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TEAD4 establishes the energy homeostasis essential for blastocoel formation

Kotaro J. Kaneko* and Melvin L. DePamphilis

SUMMARY

It has been suggested that during mouse preimplantation development, the zygotically expressed transcription factor TEAD4 is essential for specification of the trophectoderm lineage required for producing a blastocyst. Here we show that blastocysts can form without TEAD4 but that TEAD4 is required to prevent oxidative stress when blastocoel formation is accompanied by increased oxidative phosphorylation that leads to the production of reactive oxygen species (ROS). Both two-cell and eight-cell *Tead4*$^{-/-}$ embryos developed into blastocysts when cultured under conditions that alleviate oxidative stress, and *Tead4*$^{-/-}$ blastocysts that formed under these conditions expressed trophectoderm-associated genes. Therefore, TEAD4 is not required for specification of the trophectoderm lineage. Once the trophectoderm was specified, *Tead4* was not essential for either proliferation or differentiation of trophoblast cells in culture. However, ablation of *Tead4* in trophoblast cells resulted in reduced mitochondrial membrane potential. Moreover, *Tead4* suppressed ROS in embryos and embryonic fibroblasts. Finally, ectopically expressed TEAD4 protein could localize to the mitochondria as well as to the nucleus, a property not shared by other members of the TEAD family. These results reveal that TEAD4 plays a crucial role in maintaining energy homeostasis during preimplantation development.

KEY WORDS: *Tead4*, Blastocyst, Trophectoderm, Blastocoel, Oxidative stress, Anti-oxidant, Mouse

INTRODUCTION

The earliest cell differentiation event in mammalian development occurs during preimplantation development when the outer blastomeres of the embryo form a monolayer of epithelial cells [trophectoderm (TE)] that envelops the remaining blastomeres [inner cell mass (ICM)]. In mice, TE specification occurs in the 8- to 16-cell compacted morula. Developmental transition to blastocyst stage is defined by the appearance of a fluid-filled cavity called the blastocoel, which absolutely requires a functional TE (Watson and Barcroft, 2001) (supplementary material Fig. S1). Thus, TE specification is essential for blastocoel formation. Morula-to-blastocyst transition is accompanied by a major transition in metabolic energy pathways related to blastocoel formation, concomitant with changes in gene expression that are related to specification of the TE lineage. Identifying the genes that specify the TE and those that regulate changes in metabolism has been the focus of intense, but often separate, investigations. Hence, regulatory genes that directly link preimplantation development to energy homeostasis have yet to be identified. Here, we show that the TEAD4 transcription factor is one such gene.

Just prior to blastocoel formation, the embryo switches energy sources abruptly from pyruvate and lactate to glucose, concomitant with a 2.7-fold increase in oxygen consumption (Gardner, 1998; Johnson et al., 2003). This increased oxygen and glucose consumption that results in increased metabolic rate is restricted to the TE where oxidative phosphorylation (OXPHOS) drives the synthesis of ATP (Houghton, 2006; Leese et al., 2008). Na^+, K^+-ATPase within the TE consumes ~60% of this ATP in expanding the blastocoel (Houghton et al., 2003). In fact, an increase in OXPHOS activity with respect to glycolytic activity in developing blastocysts positively correlates with the capacity of the embryo to develop to term following implantation (Gardner, 1998; Gardner, 2008). However, increased energy production via OXPHOS necessarily results in increased production of reactive oxygen species (ROS) (Adelman et al., 1988; Turrens, 1997; Finkel and Holbrook, 2000), which is normally attenuated by antioxidant defense mechanisms present within the embryo and its surroundings (Johnson and Nasr-Esfahani, 1994; Guérin et al., 2001; Orsi and Leese, 2001; Favetta et al., 2007; Betts and Madan, 2008; Kawamura et al., 2010; Zhang et al., 2010). In addition, it is likely that the oxygen level in the female reproductive tract is kept hypoxic relative to the atmospheric oxygen level (3-5% versus 21%) (Gardner and Leese, 1990) in order to minimize ROS production. However, some oxygen is required, because OXPHOS is essential for blastocoel development (Thomson, 1967). Thus, embryos must carefully balance energy production, energy usage and ROS production (termed 'energy homeostasis'), because failure to do so results in increased oxidative stress, changes in the intracellular redox potential and impaired biosynthetic potential, all of which are detrimental to development (Harvey et al., 2002; Burton et al., 2003; Dumollard et al., 2007; Van Blerkom, 2009).

TEAD4 (also known as TEF3) is one of four TEAD proteins in mammals that have nearly identical DNA-binding domains and that bind the same *cis*-acting sequence (Kaneko and DePamphilis, 1998). They are >80% similar in overall amino acid sequence and bind the same transcriptional co-activators (Vassilev et al., 2001). The *Tead4* gene is first expressed at the eight-cell stage, and embryos lacking a functional *Tead4* gene arrest development at the morula stage (Nishioka et al., 2009; Nishioka et al., 2008; Yagi et al., 2007). Consequently, *Tead4*$^{-/-}$ embryos neither form a blastocoel nor implant into the uterus. Moreover, *Tead4*$^{-/-}$ embryos do not express *Cdx2*, *Gata3* and other genes characteristic of TE. These and other studies reveal that *Tead4* acts upstream of genes associated with TE specification and function (Home et al., 2009; Jedrusik et al., 2010; Ralston et al., 2010; Wu et al., 2010). They also do not produce trophoblast stem cells or trophoblast giant cells

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that are derived from TE (Tanaka et al., 1998; Yagi et al., 2007). The requirement for TEAD4 during preimplantation development is specific for TE, because *Tead4*$^{-/-}$ embryos continue to express genes characteristic of the ICM and can produce embryonic stem cells that are derived from the ICM. Thus, *Tead4* is the earliest zygotically expressed transcription factor reported to be essential for blastocoel formation and expression of TE-associated genes. *Tead4* might also be required for post-implantation development, because it is expressed selectively in extra-embryonic ectoderm as well as in developing placenta (Jacquemin et al., 1996; Jacquemin et al., 1998).

In an effort to understand how TEAD4 specifies TE, we made the surprising discovery that *Tead4*$^{-/-}$ embryos could form a blastocoel, express TE-associated genes and produce trophoblast giant cells. Therefore, TEAD4 is not essential for specifying the TE lineage. It is, however, essential for blastocoel formation and expansion, but only under conditions that promote oxidative phosphorylation and therefore oxidative stress. By managing oxidative stress *in vitro*, the requirement for TEAD4 can be bypassed. In fact, analysis of ectopic *Tead4* gene expression as well as ablation of *Tead4* gene expression suggested that *Tead4* is unique among TEAD members in that TEAD4 can localize to mitochondria and affect mitochondrial activities. Given that embryos normally respond to high-energy demands imposed during blastocoel formation by upregulating oxygen consumption (Leese et al., 2008), the role for TEAD4 during preimplantation development is to establish the energy homeostasis essential for the morula-to-blastocyst transition.

MATERIALS AND METHODS

Mice

Mice were mated naturally without hormonal stimulation, and the presence of a vaginal plug was designated embryonic day (E)0.5. *Tead4* conditional knockout mice (*Tead4*$^{lox/lox}$) and *Tead4* nullizygous mice (*Tead4*$^{-/-}$) have been described (Yagi et al., 2007). *Tead4*$^{lox/lox}$ mice were mated with B6.Cg-Tg(*CAG-cre/Esr1**)5Amc/J mice (#004682, Jackson Laboratories), which express a monohydroxytamoxifen-inducible Cre recombinase. Where indicated, *Tead4*$^{-/-}$ embryos were isolated from mating of *Tead4*$^{-/-}$ males (Yagi et al., 2007) to *Tead4*$^{lox/lox}$; *ZP3-cre*$^{Tg/+}$ females. These females were obtained by mating *Tead4*$^{lox/lox}$ mice with C57BL/6-Tg (ZP3-cre)93Knw/J mice (#003651, Jackson Laboratories), in which Cre recombinase is driven by the oocyte-specific ZP3 promoter (Lewandoski et al., 1997).

Cells

Primary mouse embryonic fibroblasts (PMEFs) were derived from E13.5 embryos (Conner, 2001). Trophoblast stem cells (TSCs) were derived from E3.5 blastocysts (Himeno et al., 2008) and propagated on mitotically arrested PMEFs (Yagi et al., 2007). PMEFs and TSCs were isolated from *Tead4*$^{lox/lox}$; *CAG-cre/Esr1*$^{Tg/+}$ embryos (supplementary material Fig. S2A). The *Tead4*$^{lox/lox}$ allele was excised efficiently in cells that contained the *CAG-cre/Esr1* allele (Hayashi and McMahon, 2002) by culturing them with 1 μM monohydroxytamoxifen (MHT; Sigma) for 48 hours (supplementary material Fig. S2B,C; data not shown). Cre eliminates exon 2, containing half of the DNA-binding domain (Yagi et al., 2007). Control cells (genotyped *Tead4*$^{lox/lox}$; *CAG-cre/Esr1*$^{+/+}$) were isolated from embryos in the same mating. *Tead2*$^{lox/lox}$; *CAG-cre/Esr1*$^{Tg/+}$ PMEFs were obtained similarly using a *Tead2* conditional knockout line (Kaneko et al., 2007).

Blastocoel formation assay

Two-cell embryos, eight-cell embryos and morula were isolated from the oviduct in M2 medium (Millipore), and blastocysts were isolated from the uterus (DePamphilis et al., 1988; Nagy et al., 2003). Embryos were passed through four 100 μl drops of culture medium overlaid with mineral oil that had been equilibrated overnight in a humidified incubator at 5% CO_2 and either atmospheric or 5% oxygen. The 5% oxygen level was maintained either by purging the incubator with 90% N_2, 5% CO_2 and 5% O_2 (tanks prepared by Roberts Oxygen) or by using a Sanyo MCO-5M tri-gas incubator. Embryos were cultured together in the indicated medium (~1 μl/embryo). Optimal blastocoel formation occurred when embryos remained undisturbed in the incubator for the time indicated. EmbryoMax KSOM medium was either purchased from Chemicon/Millipore (MR-020P-5F) or synthesized from individual components (Nagy et al., 2003) using reagents from Sigma and Invitrogen (L-glutamine). MEM essential amino acids (50×) without L-glutamine (Sigma, M5550) were used at 0.5× (Ho et al., 1995; Nagy et al., 2003). Zona pellucidae were removed in Acidic Tyrode's solution (Millipore) (Nagy et al., 2003). Embryos with or without zona pellucidae were transferred and cultured in blastocyst outgrowth medium for 3 days (Yagi et al., 2007). When available, EmbryoMax reagents from Millipore were used.

Genomic PCR

PCR primers for wild-type, lox and nullizygous *Tead4* alleles were used to genotype DNA isolated from mouse tails, cultured cells and individual embryos, as described (Kaneko et al., 2007; Yagi et al., 2007). Primers for the *CAG-cre/Esr1* allele were as described (Hayashi and McMahon, 2002). The wild-type *Tead4* allele amplifies best using Touchdown PCR (Korbie and Mattick, 2008): PCR reactions (50 μl) were incubated for 3 minutes at 95°C, followed by 15 cycles of 30 seconds at 95°C, 45 seconds at 66°C (temperature decreased 1°C per cycle) and 1 minute at 72°C, followed by 25 cycles of 30 seconds at 95°C, 45 seconds at 56°C and 1 minute at 72°C with a final extension of 7 minutes at 72°C.

RT-PCR

RNA was isolated from cells using RNeasy Mini Kit (Qiagen). Aliquots of 500 ng total RNA in 20 μl reactions were reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers. A 2 μl aliquot of cDNA was used for PCR amplification using gene-specific primers that span at least one intron (Kaneko et al., 1997; Kaneko et al., 2004; Yagi et al., 2007) (supplementary material Table S1). Individual embryos were lysed in 10 μl Cell Lysis Buffer (Ambion) for 10 minutes at 75°C. RT-PCR was performed on the entire sample, and a 2 μl aliquot of the resulting cDNA was used for PCR (Kaneko et al., 2004). To make sure that *Tead4* was not expressed in embryos identified as *Tead4*$^{-/-}$, Touchdown PCR was used with *Tead4* primers: PCR reactions (50 μl) were incubated for 3 minutes at 95°C, followed by 15 cycles of 30 seconds at 95°C, 45 seconds at 68°C (temperature decreased 1°C per cycle) and 1 minute at 72°C, followed by 25 cycles of 30 seconds at 95°C, 45 seconds at 58°C and 1 minute at 72°C with a final extension of 7 minutes at 72°C. Aliquots of 5-10 μl were analyzed as described (Kaneko et al., 2007).

Whole-mount immunostaining

Embryos were stained (Ralston and Rossant, 2008) using mouse anti-CDX2 (CDX-88, Biogenex; 1:200), rabbit anti-YAP1 (Vassilev et al., 2001) (1:400), Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen; 1:400) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen; 1:400). Stained embryos were mounted using ProLong Gold with DAPI (Invitrogen), and imaged and photographed using an Olympus FV1000 confocal microscope with 20× objective.

Mitochondrial staining

Cells cultured on glass coverslips were cultured for 30 minutes in the presence of 100 nM MitoTracker Red CMXRos (Invitrogen). Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed three times with PBS, and then cover slips were mounted onto glass slides using Prolong Gold Reagent with DAPI (Invitrogen) to stain nuclei. Cells were viewed with Nikon E600 epifluorescent photomicroscope (Vassilev et al., 2001). For JC-1 staining, live cells were stained according to the manufacturer's protocol (APO LOGIX JC-1 Kit, Cell Technologies) and viewed immediately after washing. Fluorescein and rhodamine signals were detected separately as well as simultaneously.

Immunocytochemistry

TSCs were grown on glass coverslips and in some cases induced to differentiate into trophoblast giant cells (TGCs) before being fixed in paraformaldehyde and stained with TROMA-1 and anti-p57 antibodies

(Ullah et al., 2008). Expression vectors were constructed from the pCI-vector (Promega) expressing N-terminal HA-tagged *Tead1*, *Tead2*, *Tead3* or *Tead4* (Vassilev et al., 2001). NIH3T3 fibroblasts (ATCC) were transfected with 10 ng to 1 μg of plasmid using Lipofectamine Plus according to the manufacturer's protocol (Invitrogen). Forty-eight hours post-transfection, mitochondria were stained with MitoTracker Red CMXRos and fixed as described above. Cells were permeabilized for 5 minutes at room temperature with 0.5% Triton X-100 and 10% calf serum in PBS (PBSS), washed four times with PBS, covered with monoclonal HA.11 antibody (clone 16B12; Covance) diluted 1:1000 in PBSS and incubated for 90 minutes at room temperature. Cells were washed three times with PBS and then covered with 1:1000 goat anti-mouse IgG Alexa Fluor 488 (Invitrogen) in PBSS for 90 minutes at room temperature. Cells were washed four times in PBS before mounting onto glass slides as described above then visualized with an epifluorescent microscope.

ROS assays

PMEFs were grown to near confluency, and 1 μM MHT or ethanol vehicle was added to fresh medium. Forty-eight hours later, cells were trypsinized and re-plated onto glass coverslips in 6-well dishes at 1×10^5 cells per well. Twenty-four hours later, cells were assayed for ROS using an Image-iT live ROS detection kit according to the manufacturer's instructions (Invitrogen). Control cells were treated with 100 μM tert-butyl hydrogen peroxide for 60 minutes prior to initiation of the assay. E3.5 embryos were flushed from the uterus into M2 media and immediately placed in 100 μl of 25 μM carboxy-H_2DCFDA in Hank's Buffered Saline+Ca^{+2}/Mg^{+2} that was pre-equilibrated for 3 hours in humidified 5% O_2/5% CO_2 incubator. After 30 minutes, the embryos were transferred to 10-20 μl Hank's Buffered Saline+Ca^{+2}/Mg^{+2} and visualized as described above.

RESULTS

Low O_2 promotes blastocoel formation in *Tead4*$^{-/-}$ embryos

Tead4$^{-/-}$ mouse embryos cultured in KSOM medium in atmospheric oxygen (21% O_2) arrest development prior to blastocoel formation (supplementary material Fig. S3A) (Nishioka et al., 2008). Reducing the O_2 level to 0.5% prevented even wild-type eight-cell embryos from developing past the morula stage (supplementary material Fig. S3B), because mouse preimplantation development requires some oxygen (Thomson, 1967). However, reducing the oxygen level in embryo cultures to 5% (the level *in utero*) has been reported to improve preimplantation development (Lawitts and Biggers, 1993; Erbach et al., 1994; Nagy et al., 2003). Therefore, we determined whether or not lowering O_2 levels to 5% in culture might improve the developmental potential of *Tead4*$^{-/-}$ embryos.

When two-cell embryos from a *Tead4*$^{+/-}$ mating were cultured at 5% O_2 in KSOM medium, all of the embryos developed past the morula stage by forming a blastocoel (Fig. 1A), including embryos subsequently genotyped as *Tead4*$^{-/-}$ (Fig. 1B). *Tead4*$^{-/-}$ embryos could become blastocysts even in the absence of *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ embryos because *Tead4*$^{-/-}$ embryos isolated from mating *Tead4*$^{-/-}$ adults [produced as previously described (Yagi et al., 2007)] still formed blastocoels (Fig. 2A,C). When developed under 5% O_2 in KSOM, ~90% (n=30/33) of *Tead4*$^{-/-}$ embryos isolated from either heterozygous or nullizygous matings formed a visible blastocoel (Table 1). Therefore, as blastocoel formation requires a functional TE, TE specification must have occurred in the absence of TEAD4.

TEAD4 is not essential for specification of the trophectoderm

TE specification is defined both by the ability of an embryo to produce a blastocoel, and by the expression of TE-associated genes.

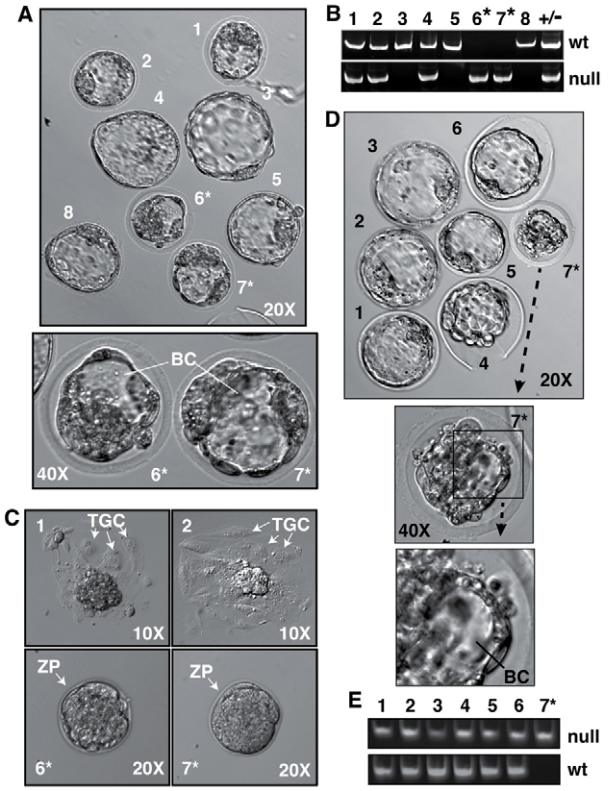

Fig. 1. *Tead4*$^{-/-}$ embryos developed a blastocoel either in 5% O_2 or in 21% O_2 with N-acetylcysteine. Two-cell embryos were isolated at E1.5 following *Tead4*$^{+/-}$ matings. (**A**) Embryos (numbered 1-8) from a single litter were cultured in KSOM and 5% O_2 for 4 days and then photographed under phase contrast. All of the embryos formed a blastocoel (BC). (**B**) Outgrowths from blastocysts 3, 5 and 8 were genotyped *Tead4*$^{+/+}$, 1, 2 and 4 were *Tead4*$^{+/-}$, and 6* and 7* were *Tead4*$^{-/-}$. *Tead4*$^{+/-}$ mouse tail DNA was used as control (+/–). (**C**) *Tead4*$^{+/+}$and *Tead4*$^{+/-}$ blastocysts produced outgrowths containing trophoblast giant cells (TGC), including blastocysts 1 and 2 (shown here), which had only partially expanded blastocoels at the time of transfer. *Tead4*$^{-/-}$ blastocysts lost their blastocoel and failed to hatch from their zona pellucida (ZP; 6* and 7*). (**D**,**E**) All of the embryos cultured in KSOM, 21% O_2 and N-acetylcysteine formed a blastocoel, including embryo 7* (D), which genotyped as *Tead4*$^{-/-}$ (E).

To determine whether or not *Tead4* was essential for TE specification, two-cell embryos from *Tead4*$^{-/-}$ matings (Yagi et al., 2007) were cultured in standard KSOM and 5% O_2 as described above, and individual embryos were assayed for TE-associated gene expression by RT-PCR. All of the embryos formed a blastocoel (Fig. 3A), despite the absence of *Tead4* transcripts (Fig. 3B). As expected, they also expressed *Atp1b1*, a subunit of the Na^+, K^+-ATPase that is essential for blastocoel expansion (Madan et al., 2007). Moreover, they all expressed *Oct4* (*Pou5f1* – Mouse Genome Informatics), a gene associated with pluripotent cells essential for ICM formation (Nichols et al., 1998). Remarkably, the TE-associated transcription factors *Cdx2* and *Gata3* were expressed in all but one embryo (Fig. 3B). Similar results were obtained when the same experiment was repeated using *Tead4*$^{+/-}$ matings (supplementary material Fig. S4). Whereas not every *Tead4*$^{-/-}$ embryo expressed either *Cdx2* or *Gata3*, some *Tead4*$^{-/-}$ embryos could express either or both genes. The same was true for other TE-associated genes, such as *Elf5* and *Eomes* (data not shown). Thus, *Tead4* expression was not essential for transcription of any of these genes.

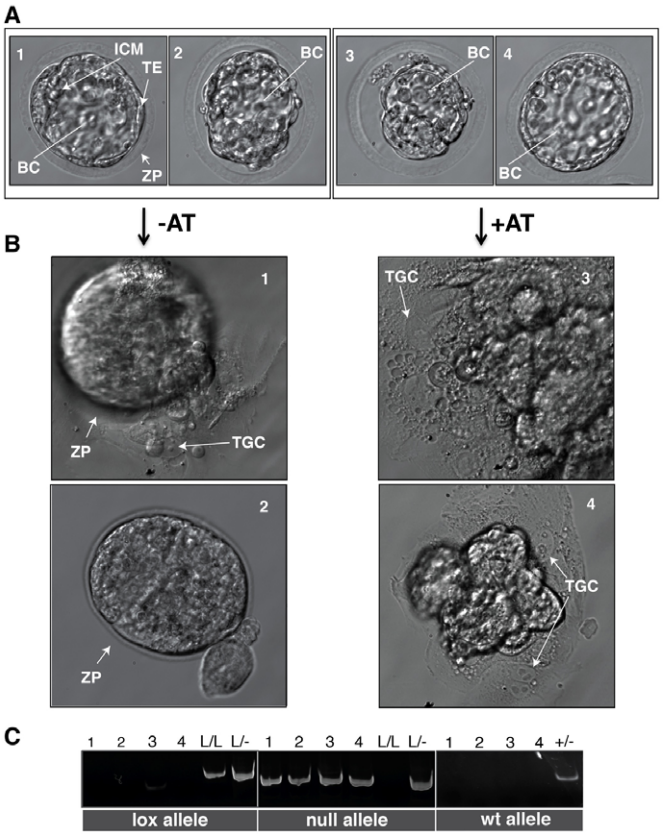

Fig. 2. *Tead4*$^{-/-}$ embryos developed a blastocoel in the absence of *Tead4*$^{+/+}$ or *Tead4*$^{+/-}$ embryos and could produce outgrowths. Two-cell embryos were isolated at E1.5 from *Tead4*$^{-/-}$ matings. (**A**) The six *Tead4*$^{-/-}$ embryos from a single litter were cultured for 4 days in KSOM and 5% O_2, as in Fig. 1A. All of them formed a blastocoel (BC). Trophectoderm (TE), inner cell mass (ICM) and zona pellucida (ZP) are indicated for four of the embryos (numbered 1-4). (**B**) Blastocysts 1 and 2 were transferred to gelatin-coated tissue culture plates and cultured in blastocyst outgrowth medium for 3 days. One blastocyst managed a partial outgrowth; one did not. Blastocysts 3 and 4 were treated with acidic Tyrode's solution (+AT) to remove their ZP prior to transfer. Both embryos produced an outgrowth. Trophoblast giant cells (TGC) are indicated. (**C**) Genotypes of blastocysts or outgrowths confirmed they were *Tead4*$^{-/-}$. Tail samples from *Tead4*$^{lox/lox}$ (L/L), *Tead4*$^{lox/-}$ (L/–) and *Tead4*$^{+/-}$ (+/–) were used as controls.

Immunostaining confirmed that CDX2 was expressed in *Tead4*$^{-/-}$ blastocysts that developed *in vitro* under conditions of low oxidative stress, as well as in *Tead4*$^{lox/lox}$ blastocysts isolated from pregnant females (Fig. 4; supplementary material Fig. S5). As expected, CDX2 was localized to the nuclei of the TE, but absent from the ICM. In both *Tead4*$^{-/-}$ and *Tead4*$^{lox/lox}$ blastocysts, the TEAD transcriptional co-activator YAP1 was localized to the cytoplasm of the ICM, and was present in both cytoplasm and nuclei of the TE, as previously reported (Hirate et al., 2012; Saha et al., 2012) (Fig. 4). By contrast, *Tead4*$^{-/-}$ embryos isolated from pregnant females at E3.5 contained neither a blastocoel cavity nor CDX2 protein, although they did express OCT4 protein (supplementary material Fig. S5, *in vivo* panels) (Yagi et al., 2007). Thus, a functional *Tead4* gene is not required either for the formation of a blastocoel or for the expression of TE-associated genes.

TEAD4 is essential *in vitro* when conditions mimic those *in vivo*

The studies described above revealed that the block to blastocoel formation in *Tead4*$^{-/-}$ embryos cultured in standard KSOM could be overcome simply by culturing the embryos in 5% O_2, the approximate O_2 concentration *in utero*. However, *Tead4*$^{-/-}$ embryos do not develop a blastocoel *in vivo*. Therefore, one or more of the other components *in utero* must prevent blastocoel development in the absence of TEAD4. To explore this hypothesis, components of the KSOM were altered while still maintaining the embryo culture in 5% O_2.

We first increased the glucose concentration in KSOM from 0.2 mM to 3.4 mM in order to match more closely the conditions *in utero* (Gardner and Leese, 1990). The increased glucose concentration, with or without glutamine (a normal KSOM component), still allowed *Tead4*$^{-/-}$ embryos to form a blastocoel (supplementary material Fig. S6A,B). We then investigated whether addition of essential amino acids (EAAs) to this formulation would prevent blastocoel formation in *Tead4*$^{-/-}$ embryos. Amino acids are normal components in the female reproductive tract, and addition of amino acids to KSOM augments embryonic development (Ho et al., 1995; Lane and Gardner, 1998; Nagy et al., 2003). Whereas adding EAA without glutamine allowed *Tead4*$^{-/-}$ blastocysts to form (supplementary material Fig. S6C), supplementing 3.4 mM glucose/KSOM with both glutamine and EAA prevented *Tead4*$^{-/-}$ eight-cell embryos from forming a blastocoel (supplementary material Fig. S7A,B). In fact, unlike *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ eight-cell embryos, which produced an expanded blastocoel within 24 hours, *Tead4*$^{-/-}$ embryos began to disintegrate by 48 hours, thereby mimicking the phenotype observed *in vivo*. Thus, *Tead4*$^{-/-}$ embryos failed to produce a blastocoel *in vitro* under conditions that most closely mimicked those *in vivo* (Table 1).

Conditions that promote oxidative stress prevent blastocoel formation in *Tead4*$^{-/-}$ embryos

Embryos normally upregulate oxygen consumption just prior to blastocoel formation in order to meet the energy demands of the trophectoderm's Na^+, K^+-ATPase pump (Houghton et al., 1996; Gardner, 1998; Johnson et al., 2003; Houghton, 2006). Therefore, TEAD4 might be essential only when embryos increase OXPHOS. For example, inclusion of amino acids in culture media, as described above (supplementary material Fig. S7), allows embryos to downregulate glycolysis and upregulate OXPHOS, thereby mimicking the metabolic energy pathway observed *in vivo* (Lane and Gardner, 1997; Gardner, 1998). Unfortunately, upregulating OXPHOS also upregulates ROS production, and the imbalance between ROS production and the ability of the cell to neutralize ROS defines the degree of 'oxidative stress' (Finkel and Holbrook, 2000). Therefore, one could determine whether or not *Tead4*$^{-/-}$ embryos were hypersensitive to oxidative stress by forcing embryos to upregulate OXPHOS while simultaneously suppressing their antioxidant defense mechanisms. This was accomplished by eliminating glucose from the culture media.

In the absence of glucose, embryos rely exclusively on OXPHOS instead of glycolysis for their energy supply, and the pentose phosphate shunt can no longer produce NADPH, a crucial component for antioxidant defense (Pandolfi et al., 1995; Dumollard et al., 2009; Jansen et al., 2009). Therefore, glucose-free conditions, which increase oxidative stress, would be expected to inhibit development of *Tead4*$^{-/-}$ embryos. Two-cell embryos from *Tead4*$^{+/-}$ matings were cultured in glucose-free KSOM media. By 48 hours, all of the embryos developed into either morulae or

Table 1. *Tead4*$^{-/-}$ embryos form a blastocoel under conditions that alleviate oxidative stress

% O_2	Glucose (mM)	Amino acids	Antioxidant	*Tead4* expressed	Blastocoel*
21	0.2	Gln	None	+	Yes** (14/14)
				–	No** (0/2)
			NAC	–	Yes (6/7)
5	0.2	Gln	None	+	Yes (22/22)
				–	Yes (30/33)
0.5	0.2	Gln	None	+	No (0/13)
5	0	None	None	+	Yes (4/4)
				–	No (2/10)
			NAC	–	Yes (6/6)
5	3.4	Gln + EAA	None	+	Yes (9/9)
				–	No (0/6)
			NAC	–	Yes (5/5)

All *in vitro* experiments used KSOM under the conditions indicated.
*Numbers in parentheses indicate the number of embryos with a blastocoel/the total number of embryos examined. For Tead4 (–) embryos, *Tead4*$^{-/-}$ embryos obtained from both heterozygous and nullizygous matings were combined. For Tead4 (+) embryos, *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ embryos were combined.
**Same conditions reported by Nishioka et al. (Nishioka et al., 2008).
EAA, essential amino acids; Gln, glutamine; NAC, N-acetylcysteine.

blastocysts (data not shown). However, by 72 hours, only those embryos with a functional *Tead4* allele developed a blastocoel (supplementary material Fig. S8A,B). By contrast, *Tead4*$^{-/-}$ embryos cultured in the absence of glucose failed to initiate blastocoel formation and began to disintegrate. Therefore, unlike *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ embryos, *Tead4*$^{-/-}$ embryos could not develop into blastocysts in the absence of glucose.

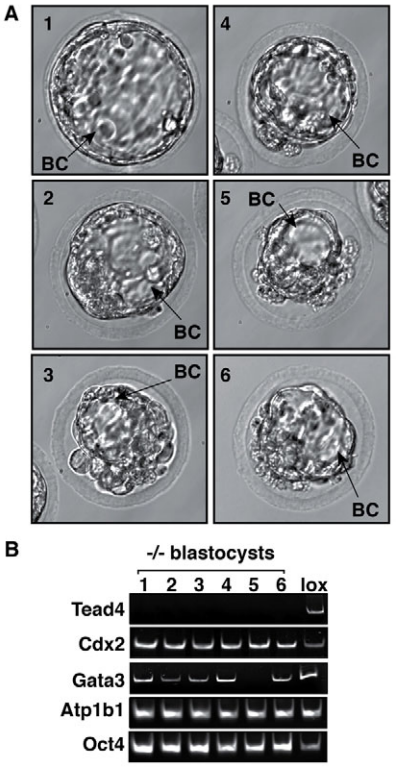

Fig. 3. Expression of TE-associated genes did not depend on TEAD4. (**A**) Six two-cell embryos (numbered 1-6) were isolated at E1.5 from *Tead4*$^{-/-}$ matings and cultured in KSOM and 5% O_2 for 4 days. Each embryo produced a blastocoel (BC). (**B**) RNA from individual embryos was assayed by RT-PCR for the presence or absence of the indicated genes. RNA from a *Tead4*$^{lox/lox}$ blastocyst was included as a positive control (lox), and H_2O was used in place of RNA to provide a negative control (not shown).

Reducing oxidative stress promotes blastocoel formation in *Tead4*$^{-/-}$ embryos

The results described above suggest that TEAD4 is essential for blastocoel formation and expansion, but only under conditions that prevent oxidative stress. To test this hypothesis, the well-characterized antioxidant N-acetylcysteine (NAC) (Zhang et al., 2011) was added to culture medium under conditions that otherwise would prevent blastocoel formation in *Tead4*$^{-/-}$ embryos (Table 1). NAC allowed *Tead4*$^{-/-}$ embryos to form a blastocoel when cultured in 21% O_2 (Fig. 1D,E). Similarly, whereas either glucose deprivation (supplementary material Fig. S8A,B) or addition of amino acids (supplementary material Fig. S7A,B) prevented *Tead4*$^{-/-}$ embryos from forming a blastocoel in 5% O_2, addition of NAC to these cultures allowed all of the *Tead4*$^{-/-}$ embryos to form a blastocoel (supplementary material Fig. S7C,D, Fig. S8C,D). These results confirmed that *Tead4* is essential for blastocoel formation and expansion under conditions expected to produce oxidative stress. Such conditions exist during the morula-to-blastocyst transition *in vivo* when O_2 consumption increases and embryos switch to glucose as their energy substrate (Benos and Balaban, 1983; Lane and Gardner, 1996; Leese et al., 2008). To determine whether or not *Tead4*$^{-/-}$ embryos that arrest development *in vivo* also have elevated levels of ROS, embryos from appropriate matings were isolated at E3.5 and assayed for the presence of ROS using a standard ROS-dependent fluorescence detection method (Brandt and Keston, 1965). These *Tead4*$^{-/-}$ embryos lacked a blastocoel (supplementary material Fig. S5C), whereas wild-type embryos isolated at E3.5 contained a well-defined blastocoel (Yagi et al., 2007). Compared with wild-type embryos, *Tead4*$^{-/-}$ embryos contained higher levels of ROS, consistent with higher levels of oxidative stress *in utero* (Fig. 5A). Thus, *Tead4*$^{-/-}$ embryos cannot maintain energy homeostasis *in vivo*, resulting in oxidative stress and developmental arrest.

TEAD4 prevents accumulation of excess ROS in embryonic cells

To determine whether or not TEAD4 is directly responsible for preventing excess ROS production in embryonic cells, *Tead4*$^{lox/lox}$ PMEFs and *Tead4*$^{-/-}$ PMEFs were constructed with a monohydroxytamoxifen (MHT)-inducible Cre recombinase system (CAG-Cre/*Esr1*$^{Tg/+}$; supplementary material Fig. S2) and assayed for ROS as above. Addition of tert-butyl hydrogen peroxide (TBHP) to these cells induced a robust fluorescent signal in all of the cells,

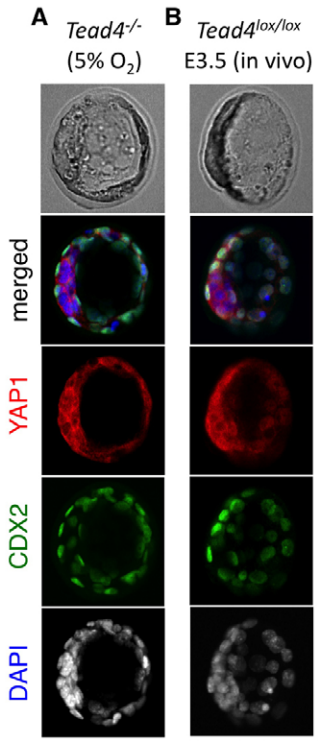

Fig. 4. *Tead4*$^{-/-}$ embryos that developed in KSOM and 5% O_2 expressed nuclear CDX2 and YAP1 in TE cells. (**A**) *Tead4*$^{-/-}$ males were mated to *Tead4*$^{lox/lox}$; *ZP3-Cre*$^{Tg/+}$ females. Seven *Tead4*$^{-/-}$ embryos were isolated from a single female at E2.5, cultured in KSOM and 5% O_2 for 48 hours, then immunostained for YAP1 (red) and CDX2 (green), and stained for nuclear DNA with DAPI (blue in merged microphotograph, gray in gray scale microphotograph). Six of the embryos developed blastocoels (representative example is shown). (**B**) Blastocysts were isolated from *Tead4*$^{lox/lox}$ mating by flushing the uterus at E3.5, fixed immediately and stained as above. All seven of the recovered embryos expressed YAP1 in cytoplasm of the ICM but nuclear YAP1 and CDX2 in the TE as previously reported (Hirate et al., 2012; Saha et al., 2012). A representative example is shown. Microphotographs of transmitted light images are shown at the top of each panel.

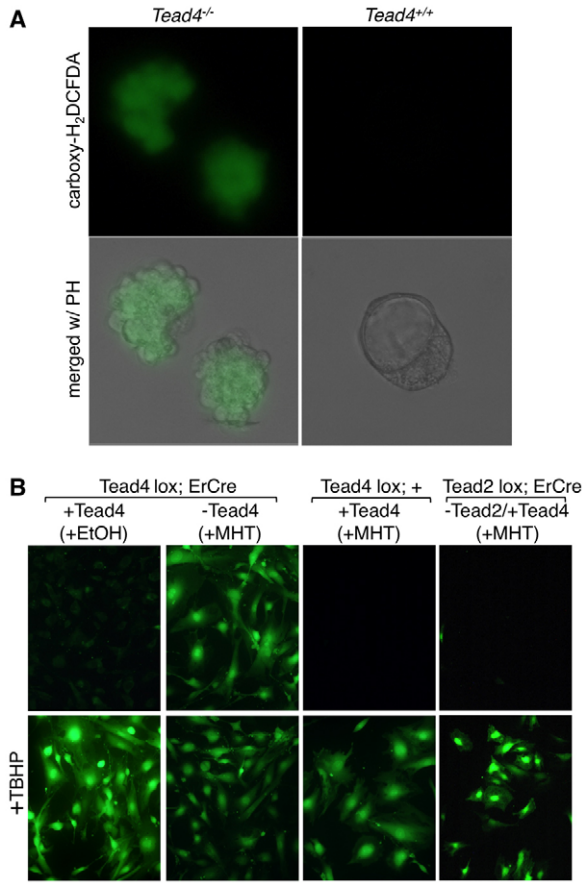

Fig. 5. TEAD4 prevents ROS accumulation in embryos and embryonic cells. (**A**) *Tead4*$^{-/-}$ males were mated to *Tead4*$^{lox/lox}$; *ZP3-Cre*$^{Tg/+}$ females, and embryos were isolated at E3.5 and immediately stained with carboxy-H_2DCFDA, a fluorogenic marker for ROS (*Tead4*$^{-/-}$ panels). Top panel is an epifluorescent photomicrograph. Bottom panel is an epifluorescent photomicrograph merged with a phase contrast photograph (PH). As a control, the same experiment was carried out in parallel with *Tead4*$^{+/+}$ males mated to *Tead4*$^{+/+}$ females (*Tead4*$^{+/+}$ panels). All images were photographed under identical conditions. (Five *Tead4*$^{-/-}$ embryos were examined and all showed increased ROS accumulation.) (**B**) *Tead4*$^{lox/lox}$ primary mouse embryonic fibroblasts (PMEFs) with (Tead4 lox; ErCre) or without (Tead4 lox; +) the *CAG-cre/Esr1*^{Tg} allele were cultured for 48 hours in the presence of either monohydroxytamoxifen (+MHT) or ethanol (+EtOH). MHT converted cells containing both *CAG-cre/Esr1*^{Tg} and *Tead4 lox* alleles into *Tead4*$^{-/-}$ PMEFs (data not shown). Cells were then replated in normal culture medium. Twenty-four hours later, control cells were treated with tert-butyl hydrogen peroxide for 1 hour (+TBHP), and then all cells were stained with carboxy-H_2DCFDA (green). The same protocol was applied to PMEFs containing *Tead2*$^{lox/lox}$ and *CAG-cre/Esr1*$^{Tg/+}$ alleles (Tead2 lox; ErCre). Whereas all of the cells showed increased levels of ROS when treated with TBHP, only MHT-treated *Tead4*$^{lox/lox}$; *CAG-cre/Esr1*$^{Tg/+}$ PMEFs showed elevated levels of ROS in the absence of TBHP.

regardless of the presence or absence of a *Tead4* allele (Fig. 5B, +TBHP). In the absence of TBHP, ROS overproduction was detected only in *Tead4*$^{-/-}$ cells (Fig. 5B). To eliminate the possibility that ROS overproduction resulted from pleiotropic effects by Cre activation, the same experiment was repeated using *Tead2*$^{lox/lox}$ PMEFs that contain the same Cre allele. *Tead4* and *Tead2* are both expressed during preimplantation development, but *Tead2* cannot compensate for *Tead4* ablation (Yagi et al., 2007). *Tead2*$^{-/-}$ PMEFs that were produced by treatment with MHT did not accumulate ROS (Fig. 5B). Therefore, accumulation of ROS resulted specifically from the absence of *Tead4*.

TEAD4 is essential for efficient blastocoel expansion, but not for hatching or formation of trophoblast giant cells

ROS production occurs primarily, although not exclusively, in the electron transport chain within mitochondria, suggesting that TEAD4 might contribute to mitochondrial energy homeostasis. In fact, during blastocoel formation, OXPHOS occurs primarily in TE. Therefore, if TEAD4 mediated mitochondrial energy homeostasis, *Tead4*$^{-/-}$ embryos would be expected to have difficulty in blastocoel expansion. The blastocyst outgrowth assay is an *in vitro* assay for examining the ability of blastocysts to hatch from zona pellucida and form TE-derived trophoblast giant cells (TGCs) (Armant, 2006; Yagi et al., 2007). When transferred to blastocyst outgrowth medium, most of the *Tead4*$^{+/+}$ and *Tead4*$^{+/-}$ blastocysts fully expanded their blastocoel, hatched, and generated outgrowths that contained TGCs (Fig. 1C; supplementary material Fig. S9A,B, left-hand panel). By contrast, expansion of the blastocoel *in vitro* was inefficient in most *Tead4*$^{-/-}$ embryos, which resulted in partial or

failed hatchings (Fig. 1C; Fig. 2B, –AT; supplementary material Fig. S9A,B, right-hand panel). Nevertheless, all of the *Tead4*$^{-/-}$ blastocysts were capable of forming TE-derived outgrowths, because partially and fully hatched embryos (Fig. 2B, embryo 1; supplementary material Fig. S9A,B, center panel) as well as those for which zona pellucida was removed artificially (Fig. 2B, +AT, embryos 3 and 4; data not shown) attached and produced outgrowths with TGCs. These results revealed that TEAD4 facilitates blastocoel expansion, but that *Tead4*$^{-/-}$ blastocysts that developed under low oxidative stress remain capable of hatching and forming TGCs.

TEAD4 is not essential for proliferation and differentiation of trophoblast cells

The results described above imply that TEAD4 is not essential for either proliferation or differentiation of trophoblast stem cells (TSCs). To test this hypothesis, TSCs containing conditional *Tead4* alleles and a CAG-Cre/*Esr1*^{Tg} (described above for PMEFs) were derived from blastocysts. Addition of MHT to *Tead4*$^{lox/lox}$; CAG-Cre/*Esr1*$^{Tg/+}$ TSCs eliminated the *lox* allele and increased the null allele with concomitant downregulation of *Tead4* mRNA (supplementary material Fig. S10A). However, no difference was detected in the proliferation of either *Tead4*$^{-/-}$ or *Tead4*$^{lox/lox}$ TSCs (Fig. 6A). Furthermore, ablation of *Tead4* in TSCs neither induced expression of genes associated with embryonic stem cells, nor suppressed genes normally expressed in TSCs. For example, ablation of *Tead4* neither activated expression of *Oct4* (supplementary material Fig. S10B), a genetic marker for embryonic stem cells, nor suppressed expression of *Cdx2*, *Eomes*, *Elf5* or *p57* (*Cdkn1c* – Mouse Genome Informatics) mRNA (supplementary material Fig. S10B,C). Moreover, ablation of *Tead4* in TSCs still allowed *Tead4*$^{-/-}$ TSC to form TGCs that expressed cytokeratin 8 (Krt8; also known as endo-A protein) with giant nuclei that contained p57 protein (Fig. 6B; supplementary material Fig. S10D,E), as previously reported (Ullah et al., 2008). Thus, TEAD4 is not essential for proliferation and differentiation of trophoblast cells.

TEAD4 supports mitochondrial energy homeostasis

The results described above suggested that TEAD4 provides important functions in trophoblast cell biology in more subtle ways, such as maintaining mitochondrial energy homeostasis. To explore this hypothesis further, TGCs were stained with Mitotracker Red, a dye that accumulates specifically in intact mitochondria (Macho et al., 1996). However, no distinguishable difference was detected between the staining patterns in *Tead4*$^{lox/lox}$ and *Tead4*$^{-/-}$ TGCs (Fig. 7A), revealing that cells that lacked TEAD4 still contained mitochondria. However, TGCs was also stained with JC-1, a dye that also accumulates specifically in mitochondria, but one that is sensitive to the membrane potential of the mitochondria (Reers et al., 1995; Mathur et al., 2000). JC-1 stains mitochondria with a strong membrane potential red, and mitochondria with a weak membrane potential green. Simultaneous detection of these fluorescent signals showed a large reduction in active mitochondria in *Tead4*$^{-/-}$ TGCs (Fig. 7B). These results revealed that TEAD4 is essential in differentiated trophoblast cells for maintaining mitochondrial homeostasis.

TEAD4 can localize to both mitochondria and nuclei

Some nuclear transcription factors, such as STAT3 and estrogen receptor, localize to the mitochondria as well as to the nucleus (Yang et al., 2004; Gough et al., 2009). To determine whether or not the same might be true for the TEAD4, NIH3T3 fibroblasts were transiently transfected with a hemagglutinin (HA)-tagged *Tead4* expression vector, and the cells stained with both anti-HA antibody and Mitotracker Red. As expected, nuclei of transfected cells stained darkly with anti-HA antibody and mitochondria with Mitotracker Red (data not shown). However, in the cytoplasm of some transfected cells (~30%), HA staining clearly colocalized with Mitotracker Red staining, revealing that some HA-TEAD4 localized to the mitochondria as well as to the nucleus (supplementary material Fig. S11A). Cytoplasmic localization of ectopically expressed TEAD4 did not result from accumulation in the endoplasmic reticulum, because co-transfection of expression vectors for HA-TEAD4 and DsRed2-ER, a fluorescent protein that

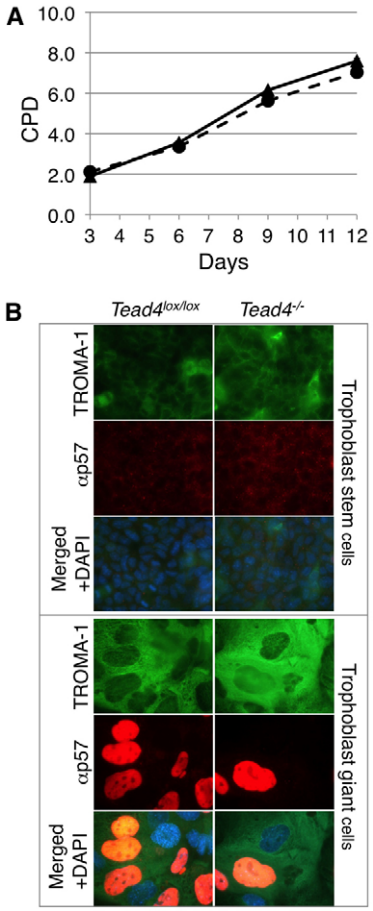

Fig. 6. TEAD4 was not required for trophoblast cells to proliferate or differentiate. *Tead4*$^{lox/lox}$; *CAG-cre/Esr1*$^{Tg/+}$ TSCs were cultured with either monohydroxytamoxifen or ethanol to produce *Tead4*$^{-/-}$ or *Tead4*$^{lox/lox}$ TSCs, respectively (supplementary material Fig. S10). TSCs were then induced to differentiate into TGCs by FGF4 deprivation for 5 days. (**A**) *Tead4*$^{lox/lox}$ (triangles) or *Tead4*$^{-/-}$ (circles) TSCs (3×10^5 cells) were plated and cultured for 3 days in TSC media without MHT, harvested and counted. This was repeated for 12 days. Cumulative population doubling (CPD) was plotted on the *y*-axis. Population doubling was calculated using the formula $\log(N_0/N_3)/\log 2$, where N_0 and N_3 denote initial number of cells plated and number of cells at day 3, respectively. (**B**) *Tead4*$^{lox/lox}$ or *Tead4*$^{-/-}$ TSCs (top panel) or TGCs (bottom panel) were stained using antibodies against a TE-specific antigen (TROMA-1, green) or a TGC-specific antigen (anti-p57, red). Merged photos include images in which nuclear DNA was stained with DAPI.

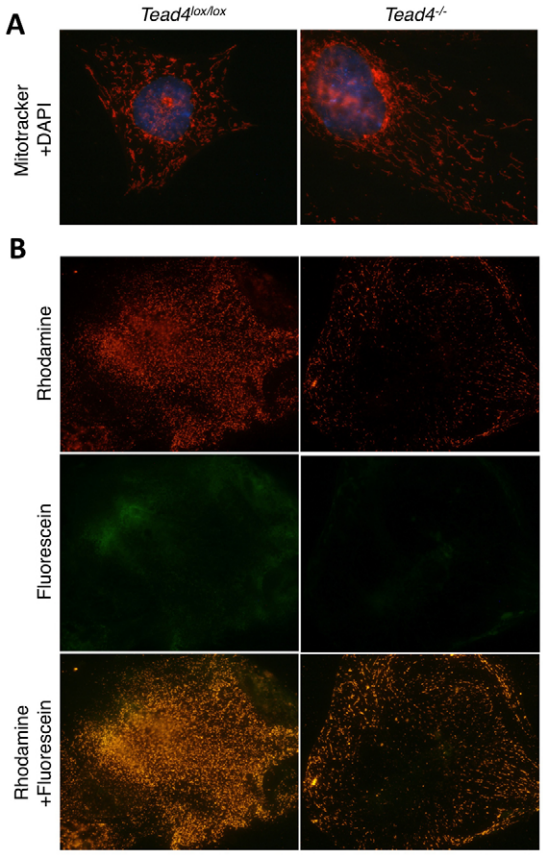

Fig. 7. TEAD4 maintains a high mitochondrial membrane potential. *Tead4*$^{-/-}$ or *Tead4*$^{lox/lox}$ TGCs were produced as described in Fig. 6 legend. (**A**) TGCs were stained with Mitotracker Red to visualize their mitochondria (red), and then the cells were fixed and stained with DAPI to visualize their nuclear DNA (blue). (**B**) Living TGCs were stained with JC-1 to distinguish mitochondria with a high membrane potential (rhodamine filter, top panels) from those with a low membrane potential (fluorescein filter, middle panels). Simultaneous collection of both red and green fluorescence is shown in bottom panels. Representative TGCs are shown.

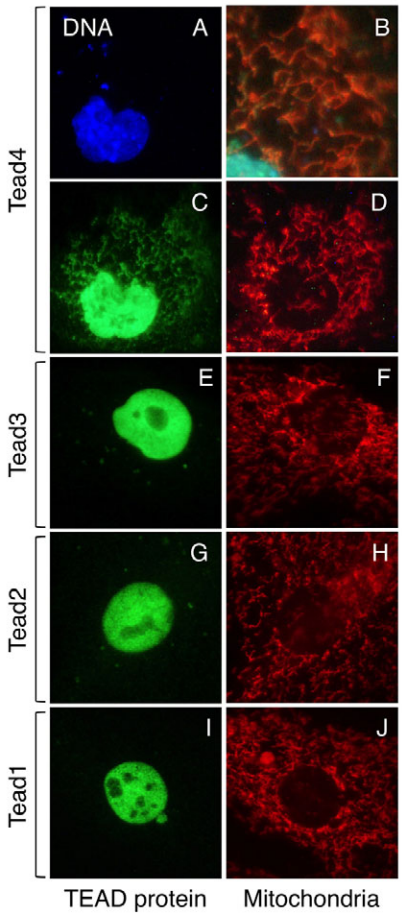

Fig. 8. TEAD4 localizes to mitochondria as well as to nuclei, whereas TEAD1, TEAD2 and TEAD3 localize only to nuclei. (**A-J**) The indicated hemagglutinin epitope (HA)-tagged TEAD protein was expressed transiently in PMEFs. Forty-eight hours post-transfection, cells were stained with Mitotracker Red, then fixed and stained with anti-HA antibody (green). Cells were also stained with DAPI to visualize nuclear DNA (blue). TEAD4 (A-D) localized to both the nucleus and the mitochondria in same cell. Superimposition of C and D confirmed colocalization of TEAD4 (green) and mitochondria (red) that appear yellow in the enlarged section (B). By contrast, none of the cells transfected with TEAD1 (I,J), TEAD2 (G,H) or TEAD3 (E,F) showed mitochondrial localization of these HA-tagged proteins.

localizes to the endoplasmic reticulum, did not produce a staining pattern similar to that observed with HA-TEAD4 and Mitotracker Red (supplementary material Fig. S11B).

To determine whether or not TEAD4 was the only one of the four TEAD transcription factors that could colocalize with mitochondria, HA-tagged expression vectors containing all four TEAD members were individually transfected into PMEFs and stained with HA-antibody and Mitotracker Red. Although all four TEAD proteins localized to the nucleus, as expected, only TEAD4 could localize to both nucleus and the mitochondria (Fig. 8). This unique ability of TEAD4 to localize to the mitochondria could account, at least in part, for the fact that *Tead4* is the only TEAD gene that is essential for preimplantation development.

DISCUSSION

The results presented here reveal that the reason *Tead4* is essential for blastocyst development *in vivo* is because *Tead4* is required to establish the energy homeostasis essential for blastocoel formation and expansion. Several lines of evidence support this conclusion. First, the inability of *Tead4*$^{-/-}$ embryos to form a blastocoel *in vivo* (Yagi et al., 2007) can be recapitulated *in vitro* (supplementary material Fig. S3A) (Nishioka et al., 2008). However, we were able to alter *in vitro* conditions that allow these embryos to form a blastocoel. Culture conditions that reduced oxidative stress facilitated blastocoel formation in *Tead4*$^{-/-}$ embryos whereas conditions that increased oxidative stress suppressed blastocoel formation in *Tead4*$^{-/-}$ embryos (Table 1). As expected, conditions that most closely mimicked those *in vivo* required either *Tead4* or antioxidant supplementation. In fact, embryos and cells that lacked *Tead4* had elevated levels of ROS. Conversely, suppressing ROS production rescued blastocoel formation in *Tead4*$^{-/-}$ embryos cultured under conditions that otherwise prevented blastocoel formation. These results confirm earlier studies that the primary block to preimplantation development is oxidative stress (Favetta et al., 2007; Dumollard et al., 2009). TEAD4 is the first regulatory gene that has been shown to link preimplantation development to energy homeostasis.

Trophectoderm specification

Tead4 was initially proposed to specify the TE lineage, because *Tead4*$^{-/-}$ embryos did not form a blastocoel and did not express crucial TE-associated genes. However, a blastocoel can form without expression of TE-associated genes, such as *Cdx2*, *Gata3* and *Eomes* (Pandolfi et al., 1995; Arman et al., 1998; Russ et al., 2000; Strumpf et al., 2005; Ralston et al., 2010; Kohn et al., 2011; Blij et al., 2012). Because blastocoel formation depends on TE, expression of these genes is not essential for TE specification. Similarly, *Tead4* is not essential for TE specification, because *Tead4*$^{-/-}$ embryos could form a blastocoel. Furthermore, *Tead4* was not essential for expression of other TE-associated genes, although some *Tead4*$^{-/-}$ embryos failed to express a subset of these genes (Fig. 3; supplementary material Fig. S4). In fact, Nishioka et al. (Nishioka et al., 2008) detected CDX2 protein as well as small blastocoel-like cavities in some *Tead4*$^{-/-}$ embryos that developed *in vitro*, whereas Yagi et al. (Yagi et al., 2007) did not make the same observation *in vivo*. By contrast, Yagi and co-workers observed expression of *Eomes* and *FGFR2* in *Tead4*$^{-/-}$ embryos, whereas Nishioka and co-workers did not. These inconsistencies can be explained by the fact that *Tead4* is not essential for TE specification, but environmental conditions determine the efficiency and extent of TE function. Thus, even *in utero*, initiation of TE specification can take place without TEAD4, but these embryos subsequently succumb to developmental failure due to oxidative stress (Betts and Madan, 2008). Whether or not *Tead4*$^{-/-}$ blastocysts that developed under low oxidative stress can implant and form a functional placenta remains to be determined.

Variability in morphology and gene expression patterns (Fig. 3; supplementary material Figs S4, S5) among a population of *Tead4*$^{-/-}$ blastocysts probably reflects differences in the ability of individual embryos to manage oxidative stress. Such differential sensitivity of individual wild-type embryos to oxidative stress has been attributed, at least in part, to the gene dosage of X-linked glucose 6-phosphate dehydrogenase, an enzyme crucial for relieving oxidative stress (Pérez-Crespo et al., 2005). Furthermore, a commonly used substrain of C57BL mice (6J substrain) contains a mutation in nicotinamide nucleotide transhydrogenase, a genetic modifier that mediates oxidative stress (Huang et al., 2006). Thus, discrepancies in the phenotype observed by siRNA suppression of genes could result from differences in the ability of individual embryos with differing genetic backgrounds to tolerate *in vitro* conditions that induce oxidative stress (Jedrusik et al., 2010; Wu et al., 2010; Blij et al., 2012). Our results suggest that care must be taken in interpretation of *in vitro* culture manipulations.

Energy homeostasis

Cleavage-stage embryos do not increase biomass and rely primarily on low-level OXPHOS using pyrvuate and lactate as energy sources (supplementary material Fig. S1) (Leese et al., 2008). At the eight-cell stage, *Tead4* RNA appears for the first time (Yagi et al., 2007). At the morula stage, embryos abruptly upregulate glucose and O_2 consumption resulting in glucose oxidation as the primary energy source (Gardner, 1998; Leese et al., 2008). Coincident with differentiation of the TE and ICM, two distinct energy pathways appear (Gopichandran and Leese, 2003; Houghton, 2006). The ICM uses aerobic glycolysis to drive cell proliferation and increase its biomass (Hewitson et al., 1996; Vander Heiden et al., 2009). The TE uses OXPHOS to efficiently drive blastocoel expansion, a prerequisite for embryo implantation during the narrow window of uterine receptivity (Dey et al., 2004). Thus, the TE has much higher density of active mitochondria than the ICM (Stern et al., 1971; Houghton, 2006). Therefore, mediating oxidative stress is more important for TE function than for ICM function.

The ability of *Tead4*$^{-/-}$ embryos to form a blastocoel *in vitro* at 5% O_2 was suppressed when KSOM was altered in two ways: (1) increasing glucose concentration to match the *in vivo* concentration and (2) including EAA and glutamine (Table 1; supplementary material Fig. S7). Whereas lowering O_2 from 21% to 5% reduces oxidative stress, culturing embryos in 5% O_2 actually increases their uptake of both glucose and amino acids, effectively increasing the ratio of OXPHOS to glycolysis (Wale and Gardner, 2012). Interestingly, EAA and glutamine have additive effects on protein synthesis and metabolism (Nicklin et al., 2009; Sengupta et al., 2010; Wise and Thompson, 2010). During blastocyst formation, these components would be expected to promote energy supply (OXPHOS) as well as demand (biosynthesis and Na^+, K^+-ATPase). Under these *in vitro* conditions, *Tead4*$^{-/-}$ embryos arrest development, as they do *in utero*, because these amino acids force them to upregulate OXPHOS for energy (Lane and Gardner, 1996; Lane and Gardner, 1998), albeit inefficiently, without decreasing energy demand. Thus, TEAD4 is essential for balancing metabolic supply and demand during blastocoel formation *in utero*.

A role for TEAD4 during OXPHOS

Although both *Tead4* and *Cdx2* affect blastocoel expansion and embryo hatching, the block to blastocoel formation *in vivo* is more severe in *Tead4*$^{-/-}$ embryos (Yagi et al., 2007) than in *Cdx2*$^{-/-}$ embryos (Strumpf et al., 2005). Nevertheless, *Cdx2*-deficient embryos also exhibit low mitochondrial activity (Wu et al., 2010), suggesting that *Cdx2* also mediates OXPHOS required for blastocoel expansion. Interestingly, *Tead4*$^{-/-}$ blastocysts that lacked *Cdx2* expression exhibited a more severe phenotype *in vitro* than those that expressed *Cdx2* (supplementary material Fig. S4). Thus, *Tead4* and *Cdx2* might play complementary roles that mediate mitochondrial function.

Among the four mammalian TEAD proteins, TEAD4 plays a unique, as yet undefined, role in preventing excess accumulation of ROS during OXPHOS. *Tead4*$^{-/-}$ embryos still expressed genes intimately involved in ROS alleviation, such as superoxide dismutase 1 and 2 and glucose 6-phosphate dehydrogenase (data not shown), and the knockout mice that prevent development beyond the morula stage are not linked to mitochondrial function per se (Kohn et al., 2011). However, the unexpected finding that ectopically expressed TEAD4, but not TEAD1, TEAD2 or TEAD3, can localize to the mitochondria strongly suggests that TEAD4 might directly interact with components within mitochondria. This finding is consistent with the recent report that TEAD4 localizes to the cytoplasm as well as to the nucleus in embryos and in primary cells derived from embryos (Home et al., 2012; Saha et al., 2012).

Hippo signaling pathway

The Hippo signaling pathway affects transcription by regulating subcellular localization of YAP1, a co-activator capable of interacting with at least 15 transcription factors, including TEAD4 (Wang et al., 2009). Some studies suggest that differential activation of the Hippo signaling pathway allows TEAD4 to function only in the outer blastomeres (Nishioka et al., 2009; Hirate et al., 2012), whereas other studies contradict this hypothesis (Home et al., 2012; Saha et al., 2012). The Hippo signaling pathway has also been linked directly to mitochondrial function (Nagaraj et al., 2012). Thus, selectively activating TEAD4-YAP1 complexes only in the TE would ensure efficient blastocoel expansion. Whether this occurs by regulating expression of nuclear genes that affect

mitochondrial function, or by affecting mitochondrial activities directly remains to be determined. *Tead1*, *Tead2* and *Tead4* are all expressed during preimplantation development (Kaneko et al., 1997; Yagi et al., 2007; Nishioka et al., 2008) and they can all mediate Yap1/Hippo function (Vassilev et al., 2001; Wu et al., 2008). Thus, the unique ability of TEAD4 to localize to the mitochondria could explain why other TEAD proteins cannot compensate for its preimplantation-lethal phenotype (Yagi et al., 2007; Nishioka et al., 2008).

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
K.J.K. designed and performed the experiments and carried out the data analyses. K.J.K. and M.L.D. prepared the figures, wrote and edited the manuscript.

Supplementary material
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