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Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in *Caenorhabditis elegans*

Arnab Mukhopadhyay, Bart Deplancke, Albertha J M Walhout, and Heidi A Tissenbaum

Program in Gene Function and Expression, Program in Molecular Medicine, Aaron Lazare Research Building, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605, USA

Abstract

In order to determine how signaling pathways differentially regulate gene expression, it is necessary to identify the interactions between transcription factors (TFs) and their cognate *cis*-regulatory DNA elements. Here, we have outlined a chromatin immunoprecipitation (ChIP) protocol for use in whole *Caenorhabditis elegans* extracts. We discuss optimization of the procedure, including growth and harvesting of the worms, formaldehyde fixation, TF immunoprecipitation and analysis of bound sequences through real-time PCR. It takes ~10–12 d to obtain the worm culture for ChIP; the ChIP procedure is spaced out over a period of 2.5 d with two overnight incubations.

Introduction

A host of biological processes such as transcription, recombination, replication and gene silencing are mediated through protein–DNA interactions. Thus, it is of paramount importance to understand where and when a protein binds to DNA. There are several experimental methods to analyze protein–DNA interactions. Most use a DNA-binding protein as the experimental starting point and seek to determine its binding site or target genes¹. These methods include chromatin immunoprecipitation (ChIP)^{2,3}, which enables the capture of protein–DNA interactions *in vivo*. Alternative methods are

- *Protein-binding microarrays*⁴. A DNA-binding protein fused to glutathione *S*-transferase is hybridized to a universal DNA microarray containing all possible 10-mer nucleotide stretches to find consensus binding sites.
- *Bacterial one-hybrid assays*⁵. Here, a DNA-binding protein is expressed in millions of bacteria, each containing a specific random DNA element. When protein binds to a particular element, the bacteria harboring the sequence are selected and a consensus binding site can be deduced.
- *Yeast one-hybrid assay*^{6,7}. This is a high-throughput gene-centered protein–DNA interaction detection method that allows the large-scale identification of proteins binding to DNA sequences of interest. Similar to protein binding microarrays and bacterial one-hybrid assays, high-throughput yeast one-hybrid assays are context independent; they are performed either *in vitro* or in a heterologous organism (bacteria or yeast).

Correspondence should be addressed to H.A.T. (E-mail: Heidi.Tissenbaum@umassmed.edu).

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- *Comparative genomics*^{8,9}. This is a complementary method used to predict protein–DNA interactions, which is based on the principle that genomic regions of biological importance are conserved across species. Therefore in this analysis, comparing DNA sequences from different species will reveal regions of conservation (motifs) that may be attributed to similar regulatory control mechanisms. Using various computational and statistical tools, one can predict the possibility of a protein binding to a DNA fragment^{8,9}. However, the main drawback of this technique is that all data are generated *in silico* and therefore each prediction needs to be verified individually.

Overview of ChIP and its applications

ChIP involves crosslinking of the protein–DNA complex within an intact cell using crosslinking agents, such as formaldehyde (Fig. 1). The DNA is then sheared to smaller pieces (~500 bp) by sonication or nuclease digestion. The sheared protein-bound DNA is then immunoprecipitated using a highly specific Ab against the protein. An aliquot of the sheared DNA before immunoprecipitation is used as a reference sample. The protein–DNA complexes from reference and ChIP samples are then reverse crosslinked. The DNA is purified and enrichment of ChIP-ed DNA over the reference sample can be analyzed using a number of techniques, such as quantitative PCR, sequencing or microarray¹⁰. Although ChIP has been widely used in other model systems, there are only a few labs that have successfully used ChIP in *Caenorhabditis elegans*^{11–14}. Previous reports of ChIP in worms mostly used embryos as the starting material and the results were analyzed in a semiquantitative manner. Recently, we have used ChIP to identify direct targets of the *C. elegans* transcription factor (TF), DAF-16 (ref. 15). Here, we outline in detail an optimized procedure for ChIP (using whole worms as the sample source) including growth and harvesting of the worms, formaldehyde fixation, TF immunoprecipitation and analysis of bound sequences through quantitative real-time PCR; see Figure 2 for an overview of the timeline involved. This protocol has been modified from our previously published studies involving DAF-16 (ref. 15) and DAF-3 (ref. 7).

Limitations of ChIP

The main drawback of ChIP is its inherent variability. This may arise due to variabilities in the crosslinking, immunoprecipitation and protein–DNA washing efficiencies. Stringent control over experimental conditions may reduce these variabilities. As indicated earlier, other limitations include the dependence on the availability of a highly specific Ab and the need for relatively high level as well as broad expression of the DNA-binding protein of interest. ChIP in whole worms produces additional challenges, including the difficulty of growing a large population of worms, contaminating DNA from the bacterial food and the inherent sample variability between biological replicates. The last problem may be overcome using a staged worm culture; however for this, advance knowledge about the expression/function of the protein of interest is required. The distinct advantage of using whole *C. elegans* worms is the availability of null mutants, thereby providing an excellent negative control (see Experimental controls).

Experimental design

Optimization of ChIP using whole worms—In order to establish the ChIP protocol using an Ab against a protein of interest, three critical steps have to be optimized: (i) fixation time, (ii) amount of input material and (iii) amount of Ab used, as discussed below. Apart from this, users need to determine whether to use mixed culture or staged culture for the assay. As a basic overview, a list of steps that require optimization is presented in Table 1. For the optimization step, it is helpful if there is a known target gene for the DNA binding protein of interest.

1. *Optimization of chemical crosslinking time*. One of the critical steps in ChIP is to capture the DNA–protein interaction within a cellular context, which is achieved

using a chemical crosslinking agent, such as formaldehyde. This step may be problematic, as the extent of crosslinking may vary from sample to sample (the reaction never reaches completion), and *C. elegans* contains an outer cuticle that is hard to penetrate with externally applied chemicals¹⁶. We homogenized the worm pellet in crosslinking buffer (CB) using a glass Dounce homogenizer that is assumed to gently create abrasions that would facilitate the entry of the crosslinking agent. The crosslinking time needs to be empirically determined when setting up ChIP for the first time. Because the handling of materials may vary significantly among various users, we suggest that each user optimizes the crosslinking time. It should be noted that we start timing the incubation of the worms in the CB after the abrasion step. So, the actual time the worms are exposed to the CB is longer than the indicated time. Follow the steps in Box 1 to optimize the crosslinking time, and refer to Figure 3 for an example of expected data.

2. *Optimization of the amount of input material.* Because DNA-binding proteins are expressed at different levels and in various tissues, the amount of protein of interest may vary tremendously. For example, for highly expressed proteins, the amount of total protein required for immunoprecipitation will be significantly lower than that of a low abundance protein. Therefore, ChIP must be optimized for the amount of input material (total protein) that is used to immunoprecipitate using Ab against the protein of interest (see Figure 4 for an example of input optimization data). Box 2 describes a miniprotocol for optimization of the amount of input material. In addition, before starting any ChIP experiment, it is important to determine the expression level or pattern of the protein of interest using western blotting (see Fig. 5 for an example) or *in situ*. A protein–DNA interaction may also take place in a few cells, although the protein may be widely expressing. In such cases, more ChIP-ed DNA may be required for the qPCR detection.
3. *Optimization of the Ab amount used for ChIP.* The crosslinked DNA–protein complex is specifically immunoprecipitated using an Ab against the protein-of-interest. The quality and specificity of the Ab contributes immensely to the outcome of the ChIP procedure. As Ab preparations differ considerably in specificity and titer, the amount of Ab used to immunoprecipitate the crosslinked TF–DNA complex needs to be empirically determined (Fig. 6) as outlined in Box 3. We used an Ab against the DAF-16 protein for optimization of this protocol. This Ab was raised against the fork-head domain and is a rabbit polyclonal Ab. As we used nonpurified Ab, the absolute concentration of the Ab could not be estimated; however, before using the DAF-16 Ab for ChIP, we performed western analysis with the Ab to confirm its specificity, as shown in Figure 5.

BOX 1

Miniprotocol for Optimization of Crosslinking Time

1. For crosslinking optimization, process three sets (or more) of samples in a manner listed in either Step 1A or 1B of the PROCEDURE.
2. Proceed as mentioned in Steps 2–5 of the PROCEDURE to crosslink the protein and DNA chemically.
3. Transfer the worm suspensions to 15-ml tubes. Wash the homogenizer with 1 ml additional crosslinking buffer and collect in the 15-ml tube. Incubate all tubes at room temperature on a Nutator shaker (25 shaking actions per minute). However, incubate each of the three (or more) tubes for different time periods (for example 15, 20 or 30 min).

4. Add 200 ml of 2.5 M Glycine to the same tube and incubate on the Nutator shaker for an additional 15-20 min.
5. Proceed as in Steps 8–33 of the PROCEDURE, to perform the chromatin immunoprecipitation and subsequent purification of the DNA. Perform quantitative PCR (Steps 34–49) to determine which crosslinking time gave the best binding ratio of the DNA-binding protein at the promoter relative to the untranslated region of the same gene. See Figure 3 for a representative result.

Real-time PCR (quantitative PCR)—A critical step in this protocol is the analysis of bound DNA sequences through real-time PCR (quantitative PCR (qPCR))^{17–19}. It provides an accurate determination of levels of specific DNA in ChIP-ed samples. The qPCR is based on detection of a fluorescent signal produced proportionally during amplification of a PCR product. The use of qPCR has several advantages: short turnaround time for data acquisition and analysis; reliable results compared to traditional PCR methods; and it is quantitative. However, as the procedure is very sensitive, there is an increased chance of variability of the results. There are several aspects of qPCR that need to be considered to get reliable and reproducible results. They are: (i) primer design using dedicated softwares, some of which are freely available on the Internet (see REAGENT SETUP); (ii) deciding on positive and negative controls (see below); (iii) technical and biological repeats (see below) and (iv) analyses of the data (detailed in PROCEDURE).

Experimental controls—As with any experiment, proper controls are absolutely essential for correct interpretation of data produced using the ChIP procedure. Positive controls are difficult to obtain, especially for *C. elegans*, as the number of experimentally defined direct protein–DNA interactions currently available is limited. However, good negative controls lead to proper interpretation of the results. The main advantage of using *C. elegans* for ChIP is the availability of null mutants, thus enabling comparison of the degree of target DNA enrichment in biological samples expressing or lacking the protein-of-interest. However, before using such a mutant, it should be verified whether the mutant worms completely lack the protein-of-interest, as a partially functional protein may still bind DNA and lead to misinterpretation of the results. When such a deletion/null mutant is not available, samples that either have prebleed serum added or from which Ab has been omitted during ChIP can be used as negative controls. Alternatively, RNAi may be used to knock down the gene, although care should be taken to judge the efficacy of the RNAi. While studying the binding profile of a TF, detecting the binding of the protein to the 3' UTR or the coding region or both may also be used as a negative control, assuming binding of the TF may be restricted to or enriched on the promoter. If a binding site is already known, it will be prudent to avoid it even in a control region, in case the protein may bind to that site. On the other hand, conditions when the protein is known to be in an inactive state may be used as a negative control.

Experimental repeats—ChIP is a long procedure involving multiple washing and precipitation steps. As such, it is important to have multiple technical as well as biological repeats. In order to assess the variability generated due to sample handling within a particular experiment, it is important to have three technical repeats. Technical repeats can be generated by performing three separate immunoprecipitation reactions and detections after the worms have been sonicated. If the handling of samples is consistent, the three technical repeats score very similar C_t (threshold cycle) values in qPCR.

BOX 2

Optimization of the Amount Input Material Used for ChIP

1. Follow Steps 1–13 of the PROCEDURE to grow and harvest worms, and chemically crosslink and sonicate the samples.
2. Centrifuge the lysate (16,000g, 4 °C, 15 min in a refrigerated tabletop microcentrifuge) to pellet the debris. Transfer the supernatant to a fresh tube, avoiding floating debris. Repeat this step if debris is carried over. Pool the supernatants from the four tubes.
3. Estimate the total protein concentration using any standard protein estimation procedure.
4. Perform each chromatin immunoprecipitation (ChIP) experiment using three technical repeats. At this step, take a different amount of precleared ‘input’ (for example 0.4, 1.0, 2.0, 5.0 and 10 mg of total protein), and dilute each to 500 ml using HEPES lysis buffer containing protease inhibitor cocktail.
5. Continue with Steps 17–33 of the PROCEDURE to perform the ChIP and purify the DNA. Follow Steps 34–49 to determine the input sample amount that gives the best binding ratio. See Figure 4 for a representative result.

Materials

Reagents

- 1× PBS (see REAGENT SETUP)
- 1× M9 buffer (see REAGENT SETUP)
- 1× TE (see REAGENT SETUP)
- 1× TBST (see REAGENT SETUP)
- Agar (Fisher, cat. no. BP1423)
- Bactopectone (Becton Dickinson, cat. no. 214010)
- Blotting grade blocker nonfat dry milk (Bio-Rad, cat. no. 170-6404)
- CaCl₂ (1 M in water; Fisher, cat. no. BP510)
- Cholesterol (10 mg ml⁻¹; JT Baker, cat. no. F676-05) (see REAGENT SETUP)
- Commercial bleach (Ultra Clorox germicidal bleach; 6.15% sodium hypochlorite)
! CAUTION Toxic and may cause severe burns. Handle in fume hood.
- Ethanol (100% vol/vol, Pharmaco AAPER, cat. no. 111000200)
- Formaldehyde (37% vol/vol; Sigma, cat. no. F1635) **! CAUTION** Highly toxic by inhalation, inflammable and carcinogenic. Should be handled with appropriate protective measures in a fume hood. Formaldehyde wastes should be discarded according to hazardous waste disposal regulations.
- Glycine (2.5 M in water; Sigma, cat. no. G8790)
- Glycogen (1 mg ml⁻¹; Roche Diagnostics, cat. no. 10901393001)
- Goat anti-rabbit secondary IgG Ab (Abcam, cat. no. 6721-1)

- HB101 bacteria. Available from The Caenorhabditis Genetics Center (CGC; <http://www.cbs.umn.edu/CGC/>), supported by the National Institutes of Health, National Center for Research Resources (see REAGENT SETUP)
- HEPES-KOH (1 M, pH 7.4) (see REAGENT SETUP)
- HEPES lysis buffer (HLB) (see REAGENT SETUP)
- KH_2PO_4 (Fisher, cat. no. P380)
- K_2HPO_4 (Fisher, cat. no. P290)
- KOH (5 N in water; VWR, cat. no. VW5040)
- LiCl (5 M in water; Fisher, cat. no. L120)
- Liquid nitrogen, dry ice **! CAUTION** Can potentially cause severe frostbite.
- LB medium (see REAGENT SETUP)
- NaCl (5 M in water; Fisher, cat. no. BP358)
- MgSO_4 (1 M in water; Fisher, cat. no. BP214)
- Na_2HPO_4 (Fisher, cat. no. S369)
- Nematode growth medium (NGM) agar (see REAGENT SETUP)
- Nystatin (Sigma, cat. no. N4014) 50 mg ml⁻¹ in water
- NP-40 (10% vol/vol in water; Calbiochem, cat. no. 492016)
- Oligonucleotide primers: user specific (see REAGENT SETUP). The lyophilized primers are resuspended in 10 mM Tris-Cl (pH 8.0) to prepare a 100 mM stock.
- Phenol–chloroform–isoamylalcohol (25:24:1, pH 7.7–8.3; Fluka, cat. no. 77617)
! CAUTION Toxic when in contact with skin or if swallowed. Causes severe burns and irritation to respiratory system, eyes and skin. Chloroform is an irritant and potentially carcinogenic. Should be handled with appropriate protective gloves, protective clothing and glasses in a well ventilated area. Phenol–chloroform–isoamylalcohol wastes should be discarded according to hazardous waste disposal regulations.
▲ CRITICAL The DNA will degrade if this reagent is acidic or old.
- PMSF (1 mM in isopropanol; Merck, cat. no. F1635)
- Protease inhibitor cocktail (Sigma, cat. no. P2714; dilute in 10 ml water and frozen in aliquots) **! CAUTION** Toxic and should be handled using gloved hands.
- Potassium phosphate buffer (PPB, 1 M) (see REAGENT SETUP)
- Proteinase K buffer (see REAGENT SETUP)
- Proteinase K (VWR International, cat. no. VW1519-01) diluted to 20 mg ml⁻¹ in water and frozen (–20 °C) in aliquots of 20 ml
- Salmon sperm DNA/protein A agarose (Upstate, cat. no. 16-157) (see REAGENT SETUP)
- S-basal (see REAGENT SETUP)
- SDS (10% wt/vol in water; Fisher, cat. no. BP166)
- Sodium deoxycholate (10% wt/vol in water; Sigma, cat. no. D5760)
- Streptomycin (Sigma, cat. no. S2522) 50 mg ml⁻¹ in water

- SuperSignal West Pico detection system (Pierce Biotechnology, cat. nos. 1856135 and 1856136)
- SYBR Green PCR Master Mix (Applied Biosystems, cat. no. 4309155) or other similar reagents
- Tryptone (Fisher, cat. no. BP1421)
- Tris-Cl (50 mM in water, pH 8.0; Fisher, cat. no. BP152)
- Triton X-100 (10% vol/vol in water; Fisher, cat. no. 492016)
- Wash buffer 1 (WB1) (see REAGENT SETUP)
- Wash buffer 2 (WB2) (see REAGENT SETUP)
- Wash buffer 3 (WB3) (see REAGENT SETUP)
- Worms. Available from CGC
- Yeast extract (EMD Chemicals, cat. no. 1.03753.500)

Equipment

- 7-ml Glass homogenizer (Kontes Glass)
- Beckman Coulter Avanti J-25 or similar centrifuge
- Beckman Coulter Allegra 6KR or similar centrifuge
- Dissecting microscope (Zeiss Stemi 2000, Nikon SMZ645 or other similar equipment)
- Nutator (Clay Adams Brand; Becton Dickinson) or similar
- Misonix Sonicator 3000 (Misonix) fitted with microtip
- Platform shaker maintained at 20 °C
- Pipette tips with microcapillary (VWR International, cat. no. 37001-150)
- Real-time PCR machine (e.g., ABI PRISM 7000 Sequence Detection System) (see EQUIPMENT SETUP) or other similar instrument
- Refrigerated tabletop microcentrifuge
- Vortex

Reagent Setup

1× M9 buffer—3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl and 1 ml 1 M MgSO_4 in 1 l of deionized water. Autoclave for 20 min. Store at room temperature (RT, ~23 °C) preferably in aliquots of 50 ml that may be discarded after each use.

1× PBS—8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 . Deionized water to 1 l. Adjust the pH to 7.4 with HCl. Autoclave for 20 min. Store at RT.

1× TE—10 mM Tris-Cl, pH 8.0, 1 mM EDTA in water. Store at RT.

1× TBST—20 mM Tris (pH 7.5), 137 mM NaCl, and 0.1% Tween-20 (vol/vol) in water. Store at RT.

Cholesterol (10 mg ml⁻¹)—Cholesterol powder is dissolved in 100% ethanol. It takes a long time to dissolve; it is convenient to let it dissolve overnight at RT on a stirrer. The solution is stored at RT.

CB 1x—PBS containing 1% formaldehyde (vol/vol). This solution is made fresh each time and discarded after use. Maintain at RT.

HEPES-KOH (1 M; pH 7.4)—Dissolve the HEPES powder in deionized water and adjust the pH with 5 N KOH at RT.

HLB—50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS, 1 mM PMSF and diluted protease inhibitor cocktail (10 ml ml⁻¹)

BOX 3

Optimization of the Ab Amount

1. Follow Steps 1–13 of the PROCEDURE to grow and harvest worms, chemically crosslink and sonicate.
2. Following sonication, centrifuge the lysate (16,000g, 4 °C, 15 min in a refrigerated tabletop microcentrifuge) to pellet the debris. Transfer the supernatant to a fresh tube, carefully avoiding floating debris. Repeat this step if debris is carried over, and then pool the supernatants from the four tubes. Quantify the total protein concentration.
3. Save two aliquots (50 ml each) of supernatant as ‘input sample’, freeze in dry ice and store at –80 °C.
4. For each Ab concentration to be used, take three technical repeats. For example, if three different Ab concentrations are to be evaluated, take 3 × 3 (9) tubes. Take supernatant equivalent to 2 mg of total protein in each tube and dilute to 500 ml using HEPES lysis buffer containing protease inhibitor cocktail and add 50 ml of prewashed salmon sperm DNA/protein-A agarose beads (Upstate; see REAGENT SETUP). Incubate for 1 h at 4 °C on the Nutator shaker (25 rockings per minute).
5. Centrifuge at 400g to pellet the preclearing agarose beads. Carefully transfer supernatant to new tubes. Add different amount of Ab to the triplicates sets (e.g., X ml in three (of nine) tubes, Y ml in three (of nine) tubes and Z ml in three (of nine) tubes) and incubate overnight on a Nutator (25 rockings per minute) at 4 °C.
6. Proceed with Steps 21–33 of the PROCEDURE to wash and purify the DNA.
7. Perform Steps 34–49 of the PROCEDURE to determine which Ab concentration will produce the best binding ratio of the DNA-binding protein to the promoter DNA. See Figure 6 for a representative result.

BOX 4

Checking Sonication Efficiency

Shearing of the chromatin is a critical step in successful implementation of chromatin immunoprecipitation (ChIP). We recommend that the chromatin be sheared to a size range of 300–1,000 bp with most of the fragments around 500 bp. If the chromatin is not sheared to this extent, it will decrease the resolution of the ChIP assay. For example, if primers are

designed against a binding site and a nonbinding site that are separated by 1,200 bp, inefficient shearing will result in detection of binding at both loci. To determine how efficient the sonication step is, follow the procedure described below.

1. Follow Steps 1–13 of the PROCEDURE to harvest and crosslink the worms.
2. Split the worm suspension into multiple 750 ml aliquots.
3. Proceed with sonication using power output ~30 W and vary the number of sonication pulses (4–8). After each pulse, collect 50 ml worm lysate from the tubes for testing on gel. Also try a few different pulse times (6–10 s), keeping the number of pulses constant. Again, collect 50 ml sample of worm lysate for testing.
4. Dilute the 50 ml worm lysate with 200 ml of proteinase K buffer, add 5 ml proteinase K and incubate at 55 °C for 4 h.
5. Reverse crosslink overnight at 65 °C.
6. Follow Steps 28–33 of the PROCEDURE to purify the DNA.
7. Run 10 ml of DNA from each optimization condition on a 1% agarose gel containing 0.05 mg ml⁻¹ ethidium bromide. Visualize on a UV transilluminator. The best condition for sonication will be the one where the DNA forms a smear, ranging from 300 to 1,000 bp, with most of the fragments averaging ≤ 500 bp.

LB medium—10 g Tryptone, 5 g yeast extract and 10 g NaCl in 1 l of deionized water. Autoclave for 20 min.

NGM agar—3 g NaCl, 17 g agar and 2.5 g bactopeptone in 975 ml of deionized water. Autoclave for 20 min. Cool to 60 °C and add 1 ml 1 M CaCl₂, 1 ml 1 M MgSO₄, 25 ml PPB and 0.5 ml cholesterol (10 mg ml⁻¹). Pour 10 ml per 45 mm plate. Let medium solidify and store at RT.

Proteinase K buffer—50 mM Tris-Cl, pH 8.0, 25 mM EDTA and pH 8.0, 1.25% (wt/vol) SDS.

PPB (1 M)—98 g anhydrous KH₂PO₄ and 48 g anhydrous K₂HPO₄ in 900 ml deionized water. Check the pH; it should be 6.0. Minor adjustments may be made with 1 M solutions of KH₂PO₄ or K₂HPO₄. Autoclave for 20 min. The solution is stored at RT.

Preparation of HB101 bacteria—Inoculate a single colony of freshly streaked *Escherichia coli* HB101 bacteria in 1 l of LB medium. Grow 16 h at 37 °C shaking at 200 r.p.m. Harvest the bacteria by centrifugation at 3,000g for 15 min (at 4 °C) in a Beckman Coulter Avanti J-25 or similar centrifuge. Resuspend bacterial pellet in 10 ml of 1× M9. The concentrated bacteria may be stored for 5–7 d at 4 °C.

Preparation of OP₅₀ bacteria and seeding NGM agar plates—Inoculate a single colony of OP₅₀ into 100 ml of LB medium and grow 16 h at 37 °C shaking at 200 r.p.m. Drop 250 ml on the NGM plates and leave at RT to dry. Store at RT and use within 1 week.

Preparation of Salmon sperm DNA/protein A agarose beads—The beads come as a 50% wt/vol solution in 70% (vol/vol) ethanol. Take 100 ml of this solution (containing essentially 50 ml of beads) in a microcentrifuge tube and wash by adding 1 ml of chilled HLB. Mix thoroughly by inverting 6–8 times, centrifuge at 400g for 1 min (at RT) and quickly remove the supernatant without disturbing the beads. Repeat the washing two more times each followed

by centrifugation and removal of supernatant. Resuspend the beads in 50 ml of HLB containing protease inhibitor cocktail.

Primer design—Primers are designed encompassing the predicted TF binding site if the latter information is available. As a control, we use primers designed to amplify the 3' untranslated region (3'-UTR) of the target gene. We regularly use Primer3 (<http://frodo.wi.mit.edu/>) or Primer Express (Applied Biosystems) for designing real-time PCR primer pairs that are typically 20–25 mer with a T_m range of 58–63 °C (maximum T_m difference 2 °C) and amplify a product of 50–150 bp. The melting temperature is calculated based on the Nearest-Neighbor thermodynamic parameters²⁰. The GC content is set between 40 and 55%; concentration of monovalent cation at 50 mM and concentration of annealing oligos is set at 50 nM; the maximum 3' complementarity is set at 3.00.

S-basal—5.8 g NaCl, 50 ml PPB and deionized water to 1 l. Autoclave for 20 min. Cool to RT and add 1 ml of cholesterol (10 mg ml⁻¹). The solution is stored at RT.

WB1—50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS and 1 mM PMSF.

WB2—50 mM HEPES-KOH, pH 7.5, 1 M NaCl, 1 mM EDTA, pH 8.0, 0.1% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) SDS and 1 mM PMSF.

WB3—50 mM Tris-Cl, pH 8.0, 0.25 mM LiCl, 1 mM EDTA, 0.5% (vol/vol) NP-40 and 0.5% (wt/vol) sodium deoxycholate.

Equipment Setup

Real-time PCR machine

The real-time PCR machine used in this study is an Applied Biosystems-manufactured ABI Prism 7000 Sequence Detection System (cat. no. 4330087). It is a real-time PCR system that detects and quantitates nucleic acid sequences. In real-time PCR, cycle-by-cycle detection of accumulated PCR product is made possible by combining thermal cycling, fluorescence detection and application-specific software in a single instrument. The instrument has a Peltier-based cycling system, uses a tungsten halogen lamp as an excitation source, four-position filter wheel and CCD camera for detection. Currently, Applied Biosystems has discontinued this instrument and replaced it with the 7500 Real-Time PCR system. Other similar machines may be used. In that case, follow the manufacturer's instructions to perform real-time PCR and compute the results.

Sonicator

The sonicator used for the experiments is the Misonix 3000, which offers powerful ultrasonic processing of samples. The instrument is microprocessor controlled and can be set to 'pulse off' automatically after a specified time (10 s). Another advantage of this system is autotuning. The sonication efficiency has been optimized using this instrument. When using other instruments, the sonication has to be optimized as outlined in Box 4.

Procedure

Worm cultures

1| Set up worm cultures as required. If mixed-stage cultures are required, follow option A. Mixed cultures are useful when it is not known when the protein-of-interest against which the Ab is raised expresses or is most likely to bind DNA. It may also be useful when identifying

all the DNA targets of a protein-of-interest is the main focus of the experiments. If stage-selected cultures are required, follow option B. Staged culture may be used when sufficient information is available regarding the expression pattern of the protein-of-interest. Also, this type of culture may reveal when the protein-of-interest is most active in binding to its target sequence. Using mixed-stage culture for stage-specific proteins-of-interest may lead to decreased detection of binding. Beginning with worms growing on a 60-mm plate, it will take 10–12 d to obtain enough worms to make extracts for the ChIP procedure. However, actively maintaining unstarved cultures on 100-mm plates or in liquid will shorten the time frame (3–5 d) for obtaining enough worms for the ChIP.

A. Mixed-stage worm culture • **TIMING** 10–12 d (3–5 d if starting with 100-mm plates)

- i.** From an unstarved 60-mm NGM plate, cut a chunk of agar and place it on the 100-mm plate with the side harboring the worms in contact with bacterial food. The chunk contains 10–15 gravid adults along with other stages. Alternatively, pick 10 gravid adults from the 60-mm plate and place in the seeded 100-mm plate. Grow until the worm population consists mostly of gravid adult worms (each plate with 4,000–6,000 gravid adults). Monitor the plates every day and make sure the plates do not get depleted of bacteria.
- ii.** Wash the worms into a 15-ml Falcon tube with $1\times$ M9. Centrifuge at 650g for 2–3 min in a Beckman Coulter Allegra 6KR or similar at RT to pellet the worms.
- iii.** Wash the pellet two times more as in Step 1A(ii) to remove excess bacteria.
- iv.** After the final wash, resuspend the worms in 3.5 ml of $1\times$ M9. Add 0.5 ml of 5 N KOH and 1 ml of 6.15% commercial Clorox bleach. Incubate for 3–7 min at RT and vortex vigorously for 30 s every minute. Proceed to the next step as soon as the worms dissolve and the eggs are released (monitor the Falcon tube under a dissecting microscope).
- v.** Pellet the eggs by centrifugation at 650g for 3 min. Discard the supernatant.
- vi.** Wash the pellet four times with 10 ml of $1\times$ M9. Centrifuge at 650g for 3 min between washes to pellet the eggs.
- vii.** In the same tube, allow the eggs to hatch overnight at 20 °C with shaking on a Nutator shaker (25 rocking actions per minute). As the worms cannot continue the life cycle in the absence of food, all the eggs will hatch and arrest at the L1 larval stage.
- viii.** The next morning, collect the hatched L1s by centrifugation at 650g for 2 min. Resuspend in 1 ml of $1\times$ M9.
- ix.** Check the viability of the L1s by transferring a few drops on to an NGM plate seeded with OP50 bacteria. Once the $1\times$ M9 is absorbed by the NGM plate, the L1 larva should start crawling in the bacteria and should grow into L2 larva within 15–24 h. Add 0.2–0.5 ml of L1s to 100 ml S-basal in a 250-ml conical flask containing 100 μ l nystatin (50 mg l^{-1}), 100 μ l streptomycin (50 mg l^{-1}) along with 1 ml of concentrated HB101 (see REAGENT SETUP).
- x.** Grow the worm culture at 20 °C with shaking (200 r.p.m.) for 2–3 d. Subculture by transferring 2 ml of the culture to fresh medium on the 4th day. Feed the worm culture every day with 1 ml of concentrated HB101. Monitor the growth every day to ensure that there is no overcrowding; transfer a drop onto a slide and estimate the number of worms in each stage.

When there are 5–8 gravid adults and 10–15 L4-young adults along with other stages (L1–L3) in that drop (~ 10 µl), the culture should be ready to harvest.

▲ **CRITICAL STEP** This culture should adequately represent all stages of worm development. However, overcrowding or shortage of food may force the worms to form dauers or arrest as L1s. This would result in skewing the balance between different developmental stages in the worm culture. Also, the growth rate may vary considerably among various mutant strains. Temperature-sensitive mutants may need to be maintained at lower temperatures (15 °C).

B. Staged worm culture ● **TIMING** 4–7 d (3–4 d if starting with 100-mm plates)

- i. Refer to Step 1A(i) on how to start the growth. Grow mixed-stage worm culture on at least three 90 mm NGM agar plates seeded with *E. coli* OP50 food until the worm population consists mostly of gravid adult worms (each plate with 4,000–6,000 gravid adults).
- ii. Wash the worms from all five plates into a 15-ml Falcon tube with 1× M9. Centrifuge at 650g for 2–3 min at RT in a Beckman Coulter Allegra 6KR or similar centrifuge to pellet the worms.
- iii. Follow Steps 1A(iii–viii) to synchronize the worms at L1 larval stage.
- iv. Add the entire 1 ml of L1s to 100 ml S-basal in a 250-ml conical flask containing 100 µl nystatin (50 mg l⁻¹) and 100 µl streptomycin (50 mg l⁻¹) along with 1 ml of concentrated HB101.
- v. Grow the worm culture at 20 °C with shaking (200 r.p.m.). Monitor the culture by aliquoting 20 µl onto a glass slide and visualizing under a dissecting microscope. Feed the worm culture every day with 1 ml of concentrated HB101. Overpopulated culture can be avoided by splitting the culture into 50 ml aliquots and adding another 50 ml of S-basal containing 100 µl nystatin (50 mg l⁻¹) and 100 µl streptomycin (50 mg l⁻¹) along with 1 ml of concentrated HB101.

Chemical crosslinking of worms ● **TIMING** 1–2 h

2| Harvest worms by centrifugation at 650g for 2 min in 50-ml tubes using a Beckman Coulter Allegra 6KR centrifuge and discard supernatant. For optimal ChIP results, ~0.4 ml of packed worms (measuring ~350 mg) should be obtained. From a mixed culture, around 50,000 or more gravid adults and 80,000 or more L4/young adults in addition to other stages should be collected.

3| Wash the worm pellet three times with 30 ml 1× PBS to remove HB101 bacteria.

4| Wash the worm pellet once with 3 ml of CB, quickly spin down for 30 s at 650g and then resuspend in 3 ml of fresh CB.

5| Use a 7-ml glass Dounce homogenizer to lyse the worms partially and allow the fixative to penetrate the worms. Use eight strokes with a 1/3 turn after each stroke.

6| Transfer the worm suspension to a 15-ml tube. Wash the homogenizer with an additional 1 ml of CB and add to the suspension in the 15-ml tube. Incubate at RT on a Nutator shaker (25 rocking actions per minute) for 20 min.

▲ **CRITICAL STEP** The amount of time the worms are incubated in the formaldehyde-containing CB buffer in Step 6 is critical in determining the signal-to-noise ratio (Fig. 3). We determined that an incubation of 20 min in CB is optimal for the Ab that we used. We recommend that the optimum fixation time be determined for each Ab (see EXPERIMENTAL DESIGN and Box 1).

7| Add 200 μ l of 2.5 M Glycine to the same tube and incubate on the Nutator shaker for an additional 15–20 min. The Glycine quenches the formaldehyde and stops the crosslinking reaction.

8| Pellet worms by centrifugation at 650g for 2 min.

9| Wash the pellet four times with 15 ml of 1 $\times$ PBS containing protease inhibitor cocktail and snap freeze in liquid nitrogen.

■ **PAUSE POINT** The crosslinked worms may be stored indefinitely at -80°C .

Sonication of worms ● **TIMING** 1-2 h

10| Thaw worm pellet from Step 9 on ice and add 2 ml of freshly prepared HLB containing protease inhibitor cocktail.

11| Pellet worms by centrifugation at 650g for 2 min (4°C), remove the supernatant and resuspend the pellet in 2 ml of HLB.

12| Incubate on ice for 10 min.

13| Split the worm suspension into 750 μ l aliquots (for a total of four prechilled microcentrifuge tubes per sample) and sonicate each tube eight times using a Misonix sonicator 3000 with output setting 8, power output of 30 W for 10 s (see EQUIPMENT SETUP).

▲ **CRITICAL STEP** In order to prevent overheating, briefly dip the tubes in a dry ice and ethanol bath just before sonication. Always incubate samples on ice for at least 2 min between each sonication. Care should be taken to prevent frothing, as this decreases the efficiency of sonication dramatically and results in loss of sample. The volume of the lysate may be maintained at 750 μ l by adding HLB containing protease inhibitor cocktail.

ChIP ● **TIMING** 20 h, performed overnight

14| Centrifuge the lysate (16,000g, 4°C , 15 min in a refrigerated tabletop microcentrifuge) to pellet the debris. Transfer the supernatant to a fresh tube carefully, avoiding floating debris. Repeat this step if debris is carried over. For each sample, pool the supernatants from the four tubes.

15| Quantify the amount of protein in the supernatant using any standard protein estimation assay.

16| Each ChIP experiment should be done using three technical repeats. For each technical repeat, take a volume of supernatant having 2 mg of total protein and dilute to 500 μ l using HLB containing protease inhibitor cocktail.

▲ **CRITICAL STEP** We recommend that the optimum amount of lysate to be used for each ChIP should be determined empirically for each Ab being used (Box 2).

17| Save three aliquots (50 μ l each) of supernatant from each sample as 'input sample'. 'Input' samples (also known as whole cell extract or WCE) represent the total genomic DNA and one

of the three is processed later along with the ChIP-ed samples (Step 26 onward). Freeze the 'input' samples on dry ice and store at -80°C . This is also a good time to check the efficiency of sonication; use one of the three input aliquots and follow the miniprotocol for 'checking shearing efficiency' (Box 4). The remaining input sample is retained frozen and serves as a standby.

18| Add 50 μl of prewashed salmon sperm DNA/protein-A agarose beads (Upstate; see REAGENT SETUP) to the diluted supernatant from Step 16. Incubate for 1 h at 4°C on the Nutator shaker (25 rockings per minute) to remove proteins that naturally stick to the beads and result in high background noise (this is the preclearing step).

19| Pellet the beads by centrifugation at 400g for 3 min. Transfer the supernatant to fresh tubes carefully without disturbing the pellet of beads. Discard the beads.

20| Add Ab or preimmune serum to the supernatant and incubate overnight at 4°C on a Nutator shaker (25 rockings per minute).

▲ CRITICAL STEP The amount of Ab should be determined empirically (Box 3). We found that 15 μl of α -DAF-16 Ab was suboptimal, while 25 and 35 μl Ab produced the best results (Fig. 6).

21| In the morning, add 50 ml of prewashed salmon sperm DNA/protein-A agarose beads to the tubes from Step 20 and incubate at 4°C on a Nutator shaker (25 rockings per minute) for 1 h.

22| Wash the beads-antibody-TF-DNA complex two times using WB1. Each time, add the WB1 directly into the tubes, place the tubes on a Nutator shaker (25 rocking actions per minute) for 5 min at 4°C , centrifuge at 400g for 2 min to collect the beads and then carefully discard the supernatant.

23| Wash the beads two times using WB2, as described in Step 22.

24| Wash the beads once with WB3, as described in Step 22.

25| Wash the beads three times using $1\times\text{TE}$, as described in Step 22. After the last wash, discard most of the supernatant and remove any excess buffer remaining at the bottom of the microcentrifuge tube using pipette tips with microcapillary endings.

Reverse crosslinking and recovery of DNA • **TIMING** 20 h, includes overnight step

26| Resuspend the beads in 250 μl proteinase K buffer and add 2 μl proteinase K (20 mg ml^{-1}). Incubate at 45°C for 2 h. At the same time, dilute the 50 μl input sample in 250 μl proteinase K buffer and incubate with 5 μl proteinase K (20 mg ml^{-1}) at 55°C for 4 h. As the amount of protein in the input samples will be significantly more than that of the ChIP samples, more proteinase K and higher temperature is used for digestion. Proteinase K has higher activity at elevated temperatures.

27| Incubate overnight at 65°C to reverse crosslink all samples.

28| Add 250 μl of phenol–chloroform–isoamylalcohol to the tubes and vortex for 10 s. Centrifuge at 14,000g for 10 min at RT.

29| Transfer the upper aqueous phase of each sample carefully to a clean tube. (Optional step: The aqueous phase may be re-extracted with 250 μl of chloroform-isoamyl alcohol, by repeating Steps 28 and 29).

30| Add 1 ml of ethanol and 1 μ l of 1 mg ml⁻¹ glycogen to each tube and mix. Incubate at -80 °C for 15–20 min.

31| Centrifuge at 14,000g for 10 min to pellet the DNA. Remove the supernatant.

32| Add 500 μ l of 70% ethanol to the tubes and centrifuge at 14,000g for 10 min. Remove the supernatant carefully and briefly air-dry the pellet.

33| Resuspend the input DNA in 200 μ l and ChIP DNA in 40 μ l of 10 mM Tris-HCl, pH 8.5. The samples are now ready to be analyzed through real-time PCR.

■ **PAUSE POINT** The DNA can be stored for up to 1 month at -20 °C.

Real time PCR • **TIMING** 4 h

34| Design primers in a way that the amplicon is 50–150 bp long (see REAGENT SETUP). Double-check whether the targeted regions have extensive secondary structures as this will significantly decrease the efficiency of the PCR (see below). For each promoter that will be assessed by ChIP, design a pair of primers for the predicted binding site as well as for the 3'-UTR and/or coding sequence that will serve as a negative control.

35| Mix each primer pair (forward and reverse of experimental or control) in the same tube and prepare a stock concentration of 25 μ M each with 10 mM Tris-Cl pH 8.5. Dilute the input DNA 10 $\times$, 20 $\times$, 40 $\times$ and 80 $\times$ with 10 mM Tris-Cl pH 8.5.

36| Set up real-time PCRs in triplicate with the diluted input DNA, as shown in the following table.

Component	Amount per reaction (μ l)	Final
Primer mix (stock 25 μ M each)	1	1 μ M each
Diluted 'Input' DNA	2	10 $\times$ to 80 $\times$ depending on the dilution used
Nuclease-free water	10.5	
SYBR Green mix	12.5	

This SYBR Green mix contains ROX as a reference dye, which minimizes fluorescence intensity variations due to differences in sample volume.

37| Mix the samples by vortexing for 2 s and briefly centrifuge.

38| Perform real-time PCR using cycling conditions as shown in the following table. Make sure that the program calculates the dissociation curve after the cycling is complete.

	Stage 1	Stage 2	Stage 3	
Temperature	50.0 °C	95.0 °C	95.0 °C	60.0 °C
Time	2.00 min	10.00 min	15 s	1.00 min
Cycles	1	1	40	

39| After the cycling is complete, first visualize the Dissociation plot. The Dissociation plot for a primer should produce a single sharp peak. More than one peak represents additionally amplified products and the amplification data should not be used.

40| Next, visualize the Amplification plot in a logarithmic scale. The x -axis will display the number of cycles while the y -axis shows the DR_n (R_n or 'normalized reporter' is the fluorescence emission intensity of the reporter dye, i.e., SYBR green, divided by that of the passive reference dye, i.e., ROX; the DR_n is calculated as R_n^+ minus R_n^- , where R_n^+ is the R_n value of a reaction containing all components while R_n^- is the R_n value of an unreacted sample. R_n^- can be calculated from early cycles of a qPCR or samples lacking template).

41| The next step is to adjust the baseline. The default baseline is set from cycles 3 to 15. To find out whether the baseline needs to be adjusted, it is important to find out which reaction emerges before the default baseline. For this, change the plot to linear view. For reactions that emerge after 15 cycles, no changes have to be made. For highly abundant targets, the end point of the baseline (15 is the default) needs to be adjusted. Usually, the baseline is adjusted to one or two cycles before the earliest amplification.

▲ CRITICAL STEP The adjustment of the baseline is critical in achieving appropriate qPCR results and might need to be manually set depending on the abundance of the template.

42| After the baseline has been adjusted, the threshold value has to be set. Threshold is defined as the average s.d. of R_n for the early PCR cycles, multiplied by an adjustable factor. The software sets a default threshold value at 10 cycles above the baseline. However, this may not always be appropriate as it should be set in the linear phase of exponential amplification that maximizes the precision of the replicates.

▲ CRITICAL STEP Setting of the threshold is critical in getting proper results with qPCR. Setting the threshold higher or lower in the amplification plot can produce variability in the replicates. Set the threshold manually to the appropriate position on the amplification curve if the default parameter (10 cycles above the baseline value) is not optimal.

43| The threshold cycle (C_t ; the fractional cycle number at which the fluorescence passes the threshold) value is determined in the experimental report. Export the data into Microsoft Excel as a Comma Separated Value (CSV) file for analysis.

44| The C_t values of the triplicates should show minimal variability, indicating proper handling of samples and optimal PCR cycling (Fig. 7). Average the C_t values of the triplicates. Plot the $\log(\text{concentration of template})$ against the C_t for each dilution to generate a standard curve. This curve can then be used to calculate the efficiency for each primer pair [$10^{(-1/\text{slope})}$]. The C_t values of the diluted 'input samples' will be used later to normalize the experimental samples. At that step, the dilution that produces a C_t value similar to the experimental sample will be used.

▲ CRITICAL STEP The efficiency of the primer pair should ideally be 2.0. Primer efficiencies between 1.8 and 2.2 are common and acceptable. However, it is critical that each primer pair yields only one amplicon, which can be determined by analyzing the amplicon Dissociation plot, which should yield a single peak. The PCR may then be loaded on a 1% agarose gel to visualize whether a single amplicon is being amplified. Also, C_t values 435 are disregarded and more concentrated DNA should be used in a reanalysis, if necessary. The input sample should ideally be in the 20–30 C_t range and should be adjusted (i.e., diluted) to the C_t value of the samples so that the DC_t between input and sample is minimal to avoid large errors.

45| After this analysis, dilute the sample DNA 4× using 10 mM Tris-Cl pH 8.5.

46| Set up the real-time PCRs for each sample as detailed in the table below. Mix by vortexing for 2 s and briefly centrifuge.

▲ CRITICAL STEP In cases where the protein–DNA interaction takes place in a few cells (although the protein is widely expressed), more sample DNA will be required. Perform the qPCR initially using the settings suggested above and follow the amplification plot. If the C_t value is 432, proceed with more sample DNA for the PCR.

Component	Amount per reaction (μl)	Final
Primer mix (stock 25 μM each)	1	1 μM each
Sample DNA (diluted)	4	1×
Nuclease-free water	7.5	
SYBR Green mix	12.5	

47| Perform real-time PCR analysis as described in Steps 39–44. Export the results into Microsoft Excel for analysis. The C_t values for the technical repeats should show minimal variation (ideally be within half a C_t value of each other; Fig. 7).

48| Calculate the DC_t value (normalized to the input samples) for each sample: $DC_t [C_t (\text{sample}) - C_t (\text{input})]$. Next, calculate the DDC_t ($DC_t (\text{experimental sample}) - DC_t (\text{negative control})$).

49| Calculate the fold difference between experimental sample and negative control using 2^{-DDC_t} . As indicated earlier, the negative control should ideally be a deletion mutant in the protein-of-interest. If no deletion mutant is available, enrichment of the ChIP target can be expressed as a fold difference between specific Ab-immunoprecipitated samples and those immunoprecipitated with prebleed serum or a no-Ab control.

? TROUBLESHOOTING

● TIMING

Timing information is summarized in Figure 2.

Step 1A, mixed-stage worm culture: 10–12 d

Step 1B, staged worm culture: 4–7 d

Steps 2–9, chemical crosslinking of worms: 1–2 h

Steps 10–13, sonication of worms: 1–2 h

Steps 14–25, ChIP: 20 h, performed overnight

Steps 26–33, reverse crosslinking and recovery of DNA: 20 h, includes overnight step

Steps 34–49, real-time PCR: 4 h

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

Anticipated Results

The success of the ChIP experiment may be determined from several perspectives. First, the three technical repeats are expected to register similar results (Fig. 7). This would indicate that the handling of the samples was appropriate and uniform. Second, a TF should bind specifically to the promoter region and not to the 3'-UTR (Figs. 3, 4 and 6). If TF binding to the 3'-UTR is high, it is possible that either the DNA has not been properly sheared by sonication, resulting in high background levels (see Table 2), or the TF binds there for some other reason. In these cases, primers designed to amplify random regions in the nearby locus could be used as a negative control. Third, there should be a significant difference between binding in the experimental sample and the deletion mutant.

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Figure 1.
Diagrammatic representation of the chromatin immunoprecipitation (ChIP) procedure.

Figure 2.
Timeline for chromatin immunoprecipitation (ChIP) protocol using whole *Caenorhabditis elegans* extract.

Figure 3.

Optimization of the crosslinking time. A well-described regulatory interaction of DAF-16 with the *sod-3* promoter^{15,21–23} was used. A mixed-stage culture of *daf-16(mu86)* null mutants²⁴ was used as a negative control to probe DAF-16-target DNA interactions in a *daf-2(e1370)* background in which DAF-16 is nuclear^{25–27}. *Caenorhabditis elegans daf-2(e1370)* and *daf-16(mu86)* were grown in liquid culture and harvested. Two milligrams of the washed pellets was resuspended in HEPES lysis buffer (see text) and worms lysed using a glass homogenizer. Immediately after the homogenization, the worm suspension was incubated at room temperature on a Nutator for 15, 20 or 30 min. The binding of DAF-16 to the *sod-3* promoter or the *sod-3* 3'-untranslated region (3'-UTR) was determined by chromatin immunoprecipitation using 25 ml of anti-DAF-16 Ab followed by detection using real-time PCR. The binding of DAF-16 to the *sod-3* is expressed as a ratio of binding in *daf-2(e1370)* versus *daf-16(mu86)*. The 20 min fixation time was determined to be optimal.

Figure 4.

Optimization of the input amount needed for chromatin immunoprecipitation (ChIP). An aliquot of 10 mg, 2 mg, 1 mg or 0.4 mg of crosslinked and sonicated worm lysate (input) was immunoprecipitated with 25 ml of anti-DAF-16 Ab. Crosslinking time was 20 min. The binding of DAF-16 to the *sod-3* promoter or the *sod-3* 3'-UTR was used to evaluate the efficiency of ChIP. Optimal results in this experiment were seen with 2 mg of input material.

Figure 5.

Western blot to determine the quality of the polyclonal Ab against DAF-16. For this analysis, 25 mg of the total protein was resolved on a 10% SDS-PAGE followed by transferring into a nitrocellulose membrane (100 V, 1 h, 4 °C). The membrane was blocked with 5% blotting grade blocker nonfat dry milk and probed with 1:1,000 dilution of the Ab (in 5% milk) for 2 h. Following 3× washes (5 min each) with 1× TBST, the membrane was incubated in 1:5,000 diluted anti-rabbit secondary Ab (Abcam) for 1 h. The membrane was washed five times with 1× TBST (5 min each) and SuperSignal West Pico detection system (Pierce Biotechnology) was used to visualize the signal (1 min exposure time). The ~70 kDa band present in both the

strains represents a nonspecific protein detected by the Ab. This highlights the importance of using a null mutant as a negative control, if possible.

Figure 6.

Optimization of the Ab amount for efficient chromatin immunoprecipitation (ChIP). A 100 ml aliquot of the sonicated worm lysate (input) corresponding to 2 mg of total protein was used for ChIP using 15, 25 or 35 ml of the anti-DAF-16 Ab. Crosslinking time was 20 min. Real-time PCR and primers specific to the *sod-3* promoter or the *sod-3* 3'-UTR were used to determine DAF-16 binding to the immunoprecipitated DNA. The data show that ChIP with 25 or 35 ml of anti-DAF-16 Ab produced optimal results. Error bars represent the standard errors between the triplicates in each experiment (see Step 16).

Stains	Technical repeat	C_t	Average C_t	ΔC_t	$\Delta\Delta C_t$	$2^{(-\Delta\Delta C_t)}$	Average $2^{(-\Delta\Delta C_t)}$	s.d.
<i>daf-2(e1370)</i>	1	22.97	23.13	-0.36	-1.86	3.63	2.83	0.77
	2	23.06		-0.27	-1.47	2.77		
	3	23.36		0.03	-1.06	2.08		
<i>daf-16(mu86)</i>	1	24.85	24.61	1.5				
	2	24.55		1.2				
	3	24.44		1.09				

Figure 7.

Example of raw data showing that the C_t values generated in triplicate technical repeats are similar, indicating that the ChIP worked uniformly and handling was optimal.

Table 1

Overview of steps needing optimization.

Procedure	Optimization steps
Worm culture	No optimization is required; however, the time needed to obtain the desired population may vary
Chemical crosslinking	The time of incubation of the worms in the crosslinking buffer (Step 6) needs to be optimized
Sonication of worms	The extent of sonication (Step 13) will require optimization depending on the sonicator used
Chromatin immunoprecipitation	The amount of lysate (input sample) used for ChIP will need optimization (Step 16). Also, the amount of Ab used for immunoprecipitation (Step 20) will have to be optimized depending on the Ab quality
Reverse crosslinking and recovery of DNA	No optimization is required
Real-time PCR	Needs optimization at: Step 39 (dissociation plot will indicate whether new primers need to be designed); Step 41 (baseline correction depending on the sample abundance) and Step 42 (setting the threshold value to maximize the precision of the replicates)

Table 2

Troubleshooting table.

Problem	Possible reason	Possible solution
Overall signal in the real-time PCR is low	The DNA may be degraded	Always autoclave or filter-sterilize all solutions and use proper molecular biology practices
	The TF is expressed at low abundance	Standardize the amount of starting material
	The DNA did not crosslink	Standardize the optimal crosslinking time
	The immunocomplex was not pulled down efficiently	Check the Ab specificity by western blot as well as immunoprecipitation
	The chosen target is not a true target of the DNA-binding protein	Known target of the DNA-binding protein may be used to optimize the procedure
Equal binding to promoter and UTR	The DNA is not sheared properly	Run the DNA on a gel following sonication to see whether shearing has occurred (see Box 4). The distance between the promoter and UTR amplicon should ideally be 41 kb
	Lack of proper crosslinking. For example in Figure 2, 15 min incubation in buffer CB results in insignificant difference of binding at the promoter vs. 3' UTR	Optimize the crosslinking time as described in Box 1
	The DNA-binding protein may actually bind to the chosen control region	Multiple other control regions should be checked
Similar binding in sample and the negative control	Nonspecific binding of the DNA to the agarose beads	Make sure that the beads used are coated with salmon sperm DNA
	Inefficient immunoprecipitation	Check the quality and efficiency of the Ab by western blotting and/or immunoprecipitation
Input dilutions do not show exponential curve	Primers not optimal	Design optimal primers using Primer Express (Applied Biosystems). Optimal primers produce an amplicon between 50 and 150 bp and have a $T_m \sim 60^\circ\text{C}$ and GC content $\sim 50\%$ (see REAGENT SETUP)