A Missense Mutation in **PPARD** Causes a Major QTL Effect on Ear Size in Pigs

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Abstract

Chinese Erhualian is the most prolific pig breed in the world. The breed exhibits exceptionally large and floppy ears. To identify genes underlying this typical feature, we previously performed a genome scan in a large scale White Duroc × Erhualian cross and mapped a major QTL for ear size to a 2-cM region on chromosome 7. We herein performed an identical-by-descent analysis that defined the QTL within a 750-kb region. Historically, the large-ear feature has been selected for the ancient sacrificial culture in Erhualian pigs. By using a selective sweep analysis, we then refined the critical region to a 630-kb interval containing 9 annotated genes. Four of the 9 genes are expressed in ear tissues of piglets. Of the 4 genes, PPARD stood out as the strongest candidate gene for its established role in skin homeostasis, cartilage development, and fat metabolism. No differential expression of PPARD was found in ear tissues at different growth stages between large-earred Erhualian and small-eared Duroc pigs. We further screened coding sequence variants in the PPARD gene and identified only one missense mutation (G32E) in a conserved functionally important domain. The protein-altering mutation showed perfect concordance (100%) with the QTL genotypes of all 19 founder animals segregating in the White Duroc × Erhualian cross and occurred at high frequencies exclusively in Chinese large-eared breeds. Moreover, the mutation is of functional significance; it mediates down-regulation of β-catenin and its target gene expression that is crucial for fat deposition in skin. Furthermore, the mutation was significantly associated with ear size across the experimental cross and diverse outbred populations. A worldwide survey of haplotype diversity revealed that the mutation event is of Chinese origin, likely after domestication. Taken together, we provide evidence that PPARD G32E is the variation underlying this major QTL.

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Competing Interests: The Jiangxi Agricultural University has applied for a patent covering the use of markers for the PPARD mutation for marker-assisted selective breeding in pigs. LH, JR, YD, RQ, and FY are listed as inventors in this application.

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Introduction

The external ear is part of the auditory system and plays a vital role in collecting sound as the first step in hearing. Multiple congenital anomalies have been documented for human external ears. For instance, microtia, characterized by a small and abnormally shaped outer ear, occurs in approximately one in 8,000–10,000 births. However, only in a minority of cases has a genetic or environmental cause been found [1]. The domestic pig services as not only an agriculturally important animal for meat production but also an important large-animal model for human medicine [2]. Thousands of years of selective breeding has created diversity of phenotypes in pigs, such as ear size in Erhualian and White Duroc breeds. Erhualian is the most prolific pig breed and exhibits unusually large and floppy ears as breed character (Figure 1). Historically, the large-ear feature of Erhualian pigs has been favored by owners for the traditional sacrificial culture [3]. White Duroc is one of worldwide-popular boar line and has small and erect ears (Figure 1). We have created a four-generation White Duroc × Erhualian resource population, in which phenotypic traits related to ear size have been recorded in 1,027 adult F2 animals and 560 adult F3 individuals (Table S1). We mapped a major QTL for ear size around 58 cM on SSC7 (Figure S1) using a genome scan on the White Duroc × Erhualian cross [4], which confirmed the previously reported QTL affecting ear size in a Large White × Meishan F2 resource population [5]. The significant QTL had a small confidence interval of 2 cM and explained more than 40% of phenotypic variance. The aim of this study was to identify the genetic determinant underlying this major QTL.

Results/Discussion

Identical-by-descent analysis defines the major QTL within a 750-kb interval

To fine map the QTL, we genotyped 1,027 adult F2 animals and their 68 parents and 19 grandparents in the White Duroc × Erhualian cross using additional 17 SNP markers and 11 microsatellite markers in the QTL region. A final set of 33 markers covering the QTL region were then explored to deduce the QTL genotypes of F1 sires by the marker-assisted segregation analysis as proposed previously [6]. We determined QTL genotypes of all 9 F1 sires (Figure S2). All 9 Q-bearing chromosomes for increased ear size shared a haplotype of ~1.2 Mb flanked by markers **HMGA1**–**TULP1**. The shared haplotype was distinct from q-bearing chromosomes (Figure 2).
Author Summary

A central but challenging objective in current biology is to dissect the genetic basis of quantitative traits. Numerous quantitative trait loci (QTL) have been uncovered in model and farm animals, providing unexpected insights into the biology of complex traits. However, only a few causal variants underlying the QTL have been explicitly identified. By using a battery of genetic and functional assays, we herein show that a major QTL effect on pig ear size is most likely caused by a single base substitution in an evolutionary conserved region of the PPARD gene. The protein-altered mutation is of functional significance and explains a proportion of variation in ear size across diverse pig breeds. A worldwide survey showed that the mutant allele for increased ear size was derived from a common ancestor in Chinese pigs, likely after domestication. These findings establish, for the first time, an essential role of PPARD in ear development and highlight the great potential of naturally occurring mutations in farm animals to gain insights into mammalian biology. Moreover, the knowledge of the PPARD causal mutation adds to the limited list of quantitative trait genes and quantitative trait nucleotides characterized in domesticated animals.

These observations strongly suggest that the QTL is located in the 1.2-Mb interval.

Given the extremely divergent ear size phenotypes between Erhualian and White Duroc animals, we assumed that Q and q alleles were alternatively fixed in Erhualian and White Duroc founder animals; hence all Erhualian founder sows could share a chromosomal segment carrying the Q allele for increased ear size. To test this assumption, we reconstructed haplotypes of all 19 founder animals (2 sires and 17 dams) using 50 markers (15 microsatellites and 35 SNPs) in the QTL region. Almost all Erhualian founder sows shared a haplotype of ~750 kb within the refined 1.2-Mb interval (Figure 2). As predicted, this shared haplotype was associated with increased ear size and presumably Q-bearing chromosomes. Two Erhualian founder sows carried a distinct haplotype (denoted as E^q), which was unexpected because it was contrast with our initial assumption. We then conducted a statistical analysis of F_2 animals in the White Duroc × Erhualian cross. The results revealed that the E^q chromosome had an effect on decreased ear size similar to the White Duroc chromosome (D^q) and significantly different from the Erhualian Q-bearing chromosome (E^Q). The least-squares means (± s.e.) of ear weight were 323.07±4.55 for E^QEQ, 266.66±10.9 for E^qE^q (P=0.04); 264.71±5.52 for D^qE^q and 236.90±17.12 for D^qD^q (P=0.06, Table 1). The shared E^q chromosome allowed us to refine the location of the major QTL to the 750-kb interval between markers UHRF1BP1 and TULP1 (Figure 2).

Selective sweep analysis refines the QTL to a 630-kb region

Historically, Erhualian pigs had undergone selection for ear size because pigs with extraordinary large and floppy ears were favored for the ancient sacrificial culture in the Taihu region of East China [3]. Reduced genetic variation in the critical region containing the QTL was therefore predicted. To define the region of reduced genetic variation, we collected 211 animals representing all lineages in 3 Erhualian nucleus populations, 216 animals from 6 Chinese indigenous breeds and 119 independent animals from 3 Western worldwide-popular commercial breeds. Using these samples, we genotyped 6 microsatellite and 32 SNP markers in the 750-kb region. We found that 18 adjacent markers in a 630-kb region between markers UHRF1BP1 and FANCE showed dramatically reduced polymorphisms in all Erhualian pigs with nearly all major allele frequencies of more than 0.90. Notably, the 18 markers in the 630-kb region are monomorphic in the Erhualian nuclear population from Xishan county (n=72). In comparison, the genetic polymorphisms of these markers were maintained in other Chinese, Western breeds, and wild boars (Figure 3). The 630-kb region showing strong selective-sweep effects on Erhualian pigs was therefore predicted to contain the responsible locus. We further genotyped the 18 markers in the 630-kb region on 188 adult animals of Sutai pigs. This breed was developed after 18-generation selection from a Duroc (50%) × Erhualian (50%) cross in 1986 [7], meaning that the breed has undergone 18 generations of meiosis reducing the extent of linkage disequilibrium between QTL and linked markers. The Erhualian-originated haplotype of 630 kb showed significant (P=0.009) association with increased ear size compared with other chromosomes in Sutai pigs (Figure S3), thereby supporting the conclusion that this region harbors the causative gene.

Figure 1. The Erhualian and White Duroc phenotypes. Erhualian pigs (right panel) are obese and short legged, have wrinkly face, extremely large and floppy ears. In comparison, White Duroc pigs (left panel) are renowned for muscularity and exhibit much smaller and half or fully pricked ears. doi:10.1371/journal.pgen.1002043.g001
### PPARD Causal Mutation for Ear Size in Pigs

| ID | Genes Affected |
|----|----------------|
| F1 | 38              |
| F1 | 39              |
| F1 | 52              |
| F1 | 53              |
| F1 | 54              |
| F1 | 55              |
| F1 | 56              |
| F1 | 68              |
| F1 | 69              |
| F1 | 74              |
| F1 | 75              |
| F1 | 80              |
| F1 | 90              |
| F1 | 91              |
| F1 | 92              |
| F1 | 93              |
| F1 | 124             |
| F1 | 125             |
| F1 | 142             |
| F1 | 143             |
| F1 | 144             |
| F1 | 174             |
| F1 | 190             |
| F1 | 194             |
| F1 | 202             |
| F1 | 206             |
| F1 | 207             |

### PLoS Genetics | www.plosgenetics.org 3 May 2011 | Volume 7 | Issue 5 | e1002043
Positional candidate gene analysis: discovery of a nonconservative missense mutation in \textit{PPARD} concordant with the QTL genotypes of founder animals

The 630-kb region encompasses 9 annotated genes (\textit{AXESIA}, \textit{DEF5}, \textit{FANCE}, \textit{PPARD}, \textit{SCUBE3}, \textit{TAF11}, \textit{TCP11}, \textit{UHR1BPI} and \textit{\textgreek{Z}NF76}) in the human homologous region. RT-PCR was performed to detect expression levels of these genes in ear tissues of piglets. Four genes including \textit{PPARD}, \textit{FANCE}, \textit{TAF11} and \textit{\textgreek{Z}NF76} were highly expressed, whereas transcripts of other genes were almost absent in ear tissues (data not shown). Of the 4 genes, \textit{PPARD} (peroxisome proliferator-activated receptor delta) is a ligand-modulated transcription factor belonging to the nuclear receptor superfamily and plays crucial roles in diverse biologically important processes [8]. For instance, \textit{PPARD} play a pivotal role in modulating cell differentiation in both keratinocytes and sebocyte of skin [9]. \textit{PPARD} also serves as a key regulator in fat metabolism; it triggers fat burning and enhances energy uncoupling in adipose tissues and skeletal muscle [10–12]. Moreover, \textit{PPARD} is a key player in Wnt/\textbeta-catenin pathway [13], which has essential roles in diverse cellular activities including chondrocyte proliferation and differentiation [14]. The external ear is composed of skin, cartilage, connective tissues and fat. Given its crucial role in skin homeostasis, cartilage development and organogenesis [14]. To demonstrate functional significance of \textit{PPARD} is involved in the Wnt/\textbeta-catenin signaling pathway that regulates diverse cellular functions. In the nucleus, \textit{PPARD} mediates down-regulation of \textbeta-catenin and its known downstream gene, \textit{c-myc} [16] genes along with \textit{GAPDH} and \textit{Sox9} in primary fibroblast cells with the lentiviral \textit{PPARD} expression vectors and monitored the mRNA levels of \textbeta-catenin and \textit{c-myc} protein levels were decreased by the mutant \textit{PPARD} treatment (Figure 5B). Western blot analysis showed that both \textbeta-catenin and \textit{c-myc} protein levels were decreased by the mutant \textit{PPARD} transfectants compared with the cells transfected with wild-type \textit{PPARD}. Western blot analysis showed that both \textbeta-catenin and \textit{c-myc} protein levels were decreased by the mutant \textit{PPARD} treatment (Figure 5B). GAPDH was used as a protein loading control for total cell lysate, which was not affected by both wild-type and mutant \textit{PPARD} treatments (Figure 5B). Altogether, we conclude that \textit{PPARD} G32E is a functional variant that mediate down-regulation of \textbeta-catenin and its

to glutamic acid substitution at codon 32 (GU565977) in the conserved intrinsically disordered domain of the \textit{PPARD} protein predicted by SMART (http://smart.embl-heidelberg.de/). The intrinsically disordered domain is a distinctive and common characteristic of eukaryotic hub proteins like multifunctional nuclear receptors and serves as a determinant of protein interactivity [15]. Comparison of amino acids of this protein domain across mammals revealed that glycine is well conserved in mammalian \textit{PPARD}s (Figure 4), while the derived glutamic acid occurs only in alleles increasing ear size in pigs. We thus speculated that the nonconservative substitution probably changes the \textit{PPARD} interactivity with other protein partners and consequently affects the gene’s regulation function. Genotypes of \textit{F1} sires (9 heterozygotes) and \textit{F0} animals (17 homozygotes and 2 heterozygotes) at the mutation site were 100% concordance with their QTL genotypes. The potentially altered function and QTL concordance of \textit{PPARD} G32E corresponded to the hypothesis that this SNP may be the causative mutation underlying the major QTL.

\textit{PPARD} G32E is a functional variant mediating down-regulation of \textbeta-catenin and its target gene expression

\textit{PPARD} is involved in the Wnt/\textbeta-catenin signaling pathway that regulates diverse cellular functions. In the nucleus, \textit{PPARD} interacts with \textbeta-catenin binding to TCF/LEF transcription factors that stimulate transcription of target genes important for multiple cellular activities including cartilage development and organogenesis [14]. To demonstrate functional significance of \textit{PPARD} G32E, we cotransfected the 293T cells with the lentiviral expression vectors of wild-type or mutant \textit{PPARD} and a TCF/LEF-driven luciferase reporter construct. A \textit{Renilla} luciferase expression vector was used for the normalization of transfection efficiency. Overexpression of mutant \textit{PPARD} led to a 40% decrease (\textit{P}=0.05 compared with the wild-type treatment) in TCF/LEF reporter activity (Figure 5A), indicating the G32E mutation mediates down-regulation of \textbeta-catenin downstream genes. To examine a direct functional role of \textit{PPARD} G32E in target genes of \textbeta-catenin, we treated pig ear-derived primary fibroblast cells with the lentiviral \textit{PPARD} expression vectors and monitored the mRNA levels of \textbeta-catenin and its known downstream gene, \textit{c-myc}. Western blot analysis showed that both \textbeta-catenin and \textit{c-myc} protein levels were decreased by the mutant \textit{PPARD} treatment (Figure 5B), thereby confirming the results of mRNA and luciferase reporter analyses. \textit{Sox9} mRNA expression in mutant \textit{PPARD} transfectants was only slightly decreased to 1.1-fold of the wild-type \textit{PPARD} treatment; the result was validated by Western blot (Figure 5B). GAPDH was used as a protein loading control for total cell lysate, which was not affected by both wild-type and mutant \textit{PPARD} treatments (Figure 5B). Altogether, we conclude that \textit{PPARD} G32E is a functional variant that mediate down-regulation of \textbeta-catenin and its

\begin{table}
\centering
\caption{Effects of Erhualian Q or q-bearing chromosomes on ear weight in the White Duroc × Erhualian F2 cross.\textsuperscript{a}}
\label{tab:1}
\begin{tabular}{lccc}
\hline
Genotype & Least square mean ± standard error (g) \\
\hline
\textgreek{D}D\textgreek{D} & 197 & 185.30 ± 4.49 \textsuperscript{a} \\
\textgreek{D}D\textgreek{E} & 443 & 264.71 ± 3.52 \textsuperscript{a} \\
\textgreek{D}E\textgreek{E} & 10 & 236.98 ± 17.12 \textsuperscript{ab} \\
\textgreek{E}\textgreek{E}\textgreek{E} & 194 & 323.07 ± 4.55 \textsuperscript{c} \\
\textgreek{E}\textgreek{E}\textgreek{D} & 7 & 266.66 ± 18.9 \textsuperscript{a} \\
\hline
\end{tabular}

\textsuperscript{a} \textgreek{E}\textsuperscript{D} represented the major chromosome and \textgreek{E}\textsuperscript{E} indicated the other distinct chromosome in Erhualian founder sows. Phenotypic values were corrected for fixed effects including sex, batch and SSC5 QTL for ear size and a covariate of carcass weight. Significance was evaluated by the t-test in the GLM procedure of SAS 9.0 (SAS Institute, Cary, NC). Values with different superscripts are significantly different (\textit{P}<0.05).
\end{table}
target gene expression in the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling has been firmly demonstrated to suppress adipogenesis [18–19]. The fact that PPARD is a key modulator of lipid production in the skin [9] and that PPARD G32E inhibits β-catenin expression led us to assume that the mutation stimulates lipid production and storage that are required for enlarged ear size.

PPARD G32E is significantly associated with ear size across the experimental intercross and outbred populations.

To confirm the effect of PPARD G32E on ear size, we performed a standard association test, a marker-assisted association test and an F-drop test [20] in the White Duroc × Erhualian cross. The SNP showed greatly significant (P<0.0001) association with ear weight and ear size in the standard association test. In the marker-assisted association test, the SNP was more significant (P<0.001) for these traits compared with the QTL effect. After fitting this polymorphism in the QTL model, the great QTL effect disappeared with F-value drop rations of less than 0.03 (Table S2). These results were in agreement with the hypothesis that the SNP is the causative mutation for the major QTL affecting ear size. Nevertheless, we cautioned the results because variants closely linked with a causative mutation also lead to strong association in F2 resource populations due to the high level of linkage disequilibrium between founder breeds [20].

To obtain additional supporting evidence, we further genotyped the G32E mutation on 667 mature pigs from 4 Chinese local breeds (Erhualian, Hang, Yushan Black and Bama Xiang) and 3 synthetic commercial lines (Sutai, Suzhong, Sujiang) with phenotypic data of ear size. These populations show a wide range of ear size and segregate for the mutation. The association analyses confirmed the effect of PPARD G32E on ear size. The 32E allele was significantly associated with increased ear size across the tested breeds (P<0.05; Table 2). Chinese local pig breeds have low levels

**Figure 4. Conservation of the intrinsically disorder domain of PPRAD protein in mammals.** The ClustalW alignment of predicted amino acids of 8 orthologous PPARD genes is shown. The sequences for the alignment were taken from the following accessions: NP_001123713 and ADF55028 (Sus scrofa), NP_001077105 (Bos taurus), NP_001041567 (Canis lupus), XP_001498920 (Equus caballus), NP_006229 (Homo sapiens), XP_001172224 (Pan troglodytes), NP_035275 (Mus musculus) and NP_037273 (Rattus norvegicus). The G32E substitution in a conserved hepta-amino acid region (grey box) is indicated by the asterisk. Glycine is the conserved amino acid at this position in the wild-type pigs (Sus scrofa, W) and other mammals, whereas glutamic acid occurs only in alleles increasing ear size in pigs (Sus scrofa, M).

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of linkage disequilibrium extending up to only 0.05 cM [21]. The concordantly significant association across Chinese breeds thereby strengthened the hypothesis that PPARD G32E is the responsible locus for ear size. The effects of PPARD G32E differ in their magnitude in the tested breeds; one reason is that the effects are context-dependent and are influenced by different genetic backgrounds and environments. Another possibility is that PPARD G32E is only responsible for part of the effect on ear size in Erhualian pigs.

PPARD G32E has a unique origin of Chinese pigs likely after domestication

To reveal the ancestral state and allele frequency of PPARD G32E in diverse pig breeds, we genotyped the mutation in a panel of 1,166 animals representing 31 domestic breeds and Chinese and European wild boars. Overall, the derived 32E allele for increased ear size occurred at high frequencies (>0.80) in Chinese breeds with large and floppy ears. In contrast, the 32G allele for normal ear size was fixed in all wild boars, European local and commercial breeds, and occurred at low frequencies (<0.30) in Chinese indigenous breeds having small and erect ears. These results indicated that PPARD G32E may occur in Chinese pigs after domestication. We detected only one heterozygote in European local breeds (Table 3). The animal was from Large Black pigs that exhibit large and floppy ears and have been influenced by Chinese breeds brought into England in the late 1800’s [22].

We further analyzed the genetic variability and haplotype structure around the G32E mutation in a worldwide pig panel. A total of 868 animals representing 34 breeds were genotyped for 32 SNPs in a 77-kb region of PPARD. Again, the Erhualian breed showed a selective sweep signal as it had negative classical selection statistics Tajimas D and much smaller nucleotide variability (πN) compared with other Chinese local breeds and Western commercial breeds (Table S3). Especially, the genetic variability at the 32 loci was wiped out in the Erhualian population from Xishan. Moreover, we plotted a distribution of the frequency of the derived 32E allele (pN) against Tajimas D index to elucidate the existence of directional selection for the G32E mutation. When

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Table 2. Effect of the PPARD G32E substitution on ear size in 7 outbred populations.

| Population     | No. | Genotype | GG   | GE   | EE   | P value |
|----------------|-----|----------|------|------|------|---------|
| Erhualian      | 105 | -        | 397.85±15.87 (n=32) | 460.17±10.51 (n=73) | 0.0014  |
| Hang           | 58  | 169.01±11.51 (n=7) | 213.49±5.88 (n=23) | 225.38±5.42 (n=28) | 0.0001  |
| Suijiang       | 80  | 258.31±5.25 (n=63) | 282.58±10.19 (n=17) | - | 0.0374  |
| Sutai          | 177 | 257.29±6.98 (n=42) | 274.62±5.19 (n=76) | 290.45±6.98 (n=59) | 0.0017  |
| Suzhong        | 81  | 214.97±6.11 (n=56) | 238.80±9.14 (n=25) | - | 0.0332  |
| Yushan Black   | 64  | -        | 172.80±5.86 (n=23) | 200.78±4.33 (n=41) | 0.0002  |
| Bama Xiang     | 102 | 55.70±1.28 (n=59) | 61.15±1.73 (n=32) | 65.41±2.96 (n=11) | 0.0027  |

a Least square mean ± standard error (cm²) is given for each genotype. Significance was evaluated by the GLM procedure of SAS 9.0 (SAS Institute, Cary, NC). doi:10.1371/journal.pgen.1002043.0002
Table 3. Frequencies of the derived 32E allele in different ear-sized outbred pig populations.

| Phenotype                     | Breed                      | Number | Allele frequency |
|-------------------------------|----------------------------|--------|------------------|
| Chinese breeds                |                            |        |                  |
| Large and floppy ears         | Erhualian, Xishan          | 67     | 1.00             |
|                               | Erhualian, Nanchang        | 67     | 0.95             |
|                               | Erhualian, Wujin           | 105    | 0.85             |
|                               | Erhualian, Changshu        | 72     | 0.85             |
|                               | Hetao Large-ear            | 55     | 0.81             |
|                               | Jiaxing Black              | 32     | 1.00             |
|                               | Meishan                    | 23     | 0.82             |
| Medium-size and floppy ears   | Hang                       | 58     | 0.68             |
|                               | Jiangquhai                 | 30     | 0.07             |
|                               | Jinhua                     | 30     | 0.00             |
|                               | Laiwu                      | 29     | 0.00             |
|                               | Lantang                    | 30     | 0.83             |
|                               | Minzhu                     | 30     | 0.67             |
|                               | Ningshiang                 | 22     | 0.14             |
|                               | Rongchong                  | 29     | 0.38             |
|                               | Tongcheng                  | 29     | 0.45             |
|                               | Yushan Black               | 64     | 0.62             |
| Small, erect or half-flicked ears | Bama Xiang               | 32     | 0.30             |
|                               | Diannan Small-Ear          | 31     | 0.00             |
|                               | Tibetan                    | 34     | 0.00             |
| Chinese wild boar             |                            | 22     | 0.00             |
| Western breeds                |                            |        |                  |
|                               | Duroc                      | 58     | 0.00             |
|                               | European domestic pigs     | 28     | 0.02             |
|                               | Landrace                   | 71     | 0.00             |
|                               | Large White                | 93     | 0.00             |
|                               | White Duroc                | 12     | 0.00             |
| European wild boar            |                            | 13     | 0.00             |

*European domestic pigs include Iberian (n = 10), Berkshire (n = 4), Large Black (n = 2), Mid White (n = 2), Chester White (n = 2), Old Spot (n = 2), Yorkshire (n = 2), Tamworth (n = 1), British Lop (n = 1), Hampshire (n = 1), Saddle Back (n = 1). All these animals are homozygous GG except a heterozygote detected in one of two Large Black pigs, which exhibits large or medium-size and floppy ears and is influenced by Chinese breeds brought into England in the late 1800’s (Kijas et al. 1998) [22].

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$P_A = 0$, Tajima’s $D$ was highly variable across breeds, likely due to demographic and/or sampling effects. In stark contrast, Tajima’s $D$ took highly negative values when $P_A > 0.8$ in Erhualian and other Chinese large-eared breeds as expected in a classical directional selection (Figure S5). We reconstructed 16 major haplotypes with frequencies larger than 0.01 from the 32 SNPs genotyped. Of the 16 haplotypes, only one carried the derived 32E allele; it was at high frequencies in Erhualian pigs and intermediate frequencies in some floppy-eared Chinese breeds whereas absent in Western pigs and wild boars (Table 4). The NJ phylogenetic tree illustrated that the typical haplotype of Erhualian pigs was generally divergent from other haplotypes (Figure S6). These observations supported the assumption that the G32E mutation has a unique origin in Chinese breeds likely after domestication and has undergone selection in Erhualian pigs. We calculated linkage disequilibrium measures ($r^2$) between all pairs of loci and inferred haplotype blocks. Three and two haplotype blocks were identified in the $PPARD$ region for Chinese indigenous pigs and Western commercial breeds, respectively. Only a single nevertheless larger block that spanned 53 kb and contained the G32E SNP was found in Erhualian pigs, reflecting a selection hitching effect (Figure S7). The G32E SNP was in high disequilibrium with very few of the SNPs analyzed (two with $r^2 > 0.8$), and there was no observable trend between physical distance and disequilibrium measures for the G32E SNP and the rest of loci (Figure S8).

Diverse pieces of evidence support the causality of $PPARD$ G32E for the QTL

The elucidation of the genetic basis of multifactorial traits in domestic animals is still a big challenge, and few successful examples have been reported [23–27]. In this study, a battery of genetic and functional assays obtained diverse pieces of supporting evidence that the $PPARD$ G32E substitution underlies the major QTL effect on ear size on SSC7. (1) The shared haplotypes of 9 F1 sires segregating for the QTL spanned a region of ~1.2 Mb containing $PPARD$. (2) All Erhualian founder chromosomal shared a ~750 kb segment spanning $PPARD$ that were associated with the Q allele for increased ear size. (3) Erhualian pigs showed an obvious selective sweep signal in a 630-kb region encompassing $PPARD$, the signal was concordant with the breeding history of the breed. (4) The 630-kb haplotype showed similar QTL effect on increased ear size in Sutai pigs that were developed after 18-generation selection in the Erhualian × Duroc cross. (5) Of the 4 genes expressed in ear tissues within the critical region, $PPARD$ stood out a prime candidate for its established essential roles in skin homeostasis, cartilage development and fat metabolism. (6) Only one missense mutation (G32E) was identified in $PPARD$ using White Duroc and Erhualian founder animals. The mutation caused a nonconservative amino acid change at the conserved intrinsically disordered domain and was of functional significance. (7) The G32E SNP was concordant with QTL genotypes of F0 and F1 animals in the White Duroc × Erhualian cross. (8) The G32E SNP showed strikingly significant association with ear size across the experimental cross and diverse outbred populations. (9) The derived allele for increased ear size occurred at high frequencies only in Chinese floppy-eared breeds. Altogether, these data led us to conclude that G32E in the $PPARD$ gene has an important contribution to ear size in pigs. The results establish, for the first time, a direct and novel role of $PPARD$ in ear development and may be of relevance for the pathogenesis of external ear abnormalities in humans.

Potential pleiotropic effects of $PPARD$ G32E on diverse traits

The genomic region harboring $PPARD$ G32E is of great interest in pig genetics, because significant QTL for diverse traits related to growth, carcass length, skeletal morphology and fat deposition have been consistently evidenced in the region using the current resource population and different crosses between Chinese Meishan and commercial breeds [28–33]. The overlapping QTL for multiple traits in the region led us to assume that there might be a single critical gene having pleiotropic effects on these traits. We herein showed the causality of $PPARD$ G32E for the QTL affecting ear size in the critical region. Given that $PPARD$ serve as a crucial and multifaceted determinant of diverse biological functions including fat metabolism, cartilage development, chondrocyte proliferation and differentiation.
Table 4. Distribution of major haplotype frequency in the PPARD gene in corresponding pig populations.

| No. | Haplotype | All | Erhualian | Chinese local breed | Chinese wild boar | Commercial breed | EU local breed | EU wild boar |
|-----|-----------|-----|-----------|---------------------|-------------------|-----------------|----------------|-------------|
| Haplo1 | CGTGGCCGACCATGAGGCGCTACACTCCAG | 0.42 | 0.91 | 0.55 | 0.00 | 0.00 | 0.00 | 0.00 |
| Haplo2 | TAC.TAGT.A.CC.GA.ACTGCGCGGT | 0.07 | 0.01 | 0.02 | 0.00 | 0.26 | 0.36 | 0.55 |
| Haplo3 | TAC.TAGT.A.CC.GA.ACTGCGCGGT | 0.07 | 0.00 | 0.02 | 0.00 | 0.28 | 0.15 | 0.35 |
| Haplo4 | .................GG....G...... | 0.06 | 0.00 | 0.07 | 0.17 | 0.01 | 0.06 | 0.05 |
| Haplo5 | ...G...A..CA....GGG...GT | 0.06 | 0.01 | 0.03 | 0.02 | 0.14 | 0.20 | 0.00 |
| Haplo6 | .................GG....G...... | 0.04 | 0.00 | 0.03 | 0.00 | 0.08 | 0.06 | 0.00 |
| Haplo7 | ....G.TGAG.CA.A...GGG..GT | 0.04 | 0.00 | 0.04 | 0.14 | 0.00 | 0.04 | 0.00 |
| Haplo8 | .................GG....G...... | 0.03 | 0.00 | 0.02 | 0.02 | 0.08 | 0.09 | 0.00 |
| Haplo9 | ....G.TGA.CA....GGG..G | 0.03 | 0.00 | 0.03 | 0.14 | 0.00 | 0.00 | 0.00 |
| Haplo10 | ....G.TGAG.CA.A...GGG..GT | 0.03 | 0.00 | 0.03 | 0.00 | 0.00 | 0.00 | 0.00 |
| Haplo11 | TAC.TAGT....CC.GA.ACTGCGCGGT | 0.02 | 0.01 | 0.02 | 0.00 | 0.05 | 0.02 | 0.00 |
| Haplo12 | G.G..GT | 0.02 | 0.00 | 0.01 | 0.00 | 0.06 | 0.00 | 0.00 |
| Haplo13 | G.G..GT | 0.02 | 0.00 | 0.02 | 0.10 | 0.00 | 0.00 | 0.00 |
| Haplo14 | G.G..GT | 0.02 | 0.00 | 0.02 | 0.10 | 0.00 | 0.00 | 0.00 |
| Haplo15 | G.G..GT | 0.01 | 0.00 | 0.01 | 0.17 | 0.00 | 0.00 | 0.00 |
| Haplo16 | A.C.TAGT....CC.GA.ACTGCGCGGT | 0.01 | 0.01 | 0.01 | 0.00 | 0.03 | 0.00 | 0.05 |

*Haplo1 is the typical haplotype of Erhualian pigs that carries the derived G32E allele; Haplo2 and Haplo3 represent European haplotypes for their predominant presence in European commercial and local breeds and wild boars; Haplo4 is an ancient haplotype that was evidenced in both Chinese and European wild boars. Haplo9 and Haplo15 are two additional ancient haplotype mainly pertain to Chinese wild boars. The PPARD G32E alleles are indicated in bold.*

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[8,10–14], we thus speculate that PPARD is a strong candidate of the multiple significant QTL on SSC7 and that PPARD G32E might have pleiotropic effects on growth, carcass and fatness traits in pigs. Further investigations will be performed to validate the hypothesis.

Methods

Ethics statement

All animal work was conducted according to the guidelines for the care and use of experimental animals established by the Ministry of Agriculture of China.

Fine mapping by identical-by-descent analysis

Microsatellite markers in the mapped interval were mined from the pig genome assembly (Build 9.2) at http://www.ensembl.org/Sus_scrofa/Info/Index and were genotyped using standard procedures. Primers for amplification of microsatellite markers are given in Table S4. QTL genotypes of F1 boars in the White Duroc × Erhualian intercross were determined by marker-assisted segregation analysis as described previously [6]. Briefly, a Z-score was calculated for each F1 sire; the score is the log10 of the H1/H0 likelihood ratio where H1 assumes that the boar is heterozygous at the QTL (Qz), while H0 postulates that the boar is homozygous QQ or qq. Boars were considered to be Qz when Qz > 2, Qz or qq when Qz < -2, and of undetermined genotype if -2 < Qz < 2. The pedigree and management of the intercross population with phenotypic data of ear size have been described elsewhere [4]. Haplotypes of founder animals were reconstructed with the SimWalk2 program.

Selective sweep detection

To detect the effects of a putative selection sweep on the genetic variation in Erhualian pigs compared with control animals, we analyzed the microsatellite and SNP genotypes of 211 Erhualian pigs and 335 control animals representing 10 different breeds (Hetao Large-Ear: 56; Laiwu: 32; Yushan Black: 31; Wuzhishan, 32; Dahan Small-Ear: 31; Tibetan: 34; White Duroc: 12; Duroc: 29; Large White: 39; Landrace: 39). SNP markers were genotyped using the ABI SNapshot protocol or PCR-RFLP assays. All primers are given in Table S4.

RT-PCR of candidate genes in ear tissues

Total RNA was extracted from pig tissues using the Rneasy Fibrous Tissue Mini Kit (Qiagen). To analyze expression of candidate genes in ears, products from the first strand-complementary DNA synthesis (TaKaRa) were amplified with primers given in Table S5. The quantification of the PPARD transcripts was performed by the comparative Ct method (2DΔCt) using the primers and TaqMan probes shown in Table S5. Real-time PCR was done with the Universal PCR Master Mix using an ABI7900 instrument (Applied Biosystem). All samples were analyzed in triplicate. The β-actin gene was used as the internal reference gene.

Resequencing of PPARD cDNA and genotyping of PPARD G32E

The entire coding region of porcine PPARD was re-sequenced using ear mRNA of two White Duroc and two Erhualian animals. Primer pairs listed in Table S6 were used to generate overlapping PCR fragments. All PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel) and sequenced using the same primers. The sequence traces were assembled and analyzed for polymorphisms using the SeqMan program (DNASTAR). The PPARD G32E mutation was genotyped using the ABI SNapshot protocol. A 385-bp DNA fragment was amplified with the F2/R2 primer pairs (F2: 5'- CCG GGT TTT TGC TGG AGG GAA GCG AGT GCT CTG 9'- AAG GA-3'; R2: 5'- CTG TAC TCA GAC CCA GAT GA-3'). SNapshot reactions were performed with Multiplex Ready Reaction Mix (Applied Biosystem) and an extension primer (5'- TTT TTT TTT TGC TGG AGG GAA GCG AGT GCT CTG GT -3') using an ABI 3130XL DNA Analyzer (Applied Biosystem).
Luciferase report assay

The coding region of pig PPARD was amplified with primers PPARD-Age-I-F (5'-GAG GAT CCC GGG GTA CCG GTG GCC ACC ATG CAG CCG CCG GAG-3') and PPARD-Age-I-R (5'-TCA TCC TTG TAG TCG GTA CCG TAG ATG TCC TTG TAG-3'). The amplified cDNA was gel-purified and digested with AgeI and HindIII. The restricted fragments were cloned to pGC-FU-EFDP-3FLAG lentiviral expression vector (Genechem). The sequence and orientation of the insert were verified by DNA sequencing. The expression of His-tagged PPARD in cultured cells was confirmed by Western blot analysis with anti-His antibody. The human 293T cells were infected with the lentiviral expression constructs of pig wild-type and mutant PPARD. The infected cells were seeded at a concentration achieving 80% confluence in 96-well plates 10 h before transfection. The cells were transiently transfected with TCF/LEF-Luc reporter vector (Cignal, SAB) along with a control Relnt luciferase vector using Lipofectamine plus reagent. The cell lysates were obtained with 1x reporter lysis buffer (Promega) 48 h after transfection. The luciferase activity was assayed in a Berthold Auto Lumar LB953 luminometer (Nashua, NH) by using the luciferase assay system from Promega. The relative luciferase activity was normalized to the Relnt luciferase activity in each sample.

Real-time RT-PCR and western blot analysis in cultured cells

The pig ear-derived fibroblast cells were transfected with pGC-FU-EGFP-3FLAG lentiviral expression vector (Genechem). Five days post-transfection, 1x10^6 cells were harvested for qPCR and Western blot analysis. Total RNA was extracted from harvested cells using Trizol (Invitrogen). Two μg of total RNA was synthesized into cDNA with M-MLV reverse transcriptase (Promega) and oligo d(T). Real-time PCR was performed on the cDNA using the SYBR Premix Ex Taq (TaKaRa) and primers listed in Table S7 in a 90°C for 1 min. Real-time PCR was performed in triplicate. The expression values were normalized with β-actin (Sigma). Rabbit anti-PPARD (Sigma), mouse anti-β-catenin (Abcam), rabbit anti-c-myc (Cellsignaling), mouse anti-Sox9 (Abcam) and mouse anti-GAPDH (Santa Cruz) antibodies were used in Western blots in a routine way. The specific immunoreactive bands were visualized using an ECL plus kit (GE Healthcare) and quantified with the Molecular Imaging Software (Kodak).

Association analysis

The entire White Duroc × Erhualian resource population was genotyped for the PPARD G32E mutation. Association of the mutation with ear size and weight was evaluated using standard association, marker-assisted association and F-drop test as described previously [20]. Association analyses were also performed on 667 pigs genotyped for the PPARD-Age-I-F (5'-GAG GAT CCC GGG GTA CCG GTG GCC ACC ATG CAG CCG CCG GAG-3') and PPARD-Age-I-R (5'-TCA TCC TTG TAG TCG GTA CCG TAG ATG TCC TTG TAG-3'). The amplified cDNA was gel-purified and digested with AgeI and HindIII. The restricted fragments were cloned to pGC-FU-EFDP-3FLAG lentiviral expression vector (Genechem). The sequence and orientation of the insert were verified by DNA sequencing. The expression of His-tagged PPARD in cultured cells was confirmed by Western blot analysis with anti-His antibody. The human 293T cells were infected with the lentiviral expression constructs of pig wild-type and mutant PPARD. The infected cells were seeded at a concentration achieving 80% confluence in 96-well plates 10 h before transfection. The cells were transiently transfected with TCF/LEF-Luc reporter vector (Cignal, SAB) along with a control Relnt luciferase vector using Lipofectamine plus reagent. The cell lysates were obtained with 1x reporter lysis buffer (Promega) 48 h after transfection. The luciferase activity was assayed in a Berthold Auto Lumar LB953 luminometer (Nashua, NH) by using the luciferase assay system from Promega. The relative luciferase activity was normalized to the Relnt luciferase activity in each sample.

Analysis of haplotype phylogenies and linkage disequilibrium

Genomic DNA pools of White Duroc (n = 2) and Erhualian (n = 2) animals were amplified with primers given in Table S6. All PCR products were purified with the Qiagen protocol and sequenced using the same PCR primers, revealing a subset of SNP markers in the genomic region of porcine PPARD. SNP markers were genotyped by iPLEX SEQUENOM MassARRAY platform. SNP genotype calls were filtered and checked manually, and aggressive calls were omitted from the dataset. Population genetics parameters including the mean number of pairwise differences across loci (Nₑ), Tajimas D, Fu and Li’s D were estimated with DnaSP v5 [34]. Haplotypes were reconstructed with PHASE v2 [35]. Haplotype phylogenetic tree based on p-distance were drawn using MEGA4 [36]. The Haploview v4.1 program [37] was used to calculate linkage disequilibrium measures (r² and D') and to identify haplotype blocks.

Supporting Information

Figure S1 Plots of F-ratios indicating the major QTL for ear size at 58 cM on pig chromosome 7. Markers and distance in cM are given on the x-axis, and F-ratios are indicated on the left y-axis. The threshold for 1% genome-wide significant level is indicated by the dashed horizontal line. The confidence interval of 2 cM is marked by the dashed vertical line. LEW: left ear weight; REW: right ear weight; LEA: left ear area; REA: right ear area. (TIF)

Figure S2 QTL genotypes of F₁ boars determined by marker-assisted segregation analysis. The number of offspring in each sire family is given above the error bars. The right ear size measured in the pedigree is marked by cm² in left axis. A Z-score is given for each sire pedigree. Q alleles associated with increased ear size are marked by a diamond, q alleles by a circle. (TIF)

Figure S3 Association of the Erhualian-originated haplotype in the critical 630-kb region with increased ear size in Sutai pigs. Rare haplotypes with frequencies of less than 0.01 were discarded for analysis. E denotes the Erhualian haplotype. (TIF)

Figure S4 Real-time RT-PCR analysis of PPARD temporal expression in ear tissues of Erhualian and Duroc pigs. Tissue samples were collected from Erhualian and Duroc pigs at days 0, 45±3, 90±3, and 300±3 for RNA extraction. Six animals were sampled from each breed at each period. Real-time PCR was performed in triplicate. PPARD expression levels normalized with β-actin are given (mean ± s.e.). No significant difference was observed in PPARD expression levels between Erhualian and Duroc pigs at each stage. (TIF)

Figure S5 Relationship between Tajima’ D and frequency of the derived 32E allele. (TIF)

Figure S6 NJ phylogenetic tree constructed with the 16 frequent PPARD haplotypes. The detail information about each haplotype is given in Table 4. Haplotype 1 is the only one containing the derived 32E allele for increased ear size and is the major haplotype of Erhualian pigs. (TIF)

Figure S7 Linkage disequilibrium (r²) plot between pairs of loci for Chinese indigenous breeds (A), Erhualian pigs (B) and Western commercial breeds (C). Haplotype blocks are underlined, and the G32E locus is indicated by arrows. (TIF)

Figure S8 Distribution of linkage disequilibrium measures (r² and D') against the distance between the G32E mutation and the rest of loci. (TIF)

Table S1 Descriptive statistics of the ear traits measured in the White Duroc × Erhualian cross. (DOC)
Table S2  Effect of the PPARD G32E substitution on ear size in the White Duroc \times Erhualian cross.

(DOC)

Table S3  Genetic variability in the PPARD gene in worldwide pig breeds.

(DOC)

Table S4  Primers for detection of SNP and microsatellite markers in the QTL region.

(DOC)

Table S5  Primers for analyzing expression of annotated genes in the refined interval using RT-PCR and real-time PCR.

(DOC)

Table S6  Primers for identification of polymorphisms in the coding and genomic regions of porcine PPARD.

(DOC)

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Table S7  Primers for real time RT-PCR analysis in cultured cells.

(DOC)

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Author Contributions

Conceived and designed the experiments: LH JR. Performed the experiments: YD RQ FY RW. Analyzed the data: JR ZZ BY LH. Contributed reagents/materials/analysis tools: YD YG SX ND ZO HA. Wrote the paper: JR LH.