Silencing of Chromatin Assembly Factor 1 in Human Cells Leads to Cell Death and Loss of Chromatin Assembly during DNA Synthesis

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In eukaryotic cells, chromatin serves as the physiological template for gene transcription, DNA replication, and repair. Chromatin assembly factor 1 (CAF-1) is the prime candidate protein to mediate assembly of newly replicated DNA into chromatin. To investigate the physiological role of CAF-1 in vivo, we used RNA interference (RNAi) to silence the 60-kDa subunit of CAF-1 (p60) in human cells. Transfection of a small interfering RNA (siRNA) directed against p60 resulted in efficient silencing of p60 expression within 24 h. This silencing led to an induction of programmed cell death in proliferating but not in quiescent human cells. Concomitantly, proliferating cells lacking p60 accumulated DNA double-strand breaks and increased levels of the phosphorylated histone H2A.X. Nuclear extracts from cells lacking p60 exhibited a 10-fold reduction of nucleosome assembly activity during DNA synthesis, which was restored upon addition of recombinant p60 protein. Nascent chromatin in cell nuclei lacking p60 showed significantly increased nuclease sensitivity, indicating chromatin assembly defects during DNA synthesis in vivo. Collectively, these data identify CAF-1 as an essential factor not only for S-phase-specific chromatin assembly but also for proliferating cell viability.

In eukaryotic cell nuclei, the genomic DNA is highly compacted into chromatin through an assembly with histone and nonhistone proteins. The structural subunit of chromatin is the nucleosome, containing 146 bp of DNA wrapped in just under two superhelical turns around an octamer of histones H2A, H2B, H3, and H4 (1). Nucleosome-packed genomic DNA assumes higher-order structures through internucleosomal contacts and association with linker histones and other proteins. This assembly serves as the natural substrate for DNA replication, repair, and recombination and for transcription in vivo.

In proliferating cells, the bulk of chromatin is assembled during DNA replication in the S phase of the cell cycle (19, 40, 43). New histones are synthesized during S phase and become incorporated into nucleosomes on the newly replicated DNA. Replication-specific nucleosome assembly is mediated by histone chaperones such as chromatin assembly factor 1 (CAF-1), which was initially purified from proliferating human cell nuclei (36). CAF-1 is a trimeric protein complex consisting of three subunits, p150, p60, and p48, which are required for replication-dependent nucleosome assembly in human cell extracts (10, 44). CAF-1 mediates the first step of nucleosome assembly, the deposition of new histone H3/H4 tetramers onto replicating DNA (37). The second step of nucleosome assembly is independent of CAF-1 and involves the association of histone H2A/H2B dimers to the precursor H3/H4 tetramer structure (37).

The two large subunits of CAF-1 physically colocalize with sites of chromosomal DNA replication throughout S phase in human cell nuclei (17), suggesting that CAF-1 is a physiologically relevant factor for nucleosome assembly during DNA replication in vivo. Furthermore, CAF-1 is substrate specific for DNA templates that undergo DNA replication or repair (7, 36). This specificity is achieved through the direct binding of the p150 subunit of CAF-1 to proliferating cell nuclear antigen (PCNA) (23, 33), a circular DNA clamp that provides a replication-dependent marking of DNA.

CAF-1 is evolutionarily conserved, and homologues have been described in yeast, insects, plants, and vertebrates. Despite the essential role of CAF-1 for chromatin assembly during DNA synthesis in human cell extracts in vitro, deletions of CAF-1 in the budding yeast Saccharomyces cerevisiae surprisingly did not yield a lethal phenotype but resulted in relatively mild physiological defects, such as UV sensitivity and impaired gene silencing at telomeres and at the mating-type loci (5, 6, 8, 11, 24). Moreover, when the two largest subunits of CAF-1 are deleted in the plant Arabidopsis thaliana, the resultant fasciata mutants are viable and show alterations only in postembryonic development, in particular a loss of cellular organization in shoot and root apical meristems (13). These results indicate the existence of redundant or alternative nucleosomal assembly pathways that provide for efficient chromatin assembly in the absence of CAF-1 in fungi and plants.

In higher eukaryotes, on the other hand, evidence for a more essential in vivo function of CAF-1 is accumulating. Severe early development defects were found in Xenopus laevis embryos upon microinjection of a dominant-negative mutant of the p150 subunit of CAF-1, which also blocked nucleosome assembly in vitro (26). A dominant-negative human p150 mutant also inactivated nucleosome assembly by CAF-1 in vitro, and its ectopic expression in vivo induced S-phase arrest, accompanied by DNA damage and S-phase checkpoint activation (45). Taken together, these studies suggest that CAF-1 is required for maintaining epigenetic information encoded in accurate chromatin assembly, and in vertebrates it has a cen-
tral role in ensuring undisturbed chromatin replication in S phase. However, as the use of ectopically expressed dominant-negative mutant proteins could lead to secondary effects, it is important to investigate the in vivo roles of CAF-1 in human cells directly by knockout experiments.

Here, we used RNA interference (RNAi) (3, 4) to silence the p60 subunit of CAF-1 in human cells. Transfection of a small interfering RNA (siRNA) directed against p60 resulted in efficient silencing of p60 expression within 24 h. This silencing led to an induction of cell death in proliferating but not quiescent human cells. Concomitantly, transfected proliferating cells displayed accumulation of double-strand DNA breaks indicative of activated programmed cell death. Nuclear extracts derived from these cells exhibited a 10-fold reduction of nucleosome assembly activity during DNA synthesis, which was restored by adding back recombinant p60 protein to the in vitro replication reactions. Collectively, these data identify CAF-1 as an essential factor for S-phase-specific chromatin assembly and, most importantly, for proliferating cell viability.

MATERIALS AND METHODS

Cell culture. HeLa-S3 and EJ30 cells were cultured as exponentially growing subconfluent monolayers in Dulbecco’s modified Eagle’s medium (DMEM) (In-vitrogen), supplemented with 10% fetal calf serum (Invitrogen), 10 U of penicillin (Sigma) per ml, and 0.1 mg of streptomycin (Sigma) per ml. EJ30 cells were made quiescent by cultivation in this medium supplemented with only 0.5% fetal bovine serum, 100 mM NaCl, 50 mM Tris-HCl [pH 7.5] in a sterile RNase-free atmosphere. To make the cells hypertonic, 10% Ca(OH)2 and 0.5 mM dithiothreitol were added to each well for 10 min. The buffer was removed, and the cells were gently scraped from each well. The cell suspension was transferred to a fresh tube, and the volume of the cells was determined with a pipette. An equal volume of 2X hypertonic buffer (20 mM HEPES-KOH, 800 mM NaCl, 5 mM KCl, 1.5 mM MgCl2, and 1 mM dithiothreitol) was added to the suspension. The cells were then homogenized by several passages through a 29-gauge syringe. The homogenate was incubated on ice for 30 min and centrifuged at 10,000 g for 10 min. The supernatant was collected and dialyzed against 20 mM HEPES-KOH/5 mM potassium acetate/0.5 mM MgCl2/0.5 mM dithiothreitol for 1 h. This dialyzed nuclear extract solution was stored in small aliquots in liquid nitrogen. Nuclear extracts contained about 1 to 5 mg of total protein per ml as determined by the Bio-Rad protein assay with bovine serum albumin as a standard.

DNA synthesis-dependent nucleosome assembly reactions. cDNA strand synthesis and nucleosome assembly reactions were performed essentially as described before (20). Standard reaction mixtures containing cytosolic extract from asynchronously proliferating HeLa cells (125 μg of protein) and 25 ng of single-stranded M13mp18 template DNA were supplemented with nuclear extracts from cells transfected with siRNA. Recombinant p60 was derived from baculovirus-infected insect S9 cells by using recombinant baculovirus particles (a gift from Bruce Stillman, Cold Spring Harbor, N.Y.) as described elsewhere (10).

Micrococcal nuclease assay. For micrococcal nuclease digestions, transfected cells were harvested after 24 h via trypsinization. The cell pellet was resuspended in hypotonic buffer (20 mM HEPES-KOH, 150 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol) per ml of the cell suspension was used for determining the cell concentration with a hemocytometer. Equivalent numbers of cells were harvested after 24 h and used for determining the cell concentration with a hemocytometer. Equivalent numbers of cells were harvested after 24 h and used for determining the cell concentration with a hemocytometer. Equivalent numbers of cells were incubated with 10 μg of RNase A (Sigma) and 0.1, 0.3, 1, 3, or 10 U of micrococcal nuclease (Roche) per reaction in a 50-μl volume at 21°C for 30 min. Nucleosome-resistant DNA was purified as described elsewhere (34). DNA purified from the stopped reactions was analyzed on 1.5% agarose gels and visualized by staining with ethidium bromide and phosphor imaging of the dried gel.

Visualization of DNA replication foci. To detect the presence of active DNA replication foci in transfected cell nuclei, cells were homogenized at 24 h after transfection exactly as described for the micrococcal nuclease assay. DNA replication run-on reactions were performed for 3 h essentially as described before (17) but with digoxigenin-11-dUTP as the tracer and propidium iodide as the counterstain for nuclear DNA. Imaging of nuclei was performed with a Leica confocal microscope as detailed elsewhere (38).

RESULTS

Silencing of the p60 subunit of CAF-1 by RNAi. We decided to investigate the role of CAF-1 in HeLa cells by selectively silencing the expression of the p60 subunit, which was previously shown to be essential for nucleosome assembly activity
the most efficient siRNA, targeting a sequence near the 3' end of the p60 mRNA, for all subsequent experimentation. With this siRNA, expression of p60 was specifically reduced by about 90% at 24 h and by more than 95% at 48 h posttransfection (Fig. 1A). These Western blot data were corroborated by immunofluorescent staining of HeLa cells at 24 h posttransfection. Expression of p60 was no longer detectable in more than 90% of the cells (Fig. 1B and data not shown). In contrast, expression of the p150 and p48 subunits of CAF-1 as well as actin was not reproducibly affected by the silencing of the p60 subunit at 24 h posttransfection. We observed a reduction of p150 protein levels of up to 20% in only two of five independent experiments (Fig. 1A and data not shown). However, at 48 h posttransfection we observed a reduction in the expression levels of p150 of up to 50% in only three of five independent silencing experiments (Fig. 1A and data not shown). In conclusion, efficient and rapid silencing of the p60 subunit by RNAi did not result in concomitant silencing of the p150 and p48 subunits.

**Induction of cell death after silencing of p60.** Following transfection with the p60-specific siRNA, we routinely observed morphological changes such as nuclear fragmentation in the majority of cells by fluorescence microscopy (Fig. 1B), suggesting an induction of programmed cell death upon silencing of p60. Therefore, we directly investigated cell viability using a quantitative colorimetric assay based on the reduction of a tetrazolium salt in living cells. We quantified cell viability at the time of transfection and at 24 h and 48 h posttransfection (Fig. 2A). Untransfected control cells grew exponentially throughout this experiment. Cells transfected with a nontarget control siRNA showed a reduced growth curve compared to that of the control as a result of the transfection procedure. In contrast, HeLa cells transfected with the p60-specific siRNA exhibited a dramatic reduction in the number of living cells during this experiment, indicating the occurrence of cell death as a result of silencing the expression of p60 (Fig. 2A). This observation was corroborated by trypan blue staining of these cells at 48 h posttransfection, confirming large numbers of dead cells after silencing of p60 (data not shown).

We also observed the morphology of these cells by phase contrast light microscopy (Fig. 2B). At 48 h posttransfection, all cell samples appeared generally healthy. After 48 h, HeLa cells transfected with the nontarget control siRNA still largely appeared healthy compared to the proliferating untransfected cells. In contrast, cells transfected with the p60-specific siRNA exhibited acute cytopathicity and cell death (Fig. 2B). We therefore conclude that CAF-1 is an essential protein for human cells and that silencing of its p60 subunit leads to cell death between 24 h and 48 h posttransfection.

**Induction of DNA damage and mechanism of cell death after p60 silencing.** To identify the pathway of cell death, we asked whether silencing of p60 expression results in DNA breaks and the induction of apoptosis. Therefore, we first checked by Western blotting for the presence of the phosphorylated form of histone H2A.X (γ-H2A.X), which has been shown to become phosphorylated adjacent to sites of double-stranded DNA breaks (28, 30). Figure 3A shows that no detectable levels of γ-H2A.X were observed in untreated cells or in cells transfected with the nontarget control siRNA at 24 or 48 h posttransfection. In contrast, transfection with the p60-specific siRNA caused a signal for γ-H2A.X at just 24 h posttransfection, which increased significantly in intensity by 48 h posttransfection. This result indicates that double-stranded DNA damage accumulates during the silencing of p60. This accumulation is suggestive of genome fragmentation during apoptosis, which also results in the accumulation of γ-H2A.X (29).

To corroborate these data independently, we used the TUNEL assay to ask whether the mechanism of cell death at 48 h posttransfection occurs through programmed cell death, or apoptosis (Fig. 3B and C). As a negative control, untreated...
proliferating human cells did not stain for fragmented nuclear DNA. Transfection with the nontarget siRNA resulted in DNA fragmentation in 11% of the cell population, perhaps due to unspecific cytotoxic insult arising from the RNAi transfection reagent or procedure. In marked contrast, 73% of the cells transfected with the p60-specific siRNA stained strongly for apoptotic genomic DNA fragmentation (Fig. 3B and C). In a positive control, 43% of the positive-staining cells were observed in the sample treated with the DNA topoisomerase poison etoposide (Fig. 3B), which causes severe DNA damage leading to the activation of programmed cell death (2, 12). It is worth noting that approximately 38% of the p60-silenced cells at 48 h posttransfection did not effectively counterstain for total DNA and appeared severely deteriorated (Fig. 3B), sug-
gesting that they had undergone apoptosis prior to this time point. In summary, we conclude that silencing of the p60 subunit of CAF-1 leads to an induction of DNA breaks and the activation of programmed cell death.

**Cell cycle effects after silencing of p60.** It has been reported that expression of a dominant-negative mutant of p150 in human cells results in S-phase arrest and activation of a DNA damage checkpoint response (45). Therefore, we tested whether the induction of DNA breaks and cell death caused by p60 silencing is preceded by an arrest of the transfected cells in S phase. An analysis of nuclear DNA content by flow cytometry of nuclei taken at 36 h posttransfection showed no significant enrichment of S-phase nuclei in either of the transfected samples (Fig. 4A). An analysis of nuclei taken at 24 h posttransfection gave virtually identical results (data not shown).

To visualize actively replicating S-phase nuclei directly, cells were harvested at 24 h posttransfection, before apoptosis became evident, and after the plasma membranes had been disrupted, the cell homogenates were subjected to nuclear run-on DNA replication in vitro (17). About 30% of the untransfected proliferating cells were found to be in S phase, whereas both of the transfected cell populations contained significantly reduced percentages of S-phase cells (Fig. 4B and C). In fact, cells transfected with the p60-specific siRNA showed the lowest proportion, only 12% replicating cell nuclei. Taken together, these data do not support an accumulation of cells in S phase upon silencing of p60. To investigate whether a requirement for CAF-1 for cell viability is specific for proliferating cells, we next analyzed the silencing of p60 in quiescent human cells.

**Silencing of p60 and viability in quiescent cells.** Human EJ30 cells were made quiescent by serum deprivation (18) and then subjected to RNAi. We were able to reduce p60 expression in quiescent cells to more than 90% at 48 h posttransfection but only after increasing the concentration of siRNA from 10 nM, as used for proliferating HeLa cells, to 25 nM (Fig. 5A). This higher siRNA concentration led to an increased nonspecific cytotoxicity in both of the transfected samples (Fig. 5B). Importantly, and in contrast to findings for proliferating cells, silencing of p60 in quiescent cells did not reduce the percentage of morphologically intact and viable cells beyond the percentage observed in the nontarget siRNA-transfected cells (Fig. 5B and C). This experiment demonstrates that cell death due to p60 silencing is restricted to actively proliferating human cells.

**Loss of CAF-1 activity in p60-silenced cell extracts.** In the last set of experiments we analyzed whether silencing of p60 removes the cell’s ability for rapid chromatin assembly during DNA replication. We prepared nuclear extracts of cells transfected with the negative-control, nontarget siRNA and with the p60-specific siRNA at 36 h posttransfection. Increasing amounts of these extracts were added to cDNA strand synthesis reaction mixtures (Fig. 6), with circular single-stranded DNA templates incubated in a cytosolic extract from human proliferating cells (20). We have shown before that addition of increasing amounts of CAF-1 to this reaction leads to increased supercoiling of the double-stranded DNA reaction products due to a stoichiometric nucleosome assembly activity of CAF-1 (20).

Addition of nuclear extract from control nontarget siRNA-transfected cells led to increased supercoiling of the reaction products.
products (Fig. 6A). In contrast, a 10-fold-higher amount of nuclear extract from p60 siRNA-transfected cells was required to achieve the same degree of supercoiling as observed with the extract from nontarget siRNA-transfected control cells (Fig. 6, compare lane 4 of panel A with lane 7 of panel B). Therefore, silencing of p60 leads to a reduction of the specific supercoiling activity of nuclear extracts by an order of magnitude. This deficiency in nucleosome assembly could be restored by addition of recombinant p60 from baculovirus-infected insect cells to the reaction (Fig. 6C). Because the nuclear extract from p60-silenced cells still contains the p150 and p48 subunits of CAF-1 (see Fig. 1A), we conclude that addition of recombinant p60 restores the functionality of CAF-1 in these reactions. This observation is consistent with a report of functional reconstitution of CAF-1 from individual recombinant subunits in vitro (10). These experiments therefore demonstrate that CAF-1 is an essential factor for nucleosome assembly during DNA synthesis in human cell extracts. They furthermore suggest that the loss of de novo nucleosome assembly activity of CAF-1 during DNA replication and the resulting perturbations of chromatin structure may be the cause of the induction of cell death in proliferating human cells.

Reduced efficiencies of chromatin assembly and DNA replication in p60-silenced cells. Finally, we investigated the chromatin structure of nascent DNA in p60-silenced cell nuclei at 24 h posttransfection (Fig. 7). Nuclei were isolated from nontarget siRNA- and p60 siRNA-transfected cells and incubated in a nuclear DNA replication run-on system (17) in the presence of their cognate cytosolic extracts and radiolabeled deoxynucleoside triphosphates. With this experimental system, it was shown before that CAF-1 colocalizes with sites of DNA synthesis in vitro in untreated S-phase nuclei (17). We therefore expected that nucleosome assembly on nascent DNA in these isolated nuclei consists of both passive transfer of parental nucleosomes and active de novo assembly from soluble histones, mediated by CAF-1 (16). Following digestion with micrococcal nuclease, we visualized the nuclease-resistant bulk nuclear DNA after gel electrophoresis by staining with ethidium bromide (Fig. 7A) and the nascent DNA by phosphor imaging (Fig. 7B). We found that undigested nuclear DNA used as a control did not enter the gel (data not shown),
indicating that at 24 h posttransfection with either the nontarget or p60 siRNA, apoptotically degraded DNA was not yet detectable. Upon digestion with micrococcal nuclease, bulk chromatin was about 10 times more sensitive to degradation in p60-silenced cells than in the nontarget siRNA-transfected cells (Fig. 7A). However, regular nucleosomal ladders were apparent in both samples, indicating that the bulk of the genomic DNA was still assembled into regularly spaced chromatin upon p60 silencing.

Nascent DNA in p60-silenced cell nuclei also was about 10 times more sensitive to digestion with micrococcal nuclease compared to the nontarget transfection control (Fig. 7B). Additionally, the p60-silenced cells were less competent in replicating their nuclear DNA. For each DNA replication reaction, the p60-silenced nuclei had incorporated 80\% ± 3\% less radioactivity than the nontarget-transfected samples (data not shown). The nuclease-resistant DNA in p60-silenced nuclei was predominantly mononucleosomal and contained fewer oligonucleosomal bands than the control (Fig. 7B). These data show that the efficiencies of DNA replication as well as of nucleosome assembly on nascent DNA were both reduced upon p60 silencing in proliferating human cells.

**DISCUSSION**

The main conclusion from our data is that CAF-1 is an essential protein for the viability of human proliferating cells, as its disruption by RNAi leads to inhibition of chromatin assembly during DNA synthesis, accumulation of DNA damage, and induction of programmed cell death. These experiments provide the strongest phenotype for a knockout of CAF-1 in eukaryotic cells described in the literature to date. We postulate that redundant factors exist in lower eukaryotes that may substitute for CAF-1 function in vivo but that CAF-1 has assumed an essential and unique regulatory role in proliferating higher eukaryotic somatic cells.

Genetic knockout studies in *S. cerevisiae* have demonstrated that deletion of CAF-1 yielded viable cells showing only a relatively mild phenotype of altered patterns of gene expression and an increased sensitivity to DNA damage (5, 6, 8, 11, 24). A similar situation has been found in *Arabidopsis thaliana*, in which deletion of CAF-1 results in viable plants which show alterations in gene expression, suggesting that CAF-1 function is required for facilitating stable maintenance of gene expression states (13). These alterations are linked to a phenotypic defect in maintaining the cellular architecture of postembryonic apical meristems (13). Taken together, these observations indicate the existence of redundant chromatin assembly factors which can bypass the requirement for CAF-1 and assemble chromatin that is epigenetically altered yet sufficiently func-
A dominant-negative approach to targeting the p150 subunit of CAF-1 in *Xenopus laevis* embryos led to an inhibition of the nucleosome assembly activity of CAF-1 during DNA repair (26). Ectopic expression of a dominant-negative p150 in vivo resulted in severe developmental defects during early embryogenesis that were indicative of major cell proliferation defects. However, when applying this dominant-negative approach to a somatic cell line from *X. laevis*, neither a loss of cell viability nor major defects were reported (26). Therefore, it is possible that CAF-1 function in *X. laevis* is required only for the early very rapid embryonic cell cycles of alternating S phase and mitosis but that other assembly factors can compensate for a loss of function in the slower-cycling adult somatic cells, as in *S. cerevisiae* and plants. In human somatic cells, introduction of a dominant-negative p150 subunit of CAF-1 resulted in reversal of epigenetic gene silencing in otherwise viable cells (39), indicating that CAF-1 might play a role that is at least similar to that in *S. cerevisiae* or plants. However, a substantially different phenotype was recently reported by Ye and coworkers (45). Ectopic expression of dominant-negative p150 in human U2OS cells to disrupt CAF-1 activity resulted in S-phase arrest accompanied by DNA damage. Furthermore, an S-phase checkpoint was activated through the ataxia telangiectasia mutated (ATM) and/or ATM-related (ATR) kinase pathways, leading to the conclusion that CAF-1 is required for the completion of S phase and that a defect in chromatin assembly during DNA replication can lead to DNA damage (45).

Our observations of induction of severe DNA damage and rapid programmed cell death as a result of silencing of the expression of the p60 subunit of CAF-1 confirm and further extend the physiological relevance of CAF-1 in proliferating human cells. We have shown here that CAF-1 is an essential factor for proliferating cell viability. In contrast to the dominant-negative approaches against p150 outlined above, our experimental strategy targeted the middle subunit of CAF-1 and used RNAi to remove the protein from the cell. Ectopic expression of dominant-negative p150 mutants does not remove the endogenous p150 but interferes with the function of the trimeric CAF-1 complex, most likely through the formation of dysfunctional p150 dimers (26) and/or through interference with the PCNA interaction (45). In contrast to a physical depletion of the protein by RNAi (Fig. 1), these dominant-negative approaches might only partially inactivate the CAF-1 complex by undefined substoichiometric mutant protein levels or by transient interactions in the transfected cells, leading to a range of relatively milder phenotypes and viable cells (26, 39, 45).

Furthermore, it is possible that the p60 and p150 subunits subtly fulfill distinct functions in linking nucleosome assembly during DNA synthesis to the cell cycle control machinery. Inactivation of p150 by binding to a dominant-negative version of itself could elicit a signal leading to S-phase arrest. Activation of the DNA damage-signaling protein kinases ATR and ATM has been shown under these conditions (45). A similar response of S-phase arrest and ATR signaling in viable cells was recently found when p150 was silenced by RNAi (9), indicating that disruption of CAF-1 activity via the p150 subunit
results in S-phase arrest. In contrast, the absence of p60 could signal through a different and as yet unknown pathway to elicit its response, i.e., programmed cell death. In this context it is worth noting that p60 interacts directly with the cell cycle control machinery (14, 22). The p60 subunit is reversibly phosphorylated by the S-phase-specific protein kinase complexes cyclin A/Cdk2 and cyclin E/Cdk2, and the nucleosome assembly activity of CAF-1 itself depends on ongoing reversible phosphorylation by these kinases (14). Interference with this control pathway by depleting p60 could rapidly result in the physiological cell response of programmed cell death, as reported here. Further experimentation is required to resolve this issue.

However, the most widely supported scenario, which is not mutually exclusive with either of the possibilities discussed above, is that any inactivation of CAF-1 leads to the inhibition of de novo nucleosome assembly during DNA replication (Fig. 6) (45), resulting in incompletely assembled chromatin on nascent DNA in vivo (Fig. 7). The extent of this defect would be influenced by the depletion protocol used and elicit physiological cell responses with effects ranging from (i) loss of a repressive chromatin structure and activated expression of a silenced transgene (39) to (ii) checkpoint activation and S-phase arrest (9, 45) to (iii) rapid programmed cell death (this paper).

The observation that extracts from p60-silenced cells exhibited a 10-fold-reduced level of nucleosome assembly and thus did not completely lose all assembly activity (Fig. 6) suggests that redundant pathways can still mediate de novo nucleosome assembly on replicating DNA. Even in the complete absence of the de novo nucleosome assembly activity of CAF-1, each nascent DNA strand would still receive half the complement of newly synthesized histones and DNA replication. Curr. Opin. Struct. Biol. 10:672–676.

Importantly, the response of induced rapid cell death upon p60 depletion depends on the physiological state of proliferating cells, because quiescent cells remain viable after depletion (Fig. 5). This lends strong independent support for a vital role of CAF-1 in S phase, which is already supported by the observation of S-phase arrest in the absence of functional CAF-1 (9, 45).

Together, these findings have identified CAF-1 as a potential target for anticancer drugs with cytostatic or, indeed, cytotoxic effects for proliferating cells.

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