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# Identification and characterization of methylation-dependent/independent DNA regulatory elements in the human *SLC9B1 gene*

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ABSTRACT

The human NHEDC1 (hNHEDC1) protein is thought to be essential for sperm motility and fertility however the mechanisms regulating its gene expression are largely unknown. In this study we have identified multiple DNA regulatory elements in the 5′ end of the gene encoding hNHEDC1 (*SLC9B1*) and have explored the role that DNA methylation at these elements plays in the regulation of its expression. We first show that the full-length hNHEDC1 protein is testis-specific for the tissues that we tested and that it localizes to the cells of the seminiferous tubules. *In silico* analysis of the *SLC9B1 gene* locus identified two putative promoters (P1 and P2) and two CpG islands – CpGI (overlapping with P1) and CpGII (intragenic) – at the 5′ end of the gene. By deletion analysis of P1, we show that the region from −23 bp to +200 bp relative to the transcription start site (TSS) is sufficient for optimal promoter activity in a germ cell line. Additionally, *in vitro* methylation of the P1 (the −500 bp to +200 bp region relative to the TSS) abolishes its activity in germ cells and somatic cells strongly suggesting that DNA methylation at this promoter could regulate *SLC9B1* expression. Furthermore, bisulfite-sequencing analysis of the P1/CpGI uncovered reduced methylation in the testis *vs.* lung whereas CpGII displayed no differences in methylation between these two tissues. Additionally, treatment of HEK 293 cells with 5-aza-2′-Deoxycytidine led to upregulation of NHEDC1 transcript and reduced methylation in the promoter CpGI. Finally, we have uncovered both enhancer and silencer functions of the intragenic *SLC9B1* CpGII. In all, our data suggests that *SLC9B1 gene* expression could be regulated *via* a concerted action of DNA methylation-dependent and independent mechanisms mediated by these multiple DNA regulatory elements.

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## 1. Introduction

Cation Proton Antiporters (CPAs) are a class of transport proteins which directly couple the transport of protons across the plasma membrane to the counter transport of monovalent cations such as $Na^+$ or $K^+$ and thereby protect cells from gradual acidification (Casey et al., 2010). On the basis of their distinct phylogenetic origins the CPA superfamily is divided into three families — CPA1, CPA2 and NaT-DC ($Na^+$-transporting carboxylic acid decarboxylase) (Brett et al., 2005; Donowitz et al., 2013). The members of the eukaryotic CPA1 family include the $Na^+/H^+$ exchangers (NHE1–NHE9) which catalyze the electroneutral exchange of extracellular $Na^+$ for intracellular $H^+$, utilizing the energy provided by the $Na^+$ gradient generated by the Na, K-ATPase (Mahnensmith and Aronson, 1985). The mammalian CPA2 family has been proposed to include NHEs with high homology to bacterial and fungal NHEs particularly the *Escherichia coli* $Na^+/H^+$ antiporter (NHA) (Padan et al., 2009; Olkhova et al., 2007). These have been named NHA1 and NHA2 but are also known as $Na^+/H^+$ exchanger domain-containing protein (NHEDC1) and NHEDC2 respectively.

In mouse sperm, four NHE isoforms have been identified: the ubiquitously expressed and most well characterized NHE1 isoform, NHE5, the sperm-specific NHE10, and the more recently identified testis-specific NHEDC1 (Woo et al., 2002; Wang et al., 2003, 2007; Ye et al., 2006; Liu et al., 2010a, 2010b). The human ortholog of NHEDC1 was partially characterized and mapped to chromosome 4q24 (Ye et al., 2006). The tissue distribution pattern of hNHEDC1, determined by reverse transcription polymerase chain reaction (RT-PCR) analysis from 18 human tissues, suggests that the transcript is testis-specific. The

*Abbreviations:* 5-azaC, 5-Aza-2′-Deoxycytidine; bp, base pair; CPA, cation proton antiporter; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagles media; DMR, differentially methylated region; DNMTS, DNA methyltransferases; ES cells, embryonic stem cells; FBS, fetal bovine serum; hCMV, human cytomegalovirus; IV methylation, *in vitro* methylation; kb, kilobase; MM, mock methylation; NFDM, non-fat dry milk; NGS, normal goat serum; NHA, $Na^+/H^+$ antiporter; NHE, $Na^+/H^+$ exchanger; NHEDC1, $Na^+/H^+$ exchanger domain-containing protein 1; NaT-DC, $Na^+$-transporting carboxylic acid decarboxylase; kDa, kilodalton; MBPs, methyl-DNA-binding proteins; ORF, open reading frame; P, promoter; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription polymerase chain reaction; SAM, S-adenosyl methionine; SDS, sodium dodecyl sulfate; TAN1, tandem reporter construct; TALEs, transcriptional activator like effectors; TSS, transcription start site; UTR, untranslated region; ZFEs, zinc finger effectors.

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open reading frame (ORF) was predicted to encode for a protein of 515 amino acids with 12 transmembrane domains. Moreover, transient overexpression of a flag-tagged hNHEDC1 in HEK 293 cells was shown to produce the flag-NHEDC1 fusion protein with a molecular weight of approximately 56 kDa (Ye et al., 2006). Subsequently, the mouse ortholog was mapped to mouse chromosome 3 in the G3 region with an ORF encoding a predicted protein of 563 amino acids with 12 transmembrane domains. The tissue distribution pattern of the mouse NHEDC1 (mNHEDC1) as determined by northern blot analysis suggests that the mNHEDC1 transcript is testis-specific (Liu et al., 2010a). Furthermore, expression analysis of NHEDC1 protein in mouse testis and sperm suggests that the protein localizes to the spermatids and mature spermatozoa in the testis and to the principle piece of the mature mouse sperm flagellum (Liu et al., 2010a). Studies in the mouse suggest that NHEDC1 might be important for sperm motility and fertility as incubation of mouse sperm with antisera against NHEDC1 decreases intracellular pH and $[Ca^{2+}]$ concentration thereby affecting sperm motility and the acrosome reaction respectively (Liu et al., 2010a). Furthermore, female mice injected with an mNHEDC1 DNA vaccine show a significantly reduced fertility rate (Liu et al., 2010b). Although studies have been published aimed at addressing the physiological role of NHEDC1 in sperm, studies understanding the mechanism of regulation and tissue-specific expression of NHEDC1 have not been reported.

Epigenetic mechanisms such as DNA methylation play important roles in the regulation of tissue-specific gene expression (Allegrucci et al., 2005). Regulation of gene expression by DNA methylation has been documented for several testis-specific genes such as the testis-specific mouse *ALF* (TFII A α/β-like factor). Methylation of the CpGs in the proximal promoter is responsible for silencing of the *ALF gene* in somatic cells (Xie et al., 2002). The expression of the cancer/testis (CT) gene, *ZNF645*, is also regulated by DNA methylation — methylation of the promoter region being correlated with silencing of *ZNF645 gene* expression in somatic tissue (Bai et al., 2010). More recently, the human *RHOX gene* cluster was shown to be regulated by DNA methylation. Members of this gene cluster have been shown to regulate target genes important for spermatogenesis and male fertility in mice (Richardson et al., 2013).

DNA methylation involves addition of a methyl group to the cytosines in CpG dinucleotides (a cytosine linked to a guanine by a phosphodiester bond) by enzymes known as DNA methyltransferases (DNMTS). CpG-rich regions, known as CpG islands, punctuate the mammalian genome. These CpG islands are on an average 1000 bp in length and are regions protected from global DNA methylation (Deaton and Bird, 2011). Based on their distribution within the genome, CpG islands have been classified as promoter CpG islands, intragenic CpG islands, (located in the gene body) and intergenic CpG islands (located between gene bodies). While methylation of promoter CpG islands is most extensively studied and has been identified as a powerful means of attaining gene silencing, the functional significance of intragenic and intergenic CpG islands is only beginning to be deciphered. Studies have shown that these intragenic/intergenic CpG islands can be involved in regulating gene expression by functioning as alternative promoters initiating expression of non-coding RNAs, by functioning as enhancer/silencer elements and/or by altering transcriptional elongation efficiency (Lorincz et al., 2004; Rinn et al., 2007; Yu et al., 2013; Sleutels et al., 2002; Illingworth et al., 2010; Maunakea et al., 2010).

In this study we show that the full-length hNHEDC1 protein is testis-specific for the tissues that we tested and that it localizes to the cells of the seminiferous tubules in the testis. Our *in silico* analysis revealed the presence of two putative promoters (P1 and P2) and two CpG islands (CpGI and CpGII) in the 5 end of the *SLC9B1 gene*, with both promoters being functional in somatic and germ cells. By deletion analysis we have determined that the region from −23 bp to +200 bp (relative to the TSS) of P1 is sufficient for optimal promoter activity in germ cells. Furthermore, we show methylation dependence of P1 activity thereby strongly suggesting the role of DNA methylation in the regulation of NHEDC1 expression. Corroborating this idea, bisulfite-sequencing analysis of the P1/CpGI revealed an inverse relationship between the methylation status of the P1/CpGI and the presence of the protein in human testis and lung. Moreover, treatment of HEK 293 cells with the DNA methylation inhibitor 5-Aza-2-Deoxycytidine (5-azaC) upregulates NHEDC1 expression. Finally, our efforts to determine the functional significance of the intragenic hNHEDC1 CpGII uncovered an enhancer/silencer activity for this CpG island.

## 2. Materials and methods

### 2.1. Western blot

Ten micrograms of human tissue lysates (brain, muscle, kidney and testis) obtained from Imgenex were mixed with standard loading buffer (1× Laemmli buffer) and boiled for 10 min and separated using 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The total protein was electroblotted onto polyvinylidene difluoride (PVDF) membrane and probed with anti-NHEDC1 primary antibody (ab106124; Abcam). The blots were blocked in 5% nonfat dry milk (NFDM) in 1× PBS, pH-7.4, 0.1% Tween-20 (PBST) for 1 h at room temperature. The blots were incubated with anti-NHEDC1 antibody (0.25 μg/ml) diluted in PBST with 5% NFDM overnight at 4 °C. After washing with PBST for 20 min (4 × 5 min) the blots were incubated with goat anti-rabbit secondary (Jackson Immunoresearch; 1:10,000 in PBST) for 1 h at room temperature. The blots were then washed for 20 min (4 × 5 min) with PBST and developed using an enhanced chemiluminescent detection system (Pierce).

### 2.2. Immunohistochemistry on human testis sections

Paraffin embedded human testis blocks were obtained from the Cooperative Human Tissue Network. The human testis sections (12 μm thick) were deparaffinized and hydrated (xylene, 100% ethanol, 95% ethanol, 75% ethanol, 50% ethanol, 30% ethanol and water). Following antigen retrieval in citrate buffer pH 6.0 (Zymed Laboratories) the endogenous peroxidase activity was quenched by incubation of the sections in 0.3% hydrogen peroxide in water. The sections were blocked in 10% normal goat serum (NGS from Vector laboratories) in PBS for 1 h at 37 °C. The sections were then subjected to avidin–biotin block and incubated overnight with anti-NHEDC1 primary antibody (ab106124; Abcam) (50 μg/ml) made in 10% NGS in PBS at 4 °C. A no primary control was included to determine the specificity of the secondary antibody. After washing the sections with PBS for 1.5 h (15 min ×6) the sections were incubated with Biotin conjugated goat anti-rabbit secondary antibody (ImmunoPure; 1:100 dilution in PBS) for 3 h at room temperature. The sections were incubated in avidin–biotin complex prepared according to the manufacturer's instructions (ABC kit Vector Laboratories) for 1 h. A filtered 3,3′-diaminobenzidine (DAB) peroxidase substrate solution was prepared and following addition of the substrate (hydrogen peroxide), the sections were incubated in this DAB solution. Following counterstaining with hematoxylin the sections were dehydrated and mounted in permount. The images were obtained using the Olympus AX70 light microscope.

### 2.3. Generation of luciferase reporter constructs

The putative *SLC9B1* promoters P1 and P2 were PCR amplified from genomic DNA isolated from HeLa cells. The *SLC9B1* P1 sequence was cloned into SacI and HindIII sites of the pGL3-Basic vector to generate pGL3-promoter1C1 and the *SLC9B1* P2 sequence was cloned into the NheI and HindIII sites of the pGL3-Basic vector to generate the pGL3-hNHEDC1-promoter2 construct. The −23 bp to +200 bp region of P1, which overlaps with the CpGI, was cloned into the NheI and Hind III sites to generate pGL3-promoter1 C2. The *SLC9B1* CpGI and CpGII were cloned into the NheI and HindIII sites of the pGL3-Basic vector to

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