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(Article begins on next page)
Loss of the Birt–Hogg–Dubé tumor suppressor results in apoptotic resistance due to aberrant TGFβ-mediated transcription

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Birt–Hogg–Dubé (BHD) syndrome is an inherited cancer susceptibility disease characterized by skin and kidney tumors, as well as cystic lung disease, which results from loss-of-function mutations in the BHD gene. BHD is also inactivated in a significant fraction of patients with sporadic renal cancers and idiopathic cystic lung disease, and little is known about its mode of action. To investigate the molecular and cellular basis of BHD tumor suppressor activity, we generated mutant Bhd mice and embryonic stem cell lines. BHD-deficient cells exhibited defects in cell-intrinsic apoptosis that correlated with reduced expression of the BH3-only protein Bim, which was similarly observed in all human and murine BHD-related tumors examined. We further demonstrate that Bim deficiency in Bhd−/− cells is not a consequence of elevated mTOR or ERK activity, but results instead from reduced Bim transcription associated with a general loss of TGFβ-mediated transcription and chromatin modifications. In aggregate, this work identifies a specific tumor suppressive mechanism for BHD in regulating TGFβ-dependent transcription and apoptosis, which has implications for the development of targeted therapies.

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Introduction

Birt–Hogg–Dubé (BHD) syndrome is a rare inherited cancer susceptibility disease characterized by benign hair follicle tumors and lung cysts in a majority of patients, and renal cell carcinoma (RCC) in approximately one third of diagnosed BHD cases (Schmidt et al., 2005). Genetic analyses of families affected by BHD syndrome previously identified germline mutations in the BHD gene on chromosome 17p11.2, most of which were predicted to prematurely truncate the encoded protein (Nickerson et al., 2002; Toro et al., 2008). Mutational analyses of BHD-related tumors from patients, as well as those from mouse and rat models of BHD syndrome, identified inactivating ‘second hits’ in the remaining wild-type BHD allele, formally establishing BHD as a tumor suppressor (Okimoto et al., 2004; Vocke et al., 2005; Hasumi et al., 2009). BHD inactivation has also been described in a subset of von Hippel-Lindau-independent RCC syndromes, in approximately one third of sporadic renal cancers, and in 60% of idiopathic cystic lung disease cases (Khoo et al., 2003; Gunji et al., 2007; Woodward et al., 2008).

While the genetic basis of BHD syndrome is well understood, the cellular and molecular mechanisms involving the protein encoded by BHD, also called Folliculin, remain unclear. Dissecting BHD’s molecular functions is particularly challenging since the BHD gene product bears no sequence or functional homology to any known protein. Studies in Schizosaccharomyces pombe have suggested a role for the putative yeast BHD ortholog in amino-acid homeostasis, while siRNA-mediated knockdown of Drosophila BHD in the fly has implicated it in male germline stem cell maintenance through Stat and/or BMP signaling (Singh et al., 2006; van Slegtenhorst et al., 2007). Finally, mammalian cell culture analyses and mouse models have linked BHD and mammalian target of rapamycin (mTOR) signaling, potentially mediated through an interaction with its binding partner, called Folliculin-interacting protein, and AMP-activated kinase (Baba et al., 2006). These studies demonstrate that BHD loss can paradoxically result in either stimulation or inhibition of mTOR depending on the system examined (Baba et al., 2006, 2009; Hudon et al., 2008, 2010).

To investigate the cellular and molecular mechanisms by which BHD suppresses tumorigenesis, we established Bhd−/− embryonic stem (ES) cell lines, since the early lethality of Bhd−/− embryos precluded the development of other cellular models. We observed that the major cellular consequence of BHD loss is resistance to apoptosis caused by decreased Bim expression. This phenomenon was independent of mTOR and ERK hyperactivation and was instead associated with a general loss of TGFβ-dependent chromatin modifications and transcription. These data present new insight into how BHD functions as a tumor suppressor and...
suggest novel targeted therapeutics for BHD syndrome, sporadic RCC, and cystic lung disease.

Results

**Homozygous mutant Bhd embryos die during early embryogenesis**

To define the basic molecular functions of BHD, we initially attempted to establish mouse embryonic fibroblasts from a gene-trap mouse model. In order to fully characterize this mutant Bhd allele (denoted as m), we mapped the insertional position of the trap cassette to intron 8 of the murine Bhd gene, and established Southern and PCR-based genotyping assays to discriminate wild-type from mutant alleles (Figures 1a–c). Using multiple quantitative real-time (qRT)-PCR probes, we observed a 40–50% reduction in Bhd mRNA expression in Bhd+/m ES cells compared with Bhd+/+ controls by qRT-PCR (Figure 1d), and confirmed expression of the BHD–βgeo fusion product by staining the ES cells with X-Gal (Figure 1e).

Bhd+/− intercrosses yielded no viable Bhd+/− embryos after e7.5; although we were unable to genotype e6.5 embryos, we observed that ~25% of these were severely abnormal or partially resorbed upon dissection, exhibiting grossly atrophied and disorganized egg cylinders, distorted visceral endoderm and lack of cavitation (Figures 1f and g). This phenotype is consistent with a recently reported embryonic lethal phenotype for an independent mutant Bhd allele (Hasumi et al., 2009).

Figure 1 Bhd+/m embryos are early embryonic lethal. (a) Schematic depicting integration site of Bay Genomics pG2Lxf gene-trap vector in murine Bhd intron 8, location of probe and restriction sites for Southern blot genotyping and location of primers (P1, P2, and P3) for PCR-based genotyping. (b) Southern blot of PvuII-digested genomic DNA and (c) PCR-based genotypes of Bhd+/+ or Bhd+/m ES cells to discriminate wild-type from mutant alleles. ‘m’ specifically designates the gene-trapped mutant Bhd allele from Bay Genomics throughout the manuscript. (d) Relative Bhd mRNA levels in Bhd+/+ versus Bhd+/− ES cells assayed by qRT–PCR using primers spanning exons just downstream of the trap cassette (exons 9–10) or across the terminal exons of the Bhd gene (exons 12–13), ruling out the presence of aberrant transcripts generated from cryptic transcriptional start sites downstream of the trap cassette. (e) X-Gal staining of Bhd+/+ and Bhd+/m ES cells showing Bhd−/m cells express the Bhd-β-galactosidase fusion product. (f) Table of progeny obtained from Bhd+/− intercrosses showing no Bhd+/− embryos could be recovered after e6.5. (g) 200 x images of H&E-stained cross-sections of paraffin-embedded e6.5 embryos from Bhd−/m intercrosses. (A) Exemplifies normal morphology while (B) and (C) demonstrate gross abnormalities, probable Bhd+/m genotypes.
Loss of BHD does not affect proliferation, but results in decreased cellular size and resistance to cell-intrinsic apoptosis

Since the early lethality of the Bhd<sup>+/−</sup> embryos precluded the development of differentiated cell types, we generated an ES cell model for in vitro studies. To do this, we used an independent mutant Bhd allele (denoted as −/−) with a gene trap in Bhd intron 1, which ablated expression of the entire Bhd open reading frame. The Bhd<sup>+/−</sup> ES cells were characterized in the same manner as the Bhd<sup>+/+</sup> cells to validate efficacy of the trap cassette (Supplementary Figures 1a–e).

Bhd<sup>−/−</sup> ES cells were generated from the parental Bhd<sup>+/+</sup> cells as shown by PCR, and western blot confirmed complete loss of BHD protein expression (Figures 2a and b). To verify BHD loss did not affect the undifferentiated state of Bhd<sup>−/−</sup> ES cells, we analyzed protein levels of Oct-4, Nanog, Sox2 and phospho-Stat3, and alkaline phosphatase activity in Bhd<sup>+/+</sup>, Bhd<sup>+/−</sup>, and Bhd<sup>−/−</sup> cells (Supplementary Figures 2a–c). We observed no difference in all parameters of ES cell pluripotency, allowing for valid comparison in in vitro studies across genotypes.

To gain insight into BHD’s tumor suppressive properties, we assessed Bhd<sup>+/+</sup>, Bhd<sup>+/−</sup>, and Bhd<sup>−/−</sup> ES cell lines for proliferation, cell growth, and apoptotic responses. Bhd<sup>+/−</sup> ES cells did not proliferate significantly faster at days 1–3 after plating, but confluent cultures analyzed at days 4 were found to contain 15–20% more cells compared with Bhd<sup>+/+</sup> and Bhd<sup>−/−</sup> counterparts (Figure 2c). Intriguingly, this difference was attributed to a 15–20% decrease in cellular volume in Bhd<sup>−/−</sup> cells (Figure 2d).

Finally, we evaluated the response of Bhd<sup>+/−</sup> cells to cell-intrinsic apoptosis by starving cells of serum, glucose, or amino acids for 24h. Bhd<sup>+/−</sup> cells clearly demonstrated a resistance to all three apoptosis-inducing stresses as revealed by light microscopy, decreased accumulation of sub-G1 populations, and reduced Caspase-3 and Parp cleavage (Figures 2e–h). This effect was specific to the intrinsic apoptotic pathway, as Bhd<sup>+/−</sup> cells showed no differential response to the death-receptor ligand Tumor necrosis factor α (Figure 2g). Thus, BHD deficiency does not confer an obvious increase in cellular proliferation or growth, but does reduce cell-intrinsic apoptosis, thereby identifying a plausible cellular role for BHD as a tumor suppressor.

Bim expression is lost in Bhd<sup>−/−</sup> cells and BHD-related tumors and contributes to apoptotic resistance

The cell-intrinsic apoptotic resistance of Bhd<sup>+/−</sup> cells could either be due to increased intracellular nutrient stores, an effect previously observed in a mutant yeast model of BHD, or result from a defect in the cell-intrinsic death machinery (van Slegtenhorst et al., 2007). To assess the former possibility, we measured levels of 13 different intracellular amino acids by high performance liquid chromatography and observed no differences between Bhd<sup>+/+</sup> and Bhd<sup>−/−</sup> ES cells, suggesting that nutrient homeostasis was not playing a role in the apoptotic-resistance phenotype (Figure 3a).

To determine if the cell-intrinsic death machinery might be impaired in Bhd<sup>−/−</sup> ES cells, we examined protein levels of several Bcl2 family members and found many pro-apoptotic BH3-only proteins to be misregulated in Bhd<sup>−/−</sup> cells. Both extra-long (EL) and long (L) isoforms of Bim, as well as Puma and Bad were significantly downregulated in Bhd<sup>−/−</sup> ES cells, while Bid and BMF levels were upregulated (Figure 2b). Bik, Noxa, and Pumaβ, as well as the multi-domain Bcl2 family members BclXL and Bak were unchanged. Similar results were obtained using three independent BHD-deficient ES clones homozygous for the Bhd<sup>b</sup> allele (Supplementary Figures 3a and b). We focused primarily on Bim regulation in subsequent experiments, given its dominant role among BH3-only proteins in mediating the cell-intrinsic death response and frequent loss in RCC (Kim et al., 2006; Zantl et al., 2007; Guo et al., 2009).

To test whether the apoptotic resistance of Bhd<sup>−/−</sup> cells was Bim dependent, we restored either BimEL or Bhd expression by retroviral transduction in Bhd<sup>−/−</sup> cells. Restoration of BimEL or Bhd expression, or treatment of cells with the BH3-only chemical mimetic ABT-737, rescued the apoptotic response of Bhd<sup>−/−</sup> ES cells to amino-acid deprivation, as assessed by FACs analysis (Figure 3c, upper panel), and Parp and Caspase-3 cleavage (Figure 3c, lower panel). Importantly, Bhd reconstitution was sufficient to restore Bim protein levels. Interestingly, autophagy inhibitors 3-methyl-adenine and chloroquine rescued the apoptotic response in Bhd<sup>−/−</sup> cells (Figure 3c), similar to apoptosis-deficient Bax<sup>−/−</sup>/Bak<sup>−/−</sup> double knockout cells (Lum et al., 2005).

To determine whether loss of BHD results in Bim deficiency in vivo, we performed immunohistochemistry on BHD-related tumors from both human BHD patients and aged Bhd<sup>+/−</sup> mice. 3/3 human-derived tumors (one fibrofolliculoma, one chromophobe-clear cell hybrid RCC, and one chromophobe RCC) and 5/5 solid murine renal tumors (one fibrofolliculoma, one chromophobe-clear cell hybrid RCC, and one chromophobe RCC) and 5/5 solid murine renal tumors (four hybrid oncocytic cell hybrid RCC, and one chromophobe RCC) and 5/5 solid murine renal tumors (four hybrid oncocytic and one papillary mass projecting from a cyst), exhibited absent or significantly reduced Bim expression, confirming that loss of Bim expression is a common event in BHD-related tumors of diverse histologies (Figure 3d).

Bim is transcriptionally downregulated in Bhd<sup>−/−</sup> cells independent of mTORC1, mTORC2, ERK, or GCN2-eIF2α pathway aberrations

Bim is regulated transcriptionally by several well-characterized signal transduction pathways, translationally by miRNAs, and on the level of protein stability (Dijkers et al., 2000; Dehan et al., 2009; Su et al., 2009). We initially assessed Bim mRNA and found it to be decreased by ~80% in Bhd<sup>−/−</sup> cells compared with Bhd<sup>+/+</sup> and Bhd<sup>−/−</sup> cells (Figure 4a). To investigate a possible additional role for enhanced proteolytic degradation of Bim, we treated Bhd<sup>−/−</sup> cells with the proteasome inhibitor MG-132, but observed no increase in Bim protein (Supplementary Figure 4a) (Dehan et al., 2009). We also examined levels of mir-19 and mir-92,
miRNAs previously shown to regulate *Bim* translation in ES cells (Su et al., 2009), but detected no differences between *Bhd*<sup>−/−</sup> and control cells (Supplementary Figure 4b).

To determine which transcriptional networks and upstream signaling pathways were involved in *Bim* misregulation, we investigated the mTOR and ERK pathways that have previously been linked to *Bim* regulation (Dijkers et al., 2000; Dehan et al., 2009), and are hyperactivated in late-stage cysts and tumors derived from a mutant *Bhd* mouse model (Baba et al., 2008). Western blot analysis of *Bhd*<sup>−/−</sup>, *Bhd*<sup>+/−</sup>, and *Bhd*<sup>−/−</sup> ES cells revealed that *Bhd*<sup>−/−</sup> ES cells exhibited hyperphosphorylation of S6K1 and 4E-BP1 (indicative of increased mTOR complex 1 (mTORC1) activity), hyperphosphorylation of Akt and the FoxO family of proteins, and increased expression of the neurotrophic factor BDNF (Brain-derived neurotrophic factor) (Dijkers et al., 2000; Dehan et al., 2009).

**Figure 2** *Bhd*<sup>−/−</sup> ES cells do not have a proliferative or growth advantage, but are resistant to apoptosis. (a) PCR genotypes verifying loss of wild-type *Bhd* allele in three independent ES clones, designated as '1', '2', and '3', after step-up selection. WT = wild-type, Mut = mutant alleles. (b) Western blot demonstrating loss of BHD protein expression in three independent *Bhd*<sup>−/−</sup> ES clones with a non-specific (NS) band shown as a loading control. (c) Growth curve showing *Bhd*<sup>−/−</sup> ES cells proliferate at similar rates to *Bhd*<sup>+/+</sup> and *Bhd*<sup>+/−</sup> counterparts, but have increased saturation density at day 4. (d) Coulter counter size measurements showing *Bhd*<sup>−/−</sup> cells are significantly smaller than *Bhd*<sup>+/+</sup> and *Bhd*<sup>+/−</sup> ES cells (*P* < 0.000623). *Bhd*<sup>−/−</sup> ES cells are resistant to stress-induced apoptosis as shown by (e) brightfield images of *Bhd*<sup>+/+</sup> and *Bhd*<sup>−/−</sup> ES cells starved of amino acids for 1 day, or (f) FACs analysis of cells containing sub-G1 DNA content after 1 day serum, glucose and amino-acid (AA) deprivation compared with *Bhd*<sup>+/+</sup> and *Bhd*<sup>−/−</sup> cells (*P* < 0.0000345), but not by Tumor necrosis factor α treatment. (g) Representative FACs plot showing *Bhd*<sup>−/−</sup> ES cells have significantly less sub-G1 DNA content following 1 day of amino-acid starvation. (h) Western blot of Caspase and Parp cleavage with and without amino-acid starvation in *Bhd*<sup>+/+</sup> and *Bhd*<sup>−/−</sup> ES cells. All bar graphs represent averages of three independent experiments with error bars showing standard error of the mean (s.e.m.).
proteins (indicative of mTOR complex 2 (mTORC2) hyperactivation) and hyperactivation of the MEK–ERK–p90RSK cascade (Figure 4b).

To determine if these pathways were playing a role in decreased Bim levels in Bhd−/− cells, we treated cells with inhibitors of each at doses that reduced signaling in Bhd−/− cells to levels observed in Bhd+/+ control cells. We found that pharmacologic inhibition of PI3K-dependent mTORC2, MEK, or mTORC1 activities, either singly or in combination, was insufficient to restore Bim protein levels in Bhd−/− cells (Figure 4c). Treatment of Bhd−/− cells with the same panel of inhibitors was similarly unable to rescue the death-resistance phenotype compared with untreated or vector only (MSCV) control wells (Figure 4d, upper panel), or Caspase and Parp cleavage following 2-day amino-acid starvation (Figure 4d, lower panel).

Bim expression is also known to be regulated by Chop, a transcription factor activated downstream of BHD in apoptosis and TGFβ pathway (Cash et al. 2018).
eukaryotic initiation factor 2α (eIF2α) kinases as part of the unfolded protein response and as an adaptation to nutrient withdrawal (Puthalakath et al., 2007). Interestingly, Bhd−/− cells exhibited decreased basal and amino-acid deprivation-induced levels of p-eIF2α levels, but not ER stress-induced p-eIF2α after thapsigargin treatment (Supplementary Figures 5a–c). However, induction of the downstream effectors ATF4 and Chop was only slightly attenuated in response to amino-acid deprivation (Supplementary Figure 5b). More importantly, reintroduction of Chop into Bhd−/− cells failed to rescue Bim expression (Supplementary Figure 5d). Collectively, these data suggest that the downregulation of Bim and apoptotic resistance observed in Bhd−/− cells are not due to signaling aberrations in the mTORC1, mTORC2, ERK, and eIF2α signaling pathways.

Bhd−/− ES cells exhibit many phenotypic and molecular defects characteristic of TGFβ-pathway mutants

We next considered the TGFβ pathway, since many reports had previously shown it regulated Bim
transcription in diverse cell types (Wildey et al., 2003; Ramjaun et al., 2007; Ramesh et al., 2008; Yu et al., 2008; Houde et al., 2009). We first verified that the TGFβ ligand family regulates Bim mRNA in ES cells by treating them with Activin, a TGFβ family ligand to which ES cells are responsive. Bim mRNA was increased within 2 h of Activin treatment in Bhd+/+ ES cells, which did not occur in Bhd−/− cells (Figure 5a). To ascertain whether this effect was specific to Bim, we analyzed the mRNA levels of several other BH3-only family members in response to Activin. Contrary to Bim, Bid, BMF, and Bad mRNAs were not induced by Activin in wild-type cells, and actually appeared elevated in Bhd−/− cells (Supplementary Figure 6a). The latter finding was consistent with increased Bid and BMF protein levels in Bhd−/− cells (Figure 3b). While Puma mRNA was induced by Activin, this was not BHD dependent as it occurred in both Bhd+/+ and Bhd−/− cells (Supplementary Figure 6a). Taken together, these data identify Bim as a specific BH3-only mRNA regulated by TGFβ in a BHD-dependent manner.

To determine if TGFβ-mediated transcriptional defects were a general phenomenon in Bhd−/− cells, we
examined the basal mRNA levels of several established TGFβ-transcriptional targets involved in diverse biological processes, mRNA levels for the cell cycle regulator p15, pro-apoptotic plasminogen inhibitor activator type I (PAI-1) and Lefty1 and Pitx2, which have developmental roles were all lower in Bhd−/− cells compared with Bhd+/+ cells (Figure 5b). Other canonical TGFβ mRNA targets such as SnoN, p21, Col1a2, and Lefty2 were similarly downregulated in Bhd−/− (Supplementary Figure 6b). Other TGFβ targets previously implicated in cell cycle regulation and apoptosis were unaffected in Bhd−/− cells, including Gadd45b and p57, while the death-associated protein kinase was actually upregulated (Supplementary Figure 6c). Overall, these data support a positive role for BHD in TGFβ-mediated transcription that governs biological processes implicated in cancer such as apoptosis, cell cycle, and differentiation.

TGFβ ligands and receptors are required for normal yolk sac vasculogenesis and embryonic hematopoiesis in vivo, and in ES cell-derived cystic embryoid body (EB) cultures in vitro (Dickson et al., 1995; Oshima et al., 1996; Goumans et al., 1999; Larsson et al., 2001). Interestingly, strong BHD expression was observed in yolk sac visceral endoderm, as revealed by X-Gal staining of E10.5 embryos heterozygous for the Bhdα allele (Figure 5c). To investigate whether BHD is required for yolk sac development, we generated day 12 EBs from Bhd+/+ and Bhd−/− ES cells. Bhd−/− ES cells failed to form expanded cystic EBs, which are reminiscent of mature yolk sacs in vivo (Figure 5d), or induce mature yolk sac markers α-fetoprotein (AFP) and Trithioredoxin (Tir) (Doetschman et al., 1985) (Figure 5e). To further evaluate the role of BHD in embryonic hematopoietic stem cell development, we generated Bhd+/+ and Bhd−/− EBs in methylcellulose cultures, which promotes the differentiation of hematopoietic lineages. Bhd−/− EBs failed to form hemoglobinized colonies at day 10 in culture (Figure 5f; Supplementary Figure 6b) and exhibited severely reduced mRNA levels of the erythroid gene Gata-1 and CD34 (a marker of both hematopoietic and endothelial lineages) (Figure 5g). Interestingly, we observed a delayed induction of the mesodermal marker Brachyury, a super-induction of Fgf-5 and attenuated maintenance of Hnfα4 at later stages (Supplementary Figure 7). However, these minor effects are unlikely to explain the dramatic hematopoietic and yolk sac defects of Bhd−/− EBs, as maximal induction of these target genes was still robust in Bhd−/− EBs, increasing by orders of magnitude.

Bhd−/− cells exhibit hypo-acetylation of TGFβ target gene promoters, resulting in their death-resistance phenotype Because BHD deficiency resulted in molecular and phenotypic defects similar to TGFβ receptor and ligand mutants, we next investigated whether the transcriptional defects occurred at the level of receptor-mediated phosphorylation and nuclear translocation of Smad transcriptional regulators. Therefore, we examined nuclear levels of phosphorylated and total receptor-regulated Smad2 and the common partner Smad4, which form a complex and translocate to the nucleus upon cellular engagement of TGFβ ligands (Zhang et al., 1996). Western blot analysis did not reveal any differences in basal or Activin-induced nuclear accumulation of phospho-Smad2, total Smad2, or total Smad4 in Bhd−/− versus Bhd+/+ ES cells (Figures 6a and b).

Since Smads have previously been shown to activate transcription via acetylation of Histone H3 at target promoters, we performed chromatin immunoprecipitation for acetylated Histone H3 at the well-characterized Lefty1 promoter (Ross et al., 2006). Whereas Bhd−/− ES cells demonstrated a significant increase in acetyl-H3 levels at the Lefty1 promoter following Activin treatment, Bhd+/+ cells exhibited no response (Figure 6c). A similar effect was observed at the promoter of PAI-1, which has been implicated in the apoptotic response (Kortlever et al., 2006; Lademann and Romer, 2008) (Supplementary Figure 8a). This difference was not due to a global reduction of acetyl-H3 levels, which appeared unaffected based on western blot assays (Figure 6d). To determine whether restoration of acetylated Histone levels rescues Bhd−/− cell defects, Bhd−/− cells were treated with the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA), which restored mRNA levels of multiple TGFβ target genes (PAI-1, p15, and Bim), Bim protein levels and cell death (Figures 6e and f). TSA appeared to have variable effects on other misregulated BH3-only proteins in Bhd−/− cells under nutrient deprivation (Supplementary Figure 8b). First, nutrient deprivation itself changed protein levels of some BH3-only proteins in Bhd−/− cells, with BMF actually decreasing and Bad equilibrating to wild-type levels, in contrast to nutrient replete conditions (Figure 3a). TSA appeared to have no effect on Puma and Bid levels abundance under nutrient limitation, while it slightly elevated Bad and BMF levels. It seems unlikely, however, that Bad and BMF induction can explain the effects of TSA, given the ability of Bim re-expression alone to completely reverse the phenotype of Bhd−/− cells. In summary, BHD loss appears to contribute to apoptotic resistance and TGFβ-dependent transcriptional defects via aberrations in chromatin modifications at target gene promoters including Bim (Figure 7).

Discussion

To date, the cellular and molecular mechanisms of tumor suppression by BHD have remained elusive. Uncovering BHD protein functions is particularly challenging, given the lack sequence homology to any known protein. In this study, we initially observed that the major cellular consequence of BHD loss is apoptotic resistance due to loss of Bim expression, a phenomenon that can be observed in BHD-related renal tumors. These findings prompted us to ask what signaling pathways and/or transcriptional networks were implicated.
in this phenotype. We initially examined the mTOR pathway, as multiple published reports have shown correlations between BHD loss and mTOR activity (Hartman et al., 2009; Hasumi et al., 2009; Hudon et al., 2010). While we did observe aberrant mTOR signaling in Bhd−/− cells, this was surprisingly unrelated to the apoptotic defects.

We turned our attention to the TGFβ pathway, given its known role in regulating Bim transcription and tumor suppression. For instance, in humans, Smad4 germline mutations are associated with juvenile polyposis syndrome, which predisposes individuals to gastrointestinal cancers, and TGFβRI, TGFβRII and activin receptor II are mutated or downregulated in a broad spectrum of sporadic cancers (Levy and Hill, 2006). Epigenetic silencing of genes involved in TGFβ signaling has also been specifically observed in RCCs (McRonald et al., 2009). Genetic ablation of these signaling components in mice have confirmed the tumor suppressive function of this pathway in models of colon, mammary, and pancreatic cancer (Massague, 2008). Our data demonstrate that BHD loss results in yolk sac and

Figure 6  Loss of BHD results in HDAC-mediated silencing of TGFβ-transcriptional targets. Western blot of nuclear extracts showing (a) basal or (b) Activin-induced levels of nuclear phospho-smad2, Smad2, and Smad4 are unaffected in Bhd−/− ES cells. Non-specific (NS) band is shown as a loading control. (c) qRT–PCR using primers located in the Lefty1 promoter on genomic DNA immunoprecipitated with acetyl-histone H3 antibody from ES cells stimulated with activin for 1 h following 24 h culture in N2B27. (d) Western blot showing total levels of acetyl-histone H3 are unaffected in Bhd+/− versus Bhd−/− cells with and without Activin treatment. (e) Treatment of Bhd−/− cells with TSA restores mRNA levels of PAI-1, p15, and Bim as shown by qRT–PCR analysis of mRNA expression levels (*P<0.0362). (f) Treatment of Bhd−/− ES cells with TSA rescues the Bhd−/− cellular death-resistance phenotype and Bim expression as shown by analysis of sub-G1 populations (above, *P=0.000145) and western blot for Bim, and Caspase and Parp cleavage (below).
While we observed increased mTOR signaling in et al., more proximal defects in TGFβ likely result from compensation and/or crosstalk with speculate that effects of BHD loss on mTOR signaling unrelated to the observed apoptotic resistance. We from a BHD mouse model, this phenomenon appeared hematopoietic defects reminiscent of TGFβ receptor and ligand mutants. These phenotypes correlate with global aberrations in TGFβ-mediated transcription, which appear to occur downstream of nuclear accumulation of phosphorylated Smads. Our data indicate this defect is most likely due to HDAC-mediated effects on chromatin at Smad-dependent promoters, given the ability of TSA to reverse the biological and molecular defects of Bhd−/− cells (Figure 7). Further work will be needed to elucidate the nature of these effects.

Previous studies have suggested a contradictory role for mTOR in BHD-related tumorigenesis, showing that BHD loss can result in either upregulation or downregulation of mTOR signaling. These studies have been largely correlative and have not demonstrated a causative relationship between the signaling aberrations and biological outcomes (Baba et al., 2006; Hartman et al., 2009; Hasumi et al., 2009; Hudon et al., 2010). While we observed increased mTOR signaling in Bhd−/− ES cells, as reported for late-stage kidney tumors taken from a BHD mouse model, this phenomenon appeared unrelated to the observed apoptotic resistance. We speculate that effects of BHD loss on mTOR signaling likely result from compensation and/or crosstalk with more proximal defects in TGFβ signaling.

Collectively, the data presented here establish a novel model whereby BHD exerts its major tumor suppressive effect through TGFβ-mediated transcriptional output to regulate cell-intrinsic apoptosis. A recent publication has further supported this model, suggesting TGFβ-dependent transcriptional defects in a human BHD-derived tumor cell line (Hong et al., 2010). This study demonstrated that BHD deficiency results in decreased levels of TGFβ pathway signaling components themselves including the ligands TGFβ-2 and Inhibin β A and Smad3. This contrasts with our data that demonstrate an effect of BHD downstream of signaling, on canonical Smad target gene promoters. Further, the report by Hong et al. suggested that TGFβ-2 stimulates cell growth and survival in the absence of BHD, while the TGFβ ligand Activin does the opposite. The latter is surprising in light of our findings, given the failure of BHD null ES cells to mount any transcriptional response to Activin treatment. This discrepancy likely derives from differential cell type, dosage and/or length of treatment, which has been well documented for TGFβ ligands (Massague, 2008). In any case, these molecular defects likely extend to other tumor-promoting effects such as cell-type specific proliferative advantages, due to p15 loss, and stromal alterations in BHD-related tumors in vivo (Massague, 2008). Although our in vitro study did not uncover a role for BHD in proliferation, this is likely due to the unusual cell cycle machinery of ES cells, which is refractory to cyclin-dependent kinase inhibitors (Savatier et al., 1996; Faast et al., 2004). It is probable that BHD loss and consequent defective TGFβ-mediated signaling in vivo results in a proliferative advantage. Collectively, these findings suggest several targeted therapeutics for the treatment of BHD-related tumors, as well as sporadic cases of cystic lung disease and RCC, including BH3-only mimetics, autophagy inhibitors, and HDAC inhibitors, which are all currently in clinical use for the treatment of other types of cancer.

Materials and methods

Sub-G1 quantification
Cells were fixed and stained as previously described, then fluorescence was measured by flow cytometry (Riccardi and Nicoletti, 2006). At least 10,000 cells were counted for each experiment and data were analyzed using FlowJo software.

X-Gal staining of tissues and embryos
X-gal staining was carried out as previously described for both cells and whole mount tissues fixed in 4% paraformaldehyde according to the Sanger Institute gene-trap protocol. X-gal stained yolk sac sections came from tissue snap-frozen in OCT.

Alkaline phosphatase staining
Cells were stained using the Alkaline Phosphatase Detection Kit from Millipore (Billerica, MA, USA) according to the manufacturer’s instructions.

RNA isolation and qRT
RNA was extracted using Qiagen (Valencia, CA, USA) RNeasy columns with DNAase treatment and cDNA was written off ~1 μg of RNA per reaction using the ABI (Foster
City, CA, USA) one-step RT-PCR Master Mix. qRT-PCR reactions were run in triplicate on an ABI 7900HT machine for each experiment, utilizing either SYBR green or Taqman primer probe sets as noted in Supplementary Materials. All target mRNA levels were normalized to β-actin or 18s expression levels. All qRT-PCR data reflect average mRNA levels from three independent RNA extractions and reverse transcription reactions with error bars showing standard error of the mean.

**Protein extraction and western blotting**

Whole-cell lysates were prepared using a buffer containing 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1% Triton, and freshly added protease inhibitor cocktail tablets from Roche (Basel, Switzerland) and 2 mM orthovandate. Nuclear extracts were prepared using the Active Motif Nuclear Extraction kit, with 500 mM NaCl added after nuclease digestion followed by sonication of nuclear extracts. Lysates were run on polyacrylamide gels, transferred to nitrocellulose, blocked using standard protocols, and visualized on film using HRP-conjugated secondary antibodies and ECL reagent.

**Cell size measurements**

Cell size was measured using a Z2 Coulter particle size analyzer. At least 10,000 cells were measured for each experiment.

**Characterization of gene-trapped ES cells and mice**

Bhd+/− ES cells were obtained from Bay Genomics and Bhd+/− ES cells were obtained from the German Gene Trap Consortium, with isogenic parental Bhd−/− lines being obtained from each respective source. Gene-trap insertions were mapped by first PCR amplifying the boundary of the fusion transcript using primers within the 3′ end of the trap cassette and within the sequence tag, on cDNA generated from heterozygous ES cells. The precise genomic location of the cassette was mapped by direct PCR amplification of the respective intronic-trap cassette boundary using primers in the exon just upstream from the insertion and within the 5′ end of the trap cassette. Southern blots were carried out on either PvuII or BamHI-digested DNA on Bhd+/− or Bhd+/− ES cell, respectively, with 5′ end-directed probes being generated by PCR amplification from genomic DNA and subsequent TOPO cloning. Bhd+/− mice were generated and intercrossed and embryo collection was performed as previously described (Covello et al., 2006; Hartman et al., 2009).

**Proliferation assay**

In all, 5 × 10⁶ cells were plated in a gelatinized 12-well plate and counted using the Countess automatic counter from Invitrogen (Carlsbad, CA, USA) each day after plating.

**Immunohistochemistry**

Paraffin-embedded tissue was immunostained using a rabbit polyclonal anti-Bim antibody from Novus following antigen retrieval in sodium citrate solution and developed using diaminobenzidine.

**Embryoid bodies**

For suspension cultures, 8 × 10⁶ ES cells were plated in 10 cm bacterial dishes in DMEM containing 10% FBS and β-mercaptoethanol. Media was changed every other day, with EBs being split 1:2 on day 3. Methylcellulose cultures and bexideni staining were performed as previously described (Cooper et al., 1974; Adelman et al., 1999).

**Northern blotting**

Northern blots for miRNAs were done as previously described (Gruber et al., 2009).

**Chromatin immunoprecipitation**

In all, 1 × 10⁶ ES cells were fixed for 10 min in 1% formaldehyde for 10 min at room temperature. Crosslinking was quenched with 125 mM glycine for 5 min, then cells were lysed and sonicated to shear DNA for 3 min with on/off pulses using a Fisher Sonic Dismembrator Model 500 at 20% power. Chromatin immunoprecipitation was carried using the Imgenex QuikChIP kit according to the manufacturer’s instructions using a pan acetyl-H3 antibody from Millipore. Eluted protein/DNA complexes were purified by phenol/ chloroform extraction and ethanol precipitation.

**Statistics**

All P-values were generated using a two-tailed Student’s t-test.

**Conflict of interest**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)