Mapping Epigenetic Divergence in the Massive Radiation of Lake Malawi Cichlid Fishes

AUTHORS

Grégoire Vernaz[1,2,3*], Milan Malinsky[4], Hannes Svardal[5,6], Mingliu Du[1,2,3], Alexandra M. Tyers[7], M. Emília Santos[8], Richard Durbin[2,3], Martin J. Genner[9], George F. Turner[10] & Eric A. Miska[1,2,3*]

* Correspondence to: gv268@cam.ac.uk (GV), eam29@cam.ac.uk (EAM)

1. Wellcome/CRUK Gurdon Institute, University of Cambridge, Cambridge, UK;
2. Department of Genetics, University of Cambridge, Cambridge, UK;
3. Wellcome Sanger Institute, Cambridge, UK;
4. Zoological Institute, University of Basel, Basel, Switzerland;
5. Department of Biology, University of Antwerp, Antwerp, Belgium;
6. Naturalis Biodiversity Center, Leiden, The Netherlands;
7. Max Planck Institute for Biology of Ageing, Cologne, Germany;
8. Department of Zoology, University of Cambridge, UK;
9. School of Biological Sciences, University of Bristol, Bristol, UK;
10. School of Natural Sciences, Sciences, Bangor University, Bangor, UK.

ABSTRACT

Epigenetic variation modulates gene expression and can be heritable. However, knowledge of the contribution of epigenetic divergence to adaptive diversification in nature remains limited. The massive evolutionary radiation of Lake Malawi cichlid fishes displaying extensive phenotypic diversity despite extremely low sequence divergence is an excellent system to study the epigenomic contribution to adaptation. Here, we present the first comparative genome-wide methylome and transcriptome study, focussing on liver and muscle tissues in phenotypically divergent cichlid species. In both tissues we find substantial methylome divergence among species. Differentially methylated regions (DMR), enriched in evolutionary young transposons, are associated with transcription changes of ecologically-relevant genes related to energy expenditure and lipid metabolism, poiting to a link between dietary ecology and methylome divergence. Unexpectedly, half of all species-specific DMRs are shared across tissues and are enriched in developmental genes, likely reflecting distinct epigenetic developmental programmes. Our study reveals substantial methylome divergence in closely-related cichlid fishes and represents a valuable resource to study the role of epigenetics in species diversification.

INTRODUCTION

Trait inheritance and phenotypic diversification are primarily explained by the transmission of genetic information encoded in the DNA sequence. In addition, a variety of epigenetic processes have recently been reported to mediate heritable transmission of phenotypes in animals and plants[1–6]. However, current understanding of the evolutionary significance of epigenetic processes, and of their roles in organismal diversification, is in its infancy.

DNA methylation, or the covalent addition of a methyl group onto the 5th carbon of cytosine (mC) in DNA, is a reversible epigenetic mark present across multiple kingdoms[7–9], can be heritable, and has been linked to transmission of acquired phenotypes in plants and animals[2,5,6,10–12]. The importance of this mechanism is underlined by the fact that proteins involved in the deposition of mC ('writers', DNA methyltransferases [DNMTs]), in mC maintenance during cell division, and in the removal of mC ('erasers', Tet methylcytosine dioxygenases [TETs]), are mostly essential and show high degrees of conservation across vertebrates species[13–16]. In addition, some ancestral functions of methylated cytosines are highly conserved, such as in the transcriptional silencing of exogenous genomic elements (transposons)[17,18]. In vertebrates, DNA methylation functions have evolved to play an important role in the orchestration of cell differentiation during normal embryogenesis/development through complex interactions with histone post-translational modifications (DNA accessibility) and mC-sensitive readers (such as transcription factors)[18–24] in particular at *cis*-regulatory regions (i.e. promoters, enhancers). Early-life establishment of stable DNA methylation patterns can thus affect transcriptional activity in the embryo and persist into fully differentiated cells[25]. DNA methylation variation has also been postulated to have evolved in the context of natural selection by promoting phenotypic plasticity and thus possibly facilitating adaptation, speciation and adaptive radiation[2,4,11,26].

Studies in plants have revealed how covarying environmental factors and DNA methylation variation underlying stable and heritable transcriptional changes in adaptive traits[2,6,10–12,27]. Some initial evidence is also present in vertebrates [2,5,28–30]. In the cavefish for example, an early developmental process - eye degeneration - has been shown to be mediated by DNA methylation, suggesting mC variation as an evolutionary factor generating adaptive phenotypic plasticity during development and evolution[28,31]. However, whether correlations between environmental variation and DNA methylation patterns promote phenotypic diversification more widely among natural vertebrate populations remains unknown.

In this study we sought to quantify, map and characterise natural divergence in DNA methylation in the context of the Lake Malawi haplochromine cichlid adaptive radiation, one of the most spectacular examples of rapid vertebrate phenotypic diversification[32]. In total, the radiation comprises over 800

endemic species[33], that are estimated to have evolved from common ancestry approximately 800,000 years ago[34]. Species within the radiation can be grouped into seven distinct ecomorphological groups based on their ecology, morphology and genetic differences: (1) shallow benthic, (2) deep benthic, (3) deep pelagic zooplanktivorous/piscivorous *Diplotaxodon*, (4) the rock-dwelling 'mbuna', (5) zooplanktivorous 'utaka', (6) *Astatotilapia calliptera* specialised for shallow weedy habitats (also found in surrounding rivers and lakes), and (7) the midwater pelagic piscivores *Rhamphochromis*[35,36]. Recent large-scale genetic studies have revealed that the Lake Malawi cichlid flock is characterised by an overall very low genetic divergence among species (0.1-0.25%), combined with a low mutation rate, a high rate of hybridisation and extensive incomplete lineage sorting (shared retention of ancestral genetic variation across species)[33,35,37,38]. Multiple molecular mechanisms may be at work to enable such an explosive phenotypic diversification. Therefore, investigating the epigenetic mechanisms in Lake Malawi cichlids represents a remarkable opportunity to expand our comprehension of the processes underlying phenotypic diversification and adaptation.

Here we describe, quantify, and assess the divergence in liver methylomes in six cichlid species spanning five of the seven ecomorphological groups of the Lake Malawi haplochromine radiation by generating high-coverage whole-genome liver bisulfite sequencing (WGBS). We find that Lake Malawi haplochromine cichlids exhibit substantial methylome divergence, despite conserved underlying DNA sequences, and is enriched in evolutionary young transposable elements. Next, we generated whole liver transcriptome sequencing (RNAseq) in four of the six species and showed that differential transcriptional activity is significantly associated with between-species methylome divergence, most prominently in genes involved in key hepatic metabolic functions. Finally, by generating WGBS from muscle tissues in three cichlid species, we show that half of methylome divergence between species is tissue-unspecific and pertains to embryonic and developmental processes, possibly contributing to early establishment of phenotypic diversity. This represents the first comparative analysis of natural methylome variation in Lake Malawi cichlids and provides initial evidence for substantial species-specific epigenetic divergence in *cis*-regulatory regions of ecologically-relevant genes. Our study represents a valuable resource, laying the groundwork for future epigenomic research in the context of phenotypic diversification and adaptation.

RESULTS

The methylomes of Lake Malawi cichlids feature conserved vertebrate characteristics

To characterise the methylome variation and assess possible functional relationships in natural populations of Lake Malawi cichlids, we performed high-coverage whole-genome bisulfite sequencing of methylomes (WGBS) from liver tissues of six different cichlid species. Muscle methylome (WGBS) data for three of the six species were also generated to assess the extent to which methylome divergence was tissue specific. Moreover, to examine the correlation between transcriptome and methylome divergences, total transcriptomes (RNAseq) from both liver and muscle tissues of four species were generated. Only wild-caught male specimens (2-3 biological replicates for each tissue and each species) were used for all sequencing datasets (Fig. 1a-c, Supplementary Fig. 1, Supplementary Tables 1-2). The species selected were: *Rhamphochromis longiceps* (RL), a pelagic piscivore (Rhamphochromis group); *Diplotaxodon limnothrissa* (DL), *a* deep-water pelagic carnivore (Diplotaxodon group); *Maylandia zebra* (MZ) and *Petrotilapia genalutea* (PG), two rock-dwelling algae eaters (Mbuna group); *Aulonocara stuartgranti* (AS), a benthic invertebrate-eating sand/rock-dweller that is genetically part of the deep-benthic group; *Astatotilapia calliptera* (AC), a species of rivers and lake margins[39] (Fig. 1b).

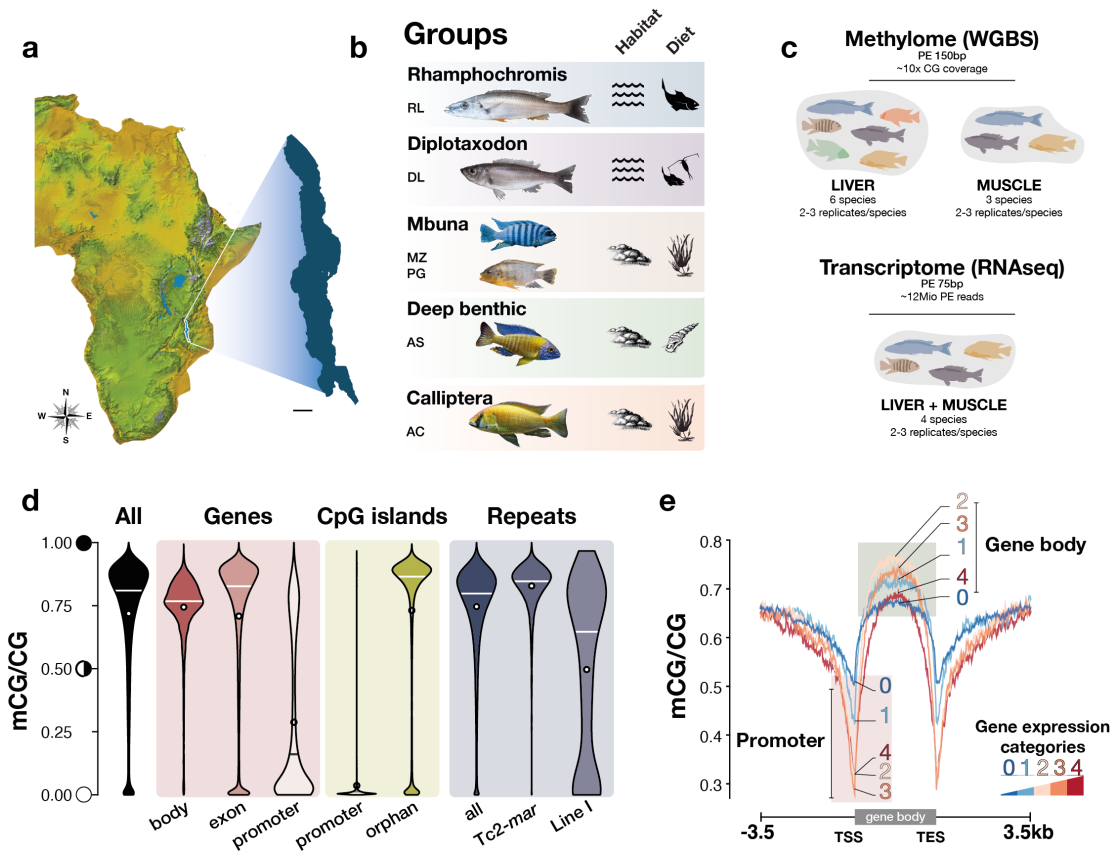

Fig. 1. The methylome of Lake Malawi cichlids. a. Satellite map of Africa and magnification of Lake Malawi (scale bar: 20km). **b**. Photographs (not to scale) of the six Lake Malawi cichlid species part of this study spanning five of the seven described eco-morphological groups. The symbols represent the different habitats (pelagic/benthic [wave symbol], rock/sand-dwelling/littoral [rock symbol]) and the type of diet (fish, fish/zooplankton, algae, invertebrates) for each group. The species representing each group are indicated by their initials. **c**. Diagram summarising the sampling and sequencing strategies for liver and muscle

methylome (whole-genome bisulfite sequencing, WGBS) and whole transcriptome (RNAseq) datasets. See Materials and Methods, Supplementary Fig.1 and Supplementary Table 2. **d**. Violin plots showing the distribution of liver DNA methylation levels in CG sequence context (mCG/CG) in different genomic regions. Average mCG levels over different genomic regions: genome-wide (50bp non-overlapping windows), gene bodies, exons, promoter regions (TSS±500bp), CpG-islands in promoters and outside (orphan) and in repeat/transposon regions. mC levels for two different repeat classes are given: DNA transposon superfamily Tc2-Mariner (*n*=5,378) and LINE I (*n*=407). **e.** Average liver mCG profiles across genes differ depending on their transcriptional activity in liver: from non-expressed (0) to genes showing low (1), intermediate (2), high (3) and highest (4) expression levels (Methods). Results shown in **d.** and **e.** are for Mbuna MZ (liver, *n*=3) and are representative of the results for all other species, and are based on average mC/C in 50bp non-overlapping windows. *RL, Rhamphochromis longiceps; DL, Diplotaxodon limnothrissa; MZ, Maylandia zebra; PG, Petrotilapia genalutea; AS, Aulonocara stuartgranti; AC, Astatotilapia calliptera.* Credits - Fish photographs: Hannes Svardal and M. Emília Santos. Satellite map, NASA WorldView.

On average, 285.51±55.6 million paired-end reads (See Supplementary Table 1) for liver and muscle methylomes were generated with WGBS, yielding ~10-15x per-sample coverage at CG dinucleotide sites (Supplementary Fig. 2a; see Methods and Supplementary notes). To account for species-specific genotype and avoid methylation biases due to species-specific single nucleotide polymorphism (SNP), WGBS reads were mapped to species-specific SNP-corrected versions of the *Maylandia zebra* reference genome (UMD2a; see Methods). Mapping rates were not significantly different among all WGBS samples (Dunn's test, with Bonferroni correction, $P>0.0.5$; Supplementary Fig. 2e), reflecting the high level of conservation at the DNA sequence level across the Malawi radiation (Supplementary Fig. 3). In parallel, liver and muscle transcriptomes were generated for four species using the same specimens as used for WGBS, yielding on average 11.9±0.7 million paired-end reads (Fig. 1c, Supplementary Table 1 and Methods).

We first characterised global features of the methylome of Lake Malawi cichlids. The genome of Lake Malawi cichlid was found to have copies of DNA methyltransferases (DNMTs) and ten-eleven translocation methylcytosine dioxygenases (TETs), the 'readers' and 'erasers' of DNA methylation respectively (Supplementary Fig. 4a-c). Like that of mammals and other teleost fish, the genomes of Lake Malawi cichlids have high levels of DNA methylation genome-wide in the CG dinucleotide sequence context, consistent across all samples in both tissues analysed (Fig. 1d and Supplementary Fig. 2a-c). Gene bodies generally show higher methylation levels than the genome-wide average, while the majority of promoter regions are unmethylated (Fig. 1d). CpG island (CGIs; i.e. CpG-rich regions - abundant in Lake Malawi cichlid genomes; Supplementary Fig. 5a-c, e, Supplementary notes and Methods), are almost entirely devoid of methylation in promoters, while 'orphan' CGIs, residing outside promoters, are mostly highly methylated (Fig. 1d and Supplementary Fig. 5 f, g). While 70% of mammalian promoters contain CGIs[40], only 15-20% of promoters in Lake Malawi cichlids harbour CGIs (Supplementary Fig. 5d), similar to frog and zebrafish genomes[40]. Notably, orphan CGIs which may have important *cis*-regulatory functions[41] compose up to 80% of all predicted CGIs in Lake Malawi cichlids (Supplementary Fig. 5e). Furthermore, repetitive regions as well as transposable elements are particularly enriched for cytosine methylation, suggesting a methylation-mediated

silencing of their transcription (Fig. 1d, Supplementary Fig. 6d), similar to that observed in zebrafish and other animals[7,17]. Interestingly, certain transposon families, such as LINE I and Tc2-*Mariner*, part of the DNA transposon family - the most abundant TE family predicted in Lake Malawi cichlid genome (Supplementary Fig. 6a-b, Supplementary notes and Ref.[37]) - have recently expanded considerably in the Mbuna genome (Supplementary Fig. 6c and Refs.[37,42]). While Tc2-*Mar* DNA transposons show the highest median methylation levels, LINE I elements have some of the lowest, yet most variable, methylation levels of all transposon families, which correlates with their evolutionary recent expansion in the genome (Fig. 1d-e and Supplementary Fig. 6d-e). Finally, transcriptional activity in liver and muscle tissues of Lake Malawi cichlids was negatively correlated with methylation in promoter regions (Spearman's correlation test, $\rho = -0.40$, $p<0.002$), while being weakly positively correlated with methylation in gene bodies ($\rho = 0.1$, $p<0.002$; Fig. 1e and Supplementary Fig. 7 a-d and Supplementary Table 3). This is consistent with previous studies highlighting high methylation levels in bodies of active genes in plants and animals, and high levels of methylation at promoters of weakly expressed genes in vertebrates[7,23]. We conclude that the methylomes of Lake Malawi cichlids share many regulatory features, and possibly associated functions, with those of other vertebrates, which renders Lake Malawi cichlids a promising model system in this context.

Methylome divergence in Lake Malawi cichlids

To assess the possible role of DNA methylation in phenotypic diversification, we then sought to quantify and characterise the differences in liver and muscle methylomes across the genomes of Lake Malawi haplochromine cichlids. Despite overall very low sequence divergence[35] (Supplementary Fig. 3), Lake Malawi cichlids were found to show substantial methylome divergence across species within each tissue type, while within-species biological replicates always clustered together (Fig. 2a). The species relationships inferred by clustering of the liver methylomes at conserved individual CG dinucleotides recapitulate some of the genetic relationship inferred from DNA sequence[35], with one exception – the methylome clusters *A. calliptera* samples as an outgroup, not a sister group to Mbuna. This is consistent with its unique position as a riverine species, while all species are obligate lake dweller (Fig. 2a and Supplementary Fig. 3a, b).

As DNA methylation variation tends to correlate over genomic regions consisting of several neighbouring CG sites, we defined and sought to characterise differentially methylated regions (DMRs) among Lake Malawi cichlid species (≥50bp-long, ≥4 CG dinucleotide and ≥25% methylation difference across any pair of species, $p<0.05$; see Methods). In total, 13,331 between-species DMRs were found among the liver methylomes of the six cichlid species (Supplementary Fig.8 a). We then compared the three species for which liver and muscle WGBS data were available and found 5,875 and 4,290 DMRs among the liver and muscle methylomes, respectively. By contrast, 27,165 within-species DMRs were found in the between-tissue comparisons (Supplementary Fig. 8 b). Overall, DMRs

in Lake Malawi cichlids were predicted to be as long as 5,000bp (95% CI of median size: 282-298bp). While the methylation differences between liver and muscle were the most prominent at single GC dinucleotide resolution (Fig. 2a), and resulted in the highest number of DMRs, we found DMRs to be slightly larger and methylation differences within them substantially stronger among species than between tissues (Dunn's test, $p<2.2\times10^{-16}$; Supplementary Fig. 8 c-d).

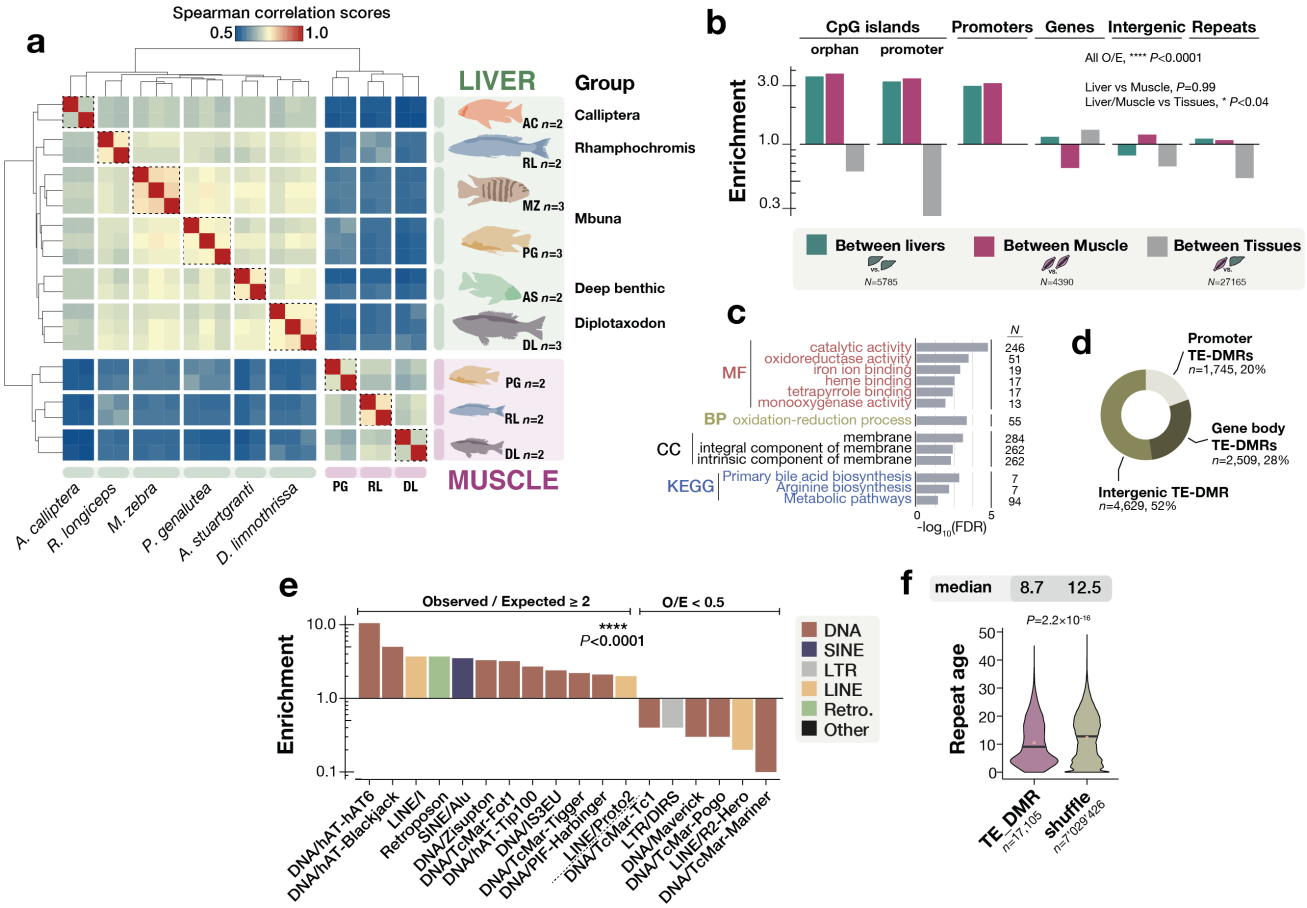

Fig. 2. Species-specific methylome divergence in Lake Malawi cichlids is enriched in promoters, CpG-islands and young transposons. a. Unbiased hierarchical clustering and heatmap of Spearman correlation scores for genome-wide methylome variation in Lake Malawi cichlids at conserved CG dinucleotides. Dotted boxes group samples by species within each tissue. **b.** Observed/Expected ratios (O/E ratio, enrichment) for some genomic localisation of differentially methylated regions (DMRs) predicted between livers and between muscles of three Lake Malawi cichlid species (green and purple, respectively) and between tissues (within-species, right, grey) - χ^2 tests, $p<0.0001$. Expected values were determined by randomly shuffling DMRs of each DMR type across the genome (1000 iterations). Categories are not mutually exclusive. **c.** Gene ontology (GO) enrichment for DMRs found between liver methylomes localised in promoters. GO terms: Kyoto Encyclopaedia of Genes and Genomes (KEGG), molecular functions (MF), cellular component (CC) and biological processes (BP). Only GO terms with FDR<0.05 shown. N indicates the number of genes associated with each GO term. **d.** Genomic localisation of liver DMRs containing repeats/transposons (TE-DMRs). **e.** O/E ratios for species TE-DMRs for each TE family. Only O/E≥2 and ≤0.5 shown. χ^2 tests, $p<0.0001$. **f.** Boxplots showing TE sequence divergence (namely, CpG-adjusted Kimura substitution level as given by RepeatMasker) in *M. zebra* genome for species TE-DMRs, TEs outside species DMRs ('outside') and randomly shuffled TE-DMRs (500 iterations, 'shuffle'). Mean values indicated by red dots, median values by black lines and shown above each graph. Total DMR counts indicated below each graph. DMR, differentially methylated region; TE, repeat/transposon regions; CGI, predicted CpG islands. *p*-values for Dunn's multiple comparison tests shown.

Next, we characterised the genomic features enriched for between-species methylome divergence in the three cichlid species for which both muscle and liver WGBS data were available (i.e., RL, PG, DL; Fig. 1c). In the liver, promoter regions and orphan CGIs have 3.0- and 3.6-fold enrichment respectively for between-species liver DMRs over random expectation (χ^2 test, $p<0.0001$; Fig. 2b) - between-species muscle DMRs show similar patterns as well ($P=0.99$, compared to liver O/E ratios). Methylome variation at promoter regions has been shown to affect transcription activity via a number of mechanisms (e.g. transcription factor binding affinity, chromatin accessibility)[20,43] and, in this way, may participate in phenotypic adaptive diversification in Lake Malawi cichlids. In particular, genes with promoter DMRs show enrichment for enzymes involved in hepatic metabolic functions (Fig. 2c). Furthermore, the high enrichment of DMRs in intergenic orphan CGIs (Fig. 2b), accounting for $n=691$ (11.94%) of total liver DMRs, suggests that intergenic CGIs may have DNA methylation-mediated regulatory functions.

The majority of between-species liver DMRs (65.0%, $n=3{,}764$) are within TE regions (TE-DMRs; Supplementary Fig. 8 a-b, e), approximately two-thirds of which are located in unannotated intergenic regions (Fig. 2d). However, a small fraction of TE-DMRs are located in gene promoters (12% of all TE-DMRs) and are significantly enriched in genes associated with metabolic pathways (Fig. 2d and Supplementary Fig. 8f). While there is only a 1.1-fold enrichment of DMRs globally across all TEs (Fig. 2b), some TE families are particularly enriched for DMRs, most notably the DNA transposons hAT (hAT6, 10.5-fold), LINE/l (>3.7-fold) and the retrotransposons SINE/Alu (>3.5-fold). On the other hand, the degree of methylation in a number of other TE families shows unexpected conservation among species, with substantial DMR depletion (e.g., LINE/R2-Hero, DNA/Maverick; Fig. 2e). Overall, we observe a pattern whereby between-species methylome differences are significantly localised in younger transposon sequences (Dunn's test, $p=2.2\times10^{-16}$; Fig. 2f). Differential methylation in TE sequences may affect their transcription and transposition activities, possibly altering or establishing new transcriptional activity networks via *cis*-regulatory functions[44–46]. Indeed, movement of transposable elements has recently been shown to contribute to phenotypic diversification in Lake Malawi cichlids[47].

In contrast to the between-species liver DMRs, within-species DMRs based on comparison of liver against muscle methylomes show much less variation in enrichment across genomic features. Only gene bodies show weak enrichment for methylome variation. Moreover, both CGI classes, as well as repetitive and intergenic regions show considerable tissue-DMR depletion, suggesting a smaller DNA methylation-related contribution of these elements to tissue differentiation (Fig. 2b and Supplementary Fig. 8e).

Methylation divergence is associated with changes in transcriptional activity and altered transcription factor activity in the livers

We hypothesised that adaptation to different diets in Lake Malawi cichlids could be associated with distinct hepatic functions, manifesting as differences in transcriptional patterns which, in turn, could be influenced by divergent methylation patterns. To investigate this, we first performed differential gene expression analysis (Fig. 1c). In total, 3,437 genes were found to be differentially expressed between livers of the four Lake Malawi cichlid species investigated (RL, DL, MZ and PG; Walt test, false discovery rate adjusted *p*-value using Benjamini-Hochberg *(*FDR)<0.01; Fig. 3a and Supplementary Fig. 9a-c; see Methods and Supplementary notes). As with methylome variation, transcriptome variation clustered individuals by species (Supplementary Fig. 9d), consistent with species specific functional liver transcriptome activity.

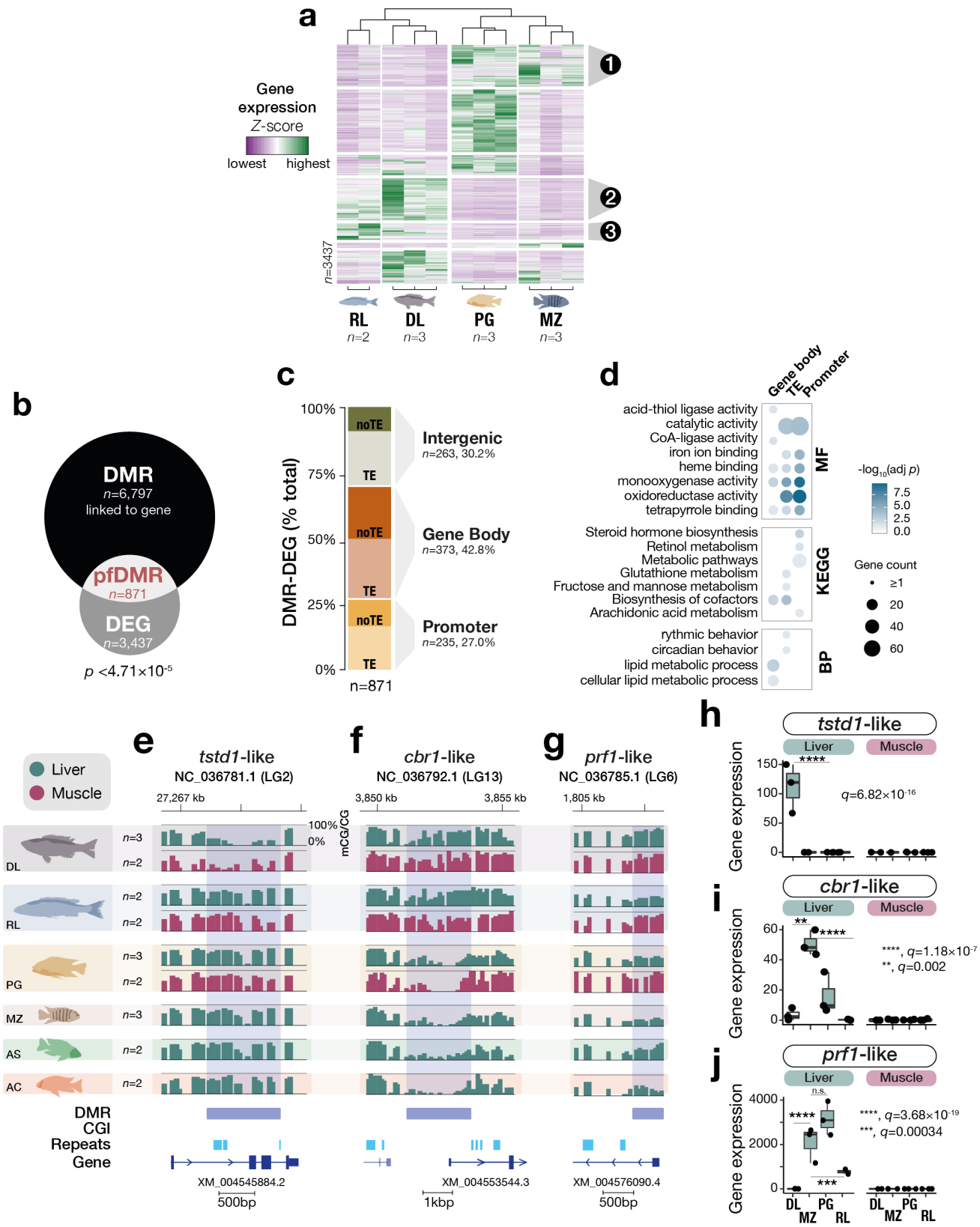

Fig. 3. Methylome divergence is associated with differential transcriptional activity in Lake Malawi cichlids. a. Heatmap and unsupervised hierarchical clustering of gene expression values (*Z*-score) of all differentially expressed genes (DEGs) found among livers of four Lake Malawi cichlid species (false discovery rate FDR<1%). GO enrichment analysis for three different DEG clusters are shown in Supplementary Fig. 9c. **b.** Overlap between DEG and differentially expressed regions (DMRs; p<0.05) that could be linked to a gene (significant overlap, exact hypergeometric test) highlighting putative functional DMRs (pfDMRs). **c.** Bar plot showing the percentage of DMRs associated with significant gene expression (pfDMRs) localised in either promoters, intergenic regions (0.5-4kbp away from genes) or in gene bodies, with proportion of TE content for each group (TE/noTE). **d.**

Heatmap representing significant GO terms for differentially expressed gene associated with DMRs, given for each DMR genomic feature. GO categories: BP, Biological Process; MF, Molecular Function. Only GO terms with *P* adj<0.05 shown. **e-g.** Examples of DMRs (highlighted in pale blue) with significant association with liver species-specific transcriptional changes for the genes thiosulfate:glutathione sulfurtransferase *tstd1*-like (LOC101468457) (**e.**), carbonyl reductase [NADPH] 1 *cbr1*-like (LOC101465189) (**f.**) and perforin-1 *prf1*-like (LOC101465185) (**g.**). Liver and muscle methylome profiles in green and purple, respectively (averaged % mCG/CG levels in 50bp bins for ≥2 samples per tissue; scale indicated above each graph). **h-j.** Boxplots showing gene expression values for each of the genes in **e-g.** in livers (green) and muscle (pink). Averaged tpm values per tissue per species. Walt-test, sleuth: *q* values are shown in graphs. CGI, CpG islands; Repeats, transposons and repetitive regions.

Next, we checked for association between liver DMRs and transcriptional changes. Of the 6,797 among-species DMRs that could be assigned to a specific gene (i.e., DMRs within promoters, gene bodies or located 0.5-4kbp away from a gene; see Methods), 871 were associated with differentially expressed genes, which is greater than expected by chance (Fig. 3a, b; $p<4.7\times10^{-5}$), suggesting that DMRs is associated with liver gene expression. Of these 871 putative functional DMRs (pfDMRs), the majority (42.8%) are localised over gene bodies, hinting at possible intronic *cis*-regulatory elements or alternative splicing[48]. The remaining pfDMRs are in intergenic (30.2%) or promoters (27%) (Fig. 3c). The majority of pfDMRs contain younger TE sequences, in particular in intronic regions, while only few contain CGIs (Supplementary Fig. 10a-c). In promoters and intergenic regions, ≥63% of pfDMR sequences contain TEs (Fig. 3c). As methylation levels at *cis*-regulatory regions may be associated with altered transcription factor (TF) activity[21,23,24], we performed TF binding motif enrichment analysis using between-species liver DMRs and found significant enrichment for specific TF recognition binding motifs. Several TF genes known to recognise some of the enriched binding motifs are differentially expressed among the livers of the three cichlid species and have liver-associated functions (Supplementary Fig. 10d-e). For example, the gene of the transcription factor hepatocyte nuclear factor 4 alpha (hnf4a), with important functions in lipid homeostasis regulation and in liver-specific gene expression[49], is >2.5x fold downregulated (qval≤ 9×10^{-5}) in the rock-dwelling algae-eater *P. genalutea* compared to the pelagic piscivores *D. limnothrissa* and *R. longiceps*, possibly in line with adaptation to different diets (Supplementary Fig. 10e).

Furthermore, genomic regions containing pfDMRs are also significantly associated in the livers with altered transcription of many other genes involved in hepatic and metabolic oxidation-reduction processes (Fig. 3d and Supplementary Fig. 10g). These include genes encoding haem-containing cytochrome P450 enzymes (such as cyp3a4, cy7b1; Supplementary Fig. 10g), which are important metabolic factors in steroid and fatty acid metabolism, as well as genes encoding other hepatic enzymes involved in energy balance processes. This enrichment is associated with significant methylome divergence among species, in particular in promoter regions and gene bodies (Fig. 3d). For example, the gene sulfurtransferase *tstd1*-like, an enzyme involved in energy balance and mitochondrial metabolism is expressed exclusively in the liver of the deep-water pelagic species *D. limnothrissa* where it shows ~80% reduced methylation levels in a gene-body DMR compared to all the other species

(Fig. 3e, h). Another example is the promoter of the enzyme carbonyl reductase [NADPH] 1 (*cbr1*) which shows significant hypomethylation (2.2kbp-long DMR) in the algae-eaters MZ and PG, associated with up to ~60-fold increased gene expression in their livers compared to the predatory *Rhamphochromis* and *Diplotaxodon* (Fig. 3f, i). Interestingly, *cbr1* is involved in the metabolism of various fatty acids in the liver and has been associated with fatty acid-mediated cellular signalling in response to environmental perturbation[51]. As a final example, we highlight the cytotoxic effector perforin 1-like (*prf1*-like), an important player in liver-mediated energy balance and immune functions[52]. Its promoter is hypermethylated (>88% mCG/CG) exclusively in the liver of the deep-water species DL, while having low methylation levels (~25%) in the four other species (Fig. 3g). This gene is not expressed in DL livers but is highly expressed in the livers of the other species that all show low methylation in the promoter (Fig. 3j). Taken together, these results suggest that species-specific methylome divergence is associated with transcriptional remodelling of ecologically-relevant genes, which might facilitate phenotypic diversification associated with adaption to different diets.

Multi-tissue methylome divergence is enriched in genes with functions related to early developmental processes

We further hypothesised that between-species DMRs that are found in both the liver and muscle methylomes could relate to functions associated with early development/embryogenesis. Given that liver is endoderm-derived, and muscle is mesoderm-derived, such shared multi-tissue DMRs could be involved in processes that find their origins prior to or early in gastrulation. Such DMRs could also have been established early on during embryogenesis and may have core cellular functions. Therefore, we focussed on the three species for which methylome data were available for both tissues (Fig.1c) to explore the overlap between muscle and liver DMRs (Fig. 4a). Based on pairwise species comparisons (Supplementary Fig. 11a-b), we identified methylome patterns unique to one of the three species. We found that 40-48% of these were found in both tissues ('multi-tissue' DMRs), while 39-43% were liver-specific and only 13-18% were muscle-specific (Fig. 4b).

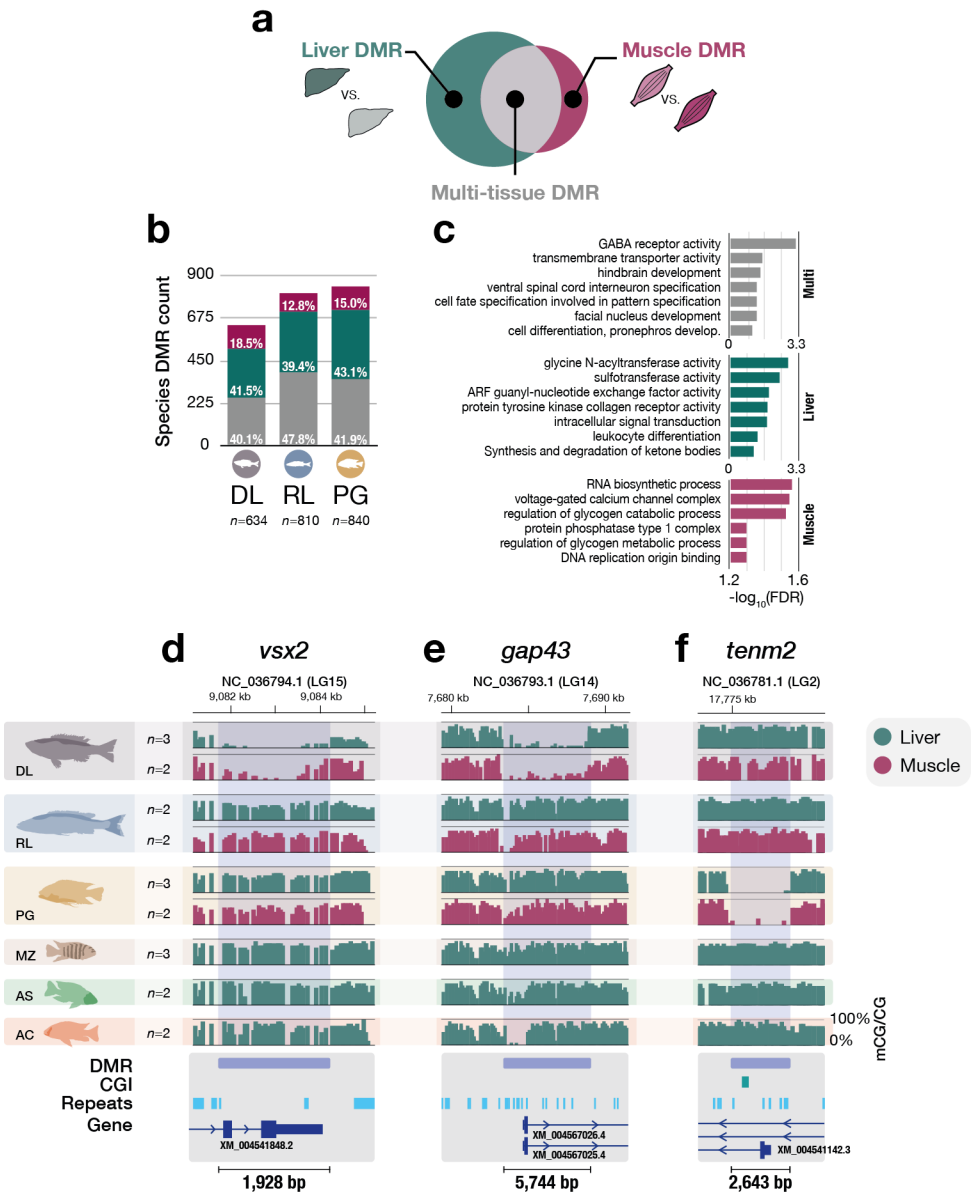

Fig. 4 Multi-tissue methylome divergence in Lake Malawi cichlids is associated with early development/embryogenesis. a. Species-specific unique methylome patterns in Lake Malawi cichlids can be found in liver- or muscle-tissues, or in both tissues ('multi-tissue'). **b.** Histograms showing the total counts of 'species' DMRs that are either liver-, muscle-specific or present in both (multi). Only 'species' DMRs showing a unique DNA methylation pattern in one species are shown. **c.** GO enrichment plots for each DMR class. **d.-f.** Examples of 'species' multi-tissue DMRs in genes related to embryonic developmental processes. Namely in the genes coding for visual system homeobox 2 *vsx2* (LOC101486458), growth associated protein 43 *gap43* (LOC101472990) and teneurin transmembrane protein 2 *tenm2* (LOC101470261). Liver and muscle methylome profiles in green and purple, respectively (averaged % mCG/CG levels in 50bp bins for ≥2 samples per tissue; scale indicated above each graph).

The relatively high proportion of multi-tissue DMRs suggests there may be extensive among-species divergence in core cellular or metabolic pathways. To investigate this further, we performed GO enrichment analysis. As expected, liver-specific DMRs are particularly enriched for hepatic metabolic functions, while muscle-specific DMRs are significantly associated with muscle-related functions, such as glycogen catabolic pathways (Fig. 4c). Multi-tissue DMRs, however, are significantly enriched for genes involved in development and embryonic processes, in particular related to cell differentiation and brain development (Fig. 4 c, d-f) and show different properties from tissue-specific

DMRs. Indeed, in all the three species, multi-tissue DMRs are three times longer on average (median length of multi-tissue DMRs: 726bp; Dunn's test, $P<0.0001$; Supplementary Fig. 11c), are more often localised in promoter regions (Supplementary Fig. 11d), and are significantly enriched for TE sequences (Dunn's test, $P\leq0.03$; Supplementary Fig. 11d) compared to liver and muscle DMRs. Furthermore, multi-tissue species-specific methylome patterns show significant enrichment for specific TF binding motif sequences. These binding motifs are bound by TFs with functions related to embryogenesis and development, such as the transcription factors Forkhead box protein K1 (foxk1) and Forkhead box protein A2 (foxa2), with important roles during liver development[50] (Supplementary Fig.10f), possibly facilitating core phenotypic divergence early on during development.

Several examples of multi-tissue DMRs are worth highlighting as generating hypotheses for potential future functional studies (Fig. 4d-f). The visual system homeobox 2 (*vsx2*) gene in the offshore deep-water species *Diplotaxodon limnothrissa* is almost devoid of methylation in both liver and muscle, in contrast to the other species (1.9kbp-long DMR; Fig. 4d and Supplementary Fig. 11f). *vsx2* has been reported to play an essential role in the development of the eye and retina in zebrafish with embryonic and postnatal active transcription localised in bipolar cells and retinal progenitor cells[53]. *D. limnothrissa* populates the deepest parts of the lake of all cichlid species (down to approximately 250m, close to the limits of oxygenation) and features morphological adaptations to dimly-lit environments, such as larger eye size[54]. *vsx2* may therefore participate in the visual adaptation of *Diplotaxodon* to the dimmer parts of the lake via DNA methylation-mediated gene regulation during development. Another example of a multi-tissue DMR specific to *D. limnothrissa* is located in the promoter of the gene coding for the growth associated protein 43 (*gap43*) involved in neural development and plasticity, and also neuronal axon regeneration[55]. The promoter of *gap43* is largely devoid of methylation (overall <5% average mC/C levels across this 5.2kbp-long DMR) in both muscle and liver tissues of *D. limnothrissa*, while being highly methylated (>86% mCG/CG) in the other species (Fig. 4 e). In *A. calliptera*, the transcription of *gap43* is restricted to the brain and embryo (Supplementary Fig. 11g), consistent with a role in neural development and in the adult brain. Finally, another multi-tissue DMR potentially involved in neural embryonic functions is located in the promoter region of the gene *tenm2,* coding for teneurin transmembrane protein (Fig. 4f). *tenm2* is a gene expressed early on during zebrafish embryogenesis as well as in cichlid brain and embryo (Supplementary Fig. 11h), and is involved in neurodevelopment and neuron migration-related cell signalling[56]. This 2.7kbp-long DMR is completely unmethylated in the algae-eating rock-dweller *Petrotilapia genalutea* (almost 80% reduction of methylation overall compared to the other species) and may mediate species-specific adaptive phenotypic plasticity related to synapse formation and neuronal networks.

DISCUSSION

The molecular mechanisms underlying adaptive phenotypic diversification are subject of intense interest[33,35,37,57,58] and the extent of the role of epigenetic processes is hotly debated[2,4,59]. However, in-depth molecular epigenetic studies remain rare in evolutionary genomics and its key model systems[2,4,28,59]. Here, we focussed on the genetically closely-related haplochromine cichlids of Lake Malawi, representing a unique system to investigate the epigenetic basis for phenotypic diversification[35,38,60]. Specifically, we describe genome-wide methylome variation at a single CG dinucleotide resolution as well as transcriptomes of two adult tissues of different embryonic origins in ecomorphologically divergent species (Fig. 1b). This work represents the first study investigating epigenetic marks in the context of rapid diversification in natural populations of cichlid fishes and provides evidence of substantial methylome divergence associated with ecologically-relevant genes and correlated with changes in transcriptional network and in TF activity . Given the resemblances we found between cichlid methylomes and those of warm-blooded vertebrates (Fig. 1. d, e), suggesting evolutionarily conserved functions, our findings are likely to be relevant to other vertebrate evolutionary model systems.

Recent large-scale epigenetic studies in natural populations of *Arabidopsis* have highlighted a functional link between local environments and methylation divergence, with possible adaptive phenotypic functions[10,12]. Yet, epigenetic variation in natural populations of vertebrates and its possible functions in the context of adaptive phenotypic diversification have scarcely been studied. Our finding of considerable among-species methylome divergence at conserved underlying DNA sequences, despite overall low among-species genome differentiation, is suggestive of a functional link between DNA methylation and local environments, which may facilitate phenotypic plasticity and diversification. The methylome divergence we found may be driven directly by the environmental differences but is also likely to have a genetic component. Our study lays the groundwork for deciphering any genetically encoded component underlying the epigenetic differences. Genetic differences in TF binding domains or in TF sequence recognition motifs, as well as in the proteins involved in the maintenance and deposition of new methyl groups, could for example lead to epigenetic divergence [10,23]. While this study provides evidence for species-specific methylome divergence associated with transcriptional changes of ecologically-relevant genes, further experimental work is required to examine the extent to which such species-specific patterns have an adaptive function in a natural context, as well as to determine the degree of plasticity and inheritance of such epigenetic patterns. Recent studies in three-spined stickleback fish have provided initial evidence for stable transmission of methylome patterns across generation associated with adaptation to salinity, some of which are inherited in a genetic-independent manner[61,62]. Moreover, epigenetic inheritance and reprogramming greatly vary among teleost fishes. Indeed, recent studies have highlighted important differences in the mechanisms of DNA methylation reprogramming during embryogenesis in teleost fishes. While the genome of the embryo in zebrafish retains the sperm methylome configuration with no global

DNA methylation resetting, possibly allowing for the transgenerational inheritance of specific epigenetic states, extensive and global DNA methylation reprogramming instead occurs upon fertilisation in medaka embryos (similar to mammals)[29,63–65]. Such DNA methylome reprogramming processes are currently unknown in cichlids, which warrants further research.

We found that that regions of methylome divergence between species (DMRs) were enriched in promoters and orphan CGIs (Fig. 2b). Methylation variation in promoter regions is known to have important *cis*-regulatory functions in vertebrates, in particular during development[19,20,23,28,30]. Such *cis*-regulatory activity is also apparent in Lake Malawi cichlids, with methylation at promoters negatively correlated with transcriptional activity (Fig. 1e and Supplementary Fig. 7a-d). This is likely mediated by the tight interaction of DNA methylation with 5mC-sensitive DNA-binding proteins, such as many transcription factors[21](see below). On the other hand, the functional roles of orphan CGIs are less well understood[41]. However, orphan CGIs have by far the highest enrichment for species methylome divergence (>3-fold over chance; Fig. 2b) - most of which are located in unannotated genomic regions. Orphan CGIs, as well as intergenic TEs (Fig. 2d), might include ectopic promoters, enhancers and other distal regulatory elements[40,41] that may participate in phenotypic diversification by reshaping transcriptional network. Such putative *cis*-regulatory regions could be validated against a full functional annotation of the genome of Lake Malawi cichlid, which is currently lacking.

We identified that in some species methylome divergence was significantly associated with differential liver transcriptome activity, especially pertaining to hepatic functions involved in steroid hormone and fatty acid metabolism (Fig. 3b, d, e-j). Consistent with a functional role of DNA methylation in *cis*-regulatory regions [20,43], we revealed significant methylation divergence in the promoters of differentially transcribed genes involved in liver-mediated energy expenditure processes and metabolism, such as gene *prf1*-like (>60-fold increase in expression; Fig. 3g, j), associated with obesity in mouse [44]. Such a functional link may promote phenotypic diversification via adaptation to different diets. Our understanding of this would benefit from knowledge of the extent to which environmental or diet perturbation might result in adaptation-associated functional methylome changes. Further work would also be required to assess the extent to which such changes may be stably inherited. Additionally, the characterisation of the methylomes of Lake Malawi cichlid species from different ecomorphological groups but sharing the same habitat/diet, would inform on the specificity and possible functions of methylome divergence at metabolic genes.

We observed that methylome divergence associated with altered transcription in livers is enriched for binding motifs recognised by specific TFs. Some of those TFs are also differentially expressed in the livers and have important roles in lipid and energy homeostasis (Supplementary Fig.10d-f). This suggests that altered activity of some TFs in livers can be associated with species-specific

methylome patterns. Methylome variation in *cis*-regulatory regions is known to affect the binding affinity of methyl-sensitive DNA-binding regulatory factors (such as TFs)[24,43,66,67]. Furthermore, methylation-associated changes in chromatin accessibility may also impede the binding affinity of such factors and could be associated with altered TF activity and changes in transcription[19,66]. Alternatively, altered TF activity, arising from species-specific mutations within the TF binding sequence motifs or in the TF binding domain, has also been reported to generate methylome divergence in *cis* and *trans*[23] and could also underlie species-specific epigenetic divergence. Our results suggest a tight link between TF activity and methylome divergence, that could participate in reshaping the transcriptional network of the livers in Lake Malawi cichlids.

TE and repetitive sequences present on average higher methylation levels than the genome-wide average (Fig. 1d), although some specific TE classes show more variable and lower levels (Supplementary Fig. 6e). DNA methylation-mediated transcriptional repression of mostly deleterious TE elements is crucial to the integrity of most eukaryote genomes, from plants to fish and mammals, and can be mediated in both animals and plants by small non-coding RNAs, such as piwi-interacting RNAs (piRNAs) in zebrafish and mammals[17,18,68]. Notably, the majority (~60%) of species differences in methylation patterns associated with transcriptional changes in liver was significantly localised in evolutionary young transposon/repeat regions, notably in intergenic retroposons in the vicinity of genes and in intronic DNA transposons (Dunn's test $p<10^{-10}$; Fig. 3c and Supplementary Fig. 10b). Even though most of TE activity is under tight cellular control to ensure genome stability, transposition events have also been associated with genome evolution and phenotypic diversification. Indeed, TE insertion may represent a source of functional genomic variation and novel *cis*-regulatory elements, underlying altered transcriptional network[44,46,47,69]. In haplochromine cichlids, variation in anal fin egg-spots patterns associated with courtship behaviour, has been linked to a novel *cis*-regulatory element, derived from TE sequences[45]. Additionally, Brawand and colleagues have revealed that most TE insertions near genes in East African cichlids were associated with altered gene expression patterns[37]. Moreover, genes in piRNA-related pathways have been reported to be under positive selection in Lake Malawi cichlid flock, in line with a fast evolving TE sequence landscape observed in cichlids [35], and these genes may also be associated with TE-related methylome variation, similar to *Arabidopsis*[10,70].

Not only can novel TE insertions participate in genome evolution, DNA methylation at TE-derived *cis*-regulatory elements has been shown to affect transcriptional activity of nearby genes[11,44]. In rodents, the insertion of one IAP (intra-cisternal A particle) retrotransposon in the upstream *cis*-regulatory region of the agouti gene is associated with considerable phenotypic variation of coat colours and metabolic changes. Differential methylation levels at this TE-derived ectopic promoter directly impacts the activity of the agouti gene[5,27], and such epigenetic patterns of methylation are

transmitted to the offspring along with the altered phenotypes in a non-genetic manner[2]. Similarly, in toadflax, the flower symmetry is associated with the variable and heritable methylation patterns in the TE-derived promoter of the *Lcyc* gene, resulting in symmetrical or asymmetrical flowers[6]. Also, in a population-scale study of more than a thousand natural *Arabidopsis* accessions, epigenetic variation was found to be associated with phenotypes, mostly arising from methylation-mediated TE silencing that was significantly associated with altered transcription of adaptive genes such as those determining flowering time[10,70]. Our work adds to this by providing further evidence that interactions between TE sequences and between-species methylome divergence might have led to altered transcriptional networks. This lays groundwork for further investigation of this issue in cichlid fishes.

Finally, we revealed that between-species methylome differences in liver tissues were greater than differences between muscle tissues (Fig. 4b), possibly highlighting a higher dependence of hepatic functions on natural epigenetic divergence. This indicates that a significant portion of the between-species methylome divergence in liver may be associated with phenotypic divergence, in particular by affecting the genes involved in tissue-specific functions, such as hepatic metabolic processes (Fig. 3c, e-j). However, almost half of the methylome divergence we observed that was driven by a single species was consistently found in both liver and muscle (Fig. 4b). This multi-tissue methylome divergence is consistent with epigenetic influences on core cellular functions, and may also be relevant to early-life biological processes such as development, cellular differentiation and embryogenesis (Fig. 4c, d-f). For example, we identified a large hypomethylated region in the visual homeobox gene *vsx2* in both liver and muscle tissues in the deep-water *Diplotaxodon* (Fig. 4d). This gene is involved in eye differentiation and may participate in long-lasting visual phenotypic divergences required to populate dimly parts of the lake, similar to the DNA methylation-mediated adaptive eye degeneration in cavefish[28]. Notably, recent studies have highlighted signatures of positive selection and functional substitutions in genes related to visual traits in *D. limnothrissa*[35,54]. Furthermore, in regions showing multi-tissue species-specific methylome divergence, we identified significant enrichment for binding motifs of specific TFs whose functions are related to embryogenesis and liver development (such as foxa2 and foxk1). This suggests that altered TF activity during development could be associated with species-specific methylome patterns (Supplementary Fig. 10f). If multi-tissue methylome divergence has been established very early during differentiation, and has important regulatory functions pertaining to early developmental stages [25] and possibly core cellular functions, then it may promote long-lasting phenotypic divergence unique to each species' adaption. Our observations suggest further characterisation of the methylomes and transcriptomes in the different cells of developing embryos may be valuable, to investigate when between-species methylome divergence is established, as well as any functional roles in early-life phenotypic diversification.

To conclude, recent large-scale genomic studies have highlighted that several mechanisms may participate in the phenotypic diversification of Lake Malawi haplochromine cichlids, such as hybridisation and incomplete lineage sorting[33,35,60,71]. Our study adds to these observations by providing initial evidence of substantial methylome divergence associated with altered transcriptome activity of ecologically-relevant genes among closely-related Lake Malawi cichlid fish species. This raises the possibility that variation in methylation patterns could facilitate phenotypic divergence in these rapidly evolving species through different mechanisms (such as altered TF binding affinity, gene expression and TE activity, all possibly associated with methylome divergence at *cis*-regulatory regions). Further work is required to elucidate the extent to which this might result from plastic responses to the environment and the degree of inheritance of such patterns, as well the adaptive role and any genetic basis associated with epigenetic divergence. This study represents the first epigenomic study investigating natural methylome variation in the context of phenotypic diversification in genetically similar but ecomorphologically divergent cichlid species part of a massive vertebrate radiation and provides an important resource for further experimental work.

Methods

Overview sampling

All cichlid specimens were bought dead from local fishermen by G. F. Turner, M. Malinsky, H. Svardal, A. M. Tyers, M. Mulumpwa and M. Du in 2016 in Malawi in collaboration with the Fisheries Research Unit of the Government of Malawi), or in 2015 in Tanzania in collaboration with the Tanzania Fisheries Research Institute (various collaborative projects). Sampling collection and shipping were approved by permits issued to GF Turner, MJ Genner R Durbin, EA Miska by the Fisheries Research Unit of the Government of Malawi (various collaborative projects) and the Tanzania Fisheries Research Institute. Upon collection, tissues were immediately placed in RNA*later* (Sigma), and were then stored at -80°C upon return. Information about the collection type, species ID and the GPS coordinates for each collection in Supplementary Table 1.

SNP-corrected genomes

Because real C->T (or G->A on the reverse strand) mutations are indistinguishable from C>T SNPs generated by the bisulfite treatment, they can add some bias to comparative methylome analyses. To account for this, we used SNP data from Malinsky et al. (2018) (Ref.[35])and, using the *Maylandia zebra* UMD2a reference genome (NCBI_Assembly: GCF_000238955.4) as the template, we substituted C->T (or G->A) SNPs for each of the 6 species analysed before re-mapping the bisulfite reads onto these 'updated' reference genomes.

To translate SNP coordinates from Malinsky et al. (2018) to the UMD2a assembly, we used the UCSC liftOver tool, based on a whole genome alignment between the original Brawand et al., 2014 (Ref.[37]) (https://www.ncbi.nlm.nih.gov/assembly/GCF_000238955.1/) and the UMD2a *M. zebra* genome assemblies. The pairwise whole genome alignment was generated using lastz v1.02[72], with the following parameters: "B=2 C=0 E=150 H=0 K=4500 L=3000 M=254 O=600 Q=human_chimp.v2.q T=2 Y=15000". This was followed by using USCS genome utilities (https://genome.ucsc.edu/util.html) axtChain tool with -minScore=5000. Additional tools with default parameters were then used following the UCSC whole-genome alignment paradigm (http://genomewiki.ucsc.edu/index.php/Whole_genome_alignment_howto) in order to obtain a contiguous pairwise alignment and the 'chain' file input for liftOver.

The 'lifted over' C->T (or G->A) SNPs were then substituted into the UMD2a genome using the evo getWGSeq command with the --whole-genome and --methylome options. The code used is available as a part of the evo package (https://github.com/millanek/evo; v.0.1 r24, commit99d5b22).

Whole-genome bisulfite sequencing WGBS

Extraction of high-molecular-weight genomic DNA (HMW-gDNA)

The main method to generate WGBS data is summarised in Supplementary Figure 1. In detail, high-molecular-weight genomic DNA (HMW-gDNA) was extracted from homogenised liver and muscle tissues (<25mg) using QIAamp DNA Mini Kit (Qiagen 51304) according the manufacturer's instructions. Before sonication, unmethylated lambda DNA (Promega, D1521) was spiked in (0.5% w/w) to assess bisulfite conversion efficiency. HMW-gDNA was then fragmented to the target size of ~400bp (Covaris, S2 and E220). Fragments were then purified

with PureLink PCR Purification kit (ThermoFisher). Before any downstream experiments, quality and quantity of gDNA fragments were both assessed using NanoDrop, Qubit and Tapestation (Agilent).

NGS library preparation for WGBS

For each sample, 200ng of sonicated fragments were used to make NGS sequencing libraries using NEBNext Ultra II DNA Library Prep (New England BioLabs, E7645S) in combination with methylated adaptors (NEB, E7535S), following manufacturer's instructions. Adaptor-ligated fragments were then purified with 1.0x Agencourt AMPure Beads (Beckman Coulter, Inc). Libraries were then treated with sodium bisulfite according to the manufacturer's instructions (Imprint DNA Modification Kit; Sigma, MOD50) and amplified by PCR (10 cycles) using KAPA HiFi HS Uracil+ RM (KAPA Biosystems) and NEBNext Multiplex Oligos for Illumina (NEB E7335S). Bisulfite-converted libraries were finally size-selected and purified using 0.7x Agencourt AMPure Beads. Size and purity of libraries were determined using Tapestation and quantified using Qubit (Agilent). Libraries were sequenced on HiSeq 4000 (High Output mode, v.4 SBS chemistry) to generate paired-end 150 bp-long reads. *A. stuartgranti* samples were sequenced on HiSeq 2500 to generate paired-end 125bp-long reads.

Mapping of WGBS reads

TrimGalore (options: --paired --fastqc --illumina ; v0.6.2; github.com/FelixKrueger/TrimGalore) was used to determine the quality of sequenced read pairs and to remove Illumina adaptor sequences and low-quality reads/bases (Phred quality score <20). All adaptor-trimmed paired reads from each species were then aligned to the respective species-specific SNP-corrected *M.zebra* genomes (see above and Supplementary Table 1) and to the lambda genome (to determine bisulfite non-conversion rate) using Bismark[73] (v0.20.0). The alignment parameters were as follows: 0 mismatch allowed with a maximum insert size for valid paired-end alignments of 500bp (options: -p5 -N 0 –X 500). Clonal mapped reads (i.e., PCR duplicates) were removed using deduplicate_bismark (see Supplementary Table 1). Mapped reads for the same samples generated on multiple HiSeq runs were also merged.

DMR calling and genome-wide methylome variation analysis

Methylation at CpG sites was called using bismark_methylation_extractor (options: -p --multicore 9 --comprehensive --no_overlap --merge_non_CpG). DMRs (>25% methylation difference, ≥50bp, ≥4 CG and $p<0.05$) were predicted using DSS[74] (v2.32.0). samtools (v1.9) and bedtools (v2.27.1) were used to generate averaged methylation levels across non-overlapping windows of various sizes genome-wide. ggplot2 (v3.3.0) and pheatmap (v1.0.12) were used to visualise methylome data and to produce unbiased hierarchal clustering (Euclidean's distances and complete-linkage clustering). Spearman's correlation matrices, Euclidean distances and principal component analyses (scaled and centred) were produced using R (v3.6.0) functions cor, dist and prcom, respectively. Minimum read overage requirement at any CpG sites for all analyses - except for DSS-predicted DMRs, for which all read coverage was used - was as follows: >4 and ≤100 non-PCR-duplicate mapped paired-end reads. mCG % levels over 50bp-long non-overlapping windows for all annotations were averaged across samples for each tissue of each species. Violin plots were generated using ggplot2 and represent rotated and mirrored kernel density plots. The genome browser IGV (v2.5.2) was used to visualise DNA

methylation genome-wide (% mCG/CG in 50bp windows). One outlier sample for *R. longiceps*, seemingly coming from a spleen instead of liver, was removed.

Additional statistics

Kruskal-Wallis H and Dunn's multiple comparisons tests (Benjamini-Hochberg correction unless otherwise specified) were performed using FSA (v0.8.25). Box plots represent interquartile range (IQR) with minimal and maximal values (black lines) and outliers (black circles).

Genomic annotations

The reference genome of *M. zebra* (UMD2a; NCBI genome build: GCF_000238955.4 and NCBI annotation release 104) was used to generate all annotations. Custom annotation files were generated and defined as follows: promoter regions, TSS±500 bp unless otherwise indicated; gene bodies included both exons and introns and other intronic regions, and excluded the first 500bp regions downstream of TSS to avoid any overlap with promoter regions; transposable elements and repetitive elements were modelled and annotated, as well as their sequence divergence analysed using RepeatModeler (v1.0.11) and RepeatMasker (v4.0.9.p2), respectively. Intergenic regions were defined as genomic regions more than 5kbp from genes. CpG-rich regions, or CpG islands (CGI), were predicted and annotated using makeCGI[75]. The following genomes were used to compare genomic CG contents across different organisms (Supplementary Figure 5a): honey bee (*A. melifera*, Amel_4.5), nematode (*C. elegans*, WBcel235), *Arabidopsis* (*A. thaliana*, TAIR10), zebrafish (*D. rerio*, GRCz10), Mbuna cichlid *Maylandia zebra* (*M. zebra,* UMD1), West Indian Ocean coelacanth (*L. chalumnae*, LatCha.1), red junglefowl (*G. gallus*, Gall_5), grey whale (*E. robustus*, v1), human (*H. sapiens*, GRCh38.p10), mouse (*M. musculus*, GRCm38.p5), tammar wallaby (*N. eugenii*, Meug1.1). pfDMRs and TE/repeat elements were assigned to a gene when they were located within gene bodies (from 0.5kbp downstream TSS), within promoter regions (TSS±500bp) and in the vicinity of genes (within 0.5-4kbp away from genes).

Enrichment analysis

Enrichment analysis was calculated by shuffling each type of DMRs (liver, muscle, tissue) across the *M.zebra* UMD2a genome (accounting for the number of DMRs and length; 1000 iterations). The expected values were determined by intersecting shuffled DMRs with each genomic category (1000 iterations). Chi-square tests were then performed for each O/E distribution. The same process was performed for TE enrichment analysis.

Gene Ontology enrichment analysis

All GO enrichments analyses were performed using g:Profiler (https://biit.cs.ut.ee/gprofiler/gost; version Sept 2020). Only annotated genes for *Maylandia zebra* were used with a statistical cut-off of FDR<0.05 (unless otherwise specified).

Sequence divergence

A pairwise sequence divergence matrix was generated using a published dataset[35]. Phylogenetic unrooted trees and heatmap were generated using the following R packages: phangorn (v.2.5.5), ape_5.4-1 and pheatmap (v.1.0.12).

RNAseq

Total RNA extraction and NGS library for long (>200nt) RNA sequencing

In brief, for each species, three biological replicates of liver and muscle tissues were used to sequence total RNA (see Supplementary Figure 1 for a summary of the method and Supplementary Table 2 for sampling size). The same specimens were used for both RNAseq and WGBS.

RNAseq libraries for both liver and muscle tissues were prepared using ~5-10mg of RNA*later*-preserved homogenised liver and muscle tissues. Total RNA was isolated using a phenol/chloroform method following manufacturer's instructions (TRIzol, ThermoFisher). RNA samples were treated with DNase (TURBO DNase, ThermoFisher) to remove any DNA contamination. Quality and quantity of total RNA extracts were determined using NanoDrop spectrophotometer (ThermoFisher), Qubit (ThermoFisher) and BioAnalyser (Agilent). Following ribosomal RNA depletion (RiboZero, Illumina), stranded rRNA-depleted RNA libraries (Illumina) were prepped according to the manufacturer's instructions and sequenced (paired-end 75bp-long reads) on HiSeq2500 V4 (Illumina) by the sequencing facility of the Wellcome Sanger Institute.

Published RNAseq data for all the tissues of *A. calliptera* sp. Itupi was taken from [35].

Mapping and gene quantification

TrimGalore (options: --paired --fastqc --illumina ; v0.6.2; https://github.com/FelixKrueger/TrimGalore) was used to determine the quality of sequenced read pairs and to remove Illumina adaptor sequences and low-quality reads/bases (Phred quality score <20). Reads were then aligned to the *M. zebra* transcriptome (UMD2a; NCBI genome build: GCF_000238955.4 and NCBI annotation release 104) and the expression value for each transcript was quantified in transcripts per million (TPM) using kallisto quant[76] (--bias -b 100 -t 1; v0.46.0). For all downstream analyses, gene expression values for each tissue were averaged for each species.

A Spearman's rank correlation matrix was produced with R function cor, and the gene expression matrix to assess transcription variation across samples. Graphs, unsupervised clustering and heatmaps were produced with R packages ggplot2 (v3.3.0) and pheatmap (v1.0.12). Heatmaps of gene expression show scaled TPM values.

Differential gene expression (DEG)

Differential gene expression (DE) analysis was performed using sleuth[77] (v0.30.0; Wald test, FDR <0.01). Only DEGs with gene expression difference of ≥50 TPM between at least one species pairwise comparison were analysed further.

Methylation variation in promoters/gene bodies associated with transcriptional activity

To study the correlation between methylome and gene expression (Fig. 1e and Supplementary Figure 7), genes were binned into 11 categories based on their expression levels (increasing gene expression levels, from category 1 to 10; cat "OFF" groups silent/not expressed genes, i.e., TPM=0 in all replicates for a particular species. RL liver (n=2): 10 'ON' categories, n=2,129; 1 'silent' category, n=5,331. MZ liver (n=3): 10 'ON' categories, n=2,199; 1 'silent' category, n=4,704. RL muscle (n=2): 10 'ON' categories , n=2,101; 1 'silent' category, n=4,622). Promoter (500bp±TSS) and gene bodies were also binned into 10 categories according to

methylation levels (0-100% methylation, by 10% DNA methylation increment; RL liver (n=2), 11 categories, n ranging from 34 to 11,202. MZ liver (n=3), 11 categories, n ranging from 28 to 11,192. RL muscle (n=2), 11 categories, n ranging from 60 to 9,946). Categories were generated using the R script tidyverse (v1.3.0) and graphs were generated using deepTools v.3.2.1. TPM values and methylation levels were averaged for each tissue and each species.

ACKNOWLEDGMENTS: We would like to thank S.M. Grant's diving team for collecting some of the fish specimens, as well as the Fisheries Research Unit of the Government of Malawi, and the Tanzania Fisheries Research Institute, for their assistance and support. We would like to thank the staff at the Gurdon Institute and the sequencing facilities at CRUK Cambridge Institute, Gurdon and Sanger Institutes for their expertise and support. We would like to thank the members of the Miska Lab as well as the Cambridge Cichlid community for fruitful discussions. We thank Navin B. Ramakrishna for critical comments on the manuscripts, as well as David Jordan, Tomás di Domenico and Konrad LM Rudolph for their support with data analysis. We are grateful to Ole Seehausen and Marcel Häsler (University of Bern, Switzerland) for providing PN tissues. We thank Alan Hudson for help with sample collection. **FUNDING**: This work was supported by the following grants to EAM: Wellcome Trust Senior Investigator award (104640/Z/14/Z and 219475/Z/19/Z) and CRUK award (C13474/A27826); to RD: Wellcome award (WT207492); to GFT and MJG, the Leverhulme Trust - Royal Society Africa Awards (AA100023 and AA130107), and NERC award (NE/S001794/1). GV thanks Wolfson College, University of Cambridge and the Genetics Society, London for financial support. The authors also acknowledge core funding to the Gurdon Institute from Wellcome (092096/Z/10/Z, 203144/Z/16/Z) and CRUK (C6946/A24843). **AUTHOR CONTRIBUTION:** GV and EAM devised the study and interpreted the results with contributions from GFT, MJG and MES; GV performed all experiments and analyses; MD, HS, MM, RD, MJG, GFT and AMT collected and provided tissues from wild-caught cichlid specimens; MD extracted total RNA samples; GV and EAM wrote the manuscript with comments and contribution from all authors. **COMPETING INTERESTS:** None declared. **DATA AVALAIBILITY:** All raw sequencing reads (WGBS, RNAseq and SNP-corrected genomes), and processed data have been deposited in the GEO database and will be made public upon publication. Sample accessions are listed in Supplementary Table 1. **CODE AVALAIBILITY:** The code used to generate SNP-substituted genomes is available as a part of the evo package (https://github.com/millanek/evo; v.0.1 r24, commit99d5b22).

SUPPLEMENTARY MATERIALS

Supplementary Figures 1 – 11

Supplementary Table 1 – 3

Supplementary notes

REFERENCES AND NOTES

1. Jablonka, E. & Raz, G. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Q. Rev. Biol.* **84**, 131–176 (2009).
2. Miska, E. A. & Ferguson-Smith, A. C. Transgenerational inheritance: Models and mechanisms of non-DNA sequence-based inheritance. *Science* **354**, 59–63 (2016).
3. Cavalli, G. & Heard, E. Advances in epigenetics link genetics to the environment and disease. *Nature* **571**, 489–499 (2019).
4. Heard, E. & Martienssen, R. A. Transgenerational epigenetic inheritance: myths and mechanisms. *Cell* **157**, 95–109 (2014).
5. Morgan, H. D., Sutherland, H. G. E., Martin, D. I. K. & Whitelaw, E. Epigenetic inheritance at the agouti locus in the mouse. *Nat. Genet.* **23**, 314–318 (1999).
6. Cubas, P., Vincent, C. & Coen, E. An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**, 157–161 (1999).
7. Zemach, A., McDaniel, I. E., Silva, P. & Zilberman, D. Genome-Wide Evolutionary Analysis of Eukaryotic DNA Methylation. *Science* **328**, 916–919 (2010).
8. Feng, S. *et al.* Conservation and divergence of methylation patterning in plants and animals. *Proc. Natl. Acad. Sci.* **107**, 8689–8694 (2010).
9. Suzuki, M. M. & Bird, A. DNA methylation landscapes: provocative insights from epigenomics. *Nat. Rev. Genet.* **9**, 465–476 (2008).
10. Kawakatsu, T. *et al.* Epigenomic Diversity in a Global Collection of Arabidopsis thaliana Accessions. *Cell* **166**, 492–505 (2016).
11. Quadrana, L. & Colot, V. Plant Transgenerational Epigenetics. *Annu. Rev. Genet.* **50**, 467–491 (2016).
12. Cortijo, S. *et al.* Mapping the Epigenetic Basis of Complex Traits. *Science* **343**, 1145–1148 (2014).
13. Best, C. *et al.* Epigenetics in teleost fish: From molecular mechanisms to physiological phenotypes. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **224**, 210–244 (2018).
14. Seritrakul, P. & Gross, J. M. Expression of the de novo DNA methyltransferases (dnmt3 - dnmt8) during zebrafish lens development. *Dev. Dyn.* **243**, 350–356 (2014).
15. Rai, K. *et al.* Zebra Fish Dnmt1 and Suv39h1 Regulate Organ-Specific Terminal Differentiation during Development. *Mol. Cell. Biol.* **26**, 7077–7085 (2006).
16. Law, J. A. & Jacobsen, S. E. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nat. Rev. Genet.* **11**, 204–220 (2010).
17. Deniz, Ö., Frost, J. M. & Branco, M. R. Regulation of transposable elements by DNA modifications. *Nat. Rev. Genet.* **20**, 417–431 (2019).
18. Bestor, T. H. DNA methylation: evolution of a bacterial immune function into a regulator of gene expression and genome structure in higher eukaryotes. *Philos. Trans. R. Soc. London. B, Biol. Sci.* **326**, 179–187 (1990).
19. Greenberg, M. V. C. & Bourc'his, D. The diverse roles of DNA methylation in mammalian development and disease. *Nat. Rev. Mol. Cell Biol.* **20**, 590–607 (2019).
20. Smith, Z. D. & Meissner, A. DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* **14**, 204–220 (2013).
21. Yin, Y. *et al.* Impact of cytosine methylation on DNA binding specificities of human transcription factors. *Science* **356**, eaaj2239 (2017).
22. Du, J., Johnson, L. M., Jacobsen, S. E. & Patel, D. J. DNA methylation pathways and their crosstalk with histone methylation. *Nat. Rev. Mol. Cell Biol.* **16**, 519–532 (2015).
23. Schübeler, D. Function and information content of DNA methylation. *Nature* **517**, 321–326 (2015).
24. Zhu, H., Wang, G. & Qian, J. Transcription factors as readers and effectors of DNA methylation. *Nat. Rev. Genet.* **17**, 551–565 (2016).
25. Hon, G. C. *et al.* Epigenetic memory at embryonic enhancers identified in DNA methylation maps from adult mouse tissues. *Nat. Genet.* **45**, 1198–1206 (2013).
26. West-Eberhard, M. J. Developmental plasticity and the origin of species differences. *Proc. Natl. Acad. Sci.* **102**, 6543–6549 (2005).

27. Kazachenka, A. *et al.* Identification, Characterization, and Heritability of Murine Metastable Epialleles: Implications for Non-genetic Inheritance. *Cell* **175**, 1259-1271.e13 (2018).
28. Gore, A. V. *et al.* An epigenetic mechanism for cavefish eye degeneration. *Nat. Ecol. Evol.* **2**, 1155–1160 (2018).
29. Skvortsova, K., Iovino, N. & Bogdanović, O. Functions and mechanisms of epigenetic inheritance in animals. *Nat. Rev. Mol. Cell Biol.* **19**, 774–790 (2018).
30. Ryu, T., Veilleux, H. D., Donelson, J. M., Munday, P. L. & Ravasi, T. The epigenetic landscape of transgenerational acclimation to ocean warming. *Nat. Clim. Chang.* **8**, 504–509 (2018).
31. Moran, D., Softley, R. & Warrant, E. J. The energetic cost of vision and the evolution of eyeless Mexican cavefish. *Sci. Adv.* **1**, e1500363 (2015).
32. Turner, G. F. Adaptive radiation of cichlid fish. *Curr. Biol.* **17**, R827–R831 (2007).
33. Salzburger, W. Understanding explosive diversification through cichlid fish genomics. *Nat. Rev. Genet.* **19**, 705–717 (2018).
34. Malinsky, M. & Salzburger, W. Environmental context for understanding the iconic adaptive radiation of cichlid fishes in Lake Malawi. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 11654–11656 (2016).
35. Malinsky, M. *et al.* Whole-genome sequences of Malawi cichlids reveal multiple radiations interconnected by gene flow. *Nat. Ecol. Evol.* **2**, 1940–1955 (2018).
36. Konings, A. *Malawi Cichlids in their natural habitat*. (Cichlid Press, 2016).
37. Brawand, D. *et al.* The genomic substrate for adaptive radiation in African cichlid fish. *Nature* **513**, 375–381 (2014).
38. Genner, M. J. & Turner, G. F. Ancient Hybridization and Phenotypic Novelty within Lake Malawi's Cichlid Fish Radiation. *Mol. Biol. Evol.* **29**, 195–206 (2012).
39. Turner, G. F., Ngatunga, B. P. & Genner, M. J. The Natural History of the Satellite Lakes of Lake Malawi. *EcoEvoRxiv* (2019). doi:10.32942/osf.io/sehdq
40. Long, H. K. *et al.* Epigenetic conservation at gene regulatory elements revealed by non-methylated DNA profiling in seven vertebrates. *Elife* **2013**, 1–19 (2013).
41. Deaton, A. M. & Bird, A. CpG islands and the regulation of transcription. *Genes Dev.* **25**, 1010–22 (2011).
42. Conte, M. A. *et al.* Chromosome-scale assemblies reveal the structural evolution of African cichlid genomes. *Gigascience* **8**, 1–20 (2019).
43. Yin, Y. *et al.* Impact of cytosine methylation on DNA binding specificities of human transcription factors. *Science* **356**, eaaj2239 (2017).
44. Chuong, E. B., Elde, N. C. & Feschotte, C. Regulatory activities of transposable elements: from conflicts to benefits. *Nat. Rev. Genet.* **18**, 71–86 (2017).
45. Santos, M. E. *et al.* The evolution of cichlid fish egg-spots is linked with a cis-regulatory change. *Nat. Commun.* **5**, 5149 (2014).
46. Fedoroff, N. V. Transposable Elements, Epigenetics, and Genome Evolution. *Science* **338**, 758–767 (2012).
47. Carleton, K. L. *et al.* Movement of transposable elements contributes to cichlid diversity. *Mol. Ecol.* **29**, 4956–4969 (2020).
48. Lev Maor, G., Yearim, A. & Ast, G. The alternative role of DNA methylation in splicing regulation. *Trends Genet.* **31**, 274–280 (2015).
49. Hayhurst, G. P., Lee, Y.-H., Lambert, G., Ward, J. M. & Gonzalez, F. J. Hepatocyte Nuclear Factor 4α (Nuclear Receptor 2A1) Is Essential for Maintenance of Hepatic Gene Expression and Lipid Homeostasis. *Mol. Cell. Biol.* **21**, 1393–1403 (2001).
50. Lee, C. S., Friedman, J. R., Fulmer, J. T. & Kaestner, K. H. The initiation of liver development is dependent on Foxa transcription factors. *Nature* **435**, 944–947 (2005).
51. Albertsson, E., Rad, A., Sturve, J., Larsson, D. G. J. & Förlin, L. Carbonyl reductase mRNA abundance and enzymatic activity as potential biomarkers of oxidative stress in marine fish. *Mar. Environ. Res.* **80**, 56–61 (2012).
52. Revelo, X. S. *et al.* Perforin Is a Novel Immune Regulator of Obesity-Related Insulin Resistance. *Diabetes* **64**, 90–103 (2015).

53. Bassett, E. A. & Wallace, V. A. Cell fate determination in the vertebrate retina. *Trends Neurosci.* **35**, 565–573 (2012).
54. Hahn, C., Genner, M. J., Turner, G. F. & Joyce, D. A. The genomic basis of cichlid fish adaptation within the deepwater "twilight zone" of Lake Malawi. *Evol. Lett.* **1**, 184–198 (2017).
55. Reinhard, E., Nedivi, E., Wegner, J., Skene, J. H. P. & Westerfield, M. Neural selective activation and temporal regulation of a mammalian GAP-43 promoter in zebrafish. *Development* **120**, 1767–1775 (1994).
56. Cheung, A., Trevers, K. E., Reyes-Corral, M., Antinucci, P. & Hindges, R. Expression and Roles of Teneurins in Zebrafish. *Front. Neurosci.* **13**, 1–13 (2019).
57. Nadeau, N. J. *et al.* The gene cortex controls mimicry and crypsis in butterflies and moths. *Nature* **534**, 106–110 (2016).
58. Marques, D. A., Meier, J. I. & Seehausen, O. A Combinatorial View on Speciation and Adaptive Radiation. *Trends Ecol. Evol.* **34**, 531–544 (2019).
59. Colot, V. & Rossignol, J.-L. Eukaryotic DNA methylation as an evolutionary device. *BioEssays* **21**, 402–411 (1999).
60. Svardal, H. *et al.* Ancestral Hybridization Facilitated Species Diversification in the Lake Malawi Cichlid Fish Adaptive Radiation. *Mol. Biol. Evol.* **37**, 1100–1113 (2020).
61. Heckwolf, M. J. *et al.* Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Sci. Adv.* **6**, eaaz1138 (2020).
62. Hu, J. *et al.* Heritability of DNA methylation in threespine stickleback (Gasterosteus aculeatus). *Genetics* **217**, 1–15 (2021).
63. Wang, X. & Bhandari, R. K. DNA methylation dynamics during epigenetic reprogramming of medaka embryo. *Epigenetics* **14**, 611–622 (2019).
64. Potok, M. E., Nix, D. A., Parnell, T. J. & Cairns, B. R. Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. *Cell* **153**, 759–772 (2013).
65. Lee, H. J., Hore, T. A. & Reik, W. Reprogramming the Methylome: Erasing Memory and Creating Diversity. *Cell Stem Cell* **14**, 710–719 (2014).
66. Thurman, R. E. *et al.* The accessible chromatin landscape of the human genome. *Nature* **489**, 75–82 (2012).
67. Bogdanović, O. & Veenstra, G. J. C. DNA methylation and methyl-CpG binding proteins: Developmental requirements and function. *Chromosoma* **118**, 549–565 (2009).
68. Houwing, S. *et al.* A Role for Piwi and piRNAs in Germ Cell Maintenance and Transposon Silencing in Zebrafish. *Cell* **129**, 69–82 (2007).
69. Cosby, R. L., Chang, N.-C. & Feschotte, C. Host–transposon interactions: conflict, cooperation, and cooption. *Genes Dev.* **33**, 1098–1116 (2019).
70. Dubin, M. J. *et al.* DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. *Elife* **4**, e05255 (2015).
71. McGee, M. D. *et al.* The ecological and genomic basis of explosive adaptive radiation. *Nature* **586**, 75–79 (2020).
72. Harris, R. S. Improved Pairwise Alignment of Genomic DNA. (The Pennsylvania State University, 2007).
73. Krueger, F. & Andrews, S. R. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* **27**, 1571–1572 (2011).
74. Wu, H. *et al.* Detection of differentially methylated regions from whole-genome bisulfite sequencing data without replicates. *Nucleic Acids Res.* **43**, gkv715 (2015).
75. Wu, H., Caffo, B., Jaffee, H. A., Irizarry, R. A. & Feinberg, A. P. Redefining CpG islands using hidden Markov models. *Biostatistics* **11**, 499–514 (2010).
76. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* **34**, 525–527 (2016).
77. Pimentel, H., Bray, N. L., Puente, S., Melsted, P. & Pachter, L. Differential analysis of RNA-seq incorporating quantification uncertainty. *Nat. Methods* **14**, 687–690 (2017).