The biological impact of blood pressure associated genetic variants in the natriuretic peptide receptor C gene on human vascular smooth muscle

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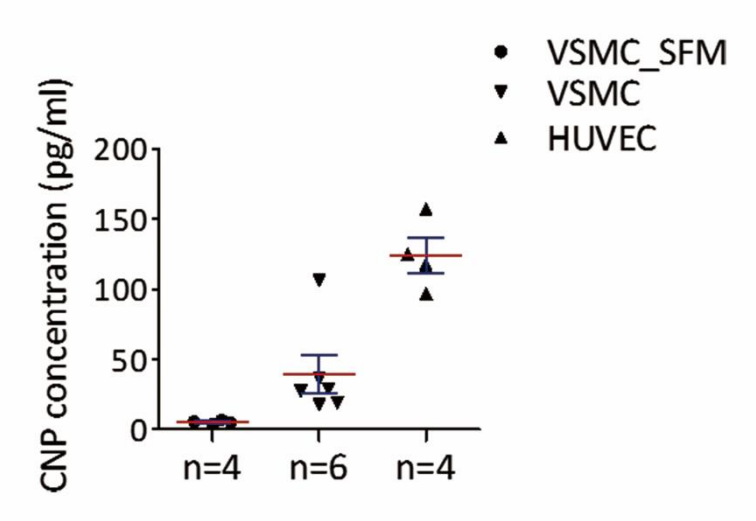
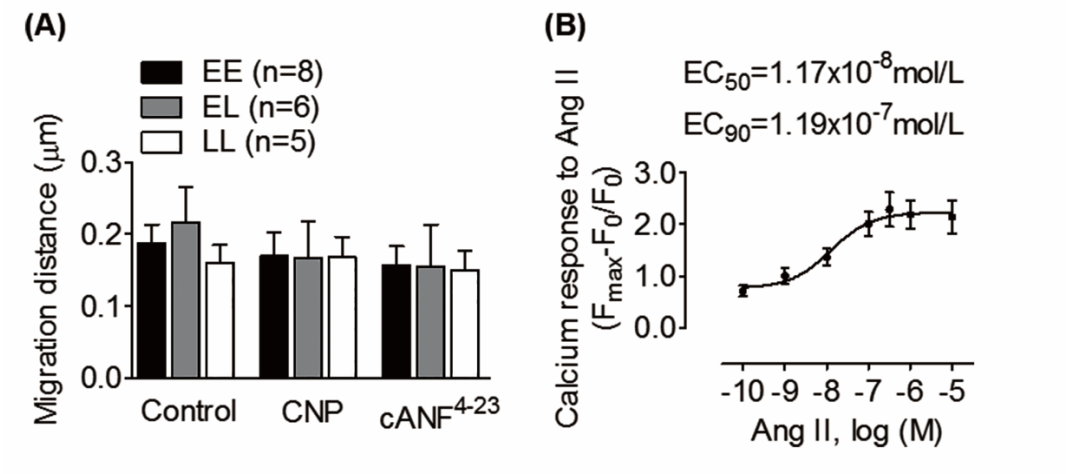
Supplementary Figures.

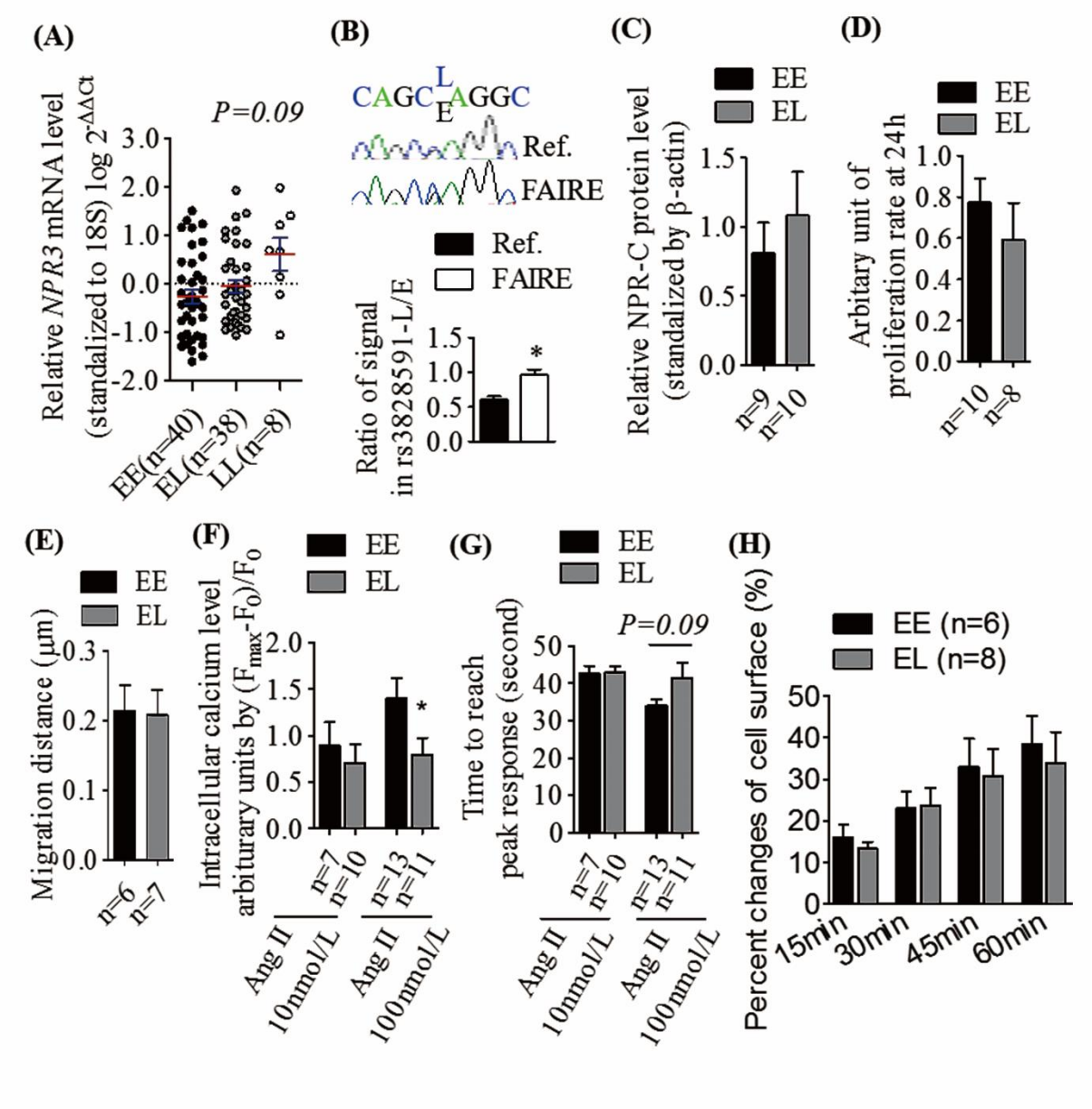


Supplementary Fig. 1. Detection of natriuretic peptides (CNP) in cell growth media. 'VSMC_SFM' indicates VSMC cultured with serum-free medium. Media from VSMC and EC (as a positive control) cultures was collected and subjected to Enzyme Immunoassay (EIA) for CNP evaluation. CNP was detected in both VSMC and EC culture media. Values shown in column charts are Mean ± SEM.



Supplementary Fig. 2. Effect of NPR3 BP-associated variants on human VSMCs migration and Ang II concentration-response curves.

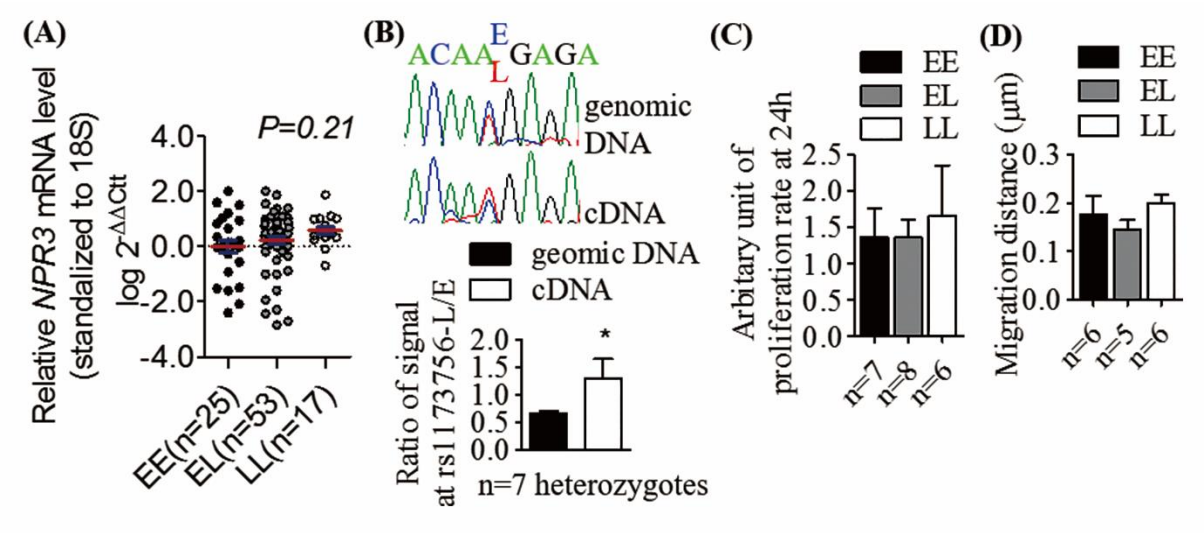
'E' indicates the BP-elevating allele, 'L' denotes the BP-lowering allele. Values are shown as Mean ± SEM of different measurements. (**A**)VSMCs were incubated in the absence or presence of C-type natriuretic peptide (CNP) (100nmol/L), the NPR-C specific agonist cANF$^{4-23}$ (100nmol/L), No difference in migration rate was observed among genotypes in untreated or treated VSMCs. (**B**) VSMCs were incubated with loading dye calcium 6 and stimulated by a range of angiotensin II concentrations. Data came from three independent experiments.



Supplementary Fig. 3. Effect of BP-associated variants in rs1421811 LD block on VSMCs NPR3 expression, and cell activities.

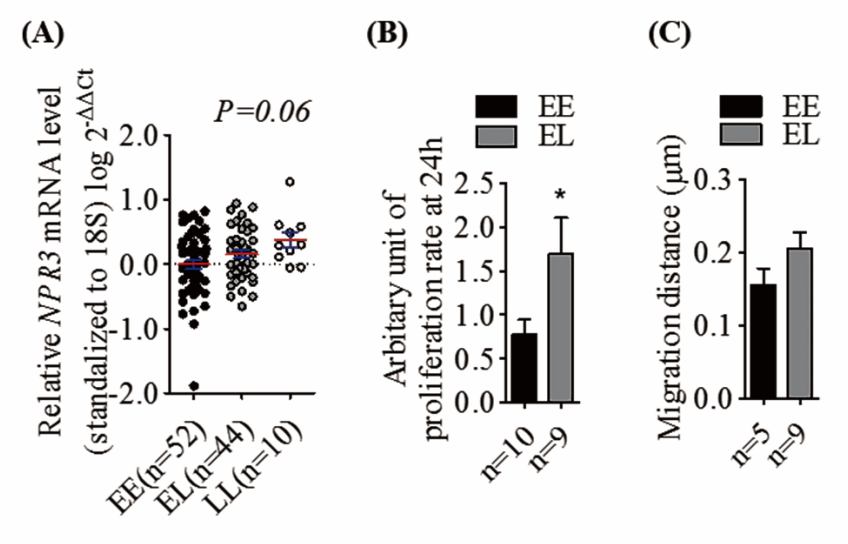
(**A-H**) 'E' indicates the BP-elevating allele, 'L' denotes the BP-lowering allele. Values are shown as Mean ± SEM of different measurements. (**A**) Relative mRNA levels of *NPR3* in VSMCs, determined by qRT-PCR and standardized against the reference gene 18S. (**B**) FAIRE followed by allelic imbalance assay and Sanger sequencing. Upper: Representative images showing different peak heights of the E and L alleles of intronic SNP rs3828591 (r^2=1.0 with lead SNP rs1421811) in chromatograms derived from reference (Ref.) DNA and FAIRE DNA,

n=5 heterozygous samples *P<0.05. (**C**) Western blot analysis of NPR-C protein in ECs with beta-actin as a loading control. (**D, E**) No genetic difference was detected in either cell proliferation or migration. (**F, G**) VSMCs were incubated with Ang II (10nmol/L or 100nmol/L). Difference between genotypes in intracellular calcium flux changes. *P<0.05 (**H**) VSMC cell contraction under stimulation of 100nmol/L Ang II.



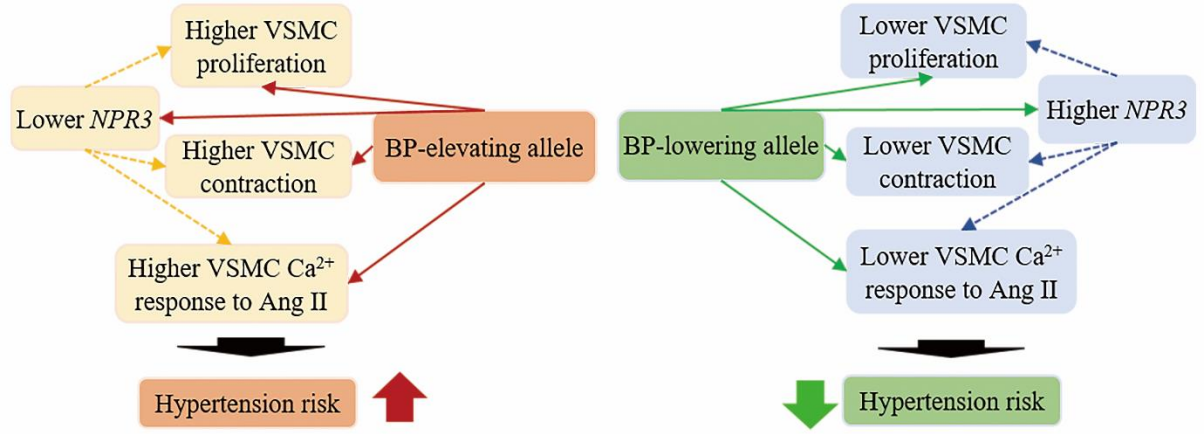
Supplementary Fig. 4. No association between BP-associated variants in rs1173771 LD block with ECs cell phenotypes.

'E' stands for the BP-elevating allele, 'L' denotes the BP-lowering allele. Values are shown as Mean ± SEM of different measurements. (**A**) Relative mRNA levels of *NPR3* in ECs, determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and standardized against the reference gene 18S. (**B**)The relative peak heights of two alleles of the 3' UTR SNP rs1173756 (r^2=0.78 with rs1173771) in sequencing chromatograms from genomic DNA and complementary DNA (cDNA) of ECs. *P<0.05 (**C, D**) ECs proliferation and migration were measured.



Supplementary Fig. 5. Effect of BP-associated variants in rs1421811 LD block on ECs *NPR3* expression, cell proliferation, migration.

'E' stands for the BP-elevating allele, 'L' denotes the BP-lowering allele. Values are shown as Mean ± SEM of different measurements. (**A**) *NPR3* mRNA detection by q RT-PCR in ECs for rs1421811 LD variants. (**B, C**) Endothelial cell proliferation and migration were measured, **P*<0.05.



Supplementary Fig. 6. Proposed model to show the systemic mechanism between NPR3 risk variants and hypertension risk.

The BP-elevating allele results in a lower *NPR3* expression and upregulates human vascular smooth muscle cell proliferation, intracellular calcium changes, and cell contraction in response to angiotensin II which could lead to higher risk for the development of hypertension.

Supplementary Tables.

Supplementary Table 1. Association between *NPR3* variants with blood pressure traits (DBP, PP) in UK Biobank cohort.

	Candidate SNPs	Positions on Chr 5 (bp)	EA	AA	EAF	Phenotypes	DBP Effect (mmHg)	*P*-value (DBP)	PP Effect (mmHg)	*P*-value (PP)
Block 1	rs1173727	32830521	C	T	0.60	SBP, DBP, PP	0.34 (0.04)	$1.64^{x}10^{-17}$	0.41 (0.05)	$2.19^{x}10^{-17}$
	rs1173743	32775047	T	G	0.52	SBP, PP	ns		0.26 (0.05)	$1.88^{x}10^{-8}$
	rs1173747	32782152	A	C	0.53	SBP, DBP, PP	0.23 (0.04)	$8.07^{x}10^{-9}$	0.29 (0.05)	$2.00^{x}10^{-9}$
	rs1173756	32789852	C	T	0.53	SBP, DBP, PP	0.22 (0.04)	$3.54^{x}10^{-8}$	0.50 (0.05)	$4.90^{x}10^{-9}$
	rs1173771	32815028	C	T	0.60	SBP, DBP, PP	0.32 (0.04)	$5.06^{x}10^{-16}$	0.41 (0.05)	$2.03^{x}10^{-17}$
	rs7733331	32828846	C	T	0.60	SBP, DBP, PP	0.32 (0.04)	$4.71^{x}10^{-17}$	0.42 (0.05)	$1.40^{x}10^{-17}$
Block 2	rs1421811	32714270	C	G	0.39	SBP, DBP, PP	-0.26 (0.04)	$2.15^{x}10^{-10}$	-0.43 (0.05)	$3.34^{x}10^{-18}$
	rs3762988	32709653	C	T	0.39	SBP, DBP, PP	-0.25 (0.04)	$4.33^{x}10^{-10}$	-0.41 (0.05)	$6.83^{x}10^{-17}$
	rs3828591	32713108	G	C	0.39	SBP, DBP, PP	-0.25 (0.04)	$4.53^{x}10^{-10}$	-0.43 (0.05)	$4.15^{x}10^{-18}$

The genetic position is based on Build 37 of the reference genome. $P<5\times10^{-8}$ was used a threshold to determine the association with BP traits.

The effect is presented as unit change (mmHg) with standard error (SE) in DBP, PP per copy of effect allele. Chr, chromosome; EA, effect allele;

AA, alternative allele; EAF, effect allele frequency. DBP, diastolic blood pressure; PP, pulse pressure. ns, not significant ($P>5\times10^{-8}$).

Supplementary Table 2. Candidate SNPs at *NPR3* locus and their bioinformatics predication annotations.

	Candidate SNPs	Positions on Chr 5 (bp)	SNP annotation	LD with lead SNP (CEU/CHBJPT/YRI)	ENCODE+ROADMAP	MotifDb/MotifbreakR	Gene-regulation	Matinspector	Published evidence
Block 1	**rs1173771**	32815028	Lead SNP/ intergenic	-	H3K27ac; H3K27M3; H3K4M1;	HNF1_3;	NA	NA	ICBP GWAS [1]
	rs1173747	32782152	intronic	0.78/1.00/0.23	H3K27ac; H3K27M3; H3K9M3	TCF7; CEBP;	GATA1; GR-α/β	TCF3; Y-box binding factor	NA
	rs1173756	32789852	3'-UTR	0.78/1.00/0.58	H3K9M3; H3K4M1; H3K27ac; H3K27M3; EZH2	SOX9; CTCF;	NA	NA	NA
	rs7733331	32828846	intergenic	1.00/0.96/0.44	EZH2; H3K9M3; H3K27M3; Poly-comb-repressed	ETS1; FOXA; FOXB1; FOXC/D2; AR; NR3C1	NA	NA	Trans-ancestry GWAS [4]
Block 2	**rs1421811**	32714270	Lead SNP /intronic	-	EZH2; Poly-comb-repressed	SETDB1; BDP1_disc3; NRF1;	-	-	Gene-centric [3]
	rs3762988	32709653	intronic	0.84/0.92/0.26	H3K27M3; Weak enhancer	HNF1_3;	-	-	NA
	rs3828591	32713108	intronic	1.00/1.00/0.31	EZH2; CTCF H3K27M3; H3K4M2 H3K4M3 Poly-comb-repressed	ESRRA;	-	-	NA

Note: NA, Not Available

Supplementary Table 3. Part 1 of summary of primers used in this study.

	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
Genotyping		
rs1173747-A	GAAGGTGACCAAGTTCATGCTCCCTCAGTTTCTGGTTAAAATGATTGTTT	GACTGGGGCTTAGGGAGCTGAT
rs1173747-C	GAAGGTCGGAGTCAACGGATTCCTCAGTTTCTGGTTAAAATGATTGTTG	GACTGGGGCTTAGGGAGCTGAT
rs1173756_C	GAAGGTCGGAGTCAACGGATTATCTATGCAATGGCCTTCATCTCG	GCATCACTTTCCTTTTAGTTATGGCTGAT
rs1173756_T	GAAGGTGACCAAGTTCATGCTGATCTATGCAATGGACTTCATCTAA	GCATCACTTTCCTTTTAGTTATGGCTGAT
rs1173771_C	GAAGGTGACCAAGTTCATGCTGGCTGCTGGTGCTTTGTGAATAAA	AGCCAACCATATGATGATCTCATACCAA
rs1173771_T	GAAGGTCGGAGTCAACGGATTGCTGCTGGTGCTGCTTTGTGAATAAG	AGCCAACCATATGATGATCTCATACCAA
rs1421811_G	GAAGGTGACCAAGTTCATGCTAAGTCAGAGACTTCCCAGGCC	CAAACCGCGGATCTCGCCCATA
rs1421811_C	GAAGGTCGGAGTCAACGGATTAAGTCAGAGACTTCCCAAGGCG	CAAACCGCGGATCTCGCCCATA
rs3828591_G	GAAGGTGACCAAGTTCATGCTGAGCTAAGTGGCGACGCCTG	GAAGCGTCCGCGGACGCCT
rs3828591_C	GAAGGTCGGAGTCAACGGATTGAGCTAAGTGGCGACGCCTC	GAAGCGTCCGCGGACGCCT
rs3762988_C	GAAGGTCGGAGTCAACGGATTAACCAGGAGCAAAAGAGACATCT	GTGTGGATTACCCTGTTTGTGGAATTAAA
rs3762988_T	GAAGGTGACCAAGTTCATGCTAACCAGGAGCAAAAGAGACATCT	GTGTGGATTACCCTGTTTGTGGAATTAAA
EMSA		
rs1173771-C*	CTCATACC AACTTATTCACAA	TTGTGAATAAGTTGGTATGAG
rs1173771-T*	CTCATACC AATTTATTCACAA	TTGTGAATAAATTGGTATGAG
rs1173747-A*	GGAGCTGATCAAACAATCATT	AATGATTGTTTGATCAGCTCC
rs1173747-C*	GGAGCTGATCCAACAATCATT	AATGATTGTTGGATCAGCTCC'

Note: * EMSA oligos were 5'-biotin labeled.

Supplementary Table 3. Part 2 of summary of primers used in this study.

	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size
q RT-PCR			
18S	CCCAGTAAGTGCGGGTCATAA	CCGAGGGCCTCACTAAACC	155bp
NPR3	GCCGCATTTCAAAACGACCT	TGCCAACGGAGACCGATATG	130bp
AEI			
rs1173756_g DNA	TTGGAATGCCCTCACTTCTC	GTGACGCCACTGGAACCTAC	220bp
rs1173756_c DNA	TGCTTTGGAATGCCCTCACT	ACGCCACTGGAACCTACTTT	221bp
FAIRE and ChIP			
rs1173771	AGTTGAGAGTTGGATGGGGA	GCTAACGTATGCTTGACCAA	165bp
rs1173747	TGTCAGGAGAAGGGGAGATGT	TTCCATGGTCATCCCTCAGTT	167bp
Cloning			
rs1173771	CACCACGAGCTCCCCATGATTCAATTACGTCCCC	GTCCACACGCGTACGTATGCTTGACCAAACACA	152bp
rs1173747	CACCACGAGCTCTTCCAAGAACCTGGGTGGGA	CGTCCACACGCGTTCTGGCCCCTGGAAATCATTA	216bp

Supplementary Table 4. Buffers' composition utilized in ChIP.

Buffers	Composition
Lysis buffer	50mmol/L Tris-HCI pH 8.0, 10mmol/L EDTA, 150mmol/L NaCl, 1% w/v sodium dodecylsulfate
ChIP dilution buffer	16.7 mmol/L Tris pH 8.0, 1.2 mmol/L EDTA, 150 mmol/L NaCl, 1.1% v/v Triton X-100, 0.01% w/v sodium dodecylsulfate
Low salt wash buffer	20mmol/L Tris-HCl pH 8.0, 2mmol/L EDTA, 150mmol/L NaCl, 0.1% w/v sodium dodecylsulfate, 1% v/v Triton X-100
High salt wash buffer	20mmol/L Tris-HCl pH 8.0, 2mmol/L EDTA, 500mmol/L NaCl, 0.1% w/v sodium dodecylsulfate, 1% v/v Triton X-100
LiCl wash buffer	250 mmol/L lithium chloride, 10mmol/L Tris-HCl pH 8.0, 1mmol/L EDTA, 1% v/v NP-40, 1% w/v sodium dodecylcholate
TE wash buffer	20mmol/L Tris-HCl pH 8.0, 1mmol/L EDTA
Elution buffer	1% w/v sodium dodecylsulfate, 100 mmol/L NaHCO3