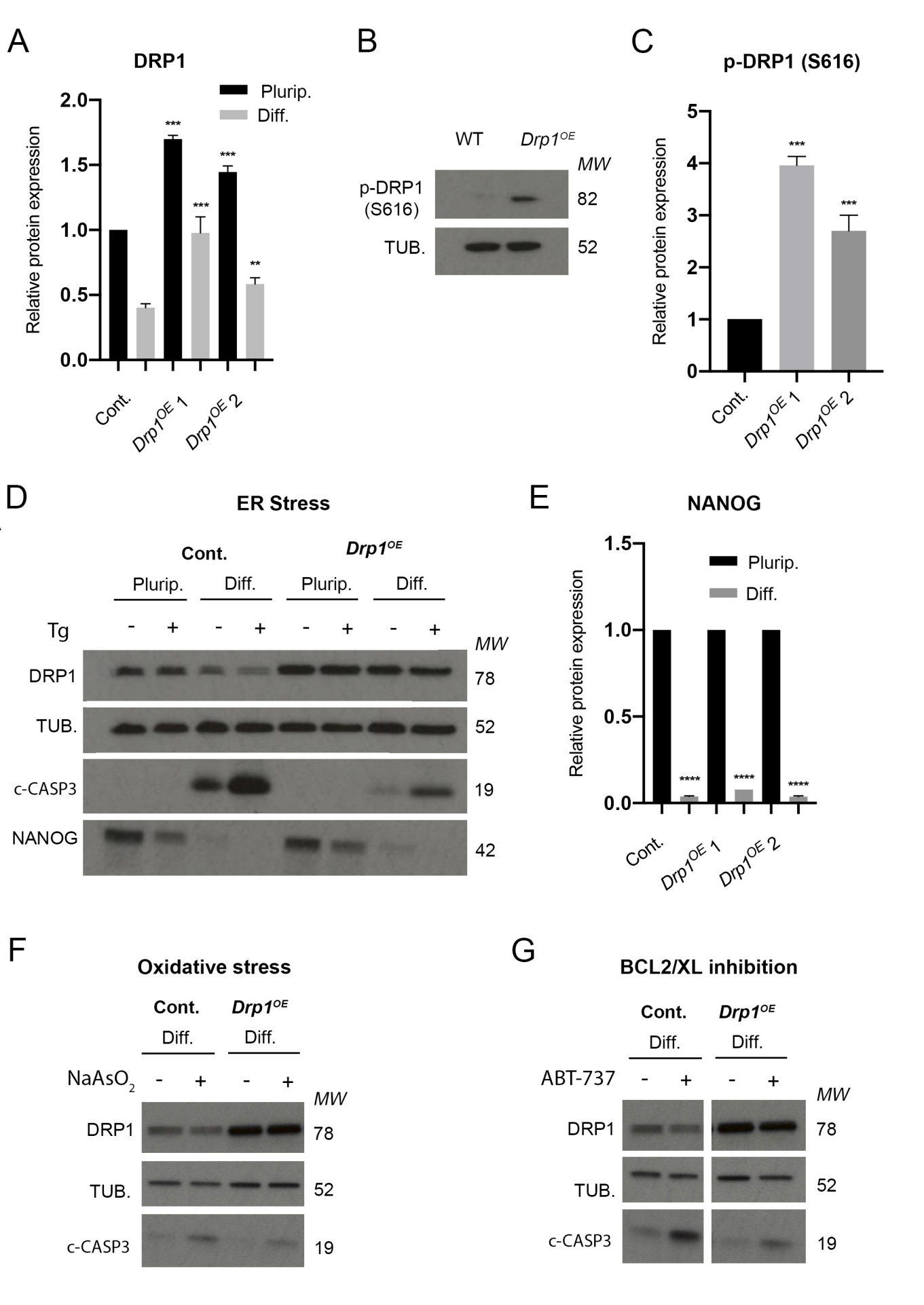
Supplementary Figure 1. Enhanced sensitivity to apoptosis in primed pluripotent stem cells. Related to Figure 1, Figure 2 and Figure 3. **A**. Levels of apoptosis as measured by cleaved-CASPASE 3 in E3.5 and E6.5 embryos cultured for 1.5h in the presence of DMSO or 1μM Etoposide. Scale bar E3.5=30 μm; E6.5=100μm. **B**. Quantification of cleaved-CASPASE 3 staining in A. DMSO: E3.5 (n= 3); E6.5 (n=4); 1μM Etoposide: E3.5 (n= 6); E6.5 (n=10). **C.** Quantitative RT-PCR showing change in miRNA relative expression 6 days after *Dicer* deletion in ESCs. Expression normalised against sno135. Fold change vs un-deleted cells is shown. **D.** Change in % of Annexin V positive ESCs at different time points after *Dicer* deletion relative to un-deleted cells. **E**. Change in % of ESCs and EpiSCs with loss of mitochondrial membrane potential (MOMP) after 24h treatment with 5μM ABT-737, relative to DMSO treated cells. **F.** Cleaved-CASPASE 3 levels in E3.5 and E6.5 embryos treated with DMSO or 2μM ABT-737 for 1.5h. E3.5 DMSO (n=5), ABT (n=5); E6.5 DMSO (n=6) ABT (n=6). Quantification of immunofluorescence images seen in Figure 2C. **G.** Levels of anti-apoptotic factors and BIM in whole cell extracts and mitochondrial extracts from ESCs and EpiSCs treated with DMSO or 5μM ABT-737 for 24h. A representative western blot is shown. **H**. Levels of anti-apoptotic proteins and BIM in whole cell and mitochondrial extracts from ESCs and EpiSC (Epi)s treated with DMSO or 5μM ABT-737 for 24h. Protein level is normalised against α-TUBULIN (TUB.) in whole cell extracts and against ATP-b in mitochondrial extracts. Fold change of ABT versus DMSO treated is shown. Average of a minimum of (B,F) 3, (D,H) 4 or (E) 5 experiments +/- SEM is shown. 2-way ANOVA with a Turkey correction (B, F); t-test1 tail unequal variance (C); t-test 2 tails unequal variance (D,E). *$p<0.05$, **$p<0.01$ or ***$p<0.001$. Where no significance is indicated it is because no statistically significant difference was observed. MW= Molecular weight.



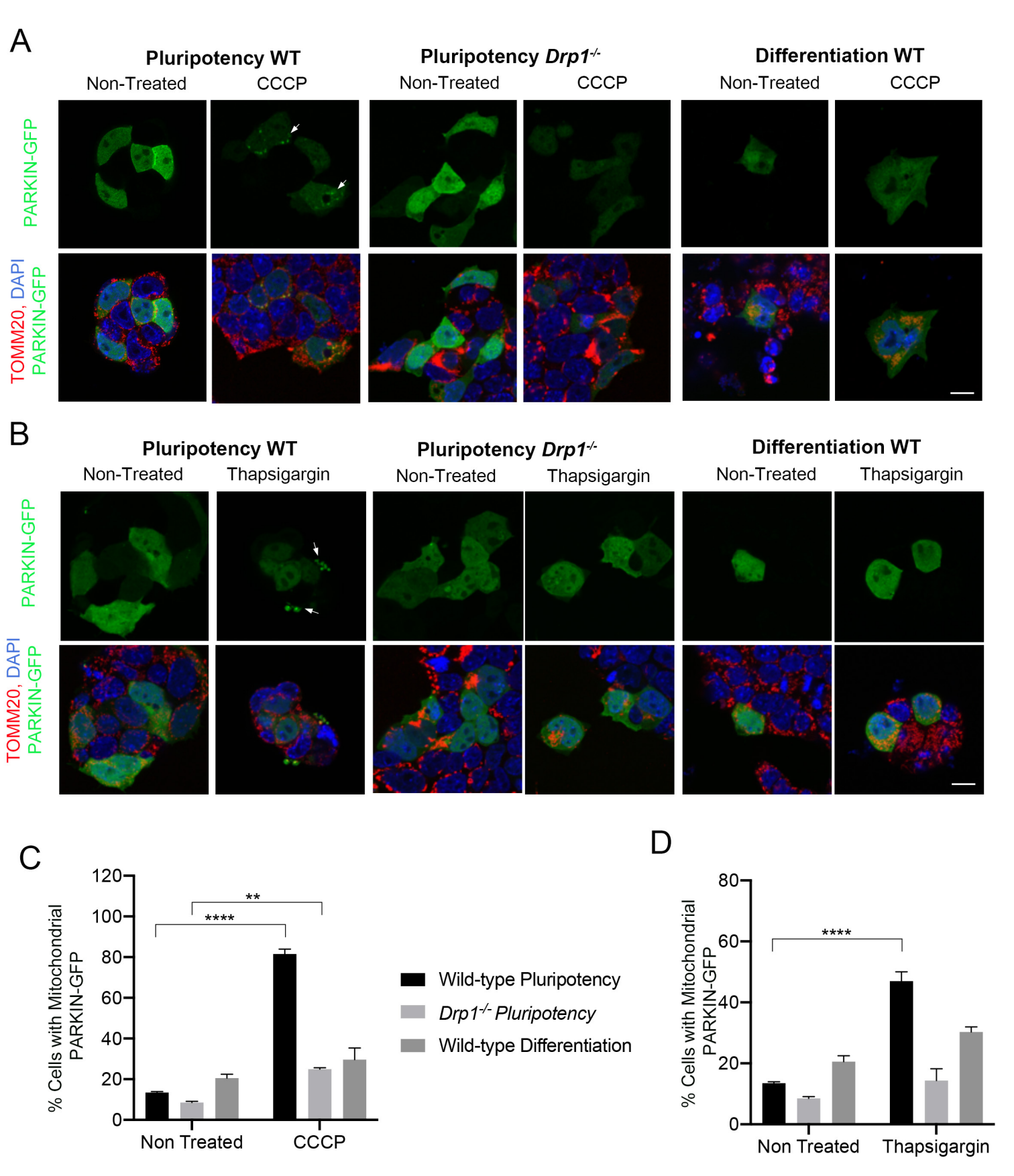
Supplementary Figure 2. pDRP1 expression in E4.5 and E5.5 embryos.
Related to Figure 4. pDRP1 immunostaining in E4.5 and E5.5 mouse embryos.
Scale bar=50 μm.


A
WT
Drp1-/-
MW
DRP1
TUB.
78
52
B
WT
Drp1-/-
C
Pluripotency
Differentiation
OCT4 Relative Levels
SOX2 Relative Levels
NANOG Relative Levels
ns
Wild-type
Drp1-/-
D
Glycolysis in pluripotency
Glu 2.5 mM
Glu 10 mM
OM 2.5 µM
2-DG 50 mM
ECAR (mpH/min per 100 000 cells)
Time (minutes)
E
Glycolysis specific parameters
Glycolysis
Glycolytic capacity
Glycolytic reserve
Non-glycolytic acidification
F
OXPHOS pluripotency
Pyr 1 mM
OM 2.5 µM
FCCP 300 nM
R&A 6 µM
OCR (pmol O2/ min per 100 000 cells)
G
OXPHOS specific parameters
Non-mitochondrial respiration
Basal respiration
Maximum respiration
H+ Leak
ATP production
Spare respiratory capacity
Response to pyruvate
Coupling efficiency
H
Cytosol
Membrane
Tg
CYT-C
ERK2
ATP-b
Normalised CYT-C levels

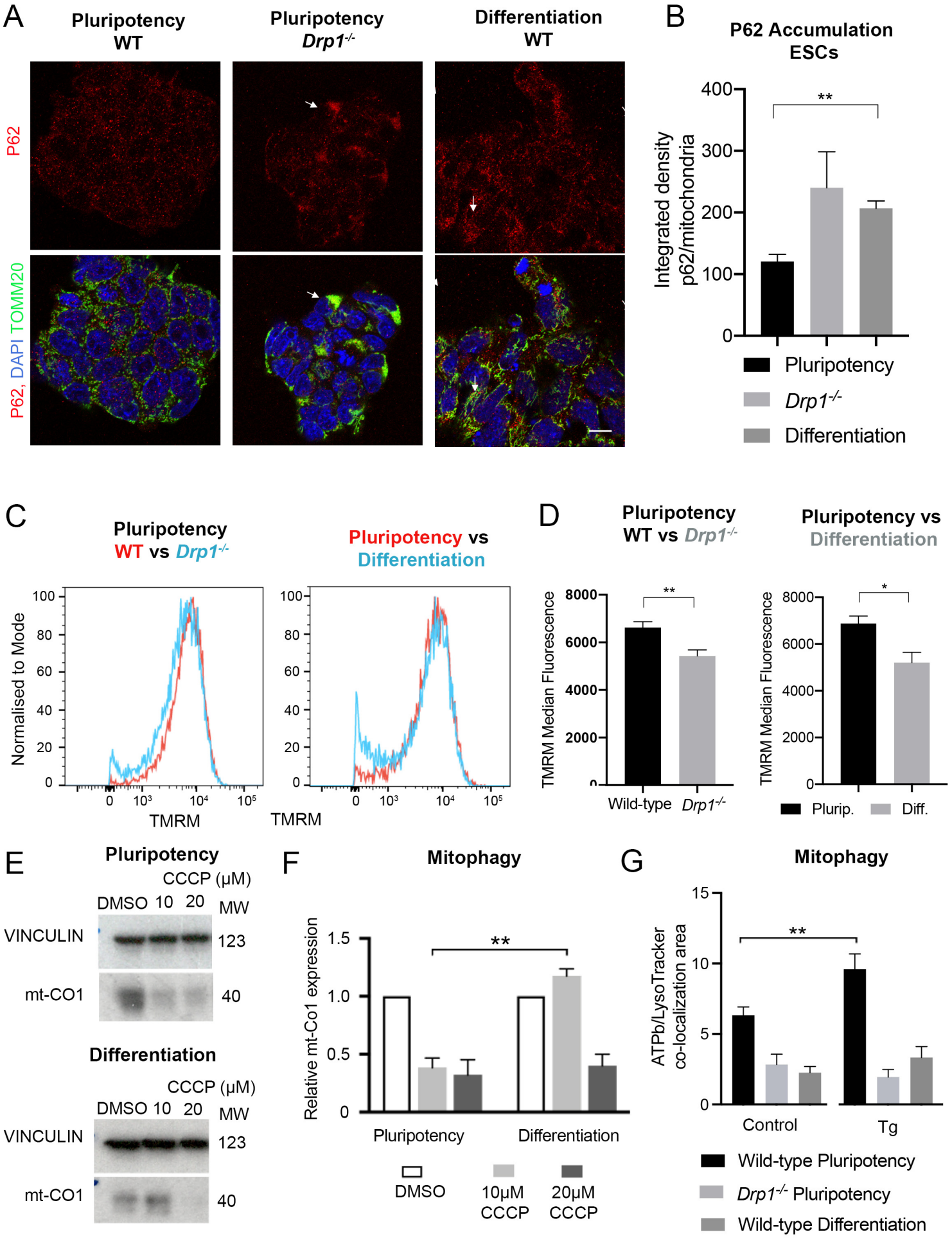
Supplementary Figure 3. Metabolic profile of wild-type and *Drp1*$^{-/-}$ ESCs. Related to Figure 5. **A.** Total DRP1 protein levels in wild type (WT) and *Drp1*$^{-/-}$ ESCs. **B.** Brightfield microscopy images showing WT and *Drp1*$^{-/-}$ ESCs morphology in pluripotency culture conditions. Scale bar=100 µm. **C.** Quantification of the western blots shown in Figure 5C. **D.** Extracellular acidification rate (ECAR) during the glycolysis stress test. **E.** Metabolic parameters assessed during a glycolysis stress test. **F.** Oxygen consumption rate (OCR) during the mitochondria stress test. **G.** Metabolic parameters assessed after the mitochondria stress test. **H.** CYTOCHROME C (CYT-C) protein levels in cytosolic and membrane fractions of wild-type and *Drp1*$^{-/-}$ ESCs un-treated or treated with 1µM thapsigargin (Tg) for 6h. Graph shows cytochrome C protein levels normalised against ERK1/2 (cytosolic fraction) or ATP-b (membrane fraction) after thapsigargin treatment. Average of 3 independent experiments +/- SEM is shown. Statistical analysis was done with a (C,E) Mann Whitney test *p<0.05, **p<0.01 or ***p<0.001. Scale bar in B=100µm. MW= Molecular weight.


A
DRP1
Plurip.
Diff.
Relative protein expression
2.0
1.5
1.0
0.5
0.0
***
***
***
**
Cont.
Drp1OE 1
Drp1OE 2
B
WT
Drp1OE
MW
p-DRP1 (S616)
82
TUB.
52
C
p-DRP1 (S616)
Relative protein expression
5
4
3
2
1
0
***
***
Cont.
Drp1OE 1
Drp1OE 2
D
ER Stress
Cont.
Drp1OE
Plurip.
Diff.
Plurip.
Diff.
Tg
- + - + - + - +
MW
DRP1
78
TUB.
52
c-CASP3
19
NANOG
42
E
NANOG
Plurip.
Diff.
Relative protein expression
1.5
1.0
0.5
0.0
****
****
****
Cont.
Drp1OE 1
Drp1OE 2
F
Oxidative stress
Cont.
Drp1OE
Diff.
Diff.
NaAsO2
- + - +
MW
DRP1
78
TUB.
52
c-CASP3
19
G
BCL2/XL inhibition
Cont.
Drp1OE
Diff.
Diff.
ABT-737
- + - +
MW
DRP1
78
TUB.
52
c-CASP3
19

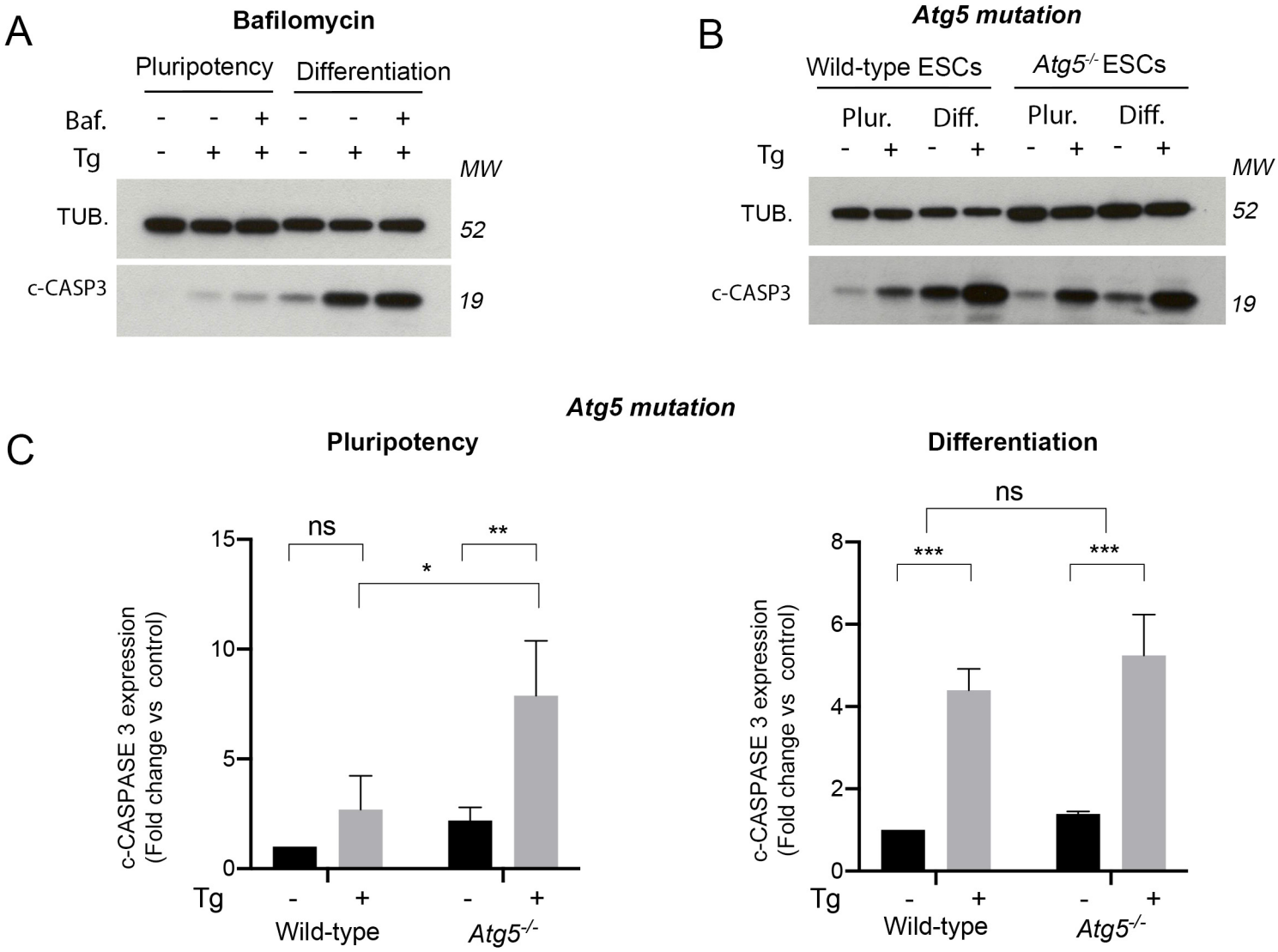
Supplementary Figure 4. Characterization of *Drp1* over-expressing cells. Related to Figure 6. **A.** Quantification of total DRP1 protein levels in wild-type (Cont.) and *Drp1*$^{-/-}$ ESCs cultured in pluripotency (Plurip.) or differentiation (Diff.) conditions. Protein levels are normalised against α-TUBULIN (TUB.) and the graph shows expression levels relative to wild-type cells quantified from Supplementary Figure 4D. **B.** Levels of phospho-DRP1 (S616) in wild-type and *Drp1*^{OE} cells in pluripotency or differentiation culture conditions detected by western blot. **C.** Quantification of (B). Protein levels are normalised against αTUBULIN and the graph shows protein expression levels relative to wild-type cells. **D**. Total DRP1, cleaved-CASPASE 3 (c-CASP3), α-TUBULIN (TUB.) and NANOG protein levels in wild-type and *Drp1*^{OE} ESCs in pluripotency or differentiation culture conditions untreated or treated with 1μM thapsigargin (Tg) for 5h. **E.** NANOG protein levels in wild-type and *Drp1*^{OE} ESCs in pluripotency or differentiation culture conditions. Protein levels are normalised against α-TUBULIN and graph shows differentiation expression levels relative to ESCs for each cell type. **F.** Total DRP1, cleaved-CASPASE 3 and α-TUBULIN protein levels in wild-type and *Drp1*^{OE} in differentiation culture conditions untreated or treated with 1μM sodium arsenate ($NaAsO_2$) for 5h. **G.** Total DRP1, cleaved-CASPASE 3 and α-TUBULIN protein levels in wild-type and *Drp1*^{OE} in differentiation culture conditions untreated or treated with 1μM ABT-737 for 4h. Average of 3 (C and E) or 5 (A) independent experiments +/- SEM is shown. Statistical comparisons are made to the control cells in the same culture condition (A and C) or compares a cell line in ESC and differentiation culture conditions for €. Students T-Test **p<0.01, ***p<0,001, ****p<0.0001. In A, C and E *Drp*1^{OE} 1 and 2 represent two independent *Drp*1^{OE} lines. MW= Molecular weight.


A
Pluripotency WT
Pluripotency Drp1-/-
Differentiation WT
Non-Treated
CCCP
Non-Treated
CCCP
Non-Treated
CCCP
PARKIN-GFP
TOMM20, DAPI
PARKIN-GFP
B
Pluripotency WT
Pluripotency Drp1-/-
Differentiation WT
Non-Treated
Thapsigargin
Non-Treated
Thapsigargin
Non-Treated
Thapsigargin
PARKIN-GFP
TOMM20, DAPI
PARKIN-GFP
C
% Cells with Mitochondrial PARKIN-GFP
120
100
80
60
40
20
0
**
****
Non Treated
CCCP
Wild-type Pluripotency
Drp1-/- Pluripotency
Wild-type Differentiation
D
% Cells with Mitochondrial PARKIN-GFP
80
60
40
20
0
****
Non Treated
Thapsigargin

Supplementary Figure 5. DRP1 regulates PARKIN localization in pluripotent stem cells. Related to Figure 7. **A.** TOMM20 immunostaining and GFP-PARKIN expression showing mitochondrial PARKIN localization in wild-type (WT) and *Drp1* mutant cells cultured in pluripotency culture conditions and wild-type cells cultured in differentiation conditions. Cells were then left untreated or treated with CCCP for 3 hours. White arrows indicate GFP-PARKIN aggregated appearing after drug treatment in control un-differentiated ESCs. **B.** TOMM20 immunostaining and GFP-PARKIN expression showing mitochondrial PARKIN localization in wild-type and *Drp1* mutant cells cultured in pluripotency culture conditions and wild-type cells cultured in differentiation conditions. Cells were then left untreated or treated with 1μM thapsigargin (Tg) for 5h. White arrows indicate GFP-PARKIN aggregated appearing after drug treatment on control un-differentiated ESCs. **C.** Quantification of GFP-PARKIN and TOMM20 co-localization from in images from (A). **D.** Quantification of GFP-PARKIN and TOMM20 co-localization from in images from (B). Average of 3 independent experiments +/- SEM is shown. 2-way ANOVA with Šidák correction **p<0.01, ***p<0.001 or ****p<0.0001. The statistical comparisons are made to untreated cells. Where no significance is indicated it is because no statistically significant difference was observed. Scale bar in A and B=10um.


A
Pluripotency WT
Pluripotency Drp1-/-
Differentiation WT
P62
P62, DAPI TOMM20
B
P62 Accumulation ESCs
Integrated density p62/mitochondria
400
300
200
100
0
**
Pluripotency
Drp1-/-
Differentiation
C
Pluripotency WT vs Drp1-/-
Pluripotency vs Differentiation
Normalised to Mode
100
80
60
40
20
0
0
10³
10⁴
10⁵
TMRM
D
Pluripotency WT vs Drp1-/-
Pluripotency vs Differentiation
TMRM Median Fluorescence
8000
6000
4000
2000
0
**
*
Wild-type
Drp1-/-
Plurip.
Diff.
E
Pluripotency
CCCP (μM)
DMSO 10 20
MW
VINCULIN
123
mt-CO1
40
Differentiation
CCCP (μM)
DMSO 10 20
MW
VINCULIN
123
mt-CO1
40
F
Mitophagy
Relative mt-Co1 expression
1.5
1.0
0.5
0
**
Pluripotency
Differentiation
DMSO
10μM CCCP
20μM CCCP
G
Mitophagy
ATPb/LysoTracker co-localization area
15
10
5
0
**
Control
Tg
Wild-type Pluripotency
Drp1-/- Pluripotency
Wild-type Differentiation

Supplementary Figure 6. DRP1 regulates mitophagy in pluripotent stem cells. Related to Figure 7. **A.** Immunostaining showing mitochondrial (TOMM20) and P62 co-localization in wild-type (WT) and *Drp1* mutant cells cultured pluripotency conditions and wild-type cells cultured in differentiation conditions. White arrows indicate mitochondrial aggregates. **B.** Quantification of co-localization area of P62 and TOMM20 from whole images taken from (A). **C.** Analysis of mitochondrial membrane potential measured by TMRM in wild-type and *Drp1* mutant cells cultured in pluripotency conditions and wild-type cells cultured in differentiation conditions. **D.** Quantification of the median fluorescence TMRM values from (C). **E.** Mitophagy assessment in wild-type cells grown in pluripotency or differentiating conditions, after 18h of CCCP treatments. **F.** Quantification of (E). Relative mt-CO1 expression upon mitophagy stimulus with CCCP, normalised to control conditions (DMSO-treated) and relative to the loading control. **G.** Quantification of co-localization area of lysotracker and ATPb from whole images taken from Figure 7A. Average of 3 independent experiments +/- SEM is shown. 2-way ANOVA with Šidák correction **p<0.01, ***p<0.001 or ****p<0.0001. The statistical comparisons are made to untreated cells. Where no significance is indicated it is because no statistically significant difference was observed. MW= Molecular weight.



Supplementary Figure 7. Inhibition of mitophagy promotes apoptosis in ESCs. Related to Figure 7.
A. Cleaved-CASPASE 3 (c-CASP3) and α-TUBULIN (TUB.) protein levels in wild-type ESCs cultured in pluripotency or differentiation conditions untreated or treated with 1μM thapsigargin (Tg) +/- 10nM bafilomycin (Baf.) for 5h. **B.** Cleaved-CASPASE 3 (c-CASP3) and α-TUBULIN (TUB.) protein levels in wild-type and *Atg5-/-* ESCs cultured in pluripotency or differentiation conditions untreated or treated with 1μM thapsigargin (Tg) for 5h.
C. Quantification of (B). Protein levels are normalised against αTUBULIN and the graph shows protein expression levels relative to wild-type cells. Average of 3 independent experiments +/- SEM is shown. Statistical comparisons are made relative to the control cells in the same culture condition. 2-way ANOVA with Šidák correction *p<0.05, **p<0.01 or ***p<0.001. MW= Molecular weight.