Mitochondrial-nuclear crosstalk, haplotype and copy number variation distinct in muscle fiber type, mitochondrial respiratory and metabolic enzyme activities

Xuan Liu¹, Nares Trakooljul², Frieder Hadlich³, Eduard Murani², Klaus Wimmers² & Siriluck Ponsuksili¹

Genes expressed in mitochondria work in concert with those expressed in the nucleus to mediate oxidative phosphorylation (OXPHOS), a process that is relevant for muscle metabolism and meat quality. Mitochondrial genome activity can be efficiently studied and compared in Duroc and Pietrain pigs, which harbor different mitochondrial haplotypes and distinct muscle fiber types, mitochondrial respiratory activities, and fat content. Pietrain pigs homozygous-positive for malignant hyperthermia susceptibility (PiPP) carried only haplotype 8 and showed the lowest absolute mtDNA copy number accompanied by a decrease transcript abundance of mitochondrial-encoded subunits ND1, ND6, and ATP6 and nuclear-encoded subunits NDUFA11 and NDUFB8. In contrast, we found that haplotype 4 of Duroc pigs had significantly higher mitochondrial DNA (mtDNA) copy numbers and an increase transcript abundance of mitochondrial-encoded subunits ND1, ND6, and ATP6. These results suggest that the variation in mitochondrial and nuclear genetic background among these animals has an effect on mitochondrial content and OXPHOS system subunit expression. We observed the co-expression pattern of mitochondrial and nuclear encoded OXPHOS subunits suggesting that the mitochondrial-nuclear crosstalk functionally involves in muscle metabolism. The findings provide valuable information for understanding muscle biology processes and energy metabolism, and may direct use for breeding strategies to improve meat quality and animal health.

Mitochondria are involved in many key cellular processes such as apoptosis, calcium homeostasis, reactive oxygen species production, and most importantly, adenosine triphosphate (ATP) generation by oxidative phosphorylation (OXPHOS). In addition, these organelles contain their own DNA distinct from the nuclear genome. In pigs, mitochondrial DNA (mtDNA) is 16,613 base pairs in length and encodes two ribosomal RNA (rRNA), 22 transfer RNA (tRNA), and 13 protein-coding genes involved in the OXPHOS system. Since OXPHOS subunits are encoded by both the nuclear and mitochondrial genome, the two genome systems interact intricately to form the fully assembled functional OXPHOS complexes.

Mitochondrial genetic variation can affect fertility, longevity, and evolutionary trajectories and acts as a human health indicator¹. mtDNA haplotype variation affects metabolic performance in many species, such as raccoons, dogs, cows, and pigs², and mitochondrial DNA (mtDNA) copy number has been associated with extensive exercise, age-related hearing impairment, disordered antioxidant capacity, and heart failure³–⁶. In Drosophila, mtDNA copy number is proposed to be modulated by mtDNA genome variation⁷. Mitochondrial DNA haplotypes are potential targets for manipulating phenotypes including tolerance to heat, growth, and milk quality in farm animals⁸. Hence, an understanding of the influence of mitochondrial haplotypes on energy metabolism in pigs would provide valuable information on mitochondrial function and inform farming practices to improve

¹Research Unit ‘Functional Genome Analysis’, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, D-18196, Dummerstorf, Germany. ²Research Unit ‘Genomics’, Leibniz Institute for Farm Animal Biology (FBN), Wilhelm-Stahl-Allee 2, D-18196, Dummerstorf, Germany. Correspondence and requests for materials should be addressed to S.P. (email: ponsuksili@fbn-dummertorf.de)
meat quality and animal health. Since pigs share many similarities with humans in terms of genetics and physiology, this knowledge is also potentially applicable to human diseases.

In addition to variation in mitochondrial density among muscle types, mitochondria are functionally optimized and specialized in glycolytic and oxidative fibers. Mitochondria isolated from muscle immediately after slaughter are similar to those found in intact muscle, whereas some mitochondria from pale, soft, exudative (PSE) muscle are already swollen and show a decreased matrix density. With an emphasis placed on glycolysis, mitochondrial content and function may be important factors contributing to postmortem muscle metabolism. Duroc and Pietrain are two commercial pig breeds known for divergent meat quality and muscular energy metabolism. Muscle from Duroc pigs typically contains more slow-twitch oxidative (STO) fibers and intramuscular fat, whereas Pietrain pigs are leaner and their muscles contain more fast-twitch glycolytic (FTG) fibers. The content of different muscle fibers types, their size and structure largely contribute to differences in growth performance and carcass traits as well as postmortem meat quality traits. Lipids are stored mainly in STO fibers, which can improve the tenderness and juiciness of the meat. The selection of a high percentage of FTG fibers may result in altered meat quality possibly due to the lower capillarization, insufficient delivery of oxygen and glycogen depletion. Indeed, the meat quality parameters such as color of the meat, drip loss and shear force were measured in muscle of the same pigs. PiPP pigs showed increased drip loss and shear force comparing to other three pig breeds. These results directly supported the association between muscle fiber type and meat quality. In Pietrain pigs, mutations within the ryanodine receptor 1 (RYR1) are associated with malignant hyperthermia susceptibility (MHS), reduced water holding capacity, and increased PSE meat. Thus, Duroc and Pietrain pigs are unique models in which to study mitochondrial properties and energy metabolism influencing muscle metabolism and meat quality.

We have previously shown the transcriptional signatures in muscle from these pigs to be related to metabolic properties and mitochondrial respiration. Furthermore, we wanted to know the mtDNA variation of these pigs and investigate whether mtDNA variation preferentially modifies the expression of mitochondria-associated OXPHOS genes from both mitochondria and nuclear genomes. Finally, we investigated mtDNA variation genes involved in mtDNA copy number in conjunction with muscle fiber types and metabolic enzyme activities in postmortem longissimus muscles (LM) from four different pig breeds: Duroc, Pietrain homozygous-negative for MHS (PiNN), Pietrain homozygous-positive for MHS (PiPP), and an F2 crossbred Duroc-Pietrain homozygous-negative for MHS (DuPi).

Results

Phenotypic differences among breeds. To illustrate breed differences in muscle metabolism, muscle fiber composition, metabolic activities, and pH were compared among the four breeds. Duroc pigs had the highest percentage of STO muscle fibers and lower fast-twitch oxidative (FTO) fibers (Supplementary Fig. S1). PiPP pigs had a significantly higher percentage of FTG fibers (80.8%) compared to PiNN pigs (74.9%, \( p = 0.02 \)), while Duroc and DuPi were in between the two Pietrain pigs.

The activities of key metabolic enzymes for energy metabolism were measured for all four pig breeds. PiNN pigs had the highest activity of phosphofructokinase (PFK), whereas no significant differences in the activities of glycogen phosphorylase (GP) and lactate dehydrogenase (LDH) were detected among breeds. Duroc pigs had the highest complex I activity (12.5 U/g protein) compared to other breeds. Moreover, PiPP had significantly lower pH than the other three breeds.

All measured phenotypic traits were compared at time 0 and 30 min postmortem (Supplementary Fig. S2). Most of the phenotypes, except complex IV activity, were significantly different between time points. The enzyme activities of PFK and LDH were increased from 475 to 859 U/g protein (\( p = 0.0002 \)) and from 10.9 to 15 U/g protein (\( p < 0.0001 \)), respectively, whereas the oxidative enzyme activities of CS, complex I and complex II, together with GP activity and pH, were decreased at 30 min postmortem compared to immediately after slaughter (\( p \) values ranging from \( < 0.0001 \) to 0.02).

Different mitochondrial haplotypes among pig breeds. The D-loop regions in 53 animals were sequenced and eight haplotypes were identified. For statistical reasons, five haplotypes identified in at least three animals were included in subsequent data analysis. Detailed haplotype information is shown in Supplementary Table 1 and Fig. S3. In brief, haplotypes 4 (Duroc: 10, DuPi: 6) and 6 (Duroc: 5, DuPi: 1) were present in mainly Duroc and DuPi pigs whereas haplotype 1 was present in five DuPi pigs only. Haplotype 7 was present in three PiNN pigs, while haplotype 8 was found in 14 PiPP and four PiNN pigs. Muscles from pigs with haplotype 7 contained significantly more FTO muscle fibers than haplotypes 4, 6, and 8 (\( p < 0.05 \), Fig. S4). Interestingly, haplotype 8 showed the lowest complex I activity among all the haplotypes and had significantly lower activity than haplotypes 4 and 6 (\( p < 0.05 \)). All other phenotypic traits were comparable between haplotypes.

Duplication of the porcine mitochondrial genome in the nuclear genome. Using BLASTN, the pig mitochondrial genome was compared to the pig nuclear genome. Many regions among 18 somatic chromosomes and the X chromosome of the porcine nuclear genome matched to the mitochondrial genome (Fig. 1).

Comparison of mtDNA copy number. PiPP pigs had the lowest mtDNA copy number (368 copies per nuclear genome) among the four breeds, especially compared to Duroc pigs (435 copies per nuclear genome, \( p = 0.02 \), Fig. 2a). Further, mtDNA copy number in longissimus muscle was decreased from 420 copies per nuclear genome at 0 min postmortem to 389 copies at 30 min postmortem (\( p = 0.01 \), Fig. 2b). Finally, muscle from pigs with haplotype 8 contained the lowest mtDNA copy number. Pigs with haplotype 8 had 375 copies per nuclear genome in their muscle tissue, which was significantly less than the 435 copies per nuclear genome seen in pigs with haplotype 4 (\( p = 0.02 \), Fig. 2c). There were no significant effects of sex on mtDNA copy number.
Correlation between mtDNA copy number, phenotype, and gene expression. As shown in Fig. 2d, mtDNA copy number was significantly correlated with muscle fiber type (STO: $r = 0.263 \ p = 0.031$; FTG: $r = -0.292 \ p = 0.017$) and enzyme activities (CS: $r = 0.249 \ p = 0.009$; complex I: $r = 0.43 \ p < 0.0001$). Moreover, the expression of mitochondria-encoded genes (ND1, CYB, COX1, and ATP6) and nuclear-encoded OXPHOS genes (NDUFA3, NDUFA11, NDUFA13, NDUFB8, NDUFS8, NDUFV1, ATP5G1, and ATP5L) were significantly correlated with mtDNA copy number ($p$ values ranged from 0.006 to 0.031 and < 0.0001 to 0.015, respectively. MtDNA copy number was weakly correlated with PPARG coactivator 1 alpha (PGC-1α) mRNA ($p = 0.156$).

Comparison of mitochondrial and nuclear encoded OXPHOS genes expression. To further examine breed and time differences in postmortem muscular energy metabolism at a molecular level, both mitochondrial and nuclear encoded gene expression was profiled at 0 and 30 min postmortem using qPCR in all four pig breeds. No mitochondrial-encoded genes were differently expressed between 0 and 30 min postmortem (Table 1). The mRNA levels of 4 nuclear-encoded genes, NDUFB8, COX7A2 and ATP5L were significantly lower at 30 min postmortem than 0 min ($p$ values < 0.0001 to 0.02).

Out of the sixteen genes investigated, ten genes including mitochondrial-encoded ND1, ND2, ND6, ATP6 were differently expressed between breeds (Fig. 3a). PiPP pigs had the most differentially expressed genes, especially compared to the Duroc and DuPi breeds: complex I subunits ND1 and ND6 and the ATP synthase subunit ATP6 were all significantly upregulated in Duroc pigs ($p$ values < 0.0001 to 0.003). Nuclear-encoded NDUFA11, NDUFB8, NDUFS8, NDUFV1, ATP5G1 and ATP5L showed significant differences among breeds ($p < 0.05$, Supplementary Table 2) (Fig. 3b). All six differentially expressed were down-regulated in PiPP pigs compared to the three other breeds.

Eleven genes including ND1, ND2, ND6, CYB, COX1, ATP6 and nuclear-encoded NDUFA11, NDUFA13, NDUFB8, ATP5G1 and ATP5L showed significant differences in haplotypes (Supplementary Table 3). Only three genes including nuclear-encoded NDUFB8, COX7A2 and ATP5L demonstrated that they were significantly influenced by time. No gene expression was affected by sex.
All PiPP pigs had haplotype 8, which showed significantly lower gene expression than haplotypes 1, 4, and 6 for ND1, ND2, ND6, CYB, COX1, and ATP6 (p values from p < 0.0001 to 0.048; Fig. 4a and b). Complex I subunits NDUFA11, NDUFA13, and NDUFB8 showed significantly lower gene expression in haplotype 8 than haplotype 1 pigs (p values < 0.0001 to 0.029; Fig. 4c and d).
Correlation between expression levels of mitochondrial, nuclear encoded genes and phenotype. Pairwise correlation was calculated and plotted for each individual OXPHOS gene (Fig. 5a). All included genes were significantly correlated with at least one of the other genes. Further, all mitochondrial-encoded OXPHOS subunits tended to be more tightly co-expressed while nuclear-encoded OXPHOS subunits were in a tight co-expressed relationship. Figure 5b shows the correlation between the expression of OXPHOS genes and phenotype. The expression of mitochondrial encoded OXPHOS subunits ND2, ND6, and ATP6 and the nuclear encoded subunit NDUFV1 were highly correlated with muscle fiber type (ND2: $R = -0.49, p = 0.0002$; ND6: $R = 0.286, p = 0.042$; ATP6: $R = 0.342, p = 0.014$; NDUFV1: $R = -0.346, p = 0.013$). The nuclear encoded OXPHOS subunits NDUFA3, NDUFA11, NDUFA13, NDUFB8, NDUFS8, and NDUFV1, complex IV subunit (COX7A2) and ATP synthase subunits (ATP5G1 and ATP5L). Relative gene expression was normalized to reference genes ACTB, RPL32 and RPS11 using 2$^{-\Delta\DeltaCT}$.

The expression of the master regulator of mitochondrial biogenesis and oxidative phosphorylation PGC-1α was significantly correlated with the phenotypes of CS, complex I, complex II, complex IV, and pH (Table 2). The mRNA levels of PGC-1α were also significantly correlated with the expression of mitochondrial-encoded genes ND1, ND2, ND4, ND6, CYB, COX1, and ATP6. Among these genes, ATP6, ND6, and ND2 showed the top three most significant correlations with PGC-1α (ATP6: $R = 0.415, p < 0.0001$; ND6: $R = 0.372, p = 0.0003$; ND2: $R = 0.361, p = 0.0004$). The expression of PGC-1α showed a significant correlation with the nuclear-encoded genes NUDFA11, NDUFA13, NDUFB8, COX7A2, ATP5G1, and ATP5L (p values < 0.0001 to 0.036).

Discussion

We compared the mitochondrial DNA content, gene expression pattern of mitochondrial and nuclear encoded OXPHOS subunits, metabolic enzyme activities and mitochondrial respiration in four pig breeds distinct in
muscle phenotype. The effect of mitochondrial haplotypes on mitochondrial DNA copy number and OXPHOS gene expression were also examined since there were different haplotypes among the investigated pig breeds. Muscle samples were collected from the fat-type Duroc pigs with a higher proportion of STO fibers and greater oxidative enzyme activities. Pietrain pigs are muscular and lean, and their muscles contain more FTG fibers. DuPi pig is a Duroc-Pietrain F2 crossbred. Mutations within ryanodine receptor 11 (RYR1) are frequently detected in Pietrain pigs. This leads to abnormal Ca\(^{2+}\) homeostasis and result in increased excitability of the muscle associated with MHS. This genetic defect consequently leads to reduction of water holding capacity, loss of stress resistance and PSE meat\(^{19-21}\).

Reduced mtDNA copy number in PiPP pigs. Previous studies have demonstrated the relative mtDNA copy number in porcine muscle\(^{25,26}\). In this study we quantified the absolute mtDNA copy number by qPCR and found that PiPP pigs contained the lowest amount of mtDNA copies in muscle cells compared to PiNN, DuPi, and particularly Duroc pigs. mtDNA copy number was positively correlated with STO fibers and negatively correlated with FTG fibers and indicated a strong association between mtDNA copy number and muscle fiber types. The underlying cause of the mtDNA copy number variation between pig breeds remains unknown. In other case, changes in cytosolic Ca\(^{2+}\) and pH could influence the amount of ROS produced at the mitochondrial respiratory complex I and III\(^{27}\). The abnormal Ca\(^{2+}\) homeostasis in PiPP pigs may result in oxidative stress associated with elevated ROS production. The elevated ROS could increase mitophagy to remove damaged mitochondria and leads to mitochondrial degradation\(^{28,29}\). The copy number depletion associated with increased mitochondrial turnover is caused by a burst of ROS production\(^{30}\). Indeed, the burst of ROS and mtDNA depletion have been observed in cell-culture experiments. All together, these may partially explain the copy number variation of mtDNA in pig breeds. However, supporting evidence under physiological situation is still needed.

Decreased transcript levels of mitochondrial and nuclear encoded OXPHOS genes in PiPP pigs. Decreased abundance of many nuclear-encoded OXPHOS subunits transcripts was found in PiPP pigs compared to other breeds. This phenomenon is very likely caused by the abnormal Ca\(^{2+}\) homeostasis in PiPP pigs since MHS knock-in mice show a clear Ca\(^{2+}\) overload in the mitochondrial matrix and a switch to a compromised bioenergetics state characterized by low OXPHOS\(^{31}\). Similarly, transcript abundance of mitochondrial-encoded genes was decreased in PiPP pigs including subunits of complex I and ATP synthase. The previous study reported that ATP concentration has been significantly reduced in PiPP pigs compared to other breeds because of their accelerated energy consumption\(^{14}\). ATP sensing by transcriptional machinery has been proposed to regulate the
initiation of mitochondrial transcription and promotor usage. Therefore, the lower ATP availability in PiPP pigs may influence their mitochondrial transcription and function.

Recent work has established that glucocorticoid receptor (GR) is translocated from cytosol to the mitochondria and binds to the D-loop control region while stress and corticosteroids have a direct influence on hippocampal mtDNA gene expression in rats. Since RYR1 mutated PiPP pigs are stress susceptible, it is speculated that the abundance of mtDNA transcripts may be decreased in those pigs.

![Figure 5. Correlation matrix of OXPHOS gene expression and phenotypes. (a) correlation matrix between mitochondrial and nuclear encoded OXPHOS gene expressions. (b) correlation matrix between OXPHOS gene expression and phenotype. Number in each cell represents the value of correlation coefficients and the corresponding p-values. Cell color indicates correlation (red, positive correlation; blue, negative correlation).](image-url)
The early postmortem period had no effect on the expression of mitochondrial-encoded OXPHOS genes, but the mtDNA copy number was decreased from 0 min to 30 min postmortem. In other words, the early phase of hypoxia triggered mitochondrial genome instability while transcript abundance was maintained in porcine muscle. This disparity is of interest and might provide valuable information toward the understanding of mitochondrial mechanisms in muscle tissue under hypoxia, which has been linked to oxidative stress, ischemia, and cancer. In addition, the identified postmortem mitochondrial properties are relevant to muscle injury, also caused by oxygen depletion.

The effect of haplotype on mtDNA copy number and OXPHOS gene expression. Our study showed for the first time the effect of different mitochondrial haplotypes on muscle fiber types and mitochondrial respiration at both the phenotypic and molecular level in pigs. Among different haplotypes, our results showed variations in complex I activity and mtDNA copy number. At the molecular level, six of the mitochondrial-encoded and three of the nuclear-encoded OXHPOS genes were differentially expressed. These findings suggest that mitochondrial haplotypes could contribute to variations in mitochondrial content and OXPHOS system function.

The mitochondrial haplotype has been found to affect mtDNA copy number, OXPHOS respiration, and mitochondrial molecular function in Drosophila. In porcine transmitochondrial cybrids, the mitochondrial haplotype is linked to metabolic traits including ROS production, ATP content, and complex II activity. The assembly kinetics of OXPHOS complexes is proposed to be modulated by mitochondrial DNA background.

Coordination of the nuclear and mitochondrial genomes contribute to phenotype. Our results showed that all mitochondrial-encoded OXPHOS subunits were highly co-expressed; moreover also the nuclear-encoded OXPHOS subunits tended to be in a tight co-expressed relationship. This observation reflects the fact that the mitochondrial genome has its own transcription machinery distinct from the nuclear genome. Although mitochondrial- and nuclear-encoded OXPHOS genes are expressed separately via transcription machineries in different cellular locations, they are not completely independent. The observed association between mitochondrial- and nuclear-encoded OXPHOS genes is in line with the theory of mitochondrial-nuclear crosstalk. PPARC coactivator 1 alpha (PGC-1α) is a nuclear-encoded transcription factor that acts as a master coordinator to mediate mitochondrial biogenesis and oxidative phosphorylation. In this study, the mRNA level of PGC-1α was significantly correlated with all investigated mitochondrial-encoded OXPHOS subunits, six nuclear-encoded subunits and enzyme activities of complex I, II, and IV. In fact, the nuclear-encoded subunits need to be imported into the mitochondria together with mitochondrial-encoded subunits to form a fully assembled functional OXPHOS system in the mitochondrial inner membrane. Accordingly, the absence of the mtDNA-encoded subunits COX1 and COX2 has been shown to affect the stability of some subunits of nuclear encoded respiratory chain proteins.

In our results, different mitochondrial haplotypes showed variation of the expression of mitochondrial-encoded subunits ND2, ND6 and ATP6 as well as nuclear-encoded subunits NDUFA11 and NDUFB8. These genes were co-expressed and not only highly correlated to PGC-1α but also highly associated with different muscle fiber types and enzyme activities of complex I and II. Therefore, we propose that mitochondrial haplotype contributes to muscle fiber type and energy metabolism in porcine via altering the gene expression of OXPHOS subunits mediated by the nuclear-mitochondrial crosstalk. In addition, different haplotypes showed variation in complex I activity. It directly supported the link between haplotype and energy metabolism.
The disrupted Ca\(^{2+}\) homeostasis affects mitochondrial membrane biogenesis and hence metabolic stress\(^{43}\). Under conditions of oxidative stress, the expression of ND6 is suppressed through methylation\(^{46}\). Consistent with our results, downregulated ND6 expression in PiPP pigs has been associated with mutant RYR1-induced mitochondrial injury and oxidative stress\(^{45}\). These evidences supported the possibility of haplotype 8 could be linked to oxidative stress with altered mitochondrial transcription in PiPP pigs.

Our results showed some of the mitochondrial and nuclear-encoded OXPHOS transcripts were significantly correlated to at least one of the phenotypes including muscle fiber type, metabolic enzyme activities and pH. The mtDNA encoded OXPHOS gene expression was highly associated with muscle fiber types, which is consistent with the fact that ST0 muscle fibers in general contain more mitochondria\(^{46}\). It is worth mentioning that the mRNA levels of ATP6, ND6, and ND2, which were the top three genes correlated with PGC-1α, also significantly correlated with muscle fiber type. Hence, it raised the possibility that mitochondrial-encoded subunits with high correlation with PGC-1α showed effects on muscle fiber types. The muscle oxidative capacity not only relies on mitochondrial function but also on mitochondrial density\(^{46}\). Our measured mtDNA copy number was correlated positively with STO fibers and oxidative enzyme activity while negatively correlated with FTG fibers. The mtDNA copy number is related to mitochondrial oxidative capacity and adipocyte lipogenesis\(^{47}\).

Conclusions

In summary, we investigated the mitochondrial DNA content and the expression of both mitochondrial and nuclear encoded OXPHOS genes in conjunction with post-mortem muscle phenotype, and metabolic enzyme activities in distinct haplotypes of pig breeds including Duroc, DuPi, PiNN, and PiPP. The most significant link between haplotypes or breeds to the muscle phenotype was found between the muscle fibers type and complex I. Specific expression pattern of mt transcript including ND1, ND6, and ATP6 and nuclear-encoded subunits NDUFA11 and NDUFB8 was identified and in turn play a role in muscle fibers type and enzyme activities of complex I. All of these changes in the PiPP haplotype 8 pigs may partially contribute to negative outcomes of meat quality such as pale soft exudative pork. PiPP pigs showed the lowest mtDNA copy number and reduced gene expression of many mitochondrial and nuclear encoded OXPHOS subunits compared to the other breeds. Our results provide valuable information on haplotype and breed-specific mitochondrial content variation as well as the molecular basis of mitochondrial respiration. Haplotypes could be linked to porcine energy metabolism at a functional level by altering gene expression of mitochondrial and nuclear OXPHOS subunits. Since haplotype 7 Pietrain pigs demonstrate a high ratio of FTO muscle fibers, while haplotype 8 pigs show the lowest complex I activity among all other haplotypes, implementing a selection of the favorable haplotype 7 along with the RYR1 locus in marker assisted selection program may further improve meat quality. Selection of the favorable haplotype can be used in marker assisted selection in pig breeding strategy.

Material and Methods

Sample collection and phenotypic measurement. The experiment and muscle collection were approved and authorized by the German and European animal welfare regulations for animal husbandry, transport, and slaughter\(^{12-14}\). All experimental procedures, including animal care and tissue sample collection, followed guidelines for safeguarding and good scientific practice in accordance with the German Law of Animal Protection, officially authorized by the Animal Care Committee and authorities [Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) 33.42502/01-47.05].

Duroc, PiNN, PiPP, and DuPi pigs were raised to the age of 180 days at the University of Bonn. Muscle samples from each breed (Duroc, n = 15; DuPi, n = 16; PiNN, n = 12; PiPP, n = 15) were collected immediately (0 min postmortem) and 30 min after stunning (30 min postmortem) from LM between the 13th and 14th thoracic vertebrae. Samples were frozen in liquid nitrogen and stored at −80°C until analysis.

We used the samples and all phenotypical traits from previously measured which were described\(^{12-14}\). In brief, the cryopreserved muscle samples were cutting into slices of 12 µm thickness. NADH tetrazolium reductase and Myofibrillar ATPase were stained to identify the muscle fiber types. 3 sections were used for calculating the percentage of the slow-twitch-oxidative (STO), fast-twitch-oxidative (FTO) and fast-twitch glycolytic (FTG) fibers by relating the number of counted fibers of each type to the total counted fiber number. For measurement of Metabolic Enzyme Activities, muscle samples were homogenized and all the experiments were finished within 2 h in duplicate. GP catalyzed the degradation of glycogen (2 mg/ml) to glucose-1-phosphate followed by the isomerization to glucose-6-phosphate (G-6-P). GP activity was determined spectrophotometrically by the reduction of NADP+ (1.6 mM) to NADPH at 340 nm and pH 6.8 when G-6-P dehydrogenase (5 U/ml) catalyzed G-6-P to gluconate-6-phosphate. PK catalyzes fructose-6-phosphate (3.0 mM) to fructose-1,6-bis-phosphate, which is split to glyceraldehyde-3-phosphate and dihydroxyacetonephosphate (DHAP). PK activity was determined by the oxidation of NADH (1.6 mM) to NAD+ at 340 nm and pH 8.0 when glycerol-3-phosphate dehydrogenase (10 U/ml) catalyzed DHAP to glyceraldehyde-3-phosphate DH activity was determined by the oxidation of NADH (150 µM) to NAD+ at 340 nm and DH activity was determined by the oxidation of pyruvate (1.2 mM) to lactate. CS catalyzes acetyl-CoA (0.1 mM) and oxaloacetate (0.5 mM) to citrate to liberate CoA. CS activity was determined by the irreversible reaction of CoA with 5,5′-Dithiobis-(2-nitrobenzoic acid; 0.1 mM) to thionitrobenzoic acid at 412 nm. Complex I was spectrophotometrically determined by following the oxidation of NADH (0.2 mM) to NAD+ at 340 nm. Complex II was determined at 600 nm following the reduction of 2,6-dichlorophenolindophenol (DCPIP) by ubiquinol resulting from this reaction. Complex IV was determined by following the oxidation of reduced cytochrome c to the oxidized form at 550 nm and pH 7.0.

DNA and RNA extraction. Genomic DNA from LM samples was extracted. Total RNA was isolated from muscle samples using Tri-reagent and RNeasy Minikit (Qiagen, Hilden, Germany) with an on-column DNase
Mitochondria-specific primer design. Primers for the detection of mitochondrial DNA (mtDNA) copy number were carefully designed avoiding co-amplification of mitochondrial duplicated regions in the nuclear genome. Duplication of the mitochondrial genome in the nuclear genome was detected using BLASTN (http://www.ncbi.nlm.nih.gov)\(^48\). The mitochondrial sequence (Sus scrofa 10.2 download from NCBI: http://www.ncbi.nlm.nih.gov/ on 1.9.2015) was split into fragments of 150 bp in length with a 50 bp overlap and blasted against the reference genome to identify a ‘unique’ mitochondrial sequence based on a significant threshold of E-value < 0.1 and length > 100 bp. The result of duplicated regions was demonstrated using R package IdeoViz\(^49\) and a cytogenetic map of pig chromosomes was extracted from ArkDB (http://www.thearkdb.org/arkdb)\(^50\).

Absolute quantification of mtDNA copy number. The absolute quantification approach was used to determine mtDNA copy number. The mitochondrial genes ND1, ND2, and COX1 were used to quantify mtDNA copy number, whereas the nuclear gene glucagon gene (GCG), which is highly conserved between species and presents as a single copy, was used as the single-copy reference gene\(^23,51\). Primer sequences were presented in Supplementary Table 4 online. Mitochondrial and nuclear DNA standards were prepared separately using PCR products in seven serial dilutions at a dilution factor of 10. The amplified DNA fragments were purified with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany). The purified products were quantified using a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). The copy number was calculated according to the following equation\(^52\):

\[
\text{copies/\mu L} = \frac{\text{ng/\mu L}}{m} \tag{1}
\]

\[
m = n \times [1.096 \times 10^{-12}] \tag{2}
\]

where \(m\) is the mass of a single copy and \(n\) is the target size in base pairs.

The absolute copy numbers of ND1, ND2, COX1 and GCG were calculated based on their standard curves using the following equation:

\[
\text{copies} = 10^{(Ct-b)/a} \tag{3}
\]

where \(a\) is the slope and \(b\) is the intercept of the regression line.

Since GCG is a single copy nuclear gene, the mtDNA copies per nuclear genome were calculated as follows:

\[
\text{mtDNA copies/nuclear genome} = \frac{\text{mtDNA copies}}{\text{nuclearDNA copies}} \tag{4}
\]

The mtDNA copy number per nuclear genome was calculated separately using ND1, ND2, and COX1. The data was reported as a mean.

Measurement of gene expression. High-throughput gene-expression analysis with EvaGreen dye on a BioMark HD real-time PCR system was used to measure gene expression according to manufacturer’s recommendations (Fluidigm, San Francisco, CA, USA). All reagents were purchased from Fluidigm unless otherwise indicated. Briefly, cDNA was synthesized from 2 \(\mu\)g of total RNA using Superscript II reverse transcriptase and oligo-dT (Invitrogen) with specific target amplification and exonuclease I (New England Biolabs) treatment. qPCR reactions were performed using a 96 \(\times\) 96 dynamic array and integrated fluidic circuit. Each sample inlet was loaded with 2.5 \(\mu\)L of 2 \(\times\) SsoFast EvaGreen supermix with low ROX (Biorad), 0.25 \(\mu\)L of 20 \(\times\) DNA-binding dye sample loading reagent, and 2.5 \(\mu\)L of specific target amplification and exonuclease-I-treated sample. Assays were performed for mitochondrial-coded complex I subunits ND1, ND2, ND3, and ND4, complex III subunit CYB, complex IV subunit COX1, ATP synthase subunit ATP6, and nuclear-encoded complex I subunits NDUF3, NDUF5, NDUF6, NDUF8, and NDUFV1, complex IV subunit COX7A2, ATP synthase subunits ATP5G1 and ATP5L, and master regulator PGC-1\(\alpha\). All measurements were performed in duplicate. Primer sequence information is available in Supplementary Table 4. Reference genes ACTB, RPL32, and RPS11 were used to normalize expression values.

Sequence analysis. DNA from muscle samples of 53 animals (Duroc: \(N = 15\), DuPi: \(N = 15\), PiNN: \(N = 9\), PiPP: \(N = 14\)) were sequenced using an ABI 3500 sequencer (Applied Biosystems Inc, Foster City, CA, USA). The D loop region was amplified using forward primer 5'‐GGCAGCCATCAGCACCCAAAG‐3' and reverse primer 5'‐GCACCTGTTTGGATTRTCG‐3'. All sequences were aligned using Clustal \(\times\) 2.1\(^14\), DNASP 5.1 software was used to analyze the haplotypes of all sequences\(^55\). The detailed information of haplotypes is shown in Supplementary Table 2. Only the haplotypes with at least three animals were included in the subsequent statistical analysis.

Statistical analysis. Data were analyzed using SAS 9.4 statistical software (SAS Institute) and the MIXED procedure. The statistical model included the effects of breed (Duroc, PiNN, PiPP, and DuPi), sex (male and female), time (0 and 30 min postmortem). With the same model, we also calculated with haplotype (Haplotypes 1, 4, 6, 7, and 8) instead of breeds. The model was combined with a repeated statement for the time component to take into account correlations among measurements made on the same animal at time 0 and 30 min postmortem.
Post hoc Tukey–Kramer method was used for multiple comparison adjustments. Results were reported as least-squares means (Lsmeans) with standard error (SE) and considered to be statistically significant if \( p < 0.05 \). Data were plotted using GraphPad Prism 5. The correlation coefficient (r) between gene expression and phenotypic measurement was calculated for all individuals. The correlation plots were generated in R.

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**Author Contributions**

X.L. analysed the data and drafted the manuscript; F.H. helped in analyzing duplicated region of the mitochondrial genome in the nuclear genome. N.T., E.M., and K.W. helped in sampling and data collection and drafting the manuscript; S.P. discussed and contributed to data interpretation and helped in drafting the manuscript. All authors have read and approved the final manuscript.

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