Stem Cell Reports, Volume 1

Supplemental Information

TBX3 Directs Cell-Fate Decision toward Mesendoderm

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Supplemental Information

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Supplemental Figure 1 (related to Figure 2). TBX3 binds lineage determining genes in the pluripotent state. Solexa ChIP-sequencing data sets, which have been generated elsewhere (Han et al., 2010), to uncover the direct regulatory targets of TBX3 during reprogramming, were reanalyzed for TBX3 binding of certain pluripotency genes (dark grey columns; *Klf5, Klf4, Oct4*) and lineage determining genes (light grey; *Sox9, Nkx6.1, Eomes*). X-axis shows fold enrichment of certain genetic regions of the respective genes upon chromatin immunoprecipitation with a TBX3-binding antibody.
Supplemental Figure 2 (related to Figure 3). Generation of a targeted, inducible TBX3-expression allele in ESCs. (A) Scheme of cloning strategy to generate a temporally regulated and dose-dependent Dox-inducible TBX3 ESC line. (B) High expression of Tbx3 mRNA in Dox-treated iTBX3 ESCs as assessed by qPCR after 24 and 48 hours. (C,D) TBX3 protein levels are increased in response to Dox-treatment after 24 hours in iTBX3 ESCs and EBs respectively. All experiments (n=3) in biological replicates. TBX3 tightly regulates certain lineages. (E,F) IF of T and SOX17 demonstrate TBX3-induction enhances differentiation towards mesendodermal derivatives respectively. All scale bars are 20 μm. (G,H) Respective quantifications of either (E) or (F). (I) WB analysis of day4 and day6 EBs, confirms TBX3 (+Dox) induces SOX17-expression levels more compared to control (-Dox) cells. All experiments (n=3) in biological replicates. (J-M) Microarray expression data depicted as log2 fold for a series of lineage specific genes in day4 -Dox or +Dox samples. TBX3-induced ESCs display upregulation of (J) early mesendodermal marker genes and (K) epiblast–associated genes while (L) neuroectodermal and (M) trophoblast genes are downregulated upon TBX3-induction. (N) qPCR analysis of certain marker genes labeling primitive endoderm. (O) Gene set enrichment plot highlights that TBX3-induction favors primitive endoderm formation.

p-values are represented as <0.001;***; <0.01, **; <0.05, *.
Supplemental Figure 3 (related to Figure 6): (A,B) Wild-type and td-tomato-labeled iTBX3 cells are mixed at the indicated ratios and chimeric EBs are formed. After differentiation (-/+/Dox) for 6 days, single cells from chimeric EBs are FACS sorted and analyzed by qPCR as shown in (B). All experiments (n=3) in biological replicates. (B) qPCR analysis for Lhx1 (upper panel) and Eomes (lower panel) FACS-purified as outlined in (A) for the indicated populations. High expression levels of Lhx1 and Eomes in both TBX3 and wild-type cells are associated with higher TBX3 expression in the origin EBs. All experiments (n=3) in biological replicates. (C) The number of beating cardiomyocyte clusters per well are counted on day14 in wild-type ESCs treated either with non-CM, with -Dox CM or with +Dox-CM from iTBX3 cells (+Dox CM day0-2 or day0-4). Higher levels of (D) the cardiac marker gene Myh6 and (E) the pancreatic progenitor marker gene Pdx1 at day14 are associated with higher TBX3 levels as assessed by qPCR in wild-type ESCs treated either with non-CM, with -Dox CM, or with +Dox-CM from iTBX3 cells (+Dox CM day0-2 or day0-4). (F) qPCR analysis for FoxA2 levels in untreated and Dox-treated iTBX3 cells at day4 of differentiation in the presence or absence of Nodal-blocking antibody at a final concentration of 3 μM (n=2) in biological replicates. p-values are represented as <0.001,***; <0.01, **; <0.05, *. 
Supplemental Figure 4 (related to Figures 5 and 7): Highly conserved promoter regions and ChIP primer localization. (A) Consensus T-box binding site generated by MEME (http://meme.nbcr.net/meme/cgi-bin/meme.cgi). Fw: forward sequence; rc: reverse complimentary sequence. (B-E) Alignment of mouse genomic loci of *Nodal* (B), *Eomes* (C), *T* (D) and *Sox17* (E) to the respective orthologous using rVista browser. The putative Tbx3 binding sites were detected using position weight matrix (PWM) of the Tbx-motif generated in (A). Two conserved binding sites for each gene promoter are shown in the respective table. The genomic location for the mouse gene is given together with the respective binding score and orientation of the respective motif (fw, rc). Angular black arrow indicates the TSS (transcriptional start site) and blue dotted box marks the genomic region containing the two depicted conserved motifs and the ChIP-PCR amplicon. Black dotted box zooms into the blue dotted box and indicates ChIP-PCR amplicon.
A

**tbx3 MO binding site**

5'-A AAG TGA ATG AAT TTA CCC ATG AGA-3'

*red* = ATG start codon

B

**tbx3 MO-gfp**

control MO  |  tbx3 MO-gfp+

C

control  |  **tbx3 MO**

D

% of the embryos

|          | WT | control MO | tbx3 MO |
|----------|----|------------|---------|
| n        | 5  | 4          | 4       |
| N        | 194| 152        | 154     |

*abnormal heart morphology*  |  

cancers

E

beats / min

|          | WT | tbx3 MO |
|----------|----|---------|
| n        | 20 | 20      |
| N        |    |         |
Supplemental Figure 5 (related to Figure 7). Testing the specificity of tbx3 morpholino oligonucleotide. (A) Xenopus tbx3 morpholino oligonucleotide- (MO) binding site. The start codon is highlighted in red. (B) The tbx3 MO-binding site is cloned in front of and in frame with gfp and injected bilaterally as RNA together with tbx3 or control MO into Xenopus embryos at 2-cell stage. GFP fluorescence was monitored at stage 13. Coinjection of tbx3 MO-gfp RNA together with the control MO has no effect on GFP fluorescence whereas the tbx3 MO efficiently blocks translation of GFP indicating the specificity of the MO used. Scale bars are 0.5 mm. TBX3 loss of function phenotype in Xenopus heart development. (C) Loss of TBX3 by bilateral injection of 40 ng tbx3 MO into 84-cell stage Xenopus embryos results in cardiac malformation and cardiac edema at stages 42 and 45, respectively. a, atrium; v, ventricle; OFT, outflow tract. Scale bars are: lateral 1 mm, ventral 0.2 mm, isolated hearts 0.2 mm. (D) Quantitative presentation of data shown in (C). (E) Loss of TBX3 results in bradycardia at stage 42. n, number of independent experiments, N, number of embryos examined. Error bars indicate standard errors of the means (SEM). p-values are calculated by a nonparametric Mann-Whitney rank sum test. * p<0.05, ** p <0.01, **** p<0.0001.
Supplemental Figure 6 (related to Figures 1–7). T-box factors and TBX3 function hypothesis. (A) Phylogenetic relationship of T-box factors. Numbers on each branch indicate the distance to the branch node (round dot). Factors in the same branch are closely related. (B) Schematic model of proposed TBX3 action. Schematic model depicting the mechanism how TBX3 may regulate early lineage commitment and mesendodermal specification. Thus, TBX3 cell-autonomously activates T, EOMES, SOX17 and NODAL signaling components thereby activating non-cell autonomously NODAL-SMAD2/3 target genes to favor the mesendoderm/endoderm specification. The latter process possibly involves the TBX3-expressing cells of the extraembryonic.
Supplemental Figure 7 (related to Figure 4). TBX3 is functionally related to other T-box family members in directing early cell fate choices. (A-B) Bilateral injection of 40 ng tbx3 MO leads to reduced t (A) and gsc (B) expression (black arrowheads). Uninjected (wild type, WT) and control MO injected embryos revealed normal marker gene expression. Vegetal and lateral views and sagittal sections of Xenopus embryos at stages 10.5 (t expression) or 10 (gsc expression) are shown as indicated. Scale bars are 0.5 mm. (C) TBX3 overexpression abolishes TBX2 and TBX6 expression on mRNA and protein level upon TBX3 induction (-/+ Dox) at day4 of differentiation. All scale bars are 20 µm. All nuclei are shown in DAPI (blue). (D) Injection of tbx2 or tbx3 MO resulted in gastrulation phenotypes. In mild cases, closure of the blastopore was delayed in comparison to control MO injected embryos. In severe cases, gastrulation was completely blocked. n = number of independent experiments. N = number of injected embryos analyzed. Error bars indicate standard error of the means (SEM). p-values were calculated by a nonparametric Mann-Whitney rank sum test. * p<0.05, ** p <0.01. Scale bars are 0.5 mm.

Movie S1. Live-cell imaging of spontaneously beating areas of cardiomyocytes derived from control ES cells (related to Figure 4). Cells were imaged at day17 of differentiation.

Movie S2. Live-cell imaging of spontaneously beating areas of cardiomyocytes derived from TBX3 overexpressing ES cells (related to Figure 4). Induction of TBX3 was started from day0 to day2 of EB generation. Note that TBX3 overexpression enhances the generation of beating cardiomyocytes. Cells were imaged at day17 of differentiation.
Supplemental Experimental Procedures.

Cell culture & ESC differentiation.

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA, www.invitrogen.com), with 10% (v/v) fetal calf serum (FCS; Lonza, Basel, BS, Switzerland, www.lonza.com) and 1% Penicillin/Streptomycin (Millipore, Billerica, MA, USA, www.millipore.com). Mouse embryonic fibroblast (MEF-Feeder) cell s were grown in DMEM with 10% (v/v) FCS (PAA, Pasching, Austria, www.paa.com), 1% Penicillin/Streptomycin, 1% GlutaMax (Invitrogen; final concentration 2mM), 1% Non-Essential Amino Acids (NEAA; Invitrogen), 1% Sodium Pyruvate (Invitrogen; final concentration 1mM), 1% β-Mercaptoethanol (Millipore) and Vitamin C (VitC; 0,05mg/ml; Sigma) in a humidified incubator containing 5% CO2 at 37°C. Feeder irradiation was performed according to standard procedures. mESCs were cultured in Knockout DMEM (KO-DMEM; Invitrogen), 15% FCS (Lonza), 1% Penicillin/Streptomycin, 1% GlutaMax, 1% NEAA, 1% Sodium Pyruvate, 1% β-Mercaptoethanol and 240 U/ml leukemia inhibitory factor (LIF; Sigma-Aldrich, St.Louis, MO, USA, www.sigmaaldrich.com). Sox 17-RFP reporter cell line was kindly provided by Douglas Melton, T-GFP/Dppa4-RFP reporter cell line was kindly provided by Hans-Jörg Fehling. In vitro differentiation of ESCs was carried out according to the standard protocol using the hanging drop method. Briefly, Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% FCS (Lonza), 1% Penicillin/Streptomycin, 1% GlutaMax, 1% NEAA, 1% Sodium Pyruvate, 1% β-Mercaptoethanol and 240 U/ml leukemia inhibitory factor (LIF; Sigma-Aldrich, St.Louis, MO, USA, www.sigmaaldrich.com). In short, 600 cells per 20 μl differentiation medium were placed on the lids of petri dishes filled with 10 ml Dulbecco’s Phosphate Buffered Saline (DPBS; Invitrogen) and were cultivated for 2 days in hanging drops. In the following, embryoid bodies (EBs) were rinsed into non-adherent bacterial dishes and were cultivated for another two days. On day4 EBs (n = 11) were plated on (0.1%) gelatin-coated 6-well dishes or cover slips for RNA or immunofluorescence analysis, respectively and assayed at specific time points as described in figure legends. Inhibitors: SB-421542 (Sigma-Aldrich, St.Louis, MO, USA, S4317, www.sigmaaldrich.com) was dissolved in DMSO and added to cell culture medium where indicated at a final concentration of 10µM. Anti-NODAL antibody (Santa Cruz, Santa Cruz, CA, USA, sc- 28913, www.scbt.com) was applied to the differentiating cells at a final concentration of 3µM.
**RNA interference.** TBX3 RNA interference was performed using a shRNA-expressing pSuperpuro (Oligoengine) construct as previously described in (Kartikasari et al., 2013). Briefly, mESCs were plated in feeder-free conditions prior to transfection using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions, followed by Puromycin (Sigma) selection at 1 mg/ml for two days. EB differentiation was performed by culturing the cells in non-adherent conditions at a density of 104 cells/ml for a day in LIF-free supplemented KO-DMEM.

**Lentiviral infection.** Lentivirus generation was implemented by using a virus containing a polycistronic expression cassette encoding LV-tdTOMATO (kindly provided by Konrad Hochedlinger) in a 70 % confluent 10 cm dish containing Lenti-X 293T cells (Clontech, Mountain View, CA, USA, www.clontech.com) by cotransfection of the polycistronic vector (8 µg), the pMD2 vector (2 µg) and the psPAX2 vector (5.5 µg) (all Addgene, Cambridge, MA, USA, www.addgene.org) using 100 µl PolyFect transfection reagent (Qiagen, Hilden, NRW, Germany, www.qiagen.com). Viral supernatant was collected at 24, 48 and 72 hours after transfection, concentrated using the Lenti-X Concentrator Kit (Clontech).

**Transfection of Reporter plasmids & Luciferase assays.** At least three independent transfection experiments in duplicates were carried out in each case. For luciferase assays, HEK-293T cells were transfected in 24-well plates with 200 ng of indicated reporter plasmids and 50 ng of pTK-RL as internal transfection control, using Lipofectamine 2000 reagent (Invitrogen). Cells were lysed in passive lysis buffer (Promega, Madison, WI, USA, www.promega.com) 24 hours post transfection and luciferase activity was measured applying firefly and renilla assay buffer (Promega) using a luminometer (GloMax 96, Promega). Light emission from the firefly luciferase was normalized to light emission from the renilla luciferase for every transfection and represented as relative luciferase units (RLU).

**Luciferase Reporter Constructs.**

**Brachyury-reporter.** The *Brachyury*-promoter luciferase reporter construct (pTPwt: T-Promoter in pGL3Basic) was previously generated by cloning a 618 bp promoter fragment of the mouse *Brachyury* gene, corresponding to region −484 to +134 of the 5′region of *Brachyury* (Clements et al., 1996), into the promoter-less luciferase reporter plasmid pGL-3Basic (Arnold et al., 2000). We obtained this plasmid from R. Kemler.
**hEOMES-reporter.** A fragment of the hEOMES enhancer region (−6943 to −6090) was previously generated by subcloning into the promoter-less luciferase reporter plasmid pGL-3Basic (Teo et al., 2011). We obtained this plasmid from L. Vallier.

**Sox17-reporter.** The luciferase reporter plasmid pSox17-5.6kb was previously constructed by amplifying a 5.6 kb fragment upstream of the translation start site from FVB/N mouse genomic DNA and was cloned into the promoter-less luciferase reporter plasmid pGL-3Basic (Lin et al., 2010). We obtained this plasmid from J. Wells.

**Oct4-reporter.** For quantifying relative Oct4-enhancer activities, the proximal and distal enhancers were previously PCR-amplified from a GOF18 plasmid (Yeom et al., 1996) and cloned into the promoter-less luciferase reporter plasmid pGL-3Basic (Greber et al., 2010).

**Immunocytochemistry.** Briefly, cells were fixed at different time points of differentiation using 4% Paraformaldehyde (PFA). Further they were treated with NH4Cl and blocked with BSA and 0.3% Triton. Primary antibodies were added: rabbit anti TBX3 (kindly provided by H.Niwa), 1:1000, 1h room temperature (RT); mouse anti OCT3/4 (Santa Cruz, Santa Cruz, CA, USA, www.scbt.com), 1:200, o.N. 4°, sc-5279; rat anti human/mouse EOMES (eBioscience, San Diego, CA, USA, www.ebioscience.com), 1:200, o.N. 4°, 14-4876-82; rabbit anti EOMES (Abcam, Cambridge, UK, www.abcam.com), 1:1000, o.N. 4°, ab23345; goat anti human Brachyury (R&D Systems, Minneapolis, MN, USA, www.mdsystems.com), 1:100, o.N. 4°, AF2085; goat anti human SOX17 (R&D Systems), 1:500, o.N. 4°, AF1924; goat anti human PDX1 (R&D Systems), 1:500, o.N. 4°, AF2419; rabbit anti mouse Albumin (FITC conjugated, Cedarlane, Burlington, ON, Canada, www.cedarlanelabs.com), 1:100, 1h RT, CLFAG3140; mouse anti α-Actinin (Sigma-Aldrich), 1:150, 1h RT, A7811; goat anti TBX6 (Santa Cruz, Santa Cruz, CA, USA www.scbt.com), 1:300, 1h RT; rabbit anti TBX2 (kindly provided by C. Goding). Finally fluorescence labeled secondary antibodies Alexa Fluor® 488 (green), Alexa Fluor® 568 (red), Alexa Fluor® 647 (magenta) (Invitrogen, all diluted 1:500) were added. Nuclei were stained with DAPI (1:20,000). Images were captured using an upright fluorescence Zeiss Axioimager Z1 microscope and analyzed using Axiovision software (Zeiss, Oberkochen, BW, Germany, www.zeiss.com). Embryos were flushed at 1.5 days post coitum (dpc) and cultured until 3.5 dpc in M16 media (Sigma, #M7292). Staining was performed as previously described (Nichols et al., 2009) with rabbit anti TBX3 (kindly provided by H.Niwa), 1:1000, o.N. 4° and mouse
anti OCT3/4, 1:200, o.N. 4°, sc-5279. All images were taken by an ApoTome fluorescence microscope (Carl Zeiss microscopy).

**Whole-mount in situ hybridization.** Whole-mount in situ hybridization (WMISH) analysis of E6.0 embryos and respective Xenopus embryo stages was performed according to standard protocols as previously described using probes for mouse and Xenopus TBX3 (Costello et al., 2011; Herrmann et al., 2011).

**Xenopus experiments.** Xenopus laevis embryos were staged according to standard protocols (Nieuwkoop and Faber, 1975). Xenopus embryos at different stages were fixed using Formaldehyde. WMISH experiments were performed following well established protocols (Gessert et al., 2007). Morpholino oligonucleotides (MOs) were purchased from GeneTools, LLC, USA. The t bx3 MO was: 5′- tct cat ggg taa att cat tca ctt t – 3′. t bx2 and t bx6 MOs were used as previously published (Cho et al., 2011; Tazumi et al., 2008). For injection control, the standard control MO from GeneTools was used.

To investigate the temporal expression of t bx3 during Xenopus development, total RNA of whole Xenopus embryos at diverse stages was isolated using the peqGOLD RNApure kit (Peqlab). cDNA was generated using random primers (Invitrogen) and Superscript II RNase H- reverse transcriptase (Invitrogen). RT-PCR was performed using the Master Amp Tm Taq PCR Core kit (Epicentre). Primer sequences were:

- **t bx3** _RT_l: 5′- ata cca acc cag gtt cca cat agt g -3′;
- **t bx3** _RT_r: 5′- cag ccc ttg aga gga aga tgc c -3′;
- **gapdh** _RT_l: 5′- gcc gtg tat gtg gaa tct -3′;
- **gapdh** _RT_r: 5′- aag ttc gtc ttc gat gac -3′.

Product length were: **t bx3**: 976 bp; **gapdh**: 230 bp. Annealing temperature was 55°C. To examine the spatial expression of **t bx3**, a **t bx3** cDNA probe with a length of 976 bp was cloned using the proof reading PfuUltra II DNA polymerase (Stratagene) and the following primers: **t bx3** _l: 5′- ata cca acc cag gtt cca cat agt g -3′; **t bx3** _r: 5′- cag ccc ttg aga gga aga tgc c -3′. The PCR product was cloned into the pSC-B vector (Stratagene). A **t bx3** antisense RNA probe was generated using NotI (NEB) and T3 RNA polymerase (Roche). To detect the spatial expression of **t bx3** during gastrulation, the specificity of the **t bx3** MO was tested by cloning the binding site in front of and in frame with GFP. *In vitro* transcribed RNA was injected together with **t bx3** or control MO at 2-cell stage and fluorescence was monitored 2 days later (**Supplemental Figure 5**). MOs were injected bilaterally into the marginal zone of each dorso-vegetal blastomere at doses indicated. To analyze marker gene expression, embryos were fixed at stage 10 (**gsc** expression) and 10.5 (**t** expression). Sagittal sections were cut using a scalpel. To analyze the heart
phenotype, tbx3 MO was bilaterally injected into 8-cell stage embryos in both dorso-
vegetal blastomeres to target cardiac tissue. Correctness of injections was verified by
gfp RNA co-injections. Heart morphology was analyzed at stage 42 and 45,
respectively. Heart beat was counted at stage 42.

**Immunoblotting.** Immunoblotting was performed according to standard procedures. Primary antibody dilutions were: rabbit anti TBX3 (Abcam), 1:1000, o.N. 4°, ab66306; goat anti TBX3 (Santa Cruz), 1:1000, o.N. 4°, sc-17871; goat anti human SOX17 (R&D Systems), 1:500, 1h RT, AF1924; rabbit anti Phospho SMAD2 (Cell Signaling, Danvers, MA, USA, www.cellsignal.com), 1:1000, o.N. 4°, #3101; ß-actin, 1:50.000, 1h RT. This was followed by incubation with secondary horse radish peroxidase–
coupled antibodies diluted 1:3000, 1h RT. Detection was performed with either ECL
or ECL+ kits (Thermo scientific, Waltham, MA, USA, www.thermofisher.com).

**FACS analysis.** On designated days, EBs were washed with PBS and dissociated
into single cell suspension by incubation with 0.25% trypsin/EDTA (Millipore). The
cells were dissolved in 5% FCS/PBS and analyzed with BD FACSaria III cell sorter.
All events were gated with forward scatter (cell size) and side scatter (cell granularity)
profiles.

**Bioinformatic analysis.**
The ECR browser (http://ecrbrowser.dcode.org) tool was used in the rVista genome
browser (http://rvista.dcode.org) to identify the evolutionarily conserved DNA
sequences within the promoters of Nodal, Eomes, Brachyury (T) and Sox17 followed
by Tbx-binding site prediction analysis. All the putative binding site sequences from
different promoters were retrieved and subjected for de novo consensus Tbx-binding
motif generation using MEME motif generation tool (meme.ncbr.net/meme/). All
genomic and transcript sequences were obtained from either UCSC browser
(http://genome.ucsc.edu/) or Ensembl (http://useast.ensembl.org/index.html). The
Tbx-motif was displayed as the motif logo by seqLogo package in R. To estimate the
Tbx3-binding of Eomes, Nodal, T and Sox17 in the mouse genome (Mouse
GRCm38/mm10 assembly), the putative Tbx-binding sites in the flanking regions
were detected for 600 up- and down-stream positions around ChIP primer binding
sites. The motif match was performed using matchPWM in Biostrings package in R.
The minimum score for counting a match was set to 0.8 indicating 80% of chance
that the predicted site is a Tbx-binding site. Afterwards the motif conservation among
human, rat and mouse genomes was assessed using Vertebrate Multiz Alignment and Conservation track in UCSC genome browser.

To perform cross-platform analysis, data from different array platforms were integrated by NCBI gene id, and further adjusted by the Combat algorithm (Johnson et al., 2007) to minimize the batch effect. Principal component analysis and hierarchical clustering were performed in R programming environment (Team, 2005). Differentially expressed genes were detected by limma t-test with criteria of p-value < 0.05 and fold change > 1.5. The p-value was adjusted by the procedure of Benjamini and Hochberg (Benjamini and Hochberg, 1995). The gene set enrichment analysis was done by customed R-GSEA script (Subramanian et al., 2005).

**ChIP detailed information.** Antibodies were applied as follows: goat anti TBX3 (Santa Cruz), o.N. 4°C, 3µg, sc- 31657; goat anti TBX3 (Santa Cruz), o.N. 4°C, 3µg, sc-17871; rabbit anti TBX3 (Invitrogen), o.N. 4°C, 3µg, #42-4800; goat IgG (Santa Cruz), o.N. 4°C, 3µg, sc-2028. TBX3 antibodies showed similar results in qPCR. The above immunoprecipitated DNA was eluted and reverse cross-linked. The purified DNA and 1% of the respective input DNA were used as templates for qPCR using Rotor Gene rtPCR Cycler (Qiagen) and SYBR Green qPCR mix (Fermentas, Burlington, ON, Canada, http://www.thermoscientificbio.com/fermentas/). Ct-values were first normalized to the respective input DNA and are represented as % of input. Appointed annealing temperatures: Nodal primer - 56°C, Eomes and T primer - 60°C, Sox17 primer - 65°C. Primer information are as follows:

| Gene   | Primer Sequence | Product length (Bp) |
|--------|-----------------|---------------------|
| Brachyury | Fwd 5' ccc ggc tgt ccc tgt ccc 3' | 348 |
|         | Rev 5' agc cgt tgt gtc atc acl 3' | |
| Eomes   | Fwd 5' agg gaa tgt ctc gta atg aat ggt 3' | 425 |
|         | Rev 5' cag agt tgt cgg tgt ctc gat g 3' | |
| Nodal   | Fwd 5' ccg cca caa ttc tgt tgt tgt ctg 3' | 123 |
|         | Rev 5' ctc cgg aag ggc cta taa cct a 3' | |
| Sox17   | Fwd 5' gag tgt tgt tgt tgt ggg tgt ggg gga 3' | 223 |
|         | Rev 5' cag cag cac tgt gct gct cct cgg 3' | |
| Fgf2    | Fwd 5' aga gaa caa cag aac cac c 3' | 125 |
|         | Rev 5' tca aag tgt tgt tgt tgt tgt a 3' | |

**Quantitative one-step real-time RT-PCR and PCR detailed Information.** Briefly, one-step real-time qPCR was carried out with the Rotor Gene RT-PCR Cycler (Qiagen) using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Each RNA preparation was tested for genomic DNA contamination by replacing reverse transcriptase with water. Internal standards (housekeeping gene) and samples were simultaneously amplified. RT-PCR was performed as recommended by supplier's
manuscript. For RNAi experiments, 0.5 µg of total RNA from each sample was denatured at 65°C and then reverse transcribed using Superscript II reverse transcriptase (Invitrogen) at 50°C for 1 hour. qPCR was performed in ABI7900HT (Applied Biosystems) using 1x FastSybrGreen Mix (Applied Biosystems), 500 nM of each primer and 100 ng cDNA. PCR conditions included denaturation at 95°C for 20 seconds, followed by 50 cycles of 94°C for 1 second and 60°C for 20 seconds, then continuation with dissociation stage. Primer sequences were listed in (Kartikasari et al., 2013).

Relative transcript expression is depicted as the ratio of target gene concentration to the housekeeping gene Hydroxymethylbilane synthase (Hmbs), cyclophilin or polymerase 2A (Pol2a) or for Xenopus PCR gapdh (glycerinaldehyde-3-phosphate dehydrogenase) concentration. Self-made (Biomer) and QuantiTect primer assays (Qiagen) were used in experiments. Primer information is available upon request.

Acknowledgements
We thank K. Kleinhans and A. Lechel for help with microarray; S.F. Katz for FACS-support; P. Mahaddalkar for support with lentivirus; and V. Sakk and J. Nichols for help with embryo stainings; L. Vallier, Eomes-reporter; J. Wells, Sox17-reporter; K. Hochedlinger, lentiviral LVdtTOMATO construct; R. Kemler, T-reporter; D. Melton, Sox17-reporter cells; H. Fehling, Dpp4- RFP/Brachyury-GFP cells; V. Christoffels, TBX3-Reporter mice; and S. Arnold, G. von Wichert, H. Friedle, K. L. Rudolph, and A. Wobus for critically reading the manuscript.

Supplemental References
Arnold, S.J., Stappert, J., Bauer, A., Kispert, A., Herrmann, B.G., and Kemler, R. (2000). Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. Mechanisms of development 91, 249-258.
Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J Royal Stat Soc Series B 57, 289-300.
Cho, G.S., Choi, S.C., Park, E.C., and Han, J.K. (2011). Role of Tbx2 in defining the territory of the pronephric nephron. Development 138, 465-474.
Clements, D., Taylor, H.C., Herrmann, B.G., and Stott, D. (1996). Distinct regulatory control of the Brachyury gene in axial and non-axial mesoderm suggests separation of mesoderm lineages early in mouse gastrulation. Mechanisms of development 56, 139-149.
Costello, I., Pimeisl, I.M., Drager, S., Bikoff, E.K., Robertson, E.J., and Arnold, S.J. (2011). The T-box transcription factor Eomesodermin acts upstream of Mesp1 to specify cardiac mesoderm during mouse gastrulation. Nature cell biology 13, 1084-1091.
Greber, B., Wu, G., Bernemann, C., Joo, J.Y., Han, D.W., Ko, K., Tapia, N., Sabour, D., Sterneckert, J., Tesar, P., et al. (2010). Conserved and divergent roles of FGF signaling in mouse epiblast stem cells and human embryonic stem cells. Cell stem cell 6, 215-226.
Han, J., Yuan, P., Yang, H., Zhang, J., Soh, B.S., Li, P., Lim, S.L., Cao, S., Tay, J., Orlov, Y.L., et al. (2010). Tbx3 improves the germ-line competency of induced pluripotent stem cells. Nature 463, 1096-1100.

Herrmann, F., Bundschu, K., Kuhl, S.J., and Kuhl, M. (2011). Tbx5 overexpression favors a first heart field lineage in murine embryonic stem cells and in Xenopus laevis embryos. Developmental dynamics : an official publication of the American Association of Anatomists 240, 2634-2645.

Johnson, W.E., Li, C., and Rabinovic, A. (2007). Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics 8, 118-127.

Kartikasari, A.E., Zhou, J.X., Kanji, M.S., Chan, D.N., Sinha, A., Grapin-Botton, A., Magnuson, M.A., Lowry, W.E., and Bhushan, A. (2013). The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and Eomes to drive endoderm differentiation. The EMBO journal 32, 1393-1408.

Lin, S.C., Wani, M.A., Whitsett, J.A., and Wells, J.M. (2010). Klf5 regulates lineage formation in the pre-implantation mouse embryo. Development 137, 3953-3963.

Nichols, J., Silva, J., Roode, M., and Smith, A. (2009). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. Development 136, 3215-3222.

Nieuwkoop, P.D., and Faber, J. (1975). Normal Table of Xenopus laevis (Daudin). North-Holland, Amsterdam.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., et al. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proceedings of the National Academy of Sciences of the United States of America 102, 15545-15550.

Tazumi, S., Yabe, S., Yokoyama, J., Aihara, Y., and Uchiyama, H. (2008). PMesogenin1 and 2 function directly downstream of Xtbx6 in Xenopus somitogenesis and myogenesis. Developmental dynamics : an official publication of the American Association of Anatomists 237, 3749-3761.

Team, T.R.D.C. (2005). R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria).

Teo, A.K., Arnold, S.J., Trotter, M.W., Brown, S., Ang, L.T., Chng, Z., Robertson, E.J., Dunn, N.R., and Vallier, L. (2011). Pluripotency factors regulate definitive endoderm specification through eomesoderm. Genes & development 25, 238-250.

Yeom, Y.I., Fuhrmann, G., Ovitt, C.E., Brehm, A., Ohbo, K., Gross, M., Hubner, K., and Scholer, H.R. (1996). Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122, 881-894.