nature portfolio

Peer Review File

Mapping the regulatory genetic landscape of complex traits using a chicken advanced intercross line

Corresponding Author: Dr Yuzhe Wang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In their manuscript “Mapping the regulatory genetic landscape of complex traits using chicken advanced intercross line” Xiaoning et al. conducted a large-scale integrative analysis on chicken growth traits to explore the underling genetic regulation mechanism using an advanced intercross line. From GWAS, 682 QTLs were identified from 43 out of 75 traits. The authors connected these QTLs and significant SNPs to genes and tissues via further integrative analysis. Finally, cross species analysis was performed to explore the conservation and differentiation between humans and chicken.

The advanced intercross line population generated in this study is of interest and indeed an important resource contribution to the genetic community, but detailed information and data about this population is limitedly provided in this manuscript.

The methods used in several steps were not clearly described or provided which prevented a smoothly evaluation of the work.

The authors were eager to demonstrate the importance and advantage of this advanced intercross line in gene mapping, but without sufficient supportive evidence. Hence, overstate and misinterpretation is inevitable. This must be revised carefully.

I have the following specific comments that the authors what to consider to improve their manuscript.

Specific comments:
Line 112. ‘no significant change’ should not be used here without statistical testing.

Line 127-129. What is your definition of ‘single-cause pleiotropic QTL’? Please provide the theorical supports for the relationship between genetic correlation and pleiotropic QTL. The mean and standard error for all heritability and genetic correlation is suggested to provide in detail.

Line 135. Change ‘A Phenotypic’ to ‘The phenotypic’.

Line 138-139. The mapping resolution, denoted as ‘QTL lengths’, might be influenced by the sample size within each generation and other factors. From F2 to F16, the sample size increased from 640 to 2269, this may cause the increase of mapping resolution—decreased QTL length. Hence the authors are suggested to clarify this point, and avoid any potential overstate or misinterpretation.

Line 145-146. Here is misinterpretation. The 79.62% newly detected may be caused by several reasons,

Line 152-160. The authors annotated each QTL to gene(s) using Bedtools solely without any validation. And the stringency of parameters are not provide and can not be evaluated. Then the authors highlighted that they ‘achieved a gene-level mapping resolution’. This is not a solid conclusion, because 52 out of 624 is not a high proportion. To support this, other evidences or results from such as integrative analysis should be provided.

Line 168. Both reference 24 and 27 are not correct.

Line 172. Can explain more heritability than what?

Line 174. How did the authors perform this enrichment analysis? In Sup. Figure 6, why not show the enrichment fold first? Why compare p values between qui and other groups? Please provide enrichment fold first, then testing significance only for those scenarios enrichment fold>1 is suggested.

Line 178. Change 'This' to '. This'

Line 193. How about the consistency of results between colocalization and the annotation from Line 152-160?

Line 209. How do you know the true causal mutation in this study? Misinterpretation!

Line 598-606. The FDR and Bonferroni correction were used for F9, F16 and F2, respectively, due to the differential LD levels as claimed by the authors. Why not use different threshold levels instead of different parameters? A comparison is suggested to prove which one is strict.
For the definition of QTL, 500kb is used for all 3 populations. Is this proper in terms of differential LD? What is the effect of this rule on the results showed in Fig 3d?

Line 763. gene annotation genes?

Fig 1. This is not well designed. Especially for b, the information is not clear. The reviewer suggested to reorganize it.

Fig 2a. How about the distribution of other generations F2 F9?

The reference list is not correctly prepared. Duplications such as ref. 14 and ref. 24 should be revised.

Fig 4j. Title for y-axis is not proper. Change gene in tissue (Nor. TMM) to Nor. TMM (gene in tissue)

Fig 5. （a）- The model and software used to estimate QTL heritability and total heritability should be provided.
(c) – what is the relationship between those Mutations showed in this Fig 5c and rs736348095 in Fig 5b? All of them are within the region of the red lines in Fig5b? Why is there a NA mutation related to PHF11 via TWAS with effect size NA on BW8?

Reviewer #2

(Remarks to the Author)
Reviewer comments:
Zhu and colleagues have established a resource population that is uncommon among chickens, significantly enhancing the mapping resolution of GWAS through multiple generations of recombination. They have also integrated functional annotation data from the Functional Annotation of Animal Genomes (FAANG) project and extensive chicken GTEx data to create a genetic regulatory landscape for complex traits. The findings not only identify a substantial number of fine-mapping genes but also present several representative examples that illustrate complex genetic regulation patterns of quantitative traits. Furthermore, they attempted to extend their findings to humans for comparative analysis, thereby underscoring the importance and significance of their results. Overall, this study synthesizes a wealth of valuable resources, employs advanced research methodologies, and uncovers numerous new genetic principles governing complex traits. This is an important topic that is under extensive investigation. I enjoyed reading this manuscript and found this work intriguing and likely to attract the interest of many peers. I have some comments that need to be addressed before its publication.

1)Why construct the AIL population using the HB and HQLA populations, and what are the characteristics of the AIL population? Please emphasize this in the introduction section.
2)The AIL population underwent multiple generations of recombination from F0 to F16. In F16, what is the ancestral composition of HB and HQLA? Has any deviation occurred?
3)Figure 7 compared functional genes related to growth traits in chickens and humans, but the selection patterns in humans and chickens are different. Please further elaborate on the conservation of growth trait functional genes between chickens and other species such as pigs and cattle.
4)Which specific QTLs correspond to Trait, Domain, and Multi-Domain in Figure 3e? Please list them in detail.
5)Figure 4i-j mentioned that the functional gene on the GGA27 chromosome was not the closest to the lead SNP. Please discuss the regulatory characteristics of the avian micro-chromosome genome.
6)Line 34: "We have developed a 16-generational advanced intercross line of chickens to enhance informative recombination and achieved a gene-level mapping resolution of 52 loci across 25 traits." Could be revised to: "We have developed a 16-generation advanced intercross line of chickens to enhance informative recombination and have achieved single gene-level mapping resolution for 52 loci across 25 traits." Additionally, does "52 loci" refer to genes? The manuscript mentions 65 single gene loci in this context. Please verify and confirm.
7)In the Introduction section, the significance of studying growth traits in chickens should be further elaborated, along with the role these traits play in the growth and development of domesticated animals and humans.
8)The description of phenotypic measurements in the methods section is too detailed and could be placed in the supplementary notes.
9)What is the sample size for each tissue in the chicken GTEx eQTL data used in the study for integration with GWAS? Please provide additional details.

10)For Figure 1a, please use the full name for “BW7”.
11)The legend for Figure 4i and the description of the “lead SNP” in the figure are inconsistent. Please verify and correct.
12)Line 140: The full name of “GGA” should be provided when it first appears.
13)Line 143: “AnimalQTL” should be changed to “Animal QTL”.
14)Line 280: The full name of “PheWAS” should be provided when it first appears.
15)Line 676 and line 796: Change “commercial Broiler LineA” to “commercial Broiler Line A” and “commercial Broiler LineB” to “commercial Broiler Line B”.
16)Line 762: Change “Distribution of QTL gene annotation genes” to “Distribution of genes annotated in QTL regions”.
17)Line 790: What does “top QTL” mean? Please provide an explanation.
18)Line 820: The spelling of “uniq” is incorrect and should be corrected to “unique”.
19)Please provide header information for each supplementary table.

Reviewer #3

(Remarks to the Author)
In this manuscript, Zhu et al. report the use of a 16-generation intercross line of chickens to investigate the genotypes underlying many phenotypes, most of them related to growth. Given the dearth of available genotype-phenotype data in chickens, the authors should be commended for the large amount of time invested in generating this dataset which will surely be useful to many future researchers. Their analyses of the data are thorough and their conclusions are sound. However, there are numerous problems with the way some of the results are reported, both in the figures and in the text. The figures are inconsistent in their use of error bars, axes are not always sufficiently defined, fonts are often too small, and many captions are not detailed enough to understand what is being displayed. In the text, numbers are not always reported well enough, with many measurements given without enough explicit information about what they represent; further, some required statistical details listed in the reporting summary are missing, and exact p-values are often left out in favor of undefined stars in the figures. Finally, although the writing is for the most part clear, there are places where it is confusing, and the manuscript overall would greatly benefit from editing by a native English speaker. I have listed many but not all examples of these issues below. Together, these flaws detract from an otherwise excellent study; therefore, if the manuscript and especially the figures are improved to address these issues, I would be happy to recommend a revised version of this manuscript for publication.

Specific comments, by line number:
75: I don't understand what "characterized by a recombination degree" means, please explain better
93-98: it is implied based on reading the full paragraph that you did low-coverage sequencing and then used this to do imputation, and the 8M SNPs were a result of imputation, but because imputation is mentioned last this is confusing
96, 98, others: be more explicit about these numbers: are they mean and standard deviation, or something else?
98: define genotype consistency
127-129: confusing sentence. If I understand what it's trying to say correctly, a better way to say this might be "Many of the phenotypic measurements were strongly correlated with each other, suggesting that they may be influenced by pleiotropic genes."
134: define "significant signals"
135: awkward construction, I suggest instead "The heritability of a phenotype and the number of QTLs found for this phenotype are significantly correlated"
136: I assume this is Pearson's R but be explicit
152: be more specific than "annotated to genes": does this mean that they overlap with coding sequence, or contain the full genes, or parts of the genes, or what?
156: how many previously reported major QTLs?
159: be more specific than "validated in mice". Were they validated to contain variants that affect the same trait, or some looser criteria?
170-172: define all these abbreviations
173: this says they are "significantly" enriched but I do not see a p-value, significance stars, or any other indication that this is based on a statistical test in the figure panel
179: no need to list numbers that are shown in the figure like this
181: can you explain a little bit why this phenotype matters?
183: what is SMR analysis?
194: what are eGenes?
193-196: I do not understand these three categories, please explain more clearly
213: define GD and TC
243: what is eviscerated weight?
505-506: "custom script" but code is not provided
534: software version and citation or link for STITCH?

Figure 1a: define BW7, add error bars
Figure 1b: I find this flowchart convoluted and hard to follow, and therefore unhelpful
Figure 2b: I do not understand what the numbers in the distributions are. Is each column the distribution of pi across the windows of a single bird from this generation, or from all the windows of all the birds together, or something else? Same thing for panels c and f.
Figure 2f: split these into two panels; it is difficult to read with error bars from the two measurements overlapping. Also, I do not understand what "number of haplotypes" or "frequency of major haplotypes" means
Figure 3a: x-axis labels are misaligned

Figure 3c: legend font is too small, but it looks like the colors are the same as in panel (b), so maybe just skip the legend in this panel
Figure 3d: define what the p-value cutoffs for stars are, give more details about the test used (one-tailed or two-tailed? paired or independent? degrees of freedom? test statistics?) and show exact p-values somewhere in text or caption as well
Figure 3g/h: fonts way too small
Figure 4a: not sure what any of these abbreviations mean
Figure 4e: I don't understand what the color scale on this is
Supplementary Figure 3: which of these correlations are significant?
Supp Fig 6: why was no multiple hypothesis correction performed given that there were 14 hypotheses? Also, what are the measurements in the distributions of p values and how was it determined whether a whole row was significant or not? Are these t tests between distributions of p values, and if so, is there any precedent for doing a t test between distributions of p-values like this? Very confused about this figure

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all my concerns in this revision. There is no further comments.

Reviewer #2

(Remarks to the Author)
The authors have done an excellent job addressing my comments as well as those of the other reviewers. I have no further questions and am pleased to recommend this outstanding work for publication.

Reviewer #3

(Remarks to the Author)
The authors have improved the manuscript and addressed all my comments to my satisfaction, so I am happy to recommend the manuscript for publication.

My only additional comment: I should have been more explicit in these cases, but when I asked a question in my review such as "what are eGenes" or "what is SMR analysis", I was asking for these terms to be defined in the manuscript the first time they are used, not explained to me in the responses. It still appears that many of these terms are not defined in the main text of the manuscript. I apologize for the confusion but still hope that the authors go through the manuscript to ensure that all acronyms and non-standard terms like these are defined to make the text as clear as possible.

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Dear editor and reviewers,

We sincerely appreciate your insightful feedback on our manuscript entitled "**Mapping the regulatory genetic landscape of complex traits using a chicken advanced intercross line**" (NCOMMS-25-05509).

We have carefully revised the manuscript based on all your valuable and constructive comments and suggestions, which have significantly improved the manuscript. We have highlighted the revised sections of the manuscript in red and provided detailed, point-by-point responses to each of your comments below.

We hope that our revisions have satisfactorily addressed all the comments. Thank you very much for your time and consideration.

Sincerely,

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Email: yuzhe891@cau.edu.cn

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In their manuscript "Mapping the regulatory genetic landscape of complex traits using chicken advanced intercross line" Xiaoning et al. conducted a large-scale integrative analysis on chicken growth traits to explore the underling genetic regulation mechanism using an advanced intercross line. From GWAS, 682 QTLs were identified from 43 out of 75 traits. The authors connected these QTLs and significant SNPs to genes and tissues via further integrative analysis. Finally, cross species analysis was performed to explore the conservation and differentiation between humans and chicken.

The advanced intercross line population generated in this study is of interest and indeed an important resource contribution to the genetic community, but detailed information and data about this population is limitedly provided in this manuscript.

Authors' response: Thank you for your valuable comments. We appreciate your interest in the advanced intercross line population. In response to your suggestion, we have incorporated more detailed information about this population in both the Introduction and Supplementary Notes. We hope that these additions provide greater clarity and enhance the overall comprehensibility of our manuscript.

Detailed information regarding this population has been added in Supplementary Notes lines 12-28: "*The Huiyang Bearded chicken (HB) is a traditional Chinese meat-type breed with a history spanning 1,600 years. It is known for its slow growth rate, exceptional meat quality, and distinctive muff and beard phenotype. Currently, conservation and breeding efforts are underway to preserve this unique genetic resource. The High-Quality Chicken Line A (HQLA) is a selectively bred population derived from a cross between the commercial Anak broiler and a Chinese indigenous chicken line. Over ten generations of intensive artificial selection, HQLA has been improved using a weight-based selection index while preserving desirable traits such as meat quality and plumage color.*

At seven weeks of age, HQLA exhibited a body weight approximately 3.2 times that of HB. Differences in growth rate and ancestral origin facilitate better study of growth phenotypes in the progeny. The F_2 population was established through reciprocal

crosses between the two parental lines, with matings of HQLA males with HB females (4♂×12♀) and HB males with HQLA females (4♂×12♀). Subsequent advanced intercross lines (AILs) from F_3 to F_{16} were derived from the F_2 generation and maintained through random mating. Each generation consisted of 655–2381 individuals (Supplementary Table 25).”

We summarized the HQLA-HB AIL information in the Introduction lines 88-92 “*The initial F_2 population was established through reciprocal crosses between Huiyang Bearded chicken (HB) and the High-Quality Chicken Line A (HQLA), which have significant phenotypic differences in growth traits (Figure 1a). Subsequent advanced intercross lines (AIL), spanning from F_3 to F_{16} were derived through random mating (detailed in Supplementary Notes).”*

In addition, we have included descriptions of the advantages of advanced intercross lines in the Introduction lines 80-85: *“AIL populations, the concept of which was first proposed in 1995, can increase recombination and improve the accuracy of QTL localization. In recent years, AIL populations have been widely established and applied in several species, and the QTLs of AIL populations have been mapped to narrower chromosomal regions and more QTLs have been identified, which helps identify functional genes and causal mutations”*.

The methods used in several steps were not clearly described or provided which prevented a smoothly evaluation of the work.

Authors’ response: We appreciate your insightful comment. For the specific methods mentioned below, such as in Q4 and Q13, we have explained the steps in the Methods section; overall, we have rechecked the Methods section for any unclear descriptions and made the necessary additions.

The authors were eager to demonstrate the importance and advantage of this advanced intercross line in gene mapping, but without sufficient supportive evidence. Hence, overstate and misinterpretation is inevitable. This must be revised carefully.

Authors’ response: Thank you for pointing this out. We recognize that our descriptions

may cause some exaggerations and misunderstandings, some of which may be because they were not explained clearly before (e.g., Q6, which we added explanations) or were overstated (e.g., Q1-Q2, Q5). We have revised the descriptions and added some analyses to support our conclusions (e.g., Q4 and Q13). Furthermore, we checked the description of AIL populations in the full text and revised the parts that might involve overstatements.

I have the following specific comments that the authors what to consider to improve their manuscript.

Specific comments:

Q1: Line 112. 'no significant change' should not be used here without statistical testing.

Authors' response: Thank you very much for your suggestion. In order to be more accurate, we have changed the section to "*Additionally, the minor allele frequency spectrum exhibited a similar distribution pattern across generations.*" (lines 124-125 in the revised manuscript).

Q2: Line 127-129. What is your definition of 'single-cause pleiotropic QTL'? Please provide the theorical supports for the relationship between genetic correlation and pleiotropic QTL. The mean and standard error for all heritability and genetic correlation is suggested to provide in detail.

Authors' response: Thank you very much for your question. The concept of "single-cause pleiotropic QTL" refers to a single QTL that affects multiple phenotypes simultaneously. Our hypothesis is based on the strong genetic positive correlation observed across multiple phenotypes, suggesting the possible existence of pleiotropic QTLs that influence multiple traits. The results in Figure 3e support this conjecture. However, positive genetic correlation alone is not sufficient to directly infer the presence of single-cause pleiotropic QTLs, as described by van Rheenen. Genetic correlation can also arise due to horizontal pleiotropy, vertical pleiotropy, or pseudo-pleiotropy caused by linkage disequilibrium (LD). Therefore, further verification of the existence of a true single-cause multiple-effect QTL is needed using methods such as

GWAS and co-localization analysis. To provide a more accurate description, also taking into account reviewer 3's suggestion. We have revised "Most phenotypes exhibited varying degrees of positive correlations, suggesting the presence of a single-cause pleiotropic QTL." to "*Many of the phenotypic measurements were strongly correlated with each other, suggesting that they may be influenced by pleiotropic genes*." (lines 140-143 in the revised manuscript). Additionally, based on your suggestion, we have added the heritability of all phenotypes and the genetic correlations between them in Supplementary Tables 5 and 6.

Reference:

van Rheenen, W., Peyrot, W. J., Schork, A. J., Lee, S. H., & Wray, N. R. Genetic correlations of polygenic disease traits: from theory to practice. *Nat. Rev. Genet.* **20**, 567–581 (2019).

Q3: Line 135. Change 'A Phenotypic' to 'The phenotypic'.

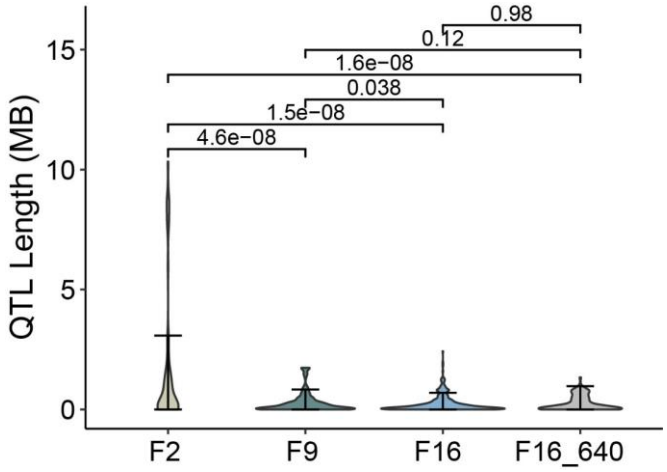
Authors' response: Thank you for pointing this out. We have revised in response to suggestions from you and reviewer 3's Q7 (lines 149-150 in the revised manuscript).

Q4: Line 138-139. The mapping resolution, denoted as 'QTL lengths', might be influenced by the sample size within each generation and other factors. From F2 to F16, the sample size increased from 640 to 2269, this may cause the increase of mapping resolution—decreased QTL length. Hence the authors are suggested to clarify this point, and avoid any potential overstate or misinterpretation.

Authors' response: Thank you for your question. Your suggestion is quite valuable. Considering the effect of sample size, we randomly selected 640 samples from the F_{16} generation for GWAS and QTL mapping and further compared the length of QTLs across different generations. We found that the length of QTLs of F16_640 was significantly greater than that of the F_2 generation but not significantly different from that of the F9 generation. Additionally, when comparing the F16_640 group to the entire F16 sample set, we observed no significant difference in QTL length, indicating that increasing the sample size does not affect QTL length. However, a larger sample size

resulted in the detection of more QTLs, suggesting that the primary advantage of increasing sample size lies in enhancing the detection of small-effect QTLs rather than altering QTL length. This finding indicates that recombination continues to occur as generations progress (New Supplementary Figure 4c).

The revised section can be found in lines 155-162: "*Considering the impact of sample size, we standardized the sample size of F_{16} to match that of F_2. A comparison of the F16_640 group with all F_{16} samples revealed no significant difference in QTL length, indicating that increasing the sample size does not influence QTL length. However, a larger sample size facilitated the detection of more QTLs, suggesting that the primary advantage of increasing sample size lies in enhancing the detection of small-effect QTLs rather than altering QTL length. This implies that recombination continues to occur across generations (Supplementary Figure 4c).*"



New Supplementary Figure 4c QTL lengths in different generations. Comparison of QTL lengths in different generations and 640 samples randomly sampled from F_{16}.

Q5: Line 145-146. Here is misinterpretation. The 79.62% newly detected may be caused by several reasons.

Authors' response: Thank you for your comment. We agree that although the continuous recombination of AILs makes them more capable of mining into independent, small-effect QTLs, we cannot attribute all the new findings to the advantages brought about by the population due to differences in statistical methods and significance thresholds, etc., across studies. We therefore removed this sentence

from the Result section.

We have changed "These newly identified QTLs highlight the advantages of fine mapping in our population, and are critical for studying growth traits in the AIL population" to "*These newly discovered QTLs allow us to more comprehensively study growth traits using AILs*." (lines 167-168 in the revised manuscript).

Q6: Line 152-160. The authors annotated each QTL to gene(s) using Bedtools solely without any validation. And the stringency of parameters are not provide and can not be evaluated. Then the authors highlighted that they 'achieved a gene-level mapping resolution'. This is not a solid conclusion, because 52 out of 624 is not a high proportion. To support this, other evidences or results from such as integrative analysis should be provided.

Authors' response: Thank you for your valuable comments. We used "bedtools intersect" to identify genes overlapping with QTL regions, considering a gene to be associated with a QTL if its genomic coordinates intersected with the QTL region. Regarding the statement on gene-level mapping, we acknowledge that our previous wording may not have been precise. Our intention was not to claim that only 52 out of 624 QTLs were mapped at the single-gene level. Rather, we aimed to highlight that, due to recombination in the F_{16} generation, we were able to identify 154 QTLs at the gene level, 52 of which have been validated in mice. This demonstrates the ability of the AIL population to refine genetic mapping to the gene level. Additionally, while these genes have been validated in mice, they were not detected in integrative analyses such as co-localization with eQTL, possibly due to relatively weak GWAS signals. To improve clarity, we have revised the phrase "achieved a gene-level mapping resolution of 52 loci across 25 traits" to "*achieved 154 single-gene QTLs*" (lines 33-35 in the revised manuscript).

Q7: Line 168. Both reference 24 and 27 are not correct.

Authors' response: We sincerely apologize for this issue. We have corrected the references and rechecked all references in the manuscript.

Q8: Line 172. Can explain more heritability than what?

Authors' response: Thank you for your question. In Figure 4a, the results are sorted from top to bottom in order of heritability, and we found that promoters (TxFlnkHet, TxFlnkWk, TssAHet, TssA, TssBiv, and TxFlnk), enhancers (EnhAHet, EnhAWk, EnhAMe and EnhA), exonQTL, lncQTL, and eQTL can explain more heritability than Qui regions without any function. We have changed "can explain more heritability" to "*could explain more heritability than Qui regions without any function*" (lines 193-194 in the revised manuscript).

For better understanding, we explained the methods of heritability calculation in more detail, and to reduce the influence of LD on the calculation of heritability to a certain extent, we utilized the GCTA bivariate GRM model for the calculation of heritability for each functionally annotated region and molQTL:

$$\mathbf{y}=\mathbf{X\beta}+\mathbf{g1}+\mathbf{g2}+\mathbf{e}$$

where **y** is the phenotype, **Xβ** is the fixed effect (sex, batch); **g1** is the genetic effect of functional annotation regions or molQTL; **g2** is the genetic effect of all SNPs upstream and downstream of the functional region beyond 100 kb; **e** is a vector of residuals.

For the heritability obtained by "gcta64 --reml-bivar --mgrm multi_grm.txt --pheno test.phen --out test", considering the different number of SNPs in each interval, we corrected for the number of SNPs and performed a log transformation. In addition, the specific analysis methods covered in this section and the specific functions corresponding to each abbreviation have been detailed in the Methods section of the revised manuscript.

The detailed description can be found in the Methods lines 601-610: "*We utilized the GCTA bivariate GRM model for the calculation of heritability for each functionally annotated region and molQTL:*

$$\mathbf{y}=\mathbf{X\beta}+\mathbf{g1}+\mathbf{g2}+\mathbf{e}$$

*where **y** is the phenotype, **Xβ** is the fixed effect (sex, batch); **g1** is the genetic effect of functional annotation regions or molQTL; **g2** is the genetic effect of all SNPs upstream and downstream of the functional region beyond 100 kb; **e** is a vector of residuals.*

For the heritability obtained by "gcta64 --reml-bivar --mgrm multi_grm.txt --pheno test.phen --out test", considering the different number of SNPs in each interval, we corrected for the number of SNPs and performed a log transformation."

The following description was added in the legend lines 962-969: *"TssA: Strongly active promoters/transcripts; TssAHet: Flanking active TSS without ATAC; TxFlank: Transcribed at gene; TxFlnkWk: Weak transcribed at gene; TxFlnkHet: Transcribed region without ATAC; EnhA: Strong active enhancer; EnhAMe: Medium enhancer with ATAC; EnhAWk: Weak active enhancer; EnhAHet: Active enhancer no ATAC (hetero); EnhPois: Poised enhancer; ATAC_Is: ATAC island; TssBiv: Bivalent/poised TSS; Repr: Repressed polycomb; ReprWk: Weak repressed polycomb; Qui: Quiescent."*

Q9: Line 174. How did the authors perform this enrichment analysis? In Sup. Figure 6, why not show the enrichment fold first? Why compare p values between qui and other groups? Please provide enrichment fold first, then testing significance only for those scenarios enrichment fold>1 is suggested.

Authors' response: Thank you for your insightful questions. In our analysis, we assessed the enrichment significance of GWAS signals in each functional region by computing the sum of squares of all SNP effect sizes and conducting a permutation-based summation test. This approach, according to Fang et al., evaluates the overall signal distribution without considering SNP significance thresholds, making it unsuitable for directly calculating enrichment fold but effective for determining whether enrichment occurs statistically. Regarding the choice of reference group, we initially used Qui regions, as these are unannotated genomic regions.

We have refined our approach in subsequent analyses to improve interpretability. To better illustrate the enrichment fold, we have now used all SNPs as the background and applied a hypergeometric test to calculate enrichment p-values. The enrichment factor was computed as:

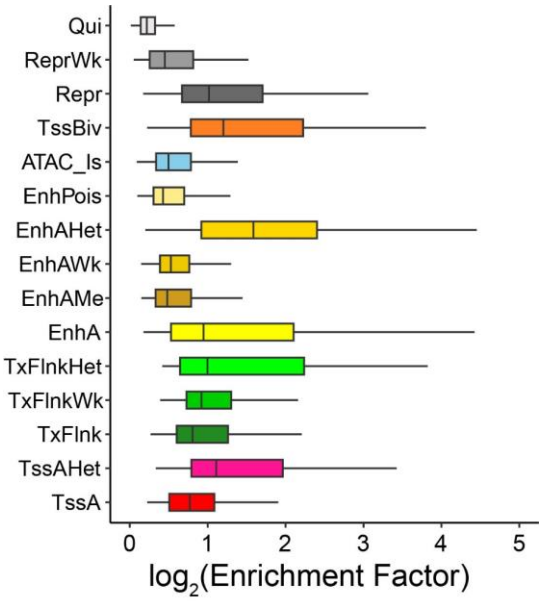
Enrichment factor = (target region significant SNPs/total significant SNPs) / (target region all SNPs/ gnome-wide total SNPs)

Additionally, we performed enrichment analysis across 40 phenotypes in 15

functionally annotated regions across 23 tissues. The updated results are presented in New Supplementary Figure 7, where we applied an FDR < 0.05 threshold for visualization. As shown in the figure, GWAS signals are highly enriched in promoter regions (TssA, TssBiv) and enhancer regions (EnhA, EnhAHet) (New Supplementary Figure 7).

Reference:

Fang, L. et al. Comprehensive analyses of 723 transcriptomes enhance genetic and biological interpretations for complex traits in cattle. *Genome Res.* **30**, 790–801 (2020).



New Supplementary Figure 7 Genome-wide association study (GWAS) signal enrichment was analyzed within chromatin states across 23 tissues and 40 complex traits in chickens. The figure illustrates the results at FDR<0.05.

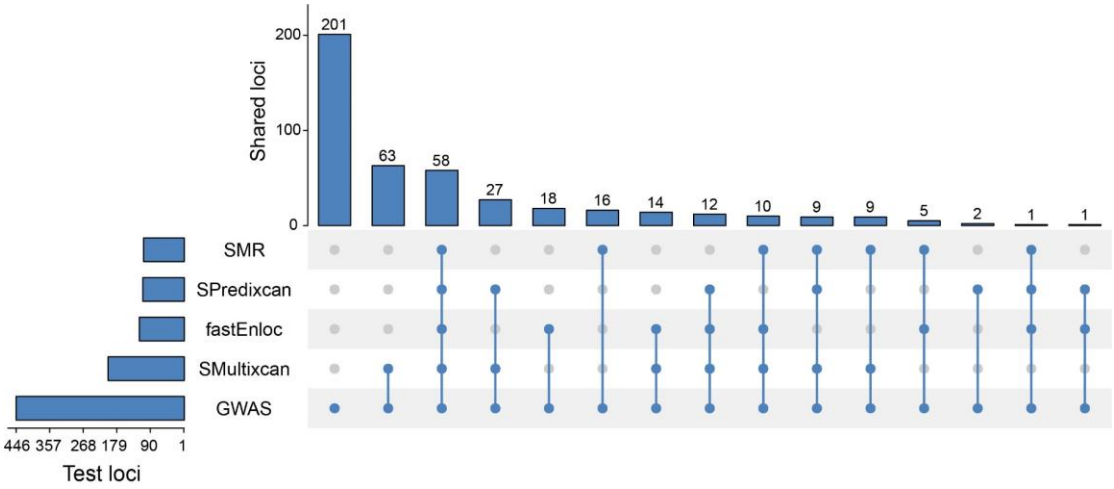
Q10: Line 178. Change 'This' to '. This'

Authors' response: Thank you for pointing this out. Taking into account the suggestions made by you and Reviewer 3, we revised "This resulted in the identification of 166, 105, 94, and 245 functional genes, respectively, yielding a total of 431 unique genes" to "*We obtained a total of 431 unique functional genes using four methods*" (lines 204-205 in the revised manuscript).

Q11: Line 193. How about the consistency of results between colocalization and the annotation from Line 152-160?

Authors' response: Thank you very much for your question. Among the 611 QTLs identified for 40 growth-related phenotypes, 446 QTLs contained significant SNPs that influenced the expression of at least one gene. We conducted a co-localization analysis of these 446 QTLs and found that 245 QTLs could be interpreted using at least one of the applied methods. This suggests that eQTLs play a valuable role in elucidating the genetic basis of complex phenotypes (New Supplementary Figure 8).

The following description was added in the Results lines 200-204: *"Of the 611 QTL identified for 40 growth phenotypes, 446 contained significant SNPs that affected the expression of at least one gene; we summarized the results of co-localization of these 446 QTLs and found that 245 QTLs could be interpreted by at least one of the methods (Supplementary Figure 8)"*



New Supplementary Fig 8 Interpretation of GWAS loci. The number of GWAS loci linked to eQTLs via four integrative methods, namely fastENLOC, SMR, SPredixcan, and SMultixcan.

Q12: Line 209. How do you know the true causal mutation in this study? Misinterpretation!

Authors' response: Your comments are highly appreciated. What we originally intended to convey is that eQTLs are more likely to help identify functional SNPs that play a real biological role. For instance, the *IGF2BP1* gene has been reported in chicken,

duck, and goat as a functional gene influencing growth traits such as body size and body weight. However, in our GWAS analysis, the most significant locus identified was the *GIP* gene. After incorporating eQTL data, SMR analysis revealed that *IGF2BP1* influences the SL6 phenotype in muscle tissue. To maintain objectivity, we have revised our wording from "true causal mutation" to "*possible functional genes and mutations*" (lines 236-237). A new description was added in the Results lines 232-233: "*The IGF2BP1 gene has been reported in chicken, duck, and goat as a functional gene influencing growth traits such as body size and body weight.*"

References:

Wang, K. et al. The chicken pan-genome reveals gene content variation and a promoter region deletion in IGF2BP1 affecting body size. *Mol. Biol. Evol.* **38**, 5066–5081 (2021).

Zhou, Z. et al. An intercross population study reveals genes associated with body size and plumage color in ducks. *Nat. Commun.* **9**, 2648 (2018).

Chen, S. et al. Identification of SNPs in the second intron of IGF2BP1 and their Association with growth traits in Nanjiang Yellow goat. *Anim. Biotechnol.* **36**, 2461176 (2025).

Q13: Line 598-606. The FDR and Bonferroni correction were used for F9, F16 and F2, respectively, due to the differential LD levels as claimed by the authors. Why not use different threshold levels instead of different parameters? A comparison is suggested to prove which one is strict.

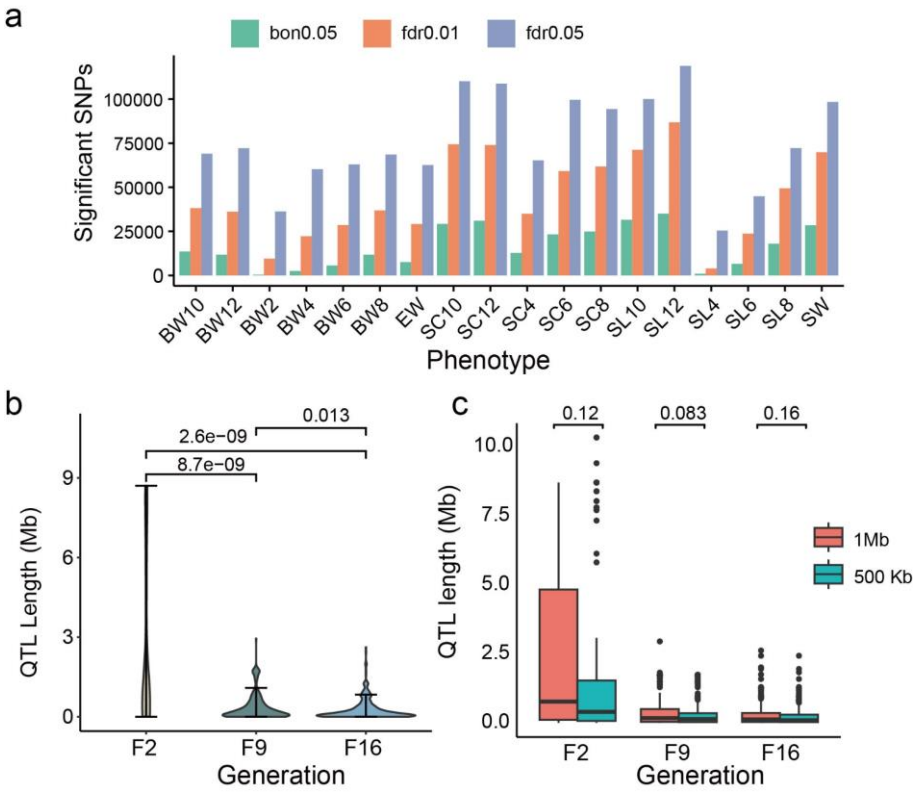
For the definition of QTL, 500kb is used for all 3 populations. Is this proper in terms of differential LD? What is the effect of this rule on the results showed in Fig 3d?

Authors' response: Thank you very much for your question. In the F_2 generation, which is prone to false positives due to LD, we should use a tighter threshold. According to your suggestion, we compared the p-value corresponding to bon0.05, fdr0.05, and fdr0.01 in F_2, the number of significant SNPs (Response Table 1; Response Figure 1a), and the number of QTLs (Bonferroni0.05: 131; fdr0.01: 505; fdr0.05: 1500), and we found that bon0.05 was the strictest. Therefore, in order to control the false positives as much as possible, for the F_2 we selected bon0.05 to correct the p-value.

Because we aimed to compare three generations, and our study mainly focused on the F_{16} generation, considering that the F_{16} generation QTL is relatively short, and in order to ensure the comparability of the three generations, we uniformly chose 500 kb in the initial definition of the QTL. In addition, we used the commonly used 1 Mb for further analysis, as shown in Response Figure 1b, the pattern of which is consistent with that of Figure 3d, and Response Figure 1c shows that the QTLs identified under the two criteria are not significantly different in the corresponding generations.

Response Table 1 P-values for different correction methods

Phe	P value (FDR0.05)	P value (FDR0.01)	P value (Bonferroni 0.05)
BW2	2.30e-04	1.20925e-05	1.26e-07
BW4	3.82e-04	2.81064e-05	1.26e-07
BW6	3.99e-04	3.6254e-05	1.26e-07
BW8	4.34e-04	4.67226e-05	1.26e-07
BW10	4.38e-04	4.84226e-05	1.26e-07
BW12	4.57e-04	4.58236e-05	1.26e-07
EW	3.97e-04	3.69543e-05	1.26e-07
SC4	4.13e-04	4.44274e-05	1.26e-07
SC6	6.31e-04	7.50752e-05	1.26e-07
SC8	5.98e-04	7.82405e-05	1.26e-07
SC10	6.98e-04	9.42613e-05	1.26e-07
SC12	6.90e-04	9.37172e-05	1.26e-07
SL4	1.61e-04	5.04958e-06	1.26e-07
SL6	2.85e-04	3.00641e-05	1.26e-07
SL8	4.58e-04	6.26631e-05	1.26e-07
SL10	6.34e-04	9.03731e-05	1.26e-07
SL12	7.53e-04	0.000110028	1.26e-07
SW	6.24e-04	8.86111e-05	1.26e-07



Response Figure 1 Comparison of F_2 with different significant thresholds and QTLs in different generations (a) Number of significant loci in F_2 GWAS at different thresholds, fdr0.01 represents a threshold of fdr<0.01, fdr0.05 represents a threshold of fdr<0.05, and bon0.05 represents p-value <1.26e-7 corresponding to a threshold of Bonferroni 0.05. (b) Comparative comparison of the lengths of QTLs in different generations; (c) Comparison of the lengths of QTLs obtained at different initial QTL thresholds (500 Kb and 1 Mb) obtained for QTL length comparison.

Reference:

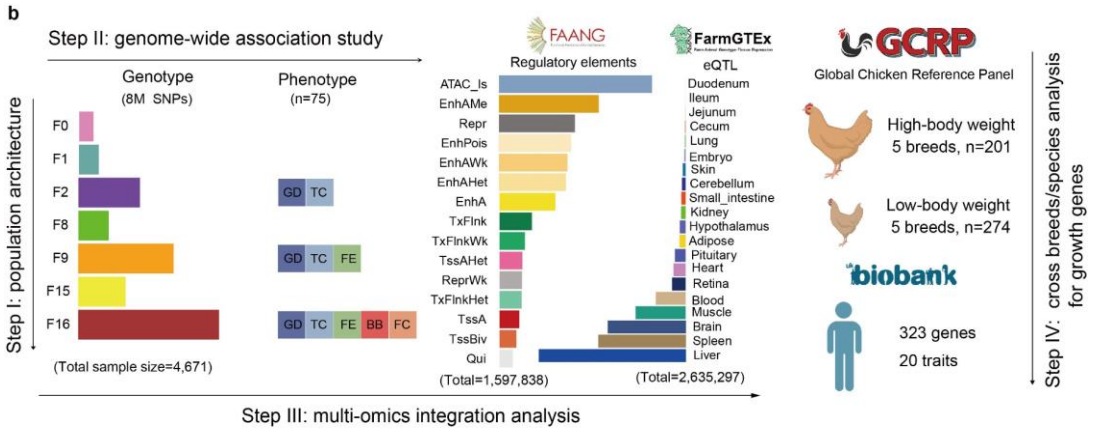
Bouwman, A. C., et al. Meta-analysis of genome-wide association studies for cattle stature identifies common genes that regulate body size in mammals. *Nat. Genet.* **50**, 362–367 (2018).

Q14: Line 763. gene annotation genes?

Authors' response: Thank you for pointing this out. We have changed "Distribution of QTL gene annotation genes" to "*Distribution of genes annotated in QTL regions*" (line 957 in the revised manuscript).

Q15: Fig 1. This is not well designed. Especially for b, the information is not clear. The reviewer suggested to reorganize it.

Authors' response: Thank you very much for your suggestion. We have made changes to Figure 1 in the revised version.

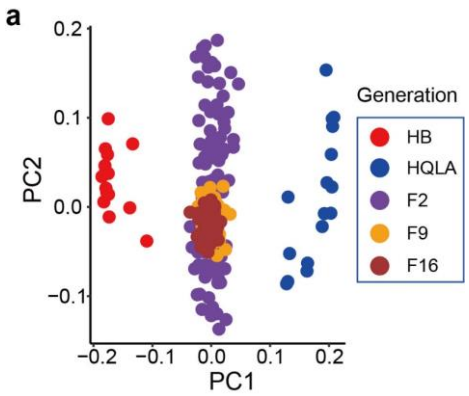


New Figure 1b The Overall workflow of the data analysis. Step I: Genetic architecture analysis of F0 to F16 using 8M SNPs. The number represents the sample size of each generation. Step II: GWAS analysis based on the genotypes and phenotypes. We collected a total of 75 traits covering five categories, including growth and development (GD), tissue and carcass phenotype (TC), feed intake and efficiency (FE), blood biochemistry (BB), and feather characteristics (FC). Step III: Integration analysis of GWAS with functional annotation of animal genomes (FAANG) and expression quantitative trait locus (eQTL). The numbers represent the count of annotated entries and the number of eQTLs in each tissue, respectively. TssA (Strongly active promoters/transcripts), TssAHet (Flanking active TSS without ATAC), TxFlank (Transcribed at gene), TxFlnkWk (Weak transcribed at gene), TxFlnkHet (Transcribed region without ATAC), EnhA (Strong active enhancer), EnhAMe (Medium enhancer with ATAC), EnhAWk (Weak active enhancer), EnhAHet (Active enhancer no ATAC, hetero), EnhPois (Poised enhancer), ATAC_Is (ATAC island), TssBiv (Bivalent/poised TSS), Repr (Repressed polycomb), ReprWk (Weak repressed polycomb), Qui (Quiescent). GTEx: Genotype-Tissue Expression. Step IV: Cross-breed analyses were conducted using high- and low-body-weight chickens from different

breeds. GCRP: Global Chicken Reference Panel (http://farmrefpanel.com/GCRP/#/). Cross-species analyses were performed using the UK Biobank database (https://www.ukbiobank.ac.uk/) and YangLab resources (https://yanglab.westlake.edu.cn/data/ukb_fastgwa/imp/). Additional analyses were performed using the Phenome-Wide Association Study (PheWAS) database (https://atlas.ctglab.nl/PheWAS). A total of 20 human traits and 323 human genes (orthologs of chicken growth-related genes) were included.

Q16: Fig 2a. How about the distribution of other generations F2 F9?

Authors' response: Thank you very much for your question. Considering the error caused by the large difference in sample size between F_0 and F_2, F_9, F_{16}, we randomly selected 100 samples in each generation of F_2, F_9, F_{16} for PCA, and the analysis results are updated in Figure 2a. The figure shows that F_2, F_9, and F_{16} are located in the middle of the HB and the HQLA, and they can be well separated.



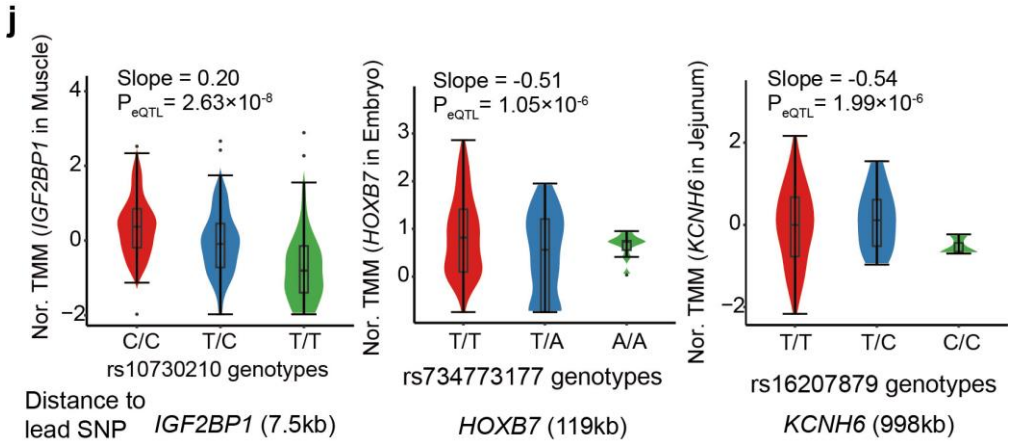
New Figure 2a Principal component analysis (PCA).

Q17: The reference list is not correctly prepared. Duplications such as ref. 14 and ref. 24 should be revised.

Authors' response: We sincerely apologize for this issue. We modified ref. 14 and ref. 24 and rechecked all references.

Q18: Fig 4j. Title for y-axis is not proper. Change gene in tissue (Nor. TMM) to Nor. TMM (gene in tissue)

Authors' response: We have modified the y-axis of Figure-4j.



New Figure 4j Differential expression of *KCNH6*, *IGF2BP1* and *HOXB7* across genotypes. Slopes and P values were from tensorqtl. We show the distance of genes from the lead SNP (rs314511712).

Q19: Fig 5. (a) - The model and software used to estimate QTL heritability and total heritability should be provided.

Authors' response: We use GCTA GRM model "gcta64 --grm genotype --pheno pheno.txt --mpheno 1 –reml" to compute the total heritability. The heritability of the QTL was calculated using the GCTA bivariate GRM model with parameters "gcta64 --reml-bivar --mgrm multi_grm.txt --pheno test.phen". We have added the software and model in lines 992-995: "*The total heritability was calculated using the GCTA GRM model, and the heritability of the QTLs was calculated using GCTA bivariate GRM model.*"".

Q20: (c) – what is the relationship between those Mutations showed in this Fig 5c and rs736348095 in Fig 5b? All of them are within the region of the red lines in Fig5b? Why is there a NA mutation related to PHF11 via TWAS with effect size NA on BW8?

Authors' response: We sincerely appreciate your question. The rs736348095 in Figure 5b is the most significant SNP in this QTL region, and the color indicates the LD of SNPs with rs736348095. The mutation in Figure 5c was identified based on the integration of GWAS with co-localization methods such as SMR and fastENLOC. The

PHF11 result corresponding to the effect of mutation is “NA” because TWAS is based on gene-level results, cannot establish a relationship between mutation-gene-phenotype, and can only determine that *PHF11* affects the BW8 phenotype in the muscle tissue. For easy understanding, we have added an explanation to the legend.

Reviewer #2 (Remarks to the Author):

Reviewer comments:

Zhu and colleagues have established a resource population that is uncommon among chickens, significantly enhancing the mapping resolution of GWAS through multiple generations of recombination. They have also integrated functional annotation data from the Functional Annotation of Animal Genomes (FAANG) project and extensive chicken GTEx data to create a genetic regulatory landscape for complex traits. The findings not only identify a substantial number of fine-mapping genes but also present several representative examples that illustrate complex genetic regulation patterns of quantitative traits. Furthermore, they attempted to extend their findings to humans for comparative analysis, thereby underscoring the importance and significance of their results. Overall, this study synthesizes a wealth of valuable resources, employs advanced research methodologies, and uncovers numerous new genetic principles governing complex traits. This is an important topic that is under extensive investigation. I enjoyed reading this manuscript and found this work intriguing and likely to attract the interest of many peers. I have some comments that need to be addressed before its publication.

Authors' response: Thank you for your thoughtful feedback and positive comments on our manuscript. We appreciate your recognition of our work and the resources and have addressed your comments carefully to improve the manuscript.

1） Why construct the AIL population using the HB and HQLA populations, and what are the characteristics of the AIL population? Please emphasize this in the introduction section.

Authors' response: Thank you very much for your question. This question is similar to Reviewer 1's first comment; we have added the relevant information to the Introduction and Supplementary Notes.

The following description was added in the Introduction lines 88-92 *"The initial F_2 population was established through reciprocal crosses between Huiyang Bearded*

chicken (HB) and the High-Quality Chicken Line A (HQLA), which have significant phenotypic differences in growth traits (Figure 1a). Subsequent advanced intercross lines (AIL), spanning from F3 to F16 were derived through random mating (detailed in Supplementary Notes).” and lines 80-85: *“AIL populations, the concept of which was first proposed in 1995, can increase recombination and improve the accuracy of QTL localization[19]. In recent years, AIL populations have been widely established and applied in several species, and the QTLs of AIL populations have been mapped to narrower chromosomal regions and more QTLs have been identified, which helps identify functional genes and causal mutations”*.

A new description was added in Supplementary Notes lines 12-28: “*The Huiyang Bearded chicken (HB) is a traditional Chinese meat-type breed with a history spanning 1,600 years. It is known for its slow growth rate, exceptional meat quality, and distinctive muff and beard phenotype. Currently, conservation and breeding efforts are underway to preserve this unique genetic resource. The High-Quality Chicken Line A (HQLA) is a selectively bred population derived from a cross between the commercial Anak broiler and a Chinese indigenous chicken line. Over ten generations of intensive artificial selection, HQLA has been improved using a weight-based selection index while preserving desirable traits such as meat quality and plumage color.*

At seven weeks of age, HQLA exhibited a body weight approximately 3.2 times that of HB. Differences in growth rate and ancestral origin facilitate better study of growth phenotypes in the progeny. The F2 population was established through reciprocal crosses between the two parental lines, with matings of HQLA males with HB females (4♂×12♀) and HB males with HQLA females (4♂×12♀). Subsequent advanced intercross lines (AILs) from F3 to F16 were derived from the F2 generation and maintained through random mating. Each generation consisted of 655–2381 individuals (Supplementary Table 25).”

2)The AIL population underwent multiple generations of recombination from F0 to F16. In F16, what is the ancestral composition of HB and HQLA? Has any deviation occurred?

Authors' response: We appreciate your insightful comment. We calculated the ancestral origin of 2,269 samples from the F_{16} generation using RFMix for chromosome 1 as an example and found it to be 0.53 ± 0.08 for the HB origin and 0.47 ± 0.08 for the HQLA origin; these results suggest no bias in the ancestral component in the progeny of this population.

Reference:

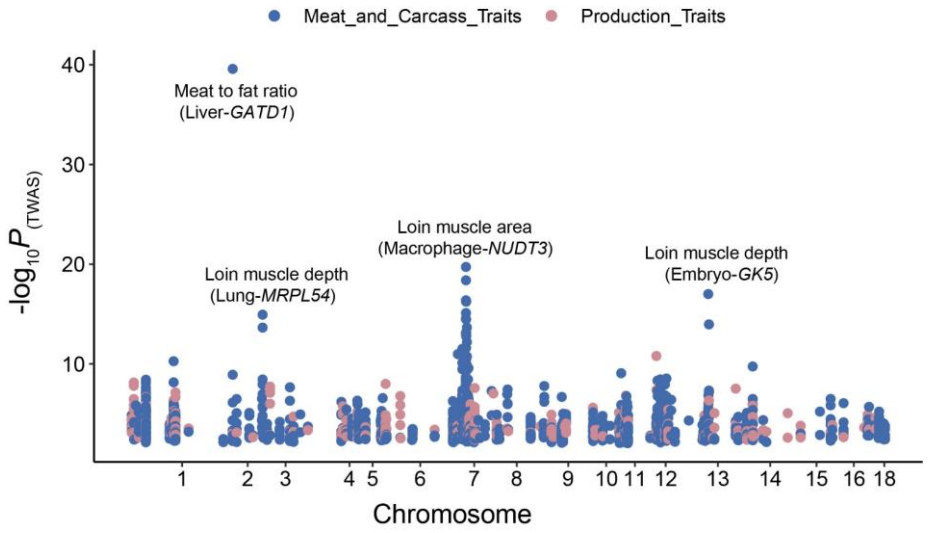
Maples, B. K. et al. RFMix: a discriminative modeling approach for rapid and robust local-ancestry inference. *Am. J. Human Genet*. **93**, 278–288 (2013).

3) Figure 7 compared functional genes related to growth traits in chickens and humans, but the selection patterns in humans and chickens are different. Please further elaborate on the conservation of growth trait functional genes between chickens and other species such as pigs and cattle.

Authors' response: We appreciate your insightful suggestions. Based on the identification of 431 functional genes in chickens, we utilized ENSEMBL Biomart to identify their homologous genes in pigs and cattle, resulting in the identification of 405 homologous genes in pigs and 447 in cattle. The FarmGTEx project facilitates the establishment of linkages between genes and phenotypes through various multi-omics methods. Upon examining the PigGTEx database, we found that 333 out of the 405 pig homologous genes are associated with growth and development-related phenotypes, such as "Production_Traits" and "Meat_and_Carcass_Traits" in pigs. This finding partially supports the conservation of functional genes between chickens and pigs. In contrast, an analysis of the CattleGTEx database revealed that only 5 out of the 447 genes are linked to complex phenotypes in cattle. Given the limited number of phenotypes available in this database, the significance of these results is constrained. As the data continue to improve, we anticipate enhanced comparative studies of functional genes across species in future (New Supplementary Figure 18).

A new description was added in the Introduction lines 315-319: *"Meanwhile, we converted 431 functional genes obtained from chickens to their homologous genes in pigs and further queried the PigGTEx database to identify 289 genes affected pigs*

Production_Traits Meat_and_Carcass_Traits and other growth- and development-related phenotypes (Supplementary Figure 18).”



New Supplementary Figure 18 TWAS results of homologs of chicken functional genes in pigs in the PigGTEx database.

Reference:

Teng, J. et al. A compendium of genetic regulatory effects across pig tissues. *Nat Genet.* **56**, 112–123 (2024).

4)Which specific QTLs correspond to Trait, Domain, and Multi-Domain in Figure 3e? Please list them in detail.

Authors’ response: We appreciate your insightful suggestion. We merged the 682 QTLs that overlapped based on their physical locations, resulting in a total of 393 unique QTLs. Furthermore, we examined the distribution of the 682 QTLs across various phenotypes and categorized them into three groups accordingly (Trait, Domain, and Multi-Domain); detailed information regarding the specific QTLs can be found in Supplementary Table 9.

5) Figure 4i-j mentioned that the functional gene on the GGA27 chromosome was not the closest to the lead SNP. Please discuss the regulatory characteristics of the avian micro-chromosome genome.

Authors’ response: Thank you for your valuable feedback. We have added

"*Furthermore, chromosome length plays a crucial role in determining overall chromosome architecture in vertebrates. For instance, chickens possess relatively short chromosomes, and their long-range contact frequency is 1.408 times higher than that of humans and mice. This finding suggests that long-range interactions may occur more frequently in chickens. Future studies should explore the impact of long-range contacts on gene regulation and their influence on complex traits via methods such as Hi-C and tensorQTL*" to the Discussion (lines 379-385).

Reference:

Li, D. et al. Comparative 3D genome architecture in vertebrates. *BMC Biol*. **20**, 99 (2022).

6) Line 34: "We have developed a 16-generational advanced intercross line of chickens to enhance informative recombination and achieved a gene-level mapping resolution of 52 loci across 25 traits." Could be revised to: "We have developed a 16-generation advanced intercross line of chickens to enhance informative recombination and have achieved single gene-level mapping resolution for 52 loci across 25 traits." Additionally, does "52 loci" refer to genes? The manuscript mentions 65 single gene loci in this context. Please verify and confirm.

Authors' response: Taking into account your comments and Q6 raised by Reviewer 1, we have revised this sentence. The 52 loci represent the 52 unique genes corresponding to the 65 single-gene QTLs.

New description was in lines 33-35 "*We have developed a 16-generation advanced intercross line of chickens to enhance informative recombination and achieved 154 single-gene QTLs.*"

7)In the Introduction section, the significance of studying growth traits in chickens should be further elaborated, along with the role these traits play in the growth and development of domesticated animals and humans.

Authors' response: Thank you very much for your question. We have added "*Chickens are an important source of protein for human beings, and analyzing the genetic*

mechanisms of growth traits in chickens is crucial for accelerating the genetic breeding of chickens and increasing the economic value of the chicken meat industry. Chickens are important model organisms for avian studies and are utilized in research on osteoporosis, obesity, metabolic disorders, and other diseases, providing important models for human medicine" to the Introduction section (lines 64-69 in the revised version).

8)The description of phenotypic measurements in the methods section is too detailed and could be placed in the supplementary notes.

Authors' response: Thank you for pointing this out. We moved the specific information to Supplementary Notes (lines 29-68 in the Supplementary Notes).

9)What is the sample size for each tissue in the chicken GTEx eQTL data used in the study for integration with GWAS? Please provide additional details.

Authors' response: We sincerely appreciate your question. We have provided sample information for each tissue in the eQTL data in Supplementary Table 12.

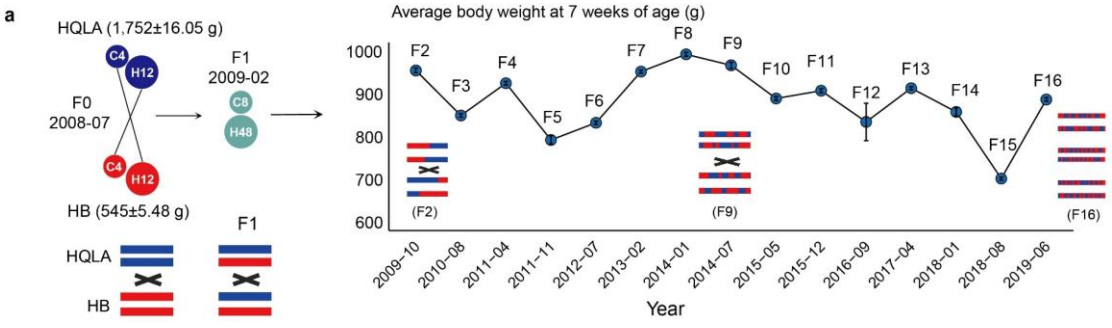
Supplementary Table 12 Sample of tissues in the chickenGTEx database

MolQTL Tissues	Samples
Adipose	115
Blood	224
Brain	479
Bursa	115
Cecum	56
Cerebellum	52
Duodenum	50
Embryo	470
Heart	258
Hypothalamus	107
Ileum	62

Jejunum	65
Kidney	78
Leukocytes	122
Liver	741
Lung	89
Macrophage	60
Muscle	517
Ovary	96
Oviduct	68
Pituitary	135
Retina	119
Skin	163
Small_intestine	169
Spleen	383
Tesits	44
Thymus	68
Trachea	92

10)For Figure 1a, please use the full name for "BW7".

Authors' response: Thank you for pointing this out. We modified BW7 to "*Body weight at 7 weeks of age (g)*" (New Figure 1a).

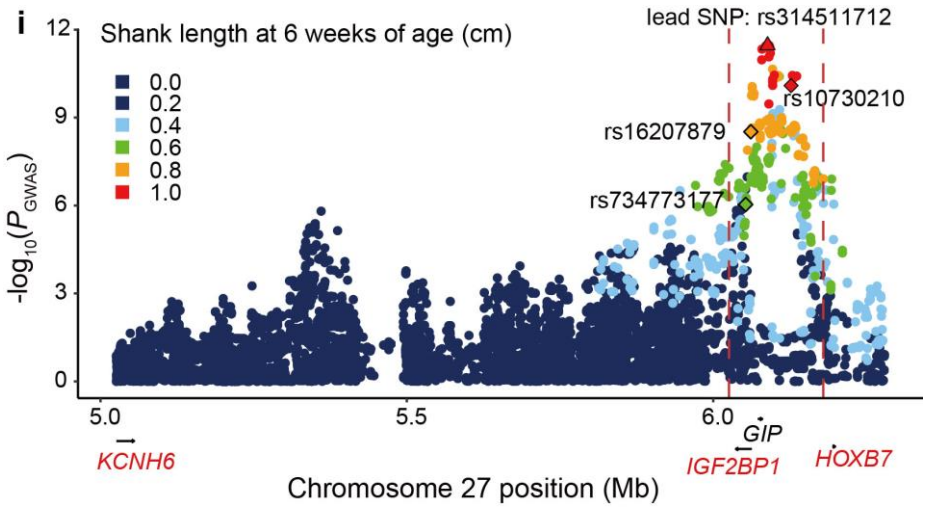


New Figure 1a A large intercross pedigree was established in 2008 from two divergent chicken lines, High Quality chicken Line A (HQLA) and Huiyang Bearded chicken (HB). The average body weight at 7 weeks of age (BW7) is represented in the figure as

mean ± standard error. 'Cx' and 'Hx' denote the number of male and female founders within each population, respectively. Starting from the F_2 generation, the figure illustrates the mean and standard error of BW7 for each generation. The horizontal axis indicates the birth time of the samples corresponding to each generation. The red and blue stripes simulate the recombination.

11)The legend for Figure 4i and the description of the "lead SNP" in the figure are inconsistent. Please verify and correct.

Authors' response: We sincerely apologize for this issue. We have verified and modified the descriptions (New Figure 4i).



New Figure 4i Shank length at 6 weeks of age (SL6) GWAS Manhattan plot. The color indicates the LD of SNPs with the lead SNP (rs314511712). The red dashed area indicates the QTL range. The gene nearest to rs314511712 is *GIP*. *KCNH6*, *IGF2BP1*, and *HOXB7* were functional genes identified via SMR.

12)Line 140: The full name of "GGA" should be provided when it first appears.

Authors' response: Thank you very much for your question. We have changed "GGA" to its full name, *"Gallus gallus chromosome".*

13)Line 143: "AnimalQTL" should be changed to "Animal QTL".

Authors' response: Thank you for your suggestion. We have modified the phrase as

suggested.

14)Line 280: The full name of "PheWAS" should be provided when it first appears.

Authors' response: Thank you for your suggestion. We have changed "PheWAS" to "*Phenome-Wide Association Study*".

15)Line 676 and line 796: Change "commercial Broiler LineA" to "commercial Broiler Line A" and "commercial Broiler LineB" to "commercial Broiler Line B".

Authors' response: We appreciate your insightful suggestion. We have made the indicated changes.

16)Line 762: Change "Distribution of QTL gene annotation genes" to "Distribution of genes annotated in QTL regions".

Authors' response: Thank you very much for your comment. We have modified the phrase accordingly (line 957 in the revised version).

17)Line 790: What does "top QTL" mean? Please provide an explanation.

Authors' response: We sincerely appreciate your question. The top QTL here refers to the one that is most significant on chromosome 1 for each phenotype, i.e., the primary QTL for chromosome 1. To make it easier to understand, we have added an explanation of the top QTL to the legend.

18)Line 820: The spelling of "uniq" is incorrect and should be corrected to "unique".

Authors' response: We apologize for this issue. We have modified.

19)Please provide header information for each supplementary table.

Authors' response: Thank you for your valuable feedback. We have added header information to the Supplementary Tables.

Reviewer #3 (Remarks to the Author):

In this manuscript, Zhu et al. report the use of a 16-generation intercross line of chickens to investigate the genotypes underlying many phenotypes, most of them related to growth. Given the dearth of available genotype-phenotype data in chickens, the authors should be commended for the large amount of time invested in generating this dataset which will surely be useful to many future researchers. Their analyses of the data are thorough and their conclusions are sound. However, there are numerous problems with the way some of the results are reported, both in the figures and in the text. The figures are inconsistent in their use of error bars, axes are not always sufficiently defined, fonts are often too small, and many captions are not detailed enough to understand what is being displayed. In the text, numbers are not always reported well enough, with many measurements given without enough explicit information about what they represent; further, some required statistical details listed in the reporting summary are missing, and exact p-values are often left out in favor of undefined stars in the figures. Finally, although the writing is for the most part clear, there are places where it is confusing, and the manuscript overall would greatly benefit from editing by a native English speaker. I have listed many but not all examples of these issues below. Together, these flaws detract from an otherwise excellent study; therefore, if the manuscript and especially the figures are improved to address these issues, I would be happy to recommend a revised version of this manuscript for publication.

Authors' response: Thank you for your valuable comments. We have carefully revised all figures to ensure consistency, clarified axis definitions, adjusted font sizes for improved readability, and expanded figure captions to provide clearer explanations of the data presented. Additionally, we have refined numerical reporting throughout the manuscript, explicitly defining all measurements. To enhance transparency, we have replaced stars with exact p-values in both the figures and text. Furthermore, any missing statistical details listed in the reporting summary have now been incorporated. Finally, we have thoroughly reviewed and refined the manuscript to improve clarity and readability. And this manuscript has also been professionally edited by Editage to ensure language and grammatical accuracy.

Specific comments, by line number:

Q1: 75: I don't understand what "characterized by a recombination degree" means, please explain better

Authors' response: Thank you for your valuable feedback. The AIL population undergoes continuous passages, recombination increases, QTLs are mapped to narrower chromosomal regions, and more QTLs are identified; this helped us in identifying functional genes and causal mutations. We have added a detailed description of the AIL group.

A new description was added in the Introduction lines 80-85: *"AIL populations, the concept of which was first proposed in 1995, can increase recombination and improve the accuracy of QTL localization. In recent years, AIL populations have been widely established and applied in several species, and the QTLs of AIL populations have been mapped to narrower chromosomal regions and more QTLs have been identified, which helps identify functional genes and causal mutations"*.

Q2: 93-98: it is implied based on reading the full paragraph that you did low-coverage sequencing and then used this to do imputation, and the 8M SNPs were a result of imputation, but because imputation is mentioned last this is confusing

Authors' response: Thank you for your question. The specific analysis process is shown in Supplementary Figure 19 and the Methods. BaseVar-STITCH is an imputation method without a reference panel for low-coverage sequencing described by Yang et al. We identified polymorphic sites using BaseVar and imputed genotypes via STITCH. In line 109 of revised version, "accuracy assessment of the genotype imputation" refers to the imputation accuracy of low-coverage sequencing. We compared the genotype consistency using samples with both WGS and low-coverage sequencing data, and results showed that the genotype consistency was very high.

Reference:

Yang, R. et al. Accelerated deciphering of the genetic architecture of agricultural economic traits in pigs using a low-coverage whole-genome sequencing strategy.

Q3: 96, 98, others: be more explicit about these numbers: are they mean and standard deviation, or something else?

Authors' response: We sincerely appreciate your question. The data indicate mean ± standard deviation; all similar data in the manuscript represent means ± standard deviation. We added a uniform description to the Supplementary Notes: "*Descriptions involving value 1 ± value 2 are the mean ± standard deviation unless otherwise stated; t-tests used are unpaired, two-tailed*" (lines 69-71 in the Supplementary Notes).

Q4: 98: define genotype consistency

Authors' response: Thank you very much for your question. We utilized the SNPs from 108 samples, each of which simultaneously had both WGS and LC data. We converted them to the 0/1/2 format, and calculated the concordance between WGS and LC data, defined as genotypic concordance; a higher concordance indicated a higher imputation accuracy of our LC data.

Q5: 127-129: confusing sentence. If I understand what it's trying to say correctly, a better way to say this might be "Many of the phenotypic measurements were strongly correlated with each other, suggesting that they may be influenced by pleiotropic genes."

Authors' response: We appreciate your insightful suggestions. Your understanding is correct, and we have changed the section according to your suggestions (lines 140-143 in the revised version).

Q6: 134: define "significant signals"

Authors' response: Thank you very much for your question. We used FDR<0.05 as the threshold of significance for GWAS for each phenotype, and 43 phenotypes had significant signals, indicating the presence of SNPs with FDR < 0.05 for 43 phenotypes.

Q7: 135: awkward construction, I suggest instead "The heritability of a phenotype and

the number of QTLs found for this phenotype are significantly correlated"

Authors' response: Thank you very much for your suggestion. We have modified the section as suggested (lines 149-150 in the revised version).

Q8: 136: I assume this is Pearson's R but be explicit

Authors' response: We appreciate your insightful comment. The value represents Pearson's correlation, which we previously clarified in the legend. We have changed "r = 0.64, p = 4.21×10^{-6}, Figure 3c" to "*Pearson's r = 0.64, p = 4.21×10^{-6}, Figure 3c*" (lines 149-150 in the revised version).

Q9: 152: be more specific than "annotated to genes": does this mean that they overlap with coding sequence, or contain the full genes, or parts of the genes, or what?

Authors' response: We apologize for the ambiguity. We used "bedtools intersect" to identify genes overlapping with QTL regions. A gene was considered annotated to a QTL if its genomic coordinates intersected with the QTL region. The statement "624 QTLs were annotated to genes" indicates that these QTLs overlapped with the full genes or parts of the genes. The description has been modified to: "*A total of 624 QTLs contained at least one gene.*" (line 173 in the revised version).

Q10: 156: how many previously reported major QTLs?

Authors' response: Thank you for your question. What we would like to show here is that the three primary QTLs previously reported to be located on chromosomes 1, 4, and 27, respectively, have also been detected by us, and we have detected their genomic locations as GGA1: 170-171 Mb, GGA4: 75-76 Mb, and GGA27: 6.02-6.17 Mb. Related references have been added (line 179 in the revised version).

References:

Wang, Y. et al. Multiple ancestral haplotypes harboring regulatory mutations cumulatively contribute to a QTL affecting chicken growth traits. *Commun. Biol.* **3**, 472 (2020).

Tu, T. C., Lin, C. J., Liu, M. C., Hsu, Z. T. & Chen, C. F. Genomic prediction and

genome-wide association study for growth-related traits in Taiwan country chicken. *Animals (Basel)* **15**, 376 (2025).

Zhong, C. et al. Age-dependent genetic architectures of chicken body weight explored by multidimensional GWAS and molQTL analyses. *J. Genet. Genomics* **51**, 1423–1434 (2024).

Xie, L. et al. Genome-wide association study identified a narrow chromosome 1 region associated with chicken growth traits. *PLOS One* **7**, e30910 (2012).

Q11: 159: be more specific than "validated in mice". Were they validated to contain variants that affect the same trait, or some looser criteria?

Authors' response: Your comments are highly appreciated. We investigated the function of these genes in mice based on the MGI database and identified 52 genes that corresponded to homologous genes in mice. These genes have been shown to produce growth- and development-related phenotypes following validation through knockout and other experimental methods. It is important to note that we did not find corresponding mutations due to the differences in genomic sequences between chickens and mice. Our study aimed to demonstrate that the AIL population can be localized to a single-gene level as recombination continues to occur. Furthermore, some of these genes have been functionally validated in mice, which underscores the accuracy of our localization efforts.

Q12: 170-172: define all these abbreviations

Authors' response: We appreciate your insightful suggestion. We have added the full name of each abbreviation in the figure legend, with specific information below: *"TssA: Strongly active promoters/transcripts; TssAHet: Flanking active TSS without ATAC; TxFlank: Transcribed at gene; TxFlnkWk: Weak transcribed at gene; TxFlnkHet: Transcribed region without ATAC; EnhA: Strong active enhancer; EnhAMe: Medium enhancer with ATAC; EnhAWk: Weak active enhancer; EnhAHet: Active enhancer no ATAC (hetero); EnhPois: Poised enhancer; ATAC_Is: ATAC island; TssBiv: Bivalent/poised TSS; Repr: Repressed polycomb; ReprWk: Weak repressed polycomb;*

Qui: Quiescent. More specific information is reported by Pan et a[4]. eQTL: expression quantitative trait locus; exQTL: exon quantitative trait locus; lncQTL: long non-coding RNA quantitative trait locus; sQTL: splicing quantitative trait locus; 3aQTL: 3' alternative polyadenylation quantitative trait locus" (lines 962-972 in the revised version).

Reference:

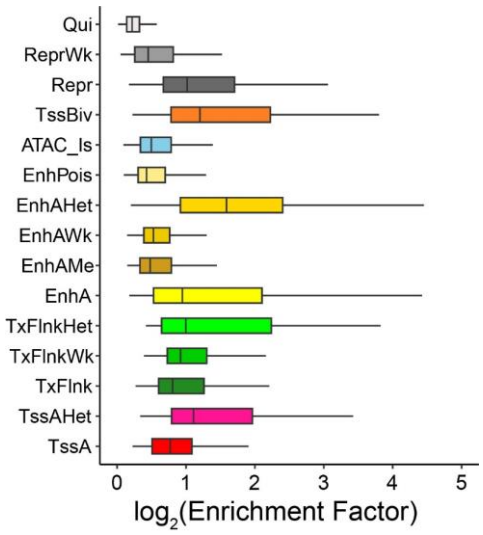
Pan, Z. et al. An atlas of regulatory elements in chicken: A resource for chicken genetics and genomics. *Sci. Adv*. **9**, eade1204 (2023).

Q13: 173: this says they are "significantly" enriched but I do not see a p-value, significance stars, or any other indication that this is based on a statistical test in the figure panel

Authors' response: Thank you for your valuable feedback. For Figure 4b, we used QTLEnrich (v2) to measure the enrichment degree between significant eQTLs and GWAS loci. We show the results for FDR < 0.05. Each GWAS phenotype is enriched by a multiple of greater than 1 in the eQTL, indicating that GWAS signaling is significantly enriched in the eQTLs. In conjunction with the question raised by you and Reviewer 1 about Supplementary Figure 7, in order to show the enrichment fold more clearly, we utilized all SNPs as a background for calculating enrichment and used a hypergeometric distribution test to calculate p-values.

Enrichment factor = (target region significant SNPs/total significant SNPs) / (target region all SNPs/ gnome-wide total SNPs)

We performed enrichment analysis using 40 phenotypes with 15 functional regions in 23 tissues, and the results are shown in Supplementary Figure 6. As shown in the figure, we filtered the results with FDR < 0.05 for plotting. We found that GWAS signaling was enriched at a high level in regions such as promoters (TssA, TssBiv) and enhancers (EnhA and EnhAHet) (New Supplementary Figure 7).



New Supplementary Figure 7 Genome-wide association study (GWAS) signal enrichment was analyzed within chromatin states across 23 tissues and 40 complex traits in chickens. The figure illustrates the results at FDR<0.05.

Q14: 179: no need to list numbers that are shown in the figure like this

Authors' response: We appreciate your insightful comment. We have removed specific numbers from the main text. We revised "This resulted in the identification of 166, 105, 94, and 245 functional genes, respectively, yielding a total of 431 unique genes" to "*We obtained a total of 431 unique functional genes using four methods*" (lines 204-205 in the revised manuscript).

Q15: 181: can you explain a little bit why this phenotype matters?

Authors' response: Alkaline phosphatase (ALP) is an enzyme widely distributed in human tissues such as the liver, bone, intestine, kidney, and placenta excreted out of bile via the liver. Total ALP activity measured in serum or plasma is the sum of different types of ALP from various tissue sources. More than 80% of ALP in the serum comes from the liver and bone, with a small amount (10-20%) coming from the intestine. The ALP assay is mainly used to diagnose diseases of the hepatic, biliary, and skeletal systems and is an important indicator of extrahepatic biliary obstruction, intrahepatic space-occupying lesions, and rickets.

ALP in intestinal tissues is mainly distributed in the villous epithelium and fibroblasts of the small intestine and regulates fatty acid transport and the absorption of metallic elements such as Ca and P from the feed. ALP also regulates intestinal phosphate secretion and pH, which serves as an alkaline chemosensor that regulates pH and helps protect the intestinal mucosa from gastric acid damage. ALP in chickens may be related to intestinal function, which in turn affects feed intake. In subsequent studies, we will also continue to focus on the relationship between ALP and growth and development as well as disease in chickens.

For ease of understanding we have added a description to lines 206-208 of the Results: "*Alkaline phosphatase (ALP) assay is mainly used to diagnose diseases of the hepatic, biliary and skeletal systems. ALP in chickens may be related to intestinal function, which in turn affects feed intake.*"

References:

Makris, K., Mousa, C. & Cavalier, E. Alkaline phosphatases: biochemistry, functions, and measurement. *Calcif. Tissue Int.* **112**, 233–242 (2023).

Lallès, J. P. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutr. Rev.* **68**, 323–332 (2010).

Lallès, J. P. Recent advances in intestinal alkaline phosphatase, inflammation, and nutrition. *Nutr. Rev.* **77**, 710–724 (2019).

Q16: 183: what is SMR analysis?

Authors' response: We sincerely appreciate your question. SMR (Summary data-based Mendelian randomization (SMR) is a method used to investigate causal relationships between gene expression and complex traits using summary-level data. Mendelian randomization (MR) is based on the principle of the "random assignment of parental alleles to offspring" according to Mendel's laws. If a genotype determines a phenotype, and the phenotype influences disease risk, the genotype will be associated with the disease through the phenotype. This allows the genotype to serve as an instrumental variable for inferring causal relationships between phenotypes and diseases. MR studies help identify true causal genes underlying complex traits in GWAS. However, due to

the polygenic nature of many complex traits, the proportion of phenotypic variation explained by a single genetic variant or a single level of gene expression is often very small. As a result, detecting gene effects on traits through MR analysis typically requires very large sample sizes. In practice, however, it is rare to obtain such large datasets where the phenotype, genotype, and gene expression are measured in the same individuals. The SMR approach overcomes this limitation by leveraging large-scale GWAS and eQTL summary data to identify genes whose expression levels are associated with complex traits due to pleiotropy.

References:

Zhu, Z. et al. Integration of summary data from GWAS and eQTL studies predicts complex trait gene targets. *Nat. Genet.* **48**, 481–487 (2016).

Meng, X. H. et al. Integration of summary data from GWAS and eQTL studies identified novel causal BMD genes with functional predictions. *Bone* **113**, 41–48 (2018).

Q17: 194: what are eGenes?

Authors' response: Thank you for your question. eGenes are defined as genes significantly regulated by at least one variant (FDR < 0.05).

Q18: 193-196: I do not understand these three categories, please explain more clearly

Authors' response: We sincerely appreciate your question. To clarify, we can take the SMR method as an example. For instance, when analyzing the phenotype of shank length at 6 weeks of age (SL6), we integrate GWAS results for SL6 with eQTL data from different tissues. Within a given QTL, if none of the genes are identified as significant by the SMR method, the locus is considered non-colocalized, implying that no colocalization is observed. If a QTL contains significant genes identified by SMR, two possible scenarios arise. If the significant genes include the nearest gene to the lead SNP within the QTL, the locus is classified as a nearest-gene locus. Otherwise, it is categorized as a not-nearest gene locus. For example, in Figure 4i, the lead SNP nearest gene is *GIP*, but SMR analysis identified *KCNH6*, *IGF2BP1*, and *HOXB7* instead. As

a result, this loci is classified as a not-nearest gene loci.

Q19: 213: define GD and TC

Authors' response: Thank you very much for your question. GD and TC are abbreviations for two major classes of phenotypes; GD stands for growth and development phenotypes, and TC stands for tissue and carcass phenotypes. In line 138, we have provided abbreviations for the five major classes of phenotypes.

Q20: 243: what is eviscerated weight?

Authors' response: The eviscerated weight is the weight of the chicken at 13 weeks of slaughter after all internal organs have been removed.

Q21: 505-506: "custom script" but code is not provided

Authors' response: Thank you for pointing this out. We uploaded the custom script "split_index.pl" to https://github.com/xiaoningZhu0506/AIL_GWAS.

Q22: 534: software version and citation or link for STITCH?

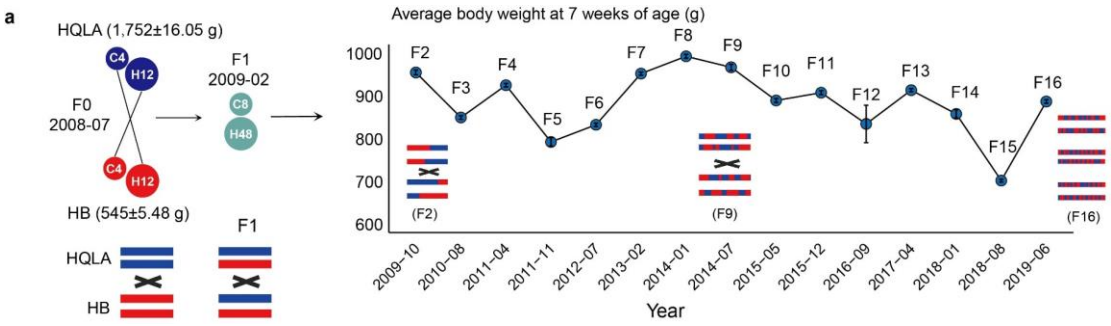
Authors' response: Thank you very much for your question. The version of STITCH is v1.1.2. We have updated it (lines 513-514 in the revised version).

Reference:

Davies, R. W., Flint, J., Myers, S., & Mott, R. Rapid genotype imputation from sequence without reference panels. *Nat. Genet*. **48**, 965–969 (2016).

Q23: Figure 1a: define BW7, add error bars

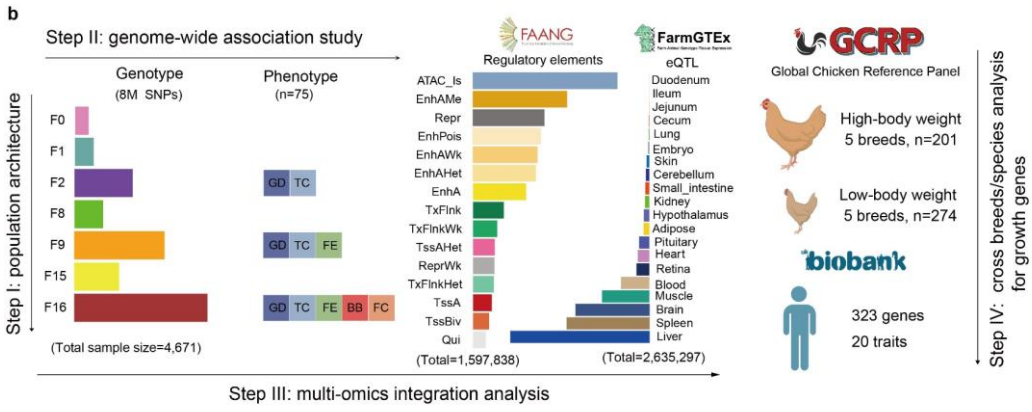
Authors' response: Thank you for your valuable feedback. We defined BW7 in the revised version and added standard error (New Figure 1a).



New Figure 1a A large intercross pedigree was established in 2008 from two divergent chicken lines, High Quality chicken Line A (HQLA) and Huiyang Bearded chicken (HB). The average body weight at 7 weeks of age (BW7) is represented in the figure as mean ± standard error. 'Cx' and 'Hx' denote the number of male and female founders within each population, respectively. Starting from the F_2 generation, the figure illustrates the mean and standard error of BW7 for each generation. The horizontal axis indicates the birth time of the samples corresponding to each generation. The red and blue stripes simulate the recombination.

Q24: Figure 1b: I find this flowchart convoluted and hard to follow, and therefore unhelpful

Authors' response: Thank you very much for your suggestion. We have made changes to Figure 1b in the revised version.



New Figure 1b The Overall workflow of the data analysis. Step I: Genetic architecture analysis of F0 to F16 using 8M SNPs. The number represents the sample size of each generation. Step II: GWAS analysis based on the genotypes and phenotypes. We collected a total of 75 traits covering five categories, including growth and

development (GD), tissue and carcass phenotype (TC), feed intake and efficiency (FE), blood biochemistry (BB), and feather characteristics (FC). Step III: Integration analysis of GWAS with functional annotation of animal genomes (FAANG) and expression quantitative trait locus (eQTL). The numbers represent the count of annotated entries and the number of eQTLs in each tissue, respectively. TssA (Strongly active promoters/transcripts), TssAHet (Flanking active TSS without ATAC), TxFlank (Transcribed at gene), TxFlnkWk (Weak transcribed at gene), TxFlnkHet (Transcribed region without ATAC), EnhA (Strong active enhancer), EnhAMe (Medium enhancer with ATAC), EnhAWk (Weak active enhancer), EnhAHet (Active enhancer no ATAC, hetero), EnhPois (Poised enhancer), ATAC_Is (ATAC island), TssBiv (Bivalent/poised TSS), Repr (Repressed polycomb), ReprWk (Weak repressed polycomb), Qui (Quiescent). GTEx: Genotype-Tissue Expression. Step IV: Cross-breed analyses were conducted using high- and low-body-weight chickens from different breeds. GCRP: Global Chicken Reference Panel (http://farmrefpanel.com/GCRP/#/). Cross-species analyses were performed using the UK Biobank database (https://www.ukbiobank.ac.uk/) and YangLab resources (https://yanglab.westlake.edu.cn/data/ukb_fastgwa/imp/). Additional analyses were performed using the Phenome-Wide Association Study (PheWAS) database (https://atlas.ctglab.nl/PheWAS). A total of 20 human traits and 323 human genes (orthologs of chicken growth-related genes) were included.

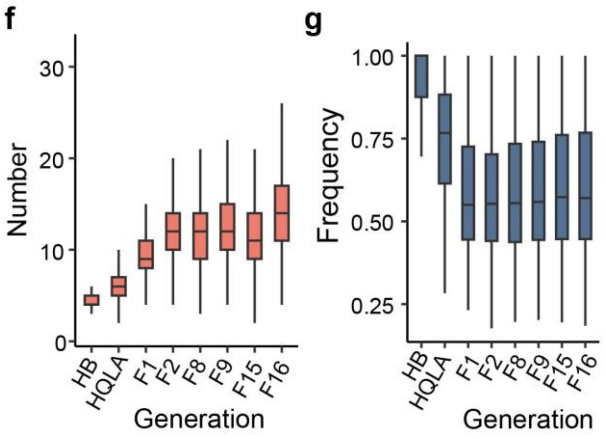
Q25: Figure 2b: I do not understand what the numbers in the distributions are. Is each column the distribution of pi across the windows of a single bird from this generation, or from all the windows of all the birds together, or something else? Same thing for panels c and f.

Authors' response: Thank you very much for your question. Figure 2b shows the nucleotide diversity calculated with "vcftools -vcf a.vcf --out a --window-pi 300000" in a 300 kb sliding window within each generation, and the pi value in each sliding window is calculated based on all samples. The pi value in each window is calculated based on all the samples, and a higher pi value represents higher nucleotide diversity.

Figure 2c shows the degree of inbreeding coefficient by “plink --file a --het”, with one value per sample. In Figure 2f, we used 18,325 SNPs after LD filtering, with a sliding window of 5 SNPs per generation; “number of haplotypes” is the haplotype species within each sliding window; and the haplotype frequency is the sum of the top 4 haplotype frequencies in each sliding window, based on all samples.

Q26: Figure 2f: split these into two panels; it is difficult to read with error bars from the two measurements overlapping. Also, I do not understand what "number of haplotypes" or "frequency of major haplotypes" means

Authors’ response: We appreciate your insightful suggestions. In the revised manuscript, we have divided 2f into 2f-g. Here, 'number of haplotypes' refers to the total count of haplotype types within each sliding window, while 'frequency of major haplotypes' indicates the cumulative frequencies of the top four haplotypes within each window. In the revised version, we have split section 2f into 2f and 2g (New Figure 2f-g).



New Figure 2f-g (f) Number of haplotypes. (g) Frequency of major haplotypes.

Q27: Figure 3a: x-axis labels are misaligned

Authors’ response: Thank you for pointing this out. We added x-axis labels as “Phenotype categories” in the revised version.

Q28: Figure 3c: legend font is too small, but it looks like the colors are the same as in

panel (b), so maybe just skip the legend in this panel

Authors' response: Thank you very much for your question. We used the abbreviation for phenotypic categorization in Figure 3c and illustrated it in the legend.

Q29: Figure 3d: define what the p-value cutoffs for stars are, give more details about the test used (one-tailed or two-tailed? paired or independent? degrees of freedom? test statistics?) and show exact p-values somewhere in text or caption as well

Authors' response: Thank you very much for your question. We have added specific p-values to the revised version. We used a two-tailed, unpaired t-test with degrees of freedom and test statistics, as shown in the table below:

Response Table 2 Summary of t-test statistics comparing the length of QTLs across generations

	group1	group2	t value	degrees of freedom
t	F2	F9	5.803639	132.1253
t1	F2	F16	6.04509	131.0648
t2	F9	F16	2.08033	648.8111

Q30: Figure 3g/h: fonts way too small

Authors' response: We appreciate your insightful comment. We have adjusted the font in the revised version.

Q31: Figure 4a: not sure what any of these abbreviations mean

Authors' response: Thank you for your valuable feedback. We have added the full name of each abbreviation in the figure legend: *"TssA: Strongly active promoters/transcripts; TssAHet: Flanking active TSS without ATAC; TxFlank: Transcribed at gene; TxFlnkWk: Weak transcribed at gene; TxFlnkHet: Transcribed region without ATAC; EnhA: Strong active enhancer; EnhAMe: Medium enhancer with ATAC; EnhAWk: Weak active enhancer; EnhAHet: Active enhancer no ATAC (hetero); EnhPois: Poised enhancer; ATAC_Is: ATAC island; TssBiv: Bivalent/poised TSS; Repr:*

Repressed polycomb; ReprWk: Weak repressed polycomb; Qui: Quiescent. More specific information has been reported by Pan et al. eQTL: expression quantitative trait locus; exQTL: exon quantitative trait locus; lncQTL: long non-coding RNA quantitative trait locus; sQTL: splicing quantitative trait loci; 3aQTL: 3′ alternative polyadenylation quantitative trait locus." (lines 962-972 in the revised version).

Reference:

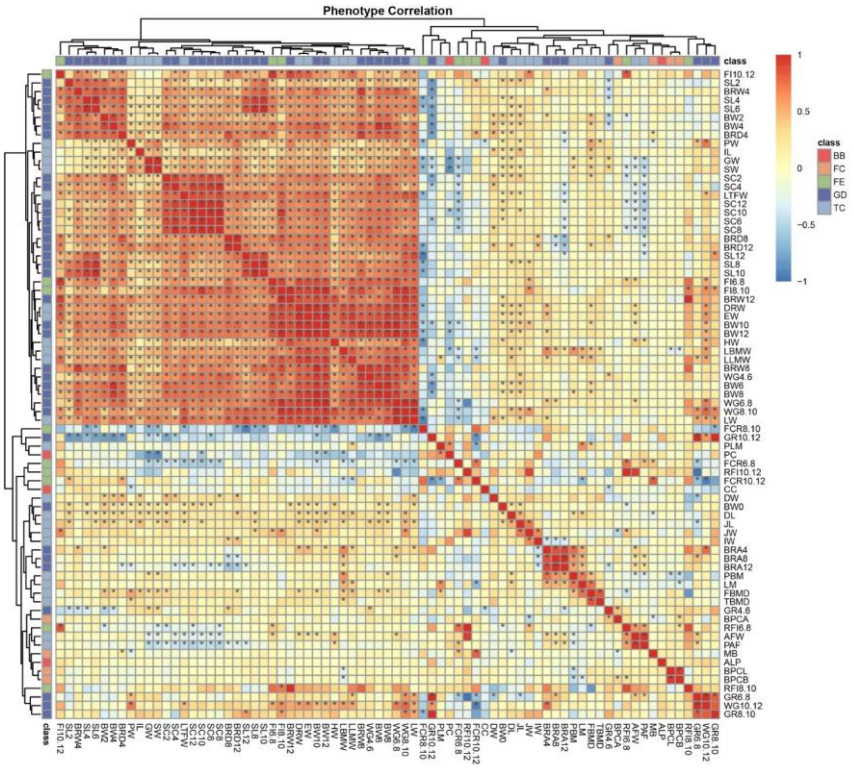
Pan, Z. et al. An atlas of regulatory elements in chicken: A resource for chicken genetics and genomics. *Sci. Adv*. **9**, eade1204 (2023).

Q32: Figure 4e: I don't understand what the color scale on this is

Authors' response: The color indicates the LD of SNPs with the colocalized SNP (rs316348444).

Q33: Supplementary Figure 3: which of these correlations are significant?

Authors' response: The GCTA "gcta64 --reml-bivar --grm test --pheno test.phen --out test" model does not output the p-value directly. Using r_g and SE, the p-value is estimated by the Wald test statistic. The specific results have been added to Supplementary Table 5 (Response Figure 2).



Response Figure 2 Genetic correlations for the 75 phenotypes in AIL F_{16}. "*" indicates p values less than 0.05.

Q34: Supp Fig 6: why was no multiple hypothesis correction performed given that there were 14 hypotheses? Also, what are the measurements in the distributions of p values and how was it determined whether a whole row was significant or not? Are these t tests between distributions of p values, and if so, is there any precedent for doing a t test between distributions of p-values like this? Very confused about this figure

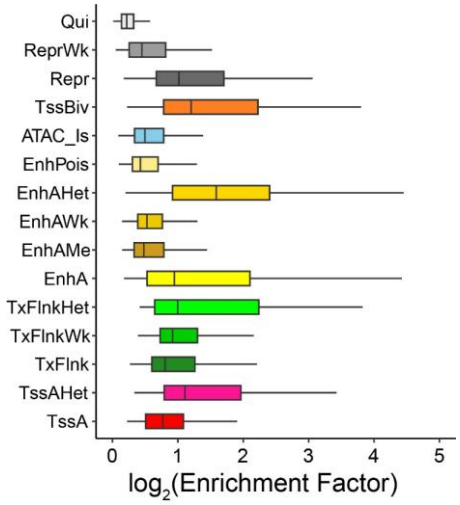
Authors' response: Thank you very much for your questions. We added the enrichment factor results and corrected the p-values for p-enrichment using FDR.

For the results presented in the figure, we assessed the significance of enrichment of GWAS signals in each functional region by calculating the summary statistic (sum of squares of all SNP effect size) and generating P-values based on the permutation test of the summation-based labeled set test; this uses the effect sizes of all SNPs, does not take into account whether the SNPs are significant or not, and is not applicable to calculating enrichment fold but rather to determine whether enrichment has occurred through statistical significance. Furthermore, the analysis method refers to Fang et al.

In order to show the enrichment fold more clearly, we utilized all SNPs as a background for calculating enrichment and used a hypergeometric distribution test to calculate p-values.

Enrichment factor = (target region significant SNPs/total significant SNPs) / (target region all SNPs/ genome-wide total SNPs)

We performed enrichment analysis using 40 phenotypes with 15 functionally annotated regions in 23 tissues, and the results are provided in Supplementary Figure 7. As shown in the figure, we filtered the results with FDR < 0.05 for plotting and found that GWAS signaling was enriched at a high level in regions such as promoters (TssA, TssBiv) and enhancers (EnhA and EnhAHet) (New Supplementary Figure 7).



New Supplementary Figure 7 Genome-wide association study (GWAS) signal enrichment was analyzed within chromatin states across 23 tissues and 40 complex traits in chickens. The figure illustrates the results at FDR<0.05.

Reference:

Fang, L. et al. Comprehensive analyses of 723 transcriptomes enhance genetic and biological interpretations for complex traits in cattle. *Genome Res*. **30**, 790–801 (2020).

Dear editor and reviewers,

We sincerely appreciate your insightful feedback on our manuscript entitled "**Mapping the regulatory genetic landscape of complex traits using a chicken advanced intercross line**" (NCOMMS-25-05509A).

We have carefully revised the manuscript based on all your comments and suggestions and comments of author checklist file, which have significantly improved the manuscript. We have highlighted the revised sections of the manuscript in red and provided point-by-point responses to each of reviewers' comments below.

We hope that our revisions have satisfactorily addressed all the comments. Thank you very much for your time and consideration.

Sincerely,

Dr. Yuzhe Wang

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Tel: +86-010-62731314

Email: yuzhe891@cau.edu.cn

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author): The authors addressed all my concerns in this revision. There is no further comments.

Authors' response: Thank you for your recognition.

Reviewer #2 (Remarks to the Author): The authors have done an excellent job addressing my comments as well as those of the other reviewers. I have no further questions and am pleased to recommend this outstanding work for publication.

Authors' response: Thank you for your recognition.

Reviewer #3 (Remarks to the Author): The authors have improved the manuscript and addressed all my comments to my satisfaction, so I am happy to recommend the manuscript for publication.
My only additional comment: I should have been more explicit in these cases, but when I asked a question in my review such as "what are eGenes" or "what is SMR analysis", I was asking for these terms to be defined in the manuscript the first time they are used, not explained to me in the responses. It still appears that many of these terms are not defined in the main text of the manuscript. I apologize for the confusion but still hope that the authors go through the manuscript to ensure that all acronyms and non-standard terms like these are defined to make the text as clear as possible.

Authors' response: Thank you for your recognition and suggestions. We have carefully reviewed the manuscript to ensure that all acronyms and non-standard terms such as "eGenes" and "SMR analysis" are clearly defined. In order to maintain a smooth flow in the main text while ensuring precision and clarity for readers seeking technical details. We have provided more detailed explanations in Methods. We hope these changes enhance the accessibility of the manuscript for a broad audience.