A haplotype variant of the human chromogranin A gene (CHGA) promoter increases CHGA expression and the risk for cardiometabolic disorders

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The acidic glycoprotein chromogranin A (CHGA) is co-stored/co-secreted with catecholamines and crucial for secretory vesicle biogenesis in neuronal/neuroendocrine cells. CHGA is dysregulated in several cardiovascular diseases, but the underlying mechanisms are not well established. Here, we sought to identify common polymorphisms in the CHGA promoter and to explore the mechanistic basis of their plausible contribution to regulating CHGA protein levels in circulation. Resequencing of the CHGA promoter in an Indian population (n = 769) yielded nine single-nucleotide polymorphisms (SNPs): G→1106A, A→1018T, T→1014C, T→988G, G→513A, G→462A, T→415C, C→89A, and C→57T. Linkage disequilibrium (LD) analysis indicated strong LD among SNPs at the −1014, −988, −462, and −89 bp positions and between the −1018 and −57 bp positions. Haplotype analysis predicted five major promoter haplotypes that displayed differential promoter activities in neuronal cells; specifically, haplotype 2 (containing variant T alleles at −1018 and −57 bp) exhibited the highest promoter activity. Systematic computational and experimental analyses revealed that transcription factor c-Rel has a role in activating the CHGA promoter haplotype 2 under basal and pathophysiological conditions (viz. inflammation and hypoxia). Consistent with the higher in vitro CHGA promoter activity of haplotype 2, individuals carrying this haplotype had higher plasma CHGA levels, plasma glucose levels, diastolic blood pressure, and body mass index. In conclusion, these results suggest a functional role of the CHGA promoter haplotype 2 (occurring in a large proportion of the world population) in enhancing CHGA expression in haplotype 2 carriers who may be at higher risk for cardiovascular/metabolic disorders.

Chromogranin A (CHGA)7 (OMIM: 118910), a 48-kDa soluble, acidic glycoprotein present abundantly in secretory vesicles of endocrine, neuronal, and neuroendocrine cells, is co-stored and co-secreted with catecholamines (1, 2). CHGA plays a crucial role in the biogenesis of these secretory vesicles (3). CHGA is also a pro-hormone that generates bioactive peptides including catecholamine release inhibitory/anti-hypertensive peptide catestatin (CST) (4, 5), dysglycemic peptide pancreastatin (PST) (6, 7), vasodilator vasostatin (8), parathormone release inhibitory peptide parastatin (PRST) (9), and myocardial β-adrenergic-like agonist serpinin (10). Plasma CHGA levels are elevated in human essential hypertension (11), rodent models of hypertension (12), and in many cardiovascular complications like myocardial infarction (13) and acute coronary syndrome (14). But the plasma level of the CHGA-derived peptide CST is diminished in hypertensive individuals and even in their normotensive offsprings (15). Chga knock-out mice displayed elevated blood pressure that could be rescued by expression of human orthologue or by administration of human CST peptide (16). Interestingly, blood pressure of mice carrying varying copy numbers (0 to 4) of the CHGA/Chga gene showed a biphasic response to CHGA/Chga gene dosage, wherein, mice with 2 copies of the gene showed normal blood pressure but a decrease/increase in copy number resulted in elevated blood pressure (17). Thus, CHGA expression plays an important role in blood pressure homeostasis.

The abbreviations used are: CHGA, chromogranin A; ANOVA, analysis of variance; BMI, body mass index; CHGA, human chromogranin A gene; Chga, mouse chromogranin A gene; CST, catestatin peptide; DBP, diastolic blood pressure; LD, linkage disequilibrium; MAF, minor allele frequency; NF-κB, nuclear factor-κB; PST, pancreastatin peptide; PRST, parastatin peptide; SDM, site-directed mutagenesis; SNP, single-nucleotide polymorphisms; CREB, cAMP-response element-binding protein; qPCR, quantitative PCR.

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A previous study in a Southern California population identified several single-nucleotide polymorphisms (SNPs) in the CHGA locus (18). Among these SNPs, the G–462A promoter polymorphism was associated with blood pressure variation in an European population (19). The G–462A variant was also associated with lower levels of body mass index (BMI) and leptin indicating its important role in creating inter-individual variation in hypertension and metabolic syndrome (20). This CHGA promoter variant resulted in an alteration of binding affinity for transcription factor COUP-II-TF (19), suggesting that differential transcriptional regulation due to genetic variation in the promoter of this candidate gene might be a predictor of cardiovascular risk. However, the status of functional SNPs in the CHGA regulatory regions (e.g. promoter) in ethnically different human populations has not yet been studied. We hypothesize that discovery and functional characterization of CHGA genetic variations in subjects of various ancestries may confirm/yield novel associations with cardiometabolic phenotypes.

In this study, resequencing of the CHGA promoter was carried out in an Indian population to identify functional polymorphisms that may govern CHGA expression in vivo. Eight common and one novel SNP were detected; their allele and haplotype frequencies were significantly different from that of the European population. Two common polymorphisms A–1018T and C–57T were observed to be in strong LD constituting a haplotype (Hap2) along with T–415C and this haplotype displayed higher promoter activity than all other haplotypes in the population. Systematic computational, cellular, and molecular analyses revealed that stronger binding of the transcription factor c-Rel with haplotype 2 underlies the enhanced expression of CHGA under basal and pathological conditions (inflammation, hypoxia). In corroboration, individuals carrying the Hap2 genotype showed higher plasma CHGA levels; these subjects also displayed elevated BMI, diastolic blood pressure, and plasma glucose levels suggesting a functional role of this haplotype in conferring cardiometabolic risk.

Results

Discovery of polymorphisms in CHGA promoter region in an Indian population

Resequencing of the CHGA promoter region (~1.2 kb at chromosome 14q32.12) in 769 unrelated subjects (i.e. 1538 chromosomes) in an Indian population revealed the presence of nine SNPs: G–1106A (rs9658628), A–1018T (rs9658629), T–1014C (rs9658630), T–988G (rs9658631), G–513A (rs550617040), G–462A (rs9658634), T–415C (rs9658635), C–89A (rs7159323), and C–57T (rs9658638) (Fig. 1A). These polymorphisms were confirmed by inspection of chromatograms (supplemental Figs. S1 and S2). Of the nine SNPs identified, the G–513A polymorphism was a novel SNP occurring at a minor allele frequency (MAF) of 0.003; the other eight were common polymorphisms with MAF > 5% and were detected in a Southern California population. The authenticity of the novel G–513A variant was confirmed by reverse-strand sequencing (supplemental Fig. S2). The frequency of occurrence of each SNP was estimated by calculating the MAF and genotypic frequency (Table 1). The T–1014C, T–988G, G–462A, and C–89A polymorphisms occurred at almost identical frequencies suggesting possible linkage. The T–415C variation was highly frequent and was present in ~57% of our study population. Variants at −1018 and −57 bp also occurred at similar frequencies in the study population (36.9 and 36.7%, respectively).

Functional chromogranin A promoter haplotypes

The presence of multiple polymorphisms in a short regulatory region led us to consider the probability that these variants might be inherited together. Accordingly, haplotype prediction of unphased genotypes was carried out using the computational program PHASE, which predicted five major haplotypes that accounted for ~97% of the study population (Table 2). The most common haplotype (Hap1: GATTGTCC) was prevalent at a frequency of 0.31 and harbored major alleles across the eight common polymorphic sites in the 1.2-kb promoter of CHGA. Minor alleles at −1018, −415, and −57 bp positions with major alleles at the other five SNP sites constituted the next frequent haplotype (Hap2: GATTGCTT) with a frequency of 0.204. The variants at −1014, −988, −462, and −89 bp positions were present in identical frequencies and formed a haplotype (Hap3: GACGATAC, MAF = 0.196). The fourth and fifth haplotypes vary from Hap1 only with respect to alleles at −1106 and −415 bp positions; whereas haplotype 1 has major alleles across all the SNP sites, haplotype 4 has a minor allele at −1106 bp (Hap4: AATTGCTC), and haplotype 5 possesses a minor allele at −415 bp (Hap5: GATTGCC). Hap4 and Hap5 were relatively less frequent in the study population with frequencies of 0.148 and 0.112, respectively.

Next, to predict the non-random association of the alleles of these SNPs in the CHGA promoter, pairwise LD analysis was carried out within the eight common variants in the CHGA promoter and with three variants in the coding region (viz. G297S, rs9658664; G364S, rs9658667; and R381W, rs729940 within the PST, CST, and PRST peptide segments). The variants at −1014, −988, −462, and −89 bp positions were in strong LD; the variants at −1018 bp (rs9658629) and −57 bp (rs9658638) positions were also in LD (Fig. 1B and supplemental Table S2). The eight common polymorphisms in the CHGA promoter constituted a haplotype block suggesting that these variants could be inherited together. The peptide variants G297S of PST and G364S of CST also indicated modest LD with the CHGA promoter variants at −1018 and −57 bp positions; the PRST variant R381W was not in LD with any of the promoter SNPs (Fig. 1B and supplemental Table S2).

Functional consequences of CHGA promoter haplotypes: Differential activities of promoter-reporter constructs under basal conditions

CHGA promoter fragments representing each of the five predicted haplotypes were cloned into a promoter-less Gaussia Luciferase reporter vector, pGLuc-basic, to test the functional role of the haplotypes in governing transcription of the CHGA gene. CHGA promoter haplotype-reporter constructs transfected in human neuroblastoma cell lines IMR-32 and SH-SY5Y showed differential promoter activities (Fig. 2). CHGA promoter Hap2 with minor alleles at −1018, −415, and
Figure 1. CHGA polymorphisms and LD. A, schematic showing the polymorphisms identified in the CHGA promoter in an Indian population. Major and minor alleles and positions of SNP (with respect to transcription start site +1) have been indicated. The G−513A indicated by a star is a novel polymorphism. B, LD plot of common polymorphisms in CHGA promoter and exon 7 region. Pairwise LD values were plotted between common SNPs in the CHGA promoter and exon 7 region (coding for catestatin, pancreastatin and parastatin) of CHGA using Haploview 4.2. D’, coefficient of LD; LOD, log of the likelihood odds ratio.

Table 1
Occurrence of CHGA promoter SNPs in Indian population
NM_001275.3 was used as the reference sequence. The frequencies of occurrence of the nine variants identified in ~1.2 kb region of CHGA promoter are indicated along with their dbSNP numbers, minor allele and genotypic frequencies. Genotypic frequencies have been indicated in % of the population.

| dbSNP No. | HGVSc Description | Promoter position | MAF in Indian population (n = 769) | Genotypic frequency in Indian population (%) (n = 769) |
|-----------|-------------------|-------------------|------------------------------------|--------------------------------------------------------|
| rs9658628 | NC_000014.9:g.92922041G>A | G − 1106A | 0.151 | 26.6 |
| rs9658629 | NC_000014.9:g.92922129A>T | A − 1018T | 0.217 | 36.9 |
| rs9658630 | NC_000014.9:g.92922133C>T | C − 1014T | 0.204 | 38.2 |
| rs9658631 | NC_000014.9:g.92922159G>T | T − 988G | 0.203 | 38.2 |
| rs9658634 | NC_000014.9:g.92922685A>G | G − 462A | 0.201 | 37.7 |
| rs9658635 | NC_000014.9:g.92922732T>C | T − 415C | 0.345 | 56.6 |
| rs7159323 | NC_000014.9:g.92923058A>C | C − 89A | 0.202 | 37.8 |
| rs9658638 | NC_000014.9:g.92923090C>T | C − 57T | 0.213 | 36.7 |
| rs550617040 | NC_000014.9:g.92922634G>A | G − 513A | 0.003 | 0.5 |
57 bp positions consistently displayed higher promoter activity than the other four haplotype constructs in both IMR-32 and SH-SY5Y cell lines (IMR-32: one-way ANOVA, $F = 96.48$, $p < 0.0001$; SH-SY5Y: one-way ANOVA, $F = 139.4$, $p < 0.0001$). This suggested that minor alleles at 1018, 415, and 57 bp may have a concomitant role in the elevated promoter activity of the CHGA Hap2 promoter. However, contribution of the 415C allele to the observed increase in promoter activity was uncertain as the Hap5 CHGA promoter varied from the consensus sequence (Hap1) only with respect to the 415 bp position and presented a decrease or no significant difference in the CHGA promoter activity in IMR-32 and SH-SY5Y cells, respectively. Therefore, the higher activity of CHGA haplotype 2 may be due to the variations at −1018 and −57 bp positions.

**c-Rel governs the higher promoter activity of CHGA haplotype 2**

To probe for possible interactions of transcription factors at −1018 and −57 bp positions that might cause enhanced activity of CHGA Hap2 promoter, stringent computational prediction of transcription factors was carried out using ConSite, MatInspector, and P-Match programs. Computational analyses were carried out at 60% cut-off and transcription factors predicted consistently by two or more programs with different scores for binding across both alleles were chosen for experimental validation (supplemental Tables S5 and S6). Each of these programs predicted preferential binding of c-Rel to both 1018T and 57T alleles as compared with the 1018A and 57C alleles. The ConSite position-weight matrix for c-Rel indicating binding scores for different nucleotides is shown in Fig. 3A.

Figure 2. Relative activities of CHGA promoter haplotypes in different cell lines. A, schematic representation of the CHGA promoter haplotype-reporter constructs cloned into pGLuc-basic vector. B and C, differential promoter activities of CHGA promoter haplotype-reporter constructs. CHGA promoter haplotype-reporter constructs were transfected into human neuroblastoma cell lines IMR-32 and SH-SY5Y along with β-galactosidase expression plasmid. Results are expressed as mean ± S.E. of triplicate values of the ratio of Gaussia luciferase/β-gal. ***, $p < 0.001$ and ****, $p < 0.0001$ as compared with Hap2–GLuc construct.

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**Table 2**

Major haplotypes in the CHGA promoter region in Indian population

NM_001275.3 was used as the reference sequence. Haplotype prediction in the CHGA promoter region was carried out using PHASE program. The alleles present at each of the eight loci for the haplotypes are indicated along with the frequency of occurrence of the haplotype in the population. 2n refers to the numbers of chromosomes analyzed.

| No. | −1106 | −1018 | −1014 | −988 | −462 | −415 | −89 | −57 |
|-----|-------|-------|-------|------|------|------|-----|-----|
| 1   | G     | A     | T     | T    | G    | T    | C   | C   |
| 2   | G     | T     | T     | G    | C    | C    | T   | C   |
| 3   | G     | A     | C     | G    | A    | T    | A   | C   |
| 4   | A     | A     | T     | T    | G    | T    | C   | C   |
| 5   | G     | A     | T     | T    | G    | C    | C   | C   |

| Frequency in Indian population (2n = 1538) |
|-------------------------------------------|
| 0.310                                      |
| 0.204                                      |
| 0.196                                      |
| 0.148                                      |
| 0.112                                      |

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**Figure 2.** Relative activities of CHGA promoter haplotypes in different cell lines. A, schematic representation of the CHGA promoter haplotype-reporter constructs cloned into pGLuc-basic vector. B and C, differential promoter activities of CHGA promoter haplotype-reporter constructs. CHGA promoter haplotype-reporter constructs were transfected into human neuroblastoma cell lines IMR-32 and SH-SY5Y along with β-galactosidase expression plasmid. Results are expressed as mean ± S.E. of triplicate values of the ratio of Gaussia luciferase/β-gal. ***, $p < 0.001$ and ****, $p < 0.0001$ as compared with Hap2–GLuc construct.
However, the Hap5 promoter construct did not exhibit any significant increase upon co-transfection of c-Rel under identical conditions. In corroboration, co-transfection of c-Rel siRNA oligo with the Hap2 promoter-reporter construct resulted in a significant reduction in promoter activity when compared with the negative control oligo (one-way ANOVA, $F = 69.85, p < 0.0001$); on the other hand, the Hap5 promoter-reporter construct did not show any significant change in promoter activity (Fig. 3D). Also, co-transfection of the c-Rel expression plasmid with the CHGA Hap1 promoter construct did not result in an increase in promoter activity of the Hap1 construct, whereas it dose-dependently augmented activity of the Hap2 promoter construct (supplemental Fig. S4).

Furthermore, to establish the extent of interactions of c-Rel individually with −1018T and −57T, site-directed mutants were generated via mutation of both major alleles (viz. −1018A and −57C) on Hap1 background: Hap1−1018T and Hap1−57T constructs that harbor the variant T allele at one of the SNP positions and a double mutant Hap1-DM that harbors the variant T allele at both SNP positions (Fig. 4A). When transfected into IMR-32 cells both Hap1−1018T and Hap1−57T mutants displayed higher promoter activities than the Hap1 construct, whereas the Hap1–DM construct exhibited the highest promoter activity (one-way ANOVA, $F = 24.38, p = 0.0002$) indicating that the increased promoter activity of the CHGA Hap2 promoter could be attributed to interaction of c-Rel with minor alleles at both sites (Fig. 4B). Consistently, co-transfection of c-Rel with these T allele constructs yielded dose-dependent enhancements (one-way ANOVA, $F = 40.76, p < 0.0001$) in promoter activity (Fig. 4C).

To study the interaction of c-Rel with the CHGA Hap2 promoter across the −1018 and −57 bp sites in vitro, electrophoretic mobility shift assays (EMSAs) were carried out (Fig. 5). Incubation of labeled CHGA promoter oligos with nuclear extract from IMR-32 cells yielded several specific complexes;
addition of molar excesses of the corresponding unlabeled oligos to the binding reactions resulted in concentration-dependent inhibitions of two specific complexes. Addition of the c-Rel antibody to the binding reactions, in general, caused complete inhibition of the higher molecular weight complex and partial inhibition of the lower molecular weight complex; on the other hand, control IgG failed to inhibit formation of the specific complexes indicating specificity of the interactions between the promoter domains and c-Rel (Fig. 5, A and B). The variant oligo (predicted to bind to c-Rel with higher affinity than the wild-type oligo) showed a greater extent of competition and interference with antibody than the wild-type oligo in the case of both SNPs. As a positive control, EMSA reactions carried out using c-Rel consensus oligo also resulted in similar interference/inhibition of the specific complexes in response to addition of c-Rel antibody as observed with CHGA −1018 and −57 variant oligos (Fig. 5C).

To assess the interactions of c-Rel with the CHGA Hap2 promoter at −1018 and −57 bp loci in the context of chromatin, chromatin immunoprecipitation (ChiP) assay was carried out in N2a cells transfected with CHGA Hap2 and Hap5 promoter constructs (Fig. 6, A and B). Quantitative PCR (qPCR) for purified, immunoprecipitated chromatin revealed that enrichment with −1018T and −57T alleles of the CHGA Hap2 promoter were ~4.2- and ~5.2-fold higher for immunoprecipitation with c-Rel when compared with the −1018A and −57C alleles of the CHGA Hap5 promoter (p < 0.05). These results strongly suggest that c-Rel binds to −1018T and −57T alleles with higher affinities thereby accounting for the higher promoter activity of CHGA Hap2 promoter.

Enhanced response of CHGA Hap2 promoter to inflammatory stimuli (TNF-α): Crucial role for c-Rel

As c-Rel activates CHGA promoter Hap2 (Fig. 3C), we probed whether TNF-α, a crucial mediator of inflammation and activator of NF-κB family of transcription factors, can modulate the activation of Hap2 promoter via c-Rel. Hap5 promoter (that harbors the wild-type alleles at −1018 and −57 bp positions) was used as a control construct. Indeed, TNF-α treatment augmented the promoter activity of the CHGA Hap2 construct in a dose-dependent manner (one-way ANOVA, F = 38.87, p < 0.0001) up to ~2.3-fold (p < 0.0001) (Fig. 7A), but no significant increase in promoter activity was observed with the CHGA Hap5 construct. Also, a proportional increase in c-Rel
Functional chromogranin A promoter haplotypes

Figure 5. Interactions of c-Rel with CHGA promoter −1018 and −57 SNPs in vitro: EMSA for −1018 (A), −57 (B), and c-Rel consensus (C) oligos. A and B, lanes 1 and 9, only labeled variant (Var) and wild-type (WT) oligos; lanes 2 and 10, IMR-32 nuclear extract with Var and WT biotin-labeled oligos; lanes 3–5 and 11–13, labeled oligos incubated with molar excesses of corresponding unlabeled oligos and nuclear extract; lanes 6 and 14, Var and WT-labeled oligos incubated with a mouse monoclonal c-Rel antibody and nuclear extract; lanes 7 and 15, Var and WT-labeled oligos incubated with control IgG and nuclear extract; lane 8, only IMR-32 nuclear extract. C, lane 1, only labeled c-Rel consensus oligo; lane 2, IMR-32 nuclear extract with labeled c-Rel consensus oligo; lanes 3–5, labeled c-Rel consensus oligo incubated with molar excesses of unlabeled oligo; lane 6, labeled c-Rel consensus oligo incubated with a mouse monoclonal c-Rel antibody and nuclear extract; lane 7, labeled c-Rel consensus oligo incubated with control IgG and nuclear extract; lane 8, only IMR-32 nuclear extract. Specific complexes are indicated.

Figure 6. Differential binding of c-Rel with CHGA promoter −1018T and −57T alleles of CHGA promoter haplotype 2. Chromatin immunoprecipitation of N2a cells transfected with the Hap2 or Hap5 constructs was carried out using antibody against c-Rel/preimmune IgG. Immunoprecipitated chromatin was subjected to qPCR using primers flanking the −1018 and −57 SNPs, respectively. Fold-enrichment in case of c-Rel antibody over preimmune IgG is shown.
levels (up to ~2.1-fold) was observed in IMR-32 cells treated with different doses of TNF-α (Fig. 7B). Additionally, in IMR-32 cells co-transfected with the CHGA Hap2 promoter-reporter construct and c-Rel followed by treatment with TNF-α (Fig. 7C), the promoter activity of the CHGA Hap2 construct was enhanced further by ~2.5-fold ($p < 0.0001$) against TNF-α treatment alone (~1.4-fold, $p < 0.0001$) or c-Rel co-transfection alone (~2.0-fold, $p < 0.01$). However, only a modest increase in promoter activity was observed in the case of CHGA Hap5 promoter (two-way ANOVA haplotype effect: $F = 85.3, p < 0.0001$; treatment effect: $F = 124.7, p < 0.0001$). Similarly, TNF-α treatment after siRNA-mediated down-regulation of c-Rel did not increase the promoter activity of the CHGA Hap2 construct (Fig. 7D) as against the ~1.5-fold increase ($p < 0.001$) in promoter activity observed after TNF-α treatment with the negative control siRNA oligos (two-way ANOVA treatment effect: $F = 124.7, p < 0.0001$).
ANOVA haplotype effect: $F = 33.24, p < 0.0001$; treatment effect: $F = 77.99, p < 0.0001$).

Consistently, ChIP assays on N2a cells transfected with CHGA Hap2 promoter constructs followed by TNF-α treatment revealed significantly higher fold-enrichment with c-Rel in the case of $−1018T$ ($−2.8$-fold) and $−57T$ ($−7.5$-fold) (present in CHGA Hap2 promoter) but not with $−1018A$ and $−57C$ alleles ($−1018: \text{one-way ANOVA}, F = 86.42, p = 0.0004; −57: \text{one-way ANOVA}, F = 67.67, p < 0.0001$) (Fig. 7, E and F). Thus, c-Rel appears to play a crucial role in mediating TNF-α-induced augmentation of CHGA Hap2 promoter activity.

**Effect of hypoxic stress on CHGA promoter haplotypes: Involvement of c-Rel**

To test if hypoxic stress (a major contributor to cardiac pathophysiology) activates the haplotypes differently and to ascertain the role of c-Rel, if any, IMR-32 cells transfected with CHGA promoter Hap2 and Hap5 constructs were subjected to hypoxia for 12 h with/without c-Rel co-transfection. Hypoxia enhanced basal promoter activity of CHGA Hap2 promoter significantly ($−2.5$-fold, $p < 0.0001$); however, no significant increase was observed in the CHGA Hap5 construct (Fig. 8A). c-Rel overexpression in cells transfected with the Hap2 CHGA promoter construct further magnified ($−3.5$-fold, $p < 0.0001$) the observed increase in promoter activity after hypoxia treatment when compared with the Hap5 CHGA promoter (two-way ANOVA haplotype effect: $F = 96.08, p < 0.0001$; treatment effect: $F = 60.35, p < 0.0001$). Western blot analysis showed overexpression of HIF-1α ($−1.3$-fold) and c-Rel ($−1.8$-fold) during these experiments (Fig. 8B).

Next, ChIP assays were carried out to probe for interaction of c-Rel with the $−1018T$ and $−57T$ alleles during hypoxia. Indeed, the fold-enrichments for c-Rel over IgG control in the case of the $−1018T$ and $−57T$ alleles after induction of hypoxia were significantly higher (Fig. 8, C and D) than the untreated control as well as the $−1018A$ and $−57C$ alleles ($−1018: \text{one-way ANOVA}, F = 31.89, p = 0.003; −57: \text{one-way ANOVA}, F = 76.8, p < 0.0001$).

**Hap2 CHGA promoter results in higher CHGA levels in vitro and in vivo**

To test whether the differential activities of CHGA promoter haplotypes result in altered CHGA levels in vitro, we generated recombinant plasmids wherein hCHGA cDNA was placed under the control of CHGA Hap1, Hap2, and Hap5 promoters (Fig. 9A) and expressed them in N2a cells. Western blot analysis of cell lysates revealed significantly higher levels of CHGA from the Hap2 promoter-driven CHGA cDNA ($−1.5$-fold, one-way ANOVA, $F = 15.89, p = 0.004$; Fig. 9, B and C) as compared to Hap1 and Hap5 promoter-driven CHGA cDNA, suggesting that in the genomic context the Hap2 promoter is likely to be more active than the Hap1 and Hap5 promoters.
Next, we analyzed the plasma CHGA levels in our subjects stratified based on their CHGA promoter haplotypes. Hap2/Hap2 and Hap2/Hap1 individuals displayed ~1.9- and ~1.4-fold, respectively, higher plasma CHGA levels than in Hap1/Hap1 individuals (one-way ANOVA, $F = 13.97, p < 0.0001$) (Fig. 9D). Likewise, plasma CHGA levels in Hap2/Hap2 individuals was higher (~1.5-fold, $p = 0.0015$) than in Hap2/Hap5 individuals (Fig. 9E). Thus, consistent with the higher promoter activity (Fig. 2) the presence of the Hap2 promoter, in general, correlated with elevated plasma CHGA levels in vivo in human subjects.

### Activities of CHGA promoter haplotype constructs in diploid combinations in cella

In view of the graded correlation of the plasma CHGA levels in humans with the copy number of Hap1 and Hap2 we sought to test whether this observation could also be demonstrated in
transfected cultured cells. We transfected IMR-32 cells with Hap1 or Hap2 promoter-reporter plasmids in three diploid combinations: Hap1/Hap1 (i.e., only Hap1), Hap2/Hap2 (i.e., only Hap2), and Hap1/Hap2 (i.e., equimolar amounts of Hap1 and Hap2). The results showed significant differences in promoter activities among the three conditions (one-way ANOVA, $F_{11.005} = 7.975$, $p < 0.02$). Specifically, the Hap2 promoter construct alone displayed 1.4-fold ($p < 0.05$) higher promoter activity as compared with Hap1 promoter (Fig. 9F) and the Hap1/Hap2 combination presented intermediate levels of promoter activity.

Similarly, we also tested the activities of diploid combinations of Hap2 and Hap5 promoter constructs. The promoter activity significantly differed among the three conditions (one-way ANOVA, $F = 7.975$, $p = 0.02$). Specifically, the Hap2 promoter construct alone displayed $\sim$1.4-fold ($p < 0.05$) higher promoter activity as compared with Hap1 promoter (Fig. 9F) and the Hap1/Hap2 combination presented intermediate levels of promoter activity.

Plausible association of Hap2 CHGA promoter with cardiometabolic traits

To understand the potential implications of CHGA promoter haplotypes on cardiometabolic traits (apart from the CHGA levels, as shown in Fig. 9, D and E) in our study population, the phenotypic data for various demographic/physiological/biochemical parameters (supplemental Table S8) were analyzed by inferential statistics. The population was stratified based on the diploid haplotype at the CHGA promoter locus and clinical parameters contributing to cardiometabolic traits were compared between Hap2 and Hap1 individuals as they represent the most frequent haplotypes in the population. These parameters were also compared in individuals of Hap5 background as they vary from Hap2 genetically only with respect to $\sim$1018 and $\sim$57 bp positions.

We detected a strong association between CHGA Hap2/Hap2 individuals and higher BMI values when compared with both Hap1/Hap1 ($\sim$1.3 kg/m²; $p < 0.05$) and Hap2/Hap5 subjects ($\sim$2.4 kg/m²; $p = 0.0011$) (Fig. 10, A and B). Interestingly, BMI levels in these subjects positively correlated with plasma CHGA levels (Pearson $r = 0.38$, $p < 0.05$) (Fig. 10C). In addition, individuals of the Hap2/Hap2 genotype displayed significantly elevated plasma glucose levels ($\sim$9 mg/dl, $p = 0.006$) when compared with wild-type subjects (i.e., Hap1/Hap1 genotype group) (Fig. 10D). Hap2/Hap2 individuals also displayed a trend toward higher plasma glucose levels (by $\sim$6 mg/dl) when compared with Hap2/Hap5 genotype subjects, although the difference was not statistically significant (Fig. 10E). However, the plasma glucose levels in Hap2/Hap2 individuals showed a modest positive correlation with plasma CHGA levels (Pearson $r = 0.31$, $p < 0.05$) (Fig. 10F). Diastolic blood pressure (DBP) was also found to be elevated in Hap2/Hap2 individuals ($\sim$6 mm Hg, $p < 0.05$) as compared to Hap2/Hap5 subjects. The DBP levels in these individuals positively correlated with plasma CHGA levels (Pearson $r = 0.39$, $p < 0.05$ for DBP).
Discussion

Discovery of CHGA promoter variations in an Indian population and comparisons with other ethnic populations

In view of the aberrant expression of CHGA in cardiovascular diseases including hypertension (21, 22), identification of genetic variants/motifs in the CHGA promoter that may regulate its gene expression has been an area of recent interest (19, 20). Of note, these studies included subjects of European ancestry alone and no data (on CHGA regulatory variants) is available for other ethnic populations including people of south Asian ancestry that have significantly increased cardiovascular risk (23, 24). However, the frequency of SNPs in different ethnic populations may differ (25–28) and different variations in the regulatory regions may contribute to differences in gene expression phenotype among ethnic groups (29). Therefore, we set out for systematic discovery and characterization of CHGA promoter polymorphisms in an Indian population.

We detected eight common polymorphisms (viz. G–1106A, A–1018T, T–1014C, T–988G, G–462A, T–415C, C–89A, and C–57T) (Table 1). The minor allele frequencies of each of these eight variants in the Indian population differed significantly as compared to the superpopulations in the 1000 Genomes project (30) with the exception of the SAS (South Asian) population indicating the contribution of ethnicity to the occurrence of SNPs (supplemental Table S3). We also discovered a novel SNP, G–513A (rs550617040) (supplemental Fig. S2); although this variation was rare (0.5%) in our study population it has now been detected in the South Asian superpopulation (viz. the Gujarati Indians in Houston, TX (2.9%), Indian Telugu in the UK (0.9%), and Punjabi in Lahore, Pakistan (1.04%)) in the 1000 Genomes project (30) indicating its specificity to the people of South Asian origin.

Pairwise LD analysis of the eight common variants showed that T–1014C, T–988G, G–462A, and C–89A variants were in strong LD (Fig. 1B and supplemental Table S2); this observation is similar to the European population (18). Additionally, our study also revealed a strong LD between A–1018T and C–57T variants (Fig. 1B and supplemental Table S2) that was not reported in the European population. In fact, the A–1018T and C–57T variants were found to be in stronger LD in the Asian populations (East Asian and South Asian) than the other populations of the 1000 genomes project study populations (supplemental Fig. S3). Haplotype analysis of the promoter SNPs inferred five different haplotypes accounting for 97% of the population. The haplotype frequencies in our study population matched with those of the South Asian population but were distinctly different from the other populations (supplemental Table S4). Notably, Hap2 (constituted by A–1018T, C–57T, and T–415C variant alleles) was the second most frequent haplotype (occurring in 20.4% of the total chromosomes) in the Indian population (Table 2). This haplotype, however, was less frequent (constituting 11.1% of the total chromosomes in population) in the 1000 genomes European superpopulation. Thus, the architecture of the CHGA locus (viz. number of genetic variations, frequency of the variants/haplotypes, and genetic linkage among the variants) appears to differ significantly between people of Indian and European origins.

Functional chromogranin A promoter haplotypes

Transient transfections of CHGA promoter-luciferase reporter in human neuroblastoma cells showed the highest promoter activity for the Hap2 construct (Fig. 2). What may be the molecular basis for the highest activity of this promoter Hap2? Analysis of the activities of CHGA Hap2 and allele-specific site-directed mutants of CHGA Hap1 constructs confirmed that the enhanced promoter activity is due to both −1018T and −57T nucleotides (Fig. 4B). Consistent with computational predictions overexpression/down-regulation of the transcription factor c-Rel in IMR-32 cells resulted in augmentation/reduction of Hap2 promoter activity (Fig. 3), suggesting synergistic binding of c-Rel at −1018T and −57T bp positions of the CHGA promoter. EMSA experiments using oligos flanking the −1018 and −57 SNPs also suggested c-Rel binding at these loci (Fig. 5). Indeed, ChIP assays confirmed preferential binding of c-Rel with −1018T and −57T alleles as compared to the wild-type alleles (Fig. 6). Of note, although important roles for the transcription factors CREB, Sp1, COUP-Tf, PPAR-γ, RXRα, LEF1 on transcriptional regulation of CHGA were previously reported (19, 31–33), this study provides evidence for the role of c-Rel for the first time.

C-Rel belongs to the NF-κB family of transcription factors that regulate a wide range of genes like vascular endothelial growth factor (VEGFC), hypoxia-inducible factor 1-α (HIF-1α), heat shock protein 70-kDa protein 1 (Hsp70), erythropoietin (EPO), α2B adrenergic receptor (ADRA2B) etc. (34–38). C-Rel was shown to promote cardiac hypertrophy and fibrosis (39). It was also identified as a key player in inflammation and was found to be up-regulated by mediators of inflammation like TNF-α (40, 41). Consistently, TNF-α-mediated overexpression of c-Rel in turn activated (up to ~2.3-fold) CHGA promoter Hap2, and ectopic expression of c-Rel only enhanced the promoter activity further (~2.5-fold; Fig. 7). In addition, siRNA-mediated down-regulation of c-Rel did not increase the promoter activity of the CHGA Hap2 construct following TNF-α treatment, thereby establishing the role of c-Rel in governing expression of the CHGA Hap2 promoter (Fig. 7). In corroboration, ChIP assays also confirmed higher promoter occupancy by c-Rel in the context of chromatin under TNF-α-treated conditions (Fig. 7). Thus, Hap2 promoter carriers may have an exaggerated response to inflammatory stimuli. It may be noted that CHGA levels are elevated in a number of inflammatory disease conditions including rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (42–44). Circulating CHGA levels also correlate with TNF receptors and TNF (45).
Hypoxia is known to develop in cardiovascular disease states like myocardial ischemia, atherosclerosis etc., where the NF-κB family of transcription factors have been known to play a key role (46). IkB phosphorylation during hypoxia leads to its degradation, which in turn activates the DNA binding and transactivation of c-Rel (47). Hypoxia also activates TNF-α and the latter up-regulates c-Rel (48). The NF-κB family of transcription factors have been shown to activate HIF-1α as well (35, 49). In line with these reports, Hap2 CHGA promoter-reporter constructs showed an enhanced promoter activity in response to hypoxia (~2.5-fold) and in the presence of ectopically expressed c-Rel, the promoter activity further enhanced to ~3.5-fold. ChIP assays also suggested increased binding of c-Rel to −1018T and −57T in response to hypoxia (Fig. 8). Thus, the polymorphisms at −1018 and −57 bp positions, constituting the CHGA promoter Hap2, have functional consequences not just by influencing basal gene expression but also by enhancing gene expression in response to stress/inflammatory stimuli.

CHGA promoter haplotypes: Genotype-phenotype correlations

The higher promoter activity of the Hap2 promoter made us consider whether the Hap2 promoter could cause activation of CHGA transcription in the genomic context as well. Indeed, expression of CHGA under influence of the Hap2 promoter was significantly higher than that of Hap1 and Hap5 in transfected cells (Fig. 9, B and C). Consistent with this observation, plasma CHGA levels were significantly elevated in individuals with a Hap2/Hap2 genotype when compared with individuals of Hap1 and Hap5 backgrounds (Fig. 9, D and E). Diploid combinations of Hap1/Hap2 and Hap2/Hap5 constructs in c ella mimicking the homozygous and heterozygous combinations of the promoter haplotypes observed in the population showed that promoter haplotype activity profiles matched with CHGA protein levels (Fig. 9, F and G).

The elevated BMI, plasma glucose levels in Hap2/Hap2 individuals (showing modest positive correlation with plasma CHGA levels) when compared with Hap1/Hap1 and Hap2/Hap5 subjects suggest a plausible association of CHGA haplotype 2 with cardiometabolic traits (Fig. 10). Hap2/Hap5 individuals also had higher diastolic blood pressure as compared to Hap2/Hap5 individuals (supplementary Fig. S5). Notably, a previous study in twin pairs of European ancestry reported an association of the CHGA promoter G−462A variation with leptin, CRP levels, and BMI; individuals with higher BMI displayed higher catecholamine, CHGA levels in this study (20). Thus, a positive correlation between BMI and CHGA levels seems to occur in ethnically distinct populations.

What could be the mechanistic basis for the higher BMI observed in haplotype 2 subjects? The activity of CHGA promoter haplotype 2 is significantly higher resulting in elevated CHGA levels that might result in higher levels of CHGA-derived anti-insulinic PST. Interestingly, CHGA levels measured by ELISA provide an indirect estimate of the PST levels too, as the antibody used in the immunoassay was raised against the PST domain of CHGA. Moreover, PST deficiency was shown to promote an anti-inflammatory environment resulting in the prevention of insulin resistance (50). Hence, higher PST levels could result in a pro-inflammatory environment, triggering effector molecules like TNF-α, which in turn activate CHGA gene expression via c-Rel. The pro-inflammatory environment also has been reported to promote insulin resistance, which is known to strongly correlate with BMI (51). Thus, the Hap2 promoter may initiate a cascade of events at the cellular, biochemical, and physiological levels that may ultimately enhance the risk of cardiometabolic disorders in its carriers (Fig. 11). Of note, our LD analysis of genetic variation at CHGA locus showed evidence of linkage of the Hap2 promoter variants at −1018 and −57 bp positions with the PST variant G297S (that was associated with elevated plasma glucose level (52)) and the CST variant G364S (that showed association with elevated blood pressure in Asian populations (53)) suggesting that the observed association of Hap2 with DBP and plasma glucose may also be contributed by these PST and CST variants.

In summary, our study identified CHGA promoter polymorphisms and haplotypes present in an Indian population and characterized the functional consequences of a highly prevalent haplotype (Hap2). The higher activity of CHGA Hap2 promoter translated to elevated CHGA levels via enhanced interaction with the transcription factor c-Rel. The CHGA Hap2 promoter also predicted higher levels of several cardiometabolic traits (plasma glucose, DBP, and BMI). This study, therefore, provides new insights on the plausible role of regulatory polymorphisms as an early control point to intermediate phenotypes predicting cardiovascular/metabolic disease risk.

Experimental procedures

The detailed methods are provided in the supplemental information.

Human subjects

The study population was comprised of 769 unrelated volunteers from an urban Chennai and Chandigarh population recruited at the Madras Medical Mission (MMM), Chennai, and at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh. Each subject gave an informed written consent and the study was approved by the Institutional Ethics Committee at Indian Institute of Technology Madras. Demographic, physiological, and biochemical parameters of the study population are shown in the supplemental Table S1.

Genotyping of polymorphisms at the CHGA locus

Genomic DNA was isolated from EDTA-anticoagulated blood samples using the Flexigene DNA kit (Qiagen, USA). The genomic region of CHGA comprised of −1185 to +76 bp (NCBI accession number: NM_001275.3) was PCR-amplified using specific primers, purified, and Sanger sequencing was carried out to detect polymorphisms from the chromatograms using Chromas software (Technelysium, Australia). The exon 7 region of the CHGA was also amplified and sequenced to genotype SNPs in the pancreastatin, catesatin, and parastatin peptide regions using specific primers.

Estimation of biochemical parameters

Standard biochemical assays were carried out to measure common parameters such as glucose, cholesterol, urea, creat-
inine, hemoglobin, sodium, and potassium in the plasma. Blood pressure readings were recorded in triplicate (and averaged) in the sitting position using a brachial oscillometric cuff by experienced nursing staff. Plasma CHGA levels were measured by a CHGA immunoassay kit (LDN, Germany).

**Cloning and mutagenesis**

The five CHGA promoter haplotype-reporter constructs were generated in pGLuc-Basic vector (New England Biolabs). Hap1–1018T, Hap1–57T, and Hap1 double mutants were generated by site-directed mutagenesis using specific SDM primers. To generate the CHGA promoter haplotype-cDNA constructs, Gaussia luciferase cDNA in Hap1–, Hap2–, and Hap5–GLuc promoter constructs was replaced with CHGA cDNA.

**Cell lines, transfection, and reporter assays**

Human neuroblastoma IMR-32, SH-SY5Y, and mouse neuroblastoma N2a cell lines were obtained from the National Center for Cell Sciences, Pune, India. All transfections were carried out using Targefect F2 transfection reagent (Targeting Systems). CHGA promoter haplotype-reporter plasmids and β-galactosidase expression plasmid (as internal control) were transfected into IMR-32 and SH-SY5Y cell lines. Gaussia luciferase and β-galactosidase assays (54) were performed after 24–30 h of transfection. Promoter activities were expressed as luciferase/β-galactosidase readings. Transfection efficiencies in experiments were calculated to be ~30–35%.

Co-transfection experiments with c-Rel expression plasmid (55), human c-Rel dicer substrate siRNA oligos (Integrated DNA Technologies, Belgium), TNF-α, and hypoxia treatments were carried out in IMR-32 cells along/after transfection with Hap2–GLuc and Hap5–GLuc promoter-reporter constructs. Gaussia luciferase activity and total protein levels were estimated and reporter activities were expressed as luciferase activity/μg of protein. Another set of experiments were carried out to imitate the homozygous and heterozygous conditions in cella with CHGA promoter-reporter constructs and to estimate CHGA levels in vitro under the influence of different CHGA promoter haplotypes. The luminescence signals were captured using Chemidoc XRS+ Chemiluminescence Detection system (Bio-Rad Laboratories).

**Western blotting**

Immunoblotting experiments were carried out using specific antibodies to detect overexpression or down-regulation of c-Rel and HIF-1α after transfection experiments/TNF-α treatment/hypoxia and to measure CHGA levels in vitro under the influence of different CHGA promoter haplotypes. The luminescence signals were captured using Chemidoc XRS+ Chemiluminescence Detection system (Bio-Rad Laboratories).

**Electrophoretic mobility shift assays (EMSA)**

EMSA experiments were carried out by using oligonucleotides spanning the A–1018T and C–57T SNP sites as well as consensus c-Rel oligonucleotide (supplemental Table S7) to detect in vitro binding of c-Rel across these SNPs in the CHGA promoter with slight modifications to our previously described method (36); specific details are included in the supplemental materials.

**Chromatin immunoprecipitation (ChIP) assays**

ChIP assays were carried out in N2a cells transfected with Hap2–GLuc and Hap5–GLuc promoter-reporter constructs with/without treatment with TNF-α (5 ng/ml) or hypoxia fol-

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**Figure 11. A schematic presentation of the plausible role and mechanistic basis behind the crucial role of CHGA promoter haplotype variants in contributing to cardiometabolic risk.** The CHGA promoter Hap2 (comprised of variant alleles at –1018, –415, and –57 bp positions), the second most frequent haplotype in Indian population, has the highest promoter activity among all the haplotypes due to transcriptional activation by c-Rel across –1018T and –57T alleles. The higher promoter activity corroborates with the elevated levels of plasma CHGA observed in Hap2 individuals. Higher plasma levels of CHGA may lead to enhanced processing of CHGA resulting in elevated levels of PST peptide. PST peptide has previously been shown to promote insulin resistance and inflammation leading to elevated blood glucose and BMI. Pro-inflammatory molecules like TNF-α may increase the promoter activity of CHGA Hap2 by activating c-Rel, and thereby confer a higher risk of cardiometabolic disease to Hap2 subjects.
allowing our recently reported protocol (56). Chromatin immunoprecipitation was carried out with c-Rel antibody; IgG was used as negative control. qPCR was carried out to amplify the DNA sequence encompassing the A→1018T and C→57T sites in the human CHGA promoter using two specific sets of primers. The amounts of DNA immunoprecipitated by c-Rel due to binding to −1018T and −57T alleles were quantified by the fold-enrichment method relative to IgG signal.

Data presentation and statistical analysis

Phenotypic parameters of the study population were expressed as mean ± S.E. Genotype-phenotype associations were tested by one-way ANOVA with post hoc tests or Levene’s test for equality of variances followed by two-tailed t test, as appropriate, using the Statistical Package for Social Sciences (SPSS). Promoter-reporter transfections results were expressed as mean ± S.E. from representative experiments. Statistical significance was calculated by Student’s t test or one-way ANOVA with Bonferroni’s multiple comparisons post-test, as applicable, using Prism 5 program (GraphPad Software Inc.). All graphs were generated using GraphPad Prism 5 software.

Author contributions—L. S. designed and performed experiments, carried out data analysis and interpretation, and wrote the manuscript; A. A. K., P. K. R. A., M. Ki., B. S. S., and S. S. performed experiments and carried out data analysis and interpretation; M. Kh. and A. A. M. provided equipments, reagents, and clinical samples; A. A. K., P. K. R. A., M. Ki., B. S. S., and S. S. performed experiments and carried out data analysis and interpretation; M. Kh. and A. A. M. provided equipments, reagents, and clinical samples; N. R. M. conceived and designed the study, carried out data analysis and interpretation, and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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