Leibniz-Institut für Nutztierbiologie (FBN)

Analysis of functional candidate genes related to ubiquitination process for meat quality in commercial pigs

Inaugural-Dissertation
zur Erlangung des Grades

Doktor der Ernährungs- und Lebensmittelwissenschaften
(Dr. troph.)
der Landwirtschaftlichen Fakultät
der Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt am 21. August 2013 von

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Tag der mündlichen Prüfung: 31.01.2014

Erscheinungsjahr: 2014

Diese Dissertation ist auf dem
Hochschulschriftenserver der ULB Bonn
http://hss.ulb.uni-bonn.de/diss_online
elektronisch publiziert.

Abstract

During the conversion from muscle to meat, the degradation of myofibrillar proteins impacts on the water holding capacity of meat. Besides the calpain/calpastatin proteolytic system, the activity of ubiquitin proteasome system also causes the degradation of intermediate filament proteins and integrins that leads to an increase of drip loss formation in the muscle cells. The current study sought to evaluate the effects on meat quality of the four functional candidate genes, *UBXN1, UBE3B, TRIP12* and *ZRANB1*, that related to the ubiquitination processes. Nine novel polymorphisms were identified in the transcribed and 3′-UTR regions of these genes. Seven of these novel SNPs were genotyped in a total of about 570 animals derived from the two populations, German Landrace (GL) and the commercial crossbreed of Pietrain × (German Large White × German Landrace) (PiF1). The SNPs of the four candidate genes exhibited strong associations with the indicators of water holding capacity, including muscle conductivity (*UBXN1, UBE3B, TRIP12*); drip loss (*UBXN1, UBE3B*), pH values (*UBXN1, TRIP12* and *ZRANB1*) and meat redness (*UBE3B*). The SNPs of the four candidate genes had also significant association with carcass traits such as loin eye area, loin fat depth and meat to fat ratio. Moreover, the variation of transcript abundances of *UBXN1, ZRANB1* and *TRIP12* were significantly associated with the respective polymorphisms. At the same time, their transcript abundances were correlated with muscle conductivity, pH and drip loss, respectively. *UBE3B* transcript abundance was associated with meat redness. The integration of association and expression data imply the existence of causal polymorphisms in the

cis-regulatory regions of these candidate genes, which are in incomplete linkage disequilibrium with the detected SNPs, and which primarily affect their transcript abundance and, in consequence, traits related to water holding capacity. Thus the study revealed the consistent triangular relationship among genotype - phenotype - transcript abundance across the four candidate genes. In fact, the detected SNPs were in linkage phase with alleles of causal sites increasing the transcript abundances, and enhancing the purge loss in the case of *UBXN1* and *UBE3B*, whereas decreasing drip loss formation in the case of *TRIP12* and *ZRANB1* genes. Moreover, interactions observed among these genes of the ubiquitination system and the ryanodine 1 receptor (*RYR1*) indicate options for further improvement of meat quality, in particular in *RYR1* heterozygous animals, by considering genotypes at these loci. The results of this study provide genetic evidences to support *UBXN1, UBE3B, TRIP12* and *ZRANB1* as the functional candidate genes for water holding capacity of pork.

Kurzbeschreibung

Während der Fleischreifung, wenn Muskel gewebe zu Fleisch wird, so wird die Wasserbindungskapazität des Gewebes durch den Abbau myofibrillärer Proteine beeinflusst. Neben dem Calpain/Calpastatin Proteolysesystem ist die Aktivität des Ubiquitin-Systems für den Abbau von Proteinen der Intermediärfilamente sowie von Integrinen mitverantwortlich. Dies verursacht einen erhöhten Flüssigkeitsverlust der Muskelzellen. Die vorliegende Studie versucht, die Auswirkungen von vier funktionellen Kandidatengenen aus dem Ubiquitinierungsprozess, *UBXN1*, *UBE3B*, *TRIP12* und *ZRANB1*, auf die Fleischqualität auszuwerten. Neun neue Polymorphismen wurden in der transskribierten Sequenz sowie im 3'UTR der Gene identifiziert. Sieben dieser neuen SNP wurden in insgesamt etwa 570 Tieren genotypisiert, die aus zwei Populationen stammen, Deutsche Landrasse (GL) und Pietrain × (Deutsches Edelschwein × Deutsche Landrasse) (PiF1). Die SNP der vier Kandidatengene wiesen eine signifikante Assoziation mit Indikatoren der Wasserbindungskapazität auf, nämlich der Muskelleitfähigkeit (*UBXN1, UBE3B, TRIP12*), dem Flüssigkeitsverlust (Drip) (*UBXN1, UBE3B*), dem pH-Wert (*UBXN1, TRIP12* und *ZRANB1*) und der Rotfärbung des Fleisches (*UBE3B*). Die SNP der vier Kandidatengene zeigten zudem signifikante Assoziationen mit Schlachtkörpermerkmalen wie der Kotelettfläche, Lendenfettdicke und dem Fleisch-Fett Verhältnis. Darüber hinaus war die Varianz der Transkriptmenge von *UBXN1, ZRANB1* and *TRIP12* signifikant mit den jeweiligen Polymorphismen korreliart. Gleichzeitig war die Transkriptmenge mit

Muskelleitfähigkeit, pH Wert und Flüssigkeitsverlust assoziiert. Die Transkriptmenge von *UBE3B* war assoziiert mit der Rotfärbung des Fleisches. Die Zusammenführung von Assoziations- und Expressionsdaten weist auf die Existenz kausaler Mutationen in den *cis*-regulatorischen Regionen der Kandidatengene hin, welche sich in einem unvollständigen Kopplungsungleichgewicht mit den entdeckten SNP befinden. Diese beeinflussen in erster Linie die Transkriptmenge und, als Konsequenz, Merkmale der Wasserbindungskapazität. Die vorliegende Studie demonstiert die konsistente dreiseitige Beziehung zwischen Phänotyp, Genotyp und Transkriptmenge bei allen vier Kandidatengenen. Tatsächlich befanden sich die detektierten SNP in einer Kopplungsphase mit Allelen kausaler Mutationen, welche die Transkriptmenge erhöhen und im Fall von *UBXN1* und *UBE3B* den Flüssigkeitsverlust erhöhen, wohingegen dieser im Fall von *TRIP12* and *ZRANB1* verringert wurde. Darüber hinaus weisen Interaktionen zwischen den Genen des Ubiquitin-Systems und dem Ryanodin 1 Rezeptor (*RYR1*) auf Möglichkeiten der weitergehenden Verbesserung der Fleischqualität hin, besonders in *RYR1* heterozygoten Tieren, indem der Genotyp an diesen Loci berücksichtigt wird. Die Ergebnisse dieser Studie unterstützen *UBXN1, UBE3B, TRIP12* und *ZRANB1* als funktionellen Kandidatengene für die Wasserbindungskapazität von Schweinefleisch.

Contents

1 Introduction

In the past decades, the meat industry sought to increase the profit due to the improvement of carcass quality that results in the increased carcass weight at reduced production costs. In order to produce the better carcass quality of pig, the processes lead to the higher incidences of the halothane gene, and subsequently the deterioration of meat quality such as PSE (pale, soft, exudative) and DFD (dark, firm, dry) meats. These kinds of pork are not accepted by the consumers, who feel confident with the meat products that had good economic values as well as satisfying palatability. Indeed, the attractiveness of pork to consumers depends on its intrinsic characteristics, such as leanness, taste, odour, tenderness and juiciness. Especially, the tenderness is assessed as one critical point of preferences of consumers for a better pork quality. In the pork chain, there are a number of factors that impact on the water holding capacity of meat and eating quality in certain directions. In particular, the loss of inherent water retained in muscle cells that occurrs due to the effects of pre-slaughter and post mortem processes, the drip loss phenomena, causes the incidence of unacceptable tenderness of pork. Before slaughter, the muscle contraction and metabolism are maintained with the energy supply of ATP and regulation of Ca^{2+} channels. In the early post mortem, the muscle tissue lacks oxygen and the metabolism changes to the anaerobic glycolysis resulting in the decreased of glycogen and ATP levels in muscle. Correspondingly, the lactic acid is accumulated to accelerate the rate of pH decline, and consequently leading to the negative

effects on meat quality characteristics, including reduced water holding capacity and meat redness. Moreover, variations in post mortem proteolysis affect meat tenderness during the conversion of muscle to meat. Indeed, the degree of degradation of cytoskeletal proteins, which was shown to be associated with the calpain/calpastatin system, cause variation of water holding capacity of meat. Pigs breeding aims to select animals that had the good quality of meat with reduced incidence of PSE. However, the performance tests are expensive and many quality traits are difficult for measurement and/or have low heritability. Molecular genetic approaches are applied to identify the variation in the DNA-sequence of genes associated with the increased of drip loss formation. Thereafter, the pork quality will be improved by the selection towards reduced frequency of unfavoured alleles of these genes in the commercial pigs.

1.1 Genetics affecting pork quality

The genetic background of pigs is evaluated as a critical factor to explain an appreciable part of the variation in meat quality (Casteels et al., 1995). It has been widely reported about several major genes, which involve many biological pathways or mechanisms, that exhibit strong effects on the meat quality such as: porcine stress syndrome *RYR1* (ryanodine receptor); increasing the glycogen content of the `white´ (fast-glycolytic) muscle types *RN* (Rendement Napole); fatty acid binding protein (*FABP3, FABP4* and *FABP5*); insulin-like growth factors *IGF-1* and *2* and leptin and leptin receptor *LEPTIN* and *LEPR* (Purslow et al., 2008). Many studies provide molecular evidences that the degradation of myofibrillar proteins in muscle related to the calpain system shows strong impact on the variation of meat characteristics. Indeed, the functional candidate gene, calpastatin (*CAST*), plays a role as an inhibitor of calpain and has high impact on the meat tenderness of pork. Moreover, the interaction between the *CAST* and the stress susceptibility (halothane gene) exhibit a strong influence on the water holding capacity of pork, these in turn indicate the combining effects of the muscle contraction and proteolysis processes on the drip loss formation.

1.2 Source of candidate genes for water holding capacity of pork

The gene expression analysis was performed to investigate the correlation between the quantitative traits and gene activity within functional networks. Since the last decade, many researches applied microarrays technique for a gene expression analysis approach to identify candidate genes for traits related to meat quality. In the previous studies of Ponsuksili and colleagues, the transcript levels of *M. longissimus dorsi* were obtained from 72 animals

(selected from 572 F2 animals of a crossbreed between Duroc and Pietrain, DuPi population) using the GeneChip Porcine Genome Arrays. Correspondingly, the transcriptional profiles and the expression QTL (eQTL) analysis revealed 1,279 transcripts with trait correlated expression to water holding capacity (Ponsuksili et al., 2008a). The expressions of several members of the ubiquitin proteasome system were associated with drip loss, conductivity, pH, or a principle component with high loadings for meat quality traits (Ponsuksili et al., 2009, Ponsuksili et al., 2010b). The comparison of the transcript levels of two extreme groups of six discordant sib pairs (selected from 572 animals of DuPi population) that had high divergence in drip loss (4.14 ± 0.77% vs. 0.9 ± 0.77%, mean ± standard deviation) ($p < 0.0001$) provided a number of differentially expressed genes, which are involved in the ubiquitination process, and that might be responsible for the different drip loss in the individuals (Ponsuksili et al., 2008b).

During the conversion of living muscle to post mortem meat, the ubiquitin-proteasome is assessed as one important proteolysis system causing the degradation of myofibrillar proteins in muscle cell. Thus, the expression studies of Ponsuksili and co-workers provided functional evidences to illustrate the strong effects of ubiquitin proteasome systems on the quality of pork. Moreover, the authors also suggested the list of candidate genes for water holding capacity of meat, in which the subset of genes related to the ubiquitination pathway was referred. Correspondingly, the four candidate genes in our study were selected from the source of promising candidate genes, depending on (i) known function of the particular gene in the ubiquitination process and/or (ii) their genetic positional information of QTL for meat quality traits.

1.3 The functions of candidate genes involve the mechanism of ubiquitination/deubiquitination processes

The ubiquitin proteasome system has many functions to control the biological events due to the degradation of specific proteins (Attaix et al., 2002; Lipford et al., 2005). In particular ubiquitination pathway affects the muscle atrophy, the process of endoplasmic-reticulum-associated degradation as well as regulates the cell adhesion and migration in skeletal muscle (Jensen et al., 1995; Ward et al., 1995; Taillandier et al., 2004; Huang et al., 2009 and 2010). Ubiquitination is an ATP-dependent proteolysis process to attach ubiquitin molecule, Ub - a seventy six amino acid polypeptide - to a protein substrate. In the presence of ATP, Ub is activated by an Ub-activating enzyme (E1), and is then transferred to one of a number of Ub-conjugating enzymes (E2s) by trans-thiol esterification. E2 binds the first Ub molecule to

protein substrates generating a monoubiquitinated protein. Several E2 are involved in the formation of polyubiquitinated conjugates due to the continued binding of Ub molecules. Subsequently, an E2 interacts with Ub-protein ligase (E3) and transfers an Ub molecule to E3, which is responsible for the recognition of substrates, thereafter an Ub is transferred to the selected proteins. The tagged proteins containing at least four Ub molecules become a target of the 26S proteasome for degradation to generate small peptides, and the ubiquitin tail is cleaved to supply the source of free Ub molecules. The ubiquitination process is disrupted by deubiquitinating enzymes (DUBs), which play roles to cleave the iso-peptide bonds within Ub molecule chains and/or between protein substrate and Ub tails, avoiding the destruction of proteins by 26S proteasome. The ubiquination pathway is shown in the Figure 1.

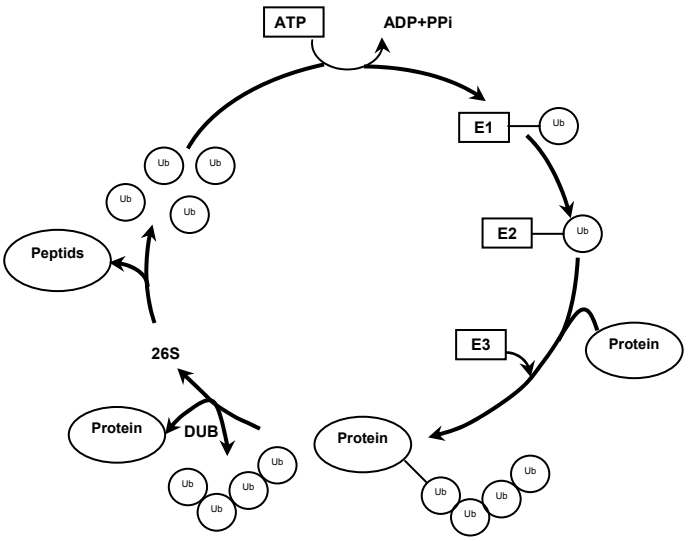

Figure 1: Schematic cycle showing the attachment of a polyubiquitin chain to the protein substrate and the degradation of tagged proteins by the 26S proteasome

In the current study, four candidate genes of the ubiquin-proteasome system were analysed, that encode proteins containing the specific domains playing roles in the ubiquination/deubiquination processes. Two candidate genes, E3 ligase (*UBE3B*) and thyroid hormone receptor interacting protein 12 (*TRIP12*), function as an E3 enzyme in the ubiquitination process. In the human genome, HECT and RING are two common types of E3s that attach Ub to the substrates following different ways (Rotin et al., 2009). The HECT domain contains the conserved Cys residue in the C-lobe and the E2 binding site in the N-lobe. It interacts with E2 to form an intermediate thioester bond between a cysteine residue in the HECT domain and C terminus of Ub molecule. Subsequently, HECT transfers Ub to a target protein. Otherwise, the RING-finger domain directly transfers Ub from E2 to a protein

substrate. A large proportion of E3s belongs to the RING family whereas only 28 E3s are members of HECT including the two candidate genes, *UBE3B* and *TRIP12*.

The linkage between the proteolysis of endoplasmic reticulum (ER) associated proteins and the regulation of cell adhesion was reported by Darom et al., 2010. Indeed, a conserved E3 ligase RING finger protein (*RNF-121*), which is expressed in the endoplasmic reticulum (ER) of cells, targeted a transmembrane glycoprotein (β-integrin) for degradation by 26S proteasome. The destruction of integrins leads to the formation of drip channels to accelerate the purge loss in muscle cells. The UBX domain containing protein 1-like gene (*UBXN1*) belongs to a UBX domain protein subfamily (SAKS1). It might be considered as an ubiquitin receptor of the valosin containing protein (VCP) that interacts with E3 ubiquitin ligase to regulate the ERAD process. In case, the *UBXN1* encodes a protein containing a UBA domain (ubiquitin - associated) and a UBX domain (ubiquitin-like); these domains recruit the ubiquitinated substrates from ER membrane to the 26S proteasome, avoiding the effects of deubiquitinating factors. Correspondingly, the UBA domain binds with polyubiquitin chain of substrate; on the other site the UBX domain interacts with VCP to protect the association of the VCP with ubiquitinated protein (McNeill et al., 2004; Schuberth et al., 2008; Dikic et al., 2009).

In the opposite aspect, the function of ubiquitin thioesterase (*ZRANB1* or *TRABID*) is to disrupt the ubiquitination process preventing the degradation of protein substrates by 26S proteasome. The *ZRANB1* consists of an ovarian tumor protease (OTU) domain that is a subclass of cysteine proteases DUBs (deubiquitinating enzymes). The function of OTU is to cleave the isopeptide bond within polyubiquitin chain and to remove polyubiquitin signals from tagged substrates (Nijman et al., 2005; Tran et al., 2008; Komander et al., 2009; Sowa et al., 2009; Bai et al, 2011; Fushman et al., 2011). The activity of *TRABID* OTU domain depends on lysine residues of ubiquitin molecules. Several authors showed that *TRABID* cleaved the Lys29- and Lys33-linked diubiquitin more efficient than the Lys63 linkages (Fushman et al., 2011; Clague et al., 2012 and Licchesi et al., 2012).

1.4 Aims of the current study

The objective of the study was to evaluate the effects of functional candidate genes on meat quality by the identification of polymorphisms in transcribed regions of these four candidate genes (*UBE3B*, *TRIP12*, *UBXN1* and *ZRANB1*), to test for associations of detected SNPs with the traits related to meat characteristics. Subsequently, we applied the gene expression analysis to address the variation of transcript abundance of the four genes affecting the quantitative traits of pork on the one hand, as well as we assessed the relationship between the transcript abundance and variation at the level of genotype on the other hand. The combination of two sites might generate the consistent of the three-way relationship among genotype – phenotype - transcript abundance, to illustrate the existence of causal polymorphisms in *cis*-regulatory regions of these functional candidate genes that primarily influence gene expression and, secondarily, water holding capacity. Integration the results of association and expression studies provided genetic envidences to support *UBE3B*, *TRIP12*, *UBXN1* and *ZRANB1* as the funtional candidate genes for pork quality.

The total ca. 570 pigs derived from two populations, including commercial herds of German Landrace (GL) and Pietrain x (German Large White x German Landrace) (PiF1), were used for this study. According to *in silico* analyse of the four genes, seven novels of SNPs were identified in the coding region; and two SNPs were detected in the 3´-UTR region of *UBE3B* gene. Based on the position in the regions with potential function and/or the causing of amino acid exchanges, seven out of nine polymorphisms were selected for genotyping, association and expression studies. The effects of these candidate genes on carcass and meat characteristics are displayed in the following sections.

2 Publications

2.1 *UBXN1* polymorphism and its expression in porcine *M. longissimus dorsi* are associated with water holding capacity

Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers

Research paper published in `Molecular Biology Reports´, (2014): DOI 10.1007/s11033-013-2985-5

2.2 Novel SNPs of the porcine *TRIP12* are associated with water holding capacity of meat

Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers

Research paper published in `Czech Journal of Animal Science´, 58, 2013 (11): 525–533

2.3 *UBE3B* and *ZRANB1* polymorphisms and transcript abundance are associated with water holding capacity of porcine *M. longissimus dorsi*

Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers

Research paper published in `Meat Science´, 95 (2013): 166–172

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Molecular Biology Reports 2014, DOI 10.1007/s11033-013-2985-5.

Conceived and designed the experiments: KW SM SP EM. Performed the experiments: TPLH EM SP KW. Analyzed the data: TPLH EM SP KW. Contributed reagents/materials/analysis tools: SM KW SP EM. Wrote the paper: TPLH KW.

Mol Biol Rep
DOI 10.1007/s11033-013-2985-5

UBXN1 polymorphism and its expression in porcine *M. longissimus dorsi* are associated with water holding capacity

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Received: 6 December 2012 / Accepted: 24 December 2013

Abstract The UBX domain containing protein 1-like gene (*UBXN1*) promotes the protein degradation that affects meat quality, in particular traits related to water holding capacity. The aim of our study was to identify *UBXN1* polymorphisms and to analyse their association with meat quality traits. Moreover, the relationship of *UBXN1* polymorphisms and its transcript abundance as well as the link between *UBXN1* expression and water holding capacity were addressed. Pigs of the breed German landrace (GL) and the commercial crossbreed of Pietrain × [German large white × GL] (PiF1) were used for this study. In GL, the novel SNP c.355 C > T showed significant association with conductivity and drip loss ($P \leq 0.05$). Another SNP at nt 674 of the coding sequence [SNP c.674C>T (p.Thr225Ile)] was associated with drip loss ($P \leq 0.05$) and pH_1 ($P \leq 0.1$). In PiF1, the SNP *UBXN1* c.674C>T was associated with conductivity ($P \leq 0.01$). Moreover, the haplotype combinations showed effects on conductivity within both commercial populations at $P \leq 0.1$. In both populations, high expression of *UBXN1* tended to decrease water holding capacity in the early post mortem period. The analysis of triangular relationship of *UBXN1* polymorphism, transcript abundance, and water holding capacity evidences the existence of a causal polymorphism in cis-regulatory regions of *UBXN1* that influences its expression.

Keywords *UBXN1* · Ubiquitin · Meat conductivity · Meat pH · Drip loss · Association · Differential expression

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Introduction

During the post mortem period, there occur a lot of biochemical reactions that affect the meat quality, in particular traits related to water holding capacity such as pH value, conductivity and drip loss, which in turn influence the sensory quality of meat and the economy of meat production. The degradation of myofibrillar proteins has been suggested as an important process affecting the water holding capacity of meat [1]. In particular, the calpain and calpastatin proteolytic system was associated with meat tenderness and water holding capacity [1, 2]. Also, the ubiquitin–proteasome contributes to the protein degradation. The ubiquitination process involves linking target proteins to chains of ubiquitin by three enzymatic components, and the subsequent recognition and degradation of the ubiquitinated small peptides by the 26S proteasome [3–7]. Holistic expression studies—either evaluating differential expression of animals divergent for meat quality traits or correlating expression and meat quality traits—revealed that mechanisms of the ubiquitination pathway affect muscle properties that are relevant for the quality of

Published online: 10 January 2014

meat. In fact, expression of several members of the ubiquitin–proteasome system was associated with meat quality traits including drip loss, pH, conductivity, water holding capacity, tenderness, or a principal component with high loadings for these traits [8–10].

The *UBXN1* gene encodes a protein that exhibits the ubiquitin regulatory X (UBX domain) and that promotes the proteolytic destruction of a subset of proteins in the course of the polyubiquitination process [11, 12]. Therefore, the *UBXN1* is considered as an important functional gene for meat quality. Moreover, the *UBXN1* gene is addressed as a promising positional gene because it locates on chromosome 2 that relates to several QTL for meat quality traits, such as drip loss, pH, conductivity and cooking loss [13–18].

The aim of our study was to identify polymorphisms in the *UBXN1* gene and to evaluate its association with traits related to meat characteristics. We found strong evidence for the role of UBXN1 for meat quality by showing a consistent triangular relationship of variation at the level of genotype, transcript abundance and phenotype in commercial pigs.

Materials and methods

Animals and phenotyping

Phenotypes and genomic DNA used for this research were obtained from animals of the breed German landrace (GL, $n = 271$), and the commercial crossbreed of Pietrain × [German large white × GL] PiF1 ($n = 317$). Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection and the experimental protocol was approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology (FBN). All the pigs were slaughtered at the abattoir of the FBN and the phenotypic data for carcass and meat quality were collected according to the guidelines of the ZDS e.V. [19]. Carcass traits addressed in the study were loin eye area (LEA), meat to fat ratio (MFR), and loin fat depth (BF). Meat quality traits analysed cover the indicators of water-holding capacity including drip loss (DRIP), pH and conductivity at 45 min post mortem (pH1, CON1) as well as at 24 h (pH24, CON24). Conductivity and pH-value were measured by Star-series equipment (R. Matthaeus Klausa, Germany) in *M. longissimus dorsi* between 13th and 14th rib. Drip loss was assessed by the bag-method with a size-standardized sample from the *M. longissimus dorsi* collected at 24 h post mortem. The samples were weighed and suspended in a plastic bag at 4 °C for 48 h before re-weighing. Drip loss percentage was calculated by expressing the change in weight as a percentage of the original weight.

SNP detection

After slaughtering, *M. longissimus dorsi* tissue samples were collected between 13th and 14th rib and frozen in liquid nitrogen for DNA and RNA isolation. Two pairs of specific primers (*UBXN1*_1FW 5′-tgatggagcacgaagacgacc-3′, *UBXN1*_1RV 5′-gaagtcccatctggcagcctg-3′; *UBXN1*_2FW 5′-caggagacaagggcaggagt-3′, *UBXN1*_2RV 5′-caatgaggac agcagaaggcac-3′) were used to amplify overlapping fragments of the coding region of the *UBXN1* gene. These primers were designed according to the sequence of the porcine gene in GenBank (XM_003353824.1), and the amplicon sizes of the two fragments were 559 and 500 bp, respectively. Polymorphisms of *UBXN1* were detected in cDNAs of eight animals from different breeds (German landrace, German large white, Pietrain and Duroc) by comparative sequencing of PCR fragments in each animal. Polymerase chain reactions (PCR) were performed in a 25 µl volume containing 2 µl cDNA of a panel animal, 1× PCR buffer (with 1.5 mM $MgCl_2$), 200 µM of each dNTP, 0.2 µM of each primer and 0.5 U of Taq DNA polymerase (GeneCraft, Münster, Germany). The PCR followed a protocol including an initial denaturing at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 62–65 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were purified and comparatively sequenced in both directions on an ABI 3130 DNA Analyzer (Life Technologies, Carlsbad, USA).

Genotyping

Pyrosequencing technique was applied for genotyping of animals of the GL and PiF1 populations. Genotyping primers (*UBXN1*_1PyFW 5′-biotin-tgatggagcacgaagacgac-3′, *UBXN1*_1PyRV 5′-ctcaggaatgtgggcttgtttc-3′; *UBXN1*_2PyFW 5′-biotin-ttatctcctttgtcctccagtatg-3′, *UBXN1*_2PyRV 5′-cactggtcatactccgctt-3′) were designed to amplify 289 and 130 bp fragments that contained the SNP c.355C>T and c.674C>T. The PCR procedure was 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Subsequent washing and capture of templates as well as sequencing was performed using the Pyro Gold Reagent Kit (Biotage, Uppsala, Sweden) on a PSQ96MA Pyrosequencing instrument (Biotage Uppsala, Sweden) according to the manufacturer's instructions as described before [20].

Genexpression analyses

In order to assess the relationship between genotypic variation and transcript abundance of *UBXN1,* ten animals per diplotype class in both segregating populations were selected for quantitative real-time RT-PCR (qRT-PCR). Total RNA was isolated from *M. longissimus dorsi* tissue

by Tri-Reagent (Sigma-Aldrich, Hamburg, Germany). After DNase treatment, RNA was cleaned up by Nucleospin RNA II kit (Macherey–Nagel, Düren, Germany). The quantity of RNA was evaluated on a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany) and RNA integrity was checked using 1 % agarose gel. First strand cDNA synthesis was performed by reverse transcription PCR of total RNA, using random and oligo d(T) primers and Superscipt III reverse transcriptase (Life Technologies, Darmstadt, Germany; Invitrogen, Karlsrhuhe, Germany). The specific primers for the qRT-PCR (*UBXN1*_qPCRFW 5′-caggagacaagggcaggagt-3′, *UBXN1*_qPCRRV 5′-ctctgctttgtccctttcgatc-3′) were designed with the online primer designing tool Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). For the normalisation of the candidate gene, *RPL32* (ribosomal protein 32) (primers: forward: 5′-agcccaagatcgtcaaaaag-3′ reverse: 5′-tgttgctcccataaccaatg-3′) and *HPRT1* (hypoxanthine phosphoribosyltransferase 1) (primers: forward: 5′-gtgatagatccattcctatgactgtaga-3′, reverse: 5′-tgagagatcatctccaccaattactt-3′) were selected as the house keeping genes [8]. The reaction mixture contained 2 µl cDNA, 0.5 µM of forward and reverse primers, 5 µl of LightCycler SYBR Green I Master kit (Roche, Mannheim, Germany) and water to obtain a final volume of 10 µl. All of the samples were run in duplicate following the program: initial denaturation of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 10 s for annealing (60 °C) and 15 s for elongation (72 °C). The qRT-PCR analysis was carried out in a BioRad iCycler iQV (BioRad, Munich, Germany). All samples were analysed by melting curve analysis and checked for the presence of any non-specific PCR product using agarose gel electrophoresis. For each assay, serial dilutions (seven orders of magnitude from 10^2 to 10^8 start molecules) of target PCR products were amplified and used to derive a standard curve and to subsequently calculate the number of copies. According to the previous study of Ponsuksili et al. [8], normalisation of variation in PCR efficiency and initial RNA input were performed using *RPL32* and *HPRT1* genes as the internal controls by dividing the calculated mRNA copy numbers by a mean normalisation factor derived from the expression of housekeeping genes.

To further address the relationship of expression and meat quality (muscle conductivity) in commercial pigs we used *UBXN1* expression levels in *M. longissimus dorsi* from a previous microarray analysis of 173 individuals of the PiF1 that represent a subset of the animals used here for the association study. In brief, 500 ng of total RNA of *M. longissimus dorsi* per animal were used for hybridisation onto GeneChip Porcine Genome Arrays (Affymetrix) to obtain muscle expression patterns. Quantitative expression levels of the present transcripts, including UBXN1, were estimated using the probe logarithmic intensity error (PLIER) algorithm as implemented in the Expression Console software (Affymetrix). The microarray data were deposited in the Gene Expression Omnibus (GEO) public repository (GEO accession no. GSE32112) [21].

Association analysis

For each population, the allele and genotype frequencies of the detected SNP were calculated separately and deviation from Hardy–Weinberg equilibrium was tested by χ^2-test. Using a mixed model (PROC Mixed, SAS v. 9.1; SAS Inc., Cary, USA), the data were analysed for association between genotypic and phenotypic variation separately in the two commercial populations:

$$Y = \mu + \mathrm{GENO} + \mathrm{SEX} + \mathrm{RYR1} + \mathrm{DAM} + \mathrm{SIRE} + \mathrm{SW} + e,$$

where Y was the observation of traits and μ was the overall mean. The fixed effects included genotype (GENO), sex (SEX) and the genotype at the ryanodine receptor 1 (RYR1; only in PiF1 population). In the model, dam (DAM) and sire (SIRE) were random effects, slaughter weight (SW) was considered as a co-variable. Moreover, haplotypes segregating within both populations were assessed by the expectation–maximization (EM) algorithm, using the 'haplotype' procedure of SAS. These estimates were then used to assign the probability that each individual possesses a particular haplotype pair. Only those animals were used, whose haplotype pairs were assigned with the probability 1. In order to refer to both SNPs at a time a corresponding mixed model was applied separately for the two populations, where the diplotype, i.e. combination of haplotypes, was used as a fixed effect instead of genotype. Least square mean values for the genotypes and diplotypes were compared by *t* test and *P*-values were adjusted by the Tukey–Kramer correction. In order to correct for multiple testing, adjusted *P*-values according to the method of Benjamini and Hochberg [22] were computed using the PROC Multtest (SAS v. 9.1; SAS Inc., Cary, USA).

The association of gene expression levels obtained from microarray analyses with meat quality traits was evaluated using a mixed model (PROC Mixed, SAS v. 9.1; SAS Inc., Cary, USA):

$$Y = \mu + \mathrm{SEX} + \mathrm{RYR1} + \mathrm{DAM} + \mathrm{SIRE} + \mathrm{SW} + \mathit{UBXN1}\mathrm{exp} + e,$$

where Y was the observation of traits and μ was the overall mean. In the model, sex (SEX) and the genotype of RYR1 were assumed as fixed effects; dam (DAM) and sire (SIRE) were considered as random effects, slaughter weight (SW) and gene expression (*UBXN1*exp) were the linear effects and were included as the co-variates for meat quality traits,

and *e* is the residual error. Furthermore, we considered the *UBXN1* expression in *M. longissimus dorsi* of 10 divergent animals (five high CON1, mean CON1 = 11.88 ± 3.01 and five low CON1, mean CON1 = 3.52 ± 0.15; mean CON1 of all 173 animals = 5.14 ± 1.72). The association of *UBXN1* genotypes or diplotypes with *UBXN1* transcript abundance as well as the differential expression among the divergent animals were addressed by unpaired *t*-tests. Moreover, Pearson correlations between gene expression levels and meat quality traits as well as between expression levels obtained by qRT-PCR and by microarray data were estimated (SAS v. 9.1; SAS Inc., Cary, USA).

Results

SNPs and trait association

According to the alignment result of cDNA sequences of 8 animals from 4 different breeds, two novel SNPs were detected including a silent SNPC>T at position 355 (c.355C>T) and SNPC>T at position 674 (c.674C>T) that causes a change of amino acid from threonine to isoleucine (p.Thr225Ile). The two SNPs were genotyped in a total of 588 animals derived from the GL and PiF1 populations by pyrosequencing.

In both populations, the genotype distribution of SNP c.355C>T was not in Hardy–Weinberg equilibrium. For SNP c.674C>T, a significant deviation derived from this principle was found only in PiF1 population. For both SNPs the alleles 'C' were more abundant in both populations. Correspondingly, the genotype 'CC' was most frequent whereas the frequency of homozygous genotype 'TT' was very low across the two populations and thus not considered for analysis (Table 1).

In the GL population, statistical analysis revealed significant associations of SNP c.355C>T with muscle conductivity (CON1) and DRIP ($P \leq 0.05$) as well as with loin fat depth (BF3) ($P \leq 0.1$). The animals of genotype; 'CT' tended to produce carcasses with lower loin fat depth, muscle conductivity and drip loss than homozygous animals. Moreover, the genotypic variation at locus c.674C>T was associated with drip loss ($P \leq 0.05$) and pH value at 45 min post mortem (pH1) ($P \leq 0.1$), the animals of genotype 'CC' had higher drip loss, but lower pH1 value than 'CT' (Table 2).

In the PiF1 population, significant associations of the SNP c.355C>T were detected for LEA and MFR ($P \leq 0.1$). Pigs with the genotype 'CT' had smaller loin eye areas but higher MFRs than pigs with genotype 'CC'. The SNP c. 674C>T showed significant association with CON1 ($P \leq 0.01$), LEA and MFR ($P \leq 0.05$), as well as with loin fat depth ($P \leq 0.1$). The animals of genotype 'CT' tended to produce carcasses with smaller LEA, but higher MFR, higher loin fat depth as well as higher muscle conductivity (Table 2).

Four different haplotypes were identified in both populations that were designated [H1] for (CC), [H2] for CT, [H3] for TC, and [H4] for TT. The frequencies of haplotype [H2] and [H4] were low (Table 1). Accordingly, the haplotype combinations (diplotypes) [H1]/[H1] and [H1]/[H3] were more abundant than others. Four haplotype combinations were found in the PiF1 and three diplotypes were detected in the GL population. The diplotypes showed an association with muscle conductivity (CON1) in the GL and PiF1 populations at *P*-value ≤ 0.1, and among these diplotype [H1]/[H3] had the lowest muscle conductivity (Table 3).

Table 1 Allele and haplotype frequencies for UBXN1 c.355C>T and c.674C>T in GL and PiF1 populations

	Allele/haplotype	Populations	
		PiF1 ($n = 317$)	GL ($n = 271$)
c. 355C>T	C	0.823	0.825
	T	0.177	0.175
c. 674C>T	C	0.90	0.974
	T	0.10	0.026
Haplotype	[H1] CC	0.781	0.798
	[H2] CT	0.035	0.023
	[H3] TC	0.113	0.175
	[H4] TT	0.071	0.004

Expression study

In order to examine the relationship of gene variation and transcript abundance ten animals per diplotype class in the segregating populations were selected for qRT-PCR. The transcription of the *UBXN1* gene was higher in the PiF1 than GL population. In the PiF1 population, the heterozygous 'CT' at SNP c.355C>T and SNP c.674C>T had significantly higher transcription than homozygous 'CC' ($P \leq 0.05$), but the results were divergent in the GL population (Fig. 1). Consistently, the animals of diplotype [H1]/[H1] had the lowest relative abundance in the PiF1, but revealed the highest transcription in the GL population.

In order to finally address the triangular relationship of genotype, expression and phenotype, in PiF1, in addition to the link between genotype and phenotype on the one hand and genotype and expression on the other hand, as detailed above, also the association and correlation between expression and phenotype was analysed. Significant association of *UBXN1* expression as obtained from microarray analyses with the muscle conductivity at 24 h post mortem

Table 2 Association analysis results of the two SNPs with carcass and meat quality traits

SNPs	Traits	Populations	Least square means (LSM) (SE/n)		*P*-value*
			CT	CC	
c.355C>T	LEA	PiF1	52.94^{e} (0.46/113)	53.95^{f} (0.37/203)	0.069
	MFR	PiF1	0.29^{e} (0.01/113)	0.27^{f} (0.01/203)	0.071
	BF3	GL	1.79^{e} (0.05/97)	1.88^{f} (0.04/174)	0.093
	CON1	GL	4.20^{c} (0.09/97)	4.44^{d} (0.08/174)	0.049
	DRIP	GL	4.06^{c} (0.26/97)	4.56^{d} (0.23/174)	0.067
c.674C>T	LEA	PiF1	52.41^{c} (0.61/64)	53.88^{d} (0.34/252)	0.065
	MFR	PiF1	0.29^{c} (0.01/64)	0.28^{d} (0.01/252)	0.069
	BF3	PiF1	1.38^{e} (0.04/64)	1.29^{f} (0.02/252)	0.069
	CON1	PiF1	5.73^{a} (0.23/64)	5.09^{b} (0.16/252)	0.037
	pH1	GL	6.45^{e} (0.07/14)	6.32^{f} (0.03/254)	0.092
	DRIP	GL	3.31^{c} (0.49/14)	4.44^{d} (0.23/254)	0.049

*Adjusted *P*-values according to the linear step-up method of Benjamini and Hochberg [22]

[a,b] $P < 0.01$

[c,d] $P < 0.05$

[e,f] $P < 0.1$

Table 3 Least square means (LSM) and standard errors (SE) for muscle conductivity across diplotypes of UBXN1 in pigs

Population	Least square means (LSM) (SE/*n*)				*P*-value
	[H1]/[H1]	[H1]/[H2]	[H1]/[H3]	[H1]/[H4]	
GL	4.44^{a} (0.09/164)	4.40^{ab} (0.23/10)	4.22^{b} (0.10/92)	–	0.08
PiF1	4.73^{ab} (0.82/181)	5.31c^{b} (0.89/20)	4.58^{a} (0.83/69)	5.22^{b} (0.86/47)	0.10

[a,b] $P < 0.1$

(CON24) ($P \leq 0.05$) was found, as well as significant positive correlation between the transcription of *UBXN1* and CON24 (Pearson's coefficient = 0.18; $P = 0.02$). Accordingly, animals divergent for the trait CON1 showed significant different transcript abundance at $P = 0.02$, with higher transcript frequency in the high CON1 group. Moreover, in the PiF1 population, significant correlation between qRT-PCR and microarray data was detected (Pearson's coefficient = 0.61; $P = 0.01$).

Discussion

The ubiquitin–proteasome system is one of the major pathways that are responsible for protein turnover or protein degradation in eukaryotes. In brief, an intracellular protein is ubiquitinated by the covalent attachment of a polyubiquitin chain, and it is destructed into small peptides by the 26S proteasome. Three classes of enzymes are required to link polyubiquitin chain onto protein substrate including E1 (ubiquitin activating enzyme) and E2 (ubiquitin conjugating enzyme) and E3 (ubiquitin protein ligase). Interestingly, the ubiquitin–proteasome system also acts during the post mortem period. Nyam-Osor [23] and Sekikawa [24, 25] detected ubiquitin and ubiquitin protein conjugates in the sarcoplasmic proteins that were extracted from bovine cardiac muscle after slaughter. Until rigor mortis, small amounts of ATP are still present in the muscle cell and activate the ubiquitin–proteasome system that promotes protein degradation and contributes to the meat quality. Ponsuksili et al. [8, 9] showed, for the first time, an association of the expression of genes of the ubiquitination system with meat quality, in particular with traits related to water holding capacity. During the post mortem glycolysis, the membrane structure of muscle fibres becomes leaky and allows the fluids to move between the intra- and extra cellular space [26, 27]. At the same time, the decline of pH value causes the denaturation of the sarcoplasmic and myofibrillar proteins. Subsequently, the contraction of filaments removes water out of the myofilament lattice leading to drip loss and the change of soluble components in the fluids causes an increased conductivity [28]. Degradation of the myofibrillar proteins promoted by the calpain/calpastatin system and/or ubiquitination reduces the shrinkage of muscle cell associated with reduced water holding.

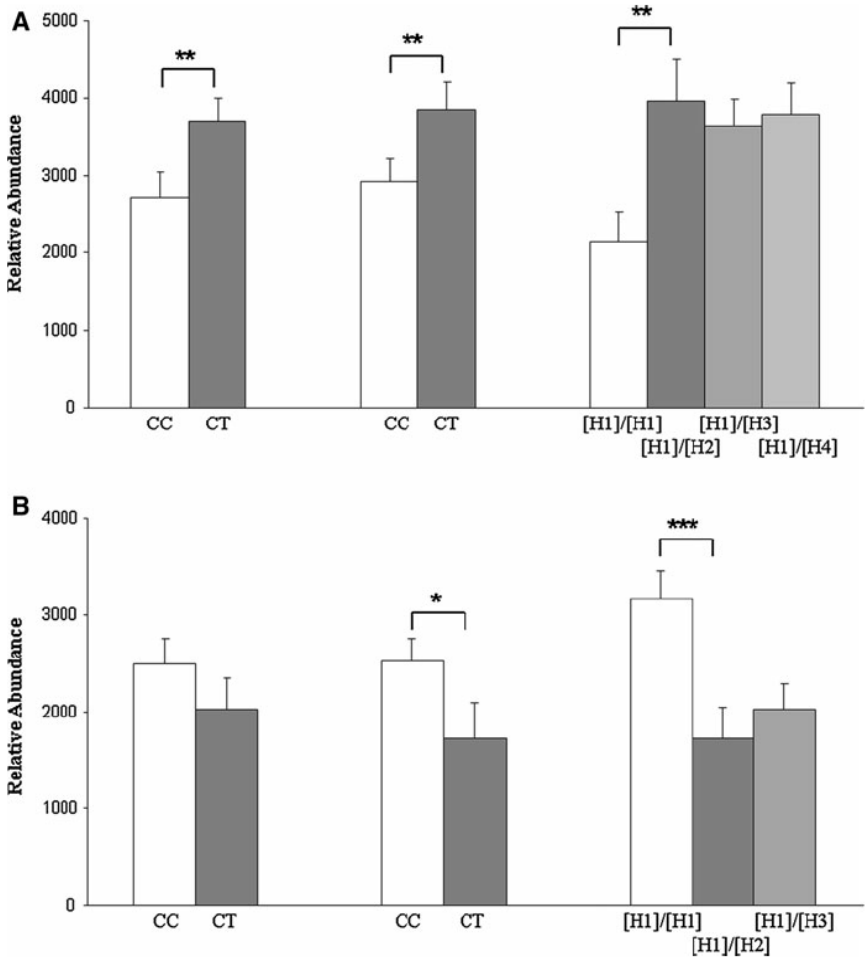

Fig. 1 The relative abundance of UBXN1 in *M. longissimus dorsi* according to the genotypes at SNP c.355C>T and SNP c.674C>T and the respective diplotypes in both populations, PiF1 (**a**) and GL (**b**). Each bar shows the mean ± standard error for each genotype/diplotype class. The stars indicate the significant differences at *P*-value levels of: $*P \leq 0.1$; $**P \leq 0.05$ and $***P \leq 0.01$

The relationship between the protein degradation, in particular of desmin, titin, as well as vinculin, and the variation in water holding capacity during post mortem has been extensively reported [1, 2, 29–31]. In the early post mortem period, degradation of the myofibrillar proteins promoted by the calpain/calpastatin system and/or ubiquitination reduces the linkage of intermediate filament proteins in muscle cells and thus the water holding capacity. During this post mortem period, before depletion of ATP and onset of muscle rigor, the impact of protein degradation systems on water holding capacity is associated with the release of immobilized water due to fragmentation of inter-myofibrillar and costameric connections by the ubiquitin–proteasome [24, 25, 32, 33] and calpain system [1, 2, 31]. However, from 24 h post mortem onward, the increased degradation of these proteins results in the decrease of muscle cell shrinkage as well as in lower drip loss, i.e. higher water holding capacity [1, 30].

In the current study, *UBXN1* gene is considered as a functional candidate gene for meat quality because its domain (ubiquitin regulatory X) binds to valsolin containing proteins, which link to Lys^{48} of ubiquitin and become the target proteins for the degradation of 26S proteasome [12, 34]. Two novel SNPs were identified in the coding region of the *UBXN1* gene, but these did not belong to the UBX domain. However, the SNP c.674C>T causes an amino acid exchange from threonine to isoleucine that might alter the function of the protein due to a change from polar to non-polar amino acid, whereas SNP c.355C>T is a synonymous nucleotide exchange. Indeed, SNP c.674C>T showed significant associations with parameters of water holding capacity across both population. An effect on meat quality of the silent SNP c.355C>T was only evident in the GL but not in the PiF1 population. Haplotype analysis revealed an effect on muscle conductivity in both populations, however, with the trend of the double homozygous haplotypes [H1]/[H1] being associated with increased conductivity in the GL, but decreased in the PiF1 population. The lack of consistency of the effects of the polymorphisms of *UBXN1* gene and the genotype combinations of the two polymorphic sites among the commercial populations might be due to the interactions

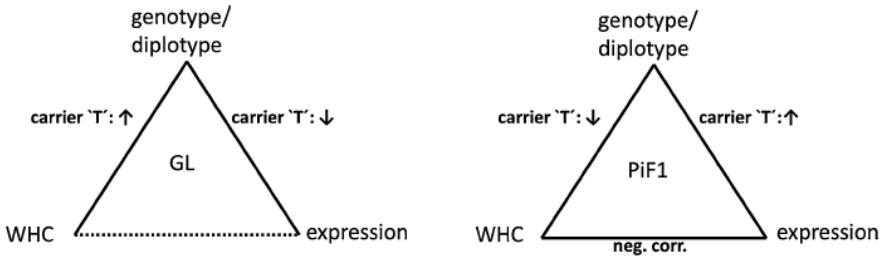

Fig. 2 Schematic outline of the triangular relationship obtained between the *UBXN1* genotypes/diplotypes, *UXBN1* transcript abundance, and traits related to water holding capacity solid lines denote relationships that were experimentally shown; dotted lines indicate suggested relationships The up or down arrows indicate the associations of the minor allele 'T' with the high or low WHC and transcript abundances, respectively

between *UBXN1* and other genes relevant to meat quality, which may differ in the different genetic background of the crossbred and purebred populations. The different directions of effects of *UBXN1* polymorphisms might be due to the fact that the detected SNPs in the coding region are not likely to be causal factors for the effects obtained, but these SNPs might be in incomplete linkage disequilibrium with other yet to be detected causal polymorphisms, which may be located in another region of the *UBXN1* gene—most likely in a cis-regulatory region of the gene.

Our findings regarding the association of *UBXN1* polymorphism and its transcript abundance consistently support the latter hypothesis in both populations.

In the GL population, the heterozygous carriers of the minor allele 'T' of the silent SNP c.355C>T showed significant lower muscle conductivity and tended to have lower transcript abundance than the homozygous 'CC'. SNP c.674C>T did not show a significant effect on the muscle conductivity, but on pH1 and DRIP with the genotype 'CT' associated with higher pH1 and lower DRIP; consistently also muscle conductivity was lower for the genotype 'CT' (data not shown). Moreover, 'CT' showed lower transcript abundance ($P \leq 0.1$). This finding implies that in GL the alleles 'T' at both SNPs are in linkage phase with an allele of a functional polymorphism decreasing the transcript abundance and increasing water holding capacity.

In the PiF1 population, the animals of the genotype 'CT' at locus c.355C>T and c.674C>T tended to exhibit increased muscle conductivity, and these had significantly higher transcription levels than the homozygous 'CC' ($P \leq 0.05$). Moreover, the effect of SNPs on the variation of gene expression was confirmed in the haplotype analysis. These results indicate that in PiF1 the alleles 'T' at both SNPs were in linkage phase with an allele of a presumed causal site increasing the transcript abundance and decreasing water holding capacity. In PiF1 also the relationship between transcript abundance and water holding capacity was evaluated and indicated a negative correlation, which is in line with the relationship of genotype and expression as well as genotype and phenotype (Fig. 2).

Conclusion

The results derived from the current study provide genetic evidence for effects of variation in the *UBXN1* gene on carcass and meat quality traits in pigs. The lack of consistency of the effects of *UBXN1* polymorphisms among the commercial populations might be due to the interactions between *UBXN1* and other genes relevant to meat quality. Moreover, the novel SNPs detected here seem to be in opposite linkage phase with a putative causal polymorphism in cis-regulatory regions of *UBXN1* that primarily influence gene expression and subsequently, water holding capacity. Indeed, the existence of this causal factor was illustrated within the both populations, due to the analysis of triangular relationship of *UBXN1* polymorphisms, transcript abundance, and water holding capacity.

Acknowledgments This research was partly supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; WI 1754/14-1) the Ministry of Education and Training of Vietnam, the German Academic Exchange Service (DAAD), and the Cantho University (Vietnam). Thanks are also due to the Pig Breeding Association of Mecklenburg-Western Pommerania.

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2.2 Novel SNPs of the porcine *TRIP12* are associated with water holding capacity of meat

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Czech Journal of Animal Science 2013, 58(11), 525–533.

Conceived and designed the experiments: KW SM SP EM. Performed the experiments: TPLH EM SP KW. Analyzed the data: TPLH EM SP KW. Contributed reagents/materials/analysis tools: SM KW SP EM. Wrote the paper: TPLH KW.

Novel SNPs of the porcine *TRIP12* are associated with water holding capacity of meat

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ABSTRACT: Degradation of proteins during maturation of meat, mediated by the calpain/calpastatin system and the ubiquitination system, largely affects the tenderness and the water holding capacity (WHC) of meat. The thyroid hormone receptor interacting protein 12 (*TRIP12*) is known as a HECT domain-containing E3 ubiquitin-protein ligase that recognizes protein substrates for ubiquitination. This study aims to identify polymorphisms of the *TRIP12* gene and to evaluate the relationship between genotype, transcript abundance, and meat quality traits in pigs. Two synonymous SNPs (XM_003484315.1:c.2211T>C, c.4957A>C) were identified that segregated among animals of herds of the breed German Landrace (DL, n = 269) and the commercial crossbreed of Pietrain × (German Large White × German Landrace) (PiF1, n = 300). Statistical analysis revealed associations between *TRIP12* polymorphisms and the organismal traits related to water holding capacity, i.e. conductivity 45 min postmortem (CON_1, $P < 0.1$) and pH 24 h postmortem (pH_{24}, $P < 0.1$). Haplotype analysis revealed consistent effects on muscle CON_1 in the two populations ($P < 0.1$). Carriers of the minor alleles *C* at the two polymorphic sites tended to have higher transcript abundance as well as higher water holding capacity. The integrated analysis of genotypic and haplotypic variation, transcript abundance, and technological parameters of WHC indicates that the XM_003484315.1:c.2211T>C and c.4957A>C of *TRIP12* are in linkage disequilibrium with a causal factor located in a *cis*-regulatory region, which affects in the first instance gene expression and in the second traits related to water holding capacity. Our results provide statistical-genetical evidence supporting *TRIP12* as a functional candidate gene for water holding capacity of porcine *M. longissimus dorsi*.

Keywords: E3 ligase; swine; drip loss; expression; ubiquitination; protein degradation; meat quality

The ubiquitination system catalyzes a non-lysozomal proteolytic pathway. Upon activation of ubiquitin-activating enzyme E1 with ATP and transfer of ubiquitin to ubiquitin-conjugating enzymes (E2), ubiquitin-protein ligases (E3) bind the first ubiquitin molecule to target proteins. After polyubiquitination of the targets by E4 the tagged proteins become degraded by the 26S proteasome (Attaix et al., 2002; Taillandier et al., 2004; Herrera-Mendez et al., 2006). In muscle, the ubiquitination system contributes to the breakdown of myofibrillar and intermyofibrillar proteins as well as costameric connections including integrins. This process precedes postmortem during the shift from muscle to meat. The degree of postmortem protein degradation in muscle affects the water holding capacity (WHC) of meat (Huff-Lonergan and Lonergan, 2005). Reduced lateral shrinkage of myofibrils, due to breakdown of myofibrillar muscle proteins, increases WHC. However, in

Supported by the German Research Foundation (Project No. WI 1754/10-14), German Academic Exchange Service (DAAD), Ministry of Education and Training of Vietnam, and Cantho University, Vietnam.

particular, the degradation of integrins leads to the formation of so-called drip channels in muscle decreasing the WHC of meat (Lawson, 2004; Zhang et al., 2006; Bee et al., 2007).

Thyroid hormone receptor interacting protein 12 (*TRIP12*) is the E3 enzyme and thus facilitates ubiquitination of substrates in conjunction with E1, E2, and E4 enzymes. *TRIP12* contains a HECT domain and thus belongs to the HECT-domain E3 ubiquitin ligases, to which is common that a conserved cysteine residue contributes to the formation of an intermediate thioester bond with the ubiquitin C terminus before transfer of ubiquitin to the target proteins (Park et al., 2008, 2009; Rotin and Kumar, 2009; Pandya et al., 2010; Kim et al., 2011). In the recent studies, the expressions of genes encoding proteins of the ubiquitination system, including several E3 ligase genes, showed significant associations with the parameters of water holding capacity as well as tenderness of meat (Ponsuksili et al., 2008a,b; Ponsuksili et al., 2009, Damon et al., 2012). Therefore, the *TRIP12* is considered as a functional gene for meat quality in our study, aiming to identify polymorphisms of *TRIP12*, to determine the mRNA expression in *M. longissimus dorsi*, and evaluate the three-way relationship of variation at the level of genotype, organismal traits related to meat characteristics, and transcript abundance in commercial pigs.

MATERIAL AND METHODS

Animals and phenotyping

Phenotypic data and genomic DNA of animals of the breed German Landrace (DL, n = 269), and the commercial crossbreed of Pietrain × (German Large White × German Landrace) (PiF1, n = 300) were used in this study. Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection and the experimental protocols were approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology (FBN). Carcass and meat quality traits were recorded according to the guidelines of German performance test (ZDS, 2004) after slaughter at the abattoir of the FBN. Technological meat traits that are indicators of water-holding capacity including drip loss (DRIP), pH, and conductivity 45 min postmortem (pH_1, CON_1) as well as at 24 h (pH_{24}, CON_{24}), were considered. Conductivity and pH-value were measured by Star-series equipment (Matthäus, Klausa, Germany) in *M. longissimus dorsi* between the 13th and the 14th rib. Drip loss was assessed by bag-method according to Honikel (1998). We collected and weighed size-standardized samples from the *M. longissimus dorsi* 24 h postmortem and re-weighed them after 24 h in a plastic bag at 4°C. In addition, meat to fat ratio (MFR) was included to the analysis as a comprehensive carcass trait.

SNP detection

DNA and RNA were isolated from *M. longissimus dorsi* tissue samples taken between the 13th and the 14th rib and snap frozen in liquid nitrogen immediately after slaughter. DNA was isolated by phenol-chloroform extraction. Total RNA was isolated with Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany). DNase (Roche, Mannheim, Germany) treatment and column based clean-up (Nucleospin RNA II kit; Macherey-Nagel, Düren, Germany). The quantity of RNA was determined on a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany), and RNA integrity was verified using 1% agarose gel. RNA (1.5 µg) was reverse transcribed to cDNA using Superscipt III reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a reaction containing 500 ng oligo $(dT)_{11}$ VN primer and 500 ng random hexamer primer according to the manufacturer's protocol.

The polymorphisms of *TRIP12* were detected in cDNAs of 8 animals from different breeds (German Landrace, German Large White, Pietrain, and Duroc) by comparative sequencing of PCR fragments of each animal. The specific primers were designed according to the published sequences (GenBank Accession No. XM_003484315.1). The two pairs of primers were: FW1 5'-gttccaactgtccaactttag-3' and RV1 5'-gcattacaccttcccttctg-3'; FW2 5'-gaaacatcccaacatggcttac-3' and RV2 5'-catactgtagactctctttggtc-3'; amplicon sizes of the two fragments were 747 and 753 bp, respectively. Polymerase chain reactions (PCR) were performed in a 25 µl volume with 30 ng of cDNA, 1 X PCR buffer (with 1.5mM $MgCl_2$), 200µM of each dNTP, 0.2µM of each primer, and 0.5 U of *Taq* DNA polymerase (GeneCraft, Münster, Germany). Cycling conditions were: initial denaturing at 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C for 30 s, and elongation at 72°C for 1 min; final extension

at 72°C for 5 min. The PCR products were purified and comparatively sequenced in both directions on an ABI 3130 DNA Analyzer using ABI kits and protocols (Life Technologies, Darmstadt, Germany).

Genotyping

A total of 569 animals derived from the DL and PiF1 populations were genotyped at the novel SNPs by pyrosequencing. Genotyping primers were designed according to the published sequences (GenBank Accession No. NW_003537404.1) to amplify 160 bp and 295 bp fragments that contained the SNP c.2211T>C and c.4957A>C, respectively. The two pairs of primers were: PyFW1 5'-cagtgaacttatgccatgtttacc-3' and PyRV1 5'-biotin-ctcaatgatccggctgtcaa-3'; PyFW2 5´-tcttagttactgggtgttgtcttc-3' and PyRV2 5'-biotin-gattttggtgatgtcagagaacac-3'. The PCR procedure was: 35 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 1 min, and final extension at 72°C for 5 min. Washing and capture of templates as well as sequencing were performed using the Pyro Gold Reagent Kit (Qiagen, Hilden, Germany) on a PSQ 96MA Pyrosequencing instrument (Biotage, Uppsala, Sweden) according to the manufacturer's instructions as described before (Srikanchai et al., 2010).

Gene expression analyses

Transcript abundance of *TRIP12* was determined by quantitative real-time RT-PCR, qRT-PCR of ten animals per diplotype class of each of the DL and PiF1 population, in order to examine the relationship between variation of genotype and expression. According to the sequence (GenBank Accession No. XM_003484315.1), the specific primers of *TRIP12* gene for the qRT-PCR were designed as qPCRFW 5'-ccaacccagaaatcaaccagtc-3' and qPCRRV 5'-gatttccaacatggcccgggag-3', using a primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Housekeeping genes, ribosomal protein 32 (*RPL32*) (primers: forward: 5'-agcccaagatcgtcaaaaag-3', reverse: 5'-tgttgctccataaccaatg-3') and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (primers: forward: 5'-gtgatagatccattcctatgactgtaga-3', reverse: 5'-tgagagatcatctccaccaattactt-3'), were considered as internal controls (Ponsuksili et al., 2008a). The reaction mixture contained 30 ng of cDNA, 0.5μM of forward and reverse primers, 5 μl of LightCycler SYBR Green I Master kit (Roche), and water to obtain a final volume of 10 μl. All of the samples were run in duplicate according to the protocol: initial denaturation of 95°C for 10 min, and 40 cycles of 95°C for 15 s, 10 s for annealing (60°C), and 15 s for elongation (72°C). The qRT-PCR analysis was performed in a BioRad iCycler iQV (BioRad Laboratories GmbH, Munich, Germany). All runs were checked for specificity by melting curve analysis and agarose gel electrophoresis of PCR products. For each assay, the serial dilutions of target PCR products were amplified and used to derive a standard curve to calculate the number of copies. Normalization for variation of RNA input was done by dividing the calculated mRNA copy numbers by a mean normalization factor derived from the expression of the housekeeping genes.

In order to address also the relationship of expression levels and traits related to the water holding capacity in commercial pigs, we obtained *TRIP12 M. longissimus dorsi* expression levels from Affymetrix GeneChip Porcine Genome Array analyses of a subset of 173 individuals of the PiF1 animals (GEO Accession No. GSE322112) (Ponsuksili et al., 2012).

Statistical analyses

Allele and genotype frequencies at the SNPs were calculated separately for DL and PiF1, respectively, and the genotype distribution was tested for Hardy-Weinberg equilibrium by χ^2. Association between genotypic and phenotypic variation in the two commercial populations was analyzed using the MIXED procedure of SAS (Statistical Analysis System, Version 9.2, 2008):

$$Y = \mu + \text{GENO} + \text{SEX} + \textit{RYR1} + \text{sire} + \text{sd} + \text{SW} + e$$

where:

Y	= observation of traits
μ	= overall mean
GENO	= genotype
SEX	= gender
sd	= slaughter date
SW	= slaughter weight
e	= residual error

Genotype, gender, and the genotype of *RYR1* (only in PiF1 population) were considered as fixed ef-

fects, whereas slaughter date and sire were random effects; slaughter weight was taken as a co-variable. The expectation-maximization (EM) algorithm ("haplotype" procedure of SAS) allowed assessing haplotypes segregating within both populations. The probability of each individual's pair of haplotypes (diplotype) was assigned and only data of animals having diplotypes with the probability 1 were selected for further analysis. Subsequently, in order to refer to both SNPs at a time, a similar mixed model was applied where the diplotype, i.e. combination of haplotypes, was used as a fixed effect instead of genotype. Least Squares Means values for the genotypes and diplotypes were compared by the *t*-test, and *P*-values were adjusted by the Tukey-Kramer correction.

The association of gene expression levels obtained from microarray analyses with meat quality traits was evaluated using a MIXED procedure of SAS (Statistical Analysis System, Version 9.2, 2008) as follows:

$$Y = \mu + SEX + RYR1 + sire + sd + SW + TRIP12exp + e$$

where:
Y = observation of meat quality traits
μ = overall mean
SEX = gender
sd = slaughter date
SW = slaughter weight
e = residual error

Sex and the genotype of *RYR1* were considered as fixed effects, slaughter date (sd) and sire as random effects. Slaughter weight and gene expression (*TRIP12*exp) were included as co-variable for meat quality traits. The genotype-dependence of transcript abundance was analyzed by unpaired *t*-tests.

Table 1. Allele and haplotype frequencies of *TRIP12* gene in DL and PiF1 populations

Polymorphic site	Allele/ haplotype	Population	
		DL (n = 269)	PiF1 (n = 300)
c.2211T>C	*T*	0.555	0.726
	C	0.445	0.274
c.4957A>C	*A*	0.924	0.941
	C	0.076	0.059
Haplotype	*CA*	0.435	0.278
	TA	0.482	0.661
	TC	0.083	0.061

DL = German Landrace, PiF1 = Pietrain × (German Large White × German Landrace)

RESULTS

In the current study, thyroid hormone receptor interacting protein 12 (*TRIP12*) is considered as a functional candidate gene for water holding capacity of meat due to its function as an E3 ubiquitin ligase, which is an important enzyme to tag proteins with a chain of ubiquitin molecules for subsequent degradation. The extent and the kinetics of postmortem protein degradation largely affect meat quality, in particular WHC.

Table 2. Least Squares Means (LSM) and standard errors (SE) for carcass and meat quality traits across genotypes of the *TRIP12* gene in DL and PiF1 populations

Position	Traits	Populations	Genotype LSM ± SE (n)			P-value
			TT	*CT*	*CC*	
c.2211T>C	MFR	DL	0.51^{a} ± 0.01 (83)	0.47^{b} ± 0.01 (137)	0.51^{a} ± 0.02 (49)	0.04
	CON_1	DL	4.65^{c} ± 0.11 (83)	4.29^{d} ± 0.09 (137)	4.21^{d} ± 0.13 (49)	0.09
	CON_1	PiF1	5.28^{c} ± 0.19 (145)	4.75^{d} ± 0.22 (135)	4.55^{d} ± 0.44 (20)	0.05
			AA	*AC*		
c.4957A>C	pH_{24}	DL	5.48^{c} ± 0.01 (226)	5.51^{d} ± 0.02 (43)		0.08
	CON_1	PiF1	5.36^{a} ± 0.22 (271)	4.74^{b} ± 0.34 (29)		0.04

DL = German Landrace, PiF1 = Pietrain × (German Large White × German Landrace), MFR = ratio of meat and fat area, CON_1 = conductivity in *M. longissimus dorsi* between the 13th and 14th rib 45 min postmortem, pH_{24} = pH value in *M. longissimus dorsi* between the 13th and 14th rib 24 h postmortem
within a row, means with different superscripts differ at $^{a,b}(P < 0.05)$, $^{c,d}(P < 0.1)$

Table 3. Least Squares Means (LSM) and standard errors (SE) for meat and carcass traits across diplotypes of *TRIP12* gene in pigs

Traits	Populations	Diplotype LSM ± SE (*n*)					*P*-value
		CA/CA	*CA/TA*	*CA/TC*	*TA/TA*	*TA/TC*	
MFR	DL	$0.51^{b} \pm 0.02$ (47)	$0.47^{a} \pm 0.01$ (117)	$0.45^{a} \pm 0.03$ (22)	$0.52^{b} \pm 0.01$ (62)	$0.48^{ab} \pm 0.02$(21)	0.06
CON_1	DL	$4.21^{a} \pm 0.13$ (47)	$4.31^{a} \pm 0.09$ (117)	$4.19^{a} \pm 0.17$ (22)	$4.76^{b} \pm 0.12$ (62)	$4.32^{a} \pm 0.17$(21)	0.07
CON_1	PiF1	$4.59^{a} \pm 0.49$ (17)	$4.73^{a} \pm 0.23$ (121)	$4.96^{ab} \pm 0.68$ (15)	$5.41^{b} \pm 0.21$ (120)	$4.26^{a} \pm 0.43$(27)	0.06

DL = German Landrace, PiF1 = Pietrain × (German Large White × German Landrace), MFR = ratio of meat and fat area, CON_1 = conductivity in *M. longissimus dorsi* between the 13th and 14th rib 45 min postmortem

[a,b] within a row, means with different superscripts differ ($P < 0.1$)

Genotype and WHC

Two novel SNPs, XM_003484315.1:c.2211T>C and c.4957A>C, located within the HECT domain of *TRIP12*, were detected. Across both populations, the genotype distributions of the two silent SNPs were in Hardy-Weinberg equilibrium. In particular, the frequency of the minor allele *C* at locus *c.4957A>C* was low in both populations. Correspondingly, homozygous carriers of the minor allele *C* were detected at low frequency (< 1%) and were not considered for further analysis. To refer to both SNPs of *TRIP12* at a time, three different haplotypes were derived segregating across the two populations: *CA*, *TA*, and *TC*. Among them, the frequency of haplotype *TC* was rare. The allele and haplotype frequencies of *TRIP12* are shown in Table 1.

The associations of the two novel SNPs and their genotype combinations with traits related to WHC, in particular conductivity and pH, were evident. In the PiF1 population, the detected SNPs showed associations with muscle conductivity 45 min postmortem (CON_1) at $P = 0.05$; at both SNPs the major alleles were associated with higher CON_1. In the DL population, animals homozygous for the major alleles *T* at *c.2211T>C* and *A* at *c.4957A>C* exhibited higher CON_1 and lower pH_{24} than other genotypes. However, the differences were only obvious at $P = 0.09$ (Table 2). Subsequently, haplotype analyses revealed consistent effects on CON_1 across both populations, at $P = 0.07$ in DL and $P = 0.06$ in PiF1, with the most frequent diplotype *TA/TA* being associated with increased muscle conductivity (Table 3).

Table 4. Relationship between *TRIP12* polymorphisms and the transcript levels

Genotype/diplotype	Transcript abundance (LSM ± SE)	
	DL	PiF1
2211_*TT*	$24\,948.37^{a} \pm 1\,673.65$	$27\,422.35 \pm 1\,954.43$
2211_*CT*	$26\,789.61^{ab} \pm 1\,565.56$	$29\,439.17 \pm 2\,118.67$
2211_*CC*	$30\,394.74^{b} \pm 1\,476.02$	$33\,075.02 \pm 1\,845.38$
4957_*AA*	$27\,077.14^{a} \pm 2\,301.43$	$29\,796.73^{b} \pm 2\,313.04$
4957_*AC*	$32\,553.06^{b} \pm 2\,217.72$	$37\,072.23^{a} \pm 2\,958.37$
Diplotype *CA/CA*	$32\,981.43^{b} \pm 1\,782.13$	$34\,082.54 \pm 2\,695.42$
Diplotype *CA/TA*	$30\,960.68^{ab} \pm 1\,756.02$	$33\,913.70 \pm 2\,584.38$
Diplotype *CA/TC*	$28\,560.85^{ab} \pm 2\,101.73$	$30\,557.87 \pm 2\,068.27$
Diplotype *TA/TA*	$25\,852.37^{a} \pm 1\,820.15$	$27\,755.69 \pm 2\,410.86$
Diplotype *TA/TC*	$27\,859.14^{ab} \pm 1\,716.05$	$29\,439.18 \pm 2\,543.76$

LSM = Least Squares Means, SE = standard errors, DL = German Landrace, PiF1 = Pietrain × (German Large White × German Landrace) crossbreed

[a,b] for the same locus, within a column, means with different superscripts differ ($P < 0.1$)

Effects on carcass traits of *TRIP12* polymorphisms were only evident in the DL for the allelic variation at *c.2211T>C* and also haplotypic variation (Tables 2 and 3). The animals with the haplotype combination *TA/TA*, of the major alleles, exhibited the highest Least Squares Means for meat to fat ration ($P < 0.1$).

Genotype and expression

To illustrate the existence of a causal polymorphism and its impact on the gene expression levels, we evaluated the relationship of *TRIP12* polymorphisms and its transcript abundance using qRT-PCR. The statistical analysis of expression data derived from selected animals (ten animals per diplotype class of *TRIP12* in the segregating populations) is shown in Table 4. Across all genotypes/diplotypes classes, the *TRIP12* transcript abundances were similar among the DL and PiF1 populations. However, mean transcript abundances among various genotype/diplotype groups in DL differed at $P \leq 0.1$. In PiF1, only transcript abundance obtained in either homozygous or heterozygous animals at *c.4957A>C* differed at $P = 0.06$. The analysis revealed that pigs carrying the major alleles *T* and *A* at both loci had lower transcript abundance in both populations. Moreover, the effect of *TRIP12* polymorphisms on the variation of gene expression was confirmed in the haplotype analysis across the two populations, with the diplotype *TA/TA* showing the lowest transcript abundance (Table 4).

Moreover, the relationship of transcript abundance and WHC was considered in PiF1. Transcript abundance detected by microarrays was compared with indicators of WHC. The *TRIP12* transcript level was associated and negatively correlated with drip loss ($P < 0.08$; Pearson's coefficient = –0.2).

DISCUSSION

Water holding capacity (WHC) of muscle cell depends on the capacity of proteins to bind water molecules and the structure of myofibrillar matrix to entrap the immobilized water (Huff-Lonergan and Lonergan, 2005). During *rigor mortis*, WHC of fresh pork is decreased due to the formation of purge loss that contains soluble components of the cells worsening nutritional and sensory properties of meat and at the same time increasing meat conductivity (Byrne et al., 2000; Lee et al., 2000; Fischer, 2007). The mechanism causing drip loss is explained by the combination of the shrinkage of muscle cells and the extent of filament proteins degradation. In the early postmortem period, in the presence of ATP, the ubiquitination process is activated in muscle cells. Subsequently, partial degrading costameric connections result in the reduced linkage of intermediate filament proteins in the muscle cell (Riley et al., 1988; Hilenski et al., 1992; Sekikawa et al., 2000, 2001). Moreover, integrins that link the cytoskeleton to the extracellular matrix, are also a target of the ubiquitin-proteasome system (Darom et al., 2010; Lobert and Stenmark, 2010). The degradation of integrins forms cracks between cell membrane that were termed drip channels. Thereby, the water is mobilized inside the myofibrillar matrix as well as expelled from the muscle cell throughout these drip channels, resulting in increased drip loss (Law-

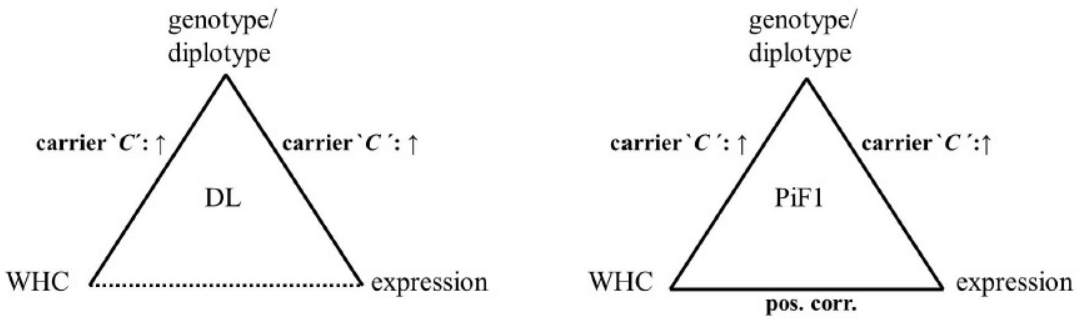

Figure 1. Three-way relationship of *TRIP12* polymorphism, transcript abundance, and water holding capacity (WHC) in commercial pigs represented by the triangle-legs. Solid lines represent relationships that were experimentally shown as indicated alongside; dotted line indicates suggested relationships

DL = German Landrace, PiF1 = Pietrain × (German Large White × German Landrace) crossbreed

son, 2004; Zhang et al., 2006; Bee et al., 2007). Accordingly, holistic expression studies revealed an association of the expression of several genes encoding enzymes of the ubiquitination system with traits related to WHC (Ponsuksili et al., 2008a, b, 2009; Damon et al., 2012). Here we considered *TRIP12*, encoding a HECT domain E3 ubiquitin ligase, as a candidate gene that might affect the ubiquitination process and thereby meat quality. Mutations occurring within the HECT domain of E3 ligases may affect the ubiquitination process. Indeed, variation disrupting the formation of ubiquitin-thioester bonds between the conserved cysteine of the HECT and the ubiquitin C terminus disturb the initial substrate-ubiquitination event as well as the elongation of the polyubiquitin chain (Wang et al., 1999; Ogunjimi et al., 2010; Kajiro et al., 2011). In this study, the two synonymous SNPs were detected in the HECT domain; both do not cause a change of the structure of *TRIP12*. There was no evidence of any non-synonymous polymorphisms, i.e. in accordance with previous microarray experiments, any association of *TRIP12* with WHC is likely to be due to variation in the transcript abundance. The SNPs detected here might be in linkage disequilibrium with polymorphism affecting the transcription level. Moreover, synonymous SNPs still might affect the function of gene due to altering the mRNA stability (Capon et al., 2004). Consequently, in order to get more insight into these possible scenarios, we addressed the relationships of genotype and WHC as well as genotype and expression here on the background of the already previously suggested association of E3 ubiquitin ligase expression levels and WHC. As illustrated in Figure 1, according to the association study, the animals carrying the minor alleles *C* at loci *c.2211T>C* and *c.4957A>C*, showed lower muscle conductivity and higher pH, which is indicative for higher WHC. At the same time, the minor alleles are associated with higher transcript abundance. The relationship between transcript abundance and WHC was evaluated and indicated a positive correlation which is in line with the relationship of gene variation and expression as well as gene variation and WHC (Figure 1). Thus, the integrated results of association and expression studies imply that the rare alleles *C* at both SNPs of the *TRIP12* gene are in linkage disequilibrium with an allele of a causal site, which is most likely located in the *cis*-regulatory region of the *TRIP12* gene and which increases the transcript abundance and decreases drip loss as well as muscle conductivity in the two commercial herds. Thus, selection for the minor alleles bears the potential of genetically improving meat quality in terms of WHC; however, the slight impact on meat to fat ratio observed in DL indicates that this might be on the cost of carcass quality. Also, any other association of *TRIP12* with production and functional traits still needs to be evaluated.

CONCLUSION

The three-way relationship among *TRIP12* polymorphisms, the transcript abundance, and water holding capacity suggests the existence of causal polymorphisms in *cis*-regulatory regions in incomplete linkage disequilibrium with the SNPs detected here. The putative causal polymorphism primarily affects the transcript abundance and, consequently, traits related to meat quality. Our study provides the statistical-genetical evidence to promote *TRIP12* as a functional candidate gene for the water holding capacity of pork.

Acknowledgement

Thanks are due to the Pig Breeding Association of Mecklenburg-Western Pommerania.

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Received: 2013–03–25
Accepted after corrections: 2013–08–15

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2.3 *UBE3B* and *ZRANB1* polymorphisms and transcript abundance are associated with water holding capacity of porcine *M. longissimus dorsi*

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Meat Science 2013, 95, 166–172.

Conceived and designed the experiments: KW SM SP EM. Performed the experiments: TPLH EM SP KW. Analyzed the data: TPLH EM SP KW. Contributed reagents/materials/analysis tools: SM KW SP EM. Wrote the paper: TPLH KW.

Meat Science 95 (2013) 166–172

Contents lists available at SciVerse ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

UBE3B and *ZRANB1* polymorphisms and transcript abundance are associated with water holding capacity of porcine *M. longissimus dorsi*

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ARTICLE INFO

Article history:
Received 7 February 2013
Accepted 10 April 2013
Available online 24 April 2013

Keywords:
Drip loss
SNP
Transcript quantification
Real time RT-PCR
Pyrosequencing
Pig

ABSTRACT

The degradation of myofibrillar proteins during meat maturation affects the water holding capacity (WHC) of meat. Our study sought to identify polymorphisms in *UBE3B* and *ZRANB1*, genes encoding proteins involved in ubiquitination, and to evaluate the relationship between genotype, transcript abundance, and WHC of pork. A single SNP of *ZRANB1*, c.552A>G (p.Ile153Val), and two silent SNPs of *UBE3B*, c.1921A>T and c.4292C>T, were associated with muscle pH, conductivity, meat colour, or drip loss in German Landrace (GL, n = 266) and Pietrain × (Large White × German Landrace) (PiF1, n = 316). Further, carriers of the minor alleles at the SNPs tended to have increased transcript abundance. Consistent with the protein degradation promoting and inhibiting effects of *UBE3B* and *ZRANB1*, respectively, and the expected impact on WHC, their expressions were positively and negatively associated with WHC. The results implicate that the SNPs in both genes are in linkage with a causal site that affects transcript abundance and WHC.

1. Introduction

The water holding capacity (WHC) of meat affects the product quality in terms of meat processing as well as sensory properties of fresh meat cuts. Moreover, WHC directly affects economic outcomes because loss of tissue fluid, or drip loss, is associated with weight loss. One important process contributing to drip loss is the degradation of myofibrillar proteins. Protein degradation, in general, occurs by several mechanisms in eukaryotic organisms. In particular, the calpain/calpastatin proteolytic system is associated with meat tenderness and WHC (Huff-Lonergan & Lonergan, 2005; Melody et al., 2004; Schäfer, Rosenvold, Purslow, Andersen, & Henckel, 2002).

More recently, the ubiquitin proteasome system and several genes related to ubiquitination were shown to affect muscle and meat properties (Damon, Wyszynska-Koko, & Vincent, 2012; Ponsuksili, Jonas, et al., 2008; Ponsuksili, Murani, et al., 2008). The expression of several members of the ubiquitin–proteasome system, including E3 ubiquitin ligase genes, is associated with traits related to WHC and tenderization. Indeed, Ponsuksili, Murani, Schwerin, Schellander, and Wimmers (2010) found a negative correlation between expression of *ZRANB1* (or *TRABID*), an ubiquitin thioesterase, and cooking loss. *ZRANB1* encodes a protein that removes ubiquitin signals from protein substrates in the deubiquitination process (Fushman & Wilkinson, 2011; Komander, Clague, & Urbé, 2009; Nijman et al., 2005). The E3 ligase UBE3B is one of three specific enzymes that link chains of ubiquitin to target proteins, tagging those proteins for degradation by the 26S proteasome (Attaix, Combaret, Pouch, & Taillandier, 2002; Taillandier et al., 2004). Interestingly, *UBE3B* and *ZRANB1* are positional genes for meat quality due to their location on chromosome 14, which contains quantitative trait loci (QTL) for meat quality traits, such as pH value, muscle conductivity, and meat colour (Duan et al., 2009; Li et al., 2010; Malek et al., 2001; Ponsuksili, Murani, Schwerin, et al., 2010; Rohrer, Thallman, Shackelford, Wheeler, & Koohmaraie, 2005).

Here, *UBE3B* and *ZRANB1* were considered as functional candidate genes for meat quality. Thus, we sought to identify polymorphisms in these genes and to evaluate the three-way relationship of variation at the level of genotype, organismal traits related to meat characteristics, and transcript abundance in pigs of commercial herds.

2. Materials and methods

2.1. Animals and phenotyping

Samples and phenotypic data were obtained from animals of the breed German Landrace (DL, n = 266), and the commercial crossbreed of Pietrain × [German Large White × German Landrace] (PiF1, n = 316). Animal care and tissue collection procedures followed the guidelines of the German Law of Animal Protection, and the experimental protocol was approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology (FBN). Slaughtering and recording

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http://dx.doi.org/10.1016/j.meatsci.2013.04.033

meat and carcass traits were performed at the slaughterhouse of the FBN. Carcass traits addressed in the study were loin eye area (LEA), meat to fat ratio (MFR), fat depth at 10th rib (FD_{mid}), and loin fat depth (LFD). Meat quality traits analysed cover the indicators of water holding capacity including drip loss (DRIP), pH, and conductivity at 45 min post-mortem (pH_1, CON_1) as well as at 24 h (pH_{24}, CON_{24}). Conductivity and pH values were measured by Star-series equipment (Matthaeus, Klausa, Germany) in *M. longissimus dorsi* between the 13th and 14th ribs. Drip loss was assessed by bag-method with a size-standardized sample from the *M. longissimus dorsi* collected at 24 h post-mortem. Samples were weighed and suspended in a plastic bag at 4 °C for 48 h before re-weighing. Drip loss percentage was calculated by expressing the change in weight as a percentage of the original weight. Meat colour scores were indicated by lightness (L^* value), redness (a^* value), and yellowness (b^* value), and measured in the loin using a Minolta device.

2.2. SNP detection

Tissue specimens were collected from *M. longissimus dorsi* between the 13th and 14th ribs and frozen in liquid nitrogen for DNA and RNA isolation. DNA was isolated by phenol–chloroform extraction. Total RNA was isolated by Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany). After DNase treatment, RNA was purified with Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). RNA quantity was determined on a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany), and RNA integrity was verified using a 1% agarose gel. First-strand cDNA synthesis was performed by reverse-transcription PCR of total RNA, using random and oligo d(T) primers and Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany).

Polymorphisms of *UBE3B* and *ZRANB1* were detected by comparative sequencing of PCR fragments amplified from *M. longissimus* muscle cDNAs of 8 animals from different breeds (German Landrace, German Large White, Pietrain and Duroc) using primers given in Table 1.

PCRs were performed in a 25 μL reaction volume with 30 ng cDNA, 1 × PCR buffer (with 1.5 mM $MgCl_2$), 200 μM of each dNTP, 0.2 μM of each primer, and 0.5 U of Taq DNA polymerase (GeneCraft, Münster, Germany). Cycling conditions were as follows: initial denaturing at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 30 s at annealing temperature for specific primer (Table 1), and elongation at 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR products were purified and comparatively sequenced in both directions on an ABI 3130 DNA Analyzer (Life Technologies GmbH, Darmstadt, Germany).

2.3. Genotyping

Animals of the DL and PiF1 populations were genotyped at the novel SNPs by pyrosequencing. Washing and capture of templates as well as sequencing were performed using the Pyro Gold Reagent Kit (Biotage, Uppsala, Sweden) on a PSQ96MA Pyrosequencing instrument (Biotage) according to the manufacturer's instructions as described before (Srikanchai, Murani, Wimmers, & Ponsuksili, 2010).

2.4. Determination of transcript abundance

In both segregating populations, to address the relationship between genotypic variation and transcript abundance, ten animals per diplotype/genotype class of *UBE3B*/*ZRANB1*, respectively, were selected for quantitative real-time RT-PCR (qRT-PCR). Reaction mixture contained 30 ng cDNA, 0.5 μM of forward and reverse primers, 5 μL of LightCycler SYBR Green I Master kit (Roche), and water to obtain a final volume of 10 μL. All samples were run in duplicate with the following conditions: initial denaturation of 95 °C for 10 min; and 40 cycles of 95 °C for 15 s, 10 s for annealing (60 °C), and 15 s for elongation (72 °C). qRT-PCR was performed in a BioRad iCycler iQV (BioRad, Munich, Germany). All samples were analysed by melting curve analysis and checked for the presence of any non-specific PCR product using agarose gel electrophoresis. For each assay, serial dilutions of target PCR products were amplified and used to derive a standard curve to calculate the number of copies. Housekeeping genes *RPL32* (ribosomal protein 32) and *HPRT1* (hypoxanthine phosphoribosyltransferase 1) were used for internal controls (Ponsuksili, Jonas, et al., 2008). Specific primers for qRT-PCR were designed by a primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Normalisation of variation in RNA input was accounted for by dividing the calculated mRNA copy numbers by a mean normalisation factor derived from the expression of housekeeping genes.

To further address the relationship of expression and meat quality traits in commercial pigs we obtained *UBE3B* and *ZRANB1 M. longissimus dorsi* expression levels from Affymetrix GeneChip Porcine Genome Array

Table 1
List of primer sequences for *UBE3B*, *ZRANB1*, and housekeeping genes.

Gene	SNP	Method	Primer sequence (5′–3′)	Size (bp)	T_m (°C)	GenBank accession
UBE3B	c.1921 A>T	Screening	F: GATGACCTGCTGCCCAAACTG R: CTCCTTGAAAACGCCGTCTTG	735	67	XM_001929210.3
		Pyrosequencing	F: biotin–GATTTAGGATCCTTGATGACATTG R: GAGGTCACAGATGGCAGAGTG Seq: AATGAAATCTGTTCTTCATA	288	63	NC_010456
	c.4292 C>T	Screening	F: AGCCAACCAAGCGTGAGATGC R: GCAAAACTCGGGGTTCTGGGG	546	65	XM_001929210.3
		Pyrosequencing	F: biotin–CACCATCTTGGGCACTCAC R: GGGGTTCTGGGGACTTTTAC Seq: TTGAGTAACGGCCAG	274	60	NC_010456
ZRANB1	c.552 A>G	Screening	F: GGTTCAAGTGATGTTGGTAGAG R: GCCAATCAGTCTTTTTCATCCTG	661	61	XM_001929009.4
		Pyrosequencing	F: CAGCATTGGACTTGTTCTATTTG R: biotin–CTCCATCGAGCTCTGTCTTG Seq: GACCTAATAATATTGAAGCA	162	63	NC_010456
UBE3B		qRT-PCR	F: GTCACACACATTACCATCCG R: CTCCTTGAAAACGCCGTCTTG	162	60	XM_001929210.3
ZRANB1		qRT-PCR	F: CTACTACGAAACGGGACTCTG R: GCCAATCAGTCTTTTTCATCCTG	141	60	XM_001929009.4
RPL32		qRT-PCR	F: AGCCCAAGATCGTCAAAAAG R: TGTTGCTCCCATAACCAATG	165	60	NM_001001636
HPRT1		qRT-PCR	F: GTGATAGATCCATTCCTATGACTGTAGA R: TGAGAGATCATCTCCACCAATTACTT	104	60	NM_001032376.2

Table 2
Allele and haplotype frequencies of the *UBE3B* and *ZRANB1* genes.

Gene		Allele/haplotype	Populations	
			PiF1 (n = 316)	DL (n = 266)
UBE3B	c. 1921 A>T	A	0.692	0.529
		T	0.308	0.471
	c. 4292 C>T	C	0.748	0.690
		T	0.252	0.310
	Haplotype	CA	0.652	0.473
		CT	0.093	0.211
		TA	0.034	0.038
		TT	0.221	0.278
ZRANB1	c. 552 A>G	A	0.904	0.884
		G	0.096	0.116

analyses of a subset of 173 individuals of the PiF1 animals (GEO accession no. GSE322112) (Ponsuksili, Du, Murani, Schwerin, & Wimmers, 2012). In addition, five male pigs (20 total) were selected for each extreme in two traits: high and low Minolta a* values (9.65 ± 0.45 vs. 5.56 ± 0.31) and high and low pH_{24} (5.68 ± 0.03 vs. 5.34 ± 0.01) were assessed to determine the relationship between transcript abundance and meat quality traits.

2.5. Statistical analyses

For each population, the allele and genotype frequencies of detected SNPs were calculated separately, and the genotype distribution was tested for Hardy–Weinberg equilibrium by χ^2. Using a mixed model (PROC Mixed, SAS v. 9.2; SAS Inc., Cary, USA), the data were analysed to observe the association between genotypic and phenotypic variation in the two commercial populations, as follows:

$$Y = \mu + GENO + SEX + RYR1 + sire + dam + sd + SW + e,$$

where Y was the observation of traits and μ was the overall mean. The fixed effects included the genotype (GENO), sex (SEX), and the genotype of RYR1 (only in PiF1 population). In the model, dam (dam), sire (sire), and slaughter date (sd) were random effects; slaughter weight (SW) was considered as a co-variable. Moreover, for *UBE3B*, haplotypes segregating within both populations were assessed by the expectation–maximization (EM) algorithm, using the "haplotype" procedure of SAS. These estimates were then used to assign the probability that each individual possesses a particular haplotype pair. Animals used had haplotype pairs assigned with the probability 1. To refer to both SNPs of *UBE3B* at once, a corresponding mixed model was applied where the diplotype, i.e., combination of haplotypes, was used as a fixed effect instead of genotype. Least squares mean values for the genotypes and diplotypes were compared by *t*-test, and *P*-values were adjusted by the Tukey–Kramer correction.

The association of gene expression levels obtained from microarray analyses with meat quality traits was evaluated using a mixed model (PROC Mixed, SAS v. 9.2; SAS Inc., Cary, USA) as follows:

$$Y = \mu + SEX + RYR1 + sire + dam + sd + GE + SW + e,$$

where Y was the observation of meat quality traits and μ was the overall mean. In the model, sex (SEX) and genotype of RYR1 were assumed as fixed effects; dam (dam), sire (sire), and slaughter date (sd) were random effects; slaughter weight (SW) and gene expression (GE) were included as covariance for meat quality traits. *e* represented residual error, and *P*-values were adjusted by the Tukey–Kramer correction. Pearson correlation between gene expression levels and meat quality traits was estimated (SAS v. 9.2; SAS Inc., Cary, USA).

The genotype-dependence of transcript abundance was analysed by unpaired *t*-tests. Also mean values of transcript abundances among animals with extreme meat colour or pH values were compared by *t*-tests.

3. Results

3.1. SNP and association analysis

According to alignment of cDNA sequences, four SNPs were detected in the *UBE3B* gene: two in the coding region (c.1597G>T and c.1921A>T) and two in the 3′-UTR region (c.4292C>T and c.5000A>G) with the two SNPs in the coding region and the two in the 3′-UTR, respectively, being in full linkage disequilibrium among the animals analysed. Thus only two polymorphisms, c.1921A>T and c.4292C>T, were selected for further analysis. A single SNP was identified in *ZRANB1*, c.552A>G, located in the coding region and causing an isoleucine to valine substitution (p.Ile153Val). These three polymorphisms were genotyped by pyrosequencing in a total of 582 animals derived from the DL and PiF1 populations; allele and haplotype frequencies are shown in Table 2.

In the PiF1 population, the genotype distributions of *UBE3B* polymorphisms were not in Hardy–Weinberg equilibrium; distributions were in equilibrium in the DL population. Significant deviation from Hardy–Weinberg equilibrium was detected for the *ZRANB1* SNP in the DL population but not in the PiF1 population. Allele A at locus *ZRANB1* c.552A>G was more abundant in both populations; correspondingly, the homozygous AA genotype was most frequent, animals homozygous for the minor allele G appeared at low a frequency (<1.5%, n = 4 in PiF1, n = 3 in DL) and were not considered for analysis.

Significant associations of *UBE3B* polymorphisms with carcass and meat quality traits are shown in Table 3. In the DL population, significant associations were observed for SNPc.1921A>T with loin fat depth (LFD) ($P < 0.05$) and fat depth at 10th rib (FD_{mid}) ($P < 0.01$). Moreover, loin eye area differed at $P = 0.08$ among genotype groups, with TT genotypes having the largest muscle areas. Differences were also found regarding traits for WHC: muscle conductivity at 24 h post-mortem

Table 3
Least squares means (LSM) and standard errors (SE) for carcass and meat quality traits across genotypes of *UBE3B* gene in pigs.

Position	Traits	Population	Genotype[1]			*P*-value
			AA	AT	TT	
c.1921 A>T	LEA	DL	42.24^{e} (0.60)	43.01^{ef} (0.54)	44.06^{f} (0.66)	0.08
	FD_{mid}	DL	2.01^{a} (0.04)	2.18^{b} (0.04)	2.07^{ab} (0.05)	<0.01
	LFD	DL	1.88^{cd} (0.05)	1.92^{c} (0.05)	1.74^{d} (0.06)	0.03
	CON_{24}	DL	3.90^{ef} (0.22)	3.80^{e} (0.20)	4.39^{f} (0.23)	0.07
	DRIP	PiF1	5.67^{cd} (0.23)	5.22^{c} (0.21)	6.12^{d} (0.50)	0.05
	a* value	PiF1	7.37^{a} (0.16)	7.37^{a} (0.15)	6.55^{b} (0.28)	<0.01
			CC	CT	TT	
c.4292 C>T	CON_1	DL	4.21^{e} (0.09)	4.37^{ef} (0.15)	4.43^{f} (0.09)	0.06
	a* value	PiF1	7.35^{c} (0.14)	7.33^{c} (0.15)	6.52^{d} (0.36)	0.05

LEA = loin eye area, FD_{mid} = fat depth at 10th rib, LFD = loin fat depth (LFD); DRIP = drip loss; a* value = Minolta a, redness.
[1] Within a row, different letters denote differences between groups at: ^{a,b} = ($P \leq 0.01$); ^{c,d} = ($P \leq 0.05$); ^{e,f} = ($P \leq 0.1$).

Table 4
Least squares means (LSM) and standard errors (SE) for carcass and meat quality traits across diplotypes of *UBE3B* in pigs.

Traits	Population	Diplotype[1]							*P*-value
		CA/CA	CA/CT	CA/TA	CA/TT	CT/CT	CT/TT	TT/TT	
FD_{mid}	DL	2.03^{ab} (0.05)	2.12^{ab} (0.06)	1.88^{a} (0.10)	2.21^{b} (0.04)	2.09^{ab} (0.09)	2.02^{ab} (0.07)	2.04^{ab} (0.08)	0.03
LFD	DL	1.93^{ab} (0.06)	1.90^{ab} (0.07)	1.71^{ab} (0.12)	1.96^{a} (0.06)	1.86^{ab} (0.11)	1.70^{ab} (0.08)	1.64^{b} (0.10)	0.03
CON_{24}	DL	3.74^{c} (0.24)	3.71^{c} (0.28)	4.39^{cd} (0.49)	3.87^{c} (0.22)	4.08^{cd} (0.45)	4.02^{cd} (0.39)	4.94^{d} (0.33)	0.08
DRIP	PiF1	5.53^{cd} (0.25)	4.83^{d} (0.34)	6.22^{c} (0.50)	5.27^{d} (0.25)	–	6.05^{c} (0.64)	6.38^{c} (0.87)	0.09
a^* value	PiF1	7.39^{a} (0.16)	7.53^{a} (0.21)	7.46^{a} (0.29)	7.34^{a} (0.16)	–	7.08^{ab} (0.50)	6.21^{b} (0.37)	0.04

[1] Within a column, different letters denote differences between groups at: ^{a,b} = ($P \leq 0.05$); ^{c,d} = ($P \leq 0.1$).

(CON_{24}) differed at $P = 0.07$ depending on the c.1921A>T genotype in DL. SNP c.4292C>T showed association with muscle conductivity at 45 min post-mortem (CON_1) at $P = 0.06$, with animals of the homozygous genotype TT having the highest muscle conductivity. In the PiF1 population, *UBE3B* polymorphisms were significantly associated with drip loss (DRIP) ($P = 0.05$) and redness (a^* value) ($P < 0.01$). Heterozygous animals at locus c.1921A>T had the lowest drip loss and had higher redness, whereas animals of TT genotype at locus c.4292C>T exhibited the lowest of least squares mean for redness ($P = 0.05$).

Four *UBE3B* haplotypes segregated across the two populations: CA, CT, TA, and TT; the frequency of haplotype TA was rare (Table 2). Six haplotype combinations were found in the PiF1, and seven diplotypes were detected in the DL population. Haplotype analysis revealed a significant association of diplotype with loin fat depth and fat depth at 10th rib in the DL animals ($P = 0.03$). Diplotype CA/TT had the highest least squares mean value. In both populations, the association of haplotype combinations was detected for indicators of WHC. Among them diplotype TT/TT had the highest muscle conductivity as well as drip loss ($P < 0.1$), but it reached lower a^* values than others ($P = 0.04$) (Table 4).

SNP c.552A>G in *ZRANB1* showed significant associations with pH value in *M. longissimus dorsi*. The heterozygous AG genotype showed the highest pH value at 45 min and 24 h post-mortem in DL and PiF1 animals, respectively ($P \leq 0.05$). Moreover, significant associations of *ZRANB1* with carcass traits were evident in the PiF1 population ($P < 0.05$); pigs with homozygous AA genotype had lower loin eye area, but higher meat to fat ratio than heterozygous AG animals (Table 5).

3.2. Expression study

To evaluate the relationship of genotype/diplotype of the two candidate genes with transcript abundance, ten animals per diplotype class of *UBE3B* or genotype class of *ZRANB1* in the segregating populations were selected for qRT-PCR. DL had higher abundance of *UBE3B* transcripts across all genotypes/diplotypes than PiF1; for *ZRANB1*, no population differences were obvious. For *UBE3B*, differences in transcript abundance among the genotypes at locus c.1921A>T were detected within commercial pigs at $P \leq 0.1$. Homozygous TT animals had the highest abundance. Pigs carrying the minor allele T at locus c.4292C>T had higher transcript abundance in both populations; however, the differences were only significant in DL at $P < 0.05$. Accordingly, haplotype analysis revealed that pigs of diplotype TT/TT tended to have the highest transcript abundances across the two populations, with $P < 0.1$ in DL. For *ZRANB1*, transcript abundance was significantly higher at locus c.552 A>G in heterozygous AG pigs than in homozygous AA pigs ($P \leq 0.1$) (Table 6).

In PiF1, the relationship between phenotype and expression was further analysed. Transcript abundance detected by microarray was compared with indicators of WHC. *ZRANB1* transcript level was significantly associated ($P < 0.01$) and positively correlated with pH_{24} (Pearson's coefficient = 0.29; $P < 0.001$). *ZRANB1* expression was also significantly associated ($P < 0.01$) but negatively correlated with drip loss (Pearson's coefficient = −0.2; $P < 0.01$). Moreover, for divergent groups of animals significantly higher transcript levels in animals with high pH_{24} were found compared to animals with low pH_{24} ($P = 0.02$). For *UBE3B*, association of transcript abundance with meat redness was detected at $P < 0.1$; animals of the high Minolta a^* value group had significantly lower mRNA expression level than the low Minolta a^* value animals ($P = 0.03$).

Table 5
Least squares means (LSM) and standard errors (SE) for carcass and meat quality traits across genotypes of *ZRANB1* in pigs.

Traits	Population	Genotype[1]		*P*-value
		AA	AG	
pH_1	DL	6.32^{c} (0.03)	6.39^{d} (0.04)	0.05
LEA	PiF1	53.03^{a} (0.34)	54.98^{b} (0.68)	<0.01
MFR	PiF1	0.28^{c} (0.01)	0.26^{d} (0.01)	0.02
pH_{24}	PiF1	5.47^{c} (0.01)	5.50^{d} (0.01)	0.04

[1] Within a row, different letters denote differences between groups at: ^{a,b} = ($P \leq 0.01$); ^{c,d} = ($P \leq 0.05$).

4. Discussion

During the ubiquitination process, an intermediate filament protein is ubiquitinated by the covalent attachment of a polyubiquitin chain by three enzymatic components. The ubiquitinated protein is subsequently degraded by the 26S proteasome. This process may be disrupted by deubiquitinating enzymes (DUBs) that remove polyubiquitin signals from tagged substrates, allowing them to avoid degradation. In the early post-mortem period, small amounts of ATP are still present in the muscle cell; these activate the ubiquitin–proteasome system, causing degradation of intermyofibrillar and costameric connections in *M. longissimus dorsi* (Hilenski, Terracio, Haas, & Borg, 1992; Nyam-Osor et al., 2009; Riley, Bain, Ellis, & Haas, 1988; Sekikawa et al., 2000, 2001). This degradation reduces the water binding capacity, thus intracellular water easily

Table 6
Relationship between *UBE3B* or *ZRANB1* polymorphisms and transcript levels. (Mean ± standard error.)

Gene	Genotype	Transcription level	
		PiF1	DL
UBE3B	1921_AA	2595.25^{cd} ± 528.12	3950.68^{cd} ± 305.22
	1921_AT	1208.24^{c} ± 566.69	3190.86^{c} ± 315.18
	1921_TT	2936.25^{d} ± 590.46	4158.22^{d} ± 325.28
	4292_CC	1594.48 ± 177.01	2752.31^{a} ± 212.54
	4292_CT	1656.16 ± 176.09	3846.86^{b} ± 300.58
	4292_TT	1854.92 ± 293.66	3086.39^{ab} ± 388.05
Diplotype	CA/CA	2233.81 ± 316.51	4316.48^{cd} ± 353.29
	CA/CT	1100.50 ± 548.21	3026.77^{c} ± 377.69
	CA/TA	2224.50 ± 540.11	4720.35^{cd} ± 706.59
	CA/TT	1653.81 ± 274.10	3875.49^{cd} ± 576.93
	CT/CT	–	3061.72^{c} ± 576.91
	CT/TT	1459.79 ± 548.21	4101.21^{cd} ± 706.59
	TT/TT	2415.75 ± 387.64	5182.26^{d} ± 446.89
ZRANB1	552_AA	16770.64^{c} ± 1313.49	16915.19^{c} ± 2399.99
	552_AG	20263.03^{d} ± 1397.25	23248.56^{d} ± 2401.69

For the same locus, different letters denote differences between groups at: ^{a,b} = ($P \leq 0.05$); ^{c,d} = ($P \leq 0.1$).

moves to the extracellular space and release of immobilized water from muscle cells is enhanced. Moreover, the ubiquitin–proteasome system also promotes the degradation of integrin, a membrane protein involved in linking the cytoskeleton to the extracellular matrix (Darom, Bening-Abu-Shach, & Broday, 2010; Lobert & Stenmark, 2010). The result of this process is the formation of drip loss channels between cell membranes, increasing purge loss (Bee, Anderson, Lonergan, & Huff-Lonergan, 2007; Lawson, 2004; Zhang, Lonergan, Gardner, & Huff-Lonergan, 2006). This change of soluble components and loss of myoglobin in fluids result in increased conductivity as well as reduced redness of meat (Lee et al., 2000; Swatland, 2004). Ponsuksili, Jonas, et al. (2008), Ponsuksili, Murani, et al. (2008) showed for the first time an association of the expression of genes of the ubiquitination system with meat quality, in particular traits related to WHC. In the current study, *UBE3B* and *ZRANB1*, encoding proteins involved in ubiquitin processing, affected meat quality.

4.1. UBE3B polymorphisms and expression associated with meat quality traits

E3 ubiquitin ligase (UBE3B) helps recognise protein substrates for ubiquitination. Its C-terminal domain (HECT, homologous to the E6-AP Carboxyl Terminus) has as a conserved Cys residue that forms an intermediate thioester bond with the ubiquitin C terminus before the transfer of ubiquitin to the target proteins (Gong, Huang, Warner, & Lomax, 2003; Kim, Steffen, Oldham, Chen, & Huibregtse, 2011; Lecker, Goldberg, & Mitch, 2006; Pandya, Partridge, Love, Schwartz, & Ploegh, 2010).

Two novel SNPs were identified in the coding and non-coding regions of *UBE3B*. Significant associations of these SNPs and the genotype combinations of the two polymorphic sites were detected for muscle conductivity in DL, as well as drip loss and redness of meat in the PiF1 population. An effect on carcass traits of SNP c.1921A>T was evident only in DL. *UBE3B* did not show significant association for a particular meat quality trait across both populations; however, genetic variation was significantly associated with a decrease in WHC in *M. longissimus dorsi* consistent with increased drip loss, muscle conductivity, and lower Minolta a* values (Byrne, Troy, & Buckley, 2000; Fischer, 2007; Lee et al., 2000; Ponsuksili et al., 2009). This might indicate that the detected SNPs are not likely to be causal factors for the effects obtained. However, these SNPs might be in incomplete linkage disequilibrium with other causal polymorphisms, which may be located in another region of the *UBE3B* gene not yet covered by the analysis. The association of *UBE3B* polymorphisms with its transcript abundance consistently illustrate the existence of a causal polymorphism in *cis*-regulatory regions of *UBE3B* that impact its expression in the two populations. According to the association study, homozygotes for the minor allele T at loci c.1921A>T and c.4292C>T showed significantly higher muscle conductivity, higher drip loss, or lower redness—all indicators of lower water holding capacity—than other genotypes. Correspondingly, the presence of the minor allele T at the two polymorphic sites resulted in increased *UBE3B* expression in both populations. Additionally, haplotype analysis revealed a trend for double homozygous haplotypes TT/TT being associated with increased expression level and decreased WHC across both populations. Moreover, in PiF1 animals transcript abundance was significantly associated with meat colour. This finding implies that in commercial pigs the T alleles at both SNPs are in linkage phase with an allele of a causal site increasing the transcript abundance and decreasing WHC. Transcript abundance and WHC were negatively correlated, in line with the relationships between genotype and expression and genotype and phenotype (Fig. 1). Interestingly, the expression QTL (eQTL) for *UBE3B* was assigned as *trans*-acting on SSC14 (Ponsuksili, Murani, Schwerin, et al., 2010); therefore, the lack of consistency of the effects of *UBE3B* on the low-heritability traits among commercial populations might be due to interactions between *UBE3B* and other *trans*-regulated genes relevant to meat quality (Ponsuksili, Murani, Phatsara, et al., 2010).

4.2. ZRANB1 polymorphism and expression associated with meat quality traits

The ubiquitin thioesterase *ZRANB1* is a major deubiquitinating enzyme (DUB) that catalyses the cleavage of the isopeptide bond within polyubiquitin chains or between ubiquitin and a target protein, thereby removing its mark for proteolytic destruction (Clague, Coulson, & Urbé, 2012; Fushman & Wilkinson, 2011). In the current study, a single SNP, c.552A>G, not belonging to a specific domain of *ZRANB1*, caused an amino acid exchange from isoleucine to valine that might alter protein function. *ZRANB1* exhibited a consistent effect on pH values (increased) within commercial pigs; its effect on carcass traits was evident only in PiF1. Heterozygous carriers of the minor allele G showed significantly higher pH values at different time points post-mortem and tended to have higher transcript abundance across both populations. Moreover, the eQTL for *ZRANB1* was assigned as *cis*-acting on SSC14, and transcript abundance was negatively correlated with cooking loss (Ponsuksili, Murani, Schwerin, et al., 2010). Here, we detected a negative correlation between *ZRANB1* transcript abundance and drip loss. In contrast, transcript abundance was positively correlated with WHC, which is in line with three dimensions of the relationship among genotype, expression, and phenotype in these pigs (Fig. 2). The results suggest that the detected *ZRANB1* SNP, c.552A>G, is in linkage equilibrium with a causal polymorphism

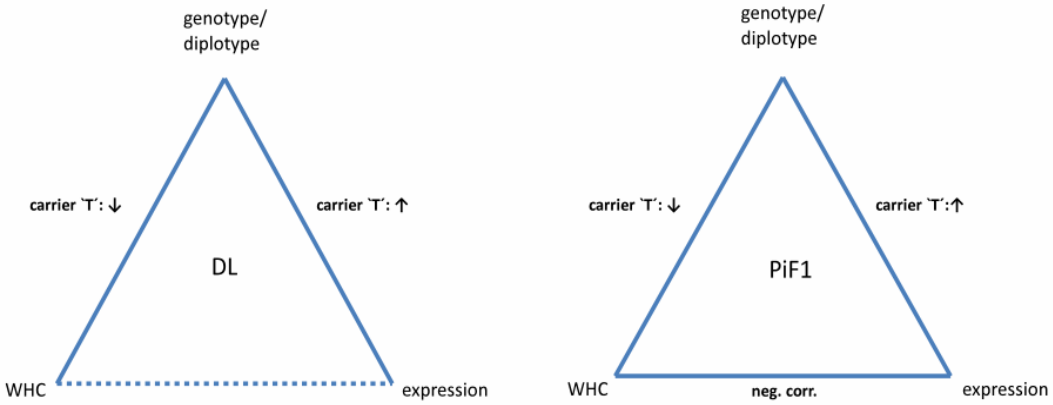

Fig. 1. The three-way relationship of *UBE3B* polymorphism, transcript abundance, and water holding capacity in commercial pigs represented by the triangle-legs. Solid lines represent relationships that were experimentally shown as indicated alongside; dotted lines indicate suggested relationships.

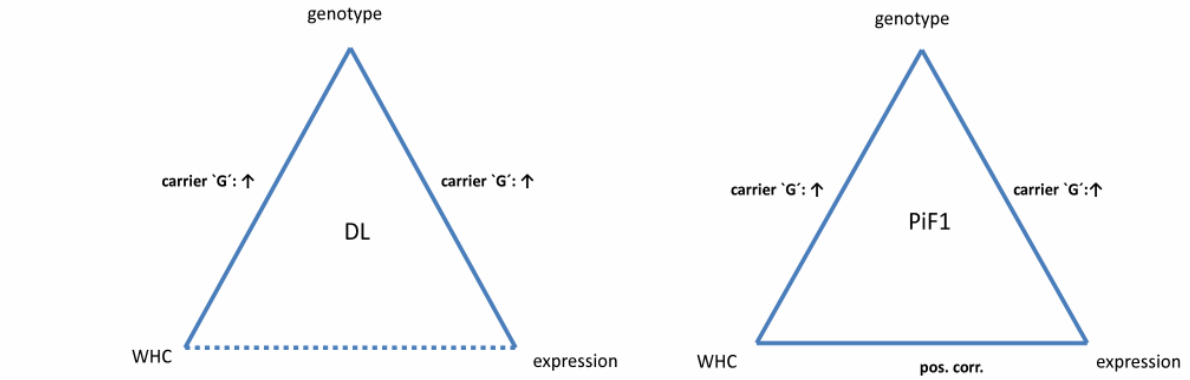

Fig. 2. The three-way relationship of *ZRANB1* polymorphism, transcript abundance, and water holding capacity in commercial pigs represented by the triangle-legs. Solid lines represent relationships that were experimentally shown as indicated alongside; dotted lines indicate suggested relationships.

located in a regulatory region of *ZRANB1*, which primarily affects its transcript abundance and, consequently, traits related to WHC.

4.3. Conclusion

Ubiquitination/deubiquitination processes regulate the degradation of myofibrils and thus affect purge loss. Indeed, *UBE3B* enhances the destruction of sarcoplasmic and myofibrillar proteins, whereas *ZRANB1* promotes protein stability. Our results provide genetic evidence for effects of variation in *ZRANB1* and *UBE3B* on the water holding capacity of pork. Analysis of the three-way relationship of *ZRANB1* and *UBE3B* polymorphisms, their transcript abundance, and water holding capacity indicates the existence of causal polymorphisms in *cis*-regulatory regions that primarily influence gene expression and, secondarily, water holding capacity.

Acknowledgements

This research was partly supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; WI 1754/10-14) the Ministry of Education and Training of Vietnam, the German Academic Exchange Service (DAAD), and the Cantho University (Vietnam). We also thank the Pig Breeding Association of Mecklenburg-Western Pommerania.

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3 Discussion

3.1 A hypothesis to explain the role of ubiquitination pathway impacting on water holding capacity of pork

In the muscle cells, water is distributed within the myofibrils and myofibrillar matrix as well as sarcolemma; moreover water is also found between muscle cells and muscle bundles (Offer & Cousins, 1992). In the recent studies, the formation of drip loss during post mortem period is explained due to the combining effects between the rigor mortis phenomenon and the proteolysis process involving the capain/calpastatin system. Indeed, the activity of the calpain system causes the degradation of myofibrillar proteins that play the roles to link adjacent myofibrils and myofibrils and the cell membrane. Morever, this system is responsible for the proteolysis of integrins, membrane proteins linking the cytoskeleton to the extracellular matrix, forming the `drip loss channels´ between cell membranes (Lawson, 2004; Zhang et al., 2006; Bee et al., 2007). In fact, the total amount of released water is depended on levels of the degradation of cytoskeletal proteins and the volume of spaces within the muscle structure. It has been widely reported about the relationship between the variation in the rate of water loss during post mortem and the quantification of intermediate filament proteins (desmin, titin, vinculin) degradation. At early post mortem, Kristensen et al. (2001) demonstrated the limited degradation of desmin causing increased drip loss of meat. At later post mortem, the

costameric connections were completely degraded by the proteolytic processes. This phenomenon can prevent the reduction of volume of muscle cells during lateral shrinkage as well as enhance the water holding capacity of meat (Joo et al., 1999; Melody et al., 2004; Huff-Lonergan et al., 2005).

The functions of the ubiquitin proteasome system during proteolysis of intermyofibrillar, costameric connections and integrins in muscle cells was reported (Riley et al., 1988; Hilenski et al., 1992; Huang et al., 2009; Darom et al., 2010; Lobert & Stenmark, 2010). However, there are no reports about the relationship between the variation of drip loss and the degree of filament protein degradation due to the effects of the ubiquitin proteasome system. In this study, we addressed the hypothesis that the activity of the ubiquitin-proteasome system in muscle cells causes a decrease of water holding capacity of meat at early post mortem. The mechanism of drip loss formation in muscle cell is described in the Figure 1.

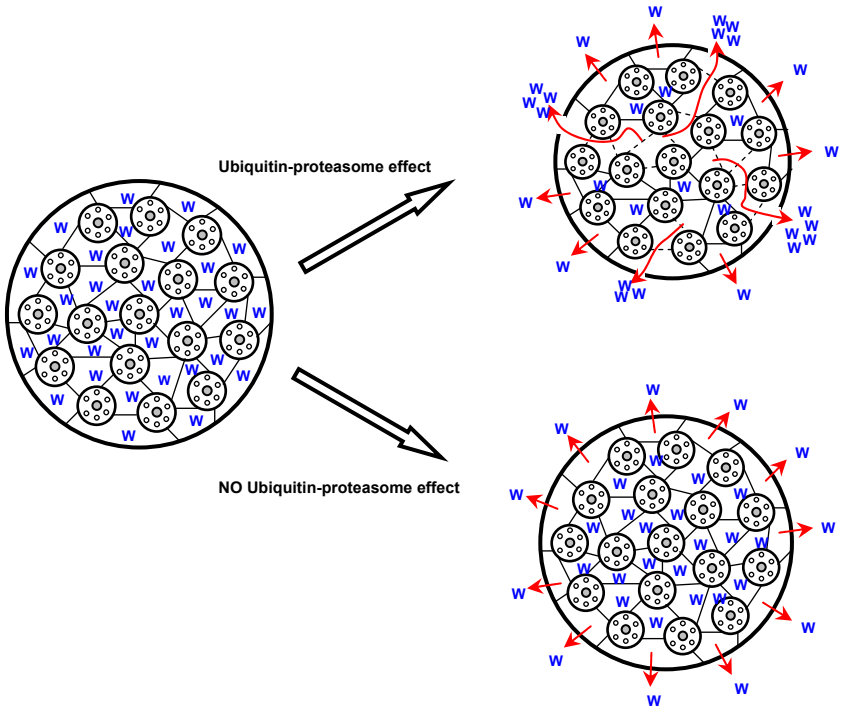


Figure 1: Schematic overview of movement of immobilised water (W) in muscle cell under the proteolysis process by ubiquitin-proteasome system.

Depending on the total amount of ATP still remaining in muscle cell during the conversion from muscle to meat, the ubiquitin-proteasome system can be activated to cause the degradation of myofibrillar proteins. Because of the limited ATP concentration in muscle cells after slaughtering, the proteolytic activity of ubiquitin-proteasome reduces only a proportion of connections inside the myofibrils matrix as well as promotes the destruction in a

part of cell membrane. Under lateral shrinkage condition, the immobilised water is easily moved from intra - to extracellular space and expelled from the weak cell structure throughout drip channels that results in the decreased of inherent water retained in muscle cell at early post mortem.

3.2 The effects of functional candidate genes on the quality of pork

3.2.1 The analysis of *UBXN1*

In the previous studies, the human UBX domain containing proteins are investigated to understand this impact on the endoplasmic-reticulum-associated protein degradation (ERAD) and the Alzheimer's disease (Liang et al., 2006; LaLonde et al., 2011). Recently, Ponsuksili et al. (2008b) found the subset of genes related to the family of UBX domain proteins that had strong effects on the drip loss formation in muscle cell. In brief, the expression of erasin, UBX domain containing protein 2 (*UBXD2*), is up-regulated in the extremely high drip loss group of pigs (Duroc x Pietrain). Subsequently, Ponsuksili et al. (2010b) determined the expression QTL (eQTL) for the several members of UBX domain proteins (*UBXN2A, UBXN2B, UBXN7* and *UBXN8*), and these genes were reported as the *trans*-acting functional candidate genes for meat quality. In this study, a candidate gene, UBX domain containing protein 1-like gene (*UBXN1*) locates on chromosome 2 that contained a number of QTL for meat quality traits, such as pH, conductivity, tenderness, drip loss and cooking loss (Wijk et al., 2006; Liu et al., 2007). Consistently, the statistical analysis revealed the high association of *UBXN1* with muscle conductivity within commercial pigs, otherwise the significant effects on pH_{45} and drip loss were found only in the purebred. The lack of consistency of the effects of *UBXN1* in both populations implied that *UBXN1* might interact with other *trans*-regulated genes that impacted on the traits related to the meat quality. Our results provided genetic evidences to suggest that the variation of *UBXN1* and its transcript levels are associated with lower water holding capacity of pork.

3.2.2 The analysis of *TRIP12*

Thyroid hormone receptor interacting protein 12 (*TRIP12*), containing the HECT domain (homologous to the E6-AP carboxyl terminus), exhibits a function as an E3 ligase in the ubiquitination process. Moreover, several researches reported the function of thyroid hormone receptors (TRs) interacting with the coregulator proteins to regulate the gene transcription (Clément et al., 2002; Moore et al., 2005; Reecy et al., 2006). In recent studies, Park et al. (2008); Keppler et al. (2010) and Kajiro et al. (2011) reported the responsibility of *TRIP12* for the

degradation of APP-BP1 and BAF57, demonstrating the interaction between *TRIP12* and the neddylation pathway as well as its effect on the regulation of SWI/SNF chromatin remodeling complex. In the current study, the *TRIP12* polymorphisms and its transcript levels were strongly associated with muscle conductivity within commercial pigs. Our findings provide evidences for the variation of *TRIP12* being associated with lower drip loss formation. Until now, the location of *TRIP12* gene is not determined in porcine genome. However, the results of association and expression studies illustrated the effects of *TRIP12* on the indicators of water holding capacity, promising the position of *TRIP12* might be in close linkage to QTL areas for meat quality traits.

3.2.3 The analysis of *UBE3B*

It has been widely reported about the function of a number of E3 ligases in the ubiquitination of many specific proteins, which are either responsible in human disease-relevant processes or in cell adhesion and migration (Nakayama et al., 2006; Scheffner et al., 2007). Especially, the transmembrane glycoproteins, integrins, are the targets of several members of E3 ubiquitin ligase family (Huang et al., 2009; Darom et al., 2010), and the proteolysis of integrins causes the formation of channels between cell membranes to increase the leakage of purge in the muscle cell. Consistently, the studies of Ponsuksili and co-workers reported the association between the expression of porcine ubiquitin ligase protein (*CHFR*) with the increased of drip loss (Ponsuksili et al., 2008b). Moreover, Ponsuksili et al. (2008a and 2010b) reported the expression of ubiquitin protein ligase (*UBE3B*) being correlated with drip loss. Its eQTL was assigned as *trans*-acting on SSC14. In our study, the *UBE3B* showed significant association with the indicators of water holding capacity, but not with particular traits across the two populations. Indeed, the statistic analysis revealed that the *UBE3B* gene exhibited strong effects on drip loss only in PiF1; otherwise it showed high impact on muscle conductivity in the DL. These findings implied that the effects of *UBE3B* on the variation of water holding capacity could be evaluated through its interaction with other *trans*-regulated genes relevant to meat quality. Moreover, the variation of *UBE3B* and its expression showed strong association and correlation with meat redness in *M. longissimus dorsi*. Our results illustrated the function of *UBE3B* results in the decreased of water holding capacity of pork.

3.2.4 The analysis of *ZRANB1*

The OTU (ovarian tumor) domain of *ZRANB1* removes ubiquitin signals from a major component of the Wnt pathway (β–catenin) to avoid the degradation by 26S proteasome

(Tran et al., 2008 and Hartley et al., 2009). Therefore, the *ZRANB1* is reported as a positive regulator of Wnt signaling in mammalian cells. Interestingly, the Wnt pathway involved the glucose metabolism in macrophage cell lines and the muscle insulin sensitivity in skeletal muscle cells, therefore it might affect on the lactic acid accumulation as well as the pH decline of meat during the post mortem (Anagnostou et al., 2008; Sethi et al., 2008; Abiola et al., 2009; Kayan et al., 2013). In our study, the *ZRANB1* polymorphism and its expression showed significant associations with pH values at different time points post mortem. Moreover, the eQTL for *ZRANB1* was assigned as *cis*-acting on SSC14, and its transcript abundance was negatively correlated with cooking loss and drip loss in *M. longissimus dorsi*. The results derived from our study promote the *ZRANB1* as a candidate gene for increasing water holding capacity of pork.

3.2.5 Consistent effects of the candidate genes on water holding capacity of pork

In the current research, the integration of association and expression studies demonstrated that the detected SNPs are in linkage disequilibrium with the causal polymorphisms, which are likely located in the *cis*- regulatory region of the candidate genes, influencing the gene expression and subsequently water holding capacity of meat. Three candidate genes related to the ubiquitination pathway, *UBE3B*, *TRIP12* and *UBXN1*, showed strong impact on the indicators of water holding capacity within commercial herds. In brief, the variations of *UBE3B* and *UBXN1* promoted the ubiquitination process; otherwise the polymorphisms of *TRIP12* disturbed the substrate-ubiquitination event and the elongation of polyubiquitin chain. Correspondingly, high transcript abundances of *UBE3B* and *UBXN1* were associated with an increase of drip loss formation; otherwise high expression level of *TRIP12* was associated with a reduction of purge loss in muscle cell. High abundance of *ZRANB1*, which promoted the deubiquitination process, showed strong association with a decrease of drip loss. The results provided genetic evidences to support for *UBE3B*, *TRIP12*, *UBXN1* and *ZRANB1* as the functional candidate genes for water holding capacity of pork.

3.3 The interaction of *RYR1* and candidate genes related to the ubiquitination process and its effects on meat quality

The previous sections present an overview of the effects of ubiquitin-proteasome system on the degradation of the cytoskeleton in muscle cells and, consequently, the drip loss formation at early post mortem. However, the drip production is enhanced by the muscle contraction,

which is promoted by the accumulation of Ca^{2+} relating to the porcine stress syndrome. Thus, in this section we sough to evaluate the interaction between the polymorphisms of the four candidate genes and the mutation of halothane gene (*RYR1*), to illustrate that potential interaction might affect the water holding capacity of pork.

The ryanodine receptor gene (*RYR1*) involves the porcine stress syndrome that exhibits negative impact on meat quality via increasing the PSE phenomena of pork. A single polymorphism of *RYR1* (c.1843 C>T) shows the impact on the function of the HPA (hypothalamic pituitary-adrenal) axis. This mutation also promotes the Ca^{2+}-release to enhance the contraction of skeletal muscle, as well as the acceleration in the rate of ATP depletion (Fujii et al., 1991; Muráni et al., 2010). Indeed, the presence of the *RYR1* sensitivity allele (T~n) in PSE meat has a strong impact on the pH decline. During the post mortem period, the weakness of cell membranes due to proteolysis of myofibrillar proteins and the muscle contraction phenomena are considered as the major factors to cause the decreased water holding capacity of meat. Association studies reported that the calpastatin gene (*CAST*), which is inhibitor of calpain (Ca^{2+}-dependent cysteine protease), plays a role in the regulation of the Ca^{2+}-channels involving the degradation of myofibrillar proteins and meat tenderness (Ciobanu et al., 2004; Meyers et al., 2008; Nonneman et al., 2011). In particular, the interaction between variants of the *CAST* and the *RYR1* gene showed strong effects on the regulation of Ca^{2+} levels as well as traits related to meat quality (Koćwin et al., 2003; Krzęcio et al., 2005; Rybarczyk et al., 2010).

The function of two mechanisms, ubiquitination proteasome system and muscle relaxation, depend on the amount of ATP that retains in muscle cell. The concentration of ATP decreases by 50% at 2 hours post mortem and thereafter falls below 0.5 mmol/kg at 24 hours (Schäfer et al., 2002). The ATP concentration depletes very fast, it reduces the activity of ubiquitination process in muscle cell, whereas it promotes the anaerobic metabolism and enhances the pH decline and muscle contraction phenomenon.

In order to further validate the effects of the four candidate genes and to address their potential interaction with *RYR1*, an association study in a PiF1 population was performed using the mixed model (PROC Mixed, SAS v. 9.2; SAS Inc., Cary, NC, USA). The data were analysed to observe the associations of *RYR1* genotype/the combination genotypes of *RYR1* and candidate genes with traits related to meat characteristics, the model is described below:

$$Y = \mu + GENO + SEX + RYR1 + (GENO \times RYR1) + sire + dam + sd + SW + e,$$

where Y was the observation of meat quality traits and μ was the overall mean. The fixed effects included the genotype (GENO), sex (SEX), the genotype of *RYR1* and the interaction between genotypes of detected SNP in each candidate gene and *RYR1* genotypes (GENO x *RYR1*). In the model, dam (dam), sire (sire), and slaughter date (sd) were random effects; slaughter weight (SW) was considered as a co-variable. Least squares mean values for the *RYR1* genotypes and the interaction effects were compared by t-test, and p-values were adjusted by the Tukey-Kramer correction.

3.3.1 The effects of *RYR1* on the meat quality traits

According to the result of genotyping, the pigs were either homozygous wild type (CC~NN) or were heterozygous carriers (CT~Nn). Statistical analysis revealed the significant associations of a single nucleotide polymorphism of *RYR1* with most of the indicators of water holding capacity, except pH value at 24 hours post mortem and Minolta a-value. Animals of the heterozygous genotype `Nn´ had the highest muscle conductivity and drip loss ($P < 0.01$), however these tended to have the lowest pH value at 45 minutes post mortem ($P < 0.0001$) and produced the paler carcasses (with higher value of Minolta L and b) (Table 1).

Table 1: Least squares means (LSM) and standard errors (SE) for meat quality traits across genotypes of the *RYR1* gene in the PiF1 population

Traits	Genotype (Lsmean ± SE)		*P*-value
	Nn (n=164)	NN (n=165)	
pH_{45}	6.05 (0.04)	6.29 (0.04)	<0.0001
$Conductivity_{45}$	5.40 (0.19)	4.77 (0.19)	<0.01
pH_{24}	5.48 (0.01)	5.50 (0.01)	ns
$Conductivity_{24}$	6.13 (0.27)	4.41 (0.26)	<0.0001
Drip loss	6.05 (0.33)	4.95 (0.33)	<0.01
Minolta L-value	49.07 (0.41)	48.27 (0.39)	<0.1
Minolta a-value	7.33 (0.26)	7.04 (0.26)	ns
Minolta b-value	1.83 (0.15)	1.52 (0.14)	<0.01

Our results are in agreement with the reports of Gispert et al. (2000); Fernandez et al. (2002) and Otto et al. (2007), the susceptible allele `n´ did not exhibit its effect on ultimate pH value due to the glycolytic potentials among animals with homozygous (NN) and heterozygous

carriers (Nn) might be similar. Moreover, this candidate gene DNA marker did not show significant effect on the Minolta a-value (meat redness), therefore Obi et al. (2010) referred that the degradation of sarcoplasmic proteins, including myoglobin, could be further investigated to explain the variation of Minolta a-value of meat at post mortem.

3.3.2 The interactions of candidate genes with *RYR1* affect the meat quality

In the current study, the four candidate genes related to the ubiquitination process, which is responsible for the degradation of proteins as costameric connections and the transmembrane glycoproteins, accelerating the expulsion of water from muscle cells. Additionally, the C1843T mutation in *RYR1* gene had the effects on intracellular Ca^{2+} level in cell to enhance the muscle contraction. The combination of proteolysis process and the shrinkage of muscle cells lead to a decrease of water holding capacity of meat. According to the results of association study, we selected detected SNPs of candidate genes, which only showed significant effects on the meat quality traits, to address the impact of its interactions with variant of *RYR1* gene on the water holding capacity of pork.

The *UBXN1* is assessed as a functional candidate gene to enhance the ubiquitination process as well as cause the decreased of water holding capacity of pork. The effects on meat quality of detected SNP at locus c.355 C>T was only evident in the DL but not in PiF1 population. However, SNP c.674 C>T (p.Thr225Ile) showed significant associations with indicators of water holding capacity across both populations. Especially, in the PiF1 population the animals of heterozygous `CT´ at loci c.674 C>T had the highest muscle conductivity at 45 minutes post mortem (CON_{45}) and transcript levels.

In the study about the influence of *UBXN1* and *RYR1* interaction on the meat quality, the significant interaction between the variation of *UBXN1* at locus c.674 C>T with the *RYR1* genotypes was detected for CON_{45} ($P < 0.05$). The values of CON_{45} were not significantly different for `CC´ and `CT´ genotypes at SNP c.674 C>T of animals that carrier `NN´ homozygote at *RYR1* locus, otherwise the result was divergent for heterozygote Nn animals. Moreover, the animals with double heterozygous `CT/Nn´ had significantly higher the least squares means for muscle conductivity than other interaction genotypes. The Figure 2 illustrated the positive interaction between the minor allele `T´ at locus c.674 of *UBXN1* with susceptible allele `n´ at locus c.1843 of *RYR1* that was being associated with an increase of muscle conductivity.

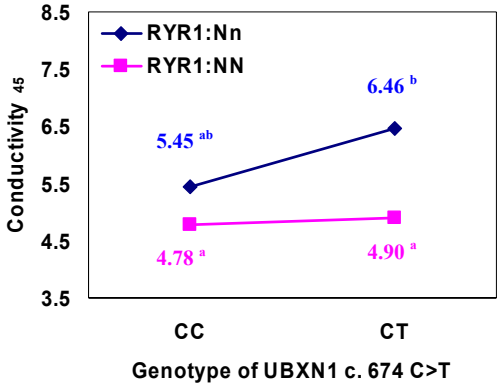
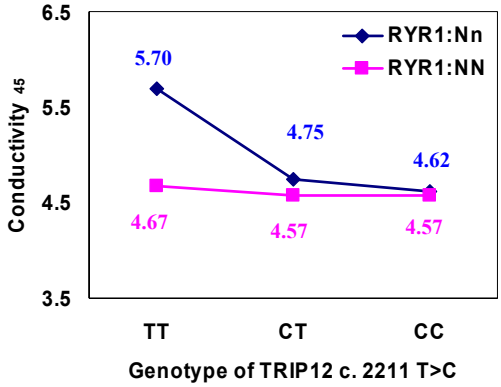
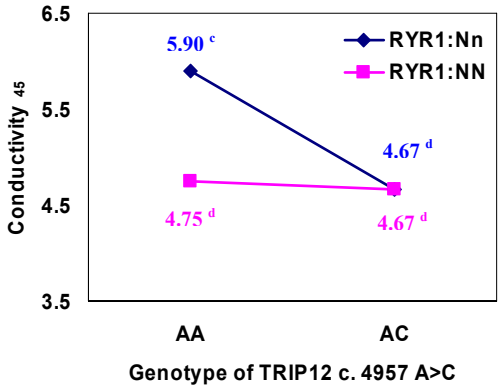


Figure 2: Interactive effects of SNP c.674 in the *UBXN1* gene and the *RYR1* genotype for muscle conductivity measured in *M. longissimus dorsi* (the mean values were shown at the plot and marked by different small letters when differing significantly at $p < 0.05$).

Belonging to the HECT family, *TRIP12* functions as an E3 ligase to transfers ubiquitin molecule to the protein substrate increasing the degradation of myofibrillar proteins in muscle cell. However, the two polymorphisms occurred within HECT domain of *TRIP12* that might cause the disturbing ubiquitination process as well as preventing the degradation of myofibrillar proteins by 26S proteasome. The minor allele `C´ of the both synonymous SNPs and their transcript levels were significantly associated with lower $CON_{45}$. The significant difference among the combination of the variant of *TRIP12* at locus (c.2211T>C) and *RYR1* genotypes was not detected for $CON_{45}$. However, the animals of `Nn´ genotype at *RYR1* locus, which carrier minor allele `C´ of SNP c.2211T>C, had lower value of CON_{45} (Figure 3).





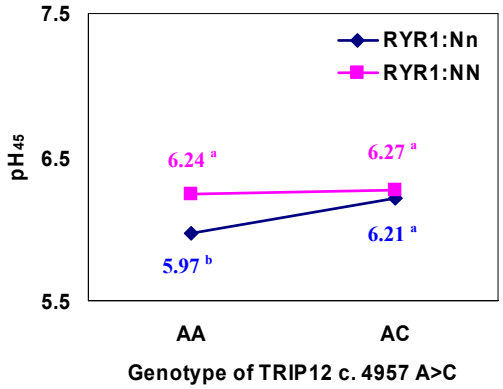


Figure 3: Interactive effects of the *TRIP12* polymorphisms and the *RYR1* genotype for the indicators of water holding capacity (the mean values were shown at the plot and marked by different small letters: a,b and c,d when differing significantly at p<0.05; p<0.1, respectively).

In the other hand, the interaction of *RYR1* genotypes and the SNP c.4957A>C exhibited the significant association with the indicators of water holding capacity. The `AC/Nn´ genotype had significantly lower muscle conductivity ($P < 0.1$) but obtains higher pH value ($P < 0.05$) than other combinations of genotypes (Figure 3). The minor allele `C´ at locus c.4957 of *TRIP12* showed a negative interaction with susceptible allele `n´ of *RYR1* that was associated with a decrease of drip loss in muscle cells.

The *UBE3B* gene encodes one of the important enzymes (E3 ligase) in the ubiquitination process. In the crossbreed population, the two polymorphisms of *UBE3B* had strong effects on the indicators of water holding capacity and the meat redness of pork (Minolta a-values). The homozygotes for the minor allele `T´ at loci c.1921A>T and c.4292C>T and its expression had significantly lower Minolta a-values but obtained higher transcript levels than other genotypes. Otherwise, statistical analysis revealed that the variation of the *RYR1* gene did not affect this particular trait (Table 1). Therefore, the least square means for Minolta

a-values were not significantly different among the combinated genotypes of *UBE3B* and *RYR1* genes. However, the positive interaction of *UBE3B* and *RYR1* genes could be expected since the carcass of animals, with the combination of the homozygote `TT´ at both *UBE3B* SNPs and the susceptible genotype `Nn´ at *RYR1*, had the lowest Minolta a-values (Figure 4).

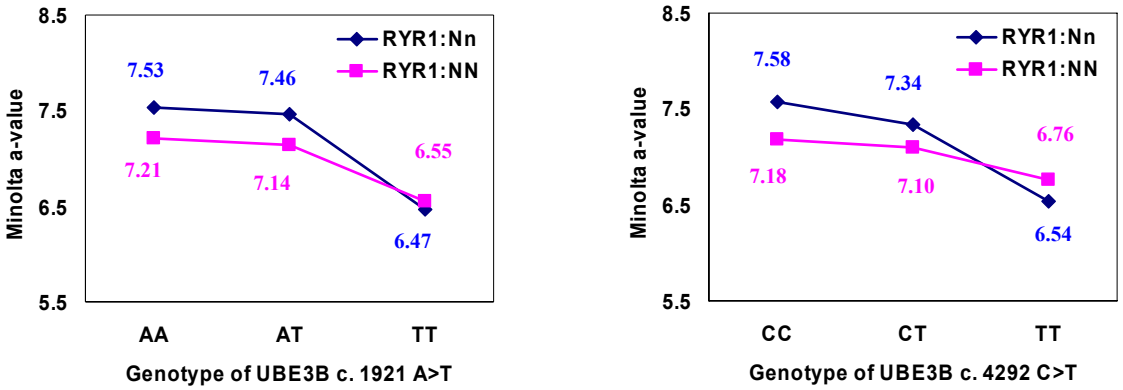


Figure 4: Interactive effects of the *UBE3B* polymorphisms and *RYR1* genotype for Minolta a-values measured in *M. longissimus dorsi* (the mean values were shown at the plot).

The member of the ovarian tumor protease family (OTU), *ZRANB1* functions in the deubiquitination process that cleavages the isopeptide bond of the polyubiquitin chain to unmark the tagged proteins of 26S proteasome. According to the association study in the PiF1 population, the detected SNP c.552A>G (p.Ile153Val) and its transcript levels were significantly associated with pH values at 24 hours post mortem. Otherwise, the significant difference between the `NN´ and `Nn´ genotypes in *RYR1* gene was detected for pH_{45}, but not for pH_{24} (Table 1). Therefore, no significant difference was found among the combinations genotypes of *ZRANB1* and *RYR1*. The Figure 5 showed the least squares means for pH_{24} of animals with double heterozygous `AG/Nn´, which is similar with the pH value of the `AG/NN´ genotype. The results implied that the negative interaction of *ZRANB1* and *RYR1* was associated with a decreased of drip loss formation.

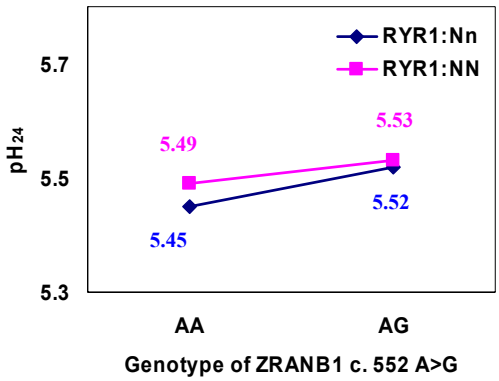


Figure 5: Interactive effects of the *ZRANB1* polymorphism and *RYR1* genotype for pH_{24} measured in *M. longissimus dorsi* (the mean values were shown at the plot).

According to the association and expression studies of the four candidate genes, the detected SNPs were in linkage phase with the alleles of causal sites of these genes impacting the transcript abundances, influencing the ubiquitination process, subsequently affecting the water holding capacity. In this section, the polymorphisms of four genes showed the interactions with *RYR1* mutation that impacted on drip loss formation in the muscle cells, consequently our findings imply that the effects of *RYR1* gene on the PSE phenomena might be influenced by the ubiquitination process.

3.4 Implication and Outlook

In the current study, the association and expression results provided genetic evidences to illustrate the physiological functions of four genes, *UBXN1*, *UBE3B*, *TRIP12* and *ZRANB1*, as well as promoted these as the candidate genes for water holding capacity of pork. The mutations in the transcribed region of these genes revealed the strong association with many carcass and meat quality traits, in particular with indicators of water holding capacity. Therefore, these polymorphisms could be integrated in the arrays of single nucleotide polymorphism (SNP chip) that is applied to the genome wide association (GWA). The advanced progress on GWA is the identification of candidate genes that had strong effects on the complex traits. The SNP chip is used to perform the association analysis in large scale. The approaches of GWA using SNP chip are faster and cheaper than genotyping methods before. Additionally, the consistent relationship between genotype, phenotype and expression provided a clear indication whether any polymorphism in regulatory regions of these genes might be relevant. Further, the analysis of regulatory regions is subsequently performed to generate strong genetically evidences, that support these candidate genes for marker assisted selection (MAS) to improve pork quality.

4 Summary

In the recent decades, the application of advances in molecular genetics is used to identify the candidate genes that play a role as the genetic associated marker for meat quality. The proteolytic systems, caspain and ubiquitin proteasome, were reported as the major factors to cause the degradation of myofibrillar proteins in muscle cell, and subsequently affecting the drip loss formation of meat. In particular, the holistic expression studies revealed the mechanisms of the ubiquitination pathway affect muscle properties that impact on the water holding capacity of pork. According to the known functions and expressions correlation with drip loss, four genes related to the ubiquitination/deubiquitination processes, *UBXN1*, *UBE3B*, *TRIP12* and *ZRANB1*, were selected to be investigated in our study. Using the *in silico* analysis, the nine polymorphisms of these genes were detected by comparative sequencing of PCR fragments, which were amplified from *M. logissimus* muscle cDNAs of eight panel animals (two each of German Landrace, German Large White, Pietrain and Duroc). Seven out of nine polymorphisms were used for association and expression studies. In total about 570 animals derived from the two populations, the purebred German Landrace (GL) and the commercial crossbreed of Pietrain × (German Large White × German Landrace) (PiF1), were genotyped at the novel SNPs by pyrosequencing.

The variation of three candidate genes involved the ubiquitination process, *UBXN1*, *UBE3B*, and *TRIP12*, exhibited strong effects on the indicators of water holding capacity, including

the muscle conductivity, drip loss and pH values. In the details, the two polymorphisms of *UBXN1* were significantly associated with CON_{45} within the two populations ($p \leq 0.01$), but the significant associations were detected for drip loss ($p \leq 0.05$) and pH_{45} ($p < 0.1$) only in the GL population. The association analysis of the two *UBE3B* polymorphisms showed the effects on muscle conductivity at different time points in the GL ($p < 0.1$), as well as meat redness and drip loss in the PiF1 population ($p \leq 0.05$). The detected SNPs of *TRIP12* had strong impacts on CON_{45} across the two populations ($p \leq 0.05$ and $p < 0.1$ in the PiF1 and GL, respectively), moreover a significant association was detected for pH_{24} in the GL ($p = 0.08$). Additionally, the polymorphisms of three candidate genes exhibited the effects on carcass quality of pork. In the GL population, the significant associations of these genes were detected for meat to fat ratio ($p < 0.05$; *TRIP12*); loin eye area ($p < 0.1$; *UBE3B*) and loin fat depth ($p < 0.05$ and $p < 0.1$ for *UBE3B* and *UBXN1*, respectively). In the PiF1 population, the *UBXN1* gene showed its impacts on variation of loin eye area and meat to fat ratio ($p < 0.1$). A candidate gene represents for the deubiquitination process (*ZRANB1*) showed the impact on pH values at different time points post mortem, within the two populations ($p < 0.05$). However, the single polymorphism of *ZRANB1* was associated with loin eye area and meat to fat ratio in the PiF1 population ($p < 0.05$).

According to the analysis of Affymetrix GeneChip Porcine Genome Array obtained from the previous study, in the PiF1 animals the expressions of three candidate genes, *UBXN1*, *ZRANB1* and *TRIP12* were significantly associated and correlated with the indicators of water holding capacity, including CON_{24} ($p < 0.05$ for *UBXN1*), pH_{24} ($p < 0.01$ for *ZRANB1*) and drip loss ($p < 0.01$ and $p < 0.08$ for *ZRANB1* and *TRIP12*, respectively). However, the association of *UBE3B* transcript abundance with meat redness was only detected at $p < 0.1$. On the one hand, these results illustrated the relationship between the transcript levels of four candidate genes and traits related to water holding capacity. On the other hand, the relationship between variation of the four candidate genes and WHC has already evaluated by the association studies. Combining these results generated the hypothesis about the linkage disequilibrium of detected SNPs and the allele of the causal sites, which may be located in another region of the candidate genes and not yet covered by the analysis that had the effects on the gene expression and WHC. Thereafter, the relationship between detected SNPs in the four genes and its transcript abundance were subsequently determined using the qPCR in the two populations. The consistent associations among polymorphisms, its transcript abundances and indicators of water holding capacity were found. The three-way relationships supported the

previous hypothesis about the existence of a causal polymorphism in *cis*-regulatory regions of these candidate genes, which primarily affects its transcript abundance and, consequently, traits related to WHC.

The results derived from association and expression studies implied that the ubiquitination process enhances the degradation of myofibrillar matrix inside of muscle cells. Under shrinkage condition the immobilized water is easily moved out of muscle cell. Moreover, the halothane gene (*RYR1*), which functions in the regulation of Ca^{2+} levels, plays an important role to regulate the metabolism and contraction of muscle. In order to evaluate the interaction of muscle contraction and proteolysis process affect on the drip loss formation, we aim to address the potential interaction of *RYR1* mutation (c.1843 C>T) and the polymorphisms of the four functional genes impacting on the water holding capacity of pork. In the PiF1 population, the susceptible allele `n´ was significantly associated with lower pH_{45} ($p<0.0001$), higher muscle conductivity and drip loss ($p<0.01$), and paler colour ($p<0.1$), these traits were the indicators of lower water holding capacity of meat. In this study, statistical analysis revealed the effects of *RYR1* gene on the meat quality might be influenced by the ubiquitination process, and the relationship between *RYR1* and the four genes was addressed as positive/negative interaction, that depended on their combination genotypes were associated with an increase/decrease of drip loss formation in muscle cells.

The functions of the three candidate genes, *UBXN1*, *UBE3B* and *TRIP12* modulate the ubiquitination process. The polymorphisms of *UBXN1* and *UBE3B* genes were in linkage phase with an allele of causal site of these genes increasing the transcript abundances, enhancing the ubiquitination process, and causing the decreased of water holding capacity. Therefore, the variant of *UBXN1* and *UBE3B* genes showed the positive interactions with *RYR1* mutation that were being associated with an increase of drip loss. Otherwise, the detected SNPs of *TRIP12* were in linkage phase with an allele of causal site of *TRIP12* increasing the transcript abundances, disturbing the ubiquitination process to protect the myofibrillar proteins from 26S degradation. Thus, the interaction of *TRIP12* and *RYR1* was assessed as a negative relationship that was associated with a reduction of purge loss. The last candidate gene, *ZRANB1*, functions in the deubiquitination process to prevent the destruction of myofibrillar matrix and membrane of muscle cell. The single polymorphism of *ZRANB1* was in linkage phase with an allele of causal site increasing the transcript abundances, decreasing drip loss formation. Indeed, the interaction between *ZRANB1* and *RYR1* genes was evaluated as a negative relationship that was associated with a decrease of purge loss.

In conclusion, our study provides genetic evidences to illustrate the polymorphisms and the expression levels of the four functional candidate genes that exhibited strong effects on the degradation of myofibrillar proteins, and affect the water holding capacity. Moreover, the relationship between DNA marker for PSE meat and these genes were performed to imply that the effects of *RYR1* on meat quality could be influenced by the activity of ubiquitin proteasome system. In the future prospect, the causal SNPs will be addressed in the regulatory regions of four genes that will provide more evidences to support these candidate genes for marker assisted selection for pork quality.

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Enclosure

1. List of abbreviations

ATP	: Adenosine triphosphate
BF3, LFD	: loin fat depth
CON_1, CON_{45}	: conductivity in *M.longissimus dorsi* at $13^{th}/14^{th}$ rib 45 minutes p.m.
CON_{24}	: conductivity in *M.longissimus dorsi* at $13^{th}/14^{th}$ rib 24 hours p.m.
DNA	: Deoxyribonucleic acid
dNTP	: Deoxyribonucleotide triphosphate
DRIP	: drip loss
eQTL	: expression quantitative trait loci
FD_{mid}	: fat depth at 10^{th} rib
GL, DL	: German Landrace
LEA	: loin eye area
LSM	: least square means
MAS	: marker assisted selection
MFR	: meat to fat ratio
PCR	: polymerase chain reaction
pH_1, pH_{45}	: pH value in *M.longissimus dorsi* at $13^{th}/14^{th}$ rib 45 minutes p.m.
pH_{24}	: pH value in *M.longissimus dorsi* at $13^{th}/14^{th}$ rib 24 hours p.m.
PiF1	: Pietrian x (German Large White x German Landrace)
PSE	: pale, soft, exudative
QTL	: quantitative trait loci
SNP	: single nucleotide polymorphism
UTR	: untranslated region
WHC	: water holding capacity

2. Acknowledgement

First of all, I appreciate Prof. Dr. Klaus Wimmers very much for supporting me the opportunity to pursue a Ph.D.degree in the Leibniz Institute for Farm Animal Biology. During four years I study in the Institute for Genome Biology, Prof. Wimmers advised me many things about research topic, suggested me the ideas to solve the problems as well as got further progress. Prof. Wimmers behaved with the best of responsibility and kindness, and he brought to me the very good memories about the `Vorbild´ of a lecturer who I want to become in my teaching life.

Subsequently, I am very grateful to Dr. Siriluck Ponsuksili and Dr. Eduard Muráni, for the explanations to help me understanding more about the knowledge and the techniques that related to my research. I thank Prof. Dr. Steffen Maak for providing the records of meat quality measurements that is important data for statistical analysis in my study. I would like to thank all technicians, Hannelore Tychsen, Angela Garve, Marlies Fuchs, Joana Bittner and Annette Jugert, for help me in various aspects of the laboratories. My grateful thanks go to my family and my friends who encourage me with the great of love, hope and belief.

Finally, I would like to express my sincere to the German Research Foundation Deutsche Forschungsgemeinschaft, the Ministry of Education and Training of Vietnam, the German Academic Exchange Service (DAAD), and the Cantho University (Vietnam), for financial supports, I also thank the Pig Breeding Association of Mecklenburg-Western Pommerania.

3. Declaration

I hereby declare that the work in this thesis is my own work and that to the best of my knowledge it contains no materials previously published or written by another person. I also declare that the intellectual content of the thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.

Rostock, 02 February, 2014

Thi Phuong Loan Huynh

4. List of publications and talks

[1] Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers, *UBXN1* polymorphism and its expression in porcine *M. longissimus dorsi* are associated with water holding capacity, Molecular Biology Reports, (2014), DOI 10.1007/s11033-013-2985-5

[2] Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers, Novel SNPs of the porcine *TRIP12* are associated with water holding capacity of meat, Czech Journal of Animal Science, 58, 2013 (11): 525–533

[3] Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers, *UBE3B* and *ZRANB1* polymorphisms and transcript abundance are associated with water holding capacity of porcine *M. longissimus dorsi,* Meat Science, 95 (2013),166–172

[4] Thi Phuong Loan Huynh, Eduard Muráni, Steffen Maak, Siriluck Ponsuksili, Klaus Wimmers, UBE3B and TRIP12 polymorphisms and transcript abundance are associated with water holding capacity of porcine *M. longissimus dorsi*, Tag des Doktoranden, FBN Dummerstorf, 23- May, 2013.

[5] Thi Phuong Loan Huynh, E.Muráni, S.Ponsuksili, K.Wimmers, Polymorphism of the porcine UBXN1 gene associated with carcass and meat quality, Vortragstagung der DGfZ und GfT, Halle – Saale, 12-13 September, 2012.