Braveheart, a Long Noncoding RNA Required for Cardiovascular Lineage Commitment

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SUMMARY

Long noncoding RNAs (lncRNAs) are often expressed in a development-specific manner, yet little is known about their roles in lineage commitment. Here, we identified Braveheart (Bvht), a heart-associated lncRNA in mouse. Using multiple embryonic stem cell (ESC) differentiation strategies, we show that Bvht is required for progression of nascent mesoderm toward a cardiac fate. We find that Bvht is necessary for activation of a core cardiovascular gene network and functions upstream of mesoderm posterior 1 (MesP1), a master regulator of a common multipotent cardiovascular progenitor. We also show that Bvht interacts with SUZ12, a component of polycomb-repressive complex 2 (PRC2), during cardiomyocyte differentiation, suggesting that Bvht mediates epigenetic regulation of cardiac commitment. Finally, we demonstrate a role for Bvht in maintaining cardiac fate in neonatal cardiomyocytes. Together, our work provides evidence for a long noncoding RNA with critical roles in the establishment of the cardiovascular lineage during mammalian development.

INTRODUCTION

The heart is the first organ to function during vertebrate embryogenesis. In mammals, cardiogenesis involves the diversification of pluripotent cells in the embryo toward mesodermal and cardiac cell types, including cardiomyocytes (Kattman et al., 2007). Coordination of this process depends on precise control of gene expression patterns, and disruption of transcriptional networks that govern cardiac commitment underlies congenital heart disease (CHD) (Bruneau, 2008; Srivastava, 2006). Notably, cardiovascular disease and aging lead to a progressive loss of cardiomyocytes, yet the adult mammalian heart has limited regenerative capacity (Mercola et al., 2011; Steinhauer and Lee, 2011). The derivation of cardiomyocytes from pluripotent stem cells in vitro is a potentially promising strategy for regenerative therapy, but the inability to generate sufficient quantities of high-quality cardiac cells has been a limitation to realizing this potential. Thus, knowledge of the molecular switches that control cardiac commitment is critical to achieve a better understanding of heart development and to design better approaches for treatment of cardiac-related diseases.

Development of the cardiovascular system, including the heart, is a multistep process that is coordinated by a network of transcription factors (Murry and Keller, 2008; Olson, 2006). For example, the transcription factor mesoderm posterior 1 (MESP1) is critical for the establishment of a multipotent cardiovascular progenitor population during gastrulation (Bondue et al., 2008; Lindsay et al., 2008). MESP1 is transiently expressed in the nascent mesoderm, and its expression marks those cells destined to give rise to the cardiovascular lineage (Saga et al., 1996, 1999, 2000). Consistent with this idea, forced expression of Mesp1 during embryonic stem cell (ESC) differentiation leads to an increase in the cardiogenic population (Bondue et al., 2008). MESP1 regulates a core network of transcription factors, including many regulators of heart development as well as genes with roles in epithelial-to-mesenchymal transition (EMT) such as Snai and Twist (Bondue et al., 2008; Lindsay et al., 2008). EMT is critical for gastrulation and morphogenetic movements during organogenesis, including heart formation (Lim and Thiery, 2012; von Gise and Pu, 2012). Thus,
identifying additional factors that promote a mesoderm-to-cardiovascular transition may provide mechanistic insights into the regulation of heart development.

Long noncoding RNAs (lncRNAs) are broadly classified as transcripts longer than 200 nucleotides that are 5’ capped and polyadenylated like most mRNAs, yet this class of transcripts has limited coding potential. lncRNAs function in a wide range of processes and can regulate gene expression by diverse mechanisms (Hu et al., 2012; Mercer et al., 2009; Ponting et al., 2009; Rinn and Chang, 2012). Though thousands of lncRNAs have been identified across eukaryotes, many are species specific and appear less conserved than protein-coding genes (Cabili et al., 2011; Derrien et al., 2012; Ulltsey et al., 2011). Importantly, lncRNAs are differentially expressed across tissues, suggesting that they regulate lineage commitment. Consistent with this idea, loss of function of two lncRNAs in zebrafish embryos, cyrano and megamind, resulted in various developmental defects (Ulltsey et al., 2011). Moreover, HOTTIP plays a role in limb formation (Wang et al., 2011), whereas other lncRNAs function to promote or suppress somatic differentiation (Hu et al., 2011; Kretz et al., 2012a, 2012b). Furthermore, depletion of a subset of lncRNAs in mouse ESCs led to upregulation of global lineage programs (Guttmann et al., 2011). Despite these promising findings, our knowledge of lncRNAs that function in lineage commitment is limited to only a few examples, and a detailed understanding of the genetic pathways that they regulate is lacking.

Here, we report the identification of AK143260, which we named Braveheart (Bvht), a lncRNA in mouse that is necessary for cardiovascular lineage commitment. Using multiple ESC differentiation strategies, we found that Bvht was necessary for activation of a core gene regulatory network that included key cardiac transcription factors (e.g., MesP1, Gata4, Hand1, Hand2, Nkx2.5, and Tbx5) and EMT genes (e.g., Snai and Twist). Further analysis revealed a significant overlap between the genes regulated by Bvht and MESP1, a master regulator of cardiovascular potential. Moreover, forced expression of MesP1 rescued the Bvht depletion phenotype, indicating that these two factors function in a similar genetic pathway. We show that Bvht interacts with SUZ12, a core component of polycomb-repressive complex 2 (PRC2), suggesting that this interaction may be critical for epigenetic regulation of network genes. We also demonstrate that Bvht is necessary for maintenance of cardiac fate in ex vivo neonatal cardiomyocytes. Together, these results indicate that Bvht functions to regulate gene expression programs that promote commitment toward the cardiovascular lineage. More broadly, our work identifies a potential additional pathway for regulation of heart development and suggests that lncRNAs represent targets that can be exploited for efficient generation of stem-cell-derived cardiac cells.

RESULTS

Identification of Candidate lncRNAs in the Cardiac Lineage

lncRNAs exhibit distinct expression patterns across tissues (Derrien et al., 2012; Dinger et al., 2008); however, we have limited knowledge of their precise molecular roles during mammalian development. We reasoned that lncRNAs whose expression becomes restricted to specific cell types during ESC differentiation represent candidate regulators of lineage commitment. We analyzed IncRNA expression as measured by RNA-seq in mouse ESCs as well as in differentiated tissues representing the three germ layers (Mortazavi et al., 2008). We identified 47 candidates that displayed high expression in ESCs and lower median expression across the tissues examined (Figure 1A and Table S1 available online). To identify lncRNAs with potential roles in cardiac commitment, we next evaluated the expression of individual candidates in each tissue, including the heart (Figure 1B).

We focused on AK143260 located on mouse chromosome 18:61,799,307–61,807,126 (+ strand, mm9) because it displayed higher expression in the heart relative to the other tissues examined in this study. Thus, we named the candidate Braveheart (Bvht).

lncRNAs are a newly emerging class of RNA, and their status as noncoding transcripts from genome annotations requires independent validation. We determined by rapid amplification of cDNA ends (RACE) that Bvht is an ~590 nucleotide transcript comprising three exons, consistent with our RNA-seq and northern analysis in ESCs (Figures 1C and 1D). We also detected an isoform that lacked the first exon (~50 bases), which represented only a minor fraction of the total transcript as determined by RACE. Cell fractionation followed by quantitative RT-PCR (qRT-PCR) showed that ~33% of the spliced Bvht transcript resides in the nucleus (Figure S1A). Notably, HOTAIR, an lncRNA with nuclear function, is also distributed across both cell compartments in human fibroblasts (Khalil et al., 2009). Though Bvht harbors short open reading frames (ORFs) (the two largest are 48 and 74 aa), ORFs of this size would be expected by chance in a transcript of this length (Figure S1B). Consistent with the recent finding that lncRNAs are rarely translated in human cells (Bánfai et al., 2012), we found no evidence of active translation or production of a protein product from Bvht (Figures S1C–S1F and Supplemmentary Information). Moreover, sequence homology searches revealed no clear orthologous Bvht sequence or transcripts in syntenic regions of the human or rat genomes, consistent with the idea that lncRNAs are less conserved than protein-coding genes (Cabili et al., 2011; Derrien et al., 2012). For example, analysis of RNA-seq data from human and rat heart, a tissue in mouse that displays strong expression of both Bvht and the conserved neighboring miR143/145 cluster, revealed robust expression of the pri-miRNA in human and rat, whereas transcripts from the Bvht region were not detected (Figure 1E). Thus, Bvht appears to be a mouse-specific IncRNA and is an example of a recently evolved rapid gain-of-function noncoding transcript.

Braveheart Is Necessary for Proper ESC Differentiation

To dissect the function of Bvht in lineage commitment, we generated mouse embryonic stem cell (mESC) lines that stably expressed short hairpin RNAs (shRNA) directed against either exon 2 or 3. We observed significant depletion (~80%) of the transcript in ESCs as measured by qRT-PCR and northern analysis (Figures 2A and 1D). Notably, Bvht depletion in ESCs did not affect colony morphology, expression of pluripotency markers, the fraction of apoptotic cells, or cell-cycle kinetics compared to control cells harboring a nontargeting hairpin (Figures 2B
Figure 1. Identification AK143260 as a Candidate Heart-Associated IncRNA

(A) Differential expression enrichment score calculated from RNA-seq data from biological replicates of two independent mESC lines and adult tissues (brain, liver, and skeletal muscle) representing the three germ layers. Stars represent the position of three example candidates and their respective GenBank accession numbers. The y axis represents the number of lncRNAs with a particular differential expression enrichment, as denoted by each bar.

(B) Transcriptome analysis in a broader range of tissues showed that AK143260 was predominantly expressed in ESCs and in heart.

(C) Depiction of transcript structure as defined by 5’ and 3’ RACE (bottom) was consistent with RNA sequencing reads in ESCs (top).

(D) Northern analysis using a full-length probe for AK143260 confirms the predicted size of the transcript (~590 nucleotides) and shows reduced levels upon shRNA-mediated depletion in mESCs.

(E) RNA sequencing reads from rat adult heart and human adult heart samples showed no evidence of a transcript expressed in a location syntenic to AK143260. Arrows mark direction of transcription.

See also Figure S1 and Table S1.
and S2A–S2C). Thus, Bvht is not required to maintain ESC self-renewal.

ESCs have the capacity to differentiate into derivatives of the three germ layers, including cardiac cell types. We next induced Bvht-depleted ESCs to differentiate by allowing cells to aggregate into embryoid bodies (EBs). Given its expression in the heart, we investigated whether Bvht-depleted cells could form cardiac tissue, which can be visualized as spontaneously contracting EBs.

**Figure 2. Bvht Is Required for Proper ESC Differentiation**

(A) Targeting of AK143260 (Bvht) in mESCs by two independent hairpins directed to either exon 2 or 3 led to significant depletion of the transcript as measured by qRT-PCR. Experiments were performed in triplicate, and error bars represent SD within one representative experiment.

(B) Bvht depletion does not affect colony morphology and OCT4 staining in ESCs. Scale bar, 100 μm.

(C) Percentage of spontaneously contracting EBs determined at days 8 and 11 of differentiation (n > 100 per time point). Bvht-depleted EBs showed a significant decrease in contracting EBs compared to controls. The number of resulting EBs was similar in each experiment. Experiments were performed in triplicate, and error bars represent SD.

(D) Expression of cardiac Troponin T (cTnT) measured by qRT-PCR at representative time points shows decreased levels in Bvht-depleted EBs. Error bars represent SD of a triplicate set of experiments.

(E) Antibody staining of paraffin-embedded sections of day 12 EBs showed decreased cTNT abundance in Bvht-depleted compared to controls. Scale bar, 300 μm.

(F) Bvht-depleted EBs can form tissues representative of all three germ layers, including gut-like epithelium (endoderm), cartilage (mesoderm), and neuroepithelial rosettes (ectoderm) as represented in hematoxylin and eosin (H&E)-stained EB sections. Scale bar, 100 μm. **p < 0.01 by two-tailed Student’s t test.

See also Figure S2 and Table S2.
contracting EBs during differentiation. In control cells, >25% of EBs beat at day 11 of differentiation, compared to ~5% of the Bvht-depleted EBs (Figures 2C, S2D, and S2E). Consistent with this observation, cardiac troponin T (cTnT), an integral component of the contraction machinery in cardiac muscle, was expressed at significantly lower levels in Bvht-depleted EBs (Figures 2D and 2E). In contrast, cells depleted of AK085506, an ESC-specific lncRNA not expressed in the heart, displayed a similar proportion of contracting EBs (Figures 1B and S2F). Examination of EB sections at day 12 of differentiation showed a range of tissues, such as neural rosettes, gut endoderm, and cartilage (Figure 2F). Together, these data suggest that Bvht is not required for global ESC differentiation and may have specific functions in cardiac commitment.

Braveheart Regulates the Core Cardiovascular Gene Network
InceRNAs can act in cis to regulate expression of neighboring genes or in trans by diverse mechanisms (Ørom and Shiekhattar, 2011; Rinn and Chang, 2012). We first analyzed the expression of neighboring genes within a 100 kb window, which included the convergent and overlapping miR143/145 cluster on the opposite strand (exons do not overlap) (Figure S3A). Expression of neighboring protein-coding genes was unaffected in Bvht-depleted cells during ESC differentiation compared to control cells; however, the pri-miRNA was not properly expressed during the time course (Figure S3B). It is likely that lack of expression of the pri-miRNA is due to depletion of cardiac cells in the Bvht-depleted EBs rather than direct regulation by Bvht. For example, miR143 and miR145 are typically transcribed in cardiovascular cell types (Cordes et al., 2009; Xin et al., 2009). Moreover, miR143/145 knockout ESCs formed contracting, cTNT-positive EBs and showed clear differences in gene expression patterns compared to Bvht-depleted cells (Figures S3C–S3E and S4 and Table S2). Thus, these data indicate that the Bvht phenotype is likely not a consequence of failure to regulate closely neighboring genes in cis, including the miR143/145 cluster.

We next reasoned that analysis of global gene expression patterns during EB differentiation might reveal further clues toward elucidating Bvht function. To this end, we analyzed gene expression patterns at various time points by RNA-seq. We found ~548 differentially expressed genes in the Bvht-depleted cells compared to controls (Figure 3A and Table S3). These genes were clustered by expression pattern, yielding several distinct clusters. Strikingly, genes in clusters that
displayed lower expression at later stages (i.e., clusters G–I) showed enrichment for genes that function in heart and blood vessel morphogenesis, heart and muscle system process, and myofibril assembly (Figure 3B). In contrast, genes in clusters that displayed higher expression in \textit{Bvht}-depleted cells (i.e., clusters A–F) were not enriched for specific gene ontology (GO) terms.

To gain further insights, we created a network diagram to illustrate how genes in the enriched GO categories changed in expression over time by computing a Pearson correlation coefficient of expression between all pairs of genes (Figure 3C). Our analysis revealed a complex network of transcription factors that failed to activate during differentiation, including key regulators of heart development, such as \textit{MesP1}, \textit{Hand1}, \textit{Hand2}, \textit{Nix2.5}, and \textit{Tbx20} as well as genes with roles in cardiomyocyte structure and function, which have been implicated in cardiovascular diseases (Bruneau et al., 2008; McCulley and Black, 2012). Though a caveat of the EB assay is that it is difficult to distinguish subtle effects among related lineages, we observed that, whereas expression of genes representing cardiovascular cell types, including cardiomyocytes, vascular endothelium, and smooth muscle, were significantly reduced, markers of paraxial mesoderm, hematopoiesis, and endoderm were expressed at normal or slightly elevated levels in \textit{Bvht}-depleted EBs (Figure SSA). Moreover, \textit{Bvht}-depleted cells displayed similar morphology upon neuronal differentiation by addition of retinoic acid and expressed similar levels of neural markers such as \textit{Sox1} and \textit{Nestin} compared to controls (Figure SSB and data not shown). Together, these data suggest that \textit{Bvht} specifically promotes activation of a core gene regulatory network in trans to direct cardiovascular cell fate.

\textbf{Braveheart Functions Upstream of MesP1}

\textit{MesP1} was among the earliest transcription factors in the gene network that showed significantly lower expression in \textit{Bvht}-depleted EBs. \textit{MesP1} marks a common multipotent cardiovascular progenitor that ultimately specifies all cell types of the heart (Bondue et al., 2008; 2011; David et al., 2008; Lindsley et al., 2008). To test whether \textit{Bvht} and \textit{MesP1} function in a similar pathway, we compared the differentially expressed genes in our EB time course with those changed upon \textit{MesP1} induction (Bondue et al., 2008). We observed a striking correlation among genes that showed lower expression in \textit{Bvht}-depleted cells and higher expression upon \textit{MesP1} induction, denoted as group II ($p < 6 \times 10^{-6}$). Genes that showed higher expression in \textit{Bvht}-depleted cells and reduced expression upon \textit{MesP1} induction also overlapped significantly and were denoted as group III ($p < 9 \times 10^{-44}$) (Figures 4A and 4B). Notably, group II genes function in transcriptional regulation, heart development, and tissue morphogenesis and included many of the transcription factors represented in the \textit{Bvht} network (Figure 4C and Table S4). These results suggest that \textit{Bvht} depletion leads to failure to activate a MESP1-driven gene expression program.

We expected that, if \textit{Bvht} functions upstream of \textit{MesP1}, then forced expression of \textit{MesP1} should rescue the depletion phenotype. We employed a doxycycline-inducible \textit{MesP1} mESC line (Bondue et al., 2008) and induced expression at day 2 of EB differentiation in \textit{Bvht}-depleted and control cells. \textit{MesP1} cardiovascular progenitors comprise a very small proportion of cell types in early stage EBs, and induction at this time point leads to expansion of the cardiogenic population (Bondue et al., 2008; Lindsley et al., 2008). Notably, \textit{MesP1} induction led to an increase in contracting EBs in both control and \textit{Bvht}-depleted cells (Figure 4D). We also found that \textit{Bvht} levels were ~10-fold higher in MESP1 population compared to negative cells using a mESC line that harbored GFP under the control of the \textit{MesP1} promoter (Bondue et al., 2011) (Figure 4E). Together, these data suggest that \textit{Bvht} functions upstream of \textit{MesP1} as a potential regulator of cardiovascular fate.

\textbf{Braveheart Promotes Cardiac Cell Fate from Nascent Mesoderm}

To further dissect \textit{Bvht} function in the cardiovascular lineage, we employed a directed in vitro cardiomyocyte (CM) differentiation assay that permits isolation of cell populations at well-defined stages (ESCs, precardiac mesoderm [MES], cardiac progenitors [CPs], and CMs) (Kattman et al., 2011; Wamstad et al., 2012) (Figure 5A). Using this assay, we observed that \textit{Bvht}-depleted EBs were smaller and displayed an irregular shape at day 4 (MES) and failed to form an adherent monolayer at day 5.3 (CP) compared to controls (Figure 5B, middle and right panels). In contrast, no visible change in morphology was apparent at day 2, a time point that precedes the addition of cardiac growth factors (Figure 5B, left). We also found an increase in apoptosis but no change in cell-cycle kinetics at day 4 in \textit{Bvht}-depleted cells, whereas day 2 cells showed no difference (Figures 5C and 5D). Notably, shRNA-mediated depletion of \textit{MesP1} showed similar effects to \textit{Bvht} depletion, and \textit{Bvht} overexpression led to an increase in the levels of cardiac genes, including \textit{MesP1}, during CM differentiation (Figures S6B and S6C and data not shown).

\textit{Brachyury} (\textit{T}) and \textit{Eomesodermin (Eomes)}, transcription factors expressed in the primitive streak, have critical roles in mesoderm induction and regulate \textit{MesP1} expression by directly binding to its promoter (Costello et al., 2011; David et al., 2011). Though \textit{T} and \textit{Eomes} were expressed normally or at elevated levels at day 4 in \textit{Bvht}-depleted and controls cells, both factors displayed higher levels in day 5.3 \textit{Bvht}-depleted cells (Figure 5D). Notably, expression of core cardiac transcription factors, including \textit{MesP1}, failed to induce at day 4 and day 5.3 in \textit{Bvht}-depleted cells despite expression of \textit{T} and \textit{Eomes} (Figures 5E and 5F). Similarly, expression of cell surface markers of early cardiac identity (i.e., \textit{PdgfrA} and \textit{Flk-1}) was lower in \textit{Bvht}-depleted cells (Figure 5G). Together, \textit{PdgfrA} and \textit{Flk-1} define an early multipotent cardiovascular progenitor population (marked by \textit{MesP1}), and their coexpression enhances cardiogenic potential (Kattman et al., 2006; Yang et al., 2008). Conversely, \textit{Fgf8} and \textit{Mix1} levels were elevated in \textit{Bvht}-depleted cells during CM differentiation, consistent with a reciprocal increase in hematopoietic markers upon loss of cardiac potential (Lim et al., 2009; Simões et al., 2011) (Figure S6D).

\textit{MesP1} also regulates EMT genes whose expression is critical for migration of precardiac cells during gastrulation (Lindsley et al., 2008). We found that expression of EMT genes such as \textit{Snai1} and \textit{Snai2}, as well as \textit{Twist1} and \textit{Twist2} (Yang et al., 2004), was not induced at day 5.3 in \textit{Bvht}-depleted cells.
compared to controls (Figure 5H). Snai1 and Snai2 repress E-cadherin, and the switch from E-cadherin to N-cadherin expression is a hallmark of EMT (Lim and Thiery, 2012). Notably, E-cadherin levels remained high, and N-cadherin was not expressed in Bvht-depleted cells (Figure 5H). Together, these results indicate that Bvht is required at a critical time during differentiation when cardiac cells are specified from nascent mesoderm.

Our observations suggest that Bvht functions in the same pathway as MesP1 in a cell-autonomous manner to regulate a core cardiac gene network. To test this idea, we generated ESC lines that harbored either the shBvht or shControl vector additionally marked with GFP or mCherry to allow us to track each population. Equal numbers of shControl-mCherry and shBvht-GFP ESCs were combined and analyzed by fluorescence-activated cell sorting FACS during CM differentiation. Although the proportion of marked cells was similar in ESCs and at day 2, we observed a dramatic overrepresentation of shControl cells at days 4 and 5.3, indicating that Bvht-depleted cells cannot efficiently contribute to cardiac cell types (Figure 5).

Similar results were obtained using reciprocal markers (Figure S6E). Notably, cardiac genes such as Gata4, Nkx2.5, Tbx5, and Is1 were expressed at normal levels at day 5.3 (Figure S6F), indicating that control cells could differentiate properly in the presence of Bvht-depleted cells. Thus, our results support a cell-autonomous role for Bvht in the transcriptional control of cardiac cell fate.

Braveheart Interacts with SUZ12, a Core Component of PRC2
Some lncRNAs are thought to act through RNA-protein interactions to effect gene expression by diverse mechanisms (Rinn and Chang, 2012). Because T and EOMES have been shown to bind to the MesP1 promoter and to regulate its expression (David et al., 2011; Costello et al., 2011), we first tested whether Bvht interacted with T or EOMES to mediate their binding at the MesP1 promoter. We performed RNA immunoprecipitation (RIP) using antibodies against T or EOMES; however, we failed to detect
Figure 5. **Bvht Is Necessary for the Transition from Nascent to Cardiac Mesoderm In Vitro**

(A) ESCs were differentiated into beating cardiomyocytes (CM) and progress through precardiac mesoderm (MES) and cardiac progenitor (CP) stages by sequential addition of cytokines.

(B) Bvht-depleted EBs were smaller than controls at day 4 and failed to attach and organize into a monolayer at day 5.3. Scale bar, 65 μm.

(C) Bvht-depleted cells showed increased levels of apoptosis at day 4 as indicated by quantification of annexin-V-positive cells, whereas no significant difference was observed at day 2.

(D) Brachyury and Eomes levels remained high at day 5.3 compared to control as measured by qRT-PCR.

(E) Expression of cell surface markers PdgfRα and Flk-1 was significantly decreased upon Bvht depletion at days 4 and 5.3 compared to control.

(F) Core cardiac transcription factors displayed lower expression at days 4 (E) and 5.3 (F) in Bvht-depleted cells compared to control.

(G) EMT genes displayed altered expression patterns in Bvht-depleted cells at day 5.3 as measured by qRT-PCR. Values for shControl samples were normalized to 1 for each individual time point.

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a specific interaction with **Bvht** (Figure S7A). We also did not detect changes in the binding of T to the **MesP1** promoter by chromatin immunoprecipitation (ChIP) followed by qPCR (Figure S7B and Supplemental Information). Thus, it is unlikely that **Bvht** directly regulates T or EOMES to control **MesP1** activation.

IncRNAs can act as molecular scaffolds by interacting with chromatin-modifying complexes (Guttman et al., 2011; Tsai et al., 2010). Using in vitro biotin-RNA pull-down, we detected a specific interaction between **Bvht** and SUZ12 in ESCs; however, other factors thought to bind IncRNAs, including hnRNPK, SNW1, RING1b, OCT4, BRG1, and MLL1, did not show a specific interaction (Figure 6A). These data were confirmed by RIP using SUZ12-specific antibodies (data not shown). SUZ12 is a core subunit of PRC2, a complex that catalyzes trimethylation of histone H3 lysine 27 (H3K27me3) and mediates transcriptional repression (Surface et al., 2010). A number of IncRNAs have been shown to interact with PRC2 to regulate target gene expression (Wang and Chang, 2011). We then constructed a series of **Bvht** deletions and found several regions of the transcript that were required for this interaction (Figure 6B), suggesting that **Bvht** directly interacts with PRC2.

Next, we tested whether **Bvht** interacted with SUZ12 during CM differentiation. Similar to ESCs, we found that SUZ12 interacted with **Bvht** by RIP at day 5.3 cells (Figure 5C). We chose to analyze day 5.3 cells because these cells comprise a largely homogenous population of >70% **NKX2.5**-positive cells and because **Bvht** is enriched in this population (Figure S7C). Many of the cardiac genes that failed to activate in **Bvht**-deleted cells at day 5.3 are known comb targets in ESCs (Boyer et al., 2006). These genes are poised for activation in ESCs, as indicated by their bivalent chromatin marks (H3K27me3 and H3K4me3) (Bernstein et al., 2006), and differentiation toward specific lineages requires the selective loss of PRC2 at subsets of genes. We found that, in **Bvht**-deleted cells, SUZ12 levels remained high at the promoters of critical genes in the cardiovascular network, including **MesP1**, **Gata6**, **Hand1**, **Hand2**, and **Nll2.5**, compared to control cells as measured by ChIP-qPCR (Figure 6D). In contrast, SUZ12 levels were lower at **T** and **Eomes**, consistent with their higher expression at day 5.3 in **Bvht**-deleted cells. SUZ12 levels were equivalent in both **Bvht**-deleted and control cells (Figure S7D). We also found that H3K27me3 levels paralleled SUZ12 enrichment in both **Bvht**-deleted and control cells. Notably, cardiac genes maintained their bivalent status at day 5.3 (Figures S7E and S7F), consistent with a failure to activate the cardiac program. Though our data do not distinguish between a direct or indirect mechanism, these results along with the demonstrated interaction between SUZ12 and **Bvht** suggest that **Bvht** functions, in part, with PRC2 to regulate cardiac commitment.

**Braveheart** Is Necessary for Maintenance of Cardiac Fate in Neonatal CMs

Our data show that **Bvht** is required for the commitment of ESCs toward a cardiac fate. Because we detected expression of **Bvht** in the adult mouse heart (Figure 1B), we investigated whether **Bvht** functions in later stages of differentiation. We
analyzed the effect of Bvht depletion in primary murine neonatal ventricular cardiomyocyte (nCMs) cultures, a model that has been used extensively for investigating drug and injury responses (Louch et al., 2011). In brief, nCMs were isolated from the newborn heart, a stage when myocytes display plasticity both in vivo and in vitro, as evidenced by increased levels of early cardiac markers such as Gata4 and Nkx2.5 (Porrello et al., 2011; Zhang et al., 2010). Within days of isolation, nCMs are able to organize into myofibrils, forming sheets of beating cardiomyocytes in explant cultures. Thus, neonatal cardiomyocytes represent an ex vivo model in which to test the role of Bvht in modulating cardiac cell fate in the heart.

nCMs were infected 1 day after explant with Bvht or control shRNA lentiviruses harboring a GFP marker. We found that Bvht-depleted nCMs as marked by GFP displayed unevenly distributed and irregularly bundled myofibrils after 5 days in culture compared to control cells as evidenced by cTNT staining (Figure 7A). Consistent with this observation, the overall cell surface area of individual GFP-positive nCMs was significantly smaller (Figure 7B), indicating that nCM structure is disrupted.
in *Brvht*-depleted cells (Figure 7C). Moreover, structural genes that were not properly activated in *Brvht*-depleted EBs, including α- and β-MHC and Smα, displayed lower expression in the *Brvht*-depleted nCMs compared to controls (Figure 7D), whereas early cardiac markers Gata4 and Nkx2.5 displayed higher expression (Figure 7E). Notably, genes involved in EMT such as *Snai* and *Twist* members, as well as *Vimentin*, showed lower expression in *Brvht*-depleted nCMs (Figure 7F). These data suggest that *Brvht* plays a critical role in promoting cardiac gene expression programs and tissue remodeling in the developing heart.

**DISCUSSION**

Dynamic regulation of gene expression programs is critical for cell fate transitions during lineage commitment, and faulty regulation can lead to developmental failure and disease. Heart development is a multistep process regulated by a network of transcription factors, and disruption of transcriptional networks leads to congenital heart disease (Bruneau, 2008; McCulley and Black, 2012; Olson, 2006; Srivastava, 2006). Though many of the genetic factors that govern heart development are known, we hypothesized that IncRNAs may represent an additional layer of regulation. Here, we identify *Braveheart* as a critical regulator of cardiovascular commitment from nascent mesoderm, representing the first example of an IncRNA with potential implications in cardiovascular development.

The temporal activation of a core transcription factor network establishes the plan for a primitive heart. The genes in this network are highly conserved during evolution, whereas the formation of more complex and species-specific substructures is likely a consequence of evolution of species-specific regulators. Analysis of sequence conservation and potential transcripts in syntenic regions in the rat and human genomes did not identify a *Brvht* homolog. The lack of conservation is likely due to both weak selective pressure on the primary sequence of IncRNAs and rapid gain and loss of entire IncRNA genes. For example, noncoding genes such as *Xist* and *Neat1* have undergone rapid sequence evolution while preserving their functional roles (Clemson et al., 2009; Nesterova et al., 2001). Increasing evidence also suggests that IncRNAs are largely species specific, with many transcripts arising from the primate lineage (Derrien et al., 2012). Thus, *Brvht* appears to represent a nonconserved lineage-specific IncRNA; however, we expect that other species with a defined circulatory system may have evolved IncRNAs with similar functions.

**Brvht May Function with PRC2 to Regulate Cardiac Gene Expression Program**

Epigenetic regulation plays an important role in development of the cardiovascular system (Chang and Bruneau, 2012). Recent studies have shown that Ezh2, the catalytic component of PRC2, is necessary for repression of the progenitor program during cardiac differentiation and for proper spatiotemporal regulation of cardiac gene expression (Delgado-Olguin et al., 2012; He et al., 2012). We found that *Brvht* directly interacted with PRC2 through SUZ12, a core component of the complex, at several stages during CM differentiation. Notably, SUZ12 and its associated modification H3K27me3 were enriched at promoters of cardiac genes, including *MesP1* in *Brvht*-depleted cells. Cardiac genes also remained bivalent in *Brvht*-depleted cells, similar to their configuration in ESCs and consistent with a failure to activate the cardiac program. Although we cannot yet distinguish between a direct or indirect role for *Brvht* at these loci, one model is that *Brvht* binds SUZ12 and competes for PRC2 binding at target sites during the transition from nascent to cardiac mesoderm, leading to activation of the cardiac program. Alternatively, *Brvht* may recruit PRC2 to a repressor of the cardiac program, where loss of *Brvht* would fail to silence the repressor. Though further studies will be required to fully elucidate the mechanism of *Brvht* function, our work suggests that *Brvht* mediates cardiac commitment, in part, by epigenetic regulation of gene expression programs.

**Implications of Brvht in Cardiovascular Development and Disease**

Cardiovascular disease is one of the most common causes of death, yet the mammalian heart has limited regenerative capacity. Thus, a detailed understanding of the pathways involved in cardiogenesis and particularly factors dictating commitment will be instrumental for recapitulating these processes for regenerative therapy. Stem cell models and the generation of specific cell types in vitro offer a potential avenue for studying diseases in a dish and ultimately for transplantation therapy (Burdridge et al., 2012). Thus, our finding that *Brvht* is required for progression of cardiac commitment suggests that IncRNAs represent a class of molecular modulators that can direct cell fate. Direct comparisons of cardiovascular-associated IncRNAs across species should contribute further insights into how these regulators are integrated into the gene regulatory networks that drive cardiogenesis and may also provide important clues into the evolution of this complex organ. Moreover, our data suggest...
that lncRNAs may represent undiscovered disease loci that, in some cases, may be identifiable by genome-wide association studies (GWAS) (Mattick, 2008; Schonrock et al., 2012). For example, the human IncRNA ANRIL has been associated with a locus implicated in cardiovascular disease (Congrains et al., 2012). In addition, human MIAT was identified as an IncRNA associated with increased risk of myocardial infarction (Ishii et al., 2006). Thus, we anticipate that future studies to identify additional IncRNAs with roles in cardiac function will lead to a better understanding of heart development and will inform strategies for cardiac repair.

**EXPERIMENTAL PROCEDURES**

Detailed experimental and analysis methods can be found in the Supplemental Information.

**ESC Lines and Growth Conditions**

E14 (ola129), V6.5 (SvJae129/C57BL/6), MesP1-GFP, A2lox-MesP1, NKX2.5-GFP, and mIR-143/145 ESCs were cultured on irradiated MEFs using standard conditions.

**Generation of ESC Lines**

shRNA-Mediated Depletion of IncRNAs

shRNAs directed against Bvht, MesP1, and control hairpins were cloned into pLKO.1 vector. Production of lentiviral particles and transduction of ESCs was performed according to protocols from The RNAi Consortium (http://www.broadinstitute.org/rnaiv/tcr).

Bvht Overexpression

Full-length Bvht cDNA was cloned into pSLIK vector, and transgenic cells were generated as described above.

**ESC Differentiation**

Embryoid Body Formation

ESCs were preplated to remove feeders, diluted to 100,000 cells/ml in complete growth medium lacking LIF, and plated on low-attachment cell culture plates.

Cardiomyocyte Differentiation

Directed differentiations and analysis were performed as described (Wamstad et al., 2012).

**Identification of Candidate IncRNAs**

Expression data from ESCs (V6.5 and E14) (this study) and adult tissues (Moritzawi et al., 2008) were collated using two annotations (Filek et al., 2011; Guttman et al., 2010) to define a set of murine IncRNAs. Candidate IncRNAs were identified by a stringent set of criteria outlined in the Supplemental Information.

**Genomic Analysis of Braveheart**

5′ and 3′ RACE was performed using FirstChoice RLM-RACE kit (Ambion) on ESC RNA, and products were cloned using TOPO TA cloning kit (Invitrogen). See Table S5 for primer sequences.

**Preparation of RNA Sequencing Libraries and Analysis**

RNA-seq was performed as described (Wamstad et al., 2012), and reads were mapped using TopHat/Bowtie. Isoform identification and quantification were performed using Cufflinks. RNA-seq quality control metrics are listed in Table S6.

**Gene Coexpression Network**

The cascade network of coexpression was generated based on the Pearson correlations of the RNA-seq expression profiles, which are described by log2-fold change between Bvht and control knockdown cells. The layout of the network was created using the Spring-Embedded layout algorithm (Cytoscape plug-in), as described in the Extended Experimental Procedures.

**Competition Assay/Cell Autonomy**

GFP and mCherry coding sequences were cloned into pLKO.1 vector containing control and Bvht hairpins (Table S5). Cells were isolated by FACS 4 days after infection to select for those expressing the fluorescent proteins. Equal numbers of ESCs were mixed, analyzed by FACS, and then plated under cardiomyocyte differentiation or ESC culture conditions. Cells were analyzed by FACS at various time points to determine the proportion of each fluorescently marked population.

**RNA Immunoprecipitation**

Native RIP was performed as described (Rinn et al., 2007). Antibodies are listed in the Supplemental Information.

**Chromatin Immunoprecipitation**

The ChIP protocol has been adapted from previous studies (Creyghton et al., 2008). Antibodies are listed in the Supplemental Information. ChIP-enriched DNA was quantified by qRT-PCR. Primers are listed in Table S5.

**Isolation and Manipulation of Primary Mouse Neonatal Cardiomyocytes**

Murine neonatal cardiomyocytes (nCMs) were isolated from C57B6 mice within the first 24 hr after birth. A detailed protocol can be found in the Extended Experimental Procedures.

**ACCESSION NUMBERS**

All RNA-sequencing data generated during the course of this study, including raw reads and analysis files, have been deposited to GEO under the accession ID GSE39656.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.003.

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