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SYMPOSIUM

Homeostasis and Dynamic Stability of the Phenotype Link Robustness and Plasticity

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Synopsis Phenotypes are remarkably robust to genetic and environmental variation. Although the general control principles of robustness are well understood in simple systems, the actual mechanisms that convey robustness in realistically complex systems have been little studied. We have studied the origins and properties of robustness in a complex metabolic system that is relevant to human health: folate-mediated one-carbon metabolism (FOCM). The FOCM network consists of several interlocking cycles, and reactions in the system contain the rate-limiting steps for DNA synthesis, the reactions for DNA methylation, and the synthesis of glutathione, the primary endogenous anti-oxidant. Defects in FOCM can arise from mutations in enzymes, or from nutritional deficiencies such as folic acid and vitamins B6 and B12, and are associated with birth defects, anemia, cardiovascular disease, and cancer. We show that this metabolic network has evolved as diverse homeostatic mechanisms that stabilize critical reactions against genetic and environmental variation. These mechanisms achieve stability dynamically, by continually altering some reaction rates in order to keep critical reactions stable. Robustness is a systems property and exists only in restricted regions of genotype space, and we show that natural standing genetic variation in human populations is concentrated in these regions. We show how genetic perturbations and/or environmental shifts that disrupt the homeostatic regime can increase phenotypic variation and the correlation between standing genetic variation and phenotypic variation. Robustness and stability are never perfect and, because they are maintained dynamically, can be readily perturbed by both genetic and environmental factors. The tightrope between stability and change sways easily and, through the release of genetic variation, may be an important enabler of rapid phenotypic evolution. Although we use examples from a metabolic system in which quantification of mechanism is particularly accessible, we note that the same principles obtain in other homeostatic systems in physiology and development.

Introduction

Phenotypes are said to be robust to genetic variation if their variation is uncorrelated, or only weakly correlated, with variation in specific genetic loci. There are two reasons why variation in a gene could have no effect on a phenotype. It could be because the gene in question is irrelevant to the ontogeny or function of that particular phenotype. Or it could be because the gene plays an important role in the function of the trait and some mechanisms exist that make the phenotype (relatively) insensitive to variation in that particular gene. Only the latter case is biologically interesting. The most common way of assessing robustness of a phenotype to mutation is to measure the correlation between a mutation and a phenotype in different genetic backgrounds or in different environments (de Visser et al. 2003; Gibson and Dworkin 2004; Kaneko 2012b). If the magnitude of the correlation depends on the genetic background then one may conclude that in some genetic backgrounds, the phenotype is more robust (or better canalized) than in others. The same result can be achieved by testing whether, and how, the correlation changes under different environmental conditions (Kaneko 2012a; Stewart et al. 2012). If the correlation does not change, no matter what the

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genetic background, it is impossible to say whether this is due to a mechanism that conveys robustness or simply because the mutation is irrelevant.

Robustness to environmental variation is ascertained similarly. Just as with genes, some environmental variables are simply irrelevant to the ontogeny or function of a trait. So when a phenotype does not change as a particular environmental variable changes that does not mean the trait is robust to that particular environment. If the correlation between environmental variation and phenotypic variation depends on genetic background, then it is possible to determine in what genetic background the trait is most robust against variation in that particular environmental variable.

Methods employing correlation and covariance have two limitations. First, they cannot say anything about the mechanism by which robustness is achieved. Second, because they can only measure the linear relationships among factors they cannot deal with the consequences of nonlinearity. This is problematic because not only is nonlinearity in biological systems pervasive, it is also essential for the development and evolution of robustness.

Nonlinearity and robustness

The processes that give rise to biological form and function are non-linear: the kinetics of biochemical and molecular reactions are non-linear, feedback mechanisms are non-linear, transport mechanisms are non-linear, the kinetics of signaling pathways are non-linear, and growth processes are non-linear; indeed, any process that is regulated is inherently non-linear.

Robustness is due to the non-linear relationship between genotype and phenotype. This is perhaps easiest to visualize by a graphical example (Fig. 1). Relationships between genotype and phenotype, or between cause and effect, or input and output, are typically sigmoidal; at low levels of input there is no output, then there is a more-or-less gradual increase in the response, which eventually saturates at very high inputs (Fig. 1A). In regulated systems, there can also be a chair-shaped relationship in which at low inputs there is a positive relationship between input and output, followed by a region in which increasing input has little effect on output, and then a region in which higher inputs are accompanied by ever higher outputs (Fig. 1B). Networks of chemical reactions, signaling cascades, and constrained growth (e.g. logistic or Gompertz growth) are examples of the first kind of nonlinearity. The second kind is found in homeostatic mechanisms. In both cases there are regions where the output (phenotype) is robust and unvarying with respect to variation in the input (genotype), and regions where it is not.

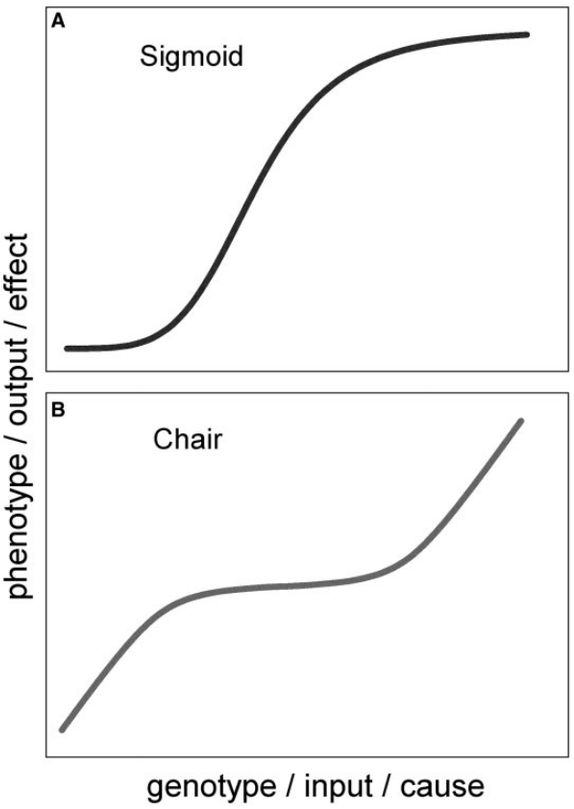

Fig. 1 Two common modes of nonlinearity. (**A**) sigmoidal relationships between cause and effect, where the output is stable at low and at high levels of input, are typical of signaling systems and systems exhibiting cooperativity. The right-hand side of the curve depicts saturating relationships characteristic of many biochemical systems. (**B**) Chair-like relationships, where the output is stable for an intermediate range of inputs but not robust when inputs are very low or very high, are found in homeostatic systems.

This is an important point: phenotypes are not unconditionally robust. Rather, their robustness is context-dependent: context being provided by the underlying mechanisms that give rise to the particular shape of the genotype/phenotype curve and by the particular value of the genotype. For some range of values there is robustness, but for adjacent values there may not be.

The general control mechanisms that can produce robustness are well known; they include negative feedback, auto-regulation, positive feedback, cooperativity, feed-forward control, lateral inhibition, source–sink gradients, oscillators, saturation kinetics, capacitance, modularity, redundancy, and parallel pathways (Nijhout 2002; Eldar et al. 2004; Kitano

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2004; Alon 2007; Whitacre and Bender 2010). How these mechanisms confer stability has been best studied in the great diversity of physiological homeostatic mechanisms, but they have also been studied in gene regulatory networks, signaling networks, metabolic networks, cell motility, morphogenesis, neural circuits, and behavior.

Mathematical models

The mechanisms that give rise to robust phenotypes are complex in the sense that they contain a very large number of interacting parts whose kinetics are nonlinear and that vary in time and space. Most recent experimental and theoretical studies on mechanisms of robustness are carried out on small systems, often called toy systems or motifs, and the principal aim has been to understand the general principles of control. However, because the control systems are nonlinear, and are, in nature, embedded within much larger nonlinear systems, it is not guaranteed that the behavior they exhibit in isolation in any way resembles their operation *in situ*.

The complexity and nonlinearity of real systems make the effect of variation in any of its components context-dependent. That is, the effect depends on the state of other components in the casual network, and as a consequence, the effect of a mutation is not a property of a gene, but rather is a systems property (Nijhout and Paulsen 1997).

Most real systems are sufficiently complex that it is impossible to do simple thought experiments to discover their probable function, or to deduce how they would be affected by mutation or by

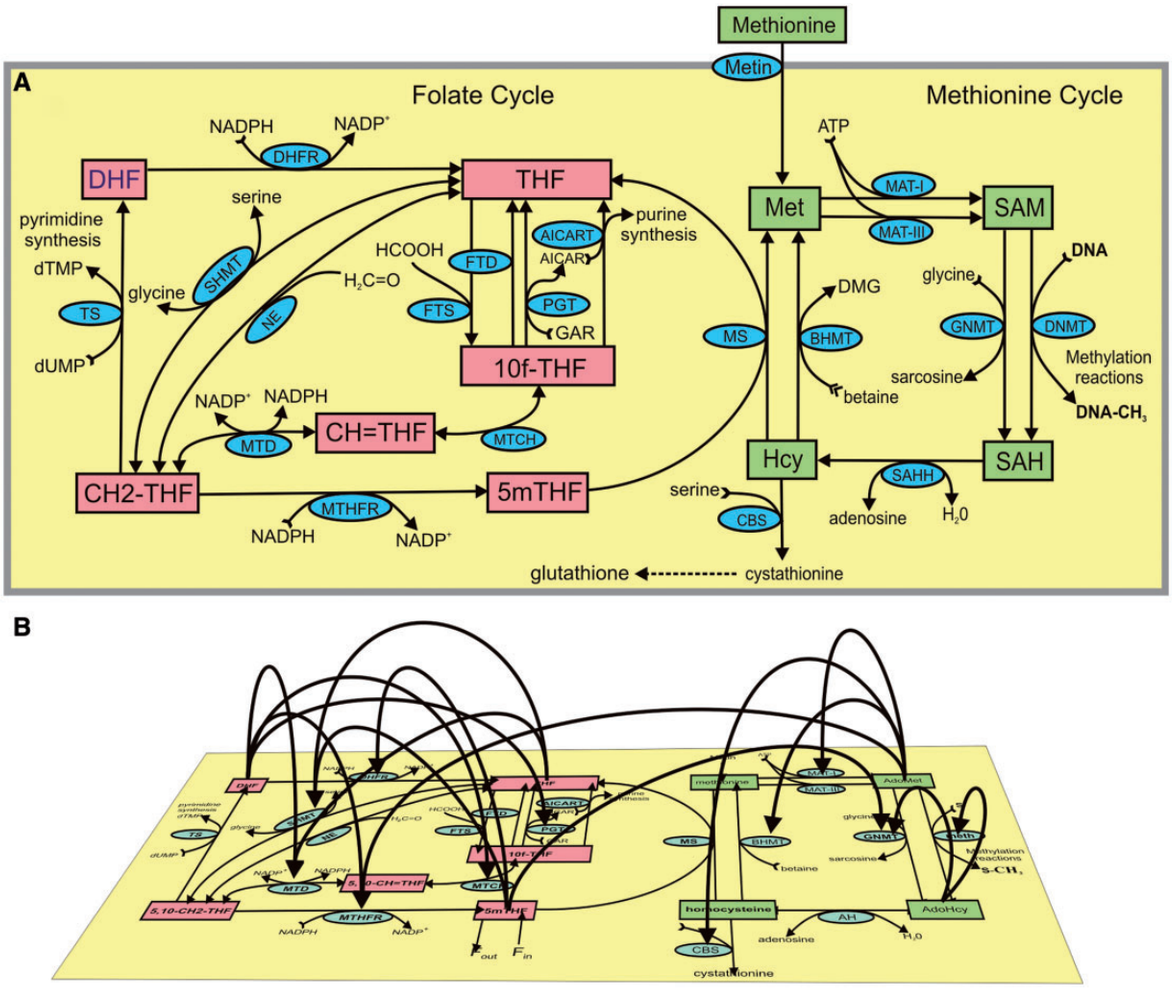

Fig. 2 (**A**) Reaction diagram of FOCM that is the foundation of the mathematical model whose results are described here. The meaning of the acronyms and the structure of the mathematical model are described in detail by Nijhout et al. (2004, 2006) and Reed et al. (2008). (**B**) Structure of some of the long-range allosteric interactions in which metabolites affect the activity of enzymes elsewhere in the network (illustrated for the cytosolic components of the methionine and folate cycles).

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environment. Moreover, most systems are incompletely understood either in terms of the component parts, their interconnections, or their kinetics. The only way in which we can test the quality of our understanding of a system is by means of a mathematical model.

A mathematical model is a quantitative description of what we believe to be the components of a system and the kinetics through which they interact. The adequacy of a mathematical model is tested against experiments and data that did not go into the original construction of the model. If the model does not give the correct results it means that something is wrong, or incomplete, in our understanding or description of the mechanism. If a model succeeds in reproducing the results of new experiments then we may conclude that the mechanism it describes is sufficient. Models are progressively modified and validated against ever more diverse and detailed experimental data until one gains confidence that the model has captured the essential parts of the mechanism.

Thus, in order to study the mechanisms that give rise to robustness in real systems it is necessary to be able to develop mathematical models for the entire causal chain between genotype and phenotype (Nijhout 2002). It is necessary to know the component parts of the network and the kinetics by which they operate. There are few systems in which this can be done well. In most developmental systems, the available data are essentially static snapshots, in most physiological systems it is difficult to deduce the effects of specific genes, and in gene regulatory networks it is difficult to deduce higher-level phenotypes. In most systems, it is necessary to evoke one or more "black boxes" for some of the internal steps, whose properties are generally assumed to be simple and linear. More often than not, the kinetics by which the different components operate are unknown.

Metabolic networks are among the few systems in which the structure of the entire system and the kinetics of the components are well enough understood to be able to develop accurate mathematical models. Moreover, the mechanisms by which many metabolites affect higher-level phenotypes are, in many cases, well understood. Metabolic systems that are relevant to human health have been particularly well-studied with an abundant literature both on the structure of the networks and the kinetics of their component parts. They are among the few systems in which it is possible to study the mechanisms of robustness in a real and non-trivial complex system.

A complex metabolic network

We have studied robustness in a variety of metabolic networks of biomedical importance (Nijhout et al. 2004, 2006, 2009; Reed et al. 2008, 2010; Best et al. 2009, 2010a, b; Gregory et al. 2013). Because defects in these networks are associated with a variety of human diseases, their components have been extremely well studied. What we have done is put all this information together into integrated models. We study these systems by building models based on differential equations. Here, we will consider only models we have developed for folate-mediated one-carbon metabolism (FOCM). The work below is based on mathematical models of these mechanisms, illustrated in Fig. 2 and described in detail by Neuhouser et al. (2011), Nijhout et al. (2004, 2006), and Reed et al. (2006, 2008).

'Folate-mediated one carbon metabolism' consists of the folate cycle, the methionine cycle, and glutathione synthesis (Fig. 2). One of the functions of this network is to take in amino acids (primarily serine and glycine) and reduce them to methyl groups (hence one-carbon) that are then used in a host of methyl transfer reactions that build complex metabolites and are used in reactions such as DNA and histone methylation, and the first steps in the synthesis of purines and pyrimidines. The thymidylate synthase (TS) reaction, for instance, is the rate-limiting step for DNA synthesis (Chen et al. 2003) and is therefore a target of chemotherapeutic anti-cancer drugs (e.g. fluorouracil) because if DNA synthesis is blocked, cell division stops.

Various forms of folate (also known as vitamin B9) are the primary methyl-group carriers in the folate cycle. Folate deficiency can lead to birth defects such as anencephaly and spina bifida because the TS reaction is slowed down such that cells cannot divide rapidly enough during dorsal closure in early embryonic development and thus fail to close the neural tube. A folate deficiency also causes the accumulation of homocysteine (Hcy, one of the metabolites in the methionine cycle), a highly reactive and toxic amino acid that stimulates expression of Vascular Cell Adhesion Molecule-1 (Silverman et al. 2002) that is a major factor in vascular plaque formation and cardiovascular disease. Deficiencies in the folate and methionine cycles can lead to mis-methylation of DNA, which results in inaccurate gene silencing and is associated with various cancers. Methylation reactions are required in the synthesis of dopamine and serotonin, and defects in methylation capacity are associated with a variety of neurological and affective disorders (Reynolds 2006). Glutathione is the

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Table 1 Selected mutations in FOCM and their effects on the activities of the respective enzymes

Protein*	Mutation	% Activity wrt Wild type*	Cofactor Vitamin	References
MS**	A2756G	50	B12 (cobalamin)	Harmon et al. (1999), Ma et al. (1999) and Tsai et al. (2000)
MS	D919G	60		
MTHFR	C677T	40	B2 (Riboflavin)	Weisberg et al. (1998) and Lievers et al. (2001)
MTHFR	A1298C	68	B9 (folate)	
TS	2rpt/3rpt	42	B9 (folate)	Trinh et al. (2002) and Kealey et al. (2005)
TS	1494del6	24		
CBS	M173V	38	B6 (pyridoxal-P)	Pogribna et al. (2001) and Urreizti et al. (2006)
CBS	A226T	19		
CBS	R548Q	60		
CBS	Down	150		

*Activity of the homozygous mutant. Many of these mutations are recessive at the phenotypic level, but we model them as co-dominant at the biochemical level. MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; TS, thymidylate synthase; CBS, cystathionine-β-synthase.
**Activity of MS alleles has not been measured; we calculate it here as the activity required to change homocysteine levels reported in the literature.

main endogenous antioxidant, and deficiencies that lead to reduced glutathione synthesis impair the ability of cells and tissues to deal with oxidative stress. Glutathione is also involved in a broad variety of detoxification reactions of metabolic by-products and ingested toxins.

The operation of FOCM depends on many genetic and environmental factors. Genetic factors include the genes for the enzymes and transporters. Environmental factors include the amino acids that serve as sources for methyl groups, and, in addition to folate, vitamins B6 and B12, which act as cofactors for a number of enzymes in the system (Nijhout et al. 2009; Kräutler 2012). Deficiencies in FOCM can arise from mutations in the genes for the enzymes or transporters, and from insufficiency in the supply of amino acids or B vitamins. The phenotypes of FOCM are the outputs, for example, purine and pyrimidine synthesis, capacity for DNA methylation, levels of *S*-adenosylmethionine (SAM, the universal methyl donor), and the rate of glutathione (GSH) production.

Robustness and homeostasis in the FOCM network

Defects in FOCM are associated with a broad range of diseases such as anemia, spina bifida and other neural-tube defects, atherosclerosis and cardiovascular disease, affective disorders, and various cancers, most prominent among which are colorectal and pancreatic cancer (Stover 2004; Bailey 2010). Yet, in spite of the importance of FOCM in human health and disease, the system is subject to a great deal of environmental and genetic variation. Environmental variation comes in the form of large hourly, daily and seasonal fluctuations in input of amino acids and B vitamins. In addition, most of the enzymes have multiple functional polymorphisms (see Table 1 for a sample) that occur at high frequencies in human populations.

Our computational models have revealed that these systems are stabilized by numerous and diverse regulatory mechanisms, many involving activating and inhibitory allosteric regulation of enzymes by metabolites far removed in the pathway (Fig. 2). Because the FOCM network is highly interconnected and multicyclical, every element is both upstream and downstream of all others, so it is difficult to characterize these allosteric interactions as standard feedback or feed-forward regulations. In addition to allosteric interactions, there are many parallel pathways and many reactions in the folate cycle that are subject to product inhibition and substrate inhibition (Nijhout et al. 2004; Reed et al. 2010). These various structural and regulatory mechanisms buffer the outputs against short-term fluctuations due to environmental variation in the inputs. They also make the outputs relatively insensitive to genetic variation. The robustness against environmental and genetic variation has some very interesting properties.

Dynamic stability against environmental variation

In our models, we can simulate natural daily fluctuations in amino acid input with meals (Nijhout et al. 2008; Reed et al. 2008). Figure 3 shows how several metabolites and reaction velocities in FOCM fluctuate with daily variation in amino acids. Many

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fluctuate severely, and some reactions (e.g. SHMT) even reverse direction, but the critical reaction in the system: purine and pyrimidine synthesis (represented by the AICART and TS reactions, respectively), methylation of DNA, and synthesis of glutathione remain very steady. The important feature here is that the overall steady-state of the system is not stable but varies dynamically with variation in input. Only the most important outputs of the network are robust, and the fluctuations elsewhere are designed to actively maintain that stability. Thus, robustness to environmental variation has the characteristics of physiological homeostasis: the dynamics of the system change continually, thereby actively maintaining one or more outcomes at a particular set-point. This stability depends on several of the allosteric interactions. When we eliminate the repression of DNA-methyltransferase (DNMT) by *S*-adenosylhomocysteine (SAH), the DNMT reaction becomes much more sensitive to variation in amino-acid input (Fig. 3).

Homeostasis and cryptic genetic variation

Many functional polymorphisms in the genes for the enzymes in FOCM have been described that have severe effects on the activities of the relevant enzymes, but the association with health outcomes is quite variable among individuals and populations (Sharp and Little 2004; Ulrich et al. 2005; Bailey 2010; Kennedy et al. 2012). We investigated whether our mathematical models could help explain why phenotypes are relatively insensitive to this genetic variation. Figure 4 illustrates several phenotypic landscapes for FOCM. Phenotypic landscapes are graphs of how the phenotype changes with variation in one or more of the underlying causal factors. In these cases, the *X* and *Y* axes represent gene (or enzyme) activities for two genes whose effect on phenotypic variation we wish to assess. The *Z* axes are the relevant phenotypes. Because the relationship between causal factors and the phenotype is nonlinear, their interaction produces rather complex nonlinear landscapes. The shape of the landscape is a systems property and is determined by all the causal factors that are not being graphed. A mutational change in one of those factors will change the shape of the phenotypic landscape and will thus alter the relationship of the phenotype to the two causal factors in the graphs.

The values of the *X* and *Y* axes are shown as percent deviations from the wild-type genotype (we assume wild-type to be the most common genotype in the population). The positions of the wild types are shown by the large white circles. In all cases, the wild-type is located in a region of the landscape that is relatively flat and normal to the *Z* axis. These are the regions where genetic variation has the least

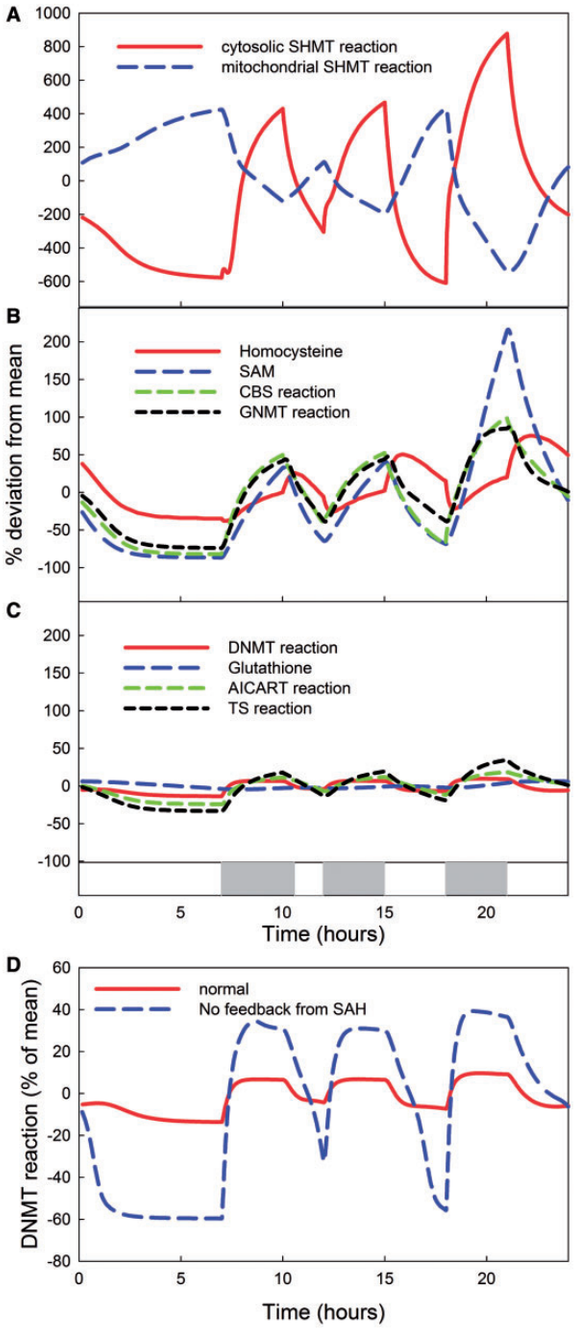

Fig. 3 (**A**, **B**, and **C**) Variation of reaction velocities and metabolite levels during a 24-h period in response to variation in amino-acid input associated with three meals (gray bars in panel C), based on Nijhout et al. (2008) and Reed et al. (2008). Reaction velocities are shown as % deviation from the mean; values below −100% signify reversals of direction of the reaction. (**D**) Eliminating feedback from SAH increases the sensitivity of the DNMT reaction to variation in amino-acid input.

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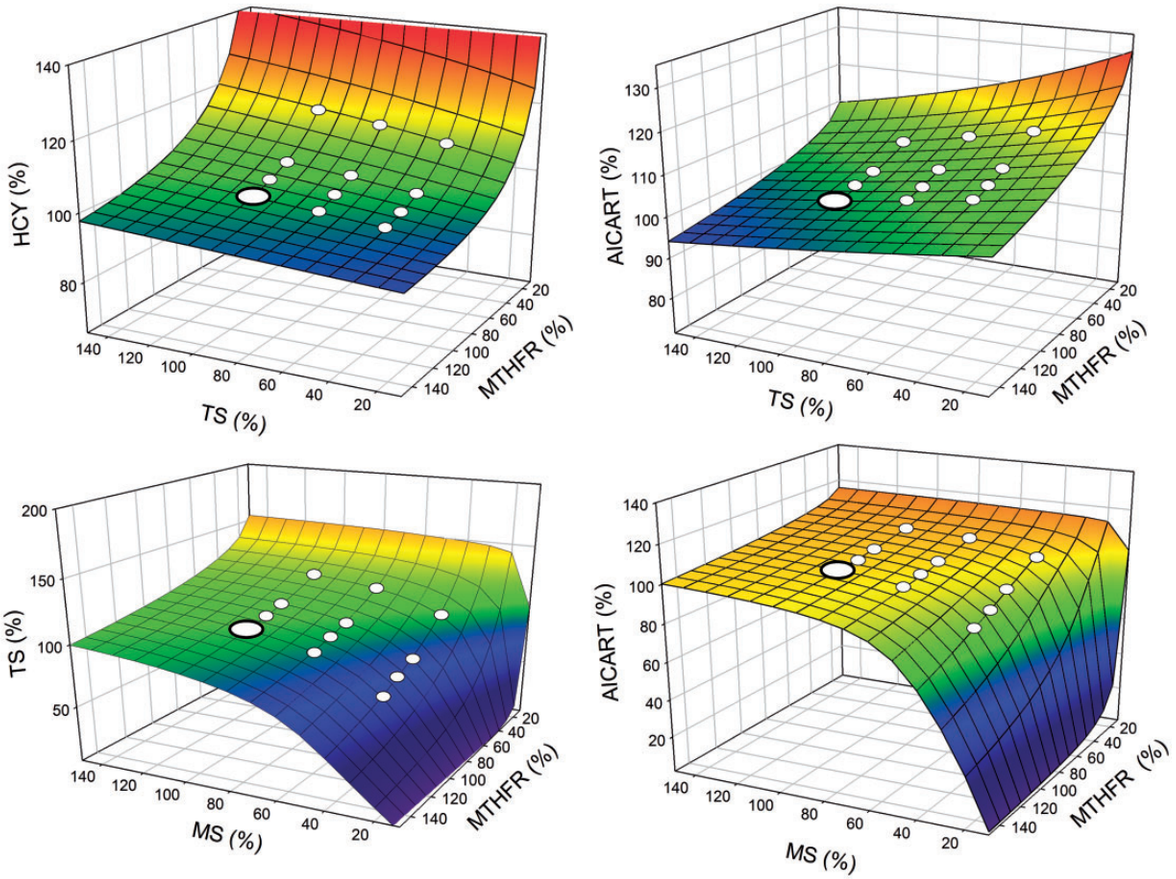

Fig. 4 Phenotypic landscapes that show the effect of variation of four pairwise combinations of enzymes on phenotypes in FOCM. Large white circles indicate location of wild types. Smaller circles indicate locations of polymorphisms of enzymes depicted on the *X* and *Y* axes, taken from Table 1. The wild type and most mutations lie in regions where the landscape is relatively flat. Although genetic variation along the *X* and *Y* axes is large, mutations have relatively little effect of the phenotype (*Z* axis). MS, methionine synthase; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; AICART, aminoimidazolecarboxamide ribonucleotide transferase; Hcy, homocysteine.

effect on the phenotype. We also plot the positions of several of the polymorphisms (from Table 1). The landscapes illustrate how it is that polymorphisms with a large effect on the activity of the enzyme nevertheless have relatively little effect on the phenotype. This is because there is a rather large ''safe zone'' around the wild type where the landscape is relatively flat. The phenotypic landscapes show that the effect of allelic variation is contingent on the genetic background. This can be seen in any of the landscapes by observing how the relationship between one gene and the phenotype changes when one holds the second gene constant at different values. The phenotypic landscapes that are illustrated can thus be seen as sections through a multidimensional space of causal factors where all other factors are held constant. Just as the relationship of one gene depends on the value of the other gene, so does it depend on the values of all other genes in the system. Mutations in the genes that are being graphed move a point across the landscape, whereas mutations in genes not being graphed have their effect by altering the shape of the landscape, as we will see below.

Destabilizing homeostasis releases cryptic genetic variation

The slope of the phenotypic landscape is a measure of robustness. The slopes around the wild-type are small, but seldom zero, so although the wild-type phenotype is robust to genetic variation, it is not perfectly so. The clustering of standing genetic variation within the safe zone where the phenotypic landscape has a low slope shows that this variation is, in effect, cryptic; it has little or no effect on the phenotype. We examined whether this robustness to genetic variation was due to the allosteric interactions within the network. Using our mathematical models, we selectively

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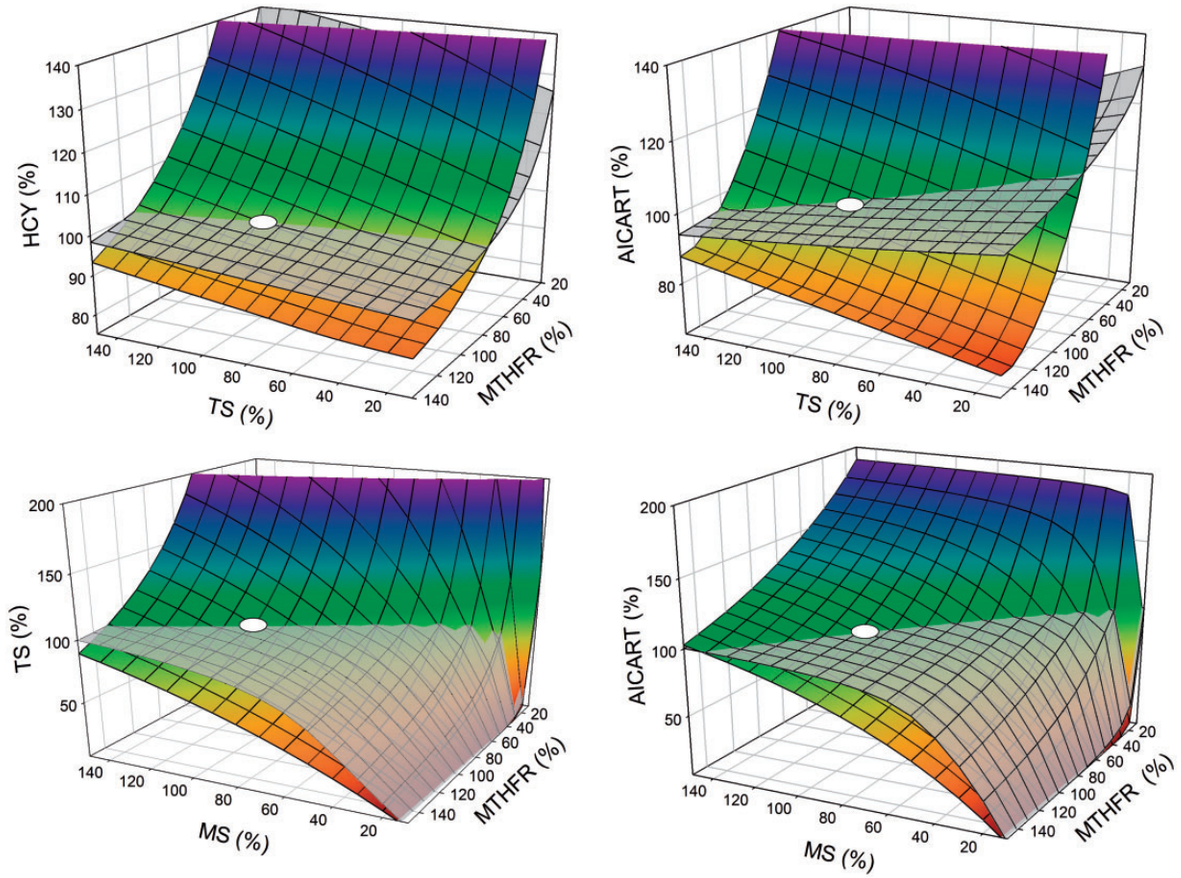

Fig. 5 Eliminating an allosteric interaction destabilizes the phenotype and makes it more sensitive to standing genetic variation. The gray landscapes are the same as the four landscapes illustrated in Fig. 4. The colored landscapes are those obtained when a regulatory feedback is eliminated.

canceled some of the allosteric interactions in FOCM. The effects on the shapes of the phenotypic landscapes were dramatic (Fig. 5). In all cases, reducing or eliminating the allosteric effects of SAM or 5mTHF increased the slope of the phenotypic landscape. The phenotypes are now extremely sensitive to what was previously cryptic genetic variation.

Thus, disruption of a stabilizing mechanism provided by the allosteric interactions releases cryptic genetic variation. This study thus supports the theoretical notion that disruption or destabilization of canalizing or homeostatic mechanisms can enhance phenotypic variability by releasing previously cryptic genetic variation (Gibson and Dworkin 2004; Suzuki and Nijhout 2006; West-Eberhard 2003, 2005).

Environmental destabilization

The *X* and *Y* axes of the phenotypic landscapes can also represent environmental variation. For instance, the enzyme methionine synthase (MS in Figs. 4 and 6) requires vitamin B12 as a cofactor. A vitamin deficiency reduces the activity of the enzyme and will thus have the same effect as a mutation that reduces its activity. This puts genes and environment on the same footing. If the effect of an environmental factor on any of the causal factors of the phenotype is known, then that effect can be graphed as a phenotypic landscape.

A folate deficiency is a well-established risk factor for birth defects, cardiovascular disease, and some cancers (Stover 2004; Bailey 2010). We examined the effect of a folate deficiency on the phenotypic landscape for FOCM and illustrated the landscape for homocysteine (Fig. 6A). The landscape is tilted and the phenotypic effects of standing genetic variation become much bigger than they were under a sufficient folate status. Vitamin B12 deficiencies are associated with a variety of illnesses, such as anemia, hyperhomocysteinemia, cardiovascular disease, and various neurological disorders. Vitamin

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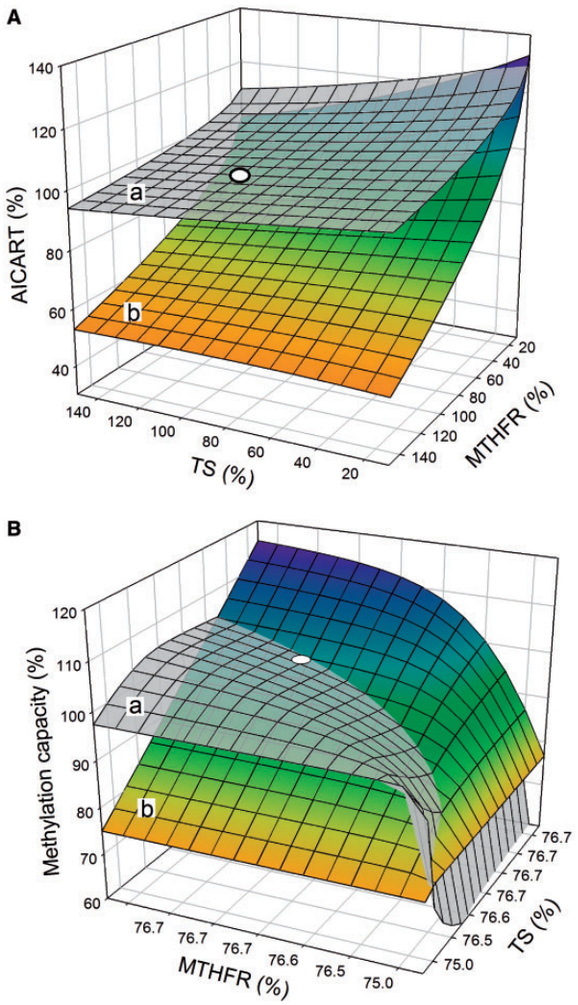

Fig. 6 Environment affects the shapes of phenotypic landscapes. (**A**) Effect of a folate deficiency. The horizontal transparent plane (a) shows the effect of variation in MTHFR and TS on the AICART reaction under normal folate. The inclined plane (b) shows the relationship under a folate deficiency. (**B**) Effect of a vitamin B12 deficiency. The more horizontal transparent plane (a) shows the effect of variation in MTHFR and TS on DNA methylation with adequate vitamin B12. The inclined plane (b) shows the relationships under a vitamin B12 deficiency. The white circle indicates the position of the wild-type.

B12 is a required co-factor for MS and a deficiency affects the kinetics of both the methionine and folate cycles. In Fig. 6B, we illustrate the effect of a vitamin B12 deficiency on the capacity for methylation, taken here as the rate of the DNMT reaction. The vitamin deficiency profoundly alters the shape of the phenotypic landscape for DNMT and, as in the case of a folate deficiency, increases the phenotypic effects of standing genetic variation.

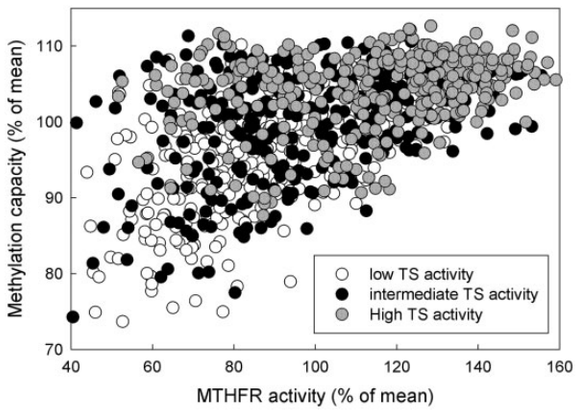

Fig. 7 Distribution of a population of virtual individuals with variation in both genetic makeup and environmental exposure based on the model described by Duncan et al. (2013), partitioned by the 2rpt/3rpt TS genotype from Table 1. High, low, and medium TS refer to the values of the two homozygotes and heterozygotes, respectively.

Population distributions

The phenotypic landscapes graphed in Figs. 4–6 are individual landscapes. This means that an individual (e.g. wild-type) is represented by a point on that landscape and it is implicit that nothing else, either environment, or genotype, varies. Individuals in a population, however, differ in both genotypic makeup and environmental exposure, and therefore each individual lives on a slightly different phenotypic landscape.

A large number of SNPs have been identified for almost every gene in FOCM, and large population samples, such as the NHANES studies (http://www.cdc.gov/nchs/nhanes.htm), have revealed a very large range of individual variation in metabolite values that must be due to individual genetic and environmental variation. We developed a variant of our FOCM model that allows us to introduce a range of variation in enzyme activities and nutrient inputs and generate a population of virtual individuals (Duncan et al. 2013). Distributions of metabolite concentrations in these virtual populations closely resemble those of the NHANES cohorts (Duncan et al. 2013). An example of such a population is shown in Fig. 7 where we plot the relationship between methylenetetrahydrofolate reductase (MTHFR) and capacity for methylation of DNA, partitioned into three cohorts of individuals that differ in TS genotypes. It is clear that there is a great deal of individual variability, that the relationship is not linear, and that this relationship is, again, context-dependent.

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Discussion and conclusions

Robustness is contingent and never absolute. We illustrate this by means of well-validated mathematical models of a complex metabolic system. The system we studied has profound implications for human health and diseases and, as a consequence, the structure of the network and the kinetics of its component parts have been exceptionally well studied. In this system, robustness is achieved by a variety of mechanisms that include feedback, substrate-inhibition, long-range allosteric regulation, and parallel pathways. All these mechanism operate simultaneously to stabilize the systems against both environmental perturbations and genetic polymorphisms. Using our mathematical models, we examined the mechanisms by which genetic polymorphisms with severe effects at the molecular level can have only minor effects at the phenotypic level.

Robustness against short-term environmental perturbations is not due to the inherent stability of the steady-state. Rather, it is achieved dynamically by often severe fluctuations in many reactions in the system that serve to stabilize a few critical reactions that are the main phenotypic outputs of these systems (Fig. 3). Thus, robustness in this metabolic system has the characteristics of physiological homeostasis, where a particular set-point of the phenotype is stabilized by a diversity of ever-changing kinetic measures and countermeasures. Interestingly, the regulation of stability in some gene-activation networks also has the characteristics of a homeostatic system (MacNeil and Walhout 2011).

Robustness to functional mutations that alter the activity of gene products depends on the same kinds of interactions that stabilize phenotypes against short-term perturbations. We illustrate robustness by means of phenotypic landscapes: graphs that show the relationships between genetic variation and phenotypic variation. The slopes of phenotypic landscapes are measures of robustness. The shapes of phenotypic landscapes are systems properties and depend on all the genetic and environmental factors that affect the phenotype. Wild-type genotypes and much of standing genetic variation occur in regions of the landscapes that have a relatively low slope with respect to the phenotype (Fig. 4). Thus, although the genetic variation is large along the genotype axes, it is, in most cases, quite small along the phenotype axes. This standing genetic variation is nearly cryptic in regard to its effect on the phenotype. It is never perfectly cryptic, because the slopes are seldom, if ever, zero. This is probably a general feature of robust systems: robustness is never absolute.

More importantly, there are many ways in which robustness can be perturbed. We showed that altering one or more of the allosteric interactions severely tilts the phenotypic landscapes (Fig. 5). These results show that the long-range allosteric interactions are the mechanisms that stabilize the phenotypes (see also Nijhout et al. 2006, 2008). Thus, mutations that alter the strength of allosteric interactions can release standing genetic variation (West-Eberhard 2003, 2005; Gibson and Dworkin 2004; Badyaev 2005). Environmental factors likewise can alter the slopes of the phenotypic landscape (Fig. 6) and make phenotypes more sensitive to a standing genetic variation.

These molecular homeostatic mechanisms, just like the well-studied heat shock protein HSP90, act as capacitors for genetic variation (Rutherford and Lindquist 1998; Milton et al. 2006; Rutherford et al. 2007), although they do so by fundamentally different mechanisms. HSP90 is a chaperone that stabilizes the structure of metastable proteins and essentially masks genetic variation that would alter their structure. Environmental stressors can deplete HSP90 and thus release accumulated genetic variation. Homeostatic mechanisms, by contrast, do not abolish the effects of genetic variation on the activity of proteins, but establish a set of systems properties that act dynamically to make the phenotypes that emerge from these complex interactions insensitive to that variation. Environmental stressors, as well as mutations, alter the internal dynamics of the system and that causes standing genetic variation to become more highly correlated with phenotypic variation.

The mathematical properties of these homeostatic mechanisms allow us to explain why genetic polymorphisms with rather large molecular effects, and which would on the face of it appear to be deleterious, can nevertheless have small phenotypic effects and thus accumulate and be maintained in a population. They also allow us to understand why the association between those polymorphisms and disease outcomes is extremely variable both within and between populations. This is because no two individuals are identical in genetic makeup or environmental exposure and will therefore have different phenotypic landscapes. Moreover, that landscape changes as environmental exposures change and somatic mutations accumulate during a lifetime. Genetic variation, therefore, does not have a fixed relationship to the phenotype. Rather, it varies among populations and among individuals, and within an individual it varies over time. For instance, many individuals develop a deficiency in vitamin B12

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as they age, and Fig. 6B illustrates that such a deficiency could lead to inappropriate methylation of DNA and therefore mis-expression of a variety of genes, which could be associated with the diverse symptoms associated with aging and senescence.

The most remarkable feature of the phenotypic landscapes we explored is that in almost all cases the wild-type phenotype/genotype is located where the slope is smallest. This suggests that past evolution must have operated to make the wild-type as robust to genetic and environmental variation as possible. Insofar as phenotypic landscapes are smooth and continuous, the genetic neighborhoods around the wild type will also be robust to genetic variation, allowing for the accumulation of standing genetic variation that has little effect on the phenotype.

Phenotypic landscapes, however, are plastic. This is because they are defined by factors that vary spatially and temporally, and they are affected equally by genetic and environmental factors. Robustness is therefore variable and context-dependent. Individuals differ in the degree of robustness of any given trait, and robust phenotypes can become less (or more) robust during the life of an individual. Organisms do not, therefore, have to ''choose'' between being robust or flexible (and evolvable); phenotypes can have different degrees of robustness that can vary throughout life.

In this study, we used examples from a complex metabolic network because we can account for all steps and reactions in the mechanisms that connect genotype and environment to phenotype, and because the quantification of the kinetics of these mechanisms is particularly accessible. We note, however, that the same principles of dynamic stability and contingent robustness obtain in all homeostatic systems in physiology and development.

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