Giant Island Mice Exhibit Widespread Gene Expression Changes in Key Metabolic Organs

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Data deposition:

RNA-sequencing data from this study are available in GEO under accession number GSE135007. Whole genome sequencing data for Gough Island mice are available in SRA under accession number PRJNA587779. Fixed SNPs used to map RNA-sequencing reads from WSB/EiJ mice were obtained from Keane et al. (2011). Supplementary Tables 3, 7, and 11 are available at https://doi.org/10.5061/dryad.cjsxksn34.
Abstract

Island populations repeatedly evolve extreme body sizes, but the genomic basis of this pattern remains largely unknown. To understand how organisms on islands evolve gigantism, we compared genome-wide patterns of gene expression in Gough Island mice, the largest wild house mice in the world, and mainland mice from the WSB/EiJ wild-derived inbred strain. We used RNASeq to quantify differential gene expression in three key metabolic organs: gonadal adipose depot, hypothalamus and liver. Between 4,000 and 8,800 genes were significantly differentially expressed across the evaluated organs, representing between 20 and 50% of detected transcripts, with 20% or more of differentially expressed transcripts in each organ exhibiting expression fold changes of at least 2X. A minimum of 73 candidate genes for extreme size evolution, including *Irs1* and *Lrp1*, were identified by considering differential expression jointly with other data sets: (i) genomic positions of published quantitative trait loci for body weight and growth rate; (ii) whole genome sequencing of 16 wild-caught Gough Island mice that revealed fixed single nucleotide differences between the strains; (iii) publicly available tissue-specific regulatory elements. Additionally, patterns of differential expression across three time points in the liver revealed that *Arid5b* potentially regulates hundreds of genes. Functional enrichment analyses pointed to cell cycling, mitochondrial function, signaling pathways, inflammatory response, and nutrient metabolism as potential causes of weight accumulation in Gough Island mice. Collectively, our results indicate that extensive gene regulatory evolution in metabolic organs accompanied the rapid evolution of gigantism during the short time house mice have inhabited Gough Island.
Keywords

Island evolution, Island rule, body size, house mouse, gene regulatory evolution
Significance Statement

Reported instances of island-colonizing organisms evolving extreme body sizes are numerous, while insights into the genetic changes responsible for this widespread phenomenon are fewer. We found genes that are expressed at different levels in metabolic organs from giant Gough Island mice and smaller mainland mice, including genes with roles in cell cycling, fat cell maturation, the inflammatory response, and nutrient metabolism. The genes and cellular processes we identified help to elucidate the gene regulatory basis of gigantism evolution.
Introduction

The repeated evolution of similar phenotypes is a hallmark of adaptation. Island populations of vertebrates often evolve extreme body sizes, either gigantism or dwarfism (Foster, 1964), a pattern known as the “island rule” (Foster 1964; Van Valen 1973). The genomic basis of body size evolution on islands remains poorly characterized, even while the ecological causes and generality of the island rule have been subject to considerable study and debate (Lomolino 2005; Meiri et al. 2008, 2011; Raia and Meiri 2011).

One striking example of extreme body size evolution comes from house mice that colonized remote Gough Island, which is located in the South Atlantic Ocean, roughly 2,100 miles from both South America and South Africa. The mice on Gough Island are the largest wild house mice on record. Adult island mice are, on average, about 45% heavier than mainland counterparts (island/mainland males: 26.5 grams (g)/18 g; island/mainland females: 22 g/15.5 g) and their skeleton is 12% longer (Gray et al. 2015; Jones et al. 2003; Parmenter et al. 2016; Rowe-Rowe and Crafford 1992). Moreover, house mice likely colonized the island within the last 200 years (Gray et al. 2014; Wace 1961), suggesting that size evolution was rapid. Gough Island mice belong to the same subspecies as laboratory mice (Mus musculus domesticus) (Gray et al. 2014), opening the door for the application of genomic and molecular genetic tools developed for this model system to the question of the genetic basis of an instance of the island rule.

Utilization of classical inbred strains selected for growth differences revealed that mechanisms of weight accumulation are complex, involving different cellular processes throughout ontogeny and underpinned by tens of quantitative trait loci (QTL) (Atchley et al. 2000; Cheverud et al. 2001; Vaughn et al. 1999). Gough island mice are unique because they provide a rare opportunity to explore the genetics of this complex trait from a population evolving in the wild. Gray et al. (2015) reported that over 16 weeks of measurement, 8 QTL contribute to body weight differences between Gough Island mice and a mainland strain from the same subspecies (WSB/EiJ), while 11 QTL contribute to differences in growth rate. The QTL display additive effects of modest magnitude, with the largest for weight being 0.66 grams. Most of the QTL begin to affect growth in the first 6 weeks after birth, a period that coincides with
augmented growth rates for laboratory mice (Cheverud 2005). These findings provide clues about the
genetic architecture of size evolution and point to candidate regions for further study, as the underlying
genetic and molecular mechanisms for differential growth are unknown.

Differential expression analyses can identify candidate genes within QTL that are responsible for
trait divergence. In mice and humans, regulatory mutations are known to contribute to standing variation
in body size and length (Castro et al. 2019; Claussnitzer et al. 2015; Oliver et al. 2005). Additionally,
many of the variants connected to size variation in humans through genome-wide association studies fall
outside of protein-coding regions (Locke et al. 2015; Shungin et al. 2015; Speliotes et al. 2010),
indicating an important role for gene regulation in the determination of this complex trait. We
hypothesized that evolution of gene expression in three metabolic organs contributed to the evolution of
gigantism in Gough Island mice: the gonadal adipose depot, hypothalamus and liver. Nutrient uptake and
distribution is modulated by extensive cross-talk between these organs, and developmental, physiological
and immunological processes in both the healthy and diseased state is well-documented for these organs
(gonadal adipose: (Björndal et al. 2011; Han et al. 2011; Lackey and Olefsky 2016; Oh et al. 2015);
hypothalamus: (Kälin et al. 2015; Lam et al. 2005; Ohlsson et al. 2009; Thaler et al. 2012); liver: (Cuezva
et al. 1997; Gunewardena et al. 2015; Hart et al. 2009; Hay 1991; Kimura 1991; Klaassen and Aleksunes
2010; Klochendler et al. 2012; Lackey and Olefsky 2016; Liechty and Lemons 1991; Ohlsson et al. 2009;
Septer et al. 2012)).

In this article, we report genome-wide patterns of differential expression between Gough Island
mice and a mainland strain, with the goal of understanding the evolution of island gigantism. While
expression differences may relate to phenotypic differences other than body size, we discovered over
4,000 differentially expressed (DE) genes each in the gonadal adipose, hypothalamus, and liver. We
found strong candidate genes for size evolution by overlapping DE genes with body size QTL and with
single nucleotide differences located in putative regulatory elements. By comparing the differential
expression of liver-specific genes across three time points we identified potential co-regulated gene
groups, which allowed us to identify genes encoding transcriptional regulators within body size QTL that
might coordinate widespread expression differences between Gough Island and mainland mice.

Functional enrichment analyses of DE genes pointed to candidate cellular and physiological processes underlying the evolution of extreme size, including differences in cell proliferation and organ maturation. This work builds upon previous studies aimed at illuminating the genetic basis of the evolution of island gigantism and reaffirms that Gough Island mice are a unique resource for investigating the evolution of extreme size in natural settings.
Material and Methods

Sample collection

Two mouse strains provided tissues for this study: mice from Gough Island (referred to as “island mice” in this report) and WSB/Eij (The Jackson Laboratory, Bar Harbor, ME; referred to as “mainland mice”). The laboratory colony of Gough Island (“island”) mice was founded from mice live-caught on the island and shipped to our laboratory in 2009 (Gray et al. 2015). RNA was obtained from individuals inbred (brother-sister matings) to the seventh generation. All island females sampled shared a most recent common ancestor in the fourth generation of inbreeding. For island females, three litters contributed to the E16.5 samples, two to the 2-week, and four to the 4-week. All island males sampled shared the same most recent common ancestor as the island females. For island males, two litters contributed to the E16.5 samples, three to the 2-week, and one to the 4-week. WSB/Eij is a wild-derived, inbred strain with a body size representative of mainland wild house mice. Among mainland mice sampled, a minimum of two litters and a maximum of four provided individuals for sampling across the five conditions. Each mouse was treated as an independent replicate of its line for analyses of differential expression. Island mice are larger than mainland mice. A general linear model that considered all mice in litters that contributed individuals for RNA collection, with predictor variables litter size, age (2 or 4 weeks), sex, strain and the interaction of strain and age showed that, after controlling for the effects of all predictor variables, strain was significantly related to body weight (F(1, 102) = 102.9, p < 0.001, partial η² = 0.50; adjusted mean weights (M) and standard errors (SE): 2-week: M_island = 10.6 g, SE_island = 0.26 g; M_mainland = 7.2 g, SE_mainland = 0.28 g; 4-week: M_island = 15.9 g, SE_island = 0.20 g; M_mainland = 10.4 g, SE_mainland = 0.20 g).

RNA was obtained from five conditions: the gonadal adipose depot (“adipose”; 4 weeks); the hypothalamus (4 weeks); and the liver at three time points - embryonic day (E) 16.5, postnatal day 14 (2 weeks), and postnatal day 28 (4 weeks). For each condition, samples from five males and five females of each strain were collected, for a total of 100 samples. All samples were snap frozen in liquid nitrogen and stored at −80°C until RNA extraction.
To collect E16.5 embryos, pregnant females were euthanized via CO₂ asphyxiation between 11:30 am and 1:30 pm. As mouse embryos are resistant to hypoxia, embryos were dissected out of uteri into fresh phosphate-buffered saline whereupon embryos were decapitated immediately, ensuring rapid retrieval of the liver for RNA preservation. To determine the sex of embryos we followed the PCR protocol in Deng et al. (2001), genotyping for the presence or absence of \textit{Sry}, using \textit{Rapsn} amplicons as a positive control.

2-week and 4-week old mice were placed in a 1 liter chamber and exposed to non-vaporized isoflurane until non-responsive. Mice were then euthanized via decapitation. This euthanasia method is rapid (≤ 45 seconds) and obviates the need for excessive handling, resulting in rapid acquisition of organs for RNA preservation. From the medial border of the liver’s left lobe an approximately 0.5 cm x 0.5 cm section was taken from 2-week and 4-week old individuals. 2-week collections occurred between 11 am and noon, except for two mainland individuals collected between 9:30 and 10 am. Collection of 4-week adipose, whole hypothalamus and liver samples occurred between 9 am and 11 am.

We collected both right and left gonadal (peri-uterine) fat depot samples from 4-week old females. Samples were approximately 0.25 cm x 0.25 cm and taken from the border between the parametrial and perivesical portions of the abdominopelvic (also called the peri-uterine or gonadal) fat depot (Vitali et al. 2012) (Supplementary Figure 1). In 4-week males an approximately 0.5 cm x 0.5 cm portion of both testicular fat depots was collected; care was taken not to collect any epididymal or testicular tissue. At the time of RNA extraction right and left fat samples were combined.

**Animal husbandry**

Pups were weaned at 3 weeks; individuals providing tissue for the 2-week time point were housed with their mother and siblings until the time of tissue collection. Weanlings were housed with two to three other individuals of the same sex. All adult and weaned mice were housed in a temperature controlled room (68-72° F) set on a 12 hour light/dark cycle. Pregnant and nursing mothers
were provided with breeder chow (Envigo 2019 Teklad Global Diet) and water *ad libitum*, while weaned mice were provided with standard rodent chow (Envigo 2020X Teklad Global Diet) and water *ad libitum*.

**RNA-sequencing library preparation**

Frozen liver tissue was homogenized in 1 mL of TRIzol Reagent (Ambion by Life Technologies) using a powered Qiagen TissueLyser II homogenizer. Liver RNA was isolated using the protocol accompanying the TRIzol Reagent; after the “RNA resuspension” step, the total RNA was treated with DNase and purified (DNA-free Kit, Ambion by Life Technologies, AM1906). Frozen adipose and hypothalamus samples were processed using the Qiagen RNeasy Lipid Tissue Mini Kit (74804). Additionally, The RNase-Free DNase Set (Qiagen, 79254) was used to treat each adipose and hypothalamus sample. Highest quality RNA samples were selected. Selected RNA samples were verified to have an A260/A280 ratio between 1.8 and 2.1 on a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific). Additionally, sample integrity was verified using a fragment analyzer (RNA Quality Number: M = 8.5, SD = 0.85). Subsequently, 1 μg of DNase-treated and purified total RNA for each sample was used for mRNA enrichment and RNA-seq library preparation.

Paired-end, 50-base pair RNA-seq libraries were generated on a Illumina HiSeq 2500 platform (using the Rapid Run mode) in the University of Wisconsin-Madison Biotechnology Center. RNA-seq library preparation and construction included the use of Illumina TruSeq RNA Sample Prep kits (v2, sets A and B; RS-122-2001 and RS-122-2202), which enabled the generation of mRNA-enriched libraries from the total RNA, and Illumina TruSeq Rapid Cluster-HS kits (PE-402-4001), which enabled the formation of clonal template clusters during the HiSeq 2500 runs, and Illumina TruSeq Rapid SBS kits (FC-402-4002) for the sequencing of reads.

**Read mapping**

We mapped the RNA-seq reads to a reference set of transcripts from the *Mus musculus domesticus* reference genome (mm10/GRCm38, Ensembl v79 annotation) and estimated transcript
abundances and read counts using the software packages RSEM (v1.2.21) (Li and Dewey, 2011) and Bowtie (v1.1.1) (Langmead et al. 2009). As the libraries were mRNA-enriched, only long transcripts were kept in the reference set. Reference transcript sequences were modified in one of two ways. When mapping reads from island mice, single nucleotide polymorphisms (SNPs) fixed in island mouse coding sequences were used to replace sites in the reference transcript sequences. The fixed island mouse SNPs were identified as those that differed between the island and mainland inbred strains (but were invariant between a male and a female founder of the island mouse strain) from whole genome sequences (average depth of coverage = 10X) (described below). When mapping reads from mainland mice, fixed SNPs within mainland mouse coding sequences (obtained from Keane et al. 2011) were used to replace sites in the reference transcript sequences.

Detection of differentially expressed genes

To determine whether a gene was differentially expressed (DE) between island and mainland mice, we used a negative binomial generalized linear model (GLM) in the DESeq2 package (Love et al., 2014), implemented in R (Bioconductor version 3.2). To identify DE genes in adipose and hypothalamus, we modeled read counts using factors strain, sex, and strain by sex interaction (strain:sex). Since we collected transcripts from three time points for the liver, we incorporated age as a factor in the model for determining read counts: strain, sex, age, sex:age, strain:sex, strain:age, and strain:sex:age. DE genes were identified as those with an adjusted p-value < 0.05 after using the Benjamini-Hochberg procedure for multiple test correction with a false discovery rate = 0.05 (Benjamini and Hochberg 1995).

Our analyses focused on sex-averaged differences between the strains for two reasons. This approach enabled straightforward comparison of the locations of DE genes and QTL (Gray et al. 2015) that confer body size differences between these strains in both sexes. Moreover, whole-body sexual dimorphism does not begin until around 3 weeks of age in mice, with accompanying sex-specific gene expression extending across puberty and into increasingly mature mice (Cheverud 2005; Waxman and Celenza 2003). We did detect minimal to modest sex-specific differential expression from the factor sex.
in our models: 4-week adipose – 1656 genes; 4-week hypothalamus – 15 genes; E16.5 liver – 34 genes; 2-week liver – 9 genes; 4-week liver – 138 genes. The relatively large number of sex-specific differential expression in the adipose may reflect the fact that female and male gonadal adipose depots are less anatomically, and perhaps functionally, homologous than other adipose depots, while the increased sex-specific differences in more mature livers may reflect the sexual dimorphism that accompanies liver gene expression in increasingly mature mice (Ohlsson et al. 2009). To obtain sex-averaged gene expression differences, contrast vectors were multiplied by the vector of GLM coefficients to obtain sex-averaged log₂ fold changes for all assayed conditions. Throughout our report, higher and lower refer to gene expression levels that were higher or lower in island mice relative to mainland mice.

To construct putative co-regulated gene groups (described below) from the expression data collected across three time points in the liver we multiplied a series of contrast vectors with the vector of GLM coefficients to obtain log₂ fold changes from one time point to another within a strain (as opposed to across strains): embryonic-to-2-week and 2-week-to-4-week.

**Functional enrichment analyses**

Utilizing the R package GOseq (version 1.24.0), we determined the enrichment of Gene Ontology (GO) annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways among the DE genes in all conditions assayed (Young et al. 2010). In GOseq, enrichment analyses were conducted with the method argument in the goseq() function set to "Hypergeometric". For each condition, separate enrichment analyses were conducted for genes expressed at higher and lower levels in island mice, and the background set of genes consisted of all genes whose transcription was detected in that condition. To obtain maximum functional information we determined the quantity of significantly enriched GO and KEGG terms returned for different magnitudes of fold change. We quantified the number of enriched GO and KEGG terms for 9 fold change cut-off classes (|log₂ fold change| > 0, or ≥ 0.5, 1, 1.5, …, 4). We report on the terms from the fold change cut-off classes from each condition that provided the greatest and second-greatest quantity of significantly enriched GO and KEGG terms (in all cases these cut-off
classes were |log₂ fold change| > 0 or ≥ 0.5 (Supplementary Figures 2, 3, 4, and 5). This approach incorporated terms associated with stringent cut-off classes and ensured that biological processes represented by relatively small expression differences were considered.

The enrichment analyses for all liver conditions, except higher expressed genes at 2 weeks, returned 115 or more significantly enriched GO terms (maximum returned GO terms = 464, for higher in island 4-week liver). While the full output of the enrichment analyses are available in Supplementary Tables 1-4, to navigate these results we used two arrangements of the significant GO terms to look for patterns in the cellular and biochemical processes that could relate to differences between island and mainland mice. First, we used REVIGO to obtain the specificity of each GO term (Supek et al. 2011). In REVIGO, output files contain a “frequency” column that indicates the percentage of genes in the GO Annotation database annotated to a GO term; we consider the REVIGO frequency score to be a measure of the GO term’s specificity. More specific or detailed GO terms are annotated to fewer genes, while increasingly general GO terms are annotated to a greater number of genes. For the purpose of illuminating differences in specific cellular, biochemical, and biological processes between the strains, we focused on the top 50% of the most specific (per the REVIGO frequency value) enriched GO terms in each condition and fold-change direction. (A visual example of how the first arrangement was implemented can be seen in Supplementary Figure 6).

In a second arrangement, we extracted the broad functional categories, or GOSlim categories, to which each of the specific GO terms is mapped from the GOSlim resource at Mouse Genome Informatics (The Jackson Laboratory, http://www.informatics.jax.org/gotools/MGI_GO_Slim.html). There are 38 GOSlim categories: 12 each are affiliated with sub-ontologies Cellular Component and Molecular Function, and 14 are affiliated with sub-ontology Biological Function. We then quantified the number of highly specific significantly enriched GO terms (from the first arrangement described above) in each of the GOSlim categories. This allowed us to make observations about the broad patterns differentiating the metabolic organs evaluated in the two mouse strains. In particular, because we assayed three time points in the liver we were especially interested in noting the dynamism in cellular processes across time. For
this purpose, in the liver, we paid special attention to GOSlim categories to which a large proportion of specific GO terms mapped from all three time points (Supplementary Table 5). Importantly, merging these two arrangements enabled us to assess the most specific biological processes (provided by the first arrangement) that differ between island and mainland mice, while retaining access to observations on broad differences in liver maturation and function (provided by the second arrangement).

**Identifying co-regulated gene groups in the liver**

We reasoned that genes whose expression levels changed from one time point to another in a similar manner in the liver might be regulated by the same transcription factor(s). Such transcription factors, we further reasoned, might be key players in the differential expression we discovered because their activity could impact the expression of the downstream genes they regulate. We used two criteria related to differential expression across the three time points in the liver to place a subset of genes into co-regulated groups. First, we looked for genes that showed coordinated expression changes throughout liver maturation *within* strains. Second, we looked for genes that showed similar patterns of differential expression *between* strains (throughout liver maturation). Below we describe each criterion in turn.

Within each mouse strain, genes expressed across all three time points could manifest significant changes in expression level within two time intervals: from E16.5 to 2 weeks, and from 2 weeks to 4 weeks. Thus, a gene could manifest one of nine possible within-strain temporal trajectories across time intervals one and two: decrease-decrease, decrease-increase, decrease-no change, increase-decrease, increase-increase, increase-no change, no change-decrease, no change-increase, and no change-no change. Placement of genes into the trajectories depended on two filters. First, the change in magnitude had to be significantly different (adjusted p-value < 0.05) in a contrast from the full DESeq2 GLM that explicitly tested for changes in expression level from one time point to another within a strain. Second, in addition to statistical significance, the change in expression magnitude had to exceed an absolute log$_2$ fold change of 0.6 from one time point to another. Note that any given gene may belong to either the same temporal trajectory class in both island and mainland mice, or to a different one. Thus, genes that are...
expressed throughout liver maturation in both strains could be mapped onto a 9 x 9 grid, which indicates the within-strain temporal trajectory to which the gene belongs in both strains (Supplementary Figure 7).

In addition to classifying liver-specific expressed genes by their strain-specific temporal trajectories (criterion 1), we also classified them according to their between-strain differential expression patterns (criterion 2). A gene that is expressed in both strains across all three time points may be differentially expressed at one, two, or all three time points. Thus, each (presumably) continuously expressed gene could be sorted into one of 27 between-strain differential expression patterns: either higher, lower, or the same at any one of the three time points \((3^3 = 27)\). As was done for criterion 1, we considered a gene to be differentially expressed if the expression level difference was both statistically significant (adjusted p-value < 0.05), per the DESeq2 GLM, and if the absolute log\(_2\) fold change exceeded 0.6.

In summary, a gene could be classified (1) by its within-strain temporal trajectories, and (2) by its between-strain differential expression pattern. By combining these two classes of patterns we could separate continuously expressed genes into putative co-regulated groups. To do this, we further divided the genes mapped onto the 9 x 9 within-strain temporal trajectory grid (from grouping criterion 1) by their classification into one of the 27 differential expression patterns (grouping criterion 2). This procedure resulted in 27 individual 9 x 9 grids, where each grid is specific to a particular differential expression pattern. We hypothesized that genes located within the same cell, on any of the 27 grids, constitute a putative co-regulated group (Supplementary Figure 7).

We sought to identify candidate transcription factors (TFs) regulating the putative co-regulated groups that contained five or more genes meeting the two criteria described above, which resulted in 4,965 genes spread across 285 co-regulated groups. We searched for enrichment of particular TF binding sites, or motifs, in the 5 kilobase (kb), 5′-upstream sequence of each of the genes within a putative co-regulated group using the R package PWMEnrich (Stojnic R., Diez, 2018). In each enrichment test, the background set of sequences used for comparison to the co-regulated group’s sequences were those 5 kb, 5′-upstream sequences from all genes expressed in both strains in any of the three time points assayed for
the liver. Assessed TF binding motifs were obtained from the R package PWMEnrich.Mmusculus.background, which provided the Position Weight Frequencies (PWF) for 637 motifs. Prior to the enrichment analyses, background sequences with strings of “Ns” were removed (single “N” sites were kept, and assigned a weight of zero), nucleotide base frequencies were determined in the set of background sequences, the 637 PWFs were converted to Position Weight Matrices (PWMs), and the distribution of the PWMs was determined in the background sequences.

Enriched TF binding sites upstream of putative co-regulated groups were identified as those with an adjusted p-value < 0.05 after using the Benjamini-Hochberg procedure for multiple test correction with a false discovery rate = 0.05 (Benjamini and Hochberg 1995). The TF gene names corresponding to the significantly enriched binding sites were obtained by matching the motif IDs provided by PWMEnrich with the geneSymbol variable drawn from the R package MotifDb, which was queried with argument “mmusculus”.

**Identifying candidate genes for gigantism by intersecting data sets**

To nominate candidate genes involved in the evolution of body size, we intersected our data set of DE genes with other publicly available data sets related to body size evolution in island mice and gene regulation in metabolic organs. For ease of discussion and interpretation, the sequence space around each DE gene of interest was divided into six sequence lengths upstream and downstream of the gene – 500 base pairs, 1.5 kb, 5 kb, 10 kb, and 100 kb – as well as 5’- and 3’-untranslated regions (UTRs), first and second introns, additional introns, and exons.

We employed the R package GenomicFeatures to create a TxDb object that contained sequence information from the mm10/GRCm38 mouse genome assembly (Lawrence et al. 2013). From this TxDb object, which maps virtual mRNA transcripts to their virtual genomic positions, we acquired the sequence content of each of the genic regions for each transcript variant of the DE genes of interest. We used the GenomicRanges function `reduce()` to merge overlapping genic region sequences from different transcript variants, or gene isoforms (Lawrence et al. 2013). To ensure that the sequence coordinates of all of the
transcripts for a given gene were considered only once per genic region, we merged genic regions
(upstream sequences, UTRs, introns, etc.) into non-redundant sets of sequences. Note that if a particular
sequence was designated a different genic region in two different transcripts then it was retained in the
merged sets of sequences for the respective genic regions (e.g. if a position was in the first intron of
several variants but in the 5’ upstream region of another, then that position would be considered once in
both the merged intronic and merged upstream sequences).

Our five-condition DE gene data set was intersected with four additional data sets to identify
strong candidates for extreme body size evolution: (1) a published set of quantitative trait loci (QTL)
mapped using the phenotypes body weight and growth rate in a cross between the island and mainland
strains (Gray et al. 2015); (2) fixed single nucleotide polymorphisms (SNPs) differentiating the island and
mainland strains; (3) Mouse Encyclopedia of DNA Elements (ENCODE) data sets and published data sets
that catalog various, though not exhaustive, indicators of putative gene regulatory elements (Yue et al.
2014); and (4) segments of highly conserved sequences taken from the Phylogenetic Analysis with
Space/Time models conservation track (phastCons) on the UCSC Genome Browser (Siepel et al. 2005).
By intersecting these data sets we crafted a way to qualitatively assess and rank candidate DE genes. For
example, a DE gene within a body weight QTL with a proximal fixed SNP in an established regulatory
element may be more causally connected to organ functions that promote gigantism than DE genes
outside of the QTL. Information on each of these data sets follows.

(1) Gray et al. (2015) employed a large cross between island mice and the mainland strain used
here (WSB/EiJ) to map 8 QTL for body weight and 11 QTL for growth rate (a total of 14 distinct QTL)
during the first 16 weeks of life. We report on those DE genes for which any of their exonic positions lies
within 2 Mb of the QTL peaks (“QTL-DE” genes; Supplementary Table 6). We performed a permutation
test (10,000 replicates) by permuting genic positions to test whether there are more DE genes in QTL than
expected by chance. The number of DE genes in the QTL (n = 363) does not differ from chance (median
= 363, mean = 362.5, min. = 325, max = 397) (Supplementary Figure 8). This result is expected under the
simple assumptions that a single mutation is responsible for the QTL and that the mutation affects gene expression.

(2) To obtain island mouse genome sequences for calling SNPs, Qiagen DNeasy Blood & Tissue kits were used to extract DNA from the livers of 16 wild-caught Gough Island mice (5 females/11 males) and four descendants of Gough Island mice (2 females/2 males) inbred to the fourth generation in the laboratory. Samples were prepared according to the TruSeq PCR Free Sample Preparation kit (Illumina Inc., San Diego, California, USA) with minor modifications. Libraries were size-selected for an average insert size of 550 bp using SPRI-based bead selection. Paired-end, 100 bp sequencing was performed, using Rapid v2 SBS chemistry on an Illumina HiSeq 2500 sequencer. Average sequencing depth was 10X per sample. Reads were mapped to the mm10 (GRCm38) mouse reference genome using the BWA 0.7.10 (Li, 2013; Li and Durbin, 2009), PCR duplicates were marked using Picard 1.119, and indel realignment was done using GATK version 3.3. For each individual, SNPs were called with SAMtools 1.0 (Li, 2011) and filtered with QUAL \( \geq 20 \) and coverage \( \geq 3 \); SNPs from these samples were then merged using BCFtools 1.0 (Supplementary Table 7).

(3) Mouse ENCODE data sets were obtained from www.encodeproject.org. We located 16 data sets mapped to the mm10 (GRCm38) genome assembly that were generated using mouse tissue collected at time points that matched our experimental design as closely as possible. We considered nine data sets produced from embryonic liver: six data sets from age E16.5 and three from age E14.5. We obtained five data sets derived from 8-week old male livers and two from 8-week old gonadal adipose depots. Despite an imperfect temporal match to our data set, the ENCODE data sets likely provide biological insight into the regulatory landscape around DE genes; the activity of temporally restricted enhancers is known to span a wide range of time points, from several days during embryonic development to weeks in post-natal mice (Nord et al. 2013). Supplementary Table 8 lists the Mouse ENCODE data sets used, including the Experiment IDs for each data set and the file that was downloaded from the website.

As most ENCODE experiments consist of more than one biological replicate, we obtained the “replicated peaks” file for 11 of the 16 data sets; these files contain high-quality overlapping peaks from
all replicates. However, replicated peaks files were not available for three data sets cataloging DNase1 hypersensitive sites in E14.5 livers from male 129 mice, and two data sets cataloging DNase1 sites in 8-week old gonadal adipose depots from male C57BL/6 mice. In these instances, we employed a conservative approach to collapse these sequence ranges into single files most likely to represent biologically relevant peaks – one for DNase1 sites in embryonic liver and one for DNase 1 sites in adult adipose depots. The coordinates for the DNase1 sites in each data set were converted into GenomicRanges objects using the R package GenomicRanges (Lawrence et al. 2013). Then the `reduce()` function was used to join overlapping peak sequences in each file. Shared peak sequences among replicates (or experiments) generated from similar conditions (three from E14.5 livers, and two from 8-week adipose) were then identified using the `intersect()` function. This function ensures the retention of only the smallest sequence range shared among the intersected data sets.

We obtained 16 data sets from publications that identified putative gene regulatory elements in mouse 4-week to 12-week liver tissue or the 3T3-L1 cell line, a model pre-adipose and adipocyte differentiation cell line (see references in Supplementary Table 9). Adipogenesis in gonadal adipose depots is highly plastic – responding to developmental age, temperature, and diet – indicating that findings from the 3T3-L1 cell line could provide insights into differences in differentiation between island and mainland adipose depots (Han et al. 2011; Wang et al. 2013). Data sets were taken directly from published tables or obtained from personal communication with contributing authors. For our analysis, we considered a wide range of gene regulatory element indicators, including histone modification marks, specific TF binding sites, general TF binding sites, and enhancer RNAs (Supplementary Table 9).

(4) Across species, conservation among non-coding sequences might indicate a gene regulatory role for the conserved sequence. We used the UCSC Genome Browser Table Browser tool to obtain coordinates for conserved non-coding sequences among vertebrates (Karolchik et al. 2004). The selected options included genome: mouse, assembly: GRCm38/mm10, group: Comparative Genomics, track: Conservation, and for the table option we selected, in turn, three options that utilize aligned sequence
information from three sets of vertebrates – Euarchontoglires (21 rodent and primate species), placental mammals (40 species), and vertebrates (60 species) (actual options selected were phastConsElements60wayEuarchontoGlires, phastConsElements60wayPlacental, and phastConsElements60way). All returned conserved sequence elements had conservation scores of 200 (default) or higher.

Results

Organ-specific patterns of gene expression evolution

Two broad patterns emerged when looking at differential expression in the adipose, hypothalamus, and liver. First, island and mainland mice exhibit substantial gene regulatory evolution – both in the number of genes and in the magnitude of differential expression. Between 21% and 48% of expressed genes are differentially expressed (DE) between strains in each condition, and approximately one quarter of these DE genes differ between strains by an absolute log₂ magnitude of 1 (≥2 X or ≤1/2 X) (Figure 1A). While certainly not all of these expression differences impact weight accumulation, the extensive divergence in transcript levels across three organs provides a rich data set from which hypotheses about the roles of specific genes and pathways in island gigantism evolution can be explored. Second, among genes that are expressed throughout liver development, while there are more DE genes of any magnitude in 4-week livers (Figure 1A) and while genes exhibiting a wide range of expression differences, from low to high, increase in number beyond the embryonic time point, it is notable that the percent increase is especially pronounced for genes manifesting large magnitudes of differential expression (Figure 1B). In other words, the elevated number of DE genes in 2- and 4-week livers is not just due to an increase in genes exhibiting relatively small magnitudes of differential expression (test for relationship between time point and number of DE genes in a log₂ fold change class: (a) all DE genes regardless of fold change, which is equivalent to no |log₂ fold change| threshold: $X^2(2) = 165.4$, p < .001; (b) |log₂ fold change| threshold = 0.6: $X^2(2) = 80.9$, p < .001; (c) |log₂ fold change| threshold = 1: $X^2(2) = 50.5$, p < .001).
42.4, p < .001; (d) |log₂ fold change| threshold = 2: \( \chi^2(2) = 16.9, p < .001 \). Among DE genes higher in island mice this trend is most pronounced from 2 to 4 weeks, where the number of DE genes with a log₂ fold change ≥ 0.6 or 1 increased by 34% (blue and gold lines in Figure 1B, upper panel). In contrast, among DE genes lower in island mice this trend is a bit more subtle; the trend is most pronounced from the embryonic to the 2-week time point, where the number of DE genes with a log₂ fold change ≤ -2 increased by 16% (green line in Figure 1B, lower panel) (see Supplemental Figure 9 for raw count of DE genes). Our temporal investigation of divergent gene expression in the liver spans two important developmental transitions for house mice (Walthall et al. 2005): the fetal-to-postnatal transition, and the parental care-to-weaning transition (includes a suckling-to-solid food shift and increased periods of fasting in the absence of parental care), which occurs at 3 weeks for mice in our colony. Though temporal resolution is low, the accumulation of genes displaying large magnitudes of differential expression at time points flanking the parental care-to-weaning transition (3 weeks) suggests that island mice livers evolved to respond differently to this transition more strongly than the earlier transition. Additionally, this pattern might simply reflect accumulating differences in transcriptional networks as the liver matures, including through key developmental transitions (Hart et al. 2009; Klaassen et al. 2010; Septer et al. 2012; Walthall et al. 2005).

Overlap of differentially expressed genes with body size QTL

To aid in the nomination of candidate genes and mutations for the evolution of gigantism, we identified the DE genes from all 5 evaluated conditions that reside within each of the 14 previously identified body weight and growth rate QTL peaks (Gray et al. 2015). We found a total of 363 DE genes within the QTL peaks (subsequently referred to as QTL-DE genes; Supplementary Table 10). While all QTL-DE genes are candidates, we reasoned that strong candidates for gigantism evolution would include those with high magnitudes of differential expression, with fixed single nucleotide polymorphisms (SNPs) in putative regulatory sequence space, and with a subset of those SNPs overlapping established conserved sequences and/or organ-specific regulatory elements (Figure 2; Table 1). These criteria point to
molecular genetic changes that could alter gene transcription in genomic regions already known to be involved in body size evolution (Gray et al. 2015) and metabolic organ function (Supplementary Tables 8 and 9).

Partitioning QTL-DE genes in this manner revealed that 180/363 QTL-DE genes exhibit high levels of differential expression with \( \geq 1.5 \times \) or \( \leq 2/3 \times \) transcripts (equivalent to an absolute log\(_2\) fold change \( \geq 0.6 \)) in one, two, or more conditions (Table 1, Supplementary Table 10). Among these 180 QTL-DE genes with substantial expression differences, 113 harbor a fixed nucleotide difference in their genic regions (Supplementary Figure 10). Among these 113 genes, 73 contain fixed differences between island mice and the mainland strain that overlap one or more putative cis-regulatory elements (including Mouse ENCODE elements and highly conserved sequences) characterized in publicly available data sets (Figure 2; Table 1; Supplementary Tables 8, 9 and 11). Moreover, 28/73 of these genes are annotated to one or more Mammalian Phenotype Ontology (MPO; Smith et al. 2004) phenotypes related to growth, body size, and metabolism (Table 2). For example, *Irs1* and *Lrp1* are annotated to tens of MPO phenotypes (Table 2), including abnormal fat pad morphology and postnatal growth retardation.

Concordantly, *Lrp1* is known to regulate many metabolic processes in the organs in which it is expressed and in which organ-specific regulatory elements overlap its proximal fixed SNPs (Figure 2), including cholesterol storage, glucose and lipid transport, and insulin responsiveness (Hofmann et al. 2007). Similarly, *Irs1* is one of two major intercellular transmitters of insulin signaling (Thirone et al. 2006).

Promising candidate genes for extreme size evolution also come from QTL-DE genes that display only a subset of the criteria we used above to learn more about their transcriptional and phenotypic contexts. Four examples illustrate this point. First, none of the fixed SNPs flanking *Egr2* overlap the published regulatory element coordinates we assessed, yet *Egr2* promotes adipocyte differentiation (Chen et al. 2005) (Table 1; Supplementary Figure 10; Supplementary Table 10). Second, while *Zbtb16* is not annotated to an MPO phenotype it harbors proximal fixed SNPs that overlap with condition-specific regulatory elements (Figure 2; Table 1). Polymorphisms in *Zbtb16* are associated with obesity-related traits in humans, while in mice this gene influences fat cell-regulated thermogenesis and body weight.
(Bendlová et al. 2017; Plaisier et al. 2012). Third, the apolipoprotein gene cluster on chromosome 9 (Apoa1, Apoc3, Apoa4, and Apoa5), which harbors high-frequency (but not fixed) island SNPs (Table 1), is associated with plasma triglycerides, obesity risk, and the metabolic syndrome in humans, while in mice this gene cluster resides within QTL for body fat and liver lipoprotein metabolism (Dallongeville et al. 2008; Mehrabian et al. 1998). Fourth, while Jmjd1c exhibits a modest significant fold change magnitude (|log2 fold change| < 0.6; Table 1; Supplementary Table 10), its knock down in the 3T3-L1 preadipocyte cell line inhibits adipocyte differentiation (Buerger et al. 2017).

The majority of QTL-DE genes do not have flanking fixed SNPs that overlap established organ-specific regulatory elements (213/363, 59%; Table 1; Supplementary Tables 8 and 9)) nor are the majority associated with an MPO phenotype (257/363, 71%; Supplementary Table 10). Nonetheless, evidence for the contribution of at least some of the QTL-DE genes to the genesis or maintenance of extreme size comes from the significant enrichment of body size-related phenotypes among the QTL-DE genes when compared to all mouse genes with annotated phenotypes (Table 3). Notably, numerous QTL-DE genes from numerous body size QTL drive the enrichment of each MPO phenotype, providing evidence that at least some of the physiological and metabolic processes that evolved to confer large size in island mice might be fostered by regulatory changes at multiple loci, and possibly in multiple organs, as would be expected for a complex trait like gigantism.

Co-regulated gene groups in the liver

We combined patterns of coordinated and differential expression in the liver across three time points to form putative co-regulated gene groups. From the perspective of gene regulatory evolution, the differential expression patterns manifested by the genes in each co-regulated group can be traced to two sources, which are not mutually exclusive: (1) differences (e.g. SNPs) in transcription factor binding sites (TFBS) in regulatory elements of a group’s genic members; and (2) differential expression of the transcription factors (TF) that modulate the expression levels of a group’s genic members. To evaluate both sources of differential expression we assessed the enrichment of transcription factor binding motifs
in the 5 kb, 5′-upstream sequences of each co-regulated group’s genes and asked whether TFs whose motifs were enriched were themselves differentially expressed. This approach permitted the construction of simple gene regulatory networks that are altered in island mice relative to mainland mice.

We parsed 4,965 genes with dynamic or consistent differential expression patterns within and between strains into one of 285 putative co-regulated groups (see Material and Methods). Among these groups, 224 show statistically significant enrichment for TFBSs in their upstream sequences (Supplementary Table 12). Overall, there are 253 significantly enriched TFBSs across the 224 co-regulated groups (note the following verbal equivalency: enriched TFBS = enriched TF). 104 of these 253 TFs are themselves differentially expressed in the liver, and 39 of these differentially expressed TFs are members of one of the co-regulated groups with significant upstream TFBS enrichment (Supplementary Table 12). This suggests that the differential expression of co-regulated group genes might be a result of both genetic variation in their putative cis-regulatory sequence space (for example, see Figure 2) and alterations to the expression levels of their upstream-binding TFs (trans-regulatory effects).

Of special interest are enriched TFs that reside within body size QTL (QTL-TF; Gray et al. 2015) as they might regulate numerous DE genes that underlie functional differences between island and mainland metabolic organs. Four enriched TFs are QTL-TFs: *Arid5b*, *Egr2*, *Sp100*, and *Tfec*. Except for *Egr2*, these QTL-TFs are themselves differentially expressed in the liver (Supplementary Table 10). *Arid5b*, *Egr2*, *Sp100*, and *Tfec* putatively regulate 40, 1, 3, and 28 co-regulated groups, respectively (Figure 3). Among all the 253 enriched TFs, *Arid5b* and *Tfec* contribute regulation to more co-regulated groups than 95% and 90% of the other enriched TFs, respectively. Moreover, considering all the genes in their co-regulated groups, *Arid5b* and *Tfec* putatively contribute to the regulation 776 and 790 DE genes, respectively (jointly, 32% of the 4,965 DE genes that were partitioned into co-regulated groups) (Supplementary Table 12). This indicates that *Arid5b* and *Tfec* might play a disproportionate role in the total differential expression observed in the maturing liver. It is known that *Arid5b* regulates numerous
other transcription factors essential for switch points in the adipocyte differentiation pathway, and is also active in hepatocytes (Baba et al. 2011; Clausnitzer et al. 2015).

The proximity of Arid5b and Tfec to the QTL peaks and their role in regulating hundreds of genes strengthens their nomination as candidate genes with prominent roles in the evolution of gigantism in island mice. Notably, Tfec belongs to a co-regulated group whose upstream sequences are enriched for Arid5b binding sites, and both QTL-TFs regulate genes with well-characterized roles in liver differentiation and metabolism (Figure 3). For example, Tfec putatively regulates two genes, Dbp and Igfbp5, that belong to the same co-regulated group and that likely participate in circadian rhythm transcription and body weight regulation, respectively (Salih et al. 2004; Wuarin et al. 1992). Arid5b regulates multiple genes from many co-regulated groups that have been linked to the liver’s ability to process glucose and lipids, including Hk2 and Prkaa2, the latter of which also resides within a body size QTL (Panasyuk et al. 2012; Ruderman et al. 2003) (Supplementary Table 10). Additional regulatory connections between the QTL-TFs and genes involved in different aspects of metabolic or transcriptional regulation in the liver, including Cebpβ and Esr1, are indicated by the TFBS enrichment analysis (Figure 3; Supplementary Table 12) (Qiu et al. 2017; Westmacott et al. 2006).

Functional enrichment throughout liver maturation

To identify cellular and metabolic processes that differ between island and mainland mice throughout liver maturation, we sorted highly specific, significantly enriched Gene Ontology (GO) terms into 38 GO Slim categories (see Material and Methods). Each GO Slim category encompasses hundreds of GO terms. Figure 4 (and Supplementary Figure 11) shows the top one quarter of GO Slim categories containing the greatest quantity of highly specific, enriched GO terms; these include, from highest to lowest, Other metabolic processes, Cell organization and biogenesis, Transport, Stress response, Cell cycle and proliferation, Other membranes, Developmental processes, Signal transduction, Mitochondrion, and Translational apparatus (Supplementary Table 5). Figure 4 simultaneously allows...
for the visualization of broad functional differences (via the GO Slim color code) and specific alterations in cellular and metabolic processes (via the enriched GO terms along the x-axes) between island and mainland livers.

When considering functional enrichment across the evaluated liver time points, three broad patterns are evident (Figure 4; Supplementary Figure 11). First, gene regulatory evolution in the liver was temporally and functionally extensive, affecting a wide range of cellular and metabolic processes from late embryonic development to sub-adult life stages. In particular, gene regulatory evolution affected a more diverse set of biological functions in embryonic and 4-week livers (as indicated by the diverse color palette exhibited for E16.5 and 4-week livers in Figure 4) than in 2-week livers (Supplementary Figure 11). Second, subsets of biological functions are either consistently associated with higher or lower expressed genes across two assayed time points or they are associated with genes that flip their direction of fold change across time points. For example, immunity and stress response-related functions are enriched among highly expressed genes at both E16.5 and 4 weeks, while mitochondrial activity is enriched among higher expressed genes in embryo but enriched among lower expressed genes at 4 weeks. Third, the average difference in expression for genes annotated to these enriched functions ranges from an approximately 20% to >400% decrease or increase in mRNA levels in island mice relative to mainland mice (Figure 4; Supplementary Figure 11).

Condition-specific functional enrichment

While some enriched functions may reflect processes that differ among island and mainland mice beyond aspects of body size, below we emphasize significantly enriched GO terms and KEGG pathways in each condition (see Material and Methods; Figure 4; Supplementary Tables 1-4) that might relate to differences in liver maturation and/or body size.

Embryonic liver
At E16.5, GO terms and KEGG pathways related to mitochondria, metabolism, and immunity are enriched among higher expressed genes in the liver (Figure 4; Supplementary Tables 1 and 2). In contrast, general developmental processes are reduced in island mouse embryonic livers, as indicated by lower expression of genes annotated to GO terms such as Organ morphogenesis and Mitotic cell cycle (Figure 4).

2-week liver

In 2-week livers, the vast majority of significantly enriched GO terms and KEGG pathways are annotated to genes manifesting lower expression in island mice (Supplementary Figure 11; Supplementary Tables 1 and 2). Prominent among these enriched GO terms and KEGG pathways are those related to amino acid catabolism (Supplementary Figure 11; Supplementary Tables 1 and 2). Complementary to this observation is that the KEGG pathway Lysosome – a general carbohydrate, lipid and protein degradation system – is enriched among lower expressed genes. On the other hand, the only enriched KEGG pathway among higher expressed genes is Ribosome, indicating potential protein construction (Supplementary Tables 1 and 2).

4-week liver

The functions carried out by differentially expressed genes in 4-week livers are spread across a wide array of GO Slim categories, indicating that many biochemical and cellular functions differ between island and mainland mice in increasingly mature livers. The majority of enriched GO categories among higher expressed genes are related to the cell cycle and to immune cell functions (Figure 4). This pattern is mirrored in the enriched KEGG pathways, which include Cell cycle, p53 signaling pathway, T cell receptor signaling pathway, and Cytokine-cytokine receptor interaction (Supplementary Table 2). Lower expressed genes provide another prominent signal, that of suppressed metabolic processes, particularly those carried out in the mitochondria. This signal can be seen in the GO terms found within the GO Slim
category *Mitochondrion*, and in KEGG pathways *Citrate cycle (TCA cycle)* and *Oxidative phosphorylation* (Figure 4; Supplementary Tables 1 and 2).

**4-week gonadal adipose depot**

Numerous significantly enriched KEGG pathways among lower expressed genes in the 4-week adipose are signaling pathways and metabolic processes associated with differentiated fat-storing cells (adipocytes), suggesting the presence of immature pre-adipocytes in island gonadal adipose depots. Concordantly, these themes – reduced pro-adipocyte signaling pathways and reduced lipid metabolism – are also manifested in the myriad enriched GO categories among lower expressed genes (Figure 5; Supplementary Tables 1 and 2). Among the 67 differentially expressed genes that contribute to the enrichment of the *Fat cell differentiation* GO term are several with negative, large magnitude fold change values (log$_2$ fold change $\leq$ -1), including *Arid5b, Klf5, Rgs2, Sfrp1*, and *Zbtb16*, all of which play documented roles in adipogenesis (Bendlová et al. 2017; Claussnitzer et al. 2015; Lagathu et al. 2010; Nishizuka et al. 2001; Oishi et al. 2005; Plaisier et al. 2012) (Supplementary Table 3). Moreover, both *Arid5b* and *Zbtb16* reside in body size QTL peaks (Table 1).

Notably, significantly enriched GO terms among higher expressed genes also contain cellular features related to fat cell differentiation, including enrichment of the Notch signaling pathway and terms related to cilia (20% of enriched GO terms) (Figure 5 and Supplementary Table 1). Both ciliogenesis and Notch signaling are implicated in early stages of adipocyte differentiation (Garcés et al. 1997; Marion et al. 2009).

**4-week hypothalamus**

Immunity-related categories comprise the vast majority of significantly enriched GO terms and KEGG pathways annotated to higher expressed genes in the four-week hypothalamus (Figure 5; Supplementary Tables 1 and 2). Higher expressed genes are also enriched for KEGG pathways *Arachidonic acid metabolism* and *Toll-like receptor signaling pathway* (Figure 5; Supplementary Table...
2). Notably, cilia-related GO categories are enriched among genes transcribed at lower levels in island mice (Figure 5). Hypothalamic cilia might aid in sensing and regulating fat stores (Kang et al. 2015; Oh et al. 2015). Of particular interest is the lower expression of Bardet-Biedl Syndrome genes, Bbs2/9/10/12, which are annotated to cilia-related terms (Figure 5 and Supplementary Table 4). Disruption of these genes in mouse models of human diseases, such as Bardet-Biedl Syndrome, result in obesity (Oh et al. 2015). Relatedly, genes contributing to a particular ciliary protein complex (GO term TCTN-B9D complex), which functions in ciliary assembly and resides at the transition of the ciliary basal body and axonemal complex (Garcia-Gonzalo et al. 2011) (referred to as the “Basal body – axoneme transition” in Figure 5), are transcribed at a lower level in island mice (Supplementary Table 3).
Discussion

Our study examined genome-wide patterns of gene expression in three key metabolic organs – the adipose, hypothalamus, and liver – to accomplish two goals. First, we sought to identify candidate genes for the evolution of gigantism in Gough Island mice. Second, we aimed to infer changes in cellular and metabolic functions that accompanied the evolution of Gough Island gigantism. Below we discuss our progress toward these goals.

Candidate genes

Numerous studies employ comparative transcriptomics (Gallant et al. 2014; MacManes and Lacey 2012; Vonk et al. 2013) and/or sequence variation in characterized gene regulatory elements (Infante et al. 2015; Kvon et al. 2016) to nominate regulatory changes involved in phenotypic evolution. Additionally, others have shown that complex genetic architectures underlie body size evolution via QTL mapping (Atchley et al. 2000; Cheverud et al. 2001; Vaughn et al. 1999). Our research revealed candidate genes for island body size evolution by combining comparative transcriptomics and sequence differentiation data within existing QTL (Gray et al. 2015). Some of the DE genes discovered within QTL for body weight and growth rate differences between Gough Island mice and mainland mice could be responsible for the evolutionary divergence of these traits. We further characterized DE genes within body size QTL by finding fixed nucleotide differences between wild-caught Gough Island mice and a mainland strain, some of which reside in putative regulatory elements. These mutations might have contributed to the expression differences we found. Among candidate genes within the island/mainland body size QTL, two genes stand out: Arid5b and Tfec. Binding-motif enrichment analysis among potentially co-regulated groups of DE genes suggested that Arid5b and Tfec transcriptionally regulate hundreds of downstream genes in the liver. Our results therefore point to specific genes whose roles in promoting the evolution of island gigantism can be further explored.
Our consideration of candidate genes is accompanied by three caveats. First, much of the differential expression we documented probably affects functions unrelated to body size. Second, some (though not all) differential expression could reflect changes in the composition or proportion of cell types in the evaluated organs. Third, our search for common transcriptional regulators of liver-specific co-regulated groups (such as Arid5b and Tfec) was restricted to 5kb of 5′-sequence space. While these sequences likely include promoter and proximal enhancer elements (Visel et al. 2009; Xie et al. 2005), they do not contain all regulatory inputs.

**Differential gene expression and organ function**

The data shared in this report are valuable for generating hypotheses about the role of specific genes and organ functions that evolved to confer Gough Island mice with their giant body size. Below we share how patterns of differential expression suggest potential mechanisms underlying an instance of the island rule on Gough Island that could be explored in future work.

**Maturational differences**

Temporal shifts in developmental processes, or heterochronic shifts, are commonly invoked as mechanisms for evolutionary change (Gould 1985). Results from GO term and KEGG pathway enrichment analyses suggest that the maturational state of both the liver and gonadal adipose differs between island and mainland mice; these differences could impact the development of body size.

Livers in island mouse embryos express genes related to organ growth and development at lower levels, while expressing higher levels of genes related to postnatally maturing livers, including mitochondrial, metabolic and immune functions (Cuezva et al. 1997; Hay 1991; Kimura 1991; Liechty and Lemons 1991; Thomson and Knolle 2010). Evidence of greater maturation is seen in 2-week livers as well. Attenuation of amino acid degradation is an established biochemical signal of neonatal and early
postnatal livers in murid rodents (Blommaart et al. 1995; Conde and Scornik 1977; Gunewardena et al. 2015). At 2 weeks, amino acid metabolism is dissimilar between island and mainland mice, as suggested by the lower expression of lysosomal genes responsible for protein degradation and of genes related to the catabolism and biosynthesis of specific amino acids (Supplementary Figure 11; Supplementary Tables 1-4). Concordantly, at this time point there is significant elevation of ribosomal gene transcription in island mice, indicating that proteins are being constructed rather than dismantled. These findings suggest that the liver matures earlier in Gough Island mice and this change may promote metabolic energy for growth and protein synthesis.

Three observations indicate that gonadal adipocytes from island and mainland mice are at different stages of differentiation (and/or proliferation) in the gonadal adipose depot at 4 weeks. First, island mice exhibit lower expression of genes contributing to signaling pathways and metabolic processes characteristic of mature, lipid-storing adipocytes (Figure 5; Supplementary Tables 1-4). Second, island mice show higher expression of genes related to ciliogenesis. During adipogenesis, the presence of a transient primary cilium is an indication of the preadipocyte stage of differentiation, when cilium-modulated signaling pathways suppress differentiation (Forcioli-Conti et al. 2015; Marion et al. 2009; Oh et al. 2015). Third, the Notch signaling pathway was the only enriched signaling pathway among higher expressed genes (Figure 5; Supplementary Table 1). In vitro experiments indicate that adipocyte differentiation is sensitive to the magnitude of Notch signaling (Garcés et al. 1997; Nichols et al. 2004). At 4 weeks we may have captured part of a population of new, yet young (in terms of differentiation status), pre-adipocytes. Notably, adipose depot enlargement via proliferation (hyperplasia), rather than expansion (hypertrophy), is correlated with the capacity to attenuate obesogenic conditions (Sun et al. 2011).

**Potential hypothalamic contributions to size evolution**

The significant enrichment of signals related to immunoreactivity and inflammation in island mouse hypothalami is akin to observations from mouse models of diet-induced obesity (Gao et al. 2014;
Kälin et al. 2015; Thaler et al. 2012). Hypothalamic immunoreactivity is a hallmark of diet-induced obesity and long-term inflammation in the hypothalamus disrupts energy balance by disproportionately reducing satiety (anorexigenic)-promoting POMC neurons (Thaler et al. 2012).

The enhanced expression of immunity-related genes in island mouse hypothalami suggests two ways this organ could have evolved to alter energy balance and increase body size. First, hypothalamic regulation of feeding (Bechtold and Loudon 2013; Turek et al. 2005) may differ between island and mainland mice. Hypothalamic neuronal signaling can promote feeding and in turn nutrient uptake stimulates hypothalamic immunoreactivity (Kälin et al. 2015), raising the possibility that Gough Island mice sampled for RNA collection fed more recently than their mainland counterparts. Second, the hypothalamic immunoreactive environment might be enhanced in island mice, either because there are proportionately more immunoreactive cells, such as microglia, or those cells are more sensitive to circulating nutrient-derived compounds. The latter hypothesis is further supported by enrichment of the KEGG pathway *Toll-like receptor (Tlr) signaling pathway* among higher expressed genes (Supplementary Table 2). Tlrs are hypothesized to be triggered by circulating saturated fatty acids (Könner and Brüning 2011) and Tlr signaling in microglia stimulates the transcription of pro-inflammatory genes (de Git and Adan 2015).

Two additional hints that hypothalamic control of food intake might be different between island and mainland mice at 4 weeks come from considering the function of primary cilia projecting from hypothalamic neurons and the way Arachidonic acid derivatives affect hunger (orexigenic)-promoting Npy/AgRP neurons. Leptin, an anorexic hormone secreted by adipose and other tissues, may instigate its signaling cascade through neuronal primary cilia in the hypothalamus (Kang et al. 2015; Oh et al. 2015). The reduced transcription of primary cilia-related genes in island mouse hypothalami indicates that leptin responsivity may be suppressed; this would reduce suppression of hunger-promoting neurons, possibly increasing food intake (Lopaschuk et al. 2010) (Figure 5; Supplementary Table 1). Relatedly, neurotransmitters synthesized from the cell membrane polyunsaturated fatty acid Arachidonic acid...
(enriched among higher expressed genes) act on hunger-promoting neurons to increase energy intake (Figure 5) (Lopaschuk et al. 2010).

**Island-mouse specific metabolic environment in post-weaning livers**

4-week livers from island mice expressed higher levels of genes related to the cell cycle, and lower levels of mitochondria-related genes. At or near the time of weaning, a subset of hepatocytes undergoes extensive proliferation and growth via endoreduplication, resulting in polyploid hepatocytes that are larger than their neighboring 2N hepatocytes (Klochendler et al. 2012; Miettinen et al. 2014; Pandit et al. 2012). Differently sized hepatocytes manifest mitochondria-related differential gene expression, with larger polyploid hepatocytes showing about a 20% reduction in transcript levels (Miettinen et al. 2014). This reduction is similar in magnitude to that of lower expressed genes annotated to GO terms related to oxidative phosphorylation and mitochondria in our study (Figure 4). Overall, these patterns could indicate distinct sizes for island mouse and mainland mouse hepatocytes or a greater population of large polyploid hepatocytes in island mouse livers, either of which could alter the storage and mobilization of metabolites for growth in island mice.

Increased expression of genes promoting immunoreactive processes was also observed in the 4-week liver. (Figure 4, see GO terms in GO Slim categories *Developmental processes, Signal transduction, Stress response* and *Transport*). This observation is noteworthy because interactions between the immune system and hepatic cellular environments are key indicators of the progression of metabolic diseases, including obesity (Kim et al. 2004; Lackey and Olefsky 2016; Mighiu et al. 2012; Schmid et al. 2004). In a study that compared hepatic gene expression between lean and obese pigs across time points, the expression of genes regulating immunity was out of sync between the two breeds, suggesting a potential role for immune system pathways in promoting body size differences (Ponsuksili et al. 2007).
Summary

Overall, our results show that extensive gene regulatory evolution in metabolic organs accompanied the evolution of gigantism in Gough Island mice. The intersection of differential expression with QTL mapping, sequence variants, and putative regulatory sequences points to promising candidate genes for body size evolution. Differences in metabolic processes, developmental status, and immunoreactivity in the adipose, hypothalamus, and liver likely promote and/or maintain the extreme body size of Gough Island mice. Our work sets the stage for functional evaluation of the candidate genes, networks, and physiological processes we identified, using the powerful tools available for house mice. Application of comparative transcriptomics to additional island mouse populations with extreme body sizes holds the potential to reveal the generality of gene regulatory mechanisms that underlie the island rule.
**Figure Legends**

**Fig. 1.** Thousands of genes expressed in metabolic organs are transcribed at different levels in island mice relative to mainland mice. (A) Reverse cumulative distribution plots showing the distribution of magnitudes of differential expression for five different conditions. For each condition the count of DE genes and the percentage differentially expressed among all detected transcripts (in parentheses) are provided. (B) Genes expressed throughout liver maturation that are DE (either higher or lower in island mice) at some or all evaluated time points are sorted into four log₂ fold change classes (absolute value of log₂ fold change): No threshold (or cutoff), 0.6, 1, and 2. For the liver, the number of genes manifesting large magnitudes of differential expression increases over time. Counts of DE genes are scaled down and given a common origin so that all magnitudes of differential expression can be viewed together. See raw gene counts in Supplementary Figure 9.

**Fig. 2.** A subset of DE genes residing within body size QTL (QTL-DE genes) exhibit fixed SNPs coincident with putative gene regulatory elements. (A) Established gene regulatory elements (RE) – drawn from 29 public data sets (Supplementary Tables 8 and 9) – overlap with fixed SNPs in 73 QTL-DE genes. 73 QTL-DE genes whose resident fixed SNPs overlap with established gene regulatory elements (RE). Counts of SNP-RE intersections in genic regions are not mutually exclusive: for example, if a SNP occurs in different genic regions in different transcript variants then its coincidence with an RE will be counted for each genic region. Counts are for any position between two adjacent genic regions on the x-axis; for example, all SNPs between upstream position 501 and 1,500 from the transcriptional start site would be included in the up500 column. Spanning over 1 Mb, gene Dab1 bears many fixed SNPs and was removed from panels (A) clarity of the heat map; likewise, genes with long alphanumerical names were removed for clarity. Red arrows in (A) indicate QTL-DE genes highlighted in (C), where genes Arid5b, Insl5, Lrp1, and Zbtb16 are shown with a subset of fixed SNPs that overlap with putative organ-specific REs from a wide array of public data sets (B), details of which can be found in Supplementary...
Tables 8 and 9. (C) does not provide the total number of SNP-RE coincidences, but rather indicates the types of putative REs that overlap with fixed SNPs. In all panels, “5’ introns” refers to either the first or second intron of a transcript; 5’ intronic counts are also included in the total count for the “intron” genic region. “dn” and “up” refer to down- and upstream, respectively, and the numbers refer to base pairs (k = kilobases).

**Fig. 3.** Transcriptional regulation of co-regulated gene groups throughout liver maturation. Transcription factors (TF) *Arid5b* and *Tfec* reside within a body size-related QTL (light blue box) mapped from an intercross between island and mainland mice (Gray et al. 2015). Colored dashed lines emanating from either *Arid5b* or *Tfec* connect to co-regulated gene groups whose transcription is putatively regulated by these TFs. Circles at the terminus of colored, dashed lines indicate transcriptional regulation as evidenced by enrichment of TF binding motifs in upstream sequences of co-regulated group members. While one representative gene is shown for each co-regulated gene group, the number of genes within a group is indicated by the vertical ellipsis and associated count within parentheses. Panels show temporal expression patterns for representative genes. Red text indicates a differentially expressed gene that also resides within a body size QTL.

**Fig. 4.** Summary of GO functional enrichment for DE genes in the embryonic (E16.5) and 4-week liver. Higher and Lower refer to gene expression levels that were higher or lower in island mice relative to mainland mice. Vertical GO terms in all panels are a descriptively specific subset of all significantly enriched GO terms for each time point and fold change direction (see Material and Methods). Colors designate GOSlim categories, broad functional categories to which individual GO terms are sorted, which highlight general biochemical and physiological processes that are different between island and mainland mice. Plots show the average log₂ fold change for differentially expressed genes annotated to an enriched GO term. The GOSlim color key applies to rectangles at the top of each plot as well; rectangles provide the number of all DE genes pertaining to a GOSlim category regardless if a gene’s annotated GO term is
represented in the plot. Black diamonds designate average log₂ fold change values that exceed the maximum value of the plots.

**Fig. 5.** GO and KEGG functional enrichment of gonadal adipose depot and hypothalamic DE genes hint at differences in organ function between island and mainland mice. (A) Subset of enriched GO and KEGG terms among gonadal adipose depot DE genes. (B) Subset of enriched GO and KEGG terms among hypothalamic DE genes. In (A) and (B) the height of the triangle (shorter vertical axes) is arbitrary and indicates the qualitative contribution of the functions encapsulated by the enriched GO and KEGG terms to the biological processes denoted along the long axes with blue arrows. Also, in both (A) and (B) green text designates significantly enriched terms among genes with higher transcription in island mice relative to mainland mice, while red text indicates enrichment among lower transcribed genes. In (B) the term Macrophage is used in place of Osteoclast as osteoclasts are bone-specific macrophages. Macrophages specific to the central nervous system (i.e. hypothalamus) are called microglia and while tissue-resident macrophages carry out tissue-specific functions and manifest distinctive transcriptional profiles they also have a subset of functions and gene expression profiles in common (Casano and Peri 2015; Gautier et al. 2012; Nataf et al. 2005).
Table 1. Differentially expressed (DE) genes (island vs. mainland) in body size QTL: Sorted according to magnitude of differential expression, SNP status, and SNP-regulatory elements

| Genes with fixed flanking SNPs: | Genes with high-frequency flanking SNPs | Remaining genes | Genes with flanking SNPs in regulatory element(s)* | SNPs in regulatory element(s)* | SNPs not in regulatory element(s) | 363 Total DE genes (± 2Mb around QT) |
|---------------------------------|----------------------------------------|-----------------|-----------------------------------------------|----------------|--------------------------------|-------------------------------------|
| Acot11                          | Btd10                                  | Ampd3           | Acsl1                                          | Nmnt           | Nrip3                           | 245                                  |
| Ank3                            | Cav2                                   | Ankrd52         | Ankrd37                                        | Ankrd7          | Nxpe2                           | 122                                  |
| Ankrd7                          | Ctdsp2//                                | Apoa1           | Bloc1s1                                        | Arhgap10        | Nxpe4                           | 133                                  |
| Arhgap10                        | Cyp4v3                                 | Apoa4           | Ccdcl10                                        | Arhgap9         | Parp8                           | 133                                  |
| Arhgap9                         | Dock10                                 | Apoa5           | Cd63                                           | Arid5b          | Pcsk9                           | 122                                  |
| Arid5b                          | Egr2                                   | Apoc3           | Dkk3                                           | Avil            | Phyhipl                         | 133                                  |
| Avil                            | Fam151a                                | Apon            | Erbb3                                          | Cadm1           | Prkaa2                          | 133                                  |
| Cadm1                           | Fam174b                                | Ccl20           | Gdf9                                           | Cdk1            | Rexo2                           | 122                                  |
| Cdk1                            | Fbno36                                 | Cenu            | Gm9945                                         | Cftr            | Rtkn2                           | 122                                  |
| Cftr                            | Fgf12                                  | Dau1            | Gpr55                                          | Cldn1           | Scube2                          | 122                                  |
| Cldn1                           | Ga1nt18                                | Emb             | Htr2b                                          | Col4a3          | Sgip1                           | 122                                  |
| Col4a3                          | Gfpt2                                  | Fstl4           | KCn4                                           | Col4a4          | Shmt2                           | 122                                  |
| Col4a4                          | Gmpl025                                | Gls2            | Kif3a                                          | Ctnna3          | S1c16a7                         | 122                                  |
| Ctnna3                          | Gmp28046                               | Il123a          | Lrig3                                          | Dab1            | S1c16a9                         | 122                                  |
| Dab1                            | Gmp29125                               | Itga7           | Lrp2bp                                         | Dhcr24          | S1c3a1                          | 122                                  |
| Dhcr24                          | Gmp4890                                | Lyve1           | Myl6                                           | Erich5          | Snx31                           | 122                                  |
| Erich5                          | Gmnc                                   | Melt7b          | Myl6b                                          | Fam13c          | St7                             | 122                                  |
| Fam13c                          | Gpr182                                 | Mical2          | Osr2                                           | Fst              | Ikr4                            | 122                                  |
| Fst                             | Ikr4                                   | Micalcl         | Pdlim3                                         | Gm26809         | Tcf7                            | 122                                  |
| Gm26809                         | Ipo7                                   | Rdh19           | Psmd1                                          | Gm9747          | Tctex1d1                        | 122                                  |
| Gm9747                          | Klkb1                                  | Rdh7            | Sarnp                                          | Gypsy           | Tm4sf20                         | 122                                  |
| Gypsy                           | Pmel                                   | Rgs22           | Serpine2                                       | Hsd17b6         | Ttc29                           | 122                                  |
| Hsd17b6                         | Rps26                                  | Sik3            | Shroom1                                        | Htr3b           | Utp14b                          | 122                                  |
| Htr3b                           | S1c16a14                               | Sp140           | Slc26a10                                       | Il1rap          | Wdr78                           | 122                                  |
| Il1rap                          | Slc19a3                                | Tagln           | Tesp1                                          | Inhbe           | Zbtb16                          | 122                                  |
| Inhbe                           | Sorbs2                                 | Tes             | Tmem26                                         | Ins15           | Zfp365                          | 122                                  |
| Ins15                           | Sphkap                                 | Tfec            | Uqcrq                                          | Irs1            | a                               | 122                                  |
| Irs1                            | Suox                                   | Timeless        | Vmn2r84                                        | Itga2           | Trp63                           | 122                                  |
| Itga2                           | U       b                               | Ttc12           | u                                              | Kif5a           | c                               | 122                                  |
| Kif5a                           | Vps13b                                 | V       v       | Inhbc                                          | Lepre1           | d                               | 122                                  |
| Lepre1                          | Zfp879                                 | p       w       | Lrp2b                                          | Lrp1            | e                               | 122                                  |
| Lrp1                            | g                                   | q       x       | Itga1                                          | March9           | f                               | 122                                  |
| March9                          | h                                   | r       y       | Jmdj1c                                         | Mctp2           | j                               | 122                                  |
| Mctp2                           | s                                   | t       z       | Lpp40                                          | Met              | j                               | 122                                  |
| Met                             | k                                   |                     | Lsm8                                           | Mroh7           | i                               | 122                                  |
| Mroh7                           |                     |                     | Ltc4s                                          | Mtnr1a          |                     | 122                                  |
| Mtnr1a                          |                     |                     | Maml1                                          |
| Gene name | Ensemble gene ID      | Chromosome/ QTL^ | ± Distance flanking QTL peak | QTL-associated trait                  | Condition^// | e |
|-----------|----------------------|------------------|------------------------------|---------------------------------------|---------------|---|
| **Met**   | ENSMUSG00000009376   | chr6             | 1Mb                          | body weight, growth rate              | wk4a, wk4h    |   |
| **Itga2** | ENSMUSG00000015533   | chr13            | 1Mb                          | growth rate                           | el, wk4l      |   |
| **Cdk1**  | ENSMUSG00000019942   | chr10            | 1Mb                          | body weight                           | wk4a, wk4l    |   |
| **Cldn1** | ENSMUSG00000022512   | chr16            | 1Mb                          | growth rate                           | el, wk2l, wk4a, wk4h, wk4l |   |
| **Prkaa2**| ENSMUSG00000028518   | chr4             | 1Mb                          | growth rate                           | el            |   |
| **Dab1**  | ENSMUSG00000028519   | chr4             | 1Mb                          | growth rate                           | el, wk2l, wk4a, wk4l |   |
| **Rexo2** | ENSMUSG00000032026   | chr9             | 1Mb                          | body weight                           | el, wk4a      |   |
| **Cadm1** | ENSMUSG00000032076   | chr9             | 1Mb                          | body weight                           | wk2l, wk4h, wk4l |   |
| **Cftr**  | ENSMUSG00000041301   | chr6             | 1Mb                          | body weight, growth rate              | wk2l          |   |
| **Ncad**  | ENSMUSG00000051359   | chr15            | 1Mb                          | growth rate                           | wk4a, wk4h    |   |
| **Utp14b**| ENSMUSG00000079470   | chr1             | 1Mb                          | body weight                           | wk4a          |   |
| **Arid5b**| ENSMUSG00000019947   | chr10            | 1Mb-2Mb                      | body weight, growth rate              | el, wk2l, wk4a |   |
| **Nab2**  | ENSMUSG00000025402   | chr10            | 1Mb-2Mb                      | body weight, growth rate              | wk2l, wk4l    |   |
| **Scube2**| ENSMUSG00000007279   | chr7             | 2Mb                          | body weight, growth rate              | wk2l, wk4h    |   |
| **Htr3b** | ENSMUSG00000008590   | chr9             | 2Mb                          | body weight                           | el, wk2l, wk4l |   |
| **Fst**   | ENSMUSG00000021765   | chr13            | 2Mb                          | growth rate                           | wk2l, wk4a, wk4l |   |
| **Shmt2** | ENSMUSG00000025403   | chr10            | 2Mb                          | body weight, growth rate              | wk2l, wk4l    |   |
| **Acot11**| ENSMUSG00000034853   | chr4             | 2Mb                          | growth rate                           | el, wk2l, wk4h |   |

http://mc.manuscriptcentral.com/gbe
| Mammalian Phenotype Ontology phenotypes | Count of QTL-DE genes linked to phenotype | Count of body size QTL (out of harboring DE genes) |
|----------------------------------------|------------------------------------------|-----------------------------------------------|
| abnormal circulating cholesterol level  | 22                                       | 8                                             |
| abnormal circulating HDL cholesterol level* | 16-17                                   | 8                                             |
| decreased circulating HDL cholesterol level* | 13-14                                   | 7                                             |
| abnormal lipid level*                  | 40-41                                    | 11                                            |
| abnormal lipid homeostasis*            | 40-41                                    | 11                                            |
| abnormal cholesterol level             | 24                                       | 8                                             |
| abnormal circulating lipid level*      | 34-35                                    | 10                                            |
| decreased cholesterol level*           | 16-17                                    | 7                                             |
| decreased circulating cholesterol level* | 15-16                                   | 7                                             |
| abnormal cholesterol homeostasis       | 24                                       | 8                                             |
| homeostasis/metabolism phenotype       | 103                                      | 14                                            |
| abnormal lipoprotein level*            | 18-19                                    | 8                                             |
| abnormal circulating lipoprotein level* | 18-19                                   | 8                                             |
| abnormal triglyceride level            | 19                                       | 9                                             |
| increased circulating triglyceride level | 11                                      | 7                                             |
| abnormal circulating triglyceride level | 17                                       | 9                                             |
| abnormal fatty acid level*             | 15                                       | 8                                             |
| abnormal body weight                   | 52                                       | 14                                            |
| abnormal brown adipose tissue thermogenesis | 3                                      | 2                                             |
| abnormal circulating free fatty acids level | 10                                    | 7                                             |
| abnormal free fatty acids level        | 11                                       | 8                                             |
| abnormal body length                   | 16                                       | 11                                            |
| decreased body length                  | 13                                       | 9                                             |
| decreased body weight                  | 45                                       | 14                                            |
| decreased total tissue mass            | 45                                       | 14                                            |
| abnormal prenatal growth/weight/body size | 27                                     | 13                                            |
| abnormal prenatal body size            | 24                                       | 13                                            |
| abnormal cell proliferation*           | 36                                       | 13                                            |
| decreased cell proliferation           | 25                                       | 12                                            |

*aEnrichment carried out in MouseMine: Motenko, H., et al. (2015). MouseMine: a new data warehouse for Mamm Genome. 26:325-330. All adjusted p-values < 0.05.

bThe number of QTL-DE genes annotated to the phenotype in a Hypergeometric Test with background equal to genes with annotation for *Mus mus domesticus*.

*Significant after multiple test correction using both Bonferroni and Benjamini Hochberg methods, otherwise significant after Benjamini Hochberg multiple test correction.
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Fig. 1. Thousands of genes expressed in metabolic organs are transcribed at different levels in island mice relative to mainland mice. (A) Reverse cumulative distribution plots showing the distribution of magnitudes of differential expression for five different conditions. For each condition the count of DE genes and the percentage differentially expressed among all detected transcripts (in parentheses) are provided. (B) Genes expressed throughout liver maturation that are DE (either higher or lower in island mice) at some or all evaluated time points are sorted into four \( \log_2 \) fold change classes (absolute value of \( \log_2 \) fold change): No threshold (or cutoff), 0.6, 1, and 2. For the liver, the number of genes manifesting large magnitudes of differential expression increases over time. Counts of DE genes are scaled down and given a common origin so that all magnitudes of differential expression can be viewed together. See raw gene counts in Supplementary Figure 9.
Fig. 2. A subset of DE genes residing within body size QTL (QTL-DE genes) exhibit fixed SNPs coincident with putative gene regulatory elements. (A) Established gene regulatory elements (RE) – drawn from 29 public data sets (Supplementary Tables 8 and 9) – overlap with fixed SNPs in 73 QTL-DE genes. 73 QTL-DE genes whose resident fixed SNPs overlap with established gene regulatory elements (RE). Counts of SNP-RE intersections in genic regions are not mutually exclusive: for example, if a SNP occurs in different genic regions in different transcript variants then its coincidence with an RE will be counted for each genic region. Counts are for any position between two adjacent genic regions on the x-axis; for example, all SNPs between upstream position 501 and 1,500 from the transcriptional start site would be included in the up500 column. Spanning over 1 Mb, gene Dab1 bears many fixed SNPs and was removed from panels (A) clarity of the heat map; likewise, genes with long alphanumerical names were removed for clarity. Red arrows in (A) indicate QTL-DE genes highlighted in (C), where genes Arid5b, Ins15, Lrp1, and Zbtb16 are shown with a subset of fixed SNPs that overlap with putative organ-specific REs from a wide array of public data sets (B), details of which can be found in Supplementary Tables 8 and 9. (C) does not provide the total number of SNP-RE coincidences, but rather indicates the types of putative REs that overlap with fixed SNPs. In all panels, “5’ introns” refers to either the first or second intron of a transcript; 5’ intronic counts are also included in the total count for the “intron” genic region. "dn" and “up” refer to down- and upstream, respectively, and the numbers refer to base pairs (k = kilobases).
Fig. 3. Transcriptional regulation of co-regulated gene groups throughout liver maturation. Transcription factors (TF) Arid5b and Tfec reside within a body size-related QTL (light blue box) mapped from an intercross between island and mainland mice (Gray et al. 2015). Colored dashed lines emanating from either Arid5b or Tfec connect to co-regulated gene groups whose transcription is putatively regulated by these TFs. Circles at the terminus of colored, dashed lines indicate transcriptional regulation as evidenced by enrichment of TF binding motifs in upstream sequences of co-regulated group members. While one representative gene is shown for each co-regulated gene group, the number of genes within a group is indicated by the vertical ellipsis and associated count within parentheses. Panels show temporal expression patterns for representative genes. Red text indicates a differentially expressed gene that also resides within a body size QTL.
Fig. 4. Summary of GO functional enrichment for DE genes in the embryonic (E16.5) and 4-week liver. Higher and Lower refer to gene expression levels that were higher or lower in island mice relative to mainland mice. Vertical GO terms in all panels are a descriptively specific subset of all significantly enriched GO terms for each time point and fold change direction (see Material and Methods). Colors designate GOSlim categories, broad functional categories to which individual GO terms are sorted, which highlight general biochemical and physiological processes that are different between island and mainland mice. Plots show the average log2 fold change for differentially expressed genes annotated to an enriched GO term. The GOSlim color key applies to rectangles at the top of each plot as well; rectangles provide the number of all DE genes pertaining to a GOSlim category regardless if a gene’s annotated GO term is represented in the plot. Black diamonds designate average log2 fold change values that exceed the maximum value of the plots.
Fig. 5. GO and KEGG functional enrichment of gonadal adipose depot and hypothalamic DE genes hint at differences in organ function between island and mainland mice. (A) Subset of enriched GO and KEGG terms among gonadal adipose depot DE genes. (B) Subset of enriched GO and KEGG terms among hypothalamic DE genes. In (A) and (B) the height of the triangle (shorter vertical axes) is arbitrary and indicates the qualitative contribution of the functions encapsulated by the enriched GO and KEGG terms to the biological processes denoted along the long axes with blue arrows. Also, in both (A) and (B) green text designates significantly enriched terms among genes with higher transcription in island mice relative to mainland mice, while red text indicates enrichment among lower transcribed genes. In (B) the term Macrophage is used in place of Osteoclast as osteoclasts are bone-specific macrophages. Macrophages specific to the central nervous system (i.e., hypothalamus) are called microglia and while tissue-resident macrophages carry out tissue-specific functions and manifest distinctive transcriptional profiles they also have a subset of functions and gene expression profiles in common (Casano and Peri 2015; Gautier et al. 2012; Nataf et al. 2005).