Connecting GCN5’s centromeric SAGA to the mitotic tension-sensing checkpoint

Emily L. Petty, Masha Evpak, and Lorraine Pillus*
Division of Biological Sciences, Molecular Biology, UCSD Moores Cancer Center, University of California, San Diego, La Jolla, CA 92103

ABSTRACT Multiple interdependent mechanisms ensure faithful segregation of chromosomes during cell division. Among these, the spindle assembly checkpoint monitors attachment of spindle microtubules to the centromere of each chromosome, whereas the tension-sensing checkpoint monitors the opposing forces between sister chromatid centromeres for proper biorientation. We report here a new function for the deeply conserved Gcn5 acetyltransferase in the centromeric localization of Rts1, a key player in the tension-sensing checkpoint. Rts1 is a regulatory component of protein phosphatase 2A, a near universal phosphatase complex, which is recruited to centromeres by the Shugoshin (Sgo) checkpoint component under low-tension conditions to maintain sister chromatid cohesion. We report that loss of Gcn5 disrupts centromeric localization of Rts1. Increased RTS1 dosage robustly suppresses gcn5Δ cell cycle and chromosome segregation defects, including restoration of Rts1 to centromeres. Sgo1’s Rts1-binding function also plays a key role in RTS1 dosage suppression of gcn5Δ phenotypes. Notably, we have identified residues of the centromere histone H3 variant Cse4 that function in these chromosome segregation-related roles of RTS1. Together, these findings expand the understanding of the mechanistic roles of Gcn5 and Cse4 in chromosome segregation.

INTRODUCTION
The eukaryotic genome is packaged into the DNA–protein complex defined as chromatin. This packaging is dynamic, ensuring that cells can make rapid alterations to gene expression, respond to DNA damage, and divide their genomes accurately. As its foundation, chromatin is made up of nucleosomes, the repeated units of DNA wrapped around octamers of histone proteins (Kornberg and Lorch, 1999). Multiple, diverse enzymes add small and large chemical groups or proteins to histone residues and opposing enzymes remove these modifications, contributing to the dynamic nature of chromatin organization necessary for life and growth in an ever-changing environment (Tessarz and Kouzarides, 2014).

Gcn5 is one enzyme that adds the small acetyl group to histones H3 and H2B within chromatin and other substrates as well (Grant et al., 1997, 1999; Downey et al., 2015). There are three biochemically distinct, multisubunit complexes that contain Gcn5 in yeast. Among these, SAGA (Spt-Ada-Gcn5 acetyltransferase) and SLIK/SALSA (SAGA-Like/SAGA altered Spt8 absent) are large cotranscriptional activator complexes that target H3K9 and K14 acetylation near gene promoters (Grant et al., 1997; Roberts and Winston, 1997; Pray-Grant et al., 2002; Sterner, Belotserkovskaya, and Berger, 2002; Rosaleny, Ruiz-Garcia, et al., 2007). The smaller ADA complex may contribute to broad H3 acetylation outside promoters (Eberharter et al., 1999; Lee, Sardiu, et al., 2011). Characterization of gcn5Δ mutants in yeast revealed late cell cycle delays and mitotic errors pointing to roles in chromosome segregation (Zhang et al., 1998; Howe et al., 2001; Vernarecci et al., 2008). Gcn5 also contributes to chromatin organization in centromeric regions (Vernarecci et al., 2008) and regulates a tension-sensing function of H3 during chromosome segregation (Luo, Deng, et al., 2016).

Successful chromosome segregation begins with genomic replication, followed by massive condensation of interphase chromatin into chromatids, and sister chromatid pairing. Sister chromatids remain paired at their centromeres, encircled by the multimeric cohesin ring complex, until all chromosomes are aligned and attached to...
opposing spindle microtubules via kinetochores (Onn et al., 2008). Of note, many of the genes encoding components of centromeres and kinetochores are essential and deeply conserved. For example, and B56 subunits, respectively (Figure 1A; Zhao et al., 1997). Initial characterization of Rts1 revealed that its cellular localization depends on the cell cycle, and that it is recruited to centromeres during

FIGURE 1: Overexpression of RTS1 rescues late cell cycle and chromosome segregation-related gcn5∆ phenotypes. (A) Rts1 is the regulatory subunit of the PP2A-Rts1 complex, which is functionally linked to Gcn5 (Petty et al., 2016). (B) RTS1 overexpression promotes chromosome stability in gcn5∆. Strains were transformed with a URA3-marked SUP11 plasmid for colony color assay (Hieter et al., 1985) along with vector control (−) and RTS1 overexpressing (+) constructs, then plated at low density to grow without selection to observe rates of SUP11 plasmid loss. From left to right, n = 1756, 1562, 1595, and 1953, with n representing the number of colonies scored. Shown are average rates of loss from three independent experiments; error bars indicate SD and n values contributing to rates of loss from three independent experiments.

D Strain RTS1 △ YPAD NOC
WT − + − +
gcn5Δ − + − +
ahc1Δ − + − +
ahc2Δ − + − +
spt8Δ − + − +
spt20Δ − + − +
tg2Δ − + − +

E Gene Complex
GCN5 ADA, SAGA, SLIK/ SALSA
AHC1 ADA
AHC2 ADA
SPT8 SAGA
SPT20 SAGA, SLIK/ SALS A
RTS1 SLIK/SALSA

mistakes in orchestrating chromosome segregation can lead to aneuploidy, a common cause of birth defects in humans that is also detected in an overwhelming majority of solid tumors (Holland and Cleveland, 2012).

In many organisms, the centromere is defined by a histone H3 variant, CENP-A, along with centromere-specific DNA sequences (McKinley and Cheeseman, 2016). In budding yeast the centromere is defined by a single nucleosome containing the essential CENP-A H3 histone variant Cse4 (Stoler et al., 1995; Meluh et al., 1998; Furuya and Biggins, 2007). Like canonical histones, Cse4 is dynamically modified, and many of the modifications that have been characterized thus far are notably involved in maintaining precise levels of Cse4 to prevent misincorporation at ectopic sites (Hewawasam et al., 2010; Hildebrand and Biggins, 2016) or in directing Cse4 deposition (Samel et al., 2012). There is a functional relationship between Gcn5 and Cse4 as well, evidenced by the extreme temperature sensitivity of the gcn5Δ cse4-1 double mutant (Vernarecci et al., 2008).

The kinetochore is a massive molecular structure that assembles onto the centromere and connects each sister chromatid to spindle microtubule(s) emanating from opposing poles. Sister chromatid cohesion is guarded by the spindle assembly checkpoint (SAC) until all kinetochores are properly attached to spindle microtubules. Dynamic phosphorylation is key for the checkpoint’s execution, with multiple kinases and phosphatases acting in opposition to one another (Nasa and Kettenbach, 2018). For example, the Mps1 kinase monitors microtubule attachment by phosphorylating unattached kinetochore proteins to recruit SAC components, with the PP1 phosphatase acting to reverse Mps1 phosphorylation (Liu et al., 2010; London et al., 2012; Shepperd, Meadowes, Sochaj, et al., 2012; Yamagishi et al., 2012; Primorac, Weir, Chirol, et al., 2013). An ongoing question is the extent to which novel interactions between enzymatic activities contribute to SAC silencing.

We previously characterized a genetic interaction between Gcn5 and the phosphoprotein phosphatase 2A (PP2A) regulatory subunit Rts1, such that overexpression of RTS1 suppresses multiple gcn5Δ phenotypes, whereas loss of Rts1 is lethal in gcn5Δ cells (Petty et al., 2016). There are two forms of PP2A in yeast that are distinguished by their regulatory subunits, Cdc55 or Rts1, which are homologous to mammalian B55 and B56 subunits, respectively (Figure 1A; Zhao et al., 1997). Initial characterization of Rts1 revealed that its cellular localization depends on the cell cycle, and that it is recruited to centromeres during...
metaphase of mitosis and meiosis by the Shugoshin protein Sgo1 (Gentry and Hallberg, 2002; Riedel, Katis, et al., 2006; Yu and Koshland, 2007; Peplowska, Wallek, and Storchova, 2014). Together, Rts1 and Sgo1 contribute to sister chromatid cohesion protection in the tension-sensing checkpoint by blocking progression into anaphase if tensionless kinetochore-microtubule attachments are present (Riedel, Katis, et al., 2006; Yu and Koshland, 2007; Nerusheva et al., 2014; Peplowska, Wallek, and Storchova, 2014; Jin et al., 2017). We report here that gcn5Δ chromosome segregation defects are alleviated by increased dosage of RTS1. Further, loss of Gcn5 reduces the centromeric localization of Rts1 in metaphase-arrested cells. We identify three residues of Cse4 that affect RTS1 dosage rescue of gcn5Δ phenotypes and specifically identify Cse4-S180 as a critical residue for localization of Rts1 to centromeres. These results broaden the evidence for a role for Gcn5 in the tension-sensing checkpoint and understanding of the deeply conserved Gcn5 acetyltransferase in the critical process of chromosome segregation.

**RESULTS**

**RTS1 is a high-copy suppressor of gcn5Δ chromosome segregation phenotypes**

We previously reported that RTS1 suppresses gcn5Δ’s histone gene expression and cell cycle entry defects (Petty et al., 2016). To determine whether RTS1 could also suppress gcn5Δ phenotypes related to chromosome segregation, we began by using the SUP11-based minichromosome assay (Hieter et al., 1985) to determine rates of loss as a proxy for genome stability. Briefly, cells carrying the SUP11 plasmid will suppress the pink colony phenotype of ade2 mutants, leading to white colonies. A colony that is half-pink and half-white indicates that the plasmid was lost in one of the daughter cells arising from the first division after plating. As expected, we observed a significantly greater proportion of half-sectored colonies in gcn5Δ mutants (Figure 1B) indicative of reduced genome stability. Overexpression of RTS1 significantly reduced SUP11 loss in gcn5Δ cells and further reduced loss in wild type. These results suggest that RTS1 positively regulates accurate chromosome segregation.

Defects in chromosome segregation can slow G2/M progression. Indeed, this is a classic gcn5Δ phenotype. To determine whether RTS1 overexpression can suppress sluggish G2/M passage, we tracked cell cycle profiles of cells initially synchronized in G1 with α-factor phenome, and collected at 20-minute intervals for flow cytometric analysis. By 2 h, wild-type cells with and without RTS1 overexpression had resumed dividing, whereas the gcn5Δ vector control cells remained predominantly in G2/M (Figure 1C). In gcn5Δ cells overexpressing RTS1, the G1 population was increased at 2 h, indicating restored and timely progression through G2/M.

**Increased RTS1 suppresses loss of SAGA function in chromosome segregation**

Mutations affecting chromosome segregation can increase sensitivity to microtubule destabilizing drugs (Ouspenski et al., 1999; Lampson and Kapoor, 2006). The SAC was first characterized by the identification of the MAD (mitotic arrest deficient) and BUB (budding uninhibited by benzimidazole) genes in genetic screens for mutants that fail to arrest growth in the presence of microtubule poisons (Hoyt et al., 1991; Li and Murray, 1991). We previously reported that RTS1 overexpression suppresses gcn5Δ sensitivity to the microtubule drug nocodazole (Petty et al., 2016). To genetically dissect whether the chromosome segregation phenotypes of gcn5Δ mutants were tied to Gcn5 function in one of its complexes and, further, whether RTS1 suppression of nocodazole sensitivity was complex specific, we transformed mutants of genes encoding a combination of distinct and structural complex subunits with RTS1 or 2 μM vector controls. Transformants were challenged by exposure to nocodazole (Figure 1D). We observed nocodazole sensitivity in gcn5Δ, ahc1Δ, spt8Δ, spt20Δ, and rtg2Δ transformants, indicating that microtubule poison sensitivity is a phenotype common to impaired function of all Gcn5-containing complexes. However, RTS1 suppression of nocodazole sensitivity was observed specifically in gcn5Δ, spt8Δ, and spt20Δ mutants, suggesting that RTS1 overexpression suppresses loss of SAGA function in chromosome segregation (Figure 1E).

SAGA is a well-defined transcriptional coactivator complex with an acute role in stress-induced gene activation (Huisinga and Pugh, 2004). There is mounting evidence for broad SAGA involvement in activation of most genes that is buffered by increased mRNA stability (Baptista et al., 2017). Therefore, we hypothesized that loss of Gcn5 function may impair chromosome segregation due to loss of expression of key genes and used reverse transcription-quantitative PCR (RT-qPCR) to determine steady-state expression levels of RTS1, GSO1, and SGE4 (Supplemental Figure S1). There was no significant change in their steady-state expression in gcn5Δ cells suggesting that the chromosome segregation phenotype is not due to loss of expression of these central players. This result is consistent with previous genome-wide analysis that identified RTS1, SGO1, and CSE4 as TFIID-dominated genes (Huisinga and Pugh, 2004).

Several studies have suggested a direct role for SAGA subunits at the centromere. The SAGA debiquitinase subunit Ubp8 promotes Psh1-directed proteolysis of excess Cse4 (Canzonetta et al., 2015). Gcn5 has been genetically identified as a regulator of a tension-sensing motif on histone H3 and found directly bound at centromeres by chromatin immunoprecipitation (ChIP; Luo, Deng, et al., 2016). To determine whether centromeric localization of SAGA subunits was broadly shared, we analyzed patterns of SAGA subunit binding at the centromere that were reported in recent studies of asynchronous cell populations (Supplemental Figure S2). FLAG-tagged Cse4 ChIP-seq binding (Hildebrand and Biggins, 2016) was used to demarcate the centromere. SAGA subunits Spt3, Spt7, and Spt8 along with debiquitinase Ubp8 were recently mapped using ChEC-seq (Baptista et al., 2017) and Sgf73 localization by ChIP-seq (Mason et al., 2017). There is a notable pattern of localization of all of the SAGA subunits at centromeric regions of chromosome III and IV (Cen III and Cen IV). As the cells used in these published studies were not synchronized to metaphase, the lower signal we observe may be due to the smaller population at this point in the cell cycle. Nonetheless, these observations collectively point to a direct role for SAGA at or near the centromere.

**Sgo1 is required for RTS1 suppression of gcn5Δ nocodazole sensitivity**

Rts1 subcellular location changes during the cell cycle, but relies predominantly on Sgo1 for localization to the centromere during metaphase (Gentry and Hallberg, 2002; Nerusheva et al., 2014; Peplowska, Wallek, and Storchova, 2014). We considered the possibility that if RTS1 suppression of gcn5Δ’s chromosome segregation phenotypes occurred at the centromere, it should be dependent on Sgo1. To test this, we used the sgo1-NS11 mutant allele that has been shown to specifically disrupt Sgo1’s binding to Rts1 (Xu, Cetin, et al., 2009; Peplowska, Wallek, and Storchova, 2014). Notably, Sgo1 plays several critical roles in chromosome segregation, therefore although sgo1-NS11 impairs Rts1 recruitment, its chromosome segregation and growth phenotypes are less severe than those observed in sgo1a (Peplowska, Wallek, and Storchova, 2014). We constructed double mutants of sgo1-NS11 with gcn5Δ
and the catalytic mutant gcn5-KQL (Wang, Liu, and Berger, 1998), and then transformed the strains with RTS1 or vector control plasmids to determine the effect of Sgo1 on RTS1 suppression of gcn5 phenotypes (Figure 2).

RTS1 overexpression suppressed gcn5Δ's temperature sensitivity as well as sensitivity to the DNA damaging agents hydroxyurea (HU) and methyl methanesulfonate (MMS), as we previously reported. We found that sgo1-T::N51I did not interfere with RTS1 suppression of either gcn5Δ or gcn5-KQL sensitivity to heat nor DNA damage (Figure 2). However, the impact of sgo1-N51I on suppression of nocodazole sensitivity was markedly different. The sgo-N51I mutant exacerbated gcn5Δ and gcn5-KQL sensitivity to nocodazole and disrupted suppression by RTS1. Sensitivity was also exacerbated by RTS1 overexpression in SGO1-TAP controls, similar to previous observations of overexpression improving gcn5Δ growth phenotypes while adversely affecting wild-type growth (Petry et al., 2016). Overall, the fact that RTS1 suppression of nocodazole sensitivity is abolished in gcn5 sgo1-N51I mutants points to Rts1 recruitment to the centromere by Sgo1 as key for the mechanism of suppression.

We considered the possibility that loss of RTS1-mediated suppression might be due to loss of Rts1 protein expression in gcn5 sgo1-T::N51I mutants. To address this, we integrated a single copy of Rts1 tagged with a triple PK epitope into the Rts1 tagged with a triple PK epitope into the RK14 integration and overexpression constructs to evaluate Rts1 centromere binding in wild type and gcn5Δ and gcn5-KQL under endogenous and overexpression conditions. We used nocodazole to arrest transformed cells in metaphase, confirmed by flow cytometry, and briefly fixed cells for ChIP. Rts1-3PK ChIP was followed by quantitative PCR (qPCR) using primers to amplify centromeric, pericentromeric, and distal loci on chromosome III as in Nerusheva et al. (2014). We observed lower Rts1 binding at both the centromere and pericentromere in gcn5Δ containing the vector control, but binding was restored to levels comparable to wild type with vector control upon RTS1-3PK overexpression (Figure 3A). The strongest signals were detected in the wild-type cells overexpressing RTS1-3PK. To determine whether this pattern was specific to Cen III, we also evaluated Rts1-3PK binding at the centromere, pericentromere, and a distal region of chromosome IV (Figure 3B). The results for Cen IV were very similar to those of Cen III, leading us to conclude that the pattern of lost Rts1 binding upon loss of Gcn5 is not specific to a single centromere. The Gcn5 catalytic mutant gcn5-KQL displayed similarly lower Rts1-3PK localization at both Cen III and Cen IV compared with wild type, indicating that it is loss of Gcn5's catalytic activity that interferes with Rts1 centromere localization in arrest conditions (Figure 3, A and B).

As with nocodazole sensitivity, it was possible that suppression of gcn5Δ's Rts1-binding defect would depend on Sgo1. We tested this by performing Rts1-3PK ChIP-qPCR in backgrounds with the sgo1-N51I mutation. Loss of Rts1-3PK was observed at the centromere and pericentromere of Chr IV in the sgo1-N51I single-mutant vector control and when combined with gcn5Δ (Figure 4). Unexpectedly, upon overexpression of RTS1-3PK in the sgo1-N51I mutant, increases in the ChIP signal were observed at the centromere and pericentromere, yet the strongest signal was at the nonspecific, distal arm locus. This result suggests that in the absence of Sgo1-specific recruitment, increased Rts1 may bind chromatin broadly and nonspecifically.

Directed Cse4 screen reveals roles for uncharacterized residues

Screens to evaluate the effects of mutations in individual histone residues are a powerful tool for uncovering new functional regions and potential sites of dynamic modification. Indeed, several sites of Cse4, H3, and H4 that contribute to chromosome segregation were originally identified in such screens (Camahort et al., 2009; Luo et al., 2010; Ng et al., 2013). We hypothesized that the centromere-specific histone, Cse4, may be a target of Rts1 or Gcn5 activity and therefore screened alanine substitution mutants of serine, threonine, tyrosine,
FIGURE 3: Gcn5 functions in RTS1 localization to the centromere. Cells were arrested for 60–90 min with nocodazole (7.5 µg/ml) with arrest confirmed by light microscopy and flow cytometry. Metaphase-arrested transformants were fixed and analyzed for Rts1 binding at the centromere by chromatin immunoprecipitation using Rts1 tagged with the PK epitope (Nerusheva et al., 2014; Verzijlbergen et al., 2014) followed by qPCR amplification of centromeric (Cen), pericentromeric (P. Cen), and distal arm (Arm) of chromosomes III (A) and IV (B). Shown is the average of three independent experiments, with error bars indicating SD.

FIGURE 4: Binding specificity of overexpressed Rts1 is lost in SGO1 mutants. Cells were grown, arrested with nocodazole (7.5 µg/ml), and fixed for Rts1-3PK ChIP as in Figure 3. In samples overexpressing RTS1 with the sgo1-N51I mutation, the strongest Rts1-3PK ChIP signal is measured at the nonspecific Chr IV Arm locus, rather than at the centromere or pericentromere. Shown is the average of three independent experiments, with error bars indicating SD.
restored growth at high temperature. By comparison, the acetyl-
mimetic cse4-K215Q mutant (Supplemental Figure S4C) reversed the
phenotypes in the wild-type background and improved growth at high temperature in the gcn5Δ background. These results sug-
gest that the charge and potential acetylation of Cse4-K215 contrib-
ute to a temperature-sensitive function of Cse4. Although found
within the conserved histone fold region of CENP-A/Cse4, K215 is
not conserved in the major model organisms that we surveyed, so
this function may be unique to Saccharomyces cerevisiae.

We were particularly interested in effects of the cse4-S180A mu-
tation due to the conservation of this residue among yeast species
and some metazoans, and its proximity to the recognition motif for
binding by Scm3, the chaperone that integrates Cse4 into chroma-
tin (Figure 5C; S180 is shown in red and Scm3 recognition motif

**FIGURE 5**: Directed screen identifies specific Cse4 residues that affect gcn5Δ rescue by RTS1. (A) Nonessential S, T, Y, and K residues of Cse4 (57; bold) were individually screened for function in rescue by increased RTS1 dosage for a battery of gcn5Δ sensitivities. The alpha helices of the histone fold domain are highlighted in blue. (B) Candidate cse4 substitution mutations of interest were integrated into the genome to confirm results of an initial plasmid shuffle screen. Cultures of freshly transformed strains were normalized, plated onto URA− plates, and challenged to grow at high temperature (37°C), and in the presence of DNA damaging agents HU (0.05 M) and MMS (0.015%). For nocodazole (2 µg/ml), YPAD medium was used. (C) Cse4-S180 is within the conserved C-terminal region of Cse4. Clustal W (Thompson et al., 1994) was used to generate an alignment of CENP-A homologues. Cse4-S180 and the corresponding conserved S residues in X. laevis, C. elegans, K. lactis, and S. pombe are shown in red bold font, and the Scm3-binding motif (Zhou, Feng, et al., 2011) is underlined. The NMR structure of K. lactis Cse4 (cyan), Scm3 (green), and H4 (blue) is shown with S180 corresponding residue highlighted in red (Cho and Harrison, 2011). (D) Integrated cse4-S180E substitution rescues nocodazole sensitivity similarly to cse4-180A. Growth of fresh transformants was challenged as above and assessed after 3 d. (E) Recapitulating phosphorylation charge change by cse4-S180EE mutation is lethal. Plasmid shuffle by plating onto media containing 5-FOA was used to select for cells having lost the CSE4-URA3 covering plasmid. Representative images of at least four independent experiments are shown.
underlined). We found that cse4-5180A alleviated gcn5\Delta no-
codazole sensitivity, and that growth was further improved by RTS1
overexpression (Figure 5B). There was also a reduction in the num-
ber of suppressor colonies among gcn5\Delta cse4-5180A growth when
challenged by nocodazole. We hypothesized that S180 could be a
site of critically dynamic phosphorylation and generated a cse4-
5180E phosphomimetic mutant to test this possibility genetically.
The effect of cse4-5180E on gcn5\Delta nocodazole sensitivity was simi-
lar to cse4-5180A (Figure 5D); therefore, disruption of this site may
be sufficient to improve a function related to microtubule stress.
Alternatively, although glutamate is commonly used to genetically
mimic constitutive phosphorylation, a single glutamate substitution
does not faithfully recapitulate the charge change of serine phos-
phorylation. Previous work has demonstrated that single serine to
alanine substitution to microtubule stress but it is specifically the loss of SAGA that can
be sufficient to improve a function related to microtubule stress.
Although serine to alanine substitution to microtubule stress,
yielding a charge change analogous to phosphorylation, resulted in
phenotypes more similar to constitutive phosphorylation (Strick-
fen et al., 2007). Therefore, we generated a second cse4 phosph-
omimetic mutant substituting two glutamate residues for S180.
Multiple failed integration attempts in haploids led us to test the
viability of the cse4-5180EE mutation using a plasmid shuffle stra-
egy. We transformed a cse4\Delta/CSE4 diploid and cse4A and gcn5\Delta
cse4A haploids containing a URA3-marked CSE4 plasmid with either
a TRP1-marked HA-CSE4 or HA-cse4-5180EE plasmid. The transform-
ants were then challenged to grow on medium containing 5-fluo-
roorotic acid (5-FOA) to select for cells that could lose the URA3
covering plasmid (Figure 5E). Haploids selected for the cse4-5180EE
plasmid as the sole source of Cse4 were inviable. We confirmed
expression of HA-cse4-5180EE in diploid transformants by immuno-
 blot, ruling out loss of expression as the cause of inviability (Supple-
mental Figure S6). Rather, these data reveal toxicity of a constitutive
change mimicking S180 phosphorylation.

Cse4-5180 contributes to centromeric localization of Rts1
The observation that cse4-5180A suppressed gcn5\Delta nocodazole
sensitivity raised the possibility that this might be due to restored
localization of Rts1 to the centromere in metaphase cells. This idea was tested by
ChIP of Rts1-3PK in the HA-tagged Cse4 and cse4-5180A strains in wild-type and
gcn5\Delta backgrounds, with and without RTS1 overexpression. During optimization
of metaphase arrest conditions, we noted that the high concentration of nocodazole
used for arrest is toxic to gcn5\Delta cse4-5180A, so a reduced concentration of 2 µg/ml
was used to delay the cell cycle and enrich for metaphase cells as shown in Wang and
Burke (1995). Interestingly, this lower no-
codazole concentration does not com-
pletely disrupt kinetochore microtubules
(Wang and Burke, 1995), suggesting that
although the cse4-5180A mutation allevi-
ated gcn5\Delta sensitivity to a cell cycle delay
by nocodazole, some microtubule structure
is required for viability. We again evaluated
Rts1 binding at and around Cen IV using
qPCR in wild-type (Supplemental Figure S5)
and gcn5\Delta backgrounds (Figure 6). Com-
pared to wild type, cse4-5180A slightly re-
duced Rts1 levels at the centromere and
pericentromere in cells containing Gcn5, but in gcn5\Delta, there was in-
creased Rts1 at the centromere. Overexpression of RTS1 led to fur-
ther increases of Rts1 at the centromere in GCNS cells, although not
to the extent as when Cse4 is wild type, and further increases at both
the centromere and pericentromere in gcn5\Delta cells. To determine
whether these differences in Rts1 binding were due to altered Rts1-
3PK expression/overexpression, we used quantitative immunoblot-
ing of lysates prepared in parallel with samples prepared for ChIP.
We observed consistent levels of Rts1-3PK in cells carrying a single
 copy and cells containing the 2 µm-RTS1-3PK high-copy plasmid
from strain to strain (Supplemental Figure S6, A and B). At the same
time, we observed levels of Cse4 expression and noted a reduction
in cse4-5180A in both Gcn5-containing and deletion backgrounds.
These results point to a role for Cse4-5180 in promoting Rts1 local-
ization to centromeres in the absence of Gcn5 and reveal a potential
role in regulation of Cse4 levels independent of transcription.

DISCUSSION
Our data reveal opposing roles for Gcn5 and Cse4-5180 in the local-
ization of Rts1 in the mitotic spindle tension-sensing pathway (Figure
7). Loss of function of any Gcn5-containing complex results in sensitiv-
ity to microtubule stress but it is specifically the loss of SAGA that can
be suppressed by RTS1 overexpression. RTS1-mediated suppression
of gcn5\Delta nocodazole sensitivity requires recruitment of Rts1 to the
centromere by Sgo1. We characterized a new role for Gcn5 acetyl-
transferase activity (Luo, Deng, et al., 2010; Ng et
al., 2013). Indeed, three residues of H3 (K42, G44, and T45) func-
tion in centromeric tension sensing and growth phenotypes
caused by mutations to these residues can be suppressed by loss
of Gcn5 acetyltransferase activity (Luo, Deng, et al., 2016). This
suggests that under normal conditions, Gcn5 positively regulates

FIGURE 6: The cse4-5180A mutant restores Rts1 binding at centromere IV in metaphase-
arsestrated cells. Integrated cse4-5180A mutant strains were transformed with vector (−) or RTS1
 (+) 2 µm plasmids and grown, arrested in metaphase, and fixed for ChIP as described. For gcn5\Delta
cse4-5180A, a nocodazole concentration of 2 µg/ml was used to enrich for metaphase cells; for
all other strains 7.5 µg/ml was used. Rts1 binding at centromeric, pericentromeric, and distal arm
loci was assessed by qPCR. Shown are average percent input values of three independent
experiments; error bars indicate SD.
Gcn5 promotes Rts1 localization to centromeres. The tension-sensing checkpoint ensures that sister chromatids do not separate before opposing spindle microtubules are properly attached. We propose that under low-tension conditions, depicted here as only one chromatid attached to a microtubule (MT), Gcn5 acts within SAGA to promote Rts1 localization to the centromere. In the absence of Gcn5 or in a catalytic mutant, overexpression of \textit{RTS1} can restore its centromeric localization, but localization specificity requires Sgo1. The centromere (shown in red) is defined by the presence of a histone H3 variant Cse4-containing nucleosome. We report a role for the conserved Cse4-S180 residue in promoting Rts1 centromeric localization in the absence of Gcn5. For simplicity, the condensin, cohesin, and chromosome passenger complexes, also active in centromeric checkpoints, are not shown, but among their subunits are potential targets of Gcn5 and PP2A-Rts1 activities.

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<th>gcn5Δ</th>
<th>+ RTS1 overexpression</th>
<th>+ cse4-S180A</th>
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<td>• Reduced viability under MT destabilizing conditions.</td>
<td>• Rescues viability under MT destabilizing conditions; Sgo1-dependent.</td>
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<td>• Low Rts1 @ centromere in arrested cells.</td>
<td>• Restores centromeric Rts1 in arrested cells. Specificity requires Sgo1.</td>
<td>• Restores Rts1 @ centromere in arrested cells.</td>
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<td>• Increased extra-chromosome instability</td>
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<td>• S180EE phosphomimetic is lethal.</td>
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FIGURE 7: Gcn5 promotes Rts1 localization to centromeres. The tension-sensing checkpoint ensures that sister chromatids do not separate before opposing spindle microtubules are properly attached. We propose that under low-tension conditions, depicted here as only one chromatid attached to a microtubule (MT), Gcn5 acts within SAGA to promote Rts1 localization to the centromere. In the absence of Gcn5 or in a catalytic mutant, overexpression of \textit{RTS1} can restore its centromeric localization, but localization specificity requires Sgo1. The centromere (shown in red) is defined by the presence of a histone H3 variant Cse4-containing nucleosome. We report a role for the conserved Cse4-S180 residue in promoting Rts1 centromeric localization in the absence of Gcn5. For simplicity, the condensin, cohesin, and chromosome passenger complexes, also active in centromeric checkpoints, are not shown, but among their subunits are potential targets of Gcn5 and PP2A-Rts1 activities.
conserved in *Kluyveromyces lactis* and Schizosaccharomyces pombe as well as metazoans Caenorhabditis elegans and Xenopus laevis lends credence to the possibility that S180 is a site of functional significance and potential dynamic phosphorylation. Further, S180 is conserved in human H3 variants, which may reflect functions beyond chromosome segregation. A need for controlled, dynamic regulation of phosphorylation at this site is supported by the lethality caused by the cse4-S180E mutation. Our observation that cse4-S180A alleviates gcn5Δ sensitivity to chronic, low-level microtubule destabilization by nocodazole, but exacerbates sensitivity to acute treatment, clearly points to the mechanism of suppression involving microtubules. Previously, N-terminal Cse4 phosphomimetic mutants were found to suppress mutations to the kinase Ipl1, a subunit of the CPC, and Ipl1 kinetochore targets, but to exacerbate growth and chromosome segregation phenotypes of kinetochore assembly mutants. These observations support a role for Cse4 N-terminal phosphorylation by Ipl1 in destabilizing kinetochore–microtubule attachments (Boeckmann, Takahashi, Au, et al., 2013). If a similar mechanism is at play with Cse4-S180, the nonphosphorylatable cse4-S180A mutant may stabilize kinetochore–microtubule attachments, whereas persistent phosphorylation may prevent stable attachments from forming, leading to lethality.

The new connections we report between Cse4 residues (S135, S180) and Rts1, and Cse4 expression are also of note considering its regulation. Ordinarily, Cse4 is tightly regulated by lysine ubiquitin-mediated proteolysis by multiple E3 ligases, most notably by Psh1 and the SUMO-targeted ubiquitin ligase, Slx5 (Hewawasam et al., 2010; Ranjitkar et al., 2010; Au et al., 2013; Ohkuni et al., 2016, 2018). Psh1 recognizes Cse4’s CENP-A targeting domain and S180 is within that key region (Ranjitkar et al., 2010), and Scm3 chaperone binding at the centromere is thought to protect from Psh1-mediated degradation (Hewawasam et al., 2010). Therefore, if cse4-S180A impairs Scm3 binding, it may be more prone to degradation, which is in line with our observation that HA–Cse4–S180A levels are lower than wild type. Moreover, constitutively stable Cse4 is toxic to yeast cells and stable or overexpressed Cse4 mislocalizes to euchromatin (Collins et al., 2004; Hildebrand and Biggins, 2016). Similarly, increased expression of CENP-A is a prognostic biomarker of human cancers and overexpression causes chromosomal instability in human cell lines (Sun et al., 2016; Shrestha et al., 2017). Alterations in levels observed in the cse4-S135A mutant may be due to increased Cse4 stability by altering proteolysis dynamics. Further phenotypic analysis of phosphomimetic mutants may yet reveal proteolytic-independent functions.

Much has been learned about the structural components and regulatory partners that ensure faithful chromosome segregation, from centromere-specific histone variant Cse4, to the dozens of subunits comprising the kinetochore, to the multiple kinase–phosphatase partners that function in the SAC. Ultimately, it seems reasonable to expect that Gcn5 and PP2A-Rts1 targeting of centromeric chromatin, including CENP-A/Cse4, and kinetochore/SAC substrates, contribute to mitotic SAC silencing through the critical tension-sensing checkpoint. The human SAGA complex is an activator of the oncoprotein c-MYC and Gcn5 acetylates regulatory regions of c-MYC targets for activation, driving cancerous transformation (Liu et al., 2003; Patel et al., 2004; Wang and Dent, 2014). The functional conservation of kinetochore components and regulatory mechanisms suggests that the work reported here may also lead to an expanded understanding of the role for SAGA in the mechanisms underlying aneuploidy that is so commonly found in human cancers.

**MATERIALS AND METHODS**

**Yeast growth, strains, and plasmids**

Yeast strains used in this work are in the W303 background as listed in Supplemental Table S1. Cells were grown in synthetic dropout or yeast extract–peptone–adrenaline–dextrose (YPAD) liquid medium under standard growth conditions (Guthrie and Fink, 1991) at 30°C except where indicated. For plate assays, cells from overnight cultures were normalized to *A*~600~ of 1 and 1.5 serial dilutions were pinned onto plates to grow for 3 d in the presence of an indicated challenge. Plates were photographed after 3 d growth. Drug concentrations are listed in corresponding figure legends. Plasmids used are listed in Supplemental Table S2. Independent lithium acetate transformations were used for biological replicates of all experiments (Amberg et al., 2005). For the minichromosome loss assay, transformants were grown under URA-LEU selection, then diluted in LEU^-1^-synthetic medium for two doublings (4–6 h of growth) before plating onto LEU^-1^-plates at a concentration of 500 cells per plate. Colony color and sectoring was assayed as described (Hieter et al., 1985). The Cse4 mutant screen was performed by plasmid shuffle using plasmids from the Cse4 plasmid library (Camahort et al., 2009). Site-directed mutagenesis of the pRB294 (HA3::His6-CSE4 construct (Baker and Rogers, 2006) was used to generate tagged Cse4 mutants for insertion into the CSE4 locus (Amberg et al., 2005). Cse4 integrants were screened by molecular genotyping and backcrossed once to wild type. Newly constructed strains generated through crosses were sporulated using minimal sporulation media (Rose et al., 1990) before dissection and genotyping.

**α-Factor arrest and flow cytometry**

Isogenic bar1Δ strains were used for α-factor sensitization. Growth, arrest, and recovery were performed at 30°C. Overnight cultures of transformants were grown in URA and diluted to 0.1 *A*~600~ in YPAD the next morning. At 0.3 *A*~600~, α-factor was added to the cultures for 90–120 min and arrest was monitored by light microscopy. To release, cells were collected, washed twice in prewarmed YPAD, and the T0 sample was collected before resuming growth at 30°C. Samples were collected every 15 min for 4 h and fixed in 70% ethanol overnight at 4°C. Fixed cells were processed and stained with propidium iodide before analyzing for DNA content with a BD Accuri C6 Flow Cytometer as in Petty et al. (2016).

**Rts1-3PK ChIP**

Transformants were grown overnight in URA^-1^- or LEU^-1^- medium, diluted into 10 ml URA^-1^- or LEU^-1^- cultures to grow to 1.0 *A*~600~. Cultures were then diluted to 0.1 *A*~600~ in 50 ml YPAD and grown to 0.2–0.3 *A*~600~ before addition of nocodazole (Sigma M1404) at 7.5 μg/ml for most strains; 2 μg/ml was used for gcn5Δ cse4-S180A RTS1-3PK::TRP1 (LPY22275) and arrested for 90–120 min. Arrest was monitored by light microscopy and subsequently confirmed by flow cytometry using samples removed at onset of treatment and just before fixation. Metaphase-arrested cells were fixed for 15 min at room temperature by addition of 1.2 ml 37% formaldehyde. Cells were lysed and sonicated for ChIP as described (Torres-Machorro et al., 2015) and 400 μg of sample was used per IP with anti-V5 (MCA1360; Bio-Rad), which recognizes the PK epitope. ChIP DNA was recovered using the Qiagen PCR purification kit. qPCR was completed using an Opticon Monitor 2 and Eurogentec qPCR MasterMix Plus. Primer pairs CEN4 150bp R, CEN4.2, CEN3 250bp R, CEN3 2.6kb R, and Chil1 103kb R from Nerusheva et al. (2014) along with a newly designed ArmlV 112kb R (Supplemental Table S3) were used to amplify centromeric, pericentromeric, and distal targets for ChIP and input DNA. ChIP signals were quantified relative to input.
RT-qPCR
Wild-type and gcn5Δ cells were grown in 20 ml cultures to log phase at 30°C before RNA extraction by the hot phenol method. Samples were treated with DNase (Ambion) and cDNA prepared with the TaqMan Reverse Transcriptase kit (Life Sciences). Primers for ADH1, CWP1, and SCR1 controls are as listed in Petty et al. (2016); GCN5, RTS1, and SGO1 primers are listed in Supplemental Table S3. Otherwise, qPCR was completed as above, normalized to RNA polymerase III transcript SCR1, an established GCN5-independent control, and gcn5Δ expression relative to wild type was determined.

Protein lysate preparation and immunoblotting
Overnight cultures of transformants were grown in selective media and diluted to 0.15 A600 the following morning. Cells were collected at 0.8 A600 and lysates were prepared as described (Clarke et al., 1999). Acrylamide gels (12%) were used for SDS–PAGE separation of HA-Cse4, Rts1-3PK, and tubulin, which were then detected by immunoblotting on nitrocellulose (0.2 μM; Prometheus). Anti-V5 was used at 1:10,000, anti-HA 1:20,000, and anti-tubulin 1:20,000 in 3% milk in Tris-buffered saline-Tween. Anti-mouse and rabbit immunoglobulin G horseradish peroxidase–conjugated secondary antibodies (Promega) were used at 1:10,000, anti-HA 1:2000, and anti-tubulin 1:20,000 in 3% milk in Tris-buffered saline-Tween. Anti-mouse and rabbit immunoglobulin G horseradish peroxidase–conjugated secondary antibodies (Promega) were used at 1:20,000. Blots were exposed to Pierce ECL Western Blot Substrate (Thermo Fisher) and images collected using a Protein Simple FluorChem E imager. Relative quantitation was determined by signal density analysis in ImageJ (Schneider et al., 2012).

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Boldface names denote co–first authors.


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