

Regulation of Skeletal Muscle Oxidative Capacity and Insulin Signaling by the Mitochondrial Rhomboid Protease PARL

Anthony E. Civitaresse,^{1,2,9,*} Paul S. MacLean,³ Stacy Carling,¹ Lyndal Kerr-Bayles,⁴ Ryan P. McMillan,⁶ Anson Pierce,⁷ Thomas C. Becker,⁸ Cedric Moro,¹ Jean Finlayson,² Natalie Lefort,² Christopher B. Newgard,⁸ Lawrence Mandarino,^{2,9} William Cefalu,¹ Ken Walder,⁴ Greg R. Collier,^{4,5} Matthew W. Hulver,⁶ Steven R. Smith,¹ and Eric Ravussin¹

¹Pennington Biomedical Research Center, Baton Rouge, LA 70808, USA

²Arizona State University, Tempe, AZ 85287, USA

³University of Colorado, Denver, CO 80045, USA

⁴Deakin University

⁵ChemGenex Pharmaceuticals

Geelong VIC 3220, Australia

⁶Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

⁷Barshop Institute for Longevity and Aging Studies, San Antonio, TX 78245, USA

⁸Duke University, Durham, NC 27708, USA

⁹Mayo Clinic, Department of Medicine, Scottsdale, AZ 85259, USA

*Correspondence: civitaee@pbrc.edu

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SUMMARY

Type 2 diabetes mellitus (T2DM) and aging are characterized by insulin resistance and impaired mitochondrial energetics. In lower organisms, remodeling by the protease *pcp1* (PARL ortholog) maintains the function and lifecycle of mitochondria. We examined whether variation in PARL protein content is associated with mitochondrial abnormalities and insulin resistance. PARL mRNA and mitochondrial mass were both reduced in elderly subjects and in subjects with T2DM. Muscle knockdown of PARL in mice resulted in malformed mitochondrial cristae, lower mitochondrial content, decreased PGC1 α protein levels, and impaired insulin signaling. Suppression of PARL protein in healthy myotubes lowered mitochondrial mass and insulin-stimulated glycogen synthesis and increased reactive oxygen species production. We propose that lower PARL expression may contribute to the mitochondrial abnormalities seen in aging and T2DM.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is the most common metabolic disease in the elderly (King et al., 1998) and is one of the leading causes of death in the U.S. (Marx, 2002). T2DM is projected to afflict more than 300 million individuals worldwide by the year 2025 (Zimmet et al., 2001). Although the primary cause of T2DM is unknown, it is clear that insulin resistance in skeletal muscle and liver plays a primary role in its pathogenesis before the progressive failure of pancreatic β cells (Lillioja et al., 1993). Studies of the etiology of insulin resistance in skeletal muscle

have implicated numerous factors including reduced mitochondrial mass (Kelley et al., 2002), lower mitochondrial oxidative phosphorylation (Befroy et al., 2007; Petersen et al., 2005), blunted lipid oxidation (Zhang et al., 2007), and elevated reactive oxygen species (ROS) production (Civitaresse et al., 2006; Patti et al., 2003; Petersen et al., 2004; Ukropcova et al., 2007). However, the molecular mechanism(s) governing mitochondrial dysfunction and insulin resistance in skeletal muscle are not completely understood.

Mitochondria carry out a vast array of cellular functions, including ATP production, biosynthesis of amino acids and lipids, cytosolic calcium transport (Frazier et al., 2006), and amplification of apoptotic stimuli (Cipolat et al., 2004). Part of the functional diversity and variability in mitochondrion ultrastructure is mediated by the interaction with the cytoskeleton (Hollenbeck and Saxton, 2005) and the balance between the forces of mitochondrial fusion and fission. In lower organisms, mitochondrial fusion is regulated, in part, by the evolutionary conserved GTPase protein *mgm1* (Herlan et al., 2003), the human ortholog of which is optic atrophy 1 (OPA1). Mutations in the OPA1 gene are the most common cause of autosomal-dominant optic atrophy in humans (Alexander et al., 2000), a progressive disease of the retinal ganglion. *Mgm1*/OPA1 participates in several biological processes, most notably fusion of the inner mitochondrial membrane, remodeling of the mitochondrial cristae (Cipolat et al., 2004), inhibition of cytochrome c release, and apoptosis (Cipolat et al., 2006). In budding yeast (McQuibban et al., 2003) and in mice (Cipolat et al., 2006) *mgm1*, is cleaved by the inner mitochondrial membrane rhomboid protease, *pcp1*. Yeast strains lacking *pcp1* exhibit decreased oxidative capacity, impaired growth, and fragmented mitochondria (McQuibban et al., 2003; Sesaki et al., 2003). Importantly, insertion of the human “presenilin-associated rhomboid-like” (PARL) gene (the *pcp1* human ortholog) into *pcp1*-deficient yeast rescues growth and restores mitochondrial morphology, suggesting that PARL may play a similar role in humans (McQuibban et al., 2003).

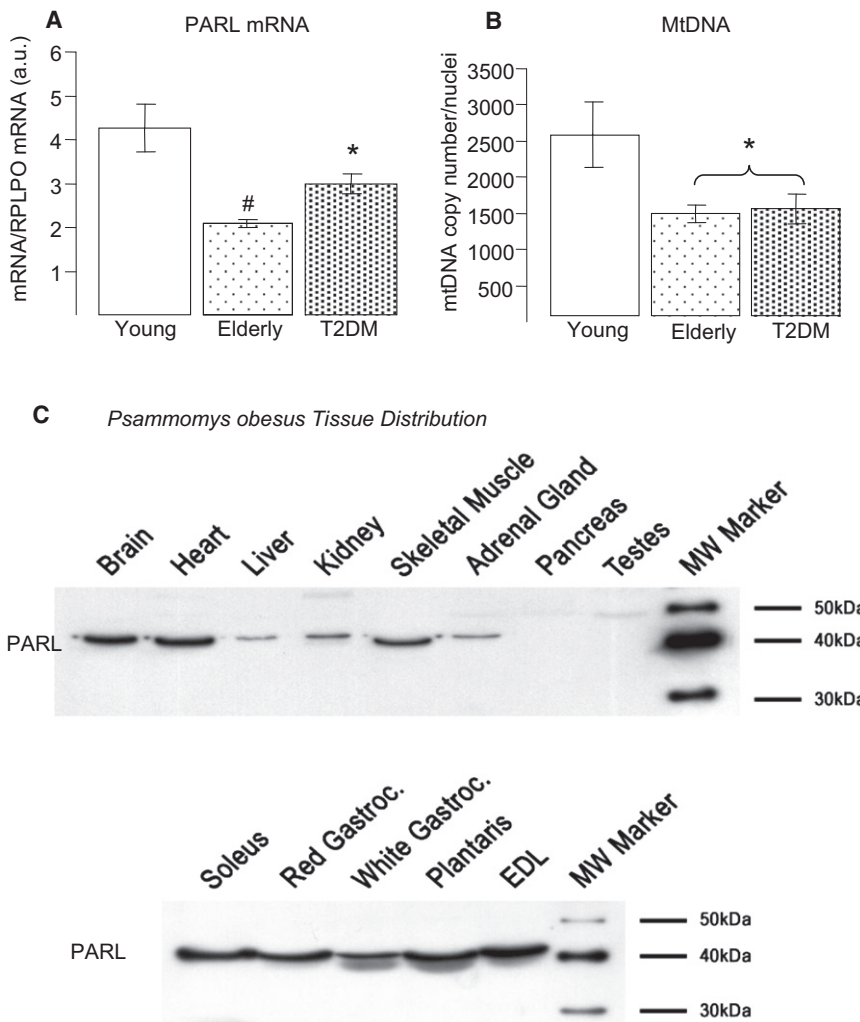


Figure 1. Human mRNA Expression and Rodent Tissue Distribution

(A and B) Presenillin-associated rhomboid-like (PARL) mRNA and mitochondrial content by mtDNA copy number (mtDNA) in lean-young ($n = 11$) and lean-insulin-resistant elderly subjects ($n = 11$) and participants with type 2 diabetes mellitus (T2DM) ($n = 32$). Data represent mean \pm SEM. (C) Western analyses with affinity-purified anti-PARL antibodies in *Psammomys obesus*. PARL protein expression is most abundant in tissues that rely heavily on oxidative metabolism (top). PARL protein expression is greater in muscle groups, including soleus and the red gastrocnemius, that contain a higher proportion of oxidative fibers (bottom). Polyclonal antisera were generated using a KLH-conjugated peptide corresponding to amino acids 53–77 of human PARL. PARL-specific antibodies were affinity purified using GST-PARL (53–77).

We have previously shown that PARL mRNA is reduced in skeletal muscle of obese diabetic *Psammomys obesus* and is positively correlated with insulin sensitivity in humans (Walder et al., 2005). Given the large body of accumulating evidence from the past decade demonstrating impaired mitochondrial energetics in states of insulin resistance, we hypothesized that dysregulation of PARL contributes to the defective lipid and glucose metabolism frequently seen in T2DM and aging.

RESULTS

PARL mRNA Is Associated with Reduced Mitochondrial Mass in T2DM and Aging

To investigate whether variation in PARL mRNA expression was associated with mitochondrial dysfunction and insulin resistance in humans, we studied healthy, young individuals (23 ± 1 years old; $n = 11$) and older subjects (64.4 ± 1.2 years old; $n = 11$) matched for body composition, plasma-free fatty acids, and glucose concentrations, as well as physical activity levels (Table S1 available online). Relative to younger participants, elderly individuals had elevated plasma insulin ($p < 0.01$), lower insulin

sensitivity ($p < 0.01$), and a 19% reduction in resting metabolic rate (RMR; $p < 0.01$) (Table S1). Analysis of the skeletal muscle transcriptome from muscle biopsies collected under fasting conditions revealed lower mRNA content of peroxisome proliferator-activated receptor γ coactivator β (PGC1 β), nuclear respiratory factor (NRF1), and mitochondrial transcription factor A (TFAM) in elderly individuals compared to young controls, suggesting a reduction in mitochondrial number and function (Table S2). Consistent with these observations, PARL mRNA, mtDNA (Figures 1A and 1B), and citrate synthase activity (Table S2) (the latter two being markers of mitochondrial content; Wang et al., 1999) were reduced in elderly subjects by 51%, 48%, and 33%, respectively (all $p < 0.05$). In addition, PARL mRNA was strongly correlated with TFAM mRNA ($r = 0.87$; $p < 0.001$) and mtDNA content ($r = 0.61$; $p = 0.001$), suggesting that PARL may be involved in the regulation of mitochondrial mass.

Functional studies by Petersen et al. (2003) and Conley et al. (2007) have observed reduced resting rates of ATP synthesis in elderly subjects with insulin resistance, suggesting that mitochondrial dysfunction with aging may be relevant to mitochondrial dysfunction seen in T2DM (Petersen et al., 2005). We therefore next identified that PARL mRNA and mtDNA content were reduced in subjects with T2DM relative to lean healthy controls (Figures 1A and 1B; $p < 0.05$) and in Pima Indians with a family history of T2DM (FH⁺; $n = 14$) compared to those without a family history (FH⁻; $n = 7$) (50 ± 6 versus 86 ± 15 PARL mRNA/cyclophilin mRNA; $p = 0.05$). Collectively, these observations establish that PARL mRNA and mitochondrial content in skeletal muscle are reduced in subjects with a genetic predisposition to develop T2DM, those presenting with T2DM, and in insulin-resistant elderly individuals.

mCK-CAR-PARL(-) Transgenic Mice Have Reduced Mitochondrial Mass in Muscle

Tissues with high demand for aerobic respiration, such as the brain, skeletal muscle, and heart, have the most prominent requirement for mitochondrial reticulum networks (Bach et al., 2003; Kirkwood et al., 1986). Accordingly, in *Psammomys obesus*, PARL protein was highly expressed in the brain, heart, and skeletal muscle and predominantly in oxidative muscle fibers of lean healthy animals (Figure 1C). Similarly, in humans, PARL mRNA is mainly expressed in skeletal muscle, brain, placenta, testis, and heart (Figure S1A). Given that skeletal muscle is the major site of insulin-stimulated glucose disposal and, therefore, a key tissue in the development of whole-body insulin resistance (Petersen et al., 2005), we set out to examine the functional consequences of reduced PARL protein on mitochondrial function and glucose metabolism in the skeletal muscle of a transgenic rodent model.

Mice expressing the human coxsackie/adenovirus receptor (CAR) under the control of the muscle creatine kinase promoter (mCK) were used to create mCK-CAR-PARL(-) mice muscle, herein referred to as PARL(-) mice. mCK-CAR mice have a dramatic enhancement of skeletal muscle transduction with site-specific injections of AdV-shuttle because they transgenetically overexpress CAR on mature myofibers (Nalbantoglu et al., 2001). We treated mCK-CAR mice (16 weeks of age; $n = 7$) with a single intramuscular transverse injection into each lobe of the gastrocnemius (gastroc) and tibialis anterior/extensor digitorum longus (TA/EDL) muscle groups with AdV-PARL(-) siRNA injected into the left leg and an “empty” vector with a GFP expression cassette in the right leg to serve as a collateral control (Figure S1B). Analysis of PARL expression in the TA/EDL muscle of the PARL(-) leg revealed lower PARL protein that was associated with reduced PGC1 α and CS protein levels relative to the control leg (mCK-GFP) (Figure 2A). Similar reductions in PARL protein were observed in gastroc muscle (data not shown). MtDNA (Figure 2D; $p = 0.03$) content and CS activity (Figure 2E; $p = 0.007$) were lower in PARL(-) muscle relative to GFP control muscle. Thus, it appears that mitochondrial content is affected by PARL protein content in muscle.

The key regulators of mitochondrial fusion, mitofusin 1 and 2 (Mfn1/2) and OPA1 (long and short isoform of OPA1; Guillery et al., 2008) were examined next. MFN2 and OPA1 were lower in PARL(-) muscle, whereas MFN1 was unchanged (Figure 2A). The reductions in OPA1 protein are consistent with previous reports demonstrating enhanced degradation of OPA1 protein (constitutive cleavage) and increased susceptibility to apoptosis (Cipolat et al., 2006; Griparic et al., 2007) with reduced PARL and DRP-1 (Mopert et al., 2009) expression. Therefore, lower OPA1 protein may result from reduced mitochondrial mass or dysfunction as observed in PARL loss-of-function myotubes (see below). OPA1 functions include mitochondrial fusion and mitochondrial cristae remodeling (Cipolat et al., 2006; Griparic et al., 2007). To determine the effects of PARL depletion on cellular morphology, we examined mCK-CAR mice muscle by transmission electron microscopy. Electron micrographs demonstrated normal mitochondrial distribution (Figures 3A and 3B) in both PARL(-) and GFP mice muscle; however, in PARL(-) muscle (Figure 3C), mitochondrial cristae appeared “disorganized,” with some mitochondria totally lacking clearly defined cristae.

This mitochondrial phenotype is characteristic of HeLa cells (Griparic et al., 2007) and “class II” remodeled mitochondria from mouse embryonic fibroblasts depleted of OPA1 (Frezza et al., 2006). We also observed increased activity of the pro-apoptotic protein caspase-9 in PARL(-) TA/EDL muscle (1.8 ± 0.4 versus 2.9 ± 0.6 rfu/mg protein; $p = 0.048$), consistent with a role for PARL in the antiapoptotic function of OPA1 (Frezza et al., 2006). Of interest, global PARL knockout mice display normal intrauterine development but from the fourth postnatal week undergo progressive multisystemic atrophy leading to accelerated cellular apoptosis and cachectic death (Cipolat et al., 2006). Skeletal muscle loss and the development of sarcopenia is a hallmark feature of the aging process and is associated with elevated levels of apoptosis (Whitman et al., 2005), suggesting that lower PARL protein may be a contributing factor to muscle atrophy during aging.

The inner mitochondrial membrane cristae are easily damaged by ROS as a large portion of protein complexes associated with oxidative phosphorylation are embedded in this membrane (Gilkerson et al., 2003). Analysis of cysteine oxidation was performed by measuring protein disulfides and TBARS concentration (marker of lipid peroxidation) in PARL(-) muscle and revealed elevated oxidative damage relative to the control muscle (Figures 2F and 2G; $p < 0.05$). Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen and is considered to be one of the primary antioxidant responses to elevated ROS production (Luo et al., 2006). SOD protein was elevated in the muscle of PARL(-) muscle when compared to control muscle, whereas there was no difference in glutathione peroxidase content (Figure 2A). Thus, PARL protein appears to influence OPA1 protein levels, and a low level of PARL protein expression is associated with increased oxidative damage in skeletal muscle. Importantly, reduced muscle PARL protein content results in abnormal mitochondrial morphology, consistent with the mitochondrial structural abnormalities observed in skeletal muscle of subjects with T2DM (Kelley et al., 2002; Ritov et al., 2005).

PARL(-) Mouse Muscle Has Elevated Lipid Content and Impaired Insulin Signaling

Insulin-resistant individuals have excess triglyceride and intramyocellular lipid (IMCL) accumulation (Forouhi et al., 1999; Jacob et al., 1999; Krssak et al., 1999; Perseghin et al., 1999) in skeletal muscle (Goodpaster et al., 2000a, 2000b; Pan et al., 1997) that has been postulated to accumulate, in part, due to deficient mitochondrial oxidative capacity (Befroy et al., 2007; McGarry, 1992; Morino et al., 2005; Petersen et al., 2003, 2005; Zhang et al., 2007). We next measured the triglyceride content in PARL(-) mice muscle homogenates. PARL(-) muscle had elevated triglyceride content relative to the GFP null muscle, although this did not reach significance (65 ± 15 versus 48 ± 9 $\mu\text{M}/\text{mg}$ protein; $p = 0.077$). Several groups have shown that the accretion of long-chain fatty acyl-CoAs (LCFCoA), diacylglycerols (DAG), and ceramides (Adams et al., 2004, 2009; Chavez and Summers, 2003; Yu et al., 2002) is more closely associated with impaired insulin signaling than triglycerides or IMCL per se. Among these bioactive lipid intermediates, there is evidence to support a direct role for fatty acyl CoAs in the

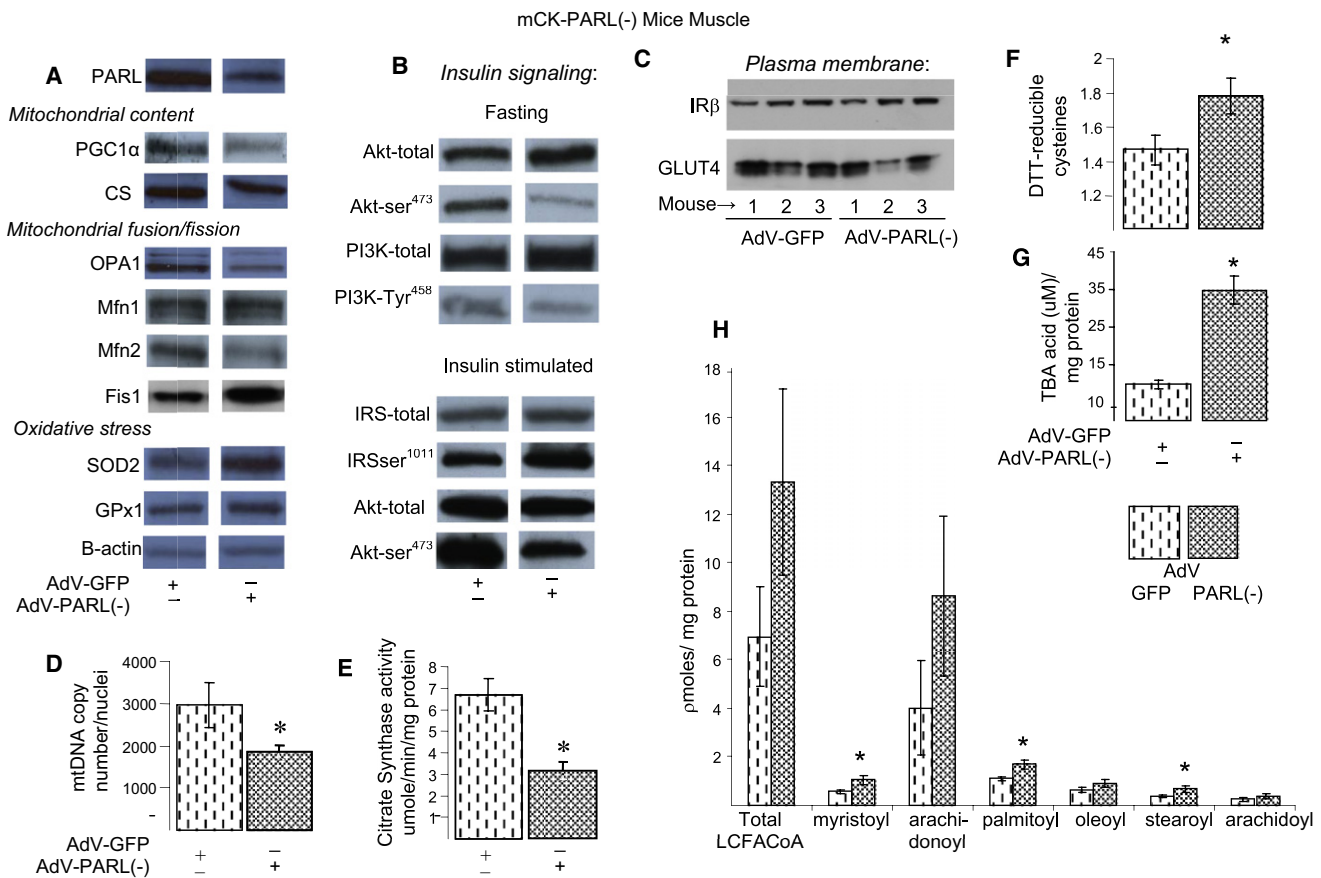


Figure 2. mCK-PARL(-) Mice Muscle

Molecular characterization of 12-week-old mice expressing the human coxsackie/adenovirus (CAR) receptor under the control of the muscle creatine kinase promoter (mCK) treated with either adenovirus (AdV)-GFP-empty cassette control in the left muscle and AdV-PARL-siRNA in the collateral leg to create mCK-CAR-PARL(-) muscle.

(A) Total amount of PARL, peroxisome proliferative-activated receptor γ coactivator 1 α (PGC1 α), citrate synthase (CS), optic atrophy 1 (OPA1), Mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), fission 1 (Fis1), superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPx1) and β -actin protein in the AdV-GFP and PARL(-) muscles. (B) Phosphorylation and total amount of protein of key insulin-signaling cascade proteins in AdV-GFP and PARL(-) muscles in the fasting (top) and insulin-stimulated condition (bottom).

(C) Total amount of insulin receptor β (IR β) and GLUT4 protein in the plasma membrane fraction of AdV-GFP and PARL(-) muscles.

(D and E) MtDNA (D) and citrate synthase (CS) activity (E) in AdV-GFP and PARL(-) muscles.

(F) Measurements of irreversible cysteine oxidation using dithiothreitol-reducible cysteines with 6-iodoacetimido-fluorescein. A low value equates to elevated rates of irreversible cysteine oxidation, whereas a high value implies a very low level of cysteine oxidation.

(G) Measurement of thiobarbituric acid (TBA)-reactive substances for estimate of muscle lipid peroxidation.

(H) Measurement of long-chain fatty acyl-CoA using high-performance liquid chromatography. Total amount of saturated long-chain fatty acyl-CoAs ($p = 0.16$), myristoyl-CoA (14:0; $p = 0.031$), arachidonoyl-CoA (20:4; $p = 0.25$), palmitoyl-CoA (16:0; $p = 0.016$), oleoyl-CoA (18:1; $p = 0.17$), stearoyl-CoA (18:0; $p = 0.004$), and arachidoyl-CoA (20:0; $p = 0.4$) in GFP and PARL(-) muscle.

(A-E) and (H) $n = 6$; (F and G) $n = 5$. (D-G) Data represent mean \pm SEM. * $p < 0.05$.

resistance to insulin action (Adams et al., 2004; Chavez and Summers, 2003; Yu et al., 2002). Consistently, knockdown of PARL protein in skeletal muscle resulted in increased concentration of all fatty acyl CoAs examined (Figure 2H, total fatty acyl CoAs). In particular, the saturated long-chain myristoyl-CoA (14:0; $p = 0.031$), palmitoyl-CoA (16:0; $p = 0.016$), and stearoyl-CoA (18:0; $p = 0.004$) were all elevated in PARL(-) muscle relative to the collateral GFP control.

We then tested whether elevated LCFCoA concentration in PARL(-) muscle was associated with impaired insulin signaling. Animal models and in vitro experiments have shown that the

phosphorylation of serine residues (³⁰⁷, ⁶³⁶, ⁶³⁹, and ¹¹⁰¹) of insulin-receptor substrate-1 (IRS-1) blocks the activity of IRS-1 on critical tyrosine sites that are required for phosphatidylinositol 3-kinase (PI3K) activation (at phosphorylation site PI3K p85-Tyr⁴⁵⁸; Luo et al., 2007; Yi et al., 2007). Downstream of PI3K, Akt phosphorylation at Ser⁴⁷³ has been shown to be a key step for the activation of glucose transport in skeletal muscle (Morino et al., 2005). In fasting conditions, we observed reduced basal phosphorylation of PI3K^{-Tyr⁴⁵⁸} and Akt-Ser⁴⁷³ in PARL(-) TA/EDL muscle relative to control muscle (Figure 2B). In the insulin-stimulated state, phosphorylation at IRS-1^{Ser-1101} was

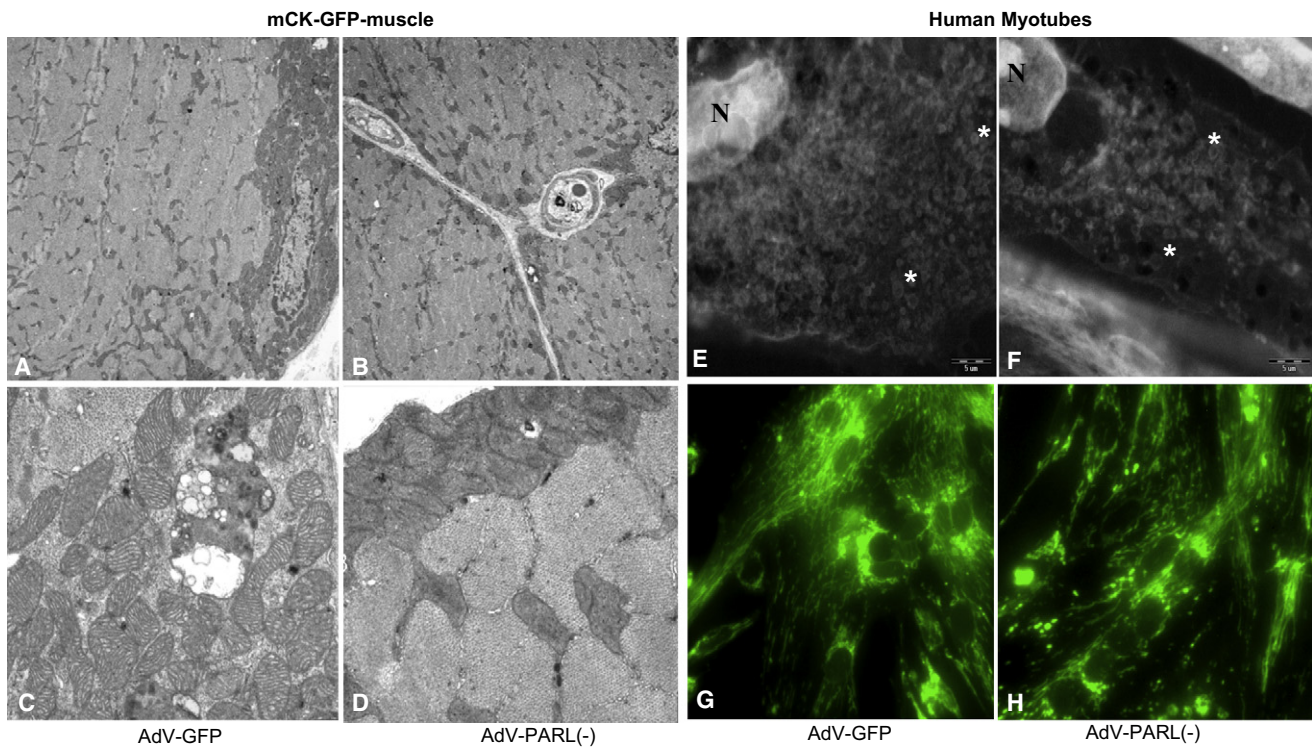


Figure 3. Mitochondrial and Cellular Imaging

(A–D) Representative transmission electron microscopy of cross-sectional slice of AdV-GFP (A and C) and PARL(–) (B and D) muscles at low ($\times 4000$, top two images) and high ($\times 20,000$, bottom two images) magnification are shown.

(E–H) Effects of PARL protein depletion on mitochondrial distribution in primary skeletal muscle cells derived from lean-young subjects and transduced with AdV-GFP control and AdV-PARL-siRNA.

(E and F) Wet-scanning electron microscopy analysis of AdV-GFP-depleted (E) and AdV-PARL(–)-depleted (F) myotubes. N, nucleus. Scale bar, 5 μm .

(G and H) Mitochondrial distribution in AdV-GFP (G) and AdV-PARL(–) (H) myotubes measured using MTG. Respiring myotubes were incubated with 100 nM MTG for 30 min.

Images were acquired within 5 min using a conventional wide-field microscope fitted with a $\times 40$ Nikon plan-apo objective. $n = 4$.

elevated in PARL(–) muscle relative to control, in parallel with lower insulin-stimulated Akt^{Ser473} phosphorylation (Figure 2B). We next isolated the cytosolic and plasma membrane fraction of PARL(–) and AdV-GFP red (oxidative) gastrocnemius muscle under insulin-stimulated conditions. No change was observed in the plasma membrane content of the insulin receptor- β between PARL(–) and GFP control muscle (Figure 2C). However, reductions in muscle PARL protein resulted in lower insulin-stimulated translocation of GLUT4 protein to the plasma membrane (Figure 2C). Together, these data indicate that skeletal muscle-specific reduction of PARL protein results in elevated saturated long-chain fatty acyl CoAs, impaired insulin signaling, and reduced GLUT4 translocation to the plasma membrane.

HFD Lowers Muscle PARL and PGC1 α mRNA, along with Glucose Tolerance in Mice

Impairments in mitochondria transcription and function (Civitarese et al., 2006; Patti et al., 2003; Petersen et al., 2004), along with lower PARL mRNA (see above), are present in the prediabetic state, suggesting that PARL and mitochondrial abnormalities may be primary factors in the development of skeletal muscle insulin resistance. Because a high-fat diet (HFD) causes insulin resistance and a decrease in the expression of multiple

genes involved in mitochondrial biogenesis and oxidative phosphorylation (Koves et al., 2008; Sparks et al., 2005, 2006; Tunstall and Cameron-Smith, 2005), we examined whether the regulation of PARL transcription precedes or coincides with changes in glucose tolerance, mtDNA, fusion, and fission gene expression in C57Bl6/J mice fed a HFD for 4 or 10 weeks. After 4 weeks of the HFD, there was no difference in fasting glucose levels between groups; however, blood glucose levels were significantly elevated in mice fed a HFD for 10 weeks (Table S3; $p < 0.05$). By 4 weeks, HFD mice had glucose intolerance and resistance to the glucose-lowering effects of insulin that worsened by the end of the study (Table S3). Glucose homeostasis remained normal in control diet mice.

Despite the insulin resistance in HFD mice, there was no change in the expression of the majority of genes examined at 4 weeks (Table S3). DRP1 was the only gene modified, with an upregulation in HFD group (Table S3; $p < 0.05$). The increase in DRP1 expression in the HFD mice may relate to the role of DRP1 in the tethering and biogenesis of peroxisomes (Koch et al., 2003) that participate in the β oxidation of fatty acids and the adaptation to nutritional stimuli that elevates lipid flux (Reddy and Mannaerts, 1994). After 10 weeks of HFD, there was a down-regulation in mtDNA that was mirrored by a suppression of PARL

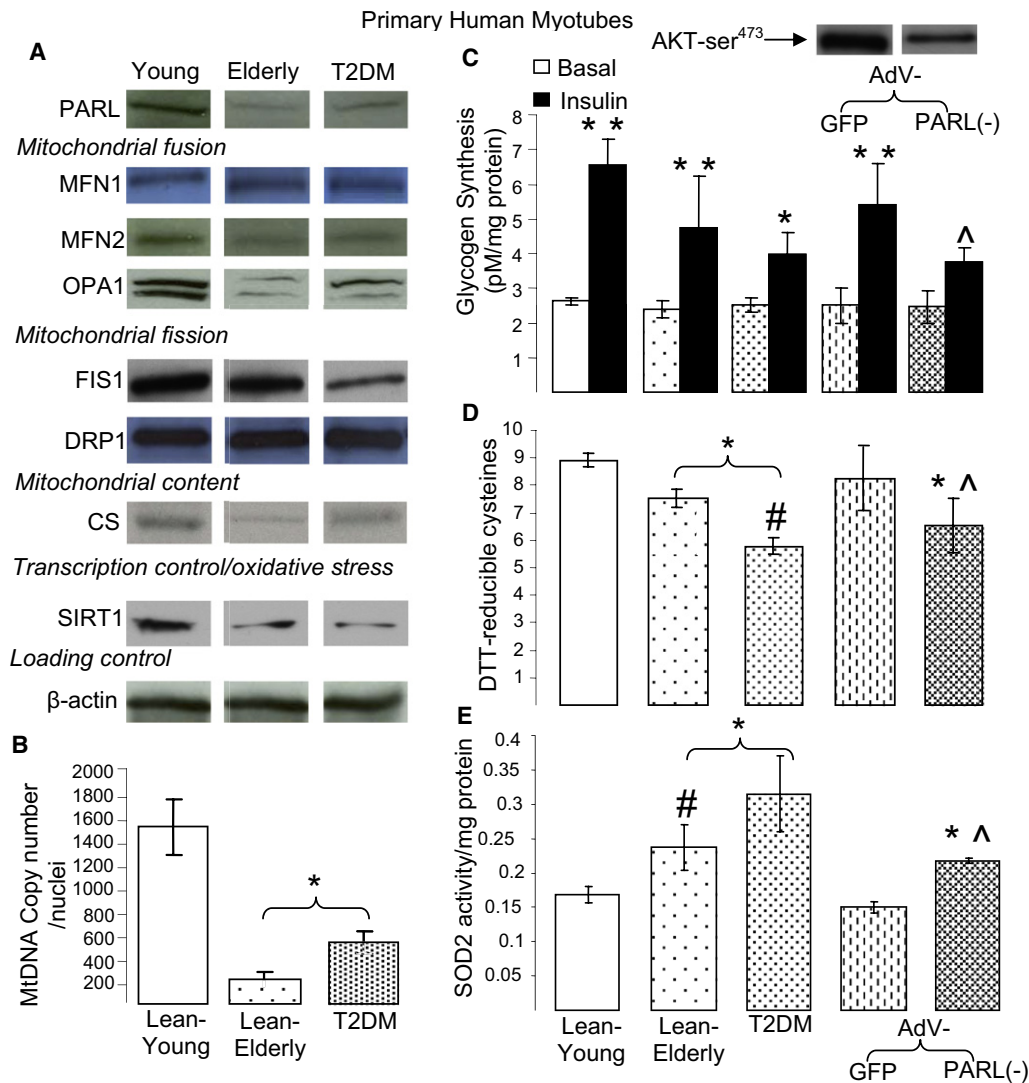


Figure 4. Human Myotubes

(A) PARL protein expression and key proteins of mitochondrial fusion (MFN1 and -2 and OPA1); mitochondrial fission, human fission 1 (FIS1) and dynamin-related GTPase 1 (DRP1); mitochondrial energetics, citrate synthase (CS); and silent mating type information regulation 2 homolog 1 (SIRT1).

(B) Mitochondrial content assessed by mtDNA copy number.

(C) Insulin-stimulated glycogen synthesis (top insert, AKT-ser⁴⁷³ phosphorylation) in AdV-GFP and AdV-PARL(-) myotubes.

(D) Cysteine oxidation and (E) superoxide dismutase 2 (SOD2) activity in primary skeletal muscle cells derived from *vastus lateralis* muscle from lean-young subjects, lean-insulin-resistant elderly subjects, and participants with T2DM. Lean-young myotubes were also transduced with (AdV)-GFP-empty cassette and AdV-PARL-siRNA. n = 5.

(B–E) Data represent mean \pm SEM. *, different to control; #, different to subjects with T2DM; \wedge , different to AdV-GFP. **, different to basal (non insulin stimulated). p < 0.05.

and PGC1 α mRNA (Table S3; p < 0.05). No other significant changes were observed, although there was a trend for elevated PGC1 β and OPA1 mRNA (Table S3). Together, these data suggest that, in the progression of insulin resistance to T2DM, the suppression of PARL and PGC1 α parallel the impairment in mitochondrial biogenesis.

Abnormal Fusion and Fission Protein Expression in Primary Cultures of Human Muscle

To address the biological significance of reduced PARL protein expression in human muscle, we first investigated mitochondrial

content and PARL protein expression and key proteins of mitochondrial energetics, fusion, and fission in primary human skeletal muscle cell model derived from *vastus lateralis* samples from our clinical cohorts. Consistent with our mRNA analysis in whole skeletal muscle, PARL mRNA (Figure S2A) and protein levels (Figure 4A) were strikingly reduced in primary myotubes derived from both T2DM and elderly subjects versus myotubes from control subjects (p < 0.001). In mammals, the separation of adjoined mitochondria by the fission process is chiefly controlled by the cytosolic dynamin-related GTPase protein 1 (DRP1) that is recruited to the mitochondrial outer membrane

by FIS1 (Stojanovski et al., 2004). FIS1 protein was lowest in myotubes from T2DM individuals relative to myotubes from elderly ($p = 0.01$), which, in turn, had lower FIS1 protein than in controls ($p = 0.005$) (Figure 4A). DRP1 was not different between the three cohorts (Figure 4A), possibly reflecting high cytosolic abundance of DRP1 (Smirnova et al., 1998). The fusion of mitochondria requires MFN-1 and -2 to conjoin the “tethered” outer membranes of juxtaposed mitochondria (Bach et al., 2003; Pellegrini and Scorrano, 2007), whereas OPA1 unifies the inner mitochondria membranes (Frezza et al., 2006). However, despite lower protein content of MFN2 ($p = 0.04$) and OPA1 (Figure 4A; $p < 0.001$) in myotubes from T2DM/elderly subjects compared to those of control subjects, there was no significant difference in MFN1 (Figure 4A) between the three groups. Similarly, reduced MFN2 protein was observed in skeletal muscle samples from obese humans (Bach et al., 2003). Thus, we show for the first time that primary skeletal muscle cells derived from elderly and T2DM subjects are characterized by low expression of PARL and mitochondrial fusion and fission proteins (FIS1, MFN2, and OPA1).

Low PARL protein expression was also associated with impaired muscle energetics, with lower levels of mtDNA (Figure 4B) and CS protein (Figure 3A), and with impaired insulin-stimulated glycogen synthesis (Figure 4C) in insulin-resistant groups versus controls. However, we observed no difference in PGC1 α and TFAM mRNAs in subjects with T2DM (Figure S2A) between the three cohorts. Despite the fact that sirtuin-1 (SIRT1) activates PGC1 α and mitochondrial biogenesis (Nemoto et al., 2005; Rodgers et al., 2005), we observed a surprising upregulation of SIRT1 mRNA (Figure S2A). However, SIRT1 protein was lower in myotubes of T2DM participants ($p = 0.04$) and elderly individuals ($p = 0.03$) relative to healthy controls (Figure 4A). The mitochondrial fractions derived from myotubes of insulin-resistant subjects (T2DM and elderly) demonstrated reduced levels of DTT-reducible cysteines (measure of irreversible cysteine oxidation; Figure 4D) and SOD2 activity (Figure 4E) compared with controls. Together, these data show that myotubes derived from insulin-resistant subjects are characterized by low expression of PARL, FIS1, MFN2, and OPA1 protein; reduced mitochondrial mass; nonoxidative glucose metabolism; and elevated oxidative stress. Furthermore, the reductions in mitochondrial mass in elderly and T2DM myotubes were independent of PGC1 α , a known regulator of mitochondrial mass.

PARL Protein Is a Determinant of Mitochondrial Mass in Primary Human Muscle Cells

To investigate whether reduced PARL may be a cause, rather than a consequence, of perturbed mitochondrial function in human-derived muscle cells, we treated myotubes from the lean-young cohort with AdV-PARL(-) siRNA and AdV-GFP control shuttles. Myotubes transduced with AdV-PARL-siRNA began to senesce (detachment of adherent cells) on day 7 of differentiation (data not shown) in conjunction with lower OPA1 protein and elevated protein expression of the stress-induced, proapoptotic protein BAX (Figure 5A), suggesting an elevated rate of apoptosis (Frezza et al., 2006). No obvious morphological defects were observed between the two types of myotubes (decreased PARL versus normal PARL). Mitochondrial mass

($p < 0.001$) and mtDNA ($p = 0.04$) were both reduced in PARL depleted myotubes (Figure 5B).

We next examined potential mechanisms by which PARL may regulate mitochondrial biogenesis. Regulated intramembrane proteolysis (RIP) is a signaling mechanism that involves the generation of biologically active peptides from membrane-tethered precursor proteins (Sik et al., 2004). PARL is cleaved by RIP to generate a smaller 33 kDa inner mitochondrial membrane protein termed PARL C-terminal product of β cleavage (PACT) and, in turn, liberates a nuclear targeted 25 amino acid peptide named PARL- β (P β) (Sik et al., 2004). Fluorescent microscopy analysis of healthy human myotubes transfected with a synthetic P β construct with a GFP (P β GFP) flag in the 5' position was used to study P β subcellular localization. Image overlay analysis demonstrated a small fraction of P β GFP localized within the nucleus (white arrows), with the majority of P β GFP in the perinuclear region (pound sign), with mitochondria evenly distributed throughout the myotubes (as indicated by Mitotracker Red; Figure S2B, i-iv). It has been hypothesized that P β may mediate mitochondria-to-nucleus crosstalk and regulate transcription factor(s) to alter the expression of mitochondrial-specific genes (Sik et al., 2004). We next treated myotubes with naive synthetic P β with no GFP flag. Consistently, P β (0.1 μ g) robustly increased the mRNA expression of PARL and genes involved in mitochondrial biogenesis (PGC1 β and NRF1) and mitochondrial fusion (MFN-1 and -2; Table S4) and OPA1 protein expression (Figure 6A). No changes were observed in PGC1 α , NRF2, and RPLPO (internal control) mRNA (Table S4). Importantly, P β increased SIRT1 protein expression (Figures 6A and 6D) in conjunction with elevated mitochondrial biogenesis (Figure 6B), suggesting a potential P β -SIRT1 regulation of mitochondrial mass. To confirm P β effects on cellular energetics and SIRT1 protein content, we treated RMS-13 human myoblasts with P β GFP plasmid containing the wild-type P β (P β WT) sequence (amino acids 53–77 of human PARL gene), P β GFP plasmid with coded mutation of the nuclear localization sequence (P β NLSM), the backbone pEGFPN1 plasmid as control, and synthetic naive P β peptide (no GFP flag). Expression of P β WT and transfection of naive P β peptide increased cellular oxygen consumption above that of P β NLSM (Figure 6E) and coincided with an increase in SIRT1 protein (Figure 6D). Consistent with the ability of SIRT1 to regulate mitochondrial mass (Lagouge et al., 2006), P β WT in RMS13 cells robustly increased mitochondrial biogenesis (Figure 6C); however, the expression of PARL cDNA in myoblasts did not alter mitochondrial mass (data not shown). Conversely, expression of PARL in myoblasts increased cellular oxygen consumption (Figure 6F). This suggests that P β is able to modulate mitochondrial energetics by increasing the transcriptional machinery involved in regulating mitochondrial mass, whereas PARL effect on cellular oxygen consumption may relate to its effects on cristae remodeling or direct interaction with OXPHOS protein(s).

PARL Protein Depletion in Myotubes Lowers Energetics and Increases ROS Production

Consistent with lower mitochondrial mass, PARL(-) myotubes displayed lower rates of oxygen consumption (Figure 5C; $p < 0.01$), diminished COX II activity (Figure 5D; $p < 0.01$), and lower rates of palmitate oxidation (Figure 5E; $p = 0.04$), indicating

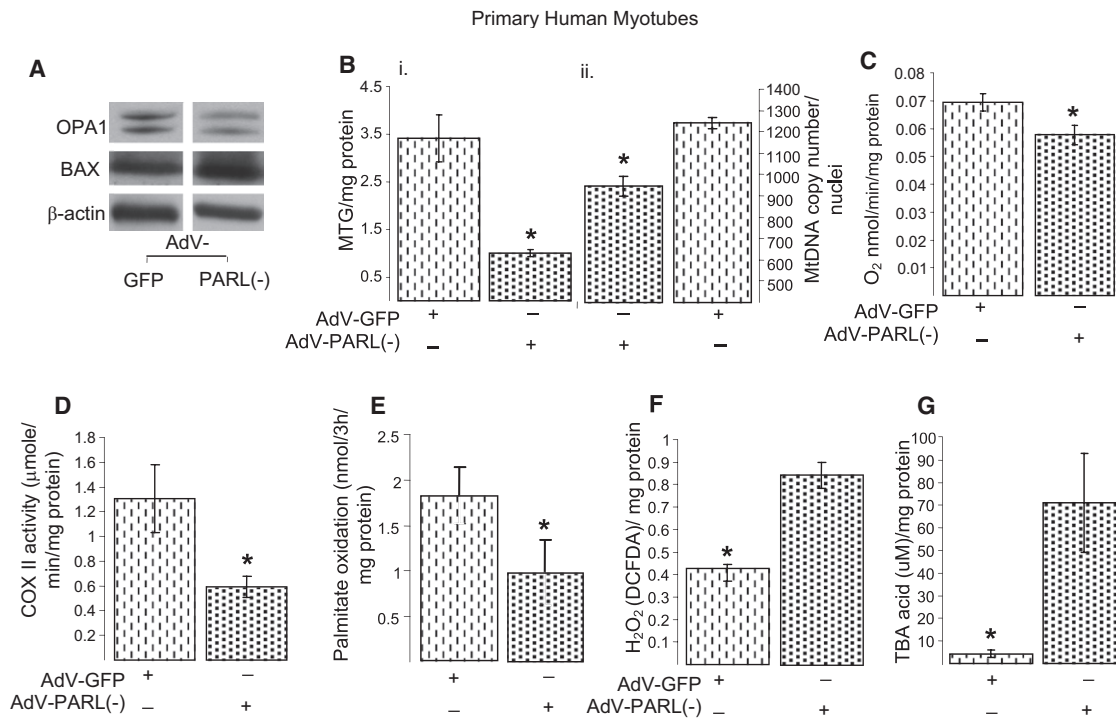


Figure 5. Mitochondrial Energetics in Human Myotubes with PARL(-)

(A and B) OPA1 and BAX protein expression (A) and mitochondrial mass using MitoTracker Green (B, i) and mtDNA copy number (B, ii) in primary skeletal muscle cells from lean-young subjects transduced with AdV-GFP control and AdV-PARL-siRNA shuttles. Mitochondrial energetics.

(C–G) Oxygen consumption (C), cytochrome c oxidase 2 activity (D), lipid oxidation (E), hydrogen peroxide (H_2O_2) production (using 1 μ M of the H_2O_2 -sensitive fluorescent probe 1 carboxy- H_2 [5-(and-6)-carboxy-2', 7'-dichlorohydrofluorescein diacetate] (DCFDA)) (F), and lipid peroxidation (TBA acid) (G) in lean-young myotubes transduced with AdV-GFP control and AdV-PARL-siRNA shuttles.

(B–G) Data represent mean \pm SEM. $n = 5$ in all groups. *, different to AdV-GFP control. $p < 0.05$.

a reduction in oxidative capacity in PARL(-) myotubes. PARL(-) myotubes were also characterized by impairments in nonoxidative glucose disposal with reduced rates of insulin-stimulated glycogen synthesis (Figure 4C) and AKT phosphorylation (Figure 4C, top). The effects on oxygen consumption and glucose metabolism are similar to lower cellular respiration and glucose oxidation in mammalian fibroblasts deficient in MFN2 protein (Bach et al., 2003; Pellegrini and Scorrano, 2007). Analysis of mitochondrial morphology by electron microscopy in PARL(-) myotubes showed mitochondria to be uniformly distributed throughout the myotubes (Figures 3E and 3F). Confocal microscopy reconfirmed distribution results with mitochondria contained within reticulum networks (Figures 3G and 3H). Therefore, PARL protein depletion in primary human muscle cells results in reduced mitochondrial mass, impaired oxidative capacity, and lower glycogen storage. However, PARL does not appear to play a role in mitochondrial distribution in human muscle cells, consistent with recent reports in other mammalian models (Cipolat et al., 2006; Griparic et al., 2007). Therefore, PARL appears to be involved in mitochondrial bioenergetics by modulating cristae remodeling and/or participation in the regulation of mitochondrial mass or cellular apoptosis.

Disturbance in mitochondrial cristae morphology can result in elevated ROS production (Yu et al., 2006), as a significant proportion of the oxidative phosphorylation machinery is embedded

in the inner mitochondrial membrane. Analysis of ROS production using carboxy-dichloro-fluorescein (Civitaresse et al., 2006) in PARL(-) myotubes revealed a 84% increase in H_2O_2 levels ($p = 0.02$) (Figure 5F), along with elevated lipid peroxidation (Figure 5G), cysteine oxidation, and SOD2 activity (Figures 4D and 4E; $p < 0.05$) relative to AdV-GFP. In addition, acute (3 hr) exposure of PARL(-) myotubes to 20 mM glucose resulted in a 96% increase in H_2O_2 production compared with basal levels ($p = 0.04$; data not shown). Together, this indicates that reduced PARL protein may explain a proportion of the elevated oxidative stress observed in myotubes from elderly and T2DM subjects.

In summary, we have shown that lowering PARL protein in human muscle cells results in lower mitochondrial oxidative capacity, coupled with reduced mitochondrial mass, increased protein oxidation and ROS production, and impaired insulin signaling—all known metabolic defects in T2DM and aging (Conley et al., 2007; Imoto et al., 2006; Kelley et al., 2002; Morino et al., 2005; Petersen et al., 2003, 2005; Ritov et al., 2005).

DISCUSSION

There is an abundance of indirect evidence demonstrating diminished oxidative phosphorylation and lipid oxidation in T2DM and aging (Civitaresse et al., 2006; Kelley et al., 2002;

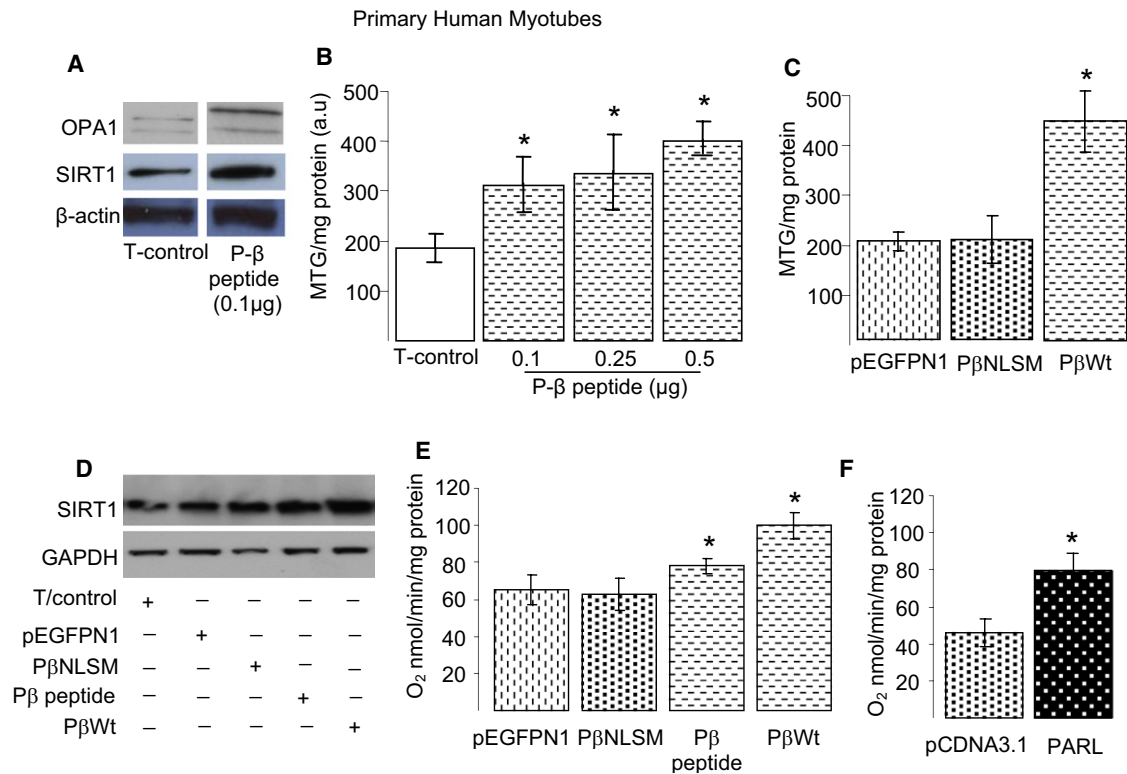


Figure 6. PARL-β

(A) OPA1 and SIRT1 protein expression (A) in human myotubes treated with 0.1 μg of naive Parl-β (Pβ). (B and C) Mitochondrial mass. Myotubes from lean-young subjects were transfected with (B) 0.1, 0.25, and 0.5 μg of naive Pβ peptide (synthetic peptide without a 5' GFP tag [FRKAPRKVEPRRSDPGTSGEAYKRS]); GenePorter transfection reagent (Genethery Systems) was used as transfection control (T-control), and (C) with the backbone pEGFPN1 plasmid as control, Pβ GFP plasmid with coded mutation in the nuclear localization sequence (PβNLSM), and Pβ GFP plasmid containing the wild-type Pβ (PβWt) sequence (amino acids 53–77 of human PARL gene). (D–F) (D) SIRT1 and GAPDH protein expression and (E) cellular oxygen consumption analysis in RMS-13 myoblasts were treated with T-control (D only), backbone pEGFPN1, PβNLSM, Pβ-peptide, and PβWt. In separate experiments (F), RMS-13 myoblasts were treated with control vector (pCDNA3.1(+)) and pCDNA3.1-PARL (PARL), and cellular oxygen consumption was measured. (B–F) Data represent mean ± SEM. (C–F) n = 6. (C and D) n = 3. *, different to control. p < 0.05.

Morino et al., 2005; Petersen et al., 2003, 2005; Ritov et al., 2005; Ukropcova et al., 2007). This has led to the widely held hypothesis that mitochondrial dysfunction can lead to insulin resistance. Elevated ROS production resulting from impaired mitochondrial metabolism and the accumulation of ectopic lipid intermediates in skeletal muscle are linked to the activation of serine kinases that inhibit insulin signaling (Adams et al., 2004; Chavez and Summers, 2003; Yu et al., 2002). Early functional studies postulated that, in combination with dyslipidemia, the elevation of the lipid intermediates (i.e., diacylglycerol, ceramides, and fatty acyl CoA) was driven by the inability of skeletal muscle and mitochondria to appropriately shift between carbohydrate and lipid as metabolic substrates, a defect referred to as “metabolic inflexibility” (Kelley and Mandarino, 2000). Later, in vitro studies demonstrated that increased PGC1α activity elevated mitochondrial mass and lipid oxidation in isolated mitochondria and, in parallel, increased insulin-stimulated glucose transport (Benton et al., 2008). Taken together, these data indicated that mitochondrial abnormalities in insulin resistance can result from either lower oxidative capacity—and therefore are reflective of a functional impairment—or lower mitochondrial

number, or a combination of both of these defects. Some studies have questioned whether decreased mitochondrial markers from insulin-resistant muscle are merely the result of less-active muscle (Holloszy and Coyle, 1984; Terjung et al., 2002) and therefore are reflective of the diminished energy requirement of the muscle.

To address this, investigators have performed direct experiments to determine the ability of isolated mitochondria from healthy and insulin-resistant muscle to respire and synthesize ATP with contradictory results. Hojlund et al. demonstrated that the rates of state 3 respiration in isolated mitochondria are lower in the skeletal muscle from subjects with T2DM relative to obese controls (Mogensen et al., 2007). In accordance with this study, a 35% lower ADP-stimulated respiration in muscle fibers from subjects with type 2 diabetes has been described (Phielix et al., 2008). Contradictory to these studies, Boushel et al., using permeabilized human skeletal muscle fibers, showed no difference in either NADH- or FADH₂-linked respiration in patients with T2DM compared to lean healthy subjects (Boushel et al., 2007). In fact, metabolic control theory modeling from ³¹P NMR spectroscopy experiments in resting skeletal

muscle (the condition in which all the above studies were performed) indicates that metabolic control lies exclusively at ATP utilization and demand and not at sites of ATP production (Jensen et al., 2000). On the other hand, reduced demand for energy supply does not exclude the coexistence of a functional abnormality in mitochondria, and, in fact, the two could be related. Given that reduced mitochondrial mass and function (lower oxygen consumption, lower lipid oxidation, and elevated ROS production) and impaired insulin signaling were observed in PARL(-) muscle and myotubes, we propose that PARL protein, insulin sensitivity status, and mitochondrial energetics (mass and substrate oxidation) are directly linked and may account for a portion of the energetic defects observed in insulin-resistant muscle. Recent evidence in β cells demonstrates that regulation of mitochondrial remodeling, assembly, and distribution are important in modulating both mitochondrial number and energetic status (Twig et al., 2008).

The mechanism that governs the transcriptional interplay between mitochondrial function in insulin resistance is still unclear. Although it is well accepted that PGC1 α is one of the chief regulators of mitochondrial biogenesis and multiple studies report lower PGC1 α expression in the skeletal muscle of insulin-resistant subjects with or without T2DM (Civitarese et al., 2006; Mootha et al., 2003; Patti et al., 2003), lower PGC1 α mRNA levels can result from chronic physical inactivity (Scarpulla, 2008), and genetic models of PGC1 α and PGC1 β deletion do not show muscle insulin resistance (Leone et al., 2005; Lin et al., 2004; Sonoda et al., 2007). Similarly, global PARL knockout mice do not develop T2DM, although failure to develop this phenotype may relate to premature mortality, as PARL null mice die before the end of the third month from cachexia (Cipolat et al., 2006). Although, mice that were fed a HFD in this study had a progressive decline in glucose tolerance that was mirrored by reduced PARL mRNA and mtDNA content in skeletal muscle, indicating the suppression of PARL mRNA tracks with lower mtDNA and a worsening of glucose tolerance. PPAR δ knockout mice develop both mitochondrial dysfunction and T2DM, with mitochondrial abnormalities predating insulin resistance (Schuler et al., 2006). This suggests that additional genetic “players” may be involved in mitochondrial dysfunction and that insulin resistance can occur in concert or independent of the PGC1 system. We propose that an alternative pathway for the regulation of mitochondrial mass may also include PARL-P β . Reduced PARL protein would be expected to result in reduced ability of P β to provide a “retrograde” signal to the nucleus to stimulate mitochondrial mass via SIRT1.

In most mammalian cells, mitochondria exist as “branched-chain” reticulum networks that enable mitochondrial matrix lumens to merge and the exchange of metabolites and mitochondrial nucleoids to occur (Civitarese and Ravussin, 2008). Cells can then adapt to varying energy requirements by coupling the mitochondrial electrochemical gradient between mitochondria to distal cellular locations where ATP demand is highest (Bach et al., 2003; Skulachev, 2001). This in situ “energy sensing” (Amchenkova et al., 1988; Frazier et al., 2006) by mitochondria is highly dependent on OPA1-mediated remodeling of the cristae scaffold. OPA1 activity in the intermembrane mitochondrial space is controlled, in part, by the proteolytic cleavage of OPA1 by PARL (Cipolat et al., 2006). Such activity can explain

our electron microscopy data showing altered mitochondrial cristae in mCK-PARL(-) mice muscle, as well as the lower energetic capacity and elevated ROS production in AdV-PARL(-) myotubes. These data provide a rationale whereby mitochondrial cristae remodeling and mitochondrial function in skeletal muscle are mediated, in part, by PARL, and lower PARL protein content results in lower energetic efficiency of the cells and contributes to cellular damage and aging (Figure 7).

P β was able to upregulate fusion-specific expression (MFN1 and 2 and OPA1), which would favor the energy and metabolite transfer between mitochondria (Civitarese et al., 2007) or clearance of dysfunctional mitochondria (Twig et al., 2008). Recent studies indicate that mitochondrial fusion and fission and OPA1 levels constitute a quality control “checkpoint” that allows for the sorting and elimination of functionally impaired mitochondria by autophagic clearance (Twig et al., 2008). Given that mitochondrial mass is in constant flux and that steady-state levels of mitochondrial mass are the product of mitochondrial biogenesis and degradation (chiefly through macroautophagy), defects in the regulators of this balance may contribute to mitochondrial dysfunction in insulin resistance. A balanced level of mitochondrial autophagy is required to maintain the quality of cellular and mitochondrial function (i.e., to eliminate the cell of damaged/aging organelles) (Meijer and Codogno, 2007). Therefore, an imbalance in fusion or fission or an overactive autophagy machinery would be expected to result in altered mitochondrial energetics or lower mitochondrial mass. This hypothesis is consistent with the lower levels of PARL, OPA1, FIS1, and MFN2 protein content measured in insulin-resistant myotubes from this study and by others (Bach et al., 2003). Collectively, these data further support our hypothesis that: (1) altered PARL function may contribute to worsening mitochondrial function, mass, and insulin resistance and (2) changes in PARL protein and the balance between fusion and fission may be involved in changes in mitochondrial energetics and mass in insulin resistance.

In summary, PARL shares commonalities with OPA1 by regulating cristae shape (Cipolat et al., 2006; Frezza et al., 2006). However, unlike bona fide fusion and fission proteins, PARL has the ability to induce the transcription of both the fusion/fission genes (including OPA1) and mitochondrial mass vis-à-vis P β . In addition, the suppression of PARL mRNA tracks with lower mtDNA and a worsening of glucose tolerance in mice fed a HFD for 10 weeks. These studies demonstrate that PARL mRNA suppression predates the suppression of FIS1, MFN2, and OPA1 in insulin resistance. Taken together, this indicates that lower PARL expression is an early defect altering mitochondrial function and insulin resistance in response to a metabolic challenge. As insulin resistance worsens, declines in FIS1, MFN2, and OPA1 protein probably occur at later stages (as observed in insulin-resistant elderly and T2DM subjects), contributing to a further decline in mitochondrial function. We hypothesize that impaired PARL function is an important risk factor for the development of insulin resistance in skeletal muscle by decreasing mitochondrial mass and energetics and increasing oxidative stress, thus contributing to impaired glucose metabolism. As insulin resistance continues to develop, mitochondrial function, oxidative damage, and PARL activity may decline further, leading to a vicious cycle that eventually

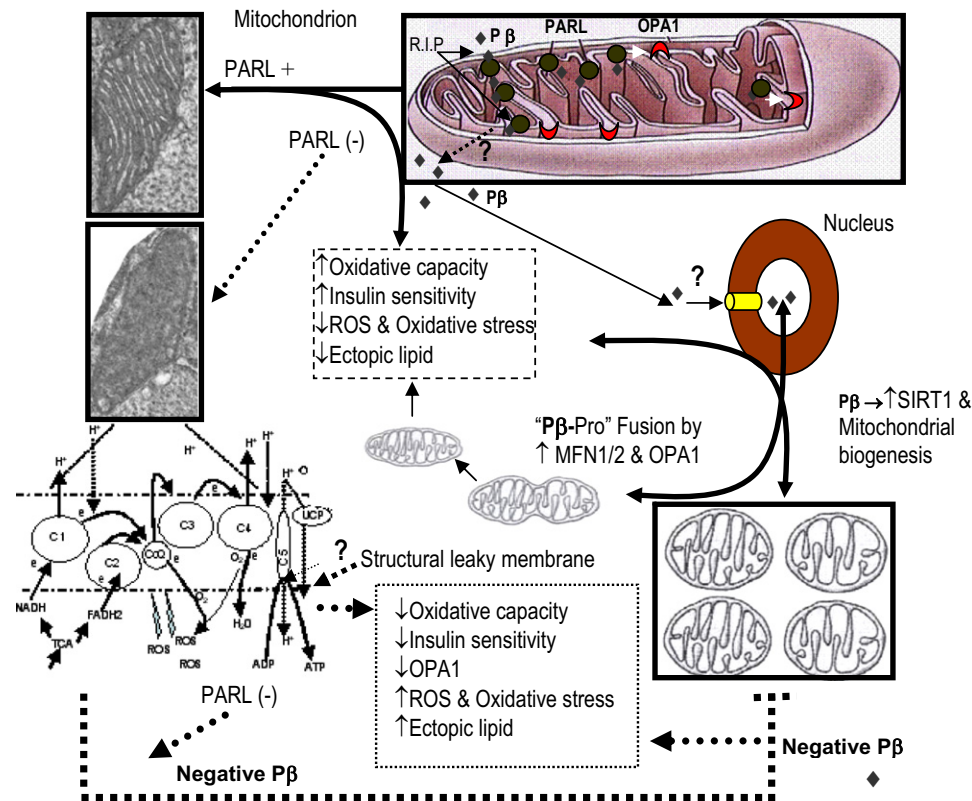


Figure 7. Overview of PARL Function and Steps that Contribute to the Formation of ROS and Insulin Resistance

PARL-mediated cristae remodeling results in maintenance of “normal” mitochondrial structure, oxidative capacity (both oxidative phosphorylation and β oxidation), and insulin sensitivity. Improvements in mitochondrial energetics are further sustained by the ability of PARL- β ($P\beta$) to induce SIRT-1 protein and mitochondrial biogenesis. Inhibition of PARL-mediated cristae remodeling results in damaged inner-membrane structure, loss of mtDNA, and reduced oxidative capacity. Under hyperglycemic conditions, the increased production of NADH and FADH₂, coupled with lower mitochondrial mass and a “structural leaky” inner membrane, would favor electron transfer to oxygen creating reactive oxygen species (ROS) and damaging of the mitochondrial membrane. If mitochondrial damage is not sufficiently compensated by increased $P\beta$ function and mitochondrial biogenesis, fusion/fission complementation of the mitochondrial genome, and ROS defense mechanism, oxidative capacity would decline further, eventually leading to a vicious cycle of increasing muscle insulin resistance and oxidative damage.

contributes to the development of T2DM or other age-associated diseases, including sarcopenia.

EXPERIMENTAL PROCEDURES

Animal Care and Use

A colony of *Psammomys obesus* was maintained at Deakin University, Geelong, Australia. Experimental animals were weaned at 4 weeks of age and given a diet of standard laboratory chow, from which 12% of energy was derived from fat, 63% from carbohydrate (CHO), and 25% from protein (Barastoc, Pakenham, Australia). Animals were housed in a temperature-controlled room (22°C \pm 1°C) with a 12-12 hr light-dark cycle (light 06:00-18:00). At 18 weeks of age, adult *Psammomys obesus* were euthanized, and selected tissues were harvested immediately for protein extraction and PARL tissue distribution analysis. All experimental animals and procedures were maintained in accordance with the Code of Practice outlined by the National Health and Medical Research Council (NHMRC) and were approved by the Deakin University Animal Welfare Committee. For high-fat feeding studies, mice were housed and maintained at Laboratory Animal Resources, Virginia Polytechnic Institute and State University, Blacksburg, VA. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Mice expressing the coxsackie/adenoviral receptor driven by the muscle creatine kinase promoter (mCK-CAR) were generously provided by

Drs. Nalbantoglu and Holland (McGill University). Heterozygous mice used in this study (age 4 months) were housed under normal conditions (12 hr-12 hr light/dark cycle) in microisolator units in the UC Denver Center for Comparative Medicine, and all treatments and manipulations were approved by the UC Denver Internal Animal Care and Use Committee.

In Vivo Knockdown of PARL Protein

Mice were placed on a high-fat ad libitum diet (HFD) (Research Diets, NJ, #12344) for 2 weeks prior to the injection protocol. Under isoflurane anesthesia, mCK-CAR mice were injected with AdV-PARL-siRNA(-) (1×10^8 plaque forming units [pfu] in 25 μ l) in each lobe of their left gastrocnemius muscle and in the tibialis/extensor digitorum longus (TA/EDL) muscle group. An additional injection of AdV-GFP (1×10^8 pfu in 25 μ l) was delivered in each lobe of the gastrocnemius muscle and the TA/EDL muscle group of the contralateral leg. All experiments were performed at 7 days posttransduction with mice remaining on a HFD during this period. An additional set of mCK-CAR mice were injected with an equivalent volume of the saline vehicle in the left gastrocnemius muscle, whereas the contralateral leg was left untreated. Following injections, mice were allowed to recover and were maintained in a biohazard-specific pathogen-free barrier facility. The mice were then fed an ad libitum high-fat diet (Research Diets, NJ, #D12344) for 7 days, after which the muscles were collected under isoflurane anesthesia and flash frozen in liquid nitrogen or placed in a fixative solution (6.25% Glutaraldehyde in 0.1 M Sodium Cacodylate Buffer [pH 7.4]) for electron microscopic

analyses. In a subset of mice, animals received insulin tolerance test or saline 15 min prior to euthanasia and tissue collection.

Using mitochondrial DNA and citrate synthase activity as endpoint measures, we found no difference between AdV-GFP, saline, and untreated controls (data not shown). Therefore, only AdV-PARL(-) and AdV-GFP transduced muscle data is shown in manuscript.

Human Subjects

Eleven young (18–29 years), 11 elderly (60–84 years), and 32 subjects with type 2 diabetes mellitus (40–67 years) were recruited into this study. All subjects underwent complete physical examination, routine medical laboratory tests, and anthropometry. Nonsmoking and healthy young individuals (5F/6M) and elderly subjects (5F/6M) with a BMI 20–25 kg/m² and fasting glucose of < 110 mg/dl were included. All young and elderly subjects were sedentary and matched for physical activity by activity index questionnaire. Subjects with type 2 diabetes diagnosed at least 6 months earlier were evaluated. All subjects had been previously maintained on dietary therapy only and did not use insulin or thiazolidinediones. They were required to have a fasting plasma glucose of \geq 125 mg/dl but of < 170 mg/dl at time of screening. Subjects on medications known to affect glucose metabolism were excluded. Physical activity in all subjects was determined by questionnaire, with participants reporting less than three times per week of aerobic exercise. The Internal Review Board of the Pennington Biomedical Research Center approved the protocol, and subjects provided written informed consent.

Skeletal Muscle Cell Culture

Vastus lateralis muscle biopsy (~100 mg) was obtained from a subset of the clinical cohort. Five healthy and young sedentary subjects (age 24.2 \pm 0.2 years old; BMI 23.7 \pm 0.5; percent body fat 19.9% \pm 3%; fasting glucose concentration 90 \pm 3 mg/dl and fasting plasma insulin 4.0 \pm 0.4 μ U/ml), five elderly subjects (age 65.1 \pm 3 years old; BMI 23.8 \pm 0.7; percent body fat 21.7% \pm 2.8%; fasting glucose concentration 96 \pm 3 mg/dl, and fasting plasma insulin 6.1 \pm 1 μ U/ml), and five individuals with T2DM (age 51.8 \pm 3 years old; BMI 24.2 \pm 0.5; percent body fat 26% \pm 2%; fasting glucose concentration 139 \pm 17 mg/dl, and fasting plasma insulin 31 \pm 5 μ U/ml). All subjects were male. Satellite cells were isolated as previously described (Civitarese et al., 2006; Ukropcova et al., 2005). Cells were seeded into 12-well and 6-well plates at a density of 20 \times 10³ cells per cm² and grown at 37°C in a humidified atmosphere of 5% CO₂. All myoblasts were differentiated into myotubes as previously described (Civitarese et al., 2006). In selected wells, myotubes were transduced with either AdV-PARL-siRNA (CCU AUC CUA UAA GGA GUC UCA UAA A) or AdV-GFP control shuttle using a low dose (4 \times 10⁶ pfu) in saline. Saline-treated cells were used as additional negative controls. All metabolic assays were performed 6 days posttransduction. Human RMS-13 myogenic and stable cell line were purchased from ATCC.

Vector Constructs

PARL cDNA clone was inserted into the pcDNA 3.1(+) vector (Invitrogen, Inc.) under the control of the CMV promoter. For P β studies, the backbone vector used was a control GFP construct-pEGFPN1 (Invitrogen, Inc.). Using this plasmid, the wild-type P β (P β WT) sequence (amino acids 53–77 of human PARL) was cloned upstream of the GFP flag. A coding methionine (in the context of a kozak sequence) was added upstream of the P β sequence to ensure initiation of translation from this site. To prevent translation of GFP alone (a consequence of “leaky” scanning by the ribosome), the coding methionine of the GFP open reading frame was mutated to isoleucine. To create the P β NLSM, the amino acids of P β WT 54 and 55 and amino acids 58 and 59 (both human PARL gene) were substituted from arginine and lysine to glycine and serine. A similar P β NLSM was used by Sik et al. (2004), with two doublets changed (54RK55 to 54TS55 and 58RK59 to 58TS59).

Mitochondrial Content and Enzyme Activity Assay

For quantification of mitochondrial content, we used mtDNA and Mitotracker Green probe (Molecular Probes) as previously described (Civitarese et al., 2006). Mitotracker Green probe preferentially accumulates in mitochondria and provides an accurate assessment of mitochondrial mass. Cells were washed with PBS and incubated at 37°C for 30 min with 100 nM Mitotracker Green FM (Molecular Probes). Cells were harvested using trypsin/EDTA and

resuspended in PBS. Fluorescence intensity was detected with excitation and emission wave lengths of 490 and 516 nm, respectively, and values were corrected for total protein (mg/ml). Citrate synthase, cytochrome c oxidase, and β -HAD activities were determined spectrophotometrically from cell homogenates using previously described methods (Civitarese et al., 2006).

Myotube Oxygen Consumption

Oxygen consumption in primary human myotubes was measured using a self-referencing microelectrode system (MacLellan et al., 2005). Myotubes, cultured in a 6 cm² dish, were suspended in Earle’s basic salt solution (117 mmol/l NaCl, 5.4 KCl, 1.5 CaCl₂, 0.8 MgSO₄, 0.9 mmol/l NaH₂PO₄, 10 mmol/l HEPES, and 5.6 mmol/l glucose) and housed within an insulated Faraday box and maintained at 37°C. The oxygen microelectrode was oscillated at a frequency of 0.3 Hz to collect alternating oxygen flux and reference measurements. Total cellular oxygen consumption was determined for a 5 min period, and values were corrected using mg protein.

For PARL- β experiments in RMS-13 cells, a Seahorse Bioscience XF24 instrument was used to measure the rate of change of dissolved O₂ and pH in medium immediately surrounding cell culture in 24-well plates. Measurements were performed using a cartridge in which 24 optical fluorescent O₂ and pH sensors are configured as individual well “plungers.” For measurements of rates, the plungers gently descended into the wells, forming a chamber that entraps the cells in ~5 μ l volume. O₂ concentration and pH were measured for 2 min. The rates of O₂ consumption and extracellular acidification were obtained from the slopes of concentration changes versus time. For preparation of the cell plate for assay with the XF24 instrument, 1 ml of Krebs-Henseleit buffer lacking bicarbonate (111 mM NaCl, 4.7 mM KCl, 2.0 mM MgSO₄, 1.2 mM Na₂HPO₄, 0.24 mM MgCl₂, and 5 mM Sodium Pyruvate) warmed to 37°C was added to each well containing 0.25 \times 10⁶ RMS-13 myoblasts.

Glycogen Synthesis

The rate of total glycogen synthesis was determined in myotubes maintained in differentiation medium for 5 days by measuring the incorporation of D-[U-¹⁴C] glucose into glycogen. Cells were incubated in 1 ml of experimental medium (136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄·7H₂O, 1.2 mM CaCl₂·2H₂O, and 20 mM HEPES [pH 7.4]), with and without insulin, containing D-[¹⁴C]glucose (1.25 μ Ci/ml, final concentration). Cells were lysed by adding 200 μ l 20% (wt/vol) potassium hydroxide and partially neutralized with 200 μ l 1 M hydrochloric acid. The cell extracts were then transferred to Eppendorf tubes and glycogen extracted by ethanol precipitation after the addition of 12 mg/ml (final concentration) carrier glycogen. Radioactivity incorporated into glycogen was determined by scintillation counting.

Electron Microscopy

mCK-CAR muscle specimens were fixed for 4 hr by immersion in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C–6°C. Bioptic specimens were then fixed in 1% osmium tetroxide and embedded in an Epon-Araldite mixture. Semi-thin sections (2 μ m) were stained with toluidine blue, and thin sections obtained with an MT-X ultratome (RMC, Tucson, Arizona) were stained with lead citrate and examined with a CM10 transmission electron microscope (Philips, Eindhoven, Netherlands). Scanning electron microscopy analysis of AdV-PARL(-) cells and AdV-GFP-transduced myotubes were performed by Quantomix Ltd. (Israel) as previously described (Thiberge et al., 2004).

Confocal Microscopy

In 12-well collagen-treated plates, fully differentiated myotubes were washed twice in PBS, and cells were incubated at 37°C for 30 min with 100 nM Mitotracker Green FM (Molecular Probes). Myotubes were washed twice with PBS, and a single in-focus optical section was acquired using a confocal microscope (Nikon Eclipse TE2000-U) with a \times 40 air objective, as previously described (Civitarese et al., 2006).

Statistical Analysis

Group means were compared by analysis of variance. In young, lean, and elderly subjects, age, sex, BMI, percent body fat, insulin sensitivity (HOMA-IR), fasting plasma insulin and glucose, plasma fatty acids, PARL, OPA1, MFN1/2, PGC1 α / β mRNA, TCAD mRNA, mtTFA mRNA, citrate synthase,

and β -HAD enzyme activity were entered in stepwise regression models to assess their contribution to the interindividual variability of mtDNA copy number and energy expenditure. Pearson correlation coefficients were calculated to determine the relationship between selected variables within each group. All data are presented as means \pm SEM. p values < 0.05 were considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five tables, and two figures and can be found with this article online at doi:10.1016/j.cmet.2010.04.004.

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