Retinoblastoma-associated protein 140 as a candidate for a novel etiological gene to hypertension

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ABSTRACT

Gene discovery in animal models may lead to the revelation of therapeutic targets for essential hypertension as well as mechanistic insights into blood pressure (BP) regulation. Our aim was to identify a disease-causing gene for a component of polygenic hypertension contrasting inbred hypertensive Dahl salt-sensitive (DSS) and normotensive Lewis rats. The chromosome segment harboring a quantitative trait locus (QTL), C16QTL, was first isolated from the rat genome via congenic strains. A candidate gene responsible for C16QTL causing a BP difference between DSS and Lewis rats was then identified using molecular analyses combining our independently-conducted total genome and gene-specific sequencings. The retinoblastoma-associated protein 140 (Rap140)/family with sequence similarity 208 member A (Fam208a) is the only candidate gene supported to be C16QTL among three genes in genome block 1 present in the C16QTL-residing interval. A mode of its actions could be to influence the expressions of genes that are downstream in a pathway potentially leading to BP regulation such as that encoding the solute carrier family 7 (cationic amino acid transporter, y+ system) member 12 (Slc7a12), which is specifically expressed in kidneys. Thus, Rap140/Fam208a probably encoding a transcription factor is the strongest candidate for a novel BP QTL that acts via a putative Rap140/Fam208a-Slc7a12-BP pathway. These data implicate a premier physiological role for Rap140/Fam208 beyond development and a first biological function for the Slc7a12 protein in any organism.

Introduction

Although its prevalence and elevated risks for cardiovascular and renal consequences are well recognized (1), essential hypertension is poorly understood as its etiology of pathogenesis eludes clarification (2,3). Despite that nearly 30 genetic loci, known as quantitative trait loci (QTLs), have been detected that are associated with systolic and/or diastolic pressures (4,5), no genes with function-changing mutations have actually been identified, which are responsible for a blood pressure (BP) QTL in humans (6). Because of the conserved homology among mammalian species, causal genes discovered in rodent models of hypertension has the potential for providing mechanistic insights (7) into the human BP homeostasis, which may lead to hypertension when disturbed.

Localization of BP QTLs in rat models of essential hypertension has been largely accomplished to various chromosome segments (3). The present challenge is to pinpoint them at the molecular level by position genetics and to elucidate their mechanisms regulating BP (7). The first step in confronting this challenge is to identify a candidate gene for a BP QTL that may be causal to hypertension and are molecularly contrasting between the two comparing rat strains used in genetic studies (8).

Previously, we localized a BP QTL, C16QTL, to a segment of chromosome (Chr) 16 in a congenic strain made by crossing hypertensive Dahl salt-sensitive (DSS) and normotensive Lewis rats (9). C16QTL behaved as if it determined a ‘monogenic trait’ (10), suggesting that a gene may be responsible for it. Due to a large number of genes contained in the segment, the single gene among them was not identifiable.

The current investigation had two objectives. First, a fine congenic resolution (FCR) of C16QTL was to be conducted to an experimental limit, and second, a candidate gene for C16QTL was to be sorted out via molecular analyses. Among three possible genes narrowed by FCR and excluded from genome sequence identities, we have identified a protein-altering genetic variant in the gene encoding retinoblastoma-associated protein 140 (Rap140)/family with sequence similarity 208 member A (Fam208a) as the candidate for C16QTL, despite its embryonic lethality in homozygous mouse mutants (11).

Methods

Animals

Protocols for handling as well as maintaining animals were approved by Comité Institutionnel de Protection des Animaux.
Construction of congenic strains

All congenic strains carry different Chr 16 segments of Lewis, which are placed on the DSS background. The earlier congenic strain C16S.L5 (9) was the basis for deriving new congenic strains utilized in the current study. Briefly, C16S.L5 was bred to DSS to beget the F1 generation. F1s were crossed to produce F2 offspring among which crossovers were sought. After screening for more than 400 F2s, five crossovers were found. Four of them gave rise to informative congenic substrains (Figure 1), which were designated as: DSS.Lewis-(D16Chm36-D16Mit2)/Lt (abbreviated as C16S.L6), DSS.Lewis-(D16Rat112-D16Chm60)/Lt (C16S.L7), DSS.Lewis-(D16Chm36-D16Chm60)/Lt (C16S.L8), and DSS.Lewis-(D16Chm48-D16Chm60)/Lt (C16S.L9).

Experimental protocols and analyses

The breeding procedure, dietary treatments, telemetry implantation, postoperative care, and BP measurement durations were essentially the same as reported previously (9,12). In brief, male rats were weaned at 21 days of age, kept on a low salt diet followed by a high salt diet starting from 35 days of age until the end of the experiment. Telemetry probes were implanted at 56 days of age (namely three weeks from the time of the high salt diet). BP of DSS was pooled from three different measurements since they were not different. In the BP presentation (Figure 1), averaged readings for the duration of measurement were given for each strain. Since systolic and diastolic pressures were consistent (data not provided) with the mean arterial pressures (MAPs) of all the strains, only their MAPs are provided for simplicity.

Repeated measures’ analysis of variance (ANOVA) followed by Dunnett’s test, which corrects for multiple comparisons and unequal sample sizes, was used to compare a parameter in MAPs between two groups as reported previously (9,12). During the BP comparison, ANOVA was first applied to assess the inter-group differences. If it was significant, Dunnett’s test ensued to identify which group

![Figure 1](image). Fine congenic resolution of C16QTL. Marker positions in megabases (Mb) are taken from [http://www.ncbi.nlm.nih.gov/mapview](http://www.ncbi.nlm.nih.gov/mapview). Solid bars under congenic strains symbolize the Dahl salt-sensitive (DSS) chromosome fragments that have been replaced by those of Lewis. Open bars on ends of solid bars indicate ambiguities of crossover breakpoints between markers. The full gene nomenclature corresponding to their abbreviations is given in Table 1 legend. Mean arterial pressures (MAPs) for DSS and congenic strains are averaged for the period of measurement and are given at the bottom of the map to facilitate strain comparisons. Significant \( p \) values are emphasized in bold and italics. \( \pm \) indicates SEM. C16S.L6, C16S.L7, C16S.L8, and C16S.L9 are newly-generated from this study. Their full names are listed in the method section. The placement of C16QTL is indicated by a bracket.
was different from DSS, and the level of significance. The power and sample size calculations in the analysis are as given previously (12).

**Independent total genome sequencing of DSS and Lewis rats**

Supplement 1 outlines the methods, procedure, interpretation, sequence calling, and data mining along with relative references that we have performed. As shown in results, our genome database has revealed relevant protein-altering mutations that the public rat genome database (RGD) did not, and thus are uniquely useful for mutation screening in identifying a candidate for the QTL in question.

Our genome sequences of DSS and Lewis became our database (Supplement 2) for identifying single-nucleotide polymorphisms (SNPs) in genes in the C16QTL-containing interval (Figure 1). Bioinformatics software NexxiaGen® developed by Max Chauvet was used to position variants resulting from the SNP analysis within the genomic sequence of each gene.

**Mutation screenings**

First, all missense and intron–exon junction mutations were initially detected from our database of the total genome sequencing of DSS and Lewis rats and are homozygous for the Lewis alleles (i.e. LL), in the congenic strain that defines C16QTL. Subsequently, the chromosome segment carrying the mutation was individually PCR-amplified and validated by Sanger sequencing. Thus, we have independently verified all mutations listed in Table 1.

Second, to eliminate false negatives in the total genome sequencings, we have amplified by PCR and sequenced every exon and intron–exon junction in all seven genes present in the C16QTL-residing segment.

Third, copy number variations (CMVs) were detected as genome deletions and duplications from our database (Supplement 2) and those of RGD (13).

Finally, the protein-coding genes contained in the C16QTL-containing interval were assessed for expression by reverse transcriptase (RT) polymerase chain reaction (PCR) in a panel of cDNAs extracted from various organs of DSS and/or Lewis rats.

**Quantitative real-time polymerase chain reactions (qPCRs)**

Expressions of genes were assessed by a standard procedure as reported previously (14).

**Results**

**C16QTL placement**

The smallest segment that defined the interval lodging C16QTL emanated from congenic strain C16S.L9 (Figure 1), which alone displayed a magnitude of the BP effect accounting for about 28% of the averaged MAP difference (88 mmHg) between DSS (183 mmHg) and Lewis (95 mmHg). C16QTL acts autonomously of other QTLs in the DSS genome and is independently confirmed four times by four congenic strains, i.e. C16S.L6, C16S.L7, C16S.L8, and C16S.L9 (Figure 1).

| Genome block | Gene     | Size of coding seq (bp) | Exon | Codons                      | Change in amino acid (AA) Lew/DSS | Mutation detected in In/Ex junction | Splicing variant |
|--------------|----------|-------------------------|------|-----------------------------|-----------------------------------|------------------------------------|------------------|
| 1            | Asb14    | 1785                    | 9    | No                          | No                                | No                                 | No               |
|              | Appl1    | 4444                    | 22   | No                          | No                                | No                                 | No               |
|              | Hesx1    | 558                     | 4    | No                          | No                                | No                                 | No               |
|              | Il17rd   | 2223                    | 13   | A 1362 C                    | T 1428 G                          | No                                 | No               |
|              | Arhgef3  | 1575                    | 10   | E11 (+30)                   | E13 (−14)                         | No                                 | No               |
| 2            | Fam208a/ | 4696                    | 22   | A666C73                     | A626/G26                          | E3 (+17)                           | No               |
|              | Rap140   |                         |      | A1161C                      | No                                | E4 (+22)                           | No               |
|              |          |                         |      | A2493G                      | No                                | E10 (−11)                          | No               |
| 2            | Ccdc66   | 2840                    | 18   | A1059G                      | No                                | E2 (−49)                           | No               |
|              |          |                         |      |                             | No                                | E3 (−47)                           | No               |

Gene locations on chromosome 16 are indicated on the map in Figure 1. The position of a mutation enumerates from the ATG start codon of that gene. The amino acid position begins from the first methionine. Arhgef3, Rho guanine nucleotide exchange factor (GEF) 3; Appl1, adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper containing 1; Asb14, ankyrin repeat and SOCS box-containing 14; Ccdc66, coiled-coil domain containing 66; Hesx1, HESX homeobox 1 (15). Il17rd, interleukin 17 receptor; Rap140, retinoblastoma-associated protein 140/family with sequence similarity 208 member A (Fam208a). LOC102548056 and LOC102548123 are designated as non-coding RNAs that are located in either the 5′-untranslated or 3′-untranslated regions in the known genes around them and are not included in the table. There are no SNPs in them. In: intron, Ex: exon, (+) and (−): nucleotide after and before a given exon, respectively. No copy number variation (CNV) had been found for those genes from the total genome sequencing of DSS and Lewis rats based on our current work and those of others (13). Genome blocks 1 and 2 are explained in the results. All the nucleotide variations were confirmed by separate sequencings of PCR-amplified genome fragments.
Molecular analyses identified retinoblastoma-associated protein 140 (Rap140)/family with sequence similarity 208 member A (Fam208a) as the sole candidate gene for C16QTL

Except for the pseudogenes, all the expressed genes present in the C16QTL-containing interval in the C16S.L9 congenic strain have been sequenced in two ways (see the following section). Among them, only Rap140/Fam208a is supported as the candidate gene for C16QTL, because it harbors non-conserved mutations (Table 1 and Supplement 3).

First, Rap140/Fam208a bears a deletion of 3 base pairs (bp) from #73 to 75, which removes a single amino acid of glycine from the coding region of Lewis rats (Fig ure 2a and b). The rest of the Rap140/Fam208a coding region is identical between DSS and Lewis (Supplement 3). The recently published RGD (13) did not reveal the 3-bp deletion by comparing our independently-generated genome sequences of DSS and Lewis. Since this 3-bp deletion in Lewis has been confirmed both in our sequencing and as a size polymorphism (Figure 2b), the Rap140/Fam208a protein of Lewis rats is truly one amino acid shorter than its DSS counterpart and the information of RGD (13) was false negative.

In contrast, all the remaining six genes in the C16QTL-lodging interval (Figure 1) carry the identical coding region in Asb14, Appl1, Hesx1, and Il17rd, and the same amino acids in Arhgef3 and Ccdc66 (Supplements 4 and 5) between DSS and Lewis. These data were derived from two categories of sequencing by us, i.e. from our total genome databases comparing DSS with Lewis, and by sequencing individual exon fragments amplified by PCR that covered the complete coding region of a gene in question. The latter method confirmed the former and no additional mutations were detected from the latter method. Thus, our two sequencing approaches produced consistent and mutually-validating data and no false negatives of coding variations were found from our total genome sequencing of the seven genes.

Second, nucleotide differences at intron–exon junctions in all three genes, i.e. Arhgef3, Rap140/Fam208a, and Ccdc66 (Table 1), were first obtained from our genome databases and then confirmed by sequencing PCR-amplified genome products. No alternative splicing (Table 1) was found. There were no copy number variations (from our sequencing data and the RGD (13)) in any of the seven genes. Thus, it appears that the single glycine deletion in the Rap140/Fam208a coding domain is necessary and sufficient to support the candidacy of Rap140/Fam208a as C16QTL.

Third, the seven genes existing in the C16QTL-residing interval can be divided into two genome blocks, i.e. block 1 consisting of four genes (Asb14, Appl1, Hesx1, and Il17rd) and block 2 consisting of three genes Arhgef3, Rap140/Fam208a, and Ccdc66. Based on genome sequences from the RGD (13) and our database, block 1 is practically identical, whereas block 2 is divergent between DSS and Lewis in C16S.L9 (Table 1). Among the three genes in block 2, only Rap140/Fam208a bears a protein-changing mutation and thus is supported as the candidate for C16QTL.

In block 1, Hesx1 is a developmentally-expressed gene restricted to embryos (15). The segment carrying Asb14, Appl1, Hesx1, and Il17rd is highly conserved between DSS and Lewis. Not only are there no single nucleotide variations in their coding domains and intron–exon junctions (Table 1), but also in their 5’UTRs and 3’UTRs and their introns (Supplement 6). Beyond the fact that very few single nucleotide polymorphisms (SNPs) appeared in non-coding regions from the total genome sequencings, a C-T SNP in 5’UTR of Asb14 (the first in Supplement 6)

**Figure 2.** Detection of three base deletions in Rap140/Fam208a. (a) The chromatograms of the Rap140/Fam208a domain containing glycine repeats in Dahl salt-sensitive (DSS) and Lewis rats. (b) A length polymorphism spanning the glycine repeats. Primers used for PCR on genomic DNAs to detect the deletion are forward 5’-atggcgactgctgcggagacg-3 and reverse 5’-gctccgcggtggagaagacgc-3. (c) Organ expressions of Rap140/Fam208a assayed by reverse transcriptase polymerase chain reaction (RT-PCR). Numbers to the left indicate the size of the fragment in base pairs. Primers are forward 5’-tggctcagcagctatgag-3 and reverse 5’-acaactgcatatgcaac-3 for Rap140/Fam208a; and 5’-ACTGCGATAGAGTATTCTTC-3 and reverse 5’-CGCTCGTACCAATGAGA-3 for β-actin.
was commonly detected in the RGD (13) as well as ours, but upon re-sequencing of a PCR-amplified segment containing it, the SNP does not exist. So far, not even one single SNP in a non-coding region of these four genes has been confirmed to occur (Supplement 6) and consequently, those detected from the total genome sequencing are false positives. Several more examples of false positives came from microsatellite polymorphisms (Supplement 6). A total of 66 microsatellites present in block 1 are identical between DSS and Lewis (data not provided). Thus, the four genes located in block 1 can be downgraded as probable candidate genes for C16QTL for a lack of genome differences between DSS and Lewis.

Fourth, since a MAP difference of 19 mmHg appeared between C16S.L8 and C16S.L9 (Figure 1), the region non-overlapping between the two congenic strains might contain another additive QTL. However, all the genes present in the segment from Zmiz1 to Dnah12 did not contain any genomic variations in the coding regions and intron-exon junctions comparing DSS with Lewis rats, except for Zmiz1 which contains two silent coding mutations that are far from splice sites (data not provided). Once again, our sequencings combined the total genomes of DSS and Lewis and gene-specific genome fragments. Moreover, no copy number variations were detected for these genes from our total genome sequencing (data not provided) and from the RGD (13). Thus, there is no supportive evidence for protein-altering variations and intron-exon junction mutations in the genes present between Zmiz1 and Dnah12. The BP difference between C16S.L8 and C16S.L9 congenic strains mostly likely reflects their phenotypic variations, rather than due to the existence of an additional QTL residing in the Zmiz1 to Dnah12 interval.

Finally, the single QTL, C16QTL, exists and Rap140/Fam208a is strongly supported to be it. The ubiquitous pattern of gene expressions across organs shown by the sensitive method of RT-PCR (Figure 2c) indicates that Rap140/Fam208a is a functional gene and is consistent with the work of other investigators (11). The pattern of 3-bp deletion as a length polymorphism among rat strains is shown in Supplement 7 and does not reflect BP phenotypes of the various strains, because different normotensive, and by inference hypertensive, strains have diverse genetic determinants on BP (14).

**Rap140/Fam208a may regulate expressions of genes downstream in a pathway**

It is known that a single glycine repeat variation in the androgen receptor affects the level of its protein product without altering the protein function itself (16). Since the Rap140/Fam208a protein is suspected as a transcription factor resembling E2F (17), it appeared to be reasonable to hypothesize that the single glycine repeat variation in it might cause a change in the quantity of the Rap140/Fam208a protein. As a transcription factor, more or less of it could up or downregulate expressions of other genes, some of which might be eventually involved in the BP maintenance.

As a first proof of principle in testing this hypothesis, we chose kidneys as the primary organ for expression studies because they play a prominent role in the long-term BP homeostasis (18), not skins and testes where Rap140/Fam208a was abundant (11). We first selected a number of genes differentially expressed in the kidneys comparing DSS and Lewis rats as culled from a preliminary gene profiling result (data not provided). Most of these genes are implicated in BP regulation (19–22), they are all located outside the C16QTL-residing interval, and their expression patterns across organs are presented in Figure 3.

We reasoned that any of these genes, if regulated by Rap140/Fam208a, could be differentially expressed in the kidneys of DSS and C16S.L9 (Figure 1). Because the genome outside the C16QTL-containing region is the same in C16S.L9 as in DSS, differential expressions of any gene not included in C16S.L9 have to be elicited by one of the genes lodging in it. Since the 3-bp deletion in Rap140/Fam208a is a prominent genetic difference between DSS and C16S.L9, Rap140/Fam208a, a transcription factor, may act as a primary trigger for differential expressions of genes elsewhere in the genome. Neither Arhgef3 nor Ccdc66 in the same genome block 1 as Rap140/Fam208a is known as a transcription factor, despite the fact that they might compensatorily affect expressions of other genes.

Indeed, among those evaluated by qPCR, the gene encoding the solute carrier family 7 (cationic amino acid transporter, γ+ system), member 12 (Slc7a12) on rat Chr 2 is differentially expressed (p < 0.05, Table 2). Its expression was nearly five times less in the C16S.L9 kidneys than that of DSS. In contrast, the other genes tested are not differentially expressed. Thus, a modulation of transporter gene

![Figure 3](image-url)  
**Figure 3.** Organ expression pattern of genes assayed by reverse transcriptase polymerase chain reaction (RT-PCR). The organs are from Dahl salt-sensitive (DSS) on the left panel and Lewis rats on the right. Numbers to the left indicate the size of the fragment in base pairs. Primers for β-actin are the same as provide in Fig. 2 legend. Two primers for all the genes are located in two different exons to avoid amplifying genomic DNAs contaminated in RNA preparations, since no products were seen when genomic DNAs were amplified (data not provided). The results given are from one rat of each strain and they have been replicated with multiple rats of the same strain (data not provided). All rats were males, 11 weeks of age, and fed a high salt diet for 6 weeks starting from 5 weeks of age.
expressions by Rap140/Fam208a appears probably and is target-specific. No evidence of differential expressions in renal gene profiling (data not provided) was found for any of the other genes well-known to be involved in BP regulation such as those for the renin–angiotensin–aldosterone system, nitric oxide system, and the endothelin system.

We next addressed the issue of whether or not the differential Slc7a12 expression being due to the varying expression of any of the three genes, Arhgef3, Rap140/Fam208a, and Ccde66, since they all show nucleotide variations within putative promoter regions (Supplement 8). qPCR showed that their levels of renal expressions were comparable in C16S.L9 and DSS (Supplement 9). Therefore, the genetic difference of the 3-base deletion in Rap140/Fam208a, not in its expression or expressions of other genes in genome block 1 in the C16QTL-residing interval, seems to be necessary and adequate to account for the differential expressions of Slc7a12.

### Discussion

Major revelations from the current work are (a) C16QTL is restricted to a chromosome segment about 1 megabase (Mb) that bears seven possible genes divisible into two genome blocks. Block 1 contains four genes that are nearly identical in DSS and Lewis rats, whereas block 2 contains three genes that are divergent between DSS and Lewis strains. (b) The Rap140/Fam208a gene in block 2 emerged to be the sole, although not exclusive, candidate gene for C16QTL with a protein-changing mutation. Thus, C16QTL seems to be represented by a new gene previously not known for BP modulation. (c) A novel pathway mediated by Rap140/Fam208a is tentatively implicated that might modulate a renal-specific expression of Slc7a12 potentially relevant for BP.

### Fine congenic resolution of C16QTL

The use of congenic strains is powerful in physically and not statistically delimiting the fragment lodging C16QTL predicated on a cause–effect relationship between a chromosome segment and its phenotypic influence. The congenic approach broadly follows a 'knock-in' strategy where a segment (e.g. of a Mb in C16S.L9) of the hypertensive DSS has been replaced by that of the normotensive Lewis rat, while maintaining the remaining genome as that of DSS. Consequently, BP has been altered due to the replaced segment (Figure 1). Of the three polymorphic genes, Rap140/Fam208a is the only gene that is different between C16S.L9 and DSS parental strains in harboring protein-altering mutations, yet, this difference seems to be adequate to change blood pressure. In so doing, the primary causal gene such as Rap140/Fam208a to hypertension has been provisionally identified and separated from secondary genes such as Slc7a12 in response to it, since Slc7a12 on Chr 2 is identical between C16S.L9 and DSS.

Rap140/Fam208a is the only candidate gene supported as C16QTL

Our genetic analysis identified Rap140/Fam208a to be a credible gene responsible for C16QTL, paving the way for future in vivo function studies such as single-gene congenics, transgenesis or targeted-gene modifications. No other genes in the C16QTL-containing interval can be bolstered, although they cannot be excluded, as C16QTL, since they are either the same between DSS and Lewis rats, or contain silent mutations (see caveats in the discussion).

The function of the Rap140/Fam208a protein is little understood. Homozygous Rap140/Fam208a mice with a nonsense or a splice donor site mutation die before gastrulation (11), indicating that development is required. The Rap140/Fam208a protein was detectable by antibodies in the skin and testis extracts, but not in other organs such as kidneys, and it is believed to be a nucleoprotein in testes (11). In that work, no RT-PCR was done as we have shown (Figure 3), which is more sensitive than western blotting. Thus, the degree of the gene expression in other organs such as kidneys may be too low to be detected at the protein level.

Rap140/Fam208a (23) belongs to a large class of proteins associated with the retinoblastoma (RB) tumor suppressor (24). Its cellular function is believed to mimic that of a transcription factor similar to E2F (17). E2F constitutes a diverse array of factors that can be transcription activators or suppressors depending on their target genes (25). Similar to the RB gene that is ubiquitously expressed but prominently

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**Table 2. Evaluation of gene expressions triggered by Rap140/Fam208a via quantitative reverse transcriptase polymerase chain reactions (qPCR) on kidney cDNAs.**

| Genes     | Chr | Mean R C16S.L9/DSS | Ratio of mean R C16S.L9/mean R DSS | p     | Primers |
|-----------|-----|--------------------|-----------------------------------|-------|---------|
| Slc7a12   | 2   | 6.49 × 10^{-3} ± 6.83 × 10^{-3} / 32.3 × 10^{-4} ± 5.47 × 10^{-4} | 0.201 | 0.05 | Fwd: 5'-cctctagctatgacagaggag-3' |
| Comtd1    | 15  | 1.05 × 10^{-1} ± 1.7 × 10^{-1} / 1.76 × 10^{-2} ± 1.34 × 10^{-2} | 0.597 | 0.53 | Rev: 5'-cgagcttttatcctgc-3' |
| Csk       | 8   | 3.82 × 10^{-2} ± 2.44 × 10^{-2} / 4.46 × 10^{-2} ± 1.26 × 10^{-2} | 0.856 | 0.77 | Fwd: 5'-acttctgctctccacgca-3' |
| Sod3      | 14  | 1.32 × 10^{-1} ± 4.55 × 10^{-2} / 2.39 × 10^{-2} ± 1.42 × 10^{-2} | 0.552 | 0.42 | Rev: 5'-gctctggatctgggtactttca-3' |

Chr, Chromosome location; R represents the ratio of the expression of the gene target relative to that of the Gapdh reference. R is the average value of triplicates from one rat. Mean R is the average value of R from three rats of the same strain. DSS, Dahl salt-sensitive rats. C16S.L9 is a congenic strain defined in Figure 1. ± refers to SEM. P, t-test. Fwd and Rev refer to the forward and reverse PCR primers for each gene, which are located in two separate exons and were designed to span at least one intron to eliminate the amplification of genomic DNAs contaminated in RNA preparations. The RNA samples were also treated with DNase before qPCRs. Catechol-O-methyltransferase domain containing 1 (Comtd1); c-src tyrosine kinase (Csk); solute carrier family 7 (cationic amino acid transporter, y+ system), member 12 (Slc7a12); superoxide dismutase 3, extracellular (Sod3). The kidneys of the two comparing strains were from male rats of 11 weeks of age fed on a 2% NaCl diet starting from 5 weeks of age.
causes retinoblastoma (24), Rap140/Fam208a is omnipresent (Figure 2), yet may cause hypertension when mutated as our current data suggest.

How RAP140/Fam208a modulates expressions of genes downstream has been poorly defined, and so far, received little attention. There is fresh evidence, however, that Rap140/Fam208a is involved in epigenetic transcriptional silencing of heterochromatin in human cells (26). From our current work, it is observed that it preferentially regulates expressions of a renal transporter gene Slc7a12. Based on the human cellular work (26), a human silencing hub contributed by Rap140/Fam208a is worth examining in the kidney cells, where Rap140/Fam208a is produced in potentially modulating Slc7a12 expressions.

A glycine repeat variant in the androgen receptor was associated with human baldness in one population (27), but not in another (28). Regardless of its role in a human disorder, based on a glycine variation-function paradigm in the androgen receptor gene (16), the length of glycine repeats is in inverse correlation with the quantity of the protein product, namely, the shorter the repeat, the more the protein is produced. Glycine repeats form a hairpin structure in the gene transcript that can interfere with the translation efficiency into the protein product. The longer the repeats, the stronger the formation of the hairpin and more powerful the impediment to the protein translation efficiency (16). Based on this analysis, Lewis Rap140/Fam208a with a shorter glycine repeat is expected to produce more Rap140/Fam208a proteins than that of DSS rats.

**Slc7a12 is a potential transcription target for Rap140/Fam208a**

Slc7a12 (29) also known as Asc-2 encodes a neutral amino acid transporter that prefers alanine, serine, and cysteine (30). Since no physiological studies have been done (29), probable mechanisms causing hypertension by SLC7A12/ASC-2 are unknown and can only be inferred from kidney functions and their BP control. Slc7a12 is specifically expressed in kidneys (Figure 3), and particularly in apical and basal plasma membranes of collecting ducts (29). SLC7A12/ASC-2 might participate in the synthesis of organic osmolytes from neutral amino acids, and/or in renal osmotic and fluid imbalances through a facilitated diffusion (31) and consequently in the BP homeostasis (18). A shorter Rap140/Fam208a transcript (Figure 2) supposedly results in producing a larger amount of the Rap140/Fam208a protein, which is associated with a lower Slc7a12 expression and lower BP in C16S.L9 (Table 2). Rap140/Fam208a seems to act as a transcription suppressor of Slc7a12. As a consequence, a slower diffusion rate/fewer osmolytes with a fewer Slc7a12 might explain lower BP.

In contrast to Slc7a12, none of the other genes tested were differentially expressed when comparing the DSS with C16S.L9 (Table 2), indicating that their differential expressions when comparing DSS and Lewis kidneys are either determined by mutations in their own regulatory regions or by genes other than C16QTL in the genome.

**C16QTL acts ‘indirectly’ on blood pressure**

Rap140/Fam208a as C16QTL resembles a transcription factor, most likely involved in regulating expressions of other genes, and consequently its identification molecularly validates the concept that a QTL can ‘indirectly’ influence blood pressure via modulating other genes downstream such as Slc7a12 in a pathway leading to BP regulation, rather than encoding an immediate and ‘direct’ BP physiology agent itself. This concept was logically inferred from the modularized actions of QTLs in influencing BP (7). Since multiple BP QTLs together affect BP via an epistatic hierarchy (9), these QTLs standing higher in the hierarchy regulate other QTLs in the lower hierarchy and therefore are not expected to influence BP directly. The avenue of Rap140/Fam208a/C16QTL regulating other QTLs, or regulated by another QTL, in the same epistatic module (9) appears to be likely and warrants attention.

**Significance and caveats**

First, the transcription regulation of Slc7a12 by RAP140/Fam208a implicates a putative novel renal mechanism potentially connecting to blood pressure. Our present work suggested, for the first time, an adult physiological function for Rap140/Fam208a beyond its involvement in development (11). Because no function was previously attributed to Slc7a12 and its expression is highly kidney-specific, the current investigation implicates a premier functional role in any organism for Slc7a12. Particularly, a novel pathway of Rap140/Fam208a–Slc7a12-BP tentatively emerged in kidneys. Nevertheless, since Rap140/Fam208a is ubiquitously expressed (Figure 2c), mechanisms other than the renal origin are probable that may have an impact on BP. It is worthwhile in future studies to incorporate detailed longitudinal and in situ renal expression analyses to further understand the involvements of Rap140/Fam208a and Slc7a12 in pre and post the onset of hypertension, even on a low salt diet when hypertension proceeds slowly.

Second, the Rap140/Fam208a–Slc7a12-BP pathway is the first to be implicated, but by no means, the only likely route to the BP control genetically triggered by differing R140/Fam208a alleles. Genes in addition to Slc7a12 downstream of it may be differentially modulated in spatial (e.g. in another organ), temporal, and environmental-specific fashions and could also be connected to BP.

Third, Rap140/Fam208a was merely predicted in the rat before our current work. Since the Rap140/Fam208a protein is not detectable in kidneys by antibodies (11), but its gene expression was found by a highly sensitive RT-PCR (Figure 2), the impact of the glycine deletion (Figure 2) on its protein production in kidneys cannot be directly assessed, and was indirectly evaluated by the downregulation of the renal Slc17a12 expressions (Table 2). At present, no Slc17a12-specific antibodies are available anywhere. Thus, it remains to be determined whether or not the differential Slc17a12 expressions can result in a differential production of the Slc17a12 protein.
Finally, although the structural 3-bp deletion supports Rap140/Fam208a as the candidate gene for C16QTL in contrast to the remaining two genes in the same genome-variant block, Arhgef3 and Ccdc66, other genetic mechanisms in them cannot be excluded such as a regulatory mutation that could have a function consequence. Despite this caution, neither Arhgef3 nor Ccdc66 has a known function connection to BP. Thus, C16QTL is represented by a brand-new gene involved in the BP regulation.

Perspective
A systematic and comprehensive genetic analysis identified C16QTL that can independently influence BP from other QTLs. Our work has provided the first evidence that Rap140/Fam208a is a novel and sole candidate gene for C16QTL, and may become a plausible target for the genetic research of essential hypertension. One of the pathways originating from it toward the BP homeostasis may be mediated by the kidney-specifically expressed Slc17a12, whose function is suggested for the first time in any organism from the present work.

Declaration of interest
The authors have no conflict of interest.

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References
1. Kearney PM, Whelton M, Reynolds K, et al. Global burden of hypertension: analysis of worldwide data. Lancet 2005;365:217–23.
2. Cowley AW, Jr. The genetic dissection of essential hypertension. Nat Rev Genet 2006;7:829–40.
3. Deng AY. Genomic basis of polygenic hypertension. Hum Mol Genet 2007;16:R195–202.
4. Kato N, Takeuchi F, Tabara Y, et al. Meta-analysis of genome-wide association studies identifies common variants associated with blood pressure variation in east Asians. Nat Genet 2011;43:531–8.
5. International BP Consortium. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature 2011;478:103–9.
6. Lim J, Shin YA, Hong KW, et al. Characterization of functional variants in 33 blood pressure loci using 1000 genomes project data. Genes Genom 2013;35:387–93.
7. Deng AY. Genetic mechanisms of polygenic hypertension: fundamental insights from experimental models. J Hypertens 2015;33:669–80.
8. Deng AY. Positional Cloning of Quantitative Trait Loci for Blood Pressure: How Close Are We?: A Critical Perspective. Hypertension 2007;49:740–7.
9. Chauvet C, Crespo K, Menard A, et al. Modularization and epistatic hierarchy determine homeostatic actions of multiple blood pressure quantitative trait loci. Hum Mol Genet 2013;22:4451–9.
10. Duong C, Charron S, Deng Y, et al. Individual QTLs controlling quantitative variation in blood pressure inherited in a Mendelian mode. Heredity 2007;98:165–71.
11. Harten SK, Bruxner TJ, Bharti V, et al. The first mouse mutants of D14Abbl (Fam208a) show that it is critical for early development. Mamm Genome 2014;25:293–303.
12. Crespo K, Menard A, Deng AY. Hypertension Suppression, Not a Cumulative Thrust of Quantitative Trait Loci, Predisposes Blood Pressure Homeostasis to Normotension. Circ Cardiovasc Genet 2015;8:610–7.
13. Atamur S, Diaz A, Maratou K, et al. Genome Sequencing Reveals Loci under Artificial Selection that Underlie Disease Phenotypes in the Laboratory Rat. Cell 2013;154:691–703.
14. Crespo K, Chauvet C, Blain M, et al. Normotension in Lewis and Dahl salt-resistant rats is governed by different genes. J Hypertens 2011;29:460–5.
15. Dattani MT, Martinez-Barbera JP, Thomas PQ, et al. Mutations in the homeobox gene HESX1/Hexx1 associated with septo-optic dysplasia in human and mouse. Nat Genet 1998;19:125–33.
16. Ding D, Xu L, Menon M, et al. Effect of GGC (glycine) repeat length polymorphism in the human androgen receptor on androgen action. Prostate 2005;62:133–9.
17. Shan B, Zhu X, Chen PL, et al. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol Cell Biol 1992;12:5620–31.
18. Guyton AC, Coleman TG, Cowley AW, Jr., et al. Brief Reviews: a systems analysis approach to understanding long-range arterial blood pressure control and hypertension. Circ Res 1974;35:159–76.
19. Berk BC, Corson MA. Angiotensin II Signal Transduction in Vascular Smooth Muscle: Role of Tyrosine Kinases. Circ Res 1997;80:607–16.
20. Goss EI, Boiselle JP, Habermeier A, Rotmann A. Structure and function of cationic amino acid transporters (CATs). J Membr Biol 2006;213:67–77.
21. Gongora MC, Qin Z, Laude K, et al. Role of extracellular superoxide dismutase in hypertension. Hypertension 2006;48:473–81.
22. Ooshima K, Ozaki S, Tabuchi M, et al. Decreased expression of catechol-O-methyltransferase in the renal cortex of malignant spontaneously hypertensive rats. Tohoku J Exp Med 2009;219:331–6.
23. Li Q, Wen H, Ao S. Identification and cloning of the cDNA of a Rb-associated protein RAP140a. Sci China C Life Sci 2000;43:637–47.
24. Murphee AL, Benedict WF. Retinoblastoma: clues to human oncogenesis. Science 1984;223:1028–33.
25. Du W, Pogoriler J. Retinoblastoma family genes. Oncogene 2006;25:5190–200.
26. Tchashovnikarova IA, Timms RT, Matheson NJ, et al. Gene Silencing. Epigenetic silencing by the HUSH complex mediates position-effect variegation in human cells. Science 2015;348:1481–5.
27. Hillmer AM, Hanneken S, Ritzmann S, et al. Genetic variation in the human androgen receptor gene is the major determinant of common early-onset androgenetic alopecia. Am J Hum Genet 2005;77:140–8.
28. Ellis J, Scurrah K, Cobb J, et al. Baldness and the androgen receptor: the AR polyglycine repeat polymorphism does not confer susceptibility to androgenetic alopecia. Lancet 1997;349:1028–33.
29. Verrey F, Closs E, Wagner C, et al. CATs and HATs: the SLC7 family of amino acid transporters presumed to be associated with an unknown heavy chain. J Biol Chem 2001;276:49390–9.
30. Verrey F, Closs E, Wagner C, et al. CATs and HATs: the SLC7 family of amino acid transporters. Pfluegers Arch Eur J Physiol 2004;447:532–42.
31. Broer S. Amino Acid Transport Across Mammalian Intestinal and Renal Epithelia. Physiol Rev 2008;88:249–86.