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Transcriptional Regulator BPTF/FAC1 Is Essential for Trophoblast Differentiation during Early Mouse Development[▽]

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The putative transcriptional regulator BPTF/FAC1 is expressed in embryonic and extraembryonic tissues of the early mouse conceptus. The extraembryonic trophoblast lineage in mammals is essential to form the fetal part of the placenta and hence for the growth and viability of the embryo in utero. Here, we describe a loss-of-function allele of the BPTF/FAC1 gene that causes embryonic lethality in the mouse. BPTF/FAC1-deficient embryos form apparently normal blastocysts that implant and develop epiblast, visceral endoderm, and extraembryonic ectoderm including trophoblast stem cells. Subsequent development of mutants, however, is arrested at the early gastrula stage (embryonic day 6.5), and virtually all null embryos die before midgestation. Most notably, the ectoplacental cone is drastically reduced or absent in mutants, which may cause the embryonic lethality. Development of the mutant epiblast is also affected, as the anterior visceral endoderm and the primitive streak do not form correctly, while brachyury-expressing mesodermal cells arise but are delayed. The mutant phenotype suggests that gastrulation is initiated, but no complete anteroposterior axis of the epiblast appears. We conclude that BPTF/FAC1 is essential in the extraembryonic lineage for correct development of the ectoplacental cone and fetomaternal interactions. In addition, BPTF/FAC1 may also play a role either directly or indirectly in anterior-posterior patterning of the epiblast.

The bromodomain plant homeodomain transcription factor (BPTF) in mammals represents the orthologue of the *Drosophila* nucleosome remodeling factor NURF301, which constitutes the largest subunit of the NURF chromatin remodeling complex (48). Human BPTF consists of 2,781 amino acids (23), and the sequence contains typical features of a transcriptional regulator, such as the DDT DNA binding domain, the PHD/LAP zinc finger (9, 52), a putative histone acetylating bromodomain of 110 amino acids (21, 51), a glutamine-rich acidic transcriptional activation domain, and nuclear import and export signals. It has been demonstrated that BPTF in *Drosophila* participates in the regulation of engrailed 1 and 2 expression as part of the NURF complex, presumably by changing the periodic alignment of nucleosomes (2). The fetal ALZ50-reactive clone 1 (FAC1) was discovered in amyloid plaques of patients with Alzheimer disease (5, 22), and it is likely to be a shorter transcript of the BPTF gene. FAC1 encompasses 801 N-terminal amino acids of BPTF but lacks the C-terminal bromodomain. It was proposed that FAC1 acts as transcriptional regulator on a DNA consensus sequence (FAC1 binding element) which is present in several genes that have been implicated in neurodegenerative disorders, including presenilin-1, amyloid precursor protein, dopamine D2 receptor, and Cu^{2+}/Zn^{2+} superoxide dismutase (25). During cortical brain development the protein is found in soma and neurites of various neurons (5). A BPTF/FAC1 orthologue was also identified in rat based on sequence conservation and similar domain organization. The biological role and in vivo functions of BPTF/FAC1 proteins are not known. Here, we describe a loss-of-function mutation of the murine BPTF/FAC1 gene by gene trap insertion in mouse embryonic stem (ES) cells from which the corresponding mutant mouse line was generated (46). We also provide evidence that BPTF/FAC1 plays a pivotal role during early mouse embryogenesis, particularly in the extraembryonic lineage.

Growth, viability, and patterning of the implanted mammalian embryo require the trophoblast from which the definitive placenta develops that mediates interactions and exchange between fetal and maternal tissues. Correct development of the trophoblast is therefore crucial for the survival of the embryo (7, 35). Segregation of the trophoblast cell lineage in the embryo becomes apparent first at the blastocyst stage when the inner cell mass (ICM) and polar trophectoderm (pTE) can be readily distinguished morphologically (34). While the ICM gives rise to the epiblast and primitive endoderm and thus forms both embryonic and extraembryonic tissues, the pTE exclusively contributes to the extraembryonic lineage, which comprises the proximal half of the egg cylinder stage embryo after implantation. The trophoblast can be subdivided into the regions of the extraembryonic ectoderm (ExE) located adjacent to the epiblast and ectoplacental cone (EPC) contacting the maternal tissues. Growth and development of extraembryonic tissues depend on a population of trophoblast stem (TS) cells present in pTE and ExE. No TS cells are present in the EPC, which contains differentiated trophectodermal progenitor cells for spongiotrophoblasts (7).

Numerous genes have been shown to be involved in the maintenance and differentiation of TS cells to give rise to cellular constituents of the placenta (35, 39). Formation of ExE and self-renewal of TS cells require synergistic signaling by

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fibroblast growth factor (FGF) and Nodal, a member of the transforming growth factor β family, first from the ICM and later from the epiblast (15, 19, 42, 43). ExE in turn produces signals, such as BMP4 and Spc proteases, that directly and indirectly act on the epiblast to promote mesoderm and posterior identity (4). The transcription factor Cdx2 initially determines trophectoderm identity (28, 34) and cooperates with Eomes (36), Errβ (27), Ets2 (14), and Elf5 (10) in the development of ExE, while formation and differentiation of the EPC depends on the basic helix-loop-helix transcription factors Mash2 (17) and Hand1 (12, 32).

Prior to the formation of the definitive placenta, the trophoblast already plays a crucial role in implantation of the blastocyst into the uterus and in signaling to the subjacent epiblast during germ cell formation (50) and embryonic patterning (4, 10, 13, 33). Evidence for the requirement of the ExE for anteroposterior (AP) patterning of the epiblast was provided by mouse mutants that lack ExE as a consequence of loss of Ets-related transcription factors (10, 14). These mutants display defects in forming the anterior visceral endoderm (AVE) and elongating the mesoderm-producing primitive streak (PS). Mutations that compromise signaling from ExE also display patterning defects (4). Similarly, surgical removal of ExE at pregastrulation stages results in loss of PS markers (33).

In this report we show that the mouse BPTF/FAC1 gene is expressed in both the embryonic and extraembryonic lineages of the conceptus. Homozygous BPTF/FAC1 mutant embryos are severely growth retarded and die in utero around embryonic day 9 (E9) to E10. Most notably, the EPC in the mutant appears substantially reduced or absent while ExE and TS cells are present. The mutant epiblast produces mesodermal cells expressing brachyury with some time delay, but the PS fails to extend correctly. Also, formation of the AVE is impaired in the mutant. Our data indicate that BPTF/FAC1 constitutes a novel key regulator of preplacental trophoblast development with a particularly critical role for differentiation of the EPC.

MATERIALS AND METHODS

Generation of the BPTF/FAC1 gene trap mouse line. Gene trap mutations were obtained in TBV2 ES cells by electroporation of 120 μg of linearized pPT1βgeo vector as described previously (46). The trapped gene locus was identified in ES cells by 5′ rapid amplification of cDNA ends (RACE) using the system kit (Gibco BRL). Briefly, poly(A)$^+$ RNA (0.5 μg) was isolated and transcribed with SuperscriptII reverse transcriptase using the *lacZ*-specific primer 5′-GGATTGACCGTAATGGGATAG-3′. Purified cDNA was extended with oligo(dC) using terminal transferase and subjected to 35 PCR cycles with the anchor primer 5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′ (Gibco BRL) and the nested *lacZ*-specific primer 5′-GTAGATGGGCGCATCGTAAC-3′. The product was reamplified with the abridged universal amplification primer (5′-GGCCACGCGTCGACTAGTAC-3′) (Gibco BRL) and another nested *lacZ* primer (5′-TGCCGGAAACCAGGCAAAG-3′), cloned into the pAMP vector (Gibco BRL), and sequenced. The BPTF/FAC1 founder mouse was generated by aggregating ES cells of the annotated clone A012C04 with morulae from superovulated CD1 females (Charles River), as described previously (44). Chimeric offspring carrying the gene trap allele were backcrossed onto C57BL/6 and CD1 mice to test for germ line transmission of the mutation and to establish the mutant mouse line BPTF/FAC1^{GT}.

Genotyping and RNA analysis. Genotyping was performed with BglII-digested genomic DNA from tail biopsies or yolk sacs on Southern blots using a ^{32}P-labeled 750-bp genomic DNA fragment of intron 1 (nucleotides [nt] 5902 to 6651) as a hybridization probe. The genomic probe was generated by PCR using the following primers: intron 1 upper primer, 5′-CGCCCAGCCAAGTAGTGTA-3′; and intron 1 lower primer, 5′-TGCCCAATCATTAACTCAG-3′. For further amplification the nested lower primer 5′-TGCCCACACTTGACTATCAG-3′ was used. Blastocysts and small embryos were genotyped by PCR with the following three primers: intron 1 upper primer, 5′-CGGGCAGAGTTCTGACAG-3′; intron 1 lower primer, 5′-TGCCCACACTTGACTATCAG-3′; and gene trap vector lower primer, 5′-CGCCATACAGTCCTCTTCA-3′. DNA for PCR was extracted by boiling the embryos in 50 mM NaOH for 15 min and neutralizing the lysate by addition of a 1/4 volume of 1 M Tris-Cl, pH 8.0.

To analyze mRNA expression, total RNA was extracted with guanidinium thiocyanate-phenol from postgastrula embryos or dissected mouse organs. RNA from gastrula embryos (E8.5) was isolated using an Oligotex Direct mRNA Micro Kit (Qiagen). For reverse transcription-PCR (RT-PCR), total RNA (2.0 μg) was transcribed with SuperscriptII reverse transcriptase (Gibco BRL) using the oligo(dT)$_{15}$ primer. BPTF/FAC1-specific fragments were amplified in 35 cycles using the following primers: exon3/4 upper primer, 5′-GCGGCAGCTAATGAGGAA-3′; and exon7 lower primer, 5′-CCGGGTTCCTCAGTCTGG-3′. LacZ amplificates were generated by use of the upper primer 5′-GGTGGCGCTGGATGGTAA-3′ and the lower primer 5′-CGCCATTTGACCACTACC-3′. All RT-PCR fragments were verified by sequencing.

Histochemistry, β-galactosidase staining, and in situ hybridization. For histological analysis of early postimplantation embryos, the decidua was embedded into paraffin, sectioned sagittally at 7 or 10 μm, dewaxed, and stained with hematoxylin and eosin. For β-galactosidase staining, embryos were rinsed in phosphate-buffered saline (PBS) and fixed with 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM magnesium chloride, 1% formaldehyde, and 0.2% glutaraldehyde in PBS for 2 h, followed by three washes for 20 min each in PBS buffer containing 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 2 mM magnesium chloride and staining in 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM magnesium chloride, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in DMF, and 20 mM Tris (pH 8.0) in 1× PBS at 30°C overnight. Whole-mount in situ hybridizations using digoxigenin-labeled probes were performed as described previously (46a). For in situ hybridization on sections, the intact decidua was embedded in paraffin, sectioned longitudinally at 7 μm, dewaxed, treated with proteinase K for 10 min at 37°C, fixed in 4% paraformaldehyde, and hybridized at 67°C overnight. The sections were then washed, treated with RNase A, and incubated with anti-digoxigenin–alkaline phosphatase Fab fragments (Roche) (1:2,000 diluted in 100 mM maleic acid, 150 mM sodium chloride, 0.1% Tween 20, 2% Boehringer blocking reagent, pH 7.5) overnight at 4°C. BM Purple substrate (Roche) was used for the color reaction. The plasmids for brachyury (T), Cerberus1 (Cer1), Foxa2, and Chordin were obtained from A. Gossler (Hannover, Germany); the plasmids for Cdx2 and Eomes were obtained from E. J. Robertson, (Oxford, United Kingdom). Oct4, Mash2, and Hand1 fragments were generated by RT-PCR and cloned into the pGEM-T Easy vector.

Rescue experiments: generation of chimeric embryos from wild-type and mutant cells. Attempts to rescue mutant embryos were performed with Rosa26 ES cells (obtained from E. J. Robertson) that were aggregated to E2.5 embryos resulting from intercrosses of heterozygous BPTF/FAC1 mutant mice. Morula aggregates were incubated in M16 medium and 5% CO_2 at 37°C for 24 h and then transferred into foster mothers. Embryos were isolated between E6.5 and E8.5, fixed, and stained for β-galactosidase activity. Embryos with a high degree of chimerism (>80%) were examined morphologically and documented. The genotypes of embryos were determined on unstained extraembryonic tissue that was dissected and subjected to PCR, as described above. Homozygous BPTF/FAC1 mutant embryos could not be genotyped but were identified phenotypically by the lack of extraembryonic tissue.

RESULTS

BPTF/FAC1 is expressed in embryonic and extraembryonic tissues during early mouse embryogenesis. Human FAC1 has been reported to be expressed in neurons of the developing brain and neural tube (5), but the complete expression profile of BPTF/FAC1 during vertebrate development is not known. To investigate the spatiotemporal pattern of BPTF/FAC1 expression during mouse development, we performed in situ hybridizations on sections of embryos using the 5′ probe that recognizes both BPTF and FAC1 mRNAs. First transcripts were found at E5.5 in the epiblast and in extraembryonic ectoderm (Fig. 1A). At E6.5 BPTF/FAC1 expression continued prominently in embryonic ectoderm as well as in the extraembryonic lineage but apparently not in visceral endoderm (VE)

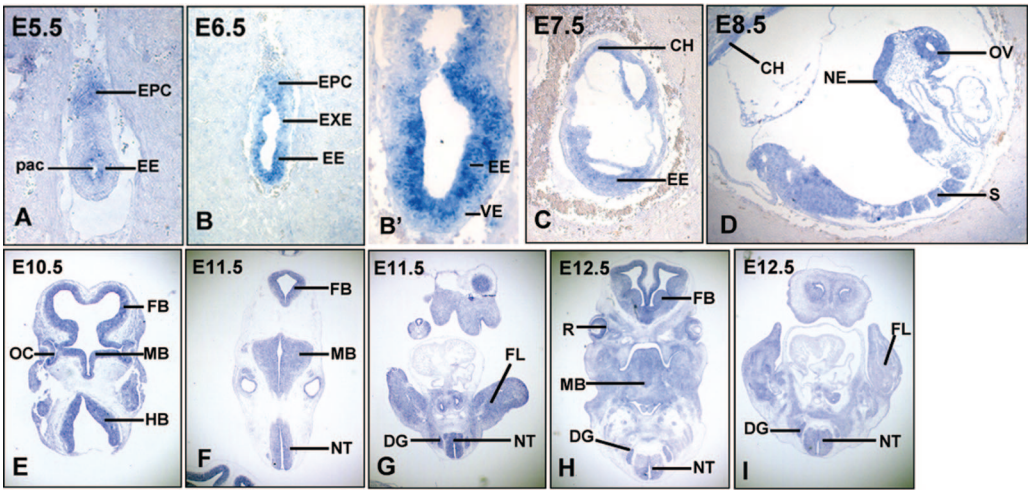


FIG. 1. BPTF/FAC1 expression profile in the mouse embryo. In situ hybridization on paraffin sections of embryos between E5.5 and E7.5 demonstrate accumulation of BPTF/FAC1 transcripts in the epiblast and extraembryonic lineage (A to C). Higher-power magnification of an E6.5 embryo reveals strong expression in embryonic ectoderm but not in VE (B'). Embryos of later stages show general but low-level expression and prominent accumulation of transcripts in distinct tissues (D to I). Early embryos were sectioned sagittally within the decidua (A to C). Panels F and G and panels H and I represent serial transverse sections of E11.5 and E12.5 embryos, respectively. EE, embryonic ectoderm; pac, preamniotic cavity; CH, chorion; NE, neural ectoderm; OV, optic vesicle; S, somites; FB, forebrain; MB, midbrain; HB, hindbrain; FL, forelimb; DG, dorsal root ganglia; R, retina; NT, neural tube.

(Fig. 1B, B'). Expression in both embryonic and extraembryonic lineages was maintained during subsequent development (Fig. 1C). At E8.5 BPTF/FAC1 transcripts were broadly expressed at low levels but accumulated to higher concentrations in chorion, neuroectoderm, optic vesicles, somites, and tail bud (Fig. 1D). Ubiquitous expression was still observed between E10.5 to E12.5 of embryogenesis; however, brain, neural tube, dorsal root ganglia, optic cups, and limb buds contained particularly high levels of BPTF/FAC1 mRNA (Fig. 1E to I). BPTF/FAC1 was also expressed in the adult mouse, where we detected different BPTF splicing variants (data not shown). While the rather broad expression pattern of BPTF in the mouse embryo obviously does not favor a particular function in specific cell lineages, the time of onset suggests that BPTF/FAC1 most likely plays a role during early stages of mouse embryogenesis.

A BPTF/FAC1 loss-of-function mutation by gene trap insertion causes embryonic lethality. The BPTF/FAC1 mutation was detected in a large-scale gene trap approach in mouse ES cells using the pPT1βgeo vector that was designed for insertion mutagenesis in introns (46). Integration of this vector usually generates a fusion transcript by splicing upstream exons of the trapped gene to the bacterial *lacZ* gene via a splice acceptor site that is part of the vector construct. One of our established ES cell clones produced a fusion transcript that was highly homologous to murine FAC1 and human BPTF mRNAs as determined by 5′ RACE analysis using LacZ-specific primers. The 5′ RACE product corresponded to the first exon of the putative mouse BPTF/FAC1 gene that we assembled from transcript sequences in various databases. Entries for human and rat BPTF expressed sequence tags facilitated RT-PCR-mediated cloning of the complete murine BPTF cDNA that is 9,420 nt long and encompasses the shorter FAC1 transcript of 2,655 nt at its 5′ end (Fig. 2A). Using the NCBI Conserved Domain Architecture Retrieval Tool on the deduced 2,822-amino-acid sequence, a domain composition for murine BPTF was predicted that closely resembled the human protein. Conserved motifs in both species include the N-terminal putative DNA-binding domain DDT, several PHD zinc finger domains, the C-terminal bromodomain, and nuclear import and export signals. Alignment of the BPTF cDNA and the mouse genomic sequence indicated that the murine BPTF gene is comprised of 31 exons extending over approximately 150 kb of DNA on mouse chromosome 11. The gene trap vector integrated after nt 6627 of the first intron (total length of 19 kb), as determined by genomic sequencing of the insertion site (Fig. 2B). The ES cell clone carrying the BPTF/FAC1 gene trap allele (BPTF/FAC1^{GT}) was used to generate a stable mouse line that transmitted the mutation through the germ line. Heterozygous BPTF/FAC1 mutant mice were normal and fertile with no obvious phenotype. They were backcrossed for more than seven generations to the inbred strains C57BL/6J and 129S2/SvHsd and to the outbred strain CD1. No major differences in phenotypes were observed on the various genetic backgrounds. When live-born offspring from heterozygous parents were genotyped, no homozygous mutants (BPTF/FAC1$^{GT/GT}$) were obtained, suggesting that the mutation resulted in recessive lethality during prenatal development (Fig. 2C). In contrast, at E7.5 and E8.5 homozygous BPTF/FAC1$^{GT/GT}$ as well as heterozygous and wild-type embryos were detected close to the expected Mendelian frequency (Fig. 2D and Table 1). Systematic analysis of embryos derived from heterozygous intercrosses confirmed the almost normal ratio of genotypes until E8.5 but showed significant loss of homozygous mutants at E9.5 and no more BPTF/FAC1$^{GT/GT}$ survivors at E11.5 (Table 1). Conversely, the number of detectable resorptions increased markedly between E8.5 and E10.5 (data not shown). To ascertain that the BPTF/FAC1^{GT} mutation actually represents a null allele that generates no BPTF mRNA, we performed RT-PCR with BPTF-specific primers located downstream of

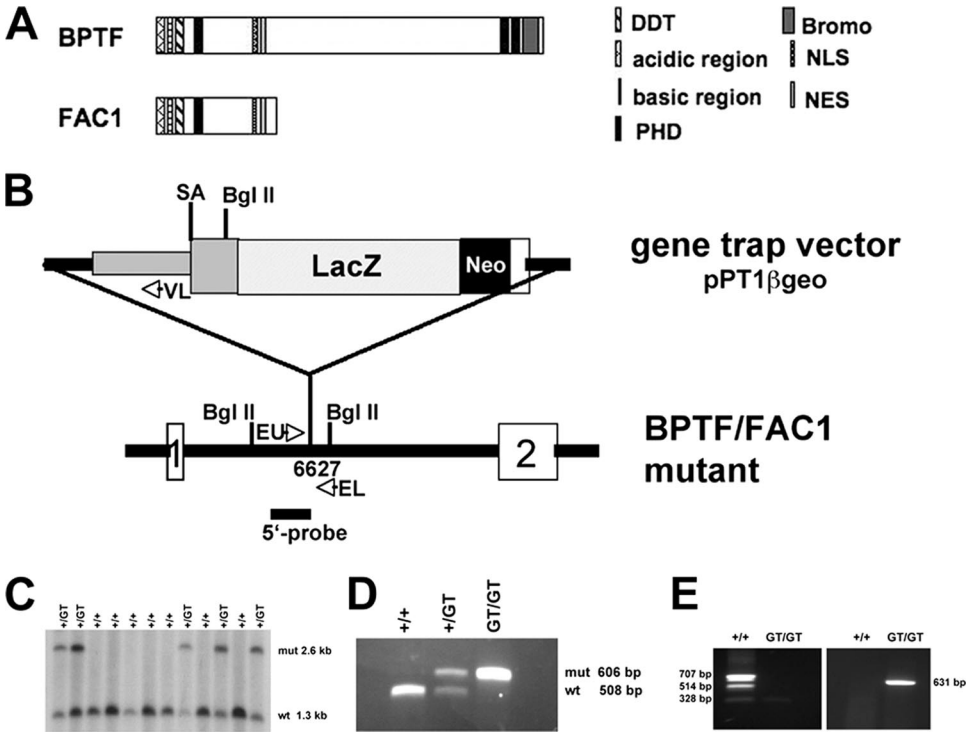


FIG. 2. Mutation of BPTF/FAC1 by gene trap integration. (A) Computational prediction of domain architecture of the two overlapping proteins BPTF and FAC1. (B) Schematic representation of the gene trap mutation. Integration of the splice acceptor (SA) gene trap vector pPT1βgeo into the first intron results in the mutant allele BPTF/FAC1^{GT} from which a fusion transcript is expressed. Genotyping was performed either by PCR using the indicated upper (EU) and lower (EL and VL) primers or by Southern blot analysis with the indicated hybridization probe. (C) A typical Southern blot analysis of litters from heterozygous parents 3 weeks after birth reveals wild-type (1.3-kb BglII fragment) and heterozygous (2.5-kb BglII fragment) animals but no homozygous mutants, whereas DNA from E8.5 embryos displays all three genotypes in a PCR-based analysis (D). (E) Expression analysis by RT-PCR detects various splicing isoforms of BPTF mRNA in E8.5 wild-type but not in homozygous mutant embryos, while LacZ transcripts are detected only in RNA from mutants. wt, wild type.

the gene trap insertion. As a control we also used LacZ-specific primers to identify the fusion transcript generated from the BPTF gene trap allele. While BPTF mRNA was readily detected in E8.5 wild-type embryos, it was totally absent from mutants that accumulated LacZ-containing fusion transcripts instead (Fig. 2E). From these observations we conclude that the gene trap insertion into the BPTF/FAC1 locus prevents normal transcripts and causes early embryonic lethality (E9 to E10).

BPTF/FAC1$^{GT/GT}$ mutant embryos are severely growth retarded and exhibit morphological defects. BPTF/FAC1-deficient blastocysts implanted normally and were morphologically indistinguishable from their wild-type counterparts (Fig. 3A and D). Following implantation, however, mutant embryos appeared to be arrested in development between E5.5 and E6.5, when they were already substantially smaller than their wild-type littermates (Fig. 3B). Progressive growth retardation during subsequent development resulted in embryos with highly abnormal morphology (Fig. 3C, E, and F). We observed very little elongation of BPTF/FAC1$^{GT/GT}$ conceptuses along the proximodistal axis and failed to detect later features characteristic of the rostrocaudal axis, such as head and tail structures (Fig. 3E and F). Hematoxylin-eosin staining of sagittal sections of embryos between E5.5 and E8.5 confirmed severe morphological defects in BPTF/FAC1$^{GT/GT}$ mutants during early postimplantation development (Fig. 4). Homozygous mutant embryos at egg cylinder stage (E5.5) displayed a dramatically shorter proximodistal axis than wild type, most likely due to the substantial reduction of extraembryonic tissue at the proximal pole (Fig. 4A and E). In contrast, the mutant epiblast at this stage appeared almost normal in size and was comprised of embryonic ectoderm and VE similar to the wild type. At E6.5 the EPC in wild-type embryos was well developed, while it was barely detectable in mutant embryos of the same stage (Fig. 4B and F). Most BPTF/FAC1$^{GT/GT}$ embryos seemed to undergo cavitation although chorion development was strongly

TABLE 1. Distribution of genotypes among offspring from heterozygous BPTF/FAC1$^{+/GT}$ parents

Stage	No. of animals	No. (%) of genotype:[a]		
		+/+	+/GT	GT/GT[b]
Adult	96	42 (43.8%)	54 (57.3%)	0 (0.0%)
E11.5	37	42 (43.8%)	21 (56.8%)	0 (0.0%)
E10.5	39	12 (30.8%)	25 (64.1%)	2 (5.1%)
E9.5	47	12 (25.5%)	32 (68.1%)	3 (6.4%)
E8.5	82	15 (18.3%)	50 (61.0%)	17 (20.7%)

[a] Mutants were analyzed after the seventh generation of backcrossing to strain C57BL/6J.
[b] All detected GT/GT embryos were malformed (round to conical shape) and smaller than those of the wild type.

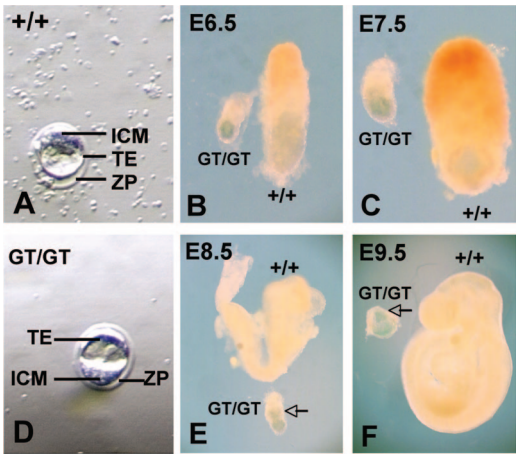


FIG. 3. Significant growth inhibition in homozygous mutant embryos. Wild-type (A) and mutant (D) blastocysts, isolated at E3.5, are essentially indistinguishable and show intact zona pellucida (ZP), ICM, and trophectoderm (TE). Altered morphology of BPTF/FAC1$^{GT/GT}$ (GT/GT) embryos can be readily recognized between E6.5 and E9.5 by their dramatically smaller size and the truncated proximodistal axis (arrows) that hardly elongates over time (B, C, E, and F), arresting development of mutants in the egg cylinder stage of approximately E6.5. There is no evidence for AP polarity in mutants, in contrast to wild-type embryos showing head, tail, and all midline structures (E and F). Note the faint LacZ staining in mutant embryos reflecting expression of the BPTF/FAC1^{GT} allele.

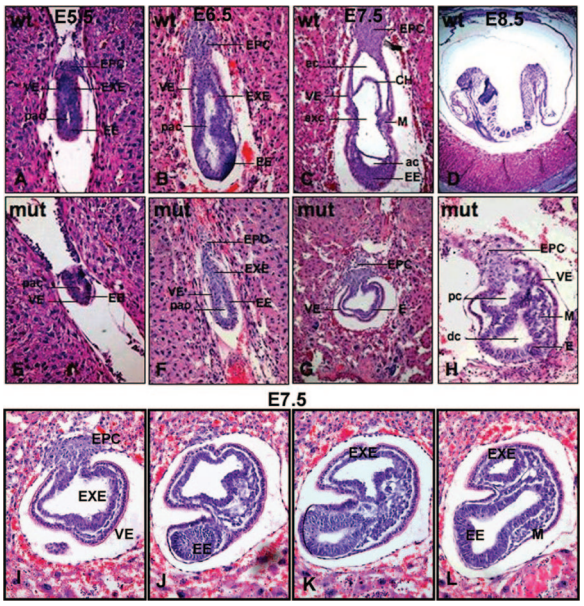


FIG. 4. Morphological defects in BPTF/FAC1$^{GT/GT}$ embryos. Hematoxylin-eosin staining of sagittal sections through the decidua containing wild-type (A to D) and mutant (E to H) embryos from E5.5 to E8.5. At E5.5 the proximodistal axis of the mutant embryo is already shorter than normal due to loss or massive tissue reduction of the proximal pole (A and E). At E6.5 and E7.5 the extraembryonic portion of mutants remains clearly smaller than in the wild type, and the EPC barely forms (B, C, F, and G). Note that the E8.5 mutant embryo contains mesodermal mesenchyme, indicating that gastrulation has been initiated (H). Development of the mutant embryo apparently stops at the egg cylinder stage (compare D and H). Serial sagittal sections of an E7.5 mutant embryo (I to L) illustrate the abnormal hook-like shape, with the distal pole representing the epiblast bent by approximately a 90° angle from the proximodistal axis. The embryonic ectoderm in the mutant appears unusually thick (J and K), and mesoderm is clearly visible (K and L). EE, embryonic ectoderm; pac, preamniotic cavity; CH, chorion; M, mesoderm; wt, wild type; mut, mutant.

reduced or absent (Fig. 4C and G). It is noteworthy that mutants at E7.5 consistently displayed a hook-like shape which deviates from the usual conical form of the egg cylinder. Serial sagittal sections through an E7.5 mutant embryo illustrated the nearly rectangular bending of the proximodistal axis, which places the distal pole including most of the embryo proper toward the anterior side of a normal embryo (Fig. 4I to L). This unusual hook-like shape of mutant embryos made it difficult to recognize all cavities on single sections. In E7.5 mutant embryos the EPC was still smaller than normal and only partly contacted the adjacent decidua (Fig. 4C and G). Homozygous mutants at E7.5 also tended to contain irregularly thick VE and embryonic ectoderm in comparison to wild type (Fig. 4I to L). Despite the early developmental arrest of mutant embryos, numerous mesenchymal cells representing mesoderm were generated, suggesting that at least early steps of gastrulation were taking place (Fig. 4H to L). At E8.5 when most body structures, such as head, heart, and somites, were established in wild-type embryos, BPTF/FAC1$^{GT/GT}$ embryos resembled much earlier stages, still lacking most structures that reflect rostrocaudal patterning (Fig. 4D and H). Even at this late stage the EPC of BPTF/FAC1$^{GT/GT}$ embryos remained very small and rudimentary. In summary, mutant embryos seemed to develop embryonic and extraembryonic ectoderm, VE, and even mesodermal cells, but they contained only a substantially reduced number of EPC cells. These observations suggest that BPTF/FAC1-deficient embryos are developmentally arrested at the pregastrula to gastrula stage, presumably due to a growth or differentiation defect in the extraembryonic lineage.

Cell-type-specific markers reveal trophoblast defects in BPTF/FAC1$^{GT/GT}$ embryos. To characterize the morphological defects in BPTF/FAC1$^{GT/GT}$ conceptuses in more detail, we performed in situ hybridizations on sections of E5.5 embryos using markers that identify epiblast and various extraembryonic cell types. Oct4 that is expressed in undifferentiated embryonic ectoderm (37) accumulated in mutant epiblasts, suggesting that at this stage it was not really affected by the mutation (Fig. 5A and H). This result confirmed the histological picture of the fairly normal E5.5 epiblast in mutants. We next examined expression of BMP4 that marks the ExE (45, 47). While ExE in both mutant and wild-type embryos was labeled by the BMP4 probe, the expression domain in the mutant appeared slightly smaller, possibly reflecting partial loss or reduction of proximal ExE (Fig. 5B and I). TS cells can be recognized by the expression of the transcription factors Cdx2 (3, 34, 42) and Eomesodermin in ExE (36). Transcripts for both markers were readily detected in the ExE of BPTF/FAC1$^{GT/GT}$ embryos, suggesting that the mutation did not impair the TS cell pool (Fig. 5C, J, D, and K). Significantly, Cdx2-positive cells in the mutant were localized to the most proximal domain of the embryo, suggesting that the EPC that is normally located in this region was missing or entirely converted to ExE. Mash2 that was specifically expressed in the EPC of wild-type control embryos (17) was not found in mu-

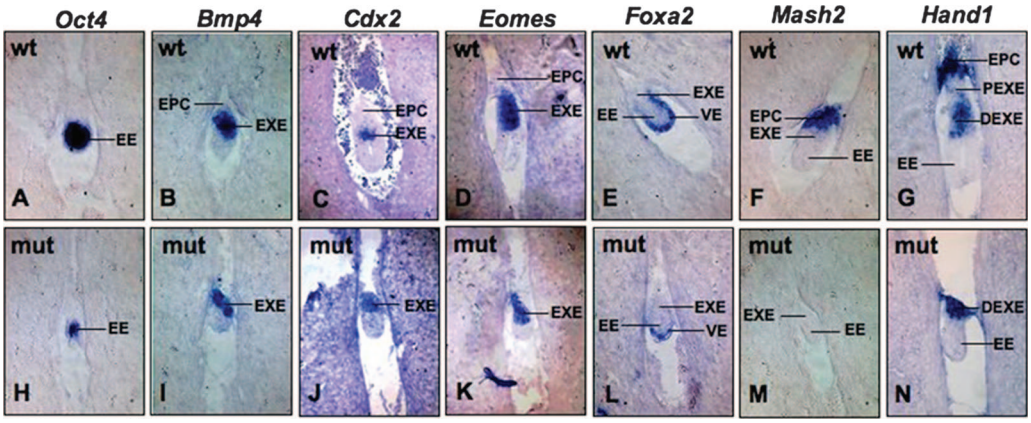


FIG. 5. BPTF/FAC1 mutant embryos exhibit defects in the extraembryonic lineage. In situ hybridizations on sagittal sections of E6.5 embryos using the molecular marker Oct4 for epiblast cells (A and H) and Bmp4 (B and I), Cdx2 (C and J), and Eomes (D and K) as markers for TS cells and ExE. All molecular probes indicate the presence of epiblast and ExE in BPTF/FAC1-deficient (H to K) and wild-type (A to D) embryos. However, mutant embryos appear smaller than those of the wild type. Foxa2, a marker for VE, is also expressed in wild-type and mutant embryos (E and L). In contrast, Mash2 (F and M) and the proximal expression domain of Hand1 (G and N), which marks the EPC, are both absent in the E6.5 mutant embryo, suggesting that EPC formation is affected by the mutation. All conceptuses were sectioned within the decidua and are presented in each panel with their proximal ends toward the top. wt, wild type; mut, mutant.

tant embryos at E5.5, reinforcing the notion that development of the EPC was strongly affected by the BPTF/FAC1 mutation (Fig. 5F and M). Finally, Hand1 that was expressed in EPC and the distal part of ExE of controls (12, 32, 38) was also missing in the presumptive EPC domain of mutant embryos while it was present in the distal ExE (Fig. 5G and N). This observation further supported the idea that the mutation caused the EPC defect. To analyze the visceral (primitive) endoderm, we utilized the Foxa2 marker (31) which was expressed in VE of both mutant and wild-type embryos (Fig. 5E and L) although Foxa2 transcripts in mutants appeared more restricted to the distal part of the epiblast. Collectively, the expression analysis of various markers in E5.5 embryos demonstrated that loss of BPTF/FAC1 primarily affects development of the preplacental trophoblast, resulting in a massive EPC defect possibly including part of the proximal ExE.

Expression of Cer1 and brachyury (T) in BPTF/FAC1$^{GT/GT}$ mutant embryos. Signaling from the trophoblast prior to the formation of the definitive placenta is important for embryonic patterning events including AP axis formation. To examine AP patterning in the BPTF/FAC1$^{GT/GT}$ mutant, we used Cer1, which constitutes a marker for the anterior definitive endoderm underlying the anterior neuroectoderm at E7.5, and brachyury, which is expressed in the PS and reflects posterior structures. In contrast to a sharply defined anterior expression domain of Cer1 in E7.5 wild-type embryos, no Cer1 transcripts were detected in E7.5 mutants (Fig. 6A and D). We also failed to see expression of brachyury in the PS of E6.5 mutant embryos while it was readily observed in the wild type (Fig. 6B and E). At E7.5 brachyury expression was found; however, no defined PS could be recognized (Fig. 6C and F). In some of the E8.5 mutant embryos, we also observed delayed Cer1 transcripts that were localized abnormally (data not shown). We interpret these findings to suggest that in the absence of BPTF/FAC1, a correct AP axis in the epiblast is apparently not formed although with some delay cells arise that express typical anterior and posterior markers. Nevertheless, the morphological AP pattern of the epiblast is disturbed.

Wild-type ES cells fail to rescue the developmental defects in BPTF/FAC1$^{GT/GT}$ mutant embryos. As BPTF/FAC1 is expressed in both the epiblast and the extraembryonic lineage, abnormal morphology and early lethality of mutant embryos could be the result of loss of function in the trophoblast or in

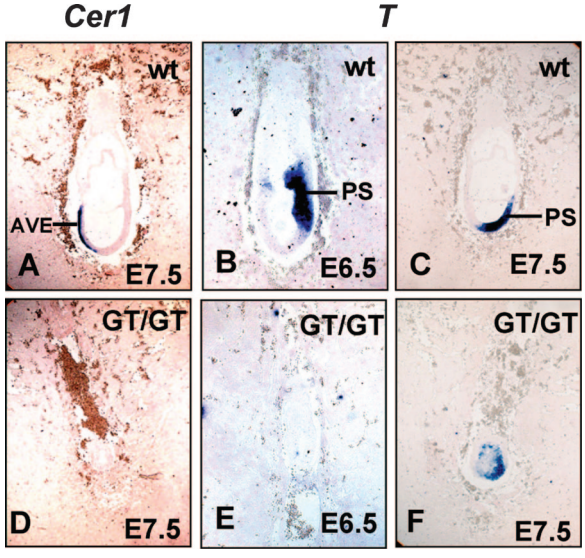
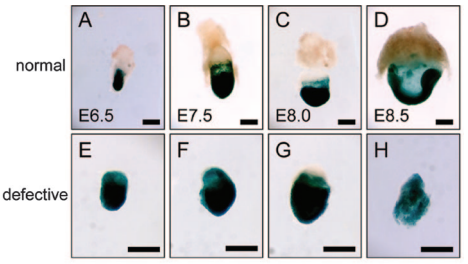


FIG. 6. BPTF/FAC1$^{GT/GT}$ (GT/GT) embryos fail to form a correct AP axis: delayed and displaced expression of markers for AVE and PS. In situ hybridization on sections through wild-type (A to C) and homozygous mutant (D to F) embryos within the decidua using Cer1-specific (A and D) and brachyury (T)-specific (B, C, E, and F) probes. Developmental stages of embryos are indicated. Note that at E7.5 Cer1 expressed in AVE of the wild type is entirely lost in the mutant (A and D). Likewise, brachyury expression in the PS of the wild type (B and C) is delayed (E) and later not exactly confined (F) in mutant embryos. wt, wild type.



total	normal morphology			defective
	+/+	+/GT	GT/GT	
53	18 (34.0%)	22 (41.5%)	0 (0%)	13 (24.5%)

FIG. 7. Wild-type ES cells (Rosa26) are unable to phenotypically rescue chimeric BPTF/FAC1$^{GT/GT}$ embryos. Chimeric embryos are intensely blue owing to the contribution of LacZ-positive Rosa26 ES cells to the epiblast but not to the extraembryonic trophoblast. Chimeras between E6.5 and E8.5 revealed about 75% morphologically normal (A to D) and approximately 25% defective (E to H) embryos. Genotyping of all embryos with normal morphology was performed on carefully dissected LacZ-negative extraembryonic tissue (EPC, ExE, and chorion). Morphologically defective embryos could not be genotyped due to insufficient extraembryonic material. Note that none of the phenotypically normal embryos is a homozygous mutant, and the number of highly chimeric, but nonetheless defective, embryos reflects the expected Mendelian ratio for BPTF/FAC1$^{GT/GT}$ mutants. Scale bars, 1 mm.

the epiblast or both. We sought to examine this issue on chimeric embryos obtained by aggregation of morulae from heterozygous parents with wild-type Rosa26 ES cells that carry the LacZ reporter gene. Since ES cells contribute exclusively to the embryo proper and most of the VE but not to the trophoblast, genetic defects in the extraembryonic lineage of mutants will not be rescued in this type of chimera, whereas loss of BPTF/FAC1 function in the mutant epiblast should be phenotypically rescued. As expected, all chimeras showed strong β-galactosidase staining in the epiblast (>80% of cells), indicating that it was derived from Rosa26 ES cells, and no LacZ-positive cells were found in trophoblasts. The genotype of the host cell contribution in these chimeras was determined by PCR analysis of extraembryonic LacZ-negative tissue that was not contaminated by wild-type ES cells. We recovered a total of 53 highly chimeric embryos, 40 of which were morphologically normal at least until E8.5 while 13 chimeras appeared grossly abnormal, containing almost no extraembryonic trophoblast tissue (Fig. 7). None of the embryos with normal morphology was derived from a homozygous mutant conceptus, suggesting that wild type ES cells were unable to rescue the BPTF/FAC1$^{GT/GT}$ embryos. The morphologically defective chimeras resembling the phenotype of natural BPTF/FAC1$^{GT/GT}$ mutants could not be genotyped due to insufficient extraembryonic material. Significantly, however, these developmentally arrested embryos represented close to 25% of all chimeras, which is in good agreement with the expected number of homozygous mutants according to Mendelian distribution. Taken together, these observations provide strong evidence that the early BPTF/FAC1 mutant phenotype primarily results from loss of BPTF/FAC1 function in the extraembryonic lineage since providing the wild-type gene to the epiblast is not sufficient to rescue the embryo. Of course, additional and later functions of BPTF/FAC1 also in the epiblast are not excluded by this experiment.

DISCUSSION

Human BPTF/FAC1, the vertebrate homolog of *Drosophila* NURF301 protein (48), has been described as a developmentally regulated DNA-binding protein in brain (5, 24, 25); however, its physiological role has not been elucidated. In this study we demonstrate that BPTF/FAC1 is already expressed in the epiblast and trophoblast of E5.5 embryos, suggesting an early requirement for BPTF/FAC1 function presumably in both the embryonic and extraembryonic lineages. A gene trap loss-of-function mutation of the BPTF/FAC1 gene in mouse provides evidence that the gene is important during early embryogenesis since development of the homozygous mutant is arrested shortly after implantation and since virtually all BPTF/FAC1-deficient embryos die prior to midgestation (Table 1). Mutant blastocysts at E3.5 appear morphologically indistinguishable from the wild type, with a regular ICM surrounded by pTE (Fig. 3). Most mutant blastocysts are implanted at E4.5, but shortly thereafter, when the pTE that covers the inner cell mass starts to proliferate and eventually form ExE, the first phenotypic differences between genotypes become readily visible. At E6.5 mutant embryos are significantly smaller compared to the wild type, and this growth retardation initially affects the proximodistal extension and later also the AP axis. Mutant embryos at E8.5 are barely elongated in the rostrocaudal direction and morphologically resemble an earlier developmental stage, approximately corresponding to E6.5 (Fig. 3 and 4). This phenotype convincingly shows that BPTF/FAC1 is absolutely essential for the progression of early postimplantation development either in the epiblast or in the extraembryonic trophoblast or in both lineages. Our attempts to phenotypically rescue mutant embryos with wild-type Rosa26 ES cells completely failed, strongly suggesting that early embryonic defects in the mutant are primarily, if not exclusively, the result of loss of BPTF/FAC1 function in the extraembryonic lineage that cannot be rescued in ES cell chimeras (Fig. 7). This experiment, of course, does not exclude the possibility that BPTF/FAC1 may perform additional or later functions also in the epiblast or embryo proper. The potential roles of BPTF/FAC1 during later embryogenesis, however, cannot be investigated in the gene trap mutant mouse due to the early and dramatic growth arrest. Consistent with a critical role of BPTF/FAC1 in extraembryonic structures, homozygous mutants at the egg cylinder stage (E5.5) are already significantly shorter at the proximal, extraembryonic pole, whereas the distal ends representing the epiblast appear very similar in the mutant and wild type.

Proliferation of ExE relies on TS cells that are maintained by FGF4 signals from the epiblast, and genetic disruption of FGF signaling in mouse results in degeneration of embryos shortly after implantation (1, 11, 16). The transcription factors Cdx2 and Eomesodermin are expressed in trophectoderm and are essential for generation and maintenance of the TS cell population (20, 40). The orphan receptor Errβ (27) and Ets2 (14) and Elf5 (10), two members of the Ets superfamily of transcription factors, are also pivotal in the trophoblast for maintaining TS cells and forming the ExE, which is required

for AP patterning of the epiblast. Ets2- and Elf5-deficient embryos display early growth arrest and embryonic lethality at developmental stages that are very comparable to those of BPTF/FAC1 null embryos, which die between E9.5 and E10.5 (10, 14). Cdx2-deficient mutant embryos die prior to implantation (6), consistent with the notion that Cdx2 is one of the earliest genes that determines trophectodermal identity (26, 34). Likewise, Eomes loss-of-function mutants arrest early at implantation, and no TS cells can be obtained from null mutant blastocysts (36). Strikingly and in contrast to the abovementioned mutant phenotypes, BPTF/FAC1-deficient embryos express the ExE markers BMP4, Cdx2, and Eomes, indicating that they are capable of forming ExE as the direct derivative of pTE (Fig. 4 and 5). These data argue that BPTF/FAC1 is not required for the formation and/or maintenance of TS cells, and the mutation seems unlikely to cause a general proliferation defect in trophectoderm and pTE. The observation that ExE is formed in BPTF/FAC1$^{GT/GT}$ mutants and that TS cells are maintained also implies that the BPTF/FAC1 null epiblast at this stage is signaling properly to the TS cells and, conversely, that the ExE provides the required inductive function for embryonic patterning in the subjacent epiblast (4). Taking these observations together, we conclude that BPTF/FAC1 protein most likely exerts its critical role in the extraembryonic lineage genetically downstream of Cdx2 and Eomes or possibly in a parallel and independent pathway.

Histological analysis and the expression of marker genes demonstrate that the EPC is not developing correctly in BPTF/FAC1$^{GT/GT}$ embryos, and part of the proximal ExE may also be reduced. In particular, the expression domains of the basic helix-loop-helix transcription factors Mash2 and Hand1 demarcating the EPC in wild-type embryos are clearly missing in mutants at E5.5 (Fig. 5) and appear to be significantly reduced in BPTF/FAC1$^{GT/GT}$ embryos of later stages (data not shown). These findings together with histology indicate that formation of the EPC in the absence of BPTF/FAC1 is delayed and in most cases drastically diminished. Thus, BPTF/FAC1 plays an essential role for differentiation of the EPC and formation of the placenta. Hitherto, only few genes have been shown to be specifically involved in EPC development. A knockout mutation of the Hand1 gene in mouse causes embryonic lethality at E7.5. Homozygous mutants contain reduced EPCs and lack differentiated giant cells, indicating that Hand1 is critical for giant cell formation (8, 32, 38). Mash2 is expressed in ExE and EPC (17, 18, 29), where it suppresses differentiation of giant cells, thereby maintaining the population of spongiotrophoblasts. Mash2 null mutant embryos die at E10.5 and contain increased numbers of giant cells on account of spongiotrophoblast cells (18, 41). A null mutation of the transcription factor Ets2 also results in reduced proliferation of EPC cells and a differentiation block leading to death of embryos at E8.5 (49). More recently, the transcriptional repressor Erf, also a member of the Ets family, was shown to be essential for differentiation of ExE since null mutants exhibit placental defects (30). Although the Erf mutation causes embryonic lethality at E10.5, it appears to have little or no effect on the developmental progression of the embryo proper until the time of death, in contrast to BPTF/FAC1$^{GT/GT}$ embryos, which exert severe growth retardation from implantation onwards. It is interesting that BPTF/FAC1$^{GT/GT}$ embryos, although they maintain TS cells in ExE, exhibit a phenotype that is more reminiscent of mutations that cause loss of TS cells and ExE than those that impair formation of EPC and placenta. This observation suggests that BPTF/FAC1, in addition to being required for EPC development, probably serves other pleiotropic functions that may contribute to the strong mutant phenotype.

Defects in trophoblast development not only affect the formation of the placenta and, hence, the maternal-fetal exchange of nutrients but may also have fundamental effects on correct AP patterning of the epiblast. In the Ets2-deficient mouse mutant, it has been demonstrated that PS and mesoderm initiation as well as the anterior shift of distal VE to form AVE are dependent on the direct role of ExE signaling to the epiblast (14). Targeted deletion of the trophoblast-specific Elf5 gene also provides evidence that ExE signaling is required for inducing posterior epiblast identity (10). Both of these mouse mutants phenocopy embryos from which ExE has been removed at pregastrulation stages, resulting in failure to express markers of the PS (33). In keeping with the signaling role of ExE, BPTF/FAC1$^{GT/GT}$ embryos generate brachyury (T)-expressing cells, albeit with some delay, suggesting that gastrulation is initiated, but an anatomically well-defined PS fails to form. The lack of Cer1 expression in the anterior definitive endoderm of BPTF/FAC1$^{GT/GT}$ mutants at E7.5 (Fig. 6) and abnormally located Cer1-positive cells in E8.5 mutant embryos (data not shown) suggest that the anterior-distal VE shift to form AVE does not occur correctly in the mutant. Thus, despite the presence of ExE and expression of signaling molecules, such as FGF4, Nodal, and Wnt3a (data not shown), BPTF/FAC1-deficient embryos do not generate a perfect AP axis although they can activate marker genes that are part of this epiblast patterning process. The observed defects in the mutant epiblast could be a direct result of the missing EPC or be due to reduced levels of the bidirectional signals between trophoblast and epiblast. Alternatively or in addition, BPTF/FAC1 may have an essential function in the epiblast.

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