Vezf1 regulates genomic DNA methylation through its effects on expression of DNA methyltransferase Dnmt3b

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The zinc finger protein vascular endothelial zinc finger 1 (Vezf1) has been implicated in the development of the blood vascular and lymphatic system in mice, and has been characterized as a transcriptional activator in some systems. The chicken homolog, BGP1, has binding sites in the β-globin locus, including the upstream insulator element. We report that in a mouse embryonic stem cell line deletion of both copies of Vezf1 results in loss of DNA methylation at widespread sites in the genome, including LINE1 elements and minor satellite repeats, some imprinted genes, and several CpG islands. Loss of methylation appears to arise from a substantial decrease in the abundance of the de novo DNA methyltransferase, Dnmt3b. These results suggest that naturally occurring mutations in Vezf1/BGP1 might have widespread effects on DNA methylation patterns and therefore on epigenetic regulation of gene expression.

[Keywords: DNA methylation; BGP1; Vezf1; Dnmt3b]

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DNA methylation is an indispensable epigenetic modification that is directly connected to gene silencing. It is important for many cellular processes including silencing of repetitive elements, X inactivation, imprinting, and development. Methylated sites on promoters provide signals that result in the recruitment of repressive histone modifications and down-regulation of gene expression [Miranda and Jones 2007]. Recent studies have suggested a role of DNA methylation during differentiation in repressing a subset of germline specific genes [Weber et al. 2007; Zilberman 2007]. Anomalous DNA methylation has been linked to several forms of cancer and other genetic diseases [Esteller 2005].

DNA methylation is established in early development and maintained throughout the cell’s lifetime by de novo DNA methyltransferases Dnmt3a and Dnmt3b and the maintenance methyltransferase [MTase] Dnmt1 (Goll and Bestor 2005). Considering the scale at which genomic expression is regulated by DNA methylation, relatively few cofactors/modifiers have been shown to be involved in this process. A number of proteins interact directly with the MTases and regulate their activity. Recently, it has been shown that Dnmt3L interacts directly with Dnmt3a2 and Dnmt3b and targets these factors to promoters carrying unmethylated H3K4, promoting de novo DNA methylation at these sites [Ooi et al. 2007]. Similarly, UHRF1 interacts with Dnmt1 and delivers it to hemimethylated CpG sites, helping in maintenance methylation [Bostick et al. 2007; Sharif et al. 2007]. LSH, a chromatin remodeling factor, also interacts directly with Dnmt3a and Dnmt3b; it has been suggested that this increases the accessibility of the heterochromatic DNA to MTase enzymes [Muegge 2005; Zhu et al. 2006]. Other mechanisms for regulation of DNA methylation act indirectly through the histone modification pathway: CGBP, a zinc finger protein, binds specifically to unmethylated DNA and associates with the Set 1 H3K4 MTase complex, restricting its activity and thereby influencing the DNA methylation pattern [Lee and Skalnik 2005]. At the level of transcription, the MTases are known to be regulated by binding of the ubiquitous transcription factors Sp1 and Sp3 to MTase promoters [Ishida et al. 2003; Jinawath et al. 2005]. However, the roles of any tissue-specific transcription factors or regulatory mechanisms controlling their expression in different cell types are unknown.

Here, we present evidence that the absence of a zinc
finger protein, vascular endothelial zinc finger 1 (Vezf1)/BGP1, causes major loss of genomic methylation at specific sites, which include certain repeat elements, some imprinted loci, and many CpG islands. We identified BGP1 some years ago as a DNA-binding protein that recognizes poly dG sequences (Lewis et al. 1988; Clark et al. 1990). Recently, we began to reinvestigate the role of BGP1 as a regulatory factor because of our identification of BGP1-binding sites (A. West, M. Gaszner, and G. Felsenfeld, unpubl.) within the compound insulator element at the 5’ end of the chicken βA-globin locus, where they play an essential role in the functioning of the insulator (Recillas-Targa et al. 2002). Cloning of BGP1 has revealed that it is the chicken homolog of the mouse gene Vezf1 and the human gene DB1 (A. West, M. Gaszner, and G. Felsenfeld, unpubl.). Reports of Vezf1 and DB1 indicate that the protein might serve as a transcriptional regulatory factor (Aitsebaomo et al. 2001; Miyashita et al. 2004). However, our earlier studies of another BGP1 site in the cβA-globin promoter had shown that BGP1 does not have a strong regulatory effect on cβA-globin expression in transient assays, and we had suggested that it might function through its effects on chromatin structure (Lewis et al. 1988).

The availability of mouse embryonic stem cells in which both alleles of Vezf1 were deleted homozygously (Kuhnert et al. 2005) made it possible to investigate further the role of Vezf1/BGP1 in gene expression. We show that loss of the gene has genome-wide effects on DNA methylation, and that these can be explained by a preferential depletion of Dnmt3b1, which carries the catalytic domain responsible for de novo DNA methylation.

**Results**

As part of a survey of gene expression patterns in mouse Vezf1−/− embryonic stem (ES) cells, we examined overall genomic levels of histone H3 acetylation and DNA methylation. Histone acetylation levels, determined by chromatin immunoprecipitation (ChIP), were lower in the null line than in wild-type controls (Supplemental Fig. S1), suggesting that the abundance of transcriptionally active chromatin might be reduced. DNA methylation levels were measured by digestion of DNA with the methylation sensitive restriction enzyme HpaII. This revealed, unexpectedly, a marked loss in DNA methylation in Vezf1−/− cells compared with wild-type ES cells (Fig. 1A).

To determine in more detail the sites of altered methylation, specific regions of the genome were probed by Southern blot analysis of HpaII restriction digests. In the mutant cells, Line1 elements and minor satellite repeats showed a substantial loss of methylation. There was partial loss at Sine B and telomeric repeats, whereas major satellite repeat sequences were not affected (Fig. 1B). We also examined methylation at HpaII sites in the upstream regions of several individual genes by methylation-dependent PCR analysis (MDPCR; see Materials and Methods): β-globin and Pgk2, both single-copy, tissue-specific genes (Singer-Sam et al. 1990; Kafri et al. 1992; Ariel et al. 1994), were examined and the results show that whereas β-globin partially lost methylation in Vezf1−/− cells, sequences upstream of Pgk-2 showed no decrease in DNA methylation (Fig. 2A). Loss of Vezf1 also had varying effects at several imprinted loci. Those
surveyed were the region upstream of the H19 gene, including the imprinted control region (ICR) with all four CTCF-binding sites (Bell and Felsenfeld 2000), the upstream regions 1 and 2 (reg1 and reg2) of the maternally imprinted Igf2r gene (Stoger et al. 1993), and the upstream region of Snrpn (Tada et al. 1997). As shown in Figure 2B, in wild-type cells the H19 ICR and reg2 of Igf2r had relatively few copies in which every site in the probed region was methylated (i.e., most copies were cut at least once by HpaII). However, these levels of methylation were further reduced in Vezf1−/− cells. There was no change in methylation at Igf2r reg1 which is unmethylated, or at the site upstream of Snrpn which is methylated in the wild-type cells. The PCR results were confirmed by Southern analysis of some of these sites after DNA had been cleaved with methylation-sensitive restriction enzymes (Supplemental Fig S2). CpG islands in the promoters of several housekeeping genes, which usually are maintained in an unmethylated state, did not show any gain of methylation as determined by MDPCR (Fig. 2C). To investigate in detail the methylation state of the individual CpG residues at the H19 ICR and reg2 of Igf2r, we used bisulfite modification of the DNA, followed by cloning and sequencing of the products (Warnecke et al. 1997). As shown in Figure 2D, individual CpGs were selectively reduced in methylation in Vezf1−/− cells, consistent with the results in Figure 2, B and C. At all but three sites there is a decrease in methylation. It should be noted that these changes are not uniformly distributed: Some CpG sites, especially at the H19 ICR sites 8–13, appear to be particularly sensitive to loss of Vezf1, while others are not. This may reflect differences in the relative contributions of different methylating enzymes to methylation of individual CpG sites within the imprinted loci.

The above observations prompted us to investigate the global methylation of CpG islands in the Vezf1−/− cells, using methylated DNA immunoprecipitation (MeDIP) followed by hybridization of the immunoprecipitated product to a 4.6K mouse CpG island microarray. Using criteria described in the Materials and Methods, analysis of the data [significance analysis of microarray (SAM)] [Tusher et al. 2001] revealed genome-wide loss of methylation at >1300 CpG islands (CpGis); there were no sites of significantly increased methylation (Fig. 3A). Among these, 500 CpGis lost 20%–60% of their wild-type methylation (q value 0%–2%) of which 76 CpGis were located within 3 Kb upstream of the promoters and 71 CpGis within 3 Kb downstream from genes (Supplemental Tables 4, 5). Many of these CpG islands were found to be associated with genes involved in tumorigenesis (Mcm7, Tcebl1, Brf1, Mtnr11, Fev, and Tnc6b), testis-specific genes (Abca14, Sart3, Tbx3), neuronal-specific genes involved in brain and craniofacial development (Ahab, Tmem24, Dscr1, Tcfap2a, Foxb1, and Ppi13), homeobox genes (Lhx1 and Pbx1), and several tissue-specific genes (Supplemental Table 4). To investigate whether this loss of DNA methylation led to a change in expression, 14 genes were randomly selected (Fig. 3B, C). A total of six genes showed an increase in levels of expression. Although this gene acti-
isoforms are catalytically inactive since the splicing re-
1999; Saito et al. 2002; Ostler et al. 2007). Some of these
expressed, in different cell types (Fig. 5A; Robertson et al.
identified, and subsets of these have been shown to be
active splicing; more than eight splice variants have been
line. Dnmt3b transcripts are known to undergo alterna-
tions from several Dnmt3b splice variants, and it
was not clear which of these were changed in the mutant
result may reflect the reduced DNA methylation, a di-
rect effect of the loss of Vezf1 on expression of some of
these genes cannot be excluded.

**Effects on DNA methylation mechanisms**

DNA methylation in ES cells involves both maintenance
and de novo mechanisms. The above results suggested
that levels of one or more of the DNA MTases might be
affected in Vezf1−/− cells. As a first step, total DNA
methylation activity in vitro was compared in nuclear
elements from wild-type and Vezf1−/− ES cells by methyl-
ating plasmid DNA in the presence of 3[H] S-adenosyl
methionine. There was no significant difference in the
methylation activity, indicating that the level of major
MTase activity (predominantly Dnmt1) was essentially
unaltered (Supplemental Fig. S3). To distinguish among
the different methylating enzymes, we next determined
the expression levels in wild-type and Vezf1−/− cells
by semi-quantitative RT–PCR analysis of genes selected from the microarray that showed significant loss of DNA methyla-
tion at CpG sites were tested for change in
expression. The asterisks indicate the
genes that show increased expression in the Vezf1−/− cells.

Figure 3. (A) MeDIP/CpG island micro-
array analysis, comparing genomic DNA
from wild-type and Vezf1−/− ES cells. Ob-
erved score [see the Materials and Meth-
ods] is plotted against expected score (based on randomized sample). The limit-
ing parallel lines are chosen to give a false
discovery rate of 29.7%. Analysis shows
that almost all the CpG is with significant
change (points lying outside the parallel
lines) have lost DNA methylation (gray
diamonds). Gray triangles represent gain
of methylation. (B,C) RT–PCR analysis of
genes selected from the microarray that
showed significant loss of DNA methyla-
tion in ES cells. The catalytically inactive forms Dnmt3b6
and weak amounts of Dnmt3b7 are also expressed; these
lack the essential MTase motifs in the catalytic domain,
which are lost by alternative splicing of exons 21 and 22
[Weisenberger et al. 2004]. To identify these splice vari-
ants, primer pairs were constructed which spanned sev-
eral exon–intron boundaries of the Dnmt3b gene allowing
them to be visualized simultaneously. The results
show that expression of both Dnmt3b1 and Dnmt3b6 is
decreased in Vezf1−/− ES cells. Interestingly, semiqua-
titative analysis of RT–PCR products revealed a
Dnmt3b6 to Dnmt3b1 ratio of 0.5:1 in wild-type cells
but 0.8:1 in Vezf1−/− cells [Fig. 5B].

Splicing events known to take place on Dnmt3b tran-
scripts were further investigated: 5′ splicing, which can
remove exon 2, and splicing of exons 10 and 11. Semi-
quantitative RT–PCR measurements [Weisenberger et
al. 2004] showed that Vezf1−/− ES cells expressed
Dnmt3b mRNAs containing exons 10 and 11, which is
results in the loss of critical MTase motifs from the pro-

Figure 4. Effect of Vezf1 deletion on expression levels of DNA
MTases and of genes coding for other proteins known to modu-
late DNA methylation levels in the genome. (A,B) One-step
RT–PCR was performed with RNA from wild-type and
Vezf1−/− cells to amplify DNA MTases, LSH, CGBP, Dnmt3L,
and β-actin as a control for the amount of RNA template used
in the RT–PCR reaction.
also characteristic of the Dnmt3b1 and 3b6 variants found as the major components in normal ES cells [data not shown]. Experiments designed to detect splicing at exon 2 at the 5′ end of the gene revealed both spliced and unspliced forms of Dnmt3b transcripts, at least 90% of the mRNA was spliced [Fig. 5C]. However, the abundance of the spliced form was 2.5-3 fold lower in Vezf1−/− cells than in wild type. We also detected in small amounts in ES cells a previously unreported variant (see the Discussion, Supplemental Fig. S7).

We further determined the expression levels of DNA MTases by quantitative RT–PCR measurements. For Dnmt3b, the primers were designed to target the 3′ end of the transcript, which detect only the catalytically active full-length form Dnmt3b1. The analysis confirmed a fourfold reduction in the Dnmt3b1 transcript in Vezf1−/− ES cells [Fig. 5D], whereas the levels of Dnmt1 and Dnmt3L remained unchanged. There was an ~1.5-fold increase in Dnmt3a transcript. However, this increase does not appear to compensate at the sites we observed for the loss of Dnmt3b activity.

The level of Dnmt3b protein was measured by Western blot, and appeared decreased in the mutant cells, consistent with the above finding [Supplemental Fig. S4]. Both the splice variant Dnmt3b6 and the full-length variant Dnmt3b1 can be seen, and both are diminished in abundance, as compared with an actin control. No other Dnmt3b variants are significantly expressed in ES cells.

As shown in Figure 6A, the genomic DNA from the Vezf1-transfected clone is more resistant to HpaII digestion than is DNA from the Vezf1−/− cells. Southern blot analysis of DNA methylation of the Line1 and minor satellite repeats from the transfected clone clearly displayed a substantial rescue of DNA methylation at both repetitive elements when compared with Vezf1−/− [Fig. 6B]. Quantitative RT–PCR showed that the level of Dnmt3b expression was also considerably restored [Fig. 6C]. Measurements of both Vezf1 and Dnmt3b protein in the transfected Vezf1−/− cells were consistent with the above results [Supplemental Fig. S5B].

**Vezf1-binding sites at the Dnmt3b locus**

Vezf1/BGP1 is a C2H2 zinc finger protein that has been characterized biochemically and shown to bind to poly G tracts as well as to some bipartite sequences containing poly Cs or Gs. The sequence in the neighborhood of Dnmt3b contains several candidate Vezf1-binding sites; the three best potential sites were found within two introns near the 5′ and 3′ ends of the gene, and within the 3′ untranslated region [UTR] of the Dnmt3b gene [Fig. 7A; sites 1, 2, and 3, respectively]. A ChIP/quantitative PCR assay was performed with primers and TaqMan probes covering all three potential binding sites, using a polyclonal antibody against chicken Vezf1/BGP1 [Supplemental Fig. S6]. The comparative analysis of Vezf1 binding in wild type versus Vezf1−/− in Figure 7B shows that Vezf1 is present at site 2. There is little or no binding to other sites.

ChIP assays using antibodies directed against several histone H3 modifications revealed about threefold reduced levels of acetylation, compared with wild-type cells, over the Dnmt3b promoter in Vezf1−/− cells [Supplemental Fig. S7]. Small changes in acetylation were observed at downstream sites 1–3. Only small
changes were seen at these sites for H3K4 dimethylation, H3K9 dimethylation, and H3K27 trimethylation, with the exception of site 3 in the 3′ UTR, where H3K4 dimethylation was somewhat increased in Vezf1+/− cells (Supplemental Fig. S7).

Discussion

Mouse Vezf1 was identified as a gene that is highly expressed in vascular endothelium during early embryonic development. Vezf1 is also expressed in neuronal and mesodermal tissues in embryos, and in endothelial and hematopoietic cell lines (Xiong et al. 1999). Mouse and human Vezf1 bind in a sequence-specific manner to GC-rich sequences of several genes, including IL-3, FLK-1, FLT-1, ET-1, and stathmin/OP18 (Koyano-Nakagawa et al. 1994; Aitsebaomo et al. 2001; Miyashita et al. 2004; D. Lemons and H. Stuhlmann, in prep.). However, transcriptional activation of at least some of the target gene promoters appears to be dependent on interactions between VEZF1 and components of the Rho GTPase machinery, including p68RacGAP (Aitsebaomo et al. 2004) and RhoB (Lebowitz and Prendergast 1998; Kuhnert and Stuhlmann 2007). The homologous protein in chicken, BGP1, has known binding sites in the chicken β-globin promoter (Lewis et al. 1988), and in the insulator at the 5′ end of the β-globin locus [A. West, M. Gaszner, and G. Felsenfeld, unpubl.].

Targeted inactivation of the Vezf1 gene in mice revealed that it is essential during embryogenesis, and that it acts in a dosage-dependent fashion to regulate the development of blood and lymphatic vascular systems (Kuhnert et al. 2005). Furthermore, embryoid bodies derived from in vitro differentiating ES cells with both Vezf1 alleles inactivated [Vezf1−/−] fail to form a well-organized vascular network and display vascular sprouting defects [Z. Zhou and H. Stuhlmann, in prep.).

Results from our present study show that Vezf1+/− ES cells display widespread loss of DNA methylation of the genome, and that this loss correlates with an approximately fourfold reduced expression of the de novo DNA MTase, Dnmt3b, compared with wild-type ES cells. Of interest, both Dnmt3b-null and Vezf1-null embryos show ectopic hemorrhage in the head region, subcutaneous edema, and die during midgestation stages (Kuhnert et al. 2005; Ueda et al. 2006); however, vascular defects have not been studied in detail in the Dnmt3b-null mice, and the mutant mice show additional defects in other tissues [Ueda et al. 2006]. Therefore, it is plausible that at least some of the defects observed in the Vezf1 mutant mice are due to the considerable loss of Dnmt3b activity.

Complete loss of Dnmt3b (Dnmt3b−/−) causes fetal lethality in mice (Okano et al. 1999). In humans, hypomorphic mutations in the DNM3B gene are found in individuals with ICF [immunodeficiency, centromere instability, facial anomalies] syndrome (Xu et al. 1999; Ehrlich et al. 2003). Patients with ICF syndrome display developmental defects that are characterized by region-specific losses of DNA methylation. These include satellite 2 and 3 repeats, other sporadic repeats like D4Z4, the inactive X chromosome, and cancer-testis (C-T) genes, suggesting that these sequences are bona fide targets of Dnmt3b [Hansen et al. 2000; Kondo et al. 2000]. Of interest, mice carrying ICF-like hypomorphic missense mutations survive to term, but most of them die shortly after birth. The mice display phenotypes that are similar to those found in ICF patients, including hypomethylation of repetitive sequences [Ueda et al. 2006]. We show here that the partial loss of Dnmt3b expression in Vezf1+/− ES cells causes methylation defects similar to those found in ICF patients, Dnmt3b−/− embryos, and ICF-like hypomorphic mutant mice. Our microarray analysis of CpG island methylation revealed a decrease in methylation levels surrounding the promoters of several genes, some of which were shown recently to have
Vezf1 regulates the expression of Dnmt3b, identified three binding motifs for Vezf1, all in the introns or 3' UTR of the Dnmt3b gene. Data from ChIP experiments (Fig. 7B) shows that Vezf1 is present at one of these sites, and may be present at a second, in wild-type cells. The mechanism by which loss of Vezf1 from binding sites within the Dnmt3b gene results in a decrease of its expression is not yet clear. Our results, however, show that the Dnmt3b6 transcript, which is an inactive splice variant of Dnmt3b, is also decreased in abundance in Vezf1−/− ES cells, but to a lesser extent than Dnmt3b1, perhaps due to improper alternative splicing. This hypothesis is also supported by a number of independent observations. First, a primer set designed across exons 4 and 7 of the Dnmt3b gene revealed a new isoform in which exon 6 is spliced out (Supplemental Fig. S8). This expressed isoform was present in wild-type ES cells, and was not detected in the Vezf1−/− ES cells. The novel splice product would result in removal of 42 amino acids N-terminal of the PHD domain of Dnmt3b protein. Second, in surveying effects of the absence of Vezf1 on expression of tissue specific genes (Fig. 3B) we noted another example of an effect on splicing: Fosb showed decreased expression of full-length transcript and increased expression of an alternatively spliced shorter isoform, ΔFosb, in Vezf1−/− cells compared with the wild-type ES cells. It remains to be determined whether Vezf1/BGP1 plays a more widespread role in regulation of splicing. Long uninterrupted poly-G sequences (n > 10), binding sites for BGP1 (Lewis et al. 1998), can be found in introns of many genes, including Fosb. One possibility is that changes in rates of transcription affect the relative probabilities of following different splicing pathways. It is interesting to note that the zinc finger-binding domain of Vezf1 has considerable homology with that of the DNA-binding factor Maz, which, it has been suggested, can slow transcript elongation (Ashfield et al. 1994), and could, in principle, alter the relative proportion of splice variants.

Whatever the mechanism, it is clear that Vezf1/BGP1 has a significant effect on Dnmt3b expression in ES cells, and consequently a major but selective effect on patterns of DNA methylation, which in turn has the potential to affect gene expression throughout the genome. We note that Vezf1 protein does not appear to interact directly with Dnmt3a, Dnmt3b, or Dnmt3l, as judged by the negative results obtained in attempts to immunoprecipitate these MTases with an antibody to Vezf1 (data not shown).

We suggest also that the possibility of defects in Vezf1 function might be explored in connection with certain genetic disorders like ICF syndrome. Sixty percent to 90% of ICF patients have a missense mutation in the catalytic domain of DNMT3B, causing a partial loss of its activity (Hansen et al. 1999; Xu et al. 1999). There are, however, known ICF patients who lack DNMT3B mutations but have a distinctive set of DNA methylation defects, pointing to the existence of distinct ICF subtypes and the possible involvement of another gene (Jiang et al. 2005). Alterations in DNA methylation are also seen in almost all cancers. Satellite 2 and 3 hypomethylation is very common in cancer cells (Narayan et al. 1998), and can cause chromosomal instability (Wong et al. 2001; Schulz et al. 2002; Tsuda et al. 2002; Ehrlich et al. 2003). It has also been proposed that cancer-associated hypomethylation of satellite DNA sequences might function in altering gene expression to favor carcinogenesis (Ehrlich 2002). It is therefore plausible that Vezf1 may be a candidate oncogene whose altered expression can result in transformation at least mediated by changes in expression/splicing of Dnmt3b.

**Materials and methods**

**Maintenance and transfection of ES cells**

Wild-type ES cells and Vezf1−/− cells were cultured in the absence of MEFs in 1× GMEM medium (Sigma, G5154) supple-
Methylation analysis

The DNA methylation activity of the wild-type and Vezf1+/− ES cells was determined by an in vitro DNA MTase activity assay. Twenty-five micrograms of wild-type and Vezf1+/− ES nuclear extract as determined by Bio-Rad protein assay (catalog no. 500-0006) were incubated with 1 µg of λ-phage DNA in the presence of 2 µCi of 3H-methyl-S-adenosyl L-methionine (15 Ci/mmol, Perkin-Elmer) in 120 mM NaCl, 10 mM Tris-HCl [pH 7.9], 10 mM EDTA, and 1 mM dithiothreitol [DTT] for 1 h at 30°C. In vitro methylated DNA was spotted on a Whatman DE-81 ion-exchange filter, washed five times with 0.2 M ammonium bicarbonate, and incorporated radioactivity was measured by scintillation counting.

For Southern blots, genomic DNA was digested, separated by electrophoresis on 1% agarose gels, and transferred on a ZetaProbe GT membrane (Bio-Rad). The rest of the procedure was according to the manufacturer’s protocol. The oligonucleotide probes used for repetitive elements have been described [Dennis et al. 2001]. A 330-bp fragment was used to analyze the Mlui site of region 2 of the Igf2r gene [Tada et al. 1997]. The H19 imprinted region was analyzed by using a 1.5-kb probe corresponding to the exon–intron boundaries (Supplementary Table 1). All the RT–PCR products were in the size range of 200–500 bp. The primer sequences are provided in the Supplemental Material. Quantitative RT–PCR procedures used TaqMan One-Step RT–PCR Master Mix and TaqMan Gene Expression Assays in the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Quantitative PCR for ChIP studies was carried out on the same instrument with primer-probe sets designed using the software Primer Express. The analysis was done as described [Litt et al. 2001].

Antibody production and ChIP

Antibody against Vezf1 was produced in rabbits at Spring Valley Laboratories, Inc. Chicken Vezf1/BGP1, which shares >95% homology with the mouse protein, was cloned and expressed as a His-tagged recombinant protein in Escherichia coli. The overexpressed protein was purified in the presence of 7 M urea on Ni NTA Agarose (Qiagen) using the manufacturer’s protocol. The Vezf1 protein was separated by PAGE. Coomassie-stained bands were excised and used as an antigen to raise a polyclonal antibody. The antibody was able to immunoprecipitate His-tagged recombinant Vezf1 from E. coli cell extracts. The specificity of the antibody was tested by using it to detect Vezf1 from wild-type and Vezf1+/− nuclear extracts in Western blot (Supplemental Fig. S6).

for wild-type and the Vezf1+/− genomic DNA and the IP fraction was amplified by random PCR using the following primers: Primer A, GTTTCCAGTCAGCATNNNNN: Primer B, GTTTCCAGTCAGCATGC, using the protocol published by University Health Network Microarray Center (UHNMC; http://www.microarrays.ca) to generate data that were tested for statistically significant changes in methylation. The IP products and inputs were sent to UHNMC for hybridization on a mouse CpG-island 4.6k array (MCG14.6k). This is a single-spotted DNA array containing 4642 CpG-island clones. The data analysis was also performed by UHNMC using the SAM program [Tusher et al. 2001] to detect changes in methylation levels. The SAM analysis calculates a score by dividing the difference between the signal-to-input ratios of the null and the wild type by the average standard deviation [Tusher et al. 2001]. The program yielded 1302 genes with decreases ranging from 5% to 60% (q value ranging from 0% to 64%) at a false discovery rate of 29.7%. We further sorted the data manually and reduced the list to 500 genes with fold change of 20%–60% and q values of 0%–2%.

RT–PCR and quantitative PCR analysis

The specified amount of DNase I-treated RNA purified using RNeasy Mini Kit (Qiagen) was reverse transcribed using the 1 Step RT–PCR kit [Invitrogen]. The primers were designed at the exon–intron boundaries (Supplemental Table 1). All the RT–PCR products were in the size range of 200–500 bp. The primer sequences are provided in the Supplemental Material. Quantitative RT–PCR procedures used TaqMan One-Step RT–PCR Master Mix and TaqMan Gene Expression Assays in the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Quantitative PCR for ChIP studies was carried out on the same instrument with primer-probe sets designed using the software Primer Express. The analysis was done as described [Litt et al. 2001].
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