SUPPLEMENTAL INFORMATION

Restriction of an intron size en route to endothermy

### SUPPLEMENTAL TABLES

#### Table S1  Synthetic nucleic acids

| Oligo(ribo)nucleotide | 5'-3' sequence$^{\text{1,2}}$ |
|------------------------|-------------------------------|
| **OGDH splicing reporter constructs** | |
| Human-F (*Homo sapiens*) | ATTACTCGAGTTTGTCTTGTTGGCATGG |
| Human-R | CAGGTTACAGACAGATGAC |
| Opossum-F (*Monodelphis domestica*) | ATTACTCGAGAAGCCTCTGGGTAAAGGT |
| Opossum-R | ATTATCTAGAGGCTAGTCCAAC |
| Platypus-F (*Ornithorhynchus anatinus*) | ATTACTCGAGTGGTACAGTCTAGGCTTGTC |
| Platypus-R | ATTATCTAGAGGCTAGTCCAAC |
| Chicken-F-large (*Gallus gallus*) | ACCACTCGAGAACCAGCAGAGATTTG |
| Chicken-F-small | ACCACTCGAGAACCAGCAGAGATTTG |
| Chicken/quail-R | ACCACTCGAGAACCAGCAGAGATTTG |
| Quail-F | ATTACTCGAGGCTCCAGACTCCCTTGCTA |
| Python-F (*Python molurus bivittatus*) | ATCACCTCGAGACGGCATGGAGGACGAGATGCC |
| Python-R | ATCACCTCGAGACGGCATGGAGGACGAGATGCC |
| Frog-F (*Xenopus laevis*) | ATTACTCGAGAATTTGTTTGGTAACTTGCC |
| Frog-R | ATTACTCGAGAATTTGTTTGGTAACTTGCC |
| Zebrafish-F (*Danio rerio*) | ATTACTCGAGGCTCCAGACTCCCTTGCTA |
| Zebrafish-R | ATTACTCGAGGCTCCAGACTCCCTTGCTA |
| Cod-F (*Gadus morhua*) | ATTACTCGAGTCAAATGAATTTATAGCGTTTTGG |
| Cod-F | ATTACTCGAGTCAAATGAATTTATAGCGTTTTGG |
| Sunfish-F1-large (*Mola mola*) | ACCACTCGAGAACCAGCCTCCGAAACCTTA |
| Sunfish-F2-small | ACCACTCGAGAACCAGCCTCCGAAACCTTA |
| Sunfish-R | ACCACTCGAGAACCAGCCTCCGAAACCTTA |
| Human OGDH-e5+-F (with exon 5) | ATCAGCTAGACACCTGGAGATGCAAGAAAA |
| Human OGDH-e5+-R (with exon 5) | ATCAGCTAGACACCTGGAGATGCAAGAAAA |
| **Cloning/subcloning of expression plasmids** | |
| PTB4-Bam | ATTAGGATCCGGCAATGAGGCACTTGTTGATCC |
| PTB4-Xho | ATTACTCGAGAATTTATAGCGTTTTGG |
| TIA-1-Bam | ATTAGGATCCGGCAATGAGGCACTTGTTGATCC |
| TIA-1-Xho | ATTACTCGAGAATTTATAGCGTTTTGG |
| TIAR-Bam | ATTAGGATCCGGCAATGAGGCACTTGTTGATCC |
| TIAR-Xho | ATTACTCGAGAATTTATAGCGTTTTGG |
| **TIA-1 RRM2 mutagenesis** | |
| F98A | CATTTCCCTTGCGTCTTTGCGTCTTC |
| F140A | GAGGAAATGCGCTTTT |
| **TIA-1 Q-domain mutagenesis** | |
| N357S | GGATGGGACCAAGTTATGGAGTGC |
| E384K | TGCCGAGGATATAAACCTTCAGGC |
| **Mapping of *H. sapiens* dbPs** | |
| R1-HS (*U2AF1*) | TCGATCTGTCCTCAGATTTT |
| R2-HS (*U2AF1*) | CACAAAAATGGAAATTCACACTGAGA |
| F1-HS (*OGDH*) | CACAGCTACTGGATCTGG |
| F2-HS (*OGDH*) | AGTGCAGGTCACCACAT |
| **Mapping of *G. gallus* dbPs** | |
| F1-GG | TCTGTATTGTTACTCCAGAG |
| F2-GG | ATTGAGAACCTGCTGCTTTC |
| R3-GG | TCCCTGGGATGATGTTGTA |
Mapping of *X. laevis* dBPs

| Primers     | Sequences                        |
|-------------|----------------------------------|
| F1-XL       | CGCGCTGGAATTAGTGCTGG             |
| F2-XL       | TCAATTCGAAAGCCTGACC              |

Mapping of *M. mola* exon 4b BP

| Primers     | Sequences                        |
|-------------|----------------------------------|
| F1-MM       | TTGATCTCTGGAAATGTT               |
| F2-MM       | TTGATCTTCCGCTGGTGA               |
| R2-MM       | ACAAAATTTCCGCTGTCGAG             |

**DBR1 depletion**

*DBR1 siRNA-1* [GCAUGCAAGGUGGGAUUA]TT

*DBR1 siRNA-2* [GCAUGCAGGCAAGGUAUAA]TT

**Depletion of TIA proteins**

*TIA-1 siRNA* [AACCAUGGAAUCACACAGAU]TT

*TIAR siRNA* [GCAUGGAAUCACACAGAU]TT

**BP mutagenesis**

| Primers     | Sequences                        |
|-------------|----------------------------------|
| OG-BP+25    | GCTGTAATAATGCTGGTTTAATGTAAT      |
| OG-BP+31    | AAGCTTAATTTGGTGTAATTTTACT        |
| OG-BP+36    | CTAATTTTAAATGTTTGTTACTTTTTT     |
| OG-BP+36+36 | AATGCTAATTTGGTGTTACTTTTTT       |
| OG-BP+25+31 | AATGCTGGTTTTGGTGTAATTTTACT      |
| OG-ins-BP28F| TGTGTGTTCTACTAACTCCCCCATCGTT    |
| OG-ins-BP28R| GAGGGGAGGTTATAGAAGCACAACACAAAAAGC |
| OG-ins-BP41F| CTTCGTCCACTACTAGTTTGGTGTTTTTCT  |
| OG-ins-BP41R| AAAACAGTTTAGGTAGACAGAAGGAAAGAAAAAGA |
| OG-ins-TA41 | TTTTTTCTTCTCGCTAATTTGTTGIGTCC   |
| OG-ins-TGA28 | TTGTGTGTTGTTGCTACTTCCCTTCICACTC |
| OG-ins-TGA41 | TTTTTTCTTCTCGCTGATTTGTTGIGTCC   |
| XL-mBP(TAA>TGG) | AGACTTGTTGTGGCGTTCTCTTTTC |

**RT-PCR primers for exogenous expression**

| Primers     | Sequences                        |
|-------------|----------------------------------|
| 35E1+PL4    | CAGGTGCTCTCGGTTGCA               |
| 35m-amplF   | GCTCGAGTCTACAGAGTCA              |
| PL4         | AGTCGAGTCTACAGAGTCA              |

**Restriction site insertions for MIR cloning**

*PstI+15* AGAATTAAGCTGCAGTTAAATGCTAATT

*EcoRV+65F* CAGTCGATATC(A/G)CAG(C/T)A(C/T)AG(C/T)ATAGTGGTTAAGAGCAC

**Amplification of the MIR library**

*MIR-F-PstI* CAGTCTCGCAGA(GCAGC/T)AC(T/G)ATAGTGTGTTAAAGCAC

*MIR-R-PstI* CAGTCGCTGCGGTTAAAAGCAGCAGAC

*MIR-F-EcoRV* CAGTCGATATC(A/G)CAG(C/T)A(C/T)AG(C/T)ATAGTGTGTTAAAGCAC

*MIR-R-EcoRV* CAGTCGATATC(A/G)CAG(C/T)A(C/T)AG(C/T)ATAGTGTGTTAAAGCAC

**Mapping of MIR BPs**

| Primers     | Sequences                        |
|-------------|----------------------------------|
| BP-MIR15-R/R1 | TACATATAATGCTAATGCTAATT      |
| BP-MIR15-F1  | ATGTAGGTCTCTAGCTAATGCTAATT    |
| BP-MIR15-F2  | GCATTGCGCATGAATGGCTTCT     |
| BP-MIR15-R2  | AATTAGCATTTAGCCTAATGCTAATT    |

**MegaPPT deletions**

| Primers     | Sequences                        |
|-------------|----------------------------------|
| OG-del1F    | TGTTAATTTCATTGTTTTTTTCTTCCTGTCCCT |
| OG-del1R    | GAAAAAAAGGATTAATACATTAAAAATTACATAGCAT |
| OG-del2F    | TTACCCCTCCCTTTGGTGTTGCTCCTCACCTCCT |
| OG-del2R    | CACACACAAAGGGAGGAGGATGAAAAAAAGAGG |
| OG-del3F    | CTTCGAGCGGTTCCCTCCCGTCCCTCCCTGAGCC |
| OG-del3R    | TAGAAGGGAGGAGGACAGAAGGAAAGAAAAAGGAGG |
| OG-del4F    | TTTTGGTGTTGTTGCTGAGCCACATAGATAGCGAG |
| OG-del4R    | TAGGTGGGGCAACACACACACAAAAGGGAGCAG |

**Exon variants**

| Primers     | Sequences                        |
|-------------|----------------------------------|
| INS-TCC(HS>XL) | TACGTTTCTCCTCCAAACCTGCGGTTGAGAAATTT |
| 4a-5T(HS>F)  | ACTGTTTCTCCTAAATGGGTTGAGAAATTT |
| 4a-2T (5’S)  | GTTTCTTCAAACGGGTTGAGAAATTTGAGAG |
| 4a-2C (5’S)  | GTTTCTTCAAACGGGTTGAGAAATTTGAGAG |
| 4b+3T        | GAGGGCCATAAGT(T/G)GACAGGCTGAGGCCC |
| 4b+3SI       | GAGGGCCATAAGT(T/G)GACAGGCTGAGGCCC |
| 4b+3SI+31    | GACAGGCTGAGGCAAA(T/G)GACAGGCTGAGGCCC |
4b+78T TCCGTCGCGCTGATATTATCTCATCCACA
4b+91T ACATTAATCTACCTTCAGCAGAAGCTTG
H>XL (insGGA) CTCCAGTAACGTGTCCTCATAACCTGCGG
H>F (insGGC) CTCCAGTAACGTGTCCTCATAACCTGCGG
XL>H (delGGA) CTCCAGTAACTGTTGGATCTTCAAACGTGGT
RT-PCR with endogenous OGDH transcripts

Human-F (Homo sapiens) GCACAGTCCCTGGTAGAAGC
Human-R GCAGAGGAAGTGCTGATTCC
Rat-F (Rattus norwegicus) AGCCTAACGTCGACAAGCTC
Rat-R GGTTGGTGGGTAGTGGAAGA
Opossum-F (Monodelphis domestica) AGTGGCACCCCTCCTACACT
Opossum-R AAGTGGAGGCGAGATTTCTT
Echidna-F (Tachyglossus aculeatus) CGCGT1AGCGCTTACAAAG
Echidna-R CCGTCCCTCAAATGAAAGT
Platypus-F (Ornithorhinchus anatinus) CCGGTTACACGCTTACAAAG
Platypus-R CCGTCCCTCAAATGAAAGT
Quail-F (Coturnix japonica) GGGCACATCTTTCGAC
Quail-R GTGTTGGGCAAATGGAAGAC
Frog-F (Xenopus laevis) GCAGTCTGTCCACCCTTACC
Frog-R (target shared by Ogdh and Ogdhl) AGTGGTCGGGAGATGGAAGA
Zebrafish-F (Danio rerio) GTGGAAGACCATCTGGCAGT
Zebrafish-R GGCAAGCGGAACACTTTATC
Ogdhl-Xenopus laevis-F GCAGTCTGTCCACCCTTTCG
Lengthening of M. mola AGEZ

molaA>G CGGTGAGAATTACTCTG
molaA>T GTGTTGATGTTGTGCTTTCTTTCTGT
molaA>G GTGTTGATGTTGGGCTTTCTTTCTGT
DADLD mutagenesis

HADLD CTGGGGATTTTCAGCACTGTAGTGACT
DAHLD GGAACGGACCGAAGCCGGGATCGA
DADLD GGAACGGACCGAAGCCGGGATCGA
Templates for RNA structural probing

T7F-Human TAATACGACCTCACTTAAGGGAGAAGGGTGAGAATTAAAGCTG
R-Human GAAACGGACCGAAGCCGGGATCGA
T7F-Chicken TAATACGACCTCACTTAAGGGAGAAGGGTGAGAATTAAAGCTG
R-Chicken GAAACGGACCGAAGCCGGGATCGA
T7F-link-Human TAATACGACCTCACTTAAGGGAGAAGGGTGAGAATTAAAGCTG
R-link-Human GAAACGGACCGAAGCCGGGATCGA
T7F-link-Chicken TAATACGACCTCACTTAAGGGAGAAGGGTGAGAATTAAAGCTG
R-link-Chicken GAAACGGACCGAAGCCGGGATCGA
Cy5-labelled universal primer GAAACGGACCGAAGCCGGGATCGA

1 Degenerate primer positions are in parentheses. 2 T7 promoter is highlighted in grey; linker sequences are underlined.

Table S2 The number of BP-like URA motifs in consensus sequences of mammalian interspersed repeats (MIRs)

| MIR subfamily | Sense orientation | Antisense orientation |
|---------------|-------------------|----------------------|
| MIR (260)     | 13 (5.0)          | 13 (5.0)             |
| MIRc (268)    | 12 (4.6)          | 11 (4.2)             |
| MIR3 (224)    | 10 (3.8)          | 5 (1.9)              |

1 Consensus sequences were retrieved from RepBase (1); their length (in nts) is in parentheses. 2 Densities (%) of URA motifs are in parentheses.
| MIR clone | Restr. site | PCR product | Insert size (nt) | MIR orientation | Sequence of transcribed MIRs (5'-3') |
|-----------|------------|--------------|------------------|-----------------|-------------------------------------|
| 1         | PsiI       | T            | 193              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 2         | PsiI       | F            | 209              | sense           | UUGUGCUCUUAACCACUAUGCUAUGCUGC       |
|           |            |              |                  |                 | AUAACCCUGUGAAGUAGAAACUAUGUAAGC       |
| 3         | PsiI       | D            | 209              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 4         | PsiI       | N            | 209              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 5         | PsiI       | R            | 209              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 6         | PsiI       | O            | 209              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 7         | PsiI       | P            | 193              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 8         | PsiI       | E            | 193              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 9         | PsiI       | A            | 193              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 1         | EcoRV      | 4_E          | 262              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 2         | EcoRV      | 3_X          | 209              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 3         | EcoRV      | 3_F          | 209              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 4         | EcoRV      | 4_I          | 249              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 5         | EcoRV      | 3_K          | 392              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 6         | EcoRV      | 3_A          | 193              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 7         | EcoRV      | 3_J          | 193              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 8         | EcoRV      | 3_D          | 193              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 9         | EcoRV      | H            | 193              | sense           | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 10        | EcoRV      | 4_B          | 241              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 11        | EcoRV      | 4_C          | 241              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 12        | EcoRV      | 3_G          | 193              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 13        | EcoRV      | 4_G          | 193              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 14        | EcoRV      | 1            | 193              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 15        | EcoRV      | 4_D          | 211              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |
| 16        | EcoRV      | 3_C          | 209              | antisense       | UUGUGCUCUUAACCACUAUACUAUACUGC       |
|           |            |              |                  |                 | AAGAGGCUCAUAUAUGUAUGCUAUACGCCG      |

1 Restriction sites are highlighted in yellow. URA motifs unique to clone 15 are underlined (UGA-181, UAA-178, UAA-165, UAA-135, UGA-100 and UGA-60; each motif is followed by the distance in nts between putative BP-A and the 3'ss). BPs determined in MIR15 transcripts (Figure 4D-F) are in red.
Table S4  Relaxation of OGDH intron 4a splice sites in vertebrates had a minimal impact on exon 4a/4b ratios

| Species       | 5'ss of intron 4a  | Max. entropy score  | Exon 4b inclusion | 3'ss of intron 4a  | Max. entropy score  | Exon 4b inclusion |
|---------------|--------------------|---------------------|-------------------|--------------------|---------------------|-------------------|
| Placentals    | TGGgtgaga          | 6.04                | -                 | tcctgtggecactcatagATA | 3.36                | -                 |
| Marsupials    | TGGgtgaga          | 6.29                | No change         | atgtggccacctcatagAT | 5.11                | No change         |
| Birds and reptiles | TGGgtgaga      | 8.51                | Potentially slightly lower | actctgtggecactcatagAT | 6.54                | No change         |

1Intron sequences are in lower case, exon sequences are in upper case. 2Maximum entropy scores (2) were computed for human, wallaby and duck splice sites. Tested mutations are in red. Splicing assays are shown in Figure 5.

Table S5 Amino acid identity between zebrafish and human TIA-1 and PUF60 RRMs

|                     | RRM1     | RRM2     | RRM3     |
|---------------------|----------|----------|----------|
| Human PUF60 versus zebrafish PUF60 | 78/79 (97%) | 74/79 (94%) | 79/88 (90%) |
| Human TIA-1 versus zebrafish TIA-1 | 67/77 (87%) | 69/79 (87%) | 67/73 (92%) |

1RRM3 is known as the U2AF homology motif or UHM (3).

Table S6  Function of human genes containing MXEs separated by dBP/megaPPT introns

| Gene symbol | Length of the intron between MXEs (nts) | SVM dBP score | 5'ss to dBP (nts) | Longest PPT U-run 3' of dBP (nts) | Gene product | Function | Human loss-of-function phenotype | Key references |
|-------------|-----------------------------------------|---------------|------------------|----------------------------------|--------------|----------|----------------------------------|---------------|
| OGDH        | 122                                     | 1.92          | 25               | 9                                | E1 subunit of the 2-oxoglutarate dehydrogenase complex | Ca\(^{2+}\)-mediated NADH supply; distinct sensitivity of MXE isoforms to mitochondrial Ca\(^{2+}\) | No reliable reports of human mutations; lethal in lower organisms | (4)           |
| KCNMA1      | 103                                     | -             | -                | 15                               | K\(^{+}\) Ca\(^{2+}\)-activated channel, subfamily M, α1 | Dampens excitatory events that elevate Ca\(^{2+}\), and/or depolarize the cell membrane; distinct gating characteristics of MXE isoforms; Ca\(^{2+}\) sensitivity of splice variants | Dyskinesia, seizures (OMIM # 609446 and 617643) | (5, 6, 7) |
| SNAP25      | 161                                     | 1.74          | 23               | 13                               | Synaptosome associated protein 25 | Ca\(^{2+}\)-triggered exocytosis and regulation of neurotransmitter release; distinct induction of primed vesicle pool by MXE isoforms | Myasthenia (OMIM # 616330) | (8, 9) |
| ACAD10      | 212                                     | 1.59          | 23               | 20                               | Acyl-CoA dehydrogenase, 10 | Mitochondrial fatty acid metabolism | Not reported\(^{2}\) | (10) |

1MegaPPT is defined here by the presence of 8 or more consecutive uridines adjacent to dBPs. This length permits cooperative binding of PUF60 and U2AF65 (11) and is close to a limit for in vitro binding of PUF60 (11 pyrimidines) (12). PPTs with longer uridine stretches generally promote the efficiency of 3'ss recognition (13,14); to achieve the same level of splicing, shorter PPT tracts require higher uridine content (15). 2Acad10-deficient mice accumulate excess abdominal adipose tissue, exhibit fasting rhabdomyolysis and have abnormal skeletal muscle mitochondria (16). OMIM #, Online Mendelian Inheritance in Man database ID.
Table S7  MXE responses to PUF60/U2AF65 knockdown in transcripts involved in Ca\(^{2+}\) signalling

| Human gene symbol | Putative dBPs | PPT in the intron that separates MXEs | ~RPKM\(^{4}\) in HEK293 cells | Effect of PUF60/U2AF65 depletion |
|-------------------|--------------|--------------------------------------|---------------------------|---------------------------------|
| ACTN2             | +            | long, partitioned into UC- and UG-rich segments; UG repeat close to 3'ss | not expressed             | -                               |
| CACNA1A           | +            | very long, GU in repeat close to 3'ss, (A) repeat further upstream | not expressed             | -                               |
| CACNA1B           | +            | very long, (C)n repeat close to 3'ss | not expressed             | -                               |
| CACNA1C           | -            | short (A)n repeat not expressed       | -                          |                                 |
| CALU              | +?           | short and lacks uridines, (U)n is the longest uridine repeat | 1000                      | no change                       |
| DNM2              | +            | long, partitioned into repeated UC- and UG-rich segments | 500                       | exon b down in PUF60- cells and up in U2AF65- cells |
| FYH               | -            | mid-size, (A)n repeat                 | 500                       | exon b up in PUF60- cells, down in U2AF65- cells |
| GNAI              | -            | NA, ATI                               | 100                       | first exons sensitive to U2AF65 depletion |
| GNAS              | -            | NA, ATI                               | 10000                     | skipping of a downstream exon in U2AF65- cells |
| GRB2             | -            | short                                 | not expressed             | -                               |
| GRIA              | -            | short                                 | not expressed             | -                               |
| GRIIA             | -            | short                                 | not expressed             | -                               |
| GRIIB             | -            | short                                 | not expressed             | -                               |
| DMDH             | -            | NA, ATI                               | 500                       | no change                       |
| MASP1             | -            | NA, APA                               | not expressed             | -                               |
| GRIH              | +            | long, partitioned into UC-, UG- and UC-rich segments | 200                       | exon b down in PUF60- cells and up in U2AF65- cells |
| OTOP              | -            | short, MXEs are terminal              | not expressed             | -                               |
| PRKCB            | -            | NA, ATI                               | 80                        | no change                       |
| PRKG1             | -            | NA, ATI                               | not expressed             | -                               |
| SCN5A             | +            | long, partitioned into multiple UC- and UG-rich regions | 15                         | not informative, exon b was not used |
| SLC32A1          | +            | mid-size                              | not expressed             | -                               |
| SLC32A2         | -            | short                                 | 3000                      | not informative, exon a was not used |
| SLC2A24         | -            | NA, ATI                               | 200                       | no change                       |
| SLC3A1         | -            | mid-size, (U)nG repeats               | 50                        | not informative, exon a was not used |
| SLC3A3         | +            | mid-size, UC-rich                     | not expressed             | -                               |
| SNAP25         | +            | long, partitioned into (U)nG-rich and GA-rich segments | not expressed | -                               |
| TPM2        | +            | long, partitioned into UC- and C-rich segments | 25                       | exon b down in PUF60- and up in U2AF65- cells |
| TPM3        | +            | long and partitioned                  | 2000                      | not informative, exon b was not used |

1 Alternative transcription initiation (ATI). 2 Alternative polyadenylation (APA). 3 Three MXEs; dBPs were predicted both for the intron between exon a (5' exon) and b (0.53 kb) and between exon b and c (0.51 kb). 4 RPKM (reads per kilobase of transcript per million mapped reads) data are rounded; full RNA-seq data are available under the ArrayExpress accession number E-MTAB-6010 (17). NA, not applicable.

Table S8  PUF60-regulated exon usage in transcripts that encode mitochondrial proteins

(Microsoft Excel table)
SUPPLEMENTAL FIGURES

Figure S1  Alignment of mutually exclusive OGDH exons 4a and 4b and intron 4a in 62 vertebrate species

Intronic sequences are in lower case; exons 4a and 4b are in upper case. dBPs mapped in this study (Figure 3 and 6) are highlighted in green. A tentative dBP+45 is in blue. Conserved adenine residues of amniote dBPs are highlighted in yellow. Variants that change their extended BP consensus motifs are in red. Exon variants examined for splicing (Figure 5) are underlined. The AGEZ spoiler in M. mola is in magenta. Asterisks denote identical nucleotides. Alignment was carried out with reference sequences (www.ensembl.org) and Clustal Omega (v. 1.2.4.) using exons 4a, introns 4a, exons 4b and 50-nt flanking intronic sequences.

human                           gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
baboon                          gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
angola_colobus                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
bola_lizard                    gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
bank_vole                      gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
kangaroo_vole                  gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
zebra_mbuna                    gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
tilapia                        gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
xenopus_trop.                  gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
anole_lizard                   gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
turtle_painted                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
turtle_softshell               gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
rahende                        gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
quail                          gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
turkey                         gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
chicken                        gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
duck                           gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
wa                           gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
Tasm.devil                     gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
armadillo                      gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
opossum                        gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
wallaby                        gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
platypus                       gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
duck                           gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
zebrafinch                     gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
chicken                        gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
turkey                         gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
quail                          gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
alligator                      gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
desert_tortoise                gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
turtle_softshell               gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
turtle_painted                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
tuatara_lizard                gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
anele_lizard                   gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
python_Burmese                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
xenopus_trop.                  gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
xenopus_laevissi              gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
ocean_sunfish                  gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
tilapia                       gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
zebra_mmbna                    gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
skinkleback                    gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
climbing_perch                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
ballian_wrasse                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
shortfin_salamander           gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
zigzag_eel                     gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
tetradon                      gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
tiger                         gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
tongue_soloe                   gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
amazon_molly                   gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
shortfin_molly                 gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
bicolor_Ramsel_elf              gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
zebrafinch                    gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
spotted_gar                    gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
channel_catfish                gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG
cod                           gttgtatgttcacGTGAGGGGTACACACATTTGCAAAACTTGAGTCCTCTGGGAATCAG

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www.ensembl.org  Kralovicova et al.  (Figure 3 and 6) and Clustal Omega (v. 1.2.4.) using exons 4a, introns 4a, exons 4b and 50-nt flanking intronic sequences.
human
bonobo
baboon
angola_colobus
bolivian_squirrel_monkey
bushbaby
bamboo_lesmur
lemur
polar_bear
panda
horse
sloth
rabbit
hyrax
treeshrew
hedgehog
miroma
megabat
mouse
kangaroo_rat
blind_mole_rat
Damaraland_mole-rat
naked_mole_rat
guinea_pig
armadillo
opossum
wallaby
platypus
duck
zebrafinch
chicken
turkey
quail
alligator
desert_tortoise
turtle_softshell
turtle_painted
tuatara_lizard
anole_lizard
python_Burmese
xenopus_laeviss
ocean_sunnfish
tilapia
zebra_mbuna
stickleback
climbing_perch
ballan_wrasse
swamp_eel
zigzag_eel
tetradon
tigertail_seahorse
fugu
tongue sole
amazon_molly
shortfin_molly
bicolor_damselfish
zebrafish
spotted_gar
channel_catfish
cod
| Species                          | Sequence                                      |
|---------------------------------|-----------------------------------------------|
| human                           | tgcctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
| Animal                  | Sequence                                                                 |
|------------------------|---------------------------------------------------------------------------|
| human                  | tttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
| Animal                  | DNA Sequence                  |
|-------------------------|-------------------------------|
| human                   | `tttggt`                      |
| baboon                  | `tttggt`                      |
| angola colobus          | `tttggt`                      |
| bolivian squirrel monkey| `tttggt`                      |
| bushbaby                | `tttggt`                      |
| bamboo lemur            | `tttggt`                      |
| lemur                   | `tttggt`                      |
| polar bear              | `tttggt`                      |
| panda                   | `tttggt`                      |
| horse                   | `tttggt`                      |
| sloth                   | `tttggt`                      |
| rabbit                  | `tttggt`                      |
| hyrax                   | `tttggt`                      |
| treeshrew               | `tttggt`                      |
| hedgehog                | `tttggt`                      |
| microbat                | `tttggt`                      |
| megalab                | `tttggt`                      |
| mouse                   | `tttggt`                      |
| kangaroo rat            | `tttggt`                      |
| blind mole rat          | `tttggt`                      |
| Damalarand mole-rat     | `tttggt`                      |
| naked mole rat          | `tttggt`                      |
| guinea pig              | `tttggt`                      |
| armadillo               | `tttggt`                      |
| Tasm.devil              | `tttggt`                      |
| opossum                 | `tttggt`                      |
| wallaby                 | `tttggt`                      |
| platypus                | `tttggt`                      |
| zebraphin               | `tttggt`                      |
| chicken                 | `tttggt`                      |
| turkey                  | `tttggt`                      |
| quail                   | `tttggt`                      |
| alligator               | `tttggt`                      |
| desert tortoise         | `tttggt`                      |
| turtle softshell        | `tttggt`                      |
| turtle painted          | `tttggt`                      |
| tuatara lizard          | `tttggt`                      |
| anole lizard            | `tttggt`                      |
| python Burmese          | `tttggt`                      |
| xenopus trop.           | `tttggt`                      |
| xenopus laevis          | `tttggt`                      |
| ocean sunfish           | `tttggt`                      |
| tilapia                 | `tttggt`                      |
| zebra mbuna             | `tttggt`                      |
| stickleback             | `tttggt`                      |
| climbing perch           | `tttggt`                      |
| ballian wrasse          | `tttggt`                      |
| swamp eel               | `tttggt`                      |
| zigzag eel              | `tttggt`                      |
| tetraodon               | `tttggt`                      |
| tiger tail seahorse     | `tttggt`                      |
| fugu                    | `tttggt`                      |
| tongue sole             | `tttggt`                      |
| amazon molly            | `tttggt`                      |
| shortfin molly          | `tttggt`                      |
| bicolor damselfish      | `tttggt`                      |
| zebraphin               | `tttggt`                      |
| spotted gar             | `tttggt`                      |
| channel catfish         | `tttggt`                      |
| cod                     | `tttggt`                      |
human
baboon
angola_colobus
bolivian_squirrel_monkey
bushbaby
bamboo_lemur
lemur
polar_bear
horse
sloth
rabbit
hyrax
treeshrew
hedgehog
microbat
megabat
mouse
kangaroo_rat
blind_mole_rat
Damalarand_mole-rat
naked_mole_rat
guinea_pig
armadillo
Tasm.devil
opossum
wallaby
platypus
duck
zebrafinch
chicken
turkey
quail
alligator
desert_tortoise
turtle_softshell
turtle_painted
tuatara_lizard
anoine_lizard
python_Burmese
xenopus_trop.
xenopus_laevius
ocean_sunfish
tilapia
tilapia
nile tilapia
zebra_mbuna
stickleback
climbing_perch
kangaroo_rat
wallaroo
swamp_eel
zigzag_eel
tetraodon
tigerpleus_seahorse
fugu
tongue_sole
amazon_molly
shortfin_molly
bicolor_damselfish
zebrafish
spotted_gar
channel_catfish
cod
human
bonobo
baboon
angola_colobus
bolivian_squirrel_monkey
bushbaby
bamboo_lemur
lemur
polar_bear
daedon
fugu
tigertail_seahorse
treeshrew
hedgehog
microbat
megabat
mouse
kangaroo_rat
blind_mole_rat
Damalaland_mole_rat
naked_mole_rat
guinea_pig
armadillo
Tasm.devil
opossum
wallaby
platypus
duck
zebrafinch
chicken
turkey
quail
alligator
desert_tortoise
turtle_softshell
turtle_painted
tuatara_lizard
anole_lizard
python_Burmese
xenopus_trop.
xenopus_laevis
ocean_sunfish
tilapia
tigerbullseye
stickleback
climbing_perch
ballian_wrasse
swamp_eel
tetraodon
tigertail_seahorse
fugu
tonguesole
shortfin_molly
bicolor_damselfish
zebra_fish
spotted_gar
channel_catfish
cod
Figure S2  Example of exon skipping in HEK293 cells overexpressing U2AF65

10+, 10-, RNA products with and without HGD exon 10. HGD minigene assay was described previously (18). EV, empty vector.
Figure S3  Usage of individual OGDH dBPs and MXE regulatory proteins

A, Alignment of 26 informative sequences across lariat junctions in control cells. Intron 4a reference sequence shown in at the top, dBP adenines are in green. Mutations around lariat junctions introduced by RT or PCR are highlighted in yellow. The 5’ss GT dinucleotide is in red. B, DBR1 knockdown does not alter exon 4a/4b ratios. C, Immunoblots (upper panels) from HEK293 cells depleted of DBR1 and TIA proteins or overexpressing PUF60. Lower panels show PCR products containing lariat junctions (red rectangles). D, Usage of individual human dBPs in HEK293 cells lacking or overexpressing OGDH MXE regulators. Clones are sorted by dBP; their numbers are summarized in Table 1. E, Chicken dBP usage in HEK293 cells lacking or overexpressing OGDH MXE regulators. Clone numbers are shown in Table 2. F, Mapping of OGDH exon 4a BP. BP is denoted by a circle. The 5′ end of the reporter intron is shown as a black rectangle. Location of exon 4a BP corresponds to a pile up of SF3B4 eCLIP reads (Figure 2B).
D

Control cells (human transcripts)

| Clone | gagaattaagctgtaaatgcta | BP+25 | BP+31 | BP+36 | BP+41 |
|-------|-------------------------|-------|-------|-------|-------|
| H2_EV_N | GAGAATTAAGCTGTAAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con4 | AGAATTAAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con2 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con20 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con9 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con22 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_R | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_D | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_C | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_B | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_E | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_P | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_T | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con30 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-4 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-6 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con13 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con36 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con29 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_S | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con7 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con14 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con27 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con31 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con8 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con33 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con12 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con3 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-7 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con18 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-3 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con16 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con17 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con26 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con28 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_T | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_P | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_E | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_B | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_C | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_D | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_K | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_R | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-2 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-5 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con1 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con32 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_O | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_I | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_L | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_M | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H-F2-1 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con10 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| con35 | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
| H2_EV_G | GAGAATTAAGCTGTAATGCTAATTTTAATGTAATTTTATCCAG | GATTGTTGCTTTTTTTCATG |
Cells overexpressing PUF60 (human transcripts)

| Clone | gagaattaagctgtaaatgctaatTTTAATGTA |
|-------|-----------------------------------|
| H2_PUF_M | GAGAATTAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP7 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP20 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP21 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP27 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP14 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP15 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| PUF-BP32 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| H2_PUF_C | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| H2_PUF_H | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| H2_PUF_O | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| H2_PUF_L | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP26 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP7 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP22 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP28 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP36 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP37 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP26 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP24 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP21 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP34 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP23 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP12 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP8 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP10 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP18 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP33 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP31 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP16 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP13 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP19 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP27 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP21 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP7 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP32 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP34 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP37 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP26 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |
| BP24 | GAGAATTAAGCTGAAATGCTAATTTTAATGTA |

Clone Cells overexpressing Kralovicova et al. -
### Cells lacking TIA-1 and TIAR (human transcripts)

| Clone  | gagaattaagctgtaaatgcta | BP+25 | BP+31 | BP+36 | BP+41 |
|--------|-------------------------|-------|-------|-------|-------|
| TIA-34 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-45 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-35 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-12 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-18 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H2_TIA_C | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| TIA-30 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-22 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-11 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H-T-6  | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H-T-7  | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-36 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-42 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-25 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H2_TIA_E | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| H2_TIA_B | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| H2_TIA_O | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| H2_TIA_S | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| TIA-24 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-44 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| T-H-4  | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-28 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-39 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-32 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-8  | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-28 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H2_TIA_G | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| H2_TIA_H | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| TIA-15 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-23 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-43 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H2_TIA_F | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
| H-T-3  | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| TIA-13 | GAGAATTAAGCTGTAATGCTA  | ------| ------| ------| ------|
| H2_TIA_N | GAGAATTAAGCTGTAATGCTA | ------| ------| ------| ------|
Clone Control cells (chicken transcripts)

BP+25 BP+31 BP+36 BP+41
Clone gtgagaattacctgcaaatattttgtgagaattacctgcaaatatttt

Cells overexpressing PUF60 (chicken transcripts)

BP+25 BP+31 BP+36 BP+41
Clone gtgagaattacctgcaaatattttgtgagaattacctgcaaatatttt

Cells lacking TIA-1 and TIAR (chicken transcripts)

BP+25 BP+31 BP+36 BP+41
Clone gtgagaattacctgcaaatattttgtgagaattacctgcaaatatttt
Figure S4  Solitary BPs: splicing activities of systematically mutated BP consensus motifs in TSC2 intron 38 and in F9 intron 1

The WT reporter construct (19) was mutated at the indicated positions of extended BP motif (denoted by a rectangle in the middle panel; BP adenine is at position 0) and transfected into HEK293 cells. Total RNA was extracted 24 hours later, treated with DNase, reversed transcribed with oligo-d(T) primers, amplified with vector-specific primers and separated by electrophoresis (upper panel). RNA products (schematically shown to the left) are denoted by boxes (exons) and lines (introns). The relative abundance of spliced products measured from two independent transfections is shown in the lower panel. US, unspliced products; IR, intron retention; CS, canonical splicing; ES, exon skipping. Error bars, SDs. Mutations with significant impact on splicing are in red. Putative silencing and enhancing mutations are in pink and blue, respectively. The first exon of the F9 minigene employs a cryptic 5' splice site, as described (19).

TSC2

| BPS position | -5 | -4 | -3 | -2 | -1 | 0 | +1 | WT |
|--------------|----|----|----|----|----|---|----|----|
| Mutation     | A  | T  | C  | G  | A  | T | C  | G  | A  | T | A |

[Image of the figure with splicing activities and mutants highlighted]

|  | ES | CS | IR | US |
|---|----|----|----|----|
| **Per cent utilization** | 100 | 90 | 80 | 70 |
| **Percent** | 60 | 50 | 40 | 30 |
| **Exon** | 20 | 10 | 0  | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 |

[Graph showing percentage utilization of exons with different mutations]
### F9

| BPS position | -5 | -4 | -3 | -2 | -1 | 0 | +1 | WT |
|--------------|----|----|----|----|----|---|----|----|
| Mutation     | G  | A  | T  | A  | T  | C | G  | A  | C  |

**Graph:**

- **ES**
- **CS**
- **US**

**Legend:**

- ES
- CS
- US

**Y-axis:** Per cent utilization

**X-axis:** Various categories

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**Note:**

- The table and graph represent specific data points for each position and mutation. The graph shows the per cent utilization for different categories, indicated by ES, CS, and US symbols.
**Figure S5** MegaPPT as a robust substructured platform for regulating *OGDH* MXEs

*Upper panel:* Exon 4b usage upon deletions (del1-4, Figure 3A) of megaPPT subregions. Columns are means of 2 transfections; error bars are SDs. Asterisks denote significant changes in isoform 4b+ usage (*P* < 0.05, one-way ANOVA with post-hoc Dunnett’s tests). *Middle panel:* PCR products digested with PvuII, which cuts only exon 4b. Spliced products are shown schematically to the right and in Figure 1G. *Lower panel:* Uncut products separated by an extended electrophoresis run.
Figure S6  Conserved regulation of OGDH MXEs by U-binding proteins in vertebrates

A, Splicing of exogenous vertebrate transcripts derived from Homo sapiens (Hs), Monodelphis domestica (Md), Gallus gallus (Gg), Xenopus laevis (Xl), Gadus morhua (Gm) and Danio rerio (Dr) in two cell lines. Spliced products are shown to the right; asterisk denotes minor products or heteroduplexes. B, A lack of isoform 4a+ in exogenous bird Ogdh mRNAs is insensitive to deletions in the upstream intron. Bird isoform 4a+ was found in viscera (Figure S9D) and was reported in chicken lymphocytes (NM_001031382.1) (20). C, Coexpression of species-specific reporters and splicing factors that regulate exon 4a/4b ratios. A corresponding immunoblot is bottom right. D, Conserved TIA-1/TIAR regulation of exons 4a and 4b in transcripts derived from the indicated species. E, Immunoblots from depleted cells for panel D.
Figure S7  Evolutionary transitions between cytosine- and uridine-rich megaPPTs in introns with dBPs

Uridine (red lines) and cytosine (black lines) fractions (y-axis) were computed for sequences between the most 3’ dBP and position -3 relative to the 3’ss. Their mean length in the indicated orthologues is at the top right corner. OGDH dBPs are mapped in Figures 3 and 6. Rat Actn1 and Tpm1 dBPs were reported previously (21,22). For KCNMA1, we selected a putative dBP that was most conserved, as none was predicted by SVM-BP (10). MegaPPTs are located between OGDH exons 4a and 4b (A), ACTN1 (actinin) exons 2a and 2b (B), TPM1 (tropomyosin) exons 2a and 2b (C) and KCNMA1 (Ca^{2+}-activated potassium channel) exons 9a and 9b (D).
**Figure S8  Gene- and exon-level expression of OGDH across human tissues**

*Gene-level* expression is based on the GENCODE 19 (http://www.gencodegenes.org/releases/19.html) annotation collapsed to a single transcript model (*upper panel*). Exons associated with transcripts annotated as “retained_intron” and “read_through” were excluded. RPKM and TPM values were produced with RNA-SeqC v1.1.8; the filters were applied using the “--strictMode” flag in RNA-SeqC. Reads overlapping introns were not counted and the TPM values were not corrected for covariates. *Exon-level* expression is quantified for exon read counts; if a read overlapped multiple exons, a fractional value equal to the portion of the read contained within that exon was allotted. *Transcript-level* expression (*lower panels*) were calculated using RSEM v1.2.22. Data were compiled from ref. (23).
Figure S9  Endogenous expression of E1 transcripts during vertebrate evolution

A, OGDH (upper panel) and OGDHL (lower panel) mRNAs in human tissues. B-F, Comparison of endogenous Ogdh expression in striated muscles, brain and viscera in the indicated vertebrate classes. Restriction sites and the size of digestion products are schematically shown for each species at the top. B, human; C, rat; D, quail; E, frog; F, opossum, echidna and platypus. Asterisks denote heteroduplexes. The alignment of Ogdhl and Ogdh in X. laevis is in Figure S11. G, Inclusion of OGDH exon 5 is regulated by U-binding proteins that control MXEs.
Figure S10  *Ogdh* isoforms in the mouse brain

Exon usage was analyzed by next-generation sequencing of RNA samples extracted from mouse brain cell populations (24) (http://jiaqianwulab.org/braincell/RNASeq.html). MO, myelinating oligodendrocytes; NFO, newly formed oligodendrocytes; OPC, oligodendrocyte precursor cells. Exons are numbered at the bottom.
Figure S11  Alignment of *Xenopus laevis* Ogdh and Ogdhl intron 4a

Exons 4a and 4b are in green and red. HindIII sites are double underlined.
Figure S12  BP mapping in *Xenopus laevis* *Ogdh* intron 4a

A, Alignment of 10 clones with lariat junctions. dBP adenines are highlighted in green. B, Mutation of the dominant exon 4b dBP (dBP+30) eliminates exon 4b usage. C, Sequencing chromatograms of informative clones. Vertical arrowheads denote the 5’ss of intron 4a. Circles represent lariat junctions and horizontal bars denote the 5’ end of the first minigene intron. The mutation pattern is consistent with a typical variability across lariat junctions introduced largely by RT (25-27).
Figure S13  Tracing the origin of OGDH dBPs and AGEZ

**A.** Mean (±SD) distances (in nts) between the 5′ss of intron 4a and adenine in the first URA motif downstream. Asterisk denotes a significant difference between tetrapod and fish. **B.** Bidirectional shortening of OGDH intron 4a en route to terrestrial life. Average delta values (intron size reduced by AGEZ, in nts) in the inset highlight the shift to a stricter 5′ss-dBP threshold in amniotes. Error bars are SDs. **C.** High positive correlation between vertebrate Ogdh intron 4a size and their AGEZ length (r=0.96, P<10^-12). The outlier is M. mola, the largest bony fish. The violation of M. mola intron 4a AGEZ by an AG dinucleotide (boxed) is shown to the right. Mutations that extend the AGEZ to its canonical size in the M. mola reporter (bottom) are tested in panel **E.** **D.** Splicing pattern of taxon-specific constructs in cells lacking two OGDH MXE regulators. Ogdh reporters derived from the indicated species and their mutated versions were transfected into HEK293 cells depleted of U-binding RBPs shown at the top. RNA products are shown to the right. MW, size markers; sc, control cells transfected with scrambled siRNAs (17). M. mola transcrips lacking both MXEs are indicated by a red arrow. Immunoblots are bottom right. **E.** Removal of the M. mola AGEZ spoiler by point mutations failed to correct exon skipping observed for the WT (P>0.1, ANOVA with Dunnett’s t-tests). Error bars are SDs of duplicate transfections.
**Figure S14**  MXE genes preferentially function in calcium and sodium channel signalling

Enrichment of human MXE genes was analyzed using the overrepresentation method and molecular function categories of updated WebGestalt ((28); accessed on 28 October 2020), with 16,671 annotated IDs to functional categories as the reference, 550 unambiguously mapped EntrezID entries, and the entire human genome annotation as a background. Molecular function categories with the corrected false discovery rate (FDR) higher than 0.05 are not shown.
Figure S15  Ca\textsuperscript{2+}-induced OGDHC activation: endotherm/ectotherm split and OGDH intron 4a size

$K_m$ values for 2OG in heart mitochondria were taken from reference (29). Y-axis shows $K_m$ ratios at <1 nM and ~30 μM Ca\textsuperscript{2+}. Endotherms (human, rat and pigeon) are in red, ectotherms (frog and fish) are in blue.

![Graph](image)

$r$ = -0.96 (P < 0.05)

Figure S16  Additional examples of PUF60-regulated exons in genes involved in mitochondrial Ca\textsuperscript{2+} signalling/ATP synthesis

A, PUF60-depletion induced retention of the last AK2 intron. This intron contains an alternative polyadenylation site (APA; vertical red triangle). The use of APA site would alter the availability of the ATP binding site at Q214 encoded by the last exon. B, Spacefill representation of the human AK2 structure. Arrow denotes the exposed residue that binds ATP. C, Downregulation of AFG3L2 exons 1-4 in cells lacking PUF60. AFG3L2 mediates degradation of SMDT1/EMRE before its assembly with the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) complex, limiting the availability of SMDT1/EMRE for MCU and promoting efficient assembly of gatekeeper subunits with MCU (30). Downregulation of AFG3L2 exons 1-4 in cells lacking PUF60 would reduce the availability of AFG3L2 isoforms with the transit peptide (encoded by exon 1) and propeptide (encoded by exon 2). PUF60 depletion would therefore reduce not only the OGDH sensitivity to Ca\textsuperscript{2+} and but also canonical AFG3L2 transcripts that encode proteins capable of reaching mitochondria. Mitochondria in cultured AFG3L2-deficient Purkinje cells are inefficient in buffering and shaping Ca\textsuperscript{2+} peaks; spinocerebellar ataxia due to AFG3L2 mutations can be rescued by reduced Ca\textsuperscript{2+}c concentrations (31). Neurons lacking AFG3L2 and the essential MCU subunit EMRE are also vulnerable to mitochondrial Ca\textsuperscript{2+} overload (30). Loss of MICU1 and mitochondrial Ca\textsuperscript{2+} overload specifically affects Purkinje cells and leads to ataxia (32). D, APA of GLS transcripts. Proximal APA site was promoted in cells lacking PUF60 while distal site was promoted in cells lacking U2AF65 (see also Figure 9). Red rectangles show differentially used exons. GLS is alternatively spliced and polyadenylated, giving rise to long (669 aa, KGA) and short (598 aa, GAC) isoforms ((33) and refs. therein). The GAC isoform is abundant in the heart whereas isoform KGA is expressed in the brain, mainly in neurons and less in astrocytes (33). The GAC isoform lacks ankyrin repeats and the KEN box and its dependence on inorganic phosphate ($P_i$) may not be identical to that of KGA (33). GLS catalyzes the hydrolytic deamidation of glutamine to glutamate and contributes to the production of the most
important neurotransmitter in the brain (33). Synaptic vesicles are, however, capable of synthesizing glutamate from 2OG (34,35), although the exclusive supply to glutamate through this pathway might deplete the TCA cycle of key intermediates (36).
**Figure S17  Evolution of the DADLD motif and site 2 in Ogdh**

A, Partial alignment of prokaryotic and eukaryotic E1 proteins. Residues encoded by mammalian exon 4 variants are underlined; residues encoded by exon 6 are double-underlined. Putative Ca\(^{2+}\)-binding motif at site 2 (4) is highlighted in yellow. In some photosynthetic eukaryotes, such as green algae, site 2 contains the DADLD motif (in red). Full alignment of E1 proteins was carried out using Clustal-Omega (v. 1.2.4) with default options. Residues encoded by neuron-specific exon 5 are not shown. B, Alignment of peptides encoded by human OGDH exon 4a and 4b (highlighted in grey).

### A

| Species                      | Site 2                                                                 | 4b                        |
|------------------------------|-----------------------------------------------------------------------|---------------------------|
| Escherichia                  | NLDPLGLWQQ-D---------------KVADLDPSFHDLTLDAPFATFVNVQSF---------------ASGKTMK | 152                       |
| Pelagibacterium              | NLDPLGLWR-E---------------IEPELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 182                       |
| Starkeya                     | KLDPLGREP-HE---------------SYNELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 186                       |
| Chelativorans                | DLDPLGLAKPME--------------SYNELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 184                       |
| Labrenzia                    | DLDPLQLATPG--------------DHEELHPSSGYFTPADWRSIFIDH---------------LGLLETAT | 182                       |
| Medicago                     | KLDPLNLAEQQ--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 199                       |
| Glycine                      | KLDPLNLERFFF--------------ICEEDLPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 195                       |
| Brachypodium                 | KLDPLGKEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 191                       |
| Zea                          | KLDPLGKEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 190                       |
| Vitis (grapevines)           | KLDPLGKEEERF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 197                       |
| Prunus                       | KLDPLGKEEERF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 190                       |
| Solanum                      | KLDPLGKEEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 197                       |
| Pelagibacterium              | NLDPLGLWQQ-D---------------KVADLDPSFHDLTLDAPFATFVNVQSF---------------ASGKTMK | 152                       |
| Starkeya                     | KLDPLGREP-HE---------------SYNELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 186                       |
| Chelativorans                | DLDPLGLAKPME--------------SYNELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 184                       |
| Labrenzia                    | DLDPLQLATPG--------------DHEELHPSSGYFTPADWRSIFIDH---------------LGLLETAT | 182                       |
| Medicago                     | KLDPLNLAEQQ--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 199                       |
| Glycine                      | KLDPLNLERFFF--------------ICEEDLPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 195                       |
| Brachypodium                 | KLDPLGKEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 191                       |
| Zea                          | KLDPLGKEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 190                       |
| Vitis (grapevines)           | KLDPLGKEEERF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 197                       |
| Prunus                       | KLDPLGKEEERF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 190                       |
| Solanum                      | KLDPLGKEEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 197                       |
| Pelagibacterium              | NLDPLGLWQQ-D---------------KVADLDPSFHDLTLDAPFATFVNVQSF---------------ASGKTMK | 152                       |
| Starkeya                     | KLDPLGREP-HE---------------SYNELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 186                       |
| Chelativorans                | DLDPLGLAKPME--------------SYNELDPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 184                       |
| Labrenzia                    | DLDPLQLATPG--------------DHEELHPSSGYFTPADWRSIFIDH---------------LGLLETAT | 182                       |
| Medicago                     | KLDPLNLAEQQ--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 199                       |
| Glycine                      | KLDPLNLERFFF--------------ICEEDLPAYGFSDEADLYEFIDNY---------------LGLLEFAT | 195                       |
| Brachypodium                 | KLDPLGKEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 191                       |
| Zea                          | KLDPLGKEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 190                       |
| Vitis (grapevines)           | KLDPLGKEEERF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 197                       |
| Prunus                       | KLDPLGKEEERF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 190                       |
| Solanum                      | KLDPLGKEEKRF--------------IFPDLDPAAYGFSDEADLYEFIDNY---------------LGLLEFAT | 197                       |

### B

| Species                      | Site 2                                                                 | 4b                        |
|------------------------------|-----------------------------------------------------------------------|---------------------------|
| Putative Ca\(^{2+}\)-binding motif - Omega alignment of E1 proteins (in red) | | |
**Figure S18**  Exon enhancing and silencing activity of 64 codons: dichotomy of codons involved in Ca\(^{2+}\)/Mg\(^{2+}\) binding versus Cu\(^{2+}\)/Zn\(^{2+}\) binding

A, B, Codon frequencies in high-confidence ESEs (A) and ESSs (B). The ESE and ESS hexamers were reported previously (37). Residues preferentially involved in binding weak Ca\(^{2+}\) and Mg\(^{2+}\) are boxed in green; residues preferentially involved in binding of competitive Cu\(^{2+}\) and Zn\(^{2+}\) are boxed in red. Asterisks denote stop codons. The UAG codon has the highest silencer activity and is completely absent in the comprehensive set of ESE hexamers (37). C, D, Average splicing activities for codons involved in metal binding sites for Mg\(^{2+}\), Ca\(^{2+}\) and Mn\(^{2+}\) (C) and Fe\(^{2+}\), Cu\(^{2+}\) and Zn\(^{2+}\) (D). Amino acids are shown at the bottom; their frequencies in metal binding sites were compiled using estimates based on fragment transformation methods (38).
Figure S19  Hexamer preference indices (HPIs) of ESE sets and residues most frequent in metal binding sites

Designation of ESE sets is as described by Cáceres et al. (39), including RESCUE ESE (40,41), PESE (42), a mixture of ESE and ESS (ESR; (43)), Ke-ESE (37) or their subset (Ke_ESE), or various intersects (INT sets). A, Codons for key residues in calcium/magnesium binding sites. B, Codons for key residues in copper/zinc protein binding sites. C, The Irving-Williams order (top) and HPI (39) gradient for the ESE sets shown at the bottom. Weighted average HPIs were normalized for amino acid frequencies in protein binding sites for the indicated metals, employing only residues within 3.5 Å of the metal ion centre (38).
SUPPLEMENTAL DISCUSSION

Evolution of endothermy and Ogdh MXEs: a hypothesis

Discussion S1  Ogdh MXEs and the energy supply face of becoming warmblooded

Ca\(^{2+}\) levels are important regulators of the TCA cycle. They can exceed 10 \(\mu\)M during cell activation, stimulating matrix DHs, increasing the cycle flux and ATP production and the release of ATP to the cytosol. The increase of ATP supply depends on the amplitude of Ca\(^{2+}\) rise and the availability of oxidative substrates ((44,45) and refs. therein). OGDH plays a key role in Ca\(^{2+}\)-activated delivery of NADH and ATP, limiting reducing equivalents, electron transport and ATP synthesis (46-50). OGDH activities provide good estimates of the maximum flux through the TCA cycle in vivo (51) and may define the maximal respiratory capacity of mitochondria (48,49,52). OGDHC may have a greater effect on the respiration rate than other TCA enzymes. For example, OGDH and isocitrate DH were estimated to control 70% and 23%, respectively, of the respiratory flux upon Ca\(^{2+}\) stimulation (53). Moreover, catalytic OGDH reactions are accompanied by a large change in free energy and, among Ca\(^{2+}\)-sensitive matrix enzymes, OGDH has the smallest K\(_m\) values in the presence of ADP ((44,54) and refs. therein).

OGDHC deficiency or inhibition leads to a significant reduction in respiration rate that may be more dramatic than inhibition of mitochondrial isoforms of other TCA enzymes, including aconitase (ACO1), malate DH, fumarase, citrate synthase and the NAD+-dependent isocitrate DH (50,52). Transgenic studies in plants showed that the TCA cycle control points were distributed among malate DH, OGDH (flux control efficient 0.79), ACO1, fumarase, and succinate DH (52). Besides ACO1, systematic inactivation of TCA cycle genes in yeast coupled with transcript analysis revealed altered expression of the largest number of genes for Ogdh mutants (55). The expression of aerobic genes, predominantly of the mitochondrial respiratory complexes, was diminished in Ogdh-mutated strains and to a lesser extent in ACO1 mutants, but not in other mutants of TCA cycle enzymes, while the expression of anaerobic and hypoxic genes was elevated (55).

Simulation studies using a human heart model of mitochondrial metabolism suggested that the effect of OGDHC inactivation on the energy status is manifested only under conditions of increased energy demand, and not at rest (56). Mice lacking the mitochondrial Ca\(^{2+}\) uniporter (MCU) cannot perform strenuous work as a result of diminished Ca\(^{2+}\) uptake in skeletal muscle mitochondria (57).

Addition of Ca\(^{2+}\) over the physiological range increased mitochondrial respiration in the wild-type mice, but no Ca\(^{2+}\)-dependent stimulation was observed in homozygous mutant mice while the basal metabolism seemed unaffected, although Ca\(^{2+}\) was not completely absent (57). The large change in ATP requirements during striated muscle activity renders Ca\(^{2+}\) transfer to mitochondria a key signalling step (58). At rest, a low MCU activity was enough to sustain basal ATP levels and heart rate, but under stress conditions the MCU-dependent Ca\(^{2+}\) uptake and ATP generation became crucial (59).

Activation of Ogdhc by Ca\(^{2+}\) can be, to a large extent, explained by the DADLD motif encoded by exon 4b (4,60). This motif as well as closely linked site 2 probably evolved on multiple occasions during evolution (Figure S17A). Expression of this motif has been promoted by the capacity of codons encoding critical residues in Ca\(^{2+}\)-binding sites to enhance exon inclusion levels in mature transcripts (Figure 10, S18-S19), by a loss of the enhancing codon in competing exon 4a during evolution (Figure 5) and by the unusual arrangement of the 3’ss, with dBP located near the opposite intron end (Figure 1-4). The DADLD motif evolved in ectotherms (4), but ectotherms have longer intron 4a (Figure 1) and their splicing pattern is not fully developed (Figure S6). Non-redundant dBP clusters in anamniotes evolved into redundant and more robust dBP clusters, followed by enrichment of megaPPTs for uridines that bind abundant splicing factors (Figure 1,2). This intron organization was completely immune to enlargements, protecting it from transposon expansions for hundreds of Myrs (Figures 1-4), in line with the importance of the MXE regulation in cell energy supply.

Taken together, Ogdh MXEs provide a critical switch for Ca\(^{2+}\)-dependent NADH and ATP supply. Fine-tuning of their alternative splicing may result in a strong selective advantage at the cellular level, as well as at the level of the organism and the species. Optimized regulation of their tissue-specific
inclusion in mRNAs at multiple levels should ensure responsive and sustained activity of striated muscles, major contributors to thermogenesis (Discussion S2), as well as maximum locomotory endurance during vertebrate evolution, a central tenet of the aerobic scope model of endothermy.

**Discussion S2  OGDHC in Ca\(^{2+}\)-dependent muscle thermogenesis**

Cell motility was intrinsically coupled to evolution of muscles (61), which evolved muscle-specific alternative splicing pathways in vertebrates (62,63) (Table S6,S7). Apart from locomotory function, striated muscles have played a key role in both activity-dependent and -independent heat production and have the largest potential to generate body heat (64) and refs. therein). Although they may not generate most body heat at rest in some species, increased muscle activity can produce ~40x as much heat as the rest of the body, including visceral organs. For example, the contractile apparatus and ion pumps together account for ~75% of ATP consumption by cardiac myocytes ((65) and refs. therein).

Striated muscles have also the highest densities of organelles that generate most of the heat. Mitochondrial as well as respiratory chain complex densities are much higher in striated muscles than in the brain or viscera and muscle mitochondria have a very distinct morphology, including cristae organization, matrix density and subcellular distribution (66). In endotherms, mitochondria physiologically operate at temperatures as high as ~50 °C, as measured by a fluorescent dye that accumulates in active organelles and may bind matrix components (67). The high temperature fell upon addition of respiratory inhibitors and rose upon pyruvate addition, although Ca\(^{2+}\)\(_{m}\) levels were not measured (67). Human respiratory enzymes also operate at higher temperatures and their thermophilic counterparts and matrix DHs can withstand even hotter temperatures without loss of activity (67-69). Thus, muscle mitochondria have been major heat suppliers to the cells.

Skeletal muscles of many partial or regional endotherms evolved into ‘heater organs’ which employ energetically costly Ca\(^{2+}\) cycling to/from sarcoplasmic reticula (70). One of the best studied heater organs are slow-twitch muscles of some fish predators, such as tunas or billfishes (70). These organs have high oxidation rates and aerobic capacity and are densely packed with sarcoplasmic reticula and mitochondria. The two organelles are intimately juxtaposed in the cell (58). The heater organs lack myofibrillar structures typical of force-producing (slow-twitch) muscles, reflecting high NADH/ATP demand and selection for rapid Ca\(^{2+}\) release and reuptake (70). The slow-twitch muscles have on average higher stamina, smaller glucogen stores and lower glycolytic activity, higher oxidative phosphorylation and reliance on oxygen, smaller fibre diameter, higher capillary density, slower build up of lactate, and a higher myoglobin concentration and oxygen storage capacity than their fast-twitch counterparts ((64,71) and refs. therein). A prominent example of a largely slow-twitch muscle is soleus, which can contain up to 100% of slow myofibres, is critical for walking and running, evolved from a common limb flexor present already in early tetrapods, including walking fish *Ambystoma mexicanum*, and may have originated from fish pelvic appendages (72). In lizards, stance-phase muscles such as gastrocnemius or soleus are better predictors of maximum performance than swing phase muscles (73). Disuse of skeletal muscles in non-hibernating mammals leads to slow-to-fast fibre type transitions (74). In contrast, muscles in hibernators (Discussion S3) undergo fast-to-slow transitions (74) and are capable of a remarkable preservation of skeletal muscle mass, which could contribute to their resistance to atrophy ((75) and refs. therein). Slow-twitch muscle fibre formation is driven by PGC-1α (76). PGC1 proteins are master metabolic regulators that may also function as RBPs since they contain a C-terminal RMM and a serine/arginine-rich domain along with N-terminal transcriptional activation domain (77). Thus, under the aerobic scope model of endothermy, slow-twitch muscles are prime candidates for selection processes acting on MMR.

Contraction-based (‘shivering’) thermogenesis is an ancient mechanism for generating heat, well documented in invertebrates (78) and long predating the *Ogdh* exon duplication. With a significant increase of skeletal muscle mass in vertebrates, striated muscles have become the primary thermogenic organ (79). At some point in evolution, however, futile Ca\(^{2+}\) cycling through sarcoplasmic reticulum Ca\(^{2+}\) ATPases (SERCAs) andryanodine receptor channels became a key mechanism for a dominant form of heat production during prolonged cold adaptation, replacing shivering thermogenesis with a ‘non-
shivering’ counterpart (64). This process had to rely on a Ca\textsuperscript{2+}-dependent ATP supply by mitochondria to match the demand of ATPase activities, particularly SERCAs, which are more important for establishing non-shivering thermogenesis than myosine ATPase or Na+/K+ ATPase (79). SERCA2 (ATP2A2) is differentially expressed in oxidative and glycolytic chicken myofibers (80). D. rerio with high levels of sustained locomotor performance had elevated levels of SERCA1, RYR and PGC-1 mRNAs than the fish with low sustained swimming speeds (81). The importance of Ca\textsuperscript{2+}-mediated pathways in both resting and activity-induced heat production is probably best illustrated by a high fraction of the muscle energetic turnover attributable to Ca\textsuperscript{2+} cycling in multiple species and close parallels between ‘tuna burn’ and malignant hyperthermia in mammals ((70) and refs. therein). Malignant hyperthermia is caused by mutations in RYR1, ryanodine receptor tightly regulated by ATP binding in a Ca\textsuperscript{2+}-dependent manner (82,83). The significance of futile cycles in T\textsubscript{h} control is highlighted by a loss of Drosophila THADA (THyroid ADenoma Associated human homologue), one of the most strongly selected gene. The THADA knockout leads to elevated activity of SERCA, diminished heat production and cold sensitivity (84). THADA binds SERCA and uncouples its ATP hydrolysis from Ca\textsuperscript{2+} pumping (84).

Many defects of Ca\textsuperscript{2+}_m pathways and ATP synthesis generate phenotypes primarily involving striated muscles. For example, MCU silencing in limb muscles leads to muscle atrophy, suggesting that the MCU is required for muscle size control (85). Loss-of-function mutations in the MICU1 gene (mitochondrial calcium uptake 1) cause proximal myopathy and extrapyramidal movement disorders (86). Mutations in the OGDH MXE regulator TIA-1 (Figure 1F) were associated with myopathy (87,88) and may affect steady-state TIA-1 expression or protein properties other than RNA binding (Figure 2D).

Muscular activity in endotherms, but not in vertebrates with cartilaginous skeletons, leads to abrupt hypercalcaemia (89). During maximal contraction, ATP consumption in skeletal muscles can increase over 100-fold (90). Muscle ATP stores are insufficient to meet this demand, necessitating a rapid and sustained supply of new molecules. Mitochondrial Ca\textsuperscript{2+} uptake is fast enough to support step changes in muscle workload: in isolated heart mitochondria, the uptake occurs within a 100 ms time-resolution limit, leading to activation of NADH production and Ca\textsuperscript{2+}-dependent DHs within 200 ms and oxidative phosphorylation within 270 ms, although the off-kinetics is much slower (91). Beat-to-beat changes in Ca\textsuperscript{2+}_m in cardiomyocytes can translate time-dependently into steady-state alterations in ATP (92,93). Even a single muscle twitch could be associated with measurable changes in Ca\textsuperscript{2+}_m in living motor fibers milliseconds later (94). Although electrical conduction via the mitochondrial reticulum as opposed to ATP or oxygen diffusion is a more effective way to quickly and uniformly distribute energy in muscle cells, metabolite-facilitated diffusion pathways become significant during maximum endurance (95). Mitochondrial ATP production is kinetically more responsive to changes in Ca\textsuperscript{2+} than ADP or P\textsubscript{i} (91). Elevation of Ca\textsuperscript{2+}_m stimulates oxidative phosphorylation more strongly than substrate oxidation to achieve homeostasis of mitochondrial membrane potential (96). Although the exon-level regulation of OGDH activation by RBPs (Figure 1) is slower than the time-scale of Ca\textsuperscript{2+} transients, the drop in ATP synthesis was considerably delayed after the transient Ca\textsuperscript{2+}_m signal had returned to basal levels (45).

The MXE organization of OGDH exons 4a/4b resembles that found in other muscle-related genes, including tropomyosin (TPM1) (21), α-actinin (ACTN1) (22,97), troponin T (TNNT) (98) and myosin light chains (MYLI and MYL3) (99). Actinin MXEs that encode isoforms with differential Ca\textsuperscript{2+} sensitivity employ dBPs of the smooth muscle-specific exon (22). ACTN3 is only expressed in glycolytic myofibers (100) and its null allele has been associated with increased aerobic capacity and endurance and higher citric synthase activities ((101,102) and refs. therein). The null allele leads to increased SERCA1 expression and Ca\textsuperscript{2+} leak from the sarcoplasmic reticulum (101,102). The futile cycle involving an increased reuptake of Ca\textsuperscript{2+} would generate heat by non-shivering muscle thermogenesis and provide an explanation for the evolutionary advantage of carrying the null allele in cold climates (101,102). Actinin and tropomyosin are also important in slow-to-fast muscle fibre transitions in hypometabolic states ((103) and refs. therein; Discussion S3).

To what extent were tissues other than skeletal muscles important for the acquisition of endothermy? Although cardiovascular characteristics of animals have been studied extensively, improved delivery of oxygenated blood is unlikely to be selected for in the absence of efficient mechanisms that can
utilize oxygen as the ultimate electron acceptor and produce ATP on demand. Rather, a greater capacity for responsive ATP production would favour improved oxygen delivery to critical tissues (striated muscles and CNS). Rapid oxygen delivery would be facilitated by the emergence of four-chamber hearts in mammals, birds and crocodilians, through-flow lungs in birds and theropods (104), blood sinuses on the top of the head of earless lizards rapidly warming the whole body, and superior capillary systems allowing efficient oxygen transport. E1 is most expressed in the left heart ventricle (Figure S8). OGDHC generates a substrate (succinyl-CoA) for synthesis of heme, a prosthetic group of heme proteins involved in electron and oxygen transport, such as cytochromes and hemo-/myoglobin. In yeast Ogdh mutants, expression of hypoxic/anaerobic genes was elevated while expression of oxidative genes was diminished, consistent with a heme signalling defect caused by inadequate levels of succinyl-CoA, the heme precursor (55). Ca^{2+} can induce a spectral shift in heme a and change in the midpoint redox potential (105,106). OGDHC is sensitive to oxidative stress and occupies a central position at the crossroads of redox pathways (107) and refs. therein. OGDHC is important for regulation of hypoxia-inducible factor HIF1α in aerobic conditions, a master regulator of genes involved in essential hypoxic responses that maintain cellular ATP levels (108). In addition, OGDHC is the main source of reactive oxygen species (ROS), which are strongly stimulated by Ca^{2+}, beating other TCA cycle enzymes, including pyruvate DH, both in cardiac and visceral tissues (109-111). OGDHC dominates ROS production regardless of whether pyruvate or succinate serves as the sole source of carbon (110,111).

Brown adipose tissue (BAT) is believed to have unique thermogenic abilities that are derived from properties of the uncoupling protein UCP1 ((112) and refs. therein) (see also Discussion S6). However, BAT has not evolved until the appearance of placentals and cannot explain more ancient examples of regional endothermy (79). Neither fish nor large mammals have BAT (64). Importantly, skeletal muscles and BAT have a common ancestor: for example, mitochondrial proteomic signatures show more similarities between muscles and BAT than between BAT and white adipose tissue ((79) and refs. therein).

The increase in the Ca^{2+} cycling across the mitochondrial membrane generates proton leak and heat. The contribution of proton leak to oxygen consumption in resting skeletal muscles was reported as high as 60% in vitro (113,114). However, this figure was estimated to be very low or close to zero in other systems or in vivo (115,116) or during intense muscle activity (117). Ca^{2+}-induced increase in state 4 respiration resulted from elevated protonmotive force and not from direct activation of proton leak (115).

Collectively, these studies show that responsive and Ca^{2+}-dependent ATP supply to striated muscles has been crucial for internal heat generation during evolution. OGDH role in Ca^{2+}-dependent NADH supply, coregulation of this reaction with and distant steps of ATP synthesis pathways (Figure 9B-D, S16), as well as the OGDHC role in glutamate (Discussion S4), heme, redox and fatty acid metabolisms make E1 a strong candidate for playing a potentially major role in shaping the aerobic scope and evolution of endothermy.

**Discussion S3  OGDHC and Ca^{2+} in hypometabolic states**

In torpor and hibernation, ATP supply and T_b are dramatically reduced in multiple tissues, particularly in skeletal muscles, and the TCA cycle enzymes such as pyruvate DH are repressed ((118) and refs. therein). For example, pyruvate carboxylation in intact mitochondria is decreased by 75% during hibernation and mitochondrial, but not cellular, ATP/ADP/AMP are also reduced (119). A recent comparative study of several hibernators showed that genes involved in mitochondrial oxidation and pyruvate metabolism in skeletal muscles were most significantly associated with hibernation phenotypes (120), but their alternative splicing was not studied. Moreover, Ogdh isoforms were among top-ranked proteins differentially expressed in the brainstem and skeletal muscle proteomes in summer active versus hibernating ground squirrels (121,122), but their exact identities and underlying mRNA isoforms were not determined. Among differentially expressed factors, including Ogdh, mitochondrial genes were highly enriched, particularly in striated muscles (122-124), but exon-level comparisons were not performed. Both Atp5c1 (Figure 9B-D) and Ogdh were identified among proteins differentially expressed in skeletal
muscles of ground squirrels, even when comparing March arousals with April activity (103), but alternatively spliced mRNA isoforms were not studied. Recent transcriptomics analysis in marsupial Dromiciops gliroides during hibernation revealed 566 transcripts that were significantly up-regulated during hibernation (369 in brain, 147 in liver and 50 in skeletal muscle) and 339 down-regulated transcripts (225 in brain, 79 in liver and 35 in muscle), including alterations of spliceosome components (125).

As compared to non-hibernating mammals, hibernators such as ground squirrels exhibit enhanced muscle contractility and remarkable ability to maintain stable Ca\(^{2+}\) levels at low temperatures (e.g.,(126,127)), suggesting that they evolved mechanisms that prevent Ca\(^{2+}\) overload. Low temperatures reduce the rate of Ca\(^{2+}\) removal from cytosol although the relative contribution of plasma and organelle channels in hibernator tissues are poorly understood (126). Cardiac sarcoplasmic reticula from ground squirrels showed faster Ca\(^{2+}\) uptake than non-hibernators, but SERCA activities did not appear to be distinct ((126) and refs. therein). Brain mitochondria of ground squirrels were able to load significantly less Ca\(^{2+}\) during torpor than in spring animals (128). Interbout arousal squirrels displayed a striking increase in intracellular Ca\(^{2+}\) concentration (129).

Rapid increase in ATP supply during arousal periods (121,122) would require a control of the cyclical activation of TCA cycle enzymes, such as Ogdhβ and its MXE regulators. For example, TIA-1 (Figure 1F) showed up to a sevenfold increase in relative protein levels in the nucleus during hibernation (130), which would repress Ca\(^{2+}\)-sensitive isofrom 4b+. When exposed to low temperatures, distal muscles trigger formation of TIA-1-containing stress granules (131).

Collectively, these studies support a profound role of Ca\(^{2+}\) and energy metabolism in hypometabolic states, and some directly point to Ca\(^{2+}\)-activated TCA cycle enzymes. As hypometabolic states in birds and mammals may share the same ancestral origin with reptiles (132) and Ogdh intron 4a in reptiles reached the same size as in endotherms, regulation of the MXE pairs could provide valuable clues about their evolution. Expanding the number of available hibernator genomes/transcriptomes coupled with exon-level transcriptomic data should facilitate future insights into molecular mechanisms underlying evolution of these phenotypes.

**Discussion S4  Dichotomy of OGDH splicing in neurons and astrocytes**

**A raison d’être?**

Presynaptic Ca\(^{2+}\) is the principal regulator of neurotransmitter release and synaptic plasticity ((133) and refs. therein). Presynaptic terminals are densely packed with mitochondria to support high demand for energy at the synapse. The absence of mitochondria in the terminals of dmiro (a GTPase that binds Ca\(^{2+}\)) fly mutants leads to locomotory defects (133,134). MCU is very sensitive to pre-synaptic Ca\(^{2+}\) levels (135), but unlike in striated muscles, the MCU deletion in brain synaptic or non-synaptic mitochondria does not lead to a complete block of Ca\(^{2+}\) uptake (136).

In eukaryotes, Ca\(^{2+}\) elevations come from intracellular and extracellular sources. In striated muscles, the bulk originates from intracellular stores, largely from the sarcoplasmic reticulum, whereas the main source of Ca\(^{2+}\) spikes in neurons, but not in astrocytes, is extracellular Ca\(^{2+}\). Elevations of Ca\(^{2+}\) in visceral organs come both from the outside and the inside ((137) and refs. therein).

Neurons and their precursors repress the Ca\(^{2+}\)-sensitive OGDH isoform, unlike non-excitable astrocytes or striated muscles (Figure S10). In response to stress, OGDHC activity fluctuates less in the brain than in striated muscles or in liver (56). Astrocytes respond to synaptic activity with Ca\(^{2+}\) elevations, which stimulate chemical transmitters, including glutamate (138). Astrocytes consume considerable energy to remove/release neurotransmitters, restore ion gradients and maintain other homeostatic processes ((139,140) and refs. therein). Excitatory neurotransmission mediated by astrocytes is a critical contributor to brain energy needs (141), consistent with their high requirement for Ca\(^{2+}\)-stimulated ATP supply and high exon 4b+/4a+ ratios (Figure S10).

Astrocyte size and number of their processes dramatically expanded during vertebrate evolution, with a single human cell employing up to 2 million synapses ((141) and refs therein). Ca\(^{2+}\) regulates mitochondrial mobility in astrocyte processes, and mitochondria in turn control Ca\(^{2+}\) signals (142).
Astrocytes are highly sensitive to physiological hypoxia, are capable of efficiently modulating vasoconstriction/dilation and can determine the exercise capacity through adaptive respiratory responses in conditions of increased metabolic demand (143,144), acting essentially as functionally specialized oxygen sensors. They respond to low oxygen levels with increased exocytosis of ATP-containing vesicles (143). Unlike a massive depletion of ATP upon inhibition of mitochondrial respiration in neurons, astrocytes are able to limit the ATP decline (141).

Apart from providing global support for neural circuits, astrocytes also exert local control over individual synapses or a small group of synapses, acting as regulatory units for the astrocyte-neuron metabolic cooperation (139,140). They express a variety of neurotransmitter receptors including glutamate receptors, which induce transient increases of Ca\(^{2+}\), particularly in localized microdomains (140). The microdomain Ca\(^{2+}\) transients colocalize with mitochondria; these organelles are highly abundant in astrocytic microdomains at a density comparable to nerve terminals, reflecting a high demand for ATP (139). Spatially restricted Ca\(^{2+}\) transients in astrocyte processes that are independent of the release from endoplasmic reticulum stores result from the Ca\(^{2+}\) efflux from mitochondria via transient openings of the permeability transition pore, generating microdomain Ca\(^{2+}\) signals (139). Enhanced neuronal firing in vivo, such as arousal during locomotion, increases the microdomain activity of astrocytes in the absence of signalling through a Ca\(^{2+}\) release channel (IP3R2/ITPR2), linking the microdomain Ca\(^{2+}\) transients to the metabolic rate (139). Astrocyte Ca\(^{2+}\) elevations may be as rapid as in neurons (145) and can propagate to astrocytic perivascular endfeet to regulate the vasomotion and microcirculation and to meet rapid energy demand in the areas of elevated neuronal activity (146). Recovery from the glutamate-induced rise of Ca\(^{2+}\) is more efficient in astrocytes than in neurons (147).

The ratio of OGDH exons 4a/4b in myelinating oligodendrocytes (MOs), but not in their precursors, is similar to neurons (Figure S10). MOs express many molecules that make them susceptible to excitotoxic cell death, including glutamate receptors (148). MOs express P2X7, which makes them prone to detrimental effects of sustained levels of extracellular ATP (148,149). ATP is a major excitatory neurotransmitter in the central nervous system, activating ionotropic (P2X) and metabotropic (P2Y) receptors. ATP-gated P2X channels are Ca\(^{2+}\)-permeable and participate in fast synaptic transmission and modulation; ATP signalling may trigger oligodendrocyte excitotoxicity via activation of Ca\(^{2+}\)-permeable P2X7 receptors (149). Glial function started to evolve in invertebrates, but reactive astrocytes are still absent in zebrafish (150), which may not yet gained an optimal MXE control (Figure S6).

Further clues to our understanding of distinct Ogdh exon 4a+/4b+ ratios in neurons and glia (Figure S10) may lie in the malate-aspartate shuttle (MAS). In MAS, glutamate and malate enter the mitochondrial matrix in exchange for aspartate through Ca\(^{2+}\)-sensitive aspartate/glutamate carriers aralar (SLC25A12) and citrin (SLC25A13; for solute carriers A12 and A13) while 2OG is transported out through the oxoglutarate carrier (SLC25A11). SLC25A12-deficient neurons showed decreased respiration levels and a failure to regulate respiration rates in response to Ca\(^{2+}\) (151). A lack of Ca\(^{2+}\) stimulation observed under workload in SLC25A12 knock-out neurons (152) indicates that SLC25A12 is a major contributor of Ca\(^{2+}\)-stimulated respiration in neurons by providing increased pyruvate supply to mitochondria. SLC25A12 is preferentially expressed in the brain and striated muscles. In the brain, the SLC25A12 expression is most abundant in neurons (24), and the protein is thought to be functional only in this cell type (reviewed in (153)). MAS reconstitution using isolated mitochondria showed that activation of OGDH by Ca\(^{2+}\) reduced efflux through SLC25A11, decreasing the MAS activity as a result of competition between MAS and TCA cycles for the shared metabolite 2OG (154). Aralar has a higher Ca\(^{2+}\) affinity than the MCU and even tiny Ca\(^{2+}\) signals can stimulate ATP (155). In plants, malate DH has a high flux control coefficient for respiration (52). A mutual exclusivity of MAS, the main NADH shuttle in the brain, and MCU-DH pathways in brain mitochondria under Ca\(^{2+}\)-stimulated conditions (155) might have facilitated Ogdh exon 4b repression in neurons. Under the sequential MAS and the MCU-DH activation model (154), the Ca\(^{2+}\) activated OGDHC may become the main NADH producing pathway when Ca\(^{2+}\) transients are high following the MCU activation. Finally, 2OG as an important source of glutamate and glutamine directly inhibits F1F0-ATP synthase by binding ATP5B, reducing ATP and
oxygen consumption and increasing the *C. elegans* lifespan (156). This link may expanded by the shared exon-level regulation of F$_{i}$F$_{o}$-ATP synthase and *OGDH* MXEs (Figure 9B-D).

Taken together, the important role of astrocytes in neuroenergetics was associated with derepression of *OGDH* exon 4b in this cell type (Figure S10). Prominent exon 4b activation in astrocyte Ca$^{2+}$ microdomains would provide responsive ATP supply and superior synaptic and locomotory control *en route* to endothermy. In contrast, exon 4b repression in neurons and MOs (Figure S10) may have been required to prevent excitotoxicity, in line with a strong pressure against energy dissipation in the brain (157). The key role of OGDHC in glutamate metabolism should provide additional clues for the dichotomy of this MXE pair in neurons and glia.

**Regulation of OGDH exon 5 splicing in neurons**

Neurons adopted an extra exon 5, which encodes additional 15 amino acids (Figure S8-S10) (4). It remains to be confirmed when exactly this event evolved, possibly before the split of cartilaginous and bony fish (4). The inclusion of exon 5 in mRNAs suggests that a simple on/off switch of MXEs to reduce or increase the Ca$^{2+}$-dependent OGDHC activation is not satisfactory in this cell type. Exon 5 inclusion is associated with exon 4a, albeit not exclusively (Figure S10), raising a possibility that it may fine-tune OGDHC inhibition. The *OGDH* 4a+5+ or 4b+5+ isoforms could modulate Ca$^{2+}$-sensitivity of OGDHC since both isoforms showed much attenuated response to Ca$^{2+}$; among tested E1, isoform 4a+5+ was associated with the lowest enzyme activation at high Ca$^{2+}$ concentrations, nevertheless the loss of Ca$^{2+}$-sensitivity was accompanied only by modest decreases in sensitivity to inhibition by NADH and elevated ATP/ADP ratios (4).

The neuron-specific exon 5 activation can be explained by the presence of an activator and/or a lack of repressor in this cell type. One candidate for the repressive regulation is U2AF35 (17). U2AF35 isoforms are expressed at similar levels as U2AF65 in skeletal muscles, but U2AF35a mRNAs are low in the brain, less than half of the U2AF65 mRNA levels (158,159). U2AF35 but neither U2AF65 nor PUF60 knockdown activated exon 5 inclusion (Figures S6 in ref. (17)). The U2AF35a/U2AF35b ratio is also low in the brain and high in skeletal muscles (159). The two U2AF35 isoforms play an important role in regulated splicing (158,160-162), which could be involved in ensuring protection of neurons against Ca$^{2+}$-dependent ATP oversupply and neurotoxicity.

**Discussion S5  Endothermy and body mass: clues from Ogdh MXEs?**

Smaller organisms have a larger surface-to-volume ratio than larger ones and heat and cool faster, suggesting that selection for the body size was important in the acquisition of homeothermy and for evolution of hypometabolic states (163-167). Mitochondrial function in relation to body mass has been studied extensively both in endotherms and ectotherms. For example, small endotherms were proposed to have a lower mitochondrial efficiency than larger species and the production of ATP and reactive oxygen species have been correlated with body mass (168,169). Mitochondrial efficiency to generate ATP is reduced by proton leak, a dissipation of the protonmotive force through mitochondrial inner membranes, diverting energy away as heat (168,169). During vertebrate evolution, however, this reduction should promote selection processes that favour activity-dependent ATP supply pathways.

Selection routes shaping body or organ size are controlled by apoptotic cellular decisions. Such choices involve Ca$^{2+}$ signalling both in extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) cell death pathways (170), including Ca$^{2+}$ spikes (171) and Ca$^{2+}$ regulators in endoplasmic reticula (reviewed by (170)). Importantly, Ca$^{2+}$ overload acts as a proapoptotic signal that induce mitochondria swelling and the release of mitochondrial apoptotic factors into the cytosol (172). Local communication between organelles can propagate Ca$^{2+}$-mediated apoptotic signals over relatively large distances, which would ensure coordinated execution of apoptosis across large cells, particularly cells with high mitochondrial densities (171). Ca$^{2+}$ is involved in multiple cell death modalities including autophagy, which can be triggered by very subtle changes in Ca$^{2+}$ distribution within intracellular compartments (170).
In large marine species, the mass-specific metabolic rate decreases with increasing body mass, suggesting that active macropredation cannot be sustained once a given body size is reached and only less active strategies such as filter feeding are physiologically affordable above a certain threshold (173). Endothermic macropredators can attain larger body masses than their ectothermic counterparts and endothermy was even proposed to play a role in the evolution of gigantism in extinct macropredatory groups (173) and refs. therein). Ocean sunfish (*M. mola*), the *Ogdh* intron 4a AGEZ outlier (Figures 6E and S13), is the world’s largest bony fish weighing up to 2.3 tons and native only to tepid and tropical waters; prolonged periods spent in water at temperatures of 12 °C or lower lead to disorientation and death (174). The AGEZ of *M. mola* *Ogdh* intron 4a is violated, but the associated exon 4b skipping was not rescued by extending the AGEZ (Figure S13). Nevertheless, this result does not completely exclude physiological significance of the reduced AGEZ in endogenous expression. If this reduction does decrease Ca"superscript+"-dependent OGDH activation by repressing authentic 3’ss of intron 4a, activity-related ATP supply by sunfish mitochondria may not be sufficient for muscle thermogenesis to cope with cold ambient temperatures, preventing predatory activities, selection for higher MMR and limiting the habitat.

Taken together, the proposed link between diversification of *Ogdh* isoforms and the acquisition of endothermy may extend beyond the Ca"superscript+"-stimulated ATP supply. OGDHC occupies a critical position between the carbohydrate, amino acid and fatty acid metabolism (50,56), which may provide clues for our understanding of the role of body mass and blubber insulation in the evolution of endothermy, both in water and terrestrial environments.

**Discussion S6 Alternative pre-mRNA splicing and endothermy: a search for candidate isoforms**

The division between endotherms and ectotherms is not absolute. Intermediate phenotypes range from mammals with lower T<sub>a</sub> and intrinsic metabolic activities (monotremes, most marsupials) or hypometabolic states (torpor, hibernation; Discussion S3) to ‘partially endothermic’ reptiles, such as Burmese python or leatherback turtles (175,176), fish, such as lamnid sharks, tunas and opah (70,177), and extinct species, including dinosaurs (178). This large body of indirect evidence and a growing support for the aerobic capacity model (179-181) strongly indicate that these regional or partial endotherms (also termed ‘mesotherms’ (178)) already harboured heritable mechanisms that promoted selection for MMR.

The acquisition of endothermy as well as other complex traits cannot be explained by a single selection event, isoform or DNA variant (165,167). Nevertheless, it is conceivable that only a limited number of molecular ‘drivers’ on the energy supply side may have been necessary for orchestrating selection processes that led to this animal innovation. This concept is compatible with the independent emergence of many ‘mesothermic’ taxa that share the same metabolic features (132,165,167,182), apparently driven largely by Ca"superscript+"-dependent muscle thermogenesis (Discussion S2). Although OGDHC activation by Ca"superscript+" is a critical step in activity-driven NADH and ATP supply (Discussion S1), activation of a single enzyme may not always produce massive increases in flux in complex metabolic pathways (183). Effective control often involves multisite modulation involving many enzymes and their isoforms (183). The TCA cycle is no exception, yet only a subset of enzymes showed positive flux coefficients (52). The multisite modulation would require coregulation of these enzymatic reactions, including tissue-specific components of the respiratory chain (66). Regulation of distant steps can be conveniently accomplished at the exon level (Figure 9B-D) and RBPs that bind accessible PPTs seem to be well-suited for this task, providing a wealth of exon activating and inhibitory effects (Figure 1).

Alternatively spliced gene segments preferentially encode peptides that are intrinsically disordered and/or contain linear interaction motifs or posttranslational modification sites (184). The resulting protein isoforms can evolve distinct function in different tissues and organisms by rewiring interaction networks through the recruitment of distinct interaction partners, contributing to the emergence of new traits (184). Which mRNA isoforms are respectable candidates for their role in the evolution of warm-bloodedness?

Assuming the validity of aerobic capacity model (179), genes encoding components of mitochondrial Ca"superscript+"-signalling pathways, many displaying Ca"superscript+"-regulated MXEs (Tables S6,S7) and tissue-specific isoform expression similar to *Ogdh*, would appear to be strong suspects. Ca"superscript+" activates
ultimately RNA processing events important in changes in particularly (de)phosphorylation humans identified in both prokaryotes and humans dissipating heat by ATP substrate cycles that occur when two metabolic pathways run simultaneously in opposite directions, response to high demand in muscles may be influenced by muscle (isometric tension differences muscles) involves alternative splicing initiation sites that produce shorter annotated transcripts (I.V., unpublished production confers high sensitivity of the subunit of F elements regulated by alternatively spliced organisms cellular ensuring the right balance between the rate and yield of ATP production investigated the enzyme NAD and CoA OGDHC is similar to the homologous MXE isocitrate DH and F many phosph the heart and skeletal muscles. This alternative splicing event is controlled by a muscle-specific exonic silencer (189), by Fox-1 (A2BP1) through GCAUG cis-elements (190), and by PUF60 (Figure 9B-D and Table S8). Fox-1 is expressed exclusively in brain and muscles (191). Examination of mitochondrial matrix proteomes in porcine hearts revealed many phosphorylation events associated with Ca$^{2+}$ signalling, including sites in OGDHC and the γ-subunit of F$_3$F$_0$-ATPase (192).

Turning to Ca$^{2+}$m transporters, a skeletal muscle- and vertebrate-specific microexon in MICU1 confers high sensitivity of the mitochondrial Ca$^{2+}$ uptake machinery and is required for sustained ATP production (193). Intronic sequences surrounding this microexon are highly conserved, including a putative UAA BP located 32 nts upstream, and the microexon is located close to alternative transcription initiation sites that produce shorter annotated transcripts (I.V., unpublished data). Impaired Ca$^{2+}$ handling was also observed for knockouts of cardiac splicing factors, including SRSF2 and SRSF10 (194).

Fourth, one of the highest fractions of tissue-specific alternative splicing was found in skeletal muscles (62). Splicing of troponin transcripts, including Ca$^{2+}$-dependent troponin C, might explain isometric tension differences of flight muscles between insect species (195) and their variable thermogenic potential. Troponin T is a central player in the Ca$^{2+}$ regulation of actin thin filament function and is essential for the contraction of striated muscles (98). The expression of slow-skeletal muscle gene (TNNT1) involves alternative splicing of exon 5, which is PUF60-dependent and is upregulated in cells lacking U2AF ((17) and J.K. and I.V., unpublished data). The hearts of dry land frogs utilize the slow-muscle TNNT1 rather than canonical cardiac TNNT2 ((98) and refs. therein). The Ca$^{2+}$-induced fast-to-slow myosin transition was associated with elevated citric synthase (196). Finally, ATP generation in response to high demand in muscles may be influenced by alternative RNA processing of transcripts encoding uncoupling proteins as well as other Ca$^{2+}$-sensitive mitochondrial enzymes, including those in the NADH glycerol-3-phosphate shuttle, a key link between lipid and carbohydrate metabolism (71).

Fifth, alternative pre-mRNA splicing is likely to play a pivotal role in the regulation of futile or substrate cycles that occur when two metabolic pathways run simultaneously in opposite directions, dissipating heat by ATP hydrolysis (157). A large number of futile cycles that consume ATP has been identified in both prokaryotes and humans (157,197), with the median length of predicted cycles in humans of ~35 and most cycles spanning several cellular compartments, often involving (de)phosphorylation (157). Selective pressure also acts against the uncontrolled dissipation of energy by avoiding the coexpression of enzymes belonging to the same substrate cycle; these selective forces are particularly strong against high-flux futile cycles (157). Futele cycles can be highly sensitive to very small changes in enzyme activities (198), which can be easily modified by alternative splicing.

In conclusion, although the candidate list is far from complete, this discussion may help identify RNA processing events important in selection processes that led to endothermy. Expanding transcriptomic and exon-level data from extant species should permit more detailed studies in the future, ultimately identifying key selection pathways en route to this highly successful animal strategy.
Discussion S7  Endothermy in monotremes and marsupials: TCA cycle clues?
Monotreme and marsupial Tₘ and metabolic rates are on average lower than those of placentals (199,200). Monotreme cell lines must be maintained at 32 °C, close to their average Tₘ, because they do not survive mean Tₘ of placentals or birds (201). Splicing of endogenous Ogdh transcripts including brain-specific exon 5 in monotremes and marsupials is similar to other mammals (Figure S9), arguing against the involvement of MXEs in reduced metabolic rates and Tₘ in this group of mammals. Except for platypus, their DNA variability within the dBP cluster is similar to other homeotherms and cannot explain a unique mammalian thermoconformity of the naked mole rat either (Figure S1), supporting the role of other molecular drivers in this group of animals.

Unlike placental mammals and birds, however, monotremes and marsupials have functional malate synthase genes, a key enzyme of the glyoxylate cycle, a TCA cycle variation present in plants, fungi, protists and bacteria (202). The glyoxylate bypass skips two rate-limiting decarboxylation steps of the TCA cycle catalyzed by IDH and OGDHC and employs isocitrate lyase and malate synthase instead (203). Mice expressing the glyoxylate shunt had reduced total liver ATP and malonyl-CoA levels compared to lacZ-injected mice and develop resistance to diet-induced obesity (204). The ATP reduction was attributed to the shunt bypassing the oxidative portion of the TCA cycle and the possibility that the NADH produced by cytosolic malate dehydrogenase was oxidized in lactate rather than shuttling back into mitochondria. The reduced ATP might explain the observed AMP-activated protein kinase activation in the HepG2 cell line constitutively expressing E. coli glyoxylate shunt genes (204). The AMP-activated kinase responds to low energy states by enhancing ATP producing pathways and inhibiting energy consuming pathways such as fatty acid biosynthesis, suggesting that despite increased fatty acid oxidation, the shunt mice generates less energy per oxidized fatty acid than the wild-type mice.

This energy dissipation resembles the mechanism by which uncoupling proteins (UCPs) regulate the whole body energy balance. UCPs decrease metabolic efficiency by dissipating the proton gradient in mitochondria, causing energy created from metabolism to be released as heat. UCPs have been studied as candidate proteins for the transition from ecto- to endothermy, with the most prominent member UCP1 proposed to facilitate non-shivering thermogenesis in mammalian BAT (205). However, an avian UCP is not involved in uncoupling of respiration and metabolic heat (206), birds do not possess BAT and there is no evidence for BAT thermogenesis in marsupials and no evidence for BAT in monotremes ((199,206) and refs. therein). The activity of UCPs appears to modulate the efficiency of oxidative phosphorylation, presumably by catalyzing proton re-entry into the matrix (207). However, at least some of the physiological effects of UCPs might be explained by Ca²⁺ transport, with UCP2/3 facilitating Ca²⁺ flux across the inner mitochondrial membrane and not directly supporting thermogenic function (208). UCPs were proposed to be essential for Ca²⁺ uptake to mitochondria, but animal knockouts revealed little or no effect (209,210). Mice overexpressing UCPs in skeletal tissue resisted diet-induced obesity and the liver-specific UCP1 expression was associated with increased energy expenditure and decreased liver triglycerides (204) and refs. therein).

Taken together, similar to UCPs, the glyoxylate shunt may alter the energy state of the cell in part by decreasing the energy produced from fatty acid oxidation. This TCA branch point could interfere with cellular energy needs in vertebrates that express relics of the shunt pathway. We speculate that the flux diversion via malate synthase and other activities could reduce ATP supply and modulate evolutionary pathways that led to typical homeothermy.

Discussion S8  A timeline of OGDH MXE regulation and evolution of endothermy in vertebrates
The DADLD motif is present in multiple fish species (4), in which tissue-specific MXE patterns and regulation do not appear to be fully developed (Figure S6, S9, S13) and may not be sufficient for optimal Ca²⁺-dependent control of Ogdh in their water milieu. Fish have the widest range of Ogdh intron 4a length diversity, only a single primordial dBP and the largest bone fish, but not other fish, have a reduced AGEZ (Figure 1B, 6E, S13; Discussion S5). However, teleost fish already show correlated mass- and temperature-adjusted BMR and MMR (180). Fish with more active lifestyles have higher BMR when comparing various taxa living at similar temperatures. In pelagic teleost predators, BMR correlates with
protein-rich skeletal musculature and caudal fin aspect ratios, but not with the brain mass (180). Fish predators exhibit regional endothermy more often than the opposite end of their life-style continuum, such as sluggish, sit-and-wait cyprinids, which have low BMR and high tolerance to hypoxia (180). This continuum was linked to body mass, with a trend toward less active life style in bigger marine species (173). Partially endothermic fish such as tunas were found to have a higher proportion of slow-twitch muscles relative to fast-twitch counterparts than other teleosts ((211) and refs. therein; Discussion S2). Some sharks such as Callorhinchus millii lack the DADLD motif and would be expected to exhibit greatly attenuated activation of OGDHC (4,60). More generally, reduced capacity to activate OGDHC could manifest as intolerance to low ambient temperatures, selective stenothermal altitude and largely coastal habitats. In any case, this vertebrate class shows convincing signs of early selection for endothermy and must have already possessed a molecular ‘gas-pedal’ mechanism for sustained energy supply to muscles. Fish will therefore continue to provide an ideal taxonomic context for studying early events leading to endothermy (182).

With only one dominant and nonredundant dBP in long intron 4a, the regulatory OGDH MXE potential in amphibians may still be suboptimal (Figure 1B, S6, S12-S13). However, primary transcripts in X. laevis already show efficient muscle-specific promotion of exon 4b (Figure S9) and frog OGDHC is activated by Ca\(^{2+}\) even if addition of Ca\(^{2+}\) decreased the K\(_m\) value for 2OG to a lesser extent than in placentals (29) (Figure S15). Amphibians also show a brain-specific repression of isoforms 4b+ together with activation of exon 5 (Figure S9) (Discussion S4). The extra codon in exon 4a in fish and amphibians, which is absent in reptiles, birds and mammals (Figure S1), promotes the inclusion levels of this exon in mature transcripts (Figure 5), which could limit maximum Ca\(^{2+}\)-mediated OGDHC activation by isoform 4b+.

A narrow range of intron 4a size typical of mammals was first achieved in reptiles, barring crocodilians (Figure 1C and S1). The length of crocodilian intron 4a is intermediate between endo- and ectotherms (Figure 1C), probably reflecting an exceptionally slow evolutionary rate of microdeletions (212). This rate was much slower than that of lizards, which have the intron 4a length typical of mammals. Nevertheless, the aerobic scope and tidal volume of alligators are close to values observed in endotherms although the total power generated by maximally active crocodiles is lower than in mammals of the same size (213,214). However, this reptile class has evolved piston lungs and four-chambered hearts, which prevent mixing of de- and oxygenated blood. This separation is typical of homeotherms and can deliver blood to tissues at higher pressure, facilitating adaptive tissue oxygen supply and oxidative phosphorylation. More generally, reptiles already exhibit many features of mammalian or avian endothermy, including the ability to enhance metabolic rate by muscular activities decoupled from locomotion, circadian T\(_b\) rhythms not associated with activity and a sophisticated peripheral vascular control, indicating that they already possess selection instruments for incipient facultative endothermy ((132,175,176) and refs. therein). Field factorial scopes, which may better reflect sustained aerobic performance than MMR, are quite similar for reptiles, mammals and birds (166). Some reptiles, such as brooding pythons, efficiently integrate shivering thermogenesis and basking, which is not dissimilar to torpidating marsupials, suggesting that reptiles rather than fish could be ‘proendothorms’ (132). Stronger skeletal muscles in terrestrial reptiles would stimulate muscle thermogenesis (Discussion S2), which has been best studied in brooding pythons and leatherback turtles (132,175,176). For example, deep T\(_b\) of leatherback turtles, the largest living marine turtles, is higher than water temperature as a result of muscular activity, which increases as surrounding temperature is lowered (176,215). Leatherback turtles have a high energy intake and are highly migratory, up to 18,000 km between tropical and northern foraging grounds (216) and refs. therein). The reptile dBP cluster resembles that of birds, however, their dBPs are upstream of megaPPTs with a lower U content (Figure S7). This might reduce the repertoire of U-bound regulators and lead to a less responsive regulation of MXE usage.

Retention of metabolic heat by feathers or fur would help conserve the energy and maintain higher T\(_b\) for longer ((166) and refs. therein). T\(_b\) of birds is on average significantly higher than mammalian T\(_b\) (217 and refs. therein). Alternative pre-mRNA splicing in birds is already extensive although chicken genes still generate, on average, fewer transcripts per gene and have shorter introns than
humans or mice (218). The *Ogdh* endogenous splicing pattern in the brain and viscera of *C. japonica* is similar to the mammalian pattern (Figure S9). The avian dBP cluster, however, largely employs efficient orthologues of low-usage human dBP+41 (Figure 3H and 6D), which has the optimal dBP motif UAA (Figure S1, S4). The dominant bird dBP is also more accessible than the human counterpart (Figures 3, 6-8) and seems to contribute to the excessive exon 4b+/4a+ ratios observed for exogenous chicken transcripts (Figure S13).

In contrast to birds, **monotremes** and most **marsupials** have lower T_b than placental mammals (Discussion S7). The length of platypus intron 4a is intermediate between aquatic and terrestrial species and the platypus dBP+31 orthologue is inefficient, but the endogenous splicing pattern of tested monotremes and marsupials was similar to placentals (Figure S9). Finally, the tentative role of OGDHC in hypometabolic states of **mammalian hibernators** is explained in Discussion S3.

Collectively, this timeline suggests that changes in the *Ogdh* MXE regulation influenced the capacity of animals to optimize Ca^{2+}-mediated responses to sustained energy demand and maximum locomotory endurance *en route* to endothermy.
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