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Banf1 Is Required to Maintain the Self-renewal of Both Mouse and Human Embryonic Stem Cells

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Abstract

Self-renewal is a complex biological process necessary for maintaining the pluripotency of embryonic stem cells (ESCs). Recent studies have used global proteomic techniques to identify proteins that associate with the master regulators Oct4, Nanog, and Sox2 in ESCs or in ESCs during the early stages of differentiation. Through an unbiased proteomic screen, Banf1 was identified as a Sox2-associated protein. Banf1 has been shown to be essential for worm and fly development but, until now, its role in mammalian development and ESCs has not been explored. In this study, we examined the effect of knocking down Banf1 on ESCs. We demonstrate that the knockdown of Banf1 promotes the differentiation of mouse ESCs and decreases the survival of both mouse and human ESCs. For mouse ESCs, we demonstrate that knocking down Banf1 promotes their differentiation into cells that exhibit markers primarily associated with mesoderm and trophectoderm. Interestingly, knockdown of Banf1 disrupts the survival of human ESCs without significantly reducing the expression

levels of the master regulators Sox2, Oct4 and Nanog or inducing the expression of markers of differentiation. Furthermore, we determined that the knockdown of Banf1 alters the cell cycle distribution of both human and mouse ESCs by causing an uncharacteristic increase in the proportion of cells in the G2–M phase of the cell cycle.

Keywords: embryonic stem cell, Sox2, Banf1, self-renewal, cell cycle

Introduction

Self-renewal is a fundamental, yet highly complex, process required for maintenance of embryonic stem cells (ESCs) in an undifferentiated pluripotent state. It is widely recognized that advancing our understanding of stem cell biology is crucially dependent on developing a much deeper understanding of the molecular machinery that controls stem cell self-renewal. Our understanding of self-renewal is also crucial for the future development of cell-based therapies that are expected to offer new and more effective treatment options for a wide-range of diseases. Although significant progress has been made during the past decade, our understanding of the molecular machinery and molecular mechanisms that control the fate of stem cells is far too limited. Thus far, the greatest strides have been made during the study of pluripotent ESCs, which under carefully controlled conditions, can self-renew without limit (Rizzino, 2009).

At the center of the self-renewal of ESCs are the transcription factors Sox2, Oct4, and Nanog, whose expression is precisely and tightly regulated in ESCs. As expected, knockdown of these master regulators either induces the differentiation of ESCs, in the cases of Sox2 and Oct4, or increases the propensity of ESCs to differentiate, in the case of Nanog (Chambers et al., 2003; Chew et al., 2005; Niwa et al., 2000). Remarkably, a small increase in either Sox2 (Kopp et al., 2008) or Oct4 (Niwa et al., 2000) induces the differentiation of ESCs, whereas an increase in Nanog eliminates the requirement for LIF by mouse ESCs (mESCs) (Chambers et al., 2003; Mitsui et al., 2003). The importance of these master regulators is further highlighted by their roles in the regulation of large networks of downstream target genes, many of which are crucial for maintaining the self-renewal and pluripotency of ESCs (Avilion et al., 2003; Boyer et al., 2005; Dailey et al., 1994; Kuroda et al., 2005; Nakatake et al., 2006; Nichols et al., 1998; Nishimoto et al., 1999; Nowling et al., 2003; Tokuzawa et al., 2003; Yuan et al., 1995). In an effort to expand our understanding of self-renewal, various global approaches have been used to identify components, in addition to master regulators, that are essential for the proper growth and differentiation of ESCs. During the past five years, ChIP-Chip and ChIP-Seq studies have identified several thousand Sox2, Oct4, and Nanog target genes (Boyer et al., 2005; Chen et al., 2008; Kim et al., 2008; Loh et al., 2006), some of which have been shown more recently to contribute to the self-renewal of ESCs (Darr and Benvenisty, 2009; Kidder et al., 2009; Lim et al., 2008; Viswanathan et al., 2008). Other studies have used global high-throughput RNA interference (RNAi) screens to continue the identification of additional players that are essential for controlling the fate of ESCs (Chia et al., 2010; Fazzio et al., 2008; Hu et al., 2009; Schaniel et al., 2010). More recently, global proteomic screens have demonstrated that the master regulators Sox2, Oct4, and Nanog (Liang et al., 2008; Mallanna et al., 2010; Mallanna and

Rizzino, 2011; Pardo et al., 2010; van den Berg et al., 2010; Wang et al., 2006) interact with large numbers of nuclear proteins, and that these master regulators are present in large diverse protein complexes (Cox and Rizzino, 2010). To date, the vast majority of proteins identified using these large-scale proteomic screens have not been tested directly for their roles in the physiology of ESCs. Nonetheless, it is probable that many of the proteins identified in these proteomic screens represent proteins essential for ESCs, in particular those that are components of large protein complexes involved in transcription and chromatin structure.

Recently, our laboratory conducted an unbiased proteomic screen to identify the proteins that associate with Sox2 (Mallanna et al., 2010). For this purpose, we used ESCs (i-Sox2-ESCs) engineered to inducibly express Flag-epitope-tagged Sox2 (Flag-Sox2) when doxycycline is added to the culture medium (Kopp et al., 2008). Because of the essential role of Sox2 in the maintenance of the self-renewal and pluripotency of ESCs, and its ability to help promote the reprogramming of somatic cells to induced pluripotent stem cells (Takahashi et al., 2007), we hypothesized that many of the identified Sox2-associated proteins also control the fate of ESCs. In fact, our proteomic screen identified several proteins known to be crucial for maintaining the properties of ESCs, including, but not limited to, Sall4, Lin28, Esrrb, and Smarca4 (Mallanna et al., 2010). Importantly, our proteomic screen identified an even larger set of Sox2-associated proteins that have not been tested for their roles in the biology of ESCs.

Barrier to autointegration factor 1 (Banf1) was identified in our proteomic screen as a Sox2-associated protein (Mallanna et al., 2010). Banf1 is an evolutionarily conserved DNA-binding protein originally described for its role in preventing destructive autointegration of retroviral genomes (Lee and Craigie, 1998; Lin and Engelman, 2003). Other studies have shown that Banf1 is part of the nuclear envelope (Furukawa et al., 2003; Haraguchi et al., 2001; Margalit et al., 2005; Shimi et al., 2004). Importantly, Banf1 is believed to carry out crucial functions during cell cycle progression, specifically in the formation of the nuclear envelope during telophase through association with proteins such as Emd (Cai et al., 2007; Furukawa, 1999; Haraguchi et al., 2008; Margalit et al., 2007; Montes de Oca et al., 2009). Equally important, knockdown of Banf1 homologs during the early stages of *Drosophila* or *Caenorhabditis elegans* development causes embryonic lethality (Margalit et al., 2007). Thus far, these findings have not been extended to mammalian development. Given the important role that Banf1 plays in cell cycle progression during the development of model organisms, and the unique features of the cell cycle checkpoints in ESCs (Boheler, 2009; White and Dalton, 2005), we suspected that Banf1 plays an important role in the physiology of ESCs, as well as during early mammalian development.

To address the role of Banf1 in maintaining the self-renewal and pluripotency of ESCs, we utilized RNAi technology, delivered by lentiviral vectors, to knockdown Banf1 in both mESCs and human ESCs (hESCs). Specifically, we focused on three questions. Does the knockdown of Banf1 alter the self-renewal of ESCs, induce their differentiation and/or alter their cell cycle? We demonstrate that cell survival, as well as cloning efficiency, decreases after Banf1 is knocked down in mESCs and hESCs. We also demonstrate that the knockdown of Banf1 promotes the differentiation of mESCs and alters the cell cycle of both

mESCs and hESCs by increasing the percentage of cells in the G2–M phase and decreasing the percentage of cells in the S-phase compartment.

Results

Knockdown of mouse Banf1 induces the differentiation of mESCs

Kopp and colleagues previously engineered mESCs for inducible expression of Flag-epitope-tagged Sox2 (Flag–Sox2) when doxycycline is added to the culture medium (Kopp et al., 2008). We recently used these ESCs to perform an unbiased proteomic screen of Flag–Sox2-associated proteins, and identified Banf1 as a Sox2-associated protein (Mallanna et al., 2010). Importantly, Banf1 protein expression did not change in our Flag–Sox2 inducible system before or after the induction of Flag–Sox2.

Given that several of the identified Sox2-associated proteins, such as Sall4 and Lin28, are essential for maintaining self-renewal of ESCs, we wanted to determine whether the expression of Banf1 is crucial for maintaining the characteristic phenotype of mESCs. For this purpose, we utilized RNAi technology to knockdown *Banf1* transcripts. Initially, an shRNA targeting the *Banf1* transcript (Mouse Banf1 shRNA) was placed into the pLL3.7 lentiviral transfer vector. Additionally, we used, as a control, a construct containing a non-specific shRNA sequence (Scrambled shRNA) described previously (Wiebe and Traktman, 2007). Scrambled and Banf1 shRNA lentiviral particles were initially used to infect D3 mESCs. Importantly, the pLL3.7 construct contains a puromycin-resistance gene that is used for positive selection of infected cells. At 72 hours after infection, western blot analysis of nuclear proteins demonstrated a substantial knockdown of endogenous Banf1 in the presence of the Mouse Banf1 shRNA construct (fig. 1A). Moreover, ESCs infected with the Mouse Banf1 shRNA viral construct began to lose their characteristic phenotype and to differentiate when subcultured at low density (400 cells per cm²) (fig. 1B). Specifically, the cells infected with Banf1 shRNA viral construct began to acquire cytoplasmic processes, a flattened morphology, and an increased cytoplasmic to nuclear ratio compared with those cells infected with the Scrambled shRNA viral vector. In addition, cells transduced with Mouse Banf1 shRNA, did not stain as intensely for the pluripotent stem cell marker alkaline phosphatase (AP). To corroborate our observations, three additional shRNA constructs that target both mouse and human transcripts for Banf1 were used to knockdown mouse Banf1 in mESCs. These shRNA constructs are referred to as Banf1 shRNA #1, #2, or #3. Constructs #1 and #2 knocked down Banf1 protein much like our engineered Mouse Banf1 shRNA construct, whereas construct #3 did not knockdown Banf1 to the same extent (fig. 1A). Additionally, mESCs infected with Banf1 shRNA constructs #1, #2, and #3 exhibited flattened morphology, reduced AP staining and an overall loss of phenotype characteristic of ESCs when grown at low density (400 cells per cm²) (fig. 1B). These data strongly suggest that maintaining Banf1 protein at appropriate levels is necessary to preserve the characteristic morphology and prevent differentiation of mESCs.

Figure 1. Knockdown of *Banf1* in mESCs causes changes in cell morphology, elevation of gene markers of differentiation and reduction in the protein levels of Oct4, Sox2, and Nanog.

(A) *Banf1* levels were examined by western blot analysis using nuclear proteins isolated from D3 mESCs 72 hours after being transduced with lentiviral constructs that express Scrambled shRNA, Mouse *Banf1* shRNA or *Banf1* shRNA constructs #1, #2 or #3. HDAC2 expression was used as a loading control. (B) Photomicrographs of representative D3 mESC colonies infected with lentiviral constructs for Scrambled shRNA, Mouse *Banf1* shRNA or *Banf1* shRNA constructs #1, #2, or #3. D3 mESCs were subcultured 72 hours after infection and selection was with puromycin. Photomicrographs were taken and AP staining was performed 72 hours after subculture. (C) qRT-PCR of RNA transcripts isolated from D3 mESCs 72 hours after transduction. Threshold cycle (Ct) values for cells transduced with the Mouse *Banf1* shRNA lentiviral vector are subtracted from Ct values for cells transduced with the Scrambled shRNA lentiviral vector and normalized against

the expression of *GAPDH*. RNA isolation, cDNA synthesis and qRT-PCR for each condition were performed on three independent cell culture preparations. Differences in Ct values were averaged between the three separate replicates and are represented on the bar graph (error bars represent s.d.). A positive value on the graph signifies an increase in the level of the transcripts assayed. (D) Western blot analyses of nuclear proteins isolated from D3 mESCs 3 days and 7 days following transduction. Separate blots were run for each protein probed. Protein expression levels, presented in parentheses beneath corresponding bands, were normalized against a corresponding HDAC1 loading control (data not shown).

To identify which cell types form when *Banf1* is knocked down, we examined the expression of genes known to be markers of ESCs, as well as markers of specific developmental cellular lineages. For this purpose, RNA was isolated from mESCs 72 hours after transduction with lentiviral constructs that express either the Scrambled or Mouse *Banf1* shRNA. Next, expression of specific genes was examined by quantitative real-time PCR (qRT-PCR), and cycle threshold (Ct) value differences between Scrambled and Mouse *Banf1* shRNA were determined. Gene markers specific to ectoderm rose modestly, whereas markers of mesoderm and trophoctoderm exhibited more substantial increases upon *Banf1* knockdown (fig. 1C). Specifically, ectoderm markers *Sox21* and *Nestin* were elevated ~1 cycle in the *Banf1* knockdown cells. Mesoderm markers *Tpm-1* and *Sm22a* were elevated ~5 and ~4.5 cycles, respectively. Trophoctoderm markers *Cdx2*, *Esx1* and *Cdh3* were all elevated >1 cycle as well. By contrast, the endoderm markers examined did not increase significantly. Markers of ESCs, *Sox2* and *Oct4* demonstrated a modest decrease (~0.5 cycles) in transcript expression, and *Nanog* and *Fgf4* were both reduced ~1.5 cycles upon *Banf1* knockdown.

We next examined the protein expression of the transcription factors *Oct4*, *Sox2*, and *Nanog* by western blot analysis, following knockdown of *Banf1*, at early (3 days after infection) and late (7 days after infection) time points (fig. 1D). *Sox2* protein levels decreased slightly 3 days after *Banf1* knockdown, and a more substantial decrease was observed at day 7. *Oct4* levels did not change 3 days following *Banf1* knockdown; however, *Oct4* levels were substantially reduced at day 7. Additionally, protein levels of *Nanog* were also reduced 7 days after transduction with *Banf1* shRNA lentiviruses. Together, these findings indicate that maintaining *Banf1* expression is crucial for maintaining the expression of pluripotency-associated transcription factors and preventing the activation of genes expressed by differentiated cells.

***Banf1* knockdown disrupts self-renewal and pluripotency of mESCs**

In addition to the maintenance of pluripotency-associated genes, a characteristic property of ESC self-renewal is the ability of ESCs to proliferate and form colonies with a characteristic morphology when plated at clonal densities. In this regard, differentiated cells derived from ESCs can be replated when subcultured; however, their plating efficiency is substantially lower than that of ESCs, and they form clusters of loosely associated cells that are morphologically distinct from colonies formed by ESCs. To determine further how the knockdown of *Banf1* affects the fate of mESCs, we examined the cloning efficiency of

mESCs after *Banf1* was knocked down. For this analysis, equal numbers of cells, previously infected with either the Scrambled shRNA or the Mouse *Banf1* shRNA viruses, were subcultured into T25 culture flasks at two clonal densities (320 cells per cm² and 80 cells per cm²). Two independent observers, unaware of sample designation, counted colonies in 20 random low-power fields (40 $\times$ magnification) 96 hours after the cells were subcultured (fig. 2B). A representative photomicrograph of a random field is presented in figure 2A. At both clonal densities, knockdown of *Banf1* reduced the self-renewal of ESCs ~six-fold compared with control cells infected with the Scrambled shRNA.

Figure 2. Self-renewal and the phenotype of ESCs are disrupted upon *Banf1* knockdown in mESCs. (A) Representative low-power (viewed at 4 $\times$) photomicrographs comparing the cloning efficiency of D3 mESCs transduced with either Scrambled or Mouse *Banf1* shRNA lentiviral constructs. Cells were transduced, selected with puromycin and passaged at a density of 320 cells per cm², and were photographed 96 hours after subculture. (B) Two observers, unaware of sample designation, counted the colonies in 20 random low-power (4 $\times$) fields, to determine cloning efficiency. Counts for each field and observer were averaged (error bars represent s.d.). (C) Representative photomicrographs of ESC-like (left) and mixed-differentiated (right) colonies of cells seeded at 200 cells per cm², 72 hours after infection. AP stain photomicrographs are also shown (bottom). (D) Quantification of ESC-like and mixed-differentiated-like colonies. D3 ESCs were seeded at 200 cells per cm², transduced with either the Scrambled or *Banf1* shRNA lentiviral vector and selected with puromycin. At 72 hours after transduction, cells were stained for AP, and two observers, who were unaware of the sample designation, scored the colonies observed in 10 random high-power (viewed at 10 $\times$) fields as being either ESC-like or mixed-differentiated.

Upon knockdown of *Banf1* levels in mESCs by multiple independent shRNA constructs, we observed that cells began to lose their characteristic phenotype and differentiate (fig. 1B). To quantify changes in cell morphology, mESCs, initially seeded at a density of 200

cells per cm², were infected with either Scrambled or Mouse Banf1 shRNA for 24 hours, followed by puromycin selection. At 72 hours after lentiviral infection, cells were stained for AP. Two independent observers, unaware of sample designation, scored the morphology of colonies as either ESC-like or mixed-differentiated (fig. 2C). ESCs infected with the Scrambled shRNA vector exhibited a 3:1 ratio of ESC-like to mixed-differentiated-like colonies, whereas the ratio was reversed when Banf1 was knocked down by the Mouse Banf1 shRNA (fig. 2D). Importantly, there was a significant decrease in the number of ESC-like colonies ($P = 0.017$) and a significant increase in mixed-differentiated-like colonies ($P = 0.002$) upon Banf1 knockdown. Taken together, our findings argue that Banf1 expression is necessary to maintain the self-renewal of mESCs.

Subcellular localization of pluripotency-associated markers and the Banf1-associated protein Emd after knockdown of Banf1

Because protein levels of core pluripotency factors Sox2 and Oct4 were only changing modestly 3 days after Banf1 was knocked down in mESCs (fig. 1D), we examined the subcellular localization of Sox2, Oct4, and Nanog. For this purpose, we conducted immunocytochemistry. Specifically, mESCs were infected with either Scrambled or Mouse Banf1 shRNA lentiviruses. Following infection, the transduced cells were selected for puromycin resistance for 24 hours, subcultured into 12-well plates, and probed for Sox2, Oct4, or Nanog by immunocytochemistry. Sox2, Oct4 and Nanog proteins were detected within the nucleus of ESC-like cells 72 hours following Scrambled shRNA lentiviral transduction. Although there was a reduction in the intensity of fluorescence associated with Sox2 and Oct4 upon Banf1 knockdown, the presence of these factors and Nanog in the nucleus did not appear to be dramatically altered (fig. 3A–C). We also used immunocytochemistry to examine the expression of SSEA1, a cell surface marker used to identify pluripotent mESCs. Although some small colonies of ESC-like cells continued to express SSEA1 upon Banf1 knockdown, cells that exhibited a differentiated phenotype had lost SSEA1 expression (fig. 3D).

Figure 3. Immunocytochemistry of Sox2, Oct4, Nanog, SSEA1, and Emd in mESCs with and without Banf1 knockdown. D3 mESCs were infected, selected with puromycin and seeded into a 12-well plate for immunocytochemistry as described in the Materials and Methods. Photomicrographs are arranged from top to bottom as: bright field, nuclear staining with DAPI, DAPI and FITC merge and FITC stain for (A) Sox2, (B) Oct4, (C) Nanog, (D) SSEA1, and (E) Emd. The scale bar is representative for all photomicrographs.

Previous reports have shown that Banf1 is able to associate with the nuclear envelope protein Emd (Haraguchi et al., 2008; Margalit et al., 2007; Montes de Oca et al., 2009). Additionally, Emd association with Banf1 in HeLa cells has been shown to be crucial for the proper reassembly of the nuclear envelope during telophase of mitosis (Haraguchi et al., 2008). This suggests that disruption of proper progression of mitosis could interfere with the ability of ESCs to exit the cell cycle. To determine whether the nuclear envelope of the cells was substantially disrupted, we examined Emd localization in ESCs following transduction with either Scrambled or Mouse Banf1 shRNA lentiviruses (fig. 3E). Interestingly, Emd continued to be localized to the nucleus. This observation suggests that the nuclear envelope is not dramatically affected in the viable cells following passage. However, the

knockdown of Banf1 might have caused subtle changes in Emd localization, specifically during telophase, not readily visible in our assay (Haraguchi et al., 2008).

Culture of CHB-4 hESCs without a feeder layer

To extend our observation that normal Banf1 expression levels are crucial for maintaining self-renewal of mESCs, we examined whether BANF1 expression is also crucial for the self-renewal of hESCs. Previous reports have described culture conditions suitable for maintaining hESCs and iPS cells without a feeder layer (Ludwig et al., 2006a; Ludwig et al., 2006b). However, no reports have described suitable feeder-free culture conditions for the CHB-4 human embryonic stem cell line. Our laboratory had previously used mTeSR1 serum-free medium supplemented with Y-27632, a Rho-associated coiled-coil kinase (ROCK) inhibitor, in conjunction with Matrigel-coated tissue culture plastic, to culture H9 hESCs and human iPS cells under feeder-free conditions (Claassen et al., 2009). Importantly, we determined these culture conditions support the growth of CHB-4 hESCs in the absence of a feeder-layer. In addition, we used immunocytochemistry to show that CHB-4 hESCs grown under these conditions express SOX2, OCT4, NANOG and SSEA4 (fig. S1A). We also determined that the CHB-4 cells were SSEA1 negative [SSEA1 expression is associated with later stages of human development (Draper et al., 2002)]. Finally, we demonstrated that CHB-4 cells, grown without a feeder-layer at clonal densities, give rise to AP-positive ESC-like colonies at a cloning efficiency of ~1% (fig. S1B). Together, these observations argue that CHB-4 hESCs grown in the absence of a feeder layer are able to retain the properties of pluripotent hESCs capable of self-renewal.

Knockdown of BANF1 in hESCs disrupts cell survival and self-renewal

Human BANF1 is 96% identical and 98% similar to mouse Banf1. Because of high conservation between human and mouse Banf1, and the crucial role of proper expression of Banf1 in maintaining the self-renewal of mESCs, we hypothesized that hESCs must also maintain normal levels of BANF1. To test this hypothesis, RNAi technology was used to knockdown endogenous BANF1 in CHB-4 hESCs. For this purpose, we used a previously validated and characterized shRNA lentiviral construct against human BANF1 (Human BANF1 shRNA) (Wiebe and Traktman, 2007). The Scrambled shRNA lentiviral construct used as a control in our mouse studies was also used in CHB-4 cells. In a manner similar to our work with mESCs, virally transduced hESCs were selected by culturing them for 24 hours in medium supplemented with puromycin. At 72 hours after transduction, nuclear proteins were isolated and used for western blot analysis. Endogenous levels of BANF1 were reduced substantially upon constitutive expression of Human BANF1 shRNA (fig. 4A).

At 4 days after viral transduction, substantial numbers of floating cells were observed in the Human BANF1 shRNA group, suggesting that cell survival was adversely affected, and cells were undergoing increased rates of apoptosis (discussed below). Moreover, cells infected with Human BANF1 shRNA lentiviruses were unable to be subcultured for more than three passages. In regard to this point, examination following subculture at 4000 cells per cm² showed that CHB-4 cells expressing the shRNA construct against BANF1 did not form colonies as readily as the hESCs infected with the Scrambled shRNA construct (fig.

4B). Interestingly, however, the hESCs infected with BANF1 shRNA lentiviral constructs still resembled, morphologically, their Scrambled-shRNA-infected counterparts.

Figure 4. BANF1 knockdown disrupts self-renewal of CHB-4 hESCs. (A) Western blot analysis of BANF1 levels in CHB-4 cells transduced with either Scrambled or Human BANF1 shRNA lentiviral constructs after selection with puromycin. (B) Photomicrographs and AP staining of CHB4 cells 72 hours after subculture of cells previously infected with Scrambled shRNA, Human BANF1 shRNA or Banf1 shRNA constructs #1, #2, or #3. (C) Photograph of transduced CHB-4 ESCs stained with AP. Cells were transduced, selected, and passaged at low density (2000 cells per cm^2) into culture flasks. Approximately 2 weeks after seeding, AP staining was conducted and photographed. (D) Quantification of

AP-positive colonies per low-power (4×) field. Two independent scorers counted AP-positive colonies in 20 random fields in the flasks shown in C. Counts were averaged and graphed (error bars represent s.d.). (E) Western blot analyses of nuclear proteins isolated from CHB-4 hESCs 3 days and 7 days following transduction. Separate blots were run for each protein probed. Protein expression levels, presented in parentheses beneath corresponding bands, were normalized against a corresponding HDAC1 loading control (data not shown).

Our initial observations were corroborated through the use of multiple shRNA constructs against human BANF1 transcripts. For this purpose, we used the shRNA lentiviral constructs described above (#1, #2, and #3), since their sequences should target both mouse and human Banf1. Each of the lentiviral constructs was used to knockdown human BANF1 in CHB-4 hESCs. Knockdown of human BANF1 was verified by western blot analysis (fig. 4A). Banf1 shRNA #1 construct knocked down human BANF1 to the same extent as our previously validated Human BANF1 construct. The Banf1 shRNA #2 construct also knocked down Banf1, although to a lesser extent, whereas Banf1 shRNA #3 construct failed to substantially knockdown human BANF1 (fig. 4A). Representative photomicrographs from 72 hours after subculture demonstrated that hESCs infected with Banf1 shRNA constructs #1 and #2 form fewer and smaller colonies, which were composed of cells whose morphology still resembled cells infected with the Scrambled shRNA construct. Importantly, cells infected with Banf1 shRNA #3 construct, which failed to knockdown human BANF1, formed colonies at a similar rate and size as cells infected with Scrambled shRNA constructs (fig. 4B). To extend these findings, we examined the cloning efficiency of CHB-4 cells after BANF1 knockdown. Specifically, CHB4 cells transduced with either the Scrambled or the Human BANF1 shRNA constructs were seeded at clonal density and allowed to grow for ~2 weeks. Importantly, cells expressing Scrambled shRNA appropriately gave rise to AP-positive colonies when seeded at clonal density, whereas cells expressing BANF1 shRNA did not form AP-positive colonies (fig. 4C,D).

BANF1 knockdown in hESCs does not significantly affect the expression of pluripotency-associated genes or markers of differentiation

Examination of markers of differentiation in mESCs infected with Banf1 shRNA lentiviral constructs demonstrated an increase in markers of differentiation, which corresponded with the changes observed in the cell morphology. To determine whether markers of differentiation increase in hESCs following knockdown of BANF1, RNA was isolated at 72 hours after infection of CHB-4 hESCs with either Scrambled or Human BANF1 shRNA lentiviral constructs. qRT-PCR demonstrated that there were no significant changes in the expression levels of the pluripotent stem cell markers (SOX2, OCT4, and NANOG), ectodermal markers (NESTIN and HES1), mesodermal markers (brachyury, NKX2.5, and FLK1), endodermal markers (FOXA2 and AFP) or trophectoderm markers (CDX2 and ESX1). However, there was a small increase in the expression of two ectoderm markers, PAX6 and SOX21 (~1 cycle increase in Ct value), which could be due to spontaneous differentiation within the cell population (data not shown). Additionally, we performed western blot analyses of the transcription factors OCT4 and SOX2 at two different time points,

3 and 7 days after infection with human BANF1 or Banf1 #1 shRNA viral constructs. This analysis determined that there was little, if any, change in the level of Sox2 three days after infection, and only a modest decrease in Sox2 protein level 7 days after infection (fig. 4E). OCT4 levels were not affected at either time point examined.

To extend this analysis, we examined whether the knockdown of BANF1 influences the cellular localization of SOX2, OCT4, EMD, SSEA4, and SSEA1. No changes were observed in the localization of the core master regulators SOX2 and OCT4 (fig. 5A,B), and no changes were evident in the localization of EMD (fig. 5C). Moreover, hESCs expressing shRNA against BANF1 did not exhibit substantial changes in SSEA4 expression (fig. 5D). Consistent with these findings, we did not observe the expression of SSEA1 (fig. 5E), a marker of neural differentiation of hESCs (Draper et al., 2002). Importantly, the number of viable cells that expanded was dramatically reduced upon knockdown of BANF1, corresponding with our previous observation that BANF1 knockdown in hESCs reduces their capacity to proliferate and self-renew. Taken together, these data argue that, despite continued expression of core transcription factors necessary to maintain the self-renewal of ESCs, knockdown of BANF1 dramatically reduces the cloning efficiency of hESCs.

Figure 5. Immunocytochemistry of SOX2, OCT4, NANOG, EMD, and SSEA1 in hESCs with and without BANF1 knockdown. CHB-4 hESCs were transduced with either Scrambled or human Banf1 shRNA lentiviral vectors, selected with puromycin to eliminate uninfected cells, and seeded into a 12-well plate for immunocytochemistry as described in the Materials and Methods. Photomicrographs are arranged from top to bottom as: bright field, nuclear staining with DAPI, DAPI, and FITC merge, and FITC stain for (A) SOX2, (B) OCT4, (C) EMD, (D) SSEA4, and (E) SSEA1. The scale bar is representative for all photomicrographs.

Banf1 knockdown enriches the proportion of cells in G2 phase and induces apoptosis in both mESCs and hESCs

Previous reports have implicated Banf1 as being necessary for proper cell cycle progression. Specifically, Banf1 is thought to play a role in the reassembly of the nuclear envelope at the end of telophase. Additionally, Banf1 associates with Emd to properly reassemble the nuclear envelope. A previous report demonstrated that knockdown of BANF1 in HeLa cells significantly disrupted the ability of Emd to properly localize with chromatin during telophase (Haraguchi et al., 2008). The immunocytochemistry experiments presented above did not demonstrate a gross change in the subcellular localization of Emd upon Banf1 knockdown in either mouse or human ESCs. However, the techniques used above might not have been sensitive enough to detect the subtle changes described in the report by Haraguchi and co-workers.

To address the possible mechanism mediating the loss of self-renewal following knockdown of Banf1 expression, cell cycle analysis was performed to check for defects in the ability of the cells to progress through the cell cycle. Disruption of Banf1 expression might cause a shift in the cell cycle distribution, which in turn could cause ESCs to lose their capacity for self-renewal and/or promote differentiation. Typically, mESCs undergoing differentiation begin to utilize the G1 checkpoint typical of somatic cells, thereby lengthening G1, causing a subsequent enrichment in the proportion of cells in G1 phase (White and Dalton, 2005). To investigate whether BANF1 knockdown disrupts the cell cycle distribution of ESCs, and whether any cell cycle changes are typical of differentiation of ESCs, we conducted cell cycle analysis in both mESCs and hESCs. Specifically, mESCs were infected with either the Scrambled or the Banf1 shRNA lentivirus and cell cycle analysis was performed 72 hours later (fig. 6A). Cell cycle analysis revealed that Banf1 knockdown resulted in a statistically significant 1.5-fold increase in the proportion of cells in G2–M phase (15.8% for the Scrambled shRNA and 23% for the Banf1 shRNA; $P = 0.042$). Additionally, there was a corresponding decrease (7.5%, $P = 0.0009$) in the proportion of cells in S phase. Interestingly, there was no change in the proportion of cells in G1 phase, which is normally enriched upon ESCs differentiation.

Figure 6. Effects of *Banf1* knockdown on cell cycle distribution, and annexin V expression in mESCs and hESCs. Cell cycle analysis by propidium iodide (PI) staining and flow cytometry is presented. Experiments for both mouse and human cell cycle analyses were conducted in triplicate using three independent cell culture preparations, and the averages of cell cycle compartment distribution are presented in the column graphs (error bars represent s.d.). Floating cells were included in the hESCs cell cycle analysis (B), and in both the mESC and hESC annexin V analyses (fig. S2C). By contrast, floating cells were not included in the mESC cell cycle analysis (A). Consequently, the cell cycle analysis of mESCs (A) does not include the entire apoptotic cell population. *P*-values are calculated using Student's *t*-tests. (A) Analysis of mESCs transduced with Scrambled or Mouse *Banf1* shRNA lentiviral constructs. (B) Analysis of hESCs transduced with Scrambled or Human *Banf1* shRNA lentiviral constructs. (C) Annexin V staining of mESCs and hESCs following knockdown of *Banf1*. Cells were infected with Scrambled or *Banf1* shRNA lentiviral constructs. At 72 hours after infection, cells were harvested for annexin V staining,

as described in the Materials and Methods. Data from two independent cell preparations were averaged (error bars represent s.d.). *P*-values were calculated using Student's *t*-tests.

These findings were then extended to CHB-4 hESCs. As noted above, the cells were transduced with the Scrambled or BANF1 shRNA lentiviruses, selected for puromycin resistance and harvested for cell cycle analysis 72 hours after viral transduction (fig. 6B). Because substantial numbers of floating cells had been observed in previous experiments with the hESCs following BANF1 knockdown, floating cells were collected and included in our cell cycle analyses. Similar to the results with mESCs, BANF1 knockdown in hESCs led to a statistically significant enrichment of the proportion of cells in G2 phase (16.5% in Scrambled, 25% in BANF1 shRNA; $P = 0.035$), and a statistically significant decrease in those in S phase (39.9% in Scrambled, 15.2% in BANF1 shRNA, $P = 0.0008$). Additionally, the proportion of cells in G1 and undergoing apoptosis also increased in hESCs following BANF1 knockdown ($P = 0.069$ and $P = 0.35$, respectively).

Because large numbers of floating cells were observed following Banf1 knockdown in hESCs, annexin V staining was conducted to examine the effects of Banf1 knockdown on cell survival and induction of apoptosis. mESCs and hESCs were infected and prepared as described above. At 72 hours after infection, cells were isolated and prepared for annexin V staining as described in the Materials and Methods. Importantly, floating cells were collected for both mESC and hESC experiments. Knockdown of Banf1 in mESCs increased the annexin-V-positive population >7.5-fold (fig. 6C; fig. S2), arguing that apoptosis increased significantly. A similar trend was also observed upon BANF1 knockdown in hESCs. In hESCs, we observed a more modest (~2.6-fold), but still significant, increase in annexin V staining (fig. 6; fig. S2). Importantly, it is probable that annexin-V-positive cells were underestimated in the population of hESCs in which BANF1 was knocked down because many dead floating cells were lost when the culture medium was changed with fresh medium daily. Taken together, Banf1 knockdown in both mESCs and hESCs disrupts the cell cycle distribution and increases the population of cells undergoing apoptosis.

Discussion

Global proteomic analyses have proven to be powerful tools for identifying proteins that are part of highly interconnected protein-protein interaction landscapes, anchored by master regulatory transcription factors that control essential gene regulatory networks (Liang et al., 2008; Mallanna et al., 2010; Pardo et al., 2010; van den Berg et al., 2010; Wang et al., 2006). Our unbiased screen of Sox2-interacting partner proteins identified a number of proteins already known to play essential roles in regulating the pluripotency and self-renewal of ESCs, such as Sall4 and Lin28 (Darr and Benvenisty, 2009; Lim et al., 2008; Mallanna et al., 2010; Viswanathan and Daley, 2010; Zhang et al., 2006). This approach also identified a number of proteins not previously implicated in the fate of ESCs. The Sox2-associated protein Banf1 has been shown to be essential for the proper development of model organisms, such as worms and flies (Margalit et al., 2007). Until the present study, the role of Banf1 in the fate of mammalian pluripotent stem cells had not been studied.

Through RNAi technology, *Banf1* was knocked down in both mESCs and hESCs. Knockdown of *Banf1* in mESCs leads to a decrease in the expression of pluripotency-associated transcription factors *Oct4*, *Sox2*, and *Nanog*, as well as an increase in the expression of gene markers associated with mesoderm and trophoctoderm development. Moreover, the cloning efficiency of mESCs decreases significantly when *Banf1* is knocked down. Thus, it is evident that *Banf1* is required to maintain the self-renewal and pluripotency of mESCs. Parallel experiments conducted in hESCs demonstrate that hESCs also require *BANF1*. Interestingly, unlike mESCs, the levels of *OCT4* changed little, if at all, and levels of *SOX2* decreased modestly following *BANF1* knockdown. Moreover, gene markers associated with hESCs differentiation did not increase significantly, unlike their mESC counterparts. Thus, it is evident that the responses of mESCs and hESCs to the knockdown of *Banf1* are not the same. Nonetheless, knockdown of *Banf1* in both mESCs and hESCs leads to significant decreases in cell survival. Currently, it is unclear why mouse and human ESCs respond differently to the knockdown of *Banf1*. This might, in part, be due to their stage of development. In this regard, hESCs and mESCs appear to be related to different stages of mammalian embryogenesis (Najm et al., 2011; Tesar et al., 2007). Moreover, these differences might be related to the differences in the signaling pathways active in mESCs and hESCs (Yu and Thomsom, 2008).

Importantly, our studies also demonstrate a significant enrichment in the proportion of cells in the G2–M phase of the cell cycle, a significant decrease in S-phase cells and significant increases in the annexin-V-positive population when *Banf1* is knocked down in both hESCs and mESCs. The cell cycle structure of most somatic cells is regulated by a G1 checkpoint that restricts the G1–S transition until the formation of activated cyclin-dependent kinases (Cdk4 and Cdk6 complexed with cyclins D and E) (Boheler, 2009; White and Dalton, 2005). By contrast, mESCs lack a G1 restriction point due to constitutively active Cdk2–cyclin-E complexes (Boheler, 2009; White and Dalton, 2005). Both ESCs and somatic cells possess a checkpoint between the G2 and M phases of the cell cycle. In the case of ESCs, cyclin B levels oscillate, and when active Cdk1–cyclin-B complexes form, the cells are able to enter M phase. Consequently, it is not surprising that cell cycle defects in ESCs lead to an accumulation of cells in the G2–M phases. Interestingly, an earlier study demonstrated that the knockdown of *B-Myb* in ESCs causes chromosomal instability and increases the proportion of cells in G2–M phases (Tarasov et al., 2008).

The disruption of the self-renewal of ESCs, coupled with the increase in the percentage of cells in the G2 phase that occurs with *Banf1* knockdown, is reminiscent of findings in HeLa cells, as well as in *C. elegans* and *Drosophila* mutants null for the *Banf1* homolog. In HeLa cells, knockdown of *BANF1* causes telophase defects owing to improper localization of Emd and lamin A, proteins that play important roles in nuclear envelope assembly (Haraguchi et al., 2008; Margalit et al., 2007). Moreover, RNAi-mediated knockdown of the *Banf1* homolog in *C. elegans* causes anaphase-bridged chromatin defects, in addition to mislocalization of lamin, Emd, and MAN1 (Margalit et al., 2005). Similar mitotic defects have been observed in *Drosophila* mutants null for the *Banf1* homolog, which lead to lethality at the larva-pupal transition (Furukawa et al., 2003). Given the early requirement for *Banf1* in *C. elegans* and *Drosophila*, we strongly suspect that *Banf1* also plays essential roles during early mammalian development. Thus, it is probable that knockdown of *Banf1*

perturbs the ability of ESCs to transit through mitosis, leading to an increase in apoptosis. Moreover, the requirement for Banf1 during the early stages of fly and worm development suggests that conditional knockouts of Banf1 during mammalian development will be far more informative than the study of simple Banf1-null embryos.

It is conceivable that Banf1 plays more than one essential role in ESCs. Thus far, a diverse set of biological functions has been described for Banf1. Banf1 dimers are believed to bind to the phosphate backbone of DNA in a sequence-independent manner (Margalit et al., 2007). Additionally, Banf1 is believed to be an important component of the nuclear envelope (Margalit et al., 2007), as well as being involved in chromatin structure and maintenance by binding to core and tail regions of histones (Montes de Oca et al., 2005). Furthermore, Banf1 has been reported to act as a transcriptional repressor by associating with the murine conerod homeobox factor (Crx) to repress Crx-dependent promoter activity (Wang et al., 2002). Thus, it is tempting to speculate that Banf1 plays a direct role in the repression of genes that must remain silent in ESCs to avoid triggering differentiation. Alternatively, Banf1 might indirectly influence gene expression within ESCs, by fine-tuning the activity of Sox2, which must be carefully regulated. Perturbation of Banf1 levels in ESCs could alter the formation and composition of Sox2-containing protein complexes, which have been shown to vary considerably in size (Cox and Rizzino, 2010). In this scenario, the knockdown of Banf1 would cause only small changes in the levels and/or activity of Sox2 in ESCs that would become crucial when amplified through the participation of Sox2 in a highly interconnected protein–protein interaction landscape (Mallanna et al., 2010). Given that over a thousand Sox2 target genes have been identified in ESCs, it will be of interest to determine whether any silent Sox2 target genes are activated when Banf1 is knocked down.

In conclusion, global proteomic approaches that have been used recently to identify proteins that associate with master regulators, such as Sox2, offer a powerful approach for significantly enhancing our understanding of self-renewal in ESCs. In this connection, we have demonstrated that the Sox2-associated protein Banf1 contributes to maintaining self-renewal in both mESCs and hESCs. Identification of additional proteins that associate with master regulators of ESC fate will continue to reveal crucial mechanisms and machinery driving the fundamental process of self-renewal. Moreover, future efforts to develop cell-based therapies for a wide range of diseases will no doubt benefit from advances in the basic understanding of the molecular machinery that controls the fate of stem cells.

Materials and Methods

Cell culture

Cultivation of D3 mESCs and HEK-293T cells has been described previously (Kim et al., 2002; Kopp et al., 2008; Ma et al., 1992). CHB-4 hESCs (Lerou et al., 2008) were obtained from George Daley (Children's Hospital, Boston, Massachusetts). CHB-4 cells were cultured in mTeSR1 medium (StemCell Technologies), supplemented with 10 μ M Y-27632 (Calbiochem), on tissue culture plastic coated with Matrigel (BD Biosciences). CHB-4 cells were subcultured by washing once with PBS and treating with Accutase (Chemicon, Temecula, California) for 5 minutes in a 37°C incubator. Accutase was then neutralized

with mTeSR1 medium, and cells were spun at 400 g for 5 minutes. Cells were resuspended in mTeSR1 medium, counted using a hemocytometer and seeded into culture dish or flask. All cells were maintained at 37°C under a humidified atmosphere of 95% air and 5% CO₂. Photomicrographs in figures 1, 2, and 4 are taken using an Olympus IMT-2 microscope and Canon Rebel XTi camera. Microphotographs in figures 3, 5, and supplementary material figure S1 were taken using a Zeiss Axiovert 200M microscope and a Hamamatsu Photonics Camera C4742-95-12ER, using identical exposure settings, with the Slidebook 4.0.2.2 microscope control program. Images were processed in Adobe Photoshop 12.0.2.

Protein preparation and western blotting

Nuclear extracts were prepared using a NE-PER kit (Pierce). The CER-I and NER reagents of the NE-PER kit were supplemented with a cocktail of protease inhibitors, and the protein concentration of the nuclear extracts was determined as described previously (Mallanna et al., 2010). Equal amounts of nuclear proteins were separated using Tris-HEPES-SDS PAGE (4–20% gradient gel). Separated proteins were transferred onto PVDF membrane (Millipore), the membrane was cut into high- and low-molecular-mass portions (for detection of loading control and protein of interest, respectively), blocked with 5% non-fat milk for 1 hour at room temperature and probed with appropriate antibody, as described previously (Mallanna et al., 2010). The dilutions of primary and secondary antibody are made in 3% BSA and Tris-buffered saline with Tween (TBST; 0.1% Tween 20), respectively. The membranes were incubated in primary antibody overnight, at 4°C, and secondary antibody for 30 minutes, at room temperature. Following incubation with primary and secondary antibodies, membranes were washed three times for 5 minutes each time with TBST (0.1% Tween 20), and developed by incubating in ECF substrate (GE Healthcare) for 5 minutes at room temperature. The fluorescent signal was scanned using a Typhoon 9410 Variable Mode Imager (GE Healthcare). The polyclonal antibody against Banf1 was raised in rabbits against a KLH-conjugated peptide consisting of residues 4–20 of human BANF1 (Bethyl Laboratories, Montgomery, Texas). Other primary antibodies used are against: OCT4 (1:500, sc-8628, Santa Cruz Biotechnology), SOX2 (1:1000, ab-75179, Abcam, Cambridge, Massachusetts), NANOG (1:500, AF2729, R&D Systems, Minneapolis, Minnesota), HDAC1 (1:5000, ab-7028, Abcam), HDAC2 (1:5000, ab-7029, Abcam). Secondary antibodies were the anti-rabbit-IgG-AP antibody conjugate (A3687, Sigma-Aldrich) and the anti-goat-IgGAP antibody conjugate (A4187, Sigma-Aldrich), and were used at a 1:10,000 dilution.

Lentivirus production and Banf1 knockdown in ESCs

Plasmids necessary for third-generation lentiviral production, pRSV-Rev, pMD2.G and pMDLg/pRRE, were obtained from the Addgene plasmid repository (Dull et al., 1998). Transfer vector for Scrambled shRNA sequence and shRNA sequence for knockdown of human BANF1 were cloned into the lentiviral vector pLL3.7/Puro as described previously (Robinson et al., 2003; Wiebe and Traktman, 2007). The Mouse Banf1 shRNA lentiviral transfer vector was produced by annealing the primers shBAFUP (5'-TGGCTTATGTGGT CCTTGGCTTCAAGAGAGCCAAGGACCACATAAGCCTTTTTGGAAAC-3') and shBAF-DN (5'-TCGAGTTTCCAAAAGGCTTATGTGGTCCTTGGCTCTCTTGAAGCCAAGGA

CCACATAAGCCA-3'), and cloning them into the *HpaI* and *XhoI* sites of pLL3.7/Puro (Rubinson et al., 2003). Banf1 shRNA lentiviral constructs #1, #2, and #3 were obtained from Open Biosystems as a set (RHS4533-NM_001038231, Open Biosystems, Huntsville, Alabama). Banf1 shRNA #1 is processed into the mature sense sequence 5'-CAGTTTCTGGT GCTAAAGAAA-3', #2 into 5'-GCCAGTTTCTGGTGCTAAAGA-3' and #3 into 5'-GCTA AAGAAAGATGAAGACCT-3'. VSV-G pseudotyped, replication-incompetent third-generation lentiviral particles were produced in HEK-293T cells. To package lentiviral particles, 293T cells were transiently transfected with the above plasmids using the calcium phosphate precipitation method described previously (Ma et al., 1992). After 24 hours, the cells were refed with DMEM + 10% FBS, 10 mM HEPES (Sigma-Aldrich), and 10 mM sodium butyrate (Sigma-Aldrich). The next day, the medium was collected, filtered (0.45 μ m), and stored at 4°C. Cells were then refed, and the following day medium was again collected and filtered. The filtered medium was combined, and viral particles were collected with an SW-28 rotor at 26,000 r.p.m. for 2 hours at 4°C. Lentiviruses were resuspended in DMEM + 10 mM HEPES, and stored at -80°C. Lentiviruses for expression of Scrambled shRNA or Banf1 shRNA was diluted in D3 mESCs or mTeSR1 medium containing 10 mM HEPES and 6 μ g/ml polybrene, and the medium was added to ESCs. After 24 hours, infected cells were selected for puromycin resistance by re-feeding the cells with fresh ESC medium containing 5 μ g/ml puromycin for 24 hours.

Determining cloning efficiency and quantification of ESC-like and mixed-differentiated colonies

To determine cloning efficiency, D3 ESCs were seeded at a density of 100,000 cells per well in a sixwell plat, infected with lentivirus for expression of either Scrambled or Banf1 shRNA and selected for puromycin resistance as described above. At 72 hours after infection, cells were trypsinized and re-seeded into T-25 flasks at 80 or 320 cells per cm². At 96 hours after re-seeding, the number of ESC-like colonies the in Scrambled and Banf1 shRNA-treated cells were scored in 20 random 4 $\times$ fields by two independent scorers who were unaware of the sample designation. For quantification of ESC-like and mixed-differentiated colonies, D3 mESCs were seeded at a density of 200 cells per cm² in a sixwell plate and infected with lentiviruses for expression of either Scrambled or Banf1 shRNA, as described above. At 72 hours after infection, the Scrambled and Mouse Banf1 shRNA-treated cells were stained for AP, and colonies in 10 random 10 $\times$ fields were scored for either ESC-like or mixed-differentiated morphology by two independent observers who were unaware of the sample designation. Statistical analysis used unpaired Student's *t*-tests.

For cloning efficiency of hESCs, CHB-4 hESCs were transduced with lentiviral particles as described above. At 72 hours after transduction, cells were passaged using Accutase and seeded at a density of 2000 cells per cm². Cells were re-fed daily, and after ~2 weeks, AP staining was conducted as described above. AP-positive colonies in 20 random 4 $\times$ fields were counted by two independent scorers, totaled and averaged.

RNA isolation and quantification

D3 or CHB-4 ESCs were infected with lentiviruses that expressed either Scrambled or Banf1 shRNA, as described above. At 72 hours after transduction, RNA was isolated from

cells. RNA isolation and cDNA was synthesized using AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene) as described previously (Kopp et al., 2008). RNA was isolated from three independent cell culture preparations. Levels of transcripts for specific genes were determined by SYBR Green qRT-PCR, using gene specific primers for mouse transcripts encoding Sox2, Oct4, Nanog, FGF4, Sox21, Nestin, Pax6, Vimentin, MyoD1, Nkx2.5, Tpm-1, Sm22a, Gata6, Sox17, Cdx2, Esx1, Cdh3, and GAPDH as described previously (Boer et al., 2007; Kopp et al., 2008; Mallanna et al., 2010). Sequences of primers for remaining human and mouse genes are provided in supplementary material table S1.

Cell cycle analysis and annexin V staining

D3 or CHB-4 ESCs were seeded at a density of 10^6 cells per 100 mm plate. After 24 hours, cells were infected with Scrambled or the Mouse or Human Banf1 shRNA lentiviral constructs, followed by selection for puromycin resistance, as described in the above section. At 72 hours after infection, cells were prepared for either cell cycle analysis by the Telford Method, as described previously (Telford et al., 1991), or annexin V staining with a TACS Annexin V-FITC Kit (4830-01-K, Trevigen, Gaithersburg, Maryland), according to the manufacturer's protocol. Floating cells were included in the hESC cell cycle analysis, and both mESC and hESC annexin V analyses, whereas floating cells were not included in the mESC cell cycle analysis. Flow analyses were performed by the UNMC Cell Analysis core facility. Experiments for both mouse and human cell cycle analyses were conducted in triplicate using three independent cell culture preparations. Experiments for annexin V staining were conducted in duplicate using two independent cell culture preparations. Statistical analyses were conducted using an unpaired Student's *t*-test in Microsoft Excel for Mac 14.0.2.

Immunocytochemistry

D3 or CHB-4 ESCs, infected with Scrambled or Banf1 shRNA lentiviruses as described above, were seeded into a 12-well plate, previously coated with 0.1% gelatin or Matrigel, respectively. At 24 hours after seeding, wells were washed three times in 1 ml PBS. Cells were then fixed with 4% formaldehyde (Sigma-Aldrich) in PBS for 20 minutes at room temperature. For SSEA1 and SSEA4 staining, cells were washed three times in PBS, before blocking. For Sox2, Oct4, Nanog, and Emd staining, cells were washed twice in PBS, permeabilized in 0.1% Triton X-100 in PBS for 10 minutes at room temperature, and washed twice in PBS, before blocking. Cells to be stained for Sox2, Oct4, or Nanog were blocked using 5% rabbit serum (Sigma-Aldrich) in PBS for 1 hour at room temperature. Cells to be stained for Emd, SSEA1, or SSEA4 were blocked using 5% goat serum (Sigma-Aldrich) in PBS for 1 hour at room temperature. Following blocking, cells were washed 1× in PBS. Cells were incubated in primary antibody overnight, at 4°C on a rocking platform. Cells were washed three times in PBS. Appropriate secondary antibody was then added and allowed to incubate for 1 hour in the dark at room temperature. Cells were then washed twice in PBS, stained with DAPI and washed two additional times in PBS. Cells are photographed as described above. Primary antibodies used were against: SSEA1 (1:10 in 1% FBS in PBS, MC-480, Developmental Studies Hybridoma Bank, University of Iowa), SSEA4 (1:5, MC-813-70, Developmental Studies Hybridoma Bank), SOX2 (1:50, sc-17320, Santa Cruz

Biotechnology), OCT4 (1:50, sc-8628, Santa Cruz Biotechnology), NANOG (1:50, AF2729, R&D Systems), Emerin (1:50, sc-15378, Santa Cruz Biotechnology). Secondary antibodies were against mouse-IgM-FITC (F9252, Sigma-Aldrich), mouse-IgG-FITC (F0257, Sigma-Aldrich), goat-IgG-Cy3 (C2821, Sigma-Aldrich), goat-IgG-FITC (F2016, Sigma-Aldrich), rabbit-IgG-FITC (F0382, Sigma-Aldrich). Nuclei were visualized using DAPI stain (D9542, Sigma-Aldrich) at 1:10,000 in PBS.

Alkaline phosphatase staining

AP staining was conducted using an AP staining kit, according to the manufacturer's protocol (00-0009, Stemgent, San Diego, California).

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Supporting Information

Table S1. Primer design for amplifying gene markers of human and mouse cellular lineage by qRT-PCR

RNA Accession	Symbol	Upstream Primer (U)	Downstream Primer (L)	Amplicon Size	Cellular Lineage	Species
NM_021784	FOXA2	CAGCTACACGCACGCAAAGCC	GCTGGTTCTGCCGGTAGAAGG	143	Endoderm	Human
NM_001134	AFP	CAGCCACTTGTGTCCAACCTCA	ACACCAGGGTTTACTGGAGTC	118	Endoderm	Human
NM_003181	T	CGTGACCAAGAACGGCAGGAG	GACGGGAGCCTTCATCCAGTG	235	Mesoderm	Human
NM_004387	NKX2.5	GTGGAGCTGGAGAAGACAGAG	GTTCTGGAACCAGATCTTGAC	207	Mesoderm	Human
NM_002253	KDR/FLK1	AAGGAGAAGCAGAGCCATGTG	TGCCAATACCAGTGGATGTGA	167	Mesoderm	Human
X65964	NES	CAGAGCCAGATCGCTCAGGTC	GGGAATTGCAGTCCAGCTTG	185	Ectoderm	Human
NM_000280	PAX6	GGGCAGGTATTACGAGACTGG	CTCTGTTTATTGATGACACGC	206	Ectoderm	Human
NM_005524	HES1	CGGCATTCCAAGCTGGAGAAG	CCCTCGCACGTGGACAGGAAG	179	Ectoderm	Human
NM_007084	SOX21	CCTACTCGCTGCTCGACCTGG	GTTGCACGGGATCATGTAGCC	212	Ectoderm	Human
NM_024865	NANOG	GGATCTGCTTATTCAGGACAG	TAGCTGAGGTTTCAGGATGTTG	234	ES Cells	Human
NM_003106	SOX2 (CDS)	ATGCACAACCTCGGAGATCAGC	CCTTCTTCATGAGCGTCTTGG	172	ES Cells	Human
NM_002701	OCT4	CTGCACTGTACTCCTCGGTC	GAACCTAATCCCAAAACCC	186	ES Cells	Human
NM_001265	CDX2	ACCACAGCCTCCCGAGCCTC	CAGCCTGGAATTGCTCTGCC	189	Trophectoderm	Human
NM_002046	GAPDH	ACAGCGACACCACTCCTCC	GAGGTCCACCACCTGTTGC	122	Trophectoderm	Human

Figure S1. Characterization of CHB-4 hESC grown without a growth-inactivated feeder-layer. (A) Immunocytochemistry of CHB-4 hESC grown in the absence of a feeder-layer. Photomicrographs are arranged from top to bottom as bright field, nuclear staining via DAPI, DAPI/protein of interest merge, and protein of interest. Cy3 (red) staining represents Sox2, while all other markers are stained with FITC (green). (B) Cloning efficiency of CHB-4 cells grown without feeder-layer. CHB-4 cells were seeded at decreasing densities in each well of a 6-well plate: 10, 5, 2, 0.5×10^3 cells per well. Cells were allowed to grow for 48 hours, and AP staining was conducted. Cloning efficiency is approximately 1%.

Figure S2. Annexin V-FITC/PI staining of mESC and hESC following knockdown of Banf1. (A) mESC and (B) hESC were infected with Scrambled or Banf1 shRNA lentiviral constructs. Cells were harvested 72 hours after infection for annexin V/PI staining and flow cytometry analysis, as described in the Materials and Methods. Distribution of cells is provided as a percentage in each quadrant. The annexin V expression values presented in figure 6C are derived from the sum of the upper-right and the lower-right quadrants.