Stem Cell Reports, Volume *10*

Supplemental Information

KLF4 Nuclear Export Requires ERK Activation and Initiates Exit from Naive Pluripotency

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Inventory of Supplemental Information

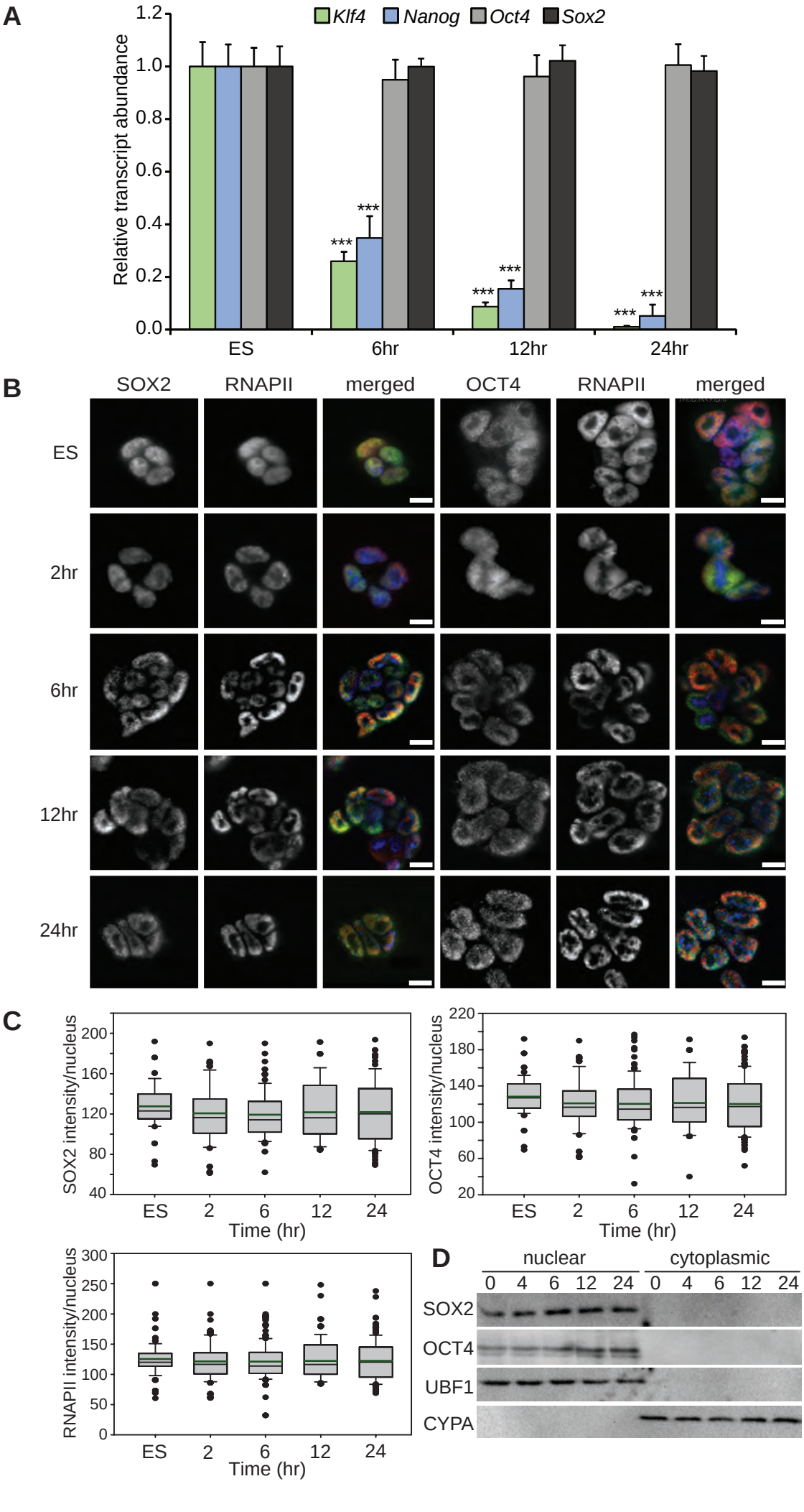
A
Klf4
Nanog
Oct4
Sox2
Relative transcript abundance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
ES
6hr
12hr
24hr
B
SOX2
RNAPII
merged
OCT4
RNAPII
merged
ES
2hr
6hr
12hr
24hr
C
SOX2 intensity/nucleus
200
160
120
80
40
ES
2
6
12
24
Time (hr)
OCT4 intensity/nucleus
220
180
140
100
60
20
RNAPII intensity/nucleus
300
250
200
150
100
50
0
D
nuclear
cytoplasmic
0
4
6
12
24
SOX2
OCT4
UBF1
CYPA

Figure S1: Changes in pluripotency factor mRNA and protein levels during the first 24hr of differentiation. Related to Figure 1. A) Real time RT-qPCR from three biological replicate samples reveals downregulation of *Klf4* and *Nanog* mRNA 6hr after LIF/2i withdrawal. Statistical differences are indicated by *** $P < 0.001$. Error bars represent standard deviation. B) Immunofluorescence images of ES cells cultured with LIF/2i and ES cells 2, 6, 12 and 24hr after LIF/2i removal. OCT4, SOX2, and RNAPII-S5P proteins were detected by immunofluorescence. Merged images display SOX2 or OCT4 in green, RNAPII-S5P in red and DAPI DNA stain in blue. Scale bar = 10μm. C) Box and whisker plots display intensities per nucleus of SOX2 (left) and OCT4 (right) and RNAPII-S5P (bottom). Green line indicates the average intensity at each time point, black line indicates the median intensity. Boxes indicate interquartile range of intensity values, whiskers indicate the 10th and 90th percentiles, and outliers are shown as black dots. Images were collected from at least three biological replicate samples and ≥100 nuclei were quantified for each. No significant differences were observed in the intensity of these proteins during this time frame. D) Immunoblot of the nuclear and cytoplasmic fractions confirmed that SOX2 and OCT4 are exclusively nuclear in ES cells.

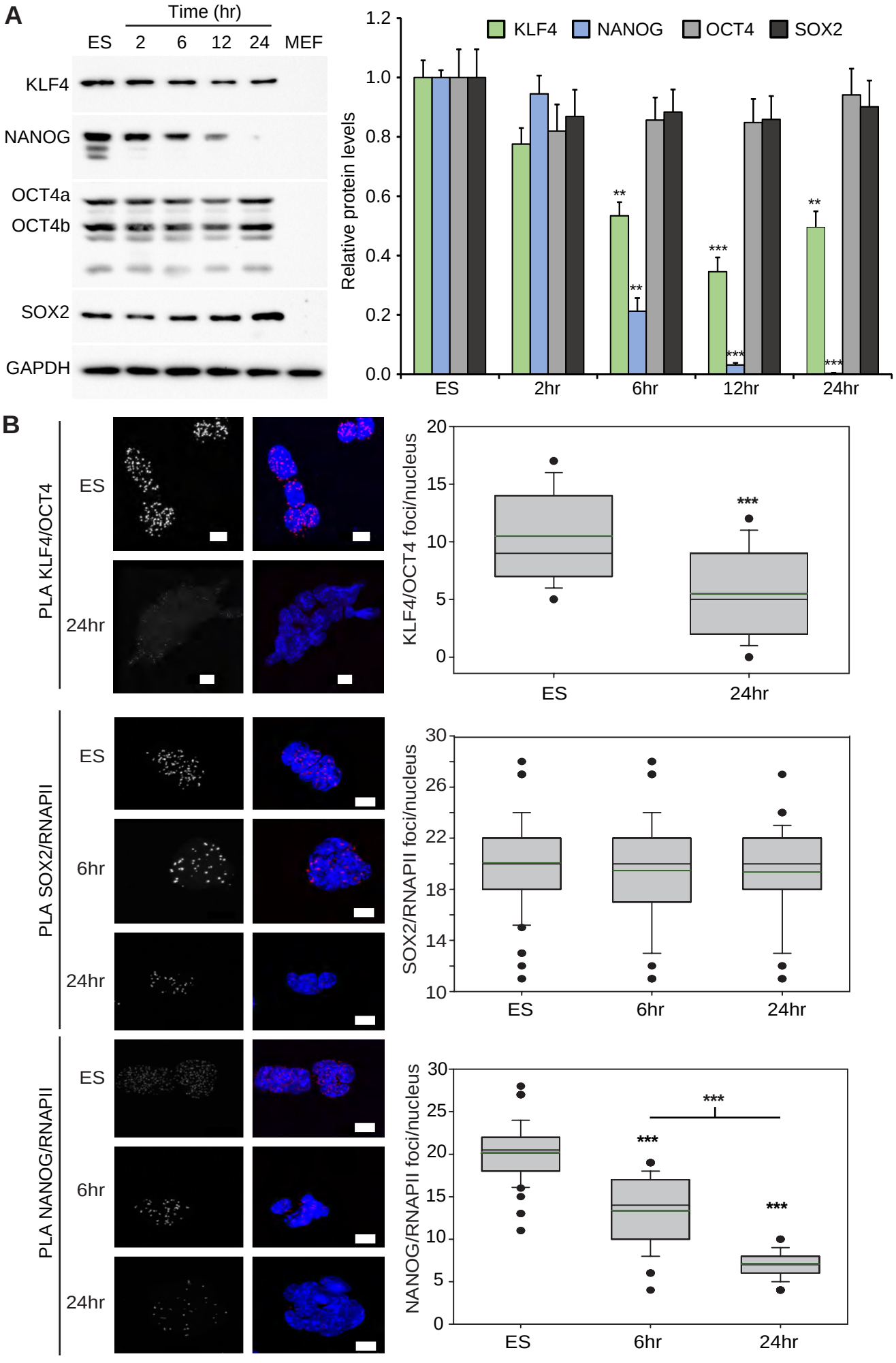
A
Time (hr)
ES
2
6
12
24
MEF
KLF4
NANOG
OCT4a
OCT4b
SOX2
GAPDH
KLF4
NANOG
OCT4
SOX2
Relative protein levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ES
2hr
6hr
12hr
24hr
B
PLA KLF4/OCT4
ES
24hr
KLF4/OCT4 foci/nucleus
20
15
10
5
0
ES
24hr
PLA SOX2/RNAPII
ES
6hr
24hr
SOX2/RNAPII foci/nucleus
30
26
22
18
14
10
ES
6hr
24hr
PLA NANOG/RNAPII
ES
6hr
24hr
NANOG/RNAPII foci/nucleus
30
25
20
15
10
5
0
ES
6hr
24hr

Figure S2: Changes in nuclear protein complexes associated with pluripotency exit. Related to Figure 2. A) Immunoblot analysis of total protein isolated from ES cells cultured with LIF/2i (ES) and cells 2, 6, 12 and 24hr after LIF/2i removal is shown on the left. GAPDH levels were monitored to evaluate protein loading. Mouse embryonic fibroblasts (MEF) were used as a negative control for the expression of the pluripotency transcription factors. On the right quantification from three biological replicates is shown as the average relative intensity for each protein compared to GAPDH. Whereas NANOG levels are dramatically reduced at 24hr compared to undifferentiated cells KLF4 levels are only reduced to about 50% compared to undifferentiated cells. Statistical differences compared to the undifferentiated ES cell values are indicated by ** $P < 0.01$ and *** $P < 0.001$. Error bars represent standard deviation. B) Proximity ligation amplification (PLA) indicating the amount of interaction between KLF4/OCT4, SOX2/RNAPII-S5P and NANOG/RNAPII-S5P in ES cells after LIF/2i withdrawal. Scale bar = 10μm. Quantification of the number of interaction foci per nucleus is shown to the right. Box and whisker plots display the number of PLA foci per nucleus. Boxes indicate interquartile range of intensity values, whiskers indicate the 10th and 90th percentiles, outliers in the 5th and 95th percentiles are shown as black dots. Images were collected from at least three biological replicate samples and ≥100 nuclei were quantified for each. Statistical differences are indicated by *** $P < 0.001$.

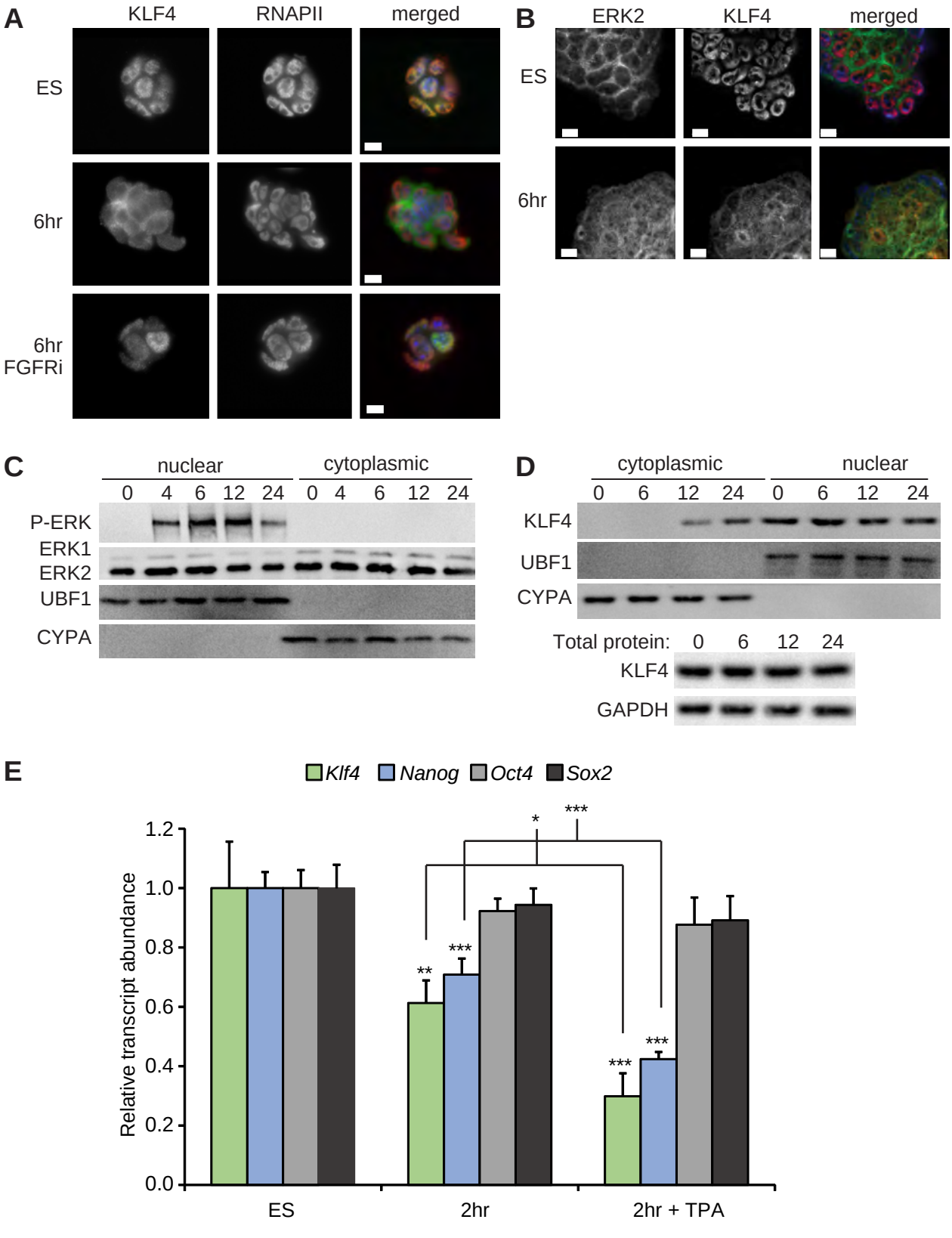
A
KLF4
RNAPII
merged
ES
6hr
6hr FGFRi
B
ERK2
KLF4
merged
ES
6hr
C
nuclear
cytoplasmic
0 4 6 12 24 0 4 6 12 24
P-ERK
ERK1
ERK2
UBF1
CYPA
D
cytoplasmic
nuclear
0 6 12 24 0 6 12 24
KLF4
UBF1
CYPA
Total protein: 0 6 12 24
KLF4
GAPDH
E
Klf4
Nanog
Oct4
Sox2
Relative transcript abundance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
***
**
***
***
***
ES
2hr
2hr + TPA

Figure S3: Signaling mechanisms regulating KLF4 nuclear export. Related to Figure 3. A) Immunofluorescence images of ES cells cultured with LIF/2i and ES cells 6hr after LIF/2i removal or after 6hr of LIF/2i removal in the presence of the FGFR inhibitor (FGFRi, PD173074). Merged images display KLF4 in green, RNAPII-S5P in red and DAPI in blue. Scale bar = 10μm. B) Removal of LIF/2i causes ERK2 to accumulate in the nucleus concurrently with KLF4 nuclear exit. Immunofluorescence images of ES cells cultured with LIF/2i and ES cells 6hr after LIF/2i removal. Merged images display KLF4 in red, ERK2 in green and DAPI in blue. Scale bar = 10μm. C) Immunoblot analysis from nuclear and cytoplasmic fractions of ES cells cultured with LIF/2i (0) and ES cells 4, 6, 12 and 24hr after LIF/2i removal. D) Immunoblot analysis from nuclear and cytoplasmic fractions of ES cells, and total protein from ES cells cultured with LIF (0) and ES cells 6, 12 and 24hr after LIF removal. E) TPA treatment causes greater decrease in *Klf4* and *Nanog* mRNA 2hr after LIF/2i withdrawal. The average of three biological replicates are normalized to the levels observed in undifferentiated ES cells and GAPDH. Statistical differences compared to the undifferentiated cells or the 2hr differentiated cells are indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars represent standard deviation.

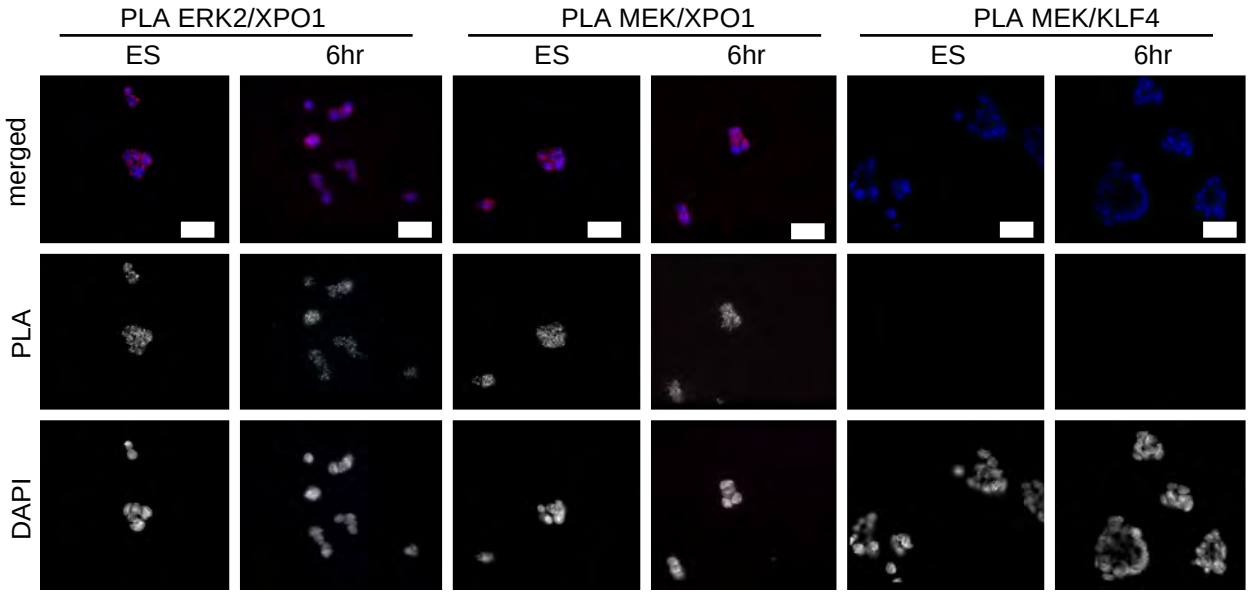
PLA ERK2/XPO1
PLA MEK/XPO1
PLA MEK/KLF4
ES
6hr
ES
6hr
ES
6hr
merged
PLA
DAPI

Figure S4: KLF4 interacts with XPO1 independently of MEK. Related to Figure 4. PLA in undifferentiated and 6hr differentiated ES cells revealed interaction between ERK2/XPO1 and MEK/XPO1 in both conditions but no interaction between KLF4 and MEK in either condition. Scale bar = 25μm.

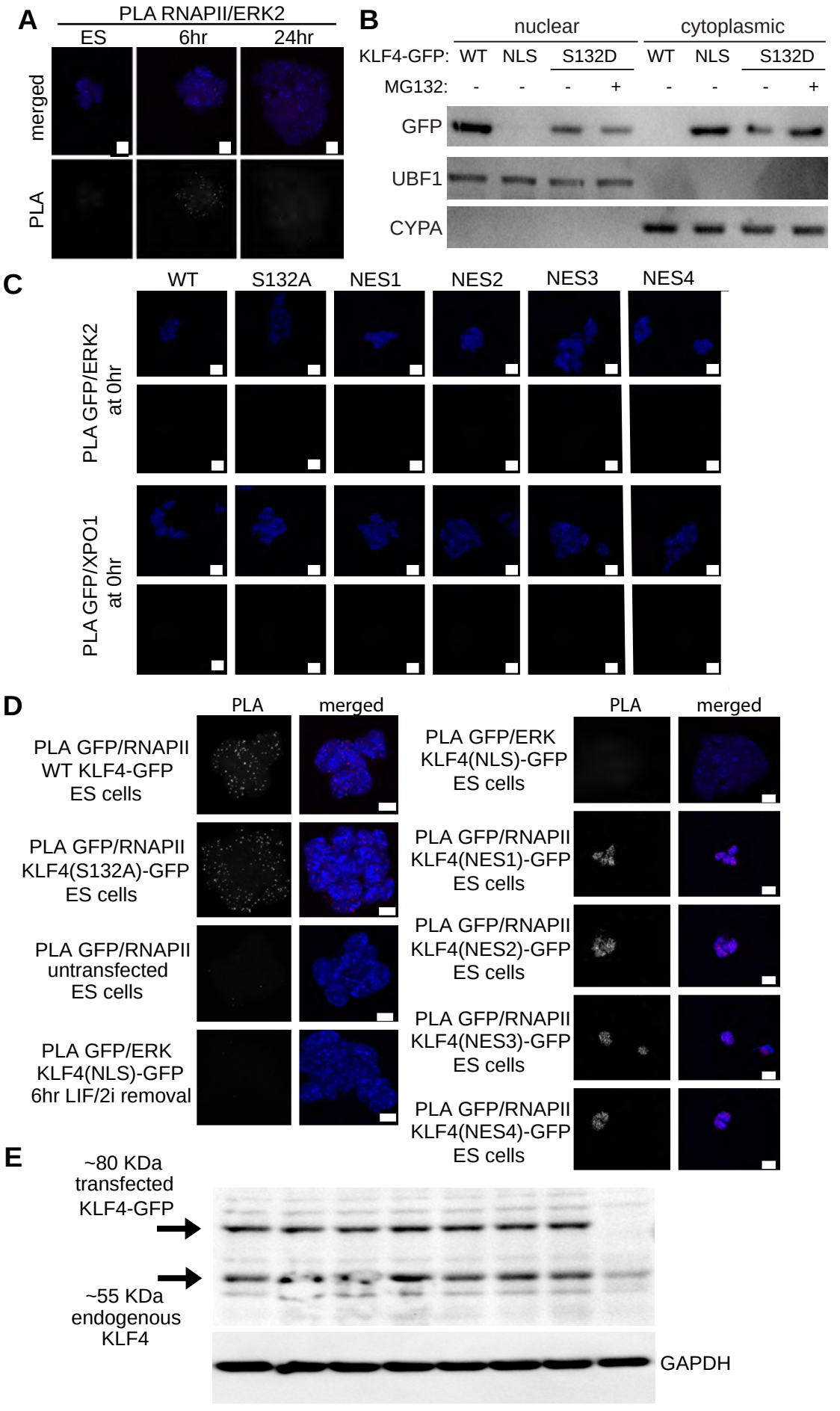
A
PLA RNAPII/ERK2
ES
6hr
24hr
merged
PLA
B
nuclear
cytoplasmic
KLF4-GFP: WT NLS S132D WT NLS S132D
MG132: - - - + - - - +
GFP
UBF1
CYPA
C
WT
S132A
NES1
NES2
NES3
NES4
PLA GFP/ERK2 at 0hr
PLA GFP/XPO1 at 0hr
D
PLA
merged
PLA GFP/RNAPII WT KLF4-GFP ES cells
PLA GFP/RNAPII KLF4(S132A)-GFP ES cells
PLA GFP/RNAPII untransfected ES cells
PLA GFP/ERK KLF4(NLS)-GFP 6hr LIF/2i removal
PLA GFP/ERK KLF4(NLS)-GFP ES cells
PLA GFP/RNAPII KLF4(NES1)-GFP ES cells
PLA GFP/RNAPII KLF4(NES2)-GFP ES cells
PLA GFP/RNAPII KLF4(NES3)-GFP ES cells
PLA GFP/RNAPII KLF4(NES4)-GFP ES cells
E
~80 KDa transfected KLF4-GFP
~55 KDa endogenous KLF4
GAPDH

Figure S5: Supplemental data related to Figure 5. A) PLA indicating the interaction between ERK2 and RNAPII-S5P in ES cells and 6 or 24hr after LIF/2i withdrawal. Images shown are maximum intensity projections. Scale bar = 10μm. B) Nuclear and cytoplasmic fractions prepared from ES cells expressing WT KLF4-GFP or KLF4-GFP mutants maintained in LIF/2i. Anti-GFP immunoblot indicated the S132D phosphomimetic is exported to the cytoplasm in LIF/2i. Treatment with the proteasome inhibitor MG132 prevents degradation of cytoplasmic KLF4(S132D)-GFP. C) PLA indicating no interaction between any KLF4-GFP (WT or indicated mutants) and ERK2 or XPO1 in ES cells maintained in LIF/2i. Scale bar = 10μm. D) PLA controls in KLF4-GFP transfected cells. PLA for GFP/RNAPII-S5P in WT KLF4-GFP expressing cells and KLF4(S132A)-GFP, KLF4(NES1, 2, 3, 4)-GFP expressing cells indicates that these mutations do not affect participation of KLF4 in RNAPII complexes. NLS mutation does affect KLF4 nuclear localization and interaction with RNAPII. PLA for GFP/ERK in cells expressing mutant KLF4(NLS)-GFP 6hr after LIF/2i removal indicates the NLS is required for KLF4/ERK interaction. Scale bar = 10μm. E) Immunoblot from ES cell lines shown from left to right with stable integration of mutant KLF4(NES4)-GFP, KLF4(NES3)-GFP, KLF4(NES2)-GFP, KLF4(NES1)-GFP, KLF4(NLS)-GFP, KLF4(S132A)-GFP, or WT KLF4-GFP and untransfected ES cells. KLF4 detection identifies the endogenous and the transfected KLF4-GFP proteins. GAPDH levels indicate relative protein amounts in each lane.

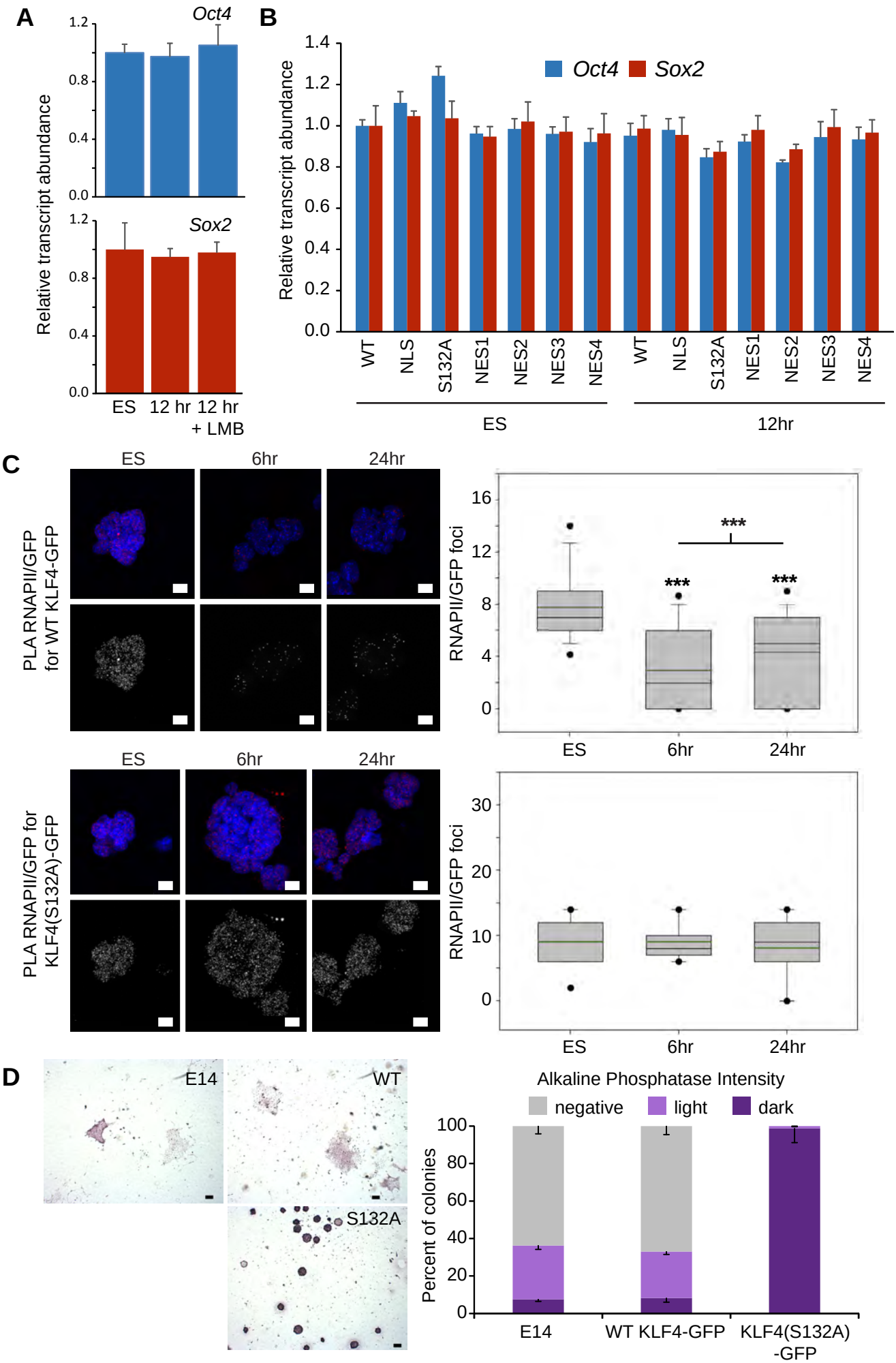
A
Oct4
Sox2
Relative transcript abundance
1.2
0.8
0.4
0.0
ES
12 hr
12 hr + LMB
B
Oct4
Sox2
Relative transcript abundance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
NLS
S132A
NES1
NES2
NES3
NES4
ES
12hr
C
ES
6hr
24hr
PLA RNAPII/GFP for WT KLF4-GFP
RNAPII/GFP foci
16
12
8
4
0
***
PLA RNAPII/GFP for KLF4(S132A)-GFP
30
20
10
D
E14
WT
S132A
Alkaline Phosphatase Intensity
negative
light
dark
Percent of colonies
100
80
60
40
20
E14
WT KLF4-GFP
KLF4(S132A) -GFP

Figure S6: Expression of KLF4(S132A) blocks ES cell differentiation. Related to Figure 6. A) Treatment with 5μg/ml LMB does not affect *Oct4* or *Sox2* transcript abundance. Average data form three biological replicates are normalized to the levels observed in undifferentiated ES cells. Error bars represent standard deviation. B) Relative transcript abundance of *Oct4* and *Sox2* is not affected by 12hr of LIF/2i removal in WT KLF4-GFP, KLF4(NLS)-GFP, KLF4(S132A)-GFP, KLF4(NES1)-GFP, KLF4 NES2)-GFP, KLF4(NES3)-GFP, or KLF4(NES4)-GFP. Average data form three biological replicates are normalized to the levels observed in undifferentiated ES cells expressing WT KLF4-GFP. Error bars represent standard deviation. C) The reduction in KLF4/RNAPII interaction during differentiation depends on KLF4 nuclear export. Proximity ligation amplification (PLA) indicating the interaction between WT KLF4-GFP and RNAPII-S5P or KLF4(S132)-GFP and RNAPII-S5P in ES cells and 6 or 24hr after LIF/2i withdrawal is shown on the left. Images shown are maximum intensity projections. Scale bar = 10μm. On the right box and whisker plots display the number of PLA foci per nucleus. Boxes indicate interquartile range of intensity values, whiskers indicate the 10th and 90^{th} percentiles, outliers in the 5th and 95th percentiles are shown as black dots. Images were collected from at least three biological replicate samples and ≥100 nuclei were quantified for each. Statistical differences are indicated by *** $P < 0.001$. D) Alkaline phosphatase staining of untransfected E14, WT KLF4-GFP, or KLF4(S132A)-GFP colonies 5 days after LIF/2i removal. Scale bar = 50μm. Positive and negative colonies were counted form at least three replicates for each revealing that expression of WT KLF4-GFP does not block differentiation whereas expression of the S132A mutant does. Error bars represent standard deviation.

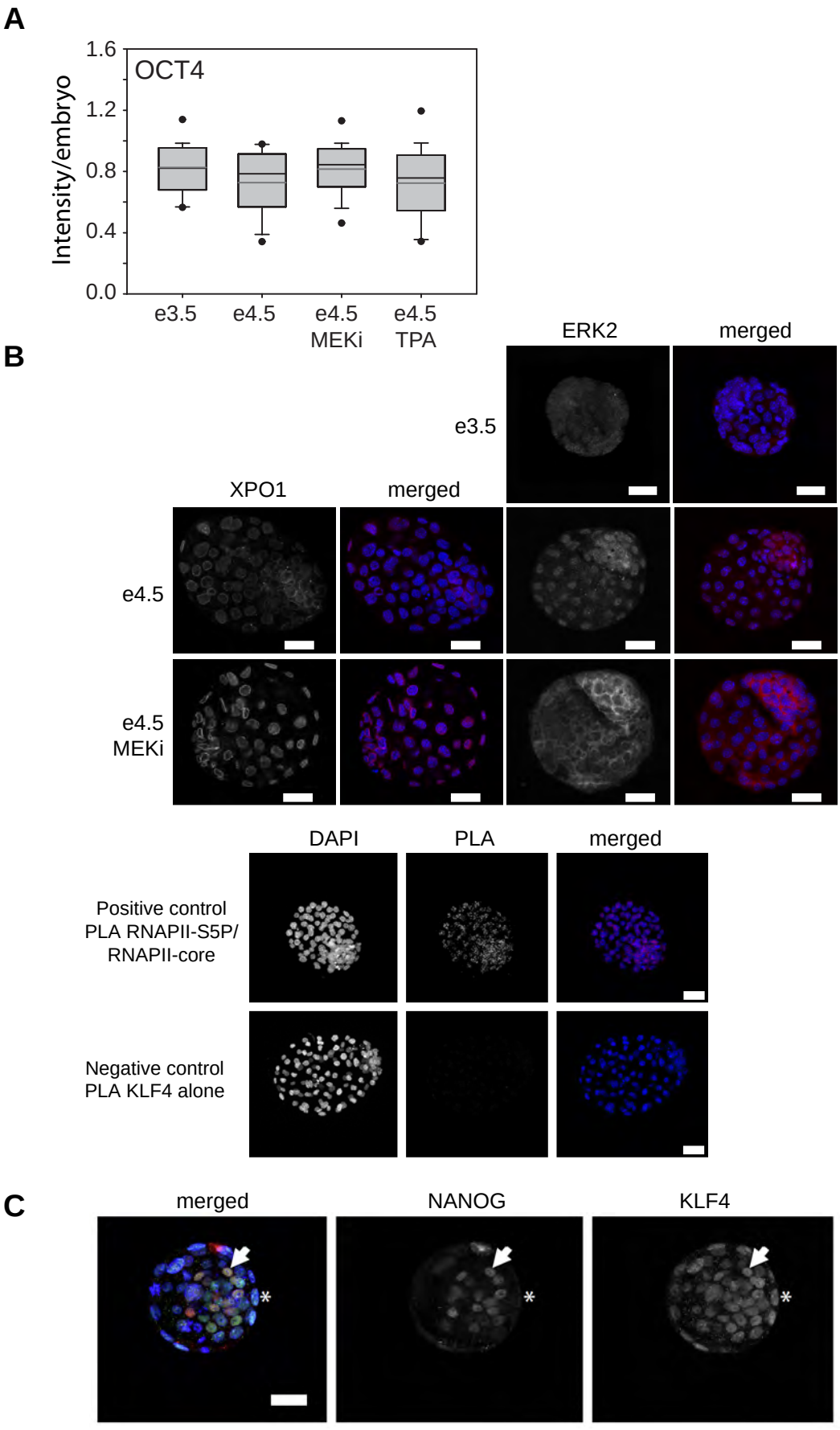
A
OCT4
Intensity/embryo
1.6
1.2
0.8
0.4
0.0
e3.5
e4.5
e4.5 MEKi
e4.5 TPA
B
ERK2
merged
e3.5
XPO1
merged
e4.5
e4.5 MEKi
DAPI
PLA
merged
Positive control PLA RNAPII-S5P/ RNAPII-core
Negative control PLA KLF4 alone
C
merged
NANOG
KLF4

Figure S7: Proximity ligation amplification and immunostaining controls in mouse embryos. Related to Figure 7. A) Quantification of immunofluorescence images from at least 30 embryos in each group reveal no significant change in OCT4 protein levels. B) XPO1 and ERK2 immunofluorescence at the indicated embryonic day and PLA control experiments. Scale = 25μm. As a positive control proximity ligation amplification (PLA) was conducted with anti-RNAPII-S5P and anti-RNAPII-core. As a negative control PLA was conducted with the anti-KLF4 antibody alone followed by both the anti-mouse and anti-rabbit oligo linked secondary antibodies. Embryos shown are at embryonic day 4.5. C) Immunofluorescence image of an e3 mouse blastocyst. KLF4 is detectable in both NANOG positive cells (arrow) and NANOG negative outer cells (*). Images shown are maximum intensity projections. Merged images display DAPI in blue NANOG in red and KLF4 in green. Scale bar = 25μm.

Table S1: Antibody list

Name	Company	Catalog #	Experiment
rabbit anti-KLF4	Abcam	AB129473	IF, PLA,WB, IP, Embryo IF and PLA
mouse anti-KLF4	Santa Cruz	sc-393462	IF, PLA, IP-WB, Embryo IF and PLA
rabbit anti-NANOG	Cosmo Bio	RCAB0002 P-F	IF, PLA,WB
rabbit anti-NANOG	Abcam	AB80892	Embryo IF
mouse anti-NANOG	BD Biosciences	560259	PLA, Embryo IF
mouse anti-SOX2	R&D Systems	MAB2018	IF, PLA
mouse anti-OCT3/4	Santa Cruz	sc-5279	IF, PLA, Embryo IF
mouse anti-RNAPII-PS5	Abcam	AB5408	IF, PLA
rabbit anti-RNAPII-PS5	Abcam	AB5131	IF, PLA, Embryo PLA
mouse anti-RNAPII core (ARNA3)	Millipore Sigma	CBL221	PLA, Embryo PLA
rabbit anti-ERK1	Santa Cruz	sc-093	WB
rabbit anti-ERK2	Santa Cruz	sc-154	IF, PLA,WB, IP, IP-WB, Embryo IF and PLA
rabbit anti-ERKpTEpY	Promega	V8031	WB
mouse anti-XPO1 (CRM1)	Santa Cruz	sc-74454	IF, PLA, IP-WB, Embryo IF and PLA
mouse anti-GFP	Thermo Fisher Scientific	A11121	IF, PLA
rabbit anti-GFP	Origene	TA150071	PLA
chicken anti-GFP	Abcam	AB13970	WB
goat anti-rabbit A488	Thermo Fisher Scientific	A-11034	IF
goat anti-mouse A594	Thermo Fisher Scientific	A11032	IF
rabbit anti-CYPA	Abcam	AB131334	WB
rabbit anti-UBF1	Santa Cruz	sc-13125	WB
mouse anti-GAPDH	Santa Cruz	sc-365062	WB
goat anti-rabbit-HRP	Bio-Rad	170-6515	WB
goat anti-mouse-HRP	Bio-Rad	170-6516	WB
mouse anti-phosphoserine	Abcam	AB9332	IP-WB

Table S2: Primer list

Gene	Forward primer	Reverse primer	Amplicon size
Klf4	GAAGACGAGGATGAAGCTGAC	TGGACCTAGACTTTATCCTTTCC	94bp
Nanog	TCCCAAACAAAAGCTCTCAAG	ATCTGCTGGAGGCTGAGGTA	165bp
Sox2	ACGCCTTCATGGTATGGTC	CGGACAAAAGTTTCCACTC	114bp
Oct4	ATGAGGCTACAGGGACACCTT	GTGAAGTGGGGGCTTCCATA	100bp
Gapdh	GCACCAGCATCCCTAGACC	CTTCTTGTGCAGTGCCAGGTG	109bp

Table S3: Site directed mutagenesis primer list

Primer name	Sequence
S132A forward	CCACCTCGGCGTCAGCTTCATCCTCGTCTGCCCCAGCGAGCAGCGGCCCTGCC
S132A reverse	GGCAGGGCCGCTGCTCGCTGGGGCAGACGAGGATGAAGCTGACGCCGAGGTGG
NLS forward	CGGGGCCACGACCCGCTTCCGCTCTTTGGCTTGG
NLS reverse	CCAAGCCAAAGAGCGGAAGCGGGTCGTGGCCCCG
NES1 forward	AAAGGATAAAGTCTAGGTCCTGTTGGTCGTTGAACTCCTCGGTC
NES1 reverse	GACCGAGGAGTTCAACGACCAACAGGACCTAGACTTTATCCTTT
NES2 forward	GTGGTCACGGTGCCGCCCACCGATTCCT
NES2 reverse	AGGAATCGGTGGGCGGCACCGTGACCAC
NES3 forward	GCCAGGGGTGGTCTGAGACGCCTTCAG
NES3 reverse	CTGAAGGCGTCTCAGACCACCCCTGGC
NES4 forward	CAAATGGGCCTCTTGGGACCGGCTGAC
NES4 reverse	GTCAGCCGGTCCCAAGAGGCCCATTTG
S132D forward	TGCTCGCTGGGTCAGACGAGGATGAAGCTGACGC
S132D reverse	GCGTCAGCTTCATCCTCGTCTGACCCAGCGAGCA