

Cellular sex throughout the organism underlies somatic sexual differentiation



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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Herault et al. re-examines the genetic mechanisms underlying somatic sexual differentiation in *Drosophila melanogaster*. By engineering and studying a series of novel and well-designed alleles of the sex determination gene transformer (*tra*), the authors show that the female-specific isoform of this gene, *traF*, is widely, if not ubiquitously, expressed in the female fly body, presumably throughout the life cycle. This contradicts the prevailing view that only a subset of cells, those expressing the sex-specific isoforms of fruitless (*fru*) or doublesex (*dsx*), the only known splicing targets of *TraF*, know their sexual identity. The authors also show that *TraF* exerts its splicing activity in a wide range of cells, including some that do not express *fru* or *dsx*. In particular, *traF* is critical for establishing sex differences in adult size and weight, and in the shape and function of the reproductive organs.

The main merit of this work is the very elegant and rigorous demonstration that almost all, if not all, the cells in the *Drosophila* body have a genetically defined sexual identity: all female cells express *TraF*, male cells do not. This is a simple but crucial and novel conclusion of this study. Furthermore, this work confirms and extends previous studies suggesting that non-canonical sex determination pathways (i.e., independent of *fru* and *dsx*) that rely on alternative splicing play a key role in establishing multiple phenotypic differences between the sexes. Finally, the results suggest that *TraF* evolution may have contributed to sex-specific trait changes between *Drosophila* species.

The results and the new genetic tools introduced in the study by Herault et al. open up many exciting questions about the mechanisms and the genes by which *TraF* shapes sex divergence in different organs and cell types. One intriguing question left open is the cell-autonomous effects of *TraF*. Previously, in Rideout et al. (2015 Plos Genetics), it was shown that the effect of *TraF* on pupal size is controlled in parallel by a cell-autonomous effect on cell size and a non-cell-autonomous effect on growth (via the fat body and insulin signaling). With the new alleles presented in this work, it would be interesting to assess the relative contribution of cell-autonomous vs. non-cell-autonomous effects of *TraF* on the sex differences in different phenotypic traits (including adult size). For example, by restricting *TraF* function to the fat body, or the wing disc; or conversely, by eliminating *TraF* function from these tissues, and measuring the effect on wing size.

Reviewer #2 (Remarks to the Author):

Frustratingly, I feel the authors have created a straw man argument, in this case, that the field is guilty of believing in a 'two-gene' model of sexual differentiation. The author's views are taken out of context, and by doing this, they ignore the various considerations at play and, by oversimplifying the opposing argument, present a small part of a model as if that part were the whole story. The field has a more refined idea of how sexual differentiation works, and the authors should have laid out a more nuanced set of contemporary ideas and arguments.

This paper is very challenging for the reader to interpret. The text simplifies the explanations, leaving the reader to jump between figures, legends, separate genotypes, and methods to

interpret each experiment. The genotypes should be unambiguous in the Figures and/or legends. The authors should be encouraged to show each novel allele made diagrammatically and what tests have been done to validate a given reagent—do they do what the authors suggest?

The authors have generated many valuable and unique reagents that could benefit the field (with the abovementioned caveat regarding validation). There are also some interesting experiments within the manuscript. The problem comes with the way they are laid out and interpreted. Without a total rewrite and rethinking of the narrative, this paper is impenetrable, particularly for non-aficionados.

Technical concerns:

The authors state in the methods; they generated a fru-Gal80 knock-in allele by inserting the plasmid pBS-KS-attB2-SA(1)-T2A-3X Gal80-Hsp70 (addgene: Plasmid#62952) into the MiMIC fru[MI05459]. This “plug-and-play” cassette exchange was first described by Diao et al., 2015. As stated in this paper, it is a “method for achieving cell-type-specific expression of transgenes that leverages the untapped potential of “coding introns” (i.e., introns between coding exons).” This method, therefore, relies on the original MiMIC being in a coding intron; however, fru[MI05459] is not in a coding intron; thus, it is incumbent upon the authors to show how this construct functions in this context. The evidence the authors show suggests their Gal80 is working in the repression of fru-Gal4, meaning there is Gal80 expression; however, this does not tell us where and when the Gal80 is expressed. The authors need to show the full expression pattern of a cassette exchange within this MiMIC site. This verification can be achieved by exchanging the MiMIC with Gal4. This same approach was later adapted for the expression of tra2 (Generation of the fru-tra2 knock-in allele); however, this has a similar problem: it is impossible to know when and where tra2 is being expressed. This technical issue makes all the experiments utilising these reagents impossible to interpret. One note of caution- it has been shown that even existing reagents to recapitulate fru expression (i.e. fru-Gal4 vs fru-LexA) have been shown to have both overlapping and unique expression patterns- thus, every reagent has its shortcomings- this will, of course, also be true for reagents capturing dsx expression.

Utilising the Gal4/UAS system, in conjunction with Gal80 or FLP, can be challenging when dealing with developmentally regulated gene expression. A lag in the Gal4 expression/UAS activation can result in missing critical developmental windows of expression. This system has been historically problematic when trying to ‘rescue’ sex differences. This system has been historically problematic when trying to ‘rescue’ sex differences. For example, in Ren et al., 2016 and Rideout et al., 2010, using dsx-Gal4 to try to inhibit cell death (via UAS-p35) is insufficient to rescue male-like neuron numbers in females. Even more problematic is using this system to turn off traF, as then you rely on Gal4/UAS expression of FLP and then the decay of endogenous traF.

The authors should be mindful of comparing different genetic variants of the white gene, as some panels compare individuals that are w- vs. w+ vs those that contain mini-white. The white gene encodes a metabolically relevant protein, an ATP-binding cassette G-subfamily half-transporter, which may affect phenotypes such as size and weight. This should be discussed or controlled for by replacing the w- allele with w+.

Notes on the text:

Line 59: Not all DsxM-expressing neurons co-express FruM

Line 66: it is not true to say that *dsx* and *fru* are no longer necessary in adulthood. See the following review: doi.org/10.1111/gbb.12772

Line 44: there is not a widely held belief in ‘the two-gene model’- the main point of Robinett et al. (2010) was to say that not all sex differences are due to one step- the sex-specific expression of *SXL*, but rather developmental and cell-specific regulation of *dsx* and *fru* give mosaic sexual differentiation.

Line 68/69: the conclusion is not that all other cells do not have a sexual identity- we know that all do- this comes down to what you mean by sexual identity.

Line 78: The phrase “phenotypic consequences of having sex chromosomes” is confusing. Is the idea here to separate the presence of sex chromosomes from the SDH? It is not clear this is what you are doing when you focus on members of the SDH to make your arguments- this just says we don’t know everything about how the SDH determines sex differences.

Line 98: These observations DO NOT suggest *TraF* lacks expression/function in most organs- that is one hypothesis, but it is equally likely it is expressed everywhere- in fact, it seems more likely this would give small changes in *fru/dsx* expression the maximum chances of evolving novel sex differences.

Line 131: if *traF* is expressed everywhere, it is not necessarily always functioning to produce sex differences- just like *SXL* and *Tra2*, a splicing target makes the differences. So, it follows that for a *Drosophila* cell to “know” its sex, it must have a mechanism for translating the *traF* into sexual identity. If a cell does not express a downstream factor such as *Dsx* or *Fru*, how does it interpret the information about *traF*, so it can develop differently in males and females. What mechanism would *traF* confer sexual identity to cells that are not expressing terminal differentiation genes like *fru* and *dsx*? Would this be in a cell- or non-cell-autonomous manner.

Line 152: What is meant by sexual identity in this context? It is well known that cells have a sexual identity based on their chromosomal makeup and *SXL* expression, so it is not clear why expressing *traF* makes a big difference in defining the sexual identity- it is important information but not game-changing.

Line 160-162: classical *SXL/tra/dsx/fru* mutants suggested long ago that *dsx* and *fru* were not ‘sufficient’ for all sex differences.

Line 204: How can it be concluded that all cells not captured are ‘monomorphic’? “The sex of organs that are sexually monomorphic is necessary and sufficient to drive sex differences” This sentence seems to contradict the whole premise- if there are still sex differences, then there must be dimorphism.

General notes for Figure 4:

Looking at splicing in the mature adult is not the same as confirming sex-specific splicing occurs at normal levels when and where it is needed during development. The authors have made a *dsxF* constitutive allele using a novel approach; given this, it is incumbent upon the authors to show it works as expected. For example, in Extended Data Fig. 4e, this mutant is

not used as the control, even though it is used in the previous panel. This approach makes interpreting the result problematic.

Figure 4f: given potential delays in fru-Gal4/UAS-FLP expression and the decay of D.mel traF, it would be hard to rule out fru cells based on this strategy.

Reviewer #3 (Remarks to the Author):

This work utilizes sophisticated genetic tools to dissect the role of the essential sex determination factor Tra in regulating sex-specific characteristics. Importantly, the evidence presented indicates that Tra has functions in non-dsx and non-fru expressing cells to control sexually dimorphic characteristics and fertility. It is particularly surprising that fertility is affected as the cells of the gonad and reproductive tract are all thought to express dsx, and sometimes fru, meaning that this is likely an effect of more distant tissues on fertility. While it has been previously demonstrated that Tra controls traits like body size independently of dsx and fru, it was not known that this is partially controlled by cell types other than those that express dsx and fru. Further, the finding that the Tra co-factor Tra2 is required for some aspects of these traits, like body size, but is not required for effects on fertility, is also of interest.

Many of the findings in this work are most appropriate for a specialized audience. It was previously thought that Tra expression is very broad and general in the animal, and this has been previously found in large-scale RNA-seq from different stages and tissues, but I'm not sure it has ever been directly shown. Therefore, it is very nice to see the general Tra expression and activity clearly presented here, but it does not change our thinking. In addition, as clearly described by the authors, it has previously been shown that Tra has dsx- and fru- independent functions, including in regulation of body size, but here they demonstrate that this is at least partly due to Tra effects in tissues other than those that normally express dsx and fru. Lastly, while it is good to know that mutating the RNA binding function of Tra acts as a loss of function allele, this was not much in doubt given that the only known functions for Tra so far involve alternative splicing. I have seen some papers that appear to be highly specialized in Nature Communications before, and so this work may be appropriate for publication here.

The work expressing Tra from other species in melanogaster is perhaps the weakest part of the paper and could be removed. While I appreciate that they try and use these different Tra's to separate dsx and fru splicing from other Tra functions, I think it is also possible that these other Tra forms might act in unanticipated ways, reducing confidence in the conclusions. In addition, I would suggest a few changes to the way the manuscript is presented. First, the genetics performed, while quite elegant, might be difficult for the general audience to grasp. They have made good headway in making this more accessible, including with the diagrams in the figures, but it could still be improved in the text. Further, I disagree with the way a few terms are used. First, they refer to all dsx- and fru-expressing cells as "sex organs". This may be confusing to people that consider the gonads and genitalia as the primary sex organs. Tissues like the fat body, which express dsx have sexually dimorphic function, would not be considered "sex organs" by most people. Second, they refer to "primary" and "secondary" sex tissues in a manner that is different from what I feel is common in the field. Normally, I would consider a "primary" sex tissue or cell as one that directly assesses the sex

determination mechanism (XX or XY in this case), while a secondary sex tissue is one that receives information from another source (e.g. a primary sex tissue) about what sexual path to follow. In this work, its not clear what they mean by secondary sex tissue, but it seems these tissues express *dsx* or *fru* and are directly responsive to the sex chromosome genotype, making them really “primary” sex tissues.

Response to Reviewers

We thank the Reviewers for their time examining our manuscript and providing valuable comments. We have followed all their suggestions and believe that the new experiments and their outcome have improved our manuscript. We are providing a point-by-point response to their specific points below. Before doing so, we also highlight the critical new data included in the revised manuscript:

- 1- As requested by Reviewer #1, we performed additional experiments to assess the relative contribution of cell-autonomous vs. non-cell-autonomous effects of TraF on the sex differences in adult size.
- 2- We addressed the technical concerns of Reviewer #2 about our new genetic reagents and reframed our hypothesis to reflect the field's current state better.
- 3- We extended our experiments using TraF proteins of different *Drosophila* species.

We present these new data in 20 new panels included in the figures and supplementary figures in a significantly revised manuscript. Changes in the manuscript are highlighted in blue.

REVIEWER COMMENTS

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The main merit of this work is the very elegant and rigorous demonstration that almost all, if not all, the cells in the *Drosophila* body have a genetically defined sexual identity: all female cells express TraF, male cells do not. This is a simple but crucial and novel conclusion of this study. Furthermore, this work confirms and extends previous studies suggesting that non-canonical sex determination pathways (i.e., independent of *fru* and *dsx*) that rely on alternative splicing play a key role in establishing multiple phenotypic differences between the sexes. Finally, the results suggest that TraF evolution may have contributed to sex-specific trait changes between *Drosophila* species.

We want to thank the Reviewer for the positive assessment of our work.

The results and the new genetic tools introduced in the study by Herault et al. open up many exciting questions about the mechanisms and the genes by which TraF shapes sex divergence in different organs and cell types. One intriguing question left open is the cell-autonomous effects of TraF. Previously, in Rideout et al. (2015 Plos Genetics), it was shown that the effect of TraF on pupal size is controlled in parallel by a cell-autonomous effect on cell size and a non-cell-autonomous effect on growth (via the fat body and insulin signaling). With the new alleles presented in this work, it would be interesting to assess the relative contribution of cell-autonomous vs. non-cell-autonomous effects of TraF on the sex differences in different phenotypic traits (including adult size). For example, by restricting TraF function to the fat body, or the wing disc; or conversely, by eliminating TraF function from these tissues, and measuring the effect on wing size.

This is an exciting point. To investigate the cell-autonomous vs. non-cell-autonomous contribution of TraF on adult size, as suggested by the Reviewer, we have conducted the following additional experiments:

1-First, specifically in the fat body, we knocked out *traF* in females and knocked in *traF* in males and measured the impacts on adult wing size. Neither of these two genetic manipulations affects wing size. Fat body-specific *traF* expression in a *traF* null mutant background also does not rescue female wing size. Correspondingly, fat body-specific

traF knock-down and *traF* over-expression do not impact wing size. From these experiments, we can conclude that *traF* expression in the fat body is not required for sex differences in adult wing size.

2-Conversely, wing disc-specific *traF* knock-out or *traF* knock-in significantly influences wing size. Furthermore, the effects obtained recapitulate and are similar to whole-body *traF* manipulations. Interestingly, wing disc-specific *traF* expression in a *traF* null mutant background completely rescues female wing size. Even though every organ except the wing pouch is masculinised in these individuals, the adult wing is even slightly bigger than in wild-type females.

Our experiments have provided unambiguous results. They indicate that TraF's contribution to adult organ size is indeed mediated through cell-autonomous mechanisms. We also want to draw attention to critical points in the publication by Rideout et al. (2015 PLoS Genetics): 1) fat body-specific *traF* over-expression had no effect on male pupal size (S5 panel B), and 2) fat body-specific *traF* knock-down or knock-out was not performed.

The revised manuscript now incorporates these experiments in three new panels of Figure 3 (**Fig. 3k-m**). These additions enhance the understanding of TraF's role in adult organ size determination. For more details, please refer to the text **lines 210-223**.

Reviewer #2 (Remarks to the Author):

Frustratingly, I feel the authors have created a straw man argument, in this case, that the field is guilty of believing in a 'two-gene' model of sexual differentiation. The author's views are taken out of context, and by doing this, they ignore the various considerations at play and, by oversimplifying the opposing argument, present a small part of a model as if that part were the whole story. The field has a more refined idea of how sexual differentiation works, and the authors should have laid out a more nuanced set of contemporary ideas and arguments.

We agree with the Reviewer's comment that our initial manuscript lacked a more thorough discussion of previous studies. We are now providing a new extended Introduction section (please see **lines 58-78, 84-88 and 91-94**) that reframes our hypothesis to better reflect the field's current state.

This paper is very challenging for the reader to interpret. The text simplifies the explanations, leaving the reader to jump between figures, legends, separate genotypes, and methods to interpret each experiment. The genotypes should be unambiguous in the Figures and/or legends. The authors should be encouraged to show each novel allele made diagrammatically and what tests have been done to validate a given reagent—do they do what the authors suggest?

We agree with the Reviewer and modified our manuscript to clarify this point. We have now included the specific genotypes and diagrams for each new allele. The following new panels incorporate these illustrations:

-Fig. 1a, 1c, 1d and 1e.

-Fig. 2 b and 2c.

-Fig. 3a, 3b, 3k and 3m.

-Fig. 4e.

-Fig. 5e and 5f.

-Extended Data Fig. 1a, 1b, 1d and 2e.

-Extended Data Fig. 2b and 2c.

-Extended Data Fig. 3e and 3k.

-Extended Data Fig. 4i and 4l.

-Extended Data Fig. 5d, 5g and 5l.

The authors have generated many valuable and unique reagents that could benefit the field (with the abovementioned caveat regarding validation). There are also some interesting experiments within the manuscript.

We want to thank the Reviewer for the positive assessment of our work.

The problem comes with the way they are laid out and interpreted. Without a total rewrite and rethinking of the narrative, this paper is impenetrable, particularly for non-aficionados.

Technical concerns:

The authors state in the methods; they generated a *fru*-Gal80 knock-in allele by inserting the plasmid pBS-KS-attB2-SA(1)-T2A-3X Gal80-Hsp70 (addgene: Plasmid#62952) into the MiMIC *fru*[MI05459]. This “plug-and-play” cassette exchange was first described by Diao et al., 2015. As stated in this paper, it is a “method for achieving cell-type-specific expression of transgenes that leverages the untapped potential of “coding introns” (i.e., introns between coding exons).” This method, therefore, relies on the original MiMIC being in a coding intron; however, *fru*[MI05459] is not in a coding intron; thus, it is incumbent upon the authors to show how this construct functions in this context.

Plasmid#62952 carries a splice acceptor site, a start codon (ATG), the Gal80 coding sequences, and a polyadenylation site. Consequently, intronic insertion of such plasmid will lead to incorporating Gal80 sequences into gene transcripts and interrupt transcription, functioning as a reporter of gene expression. The Bellen lab developed and extensively validated this approach in 2011 (PMID: 21985007). A substantial portion (20.4%, 467 lines over the 3 633 MiMIC insertions existing) of intragenic MiMICs are localised to 5'UTR introns. Bellen lab demonstrated that introducing exogenous protein-coding sequences into these insertions allows expression under the control of the endogenous gene regulatory elements (see Figure 2 in PMID: 21985007). Thus, Gal4 inserted into these MiMIC lines faithfully recapitulated the gene's expression pattern. This method has been proven to be reliable and successful, with hundreds of Gal4 derived from MiMIC insertions localised to 5'UTR introns being generated by the Bellen lab (<https://flypush.research.bcm.edu/pscreen/rmce/>, like *AstA-Gal4* (BL#77842), *Dh44-R2-Gal4* (BL#66865), *Lapsyn-Gal4* (BL#78375), *Oct-TyrR-Gal4* (BL#77735), *wdb-Gal4* (BL#78374)...). For example, Michelle Arbeitman's lab recently used the 5'UTR intronic Gal4 into the *wdb* gene (BL#78374) to visualise co-expression with *fruitless* (see Figure 7a in PMID: 33901168).

The evidence the authors show suggests their Gal80 is working in the repression of *fru*-Gal4, meaning there is Gal80 expression; however, this does not tell us where and when the Gal80 is expressed. The authors need to show the full expression pattern of a cassette exchange within this MiMIC site. This verification can be achieved by exchanging the MiMIC with Gal4. This same approach was later adapted for the expression of *tra2* (Generation of the *fru*-*tra2* knock-in allele); however, this has a similar problem: it is impossible to know when and where *tra2* is being expressed. This technical issue makes all the experiments utilising these reagents impossible to interpret. One note of caution- it has been shown that even existing reagents to recapitulate *fru* expression (i.e. *fru*-Gal4 vs *fru*-LexA) have been shown to have both overlapping and unique expression patterns- thus, every reagent has its shortcomings- this will, of course, also be true for reagents capturing *dsx* expression.

Following the Reviewer's advice, we have performed an additional swapping experiment of MiMIC insertion MI05459, used to generate the *fru*-Gal80 line, to verify its expression profile. Unfortunately, during the review, Bloomington Stock Centre could not ship us this MiMIC line (as they had too few flies). So, instead of exchanging this MiMIC insertion with Gal4, we swapped it with *traF* cDNA (**Figure for Reviewer 1a**, as this line was already generated in the lab for another project). Results are presented in **Figure for Reviewer 1** (below). We extensively characterise this new allele at the molecular, cellular and phenotypic levels.

RT-qPCR analyses established that, as expected, *fru* sex-specific splicing is reversed in the brain (**Figure for Reviewer 1b**). On the contrary, sex-specific splicing of *dsx* in the abdomen is unaffected, as the expression of its sex-specific direct targets, *Yolk protein 1* and *desatF* (**Figure for Reviewer 1b**). At the cellular level, we first examine sub-populations of sex-specific *fruP1*-positive neurons. As expected, this allele induces the elimination of the P1a male-specific neurons (visualised with the *R71G01-Gal4.DBD*, *R15A01-p65.AD* split reporters, PMCID: PMC6246841) (**Figure for Reviewer 1c**) and the appearance of the female-specific *GMR19B09*-positive neurons (PMCID: PMC4780643, **Figure for Reviewer 1d**). It also causes the sex reversal of the only structure controlled by the sexual identity of the *fruP1*-positive neurons outside the brain: the muscle of Lawrence (PMID: 38355873, **Figure for Reviewer 1e**). We then looked more broadly to visualise the entire *FruM*-positive neuronal network using two independent tools: an antibody against *FruM* (PMID: 35124238) and a V5 knock-in into the *fru* gene (*fru*^{V5}, PMID: 33463521). Both approaches clearly show that the entire *fruP1*-positive network is sex reversed in these flies, as not a single neuron is positive for *FruM* in these male brains (**Figure for Reviewer 1f**). Finally, as expected, the feminisation of the *fruP1* neuronal network triggers male sterility at the phenotypic level (**Figure for Reviewer 1g**). Together, these new experiments, combined with our old data, reaffirm the reliability of our *fru*-Gal80 and *fru*-*tra2* lines, as they faithfully recapitulated the *fru*

expression pattern, with *Gal80* and *tra2* sequence expressions under the control of the endogenous *fru* regulatory elements.

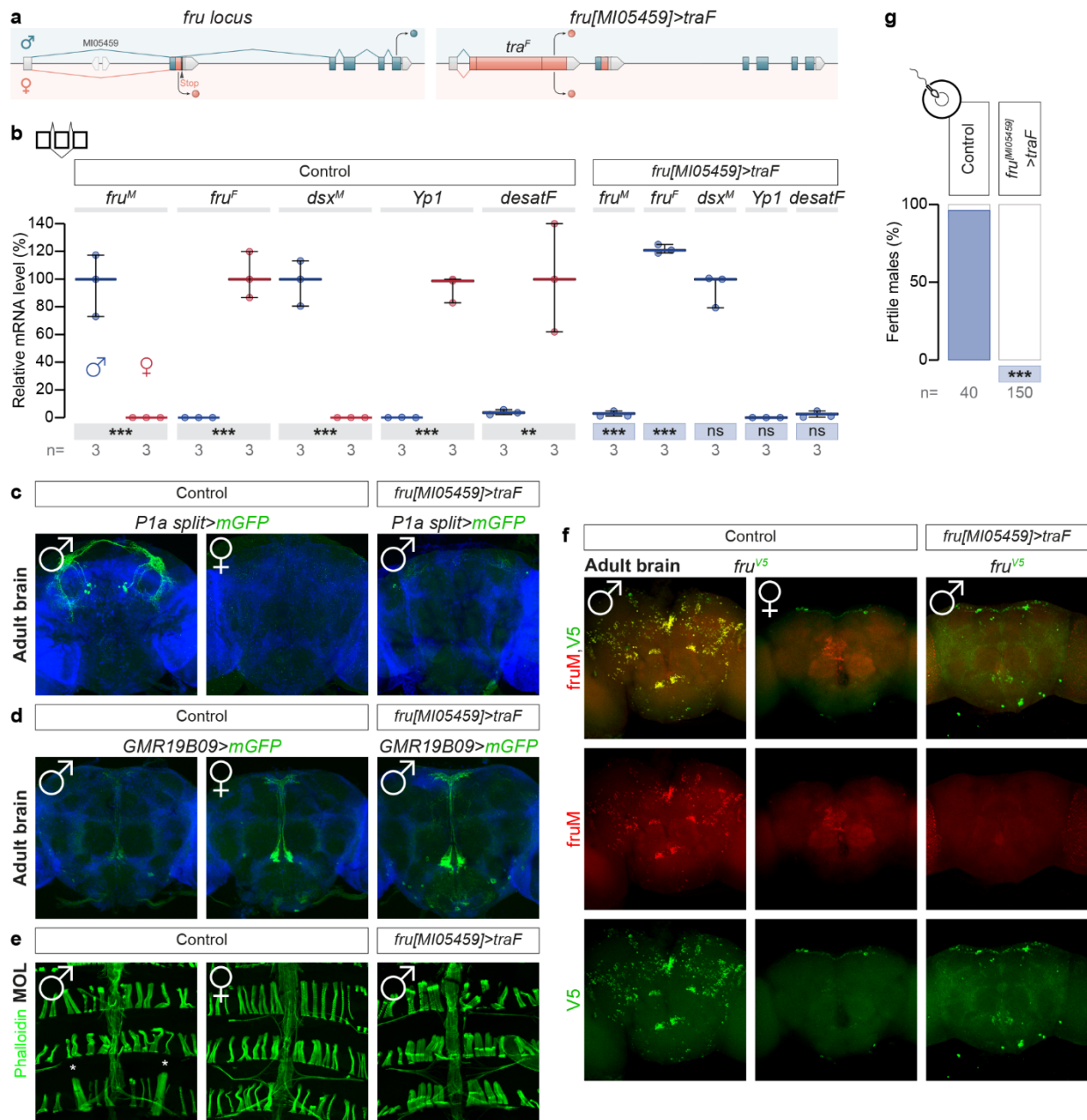


Figure for Reviewers 1.

Figure for Reviewers 1: Molecular and functional validation of *fru*[MI05459] swapping.

a, Diagrams representing the genomic *fru* locus and the new *fru*[MI05459]>*traF* allele generated by *fru*[MI05459] MiMIC swapping. **b**, RT-qPCR expression data for *fru*, *dsx* sex-specific isoforms, and the *DsxF* target genes, *Yolk protein 1* (*Yp1*) and *desatF* in *fru*[MI05459]>*traF* males in relation to relevant controls. **c**, Expression pattern of the *P1a split>mGFP* (*R71G01-Gal4.DBD*, *R15A01-p65.AD*) reporter in adult brains of control flies and *fru*[MI05459]>*traF* males (DNA labelled with DAPI, blue; the membrane-bound form of GFP, green). **d**, Expression pattern of the *GMR19B09-Gal4* reporter in adult brains of control flies and *fru*[MI05459]>*traF* males (DNA labelled with DAPI, blue; the membrane-bound form of GFP, green). **e**, Dorsal musculatures of control flies and *fru*[MI05459]>*traF* males. The male-specific muscles, called the muscles of Lawrence (MOL), are indicated by asterisks. (Actin stained with phalloidin, green). **f**, Expression patterns of the male-specific products of the fruitless (*fruM*) gene in adult brains of control flies and *fru*[MI05459]>*traF* males are visualised using an anti-FruM antibody and an anti-V5 antibody using a V5-tagged knock-in into the *fru* gene (*fruM*, red; V5, green). **g**, Male fertility quantifications of control, and *fru*[MI05459]>*traF* males. Data combined from at least three independent experiments. n = number of replicates (each repeat containing fifteen flies) in (b), and number of flies in (g). Asterisks highlighting significant comparisons across sexes are displayed in gray boxes at the bottom of graphs; those highlighting significant comparisons within male datasets are displayed in blue. p-values from Mann-Whitney-Wilcoxon tests are (ns) p > 0.05, **p < 0.01, ***p < 0.0001.

Please also note that, as previously mentioned, the Bellen lab developed and validated this approach (PMID: 21985007).

Furthermore, we used these alleles to confirm and extend previous studies suggesting that non-canonical sex determination pathways (i.e., independent of *fru* and *dsx*) that rely on alternative splicing play a key role in establishing multiple phenotypic differences between the sexes (size, weight, sex organ formation). Various independent genetic manipulations (10 overall, including bidirectional reversals) of *tra* and its cofactors *tra2* corroborate our main conclusions (for ***traF* loss-of-function**: **1/** *dsx-Gal4, fru-Gal4>UAS-flp, tra^{KO FRT traF}*, **2/** *tra^{KO}*, **3/** *tra^{ΔRS}*, **4/** *nub-Gal4>UAS-flp, tra^{KO FRT traF}*; ***traF* gain-of-function**: **5/** *dsx-Gal4, fru-Gal4>UAS-flp, tra^{FRT KO}*, **6/** *tra2-Gal4, fru-Gal80, dsx-Gal80>UAS-traF*, **7/** *nub-Gal4>UAS-flp, tra^{F FRT KO}*; for ***tra2* loss-of-function**: **8/** *traF-Gal4, fru-Gal80, dsx-Gal80>UAS-tra2^{RNAi}*, **9/** *tra2^{KO}*, *dsx>tra2, fruP1>tra2*, **10/** *tra2^{KO}*). All of them have similar phenotypes. So, our *fru-Gal80* and *fru>tra2* lines are optional to support the main messages of our works; they add supplementary evidence to support our conclusions.

Utilising the Gal4/UAS system, in conjunction with Gal80 or FLP, can be challenging when dealing with developmentally regulated gene expression. A lag in the Gal4 expression/UAS activation can result in missing critical developmental windows of expression. This system has been historically problematic when trying to ‘rescue’ sex differences. This system has been historically problematic when trying to ‘rescue’ sex differences. For example, in Ren et al., 2016 and Rideout et al., 2010, using *dsx-Gal4* to try to inhibit cell death (via UAS-p35) is insufficient to rescue male-like neuron numbers in females. Even more problematic is using this system to turn off *traF*, as then you rely on Gal4/UAS expression of FLP and then the decay of endogenous *traF*.

We fully acknowledge the Reviewer’s comment that utilising the Gal4/UAS system, in conjunction with Gal80 or FLP, can induce a lag in gene expression. However, we want to stress that, in our case, this potential lag has no functional impact. Our genetic manipulations using the Gal4/UAS + Gal80 or FLP (like **1/** *dsx-Gal4, fru-Gal4>UAS-flp, tra^{KO FRT traF}* or **2/** *traF-Gal4, fru-Gal80, dsx-Gal80>UAS-tra2^{RNAi}*) have identical impacts and fully recapitulate the phenotypes obtained with equivalence manipulations not using the Gal4/UAS system (like *tra2^{KO}*, *dsx>tra2, fruP1>tra2*). Even more striking, these manipulations yielded similar phenotypes to our three null mutant conditions (**1/** *tra^{KO}*, **2/** *tra^{ΔRS}*, **3/** *tra2^{KO}*). Therefore, we are confident that this issue does not impact our conclusions.

The authors should be mindful of comparing different genetic variants of the white gene, as some panels compare individuals that are w- vs. w+ vs those that contain mini-white. The white gene encodes a metabolically relevant protein, an ATP-binding cassette G-subfamily half-transporter, which may affect phenotypes such as size and weight. This should be discussed or controlled for by replacing the w- allele with w+.

We agree with the Reviewer's feedback, and in response, we conducted additional experiments using a *white+* genetic background. We replicated *traF* loss-of-function and *traF* gain-of-function and substituted the w- allele with w+ (see **Figure for Reviewers 2**, below). Our findings suggest that TraF's impact on organ size is unrelated to the presence of the *white* gene, as we observed an identical phenotype to that in the w- background (in comparison to Fig. 5b). Furthermore, our new data (**Fig. 3k-m**) also indicates that TraF's influence on adult organ size occurs through a cell-autonomous mechanism. The organ under consideration (wing pouch cells) does not express the *white* gene (data from PMID: 24670639).

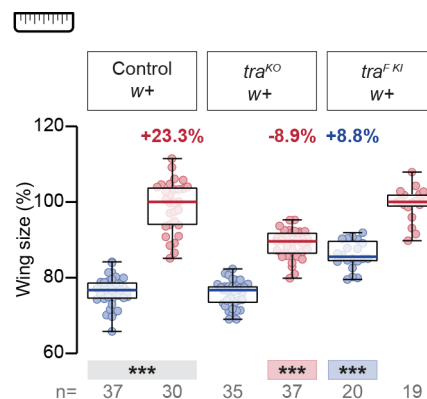


Figure for Reviewers 2.

Figure for Reviewers 2: TraF's impact on organ size is unrelated to the presence of the *white* gene.

Adult wing size quantifications following *tra* knock-out and *traF* gain-of-function in a *white+* genetic background. n = wing number measured per genotype. Asterisks highlighting significant comparisons across sexes are displayed in gray boxes at the bottom of graphs; those highlighting significant comparisons within female and male datasets are displayed in red and blue boxes, respectively. When significant, the difference compared to the median of the controls is indicated. p-values from Mann-Whitney-Wilcoxon tests are ***p < 0.0001.

Notes on the text:

Line 59: Not all DsxM-expressing neurons co-express FruM

This point is now clarify (please see text **lines 58-61**).

Line 66: it is not true to say that dsx and fru are no longer necessary in adulthood. See the following review:

doi.org/10.1111/gbb.12772

We want to thank the Reviewer for pointing out this oversight. We agree and have amended the text accordingly to reflect the field's current state better (please see text **lines 65-74**).

Line 44: there is not a widely held belief in 'the two-gene model'- the main point of Robinett et al. (2010) was to say that not all sex differences are due to one step- the sex-specific expression of SXL, but rather developmental and cell-specific regulation of dsx and fru give mosaic sexual differentiation.

We have amended the text accordingly (please see **lines 58-78, 84-88 and 91-94**).

However, the last sentence of the author's summary in the publication of Robinett et al. (2010) is: "*Strikingly, dsx is only expressed in a subset of tissues. Thus, **adult flies of both sexes appear to be mosaics of cells that do know their sex and cells that do not know their sex.***" The conclusion in the results section is: "*Drosophila males and females are mosaics composed of some cells that know their sex (express dsx and/or fru) and sexually differentiate, and other cells that do not express dsx and/or fru and thus don't sexually differentiate.*" The overall conclusion of the discussion is: "***our general conclusion that most cells that don't express dsx and/or fru don't know their sex***". Since then, the two-gene model of fly sexual differentiation is still widely dominant in the community; just a few examples to illustrate this point:

- 1- In the introduction of a recent (2020) paper from Prof. Kenta Asahina's lab (PMID: 32314964), it is indicated: "*Instead of sex hormones, **Drosophila uses two sex-determining genes, doublesex (dsx) and fruitless (fru), to specify sexual dimorphisms at the cellular level.***"
- 2- In the introduction of a recent (2021) paper from Prof. Stephen Goodwin's team (PMID: 33508219), the authors claimed: "*Sex is determined early in an animal's development and initiates many irreversible sexual differentiation events that influence how the genome and the environment interact to give rise to sex-specific morphology and behavior. In Drosophila, selective expression of **two sex determination transcription factors (TFs), Doublesex (Dsx) and Fruitless (Fru)**, define cell-type-specific developmental programs that govern functional connectivity and lay the foundations through which innate sexual behaviors are genetically predetermined.*"
- 3- In one of the last reviews of the field published the previous month (April 2024) (PMID: 38185860), the authors started their review with the following sentences: "*As we will touch on for different molecular mechanisms throughout this review, the control of sex differentiation has two orthogonal components. One set of mechanisms determine which cells transcribe fru or dsx: **If a cell transcribes one or both of these factors, it has the potential to be sensitive to cellular sex.***"

In 2007, Prof. Michael McKeown and Prof. Troy Shirangi wrote in a review (PMID: 17475234): "*It is assumed that fru and dsx are together the only genes directly regulated by tra for sexual behavior. This is consistent with inferences from the combined phenotypes and with the lack of any clustered tra regulatory sites, as identified in dsx and fru, associated with any other gene sequenced in Drosophila. **This inference is still based on lack of evidence. For example, the two-gene model for control of behavior predicts that expressing both DsxM and FruM would be sufficient to fully masculinize female behavior, even with tra active. This has not been tested, but should be possible with today's technology***". Since 2007, the two-gene model of sexual differentiation has never been experimentally tested, making our current manuscript a significant contribution to the field.

Line 68/69: the conclusion is not that all other cells do not have a sexual identity- we know that all do- this comes down to what you mean by sexual identity.

We removed the term "sexual identity" and clarified our hypothesis (please see text **lines 74-78**).

Line 78: The phrase “phenotypic consequences of having sex chromosomes” is confusing. Is the idea here to separate the presence of sex chromosomes from the SDH? It is not clear this is what you are doing when you focus on members of the SDH to make your arguments- this just says we don’t know everything about how the SDH determines sex differences.

We amended the term "*phenotypic consequences of having sex chromosomes*" and explained our hypothesis more precisely (please see text **lines 84-88**).

Line 98: These observations DO NOT suggest TraF lacks expression/function in most organs- that is one hypothesis, but it is equally likely it is expressed everywhere- in fact, it seems more likely this would give small changes in fru/dsx expression the maximum chances of evolving novel sex differences.

We have amended the text accordingly (please see text **lines 105-108**).

Line 131: if traF is expressed everywhere, it is not necessarily always functioning to produce sex differences- just like SXL and Tra2, a splicing target makes the differences. So, it follows that for a Drosophila cell to “know” its sex, it must have a mechanism for translating the traF into sexual identity. If a cell does not express a downstream factor such as Dsx or Fru, how does it interpret the information about traF, so it can develop differently in males and females. What mechanism would traF confer sexual identity to cells that are not expressing terminal differentiation genes like fru and dsx? Would this be in a cell- or non-cell-autonomous manner.

This is an interesting point. To investigate the cell-autonomous vs. non-cell-autonomous contribution of TraF on adult size, we have conducted the following additional experiments:

1-First, specifically in the fat body, we knocked out *traF* in females and knocked in *traF* in males and measured the impacts on adult wing size. Neither of these two genetic manipulations affects wing size. Fat body-specific *traF* expression in a *traF* null mutant background also does not rescue female wing size. Correspondingly, fat body-specific *traF* knock-down and *traF* over-expression do not impact wing size. From these experiments, we can conclude that *traF* expression in the fat body is not required for sex differences in adult wing size.

2-Conversly, wing disc-specific *traF* knock-out or *traF* knock-in significantly influences wing size. Furthermore, the effects obtained recapitulate and are similar to whole-body *traF* manipulations. Interestingly, wing disc-specific *traF* expression in a *traF* null mutant background completely rescues female wing size. Even though every organ except the wing pouch is masculinised in these individuals, the adult wing is even slightly bigger than in wild-type females.

Our experiments have provided unambiguous results. They indicate that TraF's contribution to adult organ size is indeed mediated through cell-autonomous mechanisms.

The revised manuscript now incorporates these experiments in three new panels of Figure 3 (**Fig. 3k-m**). These additions enhance the understanding of TraF's role in adult organ size determination. For more details, please refer to the text **lines 210-223**.

Line 152: What is meant by sexual identity in this context? It is well known that cells have a sexual identity based on their chromosomal makeup and SXL expression, so it is not clear why expressing traF makes a big difference in defining the sexual identity- it is important information but not game-changing.

We removed the term "*sexual identity*" and clarified our hypothesis (please see text **lines 159-161**).

Line 160-162: classical SXL/tra/dsx/fru mutants suggested long ago that dsx and fru were not ‘sufficient’ for all sex differences.

We searched for original research articles (in addition to the ones already cited) that provide experimental evidence of *tra* functions independent of *dsx* and *fru*. However, we were unable to find any. Of course, we are open to citing any published works that we may have overlooked.

Line 204: How can it be concluded that all cells not captured are ‘monomorphic’?

"The sex of organs that are sexually monomorphic is necessary and sufficient to drive sex differences" This sentence seems to contradict the whole premise- if there are still sex differences, then there must be dimorphism.

We removed the term "*monomorphic*" and further clarified our conclusions (please see text lines 225-229).

General notes for Figure 4:

Looking at splicing in the mature adult is not the same as confirming sex-specific splicing occurs at normal levels when and where it is needed during development. The authors have made a *dsxF* constitutive allele using a novel approach; given this, it is incumbent upon the authors to show it works as expected. For example, in Extended Data Fig. 4e, this mutant is not used as the control, even though it is used in the previous panel. This approach makes interpreting the result problematic.

Considering these comments, we now provide a more thorough characterisation of our new *dsxF* constitutive allele. In addition to the molecular characterisation of *dsx* splicing and *dsx* direct target gene expression, we performed the following experiments:

-1/ At the cellular level, we show that this allele can eliminate the expression of the male-specific *R17G11-Gal4* reporter in the ventral nerve cord (PMID: 26884206) in males and females in a *dsx* null mutant background (**Extended Data Fig. 4h**). Conversely, this allele can activate the female-specific expression of the *Yolk protein 1-Gal4* reporter in the adult fat body in males and females in a *dsx* null mutant background (**Extended Data Fig. 4g**).

-2/ At the organismal level, this new reagent also rescues the fertility of *dsx* null mutant females (**Extended Data Fig. 4j**).

Collectively, these results demonstrate that this allele is capable of executing all the crucial functions mediated by *DsxF* during both development and adulthood necessary for female differentiation and fertility (please see text lines 275-283).

Figure 4f: given potential delays in *fru-Gal4/UAS-FLP* expression and the decay of *D.mel traF*, it would be hard to rule out *fru* cells based on this strategy.

We completely agree with the reviewer's comment. We are using these experiments only to conclude that the new *TraF*-dependent mechanism is also active in *dsx*-expressing cells (please see text lines 273-275). We cannot rule out a role in *fru*-expressing cells undetected by our approach.

Reviewer #3 (Remarks to the Author):

This work utilizes sophisticated genetic tools to dissect the role of the essential sex determination factor *Tra* in regulating sex-specific characteristics. Importantly, the evidence presented indicates that *Tra* has functions in non-*dsx* and non-*fru* expressing cells to control sexually dimorphic characteristics and fertility. It is particularly surprising that fertility is affected as the cells of the gonad and reproductive tract are all thought to express *dsx*, and sometimes *fru*, meaning that this is likely an effect of more distant tissues on fertility. While it has been previously demonstrated that *Tra* controls traits like body size independently of *dsx* and *fru*, it was not known that this is partially controlled by cell types other than those that express *dsx* and *fru*. Further, the finding that the *Tra* co-factor *Tra2* is required for some aspects of these traits, like body size, but is not required for effects on fertility, is also of interest.

Many of the findings in this work are most appropriate for a specialized audience. It was previously thought that *Tra* expression is very broad and general in the animal, and this has been previously found in large-scale RNA-seq from different stages and tissues, but I'm not sure it has ever been directly shown. Therefore, it is very nice to see the general *Tra* expression and activity clearly presented here, but it does not change our thinking. In addition, as clearly described by the authors, it has previously been shown that *Tra* has *dsx*- and *fru*- independent functions, including in regulation of body size, but here they demonstrate that this is at least partly due to *Tra* effects in tissues other than those that normally express *dsx* and *fru*. Lastly, while it is good to know that mutating the RNA binding function of *Tra* acts as a loss of function allele, this was not much in doubt given that the only known functions for *Tra* so far

involve alternative splicing. I have seen some papers that appear to be highly specialized in Nature Communications before, and so this work may be appropriate for publication here.

The work expressing Tra from other species in *melanogaster* is perhaps the weakest part of the paper and could be removed. While I appreciate that they try and use these different Tra's to separate *dsx* and *fru* splicing from other Tra functions, I think it is also possible that these other Tra forms might act in unanticipated ways, reducing confidence in the conclusions.

Considering this comment (and in response to the editor's request that we keep these data), we tried to add more depth to these results. We extended our work using TraF proteins of different *Drosophila* species by performing the following additional experiments:

1- We generated a new knock-in line that replaced the coding sequence of *D. melanogaster traF* with sequences from a more distant species: the beetle *Tribolium castaneum*. TraF from this species is slightly more divergent than *D. virilis* TraF (35% sequence identity with *D. melanogaster* compared to 48% for *D. virilis*). As envisioned, this knock-in line is a negative control of our cross-species transgenic approach as it cannot rescue *D. melanogaster* functions. When heterozygous with a *tra* null mutant, this allele produced classical *tra* loss-of-function morphological phenotypes, such as female-to-male sex reversals (**Extended Data Fig. 4a-d**). This beetle protein cannot induce female-specific splicing (of *dsx* and *fru*, **Extended Data Fig. 4a**) or sustain female organ size and fertility (**Extended Data Fig. 4c and d**).

2- We independently replicated our results obtained with *D. virilis* by producing a new knock-in line. This line swaps the *traF* of *D. melanogaster* with sequences from *D. pseudoobscura*. The degree of divergence of both proteins with *D. melanogaster* is similar (50.9% versus 48%, **Fig. 4a**). As anticipated, the *D. pseudoobscura* protein triggered *dsx* and *fru* female-specific splicing (**Extended Data Fig. 4a**) and female size (**Extended Data Fig. 4c**), but it was unable to support female fertility (**Extended Data Fig. 4d**). These findings strengthen our conclusion that *D. virilis* and *D. pseudoobscura* factors can only partially activate the new TraF-based non-canonical sex determination pathways (i.e., independent of *fru* and *dsx*).

3- We described more precisely the sterility phenotype induced by the knock-in of *D. virilis traF*. Indeed, these females have normal ovaries containing ovarioles with developing egg chambers at all stages (including late vitellogenic stages, **Extended Data Fig. 4k**). However, these females cannot lay eggs and display a bloated abdomen (**Fig. 4b**), indicating egg retention. The Hox gene *Abdominal-B* (*Abd-B*) controls the formation of the posterior segments, including the cells implicated in egg-laying behaviour (like motoneurons, the oviduct, or the vaginal plate). Expressing *D. virilis traF* specifically in *Abd-B* expressing cells sterilised females (**Extended Data Fig. 4l**), indicating that the egg retention phenotype could result from defects in the sexual differentiation of these somatic cells.

The revised manuscript now incorporates these experiments in seven new panels of Extended Data Figure 4 (**Extended Data Fig. 4a-d and k-l**). These additions enhance our conclusion that divergent TraF factors can only partially activate the new TraF-based non-canonical sex determination pathways (i.e., independent of *fru* and *dsx*). (please see text **lines 253-267 and 275-291**).

In addition, I would suggest a few changes to the way the manuscript is presented. First, the genetics performed, while quite elegant, might be difficult for the general audience to grasp. They have made good headway in making this more accessible, including with the diagrams in the figures, but it could still be improved in the text.

We tried to improve the text and have included additional diagrams for each new allele, hoping to clarify the issue (please see text **lines 175-179, 186-187, 195-197, 210-229, 253-267, 275-291**).

Further, I disagree with the way a few terms are used. First, they refer to all *dsx*- and *fru*-expressing cells as "sex organs". This may be confusing to people that consider the gonads and genitalia as the primary sex organs. Tissues like the fat body, which express *dsx* have sexually dimorphic function, would not be considered "sex organs" by most people.

It's a valid point; we simplified our terminology based on this and never refer to *dsx*- and *fru*-expressing cells as "sex organs".

Second, they refer to "primary" and "secondary" sex tissues in a manner that is different from what I feel is common in the field. Normally, I would consider a "primary" sex tissue or cell as one that directly assesses the sex

determination mechanism (XX or XY in this case), while a secondary sex tissue is one that receives information from another source (e.g. a primary sex tissue) about what sexual path to follow. In this work, its not clear what they mean by secondary sex tissue, but it seems these tissues express *dsx* or *fru* and are directly responsive to the sex chromosome genotype, making them really “primary” sex tissues.

We agree, and we have removed all references to “primary” and “secondary” sex tissues.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

the authors have responded satisfactorily to all the comments and criticisms of the various reviewers. I have no other concerns.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of the reviewers' criticisms (including some organizational changes), and the paper seems better for this.

However, the new experiments, which are apparently testing cell-autonomous versus non-cell-autonomous effects of traF in panels 3k-m, and the text (210-223) are hard to follow and seem to contradict previous work from the Rideout group.

"Based on our observations, it appears that TraF exerts its splicing activity in a wide range of cells, including some that do not express fru or dsx" Given the only known role for Tra is in alternative splicing, how do the authors envisage this mechanism is working? What are the target genes?

Reviewer #3 (Remarks to the Author):

I feel the authors have addressed many of the reviewer issues and the paper is appropriate for Nat Comm

Response to Reviewers

Reviewer #2 (Remarks to the Author):

The authors have addressed most of the reviewers' criticisms (including some organizational changes), and the paper seems better for this. However, the new experiments, which are apparently testing cell-autonomous versus non-cell-autonomous effects of *traF* in panels 3k-m, and the text (210-223) are hard to follow and seem to contradict previous work from the Rideout group.

To strengthen and clarify the cell-autonomous vs. non-cell-autonomous contribution of TraF on adult wing size, we have conducted the following additional experiments:

1. First, specifically in the wing pouch cells, we knocked down *traF* in females and over-expressed *traF* in males and measured the impacts on adult wing size. These two genetic manipulations significantly affect wing size, mirroring whole-body *traF* manipulations.
2. Conversely, we knocked down *traF* from all wing disc cells except the pouch and didn't observe an effect on wing size.

These results confirm our previous findings that TraF controls the size of adult organs exclusively through cell-autonomous mechanisms. The revised manuscript now incorporates these experiments in two new panels of Figure 3 (**Fig. 3l-m**). We have also taken steps to simplify the text, making it more accessible and easier to understand (for more details, please refer to the text **lines 210-229**).

In response to the potential contradiction with previous work from the Rideout group (PMID: 26710087), we want to clarify the following points:

1. The Rideout publication measured pupal volume, while our work focused on adult wing size. Therefore, we are examining different functional readouts. We are specifically investigating the potential influence of the sexual identity of the adipocytes to eliminate a similar mechanism in the context of organ size determination.
2. Only fat body-specific *traF* over-expression was conducted, with no effect on male pupal size (S5 panel B). We obtained identical results as shown in Extended Fig. 3l-m. Fat body-specific *traF* knock-down or knock-out was not tested in the mentioned publication.
3. Our present experiments, using newly developed genetic tools, encompass the following:
 - Tissue-specific *traF* knock-out (Fig. 3k)
 - Tissue-specific *traF* gain-of-function (Fig. 3k)
 - Tissue-specific rescue of *traF* null mutant (Fig. 3k)
 - Tissue-specific *traF* knock-down (Fig. 3l-m)
 - Tissue-specific *traF* over-expression (Fig. 3l)
 - Tissue-specific *tra2* knock-out (Fig. 5g)
 - Tissue-specific rescue of *traF* null mutant with splicing-deficient TraF variant (Extended Fig. 5b)

These experiments collectively demonstrate that TraF exclusively regulates the size of adult organs through cell-autonomous mechanisms.

"Based on our observations, it appears that TraF exerts its splicing activity in a wide range of cells, including some that do not express fru or dsx" Given the only known role for Tra in alternative splicing, how do the authors envisage this mechanism is working? What are the target genes?

We have clarified this point in our manuscript (please refer to the text **lines 384-386**). Our results suggest that the mechanism involves alternative splicing of targets other than *dsx* and *fru*. Identifying these splicing isoforms and characterizing their functions in the context of specific readouts (such as wing size or adult weight) is beyond the scope of the current manuscript and will require many years of work. Interestingly, previous transcriptomic experiments by Michelle Arbeitman (PMID: 21756339) have already identified numerous sex-biased splicing isoforms downstream of TraF, in addition to *dsx* and *fru*. However, these experiments were conducted on heads and may not have identified isoforms relevant to the phenotypes investigated here (wing size, adult weight, or sex organ determination).

Nevertheless, they provide evidence of the existence of uncharacterized sex-biased isoforms downstream of TraF. Specifically, they found 44 and 93 transcript isoforms with robust female- and male-biased expression differences downstream of *traF*. Interestingly, some of these isoforms contained Tra2-TraF binding sites, for example *Psa*, like *dsx* and *fru* (PMID: 21756339).

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have responded satisfactorily to all the comments and criticisms of the various reviewers.