Contribution of CAF-I to Anaphase-Promoting-Complex-Mediated Mitotic Chromatin Assembly in Saccharomyces cerevisiae

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The anaphase-promoting complex (APC) is required for mitotic progression and genomic stability. Recently, we demonstrated that the APC is also required for mitotic chromatin assembly and longevity. Here, we investigated the role the APC plays in chromatin assembly. We show that apc5Ca mutations genetically interact with the CAF-I genes as well as Asf1p, Hir1p, and Hir2p. When present in multiple copies, the individual CAF-I genes, Caf1p, Caf2p, and Msi1p, suppress apc5Ca phenotypes in a CAF-I- and Asf1p-independent manner. CAF-I and the APC functionally overlap, as caf1Δ caf2Δ msi1Δ (caf1Δ) cells expressing apc5Ca exhibit a phenotype more severe than that of apc5Ca or caf1Δ. The Ts+ phenotypes observed in apc5Ca and apc5Ca caf mutants may be rooted in compromised histone metabolism, as coexpression of histones H3 and H4 suppressed the Ts- defects. Synthetic genetic interactions were also observed in apc5Ca asf1Δ cells. Furthermore, increased expression of genes encoding Asf1p, Hir1p, and Hir2p suppressed the apc5Ca Ts- defect in a CAF-I-dependent manner. Together, these results suggest the existence of a complex molecular mechanism controlling APC-dependent chromatin assembly. Our data suggest the APC functions with the individual CAF-I subunits, Asf1p, and the Hir1p and Hir2p proteins. However, Asf1p and an intact CAF-I complex are dispensable for CAF-I subunit suppression, whereas CAF-I is necessary for Asf1p, Hir1p, and Hir2p suppression of apc5Ca phenotypes. We discuss the implications of our observations.

Chromatin assembly, the deposition of histones onto DNA, is an essential step in chromosome compaction, a process which determines the precision of chromosome segregation during mitosis (12, 24). Imperfections in chromatin assembly and remodeling have been linked to the onset of many disease states, including premature aging and cancer (reviewed in references 4, 6, 10, 29, and 31). Chromatin is composed of a string of nucleosomes, in which 147 bp are wrapped two times around an octameric complex called the nucleosome core particle (54). The nucleosome core particle is made up of two copies of the four histones, H2A, H2B, H3, and H4. Proteins called chromatin assembly factors (CAFs) facilitate nucleosome formation, and they have been isolated from virtually all systems studied (68, 71). However, little is known regarding how CAFs ensure proper chromatin assembly or how they are regulated. Recently, we isolated the apc5Ca mutant, which compromised anaphase-promoting complex (APC) activity and impaired a novel mitotic chromatin assembly activity (20, 21). Furthermore, we demonstrated that the APC is required in Saccharomyces cerevisiae for longevity (22). Thus, the possibility exists, at least in S. cerevisiae, that defects in APC-dependent chromatin assembly impact longevity. To understand this potential correlation, it is imperative to explore the molecular mechanisms involved in APC-dependent chromatin assembly.

The APC is a large, evolutionarily conserved complex that is essential in S. cerevisiae. The APC functions as a ubiquitin-protein ligase (E3) in the ubiquitin-dependent targeting pathway (reviewed in references 23 and 51). The E3s mark the endpoint of a molecular cascade which relies on the prior activity of a ubiquitin-activating protein (E1) and one of a family of conserved and homologous ubiquitin-conjugating enzymes (E2s) (27). The E3s are structurally and functionally diverse and share the ability to select proteins for ubiquitination (65). The APC’s role is to target proteins that prevent sister chromatid separation (Pds1p) and exit from mitosis (Cdb2p) for proteasome-dependent degradation. The segregation of sister chromatids is sensitive to mitotic stress, and this depends in part on proper posttranslational modification of histones (11, 13). Thus, an efficient mechanism maintaining proper chromatin metabolism during mitosis may involve the coupling of sister chromatid separation with a chromatin repair, modification, and/or assembly machinery.

The chromatin assembly machinery potentially targeted by the APC would be expected to be mitotic specific, since the main function of the APC is to promote passage through mitosis. However, all of the CAFs identified, none have been shown to function exclusively during mitosis. The only CAF clearly linked to the cell cycle has been CAF-1, which is critical for S-phase-coupled nucleosome deposition (64). CAF-1 is a trimeric complex conserved in virtually all eukaryotic systems studied (37, 39, 55, 64, 67). In S. cerevisiae, the CAF-I subunits are referred to as Cac1p, Cac2p, and Cac3p/Msi1p, whereas in humans the subunits are called p150, p60, and p48. Biochemical studies in vitro have shown that human CAF-I is associated with newly synthesized and acetylated histones H3 and H4 (36, 69). Furthermore, the observation that the p150 subunit of human CAF-I interacts with PCNA, a DNA polymerase clamp,
sparked speculation that CAF-I is loaded onto replicating DNA via its interaction with PCNA (61, 73). Recent in vivo studies also support a role for CAF-I in S phase, as a dominant-negative p150 subunit mutation or depletion of CAF-I results in S-phase arrest with concomitant DNA damage and S-phase checkpoint activation (28, 72).

The role of CAF-I plays during S phase is not essential in S. cerevisiae, as S. cerevisiae cells lacking all three CAF-I subunits remain viable (14, 37). This may reflect redundant interactions between different CAFs. For example, yeast CAF-I has been shown to genetically and physically interact with several factors involved in chromatin metabolism, namely, Asf1p and the histone information regulators (Hir) (41, 48, 60, 66, 67). These studies revealed physical interactions between Cac2p/ p60 and Asf1p and between Asf1 and the Hir proteins. Taken together, the accumulating data have lead to the suggestion that CAF-I and Asf1p/Hir proteins function in alternative chromatin assembly pathways (41). Moreover, the CAF-I p48 subunit has been isolated from plant and animal cells in CAF-I-independent complexes involved in histone acetylation and deacetylation (26, 57, 70). Although CAF-I is clearly required for S-phase-coupled chromatin assembly, CAF-I function appears to be far more complex than originally anticipated.

CAF-I has been demonstrated to function outside of S phase, as CAF-I is required for DNA repair-coupled chromatin assembly (16, 47, 49), and can assemble bulk chromatin in a replication-independent manner (35), and p60 and p150 co-localize with sites of nucleotide excision repair sites outside of S phase (42, 47). Moreover, yeast CAF-I was recently shown to be required, along with the Hir proteins, for the formation of functional kinetochores (60). Cells lacking both CAC1 and HIR1 exhibited a slow-growth phenotype due to a delay in progression through G2/M (60). Thus, it is becoming apparent that CAF-I can function outside of S phase.

In this report, we present genetic studies that suggest the existence of a complex molecular interaction between the APC and the CAF-I subunits as well as Asf1p, Hir1p, and Hir2p. The individual CAF-I subunits suppress APC defects in a CAF-I- and Asf1p/Hir-independent manner, whereas Asf1p, Hir1p, and Hir2p all require CAF-I.

MATERIALS AND METHODS

Yeasts strains. Table 1 lists the S. cerevisiae strains used. All strains were isogenic derivatives of S288c strains. The RMY102 strain (a generous gift from M. Grunstein) was used to purify plasmid pRM102 (45). To construct the cac1Δ::LEU2 strain, a plasmid (pPK103) containing cac1Δ::LEU2 and the CAC1 flanking region was obtained from P. Kaufman. A cac1Δ::LEU2 PCR fragment was generated by using primers designed to amplify 226 bp upstream and 1,063 bp downstream of the CAC1 coding region and pPK103 as the template. This fragment was transformed into YTH3 cells, and correct transformants were confirmed by PCR. YTH1173 was subsequently used in crossing experiments. All other strains were obtained from colleagues or generated through tetrad analyses. Strains containing msi1Δ::kanMX6 and cac2Δ::kanMX6 (Research Genetics) were selected from tetrads in which the kanMX6 marker segregated 2:2. Triple and quadruple mutants containing msi1Δ::kanMX6 and cac2Δ::kanMX6 were selected by picking spores from tetrads where the kanMX6 marker segregated 2:2 and the additional desired markers segregated with kanMX6.

Media and Methods. YPD (yeast extract, peptone, dextrose), CM (complete medium), SD (synthetic dextrose), SD lacking uracil (SD-ura), and SD lacking leucine (SD-leu) were prepared according to published protocols (56). Glycerol was used at 2% in place of glucose in plates. Standard genetic techniques were performed as described (19). In vitro chromatin assembly assays were performed according to previously published protocols (20, 21). Histone add-back experiments were conducted by including histones isolated from untreated or colcemid-treated HeLa cells, following acid extraction (Uspate), in chromatin assembly reactions according to the nonradioactive protocol (21). Spot dilutions were performed with cells, adjusted to 2 × 10^6/ml, from a fresh overnight culture. A 10-fold serial dilution series was then prepared, and 5 μl of each dilution was spotted onto the appropriate medium. The plates were placed in the appropriate incubator for 3 to 8 days. The plates were then scanned with an Epson Perfection 1650.

Standard DNA manipulations. Escherichia coli strains JM109 and DH10B were used to propagate DNA plasmids. DNA manipulations such as restriction enzyme digestions, DNA minipreps, S. cerevisiae and E. coli transformations, and S. cerevisiae genomic DNA preparation have been described previously (56).

Plasmid construction. All plasmids used in this study are shown in Table 2. pH1133 was constructed by first cloning a CAC1 PCR fragment into pCR2.1-TOPO. The CAC1 PCR fragment contained 226 bp of upstream and 195 bp of downstream sequence. Both Topo-CAC1 and the S. cerevisiae 2μ expression vector, YEplac181 were digested with KpnI and PstI. The appropriate DNA fragments were ligated, resulting in plasmid pH1330 (YEp-CAC1), pRM102, which expresses histones H3 and H4 from the GAL promoter (45), was purified from RMY102.

Growth curves. Growth curves were generated by inoculating 5-ml YPD cultures with 1 × 10^6 cells from fresh cultures, followed by incubation at the indicated temperatures. Samples were removed at the indicated times, and the cell concentrations were determined by measurements of the optical density at 600 nm (OD_{600}). Some variation was observed between different growth curves of the same strain. Therefore, the curves shown were all generated at the same time and are a typical representation of the results obtained.

Flow cytometry. Yeast cells were grown overnight in YPD at permissive temperatures. The next morning, cells were diluted to an OD_{600} of 0.2 and incubated with shaking at 30°C and 37°C till an OD_{600} of 0.5 was reached. The cells were then harvested and prepared for flow cytometry according to published protocols (15).

RESULTS

Multicopy expression of CAF-I subunits suppresses apc5ΔCT phenotypes. The chromatin assembly deficiency observed in apc5ΔCT cells prompted us to examine whether the APC interacts with a specific CAF. Hence, we asked whether increased levels of CAFs would suppress apc5ΔCT mutant phenotypes. We began our investigation with an analysis of the genes encoding the CAF-I subunits, CAC1, CAC2, and MSII. Our results demonstrate that multicopy expression of CAC1, CAC2, and MSII could indeed suppress the Ts+ growth and chromatin assembly defects associated with apc5ΔCT cells (Fig. 1 and data not shown; a 2μm plasmid expressing MSII, pH149, was generously provided by M. Carlson). The finding that multicopy expression of MSII suppressed apc5ΔCT defects provides an explanation as to how MSII could suppress both snf1Δ (30) and Ras2V1419Δ (58) phenotypes; Snf1p activates the APC (22), while Ras/protein kinase A signaling inhibits it (32, 40). Taken together, these data provide evidence that CAF-I acts downstream of the APC and, when overexpressed, may be capable of repairing damaged chromatin conferred by mutations that reduce APC activity.

Apc5ΔCT and CAF-I mutations interact genetically. If increased expression of CAF-I subunits provided a benefit for apc5ΔCT cells, then perhaps decreased expression would be detrimental. This prediction was borne out, as the combination of different caf mutations with apc5ΔCT exacerbated the apc5ΔCT Ts+ growth defect (Fig. 1). Previous studies showed that disruption of one CAF-I subunit was as deleterious as deleting all three, indicating that Cac1p, Cac2p, and Ms1p acted together (14, 37). If the intact CAF-I complex was required for APC function, then progressive disruption of CAF-I subunits in apc5ΔCT cells would not be expected to create a more severe...
whether CAF-I subunits can suppress the apc5CA phenotype. However, this was not the case, as we observed that when double and triple caf mutants were given apc5C4 mutations and grown on glucose-supplemented medium, growth was increasingly impai...sion in DNA repair (16, 47, 49). To gain insight into the effects of caf mutations inter...in DNA replication-coupled chromatin assembly (64), it is necessary to understand the role of CAF-I in these processes.

**Intact CAF-I complex is not required for suppression of apc5C4 Ts phenotypes.** In order to gather supporting evidence for the results shown in Fig. 2, which suggest that the CAF-I subunits can function independently in apc5C4 cells, we asked whether CAF-I subunits can suppress the apc5C4 Ts defect in cells lacking intact CAF-I. Our results indicate that an intact CAF-I complex is not required for apc5C4 suppression, as CAC1, CAC2, and MSL2 continued to suppress the apc5C4 Ts defect in apc5C4 caf mutants (Fig. 3 and 8C; data not shown). These results clearly show that increased levels of only one CAF-I subunit are sufficient for suppression of the apc5C4 Ts phenotype in the absence of an intact CAF-I complex. These observations confirm that the apc5C4 and caf mutations interact genetically and further suggest the possibility that the CAF-I subunits may function individually in apc5C4 cells.

**Deletion of any of the CAF-I subunits does not exacerbate the apc5C4 cell cycle defect.** Although CAF-I plays a major role in DNA replication-coupled chromatin assembly (64), it is clear that CAF-I can function in a DNA replication-independent manner, such as in DNA repair (16, 47, 49). To gain insight into the effects of caf mutations on cell cycle progression in apc5C4 cells, we prepared the various CAF-I subunit mutants described in this study and determined their effects on cell cycle progression. The results summarized in Table 1 and Fig. 3 illustrate that deletion of any of the CAF-I subunits does not exacerbate the apc5C4 cell cycle defect.

### TABLE 1. S. cerevisiae strains

| Strain       | Relevant genotype | Source or reference |
|--------------|-------------------|---------------------|
| RMY102       | MATa ade2-101(Oc) his3Δ200 lys2-801(Am) trp1Δ001 ura3-52 hht1 hht2::LEU2 hht2::HIS3 PRM102 | M. Grunstein |
| S288c derivatives |                   |                     |
| YTH3         | MATa ade2 leu2-3,112 lys2Δ201 ura3-52 | W. Neupert |
| YTH6         | MATa ade2 his3Δ200 lys2Δ201 ura3-52 | W. Neupert |
| YTH457       | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4 | 20 |
| YTH1149      | MATa ade2 his3 leu2 his2(?) ura3 asf1Δ::HIS3 | This study |
| YTH1155      | MATa ade2 his3Δ200 leu2-3,112 lys2Δ201(?) ura3-52 apc5C4-PA::His5+ | 20 |
| YTH1156      | MATa ade2 his3 leu2 his2(?) ura3 | This study |
| YTH1159      | MATa ade2 his3 leu2 lys2(?) ura3 apc5C4-PA::His5+ asf1Δ::HIS3 | This study |
| YTH1173      | As YTH3 but cac1Δ::LEU2 | This study |
| YTH1275      | MATa ade2 his3 leu2 lys2 met15Δ(?) ura3 apc5C4-PA::His5+ cac22::kanMX6 | This study |
| YTH1296      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1298      | MATa ade2 his3 leu2 lys2 met15Δ(?) ura3 apc5C4-PA::His5+ ms1Δ::kanMX6 | This study |
| YTH1417      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 cac1Δ::LEU2 | This study |
| YTH1418      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1418      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1421      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1536      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1537      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 cac1Δ::LEU2 | This study |
| YTH1561      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 cac1Δ::LEU2 | This study |
| YTH1562      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 cac1Δ::LEU2 | This study |
| YTH1588      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1589      | MATa ade2 his3 leu2 lys2(?) met15Δ(?) ura3 apc5C4-PA::His5+ cac1Δ::LEU2 | This study |
| YTH1636      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 | This study |
| YTH1637      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1636      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1666      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1667      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1668      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1669      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1688      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1689      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1690      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1691      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
| YTH1817      | MATa ade2 his3Δ200 leu2-3,112 ura3-52 apc5C4-PA::His5+ | This study |
YPD to an OD_{600} of 0.5 at 30°C and 37°C prior to fixation. Our results indicate that deletion of CAF-I subunits does not cause an increase in the accumulation of \textit{apc5CA} cells in the G\textsubscript{2}/M phase of the cell cycle (Fig. 4) (2, 20). The caf mutations may in fact mollify the accumulation of \textit{apc5CA} cells in G\textsubscript{2}/M. Deletion of \textit{CAC1} had previously been shown to have no effect on cell cycle progression (60, 66), and we extended that by showing that \textit{caf1}/\textit{H9004} cells also have little trouble with cell cycle progression. Thus, the increased exacerbation of the \textit{apc5CA} Ts\textsuperscript{-}/\textit{H11002} defect as CAF-I subunits are progressively deleted (Fig. 2B) is apparently not accompanied by an increased accumulation of \textit{apc5CA} cells in G\textsubscript{2}/M.

**TABLE 2. Plasmids**

| Plasmid     | Vector | Insert\(^{a}\) | Source or reference |
|-------------|--------|-----------------|---------------------|
| YCp50       | CEN-URA3 |                 | M. Ellison          |
| YCpplac111  | CEN-LEU2 |                 | W. Neupert          |
| YEplac181   | 2μm-LEU2 |                 | W. Neupert          |
| pYEX4T-1    | 2μm-leu2-d-UAA3-CUP1-GST |                 | Clontech            |
| pTH101      | YCplac111 | \textit{APC5} plus 300 bp upstream | 20                   |
| pTH113      | YEplac181 | \textit{MSII}   | R. Wozniak          |
| pPK103      | pBlueScript | \textit{cacl1::LEU2} | M. Carlson          |
| pYEX-CAC2   | pYEX4T-1 | \textit{GST-CAC2} | Exclone library     |
| pYEX-MSII   | pYEX4T-1 | \textit{GST-MSII} | Exclone library     |
| pYEX-ASF1   | pYEX4T-1 | \textit{GST-ASF1} | Exclone library     |
| pYEX-APC5   | pYEX4T-1 | \textit{GST-APC5} | Exclone library     |
| YEplHIR1    | URA3-2μm | \textit{HIR1}   | M. Osley            |
| YEplHIR2    | URA3-2μm | \textit{HIR2}   | M. Osley            |
| YEplASF1    | YEplac181 | \textit{ASF1}   | This study          |
| pRM102      | URA3-CENIARS | GAL-H3/H4 | Isolated from RMY102 (this study) |

\(^{a}\) GST, glutathione S-transferase.

Increased expression of histones H3 and H4 suppresses \textit{apc5CA} and \textit{apc5CA} caf mutations in vivo. To investigate whether \textit{apc5CA} and \textit{apc5CA} caf phenotypes arise from defective chromatin metabolism, implied by the \textit{apc5CA} chromatin assembly defect (20), we tested whether \textit{apc5CA} and \textit{apc5CA} caf Ts\textsuperscript{-} defects could be suppressed by increased coexpression of histones H3 and H4 from a divergent \textit{GAL1}/\textit{10} promoter (pRM102 was kindly provided by M. Grunstein) (45). The results show that coexpression of histones H3 and H4 in \textit{apc5CA} cells is sufficient to suppress the Ts\textsuperscript{-} defect, whether grown under repressing (glucose) or derepressing (galactose) conditions. Thus, these results are consistent with the notion

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**FIG. 1.** Multicopy expression of individual CAF-I subunits can restore \textit{apc5CA} phenotypes. (A) Multicopy expression of CAF-I subunit genes \textit{CAC1} and \textit{MSII} suppresses the \textit{apc5CA} growth defect at 37°C. \textit{apc5CA} cells (YTH1155) were transformed with YEplac181 (2μm-LEU2), YEplCAC1, YEplMSII, and YCpAPC5. The YCp vector is CEN-ARS based. The transformants were grown for 3 to 5 days on selective medium at 30°C and 37°C. (B) Multicopy expression of \textit{CAC1} or \textit{MSII} suppresses the \textit{apc5CA} in vitro chromatin assembly defect. The transformants described above were assayed for in vitro chromatin assembly according to published protocols (20, 21). The position of uniquely labeled, circular, relaxed pBlueScript plasmid (O, R) is indicated. Efficient chromatin assembly is observed when the amount of highly supercoiled topoisomers (sup.) greatly exceeds that of the intermediate topoisomers (inter.).
that histone metabolism is aberrant in apc5CA cells and is exacerbated when CAF-I genes are disrupted.

We next asked whether we could suppress the apc5CA in vitro chromatin assembly defect with the addition of exogenous histones. Acid-extracted histones H2A, H2B, H3, and H4, obtained from untreated or colcemid-treated HeLa cells (Upstate), were added to chromatin assembly reactions with wild-type or apc5CA extracts. Histones isolated from colcemid-treated cells are rich in mitosis-specific histones. However, although addition of histones to wild-type extracts improved chromatin assembly, no effect was observed when histones were added to apc5CA extracts (Fig. 5B). It is likely that apc5CA extracts are “dead end” extracts, as we were previously unable to restore assembly activity when Apc5p protein was added back to apc5CA extracts (data not shown).

cac1Δ interacts genetically with additional APC mutations. Our data thus far indicate that at least apc5CA interacts genetically with the CAF-I subunit genes. Next, we asked whether CAF-I interactions were limited to Apc5p. We extended our analysis by creating apc10Δ cac1Δ and cac1Δ cdc26Δ double mutants. The double mutants were grown on glucose-supplemented medium at different temperatures and compared with wild-type and single-mutant cells. The results in Fig. 6 demonstrate that the disruption of CAC1 in apc10Δ and cdc26Δ cells lowers the restrictive temperature at which these mutants will grow. Thus, our results indicate the APC complex, not just Apc5p, is required for interactions with CAF-I subunits, as the cac1Δ mutation exacerbates the Ts mutation phenotype of multiple APC mutants.

apc5CA interacts genetically with ASF1. The use of a novel APC mutation, apc5CA, allowed us to characterize novel CAF-I-independent functions for each CAF-I subunit. Next, we asked whether other CAFs were capable of genetic interactions with apc5CA. CAF-I is known to physically and genetically interact with the CAF Asf1p (41, 48, 67). To assess whether Asf1p was involved in APC-dependent chromatin assembly, an apc5CA strain was crossed with asf1Δ cells and scored for
that \textit{APC4, APC5, and APC9 transcripts are extremely rare (22).}

Multicopy expression of \textit{MSI1} restored the growth of the \textit{apc5\textsuperscript{CA} asf1\textDelta} mutant at 37°C (Fig. 7D), indicating that suppression of \textit{apc5\textsuperscript{CA} Ts\textsuperscript{+}} growth defects by \textit{MSI1} did not require Asf1p or CAF-I. \textit{ASF1} could not, however, restore the Ts\textsuperscript{−} defect in \textit{apc5\textsuperscript{CA} msi1\textDelta} cells (Fig. 7E). This observation suggests that Asf1p requires at least Msi1p to suppress \textit{apc5\textsuperscript{CA} Ts\textsuperscript{−}} defects. It is also worthy of note that overexpression of \textit{APC5} could restore the Ts\textsuperscript{−} growth defect of \textit{apc5\textsuperscript{CA} cells when MSII is deleted (compare Fig. 7C with Fig. 7E)}. Together, these observations add strength to our hypothesis that the APC controls mitotic chromatin assembly, perhaps by recruiting multiple CAFs to mitotic chromatin templates.

**H\textsubscript{IR1} and H\textsubscript{IR2} suppress \textit{apc5\textsuperscript{CA}} in a CAF-I-dependent manner.** Lastly, we asked if the interaction of \textit{APC5} with the CAF-I subunit genes and \textit{ASF1} involves an additional pair of CAF-I- and Asf1p-interacting proteins, Hir1p and Hir2p. As found for the CAF-I subunits and \textit{ASF1}, the expression of \textit{H\textsubscript{IR1} and H\textsubscript{IR2}} in multicopy suppressed the \textit{apc5\textsuperscript{CA} Ts\textsuperscript{−}} defect (Fig. 8A and 8B; \textit{H\textsubscript{IR1} and H\textsubscript{IR2}} multicopy plasmids were kindly provided by M. Osley). Asf1p and Hir1p have been linked to a chromatin assembly pathway that functions in parallel with CAF-I (41, 59, 68). Our results agree with this hypothesis, as increased expression of \textit{ASF1, H\textsubscript{IR1}, and H\textsubscript{IR2}} could not suppress \textit{apc5\textsuperscript{CA}} when CAF-I subunits were deleted (Fig. 8C). We also observed that while \textit{CUP1\textsubscript{prom}-dependent overexpression of APC5 could suppress the Ts\textsuperscript{−} growth of an \textit{apc5\textsuperscript{CA} msi1\textDelta} mutant (Fig. 7E), it could not suppress the Ts\textsuperscript{−} growth of the \textit{apc5\textsuperscript{CA} caf1\textDelta} strain (Fig. 8C). Taken together, our results suggest that the APC requires only one CAF-I subunit for mitotic chromatin assembly activity, supporting a model in which the individual CAF-I subunits assemble mitotic chromatin in a CAF-I-independent manner.

The observation that all CAF-I subunits must be deleted in \textit{apc5\textsuperscript{CA}} cells to confer Ts\textsuperscript{−} growth on glycerol (Fig. 2B) provides supporting evidence for this model. The genetic complexity of this model becomes apparent when one considers that Asf1p, Hir1p, and Hir2p likely require an intact CAF-I complex to suppress \textit{apc5\textsuperscript{CA} Ts\textsuperscript{−}} defects. Thus, it remains possible that an intact CAF-I complex also contributes to APC-dependent chromatin assembly.

**DISCUSSION**

The results reported here suggest a molecular regulatory mechanism for mitotic chromatin assembly. Our genetic and biochemical experiments support a model in which the APC plays a crucial role in mitotic chromatin assembly regulation, a function mediated by interactions with subunits of the chromatin assembly factor CAF-I. In \textit{apc5\textsuperscript{CA}} cells, the CAF-I subunits can function independently in an apparently redundant manner; the \textit{apc5\textsuperscript{CA} Ts\textsuperscript{−}} phenotype becomes increasingly impaired as additional CAF-I subunits are deleted (Fig. 2B). Asf1p as well as the Hir proteins, which interact physically and genetically with CAF-I and with each other (41, 48, 59, 60, 66, 67), also play a role in APC-dependent chromatin assembly but require an intact CAF-I complex. Thus, our results suggest that CAF-I may play a dual role in mitotic chromatin assembly, both as an intact complex in conjunction with Asf1p and the
Hir proteins and as individual subunits that act independently of Asf1p and the Hir proteins.

CAF-I subunits play a role in APC-dependent mitotic chromatin assembly. Evidence exists that potentially ties CAF-I to APC activity. For example, the smallest S. cerevisiae CAF-I subunit, Msi1p, has been found to suppress phenotypes resulting from overactive protein kinase A when expressed in multicopy (58). As protein kinase A inhibits APC activity (40), Msi1p may either play a role in activating the APC by down-regulating protein kinase A, or act downstream of the APC by directly compensating for chromatin defects resulting from APC impairment. Furthermore, multicopy expression of MSI1-suppressed phenotypes associated with an SNF1 disruption (30). Snf1p is activated upon glucose limitation and acts to ensure that genes required to utilize alternate carbon sources are transcribed (9).

We recently characterized a role for Snf1p in APC activation (22). We demonstrated that Snf1p, a known aging determinant in S. cerevisiae (3, 43), was required to promote APC-dependent longevity (22). We also have evidence to suggest that protein kinase A induced life span reduction (reviewed in references 33, 44, and 63) is mediated through inhibition of the APC, as blocking Ras2p function extends apc5C4 reduced life span (C. R. Geyer and T. A. A. Harkness, unpublished data). Thus, the complex MSI1 genetic interactions could be explained if Msi1p were acting downstream of the APC and was capable of repairing damaged chromatin resulting from APC inhibition through overactive protein kinase A or reduced Snf1p signaling.

The suppression of apc5C4 defects by increased expression of CAF-I subunits could also be explained as an indirect effect, as each subunit of CAF-I can separately bind histones (36, 62, 69). Thus, suppression of apc5C4 phenotypes could result from the compensatory action of histone binding, followed by either deposition into chromatin or sequestration. This would occur if histones were stabilized or accumulating in a free form in apc5C4 cells, as excess free histones are deleterious to cell health (18). However, this is clearly not the case, as increased expression of histones H3 and H4 in apc5C4 caf mutants suppressed the Ts+ growth defects (Fig. 5A). Even low levels of H3 and H4 expression, resulting from growth of the GAL1/10prom driven plasmid in repressing conditions (glucose), are sufficient to suppress the Ts+ growth defects. Our attempts to suppress the in vitro chromatin assembly defect in apc5C4

![FIG. 5. Increased levels of histones H3 and H4 in apc5C4 cells suppresses in vivo but not in vitro phenotypes.](image-url)
extracts with the addition of purified histones demonstrated that apc5Ca extracts could not be rescued by the addition of exogenous histones (Fig. 5B). We were also unable to rescue apc5Ca extracts with purified Apc5p protein (data not shown). It is therefore likely that the defects conferred by the apc5Ca allele are permanent once an extract is generated. We conclude from these experiments that in apc5Ca and apc5Ca caf cells, defects in histone metabolism can be overcome by increased expression of histones H3 and H4 in vivo.

**CAF-I subunits play separate yet partially redundant functions in apc5Ca cells.** Our conclusion that the CAF-I subunits can function independently of an intact CAF-I complex in apc5Ca cells is supported by two observations: (i) increased temperature sensitivity of apc5Ca cells as additional CAF-I subunits were deleted, indicating that the individual subunits support limited viability of apc5Ca cells at 37°C (Fig. 2B); and (ii) the ability of individual CAF-I subunits to suppress apc5Ca Ts defects when a different CAF-I subunit was deleted (Fig. 3). Basically, if an intact CAF-I complex was interacting with the APC, then double and triple caf mutations would create the same phenotype as single caf mutations in apc5Ca cells. Msi1p had previously been shown to function independently of CAF-I (34, 74). A recent study also ascribed a CAF-I-independent function for Cac1p in histone nuclear import (17). This report demonstrates additional CAF-I-independent functions for Cac1p and Msi1p and a novel CAF-I-independent function for Cac2p.

The notion that the individual subunits are at least partially redundant in apc5Ca cells is based on the following observations: (i) only apc5Ca cells lacking all three CAF-I subunits were Ts- on glycerol, which also suggests that the CAF-I subunits provide some function that is redundant with at least Apc5p (Fig. 2B); (ii) low-level expression of histones H3 and H4 had a greater suppressive effect on apc5Ca caf1Δ cells than on apc5Ca caf1Δ cells (Fig. 5A); and (iii) overexpression of APC5 could suppress apc5Ca msi1Δ cells but not apc5Ca caf1Δ cells (compare Fig. 7E with 8C). Therefore, our results are consistent with a model in which the individual CAF-I subunits act independently and most likely redundantly with each other.

We obtained additional evidence supporting the hypothesis that the CAF-I subunits can function in the absence of an intact CAF-I complex. We performed in vitro chromatin assembly assays on extracts prepared from the mutants shown in Fig. 2B. Mild chromatin assembly defects were observed in extracts prepared from single CAF-I mutants (C. Legrand and T. A. A. Harkness, unpublished data), implying a role for intact CAF-I in mitosis-specific chromatin assembly. However, the defects were increased marginally when additional subunits...
were mutated, implying that CAF-I subunits may function outside of an intact CAF-I complex. Combining each CAF-I mutation with the apc5CA allele also slightly decreased assembly efficiency. The marginal defects that we observed in chromatin assembly efficiency were reproducible and consistent with the involvement of single CAF-I subunits in mitotic chromatin assembly. However, the marginal results clearly show that other factors are involved.

**ASF1, HIR1, and HIR2 interact genetically with the apc5CA allele in a CAF-I-dependent manner.** Work in several laboratories has demonstrated physical and genetic interactions between CAF-I, Asf1p, and the Hir proteins (38, 41, 59, 67). Asf1p has been shown to stimulate the assembly of newly replicated chromatin by CAF-I in several systems (41, 48, 59, 67) and may act by delivering histones to CAF-I in vivo (48). Consistent with Asf1p functioning as a CAF, disruption of ASF1 was recently shown to cause in vitro chromatin assembly defects (52). On the other hand, an in vivo study in *S. cerevisiae* suggests that Asf1p may mediate global chromatin disassembly (1). Disruption of ASFI was shown to increase the in vivo chromatin assembly capacity of the 2μm plasmid, as opposed to disruption of CACI, which partially reduced assembly efficiency (1).

We have been unable to observe in vitro chromatin assembly defects with asf1Δ extracts (data not shown). The differences observed with asf1Δ strains might reflect the different methods employed to measure in vitro chromatin assembly. Nonetheless, our in vivo observations are consistent with models proposing that Asf1p acts as a CAF, perhaps by facilitating the role that CAF-I plays in mitotic chromatin assembly. In the absence of intact CAF-I, however, increased ASF1 cannot suppress apc5CA defects. Perhaps under these circumstances (the absence of CAF-I), Asf1p is free to act as a chromatin disassembly factor.

Our experiments suggest that an intact CAF-I complex may play a role in mitotic chromatin assembly that is facilitated by Asf1p and the Hir proteins (Fig. 7 and 8). We have recently shown that the mitotic chromatin assembly pathway may be regulated as early as G1, as SCF (Skp1/Cdc53/F-box protein) and RSP5 ubiquitin-ligase mutants, which arrest or accumulate in G1 at restrictive temperatures, respectively, are defective in our in vitro mitotic chromatin assembly assay (2). That work also provided evidence that RSP5 interacts genetically with SCF and the APC to promote mitotic chromatin assembly.

As CAF-I is critical for replication-dependent chromatin assembly (64) and passage through S phase (28, 50), it is possible that the role that an intact CAF-I complex plays in APC-dependent chromatin assembly is limited to ensuring that cells progress through S phase. Cells must complete DNA replication and chromosome compaction in order to progress through mitosis, and this is regulated by cell cycle checkpoints that inhibit APC activity (5). Thus, our results are consistent with a model in which CAF-I assembly and disassembly are regulated at mitosis, allowing CAF-I subunits to contribute to

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**FIG. 8.** HIR1 and HIR2 suppress apc5CA Ts- defects in a CAF-I-dependent manner. (A) YTH457 (apc5CA) was transformed with YCp50 and YEprHIR1. (B) YTH1637 (apc5CA) cells were transformed with YCp50, YCp50-APC5, and YEprHIR2. The transformants were grown for 3 days at 30°C and 37°C. The plates were then scanned. (C) Suppression of apc5CA Ts- defects by ASF1, HIR1, and HIR2 requires an intact CAF-I. apc5CA caf1Δ (YTH1418) cells were transformed with the plasmids shown. The transformants were grown at 30°C and 37°C for 3 days.
both S-phase- and mitosis-specific chromatin assembly. There is evidence to support CAF-I disassembly in mammalian cells, as p60 is hyperphosphorylated as cells enter mitosis, and this coincides with release of CAF-I from chromatin, export of monomeric hyperphosphorylated p60 to the cytosol, and an inability of purified CAF-I to assemble chromatin in a replication-dependent manner (46).

In conclusion, the experiments presented in this report provide evidence that a novel molecular mechanism controls chromatin assembly during mitosis. It is feasible that this mechanism is in place to maintain functionally intact chromosomes during the segregation of sister chromatids or to establish a transcriptionally primed genome as cells exit mitosis. The possibility that this is a conserved mechanism raises several interesting questions. For example, what would be the consequences to a mammalian cell that harbored mutations in this pathway? We have recently demonstrated that S. cerevisiae APC is required for longevity (22). Premature aging in mammalian cells is often accompanied by cancer and genomic instability (7, 8). Strikingly, several reports have indicated that the APC plays a pivotal role in resistance to cancer (5, 53, 65). In addition, CAF-I mutations can lead to S-phase arrest and genomic instability (50, 72), while p48 and the protein that directs CAF-I to replication forks, PCNA, are deregulated in tumors found in patients with tuberous sclerosis (25). Future studies aimed at fully characterizing the mechanisms employed by the APC to control chromatin assembly in S. cerevisiae and higher eukaryotic cells will provide valuable insights into disease onset and progression.

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