The RSC chromatin remodeling complex has a crucial role in the complete remodeler set for yeast PHO5 promoter opening

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Received July 30, 2013; Revised November 28, 2013; Accepted December 19, 2013

ABSTRACT

Although yeast PHO5 promoter chromatin opening is a founding model for chromatin remodeling, the complete set of involved remodelers remained unknown for a long time. The SWI/SNF and INO80 remodelers cooperate here, but nonessentially, and none of the many tested single or combined remodeler gene mutations could prevent PHO5 promoter opening. RSC, the most abundant and only remodeler essential for viability, was a controversial candidate for the unrecognized remodeling activity but unassessed in vivo. Now we show that remodels the structure of chromatin (RSC) is crucially involved in PHO5 promoter opening. Further, the isw1 chd1 double deletion also delayed chromatin remodeling. Strikingly, combined absence of RSC and Isw1/Chd1 or Snf2 abolished for the first time promoter opening on otherwise sufficient induction in vivo. Together with previous findings, we recognize now a surprisingly complex network of five remodelers (RSC, SWI/SNF, INO80, Isw1 and Chd1) from four subfamilies (SWI/SNF, INO80, ISWI and CHD) as involved in PHO5 promoter chromatin remodeling. This is likely the first described complete remodeler set for a physiological chromatin transition. RSC was hardly involved at the coregulated PHO8 or PHO84 promoters despite cofactor recruitment by the same transactivator and RSC's presence at all three promoters. Therefore, promoter-specific chromatin rather than transactivators determine remodeler requirements.

INTRODUCTION

Promoters in Saccharomyces cerevisiae may be broadly divided into two classes: open and covered (1,2). Open promoters have stereotypic chromatin architecture with an ~150 bp nuclease hypersensitive site, also called nucleosome-free region (NFR), just upstream of the transcriptional start site. This provides an ‘open door policy’ (3) for transcription factor binding and assembly of the preinitiation complex (PIC). Accordingly, such promoters drive constitutive and rather steady expression of growth or housekeeping genes. In contrast, covered promoters have nonstereotypic nucleosome arrangements. Their name-coining feature is a repressed state where the position of the stereotypic NFR, i.e. sites for PIC assembly (TATA boxes), as well as other transcription factor binding sites (upstream activating sequence (UAS) elements), is covered by nucleosomes. NFRs here are usually shorter, further upstream and often contain sites for ‘pioneer factors’, which initiate promoter opening. Such promoters drive inducible or stress genes and markedly depend on chromatin cofactors, as their activation requires nucleosome remodeling. Even though they are the minority in yeast, the covered promoters are prime models for gene regulation by switching chromatin states.

This promoter class distinction was recently derived from genome-wide studies, while earlier work on individual promoters, especially the PHO promoters, established the basic principles of regulation through chromatin, e.g. the direction of causality beyond mere correlation. The PHO5 promoter was one of the first models for gene regulation through chromatin remodeling and is an archetype of covered promoters (2,4). Four positioned nucleosomes package this promoter under repressive (high phosphate) conditions such that nucleosome -1 covers the TATA box and nucleosome -2 the high affinity binding site (UASp2) for the specific transactivator Pho4 while a short NFR between the nucleosomes -2 and -3 contains a low affinity UASp1 [Supplementary Figure S1A (5)]. The PHO84 promoter is a covered promoter, too, as the TATA box and two UASp sites are occluded by nucleosomes in the repressed state while a short NFR is far upstream and contains two more UASp sites [Supplementary Figure S1B.
The PHO8 promoter has a bit blended nucleosome organization but is also not a stereotypical open and rather a covered promoter [Supplementary Figure S1C (8)]. All three PHO promoters are coregulated by Pho4 (6,8–10), induced by phosphate starvation, and their opening requires massive chromatin remodeling (5,6,8), but with markedly different cofactor requirements (6,11–13).

Historically, chromatin remodeling seemed to result from transcription or require replication. However, pioneering experiments showed that PHO5 promoter chromatin could be switched between its open and closed state in from transcription or require replication. However, pion-

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independent ways to remodel chromatin. This identified ATP-dependent chromatin remodeling enzymes (16) and their distinct subfamilies (17,18). Therefore, it seemed straight forward to identify a particular remodeler that is responsible for a given chromatin transition. However, PHO5 promoter opening proved more complicated. The SWI/SNF and INO80 complexes are involved here, but not essential, as their individual or combined lack only led to delayed but finally rather complete PHO5 promoter chromatin remodeling (13,19–23). All other remodeler ATPases encoded by nonessential genes could be removed individually without appreciable effect on PHO5 induction (13,23,24). Recently, it was reported that the combined absence of Isw1 and Chd1 remodelers caused delayed PHO5 expression upon phosphate starvation and abolished PHO5 transcription under semi-inducing conditions, but chromatin remodeling was not assessed in vivo (25). Collectively, some remodelers were shown or suggested to be involved in PHO5 promoter opening, but none were essential and a major remodeling activity seemed unrecognized. In contrast, PHO8 promoter opening depended critically upon SWI/SNF (11), which was thought to settle this case. Nonetheless, we found later that INO80 contributes here importantly, too (13). Finally, the PHO84 promoter is like a hybrid between the PHO8 and PHO5 promoters, as it harbors one nucleosome, so called ‘upstream nucleosome’ (Supplementary Figure S1C), that uses INO80 and strictly requires SWI/SNF for remodeling, just like the PHO8 promoter nucleosomes, while its neighboring nucleosome, so called ‘downstream nucleosome’, has similarly relaxed remodeler requirements as PHO5 promoter nucleosomes (6).

Apparently, at the PHO5 and part of the PHO84 promoter, several remodelers cooperate to activate wild type (wt) promoter opening kinetics, but form a redundant network with regard to eventually opening the promoter. This makes it difficult to identify all members of this network in vivo. It seemed that more remodeler ATPase genes would need to be deleted in the same cell to prevent PHO5 promoter opening than viability would allow. In particular, the question whether the remodels the structure of chromatin (RSC) complex, the most abundant (26) and only chromatin remodeling enzyme essential for viability (27), was involved and maybe even uniquely essential for PHO5 promoter chromatin remodeling was not directly assessed in vivo so far.

The RSC complex is globally involved in positioning and remodeling of nucleosomes (28–30). Especially the Kornberg group considered whether RSC was the critical remodeler for PHO5 promoter opening. They showed in vitro nucleosome disassembly by RSC (31), which fits to the observed histone eviction in vivo (22,32). As on average all but one PHO5 promoter nucleosome become disassembled at the same time in vivo (24,32,33), they suggested sliding-mediated disassembly (34), which is a mechanism proposed for the RSC complex (34,35). In agreement, we also speculated that RSC could be one or even the decisive remodeler for PHO5 promoter opening (13). However, the Kornberg group recently dismissed this notion owing to in vitro experiments and indirect in vivo evidence (25,36). Owing to the lethal phenotype of RSC inactivation, RSC’s role in PHO induction is difficult to study in vivo. Physiological PHO induction relies on intracellular phosphate depletion (20,37,38), which is only achieved by net phosphate consumption during cell growth and DNA replication. Therefore, it is difficult to fully deplete RSC and at the same time to induce PHO genes by phosphate starvation in nongrowing cells. So the question about RSC’s role remained unsolved.

In carefully controlled in vivo experiments using a temperature-sensitive allele of the gene encoding the RSC ATPase subunit Sth1 (28), we demonstrate now a clear, but likely nonessential, role for RSC in PHO5 promoter opening, both for the physiological as well as several alternative induction pathways. Upon submaximal induction, RSC became essential. In addition, we show directly that the combined absence of Isw1 and Chd1 delayed but did not prevent PHO5 promoter chromatin remodeling upon phosphate starvation. Importantly, RSC became essential even under full induction conditions if combined with the absence of Isw1/Chd1 or Snf2, indicating a major role of the RSC complex in PHO5 promoter opening. Collectively, our here presented results, together with previous findings (13,19–25,32,39), give a clear and maybe final picture of the remodelers involved at the PHO5 promoter. There is a cooperative network of five remodelers from four major subclasses: RSC, SWI/SNF, INO80, Isw1 and Chd1. This may be the first case where the complete set of remodelers involved in a physiological chromatin transition is reported.

Surprisingly, neither PHO8 nor PHO84 promoter opening depended much on RSC. So RSC is not necessary in general, and its requirement is apparently determined by a particular chromatin structure rather than by a particular transactivator. Nonetheless, at all three model promoters more than one remodeler from more than one subfamily cooperate towards chromatin opening. This suggests more complex remodeling mechanisms in general than anticipated.

MATERIALS AND METHODS

Strains, media, plasmids and strain construction

Saccharomyces cerevisiae strains used in this study are listed in Supplementary Table S1. Yeast strains were grown in YPDA [containing per liter 20g BactoTM
Peptone (Becton, Dickinson and Company, catalog no. 211677), 10 g yeast extract (Biolife, catalog no. 4122202), 20 g glucose, 0.1 g adenine and 1 g KH₂PO₄, or for plasmid-bearing strains, in YNB selection medium [containing per liter 6.7 g Difco™ Yeast Nitrogen Base without Amino Acids (Becton, Dickinson and Company, catalog no. 291920), 2 g drop-out mix as in (40) but with 6-fold more adenine, 2-fold more lysine, 10-fold less p-aminobenzoic acid and without histidine, uracil, leucine and tryptophane, 20 g glucose and 1 g KH₂PO₄] supplemented with the required amino acids and uracil (80 mg/l each), as repressive conditions (high phosphate), and in phosphate-free synthetic medium for induction by phosphate starvation (5,6,13,41). Strains containing the sth1Δ allele were always grown under minus uracil selection (5,6,13,41). For transfer to phosphate-free medium and 37°C at the same time, cells were washed in water (24°C), resuspended in phosphate-free medium prewarmed to 37°C and further incubated at 37°C. If only the temperature was changed, cells were diluted 1:1 or 2:1 with medium prewarmed to 37°C and further incubated at 37°C. For galactose induction of strains carrying the plasmid pP₄ho5v33-lacZ, cells were pregrown in YNB medium with 2% (w/v) raffinose and induced by the addition of galactose to a final concentration of 2% (w/v). The budding index was determined by counting budded cells relative to nonbudded cells under the microscope. Plasmid pP₄ho5v33-lacZ is a derivative of the pPHO5lacZ reporter plasmid (42), in which the two Pho4 binding sites at the PHO5 promoter, UASp1 and UASP2, were replaced by Gal4 binding sites (12,13,41,43). Plasmid pRS306.SthlOΔ (p1622) is derived from pRS306 (44) and carries the Ub-DHFR ts-HA-STH1 fusion (28) (kind gift of B. R. Cairns). It was integrated into the STH1 locus after cutting with PmII at the STH1 ORF nucleotide position 262 and transforming the linear fragment. The correct integration of the sth1Δ allele was verified by PCR using the primers BC2095 CAGGGGTCTTCTGAGGTCCA (universal (CUP1) degron verification) and BC2097 CTTTCGCACT TGAAATCTGCTGA (sth1Δ degron verification) and produced a ~0.7-kb PCR product in sth1Δ strains, but no product after no or wrong integration. Plasmid pPHO85F82G was constructed by transferring into pRS305 (44) a DraIII-Sall fragment from the plasmid based on pRS304 (44) containing the pho85F82G gene driven by the PHO85 promoter described previously (45) (kind gift of E. K. O’Shea). Plasmid pPHO85F82G was integrated at the LEU2 locus of YS30 (pho85Δ) by transformation after cutting with AflII at the LEU2 position 315 from ATG. 1-NaPP1 was obtained from Toronto Research Chemical Inc., stored as stock solution of 4 mg/ml in DMSO and added to a final concentration of 10 µM.

### Enzyme activity assays and chromatin analysis

Acid phosphatase assays were done as described previously (46,47) with slight modifications. Intact whole cells resuspended in 0.1 M acetate buffer pH 3.6 were assayed for acid phosphatase activity with 10 mM p-nitrophenylphosphate. The reaction was performed at 30°C, stopped by addition of NaOH to a final concentration of 0.2 M, and absorbance at 410 nm was measured. Enzyme activity was calculated as A₄₁₀ × 1000/[OD₆₀₀ × (volume/ml) × (t/min)]. OD₆₀₀ was measured using a Zeiss PMQII photometer. Activity of β-galactosidase was measured as described previously (42), but using chloroform instead of toluene. The preparation of yeast nuclei and chromatin analysis of nuclei by restriction nucleosome accessibility assays were as described previously (5,48–50). 120 U of restriction enzymes were used for chromatin analysis of nuclei, except for TaqI of which 2000 U were used. In representative experiments, we controlled that restriction enzyme concentration was not limiting by using 2-fold more enzyme (240 U). Restriction enzymes for secondary cleavage and probes for hybridization were as described previously (5,6,8,13). Quantification of the percentage of cleaved DNA was done by PhosphorImager analysis (Fuji FLA3000) with Aida Image Analyzer software v.3.52. Error bars show either the range of two or the standard deviation of more than three biological replicates.

### Western blot analysis

At the conditions indicated in the legend to Supplementary Figure S2A, cells (CY337 or CY337 sth1Δ) were resuspended in 100 µl 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 12% glycerol, 0.1% TritonX-100 (Sigma-Aldrich, #T8787), 1 mM DTT, 1 µg protease inhibitor cocktail (Complete™ without EDTA, Roche Applied Science) per 7.5 OD₆₀₀ (Zeiss PMQII), lysed by bead beating (0.5-mm glass beads, Carl Roth #N030.1; three cycles of 1-min vortexing followed by 1-min incubation on ice) and clarified by centrifugation (14000 rpm, 4°C, 4 min, Eppendorf 5417R centrifuge). Supernatants were transferred to fresh tubes and total protein concentrations determined by Bradford assay according to manufacturer’s protocol (BIO-RAD Protein Assay, #500-0006). Approximately 80 ng protein were separated by SDS-PAGE (NuPAGE® Novex® 4-12% Bis-Tris Protein Gels, Invitrogen, catalog no. NP0321BOX) and blotted over night [12 h, 12 V, 4°C, nitrocellulose membrane (Machery & Nagel, catalog no. 741280)] according to manufacturer’s (Invitrogen) protocols. Membranes were blocked in 1× TBS-Tween [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 (v/v, Sigma-Aldrich, #P7949)] and then incubated with an anti-HA antibody (Roche, catalog no. 11867423001) and a goat anti-rabbit (GE healthcare, catalog no. NA934V) and anti-rabbit (GE healthcare, catalog no. NA935V) for 1 h at room temperature and washed again three times with TBS-Tween for 10 min. Secondary antibodies were detected using ECL detection reagent (GE healthcare, catalog no. RFN 2106).
and medical X-ray films (Fujifilm, catalog no. 47410 19236) according to manufacturer’s protocols.

RESULTS

Experimental strategy

To examine the role of RSC in PHO promoter opening and activation in vivo, we used a temperature-sensitive sth1td allele (28). This allele encodes the fusion of Sth1 to a DHFR tag, which becomes unfolded at the restrictive temperature of 37°C and is targeted for degradation by the E3 ubiquitin ligase Ubr1 (51). As induction of a PHO5 promoter variant through the GAL system was part of our study, we relied on the native UBR1 instead of the GAL1 promoter for UBR1 regulation. Shifting of these sth1td cells to 37°C led to ablation of Sth1td (Supplementary Figure S2A) and to growth delay and arrest, i.e. a strong sth1 phenotype, within 6–8 h (Supplementary Figure S2B). With regard to the viability of cells after ablation of Sth1td see also below and Figure 5A.

Figure 1. Ablation of RSC activity concomitant with induction through phosphate starvation delays opening and activation of the PHO5 promoter. (A) Pho5 acid phosphatase induction kinetics for wt (CY337) and the corresponding sth1td cells after shift to phosphate-free medium and 37°C. (B) As (A) but after shift to phosphate-free medium at the indicated temperatures and time point. (C) Kinetics of promoter opening as monitored by ClaI accessibility at nucleosome -2 of the PHO5 promoter after induction as in (A). (D) As (C) but monitoring HhaI accessibility at nucleosome -1. Schematics in this and all following figures represent experiment design. Entries in parentheses apply not to all cells, e.g. Sth1td ablation occurred only in sth1td cells.

Depletion of Sth1 delayed PHO5 promoter chromatin remodeling and activation of PHO5 expression during physiological induction in phosphate-free medium

We first combined inactivation of Sth1td with strong physiological PHO induction by incubation in phosphate-free medium. Wild-type and sth1td cells were shifted from phosphate-rich medium at 24°C to phosphate-free medium at 37°C. PHO5 expression was monitored by acid phosphatase activity (Figure 1A and B), and promoter opening by ClaI accessibility (Figure 1C), which monitors remodeling of nucleosome -2 as well established proxy for remodeling of the whole promoter (5,12,13,41,50,52).

The sth1td mutant showed reduced phosphatase activity upon induction at 37°C (Figure 1A), but no effect in preinduction (Figure 1A, 0 h time point) and final levels at 24°C (Figure 1B). Chromatin opening was delayed early (Figure 1C, 2 h time point), but finally reached wt levels (Figure 1C). This was also true for the HhaI site in the TATA box-containing nucleosome -1 [Figure 1D, Supplementary Figure S1A (5)].
This suggested that the slower phosphatase induction kinetics were due to slower chromatin remodeling while the reduced final activity level was not due to incomplete chromatin opening, but probably reflected Sth1\textsuperscript{td} ablation effects downstream of chromatin remodeling, which is not followed up further here.

These results argue for a relevant, but nonessential, role of RSC in PHO5 promoter opening. However, there is a 2-fold caveat as we observed Sth1\textsuperscript{td} inactivation and PHO5 induction in parallel. First, early upon shift to 37°C some active Sth1\textsuperscript{td} was still present (Supplementary Figure S2A), which allowed at least one more round of replication (Supplementary Figure S2C). This residual Sth1 activity may have caused the observed PHO5 promoter opening, assuming that the promoter did not close again upon further Sth1 depletion. Complete Sth1\textsuperscript{td} ablation before induction may have stronger effects on promoter opening. However, this experiment is difficult to do properly by induction through phosphate starvation, as this depends on replication (14) and therefore on Sth1 (Supplementary Figure S2B and C). Second, Sth1 ablation may affect PHO signaling by compromising growth and/or some factor(s) in the signaling pathway. So the observed effects may seem either weaker (first caveat) or stronger (second caveat) than they truly are.

The effect of RSC inactivation on PHO5 promoter opening is not due to cell cycle defects or compromised PHO signaling and is exacerbated by weaker induction

Regarding the second caveat, we wondered if the difference in PHO5 induction between wt and sth1\textsuperscript{td} cells during phosphate starvation at 37°C was mainly due to the G2/M cell cycle arrest that is caused by Sth1 inactivation (53–55). To compare both strains under the same cell cycle arrest conditions, we used PHO5 induction conditions previously described by Pondugula \textit{et al.} (56). Here, the gene \textit{CDC20} encoding an essential activator of the anaphase-promoting-complex/cyclosome (APC/C) is placed under control of the \textit{GAL1} promoter (P\textsubscript{GAL}::\textit{CDC20}). Addition of glucose switches off this promoter and therefore \textit{CDC20} expression, which arrests cells in M phase. The \textit{PHO5} promoter can still be induced in such arrested cells by shift to phosphate-free medium (56). We reproduced such PHO5 induction in a P\textsubscript{GAL}::\textit{CDC20} \textit{STH1} strain and compared it with induction of P\textsubscript{GAL}::\textit{CDC20} sth1\textsuperscript{td} cells during ablation of Sth1\textsuperscript{td} by shift to phosphate-free medium and 37°C (Figure 2A). Also, under these conditions, lack of Sth1\textsuperscript{td} strongly impaired PHO5 expression arguing for a direct RSC effect and against an indirect cell cycle arrest effect.

To address the second caveat more specifically with regard to the PHO signaling pathway, we turned to the pho80\textsuperscript{ts} allele (57) encoding a temperature-sensitive version of the negative factor Pho80 (10,58). Inactivation of Pho80\textsuperscript{ts} at 37°C bypasses the physiological signaling pathway and allows switchable PHO induction in otherwise repressive phosphate-rich medium and in the absence of replication (14).

To again eliminate effects of different growth for \textit{STH1} pho80\textsuperscript{ts} versus sth1\textsuperscript{td} pho80\textsuperscript{ts} cells during incubation at 37°C, we arrested both strains prior to shift to 37°C, but now in G1 phase, by incubation in histidine-free medium. We reproduced complete opening of the PHO5 promoter after 2h at 37°C in \textit{STH1} pho80\textsuperscript{ts} cells in the absence of replication (14). In contrast, chromatin remodeling in the sth1\textsuperscript{td} pho80\textsuperscript{ts} strain was diminished even at later time points (Figure 2B). The same was observed without prior histidine removal (data not shown) or using cells in late log phase after growth plateaued (Supplementary Figure S3A). Therefore, impaired chromatin opening upon Sth1\textsuperscript{td} inactivation was a direct effect rather than indirectly caused by growth arrest, and independent of the PHO signaling cascade thus occurring downstream from Pho4 activation, i.e. downstream from nuclear accumulation of nonphosphorylated Pho4 (58,59).

Importantly, PHO induction through Pho80\textsuperscript{ts} inactivation is weaker than through pho80 deletion, which is again weaker than through phosphate starvation (14). Weaker induction leads to more stringent cofactor requirements for PHO5 promoter opening (13,20,21,41,60). Therefore, Sth1\textsuperscript{td} inactivation impaired promoter opening more strongly upon Pho80\textsuperscript{ts} inactivation than upon phosphate starvation. We could observe this aggravating effect of suboptimal induction strength side by side starting from the same cells by comparing the shift of \textit{STH1} pho80\textsuperscript{ts} and sth1\textsuperscript{td} pho80\textsuperscript{ts} cells to 37°C and into either phosphate-rich or phosphate-free medium (Supplementary Figure S3B).

At high phosphate, which corresponds to the weaker induction conditions, there was clear PHO5 activation in \textit{STH1} pho80\textsuperscript{ts}, but none in sth1\textsuperscript{td} pho80\textsuperscript{ts} cells. In contrast, there was substantial activation also in sth1\textsuperscript{td} pho80\textsuperscript{ts} cells, albeit again impaired relative to \textit{STH1} pho80\textsuperscript{ts} cells, under the stronger induction conditions in phosphate-free medium. Note that the first caveat above also relates to these experiments as PHO induction and Sth1\textsuperscript{td} inactivation occurred in parallel.

To address this first caveat, i.e. to uncouple RSC ablation and PHO induction, we used the cell-permeable small molecule inhibitor 1-\textit{Nappp1} (4-amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine), which specifically inhibits the otherwise fully functional mutant form Pho85 by inactivation of the Pho80-dependent kinase Pho85 (45). Similar to the pho80\textsuperscript{ts} allele, such induction is also weak, bypasses PHO signaling, i.e. works at high phosphate conditions, but allows depletion of Sth1\textsuperscript{td} prior to induction.

Wt and sth1\textsuperscript{td} cells were incubated for 5.5h at 37°C leading to growth arrest of the sth1\textsuperscript{td} cells and effective depletion of Sth1\textsuperscript{td} (Supplementary Figure S2A and B). To compare only arrested cells, even though in different cell cycle phases (G2/M versus G1), wt cells were arrested in uracil-free medium. Two hours of induction by addition of 1-\textit{Nappp1} were sufficient for extensive PHO5 promoter opening in \textit{STH1} cells whereas sth1\textsuperscript{td} cells hardly increased CiaI accessibility throughout the time course (Figure 2C). Therefore, Sth1 was essential for PHO5 promoter opening under such weak induction conditions. As shown in Figure 2A, the G2/M arrest due to RSC inactivation was unlikely responsible for this lack of promoter opening.

We conclude that indirect effects due to RSC inactivation, neither on cell cycle progression nor on PHO
signaling, were not responsible for the PHO5 induction defects. RSC rather has a direct role in PHO5 promoter opening.

**RSC is not essential for PHO5 promoter opening upon strong activation by Gal4 even after more complete Sth1<sup>td</sup> depletion**

A PHO5 promoter variant (variant 33) with both UASp elements replaced by high affinity Gal4 binding UASg elements (43) was activated by Gal4 with the same cofactor requirements as the native promoter (12,13,41). Ablation of Sth1 <em>in vivo</em> had no effect on GAL1 promoter activation after shift from raffinose- to galactose-containing medium (61), thus excluding effects on the GAL signaling pathway. With this PHO5 promoter variant, we could address both of the above caveats: strong ablation of Sth1<sup>td</sup> before induction and without effects on the induction pathway. Importantly, GAL induction of PHO5

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**Figure 2. PHO5 promoter opening is impaired by ablation of RSC activity also upon induction in growth-arrested cells and through alternative pathways.** (A) Kinetics of PHO5 promoter activation in <span><span><span>&#39;GAL::CDC20 STH1 (strain 565) versus &lt;span&gt;</span><span>&lt;span&gt;GAL::CDC20 sth1<sup>td</sup> (strain 565 sth1<sup>td</sup>) cells after M phase arrest in glucose- and phosphate-containing media for 3 h at 24°C followed by degron induction due to shift to 37°C and concomitant further PHO5 activation by shift to phosphate-free, or to phosphate-containing medium as control. (B) Kinetics of ClaI accessibility at nucleosome -2 of the PHO5 promoter in pho80<sup>ts</sup> (YS44) and the corresponding sth1<sup>td</sup> cells after shift to 37°C, which induces the PHO regulon by inactivation of Pho80<sup>ts</sup> and ablates Sth1<sup>td</sup>. Cells were growth arrested in G1 phase 1 h before induction by histidine removal. (C) Kinetics of ClaI accessibility in pho85<sup>F82G</sup> (YS30 pho85<sup>F82G</sup>) and the corresponding sth1<sup>td</sup> cells after preincubation for 5.5 h at 37°C for depletion of Sth1<sup>td</sup> and then PHO induction by addition of the Pho85<sup>F82G</sup> kinase inhibitor, 1-NaPP1, at 37°C for the indicated times. Removal of uracil 1 h before induction for STH1 cells led to growth arrest in G1 phase, which is also a growth arrest but distinct to the G2/M phase arrest in sth1<sup>td</sup> cells after 5.5 h preincubation at 37°C.
promoter variant 33 is stronger than via inactivation of Pho80\(^{\circ}\), Pho85\(^{\circ}\)/StH1\(^{\circ}\) or even phosphate starvation.

Wt and sth1\(^{\circ}\) cells bearing a plasmid where the PHO5 promoter variant 33 drives lacZ expression (12,13,41,43) were incubated for 6 h in raffinose medium at 37\(^{\circ}\)C leading to rather complete StH1\(^{\circ}\) depletion in sth1\(^{\circ}\) cells, as indicated by growth arrest (data not shown). Kinetics of promoter activation by galactose, monitored by \(\beta\)-galactosidase activity, were strongly affected in the absence of StH1\(^{\circ}\), both in rate as well as in final level (Figure 3A). Promoter opening was also delayed but reached almost wt levels (Figure 3B). These effects on activity and chromatin opening were reminiscent of those on the wt promoter induced by phosphate starvation (Figure 1A and C). Importantly, G\(\text{AL}\) induction worked well in cells arrested in histidine-free medium, and preincubation of nongrowing sth1\(^{\circ}\) cells in histidine-depleted medium had no effect (Supplementary Figure S4).

Thus, RSC has an important but probably not essential role in PHO5 promoter chromatin remodeling upon such strong induction, even after rather complete StH1\(^{\circ}\) depletion. Nonetheless, we cannot exclude that residual low RSC levels even after incubation at 37\(^{\circ}\)C for 6 h (Supplementary Figure S2A) were sufficient and masked a truly essential RSC requirement. The reduced final activity level despite complete promoter opening argues again for effects downstream of chromatin opening, and these are independent of the transcribed gene (PHO5 versus lacZ).

Either the RSC or the SWI/SNF complex is necessary for PHO5 promoter chromatin remodeling upon phosphate starvation

As we had now strong evidence that the RSC complex contributed to the cooperative network for PHO5 promoter opening, we tested the interplay of RSC and SWI/SNF with an snf2 sth1\(^{\circ}\) double mutant. While PHO5 became substantially induced upon phosphate starvation in both single mutants already after 6 h of induction, StH1\(^{\circ}\) depletion in the snf2 background resulted in poor induction (Figure 4A), no chromatin opening at early, and rather low levels at later time points (Figure 4B). So the presence of either the SWI/SNF or the RSC complex was necessary for eventually full PHO5 promoter chromatin remodeling under such induction conditions.

To put this result in perspective, we re-examined PHO5 activation in the previously studied snf2 ino80 and snf2 asf1 double mutants (13), but now also at 37\(^{\circ}\)C. Even here the PHO5 promoter was strongly activated (Supplementary Figure S5), just somewhat less than at 30\(^{\circ}\)C (13). Therefore, the low levels of PHO5 promoter opening and activation in the snf2 sth1\(^{\circ}\) double mutant were not simply due to the combination of a rather impaired double mutant with high temperature.

 Nonetheless, we additionally controlled for the caveat that the inability of the snf2 sth1\(^{\circ}\) mutant to open and activate the PHO5 promoter was due to lower strength of PHO induction caused by poorer growth and earlier growth arrest compared with the other mutants. First, for up to 13 h of incubation in phosphate-free medium at 37\(^{\circ}\)C, the growth rate of the snf2 sth1\(^{\circ}\) mutant was not much reduced compared with that of the snf2 mutant (Supplementary Figure S6), which could finally open the PHO5 promoter [Figure 4B (13,20–23,39)]. Second, snf2 sth1\(^{\circ}\) as well as sth1\(^{\circ}\) cells that stopped to grow during incubation in phosphate-free medium at 37\(^{\circ}\)C were divided into two aliquots: one was shifted to the permissive temperature for StH1\(^{\circ}\) (24\(^{\circ}\)C) and the other was further incubated at 37\(^{\circ}\). snf2 sth1\(^{\circ}\) cells shifted to the permissive temperature did not resume growth (Supplementary Figure S6) probably owing to lack of phosphate. Nonetheless, the incubation at the permissive temperature brought about further activation of the PHO5 promoter in the snf2 sth1\(^{\circ}\) as well as in sth1\(^{\circ}\) mutants up to the level of the corresponding STH1 cells, i.e. snf2 and wt cells, respectively (Figure 5A and B).
This argues in two ways: For one, ablation of Sth1td did not substantially kill the cells, but they were able to resume full PHO5 activation after repletion of Sth1td. Second, full reactivation was achieved without further growth and therefore without further depletion of intracellular phosphate and consequently without increase in induction strength. So it was indeed the inactivation of the RSC complex in the snf2 background that prevented appreciable PHO5 promoter opening and activation and not just weak PHO induction strength.

We did the same kind of experiment using the Gal4-activated PHO5 promoter variant 33 with the difference that Sth1td was again ablated prior to induction and that effects after repletion of Sth1td were monitored in growing cells. Also here, upon strong induction, there was not much chromatin opening after prolonged induction in the snf2 sth1td double mutant in comparison with the uninduced state and in contrast to full opening in the sth1td and snf2 single mutants (Figure 6). Upon repletion of Sth1td in the snf2 sth1td strain by shift to the permissive temperature, cell growth (Supplementary Figure S7A) as well as promoter activation (Supplementary Figure S7B) recovered to the levels of the snf2 cells. Thus, growth arrest and impaired PHO5 promoter opening were due to Sth1td ablation.

RSC becomes critically important for PHO5 promoter chromatin remodeling also in the combined absence of Isw1 and Chd1

The individual absence of the remodeler ATPases Chd1 and Isw1 caused no or only slight effects on PHO5 promoter induction (13,23,24). Nonetheless, a chd1 isw1 double mutant displayed delayed induction upon...
phosphate starvation and almost no induction under pho80 high phosphate conditions (25). The exacerbated phenotype under pho80 induction agrees again with generally more stringent cofactor requirements at lower induction strength (see above) and makes an indirect effect on PHO signaling unlikely. As a defect in chromatin opening was not directly assessed so far (25), we first assayed here PHO5 promoter opening upon induction by phosphate starvation in the chd1 isw1 double mutant. Further, we tested RSC’s role in the context of the Isw1, Isw2 and Chd1 remodelers.

Acid phosphatase induction in phosphate-free medium at 37°C was significantly delayed in the chd1 isw1 double mutant, similar as already reported (25), and even more in a chd1 isw1 isw2 triple mutant, which may speak for a slight role of Isw2 (not followed up further), but reached full final activity levels (Figure 7A). However, concomitant Sth1td depletion severely compromised PHO5 expression in these mutant backgrounds (Figure 7A). Consistently, chromatin opening was just slower in the double and triple mutant, but additional Sth1td depletion practically abolished chromatin remodeling (Figure 7B), similar to the snf2 sth1td mutant (Figure 4B). The latter was unlikely due to low PHO induction strength as the chd1 isw1 sth1td and chd1 isw1 isw2 sth1td mutants grew faster during the first six hours of phosphate starvation at 37°C and achieved similar final numbers of cell doublings (on average 2.2) as the snf2 mutant (Supplementary Figure S8), which yielded full chromatin opening (Figure 4B). Collectively, RSC has a critical role in PHO5 promoter opening in the absence of Isw1 and Chd1.

The RSC complex had hardly any role in chromatin remodeling at the PHO8 and PHO84 promoters

We asked whether the RSC complex was involved in chromatin opening also at the PHO8 and PHO84 promoters. As a perfectly internally controlled experiment, we analyzed chromatin accessibility at the PHO8 and PHO84 promoters by respective restriction enzyme accessibilities [Supplementary Figure S1B and C (6,8)] in the same nuclei that were analyzed for PHO5 promoter opening. In contrast to the PHO5 promoter, depletion of Sth1td hardly affected PHO8 or PHO84 promoter opening upon phosphate starvation (Figure 8A) or Pho80ts inactivation (Figure 8B), and just slightly under the weakest induction conditions of chemical Pho85F82G inhibition (Figure 8C). Therefore, the lack of PHO5 promoter opening under the latter conditions (Figure 2C) was not due to a general PHO induction defect. We also examined the effect of Sth1td inactivation in snf2 and chd1 isw1 backgrounds regarding remodeling of the downstream nucleosome at the PHO84 promoter (monitored via the TaqI site, Supplementary Figure S1B). Remodeling of this
downstream nucleosome was only delayed in the absence of Snf2 [Figure 8D, (6)], similar to PHO5 promoter opening (13,20) and in contrast to the neighboring upstream nucleosome and the nucleosomes at the PHO8 promoter, but not much affected by lack of Isw1 and Chd1. Sth1td inactivation in the snf2, but hardly in the isw1 chd1 background, further delayed remodeling of this downstream nucleosome, but still allowed full final opening (Figure 8D). Altogether, the RSC requirement for PHO8 and PHO84 promoter opening was much less pronounced, if at all relevant, in striking difference to that at the PHO5 promoter (Figures 1C and D, 2B and C, 4B and 7B).

**DISCUSSION**

We show here first clear in vivo evidence for a role of the RSC complex in PHO5 promoter opening. Sth1 depletions substantially delayed PHO5 promoter opening under strong (Figures 1C and D and 3B) and even abrogated opening under weak induction conditions (Figure 2C). As all remodeler ATPases encoded in the S. cerevisiae genome are now investigated in this regard, we finally settle which remodelers open the PHO5 promoter. This historically long standing question has a surprisingly complex answer. Five remodelers from four major classes form a cooperative and redundant network: RSC, SWI/SNF, Ino80, Isw1 and Chd1 (13,19–25).

Most remodeler contributions became apparent either by delayed chromatin opening upon full or by reduced chromatin opening upon submaximal induction (Supplementary Table S2 and references therein). This argues that these remodelers must cooperate together to achieve wt PHO5 promoter opening capacity and are not just back ups for each other. Isw1 and Chd1 are an exception, as the respective single mutations showed no effects in two studies (13,23), although one study reported significant effects (25), but only their combined absence substantially affected PHO5 transcription (25) and, as shown here, promoter opening (25). This leaves room for more remodelers to show a slight contribution in the context of double or triple mutations of remodeler encoding genes. However, we expect these contributions to be negligible. The remodeler network is redundant in the sense that PHO5 promoter chromatin is eventually opened in the individual or even some combined [snf2 ino80 (13) or isw1 chd1 (Figure 7B) double mutants] absence of each remodeler. Therefore, no uniquely dedicated remodeler but a complex remodeler network opens the PHO5 promoter (13). Our new results suggest that RSC is a major player in this remodeler network. The sth1td snf2 and isw1 chd1 sth1td combined mutations are the first chromatin cofactor mutations that practically prevent PHO5 promoter opening even upon strong physiological induction by phosphate starvation (Figure 4B and 7B). In contrast, the snf2 ino80 mutant could still achieve high
whether it is essential for \textit{PHO5} promoter [Supplementary Figure S5, (13)]. Thus, RSC—possibly together with Isw1 and Chd1—could still open \textit{PHO5} promoter chromatin in the absence of SW1/SNF and INO80 but not vice versa.

RSC probably also evicts histones from the \textit{PHO5} promoter, as histone eviction upon induction was demonstrated in \textit{snf2} cells (13), but promoter opening was prevented by additional ablation of RSC activity (Figure 4B). Therefore, mainly the RSC complex was responsible for histone eviction in \textit{snf2} cells.

Even though RSC is a major player, we cannot be sure whether it is essential for \textit{PHO5} promoter opening. Opening was still possible in the \textit{sth1} mutants both upon phosphate starvation with concomitant \textit{Sth1} ablation (Figure 1C and D) as well as upon strong induction of the Gal4-driven \textit{PHO5} promoter variant after preablation of \textit{Sth1} (Figure 3B). This makes an essential role for RSC unlikely. However, especially given the high cellular abundance of the RSC complex (26), we cannot exclude that more complete ablation of RSC activity than through this particular \textit{sth1} allele under our conditions (Supplementary Figure S2A) may show even stronger effects.

Does the cooperation between these five remodelers just reflect that a certain ‘sum of remodeling activity’ is necessary to achieve wt \textit{PHO5} promoter opening? Or are there remodeler-specific mechanistic contributions such that each remodeler is not absolutely necessary but irreplaceable by other remodelers (with the exception of Isw1 and Chd1, which can mutually replace each other, see above)? We favor the latter due to remodeler-specific mechanistic differences characterized \textit{in vitro} and remodeler-specific functions \textit{in vivo} (16,62,63), but the exact nature of these differences with regard to \textit{PHO5} promoter chromatin remodeling remains to be addressed.

In contrast to our conclusions, Ehrensberger and Kornberg argued recently 2-fold against a role for RSC in \textit{PHO5} promoter opening (25,36). First, RSC perturbed nucleosomes at both the promoter and coding region in an \textit{in vitro} assay in contrast to the \textit{in vivo} situation. However, this contrasts data from the same group showing that RSC is specific for promoter nucleosomes in a similar \textit{in vitro} system (36). Besides the inconsistency of these \textit{in vitro} data, we generally consider positive \textit{in vitro} evidence stronger than negative \textit{in vitro} data, as a negative outcome there may result from any shortcomings of the \textit{in vitro} system. Second, they argue that RSC was not involved in \textit{PHO5} promoter opening, as the \textit{isw1 chd1} mutant showed no induction under \textit{pho80} high phosphate conditions ‘despite the continued presence of RSC’ (25).

This conclusion does not acknowledge that many mutations prevent \textit{PHO5} promoter opening at submaximal induction (Supplementary Table S2) due to the generally more stringent cofactor dependency at lower induction strength (12,13,20,21,23,41,60). Submaximal induction does not allow to show an essential requirement of any cofactor, or to exclude the role for another cofactor in a mutant background that is already impaired for \textit{PHO5} promoter opening. Indeed, the \textit{isw1 chd1} double mutation impaired \textit{PHO5} induction under suboptimal (early induction time points) but allowed full \textit{PHO5} expression and chromatin opening under physiological full (late induction time points) induction conditions [Figure 7A and B; see also Supplementary Figure S5 in (25)].

Surprisingly, the coregulated \textit{PHO8} and \textit{PHO84} promoters did not require RSC for promoter opening (Figure 8), even though RSC is present at all three \textit{PHO5}, \textit{PHO8} and \textit{PHO84} promoters under repressed conditions \textit{in vivo} (64). Especially at the \textit{PHO8} promoter, we even anticipated a role for RSC, given RSC’s role in setting up nucleosome positioning of the repressed state (65), but this is apparently not predictive of a positive role in chromatin remodeling upon induction. All three promoters are coregulated by the same transactivator Pho4 (6,8,9,66), which may determine cofactor recruitment specificity. Nonetheless, Pondugula \textit{et al.} (56) found an additional Pho4-independent pathway that is mediated by Mcm1 and the Forkhead proteins Fkh1 and Fkh2 binding to the \textit{PHO5} promoter (Supplementary Figure S1A) and contributes to weak mitotic \textit{PHO5} induction [5–10% of full induction level (20)]. As neither the \textit{PHO8} nor the \textit{PHO84} promoter contain Mcm1 or Fkh binding sites (personal communication by anonymous referee), there is the possibility that RSC exerts its effect on \textit{PHO5} promoter opening through Mcm1-Fkh rather than through Pho4, which would explain why we did not observe respective effects at the \textit{PHO8} and \textit{PHO84} promoters. While this remains a formal possibility, we think this unlikely, as the role of the Mcm1-Fkh pathway was only demonstrated for weak mitotic but not for strong phosphate starvation induction conditions (56), which is where we saw clear effects. Further, a \textit{PHO5} promoter mutant, where the region from bp –187 to –124 relative to the start codon was deleted, encompassing the Mcm1-Fkh binding site [bp –148 to –126 (56)], showed full wt levels of \textit{PHO5} transcription upon phosphate starvation induction (67). Therefore, Mcm1-Fkh-driven RSC recruitment specifically to the \textit{PHO5} promoter is unlikely to explain the discrepancy of RSC usage at the three Pho4-regulated \textit{PHO} promoters. Consistently, this discrepancy strikingly supports a concept that we proposed earlier (6,68): inherent nucleosome stability determines the cofactor requirements for remodeling rather than recruitment specificity through transactivators. Consistently, the Gal4-driven \textit{PHO5} promoter variant showed the same cofactor requirements as the Pho4-driven wt promoter [this study and (12,13,41)], especially the requirements of SW1/SNF and SAGA (12,13), which are essentially recruited via Pho4 (21). The change from Pho4 to Gal4 thus should have changed the cofactor requirements if they depended on the specific transactivator. A related concept was recently derived from \textit{in vitro} studies by the Kornberg group (36) with the important distinction that they contrast if nucleosomes or remodelers determine specificity whereas we contrast nucleosomes versus specific recruitment.

We propose that nucleosomes may be classified into at least three classes according to their cofactor requirements. As an example for the first class, the ‘downstream nucleosome’ of the \textit{PHO84} promoter seems to be rather unstable and exemplify the most relaxed case where just one and any one remodeler would be sufficient for complete and rather
rapid remodeling. It was fully remodeled even in the combined absence of Snf2 and Sth1 as well as Isw1, Chd1 and Sth1. Members of the second class would be like the PHO5 promoter nucleosomes that require a cooperative network of many remodelers for fast remodeling, but complete remodeling may still be achieved with a more limited remodeler set. Finally, the PHO5 promoter nucleosomes and the ‘upstream nucleosome’ at the PHO84 promoter would exemplify the third class that also uses several remodelers, e.g. SWI/SNF and INO80, but essentially requires at least one of them (e.g. SWI/SNF). This specific use of a remodeler is rather puzzling, as nucleosomes of a broad inherent stability range were remodeled in vitro equally well by very different types of remodelers (69). Nonetheless, chromatin-intrinsic requirements for specific remodelers can certainly explain why eukaryotic genomes encode such a wide diversity of remodeling enzymes (17,18) and we speculate that the PHO5 promoter will again become the pioneering example of a general phenomenon: the majority of chromatin transitions in vivo involves more than one remodeler type.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank Corinna Lieleg for blot hybridizations, and Dorothea Blaschke for technical assistance. We are grateful to Brad Cairns, Timothy Parnell and Erin O’Shea for sharing plasmids and Wolfgang Zachariae for the gift of strains.

This article is dedicated by S.B. and P.K. to the memory of Wolfram Hörz, who would have loved to know what remodels PHO5 promoter chromatin.

FUNDING
Ministry of Education, Science, and Technology of the Republic of Croatia [project grant number 058-0580477-0247 to S.B.]; German Research Community (DFG) [project grant number KO 2945/1-1 and project grant within the SFB/Transregio 5] (to P.K.); Bavarian Research Network for Molecular Biosystems (BioSysNet) (to P.K.). Funding for open access charge: DFG.

Conflict of interest statement. None declared.

REFERENCE


