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Natural Antisense Inhibition Results in Transcriptional De-Repression and Gene Upregulation

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Abstract

Here we demonstrate that natural antisense transcripts (NATs), which are abundant in mammalian genomes, can function as repressors of specific genomic loci and that their removal or inhibition by AntagoNAT oligonucleotides leads to transient and reversible upregulation of sense gene expression. As one example, we show that Brain-Derived Neurotrophic Factor (*BDNF*) is under the control of a conserved noncoding antisense RNA transcript, *BDNF*-AS, both *in vitro* and *in vivo*. *BDNF*-AS tonically represses *BDNF* sense RNA transcription by altering chromatin structure at the *BDNF* locus, which in turn reduces endogenous BDNF protein and function. By providing additional and analogous examples of endogenous mRNA upregulation, we suggest that antisense RNA mediated transcriptional suppression is a common phenomenon. In sum, we demonstrate a novel pharmacological strategy by which endogenous gene expression can be upregulated in a locus-specific manner.

Introduction

Although RNA interference and other technologies have provided useful tools for downregulation of mRNA and subsequently proteins, specific upregulation of individual genes remains challenging. Natural antisense transcripts (NATs) are transcribed from the opposite strand of many protein-coding (sense) genes and overlap in part with sense RNA, promoter region and their regulatory regions[12]. Here, we demonstrate a potent mechanism by which endogenous NATs suppress transcription of their sense gene counterparts. We show that endogenous gene expression can be upregulated, in a locus-specific manner by the removal or inhibition of the NATs, which are transcribed from most transcriptional units[1,3]. Our study provides examples of functional ncRNAs that regulate protein output, by altering

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chromatin structure and we posit that this phenomenon is applicable to many other genomic loci.

Brain-derived Neurotrophic Factor (BDNF) is a member of the "neurotrophin" family of growth factors, essential for neuronal growth, maturation[4,5], differentiation and maintenance[6]. BDNF is also essential for neuronal plasticity and shown to be involved in learning, and memory processes[7]. The *BDNF* locus is on chromosome 11 and shows active transcription from both strands, which leads to transcription of a noncoding NATs[8]. Here, we characterize the regulatory role of this antisense RNA molecule, *BDNF*-AS that exerts a potent reciprocal and dynamic regulation over the expression of sense *BDNF* mRNA and protein, both *in vitro* and *in vivo*. We also report similar upregulation of Glial-derived Neurotrophic Factor (*GDNF*) and Ephrin receptor B2 (*EphB2*), by blocking endogenous NATs. We introduced a novel strategy for upregulation of mRNA expression, using antisense RNA transcript inhibitory molecules, which we term AntagoNATs.

Results

Genomic organization of the human *BDNF* locus

BDNF mRNA as well as *BDNF* antisense RNA (*BDNF*-AS, also annotated as *BDNF*-OS) displays a complex splicing pattern; however, all variants share a common sense-antisense overlapping region[8,9]. The transcription start site (TSS) of human *BDNF*-AS is approximately 200 kb downstream from the *BDNF* promoter and it is located on the positive strand of chromosome-11. Transcription from this site gives rise to 16–25 splice variant long ncRNAs with 6–8 exons[8]. Exon-5 of *BDNF*-AS, which contain 225-nucleotides of full complementarity to *BDNF* mRNA (overlapping) and exon-4 (non-overlapping) are common between all these variants (Fig. 1a). Nucleotide sequence of human *BDNF*-AS is provided in supplementary data file 1. *BDNF* mRNA is transcribed from the negative strand of chromosome-11 and shows 11 alternative splicing patterns and one coding exon. All variants of *BDNF* mRNA also share the 225-nucleotide overlapping region with the *BDNF*-AS transcript. Our next generation sequencing (deep sequencing) data confirms expression of *BDNF*-AS transcript in human brain RNA samples (Fig. 1a inset data). Therefore, *BDNF*-AS has the potential to form an *in vivo* RNA-RNA duplex with *BDNF* mRNA through 225 complementary nucleotides overlap.

Identification of mouse *Bdnf*-AS transcript

Although several human EST's have been reported to have the potential of forming sense-antisense pairs with *BDNF* transcripts[8], the mouse antisense transcript was not previously identified and thus *BDNF*-AS was erroneously reported as a primate-specific transcript by others[9]. Using 5' and 3' Rapid amplification of cDNA ends (RACE) experiments; we identified the mouse *Bdnf*-AS transcript (Fig. 1b). Based on RACE data we designed primers and probes for detection of mouse *Bdnf*-AS by real-time PCR (RT-PCR) experiments. RACE experiments followed by sequencing and RT-PCR indicate the existence of a conserved noncoding antisense transcript to the mouse *Bdnf* mRNA. The mouse *Bdnf*-AS transcript has two splice variants with 1–2 exons and 934-nucleotide complementarity to *Bdnf* mRNA (Fig. 1b). Nucleotide sequence of mouse *Bdnf*-AS is

provided in Supplementary data file-2. Although the architectures of *BDNF*-AS in human and mouse are not entirely similar, the 225 bp overlapping region is almost identical between these two species (90% homology across the two species), suggesting the presence of an evolutionarily conserved functional binding domain.

Expression analysis of *BDNF* and *BDNF*-AS

We assessed expression of *BDNF* and *BDNF*-AS transcripts in various human (adult and embryonic), as well as monkey and mouse tissues, by RT-PCR and RNA fluorescence in situ hybridization (FISH). We also measured the absolute expression of *BDNF* and *BDNF*-AS transcript by generating standard curves, using DNA vectors containing cDNA of each transcript (Supplementary Fig. 1). *BDNF* mRNA levels are generally 10–100 fold higher than *BDNF*-AS transcript, except in testis, kidney and heart, which contain equal or higher levels of *BDNF*-AS. Both transcripts are expressed in brain, muscle and embryonic tissues (Supplementary Fig. 2). *BDNF* mRNA levels were relatively low in all post-natal tissues examined except in brain, bladder, heart and skeletal muscle (Supplementary Fig. 3). We examined the expression pattern of sense and antisense transcripts in rhesus monkey (Supplementary Fig. 4) and mouse tissues by RT-PCR (Supplementary Fig. 5) and RNA FISH (Supplementary Fig. 6). Both transcripts are co-expressed in many tissues, which suggest *BDNF*-AS potential for regulation of *BDNF* mRNA.

Knockdown of *BDNF*-AS increases *BDNF in vitro*

Transfection of several human and mouse cell lines including HEK293T cells with three independent siRNA molecules that targeted to non-overlapping regions of the *BDNF*-AS transcript, shown by asterisks in Figure 1b, resulted in over 85% knockdown of *BDNF*-AS transcript, accompanied by 2 to 6 fold upregulation of the *BDNF* transcript (Fig. 2a). Sequence information of these siRNAs as well as scrambled controls, AntagoNATs and other oligonucleotides are listed in supplementary Table S1. The upregulation of *BDNF* was not related to the choice of endogenous controls (Supplementary Fig. 7). *BDNF*-AS is a very low abundance transcript and therefore, in order to reliably detect the transcript, avoiding false signals from genomic DNA contamination and as control for RT-PCR reactions, we included no reverse transcriptase (NRT) and no template (NTC) controls in our experiments. Additionally, we tested the final PCR products for each set of primers and probe on a gel to ensure that only one product was amplified (Supplementary Fig. 8).

To monitor the sequential events after administration of *BDNF*-AS-targeted siRNA, we performed a time-course study (Fig. 2b) in which we collected HEK-293 cells and assessed the (endogenous) expression of both *BDNF* and *BDNF*-AS transcripts at several time points after treatment. We observed downregulation of *BDNF*-AS at 6 h with maximum efficacy between 24–48 h and it lasting up to 72 h (Fig. 2b). Upregulation of *BDNF* mRNA only started at 18 h and reached the maximum at 48 h then decreased by 72 h, the expression returning to pre-treatment levels by 96 h. These data show that the observed increase of *BDNF* commences sequentially after reduction of the *BDNF*-AS transcript and displays full reversibility.

Moreover, we examined BDNF protein levels following treatment of HEK-293 cells with two active *BDNF*-AS siRNAs, control siRNA or scrambled siRNAs by ELISA (Fig. 2c). We measured BDNF protein level by western blot following transfection of cells with *BDNF*-AS siRNA-1 or control siRNA (Fig. 2d). Both ELISA (Fig. 2c) and western blotting (Fig 2d) experiments demonstrated that siRNA targeting *BDNF*-AS significantly increased the expression of BDNF protein. We observed that the magnitude of BDNF protein upregulation (~2 fold) was somewhat lower than with mRNA upregulation (2–6 fold); this suggests that protein upregulation kinetics lags that of mRNA in these cells or possibly an involvement of post-transcriptional regulatory mechanisms aside from antisense RNA to control BDNF protein output, including, for example, microRNA regulation[10–12] (Supplementary Fig. 9).

BDNF-AS did not alter *BDNF* sense RNA stability

In order to examine the effects of *BDNF*-AS transcript on the stability of *BDNF* sense transcript, we depleted *BDNF*-AS with siRNA then treated cells with α-amanitin. We found that the baseline half-life for *BDNF*-AS was 15.3 h, nearly 3 h longer than that of the *BDNF* sense transcript (Supplementary Fig. 10). There was no significant change in *BDNF* sense RNA stability after reduction of *BDNF*-AS transcript, suggesting that unlike some other highly abundant antisense transcripts[13], *BDNF*-AS does not alter *BDNF* sense mRNA stability. Recently it has been suggested that some NATs can produce endogenous siRNAs from the overlapping region between sense and antisense RNAs. Indeed Watanabe *et al.* published a list of endogenous siRNAs from mouse oocytes[14]. We examined this list but we were not able to find any endogenous siRNAs originating from the *Bdnf* locus.

Targeting of *BDNF*-AS by AntagoNATs

We introduce the term AntagoNAT here to describe single-stranded oligonucleotide molecules that inhibit sense-antisense interactions (with different modifications, see supplementary methods). We hypothesized that use of AntagoNATs should have a similar outcome on *BDNF*-AS as that observed with *BDNF*-AS siRNA and designed gapmer single–stranded oligonucleotides, 14 nucleotides in length, with 2'O-Methyl RNA and/or locked nucleic acid (LNA) modifications. Using this strategy, we tiled the entire overlapping region between human *BDNF*-AS and *BDNF* transcripts and identified several efficacious AntagoNATs capable of upregulating of *BDNF* mRNA. h*BDNF*-AntagoNAT1 and h*BDNF*-AntagoNAT4, targeting the first part of the overlapping region, produced the largest response (Supplementary Fig. 11). Our data suggests that blockage of *BDNF* antisense RNA, by single-stranded AntagoNATs, is sufficient in causing an increase in *BDNF* mRNA.

We then designed single-stranded gapmer LNA-modified[15] DNA oligonucleotides (AntagoNATs) 16-nucleotides in length with phosphorothioate backbone, complementary to mouse *Bdnf*-AS. Two AntagoNATs (m*Bdnf*-AntagoNAT3 and m*Bdnf*-AntagoNAT9) consistently showed a statistically significant increase in *Bdnf* mRNA levels in mouse N2a cells (Supplementary Fig. 12). In order to establish an optimal dosage for further *in vitro* studies, we performed concentration-response experiments with 11 different concentrations (1:3 serial dilutions ranging from 300 nM to 5 pM) of mBdnf-AntagoNAT9, measuring fold changes in *Bdnf* mRNA levels (Fig. 2e). A concentration-dependent increase in *Bdnf* mRNA levels at 1–300 nM with EC_{50} of 6.6 nM was determined.

Knockdown of *GDNF*-AS increases *GDNF* mRNA *in vitro*

We selected another low abundance noncoding antisense RNAs that is transcribed from opposite strand of *GDNF* at chromosome 5 and designed several AntagoNATs targeting the *GDNF* antisense transcript. We found two AntagoNATs that significantly increase the *GDNF* mRNA by 3–4 fold (Fig. 2f). Additionally, we show AntagoNAT-mediated upregulation of *EphB2*, a member of a different gene family (Supplementary Fig. 13). These results suggest that antisense RNA mediated transcriptional suppression is a widespread phenomenon in the mammalian genome.

Bdnf upregulation increases neuronal outgrowth

Consistent with many previous reports that indicate stimulatory effects of *Bdnf* on neuronal outgrowth and adult neurogenesis[16–17], we found that an increase in the endogenous *Bdnf* level due to the knockdown of *Bdnf*-AS transcript resulted in increased neuronal cell number and in neurite outgrowth and maturation at 3 and 7 days post-plating in neurospheres (Fig. 3 a–d). These data suggest that the upregulation of endogenous *Bdnf*, due to inhibition of antisense RNA, induces neuronal differentiation in neuronal progenitor cells and might cause a mature phenotype in nascent neurons.

Knockdown of *Bdnf*-AS increases *Bdnf in vivo*

We utilized osmotic mini-pumps for intracerebroventricular (ICV) delivery of mBdnf-AntagoNAT9 to C57BL/6 mice. We selected mBdnf-AntagoNAT9, which is targeting a non-overlapping region of mouse *Bdnf*-AS, over other active AntagoNATs, based on its high efficacy to increase in *Bdnf* mRNA *in vitro*. After 28 days of continuous AntagoNAT infusion, *Bdnf* mRNA levels were increased across forebrain regions adjacent to the third ventricle in mice treated with mBdnf-AntagoNAT9 as compared to levels unaltered by an inert control oligonucleotide (Fig. 4a,b). *Bdnf* and *Bdnf*-AS transcripts were unaltered in the hypothalamus, a structure that is not immediately adjacent to the third ventricle (Fig. 4c). Moreover, we find that AntagoNAT-mediated blockade of *Bdnf*-AS results in increased Bdnf protein levels (Fig 4 d,e). These findings correspond with the *in vitro* data described above and indicate that the blockade of *Bdnf*-AS results in the increase of *Bdnf* mRNA and protein expression *in vivo*.

We injected BrdU in the mice treated with mBdnf-AntagoNAT9 in the first week of the study for 5 days. After 28 days of continuous AntagoNAT infusion, we performed histological examination of brain tissues and quantified neuronal proliferation and survival using Ki67 and BrdU markers, respectively. In mice treated with m*Bdnf*-AntagoNAT9, we observed an increase in Ki67 positive (proliferating) cells as compared to control treated mice (Fig 5a,b). We quantified the number of Ki67 positive cells and found a significant increase in cell proliferation in mice treated with m*Bdnf*-AntagoNAT9 compared to control oligonucleotide (Fig. 5c). In mice treated with m*Bdnf*-AntagoNAT9, there was a significant increase in BrdU incorporation (surviving cells) as compared to the control oligonucleotide-treated mice (Fig. 5d). There were no differences in hippocampal volume between control and m*Bdnf*-AntagoNAT9 treated mice (Fig. 5e). These findings demonstrate that *Bdnf*-AS regulates *Bdnf* levels *in vivo*.

BDNF-AS induces repressive chromatin marks

We measured the association of repressive (H3K9met3, H3K27met3) and active (H3K4met3, H3K36met3) chromatin marks to the *BDNF* genomic locus (Fig. 6a). Treating with siRNA, intracellular *BDNF*-AS was depleted and chromatin immunoprecipitation (ChIP) assays were performed. DNA was extracted and analyzed using 16 primer sets matching regions along the entire *BDNF* locus including the sense-antisense (S-AS) overlapping and *BDNF* promoter region (Fig-6a). Primers were designed to span the entire *BDNF* locus, at 20 kb increments, covering more than 270 kb and extending through to neighboring genes LIN7C and KIF18A. Initially, we performed RT-PCR on RNA samples and found that the knockdown of *BDNF*-AS transcript increases *BDNF* mRNA without any effects on neurotrophic tyrosine kinase, receptor, type 2 (*TrkB*) or on neighboring genes (*LIN7C* and *KIF18A*) in both directions (Supplementary Fig. 14). Next, we studied the immunoprecipitated DNA, using individual primer sets using RT-PCR and found that the siRNA-mediated depletion of the *BDNF*-AS transcript causes a significant reduction in H3K27met3 association in both the sense-antisense overlapping region and in the upstream *BDNF* promoter region (Fig. 6b). We found similar reduction in H3K27met3 binding to the promoter of the mouse *Bdnf* gene upon treatment of N2a ells with mBdnf-AntagoNAT9 (Supplementary Fig. 15). Furthermore, we found that alterations in repressive marks were specific to H3K27met3 as we were not able to detect significant changes in H3K9met3, H3K4met3 and H3K36met3 (Supplementary Fig. 16). These data suggest that *BDNF*-AS might play a role in the guidance, introduction and maintenance of H3K27met3 at the *BDNF* locus.

We observed that the ablation of Ezh2 activity using two different siRNAs phenocopied the *BDNF*-AS knockdown effect and caused the upregulation of *BDNF* mRNA (Fig. 6c). A ChIP assay was performed using the Ezh2 antibody. ChIP data revealed a significant reduction in Ezh2 binding at the *BDNF* promoter, upon depletion of *BDNF*-AS by siRNA (Fig. 6d). However, not all 16 primer sets gave detectable PCR signals, which could be attributed to the lack of direct Ezh2-chromatin binding. Given the reduction of binding of Ezh2 and the loss of the H3K27met3 at the *BDNF* promoter when *BDNF*-AS is knocked down, we conclude that *BDNF*-AS represses chromatin by recruiting Polycomb Repressive Complex 2 (PRC2) to the *BDNF* promoter region. Removal or inhibition of *BDNF*-AS could lead to the locus-specific upregulation of *BDNF* mRNA and protein.

Discussion

The number of ncRNAs in eukaryotic genomes have been shown to increase as a function of developmental complexity[18,19] and there is, for example, a great deal of diversity in ncRNAs expressed in the nervous system[20,21]. Over the past few years, we and others have reported on functional NATs and showed their potential involvement in human disorders, including Alzheimer's disease[13], Parkinson's disease[22] and Fragile X syndrome[23]. Moreover, previously we showed that upregulation of CD97 sense gene can be attained by knockdown of its antisense RNA transcript[1]. Upregulation of progesterone receptor (PR), and other endogenous transcripts was reported following targeting of promoter-derived noncoding RNAs[24,25]. Transcriptional activation of p21 gene[26] and Oct4 promoter[27] were reported

following NATs depletion. Antisense RNA-induced chromatin remodeling seems to be a feasible and dynamic mode of action for many low-copy number NATs[2,28]. If so, antisense RNA might predominantly exert local effects to maintain or modify chromatin structure, ultimately activating or suppressing sense gene expression.

PCR2 is a protein complex that consists of four core subunits: Eed, Suz12, RbAp48 and the catalytic Ezh2, that catalyzes the trimethylation of histone H3-lysine 27 (H3K27met3)[32]. Recent studies provide evidence for direct RNA-potein interaction between Ezh2 and many ncRNA transcripts[29]. Other studies of X inactivation[30] and HOX gene cluster[31] show RNA transcripts to be involved in the PRC2-mediated induction of H3K27met3, repressive chromatin marks. PRC2 transcriptome profiling has identified over 9,000 PRC2-interacting RNAs in embryonic stem cells, many of them categorized as antisense RNA transcripts[29]. Epigenetic silencing of p15 and DM1 genes were reported to involve heterochromatin formation by its antisense RNA[3334]. The traditional binary division of chromatin into *hetero*- or *eu*-chromatin categories might not be complete as recent work has shown that there are five principal chromatin types[49] that are more dynamic and flexible than originally believed. Likely applicable to a large number of gene loci, NATs can be manipulated in order to obtain a locus-specific alteration in chromatin modification. As examples, we show here that cleavage (by siRNA) or inhibition (by AntagoNATs) of the antisense transcripts of *BDNF*, *GDNF* and *EphB2* genes leads to the upregulation of corresponding mRNAs.

Neurotrophins belong to a class of secreted growth factors that enhance the survival, development, differentiation and function of neurons and BDNF is an important molecular mediator of synaptic plasticity[35–37]. BDNF is suggested to synchronize neuronal and glial maturation[38], participate in axonal and dendritic differentiation[39] and protect and enhance neuronal cell survival[40,41]. Neurotrophin expression levels are impaired in neurodegenerative[42–45] and in psychiatric and neurodevelopmental disorders[46–48]. The upregulation of neurotrophins is believed to have beneficial effects on several neurological disorders. AntagoNATs can potentially be used as a therapeutic strategy to inhibit *BDNF*-AS and consequently enhance neuronal proliferation and survival in a variety of disease states. It cannot be excluded that the herein described approach to upregulate the synthesis of endogenous BDNF molecules, presumed to contain natural modifications and to represent all known splice forms, will prove to be distinct, and perhaps superior, to administrating synthetic BDNF molecules.

Many low abundance noncoding antisense RNAs, which are transcribed on the opposite strands of genomic loci, endogenously suppress corresponding sense gene expression; inhibition or removal of antisense transcript leads to locus-specific upregulation of sense mRNA, protein and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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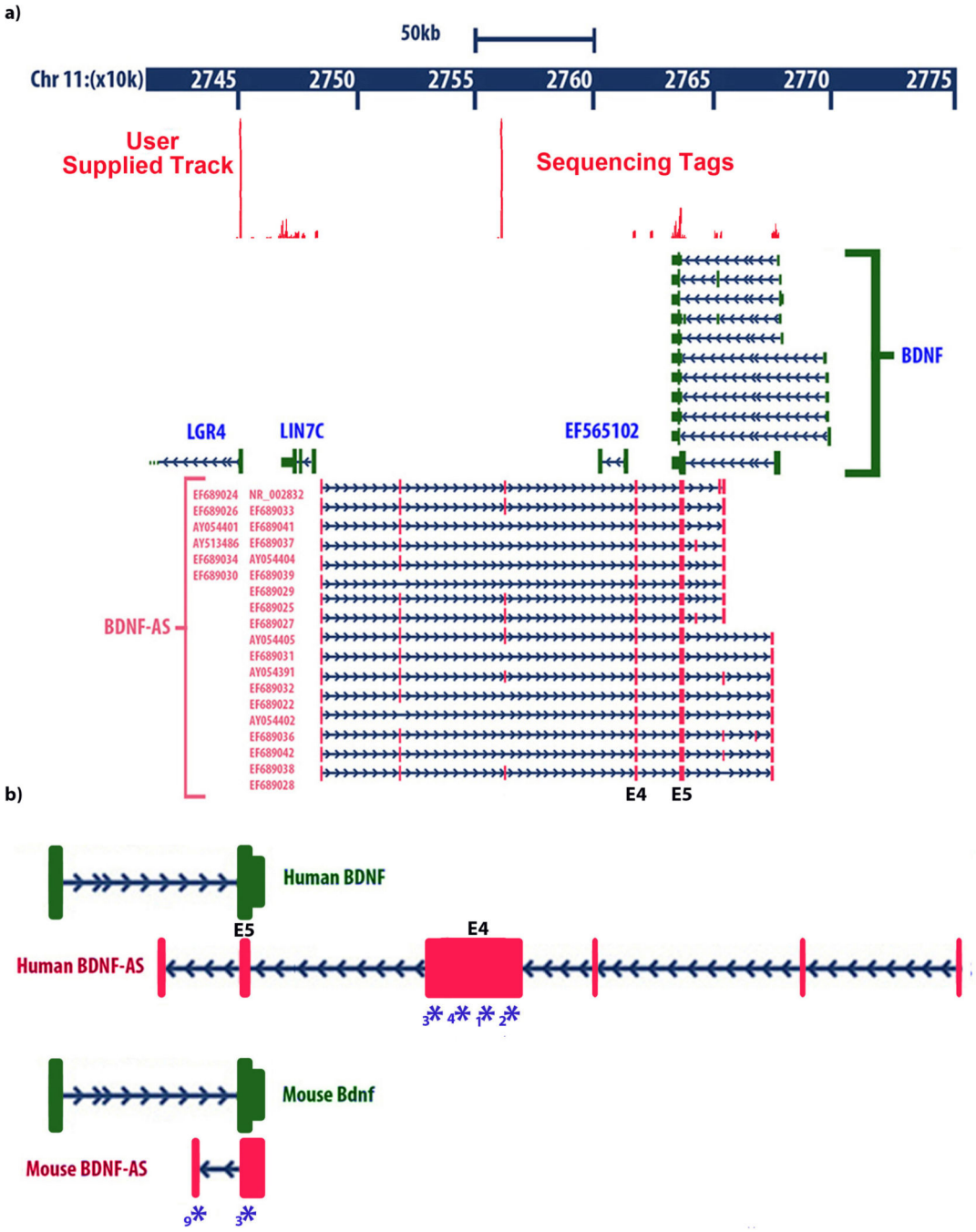

Figure 1. Genomic organization of the human *BDNF* locus showing

(A) genomic location of the *BDNF* sense and antisense transcripts and their relation to the other neighboring genes on chromosome 11. Solid boxes show exons and arrows show introns and direction of transcription. Different splice variants of *BDNF*-AS transcript are transcribed from the opposite DNA strand as that of *BDNF* mRNA. All *BDNF*-AS splice variants have a common exon that overlaps with 225 bp of all variants of *BDNF* mRNA. Inset data: Sequence tags generated by next-generation sequencing (RNA deep-seq), derived

from human entorhinal cortex are aligned to the UCSC genome browser. Peaks represent nucleotide coverage, indicating reliable detection of *BDNF*-AS exons.

(B) **Identification of mouse *Bdnf*-AS by RACE experiments followed by sequencing**. Proportional drawing representing human and mouse *BDNF* loci, showing direction of transcription for both *BDNF* and *BDNF*-AS transcripts, as well as the potential overlapping region. Mouse *Bdnf*-AS transcript is shorter than that of human and contains 1–2 exons. The potential overlapping region is bigger in mouse (934 bp compared to 225 bp in human), but the common overlap shows 90% homology across the two species. Real-time PCR (RT-PCR) primers, probes, and siRNAs are designed to target the non-overlapping parts of both transcripts. Blue Asterisks and numbers below each denote target sites of siRNAs used in this study, as well as m*Bdnf*-AntagoNAT3(*3) and m*Bdnf*-AntagoNAT9 (*9).

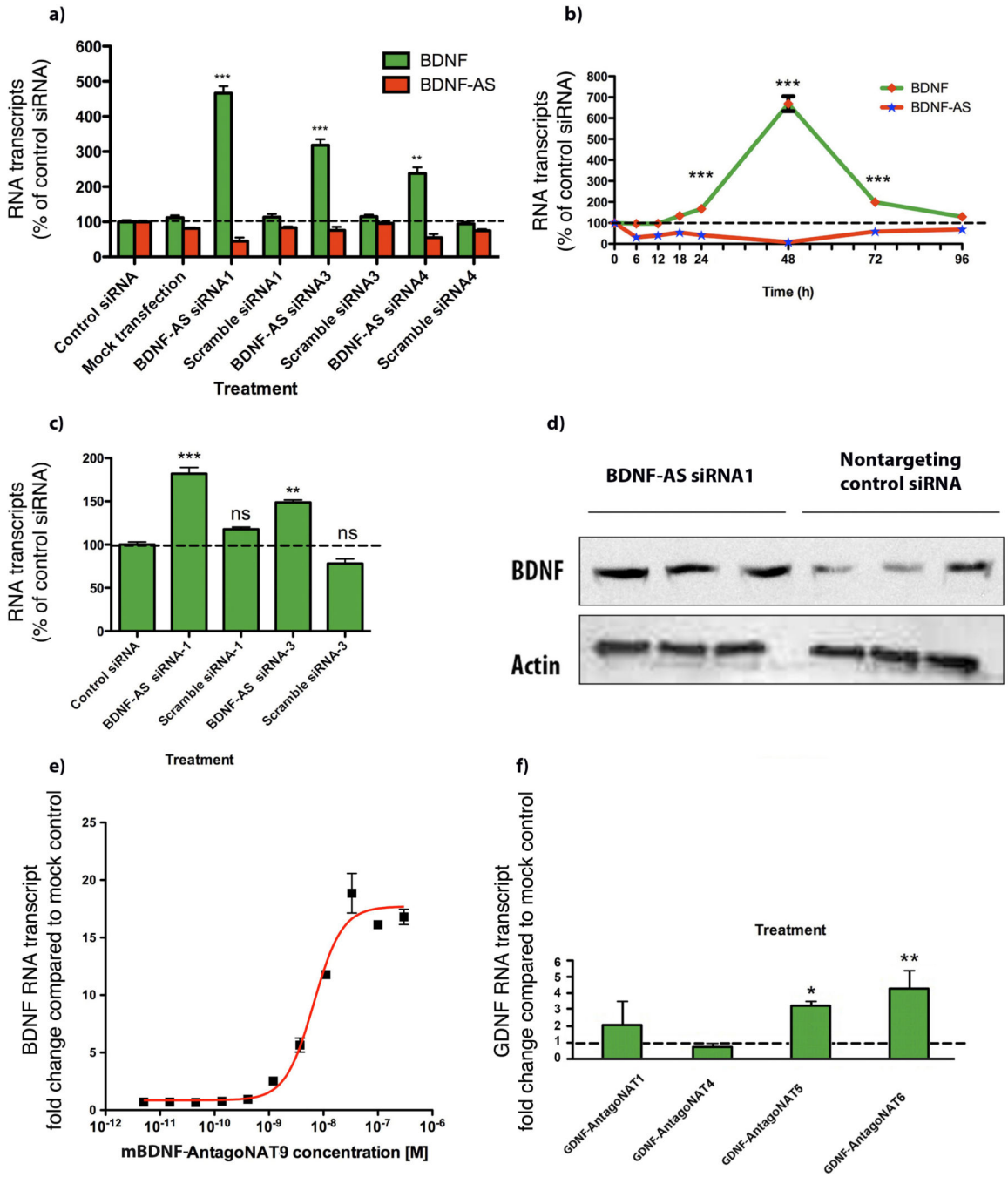

Figure 2. Antisense-mediated regulation of sense mRNA and protein

(A) Knockdown of brain derived neurotrophic factor (*BDNF*) natural antisense transcript, *BDNF*-AS, in HEK293T cells (n=12 per treatment) with each of three unique siRNAs (10 nM) targeting the non-overlapping region of *BDNF*-AS transcript, caused 2–6 fold upregulation of *BDNF* (sense) mRNA (n=6 for each data point/treatment ***= $P < 0.001$, **= $P < 0.01$). Similar results were obtained from experiments using Human cortical neuron (HCN), glioblastoma (MK059) cells, mouse N2a cells and neurospheres “data not shown”. Scrambled sequences, mock transfection and control siRNAs were used as controls. Control

siRNA for this and other experiments is an inert siRNA (CCUCUCCACGCGCAGUACATT) that does not target any known sequence in the mammalian genome. All measurements were normalized to the 18S rRNA and graphed as a percentage of each mRNA to the negative siRNA control sample.

(B) We assessed changes in *BDNF* and *BDNF*-AS transcripts over a period of time, following *BDNF*-AS knockdown (n=6 for each data point/treatment). siRNA knockdown of human *BDNF*-AS resulted in efficient and consistent downregulation of *BDNF*-AS, starting at 6 h and continuing on to 72 h. *BDNF* mRNA levels rose at 18 h, remaining high for more than 72 h, reversing to pre-treatment levels at 96 h. Note that the peak at 48 h is consistent and reproducible. Although *BDNF*-AS knockdown begins after 6 h, upregulation of *BDNF* started 18 h post-treatment. This time lag between the depletion of *BDNF*-AS and the increase of *BDNF* mRNA shows the sequential order of events indicating that the cells require time to adapt to the removal of the antisense transcript before upregulating *BDNF*.

(C) siRNA-mediated knockdown of *BDNF*-AS transcript caused an increase in BDNF protein levels measured by ELISA. Cells were transfected with 10 nM of two active siRNAs for *BDNF*-AS, scrambled siRNAs or a control siRNA for 48 hours. The supernatants of these cells were concentrated and analyzed for BDNF protein by ELISA, using a commercially available kit. BDNF protein was significantly increased (n=6 per treatment, ***=$P < 0.0001$, **= $P<0.001$) with siRNA targeting *BDNF*-AS transcript.

(**D**) Western blots confirmed that knockdown of the non-protein-coding *BDNF*-AS, with *BDNF*-AS siRNA1, but not control non-targeting siRNA transcript increased BDNF protein levels without changing the levels of beta-actin. Collectively, these data suggest that there is a discordant relationship between the sense and antisense *BDNF* transcripts in which *BDNF*-AS suppresses the expression of *BDNF* mRNA and protein. Removal of this negative regulatory effect, by *BDNF*-AS knockdown, causes upregulation of *BDNF* mRNA and protein levels.

(E) Dose-dependent increases in *Bdnf* following *Bdnf*-AS depletion: We performed dose-response experiments using 11 different concentrations (1:3 serial dilutions ranging from 300nM to 5pM) of m*Bdnf*-AntagoNAT9 (n=6 per data point/treatment) and we observed a dose-dependent increase in *Bdnf* mRNA levels at 1–300 nM concentration with an EC_{50} of 6.6 nM.

(F) Selective knockdown of GDNF-AS increases GDNF mRNA: We treated cells with various AntagoNATs targeting a low abundance noncoding antisense RNA, GDNF-AS. We observed that GDNF-AntagoNAT5 and GDNF-AntagoNAT6 increase the GDNF mRNA by 3–4 fold (n=6 per treatment *= $P < 0.05$, **= $P < 0.01$).

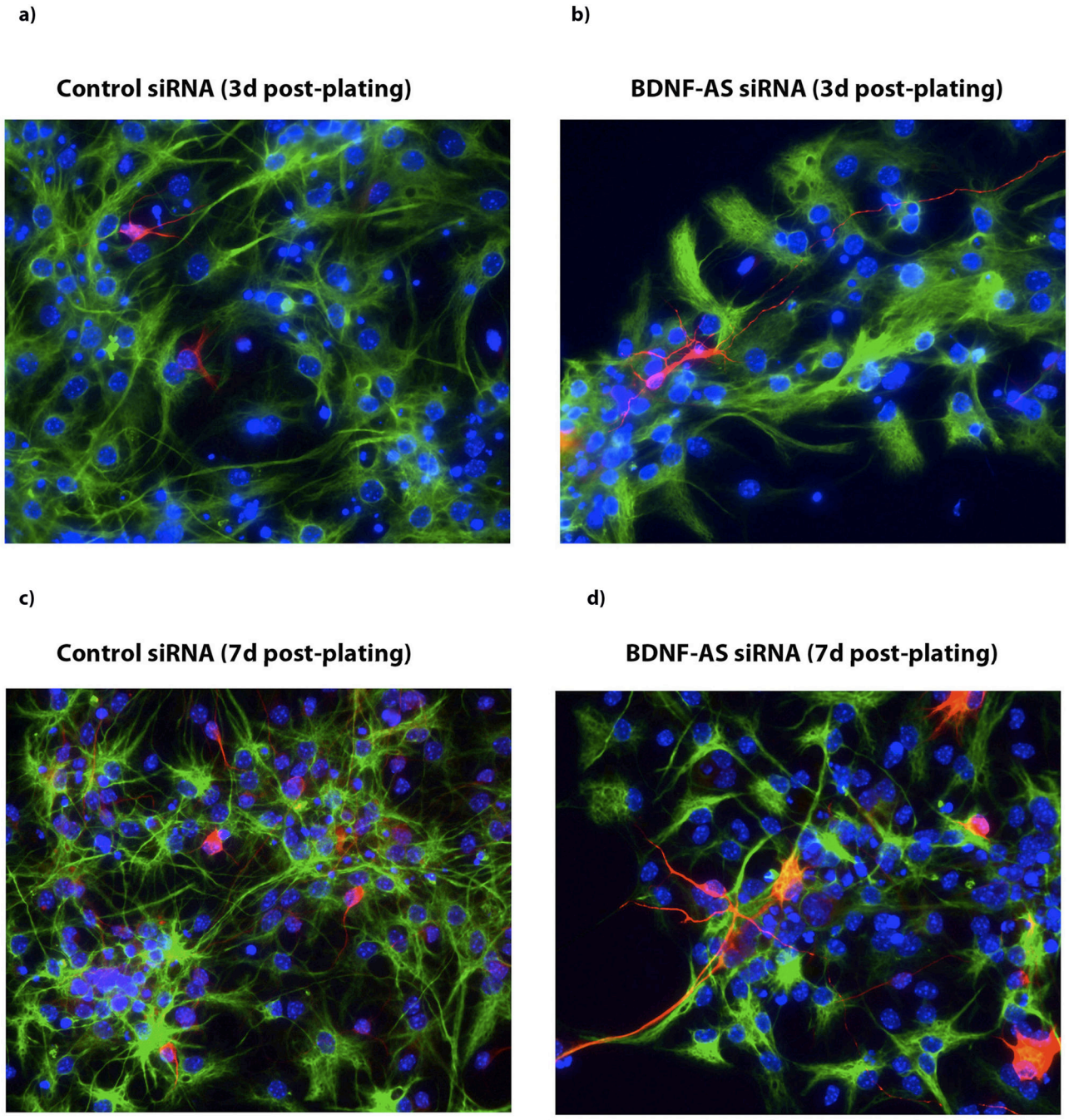

Figure 3. *Bdnf* upregulation increases neuronal outgrowth

(A–B) Immunocytochemistry images of hippocampal neurospheres treated with either control siRNA (A) or *Bdnf*-AS siRNA (B) 3 d post-platting. (C–D) Immunocytochemistry images of neuronal maturation and neurite outgrowth in hippocampal neurospheres treated with either control siRNA (C) or *Bdnf*-AS siRNA (D) 7 d post-plating. Treatment of cells with siRNA targeting the *Bdnf*-AS transcript resulted in increased neuronal cell number as well as increase in neurite outgrowth and maturation, both at 3d or 7d post-plating neurospheres. B-tubulin III stained red, GFAP stained green and DAPI stained blue.

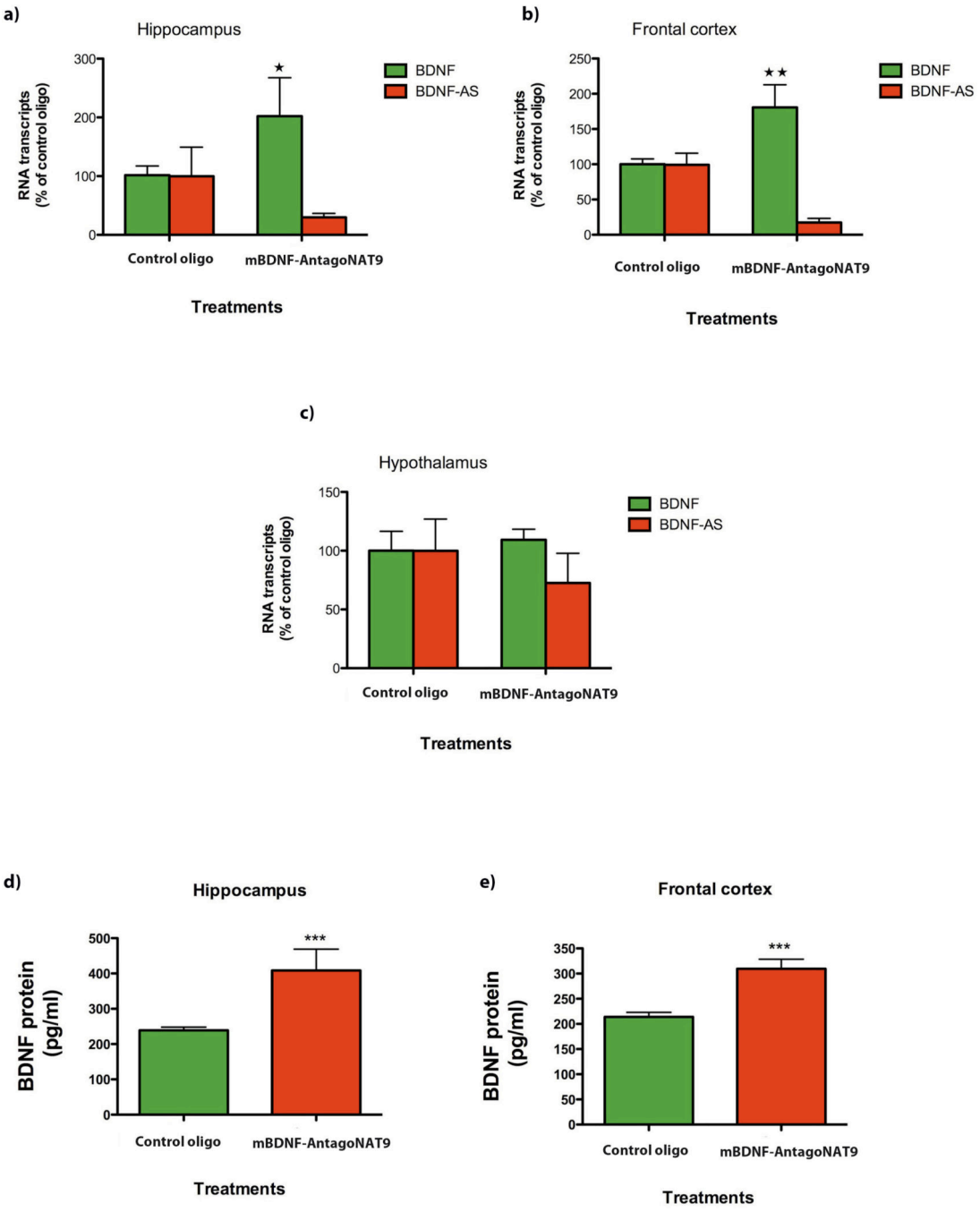

Figure 4. *Bdnf*-AS regulates *Bdnf* mRNA and protein *in vivo*

(A–C) Using osmotic mini-pumps, we infused m*Bdnf*-AntagoNAT9 (CAACATATCAGGAGCC) or control oligonucleotide (CCACGCGCAGTACATG) constantly over a period of 28 d, into the third ventricle of mouse brain (n=5 per treatment group *= P < 0.05, **= P < 0.01, ***= P < 0.001). m*Bdnf*-AntagoNAT9 directed against *Bdnf*-AS but not the control oligonucleotide resulted in an increase in *Bdnf* levels in the hippocampus (A) and frontal cortex (B). In the hypothalamus (C) both transcripts were

unchanged, as was expected for a tissue that is not directly connected to the third ventricle of the brain.

(D–E) We assessed BDNF protein levels by ELISA and found that m*Bdnf*-AntagoNAT9 treatment results in an increase in BDNF protein, both in the hippocampus (D) and frontal cortex (E), as compared to control oligonucleotide treated mice.

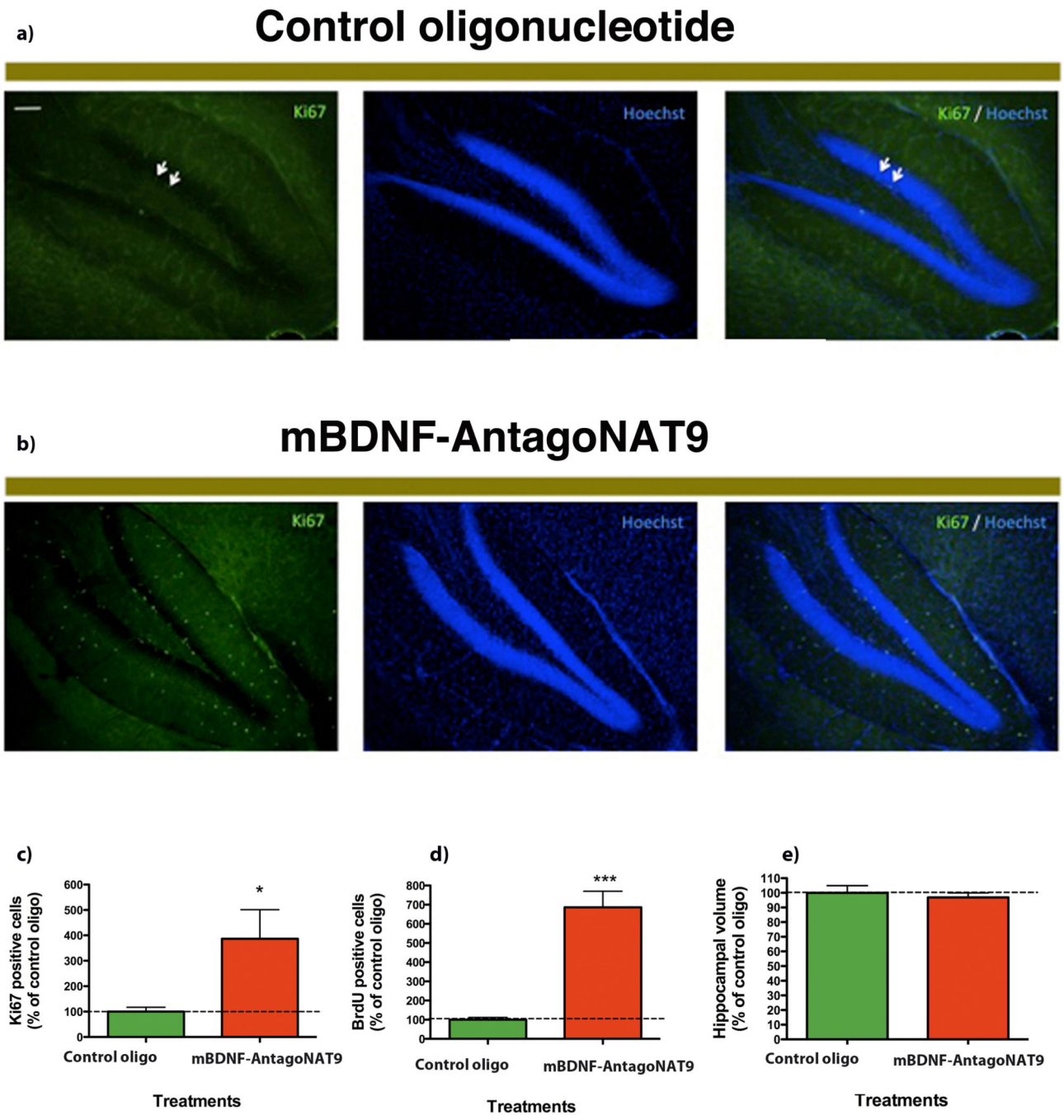

Figure 5. Blocking of *Bdnf*-AS, *in vivo*, causes an increase in neuronal survival and proliferation

(A–B) We treated mice with m*Bdnf*-AntagoNAT9 or control oligos. After 28 d of continuous m*Bdnf*-AntagoNAT9 infusion, we performed histological examination of brain tissues, using Ki67. Ki67 is the marker of proliferating cells in hippocampus and we observed an increase in the number of proliferating cells in mice received m*Bdnf*-AntagoNAT treatment compare to mice received control oligos. In mice treated with m*Bdnf*-AntagoNAT9 (B), there was an increase in Ki67 positive cells (proliferating cells), as compared to control treated mice (A).

(C) Mice treated with m*Bdnf*-AntagoNAT9 had a significant increase in the number of Ki67 positive cells as compared to control treated mice.
(D) In mice treated with m*Bdnf*-AntagoNAT9, there was a significant increase in the number of surviving cells (BrdU positive) as compared to control oligonucleotide treated mice.
(E) There were no differences in hippocampal volume between control and m*Bdnf*-AntagoNAT9 treated mice.
Together these data (n=5 per treatment group *= $P < 0.05$, ***= $P < 0.001$) demonstrates that *Bdnf*-AS regulates *Bdnf* levels *in vivo* and that blocking *Bdnf* sense-antisense interactions results in an increase in neuronal lineage, proliferation and survival.

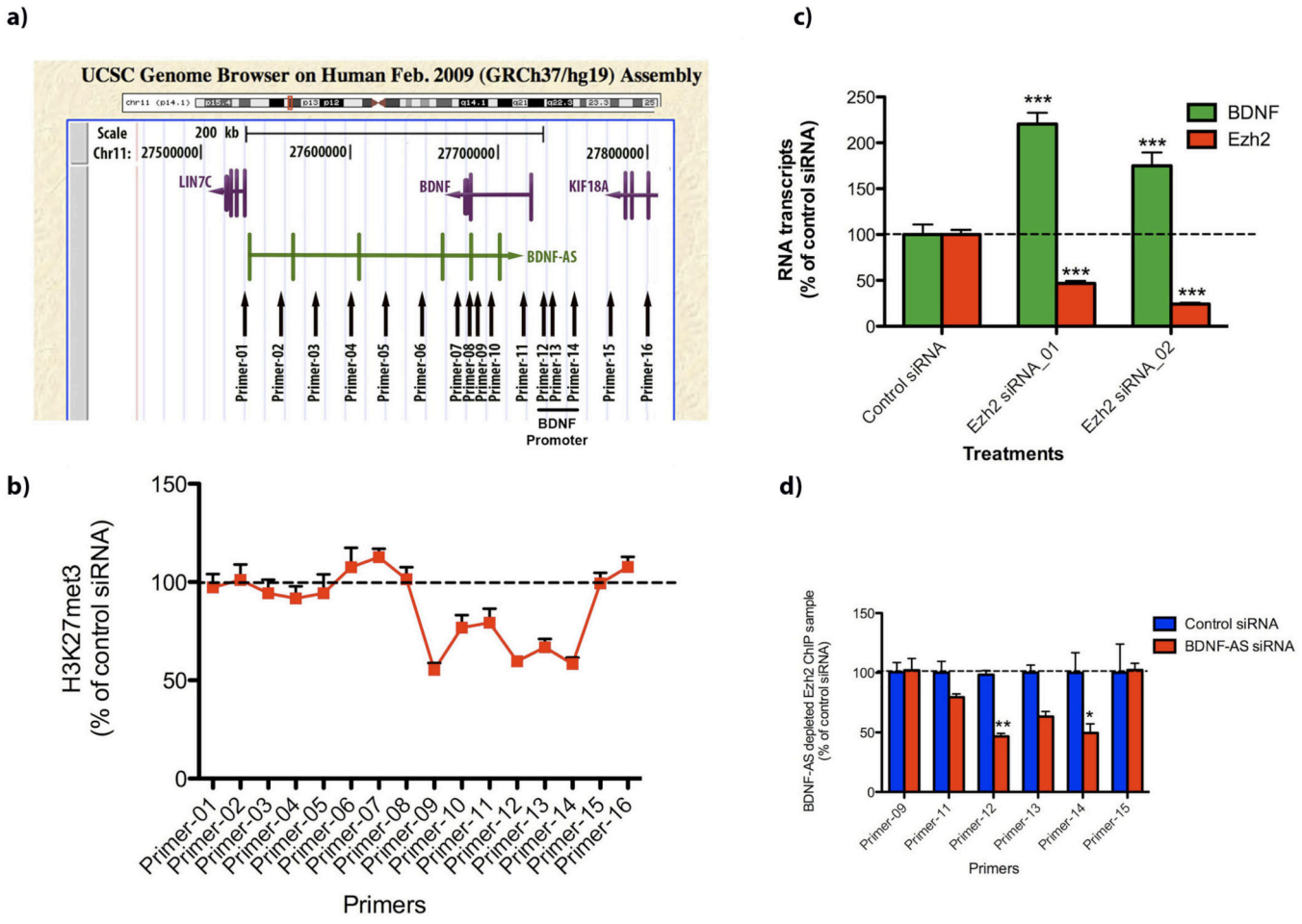

Figure 6. Removal of *BDNF*-AS resulted in the modification of chromatin marks

(A) HEK293T cells were treated with control or *BDNF*-AS siRNA. 48 h post-transfection cells were harvested, fixed with formaldehyde, sonicated and incubated with antibodies against H3K27met3. Immunoprecipitation was performed followed by DNA extraction. DNA samples were analyzed using 16 primer sets covering the entire *BDNF* gene locus, and the *BDNF* promoter region, as indicated.

(B) There was a decrease in association of the repressive chromatin marker, H3K27met3, upon treatment of the cells with *BDNF*-AS siRNA, both at the sense-antisense overlapping (primer-9) and promoter (primer12–14) regions (n=6 for each data point). The observed chromatin modification did not extend toward neighboring genes. These results suggest that local antisense-mediated chromatin modifications are occurring, beginning from the sense-antisense overlapping region and spreading in the 5' direction towards the *BDNF* promoter region.

(C) Knockdown of Ezh2, by either one of two different siRNAs, mimics or phenocopies the *BDNF*-AS knockdown and causes upregulation of *BDNF* mRNA (n=6 for each data point/ treatment ***= $P < 0.001$).

(D) ChIP assay using an Ezh2 antibody revealed that there was a decrease in Ezh2 association with the *BDNF* promoter upon depletion of the *BDNF*-AS transcript by siRNA.

Not all 16 primer sets gave detectable PCR signals, possibly due to the lack of direct Ezh2-chromatin binding (n=6 for each data point/treatment **= $P < 0.01$, *= $P < 0.05$).