Supplemental Information

Inventory of supplementary material

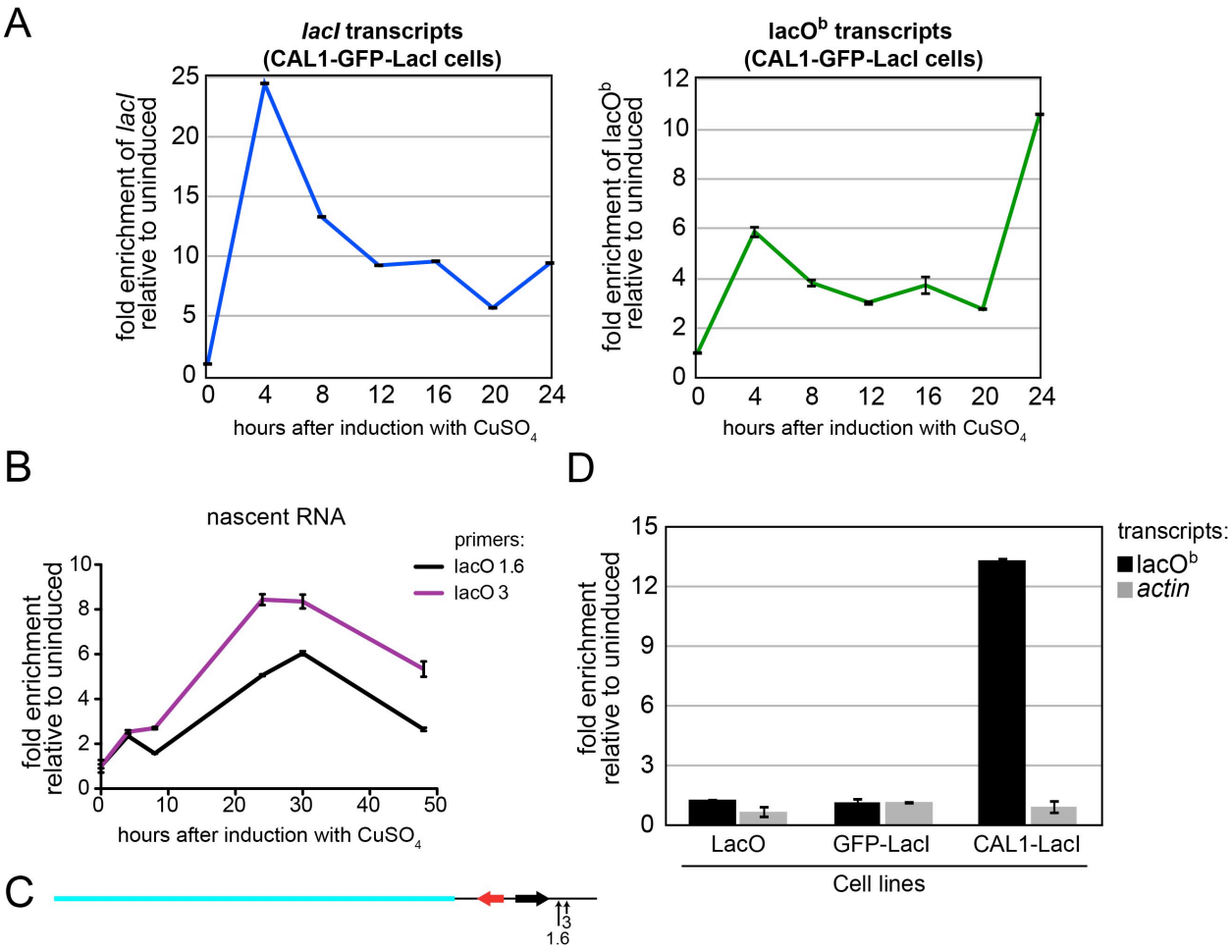

Figure S1, related to Figure 1. The transcription of lacO^b is continuous and correlates specifically with CENP-A assembly.

A) qRT-PCR analysis of CAL1-GFP-LacI transcripts (left, blue) and lacO^b (right, green) in induced CAL1-GFP-LacI cells at the indicated times. Error bars, SD of 3 technical replicates. B) qRT-PCR analysis of nascent lacO^b transcripts in a time course (0, 4, 8, 24, 30, 48h) after induction with $CuSO_4$ using two primer sets, 1.6 (black) and 3 (purple). Error bars, SD of 3 technical replicates. C) lacO diagram showing position of primer sets 1.6 and 3. D) Transcription of lacO^b (primer 1.6) and *actin* (RNAPII control gene), relative to uninduced, determined by qRT-PCR in the indicated cell lines after 24h induction with $CuSO_4$. Error bars show the 95% CI of 3 technical replicates.

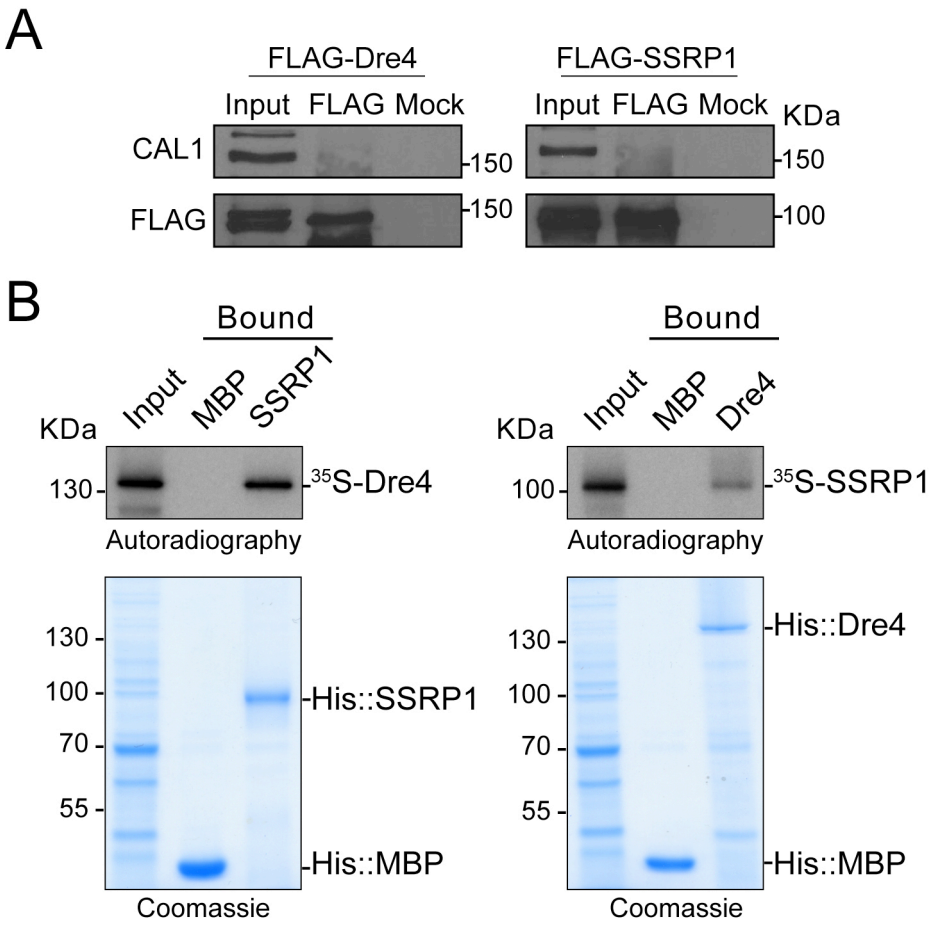

Figure S2, related to Figure 3. Interactions between FACT subunits and CAL1

A) Western blot with anti-CAL1 (top) or anti-FLAG (bottom) on input and IP performed with anti-FLAG coupled beads (FLAG) or beads alone (mock) from total nuclear extracts. Cell lines were stable S2 expressing either FLAG-Dre4 or FLAG-SSRP1. B) Direct interaction between SSRP1 and Dre4 was examined *in vitro* by incubating ^{35}S-methionine-labeled Dre4 (produced by IVTT) with recombinant His::SSRP1 (left panel) or ^{35}S-methionine-labeled SSRP1 with His::Dre4 (right panel) bound to Ni-NTA beads. His::MBP (MBP) on Ni-NTA beads was used as a negative control.

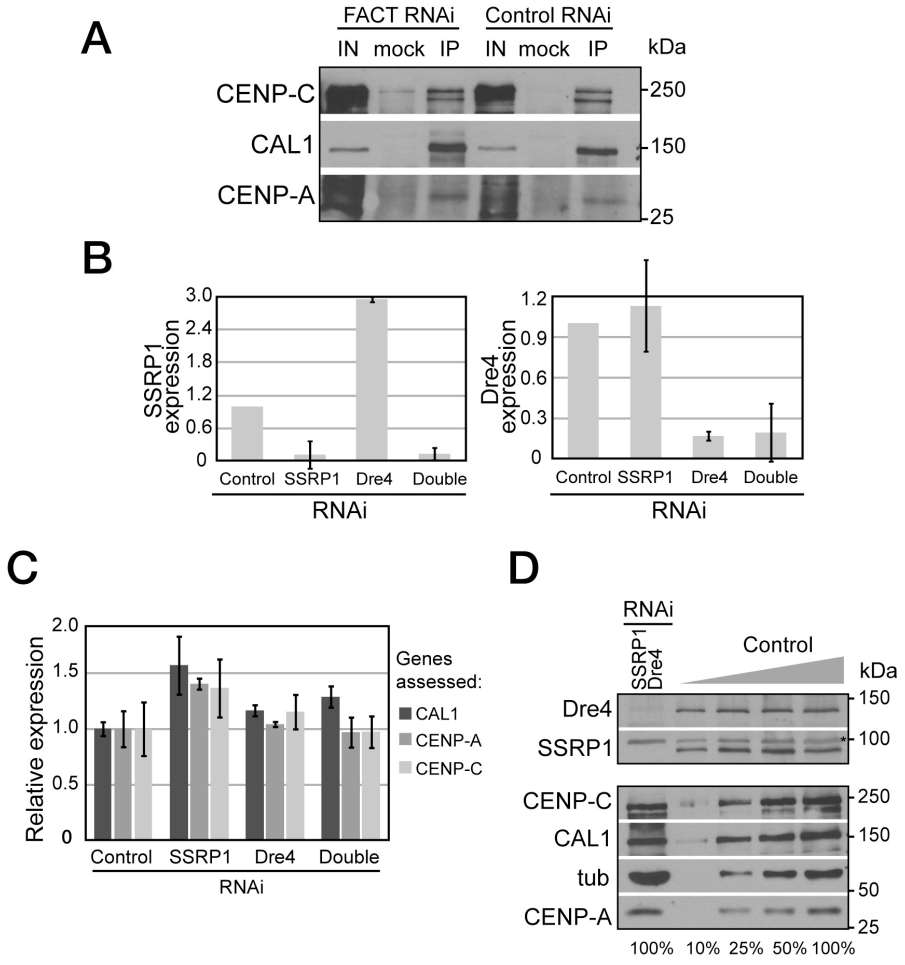

Figure S3, related to Figure 5. CAL1, CENP-A and CENP-C mRNA and protein levels, and their reciprocal association are unaffected by FACT RNAi

A) CAL1 IPs from cells in which FACT was depleted by RNAi. Western blot was performed with the indicated antibodies on total nuclear extracts (IN) and material immunoprecipitated with anti CAL1 antibodies (IP) eluted from beads from control cells and cells where Dre4 and SSRP1 were knocked-down by RNAi. The control lane (mock) shows IPs in which the antibody was omitted. B) qRT-PCR assessing the expression levels of SSRP1 (left) and Dre4 (right) after 6 days RNAi of each gene singly and together (double). Values are normalized to the Bw RNAi (control). Error bars show the 95% CI of 3 technical replicates. C) qRT-PCR with *cal1, cenp-a* and *Cenp-c* primers showing the relative expression of these genes in the indicated RNAi conditions. Error bars show the 95% CI of 3 technical replicates. D) Semi-quantitative Western blot with the indicated antibodies of total protein extracts from control RNAi and Dre4 and SSRP1 double RNAi in S2 cells. The percent of extract loaded on a 10% SDS-PAGE are shown at the bottom of the blot.* Indicates a non-specific band.

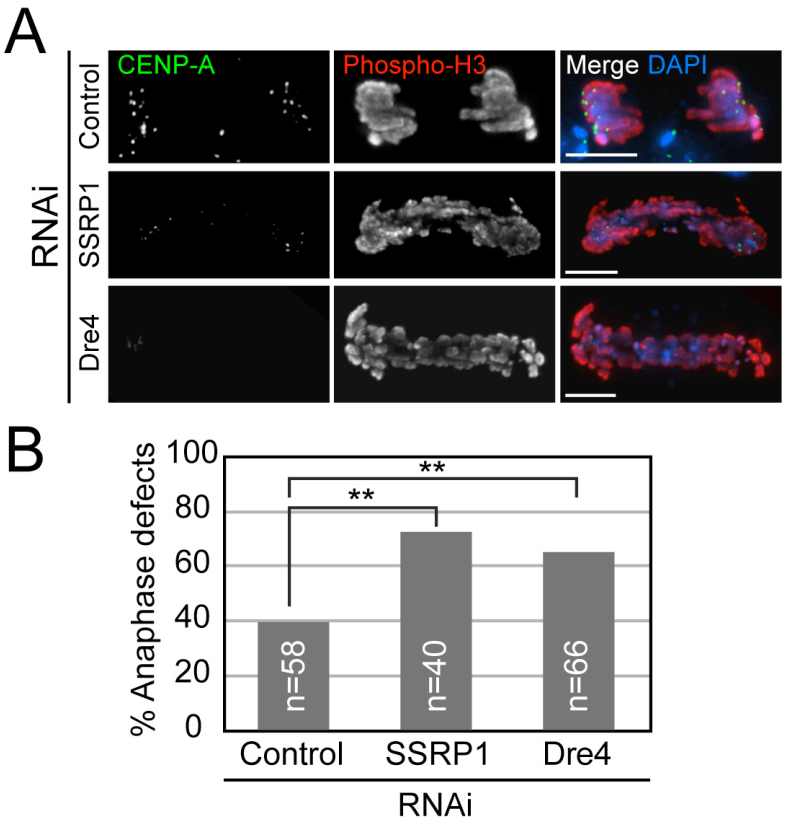

Figure S4, related to Figure 6. FACT RNAi leads to chromosome segregation defects in mitosis

A) Mitotic figures from S2 cells treated with control (Bw), SSRP1, and Dre4 RNAi. CENP-A is shown in green, phosphorylated H3-Ser10 is shown in red. Bar 5μm. B) Quantification of the frequency of chromosome missegregation in mitosis for the cells shown in A. ** $p<0.005$ (unpaired t-test). Note that SSRP1 was previously reported to affect spindle structure (Zeng et al., 2010).

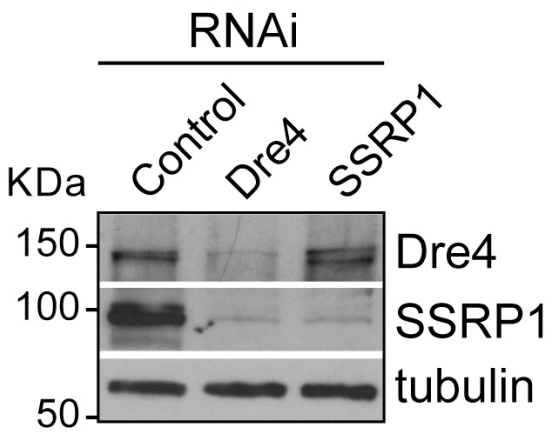

Figure S5, related to Figure 7. RNAi of Dre4 causes a reduction in SSRP1 protein levels

A) Western blot with the indicated antibodies of total protein extracts from S2 cells treated with control, Dre4, and SSRP1 RNAi. In Dre4 RNAi, SSRP1 protein levels decrease, suggesting that it becomes unstable in the absence of Dre4.

Supplementary Tables

Table S1, related to Figure 3. Summary of CAL1-FLAG mass spectrometry results

MASCOT score and % coverage are shown for the CAL1 interactors that were confirmed by IP (this work and (Chen et al., 2012)). Note that our chromatin-associated large-scale IP was less successful than the chromatin-free IP. n/a= not applicable.

	MASCOT		% Coverage	
	Chromatin Free	Chromatin Associated	Chromatin Free	Chromatin Associated
Dre4/Spt16	1099	390	47.4	21.7
SSRP1	n/a	136	n/a	16.3
CAL1 (bait)	221	n/a	19.7	n/a
CENP-A/CID	32	n/a	32	n/a
Modulo	177	n/a	33.4	n/a

Table S2, related to Figure 5. Effect of FACT RNAi on the expression of 8 genes associated with Dre4

qRT-PCR data using the indicated primer pairs (obtained from the DRSC database) and *Rp49* as a reference gene for 8 hand-picked RNAPII transcribed genes that are expressed at low, moderate, and high levels in S2 cells (as reported by modENCODE) and that are associated with Dre4/Spt16 by ChIP-chip (Kharchenko et al., 2011). Transcript levels for FACT RNAi (carried out by simultaneous RNAi for Dre4 and SSRP1) were normalized by the control. Shown are the means of three experiments ± SD. Most genes were unchanged or slightly more expressed upon FACT RNAi.

Gene	Expression level reported for S2 cells	Expression change in FACT RNAi relative to control	Primer set used (DRSC)
Rpl5	high	1.24 ± 0.06	PP16712
Ef2b	high	1.74 ± 0.02	PP35106
Pdcd4	moderate	1.92 ± 0.06	PP1031
CG9934	moderate	2.32 ± 0.07	PP6054
CG7970	moderate	1.54 ± 0.1	PP10328
Esyt2	low	4.18 ± 2.24	PP27131
Dyb	low	2.10 ± 0.18	PP24370
CG8399	low	1.23 ± 0.21	PP34732

Supplementary Experimental Procedures

Large-scale immunoprecipitations and mass spectrometry

FLAG-CAL1 complexes were isolated and purified from chromatin-free extracts generated from $2x10^6$ S2 cells, as described previously (Chen et al., 2012; Mellone et al., 2011). FLAG-CAL1 complexes from chromatin-associated complexes were generated by homogenization in 5ml ice-cold Buffer A (20mM HEPES pH 7.4; 10mM KCl; 1.5mM $MgCl_2$; 0.34M Sucrose; 0.2% Triton-X 100; 10% Glycerol; 1mM DTT), with protease inhibitors (Roche) and 1mM PMSF, followed by consecutive extractions in buffer B (Buffer A with 150mM KCl, protease inhibitor cocktail and 1mM PMSF), buffer C (0.34M Sucrose 50mM Tris pH 7.4; 1.5mM NaCl; 5mM $MgCl_2$; 1mM EGTA; 0.04% Triton-X 100; 1mM DTT, protease inhibitors and 1mM PMSF) and buffer D (10mM HEPES pH7.4; 2mM $MgCl_2$; 250mM Sucrose). 800µl of Buffer E (10mM HEPES pH 7.4; 2mM $MgCl_2$; 0.5M Sucrose) was pipetted underneath the suspension and centrifuged at 500 g for 5 min. The pellet was resuspended in 400µl Buffer F (2mM EGTA; 1mM DTT; protease inhibitor cocktail), followed by resuspension in digestion Buffer (10mM Tris pH 7.4; 150mM NaCl; 1mM $MgCl_2$; 0.025% NP-40; protease inhibitor cocktail; 1mM PMSF; 4µl Benzonase) for 1 h at 4°C. After stopping the digestion with EDTA, the extract was added to 100µl M2-FLAG agarose beads (Sigma). After washing, complexes were eluted with 400mM FLAG peptide (Sigma-Aldrich). The eluted FLAG-CAL1 complex was reduced, carboxamidomethylated, digested with trypsin and LysC and subjected to LC-MS/MS on a Waters/Micromass AB QSTAR Elite mass spectrometer (Keck Biotechnology Resource, Yale School of Medicine). MS/MS spectra were analyzed using MASCOT (Matrix Science).

Cell culture and treatments

Cells were cultured at 25°C in Schneider medium as described (Chen et al., 2014) with addition of 150µg/ml hygromycin and/or 2 µg/ml puromycin if stably transfected. Stable S2 cells containing an integrated lacO array (pAFS52; (Straight et al., 1996)) were described before (Chen et al., 2014; Mendiburo et al., 2011). Note that this line is polyclonal and approx. 65% of cells bears the lacO insertion and the CAL1-GFP-LacI transgenes. Additional stable S2 cells were generated by transfection of $1x10^6$ cells with 2µg of plasmid DNA, alongside 2µg of the pHygro plasmid using cellfectin (Invitrogen). Cells were grown for 3 days before addition of 450µg/ml hygromycin to select for transfected cells. Transfection efficiency was determined by IF and Western blotting with the appropriate antibodies. Stable S2 cells harboring the lacO array were re-thawed after one month in culture to prevent shrinking and loss of the lacO array.

Transient transfections were performed using FuGENE HD (Promega) with 2µg of plasmid DNA (pMT-CAL1-GFP-LacI; pAS52-lacO; pMT-CAL1Δ1-40 -GFP-LacI), incubated for 2 days, and induced with 0.5mM $CuSO_4$ for 24h.

Induction of CAL1-GFP-LacI or GFP-LacI was performed by addition of 0.5mM $CuSO_4$ to the growth medium and incubation at 25°C for 1-48h. Uninduced samples followed the same treatments as the induced without the addition of $CuSO_4$.

RNAi

Double-stranded RNAs (dsRNA) against *dre4, ssrp1, cal1,* and *brown* (negative control) were generated using the Ambion T7 Synthesis kit, using primers obtained from the DGRC (see Supplementary experimental procedures, primers).

RNAi was performed using 10µg of dsRNA transfected into $1x10^6$ S2 cells using DOTAP (Roche). Cells were either incubated for four days after which $1x10^6$ cells were re-transfected with 10µg of dsRNA and incubated for a further 2 days (Figure 6E-F), or were incubated for 6 days after a single transfection (Figure 6A-D). Depletion of FACT by Western blot occurred 4 days after RNAi, while complete loss of endogenous CENP-A IF signal occurred after double RNAi treatment for 6 a total of days.

To visualize H3.1 and H3.3 occupancy we performed FACT depletion by RNAi for 72h before transient transfection with either pMT-H3.1-V5 or pMT-H3.3-V5 using FUGENE (Promega). Cells were incubated for an additional 48h after which they were induced for 24h with 100µM $CuSO_4$. Cells were then processed for fiber IF; transfection efficiency and successful depletion of Dre4 and SSRP1 was confirmed by Western blotting.

Image quantification

For the quench-chase-pulse, we used manual quantification to determine signal intensity of TMR-CENP-A foci within the cell nucleus. Intensity was categorized as follows: strong signal = >800 (A.U.); medium signal = 200-800; weak/no signal = <200. 100 cells per replicate experiment were scored, three replicates were performed.

For the CENP-A IF signal we used Softworx Suite's 2D Polygon Finder function. The total intensity/per cell was averaged for 50-100 cells from each condition. The resulting averages for each condition were averaged again across three independent replicates.

CENP-A fibers were measured using the Measure Distance tool in Softworx Suite by placing one point at each end of the CENP-A signal on a single continuous fiber (defined by the presence of CENP-A signal at least 1/3 of the max intensity for that stretch).

DNA constructs

The lacO plasmid was described previously (Straight et al., 1996). *dre4* and *ssrp1* were cloned into pCopia-FLAG vector by PCR (see 'primers used in this study' section below) from cDNA clones obtained from the DGRC adding AscI/PacI restriction sites. For the *in vitro* binding assays, the coding sequences (*dre4 and ssrp1*) were inserted into pDONR221 using the Gateway System (Life Technologies) or PCR amplified to include a T7 promoter, a Kozak sequence, a start codon and 4 stop codons in the case of CAL1Δ1-40. Expression clones were then made using pDEST17. pMT-H3.1-V5 and pMT-H3.3-V5 were generated by topo-cloning PCR products amplified from genomic DNA using the DES TOPO TA Expression Kit (Invitrogen). All plasmids were verified by sequencing.

Protein expression and affinity purification

The expression of His-tagged Dre4, SSRP1 and MBP was induced in *E. coli* strain Rosetta 2(DE3)pLysS Single (Novagen) with 0.2mM IPTG at 22°C for 5h. Cells were lysed by sonication in extraction buffer containing 20mM Tris pH 8.0, 500mM NaCl, 20mM imidazole and 5mM β-mercaptoethanol supplemented with 6M Guanidine hydrochloride. Recombinant proteins were immobilized onto Ni-NTA agarose beads under denaturing conditions according to the manufacturer (Qiagen). Refolding was performed using standard procedures: beads were gradually washed in extraction buffer supplemented with 0.1% Triton X-100 and 6 to 0M urea, respectively.

For the *in vitro* binding assay ^{35}S-methionine-labelled Dre4, SSRP1 or CAL1 (from pDEST24 or PCR product for CAL1Δ1-40) were expressed *in vitro* using the TNT T7 Quick Coupled Transcription/Translation System (IVTT) according to the manufacturer (Promega, L1170). After 2h incubation at 30°C IVTT reactions were treated with 10 Kunitz units DNase I (Sigma, D4263) for further 15 min to eliminate DNA, centrifuged at room temperature (21000 g, 5 min) and supernatants were directly used in *in vitro* binding assay.

SDS-PAGE and autoradiography for *in vitro* interaction assays

Protein samples (2-6% input and 30-50% bound proteins) were separated by 8% or 9% SDS-PAGE, gels were rinsed in water and stained with Bio-Safe Coomassie (Bio-Rad), scanned, dried on Whatman 3MM filter paper and directly used for autoradiography. Exposure to hypersensitive film (Kodak BioMax MS film) was performed at -80°C.

Detection of nascent RNA

Nascent RNA was isolated using the Click-iT nascent RNA capture kit (Life Technologies). Cells were either not induced (0h) or induced with 0.5mM $CuSO_4$ for 4, 8, 24, 30 and 48h and nascent RNA was labeled with EU for the last 4h of each time point (in the case of the uninduced 0h, no $CuSO_4$ was added and EU was added for 4h). Total RNA was isolated using the TRI-reagent (Sigma) and 5µg of total RNA were subjected to biotinylation followed by EU-labeled RNA purification using 40µl of streptavidin magnetic beads. The isolated RNAs were used as templates to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad) and followed by qPCR.

ChIP-Seq and RNA-Seq mapping

Prior to mapping, all libraries were Illumina adaptor trimmed and filtered for quality scores 30 and greater using Trimmomatic version 0.32 (Bolger et al., 2014). Paired-end global alignments to the full LacO array and vector reference were performed using Bowtie2 version 2.2.2 (Langmead and Salzberg, 2012) under the --very-sensitive settings and allowing for an inter-mate distance of 100 to 300nt in the ChIP pools and 100 to 400nt for the RNA pools. Alignment files were filtered to report only read pairs that mapped concordantly with a mapping quality greater than 0.

To generate signal tracks for the ChIP-Seq, peak-calling was performed on each of the lacO mapped pools using MACS2 (Zhang et al., 2008) with the recommended broad peak-calling parameters, --no-model and --no-lambda. The coverage values in the bedgraph output files were converted to fragments per million reads to normalize coverage to the sequencing depth of each library. Signal tracks were visualized using IGV (Robinson et al., 2011). In addition, MACS2 was used to compute the fold enrichment of called peaks, and corresponding p- and q-values, for each induced pool over its corresponding uninduced control.

To calculate a p-value for differential expression in the RNA-Seq, the pools were aligned with TopHat to the LacO reference concatenated to the dm3 chromosome 2L and 3L reference sequences and processed through the Cufflinks suite (Trapnell et al., 2012). Default parameters were used, with the exception of an upper quartile normalization (-N) and multiple mapping read correction (-u). For the RNA-Seq data, a differential expression analysis was also performed to calculate the fragments per kilobase (FPKM) mapped for each pool. CAL1-GFP-LacI pool had an FPKM of 92.06, while the GFP-LacI pool had an FPKM of 45.88. This was found to be a differential expression to a p-value < 0.05%.

Co-immunoprecipitation of CAL1 and RNAPIIS2p

Chromatin associated extracts were isolated from $2x10^8$ S2 cells as described above and chromatin-containing pellets were digested either with 4µl of DNaseI (Promega) in digestion buffer (protease inhibitors,10 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 1 mM $MgCl_2$, 0.025% NP-40) for 20 min at 37°C after which 2 mM EDTA was added to the extracts, or with $20U/10^8$ cells MNase (Zymo Research) in EX100 buffer (10 mM HEPES, pH 7.6; 100 mM NaCl; 1.5 mM $MgCl_2$; 2 mM $CaCl_2$;0.5 mM EGTA; 10% v/v glycerol; protease inhibitors) for 20 min at 26°C followed by termination with 10mM EGTA. The NaCl concentration was increased to 300 mM and the chromatin was centrifuged at 12,000g for 10 min at 4°C. The supernatant was then used as the input in the IPs. For IPs, 4µg of anti-CAL1 antibody (rabbit) or PBS (mock) was coupled to 25µl of Dynabeads Protein-A (Invitrogen) beads, following the manufacturer's instructions. The input was incubated with beads for 2h with rotation at 4°C. After three washes in cold PBS, bound proteins were eluted with 20µl of Laemmli buffer (100 mM Tris, pH 6.8; 4% SDS; 20% glycerol; 0.1% bromophenol blue; 300 mM 2-mercaptoethanol) and boiled 5 min at 95°C. 1.6% of the total input and 50% of the total IP were subjected to Western blot.

Primers used in this study

RNAi	
Dre4 R	TAATACGACTCACTATAGGGCAGCGACGAGGAAGATGTG
Dre4 S	TAATACGACTCACTATAGGGCTGACAAATGTGGCCTCTGG
SSRP1 R	TAATACGACTCACTATAGGGCGAAAATCCGGGCATAAAGGT
SSRP1 S	TAATACGACTCACTATAGGGCTTGCTCTTCTTCTTGGCAGG
Bw R	TAATACGACTCACTATAGGGCGATATTATCGATGTCGATCCAG
Bw S	TAATACGACTCACTATAGGGCCTATGGCGTGACGTATATATTT
CAL1 R	TAATACGACTCACTATAGGGCTGGATGCCAGGAAAGTTAGT
CAL1 S	TAATACGACTCACTATAGGGCCTATAGGGATTGTTGATATCAGC
Cloning	
SSRP1 F (flag)	CAGTGGCGCGCCATGACAGACTCTCTGGAG
SSRP1 R (flag)	CAGTTTAATTAACTAATCACTGGCCTCATCTTCC
Dre4 F (flag)	CAGTACTAGTATGTCGAGCTTTGTGCTGG
Dre4 R (flag)	CATGGCGGCCGCCTAATGTCGCGACTTCTTCG
Dre4-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTCGAGCTTTGTG CTGGACAAGG

Dre4-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAATGTCGCGACTTC TTCGACTTG
SSRP1-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGACAGACTCTCTG GAGTACAACG
SSRP1-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTACTAATCACTGGCCTCA TCTTCCTCA
Cal1-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCGAATGCGGTG GTGGACGAGG
Cal1-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACTTGTCACCGGAA TTATTCTCG
qPCR	
Dre4 F	CCTGAAGCAGACGCAGAACATC
Dre4 R	CAAACGCTTGCCCTTCTTCGAG
SSRP1 F	AACGACATAAACGCCGAAGTGC
SSRP1 R	CCTTGCCGGTCTTGGTGTTC
CAL1 F	TGGTGGACGAGGAAACACTGG
CAL1 R	TCTATTTCCATGCCGTCTTCCAGG
CID F	TCTAGTGCGCGAGTTCATCGTG
CID R	AAGTACATCTCGCACGACTCCTG
CENP-C F	AGGGCGACCACGATCTACTC
CENP-C R	CCGTCCTTTGAAGATTTGCGTCG
Actin F	GATCTGTATGCCAACACCGT
Actin R	GCGGGGCAATGATCTTGATC
Rp49 F	CCGCTTCAAGGGACAGTATC
Rp49 R	GACAATCTCCTTGCGCTTCT
18S F	TGACGAAAAATAACAATACAGGACTCA
18S R	CAGACTTGCCCTCCAATT GG
lacO 1.6 F	CCCCGTAGAAAAGATCAAAGG
lacO 1.6 R	GCTGGTAGCGGTGGTTTTT
lacO 3 F	CGGATCAAGAGCTACCAACTC
lacO 3 R	CTTGAAGTGGTGGCCTAACT
LacI F	TATCCGCTGGATGACCAGGA
LacI R	CAGTCGCGTACCGTCTTCAT

In vitro transcription translation (IVTT)	
Cal1Δ1-40 F	GAATTAATACGACTCACTATAGGGAGAGCCGCCACCATGGCCGAGT TCGACAACTTGTTCCA
Cal1Δ1-40 R	TCATTACTATCATTACTTGTCACCGGAATTATTCTCGA

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