

Extra View

Phenotypic Activation to Discover Biological Pathways and Kinase Substrates

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functional genomics, synthetic dosage lethality, gene overexpression, dosage sensitivity, genetic interaction, synthetic genetic array, cyclin-dependent protein kinase, *Saccharomyces cerevisiae*

ABBREVIATIONS

SGA synthetic genetic array
SL synthetic lethality
SDL synthetic dosage lethality

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ABSTRACT

Observing the effects of gene perturbation on cells or organisms has long been a standard strategy in biological research. We developed a genome-wide gene overexpression library as a new tool for large-scale functional analysis in budding yeast. Previous large-scale genetic studies have focused on applications of the deletion mutant collection, which has arguably revolutionized the functional characterization of yeast genes. While extremely powerful, deletion mutant experiments are generally limited to the characterization of loss-of-function phenotypes. We have explored the potential for using the Synthetic Genetic Array (SGA) method, a platform for high-throughput genetic analysis, with a genome-wide "overexpression array", in which each strain on the array overexpresses a unique yeast gene. The overexpression array enables gain-of-function phenotypes to be examined on a large scale, providing a unique insight into gene function and a novel source of reagents for the global mapping of genetic networks and functional relationships amongst genes and pathways. Understanding the molecular bases of overexpression phenotypes should also shed new light on the nature of genetic dominance.

Phenotypic diversity can result from genetic variations such as single-nucleotide polymorphisms, insertion or deletion polymorphisms, variable numbers of repetitive sequences, and gene duplications, as well as large-scale copy number variations involving gains or losses of several kilobases of genomic DNA.¹ Such alterations can not only influence gene function but may also perturb genetic interactions, resulting in the rewiring of complex genetic networks. Alterations in gene interactions and underlying genome variation are thought to be central to both evolutionary adaptation and susceptibility to disease.

During the last five years, the focus of functional genomics has shifted from scrutinizing individual gene function to generating a global map of all genetic interactions within cells and organisms. The ultimate goal is to reveal how gene functions are integrated to regulate entire cellular processes and how perturbed genetic connections result in disease. Several disease states result from the disruption of multiple links within a network. In fact, for important biological processes, the cell exhibits remarkable resilience in the face of genetic perturbation, reflecting contributions of many genes to the same pathway or process. This 'buffering' by functionally redundant genes means that analyzing individual deletion mutants by gene knock-out or RNA interference, while incredibly valuable, can only provide a limited view of gene function in the cellular context.² Functional genomic studies in yeast, flies and worms, have made significant headway in identifying large numbers of genetic interactions and cataloguing genes as involved in particular bioprocesses.³⁻⁷ These systematic studies aim to find sets of genetic modifiers and will enable us to decipher the complete set of interactions for entire genomes. In this commentary, we highlight the value of examining gene overexpression to both uncover gene function and reveal regulatory networks in the context of sensitized genetic backgrounds. Genetic relationships related to gene overexpression will help us to understand genetic network architecture and thus will complement studies of gene deletion mutant combinations.

FROM UNDERSTANDING INDIVIDUAL GENE FUNCTION TO ENTIRE GENETIC NETWORKS

As noted above, an important step in understanding and therefore appreciating complex genetic networks has been the dissection of individual gene function by examination and classification of single-gene mutants based on their phenotypes. The ease with which the budding yeast *Saccharomyces cerevisiae* can be manipulated genetically has allowed the

systematic examination of genome-wide loss-of-function mutant collections, including gene deletion⁸ and titratable promoter alleles.⁹ Though we have gained much understanding from phenotypic assessment of these mutants, we are restricted by the lack of easily assayable or informative loss-of-function phenotypes. In fact, the yeast gene-deletion project showed that more than 80% of single-gene deletants exhibited no severe fitness defect under standard growth conditions.⁸ This could be due to genetic robustness¹⁰ and in this case the generation of double and multiple mutants can help address redundancy by revealing mutant phenotypes. Alternatively these genes may only contribute to fitness in specific conditions or at a particular developmental stage. A complementary and facile approach to the discovery of new phenotypes, used frequently for diploid systems, is the examination of gain-of-function mutants. Gene overexpression can lead to hyper- or neo-morphic effects, often due to misregulation of gene function. Understanding gene overexpression is important in deciphering the underlying causes of biological perturbations leading to disease, since several disease states are due to gene hyperactivation or amplification.

Exploiting gene overexpression phenotypes to uncover gene function. To examine gene overexpression phenotypes, we constructed an ordered array of 5280 yeast strains, each containing an inducible copy of an *S. cerevisiae* gene, covering >80% of the yeast genome. This overexpression array enables systematic analysis of gain-of-function phenotypes, including defects associated with the misregulation of essential genes. Using this array, we cataloged the spectrum of genes that affect cellular fitness when overexpressed by transferring the array to growth media to induce gene expression and examining strains for defects in colony formation. We found that, just as yeast is able to cope with single gene-deletions, it also has a tremendous tolerance for gene overexpression. Approximately 85% of the overexpressed genes had no obvious effect on growth in standard conditions. As with gene deletion, this tolerance may either reflect regulatory mechanisms which prevent the overproduction and hyperactivity of the protein products of these genes, or condition-specific gene functions. Will a similar degree of genetic tolerance be observed in other, more complex, organisms? Two studies of more than twenty phenotypically normal human beings revealed the existence of hundreds of large-scale copy number variations (LCVs) consisting of hundreds of kilobases of coding DNA present, on average, in three or more copies.^{11,12} These studies suggest that humans, like yeast, can cope with increased gene dosage; although LCVs are unlikely to be selectively neutral, given their large size and gene content. In fact, both studies found a significant association of LCVs with regions associated with human genetic disease or cancer.

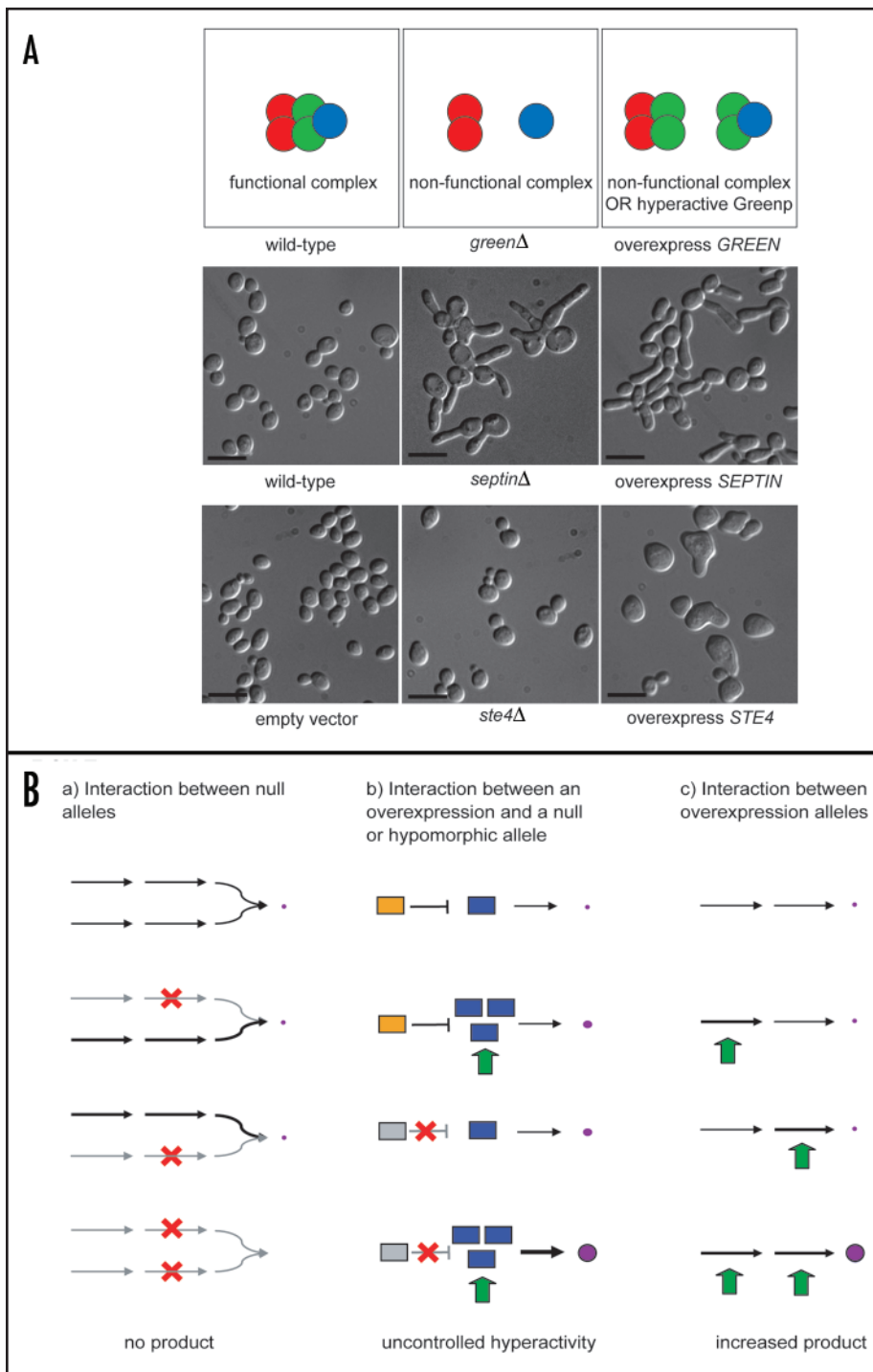
Although most genes do not cause a dramatic growth defect when overexpressed, we identified a set of 769 “toxic” genes that clearly compromise cellular fitness upon overexpression (15% of yeast genes). Our toxic gene set overlapped between 25% and 75% with other studies that searched for overexpression phenotypes using non-arrayed cDNA libraries^{13,14} or genomic libraries.^{15,16,17} In addition, our dataset overlapped 25% with a list of toxic genes generated by overexpression of a mORF (moveable ORF) library that was recently exploited to systematically examine protein glycosylation.¹⁸ We attribute our inability to achieve complete overlap with any of these datasets to: (1) an incomplete set of tagged ORFs in the libraries and arrays; (2) the various epitope tags interfering with protein function; (3) incomplete/mutated versions of nominally full-length constructs; (4) differences in the growth conditions used for the assays; and (5) comprehensiveness of the analyses.

The toxic mORF dataset, as well as ours, is highly biased towards involvement in particular bioprocesses including transport, cytoskeletal organization, and transcriptional regulation; this enrichment is also seen when only those genes represented in both datasets are considered. Both datasets are also enriched for genes annotated with specific activities, including transcription factors and transporters as well as, in the case of our dataset, signaling proteins such as phosphatases and kinases.

To ask if the prevalence of regulatory proteins reflects ectopic activation of a protein or pathway, we augmented our view of the toxic gene set by performing a systematic phenotypic analysis of all 769 strains in our collection. Our analysis involved microscopic assessment of cell morphology and measurement of DNA content to identify cell cycle defects. Of our 769 toxic strains, 184 (24%) displayed observable morphological abnormalities—this dataset allows experimental tests of hypotheses about mechanisms of overexpression toxicity. Notably, we used our phenotypic information to compare overexpression phenotypes to the deletion phenotypes reported for the same genes^{8,9,19,20} (see Fig. 1). In this analysis, we found that the majority (75%) of the morphologically defective overexpression strains had phenotypes different from the corresponding gene deletion (loss-of-function) mutant. These gain-of-function phenotypes can help assign functions to genes. For example, overexpression of *WHI4*, a gene for which deletion has no effect, led to a cell cycle arrest and allowed us to determine the role of this gene product in regulating progression through the G₁ phase of the cell cycle. Like *WHI4*, overexpression of 120 genes led to a detectable cell cycle halt and resulted in arrested growth. Consistent with this observation, the toxic gene collection is enriched for cell cycle regulated genes (i.e., those having periodic expression through the cell cycle), providing experimental evidence for a significant fitness cost associated with a failure to properly control their expression.²¹ This emphasizes the fact that gain-of-function phenotypes can result from misregulated or unscheduled expression as well as overexpression of genes.

Given the large representation of hyperactive signaling proteins (kinases, phosphatases, and transporters) within our set of toxic genes, we speculate that we could exploit this toxicity to search for enzyme inhibitors/small molecules that relieve growth inhibition. Likewise, identifying genetic suppressors (gene deletions or overexpressed genes) of this toxicity may help to dissect genetic pathways.

Addressing mechanisms of toxicity and dosage sensitivity. Understanding why cells are sensitive to the increased dosage of some genes might also shed some light on why decreased gene dosage may also produce a phenotype. Although a loss-of-function mutation in one allele of a diploid pair is recessive for most genes,^{22,23} it is nevertheless well established that certain genes show haploinsufficient phenotypes (i.e., inactivity of one allele in a diploid reduces fitness).²⁴ At least two general hypotheses have been put forward to explain haploinsufficiency: (i) according to the ‘insufficiency hypothesis’, the heterozygote produces less protein than is required to sustain normal growth;²³ in contrast, (ii) the ‘balance hypothesis’ posits that it is not the absolute level of a protein that is important but, rather, the relative dosage of proteins participating in a stoichiometric macromolecular complex.²⁵ A decrease or increase in the amount of a protein could be detrimental due to an imbalance in the concentrations of interacting subunits,²⁶ thus the balance hypothesis can explain both overexpression and haploinsufficient phenotypes. We note that, although classical genetics regards genetic dominance as a result of intralocus interactions, the notion of dosage balance is grounded on interlocus interactions.



The finding that genes encoding subunits of complexes are more likely to be haploinsufficient than other genes²⁵ is compatible with both hypotheses, but the balance hypothesis uniquely predicts that genes encoding subunits of complexes should also more frequently be toxic when overexpressed. Therefore, the overexpression array provided a unique opportunity to test this hypothesis. We failed to find: (1) an association between proteins causing toxic overexpression phenotypes and proteins annotated as components of complexes; or (2) a relationship between overexpression phenotypes and haploinsufficiency phenotypes, demonstrating that those genes sensitive to decreased dosage are not especially likely to be sensitive to increased dosage and generally have no bias towards participating in protein

complexes. This conclusion holds when data from recent comprehensive protein complex purification studies^{27,28} are used instead of literature-curated protein complexes (<http://mips.gsf.de>). Importantly, our data show that overexpression phenotypes typically reflect hyperactivity of overproduced proteins. As such, these phenotypes likely reflect a specific regulatory imbalance (see below), rather than a loss of gene function due to the disruption of protein complex stoichiometry. Thus, although the balance hypothesis may explain the dosage sensitivity of particular genes encoding subunits of protein complexes (e.g., tubulin genes),²⁹ it is unlikely to account for the majority of haploinsufficiency and overexpression phenotypes. Apparently, haploinsufficiency of genes encoding members of protein complexes could be better explained by insufficient protein production.²³

Although the balance hypothesis was originally proposed for stoichiometric macromolecular complexes, it was later extended to explain dosage sensitivity of certain signaling and regulatory pathways.³⁰ Simple mathematical models of informational pathways showed that overexpression could create problems by disrupting the concentration balance of proteins involved in the same pathway.³⁰ Therefore, it seems reasonable to hypothesize that the overrepresentation of genes encoding signaling proteins and transcription factors within our toxic dataset is the result of dosage imbalances in informational pathways. However, a systematic comparison of detailed theoretical predictions with dosage perturbation experiments will be needed to test the validity of this 'extended balance hypothesis'.

Mapping a new dimension of genetic interactions: The use of regulatory mutants to sensitize the cell to the phenotypic impact of gene overexpression. As discussed above, our systematic analysis revealed that the overexpression of most (85%) yeast genes does not cause an overt fitness defect in otherwise "wild-type" cells. More often, gene dosage effects will only become apparent in a specific genetic context that either reverses (dosage suppression)

or exacerbates (dosage enhancement) a mutant phenotype. For example, overexpression phenotypes may only be revealed in the presence of regulatory mutations that enhance gain-of-function effects. This might occur when the regulatory mutation reduces the activity of a protein that interacts with the overexpressed product, a phenomenon known as synthetic dosage lethality (SDL).^{31,32} This is one of many ways in which alterations in gene dosage might lead to disease, manifesting as an abnormal phenotype as a result of protein misregulation in a specific genetic context.

To identify toxic overexpression phenotypes specific to a particular genetic background, with the aim of exploring regulatory networks, we used the overexpression array for systematic SDL screening. We

Figure 1 (Page e3). (A) Comparison of overexpression phenotype and deletion mutant phenotype. The balance hypothesis predicts that an imbalance in the concentration of subcomponents of a protein-protein complex will be deleterious.²⁵ Therefore deletion or overexpression of a protein complex component should result in a loss of functional activity for the complex. We observed this for the septin complex for which either deletion or overexpression of components results in loss-of-function for the complex and aberrant hyperpolarized cell morphology (middle panels). In most instances, however, we found that overexpression results in a phenotype different from the known deletion mutant phenotype. In these cases, such as for the gene encoding the G protein beta subunit *Ste4* involved in mating (lower panels), overexpression results in a gain-of-function reflective of a specific regulatory imbalance rather than a disruption of complex stoichiometry. Size bar is 10 microns. (B) The role of synthetic genetic interactions in mapping genetic networks. The phenotype caused by two mutations can be substantially different from that endowed by either mutation alone; a phenomenon termed genetic interaction (nonadditivity of gene effects). One important type of genetic interaction is synthetic enhancement, in which one mutation increases the phenotypic impact of another. Although screening for synthetic enhancement (synthetic lethality or sickness) between null alleles may be used to uncover redundant pathways, synthetic genetic interactions between nonnull alleles, including overexpression, will enable the dissection of a different set of dimensions of the genetic network. (a) Interaction between null alleles (SL, synthetic lethality). Synthetic lethal interactions between null alleles typically identify genes participating in parallel or redundant pathways that converge on the same cellular process. Interactions can also result from impairment of two pathways with complementary, yet independent, functions that result in the same cellular output. Moreover, additive defects in multiple pathways contributing to a common biological process can also lead to synthetic lethal interactions if cell viability depends in a nonlinear way on the performance of the underlying biological process, e.g., there is a threshold effect (not shown). (b) Interaction between an overexpression and a null or hypomorphic allele (SDL, synthetic dosage lethality). Overexpression of a gene may have a strong effect only if another gene is inactivated (SDL). In contrast to synthetic interactions between two gene deletions, SDL relationships tend to involve genes participating in the same pathway or in opposing pathways. In general, SDL screening reveals regulatory interactions that compensate for gene overexpression. Typical SDL interactions can occur when: (1) one pathway becomes hyperactive and overwhelms a compensatory pathway; or (2) when a substrate escapes regulation, essentially mimicking a constitutively active pathway. Combining overexpression and hypomorphic alleles can also result in an enhanced dosage imbalance between the investigated genes. SDL screens could provide a straightforward way to identify those protein pairs for which the cell can tolerate a slight, but not a strong, imbalance. (c) Interaction between overexpression alleles. The combination of two silent overexpression alleles could also result in a strong phenotype in certain situations. Although such a screen has yet to be performed in a comprehensive manner, examples from the field of metabolic engineering suggest that the overexpression of genes within metabolic pathways might result in a synthetic interaction.⁴⁵ It is often observed that overexpressing genes for single enzymes in order to increase flux through a metabolic pathway has little success, but overexpressing several genes of the same pathway can significantly increase product formation. This is because the control of flux is usually distributed over numerous steps in a pathway and the degree of control of any given enzyme is therefore likely to be small.

merged our overexpression array with the synthetic genetic array (SGA) platform to allow introduction of every overexpression plasmid into any yeast deletion. The SGA method automates yeast genetics, allowing the introduction of marked alleles of a query gene (typically deletion alleles in the case of synthetic lethal (SL) screening and in our case) into any appropriately marked array of strains (typically the yeast deletion collection for SL screening and the overexpression array in our case) through a series of replica-pinning steps.^{3,33} In our case, the replica-pinning procedure effectively substitutes for thousands of yeast transformations and because the plasmids are arrayed, genetic interactors can be immediately identified by their position on the array, thus enabling rapid and systematic overexpression genetics. Briefly, a query strain possessing a hypomorphic allele of the gene of interest is mated to the haploid plasmid-based overexpression array of the opposite mating type. Diploids bearing both the marked allele and plasmid are selected and sporulated, and plasmid-bearing haploids are subsequently isolated by way of a mating type-specific marker.³⁴ Haploids are pinned onto media to induce expression and a difference in colony size between the induced and uninduced conditions indicates sensitivity to overexpression of the plasmid-borne gene. Results are compared to the effects of gene overexpression in a wild-type strain to identify overexpression phenotypes specific to the query allele.

As a proof of concept, we used the SGA method to introduce our overexpression array into a yeast strain lacking the cyclin-dependent kinase Pho85p. We reasoned that, since all known targets of Pho85p are negatively regulated by phosphorylation, overexpression of an unmodifiable substrate might be detrimental to the cell and result in an SDL phenotype. Using this approach we identified several known Pho85p targets, as well as a new target, the calcium-responsive transcription factor, Crz1p, which we confirmed as a genuine *in vivo* target of Pho85 using a variety of biochemical and genetic assays.

From our list of 65 potential Pho85p targets, we decided to focus first on Crz1p for two reasons: (1) Crz1p is one of a group of SDL hits in our Pho85p screen that overlapped with targets identified in

an *in vitro* kinase assay using proteome chip technology;³⁵ (2) Crz1p had not been previously linked to Pho85p, providing an example of a potential substrate identified through an unbiased genomics approach. In general, the integration of data generated by various independent approaches, such as SDL screening, *in vitro* kinase assays with proteome chips,³⁵ whole-cell kinase assays,³⁶ and physical interaction data^{27,28} should be hugely useful in target prioritization—a frequent stumbling block in efforts to identify *in vivo* substrates for kinases.

Our *pho85* screen validates the use of the plasmid-based overexpression array for the systematic SDL screening to identify kinase targets, and could be extended to all known protein kinases. Moreover, the same approach should be useful in identifying negatively regulated targets of other enzymes including phosphatases, acetyltransferases, and ubiquitin-conjugating and methylating enzymes. Traditional nonarray-based SDL screens have successfully identified targets of specific ubiquitin-mediated degradation machinery. For example, overexpression of *FAR1*, which encodes a cyclin-dependent kinase inhibitor, is lethal to a yeast strain defective in G₁ proteolysis,³⁷ while a strain with reduced activity of the anaphase promoting complex (APC) cannot tolerate high levels of its target Clb2p.³⁸

The full potential of high-throughput genetic screening has yet to be realized. For example, the integration of the SGA platform with high throughput microscopy to examine phenotypes other than growth, such as alterations in morphology or the localization of biomarkers, is likely to enhance our understanding of specific bioprocesses and facilitate our prediction of gene function.

SDL versus SL screening. Is information from SDL screens overlapping or complementary to that obtained from SL screens? In fact, these approaches are highly independent and lethality likely results from quite different routes. SL screens typically identify genes participating in parallel or redundant pathways that converge on the same cellular process—at least in the case of interactions involving complete gene deletions.³⁹ In fact, genes that act together in a single

complex or pathway tend to have buffering interactions with each other.⁴⁰ Synthetic lethality can also result from impairment of two pathways with complementary yet independent functions that result in the same cellular output. The collapse of distinct, non-compensatory pathways can also result in additive defects and retard a common biological process to which these pathways contribute (see Fig. 1B, part a).⁵ SDL relationships, on the other hand, tend to involve genes participating in the same pathway or in opposing pathways. Typical SDL interactions can occur when: (1) one pathway becomes hyperactive and overwhelms a compensatory pathway; or (2) a substrate escapes regulation, essentially mimicking a constitutively active pathway (see Fig. 1B, part b).

Interpretation of both SL and SDL data becomes complicated when protein complexes are considered. For example, genes encoding components of the same complex tend to have similar SL profiles, but fail to interact with each other.³ However, in some cases, SL interactions are seen between components of the same complex, if the activity of the complex is not completely impaired by the removal or reduced activity of one component.³ Genes encoding components of the same complex can also participate in an SDL interaction when the balance of the complex is perturbed due to a loss of stoichiometry.

Given the different kinds of genetic interaction addressed by SL and SDL screening, the lack of overlap between genes identified from two screens using the same query is not surprising. Among 65 SDL interactions⁴¹ and 53 SL interactions^{42,43} found with *pho85Δ*, only 2 genes, *BNII* and *RGDI*, were found to have both. We expect genes encoding components of large protein complexes will share more overlapping SL and SDL interactions due to considerable influence of these gene products on the stoichiometry of the complexes. For example, overlapping SL and SDL interactions for the yeast kinetochore⁴⁴ were more frequent (7% of all interactions) than those for the cyclin-associated kinase Pho85p (2% of all interactions) whose physical associations are likely to be more short-lived. However, the SDL screens performed using kinetochore-encoding genes asked a slightly different question: in what circumstances (i.e., genetic backgrounds) is hyperactivity of the kinetochore toxic? Measday et al. (2005) overexpressed a kinetochore-encoding gene in various deletion backgrounds, while our SDL screens addressed which genes are hyperactive in the absence of Pho85p by way of overexpressing genes in a *pho85* deletion background.⁴⁴ This difference may account for the variability in the incidence of overlapping interactions.

What does it mean when two genes are both SL and SDL? It likely indicates that the protein products encoding these two genes participate in the same complex or they function in opposing pathways to control a tightly regulated dynamic process such that balance between activation and inhibition is required. Our work highlights the utility of large-scale and systematic SDL analysis to address different types of genetic interactions from those uncovered by SL analysis, and this will undoubtedly complement reverse genetic screens aimed at locating genes or their products within regulatory networks.

WHAT LIES AHEAD

Much research activity in the communities studying yeast and other model organisms has shifted to large-scale genetic screening in an attempt to systemically define gene function and discover genetic interactions and pathways. We aim to understand how gene pathways

work together and how genetic interactions accommodate changes in the environment. The initial genetic network generated from the large-scale characterization of genetic relationships could be used to simulate the interruption of or enhanced flux of either signals or metabolic intermediates through a pathway and so predict effects (phenotypes). Ultimately, this might permit the prediction of susceptibility to disease based on knowledge of genotype and specific environmental factors (e.g., smoking or a high-protein diet). Predictions of this sort are very difficult because of the pleiotropic effects of both single and multiple mutations, and the subtle interplay between genes and environment. The polygenic nature of the problem greatly complicates the development of successful therapeutic strategies such that the era of 'personalized' medicine is still a long way off. Nevertheless, the integration of various kinds of genetic data (for example from screens examining gene overexpression and RNAi-mediated gene knockdown combinations) with reliable data on the physical interactions of proteins will greatly improve our understanding of genetic interactions in all their synergistic and antagonistic complexity. Our exploitation of gain-of-function phenotypes, together with the use of signaling mutants to increase the sensitivity of phenotypic analysis, provides an approach for the analysis of complex genetic regulatory networks. Extending this approach to metazoan systems will no doubt contribute to the modeling and understanding of entire regulatory networks in higher eukaryotes.

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