Proteomic Analysis Yields an Unexpected Trans-Acting Point in Control of the Human Sympathochromaffin Phenotype

Running title: Chiron et al.; Chromogranin A promoter G-462A polymorphism mechanisms

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Abstract:

**Background** - The secretory protein chromogranin A (CHGA) plays a necessary role in formation of catecholamine storage vesicles and also gives rise to a catecholamine release-inhibitory fragment. Since genetic variation in the proximal human CHGA promoter predicts autonomic function and blood pressure, here we explored how a common genetic variant alters transcription of the gene.

**Methods and Results** - Bioinformatic analysis suggested that the common G-462A promoter variant (rs9658634) may disrupt as many as 3 transcriptional control motifs: LEF1, COUP-TF, and PPARγ-RXRα. During electrophoretic mobility shifts, chromaffin cell nuclear proteins bound specifically to the A (though not G) allele of CHGA promoter G-462A. Upon oligonucleotide affinity chromatography followed by LC-MS/MS analysis of A-allele eluates, the transcription factor LEF1 (Lymphoid Enhancer-binding Factor-1) was identified. Interaction of LEF1 with the A-allele at G-462A was confirmed by super-shift. Upon co-transfection, LEF1 discriminated between the allelic variants, especially in chromaffin cells. Allele specificity of trans-activation by LEF1 was transferable to an isolated G-462A element fused to a heterologous (SV40) promoter. Since beta-catenin (CTNNB1) can hetero-dimerize with LEF1, we tested the effect of co-transfection of this factor, and again found A-allele-specific perturbation of CHGA transcription.

**Conclusions** - Common genetic variation within the human CHGA promoter alters the interaction of specific factors in trans with the promoter, with LEF1 identified by proteomic analysis and confirmed by super-shift. Co-expression experiments show functional effects of LEF1 and CTNNB1 on CHGA promoter. The findings document a novel role for components of the immune and WNT pathways in control of human sympathochromaffin phenotypes.

**Key words:** Chromaffin, chromogranin, catecholamine, transcription.
Glossary of abbreviations:

CHGA. Chromogranin A.

CTNNB1. Beta-catenin.

DCG. Dense Core Granule (of the regulated neuroendocrine secretory pathway).

EMSA. Electrophoretic Mobility Shift Assay.

ESI-MS/MS. ElectroSpray Ionization, followed by 2-dimensional (tandem) Mass Spectrometry.

LD. Linkage Disequilibrium.

PC12. Rat pheochromocytoma (chromaffin) cell line.
Introduction

Chromogranin A (CHGA) is a 48 kDa acidic protein directed to the regulated secretory pathway in neuroendocrine cells, and a major constituent of regulated secretory vesicles, also designated Dense Core Granules (DCGs) \(^1\). DCGs serve as a storage compartment for catecholamines in post-ganglionic sympathetic neurons and adrenal medullary chromaffin cells, from which all granule core contents are co-released upon stimulation by exocytosis. Studies have shown that CHGA may be both necessary and sufficient for the formation of a regulated secretory pathway \(^2\). In addition to this important role in DCG formation, CHGA is a pro-hormone, co-released with catecholamines from the DCGs upon stimulation \(^3\). Upon processing at dibasic sites, the pro-hormone CHGA gives rise to biologically active peptides such as the inhibitor of catecholamine release catestatin \(^4\), the vasodilator vasostatin \(^5\), or the dysglycemic peptide pancreastatin \(^6-9\).

Hypertension displays substantial heritability in family studies, but the genetic contributions to control of blood pressure (BP) are still poorly understood \(^10-12\). Based on observations in both humans and rodents, we proposed that CHGA formation and secretion may constitute “intermediate” (early or pathogenic) phenotypes for later development of hypertension \(^11\). For instance, \(CHGA\) is over-expressed in adrenal medulla of rodent hypertension, both spontaneous \(^13\) and acquired \(^14\). Moreover, ablation of the \(CHGA\) gene in mice leads to substantial hypertension \(^15\). Finally, CHGA plasma levels parallel catecholamine release in human populations \(^16,17\), and phenotypic links between \(CHGA\) and human hypertension are reported \(^17-20\).

A detailed study of naturally occurring genetic variations at the human \(CHGA\) locus revealed 20 common SNPs, including 8 variants in the 1.2-kbp proximal promoter \(^21\). From the 8 promoter
haplotypes inferred, two showed substantially different transcriptional activities in reporter gene experiments. These two haplotypes span 3 variants (T-1014C → G-988T → G-462A) in LD that govern CHGA expression. Further analysis of these 3 SNPs revealed a major contribution of the SNP G-462A (rs9658634) for modification of transcriptional activity of the isolated CHGA promoter 21. In addition, variations in this promoter region predict blood pressure increase after environmental stress, as well as basal blood pressure 20; initial analysis of the effect of the -462 position suggested involvement of the transcription factor COUP-TF 20.

In this study, we revisited the cellular mechanisms that may occur at the -462 position of the CHGA promoter, using an unbiased, hypothesis-free, proteomic approach. We find not only compelling new computational evidence of differing protein binding specificity between the wild-type and the variant promoter regions, but also biochemical as well as functional evidence for binding of LEF1 (Lymphoid Enhancer Factor-1) at this molecular switch region of the CHGA promoter.

Results

**Bioinformatic analysis indicates five putative binding motifs in the CHGA promoter region encompassing the G-462A SNP.** Because CHGA proximal promoter SNPs in the region T-1014C → G-988T → G-462A display peak predictions of human traits, including CHGA plasma level 21 and blood pressure response to environmental stress 20, and the SNP G-462A within this region was predominant in reporter gene experiments 20;21, we decided to search systematically for transcription binding sites around the G-462A position, using contemporary bioinformatic and proteomic approaches. Bioinformatic analysis of a 31-bp sequence surrounding the SNP (from -479 → -449; highly conserved across primates) revealed several degenerate potential binding
motifs, in the JASPAR and TRANSFAC databases (Figure 1A).

As expected by Chen et al, one of the first five hits for the variant (minor allele, -462A) sequence was a partial match for the previously published consensus motif of COUP-TF (MA0017). However, we also found 4 new motifs spanning -462A, with even higher scores than the COUP-TF prediction (Figure 1A). The best match corresponds to a binding motif for LEF1 (M00978) with a near-perfect homology. The second hit is a partial match for the binding sequence of the complex PPARγ-RXRα (MA0065), whereas the third hit involves a hepatic factor, HNF4-α1 (M00411). The fourth hit corresponds to a different reported binding motif for LEF1, on the reverse complement of the first hit. Of these five strongest hits, three involve the + strand of the promoter whereas the two others indicate binding on the – strand (Figure 1B).

When performed on the wild-type (major allele, G-462) sequence, the analysis failed to identify partial matches for the factors LEF1 (M00978), HNF4-α1 (M00411), or COUP-TF (MMA0017).

Therefore, our results from the bioinformatic analysis suggest a new set of proteins that putatively bind the G-462A region of the CHGA promoter, and could further explain the differences of transcriptional activity reported for the two alleles.

**EMSA:** Nuclear proteins bind to the A allele of CHGA promoter G-462A, but not to the G allele. To probe molecular mechanisms underlying the transcriptional regulation of G-462A and clearly identify the factor responsible for differences in expression, we analyzed by EMSA the binding specificity of PC12 nuclear extracts to the 31-bp sequences (alleles G-462 and -462A, Figure 2A) used in the bioinformatic analysis (Figure 1B). As shown in Figure 2B, the
Proteomic analysis of nuclear proteins binding the A allele of CHGA promoter G-462A: Identification of transcription factor LEF1. We employed oligonucleotide affinity chromatography to proceed in a hypothesis-free fashion towards identification of the trans-acting factor(s) by tandem mass spectrometry. The + strand of G or A allele DNA fragments were designed with a 3'-TEG-Biotin tag, annealed with their complementary (-) strands, and incubated with streptavidin-conjugated magnetic beads (Figure 3A). In order to minimize non-specific binding of proteins to the streptavidin or the magnetic beads, PC12 nuclear extracts were pre-cleared by incubation with streptavidin-conjugated magnetic beads alone, prior to the incubation with the magnetic beads bearing the allele-specific DNA fragments. To monitor the purification
procedure, each fraction of the oligonucleotide affinity chromatography was analyzed by SDS-PAGE and Coomassie blue staining: the SDS-PAGE analysis of the elution fractions shown in Figure 3B reveals a high MW band (Mr ~120 kDa) purified with both the A and G allele DNA fragments, as well as a lower MW band (Mr ~64 kDa; arrow), purified only in presence of the A allele DNA. Eluates of the SDS-PAGE lanes were submitted for protein identification by trypsin-LC-MS/MS analysis.

LEF1 was identified (Figure 3C and 3D) in the A allele elution fraction (Mascot scores in Supplementary Table 1), whereas we failed to detect that factor in the G allele elution. Of note, LEF1 typically migrates with an apparent MW around 60 kDa, close to the band we noted upon elution from the A-allele (Figure 3B, arrow). Abundant cellular proteins without known DNA-binding activity (presumably non-specific contaminants; list in Supplementary Table 2) were not considered further. Several known ssDNA-binding proteins and proteins involved in transcriptional machinery were also identified (Figure 3C).

Confirmation of binding of LEF1 to the A allele of CHGA promoter: EMSA with super-shift.

To confirm the binding of LEF1, we performed an EMSA super-shift assay. Antibodies directed against COUP-TF, PPARγ, or LEF1, as well as a negative control antibody (directed against Pax6) were added to the pre-incubated oligonucleotides and PC12 nuclear extract, prior to EMSA. While the control and the anti-COUP-TF and anti-PPARγ antibodies did not alter the migration pattern of the A-allele DNA fragment, anti-LEF1 antibody induced a super-shift of the A allele DNA fragment/protein complex (Figure 4A, lane 5). This result is consistent with the bioinformatic analysis identifying the LEF1 motif (Figure 1A and 4B). Not only is the LEF1 motif a perfect match with the variant -462A (as compared with more degenerate matches with
the PPARγ and COUP-TF motifs), but the -462 SNP position is also one of the bases with the strongest specificity within the motif (Figure 4B).

ChIP was also undertaken (see Methods), with an antibody directed against LEF1; after immunoisolation of nucleosomes, PCR with a 152-bp amplicon spanning G-462A detected LEF1 binding to the CHGA promoter on both alleles (G and A; data not shown). However, there is a second, non-polymorphic LEF1 partial consensus match on the human CHGA promoter minus strand at position -530→-519 (ACTTTGTTGTT; Mapper score 6.1) <http://genome.ufl.edu/mapper>. Since position -530→-519 is within the inter-nucleosomal DNA fragment size range typically obtained during sonication (500-1000 bp), we cannot rely on ChIP for specific detection of LEF1 binding at the G-462A motif.

**LEF1 functionally modifies CHGA promoter activity by discriminating between the two allelic variants at G-462A.** We attempted to trans-activate the two alleles of the CHGA promoter with putative binding factors identified previously, using plasmids wherein the human CHGA promoter (spanning -1142→+54 bp, or ~1.2 kbp) was cloned into pGL3-Basic (Promega) to control expression of the Firefly luciferase gene 21. The two CHGA promoters used in this study are promoter Hap-1 (containing the G-462 allele) and Hap-1/-462A (identical except for the -462A allele, created by site-directed mutagenesis). These plasmids were co-transfected into rat chromaffin cells (PC12 pheochromocytoma, Figure 5), along with a plasmid expressing human LEF1 under the control of the CMV promoter, or the plasmid pcDNA3.1 (empty CMV promoter vector) as a negative control. Co-expression of LEF1 resulted in a preferential decline in CHGA A-allele promoter expression as compared to the G-allele (p=0.029; Figure 5A, left).
**Allele specificity of trans-activation by LEF1 is transferable to an isolated G-462A element in a new promoter (SV40) context.** The reporter gene assays described above were performed on two haplotypes of the CHGA promoter (~1.2 kb). To probe context-dependence of the LEF1 response, we designed a new reporter construct in which the 31-bp sequence flanking the -462 polymorphic site (G-462 versus -462A, Figure 1B) was inserted just upstream of the SV40 promoter in the pGL3-Promoter/luciferase reporter plasmid (Invitrogen) (Figure 5B). These modified pGL3-Promoter plasmids (SV40, +G or +A), as well as the control pGL3-Promoter plasmid, were co-transfected with the pCMV plasmid expressing human LEF1. This experiment allows us to understand whether the differential G/A response to LEF1 can occur independent of the usual position of the G-462A region, upstream of its cognate core promoter and TATA box. Once again, co-transfection with LEF1 significantly decreased expression from the A allele as compared to the G allele (p=0.030; Figure 5A, right) in this new context.

Taken together, our results from reporter gene assays show that LEF1, identified by bioinformatics and proteomic, differentially affects CHGA promoter depending on the nucleotide at position G-462A.

**Role of the WNT/beta-catenin (CTNNB1) pathway in cooperation with LEF1.** Since LEF1 characteristically hetero-dimerizes with the WNT signal transduction pathway component CTNNB1, and thus constitutes an efferent limb of WNT pathway signaling, we tested whether CTNNB1 perturbed CHGA expression. Transfected CTNNB1 trans-activated the co-transfected human CHGA promoter in chromaffin cells (Figure 6), and the effect was greater for the A than the G allele at G-462A (A>G, p<0.001). When CTNNB1 and LEF1 were co-transfected, the A>G difference persisted (p=0.003).
Discussion

Overview. Sympathetic signaling and outflow are key components in the regulation of blood pressure. Associations of the sympathetic pathway with hypertension and “intermediate” phenotypes contributing to blood pressure control have been documented. Chromogranin A plays crucial roles in the sympathoadrenal system; because of its involvement in DCG formation, regulation of catecholamine release, and production of bioactive peptides, CHGA may represent a key candidate gene for the regulation of the autonomic contributions to blood pressure. Accumulating evidence gathered in different models now document direct links between CHGA and blood pressure regulation. The plasma catestatin (CHGA catecholamine release-inhibitory peptide) levels were reduced in subjects with established hypertension and their at-risk siblings. In addition, variations in the CHGA promoter were associated to plasma CHGA concentration and blood pressure. Although the SNP rs9658634 at position G-462A appears to be the most important functional variant in the promoter, the molecular mechanisms underlying its effects are incompletely understood.

In this study, we combined an un-biased, hypothesis-free, proteomic screening approach, along with bioinformatic analyses and gene reporter assays, in order to identify the trans-acting factor(s) affecting the -462 region of the human CHGA promoter.

Cis- and trans- mechanisms at human CHGA G-462A: Bioinformatic and experimental approaches. Bioinformatic analysis of a putative protein binding site in the promoter region spanning the -462 SNP revealed partial binding motifs for the transcription factors LEF1, HNF4-α1, COUP-TF and the heterodimeric complex PPARγ-RXRα. Whereas the putative binding of
COUP-TF was already suggested \(^{20}\), the four other binding motifs were novel. The partial motif for PPAR\(\gamma\)-RXRa in the CHGA promoter was of importance since CHGA expression responded to retinoic acid \(^{20}\), and also because PPAR\(\gamma\) has been linked to multiple complex diseases and disorders, including not only metabolic disease \(^{29,30}\) (reminiscent of the phenotypes associated to CHGA KO-mice, \(^{8,15}\)), but also hypertension \(^{31-33}\). As previously reported, COUP-TF is a putative binding protein of this promoter region that increases expression in PC12 cells; this effect appears linked to the -462 region and showed selectivity between the two alleles \(^{20}\). However, this transcription factor was not identified in the proteomic approach and an antibody directed against COUP-TF failed to super-shift DNA-protein complexes during EMSA. Our unbiased proteomic approach identified LEF1 as binding the SNP region.

Co-transfection of LEF1 acts differentially on the two alleles in gene reporter experiments in PC12 cells, and this effect is transferable to a heterologous (SV40) core promoter by the -462 region. Finally, an anti-LEF1 antibody demonstrated super-shifting of DNA-protein complexes in EMSA, identifying LEF1 as a factor binding the promoter region at the minor allele (A). Taken together, our results suggest that the factor LEF1 binds specifically the minor (A) allele at G-462A, inducing selective repression of its activity.

**Role of transcription factor LEF1.** LEF1 was first identified as a T-cell specific transcription factor (also named TCF1\(\alpha\)) regulating of expression of the TCR\(\alpha\) gene \(^{23,34}\). The LEF1 transcription factor is developmentally regulated and expressed in pre-B and T lymphocytes \(^{35}\) and is a component of the Wnt signaling pathway \(^{36}\); indeed, LEF1 bears a \(\beta\)-catenin (CTNNB1) binding domain, whereby hetero-dimerization potentiates the transcriptional activity of LEF1 \(^{37}\). However, LEF1 is also expressed in sympathochromaffin cell types, such as PC12 chromaffin
cells 36 (our data), and human pheochromocytoma 38, as well as the normal adrenal gland 39. In
the mouse adrenal gland, LEF1 expression follows a circadian rhythm 40, and LEF1 expression in
rat adrenal medulla increases after multiple rounds of immobilization stress 41. Targeted ablation
of the LEF1 locus in the mouse results in a pleiotropic spectrum of consequences, including
alterations in some populations of neural crest-derived cells 42. Here we showed that CTNNB1
expression augmented human CHGA promoter activity (Figure 6), with a A>G preference at G-
462A, either alone (p<0.001) or in combination with LEF1 co-expression (p=0.003); thus,
CTNNB1 may reverse the usual suppression of CHGA transcription by LEF1 (Figure 5), in
CHGA allele-specific fashion. The results open the way to understanding a new effect of
WNT/CTNNB1 signaling: control of the sympathochromaffin phenotype.

Conclusions and perspectives. Here, we identified LEF1 as a polymorphism-dependent
transcriptional repressor of the CHGA promoter, centering on the common functional variant G-
462A. Given our results and the observations that the BP circadian rhythm synchronizes with
changes in LEF1 expression 40, and experimental stress induces elevations in BP and LEF1
expression 40, it is plausible to postulate that LEF1 might have a role in catecholamine synthesis
and/or secretion as well as DCG formation, perhaps in concert with the WNT/CTNNB1 pathway.
Future experimentation is likely to reveal new roles of this transcription factor and its
implications for human autonomic biochemistry, physiology and disease.

Materials and Methods

Cell culture. The rat adrenomedullary chromaffin cell line PC12 43 was grown in high-glucose
Dulbecco’s modification of Eagle’s medium with penicillin G (100 U/ml) and streptomycin
sulfate (100 mg/ml), supplemented with 10% horse serum and 5% fetal bovine serum.

**Promoter/luciferase reporter plasmids.** Human CHGA promoter/reporter plasmids were constructed essentially as previously described. Haplotype-1 promoter fragment corresponding to CHGA -1142/+54 bp (with respect to the cap-site) was amplified from genomic DNA of known homozygotes, and cloned between the site Kpnl and Xhol in the pGL3-Basic vector (Promega Inc., Madison, WI, USA). The rs9658634 SNP was reintroduced by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA, USA). For modification of the pGL3-Promoter plasmid, the oligonucleotides CACTCAGACAGGGGATC\_G\_AA\_AAGGCCCCTCTAG, CACTCAGACAGGGGATC\_G\_AA\_AAGGCCCCTCTAG were annealed with their complements, and cloned into SmaI of pGL3-Promoter. The constructs were sequence-verified, and purified on columns (Qiagen, Valencia, CA, USA).

**Transfection and reporter assay.** PC12 cell line was transfected at 50–60% confluence by the liposome method (TransfectIN, Bio-Rad) according to the manufacturer; with 500 ng reporter plasmid and 2.75 μl of TransfectIN per well of a 24-well plate. For Renilla luciferase control, 50 ng of plasmid pRL-TK (Promega Inc.) was co-transfected. For trans-activation experiments, 50 ng of plasmid in pCMV vector/transcription factor or pcDNA3.1 (control) was co-transfected. Co-transfected pCMV-driven trans-activator plasmid cDNAs were: human LEF1 (Open Biosystems, Huntsville, AL), or Xenopus CTNNB1 (Open Biosystems). Cells were lysed 18-24 hr post transfection in 100 mM KHPO$_4$ pH 7.8, 1 mM DTT, 0.1% Triton X-100. For dual-luciferase measurements, Stop & Glo (Promega Inc.) as well as homemade buffers were used. Each experimental condition was repeated a minimum of four times (replicates).
EMSAs: Electrophoretic Mobility Shift Assay and supershift. PC12 nuclear extracts were obtained using nuclear extraction kit (Cayman). The oligonucleotide probes (see above promoter/luciferase reporter plasmids) were biotin-labeled using the Biotin 3’ End DNA Labeling Kit (Pierce) and annealed. Nuclear-extract-binding reactions were performed at room temperature for 20 min using 7 µg of nuclear extract and 20 fmoles of biotin-labeled oligonucleotide in 15 µl final volume, using a LightShift chemiluminescent EMSA kit (Pierce). Protein–oligonucleotide probe complexes were resolved using native 5% polyacrylamide gels, and transferred onto nitrocellulose membranes. The biotin-tagged oligonucleotides were detected using a Chemiluminescent Nucleic Acid Detection Module (Pierce), in which a streptavidin-horseradish peroxidase conjugate was hybridized to the membrane, washed, and visualized by luminol chemiluminescence. For competition experiments, unlabelled competitor oligonucleotides were pre-incubated at 100-fold excess with the nuclear extract for 5 minutes. In super-shift assays, 1 µl of antibody was added before migration and incubated 20 minutes. The antibodies were from Santa Cruz Biotechnology: PPARγ, sc-7273X; COUP-TF, sc-30180X; and LEF1, sc-8591X. Details of the LEF1 motif sequence specificity, by position weight matrix derived from experimental data45, are available at the Chip-Mapper46 interface for the Transfac database, at <http://snpper.chip.org/mapper-pages/factors.html>.

Purification of sequence-specific DNA-binding proteins by affinity chromatography. Nuclear extracts were prepared from PC12 cells as described for EMSA. Affinity purification was performed as described 47,48 with modifications. 3’-TEG-Biotinylated versions of the forward oligonucleotide probes used for EMSA were annealed to their unlabelled complements. 25
p moles of the ds-oligonucleotides were captured on 150 µg of M280 streptavidin magnetic beads for 30 min (Dynal Biotech, Oslo, Norway; Invitrogen). All purification procedures were carried out at 4°C. Crude nuclear extracts (~115 µg) were mixed with 150 µg of salmon sperm DNA in a 10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT buffer, and incubated 30 min with streptavidin beads to clear extracts from proteins binding the streptavidin or the beads. Pre-cleared extract was then incubated with beads coated with G- or A-allele oligonucleotides. Beads were washed three times with 100 mM Tris pH 7.5, 100 mM KCl buffer. The beads were eluted in 50 µl of Laemmli buffer. Elutions were separated on SDS-PAGE and Coomassie blue stained (SimplyBlue SafeStain, Invitrogen). Coomassie blue-stained protein bands were excised and in-gel digested with trypsin and analyzed by LC electrospray ionization MS as described.

Briefly, samples were loaded onto a capillary column with integrated spray tip (75 um I.D., 10 um tip, New Objective, Woburn, MA), which was packed in-house with C_{18} reversed phase material (Zorbax SB-C_{18}, 5 um particle size, Agilent, Santa Clara, CA) to a length of 10 cm. The reversed phase elution was achieved by means of a linear gradient of 0-60% acetonitrile in 0.1% formic acid within 60 minutes at a flow rate of 300 nl/min. The eluate was introduced into a Thermo LTQ-Orbitrap mass spectrometer (ThermoFisher, Waltham, MA) via a nano-spray source. Mass spectrometric analysis was conducted by recording precursor ion scans at a resolution of 60,000 in the Orbitrap Fourier-transform analyzer followed by collision induced dissociation MS/MS scans of the top 5 ions in the linear ion trap (cycle time approx. 1 s). An active exclusion window of 90 s was employed. Data were analyzed on a Sorcerer Solo system running Sorcerer-Sequest (rev11) against the IPI rat database (v3.61, RAT, 39876 entries) and by using the Mascot algorithm (V. 27 rev.11, Matrix Science, London, UK). Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein
identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by Protein Prophet. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principle of parsimony.

**Chromatin ImmunoPrecipitation (ChIP).** ChIP was accomplished by modification of procedures previously described by us. PC12 chromaffin cells were transfected with particular CHGA promoter haplotype/reporters to obtain G versus A alleles for the G-462A variant. ChIP assays were carried out using the Imprint® ChIP kit (CHIP, Sigma, St. Louis, MO). Cells (~3×10^6 in transfected 10-cm plates) were cross-linked in 1% formaldehyde for 10 min at room temp and washed x3 with ice-cold PBS, then resuspended in nuclear preparation buffer. Chromatin was sonicated to achieve inter-nucleosomal cleavage (Branson Sonifier) until DNA was fragmented to ~500-1000 bp size. After 10-min centrifugation, samples were incubated with specific or control antibodies pre-adsorbed to polystyrene wells at room temp for 1.5 hours with rotation. Characteristics of the specific antibody were: goat polyclonal anti-LEF1, SCBT sc-8591X. Control antibodies were from the Sigma ChIP kit: pre-immune normal mouse IgG (as a negative control), and anti-RNA polymerase II (as a positive control). The adsorbed immune complex washed 6-7 times and eluted by “DNA release buffer” including proteinase K digestion at 65°C for 15 min, then cross-links were reversed with “reversing solution” with heating in at 65°C for 1.5 hours. The DNA was subsequently extracted and purified with GenElute Binding Column G (Sigma). Immunoprecipitated nucleosomal DNA samples were analyzed by PCR.
using primers forming a 152-bp amplicon that bracketed the G-462A (sense: 5’-AGAGAGAAGCCTCACTCAGACAG-3’, antisense: 5’-CACCCCGTGCTATTTTTCCTA-3’) site in the human CHGA promoter. Extracted DNA from the chromatin fractions before antibody adsorption/elution was used as a positive control ("input DNA"). To ensure that the PCR amplification was in the linear range, reactions with different amounts of input DNA samples were carried out for various (typically 15-30) cycle numbers; a linear range of amplification typically occurred at ~25 cycles. After amplification, PCR products were separated on 1.5% agarose TBE gels. In this reporter system, the transfected plasmid is incorporated into the chromatin fraction of the cell\textsuperscript{54}.

**Statistics.** Analyses were performed in SPSS. Results are expressed as the mean value +/- one SEM. Cell culture experiments were typically done with 4 replicates per condition. Parametric statistics (T-test) were used to evaluate differences between experimental conditions, since descriptive statistics were consistent with approximately normal distributions of the data points (skewness from -0.8 to +0.8).

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**Conflict of interest disclosures:** None.
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**Figure Legends:**

**Figure 1:** Identification of binding motifs spanning position G-462A in the human *CHGA* promoter. *A.* Putative factors binding the *CHGA* promoter at the G-462A SNP position. A region of 31-bp spanning the -462A SNP in the variant *CHGA* promoter was analyzed using the software Mapper (http://bio.chip.org/mapper) for putative binding motifs. Shown are the hits with a score >3, the associated model and its properties, as well as the motif alignments (modelled motifs are represented between *-> <-*, with partial matches shown by +). Vertical arrows indicate the position of the G-462A variant (in italics). On the minus strand, the same variant is C/T. *B.* Schematic representation of the putative binding motifs and factors. Only the + strand (-479 to -449) of the region spanning the -462 SNP (bold) is represented, together with the putative motifs on the + strand (+, above the sequence) or on the reverse complement (-, below the sequence). *C.* LEF-1 homology match at human *CHGA* promoter variant G-462A across primate species.

**Figure 2:** *In vitro* binding specificity of the two alleles of human *CHGA* G-462A region with
PC12 nuclear extract. A. Sequences of the probes used for EMSA experiments. The SNP is indicated in red. B. Electro-Mobility Shift Assay (EMSA) of the two alleles G-462 and -462A with nuclear extract of PC12 cells. Biotinylated probes from (A) were incubated with nuclear extract and/or non-biotinylated competitor as indicated and the resulting complexes analyzed as described in M&M. PC12 nuclear extract alone shows a non-specific (NS) band (arrow). Protein:DNA complexes (asterisk) and free probes (brackets) are indicated.

**Figure 3:** Purification of sequence-specific DNA-binding proteins by affinity chromatography and identification by LC-MS/MS. A. Schematic representation of the DNA sequence used for the purification experiment. The sequence is identical to that of Figure 2, but contains a single 3’-TEG-Biotin tag. SNP G-462A allele is indicated in bold. B. Coomassie blue SDS-PAGE showing the elution fractions from both alleles, as well as beads alone, used for analysis by LC-MS/MS. The arrow indicates an Mr ~64 kDa band seen only in the “A” lane; of note, LEF1 typically migrates with a apparent Mr ~55 kDa (e.g. http://www.scbt.com/datasheet-8591-lef-1-n-17-antibody.html). C. Summary of the peptides identified by LC-MS/MS from the elution fractions. D. LC-MS/MS spectra identifying the peptide ESAAINQILGR, corresponding to amino acid residues 225-235 in rat LEF1 (<http://www.uniprot.org/uniprot/Q9QXN1>). Arrows indicate the sequence reads from MS/MS.

**Figure 4:** Identiﬁcation of the binding factor of the A-allele by EMSA. A. Supershift experiment using the biotinylated A-allele probe from Figure 2. The probe was sequentially incubated with PC12 nuclear extract and antibodies directed against PPARγ, COUP-TF or LEF1 (Santa Cruz Biotechnology; see Methods). An antibody directed against Pax6 was used as a negative control (sc-32766X). The Protein:DNA complexes (asterisk, *) and the Protein:DNA:antibody complexes (arrowheads) are indicated. B. G and A allele -462 region and recognition motifs of PPARγ-RXRalpha, COUP-TF and LEF1/TCF1. Wide, thin and dotted lines indicate respectively a perfect match with the base in the motif, a second choice base, and third or fourth choice base. The variable base is presented in bold. WebLogo profiles of consensus base preference are from Chip-Mapper (<http://mapper.chip.org/>).

**Figure 5:** Effect of LEF1 on the regulation of CHGA promoter transcriptional activity in
chromaffin (rat PC12) cells. A. Luciferase constructs containing the 1.2-kbp CHGA proximal promoter (left panel) with the -462 SNP A allele (Hap-1/-462A) or G allele (Hap-1/-G462) or the SV40 constructs illustrated in panel B were co-transfected with a plasmid expressing the LEF1 transcription factor or an empty plasmid control (No TF; pcDNA3.1) in PC12 cells. 24h post transfection, the regulatory effects of LEF1 were analyzed by gene reporter assay in as described in Methods. Each transfection experiment included a transfection efficiency control plasmid, pRL-TK (Promega). CHGA promoter transcriptional activity is expressed as the ratio of Firefly luciferase/Renilla luciferase activity with co-transfection of LEF1 plasmid over the negative control pcDNA3.1 (in %). The bars represent standard errors. B. Schematic representation of the constructs used in the assays. The 31-bp fragments spanning the position -462 (red or blue) of the CHGA promoter were inserted 19 bp upstream of the SV40p, in the plasmid pGL3-Promoter.

Figure 6: Effect of beta-catenin (CTNNB1) on CHGA promoter transcriptional activity in chromaffin (rat PC12) cells. Luciferase constructs containing the 1.2-kbp CHGA proximal promoter (left panel) with the -462 SNP A-allele (Hap-1/-462A) or G-allele (Hap-1/-G462) were co-transfected with plasmids expressing either CTNNB1 or LEF1 in PC12 cells. 24h post transfection, the regulatory effects of LEF1 were analyzed by gene reporter assay in as described in M&M. Each transfection experiment included a transfection efficiency control plasmid, pRL-TK (Promega). CHGA promoter transcriptional activity is expressed as the ratio of Firefly luciferase/Renilla luciferase activity with co-transfection of CTNNB1 or CTNNB1 and LEF1 normalized to the basal state of the promoter (co-transfection with pcDNA3.1). The bars represent standard errors.
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<th>Model</th>
<th>Strand</th>
<th>Start</th>
<th>End</th>
<th>Score</th>
<th>E-val</th>
<th>Alignment (model, match)</th>
<th>Δ Score</th>
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**Diagram B:**
- PPARγ-RXRα
- HNF4
- LEF1
- CACTCAGACAGGGGATCAAGGCCCTCTTAG
- LEF1
- COUP-TF
LEF-1 match at human CHGA promoter variant G-462A

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<td>Human minor (A)</td>
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<td>Rhesus</td>
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<td>Marmoset</td>
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<tr>
<td>Conserved</td>
<td>*** ********</td>
<td></td>
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<tr>
<td></td>
<td>G-462A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(as C/T on [-] strand)</td>
<td></td>
</tr>
</tbody>
</table>

LEF-1 = CCTTTGWWSNY; on (−) strand of CHGA.
By “Mapper”: <http://bio.chip.org/mapper>, M00978.
IUPAC: W = A or T; S = G or C; Y = C or T.
G-462A: rs9658634.
**Bold**: Polymorphic base.
### A

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### B

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<td>G</td>
<td>G</td>
<td>A</td>
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<tr>
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<td>G</td>
<td>-</td>
<td>-</td>
<td>A</td>
<td>G</td>
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</table>

![Image of gel electrophoresis](image_url)

- NS
- *Protein:DNA complexes
- Free probe
A

G-462A (rs9658634)

CACTCAGACAGGGGATCAAGGCCCTCTAG-TEG-Biotin

+ Dynabeads M280 Streptavidin

+ PC12 nuclear extract

B

MW Beads A Allele G

kDa

250 150 100 75 50 37 25 15 10

C

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D

ESAAINQLGR = LEF-1 (Q9QXN1, rat), amino acids 225-235
Oligo-biotin
Competitor
Nuclear extract
Antibody

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<td>Nuclear extract</td>
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<tr>
<td>Antibody</td>
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PPARγ, COUP, Pax6, LEF1 (control)

A

Circulation
Cardiovascular Genetics

B

PPARγ-RXRα

+ Strand: CACTCAGACAGGGGATC AAGGCCCCTCTAG

COUP-TF

- Strand: CTAGAGGGGCCTTAGATCCCTGCTGTAGTG

LEF1/TCF1

- Strand: CTGCCTTGATCCCTGCTGTAGTG
**A**

**CHGA G-462A element in PC12:**

Effect of co-expressed LEF1

Native orientation:
within 1.2-kbp CHGA promoter

31-bp CHGA element centered on G-462A, upstream of SV40 promoter

CHGA G-462A element context

% of activity compared to control (firefly luciferase/renilla luciferase)

<table>
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<th>G allele</th>
<th>p=0.029</th>
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</thead>
<tbody>
<tr>
<td>A allele</td>
<td>p=0.030</td>
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**B**

SV40 constructs

- **SV40p Firefly luc**
  - CA\_CTCA\_GAC\_AG\_GG\_G\_G\_AT\_C\_A\_A\_A\_G\_C\_C\_C\_C\_C\_T\_C\_T\_A\_G

- **SV40p Firefly luc**
  - CA\_CTCA\_GAC\_AG\_GG\_G\_G\_AT\_C\_A\_A\_A\_G\_C\_C\_C\_C\_C\_T\_C\_T\_A\_G

- **SV40p Firefly luc**
  - CA\_CTCA\_GAC\_AG\_GG\_G\_G\_AT\_C\_A\_G\_A\_A\_G\_C\_C\_C\_C\_C\_T\_C\_T\_A\_G
Effect of co-transfection plasmid
(normalized to basal state for that allele = 1.0)

Differential response to β-catenin (CTNNB1)

Human CHGA G-462A:

B-catenin
Co-transfection(s)

B-catenin + LEF-1

Basal

0.5 1 1.5 2

p < 0.001

p < 0.003
Proteomic Analysis Yields an Unexpected Trans-Acting Point in Control of the Human Sympathochromaffin Phenotype
Stephane Chiron, Zhiyun Wei, Yuqing Chen, Kuixing Zhang, Gen Wen, Wolfgang H. Fischer, Sushil K. Mahata and Daniel T. O’Connor

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SUPPLEMENTAL MATERIAL.

Chiron et al., 2011.
Supplementary Tables/Legends:

**Supplementary Table 1:** Mascot score table of possible matches for the peptide of Mr: 1170.6357

**Supplementary Table 2:** Peptide identification by LC-MS/MS of the fraction containing LEF1. All the identified peptides (minus keratins) are presented with their putative assignments.
### Supp Table 1: Mascot scores for Mr 1171.

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**Suppl Table 2: All identified peptides.**

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**Additional Peptide Information:**

- K.EEYLR.A
- K.VNIIPIIAK.S
- R.QAMLENASDIKLEKFSISAHGK.E
- K.SSLVNESEIIPASNGHEVVR.Q

**Note:** The table includes a wide range of peptides and their corresponding scores, intensities, and repeat counts, indicating the comprehensive analysis of the identified proteins.