The GTPase Activating Rap/RanGAP Domain-Like 1 Gene Is Associated with Chicken Reproductive Traits

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Abstract

Background: Abundant evidence indicates that chicken reproduction is strictly regulated by the hypothalamic-pituitary-gonad (HPG) axis, and the genes included in the HPG axis have been studied extensively. However, the question remains as to whether any other genes outside of the HPG system are involved in regulating chicken reproduction. The present study was aimed to identify, on a genome-wide level, novel genes associated with chicken reproductive traits.

Methodology/Principal Finding: Suppressive subtractive hybridization (SSH), genome-wide association study (GWAS), and gene-centric GWAS were used to identify novel genes underlying chicken reproduction. Single marker-trait association analysis with a large population and allelic frequency spectrum analysis were used to confirm the effects of candidate genes. Using two full-sib Ningdu Sanhuang (NDH) chickens, GARNL1 was identified as a candidate gene involved in chicken broodiness by SSH analysis. Its expression levels in the hypothalamus and pituitary were significantly higher in brooding chickens than in non-brooding chickens. GWAS analysis with a NDH two tail sample showed that 2802 SNPs were significantly associated with egg number at 300 d of age (EN300). Among the 2802 SNPs, 2 SNPs composed a block overlapping the GARNL1 gene. The gene-centric GWAS analysis with another two tail sample of NDH showed that GARNL1 was strongly associated with EN300 and age at first egg (AFE). Single marker-trait association analysis in 1301 female NDH chickens confirmed that variation in this gene was related to EN300 and AFE. The allelic frequency spectrum of the SNP rs15700989 among 5 different populations supported the above associations. Western blotting, RT-PCR, and qPCR were used to analyze alternative splicing of the GARNL1 gene. RT-PCR detected 5 transcripts and revealed that the transcript, which has a 141 bp insertion, was expressed in a tissue-specific manner.

Conclusions/Significance: Our findings demonstrate that the GARNL1 gene contributes to chicken reproductive traits.

Introduction

Egg number at 300 d of age (EN300), age at the first egg (AFE), and brooding behavior are valuable indices of chicken reproductive ability. In female chickens, sexual maturity is usually expressed as AFE. The AFE trait has been under artificial selection to enhance egg production efficiency [1]. EN300 is another reproductive trait of economic importance, while incubation behavior also affects egg production, as it results in the cessation of egg laying [2]. Chicken reproduction is controlled by photoperiod [3]. Generally, the process of chicken egg production is strictly regulated by the hypothalamic-pituitary-gonad (HPG) axis [4]. Gonadotrophin releasing hormone (GnRH) and its receptor (GnRHR) start the cascade and neuropeptide Y (NPY) is known to inhibit GnRH secretion via its receptor (Y1R) and to control ovulation [5]. Under photo-stimulation, GnRH is synthesized, secreted by the hypothalamus and binds to its receptor, which stimulates the pituitary gland to secret gonadotrophins that evoke steroid synthesis in the gonad, regulating ovarian follicle growth and ovulation in hens [6,7]. The hypothalamic vasoactive intestinal peptide (VIP) - pituitary prolactin (PRL) neuroendocrine pathway also controls reproductive cycles via dopaminergic neurotransmission in avian HPG system [8–10]. PRL is a key hormone that is absolutely necessary for egg laying and incubation behavior in poultry [11,12]. After stimulation by VIP, PRL inhibits the release of gonadotropins and thereby induces and maintains chicken incubation behavior [13–15].

The genetic mechanism behind incubation behavior has been widely studied because of its potential effect on egg production. This mechanism is a polygenic trait that is controlled by a set of autosomal genes [16]. Genes in the HPG axis showed high association with reproductive traits such as broodiness and egg production [9,17–30], however, this association depends on the
population used [22,30]. Aside from the genes distributed in HPG axis, other novel genes have been discovered to affect chicken reproduction traits [31–33].

Several approaches have been applied to identify the novel genes involved in chicken reproduction. A genome-wide scan is a powerful approach to understanding this complex trait. Quantitative trait loci (QTLs) for egg number, egg production rate, AFE and broodiness were identified through genome-wide scans [34–42]. Genome-wide association studies based on high density SNPs can be performed to detect QTLs that could not be detected by previous studies based on microsatellite genotyping [43–47]. A genome-wide association study attempts to obtain information on all variants, but a gene-centric SNP approach would be efficient enough to capture SNPs associated with particular traits [48,49].

Transcriptome profiling can be also used to identify new genes associated with chicken reproductive traits. Although many studies on the genetic effects of candidate chicken reproduction genes have been reported, few studies have reported transcriptomic and proteomic changes. In previous studies, transcripts related to high egg production were identified by suppressive subtractive hybridization analysis (SSH), and several of the identified transcripts were further confirmed to be significantly increased in hens with higher egg production, though they were not part of the HPG axis [50–52]. Therefore, it is valuable to identify novel genes related to chicken reproduction.

The aim of the present study is to identify novel genes involved in chicken reproductive traits using SSH analysis, an Illumina 60K chicken Beadchip GWAS, and a gene-centric GWAS, with confirmation via analysis of single marker-trait, allelic frequency spectra, and alternative splicing.

Results

**GARNL1** identified as a candidate gene underlying chicken broodiness by suppression subtractive hybridization

A subtraction library was made by subtracting cDNA from the pituitary at the egg-laying stage. As shown in Figure 1, construction of the pituitary-subtracted cDNA libraries was successful. Genes differentially expressed between brooding and non-brooding chicken pituitary glands were enriched for and sequenced, and 57 annotation transcripts and 20 unknown transcripts were characterized (Table S1). Gene ontology (GO) analysis was performed to investigate the functions of the putatively differentially expressed transcripts. Biological process accounted for the major portion of GO annotations, compared with cellular component and molecular function. Among the category of biological process, genes were involved in processes such as eye photoreceptor cell development, ovarian follicle development, epinephrine biosynthesis, regulation of small GTPase mediated signal transduction, G-protein coupled receptor protein signaling pathways, and so forth (Table S2). On the basis of biological process annotations, 10 transcripts were selected to be validated by qPCR. Among the 10 transcripts, one was identified as belonging to the chicken **GARNL1** gene (Figure S1). The **GARNL1** gene was differentially expressed between tissues (Figure 2). Low levels of mRNA expression were detected in the ovary, ovicord, liver, spleen, lung, kidney, muscular stomach, sebum, abdomen fat, and duodenum; however, higher expression levels were observed in the cerebrum, cerebellum, hypothalamus, pituitary, heart, and glandular stomach. Gene expression levels in the cerebrum, cerebellum, hypothalamus, pituitary, ovary, oviduct and spleen were significantly higher in broody chickens than in non-broody chickens (P<0.05), with the levels in tissues from broody chickens 1.6 times to 4.3 times higher than those of non-broody chickens. In contrast, **GARNL1** expression in leg muscle was 2-fold higher in non-broody chickens.

**GWAS indicates that SNPs associated with chicken reproductive traits are located in the **GARNL1** gene**

Before GWAS analysis was carried out, stratification analysis was conducted in the two-tail sample. The IBS was not significantly different between two tails sample (P_{perm}<0.05). In all, 2802 SNPs were associated with EN300 in the NDH two tail sample at the 5% genome-wide level (validated by 10000 permutation tests), and of this total, 470 SNPs were at significant at the 1% level (Table S3). On chicken chromosome 5, 118 SNPs were associated with EN300 (Table S3). Among the 118 EN300-associated SNPs, rs14533299 and GgalG282818 composed a haplotype block. The linkage distance in this block is 1691 kb, and the **GARNL1** gene was observed to be located within this block (Figure S2).

**Gene-centric GWAS reveals an association of several SNPs in the **GARNL1** gene with chicken EN300 and AFE**

Six SNPs were highly significantly associated with both EN300 and AFE (P_{perm}<0.05 and P_{perm}<0.01) (Table 1). A SNP cluster located on chromosome 5 was associated with both AFE and EN300 in another NDH two tail sample. Among the SNP cluster, 5 SNPs were located in the **GARNL1** gene.

The association of **GARNL1** SNPs with chicken EN300 and AFE was further analyzed in a NDH population comprising 1301 individuals. As showed in Table 2, corrected by SLIDE, rs15700989 was significantly associated with EN300 (P<0.01) and rs15701085 was associated with AFE (P<0.05). The block composed of rs15432778 and rs15432779 was also significantly associated with EN300 (P = 0.0088) (Table 3). In this block, there are 4 haplotypes, including H1 (TG, 70.1%), H2 (TC, 5.5%), H3 (CG, 23.3%), and H4 (GC, 1.1%). H2H2 and H2H4 had higher EN300 than the other diploymphs.

**Allelic frequency spectrum of the chicken **GARNL1** gene**

Allelic frequencies of rs15700989 were different among the 5 populations. The frequency of rs15700989 was 1.0 in Leghorn layers (Table 4), with a highly significant difference between Leghorn layer and the other 4 native Chinese chickens. The chi-square test values for the genotype distribution of rs15700989 showed significant difference between Leghorn layer and the other 4 Chinese native chickens (P<0.01) (Table 5), in accordance with their egg-production performance.
The chicken GARNL1 gene is predicted to be located on chromosome 5 and to span positions 38,617,769–38,729,036 on the reverse strand, with a total gene size of 111,268 bp. Four, six, and seven isoforms from the pituitary, ovary, and oviduct, respectively, could be detected by Western blotting. The molecular weights of these isoforms ranged from 150 KDa to 250 KDa (Figure 3).

Five alternatively spliced transcripts, GARNL1-w (NCBI accession number: JF330255), GARNL1-v1 (NCBI accession number: JF330256), GARNL1-v2 (NCBI accession number: JF330257), GARNL1-v3 (NCBI accession number: JF330258, and GARNL1-v4 (NCBI accession number: JF330259) were detected in the cDNA pool prepared from cerebrum, cerebellum, hypothalamus, pituitary, ovary, and oviduct tissues. Five transcripts were generated as a result of exon skipping and intron inclusion (Table 6 and Figure S3). The wild-type transcript, GARNL1-w, which is composed of 41 exons and 40 introns, was successfully cloned. The complete coding sequence of GARNL1-w is 6,108 bp long and encodes 2,035 amino acids. Chicken GARNL1 shares a high amino acid sequence identity with those of human (89.3% with AY596971, 89.4% with AY596970), mouse (87.4% with AY596972, 87.6% with AY596972), and zebrafish (73.2% with AB476643, 74.3% with AB476644), and it is predicted to be a nuclear protein (with 63% probability). Similar to the human GARNL1 gene and the mouse GARNL1 gene, all 5 transcripts contain a Rap/Ran-GAP domain (AA 1825–AA 2004), two transmembrane helices (AA 1203–AA 1225, AA 1385–AA 1407), and a leucine zipper motif (AA 1068–AA 1089), but have lost the N-terminal coiled coil domain (shown in figure S4).

The variant GARNL1-v2 (deduced to encode a 2134 AA peptide) skips exon 40 and includes a 141 bp intron sequence between the exon 16 and exon 17. RT-PCR showed that the 141 bp intron inclusion was tissue specific, being observed only in the cerebrum, cerebellum, hypothalamus, heart, pectoral muscle, and leg muscle. Its mRNA expression level was higher than the other isoforms without 141 bp intron inclusion (Figure 4). Similarly, the GARNL1-v4 transcript contained a 201 bp fragment of intron 19, and a single amino acid change, N (Asn) to D (Asp), occurs at the new exon-exon junction. GARNL1-v4 mRNA with the 201 bp intron fragment was present at very low levels (data not shown). The mRNA expression levels of transcripts with the 141 bp intron inclusion sequence (Figure 5) in the cerebrum, cerebellum, and hypothalamus were almost the same between brooding and non-brooding chickens. However, its expression levels in heart and pectoral muscles of the brooding chickens were 1.5 and 2 times greater than those of the non-brooding chickens, respectively. In leg muscle, the expression was 8-fold higher in the non-brooding chickens than in brooding chickens.

### Discussion

In this study, data from a SSH analyses, a GWAS, and a gene-centric GWAS indicate that the GARNL1 gene is involved in reproduction and that some GARNL1 variants are associated with chicken reproductive traits.

The SSH analysis indicated that the GARNL1 gene was involved in chicken brooding behavior. Comparing to the digital gene expression methods, such as RNA-seq, SSH is not a prevailing experimental method for detecting differentially expressed genes.
SSH have several limitations, relatively low throughput, highly false positives, and generally not statistical significance. But the following qPCR validation would help to get some good results with the following experiment validation [53–55]. This result was consistent with previous findings. Chicken *GARNL1* was identified as being potentially related to high egg production in Taiwan Country chickens [50–52]. Therefore, the expression levels of the *GARNL1* gene among the brain tissues, the cerebellum was found to have the highest expression with total egg number at 500 d of age or egg rate after the first egg were recently detected in a region within 95 cM of GGA5 [42], where the *GARNL1* gene is located. In the present study, the *GARNL1* gene is specifically associated with total egg number at 500 d of age or egg rate after the first egg [52]. The cerebellum was found to have the highest expression level of human *GARNL1* gene among the brain tissues, corresponding to its influence on 14q13-linked neurological phenotypes [56]. In zebrafish, *GARNL1* was a strong candidate gene for brain developmental delay [57]. In our study, the expression of chicken *GARNL1* gene varied at different stages. We found *GARNL1* to be predominantly expressed in the brain, and the levels of the *GARNL1* gene were consistently higher in the hypothalamus, pituitary, ovary, and oviduct of broody hens. The expression level of transcripts that included the 141 bp intron sequence suggested that the cerebellum may be an important action region in chickens and the variants of *GARNL1* do not impair their function on chicken reproductive traits. In conclusion, the expression levels of the *GARNL1* gene could reflect its functions in chicken reproduction.

Two tail samples were used to detect SNPs associated with broodiness and EN300 in this study. The first QTLs for broodiness were recently detected in a region within 95 cM of GGA5 [42], where the *GARNL1* gene is located. In the present study, the haplotype block between rs14533299 and GgaluGA282818 was also shown to be related to EN300 (data not showed). The *GARNL1* gene is located in this region. Among all 25 protein-coding genes located on this region, the *GARNL1* gene was identified as being potentially related to high egg production in Taiwan Country chickens [50], and higher *GARNL1* expression levels have been observed in high egg producing strains [51]. Furthermore, the mRNA level of the *GARNL1* gene was specifically associated with total egg number at 500 d of age or egg rate after the first egg [52]. The cerebellum was found to have the highest expression level of human *GARNL1* gene among the brain tissues, corresponding to its influence on 14q13-linked neurological phenotypes [56]. In zebrafish, *GARNL1* was a strong candidate gene for brain developmental delay [57]. In our study, the expression of chicken *GARNL1* gene varied at different stages. We found *GARNL1* to be predominantly expressed in the brain, and the levels of the *GARNL1* gene were consistently higher in the hypothalamus, pituitary, ovary, and oviduct of broody hens. The expression level of transcripts that included the 141 bp intron sequence suggested that the cerebellum may be an important action region in chickens and the variants of *GARNL1* do not impair their function on chicken reproductive traits. In conclusion, the expression levels of the *GARNL1* gene could reflect its functions in chicken reproduction.

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### Table 2. Association of 17 SNPs with chicken reproductive traits in population.

| SNP          | Information                                           | EN300 trait               | AFE trait               |
|--------------|-------------------------------------------------------|---------------------------|-------------------------|
|              | Position¹ | Location² | Allele | Pointwise-P | Corrected-P | Pointwise-P | Corrected-P | Pointwise-P | Corrected-P |
| rs15700949   | 3’ flanking | 38617982 | A/G    | 0.5737      | 1.0000      | 0.2336      | 0.9854      |
| rs16492011   | intron 39 | 38621623 | G/C    | 0.3796      | 0.9994      | 0.1031      | 0.8171      |
| rs14532750   | intron 39  | 38624284 | T/C    | 0.0191      | 0.2667      | 0.4148      | 0.9998      |
| rs15700989   | intron 37  | 38648067 | A/G    | 0.0001      | 0.0023      | 0.9498      | 0.7912      |
| rs16492027   | intron 36  | 38654577 | T/C    | 0.3907      | 0.9996      | 0.05072     | 0.5644      |
| rs16492031   | intron 32  | 38660349 | A/G    | 0.6481      | 1.0000      | 0.2189      | 0.9791      |
| rs15701085   | exon 26    | 38674045 | G/A    | 0.0621      | 0.6365      | 0.002894    | 0.0496      |
| rs13585983   | exon 20    | 38681662 | A/G    | 0.5698      | 1.0000      | 0.1158      | 0.8505      |
| rs16492034   | intron 20  | 38680830 | T/C    | 0.1092      | 0.8347      | 0.03888     | 0.4716      |
| rs14532779   | intron 15  | 38697653 | T/C    | 0.0876      | 0.7614      | 0.1711      | 0.9461      |
| rs14532787   | exon 15    | 38699909 | T/C    | 0.0316      | 0.4002      | 0.02979     | 0.3819      |
| rs16492056   | intron 8   | 38708121 | T/C    | 0.0190      | 0.2656      | 0.1233      | 0.8695      |
| rs15701119   | intron 7   | 38711935 | TTAAA   | 0.4808      | 0.9999      | 0.3975      | 0.9996      |
| rs14532824   | 5’ flanking | 38730001 | T/C    | 0.4837      | 0.9999      | 0.7553      | 1           |
| rs14532808   | intron 1   | 38724344 | A/G    | 0.9870      | 1.0000      | 0.3928      | 0.9996      |
| rs14532819   | intron 1   | 38726759 | T/A    | 0.2698      | 0.9928      | 0.6031      | 1           |
| rs14532831   | 5’ flanking | 38731847 | T/C    | 0.1901      | 0.9626      | 0.03088     | 0.3935      |

¹The position of the site on chromosome 5 in coordinates from the chicken genome database at UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start).
²The location of the variants found inside the *GARNL1* gene.
³Pointwise P indicated the P value gained by PLINK and Corrected-P means the P value corrected by SLIDE, ”” indicate P<0.05, and P<0.01, respectively.

### Table 3. The association of haplotypes composed of rs14532787 and rs14532779 with EN300 traits.

| Trait       | P value   | H1H11 (647) | H1H21 (102) | H1H31 (408) | H2H21 (5) | H2H31 (53) | H2H41 (2) | H3H31 (76) | H3H41 (1) |
|-------------|-----------|-------------|-------------|-------------|-----------|------------|-----------|------------|-----------|
| EN300       | 0.0086**  | 92.79       | 93.07       | 94.06       | 132.31    | 96.84      | 131.73    | 98.49      | 106.06    |
|             | ± ±1.09   | 2.64        | 1.32        | 11.63       |           | 3.72       | 18.44     | 3.05       | 25.87     |

Values were expressed as least-square means ± standard errors (SE).
The number in brackets was the number of chickens tested for each diplotype.
**indicate P<0.01.
The a, b or A, B values with no common superscripts within a column for each site that differed significantly (P<0.05) or highly significantly (P<0.01).

1The number in brackets was the number of chickens tested for each diplotype.

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**Table 3. The association of haplotypes composed of rs14532787 and rs14532779 with EN300 traits.**
As the GARNL1 gene might be involved in chicken reproduction, its polymorphisms could be related to chicken reproductive traits. However, no studies on the association between the mutations of GARNL1 gene and chicken reproductive traits were carried out. In humans, the GARNL1 gene was an important candidate gene for human 14q13 deletion phenotypes, and two mutations in GARNL1 were identified in a family with idiopathic basal ganglia calcification [36]. Polymorphisms of the GARNL1 gene were associated with both EN300 and AFE in a two tail sample in our gene-centric association analysis. This result confirmed our previous SSH findings and was validated in a large population. An analysis of the allelic frequency spectra of GARNL1 SNPs further supported the association. The frequencies of the rs15700989 were associated with EN300 associated with divergent egg production performance, and the frequency of predominant alleles of rs15700989 was 1.0 in Leghorn layer and was descending in Leghorn, BEH, NDH, XH, and RJF. The predominant allele of rs15700989 was related to higher EN300 trait in NDH population. Thus, the allelic frequency data supports the conclusion that the chicken GARNL1 gene contributes to chicken reproduction. The block composed of rs14532787 and rs14532779 was significantly associated with EN300 traits. Although both of them were not showed significantly relationship with EN300 after corrected by SLIDE in single marker association, the CC genotype of rs14532707 resulted in a higher EN300 and an earlier AFE than did the other two genotypes (Table S4). However, the genotype CC can be only observed in the NDH population. Compared to the variance of total egg number at 40 week between early sexual mature and later sexual mature group in Leghorn layer [50], rs14532707 might undergo artificial selection in NDH population, aiming at the increase of egg production by early sexual mature and shortening the interval of oviposition. These two SNP may contribute to EN300 by interacting each other.

Further analysis of the organization, tissue expression, and alternative splicing of the chicken GARNL1 gene was conducted. Using Western blotting, 5 alternatively spliced transcripts of the GARNL1 gene were isolated from chickens in this study. Note that none of the alternative splicing isoforms had impaired protein domains. Chicken GARNL1 is conserved with mammals, but it has some unique features. A variant of the human GARNL1 lacking exon 40, has been found and corresponded to GARNL1-v1 in chicken [56]. Chicken GARNL1 has lost the N-terminal coiled coil domain and subsequently the ability to bind to other proteins. In mice, GARNL1 plays a crucial role during brain formation and maintenance. A partial murine GARNL1 product identified as GRIP (GAP-related interacting protein to E12) binds to the helix-loop-helix domain of transcription factor E12 and regulates E12-dependent target gene transcription [59]. Similar to the murine GRIP, the region responsible for binding to HLH domains was present in all isoforms of chicken GARNL1. The Rap/Ran-GAP domain is widely distributed in signaling proteins [60–62], and two arginine residues in Rap/Ran-GAP domain are important for the GAP activity of GRIP in mice [59]. Two arginine residues were found in the Rap/Ran-GAP domain of chicken GARNL1.

In conclusion, we reveal that the chicken GARNL1 gene has an important effect on chicken reproductive traits, as determined from the data from SSH analyses, GWAS, and gene-centric GWAS. This effect was validated by analysis of allele frequency spectra, and further characterization of several aspects of the gene and its expression.

Materials and Methods

Ethics Statement

The study was approved by the Animal Care Committee of South China Agricultural University (Guangzhou, People’s Republic of China) with approval number SCAU#0011. Animals involved in this study were humanely sacrificed as necessary to ameliorate their suffering.

Table 4. Allelic frequencies of rs15700989 in the GARNL1 gene in the 5 chicken populations.

| Site          | Allele | LH (n = 60) | BEH (n = 41) | NDH (n = 82) | XH (n = 50) | RJF (n = 33) |
|---------------|--------|------------|-------------|-------------|-------------|-------------|
| rs15700989    | G      | 1          | 0.352       | 0.31        | 0.09        | 0.22        |

LH = Leghorn layers, BEH = Baier Huang chickens, NDH = Ningdu Huang chicken, XH = Xinghua chicken, RJF = Red Jungle Fowl.

The number in brackets was the number of chickens used. Hardy-Weinberg equilibrium was set at the 0.01 level.

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Table 5. Chi-square test of genotype frequency for rs15700989 in the 5 populations.

| Site          | Populations | \( \chi^2 \) Value \^ | XH | LH | NDH |
|---------------|-------------|------------------------|----|----|-----|
| rs15700989    | BEH         | 0.14                   | 27.35\*\*          | 101.00\*\* | 5.27 |
|               | RJF         | 21.22\*\*              |                | 93.00\*\* | 3.29 |
|               | XH          | 119.00\*\*             |                | 20.64\*\* |     |
|               | LH          | 120.79\*\*             |                |     |     |

\(^1\chi^2\)_{0.05}(df = 1) = 3.841; \chi^2_{0.05}(df = 2) = 5.991; \chi^2_{0.05}(df = 2) = 9.21; \chi^2_{0.01}(df = 2) = 6.635; \chi^2_{0.01}(df = 2) = 9.21; \chi^2_{0.01}(df = 2) = 12.832; \chi^2_{0.01}(df = 2) = 16.275

*\(P<0.05\);
**\(P<0.01\).

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Identifying candidate genes underlying chicken broodiness by SSH analysis

A pair of full-sib female NDH chickens was used for the suppression subtractive hybridization (SSH) experiment. One individual was broody, and its brooding lasted for more than 7 d, and the other one was continuously laying. The pituitary gland and 17 other tissues were collected after the brooding individual had been incubating for 10 d. At that time, the chicken’s incubation behavior was quite typical, and both ovary and oviducts were atrophied. The laying chickens’ tissues were also collected at the same time.

Total RNA was extracted from the tissues using Trizol reagent (Invitrogen, California, USA) according to the manufacturer’s protocol. Total RNA was treated with RNase-free DNaseI (Takara, Osaka, Japan) for 45 min at 37°C to ensure that it was free of DNA contamination. RNA quantity and quality were assessed using a Thermo Scientific Nanodrop1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware, USA) and by formaldehyde denaturation agarose gel electrophoresis.

Suppression subtractive hybridization was performed with an equal amount of tester mRNA (2 μg) from the brooding stage, as well as the driver mRNA from the egg-laying stage. Following the manufacturer’s protocol for the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA), after two subtraction hybridizations and two suppression PCRs, the subtraction efficiency was evaluated by PCR using primers for the chicken house-keeping gene G3PDH (P#1, Table S5). cDNAs were cloned and inserted into the pMD20-T vector (Takara, Osaka, Japan) and were then transferred into chemically competent E. coli (JM109) cells to generate SSH libraries.

Subtractive products longer than 300 base pairs were picked for sequencing by Invitrogen Co. Ltd (Shanghai, China). The vector nucleotide sequences were removed, and the remaining sequences clustered into contigs using DNASTar software. The basic local alignment search tool (BLAST) http://blast.ncbi.nlm.nih.gov/ was used for identifying and annotating genes.

Quantitative real-time PCR (qPCR) was performed with the Agilent Stratagene Mx QPCR CR Instrumentation (Agilent Technologies, Wilmington, DE, USA) for follow-up of candidate genes, using the SsoFast EvaGreen Supermix (Bio Rad Laboratories, Hercules, CA, USA). Four individual cDNAs were used as templates for qPCR amplification. The primers used for the qPCR were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). A housekeeping gene, the chicken beta-actin gene (accession: L08165), was used as internal control. Therefore, two sets of primers (P#2 and P#3, Table S5) were designed and used for the qPCR amplification. Each reaction mixture contained 10 μL of Eva Green PCR Master Mix, 1 μL of each primer (10 μM), 7 μL of RNase-free water and 1 μL of cDNA in a final volume of 20 μL. Standard amplification conditions were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s. Fluorescent signal was collected after an extension at 65°C in each cycle. Chicken GARNL1 gene relative expression was calculated by $2^{-\Delta Ct}$ method, and $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{G3PDH}}$.

GWAS for a two tail sample using 60K chips

The age of the first egg (AFE) and the total number of eggs at 300 d of age (EN300) were recorded in a breeding population kept in Guangdong Wens’ Foodstuff Co. Ltd (Guangdong, China). Twenty Ninghu Sanhuang (NDH) female chickens from the above population were divided into 2 groups. Group 1 was composed of the 10 individuals with the highest EN300 values (an average of 145 eggs) and no observable incubation behavior, and group 2 was composed of the 10 individuals that had the lowest EN300 values (an average of 66 eggs) as well as an average duration of broodiness of 51 d. Twenty Illumina 60K chicken chips were used for the two-tailed association study.

Stratification analysis was performed to detect the IBS of the two-tail sample before GWAS studies were carried out. SNP quality control metrics were analyzed using GenomeStudio software (version 2009.1). A SNP was removed if its call rate was less than 100%, or its minor allele frequency (MAF) was less than 5%, and its Hardy-Weinberg equilibrium (HWE) p-value was too low (P<0.00001). As a result, 54,424 SNPs were selected for use in the GWAS. PLINK was used for the single-marker association analysis. PLINK single marker basic allelic association ($X_1^2$) tests (the –assoc option) were performed for each of the post-

| Table 6. The alternative splicing types of the chicken GARNL1 gene. |
|-----------------|------------------|-----------------|-----------------|------------------|------------------|
| Sequence ID     | Length 1         | Exon Number     | Intron Number   | Amino Acid 2     | Molecular Weight | Types of AS 3     |
| GARNL1-w        | 6108             | 40              | 40              | 2035             | 230 KD           | /                |
| GARNL1-v1       | 6261             | 40              | 39              | 2086             | 235 KD           | Exon Skipping (exon 40, 31 bp) |
| GARNL1-v2       | 6405             | 41              | 40              | 2135             | 240 KD           | Intron inclusion (fragment of intron 16, 141 bp) and Exon Skipping (exon 40, 31 bp) |
| GARNL1-v3       | 5995             | 40              | 39              | 1984             | 225 KD           | Exon Skipping (exon 21, 153 bp) |
| GARNL1-v4       | 6609             | 42              | 41              | 2203             | 247 KD           | Intron inclusion (fragment of intron 16, 141 bp and fragment of intron 19, 201 bp) |

1Length of the open reading frame (ORF).
2The number of the amino acid coded by the chicken GARNL1 gene.
3The type of alternative splicing observed.

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QC SNPs. PLINK’s max (T) permutation procedure (the –mperm option) was set to 10000 for the two-tailed test, in order to get accurate P values by reducing false positives. The SNPs detected by PLINK were used to analyze haplotype structure with Haploview 4.1 software [http://www.broad.mit.edu/mpg/haploview/].

Gene-centric GWAS

Ninety-six NDH chickens were selected on the basis of AFE and EN300 measurements and genotyped for 384 SNPs using the Illumina GoldenGate™ iSelect Array genotyping platform (BGI, Shenzhen, China) via a commercial service. The 384 SNPs were located in an AFE QTL region or in 20 novel candidate genes selected according to previous reports [18,22–26,35,36,38,51,52,63] (Table S6). The DNA sample set included 3 replication pairs. Twenty-four early sexual mature individuals with an AFE of 91 to 95 d and 24 late sexual mature individuals with an AFE of 160 to 179 d, and 24 low production individuals with an EN300 of 1 to 22 eggs and 24 high production individuals with an EN300 of 140 to 163 eggs were used in the gene-centric genome-wide association study. The gene-centric GWAS association analysis was performed by quantitative trait association analysis using PLINK with 10000 permutations.

Marker-trait association analysis in NDH female population

A total of 5 site-specific primers were designed by Assay design and synthesis by the Sangon Biotech Company (Shanghai, China), a commercial service. We validated the SNPs for which associations were found using the Sequenom genotyping platform. The effects of an additional 12 SNPs on EN300 or AFE were
investigated by an association study using the PCR-RFLP method in the NDH population (P#5 to P#16, Table S5). PCR was performed in a 10 μL reaction mixture containing 1 μL of Taq polymerase (Dongsheng Co., Guangzhou, China), 5 μL of the 2×PCR buffer supplied by the manufacturer, 1 μM of each primer, 50 ng genomic DNA, with ddH2O added to a total volume of 10 μL. The PCR program used was 3 min at 94°C, followed by 32 cycles of 30 s at 94°C, 30 s at 59°C, 45 s at 72°C, and a final extension of 5 min at 72°C in a Bio-Rad Mycycles (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were digested in a 37°C or 65°C water bath overnight with MspI, HindIII (2 sites), DraI (2 sites), SacII, KpnI, StuI, PvuII, TaqI, Csp6I, and BsuRI. Genotypes were determined by electrophoresis after restriction digestion.

PLINK (version 1.07) was used to perform analysis for evaluating genetic effect of 17 sites of GARNL1 gene on EN300 and AFE. Sliding-window method for Locally Inter-correlated markers with asymptotic Distribution Errors corrected (SLIDE) program [64] was used to correct the P value, and all the pointwise P value were corrected based on 10000 sampling. A P value ≤0.05 was considered significant in the analyses. Multiple comparisons were conducted with least squares means using Fisher’s least significant difference method.

The Hardy-Weinberg equilibrium and haplotype structure were analyzed using Haploview 4.1 software [65]. Haplotypes were constructed on the basis of genotype data using PHASE 2.0 software [http://en.wikipedia.org/wiki/Phase]. The minimum haplotype frequency was set to 1%. Association analyses of haplotypes with the EN300 and AFE were carried out using SAS GLM procedure (SAS Institute Inc., Cary, NC, USA) with the following model

\[ Y = \mu + H + S + e, \]

Where \( Y \) is a trait observation, \( \mu \) is the overall population mean, \( H \) is the effect of haplotype, \( S \) is the fixed effect of sire, and \( e \) is the residual error.

Allelic frequency spectrum of the chicken GARNL1 gene

Five populations, Red Jungle fowl (RJF) (n = 33), Baier Huang chicken (BEH) (n = 41), White Leghorn (LH) (n = 60), Xinghua chicken (XH) (n = 50), and Ningdu Sanhuang chicken (NDH) (n = 82), were genotyped at the reproduction-associated SNPs of the GARNL1 gene to obtain allele frequency spectra. RJF, XH and NDH showed low egg-production ability, with 60–130 eggs per year because of their intractable incubation behavior (100% incidence of broodiness in RJF, 70–80% incidence of broodiness in XH and 50–60% incidence of broodiness in NDH). BEH chickens had a 10–15% incidence of broodiness and an egg-production of 180 per year. The Leghorn chicken is a famous layer breed with excellent egg-production ability and no incubation behavior. The primers used were the same as those used for genotyping the NDH population.

Chi-square (\( \chi^2 \)) tests performed on a 2×3 (or n) contingency table. A P≤0.05 was considered significant in all analyses.

Analysis of GARNL1 alternative splicing isoforms

Total protein was collected using Trizol reagent (Invitrogen, California, USA) according to the manufacturer’s protocol. Proteins were separated on an 8% SDS-PAGE gel by electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked using TBST containing 5% nonfat milk and 0.05% (w/v) Tween 20 overnight at room temperature. Membranes were then incubated overnight at 4°C with rabbit polyclonal anti-GARNL1 (TULIP1) in a 1:1000 dilution (Santa Cruz Biotechnology, CA, USA). Membranes were washed three times in TBST containing 0.05% Tween 20 and incubated in HRP-conjugated secondary antibody for 1 h at 37°C. Membranes were washed as before and signals were detected using Super ECL Detection Reagent (Applygen, Beijing, China) and Kodak (Kodak Film, USA).

Using cDNA transcribed from RNA pool composed of 6 chicken tissues as a template, the GARNL1 ORF was amplified by primer pairs (P#4, Table S5) on the basis of the predicted mRNA sequence (accession number: XM421244). PCR was performed in a 50 μL reaction mixture containing 1 μL of KOD FX polymerase (Toyobo, Osaka, Japan), 25 μL of the 2×PCR buffer supplied by the manufacturer, and 10 μL of 2 mM dNTPs. The PCR conditions were 4 min at 94°C, followed by 30 cycles of 10 s at 98°C, 7 min at 68°C, and a final extension of 10 min at 68°C in a Bio-Rad S1000 (Bio-Rad Laboratories, Hercules, CA, USA). The amplified fragments were cloned into a pGEM-T Easy plasmid vector (Promega, Madison, USA) and then sequenced by Invitrogen Co. Ltd (Shanghai, China) via a commercial service. The obtained sequences were assembled to obtain the full-length G:IRNL1 cDNA sequence. After sequencing, some novel alterna-
tive splice forms of the chicken GARNL1 gene were detected by Reverse Transcription-PCR.

Sequence databases were accessed and searched using the BLAST algorithm at the NCBI at http://www.ncbi.nlm.nih.gov/BLAST and the UCSC Chicken Genome Project Working Draft at http://genome.ucsc.edu/.

Protein domain predictions were obtained using the SMART program (Simple Modular Architecture Research Tool) from the European Molecular Biology Laboratory at http://smart.embl-heidelberg.de/, and transmembrane helix motif and leucine zipper domain predictions were obtained using the SOSUI algorithm at http://hp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html and the PSORT II program at http://psort ims.u-tokyo.ac.jp/form2.html. Multiple alignments of amino acid sequences were performed using ClustalW at http://www.ebi.ac.uk/clustalw/ and DNAMAN software (Lynnon Corporation, Quebec, Canada), with MEGA 4.1 software (http://www.megasoftware.net/) used for homology analysis.

Supporting Information

Figure S1 qPCR results of ten putatively differentially expressed transcripts identified by SSH.

(TIF)

Figure S2 Genes distributed in the block composed of GqalaGA282818 and rs14539299.

(TIF)

Figure S3 Partial cDNA and deduced amino acid sequence of GARNL1-v1, GARNL1-v3, GARNL1-v4.

(TIF)

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