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An active second dihydrofolate reductase enzyme is not a feature of rat and mouse, but they do have activity in their mitochondria

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ABSTRACT

The identification of a second functional dihydrofolate reductase enzyme in humans, DHFRL1, led us to consider whether this is also a feature of rodents. We demonstrate that dihydrofolate reductase activity is also a feature of the mitochondria in both rat and mouse but this is not due to a second enzyme. While our phylogenetic analysis revealed that RNA-mediated DHFR duplication events did occur across the mammal tree, the duplicates in brown rat and mouse are likely to be processed pseudogenes. Humans have evolved the need for two separate enzymes while laboratory rats and mice have just one.

1. Introduction

Dihydrofolate reductase (DHFR) is an enzyme from the folate one-carbon metabolism (OCM) pathway that has been extensively studied due to its relevance as a clinical drug target [1], in drug resistance [2,3] and in reducing the synthetic supplement folic acid and 7,8 dihydrofolate to the active form, tetrahydrofolate [4]. Despite its popularity, it has only relatively recently been realised that humans have acquired two dihydrofolate reductase enzymes during their evolution, DHFR and DHFRL1 [5,6]. This appears to fulfill the requirements of recycling dihydrofolate to tetrahydrofolate in support of *de novo* Thymidylate synthesis at the nuclear and mitochondrial genomes [6–8]. While it is clear that humans, and likely all primates, have two functional DHFR enzymes encoded by separate genes, the question remained whether non-primate species followed a similar pattern. This is especially relevant given the extensive utilisation of both rat and mouse models across a plethora of biomedical research over many years [9]. Indeed, many of the earlier research in relation to folate OCM was based on the isolation of specific folate enzyme activities from rat liver [10].

Given the central role of rodents in biomedical research and the importance of antifolate drugs as therapeutics for human disease, a full understanding of the commonalities and the more subtle differences in OCM between rodents and humans is vital to ensure the success of future/current therapeutics and nutritional supplements. Bailey and Ayling [11] previously highlighted the dramatic differences in the activity of endogenous human DHFR compared to its counterpart in the rat. DHFR activity in human liver showed wide variability between individuals and was "extremely slow" in reducing folic acid to tetrahydrofolate compared to rat liver [11].

Abbreviations: DHFR, dihydrofolate reductase; OCM, one carbon metabolism

Author Contributions: APMcD and MJOC designed the study. LH carried out all the mitochondrial purifications, enzyme assays, Western blots, RT-qPCR and Sanger sequencing. MJOC and RC designed and carried out the phylogenetic analyses. SM designed PCR assays and performed rat gene sequence analysis. GM generated a recombinant DNA clone. EED performed rat sequence analysis in wild species. APMcD performed sequence analysis of gene duplicates. All authors contributed to the drafting of this manuscript.

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This has particular relevance given the widespread use of folic acid as both a supplement and as part of mandatory (and voluntary) fortification programs to prevent neural tube defects [12]. The detection of unmetabolised folic acid in the human circulation [13,14] and the poor handling of folic acid by the human gut [15], underscores the importance of understanding DHFR activity across species and shedding light on the underlying reason(s) for such differences.

We have used a combination of phylogenetic analysis, sequence analysis, RT-qPCR, enzyme activity assays and Western blotting in liver tissue and/or cell lines of mouse and rat to address whether these species have DHFR activity in their mitochondria and whether such activity is attributable to a second functional DHFR enzyme, mirroring what we and others have previously identified in humans [5,6]. We show that while both rat and mouse do have DHFR activity in their mitochondria, we rule out that activity being due to a second DHFR enzyme in mouse and in the brown rat species *Rattus norvegicus*. We show that brown rat and mouse DHFR, not only localises to the cytoplasm, as previously known, but also localises to the mitochondria. While humans have evolved the need for two functional DHFR enzymes, mice and brown rats have just one. This data demonstrates a fundamental difference between humans and our favoured animal models and should be considered in future research in this area.

2. Materials and methods

2.1. Mitochondria extraction and purification

Four T75 flasks of NRK (normal rat kidney) and 4T1 (mouse mammary tumour) cell lines were grown in DMEM (Sigma) supplemented with 2 mM L-glutamine and FBS (Fetal Bovine Serum) (10% NRK, 5% 4T1) until 80% confluent. Cells were trypsinised and the resulting pellets washed in PBS (Phosphate Buffered Saline). The pellets from four flasks were combined and the mitochondria extracted using the Qproteome® Mitochondria Isolation kit (Qiagen) as per kit protocol with the following adaptations: pellets were lysed using 1 ml of lysis buffer. Disruption was completed using 500 µl of disruption buffer, a 1 ml syringe and a 27-gauge needle. The final mitochondrial pellet was resuspended in 100 µl of mitochondrial storage buffer. Extraction from rat and mouse tissues were also carried out with Qproteome® Mitochondria Isolation kit (Qiagen) similar to the cell line extractions except 240 mg of fresh tissue was minced with a scalpel and placed in 1 ml of lysis buffer and homogenised using the Ultra-turrax homogenizer, then a further 500 µl of lysis buffer was added, all remaining steps were as per Qiagen protocol and final mitochondrial pellet was resuspended in 200 µl of mitochondrial storage buffer.

2.2. DHFR enzyme activity assays

Endogenous enzyme activity was analysed using a Dihydrofolate Assay Kit (Sigma cat. No. CS03040-1KT). The assay was performed at room temperature and at pH 7.5 according to the manufacturer's instructions. Each reaction contained 100 µg of total mitochondrial protein combined with kit reagents as per the manufacturer's protocol up to a final volume of 500 µl in a 1 ml UVette. The absorbance was read every 15 s for 3.5 min at 340 nm using the reaction rate mode on the Biochrom Libra S12 UV/Vis spectrophotometer. Each assay was repeated with the addition of 40 nM Methotrexate to inhibit reductase activity thereby allowing deduction of background activity of reactions that also consume NADPH. The delta OD of each reaction was calculated from the linear portion of the graph.

2.3. Western blot analysis

Following denaturation of samples at 95 °C in loading buffer, a volume of 18 µl of total protein from the mitochondrial extraction (both cytoplasmic and mitochondrial fractions) were loaded onto 4–20% Precise Tris–glycine gels (Thermo Scientific) and run at 100 V in Tris–glycine SDS running buffer. Proteins were transferred onto PVDF membrane using the Pierce G2 Fast Blotter System (Thermo Scientific) and blocked in 5% non-fat milk for 2 h. Blots were probed with 1/5000 Anti-DHFR antibody produced in rabbit (Abcam ab124814) and 1/1000 Anti-GAPDH and Anti-PDH antibody produced in mouse (Sigma G8795) and incubated overnight at 4 °C with gentle rocking. Blots were washed in TBST (Tris Buffered Saline with Tween®) and probed with appropriate secondary antibodies at 1/50,000 dilution. Blots were imaged using Super Signal West Femto Max Sensitivity substrate (Thermo Scientific 34095) and the Syngene bioimaging system.

2.4. Phylogenetic analysis of DHFRLS in other species

The query sequences for our whole genome searches were extracted as canonical transcripts from the Ensembl database [16]: human DHFR (ENST00000439211), mouse DHFR (ENSMUST00000022218), rat DHFR (ENSRNOT00000018259), human DHFRL1 (ENST00000394221), putative rat DHFRLS (chromosome 4: 414,175–414,721), human DHFRP2 (ENSG00000228432). Using these sequences as queries we searched both the Ensembl database [16], Genbank (www.ncbi.nlm.nih.gov/genbank/) and the NCBI EST database (www.ncbi.nlm.nih.gov/dbEST/). The Chinese Hamster genome was also used for closer examination of the rodent clade [17]. The phylogenetic tree used in the study is well resolved [18,19]. The completed genomes used ranged in quality from 1.85X coverage to very high coverage (>6X). These genomes were selected based on their phylogenetic distribution, as we sought to examine the presence or absence of gene duplicates from early vertebrate evolution right through to the divergence of rodents and primates. To aid in the classification of a homologue as either the DHFR gene or a duplicate (pseudogene or other), we examined the syntenic location of all genes, putative duplicates and pseudogenes for all species in our dataset. Our dataset consisted of vertebrate genomes from Ensembl Genome Browser release 64 and the Chinese hamster genome [16,17]. A gene was deemed a putative duplicate if it had a sequence similarity score of 80% or higher in comparison to the DHFR transcript, an *E*-value $<10^{-30}$ and a sequence length >300 bp. The majority of subject sequences found had an *E*-value lower than 10^{-150}. To differentiate between putative duplicates we examined the syntenic information for each region. Duplicate genes that satisfied the sequence similarity criteria and that had conserved synteny across species were deemed homologous. Any putative duplicate sequence that met the strict similarity criteria outlined above, and following synteny analysis could be confirmed as unique from all previously identified DHFR pseudogenes, was classified as a DHFR-like sequence, or DHFRLS.

2.5. Sequence analysis of gene duplicates

The region containing mouse DHFRLS was retrieved from Ensembl (http://www.ensembl.org/index.html) GRCm38, chromosome 9: 31567433:31568480. Rat DHFRLS on chromosome 4 was retrieved from Ensembl Rat genome versions RGSC Rnor_5.0 (DHFRLS ORF 4: 3139757–3140178) and RGSC 3.4 (DHFRLS is within the following region, 4: 413244–415704). ORF Finder was used to search for open reading frames in all 6 frames in both mouse and rat DHFRLS. Sequence alignments were performed at http://www.ebi.ac.uk/Tools/msa/clustalw2/. A specific DNA

sequence stretch was confirmed by Sanger sequencing (Source Bioscience) of PCR products amplified using the following primers: Forward 5′ GAAAAATCAACCTTTAAAGGATAG 3′; Reverse 5′ AATAAATTTTAAAACATCATCCAGAC 3′ and template genomic DNA extracted from the livers of two different Sprague Dawley rats using the DNeasy Blood and Tissue kit (Qiagen).

2.6. Reverse transcribed-quantitative PCR

RNA was extracted from normal rat kidney (NRK) cells using the Isolate II RNA mini kit from Bioline as per the kit protocol and DNAse treated using the AMPD1 kit from Sigma–Aldrich. Samples were reverse transcribed to cDNA in two steps, 1 μg of DNAse treated RNA was added to 2 μl of random hexamers primer mix (50 ng/μl Bioline) and 4 μl Oligo (dT) 18 primer mix (50 μM Bioline) heated at 70 °C for 5 min then incubated on ice for 1 min. A master mix containing 4 μl of 5× Reaction Buffer (Bioline), 1 μl Ribonuclease Inhibitor human (Sigma–Aldrich), 1 μl Bioscript Reverse Transcriptase (200 U/μl Bioline), 1 μl 10 mM dNTPs (Sigma–Aldrich) and 1.5 μl Nuclease Free H_2O per sample, was added to each sample and they were placed on the thermocycler using the following protocol 25 °C × 10 min, 42 °C × 60 min, 70 °C × 15 min then held at 4 °C. Gene specific assays for DHFR and Ywhaz assays were designed using the Universal Probe Library (UPL) from Roche and the DHFRLS assay was hand designed to ensure primers would not amplify DHFR and the probe was designed using guidelines from IDT. Each RT-qPCR reaction contained 1 μl cDNA, 0.16 μl UPL probe, 0.2 μm forward and reverse primer, 8 μl of Sensimix™ II Probe Kit (Bioline) and was made up to a final volume of 15 μl with nuclease free water. All assays and analysis were performed on the Roche Lightcycler® 480 using the following conditions: 5 min pre-incubation at 95 °C, amplification 45 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 s at 72 °C. Primers and probes used are as follows: DHFR primers: Forward 5′ AAAGTGGACATGGTCTGGGTA 3′; Reverse 5′ CTGGCTGATTCATGGCTTC 3′; Universal Probe #16. Ywhaz Primers: Forward 5′ CTACCGCTACTTGGCTGAGG 3′; Reverse 5′ TGTGACTGGTCCACAATTCC 3′; Universal Probe #9. DHFRLS RT-qPCR primers are described in Supp. Fig. 1.

2.7. Rat sequence analysis in wild species

The region containing the frameshifted DHFRLS on RGSC Rnor_5.0 Chromosome 4: 3139757–3140178 was compared to the equivalent region in 12 wild brown rats (*R. norvegicus*) and 1 wild black rat (*Rattus rattus*) as follows: high depth Illumina sequence reads from 12 *R. norvegicus* individuals sampled from N.W. China [20] and a single *R. rattus* individual were aligned to the *R. norvegicus* reference genome version Rnor_5.0. The relevant region (chr4: 3139757–3140178) was extracted from raw bam files using (the Genome Analysis Toolkit (GATK) [21] unified genotyper with the following options – output_mode EMIT_ALL_SITES and genotype_likelihoods_model BOTH to retain maximum information. Throughout the region, the coverage of the *R. rattus* sample was close to the modal coverage for the genome as a whole (33X). The *R. rattus* sequence was called homozygous at all sites and all substitutions were supported by all or all but one read. The *R. rattus* contained a 2 bp deletion of bases 3,139,975-6, which was supported by 34 of 35 reads. The twelve *R. norvegicus* samples all had coverage close to their respective sample modes of 19–46X. All samples were called homozygous reference at all sites, with the sole exception of sample R10 (ERS215798), which was heterozygous for a C → T transition at position 3,139,847 (12 reads C, 9 reads T), part of a CpG dinucleotide.

3. Results

3.1. Rat and mouse possess dihydrofolate reductase activity in their mitochondria

Purified mitochondria from rat and mouse tissues (liver and/or kidney) or a mouse cell line were assessed for dihydrofolate reductase activity in light of what had been previously observed in human [5,6]. Cytoplasmic and mitochondrial fractions were purified from a rat NRK and mouse 4T1 cell line respectively. The same fractions were also purified from rat liver and kidney tissue and mouse liver tissue. A high level of reductase enzyme activity was detected in the cytoplasmic fraction as expected. We also observed reductase activity in the purified mitochondria in both rat and mouse (Table 1). It was essential that we demonstrated that our mitochondrial fractions were not contaminated with cytoplasm as the cytoplasm contains a very high level of Dhfr enzyme normally and mitochondrial extracts, can be frequently contaminated with cytoplasm if not executed carefully. Confirmation that the mitochondrial fractions were free from contamination with the cytoplasm was shown by Western blot (Fig. 1). This analysis demonstrated that dihydrofolate reductase activity is also a feature of the mitochondria in both rat and mouse.

3.2. Examination of DHFR gene duplication events across the vertebrate tree

The possibility that non-human species also possess a second intact Dihydrofolate Reductase-Like sequence (DHFRLS) was explored by a phylogenetic analysis. This analysis demonstrated that a number of DNA mediated and RNA mediated duplication events occurred across the vertebrate tree. Representatives from all major clades of mammals, as well as outgroup species from

Table 1
DHFR activity in rat and mouse mitochondria.

Tissue/cell line[a]	Cytoplasm SA[b] (nmol/min/mg)	Mitochondria SA[b] (nmol/min/mg)
Rat liver	7.15	6.04
Rat kidney	7.4	9.37
Mouse liver	n.d[c]	5.0
Mouse cancer line	5.39	44.0

[a] All extracts were tested for purity of mitochondria by Western blot with both rat liver and kidney shown in Fig. 4(B) and mouse cancer line in Fig. 1. Mitochondrial purity of mouse liver was also demonstrated by Western blot (data not shown).
[b] SA = specific activity per mg of mitochondrial preparation weight.
[c] n.d. = Not determined.

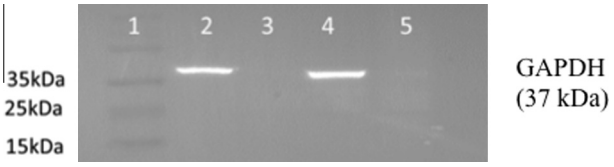


Fig. 1. Purity of mitochondrial extracts in Rat and Mouse. A representative Western blot of rat and mouse mitochondrial extracts probed with the cytoplasmic marker (GAPDH) showing purity of the mitochondrial extraction protocol. Lane order: 1: SDS PAGE ruler, 2: rat cell line cytoplasm (21.4 μg protein loaded), 3: rat cell line mitochondria (32.4 μg protein loaded), 4: mouse cancer line cytoplasm (33.1 μg protein loaded), 5: mouse cancer line mitochondria (42.1 μg protein loaded). Every mitochondrial preparation used in this study (including enzyme activity measurements) was confirmed for purity by Western blot.

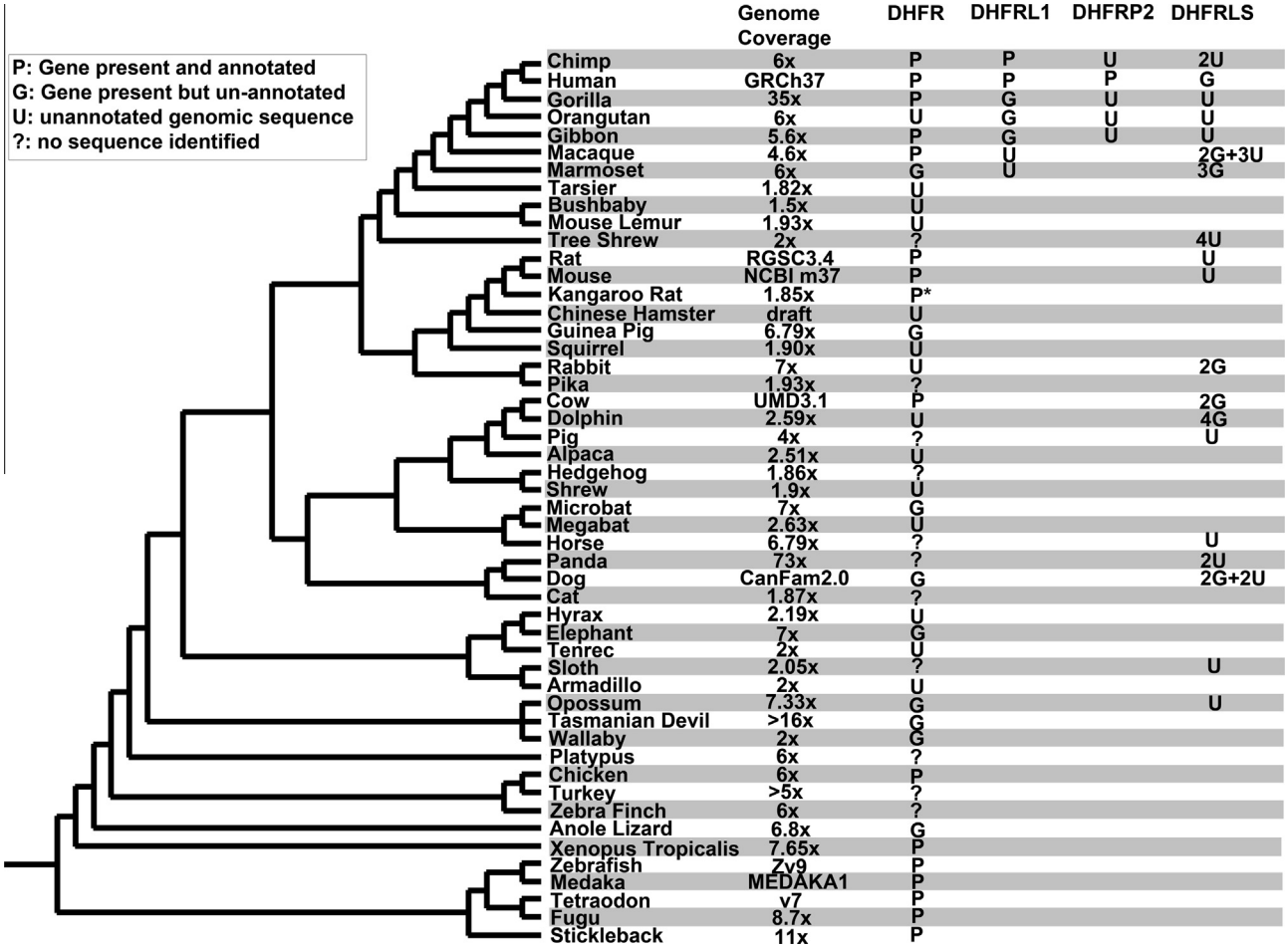
the *Aves* and *Teleostii* clades, were examined for the presence of DHFRLS. Fig. 2 shows all species tested in this analysis and their phylogenetic relationships as currently understood and summarises the distribution of DHFRLS identified [18,19]. It is clear that there is a wide phylogenetic distribution of duplicates of the DHFR gene across the mammalia, with the exception of the afrotheria (represented by Hyrax, Elephant and Tenrec) and monotremata (represented by Platypus) (although this may be due to sequence quality for these genomes rather than genuine absence from these genomes). All of the DHFRLSs identified are intronless and are most likely the result of RNA mediated duplication, i.e., retrotransposition events. We found no evidence of DHFR retrotransposition outside of the mammal clade. We also determined if there was evidence for DNA mediated duplication in the *Aves* and *Teleostii*. We tested for the presence of DHFR paralogs using the coding DNA sequence (CDS) of the native copy of DHFR for each species of *Aves* and *Teleostii* and by searching each exon individually. The only significant result for both the DHFR CDS search and the exon search was the DHFR gene itself – indicating that there are no traceable functional duplicates of DHFR outside of the mammals. Although the phylogenetic analysis identified a DHFRLS in a number of mammal species, further sequence analysis was required to assess whether they contained an intact open reading frame (ORF) and were capable of encoding a full length dihydrofolate reductase-like enzyme.

3.3. Sequence analysis identifies a putative functional DHFRLS in rat but not in mouse

We focused our attention on the mouse and rat genomes given their widespread utilisation as animal models in research. The DHFRLS identified on chromosome 9 of the Ensembl Mouse genome (chromosome 9: 31,375,437–31,375,758) was assessed for ORF content using ORF finder. Despite sharing approximately 80% sequence identity with the CDS of its parent DHFR, this sequence does not encode an intact dihydrofolate reductase ORF. Our first analysis of the Ensembl Rat genome was performed on the most updated version available, the Rat RGSC3.4 assembly. It identified a putative un-annotated DHFRLS on chromosome 4 (chromosome 4: 414,206–414629). The predicted ORF of the putative DHFRLS was a shortened version of its equivalent parental DHFR but contained all the domains required for reductase activity as defined by *PRINTS* (www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/). We next sought to test whether this putative rat DHFRLS was actually expressed.

3.4. RT-qPCR analysis shows no evidence of expression of the DHFRLS in rat

An RT-qPCR assay was carefully designed to ensure specific amplification of the rat DHFRLS and not its parental homologue



	Genome Coverage	DHFR	DHFRL1	DHFRP2	DHFRLS
Chimp	6x	P	P	U	2U
Human	GRCh37	P	P	P	G
Gorilla	35x	P	G	U	U
Orangutan	6x	U	G	U	U
Gibbon	5.6x	P	G	U	U
Macaque	4.6x	P	U		2G+3U
Marmoset	6x	G	U		3G
Tarsier	1.82x	U			
Bushbaby	1.5x	U			
Mouse Lemur	1.93x	U			
Tree Shrew	2x	?			4U
Rat	RGSC3.4	P			U
Mouse	NCBI m37	P			U
Kangaroo Rat	1.85x	P*			
Chinese Hamster	draft	U			
Guinea Pig	6.79x	G			
Squirrel	1.90x	U			
Rabbit	7x	U			2G
Pika	1.93x	?			
Cow	UMD3.1	P			2G
Dolphin	2.59x	U			4G
Pig	4x	?			U
Alpaca	2.51x	U			
Hedgehog	1.86x	?			
Shrew	1.9x	U			
Microbat	7x	G			
Megabat	2.63x	U			
Horse	6.79x	?			U
Panda	73x	?			2U
Dog	CanFam2.0	G			2G+2U
Cat	1.87x	?			
Hyrax	2.19x	U			
Elephant	7x	G			
Tenrec	2x	U			
Sloth	2.05x	?			U
Armadillo	2x	U			
Opossum	7.33x	G			U
Tasmanian Devil	>16x	G			
Wallaby	2x	G			
Platypus	6x	?			
Chicken	6x	P			
Turkey	>5x	?			
Zebra Finch	6x	?			
Anole Lizard	6.8x	G			
Xenopus Tropicalis	7.65x	P			
Zebrafish	Zv9	P			
Medaka	MEDAKA1	P			
Tetraodon	v7	P			
Fugu	8.7x	P			
Stickleback	11x	P			

Fig. 2. Phylogenetic distribution of the DHFR genes/pseudogenes across the *vertebrata*. The phylogeny of the taxa sampled in this study is shown on the left and on the right is a table representing the genome coverage and presence of DHFR and DHFRLSs. DHFR: dihydrofolate reductase, DHFRP1: dihydrofolate reductase pseudogene 1, DHFRP2: dihydrofolate reductase pseudogene 2, DHFRL1: dihydrofolate reductase like 1, DHFRLS: dihydrofolate reductase like sequence. The sequence similarity search results are classified as: "P" gene present and annotated; "G" gene present but unannotated; "U" un-annotated genomic sequence; or "?" no sequence identified. In the DHFRLS column these classifications are preceded by a number that depicts the number of copies in that category, the absence of a numeric value indicates a single copy in that category.

(Supp. Fig. 1). Steps were also taken to ensure that any amplified RT-PCR product arose from reverse transcribed mRNA and not from contaminating genomic DNA. This was particularly crucial given the intronless nature of the putative DHFRLS. Our RT-qPCR data of the putative DHFRLS showed no evidence of expression in RNA isolated from rat liver from two separate rats, and the NRK cell line (Fig. 3). We ruled out technical reasons contributing to this result such as inefficient cDNA synthesis or PCR efficiency. Integrity of our cDNA was demonstrated by the detection of the endogenous control gene *Ywhaz* [22] and parental *Dhfr* gene expression by RT-qPCR. The efficiency of our DHFRLS PCR assay was demonstrated using a serial dilution of a recombinant clone of the putative sequence (Supp. Fig. 2). This data indicated that although version RGSC3.4 of the rat genome contained a putative rat DHFRLS, we could find no evidence that it was an expressed gene.

3.5. The source of mitochondrial DHFR activity is parental DHFR in rat and mouse

The lack of an expressed and functional DHFRLS in either laboratory brown rat or mouse indicated that the source of the DHFR activity in the mitochondria in both species was in fact the 'original' DHFR itself (mouse-chromosome 13; rat-chromosome 2). We performed Western blot analysis of purified mitochondria from rat and mouse cell lines (Fig. 4) and demonstrate a clear 21kDa band with a DHFR specific antibody. Cytoplasmic fractions from the same cells were included for comparison and as a positive control. Again the purity of the mitochondrial (and cytoplasmic) fractions was verified by PDH (Pyruvate Dehydrogenase, mitochondrial marker) and GAPDH (cytoplasmic marker). This analysis demonstrates for the first time that endogenous rat and mouse DHFR localises to the mitochondria in addition to the cytoplasm.

3.6. Sequence analysis of the updated rat genome identifies a frameshift in the rat DHFRLS

While our original sequence analysis of the putative rat DHFRLS was based on the sequence retrieved from Rat genome version RGSC3.4, we subsequently retrieved the equivalent region from the updated version Rnor_5.0 released in 2013. An alignment of putative DHFRLS from both versions of the rat genome showed that version Rnor_5.0 had a single base deletion that essentially knocks the putative DHFRLS out of frame (Supp. Fig. 3). We Sanger sequenced a PCR product amplified from the region that contained the single base deletion in DNA isolated from rat liver of laboratory rat strain Sprague Dawley (Fig. 5). This confirmed that laboratory rat strains harbour a frameshift mutation that abolishes the ORF of the putative DHFRLS. As described earlier, we also found no evidence of expression of this putative DHFRLS in lab rat cell lines or liver tissue. Unlike the mouse DHFRLS, which possessed a range of base changes to extensively degrade the reductase ORF, the lab rat DHFRLS appears to have acquired a single base deletion to cause a non-sense frameshift. We thus, hypothesised that the single base deletion may potentially be a newly acquired mutational event and even be possibly a polymorphism within the rat population or potentially unique to laboratory rat strains.

3.7. DHFRLS in wild black rat has an intact open reading frame due to a 2 bp deletion

Laboratory rat strains are *R. norvegicus* species and are more commonly known as the brown rat or the Brown Norway rat [23]. *R. norvegicus* was used to sequence the rat genome [23]. Their genus cousin is the black rat known as *R. rattus* [24]. To test the hypothesis that the non-sense frameshift mutation in the laboratory rat genome is polymorphic and/or unique to lab brown rats

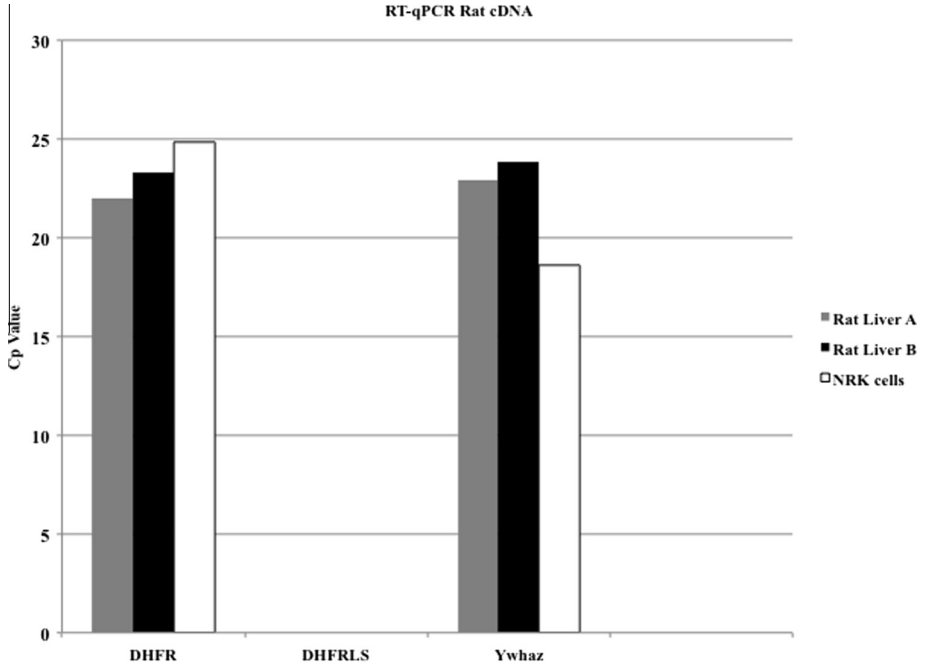


Fig. 3. RT-qPCR shows no evidence of expression of a rat DHFRLS. RT-qPCR was carried out on mRNA isolated from two separate rat liver RNA extractions (rat liver A and B) and RNA extracted from a rat cell line (NRK). The specific genes analysed are described in the *X*-axis, while the Cp value (which inversely correlates with mRNA expression level i.e., the lower the Cp the higher the level of expression) is shown on the *Y*-axis. No Cp value was detected for DHFRLS even at high cycle numbers i.e., 45. Technical reasons for non-detection of expression of the DHFRLS were ruled out due to the successful (and relatively early) amplification of 'parental' DHFR and the rat control gene Ywhaz.

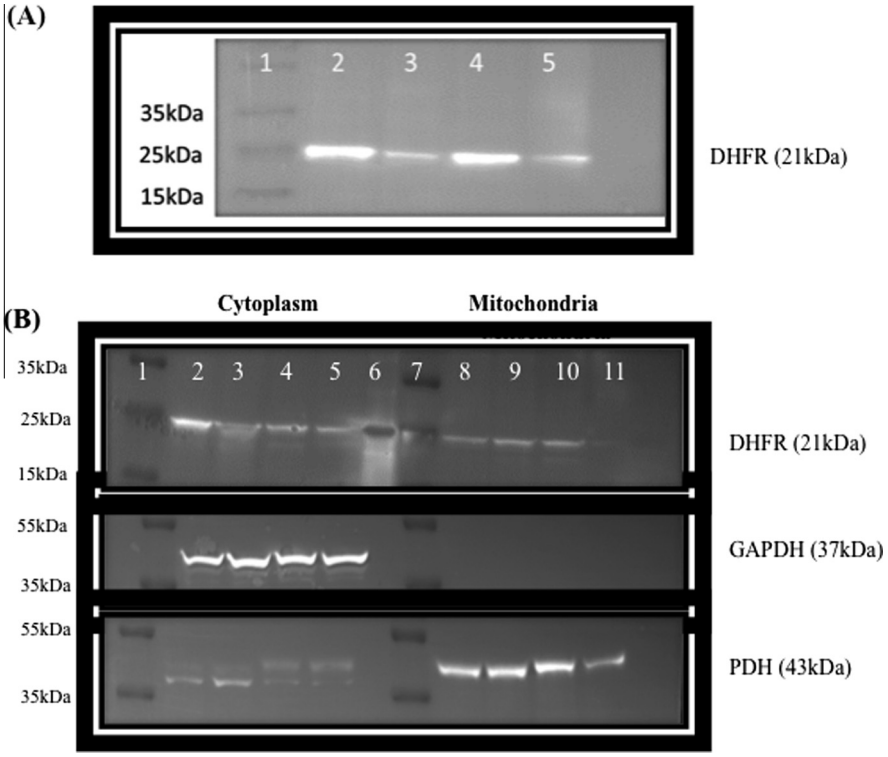


Fig. 4. Detection of endogenous mitochondrial DHFR in mouse and rat by Western blot analysis. (A) Western blot of mitochondrial and cytoplasmic protein extracts showing expression of endogenous reductase in rat (NRK) and mouse (4T1) cell lines probed with Anti-DHFR antibody: Lane order: 1: SDS PAGE ruler, 2: rat cell line cytoplasmic, 3: rat cell line mitochondrial, 4: mouse cell line cytoplasmic, 5: mouse cell line mitochondrial after probing with DHFR antibody. The mitochondrial extracts were previously shown to be free from contamination with cytoplasm as shown in Fig. 1. (B) Western blot of mitochondrial and cytoplasmic protein extracts showing DHFR enzyme present in both cytoplasmic and mitochondrial extracts of rat liver and kidney tissue. The top panel shows the samples probed with Anti-DHFR antibody. The blot was stripped twice and reprobed with Anti-GAPDH (middle panel) and Anti-PDH antibody (bottom panel). Mitochondria extracts are pure with no GAPDH detected, only PDH. The cytoplasm fractions show some leakage from the mitochondria during extraction with faint bands of PDH but this is not a concern for this experiment (the lower bands are reminants of the GAPDH signal as this was a stripped blot). Lane order: 1: SDS PAGE ruler, 2: rat 1 liver cytoplasm (29 μg protein loaded), 3: rat 2 liver cytoplasm (26.8 μg protein loaded), 4: rat 1 kidney cytoplasm (15.5 μg protein loaded), 5: rat 2 kidney cytoplasm (19.4 μg protein loaded); 6: DHFR recombinant protein (Sigma), 7: SDS PAGE ruler, 8: rat 1 liver mitochondria (25.7 μg protein loaded), 9: rat 2 liver mitochondria (21.4 μg protein loaded), 10: rat 1 kidney mitochondria (10.3 μg protein loaded), 11: rat 2 kidney mitochondria (6.8 μg protein loaded).

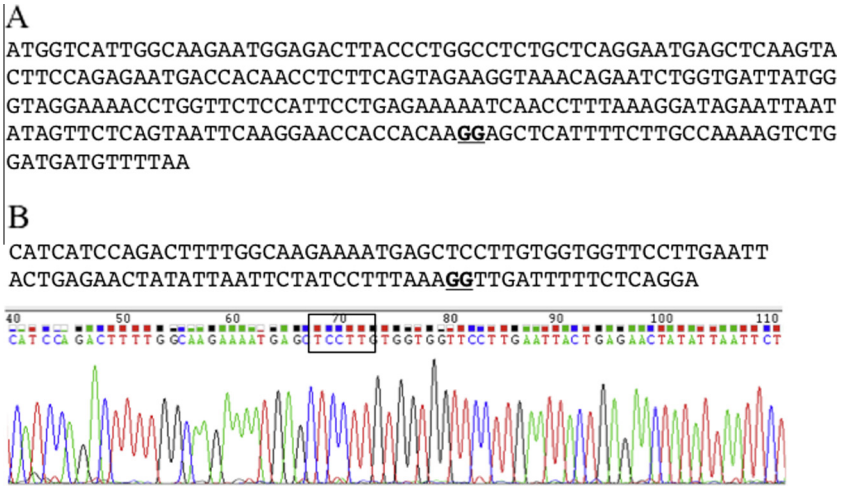


Fig. 5. Sanger sequencing confirms version RGSC Rnor_5.0 of the rat genome has the correct version of the rat DHFRLS sequence on chromosome 4. (A) Sequence of putative rat DHFRLS in version RGSC Rnor_5.0 of the rat genome. The underlined and bold 'GG' sequence highlights the site of discrepancy with RGSC 3.4 version i.e., 'GGG'. This results in a premature stop codon resulting in a shortened amino acid sequence of just 82 amino acids (see Supp. Fig. 3). (B) Sanger sequencing of PCR product amplified from genomic DNA of a Sprague Dawley rat confirms only two 'G' bases occur (RGSC Rnor_5.0) and not three (RGSC 3.4) as shown in the boxed portion of the chromatogram (sequencing with the reverse primer is shown).

we compared the rat DHFRLS on chromosome 4 (RGSC Rnor_5.0) to a sequenced wild rat population consisting of 12 brown rats (*R. norvegicus*) and 1 black rat (*R. rattus*). This analysis showed that the wild brown rat samples were similar to their lab brown rat counterparts in that the DHFRLS also contains a non-sense reading frame. This confirms that both lab and wild brown rats do not

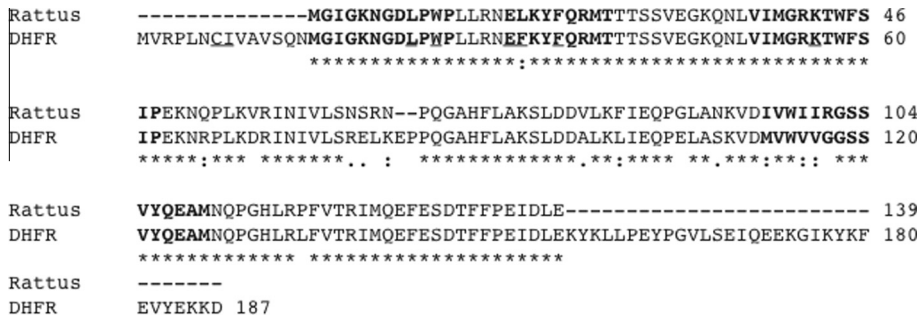


Fig. 6. Amino acid sequence alignment of the putative wild black rat DHFRLS with brown rat DHFR. The wild black (*R. rattus*) rat putative DHFRLS is labelled as 'Rattus' and the brown rat (*R. norvegicus*) functional (or 'parental' NP_569084) DHFR is labelled as 'DHFR'. Although the black rat DHFRLS is a shorter protein than the known functional rat DHFR, it does contain all four domains that have previously been identified as being necessary for dihydrofolate reductase activity (highlighted in bold). It is unclear whether this black rat DHFRLS is actually expressed.

contain a second active dihydrofolate reductase like sequence. Interestingly, analysis of the single wild black rat indicates that the equivalent DHFRLS in this genome contains a homozygous 2 bp deletion when compared to DHFRLS in brown rat. Analysis of the possible ORF in this sequence region shows that wild black rat contains an intact ORF. The ORF encodes a DHFRLS that is shorter than functional rat DHFR sequence but does contain all the domains required for dihydrofolate reductase activity (Fig. 6). It is unclear whether this putative wild black rat DHFRLS is actually expressed and produces an mRNA that is subsequently translated. This needs to be verified experimentally through the isolation of wild black rat RNA. While we cannot confirm if wild black rat DHFRLS is expressed, we can confirm that it does possess the coding capacity to produce a second active dihydrofolate reductase enzyme.

4. Discussion

We have demonstrated that dihydrofolate reductase activity is also a feature of the mitochondria in both rat and mouse (Table 1, Figs. 1 and 4), but this is not due to a second reductase enzyme. While our phylogenetic analysis revealed that RNA-mediated DHFR duplication events did occur across the mammalian tree (Fig. 2), it appears that the duplicates in brown rat and mouse are likely to be processed pseudogenes as they lack the coding capacity for an intact reductase ORF and we found no evidence of mRNA expression in lab rat tissue or in a rat cell line (Fig. 3). While, the mouse DHFRLS had accumulated numerous mutations to negate any possibility of a reductase ORF, the rat DHFRLS tantalisingly had lost its reductase like ORF to a single base shift. We showed that while both lab and wild brown rat lacked an intact ORF, wild black rat retained a reductase like ORF that has the potential to encode a second reductase enzyme (Fig. 6). If expressed and functional, the wild black rat mirrors the human DHFR scenario i.e., it also has two functional dihydrofolate reductase enzymes (albeit arising from separate gene duplication events) and may provide clues as to the relevance of this for human folate metabolism. The *Rattus* species is thought to have arisen in South-East Asia with further divergence giving rise to 66 recognised species including *R. rattus* and *R. norvegicus* [24]. While *R. rattus* was believed to have arrived in Europe first, *R. norvegicus* outcompeted *R. rattus* in the more temperature climates of Europe following their arrival during the 18th century [20]. If *R. rattus* did have a second functional reductase, it could potentially allow an interesting investigation of diet and habitat differences between the two species and how this relates to folate metabolism. However, confirmation of expression in isolated *R. rattus* RNA samples will be required to assess whether this is a possible line of investigation.

Human DHFR is known to localise to the cytoplasm and nucleus [7,8] with its paralogue DHFRL1 localising to the mitochondria [5,6]. While mitochondrial reductase activity has been detected in CHO cells [6], rat and mouse have not been proven previously. Interestingly, a publication from 1965 provided early evidence that rat mitochondria did harbour DHFR activity [25], however further investigation during this period failed to substantiate this finding [26]. Our enzyme activity and Western blot data (Table 1, Figs. 1 and 4) clearly demonstrate that endogenous mouse and brown rat DHFR co-localise to the cytoplasm and mitochondria. In contrast to humans, a single gene satisfies the dihydrofolate reductase requirements in both brown rat and mouse. A question that remains to be answered is why humans (and possible wild black rats) retained their DHFR-like retrogene (DHFRL1) for specific localisation to the mitochondria while other mammalian species did not. The answer may lie in the reduced enzyme activity of DHFRL1 [5] and its role in the mitochondria may extend beyond that of a simple enzyme. Alternatively, the 'on-site' supply of Thymidylate appears to be a feature of both the nuclear and mitochondrial genome and having the ability to differentially control thymidylate production at either genome may be the underlying selective advantage to having two rather than one DHFR enzyme. Another advantage to having a second reductase in the mitochondria might relate to its significant oxidative stress environment and the vulnerability of specific folate forms to oxidation. DHFRL1 may be better suited to the alkaline environment of the mitochondria and may slow the non-enzymatic oxidative loss of folate.

Our detailed analyses of the DHFR gene family in mammals highlights once again our need for caution in relation to the utilisation of animal models to extrapolate to the human situation. Although high profile mishaps in translating from animal models to clinical trials are rare [27], it does emphasise that attention to the detail of the differences between animal models and humans is warranted. Mouse and rat models are the cornerstone of a significant portion of in vivo research and will continue to remain so in the foreseeable future. Apart from their use in the study of folate metabolism, they are a central part of novel drug development on the road to clinical trials [28]. DHFR itself has long been a drug target [1] without any knowledge of the existence of DHFRL1 or indeed that a dihydrofolate reductase activity was a feature of mitochondria. Details such as these are crucial, particularly as it is unlikely that anti-folate drugs transport efficiently into mitochondria as oxidised forms of folate appear to transport in slowly [29], although of note, this was based on an investigation of rat mitochondria. Our data provides clarity on one of the fundamental differences between folate metabolism in humans and that of our favoured animal models. A straight forward mouse or rat model of DHFRL1 is not a possibility in light of our analyses and further investigation of the human DHFRL1 enzyme will now be restricted

to human (or potentially non-human primate) derived tissues and/or cell lines. This data will inform future studies where animal model data is being used to extrapolate to human studies and demonstrates that closer attention needs to be paid to the intricate differences between humans and rodents.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.05.017.

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