Role of FAST kinase domains 3 (FASTKD3) in post-transcriptional regulation of mitochondrial gene expression

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Running title: FASTKD3 and mitochondrial RNA metabolism

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Keywords: FASTKD3, mitochondria, RNA metabolism, translation

ABSTRACT

The FASTK family of proteins has recently emerged as a central regulator of mitochondrial gene expression through the function of an unusual RNA-binding domains named RAP, shared by all six members of the family. Here we describe the role of one of the less characterized members, FASTKD3, in mitochondrial RNA metabolism. First, we show that, in contrast to FASTK, FASTKD2 and FASTKD5, FASTKD3 does not localize in mitochondrial RNA granules, which are sites of processing and maturation of mtRNAs and ribosome biogenesis. Second, we generated FASTKD3 homozygous knockout cell lines by homologous recombination and observed that the absence of FASTKD3 resulted in increased steady-state levels and half-lives of a subset of mature mitochondrial mRNAs: ND2, ND3, CYTB, COX2 and ATP8/6. No aberrant processing of RNA precursors was observed. Rescue experiments demonstrated that RAP domain is required for FASTKD3 function in mRNA stability. Besides, we describe that FASTKD3 is required for efficient COX1 mRNA translation without altering mRNA levels, which results in a decrease in the steady-state levels of COX1 protein. This finding is associated with reduced mitochondrial complex IV assembly and activity. Our observations suggest that the function of this family of proteins goes beyond RNA processing and ribosome assembly and includes RNA stability and translation regulation within mitochondria.

INTRODUCTION

Mitochondria are thought to be descendants of endosymbiotic bacteria. During its evolution into the current “powerhouse” organelles of the eukaryotic cell, the endosymbiont transferred many of its essential genes to the nuclear chromosomes. In humans, mitochondrial DNA (mtDNA) is a circular 16.6 kb that encodes 2 rRNAs, 22 tRNAs and 13 protein-coding genes. The genome is transcribed into two long polycistronic heavy-strand and light-strand transcripts reminiscent of bacterial operons. The 2 rRNAs and most of the mRNAs are flanked by tRNAs. The polycistronic nature of the transcripts and the flanking tRNAs are the basis of the “tRNA punctuation” model. In this model, RNAse P and Z recognize the secondary structure of the tRNA precursors and cleave the RNA, leading to the release of the tRNAs and the mRNAs from the precursor RNA (1). As a number of mRNAs do
not have tRNAs flanking both ends, the so-called non canonical mRNAs, such as ATP8/6, COX3, COX1, CYTB, ND5 and ND6, the “tRNA punctuation” model is insufficient. Little is known about the mechanisms by which the 5’ and 3’ ends of these mRNAs are generated. A member of the Pentatricopeptide Repeat (PPR) protein family, PTCD2, has been reported to be involved in the processing of the pre-processed ND5-CYTB transcript (2).

The identification and characterization of a novel family of mitochondrial proteins named FASTK has shed new light on the mechanisms controlling mitochondrial posttranscriptional RNA processing and translation. This family is composed of six members: FASTK (Fas-activated serine/threonine kinase), the founding member, and its homologs FASTKD1-5 (3). All members have been found only in vertebrates (3) and were identified as RNA binding proteins on the basis of mRNA-bound proteome studies (4-6) and share 3 domains called FAST_1, FAST_2, and RAP (3). According to homology predictions the RAP (an acronym for RNA-binding domain abundant in Apicomplexans) domain is a putative RNA binding domain (7) while the function of FAST_1 and FAST_2 domains remain unknown.

FASTK and FASTKD2 were recently found to accumulate into distinct foci that colocalize with newly synthesized mitochondrial RNA in mitochondrial RNA granules (MRGs) firstly observed over a decade ago (8), and whose function has been revealed recently (9). Different proteins have been found to localize in MRGs such as GRSF1, RNase P and DDX28, among other proteins (9-12). The biological properties of the proteins present in MRGs suggest that these foci are sites of processing and maturation of newly synthesized mtRNAs and ribosome assembly. Interestingly, recent studies have shown that both FASTK and FASTKD2 are required for the expression of the ND6 mRNA, which is the only mRNA encoded on the light strand, and has no tRNA at the 3’ end (13,14). Besides that, the absence of FASTKD2 leads to aberrant processing and expression of 16S rRNA which results in impaired mitochondrial translation and OXPHOS assembly defects (14,15). It is important to note that Ghezzi et al. found homozygosity for a nonsense mutation in FASTKD2 in two siblings with familial infantile mitochondrial encephalopathy, further underlying the importance of FASTKD2 in mitochondrial function (16). A recent study has shown that endogenous FASTKD5 partially colocalizes with MRGs (15), although our previous observations with a tagged version of FASTKD5 do not support this finding (3,13). Despite conflicting data regarding the location of FASTKD5 in MRGs, it has been unequivocally demonstrated that FASTKD5 is essential for processing the three non-canonical transcripts encoded on the heavy chain (15). As a result of this property, FASTKD5 depletion renders COX1 mRNA almost undetectable, which severely reduces the synthesis of COX1 protein, resulting in a complex IV defect (15).

FASTKD1, FASTKD3 and FASTKD4 (TBRG4) do not localize in MRGs. FASTKD4 was found to modulate the half-lives of a subset of mitochondrial mRNAs and to associate with mtRNAs in vivo (17). Until now, little is known about the two members FASTKD1 and FASTKD3. We have previously reported that FASTKD3 is required for mitochondrial respiration and interacts with components of the RNA metabolism and translation machineries (3). In this manuscript, we explore the role of FASTKD3 in mitochondrial RNA metabolism and translation.

RESULTS

Generation of FASTKD3 deficient cell lines. All six FASTK family members have been annotated as RNA binding Proteins (RBPs) in independent mRNA-bound proteome studies (4-6). More recently, the three family members, namely, FASTK, FASTKD2 and FASTKD5 have been reported to localize to mitochondrial RNA granules which are considered centers for posttranscriptional RNA processing and ribosome biogenesis (13,15). However, no co-localization with BrU-labeled RNA granules was observed for FASTKD1, FASTKD3 or FASTKD4 (13). Here we confirm that FASTKD3 does not concentrate in endogenous mitochondrial RNA granules stained with the anti-FASKD2 antibody (Supplemental Figure 1), suggesting that its putative role in RNA metabolism may extend beyond these new recently described foci.

We generated FASTKD3-knockout U2OS cells to investigate the importance of FASTKD3 in mitochondrial RNA metabolism. The genomic FASTKD3 locus contains seven exons. Exon 2 contains the first ATG and represents the majority of the coding region (78%). We constructed a targeting vector designed to remove the entire
exon 2, replacing it with loxP-flanked blasticidin resistance cassette (Figure 1A). We also engineered CRISPR/Cas9n against 20 nt located at the 5’end of intron 2 in order to facilitate homologous recombination. Puromycin-resistant colonies were checked for recombination by PCR (Figure 1B). The single targeted cells obtained were treated with recombinant cell-permeant TAT-NLS-Cre recombinase to delete loxP-flanked blasticidin resistance cassette. Resultant cells were then subjected to a second round of gene targeting, and biallelic knockout cells were identified by PCR (Figure 1B). The absence of FASTKD3 in knockout cells was confirmed by western blotting (Figure 1C).

The absence of FASTKD3 leads to an increase of the steady-state levels and half-lives of a subset of mtRNAs. To explore the role of FASTKD3 in mitochondrial RNA metabolism, we first performed northern blot analysis to compare the expression of mitochondrial RNAs in wild type and FASTKD3−/− cells. FASTKD3−/− cells showed increased steady-state levels of mature mRNAs for ND2 (1.97 ± 0.14 fold increase), ND3 (2.29 ± 0.03 fold increase), CYTB (1.58 ± 0.16 fold increase), COX2 (1.85 ± 0.19 fold increase) and ATP8/6 (1.64 ± 0.11 fold increase) as compared with wild type cells (Figures 2A and 2B). The steady-state levels of all other mtDNA-encoded RNAs, including the other 7 ORFs and the ribosomal RNAs 12S and 16S were similar between wild type and FASTKD3−/− cells. As expected, FASTKD3−/− cells showed an intermediate phenotype. The increase in the steady-state levels of ND2, ND3, CYTB, COX2 and ATP8/6 transcripts in FASTKD3−/− cells was confirmed by qRT-PCR, and the results are shown in Supplemental Table 1. No alterations in the precursor mRNA levels were observed in FASTKD3−/− cells, except for a 40% decrease in RNA15 precursor (encompassing ATPase8/6-COX3 mRNA) and a non significant decrease RNA19 precursor (encompassing 16S rRNA-tRNA\text{Leu(UUA/G)}-ND1 mRNA).

Certain physiological circumstances, such as high energy needs, stimulate mitochondrial biogenesis which leads to an increase in mitochondrial transcripts associated with increased mtDNA replication and transcription (18). This mechanism seemed unlikely to contribute to the mRNA phenotype found in FASTKD3−/− cells since that would lead a global increase of steady-state levels of mtRNAs. Moreover, we have previously reported that siRNA-mediated FASTKD3 silencing does not affect mtDNA content (3). Thus, our most plausible hypothesis was that the increase in the steady-state levels of ND2, ND3, CYTB, COX2 and ATP8/6 transcripts in FASTKD3−/− cells was due to an increase in their half-lives. In order to measure the half-lives of the mitochondrial mRNAs, we blocked mitochondrial transcription with ethidium bromide. Cells were harvested at different times after the addition of the inhibitor and mitochondrial mRNAs steady-state levels were measured by qRT-PCR at each time point. The half-life of each mRNA was calculated as previously described (19). As shown in Figure 2C, ND2, ND3, CYTB, COX2 and ATP8/6 transcripts in FASTKD3−/− cells showed longer half-lives as compared to those in control cells. As expected, COX1 mRNA half-life was unaltered in the absence of FASTKD3.

We next performed rescue experiments by stably expressing either full length FASTKD3-FLAG-HA, a FASTKD3 mutant which lacks the RAP domain (FASTKD3ΔRAP-FLAG-HA), or a FASTKD3 mutant which lacks the last five amino acid residues of the RAP domain (FASTKD3Δ646-650) in FASTKD3−/− cells. FASTKD3Δ646-650 mutant was generated on the basis of a previous report showing that point mutations Y616A, L617A, K618A, K620A (NP_004740) at the C-terminus end of FASTKD4 RAP domain lead to loss of function of FASTKD4 (17). In Figure 3A, we show the sequence alignment of the RAP domain of all FASTK members and highlight the five partially conserved amino acids at the C-terminus.

As expected, overexpression of full length FASTKD3-FLAG-HA was able to rescue the FASTKD3−/− phenotype (Figure 3B), reaching steady-state levels of ND2 and ND3 transcripts similar to those seen in control wild type cells. Similarly, the levels of the transcripts CYTB, COX2 and ATP8/6 were rescued to levels comparable to those in control cells. As expected from our previous results, the levels of expression of ND5 mRNA were similar in all the cell lines. In agreement with previous work suggesting the RAP domain is required for function of FASTK proteins (13,17), expression of FASTKD3ΔRAP-FLAG-HA did not rescue the FASTKD3−/− phenotype. Finally, expression of FASTKD3Δ646-650-FLAG-HA was also unable to rescue the mRNA levels for the altered transcripts in FASTKD3−/− cells. The three
FLAG-HA recombinant proteins were expressed at similar levels as determined by western blotting (Figure 3B). We also transiently expressed GFP-tagged full length FASTKD3, FASTKD3ΔRAP or FASTKD3Δ646-650 in U2OS cells and confirmed they are all located to mitochondria and were expressed at similar levels by confocal microscopy (Figure 3C). These results ruled out the possibility that the inability of mutants FASTKD3ΔRAP or FASTKD3Δ646-650 to rescue the FASTKD3Δ−− phenotype was simply due to altered expression or location within the cell.

The absence of FASTKD3 leads to a selective decrease in COX1 translation. We next explored the role of FASTKD3 in mitochondrial translation. De novo protein synthesis was measured by metabolic labeling in the presence of emetine, which inhibits cytosolic translation. As shown in Figure 4A, increase in the steady-state levels of ND2, ND3, CYTB, COX2 and ATP8/6 transcripts did not lead to over-synthesis of the corresponding proteins. Surprisingly, the absence of FASTKD3 caused a selective >40% reduction in the synthesis of COX1 (Figure 4A). No significant alterations were detected in the de novo synthesis of other mitochondrial proteins in the FASTKD3Δ−− cells. We show representative experiments performed in two independent FASTKD3Δ−− clones.

We next explored the steady-state levels of COX1 in the FASTKD3Δ−− cells. As expected, FASTKD3Δ−− showed decreased protein levels of COX1 (approximately 2 fold reduction) compared to wild type cells (Figure 4B). The steady-state levels of other mtDNA-encoded (COX2) and nuclear-encoded (NDUFB8, NDUFA9, SDHB, UQCRCC2, COX5A and ATP5A) subunits of respiratory complexes were similar between FASTKD3Δ−− and wild type cells (Figure 4B).

We next looked at the ability of full length FASTKD3 and FASTKD3 mutants ΔRAP and Δ646-650 to restore the steady-state levels of COX1 protein. As described above, we used FASTKD3Δ−− cells stably expressing FLAG-HA tagged full length and truncated versions of FASTKD3. Only the full length construct was able to rescue the steady-state levels of COX1 protein in FASTKD3Δ−− cells (Figure 4C). Altogether, our observations demonstrate that FASTKD3 has a dual function affecting both mitochondrial mRNA stability and translation and that both activities seem to require the RAP domain.

The absence of FASTKD3 results in defective complex IV assembly and activity. The decrease in COX1 steady-state levels by deleting FASTKD3 prompted us to investigate the respiratory supercomplexes (SC) integrity by 1D-BN-PAGE using antibodies against NDUFA9 (complex I, CI), core 2 (complex III, CIII) and COX5a (complex IV, CIV) subunits which are reported to be incorporated in late steps of complexes assembly (20). Our results showed that free CIV is reduced in FASTKD3Δ−− cells. The CIII included in the SC III+IV was strongly decreased in FASTKD3Δ−− cells which in turn showed an accumulation of CIII in the SC 1+III (Figure 5A). However, there were no apparent changes in the SC 1+III+IV (the so-called respirasome).

Next, we measured the activity of the complexes of the respiratory chain in crude mitochondria isolated from FASTKD3 or the control wild type cell lines (Figure 5B). All activities were normalized to citrate synthase activity measured in the respective mitochondrial preparations. Consistent with a reduced level of assembled CIV, COX activity in FASTKD3Δ−− cells showed a 33% decrease compared to that of wild type cells. Unexpectedly, we also observed that FASTKD3Δ−− cells had a mild but significant decrease in complex II activity. Our data demonstrate that FASTKD3 is required for a fully assembled and active CIV.

DISCUSSION

Here we report the role of FASTKD3 in the regulation of mitochondrial gene expression. We show that targeted disruption of FASTKD3 gene leads to an increase in the half-lives and steady-state levels of mature mitochondrial transcripts ND2, ND3, CYTB, COX2 and ATP8/6, and that this activity requires the RAP domain of FASTKD3. In addition, we show that FASTKD3 is necessary for the translation of COX1, but not for the stability of its mRNA.

Previous work on other members of the FASTK family has shed light on the function of this emerging family of proteins in the regulation of mitochondrial RNAs. This includes the regulation of RNA processing as well as the maturation and assembly of the mitochondrial ribosomes (13-15,17). Interestingly, this is the first report that demonstrates that a FASTK family member negatively regulates the stability of a subset of
mitochondrial transcripts and selectively enhances translation. These findings raise interesting questions about the mechanism of action of FASTK proteins. FASTK has been reported to bind to ND6 mRNA and prevent its 3' end degradation by the mitochondrial degradosome, thus allowing correct ND6 processing (13). This “barrier mechanism” is not expected to be responsible for the phenotype found in FASTKD3^−/− cells since the deletion of FASTKD3 leads to the accumulation of mitochondrial mRNA rather than their depletion. However, FASTKD3 might bind to target RNAs in a way similar to how FASTK binds to ND6 and degrade the target RNAs either directly or indirectly through the recruitment of the degradosome machinery. Likewise, the apparent precursor processing defect reported for FASTKD5 depleted cells could be explained by binding to the atypical ATP8/6-COX3 junction and subsequent cleavage by FASTKD5 or by proteins recruited by FASTKD5 with nuclease activity. Therefore, it is likely that FASTK proteins have different (even opposite) molecular functions based on their ability to interact with RNA and would involve trans-acting factors such as the RNA degradation machinery (as in the case of FASTK) among others still to be defined. In this context, it is interesting to note that FASTKD2 has been recently identified in a 16S-regulatory complex together with three pseudouridine synthases that are essential for 16S stability (21).

FASTK proteins share FAST_1, FAST_2 and RAP domains. The RAP domain is particularly abundant in a phylum of parasitic protists known as apicomplexans and it is thought to be and RNA-binding domain (7). The exact biological importance of the RAP domain still needs to be clarified. For example, it seems to be required for Raa3-mediated chloroplast group II intron splicing activity in *Chlamydomonas reinhardtii* (22). In regards to its importance in the functions of FASTK proteins, we have previously reported that the RAP domain is required for the alternative splicing activity of nuclear FASTK (23). More recently, Wolf et al. have reported that four characteristic RAP domain residues located at the end of the domain are required for the ability of FASTKD4 to stabilize a subset of mitochondrial transcripts. Also, Jourdain et al. have reported that the RAP domain is essential for the interaction of FASTK with ND6 mRNA (13). Here we report that the RAP domain and also the final five residues of the domain are required for FASTKD3 function in mRNA stability. Interestingly, homology modeling of the RAP domain of FASTKD2 revealed an endonuclease-like fold that generates an interface rich in basic and aromatic residues that might be involved in RNA binding (5). The similarity between the RAP domain and endonucleases was further highlighted by structural modelling by Boulouis et al. (24). The conserved residues at the end of the RAP domain which are critical for the role of FASTKD2 and FASTKD5 in mitochondrial mRNA stability have not been identified as important for catalysis in the endonucleases based on the alignments of these domains. Further biochemical and structural studies will be required to determine if the RAP domain has endonuclease activity and the role of this domain in each member of the FASTK family. As for FAST_1 and FAST_2 domains, their function remains unknown. Interestingly, Eberhard et al. (25) suggested that FAST_1 domain is structurally related to OctotricoPeptide Repeat (OPR) domains which have been proposed to structurally and functionally resemble PentatricoPeptide Repeat (PPR) domains. Both OPR and PPR domains are predicted to fold into a pair of antiparallel α-helices. Most OPR and PPR proteins are predicted to be targeted to organelles were several have been shown to control the posttranscriptional steps of gene expression such as RNA maturation, stability and translation (24-28). It will therefore be important to study the structure of FAST_1 and FAST_2 domains and their contribution to the function of FASTK proteins.

Interestingly, the translation rate of ND2, ND3, CYTB, COX2 and ATP8/6 was not faster in FASTKD3^−/− cells despite the increase in their mRNA levels. It has been previously demonstrated that mammalian mitochondria have a great excess of transcripts under basal physiological conditions (29). We speculate that the mitochondrial translation machinery is saturated under basal physiological circumstances and therefore increased transcripts steady-state levels by 1.5 to 2 fold are not accompanied by faster translation rates.

Importantly, we also describe that FASTKD3 is required for efficient COX1 mRNA translation. Our data support a role for FASTKD3 as a translational activator of mitochondrially-encoded COX1 since its absence causes a selective defect in the translation of COX1 mRNA.
and decreased steady-state protein levels without altering its mRNA levels. TACO1 has been reported to selectively promote COX1 mRNA translation without altering mRNA levels in mammalian cells (30). Mutations in TACO1 cause cytochrome oxidase c deficiency and late-onset Leigh syndrome (30). The depletion of another FASTK protein, FASTKD5, also leads to a selective COX1 translation defect; however, and in contrast to FASTKD3$$^{-/-}$$ cells, depletion of FASTKD5 is accompanied by almost undetectable levels of COX1 mature mRNA (15). The leucine-rich pentatricopeptide repeat containing (LRPPRC) protein has been reported in in vitro studies to be necessary for the stability and translation of the COX1 and COX3 transcripts in the mitochondria and an amino-acid substitution of this protein causes the French-Canadian type of Leigh syndrome (LSFC) (31). We have previously reported that LRPPRC takes part in a protein complex with FASTKD3 (3) and we speculate that LRPPRC and FASTKD3 could act cooperatively in the regulation of COX1 translation. It will be important to unravel the molecular mechanisms underlying the ability of FASTKD3 to promote COX1 mRNA translation and to decipher the precise role that the RAP domain plays in this function.

Our data also demonstrate that FASTKD3 is required for a fully assembled and active CIV. Our 1D BN-PAGE results showed that in FASTKD3$$^{-/-}$$ cells, the free CIV content is decreased, which could be explained by the diminished COX1 protein steady-state levels. We also observed that the amount of CIII taking part in the SC III+IV was strongly decreased in FASTKD3$$^{-/-}$$ cells in which CIII seems to be incorporated into the SC I+III. We can speculate that the low steady-state levels of COX1 protein leads to both a drop in free complex IV and a reorganization of SC III+IV and SC I+III, with no apparent changes in the respirasome (SC I+III+IV). A deep debate exists on different models of supercomplexes biogenesis and assembly (32-35). However, CIV seems to be functional in free form and also being incorporated into supercomplexes (36), so the reduced levels of free CIV and SC III+IV could account for the decreased complex IV enzyme activity seen in the FASTKD3$$^{-/-}$$ cells. In vitro COX activity in FASTKD3$$^{-/-}$$ cells showed a 33% decrease compared to that of wild type cells. This finding correlates well with our earlier observation that FASTKD3 depletion causes a decrease in oxygen consumption (3). We have also observed that FASTKD3$$^{-/-}$$ cells have a mild but significant decrease in complex II activity. This result was unexpected since complex II is exclusively nuclear encoded; however, native complex II content were similar to that of wild-type cells, when an antibody against subunit SDHC, that anchor other subunits of the complex II to the internal mitochondrial membrane, was used in BN-PAGE experiments. Further studies are required to explain the differences between content and enzyme activity of CII.

In conclusion, we have established the consequences of a genomic deletion of FASTKD3 in mitochondrial RNA metabolism and our results demonstrate that FASTKD3 has dual functions: (1) as a modulator of the stability of a subset of mature mitochondrial mRNAs ND2, ND3, CYTB, COX2 and ATP8/6; and (2) promoting COX1 mRNA translation. Our observations suggest that the function of this emerging family of RNA-binding proteins goes beyond RNA processing and ribosome assembly and deeper work on FASTKD3 and the other members will help to understand the interplay between them and proteins relevant to post-transcriptional regulation of mitochondrial gene expression.

**EXPERIMENTAL PROCEDURES**

**Cells lines and antibodies.** The human osteosarcoma cell line U2OS was obtained from Dr. Paul Anderson laboratory (Harvard University). Rabbit polyclonal antibodies raised against the 350 C-terminal amino acids of human FASTKD3 and FASTKD2 were purchased from Proteintech (catalogs #18392-1-AP and #17464-1-AP, respectively). Rabbit polyclonal against actin was purchased from Sigma (catalog #AC-40). Mouse monoclonal antibodies used were as follows: anti-NDUFB8 (clone 20E9DH10C12), anti-SDHB (clone 21A11AE7), anti-UQCRC2 (clone 13G12AF12BB11), anti-COX1 (clone 1D6E1A8) and anti-ATP5A (clone 15H4C4) from Abcam, anti-NDUFA9 (clone 20C11B11B11), anti-COX2 (clone 12C4F12) and anti-COX5A (clone 6E9B12D5) from MitoSciences, anti-FLAG (clone M2) from Sigma and anti-HA (clone 16B12) from Covance. All secondary fluorescent antibodies were purchased from Molecular Probes.

**Fluorescence microscopy.** Briefly, FASTKD3-Flag transfected U2OS cells were fixed in 4% paraformaldehyde, and immunostaining with
anti-Flag and anti-FASKD2 antibodies was performed in PBS containing 0.1% Triton X-100 and 3% w/v BSA (Sigma-Aldrich). When indicated, EGFP tagged full length FASTKD3 and FASTKD3 mutants ΔRAP and Δ646-650 plasmids were transfected into cells. EGFP plasmids were generated by subcloning the respective cDNAs into the pEGFP-N1 vector (Clontech). Mitochondria were stained with 100 nM MitoTracker Red CMXRos (Thermo Fisher Scientific) and nuclei were stained with Hoechst 33258 (Sigma). Digital images were captured using a Zeiss Axioshot microscope.

Generation of FASTKD3 deficient cell lines. The targeting vector pAAV-MCS-FASTKD3 was designed to delete exon 2 of human FASTKD3 which encodes the initiator ATG, replacing it with loxP-flanked blasticidin resistance cassette. pAAV-MCS-FASTKD3 plasmid was obtained through the following steps. Firstly, the 5' homology arm (982 bp fragment) was amplified by PCR from U2OS genomic DNA using the primers 1F (5' - ATTGCAGGCCGCTGAAAGCGCTAGAA C-3', containing a NotI site) and 1R (5' - TAATGTCAGCTTATGATGGAATTA GCGAGGTTAGACGAA AGGCAAG-3'), containing a Sall site, and a FASTKD3 exon 2 sequence artificially introduced as a tool for screening). Secondly, a Sall/EcoRI fragment (1,582 bp) containing a blasticidin resistance cassette flanked by loxP sequences was obtained from a pBluescript II based plasmid previously generated in our lab. Thirdly, the 3' homology arm (1,380 bp fragment) was amplified by PCR from U2OS genomic DNA using the primers 2F (5' - ATAGAATTCTAGAAAGCTGGAAAACGTGC CCGCTGAAAGCGCTAGAA C-3', containing an EcoRI site, and a FASTKD3 exon 2 sequence artificially introduced as a tool for screening) and 2R (5' - TATGCAGGCCGCTTATGATGGAATTA GCGAGGTTAGACGAA AGGCAAG-3', containing a NotI site). The NotI/Sall-digested 5' homology arm, the Sall/EcoRI fragment containing a blasticidin resistance cassette and the EcoRI/NotI-digested 3' homology arm were cloned into the NotI site of pAAV-MCS-FASTKD3 vector as described above, in order to obtain null mutant cells for FASTKD3 (FASTKD3<sup>−/−</sup>).

Rescue of FASTKD3<sup>−/−</sup> cells and overexpression of FASTKD3 mutants. A full length FASTKD3 human cDNA (NM_024091) with C terminal FLAG-HA tag was cloned into the lentiviral vector pSin-EF2-Sox2-Pur digested with EcoRI and SpeI. pSin-EF2-Sox2-Pur was a gift from James Thomson (Addgene plasmid # 16577) (38). FASTKD3 mutants ΔRAP (deleted amino acids 593-650) and Δ646-650 (deleted amino acids 646-650) were created via QuickChange Site-Directed mutagenesis (Agilent). Amino acid positions refer to the NCBI protein database accession number NP_076996. Lentiviral stocks were produced by transient cotransfection into the human 293FT cell line (Thermo Fisher Scientific) with the appropriate lentiviral expression plasmid and lentiviral helper plasmids (psPAX2 packaging vector and pMD2.G envelope-encoding vector) using lipofectamine 2000. psPAX2 (Addgene plasmid # 12260) and pMD2.G (Addgene plasmid # 12259) were gifts from Didier Trono.
FASTKD3<sup>−/−</sup> cells were then transduced and selected with 2 µg/ml puromycin for at least 2 weeks before assaying.

**RNA isolation, qRT-PCR and Northern Blotting.** Total RNA was extracted using TRIzol (Invitrogen). DNA contamination from RNA samples was removed by treatment with DNAlase I (Ambion). RNA (1 µg) was reverse-transcribed using iScript (Bio-Rad Laboratories) to generate cDNA that was quantified by real-time PCR analysis (LightCycler 480 System; Roche Life Science) using SYBR Green PCR Master Mix (Applied Biosystems). The primers used for amplification were described in detail by Nagao et al. (19).

Northern blotting was performed as in Jourdain et al. (11), in which total RNA was extracted with TRI-Reagent (Sigma-Aldrich) and 5–15 µg RNA were separated on a denaturing formaldehyde agarose gel and transferred via electrophoresis to a Nylcon membrane (GE Healthcare). Strand-specific 32P-UTP labelled riboprobes were transcribed using T7 polymerase (Bio-Rad), and hybridization was performed at 60°C in 50% formamide, 7% SDS, 0.2M NaCl, 80 mM sodium phosphate (pH 7.4), and 100 µg/ml salmon sperm DNA. Imaging was done with a phosphorimager (Bio-Rad). The RNA bands were quantified by densitometric analysis using NIH ImageJ software.

**Measurement of mitochondrial mRNAs half-lives.** Mitochondrial transcription was disrupted with ethidium bromide (0.5 µg/ml). Cells were harvested at different time points after the addition of the inhibitor (0, 30, 60, 90, 120, 150, 180, 240, 300 and 360 min) and mitochondrial mRNAs steady-state levels were measured by qRT-PCR as described above. Half-life of each mitochondrial mRNA was calculated using the formula \( t_{1/2} = \ln 2/\lambda \), where \( \lambda \) is the slope of mRNA decay (19).

**Mitochondrial translation assay.** Cells were grown to 70% confluency in DMEM with 10% FBS and then washed two times with PBS. They were then incubated for 30 minutes with DMEM without methionine and cysteine, supplemented with 10% dialyzed FBS, Glutamax (Gibco) and 1 mM sodium pyruvate. Cytoplasmic translation was inhibited by addition of 100 µg/ml emetine and cells were incubated for 5 min. Finally, translation products were labeled by addition of 200 µCi of 35S labelled Cysteine and Methionine (Easy Tag protein labelling mix, Perkin Elmer) for 1h. Cells were washed three times with PBS, harvested and lysed. Proteins extracts were separated by SDS-PAGE in a 12-20% linear gradient gel. The gel was stained with Coomassie brilliant blue, dried and radioactive bands were visualized using a phosphorimagger.

**Respiratory chain activity.** Respiratory activity of Complexes I, II, III and IV were performed in a Shimadzu UV-1800 Spectrophotometer as previously described (39) with slight modifications.

**Blue native-PAGE (BN-PAGE).** Mitochondrial pellets were isolated from wild type and FASTKD3<sup>−/−</sup> cells and native mitochondrial proteins were prepared as described previously (33,34). Native PAGE Novex 3-12% Bis-Tris Protein Gels (Invitrogen) were loaded with 30 µg of mitochondrial protein. After electrophoresis, proteins were transferred to Nitrocellulose or PVDF membranes at 1.3 A (constant) for 10 min and probed with specific antibodies.

**Statistics.** All analyses were performed using Prism software (GraphPad). Data are expressed as mean ± SD or mean ± SE and were analyzed using one way ANOVA with Bonferroni correction, the unpaired Student t test or Mann-Whitney U test, as appropriate. *p < 0.05, **p < 0.01, ***p < 0.001.

**Acknowledgments:** We thank Roberto Cantalapiedra and Raquel Carretero for technical support.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions:** EB and MZ conducted most of the experiments, analyzed the results, made the figures and contributed with the writing of the paper. AAJ conducted some of the NB experiments and edited the manuscript. IGC has conducted BN-PAGE analyses. RTM obtained one of the FASTKD3<sup>−/−</sup> cell lines and provided excellent technical assistance in making constructs and qRT-PCR studies. ADM and MAM conducted mtDNA quantification and complexes activities measurements. JCM and AOD...
provided knowledge and infrastructure support. MSG and MAdlF conceived and coordinated the study and wrote the paper with EB and MZ. All authors analyzed the results and approved the final version of the manuscript.
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FASTKD3 and mitochondrial RNA metabolism

FOOTNOTES
This work was supported by Consejería de Sanidad JCyL (BIO/VA20/15 to MSG and BIO/VA21/15 to MaLF), Roche Diagnostics S.L (to MSG), the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (310030B_160257 / 1 to JCM), IGE3, and the State of Geneva.

The abbreviations used are: FASTKD3, FAST kinase domains 3; FASTK, Fas-activated serine/threonine kinase; LRPPRC, leucine-rich pentatricopeptide repeat containing (LRPPRC); MRGs, mitochondrial RNA granules; OPR, OctotricoPeptide Repeat; PPR, Pentatricopeptide Repeat; RAP, RNA-binding domain abundant in Apicomplexans.

FIGURE LEGENDS
FIGURE 1. Generation of FASTKD3−/− cells. (A) Schematic illustration of FASTKD3 gene targeting with pAAV-MCS-FASTKD3 plasmid. N, NotI; S, SalI; E, EcoRI. Exons are indicated by black boxes. LoxP sites are depicted with triangles, with the orientation indicated by the direction of the triangle. BSD, blasticidin-S-deaminase resistance marker. (B) Representative PCR analysis for site-specific integration. Positions of the primers used for screening are designated by arrows in (A) and expected size differences for PCR products are indicated. (C) Western blot analysis of whole-cell extracts from wild type and FASTKD3−/− with the indicated antibodies.

FIGURE 2. Mitochondrial RNA analysis in FASTKD3−/− cells. (A) RNA isolated from the indicated cell lines was analyzed by Northern blot hybridization with probes specific for the mitochondrial mRNAs, rRNAs and, as a loading control, with probes for 7SL. A representative experiment done in triplicates is shown. (B) Intensities of radioactive bands on Northern blots shown in (A) were quantified by densitometric analysis using ImageJ software. Data were normalized to 7SL RNA levels and presented relative to the wild-type control (+/+, set as 1). Values represent the mean ± SE (n=3). (C) Analysis of mtRNA half-lives and mtDNA content in FASTKD3−/− cells. Total RNA was isolated at different times up to 6 hours after treatment with ethidium bromide. Mitochondrial mRNAs steady-state levels were quantified by qRT-PCR as described in Materials and Methods. Half-life of each mitochondrial mRNA was calculated using the formula \( t_{1/2} = \frac{\ln 2}{\lambda} \), where \( \lambda \) is the slope of mRNA decay. Data are expressed as mean ± SD (n=5).

FIGURE 3. Analysis of the domains required for the decay acceleration activity of FASTKD3. (A) RAP domain protein sequence homology analysis among the different FASTK members was performed using ClustalW program. White letters on a black background highlight identical amino acids. White letters on a gray background highlight different but conserved amino acids. The red box indicates the five partially conserved amino acids at the C-terminus end of the RAP domain of FASTK family members. Amino acid position of the RAP domain boundaries are indicated. NCBI reference sequence identifiers for the aligned sequences are: FASTK (NP_006703), FASTKD1 (NP_001308975), FASTKD2 (NP_001129665), FASTKD3 (NP_076996), FASTKD4 (NP_004740) and FASTKD5 (NP_068598). (B) RNA isolated from wild type, FASTKD3−/− cells and FASTKD3−/− cells reconstituted with either full length (FL) FASTKD3-FLAG-HA, FASTKD3ΔRAP-FLAG-HA or FASTKD3Δ646-650-FLAG-HA (by lentiviral vector–mediated transduction) was analyzed by Northern blot with the indicated probes. Two lower panels show western blot analysis of whole-cell lysates using antibodies against HA or β-actin (loading control). (C) Confocal microscopy analysis of U2OS cells transiently overexpressing either full length FASTKD3-GFP, FASTKD3ΔRAP-GFP or FASTKD3Δ646-650-GFP. Mitochondria were stained with MitoTracker (CMX-ROS), and Hoechst 33258 was used for nuclei staining.

FIGURE 4. Mitochondrial protein synthesis in FASTKD3−/− cells. (A) De novo mitochondrial protein synthesis by metabolic labeling in FASTKD3−/− cells (left panel). Two independent FASTKD3−/− cell lines (clone #1 and clone #2) were incubated in the presence of 200 μCi of 35S labelled Cysteine and Methionine for 1 hour after the addition of emetine (100 μg/ml). Proteins were separated by SDS-PAGE in a 12-20% linear gradient gel. The gel was stained with Coomassie brilliant blue (CBB) and radioactivity was detected using a PhosphorImager. Complete lanes from the same PhosphorImager exposure were rearranged so that each clone and its corresponding wild type control were yuxtaposed.
This is indicated by leaving a space between the lanes. In the right panel, band intensities in FASTKD3−/− cells were determined by densitometric analysis (right panel) and data are expressed relative to those of wild type cells (set as 1). Values represent the means ± SD (n=3). (B) Representative western blot showing expression of COX1 and other MRC subunits on whole cell lysates from wild-type and FASTKD3−/− cells (left panel). Right panel shows quantification of western blot signals in FASTKD3−/− cells. Values were normalized to β-actin (loading control) and presented relative to wild-type (set as 1). Values represent the means ± SE (n=7). (C) Representative western blot of four independent experiments showing expression of COX1 in cell lysates from wild-type, FASTKD3−/− cells and FASTKD3−/− cells reconstituted with either full length (FL) FASTKD3-FLAG-HA, FASTKD3ΔRAP-FLAG-HA or FASTKD3Δ646-650-FLAG-HA (by lentiviral vector-mediated transduction).

**FIGURE 5.** Assembly and activity of MRC complexes in FASTKD3−/− cells. (A) BN-PAGE and western blot analyses of digitonin-solubilized mitochondrial extracts from wild type and FASTKD3−/− cells. Membranes were incubated with the indicated OXPHOS subunits. I+III+IV, SC containing CI, CIII, and CIV; I+III, SC containing CI and CIII; III+IV, SC containing CIII and CIV. Data are representative of two independent experiments out of four. (B) The specific activities of complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome c reductase), and complex IV (cytochrome c oxidase) were measured in cells lysates and normalized to citrate synthase activity and expressed as: (nmol/min/mg protein)/(specific activity of CS) x 100. Data are presented as means ± SE. The number of independent experiments and the p value using Mann Whitney U test are indicated.
FIGURE 1

A

Wild-type

Plasmid

Targeted

FASTKD3 locus

exon 1

ATG

P1F

P2R

P1R

P2F

N

S

E

N

S

E

P1F

P2R

P1R

P2F

+/+

+-/

-/-

5' arm

1777 bp

1270 bp

3' arm

1701 bp

1451 bp

B

C

FASTKD3 (70 KDa)

+/+

-/-

72KDa

55KDa

40KDa

β-actin
FIGURE 5

A

B

Complexes activities (corrected for Citrate Synthase)

| Complex  | Wild type | FASTKD3/f−/− |
|----------|-----------|--------------|
| Complex I | 10.8 ± 1.65 (n=7) | 11.7 ± 1.38 (n=7) | ns |
| Complex II | 18.2 ± 1.87 (n=6) | 14.4 ± 1.29 (n=6) | p=0.0051 |
| Complex III | 85.6 ± 10.13 (n=7) | 76.7 ± 8.72 (n=7) | ns |
| Complex IV | 31.9 ± 2.51 (n=10) | 21.3 ± 4.36 (n=10) | p=0.0003 |
Role of FAST kinase domains 3 (FASTKD3) in post-transcriptional regulation of mitochondrial gene expression

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J. Biol. Chem. published online October 27, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.730291

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