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Genome-Wide Identification of Haploinsufficiency in Fission Yeast

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Abnormal phenotypes resulting from haploinsufficiency (HI) are due to the loss of one allele. Recent studies in budding yeast have shown that HI originates from insufficient protein levels or from a stoichiometric imbalance between subunits of protein complexes. In humans, however, HI often involves transcription factors. Therefore, the species differences in HI and the molecular mechanisms of species-specific HI remain under investigation. In this study, HI in fission yeast was systematically surveyed. HI in fission yeast affected genes related to signaling and to basic cellular processes, as observed in budding yeast. These results suggest that there are species differences in HI and that the HI that occurs in fission yeast is intermediate to HI in budding yeast and humans.

Keywords: Genome-wide, haploinsufficiency, ontology, yeast

Heterozygous organisms with a loss-of-function allele often show no detectable change in phenotype. This observation has been attributed to the metabolic theory of dominance [1, 11], which states that one wild-type allele can mask the phenotypic consequences of a loss-of-function allele due to the redundancy of cellular physiology. There are, however, exceptions to this rule, where deletion of a single gene copy cannot mask an abnormal phenotypic change. This phenomenon, which is called haploinsufficiency (HI), is widespread among all eukaryotes from yeast to humans [18].

In budding yeast, *Saccharomyces cerevisiae*, HI has been characterized using gene-by-gene analyses [2, 5, 15]. HI of many cytoskeletal genes, including actin, tubulin, and components of the spindle pole body, suggests that a balance of protein levels is essential to the maintenance of cytoskeletal integrity. Recently, the complete heterozygous collection has been used to identify HI genes throughout the entire genome [4, 13]. The HI genes are predominantly related to basic cellular processes, such as protein biosynthesis and mRNA processing, suggesting that the majority of HI in budding yeast results from insufficient protein levels in the heterozygous deletion strains. However, most HI in humans involves transcription factors, which can affect expression of many downstream proteins [18]. Consistent with this observation, more than 65% of disease-causing mutations are dominantly inherited [8], as is the case for TWIST and GATA3. In contrast, most mutations in genes encoding enzymes are recessive and less than 25% are dominant.

As HI in humans often results in disease, such as Marfan syndrome, cleidocranial dysplasia, and cancer [6, 9, 16, 19], identification of the molecular mechanisms of HI is important and provides useful information for drug target discovery [10]. Moreover, the different molecular mechanims of HI in budding yeast and humans raises the possibility of species differences [13] – a question that is still under investigation. Despite the relevence of HI to human disease, neither the exact molecular mechanism nor the species differences are well understood. The identification of HI genes in fission yeast would be particularly informative regarding the underlying mechanisms of HI, as the fission yeast *Schizosaccharomyces pombe* is known as a micro-mammal among model organisms represented by the National Institutes of Health (http://www.nih.gov/ science/models).

The present study used heterozygous *S. pombe* deletion mutants for genome-wide identification of HI in the fission yeast. The deletion mutants were constructed using the PCR-based gene deletion method with the kanR marker as

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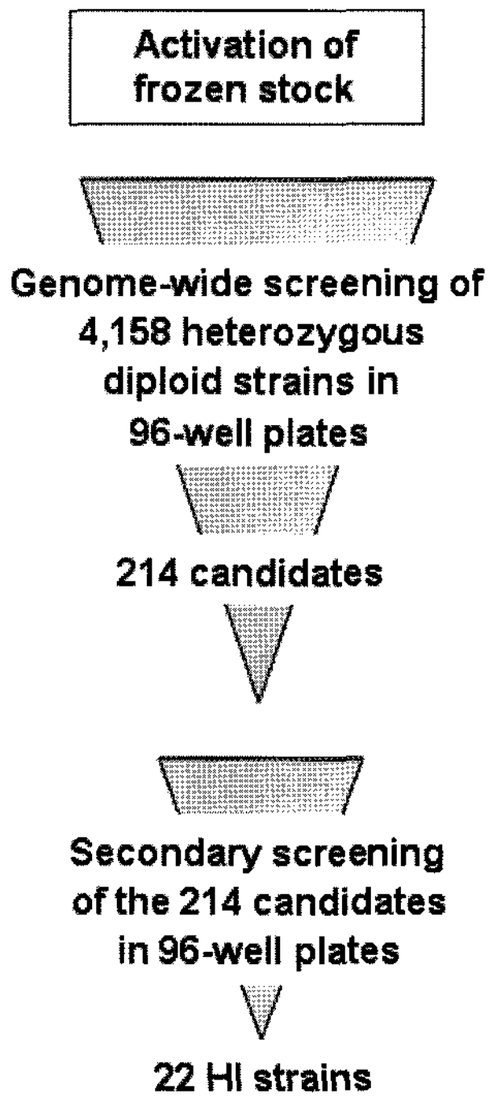

Fig. 1. Strategy used in the genome-wide screening for HI genes.

described previously [14]. In brief, the diploid S. *pombe* strain SP286 (h^+/h^+ *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32*) was transformed with the PCR deletion cassettes, and geneticin-resistant colonies were selected on YES plates containing 100 μg/ml G418. Gene deletion was confirmed by examining the colonies with check-PCR. As shown in Fig. 1, a primary genome-wide screening for HI in the collection of 4,158 heterozygous deletion mutants covering about 83% of the *S. pombe* genome was performed using the automated robotic handling system Biomek NX (Beckman Coulter). First, frozen stocks of the mutants were activated by adding 180 μl of YES-rich medium to 20 μl of mutant per well in 96-well plates, followed by incubation for 36 to 48 h until saturation. The purpose of using saturated cultures was to compensate for strain differences in growth rate and to ensure that similar numbers of cells were used in subsequent experiments. Approximately 5 μl of cells were transferred to 96-well plates containing 195 μl of fresh medium. The growth rate of each strain was then estimated by measuring OD_{620} every 3 h for a total of 30 h using a microplate reader (DTX 600, Beckman Coulter) equipped with Multimode software. This experiment was repeated eight times. As a result of the primary screening, 214 strains were identified as HI candidates. However, the 30-h culture time resulted in inconsistent medium volumes owing to different evaporation rates depending on the well-position in the 96-well plate, resulting in biased OD values. To eliminate the bias associated with position in the 96-well plates, the 214 strains were screened again after randomized positioning

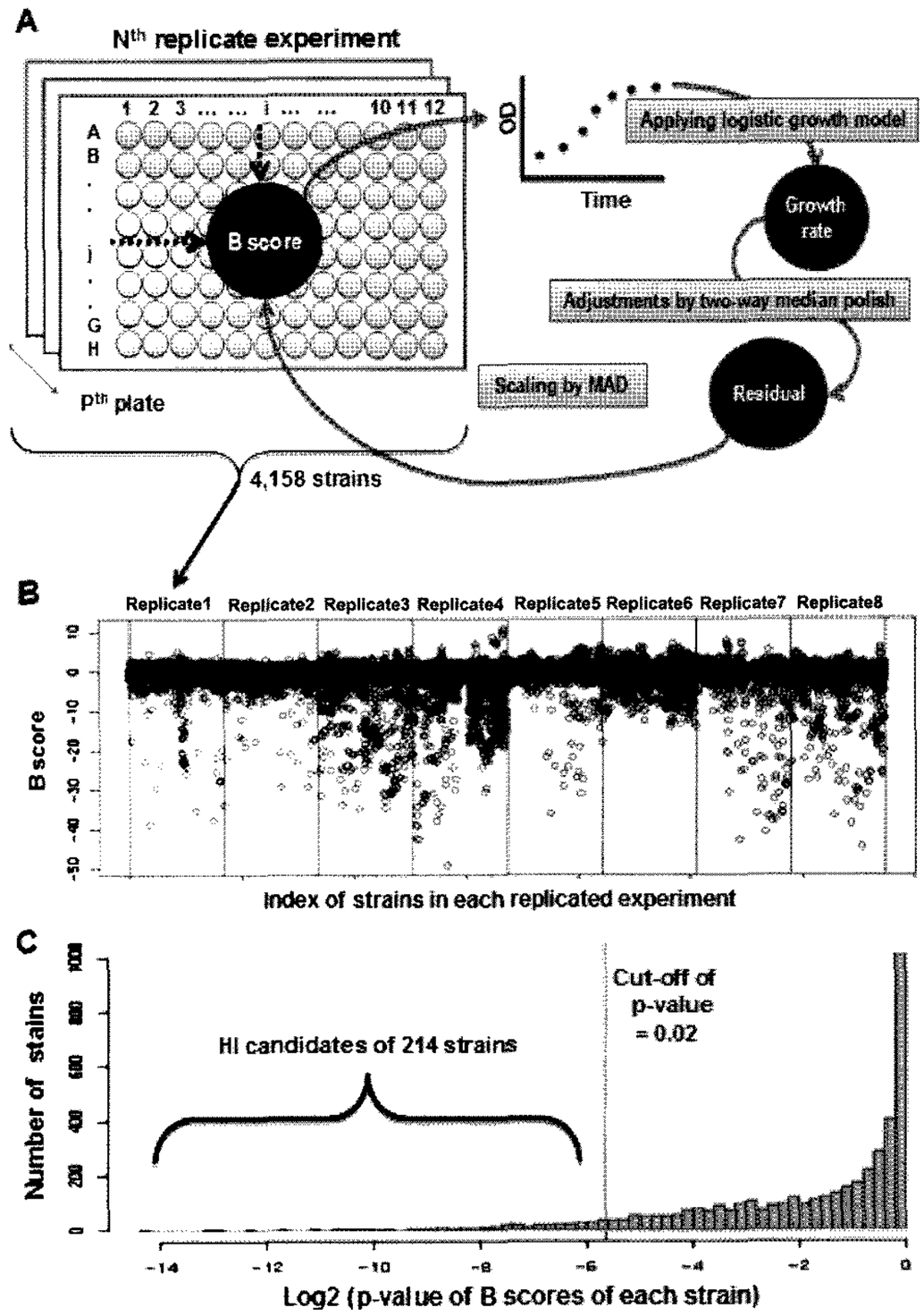

Fig. 2. The primary genome-wide screening for HI genes among 4,158 heterozygous deletion mutants.
The experiment was repeated eight times in 96-well plates using robotics, yielding 214 HI candidate strains, which were subject to a secondary screening. The procedure used to identify HI candidates genes among the 4,158 strains, including OD measurements and B-scores, are illustrated in (**A**), and B-scores from eight independent experiments are plotted in (**B**). B-scores were used to calculate *p*-values representing the probability of being statistically equal to the average growth rate using one-sample *t*-tests. The histogram of *p*-values of the 4,158 strains is plotted in (**C**). The threshold for statistical significance was set at p=0.02.

in the 96-well plates. This secondary screening was repeated three times [12], identifying 22 strains as haphoinsufficient.

For the primary genome-wide screening, it was assumed that the growth of each strain in 96-well plates followed the s-shaped (or logistic) growth model, as is observed during growth with limited resources (Fig. 2). According to this growth model, the growth rate of a strain, r, was calculated from Eq. (1), where t represents the measurement time point, N_0 is the OD_{620} at inoculation, N_t is the OD_{620} at time t after inoculation, and K is the maximal OD_{620} observed for any strain during an experiment.

$$rt = \ln\left(\frac{N_t}{N_0}\right) - \ln\left(\frac{K - N_t}{K - N_0}\right) \quad (1)$$

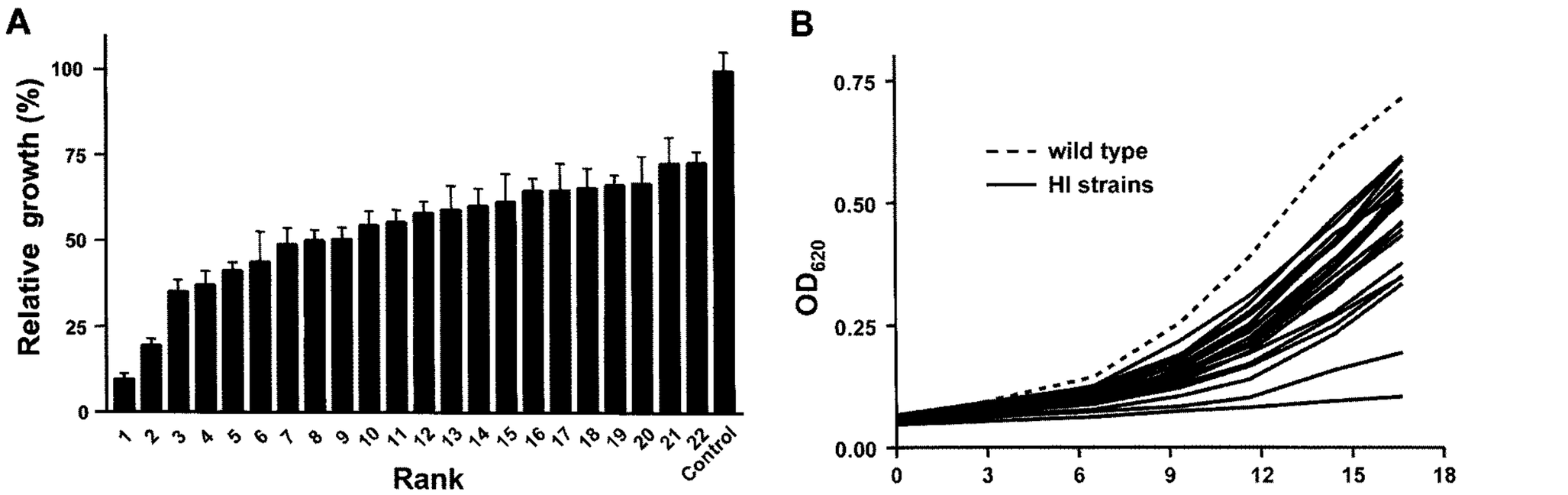

Fig. 3. The secondary screening of the 214 HI candidates and confirmation of slowed growth rate in the liquid culture assay.
A. The average growth rate of 22 HI strains was determined from three independent systematic screenings relative to the growth of wild-type control SP286 cells. **B**. Abnormal growth of the 22 HI strains was confirmed by individual liquid cultures and the order of growth rate was consistent with the results obtained from the systematic secondary screening.

The log-transformed observed values in Eq. (1) were regressed against time to estimate the growth rate, r, of each strain. The estimated growth rates were then normalized as follows. The residual (R_{ijp}) of the growth rate of the cells in row i and column j on the p^{th} plate was obtained using an adjustment based on a two-way median

Table 1. List of 22 haploinsufficient genes.

Gene name	Rank[a]	Essentiality[b]	Gene description[c]	Human ortholog[d]	*S. cerevisiae* ortholog	*S. cerevisiae* HI[e]	GO[f] (Process)
spi1	2	E	Ran GTPase	RAN	YOR185C		Signal transduction
ypt1	9	E	GTPase	RAB1A	YFL038C		Signal transduction
SPBC3F6.01c	13	V	Serine/threonine protein phosphatase	PPP5C	YGR123C		Signal transduction
SPBC25H2.03	7	ND	HEAT repeat	VAC14	YLR386W		PI signaling
mug24	19	V	RNA-binding protein	TIAL1	YPL184C		mRNA regulation
ubc16	21	V	Ubiquitin conjugating enzyme	UBE2D2	YBR082C		Ubiquitination
cyc1	1	E	Cytochrome *c*	CYCS	YEL039C		ATP synthesis
str1	3	V	Siderophore-iron transporter	SLC10A6	YKR106W		Iron homeostasis
SPCC1235.11	4	V	Conserved eukaryotic protein	BRP44L	YGL080W		Unknown (mitochondria)
nda2	66	E	Tubulin alpha 1	TUBA1A	YML085C	●	Microtubule
pcp1	11	E	Pericentrin	CENPE	YDL058W	○	Microtubule
ndc80	16	E	Spindle pole body protein	NDC80	YIL144W	○	Microtubule
SPAC4G9.04c	10	E	Cleavage and polyadenylylation specificity factor	PCF11	YDR228C	○	mRNA process
SPAC30.02c	18	V	Elongator-associated protein	KTI12	YKL110C	○	RNA polII
adk1	8	E	Adenylate kinase	AK2	YDR226W	○	Biosynthesis
rps2	15	E	40S Ribosomal protein S2	RPS2	YGL123W	●	Translation
rps401	20	V	40S Ribosomal protein S4	RPS4X	YHR203C	●	Translation
rps1802	12	V	40S Ribosomal protein S18	RPS18	YDR450W	●	Translation
rps2502	5	ND	40S Ribosomal protein S25	RPS25	YGR027C	○	Translation
rpl1801	7	ND	60S Ribosomal protein L18	RPL18	YOL120C	●	Translation
rpl2002	14	V	60S Ribosomal protein L20	RPL18A	YMR242C	●	Translation
rpl2101	22	E	60S Ribosomal protein L21	LOC653156	YBR191W	●	Translation

[a]Ranks represent the order of slowed growth rate from lowest to highest, as shown in Fig. 3A.
[b]Data of essentiality are obtained from tetrad analysis in this study. "E" and "V" represent essential and viable genes, respectively. ND, not determined.
[c, f]Description and gene ontology are from the *S. pombe* GeneDB (http://www.genedb.org).
[d]Human orthologs are from the KEGG site (http://www.genome.jp/kegg/).
[e]Filled and closed circles represent the presence of corresponding and similar orthologs in budding yeast, respectively.

polish [7, 17], and was scaled with the adjusted median absolute deviation (MAD_p) of all of R_{ij} in the p^{th} plate (Fig. 2A). The normalized score was termed the B-score [12]. Finally, a one-sample *t*-test calculated according to Eq. (2) was used to select strains with growth rates that were significantly lower than the average growth rate in the primary screening (Fig. 2B).

$$t=\frac{\bar{x}}{s\sqrt{1/N}}\bar{x} \quad (2)$$

and s are the arithmetic mean and standard deviation, respectively, of B-scores between N replicated experiments (mostly N=8). *P*-values for the above t static were determined from the t-distribution with N-1 degrees of freedom. A *p*-value ≤0.02 was considered significant in the primary genome-wide screening (Fig. 2C).

To eliminate the position effects that affected the primary screening, a second screening was performed and repeated three times. The well positions of the 214 candidate strains were randomly assigned, and growth fitness was measured as described above for the primary screening. Based on three independent secondary screenings, 22 HI strains showing significantly slower growth than the control SP286 cells were selected (Fig. 3A). The abnormal growth of the 22 HI strains was confirmed using liquid cultures of the individual strains in 15-ml conical tubes (Fig. 3B). All 22 HI strains showed lower growth rates than the control, suggesting that the 22 strains identified were not false positives.

Gene ontology of the 22 HI *S. pombe* genes was used to categorize the genes based on molecular function (Table 1). HI in budding yeast is due either to insufficient protein for basic cellular processes or to an imbalance between subunits of protein complexes. In contrast, HI in humans is due to reduced production of transcription factors, affecting various downstream molecules. Similar to HI in budding yeast, 10 out of 22 HI genes in fission yeast resulted in insufficient protein levels of basic cellular processes, such as translation (n=7), mRNA processing (n=2), and DNA synthesis (n=1). In addition, 3 of the 22 HI genes produced an imbalance in cytoskeletal proteins. The remaining 9 HI genes (shown in the upper half of Table 1) were identified as unique. Four of these genes were classified as playing a role in signal transduction and two were determined to be involved in the regulation of mRNA and ubiquitination, which can affect various downstream targets as occurs in human HI. Intriguingly, three of the HI genes identified are involved in mitochondrial function, consistent with the fact that mitochondrial function is essential in fission yeast, but not in budding yeast [3]. In conclusion, the results of the present study demonstrate that species differences in HI exist and that the mechanism of HI in fission yeast is intermediate to the mechanisms in budding yeast and humans.

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