Supplementary Table 1 | Sanger-sequencing validation of subassembled clones. A total of 40 clones were individually picked and Sanger sequenced across the targeted ORF and associated clone tag, using two reads (Gal4 DBD) or four reads each (p53). Two clones missing from the subassemblies had partially truncated tag sequences (both had single codon replacements with no additional mutations) and one was excluded after failing the allele fraction filter during subassembly. Each of the remaining 37 clone sequences was perfectly concordant with the subassembly consensus sequence bearing the same tag (i.e., no missing or extra mutations). ND, not determined; syn, synonymous mutation.

Barcode from Sanger read	Subassembly and Sanger concordant?	Clone genotype
Gal4 DBD PALS library		
AATGCTGCTGGTGATG	yes	P42D (CCC>GAT)
GTTCTCAGCCGGGCAA	yes	N34G (AAC>GGT), R51K (AGG>AAG)
TGTTAAGGAGACGCGA	yes	T55D (ACA>GAT)
GTAATGAAACTAGGGT	yes	A52X (GCA>TAA)
GAGTAGAGTCGCCGGA	yes	E56G (GAA>GGT)
TAGCATACAAATAAGA	yes	wildtype
AGTGTGGGTGGCATAG	yes	wildtype
ACGTATTAAACAACAC	ND, failed filter	in-frame deletion K18 (programmed)
AATAAGTGACCGGACC	yes	wildtype
TTCATAAAGATCACGT	yes	E8I (GAA>ATT)
TATTTTTAAAAGTGGA	yes	K33D (AAG>GAT)
TCTCAGAGAAATCGTA	yes	S47Y (TCT>TAT), E56L (GAA>TTG)
TAACGTTTTGAATGCG	yes	I7F (ATC>TTT)
p53 PALS library		
GCTTTTGGTACACAGCGTAC	yes	wildtype
ACGTATCGGAAAGCAAATGC	yes	E271S (GAG>TCT), A355F (GCT>TTT), A138syn (GCC>GCT)
CCTGAGTGGGCGACGCCTGA	yes	E2Q (GAG>CAG), Q167syn (CAG>CAA)
AGAAGCTACGTAACAAATTA	yes	wildtype
TCTTGCTTGTGAGGGTGTGG	yes	R202C (CGT>TGT), G245D (GGC>GAC)
ACCCTAAGAGAATACGAGCT	yes	K120L (AAG>TTA)

CTGCGTAGAATGAGCAGGGG	yes	S33F (TCC>TTC), E221L (GAG>TTG)
ATACTCAACATTCTGGACGA	yes	F109R (TTC>CGC), K139syn (AAG>AAA)
GTGCACTCGGGGTAGCAGGG	yes	L137V (CTG>GTC)
TGGTTCCGGACTACAGGAAG	yes	del 1bp frameshift (K371fs)
GCCGCGGGGAGGGCTAGTTA	yes	F212L (TTT>TTA)
CGAGACAATGCAGGTTAGCT	yes	Q165F (CAG>TTT)
TGATATATCGCACCGGAGAA	yes	wildtype
GCACATCCAATACCAGGCGC	yes	E271F (GAG>TTT), K373syn (AAG>AAA)
TTGAGTGGGTCGTGGCAAGA	yes	R175H (CGC>CAC), T81syn (ACA>ACT)
TCCTGACTGCAGGTAGAGGG	yes	G108V (GGT>GTA), G117R (GGG>AGG)
GAACAATGGTACCTGGGAGC	yes	P36S (CCG>TCG), N247F (AAC>TTT)
CCCAAGGTGGGTATAAGGAG	yes	L93Y (CTG>TAC), del 1bp frameshift (S89fs)
GGGAATAAGTAAATGGGCAC	yes	wildtype
GTGGAAAGAGAGGGTAAGAA	yes	A84V (GCC>GTG), A88T (GCC>ACC)
TGGAAGCGCAAAGACTCGAG	yes	P4I (CCG>ATT)
CGAAGGTCGAGTGGTGGACA	yes	del 1bp framehsift (Q191fs), R273C (CGT>TGT), G302syn (GGG>GGA)
AGCTAGGAACGTGAGAAGCC	yes	del 1bp frameshift (I231fs), I232L (ATC>CTC)
TTCTATGCGTGAGTGAGGAC	yes	syn L194:CTT>CTC
GGTATAAAGGGAGCGGGGGC	yes	wildtype
(barcode truncated)	ND, barcode <20 bp	S269P (AGC>CCG)
(barcode truncated)	ND, barcode <20 bp	T253A (ACC>GCT)

Supplementary Table 2 | Gal4 selection cultures and timepoints. SC, synthetic complete

Name	Media	Source	Collection timepoint
INPUT	SC –ura	(Original transformant pool)	0 h
NONSEL_24h	SC –ura	INPUT	24 h
SEL_A_24h	SC –ura –his	INPUT	24 h
SEL_A_40h	SC –ura –his	SEL_A_24h	40 h
SEL_B_40h	SC –ura –his + 0.5 mM 3-AT	INPUT	40 h
SEL_C_40h	SC –ura –his + 1.5 mM 3-AT	INPUT	40 h
SEL_C_64h	SC –ura –his + 1.5 mM 3-AT	SEL_C_40h	64 h

Supplementary Table 3 | Comparison of previously reported activities for Gal4 mutant alleles with effect size measurements in this study. Effect sizes measured in the present study are given as rescaled log2 values (wild-type=0). Jelicic *et al*[40] measured transcriptional activity using a GAL-responsive MEL1 reporter and introduced mutations into a Gal4 fragment containing amino acids 1-100 + 840-881 . Ferdous *et al*[19] performed a similar assay using Gal4 1-147 + 799-1082. Johnston and Dover[15] screened Gal$^-$ mutant alleles within the full-length, native Gal4 locus for activity using a LacZ reporter. ND, not determined.

Alelle	**Measured activity**	**log2 effect sizes following selection (rescaled so wt = 0)**					
		+his, 24h	**-his, 24h**	**-his, 40h**	**-his +0.5m M 3AT, 40h**	**-his +1.5m M 3AT, 40h**	**-his +1.5m M 3AT, 64h**
Jelicic et al[40]							
S5A	~ 70%	0.93	0.27	0.38	-0.42	0.19	1.09
S5D	~ 80%	-0.44	-0.35	-0.39	-0.26	0.12	1.14
S6A	~ wildtype	-1.41	-1.34	-1.56	-1.11	-0.91	-1.65
S6D	~ 75%	0.74	0.38	0.47	0.63	0.21	1.67
S22A	< 5%	0.95	-10.17	-8.90	-9.34	-7.02	-9.14
S22D	~ 25%	-4.91	-6.19	-10.57	-11.00	-11.01	-10.81
S41A	~ 40%	0.70	-0.98	-1.03	-3.42	-4.06	-3.50
S41D	< 5%	1.56	-7.46	-9.94	-11.54	-8.71	-12.35
S47A	~ 25%	0.81	-2.32	-2.69	-9.81	-7.20	-11.61
S59A	~ wildtype	-0.32	-0.85	-0.30	-0.88	-0.62	-0.45
S59D	~ wildtype	-0.81	-0.63	-0.60	-1.21	-0.89	-0.32
Ferdous et al[19]							
S22A	< 5%	0.95	-10.17	-8.90	-9.34	-7.02	-9.14
S22D	~ 20%	-4.91	-6.19	-10.57	-11.00	-11.01	-10.81
K23Q	< 5%	1.21	-2.92	-3.37	-3.94	-3.44	-2.60
K25F	~ wildtype	0.25	0.51	0.62	1.45	2.21	3.57
Johnston and Dover[15]							
C14Y	not detectable	1.30	-3.50	-3.63	-4.34	-3.99	-3.99
R15G	not detectable	1.33	-3.95	-4.56	-7.45	-8.30	-8.37
K17E	not detectable	1.25	-7.44	-10.93	-11.36	-9.56	-12.17
L19P	not detectable	1.47	-3.48	-3.61	-3.20	-2.13	-1.87
S22F	ND (mutant is Gal-)	1.08	-7.81	-12.22	-11.65	-8.75	-12.45
P26L	0.35	0.81	-4.35	-4.78	-10.56	-10.92	-11.95

	(wildtype = 355)						
L32P	not detectable	1.54	-6.17	-6.79	-7.01	-6.59	-5.95
C38G	not detectable	0.45	-2.84	-3.42	-4.12	-4.86	-4.43
S41F	not detectable	1.55	-4.48	-5.07	-6.97	-7.31	-6.65
P42L	not detectable	1.26	-4.14	-4.79	-10.40	-8.37	-10.02
P42S	not detectable	1.05	-2.34	-2.48	-5.63	-6.10	-6.04
S47F	not detectable	1.66	-7.14	-9.99	-11.59	-8.47	-12.40
P48L	ND (mutant is Gal-)	1.21	-6.78	-14.06	-11.50	-8.67	-13.30
P48T	ND (mutant is Gal-)	1.57	-7.22	-10.07	-14.51	-8.81	-13.31
T50I	not detectable	1.51	-4.39	-5.11	-5.81	-6.57	-7.30
R51S	not detectable	1.02	-3.21	-3.56	-2.90	-2.46	-2.22
V57M	1.2 (wildtype = 355)	1.24	-2.07	-2.43	-4.42	-4.63	-4.33

Supplementary Table 4 | Comparison of oligonucleotide synthesis cost, per targeted residue, between PALS and other programmed mutagesis techniques. Cost estimates based upon publicly available list prices for 12k feature 90mer array (CustomArray, Inc.) and 60mer synthesis at the smallest available scale (Integrated DNA Technologies). For both PALS and methods using individually synthesized primers, encoding codon swaps using degenerate NNN triplets required a single oligonucleotide per residue, while specifically programming each codon substitution required 20 (19+1 STOP codon) oligos per residue.

	Each residue replaced by:	
	'NNN' (degenerate, 64 codons)	19 amino acids + STOP
Array-based synthesis (PALS)	$0.28/residue	$5.67/residue
Individual column-based synthesis	$21.00/residue	$420.00/residue

Supplementary Table 5 | Estimated time required, by step, for PALS mutagenesis library construction. *first two steps can be omitted, to use only a single primer library amplification and cleanup step; QC, quality control checks not depicted on **Supplementary Fig. 1**.

Step	Hands-on time (min)	Total time required (min)	Steps from Fig. S1
Mutagenic primer library amplification I*	15	45	1
Mutagenic primer library cleanup*	5	5	
Mutagenic primer library amplification II	20	50	
Mutagenic primer library cleanup	5	5	
PCR amplify wild-type templates	15	70	2,6
Wild-type template cleanup	5	5	
Wild-type strand selection (lambda digest)	5	50	
Wild-type ssDNA template cleanup	5	5	
Qubit library and template quantification	5	15	QC
Primer library and template gel analysis	10	90	
Mutagenic primer extension on sense template	10	25	3
Wild-type template degradation	5	15	4
Primer extension product cleanup	5	5	
Primer extension product enrichment PCR	15	70	
Post-PCR gel analysis	10	90	QC
Suggested pause point, subtotal	2.25 hr	9.08 hr	
Adaptor cleavage (USER treatment)	5	20	4
PCR product cleanup	5	5	
Forward-strand megaprimer synthesis	5	20	5
PCR product cleanup	5	5	
Megaprimer extension (antisense template)	10	25	7
Wild-type template degradation	5	15	8
Primer extension product cleanup	5	5	
Full length product enrichment PCR	15	70	
PCR product cleanup	5	5	
Subtotal	1.00 hr	2.83 hr	
Total	3.25 hr	11.9 hr	

Supplementary Table 6 | Primers

Primer name	**Primer sequence**
truncL_GAL4DBD	TACCTCACGCGATCT
truncR_GAL4DBD	AGATCAATGGCAAAC
truncL_TP53	TGCCATCTTGGATCT
truncR_TP53	AGATCCGAGTTTGTT
L_TP53	GCCAAAGTCAACAAACTCGGATCT
R_TP53	TGTAGTCAGTGCCATCTTGGATCT
L_GAL4DBD	CCCTTCACGTTTGTTCTTGGATCT
R_GAL4DBD	AGGCTATGGGACTTAAAGGGATCT
R_TP53_U	TGTAGTCAGTGCCATCTTGGATC/3deoxyU/
R_GAL4DBD_U	AGGCTATGGGACTTAAAGGGATC/3deoxyU/
L_TP53_U	GCCAAAGTCAACAAACTCGGATC/3deoxyU/
L_GAL4DBD_U	CCCTTCACGTTTGTTCTTGGATC/3deoxyU/
GAL4_CLONE_F	ACTAGTGGATCCCCCGACAGAGAAGCAAGCCTCC TGAAAG
GAL4_CLONE_R	AGGTCGACGGTATCGGCGGCCGCGGGGTTTTTCA GTATCTACGATTCA
GAL4_NTERM_R	ATAACTAATTACATGACTCGAGGTCGACGGTATC GTCATCTATTCAGAACCCATTATTGTTGGGGTCC
GAL4_SENSE_F	CGTTACAGTTCTGCGATTGATCCAAGCGCGCAAT TAACCCTC
GAL4_SENSE_R	AAATCCAACGGAATTGTGGA
GAL4_ANTISENSE_F	ACGATCTATCCAGATTCATGCACTACTACAGCAT CAGTACGACACATGATCATATGGCA
GAL4_ANTISENSE_R	GAACCCATTATTGTTGGGGTCC
OUTER_F	CGTTACAGTTCTGCGATTGATC
GAL4_OUTER_R	AGGTCGACGGTATCGTCATCTATTCAGAACCCAT TATTGTTGGGGTCC
P53_SENSE_F	CAAGTCTCCACCCCATTGAC
P53_SENSE_R	/5Phos/ACGATCTATCCAGATTCATGCACTACTACA GCATCAGTCTCTCGTCGCTCTCCATCTC
P53_ANTISENSE_F	/5Phos/ GTCAGCCTCTAATGGCTCGTATGATAGTGCAGCC GCTGGTCACCAAAATCAACGGGACTT
P53_ANTISENSE_R	CCTCGTAGCGGTAGCTGAAG
SA_REV_BCFWD	ACTTTATCAATCTCGCTCCAAACCAGCTCCACGA GGCAAATGG
SA_REV_BCREV	ACTTTATCAATCTCGCTCCAAACCTATGGTCAATC GTGCATCACGC
GAL4_SA_1F	CTAAATGGCTGTGAGAGAGCTCAGGGTCTTCTCG AGGAAAAATCA
GAL4_SA_2F	GAACCCATTATTGTTGGGGTCC
GAL4_SA_3F	GACAGAGAAGCAAGCCTCCTGAAAG
P53_SA_1F	TGATGCGGCACTCGATCTC

P53_SA_2F	AATTCGTCGACTGGATCCGG
NEXV2_AD1	AATGATACGGCGACCACCGAGATCTACACTCGTC GGCAGCGTCAGATGTGTATAAGAGACAG
SHARED_BC_REV_###	Index 1: CAAGCAGAAGACGGCATACGAGATTACGAAGTC GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 2: CAAGCAGAAGACGGCATACGAGATGACGAGATT GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 3: CAAGCAGAAGACGGCATACGAGATACCGTAAGA GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 4: CAAGCAGAAGACGGCATACGAGATTAGTGGCAA GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 5: CAAGCAGAAGACGGCATACGAGATCATTAACGC GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 6: CAAGCAGAAGACGGCATACGAGATTCGTTGAAG GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 7: CAAGCAGAAGACGGCATACGAGATAAGCGTTCA GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 8: CAAGCAGAAGACGGCATACGAGATCGCAAGCGT GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 9: CAAGCAGAAGACGGCATACGAGATGCAGCGCGA GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 10: CAAGCAGAAGACGGCATACGAGATCGCGCAGCT GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 11: CAAGCAGAAGACGGCATACGAGATTCAAGCGCA GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 12: CAAGCAGAAGACGGCATACGAGATCAGTCGCAG GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 13: CAAGCAGAAGACGGCATACGAGATGCGTCAGTT GACCGTCGGCACTTTATCAATCTCGCTCCAAACC Index 14: CAAGCAGAAGACGGCATACGAGATAGTCGCGCA GACCGTCGGCACTTTATCAATCTCGCTCCAAACC
GAL4_BC_AMP_F	CTAAATGGCTGTGAGAGAGCTCAGAGCTCCACGA

	GGCAAATGG
GAL4_BC_AMP_R	ACTTTATCAATCTCGCTCCAAACC TATGGTCAATCGTGCATCACGC
ILMN_P5_SA	AATGATACGGCGACCACCGAGATCTACACACGTA GGCCTAAATGGCTGTGAGAGAGCTCAG
P416CYC_BC_CAS	CTCGAGTCTAGAAGCTCCACGAGGCAAATGGNN NNNNNNNNNNNNNNGCGTGATGCACGATTGACC ATA GGATCCGAGCTC
P416CYC_AGEMFE_TOP	/5Phos/CACCGGTGCATGTCTGGCTTTAAAATTCAA TTGGGTAC
P416CYC_AGEMFE_BTM	/5Phos/CCAATTGAATTTTAAAGCCAGACATGCACC GGTGGTAC
P416CYC_AMP_BC_CAS_F	GGCCGGTACCACCGGTCTCGAGTCTAGAAGCTCC ACGAG
P416CYC_AMP_BC_CAS_R	AATTGGGTACCAATTGGAGCTCGGATCCTATGGT CA
P53_BC_CAS	AGCTCCACGAGGCAAATGGNNNNNNNNNNNNNN NNNNNNGCGTGATGCACGATTGACCATA
P53_AMP_BC_CAS_F	GGACGTCCAGACACAGCATAGGCTACCTGGCCAT GCCCAGCGGCCGCAGCTCCACGAGGCAAATGG
P53_AMP_BC_CAS_R	GCATGAGAGGACAGTGCCAAGCAAGCAACTCAA ATGTCCCGAATTCTATGGTCAATCGTGCATCACG C

Supplementary Table 7 | PCR conditions used

Program name	**Conditions**
ADO_KHF	95ºC x 3 minutes, { 98ºC x 20 sec, 63ºC x 15 sec, 72ºC x 15 sec, plate read, 72ºC x 5 sec, repeat }.
ADO_KR	95ºC x 3 minutes, followed by 9 rounds of { 95ºC x 15 sec, 57ºC x 15 sec, 72ºC x 20 sec, plate read, 72ºC x 5 sec }
WT_STRAND_PREP	95ºC x 3 minutes, followed by 17 rounds of { 98ºC x 20 sec, 63ºC x 15 sec, 72ºC x 2 minutes, read, 72 x 5 sec}
PALS_EXTEND	95ºC x 3 minutes, 98ºC x 20 sec, 60ºC x 30 sec, 72ºC x 10 min
PALS_AMPLIFY	95ºC x 3 minutes, followed by repeated cycles of { 98ºC x 20 sec, 63ºC x 15 sec, 72ºC x 2 minutes, plate read, 72 x 5 sec} until reactions peak
PALS_SUBASSEM	95ºC x 5 minutes, followed by repeated cycles of { 98ºC x 20 sec, 60ºC x 15 sec, 72ºC x 45 sec, plate read, 72 x 5 sec} until reactions reach mid-log phase
NEXTERA_SUBASM_PCR	No initial extension step. 95ºC x 3 min, followed by repeated cycles of { 98ºC x 20 sec, 62ºC x 15 sec, 72ºC

	x 30 sec, plate read, 72 x 8 sec} until reactions reach mid-log phase
GAL4_BARCODE_PCR_ROUND1	95ºC x 2 min, followed by repeated cycles of {98ºC x 20 sec, 63ºC x 15 sec, 72ºC x 10 sec, plate read, 72ºC x 10 sec } until reactions reach mid-log phase.
GAL4_BARCODE_PCR_ROUND2	95ºC x 2 min, followed by repeated cycles of {98ºC x 20 sec, 65ºC x 15 sec, 72ºC x 15 sec, plate read, 72ºC x 10 sec } until reactions reach mid-log phase.
MAKE_BC_CAS	95'C 2:00, { 98'C 0:20, 67'C 0:15, 72'C 0:10, read, 72 0:10} repeat

Supplementary Table 8 | Summary of sequencing performed.

Purpose	**Instrument model**	**# clusters**	**Read 1 (bp)**	**Index read (bp)**	**Read 2 (bp)**
Gal4 selection tag counting	Miseq	6,717,738	50	9	NA
	GA IIx	38,979,021	36	9	NA
	Miseq	15,826,768	40	9	NA
	Miseq	15,396,976	40	9	NA
	Hiseq	128,777,071	25	9	NA
	Hiseq	90,831,159	25	9	NA
Subtotal		**296,528,733**			
Gal4 Subassembly	Miseq	8,030,018	325	9	188
	Miseq	21,561,690	325	9	200
	Hiseq	171,094,382	101	9	46
Subtotal		**200,686,090**			
TP53 Subassembly	Miseq	4,703,001	325	9	185
	Miseq	8,969,328	325	9	185
	Hiseq	90,525,981	101	9	101
	Miseq	23,788,171	375	9	104
Subtotal		**127,986,481**			

Supplementary References

40. Jeličić, B., Nemet, J., Traven, A. & Sopta, M. *FEMS Yeast Res* n/a–n/a (2013). doi:10.1111/1567-1364.12106