The Nestin neural enhancer is essential for normal levels of endogenous Nestin in neuroprogenitors but is not required for embryo development

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Abstract

Enhancers are vitally important during embryonic development to control the spatial and temporal expression of genes. Recently, large scale genome projects have identified a vast number of putative developmental regulatory elements. However, the proportion of these that have been functionally assessed is relatively low. While enhancers have traditionally been studied using reporter assays, this approach does not characterise their contribution to endogenous gene expression. We have studied the murine Nestin (Nes) intron 2 enhancer, which is widely used to direct exogenous gene expression within neural progenitor cells in cultured cells and in vivo. We generated CRISPR deletions of the enhancer region in mice and assessed their impact on Nes expression during embryonic development. Loss of the Nes neural enhancer significantly reduced Nes expression in the developing CNS by as much as 82%. By assessing NES protein localization, we also show that this enhancer region contains repressor element(s) that inhibit Nes expression within the vasculature. Previous reports have stated that Nes is an essential gene, and its loss causes embryonic lethality. We also generated 2 independent Nes null lines and show that both develop without any obvious phenotypic effects. Finally, through crossing of null and enhancer deletion mice we provide evidence of trans-chromosomal interaction of the Nes enhancer and promoter.

Introduction

Embryonic development requires precise coordinated expression of thousands of genes across space and time. Regulatory elements such as enhancers have a critical role in coordinating spatial-temporal gene expression during embryogenesis. Enhancers are typically located within introns and intergenic regions and comprise DNA motifs that can be bound by transcription factors.
factors (TF). TF binding promotes interaction of the enhancer with the target promoter via DNA looping. This process, which involves cohesins and the mediator complex [1], allows TF-associated co-activators to engage the transcriptional machinery and stimulate RNA Pol II-mediated transcription of the target gene. While enhancers are generally regarded to function as cis-acting elements, recent evidence suggests that some enhancers can act in trans to influence expression of their target gene on the homologous chromosome. Trans enhancer-promoter interaction in Drosophila, termed transvection, is relatively well characterised and has recently been visualised within developing embryos [2]. Few examples of trans interactions have been reported in vertebrates, although a recent analysis at the IGH super-enhancer indicates that trans-enhancer activity can occur in mammals [3].

The Nestin gene (Nes) encodes an intermediate filament protein and is widely expressed during embryonic development including progenitor cells throughout the neuroaxis [4, 5]. Differing reports of NES functionality have been published, with Mohseni, Sung [6] suggesting Nes is not essential for development of the central nervous system, in contrast to an earlier paper [7] indicating that loss of the gene results in embryonic lethality. The Nes neural enhancer [8] is a highly conserved element located in intron 2 and is commonly used to drive exogenous gene expression in neural progenitor cells in vivo and in vitro [9–11]. In vitro and transgenic data indicate that TFs belonging to the SOX and POU families bind the Nes enhancer and function synergistically to control the Nes expression in the CNS progenitors [12]. Consistent with these data, ChIP-seq experiments have identified robust binding of endogenous SOX3 protein at the Nes enhancer in cultured neuroprogenitor cells [13].

Traditionally, enhancers have been identified and characterized using transgenic reporter assays [14]. This has proven to be a useful approach to determine the contribution of specific enhancer elements to the spatiotemporal expression of its cognate gene. However, this strategy is incapable of recapitulating the endogenous genomic and chromatin environment in which the enhancer is usually located. CRISPR gene editing technology [15] enables rapid and efficient deletion of target sequences in vivo, providing a valuable tool to assess putative enhancer function in an endogenous context. Through the lack of enhancer activity the contribution of the enhancer to gene expression can be identified, this is an example of a ‘negative read-out’. This is an important advancement as the number of putative enhancers identified via bioinformatic and TF binding studies continues to grow, while functional studies are lagging.

Despite widespread use of the Nes neural enhancer, the contribution of this enhancer to Nes expression during development has not been studied, nor have the effects of removing the enhancer on the developing CNS. Here we show that CRISPR-mediated deletion of the Nes enhancer results in a significant reduction in mRNA expression as well as altered protein levels within the developing mouse central nervous system. Using CRISPR/Cas9, we also generate two Nes loss of function mouse lines and show that Nes KO mice are viable. Finally, we present evidence that the Nes enhancer is able to function in trans.

Materials and methods

Mouse generation

Mutant mice were generated by CRISPR microinjection as previously described [16, 17]. In brief, CRISPR guides were designed using the crispr.mit.edu tool to determine off-target scores. Guide RNA sequences (Nes enhancer deletion—TTTGCGGTCTGAAAAGGATT, AGAATCGGCCTCCCTCTCCG, Nes null lines—GGAGCTCAATCGACGCCTGG, GCACAGGAGACCCCTACTAAA) were annealed and ligated into px330 (Addgene, #42230) after digestion with BbsI (NEB) using Rapid Ligation Kit (ThermoFisher Scientific), and transformed into E. Coli using standard protocols. Plasmid was extracted from positive colonies using a Midi-Prep
kit (Qiagen). Primers were designed to incorporate T7 promoter sequence and tracR sequence, and PCR was performed on plasmid DNA with Phusion High Fidelity PCR Kit (NEB). PCR products were converted to RNA using the T7 RNA Transcription Kit (NEB) and purified with RNEasy Kit (Qiagen) to generate sgRNA. Cas9 mRNA was synthesised from the XhoI (NEB) digested pCMV/T7-hCas9 (Toolgen) using the Mmessage Mmachine T7 Ultra Transcription Kit (ThermoFisher).

BL6/2J females were superovulated with Pregnant Mare Serum Gonadotropin (PMSG) and human Chorionic Gonadotropin (hCG) prior to mating with BL6 males for zygote harvesting. Single cell zygotes were collected on the day of microinjection and treated with hyaluronidase to remove surrounding cumulus cells. Cytoplasmic injection was performed with CRISPR reagents (100ng/μL Cas9 mRNA, 50ng/μL sgRNA) before transfer into pseudopregnant CD1 females.

Genomic DNA was extracted from 3 week old tail or ear biopsies using KAPA Mouse DNA Extraction Kit (KAPA Biosystems) or High Pure PCR Template Kit (Roche).

Founder mice were genotyped using FailSafe PCR Kit (EpiCentre) and run on a 12% polyacrylamide gel for heteroduplex assay. The genotype of the founder mice was confirmed via Sanger sequencing after BigDye Terminator v3.1 (Applied Biosystems) PCR reaction using reverse primer.

Regular colony and embryo genotyping was performed with primers flanking deleted sequence (enhancer deletion line F-GCCCCAGTCAGTCTTCTGAG R-GCCACTGCGGAT CACTCTT, Nes null FS F1 -CTGCTGAGCTGGGATGATGC F2 -AGCTCAATCGACGCC TGGG R- GCATTTCTCTCCGGCTCGA, Nes null BD F- CTGCTGAGCTGGGATGATGC R- CTGCTGAGCTGGGATGATGC) using 2G Fast MasterMix (KAPA), or Buffer J (EpiCentre) with Taq Polymerase (Roche).

All mouse breeding and experimental work was performed at the University of Adelaide in accordance with relevant ethics approvals (S-201-2013 and S-173-2015) from the University of Adelaide Ethics Committee.

Tissue preparation
Heterozygous (WT/-255) males and females were time mated for embryo collection. Females were euthanised via cervical dislocation and embryos removed and stored in cold 1x PBS until dissected. Tails were removed and kept at -20˚C. Heads were removed and flash frozen on dry ice and kept at -80˚C for RNA extraction or kept overnight in 4% paraformaldehyde in PBS, washed 3x in PBS and cryoprotected overnight in 30% sucrose before flash freezing in OCT and stored at -20˚C for immunohistochemical analysis.

Immunohistochemistry
Trunks were sectioned at 16μm on a cryostat (Leica CM1900) and slides washed 3x 10mins in PBT (1xPBS, 0.25% Triton-X), blocked for 30min in Blocking Solution (1x PBS, 0.25% Triton-X, 10% Horse Serum) and then stained overnight with 20μL primary antibody diluted in Blocking Solution and kept in a humidified chamber at 4˚C. Slides were washed 3 x for 10mins in PBS. 200 μL of secondary antibody diluted in Blocking Solution was added to the slides and incubated in a dark humidified chamber for 4hrs at RT. Slides were washed 3 x for 10mins in PBS, dried, mounted with Prolong Gold Antifade + DAPI (Molecular Probes) and cover-slipped. Slides kept overnight in the dark before image acquisition using a Nikon Eclipse Ti Microscope using ND2 Elements software. Images were modified for colour, brightness and contrast using Adobe Photoshop v7 (Adobe Systems). Antibodies used were Anti-SOX3 (R&D Systems, AF2569, 1/200), Anti-Nestin (Abcam AB82375, 1/1000), Anti-CD31 (BD
Pharmingen 550274, 1/100). Secondary antibodies, Donkey anti-Goat-Cy3 (Jackson ImmunoResearch, 1/400), Donkey anti-Rat-Cy5 (Jackson ImmunoResearch, 1/400), Donkey anti-Rabbit-488 (Jackson ImmunoResearch, 1/400).

**In situ hybridization**

In situ hybridization probes were designed to target exon 4 of the Nes gene. Primers corresponding to the region of interest were used to PCR amplify WT mouse cDNA and incorporate a T7 promoter at the 5’ end. The PCR product was transcribed using the T7 IVT Kit (NEB), followed by DNase I (NEB) treatment and purification with an RNEasy kit (Qiagen). Embryo trunks were sectioned at 16μm on a cryostat (Leica CM1900) and stored at -20˚C. Prior to in situ hybridisation, slides were defrosted for 1hr at RT. The RNA in situ probe was denatured at 72˚C for 2 minutes and kept on ice. 100μL of hybridisation buffer containing 1ng/μL diluted riboprobe/slide was added to slides and kept in humidified chamber containing formamide overnight at 65˚C. Slides were washed 3 x 30 mins at 65˚C in Wash Buffer (50% Formamide, 5% 20x SSC), then 3 x 30mins washes in MABT (Maleic Acid Buffer + 0.1% Tween-20) at RT. Slides were blocked with 300μL Blocking Solution (Blocking Reagent, Sheep Serum, MABT) and kept in humidified chamber at RT for 2 hrs. 75μL of anti-DIG antibody diluted in Blocking Solution was added to slides followed by overnight incubation at RT in a humidified chamber. Slides were washed 4x 20min in MABT followed by 2x 10mins in Alkaline Phosphatase Staining Buffer (4M NaCl, 1M MgCl2, 1M Tris pH 9.5). Slides were then stained with 95μL staining solution (NBT, BCIP, Alkaline Phosphatase Staining Buffer), coverslipped, and kept in the dark at RT overnight. Staining solution was removed by washing with 3x 5mins in PBS. Slides were fixed with 300μL 4% PFA for 1hr in a sealed container. Fixative was washed off with 3x 10min PBS washes, and 50μL Mowiol added to each slide for mounting with coverslip. Slides were analysed using brightfield microscopy on Nikon Eclipse Ti Microscope using ND2 Elements software (Nikon). 3 embryos of each genotype were analysed, and representative images from 1 experiment are shown.

**qRT-PCR**

RNA was extracted from flash frozen embryo heads by using Trizol. Briefly, heads were homogenised in 500μL Trizol. 100μL chloroform was then added and centrifuged at 6000xg for 30mins. The aqueous layer was removed and an equal amount of 70% EtOH added. The solution was then loaded onto an RNEasy spin column and centrifuged at 13000 rpm for 1 minute. The column was washed with 2x Buffer RLT (Qiagen) and purified RNA eluted in 30μL of RNase free H₂O, and stored at -20˚C. RNA samples were converted to cDNA using AB Systems High Capacity RNA to cDNA Kit. SYBR Fast standard protocols were used for qPCR with samples run in quadruplicate. B-actin (F-CTGCCCTGACGCGCAGG, R- GATTC CATACCCAAGAAGGAAGG) was used to normalise cDNA levels across samples, and Nes primers used to measure expression levels across timepoints and samples (F-GCTTCTCTTGGCT TTCCCTGA; R- AGAGAAGGATGTGGGCTGA). Prism software was used for the statistical analysis of qPCR data. Unpaired t-tests were performed to determine if WT Nes expression was significantly different from enhancer deleted lines at each timepoint.

**Results**

Generating a Nes enhancer deletion mouse model

To investigate the role of the Nes enhancer in directing endogenous expression in vivo, we generated an enhancer deletion mouse model using CRISPR/Cas9 mutagenesis. Two gRNAs
flanking the *Nes* enhancer were microinjected into mouse zygotes with Cas9 mRNA. 21 founder mice were generated with a range of deletions that partially or completely deleted the *Nes* enhancer. We selected a single founder animal containing both 255bp and 208bp deletion alleles that encompassed all SOXB1 binding sites identified in the ChIP-Seq analysis (Fig 1A & S1 Fig). Independent lines were generated for each deletion (referred to hereafter as -255 and -208). Heterozygous and homozygous -255 pups and embryos were generated at expected ratios, from -255/WT breeding pairs indicating that viability was not compromised by the deletion mutation (Tables 1 and 2). No morphological abnormalities were identified in either line indicating that the enhancer deletion did not overtly impact development.

**Nes mRNA expression is reduced in enhancer deletion mice**

To determine the impact of enhancer deletion on *Nes* expression, qPCR was performed on -255 homozygous whole embryos (8.5 dpc) and embryonic heads (9.5 dpc-15.5 dpc; Fig 2A). No significant difference in *Nes* expression was detected in mutant embryos at 8.5 dpc. However, from 9.5 dpc significantly reduced levels of *Nes* mRNA were detected in the embryonic cranium. Notably, the greatest reduction in *Nes* expression was detected at 10.5 dpc, with mutant embryos expressing just 18% of *Nes* mRNA compared with WT controls. From 11.5 dpc, a gradual increase in expression was detected in mutants which by 15.5 dpc had recovered to 60% of wild type expression. A reduction in *Nes* expression was also observed in -208 homozygotes at 11.5 dpc (S2 Fig).

Next, we determined the spatial impact of enhancer deletion on *Nes* expression in the developing CNS (Fig 2B). For this experiment we analysed the spinal cord at 11.5 dpc as *Nes* is expressed in the nervous system. In non-mutant embryos, *Nes* expression is detected in the anterior spinal cord and caudal ganglia (S3 Fig). In contrast, in *Nes* enhancer depleted embryos, *Nes* expression is reduced in the anterior spinal cord but not in the caudal ganglia (S3B Fig). In addition, *Nes* expression is normal in the developing limb buds (S3B Fig).

|          | WT/WT | -255/-255 | -255/WT |
|----------|-------|-----------|---------|
| Observed | 16%(11)| 28%(19)   | 56%(38) |
| Expected | 25%(17)| 25%(17)   | 50%(34) |

p-value = 0.2437, chi-square value = 2.824.

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robustly expressed in a stereotypical pattern throughout the trunk at this stage due to the abundance of neural progenitors [18]. In situ hybridization was performed on the trunk sections of WT and homozygous enhancer deletion (-255) embryos. As expected, expression of Nes was detected throughout the spinal cord, with the highest levels confined to the lateral regions and the floor plate. In contrast, the spinal cord of enhancer-deleted embryos was virtually devoid of Nes mRNA, except for restricted expression in the floor plate and the lateral regions. Notably, lateral expression in the mesoderm was not noticeably diminished in mutant embryos, consistent with the neural-specific activity of the Nes enhancer in transgenic mice. Thus, deletion of the Nes neural enhancer results in a striking reduction in the level and extent of Nes expression.

NES protein expression is reduced in enhancer deleted mice

As Nes mRNA expression was significantly reduced in both the embryonic head and neural tube, we performed protein expression analysis in these regions to determine whether NES was similarly reduced. Both head and trunk transverse sections were prepared from WT and homozygous -255 embryos and co-stained with anti-NES (trunk and brain) and anti-SOX3 antibodies (trunk). We hypothesised that as the enhancer is controlled by the SOXB1 proteins binding to the region, that we would see little to no NES expression throughout the SOX3 expressing zones of the neural tube and brain.

The WT brain sections show the telencephalon is densely stained for Nestin, showing a long filamentous structure without nuclear staining (Fig 3A). In the homozygous enhancer deletion however, there are obvious differences in the staining pattern of the NES protein, as it is duller throughout the telencephalon, and shows regions of high reactivity that appear to be within the vasculature.

This experiment was repeated using neural tube sections, with SOX3 and NES antibodies, and similar results were obtained (Fig 3B). The WT embryos exhibit smooth filamentous NES staining from the lateral edges towards the midline. In contrast, NES signal in the -255/-255 embryos was weaker, particularly within the periluminal SOX3-positive region. Taken together with the mRNA expression analysis, these data confirm that NES expression is reduced in the developing nervous system of enhancer-deleted embryos.

Ectopic Nestin expression in vasculature of enhancer deleted embryos

Whilst analysing -255/-255 embryos for NES protein expression, we noted specific staining in discrete structures within the neural tube and cortex that appeared to be the developing vasculature. Notably, this signal was not present in WT or heterozygous embryos. To further investigate this finding, we co-stained 10.5 dpc embryo heads with antibodies to the endothelial cell marker CD31 and NES (Fig 4A). Images captured using an inverted fluorescence microscope indicated colocalisation of NES and CD31 in -255/-255 embryos but not in WT controls. Additional analysis using confocal microscopy revealed widespread expression of NES in endothelial cells lining the developing vasculature of -255/-255 embryos. In contrast, NES

| Table 2. Observed/Expected ratios of Nes enhancer deleted transient embryos. |
|-----------------------------|------------------|------------------|
|                             | WT/WT             | -255/-255        | -255/WT           |
| Observed                    | 21%(33)           | 30%(46)          | 49%(75)           |
| Expected                    | 25%(39)           | 25%(39)          | 50%(77)           |

p-value – 0.3168, chi-square value – 2.299.

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A. Nes expression in -255/-255 embryo heads

Relative Nes expression to wt/wt

| Age (dpc) | Expression | n |
|-----------|------------|---|
| 8.5       | **         | 3 |
| 9.5       |            | 5 |
| 10.5      | *          | 2 |
| 11.5      | ***        | 3 |
| 12.5      | ****       | 4 |
| 13.5      | ***        | 3 |
| 15.5      | **         | 4 |

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* p < 0.05 when compared to wt
# 8.5 dpc data from whole embryos

B. wt/wt vs. -255/-255

Images showing expression patterns for wt/wt and -255/-255 conditions.
expression was rarely detected in WT endothelial cells. Thus, deletion of the Nes neural enhancer induces ectopic expression in endothelial cells.

**Nes is not required for CNS development**

It has previously been reported that deletion of Nes causes extensive cell death in the developing CNS and embryonic lethality at approximately 8.5 dpc [7]. Given that -255/-255 mutants do not exhibit overt developmental defects, it appears that the level of Nes in these enhancer-deleted embryos exceeds the threshold required for normal development. We were therefore

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**Fig 2. Reduction of Nestin mRNA during embryonic development.**

A. Analysis of embryonic heads from aged 8.5 dpc to 15.5 dpc by qRT-PCR. All values are normalized to WT samples of the same developmental stage. Due to size constraints, whole 8.5 dpc embryos were used rather than embryonic heads. Nes expression is significantly reduced in -255/-255 embryos from 9.5 dpc-11.5 dpc. * indicates p-value <0.05, ** indicates p-value <0.01, *** indicates p-value <0.001, **** indicates p-value <0.0001. Error bars represent the standard deviation of the mean. B. In situ hybridization of Nes mRNA at 11.5dpc. Robust Nes expression is detected throughout the WT neural tube. Abbreviations; NT—neural tube, S—somite, FP—floor plate.

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**Fig 3. Immunohistochemical analysis of brain and trunk sections.**

Wildtype (WT/WT), heterozygous (-255/WT) and homozygous (-255/-255) transverse cortex (A) and trunk (B) sections labelled with anti-SOX3 and anti-NESTIN antibodies. The Nestin signal is decreased in the neural tube of the -255/-255 sections while lateral staining of NES in the somite regions also appears more distinct in the -255/-255 samples. The SOX3 remains consistent across genotypes. C. Schematic diagram of a mouse embryo, the lines indicate the approximate regions in which transverse sections are used for analysis.

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interested in assessing whether further reduction of Nes levels in -255/KO compound heterozygous embryos would compromise CNS development. To generate Nes knockout mice, we employed a dual gRNA deletion strategy (Fig 5A). The proximal gRNA targeted exon 1 immediately downstream of the start codon and the distal gRNA cut immediately upstream of the stop codon in exon 4 (S3 Fig). The rationale for this approach was that null alleles could be generated via frameshifting indels at the proximal cut site or from deletion of the ~8.7 kb intervening sequence between the proximal and distal cut sites. This approach also provided the necessary alleles for the trans enhancer interaction experiment (see below). PCR genotyping indicated that four of the six founder animals contained at least one large deletion allele. Sanger sequencing confirmed that the founder used for subsequent breeding carried the expected 8672 bp deletion that encompassed almost all of the coding region and introns of the Nes gene, including the neural enhancer in intron 2.

This null allele, Nes g.54_4518/p.L19_V150 6del/p.L19fsX, termed BD, encodes only the first 18AA of the NES open reading frame, and a frameshift causes the last 30AA of exon 4 to be incorrect. This founder also carried an 8bp frameshifting deletion, g.50_57del/p.R17fsX75, termed FS, at the proximal cut site that terminated the protein after 13 amino acids. Breeding colonies for each mutation were generated. Interestingly, FS homozygous mice were viable and did not exhibit any obvious phenotype or developmental defects such as embryonic lethality as observed in one previous report [7] (n = 2). Compound heterozygous FS/BD mice were also phenotypically normal (n = 2). These data are consistent with Mohensi et al. (6) who did not detect embryonic lethality in an independent KO line. To confirm that NES protein was not generated from the FS allele, we stained FS/FS embryonic brain sections with anti-NESTIN antibody (Fig 5B). In contrast to WT control tissue, no expression was detected in mutant tissue. We therefore conclude that Nes is not required for CNS development or viability.
Trans interactions of the Nes neural enhancer

While enhancers are generally considered to be cis-regulatory elements, previous studies have provided evidence for interchromosomal trans interaction between enhancers and their cognate promoters [19]. To investigate possible interchromosomal activity of the Nes neural enhancer in vivo, we used qPCR to measure allele-specific expression in a series of compound heterozygous embryos (Fig 6A–6C). For this experiment, we exploited the presence of the Nes enhancer in the FS allele but not the BD allele. Thus, any difference in Nes expression from the WT allele in FS/WT and BD/WT embryos would reflect trans activity of the (FS) Nes enhancer. Similarly, any difference in Nes expression from the -255 allele in FS/-255 and BD/-255 embryos would reflect trans activity of the (FS) Nestin enhancer. No detectable signal was generated from FS/BD embryos indicating that Nes mRNA is not generated from either null allele (presumably due to nonsense mediated decay for the FS allele). -255/-255 Nes expression was 23% of WT expression, consistent with our previous analysis (Fig 2A). Comparison of FS/-255 and BD/-255 expression revealed significantly higher expression in the former (17% vs 11%; p < 0.01). Similarly, WT/FS Nes expression was higher than WT/BD, although this did not reach significance (64% vs 49%; p < 0.07). Together, these data suggest that the Nes enhancer may function in trans.
Deletion of the Nestin neural enhancer in vivo

A. Controls
- FS
- BD
- WT

confirm NMD from FS/FS allele
baseline levels of 'promoter only' with no enhancer activity
confirm that a single wt allele is 50% of wt/wt

B. Experimental
- WT
- FS
- -255
- FS

Are both enhancers able to act on the same promoter?
can the enhancer from the FS allele influence the -255 allele?

C. Interchromosomal Interactions in 11.5 dpc heads

expression relative to wt/wt

WT/NT 255/255 WT/-255 WT/BD WT/FS BD/FS FSI/255 BDS/255

n=11 n=5 n=4 n=6 n=3 n=4

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Discussion

While enhancers are routinely used to drive spatio-temporally restricted expression of heterologous genes, their functional role in coordinating cognate gene expression remains poorly understood. Using CRISPR/Cas9 technology, we show that deletion of the *Nes* neural enhancer has a profound impact on endogenous *Nes* expression in the developing nervous system. Our data indicate that this region also contains a repressor element that inhibits expression in endothelial cells, underlining the ability of deletion analysis to identify both positive and negative regulatory interactions.

*Nes* is expressed within the incipient neural progenitor cells during early embryogenesis and is maintained during expansion of this cell population. Upon differentiation, *Nes* is down-regulated and is replaced by other members of the intermediate filament family [20]. At 8.5 dpc, *Nes* expression is not significantly different in -255 homozygous embryos indicating that the neural enhancer is not functionally required for initiation of *Nes* expression. Given that there is robust SOXB1 expression in neuroprogenitors at this stage, it appears that putative binding of these factors to the *Nes* enhancer at this stage is not required for expression but may nevertheless play a role in maintaining the locus in an “open for business” conformation [21]. From 9.5 dpc, *Nes* expression is significantly lower in -255 homozygous embryos indicating that the neural enhancer is not functionally required for expression within the head. The activity of the enhancer remains functionally significant until at least 15.5 dpc, although the differential between -255 and WT expression becomes less pronounced, suggesting that other neural enhancer(s) have increasingly important roles as the nervous system develops. It is interesting to compare our data with other recently published examples of developmental enhancer deletion. A study of single conserved limb enhancers showed that 90% of these had no impact on cognate gene expression [22], as well as highly conserved β-globin enhancers [23]. Computational analyses have predicted that most (96%) of enhancers within the genome are tolerant to LoF mutations, and that many essential genes will have a degree of enhancer redundancy [24]. In the vast majority of examples, deletion of a single conserved enhancer element has no impact on cognate gene expression [22, 23]. The Nestin enhancer therefore appears to be unusual in having such a profound impact on *Nes* expression during early embryonic development. The mechanism that underpins this unusually high activity remains to be determined, as well as elucidating other *Nes* regulatory elements that are responsible postnatally.

The expression of *Nes* mRNA throughout the neural tube is considerably affected in mutants lacking the 255bp enhancer, as seen in both *in situ* hybridization and qRT-PCR.
experiments. Decreased protein reactivity is seen in the -255 embryos, however the staining is still present throughout the neural tube where the mRNA is not visualized. This is possibly due to very low levels of Nestin expression within these cells, undetectable through \textit{in situ} hybridization. It is also expected that \textit{Nes} expression is controlled by other transcription factors other than SOX or POU proteins that are expressed within non-SOX/POU regions.

An unexpected finding of this study was that deletion of the \textit{Nes} enhancer resulted in ectopic expression within the vasculature. Previously, NES has been reported to be expressed within the vasculature of different tissues such as developing kidneys, and also shown to be upregulated within vasculature following focal cerebral ischemia \cite{25-27}, indicating a role in development and repair. Whilst the mechanism is unclear, it appears that the -255 deleted region also contains a repressor element that prevents NES expression in developing vasculature. While further studies are required to determine the protein-sequence interaction(s) that mediate this repressor activity, it is worth noting that unmasking of repressor elements cannot be achieved using traditional enhancer activity assays such as transgenic reporter analysis, highlighting the utility of the enhancer deletion approach we have employed.

Within the literature there are conflicting reports as to whether \textit{Nes} is an essential gene in mice. The first reported \textit{Nes} null line generated showed early embryonic lethality, and despite two further \textit{Nes} null lines showing homozygous viability, \textit{Nes} is often cited as an essential gene \cite{6, 7, 28, 29}. Through generation of two independent CRISPR KO mouse lines, we have shown that NES null mice are viable and do not exhibit overt deleterious phenotypes, consistent with two previous reports \cite{6, 28}. In contrast, the NES null mice reported by Park, Xiang \cite{7} exhibit embryonic lethality at 8.5dpc due to apoptosis of neural tube cells. The reason for this inconsistency remains unclear. Although not explored in this study, mild phenotypes such as impaired motor coordination \cite{6} in KO mice suggest that NES function cannot be entirely replaced by other members of the intermediate filament family.

It is often assumed that all enhancers only act in \textit{cis} to regulate cognate gene expression. However, it remains unclear whether some enhancers can also function in \textit{trans} to activate cognate target gene(s). \textit{Trans} enhancer interactions or transvection is well characterised in \textit{Drosophila} \cite{2, 30} but has rarely been observed in mammals \cite{3}. Utilising a genetic approach, we provide evidence that the Nestin enhancer can undergo functional \textit{trans} interactions \textit{in vivo}. While the effect is relatively weak, these data raise the possibility that transvection of developmental enhancers may be more common than is currently recognised. Further investigation using chromatin capture technology would be beneficial in characterising these putative \textit{trans} interactions. While this technology has been successfully used to characterise \textit{trans} interactions within \textit{Drosophila}, this has not yet shown interactions within the mouse \cite{31, 32}.

\section*{Supporting information}
\textbf{S1 Fig. -255/-255 mutation information.} The mutation generated via CRISPR removed 255bp of DNA within intron 2 of Nestin. (A) The deletion in shown in red text, with SOX sites in bold and POU sites in yellow. (B) Chromatogram of the Nestin deletion showing the position of the 255 deletion. (C) Genotyping gel showing band sizes of the WT/WT, WT/-255 and -255/-255 samples. Note heteroduplex band in the WT/-255.

\textbf{(TIF)}

\textbf{S2 Fig. Nestin \textit{\textit{-208 Line}.} The -208 Nestin enhancer deletion line shows a reduction in \textit{Nes} expression in 11.5 dpc embryonic heads similar to that of the -255 line (n = 2 embryos).}

\textbf{(TIF)}
S3 Fig. Nestin vasculature phenotype in neural tube. Nestin and CD31 expression within a 10.5dpc neural tube section. Within the WT sample no overlap is seen between CD31 and NES, while the -255/-255 sample shows co-localisation between the two proteins. (PNG)

S4 Fig. Nestin knockout mutation information. The Nestin ‘Big Deletion’ and ‘Frameshift Mutations’ generated by CRISPR. A. The FS mutation comprises an 8 bp deletion shown in red. B. The chromatogram file of the mutation. C. The amino acid sequence of the mutations generated by the FS mutation. D. The chromatogram file of the ‘Big Deletion’ incorporates an 8.6kb deletion that has been minimised for visualisation. E. The amino acid sequence of the BD mutation. The large 1824AA sequence within the WT sequence is not shown. (TIF)

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