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Editing activity for eliminating mischarged tRNAs is essential in mammalian mitochondria

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ABSTRACT

Accuracy of protein synthesis is enabled by the selection of amino acids for tRNA charging by aminoacyl-tRNA synthetases (ARSs), and further enhanced by the proofreading functions of some of these enzymes for eliminating tRNAs mischarged with noncognate amino acids. Mouse models of editing-defective cytoplasmic alanyl-tRNA synthetase (AlaRS) have previously demonstrated the importance of proofreading for cytoplasmic protein synthesis, with embryonic lethal and progressive neurodegeneration phenotypes. Mammalian mitochondria import their own set of nuclear-encoded ARSs for translating critical polypeptides of the oxidative phosphorylation system, but the importance of editing by the mitochondrial ARSs for mitochondrial proteostasis has not been known. We demonstrate here that the human mitochondrial AlaRS is capable of editing mischarged tRNAs *in vitro*, and that loss of the proofreading activity causes embryonic lethality in mice. These results indicate that tRNA proofreading is essential in mammalian mitochondria, and cannot be overcome by other quality control mechanisms.

INTRODUCTION

Functionality of mitochondria is central for maintaining cellular homeostasis through their involvement in multiple biosynthetic pathways and in ATP production by oxidative phosphorylation. Protein synthesis machineries in two cellular compartments, cytoplasm and mitochondria, are needed to fulfill these functions. The mitochondrial proteome is mostly synthesized by cytosolic ribosomes and imported into mitochondria. However, 13 essential subunits of the mitochondrial respiratory chain complexes are synthesized by dedicated mitoribosomes within the organelle (1,2). These 13 polypeptides, as well as the mitochondrial tRNAs and rRNAs, are encoded by mitochondrial DNA (mtDNA). On the contrary, all protein components of the mitochondrial translation machinery such as the mitoribosomal proteins and the 19 mitochondrial aminoacyl-tRNA synthetases (mtARSs) are nuclear-encoded proteins, which are imported into mitochondria (3,4).

Several mechanisms that control mitochondrial protein integrity have been identified. These include intraorganellar chaperone and protease systems (5), mitochondrial-derived vesicle pathways (6) and mitophagy, the selective autophagy of damaged mitochondria for clearing entire dysfunctional organelles (7). Furthermore, quality control mechanisms exist for proteins residing in different mitochondrial compartments as was recently demonstrated for mitochondrial membrane proteins (8). Impaired mitochondrial protein quality control is associated with multiple pathologies, especially those affecting the central nervous system (9), and also contributes to organismal stress resistance and even longevity through induction of cellular stress responses such as the mitochondrial unfolded protein response (10,11).

The fidelity of protein synthesis itself markedly contributes to protein quality. It is mediated by the aminoacyl-tRNA synthetases (ARSs) that establish the genetic code by charging tRNAs with their cognate amino acids. tRNA synthetases recognize their cognate amino acids by fitting them

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in a defined pocket of the aminoacylation site. However, some synthetases are unable to discriminate against highly similar amino acids, and mischarge tRNAs with noncognate amino acids. To avoid mistranslation, some synthetases have an editing activity for hydrolyzing the mischarged or misactivated amino acid. The editing functions of ARSs can occur pre- or post-transfer on cognate tRNAs. Pre-transfer editing is thought to take place at the synthetic active site when the non-cognate amino acid is activated by ATP, but not yet transferred to the tRNA, whereas post-transfer editing is for noncognate amino acids that are already attached to the tRNA. Synthetases with post-transfer editing activity have spatially separated editing domains with active sites that can recognize only noncognate amino acids of misacylated tRNAs (12). The post-transfer editing can be either *cis*- or *trans*-editing, depending on whether it occurs directly after the tRNA charging or after the release of the mischarged tRNA from the synthetase, respectively.

The *in vivo* significance of avoiding mistranslation is demonstrated by the spontaneous *sti* mouse mutation in the cytoplasmic alanyl-tRNA synthetase (AlaRS), causing a cerebellar ataxia with progressive degeneration of Purkinje cells (13). AlaRSs are the most conserved tRNA synthetases, consisting of an N-terminal aminoacylation domain, editing domain, and a C-terminal domain (14). AlaRSs misactivate glycine and serine, of which serine misincorporation is considered to be universally toxic (15). In *E. coli*, a severe editing mutation of AlaRS prevents cell growth in high concentrations of glycine and serine (16) and in mice the equivalent editing mutant is embryonic lethal (17). The *sti* mutation causes only a mild reduction in deacylation of Ser-tRNA^{Ala}, which then leads to the slowly progressive neurodegeneration with increased levels of misfolded proteins and activated unfolded protein response.

Defective proofreading at the level of mtDNA replication causes a premature aging phenotype in mice (18). However, the importance of mischarged tRNA editing in mammalian mitochondria has not been demonstrated. In fact, several mtARSs have been reported to lack editing function. For example, editing activity was lost during the evolution of mitochondrial phenylalanyl-tRNA synthetases (19,20), and the editing active site of human mitochondrial leucyl-tRNA synthetase is not operational (21). Therefore it was suggested that mitochondrial protein synthesis is inherently less accurate or that its protein quality control is focused on another step (20). Recently, however, the human mitochondrial threonyl-tRNA synthetase (mtThrRS) was shown to use post-transfer editing to clear cognate tRNAs mischarged with serine *in vitro* (22).

We demonstrate here that mammalian mitochondrial alanyl-tRNA synthetase (mtAlaRS), which has a highly conserved editing domain (23,24), is capable of editing mischarged Ser-tRNA^{Ala} *in vitro*. Furthermore, as an effort to investigate mitochondrial mistranslation *in vivo*, we have generated editing-deficient mtAlaRS mice, bearing either a mild or a severe mtAlaRS editing mutation, and demonstrate that editing of mischarged tRNAs by alanyl-tRNA synthetase is an essential protein quality control mechanism in mammalian mitochondria.

MATERIALS AND METHODS

Cloning and gene expression of human mtAlaRS

Human *AARS2* cDNA was cloned into pBabe-puro expression vector using EcoRI and SalI restriction sites. Point mutants for amino acid changes C749A or V760E in human *AARS2* were introduced by PCR-based site-directed mutagenesis with Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). The DNA fragments encoding mature wild type hmtAlaRS from Ser^{26}-Leu^{985} (25), or with mutations C749A or V760E were cloned between *Nde*I and *Xho*I of pET30a. All primer sequences are available on request. *Escherichia coli* BL21 (DE3) cells were transformed with the plasmids to overproduce wild type hmtAlaRS, or the C749A and V760E mutants with a C-terminal His_6 tag. The gene expression was induced with a final concentration of 100 μM IPTG at 22°C for 10 h. The first-step affinity chromatography on Ni-NTA Superflow was performed according to the method described previously (26). The protein was then purified by gel filtration chromatography with a Superdex^{TM} 200 column with the running buffer 50 mM Tris–HCl (pH 8.0) and 50 mM NaCl. The fractions corresponding to hmtAlaRS were collected.

Transcription of human mitochondrial tRNA^{Ala} (hmtRNA^{Ala})

The sequence of hmtRNA^{Ala} is 5′-AAGGGCTTAGC TTAATTAAAGTGGCTGATTTGCGTTCA GTTGA TGCAGAGTGGGGTTTTGCAGTCCTTACCA-3′.
We inserted a hammerhead ribozyme sequence (*GGGAGACCTTCTGATGAGTCCGTGAGGACGAA ACGGTACCCGGTACCGTC*) between T7 promoter and the hmtRNA^{Ala} gene to facilitate *in vitro* T7 transcription (27). Six complementary and overlapping oligonucleotides encoding T7 promotor, the hammerhead ribozyme sequence and the hmtRNA^{Ala} gene, and its complementary chain were chemically synthesized by Biosune (Shanghai, China). The fragments were then cloned between the PstI and EcoRI sites of pTrc99b with an N-terminal T7 promoter. Detailed T7 *in vitro* run-off transcription of hmtRNA^{Ala} was performed according to the method described previously (28), with an additional incubation of the transcription reaction at 60°C for 1 h after the template was digested for self-cleavage of the ribozyme. ^{32}P-labeling of hmtRNA^{Ala} by *E. coli* CCA-adding enzyme was performed as described previously (29).

ATP-PPi exchange assay

Kinetics of amino acid activation of hmtAlaRS, C749A and V760E were determined by ATP-PPi exchange reaction in a reaction buffer containing 50 mM Tris–HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, (0.5–40) mM Ala, or (100–1500) mM Ser, 2 mM tetrasodium [^{32}P]pyrophosphate and 200 nM enzyme at 37°C. A 9 μl aliquot of reaction mixture was removed into 200 μl quenching solution (2% activated charcoal, 3.5% $HClO_4$, and 50 mM tetrasodium pyrophosphate) and mixed on vortex. The solution was filtered through Whatman GF/C filter, followed by washing with 20 ml 10 mM tetrasodium

pyrophosphate solution and 10 ml 100% ethanol. The filters were dried and [^{32}P]ATP was counted by scintillation counter (Beckman Coulter).

In vitro aminoacylation assay

In vitro aminoacylation assay was performed in a reaction buffer containing 50 mM Tris–HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 100 μM [^{14}C]Ala, 5 μM hmtRNA^{Ala} and 400 nM hmtAlaRS and its two variants at 37°C. Aliquots of 9 μl reaction solution were removed at specific time-points and quenched on Whatman filter pads, equilibrated with 5% trichloroacetic acid (TCA). The pads were washed three times for 15 min each with cold 5% TCA and then three times for 10 min each with 100% ethanol. The pads were dried by a heat lamp. The radioactivities of the precipitates were quantified by scintillation counter (Beckman Coulter).

In vitro mis-aminoacylation

In vitro mis-ammnoacylation assay was performed in a reaction buffer containing 50 mM Tris–HCl (pH 8.0), 20 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 454 μM [^{14}C]Ser, 5 μM hmtRNA^{Ala} and 1 μM hmtAlaRS and its two variants at 37°C. The processing and the quantification were performed as described above.

Post-transfer editing assay

Preparation of Ser-[^{32}P]hmtRNA^{Ala} was carried out with editing-deficient C749A in a reaction mixture, which was similar to that used for mis-aminoacylation except that unlabeled Ser and [^{32}P]hmtRNA^{Ala} were added. Post-transfer editing assay was performed in a reaction buffer containing 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 2 μM Ser-[^{32}P]hmtRNA^{Ala}, 500 nM hmtAlaRS and its two variants at 37°C. Processing of samples was performed as described previously (22). Nuclease S1-generated Ser-[^{32}P]AMP (reflecting Ser-[^{32}P]hmtRNA^{Ala}) and [^{32}P]AMP (reflecting free [^{32}P]hmtRNA^{Ala}) were separated by thin layer chromatography (TLC). A control reaction represented the spontaneous hydrolysis of Ser-[^{32}P]hmtRNA^{Ala} without the addition of enzyme.

Structure analysis of mtAlaRS editing domain

The editing core of mouse mtAlaRS containing editing active site with bound zinc ion and comprising residues 619–779 was modeled on trans-editing protein AlaX (PDB id 1WNU). First, multiple sequence alignment of mAlaRS homologues retrieved from UniProt database (www.uniprot.org) was done using Promals3D web-server. Structure homology modeling was done using SWISS-MODEL (www.swissmodel.expasy.org). Analysis of the structures as well as *in silico* mutagenesis was done using Discovery Studio 4.5 (BioVIA).

Generation of C744A and V755E mtAlaRS mice and studies on embryos

All animal procedures were performed according to protocols approved by the National Animal Experiment Board and Regional State Administrative Agency for Southern Finland and all experiments were done in accordance with good practice of handling laboratory animals and of genetically modified organisms.

Nucleotide changes c.2230TG>GC (p.C744A) in exon 16 of mouse *Aars2* gene was introduced to generate the C744A knock-in mice, and nucleotide change c.2264T>A (p.V755E) in exon 17 to generate the V755E knock-in mice. For this, BAC clones containing the mouse *Aars2* gene (ENSMUSG00000023938) were obtained from BACPAC Resources Center (Children's Hospital Oakland Research Institute, Oakland (CHORI), CA, USA). A 7250 bp DNA fragment from the BAC clone, containing exons 6–22 of *Aars2* gene, was subcloned into the pACYC177 vector (New England Biolabs, MA, USA) by Red/ET recombination method according to the manufacturer's instructions (Gene Bridges GmbH, Germany). DNA fragment containing exons 15, 16 and 17 was then amplified by PCR and inserted into pGEM-4Z vector digested with SmaI or ScaI restriction enzymes. In order to generate point mutations in exons 16 and 17, mutagenesis was carried out by using QuikChange Site-Directed Mutagenesis kit (Stratagene, CA, USA). Neomycin (Neo) resistant gene flanked with two Frt or LoxP sites was introduced into intron 17 with FRT-PGK-gb2-Neo-FRT cassette (Gene Bridges) for the C744A targeting construct and with LoxP-PGK-tn5-Neo-LoxP cassette (Gene Bridges) for the V755E targeting construct, respectively. Finally, DNA fragments containing point mutations and the Neo cassette were excised from pGEM-4Z with SmaI in case of C744A mutant and SmaI and ScaI in the case of V755E mutants and replaced by using Red/ET recombination with wild type region in the previously generated pACYC177 vector containing the exons 6–22. Validity of final targeting constructs were confirmed by restriction enzyme digestion and sequencing. All primer sequences are available on request.

G4 embryonic stem cells (derived from 129S6/C57BL/6N cr mouse strain) were cultured on neomycin-resistant primary embryonic fibroblast feeder layers, and 10^6 cells were electroporated with 30 μg of linearized targeting construct. After electroporation, the cells were plated on 100-mm culture dishes and exposed to G418 (300 μg/ml; Sigma). Colonies were picked after 7–9 days selection, and grown on 96-well plates. DNA isolated from ES cell clones was screened by long-range PCR for both 5′ and 3′ homologous arms, and correct PCR products were further verified by sequencing. In order to delete the Neo cassette in the targeted ES cells, the C744A ES cells were electroporated with plasmid, pCAGGS-Flpe, and V755E ES cells with pCAGGS-Cre. After electroporation, the cells were plated on 100-mm culture dishes and colonies were picked after culturing for 3–5 days growth, and further amplified on 96-well plates. In order to detect targeted ES clones with Neo deletion, DNAs isolated from ES cells were screened by PCR with several different primer pairs, and the correct clones were further confirmed by sequencing. Morula aggregation of the ES cells was carried out at the GM Mouse unit of the Laboratory Animal Center, University of Helsinki, according to standard protocol. The chimeric mice produced from morula aggregation were crossed to mice with C57BL/6JOlaHsd genetic background.

Embryo collection was done by counting the day of vaginal plug as embryonic day 0.5 (E0.5). Embryos were collected at E8.5 and E7.

Genotyping of the mice

Genotyping of C744A and V755E mice and embryos were carried out by using primers: forward: 5′-CCTGGATCCTCGTCCTCCCTTA-3′ and reverse: 5′-TTAGAAATGTTTGCCGGTTGGG-3′. The forward primer is located upstream of the mutation sites in the exon 16 and the reverse primer downstream of the Frt or the LoxP site in the intron 17 in C744A and V755E mice, respectively. The PCR reaction results in a 401bp product from the wild type allele, 475 bp product from the C744A mutant allele and 497 bp product from the V755E mutant allele.

Protein extraction, SDS-PAGE and Western blot

Protein extraction from mouse embryos, mouse embryonic fibroblasts and inducible cells was done in RIPA buffer (Cell signaling technology) containing proteinase inhibitor (Thermo Fisher Scientific). Cells were incubated on ice for 10 min and embryos for 30 min. Fifteen μg of protein or the whole-embryo protein lysates were run on a 10% or 12% polyacrylamide gels (BIO-RAD) followed by semi-dry blotting (Thermo Fisher Scientific) to a PVDF transfer membrane (Merck Millipore). The membranes were blocked in 5% milk/TBST for an hour and the immunoblotting was done overnight at +4°C with anti-AARS2 (Sigma HPA035636), anti-COXI (Abcam ab14705), anti-GAPDH (Cell Signaling 14C10), anti-PKM1 (Proteintech 15821–1-AP), anti-SDHA (Abcam ab14715), anti-TOM40 (Santa Cruz sc-11414) or anti-Rieske (Abcam ab14746) antibodies. Bands were visualized with the ECL Prime Detection Reagent (GE Healthcare), imaged with a Chemidoc XRS+ Molecular Imager (Bio-Rad) and quantified with the ImageQuant TL software (GE Healthcare).

Protein extraction from mouse heart tissue was prepared by homogenizing the tissue in PBS (100 μl/10 mg of tissue) with Protease Inhibitor (Halt) included. 5xRIPA was diluted into the homogenized samples according to the PBS volumes. Samples were incubated on ice for 20 min after which they were centrifuged in 14 000 g for 10 min (+4°C). Twenty μg of protein was run on 12% stain-free polyacrylamide gel (Bio-Rad), followed by immunoblotting using antibodies mentioned above.

Quantitative real-time PCR (QPCR)

RNA extraction was done from freezed wild-type and heterozygous mouse kidney samples with TRIzol (Thermo Fisher scientific 15596–026) according to the manufacturer's instructions. Homogenization of the samples was done by using Precyllys 24 homogenizer (Bertin technologies). 1000 ng of RNA was used to generate cDNA by Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher scientific K1641) according to the manufacturer's instructions. QPCR reactions were done with DyNAmo Flash SYBR Green qPCR Kit (ThermoFisher scientific F-415) according to the manufacturer's instructions in CFX96^{TM} Real-Time PCR Detection System (Bio-Rad). 25 ng of cDNA was used in the reactions as a template. The primers for *Aars2* were following: forward 5′-CTCAGCTCTGTGGGGGTTTT-3′ and reverse 5′-CCATTCGCCAGGCCTTATCT-3′ (Sigma). *Rars2* gene was used as a loading control and the primers were following forward 5′- ACACGAGTTCTTCCACCGAC-3′ and reverse 5′-ACAACATTTCCCTCCGCTGT-3′. Amplification conditions were: 95°C for 7 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Dissociation curves were checked to ensure the existence of a single PCR product and each sample was run in duplicate.

In vivo aminoacylation assay

RNA extraction was carried out from mouse skeletal muscle samples with TRIzol (Thermo Fisher scientific) according to the manufacturer's instructions. RNA pellet was dissolved into 10 mM NaOAc (pH 5.2). Deacylated samples were obtained by incubation of the RNA with equal volume of 1 M Tris–HCl (pH 9.5) at 75°C for 15 min after which they were put on ice. RNA (3.2 μg in C744A and controls, and 5 μg in V755E mice and controls) was run to a 6.5% polyacrylamide gel (19:1 acrylamide/bis) including 8 M urea and 0.1 M NaOAc (pH 5). Running buffer: 0.1 M NaOAc (pH 5), 2× sample buffer: 0.1 M NaOAc, 8M Urea, 0.05 % bromophenol blue, 0.05% xylene cyanol FF. After running the gel was blotted on to a Hybond-XL nylon membrane (GE Healthcare Amersham) in 0.5× TBE after which the membrane was UV-crosslinked. Membrane was prehybridized 1 h at 37°C and hybridized overnight at 37°C with mitochondrial $tRNA^{Ala}$ and $tRNA^{Arg}$ γ-32P-labeled probes.

Culture of mouse embryonic fibroblasts

MEF cultures were prepared from E13.5 embryos. The limbs, brain, and liver were dissected out and the rest of the embryo was cut into small pieces with a scalpel. The embryo pieces were put into 10 ml of 1× Trypsin–EDTA and incubated for 30 min at +37°C and vortexed every 5 min. More trypsin (10 ml) was added and the incubation and vortexing was continued for another 30 min. Then FBS (2 ml) and media (8 ml) was added and the cells centrifuged at 500 g for 5 min. The supernatant was taken out and the cell pellet resuspended in media (20% FBS (Gibco), 1%, L-glutamate (Lonza), 1% Penicillin Streptomycin (Lonza), 0.1 mM β-mercaptoethanol (Sigma), DMEM (Lonza)) and seeded.

Chloramphenicol and serine treatments

Wild type and heterozygous C744A MEFs were treated with chloramphenicol (30 μg/ml) (Sigma C3175) for 48 h to inhibit mitochondrial protein translation. After that the cells were washed twice with PBS and then grown either in MEF-media, or in MEF-media supplemented with 10 or 20 mM of serine (Sigma S4311) for 24 h, and subjected to protein extraction for western blotting. For monitoring of cell growth, two to three independent cell lines of wild type and heterozygous C744A MEFs were grown in normal MEF

Table 1. Kinetic parameters of hmtAlaRS for Ala and Ser in ATP-PPi exchange reaction[a]

Amino acid	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})	Discrimination factor (DF)[b]
Ala	4.65 ± 0.21	5.68 ± 0.18	0.82	1
Ser	0.74 ± 0.04	515 ± 50	1.44E-03	569

[a]The results are the average of three independent repeats with standard deviations indicated.
[b]DF is calculated by equation the DF = $(k_{cat}/K_m)_{Ala}/(k_{cat}/K_m)_{Ser}$

media, or with 40 mM of added serine, and the cell proliferation was followed for over 40 h using the Cell IQ (Chip-man technologies).

Histochemistry

For hematoxylin eosin stainings mouse cerebellums were embedded in paraffin and cut into 5 μm tissue slides. For simultaneous cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) activity stainings, skeletal muscle samples were covered with fixative (Sakura) and snap frozen in isopentane/liquid nitrogen, and cut into 7 μm thick cryoslides.

Statistical analysis

All numerical data are expressed as mean ± SEM. Student's *t*-test was used for statistical analysis.

RESULTS

AlaRSs are highly conserved enzymes containing separate domains for aminoacylation and editing, and a C-terminal domain (Figure 1A). For example, the sequence similarity for the editing domains of human mitochondrial and archeon *A. fulgidus* AlaRSs, for which a full crystal structure is available (30), is 39.8% (23). However, biochemical studies using the recombinant mtAlaRS have neither been previously reported, nor the functionality of the editing domain of mtAlaRS demonstrated. We obtained the recombinant human mature mtAlaRS (Ser^{26}-Leu^{987}) (hmtAlaRS), excluding the N-terminal mitochondrial targeting sequence, from overexpression in *E. coli* (25). The requirement of editing by an ARS is generally judged from the discrimination efficiency toward cognate and noncognate amino acids. Therefore, we first measured the kinetic parameters of hmtAlaRS for alanine and serine in ATP-PPi exchange reaction. HmtAlaRS misactivated Ser with a discriminator factor of 1/569 (Table 1), which is significantly larger than 1/3300, the proposed threshold for frequency of errors in protein synthesis (31), suggesting that mtAlaRS requires editing function to prevent Ala-to-Ser misincorporation in mitochondria.

Next, we tested the effects of two editing domain mutations on the aminoacylation and editing functions of mtAlaRS *in vitro*. The first hmtAlaRS substitution C749A is equivalent to the editing-deficient *E. coli* mutant C666A (16), and to the embryonic lethal C723A in the mouse cytosolic AlaRS (13). This cysteine is fully conserved in AlaRSs, and is critical for zinc binding in the editing active site and thus for the deacylation reaction (Figure 1A). Structural prediction suggests that its substitution for alanine severely disrupts the editing activity (Figure 1B). The second hmtAlaRS mutant V760E mimics the *sti* mouse mutation A734E (13). Only hydrophobic amino acids (alanine, valine, leucine, isoleucine and methionine) are tolerated at this position (Figure 1A). This is a structural residue supporting the architecture of the editing site and forming hydrophobic interactions with conserved alanine, leucine and valine (mouse A629, L632 and V683, Figure 1B). Mutation for glutamic acid is predicted to affect the coordination of zinc ion by the conserved histidine (mouse H748, Figure 1B) resulting in a decreased deacylation rate as demonstrated for the *sti* mutation (13). In the ATP-PPi exchange reaction both hmtAlaRS mutants C749A and V760E exhibited similar alanine activation activity as the wild type hmtAlaRS (Figure 1C). *In vitro* aminoacylation assay indicated that the mutants C749A and V760E displayed only slightly decreased $tRNA^{Ala}$ charging activity compared with wild type mtAlaRS (Figure 1D). Determination of post-transfer editing of mischarged Ser-$tRNA^{Ala}$ showed that the C749A mutant was severely defective in editing while the initial velocity of the post-transfer editing activity of the V760E mutant was only slightly reduced (Figure 1E and F). *In vitro* misaminoacylation assay demonstrated that the C749A mutant generated considerably more Ser-$tRNA^{Ala}$ than the wild type synthetase, consistent with its abolished post-transfer editing activity (Figure 1G). The V760E mutant produced less Ser-$tRNA^{Ala}$ than the severe mutant, but also more than the wild type enzyme. These results indicate that while the two mtAlaRS editing domain mutants had a nearly normal aminoacylation activity, they both displayed deficiency in editing, albeit at different severities (C749A was more defective in editing than V760E).

To investigate the *in vivo* importance of editing by mtAlaRS in mammalian mitochondria, we generated two knock-in mouse models of mtAlaRS with amino acid changes corresponding to the mild and severe editing mutants described above. The mouse mtAlaRS is encoded by the nuclear gene *Aars2* (Figure 1A), and the introduced codon changing mutations were the following: c.2230TG>GC (p.C744A) and c.2264T>A (p.V755E) in exons 16 or 17, respectively (Figure 2A and D). Following morula aggregation of targeted ES cells, the chimeras were bred to C57BL/6JOlaHsd females. Crossing of heterozygous littermates failed to result in Mendelian proportions of genotypes as no homozygous C744A or V755E knock-in mice were obtained (Figure 2B). Investigation of embryonic development in either of the knock-in mouse strains revealed that the homozygous embryos were significantly smaller at embryonic day 8.5 (E8.5) than their wild type or heterozygous littermates, indicating that both mutations are embryonic lethal in the homozygous state (Figure 2C). Genotyping and sequencing confirmed the small underdeveloped embryos at E7 to be homozygous mutants, while

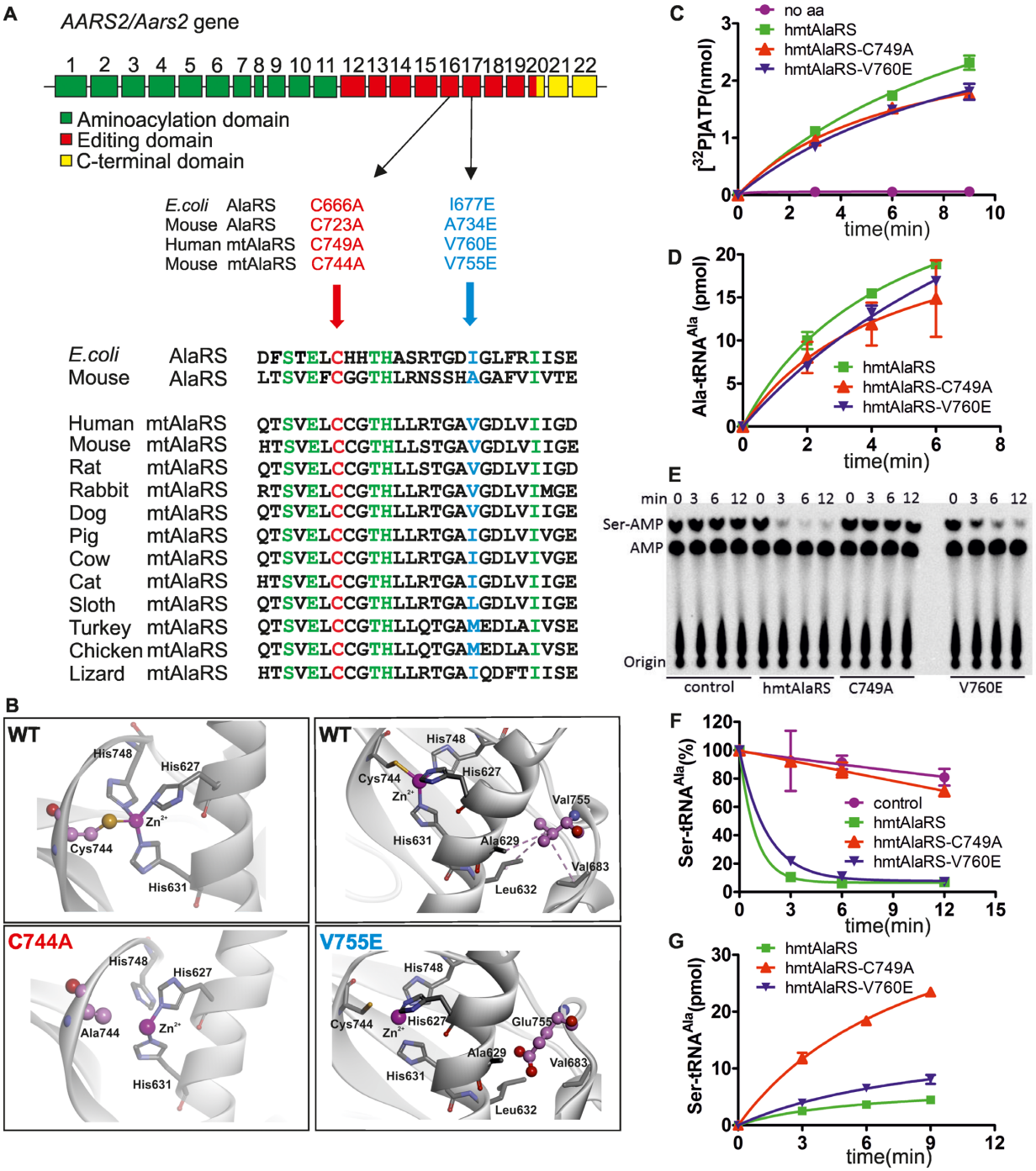

Figure 1. The mitochondrial alanyl-tRNA synthetase has a functional editing domain. (**A**) The human/mouse *AARS2/Aars2* gene, coding for mitochondrial alanyl-tRNA synthetase (mtAlaRS), is composed of 22 exons. Exons 1–11 code for the aminoacylation domain, exons 12–20 for the editing domain and exons 20–22 for the C-terminal domain. Amino acid sequence alignment is shown for a part of the editing core of AlaRS in *E. coli* and mouse, and mtAlaRS in several vertebrate species. In the alignment, green colour indicates fully conserved amino acids, and red and blue colour indicate the mutated amino acids. (**B**) Structural predictions for the mutations in the editing core are shown for mouse mtAlaRS. Cysteine 744 (pink) is critical for zinc binding (purple) in the editing active site as shown in the upper left figure. Substitution of cysteine 744 for alanine is predicted to severely disrupt the zinc binding and thus the editing activity of mtAlaRS as shown in the bottom left figure. Valine 755 supports the architecture of the editing site as shown in the upper right figure. Its change for glutamic acid affects the position of histidine 748, one of zinc binding ligands, due to repulsion forces between glutamic acid 755, alanine 629, leucine 632 and valine 683 as shown in the bottom right figure. (**C**) Alanine activation of the wild type hmtAlaRS and the C749A and V760E mutants in ATP-PPi exchange reaction. (**D**) Alanine charging of $tRNA^{Ala}$ by the wild type hmtAlaRS and the C749A and V760E mutants in *in vitro* aminoacylation assay. (**E**) A representative TLC showing hydrolysis of Ser-[^{32}P]$tRNA^{Ala}$ by hmtAlaRS, and the mutants C749A and V760E. Nuclease S1-generated Ser-[^{32}P]AMP (reflecting Ser-[^{32}P]$tRNA^{Ala}$) and [^{32}P]AMP (reflecting free [^{32}P]$tRNA^{Ala}$) were separated by TLC. A control reaction represented the spontaneous hydrolysis of Ser-[^{32}P]$tRNA^{Ala}$ without the addition of enzyme. (**F**) Graph of the post-transfer editing of Ser-[^{32}P]$tRNA^{Ala}$ by wild type hmtAlaRS and the C749A and V760E mutants. (**G**) Mis-aminoacylation of wild type hmtAlaRS and the C749A and V760E mutants with serine. In all graphs mean values with error bars indicating SD are shown.

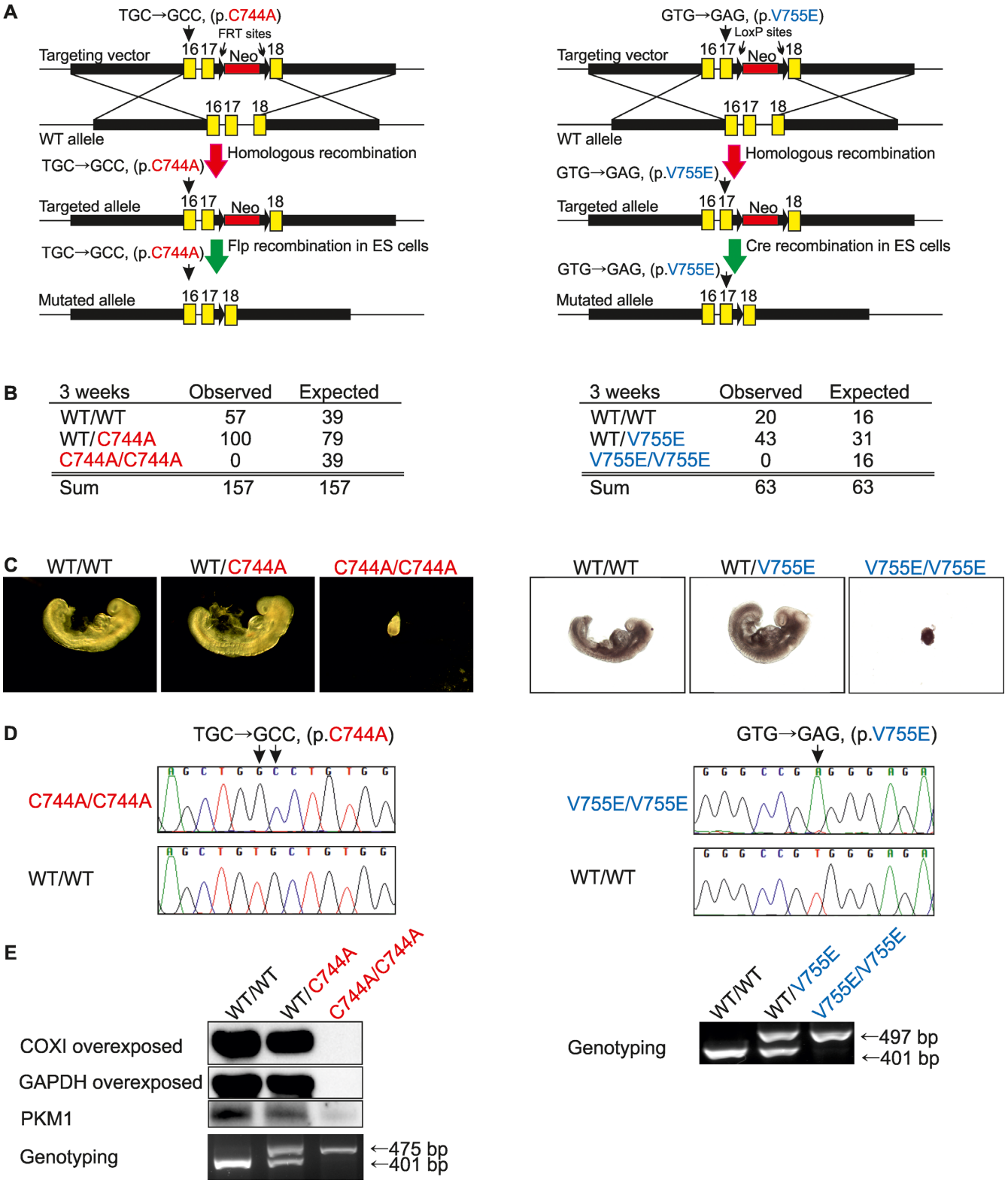

Figure 2. Editing-deficient knock-in mouse models of mtAlaRS are early embryonic lethal. (**A**) Targeting strategies for C744A and V755E knock-in mutants in mice. The mutations were introduced into exons 16 or 17 by homologous recombination. The Neo cassette that was used for clone selection was excised by Flp or Cre recombination from the ES cells. (**B**) Weaned 3-week-old pups and their genotypes from heterozygote parental crossings. WT, wild type. (**C**) Images of mouse embryos of different genotypes at E8.5. (**D**) Sequencing traces of the mutation sites from mouse embryos at E8.5. (**E**) Western blot of cytochrome *c* oxidase subunit I (COXI), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and PKM1 pyruvate kinase (PKM1), and a genotyping of E7 embryos.

proteins in them had mostly been degraded, preventing further investigation of these embryos (Figure 2D and E).

The viability of mouse embryonic fibroblasts (MEFs) homozygous for the *sti* mutation was reported to be sensitive to serine supplemented to the culture medium. Furthermore, heterozygous *sti* MEFs were shown to have intermediate serine sensitivity in comparison to the wild type and homozygous cells (13). We were unable to culture MEFs of the small homozygous C744A or V755E mtAlaRS embryos, but we prepared wild type and heterozygous MEFs. The heterozygous MEFs of either mouse line showed an amount of mtAlaRS protein comparable to the wild type cells (Figure 3A), suggesting that the editing-mutant synthetases were stable. Tissues from heterozygous animals showed no compensatory increase in *Aars2* mRNA levels, further supporting the stability and functionality of the mutated enzymes (Figure 3B). Furthermore, the levels of aminoacylated mt-tRNA^{Ala} were not substantially altered in muscle of heterozygotes of either mouse line in comparison to wild type littermates, showing that the editing mutations did not result in reduced tRNA charging or stability (Figure 3C). We then cultured the MEFs in normal medium or in medium with 40 mM of serine, and followed their proliferation for over 40 h. Increased serine reduced cell proliferation of both wild type and heterozygous MEFs, but no differences were observed between the genotypes (Figure 3D). To test the effects of supplemented serine on mitochondrial protein synthesis, we pretreated wild type and heterozygous C744A MEFs with chloramphenicol, which blocks the translation elongation step on the mitoribosome, for 48 h to deplete the cells of mtDNA-encoded respiratory chain subunits. Next, the cells were grown in normal medium or with 10 or 20 mM of supplemented serine for 24 h and monitored for the recovery of the respiratory chain subunit COXI of Complex IV by Western blot. Following the serine supplementation, the recovery of COXI protein levels was found to be comparable in wild type and heterozygous cells (Figure 3E). These results indicate that in the presence of the wild type enzyme, the expression of editing-deficient synthetase is not sufficient to induce noticeable damage to synthesized mitochondrial proteins even with increased concentrations of serine.

While the editing-deficient cytoplasmic C723A AlaRS mutation was found to be embryonic lethal in homozygous mice, even the heterozygous mice presented with a mild phenotype of cerebellar neuron loss at one year of age (13). We therefore followed the heterozygous C744A mtAlaRS mice by monitoring their weights and appearance regularly and sampled the tissues at one year of age. No differences in weight gain or behavior were observed during the one year follow-up between the different groups of mice (Figure 4A). The heart to body weight ratios of the sacrificed mice were also comparable (Figure 4B). We stained the skeletal muscle of the 1-year-old mice for the activity of COX, the respiratory chain Complex IV, and found it to be normal in heterozygous C744A mice (Figure 4C). COXI protein levels in the heart tissue were also normal as detected by western blot (Figure 4D). The cerebellums of the heterozygous C744A mice also appeared normal and no neuron loss or lack of COX activity was observed (Figure 4E, and not shown). These results further illustrate that heterozygosity for a severe mtAlaRS editing defect is not pathogenic.

DISCUSSION

The significance of editing mischarged tRNAs in mammalian cytoplasm has been undoubtedly demonstrated by the embryonic lethal phenotype of AlaRS mice carrying the severe editing mutation C723A (17), and by the progressive neurodegeneration phenotype of the milder editing mutation in *sti* mice (13). The severe cytoplasmic mutant was shown to increase mischarging 15-fold *in vitro*, whereas the *sti* mutation led to a two-fold increase. However, the importance of tRNA proofreading has not been clear in mammalian mitochondria. We show here that the hmtAlaRS is capable of editing mischarged tRNA^{Ala} *in vitro*. We further identify a severe editing domain mutation of mtAlaRS that prevents the post-transfer editing of mischarged Ser-tRNA^{Ala}, while another mutation displays a milder editing defect. Finally, we show that both of these editing mutants, mild and severe, result in early embryonic lethality in mice, demonstrating that the editing function of an aminoacyl-tRNA synthetase is essential in mammalian mitochondria.

Mitoribosomes are specialized machines for the synthesis of only 13 polypeptides that are essential subunits of the oxidative phosphorylation system. These proteins are highly hydrophobic, which requires them to be directly synthesized into the mitochondrial inner membrane. We have previously shown that misincorporation of an arginine analog, canavanine, during mitochondrial translation causes mitoribosome stalling when the nascent polypeptide containing the analog is unable to fold properly (32). The misfolding rapidly decreases the stability of the newly synthesized proteins that cannot be correctly inserted into membrane, leading to reduction in the amounts of fully assembled respiratory chain complexes (32). Similar severe outcomes could be expected when alanines in mitochondrial proteins are replaced by serines due to mischarging of tRNAs, thus leading to embryonic lethality in mice. However, it was unexpected that the editing defect causing moderate misincorporation and only a cerebellar phenotype in the cytoplasmic AlaRS mice (13), was also embryonic lethal in mtAlaRS mice. Based on this result, it could be speculated that mitochondrial protein synthesis is highly sensitive to serine misincorporation. Interestingly, both mtARSs that now have been demonstrated to have a functional editing domain, mtAlaRS (this study) and mtThrRS (22), edit serine. Recent study measuring metabolites and amino acid levels in the mitochondrial matrix of HeLa cells showed that while alanine levels were high within mitochondria, the serine levels were negligible (33), suggesting that low serine levels may be important to maintain to complement the editing activities of mtAlaRSs in preventing the toxic serine mistranslation. However, the serine concentration in rat liver was found to be greater in an earlier study (34) in which case active editing by mtAlaRS would be highly necessary, which is supported by our findings.

The phenotype of the *sti* mutant mice being restricted to the cerebellum and having a relatively slow progression as the mice were still alive at one year of age (13) suggested that the milder editing defect of the cytoplasmic synthetase

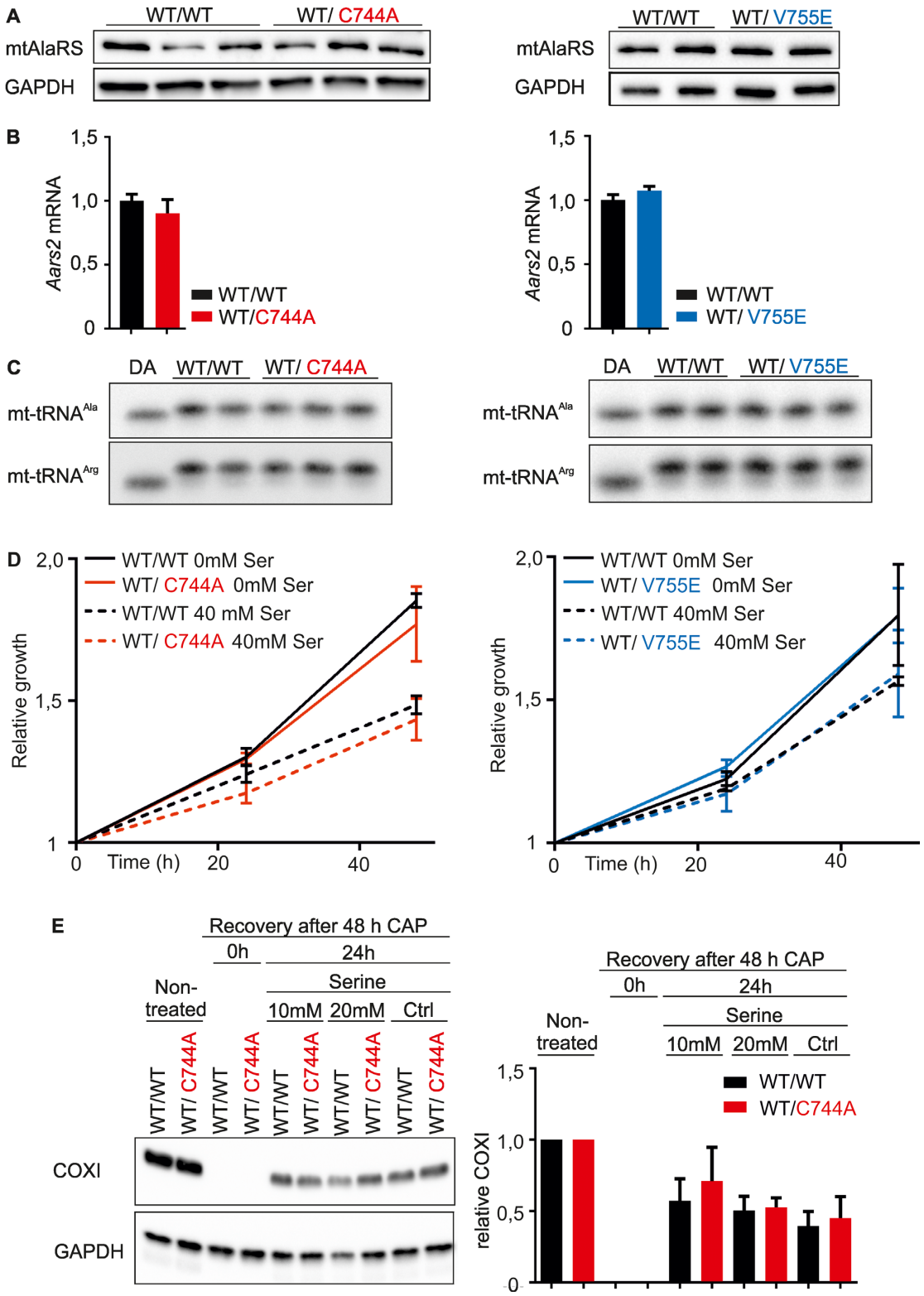

Figure 3. Wild type mtAlaRS compensates for the editing-mutant synthetase in heterozygous animals and cells. (**A**) Western blot analysis of the mtAlaRS protein levels in wild type (WT) and heterozygous C744A or V755E mouse embryonic fibroblasts (MEFs). GAPDH is shown as the loading control. (**B**) QPCR analysis of the *Aars2* mRNA levels in WT ($n = 3$) and heterozygous C744A or V755E ($n = 3$) kidney samples. (**C**) Mt-tRNA-Ala aminoacylation in WT and heterozygous C744A or V755E mouse skeletal muscle total RNA extracts. DA denotes a deacylated sample. (**D**) Proliferation of WT ($n = 2$) and heterozygous C744A or V755E ($n = 3$) MEF lines in normal media or in media with supplemented serine. (**E**) Representative Western blot for COXI and quantification of three individual blots of wild type and heterozygous C744A MEFs after 48 h of chloramphenicol (CAP) treatment, followed by a 24 h recovery in normal media or in media with increased concentration of serine (10 or 20 mM). CAP was used to deplete proteins synthesized by the mitoribosomes. In all graphs mean values with error bars indicating SEM are shown.

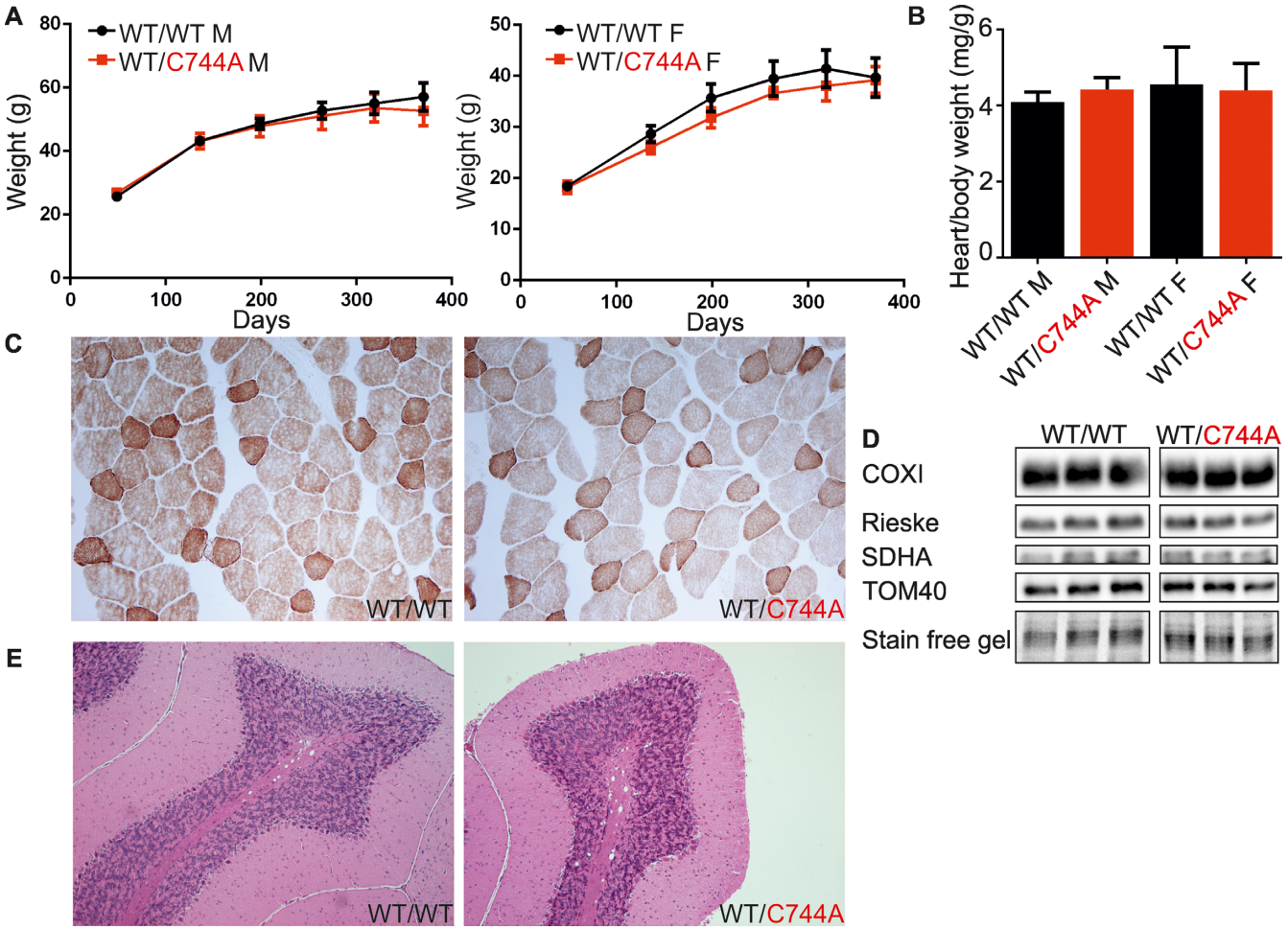

Figure 4. Normal development of the heterozygous C744A mice. (**A**) Weight gain of wild type (WT) and heterozygous C744A males (M) and females (F) (n = 5/group). (**B**) Heart/body weight ratio of the one-year-old mice (n = 5/group). (**C**) COX/SDH staining of the skeletal muscle of the one-year-old mice. (**D**) Western blot analysis of mitochondrial proteins in heart tissue protein extracts of the one-year-old mice. (**E**) Hematoxylin eosin staining of cerebellar sections of the one-year old mice. In the graph mean values with error bars indicating SEM are shown.

was tolerated in cell types other than the vulnerable Purkinje neurons. One possibility is that protective mechanisms that prevent serine misincorporation are present in other cell or tissue types but not in the cerebellar neurons. AlaXps, the free-standing editing domain homologs of AlaRSs, are found throughout evolution, and are thought to provide functional redundancy for the universal toxicity of mistranslating serine for alanine (14,35,36). Mammalian cells suppressed for AlaXp expression led to a serine-sensitive increase in the accumulation of misfolded proteins (37). The suppressive role of the mammalian AlaXp, which is found in the cytoplasm but not in mitochondria (37), may contribute to the difference between the phenotypes caused by the moderate editing defect in the two cellular compartments.

Numerous examples have shown that loss of any gene essential for mitochondrial gene expression results in lethality of mouse embryos around E8.5 (38). Of the mtARSs, this phenotype has been demonstrated for mtAspRS by *Dars2* knockout mouse (39). This suggests that the embryonal phenotype of the editing-deficient mtAlaRS mice does not differ from what is expected for a knockout of *Aars2*, completely lacking aminoacylation activity. Owing to the early death of the homozygous editing-mutant embryos, we were not able to address the stability of the mutant mtAlaRS proteins *in vivo* and their effect on aminoacylation. In heterozygous animals and MEFs the mtAlaRS protein levels were normal, without signs of compensatory increase, and the aminoacylation of $tRNA^{Ala}$ was comparable to wild type littermates, suggesting that the lethality of the homozygotes was not caused by loss of aminoacylation activity. Also the *in vitro* stability of the mutant proteins supports that the phenotypes were not due to protein instability. *In vitro*, the aminoacylation activity of the editing-mutant enzymes was slightly lower in comparison to the wild type synthetase. However, healthy parents of some of the patients with pathogenic *AARS2* mutations carry total loss of function alleles on one chromosome (23), indicating that even a 50% reduction in aminoacylation activity is sufficient for mitochondrial protein synthesis. Our results thus support that tRNA mischarging and the subsequent amino acid misincorporation was the cause of embryonal lethality in our mice. Unfortunately, the lack of viable homozygous

mice prevented more detailed studies of the consequences of amino acid misincorporation into mitochondrial proteins.

The *AARS2* gene encoding mtAlaRS has been associated with two disease phenotypes in human patients. Firstly, a recessive European founder mutation was found to cause early-onset lethal cardiomyopathy (23,24), and secondly, other recessive mutations were identified in patients with leukodystrophy and ovarian failure (40). The cardiomyopathy mutation locates to the editing domain of mtAlaRS, and was speculated to cause mistranslation. Intriguing, among the highly tissue-specific mtARS diseases, the mtAlaRS defect is the only one causing cardiomyopathy, which could suggest editing defect as a different mechanism from the other mitochondrial synthetase diseases. We have, however, previously shown that unlike the editing mutants modeled in mice in this study, the mutation causing cardiomyopathy is not located in the editing core of mtAlaRS. Rather, we predicted the mutation to have an effect on aminoacylation as part of the editing domain's contribution to tRNA binding (23,41). No human patient phenotypes have thus been described with mutations affecting the editing function of a mitochondrial synthetase. The results of this study suggest that such a mutation in recessive form would prevent embryonic development.

Our heterozygous mtAlaRS mice were completely normal, even for the duration of the one year follow-up of the severely editing-deficient C744A mice, and the heterozygous MEFs from those mice did not show a proliferation defect or a problem of synthesizing COXI even when stressed with increased concentrations of serine. The nearly normal phenotype of the heterozygous C723A AlaRS mice was suggested to indicate that the wild-type AlaRS was sufficient in *trans* to hydrolyze the Ser-tRNA^{Ala} generated by the mutant enzyme, and the trans-editing was indeed shown to occur *in vitro* (17). Based on the proposed ability of AlaRS to trans-edit mischarged tRNAs, our results suggest that trans-editing by the wild type synthetase is fully sufficient to prevent mistranslation in mitochondria of the heterozygous mtAlaRS mice.

Our study demonstrates that the editing function of mtAlaRS is essential for mouse development. Therefore, in mitochondria a high translation fidelity is required and other protein quality mechanisms are not able to overcome the damage caused by amino acid misincorporation. Thus, it is not clear why most mitochondrial synthetases do not have an editing function in place. One explanation for this could be that some mtARSs have achieved fidelity by incorporating a more specific synthetic active site that is not prone to errors (21,42). We suggest that mammalian mitochondria require a strict amino acid specificity, which is achieved by differently evolved properties of aminoacyl-tRNA synthetases and complemented by other protein quality control systems. Furthermore, the toxicity of serine misincorporation may be a particularly important problem in mitochondria.

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