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Authors
Farber, Charles R
Chitwood, James
Lee, Sang-Nam
et al.

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Overexpression of Scg5 increases enzymatic activity of PCSK2 and is inversely correlated with body weight in congenic mice

Charles R Farber1,3, James Chitwood1, Sang-Nam Lee2,4, Ricardo A Verdugo1, Alma Islas-Trejo1, Gonzalo Rincon1, Iris Lindberg2,5 and Juan F Medrano*1

Address: 1Department of Animal Science, University of California, Davis, One Shields Ave., Davis, CA 95616-8521, USA, 2Department of Biochemistry and Molecular Biology, Louisiana State University Health, Sciences Center, and Children’s Hospital Research Institute, New Orleans, LA 70112-2223, USA, 3Department of Medicine, Division of Cardiology, University of, California, Los Angeles, 695 Charles E. Young Drive South, Los Angeles, CA 90095-1679, USA, 4Research Center for Human Natural Defense System, Yonsei, University College of Medicine, Seoul, 120-752, Korea and 5Department of Anatomy and Neurobiology, University of, Maryland Medical School, 20 Penn St, Baltimore, MS 21201, USA

Email: Charles R Farber - cfarber@mednet.ucla.edu; James Chitwood - jlchitwood@ucdavis.edu; Sang-Nam Lee - snlee44@gmail.com; Ricardo A Verdugo - ricardo.a.verdugo@gmail.com; Alma Islas-Trejo - adislas@ucdavis.edu; Gonzalo Rincon - grincon@ucdavis.edu; Iris Lindberg - ilind001@umaryland.edu; Juan F Medrano* - jfmedrano@ucdavis.edu

* Corresponding author

Abstract

Background: The identification of novel genes is critical to understanding the molecular basis of body weight. Towards this goal, we have identified secretogranin V (Scg5; also referred to as Sgne1), as a candidate gene for growth traits.

Results: Through a combination of DNA microarray analysis and quantitative PCR we identified a strong expression quantitative trait locus (eQTL) regulating Scg5 expression in two mouse chromosome 2 congenic strains and three additional F2 intercrosses. More importantly, the eQTL was coincident with a body weight QTL in congenic mice and Scg5 expression was negatively correlated with body weight in two of the F2 intercrosses. Analysis of haplotype blocks and genomic sequencing of Scg5 in high (C3H/HeJ, DBA/2J, BALB/cByJ, CAST/EiJ) and low (C57BL/6J) expressing strains revealed mutations unique to C57BL/6J and possibly responsible for the difference in mRNA abundance. To evaluate the functional consequence of Scg5 overexpression we measured the pituitary levels of 7B2 protein and PCSK2 activity and found both to be increased. In spite of this increase, the level of pituitary α-MSH, a PCSK2 processing product, was unaltered.

Conclusion: Together, these data support a role for Scg5 in the modulation of body weight.

Background

Body weight, as with all complex traits, is partially regulated by the coordinate action of individual genes. One common approach for dissecting the genetics of growth is the mapping of quantitative trait loci (QTL). In the last decade numerous human and mouse growth QTL have been identified [1]; however, while this progress is important, few if any of the loci have been unequivocally resolved to the effects of a single quantitative trait gene (QTG).

Several studies involving many different mouse inbred strains have demonstrated an enrichment of growth and obesity QTL on chromosome 2 [2,3]. In our laboratory,
we have developed a number of genomic resources with the goal of discerning the molecular basis of chromosome 2 QTL segregating between the C57BL/6J (B6), C57BL/6J-hg/hg (HG) and CAST/EiJ (CAST) strains [4,5]. These include two congenic strains, B6.CAST-(D2Mit329-D2Mit457)N(6) (B62D) and HG.CAST-(D2Mit329-D2Mit457)N(6) (HG2D), constructed by introgressing an identical congenic donor region, extending from approximately 75 to 180 Mbp, from the CAST strain onto both B6 and HG backgrounds [4]. HG mice exhibit extreme body weights without becoming obese due to the high growth (hg) deletion located on chromosome 10, which eliminates the expression of the suppressor of cytokine signaling 2 (Socs2) gene, a negative regulator of growth hormone signaling [6]. The purpose of generating the HG2D and B62D strains was to capture the weight gain 2 (Wg2) QTL [7] and evaluate hypothesized interactions between Wg2 and hg. After construction, HG2D mice were not characterized due to reproductive problems, however, B62D mice displayed significant decreases in growth and obesity traits [4].

In two follow-up studies it was determined that Wg2 was actually the result of multiple genes. In the first study, an F2 cross derived from the HG2D congenic and HG progenitor strain (referred to as the HG2DF2 cross) was used to identify a strong QTL located at 112 Mbp that decreased body weight at 6 and 9 weeks of age, but did not affect fat mass. This QTL partially explained the effects of Wg2 and was referred to as weight gain QTL 5 (Wg5) [5]. In the second study, subcongenic mice derived from the B62D congenic (referred to as B62D-3), with a CAST donor region from 102–115 Mbp, were lighter and leaner due to the effects of a locus we named Wg2b [8]. Based on their coincident genomic location, Wg5 and Wg2b are likely the same QTL, however, their differing effects on adiposity suggest that an interaction with hg may eliminate or decrease the fat reducing effects of Wg5.

In the current study we have used DNA microarrays and public genomic databases to investigate the genetics of expression for genes located within the two congenic models, HG2D and B62D, to identify candidates for the Wg5 and Wg2b QTL. Based on its genomic location, differential expression and biological function, the most promising candidate is secretogranin V (Scg5; also referred to as Sgne1). The protein product of Scg5, 7B2, is a molecular chaperone for prohormone convertase 2 (PCSK2). PCSK2 is a member of the subtilisin-like proprotein convertase family and many PCSK2-generated peptides are either directly involved in glucose homeostasis (glucagon) or indirectly contribute to body weight regulation (α-MSH, CART, and nesfatin-1, to name a few examples). Knockout of peptide biosynthetic enzymes often result in mice with body weight phenotypes, most notably the fat mouse with its inactivating mutation in carboxypeptidase E [9]. While the PCSK2 knockout mouse is slightly runted but otherwise of normal weight [10], an obese PCSK1 mutant mouse has recently been described which has lowered levels of the PCSK2 product α-MSH [11], indicating possible interplay/feedback between the peptide synthesizing enzymes (also suggested by [12]). Scg5 has also been implicated in body weight homeostasis. Scg5 knockout mice (on a 129/SvEv background) exhibit a postnatal lethal phenotype in which all mice die by 5 weeks of age (due to intermediate lobe ACTH hypersecretion and rampant corticosteronemia); however, adrenalectomy rescues these animals and reveals a late-onset obesity phenotype [13]. Collectively, these results point to potential roles for peptide biosynthetic enzymes, and specifically for Scg5, in body weight regulation.

Results

Identification of differentially expressed genes in HG2DF2 mice
To identify differentially expressed genes within the HG2D congenic donor region that potentially underlie body weight and obesity QTL, we hybridized whole brain RNA from non-recombinant HG2DF2 mice (mice inheriting intact cast or b6 congenic haplotypes) from each of the three F2 genotypes (b6/b6, b6/cast and cast/cast) [5] to Agilent whole genome mouse microarrays. Comparison of normalized expression values identified 62 genes as differentially expressed at a false discovery rate (FDR) of <0.30 using an additive effects model (Additional File 1). Fifty-five of the genes (88.7%) were located within the HG2D congenic region (from approximately 75 to 180 Mbp).

Identification of expression QTL (eQTL) overlapping growth and obesity QTL
As confirmation of the microarray results, we next examined the genetic basis of expression for eleven genes (Cd44, A1451465, Fmn, Scg5, 2310032D16Rik, 3300001M20Rik, Pak7, Atr5, D93300122Rik, Sdccag33l and Rab22a) identified as differentially expressed from the microarray study (Additional File 1). These genes were selected for confirmation because they were located near the peaks of previously identified HG2DF2 body weight and obesity QTL (Table 1) [5]. Gene expression was measured in whole brain RNA from 45 recombinant HG2DF2 mice using quantitative PCR. Of the eleven genes, significant eQTL were identified for five (A1451465, Scg5, 2310032D16Rik, 3300001M20Rik and Atr5) (Table 1).

Expression of Scg5 is genetically regulated by a single local acting QTL in multiple mouse crosses
Based on its close proximity to Wg5 [5], biological function and magnitude of differential expression, Scg5 was selected as the highest priority candidate gene. From the
microarray analysis, Scg5 was the eighth most significantly differentially expressed gene, with an FDR = 0.10, and its expression was up-regulated by approximately 3.0 fold in non-recombinant cast/cast HG2DF2 mice (Additional File 1). qPCR analysis in HG2DF2 mice indicated the expression difference was due to a Scg5 eQTL located at 55.5 cM (approximately 115 Mbp) mapping with a maximum LOD score of 16.4 (Figure 1). The eQTL was primarily additive (a = -0.68 and d = -0.14), with cast alleles increasing expression. The eQTL explained 74.1% of the variation in Scg5 expression. The nearest marker, D2Mit207 (located at 9.7 cM or 111.8 Mbp), was less than

Table 1: Expression QTL for genes coincident with growth or obesity QTL and differentially expressed in HG2D mice

| Gene symbol | Mbp       | eQTL LOD | cM  | Mbp | Coincident QTL |
|-------------|-----------|----------|-----|-----|----------------|
| AI451465    | 112.272   | 9.8      | 64.0| 134 | Wg5            |
| Scg5        | 113.578   | 16.4     | 55.5| 115 | Wg5            |
| 2310032D16Rik| 132.221   | 5.5      | 55.5| 115 | Fatq1          |
| 3300001M20Rik| 132.496   | 3.0      | 66.5| 138 | Fatq1          |
| Act5        | 158.316   | 8.6      | 75.0| 155 | Fatq1, Fatq2   |

All eQTL are significant at P < 0.05

Figure 1
Scg5 expression is regulated by a strong local eQTL on chromosome 2 and is coincident with QTL for body weight and body mass index in HG2DF2 mice. The plot presents the LOD score profile across the HG2D donor region for Scg5 expression, body weight at 9 weeks and body mass index. Genetic positions in centimorgan (cM) are plotted on the x-axis. The location of Scg5 (113.6 Mbp) is indicated with an arrow.
was up-regulated in hypothesized in [4,7]), it may be mediated via alterations negatively correlated (P < 0.01) with body weight and growth traits and indeed its expression was significantly increased at 3 weeks was followed by approximately 40% increases at 4.5 (P < 0.05) and 9 weeks (P = 0.06).

Identification of Stat5b binding sites in the Scg5 promoter
The hg deletion encompasses three genes, Soc2, Raidd and Plexin C1 [19]. The extreme growth rate and mature body size of HG mice (homozygous for the hg deletion) is primarily (if not entirely) due to the absence of Soc2 [6]. In Soc2+/- mice increased growth is dependent on Stat5b [20]. The DNA sequence required for Stat5b binding has been well characterized [21]. Stat5b is the primary transcription factor responsible for mediating Gh induced gene expression changes [22] and a modest increase in its activity has been identified in Soc2+/- mice [20]. To identify putative Stat5b binding sites which may be used by Gh (via Stat5b) to regulate the increase of Scg5 expression in HG mice, we screened 72.683 Kbp of Scg5 genomic sequence for the presence of the Stat5b consensus transcription factor binding site TTCYNRGAA (Y = T or C and R = A or G) [21]. The sequence included all introns and exons and 10 kbp of 5’ and 3’ genomic sequence. In total six consensus sites were identified (Additional File 2). Interestingly, two tandem sites in the Scg5 promoter were located between -56 and -39 (relative to the transcription start site; Additional File 2). The probability that these sites occur by chance is 4.18, suggesting they are functional and may mediate the increased expression of Scg5 in HG mice.

Genomic analysis of the Scg5
It is likely that most polymorphisms giving rise to local (truly cis eQTL) will reside within promoter or intronic based regulatory elements [23]. With this in mind, we analyzed the genomic sequence of Scg5 to identify putative polymorphisms that may be responsible for the strong local eQTL. This is important since it could lead to the discovery of novel modes of Scg5 transcriptional regulation. Based on the eQTL results from multiple crosses, we hypothesized that two haplotypes should exist in the genomic region containing the eQTL, one unique to B6 (low expressers) the other shared between CAST, BALB, DBA and C3H (high expressers). To identify potential regions, haplotype blocks were identified by downloading all SNPs within Scg5 from the Mouse Phenome Database [24,25]. A total of 191 SNPs were identified between 113.571079 and 113.633403 Mbp on chromosome 2 (Additional File 3). This 62.324 Kbp region encompassed the entire Scg5 coding sequence, promoter, over 5 Kbp downstream and the 3’ end of Arhgap11a, which is located 2.4 kbp upstream of the first Scg5 exon. As predicted, two haplotype blocks were identified using the Haplovie...
A strong local eQTL on chromosome 2 regulates the expression of $Scg5$ in BXD, CXB and BXH mouse crosses. Mapping of $Scg5$ expression in A) whole brain from B6 × DBA (BXD) recombinant inbred (RI) mice, B) hippocampus from BALB × B6 (CXB) RI mice, and C) whole brain from BXH-ApoE<sup>-/-</sup> F2 mice. eQTL were identified using interval mapping tools available at [15, 16] for BXD and CXB crosses and R/qtl [38] software for BXH-ApoE<sup>-/-</sup> F2 mice.
software package [26]. Block 1 extended from the beginning of the queried region at 113.571079 Mbp to 113.581978 Mbp (10.899 Kbp) and block 2 spanned from 113.582743 Mbp to the end of the interval at 113.633403 Mbp (50.660 Kbp) (Additional File 3). Block 1 did not fit the necessary criteria of being unique to B6 (B6 and CAST were very similar across this block), however, block 2 consisted of three predominant haplotypes, one unique to B6, one unique to CAST and the other was highly similar among C3H, DBA and BALB. Although CAST differed from all other strains across the whole block, it was much more similar to C3H, DBA and BALB in the region corresponding to exon 2 and the first two introns. The haplotype similarities and differences across both blocks are shown in cladograms in Additional File 4. Therefore, since the region of haplotype block 2 contains a haplotype unique to B6 and similar among the other strains it is likely the genetic variant underlying the eQTL is located in this region.

None of the SNPs included in the above analysis were located within the proximal promoter. To comprehen-

Table 2: Correlation between Scg5 expression and body weight in five mouse crosses

| Cross          | Trait                  | Correlation | p-value | Details                                                                 |
|---------------|------------------------|-------------|---------|-------------------------------------------------------------------------|
| HG2DF2        | 9 week weight          | -0.34       | 0.02    | calculated in 45 HG2DF2 mice                                           |
| B62D-3        | 6 week weight          | -0.45       | 0.01    | calculated in 15 non-recombinant B62D-3 mice, 5 of each genotype        |
| B62D-3        | 9 week weight          | -0.62       | <0.01   | calculated in 15 non-recombinant B62D-3 mice, 5 of each genotype        |
| BXD RI        | Adult body weight      | -0.53       | 0.02    | calculated in 16 BXD RI and 2 parental strains, phenotype = BXH::10031  |
| CXB RI        | 90 day body weight     | -0.82       | <0.01   | calculated in 7 BXD RI and 2 parental strains, phenotype = BXH::10281   |
| BXH-ApoE-/- F2| Adult body weight      | -0.01       | 0.81    | calculated in 255 BXH-ApoE-/- F2 mice                                  |

Scg5 expression is up-regulated in B62D-3 F2 mice. The relative expression of Scg5 is presented for the three B62D-3 F2 genotypes (b6/b6, b6/cast and cast/cast).
sively screen this region, we sequenced 2500 bps upstream of the transcription start site (as defined by RefSeq NM_009162) in B6, HG, CAST, BALB, DBA and C3H mice. Ten SNPs were identified across all six strains (Table 3). In addition, an 11 bp deletion between -432 and -422 (del-432) was identified in DBA, C3H and BALB. This deletion was previously identified in C3H mice by Schmidt et al. [27]. Consistent with the above haplotype analysis, BALB, DBA and C3H shared an identical haplotype across the region. CAST was polymorphic at all 10 of the SNPs (but not del-432) relative to B6 and the other strains were polymorphic at SNPs -1373, -794 and -659. Interestingly, CAST mice did not possess del-432, however, position -432 was polymorphic between B6 and CAST mice. Therefore, since four SNPs are unique to B6, the low Scg5 expressing strain, it is probable that one (or more) may give rise to the Scg5 eQTL.

**Up-regulation of Scg5 increases levels of 7B2 protein and PCSK2 enzyme activity**

To characterize the molecular consequences of Scg5 up-regulation, the levels of 7B2 protein, Pcsk2 gene expression, PCSK2 activity, α-MSH and ACTH levels were quantitated in B62D-3 F2 mice. The levels of pituitary (pit) 7B2 titrated in B62D-3 F2 mice. The levels of pituitary (pit) 7B2 protein, PCSK2 activity, regulation, the levels of 7B2 protein, Table 3: To characterize the molecular consequences of PCSK2 enzyme activity

Up-regulation of Scg5 increases levels of 7B2 protein and PCSK2 enzyme activity

| Strain     | Scg5 exp. | -2475 (A/G) | -2445 (A/G) | -1540 (A/G) | -1373 (A/G) | -1095 (G/C) | -904 (G/T) | -794 (G/A) | -659 (A/G) | -432 (C/T) | del-432 (del) | -306 (A/C) |
|------------|-----------|-------------|-------------|-------------|-------------|-------------|------------|------------|------------|-------------|--------------|------------|
| C57BL/6j   | LOW       | AA          | AA          | AA          | AA          | GG          | GG         | GG         | AA         | CC          | +            | AA         |
| C57BL/6J-  | LOW       | AA          | AA          | AA          | AA          | GG          | GG         | GG         | GG         | AA          | +            | AA         |
| 6j-hg/hg   | BALB/cByJ | HIGH        | AA          | AA          | AA          | AA          | GG         | GG         | AA         | GG          | del          | del        |
| C3H/HeJ    | HIGH      | AA          | AA          | AA          | AA          | AA          | GG         | GG         | AA         | GG          | del          | N/A        |
| DBA/2J     | HIGH      | GG          | CC          | GG          | AA          | CC          | TT         | AA         | GG          | TT          | +            | CC         |

SNPs are numbered based on position upstream from transcription start site as defined by RefSeq NM_009162. SNPs with genotypes in bold italics segregate low expressers and high expressers. +, deletion not present in these strains; del, deletion is present in these strains; N/A, genotypes for -306 were not determined for strains with deletion due to the forward primer sequence overlapping the deletion.

Discussion

In the current study we have demonstrated that transcriptional variation in Scg5 across several inbred strains is mediated almost entirely by local genetic variation near the Scg5 locus. More importantly, we have demonstrated that an increase in Scg5 expression is correlated with decreases in body weight and obesity in two congenic mouse models and overexpression results in an in vivo increase in 7B2 protein levels and PCSK2 enzymatic activity.

By analyzing the expression of Scg5 in multiple segregating populations we have conclusively shown that nearly all of the variation in Scg5 expression is due to a strong local-acting variant. In all four crosses surveyed, the eQTL accounted for over 75% of the variation in Scg5 expression. Haplotype analysis using a recently generated high-density SNP collection indicated that the most probable location for the Scg5 local eQTL quantitative trait nucleotide was the promoter through the first four exons/introns. In the Scg5 promoter, four polymorphisms unique to the high-expressing strains were discovered. Of particular interest is del-432, an 11-bp deletion present in all strains except B6 and CAST. Although the deletion is absent in CAST, these mice are polymorphic at position -432 (possessing a T allele instead of the C allele in B6). It is tempting to speculate that this region is important for binding of a transcriptional repressor and that -432 is a key nucleotide.
The action of Scg5 overexpression on body weight may represent either a direct effect of 7B2 or an indirect effect mediated by PCSK2-generated peptides. In this regard, it should be pointed out that it is not yet clear that 7B2 is always regulatory for PCSK2-mediated peptide production. A recent paper showing that the varying 7B2 levels synthesized by two different mouse strains are associated with differential production of glucagon [27] supports the notion that 7B2 levels do modulate in vivo peptide production, possibly by regulation of PCSK2 activity. However, our present results do not support an obligatory association of increased peptide production by increased PCSK2 activity, since we observed no changes in pituitary α-MSH, a PCSK2 product, even though tissue PCSK2 activity increased. While two studies have shown that introduction of 7B2 into cells which lacks all 7B2 production facilitates the generation of PCSK2-mediated peptides [28,29], when cells already express some Scg5, additional 7B2 introduction does not always increase peptide production. An example is the transfection of Scg5 into AtT-20-PC2 cells, a neuroendocrine cell line which endogenously produces 7B2. The additional increase of 7B2 neither increases α-MSH production nor facilitates any other PCSK2-mediated peptide cleavage examined to date [30] and SNL and IL, unpublished results). Presumably other factors, possibly cell- or tissue-specific, can override any effects of additional Scg5 expression to limit the total amount of peptide made. In this regard it is important to note that we measured PCSK2-mediated peptide production only in pituitary; PCSK2/7B2 effects may also occur in other tissues more intimately involved in energy homeostasis, such as the hypothalamus.

If the action of 7B2 on body weight homeostasis is not mediated via generation of PCSK2-mediated peptides, then it may result from a direct effect of 7B2 itself. It is currently impossible to speculate on the mechanism for this effect; however, this interesting pan-neuronal protein may have many unexplored roles. A recent paper describes the association of human neuroblastomas with the epigenetic...
down-regulation of 7B2 [31]. In this study, loss of 7B2 expression was associated with increased tumor formation, suggesting that 7B2 expression may contribute to the maintenance of neuronal differentiation.

Although we have provided several lines of evidence supporting the role of Scg5 in body weight regulation, that is in agreement with a prior knockout study demonstrating an increase in body weight in the absence of Scg5 [13], we have not demonstrated a direct causal link between Scg5 overexpression and reductions in body weight. It is possible that the local eQTL, regulating Scg5 expression, is in tight linkage with the actual causal variant(s) responsible for Wg5. It will take additional studies, such as generating a Scg5 transgenic mouse to confirm its involvement in body weight regulation.

In the current study we have shown that in addition to the effects of the strong eQTL, Scg5 is up-regulated in HG mice, suggesting that this gene is responsive to growth hormone signaling. As discussed above, HG mice lack expression of Socs2. The absence of Socs2 leads an increase in the activity of Stat5b in Socs2−/− mice a factor which mediates the transcriptional effects of growth hormone [20]. The molecular and physiological consequences of this increase are unclear; however, it should be noted two previous studies have provided data supporting an interaction between hg and chromosome 2 QTL [4,7]. It is possible that the increase in Scg5 expression underlies this interaction.

Conclusion
We have provided data to support Scg5 as a candidate gene for body weight homeostasis. Further, our results show that expression of the Scg5 gene product, 7B2, is likely to be controlled by strain-dependent promoter polymorphisms and that this difference in expression leads to an increase in the enzymatic activity of PCSK2. The mechanism by which increased 7B2 levels contribute to body weight is unclear as yet, but may represent a PC2-independent effect of this widely-expressed neuroendocrine gene.

Methods
Mice
Phenotypic and gene expression data were generated from two inbred strains (B6 and HG [32]), a subcongenic strain (B62D-3 [8]) and two congenic-derived intercrosses (HG2DF2 [5] and B62D3 F2 [8]). In addition, Scg5 sequence data were collected from four inbred strains C3H/HeJ (C3H), DBA2 (DBA), BALB/cByJ (BALB) and CAST and Scg5 expression data were obtained from three additional crosses not produced in our laboratory, the BXD RI, CXB RI and BXH-ApoE−/− F2 [16,18]. All animal protocols were managed according to the guidelines of the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Microarray gene expression analysis in whole brain RNA from non-recombinant HG2DF2 mice
Total RNA was isolated using TRIzol reagent (Invitrogen) from homogenized whole brain samples. Whole brain total RNA from four male non-recombinant (hg/hg, hg/cast or cast/cast across the entire congenic interval) mice was used for microarray analysis. Two samples from each genotype were labeled with each dye (Cy3 or Cy5) using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Samples were hybridized on the Mouse Whole Genome Oligo Microarray (Agilent Technologies) using an incomplete loop design. Using two arrays, two hg/hg samples (one labeled with Cy3 and the other Cy5) were hybridized with two reciprocally labeled cast/cast samples (i.e. hg/hg Cy3 was hybridized along with cast/cast Cy5). Four additional arrays were used for the other two genotype combinations (hg/hg vs. hg/cast and cast/cast vs. hg/cast). TIFF images were obtained using the Agilent DNA Microarray Scanner BA (Agilent Technologies) and intensities were measured with the accompanying Feature Extraction Software. Intensities were normalized by linear and LOWESS regressions and background corrected by subtracting the trend of minimum intensities along the surface of the slide. Data were then analyzed with the maanova package [33-35] from the Bioconductor suite for R [36]. Genes were tested both for additive and dominance effects by setting the appropriate contrasts in the mixed model $y_{ijkl} = \text{Dye} + \text{Array} + \text{Genotype}_{ijkl} + \text{Error}_{ijkl}$ where Dye and Genotype are fixed and Array and Error are random effects. P-values were corrected for multiple comparisons using an FDR transformation [37].

Expression QTL analysis
Duplicate qPCR reactions were carried out with 25 ng of cDNA using ABI Gene Expression Assays (Applied Biosystems) for each gene on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). The ABI Gene Expression Assays were as follows: AI451465, Mm00505280_m1; Fmn, Mm00439021_m1; Scg5, Mm00486077_m1; Cd44, Mm01277160_m1; 2310032D16Rik, Mm00512140_m1; 3300001M20Rik, Mm00558305_m1; Pak7, Mm00556184_m1; Actr5, Mm00615134_m1; D93001122Rik, Mm00557752_m1; Sdc3a433I, Mm01248119_m1; Rab22a, Mm00508287_m1; and Psk2 Mm00500981_m1. Sdhagen (succinate dehydrogenase complex, subunit A, flavoprotein (fp)) and GUS (beta glucuronidase) were used as endogenous control genes. They are not located in the congenic region (Sdhagen is on MMU13 at 70.4 Mbp and GUS is on MMU5 at 129.2 Mbp) and both are common, stably expressed control genes. The control corrected expression
of each target gene was determined as \( \Delta Ct = Ct \) (target gene) - Ct (control gene) - Ct (control gene). These measurements \( (\Delta Ct) \) were then subjected to interval mapping using the "scanone" function of R/qtl [38]. Mouse age, litter size and dam's parity were used as additive covariates. The "fitqtl" function was used to estimate genetic effects and percent variance explained.

**qPCR in B62D-3 and HG mice**

qPCR reactions for Scg5 and Pcsk2 were performed in B62D-3 mice as described above. The expression of each target gene was determined using the expression, \( 2^{-\Delta Ct} \), where \( \Delta Ct = Ct \) (target gene) - Ct (control gene). These values were analyzed with SAS using a linear model that included the fixed effects of genotype [39]. The expression of Scg5 was also measured in whole brain from five HG and B6 mice at 3 WK, 4.5 WK and 9 WK. qPCR was carried out as described above and Sihfa normalized expression levels \( (2^{-\Delta Ct}) \) were calculated for each sample. These values were analyzed with SAS using a linear model that included the fixed effects of strain, age and strain by age interaction [39]. The mean values for HG at each age were then scaled to B6 expression levels.

**Scg5 promoter sequencing**

PCR amplicons (Additional File 5) covering approximately 2500 bp upstream of the Scg5 transcriptional start site were sequenced from the B6, HG, CAST, BALB, DBA, and C3H strains. PCR amplified fragments were gel purified using the Qiagen QIAquick gel extraction kit and sequenced at the High-Throughput Genomics Unit at the University of Washington.

**PCSK2 Enzyme Assays**

All of the following extraction steps were performed at 4°C. Pituitaries were briefly sonicated in 100 mM sodium acetate, pH 5.0, and 1% Triton \( \times \)100 in the presence of a protease inhibitor cocktail composed of 1 \( \mu \)M pepstatin, 1 \( \mu \)M trans-epoxysuccinic acid (E-64), and 1 mM phenylmethylanesulfonyl fluoride (PMSF). The extracts were centrifuged for 2 min at 15,000 \( \times \) g. The supernatants were used for PC2 enzyme assays. The assay for PC2 was carried out in triplicate in 96 well polystyrene microtiter plates using 10 \( \mu \)l of each sample in a total volume of 50 \( \mu \)l containing 200 \( \mu \)M fluorogenic substrate, pyr-Glu-Arg-Thr-Lys-Arg-methylcoumarinamide (MCA) as a substrate and 100 mM sodium acetate buffer (pH 5.0) containing 5 mM CaCl\(_2\), 0.5% Triton \( \times \)100 in the presence of a protease inhibitor cocktail above with the addition of 0.14 mM tosyllysyl chloromethyl ketone (TLCK) [40]. The activity was also separately measured in the presence of 1 \( \mu \)M 7B2 CT peptide, a specific inhibitor of PC2 [40]. The fluorescent product MCA was measured with a Fluoroscan Ascent plate fluorometer. The amount of released product was calculated by reference to the fluorescence of the free MCA standard and is given in fluorescence units (FU) per minute, in which one FU corresponds to 5.33 pmol of MCA. These values were analyzed with SAS using a linear model that included the fixed effects of genotype [39].

**7B2 Radioimmunoassays**

Pituitaries were homogenized by sonicating in 250 \( \mu \)l of ice-cold 0.1 N HCl. The samples were stored frozen, thawed, and centrifuged for 15 min at 13,000 rpm (17,383 \( \times \) g) at 4°C. The supernatant was lyophilized and resuspended in 0.5 ml of radioimmunoassay (RIA) buffer (100 mM sodium phosphate, pH 7.4, containing 0.1% heat-treated BSA, 50 mM NaCl, and 0.1% sodium azide). All samples were stored frozen at -70°C until use. For 7B2 assays, 50 \( \mu \)l of pituitary samples were subjected to assay in duplicate. The polyclonal antiserum against 7B2 (LSU113BF), directed against residues 23–39 of 7B2 [30], was used to detect 7B2. \(^{125}I\)-labeled 7B2 was prepared by the chloramine-T method originally described by Hunter and Greenwood [41] RIAs were carried out according to protocols described previously [42]. Samples were incubated with 10,000 cpm of iodinated peptide and the appropriate dilution of rabbit antiserum in a final volume of 300 \( \mu \)l at 4°C overnight. To separate the antibody-bound labeled peptide from the unbound labeled peptide, 1 ml of 25% polyethylene glycol and 100 \( \mu \)l of 7.5% carrier bovine \( \gamma \)-globulin (in PBS) were added. The samples were vortexed, kept on ice for 30 min, and then centrifuged for 20 min at 3,000 \( \times \) g at 4°C using a Sorvall RT6000B refrigerated centrifuge. The supernatant was aspirated, and the radioactivity in the pellets was determined. These values were analyzed with SAS using a linear model that included the fixed effects of genotype [39].

**\( \alpha \)-MSH and ACTH assays**

10 \( \mu \)l of a 1/200 dilution pituitary sample in RIA buffer were subjected to assay in duplicate. The polyclonal anti-\( \alpha \)-MSH antiserum was commercially purchased from Chemicon (Temecula, CA). \( \alpha \)-MSH assays were performed as described above. ACTH assays were carried out using the two-site Nichols human ACTH\(_{1-39}\) assay kit (Nichols Institute, San Juan Capistrano, CA). The \(^{125}I\)-ACTH antibody used in this kit is directed towards both N-terminal and C-terminal regions of intact ACTH molecule and does not recognize ACTH cleavage products. These values were analyzed with SAS using a linear model that included the fixed effects of genotype [39].

**Authors’ contributions**

CRF, IL and JFM conceived the study. CRF, JC, SNL, RAV and AIT performed all experiments. CRF, RAV and JFM analyzed the data. CRF, IL and JFM drafted the manuscript. JFM provided coordination of the project. All authors read and approved the final manuscript.
Additional material

Additional file 1
Differentially expressed genes in HG2DF2 non-recombinant F2 mice. These data represent a list of genes differentially expressed in HG2DF2 non-recombinant F2 mice determined using DNA microarrays. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2156-9-34-S1.pdf]

Additional file 2
Scg5 whole brain expression is up-regulated in HG versus B6 male mice at 3, 4.5 and 9 weeks of age and the Scg5 promoter contains two tandem putative Stat5b DNA binding sites. Expression analysis of Scg5 as a function of HG genotype. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2156-9-34-S2.pdf]

Additional file 3
Scg5 haplotypes based on available SNP data. Haplotype analysis of Scg5 in ‘low’ and ‘high’ expressing strains using publically available SNP data. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2156-9-34-S3.pdf]

Additional file 4
Hierarchical clustering analysis of Scg5 haplotype blocks. Illustrates the relationships of Scg5 haplotype blocks in ‘low’ and ‘high’ expressing strains. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2156-9-34-S4.pdf]

Additional file 5
Scg5 promoter sequencing primers. List of PCR primers used for Scg5 sequencing. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2156-9-34-S5.pdf]

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