Stem Cell Reports

Supplemental Information

A Dynamic Role of TBX3 in the Pluripotency Circuitry

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Supplemental Figure Legends

Supplementary Figure 1. Expression heterogeneity of *Tbx3* upon ESC differentiation. Related to Main Figure 1. (**A-D**) Violin plots of expression levels in individual cells for *Nanog, Pou5f1* and *Tbx3* at the 8-cell, 16-cell, early blastocyst and late blastocyst stage, respectively. (**E-G**) Individual cells were classified into (putative) ICM or TE group according to expression of predefined gene set (Guo et al., 2010) at 16-cell, early blastocyst and late blastocyst stage, respectively. The expressions of *Tbx3* were also included and highlighted in the heat maps. Blue, expression lower than median; white, median expression; red, expression higher than median. (**H-J**) The boxplot of expressions in ICM or TE biased cells for *Pou5f1* and *Tbx3* at three different stages, respectively. (**K**). Genes in predefined gene set (Guo et al., 2010) were ranked according to their correlation with *Tbx3* at late blastocyst stage, from most positive correlated (red) to most negatively correlated (blue).

Supplemental Figure 2. ***Tbx3* expression in wild type PSCs and differentially regulated genes between indicated groups.** Related to Main Figure 1. (**A**) Immunostaining for the TBX3 protein wild type mESCs cultured under the indicated conditions. Note heterogeneous TBX3 expression under LIF/feeder conditions. n=3. Scale bars 20µm. (**B**) Endogenous *Tbx3* mRNA levels relative to *Hmbs* in single sorted $TBX3^{GFP}$ high or low ESCs. (**C**) Venn diagram showing numbers of differentially regulated genes comparing $TBX3^{GFP}$ high or low ESCs and 2i-treated ESCs (J1_2i taken from GSE58735). (**D**) The expression profiles of selected ESC, differentiation and epiblast stem cell-specific genes (Kurek et al., 2015) in 2i-treated, $TBX3^{GFP}$ high or low ESCs ESCs are represented in a heatmap format.

Supplemental Figure 3. **TBX3 null PSC characterization.** Related to Main Figure 3. (**A**) and (**B**) Representative fluorescent images for SSEA1 of $TBX3^{+/+}$ and $TBX3^{+/-}$ iPSCs (**A**) and *de novo* derived $TBX3^{+/+}$ and $TBX3^{ven/ven}$ mESCs (**B**) under 2i/LIF conditions. Scale bars, 20 µm. (**C**) Representative phase contrast and GFP fluorescent images of *de novo* derived mESCs with the indicated genotypes cultured under 2i/LIF conditions. Scale bars, 20 µm. (**D**) Representative genotyping of mECS clones derived from blastocysts of heterozygous mating strategies as outlined in Figure **3H**. (**E**) FACS profiles of the indicated TBX3-genotypes in *de novo* derived ESCs. n=3. Scale bars 20µm.

Supplemental Figure 4. **Assessment of chimeric contribution of TBX3 null PSC.** Related to Main Figure 3. **(A)** Tetraploid (4n, Top panel) and diploid (2n, Bottom panel) embryo aggregation using LV-EGFP $TBX3^{ven/ven}$ ESCs. E2.5 Zonae pellucidae-free embryos were aggregated in pair (4n) or individually (2n) with *FAH*$^{-/-}$-iPS cells. The chimeric embryos developed to blastocyst stage in culture overnight (E3.5) and were then transferred into the uterus of pseudopregnant mice. E9 fetuses were collected and

examined for green fluorescence. Scale bars = 50 µm. (**B**) Arrested E11.5 fetuses were recovered after tetraploid embryo complementation with LV-tdTomato TBX3$^{-/-}$ iPSCs. (**C**) To assess germline development in chimeric embryos, the zonae pellucidae of the 8-cell embryos (2n, E2.5, top panel) were removed by acidified Tyrode's solution and aggregated with OCT4-GFP/LVtdTomato TBX3$^{-/-}$-iPSCs. The chimeric embryos developed to blastocyst stage in culture overnight (E3.5, bottom panel) and then were transferred into the uterus of pseudopregnant mice. Scale bars 20µm (A; left and middle panel), 1mm (A; right panel), 100µm (C).

Supplemental Figure 5. Investigation of differentiation of Tbx3 null PSCs. Related to Main Figure 4. Phase contrast view of TBX3$^{ven/ven}$ and TBX3$^{+/+}$ mESC (second clone per genotype, n=2, see also Fig. **4F**) at 0 h, 24 h, 48 h and 72 h after withdrawal of LIF and 2i to induce spontaneous differentiation. Note the delayed differentiation of TBX3$^{ven/ven}$.

Supplemental Table Legends

Supplemental Table 1. Information on the used qPCR and genotyping primers in the study.

Supplemental Table 2. Differentially regulated genes between TBX3^{GFP}-high and –low cells referred to 2i-treated ESCs. J1_2i taken from GSE58735.

Supplemental Table 3. Differentially regulated genes between TBX3^{GFP}-high and 2i treated ESCs. J1_2i Taken from GSE58735.

Supplemental Table 4. Differentially regulated genes between TBX3^{GFP}-low and 2i-treated ESCs. J1_2i Taken from GSE58735.

Supplemental Table 5. Upregulated genes between TBX3 null ESCs and wildtype ESCs cultivated under 2i/LIF conditions.

Supplemental Table 6. Downregulated genes between TBX3 null ESCs and wildtype ESCs cultivated under 2i/LIF conditions.

Supplemental Table 7. Upregulated genes between TBX3^{GFP}-high and –low ESCs cultivated under 2i/LIF conditions.

Supplemental Table 8. Downregulated genes between TBX3^{GFP}-high and –low ESCs cultivated under 2i/LIF conditions.

Supplemental Experimental Procedures

Reprogramming of Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated according to standard protocols from $TBX3^{+/-}$ and $TBX3^{-/-}$ mice. Reprogramming virus was generated according to standard protocols as described previously (Illing et al., 2013; Liebau et al., 2013; Liebau et al., 2014). $TBX3^{-/-}$ MEFs (6well-plate) were seeded one day before infection with virus. Next day, 5 µl per well of a 6 well-plate of 100 x concentrated virus (=1.2 x 10^6 proviral hOSK (Warlich et al., 2011) copies/µl) were added to the cells as a master mix. After 8 h of incubation at 37 °C, medium was changed to ES-Feeder medium and refreshed every day. At day 7, medium was changed to ESC culture conditions and changed every day. ESC culture conditions have been described previously (Kleger et al., 2012; Kleger et al., 2010; Muller et al., 2015), but for reprogramming instead of fetal calf serum (FCS), knock out serum replacement (KOSR) (Life Technologies, Germany) was used. On day 12, cultures were fixed and stained for alkaline phosphatase (AP)-expression according to standard protocols. Several independent iPSC clones derived from $TBX3^{-/-}$ MEFs were randomly picked based on typical ESC like morphology at day 13 of reprogramming. After expansion of individual clones, pluripotency analysis was carried out at early passage (1-5). All analysed clones were stained positive for pluripotency markers.

Isolation of Mouse Embryonic Stem Cells from blastocysts

TBX3-EGFP mESCs and TBX3-KI-Venus mESCs were derived using previously reported methods by breeding TBX3-BacEGFP and TBX3-KI-venus heterozygous mice respectively (Bryja et al., 2006; Czechanski et al.). Briefly, the uterine horns of E3.5 pregnant females were removed, transferred into pre-warmed M16 medium (Sigma) and the embryos were flushed out of the uterine horn under a dissection microscope. The blastocysts were collected and individually transferred to one well of a 12 well-plate containing inactivated MEFs in SR-ES Medium. Medium was replaced every second day. Seven days later, expanded blastocysts were subject to 0.25 % Trypsin/EDTA for 15 s to ease removal from surrounding MEF, picked using a 10 µl pipette, and individually transferred to 2.5 % Trypsin containing wells in a 96 well-plate. The picked blastocysts were dissociated in the well by pipetting up and down and subsequently transferred to individual wells of a 12 well-plate containing mitotically inactivated MEFs in ES-Feeder medium with daily medium changes. Once colonies were of sufficient size, cells were stocked and further expanded on inactive MEFs or transferred to feeder-free ES cell culture conditions.

***In Vitro* Fertilization**

Sperm preparation: The caudae epididymis were removed from 2 months-old OCT4-GFP/LVtdTomato iPSC chimera mouse and placed into 1-ml drops of mKSOM supplemented with 4 mM glucose and 0.4% (w/v) BSA (Sigma A9647) under oil in sperm dispersion dishes (referred to as modified-KSOM, mKSOM) (Kito and Ohta, 2005). Incisions were made in the cauda epididymis to allow spermatozoa to swim out. After 10 min of incubation at 37°C, tissue fragments were discarded. Fifty microliters of sperm suspension was added to 450-µl drops of mKSOM and capacitation was allowed to proceed for 45–60 min at 37°C in the incubator. After capacitation, oocyte-cumulus complexes were transferred to the 500-µl fertilization droplets.

Oocyte collection: Superovulated females were killed 14–16 h post-hCG administration, and the entire oviduct was dissected out and kept in 500 µl drops of M2 medium at 37°C. The ampullae were opened by tearing with a forceps and cumulus-oocyte masses were released and transferred into fertilization droplet.

Fertilization *in vitro* fertilization (IVF) was carried out in 500-µl droplets of mKSOM. Incubation was allowed to proceed for 4 h at 37°C in a 5% CO_2. Then inseminated oocytes were washed and incubated in 50-µl drops of mKSOM for 4 days and checked for GFP/tdTomato expression.

Teratoma formation and histological analysis.

$TBX3^{+/-}$ and $TBX3^{-/-}$ iPSCs were cultured under 2i/LIF conditions and subsequently harvested for injection. 1 x 10^6 cells were subcutaneously injected into the dorsal flank of male NMRI mice. Three weeks later, tumours were surgically dissected from the mice, fixed in 4 % formaldehyde and embedded in paraffin. Sections were subsequently stained with haematoxylin and eosin and subjected to histological examination.

Cell Culture

Mouse embryonic fibroblast (MEF) feeder cells were cultured in DMEM supplemented with 10%, FCS (Sigma Aldrich), 1% Penicillin/Streptomycin (Sigma Aldrich), 2mM Glutamine (Sigma Aldrich), 1% Non-Essential Amino Acids (Sigma Aldrich), 1mM Sodium Pyruvate (Sigma Aldrich), 1% β-Mercaptoethanol (Merck Millipore) and 0.05mg/ml Vitamin C (Sigma Aldrich) in humidified atmosphere containing 5% CO_2 at 37° C. mESCs and miPSCs were cultured either in feeder-dependent conditions (LIF/feeder) with Knockout-DMEM (KO-DMEM; Life-Technologies), 15% FCS (Sigma, ESC-qualified), 1% Penicillin/Streptomycin, 1% Glutamine (Sigma Aldrich), 1% NEAA (Sigma Aldrich), 1% Sodium Pyruvate (Sigma Aldrich), 1% b-Mercaptoethanol (Merck Millipore) and 240 U/ml leukaemia inhibitory factor (LIF; Cell guidance systems) or feeder-free (2i/KOSR) with KO-DMEM, 20% KOSR (Knockout Serum

Replacement, Life Technologies), 1% Penicillin/Streptomycin, 1% Glutamine (Sigma Aldrich), 1% NEAA (Sigma Aldrich), 1% Sodium Pyruvate (Sigma Aldrich), 1% β-Mercaptoethanol (Merck Millipore), 240 U/ml LIF (Cell guidance systems) and PD0325901 (1 µM) and GSK3β inhibitor CHIR99021 (3 µM) (Selleckchem, USA) were added to the culture medium.

Generation of fluorescent reporter PSCs

PSCs were infected with fluorescent reporter constructs before testing them in aggregation assays with diploid/tetraploid embryos. Therefore the lentiviral FUdGW-Tomato plasmid (Addgene #22771), psi-LVRU6MP mCherry (a gift from Alexey Ushmorov) and a lentiviral EGFP were used. Lentiviral particles were produced according to standard protocols (Wang et al., 2012) using psPax2 and pMD2 (Addgene #12260, #12259) as packaging plasmids in LentiX HEK293T cells (Clontech).

TBX3$^{-/-}$ iPSCs were infected directly with LentiX HEK293T supernatant for 8 h at 37° C. After 48 - 72 hrs, the reporter positive cells were detectable by flow cytometry and positive cells were isolated by sorting on a FACSAria II or III flow cytometer (BD).

For infection of TBX3BAC-GFP mESCs, viral supernatants from the HEK293T cells were mixed in a 50:50 ratio with ES Feeder medium + 2i and cells were infected in suspension twice over night. Infected cells were sorted according to their mCherry expression and grown in feeder free 2i/LIF conditons for making stocks. Cells were grown for at least 2 passages in Serum/LIF conditions to re-establish the TBX3 heterogeneity based on GFP expression. For embryo aggregation assays, mCherry positive TBX3BAC-GFP mESCs were FACS sorted into the respective TBX3^{GFP}-low or TBX3^{GFP}-high cell populations. To assess germline contribution of the TBX3$^{-/-}$ iPS cells, the EGFP-IRES-Pac cassette driven by the enhancer 3.5 kb upstream of Pou5f1 and the minimal human UbC promoter were introduced using the piggyBac transposon system.

Infection of PSCs with shRNA constructs

TBX3$^{+/+}$ and TBX3$^{-/-}$ iPSCs were infected with shRNAs to knock down *Dppa3*. Therefore the SFFV pGipz plasmid was used. Lentiviral particles were produced according to standard protocols (Wang et al., 2012) using psPax2 and pMD2 (Addgene #12260, #12259) as packaging plasmids in LentiX HEK293T cells (Clontech).

For infection of TBX3$^{+/+}$ and TBX3$^{-/-}$ iPSCs with shRNA viruses, the filtered HEK293T supernatant was mixed in a 50:50 ratio with ES Feeder medium + 2i and cells were infected in suspension twice over night. Two different shRNA constructs were used and cells were also infected using a pool of both

shRNA viruses. Infected cells were sorted according to their GFP expression intensity on 0.2% gelatine coated 6 well or 96 well plates. Non-infected control cells were used to set the gates.

The following shRNAs were used in this work:

shRNA1 Dppa3 5´-CAGCAGATGTGAAAGCTATTT-3´
shRNA2 Dppa3 5´-GGCTGAGGAATAAAGTAAA-3´

Western Blot

Western Blotting was performed according to standard procedures. Whole-cell extracts (50–100 μg) prepared using RIPA-lysis buffer containing 50 mM Tris HCl pH 8.0, 150 mM NaCl, 0,05% Na-Deoxycholat, 1% NP40, 0,01% SDS, 1 mM PMSF supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche) were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to PVDF membranes (Millipore Corp., USA). Membranes were blocked using 5% dry milk in PBS containing 0.2% Tween 20. For subsequent washes, 0.2% Tween 20 in PBS was used. Membranes were incubated with primary antibodies over-night at 4° C on a rotator. The following primary antibodies were used: rabbit anti-TBX3 1:1000 (Invitrogen, #424800), goat anti- TBX3 1:1000 (Santa Cruz # sc-17871). Anti-ß-ACTIN 1:10,000 (Sigma # 3101) was incubated for 1 hour at RT. This was followed by incubation with secondary horseradish peroxidase (HRP)–coupled antibodies diluted 1:3000, 1h at RT. Detection was performed with either ECL or ECL+ kits (Thermo scientific, USA).

Confocal microscopy

Pre-implantation embryos were isolated according to standard protocols. Embryos were cultured in glass bottom dishes using M16 media and kept in a humidified atmosphere containing 5% CO_2 at 37° C. Confocal images were taken of live embryos using a Leica TCS-SP8-HCS microscope. Immunostaining on wildtype embryos was performed according to standard protocols using the following primary antibodies and dilutions: rat anti-NANOG (14-5761-80, eBioscience), 1:250; goat anti- TBX3 (sc-17871, Santa Cruz), 1:250; rabbit anti-PDGFRa (sc-338, Santa Cruz), 1:100.

FACS analysis and sorting

Cells were washed with PBS and dissociated into single cell suspension by incubation with 0.25 % trypsin/EDTA (Millipore). The cells were re-suspended in 2 % FCS/PBS (FACS buffer) and analysed with a FACSAria II or III flow cytometer (BD). All events were gated with forward scatter and side scatter

profiles. The respective cell types were sorted according to fluorescent signal intensity in relation to negative controls.

Clonal formation assays

SSEA1 positive, TBX3-EGFP reporter mESCs were single cell sorted at 10,000 cells/well or as single cells onto 96 well plates containing Mitomycin C inactivated MEFs. Cells were cultured with daily medium changes until colony formation and were counted under a light microscope and subsequently expanded for further analysis. Briefly, for SSEA1 staining cells were washed with PBS and trypsinised using 0,25 % Trypsin/EDTA (Milipore) for ~ 2 min at 37° C. Detached cells were dissociated via pipetting and the cells were resuspended in serum containing medium to stop trypsin reaction. Subsequently, the cells were centrifuged at 1300 rpm for 3 min, washed twice in FACS buffer and resuspended in FACS blocking buffer for 30 min at 4°C under shaking conditions. Cells were washed with FACS buffer and incubated with SSEA1 primary antibody (Cell Signaling MC-480, 1:1600) for 1.5 h at 4° C under shaking conditions. Afterwards, the cells were washed twice with FACS buffer and then incubated with the second antibody Alexa Fluor 647 Goat anti-mouse IgM µ Chain (1:600) for 30 min at 4° C in the dark. Finally, the cells were washed again with FACS buffer, the pellet resuspended in 350 µl FACS buffer containing 1:10,000 DAPI (Invitrogen #D3571) and transferred via a cellulose filter into FACS tubes. The cells were analyzed for viability (DAPI) and SSEA1-AlexaFluor 647.

Quantitative real time PCR

RNA was extracted according to manufacturer's instructions using the GeneJet RNA Purification kit (Thermo Scientific) and eluted in 40 µl RNAse-free H_2O. Each RNA preparation was tested for genomic DNA contamination by replacing reverse transcriptase with water. cDNA was subsequently synthesized using the iScript cDNA synthesis kit (BioRad). Quantitative real time PCR was performed using SensiMix™ SYBR® No-ROX Kit (Bioline #QT650-05) according to supplier's instruction manual using 100ng cDNA as a template in a Qiagen Rotor Gene qPCR Cycler (Qiagen). Internal standards (house-keeping gene) and samples were simultaneously amplified. Details have been described elsewhere (Kleger et al., 2012; Kleger et al., 2010; Muller et al., 2012). QuantiTect Primer Assays were used throughout this study (Qiagen, Germany, http://www.qiagen.com/de/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/quantitect-primer-assays/). Primer information is provided in **Supplemental Table 1**.

Immunohistochemistry

Cells at different time points of differentiation were fixed using 4% paraformaldehyde (PFA). Samples were then subjected to treatment with NH_4Cl and blocking with 0.3-0.5% TritonX-containing BSA before incubation with the primary antibodies. Rabbit anti Nanog (Cosmo Bio Co, LTD. #REC-RCAB0002P-F), 1:250, over-night at 4° C; rabbit anti-murineTBX3 was kindly provided by Hitoshi Niwa (Toyooka et al., 2008), 1:1000, over-night at 4° C, mouse anti-OCT4 (Santa Cruz sc-5279 (Lot #3010)) 1:100, overnight at 4° C; mouse anti-SSEA1 (Cell Signalling #4744), 1:200, 1h at RT. Samples were further incubated with fluorescence labelled secondary antibodies Alexa Fluor® 488 (green), Alexa Fluor® 568 (red) (Life-technologies, all diluted 1:500). Nuclei were stained with DAPI (blue) (1:10,000). Images were captured using an upright fluorescence Zeiss Axioimager Z1 microscope and analysed using Axiovision software (Zeiss, Germany).

Cell Cycle Analysis

To analyse cell cycle distribution in detail cells were treated with 20 µM BrdU (5-bromo-2'-deoxyuridine) for 60 min in their respective medium. Cells were harvested by 0,05% Trypsin, reaction was stopped by adding respective culture medium and $2x10^6$ cells per sample were washed twice in PBS/1% BSA and resuspended in 200 µl PBS. Cell suspension was added dropwise to 70% ice-cold ethanol for fixation. After 30 min on ice cells were centrifuged for 10 min at 500 x g, pellet was loosened by vortexing and 1ml 2N HCl/0,5% Triton-X was added dropwise while vortexing. Cells were incubated at RT for 30 min to produce single stranded DNA molecules. After 10 min centrifugation at 500 x g pellet was resuspended in 1ml 0,1 M $Na_2B_4O_7$ x 10 H_2O, pH 8.5 for neutralisation. Cells were centrifuged 10 min at 500 x g and the pellet resuspended in 100 µl PBS/1% BSA/0,5% Tween20. 1 µl of BrdU-APC-Antibody (BD Biosciences, # 560209) was added to each sample to obtain a final dilution of 1:100. After 30 min incubation at RT 1 ml PBS/1% BSA was added, cells were centrifuged for 10 min at 500 x g and finally resuspended in 1 ml PBS containing 5 ug/ml Probidium Iodide (PI) (Life Technologies, # P3566). Cells were analysed on an LSR II flow cytometer (BD Biosciences) measuring the PI-signal linear. iPSCs without BrdU treatment were used as controls.

Characterisation of the Tbx3-null state

The self-renewal capacity of $TBX3^{+/+}$ and $TBX3^{ven/ven}$ ESCs was compared by mixing the cells in a 50:50 ratio and co-culturing them for 3 passages under LIF/2i conditions. FACS analysis for the venus expression was performed at the start and at the end of the experiment.

Differences in colony formation when gradually withdrawing one or two components of the LIF/2i condition, were investigated. For this purpose, cells were seeded in clonal density with 125 cells/cm^2 in a serum-free medium using the N2B27 as a backbone.

To compare if the TBX3$^{-/-}$ cells showed accelerated or delayed differentiation, TBX3$^{ven/ven}$ and WT cells were seeded in ES-Feeder + 2i and the next day LIF and 2i were withdrawn from the medium to induce differentiation. Morphology, pluripotency and lineage marker expression were checked before, 24 h, 48 h and 72 h after LIF/2i removal.

Genotyping

DNA was extracted from cells or tissue according to standard protocols. Briefly, cells/tissues were lysed using Lysing buffer + 10% SDS and 0.5 % Proteinase K for 3 h at 56 ° C, the reaction was stopped with 5 M NaCl and the sample was centrifuged for 10 min at RT at 13,300 rpm. DNA containing supernatant was precipitated with isopropanol, the pellet washed with EtOH, dried and DNA was solved in H2O for 1h at 37° C. The DNA was amplified using specific primer pairs as outlined in **Supplemental Table 1**. Finally the PCR product was separated using gel electrophoresis in a 1.2% agarose gel with Ethidium Bromide and visualized using the GenoSmart2 gel documentation system.

Microarray data analysis

The DNA microarray data were pre-processed and normalized by the limma package (Wettenhall and Smyth, 2004). The differentially expressed genes of a pair-wise comparison were detected using limma t-test with criteria of fold change > 2 and p-value < 0.05. The p-value was further adjusted by the procedure of Benjamini and Hochberg (Klipper-Aurbach et al., 1995). Additionally, the TBX3 induced genes were detected using public dataset of *Tbx3* knock down (*Tbx3* shRNA transient transfection of 72h) ESCs (GSE26520) using limma t-test with a relax fold change cut-off (> 1.5) and adjust p-value < 0.05. To perform cross-platform analysis, published microarray data (GSE11274, GSE35416, GSE58735) were retrieved from NCBI GEO and were adjusted by the Combat algorithm (Westermeyer et al., 2007) to minimize batch effects. The specific gene sets, PluriNet. Epiblast, DNA methylation (GO0006306) were collected from (Consortium, 2009; Kurek et al., 2015; MacArthur et al., 2012; Muller et al., 2008; Nora et al., 2012), respectively. The gene set enrichment analysis was performed by customized R-GSEA script (Subramanian et al., 2005).

The network, including pluripotency makers (Dunn et al., 2014) and TBX3 target genes (GSE19219, (Han et al., 2010)) were generated using the STRING database (Franceschini et al., 2013) and visualized by Cytoscape (Saito et al., 2012). The pluripotency makers were organized in the inner layer of the network, whereas TBX3 target genes being differently regulated between wild-type and TBX3 null ESCs

were selected and placed in the outer layer. The edges in the network represent the reliable interactions between two genes that had a high confidence score (>0.7) from the STRING database or between TBX3 and its target genes defined by a TBX3 ChIP-seq experiment (Han et al., 2010). The gene expression data of wild-type and TBX3 null ESCs were overlaid on to the network, reflecting the TBX3 induced or repressed changes in the network.

Single-cell gene expression analysis

Gene expression analysis of FACS-sorted single ESCs, that were kept in lysis buffer containing 9 µl RT-PreAmp Master Mix (5.0 µl Cells Direct 2× RXN Mix (Invitrogen), 2.5 µl 0.2× assay pool, 0.2 µl Superscript III Reverse Transcriptase (Invitrogen) and 1.3 µl TE buffer (Ambion), was performed for TBX3 and DPPA3 with the Dynamic Array chips (Fluidigm). The amount of targeted transcripts was quantified using TaqMan real-time PCR on the BioMark Real-Time PCR system (Fluidigm). TaqMan gene expression assays (AppliedBiosystems) and 96.96 Dynamic Arrays (Fluidigm) were applied. We analyzed the Ct values achieved from the BioMark System with GenEx software from MultiD analysis.

Analyses of single cells from published data sets

Single-cell RNA-Seq data at 8-cell, 16-cell, early blastocyst and late blastocyte stage were retrieved from Deng et al. Science 2014 (GSE45719)(Deng et al., 2014). The gene expression values (measured as RPKM) were extracted and log2 transformed. The gene expression values were represented in violin plots or boxplots to visualize the expression heterogeneity. The lineage bias (ICM or TE) of individual cells was determined using a panel of 48 genes selected by Guo et al. 2010 (Guo et al., 2010) at different stages, respectively.

Aggregation of PSCs with diploid (2n) and tetraploid (4n) embryos

All (C57BL/6 x C3H) F1 female mice for embryo collection were treated with 7.5 IU pregnant mare serum gonadotropin (PMSG) and 7.5 IU human chorionic gonadotropin (HCG) in 48 hours apart and then crossed with CD1 male mice. 2n eight-cell embryos were flushed from female mice at 2.5 days postcoitum (dpc) and placed in M2 medium (Hogan et al., 1986).

To make 4n embryos, two-cell embryos were flushed at 1.2 dpc from oviducts of (C57BL/6 x C3H) F1 female mice and fused with a Cellfusion CF-150/B apparatus in a 250-µm gap electrode chamber (BTX-450, BLS Ltd., Budapest, Hungary) containing 0.3 M Mannitol with 0.3% bovine serum albumin (Sigma-Aldrich Inc., St. Louis, MO). An initial electrical field of 2V was applied to the embryos, followed by one peak pulses of 50V for 35 µs. Embryos were transferred back into potassium simplex optimized medium (KSOM) (Ho et al., 1995) immediately to a 37°C incubator with 5% CO_2, observed for fusion

after 15 to 60 minutes. Unfused embryos were discarded after 60 minutes. The fused 4n embryos were cultured for 24 hrs to the 4-cell stage under the same conditions (Nagy et al., 1993).

Trypsin-digested PSCs (8-12 cells for 2n embryos or 15-18 cells for 4n embryos per aggregate) were transferred into a depression in the microdrop of KSOM; Meanwhile, batches of 30-50 embryos were briefly incubated in acidified Tyrode's solution (Hogan et al., 1986) until dissolution of their zona pellucida. A single 2n embryo or two 4n embryos were place in each depression. All aggregates were cultured overnight at 37C, 5% CO2. After 24 hours of culture, the majority of aggregates had formed blastocysts. We transferred 10-14 embryos into one uterine horn of a 2.5dpc pseudopregnant recipient. Seven or 9 days after embryo transfer, E9.5 and E11.5 foetuses were collected from the recipients and analysed.

Mouse strains

(C57BL/6 x C3H) F1 female mice and CD1 male mice used in aggregation experiments of PSCs with diploid or tetraploid embryos were housed in the animal facility of the Max Planck Institute of Molecular Biomedicine in Münster according to the German animal protection law. TBX3-venus mice were kindly provided by Vincent Christoffels (Kunasegaran et al., 2014) and subsequently housed in the animal facility of Ulm University according to the German animal protection law. TBX3-Bac GFP mice were also kindly provided by Vincent Christoffels (Horsthuis et al., 2009). The nomenclature $TBX3^{-/-}$ iPSCs in the current manuscript refers to the previously described TBX3 conditional allele that is a true null in the recombined state ($TBX3^{\Delta flox/\Delta flox}$ for homozygous deletion and $TBX3^{\Delta flox/+}$ for heterozygous deletion) (Frank et al., 2013).

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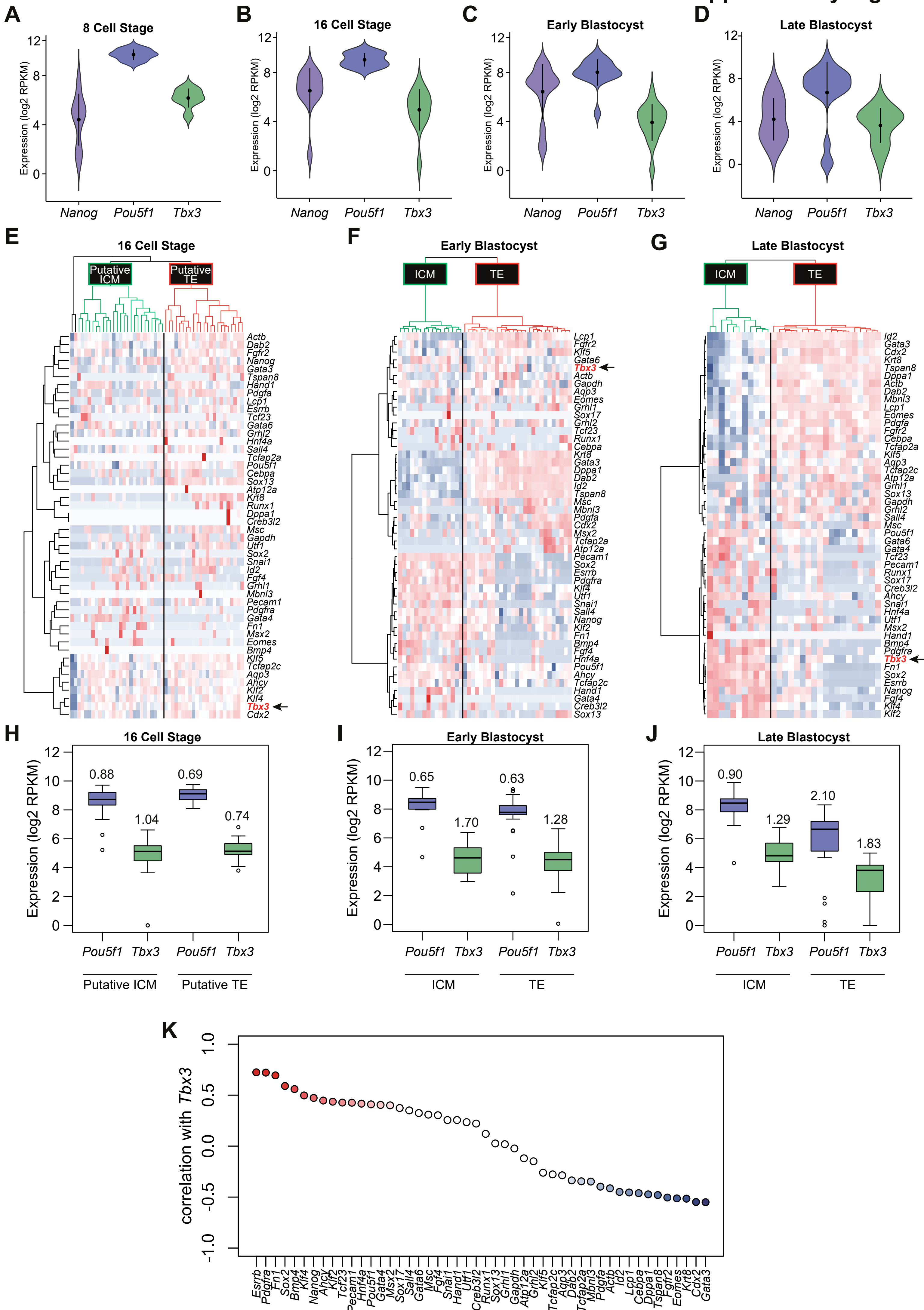
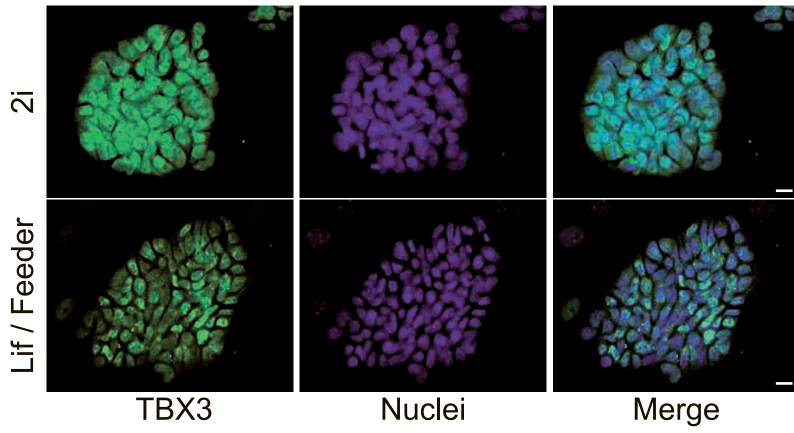
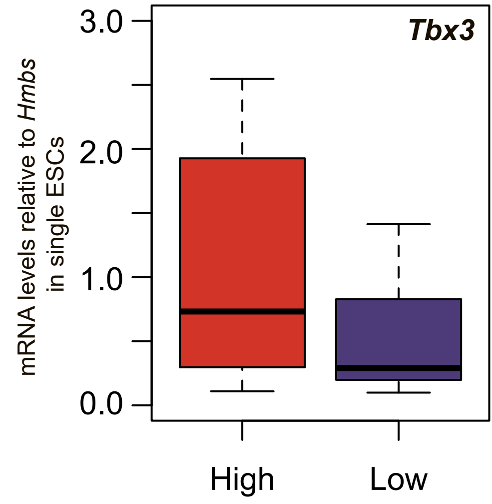
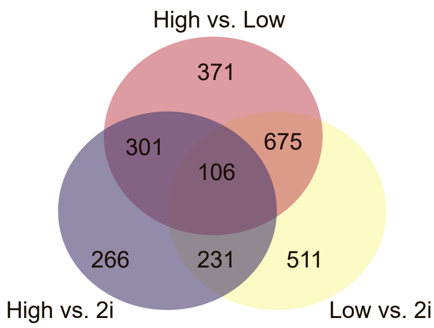


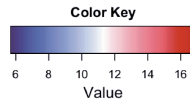
Supplementary Figure 1
A
8 Cell Stage
Expression (log2 RPKM)
Nanog
Pou5f1
Tbx3
B
16 Cell Stage
C
Early Blastocyst
D
Late Blastocyst
E
16 Cell Stage
Putative ICM
Putative TE
F
Early Blastocyst
ICM
TE
G
Late Blastocyst
H
16 Cell Stage
0.88
1.04
0.69
0.74
Putative ICM
Putative TE
I
Early Blastocyst
0.65
1.70
0.63
1.28
J
Late Blastocyst
0.90
1.29
2.10
1.83
K
correlation with Tbx3

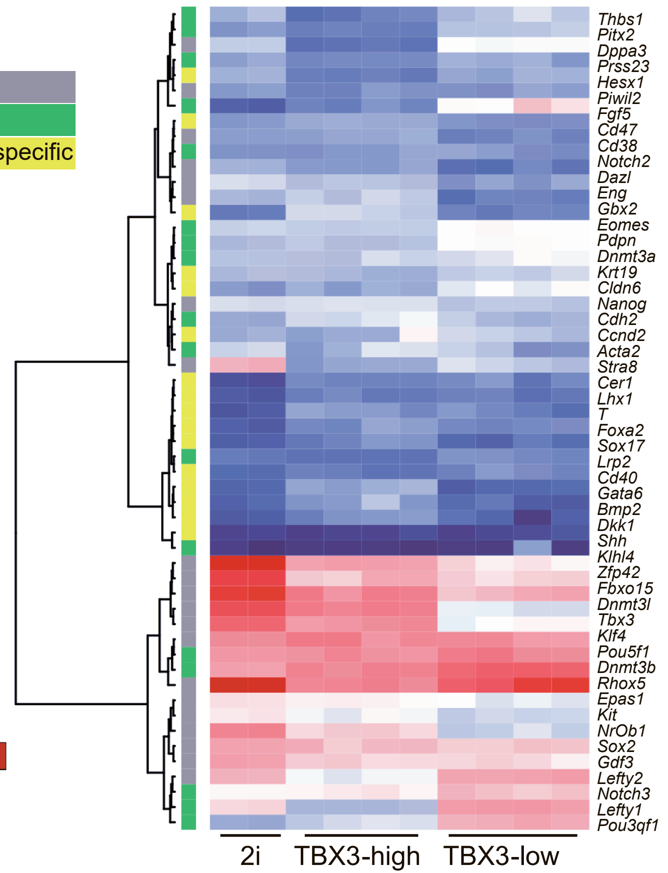
Supplementary Figure 2

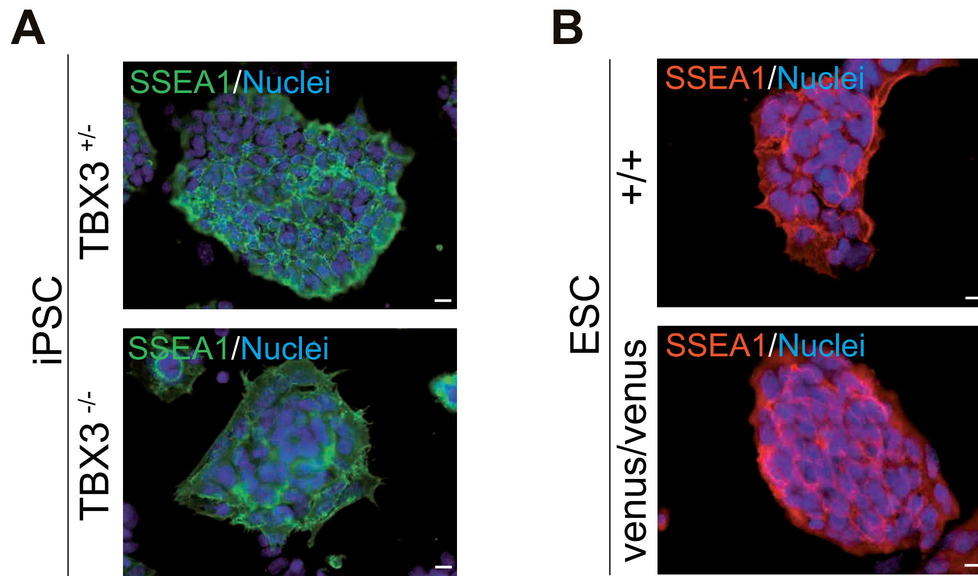


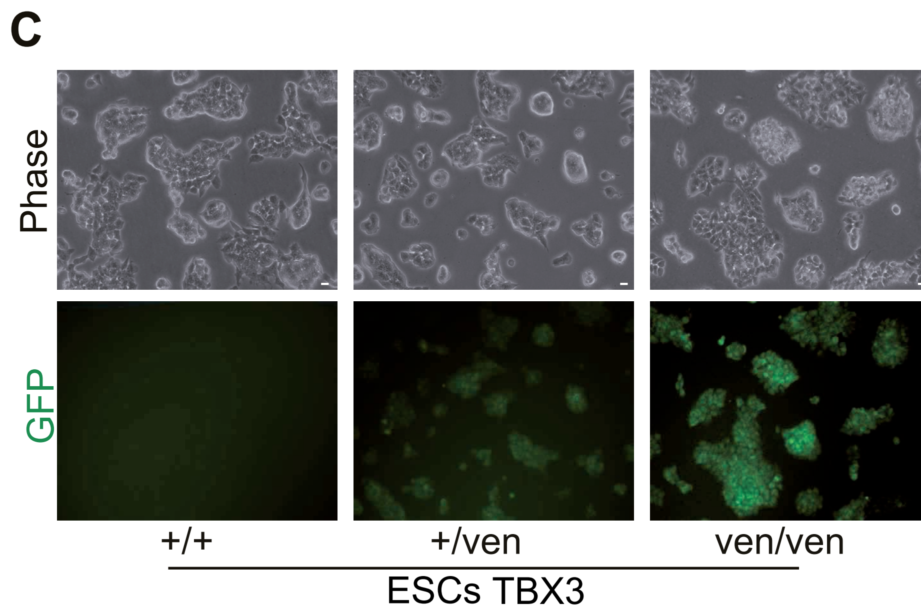


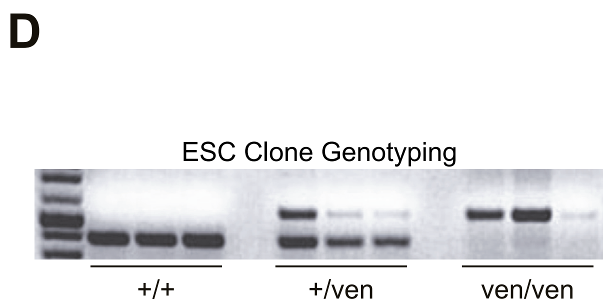


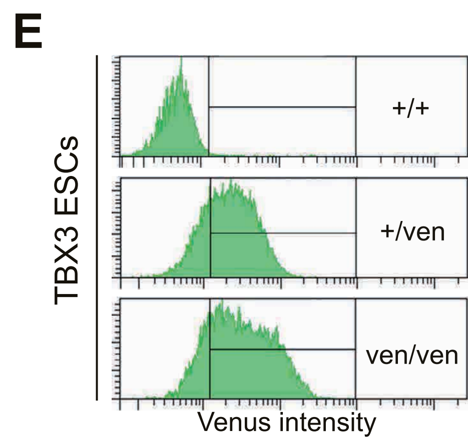




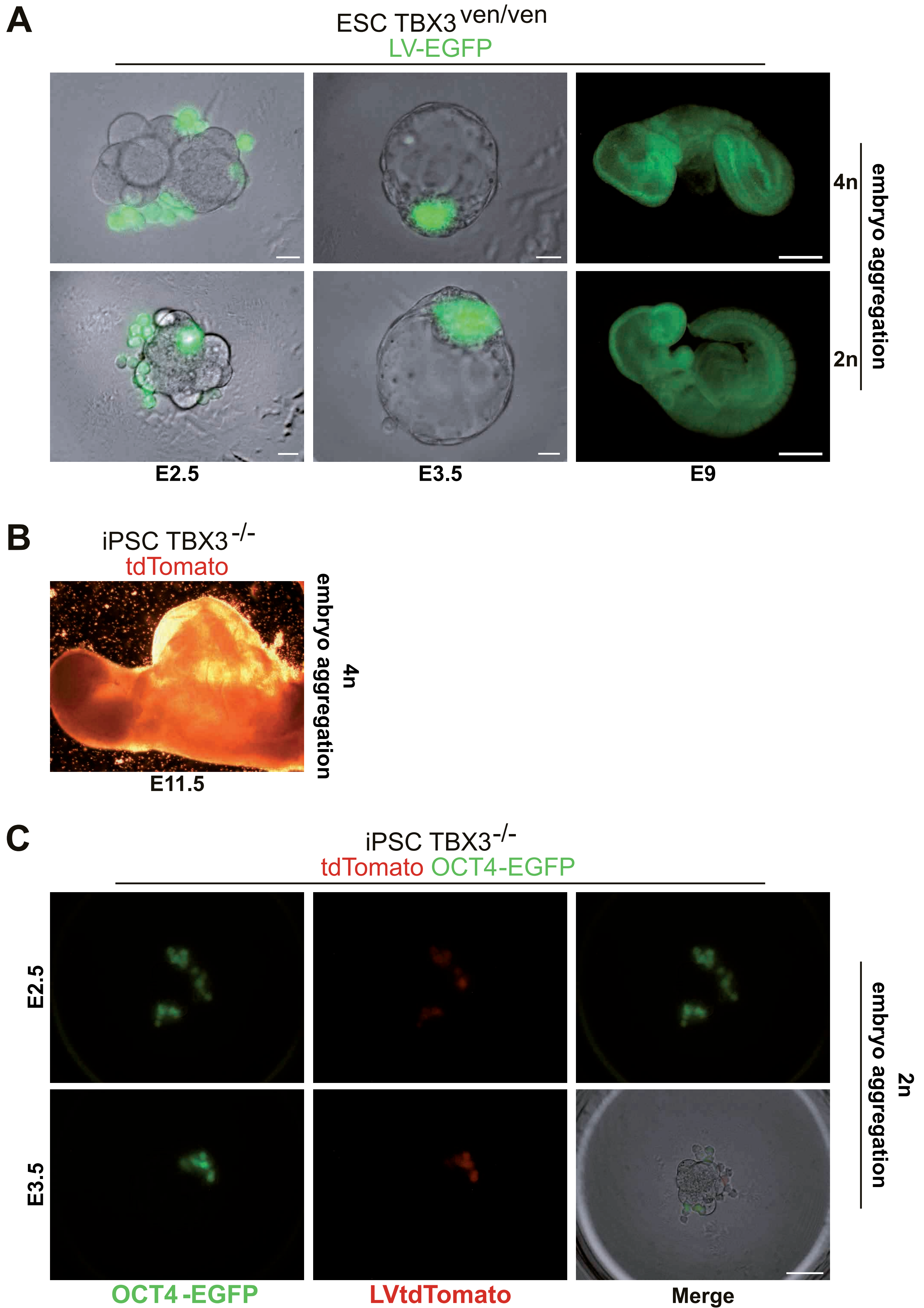

A
iPSC
TBX3 +/-
SSEA1/Nuclei
TBX3 -/-
SSEA1/Nuclei
B
ESC
+/+
SSEA1/Nuclei
venus/venus
SSEA1/Nuclei


C
Phase
GFP
+/+
+/ven
ven/ven
ESCs TBX3


D
ESC Clone Genotyping
+/+
+/ven
ven/ven


E
TBX3 ESCs
+/+
+/ven
ven/ven
Venus intensity

Supplementary Figure 4



Supplementary Figure 5