Supplemental Material

Rad9 interacts with Aft1 to Facilitate Genome Surveillance in Fragile Genomic Sites under non-DNA Damage-Inducing Conditions in *S. cerevisiae*

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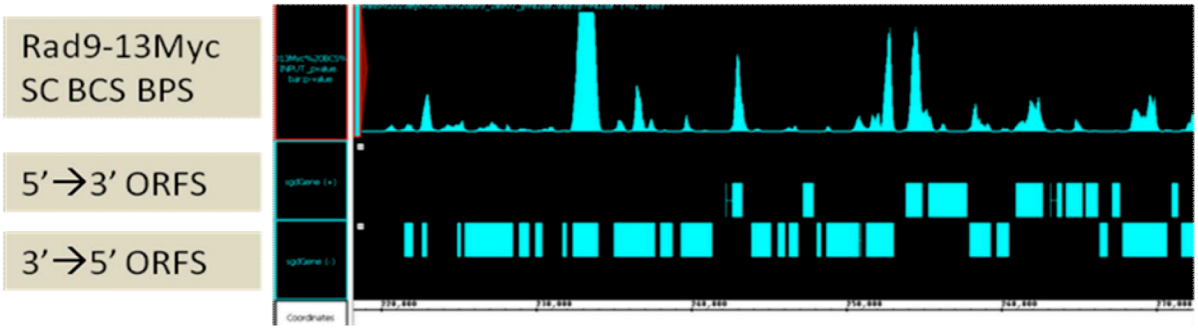
FIGURE S1

Visualisation of the enrichment of Rad9-13Myc protein in a 50kb region of ChrXII.



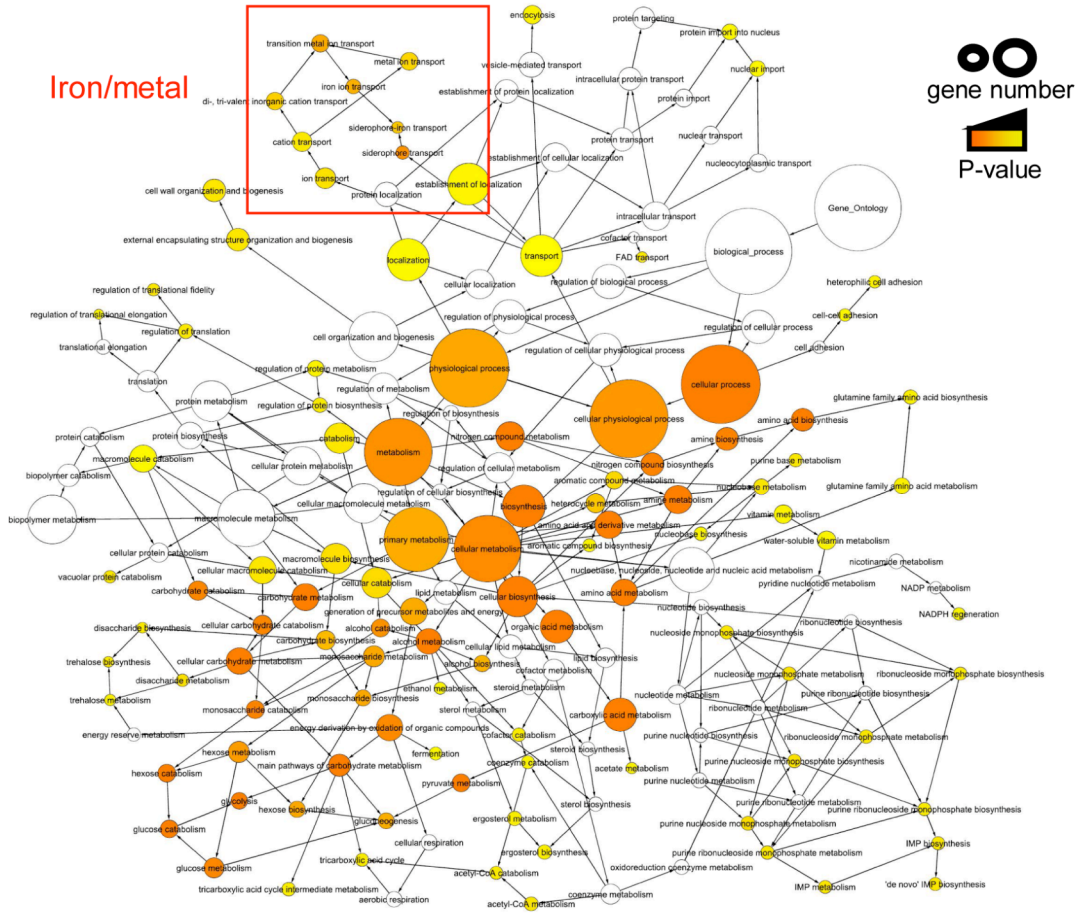
This is a typical localisation pattern of Rad9-13Myc protein, mainly in coding rather than non-coding regions.

Rectangles represent the Open Reading Frames (ORFs) of 5'→3' and 3'→5' genes included in the specific locus.

For details on the analysis, please refer to Protocol S4.

FIGURE S2

Rad9-13Myc localises to the iron regulon genes only when they are induced



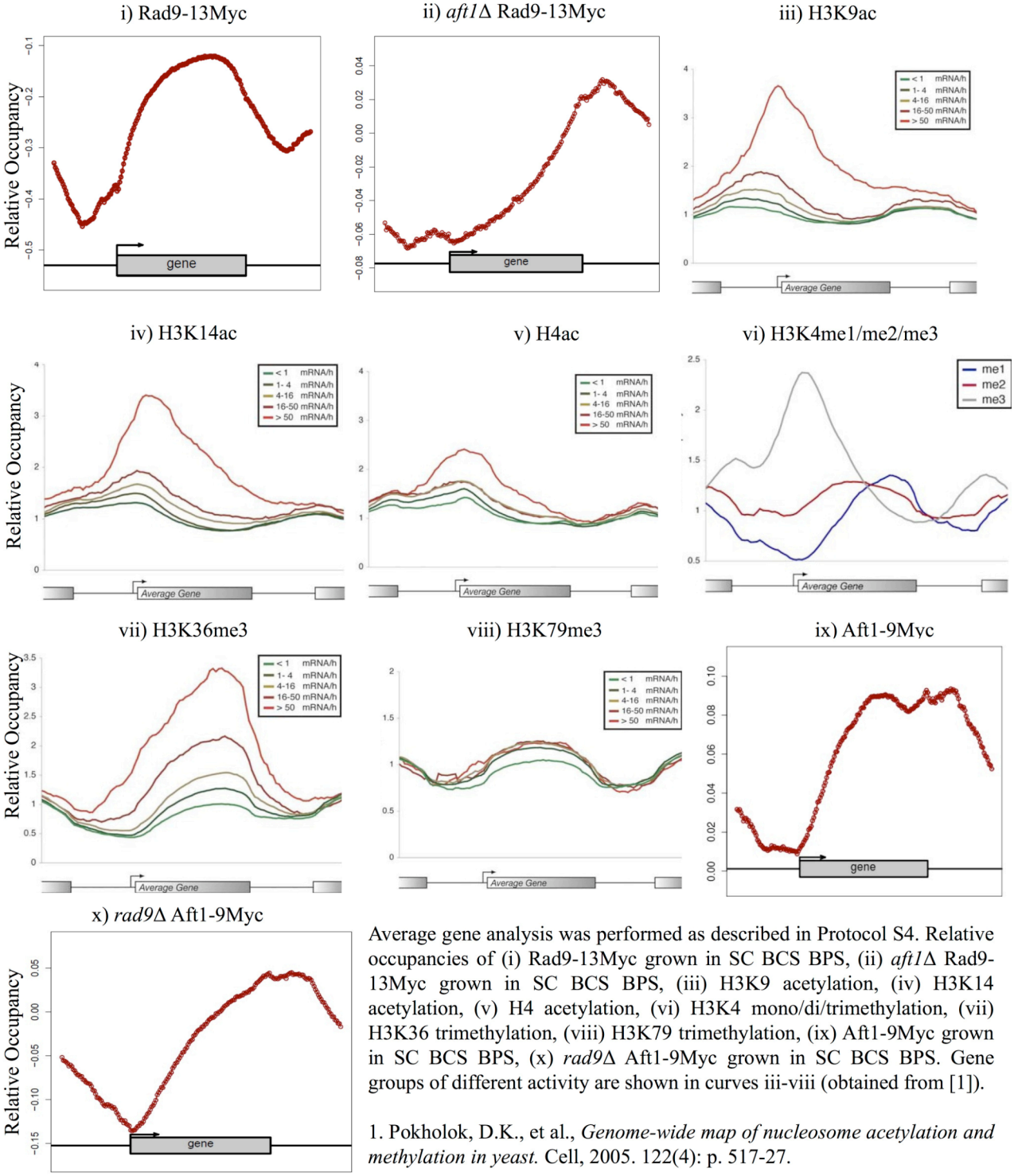
The statistically significant targets of Rad9-13Myc (See Protocol S4) when cells were grown in SC BCS BPS were used to perform functional analysis in order to determine the over-represented Gene Ontology (GO) terms with the use of the BiNGO plugin in the Cytoscape platform [1,2]. GO groups related to iron/metal metabolism are framed in a red box. Rad9-13Myc localises to the genes of the iron regulon only when they are induced, hence transcriptionally active when grown in SC BCS BPS. When these metal chelators are not added in the medium (hence, iron/copper regulons are inactive), Rad9-13Myc is not localised to these groups.
The size of nodes represents the number of genes included, while the colour corresponds to the P-value.

1. Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, et al.: **Integration of biological networks and gene expression data using Cytoscape**. *Nat Protoc* 2007, **2**:2366-2382.
2. Maere S, Heymans K, Kuiper M: **BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks**. *Bioinformatics* 2005, **21**:3448-3449.

FIGURE S3

Relative occupancy of Rad9-13Myc across an average gene in comparison to the respective occupancy of chromatin marks

Average gene analysis data of Rad9-13Myc in the presence or absence of Aft1 (i-ii) and of Aft1-9Myc in the presence or absence of Rad9 (ix-x) were compared to average gene analysis data of chromatin marks as obtained by Pokholok et al. (iii-viii) [1].



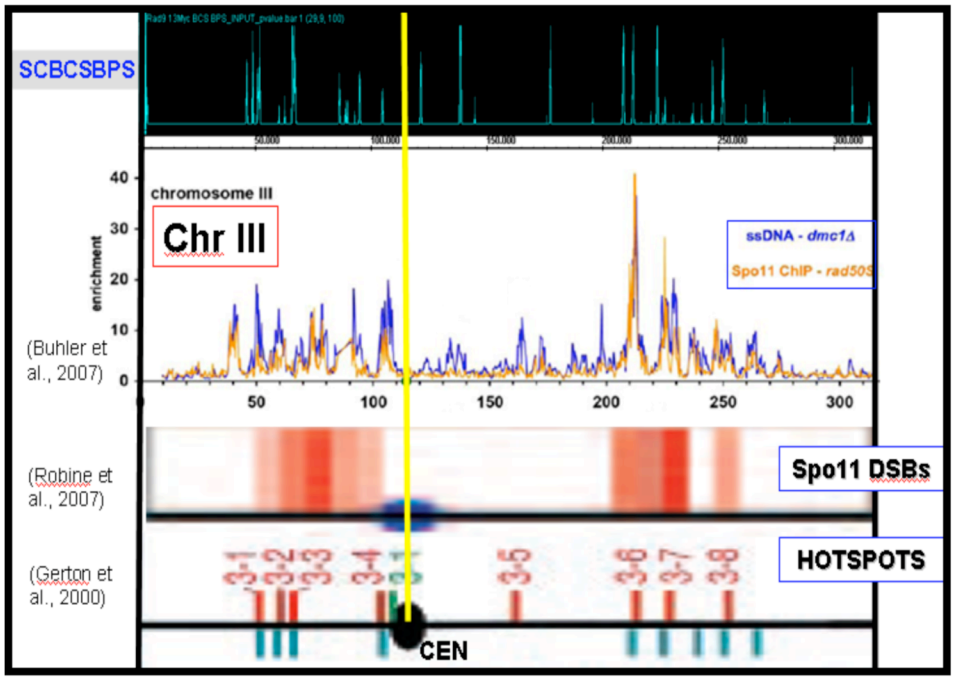
Average gene analysis was performed as described in Protocol S4. Relative occupancies of (i) Rad9-13Myc grown in SC BCS BPS, (ii) *aft1*Δ Rad9-13Myc grown in SC BCS BPS, (iii) H3K9 acetylation, (iv) H3K14 acetylation, (v) H4 acetylation, (vi) H3K4 mono/di/trimethylation, (vii) H3K36 trimethylation, (viii) H3K79 trimethylation, (ix) Aft1-9Myc grown in SC BCS BPS, (x) *rad9*Δ Aft1-9Myc grown in SC BCS BPS. Gene groups of different activity are shown in curves iii-viii (obtained from [1]).

1. Pokholok, D.K., et al., *Genome-wide map of nucleosome acetylation and methylation in yeast.* Cell, 2005. 122(4): p. 517-27.

FIGURE S4

Rad9-13Myc localisation pattern in vegetative cells corresponds to hotspots of meiotic recombination

Selected studies by three individual research groups [1-3] provided a comprehensive map of the meiotic recombination hotspots and hotORFs i.e. genomic regions in which the probability of Double-Strand Break (DSB) formation in meiosis is 100–1000 times higher than the average. Hotspot activity is primarily determined by local features of chromatin structure, notably absence of nucleosomes.



In the top panel, the binding pattern of Rad9-13Myc (grown in SC BCS BPS) in chromosome III, as obtained from our Tiling Array experiments, is visualised using the Integrated Genome Browser. The peaks correspond to genomic areas where Rad9-13Myc is enriched. The rest of the panels show the genomic regions of meiotic recombination hotspots as mapped by three different studies [1-3].

The comparison between Rad9-13Myc localisation pattern and the mapped meiotic recombination hotspots showed a rough coincidence in chromosome III, as well as in all of the yeast chromosomes (data not shown). Based on these observations, we performed a detailed analysis described in the main text of this study.

1. Gerton, J.L., et al., *Global mapping of meiotic recombination hotspots and coldspots in the yeast Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11383-90.
2. Robine, N., et al., *Genome-wide redistribution of meiotic double-strand breaks in Saccharomyces cerevisiae.* Mol Cell Biol, 2007. **27**(5): p. 1868-80.
3. Buhler, C., V. Borde, and M. Lichten, *Mapping meiotic single-strand DNA reveals a new landscape of DNA double-strand breaks in Saccharomyces cerevisiae.* PLoS Biol, 2007. **5**(12): p. e324.

FIGURE S5

A snapshot of *S. cerevisiae* non-ORF features which shows the yeast non-coding features along with the numbers of their population (Saccharomyces Genome Database)

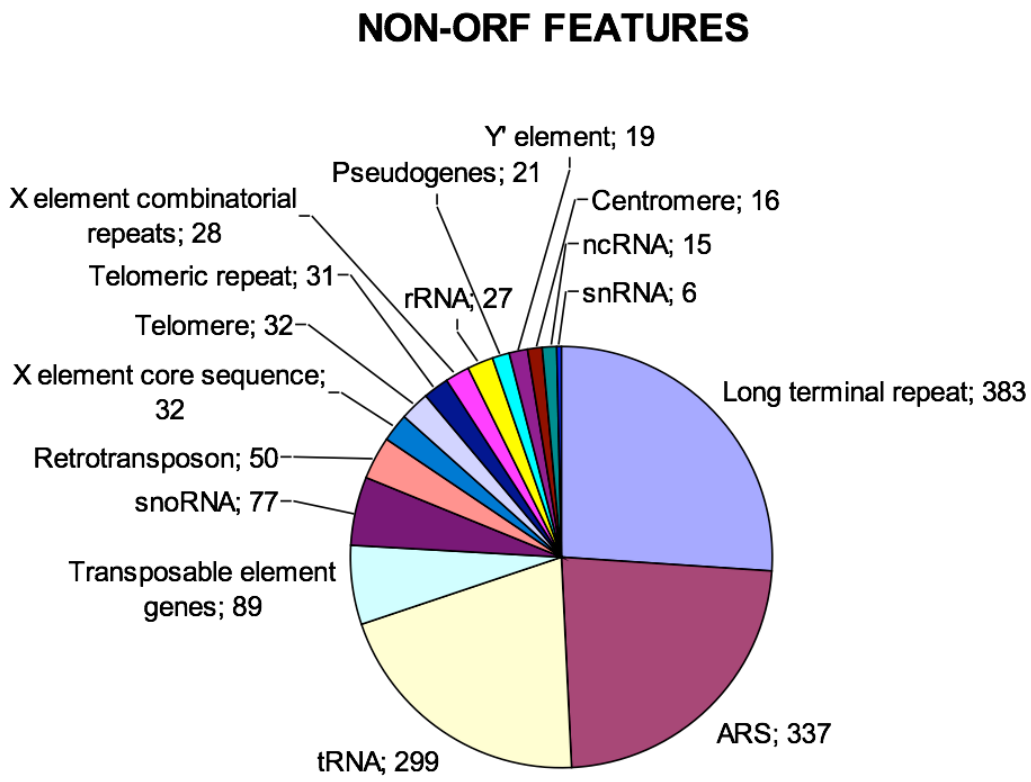
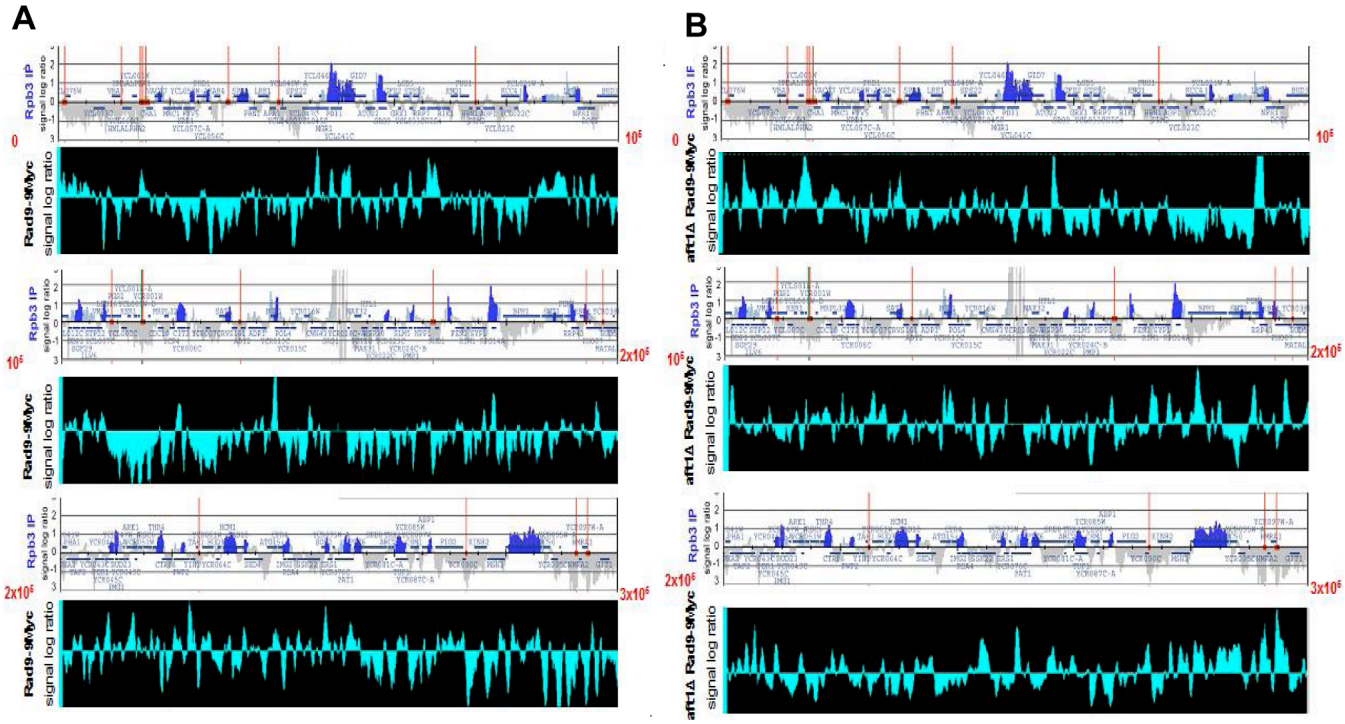


FIGURE S6

Rad9-13Myc localisation to chromosome III in comparison to the respective pattern of RNA Polymerase II (RNAPII)



The figure illustrates the signal log ratio values of RNAPII localisation pattern (Rpb3 IP, white panels) to the first $3x10^5$ base pairs of chromosome III as obtained by Bermejo et al. [1]. This pattern is compared to the respective of Rad9-13Myc signal log ratio values, which are visualised by Integrated Genome Browser as blue peaks or valleys in black background (see also Protocol S4). The same comparison was performed for the *aft1*Δ Rad9-13Myc signal log ratio values.

(A) The comparison of Rad9-13Myc cells grown in SC BCS BPS to the RNAPII genome-wide distribution shows that Rad9-13Myc has a partial overlapping localisation pattern with RNAPII. This is in agreement with the Rad9 localisation bias to transcriptionally active genes. Most of the observed overlap is lost when Aft1 is absent (B). The reduced localisation overlap in *aft1*Δ cells is in agreement with our results on the Rad9-Aft1 relation (see also main text).

1. Bermejo R, Capra T, Gonzalez-Huici V, Fachinetti D, Cocito A, Natoli G, Katou Y, Mori H, Kurokawa K, Shirahige K, et al.: Genome-organizing factors Top2 and Hmo1 prevent chromosome fragility at sites of S phase transcription. *Cell* 2009, 138:870-884.

TABLE S1

List of primers used for tagging/deletion of genes

The primers used in this study have been designed with Vector NTI software and produced by the Micro-chemistry laboratory of IMBB. They have been used for the production of tagged or deletion strains and their validation. They are listed in the table below:

NAME	SEQUENCE	Comments
RAD9 F2	5'-GATATTAC GGACAATGAT ATATACAACA CTATTTCTGA GGTTAGA CGG ATC CCC GGG TTA ATT AA-3'	For Rad9-13Myc tagging in pFA6a cassette
RAD9 R1	5'-AATCG TCCCTTTCTA TCAATTATGA GTTTATATAT TTTTATAATT GAA TTC GAG CTC GTT TAA AC-3'	For Rad9-13Myc tagging in pFA6a cassette
R1 SMALL	5'-GAA TTC GAG CTC GTT TAA AC-3'	CHECK pFA6a CASSETTE
F2 SMALL	5'- CGG ATC CCC GGG TTA ATT AA-3'	CHECK pFA6a CASSETTE
9Myc F (SmaI)	5'-TCCCCCGGGCGTACGCTGCAGGTCGACTCC-3'	pYX142 construct
9Myc R (SlaI)	5'-TCCGCTCGAGGCTAGTGGATCCGTTCAA-3'	pYX142 construct
RAD9 +3F	5'-TCAGGCCAGTTAGTTCAATGGAAAAGC-3'	Check for Rad9 tagging
KAN&HIS	5'-TGGGCCTCCATGTCGCTGG-3'	Check in pYM cassette
RAD9 -83F	5'-GCAACGATGAGCAATGTG-3'	Check for Rad9 deletion
RAD9 +4781R	5'-GCCTCGTTCAGCACATTTCC-3'	Check for Rad9 deletion
TRP primer	5'-GCTATTCATCCAGCAGGCCTC-3'	Check in pYM6 cassette
AFT1 +739F	5'-CCTGATTCCGAAGAGTAC-3'	Check of endogenous untagged gene
AFT1 +2289R	5'-ATCCTGATCTTAGCACCGAT-3'	Check of endogenous untagged gene
RAD9 disr TRP F S2	5'-AATCGTCCCTTTCTATCAATTATGAGTTTA TATATTTTTATAATTATCGATGAATTCGAGCTCG-3'	*rad9*Δ in pYM6
RAD9 disr TRP R S3	5'-GATATTACGGACAATGATATATACAACA CTATTTCTGAGGTTAGACGTACGCTGCAGGTCGAC-3'	*rad9*Δ in pYM6

TABLE S2

Primers used for the qPCR reactions in the manual Chromatin Immunoprecipitation experiments (ChIP)

NAME	SEQUENCE	Comments
FRE7 -959 F	5'-AATACAGAGACAACAAGGAC-3'	FRE7 5'-UTR
FRE7 -717 R	5'- AGAGAATATCAATCACTCCC-3'	FRE7 5'-UTR
FRE7 -355 F	5'- TCCTCGTCTGCGCAAATTG-3'	FRE7 promoter
FRE7 -111 R	5'- TCTTGAGAACAAACGAGTGC-3'	FRE7 promoter
FRE7 +1418 F	5'- GAGATGAAATGTGTACCCTC-5'	FRE7 ORF
FRE7 +1677 R	5'- AGGAATAATGGTGATGAGAC-5'	FRE7 ORF
FRE7 +1984 F	5'- TCTAGAAGACAATACAACGC-3'	FRE7 3'-UTR
FRE7 +2253 R	5'- AAGTAATGTTGCAGGAAGTG-3'	FRE7 3'-UTR
FRE1 -627 F	5'- CCCTTGATGATACTCTAAAC-3'	FRE1 5'-UTR
FRE1 -311 R	5'- TAGATTCTCACCAAGATTGC-3'	FRE1 5'-UTR
FRE1 -409F	5'-GTTGAAGAATACCCGATA-3'	FRE1 promoter
FRE1 -142R	5'-GTAGATTAATCATCGCAAGAT-3'	FRE1 promoter
FRE1 +1321F	5'-GGAGCTTTCGCATACATG -3'	FRE1 ORF
FRE1 +1804R	5'-TTGTGTCCTCAACACTGG-3'	FRE1 ORF
FRE1 +2195 F	5'- ATGCGGGTTCTAAATCTAAG-3'	FRE1 3'-UTR
FRE1 +2426 R	5'- TCCGAAGAATGATGTTGAAG-3'	FRE1 3'-UTR
FTR1 -486 F	5'-CCACGGTCACTACTAACTCC-3'	FTR1 PROMOTER
FTR1 -106 R	5'-AGCTCAAGGTCGAACTACAG-3'	FTR1 PROMOTER
FTR1 +40F	5'-TGTGTTCAGAGAGTGCTTGG-3'	FTR1 ORF
FTR1 +367 R	5'-CGCAATCTTCACTCTCCACT-3'	FTR1 ORF
FTR1 +818 F	5'-TGGACAATGGGTGGGATATC-3'	FTR1 ORF
FTR1 +1165 R	5'-TTTGAGACGAGGAATGACTG-3'	FTR1 ORF
CTR1 -220R	5'-CCTCTCGAGATGACAATAC-3'	CTR1 promoter
CTR1 -475F	5'-CCCTACTATACTACAAACCGATAC-3'	CTR1 promoter
CTR1 +118F	5'-CGATATTATCGAGCATGTCATC-3'	CTR1 ORF
CTR1 +360R	5'-ATCCAT CCCTGAAGAGCTACTG-3'	CTR1 ORF
CTR1 +360F	5'-TATGGACATGAGTATGGGAATG-3'	CTR1 ORF
CTR1 +597R	5'-GGAGTTTGCTGAAGGTAAAGTG-3'	CTR1 ORF
CTR1 +703F	5'-CTCTTTCATGACATTATAAGGGC-3'	CTR1 ORF
CTR1 +944R	5'-TGTCTACCACATTGGCAGTTAC-3'	CTR1 ORF
PHO5 F	5'-CGATATCCTAAACTTTTTGAC-3'	PHO5 ORF control
PHO5 R	5'-GTTTCAATTGGAACAACAGCA-3'	PHO5 ORF control
CMD1 +75F	5'-TGGCTCTATCTCATCAAGTG-3'	CMD1 ORF control
CMD1 +343R	5'-CACCAATGGATGTTAGCACG-3'	CMD1 ORF control

Primers for centromeric regions

NAME	SEQUENCE
CEN1 -103F	5'- TTACCAGCTTCAATAACTCG-3'
CEN1 +221R	5'- CGCCTAGTGCTTAAGAGTTC-3'
CEN2 -67F	5'- GCTATTCAACTGGCACCTAC-3'
CEN2 +261R	5'- TAAATGCGAAGAAGGTATGG-3'
CEN3 -206F	5'- TGCTACCACTTCTATTACAC-3'
CEN3 +188R	5'- ATGAGCAAAACTTCCACCAG-3'
CEN4 -63F	5'-CACGAGCCAGAAATAGTAAC-3'
CEN4 +196R	5'- TAAGCTATGAAAGCCTCGGC-3'
CEN5 -101F	5'- CCTACATGCTACATAAGTCC-3'
CEN5 +250R	5'- CCTTCAACATTGTTTAGCAG-3'
CEN6 -163F	5'- GGAAGAGGTAAAGTAGTCGG-3'
CEN6 +218R	5'- CGTCCAATATCATCGTAAAC-3'
CEN7 -178F	5'- CTTCCTGAACATTCTAATGG-3'
CEN7 +268R	5'- CATTAACTTTCATTGAACCG-3'
CEN8 -122F	5'- TGGTGCATTACATTCCTTAC-3'
CEN8 +199R	5'- ACTCTCCCTTAGTGCTTGTG -3'
CEN9 -110F	5'- CTGCGAACGGATGAATATTG-3'
CEN9 +181R	5'- CACCTGTTTTCATTACCCTG -3'
CEN10 -138F	5'- CAGTTTAGTTGTTGTGGATG -3'
CEN10 +350R	5'- CGACAAAGTATCTCAGAAGG -3'
CEN11 -60F	5'- CATTGTATTGATTGATTCTGC-3'
CEN11 +246R	5'- CTGTAATCACTTGTCAACGC-3'
CEN12 -63F	5'- CTTGTAGTACGAGGTTAAC-3'
CEN12 +238R	5'- GCTAATATCAACGAGTAAACG-3'
CEN13 -49F	5'- AGCGATTTACTACACTTGAG-3'
CEN13 +349R	5'- AGCCAGAACTTCTCATCAAC-3'
CEN14 -55F	5'- AAGCAAGTAGATGTGAAGCC-3'
CEN14 +257R	5'- TCATTCTTATCAGTCCAGCG-3'
CEN15 -141F	5'- TTTCAGAAGTCATAACGCAC-3'
CEN15 +237R	5'- AACTTCCTTGACAGAACCAT-3'
CEN16 -100F	5'- GTTGAAGCCGTTATGTTGTC -3'
CEN16 +275R	5'- TCAGTGTGGAGTAAGTCATC-3'

TABLE S4

Genes that Rad9-13Myc is localised to and affects their transcription

[Functional GO analysis (for the Biological Process) of this gene list performed in Cytoscape platform using BiNGO plugin can be found in Dataset S4]

Gene > Primary SGDID	Gene > Systematic Name	Gene > Standard Name	Gene > Name	Gene > Alias	Gene > Length
S000000018	YAL020C	ATS1	Alpha Tubulin Suppressor	KTI13 FUN28	1002
S000000070	YAR015W	ADE1	ADEnine requiring	phosphoribosylaminoimid-azolesuccinocarboxamide synthase	921
S000000379	YBR175W	SWD3	Set1c, WD40 repeat protein	SAF35 CPS30	948
S000000395	YBR191W	RPL21A	Ribosomal Protein of the Large subunit	L21A L21e ribosomal 60S subunit protein L21A URP1	871
S000000685	YCR089W	FIG2	Factor-Induced Gene		4830
S000000704	YCR107W	AAD3	Aryl-Alcohol Dehydrogenase		1092
S000000934	YER132C	PMD1	Paralog of MDS3		5262
S000001067	YHR025W	THR1	THReonine requiring		1074
S000001631	YKL148C	SDH1	Succinate DeHydrogenase	SDHA succinate dehydrogenase flavoprotein subunit SDH1	1923
S000001760	YKR052C	MRS4	Mitochondrial RNA Splicing		915
S000002867	YDR459C	PFA5	Protein Fatty Acyltransferase		1125
S000002982	YGL014W	PUF4	PUmilio-homology domain Family	YGL023	2667
S000003180	YGL212W	VAM7	VAcuolar Morphogenesis	VPL24 VPS43	951
S000003458	YGR226C				210
S000003887	YJR126C	VPS70	Vacuolar Protein Sorting		2436
S000004004	YLR014C	PPR1	Pyrimidine Pathway Regulation		2715
S000004092	YLR102C	APC9	Anaphase Promoting Complex	anaphase promoting complex subunit 9	798
S000004374	YLR382C	NAM2	Nuclear Accommodation of Mitochondria	mitochondrial leucyl-tRNA synthetase LeuRS MSL1 leucine—tRNA ligase NAM2	2685
S000004584	YML116W	ATR1	AminoTriazole Resistance	SNQ1	1629
S000004742	YMR135C	GID8	Glucose Induced Degradation deficient	DCR1 glucose-induced degradation complex subunit GID8	1368
S000005168	YNL224C	SQS1	SQuelch of Splicing suppression	PFA1	2304
S000005215	YNL271C	BNI1	Bud Neck Involved	PPF3 SHE5	5862
S000005381	YOL021C	DIS3	homolog of S. pombe dis3 (chromosome DISjunction)	MTR17 RRP44	3006
S000005480	YOL120C	RPL18A	Ribosomal Protein of the Large subunit	L18A ribosomal 60S subunit protein L18A L18e rp28A RP28A	1008
S000005565	YOR039W	CKB2	Casein Kinase Beta' subunit	CK2 subunit	777
S000005780	YOR254C	SEC63	SECretory	PTL1	1992
S000005857	YOR330C	MIP1	MItochondrial DNA Polymerase	mitochondrial DNA polymerase catalytic subunit	3765
S000005871	YOR344C	TYE7	Ty-mediated Expression	SGC1	876
S000005947	YPL026C	SKS1	Suppressor Kinase of SNF3	SHA3	1509
S000006075	YPL154C	PEP4	carboxyPEPtidase Y-deficient	PHO9 yscA PRA1	1218
S000006278	YPR074C	TKL1	TransKetoLase		2043

TABLE S6

Rad9-13Myc localisation to non-ORF features in wt and *aft1*Δ cells

Experiment ($P=5x10^{-3}$)	**Rad9-13Myc SC BCS BPS**		***aft1*Δ Rad9-13Myc SC BCS BPS**	
	#	%	#	%
ARS	20/337	5.9	31/337	9.19
Telomeres	15/32	45.3	14/32	43.75
Retrotransposons	27/89	29.77	1/89	1.12
LTRs	24/383	6.1	34/383	8.87
rRNAs	5/27	16.66	4/27	14.81

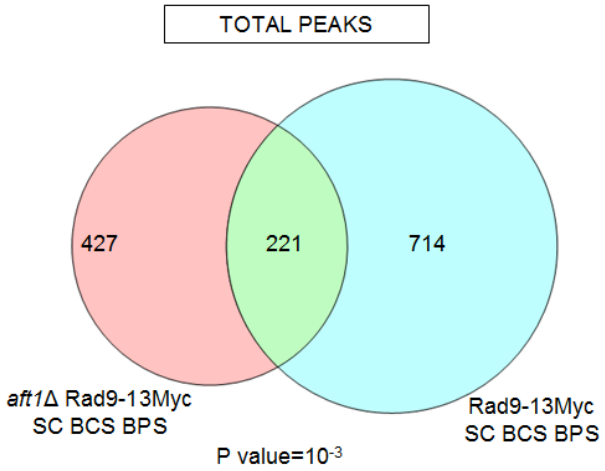
The non-ORF features that were most represented in Rad9-13Myc Tiling Arrays are shown, in the presence or absence of Aft1 transcription factor. The results for the Rad9-13Myc experiment are the average of two replicates. The number of the features which are targets in each experiment is presented, along with the corresponding percentage value of the total number of features in each category in the yeast genome. ARS: Autonomous Replicating Sequence; LTR: Long Terminal Repeat.

TEXT S1

Functional analysis of Rad9 localisation pattern in the presence or absence of Aft1

We performed genome-wide ChIP on chip analyses for Rad9-13Myc localisation in the presence or absence of Aft1 under non-induced DNA damage conditions and performed a functional analysis. To address that, we obtained the coordinates of the localisation sites of the proteins for two different P-values (10^{-3} and $5x10^{-3}$, see Protocol S4). We then clustered these sites into three groups, depending on their binding value, the highest value corresponding to the lowest P-value, in order to obtain three groups formed in a descending binding value (Group1>Group2>Group3). We concentrated on the groups of genes where Rad9 was localised with the highest binding value, which we further analysed for possible overrepresented GO categories. This analysis was performed by using BiNGO, a Java-based tool used as a plugin in Cytoscape platform (1,2).

Using a P-value cutoff of 0.001 we found that, of the 935 genes to which Rad9-13Myc was localised in SC BCS BPS, the 221 (~24%) were also detected when Aft1 was absent, while 427 genes were new targets (See Figure below).



Overlap of Rad9-13Myc targets in the presence or absence of Aft1
Venn diagram showing the overlap (green) between the targets of Rad9 in wild type cells grown in SC BCS BPS (blue) and the targets of Rad9 in *aft1*Δ cells grown in the same conditions (red).

This comparison shows that a number of different additional genes are included in each case and this indicates the role of Aft1 in directing the Rad9-13Myc localisation. We separately examined the three different GO categories (BP-biological process, MF-molecular function, CC-cellular component) in the two target groups, in order to gather functional information on the effect of Aft1 in Rad9 -13Myc localisation. The most statistically important gene clusters that appeared are presented in the following tables.

GO Molecular Function overrepresentation of gene clusters in Rad9-13Myc ChIP on chip, in the presence or absence of Aft1

GO: MOLECULAR FUNCTION	
Rad9-13Myc SC BCS BPS	***aft1*Δ Rad9-13Myc SC BCS BPS**
Translation elongation factor activity (10^{-3}) [7/14]	Adenyl nucleotide binding (10^{-1}) [71/616]
Catalytic activity (10^{-2}) [127/2123]	**ATP binding** (10^{-1}) [67/575]
Ligase activity (10^{-2}) [4/4]	**ATPase** activity (10^{-1}) [32/229]
Vitamin binding (10^{-2}) [11/61]	
Biotin binding (10^{-2}) [4/5]	
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (10^{-2}) [5/18]	
Oxidoreductase activity (10^{-2}) [29/307]	
Hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amidines (10^{-1}) [6/17]	
Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity (10^{-1}) [3/3]	
Lyase activity [11/96], NAD binding [5/19], Metal ion binding [44/812] (10^{1})	
Other clusters with higher P-values	

Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster.

In the above table, we observed firstly that the clusters formed in the presence of Aft1, are more that in its absence. Secondly, we see a qualitative difference: in the presence of Aft1, Rad9-13Myc was localised to clusters related to catalylic activity, translation elongation factor activity, ligase-, vitamin-, biotin-binding activity, oxidoreductase activity, among others. Groups like the one for oxidoreductase activity are related to high transcriptional activity. In the absence of Aft1, the most apparent gene clusters are related to ATP-binding and ATPase activity. These latter gene clusters are also formed in the presence of Aft1, but are ranked in Group 3 (lower binding values). It should also be highlighted, that a lot of clusters overrepresented in the Rad9-13Myc strain, do not appear in the *aft1*Δ Rad9-13Myc strain. These clusters include among others, the clusters related to translation elongation factor activity, ligase activity, vitamin/biotin binding activity, oxidoreductase activity, hydrolase activity, glyceraldehyde-3-phosphate dehydrogenase activity, metal ion binding, cyclohydrolase activity, lyase activity. This is consistent to our observations that in the absence of Aft1, Rad9-13Myc loses its preference to bind the mostly active genes.

GO Biological Process overrepresentation of gene clusters in Rad9-13Myc ChIP on chip, in the presence or absence of Aft1

GO: BIOLOGICAL PROCESS	
Rad9-13Myc SC BCS BPS	***aft1*Δ Rad9-13Myc SC BCS BPS**
Pyruvate **metabolic** process (10^{-8}) [14/29]	Protein **import into nucleus** (10^{-5}) [13/49]
Carboxylic acid metabolic process (10^{-8}) [46/363]	**Regulation of metabolic** process (10^{-3}) [223/1154] (Group2)
Hexose **biosynthetic** process (10^{-7}) [12/22]	Regulation of cellular metabolic process (10^{-3}) [214/1101] (Group2)
Monosaccharide biosynthetic process (10^{-7}) [13/27]	Regulation of biological process (10^{-2}) [277/1500] (Group2)
Organic acid metabolic process (10^{-7}) [46/369]	**Nuclear transport** (10^{-2}) [40/133] (Group2)
Gluconeogenesis (10^{-7}) [11/19]	Nucleocytoplasmic transport (10^{-2}) [40/133] (Group2)
Alcohol biosynthetic process (10^{-6}) [15/47]	**Regulation** of cellular **biosynthetic** process (10^{-2}) [97/436] (Group2)
Glucose catabolic process (10^{-5}) [14/44]	**Regulation** of **translation** (10^{-1}) [92/417] (Group2)
Glycolysis (10^{-5}) [12/32]	**Regulation** of cellular protein **metabolic** process (10^{-1}) [95/438] (Group2)
Hexose catabolic process (10^{-5}) [14/49]	RNA localisation (10^{-1}) [32/107] (Group2)
Glucose metabolic process (10^{-4}) [16/67]	**Posttranscriptional regulation** of gene expression (10^{-1}) [92/426] (Group2)
Regulation of cellular protein metabolic process (10^{-4}) [45/438]	Regulation of protein metabolic process (10^{-1}) [102/490] (Group2)
Posttranscriptional regulation of gene expression (10^{-4}) [44/426], more in Group 3	Regulation of macromolecule metabolic process (10^{-1}) [198/1071] (Group2)
Regulation of **translation** (10^{-4}) [43/417]	**Iron** assimilation (10^{1}) [6/8] (Group2)
Regulation of cellular biosynthetic process (10^{-4}) [43/436]	
Oxidation reduction (10^{-2}) [29/279]	
Amino acid biosynthetic process (10^{-2}) [17/128] (Group 2)	
Metal ion transport (10^{-1}) [19/84] (Group 3)	
Iron ion transport (10^{-1}) [10/32] (Group 3)	

Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster.

In the above table, we observe Rad9-13Myc localisation to genes which are clustered in groups related to glucose, hexose and organic acid metabolism. Groups for regulation of metabolic processes, translation and posttranscriptional regulation of gene expression are also present, although exhibiting higher P-value. Thus, Rad9-13Myc was localised to genes required for cell growth. The presence of metal ion/iron transport groups is indicative of the induction growth conditions used (BCS/BPS), since these groups are absent from the clustering when Rad9-9Myc strain is grown on plain rich medium (YPD). When Aft1 was absent, Rad9-13Myc was localised with higher signal value (lower P-value) to genes encoding for proteins which participate

to the import into nucleus. Furthermore, the categories for the regulation of metabolic processes, of translation, and of posttranscriptional regulation of gene expression are maintained in the absence of Aft1. Clusters related to metabolic and catabolic processes *per se* (including the highly active aminoacid biosynthetic genes), are not overrepresented in *aft1*Δ strains, which supports the notion that Aft1 assists Rad9 on its binding to genes related to cell growth.

GO Cellular Component overrepresentation of gene clusters in Rad9-13Myc ChIP on chip, in the presence or absence of Aft1

GO: CELLULAR COMPONENT	
Rad9-13Myc SC BCS BPS	***aft1*Δ Rad9-13Myc SC BCS BPS**
Fungal-type **cell wall** (10^{-7}) [21/90]	**Nuclear** lumen (10^{-1}) [114/560]
External encapsulating structure (10^{-5}) [21/107]	Nuclear pore complex (10^{1}) [19/57]
Cytosolic **ribosome** (10^{-2}) [20/156]	
Anchored to membrane (10^{1}) [9/47]	
COPII vesicle coat (ER→Golgi transport) (10^{1}) [4/9]	

Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster.

In the above table, we observe that the gene clusters to which Rad9-13Myc was localised, encode for proteins which are overrepresented primarily in cell wall and secondarily in cytosolic ribosome. Cell wall protein-encoding genes are connected to high transcriptional activity (as we found in our genome-wide expression experiments), also consistent to our previous results for Rad9. These categories are absent from the *aft1*Δ strain, also consistent to our observations that Aft1 assists Rad9-13Myc on binding to highly active regions.

1. Maere, S., Heymans, K. and Kuiper, M. (2005) BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics*, **21**, 3448-3449.
2. Cline, M.S., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, C., Christmas, R., Avila-Campilo, I., Creech, M., Gross, B. *et al.* (2007) Integration of biological networks and gene expression data using Cytoscape. *Nat Protoc*, **2**, 2366-2382.

TEXT S2

PART 1

Comprehensive functional analysis of the Rad9-13Myc binding targets concerning the localisation to coding/non-coding regions

We obtained the statistically significant loci to which each protein (Rad9-13Myc or Aft1-9Myc) was localised, corresponding to two different P-values (10^{-3} and $5x10^{-3}$), as described in Protocol S4. We then grouped the genomic loci to which each protein was localised into four categories: a) Total peaks (which included all the statistically significant loci), b) Peaks corresponding to coding regions, c) Peaks corresponding to non-coding regions and d) Peaks corresponding to coding and non-coding regions of the same gene. We compared the respective gene lists for each of these four categories between the two experiments in order to find:

i) Genes to which Rad9-13Myc was localised only when Aft1 transcription factor was absent (clustering in first Table below).
ii) Genes to which Rad9-13Myc was localised only when Aft1 transcription factor was present but was not when it is absent (clustering in secomd Table below).
iii) Genes to which Rad9-13Myc was localised regardless of the Aft1 presence (clustering in third Table below).

For each of these three categories we performed qualitative analysis using the BiNGO plugin in Cytoscape platform as described in Materials and Methods (1,2), in order to find any overrepresented gene clusters in each of the three GO categories (Molecular Function-MF, Biological Process-BP, Cellular Component-CC). The results obtained by this analysis are presented below.

Gene Clusters to which Rad9-13Myc is localised only when Aft1 is absent

TOTAL PEAKS
(MF) No clustering
(BP)
- Establishment of localization in cell (10^{-7}) [78/659]
- Intracellular **transport** (10^{-6}) [72/610]
- **Nuclear transport** (10^{-4}) [23/148]
- **rRNA metabolic** process (10^{-4}) [34/262]
- Golgi vesicle transport (10^{-4}) [26/185]

(CC)
- **Nuclear** lumen (10^{-2}) [96/546]

CODING
(MF)
- **ATPase** activity (10^{-5}) [29/242]
- Nucleoside-triphosphate activity (10^{-4}) [36/356]
- **Fatty acid synthase** activity (10^{-3}) [4/9]
- **Protein Ser/Thr kinase** activity (10^{-3}) [16/119]
- Pyrophosphatase activity/ hydrolase activity, acting on acid anhydrides (10^{-3}) [36/374]

(BP)
- Establishment of localization in cell (10^{-7}) [69/659]
- Intracellular **transport** (10^{-6}) [63/610]
- Cellular localization (10^{-6}) [73/757]
- Protein import into nucleus (10^{-6}) [13/54]
- **rRNA metabolic** process (10^{-5}) [31/262]
- **Ribosome biogenesis** (10^{-3}) [39/372]

(CC)
- **Nuclear lumen** (10^{-4}) [49/546]
- Microtubule organizing center (10^{-4}) [12/70]
- **Spindle pole** body (10^{-4}) [12/70]
- **Golgi transport** complex (10^{-4}) [4/8]
- **Mating** projection (10^{-3}) [16/121]

ONLY NON CODING
(BP) No clustering
(MF) No clustering
(CC) No clustering

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

Gene clusters to which Rad9-13Myc is localised only when Aft1 transcription factor is present but is not when it is absent

TOTAL PEAKS

(MF)

- **Catalytic** activity (10^{-15}) [343/2192]
- **Hydrolase** activity, hydrolyzing O-glycosyl compounds (10^{-7}) [17/41]
- **Oxidoreductase** activity (10^{-7}) [66/323]
- transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer (10^{-6}) [8/11]
- Ion binding (10^{-6}) [129/791]
- **Glucosidase** activity (10^{-5}) [10/20]
- **Translation elongation factor** activity (10^{-5}) [9/17]
- **Vitamin** binding (10^{-5}) [18/58]
- **Lyase** activity (10^{-5}) [25/97]
- Siderophore transporter activity (10^{-4}) [4/4]

(BP)

- **Biosynthetic/metabolic/catabolic** process (glucose, organic acid) (10^{-16}-10^{-11})
- Translation regulation (10^{-11}) [54/192]
- **Amino acid metabolic** process (10^{-10}) [63/247]
- Glucose metabolic process (10^{-10}) [34/98]
- **Posttranscriptional regulation** of gene expression (10^{-10}) [55/206]
- **Oxidation reduction** (10^{-8}) [75/363]
- **Siderophore** transport (10^{-7}) [8/9]
- **Iron-ion** transport (10^{-5}) [13/31]

(CC)

- External encapsulating structure (10^{-10}) [36/108]
- Anchored to membrane (10^{-10}) [26/59]
- Fungal-type **cell wall** (10^{-11}) [36/100]
- Cytosolic **ribosome** (10^{-6}) [40/169]
- Alpha,alpha-trehalose-phosphate synthase complex (UDP-forming) (10^{-4}) [4/4]
- **Tubulin** complex (10^{-3}) [3/3]

ONLY CODING

(MF)

- **Catalytic** activity (10^{-16}) [334/2192]
- **Oxidoreductase** activity (10^{-7}) [64/323]
- Hydrolase activity, hydrolyzing O-glycosyl compounds (10^{-6}) [16/41]
- **Cation/metal ion** binding (10^{-6}) [123/791]
- **Translation elongation factor activity** (10^{-5}) [9/17]
- **Vitamin** binding (10^{-5}) [18/58]
- Lyase activity (10^{-5}) [24/97]
- Substrate specific transmembrane transporter activity (10^{-4}) [50/274]
- **Siderophore** transporter activity (10^{-4}) [4/4]
- **Transcription repression** activity (10^{-4}) [14/51]
- rRNA binding (10^{-3}) [12/44]

(BP)

- **Carboxylic acid metabolic** process (10^{-18}) [98/377]
- Cellular ketone metabolic process (10^{-16}) [98/391]
- **Glucose catabolic** process (10^{-12}) [25/51]
- **Amine metabolic** process (10^{-11}) [73/303]
- Cellular **aminoacid and derivative metabolic** process (10^{-11} [74/315]
- Regulation of translation (10^{-11}) [53/192]
- **Post transcriptional regulation** of gene expression (10^{-10}) [54/206]
- **Siderophore** transport (10^{-6}) [7/9]
- **Iron** assimilation (10^{-5}) [6/8]
- Iron-ion transport (10^{-4}) [11/31]
- A lot of other clusters with higher P-value

(CC)

- Extracellular region (10^{-14}) [39/95]
- Fungal-type **cell wall** (10^{-12}) [37/100]
- Anchored to membrane (10^{-10}) [25/59]
- External encapsulating structure (10^{-11}) [37/108]
- Cytosolic **ribosome** (10^{-7}) [41/169]

NON CODING

(MF)

- Structural constituent of **ribosome** (10^{-7}) [25/233] (P=0.005)
- Structural molecule activity [28/375] (P=0.005)
- SSU rRNA binding (10^{-5}) [3/4]

(BP) No clustering

(CC)

- Cytosolic part (10^{-7}) [20/219]
- Cytosolic **ribosome** (10^{-6}) [16/169]
- Fungal-type **cell wall** (10^{-4}) [10/100]

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

Gene clusters to which Rad9-13Myc is localised regardless the Aft1 presence

TOTAL

(MF)

- **Nucleoside** (purine) binding (10^{-9}) [55/703]
- Adenyl nucleotide binding (10^{-7}) [50/694]
- **ATP binding** (10^{-6}) [47/652]
- Purine ribonucleotide binding (10^{-6}) [50/752]
- **Catalytic** activity (10^{-4}) [104/2192]
- Ligase activity, forming carbon-carbon bonds (10^{-4}) [3/4]
- Biotin binding (10^{-4}) [3/5]
- CoA carboxylase activity (10^{-3}) [2/2]

(BP)

- **Translation regulation** (10^{-7}) [23/192]
- Regulation of cellular **protein metabolic process** (10^{-7}) [24/215]
- **Post transcriptional regulation** of gene expression (10^{-7}) [23/206]
- **Regulation of protein metabolic** process (10^{-6}) [26/279]
- Carboxylic acid **metabolic** process (10^{-4}) [27/377]
- Protein import into nucleus (10^{-4}) [8/54]
- Fatty acid biosynthetic process (10^{-4}) [5/22]
- Iron assimilation (10^{-8}) [7/8] [P=5x10^{-3}]

(CC)

- Plasma **membrane** (10^{-7}) [54/366]
- 90S **preribosome** (10^{-5}) [17/83]
- Fungal-type **cell wall** (10^{-4}) [19/100]
- **Mating projection** (10^{-4}) [20/121]

ONLY CODING

(MF)

- **Nucleoside** binding (10^{-3}) [45/703]
- Adenyl nucleotide binding (10^{-2}) [42/694]
- **Catalytic** activity (10^{-7}) [201/2192] (P=0.005)
- ATP binding (10^{-6}) [83/652] (P=0.005)
- Ligase activity, forming carbon-carbon bonds (10^{1}) [3/4]
- Biotin binding (10^{1}) [3/5]

(BP)

- **Regulation of translation** (10^{-4}) [22/192]
- Regulation of cellular protein **metabolic process** (10^{-4}) [23/215]
- **Post transcriptional regulation** of gene expression (10^{-3}) [22/206]
- **Regulation of protein metabolic** process (10^{-3}) [25/279]
- Iron assimilation (10^{-4}) [7/8] (P=0.005)
- Carboxylic acid metabolic process (10^{1}) [25/377]
- **Siderophore** iron transport (10^{-2}) [5/6] (P=0.005)
- **Ribosome** biogenesis (10^{-2}) [46/372] (P=0.005)

(CC)

- **Plasma membrane** (10^{-2}) [47/366] (P=0.005)
- 90S **preribosome** (10^{-1}) [16/83] (P=0.005)
- **Mating projection** tip (10^{-1}) [19/110] (P=0.005)

ONLY NON CODING

(MF)

- **Helicase** activity (10^{-6}) [6/107]
- **ATPase** activity, coupled (10^{-4}) [5/172]
- Purine NTP dependent helicase activity (10^{-5}) [5/76]
- **Hydrolase** activity, acting on acid anhydrides (10^{-3}) [6/374]

(BP) Premeiotic DNA synthesis (10^{-3}) [3/6]

(CC) No clustering

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

In the above Tables, we observe once more that Aft1 is required for Rad9-13Myc localisation to highly transcriptionally active gene clusters, since in its absence

Rad9-13Myc is not localised to well known active groups such as the ones related to oxidoreductase activity, amino acid metabolic process and cell wall (second Table). Gene clusters, such as the ones related to catalytic activity, post-transcriptional regulation of gene expression, presence to cell wall, are much more represented quantitatively when Aft1 is present rather than regardless its presence (second Table versus third Table). This means that Aft1 association to Rad9 assists the localisation of the latter to the particular gene clusters.

Furthermore, we conclude that Aft1 is very important in establishing the Rad9-13Myc localisation to genes related to cell growth (such as metabolic, or biosynthetic of glucose and amino acids, encoding ribosomal proteins). Rad9-13Myc presence on genes related to cell growth, in an Aft1-dependent manner, can be of great importance since it connects a DNA damage checkpoint protein to vital cellular processes in non DNA damage-inducing conditions.

Furthermore, for the groups of genes to which Rad9-13Myc is localised only when Aft1 is absent, we observed a difference in the list of the overrepresented gene clusters, since there are clusters related to nuclear transport, rRNA metabolic process, ribosome biogenesis, ATPase activity (first Table), compared to the biosynthetic/metabolic gene clusters or the ones related to catalytic activity, regulation of translation, cell wall and ribosomes, which are apparent when Aft1 is present (second Table). This qualitative difference highlights the importance of Aft1 in directing Rad9-13Myc localisation to specific sites of the genome.

The quality of the genome-wide analysis is confirmed by the iron related gene clusters, which are significantly more apparent in Rad9-13Myc localisation pattern when Aft1 is present.

PART 2

Rad9-13Myc localisation to the coding and non-coding region of the same gene in the presence or absence of Aft1

We further examined the case in which Rad9-13Myc was localised to the coding and non-coding region of the same gene. We performed the analysis described in Part 1 for the group of genes that were characterized by this specificity and the results are presented in the following Table. The analysis was performed for the cases where Aft1 was present or absent.

Gene clusters to which Rad9-13Myc is localised to both their coding and non coding regions

Rad9-9Myc SC BCS BPS

(MF)

- **SSU rRNA** (10^{-5}) [3/4]
- Structural **constituent of ribosome** (10^{-5}) [14/233]
- **rRNA** binding (10^{-5}) [6/44]
- **Translation elongation** factor activity (10^{-4}) [4/17]
- **Structural** molecule activity (10^{-4}) [16/375]
- **Oxidoreductase** activity (10^{-3}) [14/323]

(BP)

- Cellular **metabolic** process (10^{-6}) [86/3827]
- **Regulation of translation** (10^{-5}) [13/192]
- Post transcriptional regulation of gene expression (10^{-5}) [13/206]
- Regulation of **translational elongation** (10^{-5}) [5/24]
- **Carboxylic acid metabolic process** (10^{-5}) [18/377]
- Glucose **catabolic** process (10^{-4}) [6/51]
- **Glucose metabolic** process (10^{-3}) [7/98]
- Regulation of cellular protein metabolic process (10^{-5}) [13/215]
- NADPH regeneration (10^{-4}) [4/21]
- Ribosome biogenesis (10^{-3}) [15/372]
- **Oxidation** reduction (10^{-3}) [14/363]

(CC)

- **Ribosome** (10^{-7}) [18/275]
- Cytosolic small **ribosomal** subunit (10^{-5}) [8/64]
- Extracellular region (10^{-5}) [9/95]
- **Fungal-type cell wall** (10^{-5}) [9/100]
- External encapsulating structure (10^{-5}) [9/108]
- Chaperonin containing T-complex (10^{-4}) [3/8]
- 90S **preribosome** (10^{-3}) [6/83]
- Eucaryotic translation elongation factor 1 complex (10^{-3}) [2/5]

aft1Δ Rad9-13Myc SC BCS BPS

(MF)

- **Siderophore-iron transmembrane** transporter activity (10^{-5}) [3/4] (P=0.005)

(BP)

- Transport (10^{-6}) [56/1221] (P=0.005)
- Establishment of localization (10^{-5}) [56/1226] (P=0.005)
- Di-, trivalent inorganic **cation transport** (10^{-5}) [8/45] (P=0.005)
- **Iron assimilation** (10^{-5}) [4/8] (P=0.005)
- **Siderophore** transport (10^{-5}) [4/9] (P=0.005)
- Transmembrane transport (10^{-4}) [23/381] (P=0.005)
- **Iron ion transport** (10^{-4}) [6/31] (P=0.005)
- Iron assimilation by chelation and transport (10^{-4}) [3/6] (P=0.005)
- Iron chelate transport (10^{-4}) [3/6] (P=0.005)

(CC) No clustering

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

In the case where Rad9-13Myc is localised to the coding and non-coding region of the same gene, the overrepresented clusters include genes related to ribosomal subunits, rRNA binding, translation elongation factor activity and regulation of translation. The presence of Rad9 on such genes is an indication of a

more solid role of Rad9 in cellular mechanisms related to protein synthesis. This is a further indication of Rad9 possible implication in regulation of cell growth genes.

1. Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, et al.: Integration of biological networks and gene expression data using Cytoscape. *Nat Protoc* 2007, 2:2366-2382.
2. Maere S, Heymans K, Kuiper M: BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* 2005, 21:3448-3449.

TEXT S3

Functional analysis of Aft1-9Myc localisation targets in wt and *rad9*Δ cells

We performed the same functional analysis for Aft1-9Myc localization targets in the presence or absence of Rad9 as described in Text S1 for the Rad9-13Myc. A general overview of the Aft1 binding pattern in the presence or absence of Rad9 in each of the three GO categories is presented in the following three Tables.

GO Molecular Function overrepresentation of gene clusters in Aft1-9Myc ChIP on chip, in the presence or absence of Rad9

GO: MOLECULAR FUNCTION	
***rad9*Δ Aft1-9Myc SC BCS BPS**	**Aft1-9Myc SC BCS BPS**
Structural **constituent of ribosome** (10^{-4}) [43/233]	**Iron** ion binding (10^{-6}) [20/116]
Catalytic activity (10^{-3}) [212/2192]	**Oxidoreductase** activity, oxidizing metal ions (10^{-3}) [6/10]
Structural molecule activity (10^{-3}) [55/375]	**Iron** ion transmembrane **transporter** activity (10^{-3}) [6/11]
Vitamin binding (10^{-1}) [15/58]	Transition metal ion transmembrane transporter activity (10^{-3}) [9/34]
Co-factor binding (10^{-1}) [28/162]	**Ferric chelate reductase** activity (10^{-2}) [5/8]
Purine nucleotide binding (10^{-1}) [89/793]	Di-, tri-valent inorganic cation transmembrane transporter activity (10^{-2}) [9/41]
Lyase activity (10^{-1}) [20/97]	**Translation elongation** factor activity (10^{-1}) [5/14]
rRNA binding (10^{-1}) [12/44]	**DNA helicase** activity (10^{1}) [6/30]
Oxidoreductase activity (10^{-1}) [43/323]	FAD binding (10^{1}) [6/34]
Translation elongation factor activity (10^{1}) [7/17]	**Electron carrier** activity (10^{1}) [9/77]
Transition metal ion transmembrane transporter activity (10^{1}) [9/28]	Acetolactate synthase activity (10^{1}) [2/2]
Di-, tri-valent inorganic cation	Fatty-acyl-CoA synthase activity (10^{1}) [2/2]

transmembrane transporter activity (10^{1}) [10/36]	
Ferric-chelate reductase activity (10^{1}) [5/9]	
Metal ion transmembrane transporter activity (10^{1}) [11/45]	
Copper ion transmembrane transporter activity (10^{1}) [4/6]	
ATP binding (10^{1}) [70/652]	
Iron ion transmembrane transporter activity (10^{1}) [5/11]	
siderophore-iron transmembrane transporter activity (10^{1}) [3/4]	

Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster.

In the first Table, we observe that the types of gene clusters to which Aft1 is localised are similar in the presence or absence of Rad9. These include transcriptionally active groups of genes (such as GO terms of "oxidoreductase activity", "aminoacid metabolism" and "glucose catabolism"). Nevertheless, a shift in their ranking is apparent since iron-related gene clusters rank lower when Rad9 is absent.

GO Biological Process overrepresentation of gene clusters in Aft1-9Myc ChIP on chip, in the presence or absence of Rad9

GO: BIOLOGICAL PROCESS	
***rad9*Δ Aft1-9Myc SC BCS BPS**	**Aft1-9Myc SC BCS BPS**
Regulation of translation (10^{-15}) [54/192]	**Iron** ion **transport** (10^{-8}) [13/32]
Posttranscriptional **regulation of gene expression** (10^{-15}) [56/206]	Transition metal ion transport (10^{-6}) [15/57]
Carboxylic acid **metabolic** process (10^{-14}) [78/377]	**Iron assimilation** (10^{-6}) [7/8]
Cellular ketone metabolic process (10^{-14}) [79/391]	Di-, tri-valent inorganic cation transport (10^{-5}) [13/47]
Cellular **amino acid metabolic** process (10^{-13}) [60/247]	**Siderophore** transport (10^{-4}) [6/9]
Regulation of cellular protein metabolic process (10^{-13}) [55/215]	Iron ion homeostasis (10^{-3}) [10/37]
Cellular **amine metabolic** process (10^{-12}) [61/270]	Cellular ion homeostasis (10^{-1}) [14/116]
Cellular amino acid and derivative metabolic process (10^{-11}) [65/315]	**Regulation** of biological quality (10^{-1}) [28/377]
Regulation of protein metabolic process (10^{-8}) [56/279]	**Oxidation reduction** (10^{-1}) [23/279]
Cellular nitrogen compound biosynthetic process (10^{-6}) [55/310]	**Copper** ion import (10^{-1}) [4/8]
Cellular aromatic compound metabolic process (10^{-4}) [32/146]	**Regulation** of **translation** (10^{1}) [28/417]
Transition metal ion transport (10^{-3}) [17/55]	**Post transcriptional regulation** of gene

	expression (10^{1}) [28/426]
Glucose catabolic process (10^{-3}) [16/51]	**Organic acid metabolic** process (10^{1}) [25/369]
Glutamine family amino acid metabolic process (10^{-3}) [18/64]	**Regulation** of cellular biosynthetic process (10^{1}) [28/436]
Iron-ion transport (10^{-3}) [12/31]	**Amino acid metabolic** process (10^{1}) [17/235]
Monosaccharide catabolic process (10^{-2}) [17/61]	Fatty acid biosynthetis process (10^{1}) [4/17]
Di-, tri-valent inorganic cation transport (10^{-2}) [14/45]	**Telomere maintenance** via recombination (10^{1}) [4/19]
Alcohol catabolic process (10^{-2}) [17/66]	**Amino acid** and derivative **metabolic** process (10^{-1}) [38/255] (Group 2)
Iron assimilation (10^{-2}) [6/8]	**Carboxylic acid metabolic** process (10^{-2}) [50/363]
Iron ion homeostasis (10^{-2}) [12/38]	**Glucose catabolic** process (10^{1}) [11/44] (Group 2)

Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster.

In the second Table, we can see that the absence of Rad9 does not affect the localisation of Aft1 to clusters containing highly active genes such as the ones related to oxidoreductase activity or amino acid metabolic process. Nevertheless, there is a shift in the ranking of the clusters, such as the iron related ones, which are higher in rank when Rad9 is present. "Regulation of translation" and "metabolic processes"-related gene clusters are lower in rank when Rad9 is present. It seems that the absence of Rad9 does not have a profound qualitative effect on Aft1 localisation. Rad9 effect on Aft1 localisation pattern is not as strong as Aft1 effect on Rad9 (See Text S1).

GO Cellular Component overrepresentation of gene clusters in Aft1-9Myc ChIP on chip, in the presence or absence of Rad9

GO: CELLULAR COMPONENT	
***rad9*Δ Aft1-9Myc SC BCS BPS**	**Aft1-9Myc SC BCS BPS**
Cytosolic **ribosome** (10^{-10}) [44/169]	**Eukaryotic translation elongation factor 1** complex (10^{-2}) [4/5]
Cytosolic large ribosomal subunit (10^{-5}) [25/93]	Fungal-type **cell wall** (10^{1}) [11/90]
Plasma membrane enriched fraction (10^{-4}) [23/89]	Plasma membrane (10^{1}) [25/351]
Fungal-type **cell wall** (10^{-3}) [23/100]	External encapsulating structure (10^{1}) [11/107]
Cytosolic small ribosomal subunit (10^{-2}) [17/64]	Anchored to membrane (10^{1}) [7/47]
External encapsulating structure (10^{-2}) [23/108]	High affinity **iron permease** complex (10^{1}) [2/2]
Eukaryotic translation elongation factor 1 complex (10^{1}) [3/5]	**Acetolactate synthase** complex (10^{1}) [2/2]
	Fatty acid synthase complex (10^{1}) [2/2]

Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster.

In the third Table, we can see that gene clusters related to cell wall and eukaryotic translation elongation factor 1 complex are included in the Aft1 targets regardless of the presence of Rad9, although their ranking is changed.

In all three GO categories, there is a higher overrepresentation of clusters related to ribosome structure and function when Rad9 is absent. Nevertheless, the impact of *RAD9* deletion to the gene clusters of Aft1 targets is not as strong as the impact of *AFT1* deletion to Rad9 localisation pattern.

We further performed the analysis described in Text S2 in order to find:

iv) Genes to which Aft1-9Myc was localised only when Rad9 was absent (clustering in the first Table below).

v) Genes to which Aft1-9Myc was localised only when Rad9 was present but was not when it was absent (clustering in the second Table below).

vi) Genes to which Aft1-9Myc was localised regardless of the Rad9 presence (clustering in the third Table below).

The functional analyses of these three groups are presented below.

Gene clusters to which Aft1 transcription factor is localised only when Rad9 is absent

TOTAL
(MF)

- **structural constituent of ribosome** (10^{-14}) [96/233]
- structural molecule activity (10^{-11}) [125/375]
- substrate-specific **transmembrane transporter** activity (10^{-2}) [79/374]
- substrate-specific transporter activity (10^{-2}) [91/330]
- transmembrane transporter activity (10^{-2}) [87/318]
- transporter activity (10^{-2}) [101/392]
- electron carrier activity (10^{-1}) [22/56]

TOTAL
(CC)

- Cytosolic **ribosome** (10^{-26}) [94/169]
- Cytosolic large ribosomal subunit (10^{-15}) [54/93]
- Cytosolic small ribosomal subunit (10^{-8}) [35/64]
- Ribonucleoprotein complex (10^{-7}) [156/556]
- Intrinsic to membrane (10^{-1}) [272/1295]
- Mitochondrial nucleoid (10^{1}) [12/23]

TOTAL
(BP)

- **Regulation of translation** (10^{-10}) [77/192]
- **Posttranscriptional regulation of gene expression** (10^{-10}) [80/206]
- Regulation of cellular protein metabolic process (10^{-8}) [79/215]
- **Intron homing** (10^{-4}) [10/10]
- **Regulation** of protein **metabolic** process (10^{-4}) [84/279]
- **Ribonucleoprotein** complex **biogenesis** (10^{-2}) [110/423]
- **Ribosome biogenesis** (10^{-2}) [99/372]
- Pyruvate metabolic process (10^{-2}) [16/28]
- Transmembrane transport (10^{-2}) [100/381]
- Small molecule biosynthetic process (10^{-2}) [100/390]
- Nucleotide metabolic process (10^{-1}) [61/214]
- rRNA export from nucleus (10^{-1}) [19/43]
- nucleobase, nucleoside and nucleotide metabolic process (10^{-1}) [67/245]
- Gluconeogenesis (10^{1}) [10/17]
- Amine metabolic process (10^{1}) [74/303]
- Hexose biosynthetic process (10^{1}) [10/20]
- Glycine catabolic process (10^{1}) [4/4]

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

We can see that when Rad9 is absent, Aft1 is localised to gene clusters related to protein synthesis regulation and structure and biogenesis of ribosome (to a greater extend compared to the *rad9*Δ cells). This could show a possible implication of Aft1 transcription factor in regulation of protein synthesis.

Gene clusters to which Aft1 transcription factor is localised only when Rad9 is present

TOTAL
(MF)

- **Helicase activity** (10^{-6}) [25/107]
- ATP-dependent helicase activity (10^{-4}) [18/76]
- DNA helicase activity (10^{-3}) [12/35]
- ATPase activity, coupled (10^{-3}) [27/172]
- Pyrophosphatase activity (10^{-3}) [43/374]
- Hydrolase activity, acting on acid anhydrides, in phosphorus containing anhydrides (10^{-3}) [43/374]
- Nucleoside triphosphatase activity (10^{-2}) [40/356]
- Transcription regulator activity (10^{-2}) [40/361]
- Purine ribonucleotide binding (10^{-1}) [67/752]
- ATPase activity (10^{-1}) [30/242]
- ATP binding (10^{-1}) [59/652]
- Transcription activator activity (10^{-3}) [43/374]

TOTAL
(BP)

- **Regulation of transcription** (10^{-2}) [63/669]
- **Regulation of nucleobase**, nucleoside, nucleotide and nucleic acid **metabolic process** (10^{-2}) [68/759]
- Regulation of RNA metabolic process (10^{1}) [46/489]
- Regulation of biosynthetic process (10^{1}) [72/910]
- Other with higher P-values

TOTAL
(CC)
No clustering

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

Note that Rad9 is necessary for Aft1 localisation to genes related to DNA helicase activity.

Gene clusters to which Aft1 transcription factor is localised regardless Rad9 presence

TOTAL
(MF)

- **Purine nucleotide binding** (10^{-4}) [101/793]
- Adenyl **nucleotide binding** (10^{-3}) [91/694]
- Transition **metal ion transmembrane transporter** activity (10^{-3}) [12/28]
- Purine ribonucleotide binding (10^{-3}) [94/752]
- **ATP binding** (10^{-3}) [84/652]
- **Catalytic** activity (10^{-2}) [214/2192]
- **Translation elongation factor** activity (10^{-1}) [8/17]
- **Siderophore-iron** transmembrane transporter activity (10^{-1}) [4/4]
- **Iron-ion** transmembrane transporter activity (10^{-1}) [6/11]
- Pyrophosphatase activity (10^{1}) [49/374]
- Hydrolase activity, acting on acid anhydrides, in phosphorus containing anhydrides (10^{1}) [49/374]
- Translation factor activity, nucleic acid binding (10^{1}) [14/60]
- Copper-ion transmembrane transporter activity (10^{1}) [4/6]
- Vitamin binding (10^{1}) [12/58]

TOTAL
(CC)

- **Fungal-type cell wall** (10^{-3}) [24/100]
- External encapsulating structure (10^{-2}) [24/108]
- Extracellular region (10^{-2}) [21/95]
- Anchored to membrane (10^{-1}) [15/59]
- **Eukaryotic translation elongation** factor-1 complex (10^{1}) [4/5]
- Cytoskeleton (10^{1}) [35/245]
- Cellular bud (10^{1}) [29/201]
- Cortical actin cytoskeleton (10^{1}) [13/64]
- Ribosome (10^{1}) [35/275]
- Nup82 complex (10^{1}) [3/5]

TOTAL
(BP)

- **Regulation of translation** (10^{-12}) [51/192]
- **Post transcriptional regulation of gene expression** (10^{-11}) [52/206]
- **Regulation** of cellular protein **metabolic process** (10^{-10}) [52/215]
- Regulation of protein metabolic process (10^{-8}) [57/279]
- Transition **metal ion transport** (10^{-6}) [21/55]
- Regulation of gene expression (10^{-3}) [114/874]
- Regulation of macromolecule biosynthetic process (10^{-5}) [114/878]
- Carboxylic acid **biosynthetic process** (10^{-4}) [36/172]
- Oxoacid metabolic process (10^{-4}) [60/377]
- Cellular ketone metabolic process (10^{-4}) [61/391]
- Amine metabolic process (10^{-3}) [50/303]
- **Metal ion** transport (10^{-3}) [22/86]
- Cellular aminoacid and derivative metabolic process (10^{-3}) [50/315]
- di-, trivalent inorganic cation transport (10^{-3}) [15/45]
- Cellular aminoacid biosynthetic process (10^{-3}) [28/133]
- **Iron ion** transport (10^{-2}) [12/31]
- Siderophore transport (10^{-1}) [6/9]
- More with higher P-values

See text for experimental procedure. Numbers in parentheses correspond to hypergeometric test P-value as obtained from BiNGO analysis in Cytoscape (see Protocol S4). Numbers in brackets correspond to the number of genes contained in our input fitting in each cluster, over the total number of yeast genes included in each cluster. When no clustering was possible, it is mentioned respectively. MF: Molecular Function, BP: Biological Process, CC: Cellular Component.

Here we can see that Aft1 does not need Rad9 in order to perform its transcriptional activator role. Aft1 localisation to gene clusters related to metabolic processes is strong.

TEXT S4

Rad9-13Myc localisation pattern relation with average gene occupancy, mean gene expression and GC-content

We have used the results from the genome-wide localization analyses of the Rad9-13Myc strains grown in SC BCS BPS in the presence or absence of the Aft1 transcription factor (*aft1*Δ). Figures iA and iiB correspond to Rad9-13Myc localisation in the presence of Aft1, while Figures iiA and iiB correspond to Rad9-13Myc localisation in *aft1*Δ strains.

We have incorporated in our average gene analysis the expression rates and the GC content of each gene involved. We calculated the rate of expression by taking the log2 value of the average gene expression values from our replicate genome-wide microarray experiments (wild strain grown in SC BCS BPS), in order to avoid any experimental bias (Figures iB and iiB).

Genes were divided in two ways:
a) Into six subsets through k-means clustering, taking into account the gene occupancy pattern by the protein (Figures iA and iiA).
b) Into six groups depending on the rate of expression as calculated by the method described above (Figures iB and iiB).

For each of the two cases, we have performed a three-level analysis
1: Average gene occupancy
2: Mean gene expression
3: GC content
resulting in two triple plots for each of the two experiments (Rad9-13Myc SC BCS BPS and *aft1*Δ Rad9-13Myc SC BCS BPS).

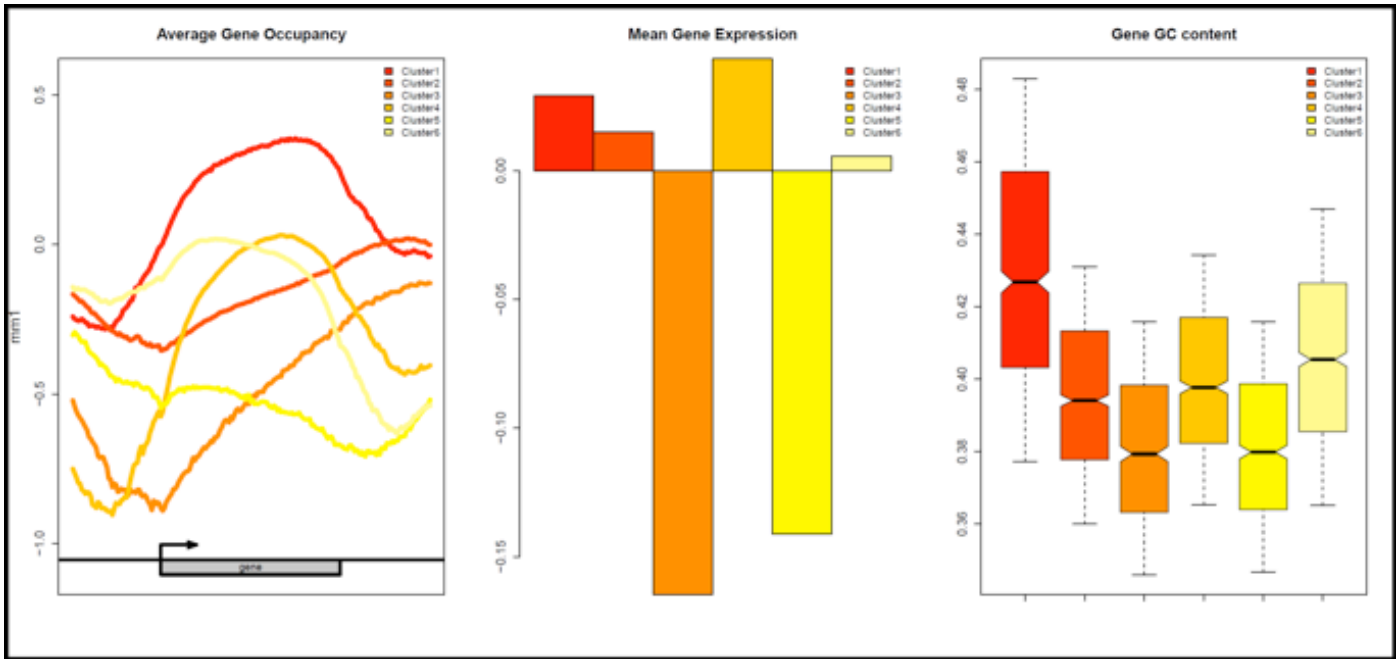


Figure iA: Rad9-13Myc SC BCS BPS

Genes were divided into six subsets through k-means clustering (Clusters 1-6), taking into account the gene occupancy pattern by Rad9-13Myc. *Left panel*: Average gene analysis (see Protocol S4) for the six gene clusters. *Middle panel*: Mean gene expression for the six gene clusters. *Right panel*: Gene GC content of the six gene clusters.

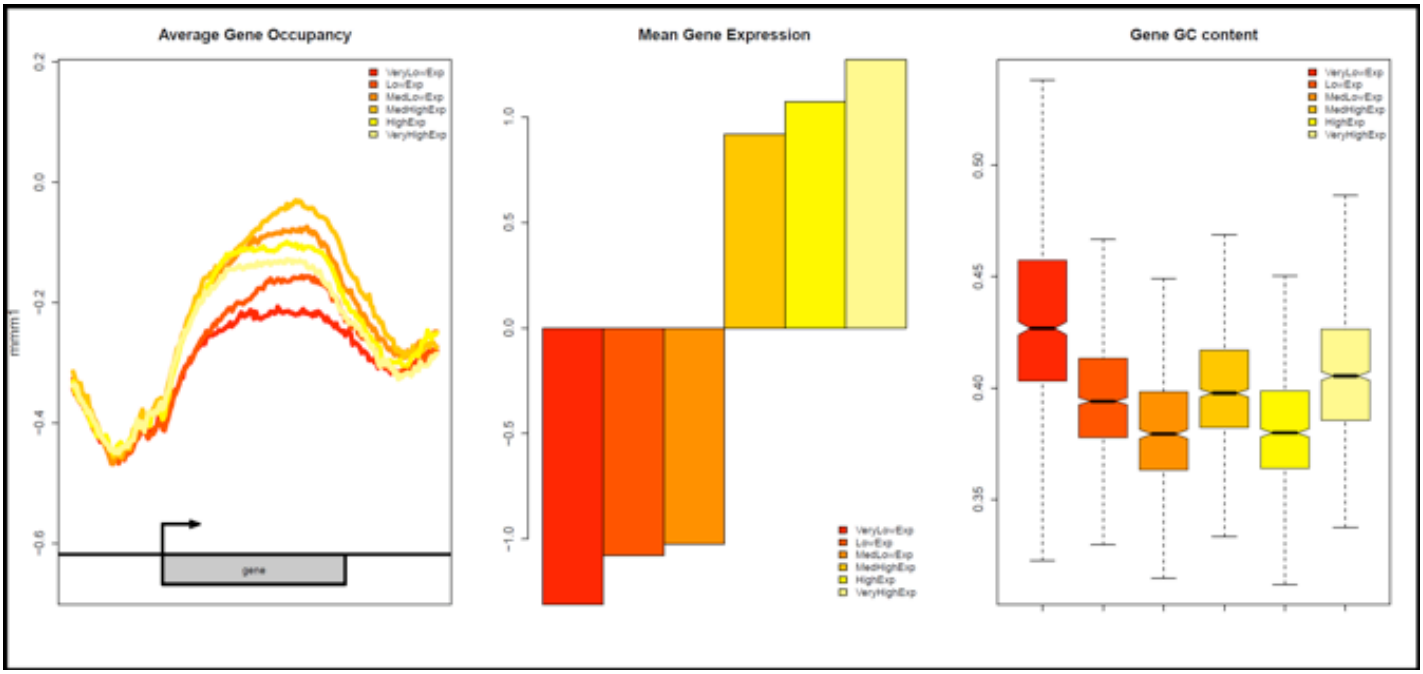


Figure iB: Rad9-13Myc SC BCS BPS

Genes were divided into six groups depending on the rate of expression (Very Low – Low – Medium Low – Medium High – High – Very High), calculated by taking the log2 value of the average gene expression values from our replicate genome-wide microarray experiments (wild strain grown in SC BCS BPS). *Left panel*: Average gene analysis (see Protocol S4) for the six gene groups. *Middle panel*: Mean gene expression for the six gene groups. *Right panel*: Gene GC content of the six gene groups.

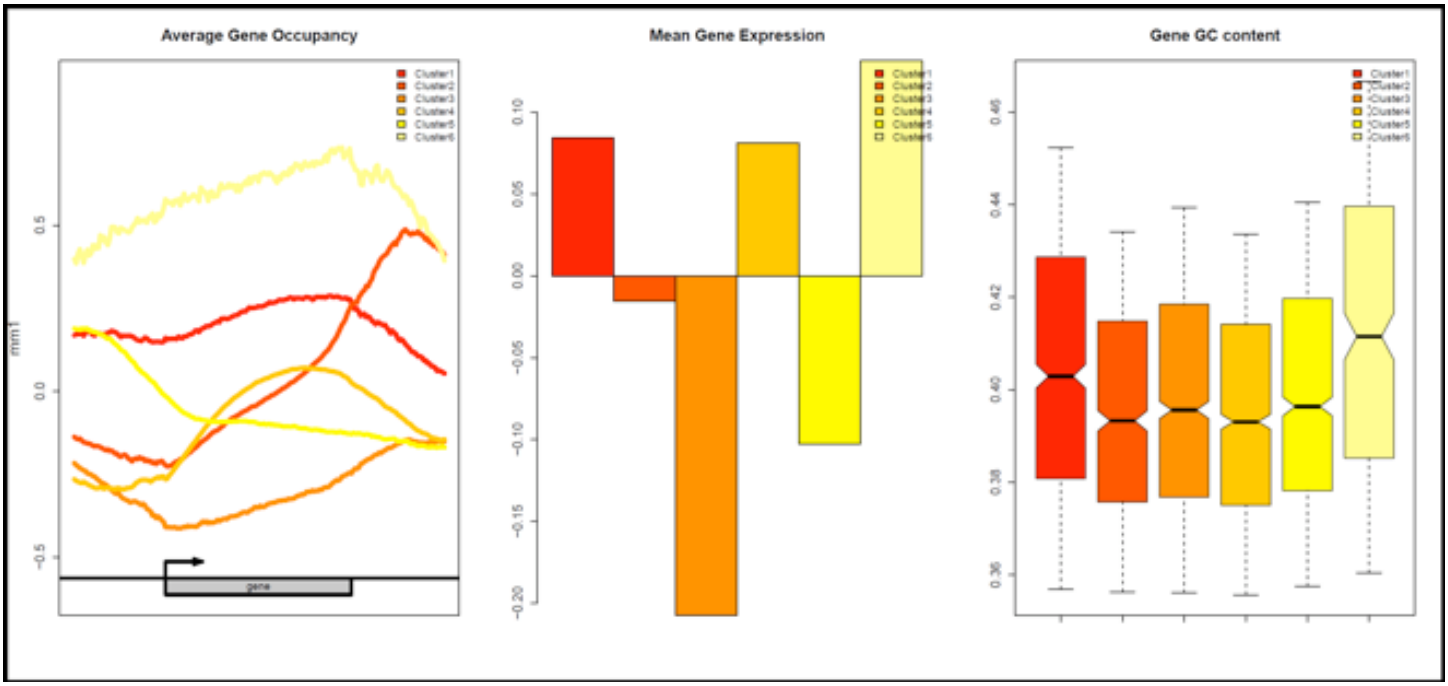


Figure iiA: *aft1*Δ Rad9-13Myc SC BCS BPS

Genes were divided into six subsets through k-means clustering (Clusters 1-6), taking into account the gene occupancy pattern by Rad9-13Myc. *Left panel*: Average gene analysis (see Protocol S4) for the six gene clusters. *Middle panel*: Mean gene expression for the six gene clusters. *Right panel*: Gene GC content of the six gene clusters.

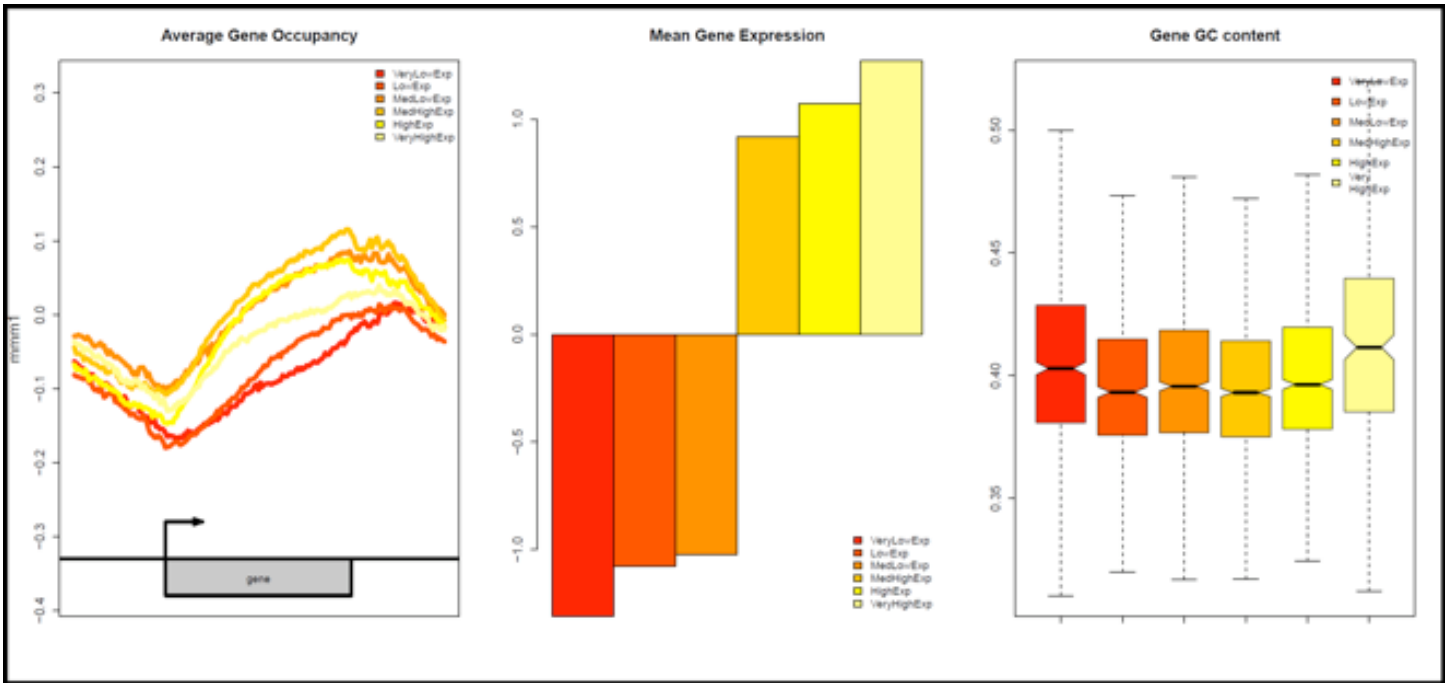


Figure iiB: *aft1*Δ Rad9-13Myc SC BCS BPS

Genes were divided into six groups depending on the rate of expression (Very Low – Low – Medium Low – Medium High – High – Very High), calculated by taking the log2 value of the average gene expression values from our replicate genome-wide microarray experiments (wild strain grown in SC BCS BPS). *Left panel*: Average gene analysis (see Protocol S4) for the six gene groups. *Middle panel*: Mean gene expression for the six gene groups. *Right panel*: Gene GC content of the six gene groups.

These analyses have led to some interesting conclusions:

There is a correlation between the rate of expression and the localisation of Rad9. Furthermore, there is high correlation between the binding intensity and the GC content. There is also a clear variety in the binding pattern of the protein (k-means clustering). This variety seems to correspond to the different GC content.

Rad9 in wild strains localises mainly to the ORF regions of an average gene regardless of the transcriptional activity of its gene-targets (Figure iB, Left panel). The respective localisation in gene groups with higher expression shows a preference to the 3' end of the average gene. Genes with very high and very low transcriptional expression have the largest percentage of GC content (Figure iB, Right panel). The typical pattern of Rad9 localisation with a bias to the 3' end of the average gene corresponds to high content in GC (Cluster 1 in Figure iA, Left and Right panels).

In the absence of Aft1, Rad9 has a biased pattern peaking at the 3'-UTR regardless of the transcriptional activity of its gene-targets (Figure iiB, Left panel). Genes with very high transcriptional expression have the largest percentage of GC content (Figure iiB, Right panel).

PROTOCOL S1

MANUAL CHROMATIN IMMUNOPRECIPITATION ASSAY AND REAL TIME qPCR

The following protocol was adapted from [1] and was used for the manual ChIP assays.

MATERIALS

TBS 20mM Tris/HCl pH 7.5 200mM NaCl (store at 4^0C)	**Proteinase K buffer** 0.01M Tris/HCl pH 7.8 0.005M EDTA pH 8.0 0.5% SDS (store at 4^0C)
FA 150mM NaCl 50mM Hepes pH 7.5 150mM NaCl 1mM EDTA pH 8.0 1% Triton X100 0.1% Sodium desoxycholat (store at 4^0C)	**FA 500mM NaCl** 50mM Hepes pH 7.5 500mM NaCl 1mM EDTA pH 8.0 1% Triton X100 0.1% Sodium desoxycholat (store at 4^0C)
Wash III 10mM Tris/HCl pH 8.0 1mM EDTA pH 8.0 250mM LiCl 0.5% NP-40 0.5% Sodium desoxycholat (store 4^0C)	**Elution buffer** 50mM Tris/HCl pH 7.5 10mM EDTA pH 8.0 1% SDS
TE 10mM Tris/HCl pH 8.0 1mM EDTA pH 8.0	

DAY 1

1) Grow an O/N culture (10ml) of your yeast strain.

DAY 2

2) On the next day dilute in 50ml growth medium in starting OD_{550} ~0.1.
3) Grow cells at 30^0 C and stop incubation when OD_{550}~0.65-0.8 (logarithmic phase).
4) Crosslink by adding 1.35ml formaldehyde, shaking for 20mins.
5) Add 0.47gr glycine and shake for 5mins to absorb formaldehyde excess.
   * Alternatively you can add 2.5ml 2.5M glycine.
6) Pellet cells by spinning at 2500rpm for 5mins at 4^0C.
7) Wash twice with ice cold TBS buffer (1/2V) -25ml here- and spin at 2500rpm for 5mins at 4^0C.
8) After the washes spin again to remove the remains of TBS buffer so that only pellet will be in the tube. You can freeze the pellets here.
9) Resuspend pellet in 400µl FA 150mM buffer and transfer in 1.5ml tubes which already have ~400µl washed glass beads (425-600µm diameter).

10) Add 16μl 25x protease inhibitor cocktail complete EDTA-free (Roche, 11 873 580 001).

* Alternatively you can add 12μl proteinase inhibitor cocktail (SIGMA P8215).

11) Adjust tube on vortex machine and vortex for 40mins at 4^0C. From now on and until the elution step, always treat your samples on ice, unless stated otherwise.
12) Collect solution and debris by spinning 1min at 3000rpm at 4^0C (by making a hole with a 0.25μm needle to the tube and placing to another one before spinning).
13) Sonicate samples 5 times for 12secs each at 40% energy and rest on ice 1min between intervals.

    * Sonication time may vary according to the machine used. Check on a control sample in order to get fragmented chromatin with a peak at 300-500 bp.
14) Spin twice for 15mins each at 13000rpm at 4^0C (change tubes) keeping the supernatant in each one.
15) Keep 50μl as IP sample, 50μl as INPUT sample (and 50μl as MOCK sample-optional). Keep the rest of the supernatant at -80^0C. Keep the INPUT samples at -20^0C until the next day.
16) PRECLEARING

    Raise the volume of your IP samples to 200μl by adding 150mM FA buffer. Add 20μl packed beads to your IP samples and rotate for 2hrs at 4^0C. *The beads must be equilibrated to the 150mM FA buffer before using them [see step 18]* (the stock is in 20% ethanol). Centrifuge for 1min at 3000rpm and collect the supernatant in a new tube.
17) To the IP sample add FA 150mM buffer until final volume ~210μl, 2μg antibody and 2μl proteinase inhibitor cocktail (SIGMA P8215) or equivalent amount of protease inhibitor cocktail complete EDTA-free (stock is 25X). Incubate O/N at 4^0C rotating.

DAY 3

18) Equilibrate 20μl G sepharose (or agarose) beads by washing 3 times with 1ml FA 150mM buffer (spin at 3-4000 rpm between the washes). Add buffer till final volume 70μl.
19) Add these 70μl of beads to IP sample (and MOCK sample if you have one). Add also 40μg sheared herring sperm DNA after you have heated it at 65^0C for 5' and cooled it on ice for 2 mins. Keep rotating at 4^0C for 1.5-2 hrs.
20) Spin at 3000rpm for 1min at RT and keep the beads.
21) Add 1ml FA 150mM buffer along with 1mM final concentration fresh PMSF and rotate for 5mins at RT.
22) Spin at 3000rpm for 1min at RT and keep the beads.
23) Repeat steps 21-22.
24) Add 1ml FA 500mM buffer along with 1mM final concentration fresh PMSF and rotate for 5mins at RT.
25) Spin at 3000rpm for 1min at RT and keep the beads.
26) Repeat steps 24-25 two times.
27) Add 1ml WASH III buffer and rotate for 5mins at RT.
28) Spin at 3000rpm for 1min at RT and keep the beads.
29) Repeat steps 27-28.
30) Add 1ml TE buffer and rotate for 5mins at RT.

31) Spin at 3000rpm for 1min at RT and keep the beads.
32) Repeat step 30.
While rotating the IP, thaw the INPUT sample (step 15) and prepare a mix of 0.2µg/µl RNAse in TE buffer (20µl RNAse 10mg/ml in 1ml TE buffer).
33) Spin at 3000rpm for 1min at RT and keep the beads.
34) Add to each of IP and INPUT samples (and MOCK if you have) 100µl of RNAse mix and incubate at 37^0C for 20mins.
35) To INPUTs: add 350µl ELUTION buffer and 40µl NaCl 2.5M. Incubate at 65^0C for at least 5hrs or O/N.
36) To IPs: add 1ml TE buffer, spin at 3000rpm for 1min at RT and keep the beads.
37) Add 250µl ELUTION buffer to the beads and rotate for 15mins at RT. Vortex 3 times in between (every 5 mins).
38) Spin at 3000rpm for 1min at RT and keep the supernatant to a new 1.5ml tube.
39) Repeat steps 37 and 38. Keep all the supernatant in one tube.
40) Add 40µl 2.5M NaCl to the eluted sample and incubate at 65^0C for at least 5hrs or O/N.
If you choose not to leave your samples O/N you can proceed to step 41 and leave at -80^0C until the next day.

DAY 4

41) Add 1ml ethanol 100% and incubate 2 hrs at -80^0C or O/N at -20^0C.
42) Spin at 13000rpm at 4^0C for 30mins and discard supernatant.
43) Wash the pellet once with 500µl 70% ethanol.
44) Spin at 13000rpm at RT for 5mins and discard supernatant.
45) Speed vac the pellets so that all ethanol is evaporated.
46) Prepare proteinase K mix (for ~6 samples):
700µl H_2O
77µl proteinase K buffer 10X
14µl proteinase K 20µg/µl
47) Add 112µl from the proteinase K mix to each of the pellets and incubate at 50^0C (water bath) for 30mins.
48) Add 390µl H_2O to increase volume at ~500µl.
49) Add 50µl sodium acetate 3M pH=5.2 (1/10 V).
50) Add 250µl phenol.
51) Add 250µl chloroform.
52) Vortex and spin at 13000rpm for 10mins at RT.
53) Keep the supernatant in new 1.5ml tubes.
54) Add 500µl chloroform, vortex, spin at 13000rpm for 5mins at RT and keep supernatant in a new 1.5ml tube.
55) Add 1ml ethanol 100% and 1µl glycogen and incubate O/N at -80^0C.

DAY 5

56) Spin at 13000rpm at 4^0C for 30mins and discard supernatant.
57) Wash the pellet once with 500µl 70% ethanol.
58) Spin at 13000rpm at RT for 5mins and discard supernatant.
59) Speed vac the pellets so that all ethanol is evaporated.

60) Resuspend the IP pellets in 50μl H_2O for injection and in 250μl the pellets of your INPUT samples. Use 5-10μl in each reaction in real time PCR.
A typical PCR reaction (V_f=25μl) is set as follows:
11.8μl H_2O
5μl IP/INPUT sample
1μl forward primer 200ng/μl
1μl reverse primer 200ng/μl
0.5μl dNTPs 10mM
2μl $MgCl_2$ 25mM
2.5μl Taq buffer 5 u/μl
0.2μl Taq polymerase
1μl SYBRE green
The typical program used in the PCR is set as follows:
1) Incubate at 94^0C for 2'
2) Incubate at 94^0C for 30''
3) Incubate at 56^0C for 30''
4) Incubate at 72^0C for 30''
5) Incubate at 80^0C for 1''
6) Plate read
7) Go to line 2 for 39 times more
8) Incubate at 72^0C for 2'
9) Perform melting curve from 60^0C to 94^0C, read every 0.5^0C, hold for 1' between reads
10) Incubate at 15^0C for 1''

The annealing temperature depends on the primers used each time. The temperature in which the machine measures the fluorescence in each cycle also depends on where the product for each primer is obtained.
The Real Time PCR that followed was performed in MJResearch or BIORAD PCR machines and analysed with the manufacturer's software. We obtained our enrichment results by performing two types of normalisation, one over the INPUT sample and one over the enrichment to the ORF of a control gene on which the protein of interest is not bound (typically *PHO5*).

1. Kuo, M.H. and C.D. Allis, *In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment.* Methods, 1999. 19(3): p. 425-33.

PROTOCOL S2

CO-IMMUNOPRECIPITATION ASSAY
We used the following co-immunoprecipitation protocol in order to detect *in vivo* interactions between proteins tagged with different epitopes.

1) Start growing cultures of the strains that you want to check for interaction. Usually there is a strain that has 2 different proteins (A and B) tagged with different epitopes,

the interaction of which you want to check. Furthermore, grow the negative and positive (if available) controls.

2) The next day dilute the starter culture in 200ml medium to an OD_{550}~0.1 and incubate at 30^0C shaking until OD_{550}~0.8.

3) Collect equal number of cells from each culture by centrifugation (3000rpm, 5mins, 4^0C) and wash twice with water. Pellets can be kept at -80^0C.

4) The next day suspend the pellets in 1ml Buffer A and transfer sample to a 2ml tube. Spin the tube and remove supernatant. Suspend the pellet in such volume of Buffer A until the V_f~400μl (cells and buffer). Add proteinase "complete EDTA free" inhibitor cocktail (Roche) in 1X final concentration and PMSF in a final concentration of 1mM.

5) Add 350μl glass beads (quantity equal to ~80-90% of the final volume).

6) Vortex 8 times, 30secs each, and rest at least 30secs between the repeats. Finally, leave the tubes on ice until glass beads are precipitated due to gravity. Collect supernatant in a new 1.5ml tube.

7) Add 200μl Buffer A in the remaining glass beads along with Proteinase inhibitor cocktail and PMSF, vortex once, leave the beads to precipitate due to gravity, collect the supernatant and combine with the supernatant from step 6.

8) Centrifuge extract at 4^0C for 15mins at 13000rpm and transfer supernatant in a new tube.

9) Keep 30μl of every sample to use later as an INPUT for the quantification of the loading on the gel (1/13-1/18 of the volume depending on the abundance of the protein).

10) In the rest of the sample add the first antibody (2μg) which will recognize and bind to one of the proteins. Leave the samples rotating at 4^0C overnight.

11) The next day spin the tubes for 1min at 13000rpm (in case of any precipitant) and collect samples to a new 1.5ml tube. Add 20-30μl packed G sepharose beads which have been equilibrated with Buffer B. Equilibration is done by adding 1ml Buffer B in the beads, spin 1min at 3000rpm and remove supernatant; repeat 3 times; add 20-30μl Buffer B, suspend and add to the samples. Leave rotating at 4^0C for 2.5hrs. The beads recognize the stable domain of the antibody which is bound to the tagged protein and the proteins which may also be present, will be isolated as a complex.

12) Spin the samples 1min at 3000rpm and discard supernatant.

13) Wash samples 4 times. The first 2 are done by 1ml Wash Buffer I and the next 2 with 1ml Buffer II. More specifically, add to the sample 1ml from the Wash Buffer along with PMSF (C_f=1mM), rotate 5mins in RT, spin 1min at 3000rpm and discard supernatant.

14) Suspend G sepharose beads in 5X protein Loading Buffer (see Western protocol). Adjust the volume so that it will not exceed 35µl (i.e. leave ~28µl of Wash II and add ~7µl of 5X protein Loading Buffer).

15) Boil the samples for 5 mins, spin and use the whole volume of IPs and INPUTs in SDS-PAGE. A typical Western experiment follows. Probe the membrane with the antibody which recognizes the other protein to detect possible interaction.

Materials
Solution 1 (100mM Hepes-KOH pH=7.5; 10mM MgAc; 200mM KAc; 0.2% NP40; 20% glycerol)
Buffer A (1/2V Solution 1; DTT 1mM; BSA 0.5mg/ml; H_2O until 1V)
Buffer B (1/2V Solution 1; DTT 1mM; H_2O until 1V)
Wash I (1/2V Solution 1; DTT 1mM; H_2O until 1V)
Wash II: as Wash I but contains 150mM KAc instead of 100mM

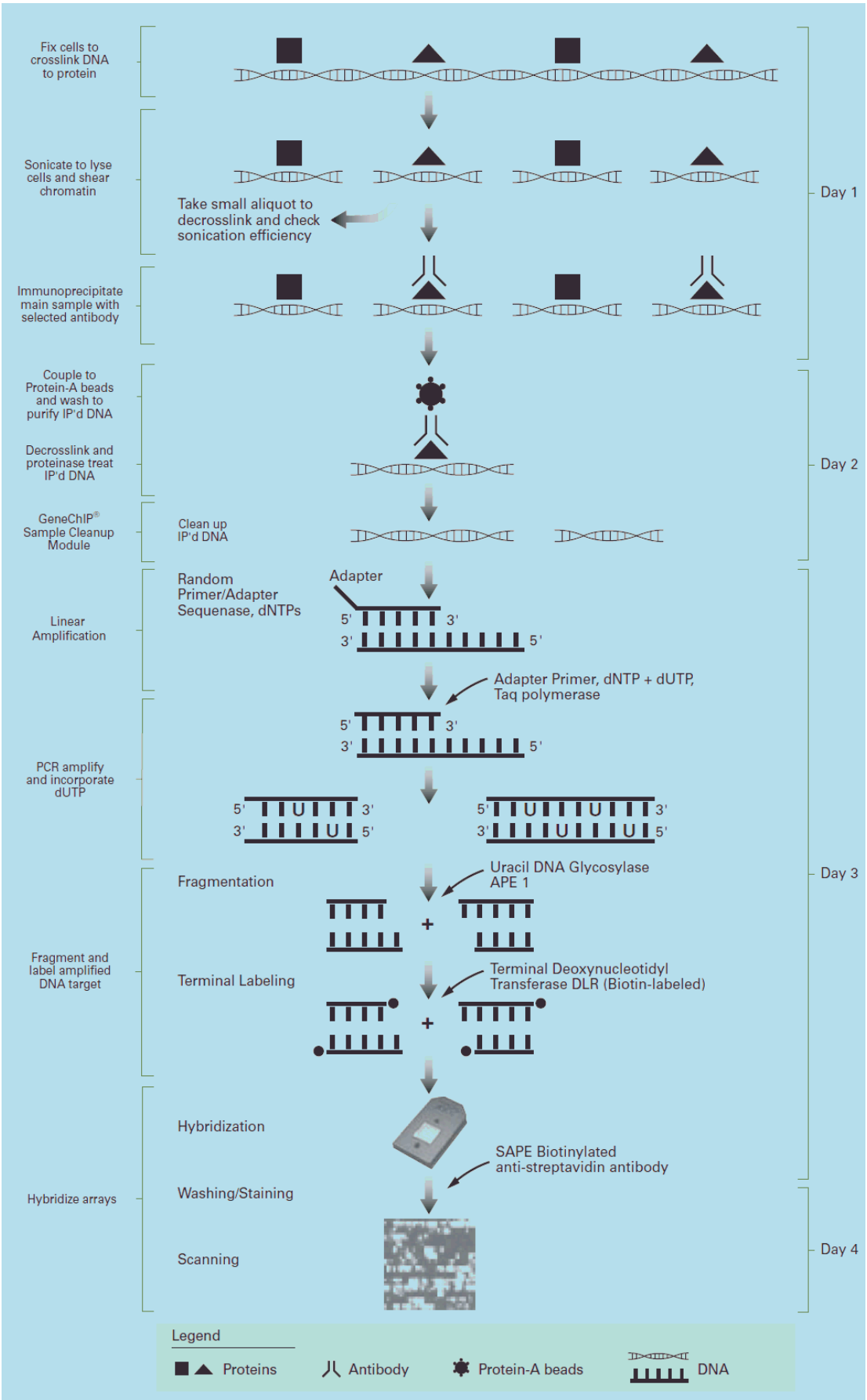
PROTOCOL S3

CHROMATIN IMMUNOPRECIPITATION ON CHIP ASSAY (ChIP on chip)
The **array** that was used for our ChIP on chip assays was GeneChip ***S.cerevisiae* Tiling 1.0R Array** manufactured by Affymetrix (900645). The GeneChip *S. cerevisiae* Tiling 1.0R Array is designed for identifying novel transcripts, mapping sites of protein/DNA interaction in chromatin immunoprecipitation (ChIP) experiments, and other whole-genome experiments. The *S. cerevisiae* 1.0R Array is a single array comprised of over 3.2 million perfect match/mismatch probe pairs tiled through the complete *Saccharomyces cerevisiae* genome. Genome sequence information for the design of the *S. cerevisiae* Tiling 1.0R Array was drawn from the October 2003 Stanford Yeast Genome Database files (www.yeastgenome.org). The array also contains probes to interrogate a 2-µm circle plasmid (NCBI Accession J01347). Centromeric regions are not included in the array; for this reason we performed manual ChIPs for these loci.
Probes are tiled at an average of 5 base pair resolution, as measured from the central position of adjacent 25-mer oligos, creating an overlap of approximately 20 base pairs on adjacent probes.
We followed the chromatin immunoprecipitation assay as proposed and described by Affymetrix in
http://media.affymetrix.com/support/downloads/manuals/chromatin_immun_ChIP.pdf
adapting it in order to fit the needs and specifications of *Saccharomyces cerevisiae*. The figures and tables included here are taken from the original protocol by Affymetrix.

The Affymetrix Chromatin Immunoprecipitation (ChIP) Assay is designed to generate double-stranded labelled DNA targets that identify sites of protein-DNA interactions or chromatin modifications on a genome-wide scale. This assay has been designed specifically for use with Affymetrix GeneChip Tiling Arrays for ChIP on chip studies in order to study transcription factor binding sites, histone protein modifications, and other chromatin-protein interactions. ChIP experiments can be used as a powerful tool to complement RNA transcription studies because they enable researchers to study the DNA-protein interactions that regulate gene expression. Following the protocol, cells are first fixed with formaldehyde to crosslink DNA to

any associated proteins. The cells are then lysed and DNA is sheared into smaller fragments using sonication. Protein-DNA complexes are then immunoprecipitated with an antibody directed against the specific protein of interest. Following the immunoprecipitation, crosslinking is reversed, samples are protease-treated and the purified DNA sample is amplified using a random-primed PCR method. Subsequently, targets are fragmented and labeled to hybridize onto GeneChip Tiling Arrays. By comparing the hybridization signals generated by an immunoprecipitated sample versus an antibodynegative or non-specific antibody control, the regions of chromatinprotein interaction can be identified. The procedure outlined in this protocol describes all the necessary steps and reagents for fixing cells, fragmenting chromatin, immunoprecipitating sheared chromatin, amplifying and labelling precipitated DNA.

An optimization must be done before the execution of the protocol because of the variability inherent in the cell type, the protein of interest, the antibody, the DNA fragmentation and the PCR conditions. Our optimizations are mentioned during the description of the protocol. A schematic representation of the protocol is presented below:



ChIP on chip assay schematic overview

A. MATERIALS

The materials required for the protocol are presented in the following tables.

Material	Source	Part Number
Formaldehyde Solution (37%), 500 mL	Sigma-Aldrich	F8775
Glycine, 1 kg	Sigma-Aldrich	50046
Phosphate Buffered Saline (PBS) pH 7.4 (1X), liquid	Various	
IGEPAL® CA-630	Sigma-Aldrich	9036-19-5
Phenylmethanesulfonyl Fluoride Solution (PMSF), 250 mL	Sigma-Aldrich	93482
Micrococcal Nuclease (MNase) (Optional)	USB	70196Y
EGTA (optional)	Sigma-Aldrich	E3889-100G
Protease Inhibitor Tablet	Roche	11873580001
Decrosslink and check sonication efficiency		
Proteinase K	New England BioLabs	P8102S
LiCl (8M), 500 mL	Sigma-Aldrich	L7026
Glycogen	Roche	10901393001
Immunoprecipitation		
Triton-X100 (non-ionic viscous liquid)	Roche	10789704001
Protein A Sepharose™ CL-4B	Amersham	17-0963-03
Antibody*	Various	

Material	Source	Part Number
PCR Amplification		
Sequenase™ Version 2.0 DNA Polymerase	USB	70775Y
Primer A: 200 µM GTTTCCCAGTCACGGTC$(N)_9$	Various	HPLC purified
Primer B: 100 µM GTTTCCCAGTCACGGTC	Various	HPLC purified
Taq Polymerase 5 U/µL	Various	
10X PCR Buffer	Various	
dATP 100 mM	Various	
dCTP 100 mM	Various	
dGTP 100 mM	Various	
dTTP 100 mM	Various	
dUTP 100 mM	Various	
BSA 20 mg/mL	Various	
DTT 0.1M	Various	
Wash Buffer		
Tris-HCl	Various	
EDTA	Various	
SDS, 100g	Sigma-Aldrich	71725
NaCl	Various	
Deoxycholate (sodium salt), 100 g	Sigma-Aldrich	D6750
$MgCl_2$, 1M	Various	
$CaCl_2$, 1M	Sigma-Aldrich	21115
Fragmentation and Labeling		
GeneChip® WT Double-Stranded DNA Terminal Labeling Kit, 30 Rxn	Affymetrix	900812
DNA Cleanup		
GeneChip® Sample Cleanup Module, 30 Rxn	Affymetrix	900371
ERC Buffer*, 85 mL	QIAGEN	1018144
Hybridization, Stain and Wash		
GeneChip® Hybridization, Wash, and Stain Kit	Affymetrix	900720
Control Oligonucleotide B2, 3nM	Affymetrix	900301

BUFFERS

Lysis Buffer (Store at 4°C)
10 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5) 10 mM NaCl 3 mM $MgCl_2$ 0.5% IGEPAL 1 mM PMSF (add fresh)
Pre-IP Dilution Buffer (Store at RT)
10 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5) 10 mM NaCl 3 mM $MgCl_2$ 1 mM $CaCl_2$ 4% IGEPAL 1 mM PMSF (add fresh)
IP Dilution Buffer (Store at RT without protease inhibitors)
20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 2 mM EDTA 1% Triton X-100 150 mM NaCl Protease Inhibitor Stock (add fresh)
Protease Inhibitor Stock
Prepare a 25X stock by dissolving 1 protease inhibitor tablet in 2 mL of nuclease-free water
ChIP Wash 1 (Store at RT)
20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 2 mM EDTA 1% Triton X-100 150 mM NaCl 1 mM PMSF (add fresh)

ChIP Wash 2 (Store at RT)
20 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 2 mM EDTA 1% Triton X-100 0.1% SDS 500 mM NaCl 1 mM PMSF (add fresh)
ChIP Wash 3 (Store at RT)
10 mM Tris-HCl (made from stock 1M Tris-HCl pH 8) 1 mM EDTA 0.25M LiCl 0.5% IGEPAL 0.5% Deoxycholate (sodium salt)
Elution Buffer
25 mM Tris-HCl (made from stock 1M Tris-HCl pH 7.5) 10 mM EDTA 0.5% SDS

THE ASSAY

Procedure A: Prepare the cells

We use a freshly streaked plate (from stab) to grow an overnight starter culture of the strain of interest. The next day we dilute the starter culture to an OD_{550}~0.1 and leave enough time for the cells to grow for 6-7 hours in order to have enough time for the induction with BCS (for 3 hours) and BPS (for 6 hours) without exceeding the exponential phase. Depending on the samples needed we grow enough cells to an OD_{550}=0.8-1 in SC or YPD medium. We use the chromatinic extract from ~$7x10^7$ cells per IP sample (equivalent to ~9ml of an exponentially growing culture of

OD_{550}=0.8). For each experiment it is vital to grow cells for at least two IP samples, one INPUT sample and one MOCK sample ($28x10^7$ cells). The INPUT sample is used for the normalization in the PCR reaction by which the immunoprecipitation efficiency is evaluated. As a MOCK sample we used an antibody(-) one, to serve as the control group in the downstream two-sample analysis and we treated it exactly as the IP experimental sample.

Procedure B: Fix cells, Lyse and Sonicate the whole cell extract

1. When the cells are ready to harvest, add formaldehyde to the culture flask to a final concentration of 1% and incubate in a fume hood for 20 minutes.

2. Add 1/20 volume of 2.5 M glycine and incubate at room temperature (RT) for 5 minutes with gentle mixing. Alternatively, add 0.47gr glycine and shake for 5mins to absorb formaldehyde excess.

3. Wash pellet with 10 mL ice-cold 1X PBS to resuspend cells, and pellet cells at 4°C, (3000 rpm) for 5 minutes, discard supernatant and repeat wash with ice-cold 1X PBS once.

4. Wash the pellet 3 times with 10 mL Lysis Buffer with fresh PMSF and pellet cells at 4^0C, 3000 rpm for 5 minutes between washes.

5. Discard supernatant and proceed to the next step or flash freeze pellet and store at –80°C.

6. Resuspend the pellet in 240µl pre-IP dilution buffer (with fresh PMSF).

7. Add to the tube:

100mM PMSF	6.4µl
25X protease inhibitor stock	16µl
Pre-IP dilution buffer	73.6µl
20% SDS	16µl
2.5M NaCl	25.6µl
Nuclease free water	22.4µl
Final Sample volume before sonication	400µl

8. Transfer in 1.5ml tubes which already have ~400µl washed glass beads (of 425-600µm diameter). Adjust tube on vortex machine and vortex for 40mins at 4^0C. Collect solution and debris by spinning 1min at 3000rpm at 4^0C (by making a hole with a 0.25µm needle to the tube and placing to another one before spinning).

9. Sonicate samples 5 times for 12secs each at 40% energy and rest on ice 1min between intervals. Sonication time may vary according to the machine used. Under these conditions samples are sheared to fragments of 300-500bp (peak).

10. Spin twice for 15mins each at 13000rpm at 4^0C (change tubes) keeping the supernatant from each one in the same tube. The sonication efficiency can be checked by taking an aliquot of this supernatant, de-crosslinking it (see Procedure C, below), and running the de-crosslinked DNA on a 1-2% agarose gel.

11. If not directly used, freeze the supernatant in -80^0C.

Procedure C: Check sonication efficiency

1. Add 40 µl 10 mM Tris pH 8.0 to a 40 µl aliquot taken from the sonicated samples.

2. Add Proteinase K (20 mg/ml) to a final concentration of 0.2mb/µl and mix well by vortexing.

3. Incubate 42°C for 2 hours, then 65°C for 6 hours to overnight. This step can be performed in a thermocycler.

4. Clean-up using Affymetrix cDNA cleanup columns, from the GeneChip Sample Cleanup Module, eluting with 20 µL Elution Buffer following the manufacturer's instructions.
5. Load 100-500 ng of purified DNA sample on an agarose gel to check sonication efficiency. Typically, sheared DNA size ranges from 100-4000 bp, with the average size fragment between 300-500 bp.

Procedure D: Incubate with specific antibody
1. If the sample (from Procedure B Step 11) was frozen, thaw.
2. Equilibrate an equal volume with the sample of G sepharose (or agarose) packed beads 3 times with IP dilution buffer (spin at 3-4000 rpm between the washes). Add buffer till there is enough for a final volume of 70µl per sample.
3. Preclear the chromatin samples: Transfer desired amount of sample chromatin supernatant to a 0.5 mL tube (~60-70µl/400µl equivalent to ~$7x10^7$ cells' extract per sample) and add the 70µl of the equilibrated G sepharose beads from step 2. If needed, raise the volume with IP dilution buffer (containing Roche complete EDTA free protease inhibitor cocktail) until enough to mix well while rotating (usually ~250µl final volume).
4. Incubate on a rotating platform at 4°C for 1 hr and 30 minutes.
6. Centrifuge at 2,000 rpm for 2 minutes at 4°C.
7. Transfer supernatant to a new 0.5 µl tube and discard beads. Aliquot the final volume of precleared chromatin so that each sample (IP, INPUT or MOCK) contains volume equivalent to the ~$7x10^7$ cells' extract.
8. Freeze the INPUT sample at -20^0C. Add 6µg of antibody per IP. MOCK sample contains no antibody but otherwise is treated as the IP sample. Add Roche complete EDTA free protease inhibitor cocktail to an 1X final concentration. Raise the final volume to ~250µl with IP dilution buffer.
9. Incubate on rotating platform at 4°C overnight.

Procedure E: Immunoprecipitate and wash
1. Pre-equilibrate protein G Sepharose beads as in Procedure D Step 2 and transfer an amount of ~60-70µl packed beads to each of the IP or MOCK samples
2. Add PMSF to each tube sample (final concentration 1mM PMSF in final volume).
3. Incubate on rotating platform at RT for 2 hours.
4. Centrifuge at 2,000 rpm at 4°C for 4 minutes, and then discard supernatant.
5. Resuspend the pellet with 700 µL ChIP wash 1 (containing 1 mM PMSF added fresh), mix and transfer to spin-X column.
6. Incubate on rotating platform at RT for 1 minute.
7. Centrifuge at 2,000 rpm at RT for 2 minutes and discard flowthrough.
8. Repeat steps 5–7.
9. Wash the beads with 700 µL ChIP wash 2 (containing 1 mM fresh PMSF).
10. Incubate on rotating platform at RT for 5 minutes.
11. Centrifuge at 2,000 rpm at RT and discard flow-through.
12. Wash the beads with 700 µL ChIP wash 3.
13. Incubate on rotating platform at RT for 5 minutes.
14. Centrifuge at 2,000 rpm at RT and discard flow-through.
15. Wash the beads with 700 µL TE (10 mM Tris-HCl pH 8, 1 mM EDTA).
16. Incubate on rotating platform at RT for 1 minute.
17. Centrifuge at 2,000 rpm at RT and discard flow-through.
18. Repeat steps 15 through 17.

19. Transfer the spin-X column with beads to a dolphin-nose tube.
20. Add 200 µL Elution Buffer to the column.
21. Incubate at 65°C for 30 minutes.
22. Centrifuge at 3,000 rpm at RT for 2 minutes.
23. Add 200 µL Elution Buffer to the column.
24. Centrifuge at 3,000 rpm at RT for 2 minutes. This 400 µL eluted sample is the "enriched" or "IP'd" sample.

Procedure F: Reverse crosslinks
1. Add 5 µL Proteinase K (20mg/mL) per 100 µL of negative control or IP sample, mix well. (20 µL for 400 µL of eluted sample.)
2. Incubate in incubator at 65°C overnight.

Procedure G: Cleanup de-crosslinked samples
i. Clean up samples using Affymetrix cDNA cleanup columns as follows:
1. Add 5X volumes of cDNA Binding Buffer to sample, and vortex for 3 seconds.
2. Apply the sample to a cDNA Spin Column sitting in a 2 mL Collection Tube (max capacity of column = 700 µL; if volume exceeds 700 µL, spin 700 µL at 10000rpm for 1 minute, discard flow-through, and repeat).
3. Spin at 10000rpm for 1 minute. Discard the flow-through.
4. Transfer the cDNA Spin Column to a new 2 mL Collection Tube and add 750 µL of cDNA Wash Buffer to the column. Spin at 10000rpm for 1 minute and discard the flow-through.
5. Open cap of the cDNA Spin Column, and spin at 13000rpm for 5 minutes with the caps open. Discard the flow-through, and place the column in a 1.5 mL collection tube.
6. Pipet 20µl of cDNA Elution Buffer directly to the column membrane and incubate at room temperature for 1 minute. Then, spin at 13000rpm for 1 minute.
7. Repeat step 6 with another 20µl of Elution Buffer. Total elution volume recovered is ~ 38 µL.
ii. IP efficiency can be checked at this stage in the protocol using polymerase chain reaction (PCR) and designing primer sets against regions that are known to be bound by the protein of interest and immunoprecipitated using the antibody being investigated. A significant (or the expected, if known) increase or enrichment for the specific target should be observed for the IP condition compared to the Ab- control (MOCK).

Procedure H: PCR amplification of the immunoprecipitated DNA targets

1) Use 9µl of IP'd or negative control sample for initial round of linear amplification.
2) Set up first round reaction:

Round A reaction Mix

DNA to be amplified and labeled	9 µl
5X sequenase reaction buffer	2.4 µl
Primer A (40µM)*	1.2 µl

*For smaller amounts of DNA increase Primer A to a concentration up to 200µM. You can test this by performing serial dilutions.

3) Cycle conditions: Random Priming.

A. 95^0C for 4 mins.
B. Snap cool samples on ice.
C. 10^0C hold.
D. Prepare first cocktail

FIRST COCKTAIL	1X (µl)	3X (µl)	5X (µl)
5X sequenase reaction buffer	1	3	5
dNTP mix (3mM each)	1.5	4.5	7.5
DTT	0.75	2.25	3.75
BSA	1.5	4.5	7.5
Sequenase	0.3	0.9	1.5
Total	5.05	15.15	25.25

Round A stocks	
dNTPs	3mM each
DTT	0.1M
BSA	500µg/ml
Sequenase	13U/µl

E. Add 5µl of the first cocktail mix.
F. Mix well by pipetting and put the sample back in thermocycler block.
G. 10^0C for 5 mins.
H. Ramp from 10^0C to 37^0C over 9 mins.
I. 37^0C for 8 mins.
J. 95^0C for 4 mins.
K. Snap cool on ice.
L. 10^0C hold.
M. Add 0.3µl of 13U/µl sequenase to each sample.
N. 10^0C for 5 mins.
O. Ramp from 10^0C to 37^0C over 9 mins.
P. 37^0C for 8 mins.
Q. Repeat from J) to P) for 1 more cycle (* this step is optional, we have found that only 2 cycles with sequenase are enough for satisfactory amplification)
R. 4^0C hold.

4) For each IP, purify with Microspin S-300 HR (GE Healthcare) columns (2 columns per reaction) as follows:

A. Add 35 µl of 10 mM TE pH 8.0 to each reaction.
B. Spin 2 columns (A & B) at 3,000 rpm for 1 minute, discard flow-through.
C. Transfer reaction volume (~ 43 µl) to column A, while equilibrating column B with 300 µl of 10 mM Tris pH 8.0.
D. Spin both columns at 3,000 rpm for 1 minute, keep flowthrough from column A (sample) and discard flow-through of column B (Tris buffer).
E. Transfer flow-through of column A to column B with new collection tube.
F. Spin at 3,000 rpm for 2 minutes.
G. Collect ~ 56 µl of first round purified DNA per reaction.

5) Prepare dNTP/dUTP mix
dCTP-25mM
dATP-25mM
dGTP-25mM
dTTP-20mM
dUTP-5mM

6) PCR Mix Setup (Round B)

	1X	4X
First round DNA from step 4	15 µl	
10X Taq buffer	10 µl	40 µl
25mM $MgCl_2$	8 µl	32 µl
dNTP/dUTP mix (step 5)	1 µl	4 µl
Primer B (100mM)	4 µl	16 µl
Taq polymerase (5u/µl)	1 µl	4 µl
Nuclease free water	61 µl	244 µl
Total	100 µl	

Set up 4 reactions of 100µl (aliquoted in 8 low tube strips, 50µl each) to get enough amount of amplified DNA sample for one hybridization.

7) Cycle conditions:
A. 16 cycles
1) 95°C 30 seconds.
2) 45°C 30 seconds.
3) 55°C 30 seconds.
4) 72°C 1 minute.
B. 16 cycles
1) 95°C 30 seconds.
2) 45°C 30 seconds.
3) 55°C 30 seconds.
4) 72°C 1 minute.
For every subsequent cycle add 5 seconds.
E.g., cycle 1: 60 seconds, cycle 2: 65 seconds, etc.
C. 4°C hold.

8) Check amplified DNA on 1% agarose gel.

9) Purify PCR samples with Affymetrix cDNA cleanup columns, provided in the GeneChip Sample Cleanup Module, eluting twice with 20 µL of Elution Buffer (see Procedure G, Part i).

10) Calculate DNA quantity by using a NanoDrop. Normally, greater than 9 µg of amplified DNA is obtained from each reaction.

Procedure I: Fragment amplified targets
1. Fragment the samples using the table below:
Fragmentation Mix for single arrays (e.g., *S. cerevisiae* 1.0R Tiling Array)

Component	Volume/Amount in 1 Rxn
Double-Stranded DNA	7.5 µg
10X cDNA Fragmentation Buffer*	4.8 µL
UDG, 10 U/µL*	1.5 µL
APE 1, 100 U/µL*	2.25 µL
Nuclease-free Water*	up to 48 µL
Total Volume	48.0 µL

** Available in GeneChip® WT Double-Stranded DNA Terminal Labeling Kit (P/N 900812)*

2. Set up fragmentation mix according to the above table. Flick-mix and spin down the tubes.
3. Incubate the reactions at:

- 37°C for 1 hour.
- 93°C for 2 minutes.
- 4°C for at least 2 minutes.

4. Flick-mix, spin down the tubes, and transfer 45 µL of the sample to a new tube.
5. The remainder of the sample is to be used for fragmentation analysis using a Bioanalyzer or agarose gel. If not labeling the samples immediately, store the fragmented DNA at –20°C.

Procedure J: Label fragmented dsDNA
1. Prepare the Double-Stranded DNA Labeling Mix as described in the table below:

Component	Volume in 1 Rxn
5x TdT Buffer*	12 µL
TdT*	2 µL
DNA Labeling Reagent, 5 mM*	1 µL
Total Volume	15 µL

** Available in the GeneChip® WT Double-Stranded DNA Terminal Labeling Kit (P/N 900812).*

2. Add 15 µl of the Double-Stranded DNA Labeling Mix to the DNA samples (45µl), flick-mix, and spin them down.
3. Incubate the reactions at:

- 37°C for 60 minutes.
- 70°C for 10 minutes.
- 4°C for at least 2 minutes.

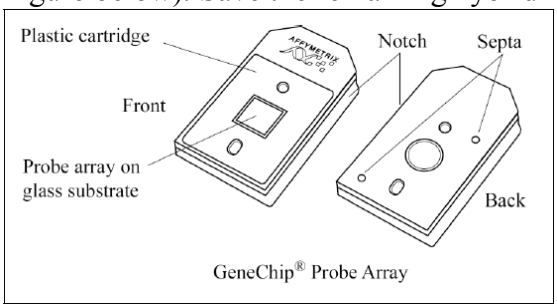
Hybridization and Array Processing

Procedure K: Hybridize labeled target on the array
This Procedure requires the use of the GeneChip Hybridization, Wash, and Stain Kit (P/N 900720).
1. Prepare the Hybridization Cocktail in a 1.5 mL RNase-free microfuge tube as shown in the table below.

Component	Volume in 1 Rxn	Final Concentration or Amount
Fragmented and Labeled DNA Target	~ 60.0 µL*	~ 7.5 µg
Control Oligonucleotide B2	3.3 µL	50 pM
2X Hybridization Mix†	100 µL	1X
DMSO	14.0 µL	7%
Nuclease-free Water	up to 200.0 µL	
Total Volume	200.0 µL	

*This volume is 56 µL if a portion of the sample was set aside for gel-shift analysis.

2. Flick-mix, and centrifuge the tube.
3. Heat the Hybridization Cocktail at 99°C for 5 minutes. Cool to 45°C for 5 minutes, and centrifuge at maximum speed for 1 minute.
4. Inject ~ 200 µL of the specific sample into the array through one of the septa (see Figure below). Save the remaining hybridization cocktail in –20°C for future use.



GeneChip® Probe Array

5. Place array in 45°C hybridization oven, at 60 rpm, and incubate for 16 hours.
6. After hybridization, remove the hybridization cocktail for future use.

Array Washing and Staining
This part of the protocol includes preparation and priming of the Fluidics station 450, selection of the appropriate protocol, wash and staining of the probe array and shutting down the station. All buffers needed can be found in the extended protocol: http://media.affymetrix.com/support/downloads/manuals/chromatin_immun_ChIP.pdf

Scanning
After the scanning a series of files are obtained, namely

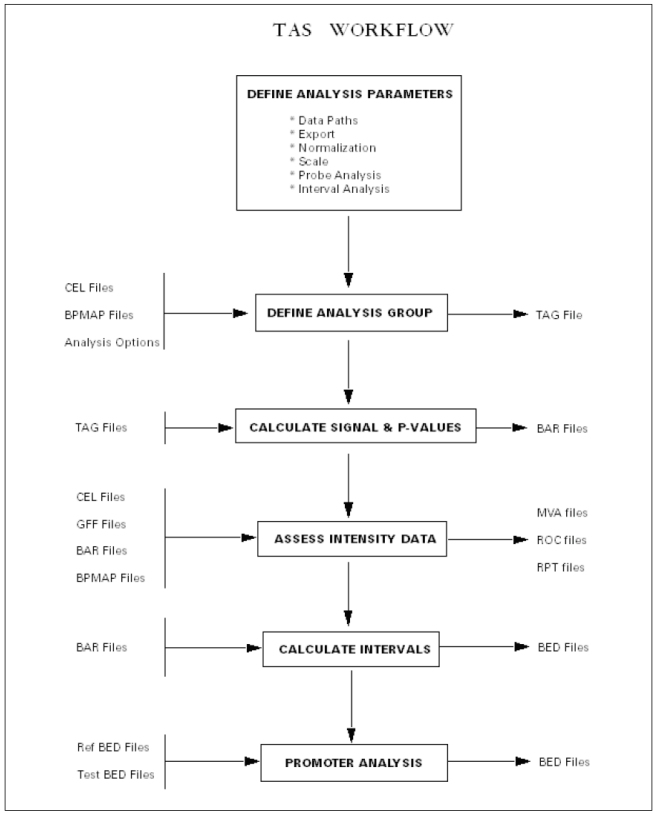
- DAT file, which contains the primary image of the scanning.
- CEL file (Cell Intensity File), which contains processed cell intensities from the primary image in the .DAT file. Intensity data is computed by the Affymetrix GCOS application and stored in CEL files. Each GeneChip Tiling Array produces a single CEL file.
- RPT (Report) file which stores report results about the quality of the experiment such as intensity threshold, median intensity, number of probes that failed a threshold set, average intensity of bright and dim probes etc.
- GRD (Grid) file, which stores the estimate of the sub-pixel location of the center of every feature in the array.
- JPG file, which provides an illustration-snapshot of the scanned array.

PROTOCOL S4

Statistical analyses of the results obtained by the genome-wide experiments
The CEL file obtained from the scanning process by the Affymetrix GCOS application is used as an input in the Tiling Analysis Software (TAS v1.1).
See also:
http://media.affymetrix.com/support/developer/downloads/TilingArrayTools/TileArray.pdf

Analysis functions provided within the TAS application include:
• Analyzing feature-intensity data stored in CEL files to produce signal and p-values for each interrogated genomic probe position
• Computation of genomic intervals based on computed signal and p-values
• Computation of summary statistics
• Visualizations for assessing the quality of array data
Analyses produced using TAS can be imported into applications such as the Integrated Genome Browser (IGB) for visualization against genomic annotations as we shall see further below.

The workflow that the TAS follows is illustrated below:



Firstly, it is important to specify the analysis settings:
Data paths: Settings on the *Data Paths* tab are used to specify the location of data files and BPMAP files. As data file we used the CEL file obtained from the scanning process. A library file BPMAP (Binary Probe Map) is also needed. BPMAP file contain the genomic probe position map. The BPMAP file maps the X/Y coordinate of a probe on a GeneChip array to a genomic position for an intended function. It designates a probe as either a perfect match (PM) or a mismatch (MM) probe.

Export: Settings on the Export tab are used to specify the type of data saved in the BAR files and whether or not analysis results (signal and p-values) should be exported along with the BAR files as text files. For our analysis we choose to export our results in both BAR and txt files and also to obtain both signal and p-values results.
Normalisation options: The setting displayed on the Normalisation Options tab is used to specify target intensity when normalising CEL data. The intensities in the CEL file are linearly scaled so that the median intensity value is equal to the target intensity.
Scale: Settings on the *Scale* tab are used to specify the scale in which the analysis results (signal and p-values) are to be stored in the output files. We choose for the Signal a Log2 scale and for the P-value the -10Log10 scale.
Probe analysis: Settings on the *Probe Analysis* tab are used to determine probe analysis levels for computing signal and p-values. Since bandwidth defines the number of bases to extend from the position being analyzed, these settings ensure that every probe in a region of *2*Bandwidth + 1* is included in the signal and p-value analysis. Bandwidth is a distance (in base pairs) used to locally group positional data and is determined by two main factors: i) Base pair (bp) tiling on the array and ii) Type of experiment. Analysis at a particular position will be based on all data aligning within ± bandwidth of the position, so that the sliding window of the analysis is of size *2*bandwidth+1*. Making bandwidth larger brings more data into each test providing more statistical power and a greater ability to detect signal. However, once the bandwidth exceeds the point where the window is larger than the signal being interrogated, power decreases to include data with no signal. The suggested BW range should be such that the window size (2 x BW +1) roughly corresponds to the median size of an exon (300 for our analysis). Furthermore, for the Test Type we choose "One Sided Upper" which derives p-values to test whether or not the treatment group has a lesser signal than the control group. In addition, we use both PM and MM probe intensities in the analysis.
Interval analysis: Settings on the *Interval Analysis* tab are used to determine how intervals are calculated. Base pair spacing of probes on the array, type of experiment (RNA mapping or ChIP-chip), and stringency of the data set to be created determine which values to use. For a typical ChIP-chip experiment, for p-value interval generation, threshold is set based on the confidence level of p-values in the two sample analysis that users select. We used p-value cutoffs between 10^{-3} and 10^{-5} for interval generation (30 to 50, respectively, on the -10log10, p-value scale). In both RNA mapping and ChIP-chip experiments, max gap and min run parameters are dependent on size of intervals that users want to identify. Calculations are performed by:
• Determining the region where a probe is positive (the signal or p-value is above or below a threshold).
• Selecting a maximum gap between positive probes. This setting is referring to the maximum tolerated gap (in base pairs) between positive positions in the derivation of detected regions. Decreasing it, results in a more stringent map; increasing it lessens the stringency. We choose a value of 80.
• Selecting a minimum length or run of adjacent probes. This setting is the minimum size (in base pairs) of a detected region. Increasing it, disallows detection of smaller regions which may be appropriate if the expected size of detected regions is large. We choose a value of 40.

After the specification of the above analysis settings (data paths, export, normalisation options, scale, probe analysis, interval analysis), the next step involves the definition of the analysis group. In this step we select the appropriate BPMAP file (Sc03b_MR_v04 in our case) and we choose also the type of the analysis that we want to perform. In our case, we selected the *two sample comparison analysis*, in which we defined the Treatment Group (CEL file from our experiment) and the Control Group (CEL file from the INPUT sample grown in the same conditions). In this step we save the group of the two files in a Tile Analysis Group (TAG) file. The TAG file stores a set of CEL files, a BPMAP file, and the normalisation option settings of our two sample analysis.

Probe Analysis

Probe analysis uses a combination of CEL files, the BPMAP file, normalisation settings specified in the TAG file, and parameters specified in the Default Properties window. Probe analysis results in a signal and a p-value for each genomic position interrogated by the array. These values are then stored in two BAR files (we also chose to obtain the txt format of these BAR files: see *Export* analysis settings).

ALGORITHMS

→Algorithms used for the probe analysis

The BPMAP file is used to associate each perfect match (PM) probe with its position in a genomic sequence. The probe position, whether its target is the forward or the reverse strand, is determined by the location of its 0-based position on the lower coordinate of the probe aligned to the target. Probe position is defined by the positions employed in the genome assembly, which is used for probe selection in the array. Mismatched (MM) probes are always paired with a PM probe and have the same convention.

Once the PM and MM pairs have been associated with sequence positions and normalised, the next step is to perform statistical analysis (in a local context for each position) to determine size and significance of the hybridization signal. In the case of PM only analysis, MM is not used. On some arrays, MM is not present. Analysis can be performed in either a one-sample or a two-sample context. A typical one-sample context might consist of using a number of biological or technical replicates for detection of regions of transcription. A typical two-sample context might consist of a treatment versus control comparison to look for regions of enrichment in a chromatin immunoprecipitation (ChIP) experiment.

Probe Analysis is focused at a single sequence position because the method is the same for all positions. The first step is to define a local data set consisting of all PM probes located within ± bandwidth base pairs of the position of interest. The value of the bandwidth should be driven by the average size in base pairs of the signal to be detected. In the case of transcription monitoring, the bandwidth would typically be on the order of half the average exon length, often about 50bp. In the case of ChIP assays, it would be half the expected fragment length in the step immediately before enrichment, which is assay dependent, but typically on the order of 500bp.

Selection of a bandwidth involves a tradeoff. On one hand, the bandwidth should be as large as possible to provide greatest statistical power for the analysis at each position; on the other hand, if the bandwidth is too large, the analysis tends to dilute signal by including background. The resulting local data set typically consists of a number of PM probes for each array being studied. The next step differs for one-sample and two-sample analysis.

→Algorithms for the two sample comparison analysis used in our experiments

In two-sample analysis, there are two data sets, which are called a treatment and a control group. Each group consists of the subset of data falling within the specified bandwidth as described above, resulting in n_t treatment pairs of probe intensities $\{PM_{t,i}\text{-}MM_{t,i};\ i=1,...n_t\}$ and n_c control pairs of probe intensities $\{PM_{c,i}\text{-}MM_{c,i};\ i=1,...n_c\}$. The log-transformed quantities $\{S_{g,i}=\log2(\max(PM_{g,i}\text{-}MM_{g,l},1));\ g=t,c;i=1,...,n_g\}$ are formed and a Wilcoxon signed-rank test is performed on the two samples $\{S_{t,i};i=1,...,n_t\}$ and $\{t_{c,i};i=1,...,n_c\}$. In the case of a PM only analysis, instead of using the log-transformed differences, the log-transformed PM signal intensities $\{S_{g,i}=\log2(PM_{g,i});g=t,c;i=1,...,n_g\}$ are used.

The default test type is a one-sided test, against the alternative that the distribution of the treatment data is shifted up with respect to the distribution of the control data. A two-sided or lower-sided test can be used instead of the one-sided lower. Similar to the one-sample p-values, by default, the $-10\log_{10}$ transform is applied to the output to enable visualization along the sequence.

An estimate of fold enrichment is also computed; the estimator used is the Hodges-Lehmann estimator associated with the Wilcoxon rank-sum test [1]. The estimator is computed by forming all n_tn_c values $\{D_{ij}=(S_{t,i}\text{-}S_{c,j});i=1,...,n_t;j=1,...,n_c\}$. The Hodges-Lehmann estimator is then the median of the D_{ij} and can be interpreted as the $\log_2$ fold change between the treatment and control group signals.

→ Algorithms used for the Normalisation

The default workflow assumes that the probe intensity data are normalised using quantile normalisation [2]. Quantile normalisation makes the assumption that the data being normalised have the same underlying distribution; this should be a reasonable assumption within biological and sample replicates. For the one-sample analysis, the assumption of equal, underlying distributions is usually reasonable. By default, all arrays are quantile-normalised together. For two-sample analysis, it is quite possible that the underlying distributions are different for the two groups; therefore, by default, quantile normalisation is performed only within each group.

Normalisation, if performed, is full quantile normalisation of all probe intensities. We can select the option to normalise TAG files separately for each group or normalise across all groups in an analysis. If the target intensity parameter is set, then the normalised intensities are further scaled to set the median intensity for every array to be the target intensity value. If normalisation is not performed, the target intensity parameter is ignored.

POST ANALYSIS OF THE BAR AND TXT FILES

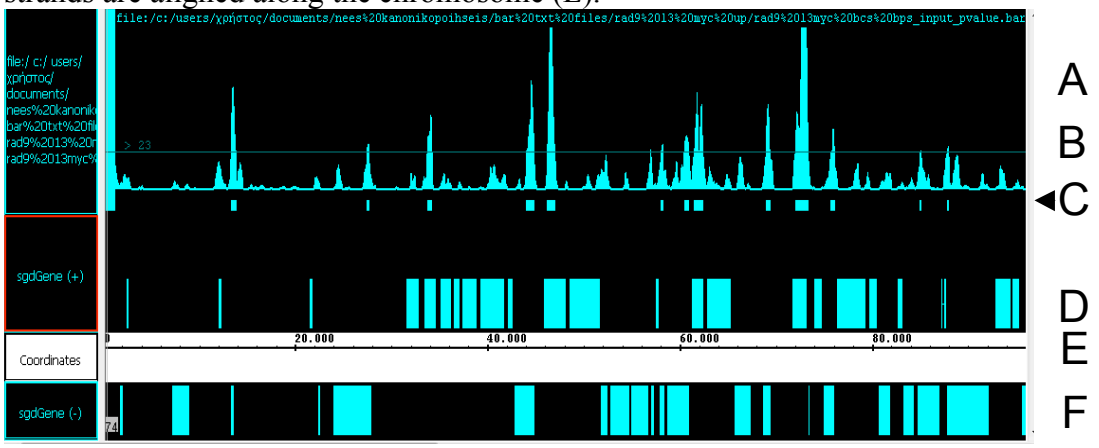
i) Visualization of the intensity values

After obtaining the BAR and TXT files which contain the normalised intensity values of our experiments we use the Integrated Genome Browser (IGB) to visualize our results and to do a further sorting. The IGB is a free software for distribution and exploration of genome-scale datasets [3] and can be downloaded following the link below:

http://www.affymetrix.com/partners_programs/programs/developer/tools/download_igb.affx

IGB can be used to visualize either p-value.BAR or signal.BAR files. We use the former, in order to be able to set a threshold (see below) above which there are the genomic areas which are significantly enriched according to our settings.

Firstly, we upload the p-value.BAR file to IGB and select the organism (*Saccharomyces cerevisie*) and the genome version used for our array which is from October 2003. We set the Y-axis scale for a minimum of 0 and a maximum of 100 (to keep a consistency between the experiments) and by using the Graph Adjuster tool we set a threshold by value corresponding to a p-value of usually 10^{-3} or $5x10^{-3}$ (value of 30 and 23 respectively). This is a common analysis step to determine meaningful regions on a sequence based on graph values being above or below a certain threshold. By using the NetAffx tool we can also visualize the ORF of genes according to Saccharomyces Genome Database (SGD). A typical image obtained from IGB is presented in the figure below. This is a visualization of Rad9 binding pattern on the first ~100000 bp of chr1. In the upper part of the plot (A) peaks represent the loci where Rad9 is significantly enriched. Also visible is the line (B) corresponding to a threshold of a p-value of $5x10^{-3}$. The genomic areas where Rad9 is bound fitting our threshold criteria are visualized in (C). The coordinates of these loci can be obtained by producing a track of them and saving it as a BED file (which can easily be transformed to an XLS file). The ORFs of the Watson (D) and Crick (F) strands are aligned along the chromosome (E).



Sorting of the results according to the intensity values and correspondence of the statistically important peaks to the genomic areas

The intensity value of each spot is incorporated in the BAR and TXT files obtained by the TAS. By using an R script (see below about R) written specifically for *S. cerevisie* genome and our data (Georgia Tsiliki) we were able to match each of the statistically important binding loci (obtained from IGB) to the coding (ORF) or non-coding areas. The output file was used in combination with the TAS TXT file in order to match each peak to an intensity value. The latter was done with specialized scripts for R and Command Promt tools (written by Christoforos Nikolaou).

R is a language and environment for statistical computing and graphics. It is a GNU project which is similar to the S language and environment which was developed at Bell Laboratories (Lucent Technologies) (http://www.r-project.org/).

ii) Cytoscape Analysis (Functional clustering)

In order to perform a functional clustering on the group of genes on which our proteins were enriched we used the Cytoscape open source software platform for visualizing complex networks [4] (http://www.cytoscape.org/). To do that, we used the BiNGO plugin [5]. The Biological Networks Gene Ontology tool (BiNGO) is an open-source Java tool to determine which Gene Ontology (GO) terms are significantly overrepresented in a set of genes. BiNGO can be used either on a list of genes, pasted as text, or interactively on subgraphs of biological networks visualized

in Cytoscape. BiNGO maps the predominant functional themes of the tested gene set on the GO hierarchy, and takes advantage of Cytoscape's versatile visualization environment to produce an intuitive and customizable visual representation of the results (http://www.psb.ugent.be/cbd/papers/BiNGO/Home.html).
BiNGO currently provides two statistical tests for assessing over- or underrepresentation in a set of genes. The basic question answered by these tests is the following : 'When sampling X genes (test set) out of N genes (reference set; graph or annotation), what is the probability that x or more of these genes belong to a functional category C shared by n of the N genes in the reference set.' The hypergeometric test (test without replacement) provides an accurate answer to this question in the form of a p-value and this is what we used for our analysis. We have also controlled the False Discovery Rate (FDR) i.e. the expected proportion of false positives among the positively identified tests by using the Benjamini & Hochberg correction, which provides strong control over the FDR under positive regression dependency of the null hypotheses. We used a significance level of 0.05.
The goal of the Gene Ontology (GO) project is to provide a structured description of known biological information at different levels of granularity. GO consists of three structured, controlled vocabularies that describe gene products in terms of their associated biological processes, molecular functions and cellular components in a species-independent manner. With BiNGO we used the *Saccharomyces cerevisiae* GO terms and annotations.

iii) Average gene analysis

The average gene analysis aimed to visualize the binding pattern results for all the genes of each experiment in one plot as an "average gene" and was performed by Christophoros Nikolaou. We used the raw values included in the signal.BAR files obtained by TAS. Each of the 5769 genes of *S. cerevisiae* (SGD version, sacCer1) was divided in 100 equal bins. For example a 1000bp gene was divided in 100 bins of 10bp, while one of 5000bp was divided in 100 bins of 50bp. The average signal value was calculated for each bin. In this way, every gene was shrunk into 100 entities regardless of size, with the first entity corresponding to TSS and the last to the TTS. Subsequently, an area of +/-500bp from the TSS/TTS respectively was divided into 50 bins of 10bp for every gene. In this way, we finally obtained for each gene 200 entities-bins (50 upstream, 100 genic, 50 downstream). In the occasions where in -500 upstream or +500 downstream there was another gene, the area ended on the spot where the neighbouring gene was met. The plots obtained represent the average value of the 200 entities-bins –for each place- for the 5769 genes. The concept of average gene analysis is also described by others [6,7].

iv) Calculation of the GC content of the genomic areas

To calculate the GC content of the sequences where the proteins of interest showed an overrepresentation of localisation, we first transformed the BED files (obtained by IGB) to XLS files. These files contain the coordinates of the areas where the proteins are bound. We transformed the XLS files to FASTA format files by using Galaxy (http://main.g2.bx.psu.edu/), an open, web-based platform for data intensive biomedical research. Subsequently, we used Galaxy's EMBOSS GeeCee tool to obtain the percentage of GC content. We compared this to a randomized sample of GC content obtained from random sequences (probes) of the same number and size as the ones from our experiments. The randomization was performed using Perl.

Using R statistical package, we calculated and plotted the median and mean percentage values of experimental and randomized samples after fitting a t-test of values lower than 10^{-5} (ranging from 10^{-5} to 10^{-120}).

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PROTOCOL S5

EXPRESSION MICROARRAY ASSAY (AFFYMETRIX PLATFORM)

The array that was used for our expression microarray experiments is **GeneChip Yeast Genome 2.0 Array** manufactured by Affymetrix (900554). The GeneChip Yeast Genome 2.0 Array contains probe sets to detect transcripts from both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, which are the two most commonly studied species of yeast. Providing comprehensive coverage of both species, the GeneChip Yeast Genome 2.0 Array includes approximately 5,744 probe sets for 5,841 of the 5,845 genes present in *S. cerevisiae* and 5,021 probe sets for all 5,031 genes present in *S. pombe*. The sequence information for this array was selected from public data sources GenBank (May 2004) and Sanger Center (June 2004) for the *S. cerevisiae* and *S. pombe* genomes, respectively. Probe sets on the array include 11 oligonucleotide pairs to detect each transcript.
We have performed one experiment (*rad9*Δ strain grown in SC BCS BPS and the wild type FT5 in the same conditions) and one biological replicate.
We followed the protocol proposed by Affymetrix.
http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf
If any changes were made, they are described below.

Briefly, the Expression Analysis includes the steps of i) target preparation, ii) target hybridization, iii) fluidics station setup, iv) probe array washing and staining, v) probe array scan and vi) data analysis.

i) RNA isolation from yeast with hot acid phenol protocol

1. Inoculate and grow yeast cultures (10ml) until exponential phase (OD_{550}~0.8).
2. Harvest the cells by centrifugation at 3000rpm (RT) for 5mins.
3. Discard supernatant and resuspend pellet in 0.45ml AE buffer (50mM CH_3COONa pH=6; 10mM EDTA pH=8). Transfer in a 1.5ml tube and add 0.5ml acid phenol prewarmed at 65^0C and 50µl 10% SDS. Vortex for 20sec. Transfer tube to 65^0C waterbath for 1hr, vortexing for 20sec, every 10mins.
4. After the final vortexing coolthe tube on ice for 10mins and add 50µl (1/10V) 3M CH_3COONa pH=5.2
5. Separate the phases by centrifugation at 13000rpm RT for 10mins.
6. Extract the supernatant once with phenol/chlorophorm and once with chlorophorm.
7. Transfer supernatant in new tube and add 1.375ml (2.5V) 100% ethanol. Store at least 2hrs at -80^0C.
8. Cenrifuge at 13000rpm for 30mins at 4^0C, wash the pellet once with 70% ethanol, dry, and resuspend in ~100µl H_2O.

ii) DNAse treatment

We treated 20µg of RNA with DNAse (Promega) for 1 hour at 37^0C

Reaction (20 µl final volume)

17 µl total RNA (15-20 µg in DEPC water)
2 µl 10x DNase buffer (Promega)
1 µl DNase (1u/ µl)

We extracted the reaction volume by phenol/chlorophorm followed by ethanol precipitation. We measured with Nanodrop the A_{260}/A_{280} ratio, ensuring that it was close to 2.0 (1.9-2.1 is acceptable) for pure RNA. We then used 7µg of DNAse treated RNA in the One-Cycle cDNA Synthesis protocol.

iii) One-Cycle cDNA Synthesis protocol

STEP 1: PREPARATION OF POLY-A RNA CONTROLS FOR ONE-CYCLE cDNA SYNTHESIS (SPIKE-IN CONTROLS)

Eukaryotic Poly-A RNA Control Kit is used for this step (Affymetrix 900433). Designed specifically to provide exogenous positive controls to monitor the entire eukaryotic target labeling process, a set of poly-A RNA controls is supplied in the GeneChip Eukaryotic Poly-A RNA Control Kit. Each eukaryotic GeneChip probe array contains probe sets for several *B. subtilis* genes that are absent in eukaryotic samples (*lys, phe, thr,* and *dap*). These poly-A RNA controls are *in vitro* synthesized, and the polyadenylated transcripts for the *B. subtilis* genes are pre-mixed at staggered dilutions. The concentrated **Poly-A Control Stock** can be diluted with the **Poly-A Control Dil Buffer** and spiked directly into RNA samples to achieve the final dilutions (referred to as a ratio of copy number) summarized in the table below.

Poly-A RNA Spike	Final Dilution (estimated ratio of copy number)
lys	1:100,000
phe	1:50,000
thr	1:25,000
dap	1:6,667

The controls are then amplified and labeled together with the samples. Examining the hybridization intensities of these controls on GeneChip arrays helps to monitor the labeling process independently from the quality of the starting RNA samples. Anticipated relative signal strength follows the order of *lys* < *phe* < *thr*< *dap*. The **Poly-A RNA Control Stock** and **Poly-A Control Dil Buffer** are provided with the kit to prepare the appropriate serial dilutions based on the table below.

Starting Amount		Serial Dilutions			Spike-in Volume
Total RNA	mRNA	First	Second	Third	
1 µg		1:20	1:50	1:50	2 µL
5 µg		1:20	1:50	1:10	2 µL
10 µg	0.2 µg	1:20	1:50	1:5	2 µL

This is a guideline when 1, 5, or 10 µg of total RNA or 0.2 µg of mRNA is used as starting material. For starting sample amounts other than those listed here, calculations are needed in order to perform the appropriate dilutions to arrive at the same proportionate final dilution of the spike-in controls in the samples.
For example, to prepare the poly-A RNA dilutions for 5 µg of total RNA:
1. Add 2 µL of the **Poly-A Control Stock** to 38 µL of **Poly-A Control Dil Buffer** for the First Dilution (1:20).
2. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
3. Add 2 µL of the First Dilution to 98 µL of **Poly-A Control Dil Buffer** to prepare the Second Dilution (1:50).
4. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
5. Add 2 µL of the Second Dilution to 18 µL of **Poly-A Control Dil Buffer** to prepare the Third Dilution (1:10).
6. Mix thoroughly and spin down to collect the liquid at the bottom of the tube.
7. Add 2 µL of this Third Dilution to 5 µg of sample total RNA.
The First Dilution of the poly-A RNA controls can be stored up to six weeks in a non-frost-free freezer at –20°C and frozen-thawed up to eight times.

STEP 2: FIRST-STRAND cDNA SYNTHESIS

One-Cycle cDNA Synthesis Kit (Affymetrxix 900431) is used for this step.
a. Briefly spin down all tubes in the Kit before using the reagents.
b. Perform all of the incubations in thermal cyclers. The following program can be used as a reference to perform the first-strand cDNA synthesis reaction in a thermal cycler; the 4°C holds are for reagent addition steps:
70°C 10 minutes
4°C hold
42°C 2 minutes
42°C 1 hour
4°C hold
1. Mix RNA sample, diluted poly-A RNA controls, and T7-Oligo(dT) Primer.
RNA/T7-Oligo(dT) Primer Mix Preparation for 1 to 8 µg of total RNA, or 0.2 to 1 µg of mRNA is presented in the following table:

Component	Volume
Sample RNA	variable
Diluted poly-A RNA controls	2 µL
T7-Oligo(dT) Primer, 50 µM	2 µL
RNase-free Water	variable
Total Volume	12 µL

A. Place total RNA (1 µg to 15 µg) or mRNA sample (0.2 µg to 2 µg) in a 0.2 mL PCR tube.
B. Add 2 µL of the appropriately diluted poly-A RNA controls
(See *Step 1: Preparation of Poly-A RNA Controls for One-Cycle cDNA Synthesis (Spike-in Controls)*.
C. Add 2 µL of 50 µM T7-Oligo(dT) Primer.
D. Add **RNase-free Water** to a final volume of 12 µL.
E. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
F. Incubate the reaction for 10 minutes at 70°C.
G. Cool the sample at 4°C for at least 2 minutes.
H. Centrifuge the tube briefly (~5 seconds) to collect the sample at the bottom of the tube.
2. In a separate tube, assemble the First-Strand Master Mix.
A. Prepare sufficient **First-Strand Master Mix** for all of the RNA samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in the Table, is for a single reaction:

Preparation of First-Strand Master Mix

Component	Volume
5X 1st Strand Reaction Mix	4 µL
DTT, 0.1M	2 µL
dNTP, 10 mM	1 µL
Total Volume	7 µL

B. Mix well by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the master mix at the bottom of the tube.
3. Transfer 7 µL of **First-Strand Master Mix** to each RNA/T7-Oligo(dT) Primer mix for a final volume of 18 or 19 µL. Mix thoroughly by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.
4. Incubate for 2 minutes at 42°C.
5. Add the appropriate amount of **SuperScript II** to each RNA sample for a final volume of 20 µL.
• For 1 to 8 µg of total RNA: 1 µL **SuperScript II**
• For 8.1 to 15 µg of total RNA: 2 µL **SuperScript II**
• For every µg of mRNA add 1 µL **SuperScript II**.
• For mRNA quantity less than 1 µg, use 1 µL **SuperScript II**.
Mix thoroughly by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.
6. Incubate for 1 hour at 42°C; then cool the sample for at least 2 minutes at 4°C.
After incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube and immediately proceed to *Step 3: Second-Strand cDNA Synthesis*.

STEP 3: SECOND-STRAND cDNA SYNTHESIS

One-Cycle cDNA Synthesis Kit (Affymetrxix 900431) is used for this step.
The following program can be used as a reference to perform the second-strand cDNA synthesis reaction in a thermal cycler.
16°C 2 hours
4°C hold
16°C 5 minutes
4°C hold

1. In a separate tube, assemble Second-Strand Master Mix (prepare immediately before use).

A. Prepare sufficient Second-Strand Master Mix for all of the samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in the table, is for a single reaction.

Preparation of Second-Strand Master Mix

Component	Volume
RNase-free Water	91 µL
5X 2nd Strand Reaction Mix	30 µL
dNTP, 10 mM	3 µL
E. coli DNA ligase	1 µL
E. coli DNA Polymerase I	4 µL
RNase H	1 µL
Total Volume	130 µL

B. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.

2. Add 130 µL of Second-Strand Master Mix to each first-strand synthesis sample from *Step 2: First-Strand cDNA Synthesis* for a total volume of 150 µL. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

3. Incubate for 2 hours at 16°C.

4. Add 2 µL of **T4 DNA Polymerase** to each sample and incubate for 5 minutes at 16°C.

5. After incubation with T4 DNA Polymerase add 10 µL of EDTA, 0.5M and proceed to *Cleanup of Double-Stranded cDNA*. Do not leave the reactions at 4°C for long periods of time.

Cleanup of Double-Stranded cDNA

Sample Cleanup Module (Affymetrix 900371) is used for cleaning up the double-stranded cDNA.

1. Add 600 µL of **cDNA Binding Buffer** to the double-stranded cDNA synthesis preparation. Mix by vortexing for 3 seconds.

2. Check that the color of the mixture is yellow (similar to cDNA Binding Buffer without the cDNA synthesis reaction).

3. Apply 500 µL of the sample to the **cDNA Cleanup Spin Column** sitting in a **2 mL Collection Tube** (supplied), and centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

4. Reload the spin column with the remaining mixture and centrifuge as above. Discard flow-through and Collection Tube.

5. Transfer spin column into a new 2 mL Collection Tube (supplied). Pipet 750 µL of the **cDNA Wash Buffer** onto the spin column. Centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

6. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube. Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation. Centrifugation with open caps allows complete drying of the membrane.
7. Transfer spin column into a 1.5 mL Collection Tube, and pipet 14 µL of **cDNA Elution Buffer** directly onto the spin column membrane. Incubate for 1 minute at RT and centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute. Ensure that the cDNA Elution Buffer is dispensed directly onto the membrane. The average volume of eluate is 12 µL recovered from the 14 µL of Elution Buffer.
8. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA for One-Cycle Target Labeling Assay*

iv) Synthesis of Biotin-Labeled cRNA for One-Cycle Target Labeling Assay
GeneChip IVT Labeling Kit (Affymetrix 900449) is used for this step.
1. Use the following table to determine the amount of cDNA used for each IVT reaction following the cDNA cleanup step.
IVT Reaction Set Up

Starting Material	Volume of cDNA to use in IVT
Total RNA	
10 to 100 ng	all (~12 µL)
1.0 to 8.0 µg	all (~12 µL)
8.1 to 15 µg	6 µL

2. Transfer the needed amount of template cDNA to RNase-free microfuge tubes and add the following reaction components in the order indicated in the table below. If more than one IVT reaction is to be performed, a master mix can be prepared by multiplying the reagent volumes by the number of reactions. Do not assemble the reaction on ice, since spermidine in the **10X IVT Labeling Buffer** can lead to precipitation of the template cDNA.

Component	Volume
Template cDNA*	variable (see Table 2.17)
RNase-free Water	variable (to give a final reaction volume of 40 µL)
10X IVT Labeling Buffer	4 µL
IVT Labeling NTP Mix	12 µL
IVT Labeling Enzyme Mix	4 µL
Total Volume	40 µL

3. Carefully mix the reagents and collect the mixture at the bottom of the tube by brief (~5 seconds) microcentrifugation.
4. Incubate at 37°C for 16 hours. To prevent condensation that may result from water bath-style incubators, incubations are best performed in oven incubators for even temperature distribution, or in a thermal cycler.
5. Store labeled cRNA at –20°C, or –70°C if not purifying immediately.
Alternatively, proceed to *Cleanup and Quantification of Biotin-Labeled cRNA.*

v) Cleanup and Quantification of Biotin-Labeled cRNA
STEP1: CLEANUP OF BIOTIN-LABELED cRNA
Sample Cleanup Module (Affymetrix 900371) is used for cleaning up the biotinlabeled cRNA.
1. Add 60 µL of **RNase-free Water** to the IVT reaction and mix by vortexing for 3 seconds.

2. Add 350 µL **IVT cRNA Binding Buffer** to the sample and mix by vortexing for 3 seconds.
3. Add 250 µL ethanol (96-100%) to the mixture, and mix well by pipetting. Do not centrifuge.
4. Apply sample (700 µL) to the IVT **cRNA Cleanup Spin Column** sitting in a **2 mL Collection Tube**. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through and Collection Tube.
5. Transfer the spin column into a new **2 mL Collection Tube** (supplied). Pipet 500 µL **IVT cRNA Wash Buffer** onto the spin column. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm) to wash. Discard flow-through.
6. Pipet 500 µL 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.
8. Transfer spin column into a new **1.5 mL Collection Tube** (supplied), and pipet 11µL of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.
9. Pipet 10 µL of **RNase-free Water** directly onto the spin column membrane. Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.
10. For subsequent photometric quantification of the purified cRNA, we recommend dilution of the eluate between 1:100 fold and 1:200 fold.
11. Store cRNA at –20°C, or –70°C if not quantitated immediately. Alternatively, proceed to *Step 2: Quantification of the cRNA*.

STEP 2: QUANTIFICATION OF THE cRNA

Use spectrophotometric analysis to determine the cRNA yield. Apply the convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA.
• Check the absorbance at 260 nm and 280 nm to determine sample concentration and purity.
• Maintain the A260/A280 ratio close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).
For quantification of cRNA when using total RNA as starting material, an adjusted cRNA yield must be calculated to reflect carryover of unlabeled total RNA. Using an estimate of 100% carryover, use the formula below to determine adjusted cRNA yield:
adjusted cRNA yield = RNAm - (total RNAi) (y)
RNAm = amount of cRNA measured after IVT (µg)
total RNAi = starting amount of total RNA (µg)
y = fraction of cDNA reaction used in IVT
Example: Starting with 10 µg total RNA, 50% of the cDNA reaction is added to the IVT, giving a yield of 50 µg cRNA. Therefore, adjusted cRNA yield = 50 µg cRNA - (10 µg total RNA) (0.5 cDNA reaction) = 45.0 µg.
Use adjusted yield in *Fragmenting the cRNA for Target Preparation.*

vi) Fragmenting the cRNA for Target Preparation

Sample Cleanup Module (Affymetrix 900371) is used for this step.
Fragmentation of cRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity. The cRNA used in the fragmentation procedure must be sufficiently concentrated to maintain a small volume

during the procedure. This will minimize the amount of magnesium in the final hybridization cocktail. Fragment an appropriate amount of cRNA for hybridization cocktail preparation and gel analysis.

1. The Fragmentation Buffer has been optimized to break down fulllength cRNA to 35 to 200 base fragments by metal-induced hydrolysis. The following table shows suggested fragmentation reaction mix for cRNA samples at a final concentration of 0.5 µg/µL. Use adjusted cRNA concentration, as described in *Step 2: Quantification of the cRNA*. The total volume of the reaction may be scaled up or down dependent on the amount of cRNA to be fragmented.

Component	169 Format
cRNA	7,5µg
5X fragmentation buffer	3µl
RNAse-free water	to 15µl final volume
Total volume	30µl

2. Incubate at 94°C for 35 minutes. Put on ice following the incubation.

3. Store undiluted, fragmented sample cRNA at –20°C (or –70°C for longer-term storage) until ready to perform the hybridization.

vii) Eukaryotic target hybridization

Materials needed are GeneChip Hybridization, Wash and Stain kit (Affymetrix 900720), GeneChip Eukaryotic Hybridization control kit (Affymetrix 900457), control oligo B2 (contained in Affymetrix 900457 or 900301).

Refer to the table below for the necessary amount of cRNA required for the specific probe array format used. These preparations take into account that it is necessary to make extra hybridization cocktail due to a small loss of volume (10-20 µL) during each hybridization.

1. Mix the following for each target, scaling up volumes if necessary for hybridization to multiple probe arrays.

Component	169 Format (Mini) Array /400 Format (Micro) Array	Final Dilution
Fragmented and Labeled cRNA†	5 µg	0.05 µg/µL
Control Oligonucleotide B2 (3 nM)	1.7 µL	50 pM
20X Eukaryotic Hybridization Controls (*bioB, bioC, bioD, cre*)	5 µL	1.5, 5, 25, and 100 pM respectively
2X Hybridization Mix	50 µL	1X
DMSO	10 µL	10%
Nuclease-free Water	to final volume of 100 µL	
Total Volume	100 µL	

2. Equilibrate probe array to room temperature immediately before use.

3. Heat the hybridization cocktail to 99°C for 5 minutes in a heat block.

4. Meanwhile, wet the array with an appropriate volume of Pre- Hybridization Mix (80µl for our array) by filling it through one of the septa.

5. Incubate the probe array filled with Pre-Hybridization Mix at 45°C for 10 minutes with rotation.

6. Transfer the hybridization cocktail that has been heated at 99°C, in step 3, to a 45°C heat block for 5 minutes.

7. Spin the hybridization cocktail at maximum speed in a microcentrifuge for 5 minutes to collect any insoluble material from the hybridization mixture.

8. Remove the array from the hybridization oven. Vent the array with a clean pipette tip and extract the Pre-Hybridization Mix from the array with a micropipettor. Refill

the array with the appropriate volume of the clarified hybridization cocktail, avoiding any insoluble matter at the bottom of the tube.
9. Place probe array into the hybridization oven, set to 45°C.
10. To avoid stress to the motor, load probe arrays in a balanced configuration around the axis. Rotate at 60 rpm.
11. Hybridize for 16 hours. During the latter part of the 16-hour hybridization, proceed to wash and staining steps immediately after completion of hybridization. From the scanning, we obtain a CEL file that contains the intensity values of the hybridization of our material to the slide in a raw form.

viii) ANALYSIS OF THE RESULTS
The intensity values were calculated by the use of Affymetrix Expression Console Software as well as other commercial software such as FlexArray using RMA algorithm to normalize our data [1]. For each of the 2 sets of experiments (*rad9*Δ vs FT5 and its biological replicate) we have de-logged the values in order to get the raw intensity values of the replicate experiments. We then calculated the average value of the replicates and obtained the exact fold ratios (*rad9*Δ/wt). We performed a t-test analysis (two sample equal variance-homoscedastic) to obtain a p-value for our results. This value determines the possibility of the two samples to have come from the same two underlying populations that have the same mean. We sorted our results depending on the fold change ($-1.2 \leq X \leq 1.2$) and the p-value obtained from the t-test ($X \leq 0.05$). The replicates had over 99% similarity in the intensity values measured. Functional analysis of the results was performed by using BiNGO plugin in Cytoscape platform as described in the ChIP on chip protocol.

1. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP: Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003, 31:e15.