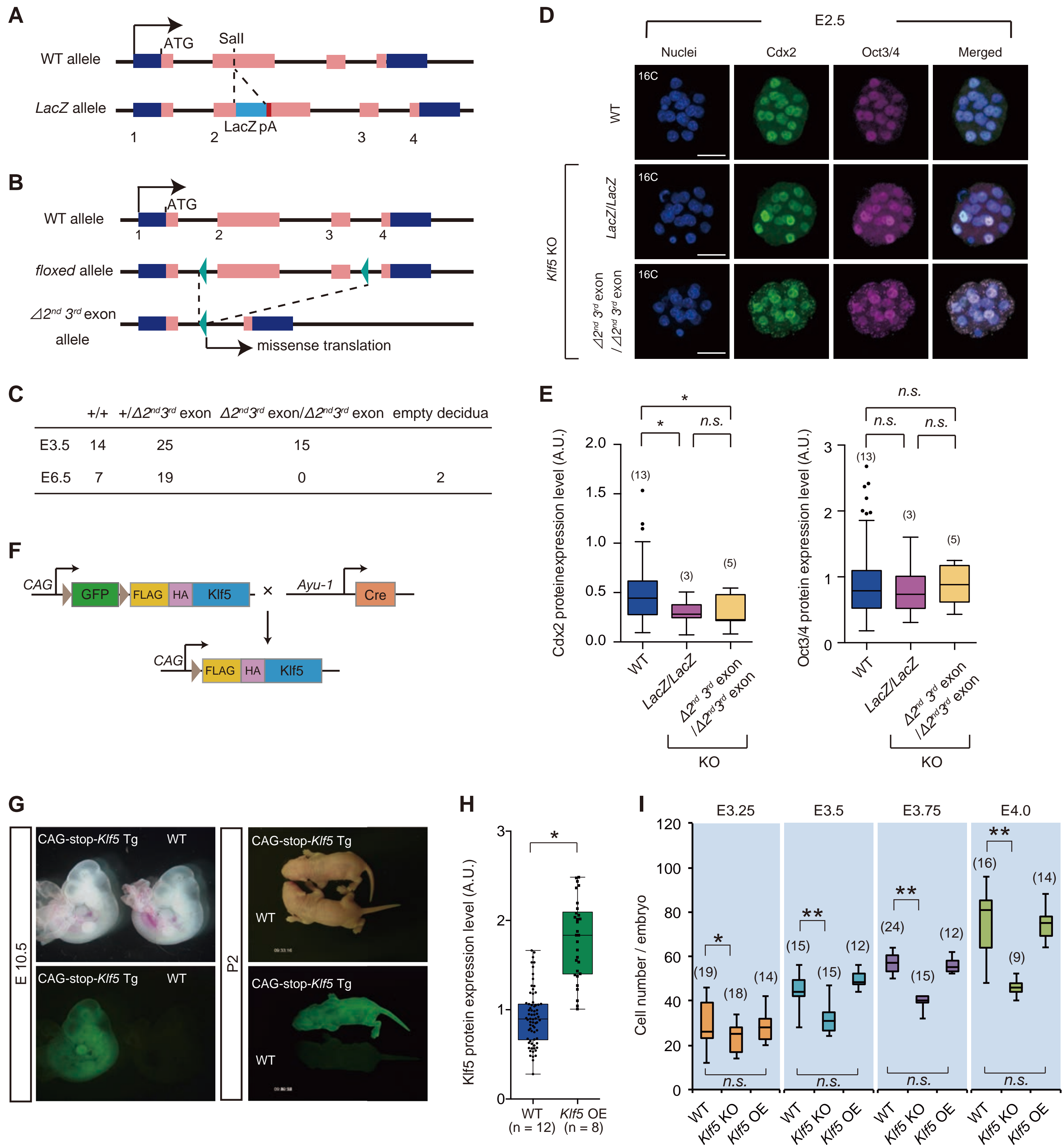
# Supplemental Figures



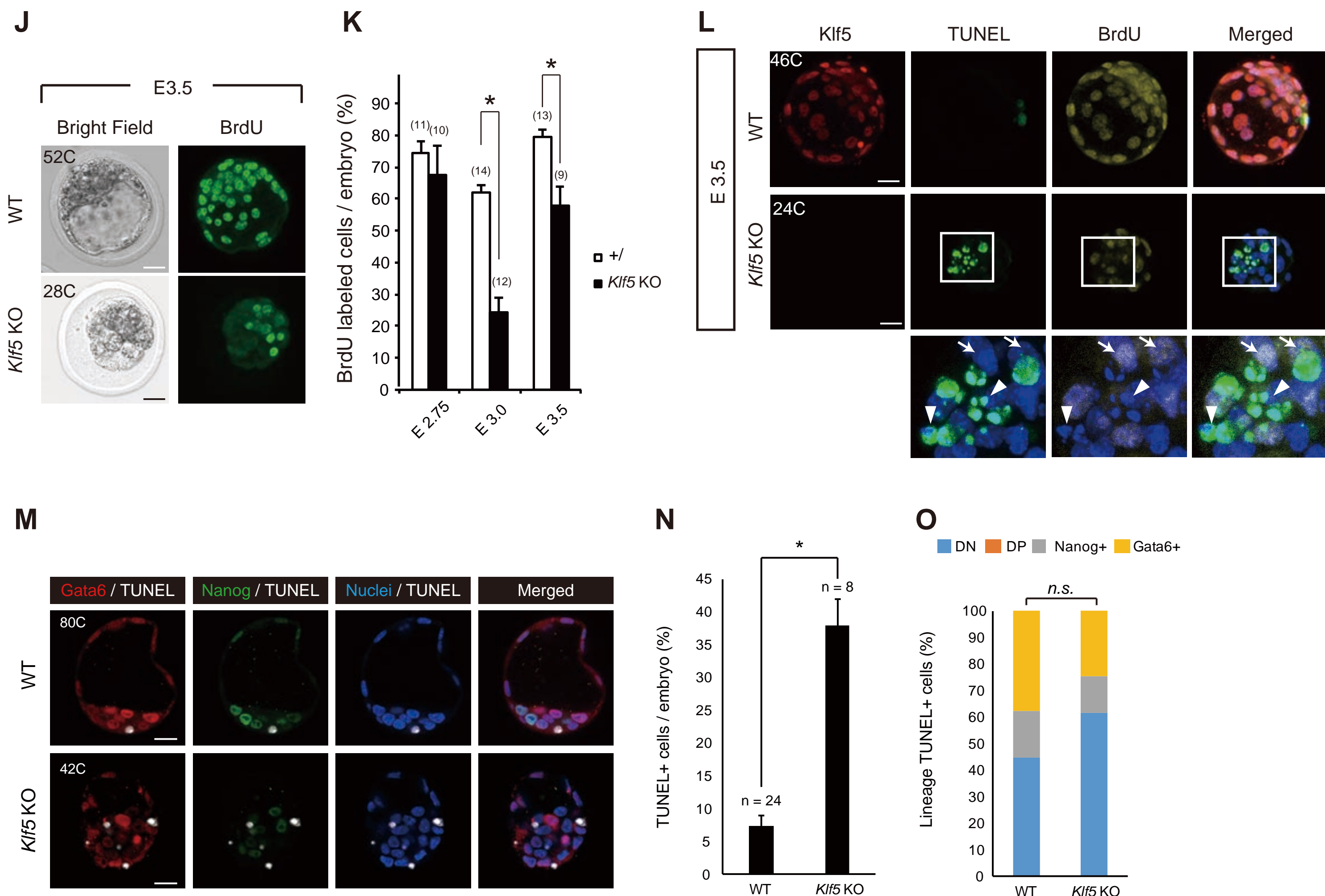



**Figure S1: The *Klf5* mutant alleles and *Klf5* conditional activation mice used for this study.** *LacZ* knock-in allele for *Klf5*. SalI is a restriction enzyme site and pA is the polyadenylation signal sequence. (B) Conditional knockout allele for *Klf5*. Two loxP sites were introduced that flanked exons 2 and 3. Exons 2 and 3 were removed by expressing Cre transiently to generate a null allele that lacked functional Klf5. ATG is the initiation codon. (C) Genotype of embryos from newly generated *Klf5*Δ2$^{nd}$3$^{rd}$ exon heterozygous mouse intercrosses at the preimplantation (E3.5) and postimplantation (E6.5) stages. (D) Expression of Oct3/4 and Cdx2 in WT, *Klf5LacZ/LacZ*, and *Klf5*Δ2$^{nd}$3$^{rd}$ exon/*Klf5*Δ2$^{nd}$3$^{rd}$ exon embryos at E2.5. C, cell number. Scale bars represent 25 µm. (E) Tukey box plots showing Cdx2 and Oct3/4 protein levels at E2.5. Embryo numbers are shown in brackets. Asterisks indicate statistical significance: *P < 0.01; n.s., not significant; Mann–Whitney U-test. (F) Generation of conditional *Klf5* OE mice. The CMV early enhancer/chicken beta actin (CAG) promoter was used to drive GFP, and FLAG/HA-tagged Klf5 after Cre excision, in the transgene (Tg). (G) GFP expression in conditional *Klf5* OE mice during embryonic and postnatal stages. (H) Tukey box plots showing Klf5 protein levels at E3.5 blastocysts. Asterisks indicate statistical significance: *P < 0.01; Mann–Whitney U-test. (I) Tukey box plots of the numbers of cells per embryo. Embryo numbers are shown in brackets. Asterisks indicate statistical significance: *P < 0.01; ** P < 0.001; n.s., not significant; Mann–Whitney U-test. (J, K) BrdU incorporation in WT and *Klf5* KO blastocysts at E3.5. Percentage of BrdU incorporated cells in WT and *Klf5* KO blastocysts is shown in (K). Embryo numbers are shown in brackets. Asterisks indicate statistical significance: *P < 0.01; Mann–Whitney U-test. C, cell number. Scale bars represent 20 µm. (L) *Klf5* KO embryos showed increased apoptosis by TUNEL assays at the blastocyst stages. Note that the cells not incorporating BrdU from E3.25 to E3.5 tended to undergo apoptosis. Arrow indicates BrdU+ cells. Arrowhead indicates TUNEL+ cells that does not incorporate BrdU. C, cell number. Scale bars represent 20 µm. (M) TUNEL+ cells in WT and *Klf5* KO embryos at E3.75. Representative confocal optical section images showing colocalization of TUNEL+ cells with Gata6+ and Nanog+ cells. C, cell number. Scale bars represent 20 µm. (N) Percentages of TUNEL+ cells in WT and *Klf5* KO embryos at E3.75. Asterisks indicate statistical significance: *P < 0.01; Mann–Whitney U-test. (O) Localization of TUNEL+ cells in Nanog+, Gata6+, Nanog/Gata6 double-positive (DP), and double-negative (DN) cells in WT and *Klf5* KO embryos at E3.75. Distributions of TUNEL+ cells are shown as percentages of total cell numbers. There was no significant difference in the distribution of TUNEL+ cells between WT and *Klf5* KO embryos. Fisher’s exact test was used for statistical analysis. n.s., not significant.

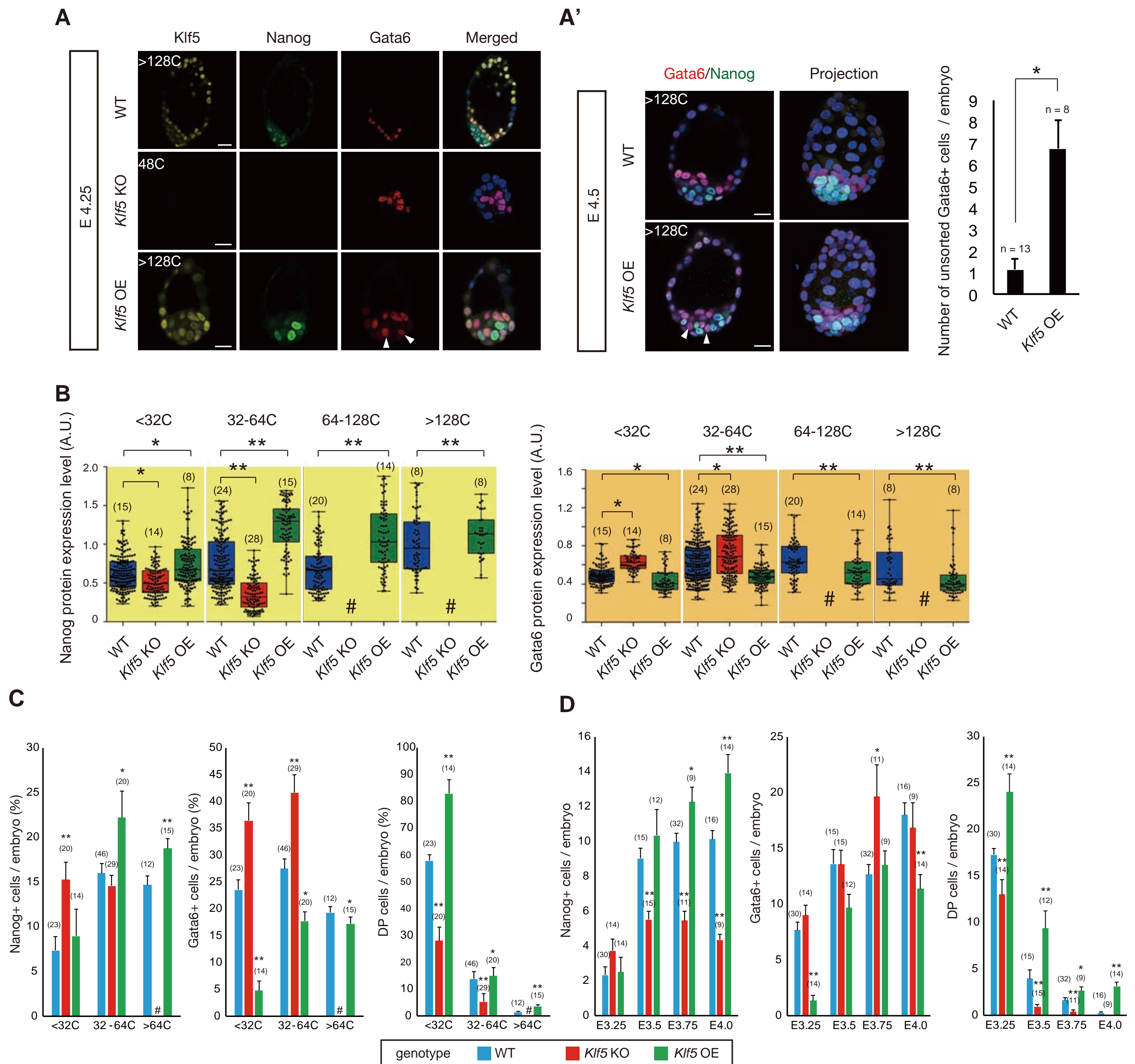


**Figure S2: Skewed EPI and PrE lineage specification in *Klf5* KO and *Klf5* OE blastocysts.** (A) Expressions of Nanog and Gata6 in WT, *Klf5* KO and *Klf5* OE embryos at E4.25. Images are confocal microscopy cross-sections. C, cell number. Scale bars represent 20 µm. (A′) Expressions of Nanog and Gata6 in WT and *Klf5* OE embryos from E4.5. Arrowheads indicate the unsorted Gata6+ cells located interiorly. Note that number of the unsorted Gata6+ cells is significantly increased in *Klf5* OE embryos at E4.5. Scale bar represent 20 µm. (B) Tukey box plots of Nanog and Gata6 protein expression levels. Embryo numbers are shown in brackets. A.U., arbitrary unit. # indicates no embryo obtained. (C) Percentages of Nanog+, Gata6+ and DP cells per embryo. # indicates no embryo obtained. (D) Absolute numbers of Nanog+, Gata6+ , and DP cells per embryo. Asterisks in (A′), (B) and (C) indicate statistical significance compared with WT: *P < 0.01; ** P < 0.001; Mann–Whitney U-test.

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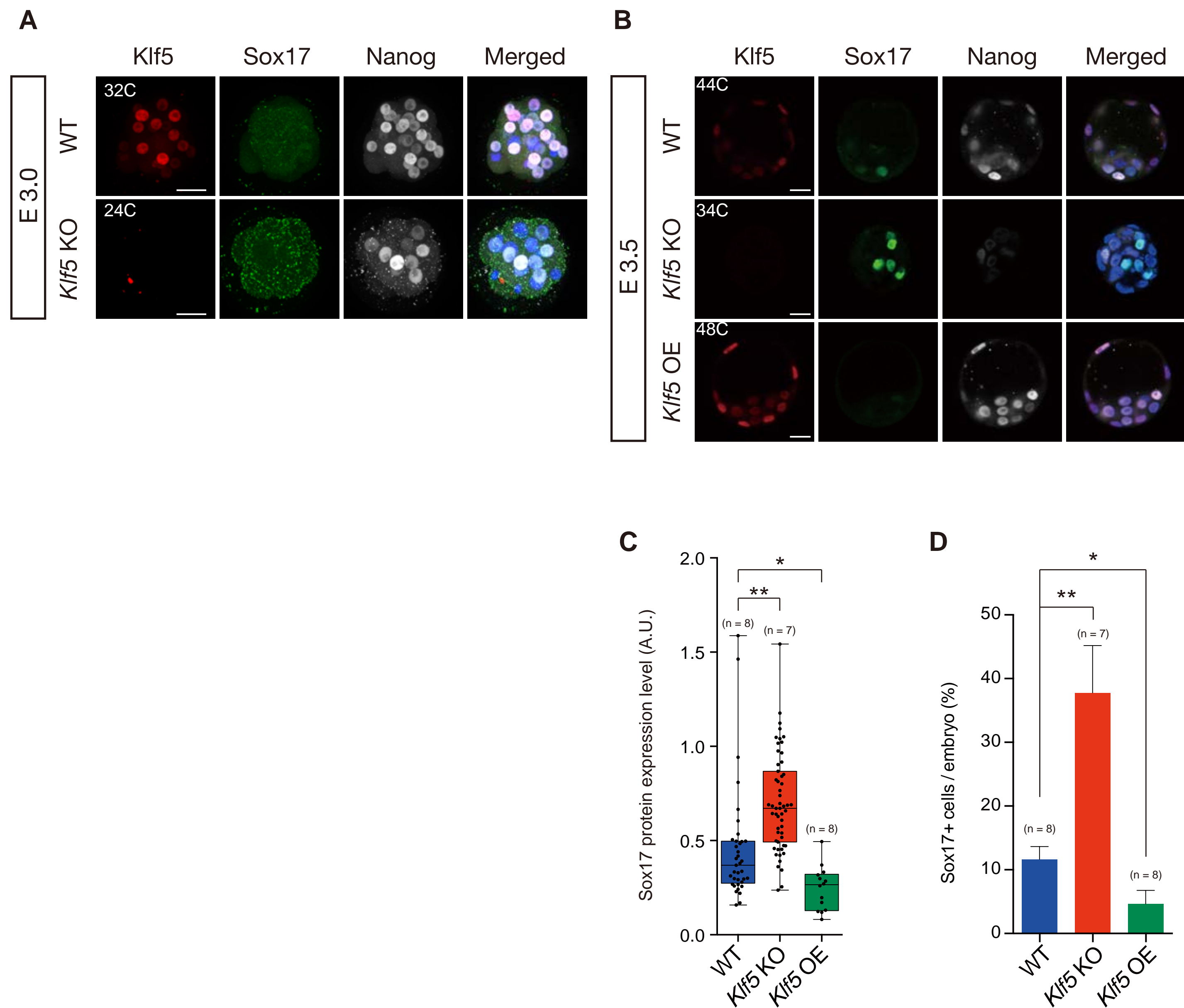


**Figure S3: Accelerated PrE lineage specification in *Klf5* KO blastocysts.** (A) Expressions of Sox17 and Nanog in WT and *Klf5* KO embryos at E3.0. Sox17 protein was not detected in the nuclear of both WT and *Klf5* KO embryos at this stage. Images are projection images from confocal microscopy. C, cell number. Scale bars represent 20 μm. (B) Expressions of Sox17 and Nanog in WT, *Klf5* KO and *Klf5* OE embryos at E3.5. Note that Sox17 protein expression levels are dramatically upregulated and downregulated in *Klf5* KO embryos and *Klf5* OE embryos, respectively. Images are confocal microscopy cross-sections. C, cell number. Scale bars represent 20 μm. (C) Tukey box plots of the levels of Sox17 expression in cells at E3.5. A.U., arbitrary unit. Embryo numbers are shown in brackets. (D) Percentage of Sox17+ cells per embryo at E3.5. Asterisks in (C) and (D) indicate statistical significance: $^{*}P < 0.01$; $^{**} P < 0.001$; Mann–Whitney U-test.

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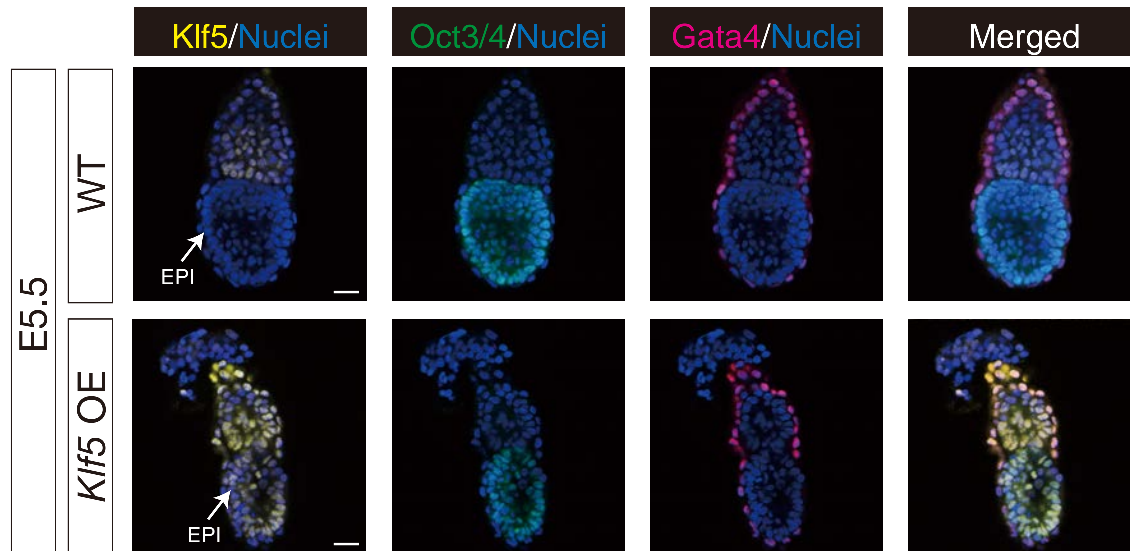


**Figure S4: Overall normal development of *Klf5* OE embryos.** Oct3/4 and Gata4 expressions in WT or *Klf5* OE embryos at E5.5. Arrows indicate postimplantation EPI. Scale bars represent 20 μm.

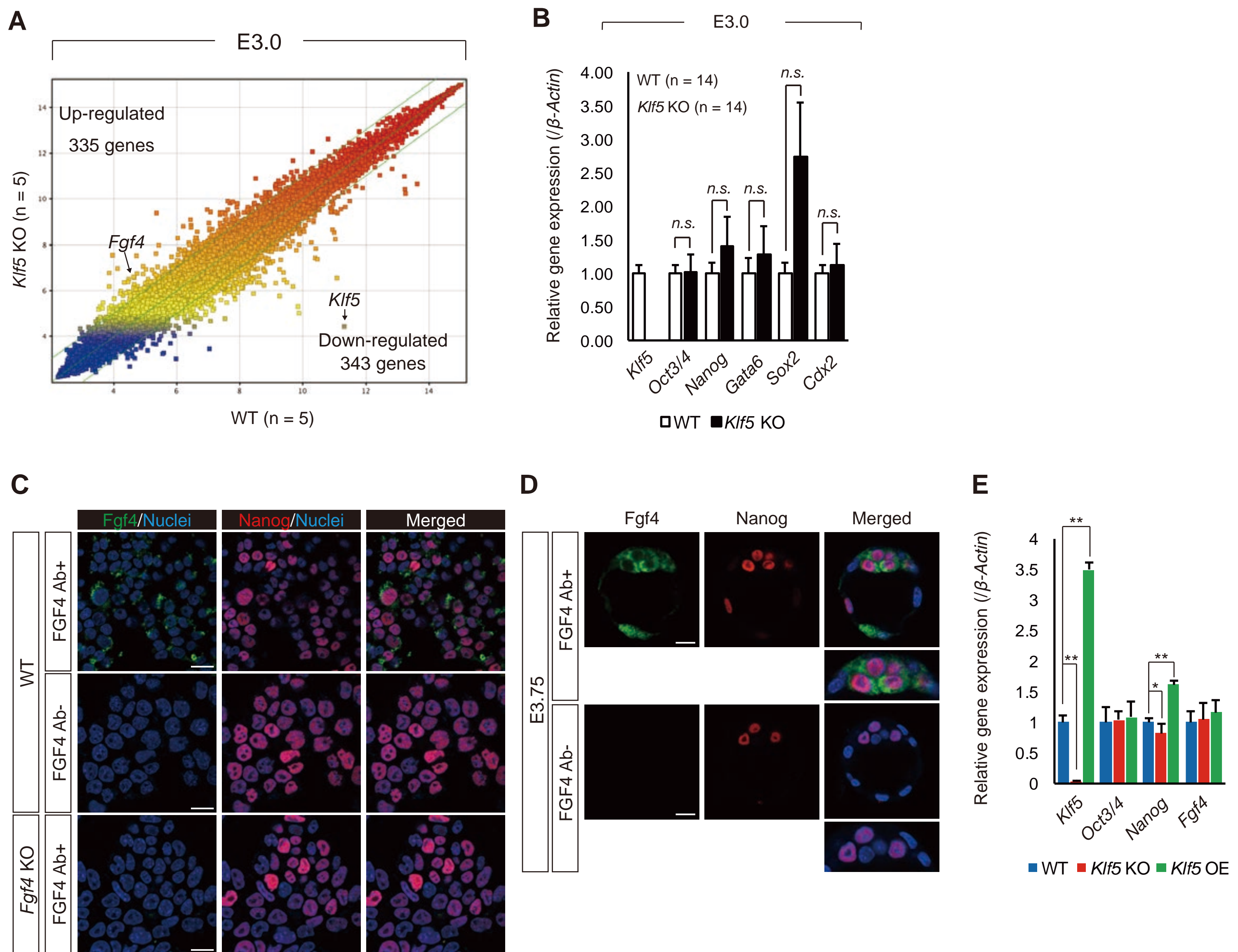


**Figure S5: *Fgf4* expression and validation of anti-FGF4 antibody.** (A) A scatter plot of genes upregulated/downregulated in *Klf5* KO embryos compared with WT embryos at E3.0. n; number of samples analysed. (B) Expression of embryonic transcription factors in *Klf5* KO blastocysts. n.s, not significant. (C) Validation of anti-FGF4 antibody with *Fgf4* KO ES cells. Note that the anti-FGF4 antibody recognises FGF4 in mouse ES cells. Scale bars represent 20 µm. (D) Detection of FGF4 expression in epiblast cells of WT embryos at E3.75 with the anti-FGF4 antibody. Scale bars represent 20 µm. (E) *Fgf4* mRNA expression in *Klf5* KO and *Klf5* OE ES cells. Asterisks indicate statistical significance: *P < 0.01; ** P < 0.001; Mann–Whitney U-test.

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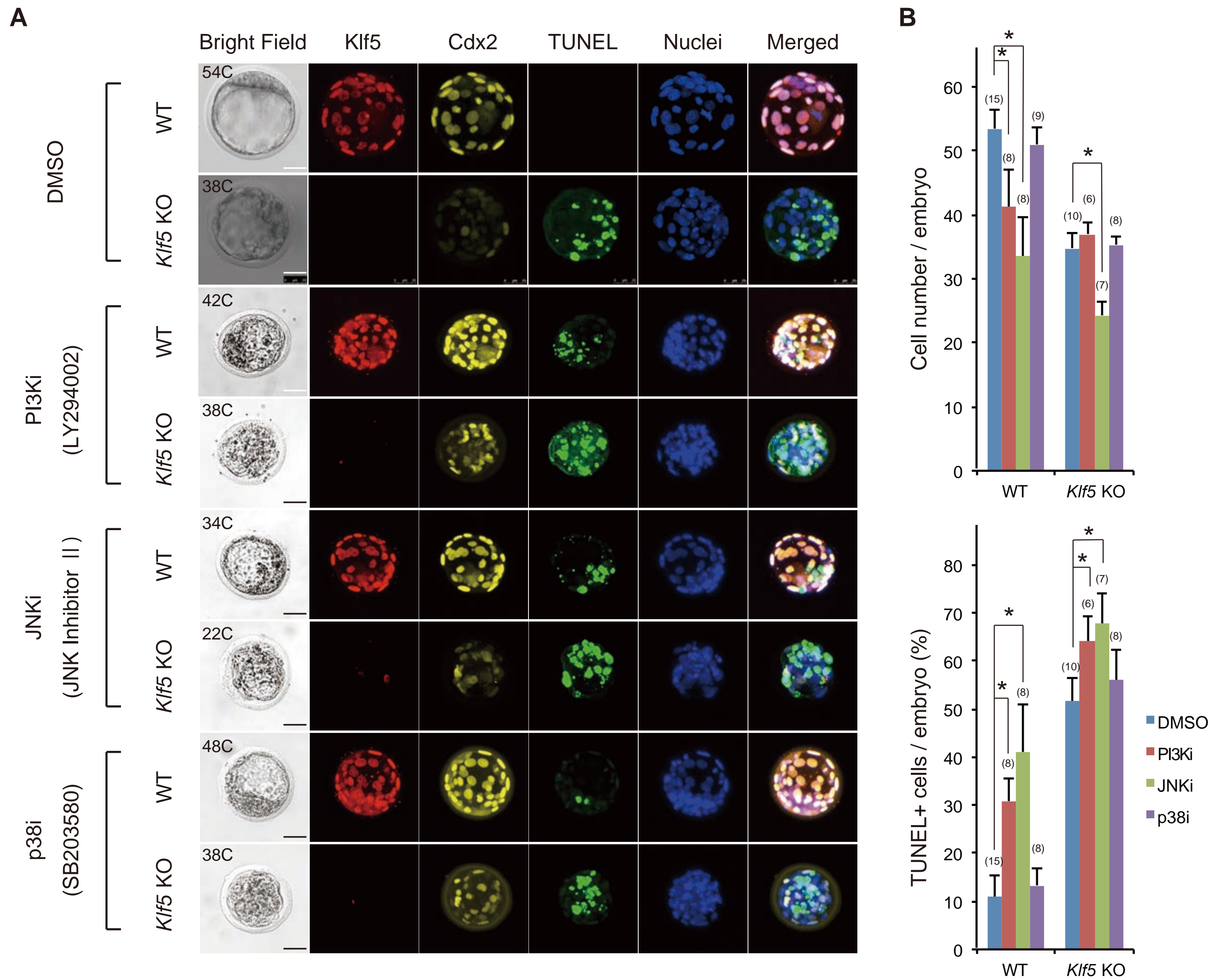


**Figure S6: Ability of chemical inhibitors (i) against JNK, p38, and PI3K to rescue the phenotype of *Klf5* KO blastocysts.** (A) Expression of Klf5 and Cdx2 and TUNEL+ cells in WT and *Klf5* KO blastocysts at E3.75. Embryos at E2.75 were collected and cultured in the presence of inhibitors of PI3K (LY294002, 10 µM), JNK (JNK inhibitor II, 5 µM), and p38 (SB203580, 10 µM) for 24 h. C, cell number. Scale bars represent 25 µm. (B) The upper panel shows the number of cells per embryo and the lower panel shows percentages of TUNEL+ cells per embryo. Embryo numbers are shown in brackets. Asterisks indicate statistical significance: *P < 0.01; Mann–Whitney U-test.

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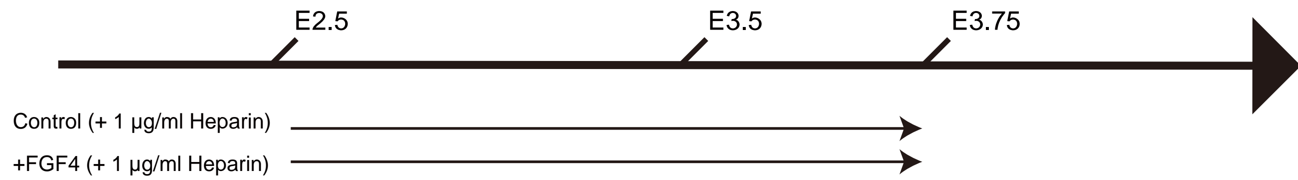


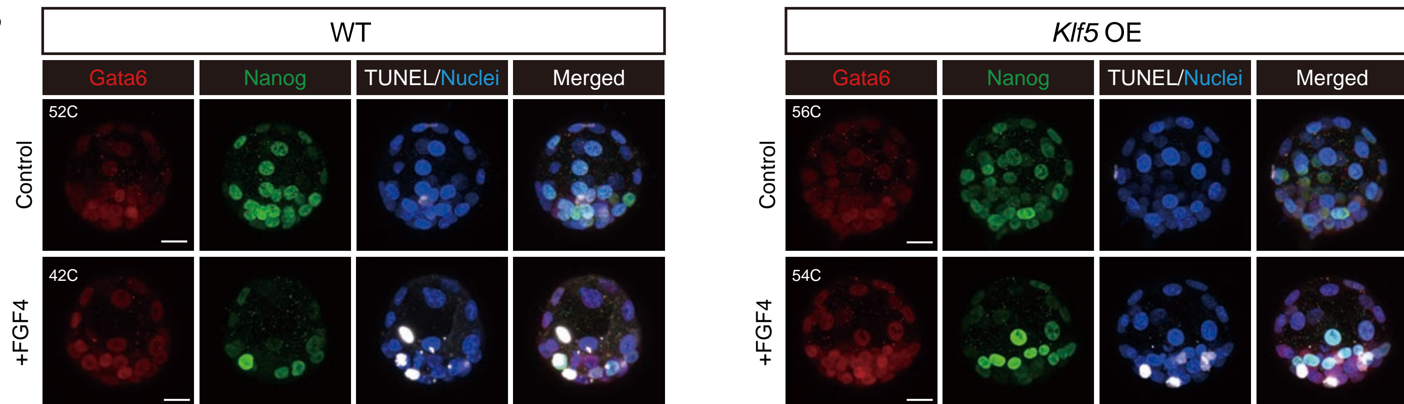


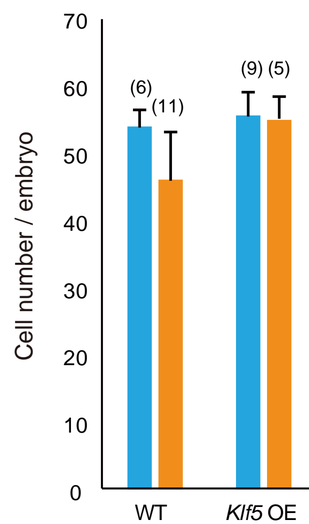


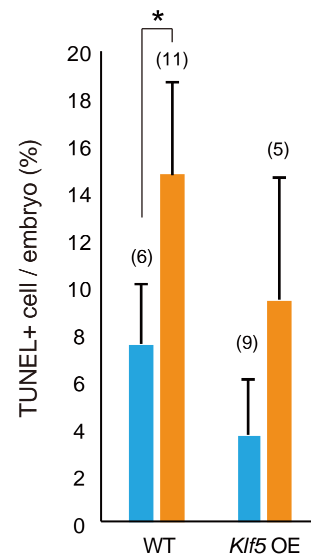


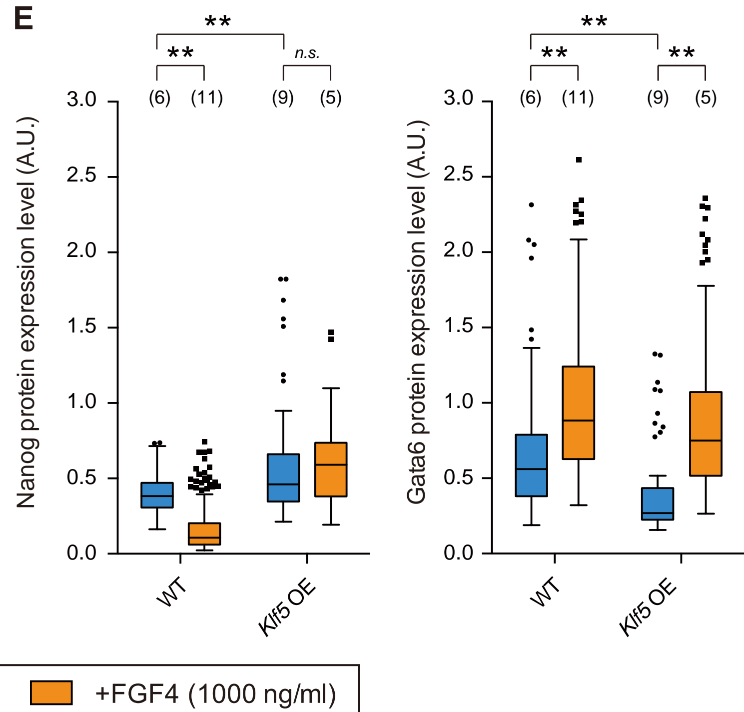


**Figure S7: Stimulation of FGF–ERK signalling by FGF4 in *Klf5* OE embryos.** (A) Outline of experiment to assess the effect of Fgf–ERK pathway stimulation by exogenous FGF4. WT and *Klf5* OE embryos were collected at E2.5 and cultured in the presence of FGF4 (1000 ng/ml) and heparin (1 µg/ml) until E3.75. (B) Effects of FGF4 on lineage specification and apoptosis induction in *Klf5* OE embryos at E3.75. Images are projection images from confocal microscopy. Scale bars represent 20 µm. (C) Average cell numbers in FGF4 treated WT and *Klf5* OE embryos. Embryo numbers are shown in brackets. (D) Percentages of TUNEL+ cells in WT and *Klf5* OE embryos cultured in the presence of FGF4. Embryo numbers are shown in brackets. (E) Turkey box plots of Nanog and Gata6 protein expression levels. Embryo numbers are shown in brackets. A.U., arbitrary unit. Asterisks in (C), (D) and (E) indicate statistical significance: *P < 0.05; **P < 0.01; Mann–Whitney U-test. n.s., not significant.

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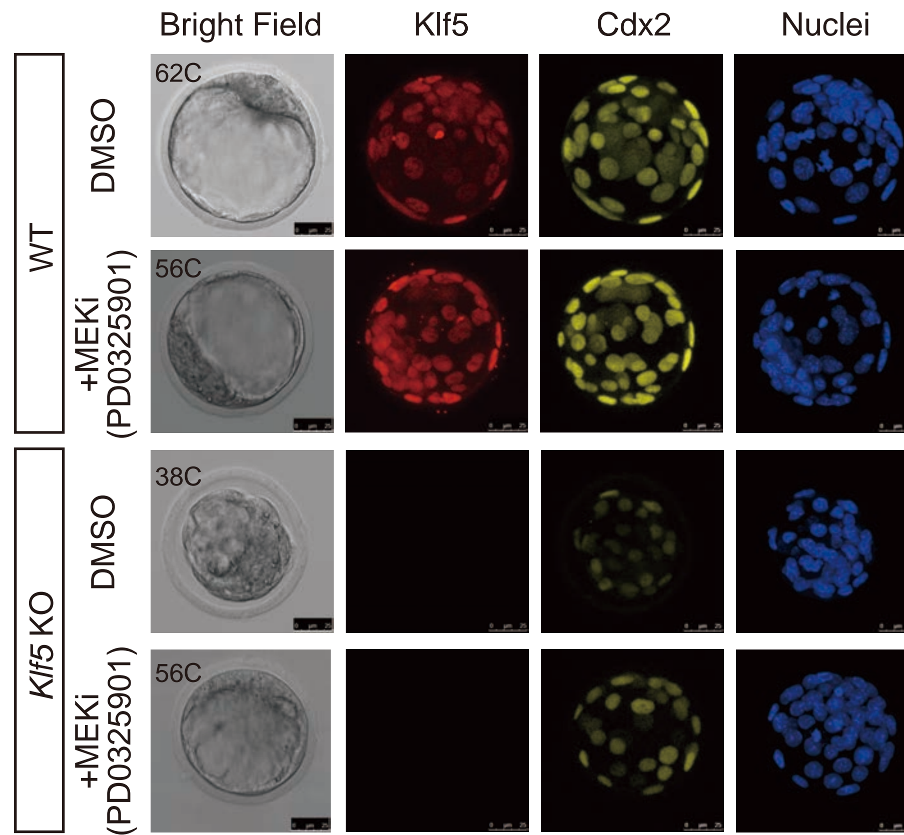


**Figure S8: Inhibition of ERK signalling does not rescue the reduced Cdx2 expression of *Klf5* KO blastocysts.** The reduced Cdx2 expression in *Klf5* KO embryos was not rescued by treatment with a MEK inhibitor (MEKi). Dimethyl sulfoxide was the solvent control. C, cell number. Scale bars represent 25 µm.

**Table S1: List of genotyping primers sequences**

| Name | Primer Sequence (Forward) | Primer Sequence (Reverse) | Usage |
|---|---|---|---|
| Klf5_WT allele | CCTGTTACCATTTTCAGCCACCAGA | TGAAGATATTGTTCACCTCCTGTGGT | for *Klf5* KO embryos |
| Klf5_deletion allele | AGGCCTGATAAAATAACCTAGTCCA | TAAGCACCGGTGCCTAACATGGTA | for *Klf5* KO embryos |
| Klf5_2-4ex | GTACACCATGCCAAGTCAGTTTCTTC | GTTGGCACACCATGCACTGGAAC | for *Klf5* OE embryos |
| Cre | GGACATGTTCAGGGATCGCCAGGCGT | GCATAACCAGTGAAACAGCATTGCTG | for *Klf5* OE embryos |

**Table S2: List of antibodies**

| Primary Antibody | Supplier | Catalogue Number | Dilution |
|---|---|---|---|
| mouse anti-Cdx2 | BioGenex | MU392A−UC | 1:300 |
| rabbit anti-Nanog | Cosmobio | RCAB0001P | 1:300 |
| mouse anti-Oct3/4 | SantaCruz | sc−5279 | 1:300 |
| goat anti-Gata6 | R&D | AF1700 | 1:300 |
| goat anti-Gata4 | SantaCruz | sc−1237 | 1:300 |
| goat anti-Fgf4 | R&D | BAF235 | 1:100 |
| rat anti-Pdgfra | eBioscience | 13−1401−82 | 1:300 |
| goat anti-Sox17 | R&D | AF1924 | 1:300 |
| rat anti-Klf5 | Kyowa KIRIN | a gift from Dr. Nagai | 1:300 |
| mouse anti-FLAG-M2 | Sigma-Aldrich | F3165 | 4 μg |
| normal mouse IgG | SantaCruz | sc-2025 | 4 μg |

| Secondary Antibody | Supplier | Catalogue Number | Dilution |
|---|---|---|---|
| Cy3-conjugated donkey anti-mouse IgG | Jackson Immunoresearch | 715−166−151 | 1:500 |
| Cy3-conjugated donkey anti-rabbit IgG | Jackson Immunoresearch | 711−165−152 | 1:500 |
| Cy5-conjugated donkey anti-rat IgG | Jackson Immunoresearch | 712−175−153 | 1:500 |
| Alexa488-conjugated donkey anti-rabbit IgG | Molecular Probes | A21206 | 1:500 |
| Alexa488-conjugated donkey anti-goat IgG | Molecular Probes | A110055 | 1:500 |
| Alexa633-conjugated donkey anti-goat IgG | Molecular Probes | A21082 | 1:500 |
| HRP-conjugated donkey anti-goat IgG | Jackson Immunologicals | 705-035-147 | 1:500 |



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# Table S3: List of qPCR primers sequences

Primers for RT-qPCR

| Gene | Primer Sequence (Forward) | Primer Sequence (Reverse) |
|---|---|---|
| Fgfr1 | CCTCAGGAAACAGAAAACGATCT | CCCTCTAAGGCTTGAGTGGTGT |
| Fgfr2 | ACAGCACCAGGAACCTACTTACACT | CTGTCCACTTATCAGGAAACAGTCT |
| Fgf4 | AAGGCACCTGCCCTGTTCTG | GGGAGCTAGCTGGCTGAAGAAA |
| Gata6 | ACTGAAGTAAGAAGAGATGGGCTTT | ATCTCTCAATCTTCCTTAGCAGACA |
| Pdgfra | CTAGCAGGTGACGCTTTGGA | GGCCAATCTGGCTCAGTCTT |
| Sox17 | CAGTATCTGCCCTTTGTGTATAAGC | GTAGTTGCAATAGTAGACCGCTGAG |
| Oct3/4 | TATTGAGTATTCCCAACGAGAAGAG | CTCAGGAAAAGGGACTGAGTAGAGT |
| Nanog | CTTTCACCTATTAAGGTGCTTGC | TGGCATCGGTTCATCATGGTAC |
| Sox2 | CATGAGAGCAAGTACTGGCAAG | CCAACGATATCAACCTGCATGG |
| Cdx2 | CACTTTAGTCGATACATCACCATCA | CTGCTTCTTCTTGATTTTCCTCTC |
| Klf5 | CGATTCACAACCCAAATTTACC | GTATGAGTCCTCAGGTGAGCTTTTA |
| β-actin | CTGAGCGCAAGTACTCTGTGTG | GTGTAAAACGCAGCTCAGTAACAGT |

Primers for ChIP-qPCR

| Gene | Primer Sequence (Forward) | Primer Sequence (Reverse) |
|---|---|---|
| Fgf4_1 | TGGGTCCTCACTCCTACCTG | AAGACAGGGAGCAGCACAGT |
| Fgf4_2 | GTGTCACAGCAGCTCCAAGA | GAGGGATCAGCCTAGCAGTG |
| Fgf4_4_2 | TCCTTAGGTGCTGGGAATTG | TGCCTGAAGCAACATAGCTG |
| Vegfr2 | CCCTGTTCACAAGTAAATACACCTC | GTTGTATATTCAGCTCCTCACGAAT |

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**Supplementary Materials and Methods.**

**Pluripotent stem cells.** *Fgf4*$^{+/-}$ (clone 342) and *Fgf4*$^{-/-}$ (clone FD6) ES cells (kind gifts from Dr. Angie Rizzino (Wilder et al., 1997)) were cultured in DMEM + 10% Serum/LIF condition onto fibronectin-coated glass bottom dishes and were subjected to immunohistochemistry using an anti-FGF4 antibody.

**Generation of *Klf5* KO mice and overexpressing transgenic mice**. To generate a conditional allele for *Klf5*, a targeting vector was created in which two loxP sequences flanking exons 2 and 3 were introduced, and the vector was introduced into E14 ES cells. ES cells that had undergone homologous recombination were selected and used to prepare chimeric animals (details are available upon request). Then, the *Klf5* conditional KO mice were crossed with FLPe deleter mice to remove the PGK-neo cassette and further crossed with Cre deleter mice to obtain the $\Delta 2^{nd}3^{rd}$ exon allele that allows removal of almost the entire open reading frame. All experiments were performed in accordance with the Declaration of Helsinki and were approved by the Ethics Committee for Animal Experiments of Shiga University of Medical Science.

Conditional *Klf5*-overexpressing mice were generated as follows. A Venus-pA cassette followed by FLAG-HA tagged mouse Klf5 cDNA was inserted into a CAG vector and electroporated into C57B6/J ES cells. An ES cell line (clone N6) expressed a similar level of FLAG-HA tagged Klf5 protein as endogenous Klf5 after Cre excision. This clone was used to create chimeric animals for germ line transmission. Conditional *Klf5*-overexpressing mice were crossed with Ayu1-Cre, a germ line deletion mouse strain to overexpress FLAG-HA-tagged Klf5.



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**Immunohistochemistry with anti-FGF4 antibody.** To obtain a clear FGF4 signal from preimplantation embryos, FGF4 staining was performed as previously described (Shimokawa et al., 2011) with modification. Briefly, embryos were fixed in 4% PFA for 15 min and then permeabilised in 30 μg/ml Proteinase K prepared in 0.1% Tween 20/PBS for 3 min at room temperature. For the blocking reaction, embryos were incubated in BlockingOne (Nacalai Tesque) for 30 min at room temperature. Embryos were incubated with goat anti-FGF4 antibody in the blocking reagent at 4 °C overnight. After the embryos were washed with 0.1% Tween 20/PBS, they were incubated with HRP-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch) for 3 h at room temperature. An Alexa 488 Tyramide SuperBoost Kit (Thermo) was used to detect the FGF4 protein according to the manufacturer's protocol.

**Confocal microscopy analysis and image data acquisition.**

For discrimination of inner and outer cells at E3.25, the distance of WT embryo nuclei (Cdx2+ TE nuclei and Cdx2- inner cell nuclei) to the nearest-neighbouring surface was measured using ImageJ. A threshold distance to distinguish Cdx2+ TE nuclei from Cdx2- inner cell nuclei was set. Subsequently, this threshold was used to manually discriminate inner and outer cells in DIC images of the embryos.

After confocal imaging, the embryos were washed in PBS and placed individually into polymerase chain reaction (PCR) tubes. To extract genomic DNA, 10 μl of 500 μg $ml^{-1}$ proteinase K (Nacalai Tesque) was added to each PCR tube and incubated at 56 °C for 45 min in a thermal cycler. After the proteinase K had been inactivated at 95 °C for 10 min. Aliquots (3 μl) of the extracted DNA were used as templates for PCR. PCR was performed with KOD FX Neo (TOYOBO) according to the manufacturer's recommendation and primers described in Table S1.

**Chromatin immunoprecipitation (ChIP) assays.** The ChIP assays were performed as described (Ito et al., 2013). Cells were cultured in 10-cm plates to approximately 80% confluence, and one plate was used for each immunoprecipitation run. The cells were fixed with 1% formaldehyde for 10 min at room temperature with swirling. Glycine was added to a final concentration of 0.125 M, and the incubation was continued for an additional 5 min at room temperature. The cells were washed twice with ice-cold PBS, harvested by scraping, pelleted, and resuspended in sodium dodecyl sulphate (SDS) lysis buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, and protease inhibitors from Nacalai Tesque). The samples were sonicated four times for 15 min each with an interval of 30 sec using a UH-50 sonicator (SMT, Tokyo, Japan). The samples were then centrifuged at 13,200 rpm at 4 °C for 15 min. After an aliquot (whole-cell extract) had been removed as an input sample, the supernatants were diluted 10-fold in ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 16.7 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS). The diluted samples were pre-cleared with 50 µl of protein G Sepharose beads (GE Healthcare) for 2 h at 4 °C with rotation, and then the supernatants were incubated at 4 °C overnight with 4 µg of normal mouse IgG (Santa Cruz Biochemicals) and anti-FLAG-M2 antibody (Sigma-Aldrich). The immunocomplexes were collected by incubation with 100 µl of protein G Sepharose beads (GE Healthcare) for 1 h at 4 °C with rotation. The beads were washed with the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris–HCl; pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, and 20 mM Tris–HCl; pH 8.1), and LiCl wash buffer (0.25 mM LiCl, 1% IGEPAL-C630, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl; pH 8.1). Finally, the beads were washed twice with 1 ml of TE buffer (1 mM EDTA and 10 mM Tris–HCl; pH 8.0). The immunocomplexes were then eluted by adding 200 µl of elution buffer (10 mM DTT, 1%



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SDS, 100 mM $NaHCO_3$). The eluate was collected and the cross-linking was reversed by adding NaCl to a final concentration of 200 mM and incubating for 6 h at 65 °C. The remaining proteins were digested by adding proteinase K. The DNA was precipitated with phenol, precipitated with ethanol, and quantified by qPCR. The sequences of the primers used for ChIP-qPCR are listed in Table S2.

**ChIP-sequencing (ChIP seq) data analysis.** ChIPseq data FASTQ files were downloaded from the EMBL-EBI site [E-GEOD-49848; Klf5, Aksoy *et al*. (Aksoy et al., 2014)]. ChIPseq reads were mapped to the mouse reference genome (mm9) using the Burrows–Wheeler Aligner software. Uniquely mapped reads were used for peak calling by CCAT3 version 3.0. Peak regions were filtered for false discovery rate values ≤ 0.05. We produced a RefSeq gene that had a Klf5 binding site within 20 kb by determining the overlap between the ChIPseq peak regions and RefSeq genes extended by 20 kb both up- and downstream. To visualise the ChIPseq tag counts in the UCSC Genome Browser, mapped reads were extended and converted into the bedGraph format by using the genomecov function of BEDTools.

## References


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