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Constraints on the evolution of a *doublesex* target gene arising from doublesex's pleiotropic deployment

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"Regulatory evolution," that is, changes in a gene's expression pattern through changes at its regulatory sequence, rather than changes at the coding sequence of the gene or changes of the upstream transcription factors, has been increasingly recognized as a pervasive evolution mechanism. Many somatic sexually dimorphic features of *Drosophila melanogaster* are the results of gene expression regulated by the *doublesex* (*dsx*) gene, which encodes sex-specific transcription factors (DSX^F in females and DSX^M in males). Rapid changes in such sexually dimorphic features are likely a result of changes at the regulatory sequence of the target genes. We focused on the *Flavin-containing monooxygenase-2* (*Fmo-2*) gene, a likely direct *dsx* target, to elucidate how sexually dimorphic expression and its evolution are brought about. We found that *dsx* is deployed to regulate the *Fmo-2* transcription both in the midgut and in fat body cells of the spermatheca (a female-specific tissue), through a canonical DSX-binding site in the *Fmo-2* regulatory sequence. In the *melanogaster* group, *Fmo-2* transcription in the midgut has evolved rapidly, in contrast to the conserved spermathecal transcription. We identified two *cis*-regulatory modules (CRM-p and CRM-d) that direct sexually monomorphic or dimorphic *Fmo-2* transcription, respectively, in the midguts of these species. Changes of *Fmo-2* transcription in the midgut from sexually dimorphic to sexually monomorphic in some species are caused by the loss of CRM-d function, but not the loss of the canonical DSX-binding site. Thus, conferring transcriptional regulation on a CRM level allows the regulation to evolve rapidly in one tissue while evading evolutionary constraints posed by other tissues.

sex determination | transcription factors | DNA binding site | evolution

How sex-specific characteristics are generated during development, what roles they have in the biology of an organism, and how they have evolved are fundamental questions in biology. In *Drosophila melanogaster*, the molecular mechanisms leading to the sex-specific and temporally and spatially restricted deployment of the transcription factors encoded by the terminal genes in the sex-determination regulatory hierarchy, *doublesex* (*dsx*) and *fruitless* (*fru*), have been well documented (1–4). In addition, although it has been established that the FRU^M and DSX^M and DSX^F proteins control nearly all aspects of somatic sexual differentiation, including anatomical and behavioral differences, much less is known about the direct target genes through which these transcription factors act (2, 3, 5–11).

It has been increasingly recognized that evolutionary significant changes in gene expression are often a result of mutations in the regulatory sequences of genes, rather than in their coding sequences (12). Furthermore, it has been noted that evolutionary changes are most likely in the *cis*-acting regulatory elements of the downstream targets of transcription factors, rather than in the genes encoding the transcription factors (13, 14). This finding is likely because changes at the transcription factors or changes at the coding sequence of the target gene could both have deleterious pleiotropic effects (15). Indeed, it was recently shown that both *dsx* and *fru* genes are very conserved in their expression and function among *Drosophila* species (16). Therefore, although closely related *Drosophila* species often differ in their sexual dimorphisms, it is likely that such rapidly evolved traits reflect changes in the expression of downstream targets of *dsx* and *fru* in these species (17). Thus, knowing the genes that are direct targets of *dsx* and *fru* would greatly facilitate studies on the evolution of sex. However, to date there are only a few validated direct gene targets for *dsx*: *Yolk Proteins* (18, 19), *bric-à-brac 1* (20), and *desatF* (also known as *Fad2*) (21). No direct target gene has been validated for *fru*, although the binding sequences for different Fru isoforms were recently identified (22, 23). The lack of known direct targets of *dsx* and *fru* places obstacles in our path to understanding the biology and evolution of sex.

To fulfill this need, we previously identified potential direct *dsx* target genes through a genome-wide screen for DSX binding sites (24). Characterization of these candidate genes should facilitate our understanding of the molecular mechanisms and evolution of the differences between males and females. As an example, here we characterized one such candidate target gene, *flavin-containing monooxygenase 2* (*Fmo-2*), a top-ranking *dsx* direct target from our screen.

Flavin-containing monooxygenases (Fmos) are present across all phyla of eukaryotes and support diverse biological functions (25–28). Sexually dimorphic expression of the Fmos is quite often observed in vertebrates. In rabbits, *Fmo-2* is expressed at higher levels in several female tissues, including esophagus, nasal mucosa, and kidney (29). In mice, hepatic expression of *Fmo1* and *Fmo3* are higher in females and are under the regulation of sex steroids (30). The male-specific repression of *Fmo-3* expression in the livers of certain *Mus* species allows the male-biased production of trimethylamine in the urine, which serves as an olfactory cue for species-specific behavior (31). Although little is known about the in vivo function or the transcriptional regulation of *Fmo-2*, the *Drosophila Fmo-2* has been characterized in vitro for its molecular properties (32), which suggest it likely functions as a terminal gene of the sex-determination pathway.

We found that *D. melanogaster Fmo-2* expression in the midgut, specifically at the posterior part of the cardia and a small

Significance

Most sexually dimorphic features of *Drosophila melanogaster* are specified by the action of sex-specific transcription factors encoded by the *doublesex* (*dsx*) gene. Evolutionary changes in such sexually dimorphic features are often a result of changes in the *cis*-regulatory sequences of the DSX target genes. When a particular target gene is directly regulated by DSX in multiple tissues, evolutionarily conflicting constraints may be generated. The research we present here reveals that such conflict can be solved by deploying different *cis*-regulatory modules (sharing the DSX-binding site) for different tissues. This mechanism could also apply to other transcription factors.

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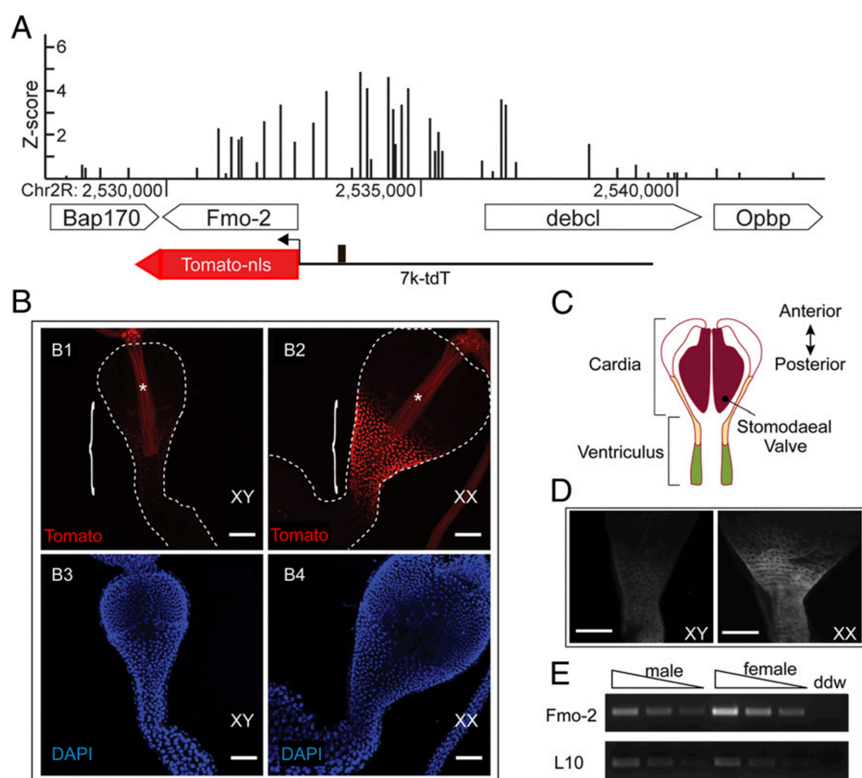

Fig. 1. *Fmo-2* expression in the cardia and anterior ventriculus is sexually dimorphic in *D. melanogaster*. (*A*) The in vivo DSX binding peak at the *Fmo-2* locus measured by DamID (24) and the Tomato reporter (*7k-tdT*) driven by the 7-kb genomic sequence, from −6994 to −61 relative to the translation initiation site of the *Fmo-2* gene. The *z*-score indicates the difference in the levels of methylation at each GATC site between the wild type DSX_Dam and its non–DNA-binding mutant control samples (24). The solid box indicates the position of the consensus DSX binding site (locating at ch2R: 2533346–2533358 of the genome). (*B*) *Fmo-2* Tomato reporter expression at the CVJ is much higher in females (*B2*) than in males (*B1*). The region where the reporter is active is bracketed. Male and female samples were imaged with the same confocal settings. Autofluorescence from the inner lining of the digestive tract is indicated by the asterisk. The dashed lines demark the borders of the samples. The DAPI staining of the nuclear DNA is also shown for reference (*B3* and *B4*). (Scale bars, 50 μm.) (*C*) A schematic drawing of a cross-section of the anterior midgut. The orange region indicates where *Fmo-2* is highly expressed in females. The muscular stomodaeal valve is indicated in dark red. The green shaded region indicates where in the ventriculus the Tomato reporter is not detected. (*D*) Representative images of the whole-mount staining of the anterior midgut of male (*Left*, XY) and female (*Right*, XX) flies with anti–FMO-2 (gray). (Scale bars, 50 μm.) (*E*) Quantitative RT-PCR using RNA of dissected cardia samples from male and female flies for *Fmo-2* (*Upper*) and ribosomal RNA L10 (as a control, *Lower*). A serial dilution for each sample was used as template to ensure the PCR is within linear range.

anterior section of the ventriculus that adjoins the cardia (together denoted CVJ, for cardia ventriculus junction), is sexually dimorphic. The manifestation of this sexual dimorphism requires both *dsx* function and the DSX-binding site at the *Fmo-2* gene. In addition, *dsx* also acts through this same DSX-binding site to activate the *Fmo-2* transcription in the fat body cells of a female-specific tissue, the spermatheca. We found that expression of *Fmo-2* in the midgut has evolved rapidly, and that DNA sequence changes upstream of the *Fmo-2* gene appear sufficient to account for differences in the expression between a number of species in the *melanogaster* group. In contrast, the expression of *Fmo-2* in the fat body cells encapsulating the spermatheca appears to be very conserved, therefore placing an evolution constraint on the DSX-binding site. We identified two *cis*-regulatory modules (CRMs) in the *Fmo-2* regulatory sequence. The more proximally (relative to *Fmo-2*) located CRM (denoted CRM-p) directs sexually monomorphic *Fmo-2* transcription across the entire anterior midgut. The more distally located CRM (denoted CRM-d) contains a DSX-binding site and directs sexually dimorphic *Fmo-2* transcription at the CVJ. Combinations of changes at these two CRMs produce diverse *Fmo-2* expression patterns in the midgut across species in the *melanogaster* group. Most notably, the reversion of *Fmo-2* expression in some species from sexually dimorphic to sexually monomorphic is caused by the loss of CRM-d function but not the loss of the DSX-binding site in CRM-d. Thus, integrating regulatory information through a CRM, but not just a mere DSX-binding site, to control sex-specific gene transcription in a specific tissue is one mechanism to get around evolutionary constraints posed by other tissues in the case of pleiotropic sex-specific transcriptional regulation.

Results

The Expression of *Fmo-2* Is Sexually Dimorphic at the Junction of the Posterior Cardia and Anterior Ventriculus in the Midgut of *D. melanogaster*. The *Fmo-2* gene was among the highest ranking candidates identified by our previous genome-wide screen for direct *dsx* targets: the region 5′ to the *Fmo-2* gene was consistently bound by DSX in vivo and this region also contains a perfect match to the 13-bp optimal DSX-binding site that is bound by DSX in vitro (Fig. 1*A* and *SI Appendix*, Fig. S1) (24).

To identify the tissues in which *dsx* might regulate *Fmo-2*, we first examined the temporal and spatial patterns of expression of the *Fmo-2* gene. For this purpose, we created a transgenic reporter in which a 7-kb genomic sequence immediately upstream of the transcriptional start site of the *Fmo-2* gene was linked to a cassette encoding a nuclear red fluorescent protein, *tdTomato-nls* (Fig. 1*A*). The 7-kb genomic sequence contains the entire intergenic region between the divergent transcriptional start sites for *Fmo-2* and *debcl*, as well as the first intron of the *debcl* gene. Flies carrying the transgene (*7k-tdT*) were examined in larval and adult stages and

Tomato fluorescence was observed in the renal system, the digestive system, and fat body cells of both larvae and adults, and in the spermatheca of adult females. These findings are consistent with transcript profiling of *Fmo-2* in FlyAtlas (33), suggesting that the reporter recapitulates the endogenous *Fmo-2* expression pattern.

When we compared the reporter expression in tissues present in both adult males and females, we only observed sexually dimorphic levels of Tomato fluorescence in part (CVJ) of the midgut (Fig. 1*B*). Specifically, in females there was a high level of Tomato fluorescence at the posterior cardia (R0 section) (34) and adjoining anterior ventriculus (R1a and maybe R1b sections) (34) (illustrated by the orange color in Fig. 1*C*), whereas fluorescence was barely detectable in males in this tissue (Fig. 1*B*). In addition, we observed Tomato fluorescence in the spermatheca, a tissue that only present in the females.

To confirm that the female-biased *Fmo-2* reporter expression at the CVJ indeed reflects the endogenous gene expression pattern, the FMO-2 protein level in adults was examined by antibody staining against FMO-2 (32). We detected a much higher level of FMO-2 immuno-reactivity at the CVJ in females than in males, consistent with what was observed for the *7k-tdT* reporter expression (Fig. 1*D*). Furthermore, quantitative RT-PCR from dissected CVJs indicated that *Fmo-2* transcript levels were higher in females than males (Fig. 1*E*). In contrast, *debcl*, the gene that shares the 5′ intergenic region with *Fmo-2,* did not show sex-biased expression in the same RT-PCR assay.

Together, our results show that expression of *Fmo-2* is sexually dimorphic in the CVJ and that this dimorphism is likely generated at the level of *Fmo-2* transcription. We also conclude that expression of the *7k-tdT* transgene faithfully recapitulates transcription of the endogenous *Fmo-2* gene.

Fmo-2 Transcription Is Directly Regulated by DSX Through an Optimal DSX-Binding Site. If *Fmo-2* is directly regulated by *dsx*, *Fmo-2*–expressing cells should also express *dsx*. Thus, we examined the simultaneous expression of *7k-tdT* and $dsx^{GAL4(\Delta 2)}$, a GAL4 knocked into the *dsx* locus that reflects the transcription of *dsx* (35), in the CVJ of females. We found that all CVJ cells that were positive for *7k-tdT* were also positive for $dsx^{GAL4(\Delta\ 2)}$, driving a nuclear GFP (*SI Appendix*, Fig. S2). In addition, there were GFP^+ cells that were not tdT^+, suggesting that factors in addition to *dsx* are required for *Fmo-2* expression in some cells of this tissue.

We then examined the effects of *dsx* mutations on the expression of the *7k-tdT* reporter in chromosomal males and females. The male-specific DSX^M and the female-specific DSX^F proteins are encoded by sex-specific mRNAs produced by alternative splicing of a primary *dsx* transcript common to both sexes. Both DSX proteins are zinc-finger transcription factors with identical DNA-binding domains, but different C termini that interact differently with cofactors, and thus can have different effects on the transcription of target genes (36–38). We found that in $XY;dsx^-/dsx^-$ flies, the *7k-tdT* reporter expression strongly increased compared with their $XY;dsx^-/+$ siblings. On the other hand, the *7k-tdT* reporter expression significantly decreased in the $XX;dsx^-/dsx^-$ flies in comparison with their $XX;dsx^-/+$ siblings (Fig. 2 *A–D*). Thus, in contrast to the sexually dimorphic expression observed in wild-type flies, *XX* and *XY* flies lacking *dsx* function exhibited monomorphic *Fmo-2* reporter expression. Similar results were observed at the FMO-2 protein level as detected by antibody staining (*SI Appendix*, Fig. S3). Therefore, the sexually dimorphic *Fmo-2* expression at the CVJ is controlled by *dsx*.

These results also revealed that each of the sex-specific DSX isoforms plays an active role in regulating *Fmo-2* transcription: DSX^F activates, whereas DSX^M represses it. To further test this conclusion, a dominant *dsx* allele, dsx^M, which produces only the male isoform of DSX regardless of chromosomal sex (39), was

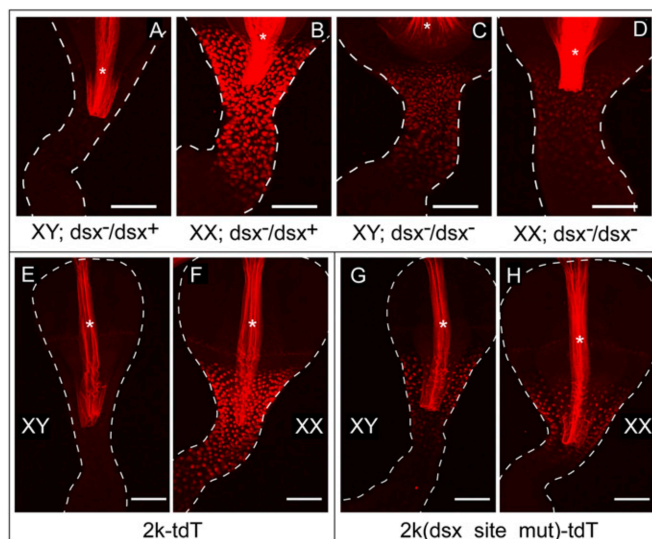

Fig. 2. *Fmo-2* transcription is regulated by *dsx* directly through the DSX-binding site. (*A–D*) The *7k-tdT* reporter expression in dsx^-/dsx^+ male (*A*) and female (*B*) flies, as well as in XY, dsx^-/dsx^- (*C*) and *XX,* dsx^-/dsx^- (*D*) flies. The dsx^- alleles used here are $dsx^{683\text{-}7058}$ and $dsx^{1649\text{-}9625}$. Similar results were observed with another *dsx* allele combination (dsx^1/dsx^{M+R13}). (*E–G*) The expression of *2k-tdT* in males (*E*) or females (*F*), and the expression of *2k(dsx_site_mut)-tdT*, in which the DSX-binding site was mutated from GCAACATTGTTGC to GgggggggggggC, in males (*G*) or females (*H*). The region where the reporter is active is bracketed. Male and female samples were imaged with the same confocal settings. Autofluorescence from the inner lining of the digestive tract is indicated by the asterisk. (Scale bars, 50 μm.)

introduced into *XX* flies to examine its effect on the *Fmo-2* expression. We found that the level of *7k-tdT* fluorescence, which was already reduced to an intermediate level in $XX;dsx^-/dsx^-$ flies, was further reduced in $XX;dsx^-/dsx^M$ flies to a barely detectable level that was similar to that seen in $XY;dsx^-/+$ flies (*SI Appendix*, Fig. S3), confirming the repressive role of DSX^M on *Fmo-2* expression.

To further delimit the genomic region that controls female-biased *Fmo-2* expression in the CVJ, a reporter driven by a smaller 2-kb fragment immediately upstream of the *Fmo-2* gene was constructed (named *2k-tdT*). This reporter also showed female-biased expression at the CVJ (Figs. 2 *E* and *F*), suggesting that regulatory sequences within this 2-kb region are sufficient to account for the sex-biased and tissue-specific *Fmo-2* expression in the gut. Within this 2-kb sequence, there is a perfect match for the optimal 13-bp DSX-binding site. We introduced mutations at this 13-bp sequence to test if it was required for the sexually dimorphic expression of *2k-tdT*. The expression of the mutated reporter [named *2k(dsx_site_mut)-tdT*] at the CVJ was comparable between males and females (Fig. 2 *G* and *H*), and at a level that was intermediate of those seen for the original *2k-tdT* in males and females (Figs. 2 *E* and *F*). These results, in combination with the observations that DSX binds both to the *Fmo-2* promoter region in vivo (Fig. 1*A*) and to the 13-bp sequence in vitro (*SI Appendix*, Fig. S1), demonstrate that DSX directly regulates the sexually dimorphic *Fmo-2* transcription at the CVJ through this 13-bp DSX-binding site.

We also found that similar to the *7kb-tdT* reporter, the *2k-tdT* reporter is also expressed in the spermatheca (Fig. 3). The fat body cells that encapsulate the spermatheca show high level of reporter expression. Mutation of the DSX-binding site in the *D. melanogaster 2k-tdT* reporter reduced the expression in these fat body cells to barely detectable levels (Fig. 3), suggesting that the DSX-binding site is required for the *Fmo-2* transcriptional activation in the fat body cells of the spermatheca. However, we

were not able to conclude confidently whether other fat body cells in the adult also show sex-specific expression of *Fmo-2*, because of the reporter expression being highly variable among fat body cells from different part of the body of the same fly.

Presence of the DSX-Binding Site in the Fmo-2 Regulatory Region Does Not Necessarily Confer Sexually Dimorphic Expression in the Midgut. We previously found that the *Fmo-2* gene of all five sequenced species in the *melanogaster* subgroup contained a sequence matching at least 12 bp of the 13-bp optimal DSX-binding site (24). To test whether the conservation of the DSX-binding site at the *Fmo-2* gene correlates with conservation of sexually dimorphic *Fmo-2* expression, we examined the *Fmo-2* expression in six species from the *melanogaster* subgroup, as well as three species in the *melanogaster* group but not in the *melanogaster* subgroup (Fig. 4 and *SI Appendix*, Fig. S5). All these species have a sequence within the 5′ regulatory region of the *Fmo-2* gene that matches at least 12 bp of the 13-bp optimal DSX binding site (*SI Appendix*, Fig. S5). As a control, we also included one species from the *obscura* group (*Drosophila pseudoobscura*), whose *Fmo-2* gene does not have an as well-matched DSX-binding sequence (Fig. 4 and *SI Appendix*, Fig. S5).

Using immunofluorescence staining, we focused exclusively on determining whether the FMO-2 protein level was sexually dimorphic in each species, because the staining intensity difference between species could be in part because of the different affinities of the antibody for the FMO-2 proteins of these species. To our surprise, despite the conservation of a well-matched DSX-binding sequence in the 5′ regulatory region, the expression pattern for the FMO-2 protein in the midgut is very diverse among the *melanogaster* group species (Fig. 4 and *SI Appendix*, Fig. S5). In five species (*D. melanogaster*, *Drosophila pseudotakahashii*, *Drosophila santomea*, *Drosophila yakuba*, and *Drosophila simulans*), we observed significant sexually dimorphic staining at the CVJ. Two species (*Drosophila erecta*, *Drosophila teissieri*) showed a monomorphic staining throughout the CVJ and the ventriculus. Three species (*Drosophila prostipennis*, *Drosophila mauritiana*, and *Drosophila eugracilis*) showed such weak staining at both the CVJ and the ventriculus that the difference between males and females could not be determined. The weak staining in the latter three species is not likely a result of either the loss of the *Fmo-2* gene in the genome or to the antibody not recognizing the FMO-2 protein in these species, because we can detect strong staining in a group of corpus cardiacum/hypocerebral ganglion neurons in the same flies. The *obscura* group species (*D. pseudoobscura*) showed monomorphic staining at both the CVJ and the ventriculus (Fig. 4 and *SI Appendix*, Fig. S5). Thus, the presence of the DSX-binding site does not necessarily confer sexually dimorphic expression in the midgut.

Changes in *cis*-Regulatory Sequences Appear Sufficient to Account for the Rapid Evolution of *Fmo-2* Expression in the Midgut Across Closely Related Species. The differences in the *Fmo-2* expression pattern in these species could be a result of: (*i*) changes in its *cis*-regulatory sequences; (*ii*) changes in the expression/properties of its up-stream transcription factors; (*iii*) posttranscriptional changes affecting the detectable levels of the FMO-2 proteins; or (*iv*) a combination of these changes. Under the first scenario, we would expect that the regulatory sequence for the *Fmo-2* gene, when moved to another species, would be able to direct an expression pattern similar to what it directs in its native species. We generated Tomato reporters driven by the 5′ genomic sequences of the *Fmo-2* genes from *D. pseudotakahashii*, *D. erecta*, *D. mauritiana*, *D. simulans*, and *D. pseudoobscura*. These transgenes were integrated into the same attP site in the *D. melanogaster* genome and their expression analyzed in males and females. We found that the midgut expression pattern for each of the reporters

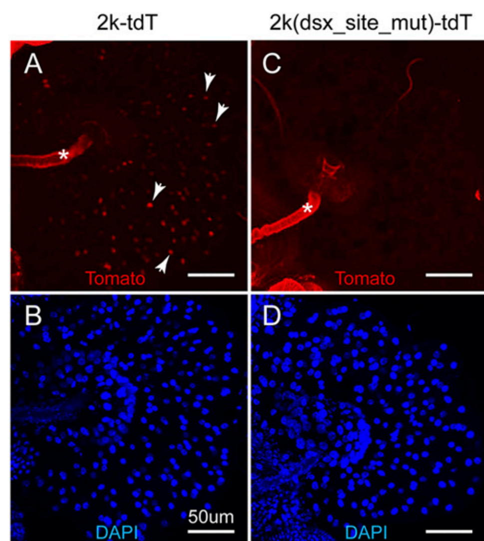

Fig. 3. The DSX-binding site is required for the *Fmo-2* transcriptional activation in the fat body cells of the spermatheca. The expression of the reporters *2k-tdT* (*A*) and *2k(dsk_site_mut)-tdT* (*C*), in the spermatheca were examined. Examples of the nuclear Tomato red fluorescence are indicated by the arrowheads. The DAPI staining showed the nuclei of these samples (*B* and *D*). Samples in *A* and *C* were processed in parallel and imaged with the same confocal settings. Autofluorescence is indicated by the asterisk.

in *D. melanogaster* closely resembled the patterns of anti–FMO-2 staining in the respective native species (Fig. 4). The reporter driven by the *D. pseudotakahashii* sequence showed highly sexually dimorphic expression at both the CVJ and the ventriculus that was quite similar to the antibody staining in its native species (Fig. 4*A*). Both reporters driven by *D. erecta* (Fig. 4*B*) and *D. pseudoobscura* (Fig. 4*E*) sequences showed a sexually monomorphic expression in both the CVJ and the ventriculus, with the expression in the ventriculus being stronger. These are close to the patterns of antibody staining in their respective native species. The reporter driven by the *D. mauritiana* (Fig. 4*C*) sequence showed no detectable expression at either the CVJ or the ventriculus, similar to the very low intensity of antibody staining in its native species. The reporter driven by the *D. simulans* sequence showed a sexually dimorphic expression at the CVJ, closely resembling the antibody staining in *D. simulans* (Fig. 4*D*). Thus, the differences in *Fmo-2* midgut expression are likely caused by changes within the *cis*-regulatory sequences of the *Fmo-2* gene.

On the other hand, we found that the *D. erecta* and the *D. mauritiana* reporters both showed expression in the fat body cells of the spermatheca (*SI Appendix*, Fig. S6), although they showed sexually monomorphic expression or no expression in the midgut. These results suggested that: (*i*) the transcriptional regulation of *Fmo-2* by *dsx* in the spermatheca is very conserved; and (*ii*) the presence of a conserved DSX-binding site in the *Fmo-2* gene is likely because of it being required for the conserved transcriptional activation in the spermatheca.

Two CRMs Control the Transcription of *Fmo-2* in the Midgut. Next we addressed how alterations in the *cis*-acting *Fmo-2* regulatory sequences might lead to the observed changes in *Fmo-2* expression in the midgut. We focused on *D. melanogaster* and *D. erecta* because the reporters driven by the *Fmo-2* genomic sequences from these two species showed very different expression patterns: the *D. melanogaster* sequence confers high, sexually dimorphic expression in the CVJ, and very low expression in the ventriculus (hence, whether ventricular expression is sexually dimorphic could not be assessed), whereas the *D. erecta* reporter

is not expressed in a sexually dimorphic pattern although it is expressed in both the CVJ and the ventriculus, with the expression in the ventriculus being much stronger than in the CVJ. To delimit the locations and properties of the sequences governing *Fmo-2* expression in the midgut, we swapped sequences between the reporters driven by the 2-kb genomic sequence immediately upstream of the *D. melanogaster Fmo-2* coding sequence and the 2.2-kb orthologous sequence from *D. erecta* (Fig. 5*A*).

For the sake of clarity, we first discuss the effects of these chimeric constructs on expression in the CVJ. Examination of the expression of these reporters showed that the combination of the distal part (~1.2 kb) of the *D. melanogaster* sequence (with respect to the *Fmo-2* transcriptional start site) and the proximal part (~0.8 kb) of the *D. erecta* sequence drove a *D. melanogaster*-like pattern (although at a higher level) of reporter expression in the CVJ (high, sexually dimorphic) (Fig. 5*A*, construct #3, and Fig. 5*B*). Notably, this distal sequence contains the DSX-binding site (Fig. 5*A*). Conversely, the combination of the distal part of the *D. erecta* sequence (~1.4 kb) and the proximal part of the *D. melanogaster* sequence (~0.8 kb) drove a *D. erecta*-like pattern of expression in the CVJ (no detectable expression) (Fig. 5*A*, construct #4, and Fig. 5 *B3* and *B4*). These results suggested that there must be at least one functional CRM in the distal portion of the *D. melanogaster* sequence that specifies sexually dimorphic expression in the CVJ. In support of this hypothesis, a smaller (~0.6 kb) substitution of *D. melanogaster* sequence into the *D. erecta* sequence is sufficient to produce a reporter with a high, sexually dimorphic expression pattern in the CVJ (Fig. 5*A*, construct #5). Conversely, when the *D. erecta* counterpart of the 0.6-kb sequence was swapped into the reporter driven by the *D. melanogaster* sequence, there was no detectable reporter expression in the CVJ of either sex (Fig. 5*A*, construct #6). In addition, two deletion constructs derived from the *D. melanogaster* sequence, one with 1 kb of the distal sequence removed and the other one with additional proximal sequence removed (Fig. 5*A*, constructs #9 and #10), can both drive sexually dimorphic reporter expression in the CVJ, further demonstrating that the *D. melanogaster* sequence around the DSX binding site contains a CRM (named CRM-d) that regulates *Fmo-2* transcription in the CVJ.

Observation of ventricular expression in the sequence swapping experiments above (constructs #3, #4, #5, and #6) suggested that there exists another CRM (denoted CRM-p) in the 0.8-kb region at the proximal end of the *D. erecta Fmo-2* sequence that is required for activation of *Fmo-2* transcription in the ventriculus (Fig. 5*A*). This CRM must be deleted or nonfunctional in the orthologous *D. melanogaster* genomic sequence. Thus, in constructs that contain this *D. erecta* sequence (constructs #2, #3, #5), we observed high levels of expression in the ventriculus that was either sexually monomorphic or sexually dimorphic (the sexually dimorphic aspect of this expression is discussed below), whereas in the constructs that contain the orthologous *D. melanogaster* counterpart (constructs #1, #4, #6), we observed little reporter expression in the ventriculus (Fig. 5*A*).

We subsequently realized that the *D. erecta Fmo-2* genomic sequence in the above reporter constructs includes extra sequence from the 5′ UTR of the *D. erecta Fmo-2* transcript (because of the different annotations for these two species in FlyBase). However, we found that addition of the extra 5′ UTR sequence into the *2k-tdT* reporter did not change the reporter expression pattern. On the other hand, when a 682-bp sequence immediately upstream of the TATA box of the *D. erecta Fmo-2* gene, was swapped into the *2k-tdT* reporter, the resulting reporter (construct #7) showed a high level of expression in both the CVJ and the ventriculus in females (Fig. 5*A*). This expression pattern is very similar to that of the reporter construct #3, suggesting that CRM-p is within this 682-bp sequence. Finally, when the distal sequence (including the DSX-binding site) was removed from the *D. erecta* reporter, the resulting construct #8 retained a sexually monomorphic expression in the ventriculus, and at a lower level, in the CVJ, similar to that in the parental construct (Fig. 5*A*). This result further demonstrates that the proximal part of *D. erecta* sequence contains a functional CRM that activates *Fmo-2* transcription in the ventriculus (and to a lower level, in the CVJ) in a sexually monomorphic manner.

Thus, the absence of CRM-p in *D. melanogaster* leads to the lack of *Fmo-2* transcription in the ventriculus, and the absence of CRM-d in *D. erecta* leads to the lack of sexually dimorphic *Fmo-2* expression.

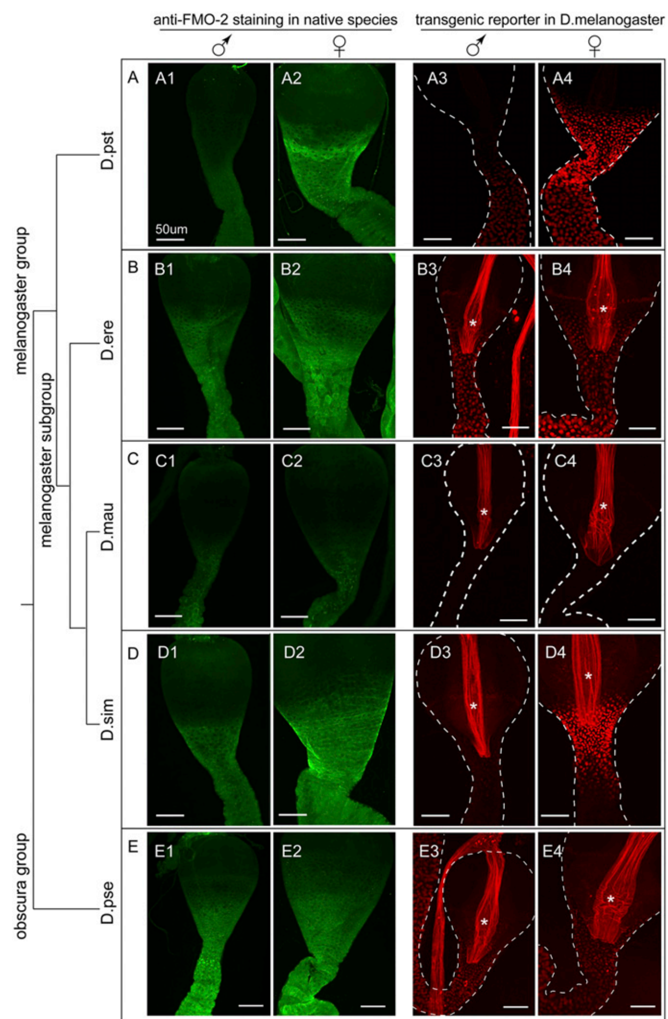

Fig. 4. Changes in the regulatory sequence appear sufficient to account for the diverse *Fmo-2* expression in closely related species. (*A*) The FMO-2 protein level in the midgut of the male (*A1*) and female (*A2*) *D. pseudotakahashii* (*D.pst*) was examined by immunofluorescence using an anti–FMO-2 antibody. Male (*A3*) and female (*A4*) *D. melanogaster* transgenic flies carrying the Tomato reporter driven by the 5′ regulatory sequence from the *D.pst Fmo-2* gene were examined for reporter expression in the midgut. (*B*) Similar to *A*, except that the species is *D. erecta* (*D.ere*). (*C*) Similar to *A* except that the species is *D. Mauritiana* (*D.mau*). (*D*) Similar to *A* except that the species is *D. simulans* (*D.sim*). (*E*) Similar to *A* except that the species is *D. pseudoobscura* (*D.pse*). The phylogenetic tree for these species is on the left (drawing not to the scale with respect to time). The region where the reporter is active is bracketed. Male and female samples were imaged with the same confocal settings. Autofluorescence from the inner lining of the digestive tract is indicated by the asterisk.

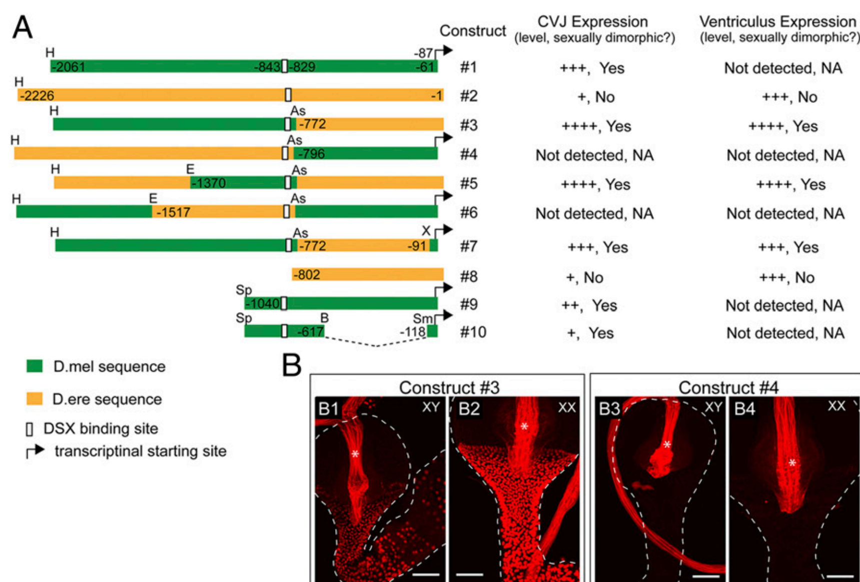

Fig. 5. Two CRMs control the *Fmo-2* transcription in the CVJ and anterior midgut (including the CVJ and the ventriculus), respectively. (*A*) Diagram of the regulatory sequences that are used to drive the tdTomato reporter in the constructs *2k-tdT* (#1) and *ere-tdT* (#2), and the derivative constructs with sequences swapped between the two. The letters denote the restriction site used for the sequence swapping: As, AseI; B, BbvCI; E, EcoRV; H, HindIII; Sp, SphI; Sm for SmaI; X, XhoI. The position of each restriction site (relative to the translation initiation site of the *Fmo-2* gene) is indicated. The translation initiation site was used as a reference for both species because the *Fmo-2* transcriptional start site has not been mapped for *D. erecta*. The expression of the each construct is summarized on the right. The level of reporter activity is indicated by the number of "+". NA indicates that the expression is too low to determine whether it is sexually dimorphic. (*B*) Examples of the sequence-swapped reporter expression in *D.mel* transgenic flies. (*Left*) Construct #3 (*B1*, male; *B2*, female); (*Right*) construct #4 (*B3*, male; *B4*, female). The region where the reporter is active is bracketed. Male and female samples were imaged with the same confocal settings. Autofluorescence from the inner lining of the digestive tract is indicated by the asterisk. (Scale bars, 50 μm.)

Changes Outside of the DSX-Binding Site Impair the Function of CRM-d in *D. erecta*. We tried to identify the sequence critical for *Fmo-2* expression in the CVJ, as this might suggest the possible causes for the loss of CRM-d function in *D. erecta* and other species. The reporter driven by the proximal 1 kb (from −1040 to −61) of the *D. melanogaster* sequence (construct #9, denoted as *1k-tdT*) has a CVJ expression pattern that is very similar to, albeit slightly lower than, that of the *2k-tdT* reporter (Fig. 5*A*), suggesting that most of the sequence comprising CRM-d is retained in this 1-kb region. The swapping experiments above (constructs #5, #6) also suggested that the *D. melanogaster* sequence between −1370 and −795 is important for the CVJ expression. Thus, CRM-d is likely located within the 246-bp sequence between −1040 and −795. Indeed, when this 246-bp sequence in the *2k-tdT* reporter was replaced with the orthologous sequence from *D. erecta*, the resulting reporter [*CRM-d(m->e)*] showed little expression at the CVJ (Fig. 5*A*). The latter result suggested that the sequence changes within this 246-bp region are sufficient to cause the loss of CRM-d function in *D. erecta*.

There are many changes scattering across the orthologous *D. erecta* sequence compared with this 246-bp *D. melanogaster* sequence (Fig. 6*B*). First, the putative DSX-binding site in the *D. erecta Fmo-2* sequence differs in 1 bp from the optimal DSX-binding site, and there are also changes immediately adjacent to the DSX-binding site (Fig. 6*B*). To test whether these particular base substitutions within and near the DSX-binding site are responsible for the absence of sexually dimorphic expression in *D. erecta*, they were mutated in the *D. melanogaster 2k-tdT* construct to those of *D. erecta.* The resulting reporter [denoted *dsxsite(m->e)*] was expressed in a sexually dimorphic pattern similar to that of its parental (*D. melanogaster)* construct (Fig. 6*A*), suggesting that other changes in the 246-bp sequence but not in the DSX-binding site or its immediate flanking sequence impair CRM-d function in *D. erecta*.

To further localize the sequences that are important for *Fmo-2* expression at the CVJ, we made progressive deletions from the 5′ end of the *1k-tdT* reporter, each deletion removing some additional sequence that has changed in *D. erecta* (Fig. 6*C*). We found that each of the deletions caused an additional decrease in the expression level of the reporter, but the sexually dimorphic expression pattern was preserved until the deletion that removed most of the sequence upstream of the DSX binding site, for which there was no longer detectable reporter expression in either sex (Fig. 6*D*). These results suggest two possibilities: (*i*) multiple transcription factors contribute to the tissue-specific activation of *Fmo-2* transcription at the CVJ, and that the loss of function for CRM-d in *D. erecta* is a result of cumulative changes at the binding sites for these transcriptional factors; or (*ii*) alternatively, there might be gain of repressors binding in the *D. erecta* orthologous sequence of CRM-d that suppress the binding/action of these activating transcription factors.

Beside the DSX-Binding Site, Other Sequences in CRM-d Are Required for Sexually Dimorphic *Fmo-2* Transcription in the Ventriculus. In the sequence-swapping experiments described above, we observed an unexpected combinatorial effect of the distal CRM-d (mel) and proximal CRM-p (ere) in regulating reporter transcription in the ventriculus. The distal CRM-d (mel) itself does not activate the reporter in the ventriculus when together in *cis* with the proximal (mel) sequence (Fig. 5*A*, construct #1). However, when together in *cis* with the proximal (ere) CRM-p (constructs #3, #5 and #7), we observed high levels of sexually dimorphic expression in the ventriculus (Fig. 5*A*). As noted above, the *D. erecta* sequence contains an intact DSX-binding site yet directs sexually monomorphic expression in the ventriculus. In addition, the positions of the DSX-binding sites (in relationship to CRM-p) in the native *D. erecta* sequence (Fig. 5*A*, construct #2) and the chimeric constructs (Fig. 5*A*, #3, #5 and #7) are identical, with only 4-bp substitutions in the spacing sequence.

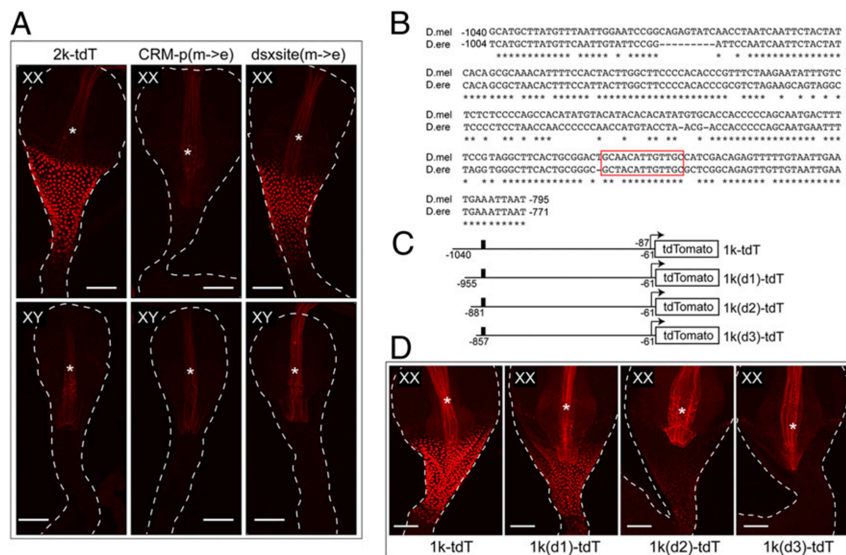

Fig. 6. Changes in other positions instead of the DSX-binding site impaired the function of CRM-d in *D. erecta*. (*A*) Mutations in other positions but not the DSX-binding site eliminated the function of CRM-d in *D.ere*. Two mutant reporters, named *CRM-d(m->e)* and *dsxsite(m->e)*, were derived from *2k-tdT*. In the reporter *CRM-d(m->e)*, a 246-bp sequence (from −1040 to −795) containing the putative CRM-d was replaced with the orthologous *D.ere* sequence. In the reporter *dsxsite(m->e)*, only the DSX-binding site (located between −842 and −830) and the flanking sequence (ACTGCAACATTGTTGCCA, the DSX-binding site underlined) were changed to the *D.ere* sequence (gc-GCtACATTGTTGCgc). Confocal images show the expression of the *2k-tdT* and the two mutated reporters in females (*Upper*) or in males (*Lower*). The reporter expression in the female CVJ was lost in *CRM-d(m->e)* but not in *dsxsite(m->e)*. (*B*) Alignment of the 246-bp *D. mel Fmo-2* regulatory sequence containing the putative CRM-d, and the orthologous *D.ere* sequence. The conserved nucleotides are indicated by the asterisk underneath them. The DNA positions relative to the translation start site are indicated. The DSX-binding sites are red-boxed. (*C*) Diagram of the *1k-tdT*, *1k(d1)-tdT*, *1k(d2)-tdT* and *1k(d3)-tdT* reporters. The DSX-binding site is indicated by the filled box. (*D*) The expression of the reporters from *C* in females. None of these constructs has detectable reporter expression in the male samples. For *A* and *D*, the region where the reporter is active is bracketed. Male and female samples were imaged with the same confocal settings. Autofluorescence from the inner lining of the digestive tract is indicated by the asterisk. (Scale bars, 50 μm.)

Thus, it is possible that a functional CRM-d, not just the DSX-binding site, is required for sexually dimorphic transcriptional activation by CRM-p in the ventriculus. To test this hypothesis, we revisited construct #3, henceforth referred to as "*mel-ere-tdT*." We deleted most sequences upstream of the DSX-binding site in this reporter construct to create a new construct (named *delta-mel-ere-tdT*) that has a DSX-binding site but not a functional CRM-d linked to the 5′ end of the CRM-p sequence (Fig. 7*A*). This new deletion construct and the parental *mel-ere-tdT* construct both contain the same DSX-binding site with the same spacing sequence relative to CRM-p. The same deletion in a purely *D. melanogaster* reporter almost completely abolishes the CRM-d function (d2 deletion) (Fig. 6 *C* and *D*). The expression of the *delta-mel-ere-tdT* reporter in the ventriculus is at similar levels in males and females, which is drastically different from that of the parental *mel-ere-tdT* reporter, but very similar to that of the *D. erecta* reporter (Fig. 7 *B–E*). Thus, the sexually dimorphic *Fmo-2* transcription in the ventriculus also requires a functional CRM-d, and the presence of just a DSX-binding site is insufficient to confer regulation by *dsx*.

We noted that the expression pattern directed by the combination of CRM-d (mel) and CRM-p (ere) is very similar to the reporter expression driven by the *D. pseudotakahashii Fmo-2* regulatory sequence. Therefore, it is very likely that in those sibling species whose *Fmo-2* expression is sexually dimorphic throughout the CVJ and the ventriculus, the *Fmo-2* regulatory sequence is composed of these two CRMs.

Discussion

We have shown that *dsx* is deployed to regulate *Fmo-2* in more than one tissue: the sexually dimorphic transcription of *Fmo-2* in the midgut is directly activated by DSX^F and repressed by DSX^M through a canonical DSX-binding site; the transcriptional activation of *Fmo-2* in the female-specific spermatheca also requires the action of DSX^F through the same canonical binding site. We further established *Fmo-2* transcription in the midgut is controlled through two CRMs, and changes within these two CRMs—but not at the canonical DSX-binding site—caused the diverse midgut *Fmo-2* expression patterns while maintaining the conserved spermathecal expression found in the *melanogaster* group. Thus, these CRMs form the foundation for the rapid evolution of *Fmo-2* transcriptional regulation in the midgut. Below we discuss some implications of these findings.

Rapid Change of the *Fmo-2* Regulatory Sequences Drive the Diversified *Fmo-2* Expression in *Drosophila* Species. We have shown that the midgut expression pattern of *Fmo-2* evolved rapidly in the *melanogaster* group. The rapid changes in *Fmo-2* expression are likely because of changes in the *Fmo-2* regulatory sequences, as the regulatory sequences of the *Fmo-2* gene from all species tested were able to drive expression of a reporter in *D. melanogaster* in a pattern similar to the *Fmo-2* expression pattern in the respective native species. This finding is consistent with the general notion that evolutionarily significant changes are most likely in the *cis*-acting regulatory elements of the downstream targets of the transcription factors, rather than in the genes encoding the transcription factors (12–14), which appears to be especially true for sexually dimorphic gene expression (40).

We found two CRMs (CRM-d and CRM-p) controlling *Fmo-2* expression in the CVJ and the ventriculus in the *melanogaster* group. CRM-d contains a DSX-binding site and can direct sexually dimorphic expression in the CVJ, whereas CRM-p directs sexually monomorphic expression in both the CVJ and the ventriculus. Because we could not find a homologous sequence for CRM-d in out-group species, such as *D. pseudoobscura*, we believe that CRM-d arose de novo in the common ancestor of

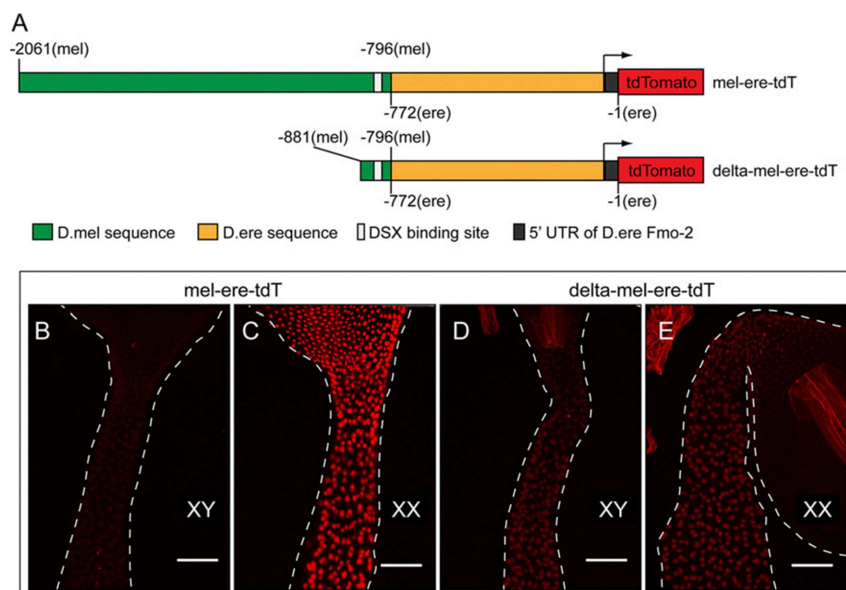

Fig. 7. The presence of the DSX-binding site itself is insufficient to confer the sex-specific regulation of *Fmo-2* by *dsx* in the ventriculus. (*A*) Diagram of the reporter *mel-ere-tdT* (construct #3, Fig. 4*A*) and its derived reporter *delta-mel-ere-tdT*. The DNA positions relative to the translation start site are indicated. (*B–F*) The reporter activity at the anterior midgut was analyzed for *mel-ere-tdT* in males (*B*) and females (*C*), and for *delta-mel-ere-tdT* in males (*D*) and females (*E*). The region where the reporter is active is bracketed. Male and female samples were imaged with the same confocal settings. (Scale bars, 50 μm.)

the *melanogaster* group. On the other hand, we did find homology for the CRM-p containing sequence in the *D. pseudoobscura Fmo-2* regulatory sequence, which can direct a monomorphic reporter expression in both the CVJ and the ventriculus in *D. melanogaster*. Therefore, we believe that the *D. pseudoobscura* sequence contains a functional CRM-p and that CRM-p represents a more ancestral CRM (arose before the branching of the *obscura* group and the *melanogaster* group). However, because of the rapid evolution of the *Fmo-2* regulatory sequence, we could not determine more precisely when CRM-p arose.

We propose that changes at these two CRMs lead to the diversified patterns of *Fmo-2* expression in the melanogaster group (summarized in Fig. 8). The gain of a functional CRM-d in the common ancestor of the *melanogaster* group had two consequences: (*i*) sexually dimorphic activation of *Fmo-2* transcription in the CVJ; and (*ii*) adding a layer of sex-specific regulation on *Fmo-2* transcription (originally directed by CRM-p) in the ventriculus. Further modifications of these two CRMs in descendent species resulted in diverse patterns of *Fmo-2* expression. There was only one event of a gain of sexually dimorphic regulation, but several events of loss of sexually dimorphic regulation in the *melanogaster* group. This finding is consistent with the "losses are easy, gains are harder" principle of regulatory evolution (12), possibly because of a functional CRM-d requires gain of multiple transcription factor binding sites in an appropriate configuration, whereas losing of the CRM-d function might require just one change.

Insight into the Tissue- and Sex-Specific Gene Regulation. There are two mechanisms that could affect the tissue- and sex-specific gene expression directly regulated by *dsx*. The first mechanism is through the changes in the *dsx* expression pattern (4, 41). The other mechanism, maybe more commonly, is through the changes at the *cis*-regulatory sequences of the *dsx* target genes. For the latter mechanism, previous studies on individual genes that are the direct regulatory targets of DSX have revealed how tissue- and sex-specific information is integrated in the transcriptional regulation of these target genes. For the *Yolk Protein* genes, the fat body- and female-specific expression is brought about through enhancers containing multiple identified protein-binding sites (42, 43). The binding of the DSX proteins provides sex-specific regulation on top of the fat body-specific transcription established by the other proteins. In the case of the *bric-à-brac 1* gene, the HOX protein Abdominal-B binds to multiple sites in the *bric-à-brac 1* regulatory sequence to produce tissue-specific transcriptional activation, which is further modified sex-specifically by the direct binding of DSX^F in females or DSX^M in males to the *bric-à-brac 1* regulatory sequence (20). Thus, a general feature for the transcriptional regulation of direct *dsx* target genes is tissue-specific transcription, established by the action of one or more tissue-specific transcription factors, in combination with DSX-binding sites recruiting the direct binding of DSX^F and DSX^M to achieve sex-specificity. We have shown that the transcriptional regulation of *Fmo-2* in the CVJ follows this theme.

However, the sex-specific regulation of *Fmo-2* transcription in the ventriculus appears to be more complex. Ventricular-specific transcriptional activation is established through CRM-p; however, it requires a functional CRM-d, not just a DSX-binding site, to confer regulation by DSX, although CRM-d itself does not activate *Fmo-2* transcription in the ventriculus. The activation may require other factors binding in CRM-d either to secure DSX to its binding site or to bridge the interaction between the two CRMs. Regardless, the tissue- and sex-specific *Fmo-2* transcription in the ventriculus is the result of the integration of regulation through two CRMs.

Overcoming the Evolutionary Constraints on the Regulatory Sequence in Pleiotropic Transcriptional Regulation. Evidence has accumulated in recent years that "regulatory evolution" is the most pervasive mechanism underlying the morphological changes during evolution (12). Under this theory, changes in the expression pattern of the genes that govern the development of a particular feature, through changes at the regulatory sequence rather than changes at the coding sequence of the gene or changes of the upstream transcription factors, lead to changes of that feature. In addition, the *cis*-regulatory elements of a particular gene are often organized as modules and the gain or loss of the trait could be a result of the independent changes of these modules (12, 44). Regulatory evolution provides great flexibility and minimizes deleterious pleiotropic effects (12, 15).

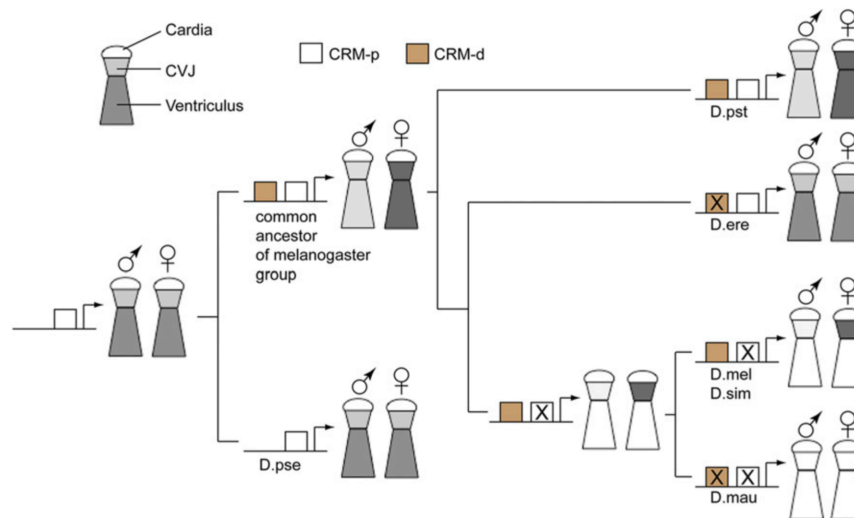

Fig. 8. A model for the evolution of *Fmo-2* transcriptional regulation at the midgut. The common ancestor for the *melanogaster* group and the out-group species started with the proximal CRM-p in the *Fmo-2* regulatory sequence; therefore the expression is monomorphic. This monomorphic transcription was retained in the out-group species (e.g., *D.pse*). The gain of distal CRM-d (indicated by the filled box) in the common ancestor of the melanogaster group led to sexually dimorphic expression in both the CVJ and the ventriculus, which was retained by some descendent species (e.g., *D.pst*). Some species (e.g., *D.ere*) lose the function (indicated by the cross) of CRM-d while maintaining CRM-p intact, causing *Fmo-2* transcription to revert to the more ancestral monomorphic pattern in both the CVJ and the ventriculus. The common ancestor of *D.mau, Dmel*, and *D.sim* lose the function of CRM-p while CRM-d was intact; therefore, *Fmo-2* transcription was highly sexually dimorphic in the CVJ but was at a minimal level in the ventriculus. This feature was maintained in some descendent species (e.g., *D.mel* and *D.sim*). *D.mau* may have further lost the function of CRM-d, thus the Fmo-2 transcription is very low in both the CVJ and the ventriculus of both sexes. The intensity of the gray shade symbolizes the level of transcription in each segment of the anterior midgut.

It is conceivable that the regulation of a direct target gene by a particular transcription factor is pleiotropic, meaning that the gene is under the transcriptional regulation of this transcription factor in multiple tissues. In some of these tissues the regulation might be more resistant to change, consequently placing an evolutionary constraint on the changes in other tissues. This theory leads to the question of how such evolutionary constraints on the pleiotropic transcriptional regulation are overcome. Under the regulatory evolution viewpoint, this conflict could be resolved by independent changes of different regulatory modules. However, very few real examples have been shown to demonstrate that this is the case. One reason might be the difficulty of clearly identifying the in vivo binding sites of that transcription factor within the tissue-specific regulatory modules of the target gene. Generally, transcription factor binding sites are short (5–8 bp) and imprecise, thus the sequence for a given transcription factor binding site occurs very often in the genome just by chance. Therefore, many of these consensus matches are not bona fide binding sites and have no effect on transcription (45).

The transcriptional regulation by *dsx* provides a great paradigm to address how evolutionary constraints on pleiotropic transcriptional regulation are overcome. First, the optimal DSX-binding site is exceptional in that it is a 13-bp palindrome, and thus rarely occurs in the genome just by chance. Second, sex-specific features evolve very rapidly (46), and such rapidly evolved traits are likely a result of changes in the expression of downstream targets of *dsx* (17).

Unfortunately, the previous identified direct *dsx* targets are all regulated by *dsx* in a single tissue (*Yolk Protein* genes in the fat body, *desat-F* gene in the oenocytes, and *bric-à-brac 1* gene in the abdominal cuticle). Here we found that *dsx* directly regulated *Fmo-2* in two different tissues, the midgut and the spermatheca. The *dsx* control of *Fmo-2* transcription in the spermatheca appears to be very conserved, whereas the *dsx* regulation of *Fmo-2* transcription in the midgut has evolved rapidly. Therefore, the *Fmo-2* transcriptional regulation by *dsx* provides a great opportunity to investigate how such a constraint shapes the evolution of *dsx* regulation. By maintaining the DSX-binding site (and possibly other sequence) required for the *Fmo-2* transcription in the spermatheca, and changing other sequence critical for CRM-d function, *Fmo-2* transcriptional regulation in the midgut could evolve without a pleiotropic effect. Thus, we provide an example at a single transcription-factor binding-site resolution to demonstrate how a constraint posed by pleiotropic transcriptional regulation is evaded during evolution.

Experimental Procedures

Reporter Constructs and Transgenic Fly Production. DNA sequences used to drive the tdTomato-nls (47) reporter were PCR-amplified from genomic DNA of the indicated species, and cloned into site-specific transformation vectors carrying the tdTomato-nls reporter. Additional information on the reporter construction, PCR primers, and examination of the reporter expression are provided in *SI Appendix, Experimental Procedures* and Table S1. All of the reporter constructs were integrated at the attP40 site.

***Drosophila* Species.** Fly stocks for other species (*SI Appendix*, Table S2) were obtained from the University of California, San Diego stock center.

Fly Stocks. The *dsx* alleles used were: dsx^{1}, dsx^{M+R13} (47), dsx^{M} (39), $dsx^{683\text{-}7058}$ and $dsx^{1649\text{-}9625}$ (48), $dsx^{GAL4(\Delta 2)}$ (35). A wild type w+ allele on the paternal X chromosome was used to distinguish *XY*, dsx^{-}/dsx^{-} from *XX*, dsx^{-}/dsx^{-}, or *XY*, $dsx^{-}/+$ from *XX*, dsx^{-}/dsx^{M}.

Whole-Mount Antibody Staining. The midgut was dissected from age-matched flies (5-d-old, unless otherwise specified) and stained with anti–FMO-2 antibody (32) diluted 1:300, followed by secondary antibody Alexa Fluor 488 (or 568) goat anti-rabbit IgG (Invitrogen) diluted 1:1,000 as described previously (49) with modifications (*SI Appendix, Experimental Procedures*). The confocal images were quantified for the intensity of the antibody staining using ImageJ (see *SI Appendix*, Fig. S5 for more details on methods).

Quantitative RT-PCR. Total RNA from 20 cardias (dissected in PBS on ice, attached with a section of ventriculus about the same length of the cardia) was isolated using TRIzol (Life Technologies). First-strand cDNA was synthesized with a SuperScript III first strand synthesis system (Life Technologies), and used as templates for PCR reactions (see *SI Appendix, Experimental Procedures* for detail PCR conditions and primers).

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