Essential Role of Chromatin Remodeling Protein Bptf in Early Mouse Embryos and Embryonic Stem Cells

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Abstract

We have characterized the biological functions of the chromatin remodeling protein Bptf (Bromodomain PHD-finger Transcription Factor), the largest subunit of NURF (Nucleosome Remodeling Factor) in a mammal. Bptf mutants manifest growth defects at the post-implantation stage and are reabsorbed by E8.5. Histological analyses of lineage markers show that Bptf⁻/⁻ embryos implant but fail to establish a functional distal visceral endoderm. Microarray analysis at early stages of differentiation has identified Bptf-dependent gene targets including homeobox transcriptions factors and genes essential for the development of ectoderm, mesoderm, and both definitive and visceral endoderm. Differentiation of Bptf⁻/⁻ embryonic stem cells lines into embryoid bodies revealed its requirement for development of mesoderm, endoderm, and ectoderm tissue lineages, and uncovered many genes whose activation or repression are Bptf-dependent. We also provide functional and physical links between the Bptf-containing NURF complex and the Smad transcription factors. These results suggest that Bptf may co-regulate some gene targets of this pathway, which is essential for establishment of the visceral endoderm. We conclude that Bptf likely regulates genes and signaling pathways essential for the development of key tissues of the early mouse embryo.

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Introduction

The packaging of eukaryotic DNA into chromatin provides a general mechanism for the modulation of gene activity and DNA metabolism through alterations of chromatin architecture. The structure and composition of chromatin can be altered by a number of distinct pathways, including post-translational modification of histones, ATP-dependent remodeling of nucleosomes, and incorporation of histone variants [1–3]. ATP-dependent chromatin remodeling is catalyzed by the large and conserved SWI/SNF super family of multi-subunit chromatin remodeling enzymes that are classified into four major subfamilies (SWI/SNF, ISWI, CHD, and INO80), and distinguished by the common presence of a SWI2/SNF2-related catalytic ATPase subunit [4,5]. The mammalian ISWI chromatin remodeling complexes contain either one of two related ISWI ATPases, Snf2l and Snf2h [6,7]. The Snf2l ATPase is contained in two assemblies—NURF (Nucleosome Remodeling Factor), which is dedicated to the regulation of transcription, and the recently reported CERF [8,9]. NURF is the founding member of the ISWI family of chromatin remodeling complexes, and was originally characterized in Drosophila [10]. Purified Drosophila NURF catalyzes ATP-dependent nucleosome sliding and promotes transcription from chromatin templates in vitro [9]. As shown by whole genome expression studies of mutants, NURF positively or negatively regulates transcription of several hundred Drosophila genes in vivo, including many genes important for fly development [11]. This is likely accomplished through recruitment of NURF301, the largest NURF subunit, by gene-specific transcription factors [11–13], and binding of a PHD finger of NURF301 to tri-methylated lysine 4 on histone H3 [14]. Human NURF contains the orthologs of three of four Drosophila NURF components—BPTF (Bromodomain PHD-finger Transcription Factor), the mammalian counterpart of NURF301, SNF2L (the ISWI ATPase) and RhAp46/48, a WD-40 repeat histone-binding protein found in several chromatin-related protein complexes [15]. Biochemical studies of human NURF have shown that it has similar properties to its Drosophila counterpart [15].
Author Summary

While the chromatin of eukaryotes provides an efficient means to compact large amounts of DNA into a small nucleus, it renders the DNA relatively inaccessible. ATP-dependent chromatin remodeling complexes mobilize nucleosomes and provide a means to gain access to DNA in chromatin. While the biochemical functions of chromatin remodeling complexes is well-characterized, less is known of their biological functions. In this manuscript, we elucidate the biological functions of Bptf, a subunit of the NURF chromatin remodeling complex. Our studies show that Bptf is required for the establishment of the anterior–posterior axis of the mouse embryo during the earliest stages of development. To understand its functions in tissue differentiation, we generated and characterized Bptf-mutant ES cells. Mutant embryonic stem cells show significant defects in the differentiation of ectoderm, endoderm, and mesoderm. Genome-wide analysis of gene expression defects during differentiation has identified many Bptf-dependent pathways including key regulators of ectoderm, endoderm, and mesoderm differentiation. Moreover, we have identified critical functions for Bptf during the TGF/Smad-induced expression of visceral endoderm and mesoderm markers, an important signaling pathway in the early embryo. These results suggest that chromatin remodeling by Bptf regulates key signaling pathways in the early mouse embryo.

The physiological functions of an increasing number of mammalian chromatin remodeling complexes have been revealed by studies of mouse mutants for the catalytic ATPase. Mutations in Btg1, Brm, Chd4, Chd2, p400 and Edil have been shown to be required for proper embryonic development, hematopoiesis or postnatal survival [16–22]. A mutant for the Snf2h, one of the two murine ISWI ATPases, revealed severe proliferation defects in the early embryo, resulting in a peri-implantation lethal phenotype [23]. Given the presence of Snf2h in chromatin remodeling complexes, the assignment of biological phenotypes to different enzyme complexes can be problematic [6,7].

By analysis of mutations of unique subunits it is possible to identify the biological functions of different Snf2h-containing complexes. This has been accomplished for the Drosophila ISWI complexes. Studies of mutants for Drosophila NURF301, which is exclusive to the NURF complex [12], have revealed a late larval-lethal phenotype and mis-expression of homeotic selector genes and genes involved in the response to heat stress, cytokine and steroid hormone signals [11,13]. These phenotypes do not overlap with those observed for mutants for Drosophila ACF1 (a component of the ISWI-containing complexes ACF and CHRAC) which are impaired in the establishment and/or maintenance of transcriptional silencing in pericentric heterochromatin and in repression by Polycomb-group genes [24].

To elucidate the biological roles of Bptf-containing complexes we have generated embryonic stem cell and mouse mutants for Bptf [15]. Our studies show that Bptf mutant phenotypes begin to manifest just after implantation stage, and mutant embryos are completely reabsorbed by embryonic day (E) 8.5. Genetic and molecular analysis in embryonic stem cells and the mouse suggest a role for Bptf in the development of visceral endoderm (VE) of the early mammalian embryo. We propose that Bptf is required for the development of the VE, and more importantly the distal visceral endoderm (DVE), in part through regulating cellular proliferation, the expression of homeobox-containing transcription factors and pathways regulated by the Smad transcription factors, a major conduit for cell signaling in development. These findings suggest a model in which the activities of Bptf-containing complexes, likely the NURF remodeling complex, regulate cell proliferation and embryonic development and therefore are essential in the post-implantation embryo.

Results/Discussion

Characterization of Gene-Trap and Conditional Alleles of Bptf

BPTF, the mammalian ortholog of Drosophila NURF301, is a large, multi-domain protein that is apparently exclusive to the mammalian NURF complex (Figure S1A) [12,15,25]. An initial characterization of Bptf showed it to be nuclear in the P19 embryonic carcinoma cell line and can exist in at least two electrophoretic variants, which we termed Bptf-H and Bptf-L (Figure S1B, Figure S1C). As previously reported, Bptf is highly expressed in testis, spleen, brain and to a lesser extent in kidney by Western blotting (Figure S1D). Interestingly Bptf is highly expressed during embryonic development, and expression substantially declines upon birth (Figure S1E). The high levels of Bptf expression in the mouse embryo suggest it may have essential functions during embryonic development.

To elucidate the biological functions of mammalian Bptf during mammalian embryonic development we generated two mutant mouse lines. One line, designated as Bptf<sup>XG022</sup>, was derived from an ES cell line carrying an in-frame gene-trap vector insertion between exons 15 and 16 of Bptf (Figure S2A, Figure S2B, S2C, S2D, S2E, S2F) [XG022; http://www.genetrap.org/] [26]. We identified the precise junction of the insertion site of the gene trap by DNA sequence analysis of the corresponding PCR products, and confirmed by RT-PCR that trapping of Bptf mRNA into vector sequences leads to loss of RNA splicing between exons 15 and 16, and reduced expression of Bptf sequences 3′ to the insertion site (Figure S3A, Figure S3B). In addition, we confirmed by 5′-RACE that the insertion resulted in an in-frame fusion between Bptf and β-galactosidase-neomycin phosphotransferase (β-Geo) sequences of the gene-trap vector. (Figure S3C). Consistent with previous findings, Northern blotting of adult tissue RNA showed that both wild-type Bptf and the Bptf-β-Geo fusion alleles are specifically expressed at high levels in the testis and at moderate levels in the lung, spleen, and brain (Figure S3D) [25]. These identical RNA expression patterns initially suggest that β-galactosidase is a faithful reporter of Bptf expression in adult tissues. These results indicate that the <sup>Bptf<sub>XG022</sub></sup> mutation creates a truncated Bptf-β-Geo fusion carrying the N-terminal 1978 residues of the 2903-residue Bptf open reading frame, which eliminates the conserved glutamine rich region, PHD finger, and bromo-domains.

The second line, designated as Bptf<sup>Albun2</sup>, was generated by targeting loxP sites flanking exon 2 of Bptf (Figure S2A, Figure S2G, Figure S2H, Figure S2I, Figure S2J, Figure S2K, Figure S2L, Figure S2M, Figure S2N). The expression of the Bptf<sup>Albun2</sup> allele was assessed by RT-PCR with the use of primer sets which amplify sequences on the 3′ end of the Bptf transcript. We found that the Bptf<sup>Albun2</sup> allele slightly decreases expression of Bptf at the RNA level (Figure S4A). However, amplification and sequencing of the Bptf<sup>Albun2</sup> mRNA from exons 1 to 8 shows an out-of-frame mutant mRNA, indicating that Bptf<sup>Albun2</sup> behaves as a loss-of-function allele (Figure S4B, Figure S4C).

Post-Implantation Phenotypes of Bptf Mutant Embryos

We intercrossed heterozygous mice to determine the biological function of Bptf during mouse development. With 210 mice...
genotyped for the \( Bptf^{XG023} \) allele and 75 mice for the \( Bptf^{4Exon2} \) allele, we did not find any surviving homozygous mice at weaning, indicating that \( Bptf \) is required for mouse development. An analysis of E6.5 to E18.5 progeny derived from heterozygous intercrosses confirmed that the homozygous mutant phenotype is embryonic-lethal between E7.5 to E8.5, with 100% penetrance (Table S1).

To identify defects in the embryonic development of \( Bptf^{XG023} \) and \( Bptf^{4Exon2} \) homozygotes, we performed whole mount examinations of mutant embryos. Growth defects increasing in severity from E5.5 to E7.5 were observed (Figure 1A) (Figure S5A). Dissections conducted at E8.5 and E9.5 were uninformative, because a majority of the embryos had been completely reabsorbed (data not shown). We crossed \( Bptf^{XG023} \) and \( Bptf^{4Exon2} \) heterozygous mice to generate the trans-heterozygous \( Bptf^{XG023}/Bptf^{4Exon2} \) embryos. The trans-heterozygote recapitulated the growth defects of the \( Bptf^{XG023} \) and \( Bptf^{4Exon2} \) homozygotes, indicating that the two mutations are functionally equivalent (Figure S5B). To determine whether the developmental defects originated before implantation, we harvested E3.5 blastocysts and performed blastocyst outgrowth assays. We observed normal E3.5 homozygous \( Bptf^{XG023} \) and \( Bptf^{4Exon2} \) blastocysts and normal outgrowths from the blastocysts after tissue culture for 5 days, suggesting that either \( Bptf \) is not essential for pre-implantation development or that the maternal Bptf protein or mRNA can mask pre-implantation phenotypes (Figure S6). The masking of pre-implantation phenotypes by maternal Bptf is possible because it is highly expressed in oocytes [27,28].

To further investigate the basis of the early embryonic lethal phenotype, we performed a histological analysis of \( Bptf^{XG023} \) mutant embryos at E5.5, E6.5 and E7.5. Mid-sagittal sections of E5.5, E6.5 and E7.5 embryos stained with hematoxylin and eosin (H&E) showed a distinct proximal-distal (P-D) axis and the development of visceral endoderm but a significant decrease in size of the embryonic and extra-embryonic tissues. This was particularly evident in the embryonic ectoderm at E6.5 and E7.5 (Figure 1B). By E6.5, mutant embryos showed clear developmental defects. Although there was a clear boundary between extra embryonic and embryonic tissues, the absence of a primitive streak and any discernable mesoderm suggests that the anterior-posterior (A-P) axis did not form (Figure 1B).

A reduction in cell number can be a consequence of decreased cell proliferation, increased cell death (apoptosis), or a combination of both processes. To ascertain the extent of programmed cell death, we performed TUNEL assays on E3.5, E6.5 and E7.5 homozygotes and found no increased numbers of apoptotic cells when compared to controls (Figure 1B). As a measure of cell proliferation we monitored phosphorylated histone H3 levels in the conceptus by immuno-histochemistry (Figure 1B). E5.5, E6.5 and E7.5 homozygotes showed ~40–50% decrease in phosphorylated histone H3 levels indicating that a decrease in cellular proliferation may contribute to the mutant phenotype (Figure S7).

Figure 1. \( Bptf \) mutants manifest early embryonic growth defects. (A) Wild type (+/+), heterozygous (+/−), and homozygous (−/−) \( Bptf^{XG023} \) embryos at E5.5, E6.5, and E7.5 were removed from their decidua and genotyped using the PCR based method. Reduced growth rates are evident in the homozygous \( Bptf^{XG023} \) embryos as early as E5.5. (B) Hematoxylin and eosin (H&E) staining of E5.5, E6.5, and E7.5 mutant embryos show reduced growth of both embryonic and extra-embryonic tissues, the presence of visceral endoderm, and an absence of anterior–posterior asymmetry when compared to controls. \( Bptf \) mutant embryos were stained for phosphorilated histone H3, a marker of cell mitosis. Mutant embryos at E5.5, E6.5, and E7.5 have significantly reduced staining when compared to controls, suggesting that they have reduced cellular proliferation. \( Bptf \)-mutant embryos were stained for apoptotic cells using the TUNEL assay. Mutant embryos at E5.5, E6.5, and E7.5 do not have any positive staining by TUNEL when compared to controls, suggesting that they are not apoptotic. Scale Bars: E5.5 and E6.5 embryos = 50 μm, E7.5 embryos = 100 μm. Abbreviations: ve, visceral endoderm; ee, embryonic ectoderm; m, mesoderm; xe, extra-embryonic ectoderm; xve, extra-embryonic visceral endoderm.

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Bptf Mutants Fail To Develop a Functional Distal Visceral Endoderm

Subsequent to implantation of the mouse blastocyst there is rapid proliferation of the egg cylinder, which consists of three cell types: the more proximal extra-embryonic endoderm, the more distal embryonic endoderm or epiblast, and an outer layer of visceral endoderm [29]. The visceral endoderm originates from the primitive endoderm, a layer of cells organized at E4.5, which is composed of cells from the ICM of the E3.5 blastocyst expressing Gata6 but not Nanog [30]. At ~E5.5, a specialized cluster of endoderm cells, the DVE, arises at the distal tip of the embryo. DVE cells migrate toward the prospective anterior, to form the anterior visceral endoderm (AVE). DVE/AVE cells secrete molecules such as cerberus (Cer1) and Lefty1, antagonists of the Transforming Growth Factor β (TGFβ)-related protein Nodal [29]. These antagonists restrict the activity of Nodal to the posterior pole of the embryo at E6.0 [31]. The primitive streak forms at E6.5, indicating that gastrulation has begun, and gives rise to the mesoderm and definitive endoderm germ layers [29].

As a first step in the molecular analysis of Bptf in embryonic development we examined the expression of Bptf using in situ RNA hybridization. We observed expression in the inner cell mass (ICM) and primitive endoderm at E4.5 and all embryonic tissues at E5.5 and E6.5. Interestingly we do not observe Bptf expression in the visceral endoderm at E5.5 and E6.5 (Figure 2A). We also monitored the activity of the β-galactosidase moiety of the Bptf–β-Geo fusion protein in heterozygous mice. Consistent with our in situ analysis a histochemical analysis of whole mounts showed that Bptf–β-Geo is expressed in the embryo proper at E5.5, E6.5 and E7.5 (Figure S8A, S8B, S8C, S8D, S8E, S8F, S8G). Further analysis of histological sections revealed that Bptf–β- Geo expression at E7.5 is primarily confined to the embryonic endoderm, with reduced levels in mesoderm and no expression in the visceral endoderm (Figure S8A, Figure S8B). At subsequent stages, from E7.5 to E13.5, histochemical analysis of whole mounts showed widespread Bptf–β-Geo expression in the developing embryo (Figure S8A, Figure S8B, S8C, S8D, S8E, S8F). This temporal correlation between the earliest stages of Bptf expression and the stages when the mutant phenotype is revealed, suggests that there could be an essential requirement for Bptf as early as E4.5.

Our histological analysis suggests that Bptf mutants are defective in establishing an A-P axis. Apparent defects in A-P axis can be due to defects in the establishment or migration of the DVE [29]. To monitor the development of the DVE and its transition to the AVE, we analyzed the markers Otx2, Lefty1, Cer1, Hex1, Hex1, Gata6, Nanog and Nodal by in situ hybridization in E4.5 to E6.5 mutant embryos [32–37].

An analysis of pre-implantation embryos suggested that Bptf mutants specify a functional primitive endoderm and ICM. The primitive endoderm of Bptf mutant embryos was found to express the primitive endoderm markers Gata6, Lefty1, and Hex1 at comparable levels to wild type controls (Figure 3B). The expression of these markers suggests that Bptf mutants are not defective in the differentiation of the primitive endoderm, the precursor of the visceral endoderm of E5.5 and later stage embryos. Consistent with a functional ICM, we observed normal expression of the pluripotency marker Nanog in Bptf mutants compared to controls (Figure 2B). Taken together, these results indicate that Bptf is not required for the specification of the primitive endoderm and the ICM in E4.5 embryos.

To assess the specification of the VE and DVE, we monitored the expression of Cer1, Hex1, Gata6, and Nodal in E5.5 Bptf mutant embryos. We observed that the markers Cer1, Hex1 are significantly reduced in Bptf mutants relative to the wild type controls (Figure 2B). The absence of expression of these markers...
indicates that the DVE does not form in the absence of Bptf. Interestingly, Gata6 expression, normally expressed only in the VE at E5.5 and E6.5, is absent in the VE but present in the epiblast in mutants at E5.5 (Figure 2B). This suggests that Bptf could also have roles in specifying the VE as well as the DVE. A key regulator of DVE specification is Nodal. Nodal expression is found as early as E4.5 but is not significantly expressed in the epiblast and VE until implantation is well underway at E5.0 [36,38]. In E5.5 Bptf mutant embryos we observe normal expression levels of Nodal in the epiblast and VE, as in wild type embryos (Figure S10A). This E5.5 pattern of Nodal expression continues into E6.5 in Bptf mutant embryos (Figure S10B).

To examine the development of the AVE we monitored the expression of Cer1, Otx2, Hesx1, Left1 and Hes1 in E6.5 Bptf mutant embryos. Otx2 expression is required for the migration of the DVE to establish the AVE. We observed lower expression of Otx2 in the epiblast of Bptf mutant embryos relative to their wild type controls (Figure S11A). As expected we did not observe expression of AVE markers Cer1, Hesx1, Left1 and Hes1 in Bptf mutant embryos (Figure S11A). Combined with our analysis of E5.5 embryos our results strongly suggest that Bptf is required for the specification of the DVE and the AVE.

Since Bptf mutant embryos are unable to form a functional DVE and AVE, we anticipated that they should be defective in specifying the primitive streak and differentiating mesoderm and definitive endoderm. Several critical transcription factors and signaling molecules such as T, Lhx1, Fgf8, Gsc, Foxa2, Nodal, and Cripto (Tdfg1) serve as effective markers for development of the primitive streak in the gastrulating embryo [34–39]. Our analyses revealed that T, Lhx1, Fgf8, Gsc, and Foxa2 were undetectable at E6.5, and in the case of T, Fgf8 but not Lhx1, were delocalized in expression at E7.5 (Figure S11A, Figure S11B). The absence of expression of primitive streak markers at E6.5 confirms our histological analyses, and further supports the observation that gastrulation and mesoderm development do not occur in Bptf mutants. Interestingly, the delocalized Nodal and Cripto expression patterns observed in the Bptf mutants at E6.5 are highly reminiscent of their expression patterns prior to the establishment of the DVE (Figure 2B) (Figure S11A) (Figure S10A, Figure S11B) [34,39]. Taken together, the data suggests that Bptf mutant embryos arrest at a stage prior to DVE formation (<E5.5).

Recent models propose that the extra-embryonic ectoderm supports the growth of the embryo and is a source of signals for A-P axis establishment [29]. To address whether the developmental defects of Bptf mutants are caused by defective extra-embryonic ectoderm or by a lack of appropriate growth signals in the epiblast, we monitored the expression of the extra embryonic ectoderm (Bmp4, Eribb2, Fgfl2), trophoderm (Mas1k2), angiogenesis (Vegf, Flk1) and a cell cycle regulator (JunB) markers [43–47]. We find Bmp4, Eribb2, Fgfl2, and Fgfl2 to be expressed normally in the mutant embryos at E6.5 and E7.5 (Figure S11A, Figure S11B). This suggests that the growth defects observed in Bptf embryos are not due to gross defects in the specification the extra-embryonic tissues. We also observe little to no expression of the angiogenesis markers Vegf and Flk1 in the extra-embryonic tissues at E7.5 (Figure S11B). However, expression of the cell cycle regulator JunB is increased in mutant embryonic ectoderm when compared to wild-type controls (Figure S11A). JunB is a member of the Ap-1 family of transcription factors which acts as a negative regulator of the cell cycle [48]. This up-regulation of JunB is consistent with our observations that Bptf mutants have reduced cellular proliferation (Figure 1B) (Figure S7).

In summary, our analysis of lineage markers by in situ RNA hybridization has revealed an essential role for Bptf in specifying the VE and the DVE of the E5.5 post implantation embryo. These defects likely lead to the observed absence of an AVE and primitive streak in E6.5 embryos. The absence of these developmental organizers arrests the growth of the embryo prior to gastrulation and is likely a major cause of the early embryonic lethal phenotype of Bptf mutant embryos.

### Bptf Is Required for Embryonic Stem Cell Differentiation

To further explore the role of Bptf in cell differentiation we generated Bptf knockout mouse ES cells and examined their development in vitro and in vivo. By gene targeting and transient Cre expression we were able to generate eight independent homozygous Bptf<sup>flk1020</sup> knockout ES cell lines with a euploid karyotype (Figure S12A). We observed varying degrees of reduced Bptf transcript levels by Northern blotting in mutant cell lines compared to that of wild type controls (Figure S12B). An analysis of Bptf<sup>flk1020</sup> mRNA from exons 1 to 8 in wild type and knockout cell lines shows the message to be out of frame resulting in no observable protein in the knockout cell lines (Figure S12C, Figure S12D) (data not shown).

To address the possibility the Bptf is essential for cell viability and proliferation we measured the doubling time of knockout Bptf ES and MEF cell lines (Figure S13). Both the Bptf knockout ES and MEF cell lines were viable but exhibited slightly reduced cellular proliferation (Figure S14). These results show that Bptf is not required for cell viability and is only marginally required for cellular proliferation.

We measured the differentiation potential of the Bptf knockout ES cells in vitro as embryoid bodies and in vivo as teratomas. Bptf wild type and two independent knockout cell lines were subcutaneously injected into NOD/SCID mice and allowed to form teratomas over 8 weeks. Wild type ES cells formed teratomas in 8/11 injected mice. Bptf knockout lines did not form any observable tumors in 10 injections (data not shown). This study demonstrated that Bptf is essential for one or more biological processes including cell viability, proliferation or differentiation in the animal.

We next utilized an embryoid body analysis to monitor differentiation in Bptf knockout cell lines to ectoderm, mesoderm and endoderm cell lineages. Analysis of the mutant embryoid bodies showed light evidence of apoptosis by TUNEL, and similar densities of PCNA and phosphorylated histone H3 positive cells (Figure S15A). We did observe that Bptf knockout embryoid bodies were slightly smaller in size and did not form any observable endoderm (Figure S15B, Figure S15C). These results indicate that Bptf is not necessary for cellular survival or proliferation under conditions of embryoid body differentiation but is essential for differentiation of endoderm and possibly other advanced tissue lineages.

To investigate the differentiation defects of Bptf knockout embryoid bodies at a molecular level we monitored the transcription of well documented markers of endoderm, mesoderm and ectoderm differentiation (Figure 3A–3C). We observed minimal Bptf dependence for the primitive ectoderm markers Fgf5 and Otx2 (Figure 3A). However, we did observe significant defects in Nestin transcription, a marker of neural stem cell progenitors derived from the primitive ectoderm (Figure 3A). These results indicate that primitive ectoderm lineages are less dependent on Bptf than the more differentiated lineages. As anticipated we observed severe defects in the expression of mesoderm and endoderm markers during the differentiation time course. We observed little to no activation of mesoderm markers T, Fgf8, Eox1, Wnt3, and Gsc which are significantly activated in wild type cultures by day 5 (Figure 3B). Similarly, endoderm markers Sox17, Cer1, Hhex, and Foxa2 show severe expression defects in Bptf mutant embryoid bodies compared to controls (Figure 3C). In contrast to the large differences in expression of mesoderm and endoderm markers we observed less than two fold changes in...
expression of cell cycle regulators and pluripotency markers with the exception of Cyclin D1 (Figure S16A, Figure S16B). These defects in transcription and differentiation were rescued for two independent knockout lines by retargeting exon 2 to the BptfΔExon 2 locus using the targeting vector. Our ability to rescue the expression defects of T, Gsc, Sox17 and Cer suggest that the observed phenotypes are due to Bptf mutation (Figure S17A, Figure S17B, Figure S17C) data not shown). Taken together these results suggest that Bptf is essential for the formation of mesoderm, endoderm and more differentiated ectoderm lineages in the embryoid body.

We chose a microarray approach to investigate any differentiation defects of Bptf knockout ES cells in undifferentiated and early differentiation states. Differentiation was induced by LIF withdrawal (LIF−) or retinoic acid (RA) for three days before harvesting RNA. A comparison of Bptf-dependent genes by Venn diagram and manual clustering identifies six categories; those being affected under only one of the growth conditions (LIF+, LIF− or RA Regulation), those being affected in all three conditions (Constitutive Regulation), a class of genes which were regulated in the same direction in two of three conditions (Complex Regulation) and genes with mixed dependence (Mixed Regulation) (Figure 4A and 4B) (Dataset S1).

From our microarray analysis we observed large changes in gene expression under conditions of maintained pluripotency (LIF+) and particularly under conditions of differentiation (LIF− and RA) (Figure 4A and 4B). As expected many of the essential markers of early embryonic tissue differentiation that are dependent on Bptf in embryos are also Bptf-dependent in ES cells (Figure 4C). These markers include; pluripotency regulators Sox2, c-Myc, Nanog, visceral endoderm markers Lefty1, Cer1, Hox1, Foxa2 and the primitive streak markers Gsc, Lhx1, Wnt3, Fgf8, T (Figure 4C). These results reinforce the view that Bptf regulates the development of ectoderm, endoderm and mesoderm in both the early embryo and in ES cells.

A gene ontology (GO) analysis of Bptf-dependent expression datasets revealed an over representation of genes with “transcription factor activity”, genes involved in the biological processes of “development” and “morphogenesis” and the cellular processes of “cell death” and “cell proliferation” (Figure S18). Notable gene clusters include the consistent activation of genes correlated with “nervous system development” in all datasets, the repression of MHC I and II receptors during LIF+ differentiation, the activation of genes correlated with “cytoskeletal components” during RA differentiation (Figure S19). In addition, we observed a striking over-representation of homeobox transcription factors within the “transcription factor activity” annotation. The homeobox-containing genes were almost exclusively up-regulated in each of the expression categories, and in some cases include almost the entire Hox gene cluster, indicating that Bptf is required for their repression in ES cells (Figure S19A, Figure S19B).

From our analysis we also observed that Bptf-dependent gene targets are more likely to be actively regulated genes, repressed in the presence of LIF or RA differentiation and conversely activated under conditions of LIF differentiation (Figure S20A, S20B, S20C). In support with these observations we observed histone modifications in Bptf knockout ES cell lines consistent with a repressed transcriptome under LIF+ growth conditions (Figure S20D). Interestingly some Bptf-dependent genes cluster together in the genome (Figure S21).

A Role for Bptf in Distal Visceral Endoderm Formation

A diagnostic defect of Bptf embryos is an inability to form the DVE. In the absence of the DVE, the AVE cannot form

![Figure 4. Analysis of Bptf knockout mouse embryonic stem cells shows severe defects in gene expression during differentiation with RA and LIF withdrawal. Bptf knockout embryonic stem (ES) cells were induced to differentiate using LIF withdrawal (LIF−) or the addition of retinoic acid (RA), and gene expression was monitored by microarray. (A) Bptf-dependent genes were defined as genes whose transcription increases or decreases more than 2-fold in the mutant compared to the wild type with a FDR value of <0.05. (B) A manual clustering analysis of Bptf-dependent genes by condition of dependence and expression values. Six expression categories were identified and include genes which are exclusively Bptf-dependent in LIF−, LIF+, or RA conditions, genes which are dependent in all conditions (constitutive regulation), genes which are dependent in 2 conditions (complex regulation), or genes which vary in direction of misregulation between conditions (mixed regulation). (C) Clustering analysis of Bptf-dependent genes important for the development of tissue lineages of the early embryo. Bptf is essential for the proper regulation of many markers of ectoderm, endoderm, and mesoderm tissue lineages.

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preventing the necessary signals for the specification of the primitive streak. To further investigate the functions of NURF in the early embryo we focused on Smad mediated signaling pathways. Smad mediated signaling pathways are essential for the formation of the DVE in the embryo and the induction of mesoderm in differentiating ES cells, two prominent phenotypes of our in vivo and in vitro studies on Bptf [49,50].

The most prominent ligand activating the Smad transcription factors in the early embryo is Nodal [51]. Nodal, and the closely related ligand activin, bind to type I and II TGFβ receptors resulting in the phosphorylation of the type I receptor. Phosphorylation of the type I receptor activates a kinase domain which phosphorylates Smad2/3. The phosphorylation of Smad2 or Smad3 transcription factors promotes interactions with Smad4 and triggers the translocation of the Smad complex into the nucleus [51]. Once in the nucleus, the Smad complex interacts with DNA sequence specific transcription factors to promote the regulation of Smad target genes [52].

Accordingly, we monitored the dependence of Smad-responsive genes on the presence of Bptf in ES cells. ES cells readily responded to activin-A as monitored by the phosphorylation of Smad2 (Figure S22A, Figure S22B). From these experiments we identified a number of Smad-dependent genes which completely or partially require Bptf for full activation. Genes requiring Bptf for full activation include Cer1, Gsc and T (Figure 5A). Genes which partially require Bptf include Fgf8, Lefty1 and p21 (Figure 5A) (Figure S23).

To understand the relationship between Bptf and CBP/p300, known co-activators of the Smad transcription factors, we knocked down both Bptf and CBP/p300 using siRNA technology and monitored the activation of the Smad responsive genes Lefty1, Fgf8, Gsc, T, and Cer in ES cells (Figure S22C). As in the Bptf knockdown ES cell lines we observed that each of these genes are dependent on Bptf for full activation (Figure 5B). While some genes differ in their requirement for Bptf, they are all dependent on CBP/p300 for activation (Figure 5B). Our results demonstrate that a genetic knockout and siRNA mediated knockdown of Bptf result in defects in the activation of Smad responsive genes to varying degrees. Taken together, these results indicate that Bptf, like CBP/p300, acts as a co-activator of Smad responsive genes in ES cells.

We also used the embryonic carcinoma cell line P19 to complement our findings with the Bptf knockout ES cells. We cotransfected P19 cells with DNA plasmids carrying four minimal Smad binding elements (SBE), or three activin response elements (ARE), linked to a core promoter and a luciferase reporter gene. The ARE and SBE elements were previously found to be the minimal Smad-responsive elements [53,54]. Under conditions of Bptf knockdown, we observed a significant reduction in luciferase activity from both reporters (Figure S24A, Figure S24B).

Figure 5. Bptf is necessary for Smad mediated gene regulation. (A) RT-PCR analysis of known Smad target genes from activin-A induced wild type (+/+). Bptf knockout (−/−) embryonic stem (ES) cells shows Cer1, T, Gsc to be dependent, and Fgf8, Lefty1 to be partially dependent on Bptf for full activation. (B) Like CBP/p300, Bptf regulates Smad-dependent genes in vitro. Bptf or CBP/p300 were knocked down in activin-A induced or uninduced ES cells. The expression of gene targets was determined by RT-PCR and is expressed as a ratio of induced/uninduced for each knockdown condition. Lefty1, Fgf8, T, Gsc, and Cer1 require both Bptf and CBP/p300 for full activin-A-dependent gene activation. (C) NURF interacts with the Smad transcription factors in vitro. Recombinant Nurf complex was subjected to GST pull-down assays using resin bound GST, GST-Smad2, GST-Smad3 and GST-β-catenin. GST-Smad2 and 3 but not GST and GST-β-catenin controls can specifically pull down the NURF complex. The GST-GF12C containing the C-terminal fragment of Smad2 specifically pulls down the NURF complex. The C-terminal domain of Smad2 interacts with native NURF from crude nuclei extracts. High salt nuclei extracts from ES cells was subjected to GST pull-down assays using resin bound GST and GST-Smad2. The GST-GF12C containing the C-terminal fragment of Smad2 specifically pulls down the Bptf and Snf2h/l components of the native NURF complex and the histone acetyl-transferase CBP from nuclei extracts. (D) Chromatin immunoprecipitation of Snf2h/l and histone modifications 3me-K4H3 and 3me-K27H3 at Lefty1 show recruitment of the Snf2h/l subunit to the neural plate specific enhancer (NPE), a region which contains Smad binding elements, in a Bptf and an activin-A-dependent manner. Snf2h/l ChIP is expressed as a ratio of induced (+activin-A) to uninduced (−activin A). Histone modifications are shown during induced (+activin-A) conditions for +/+ and −/− cells and have been normalized to a pan histone H3 pulldown. NPE = neural plate specific enhancer, LPE = lateral plate specific enhancer, RSS = right side specific enhancer.

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To simulate Smad signaling in a different way we co-transfected combinations of Smads 2,4 with constitutively active TβRI (ALK5), the type I receptor for the TGFβ signaling pathway (Figure S24D) [53]. Using this system we observed efficient reduction in the activation of the SBE regulated luciferase reporter gene with a siRNA to Bptf but not a mock siRNA control (Figure S24D). We repeated the experiments using multiple unique siRNAs and measured the transcription of endogenous TGFβ regulated genes. In these experiments we used three individual Bptf siRNAs which were effective in knocking down protein expression after 2 days of culture (Figure S24E). We then stimulated the P19 cells with TGF-β1. Like nodal and activin-A, TGF-β1 stimulates the phosphorylation of Smad 2/3 through the dimerization and activation of similar type I and II receptors. We similarly observed a significant reduction of Cer1 and T transcription in Bptf depleted cells upon Smad2/3 activation with TGF-β1 (Figure S24F). We also used the human breast cancer cell line MCF10CA1 in similar assays to determine if Bptf could play a role in Smad signaling in humans [56]. In these experiments we used BPTF siRNAs which were effective in knocking down protein expression in MCF10CA1 cells after 2 days of culture (Figure S24G). We then stimulated the MCF10CA1 cells with TGF-β1 for 1 hour. We observed a significant reduction of PAI-1 but not Smad7 induction suggesting that, as in the mouse, BPTF could regulate a subset of Smad responsive genes in humans (Figure S24H).

We next investigated whether the interaction between the BPTF-containing NURF complex and the Smads is direct or indirect using pull-down assays. Experiments with bacterially expressed GST-Smad2 or GST-Smad3 showed an interaction to a degree between recombinant NURF complex and each of the Smad transcription factors, but not the GST or GST-βcatenin controls (Figure 5C). Smad transcription factors are composed of functional domains at the N-terminus (MH1 domain) and C-terminus (MH2 domain). The N-terminal-HinR and C-terminal regions of Smad2 were used in similar pulldown experiments. We observed that recombinant NURF complex interacts specifically with the MH2 domain of Smad2 (Figure 5C). This interaction was also observed for the Bptf and Snf2l components of native NURF complex from crude ES cell nuclear extracts (Figure 5C). We also confirmed the reported interaction of the C-terminal MH2 domain of the R-Smads with the co-regulator CBP (Figure 5C) [57]. Hence, our results suggest that NURF, like p300 and CBP maybe recruited to the promoters of TGFβ responsive genes through direct interactions with the Smad transcription factors.

Our current model proposes that Bptf-containing complexes like NURF are recruited to the promoter of Smad regulated genes through direct interactions with the Smad transcription factors. To test this model, we initiated chromatin immuno-precipitation (ChIP) experiments to detect the Snf2l component of the NURF complex at Lefty1. As anticipated, we observed significant enrichment of Snf2l at the Neural Plate Specific Enhancer (NPE), a region which contains putative FAST and Smad transcription factor binding sites, of the Lefty1 promoter [58]. This enrichment was dependent on activin-A stimulation and on the presence of Bptf (Figure 5D). Snf2l enrichment correlates with the presence of the activating histone modification H3K4me3 at the 5′ UTR of Lefty1 (Figure 5D). These results are consistent with Bptf recruitment to the promoter of Lefty1 through the Smad transcription factors.

**Discussion**

In this work we report a post-implantation lethal phenotype for mutations in Bptf, the previously characterized largest subunit of the NURF chromatin remodeling complex [15]. In the early embryo Bptf is expressed by E4.5 in the ICM and primitive endoderm. At later stages of development Bptf is expressed in both embryonic and extra-embryonic ectoderm by E5.5. Following gastrulation, Bptf is widely expressed in all germ layers to E13.5. Bptf expression is essential for early development because homozygous Bptf mutant embryos are reabsorbed by E6.5. No overt defects were observed in the mutant E3.5 or E4.5 embryo or its ability to proliferate in culture, suggesting that Bptf embryos undergo a normal initial specification and proliferation of the ICM, primitive endoderm and trophectoderm. However, mutant embryos exhibit diminished proliferation post-implantation as shown by defects in size of both extra-embryonic and embryonic tissues and decreased phosphorylated histone H3 staining. A histological analysis of mutant embryos at E6.5 and E7.5 revealed that they develop a VE but do not form a primitive streak or differentiate mesoderm. To investigate the causes of the defect in gastrulation, we monitored the expression of key markers prior to and during gastrulation in the embryo. As anticipated we failed to observe expression of primitive streak markers T, Foxa2, Gsc, Fgβ or the posterior localization of Nodal and Cripto expression. Defects in the expression of primitive streak markers and the delocalized expression of Nodal and Cripto are likely due to the absence of the DVE/AVE. Defects in the DVE/AVE were confirmed by observing significantly reduced expression of the markers Cer1, Hes1, Lefty1 and Hesx1 in E5.5 and E6.5 embryos. Defects in Gata6 expression at E5.5 suggest that the defects in DVE specification are accompanied by general defects in VE specification. From these studies we conclude that a critical function for Bptf during mammalian development is directly or indirectly to specify the VE and DVE after implantation.

To identify Bptf-dependent gene targets we employed a microarray based approach on Bptf knockout ES cells during the early stages of differentiation and an embryoid body model. We discovered a role for Bptf in the regulation of gene clusters essential for development, morphogenesis, nervous system development and cell death and proliferation. Interestingly many transcription factors, primarily the homeobox-containing genes, are dependent on Bptf for their proper repression during undifferentiated and differentiated states. This dependence is interesting as NURF has been shown to be a activator of Hox gene transcription in more differentiated tissues in Drosophila and the mouse [13,15]. As expected many markers of ectoderm (Nestin, Fgf5), mesoderm (Gsc, Lhx1, Fgfβ, Tbx6, Wnt5) and endoderm (Lefty1, Cer1, Nodal, Hesx1) cell lineages require Bptf for their expression. These gene targets corroborate well with those identified from our *in vivo* studies on Bptf mutant embryos further supporting the conclusion that Bptf is essential for the development of ectoderm, mesoderm and endoderm. This also suggests that our microarray dataset obtained from ES cells is a reasonable approximation of the expression defects occurring in Bptf mutant embryos *in vivo*.

**A Model for Bptf Function in Early Mammalian Development**

The functions for Bptf in the early embryo are undoubtedly complex. Our unbiased analysis of gene targets by microarray revealed many potentially Bptf-dependent differentiation pathways. Most relevant to this study include the regulation of the cell cycle and the differentiation of mesoderm and endoderm lineages, specifically the VE and DVE. The underlying cause for these defects could largely be due to the loss of chromatin associated complexes like the NURF chromatin remodeling complex. In the case of NURF the defects could be direct, as a remodeling activity at the promoter of genes necessary for cellular proliferation and differentiation, or indirect through the deregulation of master
The challenge for the near future will be to uncover each of the mutation likely affects many different pathways and biological ovary [64]. However, we wish to emphasize that the important for transmitting Dpp/TGF embryo uses three well-known signaling pathways, the WNT/β-catenin, FGF/MAPK and Nodal/Smad pathways, to establish A-P asymmetry [29]. The three pathways can be distinguished by different A-P phenotypes. In mutants of FGF/MAPK signaling the epiblast has severe proliferation defects, do not specify primitive endoderm, are quickly reabsorbed and the blastocysts do not outgrow when grown in culture [30,44,59,60]. Mutations in the WNT/β-catenin, but not Nodal/Smad pathways, develop the DVE and in some cases the AVE [50,61–63]. The ability of the Bptf embryos to form blastocyst outgrowths, specify the primitive endoderm, but not form the DVE is reminiscent of mutants in the Nodal/Smad signaling pathway rather than a defect in FGF/MAPK or WNT/β-catenin signaling (Table S2).

Because Bptf has been associated with the NURF complex, a known regulator of transcription, the data suggest that Bptf is required for the expression of gene targets in the developing VE and DVE. In support of this hypothesis, we showed that Bptf is required for the regulation of endogenous promoters and Smad responsive promoter elements in ES, P19 and MCF10CA1 cells in tissue culture. Smad-dependent gene targets include those essential for cell proliferation (p27) and those essential for DVE function (Cer1, Lefty1 and primitive streak (T, Fgf8, Gsc). Moreover, pulldown assays showed that components of the NURF complex have direct interactions with the Smad transcription factors and it is recruited to the promoters of Smad regulated genes under conditions of activation. Taken together, our data suggest that Bptf can directly regulate Smad regulated genes, likely through the functions of the NURF remodeling complex, via recruitment by the Smad transcription factors (It is also possible that other as yet unidentified Bptf-containing complexes distinct from NURF function in this pathway). To address the possibility that the effects on the Nodal/Smad pathway are indirect we monitored the expression of key components of the pathway in embryos. We did not observe significant changes which could explain the observed defects in Smad signaling in the early embryo or during embryoid body differentiation in Bptf mutants (Figure S25).

Consistent with these findings, the ISWI ATPase, a component of Drosophila NURF, ACF and CHRAC has been reported to be important for transmitting Dpp/TGFβ signals to stem cells in the Drosophila ovary [64]. However, we wish to emphasize that the Bptf mutation likely affects many different pathways and biological processes, each of which may contribute to biological phenotypes. The challenge for the near future will be to uncover each of the many functions of Bptf in mammalian chromatin biology.

Materials and Methods

Targeting Vector Construction

The RPCI21 mouse PAC library (MRC Genomic Resource Center, England) was screened using a random hexamer labeled probe to Bptf exon 2. Blotting was performed in hybridization bags using 0.25 M sodium phosphate pH 7.2, 1 mM EDTA, 7% SDS, at 65°C overnight with rocking. Blots were washed 5 times for 10 min with 0.25× SSC, 0.1% SDS at 65°C. Eight positive clones were identified using X-ray film as; 367-I21, 373-D3, 402-C16, 402-E15, 426-P15, 540-B9, 625-D23, 502-P16. Clone 367-I21 was confirmed by Southern blotting using Eco RI and Sal I digests and Bptf exon2 probe. Genomic sequence for the construction of the targeting vector was retrieved into bluescript SKII (Stratagene) and the integration of loxP sites and Neo selectable marker was performed using recombining technology described previously [65]. The sequence of the targeting vector is available upon request.

Targeting Bptf in ES Cells

Linearized vector was electroporated into clone 7010 and conversion from Bptf/BptfFloxedNeo to Bptf/Bptflox/neo was first screened for by PCR then confirmed by Southern blotting. Clone these were identified using this strategy, and clone 7004 was used for subsequent targeting. The wild type allele in the Bptf/Bptflox/neo from clone 7004 was then retargeted using the Bptf exon 2 targeting vector. Individual conditional homozygous clones were first screened for by PCR then confirmed by Southern blotting. Two BptfFloxedNeo / Bptflox/neo clones were obtained and named H12 and B19. The pCre expression vector was electroporated into clone 1701 and conversion from Bptflox/neo/Bptflox/neo to Bptflox/neo/Bptflox/neo was first screened for by PCR then confirmed by Southern blotting. A total of 9 and 5 homozygous knockout clones were obtained from the H12 and B19 parental lines respectively. The karyotype of the knockout lines were confirmed using Giemsa staining and lines with an aneuploid karyotype were discarded. ES cells were maintained on mitomycin C treated primary mouse embryonic fibroblasts (MEFs) and ES Cell Growth Media (15% FCS (ES Cell grade, Invitrogen), DMEM, essential amino acids, 10 μM mercapto-ethanol, 2 mM glutamine, 100 U/ml LIF (Chemicon International), penicillin and streptomycin) throughout. Lines were passaged off MEFs onto gelatinized plates for 3 passages prior to any molecular analysis.

Rescue lines were made by retargeting one allele of Bptflox/neo in clones Bptf mutant clones P2-G2 and P2-B9. Individual Neo resistant and HAT resistant clones were selected on mitomycin-C treated MEF feeder layers in ES Cell Growth Media (15% FCS (ES Cell grade, Invitrogen), DMEM, essential amino acids, 10 μM mercapto-ethanol, 2 mM glutamine, 100 U/ml LIF (Chemicon International), penicillin and streptomycin) throughout. Successful retargeting events were confirmed by Southern analysis as described below.

Production of Chimera Mice and Animal Husbandry

Clone 7010 was used to make chimera mice as described previously [66]. Removal of frt-Neo-frt and deletion of LoxP-exon2-LoxP was accomplished by crossing to mice expressing Tg-CMV-Flp and Tg-CMV-Cre to Bptflox/neo to create the Bptflox/neo and Bptflox/neo lines respectively. Individual recombinants were identified by Southern blotting and maintained by backcrossing to C57B6/ CRL.

Mice used in this study were maintained in a Specific Pathogen Free environment at the National Institutes of Health at Bethesda (MD, USA). Mice were maintained on a 12 hr light/dark cycle.
Southern Blotting Genotyping Procedures

Mice were routinely genotyped by Southern blotting restriction digested tail DNA. Tail DNA was prepared using standard procedures [67]. ~10 μg of DNA was digested with the appropriate restriction enzyme and resolved by 0.5% agarose gel electrophoresis for standard electrophoresis or 1% agarose using FigE. DNA was transferred to Hybond XL (Amersham) using alkaline capillary transfer crosslinked with UV (Stratalinker) and probed with random hexamer labeled probes at 65°C using Perfect Hyb Hybridization Buffer (Sigma). Probes used for blotting were PCR amplified from pCH110 (Genbank # U02445) using primers JL510 and JL511 for Probe A, and from mouse genomic DNA using primers JL299 and JL300 for Probe A; JL60 and JL61 for probe exon2; JL235 and JL236 for probe 29–31; JL293 and JL294 for probe 25–26; JL295 and JL296 for probe 20.2–20.7 using Taq polymerase (Invitrogen) (see Table S3 for primer sequences). Blots were washed twice with 2× SSC, 0.1% SDS and twice with 0.5× SSC, 0.1% SDS for 10 min at 65°C for each wash. A Phosphorimager was used to detect the hybridization signal.

PCR Genotyping Procedures

Embryos were genotyped using a PCR based strategy. Primers used for genotyping the BpfΔm2 line were as follows, Primer A (JL511), Primer B (JL627), and Primer C (JL631) (see Table S3 for primer sequences). PCR reactions were performed in 25 μl volumes with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.2 μM each primer, 1 Unit Taq Polymerase (Invitrogen). Reactions were heated for 3 min at 94°C, then cycled for 35 cycles at 30 sec at 94°C, 30 sec at 65°C, and 45 sec at 72°C, followed by one cycle for 2 min at 72°C. PCR products were resolved on 1.5% agarose gels. The genotype of embryos was confirmed by the presence of a 375 bp BpfΔm2 or 650 bp BpfΔm2 bands.

To confirm BpfΔm2 and BpfΔm2 expression and an out of frame Bpf mRNA in the BpfΔm2 line we designed primers with homology to the 3’ end and exons 1 through 8 of the Bpf mRNA. Total RNA from wild type and BpfΔm2 homozygous embryos was converted to cDNA using superscript II according to manufacturer’s procedures. Bpf expression was estimated by first normalizing cDNA to equal concentration with GAPDH amplification. Following GAPDH normalization 3’ Bpf was amplified. Both GAPDH and Bpf were amplified in the linear range as follows. Reactions were heated for 10 min at 94°C, then cycled for 22 cycles for GAPDH and 30 cycles for Bpf at 20 sec at 94°C, 20 sec at 60°C, and 30 sec at 72°C. PCR products were resolved by native PAGE. The exon 1-8 junction was amplified using primers P3 and JL15 in 25 μl volumes with 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.2 μM each primer, 1 Unit Taq Polymerase (Invitrogen) (see Table S3 for primer sequences). Reactions were heated for 3 min at 94°C, then cycled for 35 cycles at 30 sec at 94°C, 30 sec at 65°C, and 2 min at 72°C, followed by one cycle for 5 min at 72°C. PCR products were resolved on 1.5% agarose gels. PCR fragments were cloned using TOPO TA cloning system (Invitrogen) and sequenced with Big Dye V3.1 using M13 forward and reverse primers.

The exact integration site for the BpfΔm2 insertion allele was confirmed by sequencing a PCR product generated by amplifying genomic DNA from a heterozygous mouse tail clipping. The DNA was amplified using primers JL511 and JL515 (see Table S3 for primer sequences). Reactions were heated for 3 min at 94°C, then cycled for 33 cycles at 30 sec at 94°C, 30 sec at 65°C, and 60 sec at 72°C, followed by one cycle for 2 min at 72°C. A ~1.8 Kb PCR product was resolved on 1.5% agarose and purified. The product was sequenced using Big Dye v3.1 using primer JL511 to obtain BpfΔm2 junction sequence.

BpfΔm2 disruption was confirmed by 5’ RACE according to established procedures from Bpf/BpfΔm2 total testis RNA according to procedures established by the Bay Genomics Gene Trap Consortium (http://baygenomics.ucsf.edu/). Two separate 5’ RACE reactions were sequenced giving identical results.

Embryo Procedures

Timed pregnancies used to collect embryos were preformed using standard procedures [67]. Embryonic day 0.5 was recorded the morning after an observable plug was found. Portions of later stage E18.5 to E10.5 embryos were genotyped by Southern blotting and early E9.5 to E3.5 embryos and outgrowths were genotyped by PCR.

Embryos used for β-galactosidase staining, in situ hybridizations and phenotypic analysis were dissected from their decidua in PBS at room temperature prior to staining/hybridization. Embryos were stained for β-galactosidase activity or used for in situ hybridizations using established protocols [67] (T. Yamaguchi, personal communication). Probes used for in situ RNA hybridizations were labeled with DIG according to standard procedures.
Blastocysts were isolated in 15% FCS, M2, 10% FCS, M16, 10% FCS, M16.

Blastocysts were then transferred to gelatinized plates containing 25 mM HEPES pH 7.4, 15% FCS, 2 mM glutamine, penicillin and streptomycin using standard culture conditions. After 4 days growth extra embryonic cells were removed from the ectoplacental cone and frozen in dry ice. The deciduas were then dehydrated in an ethanol series and imbedded in paraffin. Paraffin blocks were trimmed and sectioned and sections were stained with hematox-ethanol series and imbedded in paraffin. Paraffin blocks were stained by digesting the embryo proper overnight at 55°C in 10 to 20 μl PBS, 0.1% tween 20, 2 mg/ml PCR grade proteinase K (Roche). Proteinase K was heat inactivated at 100°C for 15 min and 2 μl was subsequently used for PCR genotyping.

Deciduas used for sectioning were dissected out from the uterus in PBS, washed once with PBS and immersed in 4% PFA overnight at 4°C. Deciduas were then dehydrated in an ethanol series and imbedded in paraffin. Paraffin blocks were trimmed and sectioned and sections were stained with hematox- and eosin, TUNEL assay and phosphorylated H3 according to standard procedures [67].

Embryos used for RT PCR analysis were dissected in DMEM, 25 mM HEPES pH 7.4, 15% FCS, 2 mM glutamine, penicillin and streptomycin. Embryonic and extra embryonic tissue was removed from the ectoplacental cone and frozen in dry ice. The ectoplacental cone was grown ex vivo in DMEM, 15% FCS, glutamine, penicillin and streptomycin using standard culture conditions. After 4 days growth extra embryonic cells were removed from the outgrowth and subjected to PCR genotyping as described above and qPCR as described below.

Outgrowth assays were preformed on E3.5 blastocysts. E3.5 blastocysts were isolated in 15% FCS, M2, 10 μM mercaptoethanol, penicillin and streptomycin using standard procedures. Blastocysts were then transferred to gelatinized plates containing 15% FCS, M16, 10 μM β-mercaptoethanol, 2 mM glutamine with penicillin and streptomycin. Outgrowths were cultured under standard conditions for 5 days and observed for defects. At the end of the experiment the outgrowths were removed and genotyped by PCR using conditions above.

Tissue Extraction

Mouse tissues were extracted from 3 month old male mice sacrificed by CO2 asphyxiation and immediately frozen in liquid nitrogen. Tissues were then extracted using TriReagent (Sigma) according to manufacturer's established procedures. RNA precipitate was extensively washed in 80% EtOH, re-suspended in DEPC treated water and stored at −80°C. Protein precipitate was extensively washed with 0.3 M guanadinium hydrochloride, 95% ethanol and re-suspended in 8M urea, 1% SDS and stored at −80°C. Protein concentration was determined using the DC Protein Assay (BioRad) according to manufacturer's procedure.

mRNA from embryos successfully genotyped as WT or Bptf−/− from ectoplacental growth outgrowths was isolated using Quick Prep Micro mRNA Purification Kit (Amersham).

Northern Blotting

RNA samples were denatured, resolved by formaldehyde agarose electrophoresis and blotted to Gene Screen (Dupont) by high salt capillary transfer according to standard procedures. Blots were probed with random hexamer labeled DNA probes using Prefect Hyb hybridization solution (Sigma) at 65°C according to the manufactures procedures. Probes used were the Probe B and Exon2 probe described above. Blots were washed with four times with 1× SSC for 10 minutes at 65°C. Phosphomager was used to detect the hybridization signal.

Western Blotting

Protein samples were diluted in SDS sample buffer and resolved by PAGE. 50 μg total protein was transferred to PVDF (Biorad) using 10 mM CAPS, pH 10.5, 15% methanol, 3.5 mM DTT transfer solution for 17 hours at 20 mA and 20 V limits. Membranes were blotted with affinity purified anti-Bptf at 1:10000 dilution [14], M2 anti Flag monoclonal antibody at 1:5000 (Sigma), anti Smad2 and Smad2-phos (Cell Signaling Technologies) 1:1000, anti SNP2H/L (Abcam) 1:5000, anti-CBP and p300 1:100 (Abcam) overnight at 4°C then anti rabbit or mouse HRP (Amersham) at 1:20000 dilution for 2 hrs at RT in PBS with 5% NFDM and 0.1% tween 20 through out.

Histone Western blotting was performed essentially as above. Protein samples were extracted from ES cells grown on gelatinized plates in ES cell growth media containing 100 U/ml LIF using TriReagent according to manufacturer’s procedures (Sigma).

50 μg of total protein was resolved by 15% SDS PAGE and transferred to PVDF (Biorad) using 12.5 mM Tris, 96 mM glycine pH 8.3, 20% methanol transfer solution for 1 hour at 200 mA and 25 V limits. Blots were probed with rabbit polyclonal antibodies to H4 ac-K5, H4 ac-K8, H4 ac-K12 (S erotech), H4 3me-K20, H4 acK16, H3 3me-K9, H3 3me-K27 (Upstate), g-H2AX, H3 3me-K4, H3 ac-K14, H3 ac-K18 (abcam) at 1:2000 dilutions overnight at 4°C then anti rabbit HRP (Amersham) at 1:4000 dilution for 1 hrs at RT in PBS with 5% NFDM and 0.1% tween 20 through out. Detection was preformed using Pico signal ECL according to manufacturer’s procedures (Pierce).

Tissue Culture Procedures and Luciferase Assays

ES cells were maintained on mitomycin C treated MEFs in ESC Growth Media (15% FCS, 100 U/ml LIF) (Chemicon International), penicillin and streptomycin. Culture techniques used were described previously [66]. Lines were passaged 3 times onto gelatinized plates to remove MEFs prior to experiments.

Growth rates of ES cell lines were obtained on gelatinized plates in the absence of feeder layers. Cells were plated at 1.0×10^5 in 6 well plates. Cell number was determined every day for 4 consecutive days with changes in ESC Growth media occurring every day.

Activin-A stimulation of ES cells was performed as follows. ES cell lines were started on gelatinized plates in ESC Growth Media. Cultures were then grown for 2 days in ESC Growth Media without LIF. On the third day, cells were cultured in low serum ESC Growth Media without LIF (same as ESC Growth Media except 0.1% FCS is used instead of 15% FCS) in the presence or absence of 30 ng/ml activin-A (R & D Systems) overnight. Cells were processed using TriReagent according to manufacturer's standard protocol.

p300, CBP siRNA knockdowns were performed as follows. ES cells were grown in ES Cell Growth Medium with 100 U/ml LIF on gelatinized plates. Cells were collected using trypsin and transfected using an Amaxa Nucleofector device using solution ES Cell and program A-30 according to manufacturer’s procedures. 2.0×10^6 ES cells were transfected with 400 nmoles siRNA duplex (Darmacon) or a GFP-Max nucleofection control. Post nucleofection, cultures were then grown for 2 days in ESC Growth media as described above. Cells at specified time points were lysed using TriReagent reagent (Sigma)
and RNA and protein were purified according to manufacturer’s procedures.

MCF10CA1h cells were grown in a 1:1 mixture of DMEM and Ham’s F12 medium (Gibco) supplemented with 5% horse serum. Cells were collected using trypsin and transfected using an Amazza Nucleofector device using solution V and program T-27 according to manufacturer’s procedures. 2.0×10^6 cells were transfected with 400 nmoles siRNA duplex (Dharmacon) or a GFP-Max nucleofection control. Post nucleofection 1.0×10^6 cells were plated to each well of a 6 well tissue culture plate. Cells were grown for 2 days in growth media and then serum deprived in 1% DFS, DMEM, nonessential amino acids, 10 μM β-mercaptoethanol overnight. Following serum shock cells were incubated with or without 5 ug/ml TGF-β1 (R&D Systems) for 1 hour. Cells were lysed using TriReagent reagent (Sigma) and RNA and protein were purified according to manufacturer’s procedures.

P19 cells were obtained from the ATCC (ATCC Number CRL-1825). Cells were grown in 10% DFS, DMEM, 2 mM glutamine, penicillin and streptomycin. Cells were transfected using an Amazza Nucleofector device using solution V and program C-20 according to manufacturer’s procedures. 2.0×10^6 P19 cells were transfected with 400 nmoles siRNA duplex (Dharmacon) or a GFP-Max nucleofection control. Post nucleofection 3.0×10^5 cells were plated to 12 well plates. Cells were allowed to grow for two days then were serum starved for 18 hours in 0.1% DFS, DMEM, 2 mM glutamine, penicillin and streptomycin. Cells were induced with 5 ng/ml TGF-β1 (R&D Systems) in low serum growth medium overnight. Cells were lysed using TriReagent reagent (Sigma) and RNA and protein were purified according to manufacturer’s procedures.

Luciferase assays were performed in 12 well format by transfecting 0.6 μg pGL3tk-(SBE)4 or pAR3-Luc, 0.012 μg pCH110 with or without 0.6 μg TjiRI or 0.6 μg Smad 2-2HA and 0.006 μg Smad4-2HA, 40.0 pmoles siRNA duplex per well using Lipofectamine 2000 (Invitrogen). In experiments using TGF-β1 (R&D Systems) or Activin-A (R&D Systems) cells were allowed to grow for 48 hours in 10% DFS, DMEM, 2 mM glutamine prior to adding TGF-β1 at 5 ng/ml or Activin-A at 30 ng/ml. Cells were induced for 24 hours then lysed with 250 μl SiGlow Lysis Buffer (Promega) according to manufacturer’s procedures. Transfections utilizing the constitutively active receptors were incubated for 48 hours prior to lysis as above. 50 μl or 100 μl of lysate was assayed with 100 μl Bright-Glow Luciferase Assay Reagent (Promega). Transfection efficiency was normalized to luciferase levels by assaying 10 μg of lysate. Luciferase activity was normalized by dividing the relative luciferase units obtained by dividing the luciferase activity levels by the % input using a dilution series as a standard curve. Lines were passed 3 times onto gelatinized plates to remove MEFs prior to experiments.

Activin-A stimulation of ES cells was performed as follows. ES cell lines were started on gelatinized plates in ESC Growth Media without LIF. Cultures were then grown for 2 days in ESC Growth Media without LIF. On the third day, cells were cultured in low serum ESC Growth Media without LIF (same as ESC Growth Media except 0.1% FCS is used instead of 15% FCS) in the presence or absence of 30 ng/ml activin-A (R & D Systems) overnight. Following activin-A induction cells were washed with PBS and fixed in 2 mM EGS (Pierce) in 25% DMSO/75% PBS for 30 min followed by 1% PFA in PBS for 30 min. For histone ChIP, cells were fixed in 1% PFA in PBS for 15 min. Following fixation cells were washed 3X in PBS and removed from the tissue culture dish with a cell scraper. Cell pellets were then frozen at −80°C. The following day the pellets were thawed and processed using the ChIP procedure published by Upstate biologicals. Antibodies used for pulldown were ChIP grade SNF2H/L (Abcam), H3 3me-K4, H3 3me-K27 (Upstate) and pan H3 (Abcam).

Quantitation of pulldown was performed by real time PCR using 2× DyNaMo syngropec qPCR kit (New England Biolabs) according to manufacturers procedures. Briefly, reactions were composed of 5 μl 1 μM forward and reverse primers, 5 μl diluted cDNA template and 10 μl 2× qPCR mix (see Table S3 for primer sequences). Reactions were heated for 10 min at 94°C, then cycled for 40 cycles at 20 sec at 94°C, 20 sec at 60°C, 30 sec at 72°C. After each cycle the sample was heated to 78°C for 10 sec prior to reading sample fluorescence. Pulldowns were quantified as a percentage of input using a dilution series as a standard curve. Histone modification pulldowns are expressed as enrichment relative to histone H3 occupancy under +activin-A conditions. SNF2H/L pulldowns were first normalized to signal at Gapdh and are expressed as the enrichment during +activin-A relative to −activin-A conditions.

QPCR

RT reactions were performed on RNA extracted from ES cells, embryoid bodies or P19 cells using TriReagent according to manufacturer’s procedures. 5 μg of total RNA was reverse transcribed with Superscript II using oligo dT priming according
to manufactures procedures (Invitrogen). cDNA reactions were diluted 5–10 fold and used in a template for PCR.

Real time PCR was performed using 2 × DyNamo syprogreen qPCR kit (New England Biolabs) according to manufacturers procedures. Briefly, reactions were composed of 5 μl 1.2 μM forward and reverse primers, 5 μl diluted cDNA template and 10 μl 2 × qPCR mix (see Table S3 for primer sequences). Reactions were heated for 10 min at 94°C, then cycled for 35 cycles at 20 sec at 94°C, 20 sec at 60°C or 52°C, 30 sec at 72°C. After each cycle the sample was heated to 78°C for 10 sec prior to reading sample fluorescence. Reactions were done in triplicate. ΔΔCT method was used to quantify the relative levels of expression to the Gapdh or β-actin house keeping genes. Expression levels were then normalized to un-induced cells control cells.

QPCR was performed on E7.5 embryo cDNA as follows. Briefly, reactions were composed of 5 μl 1.2 μM forward and reverse primer pair, 5 μl diluted cDNA template and 10 μl 2 × qPCR mix (see Table S3 for primer sequences). Reactions were heated for 10 min at 94°C, then cycled at 20 sec at 94°C, 20 sec at 60°C or 52°C, 30 sec at 72°C. The number of cycles to maintain linearity was determined using the real time analysis software. PCR reactions within the linear range were resolved using native PAGE.

Microarray

For microarray experiments we used one wildtype and two independent Bptf mutant cell lines in 3 culture conditions (LIF+, LIF−, and RA+). Experiments were carried out with 3 biological replications. At the day 3, Triazole (1 ml/well; Invitrogen, USA) was added to the well and total RNAs were extracted using Phase lock gel columns (Eppendorf/Brinkman) according to the manufacturer’s protocol. Total RNAs were precipitated with isopropanol, washed with 70% ethanol, and dissolved in DEPC-treated H2O. 2.5 g of total RNA samples were labeled with Cy3- CTP using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent, USA). A reference target (Cy5-CTP-labeled) was prepared from the Universal Mouse Reference RNA (Stratagene, USA). Labeled targets were purified using an RNasey Mini Kit (Qiagen, USA) according to the Agilent’s protocol, quantified by a NanoDrop scanning spectrophotometer (NanoDrop Technologies, USA), and hybridized to the NIA Mouse 44K Microarray v2.2 (whole genome 60-mer oligo; manufactured by Agilent Technologies, #014117) [66]. Transcript copy number estimation using a mouse whole-genome oligonucleotide microarray according to the Agilent protocol (G4140-90030; Agilent 60-mer oligo microarray processing protocol - SSC Wash, v1.0). All hybridizations were carried out in the two color protocol by combining one Cy3-CTP-labeled experimental target and Cy5-CTP-labeled reference target. Microarrays were scanned on an Agilent DNA Microarray Scanner, using standard settings, including automatic PMT adjustment.

Differential gene expressions in various cell lines in the standard culture condition were analyzed using the NIA Array Analysis software [http://lgsun.grc.nia.nih.gov/ANOVA/] which implements ANOVA statistics with two additional methods to reduce the number of false positives: (1) small error variances were replaced with the average error variance estimated from 500 genes with similar signal intensity, and (2) false discovery rates (FDR <0.05) were used to select genes with differential expression, instead of p-values [69]. The FDR method accounts for the effect of multiple hypotheses testing.

Gene Ontology analysis and clustering was accomplished using DAVID [http://david.abcc.ncifcrf.gov/] and the NIA Mouse Gene Index [http://lgsun.grc.nia.nih.gov/genindex/mm8] using default settings [70].

GST Puldowns

In vitro GST pulldown assays were performed in 50 μl volumes in binding buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 0.5 mM MgCl2, 0.01% NP40), ~10 μg GST protein fusion bound to a glutathione Sepharose support, 100 ng human NURF complex. Proteins were allowed to bind for 1 h at 4°C with occasional mixing. The beads were washed with 500 μl binding buffer three times at 4°C and re-suspended in a final volume of 30 μl.

NURF and CBP pulldowns from ES Cell extracts were performed essentially as described above from 500 mM KCl nuclei extractions made as previously described [71]. The extract was diluted 1:3 with 25 mM Hepes pH 7.4, 0.5 mM MgCl2, 0.01% NP40 with protease inhibitors (Roche) prior to binding to resin bound GST-Smads. The beads were washed with 500 μl binding buffer three times at 4°C and re-suspended in a final volume of 30 μl. Proteins were eluted from the beads by adding 5 μl 6× SDS sample buffer and incubating at 37°C for 30 min. Proteins were resolved on 4% or 8% polyacrylamide gels and transferred to PVDF (Biorad) and prepared for Western blotting as described above.

Supporting Information

Figure S1 Bptf is the homolog of NURF 301. (A) Mouse Bptf is highly homologous to Drosophila NURF301. Major functional domains are conserved between mammals and flies, including nucleosome and transcription factor binding sites, strongly suggesting that Bptf is the homolog of the largest subunit of the Drosophila NURF complex, NURF301. (B) Bptf is predominantly nuclear in cultured P19 cells. siRNA targeting of Bptf results in decreased Bptf signal by Western analysis and a reduction of nuclear fluorescence by anti-Bptf immuno fluorescence in P19 cells. (C) Western analysis of adult brain, testis, spleen, and embryonic stem cells reveals two migrating species, Bptf-H and Bptf-L. (D) Western analysis of total protein extracts from adult tissues using the affinity purified anti-Bptf antisera identifies a single band of high molecular weight predominately found in testis, spleen, and brain. In addition to full length Bptf, we identify a single ~100 kDa protein which reacts with the antibody exclusively in the adult brain. This protein is identical in size to the previously reported Bptf splice variant FAC1 (Bower R, Giambrone A, Daves P (1995) Dev Neuroscience 17: 20–37.). (E) A similar western analysis of protein extracts from total embryo proteins identifies high Bptf expression throughout development. The ~100 kDa cross-reacting band was only found in the adult brain loading standard and not during development.

Found at: doi:10.1371/journal.pgen.1000241.s001 (1.28 MB TIF)

Figure S2 Generation of conditional and gene trap alleles of Bptf. (A) Intron-exon map of mouse Bptf showing alternatively spliced exons and the position of the BptfΔG023 trapping vector and BptfΔExon2 floxed exon. (B) Diagram of Bptf and BptfΔG023 alleles showing tandem trapping vector insertion site between exons 15 and 16. Restriction enzyme sites and positions of Southern blotting probes used for the characterization of BptfΔG023 are shown as external probe A and internal probe B. Positions of primers used in multiplex PCR genotyping are designated A, B, C with A and B complementing genomic sequence spanning the trapping vector insertion site and C the trapping vector. (C) A Southern blot using probe A showing diagnostic restriction enzyme sites confirming the insertion of the trapping vector into Bptf. (D) A Southern blot using Bgl II and probe B showing two bands characteristic of multiple integration events by the trapping vector. Upper band contains sequences external and lower band internal to the trapping vector. (E) A Southern blot of genomic DNA digested with Nsi I, resolved
by FIGE and blotted with probe B. A single band was identified in heterozygous mice with an approximate size of 30 Kb, suggesting 3 to 4 trapping vector integration events. (F) Results from multiplex PCR analysis of wild type (+/+), heterozygous (+/−), and homozygous (−/−) Bptf alleles. The band sizes of PCR products were 250 bp and 500 bp product, respectively. (G) Diagram of Bptf, BptfExon1-Novo, BptfExon2, and BptfExon2 alleles showing the configuration of the integrated targeting vector and its Cre and Flp reaction products. Restriction enzyme sites and positions of Southern blotting probes used for the characterization of BptfExon2 alleles are shown as external probe 29–31 and internal probes 20.2–20.7, exon2 and 25–26. Positions of primers used in multiplex PCR genotyping are designated A, B, C, E, and F. (H) A Southern blot of the same mice as in (H) using EcoRI and probe 29–31. (K) Products of Cre and Flp recombination. (I) A Southern blot of the same mice as in (H) using EcoRI and probe 25–26. Positions of primers used in multiplex PCR genotyping are designated A, B, C, E, and F. (H) A Southern blot of the same mice as in (H) using EcoRI and probe 29–31. (K) A Southern blot of same mice as in (H) using EcoRI and probe 20.2–20.7. (L) A Southern blot of same mice as in (H) using Kpn I and probe 20.2–20.7. (M) Results from multiplex PCR analysis of wild type Bptf and heterozygous BptfFloxed-Novo, BptfFloxed-, and BptfExon2 alleles using primers E, F, and C. The multiplex PCR strategy yields band sizes of Bptf = 375 bp, BptfFloxed = 475 bp, and BptfExon2 = 650 bp. (N) Results from multiplex PCR analysis of wild type Bptf and heterozygous BptfFloxed-Novo, BptfFloxed-, and BptfExon2 alleles using primers A, B, and C. The multiplex PCR strategy yields band sizes of Bptf = 250 bp, BptfFloxed-Novo, BptfFloxed = 325 bp, and BptfExon2 = 500 bp.

Found at: doi:10.1371/journal.pgen.1000241.s002 (1.89 MB TIF)

Figure S3 Molecular characterization of BptfXG023 allele. (A) PCR amplification and sequencing of the upstream junction between the trapping vector and the mouse genome confirm the exact position of the trapping vector to be between exon 15 and 16 of Bptf. (B) RT-PCR results from E7.5 embryos show reduced expression of 3′-RNA sequences and severely reduced exon 15–16 splicing, confirming efficient trapping of Bptf by the trapping vector insertion. (C) DNA sequence of a 5′-RACE amplicon of the BptfXG023 allele from testis shows an in-frame fusion between exon 15 of Bptf and β-Geo from the trapping vector. (D) Northern analysis of RNA from wild type and heterozygous BptfXG023 disruption mice show predominant transcripts in testis, lungs, spleen, and brain using a probe specific for Bptf mRNA. The Bptf-β-Geo fusion RNA has the same expression patterns as the wild type allele, suggesting that the trapping vector does not interfere with Bptf regulation at the level of transcription.

Found at: doi:10.1371/journal.pgen.1000241.s003 (1.34 MB TIF)

Figure S4 Molecular characterization of BptfExon2 allele. (A) RT-PCR results from E7.5 embryos show slightly reduced expression of 3′-RNA sequences of BptfExon2 at the RNA level. (B) RT-PCR results from E7.5 embryos show deletion of exon 2 RNA sequences from BptfExon2 transcripts. Primers used were complementary to exons 1 and 8. (C) RT-PCR products from BptfExon2 transcripts in (B) were sequenced to identify out of frame splicing events, strongly suggesting that the exon 2 deletion results in efficient knockout of the Bptf protein.

Found at: doi:10.1371/journal.pgen.1000241.s004 (0.64 MB TIF)

Figure S5 BptfExon2 and BptfXG023 alleles are genetically equivalent. (A) Wild type (+/+), heterozygous (+/−), and homozygous (−/−) BptfExon2 embryos at E5.5, E6.5, and E7.5 were removed from their decidua and genotyped using a PCR-based method. Identical to the BptfXG023 allele, reduced growth rates are evident in the homozygous BptfExon2 embryos as early as E5.5. Mutant embryos from both lines maintained the gross morphology of E5.5 embryos at E6.5 and E7.5 with an overall increase in size. (B) Heterozygous BptfXG023 and BptfExon2 mice were intercrossed, and wild type, BptfExon2 heterozygous, BptfXG023 heterozygous, and BptfXG023 trans-heterozygous embryos were dissected from their decidua at E6.5, and genotypes were confirmed by PCR. Trans-heterozygous embryos phenocopy homozygous BptfXG023 or BptfExon2 embryos, suggesting that the two alleles have an equivalent phenotype.

Found at: doi:10.1371/journal.pgen.1000241.s005 (3.04 MB TIF)

Figure S6 Bptf mutants do not have significant growth defects prior to implantation. Wild type (+/+) heterozygous (+/−), and homozygous (−/−) BptfXG023 or BptfExon2 E5.5 blastocysts and 5-day blastocyst outgrowths did not reveal any significant morphological or growth differences in trophectoderm or the inner cell mass.

Found at: doi:10.1371/journal.pgen.1000241.s006 (1.36 MB TIF)

Figure S7 Bptf mutants have reduced cellular proliferation. Percent phosphorylated histone H3 positive cells from total cells of the control and mutant embryos were quantified to show reduced cellular proliferation of Bptf mutant embryos at E5.5, E6.5, and E7.5.

Found at: doi:10.1371/journal.pgen.1000241.s007 (0.15 MB TIF)

Figure S8 BptfXG023 is widely expressed in the embryo proper. (A–D) Wild type and heterozygous embryos were stained in whole mount for β-galactosidase activity at E5.5 and E6.5. BptfXG023 expression is present in E5.5 and E6.5 embryos in embryonic but not extra-embryonic tissues. (E–G) Wild type and heterozygous E7.5 embryos were stained in whole mount for β-galactosidase activity. BptfXG023 expression is present at E7.5 embryos in embryonic but not extra-embryonic tissues. (a’) Cross-section of the E7.5 heterozygous embryo shows expression exclusive to the embryonic ectoderm and little to no expression in the visceral endoderm. (b’) Cross-section of the E7.5 heterozygous embryo shows expression primarily in the embryonic ectoderm, reduced expression in the mesoderm, with little to no expression in the visceral endoderm. Abbreviations: ve, visceral endoderm; ee, embryonic ectoderm; m, mesoderm.

Found at: doi:10.1371/journal.pgen.1000241.s008 (0.34 MB TIF)

Figure S9 BptfXG023 expression during embryonic development. (A–E) Wild type (+/+), and heterozygous (+/−) BptfXG023 embryos were dissected from their decidua and stained in whole mount for β-galactosidase activity. Staining is seen predominantly in the embryonic tissues at E7.5 (A) and E8.5 (B). At later stages of development, expression is ubiquitous in the embryo (C–E). (F) Sagittal section of E10.5 embryo stained for β-galactosidase activity reveals the extent of BptfXG023 expression. Expression was observed in many tissues originating from mesoderm (notochord, myocoele, somites), endoderm (lung bud), and ectoderm (forebrain, midbrain, hindbrain, and neural tube). Scale Bar = 400 μm.

Found at: doi:10.1371/journal.pgen.1000241.s009 (4.01 MB TIF)

Figure S10 An analysis of Nodal expression at E5.5 shows Bptf mutants express Nodal at levels comparable to wild type. (A) Bptf mutants express Nodal in the visceral endoderm at E5.5 at comparable levels to the wild type control. (B) Bptf mutants express delocalized Nodal at E6.5.

Found at: doi:10.1371/journal.pgen.1000241.s010 (1.94 MB TIF)

Figure S11 Bptf mutants cannot establish an anterior–posterior axis. (A,B) Whole mount in situ RNA hybridization analysis of wild type and mutant E6.5 and E7.5 embryos for Cer1, Nodal,
Lefty1, Hex1, Ox2, Hex1, Lhx1, T, Gsc, Fgf8, Foxa2, Cripto, Junb, Mash2, Bmp4, Esh2, and Fgf2 expression. An analysis on wild type and Bptf mutant E6.5 embryos for anterior visceral endoderm (AVE) markers Gsc, Lefty1, and Hex1 show that mutants do not develop an AVE. Bptf mutants show comparable but delocalized Nodal and Cripto expression at E6.5, further suggesting a defect in the development of the AVE. Embryos showed reduced expression of the embryonic ectoderm marker Otx2 and Hex1 in mutants compared to wild type controls, suggesting defects in the embryonic ectoderm. Primitive streak markers Lhx1, T, Gsc, Fgf8, and Foxa2 also show a significant reduction in expression in mutant at E6.5 compared to wild type. The extra-embryonic markers Bmp4, Esh2, Fgf2, and Mash2 are not dramatically affected in mutant when compared to wild type embryos at E6.5. The cell cycle regulatory gene Junb was found to be over expressed in mutant relative to wild type controls at E6.5. Analysis of E7.5 embryos confirms that Bptf mutants do not establish an anterior–posterior axis. Lhx1 is not significantly expressed at E7.5 in mutant compared to wild type, suggesting that primitive streak does not form. Expression of mesoderm markers T and Fgf8 is diffuse in mutant embryos compared to wild type at E7.5, suggesting that the embryo proper becomes unorganized mesoderm-like tissue. The extra-embryonic markers Bmp4 and Mash2 maintain extra-embryonic expression in E7.5 mutant embryos. Angiogenesis markers Vegf and Fkki show little expression in the extra embryonic portion of the embryo as compared to wild type controls. However, expression is not associated with an allantoid, which is severely underdeveloped in Bptf mutant embryos. Bptf expression is largely confined to the embryonic tissues at E7.5.

Found at: doi:10.1371/journal.pgen.1000241.s011 (6.17 MB TIF)

**Figure S12** Construction of Bptf mutant embryonic stem (ES) cell lines. (A) Retargeting and subsequent transient Cre expression generated conditional heterozygous (not shown), heterozygous (+/−), conditional homozygous (Floxed-Neo−/−), and homozygous (−/−) mutant Bptf mouse ES cell clones. Genotypes were confirmed by Southern analysis using an Eco R1 digest and hybridization with Probe 20.2–20.7. (B) Northern analysis of Bptf mutant ES cell clones using a probe to the 3′ end of the gene shows reduced Bptf transcript levels. (C) RT-PCR analysis of wild type (+/+) and homozygous mutant (−/−) RNA confirms the complete deletion of exon 2 from Bptf transcripts in homozygous mutant clones. Sequence from Bptf mutant PCR products shows the Bptf transcript contains exon 1–3 and 1–4 out of frame splice events as seen in Figure S4C (data not shown). (D) Western analysis of Bptf wild type, heterozygous, conditional heterozygous, and mutant ES cells. Heterozygosity at Bptf results in reduced Bptf protein levels, raising the possibility of haploinsufficiency phenotypes. Homozygous Bptf ES cells mutants do not show Bptf protein, demonstrating that BptfExon2 is likely to be a null allele. FAC1 expression was not found in ES cells.

Found at: doi:10.1371/journal.pgen.1000241.s012 (0.71 MB TIF)

**Figure S13** Conditional deletion of Bptf in mouse embryonic fibroblasts (MEF) with adenosine expressing Cre recombinase. Conditional Bptf mutant (−/−Floxed) and control (+/+MEF) lines were infected with an adenosine expressing the Cre recombinase. Infected (+Cre) and mock infected (−Cre) cells were passaged once to deplete the cellular stores of the Bptf protein. Following growth for 4 days, cells were harvested for total protein. Bptf protein was detected by Western using mouse monoclonal anti Bptf antisera as a loading control.

Found at: doi:10.1371/journal.pgen.1000241.s013 (0.57 MB TIF)

**Figure S14** Bptf mutant embryonic stem (ES) and mouse embryonic fibroblast (MEF) cell lines show slight growth defects. (A) The doubling time of wild type and mutant ES cells was investigated. We observed that Bptf mutants but not conditional heterozygous or heterozygous ES cells showed reduced growth rates relative to wild type (+/+M) cell lines. A cell cycle analysis of slower growing Bptf knockout ES cell lines shows that they have a slight G1 to S phase delay. (B) The doubling time of wild type and mutant MEF lines was investigated. We observed that Bptf-depleted MEF lines showed slightly reduced growth rates relative to control lines.

Found at: doi:10.1371/journal.pgen.1000241.s014 (0.24 MB TIF)

**Figure S15** Bptf mutant mouse embryonic stem (ES) cells do not differentiate as embryoid bodies, are not apoptotic, and proliferate normally. (A) Day 9 embryoid bodies were sectioned and stained for gross analysis with hematoxylin and eosin (H&E), the proliferation markers PCNA and phosphorylated histone H3, and apoptosis by TUNEL. Bptf-mutant (−/−) embryoid bodies contained few differentiated and TUNEL positive apoptotic cells compared to wild type (+/+M) controls. TUNEL negative Bptf mutant (−/−) and wild type (+/+M) cells have similar staining for proliferation markers PCNA and phosphorylated histone H3. (B) Microscopy of wild type and Bptf mutant ES cells during a 9-day embryoid body differentiation time course. Bptf mutant ES cells did not develop the size and cystic structures of the wild type controls. (C) Wild type but not mutant day 9 embryoid bodies develop a prominent layer of endoderm on the exterior surface. In place of endoderm Bptf knockouth, embryoid bodies develop a thin epithelial cell layer.

Found at: doi:10.1371/journal.pgen.1000241.s015 (3.46 MB TIF)

**Figure S16** Bptf-mutant embryoid bodies show little to modest deregulation of cell cycle and pluripotency markers. Gene expression was monitored from total RNA during a 9-day embryoid body differentiation time course. RT-PCR analysis for cell cycle markers (A), pluripotency markers (B).

Found at: doi:10.1371/journal.pgen.1000241.s016 (0.93 MB TIF)

**Figure S17** Transcription and differentiation defects in BptfExon2 knockout embryonic stem (ES) cells are rescued by reintroduction of BptfFloxed-Nosac1Cre. (A) BptfFloxed-Nosac1Cre/+/BptfFloxed-Nosac1Cre ES cell lines were retargeted with the BptfFloxed-Nosac1Cre targeting vector to generate BptfFloxed-Nosac1Cre/+/BptfFloxed-Nosac1Cre rescue lines. Recombination of the targeting vector at Bptf was confirmed by Southern blotting Sca I digested DNA and probing with Probe 25–26. (B) Western analysis of total protein from parental BptfFloxed-Nosac1Cre/+/BptfFloxed-Nosac1Cre, BptfExon2/BptfExon2, and BptfExon2/BptfFloxed-Nosac1Cre rescue lines show reintroduction of the Bptf protein. (C) Expression analysis of mesoderm markers T, Gsc, and endoderm markers Sox17, Cer in a BptfExon2/+/BptfFloxed-Nosac1Cre rescue ES cell line during a 9-day embryoid body differentiation time course. Reintroduction of BptfFloxed-Nosac1Cre rescued the induction of mesoderm and endoderm markers during embryoid differentiation.

Found at: doi:10.1371/journal.pgen.1000241.s017 (1.09 MB TIF)

**Figure S18** Gene ontology clustering analysis of Bptf-dependent genes by condition. The entire array dataset from each condition was analyzed for statistically significant ontology clusters using DAVID analysis software. Genes that were repressed in expression cluster to highly similar ontology groups and include those involved in “development”, “transcription factor activity”, and “morphogenesis”. Activated gene clusters are highly dependent on condition and include “antigen processing”, “peptidase inhibitors”, and “cytoskeletal components”.

Found at: doi:10.1371/journal.pgen.1000241.s018 (0.88 MB TIF)

**Figure S19** Bptf negatively regulates many homeobox transcription factors. (A) Genes containing a homeobox but not the helix
loop helix domains are significantly enriched in all categories of up-regulated gene sets. (B) Expression levels of homeobox transcription factor gene clusters displayed as a heat map. Almost the entire a, b, and c clusters are deregulated, suggesting that Bptf plays an important function in regulating clusters of homeobox domain–containing transcription factors.

**Figure S20** Bptf preferentially potentiates dynamically regulated genes with bivalent promoters. (A) Genes whose transcription activates, represses, or is unchanged upon LIF+ conditions or the addition of retinoic acid (RA) were analyzed for Bptf dependence. Knockout of Bptf results in predominantly reduced activation (repression of activated genes with Bptf knockout) or reduced repression (activation of repressed genes with Bptf knockout) of dynamically regulated genes. (B) Bptf preferentially regulates promoters with bivalent histone modifications. The methylation state expressed as a percentage of all promoters from undifferentiated mouse ES cells. Data was retrieved from Bernstein et. al. (2006) Cell 125:315–326. Bptf-dependent genes from the microarray dataset were grouped based on the promoter’s methylation state. From this analysis, Bptf-dependent genes are predominantly enriched for bivalent histone H3 3me-K27, histone H3 3me-K4 over single modified or unmodified promoters. (C) The magnitude of deregulation was compared between activated and repressed genes for each condition of Bptf dependence. A breakdown of gene classes by condition shows that the transcriptome is repressed under LIF+ and RA growth conditions and activated under LIF−. (D) The modifications on bulk histones in Bptf mutant embryonic stem (ES) cells are consistent with a transcriptionally repressed state expressed as a percentage of all promoters from undifferentiated mouse ES cells. Data was retrieved from Bernstein et. al. (2006) Cell 125:315–326. Bptf-dependent genes from the microarray dataset were grouped based on the promoter’s methylation state. From this analysis, Bptf-dependent genes are predominantly enriched for bivalent histone H3 3me-K27, histone H3 3me-K4 over single modified or unmodified promoters. (C) The magnitude of deregulation was compared between activated and repressed genes for each condition of Bptf dependence. A breakdown of gene classes by condition shows that the transcriptome is repressed under LIF+ and RA growth conditions and activated under LIF−. (D) The modifications on bulk histones in Bptf mutant embryonic stem (ES) cells are consistent with a transcriptionally repressed state. Bulk histones were prepared from wild type (+/+), two Bptf mutant (−/−), and two Bptf mutant (−/+). (E) Total protein extracts were then probed for histone modifications by Western blotting. Bptf knockdown ES cells have an increase of the repressive H3 3me-K9 and H3 3me-K27 and a decrease of the activating marks H3 ac-K18 and H4 ac-K5. Interestingly, knockdown ES cells have an increase in levels of γ-H2AX, a mark correlated with DNA damage, and H3 3me-K36, a mark of transcription elongation.

**Figure S21** Bptf-dependent genes are frequently located in clusters. The chromosome position for each Bptf-dependent gene was identified, and statistically significant gene clusters are shown with a letter designation. Clusters include genes with similar regulation and imprinted clusters.

**Figure S22** Western analysis of Bptf, CBP/p300 knockdown and Smad2 activation during TGF-β1 and activin-A stimulation. (A) Wild type and Bptf mutant embryonic stem (ES) cells were induced with activin-A for 24 hours. Western analysis shows up-regulation of Bptf protein levels and the phosphorylation of Smad2 in wild type and Bptf mutant ES cells with the addition of activin-A. (B) TGF-β1 selectively increases protein levels of Bptf in P19 cells. P19 cells were treated with TGF-β1, BMP4, Wnt3a-conditioned media, and FGF+heparin for 24 hours. Following induction, cells were harvested and analyzed for Bptf levels by Western blotting. TGF-β1 increased and Wnt3a decreased Bptf protein levels. (C) Wild type ES cells were subjected to siRNA mediated knockdown of Bptf and CBP/p300, followed by induction with activin-A. Western analysis shows siRNA mediated knockdown of Bptf and CBP/p300 and phosphorylation of Smad2 with the addition of activin-A.

**Figure S23** Bptf is necessary for Smad mediated gene transcription of cell cycle regulators. RT-PCR analysis of Smad target genes from activin-A induced embryonic stem cell identities Bptf-dependent and -independent gene targets. The repression of p21 during stimulation with activin-A is dependent on Bptf.

**Figure S24** Bptf is necessary for activation of Smad-responsive promoter elements. (A) Knockdown of Bptf in P19 cells renders Smad responsive Smad binding elements (SBE) unresponsive to Smad activation via TGF-β. Luciferase activity was measured from stimulated or unstimulated P19 cells transfected with Bptf or mock siRNAs in addition to pGL3ti-(SBE)4 and LacZ transfection control plasmid. (B) Knockdown of Bptf in P19 cells renders Smad responsive ARE elements unresponsive to Smad activation via activin-A. Luciferase activity was measured from stimulated or unstimulated P19 cells transfected with Bptf or mock siRNAs in addition to pA3-Luc, CMV-Fast2-myc, and LacZ transfection control plasmid. (C) Co-transfection of HA-Smad3 2,4 and constitutively active TβRII results in increased activation of luciferase reporter regulated by the SBE. (D) Knockdown of Bptf in P19 cells renders SBE Smad responsive elements unresponsive to constitutively activated Smad transcription factors. Luciferase activity was measured from P19 cells transfected with Bptf or mock siRNAs and combinations of Smad transcription factors, constitutively active type I receptor TβR1*, pGL3ti-(SBE)4, and a LacZ transfection control plasmid. (E) Western analysis showing siRNA mediated knockdown of Bptf in P19 cells during TGF-β1 induction. (F) Some TGF-β1-responsive gene targets in P19 cells are Bptf-dependent. Bptf knockdown during TGF-β1 activation significantly reduces the Smad mediated gene activation of Cas and T. (G) Western analysis showing siRNA mediated knockdown of BPTF in MCF10CA1 cells during TGF-β1 induction. (H) Bptf knockdown during TGF-β1 activation significantly reduces the Smad mediated gene activation of PAI-1 but not SMAD7.

**Figure S25** Bptf-mutant embryos likely do not down regulate the Smad signaling pathway indirectly. (A) RT-PCR analysis of E7.5 embryos shows normal expression of the BMP4 ligand, Smad 1,2 and 4 transcription factors, and receptors ALK3, Alk-IIB. Expression of the TGFβ-responsive gene JoãoB is significantly increased in mutant embryos. (B) Results from quantitative RT-PCR measurements of additional markers of early embryonic development in E7.5 embryos. Expression has been normalized to GAPDH levels and is expressed as the ratio mutant/WT. (C) An RT-PCR analysis of wild type and Bptf-mutant embryoid body differentiation time course for Smad transcription factors, Bptf, and the Smad responsive gene Serpine1.

**Table S1** Disruption of Bptf results in early embryonic lethality. (A) Wild type (+/+) and heterozygous (+/−) Bptf<sup>ΔG023</sup> males and females were mated in reciprocal crosses and all progeny were genotyped by Southern blotting or PCR. Homozygous (−/−) Bptf<sup>ΔG023</sup> embryos were not viable after E7.5 to E8.5, showing that mutations in Bptf are early embryonic lethal. Curiously, more reabsorptions were observed during the dissections than could be accounted for from the death of the homozygous Bptf<sup>ΔG023</sup> embryos. To investigate if the origin of the increased lethality was caused by a parental Bptf<sup>ΔG023</sup> heterozygous contribution, we crossed heterozygous to wild type males and females in combinations and examined all embryos from E10.5 to E18.5. From these experiments, we observed an increased number of reabsorptions when the mother was heterozygous compared to the wild type controls (Table S1A, b–
d). The embryonic lethality was not biased toward heterozygous embryos, as they were found in the expected 1:1 ratio to wild type. These data suggested that BptfΔExon2 conferred a maternal effect on embryo survival. (B) As in (A), heterozygous (+/−) BptfΔExon2 males and females were intercrossed and all progeny were genotyped by Southern blotting or PCR. As with BptfΔExon2, homozygous (−/−) BptfΔExon2 embryos were not viable after E7.5 to E8.5.

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Conceived and designed the experiments: JL, AAS, HX, MSHK, TPY, CW. Performed the experiments: JL, YP, LVS, EAS, JM. Analyzed the data: JL, AAS. Contributed reagents/materials/analysis tools: JL, LT, YEZ, MSHK, TPY, CW. Wrote the paper: JL, AAS, MRK, TPY, CW.
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