

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

Inchul Choi¹, Timothy S. Carey², Catherine A. Wilson¹ and Jason G. Knott^{1,2,*}

SUMMARY

The trophoblast 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 H₂O 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-2 γ (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 trophoblast epithelium. Aggregation of control eight-cell embryos with Tcfap2c knockdown embryos rescued blastocyst formation via direct contribution to the trophoblast 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, Tcfap2c

INTRODUCTION

The blastocyst trophoblast (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 H₂O 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-2 γ (Tcfap2c; Tcfap2c – 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.

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

*Author for correspondence (knottj@msu.edu)

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% O₂, 5% CO₂, 90% N₂. 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, Holliston, 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): AAGCUGAGUCCCUAGUAA, GCACGGGACUUCGCCUAUG, AGCGGUGGCUGACUAUUUA and CCGCAGUGCAGAAUUUAU. *Tcfap2c* (pool 2): UGAAAGGUGCUACGAGUUU, CAGAUAAAG-GGAUCGAUCA, GCAAAGGACCCAUUUCGAU and UGAGAAAUG-GGGAUUCGAUU. *p21*: CGAGAACGGUGGAACUUUG, GAACAU-CUCAGGGCCGAAA, GGAGCAAAGUGUGCCGUUG and GGUGAU-GUCCGACCUGUUC. Control: UAAGGCUAUGAAGAGAUAC, AUGUAUUGGCCUGUAUUAG, AUGAACGUGAAUUGCUCUA and UGGUUACAUGUCACUAA.

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 GOrrilla (<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). *Ubt1* 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 tetroxide 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 \pm 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 (Kuckenberget al., 2010). However, the biological role of *Tcfap2c* during blastocyst formation has not been clarified. To address this, we first examined the expression of *Tcfap2c* 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 μ M *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).

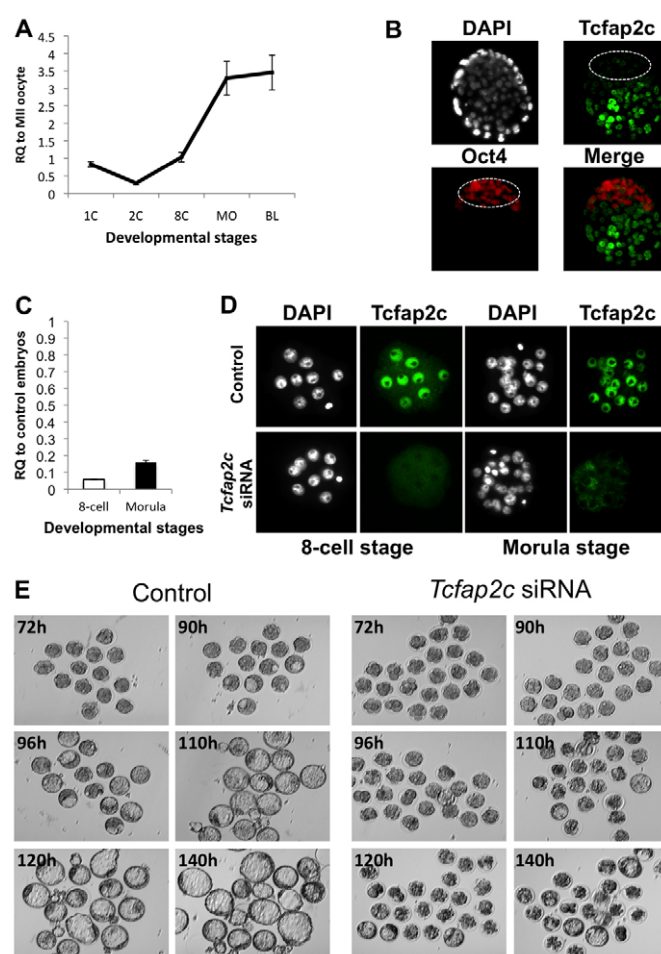


Fig. 1. Developmental expression and RNAi-mediated ablation of *Tcfap2c* in mouse preimplantation embryos. (A) qRT-PCR analysis of *Tcfap2c* transcripts in mouse MII oocytes and preimplantation embryos. Expression data from each stage were normalized to exogenous *GFP* and are relative to MII oocytes. 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 trophoderm (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 \pm 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 μ M 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).

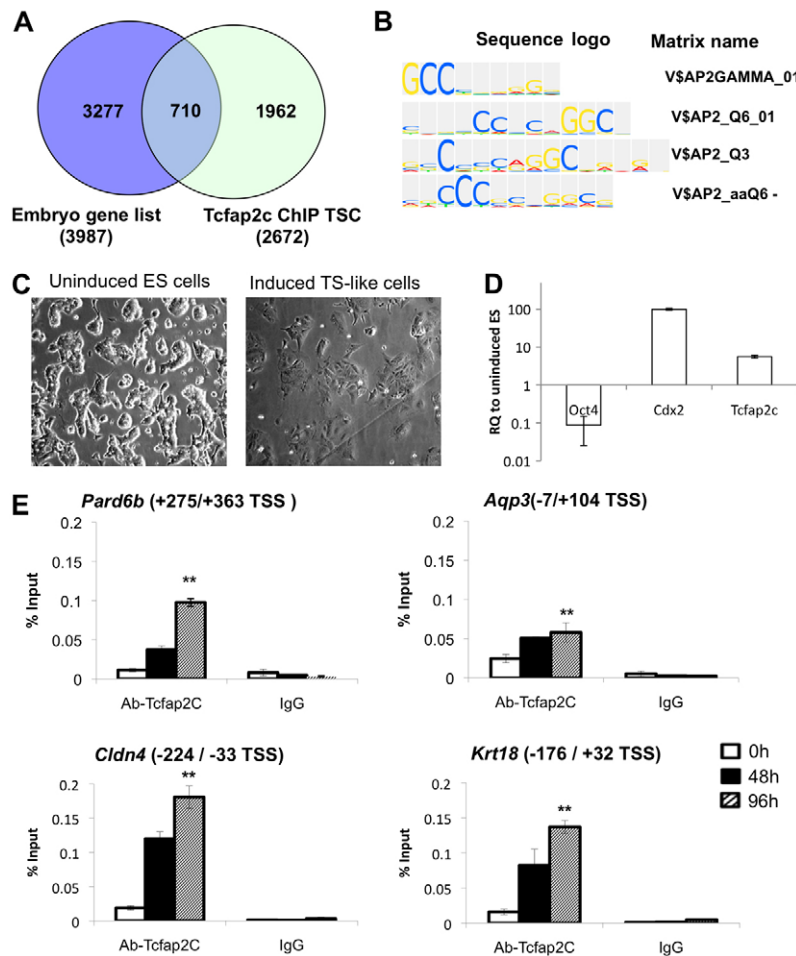


Fig. 2. Tcfap2c binding is enriched on genes that are upregulated during the morula-to-blastocyst transition.

(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 \pm 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*, *Fnl1*), TJ assembly (*Cldn4*, *Cldn6*, *Cldn7*, *Tjp1*, *Tjp2*, *Ocln*, *Inadl*, *Jam2*, *Fllr*), ion gradients (*Atp1b1*, *Atp1b3*, *Atp1a1*) and the formation of membrane H₂O 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*, *Fnl1*, *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*, *Fnl1* 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 *Fllr* (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 H₂O 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 $\geq 50\%$ 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 H₂O 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

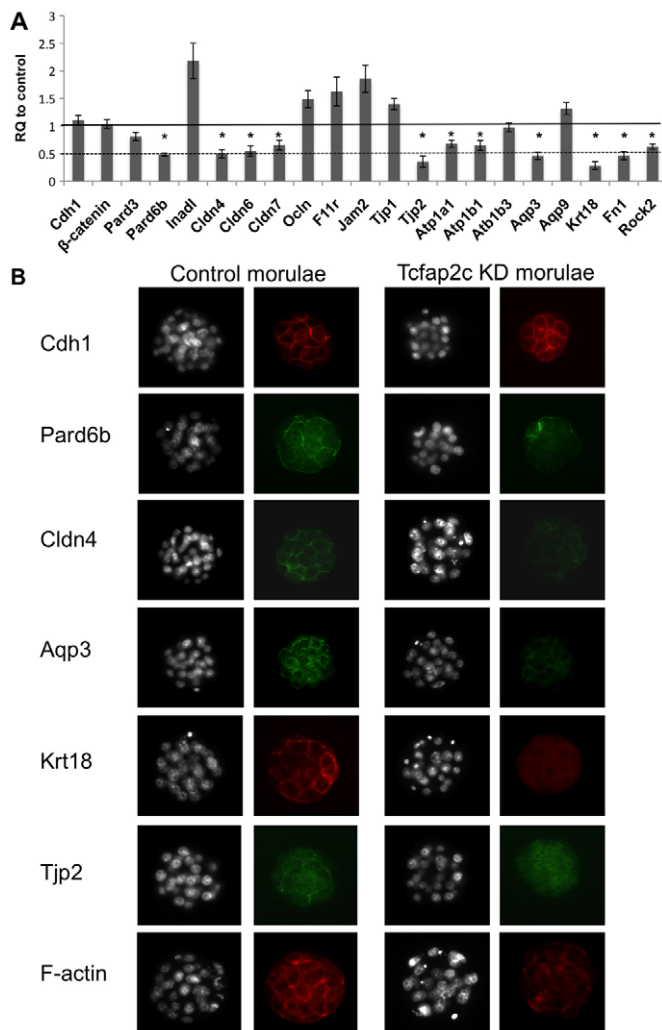


Fig. 3. Tcfap2c regulates genes that are crucial for cell polarity, TJ assembly and ion gradients. (A) qRT-PCR analysis of Tcfap2c target genes in Tcfap2c KD and control morulae (90 hph). Genes important for cell polarity, tight junction (TJ) assembly and fluid gradient were significantly downregulated in Tcfap2c KD embryos. Error bars indicate mean \pm 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.

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-

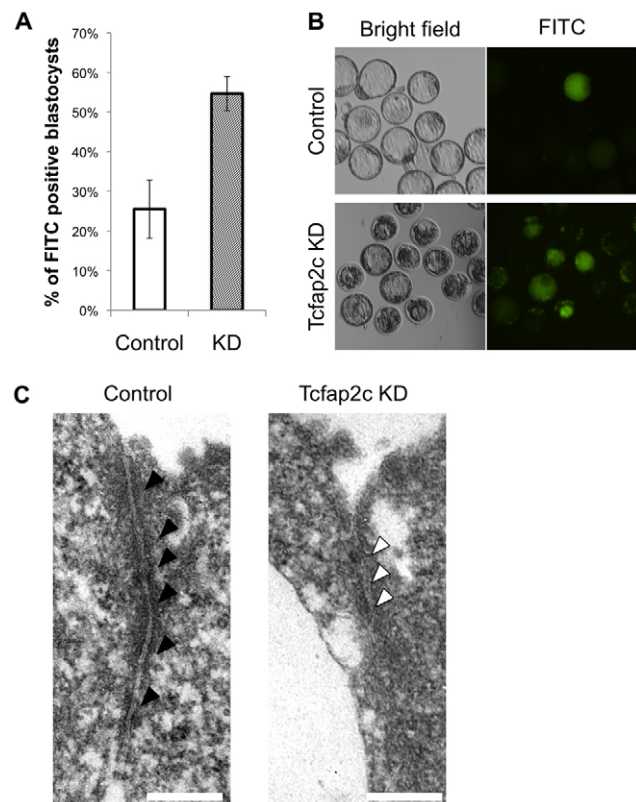


Fig. 4. Disruption of TJ complexes and paracellular sealing in Tcfap2c KD embryos. (A) A 4 kDa FITC-conjugated dextran was used to test the permeability of TJs in Tcfap2c KD and control blastocysts. A difference in permeability was observed between Tcfap2c KD and control blastocysts. Error bars indicate mean \pm 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.

dextran than control blastocysts (55 \pm 4% versus 25 \pm 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

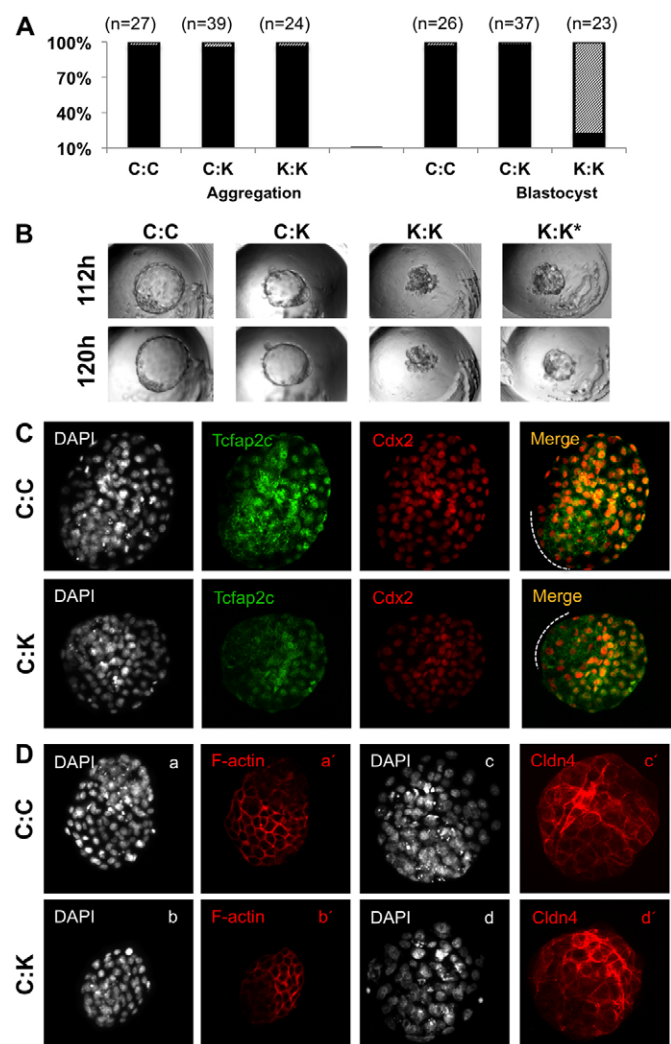


Fig. 5. Aggregation of control embryos with Tcfap2c KD embryos rescues blastocyst formation. (A) Developmental rates of control eight-cell embryos aggregated with Tcfap2c KD eight-cell embryos. To the left is shown the percentage of aggregated (black) or unaggregated (hatched) embryos and to the right 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;

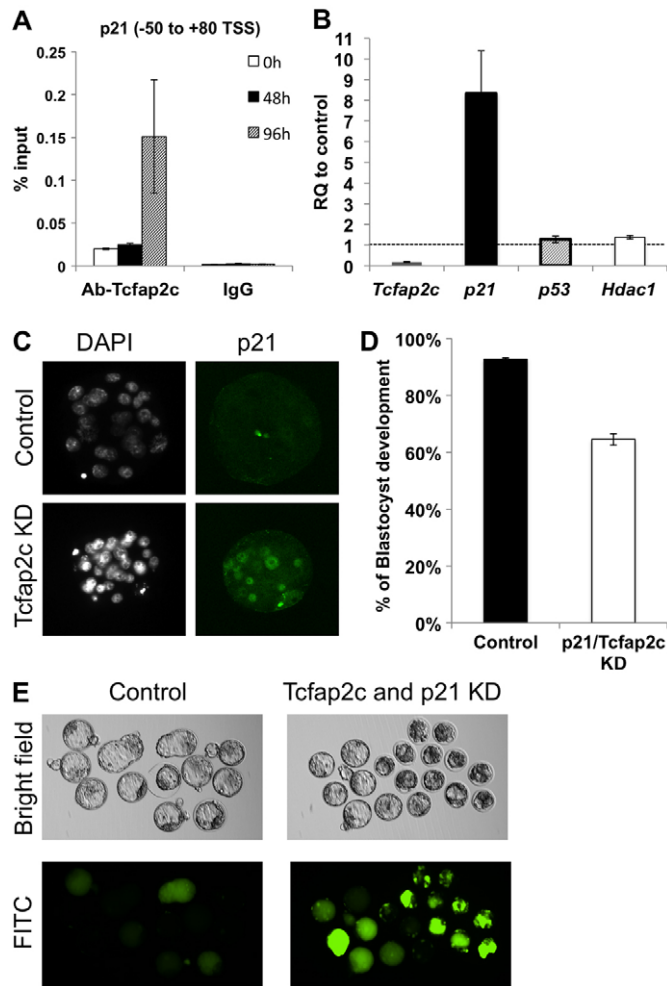


Fig. 6. Tcfap2c regulates cellular proliferation via direct repression of p21. (A) ChIP analysis of Tcfap2c binding to the p21 proximal promoter. (B) p21 is upregulated in Tcfap2c KD morulae. Upregulation of p21 is independent of p53 and Hdac1 expression. (C) Expression and subcellular localization of 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.

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

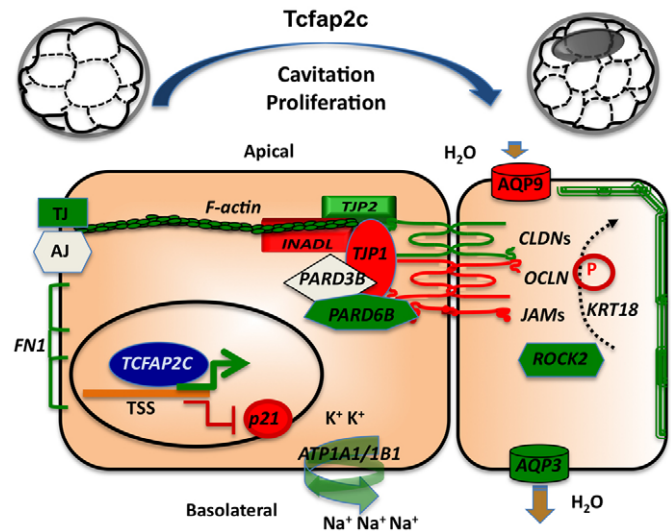


Fig. 7. Model for Tcfap2c-mediated transcriptional regulation of blastocyst formation. Tcfap2c regulates a diverse group of genes during the morula-to-blastocyst transition. Genes shown in green and red are positively and negatively regulated by Tcfap2c, respectively; genes in white are not regulated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

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) (Kuckenberger 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 (Kuckenberger 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).

Acknowledgements

We thank Dr George Smith for critically reading the manuscript.

Funding

This research was supported by a grant from the National Institute of General Medical Sciences [R01GM095347 to J.G.K.]. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.086645/-/DC1>

References

- Adiga, S. K., Toyoshima, M., Shiraishi, K., Shimura, T., Takeda, J., Taga, M., Nagai, H., Kumar, P. and Niwa, O. (2007). p21 provides stage specific DNA damage control to preimplantation embryos. *Oncogene* **26**, 6141-6149.
- Adjaye, J., Huntriss, J., Herwig, R., BenKahla, A., Brink, T. C., Wierling, C., Hultschig, C., Groth, D., Yaspo, M.-L., Picton, H. M. et al. (2005). Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells. *Stem Cells* **23**, 1514-1525.
- Alarcon, V. B. (2010). Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo. *Biol. Reprod.* **83**, 347-358.
- Auman, H. J., Nottoli, T., Lakiza, O., Winger, Q., Donaldson, S. and Williams, T. (2002). Transcription factor AP-2gamma is essential in the extra-embryonic lineages for early postimplantation development. *Development* **129**, 2733-2747.
- Barcroft, L. C., Offenberger, H., Thomsen, P. and Watson, A. J. (2003). Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. *Dev. Biol.* **256**, 342-354.
- Barcroft, L. C., Moseley, A. E., Lingrel, J. B. and Watson, A. J. (2004). Deletion of the Na/K-ATPase alpha1-subunit gene (Atp1a1) does not prevent cavitation of the preimplantation mouse embryo. *Mech. Dev.* **121**, 417-426.
- Blake, D., Farquhar, C., Johnson, N. and Proctor, M. (2007). Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst. Rev.* doi: 10.1002/14651858.CD002118.pub3.
- Brennan, K., Offiah, G., McSherry, E. A. and Hopkins, A. M. (2010). Tight junctions: a barrier to the initiation and progression of breast cancer? *J. Biomed. Biotechnol.* **2010**, 460607.
- Civico, S., Agell, N., Bachs, O., Vanrell, J. A. and Balasch, J. (2002). Increased expression of the cyclin-dependent kinase inhibitor p27 in cleavage-stage human embryos exhibiting developmental arrest. *Mol. Hum. Reprod.* **8**, 919-922.
- Cockburn, K. and Rossant, J. (2010). Making the blastocyst: lessons from the mouse. *J. Clin. Invest.* **120**, 995-1003.
- Copp, A. J. (1978). Interaction between inner cell mass and trophectoderm of the mouse blastocyst. I. A study of cellular proliferation. *J. Embryol. Exp. Morphol.* **48**, 109-125.
- de Vries, W. N., Evsikov, A. V., Haac, B. E., Fancher, K. S., Holbrook, A. E., Kemler, R., Solter, D. and Knowles, B. B. (2004). Maternal beta-catenin and E-cadherin in mouse development. *Development* **131**, 4435-4445.
- Dietrich, J.-E. and Hiiragi, T. (2007). Stochastic patterning in the mouse pre-implantation embryo. *Development* **134**, 4219-4231.
- Ebnet, K., Aurrand-Lions, M., Kuhn, A., Kiefer, F., Butz, S., Zander, K., Meyer zu Bruckwedde, M.-K., Suzuki, A., Imhof, B. A. and Vestweber, D. (2003). The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity. *J. Cell Sci.* **116**, 3879-3891.
- Eckert, D., Buhl, S., Weber, S., Jäger, R. and Schorle, H. (2005). The AP-2 family of transcription factors. *Genome Biol.* **6**, 246.

- Eckert, J. J. and Fleming, T. P. (2008). Tight junction biogenesis during early development. *Biochim. Biophys. Acta* **1778**, 717-728.
- Gardner, D. K., Surrey, E., Minjarez, D., Leitz, A., Stevens, J. and Schoolcraft, W. B. (2004). Single blastocyst transfer: a prospective randomized trial. *Fertil. Steril.* **81**, 551-555.
- Gartel, A. L. and Tyner, A. L. (1999). Transcriptional regulation of the p21(WAF1/CIP1) gene. *Exp. Cell Res.* **246**, 280-289.
- Giannatselis, H., Calder, M. and Watson, A. J. (2011). Ouabain stimulates a Na⁺/K⁺-ATPase-mediated SFK-activated signalling pathway that regulates tight junction function in the mouse blastocyst. *PLoS ONE* **6**, e23704.
- Goossens, K., Van Soom, A., Van Poucke, M., Vandaele, L., Vandesompele, J., Van Zeveren, A. and Peelman, L. J. (2007). Identification and expression analysis of genes associated with bovine blastocyst formation. *BMC Dev. Biol.* **7**, 64.
- Goossens, K., Tesfaye, D., Rings, F., Schellander, K., Hölker, M., Van Poucke, M., Van Zeveren, A., Lemahieu, I., Van Soom, A. and Peelman, L. J. (2010). Suppression of keratin 18 gene expression in bovine blastocysts by RNA interference. *Reprod. Fertil. Dev.* **22**, 395-404.
- Home, P., Ray, S., Dutta, D., Bronshteyn, I., Larson, M. and Paul, S. (2009). GATA3 is selectively expressed in the trophectoderm of peri-implantation embryo and directly regulates Cdx2 gene expression. *J. Biol. Chem.* **284**, 28729-28737.
- Katsuno, T., Umeda, K., Matsui, T., Hata, M., Tamura, A., Itoh, M., Takeuchi, K., Fujimori, T., Nabeshima, Y.-i., Noda, T. et al. (2008). Deficiency of zonula occludens-1 causes embryonic lethal phenotype associated with defected yolk sac angiogenesis and apoptosis of embryonic cells. *Mol. Biol. Cell* **19**, 2465-2475.
- Kawagishi, R., Tahara, M., Sawada, K., Ikebuchi, Y., Morishige, K., Sakata, M., Tasaka, K. and Murata, Y. (2004). Rho-kinase is involved in mouse blastocyst cavity formation. *Biochem. Biophys. Res. Commun.* **319**, 643-648.
- Keramari, M., Razavi, J., Ingman, K. A., Patsch, C., Edenhofer, F., Ward, C. M. and Kimber, S. J. (2010). Sox2 is essential for formation of trophectoderm in the preimplantation embryo. *PLoS ONE* **5**, e13952.
- Kidder, B. L. and Palmer, S. (2010). Examination of transcriptional networks reveals an important role for TCFAP2C, SMARCA4, and EOMES in trophoblast stem cell maintenance. *Genome Res.* **20**, 458-472.
- Kidder, B. L., Palmer, S. and Knott, J. G. (2009). SWI/SNF-Brg1 regulates self-renewal and occupies core pluripotency-related genes in embryonic stem cells. *Stem Cells* **27**, 317-328.
- Kim, J., Gye, M. C. and Kim, M. K. (2004). Role of occludin, a tight junction protein, in blastocoel formation, and in the paracellular permeability and differentiation of trophectoderm in preimplantation mouse embryos. *Mol. Cells* **17**, 248-254.
- Krause, G., Winkler, L., Mueller, S. L., Haseloff, R. F., Piontek, J. and Blasig, I. E. (2008). Structure and function of claudins. *Biochim. Biophys. Acta* **1778**, 631-645.
- Kuckenberger, P., Buhl, S., Woynecki, T., van Fürden, B., Tolkunova, E., Seiffe, F., Moser, M., Tomilin, A., Winterhager, E. and Schorle, H. (2010). The transcription factor TCFAP2C/AP-2γ cooperates with CDX2 to maintain trophectoderm formation. *Mol. Cell. Biol.* **30**, 3310-3320.
- Larue, L., Ohsugi, M., Hirchenhain, J. and Kemler, R. (1994). E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc. Natl. Acad. Sci. USA* **91**, 8263-8267.
- Ma, P. and Schultz, R. M. (2008). Histone deacetylase 1 (HDAC1) regulates histone acetylation, development, and gene expression in preimplantation mouse embryos. *Dev. Biol.* **319**, 110-120.
- Madan, P., Rose, K. and Watson, A. J. (2007). Na/K-ATPase β1 subunit expression is required for blastocyst formation and normal assembly of trophectoderm tight junction-associated proteins. *J. Biol. Chem.* **282**, 12127-12134.
- Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M. and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631-642.
- Moriwaki, K., Tsukita, S. and Furuse, M. (2007). Tight junctions containing claudin 4 and 6 are essential for blastocyst formation in preimplantation mouse embryos. *Dev. Biol.* **312**, 509-522.
- Ng, R. K., Dean, W., Dawson, C., Lucifero, D., Madeja, Z., Reik, W. and Hemberger, M. (2008). Epigenetic restriction of embryonic cell lineage fate by methylation of Elf5. *Nat. Cell Biol.* **10**, 1280-1290.
- Nichols, J., Zevnik, B., Anastasiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Schöler, H. and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379-391.
- Nishioka, N., Yamamoto, S., Kiyonari, H., Sato, H., Sawada, A., Ota, M., Nakao, K. and Sasaki, H. (2008). Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mech. Dev.* **125**, 270-283.
- Nishiyama, A., Xin, L., Sharov, A. A., Thomas, M., Mowrer, G., Meyers, E., Piao, Y., Mehta, S., Yee, S., Nakatake, Y. et al. (2009). Uncovering early response of gene regulatory networks in ESCs by systematic induction of transcription factors. *Cell Stem Cell* **5**, 420-433.
- Niwa, H., Toyooka, Y., Shimosato, D., Strumpf, D., Takahashi, K., Yagi, R. and Rossant, J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* **123**, 917-929.
- Parfitt, D.-E. and Zernicka-Goetz, M. (2010). Epigenetic modification affecting expression of cell polarity and cell fate genes to regulate lineage specification in the early mouse embryo. *Mol. Biol. Cell* **21**, 2649-2660.
- Ralston, A., Cox, B. J., Nishioka, N., Sasaki, H., Chea, E., Rugg-Gunn, P., Guo, G., Robson, P., Draper, J. S. and Rossant, J. (2010). Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development* **137**, 395-403.
- Riethmacher, D., Brinkmann, V. and Birchmeier, C. (1995). A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proc. Natl. Acad. Sci. USA* **92**, 855-859.
- Russ, A. P., Wattler, S., Colledge, W. H., Aparicio, S. A., Carlton, M. B. L., Pearce, J. J., Barton, S. C., Surani, M. A., Ryan, K., Nehls, M. C. et al. (2000). Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* **404**, 95-99.
- Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.-D., Fromm, M., Takano, H., Noda, T. and Tsukita, S. (2000). Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol. Biol. Cell* **11**, 4131-4142.
- Schneeberger, E. E. and Lynch, R. D. (2004). The tight junction: a multifunctional complex. *Am. J. Physiol.* **286**, C1213-C1228.
- Sherr, C. J. and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9**, 1149-1163.
- Sherr, C. J. and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501-1512.
- Sheth, B., Nowak, R. L., Anderson, R., Kwong, W. Y., Papenbrock, T. and Fleming, T. P. (2008). Tight junction protein ZO-2 expression and relative function of ZO-1 and ZO-2 during mouse blastocyst formation. *Exp. Cell Res.* **314**, 3356-3368.
- Shin, K., Straight, S. and Margolis, B. (2005). PATJ regulates tight junction formation and polarity in mammalian epithelial cells. *J. Cell Biol.* **168**, 705-711.
- Strumpf, D., Mao, C.-A., Yamanaka, Y., Ralston, A., Chawengsaksophak, K., Beck, F. and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* **132**, 2093-2102.
- Thomas, F. C., Sheth, B., Eckert, J. J., Bazzoni, G., Dejana, E. and Fleming, T. P. (2004). Contribution of JAM-1 to epithelial differentiation and tight-junction biogenesis in the mouse preimplantation embryo. *J. Cell Sci.* **117**, 5599-5608.
- Wang, K., Sengupta, S., Magnani, L., Wilson, C. A., Henry, R. W. and Knott, J. G. (2010). Brg1 is required for Cdx2-mediated repression of Oct4 expression in mouse blastocysts. *PLoS ONE* **5**, e10622.
- Watson, A. J. and Barcroft, L. C. (2001). Regulation of blastocyst formation. *Front. Biosci.* **6**, D708-D730.
- Williams, C. M. J., Scibetta, A. G., Friedrich, J. K., Canosa, M., Berlatto, C., Moss, C. H. and Hurst, H. C. (2009). AP-2γ promotes proliferation in breast tumour cells by direct repression of the CDKN1A gene. *EMBO J.* **28**, 3591-3601.
- Winger, Q., Huang, J., Auman, H. J., Lewandoski, M. and Williams, T. (2006). Analysis of transcription factor AP-2 expression and function during mouse preimplantation development. *Biol. Reprod.* **75**, 324-333.
- Xu, J., Kausalya, P. J., Phua, D. C. Y., Ali, S. M., Hossain, Z. and Hunziker, W. (2008). Early embryonic lethality of mice lacking ZO-2, but not ZO-3, reveals critical and nonredundant roles for individual zonula occludens proteins in mammalian development. *Mol. Cell. Biol.* **28**, 1669-1678.
- Yagi, R., Kohn, M. J., Karavanova, I., Kaneko, K. J., Vullhorst, D., DePamphilis, M. L. and Buonanno, A. (2007). Transcription factor TEAD4 specifies the trophectoderm lineage at the beginning of mammalian development. *Development* **134**, 3827-3836.
- Zupkovitz, G., Grausenburger, R., Brunmeir, R., Senese, S., Tischler, J., Jurkin, J., Rembold, M., Meunier, D., Egger, G., Lagger, S. et al. (2010). The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. *Mol. Cell. Biol.* **30**, 1171-1181.