

The RSC chromatin remodelling enzyme has a unique role in directing the accurate positioning of nucleosomes

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Nucleosomes impede access to DNA. Therefore, nucleosome positioning is fundamental to genome regulation. Nevertheless, the molecular nucleosome positioning mechanisms are poorly understood. This is partly because *in vitro* reconstitution of *in vivo*-like nucleosome positions from purified components is mostly lacking, barring biochemical studies. Using a yeast extract *in vitro* reconstitution system that generates *in vivo*-like nucleosome patterns at *S. cerevisiae* loci, we find that the RSC chromatin remodelling enzyme is necessary for nucleosome positioning. This was previously suggested by genome-wide *in vivo* studies and is confirmed here *in vivo* for individual loci. Beyond the limitations of conditional mutants, we show biochemically that RSC functions directly, can be sufficient, but mostly relies on other factors to properly position nucleosomes. Strikingly, RSC could not be replaced by either the closely related SWI/SNF or the Isw2 remodelling enzyme. Thus, we pinpoint that nucleosome positioning specifically depends on the unique properties of the RSC complex.

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Introduction

Eukaryotes package their nuclear DNA into a complex structure called chromatin. At the most basic level of chromatin, the DNA is wound around an octamer of histone proteins in ~1.7 turns (Luger *et al*, 1997) constituting a nucleosome core particle. Nucleosome core DNA is much less accessible to DNA-binding factors than DNA in linker regions between nucleosome cores or in nucleosome-depleted regions (NDRs). Therefore, the positioning of nucleosomes with respect to the

DNA sequence is a powerful lever for the regulation of DNA-templated processes, such as transcription or replication (Simpson, 1990; Venter *et al*, 1994; Liu *et al*, 2006; Field *et al*, 2008; Lantermann *et al*, 2010). This global importance of nucleosome positioning was underscored by the high degree of defined positions in recent genome-wide nucleosome mappings in organisms from yeast to man (Yuan *et al*, 2005; Albert *et al*, 2007; Lee *et al*, 2007; Ozsolak *et al*, 2007; Whitehouse *et al*, 2007; Field *et al*, 2008, 2009; Schones *et al*, 2008; Shivaswamy *et al*, 2008; Valouev *et al*, 2008; Mavrich *et al*, 2008a,b; Lantermann *et al*, 2010). Nevertheless, the molecular mechanism for nucleosome positioning *in vivo* is by far not fully understood.

As nucleosomal DNA is tightly bent, it is an attractive hypothesis that intrinsic features of DNA sequences have a major role in nucleosome positioning. Some sequence features, like certain dinucleotide periodicities (Satchwell *et al*, 1986), intrinsically favour, and others, like poly(dA:dT) stretches (Simpson and Shindo, 1979), disfavour nucleosome formation (Travers *et al*, 2009). Indeed, there is a significant correlation of such features with nucleosome positioning *in vivo*. For example, poly(dA:dT) stretches are enriched in *S. cerevisiae* promoter NDRs (Iyer and Struhl, 1995; Bernstein *et al*, 2004; Yuan *et al*, 2005), and a 10 bp periodicity of AA/TT/AT dinucleotides is more prevalent in strongly positioned nucleosomes flanking NDRs (Ioshikhes *et al*, 2006; Segal *et al*, 2006; Mavrich *et al*, 2008a). However, such rules are not universal. *S. pombe* NDRs, for example, are not enriched for poly(dA:dT) stretches (Lantermann *et al*, 2010), and also other yeasts do not necessarily use such sequences to establish promoter NDRs (Tsankov *et al*, 2010).

Intrinsic DNA sequence rules of nucleosome formation may be probed by *in vitro* reconstitution via salt gradient dialysis, which involves only histones and DNA mixed at initially high salt concentration that is slowly diluted until nucleosomes form spontaneously (Widom, 2001). Recently, two groups reconstituted the whole *S. cerevisiae* genome by salt gradient dialysis and found some overall correlations of *in vitro* and *in vivo* nucleosome occupancy, particularly at the promoter NDRs (Kaplan *et al*, 2009; Zhang *et al*, 2009), but individual nucleosome positions were mostly not recapitulated (Zhang *et al*, 2009). Clearly, additional factors beyond just the DNA and histones determine nucleosome positions *in vivo*.

What are these nucleosome positioning factors? Besides a role of some abundant sequence-specific DNA-binding proteins, like budding yeast Reb1 and Abf1 (Raisner *et al*, 2005; Badis *et al*, 2008; Hartley and Madhani, 2009), ATP-dependent nucleosome remodelling enzymes are implicated in global nucleosome positioning. Such enzymes enable the assembly, disassembly or relocation of nucleosomes, and in some cases they can catalyse histone exchange events. They vary in the type of ATPase subunit and in the association with different subunits (Clapier and Cairns, 2009).

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The *S. cerevisiae* Isw2 and Isw1 remodelling enzymes were shown to move nucleosomes over intrinsically unfavourable sequences at the 5' and 3' ends of genes (Isw2 (Whitehouse *et al*, 2007)) and at mid-coding regions (Isw1 (Tirosh *et al*, 2010)), which in both cases were associated with suppression of erroneous transcription. Conversely, in *S. pombe*, which does not encode a remodelling enzyme of the ISWI family, the remodelling enzyme Mit1 appears to be involved in generating regular nucleosomal arrays (Lantermann *et al*, 2010). Finally, the essential remodelling enzyme RSC appears to keep NDRs nucleosome-free in *S. cerevisiae* (Badis *et al*, 2008; Hartley and Madhani, 2009). Ablation of RSC in temperature-sensitive mutants increased nucleosome occupancy at ~55% of NDRs (Hartley and Madhani, 2009). However, such effects in conditional mutants may be indirect or confounded by cell viability issues.

Therefore, complementary to the initial identification of nucleosome positioning factors *in vivo*, there is an urgent need for an *in vitro* reconstitution system that generates *in vivo*-like nucleosome positioning in order to elucidate the molecular mechanism. Previously, we reported the establishment of such an *in vitro* system using yeast extracts that was able to successfully generate *in vivo*-like patterns of nucleosome positions at several yeast promoters (Korber and Horz, 2004; Hertel *et al*, 2005; Wippo *et al*, 2009). In this study, we describe the enrichment of the nucleosome positioning activity by chromatography and by fractionation of the yeast extract. We identify the RSC nucleosome remodelling complex and show directly by *in vitro* reconstitution that it has a specific, necessary, and in some cases even sufficient, role in nucleosome positioning at yeast promoters.

Results

The nucleosome positioning activity for the *PHO8* promoter could be enriched over four sequential fractionation steps

The *S. cerevisiae* *PHO8* promoter has promoter nucleosomes with stereotypical positioning (Yuan *et al*, 2005; Mavrich *et al*, 2008a; Jiang and Pugh, 2009), that is, an NDR of ~120 bp that is flanked by two positioned nucleosomes with the downstream nucleosome N + 1 covering the TSS (Figure 1A). Upstream of *PHO8* is the divergently transcribed *KRE2* gene with a similarly stereotypical promoter. In short, in the following sections, we call this entire region the '*PHO8* promoter'.

We assembled plasmids carrying the *PHO8* promoter into chromatin by salt gradient dialysis using *Drosophila* embryo histone octamers. As shown before (Hertel *et al*, 2005), this assembly by itself was unable to reconstitute the *in vivo* nucleosome positions (Figure 1C, lane 4, note that the pattern of salt gradient dialysis chromatin does not change in the presence of extract if no ATP is added; Hertel *et al*, 2005). However, incubation of such chromatin templates with a yeast whole-cell extract (WCE) and ATP shifted the nucleosomes to their *in vivo* positions (Figure 1C, lane 5; Hertel *et al*, 2005). Importantly, we analyse *in vivo* and *in vitro* chromatin samples side-by-side by using the same methodology and in the same gels. This way the nucleosome positioning patterns of different samples can be directly compared. Using this assay, we traced the nucleosome positioning activity during extract fractionation over four sequential

steps (Figure 1B and C). The protein complexity was greatly reduced (Supplementary Figure S1), with only a moderate loss of the nucleosome positioning activity. As our reconstitution system could also generate *in vivo*-like positioning at other loci (Korber and Horz, 2004; Hertel *et al*, 2005; Wippo *et al*, 2009; Figures 2 and 3, Supplementary Figure S2, and data not shown), we tested the 500-mM ammonium sulphate phenyl sepharose fraction and the final 350-mM KCl DEAE fraction on other promoters as well. While the former fraction was as positive as the WCE for almost all loci, the latter was mainly positive for *PHO8* (data not shown), indicating distinct nucleosome positioning activities for different loci.

LC-MS/MS analysis of the final 350-mM KCl DEAE fraction

LC-MS/MS analysis of the final 350-mM KCl DEAE fraction identified 212 proteins (Supplementary Table S2), of which 95 localized outside the nucleus and 117 localized at least partially to the nucleus or had no known localization (Huh *et al*, 2003). These 117 proteins were, in principle, the more promising candidates, but many of them were excluded from further analysis, as yeast strains harbouring deletion or temperature-sensitive alleles of the respective genes showed the wild-type DNaseI pattern at the *PHO8* promoter *in vivo* (Supplementary Table S2; data not shown).

Purified RSC repositioned nucleosomes in salt gradient dialysis chromatin, but only in few cases resulting in *in vivo*-like positions

Intriguingly, our final fraction contained 10 out of 17 subunits of the RSC complex (Supplementary Table S2), suggesting a role for this remodelling enzyme. To directly test whether the RSC complex was sufficient for proper nucleosome positioning, we chose a test set of four yeast loci in which a role of RSC in nucleosome organization had either previously been implicated (*RIM9* and *PHO8* (Badis *et al*, 2008), *CHA1* (Moreira and Holmberg, 1999; Badis *et al*, 2008; Parnell *et al*, 2008)) or not (*PHO8* (Parnell *et al*, 2008) and *SNT1* (Badis *et al*, 2008; Hartley and Madhani, 2009)). We assembled equimolar amounts of four plasmids, each carrying one of these loci, together in the same reaction by salt gradient dialysis with purified histones. This pool of pre-assembled plasmids was the common starting material for the following experiments.

Similar to the *PHO8* locus, the main NDRs and some of the positioned nucleosomes at both the *CHA1* and the *SNT1* locus were properly generated upon addition of WCE and ATP to salt gradient dialysis chromatin, whereas salt gradient dialysis by itself again did not recapitulate *in vivo*-like nucleosome positioning (Figure 2C and D, compare lanes 4–5 and 12–13 with lanes 1–2). The pattern of salt gradient dialysis chromatin and WCE without ATP was again the same as that for untreated salt gradient dialysis chromatin. So also at these loci as well, our yeast extract-based *in vitro* reconstitution system generated *in vivo*-like nucleosome organization from non-*in vivo*-like salt gradient dialysis chromatin. Nevertheless, salt gradient dialysis assembly alone could reconstitute the *RIM9* NDR2 and *ECM40* NDR3 to some extent correctly (Figure 2B, compare lanes 12–13 with lanes 1–2), while addition of WCE broadened *RIM9* NDR2. The *RIM9* locus turned out to be a rare example in which *in vivo*-like nucleosome positioning was less properly reconstituted in

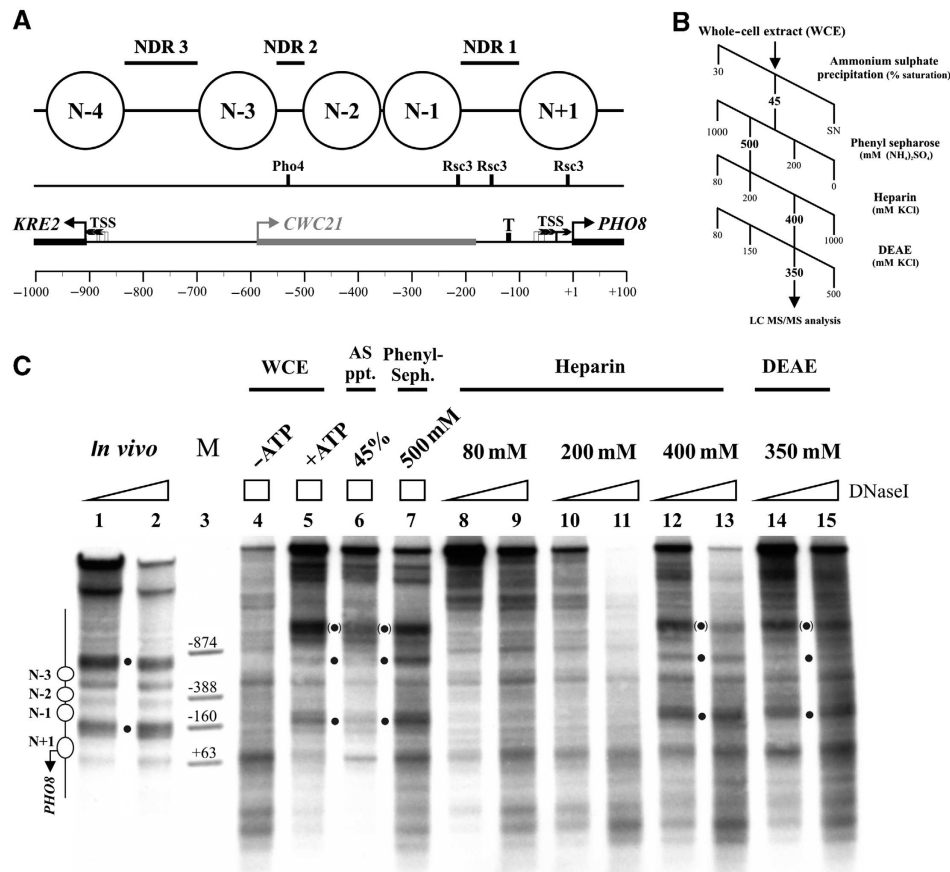


Figure 1 The nucleosome positioning activity for the *PHO8* promoter could be enriched from a yeast whole-cell extract (WCE) over four sequential fractionation steps. (A) Top panel: schematics of nucleosome positions at the *KRE2-CWC21-PHO8* locus, according to Barbaric *et al* (1992) and Jiang and Pugh (2009). Nucleosomes are numbered relative to NDR1. Middle panel: mapped Pho4 (Barbaric *et al*, 1992) or predicted Rsc3 (Badis *et al*, 2008) binding sites (Supplementary Figure S8A). Lower panel: mapped *KRE2*, *CWC21* and *PHO8* open reading frames (rectangular bars with large broken arrows), TATA box (T; Basehoar *et al*, 2004) and transcriptional start sites (TSS, small broken arrows; Miura *et al*, 2006). Scale bar: distance in base pairs from *PHO8* ORF start. All panels drawn to scale. (B) Extract fractionation scheme. Fractions positive for the *PHO8* promoter nucleosome positioning activity are labelled in bold. SN, supernatant. (C) DNaseI indirect end labelling analysis of the *PHO8* promoter region *in vivo* or *in vitro* after salt gradient dialysis assembly and incubation with either WCE in the presence or absence of ATP, or with one of the indicated fractions (see B) in the presence of ATP. Black dots: diagnostic bands, which are characteristic for the *in vivo* pattern and seen *in vitro* only in the presence of ATP and the nucleosome positioning activity. Black dots in parentheses: hypersensitive site within the lacZ ORF of the pUC19 backbone specific for the *in vitro* pattern that always co-occurred with the *in vivo*-like *PHO8* promoter pattern. The yeast sequence terminates close to the top marker band. Schematics on the left analogous to (A). Position of marker bands is labelled relative to the *PHO8* ORF start. Ramps and boxes: relative DNaseI concentrations. All samples were electrophoresed alongside in the same gel, but the *in vivo* samples migrated slightly faster, probably because of different total DNA concentration.

our yeast extract-based *in vitro* system. Moreover, the NDR1 at *AEP1* was not met under any *in vitro* conditions. A strong band close to the position of NDR1 in the presence of RSC (Figure 2B, lanes 6–9) was not at the proper position as seen by indirect end labelling using a secondary cleavage with better resolution for this region (data not shown).

Strikingly, addition of purified RSC and ATP to salt gradient dialysis chromatin already generated the proper NDR at *CHA1* to some degree (Figure 2C, compare lanes 6–9 with lanes 1–2) and could clearly position nucleosome N-1 at the *SNT1* locus as *in vivo* (Figure 2D, compare lanes 6–9 with lanes 1–2). The prominent band in the salt gradient dialysis chromatin pattern (arrow between lanes 12 and 13) at the position of the *SNT1* nucleosome N-1 was removed by the addition of purified RSC (or of WCE) in the presence of ATP, suggesting that RSC alone could move a nucleosome to an *in vivo*-like position. This was true for both tested RSC concentrations (Figure 2C and D, compare lanes 6–7 with lanes 8–9).

In contrast, addition of purified RSC was unable to reconstitute *in vivo*-like positioning both at the *PHO8* (Figure 2A, compare lanes 6–9 with lanes 1–2) and at the *RIM9* locus (Figure 2B, compare lane 6–9 with lanes 1–2), although it did change the pattern of the salt gradient dialysis chromatin (Figure 2A–D, compare lanes 6–9 with lanes 12–13) arguing for sufficient remodelling activity in the assay. Importantly, and in accordance with our earlier findings (Korber and Horz, 2004; Hertel *et al*, 2005; Wippo *et al*, 2009), the nucleosome positioning activity of both purified RSC and of the WCE was strictly dependent on the presence of ATP (Figure 2A–D, compare lanes 10–13 with lanes 4–9).

A direct and necessary role for RSC in generating *in vivo*-like nucleosome positions at *PHO8*, *RIM9*, *CHA1* and *SNT1* *in vitro*

As purified RSC could generate only a minor fraction of the proper nucleosome positioning *in vitro*, we wondered

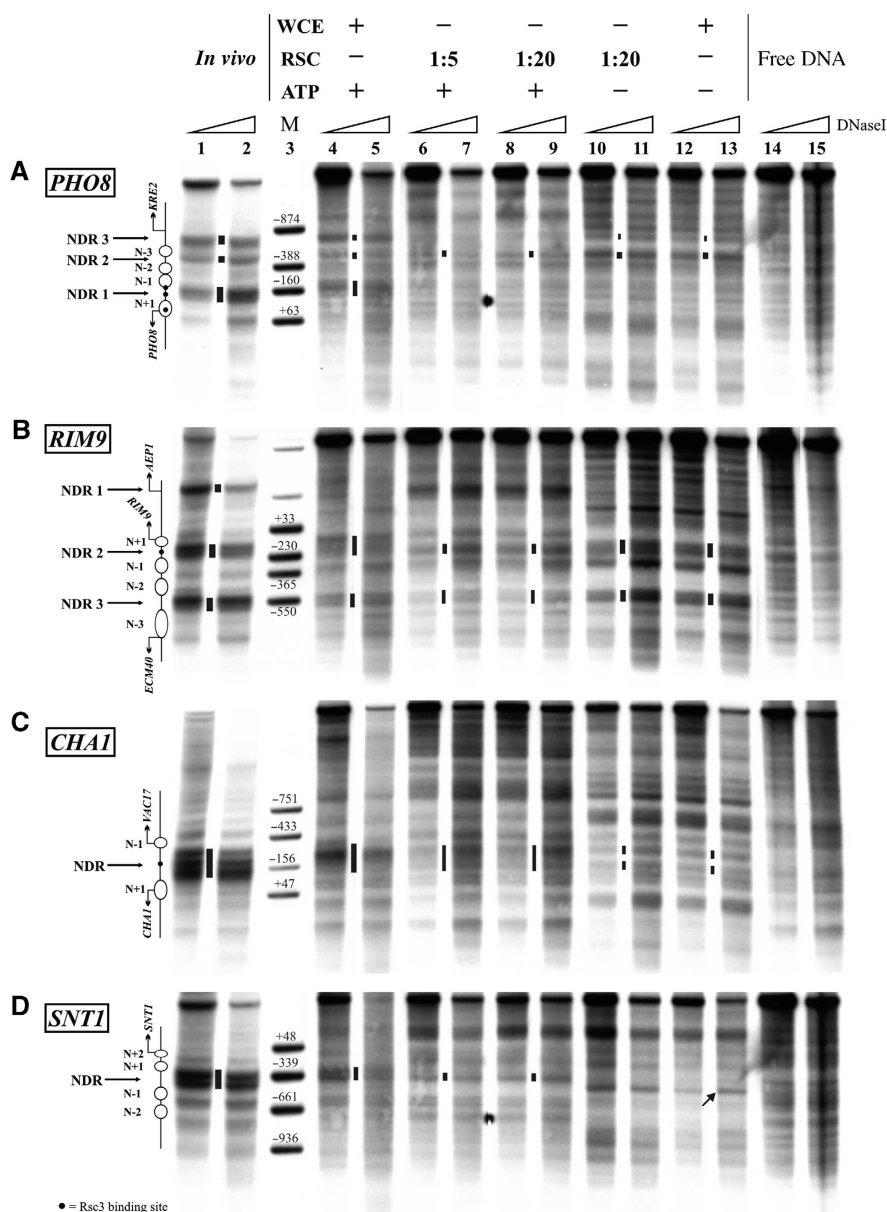
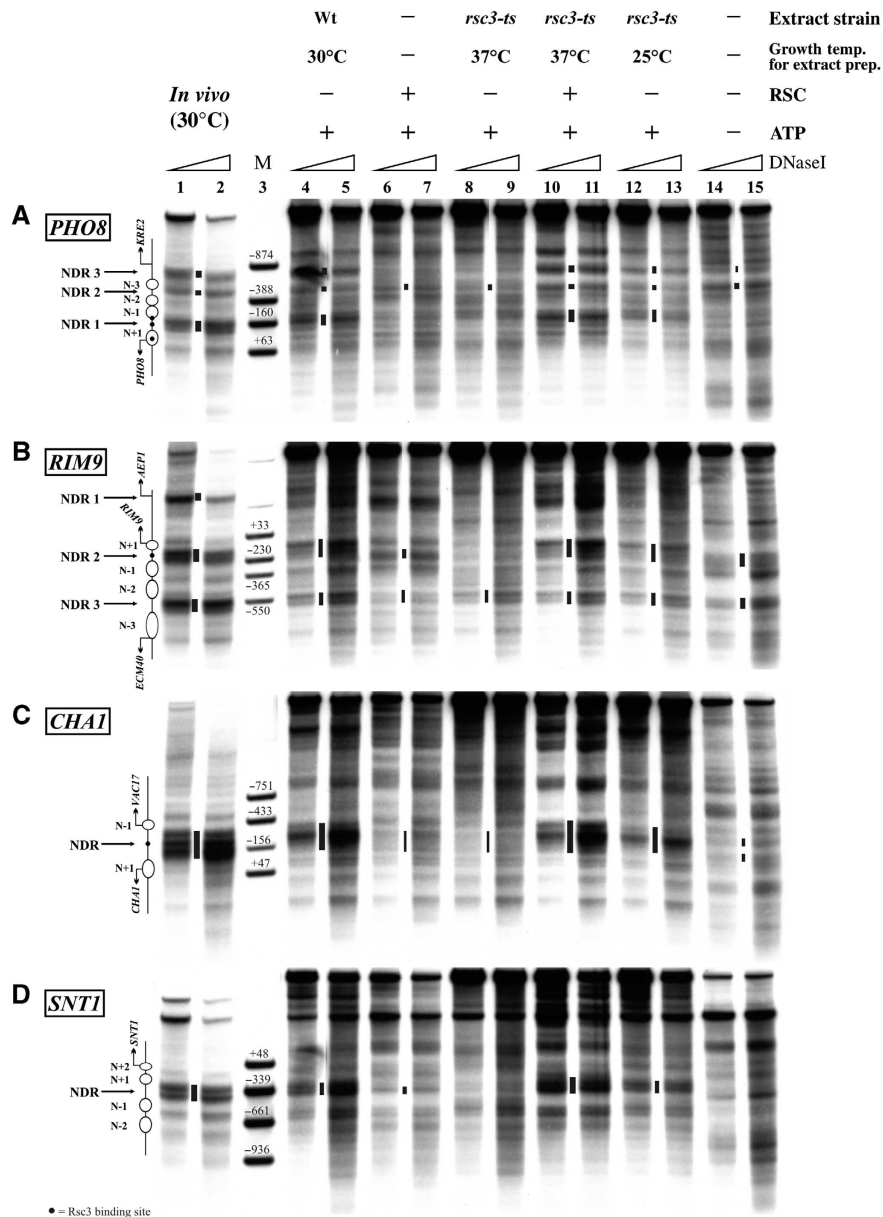


Figure 2 Purified RSC repositioned nucleosomes in salt gradient dialysis chromatin, but only in few cases, resulting in *in vivo*-like positions. DNaseI indirect end labelling analysis of the (A) *PHO8*, (B) *RIM9*, (C) *CHA1* and (D) *SNT1* promoter regions *in vitro* after assembly by salt gradient dialysis and incubation with WCE or purified RSC complex in the presence or the absence of ATP as indicated. The amount of RSC is given as the molar ratio of RSC to nucleosomes. In each panel, lanes 1 and 2 show the wt *in vivo* DNaseI pattern. Free DNA samples correspond to the respective non-assembled plasmids in the absence of WCE, RSC and ATP but under otherwise identical conditions. Bars in between lanes mark hypersensitive regions that correspond, at least to some degree, to NDRs of the *in vivo* patterns. The arrow between lanes 12 and 13 in **D** marks a nuclease-sensitive region that becomes inaccessible because of RSC activity. Ramps: increasing DNaseI concentrations. Position of marker bands is labelled relative to the ORF start of the respective locus. Schematics on the left are analogous to Figure 1A for the respective locus. Predicted Rsc3 binding sites (Supplementary Figure S8) are indicated by black dots.

whether it was even necessary. We prepared extracts from a strain carrying a temperature-sensitive allele of the gene coding for the essential Rsc3 subunit of the RSC complex (*rsc3-ts* mutant (Badis *et al*, 2008)) that was grown at the non-permissive temperature (37°C) overnight. Such an extract (Figure 3, '*rsc3-ts* 37°C' extract) was much less effective in positioning nucleosomes properly than the wild-type WCE (Figure 3A–D, compare lanes 8–9 with lanes 4–5), while an extract prepared from the *rsc3-ts* strain grown at 25°C functioned almost like the wild-type WCE (Figure 3A–D, compare lanes 12–13 with lanes 4–5). The *rsc3-ts* 37°C extract failed to

reconstitute NDR1 and NDR3 at the *PHO8* promoter, NDR2 at the *RIM9* promoter, the broad NDR at the *CHA1* locus and the strong NDR at the *SNT1* promoter (Figure 3A–D, lanes 8–9). Nevertheless, it did change the pattern of the salt gradient dialysis chromatin starting material (Figure 3A–D, compare lanes 8–9 with 14–15), arguing for residual nucleosome remodelling activity also in this extract. In summary, the *rsc3-ts* 37°C extract was sufficiently impaired in its nucleosome positioning activity to confirm the necessary role of RSC and to serve as a background for rescue experiments using purified RSC complex.



Indeed, the addition of purified RSC to the *rsc3-ts* 37°C extract, completely rescued the nucleosome positioning activity for all four tested loci (Figure 3A–D, compare lanes 10–11 with lanes 4–5) and yielded patterns that were even slightly more *in vivo*-like than those generated by the wild-type WCE. This suggests that in the wild-type WCE, RSC may even be a limiting factor for proper nucleosome positioning. The rescue by purified RSC strongly suggests that the changes observed with the *rsc3-ts*, *arp9-ts* and *sth1-td* strains, by us (see below, Figure 5 and Supplementary Figure S3) and by others (Supplementary Figure S4, Supplementary Table S3) (Badis *et al*, 2008; Parnell *et al*, 2008; Hartley and Madhani, 2009), were not caused by indirect effects. Moreover, purified RSC could generate much less of the proper nucleosome positioning than in combination with the *rsc3-ts* 37°C extract

(Figure 3A–D, compare lanes 6–7 with lanes 10–11). Therefore, both RSC and the *rsc3-ts* 37°C extract were unable to reconstitute *in vivo*-like nucleosome positioning on their own, but the combination of both reconstituted the full nucleosome positioning activity. Therefore, RSC is necessary but mostly not sufficient for proper nucleosome positioning.

Interestingly, we even found an example in which purified RSC counteracted the generation of *in vivo*-like nucleosome positioning. We published previously that almost *in vivo*-like nucleosome positioning was generated at the *PHO84* promoter by mere salt gradient dialysis reconstitution (Wippo *et al*, 2009). RSC alone disrupted this intrinsically encoded *in vivo*-like positioning, whereas the proper positioning was generated when RSC was added in the context of the *rsc3-ts* 37°C extract (Supplementary Figure S2, compare lanes 6–7 with

1–2, 4–5, 10–11 and 14–15). This further underscores the fact that additional factors from the extract are necessary to direct the role of RSC in nucleosome positioning.

The role of the RSC complex in nucleosome positioning *in vitro* is specific, as it could not be substituted by the SWI/SNF or Isw2 remodelling enzymes

We wondered whether the role of RSC was specific or whether other remodelling complexes could achieve similar results. The *rsc3-ts* 37°C extract likely still contained other remodelling enzymes. However, other remodelling enzymes might not be present in sufficient quantities to substitute for the loss of RSC function in our *in vitro* system. Most other remodelling enzymes are less abundant in the cell to start with (~2000 copies of Sth1 per cell compared to ~220 copies of Snf2; Ghaemmaghani *et al*, 2003), and they may be less stable during extract preparation or their concentration might have been affected indirectly because of the *rsc3-ts* conditions. Hence, the RSC complex might just have seemed necessary for nucleosome positioning in our *in vitro* system—and by extension also in previous *in vivo* studies—simply because it was the most abundant remodelling activity.

We added purified SWI/SNF or Isw2 remodelling enzymes in the same molar amount as previously carried out for the RSC complex to the *rsc3-ts* 37°C extract. These two remodelling complexes, whether alone or in combination with the *rsc3-ts* 37°C extract, were unable to generate *in vivo*-like positioning as achieved with RSC (Figure 4A–D, compare lanes 3–11 with lanes 1–2). Importantly, both remodelling enzymes individually (in the presence of ATP) altered the pattern of salt gradient dialysis chromatin (Figure 4A–D, compare lanes 8–9 and 10–11 with 12–13) to a certain extent, which confirmed sufficient activity to remodel the chromatin templates *in vitro*. Both remodelling enzymes did not change the pattern generated by the *rsc3-ts* 37°C extract (compare Figure 4A–D, lanes 3–6 with Figure 3A–D, lanes 8–9), possibly because both were already present in the *rsc3-ts* 37°C extract.

Loss of essential subunits of the RSC remodelling complex altered chromatin structure at the PHO8, RIM9 and other promoters *in vivo*

Our *in vitro* results strongly argue for a direct role of RSC in nucleosome positioning also *in vivo* as suggested previously (Badis *et al*, 2008; Parnell *et al*, 2008; Hartley and Madhani, 2009). Genome-scale microarray data on changes in nucleosome occupancy upon RSC ablation were already available for the temperature-sensitive *rsc3-ts* allele (Badis *et al*, 2008) and for *sth1-td* degron mutants (Parnell *et al*, 2008; Hartley and Madhani, 2009). However, a detailed comparison between different methods is often difficult and it is usually advisable to confirm genome-wide data with locus-specific techniques for regions of interest. Therefore, we monitored the *in vivo* effect of RSC on chromatin patterns at selected test loci, by the same method as used for the *in vitro* patterns, that is, by DNaseI indirect end labelling. We used the same temperature-sensitive strains as Badis *et al* and Parnell *et al* (*rsc3-ts* (Badis *et al*, 2008), *sth1-td* (Parnell *et al*, 2008)) and included an *arp9-ts* mutant (Cairns *et al*, 1998) as Arp9 came up very prominently in our LC-MS/MS analysis (Supplementary Table S2).

At the *PHO8* promoter, a broad DNaseI hypersensitive site replaced nucleosome N-3 between NDR3 and NDR2, and the short hypersensitive site at the migration position of the –160 marker band was slightly diminished, indicating increased nucleosome occupancy over NDR1. We confirmed the DNaseI indirect end labelling results by restriction enzyme accessibility. In both the *arp9-ts* and the *rsc3-ts* mutant, there was an increase in accessibility of the *HpaI* site located within nucleosome N-3 and a decrease for the *HindIII* site located within NDR1 (Supplementary Figure S3). Consistently, Badis *et al* (2008) observed increased nucleosome occupancy at NDR1 and a broad region of decreased occupancy at the upstream edge of nucleosome N-3 in the *rsc3-ts* mutant under restrictive conditions (Supplementary Figure S4A). In contrast, Parnell *et al* (2008) did not see significant changes in the *sth1-td* strain, at least for which data are available for the *PHO8* promoter region (Supplementary Figure S4A), maybe because of a shorter incubation time (2 h) at the restrictive temperature (see below).

The altered *PHO8* promoter DNaseI pattern of the three temperature-sensitive mutants resembled the pattern of the *PHO8* promoter after induction by phosphate starvation (Barbaric *et al*, 1992). This induced promoter pattern essentially depends on binding of the transactivator Pho4 in NDR2 (Barbaric *et al*, 1992; Munsterkotter *et al*, 2000). To control for inadvertent induction of the *PHO* regulon or for other Pho4-mediated effects due to ablation of essential RSC subunits, we generated *pho4 rsc3-ts* and *pho4 arp9-ts* double mutants. Importantly, the same altered chromatin structure was observed at the *PHO8* promoter under restrictive conditions as in the *ts* single mutants (Supplementary Figure S5). Further, nucleosome positioning at the *PHO84* promoter, which has a similarly low threshold of *PHO* induction as *PHO8* (Lam *et al*, 2008), was largely unchanged in the *rsc3-ts* and *arp9-ts* mutants at the restrictive temperature, arguing also against inadvertent *PHO* regulon induction (Supplementary Figure S6).

The *RIM9* NDR2 was identified as a prominent example for increased nucleosome occupancy in the *rsc3-ts* mutant under restrictive conditions (Badis *et al*, 2008; Supplementary Figure S4B). We confirmed this by DNaseI indirect end labelling and found the same effect in the *arp9-ts* and *sth1-td* strains as well. All three strains displayed significantly reduced DNaseI hypersensitivity over the *RIM9* NDR2 (Figure 5B). Notably, this effect was locus specific as the nearby NDR1 at *AEP1* and NDR3 at *ECM40* were unaffected (Figure 5B).

At the *CHA1* locus, we did not see any effect in the *sth1-td* mutant, a weakly reduced NDR in the *rsc3-ts* mutant, although only in some experiments (Supplementary Figure S7C), and a very weak effect at the NDR in the *arp9-ts* mutant (Figure 5C). Hartley and Madhani (2009) also saw only small changes in a *sth1-td* mutant, whereas both Badis *et al* (2008) and Parnell *et al* (2008) reported clear effects (Supplementary Figure S4C and Supplementary Table S3). Therefore, in our experiment as well as in the literature, the *CHA1* locus was not a clear responder to *in vivo* ablation of RSC subunits. This ambiguity is mirrored by two studies reporting RSC binding at *CHA1* while two others did not (Supplementary Figure S4C, Supplementary Tables S3 and S4). Nevertheless, in the light of all available data we consider *CHA1* as RSC target *in vivo*.

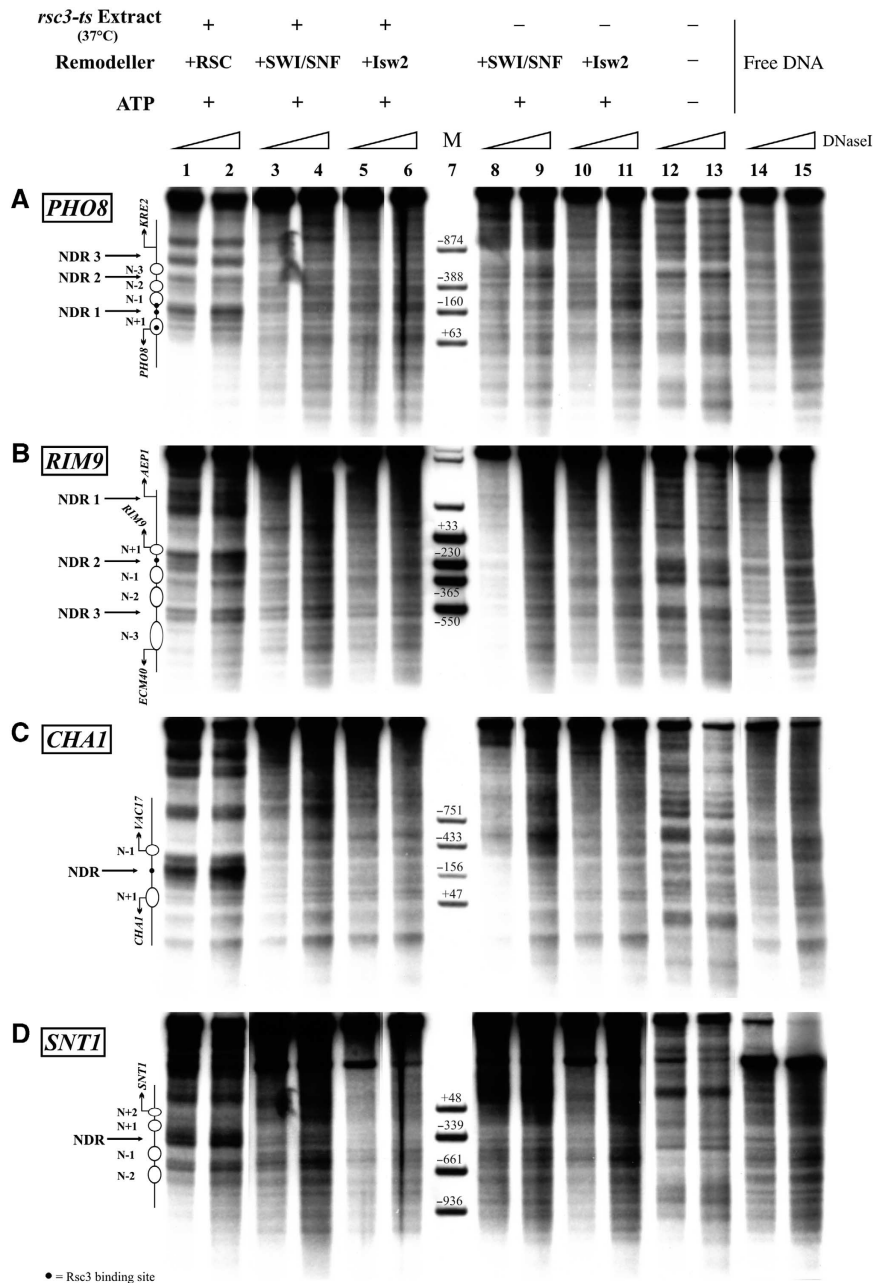


Figure 4 RSC was specifically required for nucleosome positioning *in vitro* as both SWI/SNF and Isw2 failed to rescue the *rsc3-ts* 37°C extract. DNaseI indirect end labelling analysis of the (A) *PHO8*, (B) *RIM9*, (C) *CHA1*, and (D) *SNT1* promoter regions as in Figures 2 and 3 but with addition of purified SWI/SNF or Isw2 remodelling enzymes as indicated. All remodelling enzymes were added at the same molar concentrations, corresponding to the 1:5 ratio in Figure 2.

Finally, we observed no effects at the *SNT1* locus (Figure 5D) consistent with other studies (Supplementary Figure S4D and Supplementary Table S3).

Besides the four loci that we used for our *in vitro* assays, we included six more loci in order to have a broader basis for the comparison of our data with published observations (Supplementary Figures S4 and S6, Supplementary Tables S3 and S4). To avoid missing any effects, we used rather harsh restrictive conditions (overnight incubation at 37°C), which compromised cell viability ($47 \pm 2\%$ for *arp9-ts* and $<5\%$ for *rsc3-ts* and *sth1-td* mutants). Nevertheless, it is very unlikely that this led to exaggerated or artifactual effects as our results were in excellent agreement with published data

or showed even a bit weaker effects, for example, at *ADH2* and *CHA1* (Supplementary Table S3 and Supplementary Figure S4). Further, we tested all loci in which we saw an effect in the *rsc3-ts* mutant after overnight incubation at 37°C also after 6.5 h, which are the same conditions as used by Badis *et al* (2008) for this same strain and raised the cell viability to $31 \pm 3\%$. We observed the same effects as those after overnight incubation (Supplementary Figure S7). In addition, the unchanged patterns at the *SNT1*, *ADH2* and *PHO84* loci (Figure 5D and Supplementary Figure S6) argue against globally compromised chromatin structures even under the harsh overnight restrictive conditions. The only single case in which we observed more of an effect than

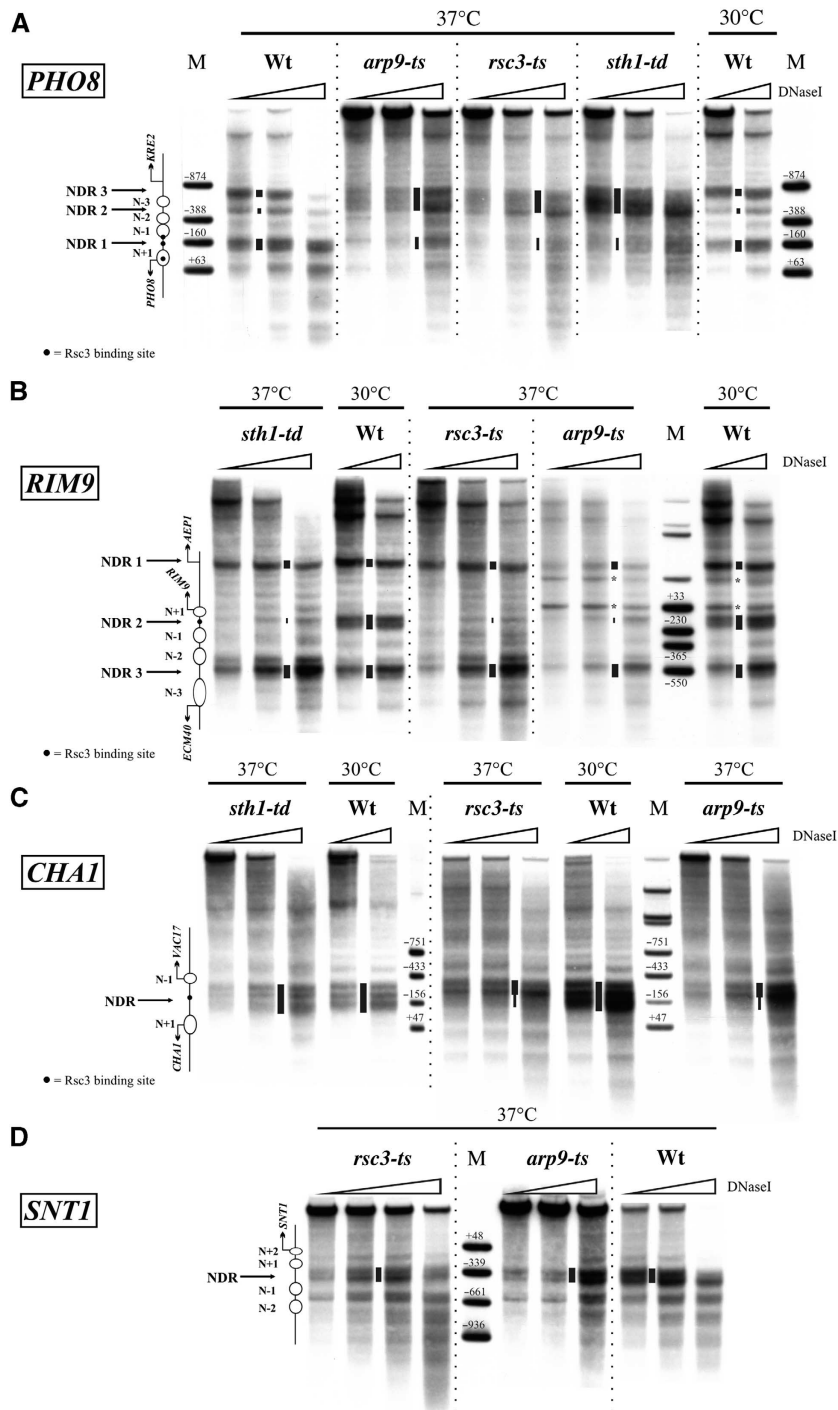


Figure 5 Loss of essential RSC subunits at elevated temperature altered chromatin structure at the *PHO8*, *RIM9* and *CHA1*, but not at the *SNT1* promoter. DNaseI indirect end labelling analysis of the (A) *PHO8*, (B) *RIM9* and (C) *CHA1* and (D) *SNT1* promoter regions *in vivo*. Nuclei were isolated from wild type (wt; BY4741) and strains carrying a temperature-sensitive (*rsc3-ts* (TH8247) and *arp9-ts* (YBC1536)) or temperature-sensitive degron (*sth1-td* (YBC2191)) allele of the indicated RSC subunits. Strains were grown logarithmically at 25°C and then shifted to the non-permissive temperature (37°C) overnight. Wt nuclei were also prepared from cells grown logarithmically at 30°C. Bars in-between lanes mark the intensity (bar width) and extent (bar length) of DNaseI hypersensitive sites. A stippled line separates samples that were not electrophoresed alongside on the same gel but combined in the figure. Asterisks indicate artefact bands. Ramps, markers and schematics as in Figure 2.

others was the altered *PHO8* promoter pattern in the *sth1-td* strain (see above). As this altered pattern was the same as that in the other two *ts* mutants (Figure 5A) and also that observed after shorter incubation times (Supplementary Figure S7A), it very likely reflects the true effect due to lack of RSC activity and could not be observed under the

milder restrictive conditions used by Parnell *et al* (2008) (2 h at 37°C).

In summary, both our own as well as published *in vivo* data confirm that ablation of RSC activity *in vivo* interferes with nucleosome positioning. Interestingly, this was only true if essential RSC subunits were ablated as deletion of the genes

encoding the non-essential RSC subunits Rtt102 or Rsc30 showed unaltered chromatin patterns at selected loci (Supplementary Figure S7 and data not shown). This clear demonstration of a role for RSC in nucleosome positioning *in vivo* argues that the RSC-dependent mechanism observed *in vitro* is not just coincidental but reflects the *in vivo* mechanism.

Discussion

In this study, we show for the first time by *in vitro* reconstitution that the RSC nucleosome remodelling complex is directly and specifically required to generate *in vivo*-like nucleosome positioning, especially to set up yeast promoter NDRs. There are a few cases in which RSC alone can properly determine nucleosome positioning. Nevertheless, RSC mostly requires other protein factors. Our findings provide strong evidence for the hypothesis that the *in vivo* nucleosome positioning machinery relies upon specific remodelling enzymes to correctly interpret nucleosome positioning cues given by the combination of DNA sequence in *cis* and other factors in *trans*. In other words, remodelling enzymes can be part of the nucleosome positioning information.

RSC is directly and specifically required, and in few cases even sufficient, to set up nucleosome positioning *in vitro*

A role of RSC in maintaining the NDRs at a large fraction of yeast promoters was suggested by three recent *in vivo* studies (Badis *et al*, 2008; Parnell *et al*, 2008; Hartley and Madhani, 2009). We confirmed these genome-scale results at the level of several individual promoters by DNaseI indirect end labelling using temperature-sensitive alleles of three different genes encoding essential RSC subunits. Very recently, a non-canonical RSC/nucleosome complex was suggested to reside within the NDR at the *GAL1-10* promoter and to have a fine-tuning role for promoter induction (Floer *et al*, 2010). We confirmed that ablation of RSC activity affected this NDR, especially in the *rsc3-ts* mutant (Supplementary Figures S6 and S7D).

However, our own *in vivo* data as well as previous reports on roles for RSC in nucleosome positioning are based on conditional mutants that are compromised cells under restrictive conditions so that indirect effects may contribute to the changes at promoter NDRs. In addition, such experiments cannot distinguish whether RSC was just necessary or also sufficient for NDR formation. To answer these questions, we tested purified RSC in our *in vitro* reconstitution system starting from salt gradient dialysis-assembled chromatin.

In most cases, purified RSC in the presence of ATP was unable to achieve the same degree of *in vivo*-like nucleosome positioning as seen with the WCE. Importantly, this was not due to a lack of RSC activity in our preparation. This RSC preparation was sufficiently active to allow remodelling of the chromatin templates as the DNaseI pattern of the starting material was clearly changed by addition of RSC and ATP. Even more to the point, the successful positioning of nucleosome N-1 at the *SNT1* and part of the NDR at the *CHA1* locus by purified RSC in the presence of ATP is proof of principle that RSC alone can be sufficient to achieve even *in vivo*-like nucleosome positioning under the assay conditions. Finally,

the same amount of purified RSC could completely rescue the proper nucleosome positioning activity of the *rsc3-ts* 37°C extract. Such an extract mimicked the *in vivo* phenotype in the sense that it was not able to generate the *in vivo*-like nucleosome positioning. As in the *in vivo* case, this could equally be caused by indirect effects of Rsc3 ablation on the activity of other factors. However, our rescue experiments strongly argue against indirect effects and show that RSC directly contributes to this nucleosome positioning activity. In addition, this experiment shows explicitly that additional factors from the extract are required in combination with RSC to generate the proper nucleosome positioning at most loci *in vitro*.

Intriguingly, both purified SWI/SNF and Isw2 remodelling enzymes failed to rescue the *rsc3-ts* 37°C extract, which argues that the RSC remodelling complex is specifically required for the nucleosome positioning activity and that only RSC can respond to the cues provided by the additional factors from the extract. Especially for the case of the SWI/SNF complex this specificity is somewhat surprising, as SWI/SNF and RSC are rather closely related remodelling enzymes with similar mechanistic properties in *in vitro* assays (Logie *et al*, 1999; Zhang *et al*, 2006), even sharing three subunits (Cairns *et al*, 1998). Nevertheless, both remodelling complexes may even have opposing roles; for example at the *PHO8* promoter. Activation of *PHO8* leads to a prominent chromatin transition at the *PHO8* promoter (Barbaric *et al*, 1992), which essentially depends on the remodelling enzyme SWI/SNF (Gregory *et al*, 1998). The enrichment of RSC over the region occupied by nucleosome N-3 (Venters and Pugh, 2009; and Supplementary Figure S4A) and the strikingly similar loss of nucleosome N-3, both upon RSC inactivation and upon promoter activation, suggests that RSC ensures the proper placement of N-3 under repressive conditions, whereas SWI/SNF overrides RSC and removes nucleosome N-3 under activating conditions.

The difference in remodelling enzyme specificity may reflect differences in recruitment specificity. The RSC complex contains two subunits, Rsc3 and Rsc30, which are able to recognize a specific DNA sequence (CGCGC). The location of this motif often overlaps with the sites of nucleosome occupancy change in the *rsc3-ts* mutant (Badis *et al*, 2008). Indeed, such Rsc3 sites are present and conserved at the *PHO8* promoter and other loci (Supplementary Figure 8A and B). However, recruitment of RSC through Rsc3/Rsc30 is unlikely the main or only reason for the specificity of RSC action. RSC was also necessary for proper formation of NDR3 at the *PHO8* promoter, both *in vivo* and *in vitro*, even though there is no Rsc3 site nearby. Further, *in vitro* nucleosome positioning at the *SNT1* locus, which does not contain an Rsc3 site, was strictly dependent on RSC.

Indeed, we were surprised that the *rsc3-ts* extract failed to reconstitute nucleosome positioning also at the *SNT1* locus, although along with others (Badis *et al*, 2008; Hartley and Madhani, 2009) we did not see significant changes here upon RSC ablation *in vivo*. Again, purified RSC was able to rescue *in vitro*. Why this discrepancy between the RSC requirement *in vivo* versus *in vitro* at *SNT1*? The *in vivo* experiment addresses the loss of properly positioned nucleosomes upon shift to the restrictive temperature, whereas *in vitro* reconstitution monitors the *de novo* generation of correct nucleosome positioning. Therefore, other factors may maintain

proper positioning *in vivo* even in the absence of RSC, while these factors are unable to set up proper positioning from scratch *in vitro*. Reb1 and Abf1 could be these factors at the *SNT1* locus, as they are redundantly involved in NDR formation. Only in an *abf1-td reb1-td* double mutant the *SNT1* NDR was compromised, but still not lost completely (Hartley and Madhani, 2009; Supplementary Figure S4D). It is also possible that nucleosome positioning at *SNT1* requires a particularly low concentration of RSC activity that is still present in the *ts* mutants even under restrictive conditions. *In vitro*, this low concentration may be even further reduced because of loss of activity during extract preparation or simply by unphysiological dilution. In any case, as a locus like *SNT1* was not scored in previous *in vivo* studies, the fraction of NDRs that depend on RSC *in vivo* may have been underestimated. Moreover, the presence of an Rsc3 site seems not to be a necessary indicator for a role of RSC.

Remodelling enzyme-intrinsic nucleosome positioning information

Our observation of the specific role for RSC in nucleosome positioning that could not be replaced by SWI/SNF or Isw2 agrees well with several *in vitro* studies showing that different remodelling enzymes have distinct sequence preferences for nucleosome positioning. These may differ significantly from the DNA-intrinsically favoured positions as determined by salt gradient dialysis (Brehm *et al*, 2000; Flaus and Owen-Hughes, 2003; Rippe *et al*, 2007; Schnitzler, 2008; Pham *et al*, 2009). We confirm this as purified RSC, SWI/SNF and Isw2 altered the salt gradient dialysis preassembled chromatin patterns at all tested loci (Figures 2 and 4). Others stressed that different remodelling enzymes moved nucleosomes with equal efficiency irrespective of the underlying DNA sequence (Partensky and Narlikar, 2009). We note that in many *in vitro* studies the patterns generated by different remodelling enzymes were mainly compared with each other and not to actual *in vivo* positions. In contrast, our system was always gauged relative to the gold standard of *in vivo* nucleosome patterns. In the light of the specificity of RSC in generating such proper patterns, we suggest that RSC not only provides the 'kinetic lubricant' for the equilibration of nucleosomes to stable positions determined by something else but also provides part of the positioning information in itself. This interpretation would also apply if the specificity of RSC function in nucleosome positioning was due to specificity of recruitment by some factor in the extract. In the case of nucleosome N-1 at the *SNT1* locus, the RSC-intrinsic information can be sufficient. Here, the combination of DNA, histones and RSC constitutes a self-organizing system yielding the exact nucleosome positioning, thus arguing against an exclusive recruitment mechanism.

Rippe *et al* (2007) suggested that remodelling enzyme-intrinsic preferences may be at the core of nucleosome positioning *in vivo* and accordingly proposed a 'remodeller code' for nucleosome positioning. Our *SNT1* data support this hypothesis to some extent. However, we showed that in most cases other factors in addition to RSC were required for proper positioning. Therefore, we think it unlikely that there is a pure 'remodeller code' for nucleosome positioning but a more diverse interplay of various factors.

A model of active non-equilibrium nucleosome positioning

Segal and Widom (2009) recently suggested an 'equilibrium model for dynamic nucleosome positioning', which assumes that nucleosomes equilibrate *in vivo* to their thermodynamically favoured positions as determined by the combined effects of intrinsic DNA features, neighbouring nucleosome exclusion, transcription factor binding, histone variants/modifications and DNA methylation. In this model, remodelling enzymes would act on nucleosome positioning only as 'kinetic lubricant', that is, as 'enzymes' (in addition to their enzymatic ATPase activity) that just help nucleosomes to overcome the activation energy barrier during the equilibration process without affecting the thermodynamics of nucleosome positions. We note that a living cell is not at equilibrium, but under steady-state conditions, so that there is no need to assume equilibrium nucleosome positioning. Accordingly, Segal and Widom (2009) explicitly remark that 'ATP-dependent chromatin remodelling complexes could actively subvert equilibrium'. As our data argue for remodelling enzyme-intrinsic nucleosome positioning in combination with other factors, we suggest that the input of energy from ATP-hydrolysis not only affects the kinetics of nucleosome positioning but also thermodynamically stabilizes positions even if they were energetically unfavourable otherwise (Korber and Becker, 2010). Indeed, we hypothesize that many if not most *in vivo* nucleosome architectures, as observed for example at promoter regions, are continuously and actively generated by ATP-dependent remodelling enzymes, and possibly other active processes, at the continuous expense of energy. The requirement for continuous energy input is incompatible with the assumption of equilibrium, but typical for the steady state of a living cell. It is to be noted that in our model as well, remodelling enzymes are necessary for nucleosome mobility on a physiologically relevant time scale. Therefore, once a nucleosome is positioned by a remodelling enzyme, it will stay there in a kinetically trapped state in the absence of remodelling activity. Hence, remodelling enzymes may determine nucleosome positions without remaining associated with the nucleosomes all the time.

Materials and methods

Strains and media

Yeast strains were as listed in Supplementary Table S1. Strains were grown in YPD with 0.1-g/l adenine and 1-g/l KH_2PO_4 , except for the *sth1-td* strain that was grown in YP with 0.1-g/l adenine and 1-g/l KH_2PO_4 containing 2% raffinose and 2% galactose. Temperature-sensitive strains were grown in 400-ml medium at 25°C to an OD_{600} of 1.2–1.5 (spectrophotometer PMQ II, Zeiss, Germany). An equal volume of medium prewarmed to 49°C was added and the cultures were placed at 37°C for the indicated temperature overnight. Viability of temperature-sensitive mutants after overnight incubation under restrictive conditions was determined by comparing the number of single colonies after plating the same number of cells for mutant and wt (BY4741 for *rsc3-ts* and *arp9-ts* or YBC2192 for *sth1-td*) treated in parallel on YPDA plates at 25°C.

Yeast WCE preparation

The WCE were prepared as described (Wippo *et al*, 2009), with the following modifications. The extract used for the fractionation was made from commercially available baker's yeast concentrate (Deutsche Hefewerke GmbH, Nürnberg, Germany). The wild-type extract for all other experiments was made from strain BY4741-grown logarithmically at 30°C. For extract preparation of TH8239

(*rsc3-ts*) at permissive conditions, cells were grown at 25°C and overnight at 37°C for non-permissive conditions.

Chromatin assembly and reconstitution

Chromatin was assembled by salt gradient dialysis, treated with WCE and analysed as described (Wippo *et al*, 2009). A measure of 0.5 µg of plasmid pUC19-*PHO8*-short per salt gradient assembly reaction was used for experiments in Figure 1C, and a mix of 200 ng each of plasmids pUC19-*PHO8*-long, pUC19-*RIM9*, pUC19-*CHA1* and pUC19-*SNT1* per assembly reaction for experiments in Figures 2–4. For a detailed description of plasmids, see the Supplementary data.

Yeast nuclei preparation

Nuclei were prepared as described (Almer *et al*, 1986).

Yeast WCE fractionation

For a detailed description of the individual fractionation steps, see the Supplementary data.

Purification of remodelling enzymes

RSC2-TAP, SWI2-TAP and ISW2-FLAG were purified as described (Smith *et al*, 2003).

Binding site prediction

The Find Individual Motif Occurrences (FIMO) program (Version 4.1.0; available at http://meme.sdsc.edu/meme4_1/cgi-bin/fimo.cgi) was used to predict sites for Rsc3 at the *PHO8*, *RIM9*, *CHA1*, *SNT1*, *RIO1*, *RNR3*, *GAL10*, *PHO5*, *PHO84* and *ADH2* promoters. The position weight matrix was obtained from Supplementary Table S6 of Badis *et al* (2008). We note that a simple search for the Rsc3 motif CGCGC identifies the same sites as the FIMO program.

Rsc3 site alignment

The orthologous sequences for *PHO8*, *RIM9*, *CHA1*, *GAL10* and *RIO1* from *S. paradoxus*, *S. mikatae*, *S. bayanus*, *S. kudriavzevii*, *S. castellii* and *S. kluyveri* were taken from Kellis *et al* (2003) and Cliften *et al* (2003). The ORF sequence plus 1000 bp upstream from each yeast species were aligned with the ClustalW2 program

(<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) using the default settings.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: CJW designed and performed the vast majority of all experimental work. LI performed LC-MS/MS analysis. SW purified RSC, SWI/SNF and Isw2 complexes in the group of CLP. AH helped with the extract fractionation. PK conceived and supervised the entire study. CJW, CLP and PK wrote the manuscript. This paper is dedicated by CJW to his parents.

Conflict of interest

The authors declare that they have no conflict of interest.

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