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SBE6, a novel long-range enhancer involved in driving Sonic Hedgehog expression in neural progenitor cells.

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Abstract

The expression of genes with key roles in development is under very tight spatial and temporal control, mediated by enhancers. A classic example of this is the sonic hedgehog gene (Shh) that plays a pivotal role in the proliferation, differentiation and survival of neural progenitor cells both in vivo and in vitro. Shh expression in the brain is tightly controlled by several known enhancers that have been identified through genetic, genomic and functional assays. Using chromatin profiling during the differentiation of embryonic stem cells to neural progenitor cells, here we report the identification of a novel long-range enhancer for Shh - Shh-Brain-Enhancer-6 (SBE6) – that is located 100kb upstream of Shh and that is required for the proper induction of Shh expression during this differentiation programme. This element is capable of driving expression in the vertebrate brain. Our study illustrates how a chromatin focussed approach, coupled to in vivo testing, can be used to identify new cell-type specific cis-regulatory elements and points to yet further complexity in the control of Shh expression during embryonic brain development.
Introduction

Enhancers orchestrate the regulation of gene expression, which is critical for cell lineage specification and differentiation, and they therefore have a pivotal role during embryonic development [1]. A well-defined example of such cis-regulatory control is seen in the case of the Sonic hedgehog (Shh) gene. Shh encodes a secreted signalling protein that imparts patterns of growth and identity to cells during many stages of embryonic development, including neural progenitors throughout ventral regions of the developing central nervous system (CNS) [2–4] (Figure 1A).

Shh is located at one end of a large (~1Mb) regulatory domain containing a number of known enhancers controlling various Shh expression domains [5–12] (Figure 1B). Precise Shh expression is critical for proper spinal cord and brain development and this is governed by a subset of floor-plate and brain enhancers, many of which were identified by reporter assays. Shh floor-plate enhancer SFPE1, located 8kb upstream of the Shh transcription start site (TSS), drives expression in the ventral spinal cord and SFPE2 and Shh-brain-enhancer 1 (SBE1), positioned in the second intron of Shh, show activity in the floor plate of the spinal cord, as well as the ventral midbrain (mesencephalon), ventroposterior region of the diencephalon and the zona limitans intrathalamica (ZLI) [5,6]. An enhancer trap assay - using BAC transgenes to screen the Shh regulatory region - identified SBE2, SBE3 and SBE4 that drive Shh expression in the diencephalon (SBE2) and in the telencephalon (SBE4) [7]. Most recently, a combined informatics and experimental study identified SBE5 that drives expression in the ZLI [13].

Perturbation of Shh cis-regulation leads to severe neural defects in mammals. Translocations separating SBE2, 3 and/or 4 from Shh, disrupt the function of these enhancers and lead to Shh haploinsufficiency causing diverse holoprosencephaly (HPE) phenotypes [14,15], Point mutation that results in the loss of SBE2 activity in the hypothalamus also leads to HPE [16]. Together these observations highlight the importance of reporting and understanding new cis-regulatory elements that control Shh expression in the CNS.

Using chromatin profiling during the in vitro differentiation of mouse embryonic stem cells (mESCs) to neural progenitor cells (NPCs) we report a new Shh brain enhancer (SBE6) that we show is necessary for proper Shh expression in NPCs and that is active in vertebrate brain and neural tube development in transgenic assays.
Material and Methods

Cell Culture and Neural Differentiation

46c mouse embryonic stem cells (mESCs), derived from E14tg2A, contain a GFP insertion into the Sox1 locus [17]. mESCs were cultured and differentiated into NPCs for five days with N2B27 medium as described previously [18]. To sort GFP\(^+\)ve cells after transfection or differentiation, cells were trypsinized and resuspended in PBS + 10% medium. Flow cytometric analysis was performed using the 488nm laser of a BD FACSAriaII SORP (Becton Dickinson) with 525/50 nm bandpass filters. BD FACSDiva software (Becton Dickinson, Version 6.1.2) was used for instrument control and data analysis.

Quantitative analysis of gene expression

RNA was prepared from approximately 1x10\(^6\) 46c mESCs using the RNeasy mini kit (Qiagen) according to the manufacturer’s protocol, including a DNaseI (Qiagen) treatment for 15 minutes at room temperature. cDNA was synthesized from 2 µg purified RNA with Superscript II reverse transcriptase (Invitrogen) primed with random hexamers (Promega). Real-time (q)PCR was carried out on a Roche LightCycler 480 Real-Time PCR System using a Lightcycler 480 Sybr Green detection kit (Roche) as described previously [19]. Primer pairs for qRT-PCR are listed in Supplementary Table 1.

The real-time thermal cycler was programmed as follows: 15 min Hotstart; 44 PCR cycles (95°C for 15 s, 55°C for 30 s, 72°C for 30 s). The relative mRNA expression for each primer set in each sample was measured by the Lightcycler software and normalized to the mean for Gapdh from at least 2 biological replicates and technical triplicates.

Native Chromatin Immunoprecipitation and Microarray Analysis

Nuclei from 3x10\(^6\) mESCs or sorted Sox1+ve NPCs were prepared and resuspended in NB-R (85 mM NaCl, 5.5% Sucrose, 10mM TrisHCl pH 7.5, 3 mM MgCl\(_2\), 1.5 mM CaCl\(_2\), 0.2 mM PMSF, 1 mM DTT) as previously described [20]. Micrococcal nuclease (MNase) digestion and native ChIP were performed as previously described [21,22]. Antibodies used for ChIP were; H3K4me1 (Abcam ab8895) and H3K27ac (Millipore 07w360).

Ten nanograms (optimal) of input or ChIP DNA were amplified using the WGA2 whole genome amplification kit (Sigma). Amplified material was labelled with Cy3 or Cy5 by random priming
according to the NimbleGen ChIP-chip protocol (Roche). In total, 2 or 3 biological replicates with dye swaps were hybridized for 20 h and washed according to manufacturer’s protocol. A custom 3x720K mouse tiling array (NimbleGen, Roche) containing 179,493 unique probes from different genomic regions, with each probe represented by 4 replicates was used. Arrays were scanned on a NimbleGen MS 200 Microarray scanner (Roche) using 100% laser power and 2µm resolution. Raw signal intensities were quantified from TIFF images using MS 200 Data Collection software.

Microarray data were analysed in R using the bioconductor packages Beadarray and Limma according to the Epigenesys NimbleGen ChIP-on-chip protocol (www.epigenesys.eu/en/protocols/bioinformatics). Scale normalization was used within replicates, to control inter-array variability. Each condition was represented by two biological replicates hybridised as dye swap experiments and enrichment scores are defined as log2 ChIP/Input signal.

Computational analysis of the SBE6 region.

Evolutionary conservation of the SBE6 region was assessed using the “Vertebrate Multiz Alignment & Conservation/Multiz Alignments and Conserved elements” tracks in the UCSC genome browser [23]. This delineated the following sub-regions for further analysis:

SBE6.1: Chr5:28889688-28890461, SBE6.2: Chr5: 28893935-28895000 (mm9)

rVISTA [24] was used to align the mouse and human orthologous sequences, with the default sequence aligner (LAGAN) and default parameters. Transcription factor binding sites (TFBS) for known forebrain TFs [25] available on the rVISTA server were selected (Arx, Maf, Dlx5, Pbx1, ER81, Six3, Vax1).

JASPAR [26] was used independently on the mouse and human core sequences, searching for potential neural activity present in the Jaspar Core Vertebrata matrices list (DLX6, PBX1, ETV1, Six3, SP8, and VAX1) with the default parameters (Relative profile score threshold 80%) (Supplementary Table 2). Hits were then highlighted on the rVista alignment.

Zebrafish enhancer reporter assay

The putative SBE6.1 and SBE6.2 enhancers were cloned by PCR amplification of the relevant fragment and flanking sequence from mouse genomic DNA, using Phusion high fidelity polymerase (NEB) and the following primers:

Sbe6.1 Fw B4 : ACGGGAAGAACCTTGTATAGAAAGGGTGCCGGCCACCTGCTTCTCTGAGGAA
Sbe6.1 Rv B1R : ACGGGAACGTGTTTTTTGTACAAACTTGCTTAGGCCATTGTGCCCAC
Sbe6.2 Fw B4 : AGGGGAGAAGCTTTGTATAGAAAAGTTGGCGCGC
Sbe6.2 Rv B1R : AGGGGACTGCTTTTTTGTACAAACTTGATCAGCCCTCCAGTTTGACT

-ve controls used were sequences 3’ of Shh, which have no suspected regulatory activity, and which are the same genomic distance from Shh as SBE6.1 and SBE6.2 are upstream (5’) of Shh.

wveCTRLs
Sbe6.1 Fw B4 : AGGGGAGAAGCTTTGTATAGAAAAGTTGGCGCGC
Sbe6.1 Rv B1R : AGGGGACTGCTTTTTTGTACAAACTTG
Sbe6.2 Fw B4 : AGGGGAGAAGCTTTGTATAGAAAAGTTGGCGCGC
Sbe6.2 Rv B1R : AGGGGACTGCTTTTTTGTACAAACTTG

attB4 and attB1r sequences (bold) were included in the PCR primers for use with the Gateway recombination cloning system (Invitrogen, 12538120). The amplified fragment was first cloned into the Gateway pP4P1r entry vector and sequenced using M13 forward and reverse primers for verification. The elements in the pP4P1r vector were combined with a pDONR221 construct containing either a Gata2 promoter-eGFP- polyA or a Gata2 promoter mCherry-polyA cassette [27], and recombined into a destination vector with a Gateway R4-R2 cassette flanked by Tol2 recombination sites.

Reporter plasmids were isolated using Qiagen miniprep columns and were further purified using a Qiagen PCR purification column (Qiagen), and diluted to 50 ng/µl with DNase/RNase free water. Tol2 transposase RNA was synthesized from a NotI-linearized pCS2-TP plasmid using the SP6 mMessage mMACHINE kit (Ambion), and similarly diluted to 50 ng/µl. Equal volumes of the reporter construct(s) and the transposase RNA were mixed immediately prior to injections. 1–2 nl of the solution was micro-injected per zebrafish embryo at the 1- to 2-cell stage for up to 200 embryos. Embryos were screened for fluorescence at 1–5 days post-fertilization i.e. 24–120 hpf (hours post fertilization) and raised to adulthood. Germline transmission was identified by mating of sexually mature adults to wild-type fish and examining their progeny for fluorescence. F1 embryos from 3–5 F0 lines showing the best representative expression pattern for each construct were selected for confocal imaging. A few positive embryos were also raised to adulthood and F1 lines were maintained by outcrossing. A summary of the independent lines analysed for each construct and their expression sites is included in Supplementary Table 3. Imaging of zebrafish reporter transgenic embryos was carried out as previously described [27].

Mouse Transgenic reporter assays
The same SBE6.1 PCR amplicon, with attB4 and attB1r sequences included as used for reporter assays in zebrafish (above), were used to generate enhancer-reporter constructs for mouse transgene assays. The amplicon was cloned directly into an hsp68-LacZ vector containing a P4-P1r entry cassette [28]. Transgenic mice were generated by micro-injection into mouse oocytes and the analysis of transgenic lines were carried out as previously described [28]. Two independently derived E11.5 SBE6.1-LacZ embryos were independently analysed; one a transient insertion, the second from a stable line. For analysis, embryos were dissected in PBS and left in LacZ fix for 1h (1% formaldehyde; 0.2% glutaraldehyde; 2 mM MgCl2; 5 mM EGTA and 0.02% NP-40 in PBS). After fixation the embryos were washed in PBS containing 0.02% NP-40, before being stained overnight at 37°C in the dark in a solution containing 5 mM K3Fe(CN)6; 5 mM K4Fe(CN)6.3H2O; 2 mM MgCl2; 0.01% sodium deoxycholate; 0.02% NP-40 and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Embryos were then fixed with 4% PFA and photographed on a Leica MZ FLIII Microscope fitted with a Hamamatsu Orca-ER digital camera and a CRI micro-color filter.

**mRNA in situ hybridization**

RNA in situ hybridization on fish embryos was performed as previously described [29]. The sequences of primers used for synthesis of Shh hybridization probes are the following:

Forward primer (5’-SP6 promoter-sequence – 3’) AAGCTGACACCTCTCGCCTA and Reverse primer (5’-T7 promoter-sequence – 3’) GAGCAATGAATGTGGGCTTT.

Mouse in situ hybridisation was performed with DIG-labelled gene-specific antisense probes as previously described [30]. The Shh probe was provided by Andy McMahon [31].

**Deletion of SBE6 from the 46c ESC genome.**

Cell line deletions were produced using the Crispr/cas9 system. SBE6.1 and SBE6.2 specific gRNA primers (Supplementary Table 4) were cloned into the cas9 plasmid pX458 following protocols from the Zhang lab [32–34]. 46C mESCs were transfected with the resulting plasmids using Lipofectamine® 2000 Reagent (Invitrogen cat. N°11668) following the manufacturer’s recommendations as described in [19]. Single transfected cells were sorted based on GFP expression from pX458 and cultured further. DNA extraction and genotyping were performed 7 days after sorting using overnight incubation at 55°C with lysis buffer (10mM Tris pH 7.5, 10mM EDTA, 10mM NaCl, 0.5% SDS, 1mg/ml ProteinaseK) followed by ethanol precipitation and washes. Genomic DNA was amplified with the following primers:

SBE6.1 Fw: TTTTGGAAGCTTAAATGCCCAT
SBE6.1 Rv: CCACCACAAGCACATTCAT
SBE6.2 Fw: GCCTCCATGAAGTCCAATGG
SBE6.2 Rv: CCACCCTTGCTACTCAGGAA

Amplification was done using DreamTaq Green PCR Master Mix (ThermoFisher K1081) following the manufacturer’s protocol and PCR products were assessed by agarose gel electrophoresis. Amplified products were later sequenced to further confirm homozygote deletions.
Results

**SBE6.1 and SBE6.2, two new putative cis-regulatory elements active in NPCs.**

We used the differentiation of 46c mESCs as a model system to identify putative regulatory elements that may become activated concomitant with the expression of *Shh* during neuronal differentiation. These cells contain a knockin of GFP into the *Sox1* locus allowing for the monitoring of neural differentiation and the purification, by fluorescence activated cell sorting (FACs), of Sox1+ve neuroepithelial progenitor cells (NPCs) (Figure 2A) [17,35,36]. Sox1+ve cells appear after day 3 of differentiation, and from day 3 to 7, expression of *Shh* and *Nestin* increase whilst *Oct4* mRNA levels progressively decrease (Figure 2B). Analysis of these NPC cells for expression of markers from different regions of the developing brain (Figure 2C) suggests that these cells do not have a distinct regional identity, though there is some evidence for a slight shift towards a more telencephalic fate (increasing *Six3* and *Enx2* expression), and away from the hindbrain (decreasing *En2* and *Gbx2* expression) by day 7 (Figure 2D).

Genome-wide chromatin immunoprecipitation (ChIP) has allowed the identification of several post-translational histone modification characteristic of active enhancers including H3K4me1 and H3K27ac [37]. The use of these two histone marks is widely employed to identify new active enhancer elements in the genome [38], though they are not comprehensive [18,39]. Using native ChIP coupled to hybridisation on microarrays (ChIP-chip) that tile the whole *Shh* regulatory region, we assessed the sites of enriched H3K4me1 and H3K27ac in mESCs (where *Shh* is not expressed) and in Sox1+ve NPC after five days of neural differentiation. Significant gains of H3K4me1 and H3K27ac were not detected at the known SBE2, 3, 4 or 5 brain enhancers (Figure 3A). However, a prominent change in the ChIP profile was seen at a small region ~ 100 kb upstream of the *Shh* transcriptional start site (TSS). This region has no evidence of active enhancer marks in mESCs but gains both H3K4me1 and H3K27ac upon neural differentiation (Figure 3A).

Analysis of sequence conservation across multiple vertebrate species indicated that this region contains two blocks of evolutionary conservation in mammals and birds and we named these putative NPC enhancers SBE6.1 (mm9 co-ordinates Chr5: 28889688-28890461, 96048bp upstream of Shh TSS) and SBE6.2. Chr5: 28893935-28895000, 100295bp away from *Shh*) (Figure 3B). Interestingly, two other sequences beyond SBE3 also show a gain of active enhancer marks (arrowheads in Figure 3A), but are not investigated further here.

*In silico* motif analysis using UCSC comparative genomics of SBE6.1 and SBE6.2 allowed us to identify two core (~1kb) regions that are highly conserved. Complementary rVista, Jaspar and RSAT scans of those regions revealed the presence of predicted binding sites for neural transcription factors such as ETV1, SP8, VAX1 and DLX6 (Supplementary Table 2).
The SBE6.1 sequence is entirely included in a recently described 1.7kb lung and gut epithelium regulatory element for Shh expression in mouse embryos called SLGE (chr5: 28889230-28890979) [40], raising the possibility either that this enhancer has multiple regulatory activities, or that SLGE is ectopically activated in NPCs. SBE6.2 has not previously been identified or studied.

**SBE6.1 drives expression in the brain of developing zebrafish and mouse embryos**

To test the regulatory potential of SBE6.1 and SBE6.2, we used a zebrafish Tol2 transposon assay in which the test element is juxtaposed to a minimal promoter driving the expression of either GFP or mCherry reporter gene expression. This assay has been shown to recapitulate the correct expression pattern for the SBE2 enhancer and to detect the loss of this enhancer activity associated with mutation of a SIX3 binding site found in cases of HPE [16,27]. In situ hybridisation for Shh mRNA in wild-type zebrafish embryos reveals expression in the forebrain at 48 and 72 hours post fertilisation (hpf) [27]. Using this assay, SBE6.1 enhancer activity was detected in the developing forebrain of the zebrafish embryos in four independent stable transgenic lines from 30 – 74 hpf (Figure 4A and B) (Supplementary Table 3). SBE6.2 however failed to consistently drive reporter gene expression in the forebrain, with forebrain specific activity noted in only one out of the four independent transgenic lines generated (Supplementary Table 3). Therefore SBE6.1 has a consistent enhancer function and is active in zebrafish forebrain development.

The ability of SLGE to drive expression in the developing mouse brain is unclear, but it is known to be capable of driving expression in the brain of transgenic rabbits [40]. We therefore made mouse transgenics to analyse the regulatory potential of SBE6.1 in mouse development. LacZ staining of transient and stable SBE6.1 transgenic embryos revealed activity in the pharyngeal endoderm, the gut and cloaca of the mouse embryo as expected due to the overlap with SLGE (Supplementary Figure 1A and B). X-gal staining could be also detected in few superficial diencephalon cells where Shh is not expressed (Figure 4C) (Supplementary Figure 1C). However, SBE6.1 also showed activity in the developing ventral mesencephalon with some cells expressing SBE6-LacZ near the hindbrain and as well as in the ventral midline of the mouse embryonic neural tube - all sites of endogenous Shh expression (Figure 4D and E) (Supplementary Figure 1D).

SBE6.1 is only active in a small number of cells in transgenic embryos, and we cannot at this stage confirm how accurately this recapitulates a subset of endogenous Shh expression. However, the strong similarities between the two mouse embryos does support our conclusion that the SBE6.1 enhancer is capable of activity in the developing vertebrate brain, from a forebrain pattern in zebrafish, transgenics to a floor plate and ventral mesencephalon expression in mouse transgenic embryos.
**SBE6.1 enhances Shh expression in neural progenitor cells.**

To determine the regulatory activity of SBE6.1 and SBE6.2 in their native context, we used CRISPR/Cas9 to delete these elements from the genome in 46c mESCs (SBE6.1<sup>−/−</sup> and SBE6.2<sup>−/−</sup>) (Supplementary Figure 2). We generated and analysed two SBE6.1<sup>−/−</sup> and three SBE6.2<sup>−/−</sup> independent cell lines. Upon NPC differentiation, the proportion of Sox1-GFP<sup>+</sup> cells remained the same between NPCs derived from wild-type and SBE6.1<sup>−/−</sup> or SBE6.2<sup>−/−</sup> cells, analysis of *Oct4* and *Nestin* mRNA expression confirmed that differentiation of mESCs into NPC was not perturbed by the loss of either SBE6.1 or SBE6.2 (Figure 5A). However, in NPCs derived from SBE6.1<sup>−/−</sup>, but not SBE6.2<sup>−/−</sup>, cells, levels of *Shh* expression were significantly reduced compared to wild-type cells (one tailed Student t-test; *p* = 0.002). Average *Shh* mRNA levels in NPCs differentiated from SBE6.2<sup>−/−</sup> ESCs were not significantly different relative to wild-type (Figure 5B).

Together these data suggest that SBE6.1 is a long-range enhancer that contributes to driving *Shh* expression during the differentiation of ESCs to neural progenitor cells.
Discussion

The regulation of Shh is a paradigm for the complex control of gene expression at different times and places in development. More than 10 discrete enhancers have been identified in the large (~1Mb) Shh regulatory domain [12] (Figure 1B). Most of these enhancers were identified using transgenic reporter assays [7]. Others have been identified through genetics in mouse and man when mutations in Shh enhancers cause phenotypes that result from aberrant control of specific aspects of Shh expression in development. Most recently, information on transcription factor motifs in known Shh brain enhancers has been used to search for other similar patterns of motifs in the Shh regulatory domain and has identified a new enhancer that drives Shh expression in a discrete region of the brain [7,13].

Here we show that analysis of histone modifications (H3K4me1 and H3K27ac), typically associated with active enhancers, in an in vitro neuronal differentiation system can be used to identify a new enhancer that is important for the activation of Shh in neural progenitor cells. This enhancer, which we have named SBE6, is located 100kb 5' of Shh and is activated during the differentiation of mESCs to Sox1+ve NPCs. Analysis of transcription factor motifs suggests that SBE6 contains consensus binding sites for a number of transcription factors expressed in the brain. Using an enhancer reporter assay in zebrafish and mouse, we show that in vivo the SBE6.1 region of SBE6, but not SBE6.2, can drive expression in the developing brain. Consistent with this, genetic ablation of SBE6.1 in mESCs, but not SBE6.2, abrogates the induction of Shh expression during in vitro NPC differentiation. Therefore, despite the presence of strong active enhancer histone modifications in NPCs, we find no functional evidence that SBE6.2 is a neural enhancer, highlighting that precise annotation and understanding of regulatory regions of the genome requires confirmation via functional enhancer assays.

Our analyses presented here add to the growing number of functionally validated enhancers directing Shh expression in different developmental contexts. Given the large size of the gene desert upstream of Shh, where many of these enhancers are located, there is the potential for this region to harbour many more cis-regulatory elements and, given the complexity of brain development, many of these may be enhancers active in the brain. Indeed, regulatory segmentation built from ChromHMM or Segway using ENCODE data from various mouse primary tissues indicates the presence of several regions with chromatin signatures indicative of enhancer activity in mouse brain at E14.5 – a period of mouse development when neurogenesis is on going (Figure 6A). This includes the genomic regions containing the known neural enhancers SBE2, 3 and 4, but also a region that corresponds to SBE6. The many other regions called as likely active enhancers using the analysis from just four tissues (brain, liver, spleen and kidney) at one embryonic stage (Figure 6A) suggests that the Shh regulatory region may harbour many tens of, as yet, un-annotated enhancers. We note that the new sites detected by these high-throughput methods, and that are marked with arrowheads as #1 and #2 in Figure 6A, correspond to peaks of H3K4me1/H3K27ac that are induced during the differentiation of 46c mESCs to NPCs (Figures 3 and 6A) (Supplementary Figure 3). A similar analysis of chromatin profiling data
from the Roadmap project also indicates the signature of an active neural enhancer at the position of SBE6 in material from different regions of the human brain and particularly in ganglionic eminence derived neurospheres (Figure 6B). This analysis also indicates many other potential regulatory elements active in different brain regions.

It is interesting that the genome coordinates of SBE6.1 are completely contained within those reported for the Shh lung and gut epithelium regulatory element SLGE [40]. Transgenic analysis in the rabbit had shown that the mouse SLGE fragment can drive expression in the rabbit brain [40]. Here we have shown that SBE6.1 can drive expression in the brain of zebrafish and mouse. Although we cannot completely exclude that our observations of SBE6.1 transgenic reporter expression in the vertebrate brain and neural tube represents ectopic activity of SLGE in these assays, our chromatin profiling indicates that this region does harbour activity regulatory potential in Sox1+ve NPCs. Consistent with this, ENCODE and Roadmap data also indicate that this region of the mammalian genome has active enhancer chromatin marks in neural tissue, as well as in the liver (Figure 6A and Supplementary Figure 3) and gastric tissue (Figure 6B). Important sequences required for enhancer function work as assemblies of transcription factor motifs [13]. SBE6.1 and SLGE motifs may be intermingled but still specific to a precise tissue and stage of development, or may be overlapping to various extents. There are several other examples of regulatory elements capable of driving expression at multiple sites during development - for example, the global control region (GCR) 5' of HoxD contains regulatory information capable of driving expression in the CNS and in the limb [41]. Moreover, for SOX9 and PAX6 there are cis-regulatory elements driving expression in multiple developmental sites, and in which disease associated variants have been identified that ablate enhancer function in one tissue but leave the other sites of expression unaltered [27, 42]. Further analysis will be necessary to determine the critical transcription factor binding sites in SBE6.1/SLGE needed to drive enhancer function in different developmental settings.

Competing Interests

The authors declare that they have no competing interests

Authors contributions

NSB conducted most of the experiments and data analysis, prepared the figures and wrote the manuscript. PG conducted the bioinformatics analysis of SBE6 sequences, BHB conducted the chromatin immunoprecipitation experiments, LAL assisted with the transient mouse transgenics, SB assisted with the study design, the zebrafish and mouse transgenics and writing of the manuscript,
WAB conceived of the study, designed the study, coordinated the study and helped draft and revise
the manuscript. All authors gave final approval for publication.

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Data Accessibility

H3K4me1 and H3K27ac ChIP-on-chip data is deposited in the GEO repository

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**Figure legends**

**Figure 1. The Sonic Hedgehog (Shh) regulatory region.**

A) Cartoon showing the sites of Shh enhancer activity in the E11.5 mouse embryo. Sites of Shh expression in the; forebrain (telencephalon, diencephalon), caudal diencephalon, zli and midbrain/mesencephalon, floor plate, epithelial linings of gut and lung, and the distal limb bud are indicated with different colours. B) Genomic map of the Shh regulatory region on mouse chromosome 5 indicating the known tissue-specific Shh enhancers, colour-coded as in A.

**Figure 2. Sox1-GFP Sox1+ve neural differentiation from ESC to NPCs.**

A) Schematic showing the differentiation of 46c mouse embryonic stem cells (ESC) – which are Sox1-GFP<sup>−</sup>ve and express high levels of the pluripotency factor Oct4 – into; first primitive ectoderm as Oct4 levels decrease and Fibroblast growth factor 5 (Fgf5) levels rise, and then further into neuroectoderm as Fgf5 levels start to decrease and Sox1 levels rise - allows for the purification of Sox1-GFP<sup>+</sup>ve neural progenitor cells (NPC). B) qRT-PCR showing mean ± standard error of the mean (s.e.m.) log2 mRNA levels for Oct4, Nestin and Shh in ESCs and in NPCs after 5 and 7 days of differentiation. Expression levels are relative to Gapdh and normalized to ESC mRNA levels. Data are from 3 biological replicates and technical triplicates. C) Schematic of an E11.5 mouse brain and gene expression markers patterning the telencephalon (tel), diencephalon (di), mesencephalon (mes) and metencephalon (met). D) qRT-PCR showing means (± s.e.m.) of log2 mRNA levels of marker genes for different brain regions in ESC and NPC differentiated for 5 or 7 days. As in (B) levels are relative to Gapdh and normalized to ESC mRNA levels. Six3 mRNA levels significantly increase in NPCs between days 5 to 7 of differentiation (one tailed Student t-test; p = 0.023). Data consist of 5 biological replicates and technical triplicates.

**Figure 3. Chromatin immunoprecipitation analysis during NPC differentiation.**

A) Log2 native ChIP/input MNase digested chromatin for H3K4me1 (black) and H3K27ac (grey) from ESCs and Sox1<sup>−</sup>ve NPCs purified after 5 days of differentiation. Averages of data from two biological replicates are shown. The position of genes (above) and known neural enhancers for Shh (below) are shown, grey arrowheads indicate two other regions that gain active enhancer signatures in NPCs. Genome co-ordinates are from the mm9 assembly of the mouse genome (chr5:28,782,000-29,711,000 bp). B) Zoom in of the region (chr5:28,887,000-28,900,000) of putative NPC enhancer activity showing conservation across multiple vertebrate species. Two smaller core conserved regions named SBE6.1 (=SLGE) and SBE6.2 are indicated.
Figure 4. Enhancer reporter assays for SBE6.1 and SBE6.2.

A) *Shh* mRNA in situ hybridisation on zebrafish embryos. A ventral view is shown at 48 hours post fertilization (hpf) and a dorsal view at 72 hpf. *Shh* expression is detected in the rostral hypothalamus (RH) and caudal hypothalamus (CH) of the forebrain (FB). B) Confocal microscopy of 48 and 72 hpf zebrafish embryos from stable transgenic lines carrying a Tol2 transposon with SBE6.1 and SBE6.2 respectively driving GFP and mCherry, respectively. Reporter gene expression is detected in the rostral hypothalamus (RH) and caudal hypothalamus (CH) of the forebrain (FB). C) External view of the LacZ staining in a stable SBE6.1 transgenic E11.5 embryos showing expression in a portion of diencephalon cells. D) Left, *Shh* mRNA in situ hybridisation in an E11.5 mouse embryo displaying expression in the forebrain (telencephalon, diencephalon), (FB), midbrain (caudal diencephalon, zona limitans intrathalamic and mesencephalon) (MB) and (zli), and hindbrain (HB). Right, Sagital section of an E11.5 transient SBE6.1-LacZ transgenic embryo with arrowhead indicating staining in a portion of the ventral mesencephalon, with some cells expressing SBE6.1 near the hindbrain. E) E11.5 transient SBE6.1-LacZ transgenic embryo with arrowhead indicating staining in the floor-plate of the spinal cord.

Figure 5. *Shh* expression levels in neural precursor cells (NPC) derived from ESC lines with SBE6.1 or SBE6.2 deletions.

A) qRT-PCR showing mean (± s.e.m.) log2 mRNA levels of *Oct4* and *Nestin* in wild-type 46c ESC, and NPCs, and in NPCs derived from SBE6.1<sup>−/−</sup> (left) or SBE6.2<sup>−/−</sup> (right) 46c cells. Levels are relative to *Gapdh* and normalized to levels in wild-type ESCs. B) As in (A) qRT-PCR showing mean (± s.e.m.) log2 *Shh* mRNA levels in wild type NPCs, and in NPCs derived from 46c cell lines deleted for SBE6.1 (left) or SBE6.2 (right). mRNA levels are shown relative to *Gapdh* and normalised to those in wild-type ESCs. *Shh* mRNA levels are significantly reduced in NPCs derived from SBE6.1<sup>−/−</sup> cell lines after 7 days of differentiation (one tailed Student t-test; p-value = 0.002). ESC data consist of 3 biological replicates, SBE6.1<sup>−/−</sup> data set are 6 biological replicates from 2 independent deletion cell lines compared with 6 biological replicates of wild-type (WT) NPC. SBE6.2<sup>−/−</sup> data are from 3 biological replicates from 3 independent deletion cell lines with 3 biological replicates of wild-type (WT) NPCs.

Figure 6. Chromatin state discovery and characterization (ChromHMM) in mouse and human *Shh* regulatory region.
A) Ensembl *Mus musculus* version 84.38 (GRCm38.p4) view of chr5:28456840-29050000 with regulatory feature tracks from primary cells (embryonic E14.5 brain, and adult liver, spleen and kidney) from ChromHMM. Grey arrowheads indicate previously described enhancer and two putative sequences indicated in Figure 3. The position of SLGE/SBE6 is indicated. B) Roadmap epigenome browser view of the human Shh locus on Hg19 (chr7:155595558-156100554), showing H3K4me1 (top) and H3K27ac (bottom tracks) ChIP-seq from a variety of brain regions, including neurospheres, as well as pancreas, gastric, small intestine, oesophagus, tissues. Arrowheads indicate corresponding enhancers in human genome. The position of SLGE/SBE6, SBE4 and SBE2 are indicated.

**Supplementary Figure legends**

**Supplementary Figure 1. Mouse SBE6.1 Transgenic reporter.**


**Supplementary Figure 2. Genomic PCR of SBE6.1** /− and SBE6.2** /− ESC lines.**

A) Illustration showing SBE6.1 and SBE6.2 sequences with the position of the gRNAs (arrowheads) used for CRISPR/Cas9 mediated targeted deletions. Half arrows indicate the position of PRC primers used for genotyping. B) Agarose gels showing PCR amplification of (left) SBE6.1 in wild-type 46C mESCs, SBE6.1** /− ESC clones 1 and 2, and (right) SBE6.2 in wild-type cells and in SBE6.2** /− ESC clones 1, 2 and 3,

**Supplementary Figure 3. Chromatin state discovery and characterization (ChromHMM) in SBE6.1, SBE6.2 and peak #2.**

Ensembl *Mus musculus* genome version 84.38 (GRCm38.p4) view of regulatory feature tracks from ChromHMM for the regions corresponding to (A) SBE6.1, (B) SBE6.2 and (C) putative peak #2 from Figure 3)
References


http://mc.manuscriptcentral.com/rsob
Figure 1

275x397mm (300 x 300 DPI)

http://mc.manuscriptcentral.com/rsob
Figure 2

209x148mm (300 x 300 DPI)
Figure 3

275x397mm (300 x 300 DPI)
Figure 4

275x397mm (300 x 300 DPI)
Figure 5

275x397mm (300 x 300 DPI)
Figure 6

275x397mm (300 x 300 DPI)