

# Hybrid Incompatibility Despite Pleiotropic Constraint in a Sequence-Based Bioenergetic Model of Transcription Factor Binding

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**ABSTRACT** Hybrid incompatibility can result from gene misregulation produced by divergence in *trans*-acting regulatory factors and their *cis*-regulatory targets. However, change in *trans*-acting factors may be constrained by pleiotropy, which would in turn limit the evolution of incompatibility. We employed a mechanistically explicit bioenergetic model of gene expression wherein parameter combinations (number of transcription factor molecules, energetic properties of binding to the regulatory site, and genomic background size) determine the shape of the genotype–phenotype (G-P) map, and interacting allelic variants of mutable *cis* and *trans* sites determine the phenotype along that map. Misregulation occurs when the phenotype differs from its optimal value. We simulated a pleiotropic regulatory pathway involving a positively selected and a conserved trait regulated by a shared transcription factor (TF), with two populations evolving in parallel. Pleiotropic constraints shifted evolution in the positively selected trait to its *cis*-regulatory locus. We nevertheless found that the TF genotypes often evolved, accompanied by compensatory evolution in the conserved trait, and both traits contributed to hybrid misregulation. Compensatory evolution resulted in “developmental system drift,” whereby the regulatory basis of the conserved phenotype changed although the phenotype itself did not. Pleiotropic constraints became stronger and in some cases prohibitive when the bioenergetic properties of the molecular interaction produced a G-P map that was too steep. Likewise, compensatory evolution slowed and hybrid misregulation was not evident when the G-P map was too shallow. A broad pleiotropic “sweet spot” nevertheless existed where evolutionary constraints were moderate to weak, permitting substantial hybrid misregulation in both traits. None of these pleiotropic constraints manifested when the TF contained nonrecombining domains independently regulating the respective traits.

**A**DAPTIVE changes in phenotype often occur through changes in gene regulation (Wray 2007; Carroll 2008). Regulatory networks map an organism’s genotype to its phenotype through developmental and physiological processes (Wilkins 2002). Such networks consist of interacting loci that can respond to selection by changing the expression levels of individual genes in space and time. In addition to gene interaction (epistasis) (Phillips 2008), gene networks are also characterized by pleiotropy, where single genetic loci have manifold effects (Gibson 1996; Paaby and Rockman 2013).

Gene interactions are pervasive in the evolution of hybrid incompatibility, an important form of reproductive isolation between species (Coyne and Orr 2004). The leading model for the evolution of hybrid incompatibility, the Bateson–Dobzhansky–Muller (BDM) model, requires interactions between at least two genetic loci (Bateson 1909; Dobzhansky 1937; Muller 1942). Interpopulation divergence in regulatory interactions may be expected to result in hybrid incompatibility due to misregulation of the traits they control (Johnson and Porter 2000, 2001; Landry *et al.* 2007; Ortiz-Barrientos *et al.* 2007; Maheshwari and Barbash 2011, 2012). Theoretical studies (Johnson and Porter 2000, 2007; Palmer and Feldman 2009) of the molecular basis of evolving regulatory interactions demonstrate that misregulation in hybrids readily arises as a byproduct of adaptation. Tulchinsky *et al.* (2014) show that the degree of this hybrid incompatibility is determined in large part by the bioenergetic [thermodynamic plus kinetic (Morowitz

Copyright © 2014 by the Genetics Society of America  
doi: 10.1534/genetics.114.171397

Manuscript received July 7, 2014; accepted for publication October 6, 2014; published Early Online October 13, 2014.

Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.171397/-/DC1>.

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1978)] details of the molecular interactions between coevolving transcription factors and the sites to which they bind.

BDM incompatibilities between species often express themselves in traits other than those involved in adaptation to divergent environments, although the traits may share a genetic basis (Schluter 2009). Pleiotropy and genetic linkage have been proposed as possible mechanisms by which selection on one trait may result in hybrid dysfunction in a different trait (Rundle and Nosil 2005; Johnson and Porter 2007; Via 2009). At the same time, opposing selective forces on coregulated traits should impose constraints on the evolution of loci with pleiotropic effects (Stern 2000; Wray 2007). For that reason, several authors have hypothesized that most adaptive changes in gene expression are due to changes in trait-specific *cis*-regulatory regions rather than in *trans*-acting transcription factors (TFs) that bind pleiotropically to multiple sites (e.g., Stern 2000; Prud'homme *et al.* 2007). Expression changes constrained to a trait's *cis*-regulatory region would not produce hybrid misregulation, which requires coevolved changes in interacting loci. Nonetheless, *cis-by-trans* incompatibility between TFs and their binding sites is commonly observed in hybrids (Wittkopp *et al.* 2004; Landry *et al.* 2005). The purpose of this study is to explore this apparent contradiction from the perspective of the molecular basis of pleiotropic regulatory interactions, to determine the bioenergetic properties of these interactions that permit BDM incompatibilities to evolve despite evolutionary constraint.

How might *cis-by-trans* divergence occur if adaptation in *trans* is constrained by pleiotropy? One possibility is that regulatory incompatibility in hybrids results from changes in the expression level of TFs rather than their coding sequence (Wittkopp *et al.* 2004). The modular structure of *cis*-regulatory regions may permit changes in expression to be confined to specific tissues or conditions and therefore affect fewer regulatory targets than would a change in DNA-binding specificity (Stern 2000; Prud'homme *et al.* 2006). Another possibility is that the sequence of a TF is often less constrained by pleiotropy than its number of downstream targets would suggest (Wagner and Lynch 2008; Baker *et al.* 2011). Transcription factors are typically modular in structure, consisting of multiple DNA-binding and cofactor-binding domains (Hsia and McGinnis 2003). As with *cis*-regulatory regions, this modularity may allow tissue-specific or condition-specific evolutionary responses that affect only a subset of the TF's potential targets (Hsia and McGinnis 2003; Wagner and Lynch 2008). This inherent limit to the extent of pleiotropy is complemented by the possibility that pleiotropic changes occur but their deleterious effects are later compensated (Haag 2007; Kuo *et al.* 2010; Pavlicev and Wagner 2012). Compensation can contribute to hybrid incompatibility if different combinations of deleterious and compensatory changes occur in diverging populations (Landry *et al.* 2005). In this study, we examine the conditions under which compensatory evolution between a TF and its binding site

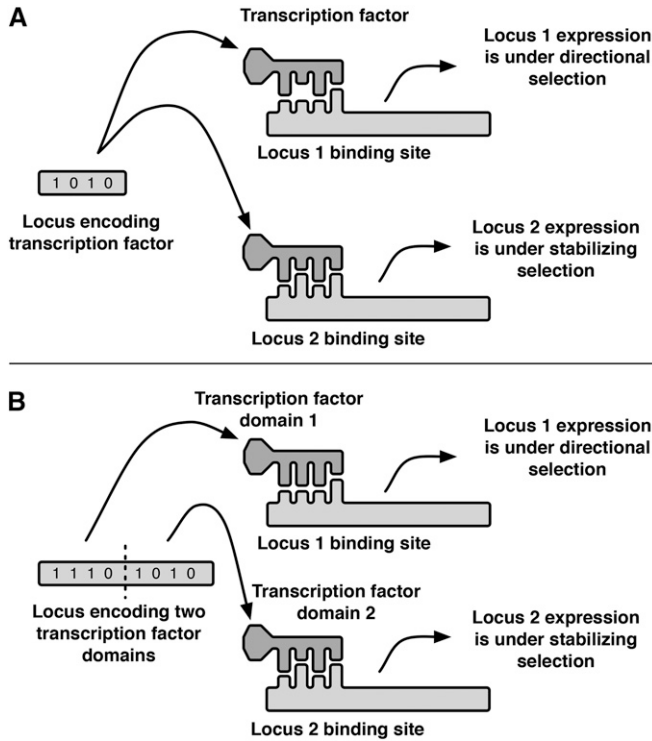
may facilitate *cis-by-trans* regulatory incompatibility despite pleiotropic constraint.

## Model

We model, in diploids, a three-locus, two-interaction regulatory network in which a pleiotropic TF regulates the expression of two downstream loci, one under directional selection and the other under stabilizing selection. Our previous results, for a two-locus interaction that regulates expression of a single trait, show that hybrid incompatibilities rapidly evolve as a byproduct of adaptation when that trait is under positive selection (Johnson and Porter 2000; Tulchinsky *et al.* 2014). Under stabilizing selection, we found that more gradual and parameter-limited evolution of incompatibilities could occur due to compensatory evolution following genetic drift (Tulchinsky *et al.* 2014). We expect the addition of a regulatory target under positive selection to accelerate evolutionary change in the TF compared to the case of a single conserved trait. Likewise, the addition of a trait under stabilizing selection should constrain TF change compared to the case of a single positively selected trait. If adaptive change occurs at the pleiotropic TF locus, it will select for compensation at the conserved regulatory target (Johnson and Porter 2007), resulting in hybrid misregulation at both interactions in the three-locus network.

To quantify the extent of pleiotropic constraint, we compare two different TF architectures. In the first, the TF consists of a single DNA-binding domain that binds to the *cis*-regulatory regions of two downstream loci associated with separate traits as illustrated in Figure 1A. In the second, the TF contains two nonrecombining DNA-binding domains, each of which binds to the regulatory region of one of the downstream loci (Figure 1B). In the terminology of Paaby and Rockman (2013), these correspond to multifunctional TF types showing “developmental pleiotropy” and “molecular gene pleiotropy,” respectively. As shorthand, we refer to these as the “pleiotropic” and “two-domain” models. Our shorthand follows a distinction made by evolutionary geneticists when offering alternative explanations, pleiotropy vs. linkage disequilibrium, for correlations between evolving traits (e.g., Hartl and Clark 2007). We characterize the one-domain TF as being mechanistically pleiotropic, while the two-domain TF serves as a control for the potential effects of maximal linkage disequilibrium between otherwise mechanistically independent regulatory sites.

We obtain a simple quantitative model of compensatory evolution by applying a bioenergetic, information-based model of transcriptional regulation (Von Hippel and Berg 1986; Gerland *et al.* 2002; Mustonen *et al.* 2008; Tulchinsky *et al.* 2014). We give an overview here and refer the reader to Tulchinsky *et al.* (2014) for details. The principle is that the phenotype is determined by the level of gene expression and that gene expression is determined by fractional



**Figure 1** Three-locus regulatory network in which a pleiotropic transcription factor (TF) regulates the expression of two downstream loci. Locus 1 is under directional selection while locus 2 is under stabilizing selection. At each locus, expression depends on the fit between the transcription factor and a binding site in the *cis*-regulatory region. The binding preference of the transcription factor and the nucleotide sequence of the *cis*-regulatory site are represented as binary strings, whose total number of matching positions determines the binding strength. For illustrative purposes, only 4 bits of the binding interaction are shown. An adaptive change in the expression of locus 1 may be accomplished either by a mutation in its *cis*-binding site or in the transcription factor. Changes in the transcription factor select for compensatory mutations in the *cis*-binding site of locus 2. Thus, if selection on locus 1 results in interpopulation divergence in the transcription factor, hybrid misregulation of both loci will occur as each binding interaction evolves a unique fit to produce the favored expression level in parent populations. (A) “Pleiotropic model” where a single domain of the TF regulates both downstream loci. (B) “Two-domain model” where two nonrecombining domains of the TF independently regulate the downstream loci.

occupancy—the probability that a TF is associated with its *cis*-regulatory site at any given moment. Fractional occupancy depends on the number of TF molecules and the energetics of their binding to the *cis*-regulatory region, which depends in turn on the number of matching positions between the TF’s recognition motif and the sequence of the binding site.

The transcription factor’s binding motif(s) and the *cis* binding-site sequences of the two downstream loci are mutable alleles, each represented by a string of bits. In a motif of length  $n$ , the number of mismatched bits  $m$  determines the energy of binding between the TF and the binding site, which determines the fractional occupancy  $\theta$ . Each matching bit increases the fractional occupancy, which is maximal when  $m = 0$ . In diploids, fractional occupancy is specific

to the allelic combination at the TF and the *cis*-regulatory site, with TF alleles competing for binding at each allelic copy of that *cis*-regulatory locus. The fractional occupancy for each allelic combination is

$$\theta' = \frac{N_{\text{TF}}}{N_{\text{TF}} + \alpha e^{-m\Delta G_1 - E_{\text{diff}}}}, \quad (1)$$

where  $N_{\text{TF}}$  is the number of molecules of the allele-specific TF in the cell,  $\Delta G_1$  is the contribution of a single matched bit to the free energy of formation between that TF and the *cis*-regulatory site allele,  $\alpha = 1 + N_{\text{TF}} e^{m_c \Delta G_1 + E_{\text{diff}}}$  accounts for the reduction in fractional occupancy of a TF variant from competition with the other variant, and  $m_c$  is the number of mismatches in the competing TF allele.  $E_{\text{diff}}$  is a heuristic parameter that measures the free energy of TF binding to the nonspecific intracellular background relative to that of a perfectly matched TF-*cis* site pair, such that  $E_{\text{diff}} < 0$  when fractional occupancy on the genomic background is higher than to the *cis*-regulatory site. This assumes that  $N_{\text{TF}}$  and  $E_{\text{diff}}$  are equal between TF alleles.

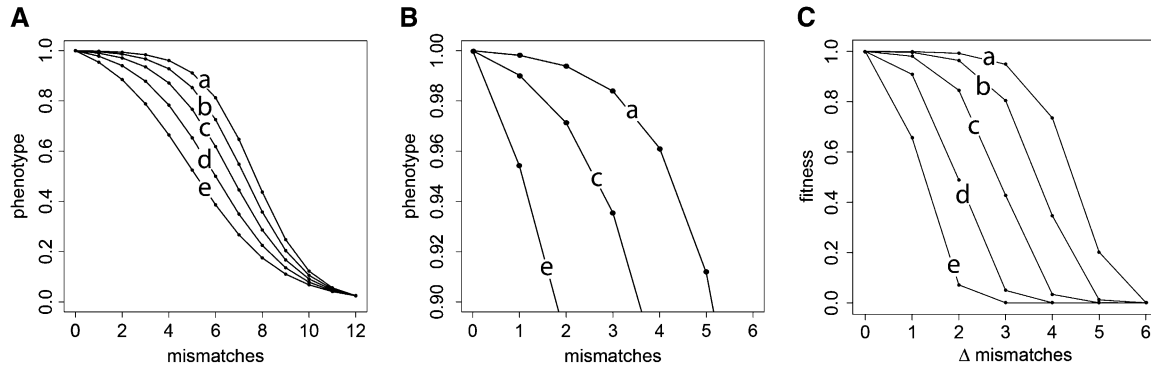
Expression proceeds when the TF is bound to the *cis*-regulatory region, so the final level of expression is proportional to the fractional occupancy (Gertz *et al.* 2009). We treat the phenotype,  $P$ , as proportional to the level of expression. We calculate the diploid phenotype as the sum of expression from each of the four allele-specific  $\theta'$  values, scaled following Tulchinsky *et al.* (2014) so that the maximum phenotype  $P_{\text{max}} = 1.0$  for a double homozygote with no mismatches ( $m = 0$ ).

Fitness is a function of an organism’s deviation from the optimal phenotypic value at each trait. The total organismal fitness is the product of the marginal fitnesses at each trait and these marginal fitnesses are

$$W = \exp \left[ \frac{-(P - P_{\text{opt}})^2}{2\sigma_s^2} \right], \quad (2)$$

where  $P_{\text{opt}}$  is the trait’s environmentally determined, optimal phenotypic value and  $\sigma_s^2$  is the variance of fitness around that optimum (a measure of the degree to which a suboptimal phenotype is tolerated by selection).

We previously found that hybrid incompatibility occurred more readily in a two-locus, single-trait model under bioenergetic parameters that yield either a shallower fitness landscape around a conserved trait or a steeper fitness landscape around a directionally selected trait (Tulchinsky *et al.* 2014). Here we examine the effect of the genotype-to-phenotype and phenotype-to-fitness functions on pleiotropically coregulated traits. In our model, the general forms of the genotype–phenotype (G-P) map and fitness landscape are sigmoid, with their explicit shapes determined by the values of  $N_{\text{TF}}$ ,  $\Delta G_1$ , and  $E_{\text{diff}}$  (and for the fitness landscape,  $P_{\text{opt}}$  and  $\sigma_s^2$  as well). Figure 2 shows this effect as well as the combinations of parameter values we used in this study. Heuristically, it is useful to characterize these shapes qualitatively



**Figure 2** G-P map shapes determined by bioenergetic parameter combinations, from figure 2 in Tulchinsky *et al.* (2014), used to assess the effects of bioenergetic parameters on pleiotropic constraint. (A) G-P maps a–e, shown with a motif length of  $n = 12$  bits. (B) G-P maps a, c, and e over the phenotypic range of 0.9–1.0, emphasizing the difference in slopes. Conditions: we held  $N_{TF}$  constant at 100 and varied  $E_{diff}$  in steps of 1.0, covarying  $\Delta G_1$  with it to enforce a constant phenotype of  $P \approx 0$  when none of the  $n$  bits matched. (C) Fitness landscapes corresponding to the G-P maps of A, at  $P_{opt} = 1$  and  $\sigma_s^2 = 2.5 \times 10^{-3}$ . The conditions are (a)  $E_{diff} = 2$ ,  $\Delta G_1 = -0.858$ ; (b)  $E_{diff} = 1$ ,  $\Delta G_1 = -0.774$ ; (c)  $E_{diff} = 0$ ,  $\Delta G_1 = -0.692$ ; (d)  $E_{diff} = -1$ ,  $\Delta G_1 = -0.610$ ; and (e)  $E_{diff} = -2$ ,  $\Delta G_1 = -0.530$ .

rather than as vectors of explicit values. To do this, we use the term “slope” as the difference in phenotype between 0 and 1 mismatches; it follows that shape “a” in Figure 2A has the shallowest slope and “e” the steepest. This characterization extends isomorphically to the fitness landscape as well, where slope is also influenced by the value of  $\sigma_s^2$ .

## Simulations

We conducted individual-based simulations to examine the effects of bioenergetic parameter combinations and fitness landscape on the evolution of net  $F_2$  hybrid misregulation. (We assess the effects of evolutionary rate and mutation effect size in Supporting Information, File S1.) A pair of independent populations was subjected in parallel to trait-specific directional and stabilizing selection. The two loci downstream from the *trans*-acting TF locus correspond to separate, *cis*-regulated traits (Figure 1). One of these traits was held under stabilizing selection at a phenotypic value of  $P_{opt} = 1.0$ , such that zero mismatched bits was optimal in its binding interaction. The other trait was placed under directional selection such that the optimal number of mismatches changed from  $m = n$  to  $m = 0$  over the course of the simulation. This was accomplished by setting the initial trait optimum to a value near  $P_{opt} = 0$ , corresponding to the phenotypic value produced by the maximum number of mismatches. The initial genotype of all individuals was set such that each trait started at its optimum. Evolutionary change in the directionally selected trait was achieved by adjusting  $P_{opt}$  by a constant amount  $\Delta P_{opt}$  each generation to arrive at a final optimum of  $P_{opt} = 1.0$ .

Each generation consisted of viability selection followed by random mating. Population size was kept constant each generation, with no overlap of generations. Thus, populations could experience bottlenecks between viability selection and mating, but returned to carrying capacity each generation. In the fitness function, we set  $\sigma_s^2$  to  $2.5 \times 10^{-3}$

at each trait; the effects of variation in this parameter are presented in File S1. In keeping with our two-locus model (Tulchinsky *et al.* 2014), mutations occurred in the offspring at a baseline rate of  $8.333 \times 10^{-5}$  per bit, equivalent to 0.001 per locus with an  $n = 12$  bit motif length. Each mutation changed one bit of information. We varied the mutation rate experimentally as described below.

We studied the effects on  $F_2$  hybrid misregulation of the G-P map and marginal fitness landscapes by varying the  $E_{diff}$  and  $\Delta G_1$  parameters, using the range of values in Tulchinsky *et al.* (2014). As binding to the genetic background increases with decreasing values of  $E_{diff}$ , TF availability at the target binding sites declines rapidly following an exponential function. We adjusted  $\Delta G_1$  along with  $E_{diff}$  to keep a constant phenotype of  $P \approx 0$  ( $P = 0$  is an asymptote and we used  $P = 0.0245$ ) when all bits were mismatched; parameter values are in the Figure 2 legend. Following Tulchinsky *et al.* (2014), we used alleles of length  $n = 12$  bits and an evolutionary rate of  $\Delta P_{opt} = 1/40,000$  generations. We held constant the upstream expression level of the TF-determining locus at  $N_{TF} = 100$ . This does not limit the generality of our results, because it is a property of Equation 1 that the effects on fractional occupancy of varying  $N_{TF}$  and  $E_{diff}$  are isomorphic, such that the effect of changing  $E_{diff}$  on a linear scale can be duplicated by changing  $N_{TF}$  on an exponential scale.

If pleiotropy constrains the substitution rate at the TF locus because of its negative side effects on the conserved trait, then mutation rate should influence the extent of that constraint. Low overall availability of mutations should cause more TF mutants to be tolerated despite their side effects. To investigate the extent to which each of the three mutable loci contributes to pleiotropic constraint, we ran simulations in which we varied the overall mutation rate, as well as mutation rates at each locus in the network, between 1/8 and 8 times the baseline rate above. For these, we used G-P map “d” of Figure 2, and the evolutionary rate was set to  $\Delta P_{opt} = 1/4000$  generations.

Net misregulation of each trait in the hybrid phenotype was assessed at the end of the final generation. Tulchinsky *et al.* (2014) showed that  $F_1$  misregulation is typically less than or equal to  $F_2$  misregulation, depending on the bioenergetic parameter combinations that determine allelic dominance in gene expression, so we report only  $F_2$  misregulation here. The parental populations were cross-mated randomly to create 50  $F_1$  hybrids, which were then randomly mated with each other to create 50  $F_2$  hybrids without prior viability selection on the  $F_1$ 's. We measured the mean deviation of  $F_2$  hybrids from the optimal phenotype at each trait. This total  $F_2$  hybrid misregulation includes a fraction representing genetic load in the parental populations. We therefore calculated the net  $F_2$  hybrid misregulation by subtracting the mean deviation of the parents at each trait from the total  $F_2$  deviation. This represents the portion of  $F_2$  misregulation that resulted solely from Bateson–Dobzhansky–Muller incompatibilities between populations. While net misregulation in the hybrid phenotype is our primary parameter of interest in assessing BDM incompatibilities, we also report the marginal fitnesses of each trait, assuming the fitness model of Equation 2. These results are based on 200 replicates. Simulations were written in C in the Xcode environment and run in Mac OS X, and graphics were produced using the statistical package R.

## Results

### **Sensitivity of pleiotropic misregulation to bioenergetics of TF binding**

When we varied the bioenergetic parameter values that determine the G-P map and fitness landscape shapes of Figure 2, net  $F_2$  hybrid misregulation occurred at both the directionally selected and conserved traits, but the patterns were different (Figure 3). Misregulation in the two-domain model's directionally selected trait (Figure 3A, shaded bars) climbed steeply as the G-P map shapes of Figure 2 proceeded from a to e and was not affected by population size. The simple two-locus model of Tulchinsky *et al.* (2014) (their figure 3) showed the same pattern, indicating that having a second TF domain under stabilizing selection does not constrain evolution at the first domain. In contrast, in the pleiotropic model, net  $F_2$  misregulation of the directionally selected trait (Figure 3A, open bars) climbed gradually, starting at shape a, and then declined rapidly by shape e. The trajectory depended on population size, peaking earlier as population size increased. Misregulation in the directionally selected trait (Figure 3A) was higher than in the conserved trait (Figure 3B) in both the pleiotropic (open bars) and two-domain (shaded bars) models.

For the conserved trait (Figure 3B), the pleiotropy model (open bars) showed the same pattern of misregulation seen in the directionally selected trait. Misregulation in the two-domain model (shaded bars) followed almost the same pattern as that in the two-locus model under stabilizing selection (Tulchinsky *et al.* 2014, their figure 3), where misregula-

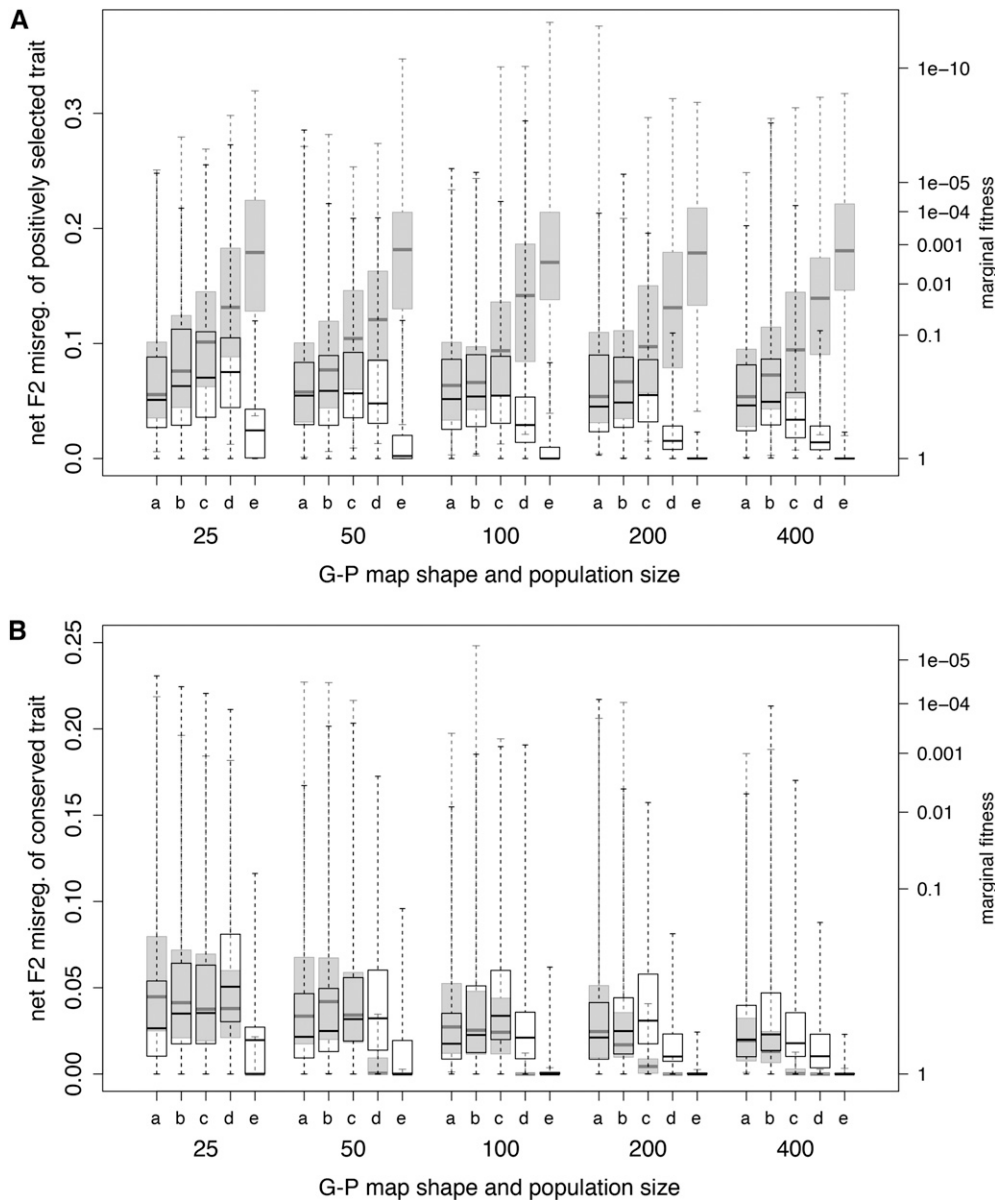
tion was associated with compensatory evolution. Here, compensatory evolution in the second domain of the two-domain TF was largely unaffected by the selection response at the first domain. Thus, the two-domain model behaved as if it were a pair of independently regulated two-locus traits.

The dynamics underlying these patterns in the pleiotropic model are influenced by the slopes of the G-P map and fitness landscape. To illustrate these influences, we can follow the substitution dynamics in the parental populations by referring to Figure 2B, looking first at G-P map e. As  $P_{\text{opt}}$  for the directionally selected trait approaches  $\sim 0.95$ , restoring the mean marginal fitness of that trait to  $W_{\text{dir}} = \sim 1$  can be accomplished by fixing a mutant having one mismatch. But, because the slope of the G-P map is steep, any mutation at the TF site will simultaneously drop the marginal fitness of the conserved trait to  $W_{\text{cons}} = 0.66$  (Figure 2C). This is a strong evolutionary constraint on the TF locus. Selection thus favors mutants in the *cis*-regulatory site of the directionally selected trait, which have no such pleiotropic fitness cost.

In the G-P map c of Figure 2B, with a moderate slope, fixation of a new mutant of the directionally selected trait is favored with a lesser change in  $P_{\text{opt}}$ , to  $\sim 0.99$ . Although a *cis*-site mutant for this trait yields the highest mean fitness, a substitution at the TF does not diminish the marginal fitness of the conserved trait very much, to  $W_{\text{cons}} = 0.98$ . Compensation is ultimately more likely because it need not occur as quickly, and over the course of the simulation, more compensation can take place. More  $F_2$  hybrid misregulation therefore accumulates at the conserved locus, and the pleiotropic constraint is relaxed under the bioenergetic parameter combinations that yield this map.

In contrast, in G-P map a of Figure 2B, the slope is very shallow and the upward trend in phenotypic misregulation reverses. The phenotypes of either trait are little affected until several mismatches become fixed, so selection for compensation at the conserved trait becomes strong only when the number of mismatches remains high (on the order of  $m > 3$  on this map, beyond which  $W_{\text{cons}} < 0.74$ ). With little to compensate for, less compensation evolves. Moreover, by the end of the simulation, even though parental populations had diverged, hybrids with several mismatches still showed similar phenotypes to those of parents, with little evidence of phenotypic misregulation. As population size increases, selection becomes more efficient, causing pleiotropic constraint to become more evident at shallower slopes.

Net  $F_2$  misregulation in the pleiotropic model was consistently lower in the conserved trait (Figure 3 and Figure S1). This traces to the dynamics of compensatory substitution in the *cis* site of the conserved trait in the later stages of the simulation. Misregulation of the conserved trait remains relatively lower until compensation is complete, whereupon it reaches that of the directionally selected trait; this holds for both total and net misregulation. Incomplete compensation is more common when the G-P map is shallow because the



**Figure 3** Median net F<sub>2</sub> hybrid misregulation as a function of bioenergetic parameters for the pleiotropic (open boxes) and two-domain (shaded boxes) models, at population sizes 25–400. Bioenergetic parameter combinations determine G-P map and fitness landscape shapes following Figure 2, with shape “a” having the shallowest and “e” having the steepest slope. (A) Misregulation of the directionally selected trait. (B) Misregulation of the conserved trait. Note the different scales. Box plots show median, quartiles, and full ranges. Simulation conditions: directional selection was applied over 40,000 generations, using a 12-bit motif.

fitness advantage of the last compensatory mutant is low (for map a of Figure 2, the advantage is only  $\Delta W = 0.005$ ), and complete remediation may not be reached until a great many generations after the bout of directional selection ends.

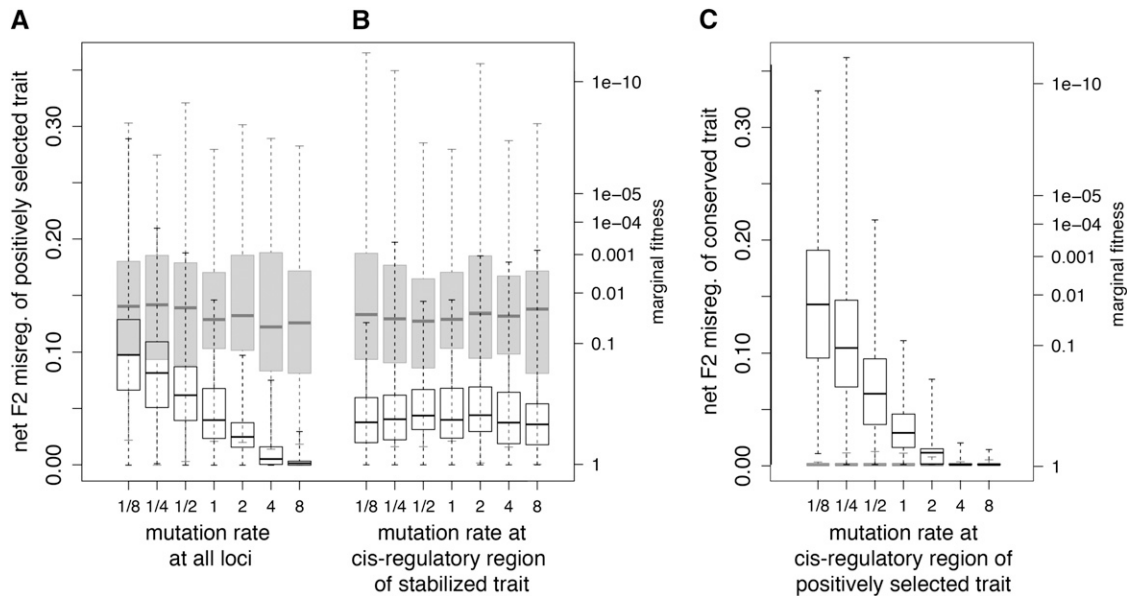
### Sensitivity to mutation rate

Decreasing the overall mutation rate increased net F<sub>2</sub> hybrid misregulation of the positively selected trait in the pleiotropic model, but not in the two-domain model (Figure 4A). In the former, where the TF is mechanistically pleiotropic, a dearth of new mutations increases the net fitness advantage of any new TF mutant that does arise, provided its favorability at the positively selected trait overcomes its disadvantage at the conserved trait. This promotes the evolution of misregulation. However, as the overall mutation rate increases, enough new mutants arise at the *cis* locus

of the directionally selected trait and more of the adaptive change can occur there. This limits the evolution of misregulation, which requires changes at both the TF and the *cis* locus.

To examine the importance of compensation in the evolution of hybrid incompatibility, we varied the mutation rate at the *cis*-regulatory locus of the conserved trait while holding it constant at the other two loci and measured the effect on misregulation at the positively selected trait. Pleiotropy constrained misregulation relative to the two-domain model, but neither model was sensitive to mutation rate at this locus (Figure 4B).

Decreasing the mutation rate at the *cis*-regulatory locus of the positively selected trait alone increased net F<sub>2</sub> hybrid incompatibility at the conserved trait (Figure 4C). A lower availability of mutations at this locus increased divergence at the TF locus by increasing the net fitness advantage of TF



**Figure 4** Median net  $F_2$  hybrid misregulation under varying mutation rates occurring at *cis*-regulatory sites of separate traits in the pleiotropic (open boxes) and two-domain (shaded boxes) models. Mutation rates are expressed as multiples (0.125–8) of the baseline mutation rate of  $8.333 \times 10^{-5}$ /bit. (A) Median net  $F_2$  misregulation of the positively selected trait as a function of varying the overall mutation rate. Misregulation was constrained to lower levels in the pleiotropic model relative to the two-domain model and the extent of constraint increased with mutation rate. (B) As in A, but instead varying the mutation rate only at the *cis*-regulatory locus of the conserved trait. Misregulation was again constrained in the pleiotropic model relative to the two-locus model, but was not sensitive to mutation rate. (C) Misregulation of the conserved trait as a function of the mutation rate at the *cis*-regulatory locus of the positively selected trait. In the pleiotropy model, misregulation was high at low mutation rates, dropping to zero as mutation rate increases, but no misregulation occurred in the two-domain model. Box plots show median, quartiles, and full ranges. Simulation conditions: directional selection was applied over the course of 4000 generations; population size = 400.

mutants, increasing hybrid misregulation. No misregulation of the conserved trait was found in the two-domain model under these conditions.

## Discussion

### *The pleiotropic sweet spot for hybrid incompatibility*

Hybrid incompatibility is predicted to arise from the disruption of regulatory genetic networks that have diverged since the hybridized populations shared a common ancestor (Johnson and Porter 2000). These disruptions are the basis of Bateson–Dobzhansky–Muller interactions responsible for hybrid dysfunction. At the same time, pleiotropy is a ubiquitous characteristic of these networks, and pleiotropic regulatory loci typically affect a diversity of traits, most of which are likely to be evolutionarily conserved. We might therefore predict strong evolutionary constraints against divergence at pleiotropic points in the network (Stern 2000; Wray 2007), and indeed, most regulatory divergence between *Drosophila* species occurs at *cis*-acting sites (Wittkopp *et al.* 2008). These predictions are at odds because transcription factors are fundamental to gene regulation and at the same time, typically pleiotropic. We tested these conflicting predictions in this study, using a bioenergetic model of gene regulation. We found that pleiotropy did indeed constrain the evolution of hybrid incompatibility to varying extents and precluded it altogether under some conditions. However, net misregula-

tion of the  $F_2$  hybrid phenotype was reasonably strong under a variety of intermediate bioenergetic parameter values, corresponding to a broad sweet spot of parameter combinations permitting the evolution of Bateson–Dobzhansky–Muller incompatibility.

Adaptation can occur at a pleiotropic locus when the selective advantage due to one trait is greater than the disadvantage due to pleiotropy with others (Otto 2004). This was the case in our pleiotropic model because the bioenergetic properties of molecular interactions yield sigmoid G-P maps (Figure 2), creating a nearly neutral region in the fitness landscape around the conserved trait. Parameters that steepen the fitness landscape decrease the advantage of a pleiotropic mutation relative to a *cis*-regulatory mutation and impose evolutionary constraint on the pleiotropic locus. These include any factors that reduce the availability of TF molecules in the region of the binding sites, particularly larger genome size, which increases  $E_{diff}$ , the extent that the genetic background attracts TFs away from the binding site, as well as factors that reduce  $N_{TF}$ , particularly the upstream regulators of TF production. The fitness landscape is also steeper if the *cis*–*trans* interaction is sensitive to mismatches (high  $\Delta G_1$ ) or if the environment is less tolerant to phenotypic variation ( $\sigma_s^2$  in the fitness function). Increased constraint on the pleiotropic TF restricted adaptive change in the directionally selected trait to its *cis*-regulatory locus and precluded compensatory evolution in the loci



underlying the conserved trait. This constrained the evolution of  $F_2$  hybrid incompatibility because both *cis* and *trans* sites must coevolve to produce it.

For a different reason, we also see reduced hybrid misregulation under bioenergetic parameter values that cause the G-P map and fitness landscape to become increasingly plateaued (e.g., shape a in Figure 2). Phenotypes near the upper end of such maps are insensitive to mutation, such that several mismatches are required to substantially change them.  $F_2$  hybrids show relatively little misregulation or incompatibility, not because pleiotropy constrains divergence in the TF but simply because insensitivity of the phenotype to underlying divergence masks it. This is the case under parameter combinations where TFs are superabundant (high  $N_{TF}$  and low  $E_{diff}$ ) or binding is robust to mutation (low  $\Delta G_1$ , in negative magnitude), so that fractional occupancy remains high even with imperfect matching.

These parameter combinations bound a broad sweet spot where hybrid misregulation is most likely to evolve despite pleiotropy. These involve G-P maps and fitness landscapes with moderate slopes (Figure 3), produced likely (but not necessarily) by a balance of moderate background binding and moderate to high TF availability, weaker selection on conserved sites (Figure S2, B and D), smaller population sizes (Figure 3 and Figure S1), and to a lesser extent longer areas of direct interaction between TFs and their binding sites (here represented by bit length; File S1). Although it is ultimately a problem to be resolved empirically, we speculate that many pleiotropic regulatory interactions will fall within these confines.

When separate but nonrecombining domains of the TF locus regulated separate traits, incompatibility always evolved in the directionally selected trait but only at small population sizes in the conserved trait. These results do not differ from those of the simple two-locus model of Tulchinsky *et al.* (2014). Thus, multifunctional molecules exhibiting Paaby and Rockman's (2013) molecular gene pleiotropy do not place significant pleiotropic evolutionary constraints on hybrid misregulation or incompatibility. More generally, Via (2009) showed that linkage within or between genes may transfer the effects of selection to a nearby hybrid incompatibility locus through genetic hitchhiking, which at the genomic level can contribute to reproductive isolation between species. However, within the relative confines of our three-locus model, this effect was minimal compared to the direct effect of functional pleiotropy at the level of individual mutations.

To what extent do additional loci under pleiotropic control increase the constraint on the evolution of hybrid incompatibility? Our experiments varying the strength of selection on the conserved trait (File S1) can provide a limited insight into the effects of adding more conserved traits. For example, following from Equation 2, doubling the baseline  $\sigma_s^2$  of a single conserved trait is equivalent to adding a second trait with the same  $\sigma_s^2$ . This is only an upper-bound approximation, as it does not consider the potential repercussions of compensation at multiple loci. If more than two

loci require compensation, it may take fewer mutations to purge a pleiotropic TF mutation and replace it with one at the *cis* site of the directionally selected trait. Whether this is the case depends on the number of loci and on the availability of potentially compensatory mutations per pleiotropic mutation. That availability was equal to one in our model, but may be higher in reality (Levin *et al.* 2000). Regardless, Wittkopp *et al.* (2008) show that TF changes are often accompanied by numerous *cis*-regulatory changes across the sites they regulate, indicating that increasing the number of loci under pleiotropic control does not seem to prohibit TF divergence altogether.

### Availability of mutations

The results of our fitness landscape experiments suggest that the constraint on substitutions at the pleiotropic TF locus is a consequence of the availability of relatively advantageous nonpleiotropic mutations and not because a pleiotropic mutation reduces fitness. Therefore, we investigated the consequences of mutational availability by manipulating the mutation rates of individual loci in the network. We found that hybrid misregulation was greatly increased if insufficient nonpleiotropic mutations were available to respond to selection (Figure 4C). Under those conditions, adaptation still occurred readily at the pleiotropic locus and was accompanied by compensatory evolution, resulting in hybrid misregulation in the conserved trait. This result demonstrates that the *trans*-regulatory TF locus is not constrained by the selective effect of pleiotropy directly.

Increasing the compensatory mutation rate may be expected to reduce pleiotropic constraint by allowing pleiotropic mutations to be compensated more quickly. We found this to be true to some extent; however, our results suggest that it is primarily the fixation probability of a pleiotropic mutation that drives the rate of compensatory evolution, rather than vice versa. Stronger selection on the conserved trait causes the population to tolerate less standing variation at the pleiotropic locus. This decreases the potential for compensatory evolution to occur, resulting in less hybrid misregulation (Figure S2, B and D). Increasing the compensatory mutation rate was not able to fully alleviate this effect (Figure 4B and Figure S3B), suggesting that the primary determinant of pleiotropic constraint is net fitness cost and that the availability of compensation is secondary, at least in the range of parameter values we tested. Furthermore, decreasing the availability of nonpleiotropic adaptive mutations (Figure 4C and Figure S3C) had a greater effect than increasing the availability of compensatory mutations (Figure 4B and Figure S3B). This also suggests that pleiotropic divergence is mainly constrained by the initial probability of a pleiotropic mutation reaching high frequency, rather than by the wait for compensation.

### Compensation and developmental system drift

The phenomenon called “developmental system drift” (DSD) exists when the underlying genetic basis for the



regulation of a phenotype differs between taxa even though the phenotype has remained (largely) unchanged since they shared a common ancestor (True and Haag 2001; Weirauch and Hughes 2010). Empirical cases of this phenomenon are found in diverse taxa (True and Haag 2001; Zinzen *et al.* 2009; Pavlicev and Wagner 2012), and DSD can be replicated in simulations (Haag 2007; Johnson and Porter 2007; Pavlicev and Wagner 2012; this study). Our results show that the evolution of DSD can occur under neutral drift, but is greatly facilitated by selection on a pleiotropic locus. This finding supports the “selection, pleiotropy, and compensation model” (Pavlicev and Wagner 2012) in which compensation for the pleiotropic side effects of adaptation drives DSD in the loci underlying conserved traits. Although we find that compensatory evolution may be constrained by the bioenergetics of the interacting loci, our results support the general conclusion of Johnson and Porter (2007) that directional selection on pleiotropic loci accelerates DSD.

An important lesson from the discovery of DSD is that it can obscure sources of selection and lessen the utility of candidate-gene approaches (True and Haag 2001). However, when the goal is to discover sources of hybrid incompatibility, DSD becomes a valuable tool. Because DSD and hybrid incompatibility go hand in hand, traits that exhibit DSD between related species are high-priority candidates for sources of hybrid incompatibility. Traits coregulated by upstream TFs are candidates as well and may help to identify the original source of adaptive evolution that drove regulatory divergence. If that divergence was recent enough that compensatory evolution has not caught up, misregulation in those traits may even be higher (Figure 3 and Figure S1).

## Acknowledgments

We thank Ward Watt, Courtney Babbitt, Jeffrey Blanchard, Ana Caicedo, Benjamin Normark, Mihaela Pavlicev, Brett Payseur, and anonymous reviewers for valuable comments and discussion.

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*Communicating editor: B. A. Payseur*

# GENETICS

Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.171397/-/DC1>

## **Hybrid Incompatibility Despite Pleiotropic Constraint in a Sequence-Based Bioenergetic Model of Transcription Factor Binding**

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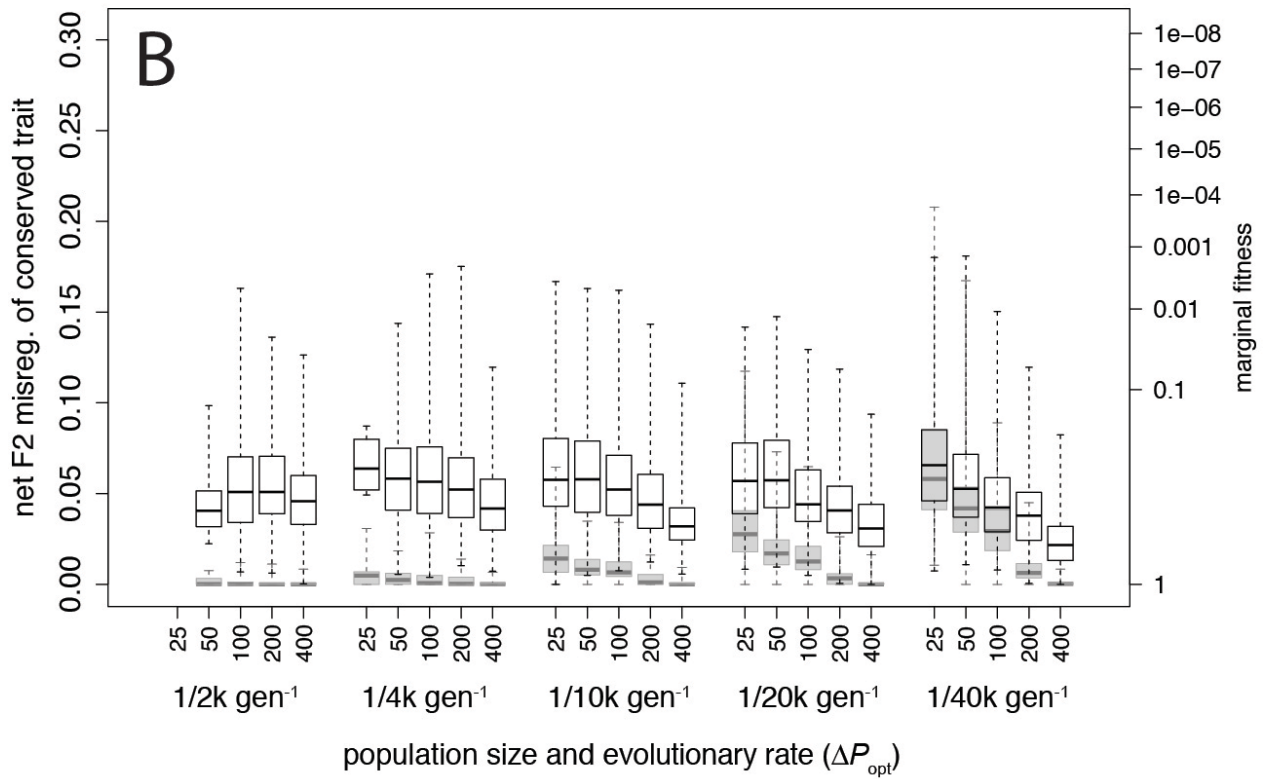
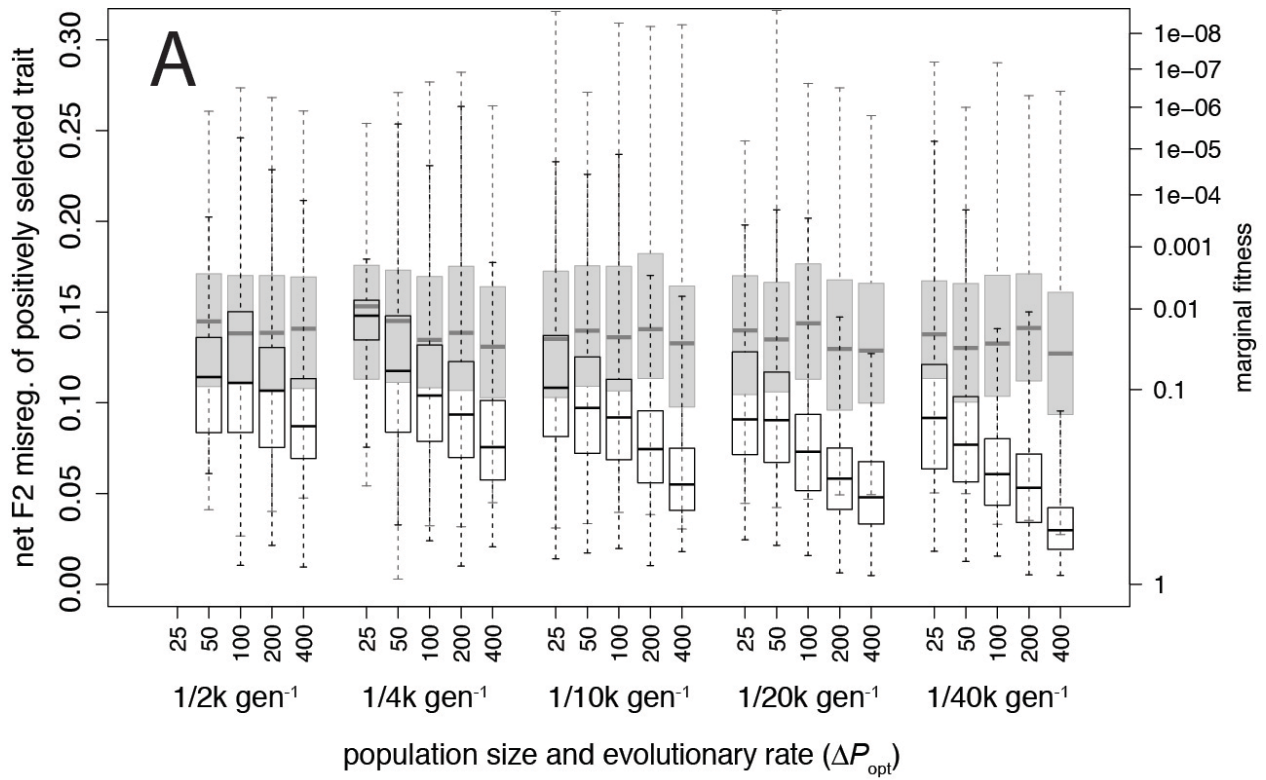


Figure S1 (Caption next page.)

**Figure S1** (Previous page.) Median net F2 hybrid misregulation in relation to population size and the evolutionary rate of the directionally selected trait ( $\Delta P_{opt}$ ), for pleiotropic (clear boxes) and two-domain (gray boxes) models. Populations evolved from  $n = 24$  to zero mismatched bits in the positively selected regulatory interaction over the course of 2000 to 40,000 generations, under population sizes ranging from 25 to 400. (A) Misregulation of the directionally selected trait. Pleiotropy constrained misregulation overall, especially when populations were large and selection was gradual. Missing data in the first column is due to population extinction. (B) Misregulation of the conserved trait. In the pleiotropic model, selection produced moderate change in the pleiotropic locus, in turn favoring a compensatory response at the *cis*-regulated locus of the conserved trait, resulting in interpopulation divergence and hybrid misregulation there. In the two-domain model, divergence occurred mainly due to neutral compensatory evolution between the conserved *cis*-regulated locus and the *trans*-regulating domain upstream of it. With pleiotropy, divergence in the conserved interaction occurred in a shorter time and at larger population sizes. Missing data in the first column is due to population extinction. Box plots show median, quartiles and full ranges.

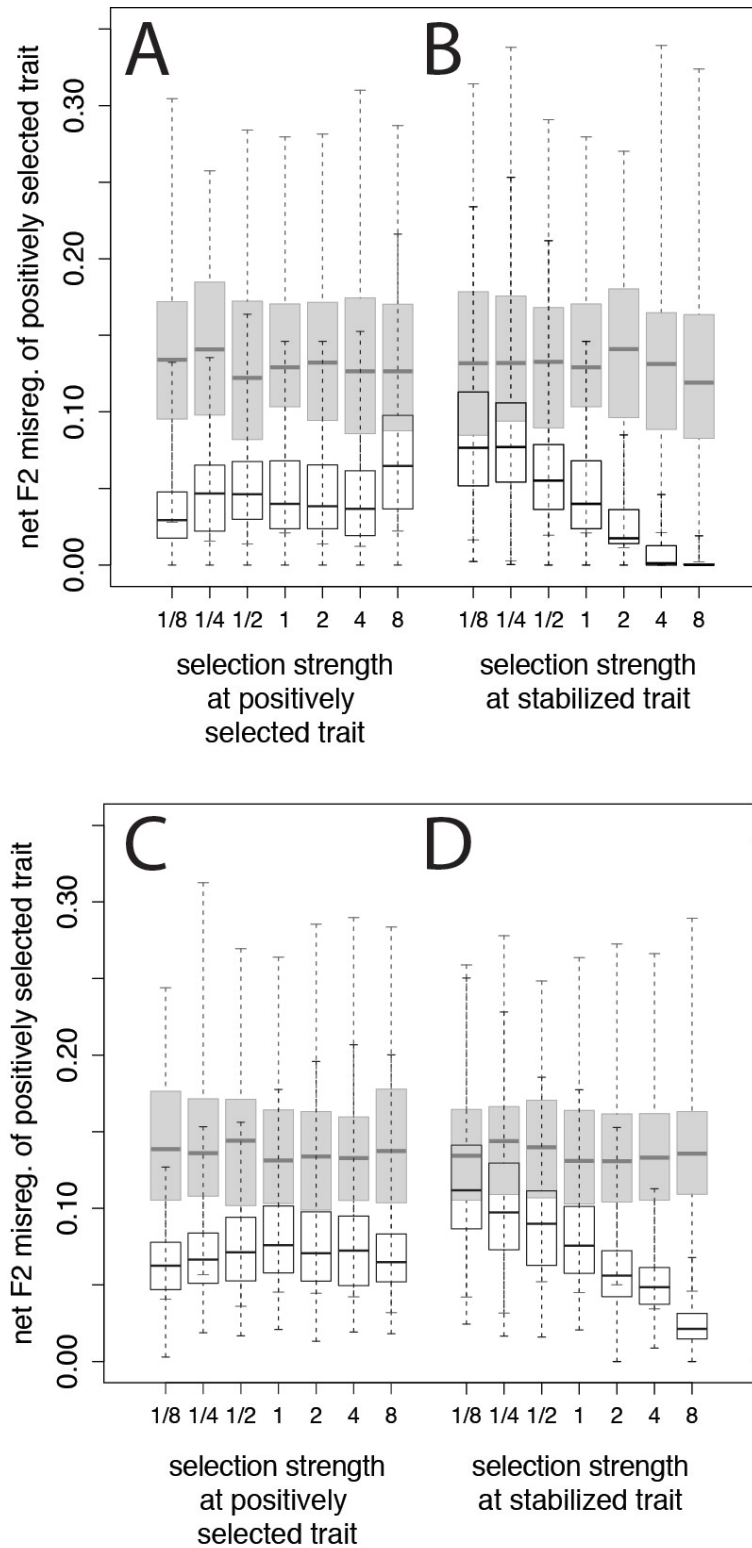
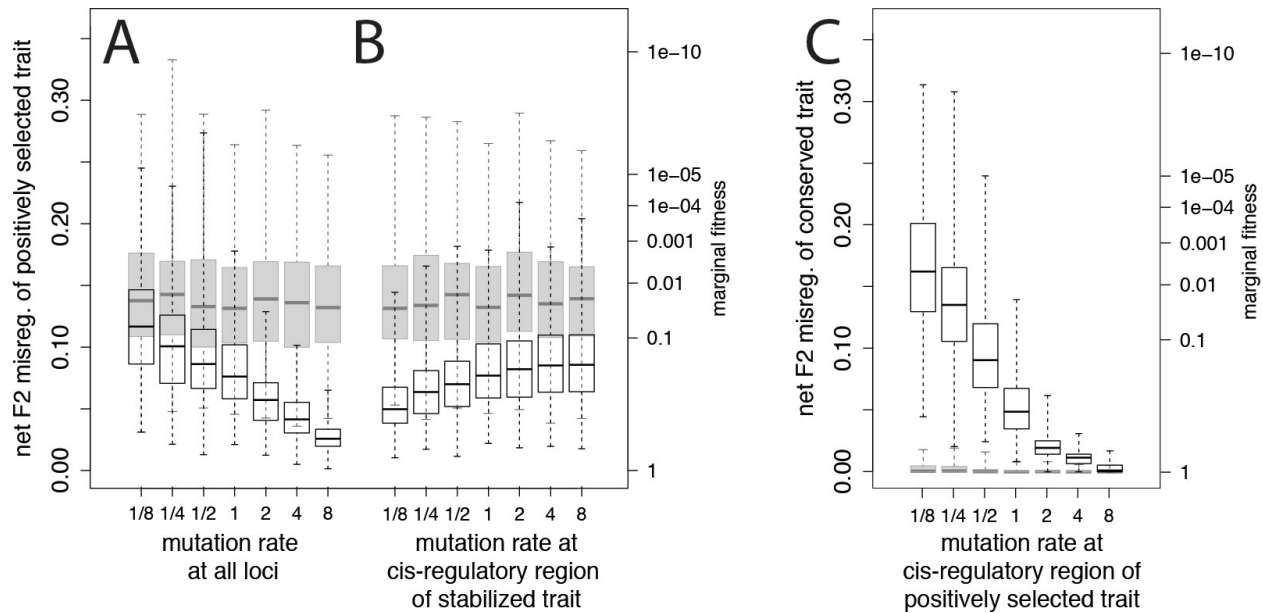


Figure S2 (Caption next page.)



**Figure S2** (Previous page.) Median net F2 hybrid misregulation of the positively selected trait under varying selection strengths ( $1/\sigma_s^2$ ) and mutation-effect sizes in the pleiotropic (clear boxes) and two-domain (gray boxes) models. (A and B) 12-bit motif, which yields a larger mutation effect size. (C and D) 24-bit motif, which yields a smaller mutation effect size. Selection strengths correspond to the steepness of the fitness function around  $P_{opt}$  (equation 2), and are expressed as multiples (0.125–8) of  $1/\sigma_s^2$ , where at baseline, the tolerance  $\sigma_s^2 = 2.5 * 10^{-3}$ . The strength of selection at the positively selected trait has no effect. Selection strength at the conserved trait has no effect in the two-domain control model, but increasingly constrains misregulation in the pleiotropic model as selection strength increases. The constraints on the evolution of misregulation are not quite as strong as in the 12-bit case. Marginal fitnesses depend on  $1/\sigma_s^2$  and are not shown. Box plots show medians, quartiles and full ranges. Simulation conditions as in text Figure 4.



**Figure S3** Median net F2 hybrid misregulation under varying mutation rates occurring at *cis*-regulatory sites of separate traits in the pleiotropic (clear boxes) and two-domain (gray boxes) models, with a motif length of 24 bits that results in a lower mutation effect size. The 12-bit case is shown in text Figure 4. Results are broadly similar, differing as noted below. Mutation rates are expressed as multiples (0.125–8) of the baseline mutation rate of  $8.333 \times 10^{-5}$  per bit. (A) Median net F2 misregulation of the positively selected trait as a function of varying the overall mutation rate. Misregulation was constrained to lower values in the pleiotropic model relative to the two-domain model and the extent of constraint increased with mutation rate. The constraint is less at high mutation rates for the 24-bit case relative to the 12-bit case. (B) As (A), instead varying the mutation rate only at the *cis*-regulatory locus of the conserved trait. Misregulation was again constrained in the pleiotropic model relative to the two-locus model, but was not sensitive to mutation rate. The constraint at low mutation rates is marginally stronger than in the 12-bit case. (C) Misregulation of the conserved trait as a function of the mutation rate at the *cis*-regulatory locus of the positively selected trait. In the pleiotropy model, misregulation was high at low mutation rates, dropping to zero as mutation rate increased; this constraint was slightly stronger in the 12-bit case of text Figure 4. No misregulation occurred in the two-domain model. Box plots show medians, quartiles and full ranges. Simulation conditions: directional selection was applied at the rate of  $\Delta P_{opt} = 1/4000 \text{ gen}^{-1}$  over the course of 4000 generations; population size = 400.

## FILE S1

### Sensitivity to evolutionary rate:

To compare evolutionary outcomes under positive selection with pleiotropy to those under stabilizing selection with genetic drift, we compared misregulation of the conserved trait in the pleiotropy model (Figure 1A) and the two-domain model (Figure 1B). We expect hybrid misregulation under pleiotropic constraint to become increasingly likely under conditions that limit the availability of timely favorable mutations at the *cis*-regulatory locus of the directionally selected trait. Mutations at the TF site, while less beneficial because of their fitness costs at the stabilized trait, could still be better than none at all. Faster change in  $P_{opt}$  and decreased population size can impose these conditions.

**Methods:** We performed simulations at population sizes of 25, 50, 100, 200 and 400, crossed with evolutionary rates ranging from 1/2000 to 1/40000 generations. For this we used alleles of length  $n = 24$  bits and G-P map 'd' of Figure 2. The 24-bit model decreases mutation effect size and allows greater opportunities for compensatory evolution. We varied the evolutionary rate,  $\Delta P_{opt}$ , by varying the duration of the simulation as  $P_{opt}$  of the directionally selected trait changed gradually from 0 to 1.

**Results:** We found net F2 hybrid misregulation of both the directionally selected and stabilized traits, with an interaction between evolutionary rate and population size that differed between the models. In the directionally selected trait (Figure S1A), misregulation was highest in the two-domain model and constrained in the pleiotropic model. Decreasing population size and increasing evolutionary rate interacted to weaken the pleiotropic constraint. Population size and evolutionary rate had no effect in the two-domain model, where only linkage could contribute to the genetic correlation. These results are for the 24-bit model, and the 12-bit model (not shown) yielded the same pattern with slightly lower HI values overall.

For the trait under stabilizing selection (Figure S1B), F2 hybrid misregulation only appeared in the pleiotropic model, unless population sizes were very small and the evolutionary rate was very slow at the directionally selected trait. At a population size  $N = 25$  and an evolutionary rate of  $\Delta P_{opt} = 1/40000$  generations, net F2 misregulation of the conserved trait in the pleiotropic and two-domain models was similar. In the pleiotropic model, high misregulation occurs because the pleiotropic TF locus provides some of the response to selection on the directionally selected trait, selecting in turn for compensatory mutants at the *cis* locus of the conserved trait. For the two-domain model, the misregulation pattern is consistent with compensatory evolution where the *cis*-regulatory locus of the conserved trait evolves essentially independently of the directionally selected locus, with perhaps a small contribution of linkage between domains at the TF site. In other words, the two-domain model behaved as if it were a simple two-locus model under stabilizing selection (TULCHINSKY *et al.* 2014). In contrast, for the pleiotropic model we found the same interaction between population size and evolutionary rate as in Figure S1A.

In some cases, the pleiotropic model showed more net F2 hybrid misregulation at high population size than at low

population size (Figure S1B). Total F2 hybrid misregulation in these cases was nevertheless high, due to a large contribution from misregulation in reconstituted parental genotypes. At low population size, genetic load accumulates and can produce substantial misregulation in the parent populations, especially when directional selection to the final optimal phenotype is more rapid ( $\Delta P_{opt} > 1/4000$  generations).

#### **Effects of fitness function and mutation effect size:**

Binding regions vary in length among types of TFs and their promoters, and we suspected this would affect evolutionary rates and pleiotropic constraints. Under a given set of bioenergetic parameter combinations, mutations with smaller effect sizes at the pleiotropic (TF) locus will also have smaller effects on both downstream phenotypes and their marginal fitnesses. That may increase the chances that substitutions will occur at that locus, and in turn, lessen the pleiotropic constraint to allow more hybrid incompatibility to evolve. Here we test that scenario.

Motif length  $n$  is a good proxy for mutation effect size. To illustrate this, consider for reference the 12-bit G-P map or fitness landscape 'd' of Figure 2, which is based on bioenergetic parameter values of  $N_{TF} = 100$ ,  $E_{diff} = -1$  and  $\Delta G_1 = -0.6125$ . A single mismatch of this map yields a phenotype of  $P = 0.978$ . By manipulating equation 2, we find that a 24-bit map with  $n = 2$  mismatches has the same shape at  $N_{TF} = 100$ ,  $E_{diff} = -1$  provided the free energy of association is  $\Delta G_1/2$ .

**Methods:** The bioenergetic parameters determine differences in expression levels between genotypes, thus the shapes of the G-P map and fitness landscape (text Figure 2). These outcomes should also depend on environmental parameters determining marginal fitness. Higher values of  $\sigma_s^2$  in the fitness function of a trait correspond to a flatter marginal fitness landscape around its optimum and therefore weaker selection on a given deviation from the optimum. Conversely, higher values of  $1/\sigma_s^2$  correspond to a steeper fitness function and stronger selection. We present our results in units of steepness ( $1/\sigma_s^2$ ) rather than flatness ( $\sigma_s^2$ ) because we find it more intuitive. To study the effect of the fitness parameters directly, we varied  $\sigma_s^2$  between 1/8 and 8 times the value used in the body of the paper,  $\sigma_s^2 = 2.5 * 10^{-3}$  at each trait.

We tested the role of mutation effect size by using a binding motif of length  $n = 12$ , which has a larger mutation effect size than a motif of  $n = 24$  bits. We used the genotype-phenotype (G-P) maps and fitness landscapes labeled 'd' Figure 2, scaled to the 24-bit case by dividing  $\Delta G_1$  by 2. The resulting G-P map shapes are identical, except that the horizontal axis extends to 24 rather than 12 mismatches. To study the interaction of mutation rate and mutation effect size, we repeated the analyses of text Figure 4, which is based on the 12-bit motif, using the 24-bit motif. We likewise examined the interaction between selection strength and mutation effect size by comparing results from the 12-bit and 24-bit motifs.

**Results:** We found that the extent that hybrid misregulation evolved under a given fitness function depended on relative strengths of selection acting through the phenotypic and fitness effects of these parameters.

The effect of the steepness of the fitness function on net F2 misregulation is shown in Figure S2. The strength of selection had no effect on misregulation in the two-domain model (gray bars), but misregulation was constrained to lower levels in the pleiotropic model (white bars). For the directionally selected trait (Figure S2, A and C), the steepness of the fitness function had only a minor effect. However, increasing selection on the stabilized trait increased the pleiotropic constraint, such that misregulation decreased and ultimately disappeared (Figure S2, B and D). Reducing the mutation effect size by adopting a 24-bit motif had no effect in the two-domain model. In the pleiotropic model, it only slightly reduced the constraint on misregulation imposed by the 12-bit motif.

A similarly small influence of mutation effect size appeared in relation to variation in mutation rate. In the pleiotropic model, decreasing the mutation effect size by setting  $n = 24$  lessened the constraint on the evolution of misregulation relative to the 12-bit case (Figure S3 A and B, vs. Figure 4 A and B). Mutation effect size and mutation rate interacted, such that median net misregulation in the 24-bit case was 1.2x higher at the lowest mutation rate and 5x higher at the highest mutation rates. The interaction effect on misregulation of the conserved trait was similar but not as pronounced (Figure S3 C vs. Figure 4C). In the two-domain model, mutation effect size again played no role in the extent of misregulation.

These results confirm our prediction that lowering mutation effect sizes will reduce the pleiotropic constraint on hybrid incompatibility, permitting more to evolve.

Earlier work by JOHNSON and PORTER (2007) examining the evolutionary dynamics of a pleiotropic locus that simultaneously regulates positively selected and conserved traits also found that increasing the strength of stabilizing selection decreased divergence at the pleiotropic locus. In that model, which did not include bioenergetic parameters, the pleiotropic locus experienced divergence sufficient to produce hybrid incompatibility even under strong stabilizing selection. The following differences in model assumptions likely explain this result. First, the mutation effect size was effectively smaller in the JOHNSON and PORTER (2007) model, in that mutation effects followed a Gaussian distribution with no minimum effect size. This allowed compensatory evolution to proceed in smaller steps. Second, because of a detail in how allelic values were represented, a large fraction of all potentially compensatory mutations successfully compensated for a given pleiotropic mutation. Though compensation is more difficult in the bioenergetic model, our results in general agree with JOHNSON and PORTER (2007) that selection due to pleiotropy drives the evolution of HI in a conserved regulatory interaction under a broad range of parameter values (Figures 3 and S1).

#### **Effects of other bioenergetic parameters:**

Motif length is a good proxy for mutation effect size as we described in the section above, provided we substitute  $\Delta G_1/2$  for  $\Delta G_1$  of the 12-bit G-P map. The same logic applies if we substitute for other bioenergetic parameters, and the same outcomes derive from those effects. The same phenotype of  $P = 0.978$  for G-P map 'd' in Figure 2 can be found for a 24-bit motif by changing any of the bioenergetic parameters. For example, a 24-bit G-P map with this

phenotype at  $\Delta G_1/2$ , but having only  $n = 1$  mismatch, exists when  $E_{diff} = -1.896$ . We also get the same phenotype using  $E_{diff} = -1$  and  $\Delta G_1 = -0.6125$  on the 24-bit map, provided that  $N_{TF} = 41$ .

The result is that the same types of mutation-effect constraints apply, but scaled differently. For example, the effect of a single substitution in a 12-bit motif with  $N_{TF} = 100$  can be achieved with a single substitution in a 24-bit motif and  $N_{TF} = 41$ . Roughly, doubling the genome size will produce the same  $E_{diff}$  for a 24-bit motif as you would find in a 12-bit motif. Thus, re-regulating to get the same evolutionary effect of a substitution at a TF or *cis*-regulatory site can be achieved by down-regulating  $N_{TF}$  through upstream evolutionary change.