Inhibition of S-phase chromatin assembly causes DNA damage, activation of the S-phase checkpoint and S-phase arrest.

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Summary.

The S-phase checkpoint protects the genome from spontaneous damage during DNA replication, although the cause of damage is unknown. We used a dominant negative mutant of CAF-I, a complex that assembles newly synthesized DNA into nucleosomes, to inhibit S-phase chromatin assembly and found that this induced S-phase arrest. Arrest was accompanied by DNA damage and S-phase checkpoint activation and required ATR or ATM kinase activity. These results show, for the first time, that in human cells CAF-I activity is required for completion of S-phase and defects in chromatin assembly induce DNA damage. We propose that errors in chromatin assembly, occurring spontaneously or caused by genetic mutations or environmental agents, contribute to genome instability.

Running title: Defective chromatin assembly induces DNA-damage.

Introduction.

Cancer cells characteristically have a high frequency of genome rearrangements (Lengauer et al., 1998), although the cause of rearrangements is poorly understood. Genome integrity during S-phase of the cell cycle depends on the S-phase checkpoint. This checkpoint is activated by DNA damage or stalled replication forks and inhibits ongoing DNA synthesis (Abraham, 2001; Osborn et al., 2002), thus giving time for DNA repair. DNA double strand breaks caused

by ionizing radiation (IR) activate ATM kinase, whereas stalled replication forks caused by hydroxyurea (HU) and lesions caused by ultraviolet (UV) light activate the related kinase, ATR. Downstream effectors of ATM and ATR include BRCA1, NBS1, Mre11, FANCD2, Chk1 and Chk2 kinases, the histone H2A variant, H2AX, and p53 (Abraham, 2001; Taniguchi et al., 2002; Redon et al., 2002). Underscoring the importance of the S-phase checkpoint, many S-phase checkpoint genes, such as ATM, NBS1, Mre11, BRCA1 (Khanna and Jackson, 2001), Chk2 (Bell et al., 1999), p53 (Vogelstein et al., 2000) and FANCD2 (Taniguchi et al., 2002) are mutated in human cancers.

The S-phase checkpoint also maintains genome stability in the absence of external genotoxic stress. Inactivation of ATR (Brown and Baltimore, 2000; de Klein et al., 2000), Chk1 (Liu et al., 2000; Takai et al., 2000), Hus1 (Weiss et al., 2000), BRCA1 (Hakem et al., 1996; Liu et al., 1996), NBS1 (Zhu et al., 2001) and Mre11 (Xiao and Weaver, 1997) in normal somatic cells is lethal and mouse cells lacking ATR exhibit high levels of chromosome abnormalities (Brown and Baltimore, 2000; de Klein et al., 2000). Depletion of xMre11 from *X. laevis* cell-free extracts accumulates double strand breaks in S-phase (Costanzo et al., 2001). In yeast, mutant alleles such as *mec1*, *mre11*, *chk1* and *rad53* (inactivated yeast homologs of human ATR/ATM, Mre11, Chk1 and Chk2 respectively) cause spontaneous "gross chromosomal rearrangements" (GCRs) (Kolodner et al., 2002). In sum, the S-phase checkpoint protects against spontaneous DNA damage that arises in a normal S-phase.

One likely source of spontaneous damage is stalled replication forks that are processed to Holliday junctions and double strand breaks (Osborn et al., 2002). A cell's response to stalled forks depends on the S-phase checkpoint. In yeast the checkpoint is required to reinitiate DNA replication after transient HU-mediated arrest (Desany et al., 1998), to maintain stable replication forks in the presence of an HU-mediated arrest (Lopes et al., 2001) and to prevent collapse of replication forks in response to methyl methanesulphonate (MMS)-mediated DNA damage (Tercero and Diffley, 2001). In the absence of exogenous DNA-damaging agents Mec1 promotes fork progression through "replication slow zones", where forks have a tendency to stall (Cha and Kleckner, 2002). Electron microscopy studies showed that in wild type yeast stalled forks retain a bifurcating, Y-shaped appearance. However, in cells lacking the S-phase checkpoint stalled forks frequently reversed to form Holliday junction-like “chickenfoot” structures that, by inappropriate processing, could give rise to double strand breaks (Sogo et al., 2002). Therefore, the S-phase checkpoint acts to prevent stalling and collapse of replication forks and, consequently, DNA damage and genome instability. The factors that influence the frequency of stalled forks are poorly understood.

In S-phase, nucleosomes are assembled onto newly synthesized DNA within a few hundred base pairs of the fork by chromatin assembly factors, including CAF-I and ASF1 (Tyler 2002). CAF-I is a heterotrimeric complex, consisting of p150CAF-I, p60CAF-I and p48CAF-I (Smith and Stillman, 1989).

Direct binding of p150CAF-I to the replication processivity protein, PCNA, targets CAF-I to sites of DNA synthesis and contributes to coupling of DNA synthesis and chromatin assembly (Krawitz et al., 2002; Marheineke and Krude, 1998; Martini et al., 1998; Moggs et al., 2000; Shibahara and Stillman, 1999).

We showed recently that repression of histone synthesis triggers S-phase arrest in human cells, suggesting that DNA synthesis and chromatin assembly are obligatorily coupled (Nelson et al., 2002). Here, we directly tested whether disruption of S-phase chromatin assembly affected DNA synthesis by inhibition of CAF-I. Indeed, inhibition of CAF-I blocked DNA synthesis, induced DNA damage and activated the S-phase checkpoint. These results suggest that errors in chromatin assembly, either spontaneous or resulting from genetic mutations or environmental agents, are likely to increase the rate of DNA mutation and genome instability.

Results.

A dominant negative mutant of p150CAF-I.

As described previously, both HA-p150CAF-I WT and HA-p150C bound stably to p60CAF-I (Figure 1a) whereas only HA-p150CAF-I WT bound stably to PCNA (Figure 1b) (Kaufman et al., 1995; Moggs et al., 2000). We reasoned that HA-p150C could behave as a dominant negative inhibitor of chromatin assembly by CAF-I via titration of p60CAF-I into non-functional complexes (Figure 1c).

During *in vitro* replication-coupled CAF-I-dependent chromatin assembly assays, incorporation of newly replicated plasmid DNA into nucleosomes causes the DNA to become negatively supercoiled (Smith and Stillman, 1989). In this assay, HA-p150C inhibited CAF-I-dependent chromatin assembly (Figure 2a, b). There was no effect on DNA synthesis (production of ^{32}P-labeled plasmid), indicating that it does not perturb the progression of replication forks directly. Importantly, inhibition of nucleosome formation by HA-p150C was abolished by excess purified, recombinant human CAF-I (Figure 2b), confirming that HA-p150C acts as a specific inhibitor of CAF-I.

We predicted that overexpression of HA-p150C in human cells would disrupt the interaction between endogenous p60CAF-I and p150CAF-I (Figure 1c). Indeed, endogenous p150CAF-I coprecipitated with endogenous p60CAF-I in the absence but not the presence of ectopically expressed HA-p150C (Figure 2c). Additionally, expression of HA-p150C in cells resulted in a dramatic reduction in the total amount of p150CAF-I (Figure 2c, lanes 5-8), suggesting that p150CAF-I is degraded when not incorporated into the CAF-I complex. Expression of p60CAF-I was unaffected by HA-p150C (Figure 2d). However, p60CAF-I was stably bound to chromatin in punctate DNA replication foci in 45% of control cells but only 15% of the cells expressing HA-p150C (Figure 2e, see note in Experimental Procedures). These data indicate that HA-p150C disrupts the interaction between endogenous p150CAF-I and p60CAF-I and

prevents tight association of p60CAF-I with chromatin and sites of DNA synthesis.

Inhibition of DNA synthesis.

We next tested whether HA-p150C affected DNA synthesis *in vivo*. As shown in Figure 3a, mock transfected cells released synchronously into S-phase progressed normally through S-phase. In contrast, cells transiently transfected with a plasmid encoding HA-p150C had a profound defect in S-phase progression. Many failed to detectably exit G1-phase and most of those that did arrested within S-phase. We also measured DNA synthesis by pulse labeling with 5'-BrdU at a time when the cells had accumulated in S-phase. Most of the HA-p150C expressing cells failed to incorporate 5'-BrdU and thus were not actively synthesizing DNA (Figure 3b, 13% of HA-p150C expressing cells were 5'-BrdU positive compared to 56% of the untransfected cells on the same coverslip. Representative of more than 5 similar experiments). Therefore both FACS analysis and 5'-BrdU labeling demonstrated that HA-p150C inhibited DNA synthesis. In contrast, full-length HA-p150CAF-IWT failed to inhibit DNA synthesis (Figure 3d and e) and coexpression of HA-p150CAF-IWT with HA-p150C abolished the arrest (Figure 3f, g). These data confirm that the effect of HA-p150C on DNA synthesis depends on its ability to perturb the endogenous CAF-I complex. Significantly, the cell cycle arrest induced by HA-p150C was indistinguishable from the arrest induced by ectopic expression of human HA-

HIRA (Figure 3c). Ectopic expression of HA-HIRA represses histone gene expression, thus indirectly inhibiting chromatin assembly (Hall et al., 2001; Nelson et al., 2002).

The model in Figure 1c predicts that overproduction of any fragment of p150CAF-I that binds p60CAF-I but not PCNA will inhibit DNA synthesis. Consistent with this idea HA-p150CAF-I(451-938), which did not bind PCNA, efficiently induced arrest but HA-p150CAF-I(250-938), which did bind to PCNA, failed to induce arrest (Figure 4a and c). These data also indicated that the PCNA-binding domain of HA-p150CAF-I WT is between residues 250-451, consistent with previous sequence analysis which identified a partial consensus PCNA-binding domain at residues 421-431 (Krawitz *et al*., 2002). As anticipated, deletion of residues 421-431 produced a polypeptide (HA-p150CAF-IΔPCNA) that bound to p60CAF-I but not PCNA and inhibited DNA synthesis (Figure 4a, b, c). Expression of HA-p150CAF-IΔPCNA did not affect localization of PCNA to DNA replication foci (Figure 4d, see note in Experimental Procedures) (Bravo and Macdonald-Bravo, 1985; Celis and Celis, 1985). Thus, perturbation of CAF-I inhibits DNA synthesis but, as far as we can tell from Figure 4d, does not affect assembly of DNA replication foci.

Additionally, we predicted that fragments of HA-p150CAF-I that fail to bind to both PCNA and p60CAF-I should fail to induce S-phase arrest. As anticipated, deletion of the C-terminal p60CAF-I-binding site (Kaufman et al., 1995) from HA-p150CAF-IΔPCNA protein resulted in a polypeptide, HA-

p150CAF-IΔPCNA(1-547), that failed to bind to either p60CAF-I or PCNA and did not induce S-phase arrest (Figure 5a and b).

We expected that the cells arrested in S-phase would have defective chromatin structure. Digestion with micrococcal nuclease (MNase) was used to probe chromatin structure of cells in S-phase (Nelson et al., 2002). Chromatin from HA-p150CAF-I(451-938)-expressing cells was indeed more sensitive to digestion than chromatin from mock-transfected control cells (Figure 5c, d). We conclude that disruption of the endogenous CAF-I complex induces chromatin abnormalities.

Induction of DNA damage.

One cause of S-phase arrest is DNA damage (Abraham, 2001). An early response to DNA damage, particularly double-strand breaks, is phosphorylation of histone H2AX in chromatin surrounding the lesion (Redon et al., 2002). To determine whether the DNA in arrested cells contained double-strand breaks we tested whether H2AX was phosphorylated. Phosphorylated H2AX (γH2AX) staining was enriched in HA-p150C and HA-p150CAF-IΔPCNA expressing cells relative to the untransfected cells or cells expressing HA-p150CAF-IWT (Figure 6a and 6b). Significantly, nuclei that had the brightest γH2AX staining often contained visibly abnormal DAPI-stained nuclear structures, consistent with a defect in chromatin assembly and/or extensive DNA fragmentation (Figure 6a).

In addition, even without MNase treatment the DNA extracted from HA-p150CAF-I(451-938)-expressing cells reproducibly migrated faster on agarose gels than DNA from mock transfected cells or cells transfected with HA-p150CAF-I (Figure 5c, 6c and data not shown). However, at the same time after transfection (36 hours), the dominant negative fragments of HA-p150CAF-I did not detectably induce three characteristic markers of apoptosis - chromatin condensation, cleavage of genomic DNA into a "nucleosomal ladder" and cells with less than 2n DNA content (Loo and Rillema, 1998) (Figures 6, 3c and data not shown). These data suggest that inhibition of CAF-I results in DNA double strand breaks but not a general apoptotic program.

Activation of the S-phase checkpoint.

Phosphorylation of H2AX in response to external genotoxic stresses is mediated by ATR and/or ATM kinases (Redon et al., 2002), suggesting that one or both of these kinases is activated in cells expressing HA-p150C. Activation of ATR is accompanied by its relocalization to discrete nuclear foci (Tibbetts et al., 2000). Expression of HA-p150C induced nuclear ATR foci in 23.2% of cells (standard deviation [SD] of 6.6% from 4 independent experiments) compared to less than 1% of HA-p150CAF-IWT expressing or untransfected cells (Figure 7). Similarly, HA-HIRA also activated ATR, as judged by induction of nuclear ATR foci (data not shown).

If the ATR foci are linked to S-phase arrest, they should exist in S-phase cells. Under the conditions used to optimally detect both ATR foci and PCNA in U2OS cells, the S-phase cells were readily identified by their "granular" PCNA staining pattern (Figure 7a, iv-vi). 73% of the HA-p150C-expressing cells that contained ATR foci were in S-phase (* in Figure 7a, i-iii) compared to 51% of the untransfected cells, suggesting that the ATR foci formed preferentially in S-phase. However, very few (less than 1%) of the HA-p150C expressing cells that contained ATR foci were actively synthesizing DNA, as measured by pulse-labeling with 5'-BrdU (Figure 7a, vii-ix). Taken together, these results are consistent with the idea that ATR foci are present in cells arrested in S-phase.

In addition to H2AX, other downstream effectors of ATR and ATM include the p53 tumor suppressor protein and BRCA1. Phosphorylation of p53 on serine 15 is catalyzed by ATR and ATM (Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999) and contributes to stabilization of the protein (Vousden, 2002). Both HA-p150C and HA-HIRA stabilized p53 (Figure 7b and 7c) and induced phosphorylation of p53 on serine 15. In the case of BRCA1, activation by genotoxic stress results in dispersal of small nuclear foci normally present in S-phase (Scully et al., 1997). Only 13% (SD=1.2%, 3 separate expts) of the HA-p150C expressing cells that contained ATR foci contained detectable BRCA1 foci (Figure 7a, x-xii), compared to 50.7% (SD=13.4%, 3 separate expts) of the untransfected cells on the same coverslip. BRCA1 activation is also frequently reflected by hyperphosphorylation and relocalization of BRCA1

to morphologically distinct nuclear foci that colocalize with PCNA, ATR and DNA repair proteins (Gatei et al., 2001; Scully et al., 1997; Tibbetts et al., 2000; Khanna and Jackson, 2001), but neither of these responses was observed in HA-p150C-expressing cells. Significantly, IR has not always been found to induce BRCA1 foci and complete phosphorylation is not essential for relocalization of BRCA1 to damage-induced foci (Scully et al., 1997; Cortez et al., 1999; Gatei et al., 2000). Conceivably, BRCA1 phosphorylation and detectable DNA damage-induced foci depend upon recruitment of BRCA1 to a specific chromatin structure that is absent from cells in which chromatin assembly is perturbed. Whatever the reason, we conclude that disruption of chromatin assembly results in ATR/ATM-mediated phosphorylation and stabilization of p53 and dispersal of BRCA1 foci, consistent with activation of S-phase checkpoint signaling.

In response to exogenous genotoxic stress, activated ATR and ATM trigger cell cycle arrest (Abraham, 2001). Caffeine, an inhibitor of ATR and ATM (Blasina et al., 1999; Sarkaria et al., 1999), abolished the HA-p150C-induced S-phase accumulation (Figure 7d) indicating that one or both kinases is required for the arrest. Caffeine abolished the arrest regardless of whether it was added before or after onset of arrest, indicating that it does act simply by blocking S-phase entry (data not shown). Significantly, when cells were harvested 60 hours after transfection HA-p150CAF-IΔPCNA decreased the viability of the cells and this was potentiated by caffeine (Figure 7e). In

contrast, expression of a dominant negative mutant of ATR (ATRkd) (Cliby et al., 1998) had no effect on the arrest induced by HA-p150C in U2OS (data not shown), suggesting that ATM also contributes. Indeed, coexpression of ATRkd with HA-p150CAF-I ΔPCNA or HA-p150C in ATM-/- cells completely abolished the inhibition of DNA synthesis (Figure 7f, data not shown). This shows that simultaneous inactivation of both ATR and ATM is sufficient to prevent a viable cell cycle arrest by HA-p150CAF-IΔPCNA and HA-p150C.

Discussion.

Our data demonstrate, for the first time, that disruption of CAF-I inhibits S-phase progression and causes DNA damage in human cells. We propose that inhibition of S-phase chromatin assembly causes stalled replication forks which are inappropriately processed to DNA double strand breaks. Most likely, stalled forks and double strand breaks are responsible for ATR and ATM-dependent checkpoint activation and cell cycle arrest.

CAF-I is required for S phase progression.

Several lines of evidence indicate that inhibition of DNA synthesis by HA-p150C and HA-p150CAF-IΔPCNA results from inhibition of CAF-I-dependent chromatin assembly. First, HA-p150C inhibits chromatin assembly but not DNA synthesis *in vitro*, disrupts the interaction between endogenous p150CAF-I and p60CAF-I *in vivo* and blocks stable association of p60CAF-I with replication

foci *in vivo* (Figure 2). Second, HA-p150C-induced inhibition of DNA synthesis is abolished by coexpression of HA-p150CAF-IWT, just as excess trimeric CAF-I complex restores nucleosome assembly to HA-p150C-inhibited reactions *in vitro* (Figures 2 and 3). Third, the ability of HA-p150CAF-IWT and HA-p150CAF-I fragments to inhibit cell cycle progression correlates inversely with their ability to bind PCNA (Figure 4). Fourth, inhibition of DNA synthesis is not due to total disruption of DNA replication foci because in arrested cells PCNA was still localized in a punctate pattern characteristic of normal replication foci (Figure 4d). Fifth, the ability of HA-p150CAF-I fragments to inhibit cell cycle progression appears to depend upon their ability to sequester endogenous p60CAF-I away from the endogenous p150CAF-I/PCNA complex, because a mutant that fails to bind to both PCNA and p60CAF-I is inert (Figure 5). Finally, chromatin from S-phase cells expressing a dominant negative HA-p150CAF-I fragment was more sensitive to MNase digestion than chromatin from control S-phase cells, showing directly that inhibition of CAF-I in S-phase induces defects in chromatin structure (Figure 5c). Together, these data strongly suggest that disruption of CAF-I-dependent chromatin assembly is responsible for inhibition of DNA synthesis.

Cell cycle arrest induced by HA-p150C and HA-p150CAF-I ΔPCNA is similar to that induced by human HIRA (Hall et al., 2001; Nelson et al., 2002) (Figure 3c). Previously we showed that repression of histone synthesis was the direct cause of HIRA-induced S-phase arrest (Nelson et al., 2002). Therefore,

direct inhibition of chromatin assembly by HA-p150C and HA-p150CAF-IΔPCNA or, presumably, indirect inhibition of chromatin assembly by HIRA-mediated repression of histone synthesis both block S-phase DNA synthesis in human cells. Importantly, the fact that HA-p150C and repression of histone synthesis have identical effects on S-phase, very strongly suggests that chromatin assembly, rather than DNA synthesis *per se*, is the target of HA-p150C.

Previous investigations suggested that inhibition of chromatin assembly would not block DNA synthesis in S-phase. First, in yeast none of the likely DNA synthesis-linked chromatin assembly factors identified to date, such as CAF-I, Asf1 or the Hir proteins, is essential for viability either alone or in combination (Kaufman et al., 1998; Sharp et al., 2001; Tyler et al., 1999). Second, *in vitro* replication of plasmid DNA in mammalian cell extracts or *D. melanogaster* embryo extracts does not require chromatin assembly (Bulger et al., 1995; Stillman, 1986). Third, yeast expressing histones from conditional promoters replicate their entire genome when new histone synthesis and chromatin assembly are blocked (Han et al., 1987; Kim et al., 1988). Fourth, in *C. elegans* and *D. melanogaster* embryos the reduced histone synthesis caused by mutant alleles of SLBP have no obvious effect on DNA synthesis (Sullivan et al., 2001; Kodama et al., 2002). Fifth, although in *X. laevis* perturbation of CAF-I blocks development past the mid-blastula transition, it has no detectable effect on a somatic cell line (Quivy et al., 2001). Therefore, our data

demonstrate for the first time that in intact human somatic cells, inhibition of chromatin assembly blocks DNA synthesis in S-phase.

Defects in chromatin assembly cause DNA damage

Inhibition of chromatin assembly induces DNA double strand breaks as measured by accumulation of γH2AX and fragmentation of genomic DNA (Figure 6). These breaks are likely to result from inappropriate processing of stalled replication forks (Osborn et al., 2002). Several scenarios that result in damage are possible. First, PCNA binds to both replication and chromatin assembly proteins, such as DNA polymerase δ and p150CAF-I respectively (Warbrick, 2000), suggesting that replication and chromatin assembly machineries function as an integrated complex in which disruption of one affects the other. Second, failure to package newly replicated DNA into chromatin might result in steric constraints that impede fork progression. Third, inhibition of chromatin assembly might not increase the rate of fork stalling, but the frequency with which stalled forks are processed to double strand breaks. Failure to incorporate the newly synthesized DNA into nucleosomes might increase the frequency of fork reversal, formation of a "chickenfoot" and resolution of this structure to give a double strand break (Osborn et al., 2002; Sogo et al., 2002).

Checkpoint activation

Several lines of evidence indicate that inhibition of chromatin assembly activates the S-phase checkpoint. First, H2AX, a known substrate of ATR and ATM kinases is phosphorylated (Figure 6a and b) (Redon et al., 2002). Second, ATR is recruited into nuclear foci, an indicator of ATR activation (Figure 7a) (Tibbetts et al., 2000). Third, p53 is stabilized and phosphorylated on serine 15, a known ATR and ATM phosphorylation site (Figure 7b and c)(Banin et al., 1998; Canman et al., 1998; Tibbetts et al., 1999). Fourth, the S-phase BRCA1 foci in HA-p150C-expressing cells that contain ATR foci are dispersed, a marker of BRCA1 activation (Figure 7a) (Scully et al., 1997). Fifth, inactivation of the S-phase checkpoint abolishes the S-phase arrest and decreases cell viability (Figure 7d, e and f), suggesting that a failure to arrest DNA synthesis is lethal to the cell. Taken together, these results show for the first time that inhibition of chromatin assembly activates the S-phase checkpoint.

Conceivably, the defect in chromatin assembly might be directly responsible for checkpoint activation. However, the simplest model to explain checkpoint activation is that defects in chromatin assembly cause stalling of replication forks and double strand breaks (Figure 6) and these structures activate the ATR and ATM-dependent checkpoints, similar to HU and IR (Abraham, 2001).

Chromatin assembly and genome stability

Cancer cells are characterized by a high frequency of genome abnormalities

(Lengauer et al., 1998), including "gross chromosomal rearrangements (GCRs)", such as translocations and large deletions. In the absence of an S-phase checkpoint, progression though a normal S-phase is associated with a high frequency of GCRs (Kolodner et al., 2002). This suggests that S-phase is an inherently mutagenic process and one function of the checkpoint is to suppress formation of GCRs. In addition, the S-phase checkpoint prevents stalling of replication forks and stabilizes them after they have stalled (Cha and Kleckner, 2002; Lopes et al., 2001; Sogo et al., 2002; Tercero and Diffley, 2001). Since stalled replication forks can be processed to double strand breaks which are a potent source of GCRs (Osborn et al., 2002), it seems likely that the S-phase checkpoint suppresses GCRs, at least in part, by protecting the integrity of replication forks and preventing conversion of stalled forks to double strand breaks (Kolodner et al., 2002). However, the processes that influence replication fork stalling and formation of double strand breaks in a normal S-phase are largely unknown.

We propose that defects in S-phase chromatin assembly cause double strand breaks due to stalling and inappropriate processing of replication forks and that the S-phase checkpoint limits the damage caused by defective chromatin assembly by stabilizing the stalled replication forks, inhibiting further DNA synthesis and promoting DNA repair. If so, defects in chromatin assembly and inactivation of the S-phase checkpoint should act synergistically to increase DNA damage. Indeed, in yeast Kolodner and coworkers have

observed a synergistic effect on accumulation of GCRs due to mutations in the S-phase checkpoint and chromatin assembly factors, such as *cac1* and *asf1* (Myung et al., Personal communication).

In human cells, errors in chromatin assembly combined with inactivation of the S-phase checkpoint might promote genome instability and neoplastic transformation. Several components of the S-phase checkpoint, such as ATM, BRCA1, NBS1, Mre11 (Khanna and Jackson, 2001), Chk2 (Bell et al., 1999), p53 (Vogelstein et al., 2000), FANCD2 (Taniguchi et al., 2002) are known to be mutated in human cancer. Errors in chromatin assembly might occur spontaneously or result from genetic mutations or environmental agents that inhibit chromatin assembly factors. Admittedly, the phenotype reported here may represent an extreme case that results from near-total inactivation of CAF-I and such a profound phenotype is, presumably, lethal in most cell contexts. However, more subtle defects in chromatin assembly that result from haploinsufficiency of chromatin assembly factors or from point mutations within the PCNA binding site of p150CAF-I (that weaken, but do not completely disrupt, the p150CAF-I/PCNA interaction) might be expected to be non-lethal but increase the error rate associated with DNA replication. Consistent with this idea, some genes encoding chromatin assembly factors, such as p150CAF1, p48CAF1, ASF1a and ASF1b, are located in regions of chromosomes reported to be deleted in some cancers [19p13, 1p34, 6q22 and 19p13 respectively (Couch and Weber, 2000; Mertens et al., 1997; Oesterreich et al., 2001; Sheng et al.,

1996)]. We are currently testing whether these genes are the targets of mutations in human cancers.

Experimental Procedures.

Cell culture and transfections. U2OS cells were cultured, transfected and synchronized as described previously (Adams et al., 1996; Nelson et al., 2002).

Plasmids. pcDNA3 Flag-ATRkd was a gift of Drs. Robert Abraham and Kathy Brumbaugh. pBOS-GFP-H2B was purchased from Becton-Dickinson. All other plasmids were generated using standard molecular biology procedures and details are available on request.

CAF-I-dependent *in vitro* chromatin assembly assays. *In vitro* SV40 DNA replication/nucleosome assembly assays were performed as described (Kaufman et al., 1995). The three subunit CAF-I complex and the p150CAF-I subunit were produced in insect cells and purified as described (Kaufman *et al*. 1995). HA-p150C was expressed by *in vitro* translation using TnT T7 Quick for PCR DNA (Promega).

Immunological techniques. Anti-HA (12CA5, mouse monoclonal) was purchased from Roche. Anti-HA (Y11, rabbit polyclonal), anti-PCNA (PC10 and FL261) and anti-ATR (C19) were purchased from Santa-Cruz Biotech. Anti-p53

(Ab6) and anti-BRCA1 (Ab1) were purchased from Oncogene Research Products and anti-p53pS15 was purchased from Cell Signaling. Anti-5'-BrdU-FITC was purchased from Becton-Dickinson. Anti-γH2AX was purchased from UBI. Anti-CD19-FITC was purchased from Caltag. Anti-p150CAF-I and p60CAF-I have been described previously (Smith and Stillman, 1991). Immunoprecipitation and western blots were performed as described previously (Adams et al., 1996). When performing anti-HA immunoprecipitations followed by anti-HA western blots, mouse (12CA5) and rabbit (Y11) anti-HA antibodies were used so as to avoid detection of antibody heavy chain in the western blot.

Immunofluorescence was performed as described previously by us or others (Hall et al., 2001; Tibbetts et al., 2000). Detailed methods of 2- and 3-colour immunofluorescence are available upon request. To optimize the detection of p60CAF-I and PCNA by immunofluorescence it was necessary to pre-extract the cells with EBC (Adams et al., 1996). Under these conditions HA-p150C and p60CAF-I not stably bound to chromatin were washed out of the cells. GFP-H2B served as an NP40-resistant marker of transfected cells (Figures 2e and 4d). GFP-H2B did not affect cell cycle nor HA-p150C induced S-phase arrest (data not shown) (Kanda et al., 1998).

Collection and FACS of CD19+ transfected cells. Collection using magnetic beads and FACS were described previously (Adams et al., 1996; Nelson et al., 2002).

Digestion with MNase and purification of genomic DNA. Transiently transfected (CD19+) cells were collected with anti-CD19 coated beads. Permeabilized nuclei were prepared, treated with MNase and genomic DNA purified as described previously (Hall et al., 2001; Nelson et al., 2002).

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References.

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases, Genes Dev *15*, 2177-96.

Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaelin, W. G., Jr. (1996). Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors, Mol Cell

Biol *16*, 6623-33.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage, Science *281*, 1674-7.

Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., *et al*. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome, Science *286*, 2528-31.

Blasina, A., Price, B. D., Turenne, G. A., and McGowan, C. H. (1999). Caffeine inhibits the checkpoint kinase ATM, Curr Biol *9*, 1135-8.

Bravo, R., and Macdonald-Bravo, H. (1985). Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication, Embo J *4*, 655-61.

Brown, E. J., and Baltimore, D. (2000). ATR disruption leads to chromosomal fragmentation and early embryonic lethality, Genes Dev *14*, 397-402.

Bulger, M., Ito, T., Kamakaka, R. T., and Kadonaga, J. T. (1995). Assembly of regularly spaced nucleosome arrays by Drosophila chromatin assembly factor 1 and a 56-kDa histone-binding protein., Proc Natl Acad Sci U S A *92*, 11726-30.

Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53, Science *281*, 1677-9.

Celis, J. E., and Celis, A. (1985). Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase, Proc Natl Acad Sci U S A *82*, 3262-6.

Cha, R. S., and Kleckner, N. (2002). ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones, Science *297*, 602-6.

Cliby, W. A., Roberts, C. J., Cimprich, K. A., Stringer, C. M., Lamb, J. R., Schreiber, S. L., and Friend, S. H. (1998). Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints, Embo J *17*, 159-69.

Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks, Science *286*, 1162-6.

Costanzo, V., Robertson, K., Bibikova, M., Kim, E., Grieco, D., Gottesman, M., Carroll, D., and Gautier, J. (2001). Mre11 protein complex prevents double-strand break accumulation during chromosomal DNA replication, Mol Cell *8*, 137-47.

Couch, F. J., and Weber, B. L. (2000). Breast Cancer. In The Metabolic and Molecular Bases of Inherited Disease, A. Beaudet, B. Childs, K. Kinzler, C. Scriver, S. Sly, D. Valle, and B. Vogelstein, eds. (New York, McGraw-Hill), pp. 999-1030.

'de Klein, A., Muijtjens, M., van Os, R., Verhoeven, Y., Smit, B., Carr, A. M., Lehmann, A. R., and Hoeijmakers, J. H. (2000). Targeted disruption of the cell-

cycle checkpoint gene ATR leads to early embryonic lethality in mice, Curr Biol *10*, 479-82.

Desany, B. A., Alcasabas, A. A., Bachant, J. B., and Elledge, S. J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway, Genes Dev *12*, 2956-70.

Gatei, M., Scott, S. P., Filippovitch, I., Soronika, N., Lavin, M. F., Weber, B., and Khanna, K. K. (2000). Role for ATM in DNA damage-induced phosphorylation of BRCA1, Cancer Res *60*, 3299-304.

Gatei, M., Zhou, B. B., Hobson, K., Scott, S., Young, D., and Khanna, K. K. (2001). Ataxia telangiectasia mutated (ATM) kinase and ATM and Rad3 related kinase mediate phosphorylation of Brca1 at distinct and overlapping sites. In vivo assessment using phospho-specific antibodies, J Biol Chem *276*, 17276-80.

Hakem, R., de la Pompa, J. L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F.*, et al*. (1996). The tumor suppressor gene Brca1 is required for embryonic cellular proliferation in the mouse, Cell *85*, 1009-23.

Hall, C., Nelson, D. M., Ye, X., Baker, K., DeCaprio, J. A., Seeholzer, S., Lipinski, M., and Adams, P. D. (2001). HIRA, the human homologue of yeast Hir1p and Hir2p, is a novel cyclin-cdk2 substrate whose expression blocks S-phase progression, Mol Cell Biol *21*, 1854-65.

Han, M., Chang, M., Kim, U. J., and Grunstein, M. (1987). Histone H2B repression causes cell-cycle-specific arrest in yeast: effects on chromosomal

segregation, replication, and transcription, Cell *48*, 589-97.

Kanda, T., Sullivan, K. F., and Wahl, G. M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells, Curr Biol *8*, 377-85.

Kaufman, P. D., Cohen, J. L., and Osley, M. A. (1998). Hir proteins are required for position-dependent gene silencing in Saccharomyces cerevisiae in the absence of chromatin assembly factor I, Mol Cell Biol *18*, 4793-806.

Kaufman, P. D., Kobayashi, R., Kessler, N., and Stillman, B. (1995). The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication, Cell *81*, 1105-14.

Khanna, K. K., and Jackson, S. P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection, Nat Genet *27*, 247-54.

Kim, U. J., Han, M., Kayne, P., and Grunstein, M. (1988). Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae, Embo J *7*, 2211-9.

Kodama, Y., Rothman, J. H., Sugimoto, A., and Yamamoto, M. (2002). The stem-loop binding protein CDL-1 is required for chromosome condensation, progression of cell death and morphogenesis in Caenorhabditis elegans, Development *129*, 187-96.

Kolodner, R. D., Putnam, C. D., and Myung, K. (2002). Maintenance of genome stability in Saccharomyces cerevisiae, Science *297*, 552-7.

Krawitz, D. C., Kama, T., and Kaufman, P. D. (2002). Chromatin Assembly

Factor I Mutants Defective for PCNA Binding Require Asf1/Hir Proteins for Silencing, Mol Cell Biol *22*, 614-25.

Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998). Genetic instabilities in human cancers, Nature *396*, 643-9.

Liu, C. Y., Flesken-Nikitin, A., Li, S., Zeng, Y., and Lee, W. H. (1996). Inactivation of the mouse Brca1 gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development, Genes Dev *10*, 1835-43.

Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A.*, et al*. (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint, Genes Dev *14*, 1448-59.

Loo, D. T., and Rillema, J. R. (1998). Measurement of cell death, Methods Cell Biol *57*, 251-64.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C. S., and Foiani, M. (2001). The DNA replication checkpoint response stabilizes stalled replication forks, Nature *412*, 557-61.

Marheineke, K., and Krude, T. (1998). Nucleosome assembly activity and intracellular localization of human CAF-1 changes during the cell division cycle, J Biol Chem *273*, 15279-86.

Martini, E., Roche, D. M., Marheineke, K., Verreault, A., and Almouzni, G. (1998). Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells, J Cell Biol *143*, 563-75.

Mertens, F., Johansson, B., Hoglund, M., and Mitelman, F. (1997). Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms, Cancer Res *57*, 2765-80.

Moggs, J. G., Grandi, P., Quivy, J. P., Jonsson, Z. O., Hubscher, U., Becker, P. B., and Almouzni, G. (2000). A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage, Mol Cell Biol *20*, 1206-18.

Myung, K., Pennaneach, V., Kats, E., and Kolodner, R. D. (Personal communication).

Nelson, D. M., Ye, X., Hall, C., Santos, H., Ma, T., Kao, G. D., Yen, T. J., Harper, J. W., and Adams, P. D. (2002). Coupling of DNA synthesis and histone synthesis in S-phase independent of cyclin/cdk2 activity., Mol Cell Biol *22*, 7459-72.

Oesterreich, S., Allredl, D. C., Mohsin, S. K., Zhang, Q., Wong, H., Lee, A. V., Osborne, C. K., and O'Connell, P. (2001). High rates of loss of heterozygosity on chromosome 19p13 in human breast cancer, Br J Cancer *84*, 493-8.

Osborn, A. J., Elledge, S. J., and Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway., Trends Cell Biol *12*, 509-516.

Quivy, J. P., Grandi, P., and Almouzni, G. (2001). Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during Xenopus early development, Embo J *20*, 2015-27.

Ray-Gallet, D., Quivy, J.-P., Scamps, C., Martini, E. M.-D., Lipinski, M., and G, A. (2002). HIRA Is Critical for a Nucleosome Assembly Pathway

Independent of DNA Synthesis, Mol Cell *9*, 1091-1100.

Redon, C., Pilch, D., Rogakou, E., Sedelnikova, O., Newrock, K., and Bonner, W. (2002). Histone H2A variants H2AX and H2AZ, Curr Opin Genet Dev *12*, 162-9.

Sarkaria, J. N., Busby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M., and Abraham, R. T. (1999). Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine, Cancer Res *59*, 4375-82.

Scully, R., Chen, J., Ochs, R. L., Keegan, K., Hoekstra, M., Feunteun, J., and Livingston, D. M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage, Cell *90*, 425-35.

Sharp, J. A., Fouts, E. T., Krawitz, D. C., and Kaufman, P. D. (2001). Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing, Curr Biol *11*, 463-73.

Sheng, Z. M., Marchetti, A., Buttitta, F., Champeme, M. H., Campani, D., Bistocchi, M., Lidereau, R., and Callahan, R. (1996). Multiple regions of chromosome 6q affected by loss of heterozygosity in primary human breast carcinomas, Br J Cancer *73*, 144-7.

Shibahara, K., and Stillman, B. (1999). Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin., Cell *96*, 575-85.

Smith, S., and Stillman, B. (1989). Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro., Cell *58*, 15-25.

Smith, S., and Stillman, B. (1991). Immunological characterization of chromatin assembly factor I, a human cell factor required for chromatin assembly during DNA replication in vitro, J Biol Chem *266*, 12041-7.

Sogo, J. M., Lopes, M., and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects, Science *297*, 599-602.

Stillman, B. (1986). Chromatin assembly during SV40 DNA replication in vitro., Cell *45*, 555-65.

Sullivan, E., Santiago, C., Parker, E. D., Dominski, Z., Yang, X., Lanzotti, D. J., Ingledue, T. C., Marzluff, W. F., and Duronio, R. J. (2001). Drosophila stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression, Genes Dev *15*, 173-87.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y. A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., and Nakanishi, M. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice, Genes Dev *14*, 1439-47.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P. R., Gregory, R. C., Kim, S. T., Lane, W. S., Kastan, M. B., and D'Andrea, A. D. (2002). Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways, Cell *109*, 459-72.

Tercero, J. A., and Diffley, J. F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint, Nature *412*,

553-7.

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53, Genes Dev *13*, 152-7.

Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. (2000). Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress, Genes Dev *14*, 2989-3002.

Tyler, J. K. (2002). Chromatin assembly, Eur J Biochem *269*, 2268-74.

Tyler, J. K., Adams, C. R., Chen, S. R., Kobayashi, R., Kamakaka, R. T., and Kadonaga, J. T. (1999). The RCAF complex mediates chromatin assembly during DNA replication and repair, Nature *402*, 555-60.

Vogelstein, B., Lane, D., and Levine, A. J. (2000). Surfing the p53 network, Nature *408*, 307-10.

Vousden, K. H. (2002). Activation of the p53 tumor suppressor protein, Biochim Biophys Acta *1602*, 47-59.

Warbrick, E. (2000). The puzzle of PCNA's many partners, Bioessays *22*, 997-1006.

Weiss, R. S., Enoch, T., and Leder, P. (2000). Inactivation of mouse Hus1 results in genomic instability and impaired responses to genotoxic stress, Genes Dev *14*, 1886-98.

Wu, X., Ranganathan, V., Weisman, D. S., Heine, W. F., Ciccone, D. N.,

O'Neill, T. B., Crick, K. E., Pierce, K. A., Lane, W. S., Rathbun, G., *et al*. (2000). ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response, Nature *405*, 477-82.

Xiao, Y., and Weaver, D. T. (1997). Conditional gene targeted deletion by Cre recombinase demonstrates the requirement for the double-strand break repair Mre11 protein in murine embryonic stem cells, Nucleic Acids Res *25*, 2985-91.

Zhu, J., Petersen, S., Tessarollo, L., and Nussenzweig, A. (2001). Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice, Curr Biol *11*, 105-9.

Figure Legends.

Figure 1. HA-p150C is a putative dominant negative inhibitor of CAF-I. (a) ^{35}S-labeled p60CAF-I was *in vitro* translated with or without cotranslation of HA-p150CAF-I WT or HA-p150C as indicated. Reactions were immunoprecipitated with an anti-HA antibody (12CA5, lanes 1-3) and fractionated by SDS-PAGE. Lanes 4-6 contain 20% of input proteins. (b) U2OS cells were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150C as indicated. Cell lysates were immunoprecipitated with an anti-HA antibody (Y11) and western blotted with antibodies to HA (12CA5) or PCNA (PC10) as indicated. (c) A model for the proposed mode of action of HA-p150C (see text for details).

Figure 2. HA-p150C inhibits CAF-I-dependent chromatin assembly *in vitro* and disrupts the endogenous CAF-I complex *in vivo*. (a) DNA replication-coupled CAF-I-dependent chromatin assembly assays were performed with purified p150CAF-I and *in vitro* translation reaction that was unprogrammed (lane 1) or expressed HA-p150C (lane 2). HA-p150C was detected by western blotting with anti-HA (12CA5, upper panel). Purified ^{32}P-labeled replicated plasmid DNA was fractionated by agarose gel electrophoresis (lower panel) to resolve relaxed (non-nucleosomal) and supercoiled (nucleosomal) plasmids. (b) DNA replication-coupled CAF-I-dependent chromatin assembly assays were performed in the absence or presence of ~4ng p150CAF-IWT, *in vitro*-translated HA-p150C and purified recombinant trimeric CAF-I complex (5.5, 11, or 22ng as indicated by the triangle). (c) U2OS cells were transiently transfected with pCMV-CD19 together with pcDNA3 HA-p150C or pcDNA3 as indicated. Transfected cells were immunopurified with anti-CD19 coated magnetic beads, lysates were prepared and immunoprecipitated with antibodies to p60CAF-I (SS24), p150CAF-I (SS1) or control (419) as indicated, fractionated by SDS-PAGE and western blotted with an antibody to p150CAF-I (SS1). Lanes 7 and 8 contain 150μg of whole cell lysate derived from immunopurified transfected cells. (d) Lysates from (c) were western blotted with antibodies to p60CAF-I (a cocktail of SS3, SS53, SS60 and SS96). (e) U2OS cells were transiently transfected with pBOS-GFP-H2B in the absence (i, ii, iii) or presence (iv, v, vi) of pCDNA3 HA-p150C. The cells were stained with antibodies to p60CAF-I

(SS75) and visualized by immunofluorescence microscopy to detect GFP-H2B and p60CAF-I. 100 cells were counted and scored as p60CAF-I positive or negative. The results of two independent experiments are plotted in panels i and iv.

Figure 3. HA-p150C blocks DNA synthesis and progression through S-phase. (a) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3 or pcDNA3 HA-p150C as indicated. 16 hours later the cells were arrested in mimosine for 20 hours, released into S-phase and at time intervals afterwards the cell cycle distribution of the CD19+ cells was determined by FACS. (b) U2OS cells were transiently transfected with pcDNA3 HA-p150C and 36 hours later pulse labeled for 30 minutes with 10μM 5'-BrdU and stained with DAPI to visualize the DNA (i), anti-HA (Y11) (ii) and anti-5'-BdU-FITC (iii). (c) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3, pcDNA3 HA-p150C or pcDNA3 HA-HIRA as indicated and processed as in a). (d) U2OS cells were transfected with pCMV-CD19 together with pcDNA3 (lane 1), 14 and 28μg of pcDNA3 HA-150CAF-IWT (lanes 2 and 3 respectively) or 1 and 3μg of pcDNA3 HA-p150C (lanes 4 and 5 respectively). 5μg of whole cell lysate was fractionated by SDS-PAGE and western blotted with anti-HA (12CA5). Lanes 1-3 and 4-5 of are non-adjacent lanes from the same exposure of the same gel. (e) The same transfections as d) processed as in a). (f) U2OS cells were transfected with pCMV CD19 together with pcDNA3

HA-p150C or pDNA3 as indicated and decreasing amounts of pcDNA3 HA-p150CAF-IWT (as indicated by the shaded triangle). The cells were processed as in a). (g) 5μg of whole cell lysate from cells in f) was fractionated by SDS-PAGE and western blotted with anti-HA (12CA5).

Figure 4. S-phase arrest correlates with failure of HA-p150CAF-I mutants to bind to PCNA. (a) U2OS cells were transiently transfected with pcDNA3, pcDNA3 HA-p150CAF-I WT or the mutants derived from HA-p150CAF-I as indicated. Cell lysates were immunoprecipitated with anti-HA antibodies (Y11) and then western blotted with anti-HA (12CA5, top panel) or anti-PCNA (PC10, middle panel). The bottom panel (WCE) shows an anti-PCNA (PC10) western blot of 10μg of the whole cell lysate. (b) U2OS cells were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150CAF-IΔPCNA as indicated. Cell lysates were immunoprecipitated with antibodies to p60CAF-I (SS24) or a control antibody of the same subclass (con., 419, IgG_1) and western blotted with anti-HA (12CA5). (c) U2OS cells were transiently transfected with pCMV CD19 together with pcDNA3, pcDNA3 HA-p150CAF-I WT or the same set of mutants used in (a) and indicated by the same numbers 1-6. The cells were processed as in Figure 3a) (d) U2OS cells grown on coverslips were transiently transfected with pBOS GFP-H2B in the absence (v-viii) or presence (i-iv) of pcDNA3 HA-p150CAF-IΔPCNA. The cells were stained with antibodies to PCNA and expression of GFP-H2B (iii and vii) and localization of PCNA (ii and

vi) were visualized by immunofluoresence microscopy. 100 GFP-H2B positive cells were scored as PCNA positive or negative and the results plotted (i and v). The results shown are the means of two independent experiments.

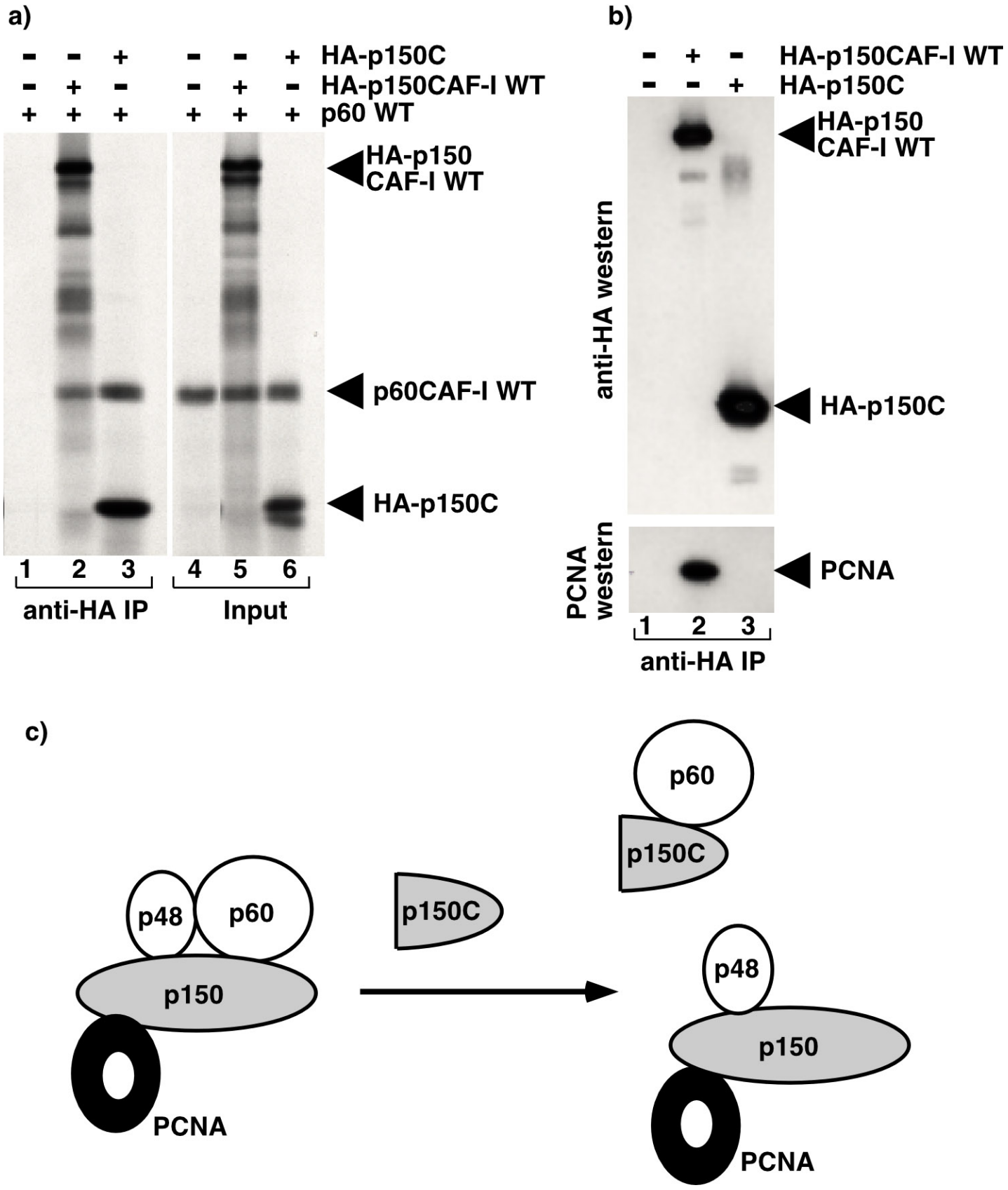
Figure 5. S-phase arrest correlates with sequestration of p60CAF-I and is accompanied by defects in chromatin structure. (a) U2OS cells were transiently transfected with pcDNA3 (lane 1), pcDNA3-HAp150CAF-IWT (lane 2), pcDNA3HAp150CAF-IΔPCNA (lane 3) or pcDNA3HAp150CAF-IΔPCNA(1-547) together with pCMV CD19. Lysates were immunoprecipitated with antibodies to anti-HA (Y11) and then western blotted with antibodies to HA (12CA5), p60CAF-I and PCNA. The bottom panel is a western blot of PCNA in the crude lysate. (b) As in a) except that the cell cycle distribution of CD19+ cells was determined as in Figure 3a. (c) U2OS cells were transiently transfected with pcDNA3 (lanes 3, 5, 7, 9) or pcDNA3 HAp150CAF-I(451-938) (lanes 2, 4, 6, 8) together with pCMV CD19. The cells were arrested in mimosine for 20 hours and then washed to release them into S-phase for 6 (pcDNA3) or 8 (pcDNA3 HAp150CAF-I[451-938]) hours to give equal proportions of cells in S-phase. CD19+ cells were immunopurified, nuclei prepared and treated with 0, 0.5, 1.5 and 5 Worthington Units/ml of MNase as indicated by the shaded boxes. Genomic DNA was purified and fractionated by agarose gel electrophoresis. Lane 1 contains 1μg of phage λ DNA digested with HindIII and lane 10 contains 1μg of a 100bp ladder. (d) The same transfection as

in c), but the cell cycle distribution of the CD19+ cells was determined as in Figure 3a. Top, pcDNA3. Bottom, pcDNA3 HAp150CAF-I(451-938).

Figure 6. Dominant negative HA-p150CAF-I mutants induce DNA damage. (a) U2OS cells on coverslips were transiently transfected with pcDNA3 HAp150C. The cells were stained with DAPI, anti-γH2AX antibodies or anti-HA antibodies, as indicated, and visualized by immunofluorescence microscopy. (b) 100 transfected HA+ cells or 100 untransfected HA- cells from the same coverslip were scored as positive or negative for γH2AX. The results are the mean of 12 (untransfected), 8 (HA-p150CAF-IWT and HA-p150C) or 2 (HA-p150CAF-IΔPCNA) independent experiments. (c) U2OS cells were transiently transfected with pcDNA3 (lane 4) or pcDNA3 HAp150CAF-I(451-938) (lane 3) together with pCMV CD19. The cells were processed and genomic DNA purified as in lanes 2 and 3 of Figure 5c (without MNase). Lane 1 contains 1μg of phage λ DNA digested with HindIII (MW markers indicated on left of gel) and lane 2 contains 1μg of a 100bp ladder.

Figure 7. S-phase arrest depends upon activation of the S-phase checkpoint. (a) U2OS cells on coverslips were transiently transfected with pcDNA3 HA-p150C. In iv-ix cells were pulse labeled for 15 minutes with 5'-BrdU prior to harvesting. Cells were stained with antibodies to HA, ATR and PCNA (i-iii), 5'-BrdU and PCNA (iv-vi), HA, ATR and 5'-BrdU (vii-ix) and HA, ATR and

BRCA1 (x-xii). (b) U2OS cells were transiently transfected with pcDNA3 (lane 1), pcDNA3 HA-HIRA(421-729) (lane 2) or pcDNA3 HAp150C (lane 3) together with pCMV CD19. CD19+ cells were immunopurified with magnetic beads and cell lysates western blotted with an antibody to p53. (c) Cell lysates from b) were western blotted with an antibody to p53pSer15. Lane 4 contains lysate from cells irradiated with UV light. (d) U2OS cells were transiently transfected with a plasmid pCMV CD19 in the absence or presence of pcDNA3 HA-p150C. 16 hours later the cells were treated with 1.7mM caffeine or PBS and 24 hours later the cell cycle distribution of the CD19+ cells determined as in Figure 3a. (e) U2OS cells were transiently transfected with pCMV CD19 in the absence or presence of pcDNA3 HA-p150CAF-IΔPCNA. 36 hours later the cells were treated with 2 or 6mM caffeine or PBS and 24 hours later percentage of the CD19+ cells determined by FACS. The number of CD19+ HA-p150CAF-IΔPCNA-expressing cells is expressed as a % of the control (CD19 alone) at each dose of caffeine. (f) ATM-/- human fibroblasts were transiently transfected with pcDNA3 HA-p150CAF-I WT or HA-p150CAF-I ΔPCNA in the absence or presence of a pcDNA3 ATRkd as indicated. 36 hours later the cells were pulse-labeled for 1 hour with 10μM 5'-BrdU and stained with anti-HA(Y11) and anti-5'-BrdU and DAPI. 100 untransfected cells (HA-negative) and 100 transfected cells (HA-positive) were scored as 5'-BrdU positive or negative and expressed as the percentage of 5'-BrdU positive. Each bar with standard deviation is the mean of 4 separate experiments.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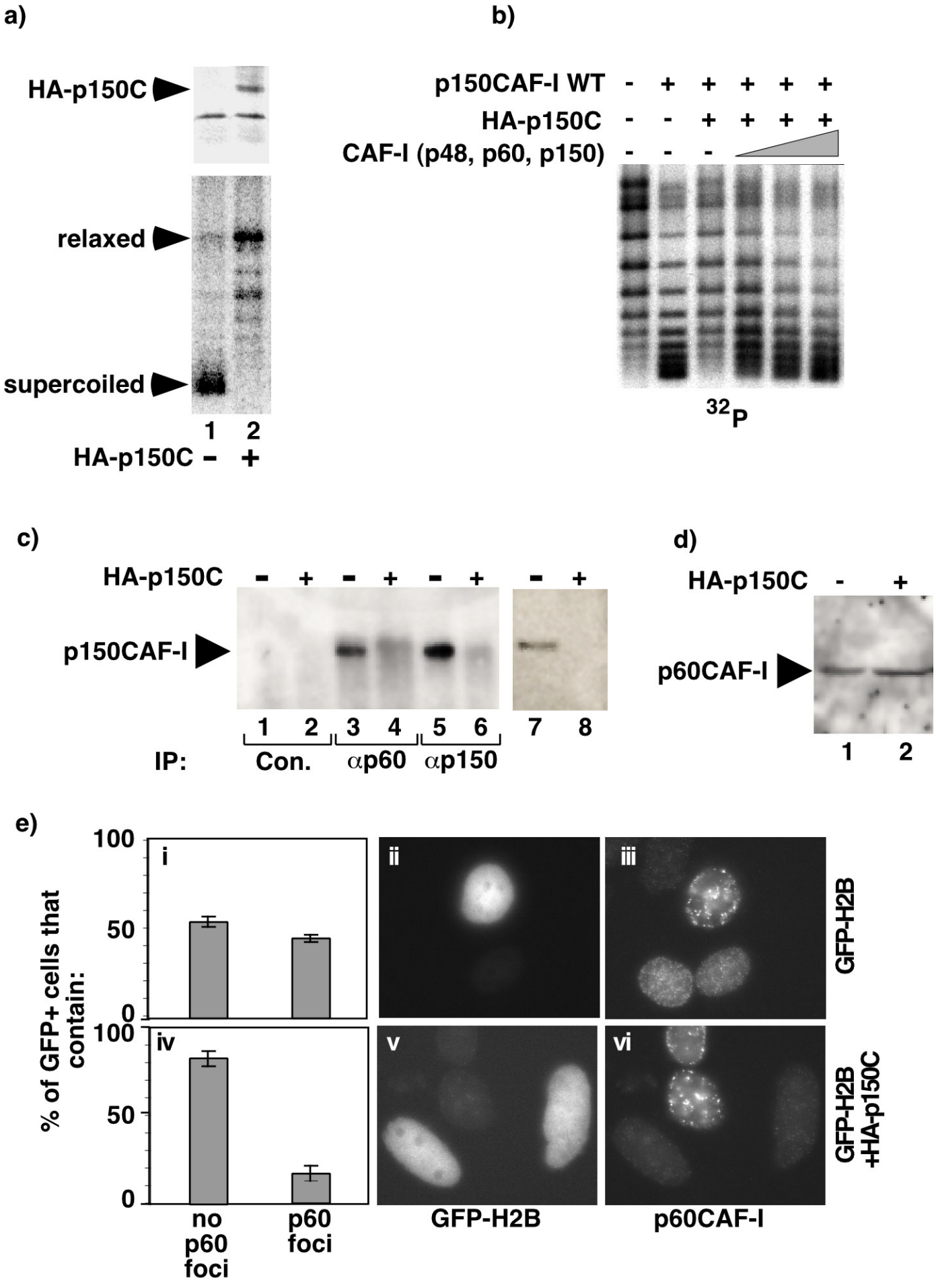
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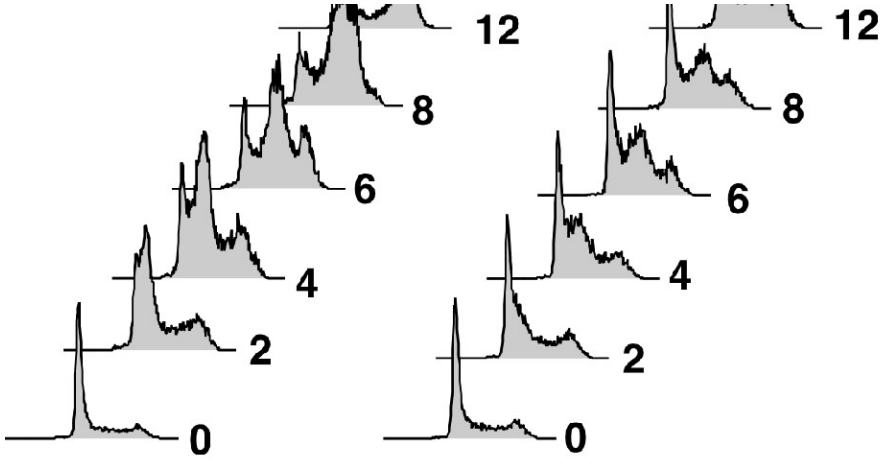
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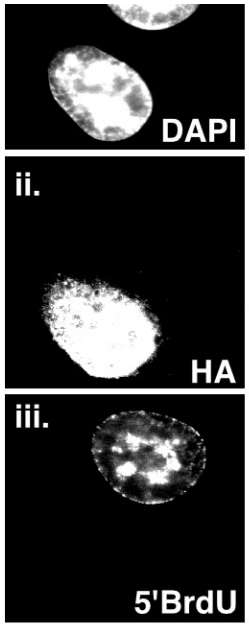
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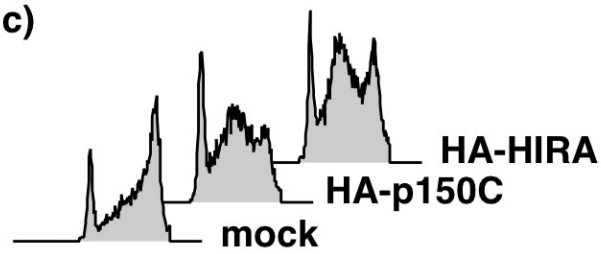
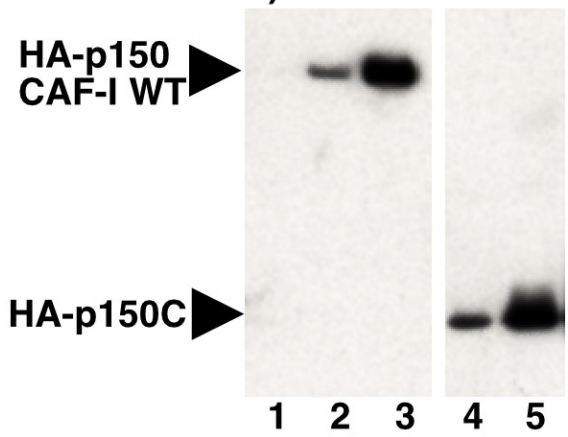
5'BrdU

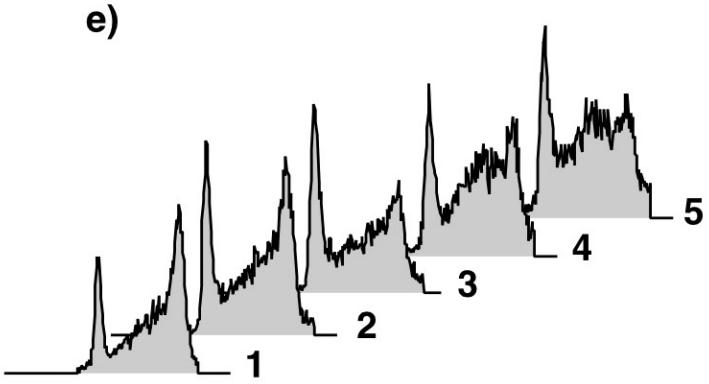
c)


HA-HIRA
HA-p150C
mock

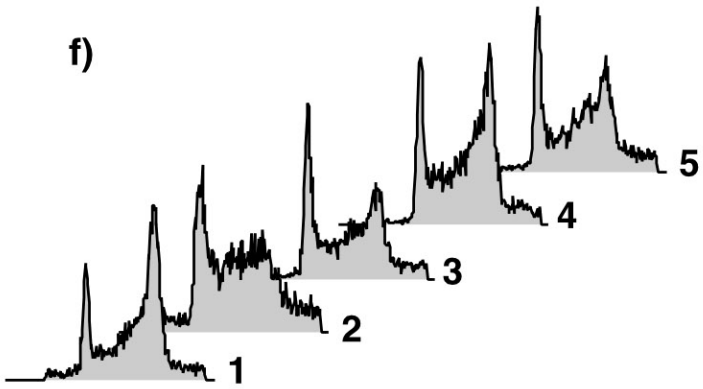
d)


HA-p150
CAF-I WT
HA-p150C
1 2 3 4 5

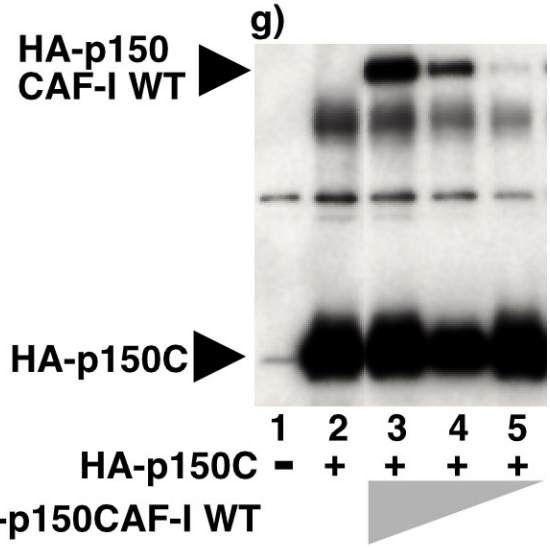
e)

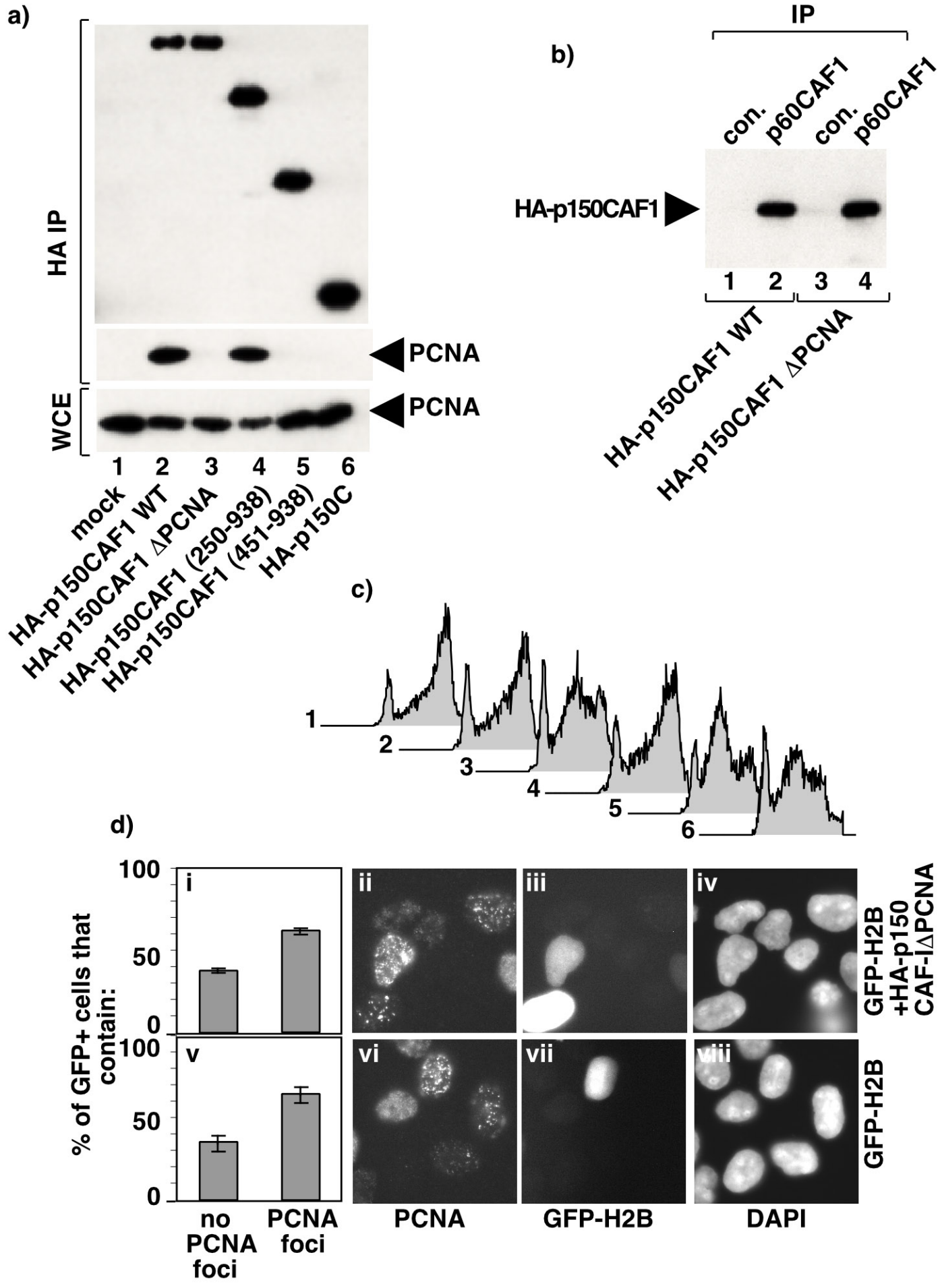

5
4
3
2
1

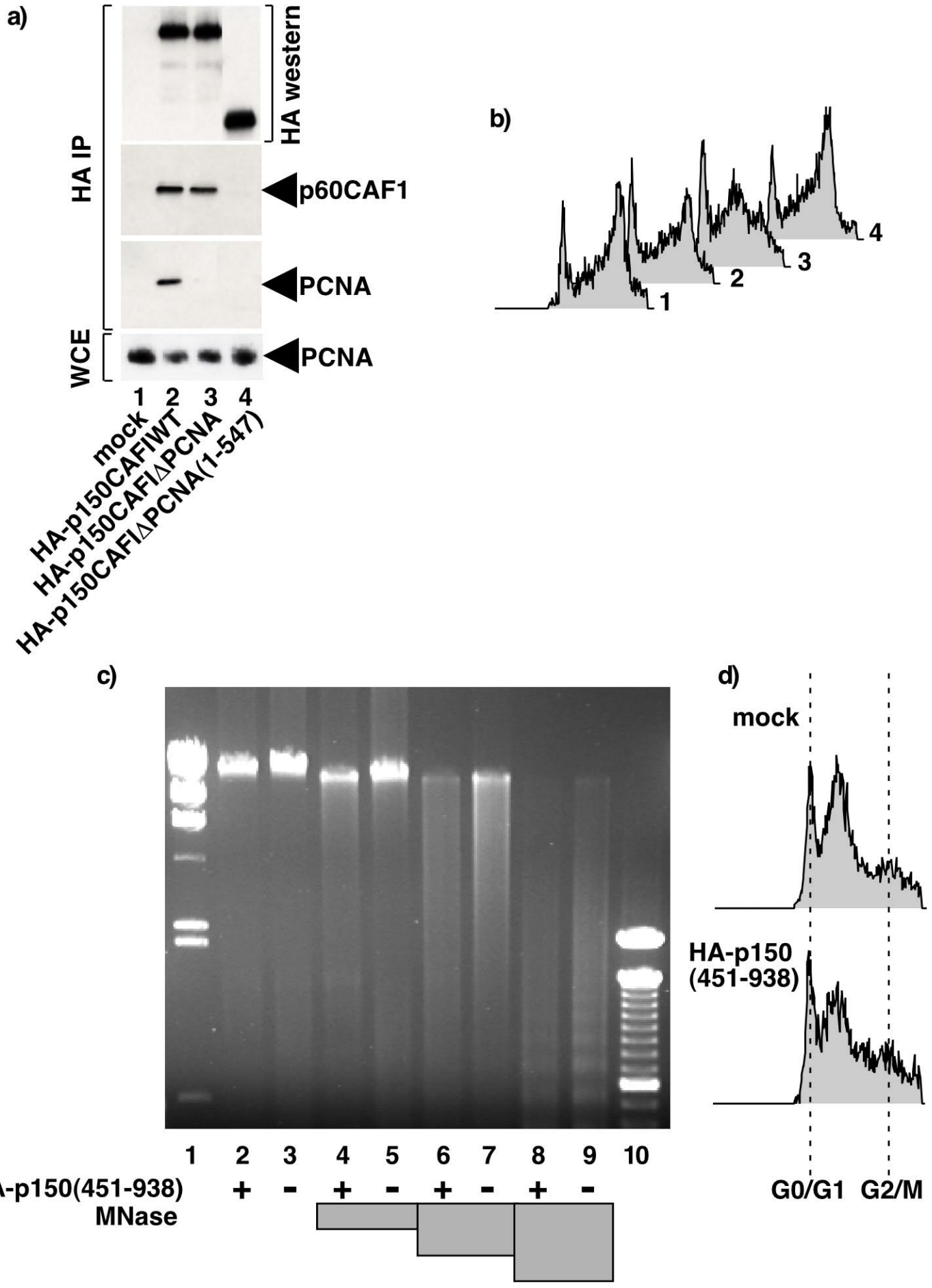
f)

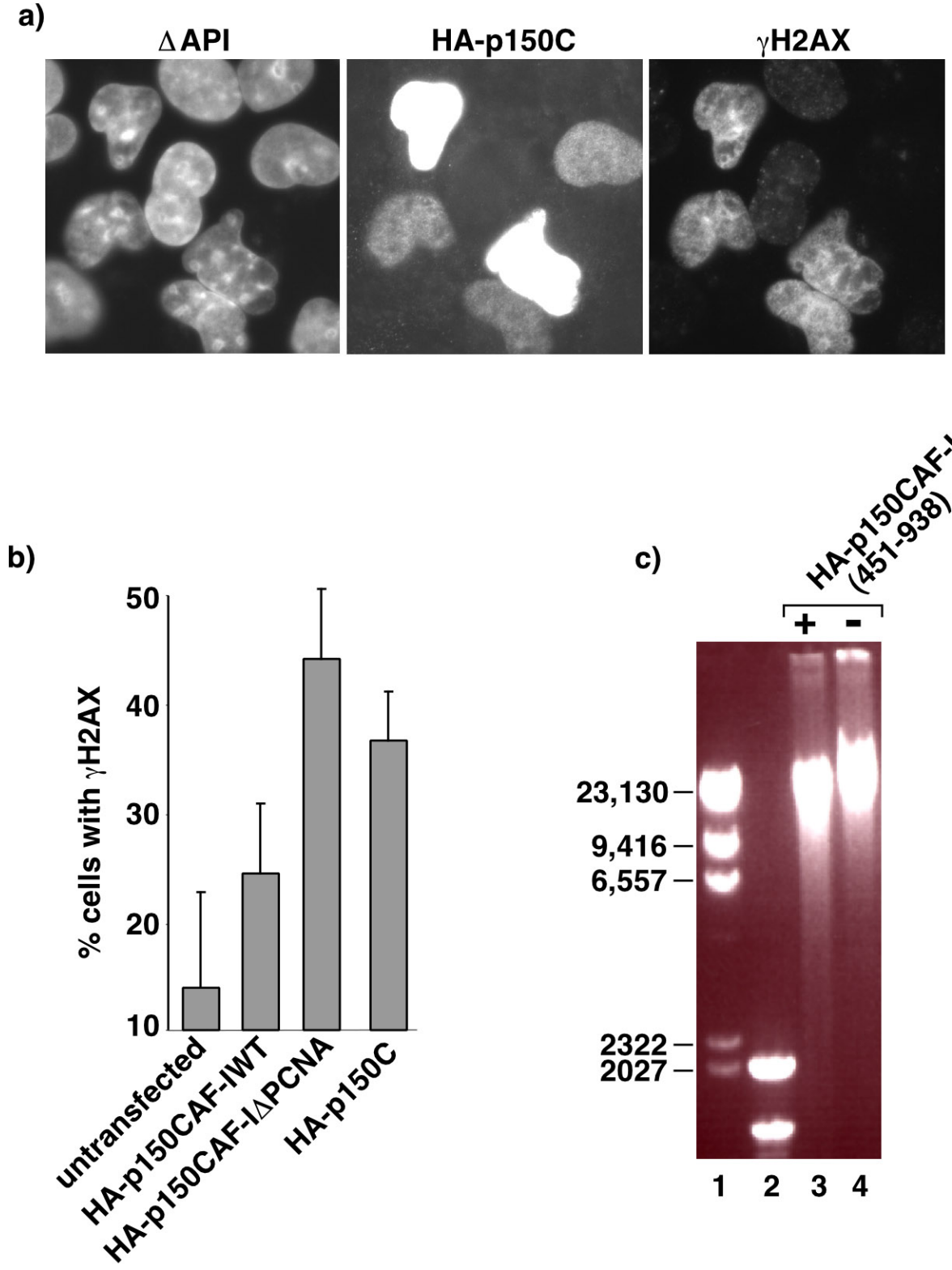

5
4
3
2
1

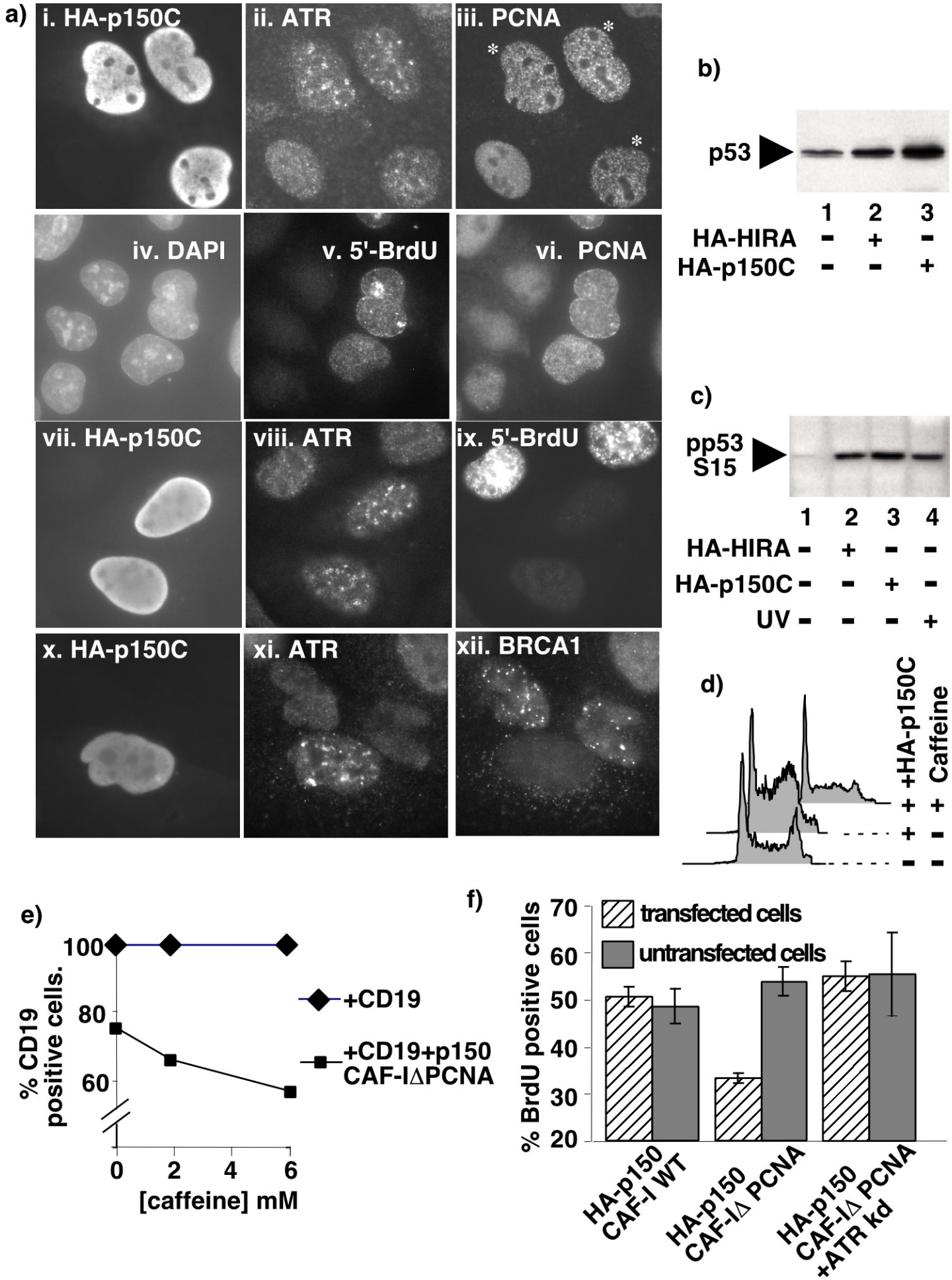
g)


HA-p150
CAF-I WT
HA-p150C
1 2 3 4 5
HA-p150C - + + + +
HA-p150CAF-I WT


a)
HA IP
WCE
PCNA
PCNA
1 2 3 4 5 6
mock
HA-p150CAF1 WT
HA-p150CAF1 ΔPCNA
HA-p150CAF1 (250-938)
HA-p150CAF1 (451-938)
HA-p150C
b)
IP
con.
p60CAF1
con.
p60CAF1
HA-p150CAF1
1 2 3 4
HA-p150CAF1 WT
HA-p150CAF1 ΔPCNA
c)
1
2
3
4
5
6
d)
% of GFP+ cells that contain:
100
50
0
i
v
no PCNA foci
PCNA foci
ii
iii
iv
vi
vii
viii
PCNA
GFP-H2B
DAPI
GFP-H2B +HA-p150 CAF-IΔPCNA
GFP-H2B


a)
HA IP
HA western
p60CAF1
PCNA
WCE
PCNA
1 2 3 4
mock
HA-p150CAFIWT
HA-p150CAFIΔPCNA
HA-p150CAFIΔPCNA(1-547)
b)
1
2
3
4
c)
1 2 3 4 5 6 7 8 9 10
HA-p150(451-938) + - + - + - + -
MNase
d)
mock
HA-p150
(451-938)
G0/G1
G2/M


a)
ΔAPI
HA-p150C
γH2AX
b)
% cells with γH2AX
50
40
30
20
10
untransfected
HA-p150CAF-IWT
HA-p150CAF-IΔPCNA
HA-p150C
c)
HA-p150CAF-I
(451-938)
+
-
23,130
9,416
6,557
2322
2027
1
2
3
4


a)
i. HA-p150C
ii. ATR
iii. PCNA
iv. DAPI
v. 5'-BrdU
vi. PCNA
vii. HA-p150C
viii. ATR
ix. 5'-BrdU
x. HA-p150C
xi. ATR
xii. BRCA1
b)
p53
1 2 3
HA-HIRA - + -
HA-p150C - - +
c)
pp53 S15
1 2 3 4
HA-HIRA - + - -
HA-p150C - - + -
UV - - - +
d)
+HA-p150C
Caffeine
e)
% CD19 positive cells.
100
80
60
0 2 4 6
[caffeine] mM
+CD19
+CD19+p150 CAF-IΔPCNA
f)
% BrdU positive cells
70
60
50
40
30
20
transfected cells
untransfected cells
HA-p150 CAF-I WT
HA-p150 CAF-IΔ PCNA
HA-p150 CAF-IΔ PCNA +ATR kd