Regulation of mitochondrial RNA expression by FASTK proteins

BOEHM, Erik

Abstract
This work focused on the regulation of Mitochondrial RNA by the FASTK proteins. CRISPR KOs were used to investigate the effects of the loss of FASTKD1, FASTKD3, and FASTKD4, while work with FASTK and FASTKD2 was done with siRNAs and MEF KOs. All FASTK proteins were found to affect mitochondrial RNA, but the specificity and effect on RNA was varied. In particular loss of FASTKD1 or FASTKD3 had opposite effects to FASTKD4 on the loss of the ND3 RNA. FASTKD4 loss showed a striking phenotype in which there was a loss of mature ND5 and CYB RNA and a large increase in the ND5-CYB precursor. Structural modeling and mutagenesis studies suggested a similarity of a domain of the FASTK proteins to an endonuclease. In contrast the N terminal region appears to resemble PPR proteins and determines if the protein is incorporated into mitochondrial RNA granules or not.

Reference


URN : urn:nbn:ch:unige-877228
DOI : 10.13097/archive-ouverte/unige:87722
Regulation of mitochondrial RNA expression by FASTK proteins

THÈSE
présentée à la Faculté des sciences de l'Université de Genève pour obtenir le grade de Docteur ès sciences, mention biologie

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Thèse n° 4929
Genève
(imprimeur)
2016
I. Acknowledgements
I would like to thank the Swiss National Science Foundation for supporting this project [310030B_160257 / 1 to J-C.M.; 31003A_140924 and 31003A_124909 to S.T.]; IGE3; and the State of Geneva for additional funding.

I would like to thank Jean-Claude Martinou, my thesis advisor, for the chance to do my PhD work in his lab. I would additionally like to thank him for creative and stimulating project discussions, and always adhering to a high ethical standard.

Furthermore, I would like to thank the members of my thesis advisory committee, Angela Kramer and Michel Goldschmidt-Clermont, as well as the members of my thesis jury, Martin Ott and Francoise Stutz for lending their expertise to my project and its evaluation.

Additionally, I would like to thank Stephane Thore and Maria Simarro for very helpful and productive collaborations, ideas, input, and motivation.

Of course, I would also like to thank members of the Martinou lab, in particular; Audrey Bellier, Benoît Vanderperre, Etienne Raemy, Gabrielle Pena, Iwona Pilecka, Kristina Cermakova, Sandra Pierredon, Sebastien Gentina, Sebastien Herzig, Sofia Zaganelli, Tom Bender, and Zeinab Ammar, for helpful discussions, advice, and pleasant conversation.

I would additionally like to thank Ratheesh Manthottam, Elisa Radosta, and Audrey Berger for their friendship and making me feel welcome in the department.

I could not have done this work without the support of my girlfriend Cristina, who has provided invaluable motivation and support to continue and succeed both in this thesis and beyond.
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III. Abstract
Mitochondria are subcellular structures that are the result of a long symbiosis between eukaryotic cells and an ancient alpha-proteobacteria. Mitochondria and the related organelles hydrogenosomes and mitosomes perform many functions, but ATP production by oxidative phosphorylation is widely considered to be one of the most important functions. A key piece of evidence for the symbiotic origin of mitochondria are the genomes these organelles contain. In humans this genome encodes for 13 proteins, as well as the tRNA and rRNA needed to express those proteins. All other proteins present in mitochondria are encoded by the nuclear genome and are imported into the mitochondria. Mutations in mitochondrial genes often cause severe disorders that primarily affect tissues with high energy demands such as the brain or muscles. As nuclear proteins are imported into mitochondria, mutations or altered expression of nuclear encoded genes can also cause mitochondrial disorders. As there are two genomes that must be correctly expressed to ensure proper mitochondrial function, a way to coordinate the gene expression of the two genomes is needed. Currently the mechanisms underlying this coordination remain ill-defined.

FASTKD2, a member of a family of 6 proteins (in humans), has been associated with mitochondrial diseases. Interestingly, deletion or silencing of FASTKD2 causes defects in the expression of the mitochondrial encoded ND6 gene. Indeed, in this work, we found that all six members of the FASTK family are involved in regulation of mitochondrial RNA levels. Different FASTK proteins can have different effects on RNA; some reduce levels of specific RNAs while others increase levels of specific RNAs, and some appear to be involved in the cleavage of precursor RNA. It is worth noting that some FASTKs affect different RNAs with no overlap in target RNA, while other FASTKs may affect the same target RNA in opposite ways. FASTK proteins are thus one way that the nucleus is able to control expression of mitochondrial genes. While FASTKs are known to be RNA binding proteins, it is not known how they accomplish their effects.

In addition to characterizing the phenotypes of the loss of human FASTKs, we also engaged in studies to try and obtain a better understanding about the mechanism of action of FASTKs. While previous work suggested that FASTKs may bind to the end of an RNA to protect it from degradation, we noted a strong structural resemblance of the C-terminal RAP domain containing regions of FASTKs to a family of nucleases known as the PD-(D/E)-XK nucleases, in particular the subfamily Very Short-patch Repair (VSR) endonucleases. This resemblance includes an aspartate at the active site of VSR endonucleases being preserved in every human FASTK, as well as FASTKs in D. melanogaster and the only FASTK we were able to identify in C. elegans. As in VSR endonucleases, mutation of this aspartate in FASTKD4 abolished protein function, suggesting conservation of the active site across the FASTKs. Based on the modeling and these results, we
suspect that the RAP domain is a nuclease domain responsible for the effects of FASTKs affect on mitochondrial RNA expression.

Previous structural predictions also suggested that two characteristic domains N-terminal to the RAP domain, FAST_1 and FAST_2 resemble Pentatricopeptide repeat (PPR) motifs involved in recognition of RNA, and these domains have been described as Octatricopeptide repeats (OPR). Due to this resemblance, we hypothesized that the FAST_1 and FAST_2 domains would be responsible for specifying the target of the FASTK protein, and bringing the RAP domain to the appropriate target.

To test this hypothesis, we generated chimeras of different FASTK proteins and found that by exchanging the RAP domain of the FASTKD4 protein with the RAP domain of FASTKD1, we changed the effect on the target RNA. Moreover, we only had an effect on mitochondrial RNA when the replacement and substituted FAST_1 and FAST_2 domains come from FASTK proteins that effect the same RNA. These results are consistent with the idea that the RAP domain carries out the effect of the FASTK protein on an RNA target specified at least in part by the region N-terminal to the RAP domain. A nuclease activity remains to be conclusively demonstrated, as is the precise mechanism by which these RAP domains can have completely different effects on the steady state levels of the target RNA.
IV. Resume
Les mitochondries sont des organites intracellulaires qui résultent d'une longue symbiose entre une protocellule possédant un noyau (eucaryotes) et une alpha-protéobactérie. Un des éléments clés de la théorie de l'origine endosymbiotique des mitochondries proposée par Lynn Margulis en 1967 est la présence et la nature du génome contenu dans ces organites. Chez l'homme, l'ADN mitochondrial code pour 13 protéines, ainsi que pour 22 ARNt et 2 ARNr nécessaires pour exprimer ces protéines. Les 13 protéines synthétisées dans la mitochondrie participent, avec l'aide d'autres protéines codées dans le noyau, à la formation de la chaîne respiratoire et de l’ATP synthase. Cet ensemble assure la production d’ATP par le processus d’oxydation phosphorylative, une des fonctions les plus importantes des mitochondries. La majorité des protéines présentes dans les mitochondries, entre 1000 et 1500, sont codées par le génome nucléaire et importées dans les mitochondries. Des mutations dans les gènes mitochondriaux, qu’ils soient portés par l’ADN mitochondrial ou par l’ADN nucléaire, sont souvent la cause de graves troubles qui affectent principalement les tissus ayant de fortes exigences énergétiques tels que le cerveau ou les muscles. L’expression des protéines mitochondriales d’origine mitochondriale et nucléaire doit être parfaitement coordonnée pour assurer une fonction mitochondriale normale. C’est vraisemblablement le noyau qui est à la manœuvre pour gouverner cette coordination.

Des mutations dans le gène codant FASTKD2, un membre de la famille de protéines FASTK comptant 6 protéines chez l’homme, ont été associées à des maladies mitochondriales. Il est intéressant de noter que ces patients présentent des défauts dans l’expression du gène mitochondrial ND6. Dans ce travail, nous avons constaté que tous les six membres de la famille FASTK sont impliqués dans la régulation des niveaux d'ARN mitochondriaux. Nous démontrons que différentes protéines FASTK peuvent avoir différents effets sur l’ARN: certaines protéines réduisent ou augmentent le niveau de certains ARN spécifiques, tandis que d’autres semblent être impliquées dans le clivage de l’ARN précurseur. Il est aussi important de préciser que certaines protéines FASTK régulent les niveaux d’ARN spécifiquement, alors que d’autres protéines peuvent affecter un même ARN cible, mais avec des effets opposés. Les protéines FASTK sont donc un moyen pour le noyau de contrôler l’expression de gènes mitochondriaux. Bien que les protéines FASTK soient connues pour lier l’ARN, nous ne comprenons pas encore comment elles sont capables d’accomplir leur rôle.

Nous avons non seulement caractérisé l’effet de la perte de protéines FASTK dans des cellules humaines, mais aussi essayé de comprendre le mécanisme d’action de ces protéines. Alors que les travaux précédents suggéraient que les protéines FASTK pouvaient simplement se lier à la fin d’un
ARN et le protéger de la dégradation, nous avons remarqué une forte ressemblance de la structure du domaine C-terminal RAP des protéines FASTK avec une famille de nucléases, les nucléases PD-(D/E)-XK, et en particulier avec la sous-famille d’endonucléases Very Short-patch Repair (VSR). Notamment, un aspartate présent dans le site actif des endonucléases VSR est conservé dans chaque protéine FASTK humaine, ainsi que dans les protéines FASTK chez D. melanogaster et dans l’unique protéine FASTK que nous avons identifié chez C. elegans. Comme c’est le cas pour les endonucléases VSR, une mutation de cet aspartate dans FASTD4 abolit la fonction de cette protéine, ce qui suggère que le site actif des protéines FASTK est conservé dans toute la famille. D’après ces résultats et la structure du domaine RAP, nous pensons que ce domaine est en fait un domaine nucléase, responsable du rôle des protéines FASTKs dans la régulation de l’ARN mitochondrial.

Selon les données de prédiction structural, les deux domaines caractéristiques à la famille FASTK en amont du domaine RAP, les domaines FAST_1 et FAST_2, sont semblables au motif répété Pentatricopeptide (PPR) impliqué dans la reconnaissance de l’ARN, et ces domaines ont été appelés Octatricopeptide repeats (OPR). Selon cette ressemblance, nous avons suggéré que les domaines FAST_1 et FAST_2 sont responsables de la reconnaissance de la cible des différentes protéines FASTK et amènerait ainsi le domaine RAP à proximité de l’ARN cible.

Pour tester cette hypothèse, nous avons généré des chimères des différentes protéines FASTK et nous avons trouvé qu’en échangeant le domaine RAP de FASTKD4 avec celui de FASTKD1, nous changions l’effet sur l’ARN cible. Aussi, nous remarquons un effet sur l’ARN mitochondrial seulement quand les domaines FAST_1 et FAST_2 et le domaine RAP proviennent de deux protéines FASTK qui affectent un même ARN cible, et cet effet dépend du domaine RAP.

Ces résultats corroborent l’idée que le domaine RAP détermine l’effet d’une protéine FASTK sur son ARN cible, qui est lui déterminé, au moins en partie, par la région en amont du domaine RAP. L’activité nucléasique des protéines FASTK, ainsi que la manière dont le domaine RAP peut avoir différents effets afin de réguler les niveaux des ARNs cible, restent à être démontrés.
V. Publication list
FASTKD1 and FASTKD4 have opposite effects on mitochondrial RNA conferred determined by their endonuclease-like RAP domain. Erik Boehm, Stéphane Thore, Jean-Claude Martinou
Manuscript in revision for Nucleic Acids Research

FASTKD3 is a novel regulator of mitochondrial mRNA stability
Erik Boehm*, María Zornoza*, Alexis A. Jourdain, Aitor Delmiro Magdalena, Rebeca Torres Merino, Maria Simarro
*Co-first Authors
Manuscript in preparation for submission to PLOSone

VI. Introduction
Abbreviations, Names, and Aliases

ATP8/6 - bicistronic mitochondrial transcript encoding the ATP synthase subunits 6 and 8
CO1 to CO3 - mitochondrially encoded Cytochrome C oxidase subunit 1 to 3
CRISPR - Clustered regularly-interspaced short palindromic repeats
CYB - mitochondrially encoded Cytochrome B

FASTK - a protein named FAS activated Serine Threonine Kinase
FASTKD1 to FASTKD5 - proteins named FAST Kinase domains 1 to 5
FASTKD4 - Also known as TBRG-4

GRSF-1 - G rich Sequence Factor-1

MRG - Mitochondrial RNA granule
Mitochondriolous - Another name for MRGs
MIOREX - mitochondrial organization of gene expression complexes - may be MRGs

mt - Mitochondrial
mt-mRNA - Mitochondrial mRNA
mt-rRNA - Mitochondrial Ribosomal RNA

MTS - Mitochondrial targeting sequence

ND1 to ND6 - mitochondrially encoded NADH Dehydrogenase 1 to 6

OPR - Octatricopeptide Repeat

PNPase - Polynucleotide Phosphorylase, also known as PNPT1

PPR - Pentratricopeptide Repeat
LRPPRC - leucine rich pentatricopeptide repeat containing protein, mitochondrial
PTCD1 and PTCD2 - pentatricopeptide repeat domain protein 1 and 2, mitochondrial

RAP - RNA-binding domain abundant in Apicomplexans

RNAse P, Z - RNases that cleave the 5' and 3' end respectively
MRPP1 to MRPP3 - Mitochondrial ribonuclease P protein 1 to 3

SUV3 - a mitochondrial ATP-dependent RNA helicase

TIM - Translocase of the Inner Membrane

TOM - Translocase of the Outer Membrane
The mitochondrion

Origin

It is widely accepted that mitochondria are the result of an ancient endosymbiotic event between eukaryotic cells and an alpha proteobacteria (Yang et al. 1985). Key evidence for the endosymbiotic theory includes the presence of a separate mitochondrial genome distinct from the nuclear genome, unique ribosomal structure and ribosomal RNA (rRNA) sequence most closely related to rickettsia (S. G. E. Andersson et al. 1998), a unique membrane lipid composition shared with prokaryotes (Mileykovskaya and Dowhan 2009), and the use of N-formyl-methionine for translation initiation (Takeuchi et al. 2001).

Structure

Mitochondria are enclosed by two membranes, and outer and an inner membrane. Between these membranes is a space known as the inter membrane space (IMS), while the interior of the mitochondria is known as the matrix. There are transport complexes on both the outer and inner membrane (TOM and TIM) that allow nuclear encoded proteins to be imported into the matrix, IMS, or membranes. The inner membrane itself contains many invaginations known as cristae, which give the inner membrane and matrix a high surface area to volume ratio. The inner membrane contains the respiratory complexes necessary for ATP production via oxidative phosphorylation that will be discussed in more detail later. The matrix of the mitochondria contains the mitochondrial DNA and ribosomes, both of which are associated with the inner membrane.
Mitochondrial Function

Mitochondria are involved in many crucial processes in the cell. These processes include Fe-S cluster formation (Lill et al. 2012), lipid biosynthesis, and ATP production. Animal mitochondria are also involved in the regulation of cell death, as cytochrome C release from their inner membrane triggers apoptosis. Animal mitochondria also appear to play a role in the immune response through the MAVS protein localized to their outer membrane. Amongst this wide array of functions, ATP production via aerobic respiration is widely regarded as one of their main functions. It should be noted that there are derivatives of mitochondria, mitosomes and hydrogenosomes that do not participate in aerobic respiration. Hydrogenosomes are important in anaerobic respiration of fungi and some metazoans (van der Giezen 2009), (Danovaro et al. 2010), while mitosomes appear to be retained for their role in Fe-S cluster formation (Goldberg et al. 2008). These diverse functions and derivatives are likely due to the complexity of the interactions and outcomes that may arise from the sort of long term and extremely close symbiosis that produced mitochondria.

ATP production

Mitochondria are the site where the majority of ATP production in mammalian cells takes place. While glycolysis can quickly produce large amounts of ATP, much more energy can be obtained by importing the resulting pyruvate into the mitochondria, and using it as a substrate in the Krebs cycle.

![Figure 2: Overview of the metabolic steps of the Krebs cycle](http://i380.photobucket.com/albums/oo241/Biolovepulse/Misc/007krebs3.gif)

The cycle takes place after import of Acetyl-CoA or import of pyruvate and conversion into Acetyl-CoA. The total reaction is Acetyl-CoA + 3NAD^+ + FAD + GDP + P_i + 3H_2O → CoA + 3NADH + 3H^+ + GTP + 2CO_2.

In addition to importing pyruvate and converting it into acetyl-CoA using pyruvate dehydrogenase, mitochondria can also directly import Acetyl-CoA derived from fatty acids through...
the action of carnitine-acyl-CoA transferase.

In addition to the production of GTP, NADH and FADH$_2$ are used as input into the electron transport chain of the oxidative phosphorylation pathway to produce large amounts of ATP (Mitchell 1961). This electron transport chain consists of four complexes. Complex I forms NADH hydrogenase, succinate dehydrogenase is complex II, CoQH$_2$-cytochrome c reductase is formed by complex III, and lastly Complex IV forms a cytochrome c oxidase. The effect of this electron transport chain is to pump hydrogen ions (protons) into the intermembrane space, and generate a gradient that is used to drive F0F1 ATP synthase as protons flow back into the mitochondrial matrix (Watt et al. 2010). This ATP synthase thus forms the last step of this process and may be referred to as complex V of the respiratory chain.

![Figure 3: The electron transport chain.](https://en.wikipedia.org/wiki/Electron_transport_chain#/media/File:Mitochondrial_electron_transport_chain%E2%80%94Etc4.svg)

Note that products of the Krebs/Citric acid cycle enter the cycle at both Complex I (NADH) and complex II (succinate). The result of this chain is the generation of an H+ gradient that can be used to drive the ATP synthase (Mitchell 1961, Green et al. 1963, Sazanov 2015). The production of H2O is also important to many species in arid environments. Water transport across the mitochondrial membranes is accomplished by aquaporins (Calamita et al. 2005).


The 4 complexes of the electron transport chain and the ATP synthase together contain over seventy different proteins (Jonckheere, Smeitink, and Rodenburg 2012, Berg, Tymoczko, and Stryer 2002), the majority of which are encoded by the nucleus. In mammals, Complexes I, III, IV, and the ATP synthase also contain proteins encoded by the mitochondria (Jonckheere, Smeitink, and Rodenburg 2012, Berg, Tymoczko, and Stryer 2002).
Iron Sulfer cluster formation

Despite the clear specialization for ATP production present in mitochondria, it appears that mitochondria play an even more essential role in Fe-S cluster formation, as evidenced by the presence of mitosomes which do not participate in aerobic respiration but are essential for their role in Fe-S cluster formation (van der Giezen 2009). Fe-S clusters assembled in mitochondria are required for many essential processes, such as DNA polymerases, DNA helicases, and proteins involved in ribosome assembly and recycling (Lill et al. 2012). While the cell can produce ATP via glycolysis without mitochondria, mitochondria are still essential and Fe-S cluster formation may be regarded as their minimal function (Lill et al. 2012). None of the proteins encoded in the mitochondrial genome are known to be involved in a mitochondrial function other than ATP production, but it should not necessarily be assumed that an imported nuclear encoded protein will have a role in ATP production.

Mitochondrial Proteome and protein import

At the time of this writing, there are 1158 proteins reported to be localized to the mitochondria with strong support (Calvo, Clauser, and Mootha 2015). Earlier estimates of the total number of mitochondrial proteins indicated that there may be as many as 1500 distinct proteins, although a number of those may be contaminants (Lopez et al. 2000). Interestingly, this is a similar number of proteins to the number found in the alpha-proteobacteria that are most closely related to mitochondria (S. G. E. Andersson et al. 1998). The vast majority of these proteins are nuclear encoded and are imported through the Translocase of the Outer Membrane (TOM) complex and the Translocase of the Inner Membrane (TIM) complex if they are localized to the inner membrane or matrix (C. M. Koehler 2000, Lithgow 2000, van der Laan, Hutu, and Rehling 2010). Protein import is generally determined, by the presence of an N-terminal signal recognition peptide (C. M. Koehler 2000, van der Laan, Hutu, and Rehling 2010, Keenan et al. 2001, Vögtle et al. 2009); but proteins, particularly those which are inserted into the outer mitochondrial membrane, have been identified which contain internal mitochondrial localization signals (Schmidt, Pfanner, and Meisinger 2010). Import normally requires a membrane potential, but membrane potential independent ways have

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<th>Complex</th>
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<tr>
<td>Complex I</td>
<td>≥ 34</td>
<td>NADH 1-6</td>
</tr>
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<td>Complex III</td>
<td>10</td>
<td>CYB</td>
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<tr>
<td>Total</td>
<td>≥ 73</td>
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Table 1: Summary of the respiratory chain complexes. (Jonckheere, Smeitink, and Rodenburg 2012, Berg, Tymoczko, and Stryer 2002)
been found (Turakhiya et al. 2016). Insertion into the membrane is generally determined by the presence of a hydrophobic signal sequence which prevents further import and instead triggers lateral release into the membrane (Carla M Koehler, Merchant, and Schatz 1999). Import of a protein, at least in the case of TOM40, begins with the precursor peptide being bound by the chaperone HSP 90 and delivered to mitochondria in an ATP dependent manner (Humphries et al. 2005). Proteins are then recognized by the receptors TOM20 (Saitoh et al. 2007) or Tom 22 (Shiota et al. 2011). The core of the TOM complex is TOM40, a pore-forming protein with a beta-barrel structure which forms multiple channels per TOM complex (Ahting et al. 2001, Model, Meisinger, and Kühlbrandt 2008). In addition to the central TOM40 component and the TOM 20 and 22 receptors there is another additional receptor TOM70, as well as several smaller associated proteins (Schmidt, Pfanner, and Meisinger 2010). Together, these proteins allow the TOM complex to physically link to the TIM transporters. TIM22 is primarily involved in the insertion of protein into the inner membrane (Rehling et al. 2003), whereas TIM 23 is involved in the import of proteins into the matrix as well as lateral release of proteins into the inner membrane (Neupert and Herrmann 2007, Chacinska et al. 2009). Transport of proteins through the TOM complex to the TIM23 complex is mediated by the protein TIM50 as well as TIM17, 21, and 23 which form a direct but short lived connection that is evident in the form of crosslinked translation intermediates (Yamamoto et al. 2002, Mokranjac et al. 2009, Albrecht et al. 2006, Chacinska et al. 2005). Beta barrel proteins are inserted into the outer mitochondrial membrane by the “Sorting and Assemble Machinery” after import into the inter membrane space by the TOM complex (Wenz et al. 2014). Import of proteins destined for the intermembrane space is primarily driven by disulfide bond formation as part of the mitochondrial disulfide relay machinery, of which the oxidoreductase Mia40 and the sulphydryl oxidase Erv1/ALR are the key components (Fischer et al. 2013).

The Human Mitochondrial Genome

In humans, the mitochondria genome is 16.6 kbp in length, and displays an unusual and highly compact organization. The 2 rRNAs and nearly all the 13 mRNAs are flanked by tRNAs (of which there are 22). All proteins encoded by the mitochondria genome are components of the respiratory chain and involved in respiration. ND1 to ND6 encode subunits of the NAD dehydrogenase complex (Complex I), while CO1 to CO3 encode subunits of the Cytochrome c Oxidase complex (complex IV). CYB encodes a subunit of the CoQH2-cytochrome c reductase (complex III). Lastly ATP6 and ATP8 are expressed in their mature form as a bicistronic transcript that encode parts of the ATP synthase (complex V) (Mercer et al. 2011). The entire genome is transcribed into long polycistronic transcripts from promoters near the D loop on the light and heavy strand (Mercer et al. 2011).
Maturation of these transcripts will be discussed further in a following section.

FIGURE 4: The human mitochondrial genome
A schematic representation of the human mitochondria genome. The tRNAs are represented by black squares with a letter according to their corresponding amino acid. Thick colored bars represent genes or open reading frames, the genes contained are annotated. Note that tRNAs punctuate most of the encoded genes, but there are bicistronic mRNA transcripts as well as genes with separate mature RNAs without flanking tRNAs. Also note that ND6 is the only protein encoding gene on its strand. (Kyriakouli et al. 2008).

As a result of encoding a complete set of tRNAs, the mitochondrial translation apparatus has its own genetic code which differs from the code of the nuclear genome (Fearnley and Walker 1987).

Other Mitochondrial genomes
Mitochondrial genomes are highly diverse and range in size considerably. In some organisms the mitochondrial genome has been completely eliminated (León-Avila and Tovar 2004), while in others the genome is intronless and extremely compact and less than 20 kbp as in the case with mammals. While mammalian mitochondrial genomes are circular, mitochondrial genomes may also be linear as is the case for chlamydomonas reinhardtii (Vahrenholz et al. 1993). While Chlamydomonas species do have relatively compact genomes, they do have introns, yet they do not encode a full set of tRNAs and must import tRNAs (Popescu and Lee 2007). The Pseudendoclonium mitochondrial genome is more complex at nearly 96kbp encoding 57 genes, and contains a high percent of introns (Pombert et al. 2004). The presence of introns and transsplicing of mRNAs is also found in the dinoflagellates phylum (Jackson and Waller 2013), making such a system in mitochondria not so unusual. Even more diversity is displayed by the mitochondrial genomes of trypanosomes which have two mitochondrial genomes termed “maxicircles” and “minicircles” that are physically linked within a single large mitochondria (Povelones 2014).
Within metazoans, there is much more similarity in mitochondrial genomes. In D. melanogaster and C. elegans, the mitochondrial genomes are intronless, compact, and display tRNA punctuation as in mammalian genomes. Although there are broad organizational similarities with human mitochondrial genomes, it is worth noting that the relative locations of genes on the genomes differs substantially.

Figure 5: C. elegans and D. melanogaster mitochondrial genomes

Note that tRNA punctuation is preserved, but atypical RNA junctions are present as in mammalian mitochondria. Additionally notes the lack of mRNA on both strands in the C elegans mt-genome. In contrast, D. melanogaster has 5 m/tRNAs on one of its strands, and 8 on the other. Thus the distribution of mRNAs and rRNAs in human mitochondria is intermediate between the distribution of genes in these genomes. Also note that the order of genes among these genomes and the human genomes is substantially different and as a result atypical junctions are often found associated with different genes (ex: the junctions of ND3 are atypical in C. elegans, while ND5 has typical junctions in d. melanogaster).

Nucleoids

Mammalian mitochondrial DNA is known to display a punctuate distribution within mitochondria with a diameter of about 100 nm (Brown et al. 2011, Kukat et al. 2011). These concentrations of mitochondrial DNA have been termed mitochondrial “nucleoids”. These nucleoids have been compared to supercoiled regions of bacterial nucleoids (Bogenhagen 2012). There are conflicting reports of the DNA content of these nucleoids, but most studies have reported an average of about 1.5 to 3 copies of mtDNA per nucleoid (Bogenhagen 2012). It has been reported that mitochondrial fission events take place immediately adjacent to mitochondrial nucleoids (Iborra, Kimura, and Cook 2004), and that if fission is blocked, large clusters of nucleoids form at sites where fission would normally take place (Ban-Ishihara et al. 2013) indicating a process that likely ensures adequate distribution of mtDNA after mitochondrial fission. While nucleoids are known to be bound to the inner membrane of mitochondria, a number of studies suggest that they may also be
localized to points of contact between the mitochondria and the endoplasmic reticulum, but this remains unclear. These nucleoids are known to contain mitochondrial Single Strand Binding protein (mtSSB) and the mitochondrial transcription factor TFAM. In addition to playing a role in transcriptional activation, TFAM is also involved in replication and packaging of the mtDNA, not unlike bacterial histone like proteins (Bogenhagen 2012). Numerous proteins involved in the expression and processing of RNA have also been detected within mitochondria, which will be discussed in following sections in more detail.

Figure 6: Mitochondrial nucleoids
Mitochondrial DNA is indicated in green, mitochondria as labelled by Tom20 staining are in red. Note that mitochondrial DNA is concentrated into foci termed nucleoids, and that these nucleoids accumulate when mitochondrial fission is blocked by knockdown of the pro-fission factor Drp1. Adapted from Ban Ishihara 2013 (Ban-Ishihara et al. 2013).

Transcription

Transcription of mitochondrial DNA is performed by the mitochondria specific RNA polymerase POLRMT. As previously mentioned, transcription starts at promoters in the D loop region. This region contains 3 promoters, two are heavy strand promoters (HSP1 and HSP2), and one light strand promoter (LSP). HSP2 and LSP generate polycistronic transcripts corresponding to both strands of nearly the entire mitochondrial genome (Fig 7). Furthermore, it is proposed that

Figure 7: Initiation of Mitochondrial transcription
Transcription begins at the d loop for both the heavy and light strands of the mammalian mitochondria, and is carried out by the mitochondrial RNA polymerase POLRMT. This enzyme is recruited by MTERF 1-3, TFAM, and TFB2M. (Peralta, Wang, and Moraes 2012)

transcription does not always proceed along the entire length of the genome, and thus transcripts closer to the transcription start site may produced at a higher rate than those distal to the promoter.
The HSP1 promoter is reported to generate transcripts which correspond only to the region of the mitochondrial genome containing the 16S and 12S rRNA sequences. Transcription of the genome is controlled by transcription factors including TFAM, TFB2M, and MTERF1-4 (Peralta, Wang, and Moraes 2012).

**Translation**

As one would expect on the basis of the mitochondrial genome encoding ribosomal RNA and a full set of tRNAs, the mitochondrial mRNAs are translated in the organelle by ribosomes specific to the mitochondria. These ribosomes are composed of nuclear encoded proteins which form two subunits, the 28S and 39S small and large ribosome subunits which respectively incorporate the 12S and 16S mitochondrial rRNAs. While cytoplasmic ribosomes additionally contain a small 5S rRNA, according to recent crystallographic studies, mitochondrial ribosomes are unusual in that a tRNA substitutes for the 5S rRNA (Greber et al. 2014). Mitochondrial ribosomes are fairly divergent from other groups of ribosomes, but share homology with eubacterial ribosomes (further supporting a symbiotic origin) to an extent that some antibiotics such as chloramphenicol and tetracyclines can inhibit mitochondrial translation (Christian and Spremulli 2012, McKee et al. 2006).

As in bacterial systems from which mitochondria derive, the mammalian mitochondria use formyl-methionyl-tRNAMet for initiation of translation (Takeuchi et al. 2001). Translation is facilitated by mIF2 and mIF3 which are homologues of the bacterial general initiation factors IF2 and IF3, with no known homologue of IF1 (Kuzmenko et al. 2014, Atkinson et al. 2012). Additionally, there have been many factors identified that regulate translation initiation of specific mRNAs in yeast mitochondria (reviewed in (Kuzmenko et al. 2014)) and it is likely that similar proteins involved in the translation initiation of specific mRNAs are present in mammalian mitochondria. Indeed, a protein which regulates the translation initiation of COX1 has already been identified, and named Translation Activator of COX1 (Weraarpachai et al. 2009).

Unlike bacterial mRNA, mitochondrial RNA contains no shine delgarno sequence. Additionally, it also contains no 5' cap unlike Eukaryotic mRNAs. In fact, most mt-mRNA have no nucleotides 5' of the first codon at all (Montoya, Ojala, and Attardi 1981). It was found that mitochondrial ribosomes preferentially initiate at start codons at the 5' terminus, and translation initiation is significantly reduced by the presence of just a few base pairs 5' to the start codon (Christian and Spremulli 2010). This “leaderless initiation” is not similar to leaderless initiation in bacteria, as mIF3 promotes leaderless initiation while bacterial IF3 negatively regulates leaderless initiation (Christian and Spremulli 2010).

Interestingly, translation of mRNA in mammalian mitochondria does not follow the standard
genetic code. Due to mitochondria having their own set of tRNA, they have a genetic code distinct from the nuclear genetic code. In brief, the differences are as follows: ATA also codes for Methionine rather than Isoleucine, TGA is not a stop codon and codes for Tryptophan, and AGA or AGG are stop codons rather than coding for Arginine (Takeo Suzuki and Suzuki 2014, Cantara et al. 2013). The 22 tRNAs encoded by mammalian mitochondrial DNA would normally be insufficient to decode 64 codons, however post transcriptional modifications of cytidine bases at the “wobble position” of certain tRNAs enables expanded codon recognition by the tRNA anticodons (Cantara et al. 2013). Mitochondria thus have the smallest genomes that specify their own genetic code, and would be an interesting model system to study genetic code alterations if they weren't so refractory to genetic modification (Lightowlers 2011).

**tRNA punctuation model**

To explain the organization of the mitochondrial genome, the “tRNA punctuation model” was proposed by Ojala in 1981 (Ojala, Montoya, and Attardi 1981). The model proposes that a polycistronic precursor is synthesized corresponding to the entire length of the genome. Base pairing interactions then lead to the folding of the intervening tRNAs, which are cleaved by RNase P and Z – thereby releasing the mature transcripts. Mitochondria contain a unique RNaseP enzyme, which is composed of three protein subunits and lacks an RNA subunit, in contrast to cellular RNaseP. The RNA component of cytoplasmic RNaseP is known to be a “persistent contaminant” of mitochondria, and it has been reported that PNPase imports this RNA into mitochondria. Furthermore, the study which identified the protein only mitochondrial RNaseP specifically used target RNA that was not a substrate for cytoplasmic RNaseP, and thus did not exclude the possibility that a second RNA containing RNaseP was present in mitochondria (Holzmann et al. 2008). The tRNA punctuation model is widely applicable to metazoans genomes, in particular nematodes and arthropods. It was shown in *D. melanogaster* that while the tRNA punctuation model accurately predicts the ends of most RNAs even when non-coding bases are present, the non-coding 5' bases are removed prior to translation of the mRNAs, and these “mRNAs begin precisely at the start codon for each gene” (Stewart and Beckenbach 2009).

**Atypical RNA junctions**

The tRNA processing model is not sufficient to explain the production and abundance of mitochondrial RNA. In human mitochondria, there are a number of mRNAs that do not have tRNAs on both the 5' and 3' ends. Briefly, these are: ATP8/6, CO1, CO3, CYB, ND5, and ND6. CO1 has a 5' end flanked by the antisense sequence of 4 tRNAs, and its processing depends upon the presence of RNaseP, and it has been proposed that the antisense strand folds in a self complimentary manner
similar to tRNAs and is processed in the same manner (Sanchez et al. 2011). ATP8/6 has no intervening sequence between it and the CO3 RNA, and thus tRNA and even anti-tRNA processing would be expected to yield an ATP8/6-CO3 precursor; which is in fact observable. In a similar fashion, ND5 and CYB have no tRNA between them, although there is a non coding region that is the antisense of a tRNA and ND6. The ND5-CYB precursor is often visible, and its processing is not dependent upon RNaseP (Sanchez et al. 2011). Instead, it has been reported that its processing depends on PTCD2 (Xu et al. 2008). Lastly, ND6 is the only mRNA encoded on the light strand, and has no tRNA at the 3’ end. Recently our lab has identified FASTK and FASTKD2 as required for the expression of this protein. It has been proposed that the 3’ end of ND6 is protected from degradation by SUV3 (along with the rest of the non-coding light strand) by FASTK and FASTKD2.

RNA Granules

Recently, our lab published two papers (Jourdain et al. 2015, Jourdain et al. 2013) describing the putative sites where RNA processing in the mitochondria occurs. Newly synthesized mitochondrial RNA (as labeled by a pulse of BrU) accumulates in foci within mitochondria, and colocalizes with RNaseP, DDX28, GRSF-1, and other RNA binding proteins (Antonicka et al. 2013, Tu and Barrientos 2015) – suggestive of the site where tRNA processing of precursor RNA occurs. FASTK and FASTKD2, also colocalize in these foci. In our lab, PTCD2 is also known to reside in these foci(un-published data). While these observations have all been made in mammalian cells, similar structures, termed the MIOREX complex, have been reported in yeast (Kehrein et al. 2015). These granules have been implicated in ribosome biosynthesis (Antonicka and Shoubridge 2015), leading some to conclude that these structures are analogous to the nucleolus (Silva et al. 2015). However this view is disputed because the apparent tRNA processing and localization of all newly synthesized RNA within these granules is unique and not found in the nucleolus. If tRNA processing and RNA maturation is the main function of these granules, ribosomal RNA would be released from the precursor within them and an association with ribosome biogenesis may be a secondary result.

PPR Proteins

Pentatricopeptide proteins are RNA binding proteins consisting of a series of repeats of 31-36 amino acid degenerate motifs which occur in tandem arrays (Lurin et al. 2004). It was shown that these repeats are able to recognize specific RNA bases, and that by combining these domains, an artificial protein could be made to specifically bind to target RNA sequences (Coquille et al. 2014). This suggests that PPR domains may determine the RNA specificity of a protein, and can be linked
to effector domains to specifically affect an RNA species. Indeed, this was found to be the case with the CRR21 and CRR4 RNA editing enzymes in plant chloroplasts which have different PPR domains but a conserved C terminal domain which is responsible for the activity of the protein (Okuda et al. 2007). These PPR proteins are found to be involved in modulating post transcriptional processes in chloroplasts and mitochondria (Delannoy et al. 2007). Of particular interest are the proteins PTCD2 and LRPPRC. PTCD2, as previously mentioned is a component of MRGs and loss of PTCD2 function results in the accumulation of ND5-CYB, suggesting that PTCD2 may be involved in processing of the ND5-CYB junction (Xu et al. 2008). Interestingly, LRPPRC has also been implicated in the processing of ND5-CYB, although higher levels of LRPPRC correlated with an increase in unprocessed ND5-CYB (Harmel et al. 2013). LRPPRC has been reported to interact with another protein called SLIRP to form a complex that suppressed RNA decay mediated by PNPase and SUV3 while simultaneously enhancing poly adenylation of bound mRNAs (Chujo et al. 2012), which raises the possibility that the increased ND5-CYB precursor seen when LRPPRC is overexpressed is not due to a processing defect, but rather a failure to degrade excess ND5-CYB precursor. As LRPPRC is often in a complex with SLIRP, activities of the complex cannot be automatically attributed to LRPPRC alone. Experiments have shown that SLIRP is dispensable for the polyadenylation of mRNAs, but SLIRP loss causes a severe reduction in mitochondrial mRNA levels (Lagouge et al. 2015). It was also found that SLIRP stabilizes LRPPRC, and that knockout of SLIRP causes severe reductions to LRPPRC levels (Lagouge et al. 2015). As LRPPRC levels were reduced when SLIRP was knocked out, it cannot be concluded if the reduction of mRNA levels is due to a loss of SLIRP, LRPPRC, or the LRPPRC-SLIRP complex.

**RNA Degradation**

Since all mitochondrial RNAs are produced from just two promoters, selective mitochondrial RNA degradation may also be an important mechanism used to modulate the relative abundance of mature RNAs, The most well characterized protein impacting RNA degradation in mitochondria is SUV3, a member of the DEAD family of RNA helicases, which forms a complex with PNPase. This complex is responsible for the degradation of mirror RNA (Borowski et al. 2013) and alters poly-A status in mitochondria (Chujo et al. 2012, Slomovic and Schuster 2008). The role of polyadenylation in RNA degradation remains unclear, as there seems to be no consistent correlation between changes in polyadenylation status and RNA stability. It has previously been suggested that SUV3 mediated degradation takes place in MRGs, and is controlled in part by the protein FASTK (Jourdain et al. 2015). As previously mentioned, this PNPase-SUV3 mediated decay is believed to be negatively regulated by the presence of LRPPRC-SLIRP binding to coding sequences of RNA
and preventing their 3’ exonucleolytic degradation (Chujo et al. 2012).

Another RNAse, REXO2, whose silencing leads to a severe phenotype, is able to degrade short segments of single stranded RNA, by an unknown mechanism (Bruni et al. 2013). Very recently, LACTB2, a metallo-lactamase protein, was identified as an endoribonuclease in mammalian mitochondria which preferentially cleaves single strand RNA substrates after two purine-pyrimidine dinucleotide sequences (Levy et al. 2016).

**Protein Homeostasis in Mitochondria**

Proper expression of mitochondrially-encoded proteins is needed for correct mitochondrial function. As with nuclear gene expression, protein expression could be regulated at the level of transcription, mRNA stability, translation of the mRNAs, or stability of the proteins. Due to the polycistronic nature of the mitochondrial genome, regulation of the level of a specific protein at the transcriptional level poses many problems. Regulation of the levels of RNA is well supported by regulation of RNA degradation as discussed above, and additionally by other proteins that will be discussed in subsequent sections. Regulation at the level of translation is also well supported. It was found that when SLIRP is knocked out, mRNA levels are dramatically decreased, yet protein synthesis is relatively unaffected leading to the conclusion that “mammalian mitochondria have a great excess of transcripts under basal physiological conditions in vivo” (Lagouge et al. 2015). The existence of excess mRNA in mitochondria, or rather that mitochondrial mRNA levels changed without corresponding changes in protein synthesis strongly suggests that mitochondria regulate translation of mRNAs. Indeed, this was found in the case of the SLIRP knock out, on the basis of multiple lines of evidence. An increase in mitochondrial ribosome content was observed in the SLIRP KO cells relative to the WT cells, as measured by 16S rRNA content and the level of ribosomal protein as measured by the amount of MRPL37 (Lagouge et al. 2015). Correspondingly, in heart tissue, it was found that mitochondrial mRNA had a higher rate of engagement with the 55S ribosomal subunit, although contrasting results were observed in the liver. Furthermore, the authors of this study detected increased steady state levels of the translation initiation factor mIF3 (Lagouge et al. 2015). Thus, in response to altered levels of mt-mRNA, mitochondria are able to alter their translation initiation factors and ribosomal content to modulate the engagement of mRNA engagement with mitoribosomes, to adjust protein synthesis rates. While this example involved a decrease in all mRNA levels, and a response of elevated mitoribosome and translation initiation factor would elevate all translation, it is likely that there is mRNA specific modulation of translation initiation. Indeed, in the example of SLIRP knockout in the heart tissue, de novo protein synthesis was normal, with the specific exception of Nd2 and Cox1/Nd4, leading the authors to conclude
“that SLIRP is involved in presenting mature mRNAs to the mitoribosome in order to promote mitochondrial translation” (Lagouge et al. 2015). As previously mentioned, in yeast there are multiple proteins involved in the translation of specific mRNAs rather than general translation, although mammalian homologues of these have not been identified (Kuzmenko et al. 2014). Mammalian mitochondria likely contain protein involved in the translation of specific mRNAs as well. Proteins which may be involved in translational activation of specific mRNAs are discussed in subsequent sections.

**RNA modifications**

Polyadenylation of is a common modification of mitochondrial RNA. Unlike cytoplasmic RNA, many mitochondrial RNAs, such as ND5, lack long poly-A tails and instead are merely “oligo adenylated” with very short poly-A tails. The deadenylase PDE12 is a negative regulator of poly-A tail length in mitochondria, while mitochondrial Poly-A polymerase (mtPAP) is obviously a positive regulator of poly-A tail length. LRPPRC has been reported to positively regulate poly-A tail length and prevent 3’ to 5’ exonuclease degradation of RNAs. The role of PNPase and mitochondrial poly-A status is unclear, and groups have reported conflicting effects when PNPase is silenced (Chujo et al. 2012, Slomovic and Schuster 2008). Interestingly, as previously noted, poly-A tail length does not strongly correlate with RNA stability - only a subset of mitochondrial RNAs are stabilized by the presence of poly A tails (Chujo et al. 2012, Takashi Nagaike et al. 2005) while for others the stability is unchanged or increased. Interestingly, when poly-A tail length is altered by different means, the poly-A status of CO1 correlates with reduced CO1 levels (mtPAP silencing) or the poly A status of CO1 does not affect its stability (PNPase silencing) (Slomovic and Schuster 2008). It has been proposed that polyadenylation at internal sites can act as a degradation signal, and that this system may exist in tandem with a system where 3’ poly-A tails stabilize mRNAs (Takashi Nagaike, Suzuki, and Ueda 2008). Furthermore it has been shown that oligo-A tails remain when mtPAP is silenced, suggesting a second poly-A polymerase, and perhaps unique functions of poly vs oligo A tails (Slomovic and Schuster 2008).

While the role of polyadenylation on RNA stability in mitochondria remains unclear, polyadenylation is known to be required for completing the UAA stop codons of mitochondrial RNAs such as ND3 and COX3 (Slomovic and Schuster 2008), as well as maturation of tRNAs (Takashi Nagaike, Suzuki, and Ueda 2008). Another type of RNA modification is known in mitochondria, and that is poly-uridylation of RNA. Poly-U modification of RNA is also known in cytoplasmic RNA, and like cytoplasmic RNA, it seems that it is mostly ribosomal RNA that is uridinylated. However, studies have detected poly-U tails on mitochondrial mRNA when RNA metabolism is
disturbed, and it is possible that this serves as a degradation signal (Slomovic and Schuster 2008) (Szczesny et al. 2010). The tRNAs of mammalian mitochondria are also extensively modified. The acceptor stem often requires the post transcriptional addition of a CCA sequence by TNT1 (T. Nagaike et al. 2001, Tsutomu Suzuki, Nagao, and Suzuki 2011). This modification is extremely important, as failure to add the CCA sequence to the tRNAs results in impaired translation and severe mitochondrial diseases (Levinger, Mörl, and Florentz 2004, Tomari et al. 2003, Sasarman et al. 2015). Additionally, as in bacteria, the methionine tRNA may be formylated (Takeuchi et al. 2001). Unlike bacterial system which have separate Met-tRNAs for elongation or modification into formyl-Met-tRNA, there is only one Met-tRNA and the ratio between modified and unmodified forms is maintained by unknown means (Tsutomu Suzuki, Nagao, and Suzuki 2011).

![Figure 8: acceptor stem completion in mitochondrial tRNAs](image)

After the pre-tRNA folded to adopt the tRNA secondary structure, the 5’ end is cleaved by RNaseP (A). This is followed by 3’ cleavage by RNaseZ (B) to yield an immature tRNA lacking the acceptor stem for an amino acid (C). The enzyme CCAs then adds the CCA sequence (D) needed to load the tRNA with an amino acid (E). The dashed line from D to B indicates that the CCA sequence prevents 3’ cleavage by RNaseZ. From Levinger 2004 (Levinger, Mörl, and Florentz 2004).

Additionally, the wobble base in tRNAs is often modified to 5-formylcytidine, 5-taurinomethyluridine, and 5-taurinomethyl-2-thiouridine (Tsutomu Suzuki, Nagao, and Suzuki 2011), which is necessary to enable the 22 tRNAs to translate 64 codons.

**PD-(D/E)-XK nucleases**

PD-(D/E)-XK nucleases are a highly diverse group of proteins involved in many aspects of nucleic acid metabolism. These nucleases are hard to identify due to extremely low sequence conservation, but are known to retain a common core fold and a few key residues (Hence the PD-(D/E)-XK name) (Knizewski et al. 2007, Kosinski, Feder, and Bujnicki 2005, Steczkiewicz et al. 2012). Even within the core active site, variation has been observed, with the Lys being replaced by Asn, Gln, or an acidic side chain (Kosinski, Feder, and Bujnicki 2005). Elements of the core often rearrange such that the spatial relationship is maintained, but the sequence is highly divergent (Fig. 9) (Kosinski, Feder, and Bujnicki 2005). This nuclease domain was initially identified in type II
restriction enzymes (site specific DNA endonucleases that act as homodimers), but they have since been found to participate in other functions such as tRNA splicing, DNA recombination, exonuclease activity, Pol II termination, Very-Short-Patch DNA repair (VSR endonucleases), cleavage of branched DNA structures. In particular cleavage of pre-mRNA messages for the purposes of “cap snatching” (Dias et al. 2009) and RNA exonuclease activity have been reported (Laganeckas, Margelevičius, and Venclovas 2011, Xiang et al. 2009). These nucleases folds are found in every domain of life: Eukarya, Eubacteria, Archea, and in the proteins encoded by numerous viruses. Many of these functions are linked with ligation of the nucleic acid products (splicing RNA caps onto viral mRNA, tRNA splicing, DNA repair, etc) suggesting that they may

Figure 9 Examples of diverse structural rearrangements of PD-(D/E)-XK nuclease folds. Circles indicate helices, triangle indicated strands, N and C indicate the amino and carboxy terminus respectively. Green indicated the conserved common. Important conserved residues implicated in catalytic activity (D,E,K) are indicated as well as the less commonly conserved proline residue. A indicates the commonly conserved topology. B illustrates an example rearrangement found in the nuclease XPF (1j24), containing an insert into the amino acid sequence which loops out of the catalytic core. C illustrates further rearrangement and addition as found in NgoMIV(1fiu). D is yet another example of structural rearrangement as found in Tt1018(1wjd). From Kosinski 2005 (Kosinski, Feder, and Bujnicki 2005).

often be involved in the addition of nucleic acids despite their apparent action being cleavage of nucleic acids.
The FASTK family

The FASTK family of proteins is at the moment poorly characterized, and but much progress is being made. Early reports characterized them as kinase domain containing proteins. It was not until 2014 that a link was published between FASTK proteins and the regulation of mitochondrial RNA (Wolf and Mootha 2014). Currently, they are known to be RNA binding proteins (Castello et al. 2012), localized to mitochondria, and to contain 3 poorly conserved domains: FAST_1, FAST_2, and RAP (Simarro et al. 2010). Interestingly, FASTK is known to localize to both the cytosol or mitochondria on the basis of alternative translation start sites (Jourdain et al. 2015), and additionally an isoform of FASTKD4 is also predicted to not be imported into mitochondrial. The non mitochondrial form of FASTK has been shown to localize to nuclear speckles and to regulate the alternative splicing of mRNA transcripts in the nucleus (Simarro et al. 2007). As briefly mentioned in Castello et al, the RAP domain bears a significant structural resemblance to known or putative nucleases (Castello et al. 2012), while the FAST domains appear to contain leucine rich alpha helical repeats, resembling pentatricopeptide repeat (PPR) proteins and OctotricoPeptide Repeats (OPR) in chloroplasts (Eberhard et al. 2011). PPR proteins appear to have a “code” reminiscent of the TAL repeats, where each repeat recognizes a specific base (Coquille et al. 2014). While no direct evidence for such code has been shown for FASTK or OPR proteins, it seems likely that the FASTK/OPR domains are involved in recognition of specific RNA structures or sequences.

The first report of a link between FASTK proteins and mitochondrial RNA was published by the lab of Mootha, where a MitoString screen identified FASTKD4 as a regulator of multiple mitochondrial RNAs (Wolf and Mootha 2014). Our lab was already working on these proteins, and was the second to publish, reporting that FASTK knockdowns result in the loss of the mitochondrial ND6 RNA (Jourdain et al. 2015). Our lab obtained the same results for FASTKD2, while another lab reported that FASTKD2 additionally downregulates 16s rRNA (Popow et al. 2015). FASTKD4 loss as a result of RNA silencing was shown to result in a reduction of multiple mitochondrial RNAs, and it was additionally shown that FASTKD4 interacts with all mitochondrial RNA (according to RNA IP data) (Wolf and Mootha 2014). Recently Shoubridge et al. reported that silencing of FASTKD5 upregulates several mitochondrial RNAs and leads to accumulation of precursor RNA that lacks tRNA punctuation (Antonicka and Shoubridge 2015). Prior to work presented in this thesis, there has been be nothing published about the effect of FASTKD1 or FASTKD3 on mitochondrial RNA. Curiously, only FASTK and FASTKD2 appear to strongly colocalize with newly synthesized mitochondrial RNA granules (Jourdain et al. 2015, Popow et al. 2015, Antonicka and Shoubridge 2015, Simarro et al. 2010).
FASTK-like proteins

Interestingly the characteristic domains of the FASTK proteins (FAST_1, FAST_2, and RAP) are found in non mammalian species such as Chlamydomonas or D. melanogaster. In particular there are proteins in the chloroplasts of chlamydomonas that could broadly be considered a FASTK-like protein due to the presence of similar domains. In addition to the identification of RAP domains, previous studies on chlamydomonas proteins also noted a homology between their PPR like OPR domains and FAST domains of mammalian mitochondria (Eberhard et al. 2011). As with FASTK proteins, these OPR containing proteins are known to be involved in regulation of RNA in an organelle, in this case the chloroplasts (Eberhard et al. 2011, Rahire et al. 2012, Wang et al. 2015, Kleinknecht et al. 2014). In Chlamydomonas, the protein TDA1 contains helical repeats which were termed Octatricopeptide repeats (OPR) due to their resemblance to PentatricoPeptide Repeats (PPR), which are known to recognize nucleic acid sequences. Interestingly, TDA1 has a RAP domain and a homology between the OPR domains and the FAST_1 domains was noted (Eberhard et al. 2011). TDA1 was reported to have a role in translational activation of a specific mRNA transcript, while another OPR and RAP domain containing protein was reported to be involved in the maturation of 16s rRNA (Kleinknecht et al. 2014). However, the characteristic OPR and RAP domains are not always found together in the same protein. Indeed, the RAP domain is named for the large amount of proteins containing this domain found in Apicomplexans - RNA-binding domain abundant in Apicomplexans (RAP)(Lee and Hong 2004), while to date a corresponding abundance of FAST-like domains in these proteins has not been reported. Proteins in both Chlamydomonas and Drosophilia which contain only RAP domains, or only FAST domains are known. In the case of Chlamydomonas, these proteins are sometimes thought to act together, and thus it seems likely that a dimer of a protein with the FAST domains and a protein containing a RAP domains may function as a FASTK protein does in mammals. In particular it has been reported that RAA1 and RAA3 participate in group two intron splicing in chloroplasts (Perron, Goldschmidt-Clermont, and Rochaix 2004), which may indicate that a complex of proteins with FAST-like domains and proteins with RAP domains may functionally constitute a FASTK-like protein. Interestingly, the FAST domain containing protein RAA1 was also shown to interact with RAA2, a putative pseudouridine synthase which was also required for chloroplast group II intron splicing (Perron, Goldschmidt-Clermont, and Rochaix 2004). As with the RAP domain of FASTKD2, structural modeling suggests that the RAP domains in OPR/RAP proteins in chloroplasts are also endonucleases (Boulouis et al. 2015).

Given the work done in chloroplasts showing proteins similar to FASTKs regulating the maturation and translation of genes encoded by an organellar genome, the reports of FASTK
proteins regulating mitochondrial RNA are not unexpected.
V. Results
This paper summarizes the results of the CRISPR KOs of FASTKD1 and FASTKD4. Additionally it summarizes the evidence in favor of the RAP domain being an endonuclease, and the N-terminal regions being involved in specifying the RNA targets and localization of the protein.

It is currently being revised for resubmission to NAR in response to reviewer comments.

I performed all experiments in this work.

Thore performed the structural modeling, most of the structural alignments, and suggested mutation of the Aspartate at a location homologous to the active Aspartate in VSR endonucleases.

Jean-Claude oversaw the entire project and helped in experimental design and interpretation of results.
FASTKD1 and FASTKD4 have opposite effects on mitochondrial RNA conferred determined by their endonuclease-like RAP domain

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ABSTRACT
FASTK family proteins have been identified as regulators of mitochondrial RNA homeostasis linked to mitochondrial diseases, but much remains unknown about these proteins. We show that CRISPR-mediated disruption of FASTKD1 increases ND3 mRNA level, while disruption of FASTKD4 reduces the level of ND3 and of other mature mRNAs including ND5 and CYB, and causes accumulation of ND5-CYB precursor RNA. Disrupting both FASTKD1 and FASTKD4 in the same cell results in decreased ND3 mRNA similar to the effect of depleting FASTKD4 alone indicating that FASTKD4 loss is dominant. Interestingly, very low levels of FASTKD4 are sufficient to prevent ND3 loss and ND5-CYB precursor accumulation, suggesting that FASTKD4 may act catalytically. Furthermore, structural modeling predicts that the RAP domains of FASTK proteins contain a nuclease fold with a conserved aspartate residue at the putative active site. Accordingly, mutation of this residue in FASTKD4 abolished function. Experiments with FASTK chimeras indicate that the RAP domain is essential for the function of the FASTK proteins, while the region upstream determines RNA targeting and localization. In conclusion, this paper identifies new aspects of FASTK protein biology and suggests that RAP domain function depends on an intrinsic nucleolytic activity.

INTRODUCTION
The human mitochondrial genome is 16.6 kbp in length, encodes 2 rRNAs, 13 open reading frames and 22 tRNAs and is packaged into discrete structures known as nucleoids(1-2). It is transcribed into two long polycistronic transcripts which account for the vast majority of RNA within mitochondria, although import of RNA has been reported(3). The sequences encoding the 2 rRNAs and most of the mRNAs are closely flanked by
tRNAs interspersed throughout the genome. To explain how the mature RNA sequences are generated, the “tRNA punctuation” model(4) was proposed, according to which excision of the tRNAs following cleavage at their 5' and 3' cleavage by RNAse P(5) and RNase Z(6) respectively, generates the mature rRNA, and most of the mature mRNA transcripts. However, a number of mRNAs, ATP8/6, CO1, CO3, CYB, ND5, and ND6, do not have flanking tRNAs at both ends and their processing cannot be explained by this model. Little is known about the mechanisms by which the 5' and 3' ends of these latter mRNAs are generated. Of particular note are the two adjacent genes, ND5 and CYB, which lack an intervening tRNA and for which an unprocessed ND5-CYB precursor is often observed. Processing of ND5-CYB is not dependent upon RNAse P(7), but has been reported to require the PPR motif-containing protein, PTCD2(8). In addition, it was recently reported that expression of the ND6 mRNA, which is the only mRNA encoded on the light strand, and which has no tRNA at the 3' end, requires the presence of the two related proteins, FASTK and FASTKD2(9-10).

FASTK and FASTKD2 are part of a family of proteins with 6 members in humans: FASTK, and FASTKD1 to FASTKD5. All are known to be mitochondrial RNA binding proteins(11-12), and display 3 poorly conserved domains: FAST_1, FAST_2, and RAP. FASTKD2 mutations have been linked to severe encephalomyopathy in human patients (13). Interestingly, modeling of the RAP domain of FASTKD2 suggested a similarity to a putative endonuclease-like protein 2 from Neisseria gonorrhoeae, a putative PD-(D/E)-XK nuclease (11, 14). On the basis of BLAST searches, there appear to be similar proteins in other branches of the metazoan tree, such as Drosophila, but their roles in non-mammalian species have not been explored. Additionally, in chloroplasts, there are multiple proteins which contain FAST-like domains characterized as Octotricopeptide repeats(OPR) and/or RAP domains, which are known to interact with each other and participate in trans-splicing reactions, ribosomal 5' end processing, mRNA stabilization, and possibly translational activation(15-20).

FASTK and FASTKD2 are two FASTK proteins shown to affect the processing of mitochondrial RNA, specifically ND6 mRNA(9-10), as mentioned above. In 2014 Wolf and Mootha published the results of a MitoString screen that identified FASTKD4 (TBRG4) as a protein which interacts with all mitochondrial RNAs and modulates the half lives of multiple transcripts(21). More recently, Antonicka et al.(22) reported that FASTKD5 upregulates several mitochondrial RNAs and leads to accumulation of precursor RNAs that lack tRNA at both ends. One of the characteristics of some members of the FASTK
family is their submitochondrial localization within distinct RNA granules (MRGs)(9-10,13,22). In addition to FASTK proteins, the MRGs contain RNAse P(9), DDX28(23), GRSF1(24-25) as well as other RNA binding proteins (9,26), and are likely to represent the sites in mitochondria where RNA processing and assembly of the small and large ribosomal subunits occurs(26). PTC2D2, apparently required for the processing of the tRNA-less ND5-CYB junction, also localizes in the MRGs (manuscript in preparation), suggesting that the RNA processing in these MRGs is not restricted to the removal of tRNAs by RNAse P. Finally, these complexes may have many similarities to the MIOREX complexes recently described in yeast and which correspond to ribosome-containing assemblies(27).

Here we report the first characterization of the effect of FASTKD1 knock-out on mitochondrial RNA content and novel aspects of FASTKD4 mitochondrial regulation. We generated several chimeras between different FASTK family members to explore the function of their protein domains, and we provide the first experimental evidence consistent with the hypothesis that the RAP domain of FASTK family members may possess nucleolytic activity.

MATERIAL AND METHODS

Cell Culture and Transfection

Cells were cultured in DMEM, 10% fetal bovine serum with 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-Glutamine, supplied by GE Healthcare. Media for ρ0 cells was additionally supplemented with 110 µg/ml pyruvate and 50 µg/ml uridine (Sigma). Transfections were performed using calcium phosphate or lipofectamine (Invitrogen).

Immunofluorescence and microscopy

Immunofluorescence, and microscopy were performed as described in Jourdain et al. 2015(9). Briefly, cells were fixed in 4% paraformaldehyde and immunostaining with antibodies to HA (Covance), FLAG (Sigma), FASTKD2 (Proteintech), FASTKD4 (Santa Cruz) was performed in PBS containing 0.1% Triton X-100 and 3% w/v BSA (Sigma-Aldrich). Imaging was performed using a Zeiss LSM700 confocal microscope or Zeiss Axiophot microscope. To induce swelling of mitochondria, cells were treated with 5µM CCCP for 1 hour prior to fixation. Antibodies are listed in Table S1.

RNA Extraction and Northern Blotting

RNA extraction and Northern blot analyses were performed as described in Jourdain et al.,
Briefly, 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 Nylon membrane (GE Healthcare). Strand-specific $^{32}$P -UTP labelled riboprobes (Table S1) 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 mg/ml salmon sperm DNA. Imaging was done with a phosphorimager (BioRad).

Cloning and Viral Production
For protein expression, cDNAs were cloned into pWPT (Addgene) in frame with a C-terminal HA-TEV-6HIS tag or 3x FLAG tag. Chimeric proteins were cloned using the Gibson Assembly system. Lentiviruses for protein expression were produced in 293T HEK by co-transfecting the constructs of interest cloned into the pWPT vector with the viral plasmids psPAX2 and pMD2G (Addgene). After 2 days, the supernatant was collected, filtered at 0.45µm and used to infect 143B or 293T HEK cells.

CRISPR/Cas9 gene disruptions
CRISPR constructs were made by subcloning 20nt guide RNAs into the pX330 CRISPR/Cas9 plasmid (Addgene). The ends of the selected guide sequences and the PAM motif formed a BstNI cut site. Guide sequences are listed in Table S1. HEK or 143B cells were then transfected with the CRISPR construct using lipofectamine, and single cells were plated using a Beckman Coulter MoFlo Astrios cell sorter. After expanding cell populations, an approximately 200 bp region containing the predicted cut site was amplified and digested with BstNI to assay for a deletion in this region. This ~200 bp region was then sequenced to confirm disruption of the gene. FASTKD4 gene disruption candidates were further confirmed by protein immunoblotting. For the double gene disruptions, FASTKD1-KO1 was transfected with the FASTKD4 CRISPR and screened for loss of FASTKD4 by protein immunoblot (Fig. S1).

Transcriptional blockage experiments
To block transcription, cells were treated with 1µg EtBr/ml for the indicated time before RNA extraction. The resulting RNA was then blotted and probed as described above.

$^{35}$S Labeling experiments
Cells in culture were washed two times with PBS. They were then incubated for 30
minutes in media consisting of: DMEM without cysteine, methionine, or glutamine; supplemented with 10% FBS, Glutamax (Gibco) 1x, and 110 mg/L sodium pyruvate. Emetine was then added to a concentration of 1µM, and after 5 minutes 200µCi of $^{35}$S labelled Cystine and Methionine(Easy Tag protein labeling mix, Perkin Elmer) was added. 1 hour later cells were thrice washed with PBS, and cells were collected and resuspended. Equal amounts of protein were loaded and run on a 12-20 percent gradient gel. The gel was then stained with Coomassie blue, destained, dried and imaged using a phosphoimager.

Structural modeling and sequence alignment of RAP domains
Protein sequences from the FASTK family from major eukaryotic organisms (human, mouse, bovine, fish) were aligned and the alignment was manually edited to optimize loop positioning based on secondary structure prediction. Protein sequences were modeled using the webserver Phyre(28). These predicted models are aligned on the original model (homing endonuclease pdb code 3r3p) and on the Vsr endonuclease (pdb code 1vsr).

RESULTS
FASTKD1 and FASTKD4 are mitochondrial, but only FASTKD1 is enriched in MRGs
In previous studies, Wolf & Mootha have shown that FASTKD4 is found in the matrix of mitochondria, and is loosely associated with the inner mitochondrial membrane since it could be solubilized by alkali treatment of the mitochondrial membranes(21). To investigate more precisely the subcellular localization of FASTKD4 and verify proper expression of our tagged constructs, we have performed immunofluorescence studies using antibodies against the endogenous protein or against the HA or FLAG tagged derivatives. We confirm that FASTKD4 is a mitochondrial protein, but in contrast to FASTK and FASTKD2, which we have previously shown to be enriched in MRGs (9-10,13), we found that FASTKD4 is diffusely distributed throughout the mitochondrial matrix and displays no preferential co-localization with MRGs (Fig. 1A-C). Unlike FASTKD4, we were unable to stably over-express FASTKD1 from the pWPT plasmid, but were able to express FASTKD1 from the pCi plasmid where it showed clear examples of localization to MRGs as labelled with anti-FASTKD2 antibody(Fig. 1D).

Disruption of FASTKD1 and FASTKD4 genes differentially affect mitochondrial gene expression
Based on our previous studies showing that FASTK is required for expression of the ND6
mRNA(9), we hypothesized that other FASTK proteins may also regulate expression of specific mitochondrial RNAs. To test this, we used the CRISPR/Cas9 system to disrupt the FASTKD1 or FASTKD4 genes in both HEK293 and 143B cells. We obtained clones containing frameshift mutations (hereafter FASTKD1-KO or FASTKD4-KO) in both genes at the predicted cut sites (Fig. S1). To control for clonal or off target effects in the FASTKD4-KO cells, we exogenously expressed FASTKD4-FLAG, and compared the resulting RNA phenotypes by Northern blotting (Fig. 2). In the case of the FASTKD1-KO cells, because we were not able to stably express an active FASTKD1 construct, and rescued with transiently expressed FASTKD1-FLAG.

As seen in Figure 2A, in FASTKD4-KO cells we observed decreased levels of ATP8/6, COX1, COX2, COX3, CYB, ND3 and ND5 relative to the rescued and wild type cells. Interestingly, all these genes have atypical junctions except for COX2 and ND3 which are flanked at both ends by tRNAs. The results are mostly consistent with the previous observations of Wolf and Mootha(21). Interestingly, in the former study reported conflicting results for ND2, and our northern blot data agrees with the MitoString data of Wolf and Mootha in contrast to their qPCR data. Moreover we found a substantial accumulation of ND5-CYB precursor RNA which was accompanied by a decrease in the mature forms of ND5 and CYB (Fig. 2A). Restoration of FASTKD4 expression in FASTD4-KO cells efficiently decreased accumulation of the ND5-CYB precursor and increased expression of mature forms of ATP8/6, CO1, CO2, CO3, ND3, ND5 and CYB (Fig. 2A). Interestingly, there was a faint band corresponding to an unknown RNA precursor when RNA from FASTKD4 KO cells was probed against COX1. We saw no effect on other precursors that could be attributed to the presence or absence of FASTKD4. These results suggest that FASTKD4 could be a general factor involved in the processing or stabilization of mRNAs which lack flanking tRNAs at both ends.

Interestingly, when we analyzed the RNA from clones of the FASTKD1-KO cells, we saw a strong increase in the level of ND3 mRNA compared to WT cells (Fig. 2A), which was reversed by transient expression of FASTKD1-FLAG (Fig. 2 A). Interestingly, we also observed increased levels of ATP8/6 in the FASTKD1-KO cells, although these differences were less striking.

As described above, the ND3 and ATP8/6 mRNAs were affected differentially by FASTKD1 or FASTKD4 removal. To test whether loss of the FASTKD1 protein act could rescue loss of FASTKD4, we used the CRISPR/Cas9 system to disrupt the FASTKD4 gene in FASTKD1-KO cells. We obtained a clone lacking FASTKD1 and FASTKD4.
(hereafter D1&D4-KO) and assessed the functional consequences on mitochondrial RNA expression (Fig. 3A). The D1&D4-KO cells showed a pattern of RNA expression that was nearly indistinguishable from the FASTKD4-KO cell line with accumulation of ND5-CYB precursor and strong reductions in mature ND3, ND5, and ATP8/6 mRNAs (Fig. 3B). We conclude that FASTKD1 and FASTKD4 function in the same pathway to regulate expression of ND3 mRNA, and that FASTKD4 loss is dominant to FASTKD1 loss.

While generating D1&D4-KO cells, a clone was identified in which the FASTKD4 protein signal was still detectable by western blot, but at dramatically reduced levels relative to wild type (Fig. 3A, clone named D1-KOlowD4). In these cells there was no obvious reduction in mature ND5 levels, yet a small amount of ND5-CYB precursor accumulated (Fig. 3B). This result shows that the presence of FASTKD4 orders of magnitude below the normal protein level, is sufficient to prevent loss of mature form of ND5 and CYB mRNAs and accumulation of the bicistronic precursor RNA and that reduction in FASTKD4 levels could compensate for the loss of FASTKD1 and result in approximately wild type levels of ND3 level. The large effect due to a small amount of protein may suggest an enzymatic model over a stoichiometric model, although we do not know the molar amounts of FASTKD4 protein relative to the RNA to which it binds. This result is consistent with a structural modeling study which was performed in parallel and which is described below.

**Mitochondrial Translation in FASTKD1 and FASTKD4 KO cells**

We wanted to know what effect these changes to mitochondrial RNA had on mitochondrial protein synthesis, and whether the observed loss of mRNA would also lead to a decrease in the abundance of the respective protein. We thus performed in vivo $^{35}$S cysteine and methionine labeling of mitochondrial encoded proteins. As expected from the Northern blot analysis, the ND3 protein levels were decreased in the FASTKD4-KO and FASTKD1D4-KO cell lines (Fig 3C). On the other hand the observed reductions in mature levels of CYB, ND5, ATP6/8, CO1, and CO3 mRNA did not appear to result in noticeably reduced synthesis of these proteins. Indeed in the case of ND5, the protein level was slightly increased in both the FASTKD1-KO and FASTKD4-KO cell lines, and even further increased increased in the double gene disruption FASTKD1D4-KO cell line. Likewise expression of the ND6 protein appears to have been increased in the cells in which FASTKD4 was disrupted despite no observed alteration in ND6 RNA levels. In contrast to the results reported by Wolf and Mootha who observed decreased steady state levels of CO2, we did not observe a decrease in the level of de-novo synthesis of CO2. On the
basis of these results, we must consider that FASTK proteins may modulate translation of RNA and not simply the amount of RNA, or that these changes are indirect effects of a compensatory mechanism.

**Specific function of FASTK family protein domains**

Structural modeling suggests that the RAP domain present in the FASTK protein family can adopt a PD-(D/E)-XK nuclease superfamily fold(14) (Fig. 4A). In contrast to the previous results of Castello et al. for FASTKD2, using an updated database we found that FASTKD1 and FASTKD4 are best modeled on the structure of the bacterial VSR endonuclease, a verified rather than putative endonuclease. Interestingly, the VSR endonuclease was also identified as a close match for the RAP domain of the chloroplast proteins (20). Previous studies showed that VSR enzymatic activity can be abolished by a single D to A mutation in its active site(29) and we note that the homologous Aspartate (D531 in FASTKD4) appears to be highly conserved in the RAP domains of the FASTK proteins (Fig. 4B), including in a distant homologue in *C. elegans*. Therefore if the RAP domain functions similarly to the VSR endonuclease, mutation of this residue should render the protein non-functional. To perform an initial test of this hypothesis, we expressed FASTKD4 with a D531A mutation in FASTKD4-KO cells, and were unable to rescue the level of mature ND3, CO3, CYB or ND5 mRNA despite the fact that the protein was expressed and did not abolish the accumulation of the ND5-CYB precursor RNA or alter the ratio of mature ND5 to ND5-CYB precursor (Fig. 5A). Thus, the residue D531 of the RAP domain is essential for the function of FASTKD4 as would be expected if it has a conserved mechanism with the VSR endonucleases.

We have shown previously that deletion of the RAP domain in FASTK impaired ND6 mRNA expression and localization to MRGs(9). To further investigate the role of the RAP domain, we constructed chimeras of FASTK (D0) and FASTKD4 (D0D4\textsuperscript{RAP} and D4D0\textsuperscript{RAP}), as well as FASTKD1 and FASTKD4 (D4D1\textsuperscript{RAP}) by swapping their RAP domain as described in Figure 5B. We found that expression of neither the D0D4\textsuperscript{RAP} nor D4D0\textsuperscript{RAP} chimeras could prevent accumulation of the ND5-CYB precursor RNA and rescue the level of mature CO3, CYB, ND3 or ND5 mRNAs in FASTKD4-KO cells (Fig. 5A). Similarly, neither D0D4\textsuperscript{RAP} nor D4D0\textsuperscript{RAP} could rescue the loss of ND6 mRNA in FASTK-KO cells (Fig. S2A). Interestingly, D0D4\textsuperscript{RAP}, which did not prevent ND5-CYB precursor RNA accumulation, did localize in the MRGs, as assessed by its colocalization with FASTKD2, while the other chimera did not (Fig. S2B). FASTKD1 and FASTKD4 both affect the ND3
mRNA, and we found that D4D1<sup>RAP</sup> expression in FASTKD1-KO cells lead to a strong reduction of ND3 signal. In FASTKD1 KO cell line expressing D4D1<sup>RAP</sup>, ND3 levels significantly reduced relative to the ND3 level observed in control FASTKD1-KO cells. Interestingly, all mitochondrial messenger RNA was reduced by the expression of D4D1<sup>RAP</sup> although rRNA and tRNA appeared unaffected (Fig 5C). These results indicate that RAP domain of FASTKD1 is involved in downregulation mitochondrial RNA and the N-terminal domain of FASTKD4 and FASTKD1 are likely compatible with targeting the ND3 mRNA. Together these results reinforce the idea that the RAP domain is the functional domain whereas the region upstream of the RAP domain is responsible for subcellular localization and target specificity. These experiments highlight two novel functional aspects of the FASTK protein family: i) The RAP domain is likely to carry an enzymatic activity possibly with a defined specificity for each FASTK family member as the RAP domains are not interchangeable; ii) The N-terminal region of the FASTK family members, which is expected to fold as PPR-like domain formed by repetition of pairs of helices, apparently plays a role in targeting each FASTK protein to appropriate RNA(s).

**DISCUSSION**

The goal of this study was to investigate the function of two members of the FASTK family, FASTKD1 and FASTKD4. We report that both proteins affect mitochondrial gene expression through regulation of specific RNA transcript levels.

FASTKD4 is required to promote expression of the ND5, CYB, COX1, COX3 and ATP8/6 genes, which are not flanked by tRNAs on both ends, and of COX2 and ND3, which are flanked by tRNAs on their 5’ and 3’ ends. In the absence of FASTKD4, the amount of these mRNA is significantly decreased. Besides depletion of the mature mRNAs mentioned above, one of the most striking phenotypes we observed in the FASTKD4-KO cells is an accumulation of the ND5-CYB precursor, which accompanied the drop in mature ND5 and CYB mRNAs. Two non-mutually exclusive hypotheses can be proposed to explain these results (See models 1 and 2, Fig. 5D).

In the first model FASTKD4 is required for efficient processing of mRNAs with atypical junctions, including ND5-CYB precursor RNA (model 1, see Fig. 5D); ii) FASTKD4 is required to stabilize mature ATP8/6, COX1-3, CYB, ND3, and ND5 (model 2, see Fig. 5D). In support of model 1, we found that all heavy strand mRNAs with atypical junctions were affected by the loss of FASTKD4, and interestingly, similar results have recently been reported for FASTKD5(22) and the authors of that study concluded that loss of FASTKD5
causes a defect in the processing of atypical junctions. This model is further supported by previous experiments showing that in *Chlamydomonas* the RAP domain-containing protein Raa3 together with the FAST domain-containing protein Raa1 play a role in RNA transsplicing(17). The processing of ND5-CYB would either require an internal cleavage (endonuclease activity) or alternately both a 5' to 3' and 3' to 5' exonuclease activity to alternately degrade ND5-CYB from either end, but there is no known mitochondrial protein with 5' to 3' exoribonuclease activity. It is thus highly likely that an endonuclease is involved in the processing of ND5-CYB. The apparent lack of cleavage of ND5-CYB when FASTKD4 is mutated at a residue that is essential for endonuclease activity in a structurally similar protein suggests that FASTKD4 may be carrying out this endonuclease activity, However, model 1 does not explain why in addition to mRNAs with atypical junctions, the level of the ND3 mRNA, which is flanked by tRNAs on both ends, is also significantly decreased in FASTKD4 KO cells. Moreover, this model also fails to explain why ATP8/6 and COX3 precursor RNAs do not accumulate in the absence of FASTKD4.

An alternative hypothesis is that the phenotype displayed by FASTKD4-KO cells is due to increased degradation of the affected mature mRNAs (model 2). We observed that in these cells the ND5-CYB precursor RNA accumulates to a level similar to that of mature ND5 mRNA in wild type cells (Fig. 2B), despite being much less stable (Fig. S2C). This accumulation to similar steady state levels, despite a significantly higher decay rate for ND5-CYB, means that ND5-CYB must be made at a higher rate than ND5 is made. It is unlikely that the transcription of ND5-CYB is specifically increased, given that the polycistronic nature of mitochondrial transcription and the lack of any global mt-mRNA upregulation. This suggests that its is likely that normally the precursor is produced in excess of the amount needed for processing into mature ND5 or CYB, and it is the rate of mature ND5 production that is modulated.

There are two possible routes for ND5-CYB precursor: processing or degradation. If mature ND5 production is lower than ND5-CYB production due to a lack of processing, significant precursor accumulation should be visible at all times under normal conditions. Another possibility is that most of the ND5-CYB precursor is degraded prior to processing to produce mature ND5. If we assume that the processing of the ND5-CYB precursor transcript occurs normally in FASTKD4-KO cells (model 2), then its accumulation may be explained by decreased degradation rather than a processing defect. Such a mechanism would serve to compensate for the loss of mature ND5 and CYB mRNAs in the absence of FASTKD4. Interestingly in the results of Shoubridge et al. the only instances of elevated
precursor to mature RNA ratios occurred when the mature mRNA was severely depleted, and thus model 2 may explain the results reported for FASTKD5 as well. As mentioned above, models 1 and 2 may be complementary and it is possible that FASTKD4 plays a role in both processing of precursor RNAs and stabilization of mature mRNAs.

Interestingly, unlike FASTKD4 which negatively influences degradation of the ND3 mRNA, we found that FASTKD1 displays the opposite effect leading to ND3 degradation. The activity of FASTKD1 is likely to occur downstream of FASTKD4, since cells lacking both FASTKD1 and FASTKD4 display a phenotype similar to the phenotype of FASTKD4-KO cells. It is intriguing to note that these two proteins display a similar domain organization. We provide evidence that the function of the RAP domain at the C-terminus of FASTK family proteins is essential for function, possibly through an intrinsic nucleolytic activity.

**RAP domain effect.** FASTK family proteins are mitochondrial RNA binding proteins (11-12) which contain 3 poorly characterized domains: FAST_1, FAST_2, and RAP. To test whether the function of these domains is conserved amongst members of the family, we generated FASTK protein chimeras. We swapped the RAP domains between FASTKD1 and FASTKD4 to generate the D4D1\textsuperscript{RAP} chimera and between FASTK and FASTKD4 to generate both the D0D4\textsuperscript{RAP} and D4D0\textsuperscript{RAP} chimeras. It was previously shown that the absence of FASTK in FASTK-KO cells leads to loss of ND6 mRNA which can be rescued upon re-expression of the FASTK protein (9). Here we show that neither the D0D4\textsuperscript{RAP} nor the D4D0\textsuperscript{RAP} chimeras were able to rescue ND6 mRNA levels in FASTK-KO cells. Similarly, the D4D0\textsuperscript{RAP} and D0D4\textsuperscript{RAP} chimeras were unable to rescue the ND5 and CYB mRNA phenotype in FASTKD4-KO cells. Together, these results indicate that the RAP domains of FASTK and FASTKD4 are not functionally equivalent. Interestingly, the RAP domain of FASTKD1 seems to carry out its function independently of its N-terminal region. Indeed, in FASTKD1-KO cells, which display elevated ND3 mRNA levels, expression of the D4D1\textsuperscript{RAP} chimera leads to reduced ND3 mRNA levels suggesting that the RAP domain is a key determinant for the function of the protein.

**N-Terminal region.** Interestingly, the chimera lead to decreased levels of note just ND3 mRNA, but all mitochondrial mRNAs tested. The IP results of Wolf and Mootha (21) suggest that FASTKD4 binds promiscuously to RNA which may result in the D1 RAP domain of D4D1\textsuperscript{RAP} being able to downregulate a much wider range of to mt-RNAs. This result parallels the results found in plant mitochondria, where it was also seen that a
change to the OPR regions upstream of a RAP domain resulted in NCC1 and NCC2 recognizing and degrading RNA targets(20).

Possible Nuclease activity. We found that the RAP domains of FASTK family proteins display structural similarity with the PD-(D/E)-XK nuclease family(Fig. 4A). This resemblance between RAP domains and these nucleases is apparently highly conserved, as RAP domains in plant chloroplast proteins also resemble PD-(D/E)-XK nucleases, specifically including the VSR endonucleases(20).

Proteins in the PD-(D/E)-XK family exhibit diverse functions, including endonuclease activity, exonuclease activity and other nucleic acid related activities(14). Interestingly, they have even been shown to be involved in the removal and attachment of eukaryotic 5’ mRNA caps(30). Alignments and structural predictions suggest that the RAP domains of FASTK proteins have an activity that is conferred by a PD-(D/E)-XK nuclease fold with a close resemblance to VSR endonucleases, as seen in some chloroplast genes (Fig. 4A). The active site of VSR endonucleases has already been described (29) and its activity is known to be abolished by mutation of a specific Aspartate residue to an Alanine residue. The hypothesis that the effect of the RAP domain depends upon a nuclease activity shared with VSR endonucleases would be disproven if mutation of the Aspartate to Alanine failed to abolish the function of FASTKD4. We showed that FASTKD4 harboring a mutation of this residue failed to rescue ND3, ND5 and CYB mRNA levels and prevented accumulation of the ND5-CYB precursor(Fig. 5A), and thus failed to disprove the hypothesis with this test.

An enzymatic activity was also suggested by the FASTKD4 phenotypic characterization, in which we showed that the protein can fulfill its function at very low, sub-stoichiometric levels (Fig. 3B). Taken together, these results are fully consistent with the conclusion that the RAP domain of FASTKD4 contains a functional PD-(D/E)-XK nuclease fold and that the function of this protein relies on a catalytic activity of the RAP domain.

Translational compensation. Interestingly, translation is relatively unaffected despite a large decrease in multiple mRNA levels in FASTKD4-KO cells and a large increase in ND3 levels in FASTKD1-KO cells. It was previously reported by Larsson et al that there is a large excess of mitochondrial mRNA relative to the levels needed to maintain protein synthesis(31). It was however reported that these reduced mRNA levels result in apparent compensatory effects to maintain rates of translation. In their study they knocked out SLIRP and the result was a substantial reduction in mRNA levels without a corresponding
decrease in protein translation rates, which is similar to what we observe for FASTKD4 knockout cells. In the case of the SLIRP knockout, the reduction of mt-mRNA was apparently compensated by increased engagement of mRNAs with the 55S ribosomal subunits in addition to elevated levels of mt-ribosomes (31). A similar compensatory mechanism is likely at work in our FASTKD4-lacking cells, although levels of ND3 mRNA would appear to fall below the levels adequate for complete compensation. We must also consider the possibility that unprocessed ND5-CYB is able to be translated as a polycistronic mRNA as ATP8/6 and ND4/ND4L are translated. In the case of FASTKD1, the compensatory mechanism is likely operating in an opposite manner in response to elevated levels of ND3. Additional changes to de-novo translation may be occurring as part of a wider compensatory response to the altered mRNA levels in these KO cells.

It is worth noting that Eberhard et al. previously reported a FAST-like and RAP domain containing protein apparently modulated the translation of specific chloroplast mRNAs (19). This raises the possibility that that loss of FASTKD4 or FASTKD1 results in less efficient translation of certain mt-mRNAs, and steady state levels of these mRNAs are increased to compensate rather than FASTKD1 and FASTKD4 having a direct effect on the stability of these mRNAs.

**Conclusion.** In summary, our results indicate that FASTKD1 and FASTKD4 regulate mitochondrial RNA expression, and in particular that they have opposite effects on the levels of ND3. Furthermore a major determinant in the activity of these proteins lies in the C-terminal RAP domain. Strong reductions in the level of FASTKD4 can compensate for the loss of FASTKD1 with regards to the steady state levels of ND3 RNA, although other mRNAs are decreased as well. However, a complete loss of FASTKD4 is dominant over the loss of FASTKD1, and results in the loss of ND3. Similar to the results for FASTKD5, we show that FASTKD4 loss leads to elevated ND5-CYB precursor, and reductions in the mature forms of mRNAs with atypical junctions as well as ND3. On the basis of analyses using chimeric FASTK proteins, as well as structural modeling and mutagenesis of the RAP domain, we conclude that the N-terminal regions of the FASTK family proteins are responsible for RNA targeting, while the C-terminal RAP domains is essential for its activity and may contain a functional PD-(D/E)-XK nuclease fold.

**ACKNOWLEDGEMENT**

We would like to thank all current and recently departed members of the JCM lab for their
technical support, comments and assistance. We would additionally like to thank the lab of Maria Simarro for helpful correspondence. ST acknowledges the support of the Ligue genevoise contre le Cancer and the Worldwide Cancer Research.

**FUNDING**

This work was supported by the Swiss National Science Foundation [310030B_160257 / 1 to J-C.M.; 31003A_140924 and 31003A_124909 to S.T.]; IGE3; and the State of Geneva. Funding for open access charge: Swiss National Science Foundation [310030B_160257 / 1 to J-C.M.; 31003A_140924 and 31003A_124909 to S.T.]

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Figure 1. Submitochondrial localization of FASTKD1 and FASTKD4
A) FASTKD4 has a mitochondrial distribution, but is not concentrated in foci. 143B cells expressing Flag-tagged FASTKD were immunostained for FASTKD4 using an anti-Flag antibody (left) and with Mitotracker to reveal mitochondria(center). Right: merged image. Flag staining is in green, and MitotrackerRed is in red.
B) FASTKD4 shows a diffuse mitochondrial staining whereas FASTKD2 is present in discrete foci.
Immunostaining of endogenous FASTKD2 (left) and endogenous FASTKD4(center) in 143B cells using anti-FASTKD2 and FASTKD4 antibodies respectively. In the right panel, the images are merged (FASTKD4 in green and FASTKD2 in Red) and enlarged (the enlarged area is indicated with a white rectangle in the center panel).
C) Specificity of the anti-FASTKD4 antibody.
FASTKD4-KO cells were labelled with mitotracker red(left) and anti-FASTKD4 antibody(right). No specific FASTKD4 signal is visible in the FASTKD4-KO cells.
D) FASTKD1 localizes to MRGs, but is not enriched in MRGs. 143B cells were transfected with pCi FASTKD1-FLAG. Cells were then immunostained with antibodies against endogenous FASTKD2 to label MRGs (middle panels) or FLAG to label FASTKD1 (left panels). The merge is shown at the right showing FASTKD1 is in MRGs.

Figure 2. FASTKD1 and FASTKD4 affect mitochondrial mRNA expression.
A) Northern blot analysis of mitochondrial RNAs. Gene disruption of FASTKD4(D4-KO) reduces levels of multiple mitochondrial mRNAs and increases levels of multiple precursors(lane 4) relative to wild type cells (lane 3). In contrast, gene disruption of FASTKD1 (D1-KO, lane 1) leads to the over accumulation of ND3, ND1, and a slight over accumulation of ATP8/6. Expression of Flag-tagged FASTKD4(lane 6) or FASTKD1(lane 2) in gene disrupted cells reverses this phenotype and restores mRNA levels, whereas expression of GFP did not(lane 5).
B) Quantification of affected RNAs from three experiments in FASTKD4 cells is displayed in the center chart showing the RNA signal in FASTKD4-KO cells as a percent of the RNA signal in the wild-type cell line.

Figure 3. FASTKD1-FASTKD4 double gene disruption.
A) Western blot with anti-FASTKD4 (top panels) or anti-TOM20 (bottom panel) antibodies on purified mitochondria.
Loading left to right: Incomplete knockout of FASTKD4 in a FASTKD1-KO cell line (D1-KO D4low), FASTKD1 & FASTKD4 gene disrupted cells (D1&D4-KO), the progenitor FASTKD1 gene disrupted cells (D1-KO), and wild type HEK cells (HEK).

B) Northern blot using antisense ND3, ATP8/6, ND5, and 7SL probes. Equal amounts of total RNA were loaded. Labels and colonies are the same as in Fig. 3A, with the addition of RNA from a FASTKD4-KO clone (D4-KO). Note that loss of FASTKD4 is dominant over the loss of FASTKD1, and small amounts of FASTKD4 are sufficient to prevent the RNA phenotype that results from FASTKD4 loss.

C) 35S labelled mitochondrial translation. Left image: Coomassie staining as a loading control. Right image: radiograph. HEK, FASTKD1 knockout cells (D1-KO), FASTKD4 knockout cells (D4-KO) and double FASTKD1&FASTKD4 knockout cells (D1&4-KO). Note that translation is significantly altered, but that these alterations do not positively correlate with the changes in mRNA abundance, with the exception of ND3.

Figure 4. The RAP domain of FASTK family members shows structural similarities with PD-(D/E)XK nuclease.

A) Structural modeling of the human FASTKD1 and FASTKD4 proteins, compared to known PD-(D/E)-XK nuclease. Models and structures are shown as a schematic colored by secondary structure elements (α-helices blue, β-strands yellow, and loops green). Key residues suspected to be involved in the enzymatic activity are shown as sticks and colored by atom type (carbon cyan, oxygen red, nitrogen blue).

B) Alignment of protein sequences of FASTK proteins from metazoans (mammals, fish, flies, nematodes). Numbers in the alignment indicate the starting and last residues shown in the alignment as well as residues removed because of poor conservation. Predicted secondary structures are indicated on the top of the alignment. Three arrows point to the three residues responsible for the endonuclease activity in the PD-(D/E)-XK phosphodiesterase superfamily (14,29). The red colored arrow indicates the Aspartate mutated in subsequent experiments. (Note: FAKL_CAEL is the C. elegans protein B0564.7).

Figure 5. Structure-function of FASTK family members.

A) Chimeric FASTK/FASTKD4 proteins, and FASTKD4 with a D531->A531 mutation do
not rescue FASTKD4 loss. Northern blot with $^{32}$P labelled antisense ND5, and CYB RNA probes (left) or anti CO3 and ND3 probes (right). Equal amounts of total RNA from the indicated cells were loaded for analyses.

B) Schematic showing the different chimeras that were generated. To the right is a summary of the RNAs affected by overexpression of the construct. An arrow pointing up indicates RNA levels are higher in the presence of the protein, while an arrow pointing down implies the opposite. * For data supporting the effect on ND6, see Jourdain et. al. (9)

C) A chimera of FASTKD1 and FASTKD4 (D4D1RAP) rescues the ND3 phenotype of FASTKD1 loss. Northern blot with $^{32}$P labelled antisense ND3 and 7SL RNA probes. Equal amounts of total RNA from the indicated cells were loaded for analysis. This reduction in ND3 levels was accompanied by reduction in other mRNAs showing the effect is not specific to ND3.

D) Possible (non-mutually exclusive) models to explain the action of FASTKD4. Model 1: FASTKD4 promotes processing of ND5-CYB precursor. In the absence of FASTKD4, the precursor remains mostly uncleaved, thus ND5 and CYB are made in low amounts. Model 2: FASTKD4 stabilizes mature RNAs. In the absence of FASTKD4, ND5, CYB (as well as ND3, CO1-3, and ATP8/6) are degraded much faster. In response to low levels of ND5 and/or CYB, degradation of the ND5-CYB precursor degradation is inhibited to favor production of ND5 and CYB.

**Figure S1. Generation of FASTKD1 and FASTKD4 knockout cells.**
A) left panel: Schematic of the PCR and restriction digest screen showing expected results for amplification of clones with: wild type sequences (WT), heterozygous deletions (-/+), homozygous deletions (-/-), and uncut sequences (-BstNI). Panel 2nd from the Right: example results obtained for an anti-FASTKD4 CRISPR/Cas9 construct treated clone (D4 CRISPR) compared to an untreated clone (WT). Right panel: example results obtained from bulk cells treated with an anti-FASTKD1 CRISPR/Cas9 construct (D1 CRISPR) or bulk wild type cells (WT). Lower panels: example screening of clones for candidate FASTKD1 or FASTKD4 knockout cell lines. Lanes corresponding to candidate cell lines are marked with an asterisk. The high cutting rate in the D1 screen is because it is a re-screen of candidates from a previous screen.

B) Confirmation of FASTKD4 knockouts by western blot with anti-FASTKD4 antibody. Left panel: Western blot of a HEK knockout cell line (HEK D4KO), compared to untreated HEK cells (HEK WT) with a marker lane in between. Top: anti-FASTKD4 signal. Bottom: anti-
TOM20 signal on the same membrane as a loading control, with weak FASTKD4 signal still present. Right panel: a western blot as in the left panel using 143B cells. Multiple knockout cell lines (143B D4-KO), compared to untreated 143B cells (143B-WT).

C) Example sequencing results of a FASTKD4 KO cell. In this specific case the cell screened as heterozygous for a deletion in the FASTKD4 that was amplified. An allele with a 1 base pair insertion was detected. Genomic DNA was amplified by PCR, and then cloned into a vector and transformed into bacteria. Individual colonies were selected and the vector insert was sequenced to determine the sequence change. 8 colonies were selected for each clone to ensure every allele was sequenced. As expected, the mutations were always detected 3 base pairs upstream of the PAM motif where CRISPR/Cas9 is known to cut. The restriction site used in the PCR and restriction digest screen is indicated; note that the screen is leaky and only detects deletion mutations and not insertions.

Figure S2. A) Chimeric FASTK proteins do not rescue FASTK loss. Northern blot with $^{32}$P labelled antisense ND6 RNA probes. Equal amounts of total RNA from the indicated cells were loaded for analysis.

B) Immunofluorescence of the DO-D4$^{RAP}$ chimeras. Top right panel: anti-FASTKD2 in green, Lower right panel anti-HA in red. HA tagged D0D4$^{RAP}$ still localizes to RNA granules.

C) Northern blot analysis with anti-ND5 probe labeling ND5 and precursor ND5-CYB. Stability was assessed by transcriptional blockage with EtBr for the indicated times.
Figure 1

A

FASTKD4-FLAG  Mitotracker Red  Merged

B

Wild type cells

Anti-FASTKD2 (endogenous)  Anti-FASTKD4 (endogenous)  Merged and enlarged

C

FASTKD4 Knock out cells

Mitotracker Red  anti-FASTKD4

D

FASTKD1-Flag  FASTKD2  Merged
Figure 2

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FASTKD1-FLAG
FASTKD4-FLAG

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ATP8/6-CO3
ATP8/6
CO1
CO2
CO3
ND5-CYB
CYB
RNA19
ND1
ND2
ND3
ND4/4L
ND5-CYB
ND5

B

D4 KO RNA level as a % of Wild Type

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Bar graph with error bars.
Figure 5

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### D

**Model 1**

- With FASTKD4
  - Efficient Processing
  - Without FASTKD4
    - Inefficient Processing

**Model 2**

- With FASTKD4
  - Processing
  - Stabilization with FASTKD4
  - Degradation
- Without FASTKD4
  - Processing
  - Degradation
Figure S1

A  WT  +/-  -/- - BstNI

Uncut DNA (209/140 bp, D1/D4)
Frag. 1 (123/85 bp D1/D4)
Frag. 2 (85/55 bp D1/D4)

```
D4 PCR: 140 bp >
80 bp >
60 bp >
D1 PCR: <200 bp
<100 bp
```

Clone PCR digested with BstN1

FASTKD1 PCR & digest Screen

< 200 bp
< 100 bp

FASTKD4 PCR & Digest screen

B

HEK-DKO Marker lane HEK-WT

anti-FASTKD4

anti-FASTKD4

anti-Tom20

<FASTKD4

C

BstN1 recognition site
PAM Sequence
CRISPR cut site
Inserted base
### Supplemental Materials and Methods

#### Supplementary Table 1. Plasmids, Primers, and antibodies.

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**Plasmids and primers**

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This paper summarizes the results of the CRISPR KOs of FASTKD3, thus completing the characterization of human FASTK proteins, as the phenotype of FASTK, FASTKD2, and FASTKD5 have previously been published, and my other paper covers FASTKD1 and FASTKD4. As with FASTKD1 and FASTKD4, mRNAs are strongly affected, but translation is barely affected.

I performed much of the IF, and all the northern blotting, and $^{35}$S labelling in this work, with the exception of the initial RNA extraction and loading of the gel for figure 3.
Dual functions of FASTKD3 in mitochondrial mRNA stability and translation

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ABSTRACT

Some members of the FASTK family of proteins have recently emerged as central regulators 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 FAST, 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 that are totally deficient in FASTKD3. 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 plays a transcript-specific role in mitochondrial translation since its absence causes a selective defect in the translation of COX1 mRNA without altering its mRNA levels, which results in a decrease in the steady-state levels of COX1 protein. 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. Nevertheless, the mitochondrion still carries hallmarks of its bacterial ancestor. For instance, many aspects of transcription and translation mechanisms of mitochondria are similar to those of bacteria. In humans, mitochondrial DNA (mtDNA) is a circular 16.6 kb that encodes 2 rRNAs, 22 tRNAs and 13 mRNAs. 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 (Ojala, Montoya, and Attardi 1981). As a number of mRNAs do not have tRNAs flanking both ends, the so-called noncanonical 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 (Xu et al. 2008).

The identification and characterization of a novel family of mitochondrial proteins named FASTK has led new light on the mechanisms controlling mitochondrial posttranscriptional RNA processing and translation. This family is integrated by six members: FASTK, the founding member, and its homologs FASTKD1-5 (Simarro et al. 2010). All members were identified as RNA binding proteins on the basis of mRNA-bound proteome studies (Kwon et al. 2013), (Castello et al. 2012), (Baltz et al. 2012) and share 3 domains called FAST_1, FAST_2, and RAP (Simarro et al. 2010). According to homology predictions the RAP domain is a putative RNA binding domain (Lee and Hong 2004) 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 (Iborra, Kimura, and Cook 2004), and whose function has been revealed recently (Jourdain et al. 2016). Different proteins have been found to localize in MRGs such as GRSF1, RNase P and DDX28, among other proteins (Jourdain et al. 2015), (Antonicka et al. 2013), (Jourdain et al. 2013), (Tu and Barrientos 2015). 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 (Jourdain et al. 2015), (Popow et al. 2015). 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 (Popow et al. 2015), (Antonicka and Shoubridge 2015). It is important to note that Ghezzi et al. found homozygosity for a nonsense mutation in FASTKD2 in two siblings with transmitted infantile mitochondrial encephalopathy, further underlying the importance of FASTKD2 in mitochondrial function (Ghezzi et al. 2008). A recent study has shown that endogenous FASTKD5 partially colocalizes with MRGs (Antonicka and Shoubridge 2015), although our previous observations with a tagged version of FASTKD5 do not support this finding (Simarro et al. 2010), (Jourdain et al. 2015). 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 (Antonicka and Shoubridge 2015). As a result of this property, FASTKD5 depletion renders mature COX1 mRNA almost undetectable, which severely reduces the synthesis of COX1 protein, resulting in a complex IV defect (Antonicka and Shoubridge 2015).

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 (Wolf and Mootha 2014). 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 (Simarro et al. 2010). In this manuscript, we explore the role of FASTKD3 in mitochondrial RNA metabolism and translation.
MATERIALS AND METHODS

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). Rabbit polyclonal antibody against full length human Tom20 was purchased from Santa Cruz (catalog # FL-145). Mouse monoclonal antibody against Flag peptide was purchased from Sigma (clone M2, catalog #F3165). 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-FASTKD2 antibodies was performed in PBS containing 0.1% Triton X-100 and 3% w/v BSA (Sigma-Aldrich). Mitochondria were stained with 100 nM MitoTracker Red CMXRos (Thermo Fisher Scientific) when indicated. Digital images were captured using a Zeiss Axiophot 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 expression 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'-ATTGCGGCCGCCTGGAAAGCGCCTAGAAC-3', containing a NotI site) and 1R (5'-TAAGTCGACGCTCTATGCATCTGAAAATCAGCGAGGTTAGAGCAAGGCAAG-3'), containing a SalI site, and a FASTKD3 exon 2 sequence artificially introduced as a tool for screening). Secondly, a SalI/EcoRI fragment (1582 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'-ATAGAATTCATAGAAAGCTGGAAAACGTGCCTGAAGAACAGATGCTGG-3', containing an EcoRI site, and a FASTKD3 exon 2 sequence artificially introduced as a tool for screening) and 2R (5'-TATGCGGCCCGATTTGGGCGTGAAACTGA-3', containing a NotI site). The NotI/SalI-digested 5' homology arm, the SalI/EcoRI fragment containing a blasticidin resistance cassette and the EcoRI/NotI-digested 3' homology arm were cloned into the NotI site of pAAV-MCS (Addgene). We also engineered CRISPR/Cas9 against 20 nt genomic sequences 5'-GACCTGAAGTTAACAGATGC-3' and 5'-TAGTGATCCGAAGATGAGAC-3' in order to facilitate homologous recombination. Annealed oligoes were cloned into the BbsI site of pX335
containing humanized S. pyogenes Cas9 (D10A) nickase (Addgene #42335) and the resulting plasmids were named as pX335-FASTKD3-sgRNA#1 and pX335-FASTKD3-sgRNA#2, respectively. U2OS cells were transfected with the targeting vector pAAV-MCS-FASTKD3, pX335-FASTKD3-sgRNA#1 and pX335-FASTKD3-sgRNA#2 using TransIT reagent (Mirus). Transfected cells were selected with 5 µg/ml blasticidin and single colonies were checked for homologous recombination. Homologous recombination at 5’ arm was verified by PCR using primers P1F (5’-CCCATGAACACATCCTGC-3’) and P1R (5’-GCTCTATGCATCTGAAAATCAG-3’), and expected fragment sizes were 1,777 bp for the wild type allele and 1,261 bp for the targeted allele. Homologous recombination at 3’ arm was verified by PCR using primers P2F (5’-TAGAAAGCTGGAAAACGTGC-3’) and P2R (5’-TTTGATTAGTGAGTCTCATTCC-3’), and expected fragment sizes were 1,750 bp for the wild type allele and 1,450 bp for the targeted allele. The single targeted cells obtained were treated with recombinant cell-permeant His-TAT-NLS-Cre enzyme to delete loxP-flanked blasticidin cassette. Resultant cells were then subjected to a second round of gene targeting with pAAV-MCS-FASTKD3 vector as described above.

**Rescue of FASTKD3 KO 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-Sox-pur digested with EcoRI and SpeI. FASTKD3 mutants delRAP (deleted amino acids 593-650) and del646-650 (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_076996.2. Lentiviral stocks were produced by transient cotransfection into the human 293FT cell line with the appropriate lentiviral expression plasmid and lentiviral helper plasmids (pPAX-2 packaging vector and pMD2.G envelope-encoding vector) using lipofectamine 2000. FASTKD3 KO 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 DNAsse 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. (Nagao, Hino-Shigi, and Suzuki 2008).

Northern blotting was performed as in Jourdain et al 2005, 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 Nylon membrane (GE Healthcare). Strand-specific $^{32}$P-UTP labelled riboprobes (Jourdain et al. 2015) 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 mg/ml salmon sperm DNA. Imaging was done with a phosphorimager (Bio-Rad).

**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 $t_{1/2}=\ln 2 / l$, where $l$ is the slope of mRNA decay (Nagao, Hino-Shigi, and Suzuki 2008).

**Mitochondrial DNA quantitation.** DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). Relative quantification of mtDNA versus nuclear DNA (nDNA) was determined by real-time PCR using TaqMan probes against *MT-RNR1* and *RNase P* in an HT7500 Real-Time PCR System (Applied Biosystems, USA) as previously described (Andreu et al. 2009).

**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 200µCi of $^{35}$S 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 blue, dried and radioactive bands were visualized using a phosphorimager.

**Respiratory chain activity.** Respiratory activity of Complexes I, II, III and IV were performed in a Shimadzu UV-1800 Spectrophotometer as previously described (Medja et al. 2009) with slight modifications.

**Statistics.** All analyses were performed using Prism software (GraphPad). Data are expressed as mean ± SD and were analyzed using one way ANOVA with Bonferroni correction or the unpaired Student $t$ test, as appropriate.
RESULTS

Generation of FASTKD3 deficient cell lines

All six FAST family members have been annotated as RNA binding Proteins (RBPs) in independent mRNA-bound proteome studies (Baltz et al. 2012; Castello et al. 2012; Kwon et al. 2013). More recently, the three family members, namely, FAST, FASTKD2 and FASTKD5 have been reported to localize to mitochondrial RNA granules which are considered centers for posttranscriptional RNA processing and ribosome biogenesis (Jourdain et al. 2015; Antonicka and Shoubridge 2015). However, no co-localization with BrU-labeled RNA granules was observed for FASTKD1, FASTKD3 or FASTKD4 (Jourdain et al. 2015). Here we confirm that FASTKD3 does not concentrate in endogenous mitochondrial RNA granules stained with the anti-FASTKD2 antibody (Figure 1), suggesting that its putative role in RNA metabolism is independent of these new recently described foci.

We generated FASTKD3-knockout U2OS cells to investigate the importance of FASTKD3 in mitochondrial RNA processing. The genomic FASTKD3 locus contains seven exons. Exon 2 contains the first ATG and represents most part of the coding region (78%). We constructed a targeting vector designed to replace the entire exon 2, replacing it with loxP-flanked blasticidin expression cassette (Figure 2A). We also engineered CRISPR/Cas9 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 2B). The single targeted cells obtained were treated with recombinant cell-permeant Cre recombinase to delete loxP-flanked blasticidin cassette. Resultant cells were then subjected to a second round of gene targeting, and biallelic knockout cells were identified by PCR (Figure 2B). The absence of FASTKD3 in knockout cells was confirmed by both qRT-PCR (data not shown) and western blotting (Figure 2C).

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 KO cells. FASTKD3 KO 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 3A and 3B). 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 KO 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 KO cells was confirmed by qRT-PCR as previously described (Nagao, Hino-Shigi, and Suzuki 2008), and the results are shown in ST1. No alterations in the precursor mRNA levels were observed in FASTKD3 KO cells, except for a 40% decrease in RNA15 precursor (encompassing ATPase8/6-COX3 mRNA) and RNA19 precursor (encompassing 16S rRNA-tRNA(Leu)-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 (Cámara et al. 2011). This mechanism seemed unlikely to contribute to the mRNA phenotype found in FASTKD3 KO 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 (Simarro et al. 2010). As expected, FASTKD3 KO cells showed mtDNA content comparable to that of control cells (data not shown). 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 KO 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 (Nagao, Hino-Shigi, and Suzuki 2008). As shown in Figure 3C, ND2, ND3, CYTB, COX2 and ATP8/6 transcripts in FASTKD3 KO 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. It is important to note that similar results were obtained when the same assays were performed in another two independent FASTKD3 KO cell clones. We next performed rescue experiments by stably expressing either wild type FASTKD3-FlagHA, a FASTKD3 mutant which lacks the RAP domain (FASTKD3 \( \Delta \) RAP-FlagHA), or a FASTKD3 mutant which lacks the last five amino acid residues of the RAP domain (FASTKD3 \( \Delta 646-650 \)) in FASTKD3 KO cells. FASTKD3 \( \Delta 646-650 \) 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 (Wolf and Mootha 2014). As expected, overexpression of wild type FASTKD3-FlagHA was able to rescue the FASTKD3 KO phenotype (Figure 4B), reaching steady state levels of ND2 and ND3 transcripts similar to those seen in control cells. Similarly, the levels of the transcripts CYTB, COX2 and ATP8/6 were rescued to levels comparable to those in control cells (data not shown). In agreement with previous work suggesting the RAP domain is required for function of FASTK proteins (Wolf and Mootha 2014;
Jourdain et al. 2015), expression of FASTKD3ΔRAP-FlagHA did not rescue the FASTKD3 KO phenotype. Finally, and as expected, expression of FASTKD3Δ646-650-FlagHA was also unable to rescue the mRNA levels for the altered transcripts in FASTKD3 KO cells. The three Flag-HA recombinant proteins were expressed at similar levels as determined by western blotting (data not shown). We also transiently expressed GFP-tagged wild type 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 (Fig. 4c). These results ruled out the possibility that the inability of mutants FASTKD3ΔRAP or FASTKD3Δ646-650 to rescue the FASTKD3 KO 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 cytoplasmic translation. As shown in Figure 5, 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 drop in the synthesis of COX1. This result was confirmed in at least two independent FASTKD3 KO cell clones.
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 (Jourdain et al. 2015; Antonicka and Shoubridge 2015; Wolf and Mootha 2014; Popow et al. 2015). 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. This 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 (Jourdain et al. 2015). This “barrier mechanism” is not expected to be responsible for the phenotype found in FASTKD3 KO 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 degradation machinery. Likewise, the apparent precursor processing defect reported for FASTKD5 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 FASTKs proteins have various 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.

FASTK proteins share FAST_1, FAST_2 and RAP domains. The RAP domain is particularly abundant in apicomplexans and it is thought to be an RNA-binding domain (Lee and Hong 2004). 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 trans-splicing activity in *Chlamydomonas reinhardtii* (Rivier, Goldschmidt-Clermont, and Rochaix 2001). 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 (Simarro et al. 2007). 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 (Jourdain et al. 2015). Here we report that the RAP domain and also the final four 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 (Castello et al. 2012). The similarity between the RAP domain and endonucleases was further highlighted by structural modeling by Boulouis et al. (Boulouis et al. 2015). 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. 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. (Eberhard et al. 2011) 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 (Boulouis et al. 2015; Barkan and Small 2014). PPR-containing proteins constitute one of the largest protein families in land plants, with more than 400 members in most species and they exhibit a diverse repertoire of molecular functions including transcription, RNA stabilization, RNA cleavage, RNA splicing, RNA editing and translation (Dahan and Mireau 2013; Shikanai and Fujii 2013; Barkan and Small 2014). However, to date, only seven PPR proteins have been identified in humans and they are all found in mitochondria, and either by prediction or experimental evidence, all are RNA binding proteins (Lightowlers and Chrzanowska-Lightowlers 2013). We propose here that proteins of the FASTK family might present the same function than PPR proteins and that, after RNA binding through their conserved domains FAST_1 and FAST_2 domains, their nuclease like RAP domain performs their molecular function, or the FASTK proteins recruit additional co-factors to perform their molecular function.

Interestingly, translation is barely affected despite a large increase in multiple mRNA levels. On the basis of a report by Lagouge et al, Mitochondria, at least in some cases, are apparently able to efficiently compensate rates of translation for varying mRNA levels (Lagouge et al. 2015). In their study, they found that mitochondria have a great excess of mRNA under basal conditions in vivo. When SLIRP was knocked out there was a substantial reduction in mRNA levels, yet protein translation was unaffected, apparently compensated in multiple ways including increased...
engagement of mRNAs with the 55s ribosomal subunits (Lagouge et al. 2015). If mitochondria are able to increase the rate of translation when mRNAs are reduced, it is likely that they are able to reduce translation rates when mRNAs become more abundant.

While FASTKD3 directly degrading the affected RNAs is an attractive mechanism that fits with previously reported structural modelling of the RAP domain, we note that it is also possible that FASTKD3 is involved in the translation of the affected RNAs. Eberhard et al. previously reported a FAST+RAP domain containing protein in chlamydomonas chloroplasts was involved in modulating the translation of certain chloroplasts mRNAs (Eberhard et al. 2011). We therefore consider the possibility that FASTKD3 loss results in less efficient translation of certain mt-mRNAs, and steady state levels of these mRNAs are increased to compensate. Further studies, in particular ribosomal engagement studies are needed to answer this question.

Our results show that increased steady state levels of multiple mt-mRNAs did not lead to significant changes in mitochondrial protein translation, supporting the existence of a robust compensatory mechanism in mammalian mitochondria which lead to only minor changes in protein translation and mitochondrial respiratory capacity. At the moment the mechanism of this compensatory effect is unclear, but we hope this study will spur further research into post transcriptional regulation of mitochondrial gene expression.
FIGURE LEGENDS

**Figure 1. FASTKD3 spatial distribution within the mitochondria.** (A) Confocal microscopic analysis of U2OS cells transfected with FASTKD3-Flag, immunolabelled with anti-Flag and stained with MitoTracker. (B) Confocal microscopy analysis of U2OS cells transfected with FASTKD3-Flag and immunolabelled with anti-Flag and anti-FASTKD2 antibodies.

**Figure 2. Generation of FASTKD3-KO 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. (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 total cell extracts from wild type and FASTKD3 KO with the indicated antibodies.

**Figure 3. Mitochondrial RNA analysis in FASTKD3 KO 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) Relative quantification of mtRNA versus nuclear-encoded RNA was determined by real-time PCR using TaqMan probes against MT-RNR1 and RNase P. Data are expressed as mean ± SD (n=3). (C) Analysis of mtRNA half-lives and mtDNA content in FASTKD3 KO 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}=\ln2/\lambda, where \lambda is the slope of mRNA decay. Data are expressed as mean ± SD (n=5).

**Figure 4. Analysis of the domains required for the decay acceleration activity of FASTKD3.** (A) RAP domain protein sequence homology analysis among the different FAST 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. (B) RNA isolated from FASTKD3+/+, FASTKD3 KO cells and FASTKD3 KO cells reconstituted with either wild type FASTKD3-FlagHA, FASTKD3\_RAP-FlagHA or FASTKD3\_646-650-FlagHA was analyzed by Northern blot with the indicated probes. (C) Confocal microscopy analysis of U2OS cells overexpressing either wild type FASTKD3-GFP, FASTKD3\_RAP-GFP or FASTKD3\_646-650-GFP. Mitochondria were stained with MitoTracker (CMX-ROS), and Hoechst 33258 was used for nuclei staining.

**Figure 5. De novo mitochondrial protein synthesis by metabolic labeling in FASTKD3 KO**
Cells. Cells were incubated in the presence of 200µCi of $^{35}$S 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 blue and radioactivity was detected using a PhosphorImager. CBB, Coomassie brilliant blue stain. A representative experiment is shown. Two independent FASTKD3 KO cell clones exhibited similar results. Experiments were carried at least twice for each clone.
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Figure 1

A

FASTKD3-Flag

Mitotracker

Merge

B

FASTKD3-Flag

FASTKD2

Merge
Figure 2

A

Wild-type

Plasmid

Targeted

ATG

FASTKD3 locus

exon 1

Blasticidin

pAAV-MCS-FASTKD3

B

5' arm

3' arm

1777 bp
1270 bp

1701 bp
1451 bp

C

55KDa

FASTKD3

40KDa

β-actin

//
Figure 3

A

B

C

Mature mitochondrial transcripts half lives (min)

<table>
<thead>
<tr>
<th></th>
<th>FASTKD3^{+/+}</th>
<th>FASTKD3^{−/−}</th>
</tr>
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<tbody>
<tr>
<td>ND2</td>
<td>94.88 ± 7.17</td>
<td>308.1 ± 66.72</td>
</tr>
<tr>
<td>ND3</td>
<td>130.92 ± 13.34</td>
<td>231.01 ± 0.023</td>
</tr>
<tr>
<td>ND4</td>
<td>130.92 ± 13.33</td>
<td>211.79 ± 33.35</td>
</tr>
<tr>
<td>CYTB</td>
<td>115.34 ± 0.29</td>
<td>211.76 ± 33.32</td>
</tr>
<tr>
<td>COX2</td>
<td>80.21 ± 5.56</td>
<td>123.08 ± 13.44</td>
</tr>
<tr>
<td>ATP8/6</td>
<td>123.21 ± 13.34</td>
<td>231.01 ± 0.023</td>
</tr>
<tr>
<td>COX1</td>
<td>130.92 ± 13.33</td>
<td>138.21 ± 0.36</td>
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Unpublished Results

Identification of a likely FASTK homologue in *C. elegans*.

BLAST searches of the translated *C. elegans* genome failed to find any significant matches with human or mice FASTKs. However, homologues were identified in drosophila with domains recognized as FAST_1, FAST_2, and RAP by the bioinformatic software. BLASTing these D. melanogaster homologues against *C. elegans* identified the protein B0564.7 as a probable homologue. Further blasting of this protein against other nematode genomes identified a number of genes with names such as “TBR4-like” (TBR4 is another name or FASTKD4), supporting the idea that FASTK homologues are present in nematodes an *C. elegans* in particular. Additional bioinformatic support for this is evident in the conservation of the aspartate residue shown in figure 4B of Boehm et al. as well as a strong prediction of a mitochondrial localization by mitoprot.

We thus predicted that this protein was a FASTK homologue, and that the lack of this protein would result in a specific mitochondria RNA phenotype. A worm strain in which B0564.7 contained an early stop mutation was available on wormbase, an this strain was backcrossed with wild type worms twice to remove other mutations. When mtRNA from these worms was compared to mtRNA from wild type worms, as expected, a subset of the mtRNA was affected - the N6 RNA level was significantly decreased while the other RNAs that were checked were not. A slight increase in ND1 RNA level was also detected, thus mimicking the phenotype of FASTK loss in mammals (Fig. 10). We further confirmed that this phenotype was due to the lack of B0564.7 by using RNAi. As seen in (Fig. 10) the worms fed with bacteria containing siB0564.7 plasmids had much lower levels of ND6 RNA relative to worms fed with bacteria containing the control plasmid (4440). On the basis of these results, we conclude that B0564.7 is a homologue of FASTK present in *C. elegans* performing a similar function to FASTK proteins in mammals.

Strains were maintained at 20C under standard conditions (Brenner 1974). The wild-type strain for this experiment was N2 Bristol. The VC41026 strain, which contains a stop mutation in the B0564.7 gene, was obtained from the Caenorhabditis Genetic Center (CGC) and was backcrossed twice. RNA interference was performed using the clone from the Arhinger library (Kamath et al. 2003).

Interaction between FASTKD4, Suv3, and PNPase.

Mass-Spec analysis on FLAG tagged FASTKD4 indicated that the only protein that was substantially enriched relative to the control was Suv3 (unpublished data). A parallel pulldown of Suv3 was not enriched for FASTKD4, however. Suv3 has been previously implicated in the regulation of RNA levels by FASTK, so we wondered if it was also involved in regulation of RNA...
by other FASTK proteins. Therefore we silenced expression of Suv3 in an attempt to rescue a loss of FASTKD1 or FASTKD4. However differences between FASTKD1 or FASTKD4 KOs persisted even after silencing SUV3 or PNPase (Fig. 11). Knockdown of SUV3 or PNPase was unable to

C. elegans B0564.7

| Probability of import to mitochondria: | 92% |
| Conserved domain detected: | FAST_1, e-value: 0.36 |
| Specific mitochondrial RNA effected: | ND6 |
| Conserved Aspartate in endonuclease like domain: | Yes |
| Closest match in Drosophila is a FASTK protein: | Yes |

Figure 10: C. elegans protein B0564.7 is likely a FASTK homologue
A) B0564.7 is predicted to be imported to mitochondria, contain a FAST_1 domain, aligns with a clear FASTK homologue in D. Melanogaster, affects a mitochondrial mRNA, and preserves the aspartate residue at the putative active site of the RAP domain.
B) Worms lacking the protein either due to an early stop mutation (Mutant) or RNA silencing (siB0564.7) have dramatically reduced ND6 RNA relative to wild type worms (N2) or worms fed a control siRNA (si Empty Vector). The differences in the mutant worms persists even after backcrossing twice to remove other mutations in the strain.
reverse ND5-CYB precursor accumulation, rescue the mature forms of ND5 or CYB, or abolish the differences in ND3 levels between the KOs and WT cells. Therefore we conclude FASTKD4 has downstream effectors other than SUV3/PNPase.

Figure 11: SUV3 knockdown does not rescue the phenotypes of FASTKD1 or FASTKD4 knockout
A) Westernblot showing reduction in SUV3 expression after silencing.
B) Knockdown of SUV3 or PNPase, do not rescue relative differences in levels of mRNAs between FASTKD1-KO, FASTKD4-KO, FASTKD1andD4-KO, and WT cells. ND5 precursor accumulation and mature form loss in FASTKD4 lacking cells is not rescued by SUV3 knockdown.

**Human and Mouse FASTK are interchangeable for the rescue of ND6**

The amino acid sequences of Human FASTK and Mouse FASTK are a 95% match, while the nucleic acid sequences of their human and mouse ND6 mRNA targets are only 55% identical. With such divergent RNA target sequences, we wondered if human FASTK could replace mouse FASTK, and vice versa. If they could not, we would suspect that the 5% amino acid sequence difference would be related to a target RNAs, while if they did, conserved sequence or structure present in human and mouse ND6 may be candidates for the features that FASTK recognizes. As seen in Fig 12, expression of human FASTK in Mouse embryonic fibroblasts cells lead to a rescue...
of mouse ND6 comparable to the expression of mouse FASTK. This result indicates that human and mouse FASTK are functionally very similar. Human and mouse FASTKs may bind to conserved elements within the ND6 RNAs, or alternately their may be another protein that recruits FASTK to the RNA. We did not find any conserved secondary structure in computational predictions based upon the sequence. There are, however, numerous micro-homologies with perfect sequence matches of 20bp or less.

Figure 12: Human FASTK can rescue the lose of Mouse FASTK in MEF knockout cells

As previously reported, ND6 is not present in amounts detectable by northern blot in MEF FAST-KO cells, but is present in wild type MEF cells (MEF WT), and MEF cells expressing mouse FASTK (mFASTK). Interestingly, human FASTK (hFASTK) is also able to rescue ND6 in MEF FASTK-KO cells (2nd lane).

FASTK H486 mutations

As mentioned in the preceding papers, FASTK RAP domains appear to contain PD-(D/E)-XK nuclease domains with a particular similarity to VSR endonucleases. FASTK appears even more similar to VSR endonucleases, in that it has a Histidine residue apparently positioned at the active site as found in VSR endonucleases. As with the substitution of the Aspartate at the active site with Alanine, we substituted the Histidine apparently at the active site with an Alanine. While FASTKD4 with a substitution of the Aspartate was unable to rescue the loss of endogenous FASTKD4, FASTK with a substitution of the “active site” histidine in FASTK was able to rescue the loss of endogenous FASTK. It should be noted that while this histidine is found in all vertebrate homologues of FASTK, it is not found in FASTKD1-D5, nor in C. elegans or D. melanogaster.
Figure 13. FASTK H486 to A rescues ND6 loss
Human FASTK or FASTKH486>A was able to rescue the knockout of mouse FASTK and restore ND6 levels ND in MEF FASTK KO cells

**MRG clustering**

While performing an IF of granules in freshly thawed cells of another cell type (A549 cells), clusters of FASTKD2 foci were sporadically seen (Fig 14). Knowing that blocking mitochondrial fission via expression of the dominant negative of Drp1K38A causes formation of mitobulbs, we wondered if we could induce the formation of these MRG clusters in other cells, and we found this to be the case, MRG clustering and mitobulb formation were strongly correlated (Fig 14). Interestingly we also noted faint signal from hoescht staining in these mitobulbs, implying that mitochondrial DNA and presumably nucleoids also accumulate in the same conditions that cause clustering of MRGs. The clustering of nucleoids under these conditions was later confirmed by a report by Ban-Ishihara (Ban-Ishihara et al. 2013), and we presented data directly showed the simultaneous clustering of MRGs and nucleoids in.
Figure 14 Formation of clusters of MRGs

Poly-A pulldowns

We attempted to discern whether there was altered poly-adenylation of RNAs which displayed aberrant phenotypes when FASTKD1 or FASTKD4 were disrupted by pulling down poly-adenylated RNAs with dT beads. We detected very little ND3 signal after a dT pulldown of polyadenylated RNAs from FASTKD4-KO cells, however, due to the low abundance of ND3 RNA in FASTKD4-KO cells, we could not discern whether this indicated a reduction in polyadenylation. Similarly, we were unable to efficiently pulldown ND5, which is known to be merely oligo-adenylated. Additionally, normalizing to input is problematic because it was unclear at what point the dT beads become saturated. We attempted to normalize to TubulinB RNA in the pulldown conditions, but we were unable to get good results.
VIII. Discussion
**FASTK proteins are regulators of mitochondrial RNA**

My PhD work has focused on a class of mitochondrial RNA binding proteins. When the work began, only two FASTKs had been shown to affect mitochondrial RNA. After the work shown in this thesis, in tandem with other published work (Antonicka and Shoubridge 2015), (Wolf and Mootha 2014), we can confidently say that all 6 human FASTKs are required for normal expression of mitochondrial RNA. Furthermore, by investigating a distant homologue in *C. elegans*, we can reasonably say that this function is likely conserved in all metazoans.

**Relationship between FASTKs and RNA granules**

The first members of the FASTK family that were known to regulate mitochondrial RNA (FASTK and FASTKD2) are known to strongly colocalize with MRGs. So far knockdown of other MRG proteins has failed to prevent FASTK or FASTKD2 from localizing in foci. To date, the only method of preventing FASTK or FASTKD2 from concentrating in foci has been to block transcription of RNA in mitochondria, which completely eliminates the entire MRG. This suggests that the incorporation into MRGs is RNA dependent, and that the MRG formation requires an RNA substrate to form. Interestingly, when the RAP domain of FASTK or FASTKD2 is deleted, the truncated protein does not concentrate in granules. The RAP domain is thought to be an RNA binding domain and this combined with the previous data suggesting an RNA interaction is needed for MRG formation, would suggest that the RAP domain is needed for incorporation into MRGs because of the of the RAPs RNA binding properties. Interestingly, in this work we found that mutations of conserved residues (H to A 486) in the RAP domain did not prevent FASTK from localizing in MRGs, as long as the mutation did not alter the structure of the protein (as is the case when histidine 486 is replaced by a proline). More interestingly, we found that the RAP domain of FASTKD4 is an acceptable substitute for the RAP domain of FASTK, for the purposes of incorporation of the protein into MRGs.

This is particularly interesting in light of the observations in this work and by others that other FASTK proteins do not strongly colocalize in these MRGs, and most do not appear enriched in these MRGs at all (Simarro et al. 2010), (Antonicka and Shoubridge 2015). In this work we never observed foci when tagged FASTKD4 was expressed in cells. We considered that high expression levels may result in a general mitochondrial staining that obscures the localization in foci, however for FASTK or FASTKD2 bright foci were still easily discernible even at very high levels of expression that resulted in the entire mitochondrial matrix. On the basis of the overexpression of FASTK and FASTKD2 failing to obscure the localization in MRGs, we doubted that FASTKD4 was in foci, but to be safe we also looked at the endogenous FASTKD4 with a very good anti
FASTKD4 antibody, and failed to see any evidence of foci. Simarro et al. and Jourdain et al. both looked at FASTKD1 to FASTKD5 by overexpression of YFP tagged constructs, and no evidence of foci was seen in anything other than FASTKD2 (Simarro et al. 2010), (Jourdain et al. 2015). While Antonicka et al report that FASTKD5 is enriched in granules, examination of the images provided in their paper does not support this conclusion, and their own quantification shows that FASTKD5 is substantially less enriched in MRGs than FASTKD2 and GRSF-1 (Antonicka and Shoubridge 2015). The apparent presence of FASTKD5 foci at all is perplexing considering the lack of such structures visible in previous studies. More perplexing is that these foci poorly colocalize with MRGs, raising the question of if these foci represent real FASTKD5 foci, or simply antibody aggregates. Regardless, on the basis of this work and previous studies, it is clear that incorporation into MRGs is not a general characteristic of the FASTK proteins, and is instead a specific property of FASTK, FASTKD2, and controversially FASTKD5. At the moment, it is not clear why FASTK does not go to MRGs when its RAP domain is deleted, but it does go to MRGs when its RAP domain is replaced with a different RAP domain from a protein that does not localize in MRGs. Clearly the RAP domain is necessary but not sufficient for incorporation of FASTKs into MRGs.

Recently MRGs have been implicated in ribosome biogenesis, leading to one commenter proposing that the structure is analogous to the nucleolus and that it should be renamed to the mitochondriolous. While previous work in our lab that primarily relied on shRNA silencing did not identify an effect of FASTK or FASTKD2 on ribosomal RNA, Popow et al. showed that FASTKD2 interacts with 16S rRNA and a complete knockout of FASTKD2 decreases levels of 16S rRNA. If the ribosome biogenesis role of MRGs is real, it may be that FASTK and FASTKD2 colocalize with MRGs because they bind and regulate ribosomal RNA. However, it is not clear that this emphasis on a link between mitoribosome assembly and MRGs is warranted. While there is increasing evidence that ribosomes are assembled inside or in close proximity to MRGs, it should be kept in mind that these structures were initially believed to be primarily RNA processing structures. If these structures are more general RNA maturation centers, then they would also be the site where mature 16S and 12S rRNA are released, and as a consequence mitoribosome assembly may occur there as well. The presence of RNAse P suggests a general role in RNA processing, and the partial colocalization of Suv3 and PNPase with these structures supports this. Thus the strength of a link with mito-ribosome formation is questionable. Furthermore, while Popow et al. did show that FASTKD2 status affects mitochondrial rRNA, there is no known role for FASTK and ribosome biogenesis. Instead FASTK appears to be required for stabilization of ND6 mRNA, possibly by defining the atypical 3’ end. Thus it remains unclear why FASTK and FASTKD2 localize with MRGs, but other FASTKs do not.
**Relationship between RNA granules and Nucleoids**

The relationship between RNA granules and nucleoids is unclear at the moment. When RNA granules were first discovered, they were found to be in close proximity to nucleoids when first formed, and to move farther away with time (Iborra, Kimura, and Cook 2004). As nucleoids would be the site of transcription and generation of the precursor RNA, an association with the RNA granules is not surprising. However the tendency to move away with time would seem to suggest that RNA leaves the nucleoids in discrete packets, and would suggest that RNA granules would be unstable structures that disappear before migrating far from the nucleoids. Bogenhagen et. al. attempted to purify these RNA granules, but did not succeed in separating them from nucleoids (Bogenhagen 2012). We noted that mitochondrial nucleoids could be concentrated by inhibiting mitochondrial fission as reported in Ban-Ishihara et. al. (Ban-Ishihara et al. 2013), and found that this affects MRG distribution in the same way (Fig. 15).

![Figure 15 clusters of MRGs colocalize with nucleoids](image)

Figure 15 clusters of MRGs colocalize with nucleoids

FASTKD2 staining is shown in purple, mtDNA is stained in green. The mitochondrial network is shown in blue. The dominant negative protein DRP1 K38->A was again used to induce clustering of MRGs and nucleoids. Not that both nucleoids and MRGS concentrate within mitobulbs. Staining and image acquisition performed by Sofia Zagenelli.

**Organization of FASTK proteins**

In mammals, the FASTK proteins all contain a FAST_1, FAST_2, and RAP domain. In other species such as Chlamydomonas or D. melanogaster, there are examples of proteins which contain only RAP domains, or only FAST domains. In the case of Chlamydomonas, these proteins are sometimes thought to act together, and thus it seems likely that cooperation between the FAST domains and the RAP domains is needed to accomplish the function of these proteins.

As mentioned in the manuscripts in preparation presented in the results sections, the RAP domain
bears a strong structural resemblance to known nucleases, while the FAST domains contain alpha helices reminiscent of PPR proteins and OctotricoPeptide Repeats (Rahire et al. 2012). While the first described cases of OPR proteins acting in Chlamydomonas and Arabidopsis (Eberhard et al. 2011), (Kleinknecht et al. 2014), (Rahire et al. 2012) involved a RAP domain and RNA splicing or 5' RNA editing, an OPR protein was recently described which stabilizes RNAs and thus far no association with a RAP or nuclease like domain has been found (Wang et al. 2015). Interestingly proteins exist in chloroplasts with both OPR domains and FAST_1 domains (Eberhard et al. 2011) (Perron, Goldschmidt-Clermont, and Rochaix 2004). It was recently shown that the OPR domains are involved in recognition of specific RNAs (Boulouis et al. 2015), and it seems likely that FASTKs are involved in this role as well.

We noted that FASTK proteins affect specific RNAs, in different ways, and thus these proteins with similar structure must contain elements able to recognize specific RNAs, and elements able to have different affects on such RNAs. Due to the modular nature of these proteins, we suspected that the different domains were specialized to either specify the target RNAs, or to specify the effect on target RNAs. Structural analysis suggests that the RAP domain is a good candidate for the “effector” part of these proteins, and likely acts as a nuclease in many cases. The PPR like FASTK domains which are N-terminal to the RAP domain would likely specify the target RNA sequence as PPR proteins do. Indeed, this was the same basic conclusion reached by Boulouis et al. in 2015 for OPR/RAP containing proteins NCC1 and NCC2. It was shown that mutations in the OPR regions would alter the RNA target of both NCC1 and NCC2, causing the degradation of the atpA and petA mRNAs, respectively (Boulouis et al. 2015). They additionally modelled the RAP domains of NCC1 and NCC2, and found that they closely matched the structure of known endonucleases as well, in particular the homing endonuclease 3r3p, and the Vsr endonuclease 1cw0.

While the results are not conclusive, the results from the chimeras generated in this thesis are compatible with this idea. Chimeras were constructed by replacing the RAP domain of a FASTK protein with the RAP domain of another FASTK protein. The RAP domain displayed its expected effect on the target RNAs only when there was an overlap in the RNA targets of the FASTK proteins which the RAP domain and N-terminal region were derived from. Notably, the effect corresponded to the effect expected for the protein which the RAP domain was derived from. FASTKD4 with the RAP domain of FASTKD1 did not stabilize the ND3 mRNA, but rather reduced the level of ND3 as FASTKD1 would. Indeed, this shows that the effect of the FASTKD4 protein is determined by its RAP domain, and the RAP domain of FASTKD1 carries the effect of the FASTKD1 protein on RNA. The lack of any observed effect on mitochondrial RNA when the N-terminal regions comes from FASTKs with different RNA targets suggests that the region N-
terminal to the RAP is required for proper targeting of the RAP. One case investigated during this thesis involved the chimera of FASTK (RNA target: ND6) and FASTKD4 (RNA targets: ATP8/6, CO1,2,3, CYB, ND3&5), expression of which did not appear to have any effect on RNA levels. The FASTK+D4RAP protein did however appear to be expressed and displayed the same MRG localization as FASTK, further supporting a role of the N terminus in determining the targeting of the protein.

It is worthwhile to note that we did not observe “off target effects” for these chimeras derived from FASTKs with no overlapping RNA targets. This may be due to RAP domains performing specific functions that are not relevant to non-target RNAs (possibilities include: cleavage of tRNAless junctions, removal of specific tags, shortening of poly-A tails on RNA that lack long poly-A tails), or it may represent a problem with this particular model of the functional organization of FASTK.

The conserved Aspartate in the putative active site

Based upon structural modeling, the D531 Aspartate of FASTKD4 is in a homologous location to the Aspartate at the active site of VSR endonucleases. We can conclude that this aspartate is essential for proper function of the FASTK proteins on the basis of not only the mutagenesis experiments, but also the high degree of conservation. We found that this Aspartate is conserved in not just every human FASTK, but also mammalian FASTKs, D. melangoster FASTKs, and even the C. elegans FASTK homologue identified in this thesis. This high degree of conservation implies a conserved function or closely related functions to maintain selection pressure for the Aspartate at the putative active site. While an apparently homologous Aspartate is essential for the function of VSR endonucleases (Tsutakawa et al. 1999), it would be premature to assume that the conserved function is that of an endonuclease. As previously mentioned, the PD-(D/E)-XK nuclease family contains exonucleases in addition to endonucleases. The very high conservation of a residue at the active site suggests some nuclease activity is retained, but it is premature to favor any specific type of nuclease activity. This aspartate may be part of a more basic conserved function, which is required for a nuclease activity; if that is the case the more basic function may be conserved without conservation of the nuclease activity.

RAP domain function

In humans ND6 is the only mRNA encoded on the light strand, with the rest of the light strand being non-coding. It was proposed by Jourdain et al. that ND6 is lost in the absence of FASTK because it is degraded along with the rest of the non-coding light strand (Jourdain et al. 2015). A mechanism was thus proposed in which FASTK regulates ND6 mRNA level by binding to and
protecting the end of the ND6 RNA from degradation, which would not require a conserved nuclease activity. Jourdain et al. showed that the reduction in ND6 levels in FASTK KO cells was accompanied by a slight increase in ND1 levels. Interestingly we observed a very similar phenotype in a C elegans strain with a nonsense mutation in a FASTK homologue (Fig 16).

In the case of C. elegans, ND6 is on the same strand as all the other coding sequences, and is not flanked by non coding sequences. Therefore another mechanism is needed to explain a nearly identical phenotype in C. elegans.

The accumulation of unprocessed precursor RNA when FASTKD4 or FASTKD5 is absent also requires another mechanism, which could be explained by a nuclease activity. Furthermore, the nuclear form of FASTK is involved in alternative splicing, and this would require a mechanism very different from protecting RNA from degradation by 3' to 5' exonucleases. Additionally Chloroplast OPR/RAP proteins, which appear to be structurally similar, are also involved in splicing reactions where a nuclease activity would better explain their effects.

As seen in figure 17 of work performed by Alexis Jourdain, there is a very strong difference in ND6 signal in WT vs shFASTK cells even when SUV3 or PNPase are silenced which indicates that there is a mechanism of ND6 loss that is independent of SUV3/PNPase.

While a band that may be close to the right size appears in shFASTK cells when PNPase/SUV3
are silenced, the apparent rescue of ND6 may be a result of an inefficient knockdown when another shRNA is strongly expressed, as it is possible for DICER to become saturated (M. G. Andersson et al. 2005), and RNA silencing can become impaired as a result (Lu and Cullen 2004).

Therefore in light of the effects of other FASTKs, the regulation of splicing by nuclear FASTK, the involvement of OPR/RAP proteins in splicing of chloroplast mRNAs, the high conservation of the Aspartate and loss of function when the aspartate is mutated, the structural similarity to known endonucleases, and the varied effects of FASTK proteins and their RAP domains, it seems unlikely that the “end protection” model is correct, and we instead favor an “end processing” model for the mechanism of action of FASTK proteins.

Figure 17: Mechanisms of ND6 Loss
Left: A northern blot showing that although there is a band in FASTK KO cells at roughly the size of ND6 when Suv3 is silenced, the signal is dramatically weaker than the signal from wildtype cells.
Right: Comparison of the position of ND6 in the mt genomes in humans vs C. elegans. Note that ND6 is not the only coding sequence on its strand in the C. elegans genome, but rather is on the same strand as all other mitochondrial encoded genes in C. elegans.

Target specificity
While it has been noted that FAST domains and OPR domains resemble PPR proteins which are
known to recognize specific DNA sequences, it seems problematic to assign a sequence specificity to FASTK proteins. FASTKD3, FASTKD4, and FASKTD5 all affect multiple RNAs, and so far no common sequence motif has been identified among the target RNAs. In the case of FASTK, it would seem to be sequence specific, as only a single target RNA is affected. However, we found that human FASTK can rescue the loss of mouse ND6 when mouse FASTK is knocked out. Mouse and human ND6 mRNA sequences are highly divergent with only 55% sequence identity. In order to explain this, we must suggest that the recognition site for FASTK lies in one of the small regions of homology between human and mouse ND6, that FASTK recognizes specific RNA secondary structures rather than primary sequence, or that there is another protein mediating this specificity. The last possibility seems plausible as hFASTK and mFASTK have 95% sequence identity (despite their apparent target RNAs having low sequence homology) and FASTK seems to have strong interactions with other proteins such as FASTKD2 and GRSF-1 which are also found in RNA granules. It would be interesting to see if human FASTK and human FASTKD2 could rescue mouse ND6 in a MEF cells where mFASTK and mFASTKD2 were both missing; perhaps hFASTK only rescues the loss of mFASTK through an interaction with mFASTKD2.

In contrast, there were no proteins that strongly co-immunoprecipitated with FASTKD4. At the moment it is difficult to postulate a mechanism by which many FASTK proteins can specifically and simultaneously affect multiple mtRNAs with widely varying sequences.

**Relationship to Chloroplast proteins**

As mentioned in the introduction, there are proteins similar to FASTK proteins in Chloroplasts, although the RAP and FAST domains are not always on the same protein, they seem to act in the same pathway (Perron, Goldschmidt-Clermont, and Rochaix 2004). Interestingly, these proteins are involved in intron splicing which does not occur in mammalian mitochondria. Intron splicing requires a nuclease activity, and thus the mechanism of action of the FAST or OPR & RAP domain containing complexes may be conserved between chloroplasts and mitochondria. While an interaction between putative pseudouridine synthases and FASTKD4 was not detected by Mass spectrometry, an interaction between multiple putative pseudouridine synthases and FASTKD2 was detected in an IP done by Alexis Jourdain (Jourdain et al, paper in preparation). This is particularly interesting, as an interaction between the FAST_1 domain containing protein RAA1 and the pseudouridine synthase domain containing protein RAA2 was detected in chloroplasts, which again suggests a similar mechanism is at work (Perron, Goldschmidt-Clermont, and Rochaix 2004). While pseudouridinylation is mostly associated with rRNA and tRNA modifications, in this case it is apparently required for intron splicing, a process requiring a nucleolytic cleavage of RNA.
followed by a ligation. This raises the possibility that pseudouridinylation can serve as a marker of a cleavage site by a nuclease, although this is highly speculative. Intron splicing in chloroplasts could also be related to a variety of speculative capping mechanisms that occur after cleavage of mitochondrial precursor RNA. The close resemblance between chloroplasts and mitochondrial RNA processing suggests a common origin for regulation of organellar RNA. In contrast FASTK proteins (containing FAST_1, FAST_2, and a RAP domain simultaneously) have thus far not been identified in mitochondria outside the metazoan clade, which is hard to reconcile with a common origin before the divergence of chloroplast containing Eukaryotes. As mentioned previously, FASTK proteins are known to be present in the nucleus as well, and thus FASTK/OPR proteins in mitochondria/chloroplasts may be an example of parallel processes repurposing a common cytosolic RNA altering complex for the control of organellar gene expression. Further characterization of the role of FASTK proteins outside of mitochondria may be enlightening.
IX. Conclusion
This PhD project started as a continuation of work on FASTK and FASTKD2 performed by Alexis Jourdain, who showed that these two FASTK proteins affect the steady state levels of mitochondria mRNA. Our lab and others have now characterized the effect of the loss of the other human FASTK proteins, and an effect on mitochondrial mRNA is apparently a general characteristic of these proteins. We have determined that FASTK proteins are able to both positively and negatively regulate mitochondrial RNA, and therefore must be able to either stabilize or enhance degradation of target RNAs. On the basis of swaps of functional domains of these proteins, we show that the C-terminal RAP domain is the key determinant of the function of the protein. Structural modeling suggests that the RAP domain may be a nuclease, and the results of mutagenesis experiments are compatible with this idea, although such an activity has not been demonstrated.

We hope our work will prove useful to others studying mitochondrial gene expression and inspire further experiments about the FASTK family.
X. Appendix
The following review on RNA granules was written primarily by Alexis Jourdain, but with my input and analysis.

Abstract

In mitochondria, DNA replication, gene expression, and RNA degradation machineries coexist within a common nondelimited space, raising the question of how functional compartmentalization of gene expression is achieved. Here, we discuss the recently characterized “mitochondrial RNA granules,” mitochondrial subdomains with an emerging role in the regulation of gene expression.

Introduction

RNA granules represent a diverse range of ribonucleoparticles that lack membrane delineation and are often visible by light microscopy. They have been observed in the nucleus and cytosol of somatic cells, neurons, and germ cells and are implicated in a variety of different functions. Nuclear RNA granule structures include the Cajal bodies, speckles, and nucleoli, sites of snRNP biogenesis and maturation, RNA splicing, and ribosomal RNA (rRNA) transcription, respectively. Cytosolic stress granules (SGs) and processing bodies (PBs) contain polyadenylated mRNAs and RNA-related enzymes and are induced under conditions in which translation is inhibited, though PBs also exist under normal conditions. SGs are rich in preinitiation complexes and contain mRNAs that can reenter polyribosomes, whereas PBs contain decapping and deadenylation factors, the RISC complex, and the nonsense-mediated decay machinery and are thought to be mostly involved in mRNA degradation (Kedersha et al., 2005). RNA granules have also been observed within organelles. In chloroplasts, SGs are induced by a variety of stimuli and like cytosolic SGs are rich in preinitiation complexes (Uniacke and Zerges, 2008). RNA granules also exist in mitochondria but appear to play a different role, as they are not induced by stress, and processes such as mRNA splicing and decapping that occur in other types of RNA granules are not relevant to gene expression in mammalian mitochondria.

The human mitochondrial genome is a highly compact circular molecule of >16.5 kb that encodes 37 genes, all of which are required for oxidative phosphorylation, including 13 highly hydrophobic membrane components of the electron transport chain, two rRNAs, and 22 tRNAs that provide the specialized translational machinery required to express these proteins. Both strands of the mitochondrial DNA (mtDNA) are almost completely transcribed from a single promoter region, giving rise to polycistronic precursors that are then processed into mono- or bi-cistronic mRNAs, in most cases by excision of flanking tRNAs interspersed throughout the genome. As with nuclear RNA, mitochondrial RNA is further modified during or after transcription by polyadenylation, methylation, aminoacylation, or pseudouridinylation. However, in contrast to nuclear gene expression, which occurs in a well-defined step-wise manner consisting of capping, splicing,
polyadenylation, and nuclear export, very little is known about the spatiotemporal organization of gene expression in mitochondria. Currently it is unclear how immature polycistronic RNAs are prevented from interacting with the translation machinery, where and how posttranscriptional modification or mitoribosome assembly occur, or what governs RNA stability. In the absence of a clear membrane delineation within the mitochondrial matrix, mitochondria appear to have developed specialized RNA-rich domains, in which these complex processes are compartmentalized and which likely represent key sites in the organization of mitochondrial gene expression. The composition and function of these mitochondrial RNA granules (MRGs) will be the focus of this paper.

MRGs

Iborra et al. (2004) first described newly made RNAs as punctate mitochondrial structures, which could be labeled with the uridine analogue 5-bromouridine (BrU). We and others later reported a specific colocalization of MRGs with GRSF1, a protein involved in mitochondrial RNA processing and translation (Antonicka et al., 2013; Jourdain et al., 2013). GRSF1 was found to interact with the mitochondrial RNase P complex, and accordingly GRSF1 depletion reduced mature RNAs, with an accumulation of RNA precursors (Jourdain et al., 2013). Not surprisingly, RNase P subunits were also enriched in MRGs, further implicating MRGs as centers of RNA processing. BrU pulse-chase experiments supported this notion, as removal of either GRSF1 or RNase P prevented the transit of newly made RNA through MRGs (Jourdain et al., 2013). Other RNA processing and maturation factors have now been shown to be present within MRGs, such as members of the FASTK family, the mitochondrial poly(A)-polymerase mtPAP, methyltransferases, RNA helicases, and the hSuv3p–PNPase complex or degradosome, which plays a central role in mitochondrial RNA degradation (Wang and Bogenhagen, 2006; Vilardo et al., 2012; Lee et al., 2013; Borowski et al., 2013; Jourdain et al., 2013, 2015; Bogenhagen et al., 2014; Wilson et al., 2014; Antonicka and Shoubridge, 2015; Tu and Barrientos, 2015; Table 1). Although some of these components are found in almost all MRGs, others are only found in a subpopulation of granules. Thus, MRGs are probably dynamic structures, which provide a platform for the spatiotemporal regulation of the numerous processes required for mitochondrial gene expression, including RNA processing, maturation, ribosome assembly, and translation initiation (Fig. 1). Their protein and RNA composition may change continuously depending on the progress of RNA processing and mitoribosome assembly.
Table 1. Composition of the MRGs

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Function</th>
<th>Conserved domains (PFAM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX28</td>
<td>Mitochondrial assembly</td>
<td>DEAD; Helicase_C</td>
<td>Antonicka and Shoubridge, 2015; Tu and Barrientos, 2015</td>
</tr>
<tr>
<td>DHX30</td>
<td>Mitochondrial assembly</td>
<td>DEAD; HA2; Helicase_C; OB-</td>
<td>Wang and Bognahan, 2006; Antonicka and Shoubridge, 2015</td>
</tr>
<tr>
<td>FASTK</td>
<td>RNA processing</td>
<td>FAST_1; FAST_2; RAP</td>
<td>Jourdain et al., 2015</td>
</tr>
<tr>
<td>FASTKD2</td>
<td>RNA processing; mitochondrial assembly</td>
<td>FAST_1; FAST_2; RAP</td>
<td>Jourdain et al., 2015; Jourdain et al., 2015</td>
</tr>
<tr>
<td>FASTKD6</td>
<td>RNA processing; mitochondrial assembly</td>
<td>FtsJ</td>
<td>Antonicka and Shoubridge, 2015</td>
</tr>
<tr>
<td>FTS12&lt;sup&gt;2&lt;/sup&gt; (MRM2)</td>
<td>rRNA methylation</td>
<td>FtsJ</td>
<td>Lee et al., 2013</td>
</tr>
<tr>
<td>GRSF1</td>
<td>rRNA methylation</td>
<td>RRM6 (3x)</td>
<td>Antonicka et al., 2013; Jourdain et al., 2013</td>
</tr>
<tr>
<td>HSD17B10 (MRP2)</td>
<td>rRNA processing; rRNA processing</td>
<td>adh_short</td>
<td>Jourdain et al., 2013; Bogenhagen et al., 2014</td>
</tr>
<tr>
<td>KIAA0351 (MRP3)</td>
<td>rRNA processing; rRNA processing</td>
<td>PRORP</td>
<td>Jourdain et al., 2013; Bogenhagen et al., 2014</td>
</tr>
<tr>
<td>MRMT&lt;sup&gt;10&lt;/sup&gt;</td>
<td>rRNA methylation</td>
<td>SpoU_methylase; SpoU_sub_bind</td>
<td>Lee et al., 2013</td>
</tr>
<tr>
<td>MTRAP (PAP1)</td>
<td>Poly(A) polymerase; RNA degradation</td>
<td>PAP_assoc</td>
<td>Wilson et al., 2014</td>
</tr>
<tr>
<td>PNPT1 (PNPase)</td>
<td>RNA degradation; RNA processing</td>
<td>KH-1; PNPass; RNASee_PH; RRSnale_PH_C; S1</td>
<td>Borowski et al., 2013</td>
</tr>
<tr>
<td>RNMTL1&lt;sup&gt;10&lt;/sup&gt; (MRM3)</td>
<td>rRNA methylation</td>
<td>SpoU_methylase; SpoU_sub_bind</td>
<td>Lee et al., 2013</td>
</tr>
<tr>
<td>S U V 3 L 1 (hSv3p)</td>
<td>RNA degradation; RNA processing</td>
<td>HeliCase_C; S UV 3_C</td>
<td>Borowski et al., 2013</td>
</tr>
<tr>
<td>TRIMT10C (MRP1)</td>
<td>rRNA processing; rRNA processing</td>
<td>tRNA_m1G_MT</td>
<td>Jourdain et al., 2013; Bogenhagen et al., 2014</td>
</tr>
</tbody>
</table>

Figure 1. MRGs and nucleoids. (A) Confocal image of a HeLa cell expressing a blue fluorescent protein in mitochondria (mitoBFP), incubated with 2.5 mM BrU for 1 h and labeled with antibodies to mitochondrial SSB (nucleoid marker, green) or BrU (MRG marker, red; Jourdain et al., 2013). (B) Model of the composition and function of MRGs and their interaction with the nucleoids.

All work on MRGs to date has been performed using human or mouse cells, and thus it is legitimate to ask whether they also exist in other species. From the original genome of the ancestral endosymbiotic bacteria, the structural organization and content of the mitochondrial genome has undergone major changes during the course of evolution. In certain species such as green plants or Saccharomyces cerevisiae, large mitochondrial genomes encode multiple promoters and introns, whereas in other experimentally tractable organisms such as Schizosaccharomyces pombe, Caenorhabditis elegans, or Drosophila melanogaster, mtDNA is shorter and, as in humans, appears to be transcribed into long polycistronic precursors that are then processed according to the so-
called “tRNA punctuation” model. In D. melanogaster, proteins related to known MRG components have been found, including members of the FASTK family, hnRNPs, and mitochondrially-targeted helicases. However, it is currently unknown whether MRGs exist in nonmammalian species, although their existence seems likely in those species with similar mtDNA architecture, mode of DNA replication, and pattern of transcription.

Dynamics of MRGs

Iborra et al. (2004) initially classified the BrU foci into two classes: those detectable after a short pulse of BrU, which colocalize with the mitochondrial nucleoids, and those detectable after a longer pulse of BrU, which are stable over time and do not colocalize with the mitochondrial genome. About 10–20% of MRGs belong to the former class and are intimately associated with nucleoids (Fig. 1 A; Iborra et al., 2004; Jourdain et al., 2013). Moreover, because this subpopulation of MRGs also contains the most rapidly labeling RNA (Iborra et al., 2004), they are most likely associated with transcriptionally active nucleoids, allowing efficient transfer of the primary transcripts. We envisage that within each MRG two distinct pathways occur in parallel (Fig. 1 B). The first involves processing and maturation of the genome-length polycistronic heavy and light strand transcripts to generate the mature mRNAs that encode the subunits of the electron transport chain, requiring tRNA excision, elimination of the noncoding “mirror” RNAs, and selective polyadenylation and posttranscriptional modifications. The second involves excision and maturation of the 12S and 16S rRNAs from the heavy strand transcript and association with nuclear-encoded MPRS and MPRL proteins, resulting in assembly of the 28S and 39S mitoribosomal subunits, respectively. In their initial work on BrU foci, Iborra et al. (2004) reported that BrU-positive foci (i.e., MRGs) are distributed in the vicinity of active cytosolic ribosomes on the mitochondrial surface and the protein import machinery, which would allow for coordinated assembly of mitoribosomes using both nuclear and mitochondrial gene products. Based on this and recent structural analyses of the mitoribosome (Greber et al., 2014; Amunts et al., 2015), the 39S subunit likely provides a membrane anchorage function, which allows release of fully assembled, active mitoribosomes from the MRGs and presentation of the nascent hydrophobic peptides to the associated membrane insertion machinery of the inner mitochondrial membrane (Fig. 1 B). According to this scheme, the compact MRG structure would provide the proximity and high concentration of reactants, together with the organizational framework and access to nuclear encoded gene products, to ensure efficient RNA processing, maturation, and mitoribosome assembly crucial for mitochondrial gene expression.
As MRGs exist independently from the nucleoids, this raises the interesting question of their dynamics and inheritance during cycles of mitochondrial fusion and fission. Nucleoids are found in close proximity to sites of fission, presumably facilitating the segregation of mtDNA throughout the mitochondrial network. During the normal cycle of mitochondrial fusion/fission, and especially during acute perturbations of mitochondrial dynamics, nucleoids may become unevenly distributed, resulting in mitochondria that lack mtDNA. Such mitochondria either re-fuse with mtDNA-containing mitochondria and reacquire the genome, or lose membrane potential and are degraded by mitophagy (Twig et al., 2008). Though MRG dynamics have not yet been investigated by live-cell imaging, mitochondria lacking nucleoids could use nonnucleoid-associated MRGs as a stock of RNA able to sustain translation temporarily and thus extend the survival time of isolated mitochondria until fusion can occur. A similar RNA storage function could be important during mtDNA replication or when transcriptional activity is otherwise decreased. Thus, MRGs could play a buffering role in mitochondrial gene expression, maintaining RNA availability during transient decreases in mitochondrial transcription and/or nucleoid loss.

**Structure and formation of MRGs**

How is the structural integrity of MRGs maintained? In the more highly studied cytoplasmic granules, proteins containing prion-like domains, Sm folds, or other motifs have been found to be essential in maintaining structural identity. In each case, deletion of the aggregation domain leads to disassembly of the granule. By analogy with the cytoplasmic granules, one or more of the protein components of MRGs might contain a similar aggregation domain. Because the only means currently known to disassemble MRGs is removal of RNA (Iborra et al., 2004; Jourdain et al., 2013), the putative scaffolding protein might contain an RNA-binding motif, in addition to other protein-interacting domains and functions. Intriguing data from Kato et al. (2012) have shown that addition of a biotinylated isoxazole compound to complex cellular extracts selectively precipitates a subset of aggregation-prone, RNA-binding proteins highly enriched in low-complexity (LC) regions, which the authors argue could provide the basis for differential assembly of various RNA granules. Searches for LC domains within known MRG proteins have so far failed to reveal clear candidates; thus, if the mode of MRG assembly resembles that of cytosolic RNA granules, the LC domain–containing proteins required to maintain the MRG architecture have yet to be identified.

Another possible mechanism of MRG assembly is suggested from a recent study of nucleoids in which assembly was shown to occur via a multistep mechanism relying on the cross-strand binding of the mitochondrial transcription factor A (TFAM) to mtDNA (Kukat et al., 2015). TFAM binding
induces loop formation in mtDNA and increasing concentrations of TFAM drive binding to multiple and sometimes distant sites, ultimately leading to compaction of the mtDNA into nucleoid-like structures. A similar mechanism could also take place in MRGs, with the putative scaffolding protein binding newly made RNA, leading to formation of foci. Site-specific binding to unprocessed RNA junctions or noncoding RNA could then ensure the controlled release of mature mRNAs to the mitoribosomes and thence to the mitochondrial matrix.

Identification of such factors that coordinate and maintain MRG architecture would be an important step forward in investigating the function of these RNA granules.

**Concluding remarks**

MRGs are emerging as discrete subcompartments within the mitochondrion, which partition the mitochondrial matrix and enable the posttranscriptional events necessary for mitochondrial gene expression. We propose that the MRGs are assembled around the newly synthesized primary transcripts in close proximity to active nucleoids with which they are transiently associated and that subsequent events in mitochondrial gene expression take place as the MRGs become detached and take up independent locations within the inner mitochondrial matrix. We view MRGs as dynamic structures whose composition changes as the primary transcript is posttranscriptionally modified, processed to mature RNAs, and assembled into mitoribosome complexes for oriented translation and insertion of the mitochondrially encoded proteins into the inner mitochondrial membrane. The changing composition of MRGs, their architecture, and their patterns of segregation during the cycles of mitochondrial fission and fusion are intriguing topics for the future that will give us greater insight into MRG function.

**Acknowledgements**
The authors thank Dr. Tom Bender for help with artwork and Sofia Zaganelli for help with the fluorescent imagery.

This work was supported by the Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung (310030B_160257/1), iGE3, and the State of Geneva.

The authors declare no competing financial interests.

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