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CONSERVED BUT NOT IDENTICAL: THE EVOLUTION OF AN ESSENTIAL DEVELOPMENTAL GENE NETWORK IN *DROSOPHILA*

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The way was long, and wrapped in gloom did seem,

As I urged on to seek my vanished dream.

– *The Lament*, Qu Yuan (c. 340 - 270BC)

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ABSTRACT

Many developmental processes are preserved evolutionarily to produce similar if not identical phenotypes across taxa. An unanswered question in evolutionary biology is whether the genes controlling developmentally regulated traits under stabilizing selection to preserve phenotypes are likewise functionally static; or conversely whether functional divergence of individual genes and/or network interactions are inevitable. In this thesis, I address this void in knowledge by investigating the evolutionary dynamics and timescale for the functional divergence of genes in a conserved gene network.

First, I ask if sequence divergence in coding and noncoding regions of the gap gene *giant* (*gt*) have fitness consequences by replacing the endogenous *giant* locus in *D. melanogaster* with transgenic orthologs from six *Drosophila* species – *D. melanogaster*, *D. yakuba*, *D. santomea*, *D. erecta*, *D. pseudoobscura* and *D. virilis* – representing a range of phylogenetic distances spanning the genus *Drosophila*. By swapping the whole locus —coding and noncoding region of the locus— I document a continuous pace of functional evolution across the *giant* locus and species-specific coding-noncoding interactions (Chapter 2).

Second, I confirmed that the coding region of *D. melanogaster giant* is responsible for causing embryonic inviability in *D. melanogaster*/*D. santomea* hybrids. Further experiments identified the involvement of a second gap gene, *tailless*, in hybrid inviability. Both *giant* and *tailless* from the two species are not functional equivalents. These findings provide additional evidence for a rapid pace of functional divergence of essential gap genes to the extent that they may contribute to hybrid inviability (Chapter 3).

Lastly, I investigate aspects of gene expression of the gap gene network in *Drosophila*. Consistent with the functional evolution of *giant*, I confirm that the gap gene network outputs are conserved across species, as others have found, but show that this is not true when *D. virilis giant* is expressed in *D. melanogaster*, a non-native genetic background. In these flies, outputs of the gap gene network both differ from wildtype and are de-canalized. These

findings refute the hypothesis that genes in this essential regulatory network with conserved outputs are themselves functionally static (Chapter 4). To the contrary, conserved outputs require native genetic backgrounds to assure phenotypic constancy, which highlights the importance of co-evolution of genes in the network in maintaining this constancy.

CHAPTER 1

INTRODUCTION

Much of our knowledge of the molecular underpinnings of evolution comes from studies that focus on adaptive changes, phenotypic novelties that arise from re-organizing the underlying genetic architectures. Facilitated by advances in genetic manipulation and genomics, the genetic basis of phenotypic change can now be pinpointed with single nucleotide precision [1–3]. But many, if not most, traits and developmental processes change little over evolutionary time. These traits are governed instead by selective constraint and stabilizing selection, the two predominant forms of selection acting on mutational variation. My thesis focuses on the problem of functional evolution of one such exemplar, the conserved gap gene network in *Drosophila*.

Essential gene networks with conserved phenotypic outputs are believed to remain static even over long evolutionary time, as disturbance of the network are invariably deleterious. Yet, the observed phenotypic conservation has never been brought to careful functional examination: Are these networks truly conserved? And, what are the functional consequences if they differ? I address these questions by carefully investigating the evolution of the essential developmental gap gene network in *Drosophila*, and show it is continuously evolving while maintaining phenotypic stasis. That is, gap gene networks from different *Drosophila* species are conserved while the genes themselves functionally (co)evolve. This apparent decoupling of phenotype and genotype is a fundamental discovery.

1.1 *Drosophila* gap gene network

Drosophila embryogenesis is a complex process whereby a fertilized egg develops into a multicellular larva. The temporal appearance of morphological landmarks throughout this developmental process are conserved among *Drosophila* species [4], indicating strong evolu-

tionary constraint on embryogenesis. Early in embryogenesis, corresponding to the syncytial blastoderm stage during which there are 13 rounds of synchronous nuclear division, a spatio-temporal cascade of interacting transcription factors (TFs) specifies segmental boundaries along length of the embryo, i.e. the anterior-posterior (A-P) axis [5, 6]. This cascade of TFs is initiated by the establishment of diffusion-mediated concentration gradients of maternally provisioned morphogens such as Bicoid and Caudal, which act on the zygotic gap genes *Kruppel*, *knirps*, *hunchback*, *tailless*, and *giant*. These gap genes, together with maternal morphogens, then specify the expression of the seven-striped pair-rule gene *even-skipped* (*eve*), and other pair-rule genes, prior to gastrulation. Because individual *eve* stripes respond in unique ways to the maternal and gap gene gradients, it is possible to infer functional upstream defects from the *eve* readout. *eve* then specifies the expression pattern of segment polarity gene *engrailed*, which together with wingless delineate the A-P boundaries for embryonic parasegments, transforming this transient cascade of gene expression pattern into stable morphological outputs [7].

Underlying the spatiotemporal cascade of maternal, gap and pair-rule gene expression is cross- and auto- regulation of one gene by another as the system of gene expression becomes more and more spatially restricted and developmentally canalized [8–10]. Not surprisingly, their roles in development are functionally conserved. Using both *in vitro* TF binding and *in vivo* ChIP-seq assays, the binding preferences of many gap genes have been shown to be largely conserved [11, 12]. Our lab and other groups have developed technology to quantify mRNA and protein expression levels at single nucleus resolution from multiple time point during embryogenesis and found striking conservation in gap genes spatio-temporal expression patterns within genus *Drosophila*. These factors together make the pattern formation network an excellent system to pursue the questions posed above: To what extent, and in what ways, do individual components of conserved pathways remain constant or functionally evolve?

1.2 The gene *giant* as a model of an ancient functionally conserved regulatory gene

Gap gene *giant* is a basic leucine zipper transcription factor which is involved in anterior-posterior patterning and segmentation of the *Drosophila* embryo [13–16]. Its role as a gap gene is conserved over 350 million years of divergence in *Oncopeltus* [17]. The spatio-temporal expression pattern of all gap genes are qualitatively conserved within the genus, including *giant*. Hence *giant*, a conserved essential gene with no signs of positive selection or relevant novel phenotypes, and whose DNA-binding affinity remains conserved over 600 million years of bilateria evolution [11], is a excellent example to study if and how conserved genes evolve.

1.3 Conservation of *even-skipped* enhancers, targets of gap gene network

even-skipped is a pair-rule gene that encodes a homeodomain-containing protein, which is expressed during embryonic development. Prior to the completion of cellularization, the *eve* expression pattern (both mRNA and protein) matures from a single broad stripe into a series of seven transverse stripes, whose boundaries are well defined, within 40 minutes [18] . Extensive studies have uncovered modular enhancers driving different *eve* stripes, and regulatory mechanisms defining different stripe boundaries, in *D. melanogaster* [19]. The *D. melanogaster eve* stripe 2 enhancer (S2E) is one of the best-characterized eukaryotic cis-regulatory sequences, whose evolution has been the paradigmatic exemplar of enhancer evolution [20]. By swapping orthologous S2E from *D. pseudoobscura* into *D. melanogaster*, as well as their chimeras, Ludwig *et. al* showed strong stabilizing selection maintaining phenotypic constancy for *eve* stripe 2 expression while allowing mutational turnover of functionally important sites [21, 22]. Sequence turnover in the apparent absence of functional evolution has been the general theme for enhancers, as exemplified by the orthologous Sepsid

S2E with nearly complete sequence divergence driving similar expression in *D. melanogaster*, a phylogenetic clade separated from the genus *Drosophila* by 100 million years [23].

This striking phenotypic conservation of the expression pattern driven by a diverged enhancer does not exclude the possibility of functional evolution of the sequence. A detailed investigation has shown that the underlying mechanisms driving similar expression patterns are indeed different for orthologous S2E's. The Sepsid S2E, for example, lacks sequence conservation with its *D. melanogaster* counterpart, and modeling studies together with experimental tests have shown Sepsid S2E activation by Caudal rather than by Bicoid, as is the case for the *D. melanogaster* S2E [24–26]. Recent experimental work by Crocker *et. al* also indicates that the *D. erecta* S2E has evolutionary substitutions outside the consensus enhancer boundary [27]. Taken together, these studies suggest more extensive cis-regulatory evolution than we postulated from simply looking at the expression patterns they drive, and suggests the need to quantitatively study *cis*-regulatory evolution in a whole-locus context.

1.4 Conflicting evidence on the evolution of essential TFs

Previous work on TF evolution has been restricted to the protein coding region; these technical limitations have confounded the interpretation of their outputs in transgenic lines. In Table 1.1, I have summarized 13 examples of ortholog rescue studies that used P-element transformation in *D. melanogaster* to test the functional conservation of the protein coding region of essential transcription factors. None of them fully rescued the mutant, yet random genomic insertions of P-element makes it difficult to distinguish whether failures to rescue result from functional divergence of the gene or from position effects of insertion.

Two genes studied are related to gap gene network, maternal gene *bicoid* and pair-rule gene *even-skipped* (Table 1.1). After generating P-element transgenes using *bicoid* coding region from Dipteran species in *D. melanogaster* [28], Gregor *et. al* found that they fail to rescue a *bicoid* mutant and fail to activate *hunchback*, a target of *bicoid*. Similarly, Fujioka *et.*

al found *eve* coding orthologs from distant species, such as worm, grasshopper and mouse, only partially rescue *eve* mutant phenotypes in *D. melanogaster* [29, 30]. As mentioned above, the results are confounded by random P-element insertion and investigation of only the coding regions.

Unlike previous work that is restricted to protein coding regions, or to small enhancers, I focus on the evolution of both coding and noncoding regions, i.e., the intact the whole locus, and eliminate uncontrolled position effects by using site-specific transgenesis. Well controlled genetic experiments reveal functional changes in both coding and noncoding regions of the gap gene *giant*, as well as epistatic interactions between them. Further investigation identified expression defects of *giant* transgenes in *D. melanogaster* background, suggesting co-evolution of gap gene network as a whole.

Table 1.1: **Summary of Ortholog Rescue Studies in *D. melanogaster***

Gene	Region	Ortholog	Protein Identity	Rescue?
eyeless[31]	Coding (Gal4)	Mouse Pax-6	44%	Ecotopic Transformation
bicoid[28]	Coding	*Lucilia sericata*	59.86%	No
bicoid[28]	Coding	*Calliphora vicina*	57.18%	No
even-skipped[29, 30]	Coding	*C. elegans*	36.89%	Partial
even-skipped[29, 30]	Coding	*Tribolium*	52.09%	Partial
even-skipped[29, 30]	Coding	mouse	36.36%	Partial
even-skipped[29, 30]	Coding	*Schistocerca*	NA	Partial
fushi tarazu[32]	Rough wholelocus	*D. hydei*	NA	Partial
otd[33]	Coding (Heat Shock)	Human OTX1	38.14%	Partial
otd[33]	Coding (Heat Shock)	Human OTX2	38.49%	Partial
Antp[34]	Coding+UTR	Mouse Hox-2.2	37.9%	Ectopic Transformation
Dfd[35]	Coding (Heat Shock)	Human Hox-4.2	21.2%	Phenocopy
labial[36]	Coding	Chicken gHoxb-1	NA	Partial

CHAPTER 2

RAPID EVOLUTION OF THE FUNCTIONALLY CONSERVED GAP GENE *GIANT* IN *DROSOPHILA*

2.1 Abstract

Developmental processes in multicellular organisms, and the outcomes they produce, are often evolutionarily conserved. Yet phylogenetic conservation of developmental outcomes is not reflected in functional preservation of the genes regulating these processes, a phenomenon referred to as developmental system drift [37, 38]. Little is known about the evolutionary forces producing change in the molecular details of regulatory genes and their networks while preserving development outcomes. Here we address this void in knowledge by systematically swapping the *Drosophila melanogaster* coding and noncoding regions of the essential gap gene, *giant*, a key regulator of embryonic pattern formation, with orthologous sequences drawn from both closely and distantly related species within the genus. Employing sensitized genetic complementation assays, the loss of a transgene's ability to restore viability occurs across phylogeny at every interspecific level of comparison and includes both coding and noncoding changes. Epistasis is present as well — both between coding and noncoding sequences and, in a dramatic example of change-of-sign epistasis, between the only two coding substitutions separating two very closely related species. A continuous process of functional divergence hidden under conserved phylotypic developmental outcomes requires reconsideration of the prevailing view that the essential genes in conserved regulatory networks are protected from the driving forces of evolutionary change.

2.2 Introduction

The preservation of molecular function is a universal theme in the evolution of life, evident in the myriad of recognizably conserved molecules, proteins, genetic pathways and biochemical processes across phylogeny. All multicellular organisms, for example, possess a shared set of Hox genes regulating cell differentiation and development [39]. Conserved molecular and gene expression phenotypes are believed to reflect intricately buffered developmental pathways that constrain functional evolution of member genes and circuits [40, 41]. Support for this view is dominated by experiments emphasizing partial activity or replaceability of a *Drosophila* gene with transgenes carrying orthologs from species as distant as chicken or even human [33, 34, 42, 43]. Yet, these orthologs (13 instances in total) never fully rescue the mutant phenotype, and they also do not restore viability (Table 1.1). Conservation of developmental outputs might belie functional changes in molecules that govern those outputs [44, 45].

Instances of this tension is apparent in the *Drosophila* gap gene network, a set of exquisitely studied transcription factors expressing early in embryonic development to orchestrate the highly conserved process of insect pattern formation [46]. Spatio-temporal expression of the gap genes are remarkably conserved across *Drosophila* phylogeny, measured at nuclear resolution in three dimensions and time [47]. So too is the cis-regulatory output of the pair-rule gene *even-skipped*, a primary target of the gap genes. When placed in *D. melanogaster*, *eve* enhancers from species in family Sepsidae, a sister group to *Drosophila*, respond to *D. melanogaster* gap proteins by driving pair-rule stripe expression nearly identically to the native *eve* expression pattern [23, 48], this despite extensive rearrangement of the relevant transcription factor binding sites. In contrast, other insect taxa, including mosquitos and moth fly, employ different maternal genes to establish head-to-tail polarity [49]. In the scuttle fly, the initiation and expression of the gap genes are, moreover, quantitatively different than *Drosophila*, though the embryos converge to a similar developmental

phenotype [50, 51].

These scattershot observations underscore the lack of a mechanistic basis for interpreting developmental system drift and highlight the need for careful systematic measurements of regulatory gene functional divergence across a phylogeny. Do these genes evolve? Is functional divergence compartmentalized to changes in cis-regulation, or do the transcription factors evolve as well? And, if so, what is the evolutionary timescale (and phylogenetic consistency) of change? We focused our experimental investigation on the gap gene *giant* (*gt*) across six *Drosophila* species whose phylogenetic ancestries range from about 1 million years ago (MYA) to about 40 MYA [52, 53]. The Giant protein (Gt), a basic leucine zipper transcription factor, is among the earliest proteins expressed zygotically in the blastoderm *Drosophila* embryo to establish landmarks for anterior-posterior patterning and segmentation [13, 15]. Its role as a gap gene is conserved over 350 million years of divergence in Oncopeltus [54], and its DNA-binding domain remains extensively conserved in *Drosophila* and across bilateria evolution [11]. Here we document the pace of *giant* functional divergence in *Drosophila*, both for coding and noncoding regions of the locus, and provide a mechanistic framework for understanding developmental system drift — how a regulatory network can evolve at the molecular level while maintaining a conserved system output.

2.3 Results

2.3.1 Experimental approach:

We employed phiC31 site-specific genetic transformation [55] to study the phenotypic output of *giant* alleles from different species when placed in *D. melanogaster* (*mel*) (Fig. 2.1). We generated *gt* whole-locus genotypes carrying sequences orthologous to the 27kb native locus — an interval that restores viability in a complementation assay with the *mel* control transgene [56] (Fig. 2.1b, c). We also generated interspecies transgene chimeras by swapping

with the *mel* whole-locus sequence either the protein coding or the noncoding region from each of the five other species (Table 2.1; a total of 30 transgenic lines). The fluorescent protein eGFP is commonly appended to proteins as a tag to visualize their cellular distribution and function [57]. As a means for amplifying possible functional differences among *gt* proteins, we added, in a parallel set of transgenes, an eGFP carboxy-terminal tag to our whole-locus and chimeric transgenes. We scored the relative viability (hereafter RV) — defined as the ratio of F1 flies carrying either an interspecies or control transgene, identified with fluorescent eye markers — in the offspring of test crosses carrying a null allele (gt^{X11}) at the native locus (Fig. 2.2, 2.3). We measured RV in both male and female separately, anticipating that transgene restoration of viability by *gt* orthologs might differ in the two sexes [56]. We also sensitized our RV measurements by analyzing flies carrying a single copy of the *gt* transgene.

2.3.2 Functional divergence of distant orthologs:

We first investigated the *gt* ortholog from *D. virilis* (*vir*), the most distant relative in the genus to *mel* (Fig. 2.1b; common ancestor 40 MYA [52]). Whole-locus RV is significantly reduced (RV = 0.56) in males and is essentially lethal in females (Fig. 2.4a, d, g). The *vir* coding region alone restores full RV in both sexes; *vir* noncoding sequences restores full RV in males but reduces RV significantly in females, though not to lethality ((RV = 0.21); Fig. 2.4f). The lethality driven by whole-locus *vir* in females, therefore, requires epistatic contributions from the *vir* noncoding and coding regions (Fig. 2.4f). A *vir* coding contribution to loss of RV is confirmed by the eGFP-tagged version of *vir* coding (RV = 0.52; Fig. 2.4h). We also observed a skewed sex ratio in adults when endogenous gt^{mel} is replaced by two copies of gt^{vir} (male:female = 3.68:1, Table 2.2). Collectively, these results identify functional differences in both coding and noncoding regions and reveal epistasis for RV between the two regions.

Next, we investigated the *gt* ortholog of *D. pseudoobscura* (*pse*). This species is estimated

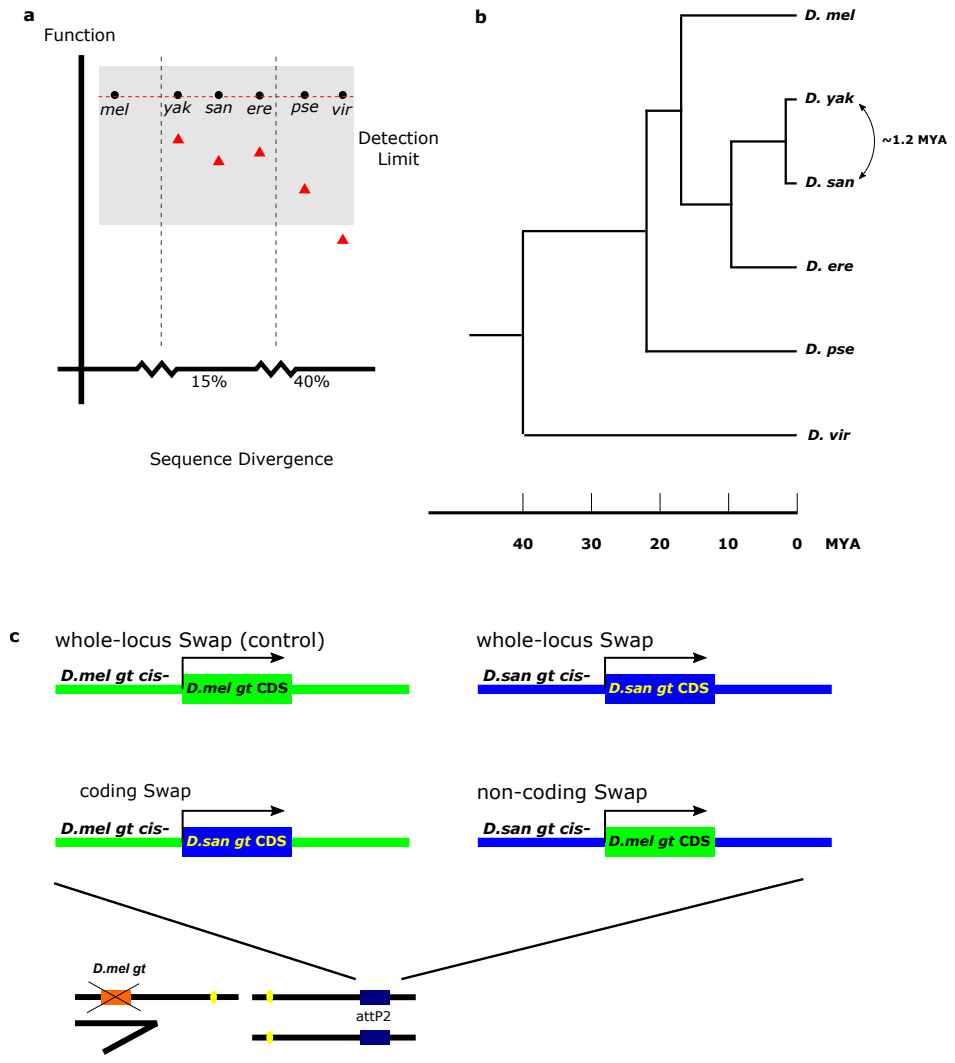


Figure 2.1: **Approach to testing for functional divergence of *gt* transgene orthologs** **a**, Competing hypotheses: Functional stasis (black dots) - *gt* orthologs will be indistinguishable; Functional divergence (red triangles) - *gt* orthologs diverge and will be distinguishable if experimental design has sufficient resolution. In this example, only *vir gt* functional divergence is detectable (shaded region depicts limits of experimental resolution). Vertical dashed lines mark sequence divergence relative to *mel* (Methods). **b**, Phylogenetic relationship of species investigated. **c**, Site-specific phiC31 transgenesis using whole-locus and chimeric *giant*.

Table 2.1: **Transgenic *giant* orthologs in attP2 docking site in *D. melanogaster***

#	Species (coding)	Species (noncoding)	Marker	Size (kb)	Source
Whole-locus Swaps					
1	*D. mel*	*D. mel*	3xP3-eGFP	29.450	This study
2	*D. mel*	*D. mel*	3xP3-DsRed	29.406	This study
3	*D. yak*	*D. yak*	3xP3-DsRed	25.252	This study
4	*D. san*	*D. san*	3xP3-DsRed	25.971	This study
5	*D. ere*	*D. ere*	3xP3-DsRed	23.848	This study
6	*D. pse*	*D. pse*	3xP3-DsRed	34.539	This study
7	*D. vir*	*D. vir*	3xP3-DsRed	28.950	This study
Coding Swaps					
8	*D. yak*	*D. mel*	3xP3-DsRed	29.401	This study
9	*D. san*	*D. mel*	3xP3-DsRed	29.414	This study
10	*D. ere*	*D. mel*	3xP3-DsRed	29.408	This study
11	*D. pse*	*D. mel*	3xP3-DsRed	29.543	This study
12	*D. vir*	*D. mel*	3xP3-DsRed	29.538	This study
Non-coding Swaps					
13	*D. mel*	*D. yak*	3xP3-DsRed	25.254	This study
14	*D. mel*	*D. san*	3xP3-DsRed	25.963	This study
15	*D. mel*	*D. ere*	3xP3-DsRed	23.843	This study
16	*D. mel*	*D. pse*	3xP3-DsRed	34.399	This study
17	*D. mel*	*D. vir*	3xP3-DsRed	28.815	This study
eGFP-tagged Whole-loucs Swaps					
18	*D. mel*	*D. mel*	3xP3-DsRed	30.150	This study
19	*D. yak*	*D. yak*	3xP3-DsRed	25.996	This study
20	*D. san*	*D. san*	3xP3-DsRed	26.715	This study
21	*D. ere*	*D. ere*	3xP3-DsRed	24.592	This study
22	*D. pse*	*D. pse*	3xP3-DsRed	35.283	This study
23	*D. vir*	*D. vir*	3xP3-DsRed	29.694	This study
eGFP-tagged Coding Swaps					
24	*D. yak*	*D. mel*	3xP3-DsRed	30.145	This study
25	*D. san*	*D. mel*	3xP3-DsRed	30.158	This study
26	*D. ere*	*D. mel*	3xP3-DsRed	30.152	This study
27	*D. pse*	*D. mel*	3xP3-DsRed	30.287	This study
28	*D. vir*	*D. mel*	3xP3-DsRed	30.282	This study
eGFP-tagged *yak-san* Chimeras					
29	*D. yak-D. san*	*D. mel*	3xP3-DsRed	30.145	This study
30	*D. san-D. yak*	*D. mel*	3xP3-DsRed	30.158	This study

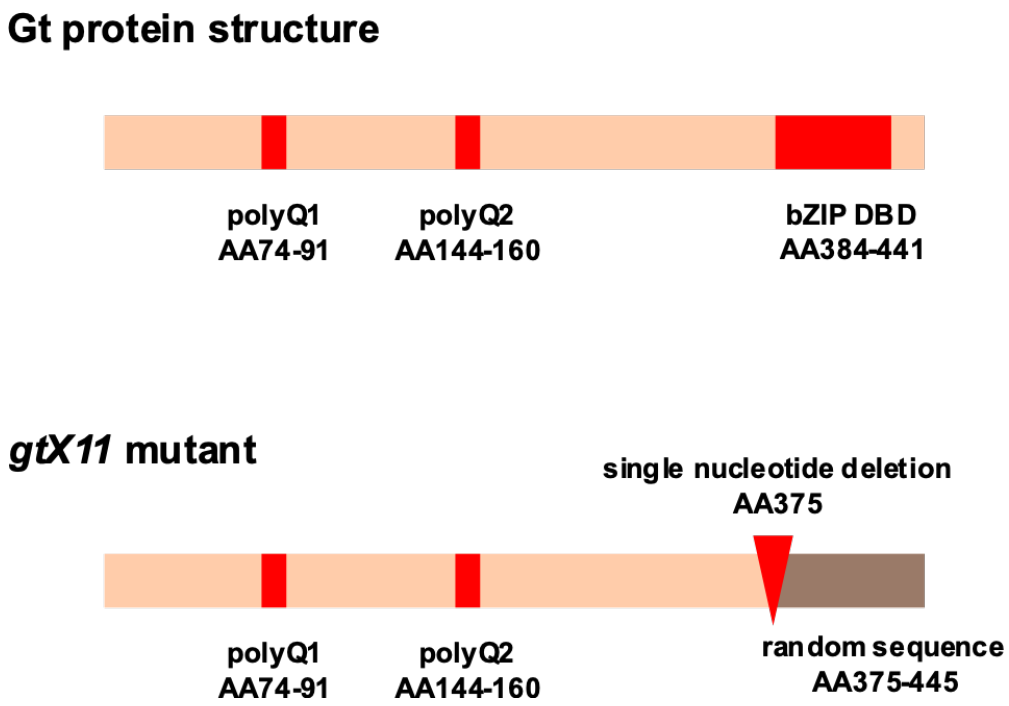


Figure 2.2: gt^{X11} **is a null allele that abolishes the DNA binding domain of Gt (bZIP DBD).**

Table 2.2: **Sex ratio in** gt^{vir} **transgenic line**

Genotype	# Male	# Female	Sex Ratio	p-value
$gt^{X11}; gt^{vir} - eGFP$	862	234	3.68	<2.2e-16

to share a common ancestor with *mel* around 20 MYA [52], half the time separating *mel* from *vir* (Fig. 2.1b). Carriers of the gt^{pse} whole-locus ortholog exhibits reduced RV in both males and females, though to a lesser extent than carriers of gt^{vir} (Fig. 2.4a, d). The reduction in RV by gt^{pse} is largely attributable to noncoding sequence (Fig. 2.4f), and, like gt^{vir}, there is also a coding contribution. Specifically, whereas the gt^{pse} coding shows reduced RV with its gt^{pse} noncoding region, the chimera carrying a gt^{mel} coding region does not (Fig. 2.4a, c). The set of experiments with *pse* and *vir* show striking parallels: strong contributions to reduced RV by the noncoding region; a contribution by the coding regions; and epistatic interaction between coding and noncoding for RV for gt^{vir} and possibly for gt^{pse} (Fig. 2.4a,c).

a

gt Relative Viability (male) (use D.san gt as example)

$$\text{♀♀}\ \frac{FM6}{gt^{X11}} \quad \times \quad \text{♂♂}\ \frac{w^{1118}}{Y};\frac{\text{P\{3xP-GFP, } gt^{mel}\text{\}attP2}}{\text{P\{3xP-DsRed, } gt^{san}\text{\}attP2}}$$

↓

Count Relative Number of F1 males

Genotype	Expectation	Marker
$\frac{gt^{X11}}{Y};\frac{\text{P\{3xP-GFP, } gt^{mel}\text{\}attP2}}{+}$	1	3xP3-GFP
$\frac{gt^{X11}}{Y};\frac{\text{P\{3xP-DsRed, } gt^{san}\text{\}attP2}}{+}$	Relative Viability	3xP3-DsRed

b

gt Relative Viability (female) (use D.san gt as example)

$$\text{♀♀}\ \frac{FM6}{gt^{X11}} \quad \times \quad \text{♂♂}\ \frac{gt^{X11}}{Y};\frac{\text{P\{3xP-GFP, } gt^{mel}\text{\}attP2}}{\text{P\{3xP-DsRed, } gt^{san}\text{\}attP2}}$$

↓

Count Relative Number of F1 females

Genotype	Expectation	Marker
$\frac{gt^{X11}}{gt^{X11}};\frac{\text{P\{3xP-GFP, } gt^{mel}\text{\}attP2}}{+}$	1	3xP3-GFP
$\frac{gt^{X11}}{gt^{X11}};\frac{\text{P\{3xP-DsRed, } gt^{san}\text{\}attP2}}{+}$	Relative Viability	3xP3-DsRed

Figure 2.3: **Crossing scheme to measure transgene relative viability (RV) with fluorescent eye markers.**

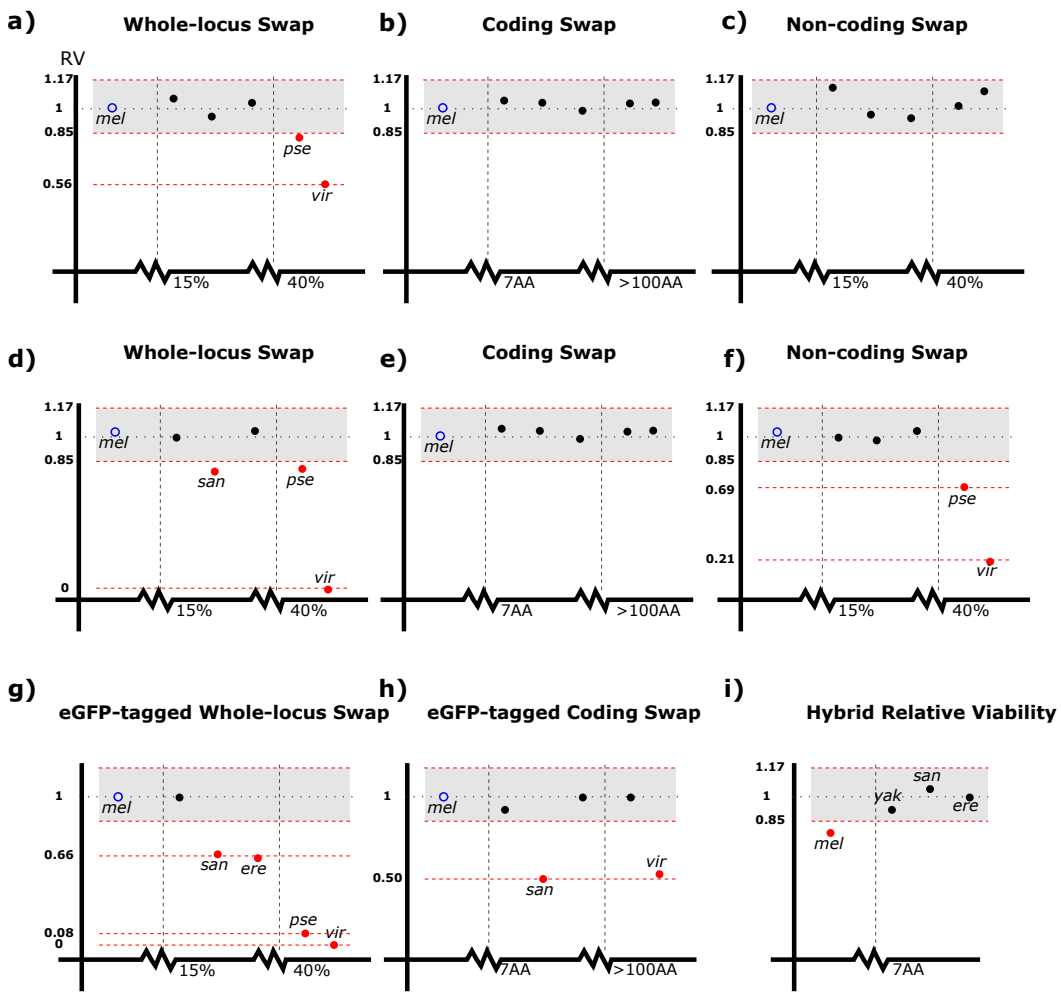


Figure 2.4: **Complementation assays reveal extensive functional divergence across *Drosophila* phylogeny**

Single copy transgene rescue in males (**a-c**) and females (**d-h**). **d**, Whole-locus *vir gt* restores *mel* female viability at a low rate (<0.2% RV). **g-h**, Female RV using eGFP-tagged *gt* transgenes. Whole-locus eGFP-tagged *vir gt* restores *mel* female viability only when two copies are present. **i**, *mel gt* coding region is deleterious in *mel/san* hybrids. For **a-h**, the species order is (left-right) *mel*, *yak*, *san*, *ere*, *pse* and *vir*. RV values significantly different from *mel* are labeled in red. For illustrative purpose, shaded region represents 80% power to detect at p¡0.05 a 15% difference in viability between control and experimental transgene (e.g., RV=0.85) for a sample of 1240 adults.

2.3.3 Functional divergence of closely related orthologs:

D. yakuba (*yak*), *D. santomea* (*san*) and *D. erecta* (*ere*) belong to the same phylogenetic clade that has a common ancestor with *mel* 10 MYA [52] (Fig. 2.1b); one of them, *san*, produces viable hybrids with *mel*. Reduced RV is observed in two of the three species: *san* — gt^{san} whole locus (Fig. 2.4d), gt^{san} eGFP-tagged whole locus, and gt^{san} coding-only (Fig. 2.4g, h); and *ere* — gt^{ere} eGFP-tagged whole locus. Thus, even on the relatively short timescale of 10MY separating this clade of species from *mel*, the experiments functionally distinguish their *gt* alleles from the *mel* ortholog.

2.3.4 Species hybrids:

Our viability assays thus far reveal functional differences between the *mel* allele and the *san*, *ere*, *pse* and *vir* *gt* orthologs. Unresolved is whether *yak*, the remaining species in our *gt* analysis, might also have functionally diverged from *mel* *gt*, albeit more subtly. We investigated this question with species hybrids. Crosses between *mel* females and *san* males produce sterile hybrid female progenies only. We have recently shown that *mel* Gt, differing by seven amino acid substitutions from *san* Gt (Table 2.3), causes reduced female viability in the hybrid [58]. Acting on this finding, we tested additional *gt* transgene orthologs in *mel/san* hybrids by crossing *mel* females hemizygous for a transgene to *san* males (Fig. 2.5). In this cross, RV is estimated from the number of hybrid F1 flies carrying either the *gt* transgene or a control chromosome bearing no transgene. Chimeric transgenes whose coding regions have been replaced by *yak*, *ere* or *san* orthologs, under the regulatory control of *mel* noncoding region, all eliminate the deleterious effect of *mel* Gt in hybrid females (Fig. 2.4i). In this sensitized hybrid genetic environment, we are thus able to place the functional divergence in the protein leading to reduced hybrid viability caused by *mel* Gt to changes on the phylogenetic branch leading to *mel* itself.

Table 2.3: **Pairwise amino acid differences between Gt protein (excluding length difference)**

	D. melanogaster	*D. yakuba*	*D. santomea*
D. melanogaster			
D. yakuba	6		
D. santomea	7	1	
D. erecta	8	6	7

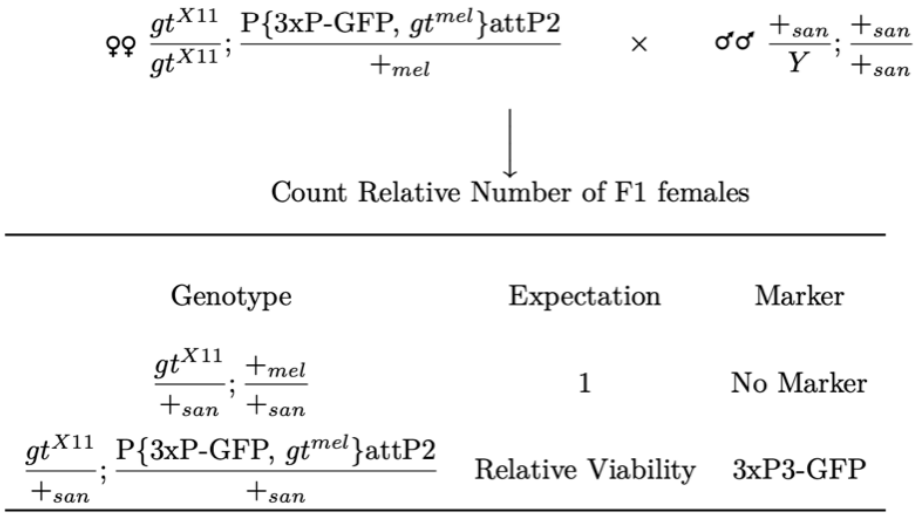


Figure 2.5: **Relative viability assay (RV) in *D. mel/D. san* species hybrid.**

2.3.5 Functional divergence of the san Gt protein:

Transgenic *yak* and *san gt* proteins under the regulatory control of *mel* noncoding sequence have significantly different RV in *mel* (Fig. 2.4h). This means that our experiments have detected *gt* functional divergence at every timescale separating the six species employed in our analysis. *san* and *yak* have a common ancestor estimated to be only 1.2 MYA [53], and Gt^{yak} and Gt^{san} proteins differ by only two substitutions — A351V and +4Q — (Fig. 2.6). Gt^{yak} is identical at both sites to the allele in *D. teissieri*, the closest outgroup species, indicating that the Gt^{yak} carries both ancestral states (Fig. 2.7). We confirmed that the *yak* and *san* Gt alleles used in this experiment are both common alleles, not unique to specific populations of either species (Fig. 2.7). With only two substitutions, both of which occurred in *san*, there are only two possible intermediate evolutionary paths. We investigated both of them in *mel* with eGFP-tagged transgenes carrying the two single-substitution genotypes under the regulatory control of *mel* noncoding sequence. Our RV assay reveals a significant increase in RV for the +4Q substitution alone and a significant decrease for the A351V substitution alone. Together, the two substitutions produce the most severe decrease in RV (Fig. 2.6b). Thus, both single substitutions have significant RV effects, the two possible trajectories differ significantly, and there is sign epistasis along one path. To summarize, no individual substitution in this sensitized experimental system is functionally inert, and together the two substitutions interact at the level of RV.

2.4 Discussion

Our experimental results reveal a continuous process of functional divergence across *Drosophila* phylogeny and timescales, a sharp refutation of interchangeability of genes regulating evolutionarily conserved developmental processes. Our sensitized assays, employing appropriate transgene controls inserted into the same chromosomal docking site, identified functional divergence attributable to both coding and noncoding regions along nearly every branch of

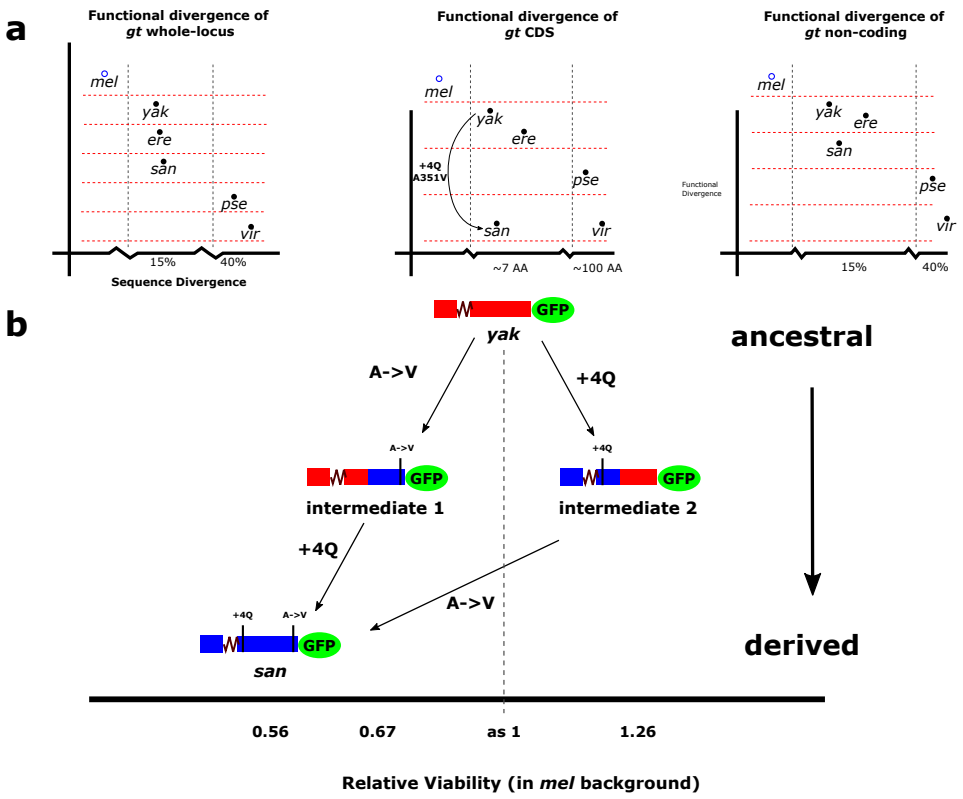


Figure 2.6: **The two possible evolutionary intermediates of Gt coding substitutions between *yak* and *san* have distinct (and opposite) relative viability effects in *mel*** **a**, Summary of *gt* functional divergence inferred from whole-locus or chimeric transgenes. Horizontal dashed lines demarcate distinguishable orthologs. **b**, Reconstructed ancestral states of *yak/san gt* protein and alternative evolutionary paths of divergence. Both alternative trajectories require non-neutral intermediates.

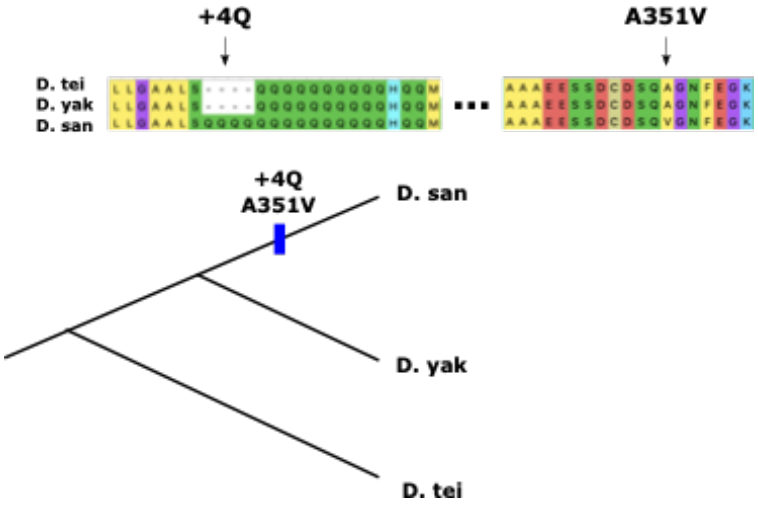
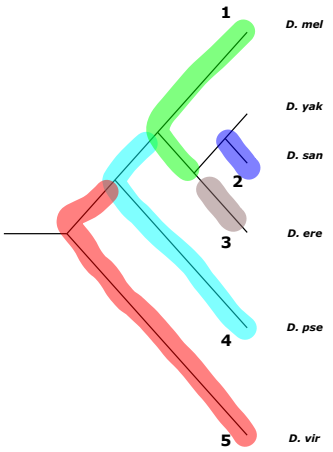


Figure 2.7: **Sequence alignment of Gt polyQ region between *D. yakuba*, *D. santomea* and *D. teissieri*.**
Of 110 *D. yakuba* alleles, 109 alleles are identical to the sequence shown. Of 18 *D. teissieri* alleles, all 18 are identical at this polyQ site. Of 9 *D. san* alleles, 8 are identical to the sequence shown while 1 is 2Q shorter.



Branch w/ Inferred Changes	Where	Evidence	Relevant Figures
1	coding	Deleterious to *mel/san* Hybrid	Fig. 2i
2	coding	eGFP-tagged *san* and *yak* CDS have different RV	Fig. 2g, h Fig. 3
3	whole-locus	eGFP-tagged *ere* and *yak* whole-locus have different RV	Fig. 2g, h
4	coding + noncoding	*pse* whole-locus, with or without eGFP, has different RV	Fig. 2a, c, g
5	coding + non-coding	*vir* whole-locus, with or without eGFP, has different RV, and is also different from *pse*	Fig. 2a-h

Figure 2.8: **Continual functional divergence of the *gt* locus in *Drosophila***
A parsimonious reconstruction of functional changes in *gt* mapped onto phylogenic branches, marked by different colors. Experimental evidence for viability differences attributable whole-locus or chimeric transgene divergence is listed alongside each branch. Functional divergence in noncoding regions inferred from species-specific epistasis is not included because of uncertainty about branch assignment.

the phylogeny (Fig. 2.8). Most published studies of orthologous gene function (see Table 1.1), i.e., interchangeability, investigate only protein coding regions and not the whole locus. Our study reveals limitations in this approach: none of protein coding regions alone from any of the five species significantly reduced RV when driven by the *mel* noncoding region (Fig. 2.4b, e). Our finding points to the whole locus as an integral target for *gt* functional evolution.

Functional changes in *gt* are relevant to understanding the genetics of interspecies hybrid incompatibility. Here, our experiments amplified on a recent finding that identified the protein coding region of *gt* in causing inviability in hybrids between *mel* and *san* [58]. We mapped those differences to substitutions in the phylogenetic branch leading to *mel* from its common ancestor with *yak/san/ere*. This functional divergence is not unique to this single lineage, however, but rather is one instance of a continuous process of functional divergence across *Drosophila* phylogeny (Fig. 2.8). Hybrid incompatibility generally results from functional divergence of two interacting genes, one in each of two species, which when brought together in a hybrid, fail to function properly. The functional divergence in gt^{mel} protein is, therefore, likely accompanied by similar functional divergence in one or more interacting partners in *san*. In a search for a partner to *gt*, we discovered that orthologs of the gap gene *tailless* from *mel* and *san*, like *gt*, also differ in their effects on viability in the hybrid [58]. In a broader context, continuous functional evolution of *gt*, as documented here, may be representative of other "conserved" genes in the gap gene network, and illustrative of the process of rapid molecular evolution leading to hybrid incompatibility.

We believe our unequivocal findings in experimental assays — a rapid, continuous process of *gt* functional evolution in coding and noncoding regions — are relevant to understanding population genetic mechanisms governing *gt* evolution. In general, one expects natural selection to be many orders of magnitude more sensitive to the fitness effects of subtle functional changes than those that can be measured in our laboratory experiment. In this

context, no organism has received more attention than *Drosophila* in a quest to understand the extent of adaptive evolution driving gene and genome evolution, and there is now near-universal agreement that natural selection is the predominant driving force in these large-population-size species [59, 60]. Our findings suggest that the very same mechanism — natural selection — may be responsible for the continuous pace of *gt* functional evolution. Especially illuminating are the two coding substitutions between the very closely related species *yak* and *san*: both evolutionary intermediates, and the combination of substitutions together, all exhibit significant viability effects in a *mel* genetic background. The fact that these *gt* intermediates are distinctly different in the same genetic background suggests to us that natural selection is likely to have been involved in their substitution, even if the fitness effects are smaller in their ancestral backgrounds.

Under this mechanistic framework, the conservation of gap gene network output is achieved both by selective constraint acting on the network, as well as by a continuous process of functional refinement to individual genes and their cross-regulatory interactions. The continuous functional divergence of gap genes also gives rise, inevitably, to changes in the detailed molecular mechanisms by which the network directs pattern formation, a characteristic of developmental system drift. Our discovery of rapid functional divergence of *gt* requires reassessment of the tempo and mode of molecular evolution of regulatory genes belonging to conserved developmental systems.

2.5 Acknowledgements

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2.6 Contributions

W.C. and M.K. conceived the project and shared in writing the manuscript. W.C. performed the transgenic and viability experiments and carried out the analysis.

CHAPTER 3

GAP GENES ARE INVOLVED IN SPECIES HYBRID INVIABILITY

3.1 Abstract

Evolved changes within species lead to the inevitable loss of viability in hybrids. Inviability is also a convenient phenotype to genetically map and validate functionally divergent genes and pathways differentiating closely related species. Here we identify the *Drosophila melanogaster* form of the highly conserved essential gap gene *giant* (*gt*) as a key genetic determinant of hybrid inviability in crosses with *D. santomea*. We show that the coding region of this allele in *D. melanogaster/D. santomea* hybrids is sufficient to cause embryonic inviability not seen in either pure species. Further genetic analysis indicates that *tailless* (*tll*), another gap gene, is also involved in the hybrid defects. *giant* and *tll* are both members of the gap gene network of transcription factors that participate in establishing anterior-posterior specification of the Dipteran embryo, a highly conserved developmental process. Genes whose outputs in this process are functionally conserved nevertheless evolve over short timescales to cause inviability in hybrids.

3.2 Introduction

The formation and persistence of species involves the buildup of barriers to gene flow as genome divergence accrues over time. These genetic barriers arise as species differentiate and involve breakdowns in a variety of cellular, developmental, and behavioral processes;

. This is in collaboration with Dr. Daniel R. Matute at UNC Chapel Hill, who performed the GWAS analysis and deficiency mapping. Details can be found in the acknowledgements section below.

eventually these barriers lead to reduced fitness of hybrids relative to pure species [61, 62]. Hybrid inviability (HI), the condition in which interspecific hybrids do not achieve adulthood because of developmental defects, is one of these barriers. The question of how natural selection could allow such maladaptive and extreme phenotypes has been a subject of intense interest to evolutionary biologists and developmental geneticists alike [63–65] . Dobzhansky [66] and Muller [67] formulated a widely regarded genetic model in which hybrid defects, including HI, arise as a collateral effect of evolutionary divergence between populations that acquire incompatible changes in interacting loci, or Dobzhansky-Muller incompatibilities (DMIs). Because the divergent alleles at the DMIs loci only have fitness costs when they are forced together in hybrids, natural selection does not oppose the changes in each species. There is substantial evidence in support of the DM model [68], including nearly a dozen instances in which HI alleles have been identified to the gene level [69–78]. Some of these alleles have been shown to evolve through positive selection [71, 72, 74, 79, 80] while others show no clear signature of selection [81]. The variety of both the gene type in HI and the processes that drive allelic divergence indicates that HI can occur in a multitude of ways [82].

Developmental processes are generally guided by interacting regulatory genes and elements, making them a rich source of potential candidates for HI. The question arises, however, as to whether they evolve functionally at a sufficient pace to fuel the rapid formation of DMIs in the speciation process. Developmental processes and their outputs are often deeply conserved phylogenetically, often displaying conserved functional attributes [45]. Large-effect mutations to developmental regulators are often incompatible with life, and these genes tend to be evolutionarily conserved both in sequence and phenotypic output [83–86]. While the developmental phenotypes in which these genes are involved generally remain similar across species, the genetic underpinnings of these crucial phenotypes may evolve [38, 87–90], and if their pace of functional evolution is sufficiently fast, could contribute to HI.

Several lines of evidence elevate this possibility and thus challenge the notion that the conservation and selective constraints on regulatory genes, and the processes they direct, immunize them from possibly contributing to HI. First, recent work by us on a canonical example of a conserved regulatory gene and pathway — the gap gene giant in *Drosophila* and the process of pattern formation— shows contrary to expectations that this gene diverges functionally at a rapid and continuous pace in the genus, as evidenced by loss of viability in carefully controlled transgenic complementation experiments [91].

Second, several cases of embryonic hybrid lethality have been identified in *Drosophila*: female hybrid inviability in hybrids between *D. montana* females and *D. texana* males [92], female lethality in hybrids of *D. melanogaster* females and *D. simulans* males [93]; and male embryonic lethality in hybrids of *D. melanogaster* females and *D. santomea* males [94] (see below).

Third, even for developmental phenotypes that remain similar across phylogeny, their genetic underpinnings change occasionally in substantial ways [38, 87–90]. Referred to as developmental systems drift —functional divergence of genes in developmental regulatory pathways with conserved outputs — has also been documented for nematode vulva induction [37, 95], and sex determination in frogs [96]. Developmental systems drift has also been proposed to lead to hybrid defects [97]. If genetic changes occur in different directions in two species, their hybrids might not have a functional pathway to produce the required developmental phenotype. This is a simple—but to date unsubstantiated— way to explain HI.

3.3 Results

We first explored whether *Drosophila* hybrids other than *mel/san* also showed embryonic hybrid inviability and abdominal ablations. We examined the embryonic lethality rates and associated cuticular phenotypes from hybrid crosses between various species within the

melanogaster supercomplex and species of the *yakuba* subgroup (Fig. 3.1). Hybrid embryos between *D. santomea* and the other species in the *yakuba* subgroup — *D. teissieri* and *D. yakuba* — are mostly viable and showed no abdominal ablations in any of the six possible reciprocal crosses (Table 3.1). Embryonic inviability is rare among crosses between collections of these species (but see [98]). Hybrid inviability is also non-existent in hybrids between collections of species of the *simulans* species group — *D. simulans* (*sim*), *D. mauritiana* (*mau*) and *D. sechellia* (*sec*). The embryonic viability of male *mel/sim* and *mel/mau* hybrids is high in all cases. The few rare embryos that failed to develop and hatch showed no abdominal ablations.

Crosses between females of two species of the *sim* clade (*sim* and *mau*) and san males showed high levels of hybrid inviability, especially of males. These dead hybrids show the characteristic abdominal ablation. This shared phenotype with *mel* in hybrids with *san* indicated that genetic changes that ultimately lead to abdominal ablations must have occurred before the split of the three species, approximately five million years ago [99, 100]. Genetic analysis with these crosses also confirm that the locus involved in HI resides in the X-chromosome. We next identified the genetic locus that causes hybrid inviability by abdominal ablation using genetic tools available in *D. melanogaster*.

3.3.1 Genetic mapping shows giant is involved in mel/san HI

To identify the X-linked allele involved in HI, we did a genome-wide association study using a panel of inbred *D. melanogaster* lines (i.e., DGRP, [101, 102]) and studied whether any genetic variant segregating in *D. melanogaster* was associated with total inviability in hybrids with *D. santomea*. In all crosses, the hybrid males die, but the females show differential rates of survival. We found a strong association between a 16.5 kb haplotype in the X-chromosome and high levels of HI (Fig. 3.2A). This haplotype harbors two genes: *CG32797* and *gt*, and overlaps with a segment of the *D. melanogaster* X-chromosome (X_{mel}) previously associated

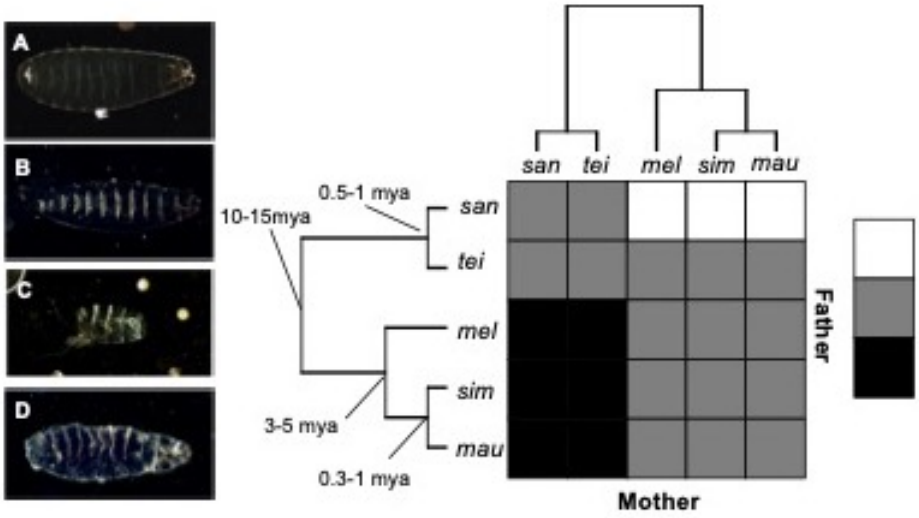

Figure 3.1: **All the X chromosomes from the *mel* supercomplex cause inviability in *mel/san* hybrids but not in *mel/tei* hybrids.**
Unlike pure species (**A**, *D. santomea*; **B**, *D. melanogaster*), F1 *mel/san* hybrid males show abdominal ablations (**C**). The nature of the defect in these hybrid males is identical to that seen in *sim/san* and *mau/san* hybrid males. Females from the same cross also show such ablations but more rarely and the majority of dead embryos show a complete abdomen. Hybrids between females from the *melanogaster* supercomplex and *D. teissieri* males show little embryonic lethality and among the few dead embryos there are no abdominal ablations (e.g., **D**, *mel/tei* hybrid male).

Table 3.1: **Rates of ablation in F1 hybrids between the species of the *yakuba* species complex.**

Cross (Female X Male)	# Embryos	# Dead Embryos	# Ablated Embryos
D. melanogaster X D. santomea	120	2	0
D. yakuba X D. yakuba	162	4	0
D. teissieri X D. teissieri	149	7	0
D. santomea X D. yakuba	102	16	0
D. santomea X D. teissieri	110	21	0
D. yakuba X D. santomea	123	19	0
D. yakuba X D. teissieri	98	16	0
D. teissieri X D. santomea	101	15	0
D. teissieri X D. yakuba	98	10	0

with male HI [103].

A similar GWAS for the incidence of abdominal ablations (Fig. 3.2B) showed the frequency of abdominal ablations in *mel/san* hybrids (both sexes pooled) is associated with an X_{mel} haplotype that contains six genes (*CG32797, gt, tko, boi, z, and trol*). This interval also overlaps with the region associated with HI. Gene(s) on the tip of X_{mel} cause both HI and abdominal ablations.

Next, we generated *mel/san* hybrid males with the X-chromosome from *D. santomea* (X_{san}) and studied whether introducing small segments of X_{mel} would cause HI. *mel/san* hybrid males with the abdominal ablation typically inherit a X_{mel} chromosome and a Y chromosome from *san* (Y_{san}). By using mel attached-X chromosomes, we manipulated chromosomal inheritance and generated hybrid F1 males that inherit a X_{san} and a *D. melanogaster* Y chromosome (Y_{mel}). These animals do not manifest the abdominal ablation and are regularly viable [94]. We obtained a similar result when we crossed *sim* attached-X females to *san* males; the cross produces viable hybrid F1 males with a X_{san} and a *D. simulans* Y chromosome. To refine the region of the X_{mel} chromosome carrying the determinant of the abdominal ablation phenotype, we introduced small segments of X_{mel} (containing *mel* alle-

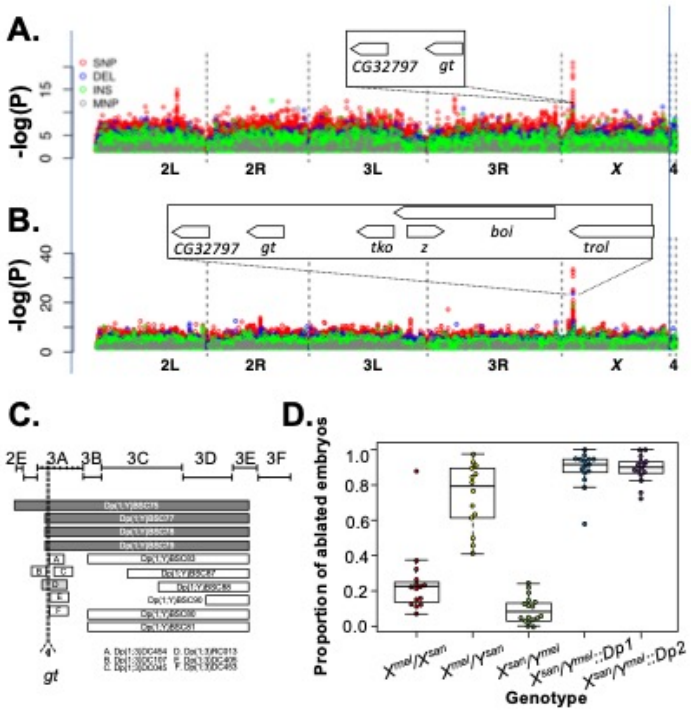

Figure 3.2: **A *D. melanogaster* X-chromosome haplotype that encompasses gt_{mel} is associated with hybrid inviability and abdominal ablations in *mel/san* hybrid males.**

A, GWAS of the genetic causes of hybrid inviability in *mel/san* hybrids (both sexes pooled) using segregating variation within *D. melanogaster*. **B**, GWAS of the genetic causes of abdominal ablations in *mel/san* hybrids (both sexes pooled). Green: insertions, blue: deletions, red: SNPs, purple: multinucleotide polymorphisms. *C*, We introduced small X_{mel} pieces attached to Y_{mel} to identify X_{mel}-linked alleles that cause hybrid inviability in in *mel/san* hybrids males. We narrowed down the allele that causes HI to an interval of X_{mel} comprising 3A3 which only contains *giant*. White bars show duplications with no abdominal ablations. The light grey bar shows a duplication with a moderate rate of abdominal ablations; dark grey show duplications with high levels of abdominal ablations. **D**, Relative frequency of abdominal defects in five different hybrid genotypes from *D. melanogaster* and *D. santomea* crosses. *mel/san* hybrid males (X_{mel}/Y_{san}) frequently show a lethal characteristic abdominal ablation that is also present in some *mel/san* hybrid females. The reciprocal *mel/san* hybrid males (X_{san}/Y_{mel}) routinely survive and the few embryos who die do not show abdominal ablations.

les ranging from approximately 10 to 100 genes each into the genetic makeup of X_{san}/Y_{mel} hybrid F1 males. Previous results had shown that the distal tip of X_{mel} contains an allele that causes inviability in hybrid males. Measuring the rates of hybrid embryonic lethality in the presence of nested Dp(1;Y) and Dp(1:3) duplications of the X_{mel} chromosome allowed us to refine the genomic interval to the region encompassing the cytological region 3A3 (dmel6: 2,410,000-2,580,000). Male hybrid embryos harboring X_{san} and Y_{mel} with duplications containing the 3A3 portion of X_{mel} fail to hatch (Fig. 3.2C). They also show the striking abdominal ablation common in *mel/san* hybrid males carrying the full-length X_{mel} and Y_{san} (Fig. 3.2C). Previous results had shown that the distal tip of X_{mel} contains an allele that causes inviability in hybrid males. Measuring the rates of hybrid embryonic lethality in the presence of nested Dp(1;Y) and Dp(1:3) duplications of the X_{mel} chromosome allowed us to refine the genomic interval to the region encompassing the cytological region 3A3 (dmel6: 2,410,000-2,580,000).

Male hybrid embryos harboring X_{san} and X_{mel} duplications containing the 3A3 portion fail to hatch (Fig. 3.2C). They also show the striking abdominal ablation common in *mel/san* hybrid males carrying the full-length X_{mel} and Y_{san} (Fig. 3.2D). Notably, the overlapping region of the duplications that cause this ablation contains only one gene: *giant*. The ablation phenotype is not found in the presence of other lethality-inducing fragments from elsewhere on the X_{mel} chromosome. gt_{mel} caused HI with abdominal ablation in hybrids with all examined lines from *D. santomea* confirming that the interaction is not a line specific effect. These experiments suggest that introducing a gt_{mel} allele in a X_{san}/Y_{mel} male hybrid background is sufficient to cause lethality with abdominal ablation.

3.3.2 The gt_{mel} allele causes inviability in both male and female hybrids

The crosses described above address the effect of the presence of a gt_{mel} allele on HI in *mel/san* hybrids. Next, we studied the absence of a functional a gt_{mel} allele using a gt_{mel}

null-allele, gt^{X11}_{mel} [13]. Hybrid male embryos carrying gt^{X11} were less likely to be abdominally ablated compared to other X_{mel}/Y_{san} hybrids (mean number of FM7/Y_{san} ablated embryos: 80.33%, Fig. 3.3A; mean proportion of gt^{X11}_{mel}/Y_{san} ablated embryos: 4.41%, Fig. 3.3B; t-test comparing the frequency of ablations in FM7/Y_{san} males and gt^{X11}_{mel}/Y_{san} males: t = 23.972, df = 21.614, P <1 x 10-10).

X_{mel}/Y_{san} males with a null allele of gt_{mel} (i.e., gt^{X11}_{mel}/Y_{san}) do not show increased viability. This result is not surprising because X_{mel} harbors at least eight more dominant (or semidominant) factors that also cause embryonic inviability [103], which may be specifically lethal to *mel/san* hybrids. In contrast, male hybrid embryos carrying a X_{mel} chromosome and null mutations of the genes adjacent to *gt* (*boi, trol,* and *tko*) show abdominal ablations. These experiments demonstrate that gt_{mel} is necessary for the abdominal ablation in *mel/san* hybrid males, but is not the only allele that can cause inviability in this hybrid individuals.

We also tested whether gt_{mel} had a deleterious effect on hybrid females by scoring whether any allele on X_{mel} between the cytological positions 2F1 and 3A4 affected the fitness of *mel/san* hybrid females. We used deficiency mapping and scored the number of df(i)/*san* hybrid females compared to their FM7/*san* sisters [70]. Deviations from 1:1 expected ratio indicate the presence of alleles involved in HI. If FM7/*san* hybrids survive at a higher rate than df(i)/*san*, then the uncovered *san* segment contains a recessive allele involved in HI. If FM7/*san* hybrids survive at a lower rate than df(i)/*san* hybrids, then the absent *mel* segment contains a dominant (or semi-dominant) allele involved in HI. The initial screening using the line *san* Carv2015.16 showed that hybrid females with a deletion for the X_{mel} region between 3A2 and 3A3 (which contains gt_{mel}) are more viable than hybrid females that carry the balancer chromosome with gt_{mel} (df/*san* to FM7/*san* ratio = 2:1; Fig. 3.3A). The minimal interval that harbors the female HI determinant contains six genes, one of them being *gt*.

We further refined the genetic analysis of this region by testing null alleles of the genes in

the interval. Of the four genetically characterized genes in the mapped interval, 3A3, only mutants of gt_{mel} lead to an increase of female hybrid viability (Table S7). Females that carry gt_{mel} (FM7::GFP/*san*) emerged at a lesser frequency than their null allele-carrying sisters (gt^{X11}_{mel}/*san* Fig. 3.3B).

The abdominal ablation defect that is characteristic of *mel/san* males is also present in some proportion of *mel/san* female embryos that die [94, 103]. We next tested whether gt_{mel} causes abdominal ablations in female as it does in male hybrids. gt_{mel}-carrying females (FM7::GFP/*san*; Fig. 3.3B) have abdominal ablations more frequently than their gt^{X11}_{mel}/*san* sisters (Fig. 3.3B; t-test comparing the frequency of ablations in FM7/X_{san} males and gt^{X11}/X_{san} females t = 6.853, df = 16.147, P = 3.699 x 10-6). These results indicate that gt_{mel}, the primary genetic determinant of the abdominal ablation in male *mel/san* hybrids, is sufficient to render some hybrid females inviable by inducing abdominal ablations. This trait varies across san lines, however, ranging from little inviability (e.g., san SYN2005, df/*san* to FM7/*san* ratio = 1:1; Fig. 3.3C) to almost complete inviability (e.g., san Rain42; df/*san* to FM7/san ratio = 4:1; Fig. 3.3C).

Two genes adjacent to *gt*, *CG32797* and *CG12496* have no available mutant stocks. The former, *CG32797*, is not expressed in embryos [104, 105] and is an unlikely candidate to cause embryonic inviability in *mel/san* hybrids. *CG12496* is expressed in the early embryos (2-14 hours after egg laying), so an undetectable role in HI cannot be excluded. However, the results for the gt_{mel} allele explain a large proportion of the inviability and abdominal ablation phenotypes we observe with the larger deletion (Fig. 3.3B).

Taken together, the mapping efforts are consistent and reveal that the gt_{mel} allele is: (1) necessary and sufficient to cause abdominal ablation defects; (2) contributes to hybrid inviability in both male and female *mel/san* hybrids; and (3) causes defects in that are specific to hybrids that have a *san* father.

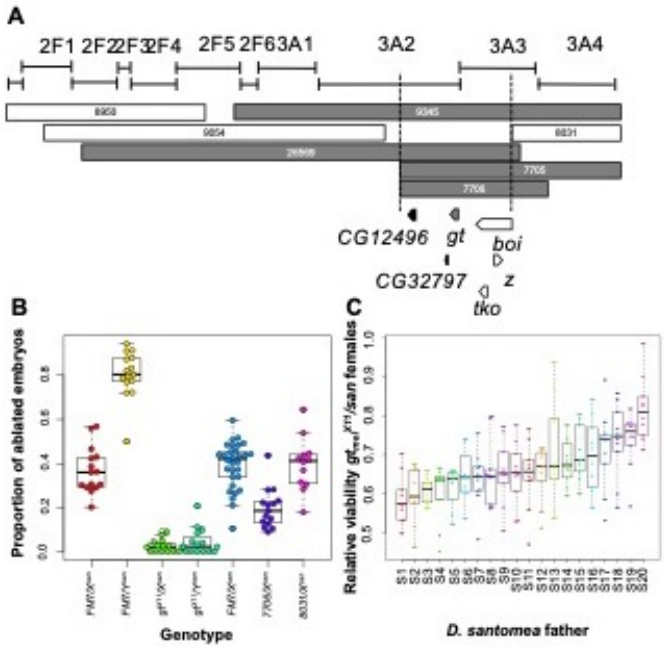

Figure 3.3: gt_{mel} **causes HI and abdominal ablations in *mel/san* females.** **A**, Deficiency mapping and null-allele mapping revealed that giant also causes hybrid inviability in hybrid females. Grey: deficiencies that increase viability of *mel/san* F1 hybrid females. White: deficiencies that do not increase viability. **B**, Relative gt_{mel}^{X11}/X_{san} female viability in twenty *D. santomea* isofemale lines. Boxes in the boxplot are ordinated from the lower median (left) to the highest (right). S1: SYN2005; S2: sanCAR1490; S3: sanCOST1250.5; S4: sanCOST1270.1; S5: sanOBAT1200; S6: sanOBAT1200.2; S7: sanRain39; S8: sanCAR1600.3; S9: Carv2015.1; S10: Carv2015.5; S11: Carv2015.11; S12: Carv2015.16; S13: Pico1680.1; S14: Pico1659.2; S15: Pico1659.3; S16: Amelia2015.1; S17: Amelia2015.6; S18: Amelia2015.12; S19: A1200.7; S20: Rain42. **C**, When females fail to hatch, it is not uncommon for them to be abdominally ablated. The presence of gt_{mel} increases the frequency of abdominal ablations.

3.3.3 Transgenic swaps confirm D. melanogaster gt causes hybrid inviability

To identify the specific region(s) of the *gt* locus responsible for causing *mel/san* hybrid inviability, we generated whole-locus *gt* transgenes from *mel* and *san*, as well as coding/noncoding chimeras between them, which we integrated into the *D. melanogaster* 3rd chromosome docking site attP2 [55, 106]; we replaced the endogenous gt_{mel} with these *gt* transgenes in flies carrying the *gt* null allele gt^{X11}_{mel}. The whole-locus gt_{mel}, also designated $gt_{mel:mel}$ to identify the species source of coding and noncoding regions respectively, is a 27kb segment of DNA that rescues lethality in the gt^{X11}_{mel} null mutant [56]. First, we asked whether the *gt* transgenes might carry cryptic functional elements, different from *gt* itself, that might affect viability. To disrupt the function of *gt* specifically, a 1.73kb removable piggyBac cassette was inserted into the 5'UTR of every *gt* transgene to conditionally eliminate the gene product (Fig. 3.4B). All of them failed to restore viability in a gt^{X11}_{mel} mutant. To restore the wildtype transgene allele, a piggyBac transposase was employed to remove the piggyBac cassette [107]. This precise excision, confirmed with sequencing, does not leave behind any additional DNA. Each pair of *gt* transgenes with or without the piggyBac cassette have identical genetic backgrounds. The restored *gt* transgenes rescue lethality in a gt^{X11}_{mel} mutant (Tables 3.2 and 3.3). Thus, transgene *gt* expression itself is necessary for viability in this rescue assay.

We carried out three sets of crosses with the pure-species and chimeric transgenes (Fig. 3.4) to measure *gt* contribution in hybrids to embryonic viability (Fig. 3.4C), and female adult viability (Fig. 3.4D). We first measured the relative viability of transgene alleles in gt^{X11}_{mel}/san hybrid embryos (both sexes pooled). *mel/san* hybrids carrying gt^{X11}_{mel} and the $gt_{mel:mel}$ transgene show a high prevalence of the embryonic lethal phenotype (Tables 3.2). $gt_{san:mel}$, an allele with the gt_{san} non-coding DNA and the coding sequence from gt_{mel}, caused the embryonic lethality in *mel/san* hybrids at a similar rate than that caused by $gt_{mel:mel}$ (Ta-

bles 3.2). In contrast, $gt_{mel:san}$, an allele with the gt_{mel} non-coding and gt_{san} coding, increased viability compared to $gt_{mel:mel}$ hybrids (Tables 3.2). Notably, the $gt_{san:san}$ allele, which has the full gt_{san} allele, shows viability comparable to that of $gt_{mel:san}$ carriers (X^2 = 0.075, P = 0.784), but higher than chimeras carrying the gt_{mel} coding sequence ($gt_{san:mel}$ and $gt_{mel:mel}$, $X^2 > 4.98$, P < 0.026; Tables 3.2). These results point to the coding region of gt_{mel} alone as being necessary for embryonic hybrid inviability.

We next evaluated the effects of these gt transgenes on a different metric of hybrid fitness – female viability. We crossed *san* males to *mel* females that were homozygous for gt^{X11}_{mel} and hemizygous for each of the four gt transgenes; the resulting female progeny from this cross was hybrid females carries gt^{X11}_{mel}/gt_{san} X-chromosomes and either a gt transgene or a wildtype 3rd *mel* chromosome lacking a transgene (Fig. 3.4D). The effect of the transgene on hybrid female viability can then be measured the ratio of flies with the transgene to flies with the wildtype 3rd *mel* chromosome. The results are largely consistent with the results from scoring embryonic lethality: one copy of the $gt_{mel:mel}$ allele reduces hybrid female adult viability compared to hybrids females without the same transgene. This finding with transgenic gt is also consistent with the deficiency mapping results using X-chromosome balancers. Hybrid females with one $gt_{san:mel}$ allele also show a reduction of viability similar to the one observed in $gt_{mel:mel}$ carriers (Tables 3.3); the reciprocal chimeric allele —$gt_{mel:san}$—, caused no reduction in relative viability. Finally, we find that the $gt_{san:san}$ transgene increases hybrid female adult viability compared to the control (Tables 3.3). This increase in viability can only be explained by an epistatic interaction between coding and noncoding regions of gt_{san}, as neither the coding nor the non-coding region of gt_{san} alone confers such an increase of hybrid female viability. These results collectively suggest that the inviability is mainly attributable to coding region of gt_{mel}, consistent with its predominant role in hybrid embryonic lethality.

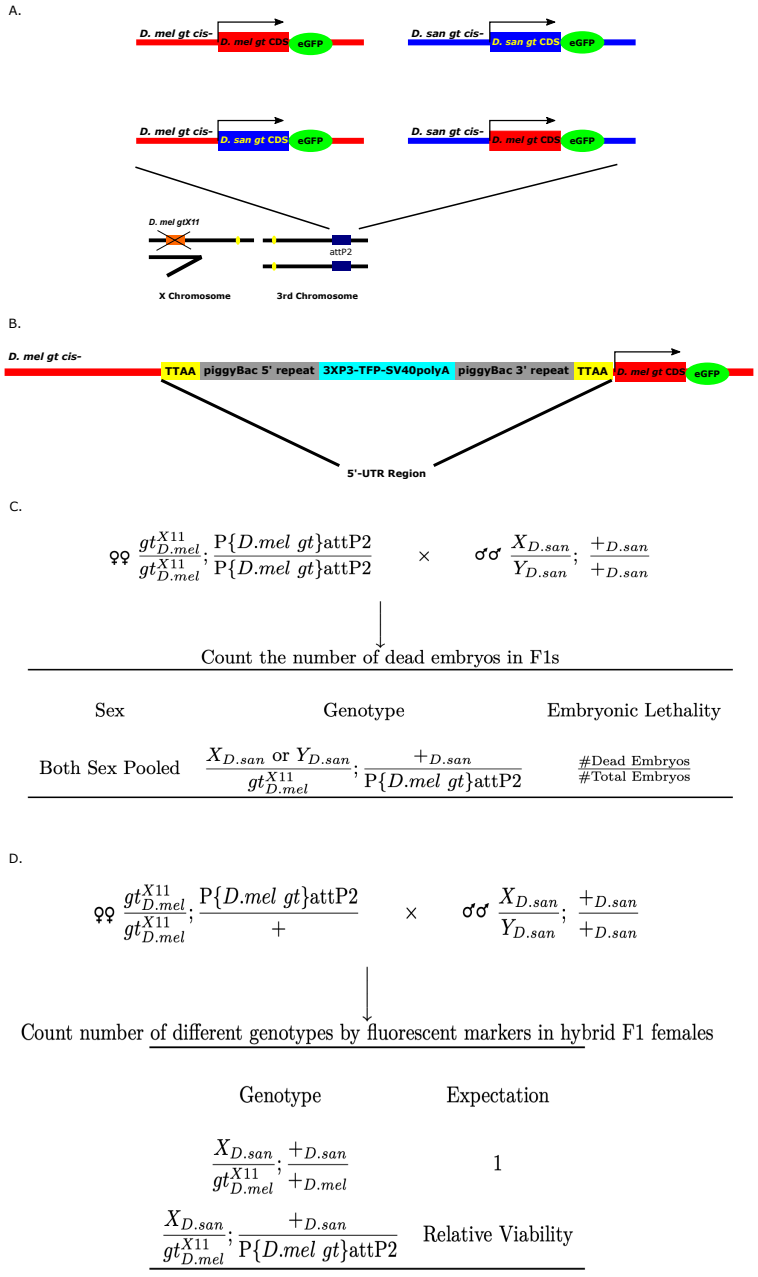

Figure 3.4: ***gt* transgene, removable piggyBac cassette design and crossing schemes involving *gt* transgenes.**
A, General experimental design to generate the four chimeric alleles included in this study and the insertion site for their integration (attP2 site in the 3rd chromosome). **B**, Details of the 5' UTR common to all the four alleles with piggyBac cassette. **C**, Crosses used in Table 3.2 when measuring *giant*'s effects on hybrid embryonic lethality. **D**, Crosses used in Table 3.3 when measuring the effects of different alleles of giant on adult hybrid female lethality.

Table 3.2: **The coding region of gt_{mel} causes embryonic lethality in *mel/san* hybrids.**

Allele	Non-coding	Coding	# Hatched Embryos	# Dead Embryos	Embryonic Lethality	χ^2	P-value
$gt_{mel:mel}$	*mel*	*mel*	40	17	0.298	NA	NA
$gt_{san:mel}$	*san*	*mel*	60	20	0.303	0.467	0.495
$gt_{mel:san}$	*mel*	*san*	90	10	0.100	8.676	3.22×10^{-3}
$gt_{san:san}$	*san*	*san*	51	4	0.091	7.922	4.88×10^{-3}

Table 3.3: **The coding region of gt_{mel} causes adult female inviability in hybrid *mel/san* females.**

Allele	Non-coding	Coding	# F1 with a *gt* transgene	# F1 with a 3_{mel} transgene	Relative Viability	χ^2	P-value
$gt_{mel:mel}$	*mel*	*mel*	86	116	0.741	4.455	0.035
$gt_{san:mel}$	*san*	*mel*	143	188	0.761	6.118	0.013
$gt_{mel:san}$	*mel*	*san*	173	180	0.961	0.139	0.710
$gt_{san:san}$	*san*	*san*	81	56	1.45	4.562	0.033

3.3.4 tll_{mel} exacerbates the hybrid inviability caused by gt_{mel}

Hybrid defects are usually caused by at least two interacting elements (reviewed in [108, 109]). *Giant* is an essential transcription factor in the gap gene regulatory network, a set of interacting genes expressed in the blastoderm embryo to establish anterior-posterior patterning [13–16]; its function in segmentation as a reciprocal transcriptional repressor of other gap genes (*Kruppel* and *knirps*) is conserved in arthropods [16, 110, 111]. *giant* is itself repressed by the gene products of *hunchback* [15, 16, 112], *tailless* [113], and *hucklebein* [114]. The proteins *Caudal* [115] and *Bicoid* [15] activate *gt*, which localizes to two broad stripes, one towards the anterior and one towards the posterior pole of the embryo (reviewed in [46]). Given this knowledge, we hypothesized that gap genes interacting with gt_{mel} could be additional candidates contributing to inviability in *mel/san* hybrids.

Even though gt_{mel} is involved in generating abdominal ablations, hybrids with no func-

tional gt_{mel} allele also show abdominal ablations but at lower frequency (Fig. 3.3B). This means that other alleles in the genome are involved in producing the maladaptive trait. We introgressed a gt^{X11}_{mel} allele into the background of 200 DGRP (Drosophila Genetic Reference Panel) lines to assess whether autosomal variants segregating within *mel*, other than those in gt_{mel}, would affect the frequency of abdominal ablations in hybrids. Using GWAS, we found a strong association between a 75.7kb haplotype in 3L which harbors nine genes: *cindr, CG15544, tll, Cpr100A, CG15545, CG15546, CG15547, CG12071*, and *Sap-r* (Fig. 3.5). Of these nine, the only gene known to interact with *gt* is *tll*.

To determine whether the two genes interact genetically in causing HI, we generated double mutants carrying loss of function mutations of *gt* and *tll* (gt^{X11}/FM7::GFP, $tll_{mel}\Delta$GFP/TM3) and crossed them to *san*. First, we scored whether the presence of tll_{mel} had any effect on hybrid female viability by itself. We found no effect of tll_{mel} in hybrid female viability in an otherwise heterozygote F1 background (FM7::GFP $/X_{san}$, $tll_{mel}\Delta$GFP/3san. FM7::GFP $/X_{san}$, TM3/3san; Table 3.4). Next, we scored whether the presence of tll_{mel} had an effect on hybrid female viability in a gt^{X11}_{mel} background. Hybrid *mel/san* females that have only a functional copy of gt_{san} (i.e., carry a gt^{X11}_{mel} allele) and are hemizygous for *tll* (i.e., only have tll_{san}) are more likely to survive to adulthood than gt^{X11}_{mel}-carrying females and a functional tll_{mel} (gt^{X11}_{mel}/X_{san}, $tll_{mel}\Delta$GFP/3san vs. gt^{X11}/X_{san},TM3/3san Table 3.4A). These results suggest that while removing tll_{mel} on its own has no major effect on HI, removing both gt_{mel} and tll_{mel} has a positive effect in viability that is larger than removing either allele individually.

tll_{mel} also has a role in the frequency of abdominal ablations. Abrogating tll_{mel} in a gt_{mel}/gt_{san} background has no detectable effect in the frequency of abdominal ablations in hybrid males or hybrid females with a functional copy of gt_{mel} (FM7/X_{san},$tll_{mel}\Delta$GFP/3sanvs. FM7/X_{san},TM3/3san, Table 3.4B and Table 3.4C). In a gt^{X11}_{mel} background, abrogating tll_{mel} decreases the proportion of male and female embryos showing abdominal ablations (Ta-

ble 3.43). These results suggest —just as occurs with female viability— the absence of gt_{mel} and tll_{mel} together has a larger positive effect than the absence of each allele individually.

Finally, we tested the effect of disrupting the tll_{san} in hybrids. $tll_{san}\Delta$dsRed did not rescue $tll_{mel}\Delta$GFP in hybrids. In *mel/san* hybrids, the $tll_{san}\Delta$dsRed deletion had no effect on female viability when tested in hybrids with multiple *mel* backgrounds (Table 3.5). This result suggests that removing tll_{san} in *mel/san* hybrids has no effect on hybrid viability in an otherwise heterozygote hybrid background. Next, we tested the effect of $tll_{san}\Delta$dsRed in the null gt_{mel}^{X11} background. We find that the tll_{san} deletion does not improve viability in gt_{mel}^{X11}-carrying *mel/san* females either (Table 3.6). These results suggests that even in the absence of a functional gt_{mel} allele, removing tllsan has no effect on hybrid female viability. Since the reciprocal deletion (removing tll_{mel} and exposing the tll_{san} allele) does improve female hybrid viability in gt_{mel}^{X11}-carriers, these results indicate that the presence of tll_{mel}, but not of tll_{san}, is involved in the HI of *mel/san* female hybrids.

3.3.5 Molecular Evolution

Gap genes *gt* and *tll* have phylogenetically conserved roles in pattern formation, as evidenced by their functionally conserved outputs in blastoderm embryos of distantly related *Drosophila* species [23] and beyond [116, 117]. Yet, they have diverged sufficiently between *mel* and *san* such that they malfunction in hybrids. We therefore conducted analyses to assess the patterns and mechanism of divergence of the *gt* coding sequence in the *melanogaster* species subgroup and across the *Drosophila* genus.

Both *gt* and *tll* are highly conserved in their coding regions. The bZip domain that confers *Gt* protein its ability to bind DNA is highly conserved across animals [11] and shows no fixed differences among *Drosophila* species (Fig. 3.6, see also [91]). *Gt* shows only thirteen single amino acid substitutions in the *melanogaster* species subgroup (Fig. 3.6), six of which occur on the branches connecting *mel* and *san*. *Giant* also contains three low complexity regions

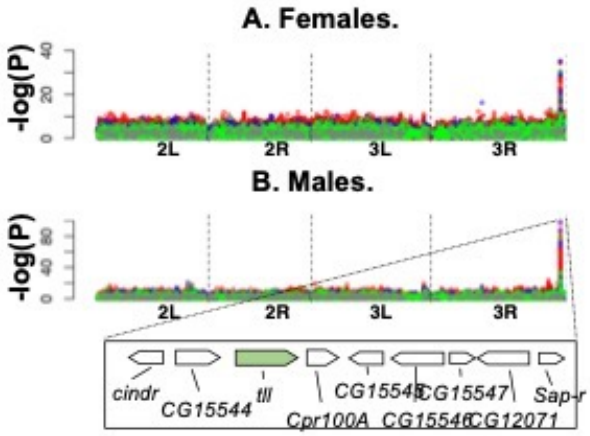

Figure 3.5: **A *D. melanogaster* third-chromosome haplotype that encompasses *tll* is associated with the prevalence of abdominal ablations in gt^{X11} mutants.** Association study of autosomal genetic variants associated with the frequency of abdominal ablations in gt^{X11}/X_{san} hybrids using segregating variation within *D. melanogaster*. A haplotype of 54kb in the tip of the X-chromosome is strongly associated with the presence of abdominal ablations in both males (**A**) and females (**B**). Each panel shows a different chromosome arm. The haplotype 9 genes: *cindr, CG15544, tll, Cpr100A, CG15545, CG15546, CG15547, CG12071,* and *Sap-r*. Of these nine, *tll* is the only one known to interact with *gt*. Green: insertions, blue: deletions, red: SNPs, purple: multinucleotide polymorphisms.

Table 3.4: tll_{mel} **exacerbates the defects caused by** gt_{mel} **in *mel/san* hybrids.**

A. Female Hybrid Viability

FM7/X_{san}; TM3/3_{san}	FM7/X_{san}; $tll\Delta GFP/3_{san}$	gt^{X11} / X_{san}; TM3/3_{san}	gt^{X11} / X_{san}; $tll\Delta GFP$ / 3_{san}
43	51	97	153
$\chi^2 = 0.681$, P = 0.4093		$\chi^2 = 12.544$, P = 3.975×10^{-4}	

B. #male embryos w/ abdominal ablations (100 embryos each)

FM7/Y_{san}; TM3/3_{san}	FM7/Y_{san}; $tll\Delta GFP/3_{san}$	gt^{X11} / Y_{san}; TM3/3_{san}	gt^{X11} / Y_{san}; $tll\Delta GFP$ / 3_{san}
94	88	23	9
$\chi^2 = 1.526$, P = 0.217		$\chi^2 = 6.287$, P = 0.012	

C. #female embryos w/ abdominal ablations (100 embryos each)

FM7/X_{san}; TM3/3_{san}	FM7/X_{san}; $tll\Delta GFP/3_{san}$	gt^{X11} / X_{san}; TM3/3_{san}	gt^{X11} / X_{san}; $tll\Delta GFP$ / 3_{san}
48	35	16	6
$\chi^2 = 2.966$, P = 0.085		$\chi^2 = 4.137$, P = 0.042	

Table 3.5: $tll_{san}^{\Delta dsRed}$ **has no effect on HI in crosses between *mel* females from four different backgrounds.**

Background	$tll_{san}^{\Delta dsRed}$ / 3_{mel}	$3_{san}/3_{mel}$	χ^2	P-value
mel AkLa	31	36	0.067	0.795
mel Zs2	62	74	1.600	0.206
mel Senegal	49	41	0.200	0.654
mel NC103	62	71	0.184	0.668

Table 3.6: **Abrogating the** tll_{san} **allele has no viability effect on female** gt_{mel}^{X11} ***mel/san* hybrids.**

FM7/X_{san}; 3_{mel} / $tll_{san}^{\Delta dsRed}$	FM7/X_{san}; 3_{mel} / 3_{san}	gt_{mel}^{X11}/X_{san}; 3_{mel} / $tll_{san}^{\Delta dsRed}$	gt_{mel}^{X11}/X_{san}; 3_{mel} / 3_{san}
12	20	41	44
$\chi^2 = 0.571$, P = 0.450		$\chi^2 = 0.006$, P = 0.939	

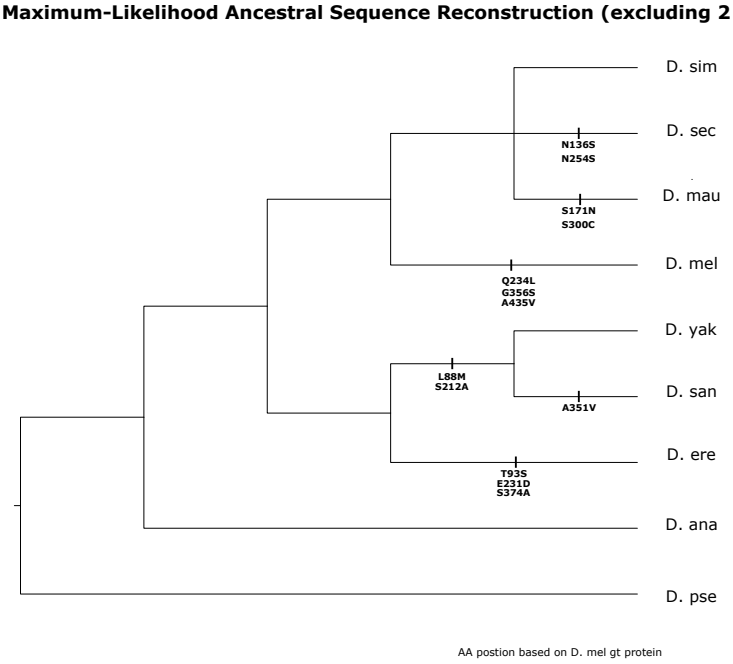

Figure 3.6: **Maximum-likelihood ancestral sequence reconstruction of Gt protein, excluding polyQ.**

All ancestral sites could be reconstructed with high confidence (posterior probability ¿ 0.98), except for polyQ tracks. Ancestral substitutions beyond *D. ere* were not displayed. AA postions based on *D. mel Gt*.

(including polyQ) that show extensive variation both within and between species (Fig. 3.7). *tll* is also conserved in the *melanogaster* species subgroup; only four residues differ between the *tll* alleles in the textityakuba clade and the *melanogaster* clade: Val509Asp, Arg1118Lys, Ser1208Thr, Leu1246Met. Only Val509Asp represents a change in the type of amino acid (a change from a nonpolar to an acidic residue).

3.3.6 DMI partner(s) of gt_{mel} is (are) unique to the D. santomea lineage

The phylogenetic occurrence of developmental defects provides an additional hypothesis to test: we next evaluated whether the unknown genetic element(s) in the *D. santomea* genome that must interact with gt_{mel} and *tllmel* to cause HI are also present in *D. teissieri* (a close relative of *D. santomea*, Fig. 3.1A). The crosses ♀ *mel* x ♂ *tei*, ♀ *sim* x ♂ *tei* and ♀ *mel* x ♂ *tei* all produce viable adult females and males that die as late larvae/early pupae. Little

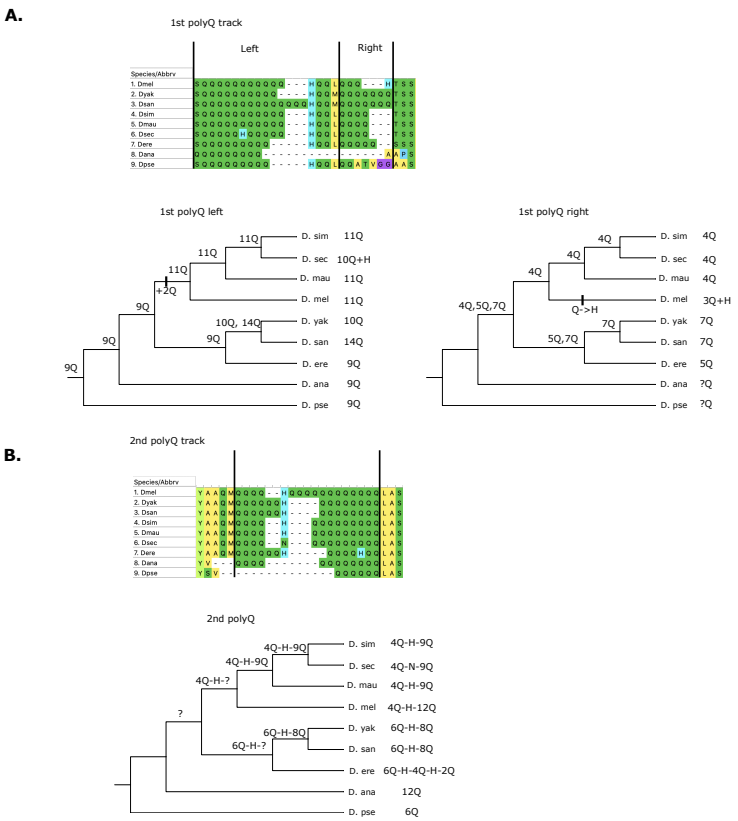

Figure 3.7: **The polyglutamine repeats (polyQ) show differences among the *melanogaster* subspecies complex species.**
Maximum-parsimony ancestral sequence reconstruction of 2 polyQ tracks of *Gt* protein. The conserved H within polyQ tracks helps to delineate both polyQ tracks to two parts. Due to the lack of proper substitution models, this maximum-parsimony based ancestral reconstruction for polyQ tracks may subject to bias due to alignment error and arbitrary choice of polyQ unit.

embryonic lethality is observed in any of these two crosses and the rare embryos that die do not show abdominal ablations (Fig. 3.1E). All *D. teissieri* lines showed similar levels of viability in each cross. These results suggest that at least one of the partners of this incompatibility is specific to *san*, and evolved after *san* and *tei* diverged.

3.4 Discussion

Hybrid inviability is a strong barrier to gene exchange between species. While it is clear that this trait is often caused by epistatic interactions between alleles from different species, few examples have been identified to the gene level. Here, we identified two genes, *gt* and *tll*, which contribute to HI in hybrids between two *Drosophila* species. The two genes belong to the gap gene network, a highly conserved pathway that is in charge of establishing embryonic polarity in insects [23, 50, 51, 116–119]. The *mel* alleles of these two genes are necessary and sufficient to cause a male abdominal ablation phenotype that is particularly common in hybrid males of the cross. We also find support for a third (or even more) elements that are exclusive to *D. santomea* and remain unidentified. Additional members of the gap gene network must have functionally diverged between the two species and contribute to HI (see also [91]. These are not the only alleles that contribute to inviability in the cross but are sufficient to cause the abdominal ablation defect that is particularly common in hybrid males of the cross [94].

The involvement of gt_{mel} and tll_{mel} in HI indicates that one or more features of their function have diverged between relatively closely related species, a direct challenge to studies of gap gene expression pattern and function that emphasize their conservation across the Diptera. Our results confirm speculation that HI can arise in phylogenetically conserved gene networks regulating development [21, 38]. The involvement of gt_{mel} and tll_{mel} in HI suggests that their function has diverged across *Drosophila* species. Consistent with this result, precise gene replacements have also shown that *gt* alleles from different species vary

in the ability to complement in a *D. melanogaster* background [91]. Natural selection has driven the evolution of regulatory elements of many developmental genes in *Drosophila* which has led to a rapid turnover [23, 48, 120]. Yet, neither *gt* nor *tll* show signatures of positive selection in their coding sequences.

Our results also suggest that the evolution of the different components involved in the DMI occurred at different times and is unlikely to have had any role on speciation. The deleterious effects caused by gtmel seem to be common to *D. melanogaster* and the species in the *D. simulans* clade, suggesting that the allele necessary for the incompatibility evolved before these species split between three and five million years ago [52, 99]. Because the presence of gt_{mel} has no quantifiable viability effect in *mel/tei* hybrids, at least one of the genetic factor(s) that interact with gt_{mel} to cause abdominal ablation in hybrid embryos must have arisen after *D. santomea* and *D. teissieri* split between 1 and 2.5 million years ago [53, 121]. An alternative divergence scenario is that at least one of the genetic components of the DMI evolved in the tei branch to suppress HI. Regardless of which of these two scenarios is correct, the components of the DMI must have evolved at different times in the two lineages, and the interactions with *giant* that cause abdominal ablation could not have been involved with any speciation event in the melanogaster species subgroup. Instead, these loci must have evolved independently in each lineage, accumulating differences as the genomes diverged after speciation, a scenario in accord with the Dobzhansky-Muller model [66, 67]. Mapping the allele(s) that interact with gt_{mel} and tll_{mel} in the *D.santomea* genome is the next step in describing how genomic divergence creates hybrid defects.

Previous comparative analyses of gap gene expression in Dipterans indicates gene network evolution in spite of a conserved developmental phenotype [91], which suggests continual fine-tuning of the genetic interactions in the gap gene network within species. Coevolved compensatory changes have been proposed to cause HI in instances in which the phenotypic output of a gene network is under moderate stabilizing selection [122, 123]. Molecular

functional evolution without phenotypic change, or developmental systems drift, has been hypothesized to underlie hybrid breakdown involving canalized traits such as embryogenesis and gametogenesis [38]. The HI involving gtmel and tllmel may exemplify compensatory changes resulting in a stable phenotype when comparing pure species, but an aberrant phenotype in hybrids.

The introduction of a developmental genetics perspective to speciation studies has the potential to shed new light on the study of hybrid inviability [124]. Hybrid inviability is a natural experiment to test genetic interactions between diverging genomes: the molecular interactions that go awry in hybrids reveal evolutionary divergence of the genes involved, or the timing, location, or amount of their expression [125]. The interactions between gt_{mel}, tll_{mel} and the unknown factor(s) in the genome of *D. santomea*, had nothing to do with setting the speciation process in motion in the *melanogaster* species subgroup. They are also not involved in currently keeping species apart as *D. melanogaster* and *D. santomea* do not naturally hybridize. The results shown here should be viewed in the broad context of genome divergence and how genomes keep evolving long after speciation has occurred. This represents a path forward in terms of how to think about stability vs. change of different functional units within the genome and different developmental processes. The identification of *giant* and *tll* as genes involved in HI is the first indication that genes involved in early embryonic development, a canonical example of a conserved developmental process, functionally co-evolve at a pace sufficient to cause hybrid inviability.

3.5 Contributions

D.R.M. conceived the project. W.C. and M.K designed the transgenic experiments. W.C. performed the transgenic and viability experiments. W.C., D.R.M. and M.K. shared in writing the manuscript.

CHAPTER 4

THE EVOLUTION OF GAP GENE NETWORK EXPRESSION IN *DROSOPHILA*

4.1 Introduction

The word "conservation" has long been used as a qualitative judgment about a trait indicating its phenotypic stasis across a phylogeny [126]. Conserved traits are generally assumed to have underlying molecular genetic mechanisms that are likewise unchanged (i.e., conserved phenotype $\rightarrow$ functionally static mechanism). In many cases, however, the genetic basis of a conserved trait is not known, or only investigated in one model organism, making it impossible to challenge this assumption. Claims of phenotypic trait conservation also overlook complications inherent in interspecific comparisons, including homology of the structure/tissue being compared and the extent of similarity required for a trait to qualify as conserved [127]. The lack of rigorous examination of "conserved" traits and their molecular evolutionary details cloud our understanding of how phenotypic conservation is achieved. Little is known, specifically, about whether (and the extent to which) genotypic evolution is decoupled from phenotypic evolution for conserved traits.

The *Drosophila* gap gene network is an excellent system for investigating this question. Composed of at least five transcription factors (Hunchback, Giant, Krupple, Knirps and Tailless), the gap genes are indispensable in establishing pattern formation during embryogenesis. Location-specific expression of the gap genes in distinct broad bands along the anterior-posterior axis in syncitial blastoderm embryos is achieved by their responsiveness to opposing concentration gradients of the maternal morphogens Bicoid and Caudal and by their cross- (and auto-) regulation of one another [46]. In addition to regulating pattern formation, the gap genes have pleiotropic activities in other developmental processes, including neurogenesis and muscle formation [128–130]. Consistent with their functional essentiality

and regulatory roles in development, the gap gene network is preserved across insect phylogeny [50, 131]. As is typical for transcription factors regulating conserved developmental processes, their protein sequences are slow-evolving. The gap genes constitute a classic model of a deeply conserved gene network.

Wunderlich *et. al* investigated the mRNA expression patterns of all gap genes in five Drosophila species spanning the phylogeny of the genus [47]. Using species-specific probes to quantify spatial patterns of gene expression they found —questionably — only subtle differences in the location of a small number of expression domains between species. Qualitatively, the expression patterns of gap genes were found to be indistinguishable for all gap genes in all species. The gap genes cross-regulate one another and also regulate the expression of downstream targets, the pair-rule genes. Ludwig *et. al* [21, 22] and Hare *et. al* [23, 48] investigated cis-regulatory DNA targets of the gap genes in the pair-rule gene even-skipped, finding that they are "swappable" from other Drosophila species and from more distant Dipteran relatives. Studies of gap gene expression across Drosophila phylogeny and transgenic complementation studies provide compelling evidence for functional conservation of the gap gene pattern formation network.

Drosophila embryogenesis is a highly conserved process: spatio-temporal morphological landmarks throughout development are preserved across the genus [4]. The earliest stage of development –the syncytial blastoderm stage— entails 14 evolutionarily conserved semi-synchronous nuclear divisions, followed by rapid cellularization to produce the blastoderm embryo. This embryo is composed of about 5000 nuclei [47] with about 100 nuclei spanning the embryo along the anterior-posterior axis. Cell membranes do not form until the end of cell cycle 14, so the syncytial activity of *eve* enhancers are direct spatio-temporal readouts of gap gene concentrations and protein functional kinetics. Importantly, expression conservation of gap genes and of *eve* in the blastoderm embryo is not subject to potential issues of tissue or cell type homology, an issue that can arise when comparing traits among distantly related

species in comparative evolutionary studies [126]. This certainty about the homology of gap genes and their conserved roles in pattern formation make this gene network an unassailable system for addressing the coupling between phenotype and genotype in a conserved trait across an evolutionary timescale. Hidden behind conserved network outputs, do genes in this network functionally evolve? And if so, what ways do they change and how are they compensated to preserved network outputs?

Beyond the simple conservation of gene expression, the gap gene network —and more broadly pattern formation— directs a robust developmental process. This system of genes has received considerable experimental attention in attempts to understand the molecular details of robustness [132–134]. Less attention has been given to mechanisms underlying the conservation of this trait. Two hypotheses have been proposed. The first proposes that robustness is an emergent property of cross-regulated gene networks and not a property specifically molded by natural selection. The second hypothesis supposes that natural selection has sculpted the network to achieve its high degree of uniform outputs in the face of stochastic molecular-level processes, as well as developmental, environmental and genetic noise. We would like to investigate whether a gap gene remains conserved both in its primary traits —its gene expression and effects on downstream target gene expression and also in its contribution to developmental robustness.

Here we refute the poorly substantiated belief that the gap gene network, a canonical example of an essential and conserved developmental network, is immune from evolutionary change, and give meaning and context to why "conserved" does not equal "identical".

4.2 Results

4.2.1 Gap gene network expression is disrupted in hybrid embryos

Previous work shows that gap genes and their downstream pair-rule genes have conserved spatial mRNA expression across the genus *Drosophila* [47, 135] and beyond in distant Dipteran species [50]. Protein expression patterns of gap genes and even-skipped are conserved in *D. yakuba*, *D. erecta* and *D. santomea*, a monophyletic sister group to *D. melanogaster* with about 15 million years of divergence, establishing that the conserved gap gene network expression is an ancestral phenotype of these species (Fig. 4.1).

The gap gene network could maintain conserved outputs by constraining functional changes of individual gap gene or by having compensatory changes in different gap genes that allows for functional divergence of individual genes [38]. If functional stasis of gap genes is true, we expect individual members from different species to be able to replace one another despite millions of years of divergence. We first tested this prediction by examining network expression in hybrid embryos between *D. melanogaster* and *D. santomea*, the most distant species that hybridize in melanogaster subgroup, and found the resultant hybrid gap gene network outputs to be disrupted (Fig. 4.2). Eve is both mis-expressed and variable, with embryos displaying a range of seven to five stripes, indicating incompatibilities and de-canalization. No such mis-expression or variability is observed in either parental species.

4.2.2 Interspecific gap gene swaps show gene-network incompatibility

We hypothesize that eve mis-expression in *D. melanogaster* and *D. santomea* hybrid embryos results as a consequence of gap gene mis-interactions, a consequence of functional divergence of one or more gene. To test this hypothesis, we generated eGFP-tagged whole-locus giant transgenes from six species, *D. melanogaster*, *D. yakuba*, *D. santomea*, *D. erecta*, *D. pseudoobscura* and *D. virilis* [91] (gt^{mel}, gt^{yak}, gt^{san}, gt^{ere}, gt^{pse}, gt^{vir}, respectively), and

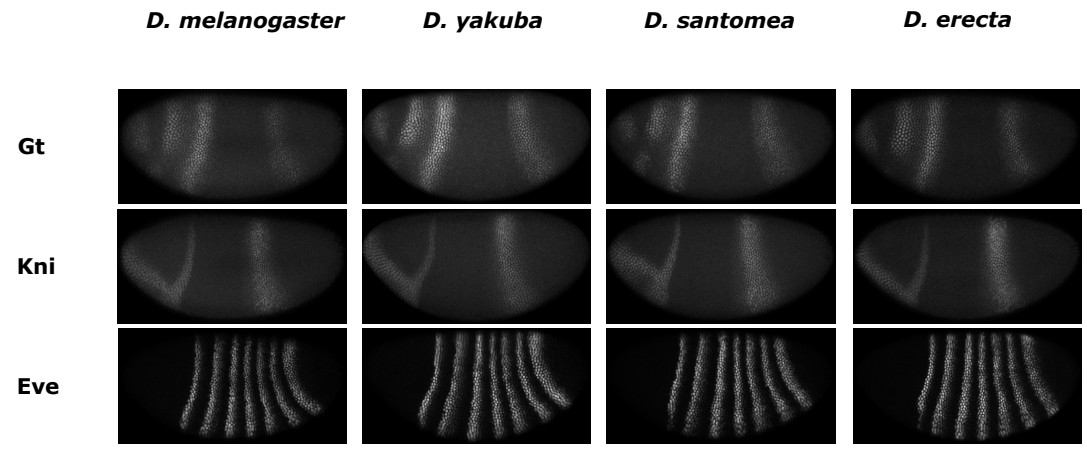

Figure 4.1: **Gap gene network outputs are conserved in *D. santomea*.** Antibody staining of cycle 14 embryos shows that expression of gap gene *giant* and *knirps* and pair-rule gene *even-skipped* from four *Drosophila* species are strongly conserved.

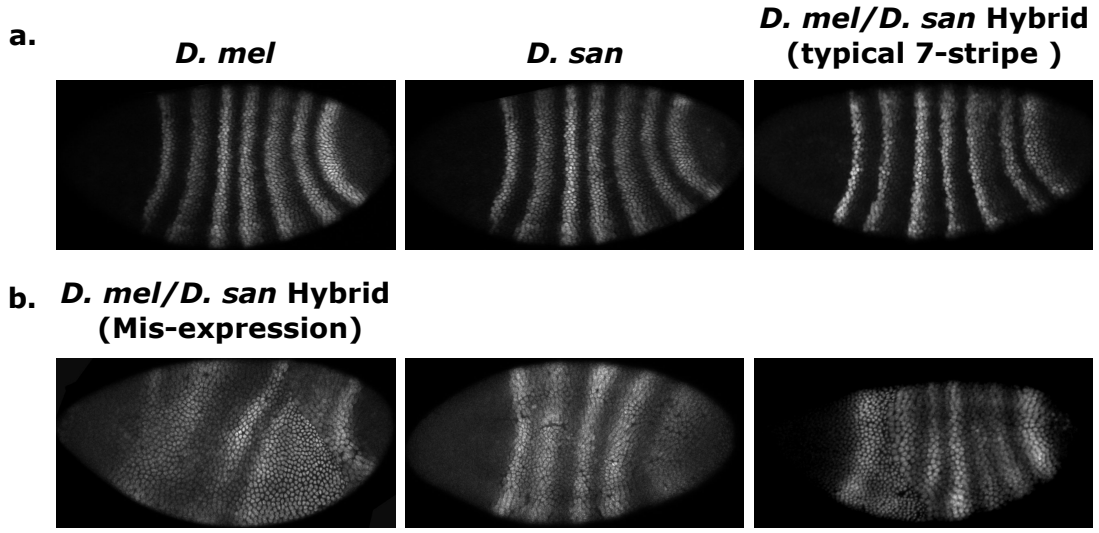

Figure 4.2: ***D. melanogaster*/*D. santomea* species hybrid reveals gap gene network incompatibility.**

a. Typical seven-stripe expression of eve in parental species and a hybrid embryo. **b**. Representative hybrid embryos displaying mis-expression of *eve*.

placed them at the same site on the second chromosome. These transgenes (25kb to 35kb in size) extend to the boundaries of neighboring genes and include all regulatory sequences [91]. The eGFP tag at the C-terminal of Gt protein enables quantifying their expression using the same antibody, making such measurements comparable. All transgenes can replace the endogenous *gt* and be maintained as stable lines [91].

We found all but gt^{vir} produce comparable indistinguishable expression patterns of gap genes and of *eve* (Fig. 4.3), consistent with their small observed fitness differences in a complementation assay [91]. gt^{vir}, in contrast, produces a weaker posterior stripe when expressed in *D. melanogaster* background, and the resultant *eve* interstripe 5 and 6 has ectopic expression. *D. melanogaster gt* is known to be indispensable for repressing *eve* interstripe 5 and 6. *D. virilis gt* has conserved posterior expression in its native genetic background, and *D. virilis eve* interstripe 5 and 6 has comparable absence of expression [47]. This suggests that gt^{vir} is mis-interacting with other *D. melanogaster* gap genes, causing its weaker posterior expression and *eve* interstripe ectopic expression. The loss of gt^{vir} posterior expression may be attributable to changes in its cis-regulatory sequence, while the expression defects of *eve* may be due to changes in both coding and non-coding regions of gt^{vir}. Consistent with these patterning defects, we have found evidence that functional divergence of both coding and noncoding regions of the gt^{vir} whole-locus region contribute to a reduced fitness in *D. melanogaster* background in a complementation experiment [91].

Our previous work showed that subtle fitness differences of giant orthologs could be amplified with sensitized genetic assays [91]. We took advantage of this and generated transgenic *D. melanogaster* embryos with only one copy of *gt* transgenes (Fig. 4.4). Consistent with the expression defects of two-copy gt^{vir}, one-copy gt^{vir} shows more severe mis-expression of *eve* — stripe 5 and 6 are fused, reminiscent of *D. mel gt* null mutant. Other *gt* orthologs produce gap genes/*eve* expression patterns comparable to the *D. melanogaster* control (Fig. 4.4).

Similar to our finding in *D. melanogaster/D. santomea* hybrid embryos, one-copy gt^{vir} produces variable expression levels of *gt* and of *eve* in *D. melanogaster* background (Fig. 4.5). This phenomenon is not observed in *gt* null mutant, indicating that evolved differences in gt causing this loss of developmental robustness is a gain of function change. In addition to the fused *eve* stripe 5 and 6, which is a classic defect in *D. mel gt* null, some embryos have only 4 *eve* stripes. No gap gene mutant has been reported to produce this *eve* expression defect. This suggests that the resultant *eve* mis-expression cannot be attributed simply to low expression level of gt^{vir}, and must instead be a consequence of mis-interaction(s) of gt^{vir} and other *D. melanogaster* gap gene(s). gt^{vir} cannot be simply considered as a low-expression *D. melanogaster gt* allele. Such effects cannot be dominant either, as two-copy gt^{vir} embryos have eve expression that is more similar to *D. melanogaster gt* null.

4.2.3 Genetic manipulation suggests gap gene network maintains conserved expression by compensatory evolution

To further explore changes in gt^{vir} that cause its mis-expression, and that of its target *eve*, we utilized a transgenic line with ubiquitous Nanos expression (nos-tub; [136]). Nanos is a maternal morphogen that is restricted to the posterior pole of *Drosophila* embryo, and it represses the translation of maternal Hunchback, which is a known repressor of *gt*. Both gt^{mel} and gt^{vir} transgene, in a ubiquitous Nanos background, show broader and more anterior expression of both the anterior and posterior *gt* stripe. The cis-regulation of gt^{vir} must be intact and functionally unchanged in its ability to respond to the forward shift of gap genes produced by ubiquitous Nanos expression.

gt^{vir} differs from gt^{mel} in one aspect — its otherwise weak expression is elevated by ubiquitous Nanos in both anterior and posterior domains. The two gt genes must differ in their sensitivity to repression by cross-regulating gap genes. The gt^{vir} transgene is capable, therefore, of driving its posterior expression, ruling out the possibility that this 29.7kb gt^{vir}

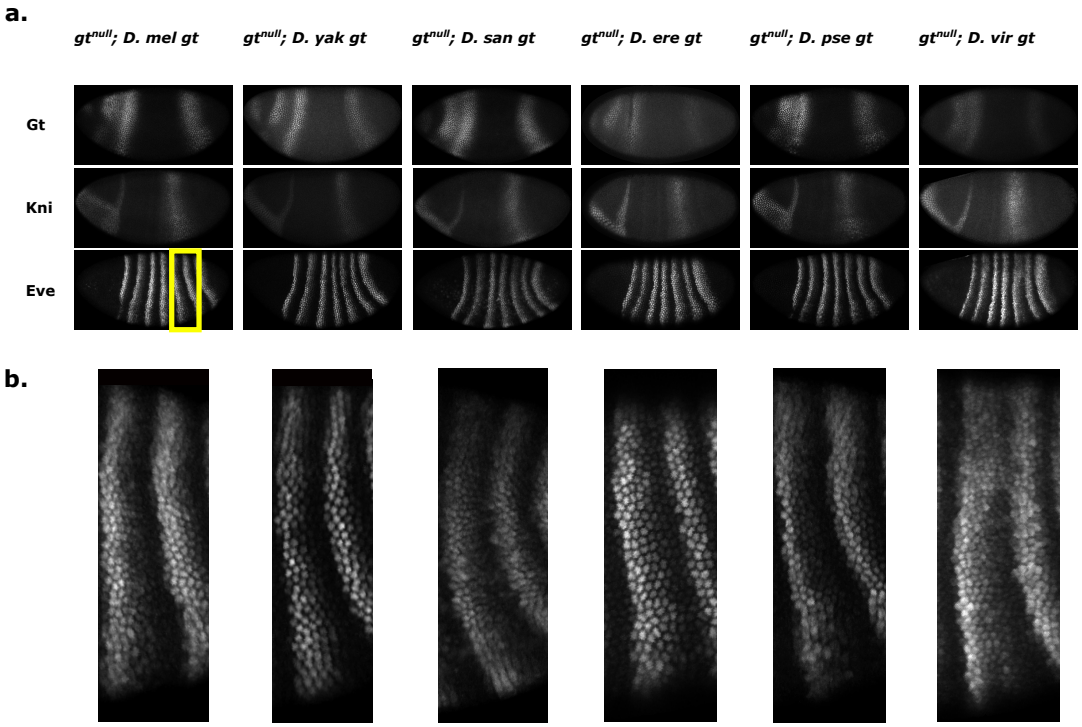

Figure 4.3: gt^{vir} **shows expression defects when two copies are present in *D. melanogaster* background.**
a. In $gt^{null}; gt^{vir}$ embryos, the posterior stripe of gt^{vir} has diminished expression and the eve interstripe between 5 and 6 has leaky expression. **b**. Enlarged panels of eve stripe 5 and 6 (yellow box in **a**) highlight inter-stripe mis-expression in gt^{vir}. All embryos shown here were stained and quantified at the same time, thus are directly comparable. Same applies to Fig. 4.4, 4.5 and 4.6.

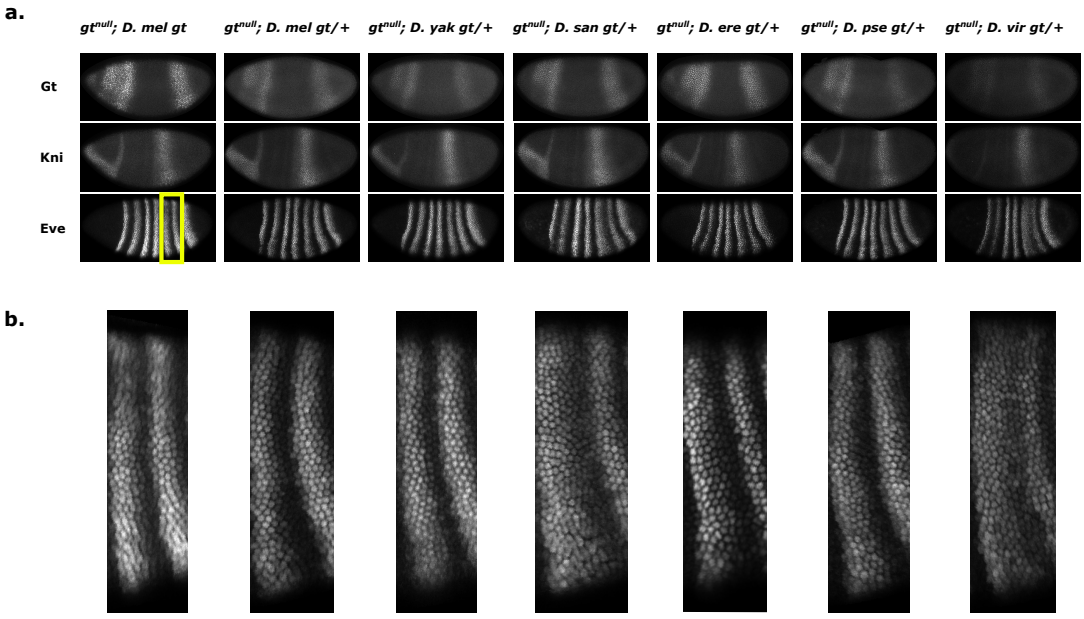

Figure 4.4: **One-copy gt^{vir} transgene expression defects in *D. melanogaster*.** **a**. In $gt^{null}; gt^{vir}/+$ embryos, eve stripe 5 and 6 are fused. **b**. Enlarged panels show eve stripe 5 and 6 inter-stripe mis-expression (yellow box in **a**).

ortholog lacks some key regulatory elements needed for posterior expression.

4.2.4 Canalization as the subject of natural selection

One striking feature observed in the gap gene network and other developmental networks is canalization: the robust phenotypic outputs produced, despite environmental and genetic fluctuations [132–134]. When gap genes from different species are artificially brought together, as shown in *D. melanogaster/D. santomea* hybrid (Fig. 4.2) and in one-copy gt^{vir} embryos (Fig. 4.5), the outputs of gap gene network become de-canalized and variable. Our results suggest that conservation of this canalizing property of gap gene network is not the result of strict conservation of gene functions; rather functional changes in gap genes occur that are de-canalizing the network when placed in a different genetic background.

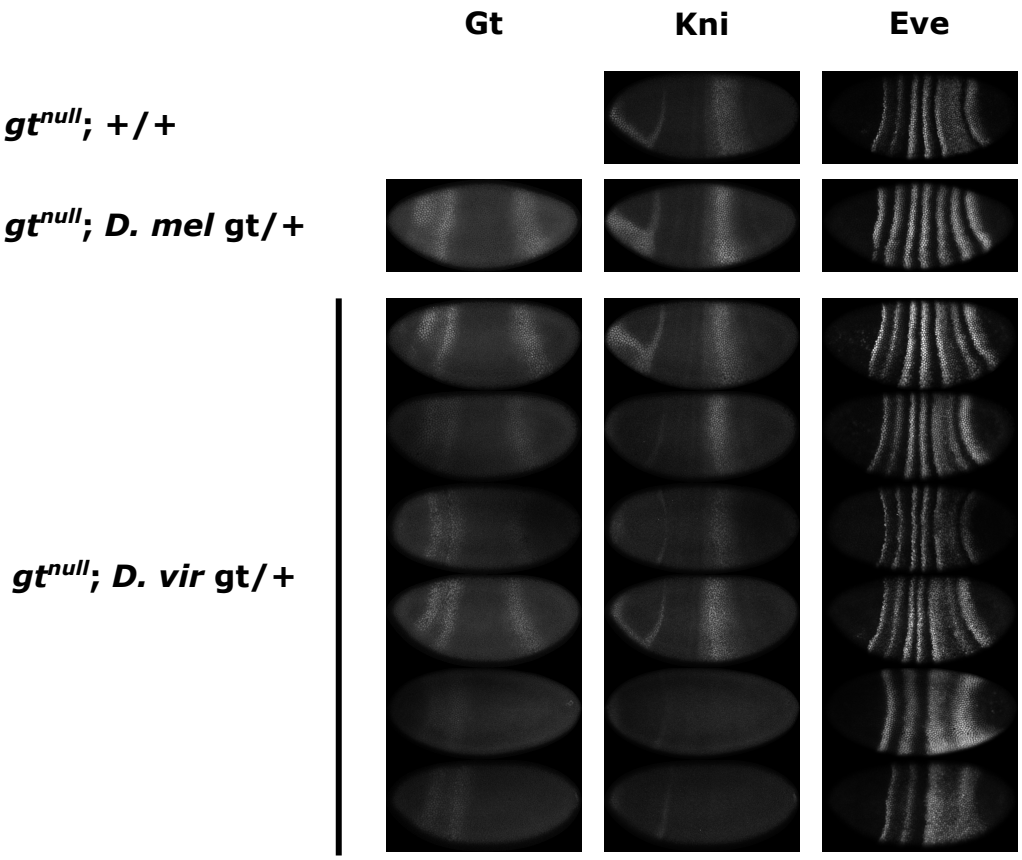

Figure 4.5: **Gap gene network shows extreme and variable defects with one copy gt^{vir} transgene.**
Some $gt^{null}; gt^{vir}/+$ embryos have fused eve stripe 5 and 6, while some only have four eve stripes. Null mutants of *D. mel gt* show fused eve stripe 5 and 6 and are not variable.

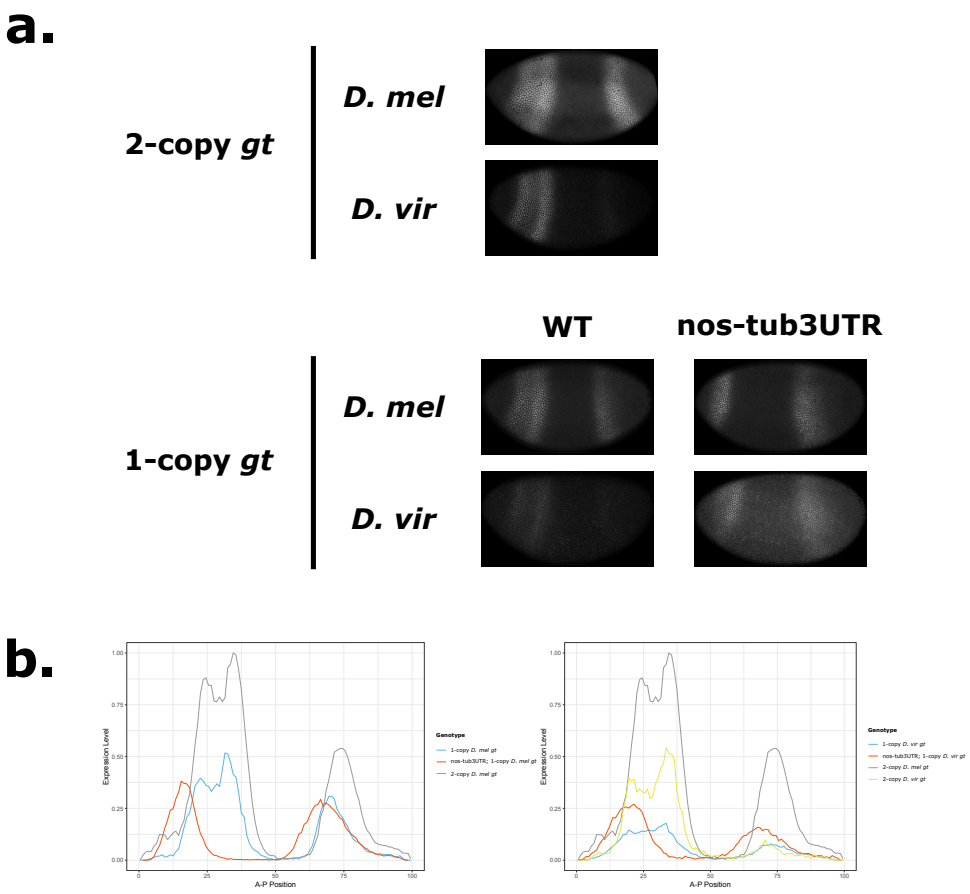

Figure 4.6: gt^{vir} **transgene is responsive to ectopic repression of Hunchback by ubiquitous Nanos.**

a. Transgenic *gt* expression in response to ubiquitous Nanos. **b**. Left panel: gt^{mel} transgene expression; Right panel: gt^{vir} transgene expression. Plotted are the averages of 10 embryos.

4.3 Discussion

The discovery of gap genes 40 years ago created a new era that attempt to understand the molecular genetic basis of development [137–139]; it provides the first clear picture of how a single fertilized embryo is programmed and segmented to be a complex multi-cellular individual, a process that is mediated by the robust expression of gap genes. Previous work that investigated the expression pattern or DNA-binding properties of gap genes in *Drosophila* [12], as well as enhancer swaps of downstream pair-rule genes [21–23], revealed conservation of gap gene networks in *Drosophila*. Here by utilizing species hybrid and site-specific transgenesis, we refute the argument that conserved outputs equates to identical functionality; to the contrary and despite all the hallmarks supporting evolutionary stasis of gap gene network, we found that gap gene networks from different species are different and incompatible. Defects observed from transgenic *gt* confirms functional changes of individual gap genes and of gap gene network as an entity.

The focus on gap gene network outputs eliminates several technical difficulties. First, DNA sequences of gap genes are highly conserved. None of the gap genes have duplicated in species studied here, and there is no ambiguity regarding gene orthology. Second, inter-specific comparisons of gap gene expression are meaningful; the embryonic structure or cell types compared are truly homologous in the same syncytial blastoderm stage. Third, most transcription factors are expressed in multiple stages and have pleiotropic effects [140], making it impossible to definitely evaluate their functional conservation or divergence as a whole [141]. We focus on the earliest developmental stage —embryogenesis— when all gap genes exert their function synchronously with few if any inputs from other developmental pathways.

Our previous work has shown fitness defects resulting from continuous functional divergence of *gt* in all of the six Drosophila species studied here [91], including contribution to hybrid inviability between *D. melanogaster* and *D. santomea* [58]. What remained unclear

was the developmental stage (or tissue) in which defects first appear. Here we pinpoint and confirmed such defects caused by functional divergence of gap genes are visible in their early expression and regulation of pattern formation.

Phenotypic canalization or robustness against environmental perturbation and genetic mutation has been documented for many developmental pathways [142, 143], and canalization itself has been proposed as a phenotype under natural selection [144, 145]. In the gap gene network, canalization is a conserved phenotype in all *Drosophila* species investigated, and gap gene shadow enhancers —separate enhancers driving similar expression patterns— partially contribute to the canalized network outputs [132–134]. We speculate that co-evolution of the gap genes (and the existence of shadow enhancers) are a consequence of natural selection for mutations that maintain the canalization properties of the network and prevent gap gene network variability.

Evidence of conserved outputs/phenotypes have been used to support underlying mechanistic identity among species. Our results show the opposite can be true: the gap gene network has highly conserved expression but its member genes are not functionally static. Together with our recent work on the continuous functional evolution of *gt*, the functional discrepancy of gap gene network from different species, camouflaged by conserved network outputs, suggest no gene/gene network is immune from evolution, regardless of its essentiality.

4.4 Contributions

W.C. and M.K. conceived the project and shared in writing the manuscript. W.C. performed the experiments and carried out the analysis.

CHAPTER 5

DISCUSSION

In stark contrast to conventional belief, I have documented in the three preceding chapters the continuous functional evolution of gap gene *giant*, demonstrated the accompanied fitness effects of evolved differences and confirmed giant's involvement of species hybrid incompatibility. Expression defects of the gap gene network in species hybrid and in *giant* transgenes disprove functional equivalence of gap gene network from different *Drosophila* species.

5.1 Gap gene network as a population genetic model

50 years ago —September 1972— Richard C. Lewontin drafted his transformational book, *The Genetic Basis of Evolutionary Change*, in the very same building I conducted my graduate research [146]. Drawing on his pathbreaking collaboration in 1966 with his University of Chicago colleague, the biochemist Jack Hubby, Lewontin was the first to bring population genetics to a stage at which it could measure genetic variation segregating in populations at single gene loci [147, 148]. It heralded in the beginning of molecular population genetics and evolution. In his book, Lewontin introduced the "modest goal" of population genetics as "providing a description of the genetic state of populations laws of transformation of state that were both dynamically and empirically sufficient", which is depicted by the following equation:

$$G_1 \xrightarrow{T_1} P_1 \xrightarrow{T_2} P_2 \xrightarrow{T_3} G_2 \xrightarrow{T_4} G_1' \xrightarrow{T_1}$$

Where G_1 and G_2, and P_1 and P_2, represent the genotypic (G) and phenotypic (P) states of the population at time t and $t + \Delta t$,respectively. The transformations of state are governed by ontogenetics and development ($T1$), natural selection other forces of differential survival ($T2$), the laws of genetics ($T3$) and reproduction ($T4$). Population genetic theory

provides the genotype-phenotype mapping and provides a transformation in the phenotype space [146]. This process is shown in Fig. 5.1a.

The continuous functional evolution of individual gap genes, such as *giant*, while maintaining phenotypic stasis of the gap gene network output, represent a special case of Lewontin's depiction (Fig. 5.1b) of the evolutionary dynamics of a trait, where a constrained phenotypic state may map to different genotypic states over time. Phenotypic stasis does not forbid genotypic transformation, and different genotypic optima in different species over millions of years of divergence could map to the same phenotypic state. The gap gene network in *Drosophila* provides a simplified system for population geneticists to focus on the mechanisms and fitness costs of genotypic transformation without complications from phenotypic transformation.

5.2 Functional divergence of *giant* with fitness effects

Giant protein is highly conserved in *Drosophila melanogaster* subgroup, with more than 98% protein sequence identity among *D. melanogaster*, *D. yakuba*, *D. santomea* and *D. erecta*. Yet careful examination of *giant* orthologs shows that their evolved sequence differences are not functionally inert: *giant* transgenes differing only in protein coding sequence (and a small intron) have distinguishable fitness effects in a *D. melanogaster* genetic background. Greater fitness loss is observed when the two most divergent orthologs —*D. pseudoobscura* and *D. virilis giant*— replaced the endogenous *D. melanogaster* locus. But I was also able to detect a fitness effect of a single amino acid difference between two closely related species —*D. yakuba* and *D. santomea*.

Giant shows no signatures of positive selection or accelerated sequence divergence. Instead it has classic hallmarks of functional conservation of all kinds – a slow sequence substitution rate, conserved expression pattern and similar DNA binding affinity. We purposely chose it as an example to determine whether, and the pace at which, such a conserved

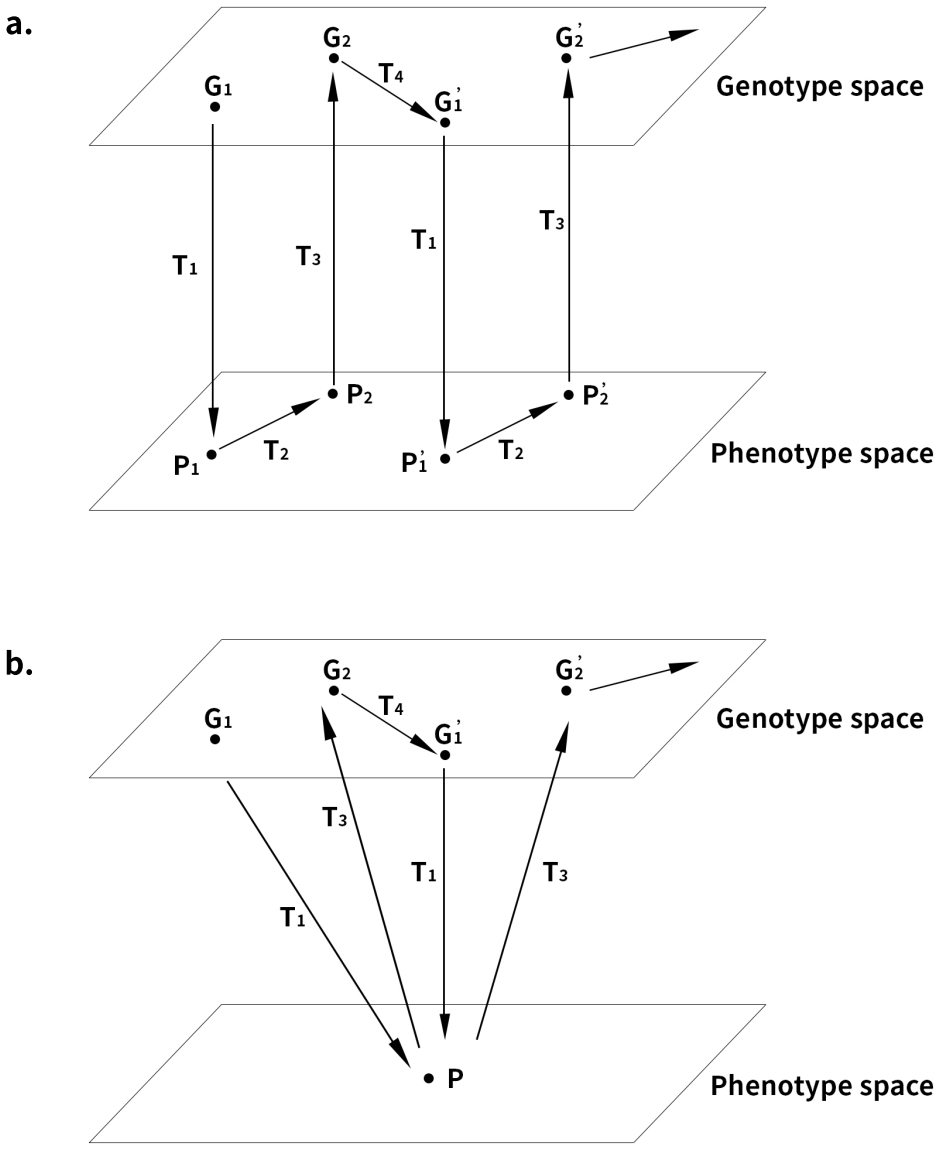

Figure 5.1: **Genotype-phenotype mapping.**
a. Schematic representation of the path of transformation of population genotype from one generation to the next (adopted from Figure 1, Lewontin 1974[146]) **b**. The evolution of gap gene network represents a special case of Lewontin's depiction, where the phenotype remains unchanged (conserved) as genotypes travel through the genotype space.

transcription factor can functionally evolve. To our surprise, I identified rapid functional evolution of *giant* throughout *Drosophila* phylogeny, as no two *giant* orthologs are functionally equivalent from the six species I investigated. *Giant* is continuously evolving despite the selective constraint imposed in maintaining conserved phenotypic outputs.

This decoupling of genotypic evolution despite phenotypic constancy demands more rigorous examination of any assumption of genotypic constancy when such a conservation claim is being made from phenotypic comparisons. Specifically, *giant* and the gap gene network (see below) show that genotypic evolution can be decoupled from phenotypic evolution for conserved traits. Essential developmental pathways can have conserved phenotypic outputs without having identical genotypic underpinnings.

5.3 Whole-locus evolves as an entity

Whether mutations and adaptive changes in developmental processes are more common in *cis*-regulatory sequences than in *trans*-regulators of gene expression has been a longstanding issue. It has been addressed in studies of gene expression in hybrids [149] and for specific trait differences that could be genetically mapped [1]. Here by swapping coding and noncoding regions of *giant* orthologs, individually or entirely (i.e., whole-locus), and monitoring both *cis*- and *trans*- effects, as well as their interactions, our results suggest that artificial separation of *cis*- and *trans*- overlooks the issue and ignores the possibility, realized for *giant*, that the whole-locus evolves as an entity.

Coding and noncoding regions from *giant* orthologs both show fitness effects, and we observed species-specific epistasis between them from at least *D. santomea* and *D. virilis giant*. This suggests that natural selection acts on the phenotypic outputs of *giant* whole-locus, not the coding or noncoding regions alone. Studying the effects of coding and noncoding regions separately may be of interest from a molecular biology perspective, but less so from the perspective of understanding natural selection; after all, selection only "sees" the phenotypic

outputs of a gene, which depend on the whole-locus as an entity.

5.4 Gap gene network evolves as an entity

For complex developmental processes under stabilizing selection the mutational target of compensatory or stabilizing selection is the entire suite of *cis*- and *trans*- components acting across the network of interacting loci. This will be especially true for the gap gene network because of the extensive self- and cross-regulation of its members. My experimental exploration of evolved interactions among gap genes provide an explicit attempt to move beyond the myopia of single-locus or single-module investigations.

I show that gap gene networks from different species maintain conserved expression outputs but are not identical: the gap gene network is incompatible between *D. melanogaster* and *D. santomea* and mis-interactions between *D. virilis giant* and the *D. melanogaster* gap gene network causees mis-expression of *D. virilis giant* and of *D. melanogaster eve*. This discrepancy between conserved phenotypes produced by the gap gene network, and evolved underlying genotypes involving interactions among gap genes, suggests that the target of selection is phenotypic outputs regulated by interactions among members of the network, which leads me to conclude that the gap gene network must evolve as a single entity.

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