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Shadows of complexity: what biological networks reveal about epistasis and pleiotropy

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Abstract

Pleiotropy, in which one mutation causes multiple phenotypes, has traditionally been seen as a deviation from the conventional observation in which one gene affects one phenotype. Epistasis, or gene-gene interaction, has also been treated as an exception to the Mendelian one gene-one phenotype paradigm. This simplified perspective belies the pervasive complexity of biology and hinders progress toward a deeper understanding of biological systems. We assert that epistasis and pleiotropy are not isolated occurrences, but ubiquitous and inherent properties of biomolecular networks. These phenomena should not be treated as exceptions, but rather as fundamental components of genetic analyses. A systems level understanding of epistasis and pleiotropy is, therefore, critical to furthering our understanding of human genetics and its contribution to common human disease. Finally, graph theory offers an intuitive and powerful set of tools with which to study the network bases of these important genetic phenomena.

Keywords

epistasis; pleiotropy; gene-gene interactions; phenotype; canalization; scale-free network; complex disease; systems biology

Introduction

Pleiotropy and epistasis were first described in the early 20th century to explain observed deviations in Mendelian inheritance patterns. German zoologist and geneticist Ludwig Plate defined pleiotropy in 1910 as one mutation resulting in multiple, independent phenotypes. For example, in Waardenburg syndrome a truncation in microphthalmia-associated transcription factor (*Mitf*) causes pigmentation abnormalities, iris defects, deafness, and reduced mast cell production. (1) Epistasis was defined by William Bateson in 1907 as one locus masking the effects of another locus. (2) As it is used now, the term refers to interactions between genes in which the contribution of one gene to a phenotype depends on

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the genotype at another locus, or more broadly, the genetic background of the organism. For example, in humans the E4 allele of apolipoprotein epsilon (ApoE) is associated with elevated blood serum cholesterol levels, but only in individuals with the A_2A_2 genotype at the low density lipoprotein receptor (LDLR) locus. (3) In other words, the contribution of the ApoE allele to cholesterol levels depends on the genotype at the LDLR locus.

Both epistasis and pleiotropy are traditionally viewed as exceptions to the Mendelian rules of genetics in which one gene affects one phenotype. We propose however that, far from being rare deviations, epistasis and pleiotropy are ubiquitous and inherent properties of biological systems, and that they are necessary byproducts of biomolecular networks.

Virtually all phenotypes are the result, not of a single gene, but of interactions between thousands of genes, as well as between genes and the environment. Because of the widespread connectivity within networks, the effects of a single mutation or variation can spread through thousands of gene-gene interactions, resulting in multiple phenotypes, or pleiotropy. The connections through which a variant's effects propagate define the molecular basis for epistatic interactions and how they translate into an observed phenotype(s).⁽⁴⁾

While the concepts of biological networks, epistasis, and pleiotropy are not new, they are widely under-appreciated. State-of-the-art genome-wide scans most often look for individual genes with large impacts on a single phenotype. Attempts to bridge the gap between a single gene and a single phenotype frequently result in spurious and irreproducible results because network interactions are not taken into account. The pervasiveness and strength of biomolecular interactions require a step back from reductionist biology and an acknowledgement of the importance of networks.

One approach already being used is the application of graph theory to biological data. Graphs offer an intuitive platform for connecting and visualizing interacting parts, as well as including an established history of analytical methods. Network theory can be used to search for system-wide patterns that help elucidate the nature and underlying mechanisms of epistasis and pleiotropy. With a more holistic view of biological systems, epistasis and pleiotropy can be seen, not as complications that interfere with the search for disease genes, but as opportunities to help explain the intricacies of biological networks, and therefore more accurate and complete genetic models.

In this essay we examine epistasis and pleiotropy from the perspective of biological networks. We demonstrate first that each is a ubiquitous phenomenon and results from pervasive network interactions. We then examine graph theory as a method of studying epistasis and pleiotropy. We assert that epistasis and pleiotropy are central characteristics of genetic architecture, and as such are fundamental in understanding genetic contributions to human disease and the effects of potential treatments.

Pleiotropy

A pleiotropic gene is one that, when mutated, gives rise to multiple, divergent phenotypes. The most striking examples of pleiotropy in humans appear in monogenic syndromes. For example, Pallister-Hall syndrome manifests with a wide range of symptoms, but is caused ultimately by a mutation in a single gene: the transcription factor *Gli3*. The symptoms of this fatal disease include extra digits, ⁽⁵⁾ webbing between digits, shortened limbs, ⁽⁶⁾ structural abnormalities in the central nervous system, ^(6,7) and kidney abnormalities. ⁽⁸⁾ Another example of a pleiotropic gene is AlphaB-crystalling (*CryAB*). Truncations in this gene have been associated with both cataracts, ⁽⁹⁾ and dilated cardiomypopathy. ⁽¹⁰⁾

When Ludwig Plate introduced the term pleiotropy in $1910^{(11,12)}$ molecular biology was completely unknown. Since that time several diverse mechanisms underlying pleiotropy have been described. As these molecular stories unfold, they reveal how tightly interconnected and complex biomolecular networks are and how critical the network connections are in understanding the effects of a single gene. The seemingly disparate effects of *Gli3*, for example, cannot be understood without considering the gene in the context of an interaction network.

Gli3 acts as a transcription factor in several organ systems during development. (6) It is a transcriptional repressor in the sonic hedgehog (*Shh*) signaling pathway, which plays a key role in vertebrate limb and neural differentiation. (13) *Gli3* also appears to play a *Shh*-independent role, and is required for expression of *Emx* genes in the mouse dorsal forebrain. (14) *Emx* genes, in turn, are required for normal formation of the dorsal telencephalon. (14) *Gli3* functions in a genetic cascade in the dorsal telencephalon that regulates expression of bone morphogenetic proteins (BMPs) and WNTs. (15) Reduced *Bmp* and *Wnt* expression in *Gli3* mutants can play a role in abnormal dorsal forebrain development. (16)

Gli3 also plays an important role in the developing limb bud. In the anterior limb bud, Gli3 expression suppresses extra digit formation, and a large part of its role in limb bud patterning is thought to be mediated through its suppression of Fgf4 and Fgf8 expression in this tissue. When Shh signaling is disrupted during development Emx1/2 and Bmp and Wnt expression in the brain are downregulated, while Fgf8 and Fgf4 are upregulated in the anterior limb bud and in the brain. The altered expression of these genes in Pallister-Hall syndrome leads to severe brain and limb abnormalities, resulting in early infant death. $^{(5,13)}$

The effects of *CryAB* mutations, while less severe, are also mediated through network interactions. *CryAB* is expressed in both the lens of the eye and cardiomyocytes, but plays different roles in the two cell types. The water-soluble CRYAB protein interacts with other CRYAB proteins as well as other crystallins in the eye to form the transparent structure of the lens.⁽¹⁷⁾ In rat cardiac tissue, CRYAB interacts with desmin and myofibrils to prevent the formation of filaments.^(18,19) In this context it acts as a chaperone, interacting with misfolded proteins to prevent their aggregation.⁽¹⁷⁾ Mutations in *CryAB* decrease its chaperone activity, and cause the filament aggregation that leads to cardiomyopathies^(19,20)

Because CryAB is widely expressed and functions in several different pathways, a single mutation in this gene can cause multiple phenotypes that seem entirely unrelated. (19,20) Hadorn⁽²¹⁾ defines this type of pleiotropy as mosaic pleiotropy. We redefine this phenomenon as horizontal pleiotropy to reflect that the multiple effects of a single gene are observable at the same physiological level--variants of CryAB affect two organs, the eye and the heart. This pleiotropy contrasts with what we define as vertical pleiotropy or relational pleiotropy in Hadorn, (21,22) in which one phenotype leads directly to the next. In this class of pleiotropy the observation of multiple phenotypes depends on the physiological level at which the organism is examined. For example, in sickle cell anemia, a mutation in the betaglobin (HBB) gene causes hemoglobin to polymerize when deoxygenated. (23,24) This polymerization deforms red blood cells into the sickle shape characteristic of the disease. Under low oxygen conditions, red blood cells cannot fit through capillaries, and the resultant backups of blood cells causes ischemia in organs and peripheral tissues. These phenotypes, polymerization of hemoglobin, the misshapen red blood cells, and ischemic organs, follow directly from each other rather than from multiple functions of HBB. In this essay we will focus primarily on horizontal pleiotropy. We assert that this class of pleiotropy is genuine pleiotropy while vertical pleiotropy is not, as it exerts physiological rather than genetic effects.

Within the class of horizontal pleiotropy molecular mechanisms vary from instance to instance. In Pallister-Hall syndrome *Gli3* is a transcription factor in a single pathway, but this pathway operates in many different cell types. CRYAB, on the other hand, performs radically different functions in different cell types. It plays a structural role in the lens and acts as a chaperone in cardiomyocytes. (25,26) These examples show some of the molecular diversity that can contribute to pleiotropy. Hodgkin (27) has defined seven classes of pleiotropy based on the number of pathways and cell types a gene is active in, as well as the evolutionary paths to the current usage. The common thread running throughout each class of pleiotropy is that the context of each gene within the larger molecular network is critical to understanding its phenotypic implications.

Pleiotropic genes offer a unique window into the complexities of biomolecular interaction networks. Because every gene in the genome is part of a vast network of interactions, the nature of a mutation's effects give clues as to what other genes and processes may be interacting with the mutated gene. Furthermore, the effects of mutating a single gene can have such pervasive consequences, demosntrating how completely connected this network is

Epistasis

William Bateson first described epistasis in 1907. (2) Like pleiotropy, this concept was developed to explain deviations from Mendelian inheritance. The term literally means "standing upon", and Bateson used it to describe characters that were layered on top of other characters thereby masking their expression. The epistatic characters had to be removed before the underlying hypostatic characters could be revealed. (2) The commonly used definition of epistasis--an allele at one locus masks the expression of an allele at another locus--reflects this original definition. (28) Eye color determination in *Drosophila* provides a classic example. The genes scarlet, brown, and white, play major roles in a simplified model of *Drosophila* eye pigmentation. Eye pigmentation in *Drosophila* requires the synthesis and deposition of both drosopterins, red pigments synthesized from GTP, and ommochromes, brown pigments synthesized from tryptophan. A mutation in brown prevents production of the bright red pigment resulting in a fly with brown eyes, and a mutation in scarlet prevents production of the brown pigment resulting in a fly with bright red eyes. In a fly with a mutation in the white gene, neither pigment can be produced, and the fly will have white eyes regardless of the genotype at the brown or scarlet loci. In this example the white gene is epistatic to brown and scarlet. A mutant genotype at the white locus masks the genotypes at the other loci.

Even before the advent of genomic and proteomic technologies there was evidence that epistasis is ubiquitous in model organisms. Gene-gene interactions are well established as integral to gene regulation, signal transduction, biochemical networks, and homeostatic, developmental, and physiological pathways. However, with the rapid development of high-throughput technologies, it has become obvious how widespread epistatic interactions are. For example, Fedorowicz et al. Hospitalisms are according to that affect olfactory behavior in Drosophila. This study was based on crosses of 12 lines derived from a common isogenic background that differed in the location of P-element insertions, each of which has homozygous effects on olfactory behavior. Eight of the 12 insertion variants defined an interactive network of genes that significantly impact the olfactory phenotype. A subsequent study that investigated the same insertions, using transcriptional profiling, showed that a total of 530 genes were significantly co-regulated with one or more of these olfactory mutations. This experiment shows first that that epistasis is common, and second that easily detectable epistatic interactions involve tens to hundreds of genes.

Other large-scale studies have shown system-wide epistasis in *Saccaromyces cerevisiae* (32) and *E. coli*. (33)

As evidence builds that epistasis is ubiquitous and can encompass hundreds or thousands of genes in a network, it is increasingly apparent that this phenomenon cannot be ignored in the analysis of gene effects. These observations bolster the hypotheses that genes operate in vast networks, and that disrupting one gene in a network has wide-ranging implications.

Biological Networks

It is increasingly possible to investigate biological networks at the system level because of the accumulation of information about individual biological interactions. Rather than studying individual gene-gene relationships, we can begin to elucidate the emergent properties of networks as a whole. Studying large biological networks has increased in recent years and offers compelling evidence that biological epistasis and pleiotropy are inherent, ubiquitous properties of biological networks.

Components of biological organisms, including DNA, proteins, and metabolites, interact with each other through complex networks. These networks create regulatory mechanisms and signaling pathways that allow organisms to respond and adapt quickly to environmental changes and genetic insults. While geneticists have traditionally acknowledged that these complex regulatory networks exist, it was not until the advent of genomic and proteomic techniques that we could examine several components simultaneously. These technologies are beginning to reveal how biological elements interact. Understanding these interactions is critical and represents the next big challenge in the biological sciences.

One of the main features commonly seen in biological networks is a tendency to exhibit a scale-free topology. (34-36) Rather than being connected randomly, the interacting elements of the network, or nodes, are linked such that most interact with only one or a few other nodes (Fig. 1). A minority of the nodes, however, known as hubs, connect to a large number of other nodes. The number of nodes that any given node is connected to is known as its connectivity (k). (34) The degree distribution P(k) of a node refers to the proportion of nodes that have a given connectivity. (34) Degree distribution is calculated by counting the number of nodes of a given k and dividing by the total number of nodes. (34) The topology of a network can be revealed by plotting the degree distribution P(k). In a random network, this plot results in a bell curve where most of the nodes have an intermediate connectivity. (34) A scale-free network, on the other hand, shows a steep curve following the power law $P(k) = Ak^{-\gamma}$, where γ is typically between 2 and 3, indicating that that there are many nodes with low connectivity and a few hubs with very high connectivity. (34) The term scale-free refers to the absence of a typical node in the network. (37)

This characteristic pattern of node connectivity is seen not only in biology, but also in social networks and the internet. (38,39) In biology, scale-free networks are the norm and have been found in protein-protein interaction networks in yeast, (40) *C. elegans*, (41) *Drosophila* (41,42) and humans, (43) as well as gene-regulatory networks, (35) gene-expression networks, (44) and even in interactions at the ecological level. (45)

Scale-free networks are also referred to as a small-world networks because the shortest path between any two random nodes is short compared to other types of networks. (37,46) This tight connectivity allows information to spread across a scale-free network much more quickly than through similarly sized networks of other structures. (47) In metabolic networks a typical path length of only three or four steps link metabolites together into an ultra-small world. (37,47) The very short average path length, or network diameter, in biological networks enables organisms to respond rapidly to environmental changes. (34,37,48)

The small-world property of metabolic networks is widely conserved, indicating that there evolutionary forces preserve a small diameter. Jeong et al. (et, 37) analyzed the average path length of metabolic networks in 43 organisms spanning the three domains of life: archea, bacteria, and eukarya. Although one might expect network diameter to increase with increasing organismal complexity or increasing numbers of enzymes, they found that it did not change across taxa.

Of biological significance network diameter remains relatively constant when random nodes are knocked out, but it increases dramatically when hubs are deleted. (37,42,49) This rapid increase in network diameter in response to hub deletion demonstrates how vulnerable scalefree networks are to directed hub attack. This can have dire phenotypic consequences in biological networks. For example, in the S. cerevisiae protein-protein interaction networks deletion of hub proteins result in lethality more frequently than the deletion of less connected nodes. (22) In a less directed attack, on the other hand, scale-free networks, including biological networks, tend to be extremely robust. (35,50) Even with 80% of its nodes randomly removed, a scale-free network will maintain its integrity, and pathways can still be drawn between any two of the remaining nodes. (35,50) In biological networks, this robustness, known as canalization, (51) manifests itself as the ability of gene networks to buffer a phenotype in the face of genetic and environmental insults. For example, Giaever et al. (2002) systematically disrupted 96% of known ORFs in the yeast genome and found that only 15% of these single deletions led to slowed growth in rich medium. (52) More complex organisms buffer developmental processes and develop normally under a broad spectrum of environmental conditions. Even when grown under a large range of temperatures, for example, wild type Drosophila larvae develop normal adult eyes with a constant number of omatidia. (53)

As Featherstone and Broadie⁽⁴⁴⁾ observe, this robustness does not result from a superfluity of useless genes, but rather the way in which genes interact. Featherstone and Broadie analyzed genome-wide expression profiles of 276 yeast deletion mutants⁽⁵⁴⁾ and found that while most gene deletions resulted in expression changes in a few other genes, a few of the deletions affected the expression of hundreds of other genes.^(44, 54) The topology of the expression network was scale-free.⁽⁴⁴⁾ This topology indicates that most genes can be deleted without widespread effects, supporting previous observations of phenotypic robustness in yeast deletion mutants.⁽⁵²⁾

Importantly, scale-free topology simultaneously suggests a mechanism for pleiotropic effects. Because the network is so highly connected, the effects of a hub deletion can propagate throughout the network influencing hundreds of genes and multiple, seemingly unrelated phenotypes.

Featherstone and Broadie assert that these findings have implications for clinical and primary genetics research. (44) They argue that because the deletion of any one gene affects the expression of many other genes, there is no way to tell the function of a single gene simply by deleting it and observing the phenotype. (44) A phenotype depends on the altered expression of many genes. This further implies that when a single gene mutation or deletion leads to what is observed as multiple phenotypes, these phenotypes cannot be attributed solely to that single gene, but to the effect that the mutation has on the entire network. Effects of the single mutation propagate across the entire network, creating many possible phenotypic changes due to compensation and downstream factors.

Significantly, genome-wide expression profiles are predictive of gene function.⁽⁵⁴⁾ This shows that the action of a gene in the context of the network is more informative about its function than simply observing a phenotype after the gene's deletion. It also implies

potential methods for more accurately determining the effects of drug treatments and disease-causing mutations in humans.⁽⁵⁴⁾ These yeast experiments imply that the context in which these genes operate, not the genes in isolation that is the most biologically significant.

It must be kept in mind, however, that relationships within a biological network are dynamic. van Swinderen and Greenspan⁽⁵⁵⁾ studied epistatic relationships between 16 interacting genes affecting coordination in *Drosophila*. Each of the 16 genes interacts with a mutation in *Syntaxin-1A*, which is involved in synaptic transmission. Flies heterozygous for the *Syntaxin-1A* mutation $Syx1A^{3-69}$ become uncoordinated at temperatures of 38°C and above. (55) van Swinderen and Greenspan analyzed the relationships between each pair of genes in the presence and absence of the $Syx1A^{3-69}$ mutation. They found that the epistatic relationships between the gene pairs in wild type flies differed dramatically from those in flies heterozygous for the $Syx1A^{3-69}$ mutation. (55) This work further supports the importance of understanding the network, and not just a single gene. (56)

Together, these observations of gene network function offer strong support for the hypothesis that epistasis and pleiotropy are the result, not of single genes or single pairs of genes, but of genes' effects on an entire genetic network. The mechanism by which a mutation in a single gene can lead to multiple phenotypes, as well as how multiple genes interact to influence phenotype, become apparent when the network is analyzed in totality. It is reasonable to conclude that although the terms epistasis and pleiotropy have been identified and hence defined as the products of known or detectable mutations, these processes are innate to all biological systems whether or not a distinct mutation is observable.

Exploring Epistasis and Pleiotropy with Graphs

High-throughput studies in yeast and E. coli have shown that the overall structure of the yeast epistatic network is a small-world⁽⁵⁹⁾ and clusters into highly connected modules that are functionally enriched ^(32,59), i.e. genetic interactions are more likely to occur between functionally related genes than unrelated genes. This feature can be used to help predict functions of individual genes.⁽⁵⁹⁾

Segrè et al. (32) furthermore found that the yeast epistatic network modules are enriched not only by function, but also by the type of epistasis. The group measured epistatic interactions between pairs of 890 metabolic genes in S. cerevisiae using a phenotype that combines growth rate with metabolic flux. The resulting interactions segregated into three categories with a trimodal distribution: aggravating epistasis, in which the double mutant phenotype is affected more strongly than predicted by the sum of the single mutant phenotypes; alleviating epistasis, in which the double mutant phenotype is less strongly affected than predicted by the sum of the single mutant phenotypes; and no epistasis. The network of these interactions organized hierarchically into function-enriched modules, for example the respiratory chain or uracil synthesis. (32) The epistatic interactions between genes within each module also tended to be of the same type, either all alleviating or all aggravating. The authors proposed that this pattern, which they named "monochromaticity," introduces a new definition of biological module that emphasizes interactions between rather than within modules, and can be used to infer functional gene modules from phenotypic epistasis measurements. (32) The group also found that the interactions within the modules were robust to changes in environmental conditions while interactions between modules were not. These results supports the biological relevance of modules formed by epistatic interactions between genes.

Epistatic networks can also be combined with networks mapping interactions of other types, for example protein-protein interactions, to uncover physical manifestations of underlying

the genetic effects. Kelley and Ideker⁽⁵⁸⁾ combined yeast epistasis data with data on protein-protein interactions, protein-DNA interactions and metabolic networks and found that ~40% of known genetic interactions in yeast could be explained by between- or within-pathway models encoded by the physical interaction network. The study most notably was able to predict new functions for 343 proteins. Integration of physical interaction data with genetic interaction data has also be used to predict protein complexes and their functional relationships more accurately than either category of data alone.⁽⁵⁷⁾

Epistatic networks have also been used to study human complex disease. A series of studies on the interaction between the renin-angiotensin, bradykinin, and fibrinolytic systems found evidence for epistasis in the regulation of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1). (60-64) t-PA and PAI-1 levels have been identified as risk factors for thromboembolic disease. (65-68) These proteins act in the fibrinolytic system to affect the formation and dissolution of blood clots, and a balance between the two proteins is critical in preventing inappropriate clot formation. (For review see Vaughan (69)) Elevated PAI-1 and reduced t-PA levels have been associated with recurrent deep-vein thrombosis. (66) The fibrinolytic system does not act as an independent unit, however. As previous studies indicate, the fibrinolytic system interacts with members of the bradykinin system and the renin-angiotensin systems. (61,62)

Moore et al. used combinatorial partitioning to analyze genotype and phenotype data and found that there was a non-additive effect of the ACE insertion/deletion polymorphism and the PAI-1 4G/5G polymorphisms on PAI-1 plasma levels in African American males and females. (63,64)

In a later study, Asselbergs et al. found that pairwise interactions between polymorphisms in the bradykinin, renin-angiotensin, and fibrinolytic systems affected t-PA and PAI-1 levels. (61) When the interactions between polymorphisms are diagramed it becomes apparent that not only are there epistatic interactions between the genes, but there are also examples of pleiotropic effects. Figure 2 shows the epistatic interactions and pleiotropic effects of the studied polymorphisms in females. These analyses indicate, for example, that the AT1R polymorphism and the PAI-1 polymorphism interacted significantly to affect plasma levels of t-PA. In addition, the AT1R polymorphism interacts with the B2R-C58T polymorphism to affect PAI-1 levels. With these and other interactions illustrated in Figure 2, AT1R affects both t-PA and PAI-1 through different epistatic interactions. Furthermore, analysis of AT1R in isolation indicates that it is a pleiotropic gene. (70) The data also indicate that there may be a correlation between the number of epistatic interactions a gene participates in and the number of phenotypes it modulates. This observation is relevant not only in the search for genes implicated in human disease, but also in selecting drug targets to maximize efficacy while minimizing side effects.

Conclusions

Historically, one of the main principles driving scientific discovery has been that any complicated problem can be solved by breaking it down into its component parts and analyzing each separately. While this reductionist research has obviously contributed enormously to our understanding of biology, we need a different approach to take the next major strides forward. This approach involves acknowledging that genes operate in widespread interaction networks and that an individual gene's effects can only be studied within the context of these networks. That epistasis and pleiotropy are ubiquitous and important in human disease reveals the great need for a more systems based approach to human genetics. Epistasis and pleiotropy reflect the complexity of underlying biomolecular

interactions and should be harnessed as tools rather than dismissed as exceptions or complications.

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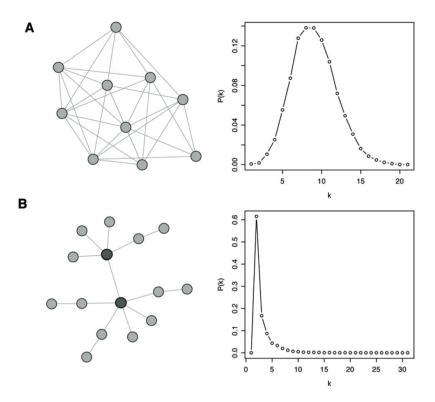


Figure 1. Examples of node connectivity in random and scale-free networks. (A) In a random network, the connectivity of the nodes follows a Gaussian distribution. A plot of log k against log P(k) follows a Gaussian distribution.(B) In a scale-free network most nodes (light gray) are connected to one or two other nodes, while a few hubs (dark gray) are connected to many other nodes. In a random network, a plot of log k against log P(k) follows a Gaussian distribution. In a scale-free network, a plot of log k against log P(k) follows a straight line following the power law $P(k) = Ak^-3$.

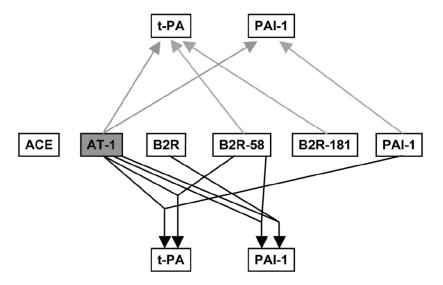


Figure 2. This figure summarizes gene-gene interactions and pleiotropic effects found statistically in a series of studies in humans.(60-64) The genes and polymorphisms examined in the studies are listed in the center row, and the phenotypes they affect are listed on the top and bottom. Downward arrows represent significant pairwise epistatic interactions between polymorphisms that affect the two phenotypes, in this case plasma levels of t-PA and PAI-1. Upward arrows in Figure 2 indicate significant main effects of each polymorphism on each phenotype. AT1R had a significant main effect on both t-PA and PAI-1. Here the angiotensin receptor is shown to be pleiotropic as it influences levels of both t-PA and PAI-1.