Gene regulation and speciation in house mice

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One approach to understanding the process of speciation is to characterize the genetic architecture of post-zygotic isolation. As gene regulation requires interactions between loci, negative epistatic interactions between divergent regulatory elements might underlie hybrid incompatibilities and contribute to reproductive isolation. Here, we take advantage of a cross between house mouse subspecies, where hybrid dysfunction is largely unidirectional, to test several key predictions about regulatory divergence and reproductive isolation. Regulatory divergence between Mus musculus musculus and M. m. domesticus was characterized by studying allele-specific expression in fertile hybrid males using mRNA-sequencing of whole testes. We found extensive regulatory divergence between M. m. musculus and M. m. domesticus, largely attributable to cis-regulatory changes. When both cis and trans changes occurred, they were observed in opposition much more often than expected under a neutral model, providing strong evidence of widespread compensatory evolution. We also found evidence for lineage-specific positive selection on a subset of genes related to transcriptional regulation. Comparisons of fertile and sterile hybrid males identified a set of genes that were uniquely misexpressed in sterile individuals. Lastly, we discovered a nonrandom association between these genes and genes showing evidence of compensatory evolution, consistent with the idea that regulatory interactions might contribute to Dobzhansky-Muller incompatibilities and be important in speciation.

[Supplemental material is available for this article.]

Forty years ago, King and Wilson argued that differences between chimpanzees and humans could not be explained by changes in protein sequences alone (King and Wilson 1975). Since then, there has been a lively debate about the relative importance of changes in gene regulation versus changes in gene structure in adaptive evolution (e.g., Hoekstra and Coyne 2007; Carroll 2008), and some recent studies have revealed a major role for regulatory changes in adaptation (e.g., Jones et al. 2012).

The role of gene regulation in speciation has received less attention. This is somewhat surprising since gene regulation requires interactions between loci, and disrupted interactions between loci in hybrids (Dobzhansky-Muller incompatibilities) are thought to underlie many examples of post-zygotic reproductive isolation. At the transcriptional level, gene expression is a consequence of the interaction of cis-regulatory elements and trans-acting factors. Cis-regulatory regions are stretches of noncoding DNA that bind trans-acting factors to regulate mRNA abundance. Thus, negative epistatic interactions between cis- and trans-regulatory elements in hybrids might be important in reproductive isolation (Landry et al. 2005; Tulchinsky et al. 2014).

One powerful way to identify cis and trans changes is to compare expression differences between species with expression differences between alleles in inter-specific hybrids (Fig. 1; Cawles et al. 2002; Wittkop et al. 2004). This approach has now been used in a number of crosses in flies, yeast, mice, and plants (Table 1). These studies have led to an emerging understanding of regulatory divergence within and between species as well as some understanding of the causes of misexpression in hybrids.

Lacking in these studies is a direct association with reproductive isolation through a hybrid sterility or inviability phenotype. House mice (Mus musculus) provide a good opportunity for making links between hybrid sterility phenotypes, misexpression in hybrids, and regulatory divergence between lineages. House mice consist of three main subspecies that diverged recently and are isolated to varying degrees by hybrid male sterility. Over the past four decades, house mice have been developed as a model system for the study of mammalian hybrid sterility (e.g., Forejt and Iványi 1974; Forejt 1985, 1996; Oka et al. 2004, 2007, 2010, 2014; Britton-Davidian et al. 2005; Good et al. 2008a, 2010; Mihola et al. 2009; Bhattacharyya et al. 2013, 2014). Genes underlying hybrid sterility are polymorphic between different laboratory strains and in natural populations (Forejt and Iványi 1974; Good et al. 2008a; Vyskočilová et al. 2009; Bhattacharyya et al. 2014). Importantly, crosses between a wild-derived inbred line of M. m. musculus (PWK/PhJ) and a wild-derived inbred line of M. m. domesticus (LEWES/EiJ) result in infertile hybrid males in one direction and fertile hybrid males in the reciprocal direction. Infertile hybrid males in this cross have significantly reduced testis weight and sperm count compared to pure subspecies (Good et al. 2008a). For simplicity, hereafter we refer to these hybrid males with lowered fertility as “sterile” though sterility is not complete in all individuals. By comparing sterile and fertile hybrid males, it is possible to disentangle missexpression that is associated with sterility from missexpression that is simply a consequence of hybridization.

In a previous study using genome-wide microarray data, hybrid male sterility in this cross was associated with widespread overexpression of the M. m. musculus X Chromosome during spermatogenesis and misexpression at a number of autosomal genes (Good et al. 2010). This work suggested that differences in gene regulation might be important in reproductive isolation. More recently, Turner et al. (2014) mapped sterility quantitative trait loci (QTL) and expression QTL (eQTL) in an F2 cross using different strains of M. m. musculus and M. m. domesticus. They identified a...
large role for trans-eQTL as well as a number of complex regulatory network interactions related to sterility (Turner et al. 2014). However, the mapping approach was not designed to identify allele-specific expression patterns in F1s and did not address the relative importance of cis and trans changes to regulatory divergence between these subspecies.

Here, we compare expression differences between house mouse subspecies with expression patterns in sterile and fertile F1 hybrids. This allows us to address a number of related issues. First, we describe the proportion of changes between subspecies that are due to changes in cis, trans, or both. Second, when both kinds of changes occur, they may occur in the same direction or in the opposite direction. If gene expression is largely under stabilizing selection, as experimental work suggests (Denver et al. 2005; Lemos et al. 2005; Gilad et al. 2006), cis and trans-variants that act in opposite directions may be more common than expected by chance. We test this prediction. Third, the identification of cis-eQTL allows us to ask whether differences in expression are driven by positive selection (Bullard et al. 2010; Fraser et al. 2010, 2011) and, if so, to identify classes of genes that are under selection. Fourth, we identify misexpression (i.e., changes >1.25-fold on a log2 scale between the hybrid and both parents) in sterile and fertile hybrids. Comparing sterile and fertile hybrids allows us to identify those genes that are misexpressed only in sterile mice and thereby associate misexpression with hybrid sterility. While this approach does not distinguish between the specific genes causing sterility from those that are misexpressed as a downstream consequence of causative genes, it does identify a set of candidate genes for reproductive isolation and it makes specific testable predictions. In particular, we test the hypothesis that these candidate genes are disproportionately governed by compensatory evolution, as expected if regulatory interactions contribute to Dobzhansky-Muller incompatibilities.

Results

Extensive cis-regulatory divergence between M. m. musculus and M. m. domesticus

To characterize the contribution of cis-and trans-acting variants to divergence between M. m. musculus and M. m. domesticus, we compared expression differences in whole testis between subspecies with allele-specific expression in their fertile hybrid using three replicates per genotype (Fig. 1A). Since hybrids inherit alleles from both parents that meet in the same trans-acting environment, differences in expression between parents that are also seen between alleles in hybrids can be inferred to be the result of one or more cis-regulatory variants (Cowles et al. 2002). Alternatively, when a gene is differentially expressed between subspecies but not between alleles in the hybrid, we can infer divergence in one or more trans variants (Wittkopp et al. 2004).

Only reads that could be assigned preferentially to either M. m. musculus or M. m. domesticus were retained for analysis (see Supplemental Table S1 for read counts). This allowed us to measure allele-specific expression in hybrids by comparing the relative number of reads mapping to the genome of each subspecies. After excluding genes with low read counts from the analysis, 9851 autosomal genes could be tested for regulatory divergence (see Supplemental Methods). Of genes that could be tested, ~24% (2349 genes) showed evidence of divergence due to one or more variants acting in cis alone, 9% (883 genes) showed evidence of divergence due to one more variants acting in trans alone, and 44% (4349 genes) showed evidence of divergence in both cis and trans (Fig. 1B).

The median regulatory divergence between subspecies in trans alone (0.58 log2 fold change) was significantly lower than the median divergence in cis alone (0.65 log2 fold change; Wilcoxon rank-sum test, P = 0.00019). Genes with an upper-quartile log2 fold change between subspecies (log2 fold change > 0.96) were also enriched for variants acting in cis alone relative to those in trans alone (40% cis alone, 9% trans alone; Fisher’s exact test, P = 0.0003).
Table 1. Studies that have identified regulatory divergence due to changes in cis and trans between species

<table>
<thead>
<tr>
<th>Species</th>
<th>Comparison</th>
<th>Divergence time</th>
<th>Tissue</th>
<th>cis vs. trans*</th>
<th>Misexpressionb</th>
<th>CAWM*</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insects</td>
<td></td>
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</tr>
<tr>
<td>Drosophila melanogaster × D. simulans</td>
<td>Inter-specific</td>
<td>2.5 mya</td>
<td>Whole fly</td>
<td>cis</td>
<td>No</td>
<td></td>
<td>Wittkopp et al. 2004</td>
</tr>
<tr>
<td>D. melanogaster × D. simulans</td>
<td>Inter-specific</td>
<td>2.5 mya</td>
<td>Whole fly</td>
<td>cis</td>
<td>Yes</td>
<td>Yes</td>
<td>Landry et al. 2005</td>
</tr>
<tr>
<td>D. melanogaster × D. simulans</td>
<td>Intra- and inter-specific</td>
<td>2.5 mya</td>
<td>Whole fly (intra-), cis (inter-)</td>
<td>No</td>
<td></td>
<td></td>
<td>Wittkopp et al. 2008</td>
</tr>
<tr>
<td>D. melanogaster × D. simulans</td>
<td>Inter-specific</td>
<td>2.5 mya</td>
<td>Head, body</td>
<td>cis</td>
<td>Yes</td>
<td>N/A</td>
<td>Graze et al. 2009</td>
</tr>
<tr>
<td>D. melanogaster; D. simulans × D. sechellia; D. melanogaster × D. simulans</td>
<td>Intra- and inter-specific</td>
<td>10,000; 250,000; 2.5 mya</td>
<td>Whole fly</td>
<td>trans</td>
<td>Yes</td>
<td>No</td>
<td>Coolon et al. 2014</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
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</tr>
<tr>
<td>Saccharomyces cerevisiae × S. paradoxus*</td>
<td>Inter-specific</td>
<td>5 mya</td>
<td>–</td>
<td>cis</td>
<td>No</td>
<td>No</td>
<td>Emerson et al. 2010</td>
</tr>
<tr>
<td>S. cerevisiae*</td>
<td>Intra-specific</td>
<td>–</td>
<td>–</td>
<td>trans</td>
<td>No</td>
<td>No</td>
<td>Schaeke et al. 2013</td>
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<tr>
<td>Plants</td>
<td></td>
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</tr>
<tr>
<td>Populus trichocarpa × P. deltoides</td>
<td>Inter-specific</td>
<td>–</td>
<td>Leaf, stem</td>
<td>cis</td>
<td>No</td>
<td></td>
<td>Zhuang and Adams 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana × A. arenosa</td>
<td>Inter-specific</td>
<td>6 mya</td>
<td>Leaf</td>
<td>cis</td>
<td>No</td>
<td></td>
<td>Shi et al. 2012</td>
</tr>
<tr>
<td>Cirsium arvense</td>
<td>Intra-specific</td>
<td>–</td>
<td>Leaf</td>
<td>cis</td>
<td>No</td>
<td>Yes</td>
<td>Bell et al. 2013</td>
</tr>
<tr>
<td>Zea mays ssp. parviglumis × Z. m. ssp. mays*</td>
<td>Intra-specific</td>
<td>9000</td>
<td>Ear, leaf, stem</td>
<td>trans</td>
<td>No</td>
<td>No</td>
<td>Combes et al. 2013</td>
</tr>
<tr>
<td>Coffea canephora × C. eugenioides3</td>
<td>Inter-specific</td>
<td>–</td>
<td>Leaf</td>
<td>trans</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammals</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>M. m. domesticus × M. m. castaneus*</td>
<td>Inter-subspecific</td>
<td>350,000–1 mya</td>
<td>Liver</td>
<td>cis</td>
<td>No</td>
<td></td>
<td>Goncalves et al. 2012</td>
</tr>
<tr>
<td>M. m. domesticus × M. m. castaneus*</td>
<td>Inter-subspecific</td>
<td>350,000–1 mya</td>
<td>Retina</td>
<td>cis</td>
<td>No</td>
<td></td>
<td>Shen et al. 2014</td>
</tr>
</tbody>
</table>

*Regulatory divergence primarily attributed to cis or trans variants in crosses.

bMisexpression tested for in crosses.

cCompensatory evolution associated with misexpression.

*Association between misexpression and compensatory evolution not formally tested.

gene-wide analysis (RNA-seq or microarray).

Hybrids are allopolyploids.

Widespread compensatory evolution

Genes with evidence of divergence in cis and trans can be further subdivided into categories based on their contribution to expression differences between subspecies and their direction of action. Genes with evidence of divergence in both cis and trans were divided into three subgroups (see Supplemental Methods and Fig. 1; Landry et al. 2005; McManus et al. 2010): (1) cis × trans, where there was significant differential expression between subspecies, significant differential expression between alleles in the hybrid, and where the subspecies with higher expression contributed the lower expressed allele in the hybrid; (2) compensatory, where the subspecies did not show differences in expression, but alleles in hybrids were significantly different; and (3) cis + trans, where there was significant differential expression between subspecies, significant differential expression between alleles in the hybrid, and where the subspecies with the higher expression level contributed the higher expressed allele in the hybrid. We further subdivided genes in this last category, cis + trans, into cases where cis and trans variants act in the same direction and cases where these variants act in opposition (Supplemental Fig. S1). Of genes with evidence of both cis and trans divergence, the majority were categorized as cis + trans (24%, or 2392 genes); in the majority of these, cis and trans variants act in opposition (1626 genes) rather than in the same direction (766 genes) (Fig. 1B). Thirteen percent of genes were categorized as compensatory (1309 genes). A minority of genes showed evidence of cis × trans divergence (7%, 648 genes) (Supplemental Table S2).

Under a neutral model, we expect an equal number of genes to show divergence due to cis and trans variants acting in opposition and cis and trans variants acting in the same direction. An excess of cis and trans changes acting to reinforce one another would be consistent with directional selection to alter expression level. Alternatively, an excess of cis and trans variants acting in opposition would be evidence for compensatory evolution and widespread stabilizing selection to maintain expression level. Genes categorized as cis × trans, compensatory, and a subset of cis + trans (where variants act in opposition) show evidence of cis and trans changes acting in opposite directions (Fig. 1B). In contrast, a subset of genes categorized as cis + trans show evidence of cis and trans changes that are acting in the same direction. By deriving neutral expectations from the number of independent cis and trans
changes acting in the same and opposite directions, we tested for bias in directionality (see Methods). The proportion of cis and trans changes that act in opposition was extremely inflated compared to the neutral expectation ($P < 0.0001$) (Table 2), providing evidence for widespread compensatory evolution.

### Adaptive evolution of cis-regulatory elements

Changes in cis variants are potentially targets for selection on gene expression level as cis-regulatory regions act as context-dependent regulators on which selection may act efficiently (for review, see Wray 2007). To test for lineage-specific selection on genes with divergent cis-acting variants between the subspecies, a gene-set approach was employed (Bullard et al. 2010; Fraser et al. 2010, 2011). Under a neutral model, an equal number of genes will be up- and down-regulated by cis variants. If a gene set associated with a biological function deviates from the null expectation by presenting a significant directional bias, we can infer lineage-specific selection. We tested this by grouping genes with only cis-acting variants by Gene Ontology (GO) terms (see Supplemental Methods). Three nonindependent biological process GO terms were identified with significant enrichment for biased directionality: (1) transcription, DNA-templated (GO:0006351, $P = 0.0004$); (2) positive regulation of transcription from RNA polymerase II promoter (GO:0045954, $P = 0.02$); and (3) regulation of transcription, DNA-templated (GO:0006355, $P = 0.02$). These inter-related gene sets collectively include 410 genes with putative evidence of selection and show biased directionality toward up-regulation in M. m. musculus (or down-regulation in M. m. domesticus).

### Misexpression in hybrids

Crosses between M. m. domesticus (LEWES/EiJ) and M. m. musculus (PKW/PhJ) result in fertile hybrid males when the mother is M. m. domesticus and sterile hybrid males when the mother is M. m. musculus. To identify differences in expression between fertile and sterile hybrids and to identify misexpression, we summed reads mapping to both the M. m. domesticus and M. m. musculus allele for each sample and then for each genotype (see Supplemental Material). Total read counts for fertile and sterile hybrids are strongly correlated with the read counts of both subspecies (Supplemental Fig. S2).

First, we compared expression patterns on the X Chromosome between sterile and fertile mice. Previous work suggests a large role for the M. m. musculus X Chromosome in hybrid male sterility (Oka et al. 2004, 2014; Storchova et al. 2004; Good et al. 2008a, 2010; Bhattacharya et al. 2013, 2014). Genes remaining in the analysis after filtering for low read counts were distributed across the X Chromosome. In fertile hybrids, the number of genes expressed above and below the level seen in M. m. domesticus was nearly equal, while in sterile hybrids the majority of genes were expressed above the level seen in M. m. musculus (Fisher’s exact test, $P < 0.0001$) (Fig. 2; Supplemental Table S3). We next compared fold changes of X-linked genes with autosomal genes. Fold changes were calculated between both subspecies and between the sterile and fertile hybrids for 10,264 genes. The ratio of genes overexpressed on the X versus the autosomes in the sterile hybrid was significant (Fisher’s exact test, $P < 0.0001$) (Supplemental Table S4), while there was no significant difference between these ratios in the fertile hybrid (Fisher’s exact test, $P = 1.0$) (Supplemental Table S4). Together, these results suggest that the X Chromosome in the sterile hybrid is uniquely overexpressed compared to the fertile hybrid and to the autosomes. Overexpression of genes on the X Chromosome in sterile hybrids is consistent with previous work based on microarrays (Good et al. 2010). It is also consistent with expression studies of germ cells that were sorted by developmental stage (Campbell et al. 2013), indicating that overexpression of genes on the X is not an artifact of differences in the cellular composition of the testes of sterile and fertile mice (see Discussion).

Next, we focused on patterns of expression of autosomal genes. Comparing the number of reads mapping to a gene in the hybrid and in the pure subspecies allowed us to identify misexpressed genes and to infer the mode of inheritance for expression for each gene (Supplemental Fig. S2). Genes that showed less than a 1.25-log$_2$ fold change between the hybrid and both subspecies were considered “similar” regardless of significance (Gibson et al. 2004; McManus et al. 2010). Since this is a conservative cut-off, we found that most genes showed similar levels of expression in hybrids and in pure subspecies (86%, or 8834 genes, and 90%, or 9300 genes, of genes in the sterile and fertile hybrid, respectively) (Supplemental Table S5). While the number of genes categorized as similar in this analysis is higher than in previous studies, this is unsurprising given the short divergence time between M. m. musculus and M. m. domesticus. Genes that did not demonstrate conserved expression patterns were divided into dominant, additive, and misexpressed (see Supplemental Methods and Supplemental Table S5). Where 28 genes were misexpressed in the fertile hybrid, 63 genes were misexpressed in the sterile hybrid (Supplemental Table S5). In the fertile hybrid, an equal number of genes were misexpressed above and below the level of both subspecies, while in the sterile hybrid, significantly more genes were overexpressed (Fisher’s exact test, $P = 0.0006$) (Supplemental Table S6). Eleven misexpressed genes were shared between the sterile and fertile hybrid, all of which were overexpressed.

Genes that are over- or underexpressed in the sterile hybrid to the exclusion of the fertile hybrid are of interest as potential candidates for hybrid incompatibilities. First, we identified genes for which the number of reads mapping to the fertile and sterile hybrid was significantly different. Then, we eliminated genes with less than a 1-log$_2$ fold difference between the sterile hybrid and both subspecies. A 1-log$_2$ fold change corresponds to an expression difference that is twofold higher or lower, so differences between the sterile hybrid and each subspecies at this threshold may be biologically meaningful. We identified 202 genes at a 5% false discovery rate (FDR) with these criteria, hereafter referred to as genes with “aberrant expression” for simplicity. These 202 genes were enriched for 39 nonindependent GO terms at a 5% FDR, the most
highly significant of which were (1) positive regulation of gene expression (FDR q-value = 0.0115), (2) positive regulation of RNA metabolic process (FDR q-value = 0.0139), and (3) regulation of cell migration (FDR q-value = 0.0236) (Eden et al. 2009). Of these aberrantly expressed genes, 17 were associated with only a cis-regulatory change and thus could be included in the test for positive selection. Remarkably, 12 of these 17 genes were identified as targets of positive selection in the analysis above, representing a highly significant overenrichment of positively selected genes among those associated with hybrid sterility (Fisher's exact test, P < 0.0001 (Supplemental Table S7).

A subset of the genes that are aberrantly expressed uniquely in the sterile hybrid are associated with male reproductive phenotypes or cell-cycle control in laboratory mice or are highly expressed in the testis relative to other tissues, making them potential candidates for reproductive incompatibilities between the subspecies (Table 3) (phenotype and expression data collected from Su et al. 2004; Wu et al. 2009; Eppig et al. 2015). Notably, five genes (Adgrl1, Itpka, Mtcl1, Myl10, and Micall2) have been identified in regions of overlap between the results of a genome-wide differentiation study between the subspecies (Phifer-Rixey et al. 2014), a QTL mapping study on measures of hybrid male sterility (White et al. 2011), and in regions of low introgression across the M. m. musculus and M. m. domesticus hybrid zone (Janoušek et al. 2012).

Compensatory evolution is associated with misexpression in sterile hybrids

If cis and trans changes interact epistatically to result in hybrid incompatibilities, we expect divergence between subspecies that involves both cis and trans changes to be associated with novel expression patterns in the sterile hybrid. Genes with both cis and trans changes in opposing directions should be particularly enriched if the breakdown of co-adapted regulatory machinery

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Associated function/ expressiona</th>
<th>Directionb</th>
<th>Regulatory category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arl8a</td>
<td>Cell cycle; chromosome segregation; mitotic nuclear division; cell division</td>
<td>+ cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Brd4</td>
<td>Positive regulation of G2/M transition of mitotic cell cycle</td>
<td>+ cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Cerp</td>
<td>Negative regulation of cell proliferation; RNA processing</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Cib4</td>
<td>Highly expressed in testis</td>
<td>− cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Cited2</td>
<td>Male gonad development</td>
<td>+ cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Crisp2</td>
<td>Testis-specific expression</td>
<td>+ cis by trans</td>
<td></td>
</tr>
<tr>
<td>Ctdsp1</td>
<td>Negative regulation of G1/S transition of mitotic cell cycle</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Cul7</td>
<td>Mitotic cytokinesis; regulation of mitotic nuclear division</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Gm5617</td>
<td>Testis-specific expression</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Hspa8</td>
<td>Heat shock protein; regulation of cell cycle</td>
<td>+ cis by trans</td>
<td></td>
</tr>
<tr>
<td>Hspb1</td>
<td>Heat shock protein; negative regulation of apoptotic signaling pathway</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Kat2a</td>
<td>Cell proliferation; chromatin binding</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Mad111</td>
<td>Mitotic nuclear division, mitotic spindle assembly checkpoint</td>
<td>+ cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Map3k9</td>
<td>Apoptotic process; cell death</td>
<td>+ Compensatory</td>
<td></td>
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<tr>
<td>Morc2b</td>
<td>Testis-specific expression</td>
<td>− cis by trans</td>
<td></td>
</tr>
<tr>
<td>Mtcl1+</td>
<td>Microtubule crosslinking factor</td>
<td>+ cis by trans</td>
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</tr>
<tr>
<td>Myl10+</td>
<td>Testis-specific expression</td>
<td>− cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Phactr4</td>
<td>Regulation of cell cycle</td>
<td>+ cis + trans, opposing</td>
<td></td>
</tr>
<tr>
<td>Pk21</td>
<td>Testis-specific expression</td>
<td>− Compensatory</td>
<td></td>
</tr>
<tr>
<td>Ppp1r42</td>
<td>Highly expressed in testis; microtubule organizing center</td>
<td>+ cis by trans</td>
<td></td>
</tr>
<tr>
<td>Pm2</td>
<td>Spermatogenesis; mutants associated with deformed sperm</td>
<td>+ cis by trans</td>
<td></td>
</tr>
<tr>
<td>Sh3bp4</td>
<td>Negative regulation of cell proliferation; positive regulation of autophagy; negative regulation of cell growth</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Usl2</td>
<td>Homozygous null mutants males are usually infertile</td>
<td>+ Compensatory</td>
<td></td>
</tr>
<tr>
<td>Zbtb16</td>
<td>Male germ-line stem cell asymmetric division; homozygous mutants develop infertility</td>
<td>+ Compensatory</td>
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</tr>
</tbody>
</table>

aPhenotype and expression data from Mouse Genome Informatics (Eppig et al. 2013) and Su et al. (2004), available through BioGPS (Wu et al. 2009).

bThe direction of change between pure species and the sterile hybrid (i.e., genes designated with a “+” are expressed above the level of both pure species in the sterile hybrid).

cGenes have been identified in regions of overlap between a hybrid zone study, a differentiation study, and a QTL mapping study between M. m. musculus and M. m. domesticus (see text for details).
contributes to misexpression in sterile hybrids. To test this hypothesis, we examined the regulatory categories associated with genes that were misexpressed in sterile hybrids (genes with a >1.25-log2 fold change between the sterile hybrid and both subspecies) (Supplemental Table S8). A number of the misexpressed genes could not be analyzed for regulatory divergence due to low read counts. Of the genes that remained in the analysis, there was a nonrandom association between cis and trans variants acting in opposing directions and misexpression in the sterile hybrid compared to genes where cis or trans variants acted alone or in the same direction (Fisher’s exact test, \( P < 0.0001 \)) (Table 4). Genes categorized as strictly compensatory, where there was no significant difference in expression between subspecies despite significant differences between alleles in the hybrid, were the most enriched in the misexpressed gene set (Fisher’s exact test, \( P = 0.0004 \)) (Supplemental Table S9). Far fewer misexpressed genes were retained for analysis from the fertile hybrid (17 genes total). No regulatory category was enriched in the misexpressed gene set of the fertile hybrid, although this may be due to lack of power given the low number of genes tested (Fisher’s exact test, \( P = 1.0 \)) (Supplemental Table S10).

Next, we repeated this analysis using the previously described “aberrantly expressed” genes (Supplemental Table S11) (i.e., a more relaxed cut-off in which expression was at least 1-log2 fold between the sterile hybrid and both subspecies). As above, genes for which cis and trans variants acted in opposition were enriched compared to genes for which cis and trans variants acted independently or in the same direction (Fisher’s exact test, \( P < 0.0001 \)) (Supplemental Table S12). Likewise, strictly compensatory changes again were especially enriched in this differentiated gene set (Fisher’s exact test, \( P < 0.0001 \)) (Supplemental Table S13). Finally, to further investigate the relationship between compensatory evolution and misexpression in the sterile hybrid, genes were binned based on log2 fold changes between the sterile hybrid and both subspecies. As fold change increased, the proportion of genes where cis and trans variants act in opposition increased (Fig. 3).

### Expression comparisons between multiple subspecies lines

The findings described above were based on a small number of wild-derived inbred lines. This limits the extent to which our conclusions speak to regulatory divergence between \( M. m. \) musculus and \( M. m. \) domesticus in general as opposed to regulatory divergence between these particular lines. To expand this analysis and look more generally at expression divergence between the subspecies, we took advantage of data from a recent study that overlapped with genes identified in our analysis as divergent in cis alone were categorized based on directionally. Genes in the three sets we identified as targets of selection (biological process GO terms GO:006351, GO:0045944, and GO:0006355; see results above) were then subjected to a hypergeometric test as in the previous analysis. Despite the reduction in genes represented in each gene set, all three sets maintained biased directionality at a 10% FDR in this new analysis based on a larger number of inbred lines.

The general concordance between these data sets suggests that many of the conclusions described above do not simply represent line effects but instead characterize regulatory divergence between these two subspecies more generally.

### Discussion

We characterized regulatory divergence in tests between \( Mus \) musculus and \( Mus \) musculus musculus as well as aberrant expression associated with sterility in hybrids. We identified evidence of widespread compensatory evolution consistent with stabilizing selection as well as evidence for lineage-specific positive selection on a subset of genes related to transcriptional regulation. Lastly, we identified genes with aberrant expression unique to sterile hybrids. These sterility-associated genes were nonrandomly associated with \( cis \) and \( trans \) changes that act in opposition to one another, consistent with the idea that regulatory changes might underlie Dobzhansky-Muller incompatibilities and be important in speciation.

### Regulatory divergence between \( M. m. \) domesticus and \( M. m. \) musculus

A large number of genes in this study showed evidence of gene expression divergence between \( M. m. \) domesticus and \( M. m. \) musculus. To mitigate the potential effects of inbreeding, we crossed two different inbred lines within each subspecies to create heterozygous individuals against which inter-subspecific hybrids could be compared. This approach, which is rarely used in studies of expression evolution, eliminates differences in gene expression that arise between subspecies as a result of differences in inbreeding depression, and it eliminates expression differences between the subspecies and hybrids as a result of heterosis. We also compared our results to an independent expression study that included more inbred lines (Phifer-Rixey et al. 2014). Without population level sampling, it is impossible to distinguish between line-specific differences.

**Table 4. Numbers of misexpressed genes in different regulatory categories**

<table>
<thead>
<tr>
<th>Regulatory categories</th>
<th>Misexpressed</th>
<th>Not misexpressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis and trans, independent or same direction</td>
<td>6</td>
<td>3992</td>
</tr>
<tr>
<td>cis and trans, together, opposing</td>
<td>29</td>
<td>3554</td>
</tr>
</tbody>
</table>

Mack et al. (2014) were represented in our data. We reanalyzed the data of Phifer-Rixey et al. (2014) for this subset of 9779 genes that were shared between the two studies. Importantly, genes that were differentially expressed in the data of Phifer-Rixey et al. (2014) overlap significantly with genes that have significant parental ratios in our analysis (hypergeometric test, \( P = 1.749 \times 10^{-16} \)). Genes categorized as cis and cis + trans where variants act in the same direction were particularly enriched in this overlap, making up 57% of the genes found to be differentially expressed between \( M. m. \) musculus and \( M. m. \) domesticus in both analyses (\( P < 0.0001 \)). Conversely, genes where cis and trans variants act in opposing directions (cis x trans and a subset of cis + trans categories) showed the lowest proportion of overlap.

We also reanalyzed the data from Phifer-Rixey et al. (2014) to see if our conclusions about cis changes subject to positive selection were general. Genes with significantly different expression between \( M. m. \) musculus and \( M. m. \) domesticus in Phifer-Rixey et al. (2014) that overlapped with genes identified in our analysis as divergent in cis alone were categorized based on directionally.

The general concordance between these data sets suggests that many of the conclusions described above do not simply represent line effects but instead characterize regulatory divergence between these two subspecies more generally.
effects and subspecific differences. However, by characterizing the intersection between these two data sets, we identified patterns that are more likely to be representative of subspecific differences. The high correspondence between the two studies despite their differences in depth and breadth suggests that we have captured a large proportion of subspecific divergence.

The majority of the regulatory divergence between \textit{M. m. musculus} and \textit{M. m. domesticus} was the consequence of \textit{cis} variants, either alone or together with one or more \textit{trans} variants. Conversely, regulatory divergence due to \textit{trans} variants alone was relatively rare, accounting for only a small proportion of genes tested. Comparisons between the median expression differences associated with variants acting in \textit{cis} or \textit{trans} alone revealed that \textit{cis} variants were of greater magnitude. Consistent with the results presented here, divergence in \textit{cis} has been demonstrated to be more common than divergence in \textit{trans} in insects and nematodes (Gordon and Ruvinsky 2012) and was previously shown to contribute to a larger proportion of differentially expressed genes in the liver between the house mouse subspecies \textit{M. m. castaneus} (CAST/EiJ) and \textit{M. m. domesticus} (C57BL/6J) (Goncalves et al. 2012). Similarly, Crowley et al. (2015) found allelic imbalance consistent with cis regulatory effects in 85\% of testable genes in comparisons between mouse subspecies. These results stand in contrast to those of McManus et al. (2010) and Coolon et al. (2014), both of whom found a large proportion of expression divergence to be the result of \textit{trans} differences in \textit{Drosophila} crosses. Elevated \textit{trans} divergence in these two studies may be due to demographic or biological differences between species or to differences in the experimental methods (e.g., the use of whole files versus specialized tissue types, number of replicates, etc.).

Studies in yeast and flies suggest that \textit{cis}-regulatory divergence typically contributes more to differences between species than to differences within species (Tirosh et al. 2009; Emerson et al. 2010) and increases consistently and proportionately with divergence time (Coolon et al. 2014). While \textit{cis}-regulatory variation is substantial in natural populations (Osada et al. 2006; Campbell et al. 2008; Genissel et al. 2008; Gruber and Long 2009; Lemmon et al. 2014), \textit{trans}-acting variation contributes more to polymorphic expression variation within species (Lemos et al. 2008; Wittkopp et al. 2008; Coolon et al. 2014). \textit{M. m. domesticus} and \textit{M. m. musculus} diverged roughly 350,000 years ago and still share some ancestral variation. Thus, some of the regulatory differences observed between inbred strains could still be polymorphic in one or both subspecies. Finally, overlap between our data and those of Phifer-Rixey et al. (2014) is greatest for genes associated with \textit{cis} changes and \textit{cis} + \textit{trans} changes (where variants act in the same direction), suggesting that these two regulatory categories may contribute disproportionately to regulatory divergence between subspecies compared to within-subspecies variation.

Stabilizing selection has been identified as a dominant force underlying gene expression evolution (Gilad et al. 2006). A widespread reduction in gene expression variation compared to neutral expectations based on intra- and inter-specific comparisons (Rifkin et al. 2003; Lemos et al. 2005) and mutation accumulation lines (Denver et al. 2005) suggests that changes in expression are frequently deleterious. The apparent reduction in expression divergence in these studies compared to neutral expectations could be the outcome of two separate processes: the elimination of \textit{cis}- and \textit{trans}-acting variants through purifying selection or compensatory evolution between regulatory elements that conserves expression levels. Our results favor the latter explanation. We identified a significantly greater proportion of instances where \textit{cis} and \textit{trans} variants acted in opposition than expected under neutrality, consistent with widespread lineage-specific compensatory evolution.

What drives this compensatory evolution? One possibility is that selection initially favors a mutation acting in \textit{trans}, perhaps because selection favors a change in expression of some downstream gene. If the initial \textit{trans} change is highly pleiotropic, it may alter the expression of other downstream genes in a
suboptimal way. Selection would then favor the restoration of optimal expression levels at these genes through compensatory cis changes (Goncalves et al. 2012; Coolon et al. 2014).

Against this background of widespread compensatory evolution involving changes in both cis and trans, we also found evidence for lineage-specific positive selection on a subset of cis-only changes. Selection is predicted to act efficiently on cis-regulatory variants (Wray 2007), and simulations suggest that natural selection is more likely to drive cis-regulatory divergence than trans-regulatory divergence (Emerson et al. 2010). In our study, hundreds of genes related to transcriptional regulation with cis changes showed biased directionality. It is clear from this result that positive, directional selection is contributing to a nonnegligible proportion of regulatory divergence.

**Misexpression in sterile hybrids**

In crosses between *M. m. musculus* (PWK/PhJ) females and *M. m. domestica* (LEWES/Eij) males, hybrid males have significantly smaller testes and lower sperm counts compared to hybrid males in the reciprocal cross (Good et al. 2008a) (see Supplemental Table S16 for phenotypes of the mice in this study). We took advantage of the asymmetrical nature of hybrid male sterility in this cross to identify genes that were uniquely misexpressed in sterile hybrids. This approach allowed us to separate misexpression that was associated with hybridization from misexpression that was associated with sterility. For example, the 28 genes that were misexpressed in fertile hybrids (Supplemental Table S5) can be excluded as contributing to reproductive isolation.

Despite the power of this approach, it is important to recognize that it does not allow us to directly identify genes causing sterility. The set of genes that are misexpressed only in sterile hybrids is expected to include causative genes, but it may also include genes that are misexpressed as downstream effects of genes causing sterility. The latter category is likely inflated by differences in the cellular composition of testes in fertile and sterile animals. Testes contain a heterogeneous mixture of cell types; sterile and fertile hybrids contain different proportions of somatic, mitotic, early meiotic, and postmeiotic cells. For example, in the well-studied cross between *M. m. domestica* (C57BL/6J) and *M. m. musculus* (PWK/PhJ), males, hybrid males have significantly smaller testes and lower sperm counts compared to hybrid males in the reciprocal cross (Good et al. 2008a) (see Supplemental Table S16 for phenotypes of the mice in this study). We took advantage of the asymmetrical nature of hybrid male sterility in this cross to identify genes that were uniquely misexpressed in sterile hybrids. This approach allowed us to separate misexpression that was associated with hybridization from misexpression that was associated with sterility. For example, the 28 genes that were misexpressed in fertile hybrids (Supplemental Table S5) can be excluded as contributing to reproductive isolation.

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Previous studies have identified an association between cis and trans changes favoring the expression of the opposite allele and misexpression in hybrids (Landry et al. 2005; Tirotch et al. 2009; McManus et al. 2010; Schaeke et al. 2013). Landry et al. (2005) first identified an association between compensatory co-evolution between cis and trans elements and misexpression in hybrids. While this initial study made powerful predictions as to how regulatory divergence could result in reproductive incompatibilities between species, a phenotypic association with this pattern that is separable from expression differences associated with hybridization has been lacking until now.

The Dobzhansky-Muller model of postzygotic isolation is one of the cornerstones in our understanding of the genetics of speciation (Coyne and Orr 2004). Despite the fact that gene regulation necessarily involves interactions between loci, there have been few systematic attempts to link disruptions in gene regulation across the genome to phenotypes underlying reproductive isolation (Turner et al. 2014). Here, we showed that genes that are misexpressed uniquely in sterile hybrid males are associated with opposing changes in cis and trans. Strictly compensatory changes (i.e., where expression levels in both subspecies are the same) were particularly enriched in genes with aberrant or misexpression. These results provide strong evidence that compensatory regulatory evolution may underlie Dobzhansky-Muller incompatibilities and contribute to reproductive isolation between M. m. musculus and M. m. domesticus.

Methods

Samples

M. m. musculus was represented by whole testis from the wild-derived inbred strains PWK/PhJ and CZECHII/EiJ (hereafter, M. m. musculusPWK and M. m. musculusCZEII), and M. m. domesticus was represented by whole testis from the LEWES/EiJ and WSB/EiJ strains (hereafter, M. m. domesticusLEWES and M. m. domesticusWSB).

Hybrids were generated from reciprocal crosses between M. m. musculusPWEK and M. m. domesticusLEWES. Male hybrids in this cross are sterile when the mother is M. m. musculusPWEK and fertile when the mother is M. m. domesticusLEWES. To circumvent the problem of inbreeding depression in pure species, we crossed M. m. musculusPWK females to M. m. musculusCZEII males and M. m. domesticusLEWES females to M. m. domesticusWSB males.

Sequencing and mapping

For each sample, 100-bp paired-end reads were sequenced from mRNA on the Illumina HiSeq 2000 platform. A mean of 7.5 Gb of sequence was obtained for each sample.

Subspecies were mapped with the program TopHat (Kim et al. 2013) to the appropriate pair of reference genomes (either M. m. musculusPWK and M. m. musculusCZEII or M. m. domesticusLEWES and M. m. domesticusWSB) as well as to the opposite maternal reference (M. m. domesticusLEWES or M. m. musculusPWSB). Hybrids were mapped to M. m. musculusPWK and M. m. domesticusLEWES, as reads that mapped preferentially to one subspecies were retained for further analysis. See Supplemental Methods for information on the reference genomes used for mapping.

On average, a greater proportion of reads mapped to M. m. musculusPWK per sample than to M. m. domesticusLEWES (see Supplemental Table S1). This difference may be due to real differences in allelic expression or due to a mapping bias; to account for the difference in the number of allele-specific reads across samples, reads were later randomly down-sampled across samples (see below).

Regulatory divergence

An equal number of reads from each parental sample were combined to create a mixed parental pool comparable to allele-specific counts in fertile hybrids. Down-sampling was chosen to equalize power across comparisons as described in Coolon et al. (2014). Reads were then pooled for the following categories: (1) M. m. musculus subspecies reads; (2) M. m. domesticus subspecies reads; (3) fertile hybrid M. m. musculus alienic reads; and (4) fertile hybrid M. m. domesticus alienic reads. Genes with fewer than 20 reads for any sample or allele were excluded. Genes were sorted into regulatory categories based on a binomial test between reads mapping to each parent, a binomial test between reads mapping to each allele in the fertile hybrid, and a Fisher’s exact test comparing these values (see Supplemental Methods for details on regulatory divisions) (Wittkopp et al. 2004; McManus et al. 2010). As described by Goncalves et al. (2012), cis + trans can further be subdivided into genes where cis and trans are acting in the same direction (hybrid ratio < pure species ratio) or opposite directions (hybrid ratio > pure species ratio).

Inheritance patterns

After reads were mapped and counted, reads mapping to M. m. domesticusLEWES and M. m. musculusPWSB were combined for each sample and then pooled. Mapped reads from pure species and hybrids were down-sampled to an equivalent number per sample and then pooled by genotype (metaSeqR) (Moulos and Hatzius 2014).

Testing for enrichment of opposing or reinforcing cis and trans changes

The expected numbers of cis and trans changes acting in the same or opposing directions were calculated based on the proportion of negative and positive cis and trans changes (Supplemental Table S1S). Expected numbers were calculated by multiplying the proportion of directional independent cis and trans changes together and then in opposition by the total number of genes with divergence in both cis and trans.

Data access

The sequencing data generated for this study have been submitted to the NCBI BioProject (http://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJNA286765.

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