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Synthetic Dosage Lethality Screen with Actin Cytoskeleton in Yeast

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Introduction:

Much of the scientific research on human diseases has been focused on genetic influences. Simple cases of monogenic disorders due to either dominant or recessive loss of function alleles have been the focus in previous work; however, dominant or recessive allele diseases are fairly rare but observable in large populations. Furthermore, researchers have been able to show a clear polygenic influence for a number of disorders. Any given individual also has two different alleles for almost all genes with ~1.8 x 10^9 possible binary complex genetic interactions (Haarer et al. 2006).

The purpose of the project was to identify new interacting pathways and functions not previously known ask how they may influence actin dynamics in ways that may relate to medically relevant problems by using an ideal eukaryotic model organism, *Saccharomyces cerevisiae.* The identification of these new genes involved using a large collection of non-essential knock-out strains to uncover binary gene interactions through whole genomic Synthetic Dosage Lethality (SDL) screening methods. Functional relationships between the essential actin gene *(ACTI)* and a collection of non-essential genes would contribute to understanding the complex genetic interactions that may contribute to human genetic disorders. The long-term goal of the research project is to define how signals that alter the cytoskeletal architecture promote cancer initiation and progression.

Saccharomyces cerevisiae, or baker's yeast, is an excellent model organism for research in biochemistry and molecular biology. Their basic

biochemical mechanisms are highly conserved and many genes are homologous to *Homo Sapiens* (humans) (Goffeau 1996). Due to this high degree of homology, molecular biological research using yeast has been crucial in fields such as advancement of anticancer research. Furthermore, *Saccharomyces cerevisiae* has been particularly useful for uncovering binary gene interactions through the use of genomic methods made possible by an ordered collection of knock-out strains.

The actin cytoskeleton is a critical and conserved organizing structure. It is an intracellular globular protein, found in the cytoplasm of our cells. Globular actin monomers (G-actin) polymerize through the use of ATP in critical concentrations in order to form filamentous actin (F-actin) and eventually actin cytoskeletons. Multiple signaling pathways provide the stimulus to accelerate polymerization on the plus end and the adhesion of F-actin to transmembrane receptors links the cell to specific matrix proteins. F-actin, which is attached to the cell membrane, works together with myosin for cell movement. Actin cytoskeletons contribute to many other central cell functions from growth and cell division, development, signal transduction, adaptation, to gene expression. It provides the cell with mechanical support and driving forces for movement. Therefore, the complexity of the structures generated during and after polymerization and the highly dynamic nature of these transitions are difficult aspects of experimenting with actin.

The experimental approach is to understand how actin regulates the dynamics of actin networks with in our cells. The actin cytoskeleton is important to all eukaryotic cells and is highly conserved among the eukaryote organisms,

including human cells. As one of the most abundant protein on earth, actin is essential for the survival of most cells (Pollard, 2009). Alpha-actin gene (*ACTA1)* is a specific human gene and its mutation has been known to cause three different congenital myopathies. "Actin myopathy" (AM) is a human disease in which the patient's biopsies reveal homogeneous filamentous inclusions containing actin in areas devoid of sarcomeres in which myofibrillar filament lattice is normally present (Sparrow et a1. 2003). Clinically, AM shows severe congenital muscle weakness and high mortality and indicates the importance of study of actin and cancer related information.

The main gene related with actin function is the essential actin gene *(ACT1)* and "the large collection of *act1* mutants display defective intracellular actin distribution and show a wide diversity of phenotypes" (Botstein 1997). Actin, as an A TP-binding protein, exists both in monomeric (G-actin) and filamentous (F-actin) forms (Pruyne 2000). Yeast cells contain three types of filamentous actin structures: actin cables, an actin-myosin contractile ring, and actin cortical patches (Pruyne 2000). Actin cables are aligned with the mother-bud axis and are composed of bundles of actin filaments. Many eukaryotic cells transport organelles along actin filaments and budding yeast cells, specifically, replicate by distributing organelles to daughter cells by directing formation of a bud (Pollard, 2009).

The philosophy underlying this work is that just as genes involved in core functions are conserved, the genetic interactions between the genes will be conserved as well (Lodish 2000). Many aspects of human genetic disease,

including cancers, are intimately tied to actin cytoskeleton's malfunction, due to their important contribution to cell function. When actin is mutated, the primary mechanism of cell motility or reorganization of the actin cytoskeleton is disrupted. Actin is critical for tumor metastasis and much research is now looking at actin reorganization to suppress the migration of invasive rumors. Cancer cells migrate in various ways and the study of the actin cytoskeleton reorganization and cell migration can contribute to appropriate therapies for treating cancer (Yamazaki et al. 2005). The laboratory of Dr. David Amberg has previously been leveraging gene dosage to uncover genetic interactions between actin and all other genes in the yeast (*S. cerevisiae)* genome. One of the gene leverage methods is called complex haploinsufficiencies (CHI) and/or altered gene dosages at multiple loci. As shown by figure 2, hemizygosity of the indicated genes causes a growth deficit if the strain is also hemizygous for the actin gene ACT1; MATa *Act1*Δ*::G418* SOG1^{WT} X MATα *sog1*Δ::NAT^{R} ACT1^{WT} (Haarer el al. 2007). Therefore, this research may contribute to identifying the genes involved in severe and complex genetic disorders such as cancers and psychiatric disorders. Given the numerous and frequently large chromosome rearrangements and aneuploidies seen in cancer cells, this work stands to be particular relevant to oncogenesis (Haarer 2007). Importantly, there has been recent increase in interest on multigenic contribution to human genetic disorders since CHI is an important contributor to oncogenesis in humans.

Instead of looking at double deficiency of CHI, Synthetic Dosage Lethality (SDL) is looking at effects of combining excess of gene 1 *(ACT1)* with

complete deficiency in other gene (yfgΔ). My project has been evolving and I retested and confirmed a large number of potential SDL interactions with actin that were initially identified in robotic screens. Using an inducible chromosome loss method, centromere-based plasmid containing the yeast actin gene *(ACT1)* was introduced into each of the ~4800 Euroscarf non-essential gene deletion mutants in the robotic screens. For each of the three robotic screens done previously in the lab, numerous deletion strains that show varying degrees of reduced (or enhanced) growth when carrying the *ACT1* plasmid versus "empty" control plasmid were identified. When the extra actin gene causes inviability or reduced growth, this is termed "synthetic dosage lethality." When deletion strains grow healthier when expressing extra actin, it's termed "synthetic dosage suppression:' In essence, the screen entailed introducing an extra copy of the actin gene to determine when the system collapses in a cell inherits one extra copy of actin and one bad copy of several different non-essential genes in the yeast genome.

In most cases, affected strains showed varying degrees of reduced growth but the overlap between the three robotic screens were less than optimal. This may be due to "escape" events, such as mutation of plasmid-borne or chromosomal *ACT1,* to generate false negatives, or to stochastic growth or pinning differences, which may generate false hits. Due to ambiguity of the data from robotic screens, an alternative test on deletion strains "one at a time" was performed by hand in order to find which strains are actually sensitive or suppressed and to what degree. Essentially, this simply involves individually

transforming candidate strains with *ACT1* and empty control plasmids and comparing relative growth of the transformants at various temperatures. Other studies done by Measday et al. (2005) laboratory has shown that systematic genetic screens are a powerful means to discover roles for uncharacterized genes and genes with alternative functions that may not be discovered otherwise.

Methods:

SDL (Synthetic Dosage Lethality) and SOS (Synthetic Dosage Suppression) Screening in S. *cerevisiae*

In previous screens in the Amberg lab, centromere-based plasmid containing the yeast actin gene *ACT/was* introduced into each of the ~4800 Euroscarf non-essential gene deletion mutants using an inducible chromosome loss method. Because there are -4800 non-essential genes, the mating of the deletion strains and the yeast actin gene *ACT1* was done robotically. The deletion strains robotically mated to a specifically engineered strain, in which each of the 16 chromosomes carries a copy of the *URA3* gene and a copy of the $Gal^{1,10}$ promoters adjacent to its centromere; this strain also carries a centromere-based plasmid with the *ACT1* and Nat^{R} genes. The modified chromosomes of this strain are stable when glucose is present; however, when switched from glucose to galactose media, mitotic transmission is greatly compromised, presumably due to the high rate of transcription across the centromere region and associated inability to assemble a fully functional kinetochore. Shifting to FOA media after growth on galactose provides a selection against strains that have retained any of the 16 modified chromosomes, effectively selecting for those strains that have gone from

a 2N (diploid) to I N (haploid) complement of chromosomes. Inclusion of drugs G418 and nourseothricin (Nat) to the media provide additional selections for the gene deletion of interest and the *ACT1* plasmid, respectively.

Lithium Acetate Transformation of Yeast

Amongst ~4800 non-essential genes tested three times with the robot, about 50 genes with more than one hit have been retested by hand to confirm their hits. Starting with those deletion strains with most frequent hits and/or greatest growth differentials, strains were picked from master plates of Euroscarf haploid deletion strains and restreaked on YPD + G418. Along with the deletion strains with previous hits, control strain yal066wΔ was also streaked.

In order to prepare a culture of yeast cells in the exponentially replicating and "pre-culture" stage instead of a stationary phase where cells stop growing after hitting a certain density, YPD yeast culture grown overnight was reinoculated into a fresh YPD media. Therefore, the yeast culture grown to an efficient transformation density was resuspended in LiOAc mix at permissive temperature to start the transformation steps. Then ssDNA and either YCp5O or pKFw29 plasmid was added to the yeast with LiOAc mixture 10 allow for DNA absorbance. This was then added to PEG mix, heat shocked, resuspended in TE, and plated on SC-ura plate for each plasmid. The plates were scored comparing growth between two different plasmids with the rating of 0-5. Rating of 0 indicates that cells with deletion plasmid did not grow compared to normal growth of the control plasmid. Rating of I was weak growth and strong hit, rating

of 3 represented half of the overall number or size of the colonies, and rating of 5 represented a strong growth but weak hit.

After 2-3 days of growth in 30°C, two transformants for each plasmid were restreaked of 25°C, 30°C, and 37°C to test for temperature-dependent effects. After growing in prospective temperatures, the temperature sensitivity was rated using a rating system “0-3". After conducting the temperature sensitivity test in the three temperatures, some of the "non-hits" from the primary transformation plates were seen to have temperature sensitivity in the restreaks. Therefore, the new "hits" were based on the difference in growth between the high temperature of 37°C or low of 25°C and yeast's most favorable temperature of 30°C. Therefore the scores were more dependent on the temperature sensitivity rather than growth difference between the control plasmid and the plasmid in question.

However, due to some similar but not completely overlapping results between the transformation colonies and restreaks, both ratings were combined to create an overall rating system of (0-5) based on both numbers and sizes. A "hit" represented a combination of lethal or reduced growth of transformation colonies and temperature sensitive colonies. Rating of 0 represented a combination of either lethal colonies with deletion plasmid from the primary transformation plates or very temperature sensitive colonies of cells. Rating of 1 was reduced growth with temperature sensitivity and a strong hit, rating of 3 represented half of the overall number or size of the colonies and/or temperature sensitivity, and

rating of 5 represented a strong growth and no temperature sensitivity, meaning a weak hit or not a hit at all.

Saccharomyces Genome Database

The "hits" or genes with a rating of 0 to 3 confirmed the binary gene interaction with *ACT* 1, so the other genes within the complex of the "hit" genes were also retested by plasmid transformation. From the original list of about 50 narrowed to 25, the potential list grew to be about 150. The potential list was assembled by searching the *Saccharomyces* Genome Database (SOD), which collects information and maintains a database of the molecular biology of the yeast *Saccharomyces cerevisiae.* Related genes were found by searching genes within the same complexes, genes with high correlation according to the CHI screen, similarities between the genetic sequences according to the sequence analysis tools such as BLAST. Furthermore, a list grouping the confirmed genes interacting with actin according to Gene Ontology terms was also created. The retested and confirmed genes were then further characterized quantitatively and qualitatively. A quantitative characterization was done by a TECAN microplate reader and a qualitative characterization was done by staining actin with rhodamine-phalloidin.

TECAN:

Quantitative data was acquired by running TECAN (Infinite F200) microplate reader. The diagnostic tool allows tracking of cell growth every 15 minutes over the 24 hour period in order to analyze the exponential growth as well as stationary phase. Diploid selection colonies grown at different

temperatures were selected and suspended in liquid media and growth of cells at 30°C over a period of 24 hours was kept track by a TECAN microplate reader. Cell suspensions result in light scatter when a light beam is passed through them. Using a microplate reader, this light scatter is detected as an increase in absorbance.

The resulting data were imported into Microsoft Excel for analysis and generation of growth curves (table 2). In order to determine if the growth rate is aligned with the ratings, some of the growth curve of the colonies was analyzed by calculating the percentage of exponential mutant growth versus wild-type at 37°C. This was done by calculating the following:

$$\frac{\text{Slope of mutant growth rate at 37°C}}{\text{Slope of wild-type growth rate at 37°C}} \times 100\%$$

Then the comparison percentage between mutant and wild-type was compared 10 the transformation and restreak ratings.

Rhodamine-Phalloidin stain:

Rhodamine-Phalloidin stain, also known as actin stain, was done to qualitatively characterize the actin filaments in order to assess the phenotype. Phalloidin binds specifically to F-actin, and fluorescent-tagged phalloidin stains the actin skeleton in cells to view under a microscope (Amberg 2006).

An overnight culture grown in SC-ura at 30°C was inoculated to grow the cells to exponential phase for three hours where the yeast cells are budding. The

cells were then grown at experimental temperatures for two hours. 4% formaldehyde was added to fix in media for 45 minutes, then washed and resuspended in I X TBS containing 4% formaldehyde was added to further fi x. The cells were washed twice and reconstituted with 1 X TBS. Rhodamine Phalloidin (6.6µM in MeOH, kept al -20°C) was added and then the cells were incubated in the dark. The cells were further washed with 1 X TBS five times and suspended in immunofluorescence mounting solution for visualizing on a coverslip.

Funspec: a web-based cluster interpreter for yeast

A web-based 1001 Functional Specification (FunSpec) is useful for analysis of large-scale data sets. It is a statistical evaluation of groups of genes with respect to existing annotations called Gene Ontology terms. FunSpec is especially helpful for interpretation of any data type that generates groups of related genes and uses information from public databases to evaluate lists of yeast genes to determine if they are enriched for particular attributes, using a well-accepted statistical model (Robinson. 2002).

Results and Discussion:

Expansion of the actin SDL (Synthetic Dosage Lethality) gene network through inference

As mentioned previously, the preliminary robotic SDL screens had low overlap; there were a total of 211 hits in three SDL screens, but only 55 strains had multiple hits and 16 strains were hit three limes. By individually testing the possible 55 strains with multiple hits, only about 25 strains confirmed to be hits.

Therefore, initially identified SDL genes were analyzed with the help of SGD to determine additional genes that could be expected to be SDL with actin. From about 25 confirmed strains, the implicated list grew to be about a 150 possible strains. The criteria included shared function and/or being present in a defined complex with the product of the primary SDL gene, having a known biological function that is related to actin, genes with high correlation according to the CHI screen, similarities between the genetic sequences according to the sequence analysis tools such as BLAST. An implicated list allowed for another 125 nonessential genes to be tested against the *act1*Δ and a total of 150 possible nonessential genes. From the implicated list of about 150, corresponding deletion were tested via plasmid transformation as done with strains found from the robotic screens. The transformation tests were done on the 150 non-essential genes to confirm that the enrichment is actually due to biological relationship to actin. After further confirmation tests, the list narrowed to 70 confirmed strains—ranging with the rating of 0 to 3.

Actin shows synthetic dosage lethality with a large number of non-essential yeast genes and the SDL interactions can be visualized through the Osprey program (figure 3). The genes are color-coded based on their primary Gene Ontology assignment and the groupings of GO terms show related genes in a clear manner. However, the grouping does not allow room for overlaps among the non-essential genes. The overlap could have shown which genes have multiple functions and ultimately which complexes may be important in actin SDL

interaction. The Osprey does show that actin is important in wide variety of function in a cell.

Characterization tests:

One of the TECAN graphs representing few of the mutant and wild-type strains grown at 37°C is shown in figure 4. The graph shows that certain strains enter log-phase growth earlier than other strains. In order to determine if the growth rate is aligned with the ratings, percentage calculated was compared to the transformation and restreak ratings (Table 2). The percentage of mutant versus wild-type growth was consistent with restreak ratings—which are strains with temperature sensitivity—but not always with the transformation ratings. The restreak rating was done with 1-3. The strains with restreak rating of 1 (weak growth of mutant at non-permissive temperature) all had a percentage less than 65% to show consistent slow growth. The strains with a restreak rating of 2 (weak growth of mutant at non-permissive temperature) all had percentage similar to 100%. One anomaly is elp2Δ, which had a percentage of 123.50%. This irregularity, however, is also consistent with data because the resteak rating of 1 was due to weak growth at 25°C. Since the reduced growth of elp2Δ was not for 37°C but at 25°C, the fact that elp2Δ did not have a reduced growth like other strains is consistent.

The yeast model employing the actin gene to determine the prevalence of multigenic influence in eukaryotic genome has proven to be tremendously useful. Since many yeast and human genes are highly conserved in function, SDL network associations could be conserved as well. Modeling these networks in

yeast could be highly informative for multigenic disorder studies in human genetics.

This study has shown that inheriting a combination of one null allele of the non-essential strains and an extra actin allele can have adverse effects on the cell function. Because actin is one of the most centrally important proteins in eukaryotic cells and shows haploinsufficiency on its own, it was predicted that a large number of SDL interactions would be displayed as well. In fact, the influence of SDL on human diseases has shown to be an important area of study. For example, simple haploinsufficiency in a large number of transcription factors has been found to contribute to severe and diverse genetic disorders, such as muscle congenital myopathies in human (Sparrow et al. 2003).

The confirmed genes from SDL screens, a number of genes were especially clustered within the same complexes. In order to find statistical analysis of the genes within the complexes or functional groups, the list of confirmed genes were entered into Funspec database. These functional enrichments in the SDL network are statistically significant given the background of ~4800 non-essential genes. The statistical evaluation of groups of genes with respect to existing annotations Gene Ontology terms was analyzed. Within the generated list of output summary, Gene Ontology (GO) terms were specifically focused upon: molecular function, biological process, and cellular component. The list only included terms with p-value lower than 0.01 in order to exclude GO terms that include genes by chance (Table 1). The list of the confirmed genes

within the GO terms looks promising because of the low p-value of the groupings to indicate the significance of the results in a statistical sense (Robinson 2002).

Many complexes found in FUNSPEC are significantly enriched: protein urmylation (*P*-value = 2.16E-l 0), proton-transporting two-sector ATPase complex (*P*-value = 7.87E-09), cyclin-dependent protein kinase regulator activity (*P*-value = 1.47E-08), elongator holoenzyme complex (*P*-value = 2.80E-07). Additionally, several of the molecular functions associated with these complexes are also statistically overrepresented.

From the complexes that have shown SDL interactions, there were two specific genes that had complete overlap within three different complexes, and eight genes that have two overlapping complexes. The three complexes were the following: elongator, urmylation, and transfer-RNA Wobble Uridine Modification complexes. The two confirmed genes present in all three complexes are elp2Δ and elp6Δ. Within urmylation and t-RNA wobble complex, four genes, ncs6Δ and uba4Δ as well as elp2Δ and elp6Δ (present in all three complexes), were overlapping. Within the elongator and t-RNA wobble complex, six genes called elp3Δ, elp4Δ, eJp5Δ, and iki3Δ as well as elp2Δ and elp6Δ (present in all three complexes) were overlapping. These genes were further investigated in their function and much is still unknown about their relationship. Due to the SDL's nature of finding new genes that have interactions with each other as well as actin, the three complexes were further examined.

<u>Elongator/urmylation/transfer-RNA Wobble Uridine Modification complexes' overlapping genes indicate similar function due to sensitivity to actin</u>

Elongator and urmylation functions have been found and established but more of the possible functional aspects of the elongator and urmylation on the transfer-RNA wobble, uridine modification are in question. Certain functions have been found, but further research on other functions as well as the relationship among the three complexes could possibly explain the relationship between the complexes and actin. Transfer RNA has distinct and important function in producing proteins. In a cell, DNA transcribes to make RNA, which in tum is translated to make an amino acid chain. The amino acid chain is what makes up proteins, which are important for cell function and survival. Translation process uses transfer RNA to make proteins by reading codons. The anticodon part of the t-RNA brings appropriate amino acids depending on the recognition of the codon on the messenger RNA and makes a chain accordingly.

Some tRNAs recognize more than one codon. A codon is composed of three bases and 61 triplet code combinations produce the 20 distinct amino acids vital for cell function. However, 40 tRNAs read the codons and some recognize more than one. This is possible due to tRNA wobble. The third base of the codon is able to be read by molecularly different third anti-codon base on the tRNA in order to bring the correct amino acid. Certain tRNA specifically has a uridine at position 34 that is altered by urmylation and elongator complex to change uridine base to $mcm^5s^2U_{34}$ (Agris et al. 2006). The urmylation pathway and elongator complex are involved in modification of tRNA bases. Urmylation pathway is specifically responsible for the thiolation of uridine (s^2U_{34}) (Pedrioli et al. 2008). Elongator complex is specifically responsible for modification of uridine to

mcm^5U_{34} (figure 5). Elongator complex's original known function was only transcriptional elongation. However, recent data links to tRNAs as well.

The molecular change at the third base of the anti-codon, or "wobble," is thought to alter folding, stability, or tum-over for the tRNAs (Agris et al. 2006). The wobble allows pairing with multiple bases by the tRNAs; therefore, reduced function of tRNAs due to lack of modified tRNAs bringing amino acids to make protein may be a cause for lethality or reduced growth of cells. Although the relationship amongst the GO functions seems likely, the SDL interaction with actin causing this reduced function in the tRNAs is still unknown. However, further studies and experiments in the future could address this unknown interaction.

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Summary:

The purpose of the project was to identify new interacting pathways and functions not previously known ask how they may influence actin dynamics in ways that may relate to medically relevant problems by using an ideal eukaryotic model organism, yeast. The identification of these new genes involved using a large collection of non-essential genes to uncover binary gene interactions through whole genomic Synthetic Dosage Lethality (SDL) screening methods. Functional relationships between the essential actin gene *(ACTI)* and a collection of non-essential genes would contribute to understanding the complex genetic interactions that may contribute to human genetic disorders.

Interest in how regulation of the actin cytoskeleton---due to actin cytoskeleton's importance to cell function-may contribute to diverse human disease states such as the rampant cell division and metastasis that occurs in cancerous tissues. The actin cytoskeleton is a vitally important organizing structure found in the cytoplasm of our cells. It contributes to essential cell functions from growth and cell division, development, signal transduction, adaptation, to gene expression. By reviewing a large number of potential Synthetic Dosage Lethality (SDL) interactions with the yeast actin gene *ACT1* initially identified in robotic screens, a smaller set of genes were reviewed, compiled, and retested. Then the list was expanded to include genes within the same complex or with the same functions using Gene Ontology terms. The implicated gene set—particularly

elongator/urmylation/tRNA-modification sets of overlapping genes—was followed up with further tests to find the most relevant complexes or functions to the observed *ACT1* SDL.

Although the three complexes were related to each other in function, the actin's relationship with the three complexes is still unknown. This relationship could further induce new findings about actin's role in the cells. Using SDL, a completely new field in biochemical pathways could potentially be found to contribute to the long-term goal. The long-term goal of the research project is to define how signals that alter the cytoskeletal architecture promote cancer initiation and progression. The method of discovering an unknown relationship between actin and non-essential genes by doing a wide-array screen analysis has proved to discover new relationships and the method could be used in other areas to uncover other findings as well.