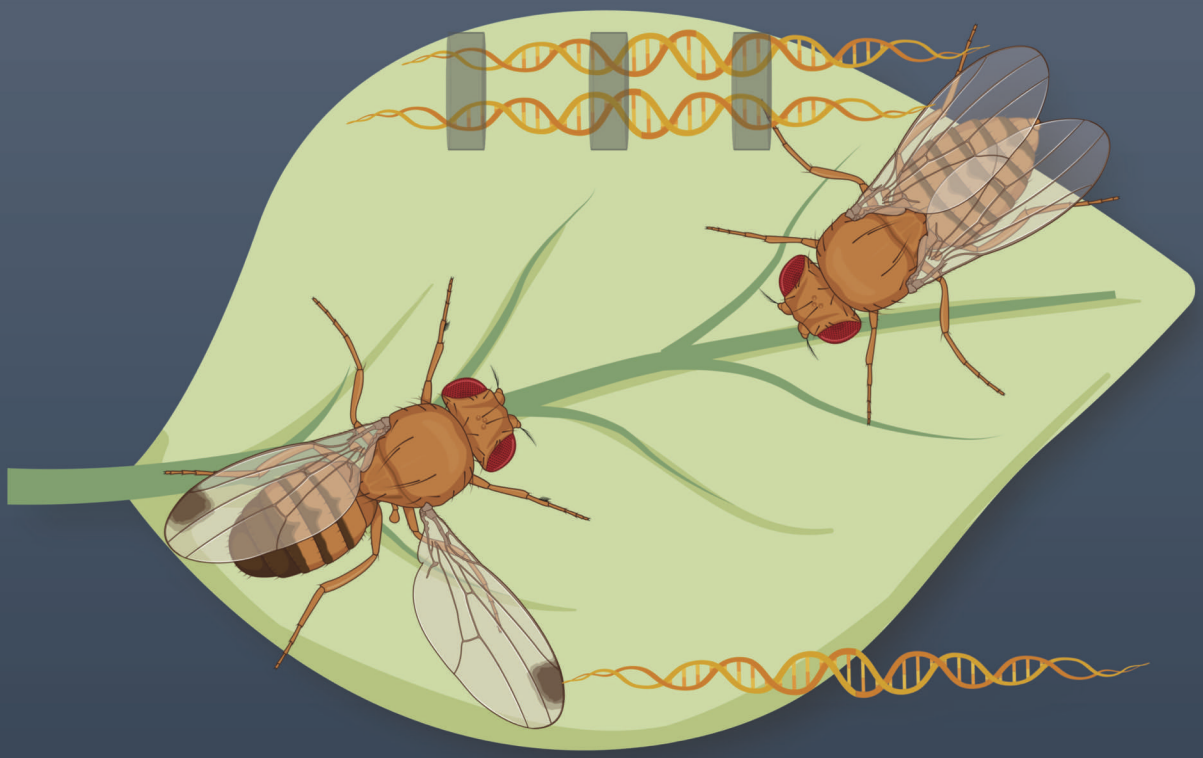

trans-homolog interaction of *yellow* controls its sex-biased expression pattern in the wing of *Drosophila biarmipes*

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Intitulée

La regulation en trans entre homologues du gène *yellow* controle son profil d'expression dimorphique entre les sexes dans l'aile de *Drosophila biarmipes*

Effectuée sous la direction du Dr. Benjamin Prud'homme

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Στην οικογένεια μου

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Enjoy your reading

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RÉSUMÉ

Les différences de morphologie, de comportement et de physiologie spécifiques au sexe sont très répandues dans le règne animal, ce qui donne lieu à un phénomène appelé dimorphisme sexuel. Le dimorphisme sexuel est largement attribué à l'expression différentielle des gènes entre les mâles et femelles, qui est elle-même contrôlée par les hiérarchies génétiques qui détermine le sexe de chaque individu. Les mécanismes moléculaires qui sous-tendent l'interaction entre ces hiérarchies et les gènes qui présentent une expression sexuée restent mal connus. J'explore ici la base génétique de l'expression sexuée du gène de pigmentation *yellow*, porté par le chromosome X. Le profil d'expression de *yellow* dans les ailes de pupes mâles de *Drosophila biarmipes* préfigure l'apparition chez les adultes d'un motif de pigmentation spécifique aux mâles. En utilisant des transgènes contenant de gène *yellow*, je montre que les interactions régulatrices entre les allèles homologues de *yellow* réduisent son expression chez les femelles. De plus, l'insertion de ces transgènes à des positions homologues sur des chromosomes autosomes récapitule, chez l'un ou l'autre sexe, l'inactivation du profil d'expression de *yellow* dans l'aile. Cette inactivation nécessite l'intron de *yellow* ainsi qu'un activateur situé dans la région 5' non-codante du gène. Un crible par interférence à l'ARN identifie la protéine architecturale Mod(mdg4) comme un composant essentiel de l'inactivation dépendante de l'homologie. En outre, je montre que Mod(mdg4) est nécessaire pour l'expression sexuée de certains gènes portés par le chromosome X dans le cerveau de *Drosophila melanogaster*. Ces résultats suggèrent que les interactions entre homologues en *trans* régulent l'expression sexuellement biaisés de certains gènes liés à l'X, indépendamment de la hiérarchie génétique de détermination du sexe. Plus généralement, ils illustrent la pertinence biologique de l'appariement des chromosomes homologues et des interactions en *trans* entre ces chromosomes homologues dans le contexte de l'expression sexuellement biaisée des gènes du chromosome X.

SUMMARY

Sex-specific differences in morphology, behavior, and physiology are widespread in the animal kingdom, shaping a phenomenon called sexual dimorphism. Sexual dimorphism is largely attributed to sex-biased gene expression, which, in turn, is controlled by the sex-determination hierarchies. The molecular details underlying the interaction between these hierarchies and genes that display sex-biased expression remains elusive. Here I explore the genetic basis of a male-biased expression pattern of the X-linked pigmentation gene, *yellow*. *yellow* expression prefigures a male-biased adult pigmentation pattern in *Drosophila biarmipes*. Using transgenes containing *yellow*, I show that regulatory interactions between *yellow* homologous alleles silence its expression in females. Furthermore, inserting these transgenes at homologous positions on autosomes recapitulates, in either sex, the homologous-dependent silencing. This silencing necessitates the intron of *yellow* as well as an enhancer located on the 5' of it. Performing and RNAi screen identifies the architectural protein Mod(mdg4) as an essential component of the homologous-dependent silencing. Additionally, I show that Mod(mdg4) is required for the sex-biased expression of some X-linked genes in *Drosophila melanogaster* brains. These results suggest that *trans*-homolog interactions regulate sex-biased X-linked genes, independently of the canonical sex-determination hierarchy. More generally, they illustrate the biological relevance of homologous chromosome pairing and *trans*-homolog interactions in the context of sex-biased gene expression.

Introduction

SEXUAL DIMORPHISM

"We should venture on the study of every kind of animal without distaste; for each and all will reveal to us something natural and something beautiful."

Aristotle

GENERAL

Mandrills' difference in size, pheasants' difference in the color of plumage, ornamentation in butterflies, and lions' mane illustrate some of the differences that sexes of the same species might display (Figure 1). These differences are not restricted to morphological traits but also can be observed in behaviors, in physiological processes, and even in life-history traits. This phenomenon is called sexual dimorphism and is widespread in the animal kingdom. A distinction has already been made by Hunter on the type of sexual characters that animals have. Primary sexual characters are the ones that are different between sexes and are directly involved in reproduction such as the gonads. Secondary sexual characters are the ones that again differ between sexes but are less involved in reproduction or not at all. For example, the breasts in female humans, although they are not directly involved in reproduction they are necessary for the growth of the progeny. Male horn beetles use their horns in fights against other males to gain access to females for reproduction. In addition, differences might be better explained by different lifestyle between sexes than by success in reproduction. For instance, the anatomical difference observed in the proboscis of male and female mosquitos can be explained by the different niches with regard to food habits. Males cannot pierce animal skin, but feed exclusively on the nectar of plants, while females, in contrast, need their blood meal for reproduction. For the scope of this thesis, my focus will be on secondary sexual characters.

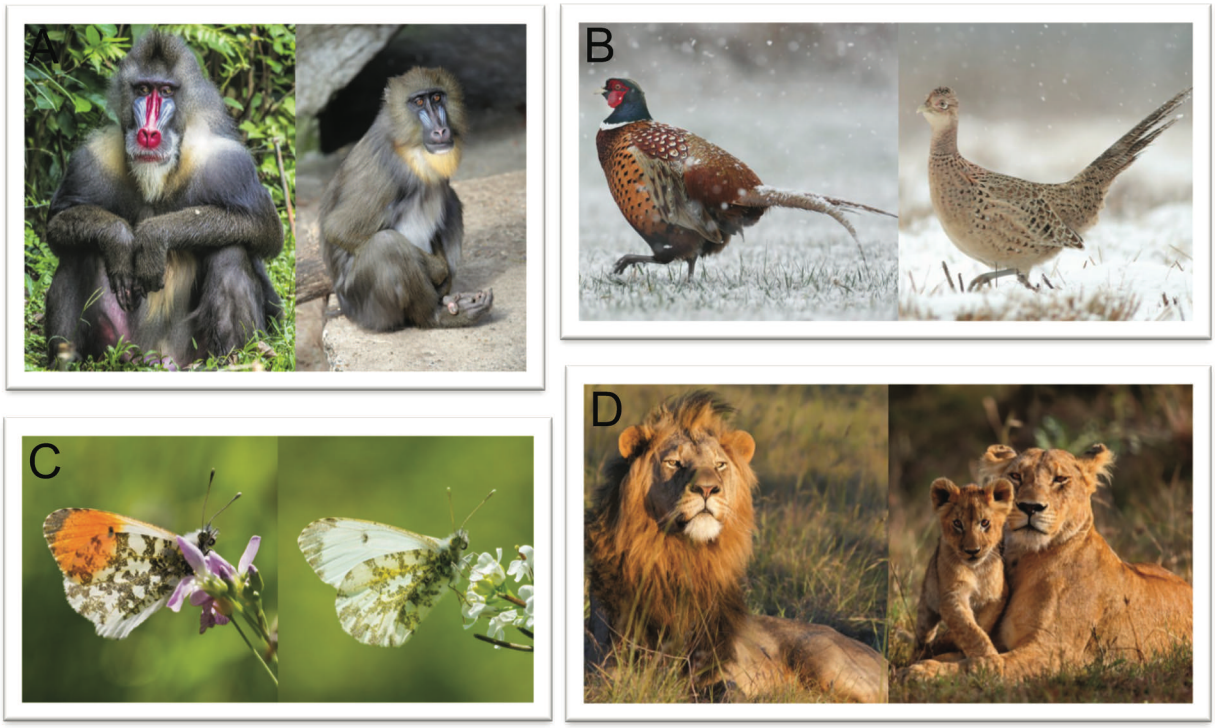

Figure 1. Males and females of mandrills (**A**), pheasants (**B**), butterflies (**C**), and lions (**D**). In all the cases differences in morphological characteristics between sexes of the same species can be observed. (Photo Credit: Curioso, Edwin Butter, Shutterstock (A), Piotr Krzeslak, Szczepan Klejbuk, Shutterstock (B), Matthijs Wetterauw, Andermeertinsphotography, Shutterstock (C), Jason Prince, Maggy Meyer, Shutterstock (D)).

A central question that dominates biology even today is how sexual dimorphism evolves. Since the principal focus of this study is not on the evolution of sexual dimorphism I will briefly discuss some important concepts in this section. Writing to Asa Gray, Charles Darwin thanked him for his positive view on the "Origin of Species" and stated the following:

> *"The sight of a feather in a peacock's tail, whenever I gaze at it, it makes me sick!"*

When C. Darwin finished his book on the "Origin of Species", where he explained the evolution of life by means of natural selection, he was purely frustrated by the fact that the evolution of sexually dimorphic characteristics cannot be explained by it. As a result, twelve years later he published a new book entitled "The Descent of Man, and Selection in Relation

to Sex", in the second part of which he describes a vast number of examples of sexual dimorphism and tries to justify their evolution through sexual selection. He stated that:

> *"Sexual selection depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction."*

In his work, he described mechanisms such as mate choice or mate competition to explain sexual dimorphism. Back in time Alfred Russel Wallace was not initially accepting the concept of sexual selection and in the first hand he was advocating for natural selection is sufficient to explain mate competition and he completely rejected the idea of mate choice (*1*). Today we know that both perspectives can explain instances of sexual dimorphism.

The first suggested mode, by the means of which sexual selection acts, is mate competition. In this case, somebody could imagine males of one species fighting each other as in the case of the sunbeam snake, *Xenopeltis unicolor* (*2*) (Figure 2A-B). Male competition can be manifested, also, by sperm competition. In the chinook salmon, *Oncorhynchus tshawytscha*, males can increase the velocity of their semen to adapt to potential competition for mating (*3*). Infanticide is observed in several species including lions, mice, and beetles (*4–6*). In this case, males are killing infants if they are the offspring of another male.

Figure 2. Males in competition of *Xenopeltis unicolor*. The two males have their bodies bridged (**A**). Subsequently, they are shaping a coiled-like structure and bite each other (**B**). (source: (*2*))

In some cases, sexual selection relies on the choice of a mate by the opposite sex. Usually, this "selection" occurs by the females although there is more and more evidence that males are, also, choosing their mate (*7*). In this case, the choice can rely on several possibilities

such as if the mate controls a specific territory with resources, if the mate is healthy or does not have parasites, etc.

Along with sexual selection, ecological mechanisms have been proposed to explain cases of the evolution of sexual dimorphism. The ecological causation hypothesis has, also, been suggested by Darwin on "The Descent of Man, and Selection in Relation to Sex", however, it was less popular at this time. Decades later received considerable attention (*8*, *9*). Intersexual niche divergence is a mechanism by which intersexual competition is decreased and sexual dimorphism develops to serve this. One of the first cases where ecological causation (intersexual niche divergence) was attributed to the evolution of sexual dimorphism was in 2000 studying tropical hummingbirds. The bills of the hummingbird, *Eulampis jugularis*, differ in length and shape in a sex-specific manner. They are feeding on two *Heliconia* plant species according to their bill morphology suggesting that niche divergence drove the evolution of this trait (*10*).

The second major arising question is what are the genetic rules governing the formation of the secondary sexual characteristics, considering that the sexes of the same species, usually, share in an almost identical manner the same genome. Even in cases in which the genome is completely identical and the sex is determined by the environment, extensive dimorphism is observed. For example, in the turtles, *Terrapene carolina* and *T. ornate*, which use environmental cues to determine their sex, females appear to be larger (*11*). Over the decades, considerable efforts have been made to address this point from pioneering embryologists studying sex determination in rabbits to modern geneticists studying worms, flies, humans and several other species. With the developing technology, concepts are reconsidered and reestablished setting new frameworks on how sexual dimorphism develops.

One of the breakthroughs towards this was the discovery of the mechanisms by which sex is determined in animals in the first place. Sex is determined by differentiating the tissues that produce the gametes. The discovery that genetic hierarchies (sex-determination hierarchies) set the sex of an individual opened an avenue towards understanding the genetic control of secondary sexual characteristics. Through the pioneering work of Calvin Bridges on the genetics of fly sex determination, it was obvious that in strains where sex was not determined properly led to the creation of intersexes in which secondary sexual characters were affected such as pigmentation or sex combs. Already then a connection between sex-determination and the development of sexual dimorphism was made. Understanding the

genetic grounds on how sex is determined in an individual was put central to understand the ontogeny of sexual dimorphism.

SOMATIC SEX DETERMINATION

There are a plethora of mechanisms underlying sex determination in animals. All living beings are a result of the interaction between genetics and environments. This is also true for the sex determination systems. So, the first classification can be made upon the usage of the genome or the environment as the onset signal. For example, some reptiles respond to specific environmental cues leading to their sex determination, and this is called environmental sex determination. In contrast, genotypic sex determination requires genetic cues as the primary signal. That is well illustrated by some insects that use a specific type of chromosomes, the so-called sex chromosomes.

In some species, the male and the female provide different gametes. These differ in the aspect that they carry either a single gene, or a non-recombining region along a chromosome, or an entire sex chromosome, and this is called heterogametic genotypic sex determination. In some other species, sex can be also determined by the number of homologous sets of chromosomes shaping a mechanism called haplodiploidy. More specifically, males develop from unfertilized eggs and as a result, are haploid. In contrast to females that develop from fertilized eggs and are diploid (*12*, *13*).

The sex chromosomes were initially discovered by Hermann Henking in 1891 studying the firebug *Pyrrhoris apterus*. While observing its chromosomes he realized that one chromosome was not behaving like the rest during meiosis and he named this as X-element (*14*). This element later on named as “accessory chromosome” was shown by Clarence Erwin McClung in 1901 to be necessary for sex determination (*15*). It is worth noting that this is one of the first cases that supported the chromosomal theory of inheritance.

In 1905, while studying mealworm beetles, Nettie M. Stevens stated in her monograph on spermatogenesis (*16*):

> *"Since the somatic cells of the female contain 20 large chromosomes, while those of the male contain 19 large ones and 1 small one, this seems to be a clear case of sex- determination, not by an accessory chromosome, but by a definite difference in the character of the elements of one pair of chromosomes of the spermatocytes of the first order, the spermatozoa which contain the small chromosome determining the male sex, while those that contain 10 chromosomes of equal size determine the female sex. This result suggests that there may be in many cases some intrinsic difference affecting sex, in the character of the chromatin of one-half of the spermatozoa, though it may not usually be indicated by such an external difference in form or size of the chromosomes as in Tenebri."*

In parallel, Edmund Beecher Wilson studying a different species came to similar conclusions. For that today the genetic basis of sex determination is credited to both of them (*17*).

INSECT SEX DETERMINATION

Studies on insect sex determination started in the earlies of the 20th century with pioneer work from Calvin Blackman Bridges in *Drosophila melanogaster* (*D. melanogaster*). Males of *D. melanogaster* are heterogametic carrying an X and a Y chromosome (XY) while females are homogametic and carry a couple of X chromosomes (XX). He suggested that the number of sex chromosomes (X chromosomes) in relation to the autosomes determines the sex of an individual fly based on results with flies carrying multiple X chromosomes and autosomes (*18–20*). However, recently obtained data, illustrate that the number of X chromosomes determines the sex independently of the autosomes. In more detail, four X-linked genes are the readout of this X-chromosome counting, *scute*, *runt*, *sisA,* and *unpaired*. These collectively are termed as X-linked signal elements (XSE) and are responsible for activating the female-specific isoform of *Sex-lethal* (*Sxl*) (Figure 3A). Looking at the structure of the gene there are two promoters, the *Pe* and the *Pm*. The first step is to sense the X chromosome dose and that is credited to the XSE that potentially activates transcription through the *Pe* promoter. Afterward, transcription is starting only at *Pm*. Because then this is maintained through an autoregulatory positive feedback loop the transcript from *Pe* directs the initial splicing of the female version of Sxl

from *Pm* finally leading to a functional protein in contrast to the male version that contains a stop codon in exon 3 (Figure 3B). In females, Sxl controls the splicing of *transformer* (*tra*) that also leads to a functional protein (TraF). In contrast, males *tra* encodes a short open reading frame that does not produce a functional protein. At last, *tra* controls the sex-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*), which are the main transcriptional effectors of the sex determination hierarchy controlling the regulation of many genes in different tissues, including the gonads. In females this produces DsxF. For FruF there is no evidence suggesting that is a functional protein. In males both DsxM and FruM are functional and have a variety of sex-specific functions (*21*, *22*).

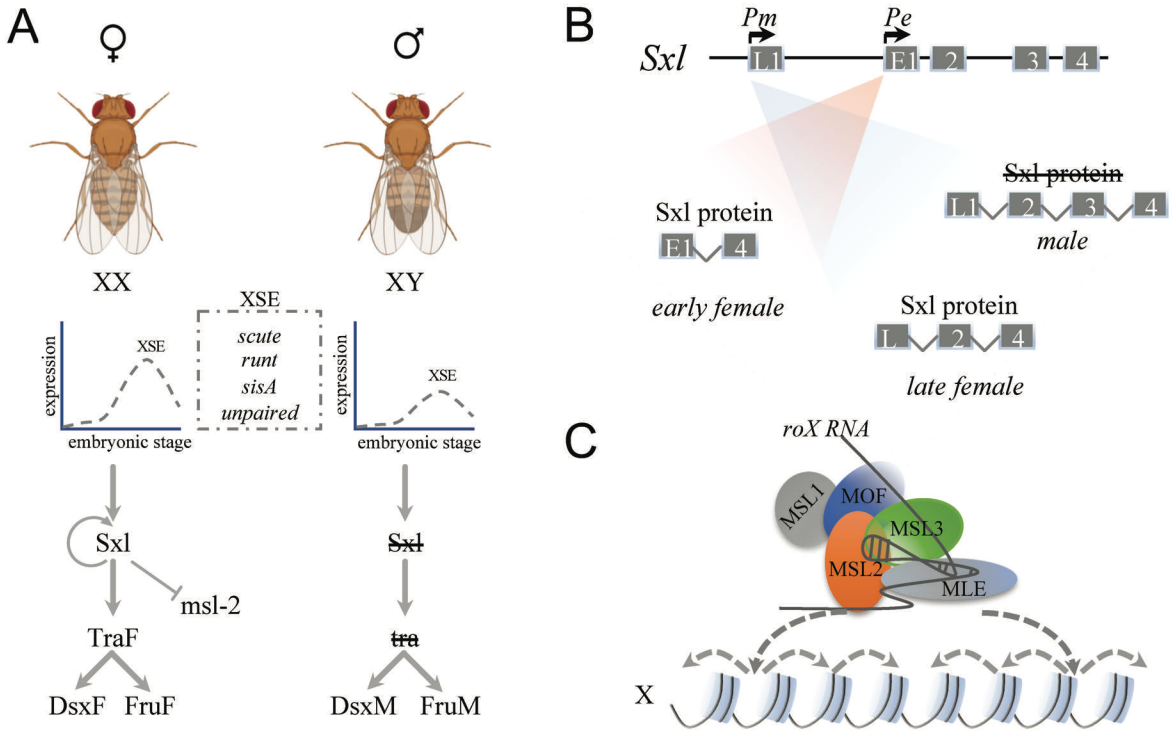

Figure 3. Sex determination and dosage compensation in *D. melanogaster*. Schematic of females and males of *D. melanogaster*. Expression levels of four XSE genes dictate the activation of *Sxl* that subsequently leads to the female-specific splicing of *tra*, *dsx,* and *fru*. In males, due to the absence of functional Sxl and Tra, *dsx* and *fru* are spliced to create the male-specific isoforms (**A**). Representation of *Sxl* gene structure. Different promoters drive distinct isoforms (**B**). Simplified schematic of the dosage compensation complex. A complex between *rox RNA* and other proteins is assembled and then targets and spreads over the X chromosome in males (**C**).

A crucial consequence of the heterogametic sex determination is that sexes do not share an equal amount of the genetic material. To equalize gene expression levels there is a variety of mechanisms that differ between species and are collectively termed as dosage compensation. There are two types of dosage compensation, the one that equalizes expression between the sex chromosomes and another one that equalizes expression between the sex chromosomes and the autosomes. Here we are focusing on the former. In *D. melanogaster*, mechanistic insight about it has initially been given by Ardhendu Sekhar Mukherjee in 1965 showing that the RNA levels produced by the single male X chromosome are equivalent to that produced by the two X in females. Over the years layers of explanations have added up on the mechanistic basis of this phenomenon. The transcriptional rate of X-linked genes in males is elevated and this is in part explained by the acetylation of histone H4 at lysine 16. This is achieved by the MSL complex that contains a subunit called msl-2 (*23*). In females, Sxl suppresses msl-2. In males, the MSL complex contains two X-linked long non-coding RNAs (*roX*). Subsequently, the complex is recruited to high-affinity sites on the X chromosome and then spreads all along it (*24*) (Figure 3C).

In the silkworm, *Bombyx mori*, sex-determination is genotypic as in *Drosophila melanogaster*, however the heterogametic sex is the female (ZW) and the homogametic is the male (ZZ). The W chromosome carries the *fem* gene coding for a piRNA. This piRNA represses the *masc* gene that is responsible for male development. These steps further lead to the generation of sex-specific *dsx* isoforms that regulate male and female development (*25*).

Another example of genotypic sex determination worth noting is haplodiploidy. It is common across Hymenoptera (bees, ants, and wasps). Males develop from unfertilized eggs and as a result, are haploid whereas females develop from fertilized eggs and are diploid. In honeybees, *Apis mellifera*, the central element in the sex determination hierarchy is a single locus, the complementary sex determiner (*csd*) gene. If an individual carries two different alleles of *csd* develops into a female. If an individual is hemizygous or homozygous for a *csd* allele then it develops to male. The absence of sex chromosomes explains the absence of a dosage compensation mechanism (*13*). Interestingly, as observed in *D. melanogaster*, through a sex-specific splicing cascade, *dsx* is spliced to its sex-specific isoforms. And this is a common node on the sex determination hierarchies between *D. melanogaster, Bombyx mori,* and *Apis mellifera*. Fascinatingly the diversity observed in the initial steps of sex determination in insects is vast, hence in all the studied cases today there is convergence on *dsx* (*13*, *26*, *27*).

NEMATODE SEX DETERMINATION

In the nematode, *Caenorhabditis elegans* (*C. elegans*), there are hermaphrodites with two X chromosomes (XX) and males with a single X (XO). In *C. elegans* sex is instructed by the ratio of X chromosomes to autosomes. For this purpose, there are four XSE identified *sex-1*, *fox-1*, *ceh-39*, and *sex-2* that repress *xol-1*. *xol-1* is the most upstream factor of the sex determination hierarchy. On the other hand, there is so far a single autosomal element identified, the gene *sea-1*, that activates *xol-1*. The balance between repression and activation leads to sex determination. In hermaphrodites (XX) the XSE outweighs the effect of the autosomal signal ultimately leading to repression of *xol-1*. The exact opposite happens in males where *xol-1* is expressed. Subsequently, XOL-1 negatively regulates *sdc-2*, leading to high levels in hermaphrodites. SDC-2 negatively regulates HER-1, a secreted protein that after binding its receptor TRA-2 orchestrates male development.

In contrast with *D. melanogaster*, the genetic imbalance in the *C. elegans* soma is compensated by decreasing by two-fold the expression of X-linked genes in hermaphrodites. This is achieved by the dosage compensation complex (DCC). For its assembly, SDC-2 is required, explaining its absence in males. In molecular terms, DCC modifies the compaction of the X chromosome. In hermaphrodites, there is an observed decrease in the levels of the acetylation of histone H4 at lysine 16, an active epigenetic mark, with a simultaneous increase of monomethylated histone H4 at lysine 20, a known repressive epigenetic mark. Additionally, the localization of the X chromosomes in the nuclei of hermaphrodites seems to be at random whereas in males it localizes preferentially towards the nuclear periphery interacting with a nuclear pore protein. Interactions with nuclear pore proteins have been shown to be activating in terms of gene expression (*23*).

MAMMALIAN SEX DETERMINATION

Mammals rely also on the XY system for primary sex determination. In mammals, the reproductive system comes developmentally from bipotential primordia. Alfred Jost removed the gonads (bipotential primordia) from embryos and as a result, rabbits developed with female

sex characteristics. This made him propose that the initial decision of the sex determination of the organism comes from the decision of developing gonad towards either ovary or testis (*28*). From a genetic point of view, today, we know that the Y chromosome carries the *Sry* gene (a member of the Sox family) that is responsible to direct testis formation. Subsequently, SRY activates *Sox9*, which is in a positive feedback loop along with FGF9 that represses *Wnt4*. In contrast, in females where SRY is absent, Wnt4 leads to the accumulation of β-catenin. Last but not least, β-catenin activates the expression of genes important for ovary development as well as follistatin (*28–30*).

As in any species with heterogametic sex determination, the existence of heteromorphic sex chromosomes leads to genetic imbalance. Studies in mammals focused mostly on humans and mice. Murray Barr in 1949 observed a dark dot in interphase nuclei of female cat neurons. Later it was demonstrated by Susumo Ohno that the dark spot is a condensed X chromosome. Following these observations Mary Lyon in 1961 hypothesized that the condensed X chromosome is inactive (*31*). Mechanistic and molecular insights though came years later and today we have a clear view of the key events of X inactivation. The initial step is the transcription of an X-linked long non-coding RNA called *Xist* (X inactive specific transcript), residing on the X inactivation center (XIC). This activation is triggered by RILM another X-linked gene whose expression level is doubled in females compared to males. It is worth noting that *Xist* is transcribed only from one allele the one from the chromosome that will be inactivated and that the choice between the two X chromosomes is random. Contrary, on the XIC of the chromosome that will remain active *Tsix* is transcribed and produces and antisense RNA inhibiting the transcription of *Xist*. Following *Xist* dispersion from the expression site to the rest of the X chromosome it alters chromatin features along with its association with the YY1 transcription factor as well as the Polycomb Repressive Complex 2 (PRC2). X inactivation is at least to a certain degree explained by the enrichment for trimethylation of histone H3 at lysine 27 that is deposited by PRC2. In addition, there is an enrichment for specific histone marks such as trimethylation of histone H3 at lysine 9, trimethylation of histone H4 at lysine 20 as well as enrichment of DNA methylation and the histone variant macroH2A. How these are targeted to the X chromosome is not known (*23*, *32–34*).

SEX-BIASED GENE EXPRESSION

Looking for the genetic causes of sexual dimorphism and taking into consideration that the genome in most animals is identically shared between sexes one must look for sexual dimorphism in gene expression. This is called sex-biased gene expression and refers to all the quantitative as well as isoform differences between sexes of a species. Today with the broad use of RNA sequencing somebody could characterize the architecture of gene expression in almost any organism in sex, tissue, and cell-type-specific resolution (*35*).

INTEGRATION OF REGULATORY INPUTS FROM THE SEX DETERMINATION HIERARCHIES

In the 1960s, in *D. melanogaster*, the first genes from the sex-determination hierarchies have been isolated as mutant alleles that create intersexes, individuals that are not typical males or females in their characteristics. Initially, for *transformer* and afterward for *doublesex*, these studies illustrated that the secondary sexual characteristics are affected by mutants of them (*36*, *37*). A few years later the most upstream known member of the sex-determination hierarchy, *Sex-lethal*, was discovered and set as the node that controls all sexual characteristics of flies (*38*). Another key aspect was the study of gynandromorphs. These are individuals that share male and female characteristics. This made the concept that sex-determination in *D. melanogaster* is by enlarge a cell-autonomous process, widely accepted. This led to the assumption that the action of the sex-determination in different cell types leads to the creation of sexual dimorphism (*39*). This part will continue with the presentation of representative examples illustrating the above idea along with the recently discovered non-cell-autonomous mechanisms of sexual dimorphism in *D. melanogaster*. At last, there is a part dedicated to relevant mechanisms in vertebrates.

In *D. melanogaster*, there is sexually dimorphic melanic pigmentation in the adult abdomen. This is characterized by the dark black pigmentation appearing in the A5 and A6 tergites of males. During pupal development, the products of genes necessary for pigment formation are deployed in a special pattern that contributes to the adult pigmentation. One crucial gene called *yellow* is prefiguring the formation of the black pigment and as a result, its expression is enriched in the A5 and A6 tergites in males. The spatial distribution of Yellow is directly controlled by a transcription factor called Bab (simplified as the product of two genes, *bric-a-brac1* and *bric-a-brac2*). Bab acts as a repressor and is itself repressed by DsxM and activated by DsxF. Collectively, the input from the sex determination pathway through Dsx in males leads to high expression of *yellow* whereas in females to repression of *yellow* expression (Figure 4) (*40–42*).

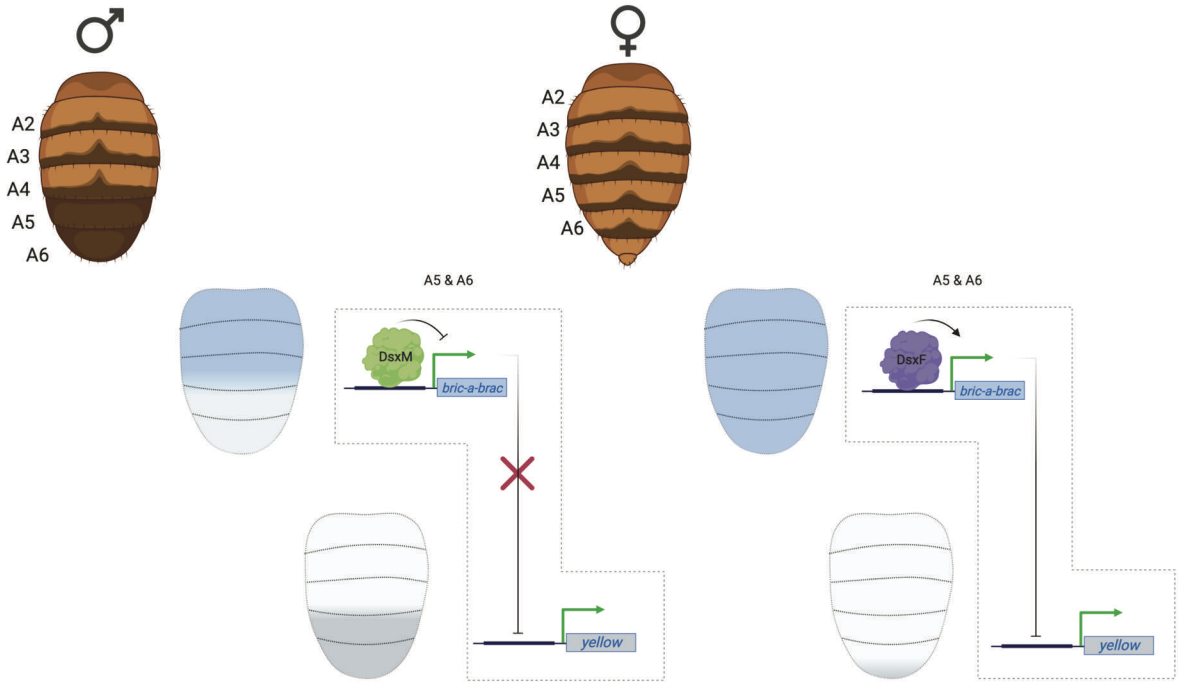

Figure 4. Genetic control of sexually dimorphic abdominal pigmentation in *D. melanogaster*. Abdomens of *D. melanogaster* displaying sexually dimorphic pigmentation with males being darker in tergites A5 and A6. The pigmentation is prefigured by the expression of *yellow* which is repressed by *bric-a-brac*. The expression between the two is complementary. That is because the male isoform of *dsx* (DsxM) is repressing *bric-a-brac* leading to the expression of *yellow*. In females, DsxF activates the expression of *bric-a-brac* leading to repression of *yellow*.

In *D. melanogaster*, there has been recent evidence for additional branches of the sex determination hierarchy. In adults the midgut display sex-specific differences in characters including size. This difference can be explained by differences in mechanisms controlling the proliferation rate of the stem cells in the enteric epithelium. Through meticulous genetic analysis, researchers were able to demonstrate that this difference is encoded in the cell-autonomous function of TraF and is independent of the known downstream factors of TraF in the canonical sex determination hierarchy that are Dsx and Fru. Instead, the action of TraF through other factors such as Idgf1, Spn88Eb, and Rdo seems to control proliferation in this context (Figure 5). These results suggested that there should be at least one additional branch of the sex determination hierarchy that controls sex-specific characteristics of at least the midgut (*43*).

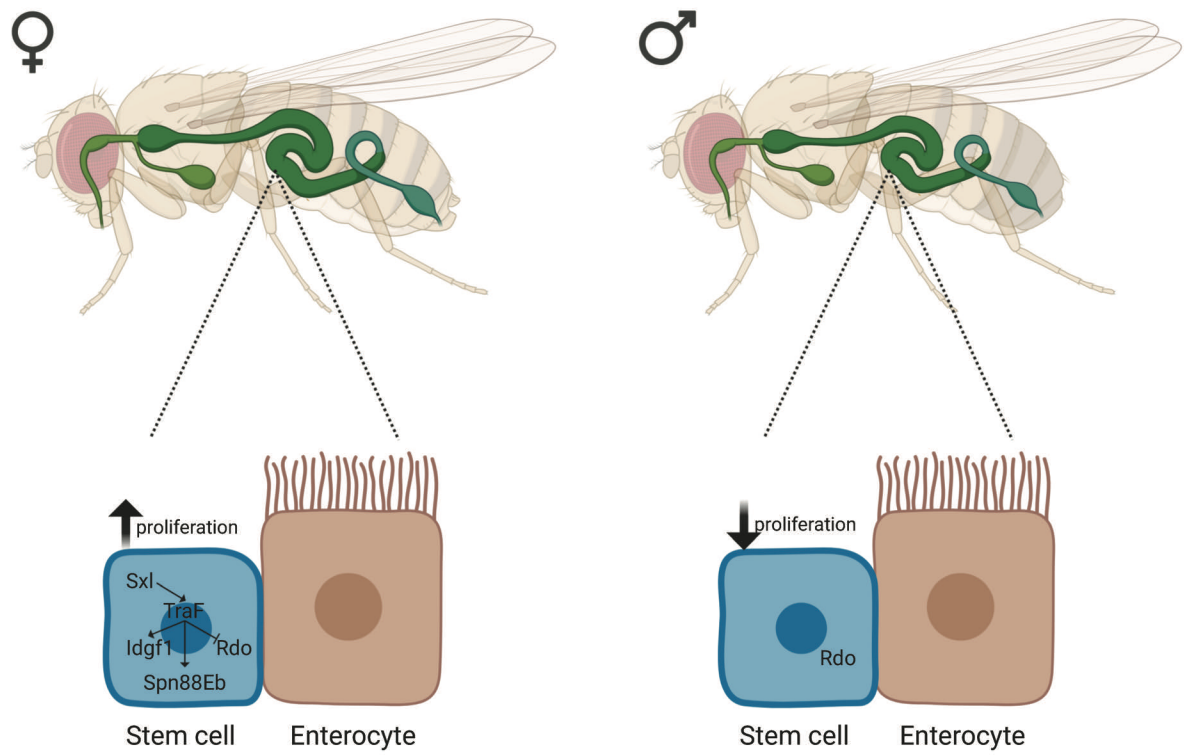

Figure 5. Sexual dimorphism in the physiology of adult *D. melanogaster* guts is conveyed by a non-canonical branch of the sex determination hierarchy. Increased proliferation in adult female gut cells is realized by the action of Sxl and TraF and independently of Dsx or Fru. TraF regulates either directly or not *Idgf1*, *Spn88Eb,* and *Rdo* and as a result, increased proliferation is observed.

The above examples illustrate the integration of the regulatory potential of the sex determination hierarchy in a cell-autonomous manner in *Drosophila*. The notion that sexual dimorphism in *Drosophila* is a result of solely cell-autonomous mechanisms has been pervasive. However, there is evidence of non-cell-autonomous mechanisms for the development of sexual dimorphism (*25*). Males and females of *D. melanogaster* differ in their size in the larval, pupal as well as adult stage, with females being larger than males. Differences in the growth trajectory reveal a sexual sign already from the larval stage. Using cell-type-specific genetic manipulations researchers were able to show that the sexual identity of specific neurons accounts for the sex-specific difference in growth. More specifically, *Sxl* expression in a couple of groups of neurons, one of which is insulin-producing cells, is necessary for the sex-specific size difference. This indicates that *Sxl* controls growth at the organismal level by acting in discrete populations of neurons (*44*).

In vertebrates, the sex-determination hierarchy indirectly controls sexual characteristics by initially controlling the differentiation of gonads that in turn produce sex-specific hormones such as estrogens and testosterone. These hormones are secreted and circulate until they reach their target receptors and modulate cell and tissue properties. For example, there is a well-characterized sex-specific difference in the hematopoiesis in mice. Blood cells originate from hematopoietic stem cells that reside in the bone marrow. The cell-cycle of these stem cells is sensitive to the estrogen levels. This property is realized by the high levels of expression of the estrogen receptor-α (ERα). ERα is bound by estradiol, a hormone produced mainly in the ovaries and accelerates the cell-cycle (Figure 6). Deploying high levels of estradiol even in males accelerated the cell-cycle of the hematopoietic stem cells. This acceleration observed in females as a result of high levels of estrogens is associated with pregnancy where there is a prominent need for blood cells (*45*).

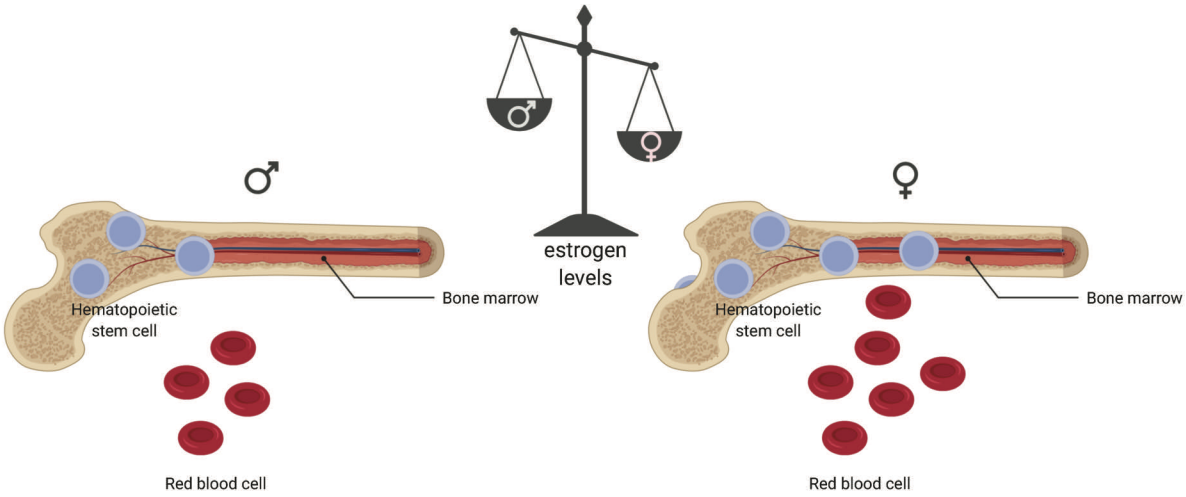

Figure 6. Sexual dimorphism in the hematopoiesis in mice. Higher estrogen levels in females bias the proliferation of hematopoietic stem cells in the bone marrow as well as the production of red blood cells.

Studies in chicken have expanded the traditional view in which sexual characteristics are established solely by the action of sex-specific hormones produced by the gonads. Bilateral gynandromorphs are cases in which organisms develop as males on the one side and as females on the other half. Mosaic situations like this one cannot easily be explained by the action of the hormones, which act in the whole organismal level. Instead, the establishment of sexual identity by cell-autonomous mechanisms better explains gynandromorphs. The existence of gynandromorphs in chicken challenged the traditional hormone-centric view in vertebrates. Researchers validated that the observed gynandromorphs are chimeras of male and female cells and through classic transplantation experiments of female tissue to a male host they were able to demonstrate that they retain their sexual identity through the expression of female-specific markers (Figure 7) (*46*). This study came to reinforce the observation initially made in zebra finch where sex-specific differences in the brain are a result of the inherent sexual identity of the brain cells (*47*).

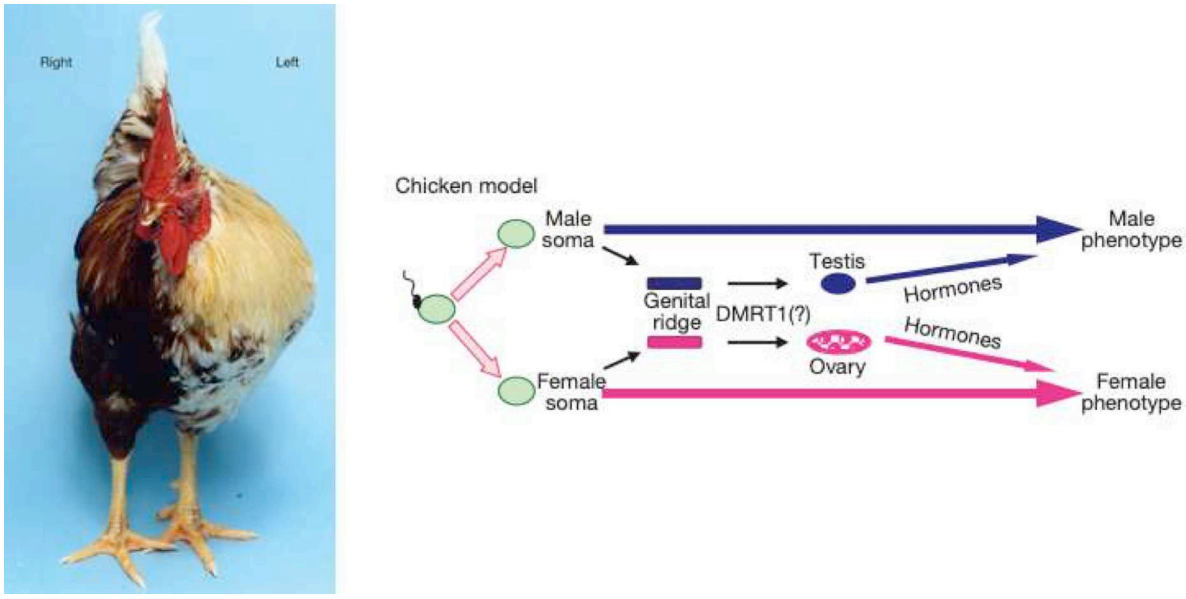

Figure 7. Cell-autonomous sex determination detected in the soma of bilateral gynandromorph chickens. The traditional model in which hormones, secreted by the gonads, confer sex-related differences in the soma is expanded. (source: (*46*))

SEX CHROMOSOME EFFECTS

The central cue for the majority of animals in sex determination is the sex chromosome complement. Core genes in the sex determination hierarchies are often on the sex chromosomes, such as *Sxl* in fruit flies of *Sry* in humans. Also, the notion that the sex determination hierarchies control the sexually dimorphic regulation of genes is widely accepted and supported from the literature (*48*). Whether sex chromosomes play additional roles in regulating the development of sexual dimorphism is a question that has drawn particular attention.

THE Y CHROMOSOME EFFECT. The Y chromosome in mammals carries the *Sry* gene, that as I described before, initiates the differentiation of the genital ridge towards testis and subsequently testis through the secretion of hormones directs the development of sexually dimorphic traits. But it has been shown that *Sry* is also expressed in other tissues and other time

points. And since it acts as a transcription factor, it may control gene expression in the somatic tissues in which it is expressed. For instance, it has been demonstrated that in a human male neuroblastoma BE(2)C cell line, *Sry*, positively regulates Monoamine oxidase A, a crucial enzyme for brain development and physiology (*49*). In addition, in PC12 rat cells, *Sry* was shown to regulate the transcription of Tyrosine Hydroxylase (*50*). These data suggest that *Sry* might regulate gene expression outside the gonad and orchestrate male-specific gene expression networks.

In *D. melanogaster*, by studying polymorphic Y chromosomes it has been demonstrated that genome-wide gene expression patterns both for X-linked but also autosomal genes, were affected to a great extent. Among the genes that were affected the most, were genes coding for components of chromatin organization and assembly complexes. These effects are not due to Y-linked gene expression since, in XXY females, where the Y chromosome genes are not transcribed, the effect exists. Up to today, there is one proposed mechanism in the ability to explain this effect of the polymorphic Y chromosome on gene expression. Since in its majority the Y chromosome is heterochromatic it could potentially act as a sink for such factors, changing their availability for the rest of the genome (*51–53*). Additionally, through the modulation of splicing and intron retention, the Y chromosome creates sexual dimorphism in gene expression (*54*).

DOSAGE COMPENSATION IN THE CREATION OF SEX-BIASED GENE EXPRESSION. Dosage compensation involves the sex chromosomes that are shared by both sexes but differ in number. There are plenty of proposed ways on how these chromosome-specific gene regulatory mechanisms might contribute to the creation of sex-biased gene expression. It is worth noting that sex-biased expression can be achieved by a variety of mechanisms that are involved in dosage compensation.

In humans, dosage compensation is achieved by the inactivation of one of the two X chromosomes in female cells. During development, in the early blastocyst, randomly one of two X chromosomes gets inactivated in the cells of the inner cell mass and the lineage of each of these cells retains this choice. Since the choice is random one would expect that almost half of the cells will have inactivated the X chromosome from the father and the rest from the mother generating mosaicism in the somatic tissues. That can have tremendous differences in the phenotype of the two sexes. On one hand, there could be a lethal recessive mutation that

due to mosaicism the effects of it might be moderate (*55*, *56*). In parallel, there is evidence of an occurring bias (skewing), in some cases, towards the X chromosome that is going to get inactivated (*57*). In humans, this seems to be common in the general population (*58*).

Studies on the X chromosome inactivation showed that the inactive X chromosome is not silenced in all its length. There is a region called PAR (pseudoautosomal region) that is a homologous region between the Y and X chromosomes. In this region the genes do not display any inequality in terms of numbers between the sexes since they are present in two copies in both sexes. So, this region is not silenced on the inactive X chromosome. Although some genes located on the PAR show male-biased gene expression (*55*, *59*).

In addition to this region, it is calculated that 15-25% of human X-linked genes in somatic tissue escape inactivation. That usually translates to a female-bias of these escapees. Although a direct association between this phenomenon and phenotypic sexual dimorphism is absent from the literature, there is an association of mutations of genes escaping inactivation and human cancers (blood, kidney, renal pelvis, and brain) that present a sex bias (*55*, *56*).

An additional mechanism that could serve as a foundation for the development of sexually dimorphic phenotypes is the genomic imprinting of the sex chromosomes. Genomic imprinting is an epigenetic mechanism that causes alleles to be expressed in a parent-of-origin manner. That would mean that if a gene is programmed to be expressed from the paternal origin it would be expressed only in half of the cells of females (XX) and not in males (XY) at all, in the case of humans. Complementary, if a gene is programmed to be expressed from the maternal origin then it would be expressed in all the cells of males but only in half of the cells of females. These can potentially have profound implications for sexual dimorphism since it can generate gene expression differences in a sex-specific manner. That can be manifested in females with Turner syndrome (XO). In this context, some females' X chromosome would be of parental and other of maternal origin. In a study in humans, an X-linked locus was identified that was imprinted and as a result, was not expressed from the maternally inherited X chromosome. Consequently, females carrying the X chromosome of paternal origin were performing better in verbal skills and cognitive tasks. That could at least partially explain why males are more susceptible to disorders involving the aforementioned skills (*60*). Similar results have been obtained while studying a mice model (39, XO). In this case, the candidate X-linked gene, *Xlr3b*, was speculated to mediate the parental effect through imprinting in related cognitive behaviors (*61*).

Last but not least, another proposed mechanism by which the X chromosome could have a leading role in the creation of sexual dimorphism is in the case of mammals in which one of the two X chromosomes gets inactive that could potentially act as a sink for *trans*-acting factors that mediate its heterochromatinization. In this case, by altering the balance of these *trans*-acting factors there could be genome-wide differences (*59*).

As I discussed also before, both sex chromosomes might serve either through different or the same mechanism achieving differences in gene expression that might serve as the foundation for the development of sexual dimorphism. Genome-wide regulatory mechanisms might act in favor of sexual dimorphism.

DISTRIBUTION OF GENES WITH SEX-BIASED EXPRESSION

The genomic distribution of genes that display sex-biased expression has been a long-standing question in sexual dimorphism. This is summarized in the following questions: is there a specific enrichment for sex-biased genes on the sex chromosomes? The sex-linked genes that are sex-biased are male or female-biased? Which are the evolutionary causes of the genomic distribution of sex-biased genes? All the above questions require an extensive study of representative animals, cell types, and developmental stages. For example, in *Drosophila* genome-wide gene expression studies have been conflicting although it is worth noting that the experimental design, tissue, and sequencing technology is different in all these cases and the conflict might arise because of these technical issues. Of course, that makes the conclusions even more complicated, requiring extra cautiousness. Initial genome-wide studies were performed in adults of *D. melanogaster*. In these studies, the genes characterized by male-biased expression were represented to a greater degree on the autosomes compared to the X chromosome. However, there were marginally more genes displaying female-biased gene expression on the X compared to the autosomes (*62–64*). More recent studies with increased resolution point out to the fact that there is an overrepresentation of male-biased genes on the brain of *D. melanogaster* (*65*). Two independent studies on third-instar larvae also produced opposite results concerning the direction of sex-bias (*66*). This reflects a discrepancy that exists in all these studies that potentially arises because of the aforementioned reasons. In mice, studies are significantly fewer. In one case, studying six different tissues the X chromosome

appeared to be enriched for female-biased genes and paucity of male-biased genes compared to the autosomes (*67*).

Taking into consideration the reported magnitude of sexual dimorphism in gene expression might explain the discrepancy between the studies. For example, if the difference of expression between two sexes is too small then little genetic or technical variation could explain variation in the results. A study in *D. melanogaster* highlighted that different genetic backgrounds display tremendous differences in sex-biased gene expression (*22*).

It is still unknown how much of the detected dimorphism in gene expression actually contributes to phenotypic dimorphism. It is clear that additional data are required to generate principles if that is possible.

CONCLUDING REMARKS

One of the most extraordinary property that shapes biodiversity is sexual dimorphism. The beauty, the elegant dances and songs of animals are characteristics that result from the evolution of sexual dimorphism. Studies especially in insects, birds and mammals have set some principles on the ontogeny and evolution of these characteristics. Undoubtedly, the role of the sex-determination hierarchies is central. Future research in model and non-model organisms will enable us expand our understanding on how these traits are built, function and evolve.

GENOME ARCHITECTURE

HIERARCHICAL GENOME ORGANIZATION. In the eukaryotic nucleus, following cell division, chromatin is organized at different levels in three dimensions. The unprecedented resolution of the architectural organization of the genome, today, has revealed a layer of regulation of gene expression. During interphase, homologous chromosomes occupy distinct territories in the nucleus. These territories tend not to be at random locations, reflected by the reproducible arrangement of territories. This localization seems to be affected by the size of the chromosome as well as its gene density (*68*, *69*). Furthermore, chromatin is organized into active and inactive compartments (*69*). Over the last ten years, chromatin conformation capture techniques uncovered additional levels of genome organization. Of these techniques, Hi-C has been quite lucrative. Hi-C contact maps led to the discovery of domains with a higher probability of intradomain interactions compared to interdomain interactions. These domains span from around 60 kb in flies till 880 kb in mice and are named Topologically Associating Domains (TADs) (*70*) (Figure 8).

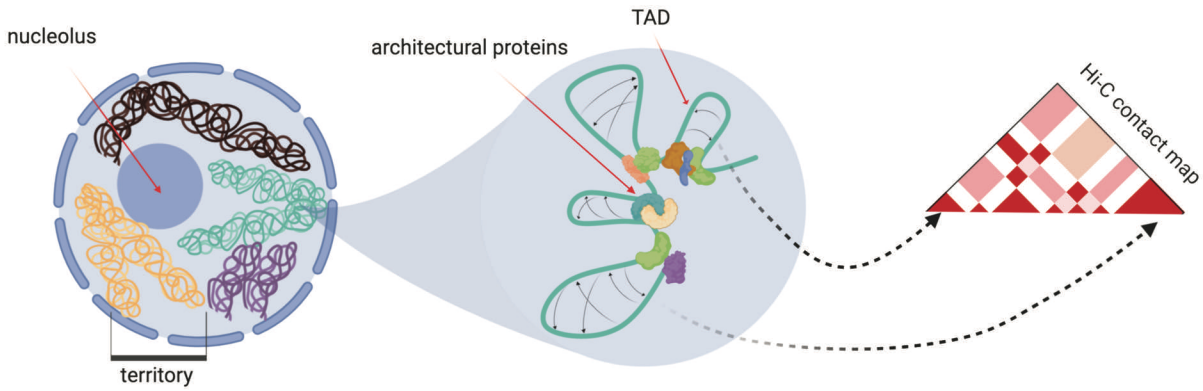

Figure 8. Two levels of chromatin organization. Different chromosomes, designated by different colors, occupy different territories in the nucleus. Each chromosome is further organized in Topologically Associating Domains (TADs), as revealed by Hi-C contact maps. The localization of architectural proteins marks the boundaries of TADs.

A core process of gene regulation is the communication between enhancers and promoters and this usually takes place exclusively inside TADs (*71*). TAD boundaries are occupied by architectural proteins. Disrupting these boundaries creates developmental defects in mammals, as a result of promoter misregulation by *cis*-regulatory elements (*72*). In contrast, large deletions of a TAD boundary in the *HoxD* cluster of mice had minor effects on expression during limb development (*73*). In flies shuffling and rearranging TADs does not dramatically impact gene expression (*74*), challenging their physiological relevance (*75*).

ARCHITECTURAL PROTEINS. Architectural proteins, also known as insulator proteins, are crucial components for both establishing genome architecture (intrachromosomal contacts) and for mediating interchromosomal contacts. In mammals, there is a single member of architectural proteins, the CCCTC-binding factor, CTCF. In *Drosophila*, in addition to CTCF, Su(Hw), Dwg, BEAF-32, Cp190, Mod(mdg4), Pita, Zipic, Dref, Trl (GAF), Ibf1 and Ibf2 are some of the components of architectural protein complexes. Architectural proteins are preferentially located in genomic regions called insulators (*76*–*78*).

The main properties of insulators are: i) the blocking of enhancer and promoter communication when interposed between them (*79*) (Figure 9A), ii) the barrier activity avoiding histone mark spreading (*80*, *81*) (Figure 9B), iii) the insulator bypass in which and enhancer can communicate with a promoter when two insulators are placed between them (*82*–*84*) (Figure 9C), iv) the realization of a specific type of *trans*-homolog interaction that regulates gene expression named transvection (*85*, *86*), and v) the facilitation of long-range regulatory interactions (*76*, *87*).

Intriguingly the functional significance for the vast disparity observed in architectural proteins between mammals and flies is not known. Hierarchically all the aforementioned levels of genomic organization are present in both groups of organisms with only minor differences. A key difference is that during interphase in somatic nuclei of flies, the homologous chromosomes are paired throughout their whole length in contrast to mammals.

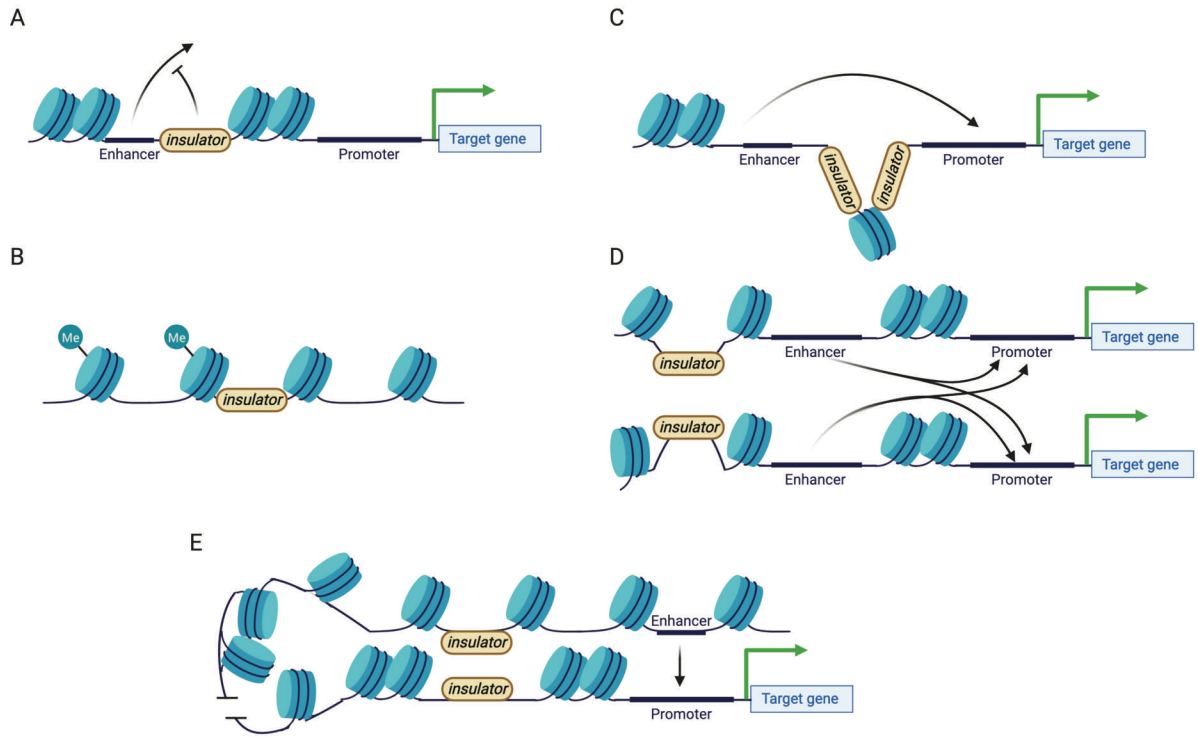

Figure 9. Properties of insulators. An insulator placed between an enhancer and a promoter blocks their communication (**A**). Also, blocks the spreading of chromatin modifications (**B**). Enhancer-promoter communication is not blocked when two insulators are placed between them (**C**). enhancer-promoter communication in *trans* is facilitated by insulators (**D**), as well as long-range chromatin interactions (**E**).

SOMATIC CHROMOSOME PAIRING

In 1908, Nettie M. Stevens studying the germ cells of Diptera, observes in mitotic cells the homologous chromosomes being paired (*88*). She states:

> *"Perhaps the most interesting point in the whole study is the pairing of the chromosomes in cells somewhat removed from the sphere of the reduction process."*

Homologous chromosomes are paired during meiosis, facilitating chromosome segregation and recombination. This phenomenon is not of interest to our study. Therefore, our focus will be on the somatic chromosome pairing observed during interphase. The non-meiotic pairing has been detected in several species including mice and yeast though to different extents. In *Drosophila* this phenomenon is exaggerated taking the lion's share in the study of the molecular foundations of it. A hundred years after the pioneering observations of N. Stevens, two factors are discovered to promote somatic pairing in *D. melanogaster*, the Su(Hw) and TopII (*89*, *90*). In addition, Cap-H2, a subunit of the condensin II complex, was shown to antagonize somatic pairing, indicating that the pairing state of a nucleus might be the output of the balance between pairing and anti-pairing factors (*91*). Indeed, performing an RNAi screen in *D. melanogaster,* more than 100 genes were found to promote or inhibit homolog pairing. This gene set was enriched for genes that have been implicated in the cell cycle control pointing to a cross-talk between the two (*92*, *93*). The ontogeny of somatic homolog pairing relies on button loci that are dispersed in the genome and are sufficient to drive pairing. These buttons are enriched for localization signals of architectural proteins (insulator proteins) such as CTCF, GAF, Mod(mdg4,) and Cp190. It is worth noting that there is no association of pairing and specific epigenetic marks (activating and repressing), Polycomb group binding sites, active transcription, or non-coding RNAs (*94*, *95*). Genome-wide studies using Hi-C technologies revealed that homolog pairing is structured as illustrated by the presence of *trans*-homolog domains and compartments as well as of regions displaying different levels of pairing, tight versus loose (*96*, *97*) (Figure 10).

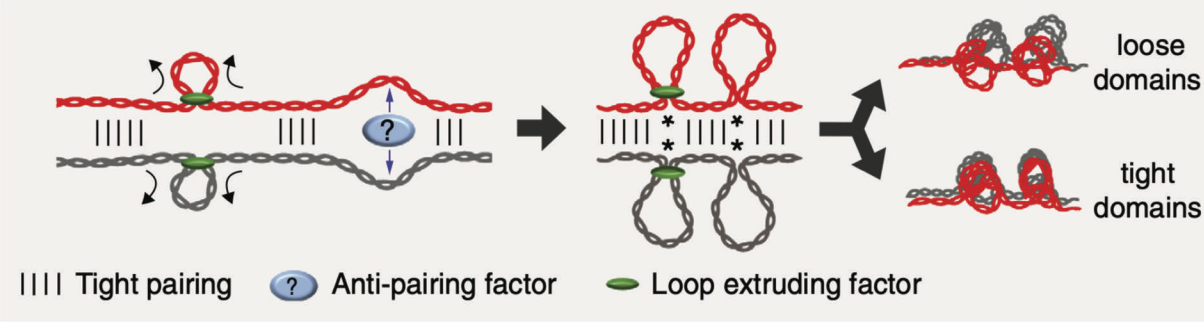

Figure 10. Structured homologous pairing. The presence of pairing and anti-pairing factors controls regions of tight and loose pairing between the homologs. Loops are created either by the action of loop extruding factors or anti-pairing. These loops bring enhancers and promoters nearby. These are further organized in loose and tight domains. (source: (*97*))

How this is interpreted in terms of genome function is still largely unknown. N. Stevens though already in 1908 stated that (*88*):

> *"One is tempted to suggest that if homologous maternal and paternal chromosomes in the same cell ever exert any influence on each other, such that it is manifest in the heredity of the offspring, there is more opportunity for such influence in these flies than in cases where pairing of homologous chromosomes occurs but once in a generation. Possibly experiments in cross-breeding of flies may bring out some interesting facts in heredity."*

Almost fifty years later, in specific loci, chromosome pairing has been associated with gene expression alteration in *Drosophila*, mainly through a phenomenon discovered by Ed Lewis and named "transvection". This phenomenon as I explain later in the manuscript was revealed using mutants (*98*).

Outside Diptera non-meiotic homologous pairing is not as extensive. Both in mice and humans continuous and widespread pairing is in general inhibited, and this is attained by distinct territorial occupancy of homologous chromosomes in the nucleus (*99*). Instead, homologous chromosome pairing is transient and local, and in certain cases, it has been associated with the regulation of gene expression. It has been documented during the differentiation of embryonic stem cells (*100*), DNA repair and V(D)J recombination (*101*).

The somatic homologous pairing as it has been suggested can underpin either local or genome-wide regulatory mechanisms. Yet, the extent to which this phenomenon contributes to gene expression regulation is still poorly known.

TRANSVECTION

HISTORICAL BACKGROUND AND INITIAL DISCOVERY. Transvection is the first case in which somatic chromosome pairing has been shown to affect gene expression. It was originally described by Ed Lewis in 1954 studying the *bithorax* complex (*98*). The discovery of transvection was driven by the observation of la ack of complementation between alleles. Tracing its history, two fundamental concepts were crucial to formulate transvection as an explanation for this observation. The first one is that genes are existing in distinct forms, the alleles and these alleles cannot get meiotically recombined between them. For example, in the early 20th century with work starting from Thomas Hunt Morgan several *white* alleles were isolated, but they were unable to recombine with one another. Finally, it was concluded that these alleles occupy the same locus. The second key concept is "pseudoallelism". This refers to tightly linked mutations that produce similar phenotypes and can also be meiotically recombined between them. These are called pseudoalleles. Many of them were described already since 1937 and it was speculated that they have evolved through duplication and accumulation of mutations (*102*). More importantly, an animal heterozygous for the two pseudoalleles does not have a wild-type phenotype but a phenotype intermediate between the wild-type and each homozygous pseudoalleles. This property distinguishes them from the classic alleles. Initially, Ed Lewis, proposed that a position effect could explain the lack of complementation between the pseudoalleles. In more detail, a mechanism was suggested in which pseudoalleles code for enzymes that convert substances and act in a consecutive manner in the same biochemical cascade. The product of each reaction of these pseudoalleles shows limited diffusion. For example, in the case that a pseudoallele (that is heterozygous) does not lead to the conversion of the first substance then little diffusion from the other pseudoallele would fuel the biochemical cascade to function with less amount of product. In the end, this might produce an intermediate mutant phenotype (*103*).

In parallel with this, Ed Lewis had already started working with mutations in the *bithorax* complex. This complex contains three genes, the *Ubx* (*Ultrabithorax*), *abd-A* (*abdominal-A*), and *Abd-B* (*abdominal-B*). These are homeobox genes that are expressed in the abdominal and thoracic segments and control their identity. The study of transvection focused on the *Ubx* gene. More specifically, *Ubx* controls the identity of the parasegments 5 and 6. Parasegment 5 gives rise to posterior T2 and anterior T3 appendages and parasegment 6 gives

rise to posterior T3 and anterior A1 appendages. Mutations affecting *Ubx* expression or activity lead to phenotypic defects reflected in the aforementioned appendages. In his landmark paper in 1954, in which he introduces the term transvection, Ed Lewis uses two pseudoalleles (as they were considered back then), the bx^{34e} and the Ubx^{1} (Figure 11). Today we know that both alleles have insertions in the *Ubx* gene, the bx^{34e} is an insertion of a transposable element that carries an insulator element (*gypsy*) and the Ubx^{1} is an insertion of a transposable element (*Doc*). The bx^{34e} is recessive and in homozygous flies the anterior T3 segment is transformed into an anterior T2-like segment, and the halteres are enlarged. The Ubx^{1} is lethal as homozygous but in heterozygous flies, the halteres are significantly enlarged. When the two mutations are linked in *cis* (bx^{34e} Ubx^{1}/+ +), they are phenotypically identical to the Ubx^{1} heterozygotes (+ Ubx^{1}/+ +). *Trans*-heterozygous flies (bx^{34e} +/+ Ubx^{1}), show a partial complementation. The halteres are larger than the bx^{34e} homozygotes but the T3 segment transformation is no longer observed. So for one phenotype, there is a less severe effect and for the other one, a much severe effect compared to the single mutants. The partial complementation, observed for one phenotype, was unanticipated and Ed Lewis reasoned that a biochemical model like the one described before could explain it. In this model, the product of the wild-type pseudoallele bx^{+} catalyzes the conversion of a substance (S) to A, and the wild-type pseudoallele Ubx^{+} catalyzes the conversion of A to B. When the two mutant pseudoalleles are in *cis* there is the production from the two wild-type pseudoalleles although the phenotype is not wild-type. That can be explained by lower production of B. When the two mutant pseudoalleles are in *trans* there is a minor diffusion of A that is produced by bx^{+} to the other chromatid and can be processed by Ubx^{+}(Figure 12). Ed Lewis argued that this diffusion is enabled due to chromosomal pairing. To address this, he induced chromosomal aberrations to disrupt chromosomal pairing. He observed that, in this configuration, the inter-allelic complementation was lost. Ed Lewis described this phenomenon of pairing-dependent interallelic complementation as transvection (*98*) (Figure 12). He expanded his findings, demonstrating that several other mutant combinations in the bithorax complex are performing transvection including abx^{1}/Ubx^{1}, abx^{1}/bx^{3}, abx^{1}/pbx^{2}, Ubx^{1}/pbx^{2}, bx^{3}/pbx^{1}, and others (Figure 11).

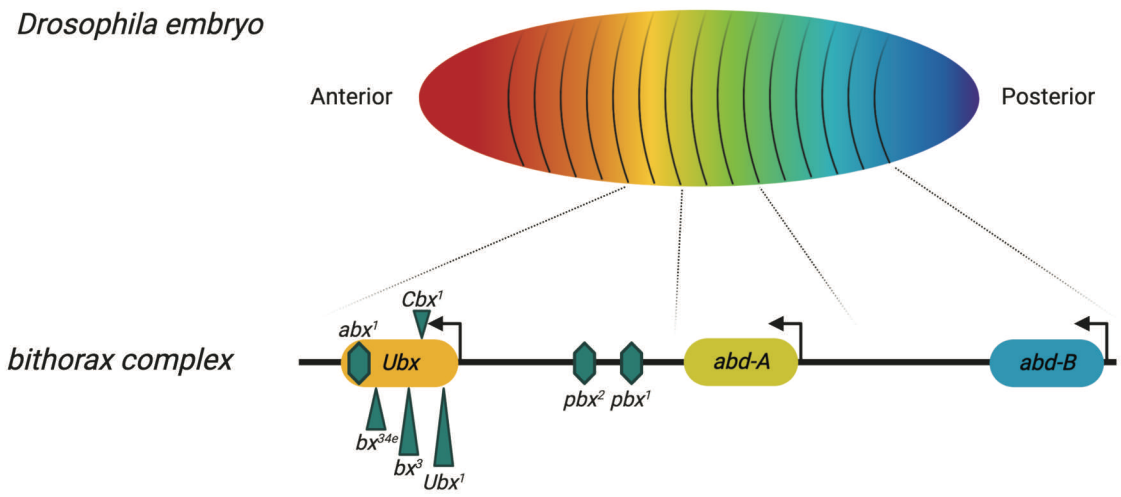

Figure 11. The bithorax complex of *D. melanogaster*. The complex contains *Ubx*, *abd-A*, *abd-B*, which are expressed in specific parasegments of the *D. melanogaster* embryo, controlling their identity. Triangles designate insertion mutants (bx^{34e}, bx^{3}, Ubx^{1}, Cbx^{1}) and hexagons designate *cis*-regulatory mutants (abx^{1}, pbx^{2}, pbx^{1}).

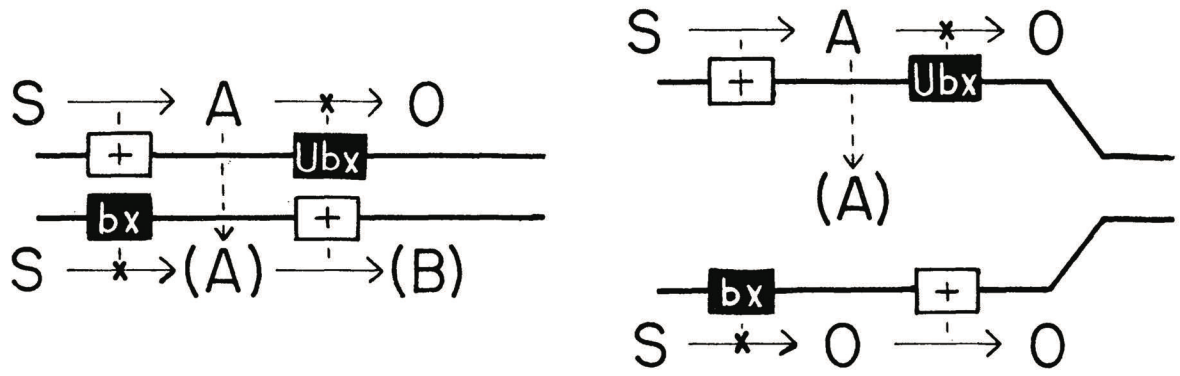

Figure 12. Model for transvection suggested and drawn by Ed Lewis. On the left, in the situation where the wild-type bx^{+} catalyzes the conversion of S to A, and Ubx^{+} catalyzes the conversion of A to B. The mutants are unable to catalyze the conversion. When the mutants are in a *trans*-heterozygous configuration, little diffusion from the produced A fuels the wild-type Ubx^{+} and as a result, there is partial complementation. On the right, chromosomal pairing is impaired and the diffusion is not efficient anymore, and as a consequence, there is no complementation. (source: (*98*))

Today, several objections exist regarding the original biochemical explanation since, for instance, translation is not a local process but instead takes place in the cytoplasm. However, the biochemical explanation has been reinforced and several others have been proposed to explain transvection phenomena like the one described by Ed Lewis. To simplify the explanation of these mechanisms we may take advantage of two alleles that represent either genes or *cis*-regulatory elements in Figure 13A-D, the wild-type being designated with a (+) and the mutant with a (-). The first possible mechanism is *trans*-splicing in which RNA produced for one pseudoallele can be spliced together and get fused with another pseudoallele requiring like this just two wild-type pseudoalleles to achieve complementation (Figure 13A). *Trans*-splicing has already been shown for two genes in *D. melanogaster*, the *mod(mdg4)* (*104–106*) and *lola* (*107*), and potentially represents a more general mechanism (*108*). The second possible mechanism is template-switching by RNA polymerase and like this RNA polymerase can change chromatid, transcribing like this the wild-type genes. Today evidence indicates that mammalian RNA polymerase II has the ability to change templates (*109*). A third potential mechanism relies on diffusing regulatory RNAs that can act as transcriptional regulators of another gene in *trans* (Figure 13C). The final mechanism relies on the communication in *trans* of a wild-type enhancer with a wild-type promoter (Figure 13D) (*110*).

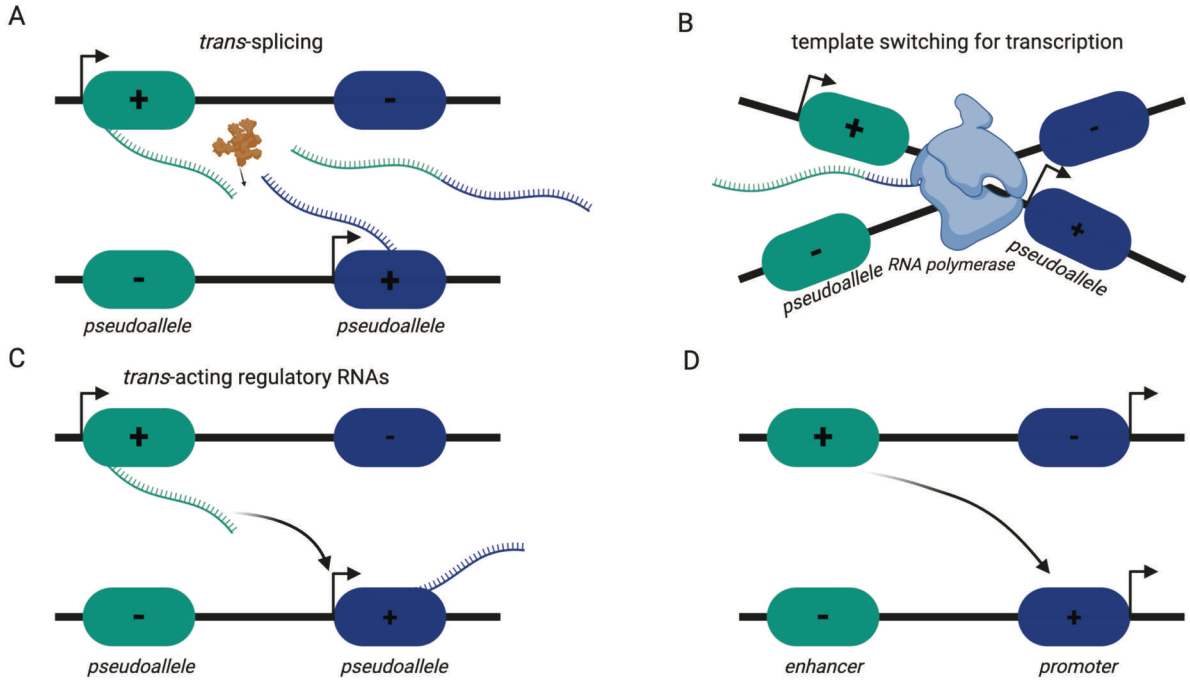

Figure 13. Models explaining transvection. Green ellipsoids represent wild-type (+) or mutant (-) genes (pseudoalleles) (**A**-**C**), and wild-type (+), or mutant (-) enhancer (D). Blue ellipsoids represent wild-type (+) or mutant (-) genes (**A**-**C**), and wild-type (+) or mutant (-) promoter (**D**). Interallelic complementation can be achieved by *trans*-splicing (**A**), template switching during transcription (**B**), *trans*-acting regulatory RNAs (**C**), and enhancer-promoter communication in *trans* (**D**).

THE EXTENT OF TRANSVECTION. Several gene regulatory phenomena that are similar to the one described, have been put under the concept of transvection. The common aspect is the existence of *trans*-homolog interactions that affect gene expression revealing the pairing-dependence of the phenomenon. Although the biological relevance of transvection in physiological conditions is not known today, it has been detected in several additional loci. *Abd-B* in the *bithorax* complex, *Sex combs reduced* (*111*), *decapentaplegic* (*112*), *white* (*113*), *apterous* (*114*), *eyes absent* (*115*), *yellow* (*116*), *vestigial* (*117*), and *spineless* (*118*) are some of the examples in *D. melanogaster*. More recently, it has been shown that regulatory fragments from numerous loci can support transvection (*119*). The recent discovery that homologous chromosome pairing occurs genome-wide (*95*, *96*) supports the idea that transvection-like phenomena might be more common and important than previously appreciated.

Although chromosomal pairing is not that extensive in organisms outside Diptera, cases of transient homolog pairing have been documented in other animals and even in plants. Paramutation (described later in the chapter) is, also, considered a special case of transvection in plants (*120*). In mammals, there are very few cases described today that resemble a transvection-like phenomenon. The first of them was described in 2002 while trying to create a deletion in the mouse germline using the *Cre/LoxP* system. In this system, *LoxP* sequences are able to recombine in the presence of CRE. In this specific case, *Cre* was under the control of a meiosis-specific promoter called *Sycp-1*. In the first generation, high efficiencies of recombination were documented. However, subsequently, in the germline of these mice, *LoxP* sequences were unable to recombine, suggesting that the initial recombination can inhibit the recombination of the other homologous region that contains the *LoxP* sequences (*121*). This

inhibition persists only for two generations, pinpointing an epigenetic mechanism (*122*). An additional case describes *Oct4* that is a major factor in embryonic stem cell pluripotency and, as a result, is expressed in pluripotent stem cells. When these stem cells transition to a differentiated state this is characterized by an increased pairing of the *Oct4* homologous alleles leading to repression of the gene, marking the exit from the pluripotent state (*100*). Transvection has, also, been detected during imprinting, an epigenetic process that causes parent-of-origin allelic expression (*123*). Unfortunately, the mechanistic insight of transvection is shallow in the case of mammals but, thankfully, immense in the case of *Drosophila*.

THE MOLECULAR BASIS OF TRANSVECTION

In this section, my focus will be on classical and modern studies in *Drosophila* that illustrate mechanistic ingredients of transvection. At the core of transvection insulators, promoter class, and architectural proteins are overrepresented. In the majority of the cases, transvection cannot be distinguished from chromosomal pairing in the context of the ingredients used. A protein that disrupts chromosomal pairing in a locus that exhibits transvection, would, by default, affect transvection. In the examples below, I intend to illustrate the aforementioned principles.

Transvection at yellow. *yellow* (*y*) is an X-linked gene in *D. melanogaster* that is necessary for black pigment formation. The *y* gene consists of two exons. In the 5' region of the gene, two enhancers are controlling its expression in the wing and the body, and in the intron, there is one enhancer controlling its expression in the bristles. A recessive *y* mutant allele, named y^2, results from the insertion of *gypsy* between the body enhancer and the promoter of *y*. As a result, *gypsy* blocks the communication between the two enhancers and the *y* promoter. Phenotypically, the y^2 flies, resemble null mutants of yellow in all the adult structures except the bristles that are wild-type (*124*). Interestingly, there is a strange observation that some null *y* alleles complement y^2 and some others not (*125*). For example, the recessive y^1 null allele is a result of a mutation of the A to C in the first ATG codon at which translation is initiated. y^2/ y^1 flies are not rescued and display the *yellow* null phenotype. Utilizing another null allele, the y^{59b}, which is a deletion of y^2 that removes a fragment that contains the first exon, part of the *gypsy* insulator, and part of the intron including the bristle enhancer, complements y^2. This

complementation relies on the activation of the y^2 promoter in *trans* by the wing and body enhancers. Besides, it was demonstrated that this interaction requires chromosomal pairing (*110*). This represents a classic case of transvection, illustrated by a pairing-dependent *trans*-action of enhancers.

The y^{3c3} null allele is a deletion of part of the intron, the first exon and part of the 5' including the body enhancer. Surprisingly, y^{3c3}/ y^2 flies have normal pigmentation. In this case, the body enhancer exists only in the y^2 allele, which, as hemi- or homozygous, does not drive *y* expression in the body. This suggests that the body pigmentation rescue relies on the action of the y^2 body enhancer by somehow bypassing the *gypsy* insulator. Taken all together, interallelic complementation between y^{3c3} and y^2 is, at least partially, explained by insulator bypass after a *trans*-homolog interaction. Insulators play a fundamental role in transvection (*126*). In addition to the *gypsy* insulator, there is another one (*1A-2*) in the 3' of *y* separating it from the *achaete-scute* complex that is downstream (*127*). Utilizing transgenes containing *y* suggests that the presence of these insulators enhances transvection. Both of these insulators, *gypsy,* and *1A-2,* are bound by the architectural protein, Su(Hw), whose presence strengthens transvection (*128*).

The role of the promoter in transvection was illustrated early on, in the first case of transvection in *y*. The $y^{1\#8}$ allele results from a *P*-element (transposable element) insertion that upon its excision removed the promoter. Remnants of the *P*-element exist in this allele. $y^{1\#8}$ complements y^2, in sharp contrast with y^1. The difference between y^1 and $y^{1\#8}$ is the absence of the promoter in the latter. This suggested that there is a promoter competition feature in transvection. On the one hand, the wing and body enhancer of y^1 act preferentially on y^1 (in *cis*) when it is over y^2 and on the other hand in the absence of a promoter in *cis,* the enhancers act in *trans* to regulate a functional promoter (*110*). Substitution of the *y* promoter with heterologous promoters from several genes (*hsp70*, *eve*, *w*) in the context of the y^2 allele creates the $y^{eve\text{-}700gin}$. This also complements the $y^{1\#8}$ allele, suggesting that activation of promoters in *trans* might be a general feature of them (*83*). It is well established that promoter is a key player establishing enhancer action in *trans*, with promoter integrity being the key feature (*129*, *130*).

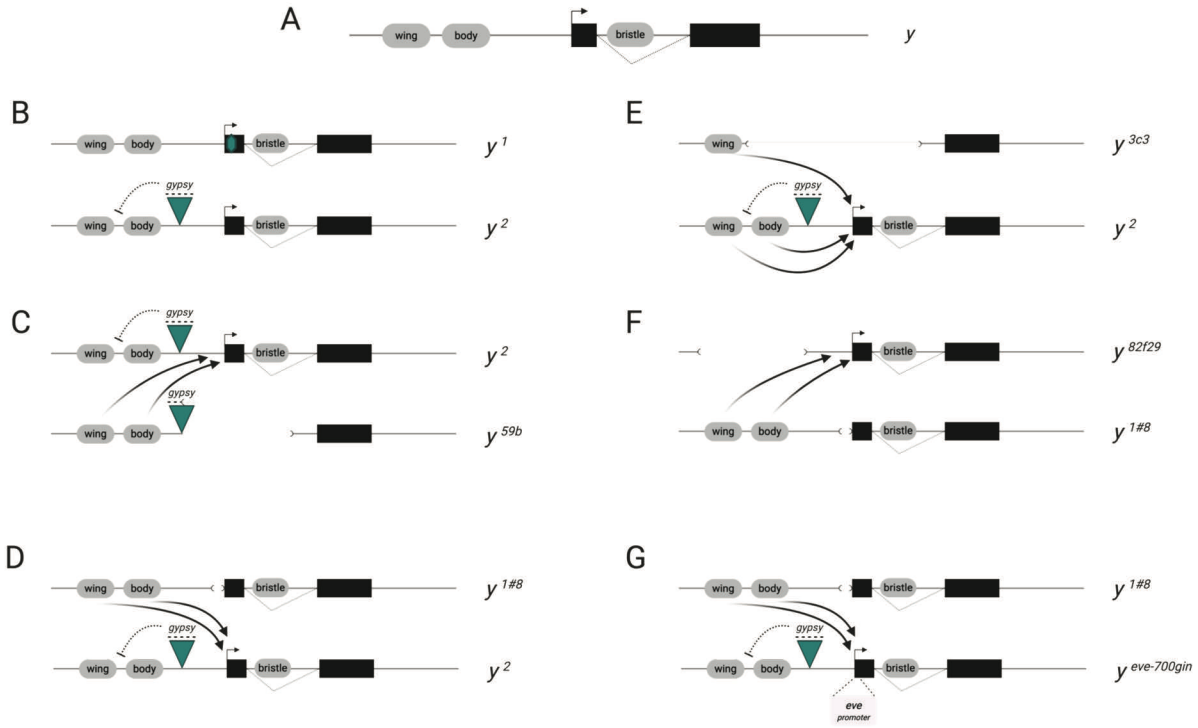

Figure 14. Modes of transvection at *yellow*. Structure of the *yellow* (*y*) gene, containing two exons. Enhancers controlling its expression in the wing, body, and bristles are annotated (**A**). y^1 null allele represents a single base mutation in the first coding triplet (ATG to CTG) and y^2 is a gypsy insulator insertion between the body enhancer and *y* promoter leading to the absence of pigmentation in the wing and body of adult flies. y^1/ y^2 are not complementing each other (**B**). y^{59b} represents a deletion of y^2 that removes part of the gypsy, the first exon, and part of the intron. y^2/ y^{59b} are complementing each other suggesting that enhancers from y^{59b} communicate in *trans* with the promoter of y^2 (**C**). y^2 is, also, complemented by $y^{1\#8}$ which carries a deletion removing its promoter (**D**). y^{3c3} represents a deletion that spans from the body enhancer until the major part of the intron. y^2/ y^{3c3}display normal pigmentation in the wing, body, and bristles suggesting that the body enhancer bypasses the *gypsy* insulator (**E**). Flies carrying $y^{1\#8}$ over the y^{82f29} that carries a deletion of the wing and body enhancers show wild-type phenotype (**F**). enhancer action in *trans* that is observed in the above cases (**C-F**), is also observed when the wing and body enhancers are tested with the *eve* promoter (**G**).

Chromosomal pairing is not homogeneous along the genome (*97*) suggesting that, also, transvection strength might be heterogeneous. Although a direct comparison between pairing and transvection does not exist in the literature, it is known that transvection effects are possible throughout the whole genome (*131*), but their strength is affected by the chromosomal environment (*132*, *133*).

Transvection at cubitus interruptus. Another noteworthy example is transvection at the cubitus interruptus (*ci*) gene. Mutations at the *ci* locus disrupt normal wing development and are mostly manifested in wing morphology. Flies that are homozygous for the recessive ci^1 allele display wing vein malformations (Figure 15B). Flies that are ci^1/ ci^+ exhibit wild-type phenotype (Figure 15C). *ci* lies normally on the fourth chromosome. Translocations of ci^+ on the fourth chromosome in flies homozygous for ci^1 can create wing phenotypes, usually, wing vein breaks, suggesting that the dominance of ci^+ is lost (Figure 15C). This phenotypic effect is caused by ectopic expression of *ci* to the posterior compartment of the wing, where under normal conditions it is repressed by *engrailed*. The insertion of the *gypsy* insulator in ci^1 blocks the communication of a silencer element, that is bound by engrailed, with the promoter leading to its expression in the posterior compartment of the wing. Through a *trans*-homolog interaction, ci^+ represses expression of ci^1 in ci^1/ ci^+ flies (*134–136*).

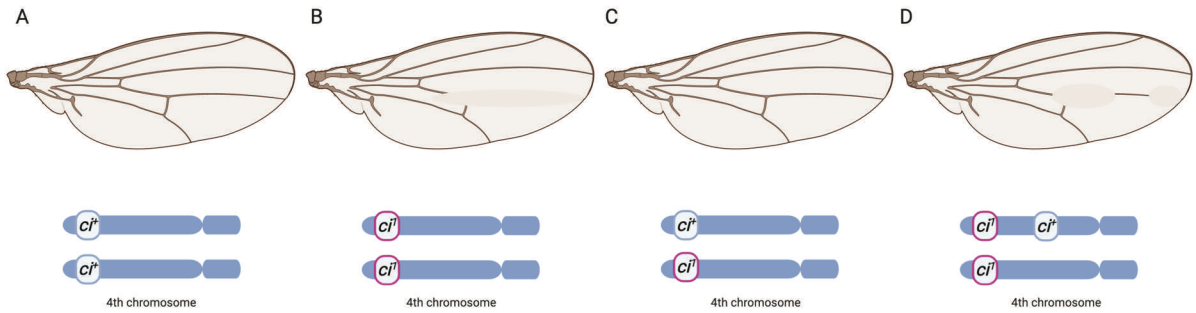

Figure 15. Transvection at the *ci* locus. Homozygous flies for the mutant ci allele, ci^1, display wing vein malformations (**B**) compared to wild-type flies (ci^+/ci^+) (**A**). ci^1 is recessive *to* ci^+ and as a result, ci^+/ci^1 flies have wild-type wings (**C**). In flies homozygous for the ci^1 allele, which carry a translocation of the ci^+ wild-type allele, wings display vein malformations (**D**) suggesting that ci^+ losses its dominance over the ci^1.

Transgenes reveal transvection features. Transgenes have been used extensively in *D. melanogaster* to rule out the specific function of sequences in transvection. As I reviewed before, insulators seem to be at the core of transvection potentially through the stabilization of homolog interactions. Traditionally, insulators have been used to protect transgenes from position effects (*137*, *138*). The *gypsy* insulator, as well as the *Wari* insulator (present at the 3' of *mini-white*), are common insulators present in vectors. A detailed examination of insulators suggests that having at least two insulators in *trans* is required to support transvection. In addition, transvection strength is affected by the position and the orientation of the insulators (*139*).

Recently, visualization of transvection using transgenes in living embryos of *D. melanogaster* revealed that indeed the predominant role of insulators is the stabilization of homolog pairing (*140*). Although promoter competition has been observed in several cases of transvection (*110*, *141*, *142*), performing quantitative imaging suggests that as soon as transvection is initiated, the enhancers have equal access to the promoter. A model in which a transcription hub, with sharing transcription components is created, is proposed today (*140*). Transcription hubs seem to generate stability during environmental and genetic perturbations illustrating a potential role for transvection (*143*).

PARAMUTATION

A special case in which *trans*-homolog interactions have been documented is paramutation. In this case, induced epigenetic changes by *trans*-homolog interactions are also perpetuated through generations. It was discovered by Alexander Brink in 1956 studying the R locus in maize (*144*, *145*). In the case of paramutation, usually, there are two genetically identical alleles but are simultaneously distinct epialleles. One of these epialleles is able to convert the second one to be identical as an epiallele also, disrupting like this Mendel's law of allele segregation. Only as hemizygous or heterozygous over other epialleles, it can convert to its original version (*146*).

A traditional example of paramutation is the *b1* locus in maize. The *b1* gene encodes a transcription factor necessary for anthocyanin pigment production. Two epialleles of the *b1* locus are the *B'* and *B-I*. The *B'* leads to low expression of the *b1* gene and the plants are lightly pigmented. In contrast, *B-I* leads to a high expression of the *b1* gene, and the plants are dark purple. When the two epialleles are together in a heterozygous configuration the *B'* has the capacity to convert *B-I* to *B'*. Afterward, this "new" *B'* has the ability to "paramutate" other alleles also (*147*). This requires a *cis*-regulatory element on the 5' of the *b1* gene that exhibits differential methylation levels as well as different nuclease hypersensitivity between the two epialleles (*148*). This *trans*-homolog interaction is potentially mediated by an RNA-dependent mechanism since it involves the production of siRNAs from the aforementioned *cis*-regulatory element and the action of *mop1*, an RNA-dependent RNA polymerase (Figure 16) (*149*).

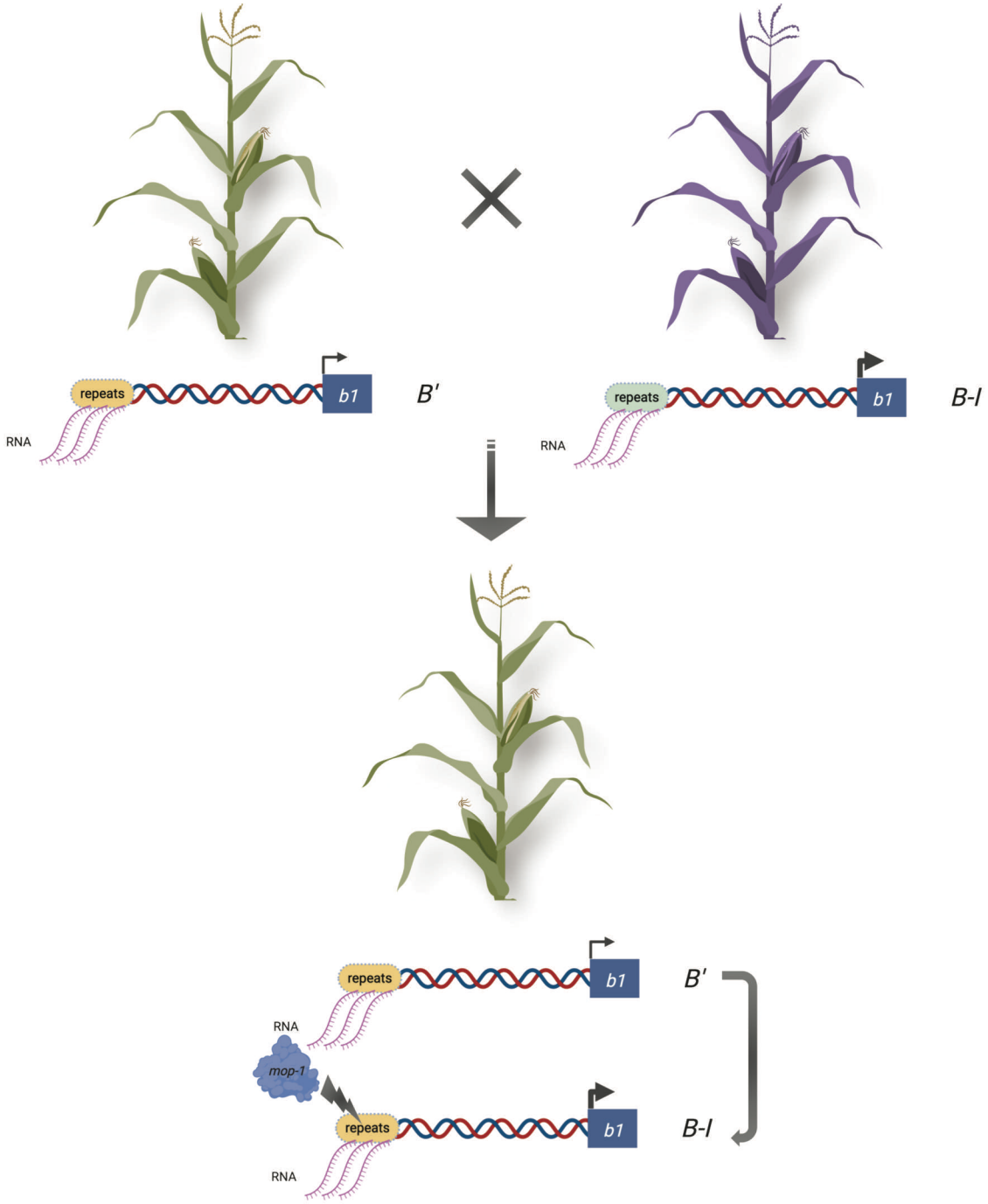

Figure 16. Paramutation on the *b1* locus in maize. The *b1* gene is necessary for pigment production. Two epialleles of *b1* are decoding differences in pigmentation. The *B'* is transcribed at a low level leading to low levels of pigmentation contrary to the *B-I* that leads to the generation of purple plants. These epialleles are identical in DNA sequence but they differ in the epigenetic properties of a *cis*-regulatory element. In heterozygous plants when *B'* faces *B-I* is able to convert it to *B'*. This relies on an RNA-dependent mechanism and the action of *mop-1*.

Paramutation has long been thought to be a plant-specific phenomenon. Relatively recent paramutation-like phenomena have been described in several organisms including mice and flies. P elements are naturally existing transposable elements that are detected in populations of *Drosophila*. Also, they are used to insert transgenes in the genome of *Drosophila*. To retain genome stability P elements are inactive in the germline. This is achieved by telomeric P elements inserted in heterochromatic regions of the telomeres. Through *trans*-homolog interactions, telomeric P elements silence homologs being in euchromatic regions (*150*). Augustin de Vanssay and colleagues demonstrated that clusters of P element transgenes were able to silence homologous clusters that had no prior ability to cause silencing. The latter was able to cause silencing to other clusters, a phenomenon transmitted for at least fifty consecutive generations. This *trans*-homolog interaction is RNA-dependent and requires maternal inheritance of piRNAs that are homologous to the transgenes (*151*). Furthermore, genes necessary for the production of piRNAs, such as *rhino*, *cutoff*, *zucchini,* and *aubergine*, are necessary for paramutation in flies (*152*).

PAIRING-SENSITIVE SILENCING

In the past, transgenesis in *D. melanogaster* was performed predominantly by exploiting transposable elements like the aforementioned P elements. *pCaSpeR* is one of these vectors that contained as a reporter a version of the *white* gene named *mini-white*, lacking the majority of the *cis*-regulatory elements for wild-type gene expression. This leads to a dose-sensitive eye pigmentation. For example, wild-type flies have dark red eyes, and the flies containing loss-of-function alleles of *white* have white eyes. Transformed flies with transgenes containing *mini-white* display orange eye color in one copy of the transgene. The number of copies and eye pigmentation can be considered as proportional variables. As a result, eye color gets darker with additional copies. In contrast with the expectations, when this vector contained a piece of regulatory DNA from the *engrailed* locus the eye color in flies homozygous for the transgene was lighter than in the heterozygous (Figure 17). The silencing of *mini-white* expression is proximity-dependent, the closer the transgenes are the stronger the effect is and also, depends on the chromosomal location (*153*, *154*). This phenomenon is named pairing-sensitive silencing.

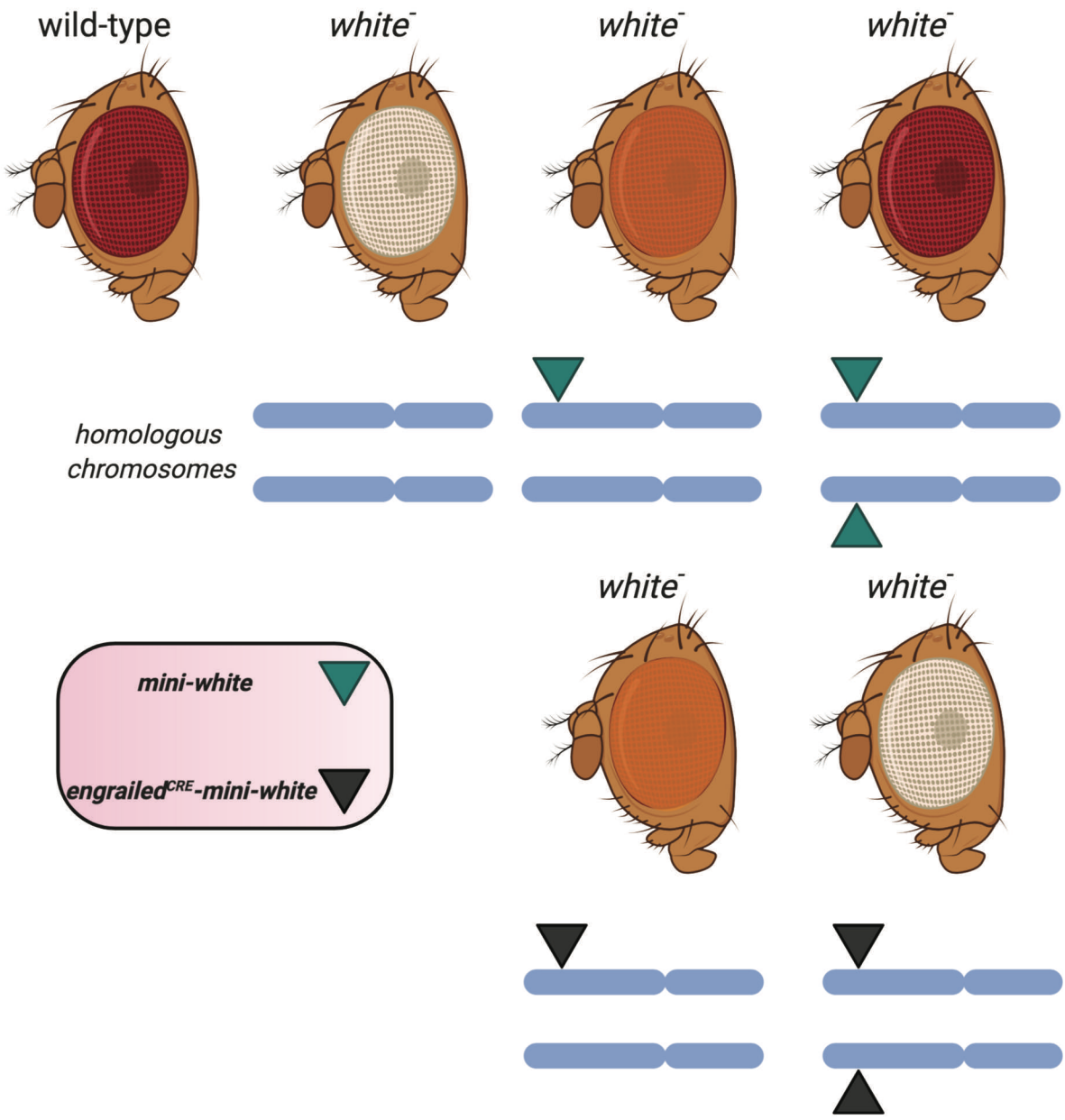

Figure 17. Pairing-sensitive silencing using mini-white transgenes in *D. melanogaster*. Wild-type individuals have dark red eyes in sharp contrast with *white* mutants. Heterozygous flies with transgenes containing as a marker *mini-white* (a shorter version of the wild-type *white* allele) have orange eyes whereas homozygous have dark red eyes. When the transgenes contain a *cis*-regulatory element from the *engrailed* locus (*engrailed*^{CRE}*-mini-white*) the eyes of heterozygous flies are light orange and eyes of homozygous are almost white illustrating the silencing of *mini-white*. Triangles depict insertion sites.

Pairing-sensitive silencing has been described today for several loci. Besides *engrailed*, fragments from *polyhomeotic*, *escargot*, *even-skipped*, *Sex combs reduced* and the *Mcp* insulator, are some of them that can induce pairing-sensitive silencing. Many of these fragments contain at least one Polycomb response element (PRE), but not all of them. PREs are *cis*-regulatory elements that can recruit complexes containing proteins of the Polycomb group. In the PRE-containing fragments, pairing-sensitive silencing is affected by mutations in genes of the Polycomb group in certain insertion sites, suggesting that the chromosomal environment is a crucial aspect for the silencing (*154–157*).

Typical features of pairing-sensitive silencing are the involvement of physical *trans*-homolog interaction, the propagation of silencing through several generations, and the spreading of silencing in chromatin on both sides of the PRE (*158*). Interchromosomal interactions involving PREs have been detected (*159–162*) for example during the early development of *D. melanogaster* to establish repressive gene expression domains (*163*). During later stages of development, chromatin looping leading to activation of gene expression requires PREs, something observed also during neural differentiation in mammals (*164*).

CONCLUDING REMARKS

A plethora of layers is known to be accountable for organizing and regulating the genome. One of them, *trans*-homolog interactions, which have been identified since 1954, appear to be widespread in animals, plants, and fungi (*165*, *166*). Several studies are describing this wide-variety of interactions that affect gene expression through diverse molecular mechanisms. *Trans*-homolog interactions are facilitated either through direct chromatin interactions or even through RNA-dependent mechanisms. Elements such as promoters, insulators, and architectural proteins appear to be critical for transvection but in the majority of the cases, they function in different ways (*86*, *167*). Future research taking advantage of genome editing technologies, quantitative imaging, and the use of non-traditional models will enable us to draw principles on the molecular foundations as well as, the biological significance of transvection.

THE WING SPOT IN *Drosophila biarmipes*

During my thesis, I addressed the question of how males and females despite sharing almost the same genome, are able to produce many sexually dimorphic phenotypes. Differences are observed both on how the animals look, behave, and function. Today our understanding is fueled by observations in a variety of species suggesting that sexually dimorphic phenotypes derive from sex-biased gene expression patterns. These patterns are governed by members of the sex-determination hierarchies (*48*). Recent observations indicate that the members of these hierarchies control genes that display sex-biased expression through independent branches, some of which are largely unknown (*43*, *44*).

It is a shared view that investigating non-traditional species will expand our understanding of how life functions and evolves. Here I am exploiting a simple and conspicuous pigmentation pattern present in males of *D. biarmipes*. This pigmentation pattern is represented as a black spot in the anterior and distal part of the wing and potentially, acts as a sexual communication signal (Figure 18).

Figure 18. Adult male (left) and female (right) of *D. biarmipes*. Sexually dimorphic pigmentation can be observed both on the wing and on the abdomen. (source: Nicolas Gompel)

In males, during pupal development, this pigmentation pattern is prefigured by the expression of the gene *yellow* (*y*) (Figure 19A-B). Although *y* precise function in pigmentation is unknown, it is necessary for the black pigment to get produced. In the female pupal wing, *y*

is expressed in a non-stereotypical dotted pattern in the spot region (Figure 19C-D). The genetic control of this sex-biased expression pattern is unknown.

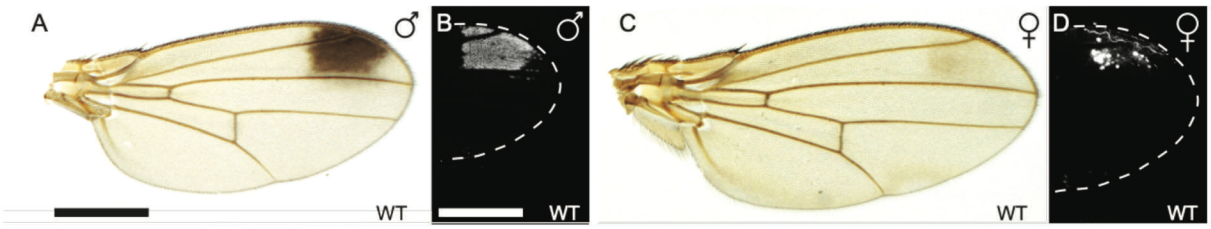

Figure 19. Sexually dimorphic pigmentation in *D. biarmipes* wings is prefigured by *yellow* expression. Wild-type male displaying a black pigmentation spot (**A**) and the associated Yellow distribution illustrated by an antibody staining (**B**). In females, pigmentation in the wing is uniform (**C**) and Yellow is distributed in an atypical dotted pattern (**D**). Scale bar corresponds to 500 µm.

The goal of my thesis was to elucidate the genetic inputs that direct *y* sex-biased gene expression in the *D. biarmipes* wing. More specifically, I have explored the contribution of the sex-determination hierarchies to *y* regulation. To achieve this, the generation of genetic tools in *D. biarmipes* was required, taking as an example the abundance of tools that exist in *D. melanogaster*.

In the next chapter, I am presenting the results of my study and discussing their implications in the context of sexual dimorphism.

Results

TRANS-HOMOLOG INTERACTION REGULATES THE SEX-BIASED EXPRESSION OF AN X-LINKED GENE

Abstract. Sex-biased gene expression in animals is generally controlled by the somatic sex-determination hierarchies. In the developing *Drosophila biarmipes* wing, the X-linked gene *yellow* is expressed in males in a spot pattern that is controlled by the *spot* enhancer, but the origin of *yellow* sex-biased expression is unknown. Here we find that a functional interaction between homologous *yellow* alleles silences specifically the *spot* enhancer, which is therefore active in males (XY) but not in females (XX), independently of the sex-determination hierarchy. We show that the chromatin architectural protein Mod(mdg4) is required for the homologous-dependent silencing of *yellow* and for the sex-biased expression of other X-linked genes in the brain. Our results demonstrate that *trans*-homolog interactions contribute to the sexually dimorphic regulation of X-linked genes.

Main text. Sexual dimorphism in morphology, physiology, and behavior is pervasive in animals. Sex-biased gene expression patterns, deployed during embryonic or adult development, direct the formation of these phenotypic sex-specific differences (*48*). It is well established that the transcriptional regulators of the somatic sex-determination hierarchies directly control sexually dimorphic gene regulation (*25*, *48*, *168*). Yet, the different tiers of these hierarchies seem to contribute to this control through a variety of regulatory mechanisms (*25*, *43*, *44*, *48*, *168–170*). To better understand the molecular mechanisms governing sexually dimorphic gene regulation, we examined the dimorphic regulation of the *yellow* (*y*) gene in *D. biarmipes*, a species that has evolved a male-specific wing pigmentation spot (Figure 20A) (*171*, *172*). During late pupal wing development in *D. biarmipes* males, Yellow spatial distribution prefigures the adult pigmentation spot (Figure 20B). In females, only a handful of cells produce Yellow, forming a typical dotted, stochastic pattern (Figure 20D). Accordingly, almost no pigmentation pattern appears in adult females (Figure 20C). We first examined the contribution of the top tier of the somatic sex-determination hierarchy, which initiates and establishes female identity in a cell-autonomous manner in *Drosophila* (*21*), to the regulation of *y* in *D. biarmipes*. For this, we feminized the male wing by overexpressing *Sex-lethal* (*Sxl*), and, conversely, we masculinized the female wing by knocking-down *transformer* (*tra*) expression, using a wing-specific driver (*173*). In both cases, this resulted in a modification of the wing pigmentation pattern (Figure 20E, G, Figure 21), revealing the conversion of the cells' sexual identity. Surprisingly, however, in both situations, the spatial pattern of Yellow was not altered and maintained its sex-specific expression (Figure 20F, H). The effects on pigmentation presumably result from the modification of expression of genes other than *y* that are involved in wing spot formation (*171*, *173*). These results suggest that the somatic sex-determination hierarchy does not control the sexually dimorphic regulation of *y* in *D. biarmipes* wing.

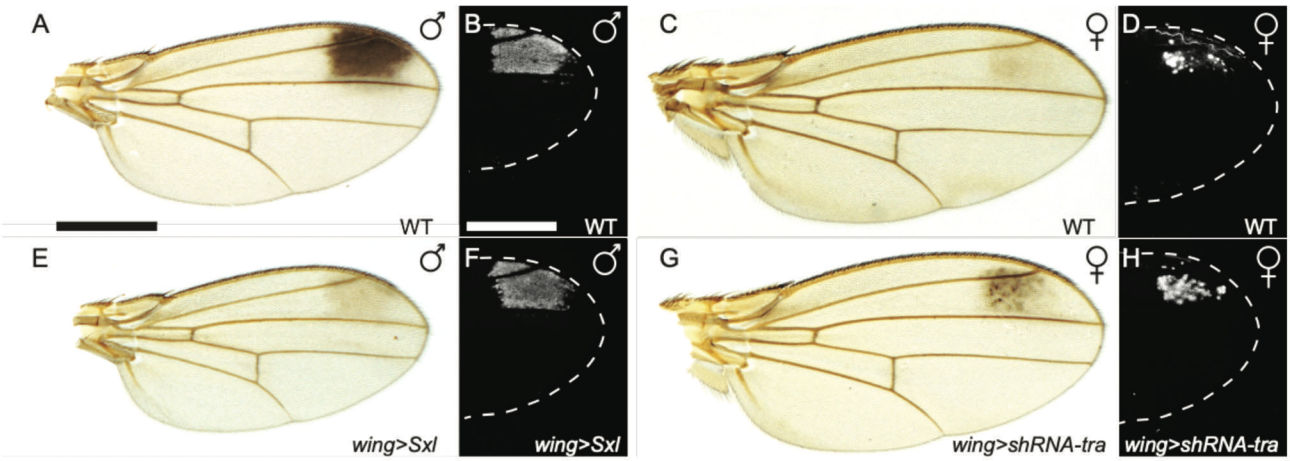

Figure 20. Yellow sex-biased pattern in *D. biarmipes* wing is independent of the sex-determination hierarchy. A male-specific wing pigmentation spot forms in *D. biarmipes* adult wings (**A**). It is prefigured during late pupal development by the spotted pattern of Yellow (revealed with an anti-Yellow antibody staining) (**B**). In female, the wing pigmentation level is almost uniform, a very faint spot appearing in some individuals (**C**), and Yellow is at a high level in just a handful of cells in the spot region (**D**). Overexpression of *Sxl* in male wings reduces the black spot intensity to levels found in females, or below, (**E**), without altering Yellow spatial distribution (**F**). Conversely, knocking-down *tra* in female wings leads to increased pigmentation in the spot region (**G**), but Yellow pattern is unaffected (**H**). White dashed lines indicate the wing contour. Scale bar, 500 μm (in all subsequent figures).

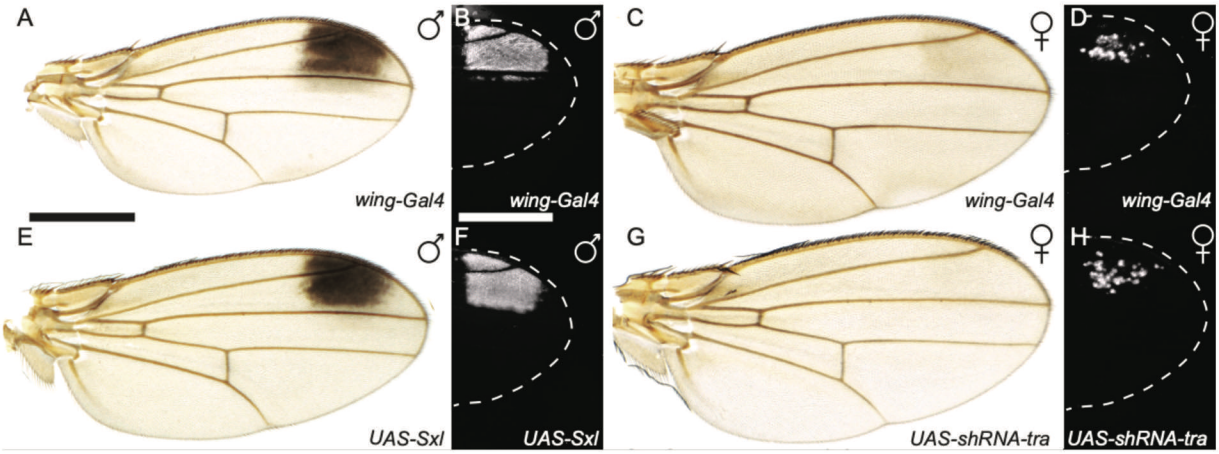

Figure 21. Yellow sex-biased pattern in *D. biarmipes* wing is independent of the sex-determination hierarchy**.** Adult pigmentation and Yellow patterns in the wings of *D. biarmipes* male *wing-Gal4* (**A**, **B**), female *wing-Gal4* (**C**, **D**), male *UAS-Sxl* (**E**, **F**), female *UAS-shRNA-tra* (**G**, **H**).

To understand how *y* is sexually regulated, we then sought to identify the *cis*-regulatory sequences directing its dimorphic expression in the wing. *y* expression in *D. biarmipes* wing is controlled by, at least, a pair of neighboring enhancers located in the 5' non-coding sequence of the locus, the *wing,* and the *spot* enhancers (Figure 22A). These enhancers are responsible for the deployment of Yellow throughout the wing at a low level, and at a high level in the presumptive wing spot area, respectively (*172*). The *spot* enhancer drives a sexually monomorphic activity in reporter assays in *D. biarmipes* (*173*) (Figure 23A, B). These results indicate that the sexually dimorphic regulation of *y* expression in *D. biarmipes* wing results from female-specific silencing of the *spot* enhancer activity. However, the *spot* sequence does not receive the female-specific regulatory inputs required for this silencing; another regulatory segment must be involved to control the sexually dimorphic expression of *y*. To identify a portion of the *y* locus that recapitulates its sexually dimorphic expression in the wing, we cloned a 25 kilobase (kb) long fragment, y^{25kb}, encompassing all the coding and non-coding

sequences of the gene. We also added a *mCherry* reporter in frame with the second exon of *y* to facilitate the detection of the protein product (Figure 22A). We then inserted this *y::mCherry*^{25kb} reporter construct on the X chromosome in a *D. melanogaster y* mutant. We anticipated that the spot patterns observed in *D. biarmipes* or *D. melanogaster* would differ slightly because of the divergence in the spatial distribution of a *trans*-regulator acting on the *spot* enhancer (*173*). Nevertheless, the *y::mCherry*^{25kb} construct fully rescued the *y* mutant phenotype in *D. melanogaster*, demonstrating its full functionality (Figure 23C-E). We then examined the distribution of Yellow::mCherry product in the wing of freshly emerged adults. In hemizygous (XY) males, Yellow::mCherry is produced moderately throughout the wing and at higher levels in the spot area, as expected (Figure 22B). This pattern, hereafter described as a spotted pattern, is similar to the activity of the sum of the *wing* and *spot* enhancers in *D. melanogaster* (*172*). In heterozygous females, *y::mCherry*^{25kb} drove spotted expression (Figure 22C), similarly to hemizygous males, an observation consistent with our result with the *spot* enhancer reporter in *D. biarmipes* (see Figure 23B). Surprisingly, in *D. melanogaster* homozygous (XX) females, Yellow::mCherry is distributed uniformly throughout the wing, but no spotted expression is detected (Figure 22D). To compare quantitatively the degree of spotted expression between genotypes we devised a Spotted-Expression-Index (S.E.I.), which measures the mean intensity of reporter expression in the spot area relative to the mean intensity in a posterior region of the wing (Figure 22E). In flies homozygous for the transgene, the S.E.I. is close to 1 while in hemi-/heterozygous individuals it is >1.5 (Figure 22F). The difference in spotted expression between hemi-/heterozygous and homozygous individuals is reminiscent of dosage compensation phenomena and could result from similar regulatory mechanisms that compensate for the imbalance of the X-linked genes expression levels between sexes (*174*). To explore this possibility, we inserted the *y::mCherry*^{25kb} reporter construct on different autosomes (chromosomes II or III) and calculated the S.E.I. for hetero- and homozygous flies. Regardless of the sex of the individuals, chromosomal insertions, or vector backbone, *y::mCherry*^{25kb} drove spotted expression when it was heterozygous, and uniform wing expression when it was homozygous (Figure 22F, Figure 23F). Together, these results ruled out that the silencing of *y* spotted expression is controlled by female-specific regulators. Instead, they suggest that a regulatory mechanism common to both sexes and relying on locus copy number difference controls the spot pattern silencing.

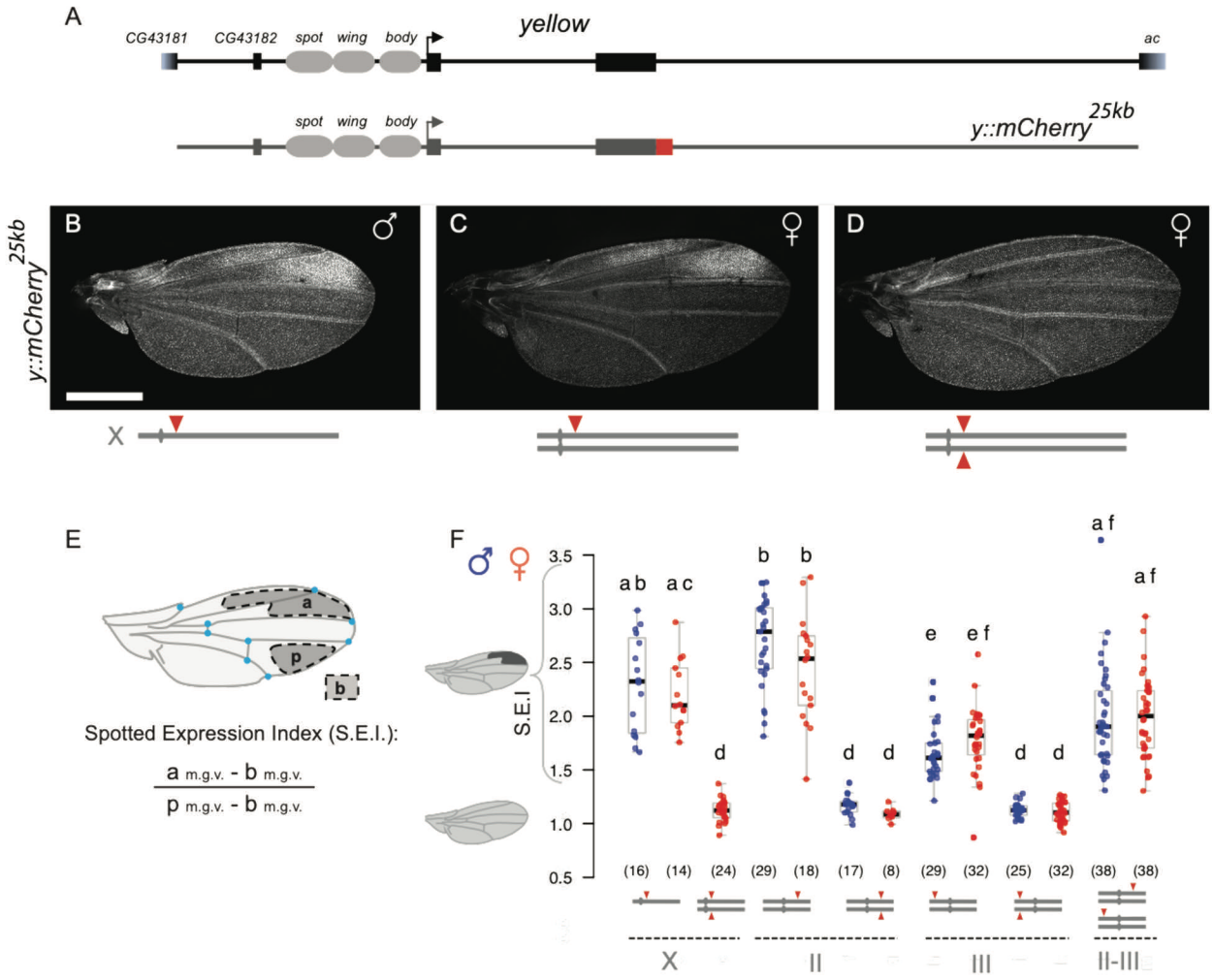

Figure 22. Sexually dimorphic regulation of *y* requires functional homolog interaction. Representation of the *D. biarmipes y* locus with the relative positions of the *spot*, *wing* and *body* enhancers, and the 25kb-long fragment cloned from the *y* locus tagged with *mCherry* (*y::mCherry*^{25kb}) (**A**). *y::mCherry*^{25kb} inserted on the X chromosome of *D. melanogaster* drives spotted expression in hemizygous males (**B**) and heterozygous females (**C**). In homozygous females, the spotted expression of Yellow::Cherry is silenced (**D**). In all figures, chromosomes are schematized below each genotype with transgene insertion(s) indicated by red triangle(s). Differences in overall intensity between (**B**) and (**C**) reflect the role of the dosage compensation complex, which enhances the transcription of X-linked genes in male. Quantification of the Spotted Expression Index (S.E.I.): after wing registration (see Methods), the nine blue reference points serve to measure the mean grey value (m.g.v.) of the three depicted regions (a, for anterior; p, for posterior; b, for background) used to calculate the S.E.I. (**E**). Quantification of the S.E.I. of *y::mCherry*^{25kb} insertions on different chromosomes in hemi-, hetero-, homo-, and *trans*-hetero-zygous configurations. For all the graphs of the study, each dot represents an individual wing (or abdomen), males are in blue, females in red; numbers in parentheses indicate sample sizes; statistically significant differences between samples are denoted by different letters (generalized linear model using a gamma distribution, followed by Tukey's contrasts for multiple comparisons of means; $p < 0.05$).

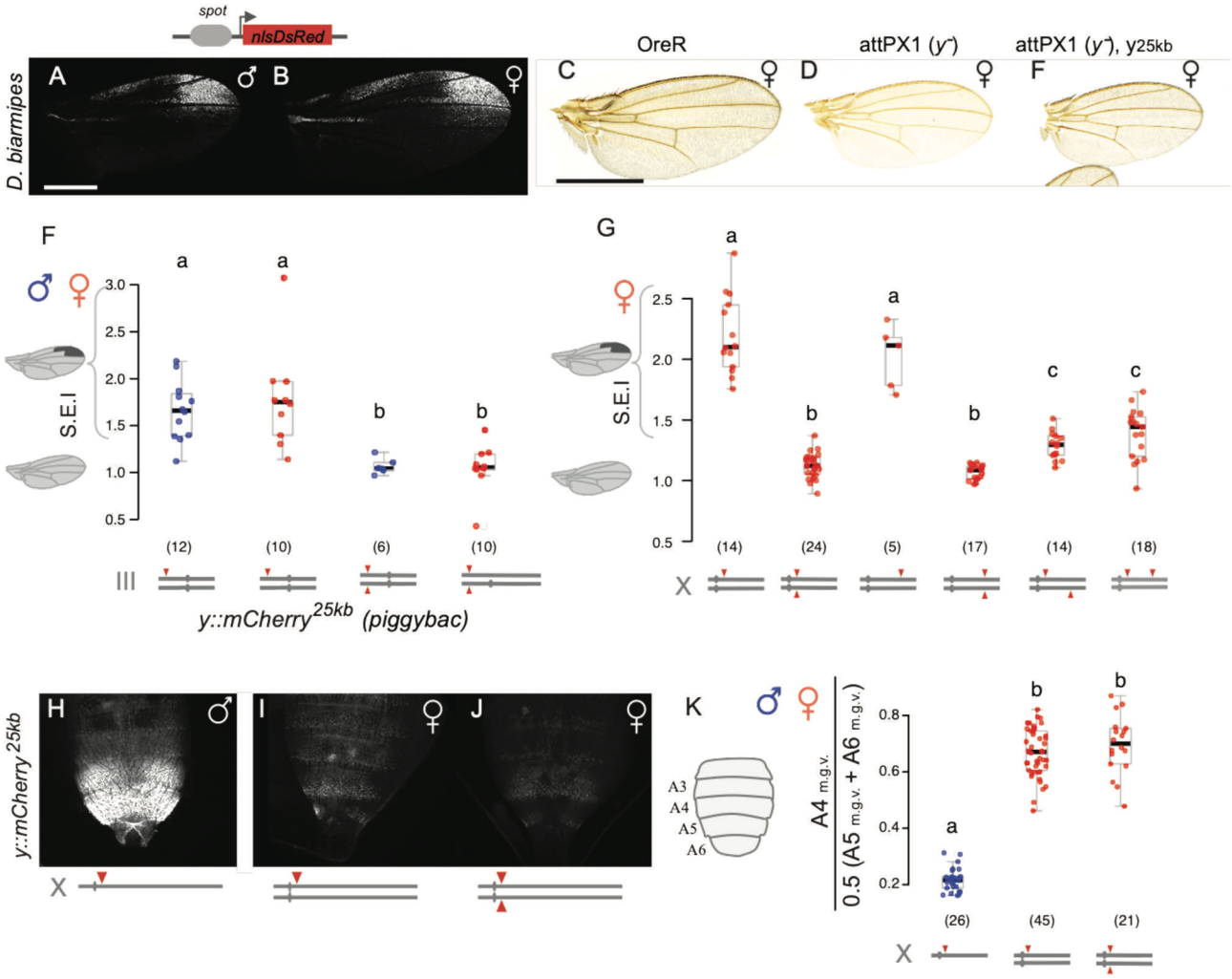

Figure 23. The *spot* enhancer drives similar reporter (nlsdsRed) activity in the wing of *D. biarmipes* males (**A**) and females (**B**) carrying a transcriptional reporter. Adult wings of *D. melanogaster* OregonR (**C**), attPX1 (**D**) and attPX1 carrying *y::mCherry*^{25kb}, which rescues the *y* mutant pigmentation phenotype (**E**). The *y::mCherry*^{25kb} construct cloned in a piggyBac vector backbone inserted on *D. melanogaster* chromosome III displays spotted expression in heterozygous males and females as indicated by the S.E.I.. In homozygous males and females, the spotted expression of *y* is silenced (**F**). *y::mCherry*^{25kb} inserted at two distinct positions on the X chromosome of *D. melanogaster* (5Mb apart) in the different configurations depicted by the schematics. When in *trans*-heterozygous configuration between the two positions, either on the same or different chromatids, the spotted expression of Yellow::mCherry is significantly decreased revealing the functional interaction between the transgenes at this distance (**G**). The data points for the attPX1 insertion (first 2 columns on the left) are the same as in Figure 20F. *y::mCherry*^{25kb} inserted on the X chromosome of *D. melanogaster* drives sexually-dimorphic expression in the posterior abdomen(**H-J**). Quantification of Yellow::mCherry, according to the schematic representation next to the y-axis, shows no significant difference between heterozygous and homozygous females (**K**).

To test whether the absolute copy number influences *y* regulation in the wing, we measured the spotted expression pattern of Yellow::mCherry with two transgenes inserted in a *trans*-heterozygous configuration on chromosomes II and III, and compared it with the pattern observed in heterozygous or homozygous situations for each insertion. When the transgenes are present in two copies inserted on different autosomes, they drove a spotted expression pattern, in both sexes, similar to either of the heterozygous transgenes, but in sharp contrast with the silencing observed when the transgenes are in homozygous configurations (Figure 22F). We concluded from this experiment that the silencing of the *y* spot pattern is not strictly due to a dose-effect. Instead, the results suggest that the transgenes interact functionally to mediate the silencing and that they can interact only when they are on the same chromosome. To test this idea, we inserted the *y::mCherry*^{25kb} transgene at two distinct sites on the X chromosome, separated by ~5Mb, and we tested their combined regulatory activity in the female wing in different configurations. As expected, females heterozygous for each transgene exhibited a spotted pattern, while homozygous females did not (Figure 23G). Remarkably, when the two copies are at different positions of the same chromosome in *trans*, or in *cis* on the same chromatid, the spotted expression is silenced, but not as strongly as in the homozygous configurations (Figure 23G). Altogether, these results indicate that a functional interaction between homologous copies of the transgenes mediates the silencing of the spot pattern. The silencing can only occur when the transgenes are on the same chromosome and is maximal when they occupy homologous positions on the chromosome pair. Therefore, we suggest that a regulatory *trans*-homolog interaction controls the sexually dimorphic regulation of *y* in *D. biarmipes* wing. Since *y* is X-linked, the *trans*-homolog-dependent silencing of the spot pattern can only occur in females, which carry two X chromosomes.

We then wondered if the *trans*-homolog interactions involved in the sexually dimorphic regulation of the spot pattern also affect other sex-biased expression patterns of *y*. We, therefore, examined the male-specific expression of *y* in the adult posterior abdominal segment (*175*). A *y body* enhancer, adjacent to the *wing* enhancer (Figure 22A) and that receives indirect inputs from the sex-determination hierarchy, directs this male-specific expression pattern (*42*). We compared the reporter activity of the *y::mCherry*^{25kb} transgene inserted on the X chromosome in hemizygous males, hetero- and homozygous females (Figure 23H-K). As expected, we observed an intense Yellow::mCherry signal in male posterior segments. By

contrast, Yellow::mCherry intensity was very low in females, both in hetero- and homozygous flies. This result reveals that the female-specific silencing of *y* expression mediated by *trans*-homolog interactions is restricted to the spot pattern and presumably only affects the *spot* enhancer activity.

To localize the sequences involved in the sex-biased spotted expression pattern, we first reduced the y^{25kb} region to a y^{10kb} fragment (Figure 25A). This fragment inserted on an autosome rescued a *y D. biarmipes* mutant (Figure 25B, C, D, F, L, N). Importantly, males heterozygous for the transgene display an increased wing pigmentation spot intensity compared to the homozygous individuals, while the overall wing pigmentation level is not affected (Figure 25D, H, F, J). This result shows that carrying a single copy of *y* matters for setting the *D. biarmipes* male wing spot intensity. Correspondingly, in both sexes, the Yellow pattern is female-like in flies homozygous for the transgene and becomes male-like in heterozygous individuals (Figure 25E, G, I, K, M, O). These results indicate that the y^{10kb} fragment contains all the necessary regulatory information for the native *y* expression, including the sexually dimorphic regulation in the spot pattern. We used this y^{10kb} fragment in *D. melanogaster*, adding a *mCherry* reporter in frame with the second exon of *y* (Figure 24A). The $y::mCherry^{10kb}$ construct behaved like the $y::mCherry^{25kb}$ both in hetero- and homozygous configurations (Figure 24B, C). Remarkably, we obtained the same results with a construct containing only *y* regulatory sequences of the y^{10kb} (5' and intron), showing that the *y* transcript or exonic sequences are not necessary for the homozygous-dependent silencing (Figure 25P-T). We then deleted the intron from $y::mCherry^{10kb}$ (Figure 24D) to assess its contribution to the spotted expression pattern. While $y::mCherry^{10kb\Delta intron}$ and $y::mCherry^{10kb}$ drive a similar expression pattern when they are present as a single copy (Figure 24B, E, G), we found that the homozygous-dependent silencing is lost when the *y* intron is missing (Figure 24C, F, G). This result reveals that the *y* intron is necessary for the homozygous-dependent silencing of the spotted expression and presumably silences the activity of the *y spot* enhancer.

Having identified that functional *trans*-homolog interactions shape the sexually dimorphic regulation of the spotted pattern, we sought to characterize the factors involved in the homozygous-dependent silencing of *y*. Therefore, we ran a genetic screen in *D. melanogaster*, using available alleles or RNAi lines for genes that have been previously associated with *trans*-homolog regulatory interactions, looking for candidates that disrupt the homozygous-dependent silencing of the $y::mCherry^{25kb}$'s spotted expression pattern (Table 1).

The only candidate gene that upon knock-down with two independent RNAi lines affected the *y::mCherry*^{25kb} sex-biased expression was *mod(mdg4)* (Figure 24H-J, Figure 27A). We confirmed this result by knocking down *mod(mdg4)* in *D. biarmipes* female wing, which resulted in a male-like Yellow pattern (Figure 24K-M). These results revealed that *mod(mdg4)* is required for the *trans*-homolog-dependent silencing of the *y spot* activity. Since Mod(mdg4) is involved in chromatin architecture and enhancer blocking (*76*, *84*, *176*), we speculate that it mediates the interactions between homologous *y* alleles, possibly bridging together the *spot* enhancers and the introns (Figure 26).

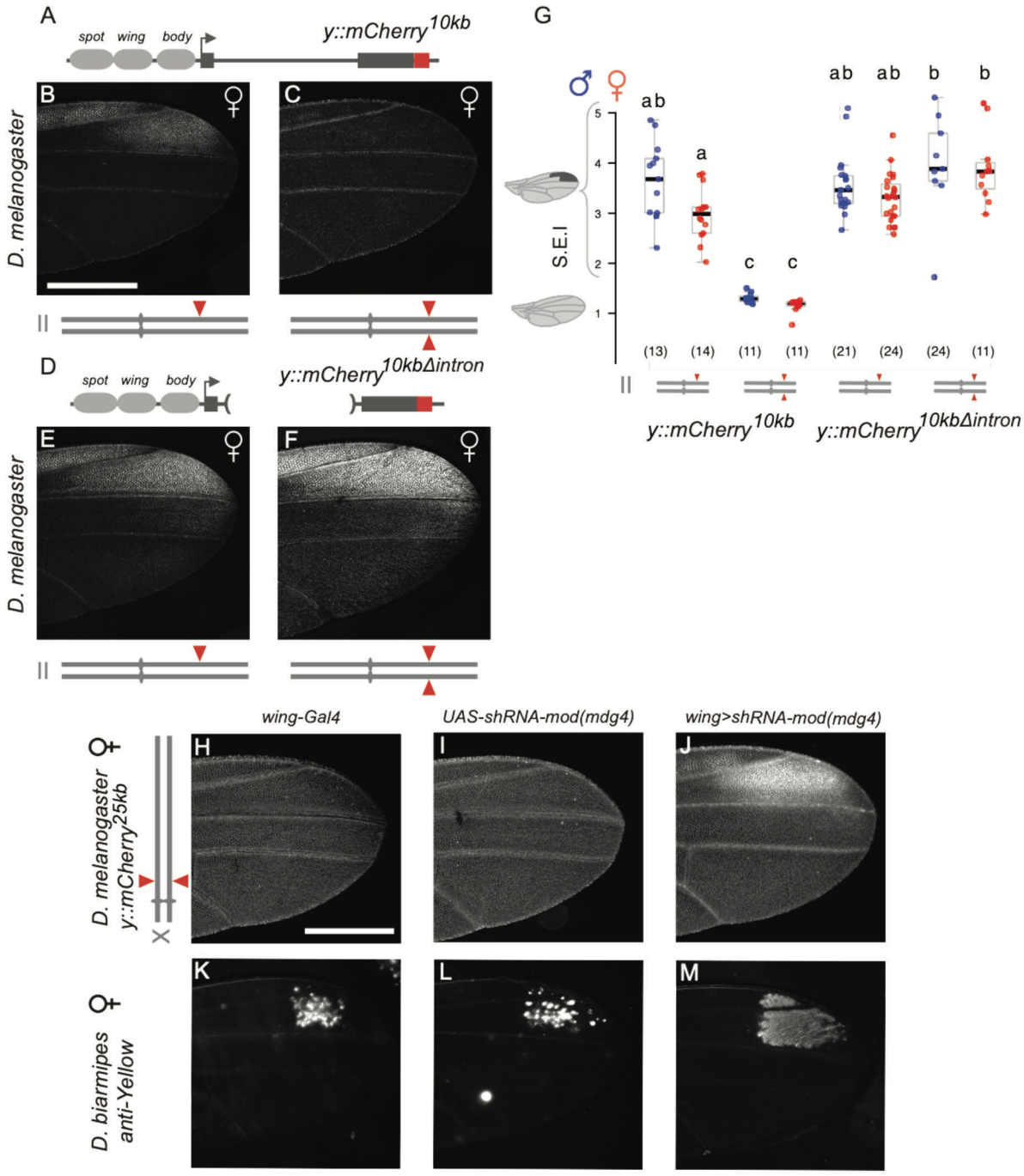

Figure 24. The *y* intron and *mod(mdg4)* are required for the *trans*-homolog-dependent silencing of the *y spot* enhancer. Females of *D. melanogaster* carrying the *y::mCherry*^{10kb} reporter (**A**) on chromosome II display spotted expression when heterozygous for the transgene (**B**) and uniform wing expression when homozygous (**C**). Females of *D. melanogaster* carrying *y::mCherry*^{25kbΔintron} (**D**) on chromosome II display spotted expression both when heterozygous (**E**) or homozygous (**F**). Quantification of the S.E.I. shows similar results in males (**G**). Knocking-down *mod(mdg4)* in females of *D. melanogaster* abolishes silencing of *y::mCherry*^{25kb} (**H-J**). Similarly, when knocking-down *mod(mdg4)* in *D. biarmipes* female wings, the native Yellow pattern (**K**, **L**) becomes male-like (**M**).

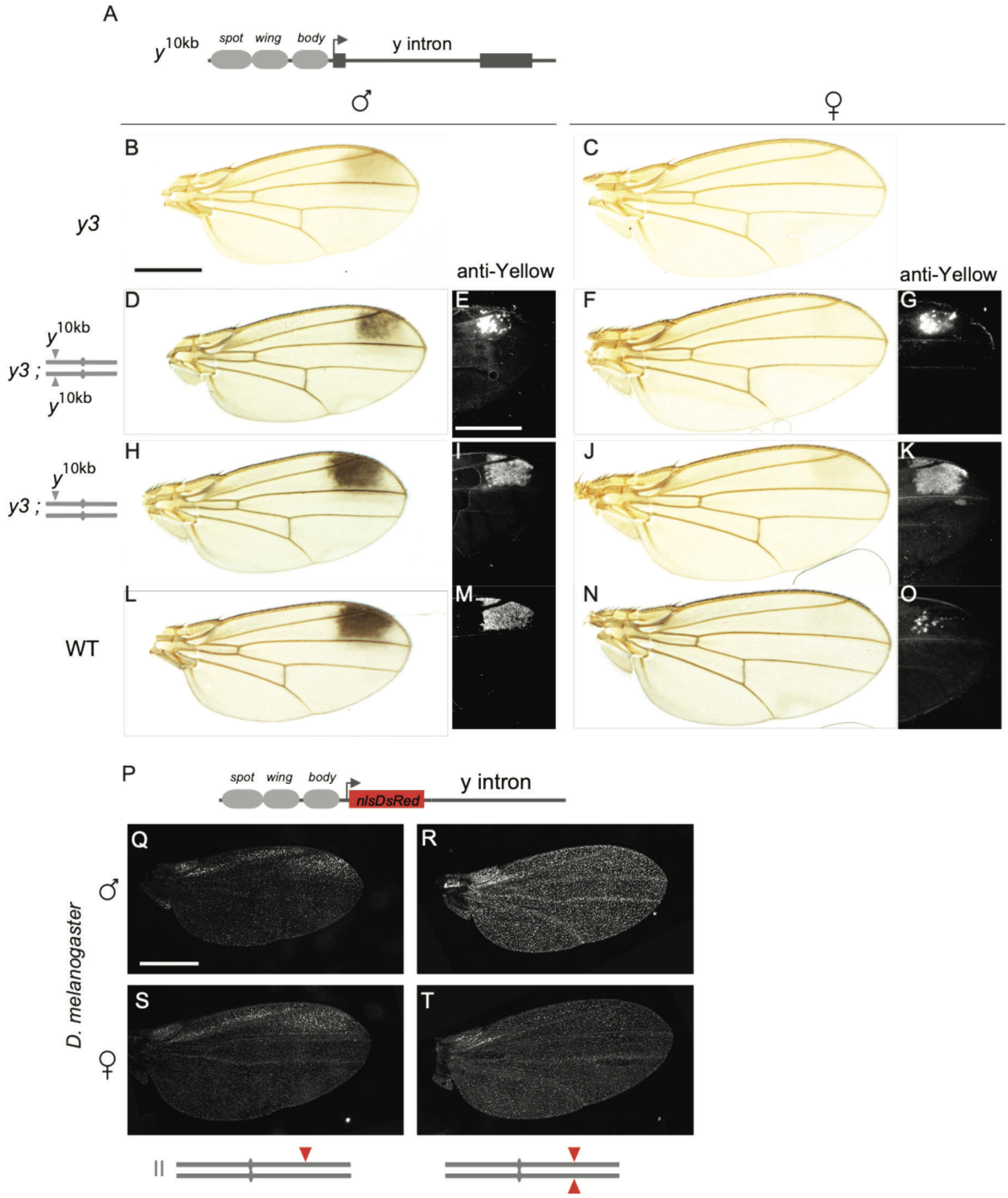

Figure 25. Schematic representation of the y^{10kb} construct (A). Adult wings of *y* mutant (*y3*) *D. biarmipes* male (B) and female (C). Adult pigmentation of *D. biarmipes y3* mutants carrying the y^{10kb} transgene in homozygous configuration in males (D) and females (F) and heterozygous configuration in males (H) and females (J) with the associated Yellow pattern (revealed by an anti-Yellow staining), respectively (E, G, I, K). Adult wings and Yellow patterns of wild-type males and females are presented for comparison (L-O). Schematic representation of the y^{10kb}-*nlsDsRed* transcriptional reporter in which the nlsDsRed is inserted upstream of the *y* intron (P). This reporter constructs behaves in *D. melanogaster* like the *y::mCherry*^{10kb} construct, it drives spotted expression when heterozygous (Q, S), and uniform expression when homozygous (R, T), in both sexes.

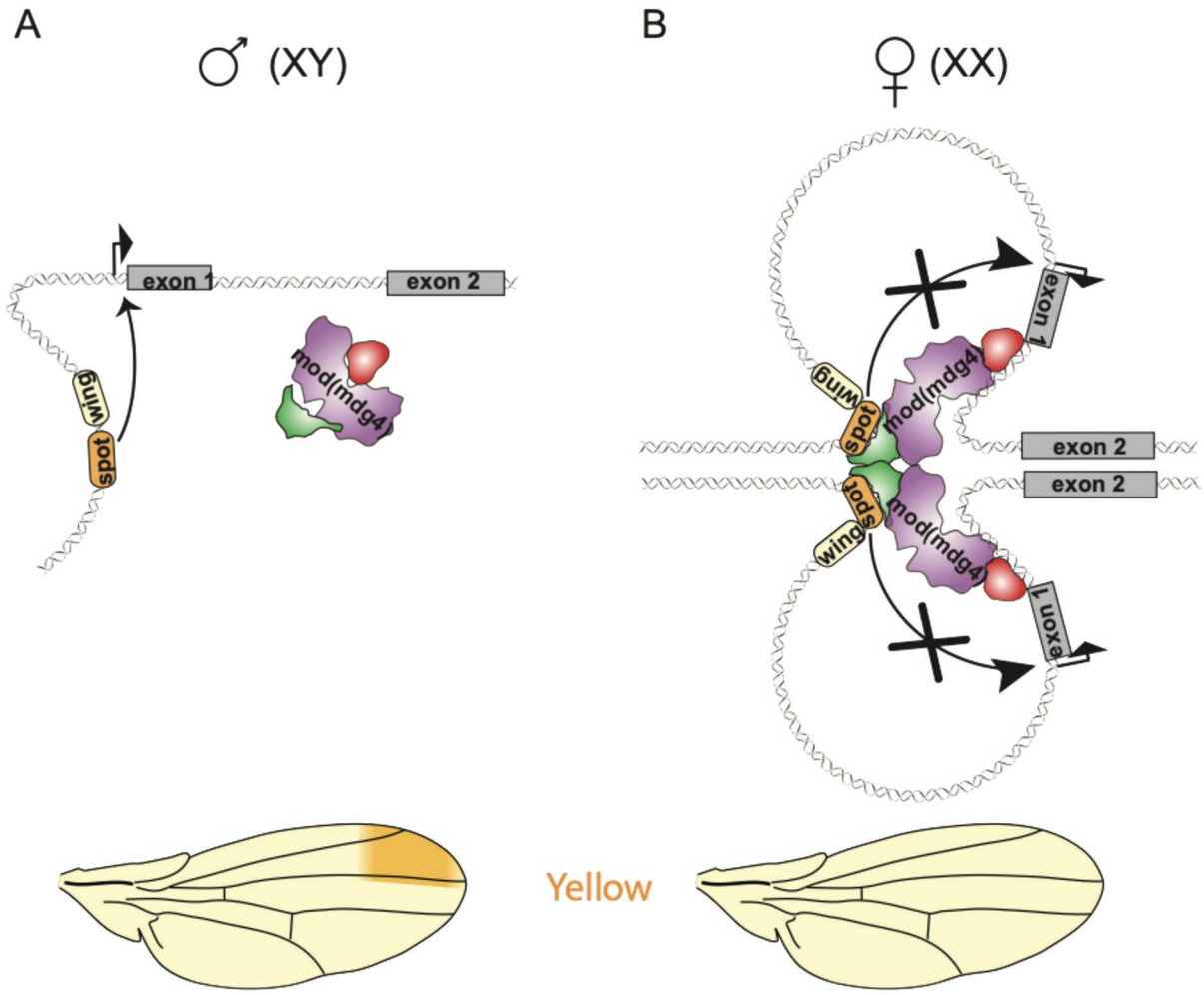

Figure 26. Regulatory model of *y* sexually dimorphic expression in the wing of *D. biarmipes*. *y* is present in one copy in males since it is X-linked and the *spot* enhancer directs its spotted expression (**A**). By contrast, in females, the two homologous copies of *y* interact through the action of the non-sex-specific Mod(mdg4) architectural protein, the *spot* enhancer can no longer activate *y* expression (**B**).

Finally, we reasoned that the *trans*-homolog regulatory interaction we identified might not be limited to the sex-biased expression of *y* in *D. biarmipes* wings. Therefore, we examined by RNA-seq the sexually-dimorphic regulation of X-linked genes in *D. melanogaster* adult brain, a tissue displaying substantial sex-biased gene expression (*65*), in wild-type and after *mod(mdg4)* neuronal knock-down. We found that 40% (6/15) of the X-linked genes showing sex-biased expression in wild-type brains displayed reduced sexual dimorphism upon

mod(mdg4) knock-down (Figure 27B, C). In contrast, genes that belong to the sex-determination hierarchy (e.g. *tra*), including X-linked ones (e.g. *Sxl*), or that are controlled by it (e.g. *Yp3*), remain sexually dimorphic (Figure 27D). These results suggest that the *trans*-homolog regulatory interaction we identified for the sex-biased expression of *y* may be a more general and non-canonical mechanism of sexually dimorphic regulation, exploiting the hemizygosity of X-linked genes.

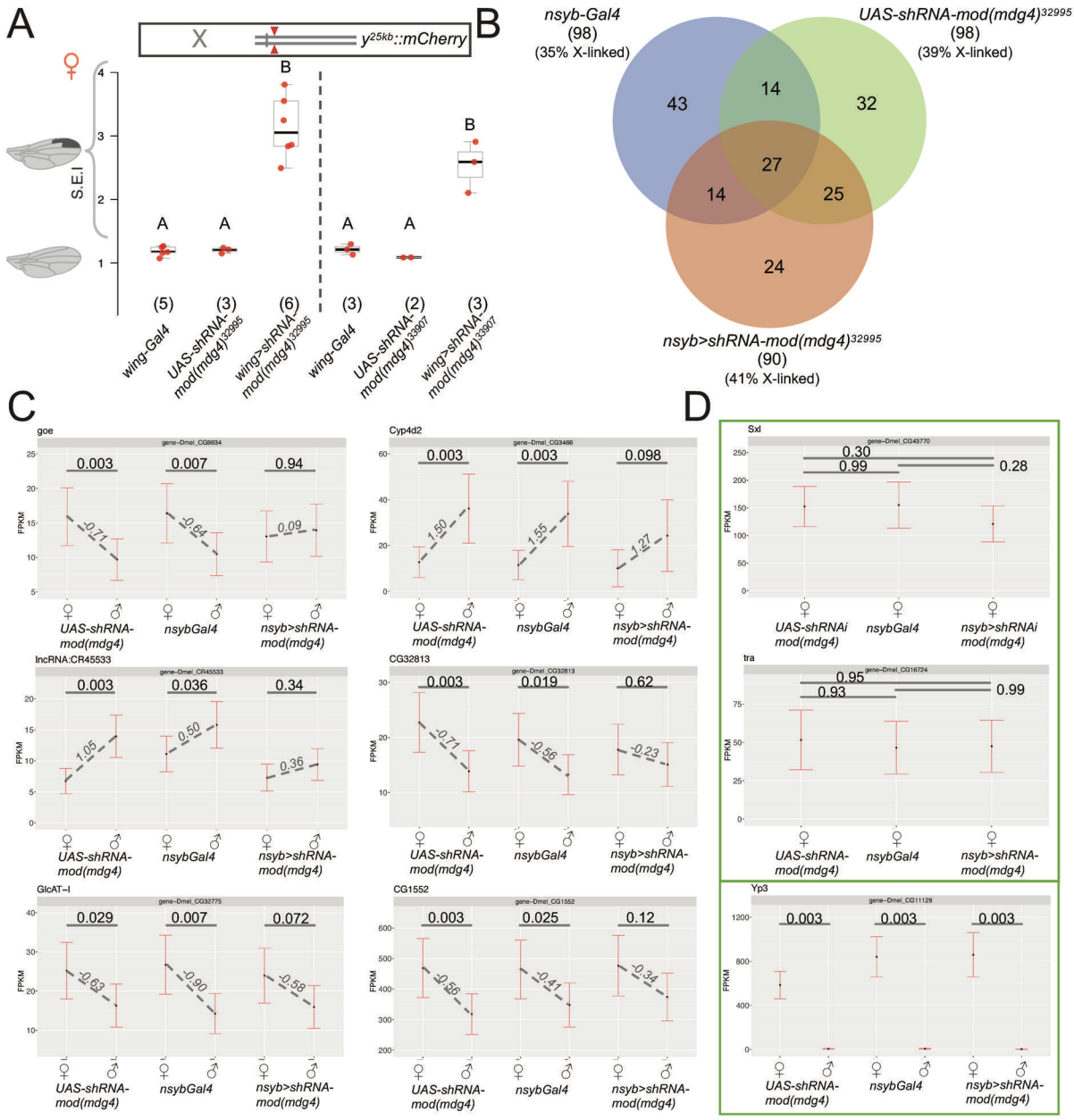

Figure 27. S.E.I. for the *mod(mdg4)* RNAi lines (Gal4 and UAS controls), raised either at 22°C (for the UAS line #32995), or at 29°C (for the line UAS line #33907) (**A**). Venn diagram showing sexually-dimorphic expression of genes in adult brains of *D. melanogaster* detected

by RNA-seq in a neuronal driver (*nsyb-Gal4*) line, a *UAS-RNAi* line against *mod(mdg4)* and the cross between them (**B**). Forty-one genes have a common sex-biased expression in the two parental lines, 15 of which are X-linked. Six of these 15 genes become monomorphic upon *mod(mdg4)* knockdown (**C**). RNA levels (FPKM) show that knocking-down *mod(mdg4)* does not alter the expression of *Sxl* or *tra*, the factors that initiate sex determination, or the expression of the *dsx*-regulated X-linked gene, *Yp3* (**D**). The fold difference between sexes are indicated for each genotype, and the statistical significance is indicated by the q values on top of the expression bars.

Altogether, these results identify a novel mechanism for the regulation of the sex-biased expression of X-linked genes that is independent of the somatic sex-determination hierarchy and relies instead on *trans*-homolog interactions. The physical proximity of homologous chromosomes, which are aligned end-to-end in all somatic cells in Diptera, can presumably facilitate functional interallelic communication (*88*, *95–97*, *177*). *Trans*-homolog regulatory interactions encompass a broad spectrum of phenomena described collectively as transvection, including Ed Lewis' initial definition (*98*), which are usually only revealed in mutant contexts or with transgenic constructs (*86*, *98*, *101*, *119*, *140*, *167*). Yet, the physiological relevance of *trans*-homolog regulatory interactions in the biology of wild-type *Drosophila* has long remained questionable. We report here the first case where it directly impinges on the sexually dimorphic regulation of an X-linked gene. Whether the *trans*-homolog regulatory mechanism we have unveiled acts specifically to control the X-linked genes, or whether it is part of a general buffering mechanism acting on a whole-genome level (*178*) remains to be explored.

BDSC #	Gene	Flybase ID	spot silencing
RNAi lines			
29734	BEAF-32	FBst0029734	Negative
34069	Caf1-55	FBst0034069	Negative
33903	Cp190	FBst0033903	Negative
42536	Cp190	FBst0042536	Negative
40850	CTCF	FBst0040850	Negative
31941	Dref	FBst0031941	Negative
27993	E(z)	FBst0027993	Negative
33659	E(z)	FBst0033659	Negative
61903	Elba2	FBst0061903	Negative
61180	HIPP1	FBst0061180	Negative
32995	mod(mdg4)	FBst0032995	Positive
33907	mod(mdg4)	FBst0033907	Positive
28343	pbl	FBst0028343	Negative
33945	Pcl	FBst0033945	Negative
33946	Pcl	FBst0033946	Negative
42926	pho	FBst0042926	Negative
35297	Psc	FBst0035297	Negative
31612	Sce	FBst0031612	Negative
33704	Set1	FBst0033704	Negative
32473	Sfmbt	FBst0032473	Negative
33906	Su(Hw)	FBst0033906	Negative
34006	Su(Hw)	FBst0034006	Negative
31191	Su(z)12	FBst0031191	Negative
33402	Su(z)12	FBst0033402	Negative
31342	Top2	FBst0031342	Negative
overexpression lines			
15026	Cap-D3	FBst0015026	Negative
17627	Cap-H2	FBst0017627	Negative
alleles			
200	z[1]/z[a]	FBst0000200	Negative
1728	Pc[1]	FBst0001728	Negative
4247	Su(Hw)[2]/Su(Hw)[e04061]	FBst0004247	Negative
1053	Su(Hw)[8]	FBst0001053	Negative
18224	Su(Hw)[2]/Su(Hw)[e04061]	FBst0018224	Negative
59959	Su(Hw)[v]	FBst0059959	Negative
1059	z[1]/z[a]	FBst0001059	Negative

Table 1. *D. melanogaster* stocks used to screen regulators of *yellow* spotted expression.

Materials & Methods

FLY HUSBANDRY

Flies were raised on a standard cornmeal-agar medium and grown at 22°C. All experiments were carried out at 25°C, except for the RNAi screen which was carried out at 29°C unless stated differently.

FLY LINES

BDSC stock number	**Genotype**
24480	y[1] M{3xP3-RFP.attP}ZH-2A w[*]; M{vas-int.Dm}ZH-102D
32107	y[1] w[67c23] P{y[+t7.7]=CaryP}attP18
24865	y[1] M{vas-int.Dm}ZH-2A w[*]; PBac{y[+]-attP-3B}VK00016
24871	y[1] M{vas-int.Dm}ZH-2A w[*]; PBac{y[+]-attP-3B}VK00033
851	y[1] w[67c23] P{y[+mDint2]=Crey}1b; D[*]/TM3, Sb[1]
-	w[*]; wg[Sp-1]/CyO ; MKRS/TM6B
33907	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00849}attP2
29734	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HM05202}attP2
31191	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01706}attP2
31342	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01300}attP2
31612	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01396}attP2
33903	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00845}attP2
32473	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00473}attP2/TM3, Sb[1]
32995	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00795}attP2

33402	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00280}attP2/TM3, Sb[1]
42536	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ02105}attP40
33659	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00066}attP2
33704	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00581}attP2
34069	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00051}attP2
33906	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00848}attP2
33945	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00896}attP2
33946	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00897}attP2/TM3, Sb[1]
61180	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMC05150}attP40
34006	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00970}attP2
35297	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL00199}attP2
61903	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ23458}attP40
40850	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02017}attP40
42926	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02619}attP40
27993	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02826}attP2
28343	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02979}attP2
31941	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02232}attP2
200	z[1] w[11E4]
1059	y[1] z[a] w[11E4]
1728	Pc[1]/TM1
51635	y[1] w[*]; P{w[+m*]=nSyb-GAL4.S}3

Constructs injected in D. biarmipes:

UAS-shRNA-tra: The shmir against *transformer* was created using the protocol from (Haley, B. et al. 2008) (*179*). The two shmirs we designed are shtra4.1: 5'-GACAGACTCCTTTCGACATAA-3' and shtra4.2: 5'-GCAAAGGAGTCCTCATCGGTA-3'. The vector (pNE3) that contained both of the transgenes was subcloned to a piggyBac vector using the In-Fusion® HD Cloning Kit from Takara with the primers pNE3_UAS_to_pBac.F: 5'-TACGCGTACGGCGCGCCGCTTCTGCAT CTCTCCGGATCCAAGC-3' and pNE3_UAS_to_pBac.R: 5'-GTCGACCTAGGCGCGCCGAT CCAGACATGATAAGATACATTGATG-3'.

UAS-Sxl: The *Sxl* coding sequence was cloned from whole fly cDNA with the primers Sxl.F: 5'-GATCATGTACGGCAACAATAATCCG-3' and Sxl.R: 5'-GATCTTATAAGTAAGGATAAT GGTACTTCCG-3' and then inserted using TOPO-TA into the pCR8 vector. It was then subcloned to a UAS-piggyBac vector with an LR reaction.

UAS-shRNA-mod(mdg4): The shmir against *mod(mdg4)* we used is 5'-TTCGTGTTGAAGTTGTTCCAG-3' and cloned in piggyBac Gateway vector ligating sh_mod(mdg4)top: 5'-CTAGCAGTCTGGAACAACATCAACACGTATAGTTATATTCAAG CATATTCGTGTTGAAGTTGTTCCAGGCC-3' and sh_mod(mdg4)bot: 5'-TCGAGGCCTGG AACAACTTCAACACGAATATGCTTGAATATAACTATACGTGTTGATGTTGTTCCA GACTG-3' between the restriction sites PspXI and NheI.

y^{10kb}: This construct was cloned at the AscI site of a piggyBac vector using the In-Fusion® HD Cloning Kit. The primers used for PCR on *D. biarmipes* genomic DNA were 1F: 5'-TACGCGTACGGCGCGCCATCGATAATCGCCC GATTACCG-3', 1R: 5'-CTTCTATTGGGTTCTTTCTTAGCCGGAAAT-3', 2F: 5'- AGAACC CAATAGAAGTTCCAGAAAAGTGAC-3' and 2R: 5'-GTCGACCTAGGCGCGAGCATACT TACAGATACTCCTCATTTTCTATTTATGATG-3'.

These plasmids were injected in *D. biarmipes* embryos at 100.0 ng/µl along with the helper plasmid (also at 100.0 ng/µl).

Constructs injected in D. melanogaster:

All the constructs have been established using the In-Fusion® HD Cloning Kit.

y^{25kb}: This construct was cloned in the piggyBac vector at the AscI site using the In-Fusion® HD Cloning Kit and the following set of primers: 3F: 5'-TACGCGTACGGCGCGCCGAGGATTCT GCCAGATCCCGG-3', 3R: 5'-ATTATCGATGGC GCGAAACAATCGCAGCGATCTCCC CA-3', 1F, 1R, 2F, 2R, 4F: 5'-GATGATAGGATATTT TAAATCACGAGGAAACGAATC TTAAACACGGG-3', 4R: 5'-TAAATAAACTTAATTTAA ATAAAAAAGCCCTTTTCCCGG-3', 5F: 5'-AATTAAGTTT ATTTAAATTAAGTGGGTTAG GTCAGAAAAAGTAAGCTGT-3', 5R: 5'-GTTTCCTCGTG ATTTCACGCTGCCGGTGGG-3'.

y::mCherry ^{25kb}: The *mCherry* with the Waldo linker was cloned from the *pJET-mCherry* vector using the primers: mCh1F: 5'- CCAGGGTTCCGCTGGCTCCGC-3' and mCh1R: 5'-GGGTTGGGTTAC TTGTACAGCTCGTCCATGCCGC-3'. This was inserted in the y^{25kb} vector to create the *y::mCherry*^{25kb} transgene using the StuI sites along with two fragments from the vector cloned with the primers: 6F: 5'- TTGAGGTGCCCAAGGCCTACATCTTCA-3', 6R: 5'- CCAGCGGAACCCTGGTGCTGGTGG-3', 7F: 5'-CAAGTAACCCAACCCGTGCACGG-3' and 7R: 5'-ATCTTAATCTTAAGGCCTCGTCTTTGGAG-3'. The *y::mCherry*^{25kb} was then subcloned into the pWalium20 vector using the sites AatII and NotI and the primers 8F: 5'-TCGAATGGCCATGGGACGTCTTTCCATAGGCTCCGCCCC-3' and 8R: 5'-TCTAGAGTCGCGGCCGCCGAATTGATCCGGAGAGC-3'.

y::mCherry^{10kb}: This was cloned from the *y::mCherry*^{25kb} at the AscI site of piggyBac using the primers: 8F: 5'- TACGCGTACGGCGCGCCATCGATAATCGCCCGATTACCG-3' and 8R:
GTCGACCTAGGCGCGAGCATACTTACAGATACTCCTCATTTTCTATTTATGATG-3' and then subcloned to pWalium20 using the same strategy as for the y^{25kb}.

y::mCherry$^{10kb\Delta intron}$: This was cloned from the *y::mCherry*^{10kb} at the AscI site of piggyBac using the primers: 8F, 8.1R: 5'- AGGGATGCCATCTCGCCAGCGGG-3', 8.2F: 5'-

CGAGATGGCAT CCCTGCCACTCT-3' and 8R. Then it was subcloned to a chimeric version of pWalium20 and piggyBac using the same strategy as previously described.

The plasmids were injected in the following stocks (BDSC stock numbers) : #24480 or #32107 (chromosome X), #24865 (chromosome II), #24871 (chromosome III). Following the injection in #24480, we used the line #851 to remove the 3xP3-RFP (flanked by two loxP sites) marking the attP site. Plasmid were injected at 100.0 ng/ul. For injection in #32107, plasmid was injected along with a helper plasmid encoding for φC31 integrase (100.0 ng/ul).

Generation of a *y* mutant in *D. biarmipes* with Crispr/Cas9

The *yellow* mutant in *D. biarmipes* was created according to (Bassett & Liu 2014) (*180*). The sgRNA used was 5'-CCCCAGAACGGCCTTCCCG-3', identified using the Target Finder of flyCRISPR (https://flycrispr.org/). The *yellow* mutant was screened based on the phenotype and confirmed by Sanger sequencing.

ANTIBODY STAINING

We performed antibody staining using the Yellow antibody and the protocol from (Hinaux, H. et al. 2018) (*181*).

IMAGING

Adult wings of five-days-old flies were mounted on Hoyer's medium according to (Arnoult, L. et al. 2013) (*173*) and imaged on a Leica Wild M420 Makroscop equipped with a ProgRes C5 ccd camera (Jenoptik, Germany).

Reporter expression and fluorescent antibody-staining were imaged on an MSV269 stereoscope with a DFC365 FX camera (Leica). Freshly hatched adults (<30 minutes) were

collected and fixed in 5% Formaldehyde/1X PBS. One wing per individual was dissected and mounted in Vectashield medium.

Image registration prior to quantification was performed by setting manually nine points described in Figure 22E using a reference wing and the Landmark Correspondences plugin of Fiji (method: least squares, alpha: 1.00, mesh resolution: 32, class: similarity).

Quantification of fluorescent wings was performed measuring mean gray value using Fiji in the a, b, and p regions defined in Figure 22E. Quantification of fluorescent abdomens was performed similarly but measuring all the A4, A5 and A6 tergites.

For each experiment, all pictures were taken under the same settings. All images were uniformly enhanced using Adobe Photoshop.

STATISTICS AND PLOTS

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment. Plots were created using RStudio (v. 1.2.1335). Each point represents an individual wing (one per individual) or abdomen. Data were analyzed using a Generalized Linear Model (GLM) with a Gamma distribution. When the GLM showed a statistically significant difference between groups, the test was followed by a multiple comparison (Tukey HSD) test with a Bonferroni correction method (R: glht function in multcomp package).

RNA-SEQ

Brains were dissected from 2 days old, male and female, unmated flies during the morning from the three following genotypes: *nsyb-Gal4*, *UAS-RNAi-mod(mdg4)*^{32995} and the cross between them. RNA was extracted using phenol/chloroform from five to eight brains per sample to generate three replicates per sex and genotype. Libraries were generated using the TruSeq library preparation kit from Illumina and sequenced using a HiSeq 4000 platform.

Indexing of the genome of *D. melanogaster* (Release_6_plus_ISO1_MT) was performed using HISAT as well as the alignment of the reads to the genome (*182*). Quantification, geometric normalization and differential gene expression analysis was performed using Cufflinks (*183*). In our experiment, we compared the differentially expressed genes (F.D.R.<0.05) between sexes in each of the three genotypes to detect genes with sex-biased expression in the two parental genotypes that are no longer sex-biased in the cross (F.D.R.>0.05).

Discussion

GENERAL CONCLUDING REMARKS

Males of *D. biarmipes* display a black pigmentation spot in the anterior and distal part of their wing, while the females do not. This sexually dimorphic pattern is reflected in the expression pattern of *yellow* in the pupal wing of *D. biarmipes*. The expression of *yellow* in the spot is controlled by an enhancer located in the 5' of the gene called the "*spot*" enhancer. During my thesis, I studied the regulatory basis of *yellow* sex-biased expression in the wing of *D. biarmipes*. Contrary to expectations, I have demonstrated that *yellow* does not receive regulatory inputs from the two central members of the sex-determination hierarchy, *Sxl,* and *tra*. Instead, its sex-biased expression is controlled by a *trans*-homolog interaction that specifically silences the *spot* enhancer in females. This silencing requires both the *spot* enhancer and the intron of *yellow*. The discovery that the architectural protein, Mod(mdg4), is necessary for this silencing leads us to the speculation that it acts through bridging the intron and the *spot* enhancer of the homologous loci. Architectural proteins are known to mediate loops (*184*). The molecular mechanism responsible for the *spot* enhancer silencing is unknown. It could be either i) through the local modification of the chromatin, ii) through disruption of the binding of transcriptional regulators or iii) structural constraints that affect the access of the *spot* enhancer to the promoter. The structural constraint could be addressed using a series of transgenes changing the order of sequences. In addition, chromatin profiling with ChIP-Seq for chromatin marks and ATAC-seq for chromatin accessibility will enable us expand our understanding regarding the nature of this silencing. At last, the use of a chromatin-conformation capture method would allow us to investigate the existance of a physical interaction between the intron and the *spot* enhancer.

Expanding the genetic framework of sexual dimorphism

It is widely assumed that sex-biased expression is controlled by the members of the sex-determination hierarchies. Here I discovered an additional mechanism that does not rely on the members of the sex-determination hierarchy. The action of this mechanism might have been masked by the parallel action of the sex-determination hierarchies in setting sexual dimorphism. Since most of the traits are controlled by the action of multiple genes, which do not reside exclusively on the X chromosome.

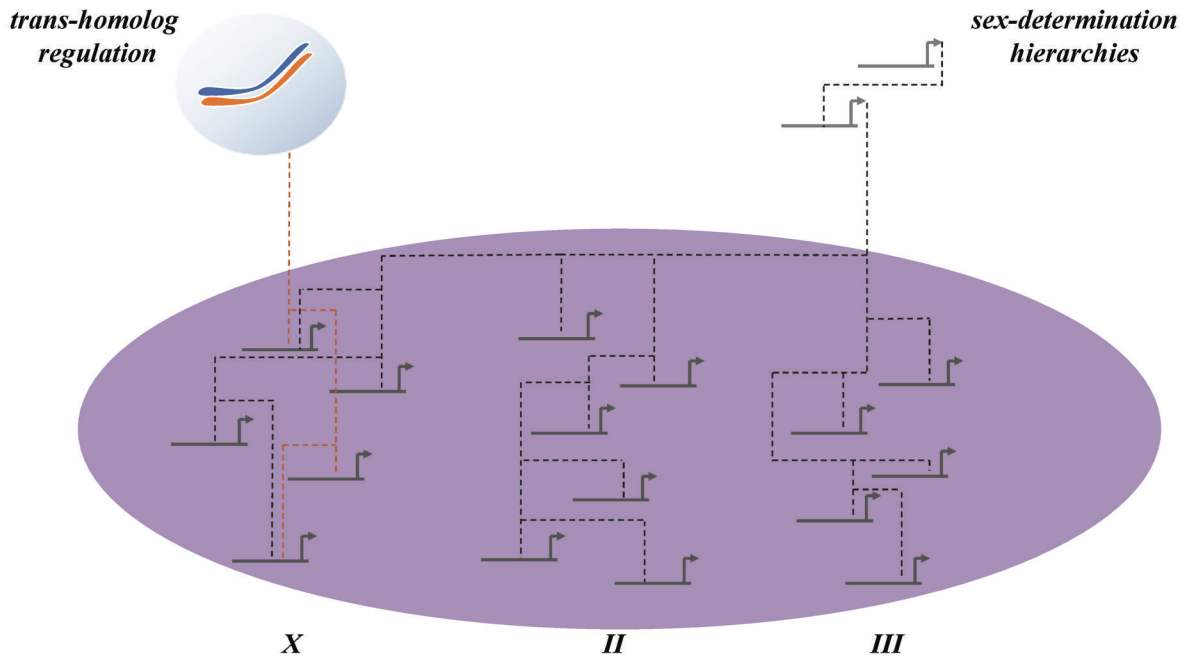

Figure 28. Two main mechanisms by which sex-biased gene expression can be generated in *Drosophila*. *Trans*-homolog interactions, taking advantage of chromosomal pairing, can regulate X-linked genes in a sex-specific manner. In parallel, in the traditional model, sex-biased gene expression is regulated throughout the whole genome by members of the sex-determination hierarchies. The X chromosome and two autosomes (II and III) of *Drosophila* are indicated.

For example, in the wing of *D. biarmipes,* the wing spot is partially under the control of the sex-determination hierarchies revealed by the overexpression of *Sxl* or downregulation of *tra*. In both cases, the phenotype is affected pinpointing that genes other than *yellow* are altered. A potential candidate gene is *ebony*, residing on an autosome, which is involved in the wing spot formation and is expressed in a sexually dimorphic manner (*171*). In the context of the wing spot, some genes of the gene network receive regulatory inputs from the sex-determination hierarchies while *yellow*, and may be others, also located on the X chromosome, rely on *trans*-homolog interactions. Other polygenic sexually dimorphic traits might be controlled in the same manner.

In my thesis, I, also, demonstrated that genes showing sex-biased expression in the brain of *D. melanogaster* might be controlled by a similar mechanism that requires the action of Mod(mdg4). From these results, we observe that it can regulate both male- and female-

biased genes. This suggested that the mechanism might be more universal than expected since in different tissue and species we can obtain similar results. Taking into consideration that this mechanism might exploit different factors in different circumstances some genes might not be caught by the analysis. We speculate that some genes that exhibit sex-biased gene expression by *trans*-homolog interaction might be independent of Mod(mdg4). Several loci of *D. melanogaster* are controlled by *trans*-homolog interactions, but the associated factors that have been found to be involved in the different cases are different (*86*, *167*).

An arising question, though, is to what extent this mechanism that relies on somatic chromosomal pairing can be in use in species outside Diptera that display a limited degree of pairing in somatic tissues. This potentially relies on the properties of chromosomal pairing. We can speculate that in one case, transient pairing might be required to establish interaction and then modify chromatin or DNA epigenetically. Although in some cases epigenetic changes are actively reinforced suggesting that transient pairing in these cases might be the onset signal. In another scenario, pairing establishes interactions and local conformations that might have to be continuous in time for gene expression control. This would mean a structure that contains both homologs and either facilitates or inhibits the interaction between enhancers, silencers, and promoters in a quantitative manner. Since gene expression, especially during development, is dynamic (*185*) then either of the two scenarios could apply in systems where pairing is not manifested in their genome in the level that is manifested in Diptera. Transient *trans*-homolog interactions have already been shown to exist during embryonic stem cell differentiation (*100*). It is worth noting that transient interactions in *trans* between non-homologous regions of the genome are widespread in mammals and affect gene expression. For example, interactions between cytokinin genes establish the subtype of CD4+ T helper cells during their differentiation (*186*). Interchromosomal associations mediate, also, olfactory receptor choice in olfactory neurons in mammals (*187*).

X-linkage in evolution

Whether genes that exhibit sex-biased gene expression are overrepresented in the sex chromosomes or which proportion of these genes are to their entity important for the development of sexual dimorphism is still an open question (*188*). Ever since the 1980s population geneticists started exploring the contribution of X-linkage in organisms with heterogametic genotypic sex determination (like flies and humans), in which the X

chromosome between sexes is different. Sexual antagonism has long been considered as a mechanism by which sexual dimorphism evolves. In this case, a phenotype, for example, is beneficial for one sex and detrimental for the other (*189*, *190*). Theory has predicted that sex chromosomes facilitate the evolution of sexual dimorphism under sexual antagonism (*191*). In addition, there is a faster rate of adaptive evolution in genes residing on the X chromosome. This can be explained by the fixation of recessive mutations (*192–194*). At least in *D. melanogaster,* X-linked genes exhibiting male-biased expression reflect this faster adaptive evolution compared to the ones on the autosomes (*195*). Our results identify a regulatory mechanism that can generate sexual dimorphism of the X-linked genes. This comes to reinforce the above theories and build a wide framework on the evolution of sexual dimorphism encompassing knowledge from population genetics and gene regulation.

The biological significance of trans-homolog interactions

Since its first description in the 20th century somatic homologous pairing has served as the foundation for *trans*-homolog interactions, collectively termed as transvection. Transvection (and as a result somatic homologous pairing) has long remained enigmatic regarding its biological significance. Revealed using mutant alleles and transgenes, transvection is widespread in the genome of *Drosophila*. Utilizing *trans*-homolog interactions at the X chromosome we were able to demonstrate, for the first time, a well-defined role for transvection in the biology of wild-type *Drosophila*. Our transgenic approach forged the idea that the molecular components for transvection at *yellow* are not restricted to the X chromosome since our transgenes function in a similar manner when placed on autosomes. This suggests that transvection might hold additional roles genome-wide. Insights come first from the differentiation of the visual system in *D. melanogaster*. Transvection at the *spineless* locus regulates the stochastic expression of photoreceptors in neurons (*95*, *118*). Besides, we suggest that transvection could act to buffer gene expression genome-wide since transcription output does not seem to correlate with gene dose. Having a single copy or three copies in *D. melanogaster* does not affect dramatically mRNA levels. Instead, these levels are closer to the wild-type levels produced by the two copies (*178*, *196*). In another study, though, measuring transcription in the *Ultrabithorax* (*Ubx*) gene, it was demonstrated that transvection increases transcription (*197*).

Undoubtedly, transvection has a considerable impact of gene expression. Examples, demonstrate that it can regulate gene expression in a variety of ways. How it regulates gene expression genome-wide, remains to be seen.

Perspectives

Following the results in this thesis, future directions are summarized in the following questions:

What are the functions of transvection in the genome? Current technologies enable us to investigate genome organization, chromatin accessibility, and gene expression in an unprecedented resolution. Exploiting in parallel the abundance of genetic tools in *D. melanogaster* would allow us to directly test hypotheses such as the role of transvection in buffering gene expression.

How general is transvection? *trans*-homolog interactions have been reported in fungi, plants, and animals although somatic homologous pairing differs dramatically between these organisms. This work reveals the value of studying non-traditional organisms with the lens of traditional models. Our principles of how life functions are directly a consequence of studying only a handful of organisms. Expanding our investigation on *trans*-homolog interactions is today feasible in closely related organisms of traditional models. This would enable us to establish tools to investigate the molecular basis and functional relevance of these interactions.

What is the molecular basis of transvection? Transvection has been described for several loci. Yet, the molecular components differ vastly between all the cases. One important common component, though, is insulators, which are shown to be crucial for transvection. What is the actual function of these insulators is unknown. Either they could form loops to facilitate communication between *cis*-regulatory elements and promoters, or they could stabilize interactions between homologs, creating transcription hubs to facilitate sharing between transcription factors, co-factors, and *cis*-regulatory elements. Current advances in genomics and imaging should help to better define the role(s) of insulators in transvection.

A new era is rising in the study of *trans*-homolog interactions. Applying new technologies in new organisms will allow us to expand our understanding of *trans*-homolog interactions and investigate their contribution in the context of development and evolution.

REFERENCES

1. M. Hiraiwa-Hasegawa, The sight of the peacock's tail makes me sick: The early arguments on sexual selection. *J. Biosci.* **25**, 11–18 (2000).

2. J. Goodyear, E. Gilbert, First record of male-male combat in Xenopeltis unicolor. *Herpetol. Bull.* **143**, 45–46 (2018).

3. M. J. Bartlett, T. E. Steeves, N. J. Gemmell, P. C. Rosengrave, Sperm competition risk drives rapid ejaculate adjustments mediated by seminal fluid. *Elife*. **6**, 1–24 (2017).

4. J. O. Wolff, D. M. Cicirello, Field evidence for sexual selection and resource competition infanticide in white-footed mice. *Anim. Behav.* **38**, 637–642 (1989).

5. A. E. Pusey, C. Packer, Infanticide in lions: Consequences and Counterstrategies. *Infanticide Parent. Care*, 277–299 (1994).

6. S. T. Trumbo, Infanticide, sexual selection and task specialization in a biparental burying beetle. *Anim. Behav.* **72**, 1159–1167 (2006).

7. D. A. Edward, T. Chapman, The evolution and significance of male mate choice. *Trends Ecol. Evol.* **26**, 647–654 (2011).

8. M. Slatkin, Ecological Causes of Sexual Dimorphism. *Evolution (N. Y).* **38**, 622–630 (1984).

9. R. Shine, Ecological Causes for the Evolution of Sexual Dimorphism: A Review of the Evidence. *Q. Rev. Biol.* **64**, 419–461 (1989).

10. E. J. Temeles, I. L. Pan, J. L. Brennan, J. N. Horwitt, Evidence for ecological causation of sexual dimorphism in a hummingbird. *Science (80-.).* **289**, 441–443 (2000).

11. R. C. St. Clair, Patterns of growth and sexual size dimorphism in two species of box turtles with environmental sex determination. *Oecologia*. **115**, 501–507 (1998).

12. T. Gempe, M. Beye, Function and evolution of sex determination mechanisms, genes and pathways in insects. *BioEssays*. **33**, 52–60 (2011).

13. H. Blackmon, L. Ross, D. Bachtrog, Sex determination, sex chromosomes, and karyotype evolution in insects. *J. Hered.* **108**, 78–93 (2017).

14. Henking, Untersuchung über die ersten Entwicklungsvorgänge in den Eiern der Insekten. II Über Spermatogenese und deren Beziehung zur Einentwicklung bei Pyrrhoris apterus L. *Zeitschrift für wissenschaftliche Zool.* **51**, 686- (1891).

15. C. McClung, Notes on the accessory chromosome. *Anat. Anz.*, 220–226 (1901).

16. N. M. Stevens, *Studies in spermatogenesis* (Carnegie Institution of Washington, Washington, D. C., 2012; http://www.biodiversitylibrary.org/bibliography/56517), vol. 36.

17. S. G. Brush, Nettie M. Stevens and the discovery of sex determination by chromosomes. *Isis.* **69**, 163–172 (1978).

18. C. B. Bridges, Direct proof through non-disjunction that the sex-linked genes of drosophila are borne by the x-chromosome. *Science (80-.).* **40**, 107–109 (1914).

19. C. B. Bridges, Non-Disjunction as Proof of the Chromosome Theory of Heredity (Concluded). *Genetics*. **1**, 107–63 (1916).

20. C. B. Bridges, Sex in Relation to Chromosomes and Genes. *Am. Nat.* **59**, 127–137 (1925).

21. H. K. Salz, J. W. Erickson, Sex determination in Drosophila: The view from the top. *Fly (Austin).* **4**, 60–70 (2010).

22. M. N. Arbeitman, F. N. New, J. M. Fear, T. S. Howard, J. E. Dalton, R. M. Graze, Sex differences in Drosophila somatic gene expression: Variation and regulation by doublesex. *G3 Genes, Genomes, Genet.* **6**, 1799–1808 (2016).

23. J. C. Lucchesi, Transcriptional modulation of entire chromosomes: dosage compensation. *J. Genet.* **97**, 357–364 (2018).

24. F. Ramírez, T. Lingg, S. Toscano, K. C. Lam, P. Georgiev, H. R. Chung, B. R. Lajoie, E. de Wit, Y. Zhan, W. de Laat, J. Dekker, T. Manke, A. Akhtar, High-Affinity Sites Form an Interaction Network to Facilitate Spreading of the MSL Complex across the X Chromosome in Drosophila. *Mol. Cell*. **60**, 146–162 (2015).

25. A. Prakash, A. Monteiro, Molecular mechanisms of secondary sexual trait development in insects. *Curr. Opin. Insect Sci.* **17**, 40–48 (2016).

26. H. K. Salz, Sex determination in insects: A binary decision based on alternative splicing. *Curr. Opin. Genet. Dev.* **21**, 395–400 (2011).

27. E. Geuverink, L. W. Beukeboom, Phylogenetic distribution and evolutionary dynamics of the sex determination genes doublesex and transformer in insects. *Sex. Dev.* **8**, 38–49 (2014).

28. B. Capel, Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. *Nat. Rev. Genet.* **18**, 675–689 (2017).

29. S. Gasca, P. Berta, Genes and Mechanisms in Vertebrate Sex Determination. *Heredity (Edinb).* **88**, 332–332 (2002).

30. A. V. Trukhina, N. A. Lukina, N. D. Wackerow-Kouzova, A. F. Smirnov, The variety of vertebrate mechanisms of sex determination. *Biomed Res. Int.* **2013** (2013), , doi:10.1155/2013/587460.

31. R. N. Chatterjee, Dosage compensation and its roles in evolution of sex chromosomes and phenotypic dimorphism: lessons from Drosophila, C.elegans and mammals. *Nucl.* **60**, 315–333 (2017).

32. J. Chery, E. Larschan, X-marks the spot: X-chromosome identification during dosage compensation. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1839**, 234–240 (2014).

33. S. Ercan, Mechanisms of X Chromosome Dosage Compensation. *J. Genomics*. **3**, 1–19 (2014).

34. T. Jégu, E. Aeby, J. T. Lee, The X chromosome in space. *Nat. Rev. Genet.* **18**, 377–389 (2017).

35. S. Grath, J. Parsch, Sex-Biased Gene Expression. *Annu. Rev. Genet.* **50**, 29–44 (2016).

36. E. H. Brown, R. C. King, Studies on the Expression of the Transformer Gene of Drosophila Melanogaster. *Genetics*. **46**, 143–14356 (1961).

37. P. E. HILDRETH, Doublesex, Recessive Gene That Transforms Both Males and Females of Drosophila Into Intersexes. *Genetics*. **51**, 659–678 (1965).

38. L. Sánchez, R. Nöthiger, Sex determination and dosage compensation in Drosophila melanogaster : production of male clones in XX females . *EMBO J.* **2**, 485–491 (1983).

39. B. S. Baker, K. A. Ridge, Sex and the single cell. I. On the action of major loci affecting sex determination in Drosophila melanogaster. *Genetics*. **94**, 383–423 (1980).

40. A. Kopp, I. Duncan, S. B. Carroll, Genetic control and evolution of sexually dimorphic characters in Drosophilia. *Nature*. **408**, 553–559 (2000).

41. S. Jeong, M. Rebeiz, P. Andolfatto, T. Werner, J. True, S. B. Carroll, The Evolution of Gene Regulation Underlies a Morphological Difference between Two Drosophila Sister Species. *Cell*. **132**, 783–793 (2008).

42. M. J. Roeske, E. M. Camino, S. Grover, M. Rebeiz, T. M. Williams, Cis-regulatory evolution integrated the Bric-à -brac transcription factors into a novel fruit fly gene regulatory network. *Elife*. **7**, 1–28 (2018).

43. B. Hudry, S. Khadayate, I. Miguel-Aliaga, The sexual identity of adult intestinal stem cells controls organ size and plasticity. *Nature*. **530**, 344–348 (2016).

44. A. Sawala, A. P. Gould, The sex of specific neurons controls female body growth in Drosophila. *PLoS Biol.* **15**, e2002252 (2017).

45. D. Nakada, H. Oguro, B. P. Levi, N. Ryan, A. Kitano, Y. Saitoh, M. Takeichi, G. R. Wendt, S. J. Morrison, Oestrogen increases haematopoietic stem-cell self-renewal in females and during pregnancy. *Nature*. **505**, 555–558 (2014).

46. D. Zhao, D. McBride, S. Nandi, H. A. McQueen, M. J. McGrew, P. M. Hocking, P. D. Lewis, H. M. Sang, M. Clinton, Somatic sex identity is cell autonomous in the chicken. *Nature*. **464**, 237–242 (2010).

47. J. Wade, A. P. Arnold, Sexual differentiation of the zebra finch song system. *Ann. N. Y. Acad. Sci.* **1016**, 540–559 (2004).

48. T. M. Williams, S. B. Carroll, Genetic and molecular insights into the development and evolution of sexual dimorphism. *Nat. Rev. Genet.* **10**, 797–804 (2009).

49. J. B. Wu, K. Chen, Y. Li, Y.-F. C. Lau, J. C. Shih, Regulation of monoamine oxidase A by the SRY gene on the Y chromosome . *FASEB J.* **23**, 4029–4038 (2009).

50. A. Milsted, L. Serova, E. L. Sabban, G. Dunphy, M. E. Turner, D. L. Ely, Regulation of tyrosine hydroxylase gene transcription by Sry. *Neurosci. Lett.* **369**, 203–207 (2004).

51. B. Lemos, L. O. Araripe, D. L. Hartl, Polymorphic Y chromosomes harbor cryptic variation with manifold functional consequences. *Science (80-.).* **319**, 91–93 (2008).

52. B. Lemos, A. T. Branco, D. L. Hartl, Epigenetic effects of polymorphic Y chromosomes modulate chromatin components, immune response, and sexual conflict. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15826–15831 (2010).

53. P. P. Jiang, D. L. Hartl, B. Lemos, Y not a dead end: Epistatic interactions between Y-linked regulatory polymorphisms and genetic background affect global gene expression in Drosophila melanogaster. *Genetics*. **186**, 109–118 (2010).

54. M. Wang, A. T. Branco, B. Lemos, The Y chromosome modulates splicing and sex-biased intron retention rates in Drosophila. *Genetics*. **208**, 1057–1067 (2018).

55. D. M. Snell, J. M. A. Turner, Sex Chromosome Effects on Male–Female Differences in Mammals. *Curr. Biol.* **28**, R1313–R1324 (2018).

56. H. Fang, C. M. Disteche, J. B. Berletch, X Inactivation and Escape: Epigenetic and Structural Features. *Front. Cell Dev. Biol.* **7**, 1–12 (2019).

57. T. Tukiainen, A. C. Villani, A. Yen, M. A. Rivas, J. L. Marshall, R. Satija, M. Aguirre, L. Gauthier, M. Fleharty, A. Kirby, B. B. Cummings, S. E. Castel, K. J. Karczewski, F. Aguet, A. Byrnes, E. T. Gelfand, G. Getz, K. Hadley, R. E. Handsaker, K. H. Huang, S. Kashin, M. Lek, X. Li, J. L. Nedzel, D. T. Nguyen, M. S. Noble, A. V. Segrè, C. A. Trowbridge, N. S. Abell, B. Balliu, R. Barshir, O. Basha, A. Battle, G. K. Bogu, A. Brown, C. D. Brown, L. S. Chen, C. Chiang, D. F. Conrad, N. J. Cox, F. N. Damani, J. R. Davis, O. Delaneau, E. T. Dermitzakis, B. E. Engelhardt, E. Eskin, P. G. Ferreira, L. Frésard, E. R. Gamazon, D. Garrido-Martín, A. D. H. Gewirtz, G. Gliner, M. J. Gloudemans, R. Guigo, I. M. Hall, B. Han, Y. He, F. Hormozdiari, C. Howald, H. K. Im, B. Jo, E. Y. Kang, Y. Kim, S. Kim-Hellmuth, T. Lappalainen, G. Li, X. Li, B. Liu, S. Mangul, M. I. McCarthy, I. C. McDowell, P. Mohammadi, J. Monlong, S. B. Montgomery, M. Muñoz-Aguirre, A. W. Ndungu, D. L. Nicolae, A. B. Nobel, M. Oliva, H. Ongen, J. J. Palowitch, N. Panousis, P. Papasaikas, Y. Park, P. Parsana, A. J. Payne, C. B. Peterson, J. Quan, F. Reverter, C. Sabatti, A. Saha, M. Sammeth, A. J. Scott, A. A. Shabalin, R. Sodaei, M. Stephens, B. E. Stranger, B. J. Strober, J. H. Sul, E. K. Tsang, S. Urbut, M. Van De Bunt, G. Wang, X. Wen, F. A. Wright, H. S. Xi, E. Yeger-Lotem, Z. Zappala, J. B. Zaugg, Y. H. Zhou, J. M. Akey, D. Bates, J. Chan, M. Claussnitzer, K.

Demanelis, M. Diegel, J. A. Doherty, A. P. Feinberg, M. S. Fernando, J. Halow, K. D. Hansen, E. Haugen, P. F. Hickey, L. Hou, F. Jasmine, R. Jian, L. Jiang, A. Johnson, R. Kaul, M. Kellis, M. G. Kibriya, K. Lee, J. B. Li, Q. Li, J. Lin, S. Lin, S. Linder, C. Linke, Y. Liu, M. T. Maurano, B. Molinie, J. Nelson, F. J. Neri, Y. Park, B. L. Pierce, N. J. Rinaldi, L. F. Rizzardi, R. Sandstrom, A. Skol, K. S. Smith, M. P. Snyder, J. Stamatoyannopoulos, H. Tang, L. Wang, M. Wang, N. Van Wittenberghe, F. Wu, R. Zhang, C. R. Nierras, P. A. Branton, L. J. Carithers, P. Guan, H. M. Moore, A. Rao, J. B. Vaught, S. E. Gould, N. C. Lockart, C. Martin, J. P. Struewing, S. Volpi, A. M. Addington, S. E. Koester, A. R. Little, L. E. Brigham, R. Hasz, M. Hunter, C. Johns, M. Johnson, G. Kopen, W. F. Leinweber, J. T. Lonsdale, A. McDonald, B. Mestichelli, K. Myer, B. Roe, M. Salvatore, S. Shad, J. A. Thomas, G. Walters, M. Washington, J. Wheeler, J. Bridge, B. A. Foster, B. M. Gillard, E. Karasik, R. Kumar, M. Miklos, M. T. Moser, S. D. Jewell, R. G. Montroy, D. C. Rohrer, D. R. Valley, D. A. Davis, D. C. Mash, A. H. Undale, A. M. Smith, D. E. Tabor, N. V. Roche, J. A. McLean, N. Vatanian, K. L. Robinson, L. Sobin, M. E. Barcus, K. M. Valentino, L. Qi, S. Hunter, P. Hariharan, S. Singh, K. S. Um, T. Matose, M. M. Tomaszewski, L. K. Barker, M. Mosavel, L. A. Siminoff, H. M. Traino, P. Flicek, T. Juettemann, M. Ruffier, D. Sheppard, K. Taylor, S. J. Trevanion, D. R. Zerbino, B. Craft, M. Goldman, M. Haeussler, W. J. Kent, C. M. Lee, B. Paten, K. R. Rosenbloom, J. Vivian, J. Zhu, A. Regev, K. G. Ardlie, N. Hacohen, D. G. MacArthur, Landscape of X chromosome inactivation across human tissues. *Nature*. **550**, 244–248 (2017).

58. E. Shvetsova, A. Sofronova, R. Monajemi, K. Gagalova, H. H. M. Draisma, S. J. White, G. W. E. Santen, S. M. Chuva de Sousa Lopes, B. T. Heijmans, J. van Meurs, R. Jansen, L. Franke, S. M. Kiełbasa, J. T. den Dunnen, P. A. C. ‘t Hoen, B. T. Heijmans, P. A. ’t Hoen, J. van Meurs, D. I. Boomsma, R. Pool, J. van Dongen, J. J. Hottenga, M. M. van Greevenbroek, C. Da Stehouwer, C. J. van der Kallen, C. G. Schalkwijk, C. Wijmenga, S. Zhernakova, E. F. Tigchelaar, P. E. Slagboom, M. Beekman, J. Deelen, D. van Heemst, J. H. Veldink, L. H. van den Berg, C. M. van Duijn, B. A. Hofman, A. G. Uitterlinden, P. M. Jhamai, M. Verbiest, H. E. D. Suchiman, M. Verkerk, R. van der Breggen, J. van Rooij, N. Lakenberg, H. Mei, J. Bot, D. V. Zhernakova, P. van ’t Hof, P. Deelen, I. Nooren, M. Moed, M. Vermaat, R. Luijk, M. Jan Bonder, M. van Iterson, F. van Dijk, M. van Galen, W. Arindrarto, S. M. Kiełbasa, M. A. Swertz, E. W. van Zwet, A. Isaacs, R. Jansen, L. Franke, L. Francioli, A. Menelaou, S. Pulit, F. van Dijk,

P. Palamara, C. Elbers, P. Neerincx, K. Ye, V. Guryev, W. Kloosterman, P. Deelen, A. Abdellaoui, E. van Leeuwen, M. van Oven, M. Vermaat, M. Li, J. Laros, L. Karssen, A. Kanterakis, N. Amin, J. Hottenga, E. Lameijer, M. Kattenberg, M. Dijkstra, H. Byelas, J. van Setten, B. van Schaik, J. Bot, I. Nijman, I. Renkens, T. Marschall, A. Schönhuth, J. Hehir-Kwa, R. Handsaker, P. Polak, M. Sohail, D. Vuzman, F. Hormozdiari, D. van Enckevort, H. Mei, V. Koval, M. Moed, K. van der Velde, F. Rivadeneira, K. Estrada, C. Medina-Gomez, A. Isaacs, S. McCarroll, M. Beekman, A. de Craen, H. Suchiman, B. Hofman, B. Oostra, A. Uitterlinden, G. Willemsen, M. Platteel, J. Veldink, L. van den Berg, S. Pitts, S. Potluri, P. Sundar, D. Cox, S. Sunyaev, J. den Dunnen, M. Stoneking, P. de Knijff, M. Kayser, Q. Li, Y. Li, Y. Du, R. Chen, H. Cao, N. Li, S. Cao, J. Wang, J. Bovenberg, I. Pe'er, P. Slagboom, C. van Duijn, D. Boomsma, G. van Ommen, P. de Bakker, M. Swertz, C. Wijmenga, Skewed X-inactivation is common in the general female population. *Eur. J. Hum. Genet.* **27**, 455–465 (2019).

59. P. J. Wijchers, R. J. Festenstein, Epigenetic regulation of autosomal gene expression by sex chromosomes. *Trends Genet.* **27**, 132–140 (2011).

60. D. H. Skuse, R. S. James, D. V. M. Bishop, B. Coppin, P. Dalton, G. Aamodt-Leeper, M. Bacarese-Hamilton, C. Creswell, R. McGurk, P. A. Jacobs, Evidence from Turner's syndrome of an imprinted X-linked locus affecting cognitive function. *Nature*. **387**, 705–708 (1997).

61. W. Davies, A. Isles, R. Smith, D. Karunadasa, D. Burrmann, T. Humby, O. Ojarikre, C. Biggin, D. Skuse, P. Burgoyne, L. Wilkinson, Xlr3b is a new imprinted candidate for X-linked parent-of-origin effects on cognitive function in mice. *Nat. Genet.* **37**, 625–629 (2005).

62. M. Parisi, R. Nuttall, D. Naiman, G. Bouffard, J. Malley, J. Andrews, S. Eastman, B. Oliver, Paucity of genes on the Drosophila X chromosome showing male-biased expression. *Science (80-.).* **299**, 697–700 (2003).

63. J. M. Ranz, C. I. Castillo-Davis, C. D. Meiklejohn, D. L. Hartl, Sex-dependent gene expression and evolution of the Drosophila transcriptome. *Science (80-.).* **300**, 1742–1745 (2003).

64. D. Sturgill, Y. Zhang, M. Parisi, B. Oliver, Demasculinization of X chromosomes in the Drosophila genus. *Nature*. **450**, 238–241 (2007).

65. A. K. Huylmans, J. Parsch, Variation in the X:Autosome Distribution of Male-Biased Genes among Drosophila melanogaster Tissues and Its Relationship with Dosage Compensation. *Genome Biol. Evol.* **7**, 1960–1971 (2015).

66. J. C. Perry, P. W. Harrison, J. E. Mank, The ontogeny and evolution of sex-biased gene expression in drosophila melanogaster. *Mol. Biol. Evol.* **31**, 1206–1219 (2014).

67. B. Reinius, M. M. Johansson, K. J. Radomska, E. H. Morrow, G. K. Pandey, C. Kanduri, R. Sandberg, R. W. Williams, E. Jazin, Abundance of female-biased and paucity of male-biased somatically expressed genes on the mouse X-chromosome. *BMC Genomics*. **13** (2012), doi:10.1186/1471-2164-13-607.

68. S. Boyle, S. Gilchrist, J. M. Bridger, N. L. Mahy, J. A. Ellis, W. A. Bickmore, The spatial organization of human chromosomes within the nuclei of normal and emerin-mutant cells. *Hum. Mol. Genet.* **10**, 211–220 (2001).

69. E. Lieberman-Aiden, N. L. Van Berkum, L. Williams, M. Imakaev, T. Ragoczy, A. Telling, I. Amit, B. R. Lajoie, P. J. Sabo, M. O. Dorschner, R. Sandstrom, B. Bernstein, M. A. Bender, M. Groudine, A. Gnirke, J. Stamatoyannopoulos, L. A. Mirny, E. S. Lander, J. Dekker, Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science (80-.).* (2009), doi:10.1126/science.1181369.

70. Q. Szabo, F. Bantignies, G. Cavalli, Principles of genome folding into topologically associating domains. *Sci. Adv.* **5**, eaaw1668 (2019).

71. B. Bonev, N. Mendelson Cohen, Q. Szabo, L. Fritsch, G. L. Papadopoulos, Y. Lubling, X. Xu, X. Lv, J. P. Hugnot, A. Tanay, G. Cavalli, Multiscale 3D Genome Rewiring during Mouse Neural Development. *Cell* (2017), doi:10.1016/j.cell.2017.09.043.

72. D. G. Lupiáñez, M. Spielmann, S. Mundlos, Breaking TADs: How Alterations of Chromatin Domains Result in Disease. *Trends Genet.* **32** (2016), pp. 225–237.

73. E. Rodríguez-Carballo, L. Lopez-Delisle, Y. Zhan, P. J. Fabre, L. Beccari, I. El-Idrissi, T. H. Nguyen Huynh, H. Ozadam, J. Dekker, D. Duboule, The HoxD cluster is a dynamic and resilient TAD boundary controlling the segregation of antagonistic regulatory landscapes. *Genes Dev.* **31**, 2264–2281 (2017).

74. Y. Ghavi-Helm, A. Jankowski, S. Meiers, R. R. Viales, J. O. Korbel, E. E. M. Furlong,

Highly rearranged chromosomes reveal uncoupling between genome topology and gene expression. *Nat. Genet.* **51**, 1272–1282 (2019).

75. M. Mir, W. Bickmore, E. E. M. Furlong, G. Narlikar, Chromatin topology, condensates and gene regulation: Shifting paradigms or just a phase? *Dev.* **146** (2019), doi:10.1242/dev.182766.

76. O. Kyrchanova, P. Georgiev, Chromatin insulators and long-distance interactions in Drosophila. *FEBS Lett.* **588**, 8–14 (2014).

77. T. Ali, R. Renkawitz, M. Bartkuhn, Insulators and domains of gene expression. *Curr. Opin. Genet. Dev.* **37**, 17–26 (2016).

78. I. Özdemir, M. C. Gambetta, The role of insulation in patterning gene expression. *Genes (Basel).* **10**, 767 (2019).

79. R. Kellum, P. Schedl, A group of scs elements function as domain boundaries in an enhancer-blocking assay. *Mol. Cell. Biol.* **12**, 2424–2431 (1992).

80. M. Fujioka, G. Sun, J. B. Jaynes, The Drosophila eve Insulator Homie Promotes eve Expression and Protects the Adjacent Gene from Repression by Polycomb Spreading. *PLoS Genet.* (2013), doi:10.1371/journal.pgen.1003883.

81. V. Narendra, P. P. Rocha, D. An, R. Raviram, J. A. Skok, E. O. Mazzoni, D. Reinberg, CTCF establishes discrete functional chromatin domains at the Hox clusters during differentiation. *Science (80-.).* (2015), doi:10.1126/science.1262088.

82. I. Comet, E. Savitskaya, B. Schuettengruber, N. Nègre, S. Lavrov, A. Parshikov, F. Juge, E. Gracheva, P. Georgiev, G. Cavalli, PRE-Mediated Bypass of Two Su(Hw) Insulators Targets PcG Proteins to a Downstream Promoter. *Dev. Cell*. **11**, 117–124 (2006).

83. A. M. Lee, C. T. Wu, Enhancer-promoter communication at the yellow gene of Drosophila melanogaster: Diverse promoters participate in and regulate trans interactions. *Genetics*. **174**, 1867–1880 (2006).

84. L. Melnikova, F. Juge, N. Gruzdeva, A. Mazur, G. Cavalli, P. Georgiev, Interaction between the GAGA factor and Mod(mdg4) proteins promotes insulator bypass in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 14806–14811 (2004).

85. M. Fujioka, H. Mistry, P. Schedl, J. B. Jaynes, Determinants of Chromosome Architecture: Insulator Pairing in cis and in trans. *PLoS Genet.* **12**, e1005889 (2016).

86. J. A. Kennison, J. W. Southworth, *13 Transvection in Drosophila* (Elsevier Inc., 2002; http://dx.doi.org/10.1016/S0065-2660(02)46014-2), vol. 46.

87. K. E. Giles, H. Gowher, R. Ghirlando, C. Jin, G. Felsenfeld, Chromatin boundaries, insulators, and long-range interactions in the nucleus. *Cold Spring Harb. Symp. Quant. Biol.* **75**, 79–85 (2010).

88. N. M. Stevens, A study of the germ cells of certain diptera, with reference to the heterochromosomes and the phenomena of synapsis. *J. Exp. Zool.* **5**, 359–374 (1908).

89. C. Fritsch, G. Ploeger, D. J. Arndt-Jovin, Drosophila under the lens: Imaging from chromosomes to whole embryos. *Chromosom. Res.* **14**, 451–464 (2006).

90. B. R. Williams, J. R. Bateman, N. D. Novikov, C. T. Wu, Disruption of topoisomerase II perturbs pairing in Drosophila cell culture. *Genetics*. **177**, 31–46 (2007).

91. T. A. Hartl, H. F. Smith, G. Bosco, Chromosome Alignment and Transvection Are Antagonized by Condensin II. *Science (80-.).* **322**, 1384–1387 (2008).

92. J. R. Bateman, E. Larschan, R. D'Souza, L. S. Marshall, K. E. Dempsey, J. E. Johnson, B. G. Mellone, M. I. Kuroda, A genome-wide screen identifies genes that affect somatic homolog pairing in drosophila. *G3 Genes, Genomes, Genet.* **2**, 731–740 (2012).

93. E. F. Joyce, B. R. Williams, T. Xie, C. ting Wu, Identification of genes that promote or antagonize somatic homolog pairing using a high-throughput FISH-based screen. *PLoS Genet.* **8** (2012), doi:10.1371/journal.pgen.1002667.

94. J. C. Fung, W. F. Marshall, A. Dernburg, D. A. Agard, J. W. Sedat, Homologous Chromosome Pairing in Drosophila melanogaster Proceeds through Multiple Independent Initiations . *J. Cell Biol.* **141**, 5–20 (1998).

95. K. Viets, M. E. G. Sauria, C. Chernoff, R. Rodriguez Viales, M. Echterling, C. Anderson, S. Tran, A. Dove, R. Goyal, L. Voortman, A. Gordus, E. E. M. Furlong, J. Taylor, R. J. Johnston, Characterization of Button Loci that Promote Homologous Chromosome Pairing and Cell-Type-Specific Interchromosomal Gene Regulation. *Dev. Cell*. **51**, 341-356.e7 (2019).

96. J. Erceg, J. AlHaj Abed, A. Goloborodko, B. R. Lajoie, G. Fudenberg, N. Abdennur, M. Imakaev, R. B. McCole, S. C. Nguyen, W. Saylor, E. F. Joyce, T. N. Senaratne, M. A. Hannan, G. Nir, J. Dekker, L. A. Mirny, C. ting Wu, The genome-wide multi-layered architecture of chromosome pairing in early Drosophila embryos. *Nat. Commun.* **10** (2019), doi:10.1038/s41467-019-12211-8.

97. J. AlHaj Abed, J. Erceg, A. Goloborodko, S. C. Nguyen, R. B. McCole, W. Saylor, G. Fudenberg, B. R. Lajoie, J. Dekker, L. A. Mirny, C. ting Wu, Highly structured homolog pairing reflects functional organization of the Drosophila genome. *Nat. Commun.* **10**, 1–14 (2019).

98. E. B. Lewis, The Theory and Application of a New Method of Detecting Chromosomal Rearrangements in Drosophila melanogaster. *Am. Nat.* **88**, 225–239 (1954).

99. L. L. Hua, T. Mikawa, Mitotic antipairing of homologous and sex chromosomes via spatial restriction of two haploid sets. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E12235–E12244 (2018).

100. M. S. Hogan, D. E. Parfitt, C. J. Zepeda-Mendoza, M. M. Shen, D. L. Spector, Transient pairing of homologous Oct4 alleles accompanies the onset of embryonic stem cell differentiation. *Cell Stem Cell*. **16**, 275–288 (2015).

101. E. F. Joyce, J. Erceg, C. ting Wu, Pairing and anti-pairing: A balancing act in the diploid genome. *Curr. Opin. Genet. Dev.* **37**, 119–128 (2016).

102. H. GRÜNEBERG, Gene Doublets as Evidence for Adjacent Small Duplications in Drosophila. *Nature*. **140**, 932–932 (1937).

103. E. B. LEWIS, Pseudoallelism and gene evolution. *Cold Spring Harb. Symp. Quant. Biol.* (1951), doi:10.1101/SQB.1951.016.01.014.

104. R. Dorn, G. Reuter, A. Loewendorf, Transgene analysis proves mRNA trans-splicing at the complex mod(mdg4)locus in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 9724–9729 (2001).

105. F. Mongelard, M. Labrador, E. M. Baxter, T. I. Gerasimova, V. G. Corces, Trans-splicing as a novel mechanism to explain interallelic complementation in Drosophila. *Genetics* (2002).

106. R. Dorn, V. Krauss, The modifier of mdg4 locus in Drosophila: Functional complexity is resolved by trans splicing. *Genetica* (2003), , doi:10.1023/A:1022983810016.

107. T. Horiuchi, E. Giniger, T. Aigaki, Alternative trans-splicing of constant and variable exons of a Drosophila axon guidance gene, lola. *Genes Dev.* **17**, 2496–2501 (2003).

108. C. J. McManus, M. O. Duff, J. Eipper-Mains, B. R. Graveley, Global analysis of trans-splicing in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 12975–12979 (2010).

109. E. S. Kandel, E. Nudler, Template Switching by RNA Polymerase II In Vivo: Evidence and Implications from a Retroviral System. *Mol. Cell* (2002), doi:10.1016/S1097-2765(02)00777-3.

110. P. K. Geyer, M. M. Green, V. G. Corces, Tissue-specific transcriptional enhancers may act in trans on the gene located in the homologous chromosome: the molecular basis of transvection in Drosophila. *EMBO J.* **9**, 2247–2256 (1990).

111. J. W. Southworth, J. A. Kennison, Transvection and silencing of the Scr homeotic gene of Drosophila melanogaster. *Genetics*. **161**, 733–746 (2002).

112. W. M. Gelbart, Synapsis-dependent allelic complementation at the decapentaplegic gene complex in Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2636–2640 (1982).

113. D. Gubb, M. Ashburner, J. Roote, T. Davis, A novel transvection phenomenon affecting the white gene of Drosophila melanogaster. *Genetics*. **126**, 167–176 (1990).

114. D. Gohl, M. Müller, V. Pirrotta, M. Affolter, P. Schedl, Enhancer blocking and transvection at the drosophila apterous locus. *Genetics*. **178**, 127–143 (2008).

115. W. M. Leiserson, N. M. Bonini, S. Benzer, Transvection at the eyes absent gene of Drosophila. *Genetics*. **138**, 1171–1179 (1994).

116. J. R. Morris, J. L. Chen, S. T. Filandrinos, R. C. Dunn, R. Fisk, P. K. Geyer, C. T. Wu, An analysis of transvection at the yellow locus of Drosophila melanogaster. *Genetics*. **151**, 633–651 (1999).

117. A. B. Coulthard, N. Nolan, J. B. Bell, A. J. Hilliker, Transvection at the vestigial locus of Drosophila melanogaster. *Genetics*. **170**, 1711–1721 (2005).

118. R. J. Johnston, C. Desplan, Interchromosomal communication coordinates intrinsically stochastic expression between alleles. *Science (80-.).* **343**, 661–665 (2014).

119. D. J. Mellert, J. W. Truman, Transvection is common throughout the Drosophila genome. *Genetics*. **191**, 1129–1141 (2012).

120. J. C. Lucchesi, *Epigenetics, Nuclear Organization & Gene Function* (Oxford University Press, 2019; http://www.oxfordscholarship.com/view/10.1093/oso/9780198831204.001.0001/oso-9780198831204).

121. M. Rassoulzadegan, M. Magliano, F. Cuzin, Transvection effects involving DNA methylation during meiosis in the mouse. *EMBO J.* **21**, 440–450 (2002).

122. J. D. Rodriguez, D. A. Myrick, I. Falciatori, M. A. Christopher, T. W. Lee, G. J. Hannon, D. J. Katz, A model for epigenetic inhibition via transvection in the mouse. *Genetics*. **207**, 129–138 (2017).

123. K. S. Sandhu, C. Shi, M. Sjölinder, Z. Zhao, A. Göndör, L. Liu, V. K. Tiwari, S. Guibert, L. Emilsson, M. P. Imreh, R. Ohlsson, Nonallelic transvection of multiple imprinted loci is organized by the H19 imprinting control region during germline development. *Genes Dev.* **23**, 2598–2603 (2009).

124. P. K. Geyer, C. Spana, V. G. Corces, On the molecular mechanism of gypsy-induced mutations at the yellow locus of Drosophila melanogaster. *EMBO J.* (1986), doi:10.1002/j.1460-2075.1986.tb04548.x.

125. M. M. Green, *Genetics*, in press (available at http://www.genetics.org/content/46/11/1385.abstract).

126. J. R. Morris, J. L. Chen, P. K. Geyer, C. T. Wu, Two modes of transvection: Enhancer action in trans and bypass of a chromatin insulator in cis. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 10740–10745 (1998).

127. A. Golovnin, I. Birukova, O. Romanova, M. Silicheva, A. Parshikov, E. Savitskaya, V. Pirrotta, P. Georgiev, An endogenous Su(Hw) insulator separates the yellow gene from the Achaete-scute gene complex in Drosophila. *Development*. **130**, 3249–3258 (2003).

128. E. Kravchenko, E. Savitskaya, O. Kravchuk, A. Parshikov, P. Georgiev, M. Savitsky,

Pairing between gypsy Insulators Facilitates the Enhancer Action in trans throughout the Drosophila Genome. *Mol. Cell. Biol.* **25**, 9283–9291 (2005).

129. J. R. Morris, D. A. Petrov, A. M. Lee, C. T. Wu, Enhancer choice in cis and in trans in Drosophila melanogaster: Role of the promoter. *Genetics*. **167**, 1739–1747 (2004).

130. A. Martínez-Laborda, A. González-Reyes, G. Morata, Trans regulation in the Ultrabithorax gene of Drosophila: alterations in the promoter enhance transvection. *EMBO J.* **11**, 3645–3652 (1992).

131. J. L. Chen, K. L. Huisinga, M. M. Viering, S. A. Ou, C. T. Wu, P. K. Geyer, Enhancer action in trans is permitted throughout the Drosophila genome. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3723–3728 (2002).

132. O. Kravchuk, M. Kim, P. Klepikov, A. Parshikov, P. Georgiev, M. Savitsky, Transvection in Drosophila: trans-interaction between yellow enhancers and promoter is strongly suppressed by a cis-promoter only in certain genomic regions. *Chromosoma*. **126**, 431–441 (2017).

133. T. D. King, J. E. Johnson, J. R. Bateman, Position effects influence transvection in drosophila melanogaster. *Genetics*. **213**, 1289–1299 (2019).

134. J. Locke, K. D. Tartof, Molecular analysis of cubitus interruptus (ci) mutations suggests an explanation for the unusual ci position effects. *MGG Mol. Gen. Genet.* **243**, 234–243 (1994).

135. C. Schwartz, J. Locke, C. Nishida, T. B. Kornberg, Analysis of cubitus interruptus regulation in Drosophila embryos and imaginal disks. *Development*. **121**, 1625–1635 (1995).

136. D. C. Slusarski, C. K. Motzny, R. Holmgren, Mutations that alter the timing and pattern of cubitus interruptus gene expression in Drosophila melanogaster. *Genetics*. **139**, 229–240 (1995).

137. R. Kellum, P. Schedl, A position-effect assay for boundaries of higher order chromosomal domains. *Cell* (1991), doi:10.1016/0092-8674(91)90318-S.

138. R. R. Roseman, V. Pirrotta, P. K. Geyer, The su(Hw) protein insulates expression of the Drosophila melanogaster white gene from chromosomal position-effects. *EMBO J.* **12**,

435–442 (1993).

139. P. Piwko, I. Vitsaki, I. Livadaras, C. Delidakis, The role of insulators in transgene transvection in drosophila. *Genetics*. **212**, 489–508 (2019).

140. B. Lim, T. Heist, M. Levine, T. Fukaya, Visualization of Transvection in Living Drosophila Embryos. *Mol. Cell*. **70**, 287-296.e6 (2018).

141. K. P. Foley, J. D. Engel, Individual stage selector element mutations lead to reciprocal changes in β- vs. ε-globin gene transcription: Genetic confirmation of promoter competition during globin gene switching. *Genes Dev.* (1992), doi:10.1101/gad.6.5.730.

142. S. Ohtsuki, M. Levine, H. N. Cai, Different core promoters possess distinct regulatory activities in the Drosophila embryo. *Genes Dev.* (1998), doi:10.1101/gad.12.4.547.

143. A. Tsai, M. R. P. Alves, J. Crocker, Multi-enhancer transcriptional hubs confer phenotypic robustness. *Elife* (2019), doi:10.7554/eLife.45325.

144. R. A. Brink, A Genetic Change Associated with the R Locus in Maize Which Is Directed and Potentially Reversible. *Genetics* (1956).

145. R. A. BRINK, Paramutation at the R locus in maize. *Cold Spring Harb. Symp. Quant. Biol.* **23**, 379–391 (1958).

146. J. B. Hollick, Paramutation and related phenomena in diverse species. *Nat. Rev. Genet.* **18**, 5–23 (2016).

147. V. L. Chandler, Paramutation: From Maize to Mice. *Cell*. **128** (2007), pp. 641–645.

148. M. Stam, C. Belele, J. E. Dorweiler, V. L. Chandler, Differential chromatin structure within a tandem array 100 kb upstream of the maize b1 locus is associated with paramutation. *Genes Dev.* **16**, 1906–1918 (2002).

149. M. Arteaga-Vazquez, L. Sidorenko, F. A. Rabanal, R. Shrivistava, K. Nobuta, P. J. Green, B. C. Meyers, V. L. Chandler, RNA-mediated trans-communication can establish paramutation at the b1 locus in maize. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 12986–12991 (2010).

150. S. E. Roche, D. C. Rio, Trans-silencing by P elements inserted in subtelomeric heterochromatin involves the drosophila Polycomb group gene, Enhancer of zeste.

Genetics. **149**, 1839–1855 (1998).

151. A. De Vanssay, A. L. Bougé, A. Boivin, C. Hermant, L. Teysset, V. Delmarre, C. Antoniewski, S. Ronsseray, Paramutation in Drosophila linked to emergence of a piRNA-producing locus. *Nature*. **490**, 112–115 (2012).

152. C. Hermant, A. Boivin, L. Teysset, V. Delmarre, A. Asif-Laidin, M. Van Den Beek, C. Antoniewski, S. Ronsseray, Paramutation in drosophila requires both nuclear and cytoplasmic actors of the piRNA pathway and induces cis-spreading of piRNA production. *Genetics*. **201**, 1381–1396 (2015).

153. J. A. Kassis, E. P. VanSickle, S. M. Sensabaugh, A fragment of engrailed regulatory DNA can mediate transvection of the white gene in drosophila. *Genetics* (1991).

154. J. A. Kassis, Unusual properties of regulatory DNA from the Drosophila engrailed gene: Three "pairing-sensitive" sites within a 1.6-kb region. *Genetics* (1994).

155. J. Americo, M. Whiteley, J. Lesley Brown, M. Fujioka, J. B. Jaynes, J. A. Kassis, A complex array of DNA-binding proteins required for pairing-sensitive silencing by a polycomb group response element from the Drosophila engrailed gene. *Genetics*. **160**, 1561–1571 (2002).

156. J. Akassis, *14 Pairing-sensitive silencing, polycomb group response elements, and transposon homing in Drosophila* (Elsevier Inc., 2002; http://linkinghub.elsevier.com/retrieve/pii/S0065266002460154), vol. 46.

157. B. Schuettengruber, H. M. Bourbon, L. Di Croce, G. Cavalli, Genome Regulation by Polycomb and Trithorax: 70 Years and Counting. *Cell*. **171**, 34–57 (2017).

158. F. Bantignies, C. Grimaud, S. Lavrov, M. Gabut, G. Cavalli, Inheritance of polycomb-dependent chromosomal interactions in Drosophila. *Genes Dev.* **17**, 2406–2420 (2003).

159. H.-B. Li, M. Muller, I. A. Bahechar, O. Kyrchanova, K. Ohno, P. Georgiev, V. Pirrotta, Insulators, Not Polycomb Response Elements, Are Required for Long-Range Interactions between Polycomb Targets in Drosophila melanogaster. *Mol. Cell. Biol.* **31**, 616–625 (2011).

160. J. Vazquez, M. Müller, V. Pirrotta, J. W. Sedat, The Mcp Element Mediates Stable Long-Range Chromosome–Chromosome Interactions in Drosophila. *Mol. Biol. Cell*. **17**,

2158–2165 (2006).

161. C. J. A. Sigrist, V. Pirrotta, Chromatin insulator elements block the silencing of a target gene by the Drosophila polycomb response element (PRE) but allow trans interactions between PREs on different chromosomes. *Genetics*. **147**, 209–221 (1997).

162. M. Muller, K. Hagstrom, H. Gyurkovics, V. Pirrotta, P. Schedl, *Genetics*, in press (available at http://www.genetics.org/content/153/3/1333.abstract).

163. Y. Ogiyama, B. Schuettengruber, G. L. Papadopoulos, J. M. Chang, G. Cavalli, Polycomb-Dependent Chromatin Looping Contributes to Gene Silencing during Drosophila Development. *Mol. Cell*. **71**, 73-88.e5 (2018).

164. V. Loubiere, G. L. Papadopoulos, Q. Szabo, A. M. Martinez, G. Cavalli, Widespread activation of developmental gene expression characterized by PRC1-dependent chromatin looping. *Sci. Adv.* **6** (2020), doi:10.1126/sciadv.aax4001.

165. C. ting Wu, J. R. Morris, Transvection and other homology effects. *Curr. Opin. Genet. Dev.* **9**, 237–246 (1999).

166. S. P. Otto, P. Yong, *Homology Effects* (2002).

167. I. W. Duncan, Transvection Effects in Drosophila. *Annu. Rev. Genet.* **36**, 521–556 (2002).

168. N. Camara, C. Whitworth, M. Van Doren, *Chapter 3 The Creation of Sexual Dimorphism in the Drosophila Soma* (Elsevier Inc., 2008; http://dx.doi.org/10.1016/S0070-2153(08)00403-1), vol. 83.

169. J. L. Rinn, M. Snyder, Sexual dimorphism in mammalian gene expression. *Trends Genet.* **21**, 298–305 (2005).

170. X. Sun, H. Yang, D. Sturgill, B. Oliver, L. Rabinow, M. L. Samson, Sxl-dependent, tra/tra2-independent alternative splicing of the Drosophila melanogaster X-linked gene found in neurons. *G3 Genes, Genomes, Genet.* **5**, 2865–2874 (2015).

171. P. J. Wittkopp, J. R. True, S. B. Carroll, Reciprocal functions of the Drosophila Yellow and Ebony proteins in the development and evolution of pigment patterns. *Development*. **129**, 1849–1858 (2002).

172. N. Gompel, B. Prud'Homme, P. J. Wittkopp, V. A. Kassner, S. B. Carroll, Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. *Nature*. **433**, 481–487 (2005).

173. L. Arnoult, K. F. Y. Su, D. Manoel, C. Minervino, J. Magriña, N. Gompel, B. Prud'homme, Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. *Science (80-.).* **339**, 1423–1426 (2013).

174. J. C. Lucchesi, M. I. Kuroda, Dosage compensation in drosophila. *Cold Spring Harb. Perspect. Biol.* **7**, 1–21 (2015).

175. S. Jeong, A. Rokas, S. B. Carroll, Regulation of Body Pigmentation by the Abdominal-B Hox Protein and Its Gain and Loss in Drosophila Evolution. *Cell*. **125**, 1387–1399 (2006).

176. M. Savitsky, M. Kim, O. Kravchuk, Y. B. Schwartz, Distinct roles of chromatin insulator proteins in control of the Drosophila bithorax complex. *Genetics*. **202**, 601–617 (2016).

177. M. S. Apte, V. H. Meller, Homologue Pairing in Flies and Mammals: Gene Regulation When Two Are Involved. *Genet. Res. Int.* **2012**, 1–9 (2012).

178. P. Stenberg, J. Larsson, Buffering and the evolution of chromosome-wide gene regulation. *Chromosoma*. **120**, 213–225 (2011).

179. B. Haley, D. Hendrix, V. Trang, M. Levine, A simplified miRNA-based gene silencing method for Drosophila melanogaster. *Dev. Biol.* **321**, 482–490 (2008).

180. A. Bassett, J. L. Liu, CRISPR/Cas9 mediated genome engineering in Drosophila. *Methods*. **69**, 128–136 (2014).

181. H. Hinaux, K. Bachem, M. Battistara, M. Rossi, Y. Xin, R. Jaenichen, Y. Le Poul, L. Arnoult, J. M. Kobler, I. C. Grunwald Kadow, L. Rodermund, B. Prud'homme, N. Gompel, Revisiting the developmental and cellular role of the pigmentation gene yellow in Drosophila using a tagged allele. *Dev. Biol.* **438**, 111–123 (2018).

182. D. Kim, B. Langmead, S. L. Salzberg, HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods*. **12**, 357–360 (2015).

183. C. Trapnell, A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn, L. Pachter, Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578 (2012).

184. D. Chen, E. P. Lei, Function and regulation of chromatin insulators in dynamic genome organization. *Curr. Opin. Cell Biol.* **58**, 61–68 (2019).

185. J. A. Briggs, C. Weinreb, D. E. Wagner, S. Megason, L. Peshkin, M. W. Kirschner, A. M. Klein, The dynamics of gene expression in vertebrate embryogenesis at single-cell resolution. *Science (80-.).* **360** (2018), doi:10.1126/science.aar5780.

186. C. G. Spilianakis, M. D. Lalioti, T. Town, G. R. Lee, R. A. Flavell, Interchromosomal associations between alternatively expressed loci. *Nature*. **435**, 637–645 (2005).

187. K. Monahan, A. Horta, S. Lomvardas, LHX2- and LDB1-mediated trans interactions regulate olfactory receptor choice. *Nature*. **565**, 448–453 (2019).

188. J. E. Mank, The transcriptional architecture of phenotypic dimorphism. *Nat. Ecol. Evol.* **1**, 0006 (2017).

189. J. E. Mank, Sex chromosomes and the evolution of sexual dimorphism: Lessons from the genome. *Am. Nat.* **173**, 141–150 (2009).

190. J. Parsch, H. Ellegren, The evolutionary causes and consequences of sex-biased gene expression. *Nat. Rev. Genet.* **14**, 83–87 (2013).

191. W. R. Rice, Sex Chromosomes and the Evolution of Sexual Dimorphism. *Evolution (N. Y).* **38**, 735–742 (1984).

192. R. P. Meisel, T. Connallon, The faster-X effect: Integrating theory and data. *Trends Genet.* **29** (2013), pp. 537–544.

193. A. Llopart, Faster-X evolution of gene expression is driven by recessive adaptive cis-regulatory variation in Drosophila. *Mol. Ecol.* **27**, 3811–3821 (2018).

194. B. Charlesworth, J. L. Campos, B. C. Jackson, Faster-X evolution: Theory and evidence from Drosophila. *Mol. Ecol.* **27**, 3753–3771 (2018).

195. J. L. Campos, K. J. A. Johnston, B. Charlesworth, K. Kunte, The effects of sex-biased gene expression and x-linkage on rates of sequence evolution in Drosophila. *Mol. Biol.*

Evol. **35**, 655–665 (2018).

196. P. Stenberg, L. E. Lundberg, A. M. Johansson, P. Rydén, M. J. Svensson, J. Larsson, Buffering of segmental and chromosomal aneuploidies in Drosophila melanogaster. *PLoS Genet.* **5** (2009), doi:10.1371/journal.pgen.1000465.

197. A. S. Goldsborough, T. B. Kornberg, Reduction of transcription by homologue asynapsis in Drosophila imaginal discs. *Nature*. **381**, 807–810 (1996).