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NFAT5 and *SLC4A10* Loci Associate with Plasma Osmolality

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ABSTRACT

Disorders of water balance, an excess or deficit of total body water relative to body electrolyte content, are common and ascertained by plasma hypo- or hypernatremia, respectively. We performed a two-stage genome-wide association study meta-analysis on plasma sodium concentration in 45,889 individuals of European descent (stage 1 discovery) and 17,637 additional individuals of European descent (stage 2 replication), and a transethnic meta-analysis of replicated single-nucleotide polymorphisms in 79,506 individuals (63,526 individuals of European descent, 8765 individuals of Asian Indian descent, and 7215 individuals of African descent). In stage 1, we identified eight loci associated with plasma sodium concentration at $P<5.0 \times 10^{-6}$. Of these, rs9980 at *NFAT5* replicated in stage 2 meta-analysis ($P=3.1 \times 10^{-5}$), with combined stages 1 and 2 genome-wide significance of $P=5.6 \times 10^{-10}$. Transethnic meta-analysis further supported the association at rs9980 ($P=5.9 \times 10^{-12}$). Additionally, rs16846053 at *SLC4A10* showed nominally, but not genome-wide, significant association in combined stages 1 and 2 meta-analysis ($P=6.7 \times 10^{-8}$). *NFAT5* encodes a ubiquitously expressed transcription factor that coordinates the intracellular response to hypertonic stress but was not previously implicated in the regulation of systemic water balance. *SLC4A10* encodes a sodium bicarbonate transporter with a brain-restricted expression pattern, and variant rs16846053 affects a putative intronic NFAT5 DNA binding motif. The lead variants for *NFAT5* and *SLC4A10* are *cis* expression quantitative trait loci in tissues of the central nervous system and relevant to transcriptional regulation. Thus, genetic variation in *NFAT5* and *SLC4A10* expression and function in the central nervous system may affect the regulation of systemic water balance.

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Abnormal water balance is an excess or deficit of total body water relative to electrolyte content, and it is determined by measuring the plasma sodium concentration.[1] Hyponatremia (relative water excess) is the most common electrolyte abnormality among hospitalized patients[2]; it is highly prevalent among the acutely ill,[3] patients undergoing surgery,[4] and the elderly.[5,6] Severe acute hyponatremia causes brain edema, seizures, and death.[7] Reversible defects in cognition, coordination, and mood occur with even subtle chronic hyponatremia.[8,9]

Water balance is regulated by thirst and aquaporin-2–dependent water reclamation in the kidney collecting duct. Both phenomena are influenced by arginine vasopressin, the secretion of which is governed by brain regions that continually monitor the osmolality of the extracellular fluid compartment. Activation of arginine vasopressin receptor-2 (encoded by *AVPR2*) by circulating arginine vasopressin mediates the insertion of preformed aquaporin-2 into the luminal membrane of principal cells of the kidney collecting duct and is permissive for water reabsorption. The degree of water reabsorption, in turn, affects the concentration of plasma sodium, the principal extracellular cation and determinant of plasma osmolality or tonicity. Changes in systemic osmolality or tonicity are almost immediately transmitted to the intracellular milieu *via* water movement. Within cells, osmoregulation and thus, water balance are governed by the tonicity-responsive transcription factor tonicity-enhancer binding protein[10], also known as the osmotic response element binding protein[11] and NF of activated T cells 5 (NFAT5).[12,13]

Identification of molecular pathways influencing systemic water balance has implications for both understanding the pathogenesis of primary disorders of water imbalance (such as the syndrome of inappropriate antidiuresis[7]) and the development of novel therapies. Exceedingly rare point mutations in *AQP2* (encoding aquaporin-2) and *AVPR2* cause Mendelian disturbances in water balance[14–19]; however, little is known about the genes influencing the interindividual variability of plasma sodium concentration (*i.e.*, water balance) in the general population. We previously showed heritability of plasma sodium concentration at the population level.[20] Genome-wide association study (GWAS)–based approaches have proven to be instrumental in identifying genetic loci associating with a wide variety of diseases and physiologic traits[21] and thus, novel biologic mechanisms[22,23]; however, the only previous GWAS of plasma sodium had limited power and did not detect any significant associations.[24] Other efforts to determine the genetic architecture of water balance have been limited to candidate gene–based studies.[25] We, therefore, performed a GWAS and replication on the phenotype of plasma sodium concentration in 45,889 and 17,637 individuals of European descent, respectively, with follow-up in 8765 and 7215 individuals of Asian Indian and African descent, respectively, and transethnic meta-analysis of replicated single-nucleotide polymorphisms (SNPs) in all 79,506 individuals.

RESULTS

Study Participants

After excluding individuals with high glucose or impaired kidney function (Concise Methods, Supplemental Table 1), 63,526 individuals of European descent from 25 cohorts were included in this analysis: 45,889 individuals from 21 cohorts in stage 1 meta-analysis and 17,637 individuals from four cohorts in stage 2 meta-analysis (Table 1). Furthermore, a total of 7215 individuals from five cohorts of African descent and 8765 individuals from four Asian Indian cohorts were included in the transethnic analyses (Supplemental Table 2). Details of each cohort's study design, genotyping methods, and quality control criteria are shown in Concise Methods and Supplemental Tables 3 and 4. Plasma sodium and glucose concentrations were within expected ranges in all cohorts (Table 1).

Stage 1 Meta-Analysis of European Descent Studies

In stage 1 meta-analysis, there was little evidence of population stratification as evidenced by the quantile-quantile plot (Supplemental Figure 1) and the low genomic inflation factor λ=1.02 with study-specific inflation factors ranging from 0.9 to 1.04. Eight SNPs met the prespecified criteria for follow-up in stage 2 meta-analysis ($P<5 \times 10^{-6}$), with minimum $P=1.9 \times 10^{-7}$. These SNPs, detailed in Table 2, showed no relevant heterogeneity ($I^2 \leq 30\%$) and good imputation quality, except for rs6565990 at the *GALR1* locus (Supplemental Table 5). Figure 1 shows the −log10 *P* value (Manhattan) plot, and Supplemental Tables 6 and 7 list all SNPs associated with plasma sodium concentration with $P<1 \times 10^{-5}$.

Stage 2 Meta-Analysis of European Descent Studies

In stage 2 meta-analysis, the minor G allele (frequency =0.15) of rs9980 in the *NFAT5* locus on chromosome 16 showed a direction-consistent significant association with higher plasma sodium concentration of similar magnitude as in stage 1 meta-analysis (β=0.06, one-sided $P=3.1 \times 10^{-5}$; significance threshold by Bonferroni method: $P<0.01$). In the combined meta-analysis of stages 1 and 2 cohorts, the association of the rs9980 minor G allele (frequency =0.14) with higher plasma sodium concentration values was genome-wide significant (β=0.06 for each copy of the minor G allele, $P=5.6 \times 10^{-10}$) (Supplemental Figure 2A, Table 2).

The minor allele G (frequency =0.10) of rs16846053 in the *SLC4A10* locus on chromosome 2 (Supplemental Figure 2B) was nominally associated with plasma sodium, and the direction of effect was consistent with stage 1 results (one-sided P=0.04). The combined stages 1 and 2 *P* value was lower than stage 1 results but did not reach strict genome-wide significance ($P=6.7 \times 10^{-8}$) (Table 2). Nonetheless, some have advocated for a more lenient genome-wide significance threshold, especially among more recently founded populations.[26]

The remaining SNPs selected for replication in stage 2 did not replicate (Table 2). However, although the SNP rs6565990

at the *GALR1* locus showed only a near-significant *P* value in stage 2 replication (one-sided $P=0.06$), the stage 2 replication meta-analysis effect estimate was direction consistent with stage 1 meta-analysis, and the *P* value of the stages 1 and 2 combined meta-analysis ($P=3.0 \times 10^{-7}$) was lower than that of the stage 1 meta-analysis ($P=1.5 \times 10^{-6}$). This SNP had poor imputation quality (Supplemental Table 5) and was available in only two studies with a sample size of $n=2912$ in stage 2 replication, thus limiting the power of the analysis.

Transethnic Analyses

In transethnic meta-analysis combining the summary statistics of a total of 79,506 individuals of European, African, and Asian Indian ethnicity, rs9980 at *NFAT5* showed direction-consistent genome-wide significant association with plasma sodium concentration across all ethnicities ($\beta=0.06$ for each copy of the minor G allele, $P=5.9 \times 10^{-12}$), with similar effect sizes in each studied ethnicity (Table 3). Results of our top SNPs ($P<10^{-5}$) for the individual African ancestry and Asian Indian cohorts meta-analyses are shown in Supplemental Tables 8 and 9, respectively.

Bioinformatic Characterization of Associated Loci

rs9980/NFAT5 Locus

NFAT5 (Supplemental Figure 2A) encodes the ubiquitously expressed tonicity-responsive transcription factor that serves as master regulator of intracellular osmoregulation. The 3′ untranslated region of *NFAT5*, containing the lead variant rs9980 (Supplemental Figure 2A), confers tonicity-dependent regulation *via* miRNA-mediated effects on mRNA stability and protein translation.[27–30] In addition, a SNP in strong linkage disequilibrium (LD) with rs9980, rs7193778 ($r^2=0.90$, $D'=1.0$, within a large LD block), is significantly associated with plasma sodium concentration in stage 1 meta-analysis (minor allele C frequency =13%, $\beta=0.05$, $P=4.5 \times 10^{-6}$,) (Supplemental Figure 2A, Supplemental Table 6) and resides in the midst of a heavily ENCODE-annotated putative superenhancer region for *NFAT5* (Figure 2, Supplemental Figure 3). A superenhancer is a group of closely spaced or even overlapping enhancers exhibiting high levels of transcription factor binding in chromatin immunoprecipitation–based approaches.[31] This 3-kb region approximately 35 kb upstream of the *NFAT5* transcriptional start site (TSS) is spanned by three dense peaks of chromatin modification emblematic of a transcriptional enhancer. Specifically, both H3K27Ac (Figure 2) and H3K4me1 (not shown) histone modifications are heavily enriched in the vicinity of this variant in ENCODE-tested cell lines. The putative superenhancer also encompasses two long DNaseI-hypersensitive regions present in 92 and 118 of 125 ENCODE-tested cell lines (Figure 2B). Moreover, ENCODE transcription factor ChIP-Seq experimental data showed binding of 115 of 161 ENCODE-tested transcription factors over the region (ENCODE data displayed in the UCSC Genome Browser) (Figure 2B). Because enhancers regulate spatiotemporal and tissue-specific gene expression, it is noteworthy that both rs9980 and rs7193778 are *cis* eQTLs for *NFAT5* expression in cerebellum and temporal cortex (HaploReg v4.0[32] citing Zou *et al.*[33]).

Ancillary data support functional relevance of rs7193778 to the CNS and glial/astrocytic cells in particular. *In silico* comparison of putative transcription factor binding sites (JASPAR 2016[34]; http://jaspar.genereg.net) affected by rs7193778 primarily identified motifs for members of the sex-determining region Y-box (*SOX*) family of transcription factors (*SRY* and *SOX2*, *-3*, *-5*, *-6*, and *-17*). Of these, *SOX2*, *-3*, *-5*, and *-6* are enriched in tissue derived from brain and/or glioma (Concise Methods). Furthermore, glial tissue (*i.e.*, astrocytes) have been proposed as the cell type conferring central sensing of extracellular sodium concentration,[35,36] in contrast to the neuronal sensing of systemic osmolality.[37,38] DNaseI-hypersensitive regions (*i.e.*, open chromatin) reported in ENCODE data further support a glial cell–specific effect for this regulatory region. Although most such regions are detected across a large number of cell types, a short DNaseI-hypersensitive region immediately upstream of rs7193778 (indicated by 2 in Figure 2C) was detected in only two tissues—the HA-sp astrocytic (glial) cell line and pancreatic islets (Figure 2C). The Regulatory Elements Database (http://dnase.genome.duke.edu/index.php) identified a DNase site (DHS1020946) physically crossing the variant (Figure 2C, orange bar) and mapping to a cluster (self-organizing map [SOM] Cluster: 977) of similar regions preferentially operative in astrocytes.[39] The SOM designation refers to SOM-based clustering of DNase sites according to their profile of DNaseI hypersensitivity across diverse cell types.[39]

The biology of other genes in this locus (Supplemental Figure 2A) (*NQO1*, *NOB1*, *WWP2*, and *CLEC18A*) is summarized in Supplemental Table 7.

rs16846053/SLC4A10 Locus

The lead variant in *SLC4A10*—a gene encoding a brain-specific member of the sodium-bicarbonate transporter family—is intronic; however, the association signal spans the entire *SLC4A10* gene as well as an additional 0.3 Mb 5′ of the gene (Supplemental Figure 2B). Importantly, this SNP, like rs9980 in *NFAT5*, is a *cis* eQTL for *SLC4A10* expression in cerebellum and temporal cortex (Haploreg v4.0[32] citing Zou *et al.*[33]). Transcriptome data in the public domain (*e.g.*, Unigene, BioGPS, and Epigenome Roadmap) support a heavily brain-enriched expression pattern for *SLC4A10*. Of 127 cell lines and tissues represented in the WashU Epigenome Browser implementation of the Roadmap Epigenomics Project data (http://egg2.wustl.edu/roadmap/web_portal/), *SLC4A10* expression is detectable *via* RNA-Seq in only hippocampus, fetal brain, cultured neurospheres derived from cortex and ganglion eminence, and pancreatic islets (data not shown). Although intronic, the vicinity of rs16846053 is annotated as an active TSS or TSS flanking region in T cells and a number of other tissues. It is annotated as weakly transcribed in brain hippocampus and only in this tissue. Therefore, a novel variant of *SLC4A10* may be expressed in the CNS. Moreover, the DNaseI-hypersensitive regions upstream of

Table 1. Study participant characteristics

Study	Sample Size, *n*	Age, yr	Sex, % Women	Plasma Sodium, mEq/L	eGFRcrea, ml/min per 1.73 m^2	Plasma Glucose, mg/dl
Stage 1 discovery						
Amish studies	1131	48.1 (15.0)	49.8	139.2 (2.2)	94.1 (16.3)	91.1 (14.0)
BLSA	594	69.6 (15.1)	42.9	141.3 (3.0)	72.5 (17.5)	90.1 (13.2)
ARIC: Europeans	8535	54.1 (5.7)	53.2	141.0 (2.3)	90.0 (17.3)	99.8 (11.0)
FHS	2494	48.1 (15.0)	49.8	139.2 (2.2)	94.1 (16.3)	91.1 (14.0)
COLAUS	2816	58.3 (10.4)	53.5	142.6 (1.8)	81.1(15.4)	107.2(21.7)
MrOS	3909	73.8 (5.9)	0	141.5 (2.6)	77.1 (16.4)	100.9 (12.5)
MICROS	1146	45.2 (16.0)	56.5	139.0 (1.9)	91.2 (16.9)	84.1 (11.7)
KORA F3	1425	62.2 (10.1)	51.7	142.9 (4.4)	80.2 (14.4)	102.4 (16.1)
KORA F4	1671	60.7 (8.8)	52.4	139.1 (2.6)	81.6 (14.5)	98.1 (12.6)
GENOA: Europeans	1064	59.0 (10.2)	43.8	138.2 (2.1)	64.4(13.6)	100.4 (14.0)
InCHIANTI	1142	67.9 (15.6)	55.7	141.8 (2.5)	79.1 (17.9)	90.2 (14.6)
LOLIPOP_EW610	881	55.8 (9.8)	27.2	140.4 (2.1)	74.4 (12.5)	94.1 (11.9)
LOLIPOP_EWA	546	54.1 (10.4)	13.3	140.5 (2.7)	82.9 (19.5)	94.6 (13.6)
LOLIPOP_EWP	574	55.4 (9.3)	0	140.5 (2.5)	81.6 (13.0)	96.7 (13.9)
LURIC	2579	62.2 (10.8)	29.7	141.4 (2.8)	83.4 (17.1)	103.5 (15.3)
Ogliastra genetic park Talana study	691	50.2 (19.1)	58.5	139.1 (2.4)	72.6 (13.6)	90.6 (12.1)
Ogliastra genetic park study	382	54.1 (13.5)	0	137.2 (3.0)	75.2 (13.1)	99.6 (14.6)
SHIP	3767	48.8 (16.1)	51.7	138.8 (2.8)	80.5 (14.1)	96.3 (14.5)
SHIP-TREND	979	50.0 (13.6)	56.2	139.3 (2.2)	91.8 (20.1)	96.8 (11.3)
The Rotterdam study	3415	69.2 (8.7)	63	140.2 (3.3)	78.0 (16.3)	109.8 (17.9)
SARDINIA	6148	46.1 (17.7)	57.4	141.7(2.6)	100.5 (26.12)	88.8 (12.5)
Stage 2 replication						
DIACORE	1151	65.8 (8.6)	42.3	139.5 (2.8)	78.5 (23.5)	111.3 (21.0)
FINCAVAS	1761	60.2 (12.1)	37.5	139.8 (2.6)	88.7 (20.7)	105.9 (15.3)
LifeLines Cohort Study	12,270	48.8 (11.4)	58.2	141.7 (1.9)	90.3 (16.3)	91.6 (15.7)
MESA	2455	62.6 (10.3)	47.4	147.2 (3.8)	74.2 (14.3)	91.1 (21.4)

Data are given as mean (SD) or percentage.

the gene disproportionately map to cell lines of CNS origin (*e.g.*, SK-N-MC neuroblastoma cells and HA-h, HA-sp, and HAc astrocytic cells). Intriguingly, the lead variant at this locus affects a canonical NFAT5 DNA binding motif as determined *via* unbiased position-weight matrix scanning of the genomic context; moreover, the minor allele reduces the fidelity score for this predicted NFAT5 binding site (Supplemental Figure 4). An additional variant at this locus, rs16845945, maps to the *SLC4A10* proximal promoter approximately 200 bp upstream of the *SLC4A10* TSS. Consistent with this role, promoter-associated H3K4me3 histone modification pattern is observed in brain and pancreatic tissue (data not shown).

The known biology of additional genes in this locus (Supplemental Figure 2B) is summarized in Supplemental Table 7.

DISCUSSION

In this GWAS meta-analysis of systemic water balance, we have identified common variants in *NFAT5* associating with plasma sodium concentration in individuals of European descent, which are further supported by tentative validation in transethnic meta-analysis. Genomic functional annotation data implicate a role for genetic variation at *NFAT5* and *SLC4A10*, the latter a locus with nominally significant association, in regulating systemic water balance through expression-level effects in glial tissue of the central nervous system. These data are the first to implicate these genes in the regulation of systemic water balance.

The NFAT5 transcription factor coordinates the response to osmotic stress at the cellular level. It transactivates genes coding for aquaporins that are permissive for water movement, and for proteins that import or synthesize osmotically protective intracellular solutes.[40] NFAT5 also increases expression of heat shock proteins,[41] molecular chaperones that stabilize protein conformation against the denaturing effect of increased intracellular ionic concentration.[42] In addition, NFAT5 participates in the immune cell response to the varying sodium content within the skin and subcutaneous tissues.[43] The novel role for genetic variation in *NFAT5* in systemic osmoregulation (*i.e.*, water balance) is likely mediated at the level of gene expression.[40] The lead variant, rs9980, functions as a *cis* eQTL for *NFAT5*, such as has been observed for other disease-associated SNPs.[44–47] This variant resides within the 3′ untranslated region, a known site of *NFAT5* regulation by osmotic stress.[27–30] *NFAT5* function is also transcriptionally regulated by changes in tonicity.[11,27,48] Importantly, the lead variant is in LD with variant rs7193778, which resides within a superenhancer region upstream of the TSS. This gene region exhibits

Table 2. Genetic association analysis results in cohorts of European descent

SNP Identification	Chromosome	Position (Build 36)	Locus	Effect/ Other Allele	Stage 1 Discovery (*n*=45,889)			Stage 2 Replication (*n*=17,637)			Stages 1 and 2 Combined (*n*=63,526)			
					Effect Allele Frequency	β	*P* Value	Effect Allele Frequency	β	One-Sided *P* Value	Effect Allele Frequency	β	*P* Value	*N*
rs16846053	2	162274291	*SLC4A10*,[a] *DPP4*	G/T	0.10	0.06	1.86×10^{-7}	0.10	0.03	0.04	0.10	0.05	6.74×10^{-8}	63,524
rs753628	3	196040762	*XXYLT1, LSG1, TMEM44*	A/G	0.37	0.04	1.75×10^{-6}	0.38	0.01	0.24	0.37	0.04	$2.87 \times x10^{-6}$	60,091
rs12677356	8	23696389	*STC1, SLC25A37*	T/G	0.05	0.09	1.41×10^{-6}	0.05	−0.01	0.58	0.05	0.08	$4.64 \times x10^{-6}$	51,254
rs10774613	12	110030548	*CUX2*,[a] *CCDC63, ATXN2*	T/C	0.57	0.03	3.77×10^{-6}	0.52	0.02	0.13	0.56	0.03	$2.40 \times x10^{-6}$	60,095
rs17074418	13	29675855	*KATNAL1*,[a] *HMGB1, UBL3, SLC7A1*	T/C	0.92	0.07	1.77×10^{-6}	0.94	0.02	0.21	0.93	0.05	$4.97 \times x10^{-6}$	63,523
rs9980	16	68294969	*NFAT5*,[a] *NQO1, NOB1, CYB5B*	G/C	0.14	0.05	$2.40 \times x10^{-6}$	0.15	0.06	3.05×10^{-5}	0.14	0.06	$5.55 \times x10^{-10}$	60,108
rs11662617	18	13243598	*LDLRAD4*,[a] *CEP192, RNMT*	A/G	0.48	−0.04	$2.02 \times x10^{-6}$	0.48	0.01	0.72	0.48	−0.02	2.06×10^{-4}	54,921
rs6565990	18	73350561	*GALR1, MBP*	T/G	0.49	0.05	$1.51 \times x10^{-6}$	0.51	0.05	0.06	0.50	0.05	$2.96 \times x10^{-7}$	39,236

The gene nearest the SNP is listed first. Coded allele equals effect allele. The SNPs rs12677356 on chromosome 8 and rs6565990 on chromosome 18 were not available in the LifeLines Cohort Study, and proxy SNPs with $r^2>0.6$ could not be identified. The SNPs rs11662617 and rs6565990 (both on chromosome 18) were not available in the MESA, and proxy SNPs with $r^2>0.6$ could not be identified. Because only directly genotyped SNP data were available from the MESA, we analyzed three proxy SNPs for stage 2 meta-analysis. For rs16846053, we used the summary statistics of rs12476631 (chromosome 2, position 162,275,182, distance =891 bp, r^2=0.91, D'=1 with rs16846053). For rs9980, we analyzed rs39999 (chromosome 16, position 68,211,197, distance =83,772 bp, r^2=1, D'=1 with rs9980), and for rs17074418, we analyzed rs1023104 (chromosome 13, position 29,675,301, distance =554 bp, r^2=1, D'=1 with rs17074418).

[a]The gene nearest the SNP if the SNP is located in the gene.

remarkable enrichment for enhancer-specific histone modification, including H3K4me1 histone methylation consistent with enhancer function,[49] H3K27ac histone acetylation emblematic of active (in contrast to poised) enhancers,[50] and robust binding of a broad array of transcription factors (Figure 2). Our findings are consistent with the frequently observed effect of functional genetic variants on enhancer regions in disease pathogenesis[51–53] and the expression of quantitative traits.[54]

Bioinformatic data are consistent with a potential glial/astrocytic locus of action for *NFAT5* variant rs7193778 and the systemic sensing of plasma sodium concentration. Although neurons of the hypothalamus and lamina terminalis are known to have osmosensing roles,[37,38] a role for non-neuronal cells in the CNS has also been proposed. Specifically, a subset of glial cells (astrocytes) senses plasma sodium concentration *via* the Na_x channel.[35,36] Interestingly, a small DNaseI-hypersensitive region immediately upstream of rs7193778 in *NFAT5* is detected in astrocytes as one of only two of 125 ENCODE-tested cell lines. Moreover, the putative transcription factor binding sites affected by rs7193778 include those for *SOX* family members with expression highly enriched in glial tissue and glioma. Because enhancer-associated epigenomic marks are over-represented in trait-relevant tissues,[55] it is plausible that rs7193778 is an eQTL for *NFAT5* in glial cells and that the central sensor of plasma sodium concentration may reside in this tissue.

SLC4A10 encodes a brain-specific member of the sodium-bicarbonate transporter family, making this gene a biologically plausible participant in systemic water balance, despite lack of formal replication in stage 2 meta-analysis. The nominally significant signal at *SLC4A10* thus similarly implicates CNS glial tissue in systemic osmosensing. The protein is expressed predominantly in brain, and epigenomic functional annotations disproportionately map to brain and in particular, astrocytic, cell lines. Therefore, similar to the case for *NFAT5*, a glial cell–specific site of action for *SLC4A10* in central osmoregulation is plausible. Furthermore the lead variant at *SLC4A10* affects an intronic putative *NFAT5* DNA binding motif. Moreover, the variant affects the position weight matrix–defined fidelity score for the motif. Intronic enhancers have long been recognized,[56] including examples in genes coding for membrane transport proteins.[57–59] Notably, other members of the SLC4 family participate in volume regulation at the cellular level.[60]

Intriguingly, rs7193778, in LD with the lead variant at the *NFAT5* locus, has previously been identified in a meta-GWAS on plasma uric acid concentration.[61] Plasma uric acid level is influenced by systemic water balance and is often used to inform the diagnosis of a water-excess state, particularly in the context of the syndrome of inappropriate antidiuretic hormone.[62–64]

The minor allele of the *NFAT5* SNP rs9980 associates with hypertonicity and thus, a reduction in either the central sensing of water loss or renal water conservation, with similar effect sizes observed in all ethnicities studied. Thus, the relative absence of this variant in African ancestry (MAF 0.03 versus 0.14 in European populations) may hint at potential selection pressure in environments where chronic or seasonal

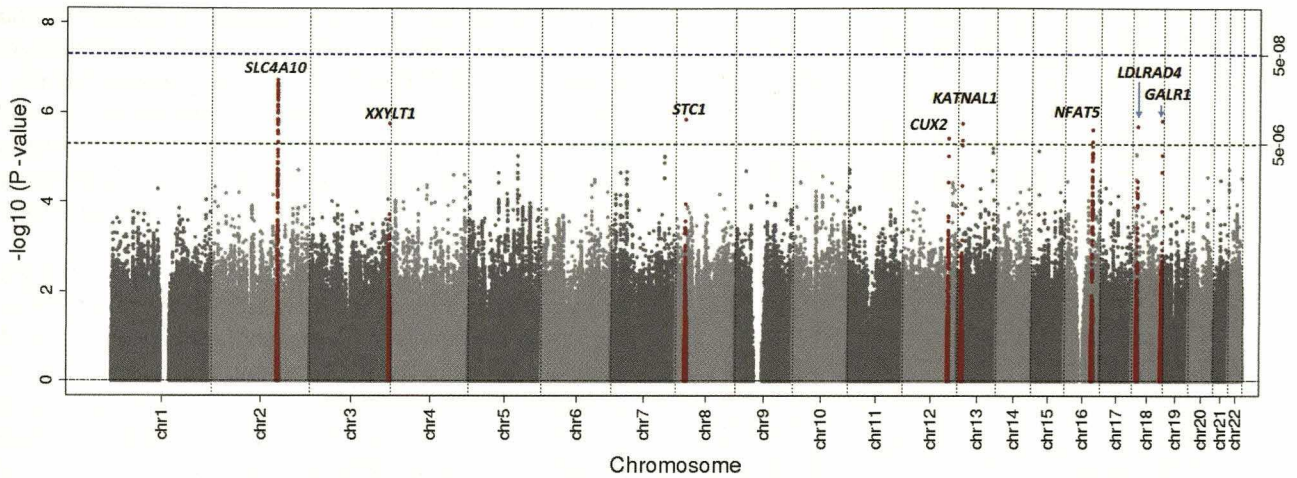

Figure 1. Stage 1 genome-wide association −log10 *P* value (Manhattan) plot identifies candidate loci. The green dotted line indicates the *P* value threshold for following SNPs in stage 2 meta-analysis ($P<5 \times 10^{-6}$). The blue dotted line indicates the genome-wide significance threshold ($P<5 \times 10^{-8}$).

water scarcity might occur. Similarly, the minor allele of rs16846053 in *SLC4A10* was under-represented in African ancestry (MAF 0.02 versus 0.10 in European populations).

Strengths of our work include the large sample size, the unbiased approach to identifying associated genetic loci by GWAS, and the bioinformatic characterization of the replicated loci. However, some limitations warrant mention. First, modest stage 2 replication and transethnic look-up sample size may have limited our ability to replicate additional loci. Second, the phenotype is based on a single measurement, potentially reducing statistical power. Third, the power for replication may have been limited by the poor imputation quality at the *GALR1* locus and the limited sample size at the *STC1* and *LDLRAD4* loci. Fourth, although the effect direction of the association of rs9980 with sodium was consistent across all analyzed ethnicities, the association was only borderline significant in Asian Indians and was not significant in those of African descent, possibly owing to limited power. Fifth, we did not directly replicate the functionally intriguing variant at the *NFAT5* locus (rs7193778), because it did not meet our *a priori* criteria for replication (*i.e.*, not independent from lead signal) and is in strong LD with the rs9980 variant ($D'=1$ and $r^2=0.9$). Finally, although we performed in-depth bioinformatic characterization of the identified loci, leading to important insights into potential mechanisms, the causal variant remains unknown, and we have not experimentally assessed the effects of the identified gene variants on gene function.

In summary, in this first well powered GWAS on plasma sodium concentration—the clinically measurable parameter of systemic water balance—we have identified genetic variants in *NFAT5* in individuals of European descent with validation by transethnic meta-analysis. Additionally, we identified a nominally significant association with an *SLC4A10* gene variant that may exert its effect through an intronic enhancer by altering its binding affinity for NFAT5. Our results and bioinformatic characterization point to a previously unknown role of genetic variation at *NFAT5* and *SLC4A10* in the regulation of systemic water balance *via* actions on gene expression within the central nervous system.

CONCISE METHODS

Data Management

An analysis plan, detailing phenotype derivation, exclusion criteria, genome-wide association testing, and data file formatting was distributed to all participating studies. Study-specific results files were uploaded to a central server for subsequent standardized central quality control and meta-analysis.[65]

Phenotype Definition

Plasma sodium concentration is the principal determinant of plasma osmolality. Plasma glucose concentration also contributes to plasma osmolality, and when elevated, it will obligate water entry into the intravascular space and render the plasma sodium concentration less reflective of true plasma osmolality. Thus, individuals with plasma glucose levels >150 mg/dl at the time of plasma sodium measurement

Table 3. Genetic association results of transethnic meta-analysis of rs9980 at *NFAT5*

Ethnic Group	Sample Size	Effect Allele	Other Allele	Effect Allele Frequency	β	*P* Value	Heterogeneity I^2, %
European descent	60,108	G	C	0.14	0.06	5.6×10^{-10}	0
Asian Indian	8760	G	C	0.12	0.05	0.02	0
African descent	5185	G	C	0.03	0.05	0.40	0
Transethnic	74,053	G	C	0.14	0.06	5.9×10^{-12}	27

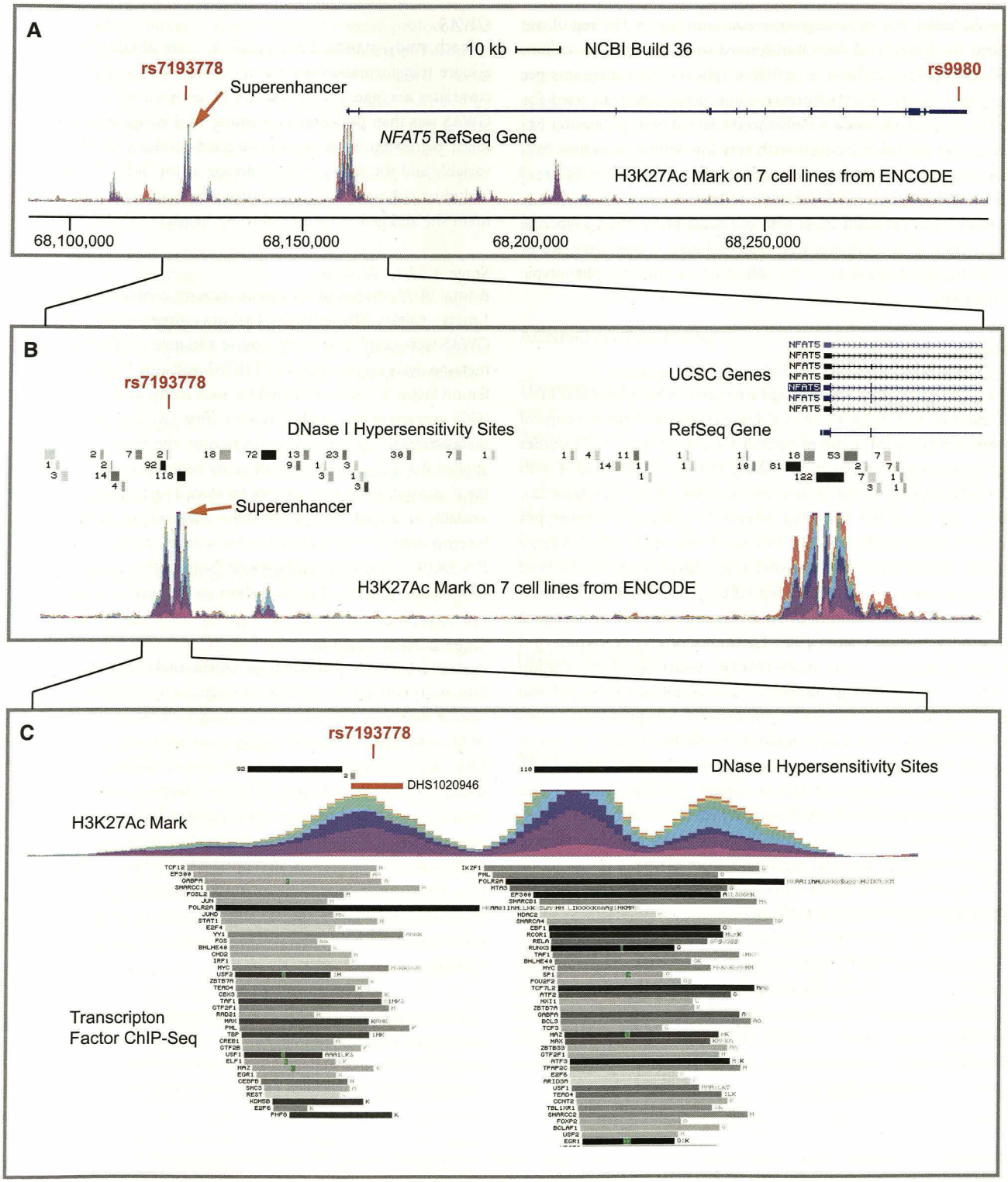

Figure 2. Sequence context of rs9980 and rs7193778 in the *NFAT5* region coincides with functional genomic annotation. Depicted are ENCODE data displayed in the UCSC Genome Browser. (A) shows *NFAT5* (exons connected by a blue line) and H3K27ac histone acetylation in the seven ENCODE cell lines (chromosome 16: 68087500–68307500 of human reference genome build NCBI build 36/hg18 and ENCODE histone modification track). Each color represents one of seven cell lines; peak height is the sum of activity in all cell types and proportional to the levels of enrichment of the H3K27ac histone mark across the genome as determined by ChIP-Seq assays. H3K27ac peaks coincide with the *NFAT5* promoter region and the vicinity of rs7193778. SNP rs9980 resides in the 3′ untranslated region. (B) illustrates an expanded view of approximately 40 kb upstream of the *NFAT5* TSS and depicts the H3K27ac triple peak of the *NFAT5* superenhancer as well as the locations of ENCODE/Epigenome Roadmap experimentally confirmed DNaseI-hypersensitive

were excluded. For plasma glucose concentration <150 mg/dl, we applied the formula of Katz: transformed sodium = plasma sodium (milliequivalents per liter) + (0.016 × (glucose [in milligrams per deciliter] −100)).[66] Transformed sodium was the trait used for the GWAS analysis. Because advanced CKD may impair water excretion, we excluded subjects with very low kidney function. We, thus, calculated eGFR on the basis of serum creatinine (eGFRcrea) using the four-variable Modification of Diet in Renal Disease Study equation[67] and excluded those with eGFRcrea below the age-specific mean minus 2 SD. Subjects were also excluded if they were not of the predominant ethnicity in the cohort or had missing phenotypic information.

Statistical Methods

Study Design, Genotypes, and Genotype Imputation

Details of each cohort's study design are shown in Supplemental Table 3. Details of each cohort's genotyping methods and quality control criteria are provided in Supplemental Table 4. In stage 1, 20 studies each imputed approximately 2.5 million SNPs on NCBI build 36 with external European haplotype reference samples (HapMap release 22). One study used the HapMap release 21 reference haplotypes on NCBI build 35 (KORA F3). In stage 2, one study (the LifeLines Cohort Study) contributed association statistics on the basis of genotypes imputed with HapMap CEU release 24 haplotypes on NCBI build 36. Two studies (FINCAVAS and DIACORE) in stage 2 imputed genotypes with 1000 Genomes reference haplotypes on GRCh build 37. One study (MESA) contributed association statistics on the basis of genotyped variants annotated on NCBI build 36. We transformed the SNP information of imputed genotypes on NCBI build 35 or GRCh build 37 to NCBI build 36 to match the data with HapMap-imputed genotypes of the other studies.

Studies of individuals of African descent and Asian Indians used cosmopolitan reference haplotypes to reflect the predominant ancestry in the study: two studies of individuals of African descent used the combined CEU and YRI haplotypes from HapMap release 22 on NCBI build 36, one study of individuals of African descent used the haplotypes from the June of 2010 release of the 1000 Genomes project on NCBI build 36 (HUFS), and one study of individuals of African descent used the 1000 Genomes Phase I interim data released in June of 2011 (on GRCh build 37) and transformed SNP information to NCBI build 36. All studies of Asian Indians used the combined HapMap release 22 CEU + CHB + JPT + YRI haplotypes on NCBI build 36. Imputed genotypes were coded as the estimated number of copies of a specified allele (dosage).

GWAS

In each study, standardized residuals were obtained by applying *z*-score transformation on plasma sodium concentration with the covariates sex, age, the interaction of sex with age, and eGFRcrea. GWAS was then performed assuming additive genetic effects using linear regression, with the standardized residuals as the dependent variable and the SNP genotype dosage as the independent variable, including cohort-specific covariates where applicable (*e.g.*, recruitment site and genetic principal components).

Stage 1 Meta-Analysis

A total of 21 studies of European ancestry contributed to the stage 1 meta-analysis. The summary statistics estimated from each cohort's GWAS were combined using inverse variance weighted fixed effects meta-analysis implemented in METAL software.[68] The genomic inflation factor λ[69] was estimated for each study, and genomic control (GC) correction was applied if $\lambda>1$ (first GC correction). After the meta-analysis, a second GC correction on the aggregated results was applied if $\lambda_{aggregated}>1$. Between-study heterogeneity was assessed by the I^2 statistic. SNPs were selected for stage 2 meta-analysis if they were available in at least 50% of all studies, they did not show an excess of heterogeneity ($I^2>50\%$), and they had a stage 1 discovery meta-analysis $P\leq 5\times10^{-6}$. The SNP with the lowest *P* value within a window of ± 1 Mb was selected for stage 2 meta-analysis and defined as the index SNP.

Stage 2 Meta-Analysis

In stage 2, SNPs identified in stage 1 meta-analysis were followed up in four study cohorts of European ancestry using the same analysis protocol as described for stage 1 meta-analysis. If the association statistics of the lead SNP in a susceptibility locus were not available, a proxy SNP with the highest LD and D' was used for the association analysis using the SNAP lookup tool in the HapMap release 22 dataset (https://www.broadinstitute.org/mpg/snap/ldsearch.php).[70] A SNP was considered to have replicated if its effect direction was consistent with stage 1 meta-analysis, it showed a significant one-sided *P* value after Bonferroni correction for multiple testing ($P<$[0.05/number of analyzed SNPs in stage 2]), and the *P* value of the stages 1 and 2 combined meta-analysis was lower than the stage 1 meta-analysis *P* value.

Genetic Associations in Studies of Individuals of African Descent and Asian Indians

For SNPs replicated in stage 2 meta-analysis, additional validation was sought among individuals of non-European ancestry (*i.e.*, African

sites above the H3K27ac peaks. The darkness of each site is proportional to the maximum signal strength observed in any cell line, and the adjacent numbers indicate numbers of tissues and cell lines in which hypersensitivity was detected (of a total of 125 tested tissues/cells). C illustrates the approximately 3-kb H3K27ac triple peak of the *NFAT5* superenhancer region, depicting rs7193778 relative to H3K27ac marks, DNaseI-hypersensitive sites, and a partial list of the 115 ChIP-Seq–confirmed transcription factor binding sites (of 161 tested) as gray boxes. The darkness of the boxes is proportional to the maximum signal strength observed in any cell line contributing to the cluster. The small DNaseI-hypersensitive region immediately 5′ of rs7193778 (marked 2) was detected in only astrocytes of the CNS (spinal cord; HA-sp cell line) and pancreatic islets (not shown: expression of *SLC4A10* is restricted to CNS and pancreatic islets). A DNaseI-sensitive region (DHS1020946) identified through the Regulatory Elements Database (http://dnase.genome.duke.edu/index.php; in the text) crosses the variant (orange bar) and maps to a cluster of motifs operative to astrocytes.

descent and Asian Indian cohorts) using the same protocols for GWAS and meta-analysis as described for the cohorts of European ancestry. Replication was defined by a significant one-sided P value after Bonferroni correction for multiple testing ($P<0.05$ divided by the number of replicated SNPs). There was little inflation in the genome-wide association meta-analysis of subjects of African and Asian ancestry ($\lambda=1.02$ in both analyses). Supplemental Tables 8 and 9 provide the summary statistics of all SNPs associated with sodium with a $P<10^{-5}$ within each ethnicity.

Transethnic Meta-Analysis

We performed a transethnic meta-analysis of replicated SNPs combining summary statistics from studies of individuals of European, African, and Asian Indian descent and following the same analysis protocol as described for stage 1 meta-analysis in individuals of European descent.

Visualization of LD Blocks

To illustrate the amount of LD between highly correlated variants, we visualized LD blocks with the R package snp.plotter (https://cran.r-project.org/web/packages/snp.plotter) with the individual genotype data of the population-based KORA F4 Study ($n=1814$).

Functional Characterization of Replicated Loci

We evaluated SNPs at replicated loci for potential relevance to gene expression by examining for overlap with functionally annotated genomic regions from the ENCODE[71] and Epigenomics Roadmap[55] projects. The former was queried *via* the UCSC Genome Browser, whereas the latter was accessed through the WashU Epigenome Browser (http://epigenomegateway.wustl.edu/) and RoadMap Epigenome Browser (http://epigenomegateway.wustl.edu/browser/roadmap/). SNPs in LD with lead SNPs ($r^2>0.8$)—and their corresponding functional annotations in ENCODE and the RoadMap Epigenome Project—were identified through HaploReg v4.0[32] citing Zou *et al.*[33] and RegulomeDB.[72] Genomic functional annotations in these public resources included the presence of DNase I-hypersensitive sites, chromatin modification (*e.g.*, histone methylation and acetylation), transcription factor binding, RNA-Seq expression data, and algorithmically assigned chromatin functional state (*e.g.*, active TSS, strong transcription, and enhancer region[55]; http://egg2.wustl.edu/roadmap/web_portal/chr_state_learning.html) in over 300 human tissues and cell lines (depending on assay platform). Putative transcription factor binding motifs were identified *via* JASPAR 2016 (http://jaspar.genereg.net/).[34] This resource uses a predefined position weight matrix to identify a potential binding motif and assign a score reflecting the fidelity of the sequence to the canonical motif. As an alternative to the more simplistic consensus sequence, the position weight matrix reflects the frequency of occurrence of each of the four nucleotides at each position in the motif.[73] *In silico* analysis of the *NFAT5* superenhancer in the vicinity of the rs7193778 sequence context (±50 bp; obtained from dbSNP) in the JASPAR 2016 screen, with the major (t) allele, identified 21 putative transcription factor binding sites crossing the variant, of which eight corresponded to SOX family members (SOX2, -3, -5, -6, -10 [$n=2$], and -17 and SRY). Apart from SRY (for which there were no expressed sequence tag (EST) data presented in the UNIGENE EST Profile Viewer), all of these SOX family transcription factors were expressed in brain and 2–28 other tissues (of a total of 45 tissues tested). For SOX2, -3, -5, and -6, brain expression was relatively enriched by up to 20-fold (for SOX3). Enrichment was quantified as normalized brain EST counts/total normalized EST counts for all 45 tissues. Expression in glioma was enriched by 2.5- to 25-fold for SOX2, -3, -6, and -10 (normalized glioma EST counts/total normalized EST counts for all 26 tumor types tested). EST counts were obtained from the UNIGENE EST profile viewer (*e.g.*, http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?UGID=155082&TAXID=9606&SEARCH=sox3).

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DISCLOSURES

None.

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