A gain of function variant of the mitochondrial matrix protease SPG7 is associated with increased risk of coronary artery disease

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Abstract

Genome-wide association studies (GWAS) have identified up to 30 loci that associate with increased risk of coronary artery disease or myocardial infarction. Here, I tested the function of one locus that changed the amino acid sequence of a mitochondrial matrix protease called paraplegin (SPG7) that performs critical quality assurance functions. Loss-of-function mutations in this protease are associated with hereditary spastic paraplegia. Here, I show that this variant that changes an arginine to a glutamine at position 688 within the protease domain is a gain-of-function. Cells bearing this variant have increased mitochondrial fusion and number, produce higher levels of reactive oxygen species and have increased cellular proliferation. Importantly, when expressed in yeast, the Q688 variant of SPG7 rescues the growth arrest caused by a protease-deficient mutation in AFG3L2. My study identifies a novel functional variant of SPG7 and highlights the need to go beyond the GWAS paradigm.

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I dedicate this thesis to the memory of my father, Ahmad Almontashiri.

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2. **Naif A. M. Almontashiri**, Allen C.T. Teng, Hsiao-Huei Chen, Brian L.M. Cheng, Robert Roberts[1], Thomas Langer, Heidi McBride, Alexandre F.R. Stewart. Mitochondrial gene variant contributing to coronary artery disease. **(in preparation)**

3. Mohammad Afaque Alam, **Naif A. M. Almontashiri** , Robbie W Davies, Sonny Dandona, Olli Raitakari, Leo-Pekka Lyytikäinen, Terho Lehtimaki, George A Wells, Ruth McPherson, Robert Roberts, Alexandre FR Stewart . Genome Wide Association Study identifies genetic variant that contribute elevated circulating oxidized LDL levels among coronary artery disease subjects. **(in preparation)**

4. Alexandre F. R. Stewart, Sonny Dandona, Meng Fan, **Naif A. M Almontashiri**, Li Chen, Robert W. Davies, George A. Wells, Wilson Tang, Stephen Ellis, Stanley L. Hazen, Muredach P. Reilly, Stephen E. Epstein, Daniel J. Rader, James C. Engert, Sonia Anand, Benjamin F. Voight, David S. Siscovick, Stephen M. Schwartz, Veikko Salomaa, Roberto Elosua, Olle Melander, L. Adrienne Cupples, Christopher J. O´Donnell, Hsiao-Huei Chen, Alistair S. Hall, Svati H. Shah,William E. Kraus, Christopher E. Granger, Nilesh J. Samani, Sekar Kathiresan, Ruth McPherson, Robert

Roberts. Identification of a Rare Variant near Neurexin 1 Associated with Coronary Artery Disease. **(in preparation)**

5. Allen C.T. Teng, **Naif A. M. Almontashiri**, Brian L.M. Cheng, Philip Lou, Pinar Ozmizrak, Hsiao-Huei Chen, and Alexandre F.R. Stewart (2011). Identification of Nuclear Localization Signal in IRF2BP2**.** . PLoS ONE, 6(8), e24100.

Published abstracts

1. Stewart AF, Assogba O, Chen L, **Almontashiri N**, Ruddy R, Dandona S, Williams K, Wells GA, McPherson R, Roberts R. Identification of SPG7 as a Novel Risk Locus For Coronary Artery Disease by Genome-wide Association. Scientific Sessions of the American Heart Association, Orlando, Florida, USA, Nov. 14-18, 2009.

2. AFR Stewart, S Dandona, M Fan, **N Almontashiri**, L Chen, RW Davies, GA Wells, W Tang, SL Hazen, S Ellis, MP Reilley, S Epstein, DJ Rader, JC Engert, S Anand, S Kathiresan, AL Cupples, CJ O'Donnell, S Shah, WE Kraus, CB Granger, R McPherson, R Roberts. Identification of a Rare Variant near Neurexin 1 Associated with Coronary Artery Disease. Scientific Sessions of the American Heart Association, Chicago, IL, Nov. 13-17, 2010.

3. **Naif A. Almontashiri**, Allen C.T. Teng, Hsiao-Huei Chen, Brian L.M. Cheng, Mohammad A. Alam[1], Matthew Ta, Robert Roberts, Thomas Langer, Heidi M. McBride and Alexandre F.R. Stewart. Mitochondrial gene variant contributing to coronary artery disease. Experimental biology meeting, Washington DC, USA, April. 9-13,2011.

4. Brian L. M. Cheng, Elizebeth D. Moher, Allen C. T. Teng, **Naif Almontashiri**, Yuhao Shi, Brandon Tam, and Alexandre F. R. Stewart. Vestigial-like 3 regulates myosin light chain and skeletal {alpha}-actin promoters. Experimental biology meeting, Washington DC, USA, April. 9-13,2011.

5. Allen Chun-Tien Teng, **Naif Al-montashiri**, Brian L.M. Cheng, Philip Lou, Pinar Ozmizrak, Hsiao-Huei Chen and Alexandre F.R. Stewart. Identification of nuclear localization signal in IRF2BP2. Experimental biology meeting, Washington DC, USA, April. 9-13,2011.

Platform presentations

1. **Naif AM Almontashiri,** Meng Fan, Hsiao-Huei Chen, Allen CT Teng, Brian LM Chen, Ruth McPherson, Robert Roberts, Alexandre FR Stewart. The 9p21.3 coronary artery disease risk genotype is associated with elevated serum levels of interferon alpha 21. ASHG/ICHG meeting, Montreal, Canada, Oct. 11-15, 2011.

2. **Naif A. Almontashiri**, Allen C.T. Teng, Hsiao-Huei Chen, Brian L.M. Cheng, Mohammad A. Alam[1], Matthew Ta, Robert Roberts, Thomas Langer, Heidi M. McBride and Alexandre F.R. Stewart. Gain of function variant in the mitochondrial protein SPG7 associated with increased risk of coronary artery disease. The Canadian Cardiovascular Congress Scientific session, Vancouver, BC, Canada, Oct.22-25, 2011.

3. **NA Almontashiri**, AC Teng, BL Cheng, M Fan,R McPherson, R Roberts, AF Stewart. Interferon alpha 21levels are elevated in peripheral blood lymphocytes and aortic smooth muscle cells with the 9p21.3 coronary artery risk genotype. The Canadian Cardiovascular Congress Scientific session, Vancouver, BC, Canada, Oct.22-25, 2011.

4. **Naif AM Almontashiri**, Meng Fan, Hsiao-Huei Chen, Allen CT Teng, Brian LM Chen, Ruth McPherson, Robert Roberts, Alexandre FR Stewart. Serum Interferon Alpha 21 is a Biomarker of the 9p21.3 Risk Locus for Coronary Artery Disease. Scientific Sessions of the American Heart Association, Orlando, Florida, USA, Nov. 12-16, 2011.

Honors, Awards and scholarships

1. 2011 Semifinalist for Trainee Research Award, American Society of Human Genetics for abstract submission to the 12th International Congress of Human Genetics.
2. Full Scholarship for the graduate studies from Taibah University, Almadinah Almunawrah, Saudi Arabia.
3. Pending patent (61/527,693) at the US patent office for discovering a new biomarker for the coronary heart disease.

List of Abbreviations

Coronary artery disease (CAD)

Single nucleotide polymorphisms (SNPs)

Genome-wide association studies (GWAS)

Cyclin dependent kinase inhibitors CDKN2A and CDKN2B

Coronary ARtery DIsease Genome-Wide Replication And Meta-Analysis (CARDIoGRAM)

Mitochondrial ribosomal protein S6 (MRPS6)

Cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1)

ATP synthase, H+ transporting, mitochondrial Fo complex, subunit C1 (ATP5G1)

Phosphatidylethanolamine N-methyltransferase (PEMT)

Reactive oxygen species (ROS)

Thioredoxin 2 (Trx2

Peroxiredoxin 3 (Prx3)

Thioredoxin Reductase 2 (TrxR2)

ATPase family gene 3-like 2 (AFG3L2)

Spastic paraplegia 7 (SPG7)

Human mitochondrial ribosomal protein L32 (hMRPL32)

Matrix-ATPases associated with diverse cellular activities (m-AAA)

Myocardial infarction (MI)

Optic atrophy 1 (OPA1)

Mitofusin 2 (MFN2)

Proliferating Cell Nuclear Antigen (PCNA)

Cytochrome c oxidase subunit 1 (COX1)

Cytochrome c oxidase subunit 2 (COX2)

Cytochrome c oxidase subunit 4 (COX4)

Cytochrome c oxidase subunit 3 (COX3)

Glyceraldehyde-3- phosphate dehydrogenase (GAPDH)

Phosphorylated retinoblastoma (pRB)

Phosphyrlated AMP-activated protein kinase (p-AMPK)

Hydroethidium (HEt)

Bromodeoxyuridine (BrdU)

Propidium iodide (PI)

2',7'-dichlorfluorescein-diacetate DCFH-DA

Soybean trypsin inhibitor (SBTI)

Mitochondrial processing peptidase (MPP)

Peripheral blood lymphocytes (PBLs)

Human embryonic kidney (HEK293)

Dynamin-related protein 1 (DRP1)

N-acetyl cysteine (NAC)

Protein kinase A (PKA)

Oocyte maturation defective metallopeptidase (OMA1)

Low density lipoprotein (LDL)

2',7'-dichlorfluorescein-diacetate (DCFH-DA)

Mitochondrial division inhibitor-1 (mdivi-1)

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Introduction

Coronary artery disease (CAD) is the leading cause of death in the western world (Murray and Lopez 1997). CAD is an age-progressive disease: although fewer than 10% of individuals show symptoms of CAD before the age of 50, intravascular ultrasound revealed that 1 in 6 adolescents and 85% of persons over the age of 50 have measurable coronary atherosclerosis (Tuzcu, Kapadia et al. 2001). Coronary atherosclerosis is a disease where coronary arteries become occluded due to the accumulation of lipids such as cholesterol in the vessel wall. It is also characterized by cell proliferation and inflammation in the wall of the coronary artery (Ross 1999). Different risk factors increase the risk of coronary atherosclerosis such as hypertension, smoking, hyperlipidemia, diabetes and obesity (Yusuf, Hawken et al. 2004).

Up to half the risk for CAD is genetic. In 2007, using microarrays of single nucleotide polymorphisms (SNPs) to genotype large numbers of cases and controls, we identified the first common genetic variant at chromosome 9p21.3 that increases the risk for CAD (McPherson, Pertsemlidis et al. 2007). Several other large genome-wide association studies (GWAS) confirmed this association with CAD and/or myocardial infarction (Helgadottir, Thorleifsson et al. 2007; Samani, Erdmann et al. 2007; 2007). Our group provided evidence that this locus promotes coronary atherogenesis, rather than plaque rupture, since the number of risk alleles are highly correlated to the severity of coronary artery disease but not to acute coronary syndrome among CAD cases defined by coronary angiography (Dandona, Stewart et al. 2010). Importantly, we and others identified regulatory sequences at this locus that controls the expression of the cyclin dependent

kinase inhibitors CDKN2A and CDKN2B (Jarinova, Stewart et al. 2009; Visel, Zhu et al. 2010; Harismendy, Notani et al. 2011). Since proliferation of vascular smooth muscle cells contributes to the formation of atherosclerotic lesions, reduced expression of the cyclin dependent kinase inhibitors would promote proliferation and be proatherogenic.

To date, more than 30 loci have been identified by GWAS, including 13 novel loci discovered in a large international consortium that our group formed called CARDIoGRAM (Schunkert, König et al. 2011). Importantly, using only 12 of these loci our group showed they could improve cardiovascular risk prediction beyond the traditional risk factors (Davies, Dandona et al. 2010). From the CARDIoGRAM consortium 4 of the 23 loci associated with CAD were found at or near genes encoding mitochondrial proteins (including MRPS6, CYP17A1, ATP5G1, and PEMT). Thus, altered mitochondrial gene expression and function are likely to be associated with increased CAD risk.

Considerable evidence shows that mitochondrial dysfunction contributes to the progression of coronary atherosclerosis (Madamanchi and Runge 2007). Mitochondria can fuse or divide by fission (McBride and Soubannier 2010), and this process is important in maintaining the mitochondrial membrane potential ($\Delta\Psi$). Mitochondria provide energy (ATP) to the cell through oxidative metabolism. During this process, mitochondria also produce reactive oxygen species (ROS) as a byproduct of respiration and energy production. ROS oxidize, denature and inactivate proteins. For this reason, mitochondria actively maintain a reduced state to eliminate ROS. In addition to SOD2 removing superoxide, mitochondria contain a specific thioredoxin (Trx2), a Trx2-dependent peroxidase (Prx3), and Trx2 reductase (TrxR2). These enzymes are critical for

maintaining the mitochondrial electrochemical potential required for ATP production (Hansen, Go et al. 2006). Indeed, cells with deficiency or knockdown of Trx2 or Prx3 accumulate endogenous ROS and are highly sensitive to exogenous oxygen radicals (Tanaka, Hosoi et al. 2002; Chang, Cho et al. 2004). Conversely, transgenic mice over-expressing Trx2 protein in endothelial cells showed increased endothelial cell capacity to scavenge ROS and reduced atherosclerotic lesions in the apolipoprotein E-deficient mouse model (Zhang, Luo et al. 2007). Protein oxidation, especially at methionines to form methionine sulfoxide, is reversible, thanks to the enzyme methionine sulfoxide reductase (MSRA) located in the mitochondrial matrix (Hansel, Kuschel et al. 2002). However, once a matrix protein is oxidized beyond a certain threshold, it becomes unfolded and targeted for degradation by the proteases AFG3L2 and SPG7. Thus, over the lifetime of an organism, differences in the ability to remove ROS or how fast oxidized proteins are degraded could affect the development of coronary atherogenesis.

ROS production is involved in many processes that play a role in the pathogenesis of atherosclerosis (Harrison, Griendling et al. 2003). Several enzyme systems produce these free radicals and involved in atherogenesis like nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxidase and nitric oxide synthase. However, the mitochondrion is the main source of ROS production. Increased levels of ROS in the mitochondria by overexpression of ROS modulator 1(Romo1) was found to be critical for the proliferation of normal and cancerous cells and its downregulation blocked cell proliferation (Na et al, 2008). The increased cellular proliferation within the atherosclerotic plaque is proved in the vascular smooth muscle and macrophages and confirmed by the animal studies (Chisolm and Chai 2000), (Lamharzi, Renard et al.

2004), (Liu, Saini et al. 1995). Interestingly, the oxidation of LDL as a risk factor for CAD was also shown to upregulate the levels of the cyclin dependent kinases (CDKs) (Zettler et al, 2003). Therefore, buffering ROS levels in the mitochondria plays an important role in controlling cell proliferation.

SPG7 (paraplegin) is a mitochondrial matrix protease that removes damaged proteins and maintains mitochondrial integrity and function. SPG7 that contains M14 type peptidase and ATPase domains (Langer 2000) encodes a mitochondrial protease located on the matrix side of the inner mitochondrial membrane whose functions are to degrade damaged mitochondrial proteins and to promote synthesis of mitochondrial proteins by controlling the processing of the mitochondrial ribosomal subunit MRPL32 and thereby controlling ribosome assembly (Nolden, Ehses et al. 2005). Mutations in SPG7 (also called paraplegin) are associated with hereditary spastic paraplegia (hence the name), a neurodegenerative disorder characterized by progressive weakness and spasticity of the lower limbs due to degeneration of corticospinal axons (Casari, De Fusco et al. 1998). SPG7 forms likely a hexameric protease complex with the related protease AFG3L2 to form the m-AAA protease (Atorino, Silvestri et al. 2003; Koppen, Metodiev et al. 2007). AFG3L2 cleaves and produces the mature active form of SPG7 before the formation of the haxameric complex (Koppen, Bonn et al. 2009). Mutations in AFG3L2 are associated with spinocerebellar ataxia (Casari, De Fusco et al. 1998; Di Bella, Lazzaro et al. 2010; Edener, Wollner et al. 2010).

The yeast orthologue of the m-AAA protease is composed of highly conserved subunits named Yta10 and Yta12 subunits that are required for healthy yeast respiratory growth (Arlt, Steglich et al. 1998). Growing on non-fermentable carbon source such as

glycerol that requires the aerobic respiration was impaired in yeast lacking these subunits (Arlt, Steglich et al. 1998). This growth arrest was rescued by expressing the human m-AAA protease that contains SPG7 and AFG3L2 subunits (Koppen, Metodiev et al. 2007).

Here, I report on the identification and characterization of a genetic variant that alters the sequence of SPG7 and associates with the risk of CAD. I show that this variant is a gain of function that increases processing of SPG7 to its mature active form, promotes mitochondrial fusion, augments ROS production, and increases cellular proliferation (the latter are biological hallmarks of atherosclerosis and coronary artery disease). My findings implicate altered mitochondrial function in the risk for coronary artery disease and show for the first time the involvement of an SPG7 variant in the risk of this disease.

Material and methods:

Genome-wide association studies:

All participants gave written informed consent according to study protocols approved for each of the 13 GWAS as described extensively in the CARDIoGRAM study (Schunkert, König et al. 2011). Our study followed the STREGA guidelines in reporting on genetic association (Little, Higgins et al. 2009). Within each study, quality control of the data was performed. This includes a check of consistency of the given alleles across all studies, deviation for Hardy-Weinberg equilibrium in the controls, the minor allele frequency, and the SNP call rate. Population stratification was evaluated using either the genomic control or a principal components analysis approach. The association between CAD (MI) and genotypes was carried out under an additive model adjusted for age and sex. Meta-analysis was performed using fixed-effect models with inverse-variance weighting. The Cochrane's Q and I^2 statistics were also calculated to test the heterogeneity across studies. Heterogeneity was assumed if I^2>50%. Random-effect models were used for the SNPs with evidence of heterogeneity.

Plasmids constructs and chemicals:

The R688 common form of human SPG7 (clone BC036104) was purchased from Open Biosystems (Huntsville, AL). The variant Q688 form was generated by site-directed mutagenesis using the Stratagene QuickChange XL site-directed mutagenesis kit according to the manufaturer's protocol (Agilent Technologies, Santa Clara, CA). The R688 and Q688 variant SPG7 cDNA was cloned in frame into the pCVM-tag4 vetor and SPG7 (R688 and Q688) cDNA was released from pCMV by NotI and Mlu I double

digestion and then subcloned into pLEX vector (Open Biosystems) using the same sites to generate pLEX-SPG7 constructs. All constructs were verified by sequencing.

Mdivi-1 (sc-215291A, Santa Cruz biotechnology) was used at a concentration of 50 μM. ROS scavengers NAC (A7250, Sigma) and Tiron (172553, Sigma) were used at a concentration of 10 mM .

Tissue culture and stable and transient transfections:

Human Embryonic Kidney cells (HEK 293) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in high glucose DMEM with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were transfected with linearized pLEX-SPG7-Flag constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 hours of transfection cells were passaged at 1:20 dilution into fresh growth medium (DMEM containing 20% fetal bovine serum) with 2 μg/ml puromycin to highly select for positive clones. Medium containing 2 μg/ml puromycin was changes every 2 days for 4 weeks. After selection of stable cells, cells were maintained in DMEM containing 20% fetal bovine serum. Stable HEK293 cells bearing the R688 and Q688 variant of SPG7 were transiently transfected with pQCXIP-N-Venus-Z-N-Rluc using Lipofectamine 2000 for the mitochondrial fusion experiments.

Protein Lysates and Western Blot Analyses:

Lysates from cells expressing either the mutant or wild type allele were collected with RIPA buffer (4.25 mM Tris, pH8.0, 135 mM NaCl, 1% IGEPAL CA-630, 1% SDS 0.5% deoxycholate) in the presence of protease and phosphatase inhibitors (Roche Molecular Systems, Branchburg, NJ).Anti-OPA1 (612607, BD Transduction Laboratories), anti-

MFN2(H00009927, Abnova), anti-cyclinD1(#2926, Cell Signalling), anti-PCNA(sc-56, Santa Cruz Biotechnology), ant-p21(sc-6246, Santa Cruz Biotechnology), anti- p27(sc-1641, Santa Cruz Biotechnology), anti-COX1(sc-58347, Santa Cruz Biotechnology), anti-COX2(sc-65239, Santa Cruz Biotechnology), anti-COX4(sc-59348), anti-GAPDH(sc-59540, Santa Cruz Biotechnology), anti-α-tubulin (sc-23948, Santa Cruz Biotechnology) were used 1:1000 dilutions in blocking solutions. Rabbit anti SPG7 (sc-135026, Santa Cruz Biotechnology), anti AFG3L2 (sc-84687, Santa Cruz Biotechnology), anti cyclinE (sc-481, Santa Cruz Biotechnology), anti- COX3 (ab81180, Abcam), anti-p-RB(ser799)(#93015, Cell Signaling), anti p-AMPK-Thr172 (#2531, Cell Signaling), anti-hMRPL32(c14071, Assay Bio Tech) were used at 1:5000 dilution. Goat anti-mouse IgG (HAF007, R&D systems, Minneapolis, MN) and goat anti-rabbit IgG (product #: 31460, Piercenet, Rockford, IL) were diluted in 1:15,000 in blocking solution before application.

Confocal imaging:

ROS production was monitored using the oxidation-sensitive dye HEt. living cells stably expressing the Q688 and R688 variant and non transfected cells (NT) were grown on coverslips in 24 well plates then incubated with 100nM MitoTracker (Green) and 5 μM HEt (Red) in the presence of 1 M HEPES buffer for 10 minutes and then were visualized by the confocal microscope. A clear staining of the nucleoli of the Q688 cells indicating high ROS level compared to R688 and NT cells.

Bromodeoxyuridine (BrdU) staining and FACS analysis:

Flow cytometry were used to determine cells proliferation on cells labeled with anti BrdU-FITC and propidium iodide (PI). Cells stably expressing the wild type or the

variant form of SPG7 and cells not transfected were plated at equal densities and grown for 48 hours at 37°C. After 48 hours, cells were synchronized for 24 hours by serum withdrawal and then released with fresh serum containing media. Then, the cells were pulse labeled with by 10uM of BrdU, for determination of newly synthesized DNA, for 45 minutes prior to harvest the cells for FACS analysis. As a negative control, no-BrdU was included to cells. For FACS analysis the number of live cells was determined by trypan blue exclusion. 1 x 106 BrdU-pulsed cells were collected and stained intracellularly with anti BrdU-FITC conjugate using the BrdU Flow kit (559619, BD Pharmingen™) according to the manufacturer's instructions. Instead of 7-amino-actinomycin D (7-AAD) I add 10 mg/ml PI. Stained cells were acquired on a CyAn ADP analyser (Beckman Coulter) and analyzed using Kaluza® software (Beckman Coulter)

Mitochondrial purification and isolation:

Stable cells were grown 10 x 100 mm dishes with DMEM medium containing 20% FBS, 100U/ml penicillin, 100 µg/ml streptomycin for 2 days. Cells were washed 3x with 1xPBS, trypsinized, centrifuged at 3000 x g at 4°C for 10 minutes. Pellets were washed and mitochondria and cytosol were isolated as described by (Schauss, Huang et al. 2010). Mitochondrial and cytosol concentrations were determined with the standard Bradford assay.

Determination of Mitochondrial H^+-ATPase activity:

This experiment was measured by fluorescence bioluminescence as described by Qian et al. (Qian, Song et al. 2004). Mitochondria were adjusted to a protein concentration of 1 µg/µl and 10 µl of mitochondria were mixed with 90 µl of the standard reaction solution that is prepared as instructed by the kit but in absence of ATP. Addition of ADP (Sigma,

A2754) to the reaction solution will trigger the conversion of ADP to ATP by H^+-ATPase of the mitochondria then the intensity of the emitted light was assayed on a luminometer (Montreal- Biotech Inc., Montreal, QC, Canada) following the manufacturer's instructions. The amount of ATP in the experimental samples was calculated from the standard curve.

Interacellular reactive oxygen species (ROS) measurement:

The OxiSelect™ ROS Assay Kit (STA-342, Cell Biolabs Inc., San Diego, CA) is used to assay ROS activity in cultured stable cells and non transfected control cell. Cells cultured in a 96-well cell culture plate (Falcon, NJ, USA) pre-incubated with DCFH-DA, which is cell permeable. After a brief incubation for 1 hour, the reaction was terminated by addition of lysis buffer for 5 minutes and then, 100 µl total volumes of the media were transferred to 96-well plate (Corning, NY,USA) and read on a standard fluorescence plate reader at 450 nm in SpectraMax M2e spectrophotometer (Molecular Devices, Sunnyvale, Ca, USA). The ROS content in the samples was determined by comparison with a predetermined DCF standard curve.

Mitochondrial fusion assay:

The *in vitro* fusion reaction was carried out in as described previously (Schauss, Huang et al. 2010). Mitochondria from HEK293 cells stably expressing either R688 or Q688 SPG7 and transfected with N-MitoVZL were combined with mitochondria of stably expressing N-MitoLZV from Hela cells (50 µg each) and were added to the reaction, where the final concentrations of reagents in the reaction were: 10mM HEPES pH 7.4, 110 mM Mannitol, 68mM sucrose, 80mM KCL, 0.5mM EGTA, 2mM $Mg(CH_3COO)_2$, 0.5mM GTP, 2 mM K_2HPO_4, 1 mM ATP(K+), 0.08 mM ADP, 5 mM Na succinate and 1 mM

DTT. Following a 30 minute fusion reaction, trypsin was added for 20 minutes, followed by SBTI for 15 minutes. Mitochondria were then lysed and placed in 96 well plates where the coelenterazine substrate was injected directly into the reaction in a Glomax Luminometer (Promega, WI, USA), following the manufacturer's instructions. The bioluminescence photon counts were detected over an integral of 10 s and the data were analyzed with Excel.

Results

Discovery of the SPG7 Q688 CAD risk variant.

I sought to identify genetic variants associated with coronary artery disease (CAD) that modify protein sequence. An early candidate that showed strong association in the Ottawa Heart Genomics Study GWAS was identified on chromosome 16 in the vicinity of the gene for paraplegin, SPG7 (Fig. 1). The OHGS was designed to discover genes that contribute to the risk of CAD independent of type 2 diabetes mellitus, so that individuals with diabetes were excluded. A consistent association with CAD was found by meta-analysis of 13 GWAS in the CARDIoGRAM consortium that revealed an locus with a combined association of $p=9.6x10^{-7}$, just below the threshold for genome-wide significance. A cluster of single nucleotide polymorphisms was identified that linked to a variant that alters an arginine residue in the protease domain of SPG7. These SNPs are linked to rs12960, a polymorphism that changes an arginine at position 688 and replaces it with a glutamine residue in the protease domain (Fig. 2). This polymorphism is unique to humans, since a search of all available vertebrate genomes failed to uncover this variant. It is also noteworthy that this sequence is highly conserved among all vertebrates.

SPG7 protein processing and maturation is enhanced in patients who carry the Q688 allele.

SPG7 undergoes 2 maturation steps. Upon import into the mitochondrial matrix, the N-terminal signal peptide is cleaved by MPP (Koppen, Bonn et al. 2009). A second proteolytic cleavage removes an additional N-terminal fragment upon assembly of SPG7 into the SPG7/AFG3L2 heterohexamer (Koppen, Bonn et al. 2009). Since SPG7 processing is

Fig. 1 SPG7 associates with coronary artery disease risk in the CARDIoGRAM consortium. **A.** Meta-analysis of 13 GWAS combining genotype and imputed SNP data from over 22,000 CAD cases and 60,000 controls identified the SPG7 locus as highly associated with CAD ($p=9x10^{-7}$). Note the consistency for association (heterogeneity, $I^2=0$). **B.** Cluster of linked SNPs around the SPG7 gene genotyped in CARDIoGRAM.

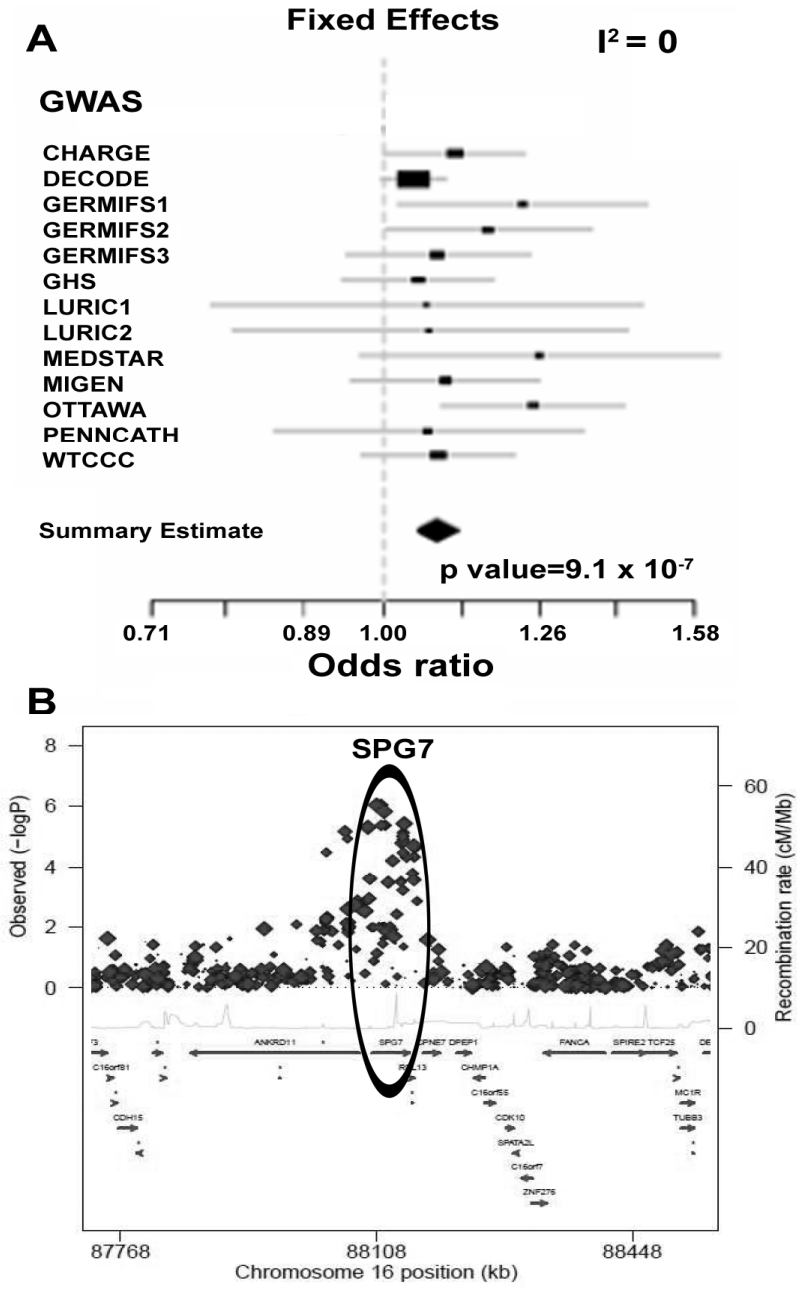
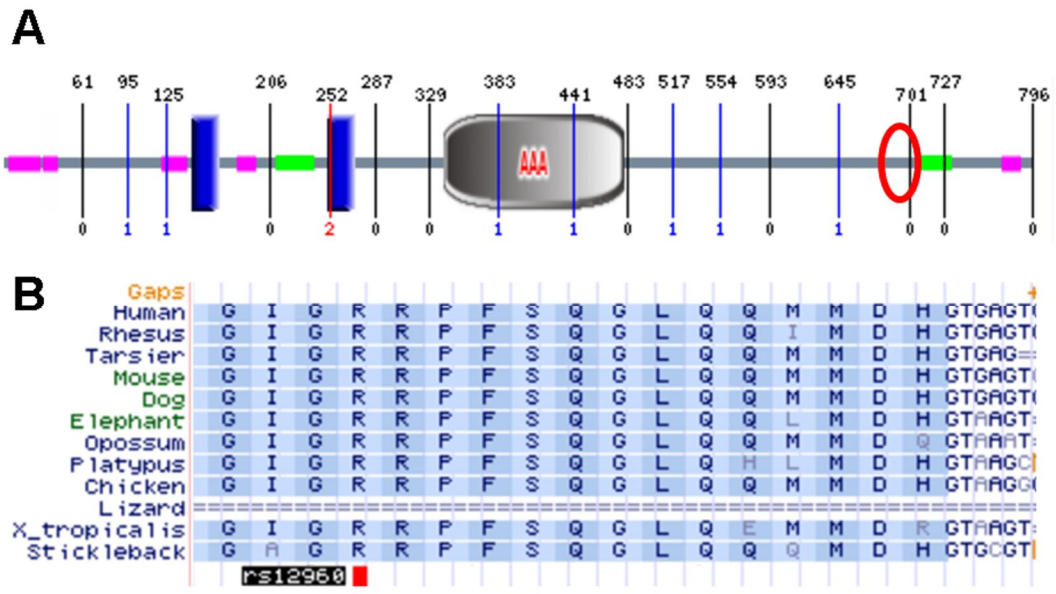

A
Fixed Effects
I² = 0
GWAS
CHARGE
DECODE
GERMIFS1
GERMIFS2
GERMIFS3
GHS
LURIC1
LURIC2
MEDSTAR
MIGEN
OTTAWA
PENNCATH
WTCCC
Summary Estimate
p value=9.1 x 10-7
0.71
0.89
1.00
1.26
1.58
Odds ratio
B
SPG7
Observed (−logP)
Recombination rate (cM/Mb)
87768
88108
88448
Chromosome 16 position (kb)

Fig. 2 Arginine 688 is conserved throughout the vertebrate lineage. **A.** Diagram of the SPG7 protease showing transmembrane domains in blue, low complexity sequence in pink, coiled coil sequence in green. Amino acid positions of the exon/intron boundaries are indicated with vertical lines. Red circle shows sequence highlighted in B. **B.** SNP rs12960 replaces arginine 688 (R688) with a glutamine, a polymorphism unique to humans.


A
61 95 125 206 252 287 329 383 441 483 517 554 593 645 701 727 796
0 1 1 0 2 0 0 1 1 0 1 1 0 1 0 0 0
AAA
B
Gaps
Human G I G R R P F S Q G L Q Q M M D H GTGAGT
Rhesus G I G R R P F S Q G L Q Q I M D H GTGAGT
Tarsier G I G R R P F S Q G L Q Q M M D H GTGAG
Mouse G I G R R P F S Q G L Q Q M M D H GTGAGT
Dog G I G R R P F S Q G L Q Q M M D H GTGAGT
Elephant G I G R R P F S Q G L Q Q L M D H GTAAGT
Opossum G I G R R P F S Q G L Q Q M M D Q GTAART
Platypus G I G R R P F S Q G L Q H L M D H GTAAGC
Chicken G I G R R P F S Q G L Q Q M M D H GTAAGC
Lizard
X_tropicalis G I G R R P F S Q G L Q E M M D R GTAAGT
Stickleback G A G R R P F S Q G L Q Q Q M D H GTGCGT
rs12960

necessary to activate its protease activity, I examined the pattern of SPG7 processing by immunoblot analysis in peripheral blood lymphocytes (PBLs) from genotyped individuals. Processing of the SPG7 protein to its shorter mature and proteolytically active form was highly significantly correlated with the gene dosage of the Q688 variant; nearly 90% of SPG7 is fully processed in patients homozygous for the Q688 variant (**Fig. 3A,B**). Primary cultures of human aortic smooth muscle cells were purchased and genotyped for the Q688 variant and the same correlation with increased processing of SPG7 was detected in the 2 samples heterozygote for the Q688 variant (**Fig. 3C**). This result strongly suggests that the Q688 variant affects SPG7 processing.

Stable HEK293 cells expressing the Q688 SPG7 variant also show increased SPG7 maturation.

To functionally characterize the Q688 variant of SPG7, human embryonic kidney (HEK293) cells were stably transfected with lentiviral vectors expressing the wild type allele of SPG7 and the CAD risk variant Q688 (**Fig. 4**). Isolated mitochondria showed that over-expression of wild type SPG7 did not affect its processing whereas over-expression of the Q688 variant markedly increased the levels of processed mature SPG7. Thus, these cells replicate the phenotype (i.e., increased levels of mature SPG7) observed in peripheral lymphocytes from CAD patients that carry the Q688 allele. Furthermore, this result makes it less likely that increased processing of Q688 SPG7 variant is due to a factor extrinsic to the variant itself. It is noteworthy that AFG3L2 levels were elevated in cells expressing the Q688 variant, compared to other loading

Fig. 3 Proteolytic processing of the Q688 SPG7 variant in human peripheral blood lymphocytes and aortic smooth muscle cells. **A**. Peripheral blood lymphocytes from CAD patients genotyped for the rs12960 SNP reveal increased proteolytic processing of SPG7 in Q688 homozygotes (QQ) compared to R688 homozygotes (RR). Note the increased levels of mature form (m) over the precursor form (p) in a representative blot. **B**. Quantification of immunoblots, normalized to GAPDH levels, reveals an allele dosage association with the level of processed SPG7. RR, (n=14), RQ, heterozygotes (n=4) and QQ, homozygotes for the Q688 allele (n=9). **C.** Primary cultures of human aortic smooth muscle cells (HAoSMC), purchased from Cell Applications, Inc. (San Diego, CA) and genotyped for the rs12960 SNP, also revealed elevated levels of mature SPG7 in heterozygotes (RQ) compared to homozygotes for the common allele (RR).

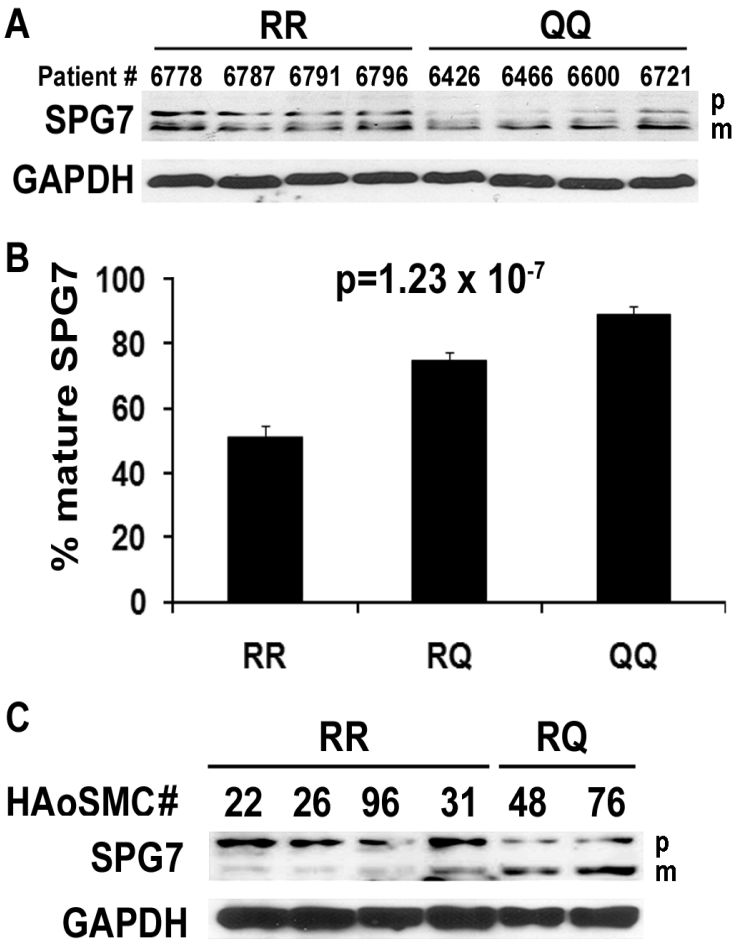
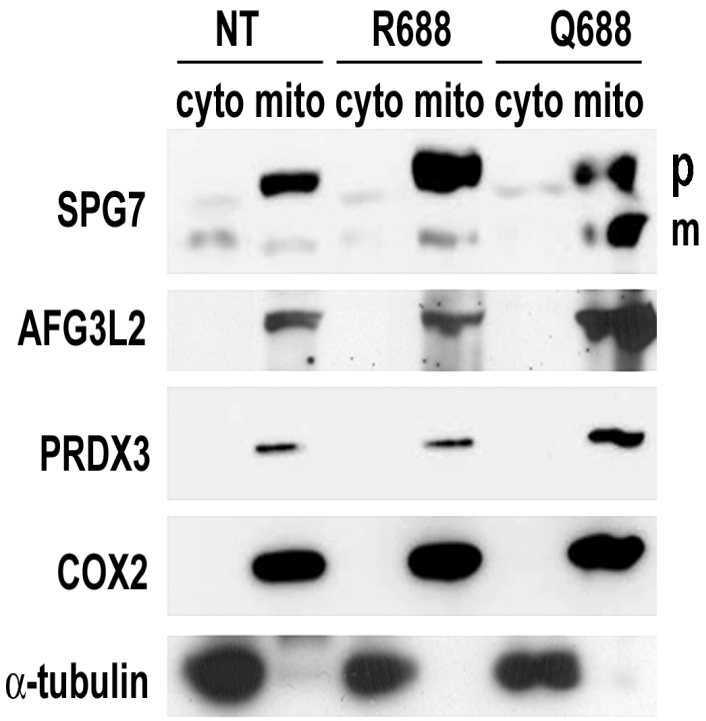

A
RR
QQ
Patient # 6778 6787 6791 6796 6426 6466 6600 6721
SPG7
p
m
GAPDH
B
% mature SPG7
100
80
60
40
20
0
p=1.23 x 10-7
RR
RQ
QQ
C
RR
RQ
HAoSMC# 22 26 96 31 48 76
SPG7
p
m
GAPDH

Fig. 4 Stably transformed human embryonic kidney cells expressing the Q688 variant show increased processing of SPG7. Mitochondria were isolated from non-transfected (NT) HEK293 cells or cells stably expressing either the R688 common allele or the Q688 allele and then quantified and analyzed on SDS-page and immunoblotted to show the level of SPG7 processing. Non-transfected HEK293 cells are homozygous for the R688 allele (data not shown). Cells expressing the Q688 variant showed increased levels of mature SPG7 compared to cells stably expressing the common allele (R688) or non-transfected cells (NT). AFG3L2 levels were increased in cells expressing the variant, as were the levels of PRDX3. Cytochrome c oxidase subunit 2 (COX 2) and α-tubulin were used as mitochondrial and cytosolic loading control protein markers, respectively.


NT
R688
Q688
cyto mito cyto mito cyto mito
SPG7
p
m
AFG3L2
PRDX3
COX2
α-tubulin

controls, as was the expression of peroxiredoxin 3 (PRDX3), a mitochondrial antioxidant protein that scavenges H_2O_2 in cooperation with thiol and peroxynitrite (Bryk, Griffin et al. 2000).

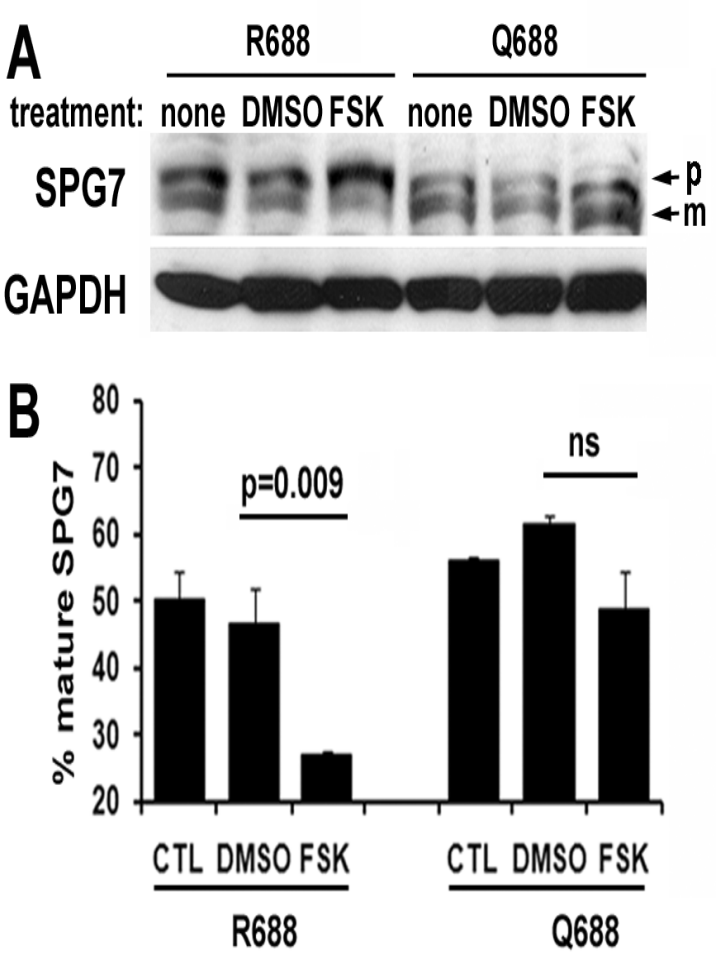
Forskolin blocks processing of R688 but not Q688 variant of SPG7

Processing of SPG7 is known to depend upon co-assembly with AFG3L2 (Koppen, Bonn et al. 2009). Since I observed increased processing of the Q688 variant of SPG7, I asked whether the metabolic state might influence the degree of SPG7 processing. Forskolin, a compound that activates protein kinase A, is known to activate lipolysis and to promote oxidative metabolism (Yehuda-Shnaidman, Buehrer et al. 2010). Cells were stimulated with Forskolin and I observed marked inhibition of SPG7 processing in HEK293 cells expressing the R688 variant (**Fig. 5**). To our knowledge, this is the first evidence to suggest that processing of SPG7 is dynamically regulated. Remarkably, Forskolin had no effect on processing of the Q688 variant, suggesting that the mechanism that blocks SPG7 processing is not effective for the Q688 variant.

The Q688 SPG7 variant increases ATP production.

Since SPG7 requires ATP for its protease activity (Langer 2000; Augustin, Gerdes et al. 2009), I asked whether increased processing and protease activity of the SPG7 Q688 variant would increase energy demand and affect ATP production. I performed immunoblot analysis of phospho-AMPK from whole cell extracts of stably transfected cells. AMPK is a cellular energy sensor that is phosphorylated when the ratio of ATP to ADP is low and ATP levels are inadequate. I observed markedly reduced levels of phosphorylated AMPK in cells expressing both the wild type R688 and the Q688 variant

Fig. 5 The Q688 variant escapes PKA-dependent regulated processing of SPG7. A. Immunoblot analysis of SPG7 processing. The PKA-activating compound Forskolin (FSK) blocked processing of R688 SPG7 from the premature to the mature form whereas processing of the Q688 variant was unaffected. This result indicates that the Q688 variant is not affected by a PKA-dependent modification controlling processing of SPG7. **B.** Quantitation of SPG7 processing. n=3 experiments.


A
R688
Q688
treatment: none DMSO FSK none DMSO FSK
SPG7
p
m
GAPDH
B
% mature SPG7
80
70
60
50
40
30
20
p=0.009
ns
CTL DMSO FSK
CTL DMSO FSK
R688
Q688

of SPG7 (**Fig. 6A**). I next measured ATP synthesis from mitochondria. Consistent with the phosphor-AMPK result, I found elevated ATP synthesis in cells over-expressing the wild type form of SPG7, and even more so in cells expressing the Q688 variant of SPG7 (**Fig. 6B**).

The Q688 SPG7 variant augments mitochondrial fusion

ATP output of networked mitochondria has been reported to be greatly increased in hyperfused mitochondria (Mitra, Wunder et al. 2009; Tondera, Grandemange et al. 2009). Thus, I asked whether increased ATP production caused by the over-expression the Q688 variant of SPG7 was accompanied by mitochondrial hyperfusion. Using the vital fluorescent dye Mitotracker®, I observed markedly fused mitochondria in HEK293 cells expressing the Q688 variant of SPG7 compared to HEK293 cells expressing the R688 form (**Fig. 7A**). Using an *in vitro* mitochondrial fusion assay (Schauss, Huang et al. 2010), I found that mitochondrial fusion was increased only in cells expressing the Q688 variant of SPG7 (**Fig. 7B**). Mitochondrial fusion can be stimulated *in vitro* with the dynamin-related protein 1 (DRP1) inhibitor mdivi-1 (Cassidy-Stone, Chipuk et al. 2008). I treated untransfected and R688 SPG7 stably-transfected HEK293 cells with mdivi-1 and found that it increased mitofusin 2 levels (**Fig. 7C**). In contrast, mitofusin 2 levels were already elevated in Q688 SPG7 expressing HEK293 cells, consistent with increased mitochondrial fusion, and were not further elevated by mdivi-1 treatment.

Fig. 6 Over-expressing Q688 and R688 alleles of SPG7 elevates ATP production in HEK293 cells. A. Immunoblot analysis showed that phospho-AMPK levels are lowest cells expressing the Q688 variant. Phospho-AMPK is inversely related to the energy status of cells (n=3 experiments). **B.** ATP synthesis was measured by a luciferase assay using isolated mitochondria from nontransfected HEK293 cells (NT), cells stably transfected with the common (R688) and the variant (Q688) forms of SPG7 incubated for 1minute with D-luciferin, ADP and luciferase. ATP synthesis was significantly higher in Q688 than R688 expressing cells. N=6 experiments. Asterisk, $p<4x10^{-7}$; double asterisks, $p<5x10^{-4}$ compared to R688.

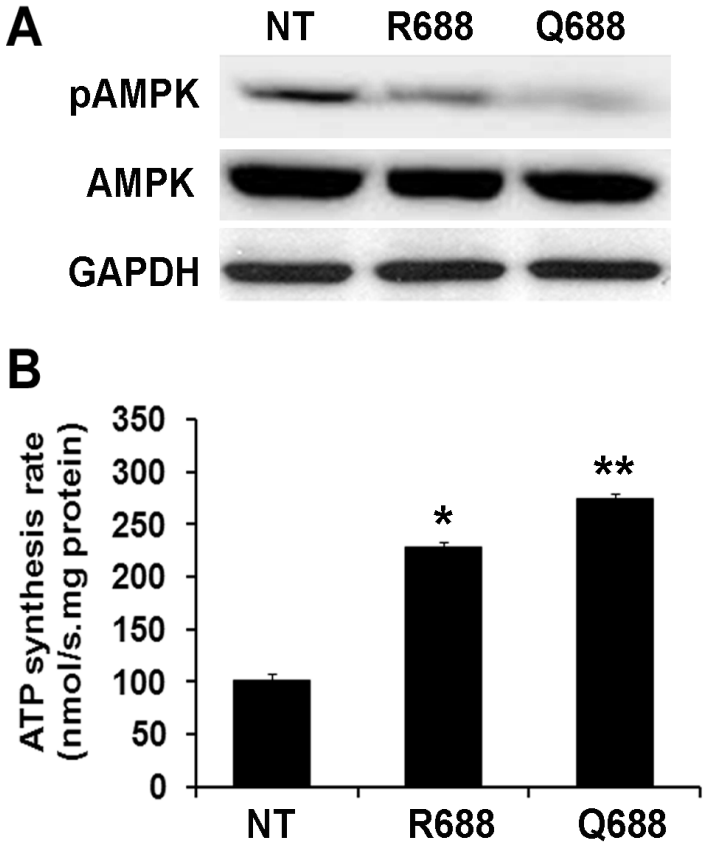
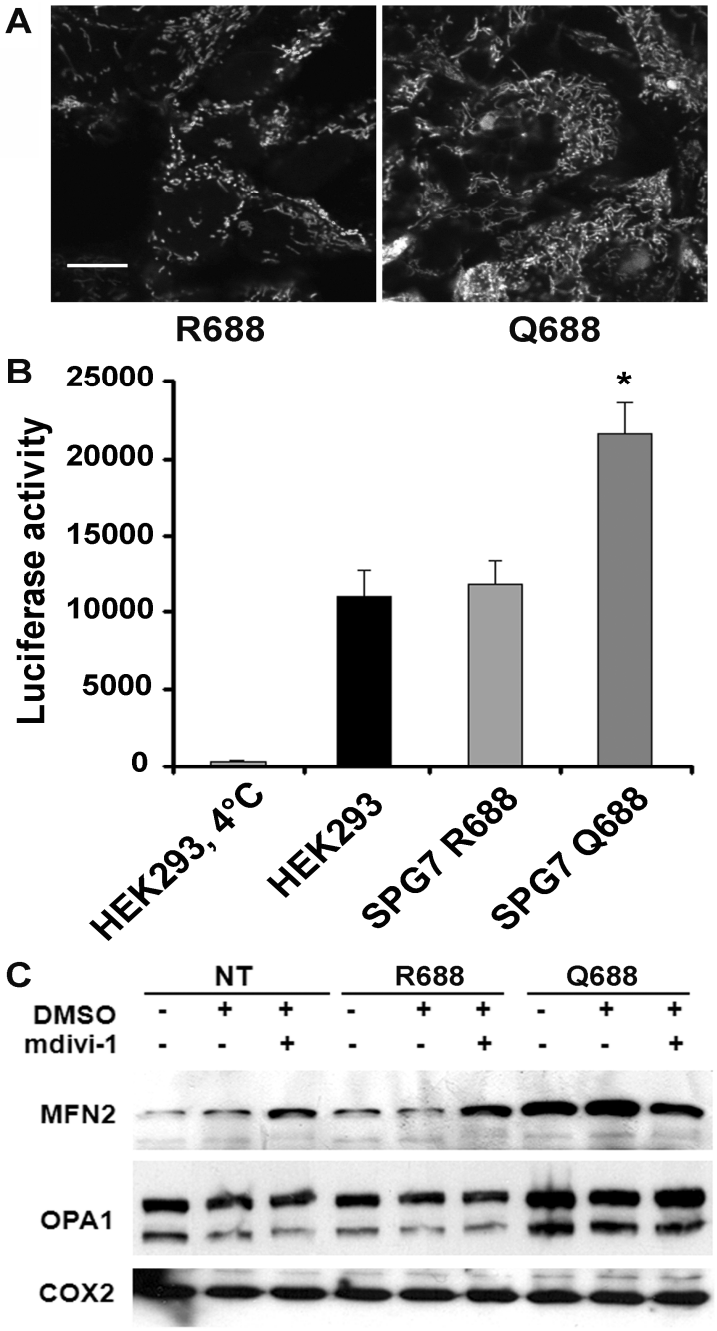

A
NT
R688
Q688
pAMPK
AMPK
GAPDH
B
ATP synthesis rate
(nmol/s.mg protein)
350
300
250
200
150
100
50
0
*
**
NT
R688
Q688

Fig. 7 Q688 SPG7 variant increases mitochondrial fusion. **A.** Mitochondria of HEK293 cells expressing the Q688 variant appeared hyperfused compared to those of cells expressing R688 or non-transfected cells (not shown). Mitochondria were labeled with Mitotracker®. Scale bar, 20 μm. **B.** A mitochondrial fusion assay (Schauss, Huang et al. 2010) revealed markedly elevated fusion in the cells expressing the Q688 variant (n=6, asterisk $p<10^{-5}$). **C.** Immunoblot analysis of whole cell extracts showed that the DRP1 inhibitor mdivi-1 (50 μM) that induces mitochondrial hyperfusion of non-transfected and R688 expressing HEK293 cells elevated mitofusin 2 (MFN2) expression. Importantly, MFN2 levels were already elevated in cells expressing the Q688 variant consistent with their mitochondria being hyperfused. OPA1 expression was elevated in the cells expressing the Q688 variant, but the short isoforms of OPA1 were similarly reduced by mdivi-1 treatment in all cells. Cytochrome c oxidase subunit 2 (COX 2) levels were unaffected and were used as a loading control.


A
R688
Q688
B
Luciferase activity
25000
20000
15000
10000
5000
0
*
HEK293, 4°C
HEK293
SPG7 R688
SPG7 Q688
C
NT
R688
Q688
DMSO
mdivi-1
MFN2
OPA1
COX2

Over-expression of the Q688 SPG7 variant alters the stoichiometry of components of the electron transport chain.

The mitochondrial ribosome protein MRPL32 is a natural target of the SPG7/AFG3L2 protease complex. Loss of function in the m-AAA protease caused by inactivating mutations in AFG3L2 halts mitochondrial protein translation as a result of impaired processing of the MRPL32 precursor into its active ribosomal form leading to deficiency of respiratory chain complex IV (Di Bella, Lazzaro et al. 2010) . The gain of function observed for the Q688 variant of SPG7 was expected to have the opposite effect. Indeed, I found that MRPL32 expression is enhanced in cells expressing the Q688 variant of SPG7 (**Fig. 8**). Surprisingly, although the mitochondrially encoded Cox1 and Cox3, as well as the nuclear encoded Cox4 subunits of complex IV were elevated in cells over-expressing the Q688 variant of SPG7, Cox2 levels were not affected.

The Q688 SPG7 variant increases ROS production.

Altered electron transport chain protein stoichiometry has been associated with elevated reactive oxygen species (ROS) release from mitochondria. ROS production was visualized by a fluorescence microscopy assay where hydroethidium (Het) is converted to ethidium and accumulates in the nucleus of cells actively producing ROS. Cells expressing the Q688 variant of SPG7 had markedly elevated ROS production compared to those expressing the wild type allele (**Fig. 9A**). Elevated ROS production was also confirmed by a colorimetric assay (**Fig. 9B**). Increased ROS production in cells expressing the Q688 variant would be expected to increase the risk of coronary artery disease.

Fig. 8 Altered stoichiometry of complex IV subunits with Q688 over-expression. Cell extracts from non transfected HEK293 cells or cells stably expressing the Q688 and R688 alleles were analyzed by immunoblot to determine the effect of SPG7 over-expression. Increased levels of MRPL32 indicated increased processing of this ribosomal subunit of mitochondria. However, expression COX2 was not coordinately upregulated with COX1, COX3, and COX4 in cells expressing the Q688 variant. Antibody to GAPDH was used to verify protein loading.

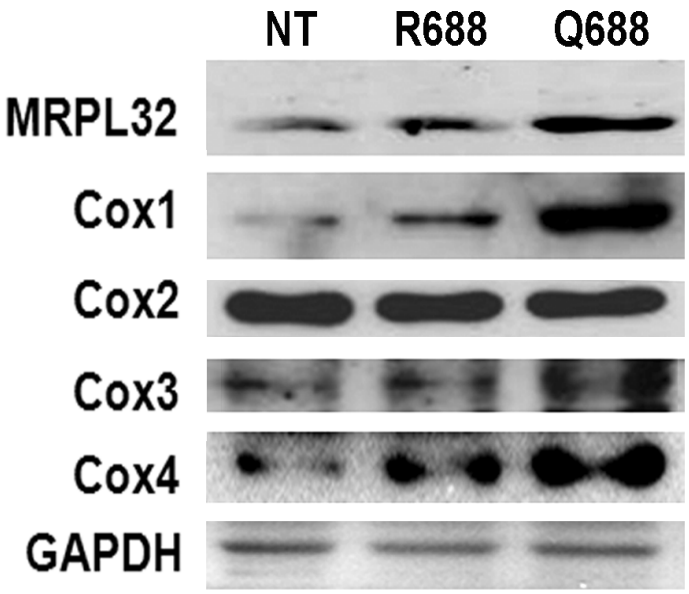
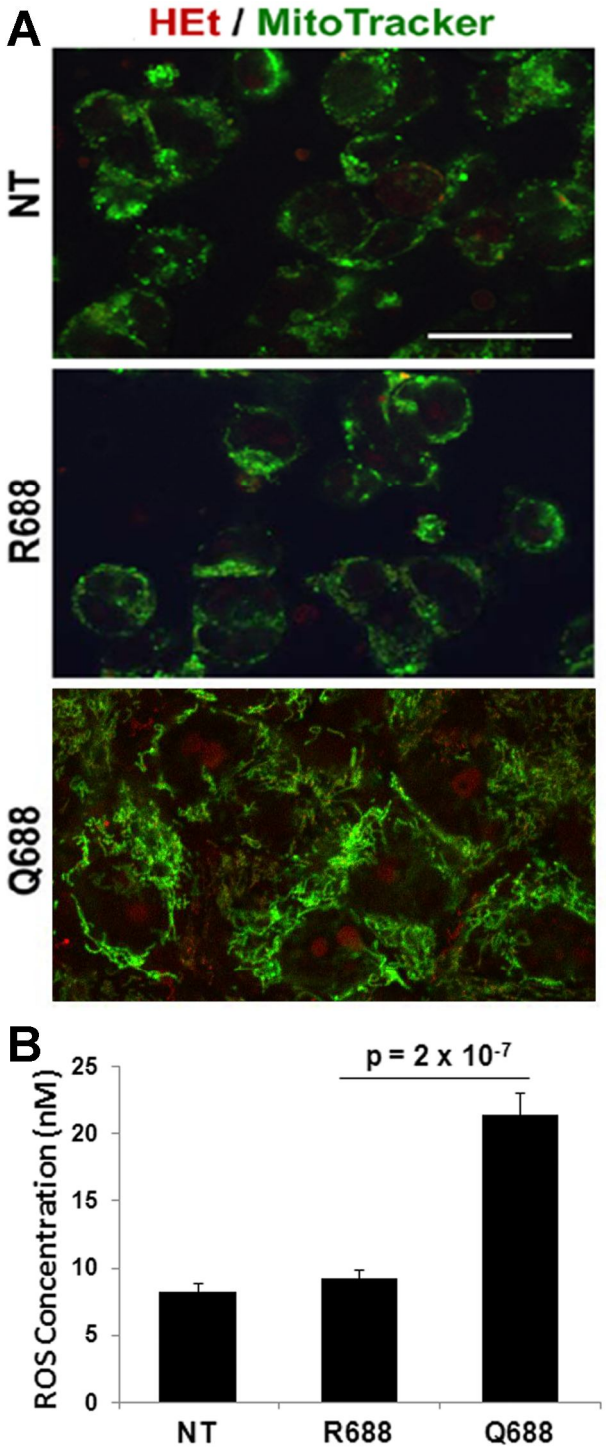

NT
R688
Q688
MRPL32
Cox1
Cox2
Cox3
Cox4
GAPDH

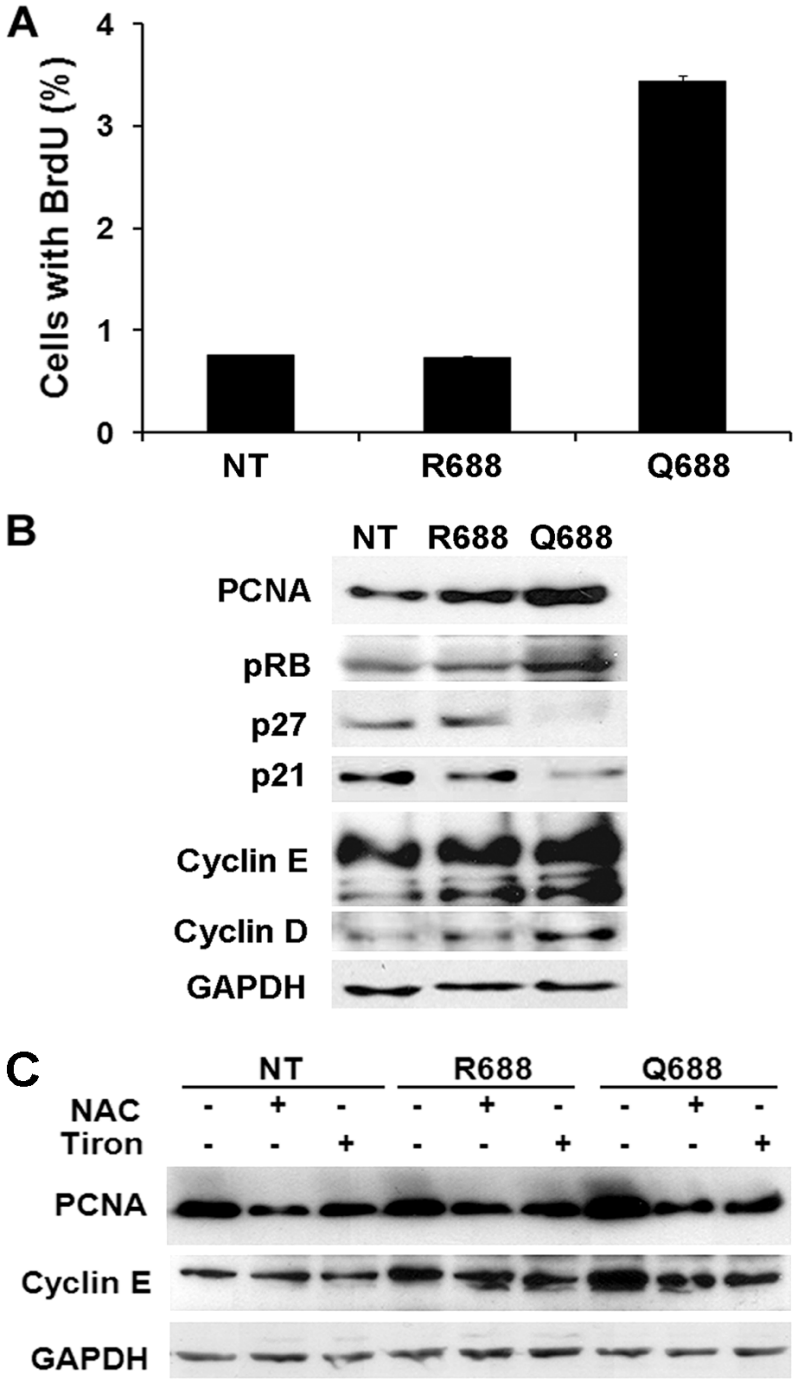
Fig. 9 Cells expressing the Q688 SPG7 variant have significantly higher production of reactive oxygen species (ROS). A. ROS release from mitochondria was visualized using hydroethidium (HEt, red) that accumulates in the nucleus upon conversion to ethidium. **B.** Intracellular ROS levels measured using the OxiSelect™ ROS Assay were highly elevated in cells expressing the Q688 variant. n=3 experiments.


A
HEt / MitoTracker
NT
R688
Q688
B
p = 2 x 10-7
ROS Concentration (nM)
25
20
15
10
5
0
NT
R688
Q688

The Q688 SPG7 variant increases cell proliferation.

A recent study has shown that inducing mitochondrial hyperfusion by acute inhibition of DRP1 causes quiescent cells to begin replicating their DNA and coincides with buildup of cyclin E (Mitra, Wunder et al. 2009). HEK293 cells expressing the Q688 variant had elevated cell proliferation, measured by flow cytometry after BrdU incorporation (**Fig. 10A**). Markers of cellular proliferation, like the Proliferating Cell Nuclear Antigen (PCNA), cyclin E and cyclin D and the level of phosphorylated retinoblastoma (pRB) were all elevated, whereas the cyclin-dependent kinase inhibitors p27 and p21 were both suppressed in cells expressing Q688 SPG7 (**Fig. 10B**). Since increased ROS production has been linked to increased cell proliferation (Deng, Gao et al. 2003), I asked whether ROS scavengers could block cell proliferation. N-acetyl cysteine (NAC) and Tiron both reduced PCNA and cyclin E levels, and the effect was most pronounced in cells over-expressing Q688 SPG7 (**Fig. 10C**). Dysregulated and increased cell proliferation are hallmarks of atherosclerotic lesions (Guevara, Kim et al. 1999).

Fig. 10 Cells expressing the Q688 variant are more highly proliferative than cells expressing the common R688 allele. A. FACS analysis of BrdU incorporation of synchronized cells showed increased proliferation of cells expressing the Q688 variant (n=2 experiments). **B.** Immunoblot analysis revealed elevated levels of cell proliferation markers (PCNA, CyclinE, Cyclin D, p-RB) and reduced levels of cell cycle suppressors (p27 and p21) in cells stably expressing the Q688 variant (n=3 experiments). Antibody to GAPDH was used to verify protein loading. **C.** ROS scavengers n-acetylcysteine (NAC, 10 mM) or Tiron (10 mM) restored PCNA and cyclin E levels, indicating that increased proliferation is caused by elevated ROS production in Q688 expressing cells (n=3 experiments).


A
Cells with BrdU (%)
4
3
2
1
0
NT
R688
Q688
B
NT R688 Q688
PCNA
pRB
p27
p21
Cyclin E
Cyclin D
GAPDH
C
NT
R688
Q688
NAC
Tiron
PCNA
Cyclin E
GAPDH

Discussion

The identification of a gain-of-function variant associating with increased risk of coronary artery disease has several implications. First, it reveals a critical requirement for mitochondrial homeostasis to prevent conditions permissive to the development of CAD. Second, it suggests that by modifying mitochondrial function it may be possible to reduce the risk in individuals who carry this allele. Third, this finding cautions against the development of therapies that augment mitochondrial fusion, for example to fend against type 2 diabetes mellitus, because they may have secondary effects promoting CAD.

Regulated processing of SPG7 is a novel finding of the present study. This could come about in several ways. If PKA phosphorylates SPG7 directly, the Q688 variant might block this PKA-dependent phosphorylation. Although I initially favored this hypothesis, tandem mass-spectrometry of immunoprecipitated SPG7 found no evidence that this protein is phosphorylated (data not shown). Similarly, phosphorylated peptides of SPG7 have not yet been identified in any of the large-scale mass spectrometry studies deposited online (http://www.phosphosite.org/). In contrast, AFG3L2 was reported to be phosphorylated at Ser^{634} (Olsen, Vermeulen et al. 2010), in the vicinity of the Q688 variant. Future studies will need to establish whether AFG3L2 phosphorylation is regulated and whether this controls SPG7 N-terminal processing. Alternatively, the Q688 variant might not depend on AFG3L2 for the processing of its N-terminal domain if, like AFG3L2, it is auto-catalytic.

One of the limitations of my study is that I used stably-transfected cells over-expressing the Q688 variant of SPG7. This is likely an exaggerated phenotype of cells in vivo.

Nonetheless, these cells replicate the increased processing of SPG7 observed in peripheral blood lymphocytes and primary aortic smooth muscle cells of individuals that carry the Q688 allele. Thus, this finding indicates that increased processing and cleavage of the N-terminal fragment of SPG7 can be accounted for by the presence of the Q688 polymorphism and not some other cellular component.

Ishihara et al. over-expressed high levels of an HA-tagged human SPG7 in HeLa cells and found that it increased mitochondrial fragmentation (Ishihara, Fujita et al. 2006). OPA1 is one of the dynamin-like GTPases required for mitochondrial fusion. When OPA1 becomes fully cleaved, mitochondrial fusion is blocked, and cells undergo apoptosis. According to Ishihara et al., over-expression of SPG7 was also asociated with increased processing of OPA1, and this was taken as evidence that SPG7 might be the protease that cleaves OPA1. However, recent evidence shows that the SPG7/AFG3L2 matrix protease interacts with the ATP-independent peptidase OMA1 and it is OMA1 that is the authentic protease that cleaves OPA1 (Ehses, Raschke et al. 2009). Head et al., 2009 Thus, the findings of Ishihara et al. likely reflect a non-physiological effect due to the high levels of SPG7 over-expression. I obtained much more modest levels of over-expression and did not replicate these findings in stably transfected HEK293 cells expressing either the R688 or Q688 variant of SPG7. Instead, I observed increased mitochondrial fusion in cells expressing the Q688 variant without overtly changing the ratio of the short form of OPA1.

Dysregulation of mitochondrial fusion and/or fission might impair mitochondrial function and contribute to many age-dependent diseases including Parkinson's Disease (Irrcher, Aleyasin et al. 2010) and coronary atherosclerosis (Davidson 2010). One of the proteins

that promote mitochondrial inner membrane fusion is the GTPase mitofusin 2 (Bach, Pich et al. 2003). The literature regarding the role of mitofusin 2 in coronary atherosclerosis is controversial, stemming largely from one laboratory. Mitofusin 2 levels decline with atherosclerotic lesion progression (Chen, Guo et al. 2004), and conversely, over-expression of mitofusin 2 was shown to inhibit vascular smooth muscle cell proliferation following balloon injury of the carotid artery (Chen, Guo et al. 2004), and in culture in response to oxidized LDL (Guo, Chen et al. 2007). Mitofusin 2 over-expression also reduced atherosclerotic lesion formation in rabbits (Guo, Chen et al. 2007). However, elevated mitofusin 2 was associated with increased smooth muscle cell apoptosis (Guo, Chen et al. 2007). It is worth noting that mitofusin 2 is phosphorylated at Ser344 (Huttlin, Jedrychowski et al. 2010), a PKA-like site, and that a Ser344Ala mutation that would prevent phosphorylation reduced cell proliferation, whereas a Ser344Asp mutation that mimics phosphorylation increased cell proliferation (Zhou, Chen et al. 2010). However, PKA activation is known to inhibit smooth muscle cell proliferation (Torella, Gasparri et al. 2009; Hewer, Sala-Newby et al. 2011), and to promote mitochondrial fusion (Schauss, Huang et al. 2010). I found that PKA activation blocked processing of the common R688 form of SPG7. So, if SPG7 processing somehow regulates cellular proliferation, I would expect the Q688 isoform that escapes PKA-dependent inhibition of processing to have increased cell proliferation. Indeed, I found that cells expressing the Q688 variant of SPG7 had increased mitofusin 2 levels associated with increased mitochondrial fusion and cellular proliferation.

The presence of a consitutively activated mitochondrial SPG7/AFG3L2 protease in cells expressing the Q688 variant of SPG7 is likely to have marked effect on matrix protein

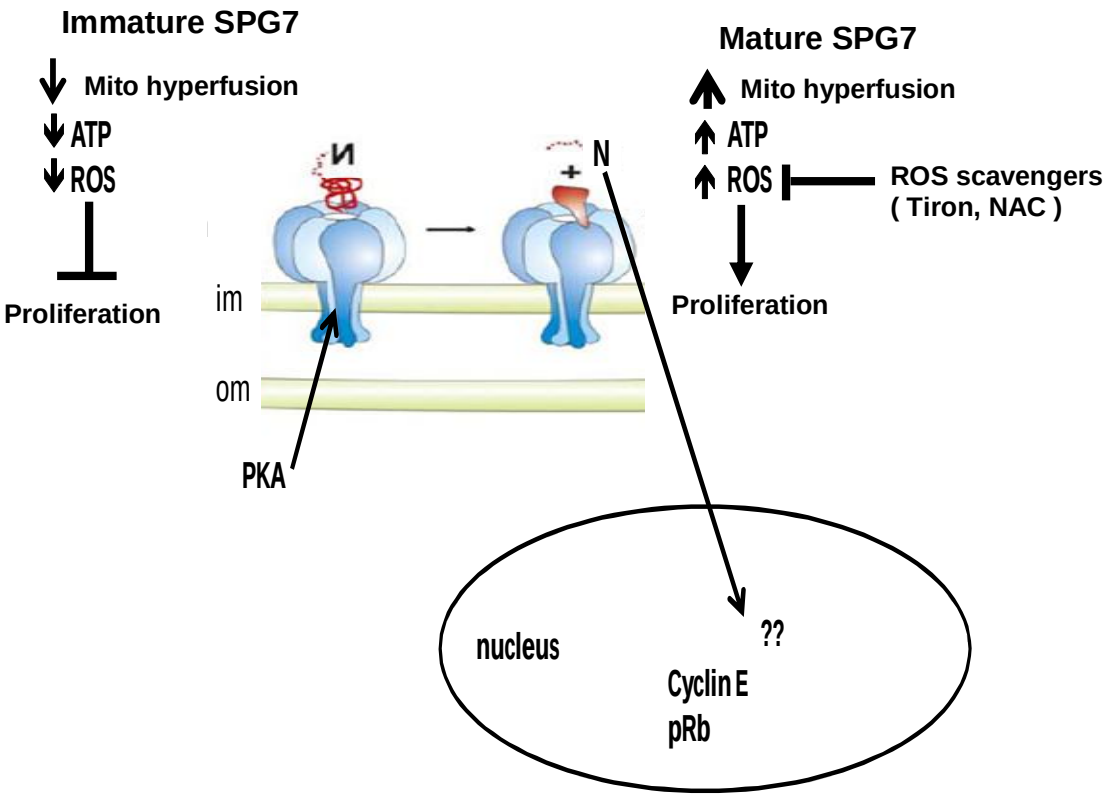
assembly. I found evidence for altered stoichiometry of components of the electron transport chain. This altered stoichiometry of subunit IV was also associated with increased ROS production. Increased ROS production has been directly tied to increased cell proliferation (Wedgwood, Dettman et al. 2001; Na, Chung et al. 2008). I also observed increased proliferation of cells expressing the Q688 variant. Elevated ROS production and increased cell proliferation are two conditions that would promote rather than protect against coronary atherosclerosis. ROS depletion by NAC is shown to have inhibitory effect on cell cycle progression (Ki-Yong Kim et al., 2001). Indeed, the upregulation of cyclin E and PCNA was reversed when ROS was quenched by NAC suggesting that ROS accumulation might be the reason behind the increased cellular proliferation seen in cells overexpressing the Q688 variant.

What might the evolutionar benefit of having a variant of SPG7 that is not regulated by a PKA-like signaling mechanism? It is of interest that a recent pharmacogenomic study of docetaxel and thalidomide toxicity in patients with castration-resistant prostate cancer found a highly significant risk associated with the R688 common variant of SPG7 (or protection from the Q688 variant) (Deeken, Cormier et al. 2010). The increased ability to detoxify xenobiotic compounds conferred by the Q688 SPG7 variant may explain how this allele has become fixed in the human population. Genes involved in xenobiotic detoxification have been shown to evolve more rapidly (Greenberg, Stockwell et al. 2008), presumably because they offer a strong selective advantage. It is interesting to note that SPG7 has a remarkably high number of coding variants, compared to AFG3L2 (Elleuch, Depienne et al. 2006). Although a variant that protects against toxic xenobiotic compounds would confer immediate advantage, the long term consequence of this gain of

protease function may be to increase susceptibility to a late onset disease like coronary atherosclerosis.

In summary, my work suggests that the protease activity of SPG7 is dynamically regulated by phosphorylation. The Q688 variant causes a loss of regulated phosphorylation, maintaining SPG7 in a hyperactive state that leads to increased processing of SPG7 to its active form. Constitutively active SPG7 increases the mitochondrial protein turnover, increases mitochondrial ATP production to cope with the increased demand for ATP required by the ATPase domain to degrade proteins, increases oxidative phosphorylation by stimulating the formation of cytochrome C oxidase complex and causes increased ROS production. The Q688 variant also increases cell proliferation as a result of the high ATP level achieved in cell expressing the Q688 variant since high level of ATP has shown to drive the cell cycle progression (Mandel S et al., 2005) or may be due to translocation of the cleaved N-terminus during SPG7 processing to the nucleus and exerts an activator effect on the cell cycle markers like cyclin E or any marker. This variant increases the risk of CAD by increasing the ROS production and therefore cell cycle proliferation which are well known biological risk factors for the development and progression of CAD (see model, **Fig. 11**)

Fig. 11 Model for Q688 variant contributing to CAD risk. Processing of the wild type form of SPG7 is inhibited by PKA, ATP and ROS production are suppressed and cell proliferation is reduced. The Q688 variant escapes PKA inhibition, ATP and ROS production are elevated, the N-terminal fragment of SPG7might be targeted to the nucleus and activates cell proliferation.


Immature SPG7
Mito hyperfusion
ATP
ROS
Proliferation
Mature SPG7
Mito hyperfusion
ATP
ROS
ROS scavengers
(Tiron, NAC)
Proliferation
N
+
im
om
PKA
nucleus
??
Cyclin E
pRb

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CONTRIBUTION OF COLLABORATORS

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STATEMENT

I hereby declare myself, Naif Ahmad Almontashiri, to have faithfully performed all experiments, excluding those mentioned in the section of CONTRIBUTION OF COLLABORATORS, and to have written this thesis under the supervision of Dr. Alexandre F. R. Stewart and in accordance with the guidelines of the Department of Biochemistry, of the Faculty of Graduate and Postgraduate Studies, and of the University of Ottawa.