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A Mutation in *PRKAG3* Associated with Excess Glycogen Content in Pig Skeletal Muscle

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A high proportion of purebred Hampshire pigs carries the dominant RN^- mutation, which causes high glycogen content in skeletal muscle. The mutation has beneficial effects on meat content but detrimental effects on processing yield. Here, it is shown that the mutation is a nonconservative substitution (R200Q) in the *PRKAG3* gene, which encodes a muscle-specific isoform of the regulatory γ subunit of adenosine monophosphate–activated protein kinase (AMPK). Loss-of-function mutations in the homologous gene in yeast (*SNF4*) cause defects in glucose metabolism, including glycogen storage. Further analysis of the PRKAG3 signaling pathway may provide insights into muscle physiology as well as the pathogenesis of noninsulin-dependent diabetes mellitus in humans, a metabolic disorder associated with impaired glycogen synthesis.

The presence of a dominant mutation (denoted RN^-) in Hampshire pigs with large effects on meat quality and processing yield was first recognized by segregation analysis of phenotypic data (*1*). Meat from RN^- pigs has a low ultimate pH (measured 24 hours after slaughter), a reduced water-holding capacity, and gives a reduced yield of cured cooked ham (*2*, *3*). These effects are due to a ~ 70% increase in muscle glycogen content in RN^- (RN^-/rn^+ or RN^-/RN^-) animals. No pathological effects of the RN^- mutation have been reported, and it does not cause a glycogen storage disease. Because the RN^- allele has been found only in Hampshire pigs, it is likely that the mutation arose in this breed and has increased in frequency due to its favorable effects on growth rate and meat content in the carcass (*3*). The RN^- allele is of considerable economic significance in the pig breeding industry, and most breeding companies would like to eliminate the mutation because of its negative effects on processing yield.

To identify the RN^- mutation, which resides on pig chromosome 15 (*4*–*6*), we screened a porcine Bacterial Artificial Chromosome (BAC) library (*7*) and constructed a 2.5 megabase pair (Mbp) contig of the *RN* region (Fig. 1C). The BAC clones were used to develop new genetic markers in the form of microsatellites (MS) and single nucleotide polymorphisms (SNPs). The markers were used to construct a high-resolution linkage map based on 1019 informative meioses (*8*) (Fig. 1A). We could exclude *RN* from the region proximal to *SLC11A1* and distal to SNP *S1010*. A porcine radiation hybrid panel was exploited for high-resolution mapping of genetic markers and coding sequences (*9*) (Fig. 1B). The corresponding region on human chromosome 2q and mouse chromosome 1 did not contain any obvious candidate genes for *RN*. Linkage disequilibrium analysis indicated complete association between RN^- and marker alleles at *S1006* and *S1007* (Fig. 1D). These marker alleles most likely define the haplotype in which the RN^- mutation arose. The two markers are present on the overlapping BAC clones 127G6 and 134C9, suggesting that *RN* may reside on the same clone or on one of the neighboring clones.

A shotgun library of the BAC clone 127G6 was constructed and more than 1000 individual sequences were determined and assembled into contigs (*10*). BLAST (*11*) searches of the National Center for Biotechnology Information (NCBI) nucleotide database (*12*) yielded three convincing matches of coding sequences. Two of these were matched to human cDNA sequences or genes (*KIAA0173* and *CYP27A1*) but did not appear to be plausible candidate genes for *RN*. The third coding sequence in BAC 127G6 showed significant sequence similarity to AMP-activated protein kinase (AMPK) γ subunits, including Snf4 in yeast. AMPK has a key role in regulating energy metabolism in eukaryotic cells and is homologous to the SNF1 kinase in yeast (*13*, *14*). AMPK (SNF1) is composed of three subunits (the analogous designations in yeast are given in parentheses): the catalytic α chain (Snf1) and the two regulatory subunits β (Sip1, Sip2, and Gal83) and γ (Snf4). AMPK is activated by an increase in the ratio of AMP to adenosine triphosphate (AMP:ATP). Activated AMPK turns on ATP-producing pathways and inhibits ATP-consuming pathways. AMPK can also inactivate glycogen synthase, the key regulatory enzyme of glycogen synthesis, by phosphorylation (*13*). Several isoforms of the three different AMPK subunits are present in

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mammals. In humans, *PRKAA1* and *PRKAA2* encode α subunits, *PRKAB1* and *PRKAB2* encode β subunits, and *PRKAG1* and *PRKAG2* encode γ subunits (*15*). The AMPK γ chain gene's localization in the region showing maximum linkage disequilibrium and the putative function of its protein product made it a strong positional candidate gene for *RN*.

The cDNA sequence of *PRKAG3* was determined by reverse transcriptase–polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) analysis with pig muscle mRNA from a rn^+/rn^+ homozygote. A BLAST search revealed that this gene is distinct from mammalian *PRKAG1* and *PRKAG2* isoforms and orthologous to a human gene represented by the expressed sequence tag (EST) sequence AA178898 (GenBank). Most of the genomic sequence of this gene was recently released as part of a "working draft" sequence (GenBank AC009974) by the Genome Sequencing Center at the Washington University School of Medicine in St. Louis, Missouri. We suggest that this gene be denoted *PRKAG3* because it is the third isoform of a mammalian AMPK γ subunit. The cDNA sequence of human *PRKAG3* was also determined by RT-PCR and 5′ RACE analysis with human skeletal muscle cDNA (*16*) (Fig. 2A). Conceptual translation of the cDNA and subsequent sequence alignment (*17*) revealed a protein with two regions: residues 1 to 159 show 65% sequence identity between pig and human PRKAG3, whereas residues 160 to 464 show as much as 97% identity. No known protein domains were detected in the former region, whereas the latter contains four cystathionine beta–synthase (CBS) domains shared with other AMPK γ sequences (*18*) (Fig. 2A).

The candidate gene status of *PRKAG3* was further strengthened by analysis of human multiple tissue Northern blots. Whereas *PRKAG1* and *PRKAG2* were widely expressed, *PRKAG3* showed a distinct muscle-specific expression (*19*) (Fig. 2B). Consistent with this, ESTs representing *PRKAG1* and *PRKAG2* have been identified in various cDNA libraries, whereas a single *PRKAG3* EST (GenBank entry AA178898) has been found in a muscle cDNA library. The muscle-specific expression is consistent with the fact that RN^- animals show high glycogen content in skeletal muscle but not in liver (*2*).

The entire *PRKAG3* coding sequence was determined from one rn^+/rn^+ and one RN^-/RN^- homozygote by RT-PCR analysis. A total of seven nucleotide differences was found, four of which were nonsynonymous substitutions (Table 1). The screening of these seven SNPs with genomic DNA from additional rn^+ and RN^- pigs of different breeds revealed five different *PRKAG3* alleles, but only the R200Q substitution was exclusively associated with RN^- (Fig. 2C). The nonconservative R200Q substitution occurs in CBS1, which is the most conserved region among AMPK γ chain isoforms, and R200 is conserved in mammalian and *Drosophila* AMPK γ isoforms (Fig. 2A). Interestingly, homozygosity for a nonconservative substitution (D444N) at the corresponding position in the regulatory domain of CBS causes loss of S-adenosylmethionine regulation and homocystinuria in humans (*20*). The 200Q allele was found in all RN^- animals but not in any rn^+ animals from Hampshire or other breeds (*21*) (Table 2). This is consistent with the assumption that RN^- originated in the Hampshire breed.

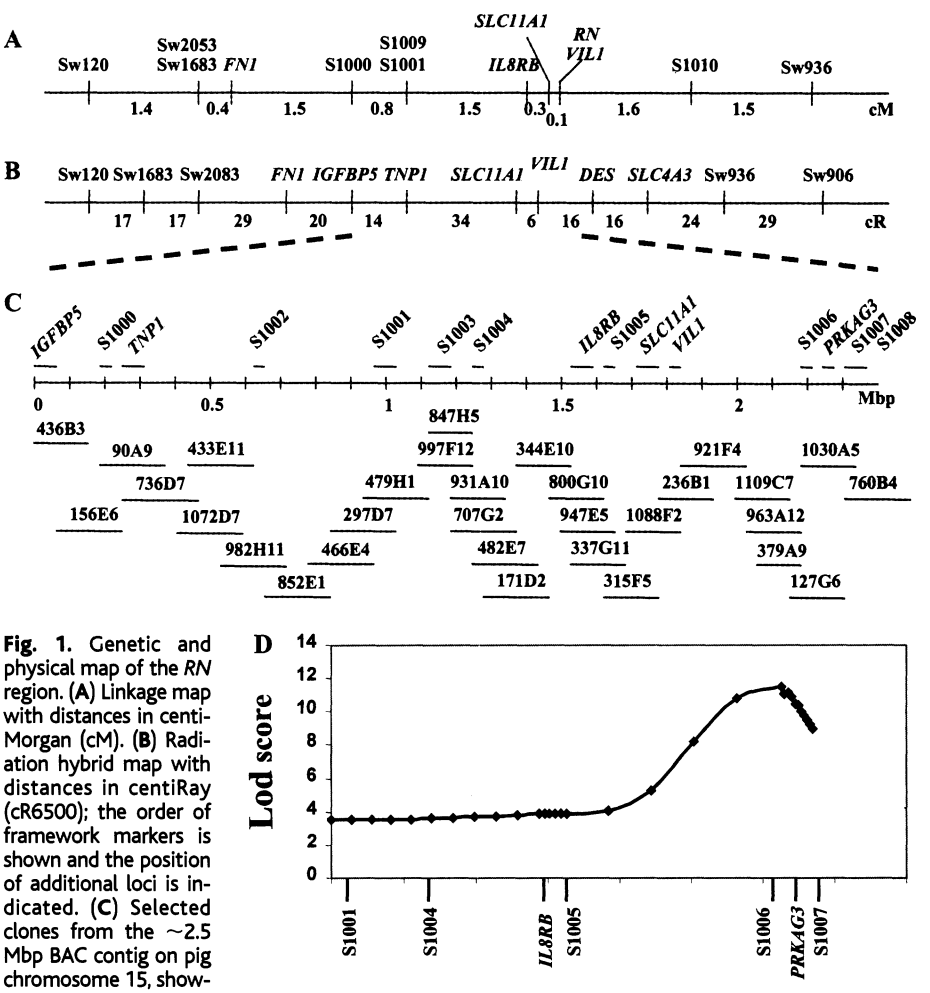

Fig. 1. Genetic and physical map of the *RN* region. (**A**) Linkage map with distances in centiMorgan (cM). (**B**) Radiation hybrid map with distances in centiRay (cR6500); the order of framework markers is shown and the position of additional loci is indicated. (**C**) Selected clones from the ~2.5 Mbp BAC contig on pig chromosome 15, showing the location of genes and genetic markers. (**D**) Linkage disequilibrium estimated with DISMULT (*39*) using a random sample of 91 Swedish Hampshire pigs.

Table 1. Comparison of *PRKAG3* sequences with associated rn^+ and RN^- alleles in parentheses for different pig populations (*37*). D, Duroc; H, Hampshire; L, Landrace; LW, Large White; M, Meishan; WB, Wild Boar. The numbering of codons differs slightly from the one in Fig. 2A, which is based on a multiple sequence alignment. Dash indicates identity to the top sequence.

PRKAG3 allele	Codon							Population
	30	53	193	194	199	200	372	
1 (RN^-)	ACC T	CTC L	GCC A	CTG L	GTC V	CAA Q	TCT S	H
2 (rn^+)	--- -	--- -	--- -	--- -	--- -	-G- R	--- -	L, LW, WB
3 (rn^+)	--- -	-C- P	--T -	T-- -	--- -	-G- R	--C -	H, L, LW, M, WB
4 (rn^+)	-A- N	-C- P	--T -	T-- -	--- -	-G- R	--C -	D, H
5 (rn^+)	--- -	-C- P	--T -	T-- -	A-- I	-G- R	--C -	H, LW, WB

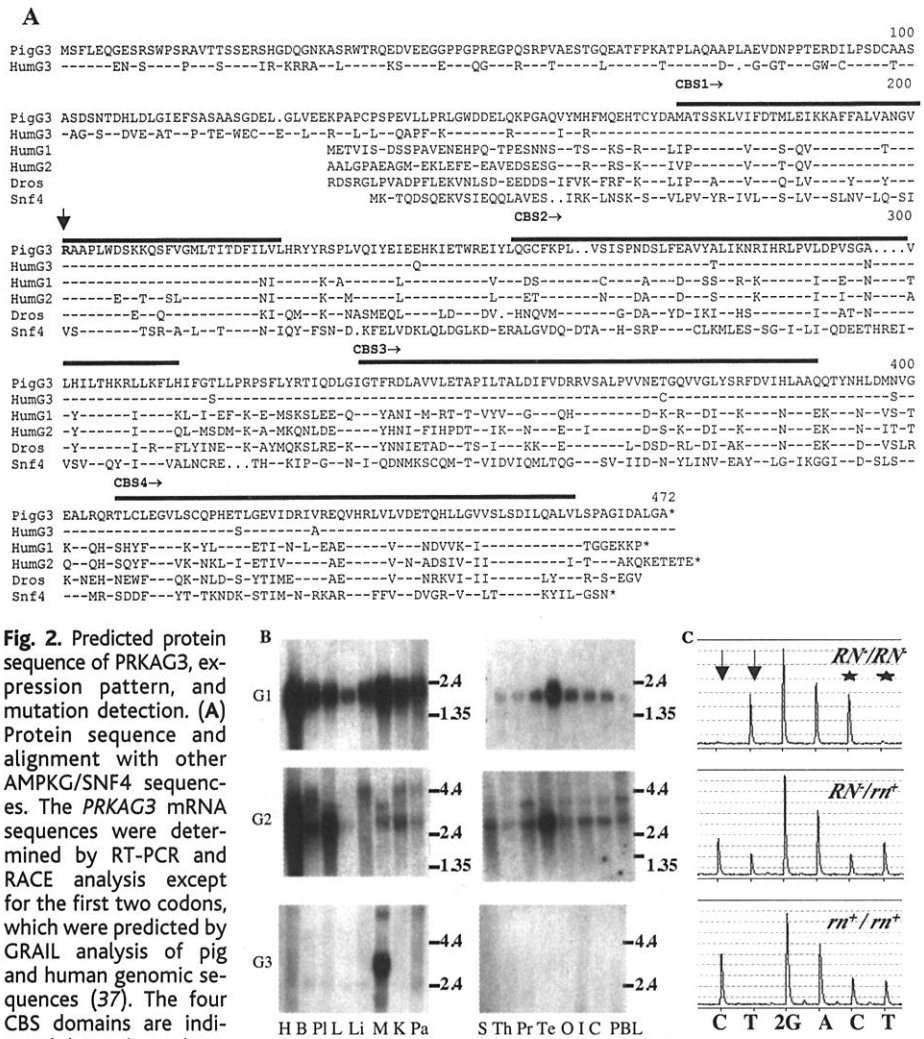

Fig. 2. Predicted protein sequence of PRKAG3, expression pattern, and mutation detection. (**A**) Protein sequence and alignment with other AMPKG/SNF4 sequences. The *PRKAG3* mRNA sequences were determined by RT-PCR and RACE analysis except for the first two codons, which were predicted by GRAIL analysis of pig and human genomic sequences (*37*). The four CBS domains are indicated by a line above the codons, and the *RN*$^-$ mutation at R200 is indicated by an arrow. PigG3, pig PRKAG3 (this study; AF214520); HumG3, human PRKAG3 (this study; AF214519); HumG1, Human PRKAG1 (U42412); HumG2, Human PRKAG2 (AJ249976); Dros, *Drosophila* (AF094764); Snf4, yeast (M30470); both the PRKAG2 and *Drosophila* sequences have longer NH_2-terminal regions, but they do not show significant similarity to the NH_2-terminal region of PRKAG3 and were not included. *, stop codon; –, identity to master sequence; ., alignment gap. (**B**) Northern blot analysis of human mRNA using human *PRKAG1*, human *PRKAG2*, and porcine *PRKAG3* probes. H, heart; B, brain; Pl, placenta; L, lung; Li, liver; M, skeletal muscle; K, kidney; Pa, pancreas; S, spleen; Th, thymus; Pr, prostate; Te, testis; O, ovary; I, small intestine; C, colon (mucosal lining); PBL, peripheral blood leukocyte. Size markers are in kilobases and are on the left. (**C**) Pyrosequencing (*40*) of the reverse strand of nucleotides 595 to 599 showing the sequence (C/T)GGA(C/T). The presumed causative SNP at nucleotide 599, which corresponds to codon 200, is marked by arrows, and the linked SNP at nucleotide 595 is marked by stars. Pyrosequencing was performed with a Luc 96 instrument (Pyrosequencing AB, Uppsala, Sweden).

Table 2. Association between the *PRKAG3* R200Q mutation and *RN* alleles among unrelated pigs from different breeds.

RN phenotype*	Genotype at nucleotide 599			
	A/A	A/G	G/G	Total
RN$^-$, Hampshire	40	87	(1)†	127
rn$^+$, Hampshire	0	0	60	60
rn$^+$, other breeds	0	0	488	488

*The RN phenotype was determined by measuring glycogen content in skeletal muscle as previously described (*4–6*). The Hampshire animals represent both Swedish Hampshire and French lines founded in part by Hampshire animals. Other breeds: Angler Saddleback, n = 31; Blond Mangalitza, n = 2; Bunte Bentheimer, n = 16; Duroc, n = 160; Göttinger Minipig, n = 4; Landrace, n = 83; Large White, n = 72; Meishan, n = 8; Piétrain, n = 75; Mangalitza, n = 12; Rotbunte Husumer, n = 15; Schwäbisch Hällische, n = 2; European Wild Boar, n = 5; Japanese Wild Boar, n = 3. †This rare exception to the complete association between the R200Q mutation and the RN$^-$ phenotype was not unexpected because RN phenotyping is not 100% accurate. Haplotype analysis with closely linked markers did not indicate that this animal carried the *RN*$^-$ allele.

Table 3. AMPK activity in muscle extracts from rn$^+$ and RN$^-$ pigs. Phosphorylation was measured as counts per minute of incorporated ^{32}P/0.1 μl muscle extract (*38*). Least square means ± standard errors are reported; the significance values were obtained by an F-test with a linear regression model; n, number of animals.

RN type	n	Kinase activity	
		−AMP	+AMP
rn$^+$	4	955 ± 134	1519 ± 323
RN$^-$	5	380 ± 122	531 ± 292
Significance		0.02	0.06

Functional characterization of the *RN*$^-$ mutation is complicated by the fact that it occurs in a regulatory subunit and by the expression of several AMPK isoforms in skeletal muscle whose functional differences have not yet been established (*22*). On the basis of the established roles of yeast SNF1 in glycogen utilization and of mammalian AMPK in regulation of energy metabolism, activated AMPK is expected to inhibit glycogen synthesis and stimulate glycogen degradation. Furthermore, AMPK activation in muscle leads to translocation of glucose transporter 4 (GLUT4) from an intracellular location to the plasma membrane, increased glucose uptake, and increased glycogen content in skeletal muscle (*23–26*). We found that AMPK kinase activity in muscle extracts was about three times higher in normal rn$^+$ pigs than in RN$^-$ pigs, both in the presence and absence of AMP (Table 3). Thus, R200Q may be a dominant negative mutation inhibiting AMP activation and glycogen degradation, but only if it interferes with multiple isoforms because the major AMPK activity in muscle appears to be associated with the PRKAG1 and 2 isoforms (*22*). Alternatively, it may be a gain-of-function mutation constitutively activating the holoenzyme, leading to an increased glucose transport and/or glycogen synthesis. If so, the reduced AMPK activity in RN$^-$ animals is likely to reflect feedback inhibition due to the high-energy status of the muscle. More detailed functional studies are needed to distinguish between these possibilities.

The distinct phenotype of the *RN*$^-$ mutation indicates that PRKAG3 plays a key role in the regulation of energy metabolism in skeletal muscle. Further characterization of PRKAG3 may shed new light on muscle physiology including the adaptation to physical exercise, which is associated with increased glycogen storage (*27*). Finally, it will be of interest to determine whether *PRKAG3* or other AMPK genes are involved in the pathogenesis of noninsulin-dependent diabetes mellitus, a disorder associated with impaired glycogen synthesis.

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9. Radiation hybrid (RH) mapping was performed with the IMpRH panel (*29*) and the RHMAP 3.0 software (*30*).
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38. Muscle tissue was collected from four rn^+ (rn^+/rn^+) and five RN^- (two RN^-/rn^+ and three RN^-/RN^-) pigs. Muscle extract was purified up to and including the DEAE-Sepharose ion-exchange step as described (*35*). AMPK activity was assayed by phosphorylation of a synthetic peptide, HMRSAMSGLHLVKRR (*36*, *37*). Assays were performed in triplicate for each animal in the presence and in the absence of 200 μM AMP. Because 0.1 μg of total protein from the 0.2 M NaCl DEAE eluates showed a linear activity both in the presence and absence of added AMP for at least 12 min (D. Milan *et al.*, data not shown), we used this amount of extract and an incubation time of 10 min in the experiments. For each assay, we included controls that lacked added peptide. The values presented represent peptide-dependent activity.
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High Frequency of Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Lung Infection

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The lungs of cystic fibrosis (CF) patients are chronically infected for years by one or a few lineages of *Pseudomonas aeruginosa*. These bacterial populations adapt to the highly compartmentalized and anatomically deteriorating lung environment of CF patients, as well as to the challenges of the immune defenses and antibiotic therapy. These selective conditions are precisely those that recent theoretical studies predict for the evolution of mechanisms that augment the rate of variation. Determination of spontaneous mutation rates in 128 *P. aeruginosa* isolates from 30 CF patients revealed that 36% of the patients were colonized by a hypermutable (mutator) strain that persisted for years in most patients. Mutator strains were not found in 75 non-CF patients acutely infected with *P. aeruginosa*. This investigation also reveals a link between high mutation rates in vivo and the evolution of antibiotic resistance.

Cystic fibrosis is a human genetic disorder caused by mutations in the CF-transmembrane conductance regulator (*1*). Mutations in the gene encoding this protein disrupt electrolyte secretion, leading to a hyperosmolar viscous mucus (*2*). The main mechanisms of lung defense against bacterial colonization are mucociliary clearance, polymorphonuclear neutrophil phagocytosis, and local production of antibacterial cationic peptides. These systems of defense are poorly effective under conditions of increased viscosity and osmolarity, resulting in chronic lung infection, most frequently by *Pseudomonas aeruginosa*, the major cause of morbidity and mortality in CF patients (*3*, *4*).

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