Tissue-specific and *cis*-regulatory changes underlie parallel, adaptive gene expression evolution in house mice

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Abstract

Changes in gene regulation have long been appreciated as a driving force of adaptive evolution, however the relative contributions of *cis*- and *trans*-acting changes to gene regulation over short evolutionary timescales remain unclear. Instances of recent, parallel phenotypic evolution provide an opportunity to assess whether parallel patterns are seen at the level of gene expression, and to assess the relative contribution of *cis*- and *trans*- changes to gene regulation in the early stages of divergence. Here, we studied gene expression in liver and brown adipose tissue in two wild-derived strains of house mice that independently adapted to cold, northern environments, and we compared them to a strain of house mice from a warm, southern environment. To investigate gene regulatory evolution, we studied expression in parents and allele-specific expression in F1 hybrids of crosses between warm-adapted and cold-adapted strains. First, we found that the different cold-adapted mice showed both unique and shared changes in expression, but that the proportion of shared changes (i.e. parallelism) was greater than expected by chance. Second, we discovered that expression evolution occurred largely at tissue-specific and *cis*-regulated genes, and that these genes were over-represented in parallel cases of evolution. Finally, we integrated the expression data with scans for selection in natural populations and found substantial parallelism in the two northern populations for genes under selection. Furthermore, selection outliers were associated with *cis*-regulated genes more than expected by chance; *cis*-regulated genes under selection influenced phenotypes such as body size, fat content, immune functioning, and skeletal morphology. These results demonstrate that parallel patterns of gene expression in mice that have independently adapted to cold environments are driven largely by tissue-specific and *cis*-regulatory changes, providing insight into the mechanisms of adaptive gene regulatory evolution at the earliest stages of divergence.

Author summary

The parallel movement of organisms into novel environments provides an opportunity to understand the molecular basis of adaptation and the repeatability of this process. Mutations affecting the expression of genes are known to underlie much of adaptive evolution. Such mutations can arise in *cis*- (near the gene of interest) or in *trans*- (at a distant locus), but the relative contribution of these different kinds of changes to adaptation is poorly understood, especially during very recent divergence. Here, we compared evolved gene expression differences between a warm-adapted house mouse strain and two different cold-adapted strains that have independently evolved similar phenotypic traits, such as increased body size and decreased extremity length during the last few hundred years. Using crosses between warm-adapted and cold-adapted mice, we found that mutations acting in a context specific manner (*cis*-regulatory and tissue-specific changes) predominate expression divergence and are more likely to be involved in parallel evolution. We used population level selection scans in wild animals to identify regions of the genome under selection and combined these findings with the gene expression data to identify candidate genes underlying adaptation to novel environments. Together, our work describes the gene regulatory dynamics of rapid environmental adaptation, and the repeatability of these patterns over multiple instances of adaptation.

Introduction

Understanding the genetic architecture of adaptive evolution is a long-standing goal in evolutionary biology. Differences in gene regulation are known to underlie adaptation to new

68 environments across a diverse set of organisms and phenotypes including armor plating and
69 pelvic spine reduction in sticklebacks, coat color differences in deer mice, and the ability to
70 digest lactose in humans [1–4].

71 Evolved changes in gene expression can occur via changes in *cis*-regulatory elements,
72 which act locally in an allele-specific manner, or *trans*-regulatory elements, which act distally
73 and can potentially affect many genes [1,5]. Since *cis*-regulatory changes can modulate gene
74 expression in a tissue-specific and temporal-specific manner, these variants are expected to be
75 less pleiotropic and may therefore evolve more easily [1,5]. In contrast, *trans*-acting changes
76 may affect the expression of many genes and thus are expected to be more constrained.
77 However, alterations to the wider regulatory environment via *trans*-acting changes may be
78 favored during early stages of adaptation, when populations are further from their fitness optima
79 [6,7].

80 Given that changes in gene expression underlie much of adaptive evolution,
81 understanding the contributions of *cis*- and *trans*-changes to gene regulation will help shed light
82 on the molecular mechanisms and genetic architecture of evolutionary change. Several studies
83 have evaluated the contributions of *cis*- and *trans*-acting mutations to gene expression by
84 studying allele-specific expression in hybrid individuals or by mapping expression quantitative
85 trait loci (eQTL) (reviewed in Signor and Nuzhdin 2018; Hill et al. 2020). One emerging pattern
86 is that *cis*- variants often contribute more to expression divergence between species, while *trans*-
87 variants dominate within-species expression variance [9–13]. This observation is usually
88 attributed to the larger mutational target for *trans*-acting variants compared to *cis*-acting variants.
89 Between species, *cis*-acting variants constitute a greater proportion of regulatory variants at
90 increasing levels of evolutionary divergence in both yeast [14] and *Drosophila* [11]. Despite

91 these patterns, some studies have found a larger role for *cis*-acting changes over short
92 evolutionary timescales. For example, comparisons between subspecies of house mice have
93 revealed a greater proportion of *cis*-regulatory changes compared to *trans*-regulatory changes in
94 liver [15] and in testes [16]. Comparisons among lines of *Drosophila melanogaster* have also
95 revealed a preponderance of *cis*-regulatory effects [17]. Different studies on the same species
96 have sometimes reached different conclusions. For example, in comparisons between marine and
97 freshwater populations of stickleback fish, Hart et al. [18] found a greater role for *trans*-acting
98 changes in governing expression differences in pharyngeal dental tissue, while Verta and Jones
99 [19] found *cis*-acting changes to predominate expression differences in the gills. In sum, it
100 remains unclear whether less pleiotropic changes (*cis*-regulated or tissue specific genes) or more
101 pleiotropic changes (*trans*-regulated or broadly-expressed genes) are most likely to contribute to
102 adaptive gene expression evolution over short time scales.

103 Recent, repeated colonization of novel environments not only provides an opportunity to
104 assess the nature of evolutionary changes over short time scales, but also to study parallel
105 evolution. When different populations have experienced similar environments and selection
106 pressures, repeated patterns provide evidence that the observed changes are driven by selection.
107 Situations in which phenotypes have evolved in parallel provide an opportunity to ask whether
108 parallelism is similarly seen at the molecular level.

109 House mice (*Mus musculus domesticus*) represent a useful system in which to explore the
110 gene regulatory basis of environmental adaptation and the degree of parallelism in expression
111 divergence. House mice are found throughout inhabited regions of the world [20] and display
112 morphological, physiological, and behavioral phenotypes reflective of their environment [21,22].
113 For example, mice from northern latitudes have larger bodies, shorter extremities, and build

114 larger nests, conforming to well-documented ecogeographic patterns in mammals [21–23].
115 Shortened extremities and increased body size are viewed as thermoregulatory adaptations since
116 they decrease the ratio of surface area to volume, thereby minimizing heat loss [24]. Importantly,
117 these phenotypes have evolved repeatedly in response to cold temperatures in multiple
118 populations of house mice [25], representing cases of parallel independent adaptation. Finally,
119 previous studies have shown that gene regulation is likely to be a primary mediator of adaptive
120 evolution in house mice, as scans for selection have identified adaptive mutations primarily in
121 non-coding regions of the genome [22].
122 Here, we analyze gene expression in inbred strains of house mice derived from two,
123 independently-colonized northern populations and one equatorial population to address several
124 key questions related to gene expression evolution, including the degree to which expression
125 divergence is shared between parallel cases of adaption, whether this expression evolution is
126 governed by primarily *cis*- or *trans*-acting changes, as well as whether those changes are largely
127 tissue-specific or broadly expressed. We also integrate expression data with scans for selection in
128 wild-caught mice to connect expression differences identified in lab-reared animals to selection
129 acting in natural populations. We find strong evidence for parallel adaptive evolution due to
130 changes in the expression of *cis*-regulated and tissue-specific genes in the early stages of
131 adaptation to novel environments.
132
133

134 Results

135 Phenotypic divergence between cold-adapted and warm-adapted mice

136 Previous work based on phylogenetic analysis of house mouse populations across eastern
137 and western North America showed that mice in upstate New York and mice in Western Canada
138 represent independent range expansions into northern habitats from southern locations and
139 independent cases of adaptation to cold climates with parallel differences in body size [25]. We
140 thus chose one strain from Saratoga Springs, New York, USA (SARA) and one strain from
141 Edmonton, Alberta, Canada (EDMA), and compared these to one strain from Manaus, Brazil
142 (MANA) (Fig 1A). Lab-reared inbred strains of mice from these localities display divergent,
143 environmentally adaptive phenotypes under common conditions, suggesting that they have a
144 genetic basis (Fig 1B-1E). In particular, mice from New York and Alberta are larger but have
145 shorter tails, ears, and feet when compared to mice from Manaus. These differences conform to
146 Bergmann's Rule (larger size in cold environments) and Allen's Rule (shorter extremities in cold
147 environments) and are thought to reflect thermoregulatory adaptations.
148

149 **Expression divergence between cold-adapted and warm-adapted mice**

150 To study expression evolution between cold-adapted and warm-adapted mice, we
151 generated RNA-seq data from the liver and brown adipose tissue (BAT) of six males each from
152 the SARA, EDMA, and MANA strains. We chose liver and BAT, as liver underlies many
153 metabolic processes involved in environmental adaptation in house mice [22,26], and BAT is
154 involved in cold adaptation via its role in non-shivering thermogenesis [27,28]. Principal
155 component (PC) analysis of expression separates samples primarily by tissue-type (S1A Fig),
156 agreeing with studies investigating divergent gene expression across multiple tissues [29,30].
157 Within each tissue, PC analysis separates individuals by strain with PC1 and PC2 two explaining
158 34.8% and 26.9% of expression variation in liver, respectively (Fig 1F), and 30.3% and 27.9% of

159 expression variation in BAT, respectively (S1B Fig). The differences in expression between
160 strains undoubtedly reflects both strain-specific differences as well as population-level
161 differences, although previous work has shown that expression variation in wild-derived inbred
162 mice from different populations mostly reflects population-level differences [22].

163 To quantify the proportion of unique and shared changes associated with independent
164 instances of adaptation to northern environments, we compared evolved gene expression changes
165 between SARA and MANA to evolved changes between EDMA and MANA using DESeq2
166 [31]. For liver and BAT, respectively, we found 4,121 and 4,160 significantly differentially
167 expressed genes (DEGs) between SARA and MANA, and 4,936 and 4,585 DEGs between
168 EDMA and MANA (FDR < 5% for all differential expression analyses). Of these genes, 2,361
169 and 2,231 were shared between SARA and EDMA comparisons for liver and BAT, respectively
170 (Fig2A, S2A Fig). Thus, roughly half of the DEGs were shared between populations and roughly
171 half were unique to each population. Nonetheless, the number of shared genes is 1.68 and 1.72
172 times greater than expected by chance in liver and BAT, respectively, as measured through
173 permutation tests (see methods for details, Fig 2B, S2B Fig). Shared changes might reflect
174 parallel evolution in New York and Alberta house mice, or unique changes along the lineage
175 leading to Manaus, as discussed in more detail in the "*Signals of selection*" section.

176 To further assess the degree of parallel expression evolution, we measured the correlation
177 between SARA-MANA expression differences and EDMA-MANA expression differences. Log_2
178 fold changes between SARA and MANA are highly correlated to those between EDMA and
179 MANA (Fig 2C, Spearman's r = 0.54 for liver, S2C Fig, Spearman's r = 0.53 for BAT).
180 Moreover, significant DEGs for both comparisons have larger correlation coefficients than the
181 transcriptome as a whole (Fig 2C, S2C Fig). Because spurious correlations can occur when

182 comparing two ratios that share the same denominator, we reanalyzed the RNA-seq data using
183 different subsets of the MANA mice to define differential expression in the two comparisons (i.e.
184 EDMA mice were compared to MANA mice 1, 2, and 3, while SARA mice were compared to
185 MANA mice 4, 5, and 6) and all significant correlations were recapitulated (S3 Fig).
186 To investigate the importance of these expression changes to phenotypic changes
187 between cold-adapted and warm-adapted mice, we next asked whether genes involved in
188 increased body size were implicated in parallel expression evolution to a greater degree than the
189 transcriptome more generally, since larger body size is a trait that has evolved independently in
190 New York and Alberta mice [25]. We defined a list of 412 "increased body size genes" using
191 phenotypes defined by the mouse genome informatics (MGI) database (MP code: 0001264).
192 Supporting this hypothesis, we found that expression divergence in increased body size genes
193 was highly correlated between the comparisons in both BAT and liver (Fig 3, Spearman's r =
194 0.82 for both tissues), showing greater correlation coefficients than for either the transcriptome
195 as a whole or for DEGs.
196

197 Enrichment of immune related functions among differentially expressed genes

198 To categorize the potential function of genes involved in gene expression evolution in
199 cold-adapted mice, we used gene ontology analyses via PANTHER [32]. We found high
200 enrichment for biological processes related to immune functioning for both the SARA and
201 EDMA comparisons in both BAT and liver, such as antigen processing, response to interferon-
202 beta, and regulation of T cells (Fig 2D-2E, S2D-S2E Fig). Additionally, we found enrichment for
203 metabolism related processes across comparisons and tissues, including lipid metabolism, small

204 molecule metabolism, and various other metabolic processes, which may be indicative of
205 metabolic evolution related to cold adaptation at the transcriptomic level.
206

207 Expression divergence is primarily governed by *cis*-regulatory changes

208 We next asked whether gene expression differences are primarily governed by *cis*-
209 regulatory changes or *trans*-regulatory changes by measuring allele-specific expression (ASE) in
210 liver and BAT from F1 hybrids between SARA X MANA and EDMA X MANA. Because F1
211 hybrids possess one allele from each parent within the same *trans*-acting environment, genes
212 showing allele-specific differences in expression in F1s are governed by a *cis*-acting change [33].
213 For both comparisons and both tissues, we found that *cis*-acting changes underlie most
214 expression divergence. While most gene expression was conserved between cold-adapted and
215 warm-adapted mice, in the liver we found that 8.6% and 8.0% of all genes were regulated in *cis*-
216 only, while 1.7% and 4.6% were regulated in *trans*- only for SARA vs. MANA and EDMA vs.
217 MANA, respectively (Fig 4A-4B). Expression in BAT showed similar trends (S4A-S4B Fig).
218 We also found that *cis*-acting changes generally have larger effect sizes, as measured by absolute
219 $\log_2$ fold change, consistent with previous studies in house mice (Fig 4C-4D, S4C-S4D Fig) [16].
220 The difference in effect size between *cis*-only and *trans*-only was significant in both SARA and
221 EDMA in BAT, but only in EDMA in liver (T-tests, Liver: SARA-MANA p=0.12, EDMA-
222 MANA p=0.01, BAT: SARA-MANA p=0.04, EDMA-MANA p=0.002). Overall, we found that
223 large-effect changes in *cis*- underlie much of expression evolution in this system.
224

225 Different degrees of parallelism in *cis*- and *trans*-regulatory changes

226 Given that most expression evolution was due to changes in *cis*-, we hypothesized that
227 *cis*-acting changes would also be more likely to contribute to expression differences that have
228 evolved in parallel in both New York and Alberta mice. To test this hypothesis, we first
229 compared the expected overlap between SARA and EDMA for DEGs in general to the observed
230 overlap for genes defined as *cis*-regulated in both comparisons via permutation tests (see
231 methods). The same test was done with *trans*-regulated genes. We focused on genes defined as
232 "*cis*-only" and "*trans*-only", and not those that contained significant levels of both *cis*- and
233 *trans*-regulation because the number of genes falling in those categories was small. Both *cis*- and
234 *trans*-regulated genes showed significant enrichment for parallel evolved DEGs (Fig 4E-4F,
235 S4E-S4F Fig, liver: $p = \sim 0$ for *cis*, $p = 0.02$ for *trans*, BAT: $p = \sim 0$ for *cis*, $p = 0.002$ for *trans*;
236 observed values of overlap that are outside of the permuted expected distribution are reported as
237 $p = \sim 0$).
238 To more directly compare the degree of parallelism in *cis*-regulated and *trans*-regulated
239 genes, we analyzed the difference in proportion of shared genes via a chi-square test of
240 independence. The average proportion of *cis*-regulated genes shared between comparisons was
241 0.25 for liver and 0.24 for BAT, while the average proportion shared for *trans*-regulated genes
242 was 0.08 for liver and 0.09 for BAT (Fig 4E-F, S4E-S4F Fig). This difference in proportions is
243 significant in both tissues ($p < 0.0001$ for liver and BAT). Additionally, for genes that were *cis*-
244 regulated in both comparisons, we found that $\log_2$ fold changes between SARA and MANA and
245 EDMA and MANA were highly correlated (S5 Fig, Spearman's $r = 0.89$ for liver, 0.96 for
246 BAT). Because *cis*-regulated genes represent independent cases of expression divergence,
247 whereas *trans*-regulated changes may influence the expression of many genes, this strong
248 correlation in *cis*-regulated genes provides further evidence of significant parallelism among

249 northern house mice. Together, these results suggest that while both *cis*- and *trans*-regulated
250 genes are more likely than expected by chance to contribute to parallel expression divergence,
251 *cis*-regulated genes are shared between parallel comparisons to a greater extent than *trans*-
252 regulated genes.
253

254 Tissue-specific genes are largely *cis*-regulated, exhibit high amounts of
255 expression divergence, and are involved in parallel evolution

256 To investigate the importance of tissue-specificity in expression evolution, we integrated
257 our liver and BAT RNA-seq data with a published adult mouse expression dataset [34] including
258 seven tissues (brain, cerebellum, heart, kidney, liver, ovary, and testis), and calculated tissue
259 specificity using the value Tau (see methods). Tau ranges from 0 to 1, with 0 corresponding to a
260 gene evenly expressed across all tissues and 1 corresponding to a gene with expression restricted
261 to one tissue [35]. For the SARA-MANA and EDMA-MANA comparisons, respectively, we
262 identified 516 and 526 liver-specific genes, 161 and 161 BAT-specific genes, and 793 and 798
263 broadly expressed genes.
264 We then investigated the mode of regulatory control (i.e. *cis*- vs. *trans*-) for genes of
265 differing tissue specificities using the regulatory categories determined via ASE in F1 hybrids.
266 These analyses were done with a smaller set of genes for which we had gene regulatory data (see
267 methods). Below we highlight the results for liver, but similar patterns were observed for BAT
268 (S6 and S8 Fig). In liver, the vast majority of broadly expressed genes have conserved
269 expression patterns between cold-adapted and warm-adapted mouse strains (SARA-MANA:
270 90.6%, EDMA-MANA: 87.4%). This proportion of conserved genes is greater than that seen
271 across all genes (SARA-MANA: 80.9%, EDMA-MANA: 74.9%) (Fig 5A, S7A Fig).

272 In contrast, for tissue-specific genes, only 46.7% and 35.8% were conserved for SARA-
273 MANA and EDMA-MANA, respectively. Moreover, we found that a higher proportion of
274 tissue-specific genes were regulated in *cis*- (SARA-MANA: 31.4%, EDMA-MANA: 33.9%) as
275 compared to all genes (SARA-MANA: 11.6%, EDMA-MANA: 11.4%) and broadly expressed
276 genes (SARA-MANA: 5.8%, EDMA-MANA: 4.6%, Fig 5A, S7A Fig). Further, using a
277 permutation approach to estimate the expected number of DEGs, we found that tissue-specific
278 genes are highly enriched for DEGs, suggesting that expression evolution is occurring in tissue-
279 specific genes to a greater extent than in broadly expressed genes or in the transcriptome as a
280 whole (Fig 5B-C, S7B-S7C Fig). Conversely, broadly expressed genes were under-enriched for
281 DEGs, which is consistent with the large amount of conserved expression in this gene set (Fig
282 5C, S7C Fig). Finally, tissue-specific genes also showed larger effect sizes, as measured as
283 absolute $\log_2$ fold change (Fig 5D, S7D Fig).
284 Given the high parallelism in *cis*-regulated genes, we hypothesized that differentially
285 expressed, tissue-specific genes would be more likely to be involved in parallel evolution as
286 compared to differentially expressed genes as a whole. Using permutation tests, we found that
287 tissue-specific DEGs are more likely to be shared between SARA and EDMA than DEGs more
288 generally for both liver and BAT (Fig 5E, S6E Fig, liver-specific: $p = \sim 0$, BAT-specific: $p = \sim 0$).
289

290 **Signatures of selection to northern environments**

291 While gene expression differences speak to patterns of regulatory evolution, they do not
292 directly address whether these differences are adaptive. To link expression variation in SARA
293 and EDMA lab mice to selection in natural populations, we used a normalized version of the
294 population branch statistic (PBSn1), which identifies significant allele frequency shifts in a focal

295 population in relation to two outgroup populations [36,37]. For focal populations analogous to
296 SARA and EDMA lab mice, we utilized published whole exome data from New Hampshire /
297 Vermont (NH/VT) (which is geographically close to Saratoga Springs, NY) and Edmonton,
298 Alberta, Canada (EDM) [22,25], and used published whole genome data from Eurasia,
299 specifically Iran and France, to serve as outgroup comparisons [38]. Iran and France are both
300 ancestral populations to house mice in North America, making them suitable populations to serve
301 as outgroups [20,25,39].

302 First, we performed two separate PBSn1 tests with NH/VT and EDM as the focal
303 population and France and Iran as outgroup populations. However, EDM mice and NH/VT mice
304 are more closely related to each other than they are to mice from Europe and the Middle East
305 [25]. Therefore, shared outliers in the two PBSn1 tests with France and Iran can either be due to
306 1) shared ancestry between EDM and NH/VT or 2) true instances of parallel adaptation to a
307 northern environment. To distinguish between these two possibilities, we performed a second
308 (nested) set of PBSn1 tests focused on identifying outliers in NH/VT and EDM that are not
309 associated with shared ancestry by using Arizona (AZ) and Iran as outgroups for EDM, and by
310 using Florida (FL) and Iran as outgroups for NH/VT. NH/VT and FL mice form a clade, and
311 EDM and AZ mice form a clade [25]. Therefore, shared outliers identified through this second
312 set of tests reflect parallel adaptation to northern environments, as opposed to evolution
313 occurring since the introduction of house mice into North America.

314 The percentage of hits retained between the EDM-France-Iran test and the EDM-AZ-Iran
315 test (or the NH/VT-France-Iran test and the NH/VT-FL-Iran test) gives an estimate of the
316 amount of shared PBSn1 outliers in the first test that are due to shared ancestry vs. true repeated
317 instances of adaptation to a similar environment. Using this nested approach, we found that

318 approximately 50% of outlier genes identified in the first tests were also identified in the nested
319 tests (S9 Fig), suggesting that half of the outlier genes identified in the first tests were due to
320 shared ancestry, while half represent instances of parallel adaptation.
321

322 Enrichment of parallelism and *cis*-regulation among genes under positive
323 selection

324 To identify selection acting on differential expression in cold-adapted house mice, we
325 focused on the second set of PBSn1 tests above using Iran and AZ or FL as outgroups. For these
326 tests we calculated PBSn1 over 82,809 and 100,602 non-overlapping 5-SNP blocks for NH/VT
327 and EDM, respectively, and we used BEDTools [40] to identify the closest gene to each SNP-
328 block (Fig 6A). Mirroring the extensive parallelism identified in the expression data, we found
329 that NH/VT and EDM PBSn1 outliers (top 5%) at the gene level were more likely to be shared
330 than expected by chance (Fig 6B, $p = \sim 0$). Second, we hypothesized that there would be
331 significant overlap between PBSn1 outliers and *cis*-regulated genes because we found *cis*-
332 regulation to largely underlie expression divergence, and because *cis*-regulatory elements have
333 long been hypothesized to be involved in adaptive evolution [1,7]. For both the SARA and
334 EDMA comparisons in liver and BAT, using permutation tests (see methods) we found that *cis*-
335 regulated genes overlapped PBSn1 outliers more than would be expected by chance (Fig 6C,
336 liver: $p = 0.0031$ for SARA, $p = \sim 0$ for EDMA, BAT: $p = 0.0033$ for SARA, $p = \sim 0$ for EDMA).
337 Importantly, there was not significant overlap of PBSn1 outliers and *trans*-regulated genes ($p >$
338 0.05 for both tissues in each comparison), which agrees well with *trans*-regulatory elements
339 being unlinked from the genes they influence. To address whether the parallelism seen among
340 *cis*-regulated genes (Fig 4E, S4E Fig) was due to shared ancestry among New York and Alberta

341 mice, we performed another test for parallelism between SARA and EDMA *cis*-DEGs using only
342 *cis*-DEGs that overlap an outlier gene in the nested PBSn1 tests. Similar to the original tests for
343 parallelism, we found significant parallelism between SARA and EDMA for *cis*-regulated genes
344 under selection in both liver (p = 0.0002) and BAT (p = 0.0134) (S10 Fig).

345 To identify specific genes that may be directly involved in parallel adaptation to northern
346 environments, we looked for overlap among genes that are *cis*-regulated in both SARA and
347 EDMA, and under selection in both comparisons (Fig 6D). Of this set of 10 genes, almost half
348 are implicated in body size and body fat variation (*Sdr9c7, Ahnak, Slc12a7, Angptl2*), and the
349 others are involved in skeletal morphology differences (*Ndc1*), coat abnormalities (*Gtpbp4*), and
350 immune functioning (*Itgb1*). Overall, we found significant parallelism in genes evolving under
351 positive selection in cold-adapted house mice, that these genes are often associated with *cis*-
352 regulatory elements, and that parallel, *cis*-regulated genes are involved in phenotypes such as
353 body size differences, immune functioning, and elements of skeletal morphology.
354

355 Discussion

356 Studying gene expression patterns in populations that have recently adapted to differing
357 climates allowed us to characterize adaptive evolution during the earliest stages of divergence.
358 Using allele-specific expression in two cold-adapted strains of mice in two tissues involved in
359 metabolic functioning and thermogenesis, we sought to understand patterns of *cis*- and *trans*-
360 regulation and tissue specificity during parallel adaptation to similar environmental conditions.
361 SARA and EDMA mice have evolved both unique and shared gene expression changes
362 compared to MANA mice, however the proportion of shared changes was greater than expected
363 by chance. Further, expression changes at genes associated with increased body size were highly

364 similar between SARA and EDMA mice, suggesting that New York and Alberta populations of
365 house mice evolved the shared phenotype of increased body size through some shared molecular
366 mechanisms. We found that tissue-specific and *cis*-acting changes formed the basis of most gene
367 expression divergence between cold-adapted and warm-adapted strains of mice, and that these
368 changes were more likely to contribute to parallel expression evolution. Lastly, comparison
369 between the expression data and scans for selection in wild populations revealed significant
370 parallelism in genes under positive selection in cold-adapted house mice and that these genes
371 were enriched for *cis*-regulated genes. Together, these results provide strong evidence of
372 adaptive expression evolution and lend insight into the nature of gene regulatory changes during
373 rapid parallel adaptation to cold environments.
374

375 Parallel gene expression and genomic changes underlie adaptation to cold 376 environments

377 Repeated phenotypic changes in response to similar selective pressures provides an
378 opportunity to study the repeatability of evolution at the molecular level. Additionally, repeated
379 phenotypic change suggests that evolution is likely adaptive, and not a result of genetic drift.
380 New York and Alberta house mice have independently evolved larger body sizes and shortened
381 extremities while adapting to a cold environment. These phenotypes are highly polygenic, and
382 thus one might expect that at the genic level the degree of parallelism involving these traits
383 would be low [41]. In contrast to this expectation, we found expression evolution to be highly
384 similar in EDMA and SARA mice in both liver and BAT, with approximately half of all
385 differentially expressed genes being shared between comparisons.

386 Importantly, mice from New York and Alberta are more closely related to each other than
387 they are to mice from Manaus (S11 Fig). Thus, some of the shared expression differences
388 undoubtedly reflect shared ancestry, while some reflect true instances of parallel evolution.
389 While we have no formal means for distinguishing between instances of parallel evolution and
390 shared ancestry in the gene expression data directly, several of the expression analyses are
391 consistent with the notion that a substantial fraction of the shared expression differences reflect
392 parallel evolution. For example, the correlation between SARA-MANA expression differences
393 and EDMA-MANA expression differences is greatest for genes involved in increased body size,
394 a trait that is known to have evolved independently in New York and Alberta mice [25] (Fig 3).
395 Additionally, we found significant overlap between *cis*-regulated genes and genes under
396 selection in the nested PBSn1 test, which was designed to identify independently evolved
397 differences in the two cold-adapted populations (Fig 6C). Lastly, we found that the signal of
398 parallelism among *cis*-regulated genes was maintained when using only *cis*-regulated genes that
399 overlapped a PBSn1 outlier identified in the nested test (S10 Fig), suggesting that *cis*-regulated
400 genes are under independent selection in both cold-adapted populations of house mice. The
401 substantial parallelism between cold-adapted house mice is consistent with selection on shared
402 standing genetic variation, which is expected to increase the degree of similarity seen between
403 independent cases of adaptation [41].
404

405 ***Cis*-regulated and tissue-specific changes dominate parallel expression**
406 **evolution**

407 We found evidence for a predominance of *cis*-regulated changes governing gene
408 expression evolution in two instances of adaptation to northern environments. *Cis*-changes are

409 generally thought to be less pleiotropic by allowing for tissue- and context-specific gene
410 expression regulation [1,5,8]. Consistent with this, we also found that tissue-specific
411 differentially expressed genes were largely *cis*-regulated and frequently contributed to expression
412 evolution.

413 These results stand in contrast to the commonly reported finding that intraspecific gene
414 expression variation is largely governed in *trans*-, while interspecific variation is largely
415 governed in *cis*- [5,8–11]. However, the proportion of *cis*-regulatory variation that is discovered
416 within species likely depends on many factors that differ among studies. First, studies that have
417 found a predominance of *trans*-changes governing intraspecific variation have often focused on
418 comparisons among individuals within a population such as inbred lab strains of *Drosophila* [9]
419 or *Saccharomyces cerevisiae* strains derived from the same isolate [10,42]. In contrast, in
420 intraspecific comparisons where the groups have experienced directional selection, such as in
421 freshwater vs. marine sticklebacks [19] or cold-adapted vs. warm-adapted *D. melanogaster* [43],
422 *cis*-changes have sometimes been found to govern the majority of expression variation. These
423 contrasting patterns can be explained if expression variation under stabilizing selection is largely
424 governed in *trans*- while expression variation under positive directional selection is largely
425 governed in *cis*-. Therefore, it seems important to consider whether the groups being compared
426 are under divergent selection when interpreting the relative amounts of *cis*- and *trans*-regulation
427 observed in intraspecific comparisons.

428 Second, the relative amount of *cis*- and *trans*-regulation that is discovered may depend on
429 whether expression is studied in a specific tissue or in a whole organism. A bias could arise in
430 two ways. First, because *cis*-acting changes are often associated with tissue-specific gene
431 expression, measuring RNA at the level of the entire organism, such as an entire fly [9,11], could

432 dampen the signal of tissue specific *cis*-changes, therefore minimizing the level of *cis*-regulation
433 detected. Second, changes in cellular composition (i.e. changes in the proportions of different
434 kinds of cells) will appear as *trans*-changes when studying expression in whole organisms. For
435 example, flies with bigger brains will express more brain-specific transcripts simply as a
436 consequence of having more brain cells. Interestingly, in studies that have analyzed the head and
437 body separately in *Drosophila*, *cis*-acting changes were found to predominate [17].
438 While there are contrasting trends represented across various divergence times and
439 organisms, here we present a case of nascent divergence (within ~500 years) facilitated largely
440 by *cis*-changes; this trend was similarly demonstrated by Ballinger et al. [44] in their comparison
441 of New York and Brazil house mice. Together, these studies lend support to the importance of
442 *cis*-regulatory elements even during very early instances of intraspecific differentiation.
443 Further supporting a large role for *cis*-acting and tissue-specific changes, we found these
444 modes of regulation to predominate in both SARA and EDMA mice, consistent with the idea that
445 less pleiotropic changes are more likely to contribute to parallel divergence. Recent studies on
446 gene regulatory patterns during parallel evolution have come to contrasting conclusions, with
447 two studies finding a greater degree of parallelism in *cis*-acting changes [19,43] and another
448 study finding a greater degree of parallelism in *trans*-acting changes [18]. Considerable previous
449 theoretical and empirical work has suggested that less pleiotropic loci are more likely to
450 contribute to parallel and convergent evolution [7,45–48]. However, some recent work in locally
451 adapted stickleback populations has suggested that genes with intermediate levels of pleiotropy
452 (identified through mapping studies and expression networks) may be more likely to underlie
453 parallel adaptive evolution [49]. Genes with intermediate degrees of pleiotropy may be favored
454 in situations where selection is acting on standing variation repeatedly in opposing directions.

455 The distinction between *cis*- and *trans*- changes in our study does not allow us to assess
456 "degrees" of pleiotropy, but future studies aimed at using gene co-expression networks could be
457 used to ask whether genes with intermediate levels of connectedness are most likely to underlie
458 parallel evolution.
459

460 **Identifying strong candidate genes for complex traits**

461 Uncovering the genetic basis of adaptation for complex traits is challenging because these
462 phenotypes are controlled by a large number of genes of mostly small effect. Here we combined
463 multiple experimental approaches including differential gene expression, scans for selection and
464 the use of parallel instances of adaptation, that, in aggregate, provide more information on the
465 genetic basis of adaptive, polygenic traits than any one method alone. Through this approach we
466 were able to identify several strong candidate genes that are differentially expressed, controlled
467 in *cis*-, and under selection in both northern populations. These genes are related to known
468 phenotypic differences in northern and southern house mice including body size (*Sdr9c7,*
469 *Slc12a7*), body fat variation (*Ahnak, Angptl2*), skeletal morphology (*Ndc1*), and coat differences
470 (*Gtpbp4*). Interestingly, both the candidate gene approach and gene ontology analyses (Fig 2D-
471 2E, S2D-S2E Fig) also revealed an enrichment for immune related genes during adaptation to
472 northern environments. This result agrees with previous work showing novel pathogens as a
473 strong selective force on organisms invading new environments, often shaping the response at
474 the genomic level [50].
475 Our results show that selection is acting on *cis*-regulatory elements in natural populations
476 to modulate gene expression levels that are then maintained in animals reared under common lab
477 conditions. This work provides a detailed account of the gene regulatory changes underlying

478 repeated instances of adaptation to cold environments and the degree of similarity between these
479 cases of repeated adaptation. Ultimately, our findings underscore the importance of *cis*-regulated
480 and tissue-specific changes in facilitating rapid adaptive evolution in organisms facing novel
481 environmental conditions.
482

483 Materials and methods

484 Animal husbandry and mice used for this study

485 All mice were housed under a 12:12 light cycle at 70-72°C and fed standard rodent food
486 *ab libitum*. This work was conducted in accordance with the University of California, Berkeley
487 Institutional Animal Care and Use Committee (AUP-2017-08-10248).
488 Wild derived, inbred strains of house mice used in this study were originally collected
489 from 43°N in Saratoga Springs, New York (SARA), 54°N in Edmonton, Alberta, Canada
490 (EDMA), and at 3°S in Manaus, Brazil (MANA). Information on the establishment of these
491 inbred strains is described in Phifer-Rixey et al. [22] and Ferris et al. [25]. In brief, live mice
492 were collected from New York, Alberta, and Manaus, brought back to the lab, and unrelated
493 individuals were mated to create the N1 generation. Inbred strains were generated via subsequent
494 sib-sib mating. SARA, EDMA, and MANA mice in this study were past generation 10 of sib-sib
495 mating and considered fully inbred.
496 We crossed SARA dams to MANA sires and EDMA dams to MANA sires to generate
497 SARA x MANA and EDMA x MANA F1 hybrids, respectively. All mice were housed singly
498 from weaning (3 weeks) until adulthood. The SARA, MANA, and SARA x MANA male F1
499 hybrids were previously generated in Ballinger et al. [44].
500

501 **Phenotyping, sample preparation and RNA-sequencing**

502 At 11 weeks of age, six males of each group [SARA, EDMA, MANA,
503 (SARAxMANA)F1, and (EDMAxMANA)F1] were sacrificed. Body weight, total body length,
504 tail length, ear length, and hind foot length were measured, and liver and brown adipose tissue
505 (BAT) were collected for RNA-sequencing. Further information for all mice used in this study
506 can be found in S1 File. Liver and BAT were immediately placed in RNAlater, kept at 4°C for
507 24 hours and then stored at -80°C until RNA-extraction. We extracted RNA using the Qiagen
508 RNeasy minikit following the manufacturer's instructions. We validated RNA integrity using an
509 Aligent Bioanalyzer and only RNA with RIN > 8 was used for library preparation. We generated
510 cDNA libraries using the KAPA Stranded mRNA-Seq kit starting with 2ug of total RNA
511 measured using Nanodrop, and uniquely indexed libraries using dual indexes from Illumina. We
512 assessed library size and quality using an Aligent Bioanalyzer with a desired insert size of 300bp.
513 Tissue-specific libraries were then pooled and sequenced across two lanes of Illumina 150bp
514 paired-end NovaSeq, one using an S4 flow cell, and one using an S1 flow cell at the Vincent J.
515 Coates Genomics Sequencing Center at UC Berkeley.

516

517 **Gene expression quantification and analysis**

518 All analyses for the liver and BAT were performed identically, but separately unless
519 otherwise noted. Resulting RNA-Seq reads were cleaned and trimmed with FastP [51] (v.0.19.6,
520 parameters = --n_base_limit 5, --qualified_quality_phred 15, --unqualified_percent_limit 30, --
521 detect_adapter_for_pe, --cut_window_size 4, --cut_mean_quality 15, --length_required 25), and
522 then mapped to the mouse reference genome (GRCm38/mm10) with STAR [52] (v2.7.7a,
523 parameters = --outFilterMultimapNmax 1, --outFilterMismatchNmax 3). After alignment, reads

524 were counted using HTseq [53], and count files from each sample were merged using the python
525 script merge_tables.py (Dave Wheeler, github link). This merged table was the input for
526 downstream differential expression analyses.

527 Differential expression between mouse strains was quantified using DESeq2 in R [31],
528 and only genes with a mean greater than 10 reads across samples were retained for analysis. This
529 resulted in a set of 14,514 and 14,703 genes that were expressed in our liver and BAT datasets,
530 respectively. The contrast parameter in the result command was used to isolate SARA vs MANA
531 and EDMA vs MANA comparisons, and differential expression between strains was determined
532 using Wald tests followed by a Benjamini-Hochberg correction for multiple testing with a false
533 discovery rate (FDR) of 5%. DESeq2 was also used to generate principal component plots (Fig
534 1F, S1 Fig).

535 To confirm that the correlation in $\log_2$ fold change observed between the SARA vs
536 MANA comparison and the EDMA vs MANA comparison (Fig 2C, S2C Fig) was not a spurious
537 correlation due to both variables having the same denominator, we re-quantified differential
538 expression between the mouse strains as described above using different subsets of MANA mice
539 in each comparison (ie. EDMA vs MANA samples 1-3 and SARA vs MANA samples 4-6).
540 Correlations were maintained (S3 Fig), suggesting parallel changes.

541

542 Gene set and gene ontology analyses

543 To test for increased gene expression parallelism associated with a parallel evolved
544 phenotype, we compiled a list of genes linked to increased body size using the Mammalian
545 Phenotype Browser on the Mouse Genome Informatics database (MP ID: 0001264). This
546 returned a list of 412 genes to be used in downstream analyses (S2 File). We conducted gene

547 ontology enrichment analyses separately for SARA and EDMA comparisons on differentially
548 expressed genes at an FDR < 5% and a $\log_2$ fold change greater than ± 1 using PANTHER [32].
549

550 **Variant calling from transcriptomic data**

551 We called single nucleotide polymorphisms (SNPs) using the Genome Analysis Tool Kit
552 (GATK) [54] on our RNA-seq data following the RNA-seq best practices for germline short
553 variant discovery. Reads were cleaned, trimmed, and aligned as described in the section above.
554 We prepared BAM files for variant calling using Picard tools MarkDuplicates and
555 AddOrReplaceReadGroups, and by running SplitNCigarReads. We then ran HaplotypeCaller on
556 each individual separately passing both the liver and BAT RNA-seq libraries as inputs. Separate
557 gVCF files were combined using CombineGVCFs and genotypes were called on all individuals
558 together using GenotypeGVCFs. We selected just SNPs using SelectVariants and filtered for
559 high quality variants using the command VariantFiltration (parameters = QD >2, FS < 60, MQ >
560 40, MQRankSum > -12.5, ReadPosRankSum > -8).
561

562 **Allele specific expression analysis**

563 To assign parental alleles for reads in F1 hybrids, we first had to identify fixed and
564 different SNPs between the parent groups for each comparison. Starting from the high quality
565 variants described above, we generated separate MANA, EDMA, and SARA VCF files using
566 bcftools and filtered for SNPs present in at least 85% of individuals using VCFtools [55]. We
567 then created two VCF files for each parent group: one that contained SNPs fixed for the non-
568 reference allele, and one that contained SNPs fixed for the reference allele. We then merged the
569 resulting VCF files to generate a file for each comparison that only contained fixed and different

570 SNPs between parent groups using vcf-merge. For example, for the EDMA vs MANA
571 comparison, fixed non-reference allele EDMA SNPs were merged with fixed reference allele
572 MANA SNPs, and vice versa for reference EDMA SNPs and non-reference MANA SNPs. These
573 two resulting files were combined using bcftools concat to generate a VCF file of only fixed and
574 different SNPs between EDMA and MANA mice.

575 To differentiate between alleles in F1 hybrids, we called variants for F1s as described
576 above, and then filtered for only heterozygous calls using bcftools. To generate a set of ASE
577 informative SNPs, we retained only variants present in each F1 individual and present in the
578 fixed and different parent group VCF files. This resulted in a set of 30,000 ASE informative
579 SNPs for the SARA vs MANA comparison and 25,229 SNPs for the EDMA vs MANA
580 comparison. We phased the resulting VCF files for each comparison to indicate whether MANA
581 or SARA/EDMA contained the reference or alternate allele using custom scripts. We then
582 generated two separate pools of reads for each F1 hybrid (one of MANA reads and one of
583 SARA/EDMA reads) by aligning the F1 reads using STAR with WASP [56], specifying the ASE
584 informative SNPs as the variant file. WASP adds a SAMtag to each read indicating its phase
585 status in the provided VCF, here corresponding to the parental group to which the read maps. We
586 selected for the different SAMtags using grep, generating two separate BAM files for each
587 individual corresponding to reads of the different parental groups, which were subsequently
588 counted using HTSeq count as described above. Reads from F1 hybrids equally mapped to both
589 the MANA and SARA/EDMA allele, suggesting that there was no mapping bias toward either
590 mouse strain (S12 Fig).
591

592 **_Cis_- and _trans_-regulatory assignment**

593 We determined the relative amount of *cis*- and *trans*-regulated genes similarly to
594 McManus et al. [57]. We performed three hierarchical statistical tests for differential expression
595 using DESeq2 in R [31] only retaining genes with a mean count greater than 10 reads across all
596 samples. Ultimately, we were able to test for ASE in 4,811 genes in liver and 4,502 genes in
597 BAT for the SARA vs MANA comparison and 4,320 genes in liver and 3,864 genes in BAT for
598 the EDMA vs MANA comparison.

599 Three separate tests were used to analyze 1) differential expression between parental samples
600 via Wald tests (P), 2) differential expression between the separate alleles of the F1 hybrids via
601 Wald tests (H), and 3) the presence of *trans*- effects (T), which was determined using a
602 likelihood ratio test comparing the ratios of mouse strain expression difference between the
603 parents and between the alleles of an F1 hybrid. A significant difference between ratios indicates
604 that the expression difference between alleles in an F1 does not recapitulate the expression
605 difference between the parents, pointing to the presence of *trans*-regulation. Genes were
606 categorized into different regulatory modes based on their significance at an FDR of 5% in the P,
607 H, and T statistical tests as described below.

608 - *cis*- only: significant in P and H, not significant in T
609 - *trans*- only: significant in P, not significant in H, significant in T
610 - *cis* + *trans*: significant in P, H and T. Expression divergence direction in P and H is the
611 same.
612 - *cis x trans*: significant in P, H and T. Expression divergence direction in P and H is not the
613 same.
614 - compensatory: significant in H, not significant in P, significant in T. No expression
615 divergence due to compensation

616 • conserved: not significant in H, P, or T. No expression divergence

617 • ambiguous: Genes not encapsulated by above parameters.

618 Genes falling into each regulatory category (excluding ambiguous) for both comparisons
619 and both tissues are listed in S3-S6 Files.

620

621 **Tissue specificity**

622 To identify liver-specific and BAT-specific genes, we integrated our RNA-seq data with
623 a published *Mus musculus* RNA-seq dataset from Cardoso-Moreira et al. [34] that analyzed
624 expression from four mice (2 males and 2 females) in seven tissues: brain (forebrain/cerebrum),
625 cerebellum (hindbrain/cerebellum), heart, kidney, ovary, testis, and liver. The Cardoso-Moreira
626 et al. [34] dataset started as EdgeR generated reads per kilobase mapped (RPKM) values. To
627 make our dataset comparable, we re-analyzed our HTSeq generated read counts (described in the
628 "*Gene expression quantification and analysis"* section) as described in Cardoso-Moreira et al.
629 [34]. Briefly, we used the TMM method in EdgeR [58] to normalize read counts, and generated
630 RPKM values using the EdgeR rpkm function. We analyzed congruence between our dataset and
631 the published dataset by comparing the separate liver RPKM values for each gene. Liver gene
632 expression was highly correlated between the datasets (Pearson's $R=0.75$, $p < 2.2e^{-16}$),
633 suggesting that our approach is reasonable.

634 We used tau to determine tissue specificity [35]. Tau captures the deviation in expression
635 level for a gene in a particular tissue compared to its average FPKM across tissues. Tau ranges
636 from 0-1, with 1 representing a gene only expressed in one tissue, and 0 representing a gene
637 evenly expressed across all tissues [35]. Genes were defined as BAT- or liver-specific if tau >
638 0.85 [59] and if the highest FPKM was in BAT or liver, respectively. Genes were defined as

639 broadly expressed if tau < 0.35. Because we had liver expression values both from the published
640 dataset and our dataset, we calculated tau twice: once including our liver data, and once with the
641 Cardoso-Moreira et al. [34] liver data. Only genes defined as liver-specific in both calculations
642 were used in downstream analyses. Liver-specific, BAT-specific, and broadly expressed genes
643 are listed in S7-S12 Files.

644 To investigate the relationship between tissue specificity and mode of regulation, we
645 determined the regulatory class for each gene using the assignments generated in the "*cis- and*
646 *trans-regulatory assignment*" section above. Genes of "ambiguous" regulatory class were not
647 included. For the SARA vs MANA and EDMA vs MANA comparisons, respectively, we were
648 able to use 169 and 174 liver-specific genes, 20 and 12 BAT-specific genes, 277 and 237 broadly
649 expressed genes in the liver dataset, and 279 and 242 broadly expressed genes in the BAT
650 dataset that had both regulatory type data and tissue specificity data.

651

652 **Permutation tests of enrichment**

653 To test for enrichment of parallelism in differentially expressed genes (DEGs), we
654 performed permutation tests using the sample function in R without replacement with 10,000
655 iterations. Specifically, to test for parallelism in DEGs in liver, we randomly sampled 4,121genes
656 from the total of those expressed in the SARA vs MANA comparison (representing the number
657 of DEGs between SARA vs MANA) and 4,936 genes from the total of those expressed in the
658 EDMA vs MANA comparison. We then calculated the overlap between the randomly sampled
659 gene sets and tabulated a p-value using the proportion of the simulated distribution that was
660 outside the observed value. We calculated the degree of enrichment by dividing the observed
661 overlap by the mean of the simulated overlap. Parallelism in *cis*- and *trans*-regulated genes was

662 done in a similar manner by randomly sampling gene sets of the appropriate size for each
663 comparison and each tissue from the total of those significantly differentially expressed (FDR <
664 0.05). We also tested for parallelism in *cis*-regulated genes that showed signatures of
665 independent selection in the nested PBSn1 test for each tissue separately by randomly sampling
666 gene sets of the same size as the number of *cis*-regulated genes under selection in EDMA and
667 SARA from the total of those significantly differentially expressed (FDR < 0.05). Lastly, we
668 tested for parallelism in differentially expressed tissue-specific genes analogously to that
669 described above by randomly sampling from the set of genes significantly differentially
670 expressed (FDR < 0.05).

671 We also tested for enrichment of expression divergence in tissue-specific and broadly
672 expressed genes by comparing the observed vs. expected amount of DEGs in each gene set using
673 a similar permutation approach as described above. This was carried out separately for the
674 SARA vs MANA and EDMA vs MANA comparisons. More specifically, for liver-specific
675 genes, we randomly sampled 230 genes (representing the number of liver-specific DEGs) from
676 the total genes tested for tissue specificity, and then tabulated how many were significantly
677 differentially expressed at an FDR < 5%. We repeated this analysis for BAT-specific and broadly
678 expressed genes and calculated p-values and degree of enrichment as described in the paragraph
679 above.

680

681 **PBS test for selection**

682 To identify loci under selection in cold-adapted populations of house mice, we used a
683 normalized version of the population branch statistic (PBSn1), which identifies significant allele
684 frequency shifts in a focal population in relation to two outgroup populations [36,37]. We

685 conducted two sets of PBSn1 tests for both cold-adapted populations, for a total of four tests. For
686 the first test we used previously published population level exome data from Edmonton, Alberta,
687 Canada (EDM) [25], New Hampshire/Vermont (NH/VT), which is geographically close to
688 Saratoga Springs, NY [22], and genomic data from European *M. m. domesticus* mice,
689 specifically from France and Iran downloaded from
690 https://wwwuser.gwdg.de/~evolbio/evolgen/wildmouse/ [38]. France and Iran were chosen as
691 comparison populations as they are ancestral to house mouse populations in the Americas
692 [20,25,39]. SNPs were called on the North American exomes together using GATK [54]. We ran
693 HaplotypeCaller on each individual separately, and then combined gVCF files using
694 CombineGVCFs. Genotypes were called on all individuals together using GenotypeGVCFs. We
695 selected just SNPs using SelectVariants and filtered for high quality variants using the command
696 VariantFiltration (parameters = QD >2, FS < 60). We then selected for only the autosomes and
697 combined the resulting VCF with the published VCF containing France and Iran mice. We
698 filtered variants for those present in more that 80% of individuals and excluded those with a
699 minor allele frequency < 5%, resulting in a set of 558,934 biallelic SNPs.
700 We used VCFtools [55] to calculate pairwise Weir and Cockerham Fst for each
701 population comparison (see details below), and calculated PBSn1 in non-overlapping 5-SNP
702 blocks as in Crawford et al. [37]. This SNP block approach results in a less diffuse signal,
703 allowing us to identify regions of clear differentiation between the populations of interest. We
704 then used BEDTools closest [40] to identify the closest gene in the mm10 Ensembl mouse
705 genome annotation to each SNP block.
706 We calculated the parameters of the PBSn1 test shown below separately for EDM and
707 NH/VT as the focal population with France and Iran as the outgroup populations.

708

$$T = -\log(1 - F_{ST}) \qquad PBS = \frac{T^{pop1pop2} + T^{pop1pop3} - T^{pop2pop3}}{2}$$

709

710

$$PBS_{n1} = \frac{PBS_1}{1 + PBS_1 + PBS_2 + PBS_3}$$

711

712 In the PBS calculation, pop1 refers to the focal population, and in the PBSn1 calculation,
713 PBS1 refers to the PBS value when EDM or NH/VT is the focal population, while PBS2 and
714 PBS3 refer to the PBS value when France and Iran are used as the focal populations,
715 respectively.

716 To address the confounding factor of shared ancestry of EDM and NH/VT, and to
717 identify independent, parallel allele frequency changes in these two populations, we performed a
718 "nested" PBSn1 test with two additional previously published North American population level
719 exome samples, one from Arizona and one from Florida [22,25]. This second set of tests
720 consisted of one with EDM as the focal population and Arizona and Iran as outgroups, and one
721 with NH/VT as the focal population with Florida and Iran as outgroups. Because mice from
722 EDM and NH/VT are more closely related to mice from Arizona and Florida, respectively, than
723 they are to one another [25], PBSn1 hits identified in this second test that are shared between the
724 EDM and NH/VT comparisons are most likely to be independent cases of parallel adaptation as
725 opposed to shared ancestry. SNP calling and PBSn1 calculation was done similarly to that
726 described for the first test. After all filtering steps, we retained a set of 667,423 biallelic SNPs.
727 Ensembl IDs for genes overlapping outlier SNPs identified using BEDTools and for *cis*-
728 regulated genes can be found in S13 File.

729 Tests for enrichment for parallel evolved PBSn1 hits (FDR <0.05) between EDM and
730 NH/VT and overlap between PBSn1 hits and *cis*- and *trans*-regulated genes were performed at
731 the gene level analogously to that described in the "*Tests of enrichment*" section.
732

733 **Phylogenetic tree construction**

734 To describe the phylogenetic relationship between focal mouse groups, we used whole
735 genome data from Iran and France from Harr et al. [38] as described above, the whole exomes of
736 EDM as NH/VT as described above, and 10 samples from Manaus, Brazil (Gutierrez-Guerrero et
737 al. *In prep*) prepared analogously to the other whole exome data. We constructed a neighbor
738 joining tree with these five populations, specifying Iran as an outgroup, using vcfR [60], poppr
739 [61], and adegenet [62] in R, and visualized with iTOL [63].
740
741

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748

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Author contributions

M.W.N., M.A.B., and S.M.D. designed the research. S.M.D. and M.A.B. collected data and generated RNA-seq libraries. S.M.D. performed statistical and computational analyses with help from M.A.B. S.M.D. and M.W.N. wrote the manuscript with input from all authors.

Data availability statement

All sequencing reads generated in this study are available on the NCBI Sequence Read Archive under accession BioProject ID PRJNAXXX. All other data are within the paper and Supporting Information files.

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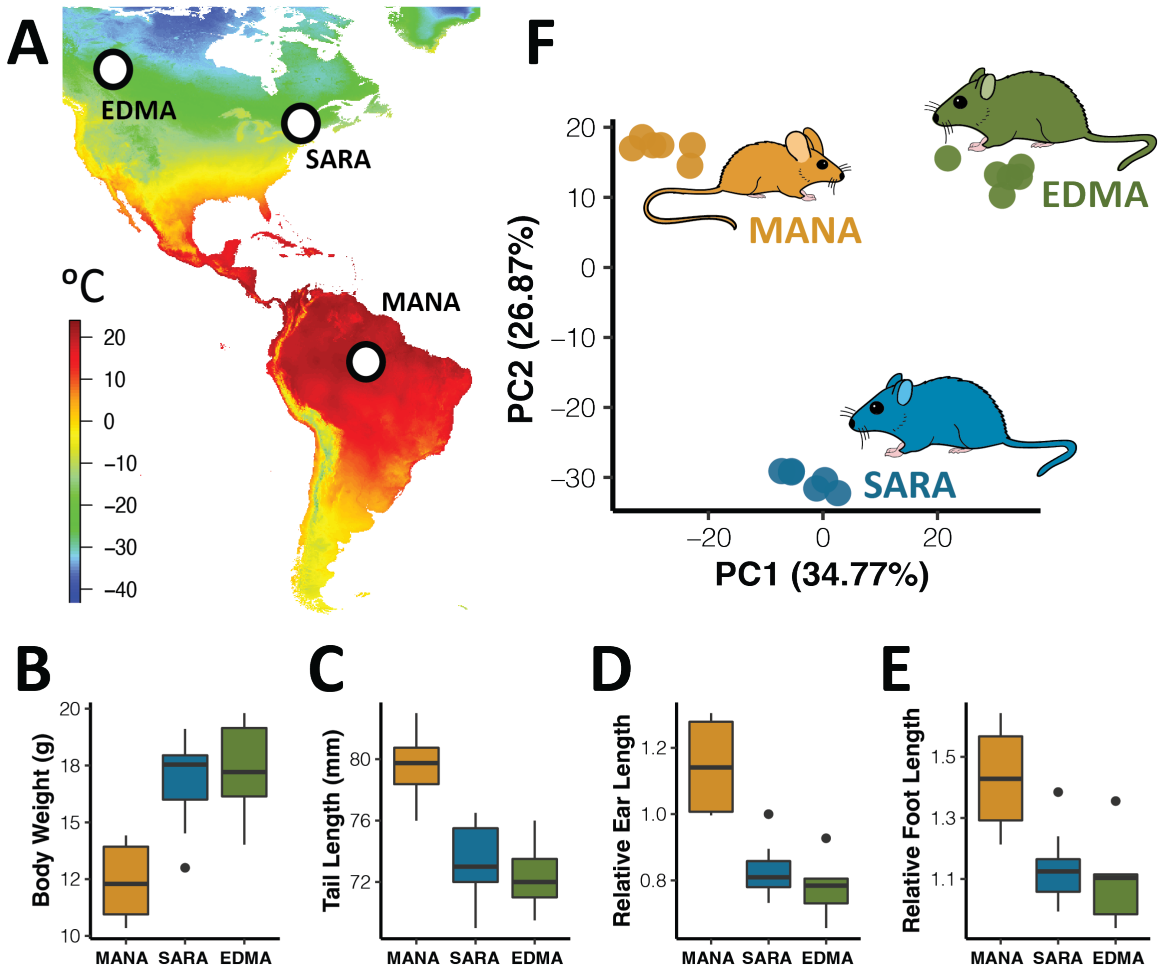


Fig 1: Morphological and gene expression variation in focal mouse strains. A). Variation in minimum annual temperature of original sampling localities for focal mouse strains. Data from WorldClim. B-E). Morphological variation in male mice collected for RNA-seq reflects environmental adaptation to differing climates. F). Principal component analyses for liver RNA-seq data separates samples based on original sampling locality. BAT data shown in S1 Fig.

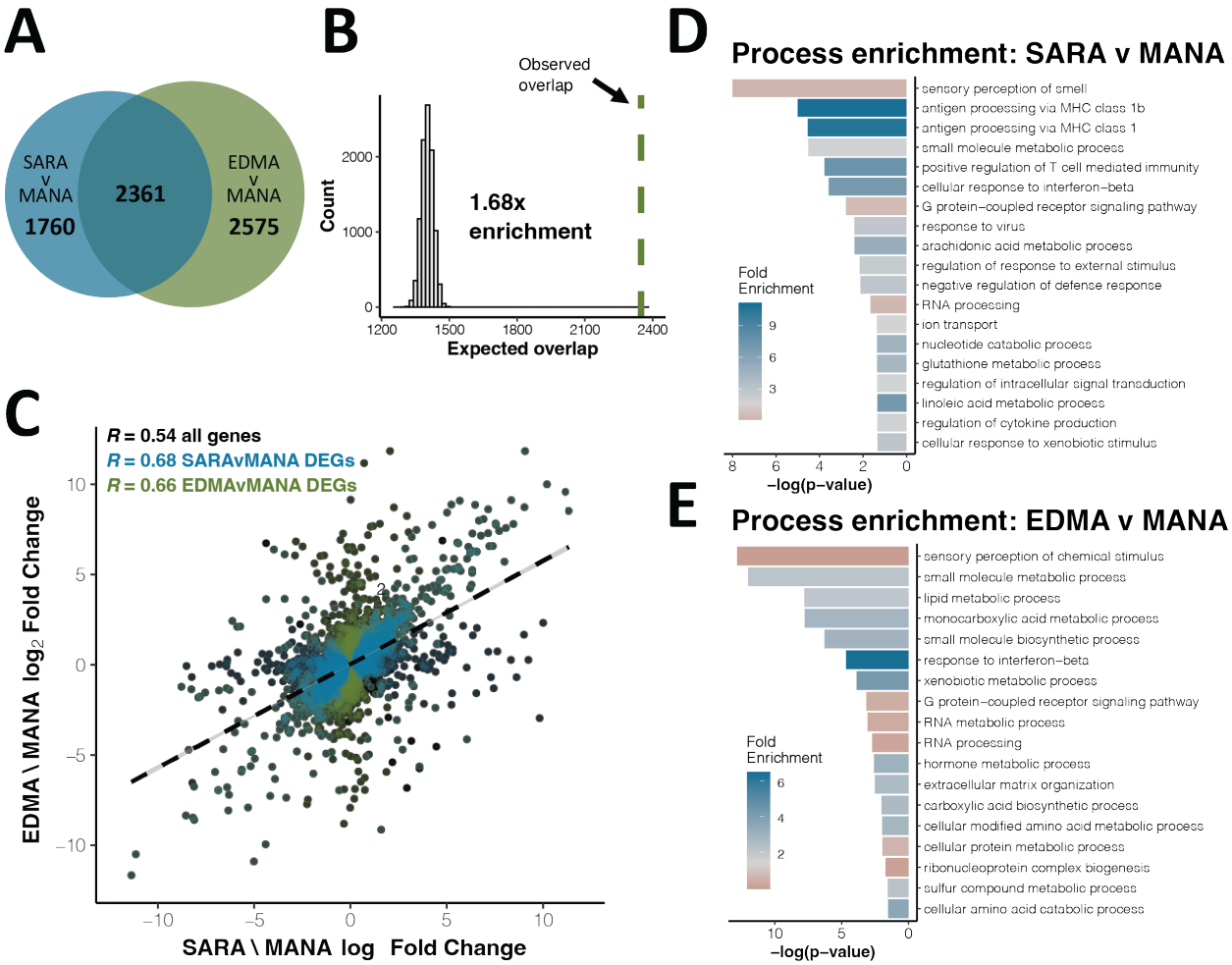


Fig 2: Parallel gene expression evolution in liver.
A). Overlap of significantly differentially expressed genes (DEGs) between SARAvMANA and EDMAvMANA comparisons (FDR < 5%). B). Expected vs. observed overlap between SARAvMANA and EDMAvMANA DEGs. C). Correlation between SARAvMANA $\log_2$ fold change and EDMAvMANA $\log_2$ fold change for all genes (black), SARAvMANA DEGs (blue) and EDMAvMANA DEGs (green). Correlation coefficients are Spearman's R. D-E). Gene ontology enrichment for SARAvMANA DEGs (D) and EDMAvMANA DEGs (E).

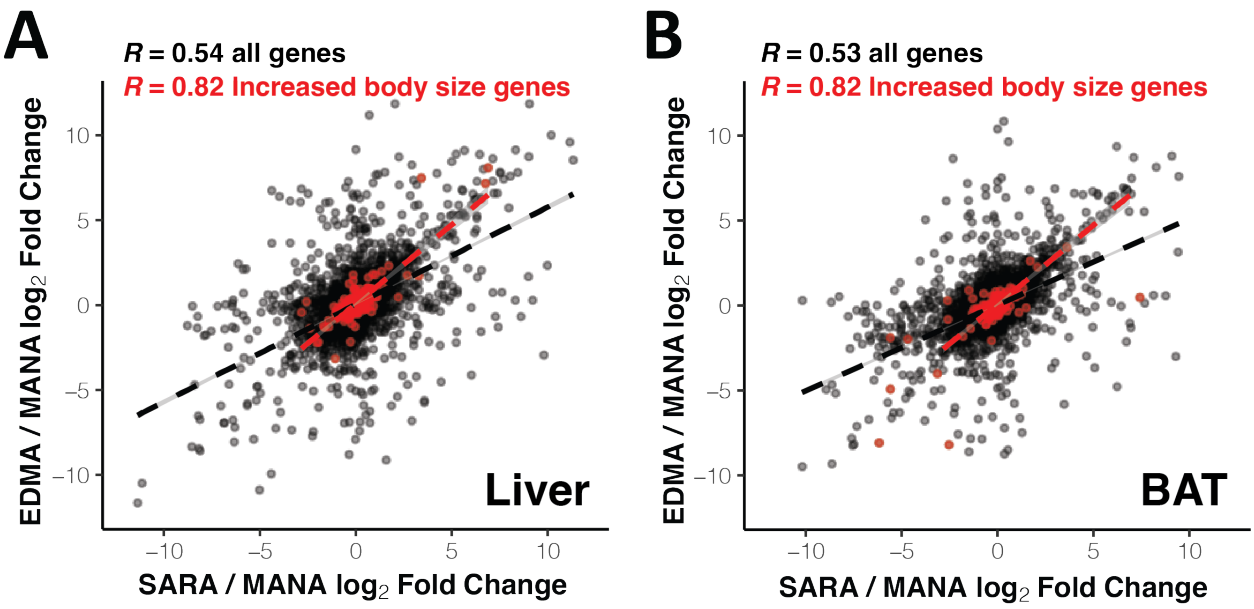


Fig 3: Parallel expression evolution in increased body size genes.
Correlation between SARAvMANA $\log_2$ fold change and EDMAvMANA $\log_2$ fold change in liver (A) and BAT (B) for all genes (black) and increased body sized genes (red).

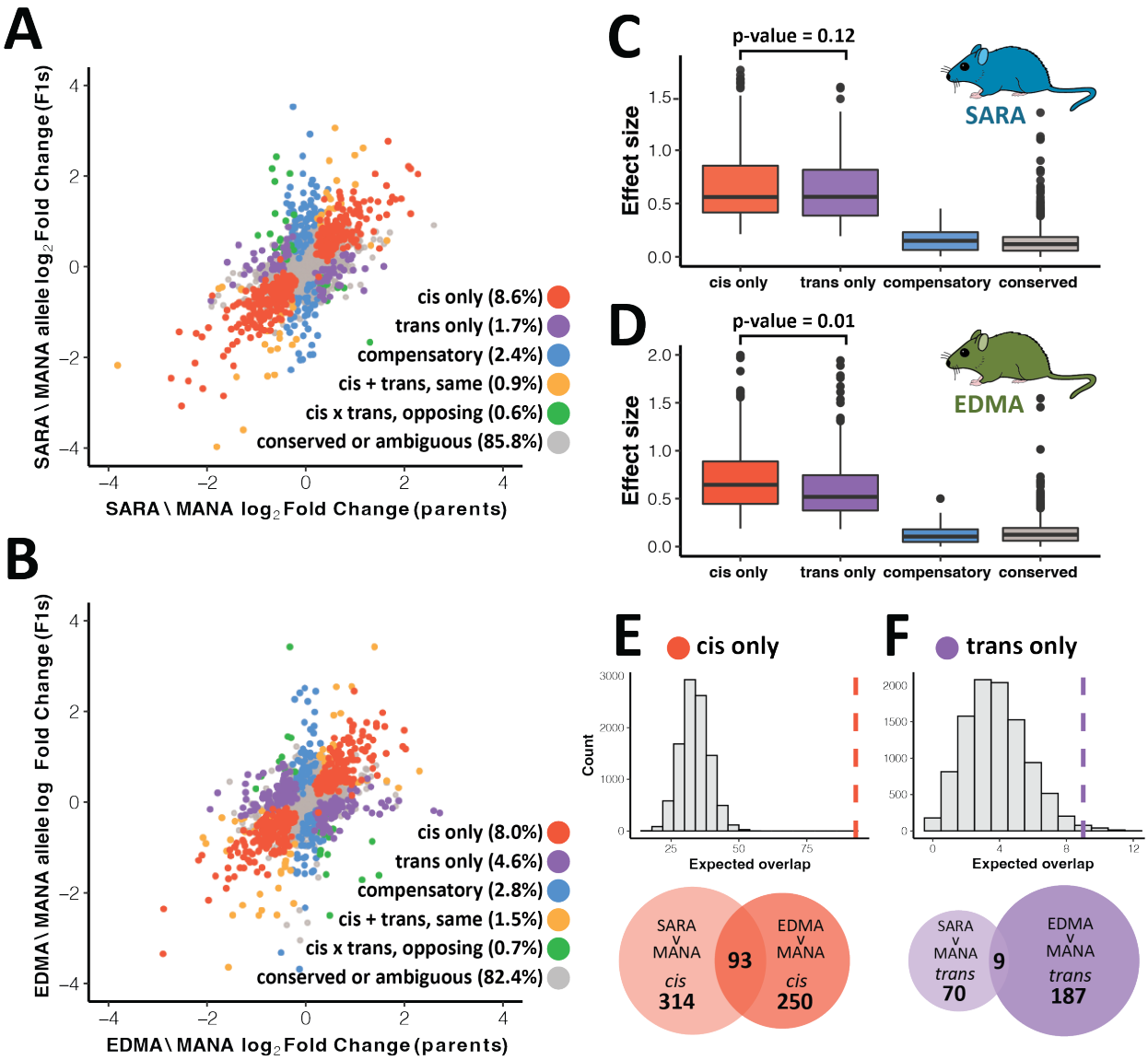


Fig 4: Parallel gene regulatory evolution in liver.
A-B). Correlation between parental differential expression and differential expression between alleles in F1 hybrids for SARAvMANA (A) and EDMAvMANA (B) comparisons. Genes are colored based on regulatory mode (see methods of details). C-D). Effect size as measured as $\log_2$ fold change for different regulatory classes in SARAvMANA (C) and EDMAvMANA (D) comparisons. P-values calculated using paired t-tests. E-F). Expected vs. observed overlap between SARAvMANA and EDMAvMANA *cis*-regulated (E) and *trans*-regulated (F) genes. Dashed line indicates observed overlap. Proportion of overlap shown in venn diagrams is significantly higher for *cis*-regulated genes as compared to *trans*-regulated genes (Chi-square test of independence, $p < 0.0001$).

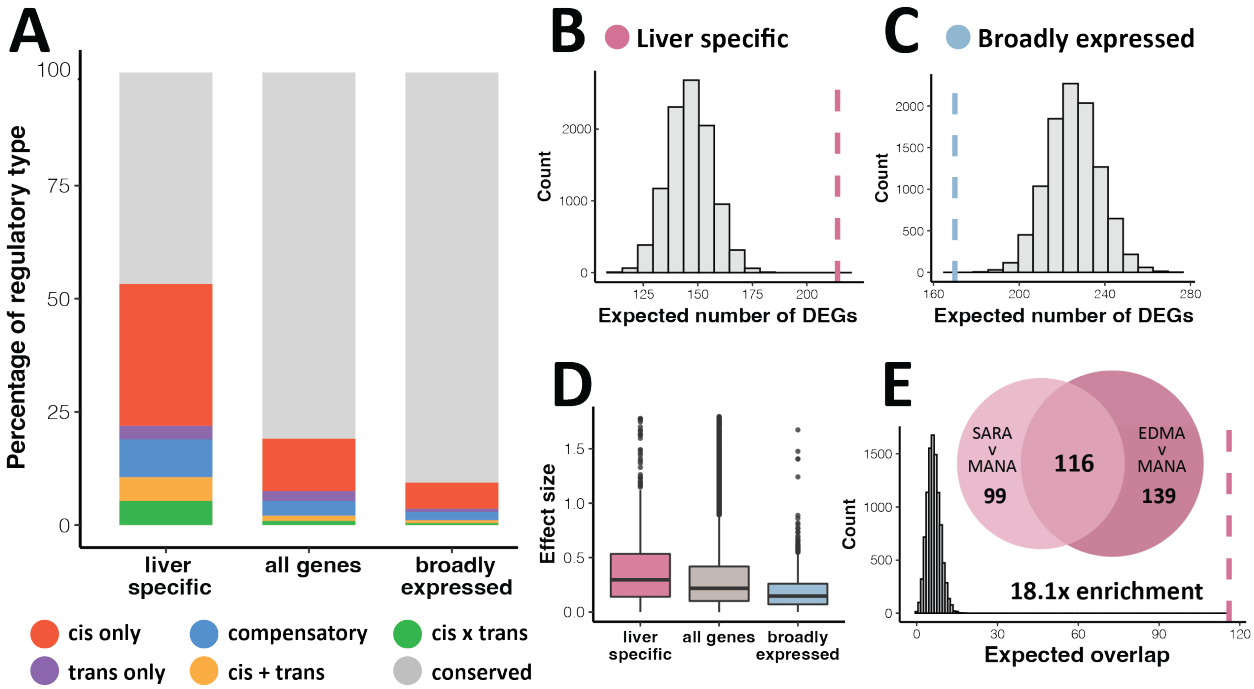


Fig 5: Patterns of tissue-specific gene expression evolution between SARA and MANA mice in liver.

A). Percentage of each regulatory type in liver-specific, broadly expressed, and all genes. Genes of ambiguous regulatory type are not included. EDMAvMANA and BAT results can be found in S6-S8 Figs. B-C). Expected vs. observed number of significantly differentially expressed genes (DEGs) in liver-specific (B) and broadly expressed (C) genes. Dashed line indicates observed value. D). Effect size as measured as $\log_2$ fold change for liver-specific, broadly expressed, and all genes. E). Overlap of liver-specific DEGs between SARAvMANA and EDMAvMANA comparisons (FDR < 5%) and expected vs. observed overlap between SARAvMANA and EDMAvMANA liver-specific DEGs. Dashed line indicates observed value.

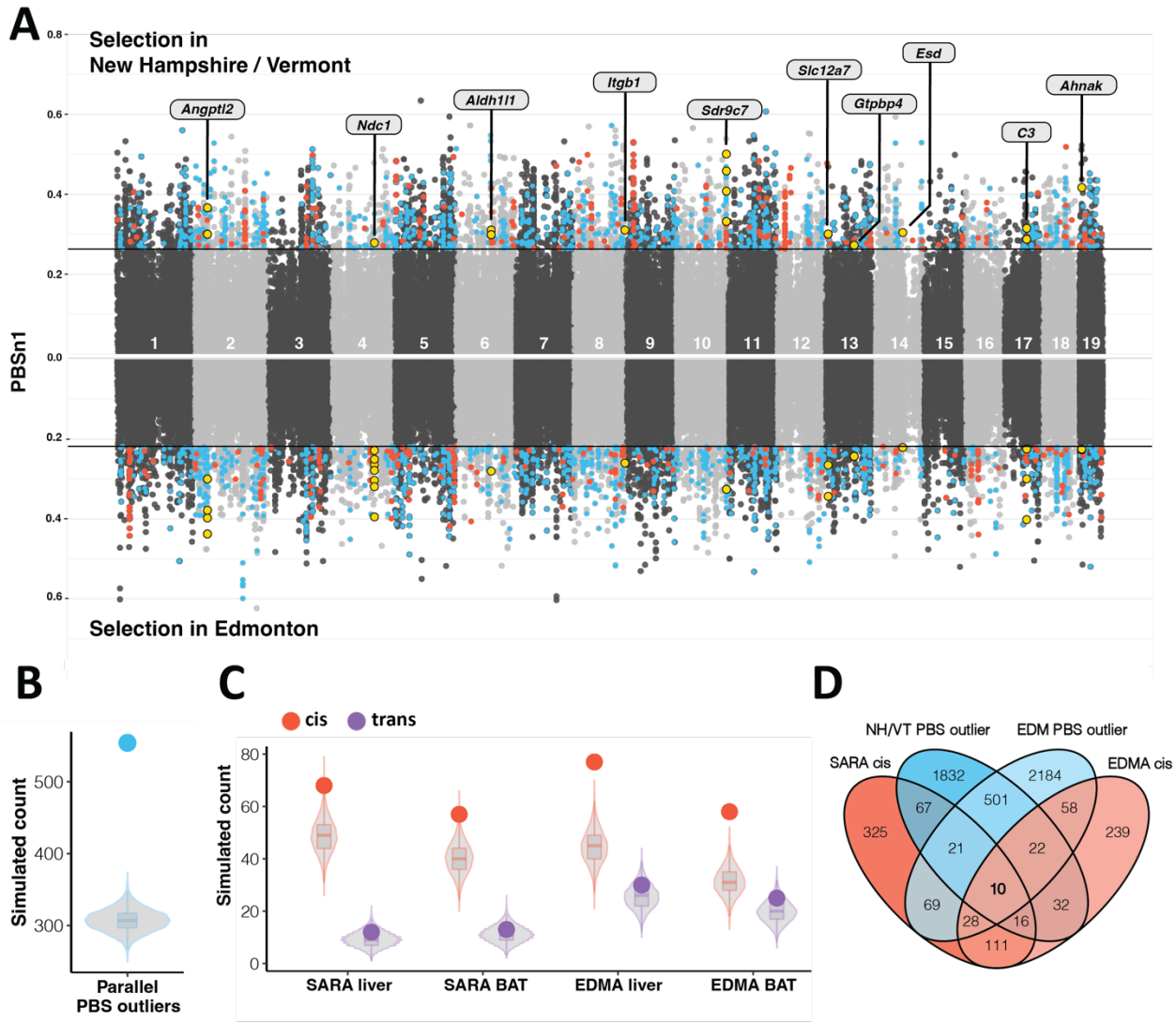
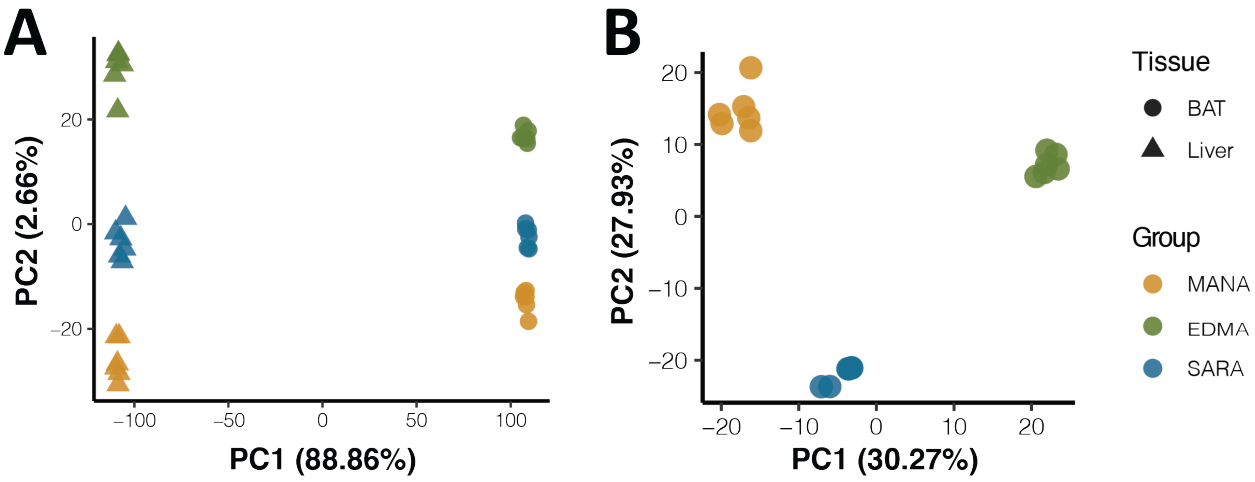
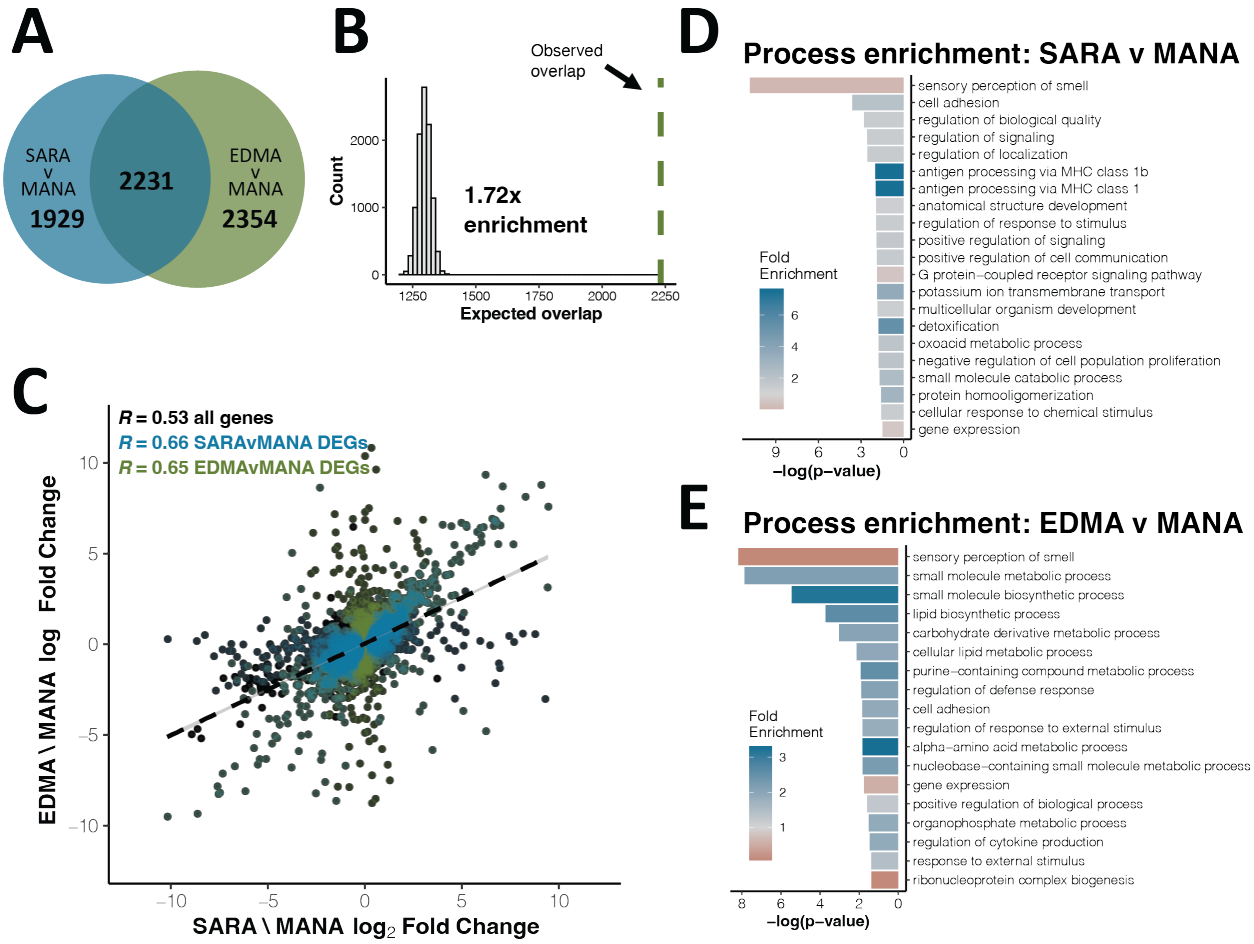


Fig 6: Natural selection on *cis*-regulated genes and parallel expression divergence. A). PBSn1 values for selection scans with New Hampshire/Vermont (NH/VT) (top) and Edmonton (EDM) (bottom) as the focal population. Line indicates the top 5% of 5-SNP blocks. Red points are PBSn1 outliers that overlap with a *cis*-regulated gene. *cis*-regulated genes identified in either liver or BAT are shown. Blue points are PBSn1 outliers that are present in both the NH/VT and EDM tests. Yellow points indicate SNPs associated with the 10 genes that are *cis*-regulated and PBSn1 outliers in both comparisons. B). Expected vs. observed overlap between NH/VT and EDM PBSn1 outliers. Blue dot indicates observed value. C). Expected vs. observed overlaps between PBSn1 outliers and *cis*-regulated genes (red) and *trans*-regulated genes (purple). Colored dot indicates observed value. D). Venn diagram of PBSn1 outliers and *cis*-regulated genes (in either tissue) for both comparisons.

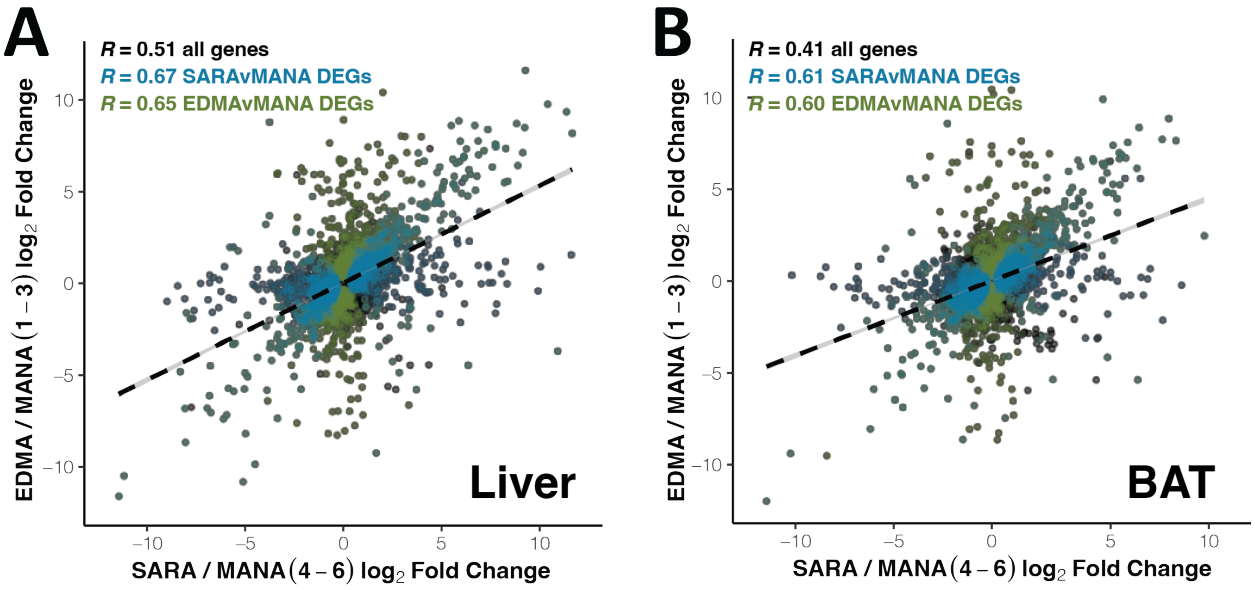
Supporting Information



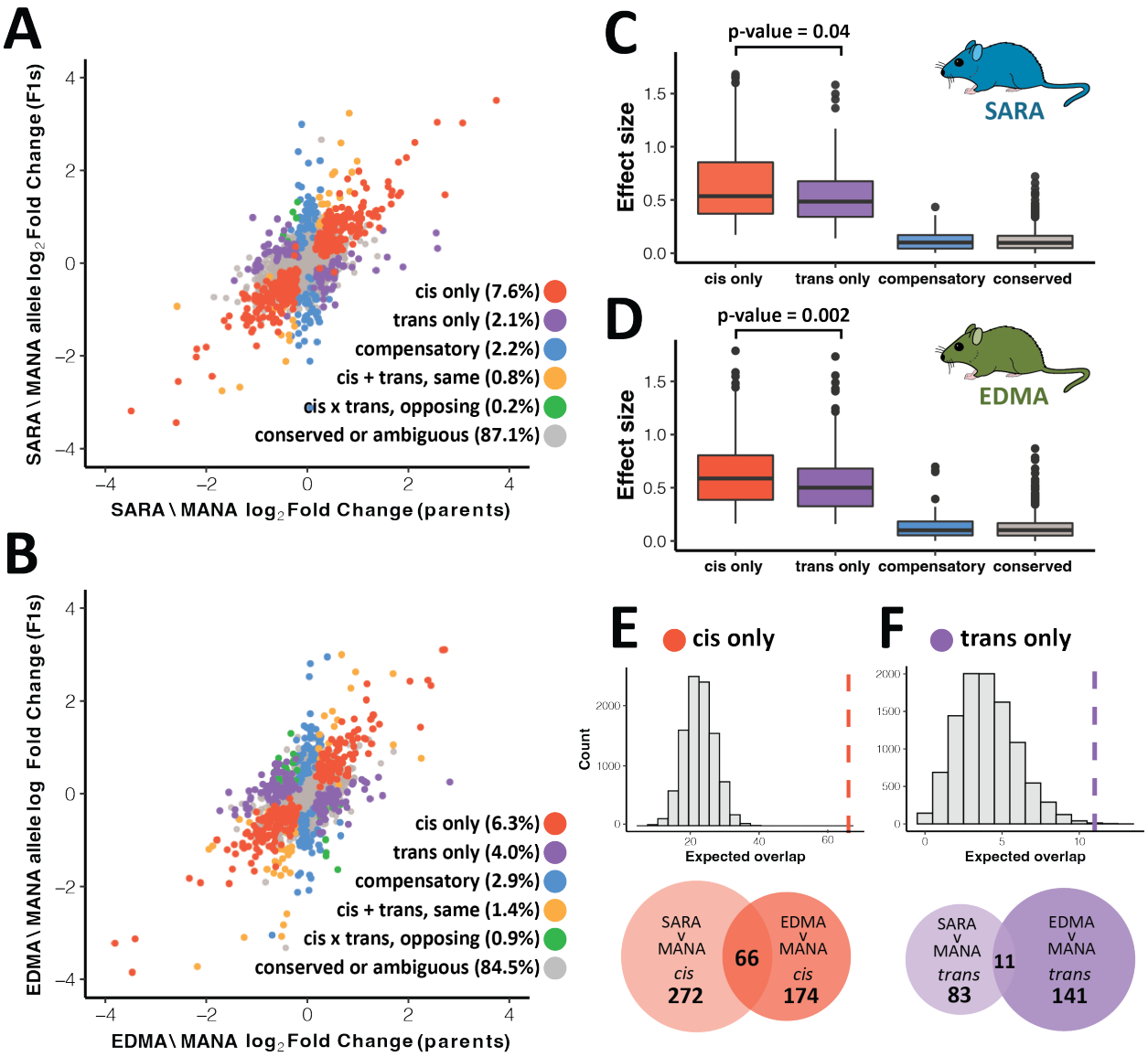
S1 Fig: Gene expression variation across tissues and samples.
A). Principal component analyses for all RNA-seq data separates samples first on tissue type, and second on original sampling locality. B). Principal component analyses for BAT RNA-seq data separates samples based on original sampling locality.



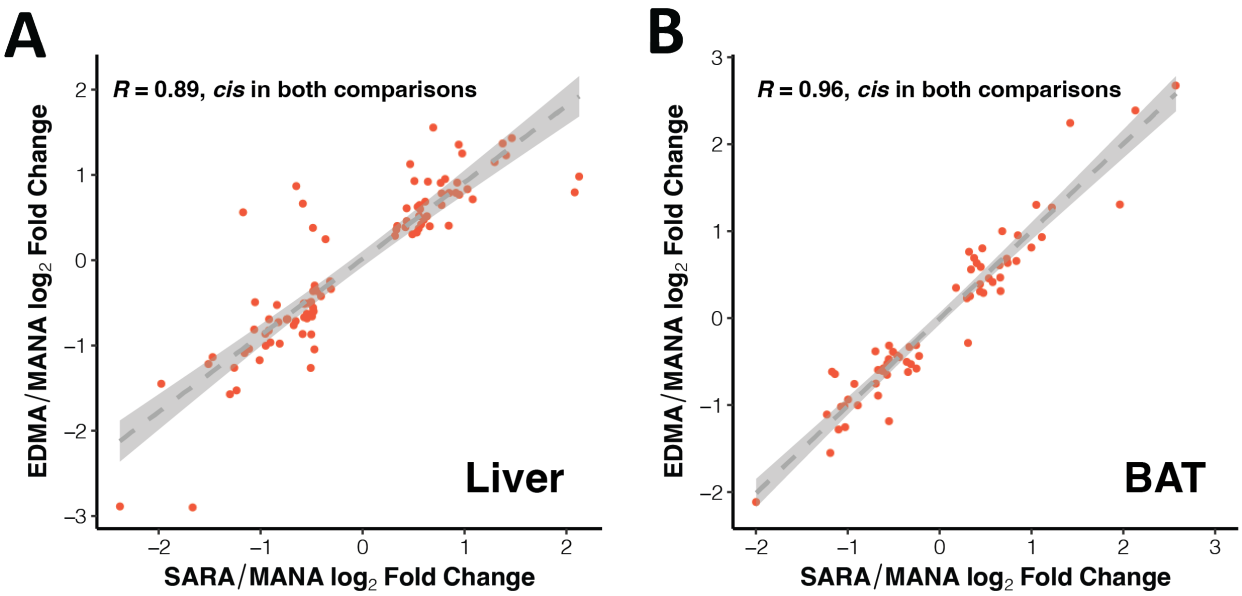
S2 Fig: Parallel gene expression evolution in BAT.
A). Overlap of significantly differentially expressed genes (DEGs) between SARAvMANA and EDMAvMANA comparisons (FDR <5%). B). Expected vs. observed overlap between SARAvMANA and EDMAvMANA DEGs. C). Correlation between SARAvMANA $\log_2$ fold change and EDMAvMANA $\log_2$ fold change for all genes (black), SARAvMANA DEGs (blue) and EDMAvMANA DEGs (green). Correlation coefficients are Spearman's R. D-E). Gene ontology enrichment for SARAvMANA DEGs (D) and EDMAvMANA DEGs (E).



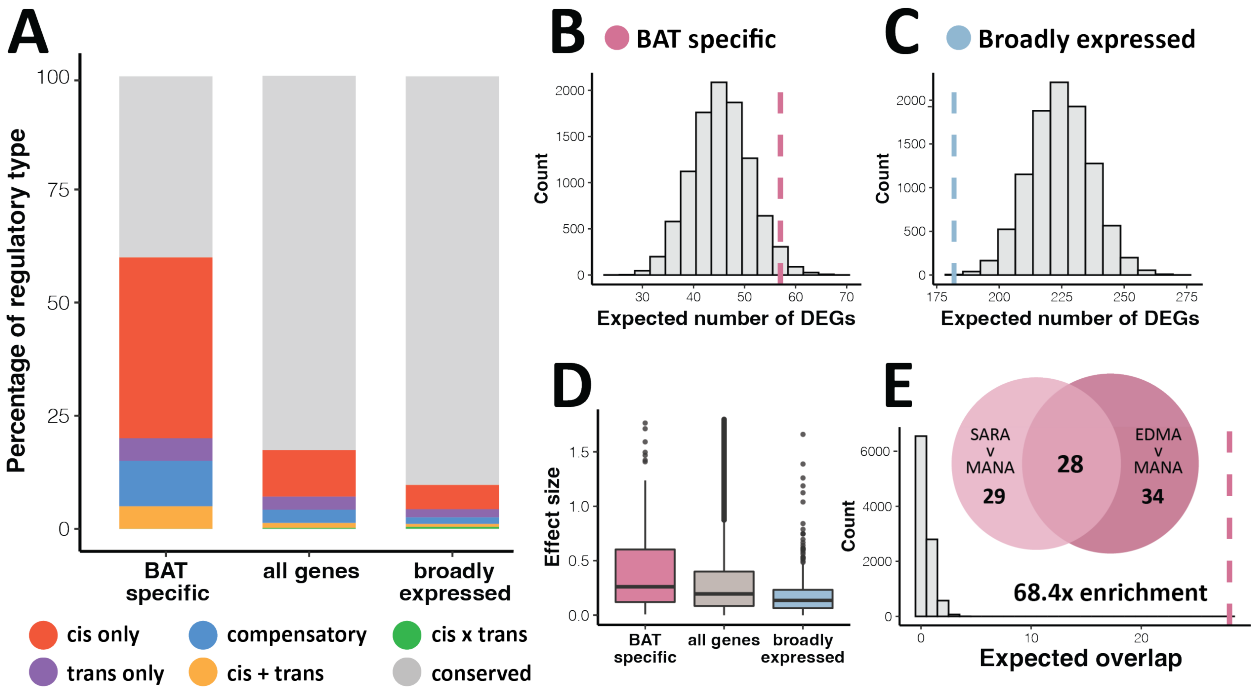
S3 Fig: Parallel expression evolution with subsets of MANA data.
A-B). Correlation between SARAvMANA (MANA samples 4-6) $\log_2$ fold change and EDMAvMANA (MANA samples 1-3) $\log_2$ fold change in liver (A) and BAT (B). All expressed genes are in black, SARAvMANA DEGs are in blue and EDMAvMANA DEGs are in green. Correlation coefficients are Spearman's R. All significant correlations found using the full MANA data for each comparison are recapitulated.



S4 Fig: Parallel gene regulatory evolution in BAT.
A-B). Correlation between parental differential expression and differential expression between alleles in F1 hybrids for SARAvMANA (A) and EDMAvMANA (B) comparisons. Genes are colored based on regulatory mode (see methods of details). C-D). Effect size as measured as $\log_2$ fold change for different regulatory classes in SARAvMANA (C) and EDMAvMANA (D) comparisons. P-values calculated using paired t-tests. E-F). Expected vs. observed overlap between SARAvMANA and EDMAvMANA *cis*-regulated (E) and *trans*-regulated (F) genes. Dashed line indicates observed overlap. Proportion of overlap shown in venn diagrams is significantly higher for *cis*-regulated genes as compared to *trans*-regulated genes (Chi-square test of independence, $p < 0.0001$).

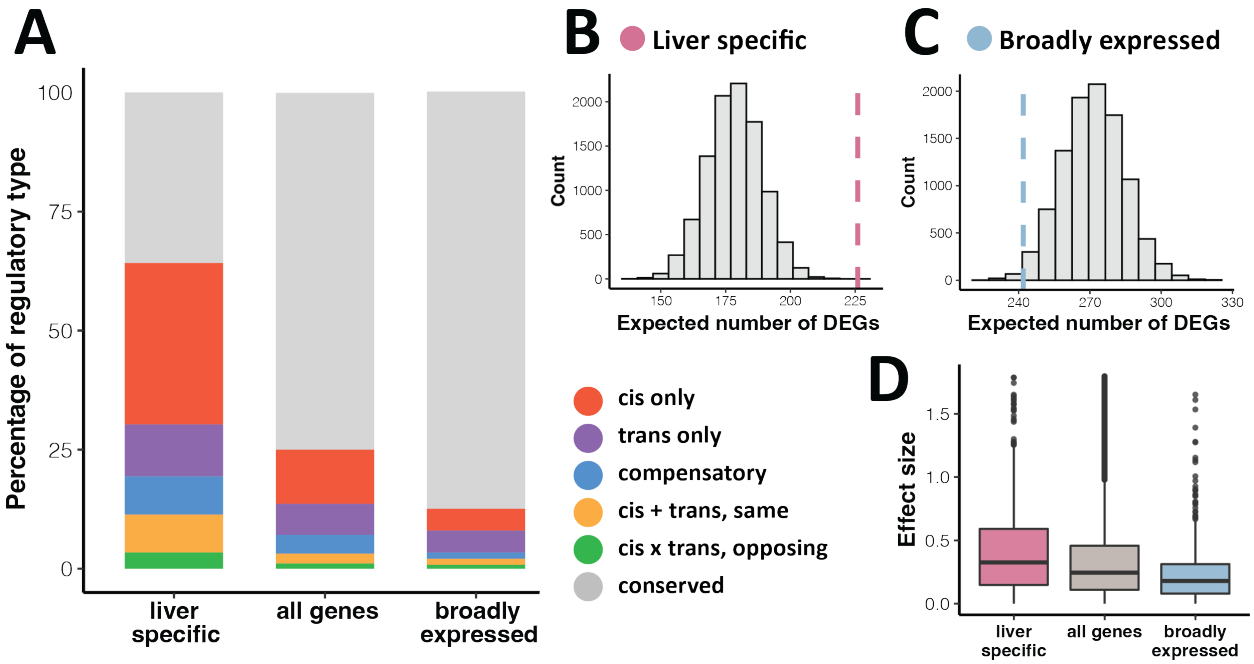


S5 Fig: Parallel expression evolution in *cis*-regulated genes.
Correlation between SARAvMANA $\log_2$ fold change and EDMAvMANA $\log_2$ fold change in liver (A) and BAT (B) for genes that are *cis*-regulated in both comparisons. Correlation coefficients are Spearman's R.

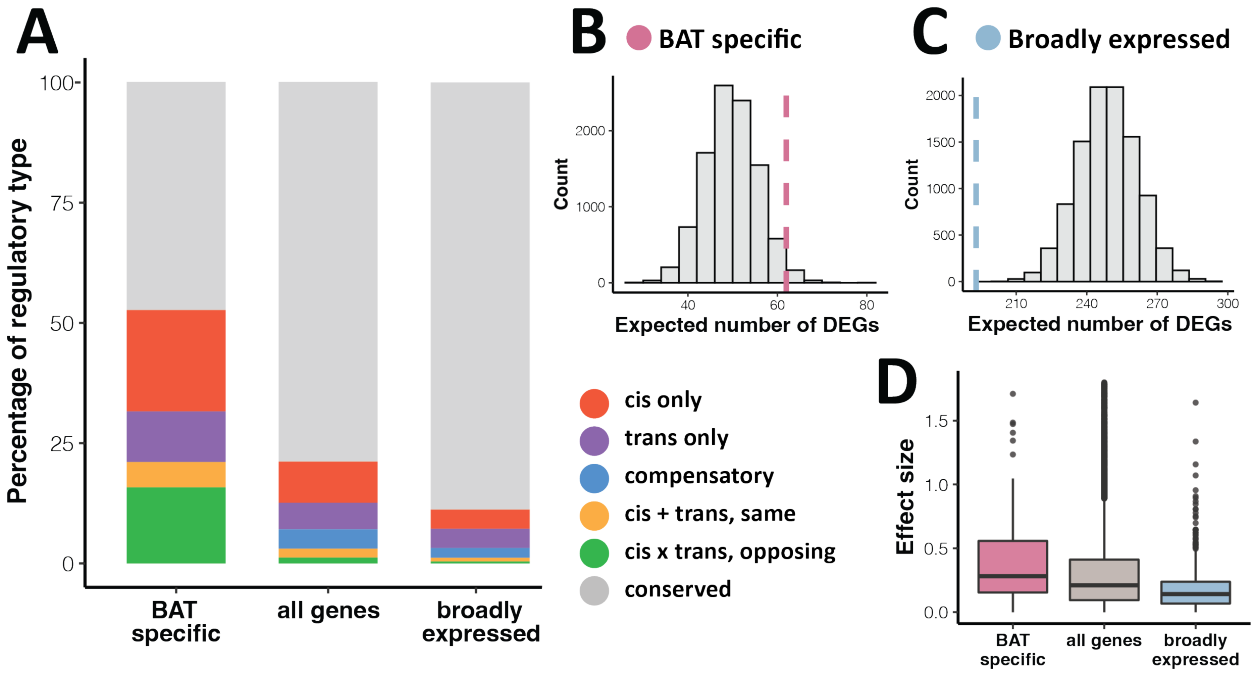


S6 Fig: Patterns of tissue-specific gene expression evolution between SARA and MANA mice in BAT.

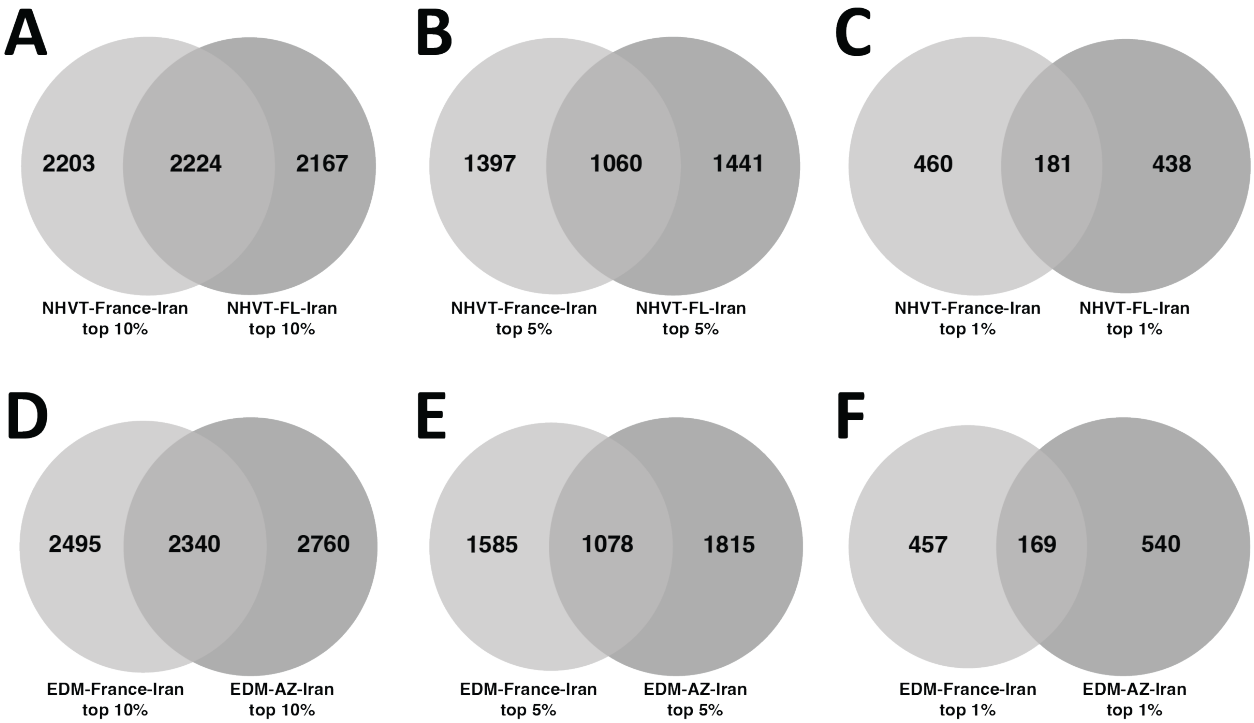
A). Percentage of each regulatory type in BAT-specific, broadly expressed, and all genes. Genes of ambiguous regulatory type are not included. B-C). Expected vs. observed number of significantly differentially expressed genes (DEGs) in BAT-specific (B) and broadly expressed (C) genes. Dashed line indicates observed value. D). Effect size as measured as $\log_2$ fold change for BAT-specific, broadly expressed, and all genes. E). Overlap of BAT-specific DEGs between SARAvMANA and EDMAvMANA comparisons (FDR < 5%) and expected vs. observed overlap between SARAvMANA and EDMAvMANA BAT-specific DEGs. Dashed line indicates observed value.



S7 Fig: Patterns of tissue-specific gene expression evolution between EDMA and MANA mice in liver.
A). Percentage of each regulatory type in liver-specific, broadly expressed, and all genes. Genes of ambiguous regulatory type are not included. B-C). Expected vs. observed number of significantly differentially expressed genes (DEGs) in liver-specific (B) and broadly expressed (C) genes. Dashed line indicates observed value. D). Effect size as measured as $\log_2$ fold change for liver-specific, broadly expressed, and all genes.



S8 Fig: Patterns of tissue-specific gene expression evolution between EDMA and MANA mice in BAT.
A). Percentage of each regulatory type in BAT-specific, broadly expressed, and all genes. Genes of ambiguous regulatory type are not included. B-C). Expected vs. observed number of significantly differentially expressed genes (DEGs) in BAT-specific (B) and broadly expressed (C) genes. Dashed line indicates observed value. D). Effect size as measured as $\log_2$ fold change for BAT-specific, broadly expressed, and all genes.



S9 Fig: Shared outlier genes in nested PBSn1 tests.
A-C). Overlap of the top 10% (A), 5% (B) and 1% (C) of outlier genes shared between the NH/VT-France-Iran and NH/VT-FL-Iran PBSn1 tests. D-F). Overlap of the top 10% (D), 5% (E) and 1% (F) of outlier genes shared between the EDM-France-Iran and EDM-AZ-Iran PBSn1 tests.

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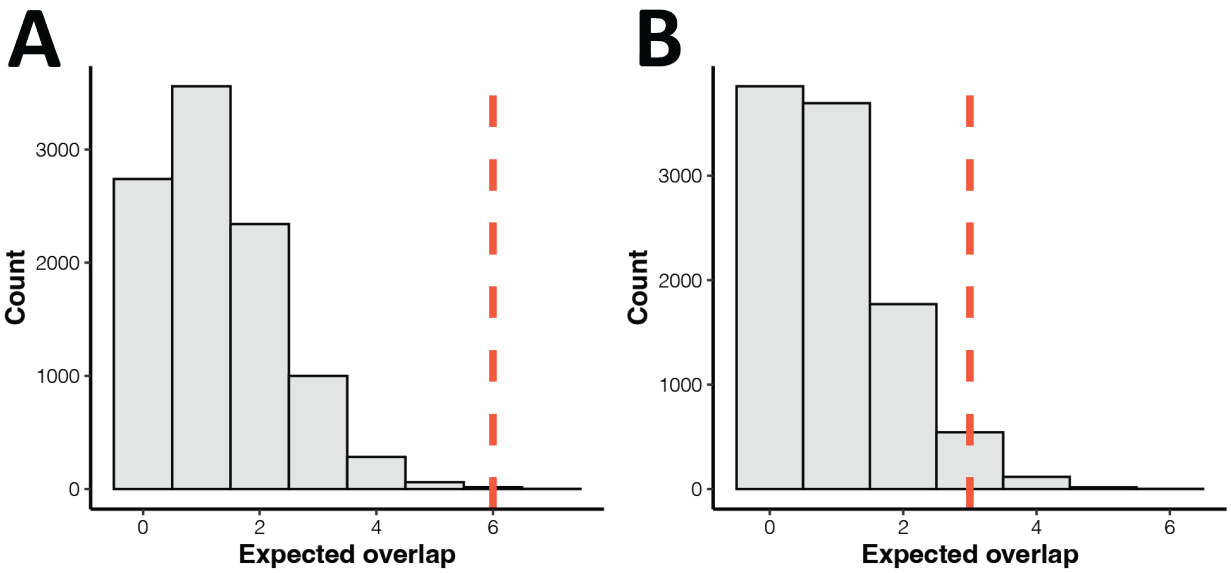

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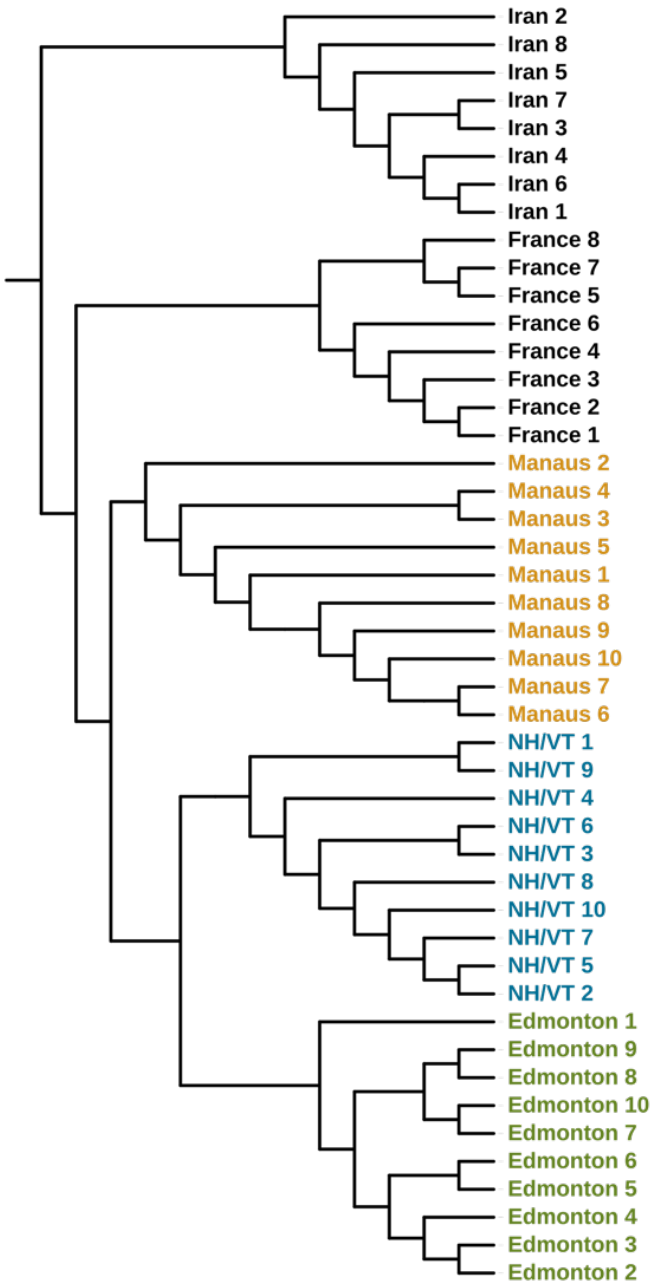
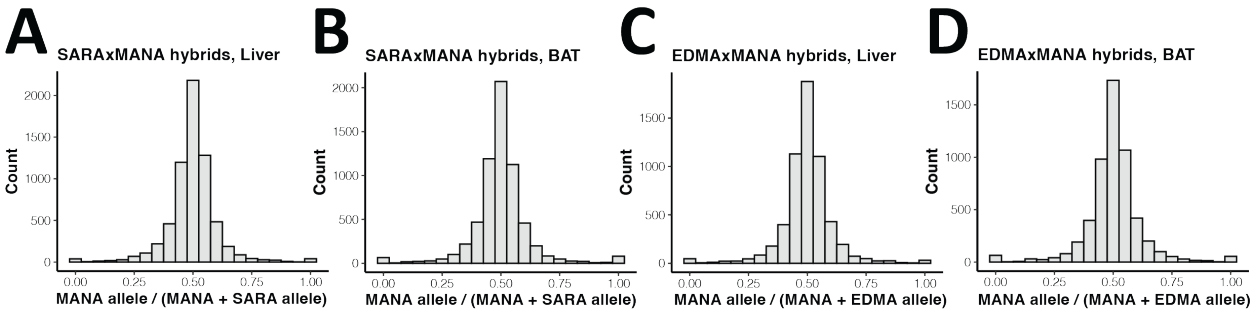
1062 **S10 Fig: Parallelism in independently evolved *cis*-regulated genes.**
1063 A-B). Expected vs. observed overlap between SARAvMANA and EDMAvMANA for
1064 independently evolved *cis*-regulated genes (*cis*-regulated genes that overlap a PBSn1 outlier hit
1065 in the nested test) in liver (A) and BAT (B). Dashed line indicates observed overlap.



S11 Fig: Relationship among focal populations.
Neighbor joining tree of focal house mouse populations from the ancestral range and North and South America. The tree is rooted using eight samples from Iran.



S12 Fig: Lack of mapping bias in F1 hybrids.
A-D). Ratio of F1 hybrid reads mapping to the MANA allele vs. all mapped reads is centered around 0.5 in each tissue for SARAxMANA (A-B) and EDMAxMANA (C-D) F1 hybrids, suggesting there is no mapping bias for reads preferentially mapping to the allele of one parent.

S1 File: Phenotypic data and metadata for mice used for RNA sequencing. Unique identifiers, sample names, other metadata, and phenotypic measurements of mice used for RNA sequencing.

S2 File: Increased body size genes. Ensembl IDs for increased body size genes identified using the Mammalian Phenotype Browser on the Mouse Genome Informatics database (MP ID: 0001264).

S3 File: SARAvMANA liver gene regulatory categories. Genes (Ensembl IDs) falling into different regulatory categories. Log2 fold changes and adjusted p-values for DESeq2 tests on parental differential expression, F1 allele differential expression, and trans expression. Parental base mean also listed.

S4 File: SARAvMANA BAT gene regulatory categories. Genes (Ensembl IDs) falling into different regulatory categories. Log2 fold changes and adjusted p-values for DESeq2 tests on parental differential expression, F1 allele differential expression, and trans expression. Parental base mean also listed.

S5 File: EDMAvMANA liver gene regulatory categories. Genes (Ensembl IDs) falling into different regulatory categories. Log2 fold changes and adjusted p-values for DESeq2 tests on parental differential expression, F1 allele differential expression, and trans expression. Parental base mean also listed.

1109 **S6 File: EDMAvMANA BAT gene regulatory categories.** Genes (Ensembl IDs) falling into
1110 different regulatory categories. Log2 fold changes and adjusted p-values for DESeq2 tests on
1111 parental differential expression, F1 allele differential expression, and trans expression. Parental
1112 base mean also listed.
1113
1114
1115 **S7 File. Liver-specific genes in the SARAvMANA comparison**. Published RPKM means
1116 (prefix "pub"), liver and BAT RPKM means from this study (prefix "local"), and calculated tau
1117 values using both published and locally generated liver mean expression values.
1118
1119 **S8 File. BAT-specific genes in the SARAvMANA comparison**. Published RPKM means
1120 (prefix "pub"), liver and BAT RPKM means from this study (prefix "local"), and calculated tau
1121 values using both published and locally generated liver mean expression values.
1122
1123
1124 **S9 File. Broadly expressed genes in the SARAvMANA comparison**. Published RPKM means
1125 (prefix "pub"), liver and BAT RPKM means from this study (prefix "local"), and calculated tau
1126 values using both published and locally generated liver mean expression values.
1127
1128
1129 **S10 File. Liver-specific genes in the EDMAvMANA comparison**. Published RPKM means
1130 (prefix "pub"), liver and BAT RPKM means from this study (prefix "local"), and calculated tau
1131 values using both published and locally generated liver mean expression values.
1132
1133
1134 **S11 File. BAT-specific genes in the EDMAvMANA comparison**. Published RPKM means
1135 (prefix "pub"), liver and BAT RPKM means from this study (prefix "local"), and calculated tau
1136 values using both published and locally generated liver mean expression values.
1137
1138
1139 **S12 File. Broadly expressed genes in the EDMAvMANA comparison**. Published RPKM
1140 means (prefix "pub"), liver and BAT RPKM means from this study (prefix "local"), and
1141 calculated tau values using both published and locally generated liver mean expression values.
1142
1143
1144 **S13 File: Summary of genes overlapping PBSn1 outlier hits and *cis*-regulated genes.**
1145 Ensembl IDs for NH/VT and EDM PBSn1 outliers (FDR <0.05), parallel outliers, outliers
1146 overlapping *cis*-regulated genes, and *cis*-regulated genes generally.