RESEARCH ARTICLE

Yeast Augmented Network Analysis (YANA): a new systems approach to identify therapeutic targets for human genetic diseases [version 1; peer review: 2 approved]

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V1 First published: 02 Jun 2014, **3**:121
<https://doi.org/10.12688/f1000research.4188.1>
 Latest published: 02 Jun 2014, **3**:121
<https://doi.org/10.12688/f1000research.4188.1>

Abstract

Genetic interaction networks that underlie most human diseases are highly complex and poorly defined. Better-defined networks will allow identification of a greater number of therapeutic targets.

Here we introduce our Yeast Augmented Network Aalysis (YANA) approach and test it with the X-linked spinal muscular atrophy (SMA) disease gene *UBA1*. First, we express *UBA1* and a mutant variant in fission yeast and use high-throughput methods to identify fission yeast genetic modifiers of *UBA1*. Second, we analyze available protein-protein interaction network databases in both fission yeast and human to construct *UBA1* genetic networks. Third, from these networks we identified potential therapeutic targets for SMA. Finally, we validate one of these targets in a vertebrate (zebrafish) SMA model. This study demonstrates the power of combining synthetic and chemical genetics with a simple model system to identify human disease gene networks that can be exploited for treating human diseases.

Open Peer Review

Approval Status

	1	2
version 1
02 Jun 2014	view	view
1. Corey Nislow , University of British Columbia, Vancouver, BC, Canada		
2. Jasper Rine , University of California Berkeley, Berkeley, CA, USA		
Any reports and responses or comments on the article can be found at the end of the article.		

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Competing interests: No competing interests were disclosed.

Grant information: This work was supported by a grant from the Muscular Dystrophy Association (MDA186435). (Christine Beattie grant support RO1NS050414 (C.E.B.) with additional support from P30NS045758).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Wiley DJ, Juan I, Le H *et al.* **Yeast Augmented Network Analysis (YANA): a new systems approach to identify therapeutic targets for human genetic diseases [version 1; peer review: 2 approved]** F1000Research 2014, **3**:121 <https://doi.org/10.12688/f1000research.4188.1>

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Introduction

Many disease-associated genes have been identified, yet most genetic diseases remain untreatable. One path to treatment is to develop extensive genetic networks in which human disease genes function (or dysfunction) and then target therapies to the genes identified in those networks. Eventually, an individual's own genotype for proteins in the network may also be considered in the therapeutic options. Developing gene networks is already widely recognized as a powerful approach to identify new drug targets¹⁻⁶. Gene networks are based on the principle that networks contain proteins that interact (physically and/or functionally) and that these interactions govern most, if not all, cellular functions. Importantly, gene network interactions are often conserved in different organisms even though the output of the networks may differ⁷. Candidate networks can be generated from studies in model organisms and then extrapolated to human cells. One can expect that, for disease gene networks, some of the interacting genes might modulate the disease phenotype thus representing potential therapeutic targets.

The direct identification of disease networks from studies in human cells is, of course, challenging because of the large number of potential proteins and incomplete knowledge of how the activity of any one protein may affect the network output. Analysis of networks would benefit from studies in a tractable model organism amenable to high throughput methods. Given that network interactions may be conserved between humans and model organisms, even while the outputs may differ, we hypothesized that we can identify human disease networks in the fission yeast *Schizosaccharomyces pombe*, a simple, genetically tractable model organism, coupled to existing protein-protein interaction databases. We can then transfer that network knowledge to human cells with the goal of identifying therapeutic target genes.

How valid is the approach of using fission yeast networks to identify human disease gene networks? First, the proteins that control most core cellular functions are in fact evolutionarily conserved, underscoring the “deep homology” that exists between all living organisms^{8,9}. For example, in *S. pombe* >65% of the genome is orthologous to human (<http://orthomcl.org/orthomcl/> and <http://www.pombase.org>). Second, many of the cellular processes implicated in human disease, e.g. vesicular transport, protein folding, metabolism, and RNA processing, are also evolutionarily conserved and highly interconnected¹⁰. Importantly, the value of a simple model organism for discovering “druggable” genetic pathways has recently been demonstrated for the budding yeast *Saccharomyces cerevisiae*¹¹, and thus extending the study of human disease genes to fission yeast seems promising.

Our approach (Figure 1) involves the expression of human disease associated genes in *S. pombe* and then the analysis of their effect on yeast fitness (growth). We performed high-throughput synthetic genetic array (SGA) screens to identify the fission yeast genetic modifiers that alter this effect, as measured by a simple yeast growth assay. The genetic modifiers are then assembled into human disease gene networks or clusters (using protein-protein interaction datasets from both *S. pombe* and humans). Any modifiers or genes in the networks or clusters represent potential therapeutic targets. This unbiased high-throughput approach could be widely and rapidly applied to many different disease-associated genes at relatively low cost.

To demonstrate the power of YANA, we considered spinal muscular atrophy, a common neurodegenerative disease affecting approximately 1/6000 births worldwide and the number one genetic cause of infantile death in the United States¹². Most cases (>90%) are caused by deletion of *SMN1*, a gene encoding the survival motor neuron (SMN), a protein involved in the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs)¹³. In contrast, X-linked spinal muscular atrophy is caused by mutations in *UBA1*, a gene encoding the ubiquitin activating (UBA) enzyme 1, an E1 ubiquitin ligase^{14,15}. Consistent with a role of ubiquitination in spinal muscular atrophy and SMN biology, it was recently shown that ubiquitin-mediated proteolysis regulates SMN stability^{16,17}. Therefore, we systematically investigated the genetic network for *UBA1* to better understand the potential connections between UBA1, its modifiers and the spinal muscular atrophy phenotype. Using YANA we identified several potential therapeutic targets and validated one of these targets in a SMA vertebrate (zebrafish) model.

Methods

Query Strain Creation

UBA1 was cloned into a pENTR/D-TOPO vector (Life Technologies, Cat # K2400-20) from cDNA (Origene, Cat. # SC320329) following the manufacturer's protocols. The following primers were used: forward primer: 5'- CACCATGTCCAGCTCGCCGC-3'; reverse primer: 5'- TCAGCGGATGGTGTATCGGAC-3'. Genetic insertion was confirmed by sequencing (<http://sylvestor.org/shared-resources/oncogenomics>). *UBA1* (G1617T), *mutUBA1*, was created by site directed mutagenesis using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Cat. # 210518; the detailed protocol is available in the kit). The primers used were: forward primer: 5'-GCAGCTGTGCGCCAAATTAATCCACAT-ATCCGG-3'; reverse primer: 5'-CCGGATATGTGGATTAATTTG-GCGCACAGCTGC-3'. LR Gateway reactions (Gateway Cloning Protocols: <http://www.lifetechnologies.com/us/en/home/life-science/cloning/gateway-cloning/protocols.html#lr>) were then performed to flip the *UBA1* genes into destination vectors, to create N-terminal HA-tagged *UBA1* under the control of the *nmt1* promoter (LR Clonase II from Life Technologies, Cat # 11791020). The newly generated expression vectors were then integrated into an *h⁺ leu1-32 ura4-D18 Ade6-M210 S. pombe* strain (PN572) to create a *wtUBA1* query strain (*h⁺ integrated pjkl48-nmt1^{3X}-HA-UBA1-nmt1^{term}. leu1-32 ura4-D18 Ade6-M210*) and a *mutUBA1* query strain (*h⁺ integrated pjkl48-nmt1^{3X}-HA-UBA1(G1617T)-nmt1^{term}. leu1-32 ura4-D18 Ade6-M210*). All media, growth conditions, and genetic manipulations were as previously described¹⁸.

Western Blot and Growth Curves

Strains containing *wtUBA1* and *mutUBA1* were grown exponentially in PMG media (Sunrise Scientific Cat. #2060, keeping the OD₅₉₅ below 0.4) or eight generations at 32°C and then induced for expression of the HA-tagged *wtUBA1* and *mutUBA1* by washing the cells three times with sterile water to remove thiamine. Cells were then grown exponentially for 16 hours and then lysed using a FastPrep 120 bead beater (MP Biomedical), followed by boiling in sample buffer (2x Laemmli Sample Buffer, Bio-Rad Cat. #161-0737) and then clarified by centrifugation. The expression of *UBA1* was confirmed by Western Blot analysis using an anti-HA antibody at 1:2000 dilution (Covance, Cat #MMS-101P, AB_10063488) and standard procedures¹⁹. Growth curve analysis was completed in the

Figure 1. YANA analysis of *UBA1* genes. Numbers illustrate various steps in our analysis. (1) Construction of query strains by integrating wildtype and mutant variants of the *UBA1* gene into *pombe*. (2) Crossing the query strains (mutant or wildtype) to a complete non-essential deletion collection. (3) Bioinformatic analysis to generate the top 'gene hits'. (4) Validation of the top hits in yeast. (5) Confirmation of the top hits in zebrafish.

presence and absence of thiamine (final concentration, 15 μ M; Sigma, Cat. #T4625). Removal of thiamine was achieved by washing the cells three times with sterile water. Cells were incubated in the absence of thiamine for 22 hours under exponential growth, diluted and 120 μ l added to the Tecan Infinity F200 plate readers (starting OD₅₉₅ of 0.05) for growth curve analysis with an n=5 for each sample. For validation experiments, *cul3* and *gsk3* were not pre-induced, while *pub1* was pre-induced for 22 hours. The deletions of *cul3*, *pub1*, and *gsk3* were confirmed by PCR analysis of genomic DNA from each strain, as previously described²⁰.

SGA screening

Query strains were grown in liquid media and then pinned to agar in a 384-format using a RoToR HDA (Singer Instruments). The query was then crossed to the *S. pombe* haploid deletion library (Bioneer, Version 3.0 equivalent) on SPAS media (details can be found at <http://www-bcf.usc.edu/~forsburg/media.html>) using a modified SGA procedure²¹. For germination, four replicates of each cross were pinned to a 1536 format on selective PMG media containing thiamine, adenine (225 mg/L, Sigma Cat. #A8751), leucine (225 mg/L, Sigma Cat. #L8912), and the antibiotic G418 (150 μ M, Gold Bio

Cat. #G-1418) (PAUT+G418). After three days, the plates were then pinned to both PAUT+G418 (non-inducing) and PAU+G418 (inducing) plates. The colonies growing on the PAUT+G418 plates were documented on a flatbed scanner for the next three consecutive days. The PAU+G418 plates were grown for two days, and then re-pinned to fresh PAU+G418 plates and documented over the next three consecutive days. Based on the growth characteristics of the wt*UBA1* strain, the plates were then pinned to fresh PAU+G418 plates and documented for an additional three days.

Hit analysis

The documented plates were analyzed for 'Hits' representing a growth defect (SL) or growth suppressor (SS) using ScreenMill software²². The software is used to quantify colony size for each individual cross and then to normalize the quantified plates with and without replicate exclusion for each quadruplicate of the query crossed to a specific deletion strain. Data was then compared (between non-induced versus induced) and ranked in Excel ($P \leq 0.05$) (Microsoft). The orthologs were then identified based on curated data from PomBase (www.pombase.org; build 2013-11-11-v1), OrthoMCL (<http://orthomcl.org/orthomcl/>; Version 5), InParanoid8

(<http://inparanoid.sbc.su.se/cgi-bin/index.cgi>; Version 8.0), and Homologene (<http://www.ncbi.nlm.nih.gov/homologene>; build 67).

Bio-Informatics

The *S. pombe* and human 'Hits' were analyzed in String (www.string-db.org) to map protein-protein interactions limited to data from experiments at the highest confidence level (0.900), and named *pombe* primary and human primary. The *S. pombe* primary was then extended by adding first degree neighbors to the original *pombe* 'Hits', keeping all interactions based on data from experiments at the highest confidence level (0.900). From the extended *pombe* network interaction map, new 'Hits' were extracted that corresponded to at least two previous 'orphans' ('Hits' that were not previously mapped) that interact through a nearest neighbor. All 'Hits' were then analyzed through PubMed (<http://www.ncbi.nlm.nih.gov/pub-med/>) for relevance in terms of SMA, with special focus on the 'Hits' in the interaction maps.

Morpholino (MO) injection and drug test

Transgenic *Tg(mnx1:0.6hsp70:GFP)os26* embryos that express GFP in ventrally projecting motor axons, referred to as *Tg(mnx1:GFP)* embryos, were used for all zebrafish experiments. Embryos were staged according to Fritz *et al.*²³ All fish were grown and maintained in the Ohio State University (OSU) zebrafish facility following established protocols and OSU animal welfare guidelines as stated in Dr. Beattie's animal protocol (On file at OSU: 2009A0141-R1). *Tg(mnx1:GFP)* embryos were injected with 4 ng *smn* MO at the one- to 2-cell stage to knock down Smn as previously described²⁴. At 10 hours post-fertilization (hpf) injected embryos (in their chorions) were placed in Petri dishes in fish water (60 µg/ml Instant Ocean® sea salts) containing compounds in 0.75% dimethyl sulfoxide (DMSO) or 0.75% DMSO only and incubated at 28.5°C in incubator until 28 hpf.

Tg(mnx1:GFP) embryos at 28 hpf were anesthetized with tricaine (250 µg/ml, Sigma A-5040) and fixed overnight at 4°C in 4% formaldehyde/PBS. After removing embryos from fix, they were mounted

on glass coverslips for observation under a Zeiss Axioplan microscope. Motor axons innervating the mid-trunk (myotomes 6–15) on both sides of the fish were scored as described²⁵.

The control *topped*^{b458} embryos were treated and fixed as described above for *Tg(mnx1:GFP)* embryos; the *topped* mutants, however, were processed for znp1 antibody (Hybridoma Bank Cat#znp-1, AB_531910) labeling as previously described²⁶ to visualize motor axons for scoring as they did not have the *Tg(mnx1:GFP)* in the background.

Zebrafish western blot

Samples of zebrafish embryos injected with 4 ng *smn* MO at the one- to two-cell stage and uninjected embryos were treated from 10 to 28 hpf with DMSO or with DMSO and compounds as described above were collected for western blot.

Samples were generated by boiling 25 identically treated embryos in 75 µl blending buffer (63 mM Tris (pH 6.8), 5 mM EDTA, 10% SDS). 10 µl (equivalent to 3 embryos or 75 µg of protein) were added to 10 µl of sample buffer (100 mM Tris (pH 6.8), 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol) and run on a 10% polyacrylamide gel, blotted to nitrocellulose, probed with mouse anti-Smn (1/500; MANSMA12, a gift from Dr. G.E. Morris or anti HuD (1/1000; Santa Cruz Cat#sc-28299, AB_627765) and detected by chemiluminescence of bound HRP-conjugated mouse antibody. Blots were stripped and re-probed with mouse anti-β-actin (1/1000; Santa Cruz Cat #sc-47778, AB_626632).

Results

We expressed the human wildtype *UBA1* (wt*UBA1*) and the disease-causing variant 1617*UBA1* (mut*UBA1*) in *S. pombe* under the control of a regulatable *nmt1* promoter²⁷. We identified strains with stable integrated human *UBA1* genes expressing equivalent levels of UBA1 proteins (Figure 2A). Under non-inducing conditions (+ thiamine) all *UBA1*-containing strains had a similar growth rate to the control non-*UBA1*-containing wildtype strain (Figure 2B). We

Figure 2. Construction of *S. pombe* strains integrated with human *UBA1*. Wildtype and mutant forms of *UBA1* were integrated into fission yeast under the control of the *nmt1* thiamine-repressible promoter. (A) Western blot analysis of protein expression levels shows that both forms of *UBA1* (wildtype and mutant) are expressed to equivalent levels. (B) Expression of wild-type *UBA1* inhibits *S. pombe* cell growth.

then found that cells expressing human wt*UBA1* (fission yeast cells grown in media lacking thiamine to induce *UBA1* expression), experienced a decrease in cell growth. No growth defect was observed for yeast cells expressing mut*UBA1* when compared to the control (Figure 2B).

We set out to identify yeast gene mutations that either enhance or suppress cell growth dependent upon expression of either wt or mut*UBA1* (i.e., epistatic modifiers). We used automated genetics to introduce the wt and mut*UBA1* genes into a deletion strain collection

corresponding to more than 90% of the non-essential genes in *S. pombe* (ca. 80% of the complete genome). We then tested each of the >7000 unique strains expressing either wt or mut*UBA1* combined with a specific gene deletion, for growth properties. Each strain was scored for significance of growth difference compared to a control, and the top hits ($P < 0.05$) were assembled into a database (Table 1). The categories of growth phenotypes were those that were synthetic lethal for wildtype or mutant (wt-SL, or mut-SL) or synthetically suppressed for wildtype or mutant (wt-SS, or mut-SS). We identified 173 *UBA1* or mut*UBA1* modifiers. Notably,

Table 1. *S. pombe* SGA top hits. The tops hits for wt*UBA1* SL, wt*UBA1* SS, mut*UBA1* SL, and mut*UBA1* SS were identified by bioinformatic analysis as described in the materials and methods section.

	wt <i>UBA1</i> SL	wt <i>UBA1</i> SS	mut <i>UBA1</i> SL	mut <i>UBA1</i> SS
1	SPAC24H6.03	SPBC365.06	SPBC8D2.01	SPAC11G7.02
2	SPAC1687.15	SPAC11G7.02	SPBC6B1.10	SPCC13B11.01
3	SPAC3G9.07c	SPCC31H12.05c	SPBC216.05	SPBC2G2.03c
4	SPBC6B1.10	SPBC31F10.13c	SPCC1795.01c	SPBC31F10.13c
5	SPAPYUG7.04c	SPCC1620.14c	SPBC24C6.11	SPCC1620.14c
6	SPBC24C6.11	SPAC57A10.02	SPBC1734.08	SPAC3G6.02
7	SPBC1734.08	SPAC1556.01c	SPAC9.13c	SPAC1556.01c
8	SPAC23D3.09	SPAC30D11.13	SPAC23D3.09	SPAC821.05
9	SPAC17A5.11	SPCC970.07c	SPAC57A10.02	SPBC6B1.04
10	SPAC16A10.05c	SPAC23H4.12	SPAC16A10.05c	SPCC1919.15
11	SPCP1E11.07c	SPCC11E10.08	SPCC895.07	SPBC23E6.09
12	SPBC146.13c	SPBC21D10.12	SPCP31B10.05	SPBC21D10.11c
13	SPAC821.09	SPCC1672.06c	SPCC550.12	SPAC22H10.07
14	SPBC23E6.09	SPAC3C7.03c	SPAC23H4.12	SPCC338.08
15	SPCC1682.16	SPBC32F12.02	SPBC11C11.02	SPBP35G2.08c
16	SPBC106.01	SPCC736.04c	SPBC1289.11	SPBC29A3.14c
17	SPCC622.16c	SPBC30D10.13c	SPCC126.02c	SPAC29A4.18
18	SPAC22H10.07	SPCC737.09c	SPBC13G1.13	SPBC1921.03c
19	SPBC13G1.13	SPBC56F2.01	SPCC188.13c	SPBC2F12.11c
20	SPBC16H5.06	SPBC2F12.11c	SPCC663.12	SPAPB1E7.02c
21	SPBC29A10.05	SPBC16A3.07c	SPCC338.05c	SPAC17A5.16
22	SPBC28F2.07	SPAC5D6.05	SPAC343.11c	SPAC20H4.03c
23	SPCC1827.08c	SPAC18G6.02c	SPCC1919.03c	SPCC1259.03
24	SPAC9G1.10c	SPAC19D5.01	SPAC343.18	SPAC18G6.02c
25	SPBC83.03c	SPAC688.11	SPCC737.09c	SPAPB1E7.06c
26	SPBC342.04	SPAC227.07c	SPBC106.16	SPAC1851.04c
27	SPCC4G3.19	SPAC1851.04c	SPAC3A11.14c	SPAC13G7.06
28	SPAC9G1.05	SPBC16H5.07c	SPAC589.02c	SPBC725.11c
29	SPCC4E9.02	SPAC17A5.18c	SPCC1450.05c	SPCC1739.12
30	SPCC794.11c	SPBC725.11c	SPAC8F11.03	SPCC663.01c
31	SPBC216.06c	SPAC23C11.08	SPAC13A11.04c	SPBC21C3.02c
32	SPCC1450.05c	SPBC3B8.02	SPAC1952.07	SPAC1F7.09c

	wtUBA1 SL	wtUBA1 SS	mutUBA1 SL	mutUBA1 SS
33	SPAC22A12.16	SPBC17D1.06	SPAC14C4.03	SPBC365.11
34	SPAC12B10.07	SPAC11E3.06	SPAC19G12.06c	SPAC144.04c
35	SPAC4A8.05c	SPCC663.01c	SPAC2F7.04	SPCC4F11.03c
36	SPAC1B9.02c	SPAC167.04	SPAC1006.09	SPAC4G8.03c
37	SPAC16C9.05	SPAC14C4.12c	SPAC17G6.04c	SPBP8B7.06
38	SPAC23H3.08c	SPBC2A9.06c	SPAC22H12.02	SPCC1223.05c
39	SPAC2F7.04	SPAC24H6.13	SPBC725.02	SPAC1556.05c
40	SPAC22H12.02	SPAPB1A10.03	SPAC140.01	SPBC16C6.03c
41	SPBC725.02	SPAC12G12.03	SPAC1805.04	SPBC2G2.07c
42	SPAC21E11.03c	SPCC663.03	SPBC1709.10c	SPAC31G5.12c
43	SPBC12C2.02c	SPBC1539.10	SPBC577.15c	SPAC4G9.15
44	SPBC21B10.13c	SPCC1223.05c	SPAC1420.03	SPAC25B8.05
45	SPAC1687.13c	SPCC31H12.04c	SPBC29A10.03c	SPAC1071.07c
46	SPBC3F6.05	SPCC1739.08c	SPAC4F10.04	
47	SPBC4B4.03	SPAC2C4.07c	SPAPB1E7.12	
48	SPAC222.16c	SPAC1142.08	SPCC320.08	
49	SPAC4F10.04		SPCC830.07c	
50	SPAC19B12.10		SPAC4G9.05	
51	SPBC2G2.02		SPBC13G1.14c	
52	SPAC1834.04		SPCC24B10.08c	
53	SPAPB1E7.12		SPAC4H3.03c	
54	SPCC320.08		SPAC1805.05	
55	SPCC830.07c			
56	SPAC4G9.05			
57	SPBC13G1.14c			

145 of the modifiers (83.8%) were orthologous to human genes, and thus are potential drug targets to modify the SMA phenotype.

To generate our candidate human disease gene networks (that we shall call network clusters) we relied on two complementary approaches. Both approaches rely on networks constructed from published protein-protein interaction data from either human (Figure 3 and Figure S3) or fission yeast (Figure 4, Figure S1 and Figure S2). By including two types of genetic interactions, synthetic lethals or synthetic suppressors, yeast augmented network analysis (YANA), yields the most complete unbiased list of potential modifiers. In a first approach, we converted all 145 *S. pombe* modifier genes to their corresponding human orthologs. All the data were combined to create network cluster diagrams. Individual datasets were delineated using a color key. (Note that we designate each interaction as a synthetic lethal or synthetic suppressor *a priori*, and include them both as it is difficult to predict which of these classes will have potential therapeutic value). Using the highest confidence (0.9) experimentally confirmed protein-protein interaction data (from www.string-db.org), we used cytoscape to draw network cluster modules that include all modifier genes. We identified 22 clusters using this approach (Figure S3). We then limited the number of networks by focusing on those modifiers that shared at least two interactions with

other modifiers shrinking the number of clusters to 10 (Figure 3). Within our human primary network clusters, we identified several genes that represent compelling therapeutic targets. These included GSK3 (Glycogen Synthase Kinase-3), CUL3 (Cullin-3) and NEDD4 (E3 ubiquitin ligase) along with SUMO1 (Small Ubiquitin Modifier-1) and HDAC (Histon deacetylase), a known chromatin modifier. Interestingly, GSK3 inhibitors increase SMN levels in spinal muscular atrophy patient-derived fibroblasts and mouse motor neurons²⁸. The HDAC inhibitor Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy²⁹. Therefore, evidence already exists that genes within our clusters affect SMA biology.

In our second approach, we constructed networks from just the *S. pombe* modifier genes using high confidence *S. pombe* protein-protein interaction data from STRING. For this analysis, we identified network clusters that were expanded to include all the so-called “first-degree (1°) interacting proteins”, proteins not identified in our SGA screens but that physically interact with a genetically identified modifier (Figure S1). The rationale underlying our approach to generate larger clusters comes from the facts that the 1° neighbor may be an essential gene (and therefore it is not present in our deletion library) or may fail to be detected in our screens for other,

Figure 3. Human *UBA1* primary gene interaction network. Human orthologs of yeast genes identified from four independent SGA screens were used to map the corresponding protein-protein interaction network. Only proteins that have experimentally verified data (STRING) supporting a physical interaction with at least two additional genes/proteins identified in the *UBA1*-SGA screens are displayed. Of particular interest is the discovery of NEDD4, CUL3, GSK3, SUMO and HDAC in distinct network clusters. Proteins of interest are highlighted by a larger node size and indicated with an arrow.

Figure 4. *S. pombe* extended *UBA1* gene interaction network (extracted from Figure S1 on the basis of identifying new primary hit interactions through a 1° neighbor). Primary yeast gene hits from all four SGA screens were combined and mapped based on highest confidence experimentally confirmed protein-protein interaction data. For this extended analysis, we also included first-degree neighbors of modifiers that were not directly identified in the SGA screens. Only selected gene clusters are shown here; the complete extended network is shown in Figure S1. When extrapolated to human, the yeast extended network analysis identified Csn5 and Cul3, two modifiers also identified in our human *UBA1* primary gene interaction network (boxed; see also Figure 3).

unknown reasons. This “Cluster with 1° neighbor” analysis reveals new interactions not observed in the yeast primary *UBA1* gene interaction network (Figure S2). A few examples of these types of clusters are shown in Figure 4. The clusters include several genes all of which were uniquely identified in the wildtype screen, including both synthetic lethals and synthetic suppressors. Of particular interest is the cluster of proteins surrounding Skp1 (S-phase kinase associated protein-1), a protein that interacts and stabilizes F-box proteins, additional F-box proteins, and proteins involved in cul-1 deneddylation and neddylation. In addition, we found that the RNA-binding protein Cwf2 interacts directly with three of our modifiers, and the plastin ortholog Fim1, interacts with two. Interestingly, Plastin 3 (PLS3) has been identified as a disease modifier in animal models of spinal muscular atrophy^{30–32}.

We then identified correlations between the extended *S. pombe* network clusters (Figure 4) and the human primary network clusters (Figure 3). In both networks, we identified components of the COPS complex, a known regulator of E3 cullins, and the E3 cullin Cul3. Note that Cul3 was not present in our primary *S. pombe* network (Figure S2) yet was present in the more extended network

clusters that incorporate 1° neighbors; this illustrates the importance of adding 1° neighbors to our analysis of the *S. pombe* data.

Together, these data suggest a prominent role for E3 ubiquitin ligases in modulating UBA1 and potentially SMN1, making Cul3 the primary target for further analysis. The E3 ubiquitin ligase might inhibit SMN function, as ubiquitination often leads to protein degradation. If so, we reasoned that inhibition of E3 ubiquitin ligase might enhance SMN activity and thus suppress loss of function mutations in SMN1. To directly test the hypothesis that inhibition of E3 ubiquitin ligases can suppress a SMN1-spinal muscular atrophy phenotype, we turned to a vertebrate model of SMN deficiency previously reported in zebrafish³³. Knockdown of *Smn* in zebrafish embryos causes developmental defects in motor neuron axonal outgrowth that include truncations and abnormal branching of neurons²⁴. Motor neuron axon defects can be corrected by injection of mRNAs encoding wildtype human SMN²⁵. We first confirmed that injection of *smn* morpholino (MO) into wildtype zebrafish causes severe motor axon abnormalities as compared to control uninjected embryos (Figure 5A and 5B). We then tested whether an inhibitor of E3 ubiquitin ligases (MLN4294) would

Figure 5. MLN4294 rescues the abnormal neuron outgrowth in *Smn*-depleted zebrafish. (A) Representative lateral views of motor axons in *Tg(mnx1:GFP)* zebrafish embryos expressing GFP in motor neurons and injected with control MO and then grown in 10 or 15 μ M MLN4294. (B) Quantification of the effects of MLN4294 on motor axon development in zebrafish. Motor axons were scored in *Tg(mnx1:GFP)* embryos injected with control MO, and subsequently (10 hrs post-injection) incubated in 10 or 15 μ M MLN4294. Embryos were classified as severe, moderate, mild, or no defects based on the severity of motor axon defects, and the percentage of each group is shown. Data in all graphs are represented as mean and SEM. (C) Western blot analysis of *Smn* and *HuD* protein following treatment with MLN4294. Quantification of the results is shown in Figure S5.

suppress the motor axon abnormalities. Addition of the Nedd8-E1 activating enzyme inhibitor MLN4294 (that blocks cullin-RING E3 ligases), at concentrations ranging from 10 μ M to 15 μ M, caused a concentration-dependent reduction in the degree of abnormal motor axon branching (Figure 5A and 5B). At 15 μ M inhibitor we found motor axon abnormalities that were completely suppressed: defects were not significantly different to those observed in

uninjected embryos ($P=0.379$). At higher concentrations of drug (20 μ M), the rescue was less pronounced (Table 2) probably because higher levels of drug led to defects in development. Thus, E3 ligase inhibition can rescue neuronal defects caused by Smn protein depletion in zebrafish. In control experiments, MLN4294 failed to rescue the zebrafish mutant *topped* (Figure 6), a mutant defective in neuronal axon guidance³⁴. We also tested a drug that inhibits sumoylation

Table 2. Complete dataset for zebrafish experiments.

	Control MO	smn MO	smn MO + 10 μ M	smn MO + 15 μ M	smn MO + 20 μ M
Severe	0 ($\pm$ 0)	10.1 ($\pm$ 6.3)	3.5 ($\pm$ 2.3)	0 ($\pm$ 0)	2.8 ($\pm$ 2.4)
Moderate	4.4 ($\pm$ 1.7)	39.7 ($\pm$ 1.2)	19.6 ($\pm$ 7.2)	16.4 ($\pm$ 5.6)	11.3 ($\pm$ 7.1)
Mild	44.7 ($\pm$ 6.4)	39 ($\pm$ 7.2)	53.1 ($\pm$ 5)	40.6 ($\pm$ 3.4)	46.8 ($\pm$ 9.6)
No Defects	50.9 ($\pm$ 7.2)	11.2 ($\pm$ 3.2)	23.9 ($\pm$ 5.5)	43 ($\pm$ 7.9)	39.1 ($\pm$ 19)
n (per exp)	23/19/27/25	23/20/28/27	21/23/27/26	19/17/26/26	22/25/26

A

***: $p < 0.001$ NS: $p = 0.4697$**

B

	WT DMSO	<i>topped</i> mutant	<i>topped</i> mutant + 10 μ M	<i>topped</i> mutant + 15 μ M
Severe	1.6 ($\pm$ 2.7)	17.8 ($\pm$ 3.2)	21.4 ($\pm$ 4.4)	13.0 ($\pm$ 3.1)
Moderate	7.7 ($\pm$ 4.1)	10.7 ($\pm$ 2.9)	5.3 ($\pm$ 2.1)	8.7 ($\pm$ 3.4)
Mild	14.0 ($\pm$ 3.5)	22.1 ($\pm$ 4.2)	22.8 ($\pm$ 4.0)	27.4 ($\pm$ 4.8)
No Defects	43.7 ($\pm$ 5.7)	16.5 ($\pm$ 2.8)	17.5 ($\pm$ 3.0)	17.8 ($\pm$ 4.7)
n (per exp.)	25/21/25	25/20/34	25/25/25	22/25/25

Figure 6. Treatment of zebrafish with MLN4294 fails to rescue defects in ventral motor axon guidance in the *topped* mutant. (A) Quantification of the effects of MLN4294 on motor axon guidance in the zebrafish *topped* mutant. Motor axons were scored in embryos injected with DMSO, 10 or 15 μ M MLN4294. Embryos were classified as severe, moderate, mild, or no defects based on the severity of motor axon defects, and the percentage of each group is shown. Data in all graphs are represented as mean and SEM. (B) Experimental data.

corresponding to an unrelated target (SUMO) identified in our human primary network clusters (Figure 3); addition of this compound had no effect on the spinal muscular atrophy model (Figure S6) necessarily surprising since *pmt3*, which encodes the yeast ortholog of *SUMO1*, has the opposite effect of *cul3* when deleted. Having been identified as a synthetic suppressor rather than a synthetic lethal, perhaps compounds that activate rather than inhibit sumoylation would be beneficial as a therapeutic.

To determine how E3 ubiquitin ligases might influence Smn1 function, we examined the protein levels for both Smn and HuD. HuD is a known SMN interacting protein that binds RNAs controlling their translation and stability and functions in neural development and plasticity^{34–36}. We observed greater than a 2-fold increase in both Smn and Hud protein levels following treatment with MLN4294, a NEDD8 activating enzyme (NAE) inhibitor that prevents activation of E3 ligases (Figure 5C and Figure S5). It is therefore possible that rescue of the neuronal defects in our zebrafish model might involve stabilization of Smn as well as of additional proteins within the Smn network.

Growth rate data of expression of UBA1 in *S. pombe*

1 Data File

<http://dx.doi.org/10.5256/f1000research.4188.d28505>

UBA1 was expressed in *S. pombe* under inducing (- thiamine) and non-inducing conditions (+ thiamine) in wildtype strains (wtUBA1) and the disease-causing variant wildtype 1617UBA1 (mutUBA1), under the control of a regulatable nmt1 promoter. Growth rate data of expression of UBA1 under inducing (- thiamine) and non-inducing conditions (+ thiamine) in wtUBA1 and mutUBA1 are shown. For each condition there are six replicates.

Discussion

In this paper we introduce YANA, a fission yeast genetic assay that that when applied to a specific human disease gene can leverage protein-protein interaction data to characterize human disease networks. From these networks, we can identify and prioritize genetic pathways likely to modify the disease-associated gene activity, and predict those genes that can be exploited as therapeutic targets. Using *UBA1*, a gene associated with X-linked spinal muscular atrophy (XL-SMA), we found a high degree of homology between our yeast modifiers and their corresponding human genes (>80%), underscoring the extensibility of the assay. Specifically, we identified several network clusters and high priority targets for therapeutic intervention,

including GSK3 and HDAC both of which are potential therapeutic targets for SMA. In addition, we found two novel related targets Cul3 (a cullin required for E3 ubiquitin ligase activity) and NEDD4, an E3 ubiquitin ligase.

Our yeast system, with its small but complex eukaryotic genome, and complete deletion library, is unique in allowing unbiased genome-wide screening of deletions that alter human disease gene activity. Moreover, YANA can be applied to any human gene, regardless of the phenotype or availability of endogenous mutations. The number of candidate genes identified by YANA for *UBA1* represents a fraction of the approximately 30,000 genes in the human genome, providing a significant enrichment of potential modifiers. Therefore, YANA offers a simple, cost-effective, and relatively rapid technology that could be applied to all human genetic diseases.

Data availability

F1000Research: Dataset 1. Growth rate data of expression of UBA1 in *S. pombe*, [10.5256/f1000research.4188.d28505](https://doi.org/10.5256/f1000research.4188.d28505)³⁷.

Author contributions

G.D. conceived the project. D.W., I.J., L.T.H performed all experiments. X.C. assisted in the bioinformatics analysis. L.B. identified the UBA1 mutation linked to XL-SMA and was co-PI on the MDA grant that supported this research. C.B. was responsible for designing the zebrafish experiments and scoring the results.

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by a grant from the Muscular Dystrophy Association (MDA186435). (Christine Beattie grant support RO1NS050414 (C.E.B.) with additional support from P30NS045758).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank Christian Romero for his expert technical assistance. Special thanks to W. Burhans, C. Boone, B. Andrews, M. Constanzo, C. Myers, C. Nislow, L. Hartwell, J. Dallman, and P. Goldschmidt for their encouragement and discussions during the early phase of these studies.

Supplementary materials

Figure S1. *S. pombe* extended *UBA1* interaction network (complete). The full protein-protein interaction network including first-degree neighbors for all fission yeast gene modifiers identified from our two independent SGA screens.

Figure S2. *S. pombe* UBA1 primary gene interaction network. Fission yeast epistatic modifiers from all four SGA datasets were combined and used to map a protein-protein interaction network using the highest confidence protein interaction data (STRING-db).

Figure S3. Complete human UBA1 primary gene interaction network representing 22 clusters. Human orthologs of yeast genes identified from four independent SGA screens were used to map the corresponding protein-protein interaction network.

Figure S4. Validation of SGA data for *cul3*, *pub1* and *gsk3*. Growth curves of the 3 double mutants confirmed our original SGA data showing that the deletions of *cul3* and *gsk3* are synthetically lethal with OE of *UBA1*, and the deletion of *pub1* (*NEDD4*) suppresses the toxicity associated with OE of wildtype *UBA1*. For *gsk3* and *cul3*, the cells were washed to remove thiamine and immediately placed in the plate reader whilst for *pub1*, cells were first pre-induced for 22 hrs. Deletions of *cul3*, *pub1*, and *gsk3* were confirmed by PCR.

Figure S5. Expression of Smn and HuD in zebrafish embryos. Quantification of western blot results shown in Figure 5C. Treatment of zebrafish embryos with MLN4294 results in approximately a 3-fold increase in both Smn and HuD levels 18 hrs following treatment.

A

	Not Injected				smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO	smn MO
MLN4294	-	-	-	-	-	-	-	-	1 μ M	5 μ M	10 μ M	10 μ M	10 μ M	10 μ M	10 μ M	15 μ M	15 μ M	15 μ M	15 μ M	20 μ M
EXP #	1	2	3	4	1	2	3	4	1	1	1	2	3	4	1	2	3	4	1	2
sev	0	0	0	0	0	0	4	7	1	4	2	1	0	0	0	0	0	0	1	0
mod	1	1	0	2	9	8	12	10	7	7	6	8	3	1	4	5	3	1	4	1
mild	8	12	10	11	13	9	7	8	12	10	11	9	16	16	8	8	8	11	12	9
no defects	14	6	17	12	1	3	5	2	4	0	2	5	8	9	7	4	15	14	5	15
n	23	19	27	25	23	20	28	27	24	21	21	23	27	26	19	17	26	26	22	26

B

	topped Mutant									
MLN4294	-	-	-	10 μ M	10 μ M	10 μ M	15 μ M	15 μ M	15 μ M	15 μ M
Exp #	1	2	3	1	2	3	1	2	3	3
sev	7	5	10	9	7	2	5	4	5	5
mod	3	4	5	2	2	1	4	2	1	1
mild	9	6	6	9	8	7	11	8	8	8
no defects	6	5	13	5	8	15	2	11	11	11
n	25	20	34	25	25	25	22	25	25	25

C

	Not Injected	smn MO	smn MO	smn MO	smn MO
Anacardic Acid	-	-	1 μ M	5 μ M	10 μ M
sev	0	3	0	3	1
mod	0	9	13	13	14
mild	4	13	7	7	6
no defects	15	0	1	0	0
n	19	25	21	23	21

Figure S6. Zebrafish raw data. **A)** MLN4294 treatment in smn MO zebrafish. **B)** MLN4294 treatment in topped mutant zebrafish. **C)** Anacardic acid treatment in smn MO zebrafish.

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Current Peer Review Status:

Version 1

Reviewer Report 23 June 2014

<https://doi.org/10.5256/f1000research.4484.r5211>

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The Wiley *et al* manuscript describes a beautiful synthesis of contemporary genetic approaches to, with astonishing efficiency, identify lead compounds for therapeutic approaches to a serious human disease. I believe the importance of this paper stems from the applicability of the approach to the several thousand of rare human disease genes that Next-Gen sequencing will uncover in the next few years and the challenge we will have in figuring out the function of these genes and their resulting defects. This work presents a paradigm that can be broadly and usefully applied.

In detail, the authors begin with gene responsible for X-linked spinal muscular atrophy and express both the wild-type version of that human gene as well as a mutant form of that gene in *S. pombe*. The conceptual leap here is that progress in genetics is driven by phenotype, and this approach involving a yeast with no spine or muscles to atrophy is nevertheless and N-dimensional detector of phenotype.

The study is not without a small measure of luck in that expression of the wild-type *UBA1* gene caused a slow growth phenotype which the mutant did not. Hence there was something in *S. pombe* that could feel the impact of this protein. Given this phenotype, the authors then went to work and using the power of the synthetic genetic array approach pioneered by Boone and colleagues made a systematic set of double mutants combining the human expressed *UBA1* gene with knockout alleles of a plurality of *S. pombe* genes. They found well over a hundred mutations that either enhanced or suppressed the growth defect of the cells expressing *UBI1*. Most of these have human orthologs. My hunch is that many human genes expressed in yeast will have some comparably exploitable phenotype, and time will tell.

Building on the interaction networks of *S. pombe* genes already established, augmenting these networks by the protein interaction networks from yeast and from human proteome studies involving these genes, and from the structure of the emerging networks, the authors deduced that an E3 ligase modulated *UBA1* and made the leap that it therefore might also impact X-linked Spinal Muscular Atrophy.

Here, the awesome power of the model organism community comes into the picture as there is a zebrafish model of spinal muscular atrophy. The principle of phenologs articulated by the Marcotte group inspire the recognition of the transitive logic of how phenotypes in one organism relate to phenotypes in another. With this zebrafish model, they were able to confirm that an inhibitor of E3 ligases and of the Nedd8-E1 activating suppressed the motor axon anomalies, as predicted by the effect of mutations in *S. pombe* on the phenotypes of the UBA1 overexpression.

I believe this is an important paper to teach in intro graduate courses as it illustrates beautifully how important it is to know about and embrace the many new sources of systematic genetic information and apply them broadly.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 05 June 2014

<https://doi.org/10.5256/f1000research.4484.r4970>

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This paper addresses the vexing problem of how to unravel the complexity of human diseases. Such diseases often involve interactions between genes, and the question is therefore well-suited to an automated genetic approach, such as Synthetic Genetic Array technology, which was pioneered in bakers yeast and more recently adopted to another well-characterized model system, namely *S. pombe*. In this study the authors examine conserved genetic interactions between a fission yeast genes and a human query gene (both wildtype and loss-of-function mutants) then combine their experimental data with bioinformatics to characterize the network of genetic interactions and protein-protein interactions. They then leverage this network to identify interacting partners that comprise novel therapeutic targets.

The example presented in this paper is the human *UBA1* gene which encodes a gene that, when mutated, results in human X-linked spinal muscular atrophy disease. They perform whole genome genetic interaction mapping for digenic interactions with both wild type and mutant versions of the gene, build a network with the observed interactions, and then expand that primary network by adding the known partners that interact with members of the primary network. They then turned to the zebrafish vertebrate model system to validate their newly identified therapeutic targets.

This study adds another tool to the armamentarium of systems biology approaches with which the

complexity of human diseases can be dissected. Because the system is new in the sense that fission yeast has been largely unexplored (compared to its budding yeast relative from which it has diverged by several hundred million years) the observations on fission yeast, when combined with those from other model systems, provide an attractive complementary approach to understanding complex human biology.

Given that this study builds on earlier approaches, the authors should cite the previously published studies on genetic interactions, chemical genetic interactions and protein-protein interactions using budding yeast, fly and other models. Similarly, the comprehensive gene expression profiling approaches used in cultured mammalian cells as well as more recent whole genome RNA interference and CRISPR-based disruption screens should be cited.

From a methodological perspective, in the interaction maps presented in Figures 3 and 4 it would be useful to describe or cite the methods used to define the links between genes. In the Results, where the authors describe the results of the different screens which include wild type and mutant human protein interactions as well as primary and secondary interactions it would be instructive to provide a graphic or flow chart that depicts how all the key pieces of data from each independent screen in fission yeast (and their analysis) were collapsed and presented together. It would also be instructive to provide background information or appropriate citations on the two compounds used in the zebrafish experiments, specifically their known or suspected mechanisms of action as well as the data to support these mechanisms.

In the Discussion, the authors have room to put the YANA screening platform into context, comparing it to other orthologous genome-wide approaches.

A key innovation in this study arises from the unique approach used in analyzing the data. Specifically, the conserved yeast genes that modify the phenotype induced by expressing a human gene of interest are assembled into human disease gene networks which are then augmented by additional protein-protein interaction data. Augmenting traditional network analysis in this manner may permit the identification of new therapeutic targets and target pathways. In summary, this comprehensive, genome-wide work represents an important addition to the systems biology toolkit to understand the complexities of human biology and the impact of this and subsequent data sets will surely increase as additional screens are added to the compendium.

Minor points

- Western blot and growth curve: "*or eight generations*" is a typo, also the type of media the cells were grown in "*exponentially for 16 hours*" should be listed.
- In Figure 2 it is not clear which band the tubulin arrow is pointing to- the loading control bands should be indicated directly on the blot/gel. Also, it is apparent from this loading control that the amount of UPA1 expressed is extremely high, the authors should comment on the fact that the overexpressing lanes are actually UNDERloaded relative to the non-expressing lanes.
- Third paragraph of the Results: the software programme cytoscape should include a reference.

- Third paragraph of the Results: histone is misspelled "*histon*".
- Sixth paragraph of the Results: the authors should clearly distinguish between the published data in zebrafish and their new data collected in this study
- Sixth paragraph of the Results: as mentioned be inhibitor's mechanism of action should be described in more detail
- Second paragraph of the discussion: approximately 30,000 genes in the human genome should be cited and probably corrected to the conventional number of 21,000.

Competing Interests: I was involved in some of the preliminary discussions with the authors about this work in the early phases when these studies were being conceived. However, I can confirm that I was not involved in carrying out any of the research nor in the writing of the final article.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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