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A genetic approach to study polyubiquitination in *Saccharomyces cerevisiae*

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Abstract

Ubiquitin has seven lysines, all of which are used to generate polyubiquitin chains in the yeast *Saccharomyces cerevisiae*. While the biology associated with chains formed through lysines 48 and 63 is well studied, other chain types are more poorly characterized. We outline a methodology for using synthetic genetic analysis to examine ubiquitin mutants. Ubiquitin is encoded by four loci, necessitating several alterations to standard protocols, including the use of the SK1 strain background, which sporulates with very high efficiency. The methods described here could be used to examine other ubiquitin mutants, including those that do not support viability.

1. Introduction

Protein polyubiquitination is a highly conserved posttranslational modification that can alter the stability, localization, and function of substrates (Finley, Ulrich, Sommer, & Kaiser, 2012). This functional versatility arises from the structural diversity of ubiquitination. Indeed, lysine residues on substrates can be modified with single ubiquitins, known as mono-ubiquitination, or with a chain of polyubiquitin, consisting of ubiquitin protomers linked to each other through one of ubiquitin's seven acceptor lysines (K6, K11, K27, K29, K33, K48, and K63) or the N-terminal amino group of methionine 1 of ubiquitin. While all ubiquitin linkages exist in vivo in widely varying amounts (Xu et al., 2009), the functional significance of most remains enigmatic.

A large number of studies over the last 4 decades since the discovery of ubiquitin has elucidated some functions of specific ubiquitin linkage types. K48-linked chains were the first to be studied in detail and are considered the canonical ubiquitin chain that leads to proteasomal degradation (Finley et al., 1994). K63 linkages have been the subject of intense study following the recognition of their nondegradative functions in various signaling pathways, including the DNA damage response (Harper & Elledge, 2007; Hoege, Pfander, Moldovan, Pyrowolakis, & Jentsch, 2002; Spence, Sadis, Haas, & Finley, 1995; Wang & Elledge, 2007; Xu et al., 2009), protein trafficking (Lauwers, Jacob, & André, 2009; MacGurn, Hsu, & Emr, 2012), mitophagy (Ordureau et al., 2015), inflammation (Deng et

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al., 2000; Grabbe, Husnjak, & Dikic, 2011; Wang et al., 2001), and immune responses (Manzanillo et al., 2013). The importance of K11-linked polyubiquitin chains has only emerged in the last decade with the discovery of their degradative functions during mitotic progression in metazoans (Matsumoto et al., 2010). Proteomics studies suggest that K6, K27, K29, and K33 ubiquitin linkages are rare in cells (Dammer et al., 2011; Xu et al., 2009). Nonetheless, some pioneering studies have identified specific physiological functions of those linkages (Akutsu, Dikic, & Bremm, 2016). K6-linked chains, for example, are reportedly synthesized by the BRCA1–BARD1 E3 ligase to regulate the DNA damage response in a proteolysis-independent manner (Christensen, Brzovic, & Klevit, 2007; Nishikawa et al., 2004). These linkages, along with K27-linked chains, are also generated by the E3 ligase Parkin, which functions in mitophagy (Birsa et al., 2014; Geisler et al., 2010; Ordureau et al., 2015, 2014). In an interesting mechanism, K29-linked chains on the mRNA-binding protein HuR are recognized by UBXD8, an adaptor for the p97 ATPase, thereby promoting the release of HuR from its association with specific mRNAs (Zhou, Geng, Luo, & Lou, 2013). Finally, a study of post-Golgi protein trafficking provided an elegant example of how polyubiquitin linked through K33 can mediate a protein–protein interaction between Coronin-7 and Eps15 (Yuan et al., 2014).

A comprehensive examination of the functional significance of all ubiquitin linkage types is lacking and would serve to accelerate the pace of discovery of the physiologically important functions of ubiquitin chain types and the ubiquitin-proteasome system. This is particularly necessary for the poorly understood polyubiquitin chains linked through K6, K11, K27, K29, and K33 of ubiquitin. The analysis of genetic interactions is a powerful tool for establishing functional relationships between genes and uncovering novel gene functions (Braberg et al., 2013; Costanzo et al., 2010, 2016; Dixon, Costanzo, Baryshnikova, Andrews, & Boone, 2009; Sarin et al., 2004; Schuldiner et al., 2005; Tong et al., 2001, 2004). When two genes function in parallel or redundant pathways, mutating or deleting both genes in a cell can lead to a negative (synthetic) genetic interaction, wherein the double mutant strain has a more severe phenotype than each of the single mutant parent strains. In contrast, mutation of two genes that function in the same linear pathway would not lead to phenotypic enhancement in the double mutant (epistasis). A comprehensive study of such genetic interactions through synthetic genetic array (SGA) analysis is typically performed by crossing individual strains, each deleted for a single gene, to a large arrayed collection of strains, each with a deletion of a different gene. By mating, and subsequently selecting for the double mutant, one can analyze the growth rate of that double mutant relative to not only each of the single mutants but also to all other double mutants with that deletion. Data generated from this analysis provides two types of highly useful data. First, it provides the interaction data itself; that is, it tells one which deletion mutants cause either synthetic sickness with, or are rescued by, other deletion mutants. Second, clustering analysis allows one to determine which genes have similar sets of interactions. For example, if loss of a particular ubiquitin linkage causes defects similar to deletion of gene X, it suggests that the ubiquitin linkage and gene X both either positively or negatively function in the same pathway.

Here we describe a high-throughput method to uncover genetic interactions of individual ubiquitin linkage types by combining lysine-to-arginine (K-to-R) ubiquitin mutations with

single gene deletions. While we have used this approach to examine ubiquitin linkages, it could similarly be used to examine ubiquitin's hydrophobic patch, phosphorylation, or other domains or surfaces. A general approach to validate novel genetic interactions arising from the screen is also detailed. Aside from its utility for the study of ubiquitin in yeast, we propose that the methodology we have developed will prove broadly applicable to the analysis of complex relationships between genes, including multigenic redundancies, in many biological pathways.

2. Strain engineering

Previous high-throughput genetic interaction screens have examined the synthetic phenotype of two (Braberg et al., 2013; Collins, Roguev, & Krogan, 2010; Costanzo et al., 2016; Schuldiner et al., 2005; Tong & Boone, 2006; Tong et al., 2004), or in a few cases three (Haber et al., 2013), mutations. The genetic analysis of ubiquitin, however, is challenging due to the fact that ubiquitin is encoded at four genomic loci, *UBI1–4*, in *Saccharomyces cerevisiae*, thus requiring an examination of five loci to probe genetic interactions with a given gene deletion. At *UBI1*, *UBI2*, and *UBI3*, ubiquitin is expressed as a fusion to ribosomal proteins RPL40A, RPL40B, and RPS31, respectively. An additional five copies of ubiquitin are encoded at *UBI4* as head-to-tail fusions. In all cases, the ubiquitin fusion proteins are proteolytically processed to release mature ubiquitin monomers that are competent to be conjugated to lysine residues of proteins. The analysis of genetic interactions normally requires the deletion of two genes in the same cell. In contrast, to study the genetic interactions of ubiquitin mutants, along with deletion of a gene of interest, all four ubiquitin genes must be modified to express the same desired ubiquitin variant (or no ubiquitin at all) while also preserving expression of the ribosomal proteins encoded at *UBI1*, *UBI2*, and *UBI3*. The relatively large number of loci that must be modified to carry out genetic analyses of ubiquitin mutants required key optimizations of the conventional SGA protocol described later.

Most genetic interaction screens have utilized the S288C yeast strain, which exhibits poor sporulation efficiency. In those studies, the low efficiency of sporulation was acceptable because only a total of five loci were under selection: the two loci of interest and three haploid selectable markers necessary to generate the desired haploid double mutant cells. In contrast, to study ubiquitin, eight total loci must be selected to generate haploid cells that carry modified *ubi1–ubi4*, a given gene deletion, as well as all necessary haploid selectable markers. The efficiency of sporulation becomes a limiting factor, as an exceedingly small percentage of spores will have the final desired genotype. The SK1 yeast strain has a naturally high sporulation efficiency (about 92%), a stark contrast to the 12% efficiency exhibited by S288C (Ben-Ari et al., 2006). This key advantage motivated the generation of all the ubiquitin mutant strains and a novel single gene deletion library in the SK1 strain background.

While the SK1 strain provided a crucial advantage over S288C, two further modifications were made to decrease the number of loci under selection, thereby increasing the percentage of spores with the final desired genotypes. First, the *UBI1* locus was replaced with a construct expressing Rpl40A, a ribosomal protein, under the control of the constitutive GPD

promoter in the entire SK1 deletion library and all K-to-R ubiquitin mutant strains to which the deletion library will be crossed. Because the locus is equivalently modified in the ubiquitin mutant strains and gene deletion array, no marker was necessary (see Section 2.1). Second, unsporulated diploid cells are a significant source of background in SGA studies. This is eliminated with two separate selections for haploid cells. The high sporulation efficiency of SK1 leads to significantly fewer unsporulated diploid cells, thus permitting the omission of one haploid selection step, *lyp1* selection. Ultimately, in the SK1 ubiquitin SGA, six loci were subject to selection: three ubiquitin loci (modified *UBI2-UBI4*), one gene deletion, and two haploid and mating type selection markers.

2.1 Deletion of ubiquitin sequences from *UBI1* and *UBI2*

Haploid strain DS1 of the genotype *MAT α his3 ura3 CAN1 ubi1 ::LoxP-GAL1pr-Cre-URA3-LoxP-GPDpr-RPL40A* was cultured in rich liquid media overnight and spread on a synthetic media plate lacking uracil to generate a lawn (Fig. 1). The cells were then pinned into a 384 colony format to allow mating to the SK1 gene deletion library on YPAD plates using a Singer Instruments Pinning Robot (Robot: Singer Instruments PLU-001; Pinning pads: Singer Instruments RePad384A; Plates: Singer Instruments PlusPlates). The GAL1pr-Cre construct (*GAL1pr* is the galactose-inducible *GAL1* promoter; Cre is a recombinase that cleaves at *LoxP* DNA sites) was amplified from yeast genomic DNA because it could not be maintained in bacteria, likely due to promoter leakiness. The mating mixes were transferred to C-uracil+G418 plates to select for diploids; G418 (Geneticin) allows selection for the *kanMX* marker used to make the library gene deletions, while the absence of uracil selects for the *URA3* marker from DS1.

Diploids were then pinned onto sporulation plates and cultured for 2 days. Haploid recombinants were selected first on C-uracil for 24h, then on C-uracil containing G418 for 2 days. To induce the Cre recombinase, the haploids were plated on 2% galactose-containing plates for 2 days. The positions of the *LoxP* sites in the construct led to deletion of the *URA3* marker and Cre recombinase. The cells were transferred to plates containing 5-fluoro-orotic acid (FOA) and incubated at 30°C for 24h to select against cells still carrying the *URA3* marker (Boeke, LaCrute, & Fink, 1984). This negative selection was performed a total of three consecutive times to ensure deletion of *URA3*. The final plates were replicate-plated onto C-uracil plates to verify loss of *URA3*. To confirm the successful recombination event, the modified *ubi1* locus was amplified by PCR and sequenced. No significant changes in ubiquitin levels or doubling time were observed following deletion of *UBI1* (Fig. 2), as has been previously reported (Hanna, Leggett, & Finley, 2003).

At the modified *ubi2* locus, two copies a cassette driving expression of the ribosomal protein Rpl40 from the GPD promoter were inserted and marked with the hygromycin resistance gene. Cells with the modified *ubi2* locus exhibited a normal doubling time.

2.2 Design of ubiquitin loci encoding ubiquitin variants

The endogenous *UBI3* locus encodes a fusion protein consisting of ubiquitin fused to Rps31, a ribosomal protein. Deletion of *UBI3* with a construct expressing a triple ubiquitin fusion protein along with expression of *RPS31* from a separate GPD promoter resulted in a very

slow doubling time (7 h). Multiple modifications to *UBI3* were attempted to ameliorate the observed growth defect. Increasing the expression of *RPS31* by including in the construct up to five copies of *GPDpr-RPS31* could not decrease the doubling time below 3h. It has been suggested that ubiquitin acts as a folding chaperone for Rps31. To address this possibility, the triple ubiquitin sequence was fused to *RPS31*, thereby leading to expression of a triple ubiquitin-Rps31 hybrid protein (Fig. 1). In addition, a *GPDpr-RPS31* cassette was included in the *URA3*-marked construct, although this may not be required to produce sufficient levels of Rps31. The resulting strains exhibited growth rates similar to wild type.

To ensure sufficient ubiquitin levels, we placed two additional copies of modified ubiquitin at the *UBI4* locus. The *UBI4* locus encodes five copies of ubiquitin expressed as single polypeptide that is proteolytically processed to release free ubiquitin. *UBI4* was replaced with a two copies of a construct expressing ubiquitin from the constitutive GPD promoter, including the C-terminal asparagine found in endogenous *UBI4* that is proteolytically processed to release mature ubiquitin (Finley et al., 2012; Ozkaynak, Finley, Solomon, & Varshavsky, 1987). This locus was marked with a Nourseothricin (NAT) resistance cassette.

The constitutive GPD promoter (which drives the *TDH3* gene) was used at all loci, as it resulted in the highest level of ubiquitin expression relative to other tested promoters, including *TEF1*, *HYP2*, *PYK1*, and *PDC1* (Partow, Siewers, Bjørn, Nielsen, & Maury, 2010). To control for the potential effects of altered ubiquitin levels, a strain expressing low levels of ubiquitin was constructed by replacing *UBI4* only with the NAT resistance cassette. In this strain, ubiquitin is just expressed from the modified *ubi3* locus.

Because lysine 48 of ubiquitin is essential, strains expressing the K48R mutation had to be supplemented with wild-type ubiquitin. In these strains, two of the ubiquitins in the triple ubiquitin fusion encoded at *ubi3* are mutated at K48. At *ubi4*, both ubiquitins are mutated at K48. Strains expressing an ubiquitin mutated at K48 and another lysine had one copy with the double mutant ubiquitin (K29R K48R, for example) and two copies of single mutant ubiquitin (K29R, for example) at *ubi3*. Exclusively double mutant ubiquitin was expressed at *ubi4* in the K48 double ubiquitin mutant strains.

2.3 Construction of strains expressing ubiquitin variants

The endogenous *UBI2-UBI4* loci were replaced with the constructs detailed earlier as follows: A diploid strain homozygous for modified *ubi1* was transformed with the engineered *ubi2* cassette. Transformants were selected by their resistance to hygromycin and then transformed with the panel of ubiquitin mutant *ubi3* constructs. Transformants were selected upon growth on plates lacking uracil. These diploid *ubi1 ubi2 ubi3* mutants were sporulated to generate haploid cells of mating type alpha carrying all three modified loci (hygromycin resistant, Ura⁺). In parallel, the panel of mutant ubiquitin *ubi4* constructs was used to transform diploid SK1 cells. Following sporulation of transformants that were resistant to nourseothricin and of mating type α were crossed to the haploid *ubi1-3* mutants. Diploids were sporulated to generate haploids of mating type alpha that carried all modified ubiquitin loci, which were genotyped by PCR.

3. Modification of the conventional SGA method to establish an SK1 4-marker ubiquitin SGA protocol

The 4-marker SGA was largely carried out as has been previously reported for conventional 2-marker SGA screening in the S288C strain. As detailed SGA protocols have been published elsewhere, a brief overview of the 4-marker ubiquitin SGA method is provided here with attention to notable modifications to the conventional SGA protocol that were key to establish a robust SGA process in the SK1 strain background and permit the simultaneous analysis of multiple mutant loci (Fig. 1B).

3.1 Drug concentrations

G418—Selection with G418 is required to select for the specific gene deletion that comes from the SK1 deletion library. Previous SGA studies in the S288C strain used up to 200mg/L of G418, although 100mg/L was found to be sufficient (Collins et al., 2010). The SK1 strain requires higher concentrations of the drug. Careful titration of G418 revealed that a concentration of 350mg/L is optimal for SGA screening using the SK1 strain.

Canavanine—Resistance to canavanine is a recessive marker that is useful to kill unsporulated diploid cells. Previous screens in S288C have used canavanine at a concentration of 50mg/L (Wang & Elledge, 2007). This concentration of canavanine proved excessive when combined with other selection steps (e.g., 350mg/L G418) for SK1 as the cells showed obvious growth defects. Titration of canavanine was done in combination with other drugs in the screen. The much lower concentration of 10mg/L was sufficient to kill diploids and did not cause obvious growth defects in *can1* haploids. Nonetheless, 50mg/L of canavanine was used for the initial haploid selection following sporulation to ensure complete selection against unsporulated diploids, but the lower concentration of 10mg/L was included in all subsequent selection plates.

Nourseothricin (NAT)—100mg/L as used in conventional S288C SGA screening.

Hygromycin—During conventional SGA screening, hygromycin is not normally used. In this screen, *ubi2* is marked with a hygromycin-resistance marker. The drug was successfully used at the commonly used concentration of 200mg/L.

3.2 Sporulation

Sporulation of the SK1 strain is about 92% efficient, compared to the approximately 12% sporulation efficiency of S288C strain (Deutschbauer & Davis, 2005). The poor sporulation efficiency of S288C necessitates a long sporulation time of 5 days. In contrast, sufficient sporulation of SK1 is obtained after only 2 days at 25°C.

3.3 Overview of the 4-marker SGA workflow

Biological replicates of the screen were carried out on different days. Each biological replicate also had three technical replicates. Lawns of each query strain were mated to the SK1 deletion array using the Singer Pinning Robot system for 1 day at 30°C. Diploids were selected on plates lacking uracil and containing NAT, G418, and HYG. The colonies were

then transferred to sporulation plates for 2 days at 25°C. Haploids of mating type α were selected on plates lacking histidine and containing canavanine. Haploid mutant selections were performed in the following order: (1) C-uracil plates (*ubi3*), 200mg/L hygromycin (*ubi2*); (2) 100mg/L Nourseothricin (*ubi4*); and (3) 350mg/L G418 (single gene deletions). To ensure effective mutant selections, in each step, the previous selection condition was also included. Of note is the fact that quality control analysis revealed that the modified ubiquitin loci must be selected for prior to the *kanMX*-marked gene deletion to achieve precise mutant selections. The final plates were grown at 30°C for 48h and then photographed.

4. Analysis of data quality

4.1 Calculation and characterization of genetic interaction scores

Using established SGA protocols and methods, colony sizes were recorded and normalized to calculate genetic interaction scores (*S*-scores) (Collins et al., 2010; Collins, Schuldiner, Krogan, & Weissman, 2006). *S*-scores were averaged among replicates to obtain the final SGA dataset. As has been observed in previous SGA studies, and given that genetic interactions are rare, the *S*-scores in the 4-marker SGA were centered around zero, with about 53% of scores being negative and 47% positive values and 95% of scores lying within two standard deviations of the mean *S*-score of -0.21 (Fig. 3). Strong negative *S*-scores were more common than positive *S*-scores. The strongest *S*-score in the screen was -20.97 while the largest positive *S*-score value was 6.68 (Fig. 3).

4.2 Quality determination

To determine the reproducibility and robustness of the data, the Pearson correlation coefficient between two biological replicates of the screen was calculated and found to be within the range of previous SGA screens, albeit somewhat lower than some SGA studies. This fact is an important consideration for the design and interpretation of SGA screens. In contrast to many previously reported SGA studies which analyze the genetic interactions of hundreds or thousands of functionally related gene deletions in a symmetrical fashion, the 4-marker SGA described here consisted of only 17 query strains that were mated to an unbiased array of deletion strains. Both of these peculiarities of the screen have the effect of diminishing the number of true genetic interactions and increasing noise in the dataset relative to other SGA studies, thereby leading to a slightly smaller Pearson correlation coefficient.

An additional comprehensive approach to evaluate data quality was therefore employed. Genes whose protein products physically interact are more likely to have similar genetic interactions due to their related biological functions. The enrichment of genetic interaction similarities was compared between gene pairs that are known to interact physically and those that do not have reported physical interactions. Proteins were considered to physically interact if they had a 'PE' score greater than 2 in Collins et al. (2007). As expected, the genetic interactions of physically interacting gene pairs are significantly higher than among noninteracting gene pairs. This is striking result when the relatively small size of the ubiquitin SGA is taken into account. Although the genetic interaction profiles of gene deletions in the ubiquitin SGA include only 17 scores, the screen is similarly enriched for

genetic similarity within physically interacting gene pairs as the published chromosome biology epistasis map (eMAP) (Collins et al., 2007), wherein the genetic interaction profiles contain over 700 scores and is biased for genes with known related functions.

Unbiased hierarchical clustering orders the mutant strains relative to each other based on the similarity of their genetic interactions. Typically, hierarchical clustering is visualized with a dendrogram or tree diagram, where the most similar strains are placed on branches next to each other. Genes that function in the same pathway or have similar functions are expected to have similar genetic interactions due to their related biological function. They are therefore more likely to cluster in the dataset. Evaluation of the degree to which known gene modules is clustered in the SGA can serve as a qualitative measure of the robustness and biological significance of the data. The ubiquitin SGA dataset was clustered using the open source software Cluster 3.0 (Settings: uncentered correlation, average linkage), which is freely available for download at <http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv> for use with Windows, Macintosh, and Linux operating systems.

5. Identification and characterization of genetic interactions

Large screens such as the one described here uncover thousands of candidate genetic interactions. Comprehensive approaches to estimate the quality and biological significance of the dataset as whole are described in Section 4. It can be an overwhelming endeavor to interpret the biological significance of the myriad of interactions for each mutant in the screen. Approaches to extract functional and biological significance from genetic interaction datasets are proposed later.

5.1 Identification of hits specific to individual ubiquitin lysine mutants

We were most interested in genes whose deletions had genetic interactions with individual ubiquitin lysine mutants, rather than with multiple lysines. To identify those gene deletions, all the S -scores for each single ubiquitin lysine mutants were compared to the average S -scores for all other ubiquitin mutants not carrying the individual lysine mutation being analyzed. For example, the S -scores of the K11R ubiquitin mutant were compared to the average S -scores of every other ubiquitin mutant not carrying mutations of K11 (K6R, K27R, K33R, etc.). This analysis revealed genes whose deletions led to genetic interactions specifically with the K11R ubiquitin mutation.

5.2 Functional characterization of individual genetic interactions

A powerful method to rapidly characterize the genetic interactomes of the ubiquitin mutants is the use of Gene Ontology (GO) enrichment analysis. An arbitrary cutoff of S -scores of -2 or smaller was applied, and all gene deletions having S -scores meeting that criterion for each ubiquitin mutant were analyzed for the enrichment of biological process GO terms (BP-direct) using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool which is freely available and can be accessed online at <https://david.ncifcrf.gov/> (Huang, Sherman, & Lempicki, 2009a, 2009b).

5.3 Functional analysis of genetic interactomes

The analysis of individual genetic interactions can be a powerful approach to generate hypotheses about the functions of ubiquitin linkage types. For example, a negative genetic interaction between a gene deletion and a specific ubiquitin lysine mutant would point to functional redundancy between the gene and the ubiquitin linkage type. However, the interpretation of isolated genetic interactions can be complicated due to pleiotropy and nonspecific effects of the mutations being studied. Thus, an approach that takes into account all the genetic interactions for a particular ubiquitin mutant in the screen would provide a more comprehensive functional analysis. The deletions of genes that function with a specific ubiquitin linkage in a given pathway are likely to have genetic interactions that are similar for that linkage type. For example, if K6 linkages activate pathway X, then K6R mutants would be lethal with the same genes as would deletions of genes in pathway X. Thus, the genetic interactions of K6R and pathway X genes would be highly correlated.

To identify gene deletions that had similar genetic interactions to the ubiquitin mutants in the 4-marker SGA, the genetic interactomes of the ubiquitin mutants were compared to a large yeast genetic interaction network containing comprehensive interactomes of thousands of gene deletions in yeast (Ryan et al., 2012). Briefly, the genetic interaction scores of the 4-marker SGA were merged with the dataset S4 from Ryan et al. (2012). Correlation coefficients between the genetic interactomes of each ubiquitin mutant strain and the 4456 mutant strains in Ryan et al. (2012) dataset were then systematically calculated. Only gene pairs for which data existed in both datasets were included in the calculation of correlation coefficients. The number of gene pairs included in the calculation of each coefficient was recorded for subsequent analysis due to the fact that correlation coefficients derived from larger numbers of gene pairs are expected to be more reliable.

6. Validation of genetic interactions

Prior to investing time and resources to study the molecular basis of any given genetic interaction, it is advisable to independently validate genetic interactions of interest. While high-throughput methods to independently validate interactions are possible, a low-throughput approach to validate a small number of genetic interactions of interest is outlined later.

6.1 Mating

To recreate the double mutant strains (carrying a gene deletion and ubiquitin mutant) that exhibited a genetic interaction of interest in the screen, ubiquitin mutant strains were crossed to the relevant gene deletions picked from the SK1 deletion array. This was done by mixing roughly equal amounts of cells onto rich plates, using toothpicks (supplemented with 2% dextrose), and incubating at 30°C overnight. Importantly, prior to mating, cells were taken directly from frozen stocks and grown at 30°C on YM-1 plates supplemented with 3% glycerol to avoid petites, which occur at a high rate in the SK1 strain. As in the screen, single colonies were not used for mating.

6.2 Sporulation

The high efficiency of sporulation exhibited by the SK1 strain obviates the need to select for diploids when looking at individual genetic interactions (assuming sufficient mating occurs) and also simplifies the sporulation protocol relative to other common laboratory yeast strains. Following overnight incubation of mating mixes as described earlier, a small patch of the mixture was transferred to 2mL of sporulation media. Sporulation cultures were incubated at 23°C with light rotation. Spores appear in the cultures relatively quickly, and in sufficient numbers to allow tetrad dissection in as little as 16h in our experience. However, usually, the cultures were incubated at 23°C for 3 days prior to tetrad dissection to facilitate the dissection process.

6.3 Tetrad dissection

The efficiency of sporulation was determined by examining the cultures under a light microscope. Tetrads are easily identifiable by their characteristic shape, which includes four small spores attached to each other, usually in tetrahedral organization. Two hundred microliters of each sporulation culture were centrifuged on a tabletop microcentrifuge at maximum speed for 30s. One hundred and eighty microliters of supernatant were removed, and the cell pellet was resuspended in the remaining 20 µL of medium. Two microliters of zymolase (US Biologicals Cat. no. Z1004 at 20mg/mL stock) were mixed with the cell suspension to digest the ascus that contains the four spores. The mixtures were incubated at room temperature for 10min. One milliliter of cold water was then added to halt digestion. Thirty microliters of the mixture were spread in a straight line across the middle of a YM-1 plate supplemented with 2% dextrose. The liquid was allowed to dry prior to manually dissecting tetrads. Dissection plates were incubated at 30°C for 3 days.

The final haploid spores needed to carry all the modified ubiquitin loci and a gene deletion (when analyzing double mutants). In addition to those loci, a mating type and haploid selectable marker also segregated in the crosses. To simplify analysis and facilitate downstream experiments, only spores carrying all modified ubiquitin loci and the gene deletion (when desired) were analyzed, and any spores also carrying the mating type and haploid selectable marker cassette (disruption of the *CAN1* locus with the spHis5 driven by the Mat a-specific *STE2* promoter) were discarded. A small percentage of spores carried the desired genotype, which was determined by replica-plating onto appropriate plates. Importantly, the dissection plates were also replica-plated onto glycerol-containing YM-1 plates to discard petites.

Because such a small percentage of spores have the desired genotypes, the work of dissecting tetrads can rapidly become overwhelming and tedious. We therefore took an alternative approach when validating multiple interactions rapidly. We generated diploid strains homozygous for the modified ubiquitin loci engineered for the screen by mating strains of the opposite mating type and selecting diploids. Because the parental haploid ubiquitin mutant strains used were isogenic (except for the mating-type locus), diploids were picked manually, based by their distinctive appearance under a light microscope, and confirmed by their lack of an ability to mate to mating type-tester strains. Gene deletion cassettes were then amplified by PCR from the SK1 deletion array and used to transform

desired homozygous ubiquitin mutant diploids. Mutants were selected on YM-1 plates containing 2% dextrose and the appropriate selection drug (usually G418). *UBI4* is known to be essential for meiotic progression. Surprisingly, the engineered ubiquitin loci, which express high levels of ubiquitin under the control of the GPD promoter, were unable to support meiotic progression. This was true even for diploids expressing wild-type ubiquitin. To allow the diploids to undergo meiosis, they were transformed with a 2- μ m plasmid, FMGp25, which carries the *UBI4* ORF cloned into PRS423, which is marked with *HIS3*. Following sporulation, the cells rapidly lost the plasmid in nonselective media, thus generating strains expressing only the integrated ubiquitin allele. Plasmid loss was verified by replica plating to plates lacking histidine.

6.4 Spot dilution assays to validate genetic interactions

Genetic interactions are defined as a deviation from the expected double mutant phenotype, which is estimated by taking the product of the single mutant parent phenotypes. In the SGA protocol, the phenotypes of all the single mutant strains are not actually measured as that would be labor-intensive and introduce significant error. Instead, the phenotypes of the single mutant parent strains are estimated by taking into account the growth phenotypes of all double mutant strains in the screen carrying any given mutation. This estimate is valid due to the empirical fact that genetic interactions are rare. For example, the phenotype of the K11R ubiquitin mutant can be estimated by determining the typical phenotype of the thousands of double mutants in the screen carrying a gene deletion along with mutation of K11. When validating a specific genetic interaction, however, it is possible to compare the double mutant strain of interest to the single mutant parent strains.

For any given genetic interaction of interest, the appropriate double mutant strain, carrying all the mutant ubiquitin loci and the specific gene deletion, was generated as described earlier. Likewise, the corresponding single mutant strains (i.e., a strain carrying only the mutant ubiquitin loci, and a strain carrying the gene deletion and modified ubiquitin loci expressing wild-type ubiquitin) were generated as described earlier. To compare the growth of the double mutant strain to that of the single mutant, overnight cultures were grown at 30°C. The optical density of the saturated cultures was then measured, and their densities normalized to an OD₆₀₀=1. Fivefold dilutions were then generated, and small volumes (3–6 μ L) were transferred onto agar plates using a multichannel micropipette. Plates were then allowed to dry prior to placing in a 30°C incubator. Images of the plates were recorded by scanning the plates on a Hewlett–Packard scanner every 24h up to 3 days after initial plating.

An important consideration when validating genetic interactions from a screen such as the 4-marker ubiquitin SGA described here is the fact that the screen is carried out on selective plates lacking certain nutrients and containing drugs. Even though the final cells carry all necessary markers to survive and grow on the selective media, the growth conditions are inherently stressful. Therefore, some true genetic interactions will not be detected when cells are grown on rich YM-1 plates with dextrose (2%) at 30°C. To rule out any given genetic interaction as being a false positive, it is necessary to reproduce the conditions of the screen as faithfully as possible. When validating genetic interactions, it is often convenient to plate the fivefold dilutions of the strains of interest on several plates containing different nutrients

or drugs, and also incubating various plates at different temperatures (30°C and 37°C). An example is shown in Fig. 4, wherein the strong synthetic interaction between the K6R ubiquitin mutant and deletion of *UBP6* identified in the screen (*S*-score of -7.05) is detectable only with increasing amounts of canavanine.

The interpretation of spot dilution assays is done by comparing the growth of each single mutant to the growth the double mutant strain. Because the cultures are normalized to the same number of cells prior to generating fivefold dilutions and plating, the number of colonies observable at higher dilutions can be compared to determine whether more (or less) cell death is observed in the double mutant relative to the single mutant strains. The size of the colonies is also analyzed to determine whether the single and double mutant cells grow at different rates.

7. Single-lysine ubiquitin variants to study ubiquitination in vivo

7.1 Conditional expression of single-lysine ubiquitin variants as the sole ubiquitin source in vivo

We propose that the conditional expression of ubiquitin mutated at all but one lysine (single-lysine ubiquitin) could be a powerful approach to study the ubiquitination of substrates with specific ubiquitin linkage types in vivo. However, constitutive expression of any single lysine ubiquitin variant (e.g., K11-only ubiquitin) as the only ubiquitin source is lethal. To conditionally express single lysine ubiquitin variants as the sole source of ubiquitin in cells, we took advantage of the well-characterized ubiquitin replacement method developed by Finley et al. (1994). Briefly, SUB280 is a yeast strain wherein the ubiquitin sequences at all four ubiquitin loci are deleted. It is complemented with a plasmid encoding wild-type ubiquitin under the control of the *GAL1* promoter. Hence, when this strain is cultured in medium containing 2% galactose, wild-type ubiquitin is expressed sufficiently to support growth.

SUB280 can be transformed with plasmids that encode any desired ubiquitin variant under the control of the copper-inducible *CUP1* promoter. Importantly, derivatives of SUB280 carrying plasmids encoding copper-inducible ubiquitin variants must be maintained in low-copper medium (LCM; 0.17% yeast nitrogen base without copper or iron, 1μM FeCl₃, 0.1% ammonium sulfate) supplemented with 2% galactose. To express ubiquitin variants as the only source of ubiquitin, cells are cultured in LCM containing 2% dextrose and 100μM CuSO₄. The dextrose in the medium suppresses the *GAL1* promoter, thereby leading to depletion of wild-type ubiquitin, while the copper induces expression of the desired ubiquitin variant under the control of the *CUP1* promoter.

We found that culturing cells for 8h in 2% dextrose and 100μM CuSO₄ led to a strong depletion of wild-type ubiquitin, with simultaneous strong induction of the ubiquitin under the control of the *CUP1* promoter (Fig. 5A). Significantly, this method permits the conditional expression of ubiquitin variants that are lethal for cells, such as ubiquitin that has been mutated at all lysines (K0 ubiquitin) or at all but a single lysine residue (e.g., K11-only, K33-only) (Fig. 5B). Remarkably, even after 8h of ubiquitin depletion, cells remained viable and could be recovered by reexpression of ubiquitin.

This system permits the expression of any desired ubiquitin variant, including variants that would be lethal if expressed continuously as the only source of ubiquitin. In particular, we suggest that the conditional expression of ubiquitin mutated at all but one lysine (single lysine ubiquitin) could be a powerful approach to study the ubiquitination of substrates with specific ubiquitin linkage types.

8. Concluding remarks

The redundancy between ubiquitin linkage types, the lack of biochemical tools, and the relatively low abundance of some linkages have all hampered the study of atypical polyubiquitin chains (Xu et al., 2009). The analysis of genetic interactions is a powerful approach to study functional relationships between genes, thereby facilitating the discovery of physiologically important roles of genes that not apparent in single mutants. The SGA described here represents the first comprehensive genetic analysis of lysine-to-arginine ubiquitin mutant alleles. Furthermore, the methodology that was developed to carry out the SGA and follow-up of individual interactions can be applied to the study of other pathways with complex relationships between genes.

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Fig. 1.

(A) The four modified ubiquitin-encoding loci. *UBI1* was engineered to initially contain a Floxed *URA3*, to allow it to be selected for while it was crossed into the deletion collection. After backcrossing to the deletion collection, CRE was induced with Galactose, and *ura3* cells were selected for with 5-FOA. (B) The eMAP (epistasis map) regime used for the ubiquitin SK1 analysis.

Fig. 2.
The levels of ubiquitin were analyzed by Western blotting (polyclonal antibody from Santa Cruz, SC-9133) in the indicated strains. The GPDpr-Ub construct encoding three tandem wild-type ubiquitins was integrated at *UBI4*.

Fig. 3.

A plot of all the S-scores in the 4-marker ubiquitin SGA. All the S-scores in the 4-marker ubiquitin SGA were plotted in increasing order from *left to right* along the *X*-axis.

Fig. 4.

The conditional nature of some genetic interactions. A synthetic interaction between mutation of K6 of ubiquitin and deletion of *UBP6* was identified in the 4-marker SGA. The interaction was validated by spot dilution assays on CSM plates containing the indicated concentrations of canavanine.

Fig. 5. Conditional expression of single lysine ubiquitin variants. (A) Cultures of SUB280 derivatives carrying either an empty vector or a plasmid encoding wild-type ubiquitin under the control of the *CUP1* promoter were cultured for 8h in LCM medium supplemented, as indicated, with 2% galactose, 2% dextrose, or 2% dextrose and 100 μ M CuSO₄. Total extracts were analyzed by Western blotting with antiubiquitin antibody (monoclonal antibody P4D1). (B) SUB280 derivatives carrying plasmids encoding the indicated ubiquitin variant under the control of the *CUP1* promoter were cultured for 8h in LCM medium supplemented with 2% dextrose and 100 μ M CuSO₄. Total extracts were analyzed by Western blotting with antiubiquitin antibody (*top*) or histone H2B as loading control (*bottom*).