Characterization of the Goose *CAPN3* Gene and its Expression Pattern in Muscle Tissues of Sichuan White Geese at Different Growth Stages

Hengyong Xu*, Yahui Zhang*, Quan Zou, Liang Li, Chunchun Han, Hehe Liu, Jiwei Hu, Tao Zhong and Yan Wang

Farm Animal Genetic Resources Exploration and Innovation Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu 611130, China

Running Title: Goose *CAPN3* gene cloning and expression

Correspondence:

Yan Wang, Institute of Animal Genetics and Breeding, Farm Animal Genetic Resources Exploration and Innovation Key Laboratory of Sichuan Province, Sichuan Agriculture University, Chengdu 611130, China.

(E-mail: as519723614@163.com)

*These authors contributed equally to this work.
Abstract

Calpain 3 (CAPN3), also known as p94, is associated with multiple production traits in domestic animals. However, the molecular characteristics of the CAPN3 gene and its expression profile in goose tissues have not been reported. In this study, CAPN3 cDNA of the Sichuan white goose was cloned, sequenced, and characterized. The CAPN3 full-length cDNA sequence consists of a 2,316-bp coding sequence (CDS) that encodes 771 amino acids with a molecular mass of 89,019 kDa. The protein was predicted to have no signal peptide, but several N-glycosylation, O-glycosylation, and phosphorylation sites. The secondary structure of CAPN3 was predicted to be 38.65% α-helical. Sequence alignment showed that CAPN3 of Sichuan white goose shared more than 90% amino acid sequence similarity with those of Japanese quail, turkey, helmeted guineafowl, duck, pigeon, and chicken. Phylogenetic tree analysis showed that goose CAPN3 has a close genetic relationship and small evolutionary distance with those of the birds. qRT-PCR analysis showed that in 15-day-old animals, the expression level of CAPN3 was significantly higher in breast muscle than in thigh tissues. These results serve as a foundation for further investigations of the function of the goose CAPN3 gene.

Key words: CAPN3 gene, clone, expression, goose
Introduction

Goose meat is rich in protein and several trace elements, such as calcium, phosphorus, potassium, and sodium, but low in fat and cholesterol. Thus, goose meat is a nutritional and healthy food. However, the tenderness of goose meat restricts consumer acceptance; therefore, candidate genes that affect goose meat tenderness have received great research interest.

Previous studies have shown that the calpain system is closely related to meat quality traits in pigs (Gandolfi et al., 2011), sheep (Grochowska et al., 2017), and chickens (Zhang et al., 2009). Calpains are Ca$^{2+}$-dependent intracellular cysteine proteases that are involved in cell motility (Glading et al., 2002), and apoptosis regulation (Liu et al., 2004), muscle atrophy (Richard et al., 1995), myoblast fusion (Honda et al., 2008), and muscle growth and development (Sultan et al., 2000; Zhang et al., 2009). To date, 15 isoforms have been identified in humans (Sorimachi and Ono, 2012), and in vertebrates, calpain 1 and calpain 2 have been widely studied among calpain isoforms (Macqueen et al., 2010; Zhang et al., 2017). Among the calpain family members, calpain 3 (CAPN3, previously named p94) is particularly interesting, because in humans, limb-girdle muscular dystrophy type 2A (LGMDA2) is mainly caused by loss-of-function mutations of *CAPN3* (Richard et al., 1995). In mammals, *CAPN3* is the most highly expressed in skeletal muscles, especially in the fast (type II) fibers (Jones et al., 1999), but *CAPN3* mRNA has also been detected in the heart (Fougerousse et al., 2000). In chickens, Zhang et al. (2012) and Sorimachi et al. (1995) reported that CAPN3 is abundantly expressed in the skeletal muscle and is...
also expressed in the liver, heart, and brain. Subsequent studies by Ilian et al. (2001, 2004) and Felício et al. (2013) revealed significant correlations between meat quality and the expression or polymorphism of CAPN3. However, the gene and cDNA sequences of the goose CAPN3 gene have not been published in GenBank. The expression level and pattern of CAPN3 in goose are less clear, and its precise physiological functions are not well characterized in goose.

In this study, we first cloned the cDNA of the Sichuan White goose CAPN3 gene, predicted the corresponding protein sequence, and performed phylogenetic and structural analyses. Then, we detected its expression patterns in muscle tissues of different growth stages using quantitative reverse transcription PCR (qRT-PCR). By comparing the cDNA sequences and mRNA expression patterns in goose breast and thigh muscle tissues, we sought to unravel potential roles of CAPN3 in goose muscle tissues. The results of this analysis will aid in understanding the function of the goose CAPN3 gene.

**Materials and Methods**

**Ethics Statement**

All geese were fed according to Chinese local goose breeding standards. All experimental protocols were reviewed and approved of by the Sichuan Agricultural University Institutional Animal Care and Use Committee in College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China (DKY-B201000805). Sampling procedures used strictly complied with the guidelines
animals and sample collection

In this study, 30 Sichuan white geese were kept in the Experimental Farm for Waterfowl Breeding at Sichuan Agricultural University, with ad libitum access to water and a commercial corn-soybean-based diet. For CAPN3 cloning and mRNA expression analysis, 15 female geese at different ages (15 and 22 days) were randomly selected, of which 3 geese aged 22 days old were used for cloning, and the remaining 12 were used for mRNA expression testing. All of these geese were healthy and of moderate weight, and they were fed in their housing. In the feeding room, the temperature was 15-19°C, and the environment was exposed to natural light. All geese were terminally killed by bleeding within 10 min of capture, and breast muscle and thigh muscle tissues were removed (within 5–10 min after death), frozen immediately in liquid nitrogen, and stored separately at −80°C until total RNA extraction.

RNA extraction and reverse transcription

Total RNA was isolated from the collected tissues using TRIZol reagent (Takara Bio, Dalian, China) according to the manufacturer’s instructions and treated with 30 μl DNase/RNase-Free water (Takara Bio) to remove DNA contamination. Before reverse transcription, RNA integrity and quality were checked by electrophoresis (1% agarose gels stained with ethidium bromide) and with a Nanodrop spectrophotometer (Nanodrop 2000C; Thermo Scientific, MA, USA), respectively. Reverse transcription
was carried out using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio) according to the manufacturer’s instructions. Briefly, each 10-μl reaction contained 2 μl of 5× PrimeScript™ buffer, 0.5 μl of PrimeScript™ RT Enzyme Mix I, 0.5 μl of OligodT Primer, 0.5 μl of random hexamers, 5.5 μl of RNase-free water, and 1 μl of total RNA. The reaction was carried out at 37°C for 15 min, terminated by 5 s at 85°C, and the cDNA was stored at 4°C.

First-strand cDNA of the breast muscle was used to clone the full-length cDNA of \textit{CAPN3}. First-strand cDNA of other tissues was used for gene expression analysis.

\textbf{Molecular Cloning of the Goose \textit{CAPN3} cDNA}

The goose \textit{CAPN3} gene was amplified from cDNA using PCR. The specific PCR primers were designed by using Primer 5.0 and Oligo 6.0 software based on predicted \textit{Anser cygnoides} \textit{CAPN3} sequence (GenBank ID: XM_013192108). Primers used for cloning are shown in Table 1. The 10-μl PCR reaction mixture contained 5 μl of 2× MasterMix (Tiangen, Beijing, China), 0.8 μl of each primer (10 mM each), 1 μl of cDNA (50–500 ng), and 2.4 μl of ddH$_2$O. Thermal cycles were as follows: initial denaturation at 94 °C for 5 min, 34 cycles of 94 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min, and final elongation at 72 °C for 10 min. The PCR fragments were purified with the Gel Extraction Mini Kit (Qiagen, Hilden, Germany) and were cloned into the pMD-19T vector (Takara Bio). The plasmid DNA was isolated using a Plasmid Mini Kit (Qiagen) and was sequenced by BGI (Beijing, China).
**Sequence, Structure, and Phylogenetic analyses**

The obtained nucleotide sequence of the goose *CAPN3* gene was analyzed at NCBI (http://blast.ncbi.nlm.nih.gov/) and was compared to the sequence database using the BLAST server (http://www.ncbi.nlm.nih.gov/blast). The open reading frame was predicted with the NCBI/ORF Finder program (http://www.ncbi.nlm.nih.gov/orf/gorf.html/). Protein domains were predicted using the Conserved Domain Architecture Retrieval Tool at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software (http://align.genome.jp) was used for multiple alignment. The fundamental characteristics of the predicted protein including molecular weight (Mw) and isoelectric point (pI) were analyzed with ProtParam (http://www.expasy.org/tools/protparam.html). TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0) were used to predict transmembrane domains. Signal peptides were predicted by the SignalP4.0 server (http://www.cbs.dtu.dk/services/SignalP). NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/NetOGlyc) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) were used to predict potential O- and N-glycosylation sites, respectively. Disulfide bonds were predicted using the SCRATCH protein predictor (http://scratch.proteomics.ics.uci.edu). Phosphorylation sites were predicted using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos). Secondary structures of deduced amino acid (aa) sequences were predicted by using
the HNN method (http://npsa-pbil.ibcp.fr/). The protein 3D conformation was predicted using the SWISS-MODEL server (http://www.expasy.org/swissmod/SWISSMODEL.html). A phylogenetic tree was constructed using the MEGA 7.0 software. GenBank accession numbers of CAPN3 from different species for the phylogenetic analysis are listed in Table 2.

**qRT-PCR**

qRT-PCR was used to evaluate changes in CAPN3 expression in muscle tissues of Sichuan white geese in different growth stages. The primers used for qRT-PCR are listed in Table 1. The housekeeping gene β-actin was selected as the endogenous control gene. qPCR was performed on a CFX-96 qPCR Real-Time PCR Detection System (Bio-Rad) and was carried out in a total volume of 25 μl, with 2.0 μl cDNA, 0.8 μl of each specific primer, 12.5 μl SYBR Premix EX Taq™ (Takara Bio), and 8.9 μl of ddH2O. The thermal protocol included one cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 10 s and 60°C for 15 s. A 45-cycle-based melting curve was generated, starting at a temperature of 55°C and increasing by 0.5°C every 10 s, to determine primer specificity. Each sample was analyzed in triplicate. mRNA levels were normalized to β-actin mRNA and expressed as a fold change relative to the expression level in the control by using the $2^{-\Delta\Delta CT}$ method.

**Statistical Analysis**

Means were compared by one-way ANOVA using the SAS 6.12 software, a
multiple comparison test was performed using Duncan’s method, and $P < 0.05$ was considered statistically significant. Means ± SEMs were plotted using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

**Results**

*Cloning and Characteristics of Goose CAPN3 cDNA*

A 2,453-bp sequence of goose *CAPN3* was obtained by cloning and splicing using cDNA generated from RNA isolated from breast muscle of Sichuan White goose as the template, which is in line with the predicted *CAPN3* gene sequence of *A. cygnoides* (GenBank: XM_013192108). The cDNA contains a 2,316-bp ORF, encoding a 771 aa protein.

The deduced aa sequence of Sichuan White goose *CAPN3* was compared to the *CAPN3* sequences from 14 other animals using DNAMAN. The coding sequence of Sichuan White goose *CAPN3* was 99.87% identical to *A. cygnoides* *CAPN3*, and 93.42%, 93.16%, 93.16%, 92.92%, 89.18%, 88.94%, 86.65%, 71.79%, 71.51%, 70.99%, 70.87%, 70.43%, 70.17%, and 70.10% identical to that of helmeted guineafowl, Japanese quail, turkey, duck, chicken, pigeon, medium ground-finch, macaque, human, Norway rat, pig, house mouse, cattle, and sheep, respectively. The aa sequence was 100%, 97.23%, 96.85%, 96%, 93.41%, 92.57%, 92.21%, 89.11%, 74.11%, 74.05%, 73.93%, 73.93%, 73.73%, 73.02%, and 72.66% identical to that of *A. cygnoides*, Japanese quail, turkey, helmeted guineafowl, duck, pigeon, chicken, medium ground-finch, macaque, Norway rat, human, house mouse, pig, cattle, and
sheep, respectively (Table 2 and Fig. 1).

**Analysis of the AA Sequence of Goose CAPN3 and Phylogenetic Analysis**

The physicochemical properties of the Sichuan White goose CAPN3 protein were predicted using ProtParam software (Table 3). The results showed that the CAPN3 protein molecular formula is C$_{3971}$H$_{6105}$N$_{1075}$O$_{1185}$S$_{36}$, and the molecular weight is 89,019 kDa. The theoretical pI is 5.54, and the instability index is 36.75. The grand average of hydropathicity of the protein was $-0.531$. The Sichuan White goose CAPN3 protein was predicted to be non-secretory, as it does not contain signal peptide sequences, and a transmembrane domain was not predicted. In addition, goose CAPN3 was predicted to have 13 O-glycosylation sites (Thr$^{13,21,22,24,25,26,30,34,310}$ and Ser$^{17,20,588,591}$), four N-glycosylation sites (Asn$^{78}$, Asn$^{112}$, Asn$^{218}$, and Asn$^{409}$), 66 phosphorylation sites (Table 4), and no disulfide bonds. The hydrophobicity profile of the protein was calculated by ProtScale of ExPASy (Fig. 2). The ordinate represents the hydrophobic score—the higher the score, the more hydrophobic, the lower the score, the lower the hydrophobicity. The abscissa represents aa position. As shown in Fig. 2, the aa at position 474 is highly hydrophobic and that at position 484 is highly hydrophilic. Moreover, the protein has substantially more hydrophilic than hydrophobic amino acids.

Secondary structural analysis indicated that the putative goose CAPN3 protein comprised 38.65% alpha helix, 18.55% extended strand, and 42.80% random coil. The fully automatic procedure on the SWISS-MODEL server was used to construct a
3D structural model of a segment of the goose CAPN3 sequence (aa 47–770). The result showed that the segment was similar to that of the calpain-2 catalytic subunit 3bow.1.A, which is the template of this 3D model in the SWISS-MODEL library (Arnold et al., 2006; Benkert et al., 2011; Biasini et al., 2014) (Fig. 3). Meanwhile, in the predicted 3D structure, the QMEAN score was –2.23, and the GMQE score was 0.73.

A phylogenetic tree was constructed from the deduced Sichuan White goose CAPN3 and CAPN3 sequences from other animals by the Neighbor Joining (NJ) method in MEGA 7.0 (Fig. 4). As shown in Fig. 4, the Sichuan White goose CAPN3 protein evaluated in this study had a position close to A. cygnoides and Anas platyrhynchos CAPN3 proteins, while it was distant from Bos Taurus and Ovis aries CAPN3 proteins.

**Expression Profile of Sichuan White Goose CAPN3 mRNA**

The mRNA levels of CAPN3 in the breast muscle and thigh muscle of Sichuan white geese at 15 and 22 days of age were determined by qRT-PCR. As shown in Table 5, CAPN3 mRNA expression in thigh tissue increased from the 15-day to the 22-day stage, but there was not significant difference ($P > 0.05$). However, in the breast muscle, the expression of CAPN3 was significantly higher in 15-day-old than in 22-day-old geese ($P < 0.05$). Meanwhile, at 15 days, the CANP3 mRNA level in thigh muscle was lower than that in breast muscle, although the difference was not significant ($P > 0.05$). In contrast, at 22 days, the expression of CAPN3 mRNA in
thigh muscle was significantly higher than that in breast muscle ($P < 0.05$) (Table 5 and Fig. 5).

**Discussion**

CAPN3 is involved in multiple important functions (Zhang *et al*., 2017) and has drawn great research interest because of its three specific regions (N-terminal, IS1 and IS2) (Ono *et al*., 2016). For example, defects IS1/2 in CAPN3 lead to limb girdle muscular dystrophy type 2A (LGMD2A) symptoms and other disease (Beckmann and Spencer, 2008). Meanwhile, the rapidity of CAPN3 autodegradation was also thought to through regulate its activity in N-terminal (Ono *et al*., 2014). Although it has also been demonstrated to be a candidate protein for meat tenderness in mammals, including cattle (Nattrass *et al*., 2014; Robinson *et al*., 2012) and pigs (De Smet *et al*., 2003), its roles, particularly its physiological activities, in geese have not been sufficiently examined. Therefore, this work will have the potential to increase our understanding of the functional roles of CAPN3 gene during improving goose meat quality provides a molecular basis. This study is the first to report the full-length cDNA of Sichuan White goose *CAPN3*. It contained a 2,316-bp coding sequence (CDS) that encodes 771 aa. Sequence alignment revealed that the aa sequence of the Sichuan white goose CAPN3 protein was highly similar to those of species including Japanese quail, turkey, helmeted guineafowl, duck, pigeon, and chicken (greater than 90% sequence identity for all analyzed bird species). This result is consistent with the zoological classification and suggests that CAPN3 protein is relatively evolutionarily conserved. Meanwhile, consistent with CAPN3 protein in other animals, such as cattle
and chickens (Pan et al., 2013; Wang et al., 2016; Zhang et al., 2012), the Sichuan white goose CAPN3 protein has no signal peptide.

Previous research has shown that disulfide bonds play an important role in maintaining the stability of protein conformations (Hatahet and Ruddock, 2009), and the instability coefficient is used as an indicator of protein stability. Usually, when the instability coefficient is greater than 40, the protein structure is considered unstable (Emanuela and Marco, 2011). In this study, the Sichuan white goose CAPN3 was predicted to lack disulfide bonds, and its instability coefficient was less than 40, indicating that CAPN3 is relatively stable.

Hägglund et al. (2004) reported that N-glycosylation occurs in the rough endoplasmic reticulum and the Golgi apparatus, whereas O-glycosylation occurs only in the Golgi apparatus. Our results indicated that goose CAPN3 might be regulated by glycosylation. Because goose CAPN3 was not predicted to have signal peptides, it likely cannot enter the rough endoplasmic reticulum to be glycosylated. Currently, there are no reports on the glycosylation of CAPN3 protein; thus, we speculate that CAPN3 is translated and synthesized on the ribosomes of the rough endoplasmic reticulum and then enclosed in vesicles that merge with the Golgi apparatus, allowing glycosylation. This hypothesis regarding the process of CAPN3 glycosylation requires verification.

In addition to glycosylation, phosphorylation is considered an important modification. Huttlin et al. (2010) and Cohen-Kaplan et al. (2012) both considered that protein phosphorylation affects various biological processes, such as
enzyme-activity regulation, cell division, and signal transduction. In this study, the
goose CAPN3 protein was predicted to contain several phosphorylation sites, and the
number of serine phosphorylation sites was larger than that in other species, such as
cattle (Wang et al., 2016). The different results might be due to the species being
different.

To better understand the expression pattern of the goose CAPN3 gene, CAPN3
mRNA levels were analyzed in breast and thigh muscle tissues collected from Sichuan
white geese. Our results showed that CAPN3 was expressed in both muscle tissues,
which is consistent with a report that the level of CAPN3 gene expression in the breast
muscle and leg muscle was significantly higher than that in other tissues in chickens
(Zhang et al., 2012). Wu et al. (2015) reported high levels of CAPN3 expression in
the muscle atrophic phase after denervation. The expression level of CAPN3 in
skeletal muscle is reportedly associated with carbohydrate oxidation and insulin
concentrations (Walder et al., 2002). In the current study, CAPN3 mRNA was
abundantly expressed in goose muscle at the two developmental stages examined, and
the mRNA levels tended to increase at the later growth stage (22 days) in thigh
muscle and maintain a high level at the earlier growth stage (15 days) in breast muscle.
This finding is in partial accordance with previous findings in duck, in which the
CAPN3 mRNA level is low at the embryonic stage, but increases at neonatal stages (7
days) (Zhu et al., 2014). Presumably, the abundant expression of CAPN3 in
developing goose muscle suggests that CAPN3 is involved in the development and
functions of goose muscle, such as controlling skeletal satellite cell
proliferation/differentiation. Interestingly, the CAPN3 expression level in thigh muscle was lower than that in breast muscle at 15 days, while at 22 days, the level in thigh muscle was higher than that in breast muscle. We speculated that through long-term breeding, the goose has lost its ability to fly, and therefore, the thigh muscle, primarily used for moving and swimming, has a higher level of activity, resulting in high CAPN3 expression. CAPN3 is not inhibited by calpastatin (Ono et al., 2004) and its expression pattern has been a matter of debate. In chickens, the CAPN3 mRNA level in breast muscle is higher than that in thigh muscle (Zhang et al., 2012). Yang et al. (2012) reported that the CAPN3 mRNA level in longissimus muscles of pigs was correlated with tenderness. Conversely, tenderization processes were not affected in CAPN3 knockout mice (Geesink et al., 2005). Additionally, Richard et al. (1995) found that inactivating mutations in human CAPN3 cause recessive limb girdle muscular dystrophy type 2. These results suggest that CAPN3 expression might have an effect on the tenderness of skeletal muscles, but unknown roles of CAPN3 in different muscle types should be investigated further.

In conclusion, a full-length cDNA of CAPN3 from the Sichuan white goose (2,316 bp long, encoding a 771 aa protein) was cloned and characterized. The protein was predicted to have 4 N-glycosylation, 13 O-glycosylation, and several phosphorylation sites. Homology analysis revealed that the aa sequence of goose CAPN3 protein was highly similar to those of other species. qRT-PCR analysis showed that CAPN3 expression was higher in breast muscle in 15-day-old than in 22-day-old animals. These results provide an important theoretical basis for further
research into the functions and regulatory mechanism of CAPN3 in geese.

Acknowledgements

This work was supported by the Key Technology Support Program of Sichuan Province (2016NYZ0027, 2016NZ0055), a Project Supported by Scientific Research Fund of Sichuan Provincial Education Department (15ZA0025) and an interest training program for undergraduates of Sichuan Agricultural University (2017085).
References

Arnold K, Bordoli L, Kopp J and Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics, 22: 195-201. 2006.

Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L and Schwede T. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. Nucleic acids research, 42: W252-8. 2014.

Beckmann JS and Spencer M. Calpian 3, the “gatekeeper” of proper sarcomere assembly, turnover and maintenance. Neuromuscular Disorders, 18: 913-921. 2008.

Benkert P, Biasini M and Schwede T. Toward the estimation of the absolute quality of individual protein structure models. Bioinformatics, 27: 343-350. 2011.

Cohen-Kaplan V, Jrbashyan J, Yanir Y, Naroditsky I, Ben-Izhak O, Ilan N, Doweck I and Vlodavsky I. Heparanase induces signal transducer and activator of transcription (STAT) protein phosphorylation: preclinical and clinical significance in head and neck cancer. Journal of Biological Chemistry, 287:6668-6678. 2012.

De Smet S, Herman L, Dedain V, Depuydt J, Jacobs K and Peelman L. Polymorphisms of candidate genes for meat and carcass quality in Belgian pig populations. Communication in Agricultural and Applied Biological Sciences, 68(2 Part B): 617-624. 2003.

Emanuela C and Marco T. Bayesian analysis of coefficient instability in dynamic
regressions. Ssm Electronic Journal, 836:1-64. 2011.

Felicio AM, Boschiero C, Balieiro JCC, Ledur MC, Ferraz JBS, Michelan Filho T, Moura ASAMT and Coutinho LL. Identification and association of polymorphisms in \textit{CAPN1} and \textit{CAPN3} candidate genes related to performance and meat quality traits in chickens. Genetics and Molecular Research, 12(1): 472-482. 2013.

Fougerousse F, Anderson LVB, Delezoide AL, Suel L, Durand M and Beckmann JS. Calpain3 expression during human cardiogenesis. Neuromuscular Disorders, 10: 251-256. 2000.

Gandolfi G, Pomponio L, Erbbjerg P, Karlsson AH, Nanni Costa L, Lametsch R, Russo V and Davoli R. Investigation on \textit{CAST}, \textit{CAPN1} and \textit{CAPN3} porcine gene polymorphisms and expression in relation to post-mortem calpain activity in muscle and meat quality. Meat Science, 88:694-700. 2011.

Geesink GH, Taylor RG and Koohmaraie M. Calpain 3/p94 is not involved in postmortem proteolysis. Journal of animal science, 83(7): 1646-1652. 2005.

Glading A, Lauffenburger DA and Well A. Cutting to the chase: calpain proteases in cell motility. Trends in Cell Biology, 12:46-54. 2002.

Grochowska E, Borys B, Grzéskowiak E and Mroczkowski S. Effect of the calpain small subunit 1 gene (\textit{CAPNS1}) polymorphism on meat quality traits in sheep. Small Ruminant Research, 150:15-21. 2017.

Hatahet F and Ruddock LW. Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. Antioxidants and Redox Signaling,
Hägglund P., Bunkenborg J, Elortza F, Jensen ON and Roepstorff P. A new strategy for identification of N-glycosylated proteins and unambiguous assignment of their glycosylation sites using HILIC enrichment and partial deglycosylation. Journal of Proteome Research, 3:556-566. 2004.

Honda M, Masui F, Kanzawa N, Tsuchiya T and Toyo-oka T. Specific knockdown of m-calpain blocks myogenesis with cDNA deduced from the corresponding RNAi. American Journal of Physiology Cell Physiology, 294:C957-C965. 2008.

Huttlin EL, Jedrychowski MP, Elias JE, Goswami T, Rad R, Beausoleil SA, Villén J, Haas W, Sowa ME and Gygi SP. A tissue-specific atlas of mouse protein phosphorylation and expression. Cell, 7:1174-1189. 2010.

Ilian MA, Morton JD, Bekhit AED, Roberts N, Palmer B, Sorimachi H and Bickerstaffe R. Effect of preslaughter feed withdrawal period on longissimus tenderness and the expression of calpains in the ovine. Journal of Agricultural and Food Chemistry, 49(4):1990-1998. 2001.

Jones SW, Parr T, Sensky PL, Scothern GP, Bardsley RG and Buttery PJ. Fibre type-specific expression of p94, a skeletal muscle-specific calpain. Journal of Muscle Research and Cell Motility, 20:417-424. 1999.

Liu X, Van Vleet T and Schnellmann RG. The role of calpain in oncotic cell death. Annual Review of
Pharmacology and Toxicology, 44:349-370. 2004.

Macqueen DJ, Delbridge ML, Manthri S and Johnston IA. A newly classified vertebrate calpain protease, directly ancestral to CAPN1 and 2, episodically evolved a restricted physiological function in placental mammals. Molecular Biology and Evolution, 27(8):1886-1902. 2010.

Nattrass GS, Cafe LM, McIntyre BL, Gardner GE, McGilchrist P, Robinson DL, Wang YH, Pethick DW and Greenwood PL. A post-transcriptional mechanism regulates calpastatin expression in bovine skeletal muscle. Journal of Animal Science, 92(2):443-455. 2014.

Ono Y, Kakinuma K, Torii F, Irie A, Nakagawa K, Labeit S, Abe K, Suzuki K and Sorimachi H. Possible regulation of the conventional calpain system by skeletal muscle-specific calpain, p94/calpain 3. The journal of biological chemistry, 279:2761-2771. 2004.

Ono Y, Ojima K, Shinkai-Ouchi F, Hata S and Sorimachi H. An eccentric calpain, CAPN3/p94/calpain-3. Biochimie, 122:169-187. 2016.

Ono Y, Shindo M, Doi N, Kitamura F, Gregorio CC and Sorimachi H. The N and C-terminal autolytic fragments of CAPN3/p94/calpain-3 restore proteolytic activity by intermolecular complementation. Proceeding of the National Academy of Sciences of the United States of America. 111: E5527-E5536. 2014.

Pan H, Luo Y, Hu J, Liu X, Li S and Zhang L. Cloning and bioinformatics analysis on CDS of CAPN3 gene in Yak. Journal of Gansu agricultural university (In Chinese), 5:1-7. 2013.
Robinson DL, Cafe LM, McIntyre BL, Geesink GH, Barendse W, Pethick DW, Thompson JM, Polkinghorne R and Greenwood PL. Production and processing studies on calpain-system gene markers for beef tenderness: consumer assessments of eating quality. Journal of Animal Science, 90(8):2850-2860. 2012.

Richard I, Broux O, Allamand V, Fougerousse F, Chiannilkulchai N, Bourg N, Brenguier L, Devaud C, Pasturaud P, Roudaut C, Hillaire D, Passons-Bueno MR, Zatz M, Tischfield J, Fardeau M, Jackson CE, Cohen D and Beckman JS. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell, 81:27-40. 1995.

Sorimachi H, Tsukahara T, Okada-Ban M, Sugita H, Ishiura S and Suzuki K. Identification of a third ubiquitous calpain species—chicken muscle expresses four distinct calpains. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1261:381-393. 1995.

Sorimachi H and Ono Y. Regulation and physiological roles of the calpain system in muscular disorders. Cardiovascular Research, 96: 11-22. 2012.

Sultan KR, Dittrich BT and Pette D. Calpain activity in fast, slow, transforming, and regenerating skeletal muscles of rat. American Journal of Physiology-cell physiology, 279: C639-C647. 2000.

Walder K, Mcmillan J, Lapsys N, Kriketos A, Trevaskis J, Civitarese A, Southon A, Zimmet P and Collier G. Calpain 3 gene expression in skeletal muscle is associated with body fat content and measures of insulin resistance. International journal of Obesity, 26(4):442-449. 2002.
Wang Y, Yan P, Pan H, Wu X and Li M. Cloning and tissue-specific expression of CAPN3 gene in Yak. Biotechnology bulletin (In Chinese), 32(1):149-155. 2016.

Wu R, Yan Y, Yao J, Liu Y, Zhao J and Liu M. Calpain 3 expression pattern during gastrocnemius muscle atrophy and regeneration following sciatic nerve injury in rats. International Journal of Molecular Sciences, 16:26927-26935. 2015.

Yang X, Chen J, Jia C and Zhao R. Gene expression of calpain 3 and PGC-1α is correlated with meat tenderness in the longissimus dorsi muscle of Sutai pigs. Livestock science, 147:119-125. 2012.

Zhang Y, Liu NM, Wang Y, Youn JY and Cai H. Endothelial cell calpain as a critical modulator of angiogenesis. BBA-Molecular Basis of Disease, 1863:1326-1335. 2017.

Zhang ZR, Liu YP, Yao YG, Jiang XS, Du HR and Zhu Q. Identification and association of the single nucleotide polymorphisms in calpain 3 (CAPN3) gene with carcass traits in chickens. BMC Genetics, 10:10. 2009.

Zhang ZR, Zhu Q, Yao YG, Jiang XS, Du HR and Liu YP. Characterization of the expression profile of calpain-3 (CAPN3) gene in chicken. Molecular Biology Reports, 39: 3517-3521. 2012.

Zhu WQ, Xu WJ, Shu JT, Chen WF, Shan YJ, Liu HX, Hu Y, Li HF, Wang JY. Expression of CAPN3 gene in skeletal muscles and its association with myofiber traits during embryonic and early post-hatching development in ducks. Acta veterinaria et zootechnica sinica (In Chinese), 45(3): 385-390. 2014.
| No | Gene     | Type     | Sequence (5´-3´)       | Product size (bp) | Tm (°C) |
|----|----------|----------|------------------------|-------------------|---------|
| 1  | CAPN3    | Clone    | F: CGCTTACAACTTTGACTAG R: CTGGCTGTAGAAGAGGGA | 390               | 56      |
| 2  | CAPN3    | Clone    | F: ATCCTGATTCCCCACCTA R: AACGTCCACCAGTCTCC | 229               | 55      |
| 3  | CAPN3    | Clone    | F: GCTGGCATCTTTCACTTT R: CGTTCCATTCACCTGTC | 619               | 56      |
| 4  | CAPN3    | Clone    | F: AGGTACCGCTACTCTGT R: TGTCATCTCTGCAATCT | 869               | 56      |
| 5  | CAPN3    | Clone    | F: AGTCCTATCTTTGAGGGT R: TGCTACTCCTGCAATCT | 605               | 55      |
| 6  | CAPN3    | Expression | F: AAGCGACATTGGGAGA R: CCAGCCCACAAGACATCC | 152               | 60      |
| 7  | β-actin  | Expression | F: CAACGAGAGGTCAGTG R: TGGAAGTTGAAGGTGTCTCG | 92                | 60      |
Table 2. Accession numbers of CAPN3 nucleic acid and protein sequences used for multiple sequence alignments and for reconstruction of the phylogenetic tree in this study

| Species                             | Accession no. (nucleic acid) | Accession no. (protein) |
|-------------------------------------|------------------------------|-------------------------|
| Anser cygnoides domesticus          | XM_013192108                 | XP_013047562            |
| Duck (Anas platyrhynchos)           | XM_005021679                 | XP_005021736            |
| Chicken (Gallus gallus)             | NM_001004405                 | NP_001004405            |
| Cattle (Bos Taurus)                 | NM_174260                    | NP_776685               |
| Medium ground-finch (Geospiza fortis)| XM_005419401               | XP_005419458            |
| House mouse (Mus musculus)          | NM_007601                    | NP_031627               |
| Human (Homo sapiens)                | NM_000070                    | NP_000061               |
| Japanese quail (Coturnix japonica)  | XM_015864332                 | XP_015719818            |
| Macaque (Macaca fascicularis)       | NM_001287701                 | NP_001274630            |
| Norway rat (Rattus norvegicus)      | NM_017117                    | NP_058813               |
| Helmeted guineafowl (Numida meleagris)| XM_021403511               | XP_021259186            |
| Pigeon (Columba livia)              | XM_021287237                 | XP_021142912            |
| Pig (Sus scrofa)                    | NM_214171                    | NP_999336.2             |
| Sheep (Ovis aries)                  | NM_001009212                 | NP_001009212            |
| Turkey (Meleagris gallopavo)        | XM_019615582                 | XP_019471127            |
Table 3. Physiochemical characteristics of the Sichuan White goose CAPN3 protein

| Physiochemical characteristics          | CAPN3     |
|-----------------------------------------|-----------|
| Amino acids                             | 771       |
| Number of atoms                         | 12,372    |
| Molecular weight (Da)                   | 89,019.81 |
| Isoelectric point                       | 5.54      |
| Asp+Glu                                 | 116       |
| Arg+Lys                                 | 97        |
| Extinction coefficients                 | 136,095   |
| Aliphatic index                         | 73.90     |
| Instability index                       | 36.75     |
| Grand average of hydropathicity         | -0.531    |
Table 4. Putative phosphorylation sites identified in the goose CAPN3 protein

| Putative phosphorylation sites | Quantity | Position |
|-------------------------------|----------|----------|
| Serine                        | 33       | Ser3, Ser17, Ser20, Ser85, Ser189, Ser190, Ser210, Ser255, Ser259, Ser260, Ser268, Ser271, Ser275, Ser321, Ser331, Ser364, Ser367, Ser394, Ser426, Ser437, Ser524, Ser528, Ser538, Ser546, Ser553, Ser569, Ser573, Ser575, Ser588, Ser591, Ser659, Ser676, Ser694 |
| Threonine                     | 22       | Thr24, Thr25, Thr30, Thr34, Thr55, Thr134, Thr220, Thr231, Thr310, Thr333, Thr338, Thr403, Thr411, Thr422, Thr424, Thr450, Thr495, Thr530, Thr554, Thr639, Thr686, Thr717 |
| Tyrosine                      | 11       | Tyr56, Tyr70, Tyr211, Tyr234, Tyr244, Tyr316, Tyr531, Tyr548, Tyr684, Tyr695, Tyr713 |
Table 5. *CAPN3* mRNA expression levels in muscle tissues during different growth stages

| Animal age | Number | Breast muscle | Thigh muscle |
|------------|--------|---------------|--------------|
| 15 days    | 6      | 0.319 ± 0.025<sup>a</sup> | 0.259 ± 0.028 |
| 22 days    | 6      | 0.113 ± 0.025<sup>b</sup> | 0.506 ± 0.031<sup>A</sup> |

<sup>a,b</sup> Means within a column with no common superscript differ significantly (*P* < 0.05);

<sup>A,B</sup> Means within a row with no common superscript differ significantly (*P* < 0.05).

The results are shown as the mean ± SEM.
Fig. 2
Fig. 3
Fig. 4

Tree showing relationships between different species. The numbers represent the bootstrap support for each branch.

- Anser cygnoides
- Sichuan White goose
- Duck
- Pigeon
- Japanese quail
- Turkey
- Chicken
- Helmeted guineafowl
- Medium ground finch
- Human
- Macaque
- House mouse
- Norway rat
- Pig
- Cattle
- Sheep

Scale bar: 0.020
Legends to figures

Figure 1 Multiple alignment of deduced aa sequences of CAPN3 from Sichuan White goose and the indicated species. Absolutely conserved aa are highlighted in dark; highly conserved sequences are highlighted in gray. GenBank accession numbers of the CAPN3 sequences are listed in Table 2.

Figure 2 Hydrophobicity profile of Sichuan White goose CAPN3 generated with the ProtScale program.

Figure 3 3D structure of Sichuan White goose CAPN3 based on homology modeling using SWISS-MODEL.

Figure 4 Phylogenetic tree based on aa sequences of CAPN3 from goose and the indicated species. UPGMA analysis based on the Poisson correction model was performed using Mega 7.0 software. Numbers at each branch indicate the bootstrap percentage.

Figure 5 mRNA expression levels of CAPN3 in two different muscle tissues at the indicated growth stages. Error bars show the SEM of triplicate measurements. *P < 0.05.