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Quantitative genome-wide enhancer activity maps for five *Drosophila* species show functional enhancer conservation and turnover during *cis*-regulatory evolution

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Abstract

Phenotypic differences between closely related species are thought to arise primarily from changes in gene expression due to mutations in *cis*-regulatory sequences (enhancers). However, it has remained unclear how frequently mutations alter enhancer activity or create functional enhancers *de novo*. Here we use STARR-seq, a recently developed quantitative enhancer assay, to determine genome-wide enhancer activity profiles for five *Drosophila* species in the constant *trans*-regulatory environment of *Drosophila melanogaster* S2 cells. We find that the functions of a large fraction of *D. melanogaster* enhancers are conserved for their orthologous sequences owing to selection and stabilizing turnover of transcription factor motifs. Moreover, hundreds of enhancers have been gained since the *D. melanogaster*–*Drosophila yakuba* split about 11 million years ago without apparent adaptive selection and can contribute to changes in gene expression *in vivo*. Our finding that enhancer activity is often deeply conserved and frequently gained provides functional insights into regulatory evolution.

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URLs. Study data are available at http://stark.imp.ac.at/data/arnold_gerlach_nature_genetics_2014/.

Accession codes. All deep sequencing data reported are available at <http://www.starklab.org/> and have been deposited in the Gene Expression Omnibus (GEO) database under accessions GSE40739 and GSE48251.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

C.D.A. and A.S. conceived the project. C.D.A., D.S. and M.P. performed the experiments. J.A.M., Y.A.S. and N.C.L. generated the follicle cell RNA-seq data. D.G. and A.S. conducted all computational experiments and bioinformatics analyses. C.D.A., D.G. and A.S. wrote the manuscript.

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Ever since Darwin's model of evolution became widely accepted, scientists have been intrigued by the question of how random mutations could have led to the phenotypic differences between species. It is commonly thought that mutations in *cis*-regulatory sequences are responsible for changes in gene expression and phenotype, especially among closely related species¹⁻⁴. Individual examples of phenotypic differences caused by sequence changes indeed exist in insects (for examples, see refs. ⁵⁻⁷) and vertebrates (for example, see ref. 8). However, it has remained unclear how often mutations alter enhancer activity and, in particular, how frequently they can create functional enhancers *de novo* from non-functional sequences. Conservation and divergence of enhancer function have been studied across genomes by indirect means (for example, via regulator binding or chromatin marks⁹⁻¹²) rather than directly because no method existed to measure enhancer activity and strength across entire genomes. Thus, genome-wide estimates of conservation, divergence or gain of enhancer function remained elusive. Here we use STARR-seq (self-transcribing active regulatory region sequencing), a recently developed quantitative enhancer assay¹³, to determine enhancer activity profiles for the entire genomes of five *Drosophila* species^{14,15} in the constant *trans*-regulatory environment of *D. melanogaster* S2 cells. We find that a large fraction of sequences orthologous to *D. melanogaster* enhancers are also active, indicating functional conservation of enhancer activity. We also find that hundreds of sequences have gained activity compared to their inactive orthologs within short evolutionary timespans and without apparent adaptive selection. The differences in enhancer activity we observe between different species can contribute to changes in gene expression *in vivo*. Our finding that enhancer activity is often deeply conserved and frequently gained provides functional insights into regulatory evolution and its molecular mechanisms, which are of fundamental importance for understanding evolution.

RESULTS

High conservation of *D. melanogaster* enhancer function

To study the evolution of enhancer sequences and their function (activity and strength) across the genomes of closely related species, we performed STARR-seq with the genomic DNA of five *Drosophila* species (*D. melanogaster*, *D. yakuba*, *Drosophila ananassae*, *Drosophila pseudoobscura* and *Drosophila willistoni*) in *D. melanogaster* S2 cells, a widely used *Drosophila* cell line. These species span an evolutionary distance of 30–40 million years and a range of neutral sequence divergence equivalent to a large part of the vertebrate phylogeny^{14,15}. Notably, by determining the enhancer activities for the genomes of all five species in a single cell type, we can ensure that differences in activity are due to sequence changes (in *cis*) rather than changes in the cellular *trans*-regulatory environment¹⁶. Excluding such *trans* effects was also a main goal in previous studies that assessed differences in gene expression or regulator binding and chromatin marks (for example, see refs. ^{17,18}). We mapped the reads from paired-end sequencing of the input and STARR-seq samples to the genome of each species and translated them to *D. melanogaster* coordinates for further analysis (Fig. 1a). For each of the species, STARR-seq results were highly reproducible, with Pearson correlation coefficients (PCCs) of >0.9 between biological replicates (Supplementary Figs. 1 and 2).

We will refer to enhancer activity that is shared by orthologous sequences as ‘functional conservation’, independent of the similarity of the sequences (that is, independent of ‘sequence conservation’). This definition can, for example, account for the conservation of enhancer function by dissimilar sequences, which has been observed frequently for enhancers (for examples, see refs. ^{19–21}). We found that the 2,325 *D. melanogaster* enhancers (enrichment of 3-fold and P value = 0.001; false discovery rate (FDR) < 0.1%; Supplementary Table 1 and Supplementary Data Set 1) were highly functionally conserved (enrichment P = 0.001; Fig. 1b): 58% of the *D. melanogaster* enhancers were functionally conserved in *D. yakuba* and 46% were conserved in *D. ananassae* and *D. pseudoobscura*, which are at evolutionary distances from *D. melanogaster* comparable to the distances between human and mouse and between human and chicken, respectively¹⁴. Even in *D. willistoni*, which is evolutionarily more distant from *D. melanogaster* than lizard is from human, 34% of the *D. melanogaster* enhancers were functionally conserved (compared to 4% of control sequences; Fig. 1c). Using more sensitive settings that took all detectable enhancer activities (enrichment P = 0.05) into account²², between 42% (*D. willistoni*) and 70% (*D. yakuba*) of the *D. melanogaster* enhancers were functional across species (Fig. 1c).

Overall, the conservation rate was strongly correlated with evolutionary distance as measured by the number of substitutions at neutral sites¹⁴, suggesting that the evolution of enhancer function might follow a molecular clock, with the activity of about 4% of the enhancers being lost every 10 million years (Fig. 1d).

STARR-seq measures enhancer activity for sequences that in their endogenous genomic context can be ‘open’ (located in DNase I-hypersensitive regions) or ‘closed’ (ref. 13). Whereas closed enhancers function in reporter assays and are specifically labeled by the general enhancer mark monomethylation of histone H3 at lysine 4 (H3K4me1), they are not accessible to DNase I and lie in broad regions of repressive trimethylation at lysine 27 of histone H3 (H3K27me3), suggesting that their genomic loci are silenced at the chromatin level¹³. We wanted to compare the functional conservation of open and closed enhancers—enhancers that *in vivo* are likely active or silenced, respectively. Interestingly, whereas open and closed enhancers were about equally well reproducible in independent biological replicates, closed enhancers were functionally conserved only about half as frequently (Supplementary Fig. 3). This finding suggests that the activity of open enhancers in S2 cells is preserved during evolution, whereas the silenced endogenous state of closed enhancers presumably means that their enhancer function in S2 cells cannot be efficiently selected for (even if the sequences maintained other putative functions). This hypothesis argues that the frequently observed conservation of enhancer function over large evolutionary distances (for examples, see refs. ^{19,20,23,24}) likely stems from specific stabilizing selection, presumably acting to maintain functional sequence elements.

Extending enhancer conservation to gene loci

In addition to positional conservation of enhancers at orthologous positions, we also found examples of apparent enhancer activity turnover and compensatory activity changes within specific gene loci. For example, the *D. melanogaster pyramus* (*pyr*) gene contained an enhancer in its first intron ~3.3 kb downstream of the transcription start site (TSS). In

contrast, the orthologous gene locus in all other species contained an intergenic enhancer ~1.5 kb upstream of the TSS, while the *D. yakuba* sequence orthologous to the functional *D. melanogaster* enhancer was only weakly active in S2 cells and the sequences for the other species were entirely inactive (Fig. 2a). Overall, the fraction of enhancers that were shared by orthologous gene loci and might contribute to gene expression yet were not positionally aligned increased from ~22% in *D. yakuba* to ~46% in *D. willistoni* (Fig. 2b and Supplementary Fig. 4). The enhancers that appeared to functionally complement each other had similar motif compositions, as expected for enhancers with similar or equivalent functions (Fig. 2c). This finding suggests that enhancer turnover and compensatory changes are common and substantially contribute to the evolution of transcriptional regulation, consistent with reports based on individual gene loci, transcription factor binding and enhancer-associated chromatin features^{9–12,25}.

Specific selection of transcription factor sequence motifs

Consistent with previous studies on individual enhancers^{19–21}, pairs of orthologous enhancers that were equally strong (<1.5-fold difference in activity) showed no substantial increase in overall sequence similarity compared to enhancers with diverged (lost) function (4–6% difference in sequence identity between medians) (Fig. 3a). In contrast, when considering only positions that corresponded to binding motifs for Serpent (Srp), a transcription factor that is important for S2 cell morphology and growth²⁶, differences were up to twofold greater between the two classes of enhancers (6–12% difference in sequence identity; Fig. 3b), a substantial difference that we did not observe for binding motifs for the Buttonhead (Btd) transcription factor, which is not expressed in S2 cells (Supplementary Fig. 5). This finding links functional conservation of enhancer activity to the sequence conservation of transcription factor motifs rather than the overall enhancer and suggests a means to identify transcription factor motifs that are important in S2 cells. When we scored all known transcription factor motifs by their increased conservation in functionally conserved enhancers, motifs for mesodermal transcription factors such as Srp and Mef2 scored highly, suggesting that transcription factors with highly scoring motifs might be active in S2 cells. Indeed, eight of the ten highest scoring motifs corresponded to transcription factors that are expressed in S2 cells (reads per kilobase per million (RPKM) > 1; Fig. 3c and Supplementary Table 2). Overall, such increased motif conservation was observed frequently for transcription factors expressed in S2 cells (RPKM > 1) but not for non-expressed transcription factors or shuffled control motifs (> 2.9-fold difference; Fig. 3d).

Compensatory changes stabilize regulatory output

In addition to conservation of transcription factor motifs at orthologous (aligned) positions, enhancers showed substantial motif turnover, and motif losses were compensated by motif gains at non-orthologous positions (for an example, see ref. 21). The fraction of compensatory motifs among all motifs shared by orthologous enhancers with equal strengths increased from ~4% between *D. melanogaster* and *D. yakuba* to 32% between *D. melanogaster* and *D. willistoni* (Fig. 4a) and substantially contributed to maintaining a similar number of motifs (Fig. 4a,b and Supplementary Fig. 6). Indeed, 48% of all enhancers that had an identical number of Srp motifs in *D. melanogaster* and *D. willistoni* showed motif turnover and the presence of compensatory motifs. The fractions of compensatory

motifs were consistent with estimates based on individual enhancer loci, genome sequence alignments and/or transcription factor binding across species (for examples, see refs. ^{9,10,12,27,28}).

We experimentally assessed the role of motif turnover and compensatory motifs for the maintenance of enhancer function during evolution using hybrid enhancers^{21,28}. For this analysis, we fused one-half of a *D. melanogaster* enhancer to the other half of the orthologous enhancer from another *Drosophila* species and vice versa, joining the two sequences seamlessly via sequence stretches that were identical in the two species (Fig. 4c–e). If all functionally relevant sequence features were exclusively conserved at orthologous positions, wild-type enhancers and both hybrid enhancers should all have the same activity. In contrast, the hybrid enhancers would have different activities if motifs that were lost from one half of an enhancer were compensated for by motifs gained in the other half^{21,28}. Indeed, for all three *D. melanogaster* enhancers and their orthologous enhancers from *D. yakuba*, *D. ananassae* or *D. pseudoobscura*, respectively, we observed that the hybrid enhancers had very different activities in luciferase assays, in contrast to the wild-type enhancers for which the activities were similar (Fig. 4c–e). This finding illustrates the key role of compensatory motif turnover in the functional conservation of enhancer activity, even at close evolutionary distances.

Frequent enhancer gains between closely related species

We next defined enhancers in each species separately by thresholding the respective STARR-seq enrichment values (enrichment ≥ 3 -fold and $P \leq 0.001$; FDR $< 0.1\%$). This analysis identified a comparable number of enhancers in each species (*D. melanogaster*, 2,325; *D. yakuba*, 2,293; *D. ananassae*, 2,096; *D. pseudoobscura*, 3,469; *D. willistoni*, 2,860; Supplementary Table 1 and Supplementary Data Set 1) with a similar genomic distribution (Supplementary Fig. 2). We mapped the binary activity status (0 or 1) of each of the 8,180 non-redundant enhancer regions found in at least 1 species to the phylogenetic tree for these species (Supplementary Fig. 7a) and inferred evolutionary enhancer gain and loss events by parsimony (Online Methods and Supplementary Fig. 7b). This analysis identified a substantial number of enhancer gains in each of the species, especially in the evolutionarily distant *D. pseudoobscura* (1,248 gains) and *D. willistoni* (1,216 gains) (Fig. 5a,b).

Even since the recent split between *D. melanogaster* and *D. yakuba* about 11 million years ago, 525 and 472 enhancers were gained in these species, respectively (Fig. 5c), for which the orthologous sequences in all other species were not active. Of the enhancers gained in *D. melanogaster*, 140 were next to genes with other, evolutionarily older enhancers, 6 appeared to compensate for the *D. melanogaster*-specific loss of an ancestral enhancer, and 275 were the only enhancers in the vicinity of 261 genes (Fig. 5d). The majority of the gained enhancers in *D. melanogaster* and *D. yakuba* (70%; 365/525 and 331/472, respectively) arose *de novo* from non-functional sequences and did not constitute relative gains in enhancer activity between species, as none of the orthologous sequences were detectably active, even at a more sensitive cutoff ($P \leq 0.05$). The same was true for enhancers gained in *D. pseudoobscura* and *D. willistoni* (919/1,248 = 74% and 903/1,216 = 74% *de novo* gains,

respectively). This finding excludes the possibility that enhancer gains arose from thresholding issues²² and were more quantitative in nature, potentially related to changes in the *trans*-regulatory environments of the respective species *in vivo* (for example, see ref. 29).

Enhancers gained in *D. melanogaster* had 113 nucleotide differences on average (along the 501-bp enhancer regions; 22.5%) compared to their inactive orthologous sequences in *D. yakuba*. Similarly, *D. yakuba*-specific enhancers differed from their orthologous sequences in *D. melanogaster* by 120 nucleotides (24%) on average (Fig. 5e and Supplementary Fig. 8). These numbers are comparable to the frequency of sequence mutations at fourfold-degenerate sites in aligned protein-coding regions¹⁴. For comparison, there was a difference of 80 nucleotides (16%) for enhancers that functioned in all five species and a difference of 85 nucleotides (17%) for enhancers that lost activity specifically in *D. melanogaster* (84 nucleotides for losses in *D. yakuba*). These observations suggest that hundreds of sequences can gain enhancer activity during only about 10 million years of evolution. More than half of all enhancers gained in *D. melanogaster* (265 or 50.5%) were next to genes that are expressed in S2 cells, as determined by RNA sequencing (RNA-seq), and might thus contribute to the expression of these genes. This was especially true for the 149 gained enhancers that were the only enhancers in the vicinity of 143 expressed genes, including *Snx6*, *Hira* and *roq* (Supplementary Fig. 9).

Evolution of OSC enhancers and *in vivo* gene expression

To study the relationship between the evolution of enhancers and gene expression, we chose ovarian somatic cells (OSCs), which have only recently been derived from adult ovaries and for which a close *in vivo* counterpart (ovarian follicle cells) exists³⁰. OSCs retained marker gene expression and other functional aspects³⁰, including the function of OSC enhancers *in vivo*¹³.

We performed STARR-seq in *D. melanogaster* OSCs with genomic DNA from *D. melanogaster*, *D. yakuba* and *D. ananassae*, which we treated as an outgroup in the identification of enhancer gains in *D. melanogaster* and *D. yakuba* (Supplementary Figs. 10 and 11a–c, Supplementary Table 1 and Supplementary Data Set 1). Similar to the results in S2 cells, this analysis identified a high degree of functional conservation for *D. melanogaster* OSC enhancers, ranging from 69% in *D. yakuba* to 46% in *D. ananassae* (Fig. 6a), and confirmed the substantial difference in functional conservation for open and closed enhancers (Supplementary Fig. 11d,e). We found that 1,012 enhancers were specific to *D. melanogaster*, a number comparable to the 890 *D. melanogaster*-specific enhancers we identified in S2 cells when we evaluated S2 cell data across the same three species.

Next, we performed RNA-seq on ovarian follicle cells³¹ isolated from *D. melanogaster* and *D. yakuba* adult females (Supplementary Tables 3 and 4). Gene expression levels were very similar in both species (PCC = 0.89), consistent with the high conservation of gene expression reported for fly embryos³² and mammals¹¹.

The *CG1620* locus contains a *D. melanogaster*-specific OSC enhancer gain, which increased the overall enhancer activity of the gene locus in OSCs by 1.6-fold in *D.*

melanogaster compared to *D. yakuba*, matching the 1.6-fold increase in expression of *CG1620* in *D. melanogaster* compared to *D. yakuba* follicle cells as measured by RNA-seq (Fig. 6b). Overall, the expression of 38 genes with enhancer activities that were at least 2-fold higher in *D. melanogaster* was also upregulated by at least 2-fold in *D. melanogaster*, and the expression of 16 genes was even upregulated by 4-fold or more, significantly more than expected (binomial $P = 0.032$ and 0.002 , respectively). Moreover, differences in OSC enhancer activity and *in vivo* gene expression levels between *D. melanogaster* and *D. yakuba* follicle cells agreed on average across a wide range of cutoffs (Fig. 6c and Supplementary Fig. 12), suggesting that the differences in enhancer activity we observed can more generally cause differences in gene expression between closely related species.

We also observed clear examples of compensatory enhancer evolution around several genes, including *jumeau* (*jumu*). In the *jumu* locus, a *D. melanogaster*-specific enhancer and a *D. yakuba*-specific enhancer appeared to compensate for each other to balance the overall enhancer activity of the locus in OSCs (Fig. 6d), in agreement with the gene expression levels for *jumu* in follicle cells of both species. Overall, 291 of 492 gene loci (59%) with unchanged enhancer activity in OSCs (<1.5-fold difference) showed unchanged gene expression (<1.5-fold difference) in follicle cells (for 401 genes (82%), expression changed by <2.0-fold). Among these gene loci, 21% (61/291) showed compensatory changes, with individual enhancer activities differing by more than 4-fold between the 2 species (Fig. 6c and Supplementary Fig. 12).

These results suggest that the enhancer gains and quantitative differences in enhancer activity we observed using STARR-seq can underlie differences in gene expression between species *in vivo*. They also emphasize the relevance of compensatory enhancer evolution for the stabilization of gene expression levels that we and others^{11,32} find to be highly conserved.

DISCUSSION

We assessed the extent of functional enhancer conservation across an entire animal genome, identifying frequent and deep functional conservation. Enhancer strength evolves according to a molecular clock, with quantitative changes linearly related to sequence divergence (Supplementary Fig. 13) but with largely overlapping distributions of sequence identity for functionally conserved or divergent enhancers, as estimated across the genome (Supplementary Fig. 14).

To our surprise, even within only 10 million years of evolution, hundreds of non-functional sequences appeared to have acquired enhancer activity. This *de novo* emergence of function can be attributed solely to *cis*-regulatory mutations, as we assayed all enhancer activities in the constant *trans*-regulatory environment of individual cell types. This situation might resemble the initial step of regulatory evolution, before cellular *trans*-regulatory environments change more globally¹⁶.

Interestingly, the frequencies of sequence changes between gained enhancers and non-functional sequences were similar to those at fourfold-degenerate sites¹⁴ (Fig. 4e and

Supplementary Figs. 8 and 11f,g), which are often used to estimate mutation frequencies for neutrally evolving sequences^{14,15} (also note, however, ref. 33). Even though individual transcription factor binding sites could be under selection that is not apparent when comparing longer sequences, it is interesting to speculate that neutrally evolving sequences might frequently gain enhancer activity, a hypothesis consistent with the observation that even random DNA sequences can be active in enhancer assays³⁴. As such gains can influence gene expression (Fig. 6), our combined results suggest how randomly occurring sequence changes might create variability in gene expression and potentially phenotype, which in turn might be selected for during evolution and ultimately lead to differences between species¹.

The high frequency of enhancer gains in single cell types and their near additivity across the two cell types studied (Supplementary Fig. 15) suggest that, across the many cell types of an animal, hundreds to thousands of enhancer activities might arise within short evolutionary timespans. These numbers estimated in insect genomes are likely substantially higher in the much larger mammalian genomes. With their ability to alter gene expression and potentially influence cellular functions and phenotypes even in closely related species, *cis*-regulatory mutations and changes in enhancer activities should be powerful drivers of evolution¹.

METHODS

Methods and any associated references are available in the online version of the paper.

ONLINE METHODS

STARR-seq screens

STARR-seq was performed in two biological replicates (independent transfections) per species as described previously¹³, with the following exceptions. (i) Input DNA for library cloning for the respective species-specific screens was the genomic DNA of *D. yakuba*, *D. ananassae*, *D. pseudoobscura* and *D. willistoni*, which we obtained from the *Drosophila* Species Stock Center (DSSC) at the University of California, San Diego (for *D. melanogaster*, we used the data from ref. 13). (ii) We transfected 1.6×10^9 S2 cells and OSCs per biological replicate. (iii) First-strand cDNA synthesis was performed in 30–60 reactions with the STARR-seq RT primer (Supplementary Table 5). (iv) Illumina sequencing was performed on an Illumina HiSeq 2000 machine using multiplexing according to the manufacturer's instructions. The STARR-seq library cloning construct is available subject to a Material Transfer Agreement (MTA).

Luciferase reporter assays

Luciferase assays were conducted as described previously¹³, and constructs are available subject to an MTA.

Hybrid enhancer constructs for luciferase reporter assays

We selected three pairs of orthologous enhancers from *D. melanogaster* and *D. yakuba*, *D. ananassae* or *D. pseudoobscura* that had comparable pairwise enhancer activities and a central stretch of identical sequence (IDS) that allowed a seamless transition from the

sequence of one species (first half of the hybrid) to the sequence for the other species (second half of the hybrid). For each such pair, we cloned the two orthologous wild-type sequences and two complementary hybrid constructs of the forms half 1 (species 1)–IDS–half 2 (species 2) and half 1 (species 2)–IDS–half 2 (species 1), also illustrated in Figure 4c–e. Fusion of the two halves was carried out by Gibson assembly³⁵, using NEB Gibson Assembly Master Mix (E2611S) according to the manufacturer's instructions. Each of the halves was amplified by PCR (incubation at 98 °C for 45 s followed by 35 cycles of 98 °C for 15 s, 65 °C for 30 s and 72 °C for 30 s) with 50 ng of genomic DNA for the respective species as template, using KAPA Hifi Hot-Start Ready Mix (KAPA Biosystems, KK2602). The homology arms needed for Gibson assembly were introduced by PCR with appropriate PCR primers (Supplementary Table 5): to each half 1, a 20-nt extension homologous to the 5' end of half 2 of the orthologous enhancer was added at the 3' end, and, to each half 2, a 20-nt extension homologous to the 3' end of half 1 of the orthologous enhancer was added at the 5' end. Wild-type sequences were PCR amplified using the forward primer for half 1 and the reverse primer for half 2 from the same species as described above, with the exception that a longer elongation time was used (72 °C for 60 s). Luciferase vector construction for hybrid and wild-type PCR products was carried out as described previously¹³.

RNA-seq from enriched *Drosophila* follicle cells

Samples highly enriched in follicle cells from *D. melanogaster* (OregonR) and *D. yakuba* (WT Liberia; obtained from DSSC) were prepared as described previously³¹. Fly strains were maintained under standard fly culture procedures, but *D. yakuba* were given a moist substrate in bottles to facilitate pupation. PolyA-selected mRNA was subjected to a custom mRNA-seq library construction method as described in ref. 36 and sequenced on an Illumina HiSeq 2000 machine as 50-bp single-end reads.

STARR-seq read mapping

Paired-end STARR-seq and input reads from all STARR-seq screens for the *Drosophila* species (*D. yakuba*, *D. ananassae*, *D. pseudoobscura* and *D. willistoni*) were trimmed to 36 bp and mapped to the respective genome assembly (droYak2, droAna3, dp4 and droWil1) using Bowtie³⁷ 0.12.9 as in ref. 13. Paired-end STARR-seq and input reads for *D. melanogaster* were obtained from our previous screens¹³ and processed the same way. As the trimmed forward and reverse reads correspond to the 5' and 3' ends of the respective DNA fragments, their respective genomic locations indicate the identity and length of each fragment (about 500 bp on average). We collapsed the mapped paired-end data into unique fragments (on the basis of identical chromosome, start, end and strand information) to remove biases from PCR duplicates as before¹³. We additionally introduced a heuristic approach (redundancy filter) to remove clustered paired-end reads that manual inspection showed were due to sequence artifacts such as homopolymer runs. For this approach, we grouped paired-end reads that had identical start positions for the forward read into clusters. For each of these clusters, we retained the longest paired-end read and discarded paired-end reads within the same cluster for which the reverse reads had 2 sequence differences within read positions 2–11. We then repeated the same filtering for groups defined on the basis of identical end positions for the reverse read.

Translating fragment coordinates to the dm3 assembly

To have all data in common reference genome coordinates, the genomic coordinates for all fragments obtained from mapping the paired-end data to the genomes of the respective species (paired-end sequencing allows the determination of identity and length for each fragment) were translated into dm3 coordinates using the UCSC liftOver tool³⁸. Only fragments that were uniquely liftable to dm3 and that had a final lifted length of 10–200% of the original fragment length were considered for further analysis. Depending on the species and deep sequencing library, between 70 and 93% of all fragments could be lifted to dm3 (Supplementary Table 1).

Density profiles of translated fragments

Density profiles of fragments translated to the dm3 assembly were computed with the BEDTools suite³⁹. All profiles were normalized to 1 million mapped fragments (FPM, fragments per million).

Enhancer peak calling and false discovery rate

We called peaks from the STARR-seq data as before¹³ with the following cutoff settings: P 0.001 and 3-fold enrichment over input. FDRs for STARR-seq peaks were computed as in ref. 13.

Assignment of enhancer peaks to genes

By default, peaks were uniquely assigned to the gene with the closest TSS to the peak summit. For gene-centric analyses (for example, compensatory peak changes), we assigned all peaks to extended gene loci. We defined gene loci as regions between 15 kb upstream of annotated genes to at most 5 kb downstream but not across neighboring genes. Two genes could share the same intergenic region, but overlapping genes and genes that were fully contained within another gene were excluded. All peaks that were located within these unique gene loci were assigned to the respective genes. Peaks that were assigned to two different genes (for example, overlapping intergenic regions) were reassigned to the gene with the closest TSS, resulting in unique peak-to-gene-locus assignment.

Genomic distributions of enhancers

Enhancer summit positions for peak calls in each of the five species were intersected with a set of uniquely assigned genomic regions (for example, coding sequences and introns) on the basis of *D. melanogaster* genome annotation from FlyBase r5.31 (ref. 40). Both the fraction of enhancer summits distributed over the genomic regions and enrichment or depletion over the expectation based on region size were computed.

Heat maps of enhancer enrichment

Heat maps were based on normalized read density profiles from the STARR-seq screen for each of the five species and were centered on enhancer summit positions. As exact positions could vary for conserved enhancers in the different species, the summit position in the first species along with the phylogenetic tree starting with *D. melanogaster* was kept.

Conservation rates of enhancers across replicates and species

Conservation rates of enhancers (STARR-seq peaks)—either between replicates or species—were computed by evaluating STARR-seq enrichments at summit positions (independent of the fixed lengths of the delineated enhancers). We called enhancers in a first set ‘conserved’ in a second set if STARR-seq enrichment in the second set was significant with hypergeometric $P = 0.001$ (or $P = 0.05$ for relaxed settings, as indicated in the text and figure legends). Conservation rates within one species were based on two biological replicates and evaluated replicate 1 versus replicate 2 and vice versa. The rates of conserved peaks with regard to *D. melanogaster* peaks were based on the combined *D. melanogaster* replicates evaluated separately against replicate 1 and 2 in each *Drosophila* species. As controls, we randomly distributed *D. melanogaster* replicate 1 peaks across the genome (shifted their coordinates to random genomic positions) while preserving the overall peak number in each of the different genomic regions (for example, introns and intergenic regions). We then assessed conservation against the original *D. melanogaster* replicate 1 peaks with the protocol above and the same respective P -value cutoffs (0.001 and 0.05). We repeated the same procedure for the *D. melanogaster* replicate 2 peaks. We also assessed all conservation rates for open and closed *D. melanogaster* enhancers in S2 cells and OSCs separately, using the definition of open versus closed enhancers from ref. 13.

Alignments of enhancer regions

Alignments of all enhancer sequences in the five species were extracted from Multiz 15-way insect alignments obtained from UCSC^{14,15,41}. Some analysis required a stringent set of well-aligned sequences. For such analysis, we considered only regions for which the alignments had no undefined nucleotides (Ns) and had orthologous non-gapped 5' and 3' ends.

Motif analysis and compensatory motif changes

We extracted alignments of enhancer regions and used a position weight matrix (PWM) for the transcription factors Srp⁴² and Btd⁴³ to scan for occurrences within each of the sequences independently using MAST⁴⁴. We used a PWM matching cutoff of $4^{-5} = 9.8 \times 10^{-4}$. Coordinates of Srp matches within individual sequences of the alignment were translated into alignment coordinates to differentiate between positionally conserved and compensatory motifs. To test for transcription factor motifs that showed preferential conservation in functionally conserved enhancers, we assessed the fraction of motif matches that were conserved at orthologous genomic positions across all five species (according to UCSC whole-genome alignments) in functionally fully conserved enhancers versus *D. melanogaster*-specific enhancers for all *Drosophila* transcription factor motifs from ref. 45. For each transcription factor motif, we calculated the increased conservation rate (fold difference corrected to the 95% confidence interval) and binomial P values, which we converted to FDRs to correct for multiple testing. Control motifs were generated by shuffling the columns (motif positions) for the motif PWMs. We also counted the fraction of motifs that showed significant preferential conservation in functionally conserved enhancers ($P = 0.01$) for transcription factors that are expressed in S2 cells (RPKM > 1) and

transcription factors that are not expressed in S2 cells and for the respective shuffled control motifs.

Sequence conservation of enhancers and motifs

To estimate pairwise sequence identity between orthologous STARR-seq enhancers, pairwise alignments of the *D. melanogaster* enhancer sequence with the sequence for the other species were extracted from a whole-genome multispecies alignment obtained from UCSC. Conservation was computed as the fraction of identical nucleotides over the length of the *D. melanogaster* enhancer sequence or only over Srp and Btd motif matches within the enhancer sequence.

Compensatory enhancer changes

To compute positional and compensatory enhancer conservation, unique gene loci were defined and the number of all enhancers falling into these regions was determined in each of the five species independently. Each *D. melanogaster* enhancer (3-fold enrichment and $P < 0.001$) was then uniquely assigned to a single enhancer for a *Drosophila* species ($P < 0.001$) in a 1:1 manner to prevent double counting. Afterward, enhancers were classified as positionally conserved if the distance to the peak summit was less than 250 bp. Enhancers that overlapped the same gene locus but that were not positionally conserved were defined as showing compensatory conservation.

Motif similarity of compensatory enhancers

For *D. melanogaster* and each of the other species in turn, we identified putatively compensatory enhancer pairs as two enhancers assigned to the same gene, one of which was found only in *D. melanogaster* and the other of which was found only in the other species (*D. yakuba*, *D. ananassae*, *D. pseudoobscura* or *D. willistoni*). We further selected active enhancers with aligned orthologous (yet inactive according to STARR-seq) sequences that allowed unique read mapping by requiring that they had 100% input coverage. This excluded alternative explanations such as alignment gaps or errors and ensured that the orthologously aligned sequence was present yet inactive, presumably owing to specific sequence changes. For *D. melanogaster* and each of the other species in turn, we then computed motif enrichment vectors for each pair of active sequences and the corresponding orthologous inactive sequences, using the transcription factor motifs that were informative for S2 enhancers (enriched at $P = 0.05$), and compared the concatenated vectors by PCC.

Non-redundant enhancer loci

Enhancer loci for all five species were combined to create a set of non-overlapping (non-redundant) enhancer loci. First, we called enhancers in each species as regions with significantly enriched STARR-seq signal ($P = 0.001$). We then intersected the enhancer loci for all species and kept only loci that had a strong enhancer ($P = 0.001$ and 3-fold enrichment) in at least one species. As summit positions for these non-redundant enhancers, we used the summit of each isolated peak and the summit of the peak in the species closest to *D. melanogaster* for each group of mutually overlapping peaks. We then assessed STARR-seq enrichment at these non-redundant peak summit positions or, for overlapping

peaks, at the peak summit position in the respective species. This approach allowed a balancing out of variation in the positions of maximal enhancer enrichment.

Enhancer gains and losses

Using the combined data set of non-redundant enhancer loci, putative gain and loss events were mapped to the phylogenetic tree (Supplementary Fig. 7). Enhancers that were exclusively found in only one species were classified as confident gain events (Fig. 5c and Supplementary Fig. 7). For analysis only, *D. melanogaster* or *D. yakuba* were taken into account. Confident loss events, as shown in Figure 5c, required the enhancer to be conserved in four of five species (either not in *D. melanogaster* or *D. yakuba*).

Follicle cell RNA-seq data analysis

We mapped the RNA-seq reads for *D. melanogaster* and *D. yakuba* follicle cells to respective genomic sequences using Bowtie and translated the droYak2 genomic coordinates into dm3 coordinates so that the analysis could benefit from the quality of the *D. melanogaster* genome annotations. To assess gene expression for both species, we intersected read coordinates with the coordinates of FlyBase gene models (FlyBase version FB2010_08 (dmel_r5.31)) and calculated *D. melanogaster* and *D. yakuba* RPKM values for each gene from the gene's combined exon length, the number of reads mapping to these exons and the total number of reads per species.

Correlation between enhancer activity and gene expression

We assigned the OSC enhancers to unique, non-overlapping gene loci and computed the sum of enhancer activities (STARR-seq enrichments) for each gene locus for each gene in *D. melanogaster* and *D. yakuba*. We then compared the changes in enhancer activity between *D. melanogaster* and *D. yakuba* with the changes in the RPKM expression values in *D. melanogaster* and *D. yakuba* follicle cells for genes that had an enhancer in at least one of the species, were expressed in at least one of the species (not necessarily the same species; i.e., these two filters were applied independently to avoid an ascertainment bias) and were expressed in OSCs. We then counted the number of genes with different changes in enhancer activity and gene expression between *D. melanogaster* and *D. yakuba* at cutoffs between 1.5-fold and 4-fold. We finally assessed the relative enrichment (fold enrichment and binomial *P* value) of genes in each enhancer activity and gene expression category by comparing genes with higher expression in *D. melanogaster* to those with higher expression in *D. yakuba* at each cutoff.

Gene expression levels in S2 cells and OSCs

We used the gene expression levels in *D. melanogaster* S2 cells and OSCs determined by RNA-seq in ref. 13.

Computations on genomic features and coordinate intersection

All computational analysis and intersections of genomic features or coordinates were performed with the BEDTools suite³⁹.

Statistics

All statistical analyses were performed using R⁴⁶.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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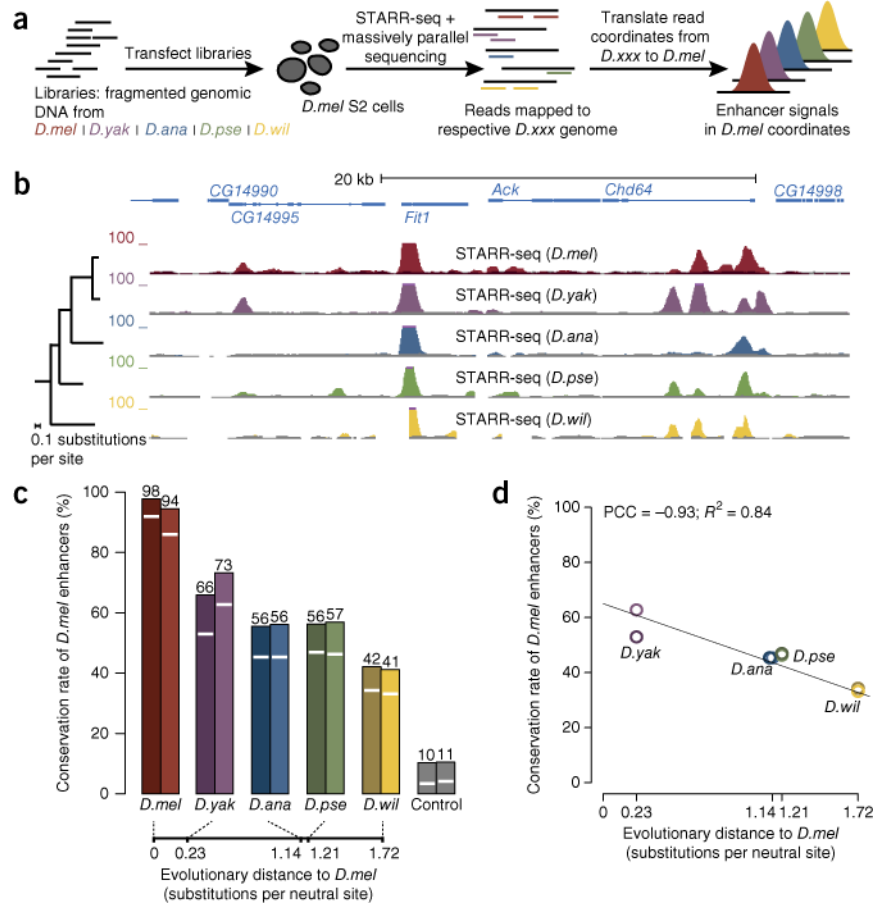


Figure 1.

Functional conservation of *D. melanogaster* S2 cell enhancers. **(a)** Schematic overview of STARR-seq enhancer screens for the genomes from different *Drosophila* species (*D.mel*, *D. melanogaster*; *D.yak*, *D. yakuba*; *D.ana*, *D. ananassae*; *D.pse*, *D. pseudoobscura*; *D.wil*, *D. willistoni*; *D.xxx*, any *Drosophila* species) in a single cell type (here *D. melanogaster* S2 cells). **(b)** UCSC Genome Browser screenshot depicting a 30-kb genomic locus with STARR-seq tracks for each species (inputs in gray; y axes depict normalized fragment counts). **(c)** Functional conservation rates of *D. melanogaster* enhancers in the four other *Drosophila* species (white lines, $P < 0.001$; bar heights, $P < 0.05$; $n = 2,325$ enhancers). Background conservation levels were assessed using shifted genomic coordinates as controls. For *D. melanogaster*, conservation rates between biological replicates are shown ($n = 2,139$ and $2,361$ enhancers; see supplementary Fig. 1 for replicates in the other species and supplementary Fig. 3 for the separate analysis of open and closed enhancers). The evolutionary distances of each species from *D. melanogaster* are indicated (bottom). **(d)** Conservation rates from **c** (stringent cutoff of $P < 0.001$) versus the pairwise evolutionary distance of each species to *D. melanogaster* (darker and lighter colors depict replicates 1 and 2, respectively).

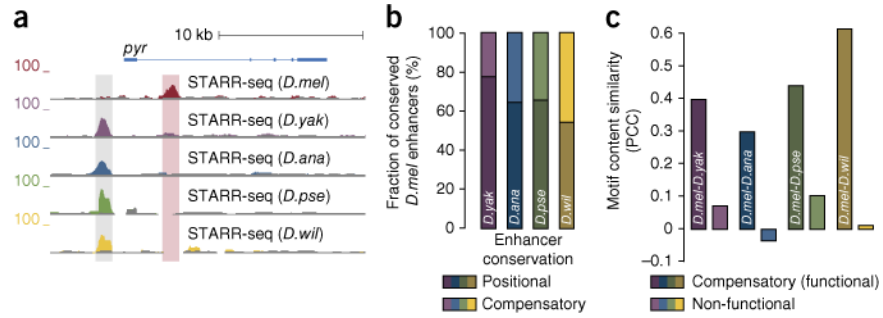


Figure 2. Compensatory enhancers stabilize total enhancer strengths for gene loci. **(a)** *D. melanogaster* lost a deeply conserved enhancer upstream of the *pyr* gene (shaded in gray) but gained an intronic enhancer in the same gene locus (shaded in red; UCSC Genome Browser screenshot; details as in Fig. 1b). **(b)** The fraction of compensatory versus positional enhancer conservation increases with evolutionary distance. Shown are the relative contributions of positional (bottom) and compensatory (top) enhancer conservation for all pairwise comparisons of *D. melanogaster* with each of the other species (for absolute contributions, see supplementary Fig. 4). **(c)** Motif similarity of compensatory enhancers. Pairs of putative compensatory enhancers have motif content that is more similar than their respective non-functional orthologous sequences (measured by PCC for motif enrichment; numbers of compensatory enhancer pairs considered (left to right): 28, 37, 74 and 69).

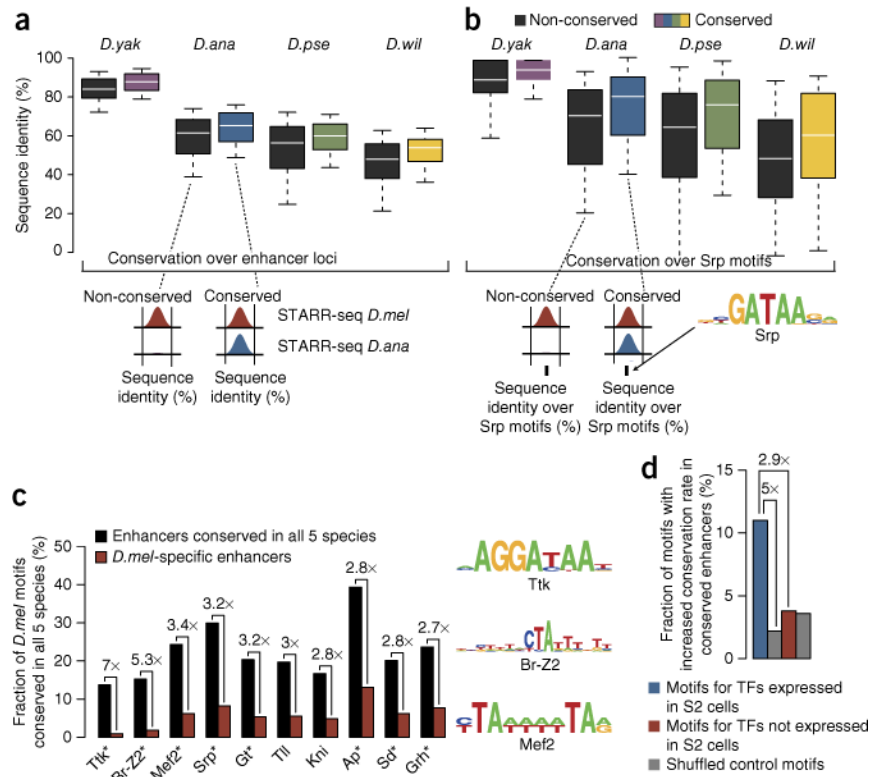


Figure 3. Motif conservation by positional sequence constraints. **(a)** Pairwise sequence identity for functionally non-conserved (black) and conserved (colored) enhancers along the entire 501-bp *D. melanogaster* enhancer sequences (boxes depict the median and interquartile range, and whiskers depict the 10th and 90th percentiles). **(b)** Sequence identity as in **a** but restricted to positions within the 501-bp enhancer sequences that match motifs for the Srp transcription factor. The number of enhancers examined was (left to right) 214, 338, 413 and 196 in **a** and 361, 216, 366 and 174 in **b**. (For equivalent analyses of 101-bp core enhancer regions and the corresponding analyses for an unrelated transcription factor motif, see supplementary Fig. 5). **(c)** Transcription factor motif conservation in functionally conserved versus non-conserved (*D. melanogaster*-specific) enhancers. Shown are the ten transcription factors for which motif conservation in functionally conserved enhancers was most strongly increased (numbers on top indicate fold increase; all increases are significant, $P < 0.05$); eight of these are expressed in S2 cells (an asterisk indicates RPKM ≥ 1 ; supplementary table 2). **(d)** Transcription factors (TFs) that are expressed in S2 cells more frequently show increased motif conservation than transcription factors that are not expressed or shuffled control motifs (cutoff $P < 0.01$; n (left to right) = 91, 91, 160 and 160 motifs).

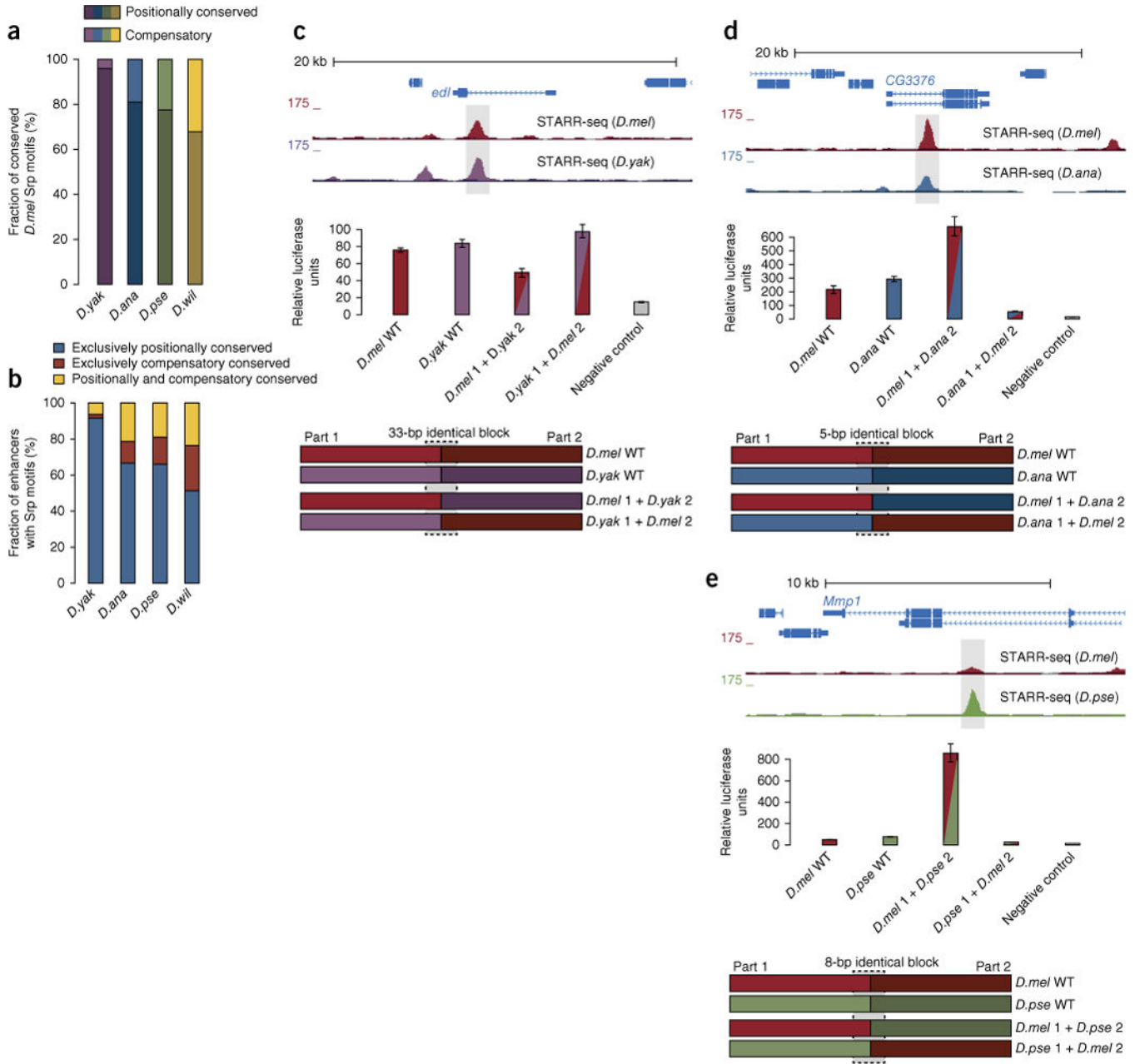


Figure 4. Compensatory motif turnover in functionally conserved enhancers. **(a)** Fraction of compensatory motif conservation (top) by turnover compared to positional motif conservation (bottom) for motifs of the Srp transcription factor. **(b)** Fraction of functionally conserved enhancers with the same number of Srp motifs between species for which all motifs are positionally conserved, all motifs are conserved within the enhancer sequence but not at identical positions (compensatory), or some motifs are positionally conserved and others are compensatory. (For absolute conservation rates, see supplementary Fig. 6.) **(c)** UCSC Genome Browser screenshot for a *D. melanogaster* enhancer in the *ETS-domain lacking (edl)* intron that is functionally conserved in *D. yakuba* (top; candidate enhancers are

shaded in gray) and corresponding wild-type and hybrid enhancer constructs (bottom; details as in Fig. 1b). Highlighted is a sequence block that is identical in both species and allowed a seamless transition between the two halves in the hybrids. Results are shown for luciferase assays of corresponding wild-type (WT) and hybrid enhancer constructs (middle). Negative control refers to a non-enhancer sequence as in ref. 13. **(d, e)** Hybrid enhancer tests as in **c** for two additional enhancers in *D. melanogaster* and *D. ananassae* (**d**) and *D. melanogaster* and *D. pseudoobscura* (**e**). All wild-type enhancers show similar STARR-seq and luciferase activities, whereas the activities of the hybrids deviate substantially from wild-type levels and from each other. Error bars show s.d. from three biological replicates; relative luciferase units were normalized to *Renilla* luciferase signal.

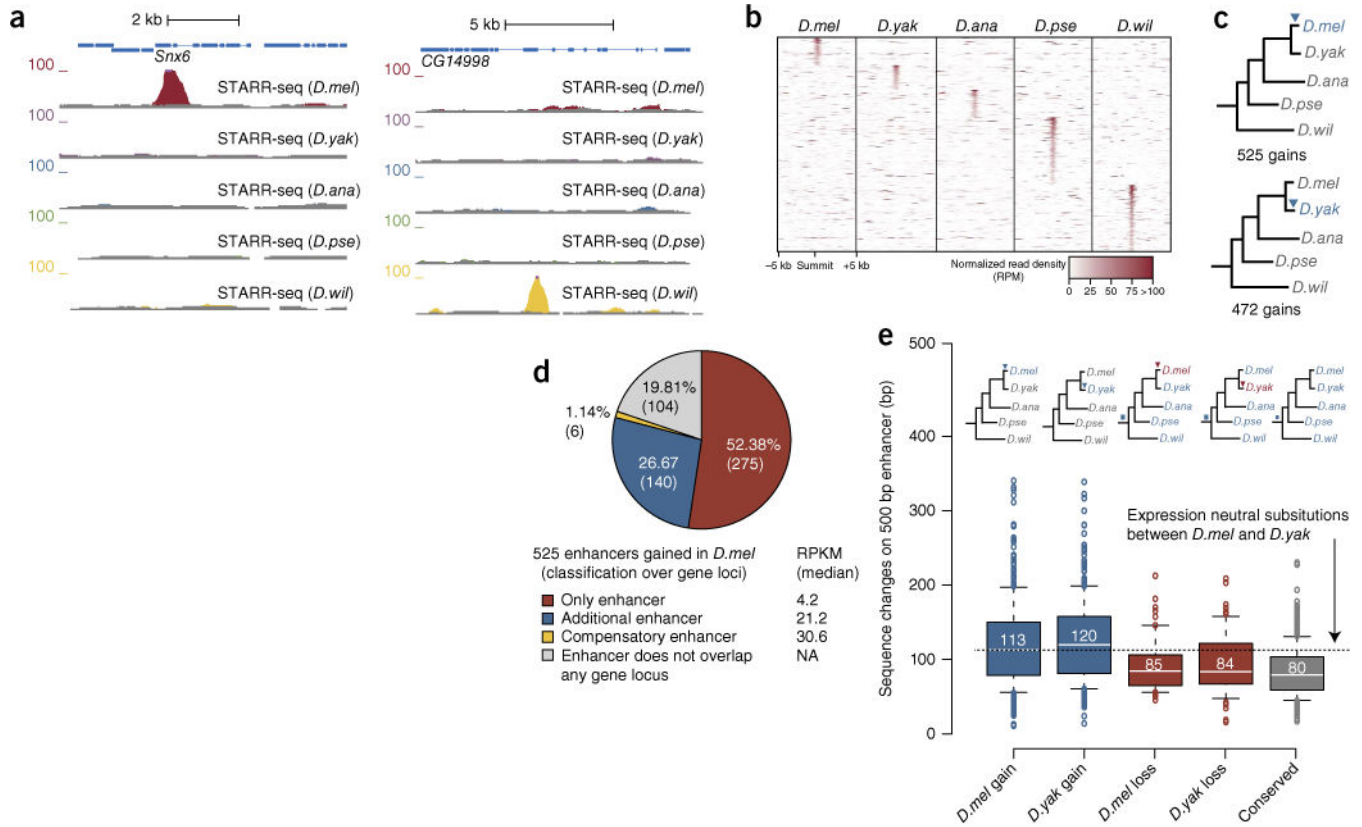


Figure 5. Species-specific gained enhancers and associated sequence changes. **(a)** UCSC Genome Browser screenshots with a gained enhancer in *D. melanogaster* (left) and *D. willistoni* (right; details as in Fig. 1b). **(b)** Heat map centered on the enhancer summit positions of species-specific enhancers that show read fragment densities at orthogonal positions across all five screened species. The distant *D. pseudoobscura* and *D. willistoni* species in particular contribute a substantial number of species-specific enhancers. RPM, reads per million. **(c)** Enhancers gained in *D. melanogaster* or *D. yakuba*. Shown are the number of gains and the branch in which the gains occurred (blue triangles on phylogenetic trees). **(d)** Classification of newly gained enhancers that are specific to *D. melanogaster*. NA, not applicable. **(e)** Sequence changes between *D. melanogaster* and *D. yakuba* across enhancers gained in *D. melanogaster* or *D. yakuba* (blue) versus ancient enhancers lost specifically in either *D. melanogaster* or *D. yakuba* (red) or deeply conserved ancient enhancers (gray). The dashed line indicates the expected number of sequence changes based on estimates from fourfold-degenerate sites in protein-coding sequences¹⁴. Boxes depict the median and interquartile range, and whiskers depict the 10th and 90th percentiles; outliers are shown individually. The number of enhancers examined (from left to right) was 525, 472, 69, 84 and 370.

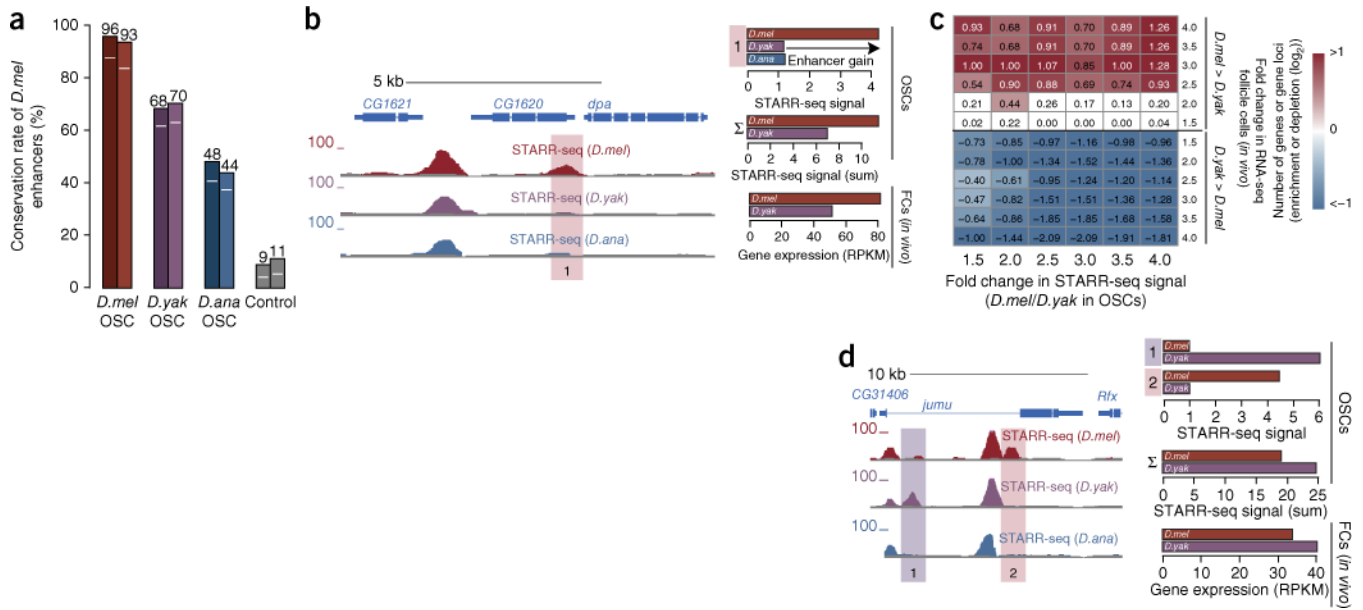


Figure 6. Evolution of enhancer activity in OSCs and gene expression in follicle cells *in vivo*. **(a)** Functional conservation rates of *D. melanogaster* OSC enhancers (as in Fig. 1c; $n = 3,342$ enhancers; $n = 3,077$ and $3,313$ enhancers for *D. melanogaster* replicates; see also supplementary Fig. 11d,e). **(b)** A *D. melanogaster*-specific OSC enhancer gain correlates with higher gene expression in *D. melanogaster* follicle cells *in vivo*. Shown is a screenshot (details as in Fig. 1b) of the *CG1620* locus with a *D. melanogaster*-specific enhancer gain (1; orange) and bar plots that display OSC enhancer activity (STARR-seq signal) at the position of the gained enhancer (top), the sum of OSC enhancer activities for the *CG1620* gene locus (middle) and the expression of *CG1620* in *D. melanogaster* and *D. yakuba* follicle cells (FCs) *in vivo* (bottom). **(c)** Changes in OSC enhancer activities and follicle cell *in vivo* gene expression between *D. melanogaster* and *D. yakuba* correlate globally. Genes that are more highly expressed in *D. melanogaster* (toward the top) also have higher OSC enhancer activities in *D. melanogaster* on average (their enrichment among genes with 1.5- to 4-fold higher enhancer activities in *D. melanogaster* is coded in shades of red). In contrast, genes that are more highly expressed in *D. yakuba* (toward the bottom) are depleted among genes with high *D. melanogaster* OSC enhancer activities (shaded in blue). Fold-enrichment values (\log_2) are depicted in each matrix cell, and cells with enrichments that are not significant (binomial $P > 0.05$) are set to white (supplementary Fig. 12). **(d)** A pair of compensatory OSC enhancers in *D. melanogaster* and *D. yakuba* stabilizes total enhancer activity, in agreement with similar *in vivo* gene expression in both species (screenshot as in **b**). The activities of OSC enhancers in the *jumy* locus (purple and red shading) change substantially between *D. melanogaster* and *D. yakuba* (top), but total OSC enhancer activity for the orthologous loci is balanced (middle), which agrees with similar *jumy* expression levels in *D. melanogaster* and *D. yakuba* follicle cells *in vivo* (bottom).