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Article *in* Nature Protocol · June 2013

DOI: 10.1038/nprot.2013.077 · Source: PubMed

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Genome-wide measurement of protein-DNA binding dynamics using competition ChIP

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Published online 13 June 2013; doi:10.1038/nprot.2013.077

Competition chromatin immunoprecipitation (competition ChIP) enables experimenters to measure protein-DNA dynamics at a single locus or across the entire genome, depending on the detection method. Competition ChIP relies on a cell containing two copies of a single DNA-associated factor, with each copy of the factor differentially epitope tagged. One of the copies is expressed constitutively and the second is induced as a competitor. The ratio of isoforms associated with discrete genomic locations is detected by ChIP-on-chip (ChIP-chip) or ChIP-sequencing (ChIP-seq). The rate at which the resident isoform of the protein is replaced by the competitor at each binding location enables the calculation of residence time for that factor at each site of interaction genome wide. Here we provide a detailed protocol for designing and performing competition ChIP experiments in *Saccharomyces cerevisiae*, which takes ~5 d to complete (not including strain production and characterizations, which may take as long as 6 months). Included in this protocol are guidelines for downstream bioinformatic analysis to extract residence times throughout the genome.

INTRODUCTION

The dynamics of interactions between proteins and DNA is likely to be central to understanding the regulation of transcription, replication and other DNA-dependent processes in contexts ranging from development to environmental response[1]. ChIP-chip and ChIP-seq experiments provide insight into the genome-wide binding pattern of proteins[2,3]. However, the measurements made by a standard ChIP experiment are largely blind to the underlying binding dynamics[4,5] and reflect a measurement of factor occupancy over time and a cell population. For example, the same apparent occupancy level could be reached by a factor that is stably bound to a given locus in a few cells as that reached by one that is more transiently bound to the same locus in many cells[1,6]. Thus, occupancy as measured by traditional ChIP is not equivalent to the residence time of a factor on DNA[5]. We have found that residence time measurements can be more predictive of biological function than occupancy[4].

Measuring the binding dynamics of factors on DNA genome-wide has been challenging. Recently, competition assays have been developed that have improved the ability to measure dynamic nuclear events[4,5,7–11]. These methods rely on two copies of the same factor: one that is expressed constitutively and one that is induced. The incorporation of the competitor relative to the constitutive copy is then compared over time at binding sites throughout the genome. The two versions of the proteins are distinguished by different epitope tags. These competition assays promise to provide a new temporal dimension for genome-wide occupancy maps of molecules associated with DNA. In theory, these assays are applicable to any DNA-protein interaction, including proteins associated with DNA repair, replication, transcription, transcription factor binding, nuclear organization, chromatin-modifying enzymes, epigenetic modifications and histones.

In competition ChIP[4,5], the specific approach covered in this protocol, induction of the competitor copy is followed by a traditional ChIP protocol to measure the enrichment of both the constitutive and competitor isoforms over time (**Fig. 1a**,**b**). Whole-genome microarrays (ChIP-chip) or next-generation sequencing (ChIP-seq) then provide relative enrichment information for both isoforms at all binding loci in the genome. At each locus, the rate at which the induced competitor isoform replaces the constitutive isoform provides a measurement of the residence dynamics of the assayed factor (**Fig. 1c**; refs. 4,5).

No conceptual barrier exists to performing a competition ChIP in any organism, cell type or environment, although here we provide a protocol for competition ChIP in *S. cerevisiae*. We also provide general advice for the design and analysis of competition ChIP experiments.

Applications

Genome-wide competition ChIP is a relatively new approach and has been mostly applied to histones[4,8,9] and transcription factors[4,10] in budding yeast. As induction systems and analysis methods improve, the ability to assay the residence dynamics of any DNA-associated factor in multiple conditions and cell types should be possible. DNA-associated factors can include chromatin remodelers, histone variants, RNA polymerases, DNA repair enzymes, nuclear pore components, cohesins and many other classes of proteins. Although the full range of factors that can influence the residence time of DNA-associated proteins is still unknown, nucleosomes have been implicated in regulating the dynamics of transcription factor binding[4,5,10,12]. Therefore, analysis of residence dynamics in cells with mutant forms of important chromatin remodelers or at binding sites with altered sequence affinity for nucleosomes will be illuminating[4]. Altering the sequence of transcription factor–binding sites or eliminating cooperating transcription factors may also reveal the principles and regulators of binding dynamics. By using tightly regulated promoters, competition ChIP could also be used to measure protein-DNA dynamics for developmentally important, as well as cell type–specific and tissue-specific processes.

Comparison with other methods

Single-cell fluorescence imaging. Fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and single-molecule tracking (SMT)[13] are common methods for studying protein-DNA binding interactions within cells based on fluorescence imaging. These approaches have the advantage of being applied to single cells, rather than to a population of cells. These approaches can thus provide information about how the protein diffuses in the nucleus and its dynamic binding interactions with DNA. These measurements are usually recorded at random locations in the nucleus, and the extracted binding behavior cannot be attributed to specific target sites. The only exceptions are a few FRAP studies conducted on artificial and natural gene arrays containing repeated genomic sequences[14] and a pioneering SMT study using the *lac* repressor[15]. The biological interpretation of the data obtained with these approaches depends on quantitative analysis methods, which are still being developed and improved[13,16].

CATCH-IT. Covalent attachment of tags to capture histones and identify turnover (CATCH-IT) estimates histone dynamics throughout the genome. Newly synthesized proteins are briefly labeled with a methionine surrogate that is subsequently coupled to biotin[11]. Total chromatin is then MNase-digested to enrich for both biotin-coupled and unmodified mononucleosomes. Streptavidin is used to enrich for mononucleosomes that have incorporated newly synthesized histones. At a given location, the abundance of a nucleosome after enrichment is then compared to the total nucleosome abundance by microarray or by next-generation sequencing. Currently, the use of a mononucleosome-enriching step limits CATCH-IT to the measurement of general histone dynamics. However, a similar labeling and enrichment strategy combined with ChIP could be developed to measure the binding dynamics of any DNA-associated protein.

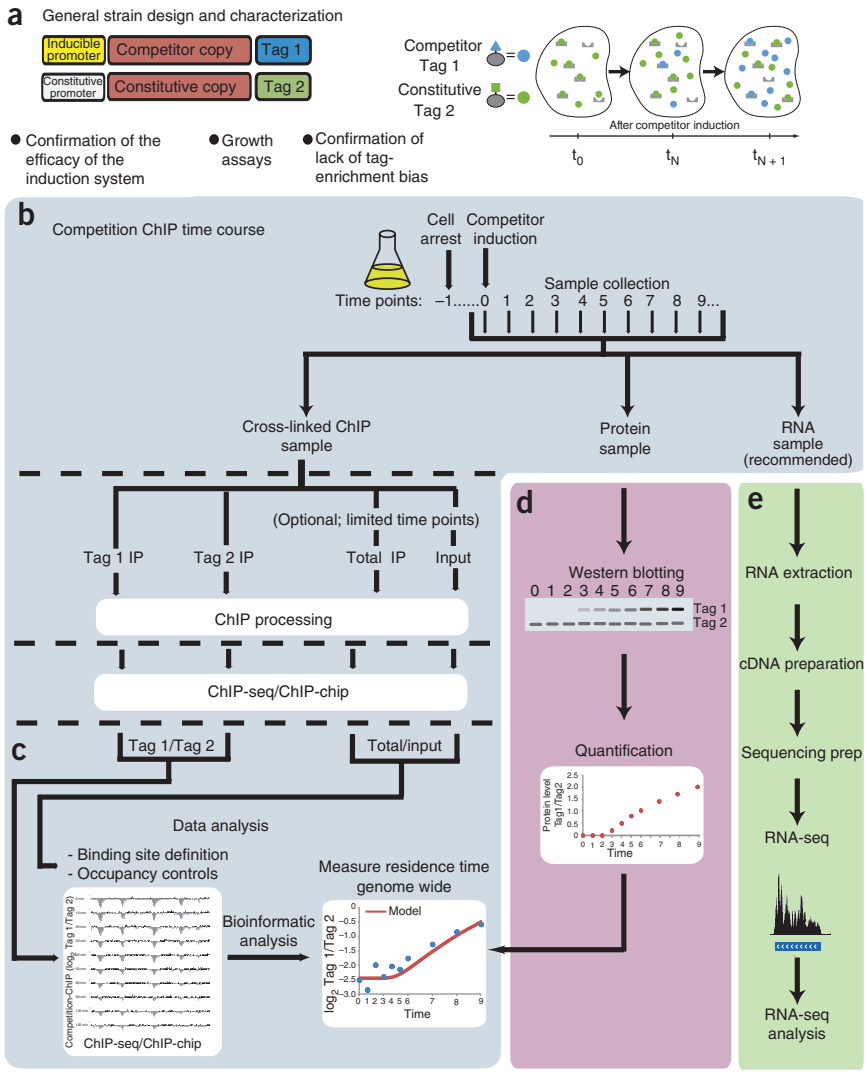

Figure 1 | General competition ChIP workflow. (**a**) Schematic of competition ChIP gene constructs (left) and competition ChIP experimental strategy (right). Initial characterizations for determining the feasibility of the competition ChIP experiment are in bullet points. (**b**) Experimental workflow for competition ChIP. (**c**) Computational analysis. (**d**) Western blotting and quantification. (**e**) Recommended RNA sample preparation.

Competition ChIP with epitope tag swapping. An alternative version of the competition ChIP method described in this protocol uses inducible site-specific recombination to replace one epitope tag with another at a single locus that encodes the factor under study[17]. Conceptually, this 'tag swapping' creates an instantaneous distinction between resident and competitor proteins. Combined with ChIP-chip or ChIP-seq, this method enables the experimenter to measure protein-binding dynamics over multiple generations of cells. Because of the incomplete synchronicity of epitope tag exchange, this method is most effective when it is applied to the measurement of binding dynamics of proteins with residence times that can last for an entire cell cycle[7]. The epitope tag-swapping method is distinct from the competition ChIP strategy described here. Competition ChIP relies, in fact, on rapid induction of expression of a new competitor protein, the expression of which continues throughout the experiment.

Advantages of competition ChIP

Resolution of residence time. Competition ChIP provides the benefit of generating high-resolution, genome-wide maps of the residence dynamics of any DNA-binding protein. These residence times can then be directly correlated to other genomic data sets for each locus in the genome.

Adaptability to different organisms. Although we provide a specific *S. cerevisiae* competition ChIP protocol, competition ChIP should

be readily applicable to any other organism in which protein expression induction systems exist (See also 'Future improvements').

Adaptability to new detection technologies. This protocol is based on detection by microarray, but it should be readily adaptable to changes in ChIP/enrichment protocols and to other detection platforms (See also 'Future improvements').

Limitations of competition ChIP

Temporal resolution. A major limitation of competition ChIP is that currently it can only measure relatively long (> 500 s) residence times[4]. This limitation arises from two main factors: the relatively slow expression induction of the competitor protein and the interval between assay time points. Because only binding kinetics that are slower than the expression induction of competitor protein can be accurately measured, events that are faster than this rate appear to have a profile that is identical to the relative protein levels[4,5]. As a result, it is important to both rapidly induce the competitor and sample the data fast enough to capture the replacement of the resident with the competitor protein (**Fig. 1b–e**; see also 'Future improvements').

Mixed cell populations. To eliminate complications caused by the cell cycle, cells are typically arrested at a specific stage in the cell cycle for the duration of a competition ChIP experiment. This provision can restrict the types of conditions and proteins that can be assayed by competition ChIP. In addition, the use of a *GAL1* promoter to induce expression of the competitor protein requires the experiment to be performed in media devoid of glucose to prevent unwanted expression induction[4,5,10]. Overexpression by non-native promoters can generate physiologically inappropriate concentrations of the factor, which may in turn cause changes in a protein's ChIP occupancy and results that are not representative of a normal condition. It should also be noted that competition ChIP measures the average residence time from a population of cells. However, residence times, protein abundance or the dynamics of competitor expression induction can vary from cell to cell. The influence of these factors on the interpretation of competition ChIP has not been formally studied.

Factor occupancy can vary across the competition ChIP time course. Initial competition experiments were designed with a Gal4 isoform whose binding was induced by the addition of estradiol. However, the results of these experiments were difficult to interpret owing to changes in total Gal4 occupancy at the *GAL1/10* promoter caused by the addition of estradiol[18,19]. To readily interpret competition between the isoforms, it is preferable that no change in overall occupancy of the factor assayed occurs during the experiment (see also Experimental design)[19]. Similarly, sites at which the factor of interest has low occupancy are difficult to characterize by competition ChIP. This difficulty arises both because such sites are difficult to distinguish as bona fide binding sites by traditional ChIP and because the signal from such loci is dominated by noise (see also Experimental design).

Closely spaced binding sites are problematic. Careful consideration is warranted when applying competition ChIP to the measurement of residence dynamics for a factor with multiple binding sites that are in close proximity[4]. Competition ChIP relies on interpreting the change in ChIP levels of two different isoforms that bind to a specific site. Traditional ChIP relies on sonication of chromatin, which typically results in DNA fragments of 150–500 bp in size. Neighboring binding sites within this range of the sonicated chromatin length distribution (e.g., 500 bp) will therefore influence each other. An increasing number of clustered binding sites would theoretically generate initially artificially fast rates at these loci, because a given chromatin fragment would be more often occupied by both the resident and competitor transcription factor isoforms[4].

Experimental workflow

The main stages of a competition ChIP experiment are summarized in **Figure 1,** and they can be described as follows: design and characterization of a strain or cell line, adjustment of sampling time points and measurement of competitor induction expression kinetics by western blotting, time-course ChIP experiment on cell cycle–arrested cells, ChIP-chip or ChIP-seq experiment to determine relative enrichment of isoforms and to identify the location of binding sites, data normalization and analysis, fit of the data with the competition ChIP model and extraction of turnover rate at binding sites to measure residence times.

Experimental design

The initial generation of the competition ChIP strain or cell line can be challenging, and the resulting strain requires characterization before a full competition ChIP experiment can be performed (**Fig. 1**).

Competition ChIP strain design and characterization. To generate a strain capable of competition ChIP, the same factor is expressed from two different promoters and tagged by two different epitope tags in the same cell. In yeast, a combination of standard cloning and transformation by homologous recombination is the preferred method for engineering the appropriate molecular transformations necessary to create a competition ChIP strain (**Fig. 1a**; refs. 4,20). We recommend beginning strain construction with a yeast strain that has *BAR1* deleted, so that a lower concentration of α-factor can be used to arrest the cell cycle.

During initial strain construction, it is advisable to consider generating multiple alternate versions (e.g., with different promoters or epitope tags) of the competition ChIP strains, because strains may not always behave as desired. Intermediate versions of the competition ChIP strain can include strains that contain one or two differentially tagged copies of the factor, or different promoters. These intermediate versions can be used to test the appropriateness of epitope tags (**Boxes 1** and **2**; see 'Competition ChIP controls'), promoter expression dynamics (see 'Considerations regarding the induction system'), or ChIP protocols before constructing a fully functioning competition ChIP strain. In other experimental systems, related methods for generating transformants and engineering DNA constructs are available.

Construction of the constitutive allele. Ideally, for competition ChIP, the DNA-binding protein to be studied will be actively binding and expressed relatively constitutively throughout the time-course conditions. The endogenous promoter of the factor being assayed is often sufficient to drive constitutive expression during this time[4,5,10]. In haploid yeast, a single copy of the promoter and coding region exist, and we recommend simply tagging the C terminus of this endogenous copy with an epitope tag using

Box 1 | Tag control 1

The purpose of this control is to determine whether the pattern of enrichment is identical for the two tagged alleles and whether both tagged alleles have an equal capacity to bind when in competition. This control is important because competition ChIP relies on the relative enrichment of these isoforms to extract residence time. Any biases in the ability to enrich for either isoform can influence the accurate measurement of dynamics.

Tag control 1 requires a cell strain that expresses both tagged copies of the factor and expresses them in equal amounts. In generating a full competition ChIP strain, this strain can be conveniently generated as an intermediate. For example, a strain encoding two copies of the factor to be assayed, each with a different tag but with identical promoters, will be the strain produced immediately before the addition of an inducible promoter. This strain can be grown in competition ChIP-like conditions (e.g., 2% (wt/vol) galactose and 5 μM α-factor) and should equally express both forms of the factor (e.g., Rap1-Flag and Rap1-Myc). Comparative ChIP-chip in this single condition will ideally identify a strong positive and linear relationship for both isoforms' enrichment throughout the genome. This observation indicates that neither isoform has an inherent advantage in binding at a locus. In practice, it is difficult to determine complete enrichment equality of any competing binding proteins *in vivo*, but a successful tag control 1 experiment is currently a strong indication that the isoforms tested will perform as desired in a full competition ChIP experiment.

homologous recombination. Suitable epitope tags include human influenza hemagglutinin (HA), FLAG and c-Myc[21]. Biotin tags are also suitable for this purpose, although they require a separate enrichment protocol[10,22]. Generally, any commonly used epitope tag is appropriate for either the constitutive or inducible copy, as long as the epitope resides solely on either copy. This requirement applies also to endogenous or exogenous copies of the epitope that are not connected to competition ChIP strain construction.

Construction of the inducible allele. In diploid organisms, the endogenous second copy of the factor to be assayed can be used to generate the inducible allele[10]. In haploid yeast, standard cloning methods can be used to generate the second copy[4]. For example, the wild-type version of the factor, including promoter and 3′-flanking untranslated regions, can be cloned by PCR into a vector containing a selection marker capable of rescuing an amino acid auxotrophy. This construct can then be transformed by homologous recombination specifically at the chosen auxotrophic locus. This transformation will both abolish the auxotrophic phenotype, allowing transformants to be selected for on medium lacking the corresponding amino acid, and integrate the second copy of the factor to be studied. The integration of the second copy of the factor into this unique location in the genome and all subsequent transformations should be confirmed by PCR and sequencing. Epitope tagging by homologous recombination of the second copy of the factor can then be performed. The desired inducible promoter can be inserted by homologous transformation immediately upstream of the factor's transcription start site at a single locus, replacing the wild-type promoter. Alternatively, if a suitable number of selection markers is not available, the construct for the inducible epitope-tagged allele can be generated through standard cloning methods, and the complete construct (promoter, coding region and epitope tag) can be transformed into a unique locus. All final strains should be sequenced to confirm that the correct transformations have been obtained.

Overexpression of proteins or epitope tagging of proteins can alter protein function and cause gross phenotypes[23]. We suggest characterizing the growth of the strain under normal and induced conditions in liquid culture, as these will be the conditions under which the competition ChIP experiment will be conducted[4]. The use of strains showing phenotypes due to tags or expression of the competitor isoform is not recommended, although not all gross phenotypes are indicative of altered residence kinetics, and any phenotypes or defects may not manifest during the relatively short duration of a competition ChIP experiment.

Considerations regarding the induction system. Although induction of the expression of the competitor isoform can be accomplished in many ways, up to this point a weakened or full-strength galactose-inducible *GAL1* promoter has been used[4,5,10]. The rate of increase in competitor concentration is critical because it sets the lower limit of the residence time that can be resolved[4,5] (see also 'Limitations of competition ChIP'). Ideally, the induction of competitor would be rapid, titratable, compatible with any growth conditions and amenable to precise measurement of the nucleoplasmic pool of competitor and constitutive isoforms. Furthermore, it would not influence the underlying physiology of the cell. We recommend using promoters that have very low basal expression levels in order to avoid expression of the competitor allele in the absence of an inducing agent.

In some cases, increases in concentration of the competitor could lead to increased overall occupancy as measured by ChIP[24] (although we have not observed this in our competition ChIP experiments). With this in mind, an induction system that produces

Box 2 | Tag control 2

An optional control that might partly compensate for differences in the capacity for factor isoforms to bind would be to generate a second competition ChIP strain in which the epitope tags have been swapped between the constitutive and competitor allele. Comparison of competition ChIP experiments between the strains with reciprocal epitope tag orientation may partly account for any differences in binding or activity caused by the epitope itself. Ideally, switching the tags between the factor isoforms would have no influence on the measurement of factor dynamics.

a large excess of competitor protein may not always be appropriate (see also 'Limitations of competition ChIP'). Suitable promoters for competition ChIP in yeast include *GAL1* (ref. 21), *CUP1* (ref. 25) and the Tet-On system[26].

Cell cycle arrest. We have performed competition ChIP experiments in cell cycle–arrested cells to prevent complications in residence dynamics caused by DNA replication and progression of the cell cycle[5]. In *S. cerevisiae*, this goal was achieved by adding α-factor to MAT*a* cells that have increased sensitivity to α-factor owing to deletion of the *BAR1* gene. The addition of α-factor results in MAT*a S. cerevisiae* cells arresting in the G1 phase of the cell cycle[4,5,8]. Of course, α-factor causes changes in transcription and cell physiology relative to other conditions and to asynchronous populations that contain cells in different stages of the cell cycle.

Currently, it is not clear how much influence DNA replication and the stage of the cell cycle have on protein-DNA dynamics. However, histone H3 competition ChIP experiments were conducted on both unsynchronized cell populations and cells arrested in G1 by α-factor[5]. In these experiments, a strong, positive correlation was observed between histone H3 dynamics at most loci in the two different conditions**.** However, unsynchronized cells did appear to have faster histone H3 dynamics[27]. Competition ChIP may thus be performed on asynchronous cell populations that are grown without cell-arresting agents such as α-factor. However, the potential influence of DNA replication and an asynchronous cell population on the measurement of the binding dynamics of the factor being assayed should be carefully considered[5,10].

Determining time-point frequency. We recommend the collection of ChIP, western blot and RNA-seq samples (**Fig. 1b**) to be spaced at time points that are spread throughout the representative range of the relative protein levels of the two isoforms after expression induction (**Fig. 1c**). In the first two or three time-point collections, no competitor presence should be detected. This allows for locus-specific estimation of ChIP background in the absence of the competitor[4]. Several time-point collections (we recommend a minimum of 6–8) should be carried out during the period in which the competitor is detectable. If the relative isoform levels reach a steady state after induction within the time course, 2–3 additional measurements should be recorded during this steady-state stage. Computational simulations can be performed to optimize the placement of time points, to minimize cost and to improve temporal resolution.

Competition ChIP replicates. We recommend carrying out a minimum of two biological replicates with confirmation of proper expression induction of the competitor isoform by western blotting for every experiment. We also recommend performing one of the biological replicates as a dye swap if experiments are conducted using a microarray platform. For analysis, biological replicates can simply be averaged.

Defining sites of binding for downstream analysis. To measure residence time at individual binding sites throughout the genome for a factor, those binding sites must first be identified. We recommend identifying a particular factor's binding using material generated during the time-course experiments (**Fig. 1b**,**c**). Traditional ChIP-chip or ChIP-seq can be performed at time zero of the experiment using an antibody that detects the constitutively expressed and the competitor isoforms simultaneously (**Figs. 1c** and **2a**). Measuring total occupancy levels directly provides independent verification of factor enrichment using an antibody specifically raised to study the protein of interest.

If an antibody that recognizes both isoforms is not available, we recommend adding competition ChIP enrichment levels from the competitor (Tag 1) and the constitutive (Tag 2) isoforms, and comparing it with 2 × input (i.e., Tag 1 ChIP + Tag 2 ChIP/(2 × input)). Alternatively, time-zero ratios of constitutive-to-competitor binding can be used to identify binding sites[10]. In this case, the competitor ChIP replaces genomic input. This strategy should only be used if the experimenter is certain that there is no competitor protein detectable by western blotting or ChIP at time zero. Standard peak-calling methods (e.g., MA2C (ref. 28) and MACS[29,30]) can then be used on any of the genome-wide data sets described above to identify regions of factor enrichment (**Fig. 2a**,**b**). Occupancy measurements generated to define factor binding sites can also be used for the occupancy control and occupancy cutoffs (see below).

Competition ChIP controls. At each locus, it is important to ensure that differences in the enrichment of the two differently tagged isoforms are not due to altered binding properties or enrichment efficiencies caused by the addition of epitope tags. For this purpose, we recommend performing a control experiment called 'tag control 1' (**Box 1**), in which a strain expressing both factor isoforms at an equal level from an identical promoter can be used to assay the relative ChIP enrichment of each isoform at all binding sites under steady-state conditions. A strong positive linear relationship of ChIP enrichment for both isoforms is desired at all binding sites.

We also recommend implementing a control experiment called 'tag control 2' (**Box 2**). In this case, competition ChIP replicates are also performed on strains in which the epitope tags have been swapped between the two different versions of the assayed protein. Competition ChIP results should be consistent independently of the orientation of the epitope tags. Competition ChIP experiments conducted so far have not reported any problems related to differences among tags used for the resident or induced form. Nevertheless, conducting these control experiments (tag controls 1 and 2) should be considered when a new system is probed[4,5,8,10] (**Boxes 1** and **2**).

Competition ChIP relies on the assumption that both isoforms are competing with each other directly at each locus. A mandatory occupancy control is to determine whether the total ChIP occupancy of the factor being assayed increases at existing binding sites during the course of the experiment. Such increases in occupancy indicate that the proportion of a particular binding site bound by the factor increases during the experiment within the population[4,18,19]. In this event, these new sites will be instantaneously bound in proportion to the available pool of the two factor isoforms. This behavior will skew the interpretation of competition ChIP results that are based on the assumption that changes in isoform enrichment are solely the result of competition at existing binding sites in the population. An increase in occupancy during the competition ChIP time course would lead to an artificially high competitor-to-constitutive binding ratio and artificially faster factor replacement rates.

Two different methods can be implemented to compare estimated ChIP occupancy at different points within the time course.

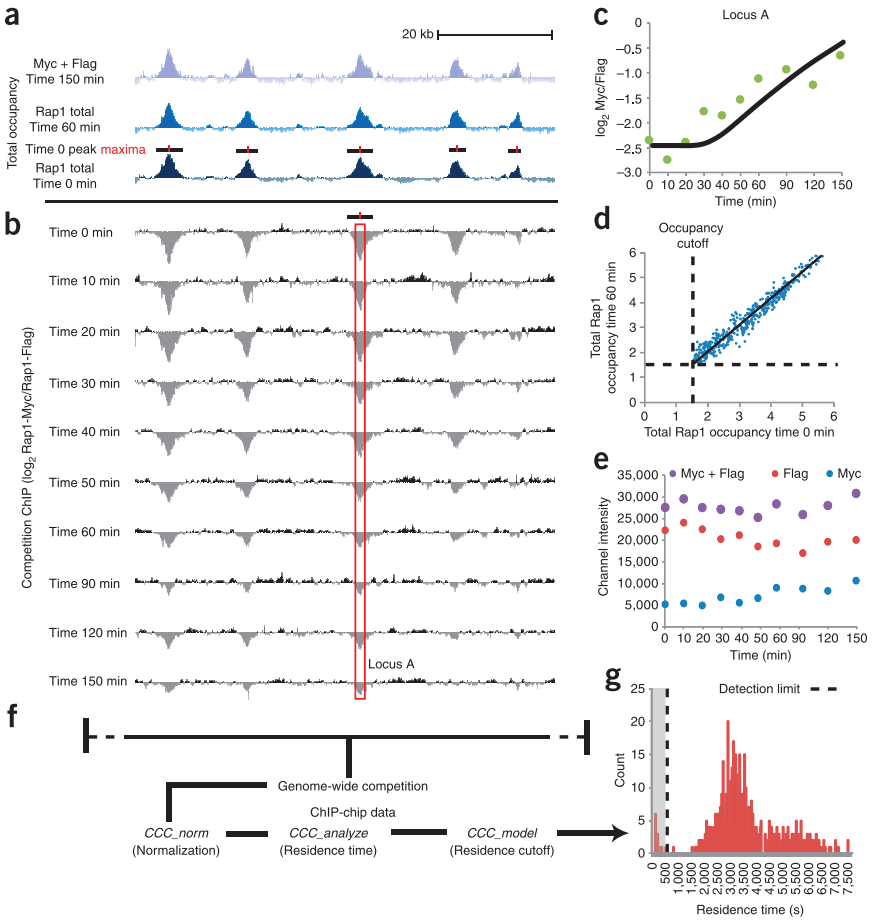

Figure 2 | Anticipated results from a competition ChIP experiment. (**a**) Example occupancy tracks from the UCSC browser show consistent total enrichment of the assayed factor at different times within the time course. Shown is total occupancy measurements for (Rap1-Myc + Rap1-Flag)/2 × input for time 150 min of the competition ChIP time course (top; light purple), (Rap1 total occupancy)/input using total Rap1 antibody (y-300) at time 60 min (middle; blue) and (Rap1 total occupancy)/input using total Rap1 antibody (y-300) at time 0 min (bottom; dark blue). Black horizontal bars in the bottom contour show regions of factor enrichment as defined by peak-calling programs on total occupancy time 0 min. The maxima of peak enrichment are reported in red. For a description of the generation of occupancy tracks see 'Competition ChIP controls' in the INTRODUCTION. (**b**) Example from the UCSC browser of Rap1-Myc/Rap1-Flag $\log_2$ values for a competition ChIP experiment. As the occupancy of competitor (Rap1-Myc) increases through the time course at different sites, the Rap1-Myc/Rap1-Flag $\log_2$ occupancy values increase. Probes ± 150 bp around a peak maximum (red box) are averaged for all time points to give a competition ChIP profile for this locus, as seen in **c**. (**c**) Example average competition ChIP signal for the locus specified in **b**. The black curve shows a model fit suitable for extracting Rap1 residence time. (**d**) Example occupancy plot showing a high correlation between initial (time 0 min) and subsequent (time 60 min) time points for Rap1 total occupancy *z*-score data from **a**. The black diagonal line represents a trend line with a slope of ~1. The black dashed lines represent occupancy cutoffs that are applied to restrict measurement of residence time to sites of confident binding (see also 'Applying occupancy cutoffs' in the INTRODUCTION). (**e**) Example occupancy control showing that the relative increase in Myc (blue) enrichment levels is coordinate with a decrease in Flag enrichment (red), whereas the total levels of the factors combined remains relatively constant (Myc + Flag; purple). (**f**) Example workflow for computational analysis. (**g**) Histogram of example residence times for a competition ChIP experiment as calculated by *CCC_analyze*. Dashed line and gray shading represent the detection limit determined from protein induction kinetics with the *CCC_model*.

The first measures the total occupancy of the factor by traditional ChIP-chip or ChIP-seq at all sites using an antibody that recognizes both isoforms of the factor[4]. Total occupancy levels are compared at time points before and after induction of the expression of the competitor. In the second method, ChIP enrichments of each isoform, as detected by separate ChIP-chip or ChIP-seq experiments, are added together[4]. For ChIP-chip, raw intensity values obtained under consistent array scanning parameters should be used to compare the added intensities of both tags at sites of enrichment across the time-course experiments. For ChIP-seq, read-normalized counts within sites of enrichment can be added directly to estimate total occupancy. Comparison of traditional occupancy measurements (ChIP/Input) and those made by adding the levels of the two tags (Tag 1 ChIP + Tag 2 ChIP/(2 × input)) can also be made to further confirm consistent occupancy levels (**Fig. 2a**). Comparisons of occupancy values for each epitope tag should show high linear correlation across all levels of ChIP enrichment, especially in regions that are bound by the factor (**Fig. 2a–e**).

Applying occupancy cutoffs. A low signal-to-noise ratio causes the competitor-to-constitutive binding ratio to tend to assume the value of 1 at all time points, as the noise overwhelms the signal in both the competitor and constitutive genome-wide enrichment experiments. The signal-to-noise ratio can vary between experiments and detection platforms (e.g., microarray or next-generation sequencing). Visual examination of turnover curves (**Fig. 2c**) at binding sites with different occupancies can be used to estimate when the turnover signal is overwhelmed by noise. For example, one cannot reliably quantify low-occupancy binding sites, which often have a ~1:1 time-zero ratio of competitor to constitutively expressed protein and do not follow an occupancy trend that tracks the protein isoform ratio (**Fig. 1d**). Sites without any enrichment for the factor assayed should show a flat turnover curve[4]. We recommend using an occupancy cutoff to exclude from further analysis sites that cannot be reliably quantified owing to a low signal-to-noise ratio. Previously, we only analyzed sites with an average *z*-score of >1.5 for microarray probes within 150 bp of the maxima of an individual site of factor enrichment, as defined by peak-calling programs, and we suggest this *z*-score cutoff value as a starting point for most analyses[4]. Please note that a *z*-score is the number of standard deviations an observed value is away from the mean of all observations. This parameter enables the comparison of data across experiments that potentially have a different distribution of values. Variations in enrichment and signal-to-noise ratio also make direct

Box 3 | Summary of modified turnover model to analyze competition ChIP data

To quantitatively interpret the turnover data, we used a modified version of a histone H3 turnover model[5] to consider the observed nonspecific measurement background of the microarrays. In the original model, $P(t)$ denotes the probability that the competitor protein occupies a given binding site, whereas $1–P(t)$ denotes the probability that constitutive protein occupies this locus. Dion *et al.*[5] show that $P(t)$ satisfies the following differential equation:

$$\frac{d}{dt}P(t) = \lambda\left(\frac{A(t)}{A(t)+B(t)} - P(t)\right) \quad (1)$$

where λ is the turnover rate at each locus. $A(t)$ and $B(t)$ are the cellular concentrations of the free competitor and constitutively expressed protein, respectively. The ratio of these two concentrations can be measured at all time points using western blotting. The occupancy ratio $R(t)$, as measured by competition ChIP, is the ratio of binding of the inducible competitor protein versus the constitutively expressed protein, and is determined by

$$R(t) = \frac{p(t)}{1-p(t)} \quad (2)$$

With regard to the nonspecific background, we assumed that this background is approximately the same for the constitutive and competitor signal and that it is time invariant. We then calculated the measured occupancy ratio $mR(t)$, including background signal, as

$$mR(t) = \frac{R(t)+C_0(1+R(t))}{1+C_0(1+R(t))} \quad (3)$$

where $R(t)$ is the occupancy ratio calculated in the absence of background equation (2) and C_0 is a constant defined as

$$C_0 = \frac{mR(0)}{1-mR(0)} \quad (4)$$

where $mR(0)$ is the measured occupancy ratio at time 0. In practice, we calculate C_0 by averaging results from samples collected at the initial (e.g., first three) time points, at which no competitor protein is detectable.

We vary λ in equation (1) such that equation (3) yields the best fit to our measured occupancy ratio. We further sample a range of different starting guesses for λ to avoid the detection of local minima.

Turnover rate is dissociation rate

Protein-DNA binding interactions are routinely modeled as reversible events with an association and disassociation rate. We have implemented Monte Carlo simulations that showed that, under our experimental conditions, the turnover rate is equivalent to the dissociation rate, which allows us to measure residence times[4].

comparison of factor dynamics at different binding sites with different factor occupancies difficult. Therefore, competition ChIP data need to be computationally processed before residence time calculations are made (**Box 3**).

Future improvements

Competition ChIP has only been performed throughout the genome on microarray platforms[4,5,8,10]. Although the competition ChIP signal for a two-color microarray is quite similar to two ChIP-seq experiments, in practice the conceptual framework and bioinformatics analysis for competition ChIP-seq still needs to be developed. We anticipate that our existing bioinformatics pipeline (http://code.google.com/p/ccc-process/) can be adapted to use competition ChIP-seq data[4,5]. ChIP-seq also enables the multiplexing of samples, potentially reducing the cost of competition ChIP or increasing the resolution of time points. The dynamic range of ChIP-seq data is generally greater than that of microarrays, which probably makes the estimation of isoform enrichment levels more accurate when they are measured by ChIP-seq. New technologies, such as ChIP-exo[3], could also be combined with competition ChIP to increase spatial resolution, with the important benefit of resolving the measurement of closely spaced binding sites (See also 'Limitations of competition ChIP'), and further reducing the noise associated with ChIP protocols.

Currently, competition ChIP can only measure long (>500 s) residence times[4]. This limitation descends from the rate of competitor expression induction and frequency of time points during the competition ChIP experiment (see also 'Limitations of competition ChIP'). Achieving faster induction kinetics is important because otherwise only steady-state ratios at each time point will be measured, although those ratios will change over time. For example, assume a situation in which competitor levels increase slowly over time but a binding site has a short residence time. After induction, the binding site has time to adjust dynamically to the changing protein levels, such that at each time point the measured competition ChIP ratio will correspond to the current concentration of the competitor and constitutive proteins. In this scenario, the turnover curve follows the kinetics of competitor expression induction, and simply sampling more time points does not alter the outcome. Although their residence times may differ,

all loci with relatively fast residence times can adjust to the changing protein levels and will have the exact same apparent turnover curves, which will overlap with the kinetics of competitor expression induction. The only way to observe different results is to increase the speed of expression induction.

Many induction systems rely on the *de novo* synthesis of a protein and are therefore relatively slow. Faster alternatives would be systems in which the competitor is already present in cells and only its activity is regulated. A host of hormone-inducible transcription factors (factors that can be induced by androgen, estrogen or glucocorticoid) may serve to induce the import or binding of a transcription factor without necessarily relying on the induction of transcription[6,18,31]. Induction could also be achieved by activating proteins by light[32] or influencing transcription factor localization by regulation of nuclear localization signal access[33]. Shutting off an allele and measuring the rate of replacement of the constitutive allele could also theoretically explore protein-DNA interaction dynamics.

Even though this protocol describes competition ChIP for yeast by inducing the expression of the competitor protein from a *GAL* promoter, competition ChIP is readily adaptable to other organisms, in which similar induction systems exist. In *Drosophila*, the Q system[34] and the Gal4-UAS system[35] should be effective for inducing the expression of a competitor allele. In mammals, the Tet-On or other tetracycline-inducible systems are a suitable starting point to design competition ChIP strategies[26]. Although technically challenging, inducing expression of the competitor with a developmentally regulated or tissue-specific induced promoter may enable the exploration of important protein-DNA interaction dynamics in a cell-specific and tissue-specific manner.

To model the competition ChIP data, we assume that protein-DNA cross-linking by formaldehyde is instantaneous in *S. cerevisiae*. A lag in the actual time from formaldehyde addition to protein-DNA cross-linking would lead to a delay in the detection of the ratio of the protein isoforms. Although we did not test this possibility explicitly, we speculate that the presence of this lag would lead to an overestimation of residence times. However, this lag would be consistent at all binding sites, and thus the relative turnover rate between different binding sites will not be affected, and their relative comparison will still be valid.

A recent study has shown that cross-linking and ChIP fail to capture transient binding interactions that last for less than a few seconds[36]. Depending on the nature of DNA-protein interactions, the lag time of formaldehyde-based cross-linking might also limit the perception of rapid interactions by both ChIP and competition ChIP in future studies. Alternative cross-linkers, such as UV light[37], ethylene glycol disuccinate[38,39] and others, may be explored to reduce the temporal resolution limitations of cross-linking. In addition, cross-linking information could be included in the mathematical model used to extract residence times at each binding site.

MATERIALS

REAGENTS

- Formaldehyde, 37% (vol/vol; Fisher Scientific, cat. no. F79-500) **! CAUTION** Formaldehyde is toxic on inhalation or if swallowed. It is irritating to the skin, eyes and respiratory system, and it may be carcinogenic. Formaldehyde should be used with appropriate safety measures such as protective gloves, glasses, clothing and sufficient ventilation. All waste should be handled according to hazardous waste regulations.
- Glycine, 2.5 M (Fisher Scientific, cat. no. BP381-500)
- Tris-HCl, pH 8.0 (Fisher Scientific, cat. no. BP152-500)
- Tris-HCl, pH 7.4 (Fisher Scientific, cat. no. BP152-500)
- Tris-HCl, pH 6.8 (Fisher Scientific, cat. no. BP152-500)
- Dulbecco's PBS (1×; Cellgro, cat. no. 21-031)
- SDS (Fisher Scientific, cat. no. BP166-500)
- NaCl (Mallinckrodt, cat. no. 7581)
- EDTA (Fisher Scientific, cat. no. BP120-500)
- KCl (Fisher Scientific, cat. no. BP366-500)
- IGEPAL CA-630 (Sigma-Aldrich, cat. no. I8896-100)
- Protease inhibitor cocktail set I (Calbiochem, cat. no. 539131) **▲ CRITICAL** Add 1 ml of DNase-free H_2O to create a 1,000× stock.
- DNase-free RNaseA (10 mg ml^{-1}; e.g., Roche, cat. no. 11119915001)
- Proteinase K (20 mg ml^{-1}; Roche, cat. no. 03115836001)
- Ethanol, 95% (vol/vol) (Decon, cat. no. 2801)
- Glycogen (20 mg ml^{-1}; Roche, cat. no. 901393)
- Double-distilled water
- Yeast extract (BD, cat. no. 212750)
- Peptone (BD, cat. no. 211677)
- Dextrose (Mallinckrodt, cat. no. 4908-04)
- Raffinose (Sigma-Aldrich, cat. no. R0250)
- Galactose (Sigma-Aldrich, cat. no. G0625)
- α-factor (GenScript, cat. no. RP01002)
- Glass beads, 0.5 mm (Soda lime, BioSpec, cat. no. 11079105)
- Bradford reagent (protein assay dye reagent concentrate; Bio-Rad, cat. no. 500-0006)
- Glycerol (Fischer, cat. no. G33)
- DTT (Sigma-Aldrich, cat. no. 43815)
- HEPES, pH 7.5 (Sigma-Aldrich, cat. no. H3375)
- KOH (Sigma-Aldrich, cat. no. P1767)
- 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148)
- Bromophenol blue (Sigma-Aldrich, cat. no. 114391)
- $MgCl_2$ (Mallinckrodt, cat. no. 5958-04)
- Protein G–Sepharose beads (GE, cat. no. 17-0618-01)
- Yeast-tagging PCR toolbox (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/Knop.html?ref=klasshop.com) (c-Myc tag and *GALL* promoter source)[21]
- p3Flag-KanMX (Flag tag source)[40]
- Rap1 competition ChIP strain[4]
- BY4741 parental strain (Thermo Scientific, Open Biosystems, cat. no. YSC1048)
- BY4741 Δbar1 (ref. 4; Thermo Scientific, Open Biosystems, cat. no. YSC6273-201920294)
- Anti-Myc clone 9e10 (Millipore, cat. no. 05-419)
- Anti-Flag M2 (Sigma-Aldrich, cat. no. F1804)
- Anti-Rap1 y-300 (Santa Cruz Biotechnology, cat. no. sc-20167)
- WGA amplification kit (Sigma-Aldrich, cat. nos. WGA2-50RXN, WGA3-50RXN)
- Exo-Klenow (Life Technologies, cat. no. 18095-012)
- CyDye labeled UTPs (Sigma-Aldrich, cat. nos. PA53022, PA55022)
- Tru-Seq amplification kits (Illumina, cat. no. FC-121-2001 or IP-102-1001)
- RNA-seq preparation kit (RS-122-2001, Illumina)
- Zymo DNA binding buffer (D4004-1-L)
- Zymo wash buffer (D4003-2-4) **▲ CRITICAL** This buffer requires the addition of user-supplied 95% (vol/vol) ethanol
- Reagents for agarose gel electrophoresis and ethidium bromide staining

EQUIPMENT

Lab equipment

- Liquid nitrogen and an appropriate container
- Swinging bucket centrifuge with variable temperature (such as Fisher Scientific Accuspin 1R)
- Beadbeater (Biospec Mini-BeadBeater-8)
- Nutator (such as BD/Clay Adams, cat. no. 421105)
- Bioruptor sonicator (Diagenode, cat. no. UCD-200)
- Zymo-I spin columns (C1003-250)
- Tubes, 1.5 ml (Costar, cat. no. 3620)
- Screw-top tubes, 2 ml (Fisher, cat. no. 02707360)
- Conical tube, 15 ml (Falcon, cat. no. 352097)
- Conical tube, 50 ml (Falcon, cat. no. 352070)
- Spectrophotometer (e.g., ThermoSpectronic GenSys20)
- Fluorometer with DNA quantification reagents and standards (e.g., Invitrogen, cat. no. Q32866) or NanoDrop ND-1000 ▲ **CRITICAL** Quantification of DNA is necessary, and a fluorescence-based system is recommended, as it is much more accurate than possible alternatives.
- Light microscope (e.g., Nikon AphaPhot2 YS-2)
- Needle, 18 gauge 1.5 inches (BD, cat. no. 305196)
- Orbital shaker (New Brunswick C2 Platform Shaker)
- Imaging system with a large dynamic range that is capable of quantification of western blots (e.g., Li-Cor, Typhoon)

Computer and software

- The source code for array normalization and data fitting is implemented in MATLAB (The MathWorks) and requires the additional 'Optimization Toolbox'; the 'Parallel Computing Toolbox' is optional. Code is available from http://code.google.com/p/ccc-process/ ▲ **CRITICAL** MATLAB programs were tested on MATLAB R2011a running on a Mac Pro with 8 2.66 GHz CPUs and 14 GB memory (Mac OS 10.6.8.). MATLAB is platform independent, and the programs will run on other operating systems as well.

REAGENT SETUP

Beadbeater lysis buffer Combine 50 mM HEPES (pH 7.5) with KOH, 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, 10% (vol/vol) glycerol, 0.1% (vol/vol) IGEPAL CA-630, 1 mM DTT and 1× protease inhibitors (from the 1,000× protease inhibitor cocktail set I stock). The volume of the buffer needed is ~2.5 ml per ChIP. The protease inhibitors should be added immediately before use. Beadbeater lysis buffer without protease inhibitors can be stored for 6 months at room temperature (21–24 °C).

IP buffer Combine 25 mM HEPES (pH 7.5) with KOH, 150 mM KCl, 1 mM EDTA, 12.5 mM $MgCl_2$, 0.1% (vol/vol) IGEPAL CA-630, 1 mM DTT and 1× protease inhibitors. The volume of buffer needed is ~5 ml + ~4 ml per ChIP. The protease inhibitors should be added immediately before use. IP buffer without protease inhibitors can be stored for 6 months at room temperature.

IP elution buffer Mix 50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% (wt/vol) SDS. The volume of buffer needed is ~150 µl per individual ChIP or input sample. IP elution buffer without protease inhibitors can be stored for 6 months at room temperature.

Protein extraction buffer (PEB) PEB contains 0.06 M Tris-HCl (pH 6.8), 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol and 0.0025% (wt/vol) bromophenol blue. This buffer can be stored for 3 months at –20 °C.

YPD Mix 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) dextrose. Autoclaved YPD can be stored for 1 year at room temperature; however, we recommend using recently prepared YPD.

YPR Mix 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) raffinose. Autoclaved YPR can be stored for 1 year at room temperature; however, we recommend using recently prepared YPR.

TE (pH 7.4) TE buffer contains 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. Autoclaved TE (pH 7.4) can be stored for more than 1 year at room temperature.

EQUIPMENT SETUP

Equipment preparation Equipment should be organized in order to ensure rapid collection and processing of competition ChIP time-course samples. Closely spaced time points may require additional people to ensure that the samples are processed promptly. Pre-label all tubes with a permanent ethanol-resistant lab marker (VWR).

PROCEDURE

Initial strain production and characterizations ● TIMING 1–6 months

1| *Obtain or generate competition ChIP strains*. For guidelines in designing the competition ChIP strain, see 'Competition ChIP strain design and characterization'. For engineering transformants, refer to relevant cloning and transformation protocols in your organism of interest[20,41]. The provided protocol uses a Rap1 competition ChIP strain[4]. Construct the strain to be used in this protocol on a haploid BY4741 background. Delete *BAR1* by homologous recombination using *LEU2* from pRS405 (ref. 42). Generate the constitutive *RAP1* allele by using the endogenous *RAP1* locus tagged with a 3× Flag tag from p3Flag-KanMX[40]. Generate the competitor *RAP1* allele by introducing a second *RAP1* copy at the *his3-1* locus. This copy is tagged with a 9× Myc tag from pYM20 and driven by a weakened galactose-inducible promoter *GALL* from pYM-N27 (ref. 21). For additional information on the characterization of competition ChIP strains, see **Box 4**.

▲ **CRITICAL STEP** Strains should be *Δbar1* to increase sensitivity to α-factor; see Step 8.

Initial yeast growth ● TIMING 18 h

2| Inoculate 50 ml of YPD with the Rap1 competition ChIP strain and allow it to grow overnight at 30 °C and 225 r.p.m.

▲ **CRITICAL STEP** Make sure to start the culture early in the day and with sufficient inoculum so that the culture is of a sufficient size (e.g., at least to a total attenuance at 600 nm as measured by a spectrophotometer (D_{600}) of 160; see Step 3) to perform the experiment the following day.

? TROUBLESHOOTING

Time-course experiment ● TIMING 10–13 h

3| After checking whether the culture has reached at least a total of 160 D_{600} units, pellet the culture by centrifugation for 3 min at 1,500*g* and at room temperature.

4| (Optional) If complete sedimentation of yeast is not achieved, split the culture into two 50-ml conical tubes and repeat the centrifugation procedure described in Step 3 or increase the centrifugation time to 5 min.

5| Wash the pellet twice with half a volume (25 ml) of YPR.

Box 4 | Characterization of a competition ChIP strain

The three characterizations described below can be carried out by performing a small-scale mock competition ChIP experiment with a competition ChIP and a control wild-type strain and only obtaining protein and samples to assay growth (see PROCEDURE for general guidelines).

1. Before performing a full competition ChIP experiment, confirm successful tagging of both proteins by western blotting.
2. Confirm the efficacy of induction of competitor allele and constitutive nature of constitutive allele in competition ChIP time-course conditions by western blotting.
3. Identify growth characteristics of competition ChIP strain relative to the wild type (BY4741). This objective can be achieved by performing dilution plating and liquid growth assays. We recommend testing growth in a range of conditions that will be representative of competition ChIP experimental conditions.

6| Use the pellet to inoculate 800 ml of YPR in a 4-liter Erlenmeyer flask to a D_{600} of 0.2 and confirm attenuance. Ultimately, 50 ml of yeast culture at a D_{600} of at least 0.4 is needed per time point. Please note that the total culture volume should be 25% larger than the culture volume consumed in the time-course experiment. For example, 13 time points (as in the present protocol) × 50 ml = 650 ml; 25% of 650 ml = 162.5 ml; 650 ml + 162.5 ml = ~800 ml of total culture volume. Flask volume should be five times that of the culture. In the present case, 800 ml × 5 = 4,000 ml.

7| Allow the cell number to double to a D_{600} of 0.4 by culturing yeast cells at 30 °C and 225 r.p.m. It should take ~4 h for the D_{600} to reach 0.4, but this time may vary for different strains and growth conditions.

8| Arrest cells by adding α-factor to the culture medium to a final concentration of 5 μM (400 μl of 10 mM α-factor) and continue shaking at 225 r.p.m. and 30 °C until 95% of yeast cells are in unbudded 'shmoo' conformation (~3 h), as determined by standard light microscopy. Please note that the term shmoo refers to the yeast morphology during perception of mating pheromone (e.g., α-factor). This morphology is characterized by an elongated rounded projection from the rounded yeast body. Shmoos are distinct from a yeast bud, which usually have an isthmus. Arrest will typically take one full doubling time, and this time may vary from strain to strain.

9| Confirm arrest by microscopy and by counting cells to ensure that 95% are in shmoo conformation.
? TROUBLESHOOTING

10| Collect three samples of different volumes for use in subsequent steps (15 ml for Step 11, 2 ml for Step 16 and 35 ml for Step 20) in order to carry out RNA-seq, western blotting and ChIP.

Collection of sample for RNA-seq (optional) ● **TIMING ~4 min**
▲ **CRITICAL** Steps 11–15 are optional, but we highly recommend their implementation; see 'RNA analysis' (Step 60).

11| Collect 15 ml of culture (from Step 10) in a 15-ml screw-top conical tube.

12| Pellet the sample by centrifugation for 1.5 min at 1,500*g* at room temperature. Pour off the supernatant.

13| Resuspend the cell pellet in 1 ml of ice-cold water and transfer it to a prelabeled 2-ml screw-top tube.

14| Quickly spin (~5 s at 15,000*g* and room temperature) the sample and pour off the supernatant.

15| Quick-freeze the tube in liquid nitrogen (over ~10 s) for subsequent RNA preparation.
■ **PAUSE POINT** The resulting sample can be stored at −80 °C for at least 3 months.

Collection of sample for western blotting ● **TIMING ~7 min**
16| Transfer 2 ml of culture (from Step 10) to a 2-ml screw-top tube and pellet it quickly by centrifugation (~5 s at 15,000*g* at room temperature).

17| Pour off the supernatant and resuspend the pellet in 80 μl of PEB.

18| Heat the suspension at 95 °C for 5 min to liberate the protein extract. Quickly spin the tube (~5 s at 15,000*g* at room temperature).

19| Quick-freeze the pellet and PEB in liquid nitrogen for ~10 s.
■ **PAUSE POINT** The resulting sample can be stored at −80 °C for at least 3 months.

Collection of samples for ChIP ● TIMING 30 min
20| Collect 35 ml of culture (from Step 10) in a 50-ml conical tube to which had been added 1 ml of 37% (vol/vol) formaldehyde (final concentration ~1%).

21| Shake the tube on an orbital shaker (~60 r.p.m.) for 20 min at room temperature to cross-link interacting proteins and DNA present in the sample.

22| Quench the formaldehyde cross-linking reaction by adding 1.75 ml of 2.5 M glycine to a final concentration of 125 mM.

23| Shake the tube on an orbital shaker (~60 r.p.m.) for 5 min at room temperature.

24| Pellet the sample by centrifugation for 2.5 min at 1,500*g* at room temperature.

25| Pour off the supernatant and resuspend the pellet in 1 ml of ice-cold Dulbecco's PBS (1× PBS).

26| Transfer the suspension to a 2-ml screw-top tube. Quickly spin the mixture (10 s at 15,000*g* at room temperature) to pellet it, and pour off the supernatant.

27| Quick-freeze the pellet in liquid nitrogen for ~10 s.
■ **PAUSE POINT** The resulting sample can be stored at −80 °C for at least 6 months.

28| Immediately after the removal of the time-zero samples, add 40% (wt/vol) galactose to the remaining 750-ml culture to a final concentration of 2% (wt/vol) galactose.
▲ **CRITICAL STEP** Ensure complete mixing of culture after initial addition of galactose. Incomplete mixing may lead to inconsistent induction of competitor expression from experiment to experiment.
? TROUBLESHOOTING

29| Take samples for RNA-seq, western blotting and ChIP for the 10-, 20-, 30-, 40-, 50-, 60-, 90-, 120-, 150-, 180-, 210-, and 240-min time points as in Steps 10–27. Typically, not all time points are fully processed. In the actual experiment on which this protocol is based, we only fully processed competition-ChIP data for 0, 10, 20, 30, 40, 50, 60, 90, 120 and 150 min. Excess time-point samples can be processed later if they are potentially informative.
▲ **CRITICAL STEP** Samples for RNA-seq, western blotting and ChIP need to be taken essentially simultaneously and consistently from time point to time point and for each separate biological replicate (see 'Competition ChIP replicates' for more details). Initially, and for closely spaced time points, multiple people may be needed to perform the time-course experiment. Carefully organizing the workspace and prelabeling the tubes is critical for a successful and reproducible experiment. The spacing and number of time points taken may vary for different experimental designs; see 'Determining time-point frequency' for more information.
? TROUBLESHOOTING

Western blotting ● TIMING ~1–2 d
30| Thaw the protein sample from Step 19 on ice. Heat the sample at 95 °C for 5 min, and then spin it at 15,000*g* for 5 min at room temperature.

31| Blot the sample according to existing protocols[20], by loading 15 µl of sample per lane. For western blotting, use anti-Rap1 y-300 (1:200 dilution) as a primary antibody. If determination of Rap1 isoform levels is desired separately, use anti-Myc clone 9e10 (1:1,000 dilution) and anti-Flag M2 (1:1,000 dilution) as a primary antibody.

32| If the different tag compositions of the constitutively expressed and competitor isoform can be distinguished by size, quantify the relative isoform concentration by using a single antibody against the protein of interest[4,10]. When isoforms are not easily distinguishable based on size, to achieve loading normalization and quantification, quantify isoforms using antibodies for their unique epitope, relative to a loading control (e.g., actin, histone H3, Gapdh). To ensure accuracy, we advise using a western blot quantification method with a dynamic range and a ceiling for saturation that is superior to that of film (e.g., Li-cor Odyssey, GE Typhoon 9410).
? TROUBLESHOOTING

ChIP sample preparation ● TIMING 2–3 h

33| Thaw ChIP sample pellets in 1 ml of ice-cold beadbeater lysis buffer. Re-pellet the sample by spinning it at 15,000*g* for 10 s at room temperature. Discard the supernatant.

34| Resuspend the pellet in 0.5 ml of ice-cold beadbeater lysis buffer. Add ~1 ml of 0.5-mm glass beads.

35| Lyse cells in the Mini-BeadBeater-8 with four 1-min sessions on 'high', with 2-min pauses between sessions. Samples should be kept on ice as well as iced between sessions.

36| Recover the extract by puncturing the bottom of the 2-ml screw-top tubes with a needle (18-gauge, 1.5 inches), unscrewing the tube top and allowing the lysate to drip into 15-ml conical tubes placed on ice. The extract will appear cloudy. We recommend heating the needle with a Bunsen burner to facilitate penetration of the screw-top tube. Puncturing the top of the tube with a heated needle may also facilitate the flow of the extract.
! CAUTION Needles are sharp and substantial pressure may be needed to puncture the tubes. Do not use a dull needle, as it increases the amount of pressure needed to puncture tubes, which can increase the likelihood of injury.

37| Add an additional 0.1 ml of beadbeater lysis buffer to each 2-ml tube to wash out additional extract as in Step 36. Discard the 2-ml tube and glass beads.

38| Transfer the lysates (~400–500 µl per sample) to 1.5-ml tubes. Keep the samples on ice.

39| Sonicate the samples in 1.5-ml tubes using a bioruptor sonicator. Set the sonicator on high with a cycle of 5 s on–30 s off, for 10 min of total reaction time (13 sonication cycles) in a 4 °C water bath.
? TROUBLESHOOTING

40| Spin the samples at 15,000*g* at 4 °C for 5 min. Transfer the supernatant lysate to new 1.5-ml tubes and repeat centrifugation. Discard the pellets. Depending on the extract concentration, the supernatant will appear from almost clear to cloudy, in which case the supernatant will be slightly viscous.

41| Remove an aliquot of lysate to test the sonication efficiency by agarose gel electrophoresis. For each chromatin sample, set up one lane containing 10 µl of cleared, sonicated lysate is then incubated at 37 °C for 30 min with RNase (1 µl of 10 mg ml^{-1}) and proteinase K (1 µl of 20 mg ml^{-1}) to quickly digest RNA and protein associated with the lysate.

42| After digestion by RNase and proteinase K, run the entire 12 µl of all representative samples on a gel to determine their sonicated DNA size distribution. Sonication should result in a DNA fragment size ranging from 150 bp to 500 bp for each sample. If necessary, perform an additional sonication at 2-min intervals, until essentially all fragments are below 500 bp in size.
▲ CRITICAL STEP Sonication size determines the minimum distance by which two binding sites can be distinguished by ChIP; see 'Limitations in competition ChIP' for full details.
? TROUBLESHOOTING

43| Determine protein concentrations of each sample by the Bradford assay.
■ PAUSE POINT Lysates may be stored at −80 °C indefinitely.

ChIP lysate preparation ● TIMING 1 h

44| *Prepare lysates for ChIP*. For each time point's ChIP sample, prepare two different new 1.5-ml tubes with 1 mg of lysate (as quantified in the previous step), and bring each tube to a volume of 500 µl with ice-cold beadbeater lysis buffer.

45| (Optional) If desired, for total occupancy ChIPs, prepare a new 1.5-ml tube with 1 mg of lysate (as quantified in Step 43) for time points 0 and 60 min, and bring the volume to 500 µl with ice-cold beadbeater lysis buffer (see 'Competition ChIP controls' and 'Defining sites of binding for downstream analysis' for additional information on deciding when and whether to perform total occupancy ChIPs).

46| (Optional) If desired, collect 50 µg of lysate from Step 43 as an input control and store it at −20 °C in a 1.5-ml tube until Step 60 (see 'Competition ChIP controls' and 'Defining sites of binding for downstream analysis' for additional information). Variations in sonication, processing and so on can result in altered distribution of fragments that are not indicative of the enrichment for the factor-bound DNA. Input refers to the distribution of DNA fragments that are present without enrichment by ChIP, and its measurement serves as a control for ChIP.
■ PAUSE POINT The input control can be stored at −20 °C for at least 1 month.

ChIP ● TIMING ~16 h

47| For each time point, prepare an epitope-specific Myc ChIP by adding 1 µg of anti-Myc (clone 9e10) antibody to one of the 1.5-ml tubes containing 500 µl of lysate and beadbeater lysis buffer from Step 44.

48| For each time point, prepare an epitope-specific Flag ChIP, and add 1 µg of anti-Flag (M2) antibody to each time point of the 1.5-ml tubes containing 500 µl of lysate and beadbeater lysis buffer from Step 44.

49| For total-occupancy Rap1 ChIPs, add 7 µl of anti-Rap1 (y-300) to each of the 1.5-ml tubes containing 500 µl of lysate and beadbeater lysis buffer for time points 0 min and 60 min from Step 45.

50| Nutate or rock the ChIP samples at 4 °C overnight. Nutation gently rocks the samples to ensure equal mixing of samples.

ChIP sample enrichment and washes ● TIMING 3–4 h

51| To estimate the volume of protein G–Sepharose beads, which are supplied as a slurry in ethanol, centrifuge the slurry for 30 s at 1,500*g* and 4 °C. Wash the beads four times with two volumes of ice-cold IP buffer. For each wash, spin down beads at 1,500*g* for 30 s at 4 °C and remove the supernatant with a pipette. In the end, the volume occupied by the 'pellet' after removal of the supernatant is the total desired volume of the beads, although sufficient supernatant should be left in order to prevent beads from drying out.

▲ **CRITICAL STEP** Sepharose bead type must have a matching affinity for the antibody used. Information for Sepharose bead and antibody compatibility can be found on information sheets provided with the products and on product websites.

▲ **CRITICAL STEP** Do not spin Sepharose beads at speeds higher than 1,500*g* or the beads will warp.

▲ **CRITICAL STEP** Do not allow the beads to dry out, as it will negatively influence their effectiveness in ChIP.

52| Resuspend the beads in 1 volume of ice-cold IP buffer.

53| Prepare enough of the immunoprecipitation mixture so that for each ChIP sample there is 50 µl of the 1:1 protein G beads:IP buffer mixture prepared in Step 52. Add 50 µl to this projected volume to account for pipetting error.

54| Add 50 µl of the mixture prepared in Step 53 to each ChIP sample.

▲ **CRITICAL STEP** To ensure equal mixing of beads, cut off the end of the pipette tip and mix beads before addition to each sample.

55| Nutate or rock ChIP reactions at 4 °C for 1.5 h.

56| Wash the beads four times for 15 min with 1 ml of ice-cold IP Buffer. To wash beads, first spin the tube briefly at 1,500*g* at 4 °C, and remove the supernatant with a pipette.

57| Elute immunoprecipitated material by adding 100 µl of IP elution buffer to the washed beads. Incubate the beads in IP elution buffer at 65 °C for 30 min.

58| Spin down the beads for 30 s at 1,500*g* and room temperature, and then transfer the supernatant to a new 1.5-ml tube.

59| Repeat Steps 57 and 58 using 50 µl of IP elution buffer. Pool the first and second eluate for each sample and discard the beads.

ChIP sample cleanup ● TIMING 16 h

60| Incubate the ChIP eluate and input controls from Step 46 at 65 °C overnight to reverse cross-links. Input control samples (from Step 46) should be diluted to 150 µl with IP elution buffer and processed as ChIPs.

61| To each sample, add an equal volume (~150 µl) of TE (pH 7.4), 1 µl of 20 mg ml^{-1} glycogen, and proteinase K to a final concentration of 100 µg ml^{-1} for each sample. Mix the sample well by pipetting, and incubate it at 37 °C for 2 h.

62| Clean up the samples using Zymo columns, wash them with wash buffer and elute them with 25 µl of TE (pH 7.4). Discard the columns after elution. Refer to the instruction manual for Zymo columns for additional information.

63| Add 0.5 µl of RNase A to each eluted sample and incubate the resulting mixtures at 37 °C for 30 min.

64| Clean up the mixtures from Step 63 using Zymo columns. Next, elute their contents with 25 µl of 10 mM Tris-HCl (pH 8.0).

▲ **CRITICAL STEP** We have found that some downstream amplification protocols are sensitive to EDTA. The final elution should therefore be performed using only Tris buffer (not containing any EDTA).

? TROUBLESHOOTING

■ **PAUSE POINT** After collection, samples can be stored at −80 °C. We have found that the quality of the eluted ChIP samples often decreases within 1 month. Process the samples as quickly as possible.

Preparation of samples for ChIP-seq or ChIP-chip ● TIMING 1–2 d

65| Follow standard protocols for the preparation of ChIP samples for ChIP-chip or ChIP-seq according to the platform used. Typically, we use WGA amplification (WGA2-50RXN, WGA3-50RXN; Sigma-Aldrich) followed by exo-Klenow random primer incorporation (18095-012; Life) of CyDye-labeled UTPs (PA53022, PA55022; Sigma-Aldrich) for ChIP-chip. For ChIP-seq, we use Tru-Seq amplification kits (FC-121-2001 or IP-102-1001; Illumina). To carry out this step, follow the manufacturers' instructions. Reagents for ChIP amplification, ChIP labeling and sequencing preparation are frequently improving. Evaluate the available options.
? TROUBLESHOOTING

RNA analysis ● TIMING ~3 d

66| We recommend determining genome-wide RNA levels at least before and after competitor induction (e.g., at 0 and 60 min) to identify whether any genes change their expression pattern and to correlate residence time with transcription[4]. The number of time points at which the experimenter chooses to assay RNA levels will depend on the nature of the experiment performed and analysis desired. For RNA-seq, extract RNA using a hot phenol extraction[43] and prepare RNA for sequencing using an RNA-seq preparation kit (e.g., RS-122-2001, Illumina).

Next-generation sequencing ● TIMING ~7 d

67| If microarray chip technology is not used to analyze samples, perform next-generation sequencing (ChIP-seq/RNA-seq) on all samples for which data are desired (e.g., Myc and Flag ChIPs for time points 0, 10, 20, 30, 40, 50, 60, 90,120 and 150 min; total Rap1 ChIPs, input and RNA-seq for time points 0 min and 60 min) either in-house or by sending the samples to an external facility. In either case, the minimum turnaround time is 7 d. See 'RNA analysis' and ANTICIPATED RESULTS for additional guidance.

Data analysis ● TIMING ≥1 d

68| Generate an average wiggle track format (WIG) or .bedGraph file of Myc/Flag $\log_2$ values for each time point from competition ChIP replicates (**Fig. 2a**,**b**).

69| Examine the quality of the data and confirm the successful incorporation of the competitor protein by visualizing all time points and occupancy tracks in the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) (**Fig. 2b**,**c**,**e**).

70| Use a peak-calling algorithm (e.g., MACS) on the total occupancy average at time zero to identify chromatin sites to which the factor binds or with which it interacts, and to determine the location of peaks (regions of protein enrichment) and their maxima.

71| Apply occupancy cutoff at a *z*-score of 1.5 and exclude binding sites with a *z*-score below this threshold from further analysis (**Fig. 2d**). Occupancy cutoff may vary; see 'Competition ChIP controls' for further details.

72| Perform total occupancy comparisons between 0 and 60-min time points within regions of factor enrichment (e.g., ± 150 bp from peak maxima; **Fig. 2d**,**e**).

73| These initial assessments (Steps 68–72) enable experimenters to obtain a qualitative visualization of the total genome-wide occupancy enrichment of the factor studied across the time course (**Fig. 2a**,**c**,**d**), regions of factor enrichment (**Fig. 2a**,**b**) and confirmation of isoform incorporation at the chromatin level (**Fig. 2b**,**e**).
? TROUBLESHOOTING

74| After the preliminary assessment in Step 73, use the provided script *CCC_norm* in MATLAB to normalize microarray competition ChIP-chip data (**Fig. 2f**). Many sources of systematic variation exist in microarray experiments, and normalization is a mathematical operation that reduces these artifacts within each array and ensures that the two channels recorded can be compared. The required input files are the average single-channel microarray intensities for each time point from the competition ChIP experiment, the annotation file describing the probes on the microarray and the group of enriched probes surrounding peak centers. Please note that the source code for array normalization and data fitting is available online together with detailed user manuals and test data at http://code.google.com/p/ccc-process/.

75| Use the provided program *CCC_analyze* to analyze the normalized competition ChIP data in MATLAB. First, the program extracts isoform ratios at the identified binding sites at each time point. The resulting curves are then fit with a mathematical model (**Box 3**) to estimate protein residence times at sites under consideration of genomic occupancy background. This genomic occupancy background probably reflects the nonspecific noise of the microarrays, including nonspecific pull-down from the beads, site-specific variations in the DNA amplification or nonspecific binding bias in hybridization[4].

Required input files for this purpose are the normalized competition ChIP data from *CCC_normalize,* factor peak maxima (i.e., binding sites) and relative protein isoform levels from quantitative western blotting experiments.
▲ **CRITICAL STEP** We caution readers against interpreting competition ChIP data without normalizing them beforehand and without considering the genomic occupancy background (**Box 3**).

76| Use the provided script *CCC_model* to determine the limit of residence time measurements based on the rate of competitor induction in MATLAB (**Fig. 2f**). The required input file for this step is the relative protein isoform levels, as determined from quantitative western blotting experiments.
▲ **CRITICAL STEP** Sites that have residence times that are too short to measure (fall below the quantification limit) should be considered as a single fast turnover group without specific residence times (**Fig. 2g**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Poor growth of competition ChIP strain	Epitope tag is toxic	Use an alternate epitope tag
		Overexpression is toxic	Reduce the amount of the inducing agent
9	Incomplete cell cycle arrest	*BAR1* is not deleted	Delete *BAR1* from strain
		α-factor concentration is too low	Confirm α-factor concentration
			Consider performing experiment without α-factor in an asynchronous population
28, 32	Competitor expression is not induced consistently	Inducing agent is not properly applied	Check to make sure the concentration of the added inducing agent is correct and the inducing agent is fully homogenized in the culture before collecting samples at subsequent time points
		Promoter is not effective enough for the experiment	Test alternative inducible promoters
28, 29, 73	Despite monotonic increase in the extent of competitor binding relative to that of the constitutive protein, array values are not monotonic	Sample variation and technical noise	Collection of samples at multiple time point enables robust measurements of isoform ChIP levels to be performed across the time course of the reaction
			Additional replicates may reduce the influence of noise
		The experiment is not performed in a technically correct fashion	Ensure proper processing of samples, use more people or improve the organization of the workspace
39, 42	Sonicated chromatin has incorrect fragment length distribution	The sample was insufficiently sonicated	Perform additional sonication cycles. Polystyrene or polymethylpentene tubes are recommended by the sonicator manufacturer. (e.g., Diagenode M-50001) for sonication steps due to their superior ultrasound conductivity
64, 65	Poor ChIP amplification	EDTA may be present in the ChIP sample	Elute off column using only Tris buffer before ChIP amplification
		Samples get degraded over time	Fully process ChIP samples promptly
73	Total occupancy changes after the induction of competitor expression	Overexpression may drive increased occupancy	Reduce competitor levels by adding less inducing agent, use data from time points before occupancy changes
		Inducing agent may influence factor occupancy	Use an alternate inducing agent, if available[19]. Use a promoter that requires a different inducing agent

● TIMING

Step 1, initial strain production and characterizations: 1–6 months
Step 2, initial yeast growth: 18 h
Steps 3–10, time-course experiment: 10–13 h
Steps 11–15, collection of sample for RNA-seq (optional): ~4 min
Steps 16–19, collection of sample for western blotting: ~7 min
Steps 20–29, collection of samples for ChIP: 30 min
Steps 30–32, western blotting: ~1–2 d
Steps 33–43, ChIP sample preparation: 2–3 h
Steps 44–46, ChIP lysate preparation: ~1 h
Steps 47–50, ChIP: ~16 h
Steps 51–59, ChIP sample enrichment and washes: 3–4 h
Steps 60–64, ChIP sample cleanup: 16 h
Step 65, preparation of samples for ChIP-seq or ChIP-chip: 1–2 d
Step 66, RNA analysis: ~3 d
Step 67, next-generation sequencing: ~7 d
Steps 68–76, data analysis: ≥1 d

ANTICIPATED RESULTS

Total occupancy should be highly correlated across different time points of the competition ChIP experiment time course (**Fig. 2a**). Occupancy experiments can also be used to define centers of factor enrichment (i.e., peaks; **Fig. 2a**,**b**). Ideally, there should be no changes in total occupancy across the time course of a competition ChIP experiment, especially at sites where factor enrichment is identified (**Fig. 2a**,**d**,**e**). As the experiment time course progresses, the Myc/Flag occupancy ratio should increase in a manner consistent with protein levels (**Fig. 1d**), as the protein competitor copy (Myc) replaces the constitutively expressed copy (Flag) (**Fig. 2b**,**c**,**e**). By averaging the occupancy ratio values within 150 bp of peak centers, individual competition ChIP curves can be generated for each binding locus (**Fig. 2c**). To generate residence times, competition ChIP data are used as input for a group of MATLAB-based programs (see Steps 68–76 of the PROCEDURE and **Fig. 2f**). The fit of the model can be evaluated by the squared sum of the residuals (SSR) that is output from *CCC_analyze*. Please see the source code for array normalization and data fitting, detailed user manuals and test data at http://code.google.com/p/ccc-process/. A distribution of residence times and a detection limit should be generated from the programs (**Fig. 2g**). Residence times for each binding locus can be used for subsequent downstream analysis[4,5].

ACKNOWLEDGMENTS We thank L.S. Kovacs and T. Jeffers for critically reading the manuscript, K. Ikegami for suggesting alternative cross-linkers and promoter systems and D. Mckay for suggesting inducible expression systems in *Drosophila*. J.D.L. and C.R.L. were supported by National Institutes of Health grant no. R01 GM072518. F.M. has been supported by the Région Ile-de-France under C'Nano IdF (the Center of Competences in NanoSciences for the Paris region) and the Fondation pour la Recherche Médicale en France (FRM).

AUTHOR CONTRIBUTIONS C.R.L. and F.M. performed the analysis and refined the competition ChIP protocol with input from J.D.L. C.R.L., F.M. and J.D.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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