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Transcription factor AP-2γ is a core regulator of tight junction biogenesis and cavity formation during mouse early embryogenesis

Inchul Choi[1], Timothy S. Carey[2], Catherine A. Wilson[1] and Jason G. Knott[1,2,*]

SUMMARY

The trophectoderm epithelium is the first differentiated cell layer to arise during mammalian development. Blastocyst formation requires the proper expression and localization of tight junction, polarity, ion gradient and H_2O channel proteins in the outer cell membranes. However, the underlying transcriptional mechanisms that control their expression are largely unknown. Here, we report that transcription factor AP-2γ (Tcfap2c) is a core regulator of blastocyst formation in mice. Bioinformatics, chromatin immunoprecipitation and transcriptional analysis revealed that Tcfap2c binds and regulates a diverse group of genes expressed during blastocyst formation. RNA interference experiments demonstrated that Tcfap2c regulates genes important for tight junctions, cell polarity and fluid accumulation. Functional and ultrastructural studies revealed that Tcfap2c is necessary for tight junction assembly and paracellular sealing in trophectoderm epithelium. Aggregation of control eight-cell embryos with Tcfap2c knockdown embryos rescued blastocyst formation via direct contribution to the trophectoderm epithelium. Finally, we found that Tcfap2c promotes cellular proliferation via direct repression of *p21* transcription during the morula-to-blastocyst transition. We propose a model in which Tcfap2c acts in a hierarchy to facilitate blastocyst formation through transcriptional regulation of core genes involved in tight junction assembly, fluid accumulation and cellular proliferation.

KEY WORDS: Blastocyst formation, Tight junction, Tcfap2c, Tfap2c

INTRODUCTION

The blastocyst trophectoderm (TE) epithelium is the first differentiated cell layer to emerge during mammalian development. A key function of the developing TE layer is to act as a barrier and to regulate the exchange and accumulation of small molecules and fluid during blastocoel formation (Cockburn and Rossant, 2010). This property is mediated primarily by the action of tight junction (TJ) complexes, ion gradient pumps and H_2O channels that assemble on the apical and basolateral membranes of the outer cell layer. Members of several gene families are important for blastocyst formation, including claudins, zonal occludins (TJ proteins), occludins, jam proteins, cell polarity proteins, Na/K-ATPases and aquaporins. The proper expression and localization of these gene products is crucial for blastocyst development (Watson and Barcroft, 2001; Eckert and Fleming, 2008). However, the underlying transcriptional mechanisms that control the spatial and temporal expression of these genes are largely unknown.

Recent studies established a role for developmental transcription factors (TFs) such as Oct4 (Pou5f1 – Mouse Genome Informatics), Nanog, Sox2, Tead4, Gata3, Cdx2, Eomes and Elf5 in cell fate specification, pluripotency and trophoblast development (Nichols et al., 1998; Russ et al., 2000; Mitsui et al., 2003; Niwa et al., 2005; Strumpf et al., 2005; Dietrich and Hiiragi, 2007; Yagi et al., 2007; Ng et al., 2008; Nishioka et al., 2008; Parfitt and Zernicka-Goetz, 2010; Ralston et al., 2010). Interestingly, many of these TFs are not essential for blastocyst formation per se, but are necessary for implantation or postimplantation development. Functional studies uncovered a potential dual role for a subset of these TFs in blastocyst formation and cell fate specification. For example, ablation of *Tead4*, *Gata3* or *Sox2* causes developmental arrest at the morula stage and defects in cell fate specification (Nishioka et al., 2008; Home et al., 2009; Keramari et al., 2010). Nonetheless, their precise role in the cellular processes associated with blastocyst formation is unknown. Thus, a fundamental question persists: which developmental TFs directly govern the cellular processes associated with blastocyst formation in mammals (i.e. TJ and fluid accumulation)?

One such TF is AP-2γ (Tcfap2c; Tfap2c – Mouse Genome Informatics), a DNA-binding protein that acts as both an activator and repressor of gene transcription (Eckert et al., 2005). Tcfap2c is expressed in both the oocyte and preimplantation embryo (Winger et al., 2006). During blastocyst formation, Tcfap2c is enriched in the TE lineage (Kuckenberg et al., 2010). Loss of zygotic Tcfap2c results in abnormal placental development and embryonic lethality between days 7.5 and 8.5 (Auman et al., 2002; Winger et al., 2006). To test whether maternally derived Tcfap2c was required for preimplantation development, Winger et al. (Winger et al., 2006) crossed mice with a *ZP3-Cre* transgene and floxed *Tcfap2c* allele to generate female mice lacking Tcfap2c in their oocytes. Subsequently, two of these females were mated with *Tcfap2c*-null males to generate embryos that lacked both maternal and zygotic *Tcfap2c* and no preimplantation phenotype was observed. However, the results of this experiment are inconclusive because only seven preimplantation embryos from a total of two mice were evaluated and studies to confirm that Tcfap2c protein was effectively ablated using this approach were not performed. Therefore, we further investigated whether Tcfap2c is required for preimplantation development.

[1]Developmental Epigenetics Laboratory, Department of Animal Science, and [2]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA.

*Author for correspondence (knottj@msu.edu)

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Here, we report that Tcfap2c is essential for the morula-to-blastocyst transition in mouse preimplantation embryos. An RNAi approach was employed to effectively ablate maternally and zygotically derived Tcfap2c protein. Using a combination of bioinformatics and transcriptional analysis, we found that Tcfap2c is required for the proper expression of a diverse family of genes involved in cell polarity, TJ biogenesis, fluid accumulation and cellular proliferation. Furthermore, functional and ultrastructural analyses revealed that Tcfap2c is obligatory for the formation of TJ complexes and paracellular sealing in the TE epithelium crucial to blastocyst formation. To our knowledge, this is the first study to describe the function of a single TF in controlling TJ assembly, fluid accumulation and cell proliferation in early mammalian embryogenesis.

MATERIALS AND METHODS

Embryo collection and microinjection

Mouse embryos were derived from superovulated B6D2/F1 females mated with B6D2/F1 males (Charles River Laboratories, Wilmington, MA, USA). Fertilized one-cell embryos were collected at 16-17 hours post-human chorionic gonadotropin treatment (hph) and then cultured in modified KSOM medium (EMD Millipore, Billerica, MA, USA) under mineral oil at 37°C in a humidified atmosphere of 5% O_2, 5% CO_2, 90% N_2. Microinjection was carried out as described previously (Wang et al., 2010). Briefly, 5-10 pl 100 μM *Tcfap2c* siRNA (siGenome and On-target plus; Dharmacon, Lafayette, CO, USA), 100 μM *p21* siRNA (Dharmacon), 100 μM control siRNA (Dharmacon) or 0.7 μg/μl *Tcfap2c* mRNA was injected into the cytoplasm of one-cell zygote embryos using a PL100 picoinjector (Harvard Apparatus, Hollistan, MA, USA) at 19-21 hph. Animal care was in accordance with the institutional guidelines of Michigan State University.

siRNA sequences

siRNA sequences (5′-3′) were as follows. *Tcfap2c* (pool 1): AAGCUGAGUUCCCUAGUAA, GCACGGGACUUCGCCUAUG, AGCGGUGGCUGACUAUUUA and CCGCAGUGCAGAAUUAUAU. *Tcfap2c* (pool 2): UGAAAGGUGCUACGAGUUU, CAGAUAAAGGGAUCGAUCA, GCAAAGGACCCAUUUCGAU and UGAGAAAUGGGAUUCGAUU. *p21*: CGAGAACGGUGGAACUUUG, GAACAUCUCAGGGCCGAAA, GGAGCAAAGUGUGCCGUUG and GGUGAUGUCCGACCUGUUC. Control: UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, AUGAACGUGAAUUGCUCAA and UGGUUUACAUGUCGACUAA.

Embryonic stem (ES) cell culture and differentiation

A Cdx2-inducible ES cell line provided by Coriell Cell Repositories (Camden, NJ, USA) was cultured on mitomycin-treated puromycin-resistant mouse embryonic fibroblasts in ES cell medium supplemented with 0.2 mg/ml doxycycline and 1.0 mg/ml puromycin (Nishiyama et al., 2009). Prior to Cdx2 induction, cells were switched onto gelatin and cultured in the presence of 1.5 mg/ml puromycin for 3 days. Transgene expression was induced by removal of doxycycline and was verified by immunocytochemistry and quantitative (q) RT-PCR analysis (Wang et al., 2010).

Microarray and bioinformatics analysis

Microarray analysis was carried out utilizing Affymetrix 430v2 gene chips as described (Kidder et al., 2009). Blastocyst array data were compared with eight-cell embryo array data using GeneSpring software (Agilent Technologies, Santa Clara, CA, USA). Genes that were upregulated ≥2-fold (P<0.05) in blastocysts were cross-referenced with a published Tcfap2c ChIP-Chip dataset in trophoblast stem (TS) cells (Kidder and Palmer, 2010). Genes that were both upregulated in blastocysts and bound by Tcfap2c in TS cells were then subjected to Tcfap2c binding motif analysis using TRANSFAC and ExPlain 3.0 (BIOBASE, Wolfenbüttel, Germany). Functional annotation and gene ontology analysis were conducted using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) and GOrilla (http://cbl-gorilla.cs.technion.ac.il/). Microarray data are available at GEO under accession number GSE41925.

Gene expression analysis by qRT-PCR

Total RNA was isolated using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA, USA) or an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis was carried out using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis was conducted utilizing TaqMan probes (Applied Biosystems, Foster City, CA, USA) or gene-specific designed primers (SYBR Green detection) and a StepOnePlus real-time PCR system (Applied Biosystems). *Ubtf* and *Eef1a1* were used as endogenous controls for embryos and Cdx2-inducible ES cells, respectively. To determine the developmental expression of *Tcfap2c* transcripts in metaphase II (MII) eggs to blastocysts, samples were normalized to exogenous *GFP* that was spiked into each sample prior to RNA isolation. SYBR Green PCR primer sequences are listed in supplementary material Table S1.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as previously described (Wang et al., 2010). Briefly, at 0, 48 and 96 hours after Cdx2 induction, samples were collected for ChIP and gene expression analysis. ES cells were sonicated and chromatin extracts were frozen at –80°C. ChIP was carried out using a Tcfap2c antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a rabbit IgG (Millipore) and chromatin extracts equivalent to 2×10^6 cells. ChIP samples were quantified by qPCR (SYBR Green Master Mix; Applied Biosystems) and ChIP-qPCR data were normalized using the percent input method.

Immunocytochemistry

Preimplantation embryos were fixed with 3.7% paraformaldehyde for 20 minutes, permeabilized with PBS containing 0.1% Tween 20 for 15 minutes, blocked with PBS containing 0.1% BSA for 1 hour at room temperature, and incubated with primary antibodies in blocking solution overnight at 4°C, followed by incubation with Alexa Fluor 488 and 594 (Molecular Probes, Eugene, OR, USA) secondary antibodies. Embryos were then mounted in Vectashield containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA) and imaged using a spinning disc confocal module (CARV; Atto Bioscience, Rockville, MD, USA) with MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). Validated primary antibodies for Tcfap2c, Oct4, p21, and Pard6b were obtained from Santa Cruz. Antibodies for Cdh1, Cldn4 and Tjp2 were from Invitrogen. Aqp3 and Krt18 antibodies were purchased from Alpha Diagnostic (San Antonio, TX, USA) and Abcam (Cambridge, MA, USA), respectively. Cdx2 antibody was obtained from Biogenex (San Ramon, CA, USA). F-actin was stained using Alexa Fluor 586 phalloidin (Molecular Probes).

TJ permeability assay by FITC-dextran uptake

To investigate the effects of Tcfap2c depletion on TJ permeability, control and Tcfap2c knockdown (KD) embryos were cultured until 120 hph, and then the blastocysts were incubated in modified KSOM medium containing 1 mg/ml 4 kDa FITC-dextran (Sigma-Aldrich, St Louis, MO, USA) for 10 minutes. Following the incubation, the blastocysts were immediately washed and visualized under an inverted fluorescence microscope.

Transmission electron microscopy (TEM)

Control and Tcfap2c KD morulae at 90 hph were washed with 0.1 M cacodylate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer for 2 hours at room temperature. The fixed embryos were embedded in 1% agarose. The agarose blocks were postfixed in 1% (w/v) osmium tetraoxide in 0.1 M cacodylate buffer for 30 minutes. The postfixed specimens were dehydrated through a graded ethanol series in the cacodylate buffer, transferred into mixtures of Spurr's resin and ethanol at room temperature and polymerized for 2 days at 60°C, followed by preparation of ultrathin sections using a Power Tome XL ultramicrotome (RMC, Boekeler Instruments, Tucson, AZ, USA) and examination by a JEOL100 CXII transmission electron microscope (Japan Electron Optics Laboratories, Tokyo, Japan).

Generation of chimeras by aggregation

The zona pellucida of control and Tcfap2c KD embryos at the compacted eight-cell stage was removed by acid Tyrode solution (Sigma) and two zona pellucida-free embryos were paired in microwells to generate chimeric embryos that consisted of: control-control, control-Tcfap2c KD, or Tcfap2c KD-KD embryos. To track the fate of Tcfap2c KD blastomeres, *GFP* mRNA and *Tcfap2c* siRNA were co-injected at the one-cell stage. Chimeric embryos were visualized using an inverted microscope equipped with UV light and an FITC filter.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) or χ^2 test using INSTAT3 (GraphPad Software, San Diego, CA, USA). The data are presented as mean ± s.e.m. $P<0.05$ was considered statistically significant unless otherwise stated.

RESULTS

Combined depletion of maternal and zygotic *Tcfap2c* blocks blastocyst formation

A recent study demonstrated that forced expression of Tcfap2c in mouse ES cells could induce a TE cell fate, indicating that Tcfap2c may promote development of the TE epithelium (Kuckenberg et al., 2010). However, the biological role of Tcfap2c during blastocyst formation has not been clarified. To address this, we first examined the expression of *Tcfap2* during preimplantation development. Consistent with previous studies (Auman et al., 2002; Winger et al., 2006), *Tcfap2c* transcripts were detected at all stages of preimplantation development (Fig. 1A). Notably, after fertilization, the levels of maternal transcripts were significantly reduced. Furthermore, from the four-cell stage onward there was a dramatic increase in zygotic *Tcfap2c* mRNA, peaking at the morula stage. Likewise, Tcfap2c protein was detected at all stages of preimplantation development, and at the blastocyst stage the levels of Tcfap2c protein were much greater in the TE than in the Oct4-positive inner cell mass (ICM) (Fig. 1B). Interestingly, the localization of Tcfap2c was heterogeneous in the TE; Tcfap2c was expressed in the mural TE, but was not detectable in the polar TE (supplementary material Fig. S1).

Next, we examined the function of Tcfap2c during preimplantation development. Previously, it was demonstrated that knockout of zygotic *Tcfap2c* resulted in embryonic lethality at around day 7.5 of development (Auman et al., 2002; Winger et al., 2006). However, in these studies expression of maternal Tcfap2c protein might have masked any potential phenotype during preimplantation development. To circumvent this, we used an RNAi approach to deplete both maternally and zygotically derived Tcfap2c. One-cell embryos were injected with either 100 μM *Tcfap2c* or control siRNA and cultured for 2 to 4 days. Microinjection of *Tcfap2c* siRNA resulted in a 96% and 80% reduction in *Tcfap2c* mRNA and in the complete loss of protein as assayed by immunocytochemistry (ICC) at the eight-cell and morula stages, respectively (Fig. 1C,D). Importantly, *Tcfap2c* siRNA did not target *Tcfap2a* transcripts, demonstrating that the siRNAs were specific to *Tcfap2c* mRNA (supplementary material Fig. S2). Remarkably, Tcfap2c knockdown (KD) embryos underwent compaction and developed normally to the morula stage (83.71% for KD versus 93.64% for control; $P>0.05$). However, only a small percentage of Tcfap2c KD morulae formed blastocysts compared with control embryos injected with scrambled siRNA (Fig. 1E; 13.73% versus 92.64%; $P<0.001$). Moreover, Tcfap2c KD embryos that developed to the blastocyst stage were not able to fully expand and hatch after extended culture (data not shown).

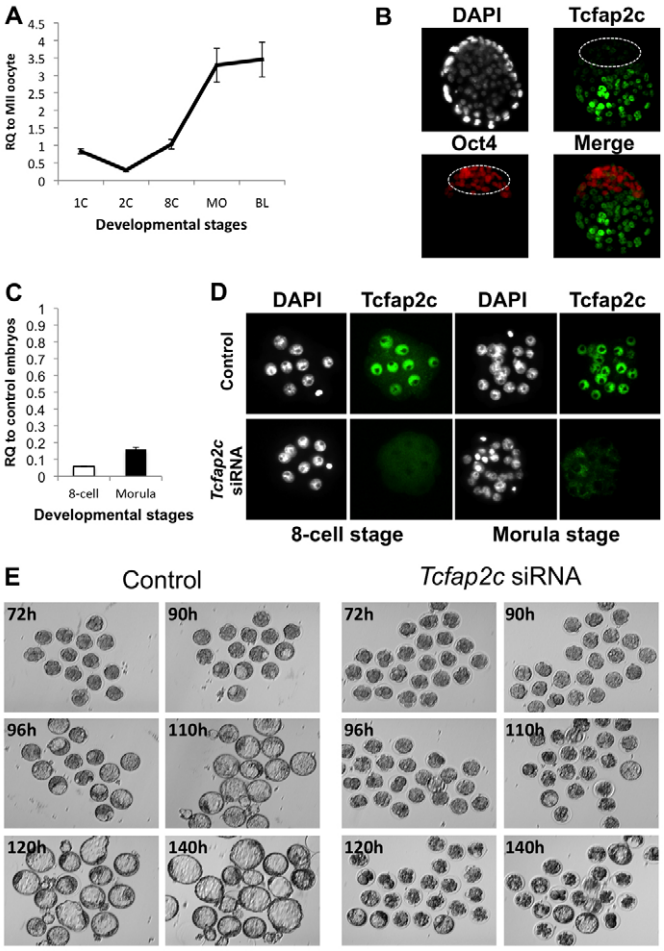

Fig. 1. Developmental expression and RNAi-mediated ablation of Tcfap2c in mouse preimplantation embryos. (**A**) qRT-PCR analysis of *Tcfap2c* transcripts in mouse MII oocytes and preimplantation embryos. Expression data from each stage were normalized to exogenous *GFP* and are relative to MII oocytes. 1C, one-cell zygote; 2C, two-cell; 8C, eight-cell; MO, morula; BL, blastocyst. (**B**) Immunocytochemistry (ICC) analysis revealed that Tcfap2c protein is enriched in the blastocyst mural trophectoderm (TE). Blastocysts were double stained for Oct4 to label the inner cell mass (ICM; outlined). Nuclei were counterstained with DAPI. (**C**) Validation of siRNA-mediated knockdown (KD) of *Tcfap2c* transcripts in eight-cell and morula stage embryos by qRT-PCR. (**D**) Confirmation of Tcfap2c protein ablation in eight-cell and morula stage embryos by ICC. (**E**) Depletion of Tcfap2c blocks blastocyst formation. Representative images of Tcfap2c KD and control embryos cultured for 72 to 140 hph. Error bars indicate mean ± s.e.m. RQ, relative quantification.

To rule out any potential siRNA off-targeting effects, several experiments were carried out. First, we showed by qRT-PCR that the control siRNA did not target *Tcfap2c* transcripts in injected embryos (supplementary material Fig. S2). Second, different concentrations of *Tcfap2c* siRNA were injected into one-cell embryos and KD efficiency and effects on blastocyst formation were assayed (supplementary material Fig. S2). These experiments revealed that 100 μM was the most effective concentration for depleting Tcfap2c and blocking blastocyst formation. Third, rescue experiments were performed by co-injection of *Tcfap2c* mRNA and *Tcfap2c* siRNA (pool 1) into one-cell embryos (supplementary material Fig. S3).

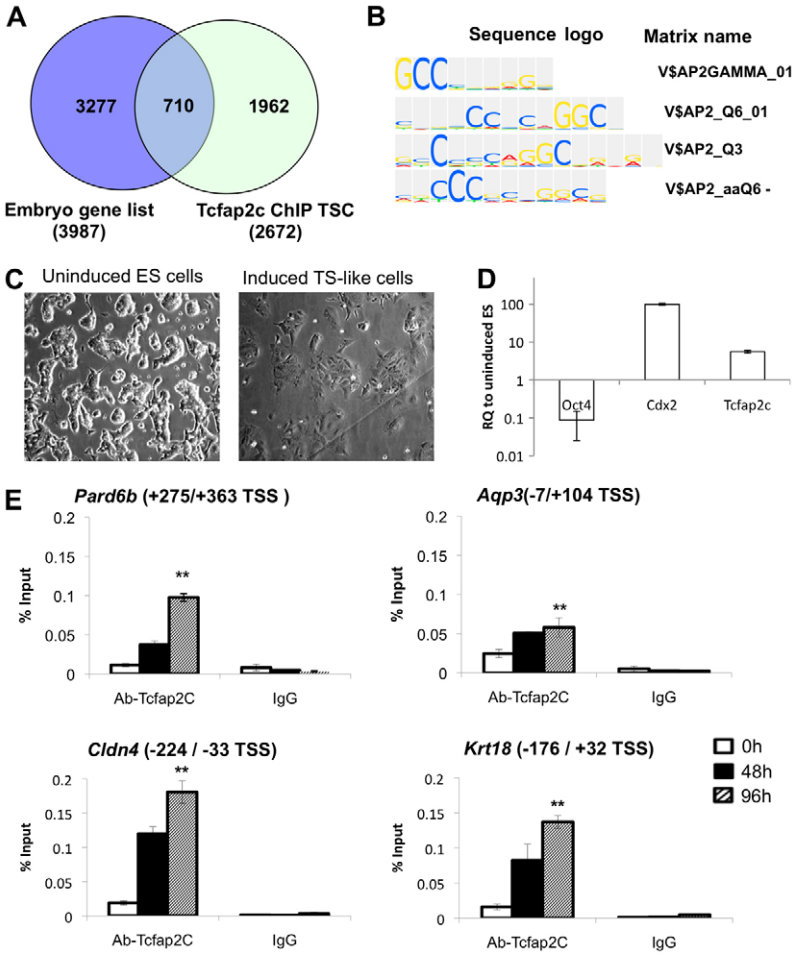

Fig. 2. Tcfap2c binding is enriched on genes that are upregulated during the morula-to-blastocyst transition. (**A**) Venn diagram illustrating genes that are both upregulated during blastocyst formation (embryo gene list) and enriched for Tcfap2c binding in trophoblast stem cells (TSC). (**B**) Tcfap2c motif analysis of putative Tcfap2c target genes using ExPlain 3.0. (**C**) Representative images of Cdx2-inducible ES cells (0 hours) differentiating into TE-like cells (96 hours post-induction). (**D**) qRT-PCR analysis of Cdx2-inducible ES cells at 0 and 96 hours post-induction. *Oct4* (ICM marker) was downregulated, but *Cdx2* (TE marker) and *Tcfap2c* transcripts were upregulated in TE-like cells at 96 hours post-induction. (**E**) ChIP analysis in Cdx2-inducible ES cells demonstrated that Tcfap2c is recruited to target gene promoters during differentiation into TE-like cells at 48 to 96 hours. Error bars indicate mean ± s.e.m. $^{**}P<0.01$. TSS, transcription start site.

These experiments revealed that a higher percentage of one-cell embryos co-injected with *Tcfap2c* siRNA and mRNA developed to the blastocyst stage compared with embryos injected with *Tcfap2c* siRNA alone (57.69% versus 13.73%; $P<0.05$). Fourth, a second pool of *Tcfap2c* siRNA was injected into one-cell embryos (supplementary material Fig. S3). Targeting of *Tcfap2c* via this second pool also reduced blastocyst formation.

As stated above, a major difference between Tcfap2c KD and control embryos was the ability to form a blastocoel cavity during the morula-to-blastocyst transition. Therefore, we monitored the timing of cavity formation in Tcfap2c KD and control morulae from 76 to 120 hph. As shown in supplementary material Fig. S3, ~25% of control embryos formed cavities at 90 hph, and by 120 hph over 90% of embryos formed a blastocoel cavity. By contrast, only 3% of Tcfap2c KD embryos formed a cavity at 90 hph, and by 120 hours only 36% of embryos formed a small cavity. To better understand the etiology of this phenotype, we used a time-lapse camera to record Tcfap2c KD and control embryos undergoing cavity formation in real-time (supplementary material Movie 1). This revealed that Tcfap2c KD morulae repeatedly initiated cavity formation but failed to form an expanded blastocoel cavity. Altogether, these data demonstrate that Tcfap2c is required for blastocyst formation.

Tcfap2c target genes in the preimplantation embryo

Because Tcfap2c KD embryos arrest at the morula stage, we hypothesized that Tcfap2c is required for transcriptional regulation of key genes involved in the morula-to-blastocyst transition. To address this, we (1) conducted transcriptome analysis on normal embryos to identify genes upregulated between the eight-cell and blastocyst stage and (2) cross-referenced this gene list with a published Tcfap2c ChIP-Chip dataset (n=2673 genes) in TS cells (Kidder and Palmer, 2010). We identified a total of 3987 genes that were upregulated ≥2-fold between the eight-cell and blastocyst stages. When cross-referenced with the Tcfap2c ChIP-Chip dataset we found a total of 710 common genes that were both bound by Tcfap2c in TS cells and upregulated in blastocysts (Fig. 2A; supplementary material Table S2). To confirm that these genes were enriched with Tcfap2c binding motifs, we used TRANSFAC and ExPlain 3.0 (Fig. 2B). This analysis confirmed that Tcfap2c binding motifs were enriched in this gene list.

Next we used a web-based application tool called GOrilla (Gene Ontology Enrichment Analysis and Visualization) to obtain meaningful and systematic biological information from the upregulated/Tcfap2c-bound gene list. This analysis revealed that epithelial cell differentiation, cellular response to cytokine stimulus, and cytokine-mediated signaling pathway presented a unique set of enriched GO terms ($P<10^{-3}$), suggesting that Tcfap2c might regulate genes involved in epithelial differentiation (supplementary material Table S3). Moreover, we utilized the DAVID (Database for Annotation, Visualization and Integrated Discovery) annotation pathway analysis tool to identify possible functions or molecular interactions of the listed genes. According to the KEGG (Kyoto Encyclopedia of Genes and Genomes) database, we found that these genes are highly involved in cell communication, including

TJs, focal adhesion and adherens junctions (supplementary material Table S4). This analysis identified putative Tcfap2c target genes involved in TJ assembly and paracellular sealing (supplementary material Fig. S4). Finally, in line with this systematic data mining and morphological observation, such as the delayed or retarded cavity formation, we selected 19 genes that are responsible for cell polarity, paracellular sealing and fluid accumulation. This list contains genes that are important for adherens junctions (*Cdh1*, also known as E-cadherin), cellular polarization (*Pard6b*, *Rock2*), cytoskeleton organization (*Krt18*, *Fn1*), TJ assembly (*Cldn4*, *Cldn6*, *Cldn7*, *Tjp1*, *Tjp2*, *Ocln*, *Inadl*, *Jam2*, *F11r*), ion gradients (*Atp1b1*, *Atp1b3*, *Atp1a1*) and the formation of membrane H_2O channels (*Aqp3*, *Aqp9*). This list of selected genes with a brief description of their known function/phenotype is presented in supplementary material Table S5.

To test whether Tcfap2c can directly bind to target gene promoters during TE formation, ChIP analysis was conducted in ES cells differentiating into TE-like cells. To accomplish this, we utilized a doxycycline-controllable, Cdx2-inducible ES cell line as a model system for the developing TE epithelium (Nishiyama et al., 2009; Wang et al., 2010). ChIP primers flanking Tcfap2c binding motifs were designed for a subset of genes. As a negative control, primers were designed for an intergenic region that does not contain binding motifs. At 0, 48 and 96 hours after Cdx2 induction, cells were isolated for ChIP and gene expression analysis (Fig. 2C,D). We found that at 48 and 96 hours post-induction, Tcfap2c was largely recruited to the promoters of *Tjp2*, *Pard6b*, *Cldn4*, *Krt18* and *Aqp3* ($P<0.01$), whereas Tcfap2c enrichment was not observed at an intergenic region (Fig. 2E; supplementary material Fig. S5). Moreover, at these same time points there was a significant increase in mRNA expression for these genes ($P<0.05$) (supplementary material Fig. S6). Furthermore, examination of the developmental expression of *Tjp2*, *Pard6b*, *Cldn4*, *Krt18* and *Aqp3* in preimplantation embryos revealed that these genes were upregulated between the eight-cell and blastocyst stage (supplementary material Fig. S6). Altogether, these data suggest that Tcfap2c might play an important role in the transcriptional regulation of genes that potentiate blastocyst formation.

Tcfap2c is required for transcriptional regulation of genes important for cell polarity, TJ assembly and fluid accumulation

As described above, a significant proportion of Tcfap2c KD embryos arrested at the morula stage and failed to form and/or maintain a blastocoel cavity. Importantly, the phenotype of Tcfap2c KD embryos resembles that observed in several other loss-of-function studies in mice and other mammalian species, implying that Tcfap2c might function as a core regulator of blastocyst formation genes. To test this, we utilized qRT-PCR analysis to determine the expression levels of 20 putative Tcfap2c target genes in Tcfap2c KD embryos (Fig. 3A). We first evaluated the expression of *Pard6b*, *Pard3*, *Fn1*, *Rock2* and *Krt18*. These genes are implicated in cell polarity and cytoskeleton organization and are important for blastocyst formation in mouse and/or bovine embryos (Kawagishi et al., 2004; Goossens et al., 2007; Alarcon, 2010; Goossens et al., 2010). The expression of *Pard6b*, *Rock2*, *Fn1* and *Krt18* mRNA was significantly reduced in Tcfap2c KD embryos ($P<0.05$), suggesting that Tcfap2c is required for the correct expression of genes during the morula-to-blastocyst transition. By contrast, the levels of *Pard3* transcripts were unchanged in Tcfap2c KD embryos.

To further examine the molecular basis of the Tcfap2c KD phenotype, we examined the expression of genes necessary for TJ assembly and fluid accumulation (Fig. 3A). The assembly of TJ complexes is crucial for paracellular sealing of the TE epithelium and formation of a fluid-filled cavity (Saitou et al., 2000; Kim et al., 2004; Thomas et al., 2004; Moriwaki et al., 2007; Katsuno et al., 2008). We observed a significant reduction in the expression levels of *Cldn4*, *Cldn6*, *Cldn7* and *Tjp2* in Tcfap2c KD morulae ($P<0.05$). By contrast, the levels of *Tjp1*, *Ocln* and *Jam2* mRNA were increased in Tcfap2c KD morulae ($P<0.05$). The expression of *F11r* (previously known as *Jam1*) did not differ between Tcfap2c KD and control morulae ($P=0.07$). In addition to the barrier function of TJs, the establishment of a trans-TE ionic gradient by Na/K-ATPase and H_2O transporters is essential for the accumulation of fluid within the blastocoel cavity. We found that *Atp1a1* and *Atp1b1* transcripts were reduced ($P<0.05$), whereas the expression of *Atp1b3* was unaffected ($P>0.05$), in Tcfap2c KD embryos. Likewise, the expression of *Aqp3* was significantly reduced ($P<0.05$), whereas *Aqp9* expression was unchanged ($P>0.05$), in Tcfap2c KD embryos. Finally, we examined the expression of *Cdh1* and *Ctnnb1* (β-catenin), two genes required for adherens junctions. These genes were unchanged in Tcfap2c KD morulae.

Subsequently, we analyzed the expression and localization of Tcfap2c target genes by ICC analysis. We selected genes that were downregulated at the transcript level by ≥50% in Tcfap2c KD embryos. Consistent with the reduction in target gene mRNA there was a dramatic reduction in the expression of Pard6b, Tjp2, Cldn4, Krt18 and Aqp3 proteins (Fig. 3B). For example, in control morulae Pard6b expression was observed as a continuous band, and at the apical pole of the outer cells Pard6b accumulated at the cell-to-cell contact sites. By contrast, in Tcfap2c KD morulae the overall expression of Pard6b was reduced and lost altogether at the cell-to-cell contacts. Likewise, in control morulae the TJ proteins Cldn4 and Tjp2 were distributed as a continuous belt around each blastomere and were concentrated at the cell-to-cell contacts in the apical region. By contrast, in Tcfap2c KD morulae the expression of Cldn4 and Tjp2 was severely reduced and not visible in the apical region at the cell-to-cell contacts. The intermediate filament protein Krt18 was highly expressed and localized to the cell membrane in control morulae, whereas in Tcfap2c KD embryos it was lost completely. Expression of Aqp3 was localized to the basolateral region in control morulae, whereas in Tcfap2c KD morulae Aqp3 expression was diminished. Finally, we stained control and Tcfap2c KD morulae for Cdh1 and F-actin (Fig. 3B). Consistent with the qRT-PCR and morphological data, the expression and localization of Cdh1 were unimpaired in Tcfap2c KD embryos. However, F-actin was highly disorganized in Tcfap2c KD embryos compared with control embryos. This is consistent with observations made in several other loss-of-function studies on TJ and cell polarity-related proteins (Madan et al., 2007; Sheth et al., 2008; Alarcon, 2010). Altogether, these results demonstrate that Tcfap2c is required for the proper expression and localization of key TJ, cell polarity, cytoskeletal and H_2O channel proteins during the morula-to-blastocyst transition.

Functional evidence that Tcfap2c is required for TJ assembly and paracellular sealing during blastocyst formation

During blastocyst formation TJ complexes are essential for paracellular sealing, which excludes the transport of higher molecular weight molecules from the apical region into the

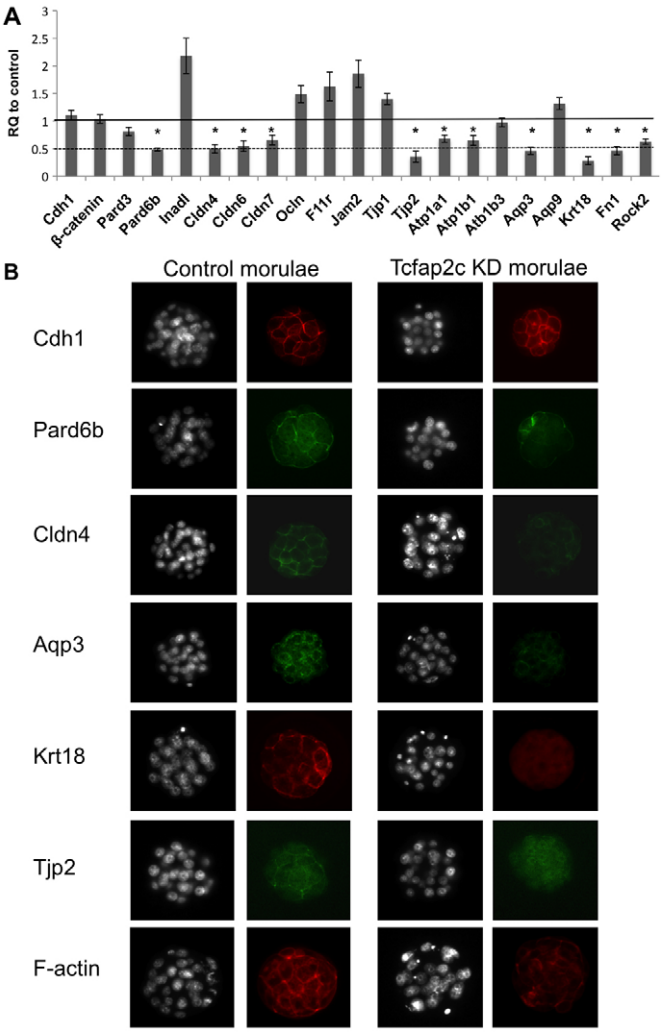

Fig. 3. Tcfap2c regulates genes that are crucial for cell polarity, TJ assembly and ion gradients. (**A**) qRT-PCR analysis of Tcfap2c target genes in Tcfap2c KD and control morulae (90 hph). Genes important for cell polarity, tight junction (TJ) assembly and fluid gradient were significantly downregulated in Tcfap2c KD embryos. Error bars indicate mean ± s.e.m. **P*<0.05. (**B**) Protein expression and localization of Tcfap2c target genes in Tcfap2c KD and control morulae. Depletion of Tcfap2c disrupted the expression and subcellular localization of Pard6b, Cldn4, Aqp3, Krt18, Tjp2 and F-actin. Embryos were counterstained with DAPI.

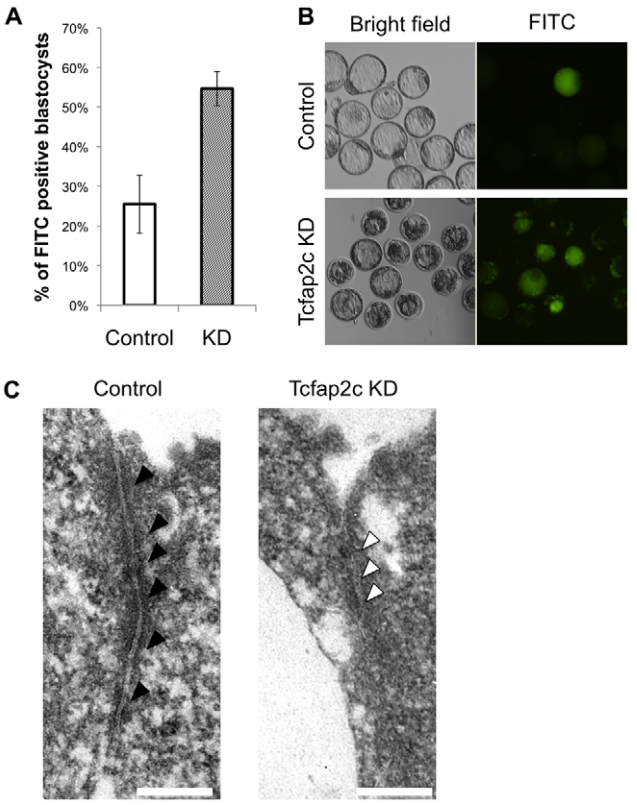

Fig. 4. Disruption of TJ complexes and paracellular sealing in Tcfap2c KD embryos. (**A**) A 4 kDa FITC-conjugated dextran was used to test the permeability of TJs in Tcfap2c KD and control blastocysts. A difference in permeability was observed between Tcfap2c KD and control blastocysts. Error bars indicate mean ± s.e.m. (**B**) Representative brightfield and fluorescence images of Tcfap2c KD and control blastocysts subjected to the FITC-dextran assay. (**C**) TEM analysis of TJ complexes in Tcfap2c KD and control morulae. At 90 hph, several electron-dense plaques (black arrowheads) were observed at apical cell-to-cell contact sites in control embryos. By contrast, in Tcfap2c KD embryos the electron-dense regions (white arrowheads) at the apical cell-to-cell contact site were absent. Scale bars: 200 nm.

basolateral region and allows the formation of a fluid-filled cavity (Watson and Barcroft, 2001). These complexes are assembled during the morula-to-blastocyst transition and become functional at the blastocyst stage (Eckert and Fleming, 2008). Because key TJ and cytoskeletal proteins are downregulated in Tcfap2c KD morulae we hypothesized that the function and/or integrity of these complexes would be compromised. To test this we analyzed the permeability of TJ complexes using a 4 kDa FITC-dextran assay (Moriwaki et al., 2007; Giannatselis et al., 2011). Since most Tcfap2c KD embryos do not form blastocysts, we confined our analysis to partially recovered blastocysts from Tcfap2c KD embryos; despite developing to the blastocyst stage this subset of embryos contained a reduced complement of Tcfap2c, Pard6b, Krt18 and Cldn4 (supplementary material Fig. S7). A higher percentage of Tcfap2c KD blastocysts were permeable to FITC-dextran than control blastocysts (55±4% versus 25±7%; Fig. 4A,B), suggesting that the function of TJ complexes was impaired in Tcfap2c KD embryos. In addition, we analyzed a subset of Tcfap2c KD embryos that had been rescued by *Tcfap2c* mRNA injection. Importantly, Tcfap2c KD embryos that were rescued by mRNA injection exhibited functional paracellular sealing in the TE epithelium, with normal expression and localization of Cldn4 and F-actin (supplementary material Fig. S8). The average total cell number of the *Tcfap2c* mRNA-rescued group was lower than that of control blastocysts (63.20 versus 71.38; $P<0.05$).

To examine the ultrastructural integrity of TJ complexes in Tcfap2c KD morulae, we performed TEM in Tcfap2c KD and control embryos at the morula stage. These experiments revealed that Tcfap2c KD morulae failed to form TJ complexes at the apical cell boundaries, consistent with the downregulation and/or mislocalization of TJ complex proteins in these embryos (Fig. 4C). Collectively, these results demonstrate that Tcfap2c is required for the correct expression and assembly of TJ complex proteins during blastocyst formation and that dysregulation of these genes results in a failure to establish paracellular sealing.

Aggregation of control with Tcfap2c KD eight-cell embryos rescues blastocyst formation

Since Tcfap2c is required for the proper expression of TJ proteins and assembly of functional TJ complexes, we hypothesized that the aggregation of control eight-cell embryos (Tcfap2c^{+}) with Tcfap2c KD eight-cell embryos would restore blastocoel formation in Tcfap2c KD embryos. We produced three types of chimeras by aggregation: (1) control-control (C:C) embryos; (2) control-Tcfap2c KD (C:K) embryos; and (3) Tcfap2c KD-Tcfap2c KD (K:K) embryos. Regardless of the type of chimera, over 90% of the paired embryos successfully aggregated and formed chimeras (Fig. 5A). More importantly, over 90% of C:K paired chimeras overcame the morula-to-blastocyst transition block and developed to the blastocyst stage at a rate that was similar to that of C:C chimeras (P>0.05) (Fig. 5A,B). By contrast, only 15% of K:K paired chimeras formed blastocyst-like structures and the majority of chimeras arrested at the morula stage (P<0.05). To confirm whether features of the TE epithelium were restored in rescued blastocysts, C:K and C:C chimeras were stained for F-actin, Cldn4 and Cdx2 (Fig. 5C,D). This revealed that in C:K blastocysts the expression and localization of Cldn4 and F-actin were restored in the Cdx2-positive TE epithelium.

To determine whether control blastomeres contribute to the TE epithelium of C:K blastocysts two experiments were carried out. First, ICC analysis revealed that Tcfap2c was localized mainly in the TE of C:C and C:K chimeras (Fig. 5C). Second, *GFP* mRNA was co-injected with *Tcfap2c* siRNA into embryos prior to aggregation with control eight-cell embryos (C:K-GFP labeled). In C:K blastocysts, GFP expression was predominantly localized to the Oct4-positive ICM and not the TE, demonstrating that the control cells contributed predominantly to the TE lineage (supplementary material Fig. S9). Collectively, these data demonstrate that developmental arrest of Tcfap2c KD morulae can be reversed through cell-to-cell aggregation.

Tcfap2c negatively regulates *p21* transcription to promote cellular proliferation during the morula-to-blastocyst transition

In addition to TJ assembly and establishment of trans-TE ion gradients, blastocyst formation requires an increase in cellular proliferation for blastocoel expansion (Copp, 1978). The observed developmental delay or arrest in Tcfap2c KD embryos suggested that cell cycle regulation was altered. Moreover, in blastocysts derived from C:K chimeras there was a reduction in total cell number (106.31±3.42 for C:K versus 132.98±7.79 for C:C; P<0.05). To more accurately assess this phenotype we calculated the total cell number in Tcfap2c KD and control embryos at the morula stage, which differed significantly (26.52±0.64 for control versus 21.21±0.49 for KD; P<0.01). To identify potential target genes involved in proliferation, we focused on genes that negatively regulate proliferation, such as *p21*, *p27* and *p57* (*Cdkn1a*, *Cdkn1b* and *Cdkn1c* – Mouse Genome Informatics), which are important in the regulation of cell cycle progression (Sherr and Roberts, 1995; Sherr and Roberts, 1999). A previous study demonstrated that increased levels of *p21* expression during preimplantation development was associated with arrest around the morula stage (Adiga et al., 2007). Using TRANSFAC and ExPlain and ChIP assays, we identified a Tcfap2c binding site in the *p21* promoter (Fig. 6A). Remarkably, qRT-PCR analysis revealed that *p21* transcripts were upregulated more than 8-fold in Tcfap2c KD morulae compared with control embryos (Fig. 6B), whereas expression of *p27* and *p57* was unaffected (data not shown). ICC

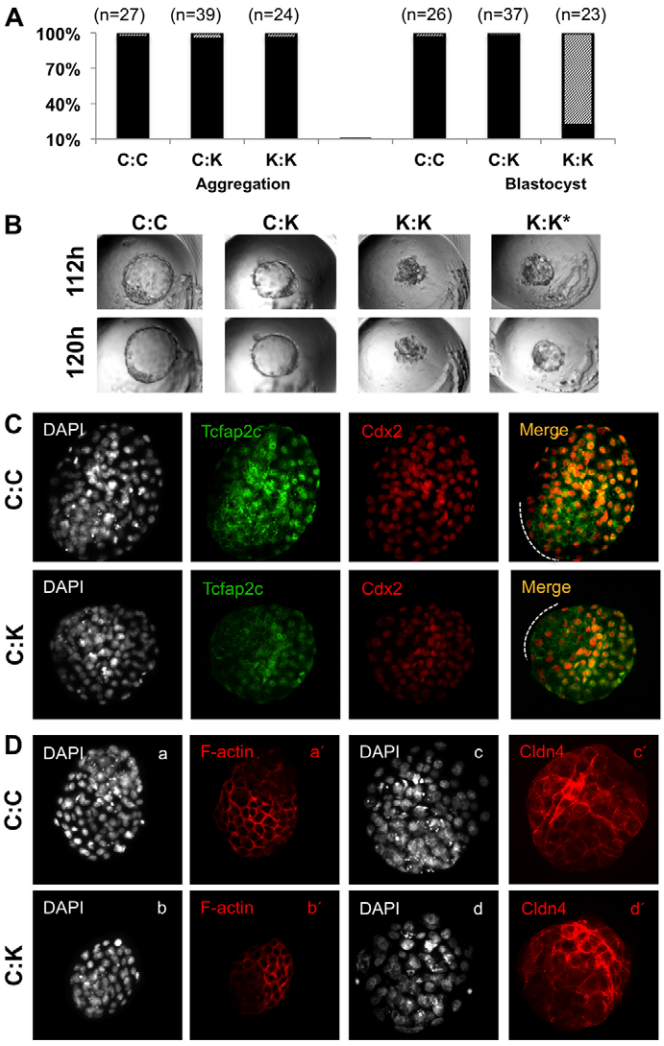

Fig. 5. Aggregation of control embryos with Tcfap2c KD embryos rescues blastocyst formation. (**A**) Developmental rates of control eight-cell embryos aggregated with Tcfap2c KD eight-cell embryos. To the left is shown the percentage of aggregated (black) or unaggregated (hatched) embryos and to the right is shown the percentage of chimeric embryos that developed to blastocyst stage (black) or failed to reach blastocyst (hatched). (**B**) Representative brightfield images of chimeric embryos produced by aggregation of C:C, C:K and K:K embryos at 112 and 120 hph. Asterisk denotes blastocyst-like vesicle in K:K chimeras. (**C**) ICC analysis of the subcellular localization of Tcfap2c and Cdx2. Orange indicates merge of Tcfap2c and Cdx2. Dotted line indicates the polar TE. (**D**) ICC analysis of F-actin and Cldn4 in C:C and C:K embryos (lowercase letters denote different groups of C:C and C:K aggregated embryos). C:C, aggregation of two control embryos; C:K, aggregation of control and Tcfap2c KD embryo; K:K, aggregation of two Tcfap2c KD embryos.

revealed increased expression and nuclear localization of p21 in Tcfap2c KD morulae versus controls (Fig. 6C). Because *p21* expression can be regulated by p53-dependent and -independent mechanisms (Gartel and Tyner, 1999), we ascertained whether upregulation of *p21* expression was mediated by p53 (Trp53 – Mouse Genome Informatics). qRT-PCR analysis revealed that *p53* transcript levels did not differ between Tcfap2c KD and control morulae. Furthermore, we evaluated the expression of *Hdac1*, a negative regulator of *p21* expression (Ma and Schultz, 2008;

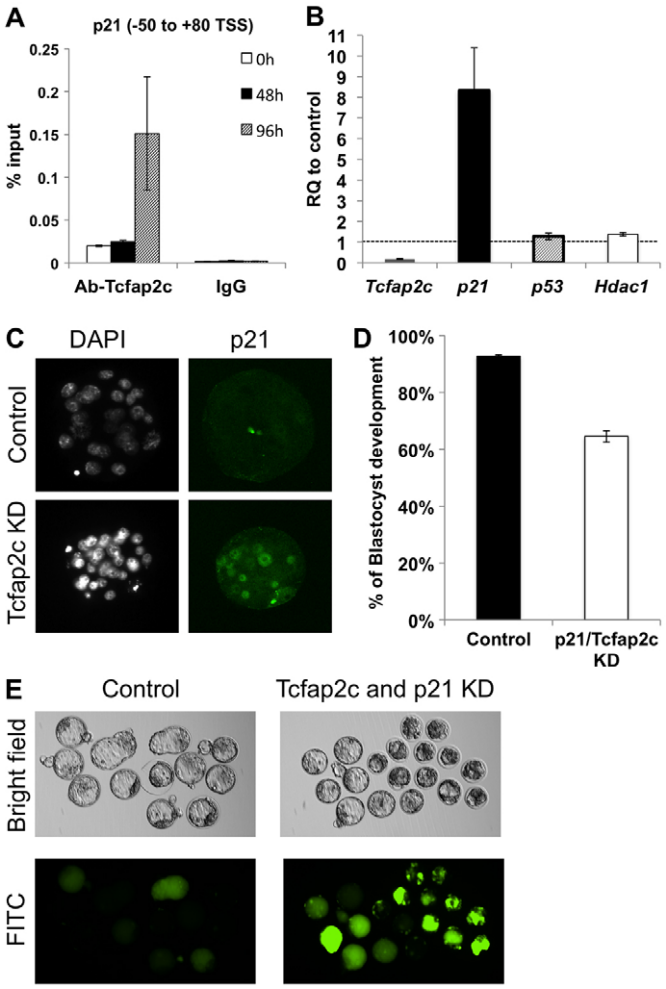

Fig. 6. Tcfap2c regulates cellular proliferation via direct repression of *p21*. (**A**) ChIP analysis of Tcfap2c binding to the *p21* proximal promoter. (**B**) *p21* is upregulated in Tcfap2c KD morulae. Upregulation of *p21* is independent of *p53* and *Hdac1* expression. (**C**) Expression and subcellular localization of p21 protein in Tcfap2c KD and control morulae. (**D**) Combined KD of p21 and Tcfap2c partially rescues blastocyst formation. (**E**) Representative brightfield and fluorescence images of p21/Tcfap2c KD and control blastocysts subjected to the FITC-dextran assay. Error bars indicate s.e.m.

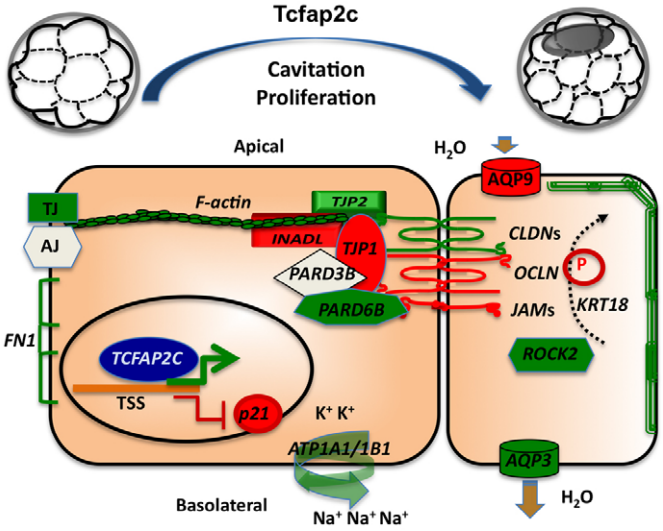

Fig. 7. Model for Tcfap2c-mediated transcriptional regulation of blastocyst formation. Tcfap2c regulates a diverse group of genes during the morula-to-blastocyst transition. Genes shown in green and red are positively and negatively regulated by Tcfap2c, respectively; genes in white are not regulated by Tcfap2c. TJ, tight junction; AJ, adherens junction; P, phosphorylation.

Zupkovitz et al., 2010). The levels of *Hdac1* did not differ between Tcfap2c KD and control embryos (Fig. 6B), suggesting that the increased expression of *p21* is mediated via Tcfap2c and not by other mechanisms.

To test whether inhibiting the increase in *p21* expression could rescue the preimplantation arrest phenotype of Tcfap2c KD embryos, we co-injected *p21* siRNA and *Tcfap2c* siRNA at the one-cell stage and cultured them for 4 days. Importantly, *p21* siRNA efficiently reduced the levels of *p21* mRNA without compromising the efficacy of the *Tcfap2c* siRNA (data not shown). A higher percentage of p21/Tcfap2c KD embryos developed to the blastocyst stage (65%, versus 93% in control embryos; Fig. 6D). However, after extended culture these embryos failed to fully expand and exhibited increased uptake of FITC-dextran into the blastocoel cavity (Fig. 6E), suggesting that the barrier function in these blastocysts was still impaired. Collectively, these results demonstrate that, in addition to regulating the expression of TJ and cell polarity genes, Tcfap2c negatively regulates *p21* transcription to promote blastocyst formation in mice.

DISCUSSION

Results presented here reveal a previously unknown role of Tcfap2c in early embryonic development. Our results in preimplantation mouse embryos demonstrate that: (1) Tcfap2c is required for blastocyst formation; (2) Tcfap2c binds to and regulates a diverse set of genes involved in cell polarity, TJs and fluid accumulation; (3) Tcfap2c is necessary for the assembly of functional TJ complexes in TE apical cell membranes; and (4) Tcfap2c controls cellular proliferation via negative regulation of *p21*. We propose a model in which Tcfap2c acts in a hierarchy to facilitate blastocyst formation via transcriptional regulation of core genes involved in TJ assembly, fluid accumulation and cellular proliferation (Fig. 7).

In the present study we identified a diverse group of Tcfap2c-regulated genes with established roles in blastocyst formation. These include genes that are important for TJ assembly (*Cldn4*, *Cldn6*, *Tjp2*, *Tjp1*), cell polarity (*Pard6b*) and fluid accumulation (*Atp1b1*, *Aqp3*). Claudin family members encode tetraspanin membrane proteins that serve crucial roles in TJ assembly and epithelial cell barrier function (Krause et al., 2008). In preimplantation embryos, disruption of Cldn4 and Cldn6 function via an inhibitory peptide impairs blastocyst development (Moriwaki et al., 2007). The TJ proteins Tjp1 and Tjp2 play an important role in connecting the actin skeleton with TJ complexes at the apical membrane (Schneeberger and Lynch, 2004). Embryos that lack Tjp1 or Tjp2 exhibit defects in blastocyst formation and/or undergo early embryonic lethality (Katsuno et al., 2008; Sheth et al., 2008). Likewise, in mouse preimplantation embryos the cell polarity regulator Pard6b is essential for blastocyst formation and proper localization of Tjp1 in the apical cell membrane (Alarcon, 2010). Atp1b1 and Aqp3 play major roles in fluid accumulation. For example, RNAi-mediated KD of Atp1b1 and pharmacological

inhibition of aquaporins blocks blastocyst formation and expansion, respectively, in mouse preimplantation embryos (Barcroft et al., 2003; Madan et al., 2007). These results in combination with our findings demonstrate that the proper expression of these genes is required for blastocyst formation.

Consistent with the altered expression of TJ, cell polarity and ion gradient-related genes, the barrier function of the TE epithelium was impaired in Tcfap2c KD embryos. Previous studies showed that TJ complexes are assembled at the morula stage and become functional near the time of blastocyst formation (Eckert and Fleming, 2008). In the present study, ultrastructural and functional analyses revealed the absence of TJ complexes and defective paracellular sealing in Tcfap2c KD embryos. In line with these findings, inhibition of Cldn4 and Cldn6 has been shown to disrupt paracellular sealing in mouse preimplantation embryos (Moriwaki et al., 2007). Altogether, our data demonstrate that Tcfap2c is a core regulator of TJ assembly and paracellular sealing in the TE epithelium.

To address whether blastocyst formation could be rescued in Tcfap2c KD embryos, chimera experiments were performed. We found that aggregation of control eight-cell embryos with Tcfap2c KD embryos could fully restore blastocyst formation. Interestingly, in chimeric blastocysts, Tcfap2c KD blastomeres (GFP^+) contributed predominantly to the ICM epithelium, whereas control blastomeres contributed to the TE epithelium. This finding, in combination with the observed enrichment of Tcfap2c in the TE epithelium (this study) (Kuckenberg et al., 2010), suggest that Tcfap2c might have a potential role in cell fate specification. In support of this idea, forced expression of Tcfap2c in mouse ES cells triggers differentiation into TE-like cells by Cdx2-dependent and -independent mechanisms (Kuckenberg et al., 2010). In Tcfap2c KD embryos we observed a significant decrease in *Cdx2* expression, suggesting that Tcfap2c might play an important role in regulating *Cdx2* expression during preimplantation development (our unpublished data). Additional studies are necessary to establish a regulatory relationship between Tcfap2c and Cdx2 in the preimplantation embryo.

Our data also provide some tantalizing clues as to the role of Tcfap2c in cellular proliferation. Molecular analysis of Tcfap2c KD embryos revealed that *p21* expression was upregulated greater than 8-fold. Several lines of evidence implicate p21 in preimplantation embryo arrest. First, mouse embryos fertilized with γ-irradiated sperm exhibit an increase in *p21* expression and developmental arrest around the morula stage, whereas, *p21*-null embryos fertilized with irradiated sperm develop normally to the blastocyst stage, demonstrating that p21 is required for DNA damage-induced arrest at the morula stage (Adiga et al., 2007). Second, depletion of Hdac1, a negative regulator of *p21* transcription, resulted in increased levels of *p21* expression and lower rates of blastocyst formation (Ma and Schultz, 2008). Third, in growth-arrested human preimplantation embryos, the p21-related family member *P27* is upregulated (Civico et al., 2002). To address the relationship between Tcfap2c and p21 we performed double KDs and found that ablation of p21 in Tcfap2c KD embryos could improve blastocyst formation. Interestingly, in other cellular contexts, *p21* expression is regulated by Tcfap2c. For example, in MCF-7 breast cancer cells Tcfap2c promotes cellular proliferation via direct repression of *p21* transcription (Williams et al., 2009). Because Tcfap2c expression is dysregulated in breast cancer it is tempting to speculate that Tcfap2c confers a dual function in controlling both cellular proliferation and TJ assembly. Collectively, our data demonstrate that Tcfap2c plays a crucial role in negatively regulating *p21* expression during the morula-to-blastocyst transition in mouse preimplantation embryos.

In summary, results reported here provide strong evidence that Tcfap2c is a core regulator of blastocyst formation in mouse embryos. These results have significant implications in understanding preimplantation embryo failure in humans, where 50-70% of embryos produced via assisted reproductive technologies fail to develop to the blastocyst stage (Gardner et al., 2004; Blake et al., 2007). From a broader perspective, our findings might have an impact on understanding the etiology of more invasive forms of cancer in which TJ complexes are lost (Brennan et al., 2010).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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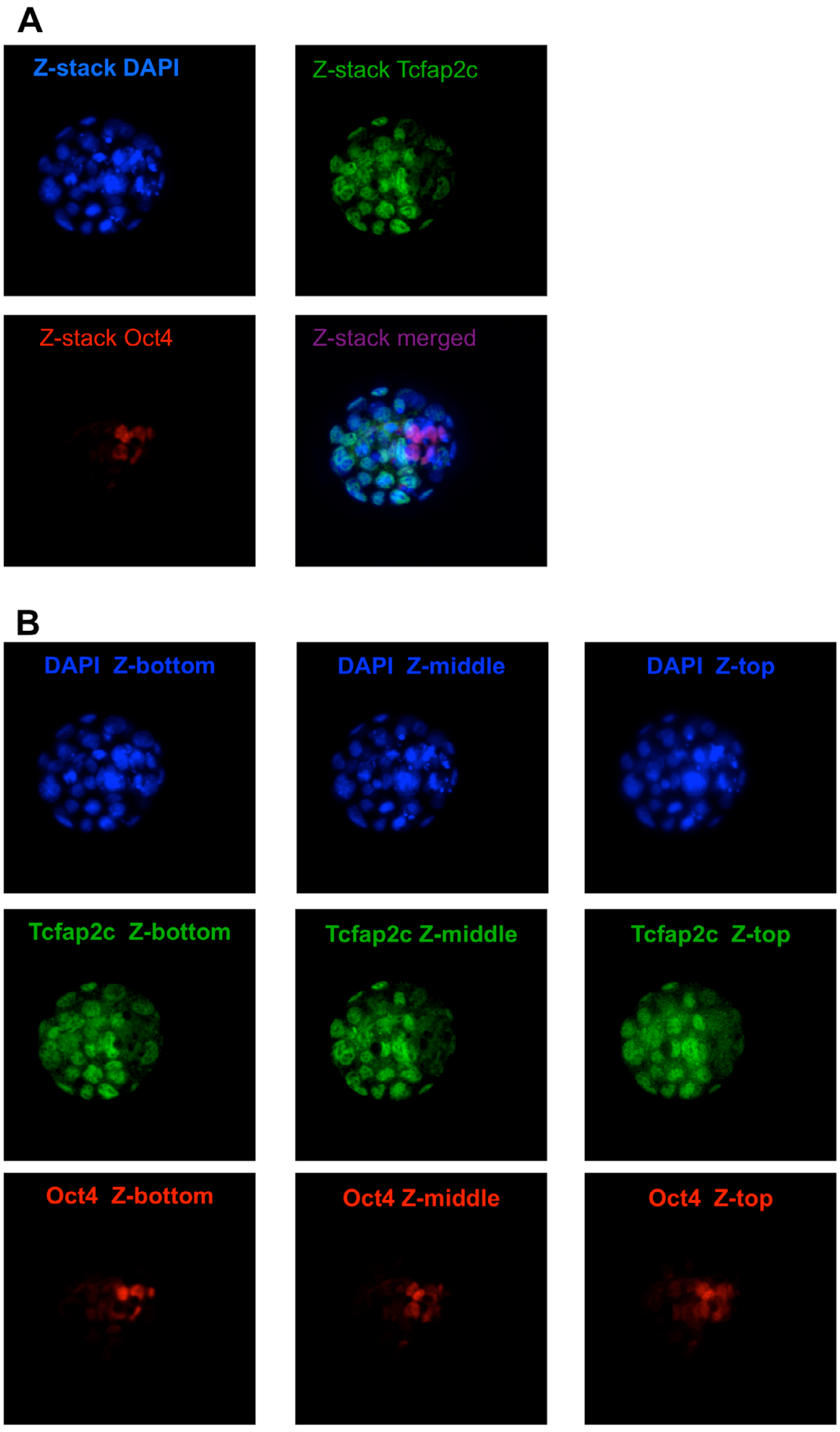

Fig. S1. Heterogeneous localization of Tcfap2c protein in mouse blastocysts. (**A**) Combined *z*-stack showing the localization of Tcfap2c in the mural TE. Tcfap2c is absent in the Oct4-positive ICM and surrounding polar TE. (**B**) Individual optical sections of a blastocyst stained for Tcfap2c, Oct4 and with DAPI.

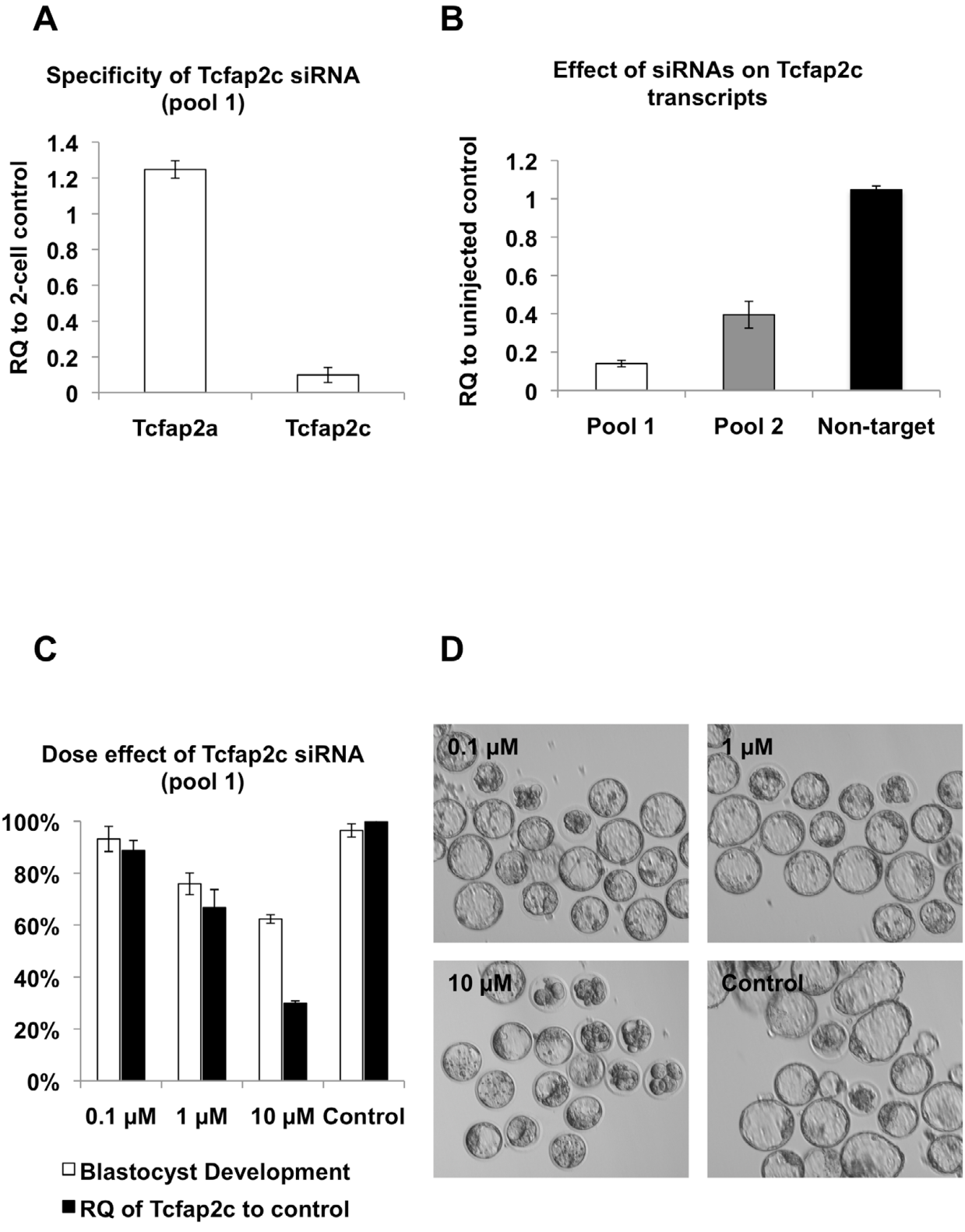

Fig. S2. Control experiments demonstrating specificity of siRNA. (**A**) qRT-PCR analysis of *Tcfap2a* and *Tcfap2c* transcripts in two-cell embryos microinjected with 100 µM *Tcfap2c* siRNA. (**B**) qRT-PCR analysis of *Tcfap2c* transcripts in embryos injected with non-targeting siRNA, *Tcfap2c* siRNA (pool 1), or *Tcfap2c* siRNA (pool 2). Expression is relative to control uninjected embryos. (**C**) Effect of different concentrations of *Tcfap2c* siRNA on *Tcfap2c* transcripts and blastocyst formation. (**D**) Brightfield images of embryos injected with different concentrations of *Tcfap2c* siRNA at 120 hph. Error bars represent mean ± s.e.m.

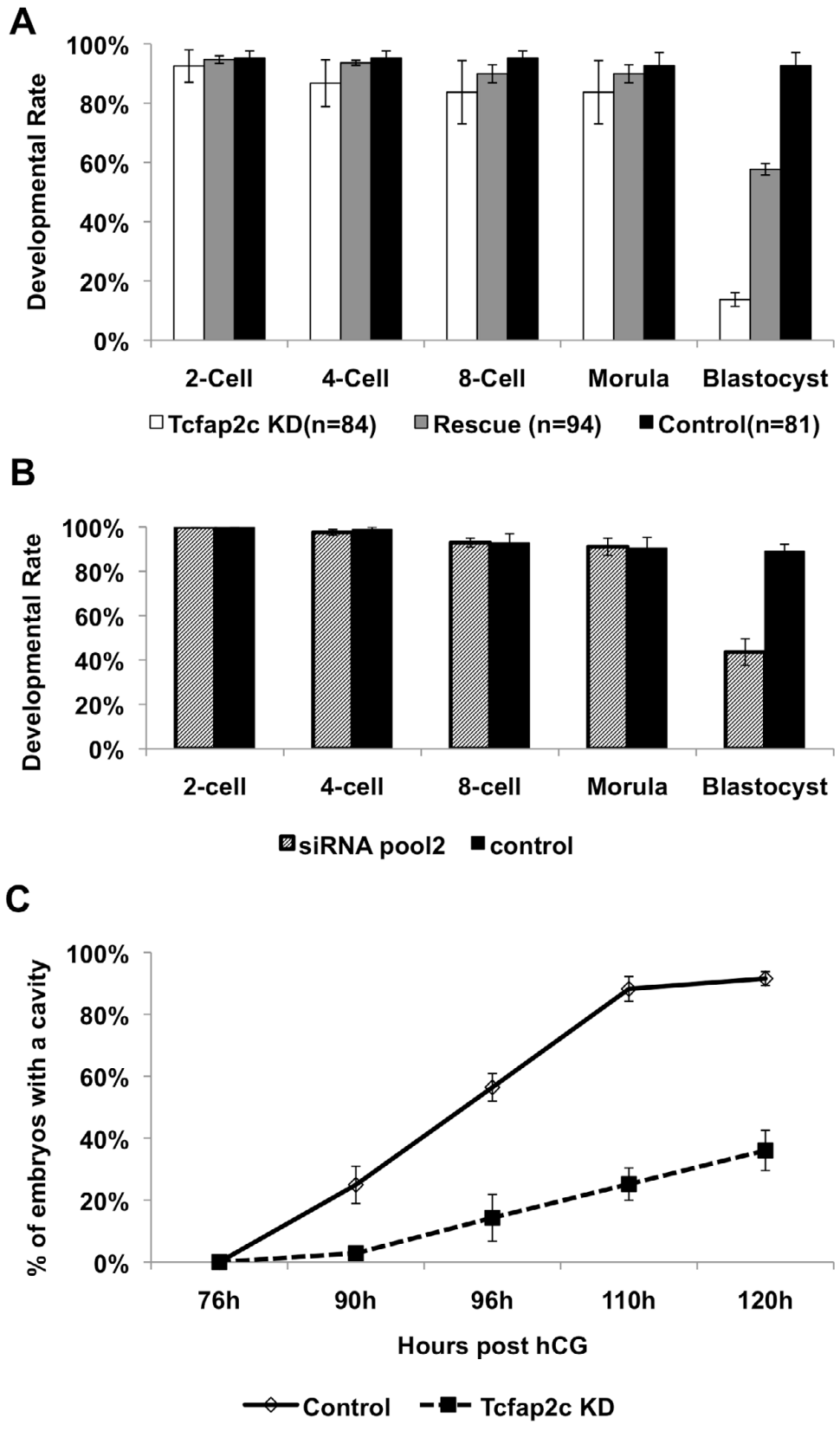

Fig. S3. siRNA-mediated knockdown of Tcfap2c in mouse preimplantation embryos. (**A**) Effect of *Tcfap2c* siRNA on mouse preimplantation development (*Tcfap2c* siRNA pool 1). A second set of embryos was co-injected with both *Tcfap2c* siRNA (pool 1) and *Tcfap2c* mRNA (rescue). (**B**) Developmental rates of preimplantation embryos injected with a second pool of *Tcfap2c* siRNA. (**C**) Timing of cavity formation in Tcfap2c KD embryos (*Tcfap2c* siRNA pool 1). Error bars represent mean ± s.e.m.

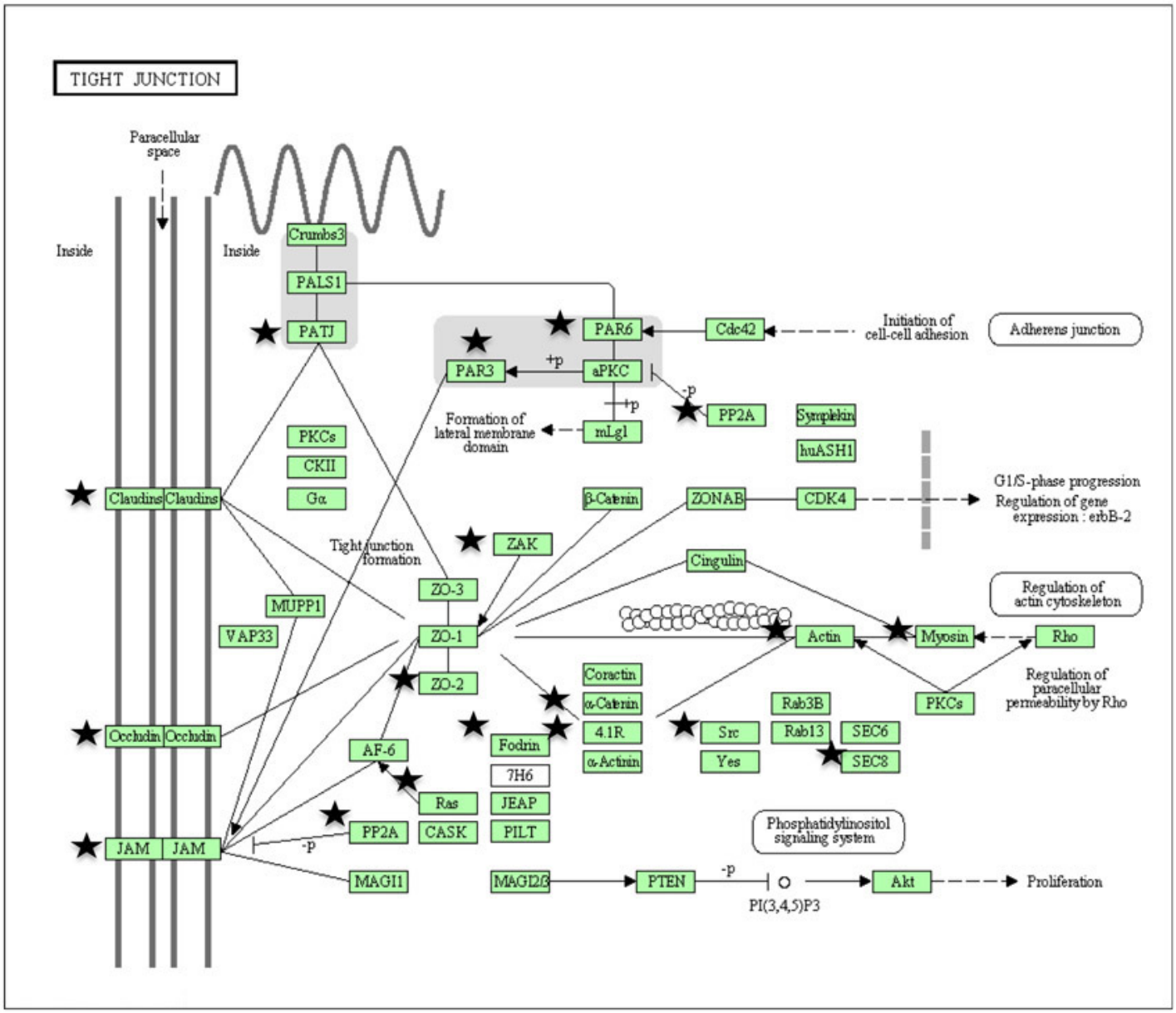

Fig. S4. Tight junction pathway map. Tcfap2c target genes are found in TJ and paracellular sealing pathways. Genes selected using the DAVID pathway analysis tool are marked with a star. Pathway map 04530 is adapted with permission from the KEGG database (http://www.genome.jp/kegg-bin/show_pathway?map04530).

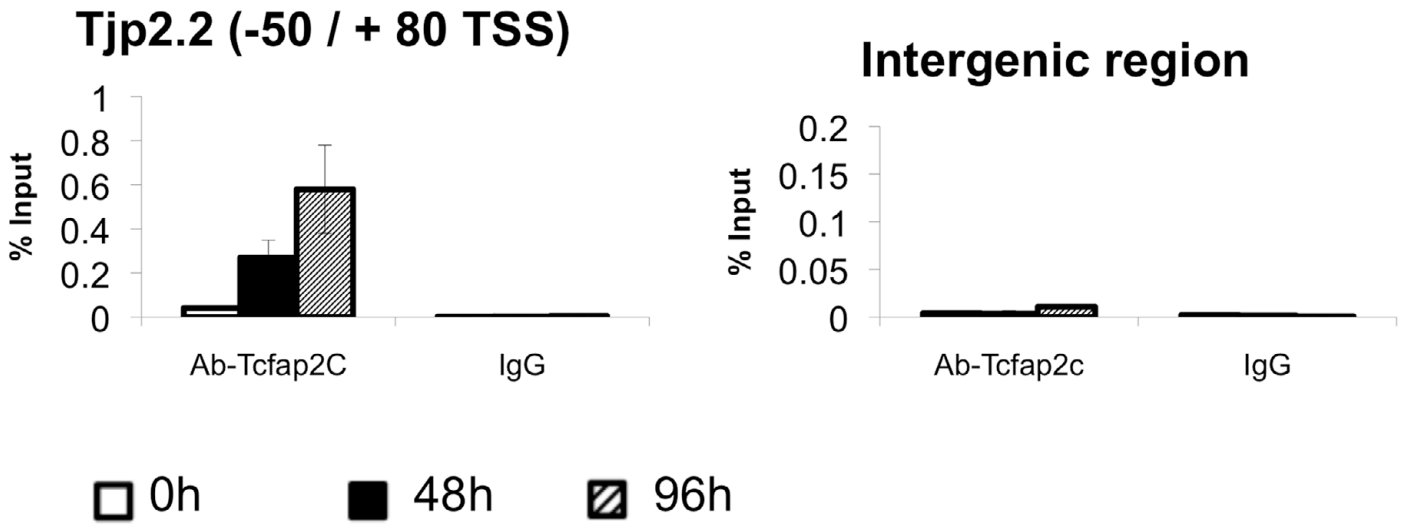

Fig. S5. ChIP analysis of Tcfap2c-bound genes. The promoter regions of *Tjp2* variant 2 and *Fn1* were occupied by Tcfap2c in Cdx2-inducible ES cells, but enrichment was not observed at an intergenic region (negative control). Error bars represent mean ± s.e.m.

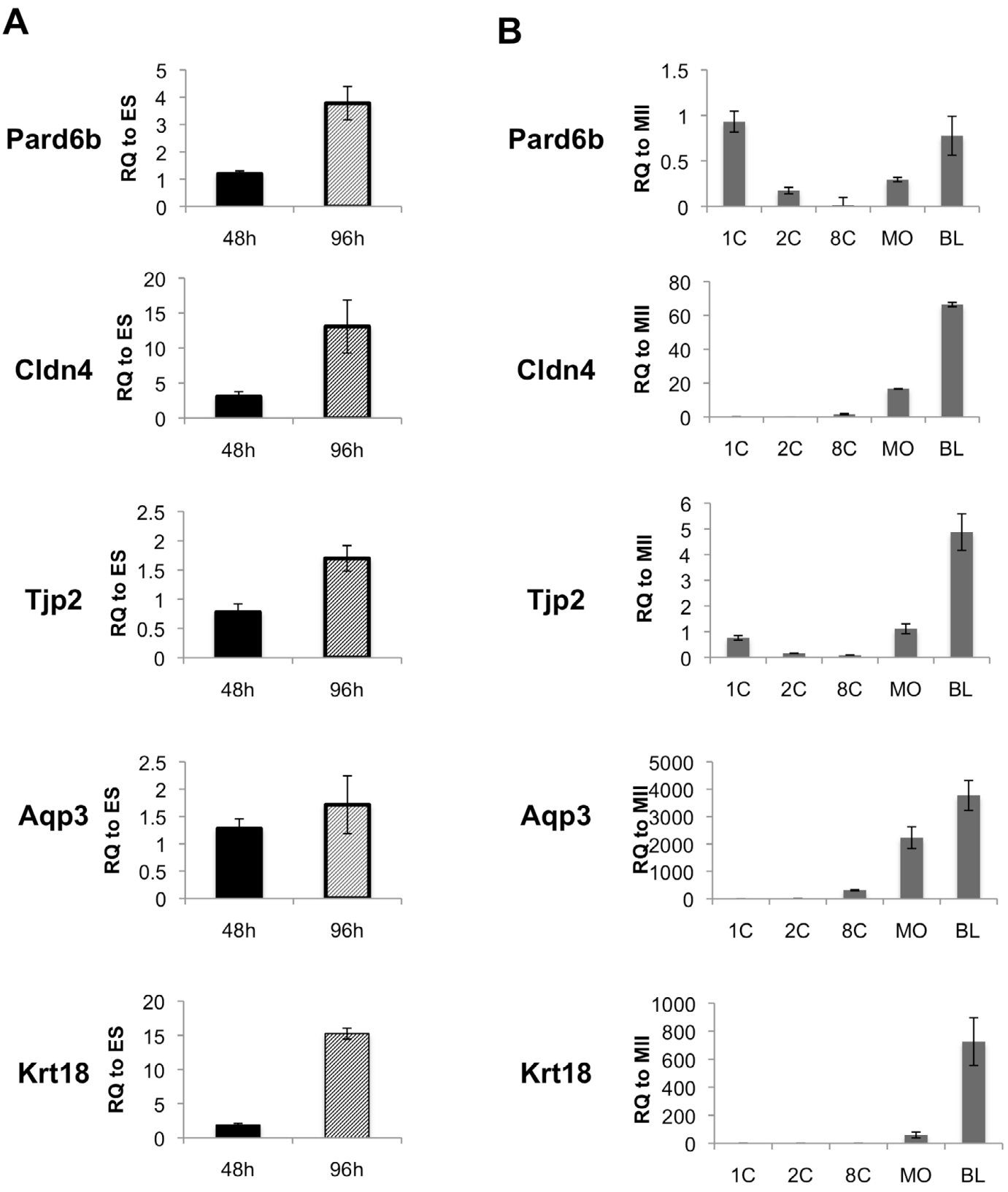

Fig. S6. Tcfap2c target genes are upregulated during ES cell differentiation into TE-like cells and during blastocyst formation. (**A**) Real-time RT-PCR analysis of Tcfap2c target genes in Cdx2-inducible ES cells at 0, 48 and 96 hours post-induction. Key blastocyst formation genes are upregulated during ES cell differentiation into TE-like cells. (**B**) Real-time RT-PCR analysis of *Pard6b*, *Tjp2*, *Cldn4*, *Aqp3* and *Krt18* transcripts in one-cell, two-cell, four-cell, eight-cell, morula, and blastocyst stage embryos. Error bars represent mean ± s.e.m.

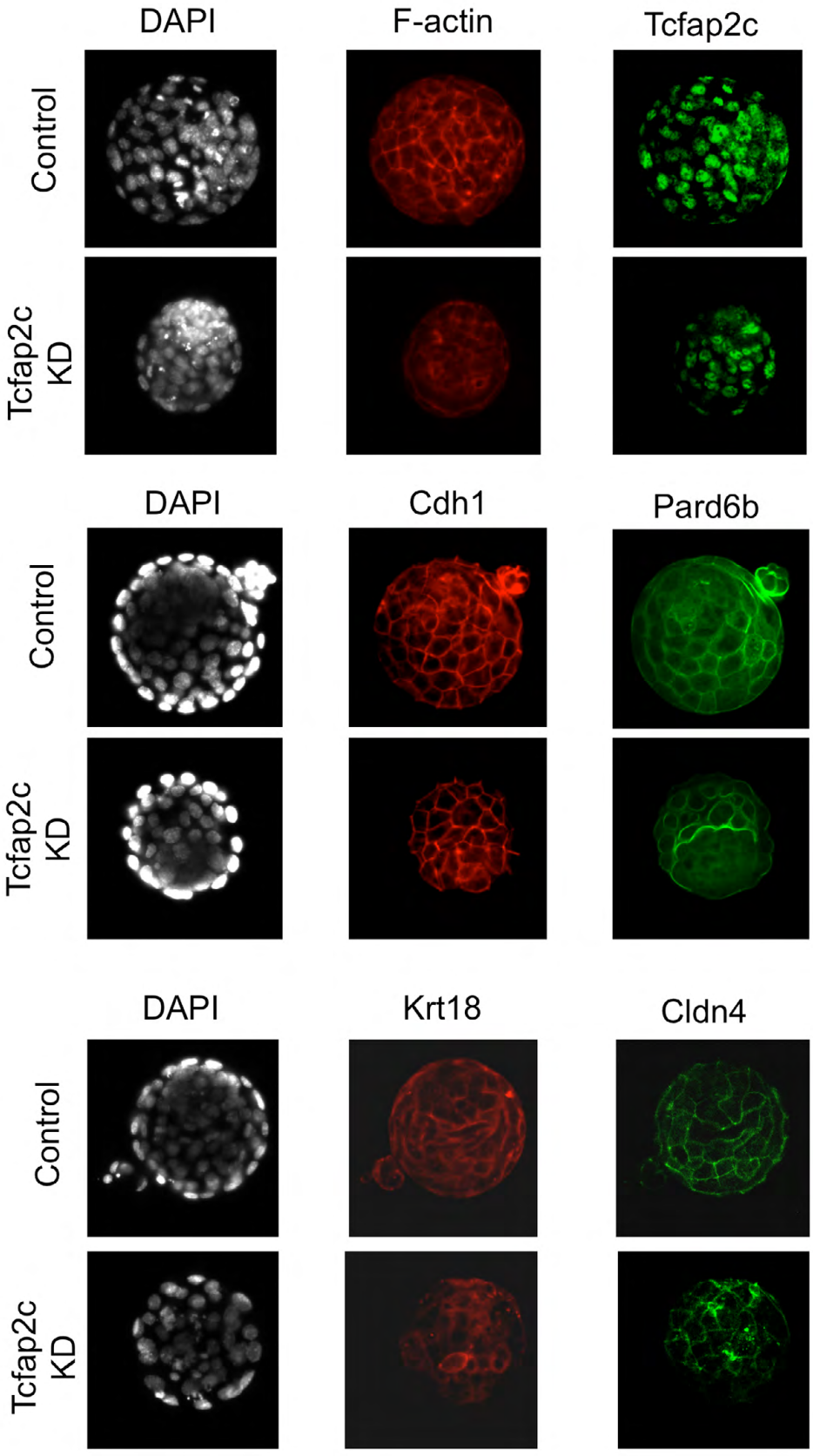

Fig. S7. Expression and subcellular localization of Pard6b, Cldn4 and Krt18 are disrupted in Tcfap2c KD blastocysts. Tcfap2c KD embryos that developed into blastocysts were stained for Tcfap2c, Pard6b, Cdh1, Cldn4, Krt18 and F-actin. Embryos were counterstained with DAPI.

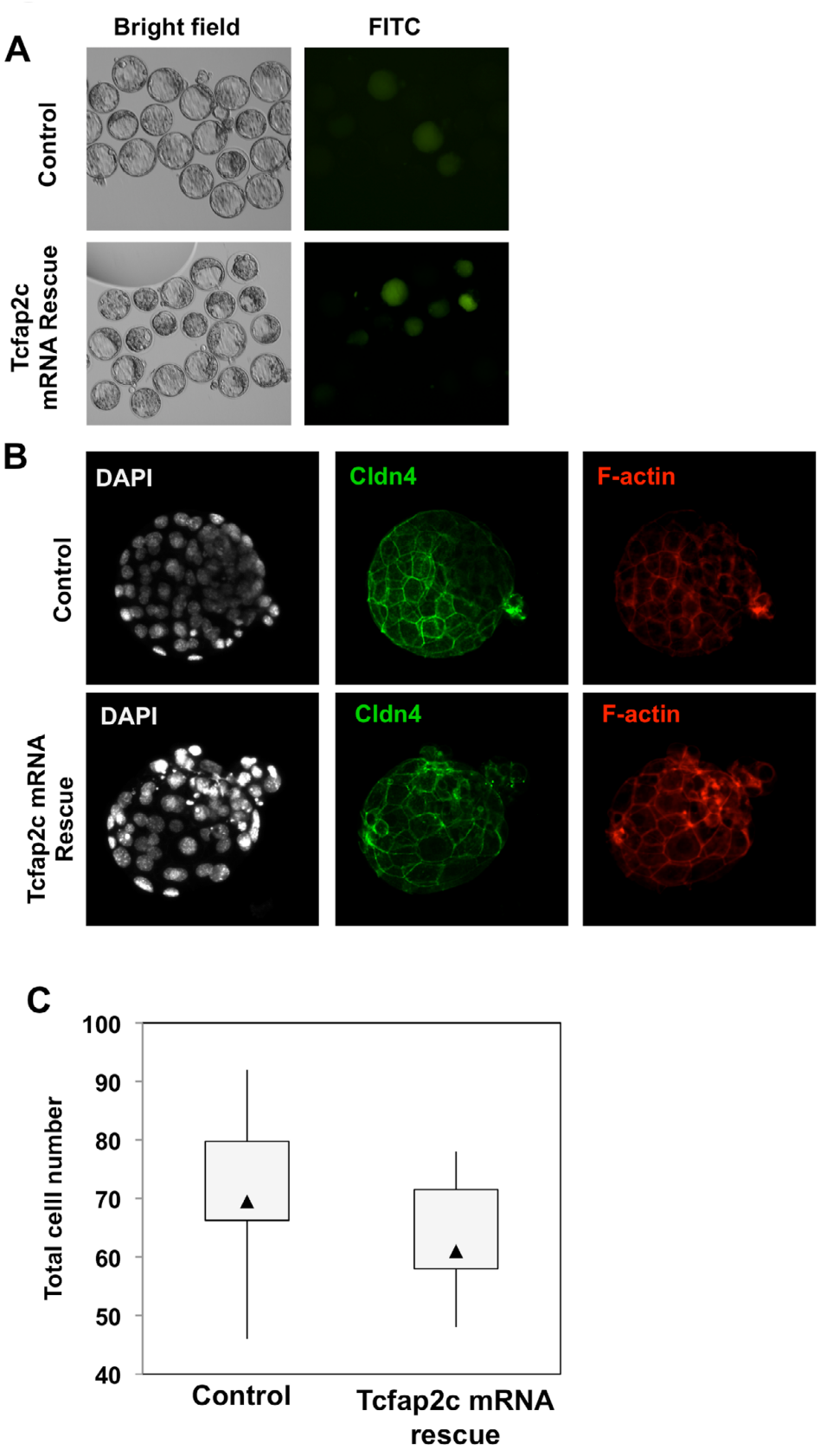

Fig. S8. Injection of *Tcfap2c* mRNA into Tcfap2c KD embryos restored paracellular sealing and TJ assembly. *Tcfap2c* mRNA-rescued blastocysts were subjected to an FITC-dextran assay and stained for TJ and cytoskeleton proteins. (**A**) Brightfield and FITC images of control and rescued blastocysts. (**B**) ICC analysis of Cldn4 and F-actin in control and rescued blastocysts. Embryos were counterstained with DAPI. (**C**) Box plot analysis of total cell numbers in *Tcfap2c* mRNA-rescued and control blastocysts. Small triangle denotes median; bars represent maximum and minimum.

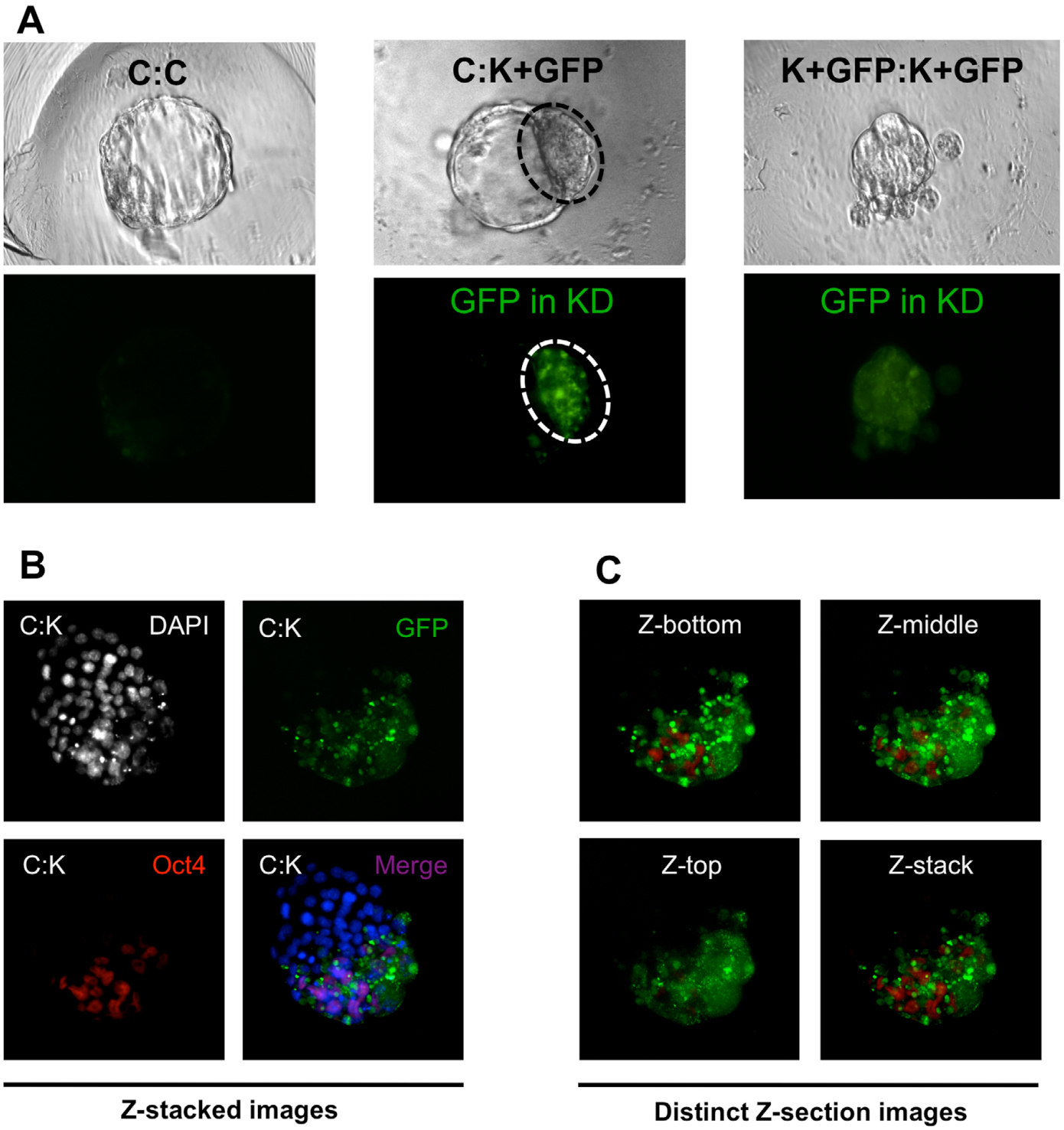

Fig. S9. Tcfap2c KD blastomeres allocate to the ICM in chimeras. GFP$^+$/Tcfap2c KD eight-cell embryos were aggregated with control eight-cell embryos (C:K) and cultured to the blastocyst stage. (**A**) GFP$^+$/Tcfap2c KD blastomeres contribute to the ICM. Dashed oval denotes GFP$^+$ ICM. (**B**) Combined *z*-stack showing the localization of GFP (Tcfap2c KD cells) and Oct4 in the ICM. Embryos were counterstained with DAPI. (**C**) Individual optical sections showing the colocalization of GFP (Tcfap2c KD cells) and Oct4 in the ICM of C:K blastocysts.

Table S1. Primer sequences

Gene	Forward	Reverse
Cdh1	TCCTTGTTCGGCTATGTGTC	GGCATGCACCTAAGAATCAG
beta-catenin	GTTCGCCTTCATTATGGACTGCC	ATAGCACCCTGTTCCCGCAAAG
Pard6b	ATGATGACAACTACCACAAGGCGG	GGACGAGCCGAGATATGAAGATGC
Pard3	GAGACTCTACGGAGGTCCATGT	TCGGTCATCCAGTTCTGTCTCG
Inadl	CAGCAAGTATGCCTTGGTCT	GTGGTCTAGGGTTTCCACCT
Cldn4	CGCTACTCTTGCCATTACG	ACTCAGCACACCATGACTTG
Cldn6	CATTACATGGCCTGCTATTC	CACATAATTCTTGGTGGGATATT
Cldn7	AGGGTCTGCTCTGGTCCTT	GTACGCAGCTTTGCTTTCA
Ocln	ACGTCGTGGACCGGTATC	AAAAACAGTGGTGGGGAAC
F11r	ACTGGGATTCTCCTGGATTC	CCACCAAAGAGCCAAGACTA
Jam2	TGAGGCAGCTCTAGCAGTTT	CTGTGTTCAGCTCCTCCAGT
Tjp1	TTTGGGCTGTGCATCTGA	TGCTTTATTGCTGCAGAGG
Tjp2	CCCCGAGGAGGAGGAGTA	GGGTCCCCACCGTATCTC
Atp1b3	CTGCACTCTTCACATTCACA	CACAGAATATGGCTTTAGGA
Aqp3	GTGGCTCAGGTGGTGCTCAG	CACATTGCGAAGGTCACAGCG
Aqp9	GAAGGACCGAGCCAAGAAGAAC	AGCAATAGAGCCACATCCAAGG
Krt18	GGGTCCTTCTGCATCTGGAG	GGCATCGTTGAGACTGAAATC
Fn1	TGTAGGAGAACAGTGGCAGAAA	CAGGTCTACGGCAGTTGTCA
Rock2	GGTACCTGTACATGGTGATGGAGT	TGCAAGCACTACTTCAGCAGTG
P21	CCGTTGTCTCTTCGGTCCC	CATGAGCGCATCGCAATC
P53	GCGTAAACGCTTCGAGATGTT	TTTTTATGGCGGGAAGTAGACTG
Hdac1	GTGCCTGCTTAGGAGCTCTG	CCTCCACCCTACAGAATTGG
ChIP primers	Forward	Reverse
Pard6b	GGGCACTCAGCATGAACC	GCCACTCTGCGTCCTCAC
Cldn4	GGCTGGGGGAGATGATAGTT	CCACAGGTGCTGCAGTTAAA
Tjp2	ATAGGCACTTGTCCCCCTTT	CAGGTACTCGCCTGGAGAAG
Aqp3	GTGCCTTGCGCTAGCTACTT	GTGAAGCATCTCCCCACAAC
Krt18	GTGAGGATGGCAGGTATGCT	GCGAGAGACCAGAACAGGAG
p21	TGTCTGGATATCGCTGTGGA	AAGGAGTGGGTTGGTCCTG
Intergenic negative control	TTTTCAGTTCACACATATAAAGCAGA	TGTTGTTGTTGTTGCTTCACTG

Table S2. Putative Tcfap2c target genes

Table S3. Enriched GO terms and biological processes of putative Tcfap2c target genes in blastocysts

GO term	Description	*P*-value	Enrichment
GO:0060644	Mammary gland epithelial cell differentiation	1.65E–04	101.86
GO:0071345	Cellular response to cytokine stimulus	2.37E–04	10.8
GO:0019221	Cytokine-mediated signaling pathway	8.53E–04	11.88

Table S4. Pathway analysis of genes bound by Tcfap2c and upregulated at the blastocyst stage

Pathway	Gene counts	*P*-value
Tight junction	24	4.83E–07
Regulation of actin cytoskeleton	26	1.68E–04
Focal adhesion	24	4.04E–04
Amino sugar and nucleotide sugar metabolism	9	0.002641929
Adherens junction	12	0.002738967
Pathways in cancer	30	0.006226075

Table S5. Tcfap2c target genes with known functions/phenotypes

Gene	Type of protein	Function/phenotype	Manipulations/methods	References
Pard6b	Regulation of junction assembly and polarization/cytoplasmic adaptor protein	Embryos failed to form blastocyst cavity; abnormal Tjp1 localization; Prkcz absent	shRNA plasmid injection	(Alarcon, 2010)
Cldn4	Tight junction-associated transmembrane protein	Absent or immature blastocoel cavity without expansion	Specific inhibitor treatment	(Moriwaki et al., 2007)
Cldn6	Tight junction-associated transmembrane protein	Absent or immature blastocoel cavity without expansion	Specific inhibitor treatment	(Moriwaki et al., 2007)
Cldn7	Tight junction-associated transmembrane protein	Expressed during preimplantation development; function in preimplantation development?	RT-PCR	(Moriwaki et al., 2007)
Tjp1	Cytoplasmic adaptor protein/regulation of junction assembly	KO embryos failed to develop beyond E11.5; KD embryos exhibited a decrease in blastocyst formation	Knockout, siRNA microinjection	(Katsuno et al., 2008; Sheth et al., 2008)
Tjp2	Cytoplasmic adaptor protein/regulation of junction assembly	KO embryos die around gastrulation; KD embryos showed delayed blastocoel formation and upregulation of Tjp1	Knockout, siRNA microinjection	(Katsuno et al., 2008; Sheth et al., 2008; Xu et al., 2008)
Atp1b1	Ion gradient	KD embryos fail to develop to the blastocyst stage; abnormal distribution of Ocln and Tjp1	siRNA injection	(Madan et al., 2007)
Atp1b3	Ion gradient	A gene preferentially expressed in human TE and associated with blastocyst formation in bovine	Human microarray data; a subtractive cDNA library in bovine	(Adjaye et al., 2005; Goossens et al., 2007)
Atp1a1	Ion gradient	KO embryos underwent compaction and cavitation, but showed aberrant blastocyst formation such as cell dissociation in vitro and peri-implantation lethality in vivo	Knockout	(Barcroft et al., 2004)
Apq3	Water channel protein	Aqp3 is localized on the basolateral membrane domain of the TE; mediates trans-trophectodermal water movement during cavitation	Hyperosmotic treatment	(Barcroft et al., 2003)
Apq9	Water channel protein	Aqp9 is localized at the apical membrane of the TE; mediates trans-trophectodermal water movement during cavitation	Hyperosmotic treatment	(Barcroft et al., 2003)
Cdh1	Adhesion	KO of zygotic Cdh1 results in failure to cavitate after compaction; maternal KO showed delayed compaction but normal development by zygotic expression	Knockout	(Larue et al., 1994; Riethmacher et al., 1995; de Vries et al., 2004)
Ocln	Tight junction-associated transmembrane protein	Inhibition of blastocoel formation and impaired paracellular sealing of TE; Occludin–/– mice born with no gross phenotype in the expected Mendelian ratios	Neutralizing antibodies	(Saitou et al., 2000; Kim et al., 2004)
Inadl/Patj	Regulation of junction assembly and polarization/cytoplasmic adaptor protein	Disruption of Patj expression in mice leads to delayed tight junction formation as well as defects in cell polarization	siRNA transfection	(Shin et al., 2005)
Jam2	Tight junction-associated transmembrane protein	Jam2 interacts with Tjp1 and Par3 in endothelia cells; role in blastocyst formation?	In vitro GST-pulldown assay	(Ebnet et al., 2003)
F11r/Jam 1	Tight junction-associated transmembrane protein	Compacted embryo treated with neutralizing antibodies showed delayed blastocoel cavity formation	Neutralizing antibodies	(Thomas et al., 2004)
Fn1	Tight junction-associated protein	Expression is upregulated during blastocyst formation in bovine; role in preimplantation development?	A subtractive cDNA library in bovine	(Goossens et al., 2007)
Rock2	Downstream effector of small GTPase	Mouse embryos treated with an inhibitor developed to the morula stage, but fail to form blastocysts	Specific inhibitor of Rho-kinase treatment	(Kawagishi et al., 2004)
Krt18	Cytoskeleton, intermediate filament	KD bovine embryos exhibit a decrease in blastocyst formation	dsRNA injection	(Goossens et al., 2007)