Sengers Syndrome-Associated Mitochondrial Acylglycerol Kinase Is a Subunit of the Human TIM22 Protein Import Complex

Graphical Abstract

Highlights

- Dysfunction in the mitochondrial acylglycerol kinase, AGK, causes Sengers syndrome
- AGK is a subunit of the TIM22 complex, which imports mitochondrial carrier proteins
- AGK has kinase-independent and kinase-dependent functions
- The TIM22 complex and carrier import are affected in Sengers syndrome cells and tissues

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In Brief

Sengers syndrome is a mitochondrial disorder caused by mutations in the lipid kinase AGK. Kang et al. show that AGK is a subunit of the mitochondrial TIM22 complex, where it functions in the import of carrier proteins in a kinase-independent manner. These findings link mitochondrial protein import to Sengers syndrome.
Sengers Syndrome-Associated Mitochondrial Acylglycerol Kinase Is a Subunit of the Human TIM22 Protein Import Complex

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SUMMARY

Acylglycerol kinase (AGK) is a mitochondrial lipid kinase that catalyzes the phosphorylation of monoa- cylglycerol and diacylglycerol to lysophosphatidic acid and phosphatidic acid, respectively. Mutations in AGK cause Sengers syndrome, which is characterized by congenital cataracts, hypertrophic cardiomyopathy, skeletal myopathy, exercise intolerance, and lactic acidosis. Here we identified AGK as a subunit of the mitochondrial TIM22 protein import complex. We show that AGK functions in a kinase-independent manner to maintain the integrity of the TIM22 complex, where it facilitates the import and assembly of mitochondrial carrier proteins. Mitochondria isolated from Sengers syndrome patient cells and tissues show a destabilized TIM22 complex and defects in the biogenesis of carrier substrates. Consistent with this phenotype, we observe perturbations in the tricarboxylic acid (TCA) cycle in cells lacking AGK. Our identification of AGK as a bona fide subunit of TIM22 provides an exciting and unexpected link between mitochondrial protein import and Sengers syndrome.

INTRODUCTION

Members of the mitochondrial carrier family (or solute carrier 25 family [SLC25] in humans) facilitate the continuous flux of diverse metabolites, nucleotides, and cofactors into and out of the mitochondrion (Gutiérrez-Aguilar and Baines, 2013; Kunji, 2004; Palmieri et al., 1996). These multi-transmembrane-spanning carriers are embedded in the mitochondrial inner membrane and are essential for a variety of processes, including oxidative phos- phorylation, the tricarboxylic acid (TCA) cycle, fatty acid oxidation, protein synthesis, and ion transport (Palmieri, 2014; Palmieri and Monné, 2016). Human mitochondria contain >50 protein subunits, each of which need to be correctly targeted and integrated into the mitochondrial inner membrane following synthesis in the cytosol. This is achieved through the “carrier” import pathway, which utilizes the translocase of the outer membrane (TOM) machinery, followed by the action of the translocase of the inner membrane 22 (TIM22) complex for insertion into the inner membrane (Chacinska et al., 2009; Koehler, 2004; Stojanovski et al., 2012; Wagner et al., 2009).

The TIM22 complex has been primarily studied in yeast and has been shown to be a 300-kDa molecular machine comprising four membrane-integral subunits, Tim22, Tim54, Tim18, and Sdh3, and a peripherally associated chaperone complex consisting of Tim9-Tim10-Tim12 (Adam et al., 1999; Gebert et al., 2008, 2011; Jarosch et al., 1996, 1997; Kerscher et al., 1997, 2000; Koehler et al., 1998, 2000; Kovermann et al., 2002; Rehling et al., 2004; Wagner et al., 2009). In human cells, the TIM22 complex is known to contain the translocase subunit Tim22 and the small TIM chaperone complex formed by Tim9-Tim10b-Tim10a (Tim10b is the Tim12 equivalent) (Bauer et al., 1999; Mühlénbein et al., 2004). Recently, we identified a metazoan-specific subunit of the TIM22 complex, which we termed Tim29 (Callegari et al., 2016; Kang et al., 2016). Tim29 functions in the assembly of the human TIM22 complex and links the complex to the TOM complex (Kang et al., 2016). Interestingly, our data indicated that another protein, identified as acylglycerol kinase (or AGK), was also associated with the human TIM22 complex (Kang et al., 2016).

AGK is a mitochondrial protein previously described to be a lipid kinase involved in the conversion of monoaucylglycerol (MAG) and diacylglycerol (DAG) to lysophosphatidic acid (LPA) and phosphatidic acid (PA), respectively (Bektas et al., 2005). PA feeds into the synthesis of the mitochondrion-specific lipid cardiolipin (CL), which is essential for mitochondrial ultrastructure and function (Tatsuta et al., 2014). Despite containing
Figure 1. AGK Is a Subunit of the TIM22 Complex

(A) Schematic of acylglycerol kinase (AGK) protein, depicting the predicted position of the transmembrane domain (TMD) and diacylglycerol kinase (DGK) domain. Bottom: immunofluorescence images of AGK 3XFLAG transiently expressed in HeLa cells. Cells were immunostained with antibodies against the FLAG epitope to visualize the FLAG-tagged AGK, NDUFAF2 (mitochondrial marker protein) and Hoechst 33258 dye (nuclear stain). Scale bars, 10 μm.

(B) Isolated mitochondria from cells stably expressing AGK 3XFLAG were subjected to submitochondrial fractionation. Untreated mitochondria (lanes 1 and 2); hypotonic swelled mitochondria (lanes 3 and 4), and TX-100 solubilized mitochondria (lanes 5 and 6) were treated with (+) or without (−) Proteinase K (PK).

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a highly conserved diacylglycerol kinase (DGK) domain, phylogenetic analysis suggests that human and murine AGK are not members of any previously described kinase family (Waggoner et al., 2004). Notably, mutations in AGK cause Sengers syndrome (Calvo et al., 2012; Haghighi et al., 2014; Mayr et al., 2012; Siriwardena et al., 2013), a mitochondrial disease characterized by congenital cataracts, hypertrophic cardiomyopathy, skeletal myopathy, exercise intolerance, lactic acidosis, and delayed motor development. The pathological mechanisms resulting in Sengers syndrome are unclear but have been attributed to AGK’s role in lipid metabolism (Mayr et al., 2012), although direct evidence for this hypothesis is missing.

Here we show that AGK is a subunit of the human TIM22 complex and functions in stabilizing the TIM22 complex and regulating the import and assembly of mitochondrial carrier proteins. These functions do not rely on AGK’s kinase domain, suggesting that AGK has a kinase-independent and kinase-dependent function within mitochondria. Cells lacking AGK have reduced mitochondrial maximal respiration, unstable respiratory chain supercomplexes, and metabolic reprogramming, resulting in impaired central carbon metabolism and reduced TCA flux through suppression of glucose-dependent anaerolysis. Furthermore, mitochondria isolated from Sengers syndrome patient cells and tissues have destabilized TIM22 complexes and reduced levels of carrier substrate proteins. We conclude that defects in protein import into mitochondria via the TIM22 complex represent a new pathogenic mechanism for Sengers syndrome.

RESULTS

AGK Is a Component of the Human TIM22 Complex

Immunoprecipitation of human Tim10b (hTim10b) from isolated mitochondria and proteomic analysis of the associated complex led to the identification of Tim29 (C19orf52) as a component of the TIM22 complex (Kang et al., 2016). Using Tim22 as bait, Coll egregi and colleagues (2016) also identified Tim29. Interestingly, both studies showed that AGK was also enriched with the TIM22 complex. Although AGK is a mitochondrial protein (Bektas et al., 2005), its sub-mitochondrial location has not been clearly established. AGK is predicted to contain a single N-terminal transmembrane domain (amino acids 11–30) (Figure 1A). Proteomic mapping of the mitochondrial intermembrane space identified the presence of AGK (Hung et al., 2014), suggesting that the 47-kDa protein has a large soluble domain in the intermembrane space. Transient expression of a C-terminal 3XFLAG-tagged AGK (AGK3XFLAG) in HeLa cells showed that the tagged protein was exclusively localized to mitochondria (Figure 1A). This construct was used to generate a stable tetracycline-inducible cell line using Flp-in T-Rex HEK293 cells. Mitochondria were isolated from AGK3XFLAG-expressing cells induced with tetracycline for 24 hr and subjected to hypo-osmotic swelling and Proteinase K (PK) treatments or carbonate extraction and high salt extraction to address the protein’s mitochondrial sub-localization and membrane integration. AGK3XFLAG became accessible to external protease when the mitochondrial outer membrane was disrupted (Figure 1B, compare lanes 2 and 4), indicating that the C-terminal FLAG tag is exposed to the intermembrane space. The outer membrane proteins Bak and Mn2, intermembrane-space exposed Opa1, and matrix-located NDUFaF2 served as controls. AGK3XFLAG was largely resistant to carbonate treatment (pH 9.5–11.5), like the membrane integrated Mn2 and ANT3, suggesting that it is an integral membrane protein (Figure 1C). Although high salt extraction of isolated mitochondria also suggested AGK to be an integral inner membrane protein (Figure 1D), carbonate extraction at pH 12.5 (Figure 1C, lanes 8 and 9) did result in the release of AGK into the supernatant fraction. This suggests that the protein is either partially embedded into the inner membrane or has a moderately hydrophobic transmembrane domain because mitochondrial proteins with moderate hydrophobicity have been shown to be released from the membrane in sodium carbonate (Kim et al., 2015). The endogenous AGK protein (data not shown) displayed an identical profile upon sub-fractionation and carbonate extraction, confirming that the protein is an integral inner membrane protein, with its C terminus facing the intermembrane space. Thus, AGK is in the correct location to be a component of the human TIM22 complex.

To examine whether AGK is a genuine subunit of the TIM22 complex, we performed immunoprecipitation and antibody shift analyses. For immunoprecipitation, mitochondria isolated from control, the AGK3XFLAG, and hTim10b3XFLAG cell lines were solubilized in digitonin-containing buffer and subjected to immunoprecipitation with FLAG affinity gel. Analysis by western blotting revealed enrichment of the known TIM22 complex subunits hTim9 and Tim29 with both hTim10b and AGK (Figure 1E), strongly suggesting that they are all components of the same complex. For antibody shift analysis, in vitro-translated [35S]-hTim22 was incubated with mitochondria isolated from control cells or cells expressing AGK3XFLAG, Tim293XFLAG, or hTim10b3XFLAG to allow import and assembly into the mature TIM22 complex. Following import, mitochondria were re-isolated, solubilized in digitonin-containing buffer, and incubated with FLAG antibodies prior to blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis. A ‘shift’ or delayed migration of the TIM22 complex because of binding of FLAG antibodies was observed in mitochondria expressing either
Figure 2. AGK Is Required for the Stability of the TIM22 Complex and Biogenesis of Carrier Proteins

(A and B) Mitochondria isolated from wild-type (control) or AGK KO (AGK<sup>ko</sup>) HEK293T cells were subjected to (A) SDS-PAGE or (B) BN-PAGE and western-blotted using the indicated antibodies. Protein levels on SDS-PAGE were quantified and normalized to the loading control (Mfn2). The amount of protein in control mitochondria was set to 100%, and data represent mean ±± SD (n = 3).

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Loss of AGK Destabilizes the TIM22 Complex

To investigate the function of AGK at TIM22 and confirm a potential role in regulating the import of TIM22 complex substrates, we used CRISPR/Cas9 to introduce mutations resulting in permanent knockout (KO) of protein expression. We targeted the third coding exon and confirmed the presence of open reading frame-disrupting insertions or deletions (indels) by DNA sequencing (Figure S1) in both HEK293T and Flp-IN T-REx HEK293 backgrounds. SDS-PAGE and immunoblot analysis confirmed the loss of AGK protein in AGK KO mitochondria compared with control HEK293T mitochondria (Figure 2A). In addition, AGK KO mitochondria also showed reduced levels of hTim22 and Tim29 and a reduction in the steady-state levels of TIM22 complex substrates, the glutamate carrier (GC1) and adenine nucleotide transporter (ANT3) (Figure 2A). Substrates of the TIM22 complex also include multi-spanning inner membrane proteins such as the subunits of the inner membrane TIM23 complex hTim17 and hTim23 (Chacinska et al., 2009; Kaldi et al., 1998; Koehler, 2004; Sirrenberg et al., 1996; Stojanovski et al., 2012). However, deletion of AGK had no significant effect on the steady-state levels of hTim23 and hTim17b (Figure 2A, quantified in the graph). When mitochondrial lysates from control and AGK KO were analyzed by BN-PAGE and immunoblotting, a discernible reduction in the level of the TIM22 complex was apparent in the absence of AGK (Figure 2B, lanes 1 and 2), whereas complex II, another inner membrane complex, remained unaffected (Figure 2B, lanes 3 and 4).

To confirm that the observed effects on the TIM22 complex and substrate carrier proteins were genuine and not because of off-target effects, we undertook a complementation analysis using the Flp-In T-REx HEK293 AGKKO cell line (Figure S1). Stable tetracycline-inducible cell lines expressing wild-type and untagged AGK (AGK WT), or FLAG-tagged AGK kinase-dead mutant (AGKG126E) (Bektas et al., 2005) were generated. AGKG126E allowed us to dissect whether AGK function at TIM22 is dependent on the protein’s kinase activity. We confirmed that the kinase-dead mutant was targeted to mitochondria (Figure S2A) and integrated into the inner membrane like wild-type AGK by mitochondrial sub-fractionation and carbonate extraction (Figure S2B). Mitochondria were isolated from control, AGK KO, and the complemented cell lines and analyzed by SDS-PAGE and immunoblotting (Figure 2C). Re-expression of AGK WT or AGKG126E restored the steady-state levels of hTim22 and Tim29 and the carrier substrates GC1 and ANT3 (Figure 2C, quantified in the graph). When the same mitochondrial lysates were analyzed by BN-PAGE, we observed restabilization of the TIM22 complex in the presence of AGK WT or AGKG126E (Figure 2D, compare lane 2 with lanes 3 and 4). Likewise, the assembly defects seen for GC1 and ANT3 in AGK KO cells (Figure 2E, compare lanes 1 and 2) were rescued upon re-expression of AGK WT and AGKG126E (Figure 2E, compare lane 2 with lanes 3 and 4). Taken together, these data indicate that AGK is required for the stability of the TIM22 complex and import/assembly of mitochondrial carrier proteins in a kinase-independent manner.

AGK Functions in the Biogenesis of Carrier Proteins

We previously showed that Tim29 functions in the assembly of TIM proteins such as hTim22 and hTim23 but not in the import and assembly of the carrier protein ANT (Kang et al., 2016). This is contrary to what we observed in the AGK KO cells, where more pronounced defects were observed in the steady-state levels of carrier proteins. We interrogated this further by using mitochondrial in vitro import assays and looked at the import and assembly of the TIM substrates, including hTim22 and hTim23; the carrier substrates, including ANT1, GC1, and the phosphate carrier (PIC); and hTom22, a subunit of the TOM complex as a control. Radiolabeled precursor proteins were incubated with isolated mitochondria (control, AGK KO, AGK KO+WT, and AGK KO+G126E) for 15 and 60 min and analyzed by BN-PAGE and autoradiography. The lack of AGK abolished the assembly of [35S]-hTim22 into the mature TIM22 complex (Figure 3A), and this assembly defect was restored by the reintroduction of AGK WT or AGKG126E (Figure 3A, lanes 7–12). In contrast, assembly of [35S]-hTim23 was not significantly affected in the absence of AGK (Figure 3B; see Figure S4B for quantification), like [35S]-Tom22 (Figure 3F; see Figure S4F for quantification). However, all carrier proteins tested ([35S]-ANT1, [35S]-GC1, and [35S]-PIC) displayed severe assembly defects in isolated mitochondria from cells lacking AGK, and assembly was restored to wild-type levels in AGK KO mitochondria re-expressing AGK WT or AGKG126E (Figures 3C–3E; see Figures S4C–S4E for quantification).

We next determined whether the reduction in carrier protein assembly was due to AGK having an active role in substrate import into the inner membrane or in subsequent assembly. [35S]-labeled hTim22, hTim23, ANT1, GC1, PIC, and hTom22 precursors were incubated with mitochondria isolated from control and AGK KO cells for 15, 30, or 60 min to allow import. Following this, samples were treated with PK to remove non-imported protein (with the exception of hTom22 because it is an outer membrane protein) and analyzed by SDS-PAGE (to monitor import kinetics; Figure S3) or BN-PAGE (to monitor protein assembly; Figure S4). A further sample taken at the 60-min time point was processed by carbonate extraction to assess membrane integration of the imported species (Figure S5).
Even though [35S]-hTim22 displayed a severe assembly defect in mitochondria lacking AGK (Figure S4A; Figure 3A), protein translocation into mitochondria was not affected (Figure S3A), and [35S]-hTim22 was efficiently integrated into the inner membrane (Figure S5A). [35S]-hTim23 displayed no significant reduction in import, assembly, or integration into the inner membrane (Figures S3B, S4B, and S5B), whereas the import (SDS-PAGE) and assembly (BN-PAGE) of all tested carrier proteins were affected in the absence of AGK: [35S]-ANT1 was reduced to 58.8% and 18.5% (Figures S3C and S4C), GC1 was reduced by 53.6%
and 30.2% (Figures S3D and S4D), and PiC to 75.1% and 56.8% (Figures S3E and S4E), respectively, at the 60-min time point. In line with this, mitochondrial oxygen consumption rates measured on control and AGK KO cells by high-resolution respirometry indicated no significant effect on basal respiration (residual basal respiration of 84% of control, p > 0.05) (Figure 4B), although a reduction in maximal respiration was detected (residual maximal respiration of 52% of control, p < 0.05) (Figure 4B). Given that maximal respiration is an indicator of energetic reserve capacity and that some Sengers syndrome patients have been reported to have combined complex I, III, and IV deficiency (Calvo et al., 2012), we took a closer look at the effect of AGK deletion on the mitochondrial respiratory chain. The steady-state protein levels of complex I (NDUFA9, NDUFAF2, NDUFV1, and NDUFV2) and complex IV (COX4) subunits revealed no apparent difference in AGK KO mitochondria relative to the control (Figure 4C). However, there was a modest reduction in the amount of the complex I+III+IV supercomplex (SC) when AGK KO mitochondrial samples were solubilized in digitonin (to maintain supercomplex integrity) and assessed by BN-PAGE and immunoblotting using the indicated antibodies. SC indicates CI+III+IV respiratory supercomplexes, CIV refers to complex IV, and CII to complex II. High and Low refer to high and low exposure time, respectively. # indicates cross-reactive band observed with AGK antibody.

**Figure 4. Loss of AGK Affects Maximal Respiration Rates and Supercomplex Stability**

(A) Control and AGK KO cells were seeded at equal density and grown in glucose- or galactose-containing cell culture medium for 24 hr. Cell proliferation was then assessed by growing cells in the presence of BrdU for 24 hr and measuring absorbance at 450–550 nm. Data represent mean ± SD (n = 3).

(B) Mitochondrial basal and maximal respiration rates in control and AGK KO cells. Data are mean ± SD (n = 3).

(C) Control and AGK KO mitochondria were analyzed on SDS-PAGE, followed by immunoblotting using the indicated antibodies.

(D) Mitochondria isolated from control, AGK KO, and AGK KO-K126E cells were solubilized in digitonin buffer, and complexes were resolved using BN-PAGE and immunoblotting using the indicated antibodies. SD (n = 3).

**Loss of AGK Affects Cellular Metabolism**

Carrier proteins regulate cellular metabolism by mediating solute exchange between mitochondria and the cytosol. Given the association between AGK and the TIM22 complex and the link between mutations in AGK and Sengers syndrome, we explored whether metabolism is reprogrammed in cells lacking AGK. First we monitored cell proliferation by seeding control and AGK KO cells at an equal density and growing them in glucose- or galactose-containing cell culture medium for 24 hr. Following this, cell proliferation was assessed by bromodeoxyuridine (BrdU) incorporation (incubated with cells for a further 24 hr), and no change was apparent in AGK KO compared with control cells in glucose or galactose medium (Figure 4A), indicating no major defects in cellular proliferation in the absence of AGK. In line with this, mitochondrial oxygen consumption rates measured on control and AGK KO cells by

[Image 335x365]
We next performed a metabolomics analysis to further understand the metabolic consequences upon loss of AGK. An untargeted metabolomics analysis of control and AGKKO cells revealed significant changes in the abundance of many intracellular metabolites, including glycolytic intermediates, TCA cycle intermediates, and numerous amino acids (Figure 5A; Table S1). Strikingly, the levels of TCA cycle intermediates were markedly reduced, suggesting that mitochondrial central carbon metabolism is disrupted/alterned in the absence of AGK. Interestingly, amino acids such as lysine, tyrosine, tryptophan, methionine, and proline, which can provide carbon skeletons for TCA metabolism, were significantly elevated in AGK KO cells, consistent with inhibition of the TCA cycle leading to an accumulation of TCA substrates. To further understand mitochondrial substrate utilization in AGKKO cells, we cultured control, AGKKO, and AGKKO+WT complemented cells in the presence of [U-13C]-glucose for 2 hr and analyzed intracellular metabolites by gas chromatography-mass spectrometry (GC-MS). Labeling of all TCA intermediates and closely connected amino acids that are generated from the TCA cycle (aspartate and glutamate) was significantly reduced in AGKKO compared with control cells (Figures 5B and 5D), consistent with AGK KO cells having a pronounced defect in TCA cycle flux. The labeling of these metabolites was largely restored upon re-expression of AGKWt in the AGKKO background (Figure 5D), suggesting that the metabolism defect is specific to the lack of AGK protein. The abundance of fully labeled glycolytic intermediates and alanine (a product of glycolysis) remained unchanged in AGKKO cells after 15 min (Table S2) and 2 hr of labeling (Figure 5C; Table S2), indicating no major changes to glycolysis or that glycolytic intermediates have hit steady-state levels because of saturation at the 15-min time point. These findings suggest the AGK has an important role in regulating mitochondrial metabolism.

**The TIM22 Complex and Carrier Import Pathway Are Affected in Sengers Syndrome Patient Cells and/or Tissues**

It has previously been speculated that Sengers syndrome is caused by a loss of kinase function in AGK. However, our identification of AGK as a component of the TIM22 complex suggested the alternative possibility that protein import via TIM22 could play a role in the pathogenesis of Sengers syndrome.
To test this hypothesis, we assessed the assembly of carrier proteins and the overall stability of the TIM22 complex in tissues and fibroblasts from Sengers syndrome patients. Calvo et al., (2012) described two unrelated patients (patients 41 and 42) who harbored three severe mutations in AGK (Figure S6). Patient 41 (referred to here as patient 1 or P1) harbored a compound heterozygous nonsense variant (p.Y390X) and splice variant that caused a shortened transcript with a premature stop codon (c.297+2T > C, p.K75QfsX12) and survived for 18 years (Calvo et al., 2012). Patient 42 (referred to here as patient 2 or P2) possessed a homozygous splice variant that caused a shortened transcript with a premature stop codon (c.1131+1G > T, p.S350EfsX19) (Calvo et al., 2012) and survived for 4 days. Mitochondria isolated from patient fibroblasts showed no detectable AGK protein relative to control fibroblasts (Figure S6) and a reduction in the steady-state levels of carrier proteins (GC1 and ANT3) and TIM22 subunits (hTim22 and Tim29), in particularly in P2 (Figure 6A). Mitochondrial lysates from control and patient fibroblasts were also analyzed by BN-PAGE to assess the integrity of mitochondrial protein complexes. The TIM22 complex was completely absent (or beyond the detection limit of the western blot) in both P1 and P2 (Figure 6B, lanes 1–3). In a similar manner, we observed a significant reduction in the levels of assembled ANT3 (Figure 6B, lanes 4–6) and GC1 dimers (Figure 6B, lanes 7–9), whereas control complex II (SDHA) remained unaffected (Figure 6B, lanes 10–12). We complemented these observations by looking at the in vitro import and assembly of [35S]-hTim22, [35S]-ANT1, and [35S]-hTim23 into mitochondria isolated from control, P1, and P2 fibroblasts. Similar to what was observed in AGK KO cells, [35S]-hTim22 and [35S]-ANT1 displayed assembly defects in mitochondria isolated from both P1 and P2 fibroblasts, whereas assembly of [35S]-hTim23 remained largely unaffected (Figures 6C–6E, respectively).

To determine whether the defects observed in fibroblasts are translated into higher-energy-demanding and in disease-relevant tissues, we analyzed mitochondria that were isolated from liver and muscle tissue biopsies from P2. SDS-PAGE analysis revealed a severe reduction in the levels of the TIM22 complex subunits hTim22, Tim29, and hTim9 in liver mitochondria (Figure 7A) in addition to carrier proteins (GC1 and ANT3), whereas the control proteins hTim23 and SDHA remained unaffected (Figure 6B, lanes 10–12). We complemented these observations by looking at the in vitro import and assembly of [35S]-hTim22, [35S]-ANT1, and [35S]-hTim23 into mitochondria isolated from control, P1, and P2 fibroblasts. Similar to what was observed in AGK KO cells, [35S]-hTim22 and [35S]-ANT1 displayed assembly defects in mitochondria isolated from both P1 and P2 fibroblasts, whereas assembly of [35S]-hTim23 remained largely unaffected (Figures 6C–6E, respectively).

In this study we show that mitochondrial AGK is a subunit of the human TIM22 complex. Like the recently identified Tim29 (Callegari et al., 2016; Kang et al., 2016), AGK is a metazoan-specific protein and, hence, absent in yeast models traditionally used to study mitochondrial protein import. These proteins appear to have evolved new functions in the TIM22-mediated import pathway in human mitochondria. AGK is integrated into the mitochondrial inner membrane and oriented with its large C-terminal domain to the intermembrane space. It is required for the overall integrity of the TIM22 complex and the import and assembly of mitochondrial carrier proteins in the inner membrane. Interestingly, Tim29 and AGK appear to display distinct preferences for TIM22 substrates, with Tim29 favoring multi-spanning membrane proteins belonging to the TIM family (such as hTim23 and hTim17) (Kang et al., 2016), whereas AGK favors carrier protein substrates (such as ANT). One difference between these two classes of substrates is the number of transmembrane domains, with the TIM family predicted to have four transmembrane domains (Meier et al., 2005; Wrobel et al., 2016), whereas the carrier proteins typically have six transmembrane domains (Monné and Palmieri, 2014; Palmieri, 2004). In yeast, TIM22 substrates are transported across the intermembrane space in association with the small TIM chaperones and show substrate specificity at this stage, with TIM substrates engaging with the Tim8-Tim13 chaperone complex (Paschen et al., 2000), whereas carrier substrates favor the Tim9-Tim10 complex (Endres et al., 1999; Truscott et al., 2002). Indeed, Tim29 and AGK could serve as distinct substrate docking sites at the human TIM22 complex. Analysis of a larger cohort of TIM22 substrates in cells lacking Tim29 or AGK will help to tease out the true molecular mechanisms overseeing the biogenesis of these different TIM22 substrate proteins.

Although AGK contains a highly conserved DGK domain, the human and murine proteins segregate away from the other lipid kinase families (Waggoner et al., 2004). However, several lines of evidence suggest that AGK has a dual role as a genuine acylglycerol kinase: AGK can catalyze the phosphorylation of both MAG and DAG in vitro, producing LPA and PA (Bektas et al., 2005); and overexpression of a kinase-dead mutant of AGK did not increase AGK activity or produce LPA or PA (Bektas et al., 2005); and overexpression of a kinase-dead mutant of AGK did not increase AGK activity or produce LPA (Bektas et al., 2005). However, expression of a kinase-dead mutant in AGKKO revealed that AGK’s kinase activity is not required for the protein’s function at the TIM22 complex. AGK3126E-expressing cells displayed a restoration in the steady-state levels of the TIM22 complex and the import and assembly of mitochondrial carrier proteins in the inner membrane. This dual function is not a unique feature to AGK. For example, diacylglycerol kinase δ (DGKδ) has been shown to promote...
endoplasmic reticulum (ER)-to-Golgi retrograde protein transport independent of its kinase activity (Nagaya et al., 2002). At present, we have only uncovered a role for the kinase-independent function of AGK at TIM22, but we cannot exclude the possibility that AGK kinase activity at TIM22 may be required in other cell types or under certain physiological conditions. For instance, bacterial DGK plays an important metabolic role by converting DAG produced under conditions of environmental stress into PA (Walsh and Bell, 1992; Yamashita et al., 1993). It should also be noted that we did not observe any effect on cell proliferation in AGK HEK293T KO cells, but PC-3 cells (a human prostate cancer cell line) show a reduction in cell proliferation upon AGK knockdown by small interfering RNA (siRNA) (Bektas et al., 2005). Thus, there are likely cell-specific differences that need to be taken into account when dissecting the role of AGK in both lipid metabolism and protein transport.

The importance of AGK to mitochondrial function and cell health is demonstrated by its link with Sengers syndrome. This metabolic disorder can present at birth, childhood, or early adulthood, and its clinical manifestations range from severe, causing death in infancy, to mild, allowing survival into adulthood (Calvo et al., 2012; Mayr et al., 2012; van Ekeren et al., 1993). Interestingly, the disease was originally thought to be due to deficiencies in...
the expression and/or function of adenine nucleotide transporter 1, ANT1 (SLC25A4) (Jordens et al., 2002). However, genetic analyses excluded mutations in the ANT1 gene, and subsequent exome sequencing revealed the AGK locus as the causative gene of Sengers syndrome (Calvo et al., 2012; Mayr et al., 2012; Palmieri and Monné, 2016). Based on these findings and the previously reported association of the mitochondrion-specific phospholipid cardiolipin with ANT (Beyer and Klingenberg, 1985; Pebay-Peyroula et al., 2003), it was concluded that transcriptional, translational, or post-translational events were responsible for the lower amounts of ANT1 observed in patient cells (Jordens et al., 2002). However, our data suggest that the observed ANT1 deficiency is due to defects in the import and assembly of this carrier protein via the TIM22 complex. Importantly, we observed a reduction in import and assembly of other carrier proteins and TIM22 complex integrity in both AGKKO cells as well as mitochondria isolated from cells/tissue of two previously reported Sengers syndrome patients (Calvo et al., 2012). It is important to highlight that the assembly of other inner membrane complexes such as TIM23 and complex II remain unaffected in mitochondria isolated from AGKKO and patient cells, indicating this is not a global effect on mitochondrial inner membrane complexes.

Although oxidative phosphorylation (OXPHOS) defects in patient samples have been variably reported (Aldahmesh et al., 2012; Calvo et al., 2012; Haghighi et al., 2014; Jordens et al., 2002; Mayr et al., 2012; Morava et al., 2004; Pitkanen et al., 1996; Siriwardena et al., 2013), we did observe a reduction in the amount of respiratory supercomplex in mitochondria isolated from AGKKO cells and tissue from patients with Sengers syndrome. These observations suggest that mitochondrial respiration and metabolism are affected in the absence of AGK. Indeed, untargeted metabolomics and 13C-glucose labeling studies in control and AGKKO cells revealed a general perturbation in central carbon metabolism in the absence of AGK as well as a more specific defect in the catabolism of pyruvate to feed into the mitochondrial TCA cycle. We postulate that the lack of AGK affects the import and assembly of mitochondrial carrier proteins, compromising metabolite transport into and out of mitochondria, ultimately affecting central carbon metabolism. Interestingly, oxidative TCA flux was maintained upon depleting a single mitochondrial carrier, the pyruvate carrier (Vacanti et al., 2014), suggesting that AGKKO has a profound metabolic effect on cells.

In summary, this work provides an exciting link between the TIM22 translocase of the inner membrane and Sengers syndrome. Given this role of AGK in mitochondrial protein import, it will be important to now dissect whether certain cases of Sengers syndrome manifest because of defects in protein transport or lipid metabolism alone or, indeed, a combined defect in both. This is not the only report of a disease linked to the TIM22 complex, with mutations in hTim8a (or DDP1) causing Mohr-Tranebjaerg syndrome, a rare X-linked progressive neurodegenerative disorder that presents with sensorineural deafness, blindness, mental retardation, and a complex movement disorder (Roesch et al., 2002). This highlights the importance of TIM22 and mitochondrial protein import into the inner membrane to human biology. Additionally, AGK overexpression has been linked to prostate and breast cancer (Bektas et al., 2005; Wang et al., 2014), and it will be fascinating to tease out whether the kinase-dependent and/or kinase-independent functions of the protein are linked to cell proliferation and tumorigenicity.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2017.06.014.

**AUTHOR CONTRIBUTIONS**


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