Network biology concepts in complex disease comorbidities

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Abstract | The co-occurrence of diseases can inform the underlying network biology of shared and multifunctional genes and pathways. In addition, comorbidities help to elucidate the effects of external exposures, such as diet, lifestyle and patient care. With worldwide health transaction data now often being collected electronically, disease co-occurrences are starting to be quantitatively characterized. Linking network dynamics to the real-life, non-ideal patient in whom diseases co-occur and interact provides a valuable basis for generating hypotheses on molecular disease mechanisms, and provides knowledge that can facilitate drug repurposing and the development of targeted therapeutic strategies.

Pleiotropy
The property of a genetic locus that affects more than one trait.

Robustness
The property that allows a system to maintain its functions against internal and external perturbations.

Rewiring
Restructuring of interactions between biological components due to conditional changes.

Complex disease
A disease that is a result of complex interactions between genetics and environment that is hard to explain by a few factors.

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Disease progression patterns of patients with more than one disease have recently received increasing attention within both molecular level systems biology and epidemiology. Diseases progress and co-occur according to the dynamics of underlying mechanisms. All biological systems are inherently dynamic[1] and entail dynamic features such as pleiotropy[2–6], robustness[7,8] and rewiring[9,10]. These features are of relevance both in the healthy state of an organism and in disease processes. Owing to this complexity, network biology has become a preferred model for rationalizing complex disease at the level of genes and proteins. Genetic or environmental perturbations of disease genes and their products can alter network robustness and wiring topologies and can in turn change network output. Some disease genes even have more than one functional role. It is increasingly being acknowledged that not only gene loci, but also proteins and pathways, can have multiple context- and time-dependent roles[11,12]. Such multifunctionalities can be involved in pleiotropy (the effect of a genetic locus on more than one trait), resulting in comorbidities in which two disease states coexist in the same individual.

Network models, such as genetic interaction networks and physical interaction networks, have been constructed to study complex disease. Traditionally, these interaction maps have been created under a single condition, making them 'static', and do not allow insight into dynamic disease states that are altered gradually by either internal regulation and environmental perturbations or treatment in the clinic. With recent advances in high-throughput quantitative 'omics' technologies (for example, single-cell technologies), which enable comprehensive network mapping across multiple conditions, a more in-depth characterization of dynamic disease patterns is now possible[13,14]. One example is the concept of differential networks, which can be used to identify network changes between two states by mapping molecular profiles onto static networks (reviewed in REF. 10). Differential networks can reveal context-specific interactions and can filter out unspecific dominant interactions of the system, such as processes that drive housekeeping functions[15]. Others use quantitative techniques, such as time- and stimuli-resolved mass spectrometry, to measure network dynamics directly[11,16]. For example, this approach was used to observe the rewiring of the hub protein GRB2 (growth factor receptor bound protein 2) in HEK293T cells after stimulation with epidermal growth factor (EGF) over time, revealing that EGF has an important role in the protein complex formation of GRB2 (REF. 17).

Whereas most dynamics studies have been carried out in model organisms, more recent studies have addressed human disease. For example, Lee *et al.*[18] monitored gene expression profiles and cell phenotype responses of triple-negative breast cancer cells *in vitro* and *in vivo* for various time- and administration order-dependent drug combinations. Time-staggered EGF receptor inhibition was able to push cancer cells into states that were more vulnerable to DNA-damaging drugs[18], suggesting that the most effective therapy against triple-negative breast cancer cells greatly depends on the right combination of, and time of administration of, multiple drugs. Two other studies also highlight the importance of rewiring in disease. One study focused on classifying functional cancer mutations that rewire signalling networks according to six different mechanisms, stressing that rewiring-based analyses can increase the number of driver mutations to be identified[19]. The second study reported a comprehensive library of tissue-specific

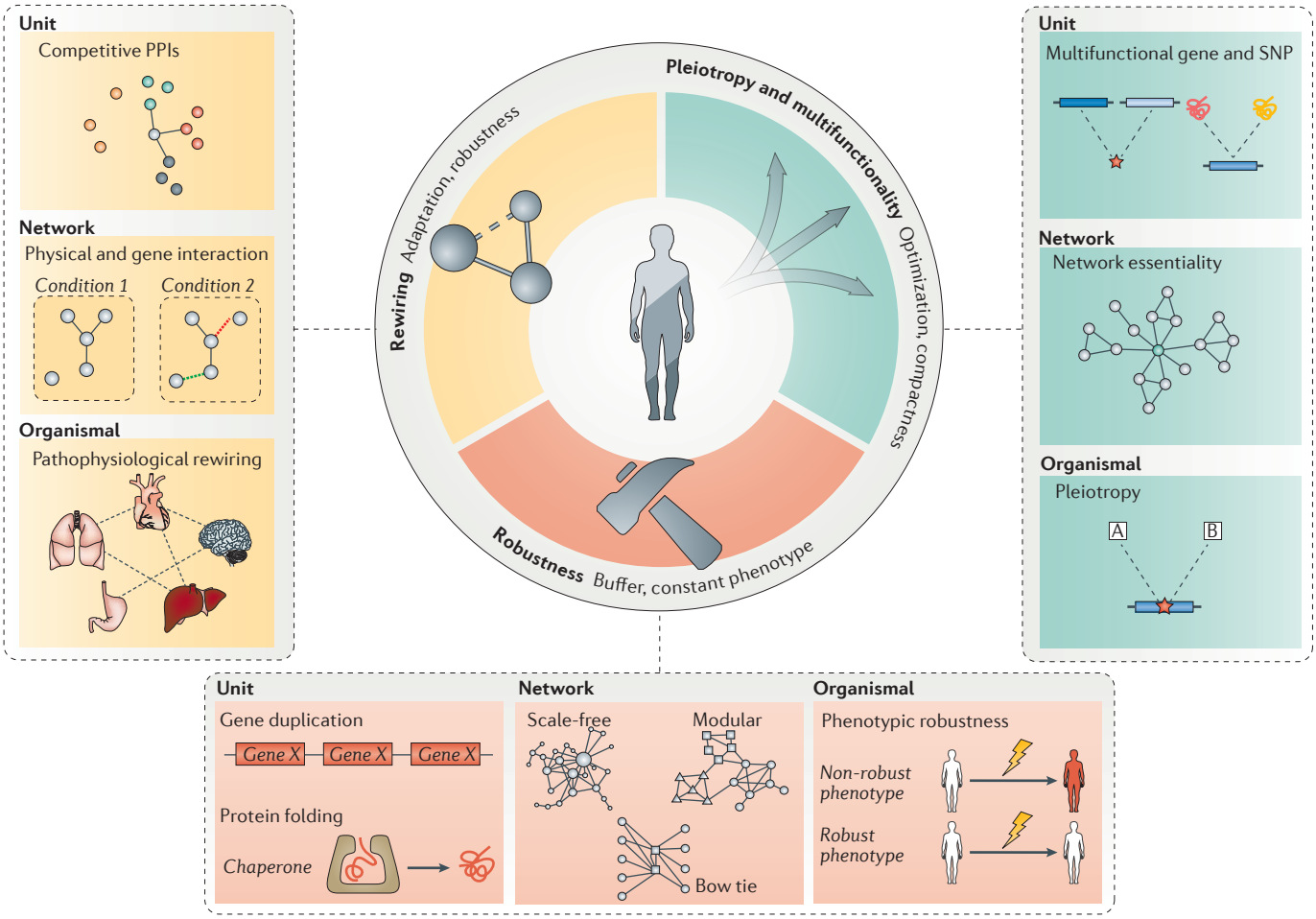


Figure 1 | **The pervasiveness of dynamic concepts.** The unit, network and organismal levels are relevant when studying pleiotropy, robustness and rewiring. The rewiring of a protein depends on the abundance of partner proteins owing to competitive binding and stoichiometry. Rewiring on a network level can be measured for physical and genetic interaction networks across conditions, and, at the physiological level, inter-organ rewiring can occur. Robustness can be maintained through, for example, chaperone activity and gene duplications, at a unit level, and robust topology structures at the network level. At the organismal level, phenotypic robustness is a measure of the capability of an organism to uphold a constant phenotype. Multifunctionality at different organizational levels can lead to pleiotropy, in which a gene or a mutation affects multiple phenotypes. At the network level, pleiotropic genes and their products often occupy central positions. PPI, protein–protein interaction; SNP, single-nucleotide polymorphism.

Multifunctionalities
Properties of a biological component that have multiple distinct roles.

Comorbidities
Diseases that co-occur on top of a primary disease of interest in an individual.

Genetic interaction networks
Networks in which nodes are genes and edges are their epistatic interactions.

Physical interaction networks
Networks in which nodes physically interact. In biology interactions may be between and among, for example, proteins, DNA and RNA.

Differential networks
Analytical approaches to identify edge changes between two static network states.

Hub
A hub node in a network has a high degree of edges, meaning that it interacts with many other nodes in the network.

functional interaction networks for 144 different human tissues and cell types to predict how perturbations may differentially affect protein function across different cell and tissue types[12].

The use of dynamic concepts in complex disease has been hampered both by technological constraints and by confusion and inconsistent terminology. Many other reviews have covered the intricate — and not always self-explanatory — dynamic concepts: pleiotropy[2–4,6], robustness[7,8] and rewiring[9,10]. However, these concepts have typically been described separately rather than as parts of a coherent framework, and almost no studies have considered the interplay between these concepts in dynamic systems.

In this Review, we delineate and define dynamic concepts from a complex disease and comorbidity angle at different organizational levels: at the level of the unit, the network and the organism (FIG. 1). We highlight the importance of considering dynamic network concepts jointly and in relation to disease co-occurrences in an individual (comorbidity and multimorbidity) for a number of complex diseases, including cancer and type 2 diabetes (T2D) mellitus, with a particular focus on immune-mediated diseases.

Comorbidity in systems biology

Comorbidities pose a major problem in the clinic, as they increase patient mortality and complicate the choice of treatment strategies[20]. For example, in a patient with both cancer and sepsis, cancer treatment needs to be adapted to the presence of the more acute condition of sepsis. Another issue is that comorbidity cases are typically associated with polypharmacy (the use of multiple drugs), which can decrease treatment efficacies and can cause unexpected adverse effects, further adding to the disease spectrum in a given patient[21].

The concept of comorbidity. Comorbidity was defined in 1970 as the presence of one or more diseases in addition to a primary disease in the same individual[22]. However, since then, comorbidity has gained multiple meanings, instigating much debate on the correct use of the term[23,24]. Difficulties in defining the concept mainly revolve around two issues: inadequate disease classification systems and problematic co-option of clinical terms in basic research.

The definition of comorbidity assumes a correct disease classification system with proper disambiguation of pathologies[25]. This is far from the case, for example, in psychiatry, where a high comorbidity rate has been criticized as being a diagnostic artefact owing to the inability to apply an existing diagnosis for the many symptoms exhibited[26]. Additionally, a lack of clarity about how comorbid diseases causally relate to each other plays a major part in classification ambiguities.

The terms comorbidity and multimorbidity are often used interchangeably, which can further complicate the relationship between network biology concepts and complex disease. Comorbidity and multimorbidity were both originally defined in order to guide decision-making in the clinic. Whereas treatments of comorbidities revolve around a primary disease, multimorbidity has a more patient-centred view, in which no disease is given more consideration[27,28]. Thus, the concepts are not mutually exclusive but consider co-occurrence of disease in the same patient from different perspectives. An example of comorbidity is kidney failure and retinopathy in patients with T2D[29,30]. Multimorbidity can arise owing to environmental exposure, such as smoking, causing damage to multiple organs and resulting in diseases, for example, chronic obstructive pulmonary disease (COPD), lung cancer, cardiovascular disease and osteoporosis[31–33].

Multiple approaches for the systematic investigation of comorbidity and multimorbidity have been used and often involve health transaction data. Some of these approaches aim to create networks of disease co-occurrence[34], whereas others add a temporal dimension and focus on disease trajectories either for a wide spectrum of diseases[35,36] or for a specific disease[37]. Additionally, approaches for moving beyond pairwise disease correlations and distinguishing between correlative and causal relationships have been proposed[38,39].

Comorbidity and multimorbidity suggest an underlying shared disease aetiology, which can be both genetic and environmental[40]. More studies are now investigating comorbidity and multimorbidity from a molecular point of view[41–44] and are co-opting these clinical concepts to the molecular level, which can lead to some confusion. Some authors define comorbidity as diseases that are associated through aetiology and define multimorbidity as diseases that coexist independently of each other, whereas others use the clinical definition mentioned above[23,24]. In this Review, we also use the clinical definition.

Emerging evidence points to the fact that diseases can be inversely comorbid[45–47], meaning that there is a lower than expected probability that they will co-occur given their individual frequencies. Comorbidity or inverse comorbidity are not necessarily due to the pathophysiological effect of one disease on another disease, but can also be due to shared disease pathways or the 'off-target' effect of a clinical treatment such as chemotherapy. One example of an inverse comorbidity is represented by T2D and cancer, as the anti-diabetic drug metformin was found to substantially lower the risk of several types of cancers[48].

Multimorbidity space. Many patients have multiple diagnoses and unrecognized conditions at varying severities at any given time. The patient state can be viewed as a point in a 'multimorbidity space', in which each dimension corresponds to a quantitative phenotype (FIG. 2a), such as laboratory measures (for example, glycated haemoglobin (HbA_{1c}) levels in T2D) or staging scores (for example, the tumour node metastases (TNM) grades in cancer). Patients who are similar in one phenotypic dimension may differ widely in other dimensions, thus reducing the power to detect, for example, genetic variants in classical case–control studies. Power can typically be increased by grouping patients on multiple dimensions[49], as is commonly done when stratifying by factors such as age and gender. Using deep phenotyping, patients can be stratified even more comprehensively on the basis of the many fine-grained features of their phenotype, such as symptoms, diagnoses, genetics and molecular profiles[50], to find a more precise location for each patient in the multimorbidity space. A recent study used deep learning to create compact versions of such rich patient representations while capturing hierarchical relationships and dependencies between diagnoses, treatments and laboratory tests[51]. Even though deep phenotyping comes at the cost of increased dimensionality of the multimorbidity space, it can also reduce the cohort size that is needed to detect, for example, rare variants[49].

Patients will follow trajectories in the multimorbidity space during their lifetime as they receive new diagnoses or are cured or treated for other diseases[34,36]. Germline variation, somatic mutations and environmental effects can influence such transitions, and can be either detrimental or protective. Environmental effects can include drugs, changes in lifestyle and exposure to a pathogen. Transitions from one point in the multimorbidity space to another point can be viewed as the interplay between robustness, rewiring and pleiotropy (FIG. 2b). Environmental and/or genetic perturbations might change the internal wiring processes within an individual (such as molecular interactions, protein levels and gene expression) at one point, leading to a transition into a new disease state. Owing to an individual's inherent robustness and genetic variants, including those with potential pleiotropic effects, some transitions are more likely to occur than others, which in turn will be mirrored in the disease-pair statistics that are obtained from electronic health data. For example, in a recent study that investigated the development of psychosis in patients with Alzheimer disease, patients without psychosis exhibited a significantly higher quantity of copy number duplications, including a protective duplication in the *APC2* gene[52].

Organizational levels
Levels in the hierarchy of biological structures and systems such as protein, cell, tissue, organ or organism.

Dynamic network
A network that continuously changes topology over time.

Multimorbidity
The coexistence of two or more diseases in the same individual without disease prioritization.

Health transaction data
Data describing patients' contacts with the health care system. Data accumulates in electronic patient records and registries.

Inversely comorbid
Diseases that co-occur less often in an individual than expected given their individual frequencies in the population.

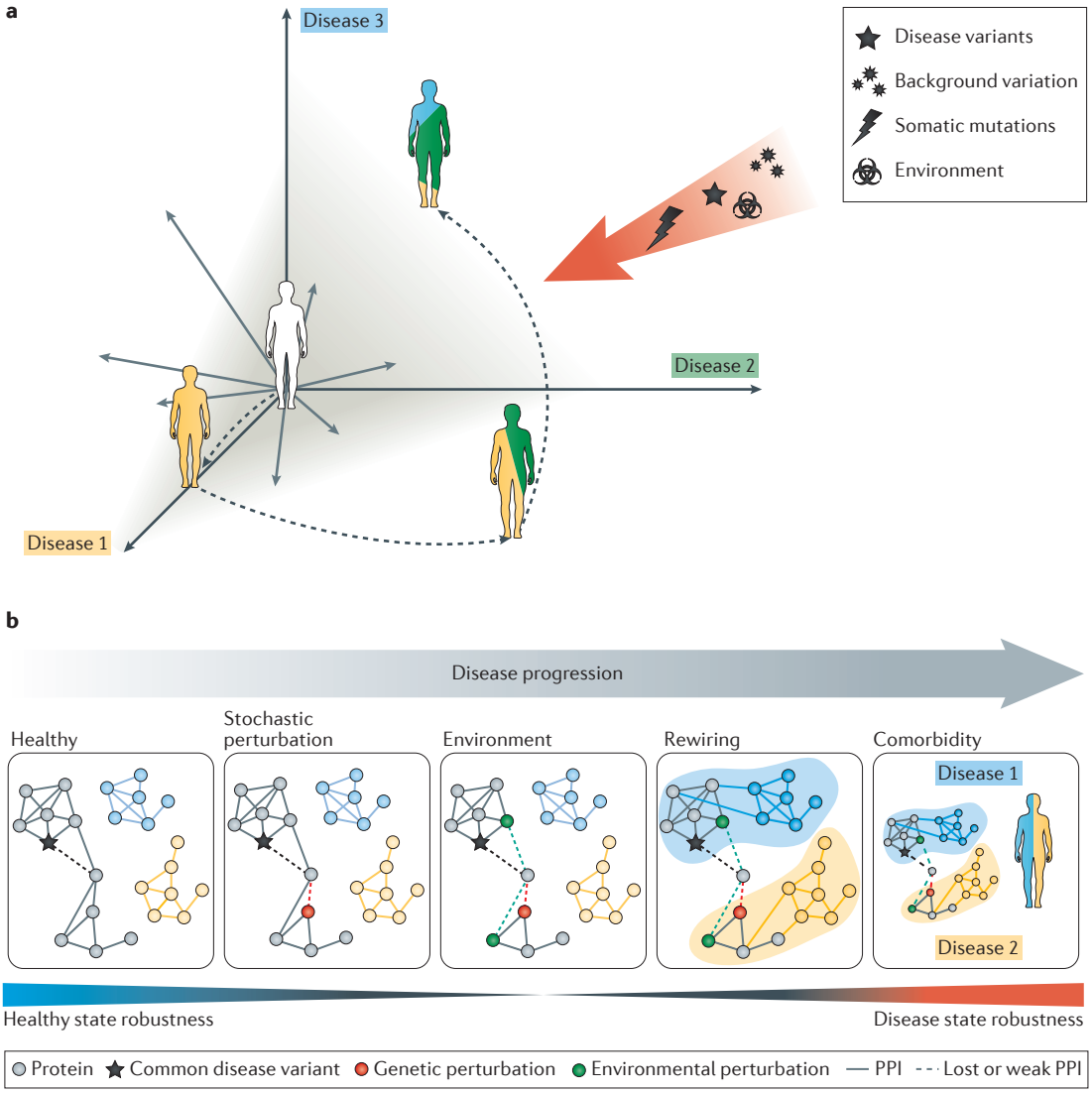


Figure 2 | **Multimorbidity space and dynamic disease progression. a** | An individual's disease state can be represented as a point in a multimorbidity space, where the axes represent quantitative measures of different diseases. The grey axes represent the fact that the multimorbidity space is not limited to only three disease dimensions but can instead span numerous dimensions. A patient's temporal disease trajectory can be described as transitions (represented by dashed arrows) between points in this space. These transitions will be facilitated by the effects of genetic and environmental parameters (red arrow). **b** | Disease progression and comorbidity can be understood in light of robustness, rewiring and pleiotropy. The networks represent protein–protein interaction (PPI) networks, and the sequential network changes depict physical protein–protein interaction rewiring during disease progression. Combinations of disease risk variants and genetic and environmental perturbations can alter protein abundances and can, in turn, rewire network topology (as shown in the first four boxes). Transitional rewiring, as a result of, for example, altered protein abundance or stoichiometry, can activate specific disease-associated modules (as shown in the blue and yellow modules). If the disease modules are bridged by pleiotropic genes (grey central node) mutations in these genes can activate several disease modules, resulting in comorbidity. In some cases, disease progression can lead to a new disease state, which can be robust, for example, towards therapeutic intervention. It should be noted that disease progression could occur either by perturbations that lead to higher vulnerability to environmental perturbations (as shown in the figure) or vice versa, or in most cases a combination of the two.

Pleiotropy and multifunctionality

The concept of pleiotropy was formally defined in the literature by the developmental geneticist Ludwig Plate in 1910 (REF. 53) as the phenomenon of a unit of inheritance that several characteristics depend upon. Since then it has been shown that many diseases share genetic architecture[54,55]. For example, a notable overlap of shared disease risk variants and pathways has been observed in immune-mediated diseases, suggesting extensive pleiotropic effects[43,56,57].

Though pleiotropy probably has an important role in complex disease and comorbidities, it is a phenomenon that has been much debated for more than 100 years. Often, the pleiotropic mechanism is presented as a black

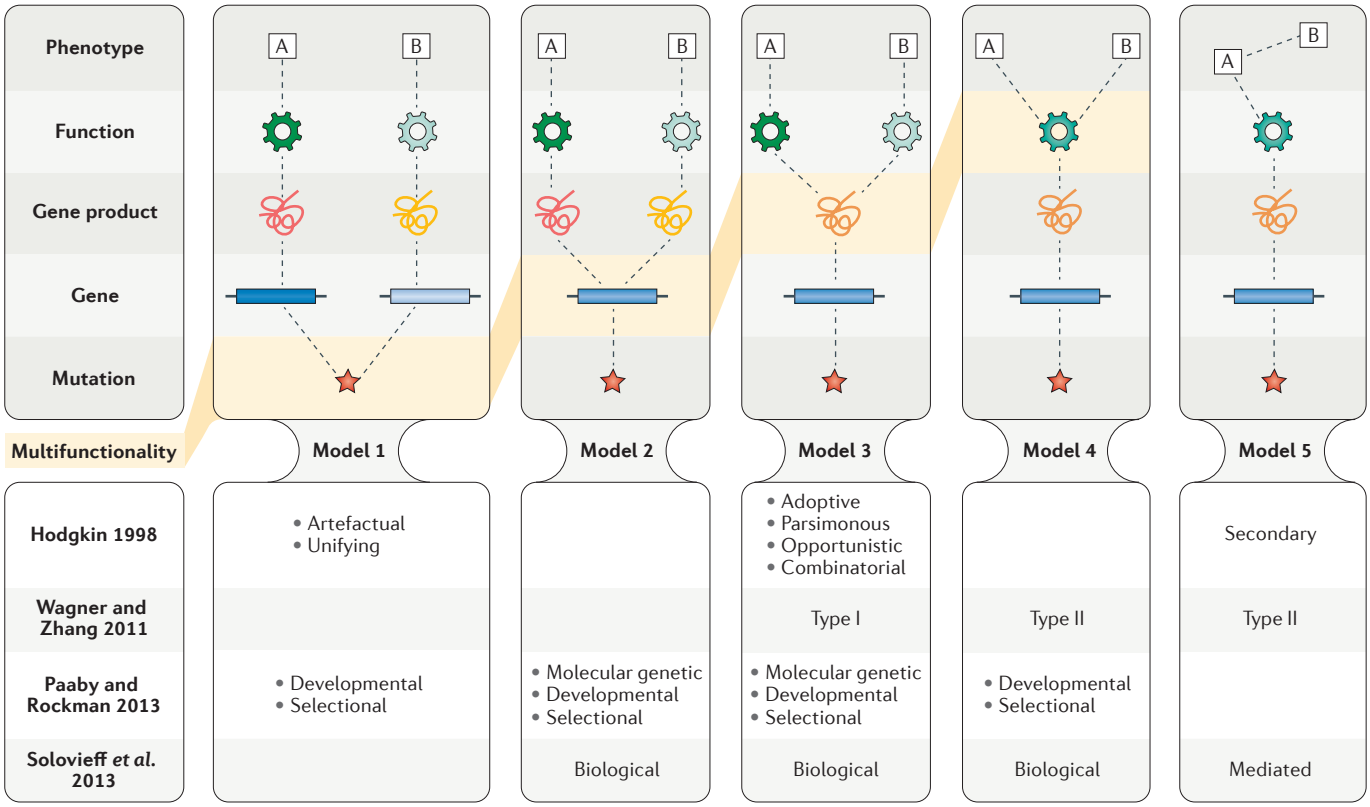


Figure 3 | **Five models of multifunctionality and pleiotropy.** These models delineate the mechanisms of how one genotype can lead to multiple observed phenotypes. The lower part of the figure summarizes the previous definitions of pleiotropy and how they relate to these models. Models 1–4 cover mechanisms that have been described as 'true' pleiotropy in the literature. Model 1: a mutation that affects multiple genes through, for example, insertions or deletions that span multiple genes or variants that affect the expression of more than one gene. Model 2: a gene with multiple functions through, for example, alternative spicing[58] or exon shipping[59]. Model 3: a gene product, such as a protein, that contains multiple domains with separate functions[60] or that has different functions in different tissues[12,61]. Model 4: a gene product with one function that is involved in multiple phenotypes, for example, by being present in multiple tissues where it is involved in the same biological process. Model 5 describes the case in which one phenotype leads to a second phenotype through physiological means. This mechanism is often termed mediated pleiotropy, as the genetic factor only has a direct effect on one of the phenotypes.

box when linking gene and phenotype[2,3]. We delineate below the many definitions of pleiotropy and discuss its relation to complex disease comorbidities and dynamic networks.

Five models of pleiotropy and multifunctionality. Pleiotropy has gained multiple disparate meanings over time, especially since the emergence of genomic techniques, which has made it possible to better investigate pleiotropy at the molecular level. Reviews in the past two decades have aimed to define pleiotropy consistently in a molecular context[2–4,6]. However, no clear consensus has yet been reached. For example, Hodgkin defines seven types of pleiotropy that each relate to a different underlying mechanism of pleiotropy[4], whereas the distinction between the definitions made by Wagner and Zhang lies in the number of functions of a gene product[6]. By contrast, Paaby and Rockman's definitions originate from different biological fields[2], and, finally, Solovieff defines pleiotropy from the perspective of genome-wide association studies (GWAS)[3]. Although each of these definitions can be individually useful, they highlight different aspects of pleiotropy, making it a challenge to amalgamate them.

We propose a consolidated view to facilitate such amalgamation that consists of five basic models of how a genetic unit can lead to multiple phenotypes (FIG. 3); that is, fundamentally, models of the mapping between genotype and phenotype. The main disagreements in the literature on pleiotropy concern how to distinguish between these models and which of the models constitute 'true' pleiotropy as opposed to 'spurious' or 'mediated' pleiotropy. All of the models rely on multifunctionality on at least one organizational level. Starting at the genetic level, model 1 describes a mutation that affects multiple genes. For example, large deletions or insertions that span multiple genes or regulatory variants that affect multiple genes. In model 2, a gene can itself have multiple functions via mechanisms such as alternative splicing[58] and basal exon skipping[59]. For example, mutations in the *CEP290* gene, which encodes a centrosomal protein that is involved in ciliary assembly and ciliary trafficking. These mutations

differentially affect protein expression, leading to multiple phenotypically distinct ciliopathies, including phenotypes such as retinal degeneration and intellectual disability[59]. Model 3 states that a gene product (such as a protein) contains multiple domains with separate functions[60] or that a gene product has different functions in different tissues[12,61]. Model 4 describes a gene product with one function that affects multiple phenotypes, for example, by being present and exhibiting the same function in multiple tissues[62]. For instance, the same genetic variants and molecular pathways have been associated with a number of immune-mediated diseases despite their great phenotypic diversity[57]. Another example is Marfan syndrome, in which mutations in the *FBN1* gene affect connective tissue in multiple organs, which leads to skeletal, ocular, skin and cardiovascular symptoms[62]. Model 5 posits that one trait (such as a complex disease) may lead to the appearance of another phenotype through physiological changes. For example, mutations in the *SERPINA1* gene cause α1 antitrypsin deficiency; this disorder leads to lung inflammation that can cause emphysema, which increases the risk of COPD[63]. Yet another example is that almost all patients with type 1 diabetes (T1D) will develop diabetic retinopathy owing to pathophysiological mechanisms that are triggered by hyperglycaemia[64]. All of the above models, except model 5, have in some context been deemed 'true' pleiotropy.

Difficulties in defining a phenotype. Issues stemming from defining what is meant by a 'trait' or a 'phenotype' are major factors that contribute to discussions on what constitutes 'true' pleiotropy[6]. A trait is defined relative to the system that harbours it. Thus, at the level of the individual, some consider a genetic variant pleiotropic if it is implicated in multiple diseases. However, others may only consider this variant to be pleiotropic if it also contributes to multiple phenotypes within the same cell, which in many instances will not be the case. Thus, what is considered 'true' pleiotropy is entirely dependent on the system and the traits under study. Difficulties related to disease classification complicate this further[25].

Pleiotropy and its role in comorbidities. When considering comorbidities, all of the above-mentioned models are relevant, as they can contribute to categorizing the mechanisms behind different comorbidities. These models are especially relevant when considering the aetiological relationship between two comorbid diseases and the risk profiles associated with them. If two diseases are comorbid owing to one disease causing the other through physiological means (model 5), then treating the first disease may prevent the second disease from ever occurring. For example, proper glycaemic control can prevent the occurrence of some T2D comorbidities, such as diabetic retinopathy. However, this will not necessarily be the case if two diseases are comorbid owing to shared genetics (models 1 and 2) or shared disease pathways (models 3 and 4). Therefore, it is crucial to unravel whether disease co-occurrence can be explained by one of the models. However, typically, comorbidities will be a mix of these models (as discussed above). Notably, although it may seem obvious, it is important to appreciate that it is impossible to distinguish between models 1, 2, 3 and 4 without knowing the specific molecular mechanism of a disease.

Finally, shared genetics or pathways are especially interesting for understanding exactly how the aetiology of two diseases overlap, which can be helpful for drug repurposing. Phenome-wide association studies (PheWAS), which combine detailed clinical data from health records with linked genotype data to detect genotype-to-phenotype associations[65,66], can identify pleiotropic associations and hold great promise in proposing novel drug repurposing candidates, such as the antiretroviral drug zidovudine used to treat HIV and AIDS, which has been repurposed for the treatment of diabetes mellitus[67].

Methods for studying pleiotropy and multifunctionality. Multiple experimental and computational methods exist for studying pleiotropy and multifunctionality (TABLE 1). Studying diseases together using GWAS can help to detect pleiotropic risk variants that are not otherwise found in single-disease studies[43]. Certain variants may be hidden in single-disease studies, because they confer a general disease risk, but they can be apparent in multi-disease studies[68]. Analytical strategies for appropriately using GWAS to identify pleiotropy have been described previously[3]. A commonality of most PheWAS and GWAS is the finding of 'potentially pleiotropic effects' without further investigation of whether these associations represent true pleiotropy. A recently developed online tool known as BUHMBOX[69] embarks on the challenge of distinguishing between pleiotropy and heterogeneity. In this case, heterogeneity is defined as the instance in which only a subgroup of cases in one disease is genetically identical to those of another disease, for example, owing to misclassification (cases that are misdiagnosed due to the presentation of atypical symptoms), ascertainment bias (cases that are affected by additional diseases that have higher chances of receiving clinical attention) or model 5 (cases that also have the causal disease). In a recent study of five chronic inflammatory diseases, BUHMBOX revealed that comorbidities among the five diseases could be explained by pleiotropy as opposed to heterogeneity. Additionally, three unreported shared risk loci were identified that would have been missed using single-disease studies alone[43].

Robustness

Robustness has mainly been approached from two angles in relation to disease. The first angle stems from systems biology, which has adopted it from physics, and focuses on robustness as a feature of a physical biological system. The other angle stems from evolutionary genetics, and mainly focuses on how the phenotypic robustness of individuals can mask genetic variation[7]; this has long been a focus of the research of model organisms, but is now also gaining more attention in human research[70].

Defining robustness. Robustness of a system has been defined as 'a property that allows a system to maintain its functions against internal and external perturbations' (REF. 71). In other words, robustness is an inherent,

Drug repurposing
The application of a known drug to new indications. Synonymous with the term drug repositioning.

Table 1 | **Approaches for investigating multifunctionality and pleiotropy, robustness and rewiring**

Dynamic term	Approaches	Description	Refs
Comorbidity	Comorbidity-View	A visualization tool for comorbidity networks, showing data from the SEPR corpus	165
Pleiotropy and multifunctionality	GWAS and joint GWAS	Identifying candidate pleiotropic variants by GWAS	3,166
	PheWAS	Identifying candidate pleiotropic variants using genome-phenome-linked data	66,167
	NetWAS	Identifying proteins with tissue-specific functions	12
	BUHMBOX	Distinguishing pleiotropy and clinical heterogeneity	69
	eQTL and QTL	Empirical data to estimate genome-wide pleiotropy	6
	Mendelian randomization	Identification of mediated pleiotropy	168,169
Robustness	Quantitative trait analysis	Experimental approach to measure trait variation across different conditions or perturbations	7
	Network analyser	Cytoscape plugin to calculate network topology parameters, such as node degree, betweenness and so on	170
	PerturbationAnalyzer	Cytoscape plugin that simulates user-defined perturbations to evaluate network robustness in PPIs	171
Rewiring	GIANT	An online webserver to visualize human tissue networks	12
	DDN	Analytical tool for differential network analysis	172
	dE-MAP	Experimental approach to map changes in genetic interaction networks	15
	AP-SRM and AP-SWATH	Targeted proteomics approach to map changes in PPIs	11
	LUMIER	A high-throughput interactome assay	164

AP, affinity purification; DDN, differential dependency network; dE-MAP, differential epistasis mapping; eQTL, expression quantitative trait locus; GWAS, genome-wide association studies; LUMIER, luminescence-based mammalian interactome mapping; NetWAS, network-guided GWAS analysis; PheWAS, phenome-wide association studies; PPI, protein–protein interaction; SEPR, Stockholm Electronic Patient Records; SRM, selected reaction monitoring; SWATH, sequential window acquisition of all theoretical spectra.

Scale-free
A network structure that has a degree distribution following a power law.

Bow tie
A multi-layered network structure where intermediate layers have far fewer components than input and output layers.

Modularity
A network structure with dense connections between clusters of nodes and sparse connections between nodes in different clusters.

Homeostasis
The ability to sustain various physiological parameters in a steady state.

Plasticity
Variation of a phenotype as a response to a given environmental exposure.

Epistasis
A phenomenon in which the function of one gene affects the function of another gene in a non-additive manner.

Penetrance
From a genome-wide association study perspective, penetrance describes the proportion of individuals for which a genetic variant results in a changed phenotype.

quantifiable feature of biological systems that can buffer against fluctuating perturbations through adaptation mechanisms[8]. In biological systems, robustness exists at multiple organizational levels. It can be maintained through molecular buffering such as gene duplication[72], non-linearity in dose–response curves[7] and the functionality of specific proteins (for example, chaperones[73]). Additionally, network architectures and properties, such as scale-free, bow tie, modularity, redundancy and degeneracy (BOX 1), uphold network functionalities when faced with perturbations. These properties have been reviewed extensively elsewhere[71,74].

Two main issues have accompanied the use of the concept of robustness (as reviewed in REFS 7,8). First, it has not always been properly distinguished from related concepts, including homeostasis, stability and plasticity. Second, the term robustness has often been used generically without proper specification of the system studied, the phenotype under study, how robustness is quantified and the nature of the perturbations considered. This is crucial in order to interpret results and compare results between studies.

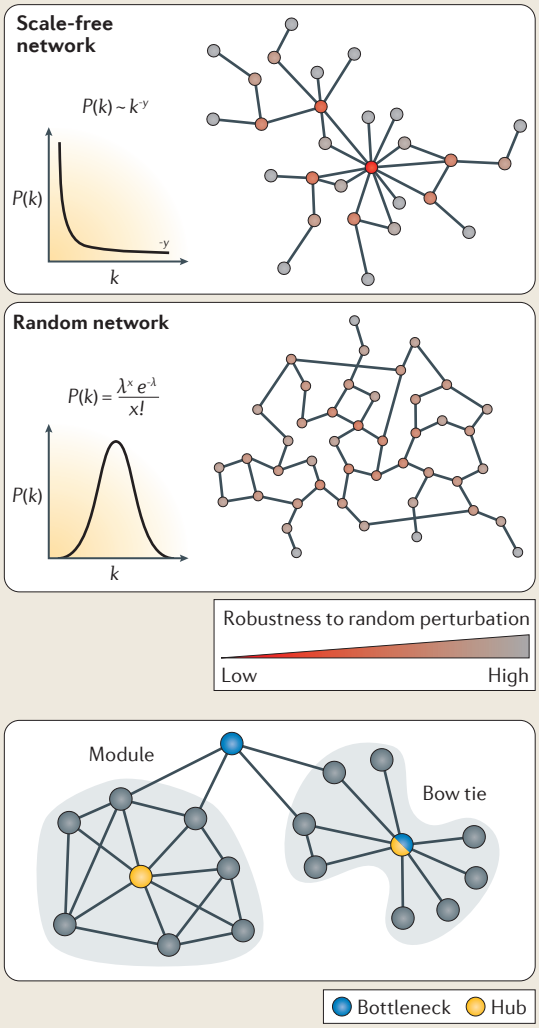
Robustness of a system. When a biological system is exposed to perturbations, either it may be robust against them and maintain its functionality (for example, phenotype) or it may change functionality[8]. In disease development, a system (for example, a cell or an entire organism) that is not robust to a specific perturbation will be pushed to a new disease state when exposed to that perturbation. However, even if the system is robust to that perturbation, its internal physical state (such as molecular network wiring, protein levels and gene expression) may still change in a way that makes the system more fragile towards other perturbations. For an individual this means that a perturbation may not necessarily lead to a new position in the multimorbidity space, but that it may still increase the individual's susceptibility to becoming ill at a later stage. For example, the physiological dynamics of the body may have evolved to ensure robustness against an unstable food supply and pathogenic infections, but at the same time these dynamics may be susceptible to environmental changes such as over-nutrition and thereby lead to complex disorders such as metabolic syndrome[75].

Phenotypic robustness and missing heritability. GWAS have so far only been able to partly explain how genetic variance relates to phenotypic variance (giving rise to the concept of 'missing heritability')[76], suggesting that other factors such as rare mutations, variants with weak effects and epistasis also have an important role[77–80]. This has led to the hypothesis of 'phenotypic robustness', which addresses the susceptibility of an individual to perturbations[70,81]. Phenotypic robustness is closely related to penetrance. However, where penetrance is a feature of one genetic variant, phenotypic robustness is a feature of the

Network topology
The layout of nodes and edges in a network.

Box 1 | Network topology and properties

Networks consist of nodes and the edges that connect them (see the figure). In biology, nodes can be, for example, proteins, genes and diseases, and edges can be the functional or physical relationships between them. Networks of physical interactions between proteins, epistatic interactions between genes and aetiological relationships between diseases are commonly found in the literature. Network topology is defined as the layout of nodes and edges, and the topological properties determine the functional aspects of the relationships.



Quantifying node and edge relationships

Multiple quantitative measures can be used to describe the characteristics of a network and its components. For example, node degree (k) is defined as the number of edges connected to a node. High-degree nodes are known as hubs. The overall distribution of node degrees in a network is known as the degree distribution ($P(k)$), and represents the probability that a node has exactly k edges. The degree distribution is often used to distinguish between different classes of networks[74].

Distances in a network can be measured by counting the number of edges that must be traversed to get from one node to another. The shortest path between two nodes is the minimum number of edges connecting them. The mean path length is the average shortest path between all pairs of nodes and is a measure of the navigability of the network[74]. Betweenness centrality is a measure that indicates how central a node is in a network. It is inferred by the fraction of shortest paths between all pairs of nodes in a network that go through a given node. Thus, the removal of a node with high betweenness centrality will increase the mean path length more than the removal of nodes with lower betweenness centrality.

Scale-free versus random networks

Most biological networks are close to being scale-free, meaning that their node degrees follow a power law. Scale-free networks are robust against random perturbations, as they are most likely to only affect a small part of the network. Most biological scale-free networks have exponents (γ) between 2 and 3 (REF. 155). The notion of scale-free refers to the lack of a characteristic degree or scale.

In contrast to random perturbations, perturbations that target highly connected nodes have a large effect. As opposed to scale-free networks, the degree distribution in a random network follows a Poisson distribution. In these, most nodes have a degree close to the average, and nodes significantly deviating from the average are extremely rare[74].

Modularity

A module is a network substructure with densely connected nodes and sparser connections to other nodes. Such modules are often specialized for a certain subtask. Networks for which most nodes are organized in modules are referred to as highly modular, indicating more limited connections between subparts of the network[156].

Bow tie

Bow tie is a multi-layered network structure (for example, in directed networks) in which intermediate layers have far fewer nodes than input and output layers. Such structures can provide robust and flexible responses when input and output layers have redundant nodes. However, they are fragile towards perturbations of the intermediate node or nodes[157].

Hubs and bottlenecks

Both hub nodes and bottleneck nodes have special roles in a network. Hub nodes interact with many other nodes in the network, and thus often occupy central positions[115]. By contrast, a bottleneck node does not necessarily have many interactions but has a high degree of betweenness centrality, meaning that it will often be a linker between different subnetworks[113]. A node can be both a hub and a bottleneck. The removal of a bottleneck node will often lead to higher fragmentation of a network than the removal of a hub node.

Redundancy and degeneracy

Redundancy and degeneracy can contribute to upholding the robustness in a system. In networks, redundancy exists, for example, if two nodes are connected via multiple paths. If one path is removed, then the nodes are still connected. Degeneracy is a special type of redundancy in which processes, for example, different pathways, lead to both overlapping and separate effects[158].

Box 2 | **Mapping network changes**

Three main strategies and techniques have been used to analyse network rewiring from a systems-level perspective (TABLE 1). One of the most common approaches to studying rewiring is an integrative 'static-temporal approach', in which static network data are overlaid with high-throughput molecular profiles (such as gene expression) for various conditions or time points[159–161]. Even though the approach can extract condition-specific static interactions, findings are limited by the original static interactions and cannot detect new dynamic interactions.

Another method is the more recent 'differential network mapping approach', in which networks are generated under different conditions and then 'subtracted' to highlight the parts of the system that are most affected by the altered condition[10]. Genetic interactions, based on the phenomenon of epistasis, can reflect the functional relationships between genes and the pathways in which their related proteins participate[162]. The differential epistasis mapping (dE-MAP) approach can reveal tailored response-specific interactions, filtering out house-keeping interactions[15]. However, the dE-MAP approach can be very labour-intensive and expensive to carry out for higher eukaryotes owing to the experimental interrogation of interactions. An alternative is differential co-expression networks, in which gene expression data are used to contrast disease and control sample co-expression networks. Differential mapping is also possible for physical maps of protein–protein interactions using targeted proteomics techniques (such as AP-SRM (affinity purification–selected reaction monitoring) and AP-SWATH (affinity purification–sequential window acquisition of all theoretical spectra)) that can map dynamic interactomes through multiple time points and conditions[11,16,17,163].

Finally, the high-throughput interactome assay technique known as luminescence-based mammalian interactome mapping (LUMIER)[164] can measure the binary interaction between a luciferase-fused 'bait' protein of interest and its 'prey' protein under various conditions. The technique has been used to study tissue-specific rewiring in brain cells, in which bait proteins were altered to include specific exons to study the exon-dependent effects on protein–protein interactions[58].

individual. A potential disease-causing variant may be masked (cryptic) in phenotypically robust individuals but may have an effect in less robust individuals[81,82], and such variants might be hard to detect. Even Mendelian variants can show complex genome–phenome relationships. This was recently evidenced by an analysis of 589,306 genomes in which 13 adults harboured mutations for severe Mendelian conditions but showed no signs of disease manifestation[83]. Another example is the exome sequencing of 3,222 British adults of Pakistani heritage, which revealed that a higher prevalence of missing genes was not associated with a significantly increased level of clinical consultations or prescription rates[84]. This could indicate the existence of protective genomic features (recently reviewed in REF. 85) that are possibly associated with higher phenotypic robustness. Rare variants can thus confer phenotypic robustness towards a specific disease or comorbidity, rendering specific trajectories in the multimorbidity space more likely than others. Individuals that are phenotypically robust against comorbidities such as T2D retinopathy[86] and diabetic nephropathy[87,88] have been used to identify protective variants. This suggests that a protective variant in some individuals will, despite perturbations increasing the chance of comorbidity, retain them in the same state in multimorbidity space, whereas others will transition.

Readouts of phenotypic robustness. It has been proposed that phenotypic robustness is a general feature that is not specific to one disease, and that less robust individuals have a higher risk of multiple complex diseases[70,89]. Even though the sources that contribute to the phenotypic robustness spectrum, such as rare protective variants, remain unclear, it might be possible to measure the effect of lowered robustness indirectly. For example, factors that are associated with genome instability, such as recurrent rare copy number variants (CNVs) and microsatellites, can be readouts of lowered robustness. Several CNVs have been implicated in disease susceptibility as either Mendelian or complex disease variants[90]. However, rare CNVs that have been observed in a number of complex diseases, such as schizophrenia, autism, epilepsy, diabetes mellitus and mental retardation, among others, are also found in control populations[91,92]. As these CNVs are not necessarily disease-specific, their accumulation could be a consequence of decreased phenotypic robustness in some individuals. Another example is that disruption of the heat shock chaperone protein HSP90 can induce genetic instability, for example, by increasing the mutation rate of microsatellites[93], and can reveal cryptic variation[73,94]. Readouts of decreased phenotypic robustness, such as CNVs and microsatellites, can be specific to the individual, and thus identification at a genome-wide scale using next-generation sequencing analysis is required. Quantification of phenotypic robustness, as already commonly done for model organisms[70], could be harnessed as a dimension in the multimorbidity space for the stratification of individuals. Such stratification might improve the power to detect variants that only have effects in less robust individuals.

Rewiring

Rewiring has become a commonly used term, as it is an inherent feature of most (if not all) biological networks. Studies use the term generically almost as a synonym for 'change', often without further interpreting rewiring results. It is thus important to delineate the different approaches for measuring rewiring to acknowledge the different meanings of the term.

Defining rewiring. Rewiring is the inherent restructuring of interactions between biological units owing to conditional change. This can reflect either adaptation needs, such as a change in nutrient availability, or cellular processes, such as cell division. Studies have mainly measured rewiring for genetic and protein–protein interactions by mapping differential network changes across conditions. Whereas the rewiring of protein–protein interactions is more intuitive for understanding dynamic state changes[9,16], genetic interaction rewiring can help to detect differential gene essentiality between conditions[15]. For example, when measuring cancer cell rewiring over many time points it might be more intuitive to observe changes in the cellular interactome using mass spectrometry approaches (BOX 2) than carrying out genome-wide epistasis mapping for each time point. Importantly, a dynamic network is the ideal representation of continuous network states that rewire over time, whereas a differential network is an analytical approach to identify edge changes between static network states that are monitored through repeated measures (FIG. 4a). Therefore, differential networks cannot necessarily catch all dynamic shifts that occur in networks, for example, during disease

Microsatellites
Polymorphic DNA loci containing repeated nucleotide sequences of typically 2–7 nucleotides per unit.

Cryptic variation
Genetic variation that has little or no effect on phenotypic variation under normal conditions, but can generate heritable phenotypic variation when circumstances change.

Edge
An edge represents the interaction between nodes in a network. In biological systems an edge can represent a physical interaction between two proteins or the co-occurrence of two diseases.

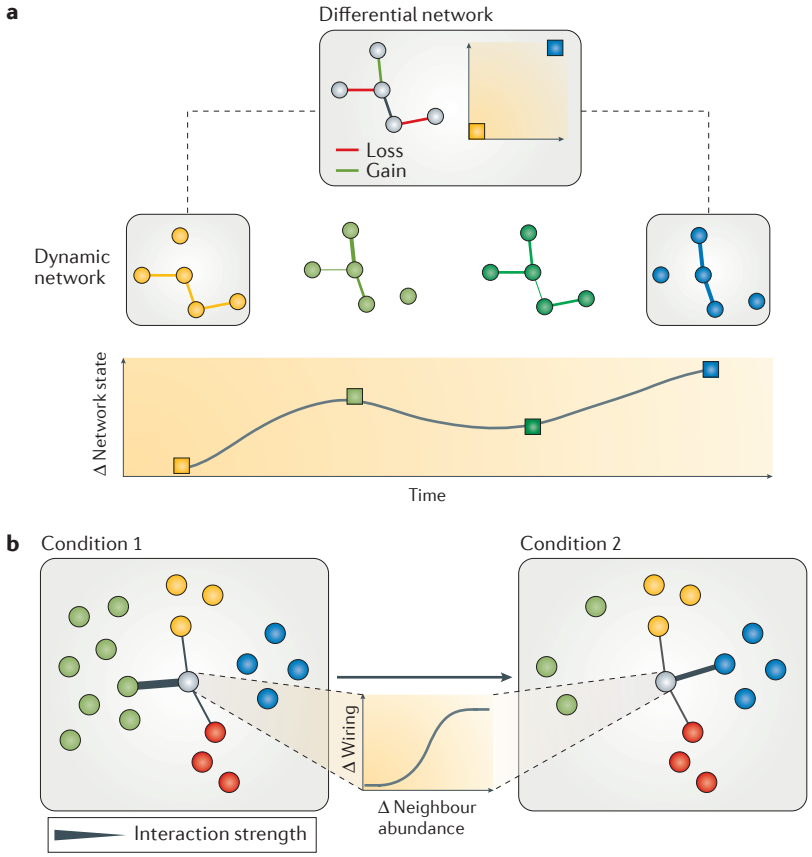


Figure 4 | **Rewiring in differential versus dynamic networks. a** | A dynamic network represents multiple network states that rewire continuously through time, whereas a differential network is an analytical approach to identify changes between two static network states. A differential network might not be able to detect all state changes between the two measured conditions. **b** | Protein networks can rewire to different states depending on the abundance of interacting proteins[107]. The figure illustrates that when the interaction partners (green) of a central protein (grey) are abundant, binding to these interaction partners will be favoured through competitive binding. But when the abundance of these interaction partners decreases, binding to alternative interaction partners (blue) will be favoured. This can be plotted as a sigmoid curve, where rewiring occurs through a non-linear switch-like mechanism, dependent on the change in partner protein abundances. This ensures that rewiring only occurs when a certain abundance threshold has been reached, providing robustness to the system.

progression. With the advent of real-time measures, such as wearables[95–97], and more sensitive technologies to measure personalized omics data, improved representations of dynamic networks become achievable.

In terms of robustness of a system (for example, a cell or an individual) rewiring can act differently to maintain robustness. As a response to perturbations, adaptational rewiring can retain the system in the same state in the multimorbidity space, whereas transitional rewiring can drive the system into a disease state. T2D is an example in which a modern lifestyle has led to transitional rewiring in multiple tissues and has thus resulted in a new disease state, such as insulin resistance in target tissues and loss-of-function of insulin-producing cells. In addition, accumulating evidence has suggested that inter-organ rewiring, which involves peptide hormones and cytokines, can play a part in insulin resistance by regulating pathways such as triglyceride synthesis, fatty acid catabolism and insulin action[98]. T2D is a case in which multiple organs progressively enter a vicious circle through destructive rewiring.

It is becoming more evident that rewiring is an inherent property of genes that are affected by disease-causing mutations and variants[19,99]. This can be used to prioritize and to detect novel network-based disease genes and biomarkers, supplementing traditional approaches, for which differentially expressed or highly mutated genes are the focus. More studies are moving from a 'guilt-by-association' principle, in which disease genes are assumed to locate in close proximity in static networks, to a 'guilt-by-rewiring' principle[100]. Rewiring can also be useful for analysing dynamic drug sensitivity[101], and drug-induced rewiring can push cells towards a wanted state, making them more susceptible to treatment[18].

Rewiring in context. Rewiring can only be properly interpreted in context, such as time-scale and condition. One study in *Saccharomyces cerevisiae* found that more than 70% of positive genetic interactions differed when testing 80,000 genetic interactions in two conditions (either untreated or methyl-methanesulphonate-treated)[15]. Also, different types of biological networks can rewire at different rates[102] — for example, transcription regulatory interactions rewire faster than protein interactions — which should be taken into consideration when either comparing rewiring studies or integrating network data[103]. Finally, it is important to underline what type of rewiring analysis is considered, given that rewiring can compare states within one system, for example, healthy versus diseased state, or between systems, for example the interaction partners of a protein in different tissue types[12]. Rewiring in one system thus answers questions related to how the system evolves, whereas rewiring between systems can emphasize differences across systems.

Rewiring of multifunctional proteins. The consequences of the rewiring of a protein depend on its position in a network[104]. Proteins with multiple interaction partners play an important part in dynamic networks, as they often have multiple functions. Such multifunctionality at the protein level can lead to multiple phenotypes, as described by model 3 (FIG. 3). A multifunctional protein may change partner affinities in altered conditions and thus may acquire different functions under different environmental or genetic conditions[11,61,105,106]. Additionally, a change in the abundance of interacting partners affects the rewiring of a multifunctional protein through competitive interactions[107], dictating its participation in different complexes or pathways. The concepts of rewiring and systems robustness also link to the stoichiometric properties of protein complexes, which are increasingly being studied using advanced proteomics techniques[11,17,108]. This has been shown for the RAS oncogene, the interactions of which with CRAF can be modified by the overexpression of its alternative interaction partner RIN1 (REF. 107). This finding suggests that the rewiring potential occurs via a non-linear biological switch-like mechanism, depending on the concentration of competing interacting partners (FIG. 4b). This idea is further supported by the finding that

Wearables
Personal portable devices that monitor the state of an individual.

sustained signalling can dictate rewiring and cell fate, whereas pulsed signalling cannot[109]. The effects of local rewiring in the cell can be thought to propagate changes through the system via changes in stoichiometry, thus leading to rewiring in other parts of the network.

Dynamic elements as network bottlenecks

The importance of proteins at central network positions in disease development has been underscored in studies of rewiring, robustness and pleiotropy. For example, a study in inflammatory diseases has shown how hub proteins rewire overlapping pathways at different disease states, thus accumulating multimorbidities[110]. These hubs might be pleiotropic genes that serve as a bridge to propagate the effect of a perturbation throughout the pathways that they span. Biological networks are often scale-free structures that are robust against random perturbations but are vulnerable towards perturbations that hit central nodes, such as a hub[111,112] or a bottleneck node[113] (BOX 1). Hub nodes have a high degree of edges, whereas bottleneck nodes have a high degree of intersections (high betweenness). Bottleneck proteins can be thought of as key connectors for functional and dynamic properties, and have been found to be highly important genes for cell survival (gene essentiality), even more so than hubs[113–115]. Rewiring of a hub gene can lead to the simultaneous rewiring of many edges, but does not necessarily lead to different cellular processes, whereas rewiring of a bottleneck gene can affect multiple processes.

Rewiring has recently been related to gene essentiality, showing that highly connected genes gain in essentiality when considering their network rewiring[104]. As bottleneck proteins are the main connectors in a network, they might be major rewiring transition points that are used by the cell for switching between states. Proteins at such positions are often disrupted in disease[116,117].

In addition, pleiotropy has been linked to gene centrality in a network. Gene knockout studies have shown that genes that are situated in central network positions tend to be enriched for pleiotropic effects and effects on phenotypic variation[118–120]. It has also been suggested that disease bottleneck proteins that link complex diseases can help to infer pleiotropy[121]. Thus, pleiotropic genes and their products may have network and wiring characteristics that place them in bottleneck positions. Additionally, products of pleiotropic genes tend to be more frequently multifunctional by having an increased number of protein–protein interactions and are more often present in more cellular components (subcellular structures and macromolecular complexes) compared with less pleiotropic genes[122].

As diseases tend to target genes that are in centralized network positions[117,123], dynamic transition states in molecular networks may represent the key to unravelling the aetiology underlying disease progression and comorbidities, and they are essential for strategies to identify novel drug targets. Strategies to manipulate rewiring[18] and robustness[124] to provoke network vulnerability and thereby to enhance chemo-sensitivity in cancer cells have already highlighted the importance of utilizing dynamic mechanisms as therapeutic targets in cancer. The same strategies can be applied to other complex diseases.

Nodes
In biological networks nodes are connection points, for example, of proteins, genes or diseases. They may or may not directly interact.

Bottleneck
A bottleneck node in a network has a high degree of intersections (high betweenness), meaning that it will often be a linker between different subnetworks.

Dynamics of immune-mediated disease

Analysing diseases from a single-disease perspective or from a single conceptual aspect ignores insights that might be detected only across multiple levels of disease dynamics. As a systems biology approach to investigating comorbidities is only just emerging, no real-life examples yet exist that unify all three network concepts. Nonetheless, immune-mediated diseases, which have many shared disease risk variants and diverse molecular and cellular phenotypes (reviewed in REF. 125), illustrate to some extent how analysing robustness, rewiring and pleiotropy together might be useful for acquiring a more complete picture of disease dynamics.

Immune-mediated diseases have very diverse phenotypes, even within each disease type, and their aetiology is not fully known[57]. It is thought that immune-mediated diseases arise due to the malfunctioning of mechanisms that keep the immune system of a host from attacking the host itself, such as for altered thymic selection, which is crucial for filtering out auto-reactive cells. Disease onset can occur years after pre-disease establishment, which makes it difficult to delineate the cause of a disease. However, some environmental triggers of immune-mediated diseases have been identified, such as gluten in Coeliac disease[126], smoking in rheumatoid arthritis[127,128] and Epstein–Barr virus in systemic lupus erythematosus (SLE)[129].

As noted above, immune-mediated diseases exhibit extensive overlap of disease risk variants and pathways, which is suggestive of pleiotropic effects. Recently, Ellinghaus *et al.*[43] concluded that clinical co-occurrences of low-inflammation conditions are associated with overlapping pathophysiological pathways that project to shared loci, and that patients with related syndromes are genetically different from other patients. The researchers carried out a pathway enrichment analysis of the candidate genes using 70,840 microarray expression profiles from humans, rats and mice, showing that comorbidities of at least some immune-mediated diseases share common risk variants that manifest at the pathway level. Pleiotropic variants have a role in essential immune-related processes, such as those related to the major histocompatibility complex (MHC), and can, in combination with rare variants, determine the specific disease phenotypes of patients[130].

Several CNVs and single-nucleotide polymorphisms have been associated with immune-mediated diseases, and immune-related genes are enriched for these variants[91]. Many of the detected disease risk variants have low effect sizes[131] but their molecular consequences are substantial[132–134]. Haplotypes of variants in immune-related genes such as *IRF5*, *IL2RA* and *CD6* have been shown to disturb the gene expression homeostasis of proximal genes in SLE, T1D and multiple sclerosis, respectively. In other words, these variants can manifest at the molecular level, suggesting that the molecular networks in immune cells undergo rewiring, propagating defects in the cell–cell network of the immune response. One example of such propagation is the association between the risk haplotype *UBE2L3* and increased protein levels of UBE2L3 in B cells, which promote the activation of nuclear factor-κB (NF-κB), leading to higher levels of circulating plasmablasts and plasma cells in patients with

SLE[135]. Another example is the *cis* expression quantitative trait locus (eQTL) *IFNB1*, which encodes the cytokine interferon-β. By stimulating monocytes with lipopolysaccharide (LPS), *cis* eQTL *IFNB1* shows both temporal and cell state-dependent activation, demonstrating that genetic regulatory variants can display functionality in a dynamic context-dependent manner[136]. These two examples highlight the importance of assessing the role of regulatory variants in a context-specific manner, taking both molecular and cellular phenotypes (immune cell abundances, cytokine levels, signalling responses and so on) into account.

More immune pathways are thought to rewire in a similar way through cross-regulation between the molecular and cellular responses. The rewired state may have a lowered phenotypic robustness towards certain environmental perturbations, increasing the risk of triggering an autoimmune response, but this is not necessarily sufficient to cause disease. This may be due to adaptational rewiring, in which counteracting processes maintain the homeostasis of the system. An example is the activation of WNT and β-catenin signalling in dendritic cells that has been observed in several inflammatory diseases[137–140]. Activation of these pathways *in vitro* and *in vivo* has been shown to suppress autoimmunity and inflammation by enhancing the survival of regulatory T cells and arresting effector T cell differentiation[138,139].

Because individuals are exposed to different combinations of common variants, rare variants, levels of genomic instability and environmental factors, each combination will result in a distinct rewiring, phenotypic robustness and disease phenotype and risk. The difficulty in detecting a systematic disease onset pattern might be due to the uniqueness of each person's phenotypic robustness. For example, smoking can trigger disease in some people, whereas others remain healthy. Another example of phenotypic robustness is found in spinal muscular atrophy (SMA): individuals with few CNVs in the *SMN2* gene, a functional substitute for the SMA-mutated disease gene *SMN1*, usually die before the age of two, whereas people with three or more CNVs survive to adulthood[141–143].

Immune-mediated diseases are thus an example of a group of diseases for which individuals can display a healthy phenotype, despite genetic perturbations, through complex interrelated rewiring at the molecular and cellular levels. Such rewiring can at times preserve an individual's position in the multimorbidity space, although environmental factors can still trigger the manifestation of disease over time, leading to the transition of an individual to a new position.

Challenges and future perspectives

Precision medicine initiatives are currently being initiated all over the world. These projects collect both electronic health data and molecular-level data for millions of individuals. The molecular-level data often reflect dynamic changes, such as changes in a cancer genome from a specific tissue over time. Many other types of temporal, molecular-level data will in the future be systematically produced using, for example, technologies from clinical proteomics or metabolomics, areas that seem to undergo the same cost-lowering industrialization as next-generation sequencing. Data generation will also increasingly include the healthy state, as data obtained from wearables and other devices will provide new opportunities for understanding transitions from health to disease[95].

As these approaches reach the clinic, the opportunities for producing dynamic models at the level of molecular network biology will drastically increase. This will be the case for *n*-of-1 studies[97,144], as well as for larger cohorts. As the amount of longitudinal health data available is also likely to grow exponentially, these data sets will allow for a more fine-grained characterization of comorbidity spectra and will provide opportunities for linkage to the molecular level. The possibilities for understanding the molecular underpinning of disease co-occurrences will therefore change considerably — both in depth ($n = 1$) and in breadth in terms of discriminating between more stochastic, non-recurrent disease development and repetitive, time-ordered disease trajectories. This will also change the landscape around clinical trials, which have traditionally enrolled patients in whom the crosstalk between diseases is either none or minimal. Huge population-wide data sets[68,145] will also allow for a much better differential quantification of comorbidities across ethnic groups. These are likely to be further related to regional reference genomes and will allow investigations of whether certain patterns of rewiring are specific to certain ethnic groups or regions.

Compared with several pre-human genome project estimates of the number of human protein-coding genes (ranging from millions in 1964 (REF. 146) to ~100,000 in 1990 (REF. 147)), the human species is now known to be a fairly gene-poor organism[148]. It is likely that many examples of multifunctionality and pleiotropy await discovery. These multifunctionalities may contribute to a better explanation of how human organismal complexity can be realized with relatively few genes (along with many other contributing factors from the regulatory domain)[149]. Higher levels of multifunctionality and pleiotropy will affect the way network biology models need to be designed in the future. With advances in quantitative proteomics[11,150–153] and single-cell technologies[154], which are expected to generate better dynamic data, more complete and even dynamic interactomes require a further concerted view on how robustness, rewiring and pleiotropy come together in frameworks that can rationalize comorbidities and their relationships at the molecular level.

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Competing interests statement
The authors declare no competing interests.

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