TRANSVECTION IS A PLASTIC PHENOTYPE

by

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Abstract

Transvection, a chromosome pairing-dependent form of *trans*-based gene regulation, is widespread in the *Drosophila melanogaster* genome. Recent studies demonstrate that transvection is sensitive to cell environment and type in *D. melanogaster*, implicating transvection as a complex trait. To test this possibility, we first established that *trans*-interactions previously documented at the *Malic enzyme* (*Men*) locus are transvection (*i.e.*, pairing-dependent). We then characterized the sensitivity of transvection at the *Men* locus to changes in the environment (temperature) and genetic background (third chromosome). Transvection varied significantly across genetic backgrounds and was significantly reduced by changes in temperature, and the two factors interacted to further modify transvection, while *cis*-based gene regulation remained unchanged by temperature. To determine if differences in transvection observed across genetic background and temperature are related to their effects on transcription factor expression, and possibly the presence or absence of binding sites for these transcription factors within the *Men* locus, we tested the relationship between *Men* expression and five transcription factors with binding sites near the *Men* transcription start sit (TSS). We found correlations between the expression of at least one transcription factor, *Abd-B,* and the presence of binding sites for that factor, and *Men* expression across changes in the environment. We also determined that changes in *Abd-B* expression can directly affect *Men* expression in *cis*, suggesting that *cis* and *trans*-regulation can share regulatory components in at least some cases. Together, our findings stress the importance of studying genetic interactions from a dynamic perspective by incorporating both genetic and environmental variation.

Keywords

Transvection, *malic enzyme* (*Men*), phenotypic plasticity, GXE interactions, genetic background, *Drosophila melanogaster*.

Co-Authorship Statement

Chapter 1 will form part of a review on the phenomenon of *trans*-interactions and transvection, importance of population genetics and understanding how changes in the environment can affect gene expression and phenotype, with Thomas J.S. Merritt (TJSM), who contributed to the conception of many of the ideas and provided editorial comments on the writing.

Chapter 2 is being submitted in similar form in *Genetics.* I am the first author, with co-author Teresa Z. Rzezniczak (TZR), Jack Bateman (JB), and TJSM. TZR provided expert technical assistance with the enzyme assays and experimental design, JB contributed to the conception of the study, TJSM contributed to the conception of the study, provided advice on the laboratory assays, contributed to experimental design, and helped write the manuscript.

Chapter 3 will be a summary of the general conclusions of the study, and conceptualization of future work that is currently ongoing or may be conducted in the lab in the near future.

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List of Abbreviations

3C: Chromosome Conformation Capture

Abd-B: *Abdominal-B*

BDSC: Bloomington Drosophila Stock Centre

Bp: base pair

ChIP: Co-immunoprecipitation

CRMs: *cis*-regulatory modules

DNA-FISH: DNA Fluorescence *in situ* Hybridization

Drosophila melanogaster: *D. melanogaster*

GXE: genotype by environment

Idh: *Isocitrate dehydrogenase*

Men: *Malic enzyme*

mirr: *mirror*

NADP: Nicotinamide adenine dinucleotide phosphate (reduced: NADPH; oxidized: NADP+)

pQTL: plasticity Quantitative Trait Loci

qPCR: quantitative reverse-transcriptase Polymerase Chain Reaction

QTL: Quantitative Trait Loci

RNA: Ribonucleic Acid

RPM: revolutions per minute

SNPs: Single Nucleotide Polymorphisms

slbo: *slowbordercells*

Su[Hw]: *Suppressor of Hairy wing*

TFBS: Transcription Factor Binding Sites

TRiP: Transgenic RNAi Project

Tris-HCL: Trisaminomethane hydrochloride

TSS: Transcription Start Site

Trl: *Trithorax-like*

Tpi: *Triose-phosphate isomerase*

y: *yellow*

z: *zeste*

Chapter 1: *Trans*-interactions, transvection and phenotypic plasticity

1.1 Overview of the thesis

Eukaryotic nuclei are organized but dynamic, with a plastic genome topology that allows for variation in gene expression in response to environmental changes, which in turn leads to changes in an array of complex phenotypic traits that affect the fitness of the organism. However, accounting for this phenotypic plasticity is not straightforward for complex traits, which arise from multiple segregating genes and their interactions with the environment and each other in both *cis* and in *trans* (Mackay *et al.* 2009). To begin to understand the genetic basis of phenotypic plasticity, it is important to understand how *cis* and *trans*-interactions differentially respond to environmental changes. One common environmental feature is temperature, one of the most important abiotic factors influencing the genetics, physiology, ecology, and evolution of organisms. Thus, in my thesis, I used *Drosophila melanogaster* as a model for genetic interactions in eukaryotes, focusing particularly on *cis* and *trans*-interactions at the *Malic enzyme* (*Men*) locus, and how these interactions could be affected by changes in temperature. My examples throughout the literature review are focused on *D. melanogaster* and transvection, a phenomenon that we show to be occurring at *Men.* The temperatures chosen represent a biologically relevant temperature range. In addition, inbred lines were originally collected from populations across a range of temperature-clines (see Materials and Methods), lending genetic complexity and biological significance to the study.

I took an integrative approach, measuring responses at several levels of biological organization. In particular, I focused on the impact of adult acclimation to different temperatures on transvection in a variety of genetic backgrounds, with consideration of the role of environmentally responsive transcription factors in mediating these effects. As background for this work, I will briefly review current knowledge of transvection, *trans*-interactions, and the effects of changing thermal conditions on eukaryotic phenotype, chiefly from a genetic standpoint.

1.2 **A three-dimensional view of the nucleus**

Given the space limitation of the nucleus, DNA must be compacted and folded hierarchically into higher-order structures that eventually form chromosomes (Woodcock 2006). These chromosomes in turn form functional compartments, within the three-dimensional context of the nucleus, in a way that allows for proper regulation of genome function. The three-dimensional nature of chromosomal organization can bring together widely separated functional genetic elements within the genome into close proximity. Detailed studies primarily using two techniques, DNA fluorescence *in situ* hybridization (DNA FISH), and chromosome conformation capture (3C: captures spatially related chromatin fragments *in vivo*), have provided insight into how distant loci are brought into close proximity *in vivo* (reviewed by Gondor and Ohlsson 2009; Naumova and Dekker 2010; Williams *et al.* 2010). These long-distance interactions can involve loci on the same chromosome (commonly referred to as "in *cis*"), and also on different chromosomes ("in *trans*"), in a spatial and temporal manner. Long-distance interactions can influence all aspects of genome function, including transcription, replication, DNA repair and mutagenesis (Dekker 2008; Misteli 2007; Sexton *et al.* 2007). With the recent introduction of high-throughput genome-wide variations of these two techniques, it has become apparent that long-distance interactions are extensive in a variety of eukaryotes, including yeast (Duan *et al.* 2010), *Drosophila* (Sexton *et al.* 2012), mice (Zhang *et al.* 2012), and humans (Kalhor *et al.* 2011; Lieberman-Aiden *et al.* 2009). Studies have also shown that genome organization is non-random, and long-distance interaction patterns are predictable and differ in a tissue- and developmental-stage specific manner (Biran and Meshorer 2012; Meister *et al.* 2010; Parada *et al.* 2004; Sanyal *et al.* 2012). As with genome sequencing, our ability to catalogue these long-distance interactions between genomic regions has exceeded our ability to determine the significance of these interactions. However, the extensiveness and deterministic nature of inter-chromosomal interactions stresses the importance of understanding how three-dimensional genomic architecture can influence genome function *in vivo.*

1.3 **Gene regulation through *trans*-interactions**

Regulation of gene expression is an important part of genome function. Cells of higher eukaryotes must tightly regulate the expression of their genome to maintain proper function. This process is regulated by a combination of classical enhancer-promoter interactions occurring in

linked *cis*-regulatory sequences within each locus, and extensive long-distance *cis* and *trans*-interactions. *Drosophila* geneticists have long postulated that chromosome architecture must play an important role in gene regulation, including gene inactivation through chromatin loops (reviewed by Bulger and Groudine 1999; Gondor and Ohlsson 2009), and gene complementation through pairing of homologous loci (Lewis 1954; reviewed by Duncan 2002). The advent of new technologies such as 3C on ChIP (Simonis *et al.* 2006: identifies chromatin interactions mediated by specific transcription factors) have provided further evidence that interactions between unlinked genomic regions at specific chromatin domains can directly impact gene expression (reviewed by Cavalli and Misteli 2013). A variety of transcriptional mechanisms, either repressive or inductive, can be involved at these domains (reviewed by Bulger and Groudine 2011; Gondor and Ohlsson 2009). Repressive chromatin domains, or closed chromatin, lead to gene silencing, which is commonly driven by interactions between insulator proteins (*e.g.*, Polycomb Group proteins, PcG's) bound to distal insulator elements (reviewed by Bantignies and Cavalli 2011; Yang and Corces 2011). Active chromatin domains, or open chromatin, lead to gene activation, driven by co-localization of shared regulatory enhancers between un-linked genes through looping (*e.g.*, *β*-globin locus control region in mammalian organisms; reviewed by Bartkuhn and Renkawitz 2008; Bulger and Groudine 2011; Cavalli and Misteli 2013).

Gene regulation through long-distance interactions has now been implicated in a variety of contexts during normal cell development of eukaryotes. For example, *trans*-interactions between homologous loci that lead to heritable changes in phenotype, termed paramutation, have been extensively studied in plants, *Drosophila*, and mice (de Vanssay *et al.* 2012; reviewed by Chandler 2007; Chandler and Stam 2004; Grant-Downton and Dickinson 2004; Stam 2009). In mammalian cells, *trans*-interactions between homologous X-inactivation centers lead to epigenetic asymmetry that leads to a mutually exclusive inactivation of one of the X-chromosomes (reviewed by Lee 2011; Xu *et al.* 2006). In *Drosophila*, proper olfactory neuron targeting is mediated by co-regulation of genes through nuclear co-localization (Clowney *et al.*2012). *Cis* and *trans*-interactions have also been shown to be involved in *Ifng* expression in naïve T cells in mice (Hakim *et al.* 2013; Spilianakis *et al.* 2005), and the activation of *IFN-β* expression in response to viral infection in HeLa cells (Apostolou and Thanos 2008). In sum, long-distance interactions in *cis* and in *trans* play a key role in driving normal cellular response and developmental gene expression in eukaryotes.

Long-distance interactions have also been implicated in a variety of diseased states. For example, parental imprinting, implicated in human diseases such as Prader-Willi syndrome (Lasalle and Lalande 1996) and autism (Hogart *et al.* 2007; 2009; Thatcher *et al.* 2005), is thought to be established by *trans*-interactions. In addition, abnormal *trans*-interactions have been implicated in the deregulation of gene expression in cancer cells. Abnormal somatic chromosomal pairing of homologous loci may drive abnormal gene expression in certain renal cancers (Koeman *et al.* 2008) and lymphomas (Liu *et al.* 2008). Long-distance interactions can also induce deregulation of genes implicated in breast cancer, although a direct link between these interactions and tumorigenesis has not been established (reviewed by Betts *et al.* 2013). Interestingly, over-expression of a specific oncogene, ERG, can trigger global chromatin remodelling in addition to establishment of nascent *trans*-interactions through binding of ERG, which then drives abnormal expression of a variety of transcription factors that lead to cancer (Rickman *et al.* 2012). Aberrant interactions between unlinked loci could very well lead to disease, and a better understanding of the mechanisms involved in establishing long-distance *cis*- and *trans*-interactions will help in uncovering the genetic basis of human diseases.

A unique type of *trans*-interaction involves pairing of homologous loci in somatic cells that can regulate gene expression, a phenomenon termed transvection (Lewis 1954; reviewed by Duncan 2002; Kennison and Southworth 2002). In most organisms, pairing of homologous chromosomes is an essential part of nuclear re-organization and "cross-over" events during meiosis. Although long-distance interactions between homologous loci appear to be important in somatic cells of most eukaryotes, they are rare and transient (McKee 2004). However, in Dipteran insects, this homologous pairing occurs in somatic cells throughout development (Metz 1916; Stevens 1907; reviewed by McKee 2004). In *Drosophila melanogaster*, somatic chromosomal pairing of homologues can impact gene regulation through a phenomenon termed transvection. Recently, it has been established that transvection can occur almost anywhere in the *Drosophila* genome (Bateman *et al.* 2012a; Mellert and Truman 2012). Although mechanisms of *trans*-interactions as pertaining to the regulation of gene expression in *Drosophila* are thought to be conserved in eukaryotes (White 2009), the extensive pairing of somatic homologues in Dipterans appears to be unique. Nevertheless, the extensive nature of this unique chromosomal architecture makes *Drosophila* a great model to study the impact of *trans*-interactions on gene regulation.

1.4 **The mechanism underlying transvection in *D. melanogaster***

Somatic chromosomal pairing between homologous chromosomes has been described in essentially all tissues and developmental stages of Dipteran organisms (Metz 1916; Stevens 1908). This process can regulate gene expression, termed transvection (Lewis 1954). Transvection is a unique type of pairing-dependent *trans*-interaction that involves interactions between homologous loci, and has been described in detail at 15 distinct loci in *D. melanogaster* (Table 1). The phenomenon is currently loosely defined as long-distance interallelic complementation, usually between two mutant alleles, that is dependent on somatic homologous chromosomal pairing (reviewed by Duncan 2002; Kennison and Southworth 2002). These interactions can lead to gene activation (*e.g., Abdominal-B* and *yellow*) or silencing (*e.g., brown and zeste-white*). Although the mechanisms underlying transvection at most loci are well-studied (Duncan 2002; Kennison and Southworth 2002), they remain ambiguous for certain loci (*e.g., brown*). Overall, most cases of transvection appear to fall under two models – enhancer action in *trans* and the topology or looping model.

Table 1 Genes that exhibit transvection (or transvection-like *trans*-interactions), in *Drosophila melanogaster*.

Gene	**Model of transvection**	**Author**[1]
Abdominal-A/B (*Abd-A/B*)	Positive enhancer action in *trans*	Hopmann *et al.* 1995
decapentaplegic (*dpp*)		Gelbart 1982
eyes absent (eya)		Leiserson *et al.* 1994
sn-glycerol-3-phosphate dehydrogenase (Gpdh)		Gibson *et al.* 1999
vestigial (*vg*)		Coulthard *et al.* 2005
Salivary glue secretion-4 (*Sgs4*)		Korge 1981
Malic enzyme (*Men*; transvection-like)		Merritt *et al.* 2005; Lum and Merritt 2011
wings-up A (*wup A*)		Marin *et al.* 2004
yellow (*y*)	Enhancer blocking insulator (*gypsy*, *mdg4* dependent),	Geyer *et al.* 1990

	topology model (insulator bypass)	
white (w) and zeste (*z*)	Zeste aggregation in *trans*, recruitment of Polycomb Group Proteins (PcG's)	Gelbart and Wu 1982
apterous (*ap*)	Enhancer blocking insulators [*su*(*Hw*), *mdg4* dependent]	Gohl *et al.* 2008
brown (*bw*)	Repression by heterochromatin pairing causing position-effect variegation	Henikoff *et al.* 1989
cubitus interruptus (*ci*)	Enhancer blocking insulator (*gypsy*)	Locke 1994
Sex comb reduced (*Scr*)	Enhancer blocking insulator	Kennison and Southworth 2002
Bithorax Complex (BX-C) *Ultra-bithorax* (*Ubx*)	Extremely complex; mutant alleles exhibit a variety of mechanisms (*gypsy*, *zeste*, *etc.*)	Lewis 1954

*Since Bateman *et al.* (2012a) and Mellert and Truman (2012) used synthetic transgenes with fragments from a variety of loci, with multiple combinations of enhancer and promoter modules tested, their examples are not shown here.

[1]Authors of the original study.

The original examples of enhancer activation of *trans* promoters were unexpectedly revealed in studies at the *Ubx* locus by E.B. Lewis in 1954. The model has since been extensively studied at *Abdominal-B* (*Abd-B*; Hendrickson and Sakonju 1995; Hopmann *et al.* 1995; Sipos *et al.* 1998), *eyes absent* (Leiserson *et al.* 1994), and *yellow* loci (*y*, Morris *et al.* 1998). The model usually describes intragenic complementation involving specific types of mutant alleles. Although enhancers predominantly act on the *cis*-linked promoter, mutation of core promoter elements in the *cis*-linked promoter releases enhancers to act in *trans* (Bateman *et al.* 2012a; Morris *et al.* 2004). For example, in certain cases of transvection at the *yellow* locus, enhancers of a promoter-less allele can act in *trans* on the promoter of an enhancer-less allele on the homologous locus, driving higher than expected gene expression and thus phenotypic rescue (Chen *et al.* 2002;

Geyer *et al.* 1990; Morris *et al.* 1998). The simplest mechanistic model for this phenomenon is that enhancers interact with a promoter in *trans* simply through physical proximity, as homologues are extensively paired in *Drosophila.* Support for this model comes from *in vitro* studies with artificial protein bridges (Muller and Schaffner 1990) and catenation of plasmids (Dunaway and Droge 1989; Rothberg *et al.* 1991) that bring enhancers and promoters on separate DNA molecules into close proximity. The action of these enhancers in *trans* is generally much reduced, however (Geyer *et al.* 1990; Leiserson *et al.* 1994; Lewis 1954) – certain enhancers in *cis* drive up to 100 fold more gene activity than when they act in *trans* (Bateman *et al.* 2012a). However, this is not always the case, as *trans*-interactions at *Malic enzyme,* which is thought to be mediated by pairing of enhancers in *trans* (or transvection-like), can lead to higher than 100% normal *cis*-gene activity (Lum and Merritt 2011). Therefore, the specific mechanisms and extent of transvection at each gene system may be different depending on enhancer and promoter identities. In addition, since this model proposes that enhancer action in *trans* is simply a by-product of extensive homologous chromosome pairing, differences in the amount of transvection observed could also be due to differences in the frequency of homologous pairing across cell types or developmental stages (*e.g.*, position-effect variegation; proposed by Duncan 2002). In short, enhancers are able to act in *trans* to drive transvection as a consequence of the synapsis of the homologous chromosomes in somatic cells of *D. melanogaster.*

The topology model complements the enhancer action in *trans* model, and can better explain certain cases of transvection. It proposes that in order to achieve proper pairing between mutant alleles with only one functional promoter, the promoter and certain elements that differ between the alleles are looped out, allowing the promoter to bypass transcriptional constraints (*i.e.*, from insulators; Morris *et al.* 1999a). Therefore, transvection as described by the topology model usually involves occurrences where unique chromatin insulator insertions are looped out, leading to higher than expected gene activity in *trans.* However, if unique enhancers are present on one of the complementing alleles, looping out of this element to attain pairing could also be possible, which would lead to less than expected amount of gene activity in *trans.* In *cis,* looping to bring enhancers and promoters in close contact is a well-accepted model of classical gene regulation that is evolutionarily conserved (Bartkuhn and Renkawitz 2008; Bulger and Groudine 2011). Given the conserved nature of looping in gene regulation and the complex array of regulatory elements and promoter identities in *Drosophila*, a combination of the topology and enhancer

action in *trans* models, whereby enhancers in *trans* are brought into close proximity to the functional promoter through looping, likely explains most or all examples of transvection.

Although examples of transvection-like phenomena have been uncovered in other organisms, transvection appears to be prevalent in *Drosophila* due to its extensive pairing of homologues. This point is further supported by the knockdown of a nuclear organizing protein involved in somatic chromosomal pairing in *Drosophila* that disrupts transvection (Hartl *et al.* 2008). Recent studies have identified numerous candidate genes important for somatic chromosomal pairing in *Drosophila* (Bateman *et al.* 2012b; Joyce *et al.* 2012), and further study of the role these genes will better our understanding of the molecular basis of transvection. Although not mutually exclusive, mechanistic differences should exist between looping and pairing, and further dissection of these differences will likely improve our understanding of differences in mechanisms underlying transvection. However, regardless of the topology or pairing mechanisms by which *trans*-interactions are mediated, the types of regulatory elements and promoters present in a locus appear to be important modulators of transvection. For example, certain transcription factors bound to regulatory elements may have a "homing" effect (through preferential interactions of binding partners) on transcriptional machinery bound to promoters, and are therefore better able to interact across long distances (*i.e.,* in *trans*; Xu and Cook 2008). This "homing" effect can help explain how certain enhancers can generate higher gene activity in *trans* than in *cis* (Gibson *et al.* 1999; Lum and Merritt 2011). It could also explain why certain regulatory elements are recurrent in loci implicated in transvection (*e.g., zeste, gypsy,* Polycomb response elements), and why loci are differentially sensitive to pairing-disruption through chromosomal rearrangements (*i.e.,* they present different sizes of genomic areas critical for transvection). Recent identification of transcription factories bound to enhancers across un-linked (*i.e.* distant) genomic regions provide further support for the importance of transcription factors in driving long-distance *trans*-interactions (Melnik *et al.* 2011; Papantonis and Cook 2010). Further identification of transcription factors that are important for *trans*-interactions will improve our understanding of the molecular mechanisms underlying transvection and long-distance gene regulation.

Altogether, the specific mechanisms of transvection involved at a specific locus depend on a combination of factors. These factors include the functional *cis*-genetic elements of the locus that

remain in specific mutant alleles, their order within the locus, and the local chromosomal environment – which depends on the cell type and developmental stage at which the phenotype is being observed (Bateman *et al.* 2012a; Duncan 2002; Kennison and Southworth 2002; Mellert and Truman 2012; Morris *et al.*1998). Importantly, two recent studies using transgenic approaches have significantly contributed to our understanding of the mechanisms underlying transvection (Bateman *et al.* 2012a; Mellert and Truman 2012). Transvection appears to be possible throughout the *Drosophila* genome if the gene contains the proper enhancer elements, or *cis* regulatory modules (CRMs), and the ability of an enhancer to bind a transcriptional activator is sufficient for transvection. Interestingly, these studies also confirm previous reports that enhancers may act on promoters in *trans* even in the presence of a functional *cis* promoter (Goldsborough and Kornberg 1996; Casares *et al.* 1997; Sipos *et al.* 1998), and that transvection shows position-specific variegation and stochasticity (reviewed by Duncan 2002). The variegated transvection phenotype (*i.e.*, cell-cell variability within the same tissue) suggests that local pairing dynamics, efficiencies of promoter activation and transcriptional elongation, and mRNA or protein stability may strongly influence the amount of transvection observed within a single cell type (Bateman *et al.* 2012a). In addition, stochasticity of transvection (*i.e.*, cell-type specificity) further supports the idea that transcription factors combinations, which maybe only available in certain cell types, differ in their ability to activate the *trans* promoter (Mellert and Truman 2012). In sum, these two studies strongly suggest that transvection is a complex trait that can show variation not only across model systems of transvection in *Drosophila*, but within the genetic configurations of a single system as well.

1.5 Taking a different approach to transvection

As discussed above, transvection shows significant variation across cell types (Mellert and Truman 2012), and even variegation within cells of the same tissue (Bateman *et al.* 2012a). Differences in cellular environment in multi-cellular organisms are synonymous to differences in environmental conditions in unicellular organisms such as yeast (Ramani *et al.* 2012). Therefore, if transvection is sensitive to cellular environment, it is possible that transvection would be sensitive to changes in external environmental conditions, characteristics of a complex trait. In addition, although changes in gene expression in response to environmental change have been extensively studied across a diverse range of organisms (Gasch *et al.* 2000; Grishkevich *et al.*

2012; Zhou *et al.* 2012), how this change in gene expression is driven by changes in genome topology is poorly understood.

Active and inactive domains tend to cluster separately in the genome, inhabiting distinct chromosomal territories (reviewed by Cremer and Cremer 2010; Cavalli and Misteli 2013). Co-regulated active domains can co-localize at transcription factories through long-distance *cis* and *trans*-interactions, and inactive domains tend to be condensed within their chromosomal territory in more peripheral regions of the nucleus. In addition, changes in the expression of chromatin regulatory network genes in different environments may drive strong phenotypic changes (Gibert *et al.* 2007). Therefore, large-scale changes in gene expression in different environments may be driven by global modifications of chromosomal architecture that shuffle active and inactive domains into their respective chromosomal environments in a given environmental context. This global chromosomal conformation change should then affect pairing between homologous somatic chromosomes, and thus lead to modifications of transvection, a pairing-dependent phenomenon. Therefore, measuring how transvection varies across environments could provide an excellent model system through which we can better our understanding of how changes in the environment can impact gene expression driven by topology.

1.6 **Complex traits and phenotypic plasticity**

Naturally occurring complex traits can demonstrate plasticity across environments and genetic background (Lander and Schork 1999). The plasticity of phenotypes allows organisms to respond rapidly to changing environmental conditions, and plays a key role in adaptation and evolution of organisms (Price *et al.* 2003; Via *et al.* 1995; Via and Lande 1985; Wagner and Altenberg 1996; West-Eberhard 2003). This variation results from interactions between the environment and multiple segregating genes (Mackay *et al.* 2009).

Interestingly, background effects can become amplified during changes in environmental conditions through genotype-by-environment interactions, or GXE interactions. These GXE interactions are observed as differences in the magnitude or direction of a phenotype under different environments (Rzezniczak and Merritt 2012; Mackay *et al.* 2009). GXE interactions have been observed in a variety of traits, appears to be evolutionarily conserved (Grishkevich *et al.* 2012; Li *et al.* 2006; Tirosh *et al.* 2006; Tirosh and Barkai 2008; Valdar *et al.* 2006), and impacts transcriptomic changes in response to environmental stress (Gasch *et al.* 2000;

Grishkevich *et al.* 2012; Zhou *et al.* 2012). Since genome topology regulates gene expression, changes in topology in response to environmental changes, as previously discussed, may contribute to the transcriptomic changes observed. However, how this plasticity of genome topology could drive the changes observed in the transcriptome is unclear.

1.7 Context-dependent effects of mutations

The study of the effects of a lab-derived mutation on an observable phenotype is a popular method of experimentation in most fields of biology. Like natural traits, the effect of a lab-derived mutation on phenotype can be also significantly different, depending on the context in which the mutation is placed (Chandler *et al.* 2013). The context in question could be genetic background, the environment, or other factors.

Within a given species, significant sequence variation is present across genetic backgrounds, including: single nucleotide polymorphisms (SNPs), polymorphic insertions or deletions (indels), and simple sequence repeats (microsatellites; Mackay *et al.* 2009). All of these genotypic variations can be observed across "wild-type" genetic backgrounds, leading to natural phenotypic variation, without having drastic effects on the fitness of those genetic backgrounds under normal conditions. However, when mutations are placed into these "wild-type" genetic backgrounds, the phenotypic consequences of the mutant allele may be profoundly affected, particularly when the given phenotype is not robust (Chandler *et al.* 2013). These cryptic genetic variations have been a recent subject of extensive study, and have been found to play a role in a variety of phenotypes – from body size to sex determination – in a wide variety of organisms (Chandler 2010; Dworkin *et al.* 2009; McGuigan *et al.* 2011), and may be involved in evolutionary change (Le Rouzic and Calborg 2008).

Further complicating the issue, environmental factors can alter how a mutant allele influences phenotype, and the impact of these factors differs across genetic backgrounds (Chandler *et al.* 2013). Many studies treat these GXE interactions on the effect of a mutation, context-dependent effects, as a "nuisance" since effects of a given allele can differ drastically across the contexts mentioned. Many researchers choose, inappropriately, to conduct experiments using one genotype and one environmental condition to reduce "variability" (Chandler *et al.* 2013). However, this variability is important since it can identify potential mechanisms that maintain genetic variation of complex traits in natural populations (Gillespie and Turelli 1989; Levene

1953). Maintenance of genetic variation in turn allows for organismal adaptation in the face of environmental change (*i.e.*, phenotypic plasticity; Price *et al.* 2003; West-Eberhard 2003).

1.8 Transvection as a context-dependent complex trait

As previously alluded to, transvection appears to be a complex trait. The amount of transvection observed, or the effect of a transvection-driving gene, may be dependent on genetic background (pertaining to *trans*-interactions (transvection-like) at *Men,* Lum and Merritt 2011), cell type (Mellert and Truman 2012), and even cellular environments of the same cell type (Bateman *et al.* 2012a). These variable, or context-specific, results are characteristic of traits that lack canalization (the ability of a population to produce the same phenotypes regardless environmental or genotypic variability, Waddington 1942). Therefore, transvection, a phenotypic consequence of lab-derived mutations, should be treated as a context-dependent complex trait.

The power of the transvection system is that it depends on the effect of the mutant alleles that interact in *trans* (or local genetic effects), within the context of a naturally occurring plastic trait – somatic homologous chromosomal pairing. Changes in the genetic background and environment may modulate non-local factors that regulate somatic chromosomal pairing and transcription machinery availability, which may in turn modify the ability of mutant alleles to interact in *trans.* If transvection is a context-dependent complex trait, it should show significant variation across genotypes and environments (or other contexts). Understanding this variation across genotypes (multiple segregating genes) and environment, and how interactions between these factors can further drive variability in transvection, will better our understanding of transvection and *trans*-interactions from an evolutionary perspective.

1.9 Trans-interactions at *Men* in *D. melanogaster*

The *Men* locus provides an ideal system for studying the plasticity of *trans*-interactions in *Drosophila.* Cytosolic malic enzyme (MEN) is an important metabolic enzyme, reversibly oxidizing malate to pyruvate with the concurrent reduction of the cofactor $NADP^+$ to NADPH (Wise and Ball 1964). NADPH is a key reducing agent in a number of cellular processes such as lipogenesis and anti-oxidation (reviewed by Ying 2008). MEN helps maintain a homeostatic concentration of NADPH, contributing to the reduction of approximately 30% of the cellular NADPH pool (Wise and Ball 1964; Ying 2008). Although MEN is physiologically important, its

strength as a model system to understanding *trans*-interactions in *D. melanogaster* lies in the sensitivity and accuracy of the experimental approach used to quantify its activity.

While examining *P*-element excision-derived knockout alleles of the *Men* gene, Merritt *et al.* (2005, 2009) found higher than expected (*i.e.,* 50%) MEN activity when knockout alleles were in *trans* to a wild-type allele. This non-additive up-regulation was then shown to be due to *trans*-interactions of the mutant allele with the functional allele, and not a simple physiological up-regulation of the functional allele (Merritt *et al.* 2005). To further understand *trans*-interactions at the *Men* locus, Lum and Merritt (2011) used *P*-element mediated excision to generate and characterize a suite of 19 *Men* knockout (*MenExi*$^{-}$) alleles. The size of the *MenExi*$^{-}$ allele deletions explained some, but could not account for the majority, of the variation in their ability to drive *trans*-interactions (*i.e.,* up-regulate MEN activity). Instead, this variation was attributed to specific intergenic regions containing high density of transcription factor binding sites (TFBS) that were present or absent in each *MenExi*- allele. Lum and Merritt (2011) also noted that certain excisions have a different impact on *trans*-interactions depending on the genetic background. To determine whether this variation is caused by distal, or non-local, genetic factors (*e.g.,* differences in transcription factor expression), six of the knockout alleles were examined in five different genetic backgrounds varying at the third chromosome only, the chromosome on which *Men* is located. This limited genetic variation also provides an experimentally manageable and biological relevant model for variation in natural populations. Specific interactions between genetic background and knockout alleles were identified, suggesting that variations in *trans*-genetic elements (*i.e.,* expression of transcription factors, or the presence or absence of binding sites for specific transcription factors) might strongly affect the level of *trans*-interactions observed. However, it is unclear what these *trans*-genetic elements might be and how their variation results in changes in the levels of *trans*-interaction at the *Men* locus. In addition, it is not known whether these *trans*-interactions are indeed driven by physical pairing between the homologous copies of *Men.* Thus, further studies are needed to elucidate the precise mechanisms underlying *trans*-interactions at the *Men* locus.

1.10 Objectives and Hypotheses

The main objective of my research project was to determine whether transvection is sensitive to differences in the environment, (specifically adult acclimation to three temperatures) and/or genetic background (specifically variation of the 3rd chromosome). To do this, my first objective was to determine whether the *trans*-interactions observed at *Men* are indeed pairing-dependent (*i.e.*, transvection), using two approaches to alter both large-scale and local genetic architecture surrounding the *Men* locus, which we expected to perturb somatic homologous chromosomal pairing. I then used the model of transvection at *Men* to test my main hypothesis that transvection at *Men* in *D. melanogaster*, as a complex trait, will demonstrate phenotypic plasticity and GXE interactions. Since phenotypic plasticity across genetic background and environmental conditions can be driven by *trans*-regulatory effects, I next investigated the nature of these *trans* effects. I focused on the relationship of transvection at two *MenExi*$^{-}$ alleles (*MenEx58*$^{-}$ and *MenEx60*$^{-}$) and the expression of transcription factors that have predicted binding sites around the deletion sites of these two alleles. I hypothesized that these *trans*-regulatory effects are dependent upon changes in transcription factor expression across genetic backgrounds and environmental conditions that regulate transvection at these two alleles. Finally, I investigated whether the transcription factors that have been implicated in the regulation of *Men* expression in *trans* also regulate *Men* in *cis*, with the hypothesis that mechanisms of co-regulated genes in *trans* are different from that of *cis*-interactions.

Chapter 2 – Transvection is sensitive to environment and genetic background

2.1 INTRODUCTION

Genome function is regulated through linear sequence and three-dimensional conformation (Cavalli and Misteli 2013). Gene expression is largely regulated through *cis*-interactions, but this regulation can be substantially modified by larger-scale features of genome topology (reviewed by Bartkuhn and Renkawitz 2008; Cavalli and Misteli 2013; Williams *et al.* 2010). Genome topology can mediate gene regulation through long-distance interactions between un-linked genomic regions both on the same (*cis*-interactions) or separate chromosomes (*trans*-interactions). Interestingly, in *Drosophila* homologous chromosomes are extensively paired in the somatic nucleus of all cell types (reviewed by McKee 2004). This distinct chromosomal conformation drives transvection, a type of pairing-dependent *trans*-interaction regulating gene expression of homologous loci. Since its original discovery by Ed Lewis in 1954, studies have shown that transvection can lead to up-, or down-, regulation of gene expression (reviewed by Duncan 2002; Kennison and Southworth 2002). Most cases of transvection involve intragenic complementation between two loss-of-function or hypomorphic alleles in a pairing-dependent manner (*i.e.*, enhancer action in *trans*), but can also involve looping of insulators and enhancers (*i.e.*, topology model). Recently, the *Drosophila* genome was found to be generally permissive to transvection (Bateman *et al.* 2012a; Mellert and Truman 2012), although a comprehensive model of the molecular, or genomic, mechanisms of these *trans*-interactions is still being developed. The strength and widespread occurrence of transvection makes *Drosophila melanogaster* an excellent model system for studying the role of *trans*-interactions in gene regulation, particularly important because pairing-dependent *trans*-interactions have been implicated in regulation of gene expression both during normal cell development (*e.g.* X-chromosome inactivation; Xu *et al.* 2006) and in various disease states (*e.g.* cancer; Koeman *et al.* 2008).

The amount of transvection, the degree of up-, or down-, regulation of gene expression, varies between loci and alleles (reviewed by Duncan 2002). Recent studies have shown that

transvection is also sensitive to cell type and cellular environment, and can be stochastic, leading to variegated gene expression within a tissue (Bateman *et al.* 2012a; Mellert and Truman 2012). Similar complex traits are generally not canalized (robust) phenotypes, and often involve multiple segregating genes and their interactions with the environment (Mackay *et al.* 2009; Chandler *et al.* 2013). Changes in environmental conditions (temperature, light, etc.) can lead to changes in phenotype (*i.e.*, phenotypic plasticity), through global shifts in gene expression often driven by changes in chromosomal architecture (Choi and Kim 2007; Gibert *et al.* 2007; Li *et al.* 2006; Tirosh *et al.* 2010; Zhou *et al.* 2012). Given that transvection appears to be a complex phenotype, and given the role of chromosomal architecture in this phenomenon, it seems likely that transvection is not canalized, and is sensitive to both genetic background and environment, presumably through changes in chromosomal conformation. In fact, an earlier study by our research group found that genetic background did have a significant impact on *trans*-interactions at the *Malic enzyme* (*Men*) locus, a possible case of transvection. Understanding how changes in the environment and genetic background can impact transvection, and *trans*-interactions in general, will therefore provide insight into the plasticity of chromosomal architecture and its influence on gene regulation.

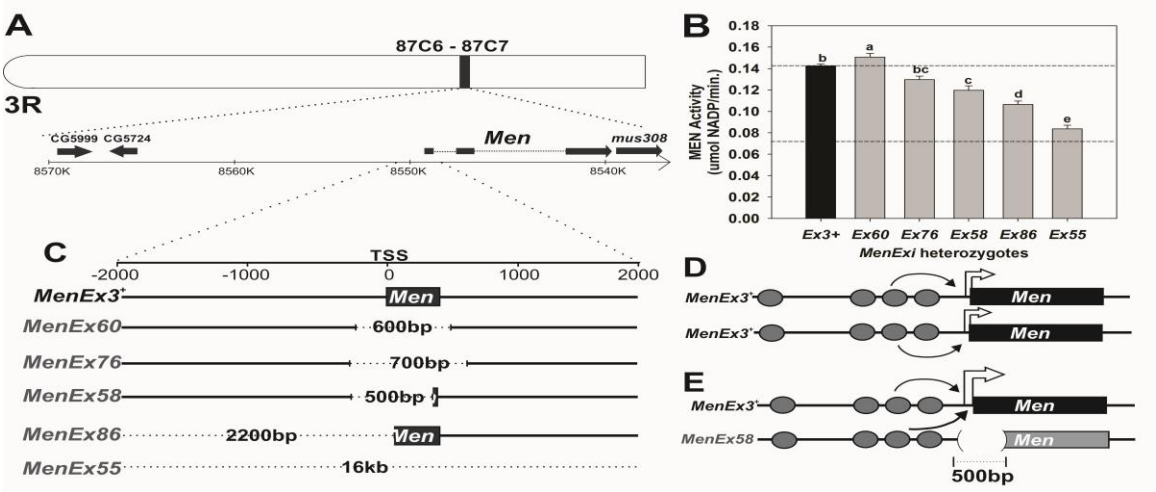

Figure 1 Model of *trans*-interactions at the *Malic enzyme* (*Men*). (A) The *D. melanogaster* *Men* locus is on the right arm of the 3rd chromosome (3R) with ORF-less 5' region of ~17kb. (B) Relative MEN activity of $MenExi^{-}/MenExi^{+}$ heterozygotes. We investigated *trans*-interactions at this locus using a suite of *P*-element excision derived knockout alleles, $MenExi^{-}$,that drive higher than expected amounts of MEN activity when heterozygous with a functional copy ($MenExi^{-}/MenExi^{+}$; *g*raph redrawn from Lum and Merritt 2011). (C) Details of $MenExi^{-}$ allele excision sites: $MenEx3^{+}$ is a perfect excision (used a "normal" or wildtype), the other excision alleles have deletion sizes that range from 500bp to 16kb around the TSS of *Men*. (D) Model of gene regulation at *Men* with two functional alleles of the *Men* gene, interactions are predominantly *cis*-based. (E) Model of gene regulation at Men with one functional and one knockout *Men* allele, interactions are now a combination of *cis* and *trans*.

Trans-interactions at the *Men* locus provide a good model system for understanding the plasticity of *trans*-interactions. Malic enzyme (MEN; Figure 1A) oxidizes malate to pyruvate, with the concurrent reduction of the cofactor NADP+ to NADPH (Wise and Ball 1964). This reaction can easily be followed in the lab with very high resolution and sensitivity; resolution of 5% differences in MEN activity is routine, allowing us to easily distinguish very small differences in regulation of the *Men* gene (Rzezniczak and Merritt 2012; Lum and Merritt 2011). Previous work has documented that regulation of the *Men* gene is sensitive to allelic differences in *trans.* Merritt *et al.* (2005, 2009) found unexpectedly high amounts of MEN activity in heterozygotes of *Men* knockout and wild-type alleles. Although flies heterozygous for a large-scale chromosomal deficiency had 50% wild-type MEN, heterozygotes with smaller deletions were found to have 80% wild-type activity. This dependence of MEN activity on chromosomal architecture suggested that the observed up-regulation was through *trans*-effects, possibly transvection. Further investigation using a larger suite of *P*-element derived knockout alleles ($MenExi^{-}$) determined that the up-regulation of MEN activity was allele-specific *trans*-

interactions (Lum and Merritt 2011). The alleles were derived through *P*-element mediated excision and all had slightly different lesions near the *Men* transcription start site (TSS; Figure 1B,C). Lum and Merritt (2011) attributed the excision-specific characteristics of the *trans*-effects to differences in the presence and absence of putative regulatory elements in each excision allele (Figure 1B,C), driving the differences in *trans*-interactions through a mechanism similar to enhancer action in *trans* (Figure 1D,E). Lum and Merritt (2011) also showed that the *trans*-interactions at the *Men* locus are sensitive to genetic background and the interaction of genetic background with specific excision alleles (*i.e.*, regulatory elements present or absent in the different excision alleles). Although the absolute magnitude of the differences in *trans*-interactions observed between heterozygotes of various genetic backgrounds were small, they were significant and repeatable, suggesting a subtle system of modulation of *trans*-regulation dependent on both local (excision site) and distant (loci coded elsewhere in the genome) factors.

The molecular mechanisms behind *trans*-interactions at *Men* are still unknown. First, although the observed up-regulation through *trans*-interactions is consistent with transvection, it is not unambiguously so. Transvection is by definition, chromosome-pairing dependent complementation, and is typically studied by generating heterozygous chromosome rearrangements that disrupt pairing, and the *trans*-interactions, at the locus of interest (Golic and Golic 1996; Lewis 1954). No such pairing dependence has been demonstrated at *Men.* Second, the molecular mechanisms underlying the sensitivity of *trans*-interactions at *Men* to genetic background are unknown. Lum and Merritt (2011) proposed that the variation in *trans*-interactions observed between alleles and genetic backgrounds may result from the presence or absence of binding sites for particular TFs on a *MenExi*$^{-}$ allele and differences in TF expression across backgrounds, but these have yet to be demonstrated. Uncovering the identity of binding sites and these non-local factors that contribute to the differences in *trans*-interactions observed across alleles and genetic backgrounds will help explain molecular mechanisms of *trans*-interactions at the *Men* locus.

Here, we demonstrate that the *trans*-interactions at the *Men* locus are transvection as classically defined, and show that this transvection is plastic with respect to both genetic background and environment. Using a subset of *MenExi*$^{-}$ alleles (Figure 1B) from our earlier study, intact *P*-element insertion alleles and chromosomal inversions, we show that *trans*-interactions at the

Men locus are pairing-dependent. We show that the magnitude of this transvection can be modified by genetic background and environment (temperature). Our demonstration that transvection is a plastic phenotype, while *cis*-based regulation is more robust, suggests a dynamic interplay between environment and genetic background in shaping transvection effects in *D. melanogaster*. We also begin to uncover the non-local factors which appear to contribute to variation in transvection across excision alleles, environment and genotype, finding correlations between *Men* and *Abdominal-B* (*Abd-B*) expression in an excision- and tissue-specific manner, across the genetic backgrounds and environmental conditions. Finally, we show overlap between elements involved in *cis* and *trans*-regulation demonstrating that changes in *Abd-B* expression can modify *Men* expression and MEN activity in *cis*, implicating *Abd-B* in the regulation of *Men* in *cis* and in *trans*.

2.2 MATERIALS AND METHODS

Fly stocks and rearing conditions

Isothird chromosome lines were a subset of nonlethal third chromosomes extracted from isofemale lines: *CT21, HFL53, JFL12, MD76, VT26* (see Sezgin *et al.* 2004). *P*-element transposon lines, GAL4 driver lines, inversion chromosome lines, and inbred lines used as common backgrounds were obtained from Bloomington *Drosophila* Stock Center (BDSC) at Indiana University (See Table S1 for a list and descriptions of all lines used). *Men* excision alleles, both knockout (*MenExi*$^{-}$) and wild-type (*MenEx3*$^{+}$) were generated by *P*-element mediated excision and described previously (Merritt *et al.* 2005; Lum and Merritt 2011). Five *MenExi*$^{-}$ allele lines were used in this experiment (see Table S1 for the list and description; Figure 1 for graphical representations). The wild-type control line used for all experiments was *MenEx3*$^{+}$, a perfect excision of the *EP517 P*-element with typical wild-type MEN activity. RNAi lines were acquired through the BDSC and had been constructed as part of Transgenic RNAi Project (TRiP) at Harvard Medical School (Table S1). All lines were kept over a TM8, *Sb* balancer chromosome. All parental flies (F_0) were maintained on a standard cornmeal medium at 25°C, 50% humidity, and 12:12 light:dark cycle, the same conditions as the experimental rearing conditions.

Essentially all experimental genotypes were created by crossing 10 male five-day old adult flies from one line (*e.g.*, a *MenExi*$^{-}$ allele line) to 10 female five-day old adult flies from another line (*e.g.*, an isothird chromosome line). Crosses were always set up in at least duplicate (two or more vials) and flies were allowed to lay eggs in standard fly vials for approximately three days at 25°C before adults were discarded. Emerging male flies were collected and aged for four days post-eclosion on fresh medium. For enzyme activity assays, at least four samples of four flies, two samples from each vial, were collected and stored at -80°C until assayed. For quantitative reverse-transcription PCR (qPCR) experiments, samples were pooled across two or more vials, and at least three samples of 15 flies were collected, snap frozen in liquid nitrogen and stored at -80°C until needed for RNA extraction.

The effect of temperature on transvection: The *MenExi*$^{-}$ allele lines were crossed to five isothird chromosome lines, emerging male flies were collected every second day and transferred to either 21°C or 29°C, or kept at 25°C (±1°C), under similar humidity (40-60%), and aged for four days. Each cross was done in at least six vials (replicates).

The effect of RNA inhibition of gene expression and *cis*-interactions: Experiments ivolving RNAi lines were conducted by crossing males from a heat shock promoter-driven *Gal4* line to females from a TRiP line (specific lines used for each gene are listed in Table S1). *Gal4/*TRiP progeny were exposed to heat-shock (47°C for 30min) five times throughout development (1st instar, 2nd instar, 3rd instar, pupae, second day post-eclosion) to induce *Gal4* expression. Each cross was done in four separate vials (replicates).

Fly homogenization and enzyme kinetic assays

Fly samples were first weighed to the nearest 0.01 mg using a MX5 microbalance (Mettler Toledo, Mississauga, Ontario), then homogenized in 100 µL of grinding buffer (50mM Tris-HCl, pH 7.4) per fly, and centrifuged at 13,000 rpm for 10 mins at 4°C to pellet insoluble residues. In general, four flies per sample were homogenized; however, if there were insufficient flies, fewer were assayed and the buffer volume was adjusted accordingly.

Malic enzyme (MEN) activity was measured using 10 µL of whole-fly homogenate in 100 µL of assay solution (described below) in a SpectraMax 384Plus 96-wellplate spectrophotometer (Molecular Devices, Sunnyvale, California). Absorbance at 320 nm was measured every nine seconds for three minutes at 25°C, and activity was quantified as the slope of the line of absorbance. Each sample was assayed three times and the mean used for statistical analysis. The assay solution consisted of 100 mM Tris-HCl, 0.34 mM $NADP^{+}$, 50 mM $MnCl_2$, and 50 mM malate, pH 7.4.

Total soluble protein concentration in the fly homogenates were measured by the bicinchoninic acid (BCA) assay, using a commercially available kit (Pierce, Thermo Fisher Scientific, Rockford IL), following the manufacturer's directions.

RNA extraction and quantitative RT-PCR

For each genotype, RNA was extracted from at least three groups of 15 male flies. Total RNA was isolated from flies using the RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Extracted RNA was eluted in RNAse-free water, and the purity and quantity of RNA was assessed using a Nanodrop ND-1000 spectrophotometer (A260/A280 and 260/230 > 1.8; Nanodrop Technologies, Wilmington, DE, USA). High quality isolated RNA was stored at -80°C until needed for reverse transcription. One microgram of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

Quantitative reverse-transcription PCR (qPCR) was performed using the Quantitect Probe PCR Master Mix (Qiagen) on a Mastercycler ep realplex Thermal Cycler (Eppendorf, Mississauga, ON) with the following program: 15 mins at 95°C; up to 45 repeats of 15 s at 94°C and 1min at 60°C. All analyses were performed in technical triplicate, alongside a non-template control. The expression of all genes of interest for cross-temperature experiments was quantified relative to the average expression of all samples in the experiment. For RNAi samples, expression was quantified relative to a combination of RNAi control lines with over-expression of mCherry in the vector, and background lines without a vector (see Table S1). Gene expression was normalized to two reference genes (*Actin-79B* and *Rpl32*; see Table S2 for all genes analyzed and their respective primers/probes). Both the target gene and two reference genes were amplified in the same run for each sample. Variation in threshold cycle (Ct) values of technical replicates did not exceed 0.9 cycles in any sample, and the average difference in Ct value was < 0.3 cycles per sample. Ct's were normalized using Biogazelle's qbase^{PLUS} software version 2.0 (http://www.qbaseplus.com) using calibrated normalized relative quantification, which removes inter-run variation (Hellemans *et al.* 2007).

All primers and probes were designed to amplify transcripts only. For each gene, primers were designed to sit in exon sequences flanking an intron, based on Flybase annotations (Table S2), using the PerlPrimer software (Marshall 2004). When annotation suggested differential splicing, primers and probes were designed to match exons present in all putative splice variants.

Data analysis

Multivariate analysis of variance tests were conducted to ascertain possible significant differences in MEN activity across genotypes. Sample wet weight and the protein concentration of each homogenate were used as covariates in statistical analyses of MEN activity to account for differences in fly size or degree of homogenization. Analysis of Covariance (ANCOVA's) and Tukey's Honestly Significant Difference (HSD) multiple-comparison tests were performed using JMP version 7 software (SAS Institute Inc., Cary, NC, 1989-2007). Tukey's HSD tests were also performed to determine whether gene expression was significantly different for genes of interest across various conditions/groups. Correlations between MEN activity, *Men* expression, and expression of genes of interest were performed using SigmaPlot version 11.0 software (Systat Software, Inc., San Jose California USA).

Transcription factor binding site predictions

Transcription factor binding sites (TFBS) were predicted using MatInspector with an optimized core matrix similarity of 0.90 (Cartharius *et al.* 2005). *MenEx58*$^{-}$ and *MenEx60*$^{-}$ were examined with the insertion sequences (*P*-element remnants) included in the analysis. TFBS were considered for analysis if found within highly conserved regions as previously predicted by phylogenetic footprinting (Lum and Merritt 2011).

2.3 RESULTS

Trans-interactions at *Men* are pairing dependent (transvection)

To test whether the up-regulation of MEN activity through *trans*-interactions at the *Men* locus (Lum and Merritt 2011) is pairing-dependent (*i.e.* transvection), we challenged flies with genomic architecture that we expected to modify the ability of homologous chromosomes to pair, by creating *Men* deletion heterozygotes with either an intact *P*-element or either of a pair of chromosomal inversions.

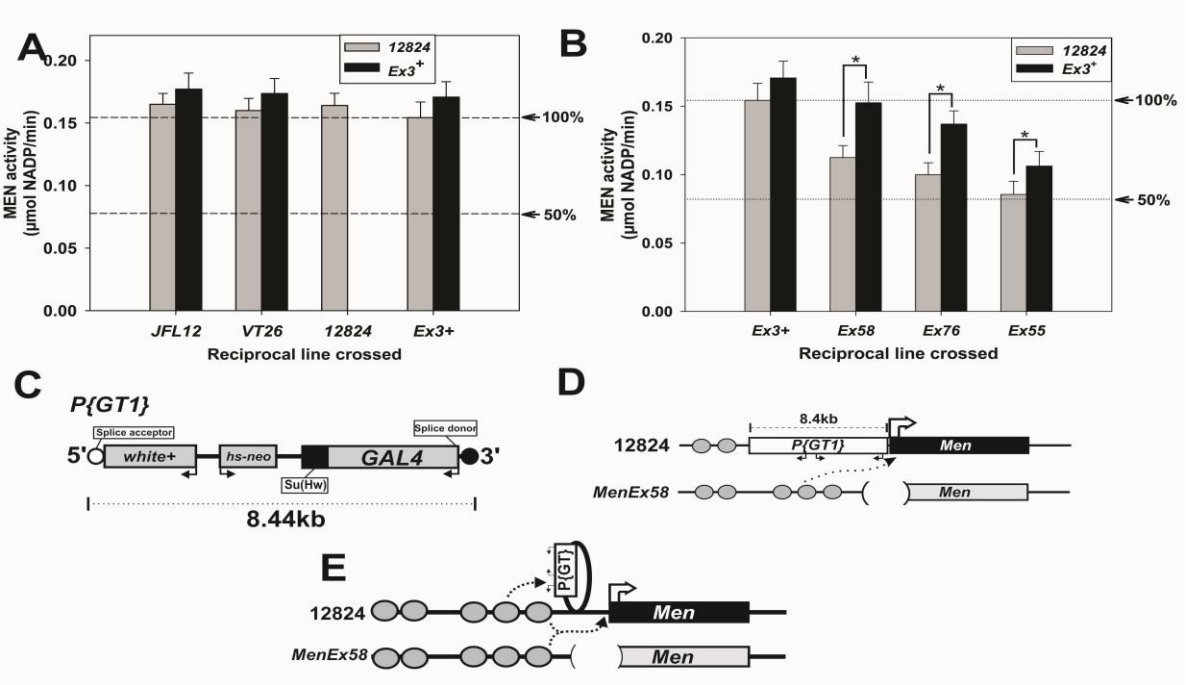

Figure 2 An 8.44kb *P*-element *P{GT1}* can significantly reduce *trans*-interactions at the *Men* locus. (A) MEN activity in flies heterozygous for two wildtype 3rd chromosomes (*JFL12* and *VT26*) over either *12824* (intact *P*-element line containing *P{GT1}*, grey bars) or *MenEx3*$^{+}$ (black bars). (B) Heterozygote MEN activity of *MenExi*$^{-}$ alleles over either *P{GT1}* or *MenEx3*$^{+}$. Asterisks indicate significantly different levels of MEN activity between groups (Tukey's *post hoc* $P < 0.05$). (C) Details of *P{GT1}* transposon (redrawn from Bellen *et al*. 2004). Arrows signify promoters for the genes within the transposon. (D) Model of gene regulation at *Men* with one functional *P{GT1}* intact allele (12824) and one knockout *Men* allele, pairing is precluded by the shift of genomic architecture caused by the *P{GT1}* element insertion, inhibiting interactions in *trans*. (E) Model of gene regulation at *Men* as in (D), here, the topology model can account for interactions in *trans* in the smaller *Men* knockout alleles (*MenEx58*$^{-}$).

***P*-element heterozygotes**: Heterozygote flies containing either a *MenExi*$^{-}$ or *MenEx3*$^{+}$ allele, and a functional *Men* allele that included an intact *P*-element (Figure 2A) were created: we reasoned

that the presence of this ~8.44 kb length of DNA would disrupt local chromosome pairing. It has been suggested that pairing disruption may be achieved by simply increasing the distance between the promoter and the *cis*-linked enhancer, thus decreasing the amount of interaction between promoter and the enhancer in *trans* (Bateman *et al.* 2012a; Kwon *et al.* 2009). The *12824 P*-element line, and not the *EP517* line that the excision alleles were derived from, was used in these crosses because the *12824 P*-element, *P{GT1},* contains no internal promoters that could modify local gene expression and possibly confound our results (Figure 2C). To account for possible differences in MEN activity between chromosomes that have been previously described (Merritt *et al.* 2005; Lum and Merritt 2011; Rzezniczak and Merritt 2012), we defined the activity of *MenEx3*$^{+}$*/P{GT1}* heterozygotes as 100%, or normal, MEN activity. We tested for chromosome-specific differences in MEN activity by crossing two wild-type 3rd chromosome lines (*JFL12* and *VT26*) to the intact *P*-element line, *P{GT1}*, and the wildtype *MenEx3*$^{+}$ line. MEN activity was slightly (*i.e.* <10%), but not significantly, lower in *P{GT1}* heterozygotes (first two grey bars; Figure 2A) than *MenEx3*$^{+}$ heterozygotes with the same isothird chromosomes (first two black bars; Figure 2A). Similarly, *MenEx3*$^{+}$*/P{GT1}* heterozygotes had slightly, but not significantly, lower MEN activity than *MenEx3*$^{+}$ homozygotes. Combined, these results suggest that the *P{GT1}* line does have slightly lower MEN activity than the *EP517* line, but that these differences are not statistically significant.

Next, we crossed the *P{GT1}* line (grey bars) and the *MenEx3*$^{+}$ line (black bars) to three knockout *EP517* excision lines (*MenEx58*$^{-}$, *Ex76*$^{-}$, and *Ex55*$^{-}$). Based simply on the number of functional alleles, *MenExi*$^{-}$*/MenEx3*$^{+}$ heterozygotes are expected to have 50% activity. However, consistent with previous results (Merritt *et al.* 2005, Lum and Merritt 2011, Rzezniczak and Merritt 2012), all *MenExi*$^{-}$*/MenEx3*$^{+}$ heterozygotes have significantly higher MEN activity than the expected 50% (Figure 2B; these are the previously reported *trans*-interactions). Strikingly, MEN activity levels were significantly lower in the *MenExi*$^{-}$*/P{GT1}* heterozygotes (grey bars) than in the *MenExi*$^{-}$/*MenEx3*$^{+}$ heterozygotes (black bars; Figure 2B), consistent with our expectations of pairing disruption by the intact *P*-element in the 12824 line. *MenEx76*$^{-}$*/P{GT1}* and *MenEx55*$^{-}$*/P{GT1}* heterozygotes were not significantly different from the expected 50% activity, *i.e.*, they showed no up-regulation through *trans*-interactions. These results suggest that the ~8.44kb *P*-element significant reduced *trans*-interactions through disruption of pairing (Figure 2D), and that the trans-interactions are, in fact, transvection. Interestingly, although

MEN activity for *MenEx58*$^{-}$/*P{GT1}* heterozygotes was significantly lower than *MenEx58*$^{-}$/*MenEx3*$^{+}$ heterozygotes (*i.e.* 26% lower), the activity was still higher than the expected 50% wildtype MEN activity. In other words, the element reduces, but does not eliminate, transvection at this locus.

Inversion crosses: We also created heterozygote flies that we expected to have reduced or non-existent homologous chromosome pairing, by crossing either a *MenExi*$^{-}$ or the *MenEx3*$^{+}$ allele to inversion chromosomes with break points either proximal or distal to *Men* (Figure 3). As above, we reasoned that these chromosomal anomalies could disrupt pairing between homologous chromosomes. This second set of crosses is similar to the "standard" tests of transvection effects (Lewis 1954; reviewed by Duncan 2002). Figure 3A shows the expected amount of *trans*-interactions, when *MenExi*$^{-}$ alleles are heterozygous to *MenEx3*$^{+}$. For comparison of relative MEN activity, we denoted *MenEx3*$^{+}$/*In(i)* as 100% activity for each set of crosses (Figure 3B,C). In these experiments, the meaningful comparisons are all between heterozygotes containing the same chromosomes, the suite of excision alleles (all derived from the same chromosome) and one of the two inversion chromosomes, so chromosomal differences in MEN activity are not an issue (*e.g.*, we never compare across heterozygotes of different inversions).

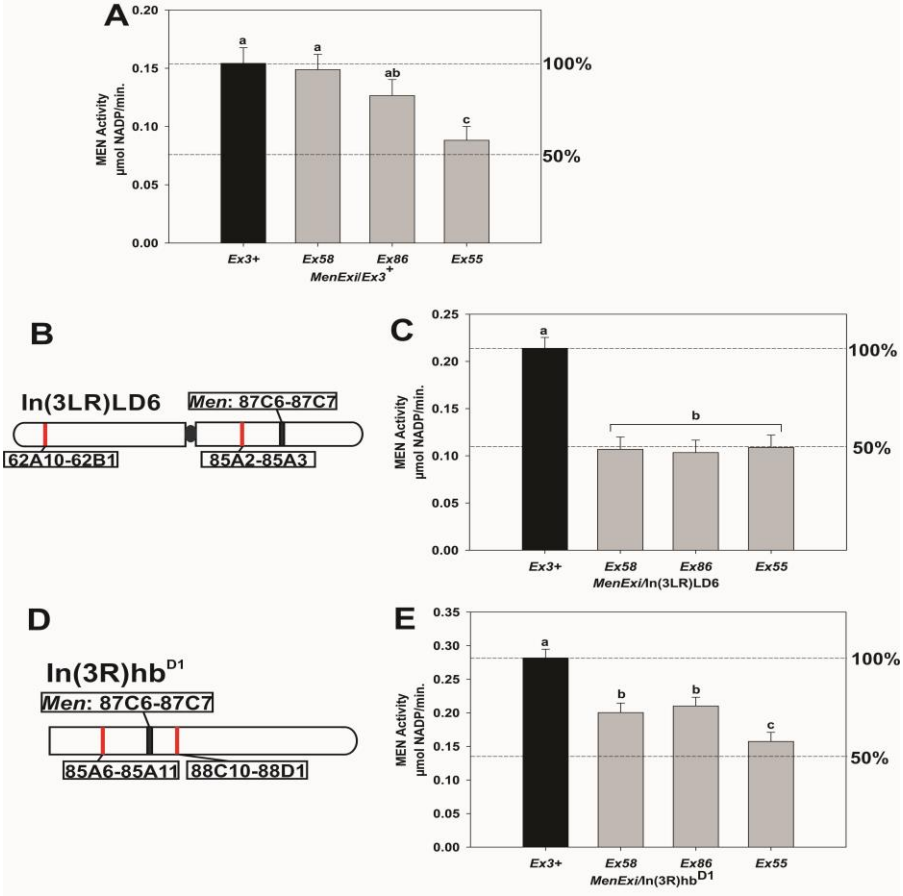

Figure 3 The *trans*-interactions at the *Men* locus are pairing-dependent; *i.e.* transvection. MEN activity in flies heterozygous for *MenExi*$^{-}$ alleles and either *MenEx3*$^{+}$ or inversion chromosomes. (A) MEN activity for *MenEx3*$^{+}$/ *MenEx3*$^{+}$ homozygotes and *MenEx3*$^{+}$ heterozygous with three *MenExi*$^{-}$ alleles. There are significant differences in MEN activity between columns with different letters ($F_{3,15}$ = 19.486, $P < 0.001$; Tukey's *post hoc* $P < 0.05$). (B) Cartoon of the third chromosome containing the *In(3LR)LD6* inversion. Red lines signify chromosomal locations of inversion breakpoints. (C) MEN activity for *MenExi*$^{-}$*/In(3LR)LD6* heterozygotes. There are significant differences in MEN activity between columns with different letters ($F_{3,16}$ = 31.210, $P < 0.001$; Tukey's *post hoc* $P < 0.05$). (D) Cartoon of the third right arm of containing the *In(3R)hb*^{D1} inversion.(E) MEN activity for *MenExi*$^{-}$ /*In (3R)hb*^{D} heterozygotes. There are significant differences in MEN activity between columns with different letters ($F_{3,15}$ = 24.313, $P < 0.001$; Tukey's *post hoc* $P < 0.05$).

The first chromosomal inversion (*In(3LR)LD6*) is an inversion involving the centromere, with the distal breakpoint 62A10-62B1 on 3L, and proximal breakpoint 85A2-85A3 on 3R between the *Men* locus (87C6-87C7) and the centromere (Figure 3D). All *MenExi*$^{-}$*/In(3LR)LD6* heterozygotes had essentially half of the MEN activity of the *MenEx3*$^{+}$*/In(3LR)LD6* heterozygotes, indicating complete loss of the *trans*-driven up-regulation of the *Men* locus (Figure 3B). These results indicate that the *trans*-interactions at the *Men* locus are transvection as

classically defined. The second inversion (*In(3R)hb^{D1}*) is smaller, with both breakpoints on 3R, 85A6-85A11 and 88C10-88D1, flanking the *Men* locus (Figure 3E). While the mechanism underlying somatic chromosomal pairing is still a topic of debate, inversions not involving the centromere usually do not disrupt transvection (Duncan 2002), and we expected that heterozygotes containing these inversions would not display significant reductions in relative MEN activity. *MenEx58^{-}/In(3R)hb^{D1}* and *MenEx86^{-}/In(3R)hd^{D1}* heterozygotes showed significantly greater than 50% activity, *i.e.*, *trans*-driven up-regulation, while *MenEx55^{-} /In(3R)hb^{D1}* did not (Figure 3C), consistent with earlier studies of these alleles (*e.g.*, Figure 3A). Interestingly, *MenEx58^{-}/In(3R)hb^{D1}* heterozygotes had lower relative MEN activity, *i.e.*, less up-regulation, than *MenEx58^{-}/MenEx3^{+}*, suggesting that *trans*-interactions are reduced in these *In(3R)hb^{D1}* heterozygotes. These results – apparently fine scale modulation of transvection – highlight the sensitivity of our system in dissecting the molecular architecture of *trans*-interactions.

In summary, we found reduced *trans*-interactions in both the intact *P*-element and inversion heterozygotes, indicating that the *trans*-interactions at *Men* are indeed pairing dependent, *i.e.*, they are transvection.

Transvection at the *Men* locus is sensitive to environmental conditions

To test whether transvection at this locus is sensitive to changes in the environment, we placed sets of adult *MenExi^{-}* heterozygotes at different temperatures and measured MEN activity. In previous work from our laboratory, we crossed six excision alleles to five genetic backgrounds, and it was found that *trans*-effects at *Men* were sensitive to both the excision allele and 3rd chromosome genetic background (Lum and Merritt 2011). Here, we set up these crosses with a slightly different set of excision alleles, and exposed the F_1 heterozygote males to one of two experimental temperatures, 21°C or 29°C, maintaining a control group at 25°C.We limited temperature shifts to adult flies (*i.e.*, instead of rearing flies at three temperatures) to reduce rearing effects that could lead to differences in MEN activity or overall metabolism, possibly confounding our results.

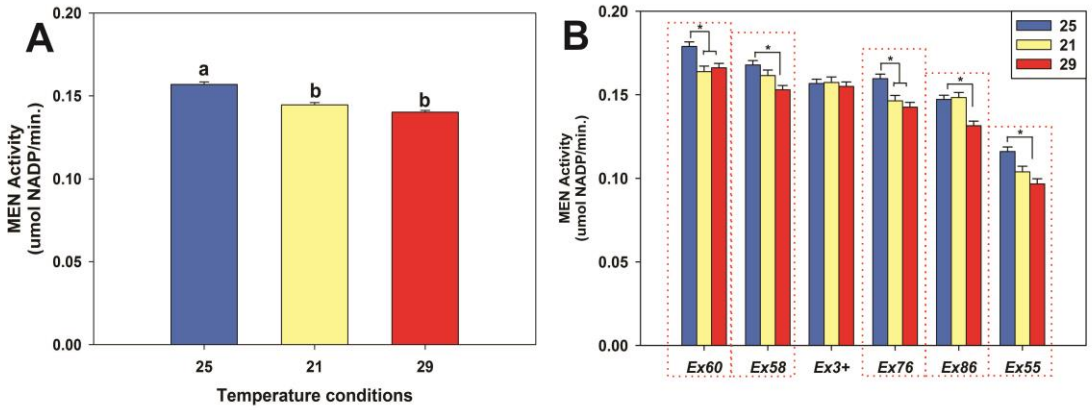

Figure 4 Change in environment (temperature) reduces transvection at *Men.* (A) MEN activity of all genotypes, all *MenExi* alleles across five genetic backgrounds, at each temperature condition: 25°C (control; blue bars), 21°C (yellow bars), 29°C (red bars). Both 21°C and 29°C groups are significantly lower than 25°C, see text for exact *P* values. (B) MEN activity at 25°C, 21°C and 29°C of *MenExi*$^{-}$ allele heterozygotes across five genetic backgrounds. Asterisks indicate groups that were significantly different according to Tukey's HSD test ($F_{10,2288} = 1.775$, $P < 0.001$, Tukey's *post hoc* $P < 0.05$).

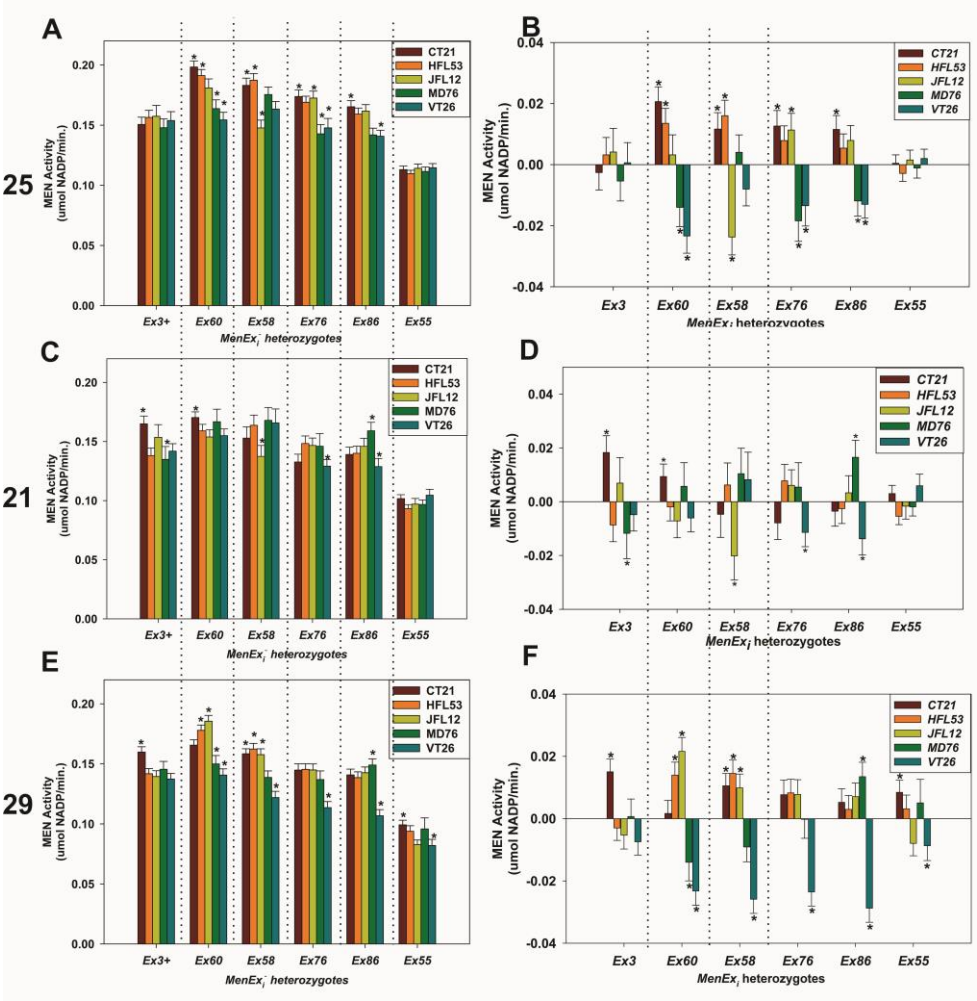

Figure 5 Genetic background significantly impacts transvection at *Men*. MEN activity of *MenExi*$^{-}$/isothird chromosome heterozygotes at (A) 25°C, (C) 21°C, and (E) 29°C. Within each excision group, activities are standardized by both average excision allele and third chromosome activities, yielding significant interactions between excision alleles and genetic background at (B) 25°C, (D) 21°C, (F) and 29°C. Asterisks indicate lines that are significantly different from the standardized average at a 0.95 threshold (according to a T-test).

Overall in the *MenExi*$^{-}$*/Men*$^{+}$ heterozygotes, transvection was significantly lower in flies moved to either experimental temperatures ($F_{2,2288}$ = 46.998, P < 0.0001; Figure 4A). Across all three temperatures, transvection was highest in *MenEx58*$^{-}$ and *MenEx60*$^{-}$ heterozygotes (the smallest deletions), and their MEN activity was significantly higher than *MenEx3+* heterozygotes at any temperature (Figure 4B). Transvection was lowest in *MenEx55*$^{-}$ heterozygotes (the largest deletion; Figure 4B), consistent with earlier results from these deletions (Lum and Merritt 2011).

MenEx55^{-} is missing the majority of the 5' intergenic region (and presumably most of the regulatory elements). The initial study by Lum and Merritt (2011) found no transvection in *MenEx55*$^{-}$ heterozygotes, while a later study (Rzezniczak and Merritt 2012) found small amounts of transvection. We found that the *MenEx55*$^{-}$ heterozygotes did show low levels of transvection; MEN activity was low, but was significantly higher than 50% of wild-type activity. Overall, *MenEx55*$^{-}$ does drive some transvection effects, although at a much lower level than any other alleles in our study. We are currently creating more excision alleles in an attempt to further examine the impact of similar, but distinct, large-scale deletions.

In contrast, exposure temperature did not impact MEN activity in the *Men*$^{+}$*/Men*$^{+}$ flies. MEN activity in heterozygotes of the wild-type excision line *MenEx3*$^{+}$ (*i.e.*, *Men*$^{+}$*/Men*$^{+}$ flies, with intact promoters at both loci) is likely predominantly regulated by *cis*-interactions, through "*cis*-preference" (Geyer *et al.* 1990; Figure 1D). Interestingly, MEN activity in the *MenEx3*$^{+}$ heterozygotes was unchanged by the temperature shifts (Figure 4B, centre columns). This lack of response suggests that, in contrast to the *trans*-interactions, *cis*-regulatory interactions are unaffected by the change in environmental temperature.

The amount of transvection was sensitive to both the excision allele and genetic background (Figure 5), consistent with earlier work (Lum and Merritt 2011). Figure 5A shows the activity of all heterozygote flies grouped by excision maintained at 25°C. Differences between excision alleles were both statistically significant ($F_{5,711} = 39.312$, $P < 0.001$) and immediately apparent; for example, *MenEx55*$^{-}$ heterozygotes have lower MEN activity than heterozygotes from any other excision. Genetic background effects on *MenExi*$^{-}$ heterozygotes were also significant under control conditions ($F_{4,711} = 32.827$, $P < 0.001$), although less obvious. We also found significant interaction effects between excision alleles and genetic backgrounds (Figure 5B; $F_{20,711} = 2.994$, $P < 0.001$), that is to say that, an excision allele could drive different amounts of transvection in two different backgrounds. To visualize these interactions, we followed Lum and Merritt (2011) and standardized all crosses by both excision allele and background, and looked for statistical outliers (Figure 5B). After standardization, MEN activity in samples that show no interactions will not be significantly different from zero (*i.e.*, the average MEN activity of that excision allele by background group). All *MenExi*$^{-}$ alleles except *MenEx55*$^{-}$ had significant interactions with at least one genetic background (Figure 5B). Importantly, *MenEx3*$^{+}$ showed no deviation across the

five backgrounds at 25°C (Figure 5A). These results are consistent with previous findings (Lum and Merritt 2011), highlighting the reproducibility of our assay across experiments.

Strikingly, we found that changes in temperature significantly impacted the excision by background effects on transvection (Figure 5). Comparison of Figure 5A-B with Figure 5C-D and E-F shows the effects of changes in temperature on these interaction effects. We found significant interactions in both experimental conditions (21°C: $F_{20,775} = 3.456$, $P < 0.001$; 29°C: $F_{20,800} = 3.444$, $P < 0.001$), and the interactions were visibly different across the three temperatures (Figure 5B,D,F). Overall, the number and magnitude of significant *MenExi*$^{-}$ allele by background interactions appear lower in flies moved to 21°C than those moved to 29°C or kept at 25°C. There were marginally fewer significant interactions, 7 in the 21°C flies (Figure 5D) and 14 in either the 29°C or 25°C flies (Figure 5B, F; Fisher's Exact Test $P = 0.054$). The absolute magnitude of the interactions (*i.e.*, standard deviation of excision by background interactions) was also slightly smaller in the 21°C flies (0.029 µmol $NADP^+$/min) than the 25°C and 29°C (0.033 µmol $NADP^+$/min and 0.040 µmol $NADP^+$/min, respectively). In addition, significant background effects were detected in flies exposed to 29°C ($F_{4,800} = 26.967$, $P < 0.001$) and 25°C (above), but not in flies exposed to 21°C. Overall, exposing flies to a 21°C ambient temperature seems to have reduced transvection and the variability in transvection across backgrounds and excision alleles.

Changes in temperature also resulted in significant background by excision interactions for both *MenEx3*$^{+}$ and *MenEx55*$^{-}$, two alleles that we expected, for different reasons, to have minimal *trans*-effects. While *MenEx55*$^{-}$, the largest deletion, showed minimal transvection overall (Figure 5), significant interactions were detected in flies exposed to 29°C (Figure 5E,F). Interestingly, we detected a significant impact of background on the wild-type allele *MenEx3*$^{+}$ under experimental, but not control, conditions. *MenEx3*$^{+}$ is a perfect excision and its expression is expected to be predominantly driven by *cis*-interactions (Geyer *et al.* 1990). The identified significant interactions between *MenEx3*$^{+}$ and background at 21°C and 29°C suggest that changes in environment, here temperature, may expose underlying differences not apparent under more constant or benign conditions.

Altogether, our results suggest that transvection is sensitive to local (excision site) and distant or regional (third chromosome background) genetic effects, the interactions between these two genetic effects, and to changes in environmental temperature.

qPCR reveals correlation between *Men*, *Abd-B*, and *Mirror* expression

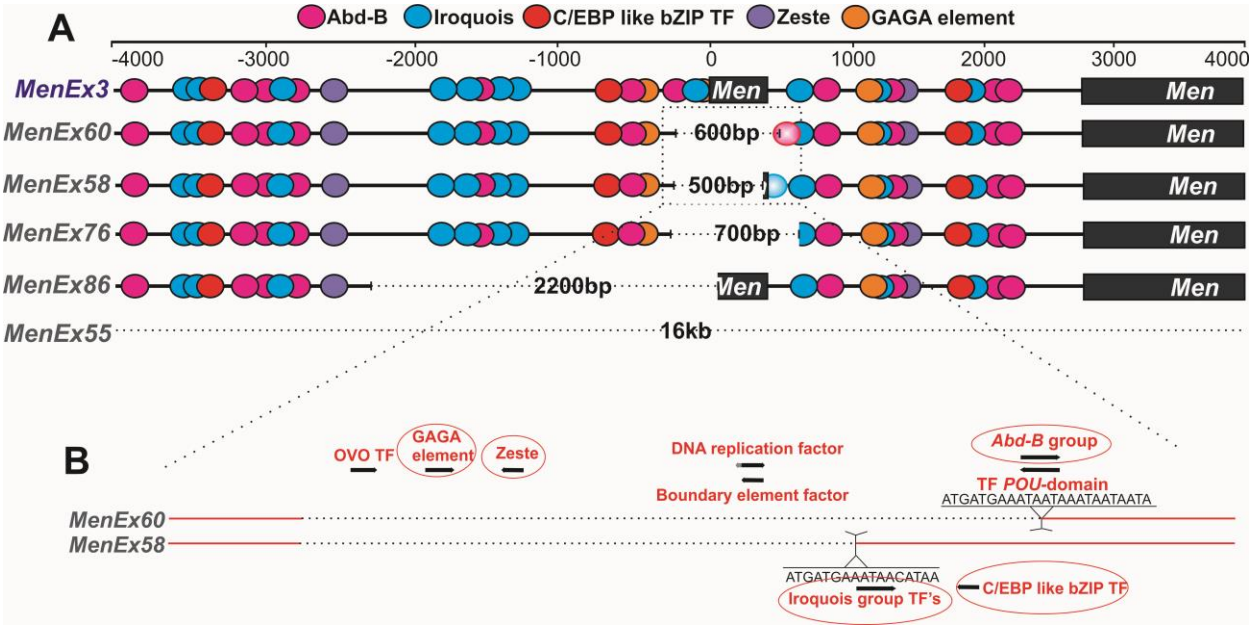

Figure 6 Putative transcription factor binding sites (TFBS) that might participate in gene regulation and transvection at the *Men* locus. (A) Colored circles indicate putative TFBS for the five genes we have analyzed in our study: Abd-B, Iroquois, C/EBP like bZIP TF, GAGA element, and Zeste. For each *MenExi*$^{-}$ allele, we indicate the excised region with a bracket. Faded circles represent TFBS unique to an excision allele (in *MenEx58*$^{-}$ and *MenEx60*$^{-}$). (B) Details of the excision site of *MenEx58*$^{-}$ and *MenEx60*$^{-}$, two alleles which differ in deletion size by ~100bp, and significantly differ in MEN activity. Each has a unique insertion at the excision site adding either an additional Iroquois, *MenEx58*$^{-}$, or Abd-B TFBS, *MenEx60*$^{-}$. TFBS circled in red correspond to TF genes analyzed with qPCR.

We wanted to determine if differences in transvection observed across excision alleles, genetic backgrounds, and ambient temperatures, could be explained by the presence or absence of TFBS (local) and differences in the activity of TFs (distant). A number of predicted TFBS near the *Men* TSS are either deleted, retained, or inserted in various *MenExi*$^{-}$ alleles, and differences in these regulatory elements may contribute to the observed variation in *trans* activity (Figure 6A; Lum and Merritt 2011). We focused on two excision alleles, *MenEx60*$^{-}$ and *MenEx58*$^{-}$, which differ

significantly in their ability to drive transvection (Figure 1; Lum and Merritt 2011), but only differ by approximately 100 bp in excision size. *MenEx60*$^{-}$ is also of particular interest because it can drive higher than 100% wild-type MEN activity (*i.e.*, >50% up-regulation). Figure 6B illustrates the excision sites of these two alleles, indicating TFBS with high matrix similarity (> 0.90, *i.e.*, confidently predicted TFBS). Of these TFBS, two sites are only found in *MenEx58*$^{-}$, but not *MenEx60*$^{-}$: C/EBP like bZIP and Iroquois, bound by *slowbordercells* (*slbo*) and *mirror* (*mirr*), respectively. One TFBS is found only in *MenEx60*$^{-}$, but not *MenEx58*$^{-}$: Abd-B, which is bound by *Abdominal-B* (*Abd-B*). The Iroquois and Abd-B binding sites may result from insertions created during the *P*-element excision event or are possibly remnants of the *P*-element itself. Because *P*-element remnants have been shown to impact transvection at other loci (*y*, Geyer *et al.* 1990; *Gpdh*, Gibson *et al.* 1999), we reasoned that these distinct sites could lead to differential transvection ability of the two alleles, or differential responses of the two alleles to levels of the respective binding proteins. Finally, two TFBS are deleted in both *MenEx60*$^{-}$ and *MenEx58*$^{-}$: GAGA element and Zeste, bound by *Trithorax-like* (*Trl*) and *zeste* (*z*), respectively. Given the differences in C/EBP like bZIP, Iroquois, and Abd-B, but not GAGA and Zeste binding sites between *MenEx60*$^{-}$ and *MenEx58*$^{-}$, we predicted that, if these binding sites are functional, the observed differences in transvection between the two alleles would correlate with differences in *slbo*, *mirr*, or *Abd-B* expression, but not *Trl* or *z* expression, across the genetic backgrounds or temperatures. To test for such a relationship, we quantified relative *Men* expression and the relative expression for five TFs that bind to the aforementioned predicted TFBS, in *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygote flies, across the same five genetic backgrounds and three temperature conditions as the previous set of experiments.

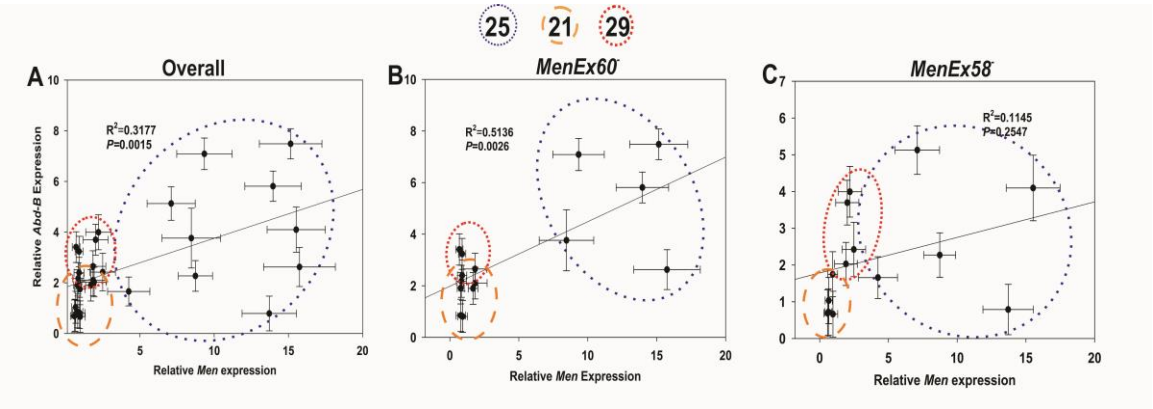

Figure 7 Correlation between *Abd-B* and *Men* expression. *Abd-B* vs. *Men* expression in (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone. Each data point represents expression in a line with a *MenExi*$^{-}$ allele heterozygous with one genetic background at a single temperature condition (*e.g.*, *MenEx58*$^{-}$*/CT21* at 25°C). Significance of correlation in expression of the two genes was determined by SigmaPlot.

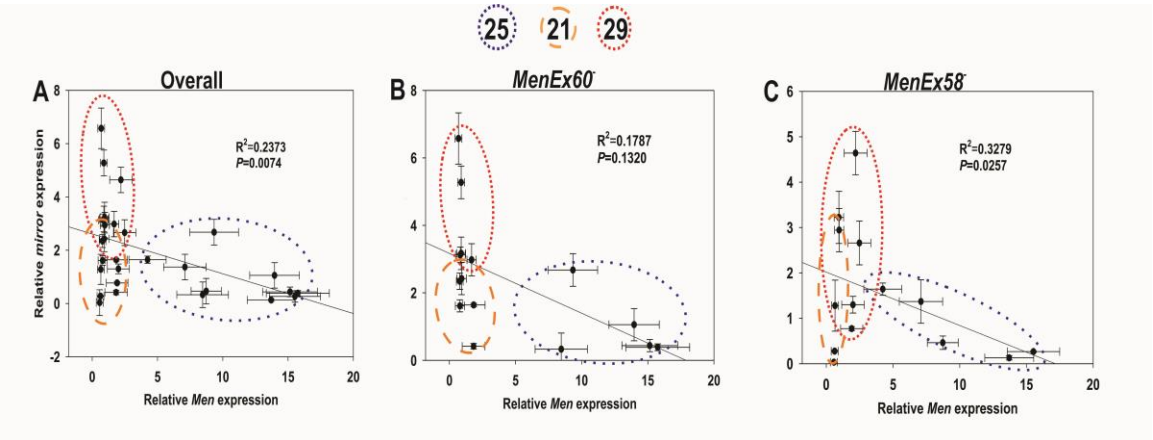

Figure 8 Correlation between *mirr* and *Men* expression. *mirr* vs. *Men* expression in (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone. Each data point represents expression in a line with a *MenExi*$^{-}$ allele heterozygous with one genetic background at a single temperature condition (*e.g.*, *MenEx58*$^{-}$*/CT21* at 25°C).

We found significant correlations between the expression of *Men* and two of the TFs analyzed – *Abd-B* and *mirr* (Figures 7,8). There was a significant temperature effect on the expression of all three genes: *Men* ($F_{2,89}$ = 89.280, $P < 0.0001$), *Abd-B* ($F_{2,89}$ = 22.350, $P < 0.0001$), and *mirr* ($F_{2,89}$ = 46.322, $P < 0.0001$). *Men* and *Abd-B* expression were up-regulated at 25°C, and were expressed at low levels at the other two temperatures (Figure 9), while *mirr* expression was up-

regulated at 29°C (Figure 9). The higher *Men* expression at 25°C, and lower expression at 21°C and 29°C, is consistent with changes in MEN activity observed in the previous experiment (Figure 4). There was also a significant background effect on *Abd-B* expression ($F_{4,89}$=21.624, *P*=0.011), *i.e., Abd-B* expression varied across genetic background, but there was no significant background effect on the expression of any other gene analyzed. Although not statistically significant, there was a trend of background effects being higher for *Men, mirr,* and *Trl*, genes residing on the third chromosome, than *z* and *slbo* (data not shown), consistent with our expectations. No significant excision effects on expression of the TF genes were found. Overall, the correlations between *Abd-B, mirr,* and *Men* appear to be driven by differences across the three temperatures (Figure 7A, 8A). *Abd-B* expression showed a strong positive correlation with *Men* expression, driven by high *Abd-B* and *Men* expression at 25°C (Figure 7A). Strikingly, although the trend is apparent in both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, it is only statistically significant in the *MenEx60*$^{-}$ heterozygotes (Figure 7B,C). In addition, while overall sensitivity of *Men* to *Abd-B* (slope of linear regression) was weak, it was much stronger in *MenEx60-* heterozygotes alone (Table S3). It is interesting to note that the *MenEx60*$^{-}$ allele has one more Abd-B binding site than *MenEx58*$^{-}$ (Figure 6B). Similarly, overall relative *mirr* expression had a strong negative correlation with *Men* expression, driven by high *Men*, and low *mirr*, expression at 25°C (Figure 8A). Again, this pattern is apparent in both sets of flies, but only statistically significant in *MenEx58*$^{-}$ heterozygotes (Figure 8B,C). Interestingly, *MenEx58*$^{-}$ alleles have one more Iroquois site, the binding site for the Mirror protein, than *MenEx60*$^{-}$ alleles (Figure 6B). These correlations suggest that temperature specific differences in *Abd-B* and *mirr* expression may be driving the observed differences in transvection between these two alleles, through binding of their respectively distinct TFBS, and thus activation of *Men* expression in *trans.* The expression of the other three TFs analyzed in this study showed no significant correlations with *Men* expression, when the data was pooled from either both excision alleles, or when the excision alleles were tested separately (Figure S1).

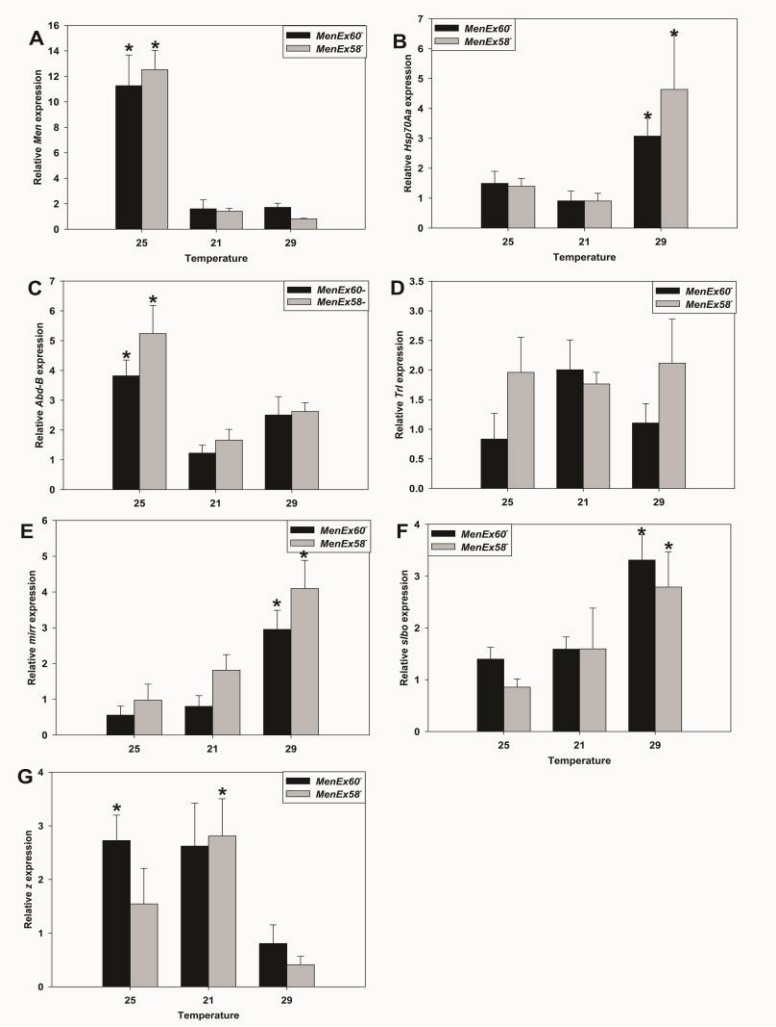

Figure 9 Average relative expression of transcription factors across temperatures. Relative gene expression of heterozygotes of each excision allele over five genetic backgrounds, of (A) *Men*, (B) *Hsp70Aa*, (C) *Abd-B*, (D) *Trl*, (E) *mirr*, (F) *slbo*, and (G) *z*. Asterisks indicate groups that were significantly different from other excision-temperature groups according to Tukey's *post hoc* ($P < 0.05$). See text for description of data analysis.

In short, differences in *Abd-B* and *mirr* expression were correlated with differences in expression of, *i.e.* transvection at, the *Men* locus, and the correlation appears to be driven by the presence of an additional binding site for each TF within their respective excision alleles. These results suggest that the additional binding sites for both Abd-B and mirr are functional in *MenEx60*$^{-}$ and *MenEx58*$^{-}$, respectively, and the two TFs may play a role in driving transvection at *Men.*

Anatomical correlations between *Men*, *Abd-B* and *mirr* expression

If differences in Abd-B and mirr binding are driving the observed differences in transvection observed between *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, then we expect that their observed correlations with *Men* expression should be more robust in tissues where their respective gene

expression levels are highest. Both Abd-B and mirr are homeodomain factors, and their expression in adult flies are relatively higher in the abdomen than the head/thorax (Chintapalli *et al.* 2007). To test whether the correlation of *Men, Abd-B* and *mirr* expression is more robust in the abdomen, we repeated the previous experiment, this time measuring *Men*, *Abd-B* and *mirr* expression in the abdomen and the head/thorax.

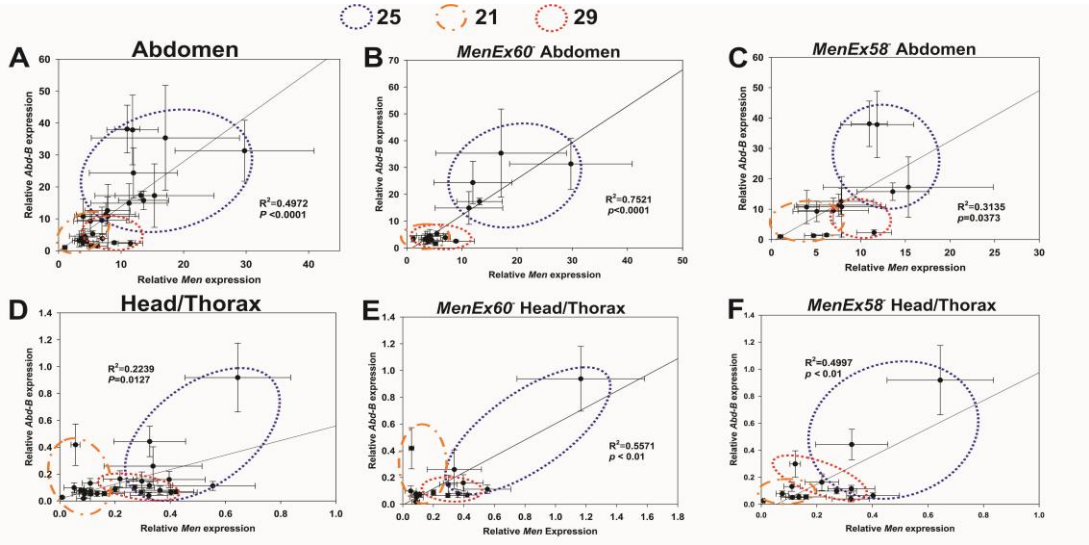

Figure 10 **Tissue-specific correlations between *Abd-B* and *Men* expression.** *Abd-B* and *Men* expression in the abdomen of (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone; in the head/thorax of (D) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (E) in heterozygotes of *MenEx60*$^{-}$ alone, and (F) in heterozygotes of *MenEx58*$^{-}$ alone.

As expected, we found tissue- and excision-specific correlations of expression between *Men* and *Abd-B* (Figure 10). Consistent with results from the whole fly, there was a significant temperature effect on the expression of both the *Men* ($F_{2,189}$ = 11.653, $P < 0.0001$) and *Abd-B* ($F_{2,189}$ = 3.512, $P < 0.05$): *Abd-B* and *Men* showed significantly higher relative expression in control conditions than in flies exposed to 21°C and 29°C. Importantly, while the correlation between *Abd-B* and *Men* expression was significant in the abdomen overall (Figure 10A), the correlation was much higher in *MenEx60*$^{-}$ heterozygotes (Figure 10B) than in *MenEx58*$^{-}$ heterozygotes (Figure 10C). Consistent with the whole fly, the correlation of the two genes appears to be driven by differences in expression across the temperature conditions. We also found a similar pattern of correlation in the head/thorax (Figure 10D,E,F). Note that relative expression of *Abd-B* and *Men* were much higher in the abdomen than head/thorax of the excision

heterozygotes (compare axes of Figure 10A&D). Sensitivity of *Men* to *Abd-B* expression was also much higher in the abdomen than in head/thorax (Table S3).

In contrast, we found that *mirr* expression patterns were drastically different in the two regions of the adult fly, and failed to replicate the correlations we saw in the intact fly (Figure S2). We found no significant effects of temperature or background on *mirr* expression, although *mirr* expression was lower in control flies and higher in flies transferred to 21°C and 29°C, consistent with our results from whole flies. However, contrary to whole fly samples, *mirr* expression showed a significant positive correlation with *Men* expression in the head/thorax, but not in the abdomen, across all genotypes (Figure S2A,D). While this positive relationship between *mirr* and *Men* expression was observed in head/thorax samples of both $MenEx60^{-}$ and $MenEx58^{-}$ heterozygotes, the correlation was stronger in $MenEx58^{-}$ heterozygotes (Figure S2D-F). There was no correlation between relative *mirr* and *Men* expression in the abdomen of both $MenEx60^{-}$ and $MenEx58^{-}$ heterozygotes (Figure S2A-C). Because relative expression of *mirr* and *Men* was much higher in the abdomen than head/thorax, we are confident in the lack of relationship in expression of the two genes in the abdomen. These observations are contrary to our findings from the previous section, and suggest that relationships between *mirr* and *Men* may not be robust.

Altogether, *Abd-B* and *Men* expression was correlated in both the abdomen and head/thorax of transvection heterozygote flies, although the relationship was stronger in $MenEx60^{-}$ than $MenEx58^{-}$ heterozygotes, in both the abdomen and head/thorax. These findings are consistent with our results from the previous section, suggesting that *Abd-B* may indeed regulate transvection at $MenEx60^{-}$. On the contrary, the relationship between *mirr* and *Men* expression was inconclusive upon tissue-specific analysis.

RNAi knockdown of *Abd-B* reduces MEN activity

The predicted TFBS described above are present in multiple copies within highly conserved regions across the *Men* locus (Figure 6A) and we suspected that TFs bound to these sites may regulate *Men* expression in *cis*, in addition to any role in *trans*-regulation. We used RNAi to test if differences in TF expression could drive changes in *Men* expression and MEN activity. We obtained RNAi constructs targeting three TF genes, *Abd-B*, *mirr*, and *slbo*, in order to drive

significant reductions in abundance of their respective mRNA levels (Figure 11A), and tested Men expression and MEN activity in these constructs. *Abd-B* and *mirr* were selected because of their correlations with transvection at *Men* described above, and *slbo* because no correlations were found in *trans*. MEN activity was significantly reduced only in flies that had *Abd-B* expression knocked down (Figure 11B), although we also observed a slight (~10%) but not statistically significant, reduction in MEN activity in the flies with reduced *mirr* expression. The reduction of MEN activity in *Abd-B* knock-down flies was reflected by a corresponding reduction in *Men* expression (Figure 11C). Overall, differences in *Men* expression across flies with the three TFs knocked down were not significant, although the trend was similar to that of MEN activity. Combined, these results suggest that Abd-B may regulate *Men* expression from binding sites within the local regulatory region in *cis*, but not *slbo* and *mirr*. Along with the tissue-specific correlation between relative *Abd-B* and *Men* expression in *trans*, our results suggest that *Abd-B* may play a role in the regulation of *Men* expression both in *cis* and in *trans*.

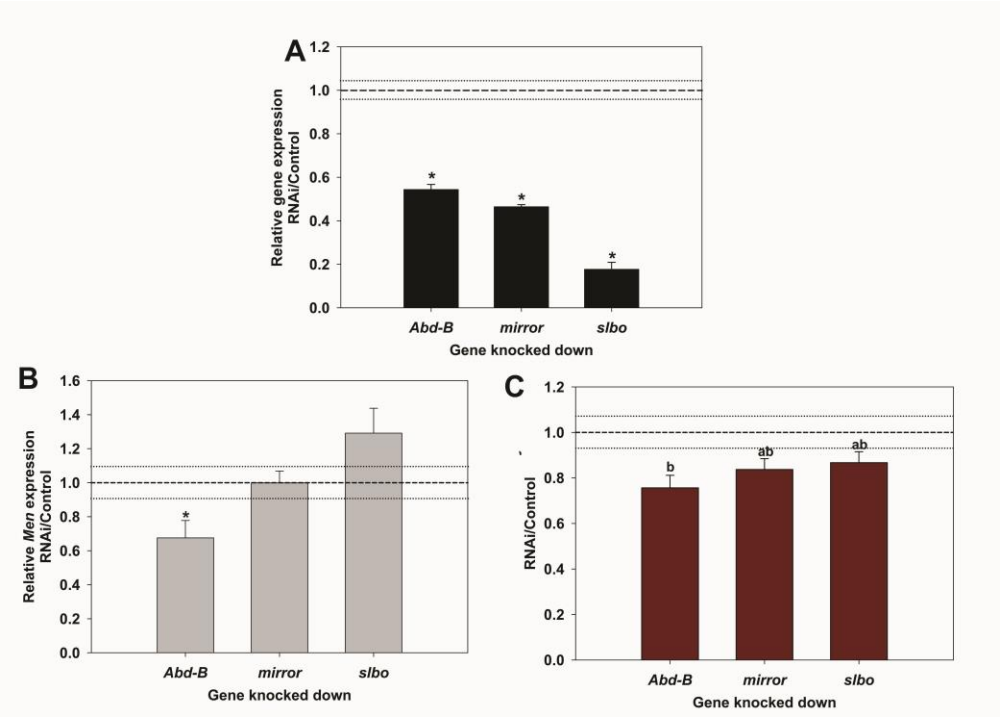

Figure 11 **Effect of RNAi knockdown of transcription factors on *Men* in *cis*.** (A) RNAi resulted in significant knockdown of expression for all three genes analyzed. (B) MEN activity in *Abd-B*, *mirr*, and *slbo* knocked down lines, relative to control lines. There are significant differences in MEN activity between columns with different letters ($F_{3,24} = 10.0164$, $P < 0.0001$, Tukey's $P < 0.05$). (C) Relative *Men* expression in the same TF knocked down lines. In all panels, mean expression/activity of the control lines is indicated by the dotted line with the flanking faint lines indicating standard error.

2.4 DISCUSSION

Transvection is a variable and plastic phenotype, subject to modification by the environment and complex interactions between local genomic architecture and elements coded elsewhere in the genome. Here, we demonstrate that *trans*-interactions at the *Men* locus are transvection as classically defined and show that this transvection is sensitive to the environment and to the overall genetic architecture of the flies. Interestingly, *cis*-interactions at *Men* were more robust to these changes, suggesting that *trans*-interactions may be more sensitive to changes in the environment and genetic architecture than *cis*-interactions, even at the same locus. In addition, we demonstrate a correlation between levels of transvection, transcription factor (TF) expression, and the presence or absence of a binding site for at least one TF, Abd-B. This correlation suggests that this factor may play a role in the allele-specific differences in transvection that we observe. Our results also suggest some overlap in the ability of *cis*- and *trans*-regulatory elements to regulate gene expression, again at least for Abd-B. The complexity of interactions that we find underscores the importance of examining genetic interactions across conditions and genetic backgrounds, *i.e.*, of using the variability within a system to uncover novel molecular mechanisms.

Trans-interactions at *Men* are pairing-dependent

We demonstrate that *trans*-interactions at the *Men* locus are significantly reduced by changes in genomic architecture expected to disrupt somatic chromosomal pairing, *i.e.*, these interactions are transvection as classically defined. Creating heterozygotes with *MenExi*$^{-}$ alleles and a large inversion completely eliminated the up-regulation of MEN activity (Figure 3B,C), unambiguous evidence that the *trans*-interactions driving the up-regulation are pairing dependent. Interestingly, we also saw a significant reduction, but not elimination, in *trans*-interactions when these alleles were paired with a locally inserted *P*-element (*P{GT1}*; Figure 2) or a smaller chromosomal inversion (Figure 3D,E). These more subtle reductions in MEN activity suggests fine scale differences in transvection in response to moderate or small changes in local genomic architecture, and underscore the strength of the sensitivity of this MEN system in fine-scale examination of regulation of transvection.

The large inversion with breakpoints distal to the *Men* locus involving the centromere, [*In(3LR)LD6*], abolished the *trans*-interactions driven up-regulation, consistent with transvection at other loci (Gelbart 1982; Golic and Golic 1996; Leiserson *et al.* 1994; Lewis 1954). Interestingly, the smaller inversion flanking the *Men* locus, $In(3R)hb^{D1}$, only decreased transvection in $MenEx58^{-}/In(3R)hb^{D1}$ heterozygotes, and not in heterozygotes with other *MenExi*$^{-}$ alleles. Chromosomal inversions with a proximal breakpoint between the locus of interest and the centromere, and a distal breakpoint anywhere on the other arm of the same chromosome, disrupt pairing in structural heterozygotes in *D. melanogaster* (Golic and Golic 1996), likely because homologous pairing is initiated at the centromere, spreading from there towards the distal ends of the chromosome (Duncan 2002). Conversely, chromosomal inversions not involving the centromere are not expected to disrupt transvection. Our findings are generally consistent with this model of pairing, the inversion involving the centromere eliminates the *trans*-interactions driven up-regulation of *Men,* and suggest that *Men* has a large critical region for pairing. Interestingly, however, although there was a significant reduction in transvection in $MenEx58^{-}/In(3R)hb^{D1}$ (~30%), transvection at *MenEx86*$^{-}$ was not perturbed by the small inversion (Figure 3C). *MenEx58-* and *MenEx86-* are very similar alleles, differing only at the excision site near the *Men* TSS. This sensitivity of the *trans*-effects to the size and/or specific sequence of the excision alleles suggests that certain regulatory elements, retained in *MenEx86*$^{-}$ but deleted in *MenEx58*$^{-}$ (Figure 6A), may be better able to interact in *trans* across long-distances. Further studies with a larger, more diverse, set of inversion chromosomes and *MenExi*$^{-}$ alleles may provide a clearer picture of chromosomal pairing kinetics at the *Men* locus (*e.g.*, Lewis 1954; Ou *et al.* 2009).

The reduction in transvection in the *MenExi*$^{-}$*/P{GT1}* heterozygotes suggests that simply shifting local chromosomal architecture is sufficient to modify transvection. *MenEx58*$^{-}$*/P{GT1}* heterozygotes still showed significant levels of transvection (Figure 2B), while heterozygotes of larger deletions did not (*e.g., MenEx76*$^{-}$). We speculate that this reduction, but not elimination, of *trans*-interactions reflects an overall decrease in interaction frequency between enhancers in *trans* and the functional *Men* promoter in the intact *P*-element line. A similar decrease in *trans*-interactions has been shown to lead to a “variegated” or “delayed” form of gene activation in *trans* at other loci (Kwon *et al.* 2009; Bateman *et al.* 2012a). Similar, variegated expression of *Men,* due to increased distance between enhancers and promoters in *trans* could be driving the

overall decrease in transvection we observe in the whole fly. However, in addition to the increased distance between the interaction partners in *trans*, we must also consider the presence of three other genes within the intact element in *cis* (Figure 2C,D), possibly competing for enhancers both in *cis* and in *trans*. We do note that the *P{GT1}* element does not contain any elements (*e.g.*, enhancers) that might augment *cis*-regulatory activity. Lastly, increases in the genomic distance between an enhancer and its target promoter have been shown to significantly reduce transcription activation in *cis* in other systems (Kwon *et al.* 2009; Lukacsovich *et al.* 2001). If simple genomic distance was driving the effects we see, we would expect MEN activity to be significantly lower in *P{GT1}/P{GT1}* homozygotes than *MenEx3^{+}/MenEx3+* homozygotes, and we see no such difference. Because significant differences were not observed, *cis*-enhancers in the *Men* locus displaced by the intact *P*-element may be able to maintain *cis*-interactions by looping out the intact *P*-element via the topology model (Morris *et al.* 1999a). This looping mechanism may also be involved in maintaining *trans*-interactions in *MenEx58^{-}/P{GT1}* heterozygotes (Figure 2B,E), although certain enhancers appear less able to interact across longer distances through looping in *trans* (*e.g., MenEx76^{-}/P{GT1}* heterozygotes).

Transvection is not canalized

Our demonstration that transvection is a plastic phenotype expands the classical view of this mode of gene regulation in *D. melanogaster*, establishing it as a much more dynamic and variable phenomenon, and opening the door to intriguing questions regarding further variability of *trans*-interactions across genetic backgrounds and environments. Our results suggest that transvection, and by extension somatic chromosomal pairing and other *trans*-interactions, vary not only within tissues (Bateman *et al.* 2012a) and across cell types (Mellert and Truman 2012), but also across genetic background and environments. These variations in pairing and pairing-related *trans*-interactions also exist across species; differences in chromosomal conformation between *Drosophila* and other organisms have been suggested to reflect a shift in the balance of genes involved in somatic chromosomal pairing (Joyce *et al.* 2012). Further unravelling of the mechanisms underlying this variability will better our understanding of the mechanisms underlying transvection, and *trans*-interactions, in eukaryotes in general. These questions of the variability or consistency of *trans*-interactions, and the factors driving them, are particularly important given the role that transvection-like, pairing-dependent, *trans*-interactions have been

demonstrated to play in at least one form of human cancer (Betts *et al.* 2013; Koeman *et al.* 2008; Rickman *et al.* 2012).

Why did changes in environmental condition (here temperature) perturb transvection (*MenExi*$^{-}$ heterozygotes), but not *cis*-interactions (*MenEx3*$^{+}$ heterozygotes), and why were slight differences in response observed between the two experimental temperatures? The two test temperatures lead to different responses in the physiology and gene regulation of *D. melanogaster*. We found that exposure to 29°C led to up-regulation of *Hsp70Aa* expression (Figure 9B), suggesting a heat-shock response (HSR) was elicited, consistent with previous results in yeast and *Drosophila* (Gibert *et al.* 2007; Herreur *et al.* 1988; Lindquist 1986; Yao *et al.* 2006). HSR triggers a genome-wide gene expression response coinciding with chromatin remodelling in a variety of organisms (Bell et al. 1988; Gasch et al. 2000; Mittal et al. 2009; Petesch and Lis 2008; Zhao et al. 2005). This remodelling leads to reshuffling of genomic architecture, changing access of transcriptional machinery, and modifying gene expression (Aalfs and Kingston 2000). Transferring flies to 21°C or 29°C also impacts the physiology of the fly. Transferring flies reared at 25°C to 21°C can increased lifespan and metabolic rate (Celotto *et al.* 2006; Gnerer *et al.* 2006; Terblanche and Chown 2006), while transferring flies to 29°C shortens life spans and lowers metabolic rates. Although mechanisms underlying the physiological changes in flies transferred to 21°C are not clear, they are likely similar to those observed at 29°C (*i.e.* gene expression changes coincident with chromatin remodeling, with the exception that heat-shock proteins are not activated). Environmental changes have been shown to impact TF expression, which in turn impacts global gene expression patterns (Gasch *et al.* 2000; Zhou *et al.* 2012). We found significant differences in the expression of most TFs analyzed in flies transferred to 29°C and 21°C, from that of the 25°C controls (Figure 9). Since the TFs we analyzed reside in different regions of the *Drosophila* genome, we hypothesize that their differential activation at different temperatures represents the effect of a global chromatin reshuffling driving changes in gene expression. This hypothesis suggests that changes in temperature may result in both significant physiological effects (*i.e.*, phenotypic plasticity; Zhou *et al.* 2012), and the observed reduction in transvection, through reduced somatic chromosomal pairing during chromatin remodelling. Consistent with this idea, *cis*-interactions (*MenEx3*$^{+}$ heterozygotes) did not show significant changes across temperatures (Figure 4). Expression of *Men* with two intact copies of the gene should be overwhelmingly *cis*-regulated, due to *cis*-

preference (Geyer *et al.* 1990). Given that *cis*-regulation is not pairing-dependent, while up-regulation of *Men* expression (*i.e.*, transvection) in *MenExi*$^{-}$ heterozygotes is, a reduction in somatic chromosomal pairing in response to changes in temperature would decrease transvection, but not *cis*-interactions. Further work using FISH, or other techniques to more directly visualize chromosome pairing, across environmental conditions could possibly help explain the environmental dependence of somatic chromosomal pairing and pairing-dependent gene regulation.

Our results support previous findings that genetic background can significantly modify the amount of *trans*-interactions driven by different excision alleles (Lum and Merritt 2011) and extend these conclusions to show that *trans*-interactions are also sensitive to the environment. The significant background by allele ($F_{20, 2288} = 13.906$, $P<0.001$), and background by allele by temperature interactions (GXE interactions on transvection, $F_{40, 2288} = 2.5448$, $P<0.001$) suggest that the overall level of transvection is a function of a complex interplay between local and non-local genetic effects and the environment. For example, comparison of the absolute magnitude of background by excision interactions, and the number of significant interactions, shows that genetic background had a much stronger effect on transvection at 29°C than at 21°C (Figure 5D,F). Our observation that the amount of transvection for certain *MenExi*$^{-}$ alleles within a given background changed drastically from one condition to the next (*i.e.*, the excision allele by background interactions shifted in terms of both direction and magnitude across the conditions) further supports the assertion that transvection is plastic and not canalized (Mackay *et al.* 2009). These significant interactions further stress the importance pointed out by ourselves and other authors of studying mutations across genotypes, and highlights the sobering fact that results from a single background or environment may not necessarily hold true across other backgrounds and conditions (Chandler *et al.* 2013). Interestingly, we found that background had relatively little effect on MEN activity of heterozygotes of *MenEx3*$^{+}$ and *MenEx55*$^{-}$, two alleles (our wild-type control and our largest excision allele) with little or no *trans*-effects at the control temperature of 25°C (Figure 5B). Background had, however, a larger effect on MEN activity of the heterozygotes at both 21°C and 29°C (Figure 5D,F), suggesting that genetic effects controlling *cis*-regulation are also affected by changes in temperature. This exaggeration of background effects under stressful conditions is consistent with the phenomenon “cryptic genetic variation” (reviewed by Chandler et al. 2013). Given the complexity we observe in this system, but the

apparent widespread influence of transvection on gene regulation of flies, mapping studies to identify the QTL's (*i.e.*, non-local effects interacting with the environment) driving these *cis*- and *trans*-regulatory effects across genotypes and environments would be particularly interesting.

Importantly, we begin to demonstrate the specific non-local and local genetic elements that interact with the environment to contribute to the observed overall difference in transvection (*i.e.*, the interactions of *Abd-B* and *MenEx60*$^{-}$). Changes in transvection of *MenEx60*$^{-}$ are correlated with, and could be driven by, changes in *Abd-B* expression, which was modified by both genetic background and environment. Therefore, changes in the availability of Abd-B across background and environment (non-local effect) may lead to changes in the ability of *MenEx60*$^{-}$, which has an extra binding site for Abd-B (local effect), to act in *trans*, leading to differences in the amount of transvection observed in *MenEx60*$^{-}$ heterozygotes across these conditions (GXE interaction on transvection). The addition of this binding site in this allele may also contribute to the ability of *MenEx60*$^{-}$ to consistently drive higher than 100% wildtype MEN activity, or more than 50% higher than expected gene expression due to transvection.

We speculate above that the background effects that manifest as cryptic genetic variation are a combination of non-local (TFs) and local effects (availability of binding sites), the former being sensitive to changes in the environment. To identify possible local factors within the *Men* locus that may influence TF binding, we sequenced ~5kb upstream of the *Men* locus across the five genetic backgrounds, with the hope of uncovering SNPs occurring in important putative regulatory regions. While we identified 52 SNPs across the five genetic backgrounds, none of the SNPs were situated within the predicted TFBS motifs we tested in this study. The SNPs may, however, influence or modify other TFBS we did not identify: we found that genotypes which showed similar levels of transvection clustered together according to neighborhood-joining analysis of the ~5kb region (data not shown). This clustering is consistent with our hypothesis that genetic changes in the regional *cis*-regulatory region of *Men* may further interact with non-local genetic effects that differ across environmental conditions (*e.g.*, Abd-B), leading to genetic background effects on both transvection and *cis*-regulation.

Abd-B regulates *Men* expression in *cis* and in *trans*

Our RNAi results suggest that *Abd-B* can regulate, directly or indirectly, *Men* expression in *cis*, and the correlations we observe between *Abd-B* and *Men* expression in the *MenExi*$^{-}$ experiments suggest that *Abd-B* can also regulate *Men* in *trans. Abd-B* is a HOX gene within the *Bithorax* (*BX-C*) gene cluster that is regulated via long-range intra-chromosomal interactions mediated by the *Polycomb group* (*PcG*) complexes and chromosomal architecture (Bantignies *et al.* 2011; Tolhuis *et al.* 2011). Previous work has suggested that *Abd-B* expression is sensitive to rearing temperature, and implicated this sensitivity in the phenotypic plasticity of adult abdominal pigmentation in *D. melanogaster* (Gibert *et al.* 2007). Additionally, these same authors demonstrated that *Abd-B* interacts with numerous chromatin regulators and may be involved in modulating chromatin architecture (Gibert *et al.* 2007). It seems reasonable that the correlations we observe between *Abd-B* expression and the amount of transvection at *Men* are the result of similar modulation of chromosomal architecture altering somatic chromosomal pairing in response to temperature-, and possibly genetic background-, driven changes in *Abd-B* expression. This *cis*- and *trans*-regulation of gene expression may represent a novel regulatory function for the TF *Abd-B*, which is more commonly known through its involvement in development of posterior abdominal segments in *D. melanogaster* (Akbari *et al.* 2006).

We find less support for a role in regulation of *Men* in *trans* by *mirr* and none for regulation in *cis.* The RNAi experiments show no indication of modulation of MEN activity in *cis* by *mirr* expression. Our initial observation of significant correlations between *mirr* and *Men* expression in the *MenEx58*$^{-}$ heterozygotes suggested that *mirr*, like *Abd-B*, may play a role in modulation of transvection at *Men*, but our failure to see the same patterns in our tissue-specific expression experiments calls this role into question. However, at least two other proteins are known to bind to the Iroquois motif in *Drosophila* (coded by the *araucan* and *caupolican* genes; Gomez-Skarmeta *et al.* 1996). The inconsistent relationship between *mirr* and *Men* expression could result from confounding effects from these two TFs. In the fly embryo, the mirr protein acts as a negative transcription regulator in the nervous system and eye development (Andreu *et al.* 2012; Cavodeassi *et al.* 2000). Like *Abd-B*, *mirr* expression is also targeted by *PcG* complexes (Tolhuis *et al.* 2011), and *mirr* is known to interact with *Trl*, *twist*, and *dorsal* (Ozedmir *et al.* 2011), TFs that were identified as potential regulators of *Men* expression in *cis* via HOT-spot analysis (Celniker *et al.* 2009). Interestingly, we found *Trl* expression to be significantly correlated with both *Abd-B* and *mirr*, although not with *Men* (data not shown), in adult flies,

suggesting possible regulatory connections. Further work will explore whether changes in these additional TFs play a role in the regulation of *Men* expression in *cis* or *trans.*

2.5 CONCLUSION

We have conclusively shown that *trans*-interactions at *Men* are pairing-dependent, are by definition transvection, and that this transvection is strongly influenced by variation in environmental conditions and genetic background; further supporting conclusions (Lum and Merritt 2011; Bateman *et al.* 2012a; Mellert and Truman 2012) that transvection is a complex and dynamic phenomenon. We have demonstrated that this plasticity in transvection is associated with changes in TFs coded elsewhere in the genome (*i.e.* non-local factors), in addition to local changes in genomic architecture, and propose that these factors interact to modulate transvection through modification of somatic chromosomal pairing. Although *trans*-interactions encompass a broader array of gene regulation mechanisms in *trans*, the phenomenon hinges on chromosomal architecture under specific conditions, and should be influenced by genetic background and environment in a way similar to transvection. These results strongly suggest that transvection, and *trans*-interactions in general, should be viewed as a dynamic interplay between three factors: local (intragenic), regional or distant (TFs and chromosomal pairing dynamics), and external (environmental conditions). Finally, our findings stress the importance of studying genetic interactions from a dynamic perspective, incorporating both genetic and environment variation.

Chapter 3 – General conclusions and future work

Transvection, pairing-dependent *trans*-regulation of gene expression, appears to be common in the *Drosophila* genome, driven by the extensive somatic chromosomal pairing of homologous chromosomes (Mellert and Truman 2012). However, extensive somatic chromosomal pairing appears to be limited to *Dipteran* insects, and long-distance interactions in other eukaryotes are predominantly transient pairing between non-homologous loci that appear to be co-regulated (reviewed by Cavalli and Misteli 2013). To achieve a better understanding of the mechanisms underlying this unique chromosomal architecture, one approach is to unravel how gene regulation mediated by genome topology has evolved. In my thesis, I have made a step toward understanding of the evolution of transvection, by first dissecting how diversity of local genomic architecture can influence transvection, and the mechanisms by which this variation is achieved. I will now place my data into the context of understanding the evolutionary significance of transvection in *D. melanogaster*, and *trans*-interactions in eukaryotes.

3.1 Pairing dynamics at *Men*

I found evidence supporting *trans*-interactions at *Men* as pairing-dependent, through disruption of both local and large-scale changes in genome architecture. Previous studies in other transvection systems have found that chromosomal rearrangements involving "critical regions" disrupt transvection, whereas rearrangements outside these regions do not. Lewis pointed out in 1954 that these critical regions usually involve the centromere, and suggested that pairing of homologous chromosomes may be initiated there. This hypothesis has been supported by numerous studies at other transvection systems (Gelbart 1982; Golic and Golic 1996; Leiserson *et al.* 1994). However, different genes have very different sizes of critical regions (reviewed by Duncan 2002). Interestingly, transvection loci with smaller critical regions tend to be near the distal end of the chromosomal arm (Lewis 1954; Ou *et al.* 2009).

Despite these hypotheses, pairing dynamics and kinetics in somatic nuclei remain poorly understood (McKee 2004). It has been argued that rather than being established at any particular region, homologous pairing in somatic nuclei is dynamic and cell-stage dependent (Duncan

2002; Golic and Golic 1996). The amount of homologous pairing may fluctuate across various stages of the cell cycle. Therefore, genes may have smaller critical regions because they are only expressed during cellular stages where homologous pairing is not extensive, while genes with larger critical regions are ubiquitously expressed (Duncan 2002). This hypothesis certainly fits with my data, in which *Men*, a ubiquitously expressed gene, appears to have a large critical region. At *Men*, I have found that large chromosomal rearrangements involving the centromere appear to be better disrupters of transvection than those that do not. Surprisingly, I also found that chromosomal rearrangements between the distal right arm of the third chromosome and the second chromosome can also disrupt transvection at the *Men* locus, albeit to lesser degrees (data not shown). Thus, the critical region of *Men* appears to be large, involving both the centromere and the distal right arm of the third chromosome.

Much work remains to be done to understand the mechanisms of homologous chromosome pairing dynamics and kinetics in somatic nuclei of *D. melanogaster*. A better understanding of how differences in critical region size and location can impact transvection will improve our understanding of the mechanisms of transvection overall, and the local pairing dynamics of the chromosomal region (Duncan 2002; Golic and Golic 1996). Further work could more systematically generate a suite of chromosomal rearrangements that will help clearly define the critical region of *Men*, the exact pairing dynamics of the third chromosome, and how the pairing here mediates transvection at the *Men* locus. The sensitivity of our transvection system also allows us to gain knowledge on pairing dynamics at a level of accuracy previously unachieved in other transvection models in *D. melanogaster*.

3.2 Specific mechanisms of transvection at *Men*

In addition to studying the effects of chromosomal aberrations on transvection, previous work on other transvection systems have implicated a variety of gene products that may play a role in mediating transvection. Mutations in the *Zeste* gene have been shown to disrupt transvection at *white*, *yellow*, *Ubx*, *dpp* and *eya* (Duncan 2002). The Zeste protein appears to be a DNA-binding protein that when mutated, hyper-aggregates, thus recruiting PcG proteins and forming a PcG repressive complex (Lifschytz and Green 1979; Wu *et al.* 1989). Since PcG repressive complexes play a role in long-distance chromosomal interactions leading to suppression of gene expression (Bantignies *et al.* 2011), Zeste mutations may disrupt transvection through the

recruitment of PcG complexes. PcG complexes also regulate *Abd-B* expression, a gene we implicated in the regulation of *Men* expression in *cis* and *trans.* We did not expect that *zeste* would play a role in transvection at *MenEx60*$^{-}$ and *MenEx58*$^{-}$ since both deletions excise the local Zeste binding site. However, Zeste is not deleted in *MenEx86*$^{-}$, an allele we found to be differentially affected by chromosomal rearrangements as compared to *MenEx58*$^{-}$. Therefore, examination of whether *zeste* plays a role in mediating *trans*-interactions at *MenEx86*$^{-}$ and transvection at *Men* in general could provide further insight into the mechanisms of transvection at the locus.

Topoisomerase 2 (*Top2*; Williams *et al.* 2007), *Suppressor of Hairy wing* [*Su(Hw)*; Savitskaya *et al.* 2006], and *Chromosome-associated protein H2* (*CapH2*; Hartl *et al.* 2008) have also been shown to influence somatic chromosomal pairing, important for transvection. These studies have described in detail the mechanisms by which these genes are involved in somatic chromosomal pairing. In addition, recent genome-wide RNAi FISH-based screens revealed 105 candidate genes which impact somatic chromosomal pairing in *D. melanogaster* (Joyce *et al.* 2012), 13 of which were confirmed by an independent group using different methods (Bateman *et al.* 2012b). Interestingly, most of these genes have orthologues in higher eukaryotes such as mice and humans, and play a similar functional role in these organisms. It is not yet clear how differences in the expression and function of these genes could contribute to the extensive pairing of homologues in *Drosophila*. In addition, since these high-throughput experiments were conducted in cell lines, further work is needed using whole-organism transvection systems to validate the role which the candidate genes play in not only somatic chromosomal pairing, but also transvection. Our transvection system at *Men* provides an excellent model for these candidate genes to be tested in whole adult flies. Thus, further work could explore the effect of the down-regulation of these genes on transvection at *Men*, which would allow an accurate quantification of the ability of each candidate gene to drive somatic chromosomal pairing and thus transvection. Certain hurdles remain in testing the effect of knockdown of genes on *Men* expression in *trans*, however, described below.

3.3 Genetic cloning of *MenExi*$^{-}$ alleles

In my work, I was able to implicate *Abd-B* and *mirr* as potential *trans*-regulatory factors that may influence transvection at *MenEx60*$^{-}$ and *MenEx58*$^{-}$, respectively, and *Abd-B* as a regulator of

Men expression in *cis.* However, to further confirm the role of *Abd-B* and *mirr* in transvection at these alleles, fly lines could be constructed containing both the UAS-RNAi vector and *MenExi*$^{-}$ alleles in order to directly test the effects of knocking down these transcription factors on transvection. In this way, we could directly observe whether *Abd-B* and *mirr* actually regulate *Men* expression in *trans*, but also test the effect of other candidate genes involved in somatic chromosomal pairing on transvection at *Men.* Future work could also include the over-expression of *Abd-B* and *mirr* in *cis* and *trans*, in tissues where these genes are not highly expressed, and observing the effect of these manipulations on *Men* expression in these tissues. Alternatively, gel shift mobility assays could definitively establish the binding sites for *Abd-B* and *mirr* as functional. This work will improve our understanding of how differences in expression of *trans*-factors across tissues can impact transvection. Fortunately, this is possible in *Drosophila* since a wide variety of tissue and developmental stage specific Gal4 drivers are available through the BDSC.

Only certain enhancer/promoter combinations, or *cis*-regulatory modules (CRMs), appear to lead to transvection (Mellert and Truman 2012). In other words, only certain enhancers, which can activate a given promoter in *cis,* can activate the same promoter in *trans*, suggesting that only certain mechanisms of gene activation in *cis* are compatible with gene activation in *trans.* This idea was further supported by my findings: although *Abd-B* and *mirror* both appear to regulate *Men* in *trans,* only *Abd-B* was found to regulate *Men* in *cis.* Further work is needed to determine the specific sets of CRMs that allow for *trans*-activation. By determining the differences between *cis*- and *trans* gene activation mechanisms, we can then understand the pairing dynamics of the *Drosophila* genome that could potentially lead to this divergence of the two types of interactions.

To achieve a better understanding of the differences in *cis*- and *trans*-regulation mechanisms, future work could involve the cloning of the two excision alleles, including designing variants of these alleles that do not have the extra insertion remnant containing the predicted binding sites for *Abd-B* and *mirr*. These clones can then be stably inserted into whole flies using the site-specific *phiC31* integrase-mediated cassette exchange system (Bateman *et al.* 2006). Using this method, which would allow *MenExi*$^{-}$ allele clones, and variants of these clones, to be inserted anywhere in the genome, the *MenExi*$^{-}$ alleles could be inserted near a functional *Men* allele, on different regions of the third chromosome, or even on different chromosomes. Thereafter, many

experimental possibilities exist: we could observe whether transvection occurs in the presence of these clones, either in isolation or in *trans* to other *MenExi*$^{-}$ alleles, in the presence of multiple copies of Men, on the same or on different chromosomes, *etc.* Using this transgenic approach would significantly improve our understanding of *trans*-interactions and gene regulation in general since our *Men* system captures the exact amount of *trans*- or *cis*-interactions that are occurring within the whole fly.

3.4 Genetic underlyings of GXE interactions at *Men*

My research demonstrates that transvection is a context-dependent complex trait. Since I found that transvection is sensitive to both changes in genotype and environment, and these two variables interact significantly to drive differences in transvection, it could be possible to map the QTLs that contribute to variation in that trait (Flint and Mackay 2011). QTL mapping in *Drosophila* is relatively straight-forward, and various genome-sequenced lines are commercially available in the fly community. It is particularly important to determine not only loci that contribute to transvection at *Men*, but the loci contributing to the variation of the transvection trait across environments, since it has been previously observed that different loci influence trait variation in different environments (pQTLs; Gutteling *et al.* 2007; Li *et al.* 2006). Mapping these pQTLs would further our understanding of the mechanisms underlying variation in transvection at *Men.* In addition, it would be interesting to see whether mapped pQTLs overlap with *Abd-B* and *mirr,* and the candidate genes found by Joyce *et al.* (2012) and Bateman *et al.* (2012b), which would further confirm the function of these genes in regulating transvection at *Men,* and transvection and somatic chromosomal pairing in general in *D. melanogaster.*

3.5 Pairing dynamics in response to changes in temperature and background

I found that acclimation to temperature in adult flies reduced the amount of transvection at *Men,* and that genetic background can significantly modify the amount of transvection observed for each *MenExi*$^{-}$ allele, in each temperature condition. The simplest explanation for this reduction in transvection, and lack of change in *cis*-interactions at *MenEx3*$^{+}$ across the temperature conditions, is that somatic chromosomal pairing is reduced during changes in temperature to adults in *D. melanogaster.* A better understanding of variation of somatic chromosomal pairing across temperatures and environmental conditions in general will shed light on how somatic chromosomal pairing and chromosomal conformation in *Drosophila* respond to changes in the

environment. Future work could confirm the reduction in somatic chromosomal pairing across temperature conditions using FISH. In addition, the amount of transvection observed in other stressful conditions could be analyzed. Alternatively, studies could compare the amount of transvection in deletion heterozygote flies carrying genetic constructs expected to disrupt somatic pairing, across temperature conditions.

I also found that the amount of somatic chromosomal pairing in each genetic background may be quantitatively and qualitatively different. In other words, variation in local genomic architecture may influence the pairing dynamics in *D. melanogaster*. The variability of transvection across genetic background also increases during more stressful conditions, reminiscent of "cryptic genetic variation" (Chandler 2010; reviewed by Chandler *et al.* 2013). Further work could continue to identify the factors (*e.g.,* transcription factors, QTLs) that are differentially expressed across environmental conditions in each genetic background that contribute to this variation across environments within *D. melanogaster*. QTL mapping will assist in the identification of these factors. In addition, FISH studies of various genetic backgrounds to quantitatively analyze intra-species variation in somatic chromosomal pairing will help us gain insight into whether differences in transvection is driven by changes in somatic chromosomal pairing or transcription factor expression, or a combination of both.

3.6 Assessing transvection in an evolutionary context

Not only do the mechanisms underlying transvection remain unclear in *Drosophila*, controversy remains as to whether transvection occurs in organisms other than Dipterans (McKee 2004). As discussed in Chapter 1, somatic *trans*-interactions do occur in mammalian cells, but are usually limited to certain chromosomal regions, and extensive homologous chromosomal pairing only occurs during meiosis (Apte and Meller 2012; McKee 2004). Previous results suggesting that yeast, *C. elegans*, and certain plants exhibit transvection are also now heavily disputed (McKee 2004; Stam 2009). These examples are now thought to be mostly driven by microRNAs or silencing RNAs, or via other epigenetic mechanisms such as methylation. The strongest support for transvection outside the Diptera is in the fungus *Schizosaccharomyces pombe* (*S. pombe*), although the peculiar cell cycle of this organism makes comparison of somatic chromosomal pairing between *Drosophila* and *S. pombe* difficult (McKee 2004).

From these previous observations, it remains unclear whether somatic chromosomal pairing occurs extensively in any organism other than Dipteran insects. In other words, did somatic chromosomal pairing evolve independently in Dipterans, or has the unique genome topology been lost over evolutionary time in other eukaryotes? This question is particularly important since unexpected somatic chromosomal pairing in certain human chromosomal regions induced by chromosomal rearrangements often lead to cancer and disease (Faruqi *et al.* 1994; Lupski and Stankiewicz 2005), abnormal somatic chromosomal pairing can be deleterious to an organism. In order to answer this question, we need to understand the evolutionary history, and the advantages of transvection and somatic chromosomal pairing in *Drosophila.*

A straightforward approach to addressing this question could be to microscopically observe somatic nuclei of cells from other species within the insect class for evidence of homologous pairing using FISH. An updated and detailed study of Dipteran insects and insects from various orders could be conducted to fulfill the gap of knowledge in the literature, particularly since this gap makes it impossible to assess the evolutionary history of somatic chromosomal pairing in insects. Furthermore, classification of somatic chromosomal pairing in Arthropods, and eventually organisms from other phylums will help retrace the evolutionary history of somatic chromosomal pairing. Only by understanding the evolutionary history of somatic chromosomal pairing can we begin to understand the evolutionary pressures which may have either led to independent evolution of somatic chromosomal pairing or loss of somatic chromosomal pairing in organisms other than Dipterans.

3.7 Understanding the players involved in chromosomal pairing

Determining the unique mechanisms that drive somatic chromosomal pairing in the organism would help us better understand the evolutionary advantages of transvection and somatic chromosomal pairing in *Drosophila.* As previously mentioned, Joyce *et al.* (2012) and Bateman *et al.* (2012b) have provided us with a large set of candidate genes that are important for somatic chromosomal pairing in *D. melanogaster*. Joyce *et al.* (2012) further hypothesized that whether a species exhibits somatic chromosomal pairing may be a result of shift in the balance of the production and function of these genes. However, further work is needed to determine the specific roles that each of these genes play in modifying somatic chromosomal pairing.

Since we can now observe the amount of transvection accurately to a single-cell level in whole organisms (Bateman *et al.* 2012a) rather than inferring transvection in cell cultures (Joyce *et al.* 2012), we could validate the biological function of the candidate genes identified by Joyce *et al.* (2012) that are involved in somatic chromosomal pairing on transvection. As a first pass, it will be important to confirm the effects of these genes on somatic chromosomal pairing by observing the effects of up- or down-regulating the expression of these genes in *D. melanogaster* on transvection using the transgenic approach developed by Bateman *et al.* (2012a). Systematically observing the effects of up- or down-regulating the expression of these genes, individually and collectively, will shed light on how the dynamic interplay of these genes can mediate variability in somatic chromosomal pairing and transvection at the cellular level. Repeating these experiments with different sets of enhancer/promoters that require different sets of transcription factors using the methods described by Mellert and Truman (2012) and our MEN transvection system, will shed light on the variability of transvection at various levels of biology in *Drosophila.*

Further detailed study of these candidate genes and their evolution using bioinformatic approaches such as test the Ka/Ks ratio test and MacDonald-Kreitman test could shed light on whether these genes are being positively selected in Dipterans, particularly in *D. melanogaster*, as compared to other species that do not exhibit pairing-mediated gene regulation. An alternative approach would be to experimentally test the effect of integrating these pairing-related genes in organisms that do not exhibit somatic chromosomal pairing (*e.g., C. elegans,* yeast), using transgenic approaches, and observing the effects of integrating these genes on somatic chromosomal pairing using FISH. Future work could also involve the generation of transvection loci in *Drosophila* species other than *D. melanogaster.* Hybridization of transvection lines in *D. melanogaster* with *D. simulans* and observing whether transvection is retained may be one way of addressing this question. Altogether, the aforementioned experiments will provide us with a better understanding of the unique mechanisms underlying somatic chromosomal pairing in *Drosophila* as compared to other non-pairing species. By understanding the loci which mediate variability in somatic chromosomal pairing and transvection in *Drosophila*, we can then better understand the changes in the degree of somatic chromosomal pairing and transvection through evolution in relation to these genetic pathways.

3.8 Conclusion

The findings described in my thesis provide insight into how transvection may be affected by changes in the environment and genetic background, and begin to address the mechanisms by which these changes in transvection occur. Perhaps the most surprising finding of my thesis is that transvection can be such a dynamic and context-dependent trait, and that previous observations in transvection may be only specific to certain environmental conditions and genetic backgrounds. Importantly, my work has provided many interesting questions regarding transvection, on scales ranging from the *Men* locus to inter-species, and has provided clues as to how we should address these questions. First, we must better our understanding of transvection at *Men*, and the mechanisms by which variation is achieved across environmental conditions and genetic backgrounds at this locus. With a better understanding of the mechanisms underlying the dynamic nature of transvection in *D. melanogaster*, we can then begin to understand why transvection and somatic chromosomal pairing occurs in *Drosophila* and Dipteran insects in general, and what evolutionary advantages these organizational characteristics may provide to this order of organisms. Finally, consolidating our knowledge in transvection and somatic chromosomal pairing will improve our understanding of how genome topology can regulate genome function, and how its mis-regulation can lead to disease.

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Appendix

Table S1. Fly lines used in this paper.

P-element line	**Insertion site(s) (~bp from TSS)**		**Type of transposon inserted**	**Type of trapping system**
12824	184bp		*P{GT1}*	Gene trap
EP517	214bp		*P{EP}*	Enhancer trap
***MenExi* allele**	**Deletion size (bp)**	**Insertion**	**5' start site (relative to TSS)**	**3' end site (relative to TSS)**
MenEx3+	0 (perfect excision)		NA	NA
MenEx60^{-}	646	TGATGAAATAATAAATAATAATA	-215	433
MenEx58^{-}	535	AACAATTCGCAGAGTCCT	-215	320
MenEx76^{-}	669	CATGATGAAATAACATAA	-215	454
MenEx86^{-}	2,239	NA		
MenEx55^{-}	16231	NA	-10245	5986
Other lines used (BDSC #)	**Description**			
(2177)	*Hsp70* promoter Gal4 driver			
(2045)	Inversion chromosome (*In(3R)hb^{D1}*)			
(106300)	Inversion chromosome (*In(3LR)LD6*)			
***w;* 6326; *x*/TM8,sb**	Common inbred 2nd chromosome, balancer for 3rd chromosome			
RNAi lines: BDSC#	**Gene name**		**Type of vector (site)**	
26746	*Abdominal-B* (*Abd-B*)		Valium 10 (attP2)	
31907	*mirror* (*mirr*)		Valium 10 (attP2)	
27043	*slowbordercells* (*slbo*)		Valium 10 (attP2)	
36303	attP2 (vector-less control)			
35787	Overexpression of mCherry in VALIUM10 attP2 (control)			

Table S2. Primers/probes for quantitative real-time PCR in this paper. Primers were obtained from literature or designed from database sequences (Flybase; ww.flybase.org) using PerlPrimer (Marshall 2004).

Gene name (Annotation symbol)	Primer Sequence (5' – 3')	Probe sequence (5' – 3')	Product length (bp)
Abd-B *(CG11648)*	(Left) CTATCCCAGCGAGAACTACTC (Right) GAAACTCCTTCTCCAGCTCC	/56-FAM/GTGGATTAT/ZEN/CCGTGGGAGC AGTGG/3IABkFQ/	132
Actin 79B *(CG7478)*	(Left) CCAGGTATCGCTGACCGTAT (Right) TTGGAGATCCACATCTGCTG	/5HEX/CCACCATCA/Zen/AGATCAAGAT CATCGCC/3IABkFQ/	158
Hsp70Aa *(CG31366)*	(Left) CAAGTACAAGAAGGATCTGCG (Right) GCACAGCTCCTCAAACCT	/56-FAM/AAGCGCACA/ZEN/CTCTCCTCCA GCAC/3IABkFQ/	139
Malic enzyme *(CG10120)*	(Left) GTATTGCCAACCTGTGCC (Right) AGCTTGTGTTCGGTGAGT	/56-FAM/ATGGTGGATAGCCGTGGTGT CA/3IABkFQ	159
Mirror *(CG10601)*	(Left) GATCAGCCGCCGTTCTAC (Right)CCATTCAAATCCATGCCATAGC	/56-FAM/CCCTGGCCA/ZEN/TATCCATCCA TGTATCATCC/3IABkFQ/	115
Rpl32 *(CG7939)*	(Left) CCATTTGTGCGACAGCTT (Right) ATACAGGCCCAAGATCGT	/56-FAM/ACCAAGCACTTCATCCGC CAC/3IABlk_FQ/	105
Slow border cells *(CG4354)*	(Left) CACAAGCAGATCTACATGCAG (Right) CGAGCACTCAAGCATTCAAG	/56-FAM/TTCCTCAAC/ZEN/ACCAACGAAC ACTCGC/3IABkFQ/	125
Trithorax-like *(CG33261)*	(Left) CTGCTGGACTTGCTAAAGAATAC (Right)TCCTTGGTAACCGTCTGC	/56-FAM/AGTGGTTAT/ZEN/GTTGGCTGG CGTCAATG/3IABkFQ/	159
Zeste *(CG7803)*	(Left) CTAAACTCCAGACTCCGCAA (Right)GCATCGGGACTAATCTGGTAC	/56-FAM/GATGGCAGC /ZEN/TCCTCAAAT CTCAGCTCT/3IABkFQ/	150

Table S3. Sensitivity of *Abd-B* vs. *Men* expression in correlations across genetic backgrounds and environments.

Group	Sensitivity/slope of curve	Correlation R^2 (*P*-value)
Overall	0.1928	0.3177 (0.0015)
***MenEx60*- overall**	0.2502	0.5136 (0.0026)
***MenEx58*- overall**	0.0967	0.1145 (0.2547)
Overall abdomen	1.4029	0.4972 (<0.0001)
***MenEx60*- abdomen**	1.3545	0.7521 (<0.0001)
***MenEx58*- abdomen**	1.6581	0.3135 (0.0373)
Overall head/thorax	0.5412	0.2239 (0.0127)
***MenEx60*- head/thorax**	0.6080	0.5571 (0.0014)
***MenEx58*- head/thorax**	1.0308	0.4997 (0.0047)

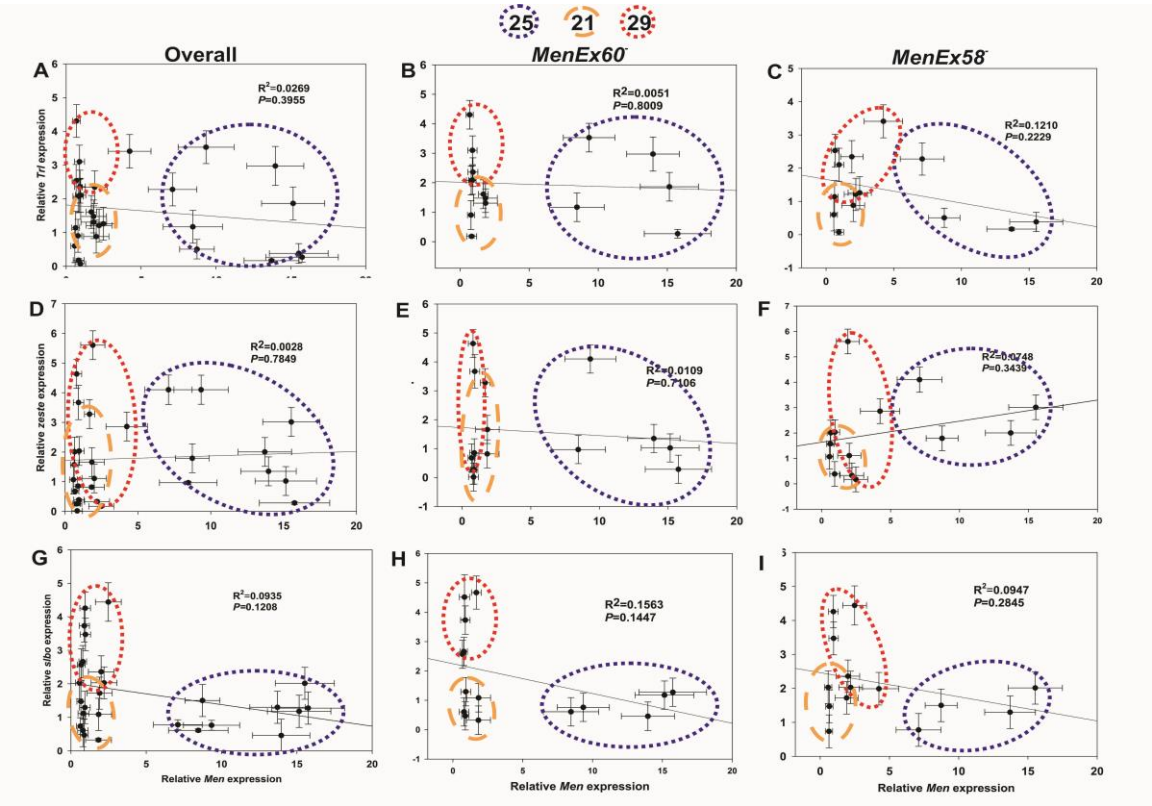

Figure S 1 Correlation between expression of other transcription factors analyzed and *Men*. *Trl* vs. *Men* expression in (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone. *z* and *Men* expression in (D) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (E) in heterozygotes of *MenEx60*$^{-}$ alone, and (F) in heterozygotes of *MenEx58*$^{-}$ alone. *slbo* and *Men* expression in (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone.

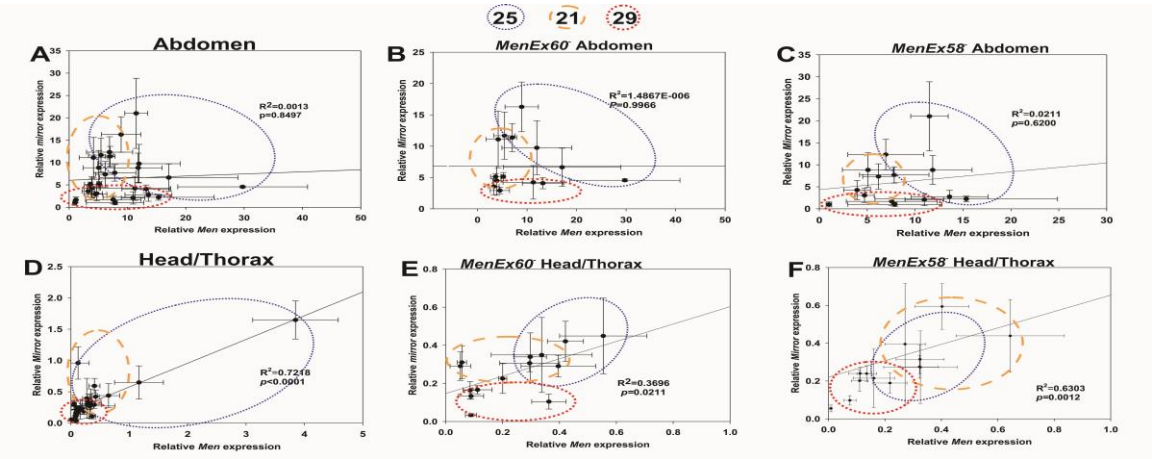

Figure S 2 Tissue-specific correlations between *mirr* and *Men* expression. *mirr* and *Men* expression in the abdomen of (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone; in the head/thorax of (A) both *MenEx60*$^{-}$ and *MenEx58*$^{-}$ heterozygotes, (B) in heterozygotes of *MenEx60*$^{-}$ alone, and (C) in heterozygotes of *MenEx58*$^{-}$ alone.