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ABSTRACT

Mammalian Host-Cell Factor 1 (HCF-1), a transcriptional co-regulator, plays important roles during the cell-division cycle in cell culture, embryogenesis as well as adult tissue. In mice, HCF-1 is encoded by the X-chromosome-linked Hcfc1 gene. Induced Hcfc1\(^{lox}\)\(^{-}\) heterozygosity with a conditional knockout (cKO) allele in the epiblast of female embryos leads to a mixture of HCF-1-positive and -deficient cells owing to random X-chromosome inactivation. These embryos survive owing to the replacement of all HCF-1-deficient cells by HCF-1-positive cells during E5.5 to E8.5 of development. In contrast, complete epiblast-specific loss of HCF-1 in male embryos, Hcfc1\(^{lox}\)\(^{-}\)\(^{Y}\), leads to embryonic lethality. Here, we characterize this lethality. We show that male epiblast-specific loss of Hcfc1 leads to a developmental arrest at E6.5 with a rapid progressive cell-cycle exit and an associated failure of anterior visceral endoderm migration and primitive streak formation. Subsequently, gastrulation does not take place. We note that the pattern of Hcfc1\(^{lox}\)\(^{-}\)\(^{Y}\) lethality displays many similarities to loss of β-catenin function. These results reveal essential new roles for HCF-1 in early embryonic cell proliferation and development.

Original research article

Epiblast-specific loss of HCF-1 leads to failure in anterior-posterior axis specification

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1. Introduction

In animals, early embryonic development is associated with rapid rounds of cell division, which allow the multicellular embryo to acquire cell numbers sufficient to support cell differentiation and development. These rapid rounds of cell division often short circuit cell-cycle regulators particularly of the G1 phase. Consequently, many G1-phase cell-cycle regulators such as transcriptional activators (e.g., E2Fs), repressors (Retinoblastoma protein (pRb) pocket-protein family), and repressors of repressors (e.g., cyclin–CDK complexes) are not required for early developmental events before embryonic day (E) 8.5, including gastrulation (reviewed in Ciemerzych and Sicinski (2005)).

Here, we study a broadly active transcriptional co-regulator called HCF-1 encoded by the X-chromosome-linked Hcfc1 gene in mice (Frattini et al., 1996; Kristie, 1997). HCF-1, in human a 2035 amino acid protein first identified as a host-cell factor for herpes simplex virus infection (reviewed by Wysocka and Herr (2003)), is required for the proliferation of cells in culture (Goto et al., 1997; Julien and Herr, 2003), at least in part, by its ability to associate with both DNA sequence-specific (e.g., E2F1 and E2F4, THAP11/Ronin, Myc) and chromatin-modifying (e.g., MLL and Set1 histone H3 lysine 4 methyltransferase, Sin3 histone deacetylase and BAP1 deubiquitinase) transcriptional regulators (reviewed by Zargar and Tyagi (2012); see also Thomas et al. (2015)). In culture, HCF-1 is required for both passage from G1 to S phase (Goto et al., 1997) and proper passage through M phase (Reilly and Herr, 2002); promotion of G1-to-S phase passage is linked to the ability of HCF-1 to associate with E2F proteins (Knez et al., 2006; Tyagi et al., 2007; Tyagi and Herr, 2009) and THAP11 (Parker et al., 2014). We have recently described a conditional knock-out (cKO) mouse allele called Hcfc1\(^{lox}\)\(^{tho}\), where the presence of Cre recombinase induces deletion of two essential exons leading to the predicted synthesis of a small inactive truncated 66 amino acid HCF-1 peptide (Minocha et al., 2016). Hcfc1 expression is ubiquitous in embryonic and extraembryonic tissues (Minocha et al., 2016). Because the Hcfc1 gene resides on the X chromosome, female offspring carry two Hcfc1 alleles of which one or the other is randomly inactivated at around E4.5–E5.5 (Clerc and Avner, 2011), whereas male offspring only possess one allele, which remains active throughout development. Epiblast-specific inactivation of the Hcfc1\(^{lox}\)\(^{tho}\) allele (generating an Hcfc1\(^{lox}\)\(^{-}\)\(^{tho}\) allele) by E5.5 does not reduce the viability of heterozygous females but is embryonic lethal in male embryos (Minocha et al., 2016). In the surviving
heterozygous female embryos, HCF-1-deficient cells are progressively and by E8.5 entirely replaced by HCF-1-positive cells carrying the deleted Hcf1<sup>frpkdo</sup> allele on the inactive X chromosome (Minocha et al., 2016).

The progressive loss of HCF-1-deficient cells in an environment in which half the cells remain positive for HCF-1 could be owing to cell competition if HCF-1-deficient cells cannot replicate as efficiently as their wild-type neighbors (Baillon and Basler, 2014). Such a cell-competition effect has been observed in the mouse epiblast as a result of variable levels of Myc oncoprotein (Claveria et al., 2013). Alternatively, HCF-1-deficient cells may simply fail to replicate. These two possibilities can be distinguished by examining the Hcf1<sup>frpkdo</sup>/Y male embryos where no potentially competing embryonic HCF-1-positive cells are present. If heterozygous Hcf1<sup>frpkdo</sup> females eliminate HCF-1-deficient cells by cell competition, the absence of HCF-1-positive compensating cells should rescue epiblast cell replication in Hcf1<sup>frpkdo</sup>/Y males at least transiently.

Here, by analyzing Hcf1<sup>frpkdo</sup> male embryos, we describe the specific requirement for HCF-1 in early mouse embryonic development. Generation of the epiblast-specific Hcf1<sup>frpkdo</sup> allele around E5.5 rapidly halts cell-proliferation, leading to developmental arrest by E6.5 prior to gastrulation. Thus, unlike the many aforementioned G1-phase cell-cycle regulators, which are not essential until after gastrulation, HCF-1 function is required for early embryonic development. Indeed, Hcf1<sup>frpkdo</sup> embryonic cells exit the cell cycle earlier than in heterozygous Hcf1<sup>frpkdo</sup> female embryos, suggesting that loss of HCF-1-deficient cells in Hcf1<sup>frpkdo</sup> heterozygotes is not due to competition. Rather, in the Hcf1<sup>frpkdo</sup> heterozygotes, HCF-1-positive cells appear to support the proliferation of their HCF-1-deficient neighbors.

2. Materials and methods

2.1. Mice

All experimental studies have been performed in compliance with the EU and national legislation rules, as advised by the Leemanic Animal Facility Network (Resal), concerning ethical considerations of transportation, housing, strain maintenance, breeding and experimental use of animals.

Homozygous mice bearing the Hcf1 conditional (lox) allele are referred as Hcf1<sup>frpkdo</sup> in this study (Minocha et al., 2016). The Hcf1<sup>frpkdo</sup> allele contains two loxP sites, one in intron 1 and another in intron 3 that undergo recombination in the presence of Cre recombinase. This removes exon 2 and 3 to generate the conditional knockout (cKO) allele encoding a highly truncated 66 amino acids long HCF-1 protein.

Conditional (lox) allele are recombined (Minocha et al., 2016). Subsequent DNA extraction was done at 62 °C for 15 s with an extension at 72 °C for 10 s. Primers for genotyping are listed below.

For Cre: Sense (5′-AGGGTACAGACGCTTCTAGG-3′) and Anti-sense (5′-CTAGCGCAGCTTCCGGACG-3′) (Le and Sauer, 2000).

For mouse Y chromosome: Sry-1 (5′-AACACTGGGGTCGAGGGAGGG-3′) and Sry-2 (5′-TGGTATACGGGGTCTCTCTAGG-3′) (Sleeve-Perkins et al., 2005).

2.3. BrdU incorporation

To label embryonic cells during S phase, pregnant mice were injected intraperitoneally 5-bromo-2-deoxyuridine (BrdU; BD Biosciences, cat. # 5580891) to a final concentration of 50 mg/kg body weight, sacrificed 24h post-injection, and BrdU incorporation revealed by immunofluorescence staining (see below).

2.4. Tissue histology and immunohistochemistry

Intact E5.5 to E8.5 embryos were paraffin-embedded and sectioned within their decidua along a sagittal axis to generate 4 μm thick sections using a microtome (MICROM HM325). The paraffin-embedded sections were prepared for hematoxylin and eosin (H&E) staining, and immunohistochemical detection of proteins.

Paraffin-embedded sections were (i) deparaffinized in xylene, (ii) rehydrated through graded alcohol washes, (iii) rinsed twice with PBS, (iv) antigen revealed by heating in a 750 W microwave oven until boiling (approximately 10 min) in citrate buffer (10 mM, pH 6.0), (v) allowed to slowly cool down at 4 °C, (vi) washed twice with PBS, (vii) blocked for 30 min with 2% normal goat serum (NGS) (Sigma-Aldrich, cat. #G9023) in PBS at room temperature (RT), (viii) incubated with specific primary antibody diluted in 2% NGS overnight at 4 °C, (ix) washed thrice with PBS, (x) incubated with secondary antibody for 30 min in the dark at RT, (xi) washed thrice with PBS, (xii) counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, CAS # 28,718-90-3), (xiii) washed twice with PBS, (xiv) incubated with Mowiol mounting medium (Sigma-Aldrich, CAS # 9002-89-5), and (xv) analyzed using an AxioImager M1 microscope with AxioCam MRm monochrome and AxioCam MRC color cameras (Carl Zeiss AG, Oberkothen, Germany). Images were processed using AxiosVision 4.8 software (Carl Zeiss AG, Oberkochen, Germany).

Primary antibodies used were: rabbit anti-HCF-1 (1:1000, H12 (Wilson et al., 1993)), rat anti-Ki67 (1:60, ebioscience cat. # 41-5698), mouse anti-HNF4α (1:100, R&D Systems cat. # PP-H1415-00), rabbit anti-Histone H3 phospho Ser10 (1:100, Abcam cat. # ab5176), rat anti-BrdU (1:250, AbD Serotec cat. # OBT0030), and mouse β-catenin (1:50, BD Biosciences, cat. # 610153).

Secondary antibodies used were: Goat anti-rabbit Alexa 488 (1:400, Molecular Probes cat. # A11034), goat anti-mouse Alexa 568 (1:500, Molecular Probes cat. # A11019), goat anti-rabbit Alexa 568 (1:1000, Molecular Probes cat. # A21206), goat anti-mouse Alexa 488 (1:400, Molecular Probes cat. # A11029), and donkey anti-mouse Alexa 594 (1:500, Molecular Probes cat. # A11005).

2.5. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed on paraffin-embedded embryo sections with the in situ cell death detection kit (Roche Applied Science, product # 11684795910), according to the
The male Hcft1<sup>lox/ytg</sup> progeny lacking the Sox2Cre<sup>tg</sup> express Hcft1 normally and possess a wild-type phenotype (Minocha et al., 2016), whereas in those carrying the Sox2Cre<sup>tg</sup> allele the epiblast-specific epiKO allele is generated, leading to loss of anti-HCF-1 immunostaining in approximately 70% of epiblast cells by E5.5 (Minocha et al., 2016).

3.1. Epiblast-specific HCF-1 depletion leads to epiblast-specific developmental arrest by E6.5

As shown in Fig. 1, in Sox2Cre<sup>tg</sup>; Hcfc1<sup>epiKO/Y</sup> embryos (henceforth referred to simply as Hcft1<sup>epiKO/Y</sup>), the percentage of HCF-1-deficient cells increases to 87% of epiblast cells by E6.5 and then varies between 85–90% of cells at E7.5 and E8.5. In contrast, the levels of HCF-1 in extraembryonic tissues, such as extraembryonic ectoderm (ExE) and visceral endoderm (VE), appeared unchanged (Fig. 2B3–B6). The major disappearance of HCF-1-positive epiblast cells at E6.5 was accompanied by a noticeable reduction in the size of the Hcft1<sup>epiKO/Y</sup> epiblast relative to the corresponding extraembryonic tissue (Fig. 2E–G). Nevertheless, immunostaining of Oct4 revealed no overt change in epiblast fate at this stage (Fig. 3A–C, compare also Fig. 2A and B1).

At E7.5, the Hcft1<sup>epiKO/Y</sup> epiblast, identified as Oct4 positive (Fig. 3D–F), did not increase in size compared to E6.5 (compare Fig. 2B1 and D1; Fig. 2F). The size of mutant epiblasts also did not noticeably increase at E8.5 (Supplemental Fig. 1A and B), indicating that their development is arrested at E6.5 and not simply delayed. At E9.5, Hcft1<sup>epiKO/Y</sup> embryos were largely deformed and the epiblast region could not be identified easily (Supplemental Fig. 1C and D), probably owing to extensive embryonic cell death (see below).

In contrast to the epiblast, the ExE and VE of Hcft1<sup>epiKO/Y</sup> embryos remained HCF-1-positive throughout the E6.5–E9.5 period (Fig. 2 and Supplemental Fig. 1). The ExE continued to grow until E8.5 despite the arrest of epiblast development, albeit less than in the Hcft1<sup>lox/ytg</sup> control littermates (compare Fig. 2C and D1, Fig. 2E and Supplemental Fig. 1B). In contrast, the embryonic VE (EmVE), which envelops Hcft1<sup>epiKO/Y</sup> epiblasts, failed to grow and did not transition from a cuboidal to a simple squamous epithelial shape (VE arrowhead, Fig. 2D2) despite the continuous presence of nuclear HCF-1 (Fig. 2B5–B6 and D5–D6), suggesting a deficiency in epiblast-derived growth signals.

Altogether, these results demonstrate that HCF-1 is critical during early mouse embryonic development and that its epiblast-specific loss leads to a rapid growth arrest at E6.5 followed by cell non-autonomous secondary defects in extraembryonic lineages.

3.2. Loss of HCF-1 leads to cell-cycle exit around E6.5

The E5.5 to E7.5 phase of mouse development is characterized by rapid cell proliferation with doublings up to every 4–5 h (Snow, 1977) that largely skip the G1 phase and indeed lack elements of G1-phase regulation (see Introduction). HCF-1, like other G1-phase regulators, is essential for proper G1-phase progression in tissue-culture cells. In contrast, in Hcft1<sup>epiKO/Y</sup> females, HCF-1-deficient cells surrounded by HCF-1-positive cells continue proliferating at E6.5 and only disappear at a later stage owing to apoptosis (Minocha et al., 2016). We therefore asked here whether, in the absence of large numbers of surrounding HCF-1-positive cells, HCF-1-deficient cells exit the cell cycle and, if so, when.

Epiblast cells undergo multiple rounds of cell division both between E5.5 to E6.5 and between E6.5 to E7.5 (Snow, 1977). To reliably identify cells that have exited the cell cycle before these time windows, we measured the percentage of cells resistant to BrdU incorporation over a 24 h labeling period (Fig. 4). We also identified replicating cells at E6.5 and E7.5 by histone H3

![Fig. 1. Significant depletion of HCF-1-positive cells in the Hcft1<sup>epiKO/Y</sup> male epiblasts by E6.5. Boxplot showing the percentage of HCF-1-negative cells in the epiblast of Sox2Cre<sup>tg</sup>; Hcft1<sup>epiKO/Y</sup> male embryos from E5.5 to E8.5 (E5.5 n=3; E6.5 n=6; E7.5 n=12; E8.5 n=5). The difference between the percentage of HCF-1-negative cells in Sox2Cre<sup>tg</sup>; Hcft1<sup>epiKO/Y</sup> male embryos at E5.5 and all other time points was highly significant (E5.5 versus E6.5 p-value 1.34 x 10<sup>-5</sup>; E5.5 versus E7.5 p-value 3.45 x 10<sup>-3</sup>; E5.5 versus E8.5 p-value 1.3 x 10<sup>-5</sup>). The difference between the percentage of HCF-1-negative cells in the epiblast of Sox2Cre<sup>tg</sup>; Hcft1<sup>epiKO/Y</sup> male embryos at E6.5, E7.5, and E8.5 was not significant (E6.5 versus E7.5 p-value 0.81; E7.5 versus E8.5 p-value 0.45). Note: Data for the time point E5.5 shown in the boxplot has been reproduced from Minocha et al. (2016).](image-url)
phosphoserine 10 (H3S10P) immunostaining of interphase and condensed mitotic chromosomes (Fig. 5). Identification of the epiblast region was aided by co-staining with Oct4 (Fig. 3). The results are quantitated in Fig. 6.

As expected, control E6.5 and E7.5 Hcfc1lox/lox embryos displayed considerable BrdU incorporation in both embryonic and extraembryonic tissues (Fig. 4A and C). In contrast, Hcfc1epiKO/Y embryos displayed reduced BrdU incorporation in the embryonic lineage by 56% at E6.5 (Figs. 4A and B, and 6A) and by 77% at E7.5 (Fig. 4C and D, and Fig. 6A), suggesting a pronounced failure to enter S phase already by around E5.5, i.e. even before BrdU was injected for analysis at the E6.5 time point. Interestingly, compared to control Hcfc1lox/lox embryos, the BrdU labeling intensity was also reduced in the extraembryonic tissues (compare Fig. 4A2 with B2 and C2 with D2; see Supplemental Fig. 2 for quantitation), including the decidua (see asterisk). The EmVE was particularly deficient in BrdU labeling in Hcfc1epiKO/Y embryos (compare Fig. 4A5 and B5; see Supplemental Fig. 2 for quantitation), consistent with a cell non-autonomous role for HCF-1 in the epiblast to maintain EmVE growth.

The H3S10P marker was readily detected both in embryonic and extraembryonic tissues in E6.5 and E7.5 Hcfc1lox/lox embryos (Fig. 5A4 and C4, magenta arrows). The number of H3S10P-labeled cells was, however, reduced in the Hcfc1epiKO/Y embryos compared...
to \textit{Hcfc1}^{lox/Y} embryos both at E6.5 (compare Fig. 5A and B; Fig. 6B) and E7.5 (compare Fig. 5C and D; Fig. 6B), in agreement with the reduced BrdU staining. Together, these results suggest that, although HCF-1-deficient cells display some proliferation, the large majority exits the cell cycle and arrests proliferation by E5.5–E7.5.

To examine the fate of HCF-1-deficient cells, we assayed for apoptotic cells in \textit{Hcfc1}^{epiKO/Y} embryos at E6.5 to E9.5 (Fig. 7 and Supplemental Fig. 3). In the epiblast, we observed an increase in the number of apoptotic cells in \textit{Hcfc1}^{epiKO/Y} embryos between E6.5 (8%) and E7.5 (27%) (Fig. 6C and Fig. 7B, D; magenta arrows). Interestingly, in five out of six embryos, we also observed apoptotic cells in the EmVE (Fig. 7D; light blue arrows). By E8.5 and E9.5, most cells stained positive for apoptosis (Supplemental Fig. 3B and D). Overall, these results suggest that the majority of the HCF-1-deficient cells exit the cell cycle around E6.5 and, after a delay, are eliminated via apoptosis.

3.3. Loss of HCF-1 in the epiblast impairs AVE formation and anterior-posterior (A-P) axis specification

Already before the widespread cell-cycle exit observed at E6.5, reciprocal signals between the epiblast and EmVE are essential to
specify anterior VE (AVE) and to pattern the future anterior-posterior (A-P) body axis. To test whether Hcf1 is required for A-P axis specification, we first monitored VE patterning by labeling the general VE marker HNF4α in Hcfc1lox/Y and Hcfc1epiKO/Y embryos. HNF4α levels began to decrease specifically in the EmVE at E5.5 both in Hcfc1lox/Y and Hcfc1epiKO/Y embryos and continued to do so in Hcfc1lox/Y controls until E6.5 as expected (Supplemental Fig. 4; Duncan et al., 1994; Morrissey et al., 1998). In Hcfc1epiKO/Y embryos, however, HNF4α failed to become restricted to the extraembryonic VE (ExVE) and HNF4α levels even increased ectopically throughout the VE by E7.5 (Fig. 8), indicating a marked defect in EmVE patterning.

To further investigate defects in EmVE patterning, we probed the expression of markers of AVE and A-P axis formation. Prior to E6.5, the distal-most cells of the EmVE (called DVE) normally migrate to the prospective anterior side of the egg cylinder ahead of future AVE cells that are accrued from nearby EmVE cells (Takahata et al., 2011) and induced to express the genes encoding the activity of the posterior determinant Wnt3 (Kimura-Yoshida et al., 2005). Although a porcupine mutant deficient in Wnt secretion and signaling did not show AVE migration defects (Biechele et al., 2013), we performed whole mount in situ hybridization (wISH) for Dkk1 and for the AVE markers Lefty1 and Hhex at E6.5 to visualize possible effects of HCF-1 loss on AVE formation (Fig. 9). While expression of Dkk1 appeared normal in the Hcfc1epiKO/Y embryos (Fig. 9A, compare panels c and c’). Lefty1-expressing cells ectopically accumulated distally in E6.5 Hcfc1epiKO/Y embryos (Fig. 9A, panels d and e). Moreover, Hhex expression, which normally marks both DVE and AVE was absent (Fig. 9A, panel b and b’). These results suggest that HCF-1 is required in the epiblast for AVE migration and normal EmVE patterning.

AVE migration and Lefty-1 expression depend on Nodal and its coreceptor Cripto (Brennan et al., 2001; Ding et al., 1998; Kimura-Yoshida et al., 2005; Trichas et al., 2011; Yamamoto et al., 2004). To assess whether epiblast depletion of HCF-1 affects Nodal or Cripto expression, their corresponding mRNA levels were analyzed by wISH. Nodal and Cripto mRNAs were clearly present in the epiblast of both Hcfc1lox/Y and Hcfc1epiKO/Y embryos (Fig. 9A, panels d and d’, and e and e’). In Hcfc1epiKO/Y embryos, however, they failed to become restricted to the posterior side (panels d’ and e’), indicating a lack of an A-P axis formation.

Graded Nodal expression along the A-P axis requires feedback regulation mediated by the mutual inhibition between Otx2 and Wnt3: Induction of Wnt3 in the posterior epiblast by Nodal and
possibly BMP4 inhibits Otx2 expression (Beddington and Robertson, 1999; Ben-Haim et al., 2006; Lawson et al., 1999; Winnier et al., 1995), whereas upregulation of Otx2 at the anterior pole induces AVE migration and thereby restricts Nodal and Wnt3 signaling to the posterior (Ang et al., 1994; Ding et al., 1998; Kimura et al., 2000; Kimura-Yoshida et al., 2005; Liu et al., 1999; Simeone et al., 1993; Trichas et al., 2011; Yamamoto et al., 2004). Wnt3 was readily detected in both wild-type and Hcfc1 epiKO/Y embryos, confirming that Nodal and Cripto were active. In Hcfc1 epiKO/Y embryos, however, Wnt3 mRNA failed to be enriched at the prospective posterior pole and instead ectopically accumulated throughout the proximal epiblast (Fig. 9A, panel f). Consistent with this ectopic Wnt3 expression, expression of Otx2 mRNA was confined to distal epiblast and only maintained at reduced levels in Hcfc1 epiKO/Y mutants. Furthermore, Otx2 expression in the EmVE and its A-P polarity in the epiblast were lost (Fig. 9A, panel g). Altogether, these results indicate that proximal-distal patterning still occurs in Hcfc1 epiKO/Y embryos but fails to be converted into a functional A-P axis at E6.5, probably owing to a defective anterior migration of the AVE.

3.4. Lack of AVE development and A-P axis formation is followed by a failure of primitive streak formation

Because Hcfc1 epiKO/Y embryos lacked A-P axis polarity at E6.5, we asked whether subsequent primitive streak formation and gastrulation movements might be inhibited. To address this question, we performed wISH of genes expressed in the primitive streak at E7.5, including Cripto, Wnt3 and Otx2. In addition, we monitored the expression of the general epiblast-specific marker Oct4. Even though the Hcfc1 epiKO/Y embryos were deformed with only tiny epiblasts, Oct4 remained normally expressed (Fig. 9B, compare panels e and e'). Consistent with the patterning defects observed at E6.5, however, the levels of Cripto, Wnt3 and Otx2 mRNAs appeared to be reduced, and their graded distribution which marks the A-P axis of wild-type E7.5 embryos was impaired (Fig. 9B, panels a–c).

Wnt3 and Nodal are critical for primitive streak formation (Conlon et al., 1994; Liu et al., 1999). To directly monitor primitive streak formation, we analyzed the expression of two of their downstream targets, Brachyury and Fgf8 (Crossley and Martin, 1995; Inman and Downs, 2006). Brachyury and Fgf8 mRNAs were
markedly decreased in Hcfc1\textsuperscript{epiKO/Y} embryos (Fig. 9B, panels f and g) compared to Hcfc1\textsuperscript{lox/Y} littermate embryos (panels f and g).

Cells that emanate from the primitive streak to form definitive endoderm eventually intermingle with the overlying HNF4α-positive VE. Interestingly, immunostaining of HNF4α protein, which marks the extraembryonic yolk sac endoderm of wild-type E8.5 embryos (Duncan et al., 1994; see Supplemental Fig. 5A2–4), revealed persistent presence of ectopic HNF4α in EmVE of Hcfc1\textsuperscript{epiKO/Y} mutants (see Supplemental Fig. 5B3). Besides confirming a defect in EmVE maturation, this result is consistent with a lack of germlayer formation.

Overall, these findings suggest that improper A-P axis formation leads to defective primitive streak formation upon loss of HCF-1 in the epiblast.

3.5. Hcfc1\textsuperscript{epiKO/Y} embryos display defective β-catenin activation

β-catenin is a dual function protein that serves as both a nuclear transcriptional co-regulator and a regulator of cell-cell adhesion by associating with the plasma membrane. In β-catenin-dependent signaling, the transcriptional regulatory functions of β-catenin are activated by its release from an APC destruction complex in the cytoplasm followed by translocation to the nucleus where it associates with the TCF/LEF family of promoter-specific DNA-binding transcriptional regulators (Huelsken and Behrens, 2002).

As Wnt3 was expressed but failed to induce primitive streak formation in Hcfc1\textsuperscript{epiKO/Y} embryos, we asked whether the loss of HCF-1 affects β-catenin activation (i.e., nuclear localization). Immunostaining of Hcfc1\textsuperscript{lox/Y} embryos between E6.5–7.5 stages readily detected β-catenin at the plasma membrane and nucleus of both embryonic and extraembryonic cells (Fig. 10; see also with DAPI co-staining in Supplemental Fig. 6; Mohamed et al., 2004). In contrast, Hcfc1\textsuperscript{epiKO/Y} embryos displayed less intense overall β-catenin staining of plasma membrane and particularly of the nuclei (Fig. 10; see also Supplemental Fig. 6), indicating a possible effect on cell–cell adhesion but certainly a defect in the activation of β-catenin transcriptional regulatory functions.

4. Discussion

We have shown that epiblast-specific loss of HCF-1 in the early mouse embryo leads to a developmental arrest at E6.5 as well as cell non-autonomous effects on extraembryonic lineages. As a result, shortly after proximal-distal patterning, there is a failure of A-P axis specification and gastrulation possibly owing to reduced epiblast growth combined with impaired β-catenin-dependent signaling. We additionally found that HCF-1 plays a key role in the rapid cell proliferation phase of embryonic development around E6.5, a time when many other G1-phase regulators (e.g., E2Fs, Retinoblastoma pocket-protein family, cyclin–Cdk complexes) are not functional (see Introduction). These results contrast with those of loss of HCF-1 function in the worm Caenorhabditis elegans, where germ-line disruption of the Ce hcf-1 gene is viable under normal growth conditions (Lee et al., 2007). It should be noted that an earlier embryonic-specific disruption of Hcfc1 expression may well cause earlier developmental defects than those
described here. Whether the case or not, the essential nature of HCF-1 for development has clearly varied significantly during metazoan evolution.

4.1. HCF-1 in early embryonic cell proliferation

As an X-linked gene, Hcfc1 is randomly inactivated in the female mammalian embryo around E4.5–E5.5 (Clerc and Avner, 2011). In heterozygous Hcfc1epiKO/+ female embryos, random X inactivation results in an approximately 50:50 mixture of HCF-1-positive and -deficient cells after which the HCF-1-deficient cells disappear (Minocha et al., 2016). To distinguish whether HCF-1-deficient cells are outgrown by competing HCF-1-positive cells, or instead simply fail to replicate, we here monitored cell proliferation in male Hcfc1epiKO/Y embryos. We observed widespread cell-cycle exit after E5.5 specifically in the mutant epiblasts, followed — after a delay — by apoptosis. This Hcfc1epiKO/Y cell-cycle exit is earlier than observed for HCF-1-deficient cells in Hcfc1epiKO/+ heterozygotes (Minocha et al., 2016), suggesting that, in the Hcfc1epiKO/+ heterozygous female embryos, HCF-1-positive cells help sustain the proliferation of their HCF-1-deficient neighbors. These results suggest a noncompetitive model for loss of HCF-1-deficient cells in Hcfc1epiKO/+ heterozygous female embryos (Minocha et al., 2016).

Despite essential roles of HCF-1 in cell proliferation and cell survival, the analysis of Hcfc1epiKO/Y mutants revealed significant residual epiblast growth between E5.5–E6.5 and no increase in apoptosis until later stages. Indeed, cell proliferation did not cease instantly after HCF-1 loss. A similar phenotype has been observed in the temperature-sensitive Hcfc1 mutant hamster cell line tsBN67: When transferred to non-permissive temperature, tsBN67 cells continue the cell cycle for about two cell divisions before

![Fig. 7. Percentage of apoptotic cells significantly increases in Hcfc1epiKO/Y epiblasts by E7.5. TUNEL assay was performed on paraffin-embedded sections of (A and C) control Hcfc1lox/Y and (B and D) Sox2Cre;Hcfc1epiKO/Y male embryos co-stained with DAPI (blue) at (A and B) E6.5 and (C and D) E7.5. TUNEL-positive apoptotic cells are shown in green. The boxed regions in immunostained control (A2 and C2) Hcfc1lox/Y and (B2 and D2) Sox2Cre;Hcfc1epiKO/Y male embryonic sections are shown at higher magnification in subsequent panels in A3–A4 and C3–C4, and B3–B4 and D3–D4, respectively. The yellow arrowheads identify the boundary between extraembryonic and embryonic portions of the embryo. The magenta arrows point to TUNEL-positive nuclei and white arrows point to TUNEL-negative nuclei in the epiblast. The light blue arrows point to TUNEL-positive nuclei in the EmVE. Scale bar: 50 μm.](image-url)
entering a stable arrest in which there is little apoptosis (Goto et al., 1997; Reilly and Herr, 2002). In both tsBN67 and embryonic cells, cell-cycle arrest may be delayed for multiple reasons. For example, it may take time to deplete specific gene products that depend on HCF-1’s function as a transcriptional regulator for synthesis. Additionally, the lack or delay of apoptosis in $Hcfc^{	ext{epiKO/}}$ male embryos after cell cycle exit may reflect an HCF-1 role in promoting apoptosis as described previously (Tyagi and Herr, 2009).

Fig. 8. Defective visceral endoderm patterning in $Hcfc^{	ext{lox/Y}}$ male embryos at E6.5 and E7.5. Immunofluorescence analysis of paraffin-embedded sections of (A and C) control $Hcfc^{	ext{lox/Y}}$ and (B and D) Sox2Cre$^{tg}$ ; $Hcfc^{	ext{epiKO/}}$ male embryos at (A and B) E6.5 and (C and D) E7.5. Two consecutive paraffin-embedded sections were taken for immunostaining with (i) anti-HCF-1 antibody (green), and (ii) VE marker anti-HNF4α (red) and DAPI (blue). The boxed regions in (A2 and C2) control $Hcfc^{	ext{lox/Y}}$ and (B2 and D2) Sox2Cre$^{tg}$ ; $Hcfc^{	ext{epiKO/}}$ male embryonic sections are shown at higher magnification in A3–A4 and C3–C4, and B3–B4 and D3–D4, respectively. The yellow arrowheads identify the boundary between extraembryonic and embryonic portions of the embryo. The magenta arrows point to HNF4α-positive nuclei. EmVE, embryonic visceral endoderm; ExVE, extraembryonic visceral endoderm. Scale bar: 50 μm.
4.2. HCF-1 in early embryonic patterning

Upon epiblast-specific deletion of Hcf1 around E4.5–E5.5, there is a clear failure of A-P axis specification and subsequent gastrulation. The impaired cell proliferation after E6.5 in the Hcfc1epiKO/Y embryos may, at least in part, account for this phenotype since the presence of a certain number of epiblast cells and active cell proliferation are critical to initiate and sustain germ layer formation during gastrulation (reviewed in Tam and Behringer (1997)). Normal proliferation in the epiblast is also necessary for AVE formation during A-P axis specification (Stuckey et al., 2011). The number of epiblast cells increases approximately 4.5- to 5-fold (to around 660 cells) between E5.5 and E6.5 (Snow, 1977), which is important for gastrulation to initiate. The developmental arrest of Hcfc1epiKO/Y embryos just prior to gastrulation thus likely reflects their failure to attain the necessary threshold number of epiblast cells. According to a computational model, such a community effect may involve the enlargement of the pool of cells that produce Nodal since two kinetically distinct feedback loops that drive Nodal autoinduction cannot account for the elevated signaling thresholds that specify mesoderm and endoderm, except if the source of Nodal-producing cells increases substantially over time (Ben-Haim et al., 2006). In particular, the relatively early time of onset of Wnt3 expression at around E6.0 in the epiblast, which is critically required downstream of Nodal to induce Brachyury expression in the primitive streak could not be explained by computational modeling without an epiblast cell community effect (Ben-Haim et al., 2006; Rivera-Perez and Magnuson, 2005; Tortelote et al., 2013).

Interestingly, we observed that Hcfc1epiKO/Y embryos, despite a noticeable size reduction, still induced Wnt3 and yet failed to accumulate nuclear β-catenin on time at E6.5, or the Wnt/β-catenin transcriptional target Brachyury (Arnold et al., 2000). Since all Wnts rely on Porcupine function to signal through β-catenin-dependent or -independent pathways (Najdi et al., 2012) and both Porcupine and Wnt3 mutant embryos display a proper DVE to AVE conversion (Biechele et al., 2013; Tortelote et al., 2013; Yoon et al., 2015) unlike Hcfc1epiKO/Y embryos, it appears that defects in Hcfc1epiKO/Y embryos are due to loss of a Porcupine/Wnt-independent function of β-catenin. Indeed, the Hcfc1epiKO/Y embryos strikingly resembled embryos lacking β-catenin in various respects (see Supplemental Table 1 for a detailed comparison): Both Hcfc1epiKO/Y and β-catenin mutant embryos show (i) embryonic lethality around E8.5, (ii) normal signs of proximal-distal

![Fig. 9. Lack of AVE migration and primitive streak formation in Hcfc1epiKO/Y male embryos.](image-url)
patterning but defects in VE differentiation and A-P axis formation, and (iii) an absence of primitive streak formation and gastrulation (Haegel et al., 1995; Huelsken et al., 2000; Mohamed et al., 2004; Rudloff and Kemler, 2012).

These observations suggest a role for HCF-1 in β-catenin-dependent signaling. Such a role could be transcriptional. Indeed, HCF-1 associates with various transcription factors such as E2Fs, Ronin/Thap11, YY1, GABP, and ZNF143 to bind many promoters in mouse embryonic stem (ES) cells (743 promoters) and human HeLa cancer cells (5400 promoters), including promoters for components that regulate β-catenin stability (Dejosez et al., 2010; Michaud et al., 2013; Parker et al., 2014; Tyagi et al., 2007). Among the HCF-1-bound promoters in ES cells (Dejosez et al., 2010) are those for gene products that destabilize β-catenin (e.g., β-transducin repeat containing protein (Btrc) and F-box and WD-40 domain protein 11 (Fbxw11), which are both part of the SCF β-catenin degradation complex, and glycogen synthase kinase 3α (Gsk3α), which triggers destabilization of β-catenin through phosphorylation dependent mechanisms) and stabilize β-catenin (e.g., casein kinase 2β; Csnk2β). Thus, HCF-1 may activate or inhibit β-catenin signaling, depending on the context.

4.3. HCF-1 in non-autonomous cell signaling

At E6.5, the Hcf1\textsuperscript{eplKO/Y} embryos, displayed incomplete extraembryonic EmVE differentiation, suggesting non-autonomous cell effects of the epiblast-specific loss of HCF-1 on surrounding extraembryonic tissue, including absence of anteriorly located Lefty1 and Hhex expression. Epiblast cells stimulate VE cell proliferation and patterning and the anterior movement of DVE cells at least in part by secreting Nodal (Yamamoto et al., 2004). Nodal may promote DVE migration by several mechanisms, including upregulation of its own proprotein convertase Furin (Mesnard et al., 2006) and of the paired-like homeobox transcription factor Otx2 (Kimura et al., 2000) in the VE and by stimulating the localization of dishevelled-2 (Dvl2) on VE cell membranes (Trichas et al., 2011).

The downregulation of the Nodal target Otx2 observed in the VE and in the epiblast of Hcf1\textsuperscript{eplKO/Y} embryos is consistent with the observed DVE cell migration defect (Kimura et al., 2000). However, as Nodal was able to induce Lefty1 but not Otx2 in the VE, epiblast-specific loss of HCF-1 might affect additional transcriptional activators upstream of Otx2 such as Lhx1 or Foxa2 (Costello et al., 2015).

In conclusion, HCF-1 plays important roles in early embryonic cell proliferation and signaling events. Possessing such fundamental and essential functions in development may explain in part why it was targeted by herpes simplex virus as a key host-cell factor to coordinate its infection cycle.

Competing interest statement

The authors declare that they have no competing interests.
**Author contributions**

The experiments were conceived and designed by S.M., T.-L.S., D.C., and W.H. The experiments were performed by S.M., S.B., T.-L.S., and C.M.S.M., S.B., T.-L.S., D.C., and W.H. analyzed the data. S.M., D.C., and W.H. wrote the paper. S.M., S.B., T.-L.S., D.C., and W.H. participated in the discussion of the data and in production of the final version of the manuscript.

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2016.08.008.

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