1 Multiple causal DNA variants in a single gene affect gene 2 expression in *trans*

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9 Abstract

10 Identifying the specific causal DNA differences in a genome that contribute to variation in
11 phenotypic traits is a key goal of genetic research. *Trans*-acting DNA variants that alter gene
12 expression are important sources of genetic variation. Several genes are known to carry single
13 causal variants that affect the expression of numerous genes in *trans*. Whether these single
14 variants are representative of the architecture of *trans*-acting variation is unknown. Here, we
15 studied the gene *IRA2*, which carries variants with broad, *trans*-acting effects on gene expression
16 in two strains of the yeast *Saccharomyces cerevisiae*. We found that *IRA2* contains at least seven
17 causal nonsynonymous variants. The causal variants were located throughout the gene body and
18 included a pair of neighboring variants with opposing effects that largely canceled each other
19 out. The causal variants showed evidence for non-additive epistatic interactions, in particular
20 among variants at the 5' end of the gene. These results show that the molecular basis of
21 *trans*-acting variation can involve considerable complexity even within a single gene.

22 Introduction

23 Genetic differences among individuals contribute to variation in phenotypic traits. The
 24 identification of causal DNA variants that influence a given trait among the vast majority of
 25 variants thought to have no effect is a central challenge in genetics (Rockman, 2012).
 26 Quantitative trait locus (QTL) mapping has been used in thousands of studies to identify
 27 genomic regions that contain causal variants (Mackay et al., 2009). However, the resulting QTLs
 28 are usually broad and span multiple genes and numerous variants. Efforts to identify the
 29 molecular cause of a QTL often implicitly assume that the QTL contains only a single causal
 30 variant that can be “fine-mapped” through a series of experiments, aided by annotations of gene
 31 function and, more recently, computational predictions of variant effect (Adzhubei et al., 2013;
 32 Choi and Chan, 2015; Kircher et al., 2014; Sim et al., 2012). Well-known success stories
 33 demonstrate that single causal genes and variants can be identified in QTLs (Carneiro et al.,
 34 2021; Sutter et al., 2007; Van Laere et al., 2003).

35 At the same time, the assumption that a typical QTL harbors a single causal variant has
 36 frequently turned out to be incorrect (Flint and Mackay, 2009). For example, a single QTL
 37 influencing the ability of yeast to grow in high temperature was shown to harbor three different
 38 causal genes (Steinmetz et al., 2002). A single chromosome region in *C. elegans* contained
 39 multiple independent effects on growth and reproduction (Bernstein et al., 2019). Approximately
 40 40% of QTLs for various physiological traits identified in an outbred rat population were
 41 estimated to be due to multiple causal variants (Rat Genome Sequencing and Mapping
 42 Consortium, 2013). Some of the most finely dissected QTLs affect traits in the yeast
 43 *Saccharomyces cerevisiae* (Fay, 2013). In several yeast QTLs, single genes were found to carry
 44 multiple causal variants (Fidalgo et al., 2006; Gerke et al., 2009). Whether typical QTLs tend to
 45 be caused by one or multiple variants in one or multiple genes remains an open question,
 46 especially given experimental biases that benefit identification of single variants with strong
 47 effect compared to multiple variants with smaller effects (Rockman, 2012).

48 Gene expression variation is a key bridge between DNA variation and organismal phenotypes
 49 (Albert and Kruglyak, 2015). In particular, work in yeast crosses (Albert et al., 2018; Brem et al.,
 50 2002) and human populations (Grundberg et al., 2012; Wright et al., 2014) revealed important
 51 contributions of variants with *trans*-acting effects on gene expression. *Trans*-acting DNA
 52 variants change the activity or abundance of a regulatory factor. This factor influences the
 53 expression of other genes, which can be located anywhere in the genome. In yeast crosses,
 54 *trans*-acting variation arises almost exclusively from “hotspot” regions (Albert et al., 2018) that
 55 contain DNA variants that alter the abundance of many genes. *Trans*-acting hotspots are
 56 commonly identified by QTL mapping of mRNA abundance as the phenotype of interest.
 57 Therefore, like QTLs affecting other traits, the resulting “expression QTLs” (eQTLs) are wide
 58 and contain numerous DNA variants.

59 Due to their broad effects on expression, *trans*-eQTL hotspots have served as models for
60 understanding *trans*-acting regulatory variation. Several yeast hotspots have been dissected to the
61 causal gene (Brion et al., 2013; Lewis and Gasch, 2012; Smith and Kruglyak, 2008) and to single
62 causal variants per gene (Brem et al., 2002; Brown et al., 2008; Fehrmann et al., 2013; Kim et
63 al., 2009; Lutz et al., 2019; Sudarsanam and Cohen, 2014; Yvert et al., 2003; Zhu et al., 2008).
64 Whether these results are representative of most *trans*-eQTL hotspots is unknown.

65 One particularly prominent *trans*-eQTL hotspot is caused by variation in the *IRA2* gene (Smith
66 and Kruglyak, 2008). *IRA2* encodes a regulator of RAS signaling. Together with *IRA1*, it is one
67 of two paralogous genes that encode the yeast homologs of the human Neurofibromin (NF1)
68 gene, mutations in which can cause neurofibromatosis 1, a disease characterized by uncontrolled
69 cell growth (Ballester et al., 1990; Ratner and Miller, 2015). In a cross between the laboratory
70 strain “BY” and the vineyard isolate “RM”, *IRA2* resides in a *trans*-eQTL hotspot that affects up
71 to 1,240 genes (Albert et al., 2018). Previous work showed that *IRA2* is a causal gene in this
72 hotspot (Smith and Kruglyak, 2008). In addition to mRNA abundance, the *IRA2* locus also
73 affects protein levels for many genes (Albert et al., 2014; Großbach et al., 2019), as well as yeast
74 growth in several different environmental conditions (Bloom et al., 2013; Breunig et al., 2014;
75 Wang and Kruglyak, 2014). Variation in *IRA2* may also underlie QTLs for a variety of traits in
76 strains other than BY and RM (Stojiljkovic et al., 2020; Wang et al., 2019).

77 In spite of these broad effects on many traits, the causal variant or variants in *IRA2* remain
78 unknown. In part, this is due to the large size of *IRA2*. With a length of 9.24 thousand bases (kb),
79 the protein-coding *IRA2* open reading frame (ORF) is one of the ten longest ORFs in the yeast
80 genome (Cherry et al., 2012), complicating allelic engineering.

81 Here, we used CRISPR-Swap, an efficient method for yeast genome engineering we recently
82 developed (Lutz et al., 2019), to dissect the molecular basis of the *IRA2* hotspot in the BY / RM
83 yeast cross. We show that the *IRA2* protein coding sequence contains at least seven causal
84 nonsynonymous variants and present evidence that these variants interact with each other in a
85 non-additive, epistatic manner. These results are in contrast to the simpler molecular basis of
86 other *trans*-eQTL hotspots dissected so far, highlighting that even a single causal gene can
87 harbor a complex genetic architecture.

88 Results

89 Protein-coding variation in *IRA2* affects gene expression in *trans*

90 The profound *trans* effects of the *IRA2* locus on the mRNA and protein levels of many genes
 91 motivated us to identify the nucleotide variant(s) causing these effects. Variants in the coding
 92 region of *IRA2* were previously shown to affect the expression of numerous genes (Smith and
 93 Kruglyak, 2008). However, the specific coding variants that are responsible for these effects are
 94 unknown.

95 Comparison of the *IRA2* alleles of BY and RM identified 26 nonsynonymous and 61
 96 synonymous variants spread throughout the coding region (**Figure 1A**). Comparison with the *S.*
 97 *cerevisiae* sister species *S. paradoxus* and an *S. cerevisiae* isolate from Taiwan that is presumed
 98 to be an evolutionary outgroup to most other strains in this species (Peter et al., 2018) indicates
 99 that 19 of the nonsynonymous variants are derived in RM, while 7 are derived in BY (**Table 1**).
 100 Causal variants may be expected to occur at sites that are conserved across evolution and encode
 101 amino acids with different chemical properties. To ask if any of the 26 nonsynonymous variants
 102 are candidates for causing the *IRA2* effect based on these criteria, we analyzed them using the
 103 Protein Variation Effect Analyzer (PROVEAN) tool (Choi and Chan, 2015). Four of the derived
 104 nonsynonymous variants were marked as “deleterious” (**Table 1**). Variants with strong effects on
 105 phenotypes tend to be at low population frequency (Bloom et al., 2019; Fournier et al., 2019).
 106 However, only two of the nonsynonymous variants are relatively rare in the population, with a
 107 derived allele frequency of 10% or less, and neither of these were predicted to be deleterious
 108 (**Table 1**). Together, this information did not pinpoint an individual variant as the obvious source
 109 of the effects arising from *IRA2*.

110 The Ira2 protein negatively regulates the cAMP-PKA signalling pathway by stimulating
 111 conversion of RAS GTPase (Ras1,2) from the active GTP-bound form to the inactive
 112 GDP-bound form (**Figure 1B**). RAS-GTP activates adenylate cyclase (Cyr1) to produce cAMP.
 113 High cAMP results in activation of Protein Kinase A (PKA) and subsequent phosphorylation of
 114 its targets (Thevelein and de Winde, 1999). For example, high levels of cAMP reduce the stress
 115 response by increasing the phosphorylation and cytoplasmic retention of Msn2,4, which in turn
 116 leads to decreased expression of genes containing stress-response elements (STRE) (Görner et
 117 al., 1998; Martínez-Pastor et al., 1996). One such gene, *GPH1*, encodes glycogen phosphorylase,
 118 an enzyme required for the breakdown of glycogen (Wohler Sunnarborg et al., 2001). The
 119 expression of *GPH1* mRNA and Gph1 protein is strongly affected by the *IRA2* locus, with the
 120 RM allele resulting in higher expression (eQTL LOD = 21, protein QTL LOD = 60) (Albert et
 121 al., 2018, 2014). Therefore, we decided to use *GPH1* tagged with green fluorescent protein
 122 (GFP) as a phenotypic readout for experiments aimed at dissecting which variants at the *IRA2*
 123 locus affect the expression of other genes in *trans*.

Figure 1. RM variants within the coding region of *IRA2* increase *Gph1*-GFP expression in *trans*. A: Schematic showing nonsynonymous variants (lollipops) and domains in the *Ira2* protein. Defined domains are depicted with colored boxes. The matching dark gray region indicates the continuous homologous region between *IRA2* and NF1. The NF1 schematic is modified from (Bergoug et al., 2020). Details on domain designations for *Ira2* can be found in Materials and Methods. B: The role of *IRA2* in the cAMP-PKA pathway including *GPH1*. C: Measurement of the effect of the *IRA2* coding variants in the BY and RM backgrounds. Different shapes represent different plate reader runs. Lines connect measurements of the same transformant. For each strain, 3 – 6 independent transformants were phenotyped 10 – 11 times during two plate reader runs. The boxplots show the median as thicker central lines and the first and third quartiles computed on averaged phenotypes per transformant. D: The *IRA2* allele effect in the BY and RM backgrounds. The allele effect is the $\log_2$ of the average *Gph1*-GFP expression in the presence of the RM allele minus that in the presence of the BY allele. Error bars are the standard error of the estimate.

138 Table 1 – The 26 nonsynonymous BY/RM variants in *IRA2*

Variant	Block	Location on chr XV	BY/RM codons ¹	BY/RM amino acids & position	Taiwanese variant	S. <i>Paradoxus</i> variant	Derived allele frequency	Derived allele PROVEAN prediction	Derived allele PROVEAN score	RM allele effect	p-value
1	1	171511	acC/acT	N148H	BY	BY	0.53	Neutral	-1.23	-0.01	0.6
2	1	171515	Aac/Cac	S149N	RM	BY	0.35	Neutral	-0.105	-0.017	0.35
3	1	171671	aGt/aAt	N201S	BY	BY	0.18	Neutral	-1.94	0	0.99
4	1	171973	Tat/Cat	Y302H	BY	BY	0.52	Deleterious	-3.12	0.016	0.32
5	1	171985	Cat/Gat	H306D	BY	BY	0.52	Neutral	0.3	-0.01	0.58
6	1	172102	Gct/Act	A345T	BY	BY	0.29	Neutral	-1.85	-0.012	0.5
7	1	172468	Aat/Gat	N467D	BY	BY	0.23	Neutral	-1.24	-0.011	0.57
8	1	172768	Gtc/Atc	V567I	BY	BY	0.3	Neutral	-0.07	0.008	0.6
9	2	173080	Gtg/Atg	V671M	RM	RM	0.06	Neutral	-0.15	0.009	0.66
10	2	173105	aAt/aTt	N679I	BY	BY	0.5	Deleterious	-5.64	-0.027	0.17
11	2	173340	caA/caC	Q757H	BY	BY	0.13	Neutral	-0.73	0.043	0.057
12	2	174019	Agc/Tgc	S984C	BY	BY	0.38	Deleterious	-2.83	-0.032	0.073
13	2	174364	Ccg/Tcg	P1099S	RM	RM	0.42	Neutral	-2.323	-0.016	0.4
14	2	174472	Ctt/Ttt	L1135F	BY	BY	0.51	Neutral	-1.85	0.049	0.0065
15	3	175135	Ata/Gta	I1356V	BY	BY	0.5	Neutral	-0.39	0.028	0.17
16	3	175142	tCt/tTt	S1358F	BY	BY	0.51	Neutral	2.53	-0.034	0.066
17	3	175588	Atc/Gtc	I1507V	RM	RM	0.36	Neutral	-0.43	0.069	0.0027
18	3	176239	Gca/Tca	A1724S	RM	RM	0.41	Neutral	-1.057	-0.034	0.06
19	3	176268	aaA/aaT	K1733N	BY	BY	0.6	Neutral	0.56	0.026	0.163
20	3	176348	aAc/aGc	N1760S	RM	BY	0.33	Neutral	0.653	-0.007	0.69
21	3	176441	tCa/tTa	S1791L	BY	BY	0.52	Neutral	-1.65	-0.003	0.89
22	4	177067	Ttt/Gtt	F2000V	BY	BY	0.39	Neutral	-0.38	-0.066	0.00047
23	4	178159	Ccc/Tcc	P2364S	BY	BY	0.41	Deleterious	-4.08	0.042	0.02
24	4	179291	gCc/gTc	A2741V	BY	BY	0.25	Neutral	0.88	0.006	0.74
25	4	179652	ttT/ttG	F2861L	BY	BY	0.1	Neutral	2.56	0.006	0.74
26	4	180056	aAt/aCt	N2996T	RM	RM	0.23	Neutral	0.072	0.009	0.62

139 ¹The base in the codon altered by the variant is indicated by a capital letter.

140 To test if *IRA2* is indeed the causal gene at this locus, we exchanged alleles of the *IRA2* ORF in
141 strains carrying Gph1-GFP using our CRISPR-Swap strategy for rapid genome engineering
142 (Lutz et al., 2019). We then measured Gph1-GFP fluorescence and optical density (OD₆₀₀) of the
143 engineered strains during 24 hours of growth in a plate reader. The Gph1-GFP expression level
144 was extracted at the inflection point of the growth curve and normalized using the OD₆₀₀ value at
145 this time (see Materials and Methods). The presence of the RM allele compared to the BY allele
146 resulted in significantly higher Gph1-GFP expression, confirming that coding variants in *IRA2*
147 contribute to the effects of this locus (**Figure 1C**). The effect was present in both the BY and RM

148 strain backgrounds. However, the effect was larger in RM than in BY (Analysis of variance
149 [ANOVA], p-value for the interaction between *IRA2* genotype and strain background: 5×10^{-8} ;
150 **Figure 1C and D; and Table S3**), suggesting the presence of epistatic interactions between
151 variation in the *IRA2* coding region and other variants elsewhere in the genome.

152 Increased expression of Gph1-GFP in the presence of the *IRA2*-RM allele is consistent with the
153 direction of known effects of this locus on *GPH1* mRNA and protein (Albert et al., 2018, 2014).
154 Based on the function of Ira2 (**Figure 1B**), increased *GPH1* expression is expected in the
155 presence of a more active *IRA2* allele. Thus, in accordance with previous results (Smith and
156 Kruglyak, 2008), our findings suggest that the RM allele of *IRA2* has higher activity than the BY
157 allele.

158 ***IRA2* harbors multiple causal variants that show epistatic interactions**

159 To narrow in on the causal variant in the large *IRA2* gene, we divided the *IRA2* ORF into four
160 blocks balancing size and number of nonsynonymous variants (**Figure 2A**). We performed these
161 experiments in BY due to the higher efficiency of genome engineering in this strain background
162 (Lutz et al., 2019). We created 16 chimeric alleles representing all combinations of the four
163 blocks to also allow testing for non-additive (“epistatic”) interactions between the blocks. Each
164 block combination was represented by five or six independent transformants that were each
165 assayed five times for their effect on Gph1-GFP expression.

166 At each of the first three blocks, the *IRA2* RM sequence significantly increased Gph1-GFP
167 expression, with the largest effect resulting from block 1 (**Figure 2B; Table 2; Figure S1 and**
168 **Table S4**). Thus, there must be at least three causal variants in the *IRA2* RM allele, with at least
169 one variant in each of blocks 1, 2 and 3.

170 We noticed that one combination of blocks (with the RM at the first 3 blocks and the BY allele at
171 the last block, “RRRB”) resulted in significantly higher Gph1-GFP expression than the allele
172 carrying RM alleles at all four blocks (One-way ANOVA: $p = 0.035$, **Figure 2B**). Thus, higher
173 Gph1-GFP levels than those driven by the full RM allele can be achieved by combinations of BY
174 and RM variants. Considering that block 4 had no effect when swapped in isolation (**Figure 2B;**
175 **Table 2 and Table S4**), this difference between the RRRB construct and the full RM allele also
176 suggests that there are epistatic interactions between variants in block 4 and variants in the other
177 blocks.

178 To comprehensively test for epistatic interactions among blocks, we performed ANOVA on our
179 fully crossed set of block chimeras. Three combinations of blocks showed nominally significant
180 non-additive interactions (**Table 2**), which exceeds the expectation of 0.6 out of 12 interaction
181 tests under a null model without epistasis. The strongest interaction was seen between block 1
182 and block 2 and is visualized in **Figure 2C**. In all three significant interactions, the observed

Gph1-GFP expression driven by the combined blocks was less than that expected from the sum of the individual block effects (note negative effect signs in **Table 2**), indicating negative epistasis between these blocks of variants. In summary, *IRA2* harbors multiple variants that affect Gph1-GFP expression in *trans*, some of which interact in a non-additive manner.

Figure 2: Effect of blocks of *IRA2* variants on Gph1-GFP expression. A: Schematic of *Ira2* showing the nonsynonymous variants (lollipops) and block junctions (red vertical lines). B: Allele effect of each of the sixteen block combinations compared to an allele with BY in all blocks. P-values are for a comparison between the given allele and the BY allele, only significant values are displayed. Error bars are standard errors of the given estimate. C: Epistasis between blocks 1 and 2. The figure shows residual Gph1-GFP expression after removal of random effects of plate and transformant identity (Materials and Methods). Measurements are grouped by their alleles at blocks 1 and 2. The remaining two blocks are any combination of BY or RM. The horizontal black lines connect the average allele effect for the indicated genotype. Epistasis is highlighted by the steeper slope of the line when block 1 is BY rather than RM.

197 **Table 2 – ANOVA of the effects of blocks 1 to 4 and all possible interaction terms**

	Effect	95% effect confidence interval	F-value	p-value
b1	0.13	0.08 – 0.19	85.57	3.9 x 10⁻¹⁴
b2	0.06	0.01 – 0.12	16.86	9.9 x 10⁻⁵
b3	0.05	0.001 – 0.11	21.93	1.2 x 10⁻⁵
b4	0.004	-0.05 – 0.06	2.96	0.09
b1:b2	-0.08	-0.16 – -0.004	8.51	0.005
b1:b3	-0.05	-0.13 – 0.03	0.04	0.84
b1:b4	-0.01	-0.10 – 0.07	0.04	0.85
b2:b3	-0.01	-0.08 – 0.05	6.34	0.01
b2:b4	-0.03	-0.11 – 0.05	1.16	0.28
b3:b4	-0.005	-0.12 – 0.03	0.00	0.97
b1:b2:b3	0.1	-0.002 – 0.22	0.66	0.42
b1:b2:b4	0.009	-0.11 – 0.12	3.36	0.07
b1:b3:b4	0.09	-0.03 – 0.20	0.05	0.82
b2:b3:b4	0.09	-0.004 – 0.19	0.18	0.67
b1:b2:b3:b4	-0.16	-0.32 – 0.0002	4.22	0.04

198 *Effect estimates are polarized such that positive values correspond to higher expression linked*
199 *to the respective RM allele. Effects were computed using a mixed-effects linear model and*
200 *p-values computed by type I ANOVA. Effect size confidence intervals were estimated using a*
201 *bootstrap procedure. The displayed p-values were not corrected for multiple testing. P-values*
202 *less than 0.05 are shown in bold.*

203 **The effect of each nonsynonymous RM variant is small compared to the full RM allele**

204 To examine the effects of single variants in *IRA2* on Gph1-GFP expression, we engineered 26
205 *IRA2* strains. Each strain carried the RM allele at a single nonsynonymous variant resulting in a
206 single amino acid change, with all other variants carrying the BY allele. We measured the effect
207 of each single variant allele on Gph1-GFP expression (**Figure 3A; Figure S2; and Table S5**).

Figure 3: Analysis of the effects of the nonsynonymous variants in *IRA2*. A: Effect of each nonsynonymous RM variant allele on Gph1-GFP expression. Each strain was represented by nine transformants that were each measured five times. The first eight variants (N148H through V567I) were measured three additional times in an additional plate configuration. P-values are shown for a comparison between the given allele and the BY allele. Only significant p-values less than 0.05 are displayed. B: Bootstrap analysis of the effect of the full RM allele compared to the summed effects of the twenty-six variants. The same-colored vertical lines mark the central 95% quantile range for each bootstrap distribution.

Four of the single-variant RM alleles (L1135F, I1507V, F2000V, and P2364S) resulted in Gph1-GFP expression significantly different from the BY allele. Three of these causal variants were predicted to have a neutral effect by PROVEAN, while the fourth, P2364S, was predicted to be deleterious (**Table 1**). None of the four variants were rare in the population, with derived allele frequencies ranging from 0.36 to 0.51. The causal variants are located in three different blocks: L1135F is in block 2, I1507V is in block 3, and F2000V and P2364S are in block 4. Alignment of *IRA2* with its human homolog NF1 revealed that L1135F is in a region that does not have homology with NF1, while I1507, F2000V and P2364S are in regions that are conserved with humans (**Figure 1**). While three of the single RM variants increased Gph1-GFP expression, the F2000V variant reduced it. This direction of effect of F2000V is in the opposite direction of that of the full *IRA2*-RM allele, revealing transgressive segregation of causal variants in a single gene. The effect of F2000V is likely canceled out by its neighboring variant P2364S, at which the RM allele significantly increased Gph1-GFP. The canceling of these variant effects is in agreement with the fact that block 4 as a whole did not significantly alter Gph1-GFP expression (**Figure 2B**). These results show that no single variant underlies the *trans* effect of *IRA2*-RM on Gph1-GFP expression. Instead, a combination of multiple variants must underlie the overall effect of the full RM allele at *IRA2*.

234 The effects of the single variants were small compared to the effect of the entire *IRA2*-RM allele
 235 (**Figure 3A**). To test whether their joint effect can account for the entire *IRA2* effect in an
 236 additive manner, we used a bootstrap strategy that accounted for measurement error. We created
 237 10,000 bootstrap datasets by randomly sampling with replacement from our individual
 238 measurements. In each dataset, we computed the observed difference between Gph1-GFP
 239 abundance for the *IRA2*-BY and the full *IRA2*-RM allele, as well as the sum of the effects driven
 240 by each of the 26 single variants. The central 95% of the resulting bootstrap distributions
 241 overlapped, such that we cannot formally rule out that the sum of the single variant effects
 242 accounts for the effect of the full RM allele in an additive manner (**Figure 3B**). However, the
 243 overlap was mostly due to the very long tail of the summed single variant distribution, such that
 244 only 3.6% of this distribution exceeded the 2.5% quantile of the full RM allele effect. As also
 245 suggested by the significant epistatic interactions among the four blocks above, it remains
 246 possible that epistatic interactions among variants across the length of *IRA2* are required to
 247 generate the effect of the full *IRA2*-RM allele.

248 **Multiple, epistatic causal variants are near the 5'-end of *IRA2***

249 The results above suggest considerable complexity of genetic variation within *IRA2*, with
 250 multiple causal variants and non-additive interactions between different regions of the ORF. To
 251 dissect this architecture further, we focused on block 1. Although block 1 had the largest effect
 252 on Gph1-GFP expression, none of the eight single variants in this block had a significant effect
 253 on their own. The variants in block 1 could have real but subtle effects, such that their combined
 254 effect could account for the block 1 effect even if no individual variant reached statistical
 255 significance. However, bootstrap analysis showed that the additive effects of these variants
 256 cannot account for the effect of block 1 (**Figure 4A**), suggesting a non-additive interaction
 257 between at least two of the eight variants within block 1.

258 We further divided block 1 into sub-block 1, containing the first five nonsynonymous variants
 259 and assayed its effect on Gph1-GFP expression (**Figure 4B; Figure S3 and Table S6**). The
 260 effect of sub-block 1 was significant ($p = 4 \times 10^{-7}$), but was smaller than the entire effect of block
 261 1 ($p = 0.05$). This result suggests that the effect of block 1 is caused by multiple variants: at least
 262 one among the first five variants and at least one among the following three variants.
 263 Furthermore, bootstrap analysis showed that the summed effects of the first five variants are
 264 unlikely to account for the effect of sub-block 1 (**Figure 4C**). Thus, further division of block 1
 265 caused its effects to splinter and uncovered the existence of non-additive interactions among the
 266 five variants closest to the 5'-end of the gene.

Figure 4: Analysis of the effects of the variants in block 1 and sub-block 1. A: Bootstrap analysis of the effect of the block 1 RM allele compared to the summed effects of the eight variants in block 1. The same-colored vertical lines mark the central 95% quantile range for each distribution. B: Allele effects of the designated *IRA2* genotypes. P-values are for a comparison between the given allele and the BY allele. Only significant p-values less than 0.05 are displayed. Each strain was represented by nine or ten transformants that were each phenotyped three (block 1 RM and sub-block 1 RM) or eight (variants 1–8) times. C: Bootstrap analysis of the effect of the sub-block 1 RM allele compared to the summed effects of the five variants in sub-block 1.

No evidence for an effect of synonymous variants in block 1

Instead of epistasis among nonsynonymous variants, a possible alternative explanation for the differences between the large effects we measured for the blocks and the smaller additive effects of their single nonsynonymous variants could be effects caused by the synonymous variants within the blocks. Specifically, the engineered blocks were generated by amplifying the block regions from BY and RM genomic DNA. Therefore, the blocks include synonymous variants that are not present in our single-variant experiments. Although they do not change protein sequences, synonymous variants have been reported to affect complex traits (Sharon et al., 2018; She and Jarosz, 2018). For example, their effects could arise from changes in translation that could affect *Ira2* expression or function, especially for variants close to the 5'-end of the gene (Plotkin and Kudla, 2011; Tuller et al., 2010). We chose to test the combined effect of the eleven synonymous variants in block 1. Block 1 had the largest effect of the four blocks, while none of its eight nonsynonymous variants had a significant effect on their own. We engineered alleles of block 1 that carried each of the four different combinations of the synonymous variants and

nonsynonymous variants. These alleles showed a strong effect of the nonsynonymous variants in block 1 (ANOVA $p = 8 \times 10^{-7}$). The synonymous variants had no discernible effect ($p = 0.20$; **Figure 5**). Thus, the overall effect of block 1 must be due to multiple nonsynonymous variants with non-additive interactions.

Figure 5: Measurement of the effect of the nonsynonymous variants in block 1. Different shapes represent different plate reader runs. Lines connect measurements of the same transformant. For each strain, seven independent transformants were phenotyped six times. The boxplots show the median as thicker central lines and the first and third quartiles computed on averaged phenotypes per transformant.

Discussion

Our fine-mapping experiments in the *IRA2* gene revealed multiple causal variants spread throughout the *IRA2* ORF that likely act together to create the *trans*-eQTL hotspot at this gene. Altogether, there must be at least seven causal variants in this gene. In our single-variant experiments, we directly identified one variant each in block 2 and block 3, and two variants in block 4. In addition, there must be at least three causal variants in block 1, with at least two in sub-block 1, that epistatically interact to create the strong increase in Gph1-GFP expression caused by this block. In addition to this localized epistasis within block 1, which covers a poorly conserved region of the ORF with unknown function, we also found evidence for epistasis between variants spread further across the gene body. Thus, this single gene harbors a complex molecular genetic architecture.

We were able to detect the small effects of the causal variants in *IRA2* because in conjunction they create a large effect that manifests as a highly pleiotropic *trans*-eQTL hotspot that drew our

312 attention to this region. It is interesting to speculate how many variants of similar effect may go
313 unnoticed by QTL mapping because they fail to co-occur in a fashion that creates a detectable
314 QTL (Bernstein et al., 2019; Kroymann and Mitchell-Olds, 2005; Metzger and Wittkopp, 2019).

315 Our work has several limitations. First, we used a single, GFP-tagged gene as a phenotype for
316 fine-mapping *trans* effects of *IRA2*. We cannot rule out that other traits affected by the *IRA2*
317 locus are shaped by different causal variants, possibly in genes close to *IRA2*. However, we deem
318 it unlikely that traits that are truly influenced by the *IRA2* gene are shaped by different variants
319 within *IRA2*, given that Ira2 acts high in the RAS signaling pathway (**Figure 1B**). Assuming that
320 *IRA2* variants ultimately affect phenotypes via altered cAMP/PKA signaling, it is unclear how
321 variants could have trait-specific downstream effects. A possible exception may occur if Ira2
322 performs unknown functions other than regulating RAS. Second, while we have provided
323 evidence for the existence of epistatic interactions between variants in *IRA2*, we have not
324 determined the specific variants that engage in these interactions. Because epistatic interactions
325 are probably required for explaining the full difference between the BY and RM alleles, the
326 precise molecular basis for this strain difference remains open.

327 In conclusion, our result of multiple causal variants in the *IRA2* gene mirrors observations about
328 genetic architectures at coarser scales. Quantitative traits including gene expression can be
329 highly polygenic (Bloom et al., 2013; Boyle et al., 2017; Flint and Mackay, 2009; Visscher et al.,
330 2017), and the expression of a given gene is shaped by many loci across the genome (Albert et
331 al., 2018; Brion et al., 2020; Metzger and Wittkopp, 2019). Each of these loci can harbor
332 multiple causal genes (Bernstein et al., 2019; Steinmetz et al., 2002). In turn, these genes can
333 have multiple causal variants, extending the pattern of multifactorial complexity to the finest
334 possible scale of individual nucleotides in a single gene. It remains to be explored whether and
335 why certain genes are more prone to carrying single, strong variants versus multiple variants
336 with smaller effect.

337 Materials and Methods

338 Yeast strains, primers, and media

339 The *S. cerevisiae* strains used here are derived from S288C (BY4741 (MATa, his3Δ1 leu2Δ0
340 met15Δ0 ura3Δ0), referred to as “BY” in the text) and RM-11a, (RM HO(BY) (MATa,
341 his3Δ1::CloNAT, leu2Δ0, ura3Δ0 HO(BY allele) AMN1(BY allele), referred to as “RM”). A
342 complete listing of the strains used in this study can be found in **Supplementary Table 1**. All
343 primers are listed in **Supplementary Table 2**.

344 We used the following media (recipes are for 1L):

345 For general yeast strain growth and storage:

346 YPD (10 g yeast extract, 20 g peptone, 20 g glucose)

347 For selection of the GFP-HIS3MX6 cassette in *GPH1* tagging:

348 SDC-His (1.66 g SC -His -Leu -Ura, 100 mg leucine, 200 mg uracil, 20 g glucose)

349 For selection of the KanMX4 cassette in *IRA2* gene deletions and verifying allele exchanges by
350 CRISPR-Swap:

351 YPD +G418 (G418 sulfate (Fisher Scientific cat# BP6731); 200 µg/ml)

352 For selecting for the CRISPR-Swap plasmid in cells after transformation:

353 SDC-Leu (1.66 g SC -His -Leu -Ura (Sunrise Science; cat# 1327-030), 50 mg histidine, 200 mg
354 uracil, 20 g glucose)

355 For phenotyping Gph1-GFP expression:

356 YNB LowFlo (6.7 g yeast nitrogen base -folic acid -riboflavin with ammonium sulfate and
357 without amino acids (Sunrise Science; cat# 1536-050), 20 g glucose, 50 mg histidine, 100 mg
358 leucine, 50 mg methionine, 200 mg uracil). Sterilized by filtration.

359 For iodine staining:

360 YNB (6.7 g yeast nitrogen base with ammonium sulfate and without amino acids (BD
361 Biosciences cat# 291940), 20 g glucose, 50 mg histidine, 100 mg leucine, 50 mg methionine, 200
362 mg uracil)

363 For solid media, 20 g/L agar was added prior to autoclaving. Yeast were grown at 30°C.

364 ***GPH1*-GFP tagging and *IRA2* gene deletions**

365 Insertions of cassettes for genome modification were performed using a standard PCR-based
366 one-step method (Longtine et al., 1998). Yeast transformations were performed using a standard
367 LiAc procedure (Gietz and Schiestl, 2007). Transformants expressing the selectable marker were
368 single colony purified and insertion of the cassette into the correct location and absence of the
369 unmodified wildtype allele were verified by colony PCR.

370 Strain BY *GPH1*-GFP:HIS3MX6 (YFA0644) was obtained from the GFP collection (Huh et al.,
371 2003). RM HO(BY) *GPH1*-GFP:HIS3MX6 (YFA0649) was created as follows: the *GPH1*-GFP
372 allele was PCR amplified from YFA0644 genomic DNA using primers OFA0471 and OFA0472
373 and integrated into RM HO(BY) (YFA0254).

374 Strains BY *GPH1*-GFP *ira2* Δ ::KanMX (YFA0650 and YFA01430) and RM *GPH1*-GFP
375 *ira2*::KanMX (YFA0654) were created by integration of the *ira2* Δ ::kanMX allele, which was
376 PCR amplified from YFA0666 genomic DNA using primers OFA0468 and OFA0469. BY
377 *GPH1*-GFP *ira2* Δ ::KanMX was created twice because glycerol stock storage at -80°C of
378 YFA0650 resulted in a stark reduction in CRISPR-Swap efficiency.

379 Strain BY *GPH1*-GFP *ira2_block1* Δ ::KanMX (YFA1443) was created by replacing *IRA2* block
380 1 sequence with the KanMX cassette PCR amplified from *nej1* Δ ::KanMX (YFA0007) genomic
381 DNA using primers OFA1079 and OFA1080.

382 **Creation of *IRA2* allele fragments used as repair templates for CRISPR-Swap**

383 All *IRA2* allele fragments were PCR amplified from genomic DNA or commercially synthesized
384 DNA using Phusion Hot Start Flex DNA polymerase (NEB). Genomic DNA was isolated using
385 the ten-minute preparation (Hoffman and Winston, 1987). All PCR fragments were analyzed by
386 agarose gel electrophoresis, excised, purified using Monarch DNA Gel Extraction Kit (NEB),
387 and quantified using Qubit fluorometric quantification (Thermo Fisher Scientific).

388 The *IRA2*(BY) allele was amplified from BY4741 or YLK1879 genomic DNA and the
389 *IRA2*(RM) allele (sometimes referred to as “full-RM”) from YFA0254 or YLK1950 genomic
390 DNA using primers OFA0468 and OFA0469. These primers amplify a 9,431 bp *IRA2* fragment
391 with termini (123 bp at the 5’ end and 68 bp at the 3’ end) that have identical sequence to the
392 region flanking the *ira2* Δ ::KanMX cassette.

393 The *IRA2*-block alleles were created using gene splicing by overlap extension (SOEing) (Horton
394 et al., 1989). Each of the four block regions was PCR amplified from BY (BY4741 for blocks 1
395 and 2 and YLK1879 for blocks 3 and 4) and RM (YFA0254) genomic DNA. Primers used were:
396 block 1, OFA0467 and OFA0455; block 2, OFA0456 and OFA0457; block 3, OFA0458 and
397 OFA0459; and block 4, OFA0460 and OFA0544. Pairwise fusions of block 1 and block 2 (4

combinations) were amplified with primers OFA0467 and OFA0457 and block 2 and block 3 (4 combinations) were amplified with primers OFA0458 and OFA0544. Finally, all pairwise fusions of block 1 and 2, and block 3 and 4 (16 combinations) were fused by amplification with primers OFA0468 and OFA0469.

Single-variant alleles were created by PCR SOEing using complementary primers containing the desired RM variant (see **Table S2**). In the first step, two PCR amplifications were performed using BY (YLK1879) genomic DNA as a template. The first amplification used the variant-specific reverse primer and OFA0467, and the second amplification used the variant-specific forward primer and OFA0544. In the second step, the two PCR fragments were fused using primers OFA0468 and OFA0469.

The *IRA2* block1 RM allele was created as described above for the *IRA2*-block alleles. The *IRA2* sub-block 1 RM allele was created by PCR SOEing of two PCR fragments. Fragment 1 was amplified with primers OFA0467 and OFA0589 from RM genomic DNA (YLK1950), and fragment 2 was amplified with primers OFA0536 and OFA0544 from BY genomic DNA (YLK1879). The two PCR fragments were fused using primers OFA0468 and OFA0469.

The *IRA2*-block 1 synonymous and nonsynonymous variant alleles were PCR amplified using primers OFA0468 and OFA1124. Amplification with these primers creates a 1,978 bp *IRA2* fragment with termini (123 bp at the 5' end and 54 bp at the 3' end) that are identical to the region flanking the *ira2_block1Δ::KanMX* cassette. The synBYnonsynBY and synRMnonsynRM alleles were amplified from BY and RM genomic DNA (YLK1879 and YLK1950, respectively) and the synBYnonsynRM and synRMnonsynBY alleles were amplified from synthetic DNA (Twist Bioscience).

CRISPR-Swap

We followed the protocol of (Lutz et al., 2019). For each CRISPR-Swap transformation, the amount of the *IRA2* allele repair fragments ranged from 1000-1500 ng, and the amount of plasmid pFA0055 (Addgene #131774) was 330 ng. pFA0055 expresses Cas9 as well as the guide RNA (gCASS5a) that directs Cas9 to the 5'-end of the KanMX cassette. We recovered 10 to 320 transformants when engineering in BY using the 9,431-kb *IRA2* repair template and 504 to more than 900 transformants when using the 1,978 kb *IRA2* block 1 repair template. Of single colony streaked transformants, 98 – 100% had lost G418 resistance, indicating a successful allele exchange.

The sixteen chimeric block strains were engineered in one batch, as were the strains with the four arrangements of synonymous and nonsynonymous variants in block 1. The BY and RM background strains were engineered in two batches: one created the BY GPH1-GFP *IRA2*(BY) and RM GPH1-GFP *IRA2*(RM) strains and the other the BY GPH1-GFP *IRA2*(RM) and RM

433 GPH1-GFP IRA2(BY) strains. The *IRA2* single-variant strains were engineered in four batches,
 434 corresponding to the four *IRA2* blocks. In each batch, a new IRA2(BY) strain was also
 435 engineered, and in batches 1, 2 and 4 a new IRA2(RM) strain was engineered. The IRA2(BY)
 436 strains from these four batches were similar in their effect on Gph1-GFP expression and
 437 therefore were grouped into one IRA2(BY) genotype that served as the wildtype baseline for
 438 determining the effects of the other alleles. The IRA2(RM) strains were similarly grouped. The
 439 IRA2 block1 RM strain and the IRA2 sub-block 1 RM strain were created in the same batch
 440 (block1) as variants 1 – 8.

441 **Verification of *IRA2* alleles after CRISPR-Swap**

442 All G418 sensitive transformants after CRISPR-Swap were assumed to have exchanged the
 443 KanMX allele for the provided *IRA2* allele. To verify the presence of the correct *IRA2* allele and
 444 that strain mix-ups did not occur during the experimental procedures, some of the strains were
 445 partially genotyped at different stages (see below for details). Verified strains are designated in
 446 **Supplementary Table 1.**

447 The *IRA2* alleles in the BY GPH1-GFP IRA2(BY) (YFA0658 and YFA0659) and RM
 448 GPH1-GFP IRA2(RM) (YFA0662 and YFA0663) strains were genotyped as BY or RM based on
 449 an EcoRI site present in the RM but not the BY allele. Genomic DNA isolated from these strains
 450 was PCR-amplified with OFA0068 and OFA0070. The PCR product was then digested with
 451 EcoRI and analyzed by agarose gel electrophoresis.

452 A representative strain, either transformant #1 or #2, from each of the sixteen *IRA2* block alleles
 453 was confirmed to have the expected arrangement of blocks by verification of the expected
 454 variants at the block borders after Sanger sequencing. Genomic DNA was isolated from cultures
 455 that were started from glycerol stock plates (YPFA010 and YPFA011) and used for PCR
 456 amplification. For each strain, the junction between blocks 1 and 2 was amplified using primers
 457 OFA0585 and OFA0905 and sequenced with OFA0904, and the junction between blocks 3 and 4
 458 was amplified using primers OFA0906 and OFA0907 and sequenced with OFA0903. Prior to
 459 sequencing, the fragments were analyzed by gel electrophoresis and purified using a Monarch
 460 DNA Gel Extraction Kit (NEB). All strains had the expected variants in the sequenced fragment.
 461 One strain, RRBR #1 had a *de novo* variant in the sequenced region resulting in an aGc to aTc
 462 (S1860I) amino acid change. This strain was not removed from phenotyping and its Gph1-GFP
 463 expression was similar to that of the other RRBR transformants. None of the RBRR
 464 transformants were sequenced.

465 To identify potential *ira2* loss of function alleles among the transformants of the sixteen different
 466 *IRA2* block strains, cells were spotted onto YNB agar plates and stained with iodine vapors.

467 Iodine stains wildtype yeast cells containing glycogen a dark reddish brown. Cells with elevated
468 RAS-GTP levels, as in the case of an *ira2Δ*, are unable to store glycogen and therefore stain pale
469 yellow in the presence of iodine (Gil and Seeling, 1999). For each of the sixteen strains, 7 – 8
470 transformants were stained with iodine and 7 / 127 stained pale yellow, possibly due to errors
471 created during the multiple-round PCR amplification of the *IRA2* alleles. Transformants that
472 stained pale yellow were excluded from phenotyping.

473 In the *IRA2* single-variant strains, the expected amino acid change was verified for transformants
474 of N148H, S149N, N201S, Y302H, H306D, A345T, N467D, V567I, Q757H, and I1507V.
475 Genomic DNA was isolated from cells taken from the randomly arrayed starter culture plates and
476 PCR amplified using primers OFA1053 and OFA1054. The PCR fragment was then Sanger
477 sequenced in the region of the expected variant using the following primers: OFA0592 for
478 N148H and S149N; OFA0589 for N201S; OFA0591 for Y302H, H306D, and A345T; OFA0536
479 for N467D; OFA0904 for V567I; OFA0539 for Q757H; and OFA0540 for I1507V.

480 **Phenotyping of Gph1-GFP expression in the plate reader**

481 Starter cultures were inoculated with cells from glycerol stocks and grown overnight at 30°C in
482 800–1000 µl of YPD medium in a 2-ml deep-96-well-plate. The plates were sealed with a
483 Breathe Easy membrane (Diversified Biotech) and placed on an Eppendorf Mixmate set at 1100
484 rpm. After overnight growth, the starter culture plates were sealed with aluminum foil and stored
485 at 4°C for the duration of phenotyping.

486 For each phenotyping plate run, 10 µl of resuspended cells from the starter culture plate was used
487 to inoculate 600 – 800 µl of YNB LowFlo medium in a 2-ml deep-well plate. These pre-cultures
488 were grown overnight as described for the starter cultures. After overnight growth, the
489 pre-cultures were diluted to an $OD_{600} = 0.05$ in 100 µl of YNB LowFlo medium in a 96-well flat
490 bottom plate (Costar) and the plates were sealed with a Breathe Easy membrane (Diversified
491 Biotech).

492 The strains were grown for ~24 hours in a Synergy H1 (BioTek Instruments) plate reader at 30°C
493 with readings taken every 15 min for 97 cycles with 10 sec of orbital shaking between reads and
494 11 – 13 min between cycles. Cell growth was characterized using absorbance readings at 600 nm
495 and Gph1-GFP expression was measured from the bottom of the plate using excitation at 488 nm
496 and emission at 520 nm.

497 Data was processed as described in (Lutz et al., 2019). Briefly, we fit growth curves to each well
498 to identify the inflection point at which the yeast culture begins to exit exponential growth. We
499 extracted the fluorescence and OD_{600} values at this point as well as the two time points flanking
500 it, took their respective averages, and calculated the $\log_2$ of the ratio between the average

fluorescence value and the average OD₆₀₀ value as the Gph1-GFP expression level for the given well.

Designation of *IRA2* domains

Conserved regions between Ira2 and NF1 were determined by multiple sequence alignment of Ira2/Ira1/NF1 sequences from *Candida glabrata* (KTB00138.1), *Saccharomyces paradoxus* (translated from CP020290.1) *Kluyveromyces lactis* (translated from CR382125.1), *Homo sapiens* (P21359.2) and *Saccharomyces cerevisiae* (AAA34710.1 and AAA34709.1) using Clustal Omega (Sievers et al., 2011), as provided through the EMBL-EBI analysis tool API (Madeira et al., 2019).

The CHD (Neurofibromin CTD-homology domain) and Sec-PH (Sec14 homologous and pleckstrin homology-like domain) were defined by previous studies (D'angelo et al., 2006; Luo et al., 2014). We defined here the Threonine-Serine Rich Domain (TSRD). The TSRD extends from amino acid 399 to 1021 and is enriched for serines and threonines, but not enriched for cysteines. This region has 23 / 24 of the detected phosphorylation sites in Ira2 (Holt et al., 2009; Lanz et al., 2021; Swaney et al., 2013). We chose the start of the TSRD at a cluster of serines and the end at the last detected phosphorylated residue. The TSRD is 16.5% serine and 10.6% threonine, while the flanking amino acids 1 – 398 and 1022 – 1645 have 13.1% and 6.3% serine and 8.5% and 5.3% threonine residues, respectively.

Ancestral alleles and BY and RM allele frequencies

Ancestral alleles were determined by comparison to two different evolutionary outgroups to BY and RM. First, we determined the nucleotide allele present in the closely related species *S. paradoxus* after an alignment of *IRA2*(BY) to GenBank #AABY01000044.1. Second, we obtained the nucleotide allele present in the Taiwanese *S. cerevisiae* isolate EN14S01 ('standardized name': 'AMH') from (Peter et al., 2018). EN14S01 is a member of the highly diverged clade 17 thought to have branched early from all other *S. cerevisiae* isolates. Ancestral alleles defined by these two outgroups are in good agreement (Table 1). The population allele frequencies of the BY and RM variants across 1,011 *S. cerevisiae* species were obtained from (Peter et al., 2018). Predictions of variant effects were obtained using PROVEAN (Choi and Chan, 2015) (<http://provean.jcvi.org>) and are based on amino acid conservation and properties. Variant changes resulting in scores of less than -2.5 are predicted to be deleterious.

531 Statistical analyses of allele effects

532 All statistical analyses were conducted in R (<https://www.r-project.org>) version 4.0.4. The effect
533 of RM alleles on the expression of GFP-Gph1 (y) was estimated using the “lmer” function in the
534 lme4 package (Bates et al., 2015) for fitting mixed-effects linear models. In each case, one RM
535 allele (such as a block or a single variant, see below) was compared to strains with the BY allele
536 that were measured during the same runs in the plate reader. The models included the genotype
537 (BY vs. the given RM allele) as a fixed effect, plate and transformant as random effects (denoted
538 in parentheses), and the residual error (ε):

$$539 \quad y = (\text{plate}) + (\text{transformant}) + \text{genotype} + \varepsilon$$

540 The effects of genotype were estimated as slopes of the linear models. Significance was
541 calculated by using type I ANOVAs, as provided by the lmerTest package (Kuznetsova et al.,
542 2017). These linear models and ANOVAs were performed to test the following effects:

- 543 1. The RM vs BY allele in both the BY or RM background
- 544 2. The various BY / RM combinations of the four blocks
- 545 3. The effect of each individual nonsynonymous variant compared to BY

546 Testing for epistasis among the four “block” regions of *IRA2*

547 We tested for epistasis between blocks with a linear model. The model fitted the effects of all
548 four blocks simultaneously, along with all two-way, three-way, and the four-way interactions:

$$549 \quad y = (\text{plate}) + (\text{transformant}) + b1:b2 + b1:b3 + b1:b4 + b2:b3 + b2:b4 + b3:b4 + b1:b2:b3 + \\ 550 \quad b1:b2:b4 + b1:b3:b4 + b2:b3:b4 + b1:b2:b2:b4 + \varepsilon$$

551 We tested the significance of each term using a Type I ANOVA through sequential addition of
552 terms to the model above.

553 Testing for epistasis between single nucleotide variant effects

554 The single nucleotide variants were not engineered in all combinations, precluding the use of
555 interaction terms in a linear model. Instead, we asked whether the effects of single variants could
556 sum to the observed effect of their given multi-variant block or the full *IRA2* RM allele. To
557 properly take into account measurement error across our experimental design, in which
558 individual transformants were run multiple times in several different runs of a plate reader, we
559 used bootstrapping. For each set of variants, we performed stratified sampling with replacement
560 across measurement plates, ensuring that each genotype was represented at the same sample size

as in the full data. For each bootstrapped experiment, we fit the same models as above and extracted the effect estimate. Single-variant effect estimates were summed to calculate the effect they would be expected to have together under an additive model. We performed 10,000 of these bootstraps to generate two distributions. The first distribution represented the difference between BY and the given multi-variant RM allele, and the second distribution represented the sum of the respective single-variant effects. Significance testing was performed by calculating the overlap between the two distributions. If the central 95% quantile range of the two distributions did not overlap, we considered it significantly unlikely that the additive effects of the single variants are able to account for the observed effect of the multi-variant allele. Thus, the less the two bootstrapped distributions overlap, the stronger the evidence for epistatic interactions among the given set of single variants.

Code availability

All analysis code is available at:

<https://github.com/Krivand/Multiple-causal-DNA-variants-in-a-single-gene-affect-gene-expression-on-in-trans>

Strain availability

All strains constructed in this work are available on request.

Supplementary Materials

Figures

Figure S1: Gph1-GFP expression in chimeric *IRA2* block alleles

Figure S2: Gph1-GFP expression in single-variant alleles

Figure S3: Gph1-GFP expression in block 1 alleles

Tables

Table S1: Strains

Table S2: Primers/Oligonucleotides

Table S3: Allele effects and linear models of full RM in BY or RM background

Table S4: Allele effects and linear models of all block combinations compared to BY

Table S5: Allele effects and linear models of single RM variants

Supplementary Table 6: Allele effects and linear models of block 1 RM, sub-block 1 RM, and their single variants

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817 Supplementary Figures

818 **Figure S1** Measurement of Gph1-GFP expression in each of the 16 chimeric *IRA2* block alleles.
819 Chimera alleles are indicated as four letters that are either B or R, indicating the BY or RM
820 allele, respectively, at blocks 1 through 4. Different shapes represent different plate reader runs.
821 Lines connect measurements of the same transformant. For each strain, five or six independent
822 transformants were phenotyped six times. The boxplots show the median as thicker central lines
823 and the first and third quartiles computed on averaged phenotypes per transformant.

Figure S2 Measurement of Gph1-GFP expression in each single-variant strain. Lines connect measurements of the same transformant. The *IRA2*(BY) allele is represented by 36 transformants, each measured 8 times. The *IRA2*(RM) allele is represented by 27 transformants, 9 of which were measured 8 times and 18 of which were measured 3 times. Single-variant alleles 1–8 are represented by 9 different transformants, each measured 8 times. Single-variant alleles 9–26 are represented by 9 transformants, each measured 5 times. The boxplots show the median as thicker central lines and the first and third quartiles computed on averaged phenotypes per transformant.

Figure S3 Measurement of Gph1-GFP expression of block1 variants, alone and in combination. Lines connect measurements of the same transformant. The *IRA2*(BY) allele is represented by 36 transformants, each measured 8 times. The *IRA2*(RM) allele is represented by 27 transformants, 9 of which were measured 8 times and 18 of which were measured 3 times. Single-variant alleles 1–8 are represented by 9 different transformants, each measured 8 times. The block 1 and sub-block 1 alleles are each represented by 10 transformants measured 3 times. The boxplots show the median as thicker central lines and the first and third quartiles computed on averaged phenotypes per transformant.