

# **FASTKD5 processes mitochondrial pre-mRNAs at non-canonical cleavage sites**

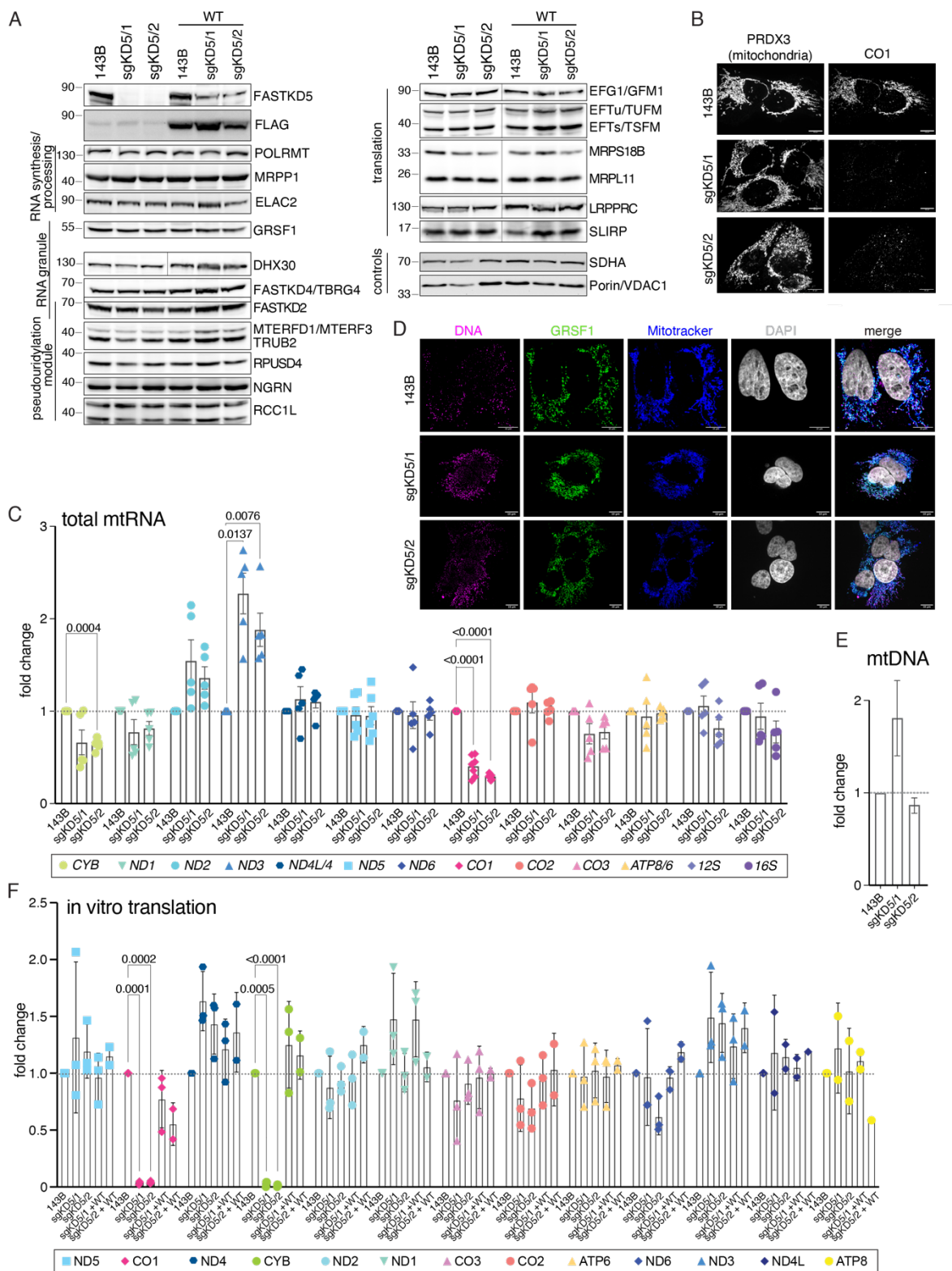
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**Supplementary Materials:**

**Supplementary Figures S1-S7**

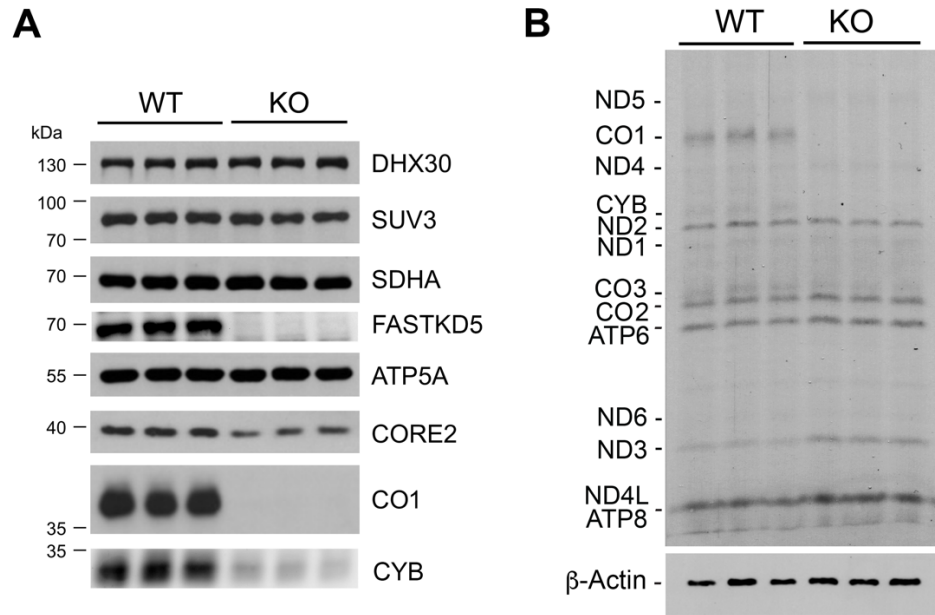
**Supplementary Table S1**



**Supplementary Figure S1 (related to Figure 1)**

**Characterization of FASTKD5 KO clones and the rescue in the 143B background.**

(A) Steady-state levels of FASTKD5 protein, FLAG and proteins involved in mitochondrial RNA metabolism or mitochondrial translation were assessed by immunoblotting in 143B cells, two KO clones (sgKD5/1 and sgKD5/2) and same cells rescued with a wild-type (WT) FASTKD5-3xFLAG. SDHA and VDAC1 were used as loading controls. Molecular weight markers (in kDa) are indicated on the left. (B) Immunofluorescence analysis of 143B cells and FASTKD5 KO clones (sgKD5/1 and sgKD5/2) indicating the levels of CO1 protein. PRDX3 was used as a mitochondrial control. (C) RT-qPCR quantification of total mtRNA levels (processed + unprocessed) for all mitochondrial transcripts, as well as 12S (*RNR1*) and 16S (*RNR2*) rRNAs in 143B cells and two KO clones (sgKD5/1 and sgKD5/2). Two-way ANOVA with a Dunnett correction for multiple comparisons was performed (compared to 143B cells, which were set to 1), and significant p-values are indicated. (D) The quantity of mitochondrial nucleoids and RNA granules was determined by immunofluorescence analysis using anti-DNA antibody (nucleoids) and anti-GRSF1 antibody (RNA granules) in 143B cells and two KO clones (sgKD5/1 and sgKD5/2). (E) mtDNA levels were measured by qPCR in 143B cells and two KO clones (sgKD5/1 and sgKD5/2) in duplicate. (F) Quantification of mitochondrial translation assay performed in biological triplicates (one example is shown in **Figure 1D**) normalized to Coomassie total protein staining. Two-way ANOVA with a Dunnett correction for multiple comparisons was performed (compared to 143B cells, which were set to 1), and significant p-values are indicated.



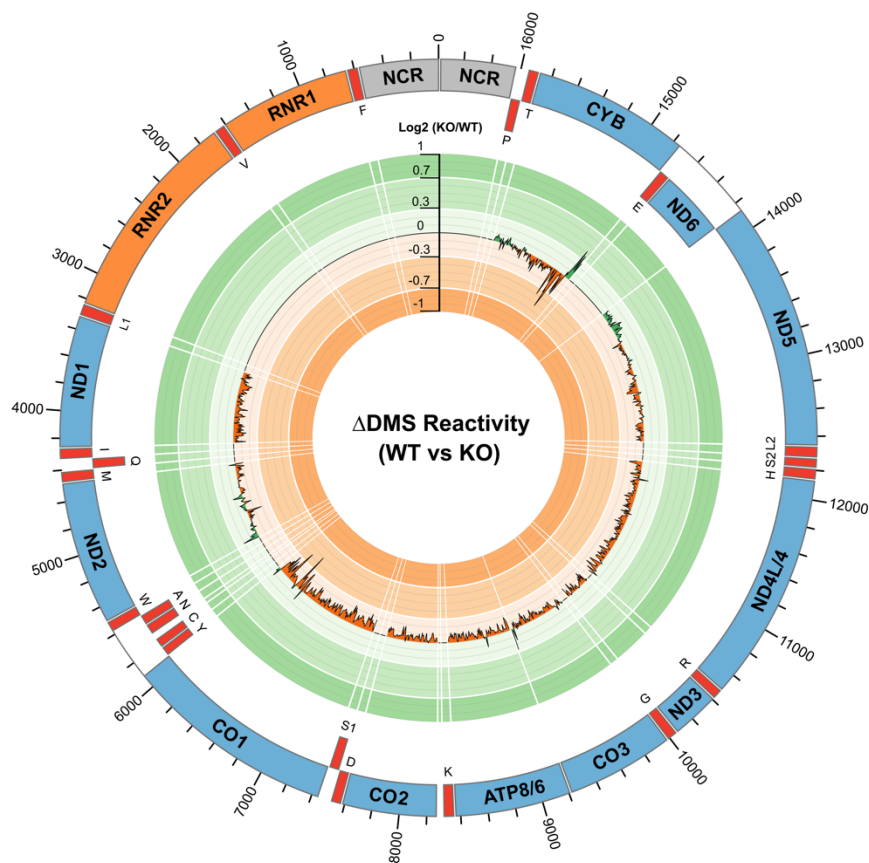
### Supplementary Figure S2 (related to Figure 2)

#### Characterization of a *FASTKD5*-KO cell line in the HEK293T background.

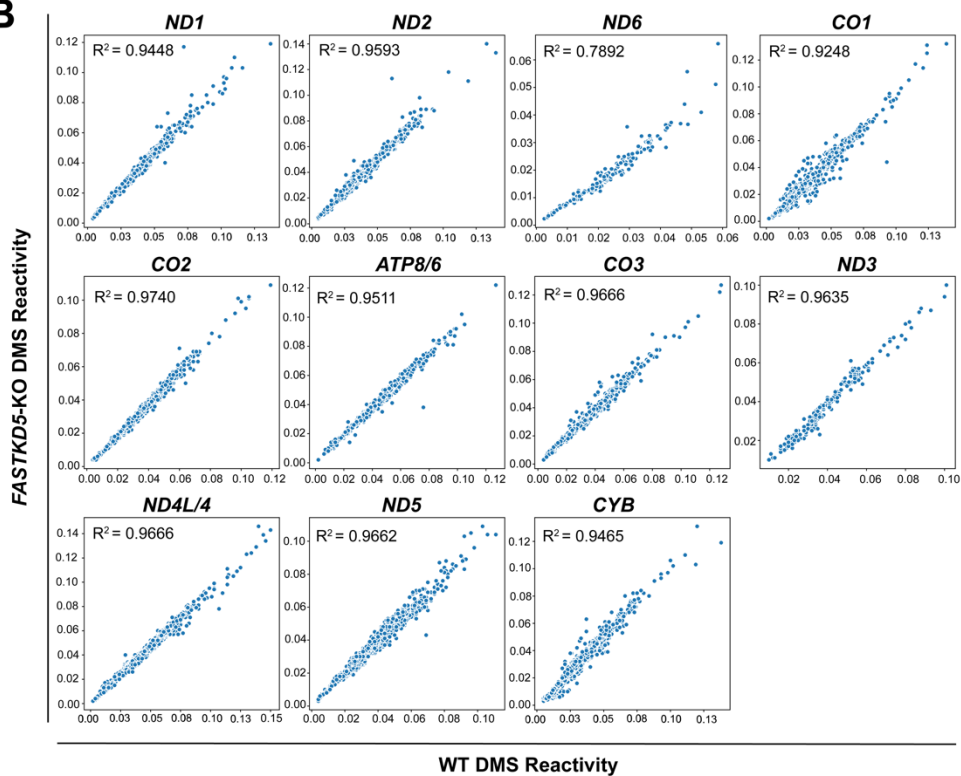
(A) Immunoblot analysis of the *FASTKD5*-KO (KO) clone showing the steady-state protein levels of the indicated mitochondrial proteins. The cell lines were tested in triplicate.

(D) Metabolic labeling of newly synthesized mitochondrial translation products with  $^{35}\text{S}$ -methionine in the presence of emetine to inhibit cytosolic protein synthesis in WT, and *FASTKD5*-KO (KO) HEK293T cells. The individual mitochondrial translation products are labeled on the left. CO1 and CYB, which are undetectable or profoundly decreased in *FASTKD5*-KO cells are highlighted with red asterisks. Immunoblot analysis against  $\beta$ -Actin is provided as a loading control.

**A**



**B**

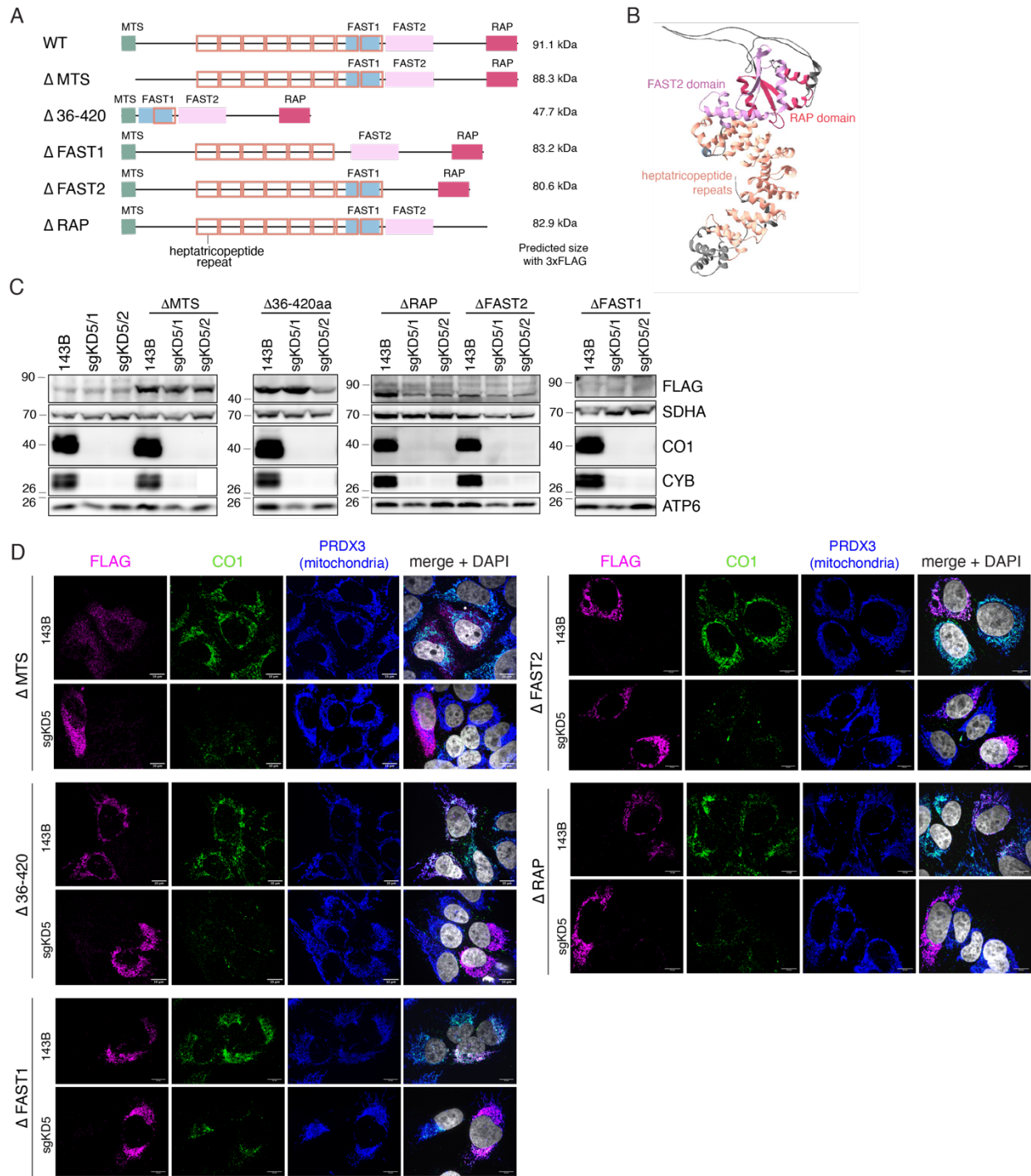


**Supplementary Figure S3 (related to Figure 3)**

**DMS reactivities are largely similar in isolated mitochondria from wild type and *FASTKD5*-KO HEK293T cells.**

(A) Circular representation of the mitochondrial genome H-strand displaying log<sub>2</sub> fold change in DMS reactivity across all mRNAs, comparing wild type (WT) and *FASTKD5*-KO (KO) HEK293T cells. rRNA, tRNA, non-coding RNAs were excluded in the analysis. The green area indicates regions with higher DMS reactivity in WT relative to *FASTKD5*-KO, whereas the orange area represents regions with higher DMS reactivity in *FASTKD5*-KO compared to WT.

(B) Scatter plots correlating DMS reactivity between wild type (WT) and *FASTKD5*-KO (KO) for each mitochondrial transcript. Each plot represents one mitochondrial transcript, with the coefficient of determination ( $R^2$ ) provided in the top left of each figure.



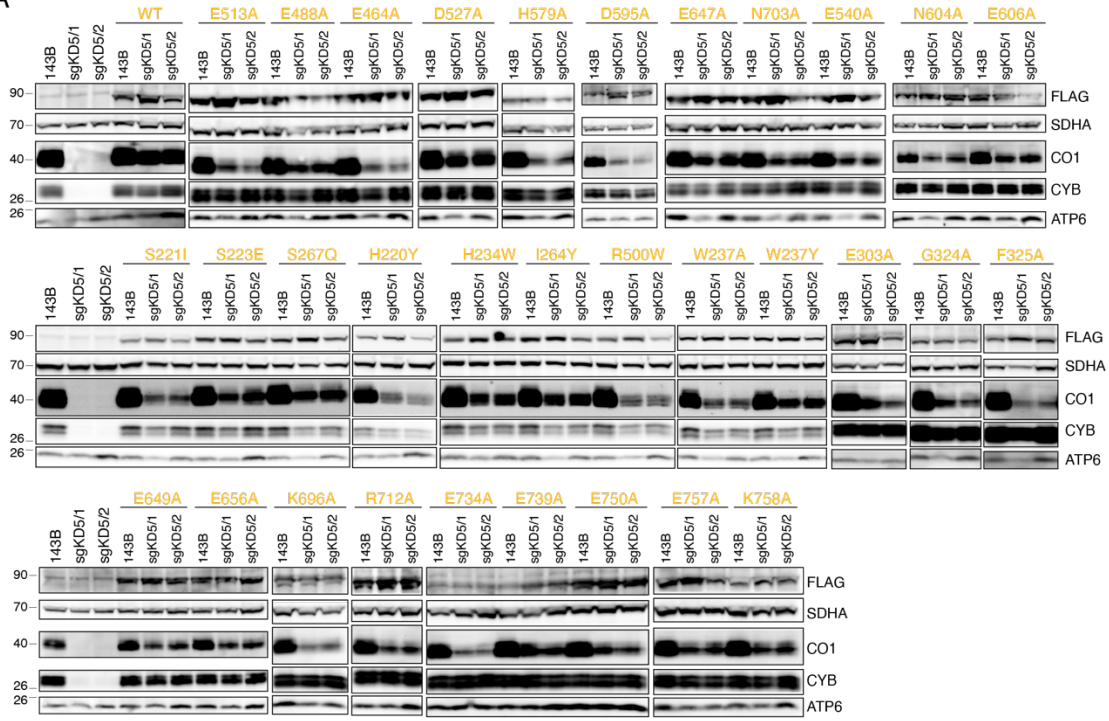
**Supplementary Figure S4 (related to Figure 4)**

**FASTKD5 deletion variants do not rescue the expression of CO1 or CYB.**

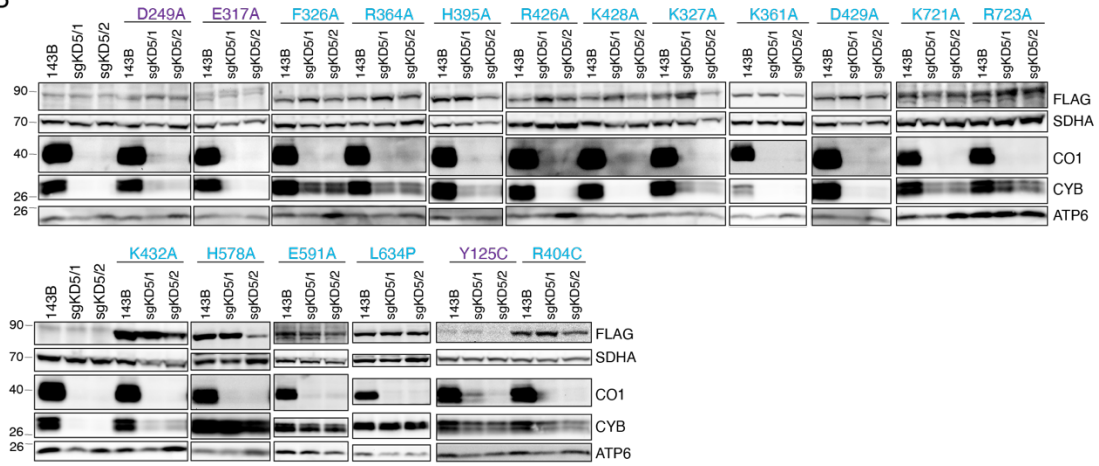
(A) Schematic representation of WT-FASTKD5 protein and individual deletion variants, indicating mitochondrial targeting sequence (MTS), previously described protein domains (FAST1, FAST2 and RAP) and predicted heptatricopeptide repeats. Expected molecular weight

of the deletion variants is indicated on the right. **(B)** Modelling of indicated domains on AlphaFold-predicted FASTKD5 structure. For clarity, the unstructured sequence of 1-100 amino acids was omitted from the picture. **(C)** Western blot analysis of 143B cells and two KO clones (sgKD5/1 and sgKD5/2) and the same cells reconstituted with the indicated FASTKD5-3xFLAG protein deletion variants. SDHA was used as a loading control. Molecular weight markers (in kDa) are indicated on the left. **(D)** Immunofluorescence analysis of 143B cells and one FASTKD5 KO clone (sgKD5) over-expressing the indicated FASTKD5-3xFLAG deletion variants. FASTKD5 expression was determined by anti-FLAG antibody. PRDX3 was used as a mitochondrial marker.

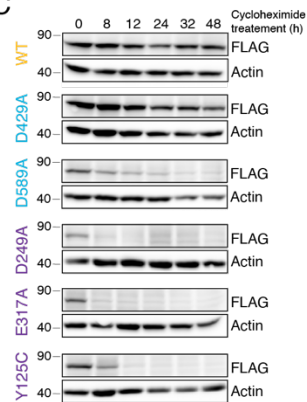
A



B



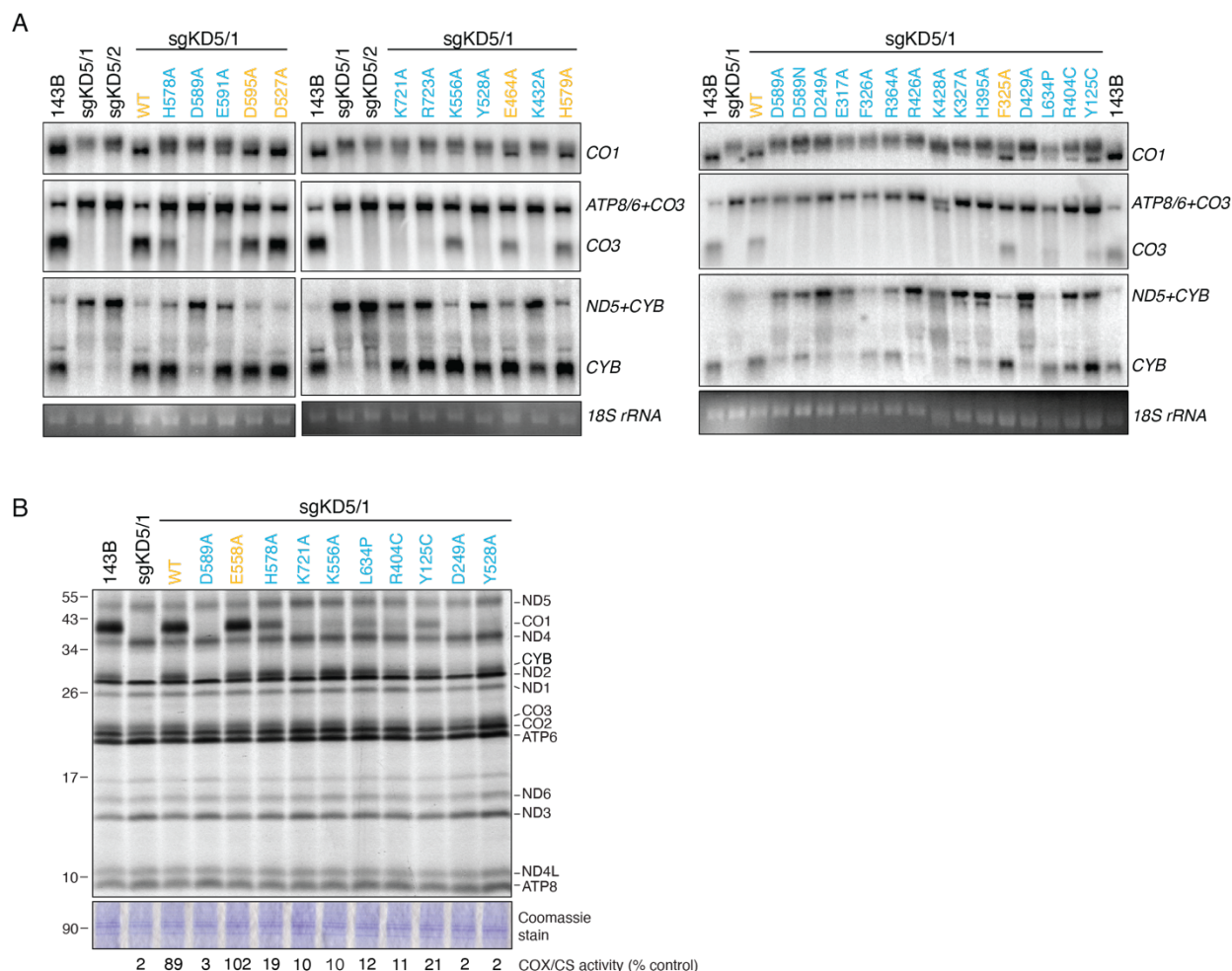
C



**Supplementary Figure S5 (related to Figure 4)**

**Analysis of single amino acid FASTKD5 variants.**

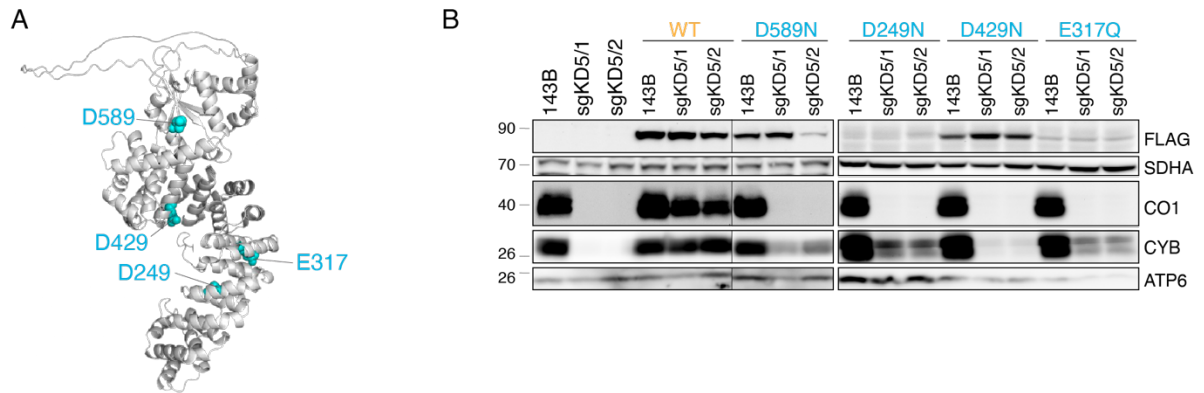
(A-B) Western blot analysis of 143B cells and two KO clones (sgKD5/1 and sgKD5/2) and the same cells reconstituted with the indicated FASTKD5-3xFLAG protein variants. SDHA was used as a loading control. Molecular weight markers (in kDa) are indicated on the left. (A) Indicated variants rescue the expression of CO1 and CYB and are denoted as dispensable amino acids. WT- wild-type FASTKD5. (B) Indicated variants do not rescue the expression of CO1 and are denoted as essential amino acids. Note, that some of these variants rescue the expression of CYB. The three variants in purple (D249A, E317A and Y125C) are expressed at much lower level than all other variants and play a role in the stability of FASTKD5 protein. (C) Western blot analysis of 143B cells reconstituted with the specified FASTKD5-3xFLAG protein variants after the treatment with cycloheximide for the indicated time to measure the stability of FASTKD5 protein, determined by anti-FLAG immunoblot. Actin was used as a loading control. WT- wild-type FASTKD5.



### Supplementary Figure S6 (related to Figure 4)

#### Analysis of FASTKD5 essential variants.

(A) Processing of non-canonical transcripts in 143B cells and two KO clones (sgKD5/1 and sgKD5/2) and in sgKD5/1 cells reconstituted with essential (blue) and dispensable (orange) variants for CO1 expression was assessed by Northern blot analysis. UV-stain of 18S rRNA was used as a control. Image of variants K556A and Y528A plus the corresponding 143B, sgKD1 and sgKD2 are identical in **Figure 4E**. (B) Mitochondrial translation assay in selected cells confirming the lack of synthesis of CO1 and CYB polypeptides. A Coomassie total protein staining served as a loading control. COX/CS activity was measured in individual samples and is indicated as a % control (143B cells) under the gel.



### Supplementary Figure S7 (related to Figure 4)

(A) Modelling of aspartate/glutamate residues essential for processing of all three non-canonical pre-mRNAs on AlphaFold predicted FASTKD5 structure. (B) Western blot analysis of 143B cells and two KO clones (sgKD5/1 and sgKD5/2) and the same cells reconstituted with the indicated FASTKD5-3xFLAG protein variants. The four aspartate/glutamate residues essential for processing of all three non-canonical pre-mRNAs were substituted to asparagine/glutamine to preserve the polarity of the individual amino acids. SDHA was used as a loading control. Molecular weight markers (in kDa) are indicated on the left.

**Supplementary Table S1**

REAGENTS	SOURCE	IDENTIFIER
<b>Antibodies</b>		
FASTKD5	Sigma	Cat# SAB2700438
COI	Abcam	Cat# ab14705 RRID: AB_2084810
CYB	Proteintech	Cat# 55090-1-AP RRID:AB_2881266
ND1	a kind gift of Anne Lombes	
ATP6	Proteintech	Cat# 55313-1-AP RRID:AB_2881305
SDHA	Abcam	Cat# ab168536 RRID:AB_2857979
NDUFA9	Abcam	Cat# ab55521 RRID:AB_2150762
ATP5A1	Abcam	Cat# ab110273 RRID:AB_10858175
UQCRC1	Abcam	Cat# ab110252 RRID:AB_10863633
UQCRC2	Abcam	Cat# ab14745 RRID:AB_2213640
COX4I1	Abcam	Cat# ab110261 RRID:AB_10862101
FLAG	Sigma	Cat# F1804 RRID:AB_262044
PRDX3	in house	
POLRMT	Thermo Fisher Scientific	Cat# PA5-28196 RRID:AB_2545672
MRPP1 (TRMT10C)	Proteintech	Cat# 29087-1-AP RRID:AB_2881239
ELAC2	Proteintech	Cat# 10071-1-AP RRID:AB_2096551
GRSF1	Sigma	Cat# HPA036985 RRID:AB_10672785
DHX30	Abcam	Cat# ab85687 RRID:AB_1860273
TBRG4 (FASTKD4)	Sigma	Cat# HPA020582 RRID:AB_1857804
FASTKD2	Proteintech	Cat# 17464-1-AP RRID:AB_2101119
MTERF3 (MTERFD1)	Sigma	Cat# HPA002966 RRID:AB_2147359
RPUSD4	Sigma	Cat# HPA039689 RRID:AB_10673537
NGRN	Proteintech	Cat# 14885-1-AP RRID:AB_2878090
RCC1L (WBSCR16)	Proteintech	Cat# 13796-1-AP

		RRID:AB_2214934
GFM1	in house	
TUFM/TSFM	a kind gift of Linda Spremulli	
MRPS18B	Proteintech	Cat# 16139-1-AP RRID:AB_2146368
MRPL11	Sigma	Cat# HPA057685
LRPPRC	in house	
SLIRP	Abcam	Cat# ab51523 RRID:AB_2066704
VDAC1	Abcam	Cat# ab14734 RRID:AB_443084
DNA	Millipore	Cat# CBL186 RRID:AB_11213573
TRUB2	Proteintech	Cat# 19891-1-AP RRID:AB_10640900
Actin	GenScript Proteintech	Cat# A00702 RRID:AB_914102 Cat# 60008-1-Ig RRID:AB_2289225
DHX30	Abcam	Cat# ab85687 RRID:AB_1860273
SUPV3L1	Bethyl Laboratories	Cat# A303-056A RRID:AB_10895649
Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L) antibody	Jackson ImmunoResearch Labs	Cat# 115-035-146 RRID:AB_2307392
Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) antibody	Jackson ImmunoResearch Labs	Cat# 111-035-003 RRID:AB_2313567
Goat Anti-Mouse IgG (H+L) Highly Cross-adsorbed Antibody, Alexa Fluor™ 488 Conjugated	Thermo Fisher Scientific	Cat# A-11029 RRID:AB_138404
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Thermo Fisher Scientific	Cat# A-11037 RRID:AB_2534095
Goat anti-Mouse IgG2a Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 594	Thermo Fisher Scientific	Cat# A-21135 RRID:AB_2535774
Donkey Anti-Rabbit IgG (H+L) Polyclonal Antibody, Alexa Fluor™ 647 Conjugated	Thermo Fisher Scientific	Cat# A-31573 RRID:AB_2536183
Goat Anti-Mouse IgG1 Antibody, Alexa Fluor™ 488 Conjugated	Thermo Fisher Scientific	Cat# A-21121 RRID:AB_2535764
<b>Recombinant Proteins</b>		
recombinant WT and D429N FASTKD5 proteins	this paper	
<b>Recombinant DNA</b>		
FASTKD5 (wild-type and all mutants) in pBABE-3xFLAG-puro	this paper	
pBABE-3xFLAG-Puro-gtw	in house	PMID: 34785538

pSpCas9(BB)-2A-Puro (PX459) V2.0	Addgene	Cat# 62988 RRID:Addgene_62988
FASTKD5 in pDONR™221	in house	PMID: 32877691
pET His6 MBP TEV expression vector with BioBrick polypromoer restriction sites (14-C)	Addgene	Cat# 48309 RRID:Addgene_48309
pFastBac His6 MBP Asn10 TEV cloning vector with BioBrick PolyPromoter LIC Subcloning (438-C)	Addgene	Cat# 55220 RRID:Addgene_55220
<b>Experimental Models: Cell Lines</b>		
143B	ATCC	CRL-8303
HEK293T	ATCC	CRL-3216
Phoenix	a gift from Garry P. Nolan	
<b>Oligonucleotides</b>		
sgKD5-F	caccgcagtcggaagtgtgtcatcac	
sgKD5-R	aaacgtatgacacacttcggactgc	
gRNAs for FASTKD5 KO in HEK293T cells	GUCUUGCUGAAUCCAAACC CUUUUUUGGCAGAAUGGCAG CAGUCCGAAGUGUGUCAUAC	
FASTKD5-E558A-a1673c	gaaggatatgaattcaaagcctgcattcttagaaactgtcttttac	
FASTKD5-E558A-a1673c_antisense	gtaaaaagacagtttctaagaatgcaggcttgaattcatatccttc	
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FASTKD5-N604A-a1810g_a1811c	tgatgttaacctgaagccattaccattgttagagaagccacgcc	
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FASTKD5-R404C-c1210t	cttactgctcgcccttatgcttctgaatgaag	
FASTKD5-R404C-c1210t-antisense	cttcattcaggaagcataaggccgagcagtaag	
FASTKD5-Y125C-a374g	tttctacagctaagaccagaatgccgtgttcacag	
FASTKD5-Y125C-a374g-antisense	ctgtgaacacggcattctggtcttagctgtaggaaa	
FASTKD5-H220Y-c658t	gaaagctttgtcatttttaggaatcccttactcccattcaatgc	
FASTKD5-H220Y-c658t-antisense	gcattgaatgggagtaagggttctctaaatgacaaaagctttc	
FASTKD5-H234W-c700t_a701g_t702g	tgtgatgagaccaagtgttctgtggcaggtatgggagatgaatatgg	
FASTKD5-H234W-c700t_a701g_t702g-antisense	ccatattcatctccataacctgccagaacacttggtctatacaca	
FASTKD5-I264Y-a790t_t791a	gccgcaaagtacctaggttttaactattttctagtattcttaattgcaact	
FASTKD5-I264Y-a790t_t791a-antisense	agtgcataaataagataactagaaaaatagtttaaaacctaggtactttgcggc	
FASTKD5-R500W-a1498t	cagtcagggtttgtctggttagctcaggagag	
FASTKD5-R500W-a1498t-antisense	ctctctgagctaaccagacaaacctggactg	

FASTKD5-S221I-t661a_c662t	ttgtcatttttaggaatccctcacatccattcaatgctagatgtgtatg	
FASTKD5-S221I-t661a_c662t-antisense	catacacatctagcattgaatggatgtgagggattcctaaaatgacaa	
FASTKD5-S223E-t667g_c668a	ttaggaatccctcactcccatgaaatgctagatgtgtatgagac	
FASTKD5-S223E-t667g_c668a-antisense	gtctcatcacatctagcatttcattggagtgagggattcctaa	
FASTKD5-S267Q-a799c_g800a_t801a	cgcaaagtaccttaggtttttaacattttttcacaatatcttaatttgactggaaggatcta	
FASTKD5-S267Q-a799c_g800a_t801a-antisense	tagatccttccagtgcacaattaagatattgagaaaaatgtttaaaacacctagggtactttgcg	
FASTKD5-W237A-t709g_g710c	ccaagtggtccatcaggtagcggagatgaatatggatca	
FASTKD5-W237A-t709g_g710c-antisense	tgatccataattcatctccgctactgtatggcaacacttgg	
FASTKD5-W237Y-g710a_g711t	ccaagtggtccatcaggtatatgagatgaatatggatcagct	
FASTKD5-W237Y-g710a_g711t-antisense	agctgatccatattcatctcatatactgtatggcaacacttgg	
FASTKD5-D249N-g745a	agctccttttggtggctaactctctggaggtactta	
FASTKD5-D249N-g745a-antisense	taagtacctccagagattagccacaaaaggagct	
FASTKD5-D429N-g1285a	agagtgccacactgtcgaagtaaaatgttgccaagat	
FASTKD5-D429N-g1285a-antisense	atcttggaacatttttacttcgacagtgtgccactct	
FASTKD5-E317Q-g949c	tagatttgatcaatttgagcaggttggtaccatctgtttg	
FASTKD5-E317Q-g949c-antisense	caaacagatggtaccaacctgtccaaattgatcaaatcta	
FASTKD5-D589N-g1765a	gcctcataccgatcttctaacttagaggtccagc	
FASTKD5-D589N-g1765a-antisense	gctggacctctaagttagaagatcgggtatgaggc	
FASTKD5-noRAP	cagacccaagaatgaaggaccagctttctgtac	
FASTKD5-noRAP-antisense	gtacaagaaagctgggtccttcttctggggtctg	
FASTKD5-noFAST_1	cgaagtaaagatgttgccaggttagctcaggagaga	
FASTKD5-noFAST_1-antisense	ttctcctgagctaacctggcaacatctttacttcg	
FASTKD5-noFAST_2	gaactaagtttgacctccttttaatagagaagccacgcc	
FASTKD5-noFAST_2-antisense	ggcgtggcttctctattaaaaaggaggtcaaacttagttc	
FASTKD5-delta2-27	agcagggtccaccatggtgtcatactggaatg	
FASTKD5-delta2-27-antisense	cattccagtatgacaccatgggtggagcctgct	
FASTKD5-delta36-420	ctggaatgtgagcagcagagtggtgcacactgtc	
FASTKD5-delta36-420-antisense	gacagtgtgccactctgtctcattccag	
FASTKD5_D429N_for_Protein	/5Phos/ctgtcgaagtaaaatgttgccaagattctg	
FASTKD5_D429N_rev_Protein	/5Phos/tgtgccactctaggaggcaaaagacgc	
HH251_for for 14C and 438C cloning	TACTTCCAATCCAATGCAgtgtcactatggaatgtgagc	
HH252_rev for 14C and 438C cloning	TTATCCACTTCCAATGTTATTAgagagcagaggtgaatacttc	
MPAT linker	5'-phospho-ATGTGAGATCATGCACAGTCATA-3'-NH2	
Anti-linker primer	TATGACTGTGCATGATCTCACAT	
CO1 MPAT primer	attttacctcacc	
CO3 MPAT primer	ctgcacgacaacaca	
CYB MPAT primer	caactacaagaacac	
<b>Oligoribonucleotides</b>		
5'-end-CO1-3'/Cy3/ or 3'/Cy5/	AUUUUUACCUCACCCCCACUGAUGUUCGCCGACCGUUGA CUA	
ATP8/6-CO3-3'/Cy3/ or 3'/Cy5/	CUGCACGACAACACAUAUAUGACCCACCAAUCACAUGCC UAU	
ND5-CYB-3'/Cy3/ or 3'/Cy5/	CAACUACAAGAACACCAAUGACCCCAAUACGCAAAACU	

	AAC	
CO1_309-349-3'/Cy3/	ACUCUUACCUCUCCUCUCUCCUACUCCUGCUCGCAUCUG CUA	
long 5'end CO1-3'/Cy5/	UCGGAGCUGGUAAAAAGAGGCCUAACCCCUGUCUUUA GAUUUACAGUCCAAUGCUUCACUCAGCCAUUUUACCUC ACCCCCACUGAUGUUCGCCGACCGUUGACUA	
mut 5'end-CO1 AUG>AUA-3'/Cy5/	AUUUUACCUCACCCCCACUGAUUAUUCGCCGACCGUUGA CUA	
mut 5'end-CO1 CCA>CCG-3'/Cy5/	AUUUUACCUCACCCCCGCUGAUGUUCGCCGACCGUUGA CUA	
mut ND5-CYB AUG>AUA-3'/Cy5/	CAACUACAAGAACACCAUAACCCCAAUACGCAAAACU AAC	
mut ND5-CYB CCA>CCG-3'/Cy5/	CAACUACAAGAACACCGAUGACCCCAAUACGCAAAACU AAC	
mut CO1_309 CCU>CCA-3'/Cy5/	ACUCUUACCUCUCCUCUCCUACUCCAGCUCGCAUCUG CUA	
5'/56-FAM/5'end-CO1	CAGCCAUUUUACCUCACCCCCACUGAUGUUCGCCGACC GUUGACUAUUCU	
5'/56-FAM/ATP8/6-CO3	CCUCUACCUGCACGACAACACAUAUGACCCACCAAUC ACAUGCCUAUCA	